NEW NEMERTEAN DIVERSITY DISCOVERED IN THE NORTHEAST PACIFIC, USING SURVEYS OF BOTH PLANKTONIC LARVAE AND BENTHIC ADULTS

by

TERRA CELESTE HIEBERT

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Student: Terra Celeste Hiebert

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This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Biology by:

Barbara “Bitty” Roy	Chairperson
Svetlana Maslakova	Advisor
Richard Emlet	Core Member
Alan Shanks	Core Member
Kelly Sutherland	Institutional Representative

and

Scott L. Pratt	Dean of the Graduate School

Original approval signatures are on file with the University of Oregon Graduate School.

Degree awarded March 2016.

DISSERTATION ABSTRACT

Terra Celeste Hiebert

Doctor of Philosophy

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Title: New Nemertean Diversity Discovered in the Northeast Pacific, Using Surveys of Both Planktonic Larvae and Benthic Adults

This study doubles the known diversity of nemertean species in one region along the northeast Pacific coast by utilizing the often over-looked larval life-history stage. Prior to this work, the nemertean fauna in this region was believed to be well described; however, previous assessments were based on adult life-history stages only and significantly underestimated the real diversity. With this dissertation, we update what is known about nemertean diversity and expand upon this "life-history" approach to describe new species, identify and describe larval forms, and speculate on the phylogenetic relevance of nemertean larvae.

A considerable amount of new diversity takes the form of cryptic species complexes, where existing descriptions include characteristics of several species. *Micrura alaskensis,* a common intertidal nemertean and an emerging model system for developmental studies, existed as a species complex consisting of five species. In this dissertation we designate a new genus, re-describe *M. alaskensis,* and describe four new species in this complex. In doing so we make accurate identification possible for future comparative research.

The complete development of few nemertean species was known before this project began, thus few species could be identified as larvae. We have identified over 30 nemertean larvae using both embryological and DNA barcoding approaches in this work. Intriguingly, many wild-caught larvae could not be matched to species previously reported from this region and instead contribute to previously unknown diversity. This new diversity includes species previously reported only from distant geographic regions as well as species new to science. The first record of a hubrechtid on the west coast of North America and the identification of two new species in the currently monotypic genus *Riserius* were revealed in larval assessments.

Aside from increasing known species-level diversity, we revealed novel larval types. Barcoding larvae allowed us to place larval morphotypes into a phylogenetic context and identify potentially useful larval synapomorphies for nemertean phylogenies. Our results emphasize the importance of a life-history approach to biodiversity assessments for all species with biphasic life-cycles.

This dissertation includes published and unpublished co-authored material.

CURRICULUM VITAE

NAME OF AUTHOR: Terra Celeste Hiebert

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene OR
Western Washington University, Bellingham WA
University of Hawaii, Hilo HI

DEGREES AWARDED:

Doctor of Philosophy, Biology, 2016, University of Oregon
Master of Science, Environmental Science, 2009, Western Washington University
Bachelor of Science, Marine Biology, 2004, University of Oregon

AREAS OF SPECIAL INTEREST:

Invertebrate Zoology
Taxonomy, Systematics, and Phylogenetics
Species Delimitation
Larval Biology and Evolution

PROFESSIONAL EXPERIENCE:

Graduate Research Assistant, Oregon Institute of Marine Biology, University of Oregon, Charleston, Oregon, 2011–2016

Graduate Teaching Assistant, Oregon Institute of Marine Biology, University of Oregon, Charleston, Oregon, 2011–2014

Administrative Graduate Teaching Fellow, Oregon Institute of Marine Biology, University of Oregon, Charleston, Oregon, 2012

Graduate Research Assistant, Shannon Point Marine Center, Western Washington University, Anacortes, Washington, 2009–2011

Graduate Teaching Assistant, Western Washington University, Bellingham, Washington, 2009–2011

Research Assistant, Institute of Neuroscience, Zebrafish International Resource Center, University of Oregon, Eugene, Oregon, 2005–2009

Teaching Assistant, Oregon Institute of Marine Biology, University of Oregon, Charleston, Oregon, 2004

GRANTS, AWARDS, AND HONORS:

Women in Graduate Sciences Parenting Award, University of Oregon 2015

Graduate Student Assistance Award, University of Oregon, 2015

Clarence and Lucille Dunbar Scholarship, University of Oregon, 2014

Donald E. Wimber Travel Award, University of Oregon, 2014

Travel Award, Western Association of Marine Labs, 2013

Student Support, Society for Integrative and Comparative Biology, 2013

Neil Richmond Scholarship, University of Oregon, 2011

Dean J. Alan Ross Travel Fund Award, Western Washington University, 2010

Huxley College Small Grant, Western Washington University, 2010

Research Sponsored Programs Award, Western Washington University, 2009

PUBLICATIONS:

Hiebert TC, BA Butler, and AL Shanks (*Forthcoming*) Oregon Estuarine Invertebrates: Rudy's Illustrated Guide to 140 Common Species, 3rd Edition. http://hdl.handle.net/1794/18839. Oregon Institute of Marine Biology, Charleston, OR.

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TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

Figure Page

Figure Page

Figure Page

Figure Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Recent estimates suggest that as much as 90% of marine species remain undescribed (Mora et al. 2011). While a large portion of unknown diversity lies in regions of especially high diversity and those that are poorly characterized, recent DNA-barcoding studies illustrate that even in areas where the fauna is thought to be well known the fraction of undescribed diversity remains high (e.g., Barber and Boyce 2006). In this dissertation, we concentrate on the previously unknown diversity of the phylum Nemertea, but our methods could be employed to reveal new diversity in other marine invertebrate phyla. The nemertean fauna in our study region (see below) was thought to be among the best studied in the world, and thus least likely to offer any biodiversity surprises or discoveries. Yet, this study, whereby we utilize DNA sequence data of both planktonic larvae and benthic adults, suggests that the real species-level diversity is at least double that which was previously known.

Hidden diversity revealed in a supposedly well-studied region

The northeast Pacific region extends along the coast from the Arctic to the southern tip of Baja California and includes, at is farthest reaches, northern (cold water) and southern (warm water) provinces. Between these two provinces is a temperate region that includes the Oregonian Biogeographic Province extending from Point Conception in California to as far north as the Gulf of Alaska (Briggs 1974; Niesen 2007). The Oregon Institute of Marine Biology (OIMB), on the southern Oregon coast, is situated in the heart of the Oregonian Biogeographic Province. This region is characterized by various intertidal habitats and diverse marine fauna with a high degree of endemism (Niesen 2007). The most comprehensive guide to intertidal invertebrates in this region to date is *The Light and Smith Manual: Intertidal Invertebrates from central California to Oregon, 4th Edition* edited by Carlton in 2007. Although this guide does not claim to extend to the northern reaches of the Oregonian Biogeographic Province, many of the species therein have ranges that include the entire northeast Pacific coast.

The area of focus for this dissertation is the Oregonian Biogeographic Province. The majority of our samples were gathered on the southern Oregon coast, in a relatively small area from Coos Bay (43.3445˚N, 124.3215˚W) to Cape Arago (43.3096˚N, 124.3991˚W), near the OIMB. In this area we uncovered an extraordinary amount of new diversity that suggests that actual diversity in the entire Oregonian Biogeographic Province or northeast Pacific is even greater. Additional samples analyzed in this dissertation were collected north and south of OIMB within the Oregonian Province (including northern California, Northern Oregon, Washington, and Alaska), as well as other parts of the world (e.g., Sea of Okhotsk, Russia; Victoria, Australia; Panama).

Prior to this study, the nemertean fauna along the entire northeast Pacific was considered among the best characterized in the world, with several monographs focussing on it (Stimpson 1857; Coe 1899, 1901, 1904, 1905, 1940, 1943; Griffin 1898; Corrêa 1964). Interestingly, although Oregon is in the center of this biogeographic region, only one study of nemertean diversity actually included a site from the Oregon coast (Sunset Bay, Charleston, Oregon, Corrêa 1964). Instead, most species reports are based on findings from adjacent geographic regions (e.g., in Southern California or Alaska). In the most recent compilation, the intertidal nemertean fauna from central California to Oregon included 65 species (Roe et al. 2007). This dissertation shows that species diversity for this phylum on the southern Oregon coast alone is almost double that number (up to 113 species).

Attempts to identify larvae with DNA barcoding reveal cryptic diversity

Most benthic marine invertebrates have a biphasic life cycle with a planktonic larval stage. The larvae often greatly outnumber adults, e.g., in free-spawning species each female may produce thousands to millions of free-swimming planktonic larvae per spawning event (Young et al. 2002). The larvae also may have long pelagic duration, and have a distinctly different morphology from adults, yet species descriptions and

biodiversity assessments are typically based on adult morphology. Since the development of most invertebrate species is not yet known, most larvae cannot currently be identified to species using morphology alone. Identifying larvae is important because larvae connect populations of marine invertebrates in both space and time (Cowen and Sponaugle 2009). 'DNA barcoding' (Hebert et al. 2003; Bucklin et al. 2011) provides a much needed short-cut for identification of larval stages. But it also has another unexpected benefit: the planktonic environment provides an additional habitat to sample diversity, and some species are more commonly encountered as larvae than as benthic adults (e.g., rare or subtidal species). Thus DNA barcoding of larvae and adults uncovers a greater species diversity than sampling adults alone.

Of the 65 intertidal nemertean species thought to occur on the Oregon coast, most are expected to have planktonic larvae and, despite their sometimes distinct morphology, the larvae of very few species could be identified prior to this study (Johnson 2001). The development of only a handful of northeastern Pacific nemertean species was known (e.g., *Carcinonemertes epialti*, Roe 1979; *Tubulanus polymorphus*, *Amphiporus formidabilis*, Stricker 1987; *Pantinonemertes californiensis*, Roe 1993; *Paranemertes peregrina*, Maslakova and von Döhren 2009; *Micrura alaskensis,* Maslakova 2010a; *Pantinonemertes californiensis*, L. Hiebert et al. 2010) and partial larval descriptions were available for some additional species, mostly from other parts of the world (e.g., Desor 1848; Bürger 1895; Coe 1899; Wilson 1900; Salensky 1912; Schmidt 1930; Dawydoff 1940; Iwata 1958; Cantell 1969; Stricker 1987; Schwartz and Norenburg 2005; Maslakova 2010a, 2010b; Maslakova and von Dassow 2012; T. Hiebert et al. 2013). As the adult nemertean fauna in this region was believed to be well known and the larval fauna was not, the initial goal of this project was to connect planktonic larvae of local species to their respective adults through DNA barcoding for the purposes of larval identification. While we successfully identified the larvae of over 30 species, these efforts also unexpectedly revealed that the local nemertean diversity was underestimated by at least 50%.

The use of DNA sequence data for species delimitation has been debated (Sites and Marshall 2003; Blaxter 2004; DeSalle et al. 2005; Rubinoff et al. 2006), however, there is considerable evidence that it is an excellent tool for species identification, and also for revealing previously undetected diversity (e.g., Hebert et al. 2004a; Fukami et al. 2004; Bickford et al. 2006; Hyde et al. 2008; Schulze et al. 2012). In this dissertation, DNA barcoding uncovered new and cryptic species in two ways. First, attempts to identify larvae require an extensive reference database of adult sequences, and by compiling and analyzing these data we often discovered multiple morphologically similar or indistinguishable species that were thought to represent just one species. In these cases, DNA barcoding prompted a more thorough investigation into additional characters and an integrative approach to species descriptions. The second way in which we identified previously unknown diversity is by identifying "orphan larvae" through DNA barcoding. Despite assembling an extensive database of reference sequences from morphologically identifiable adults, many of the locally collected larvae could not be matched to any known species. Instead, these larvae represent species that we have yet to find as benthic adults. Intriguingly, there are also several species expected to have a planktonic stage that we find as adults and have yet to find as planktonic larvae. This emphasizes the importance of what we refer to as the "life-history approach" to biodiversity assessments:using both adult and larval stages to sample diversity.

DNA sequence data renew nemertean phylogenetics and larval evolution

Adult morphological characters are often not sufficient for differentiating between closely related species, especially in soft-bodied groups like nemerteans which possess few morphological characteristics that can be objectively measured or described (Schwartz and Norenburg 2001; Sundberg and Strand 2010). Nemertean taxonomy is notoriously problematic, with species and genus descriptions based on combinations of non-unique or plesiomorphic characters, resulting in phylogenies that lack resolution (e.g., Maslakova and Norenburg 2001). Molecular phylogenies, on the other hand, may find clades that are well-supported but lack associated morphological synapomorphies.

By assembling DNA sequence data from nemertean larvae and using these to build molecular phylogenies, we were able to identify certain characters of larval morphology, that work as synapomorphies and can offer morphological support to clades of closely related species (e.g., genera). This helps to define new (monophyletic) nemertean genera and aid in revising large traditionally established non-monophyletic 'mega-genera' (such as *Micrura*, *Cerebratulus, Lineus*). Finally, by sampling and identifying larvae regularly and frequently over the period of several years, we found new larval forms, including many previously unknown species of pilidiophoran nemerteans that possess lecithotrophic larvae. By placing these discoveries into a phylogenetic context we were able to show convergent evolution of lecithotrophy, and larval pigment spots in this group. Thus, what began as an exercise in larval identification by DNA barcoding became an assessment of species diversity, and ultimately lead to a better understanding of the phylogeny, systematics, and larval evolution of an entire phylum.

Dissertation content

This study advances our knowledge of a fascinating but understudied phylum of marine invertebrates and, more generally, emphasizes how much there is still to learn about marine biodiversity. The results of our study illustrate that theoretical estimates of the vast hidden eukaryotic diversity in the marine environment (e.g., Mora et al. 2011; Appeltans et al. 2012) are not far from truth, given how much nemertean diversity we have found in a region that was thought to be well characterized. Finally, our study emphasizes an especially useful approach to biodiversity assessments, one that combines DNA barcoding of adults and larval stages adapted to and occupying different ecological niches. Chapter II, an updated assessment of nemertean diversity of the Oregonian Biogeographic Province, highlights the power of this two-pronged approach (using larval and adult stages) to assessments of diversity of taxa with bi-phasic life history (in preparation for *Zootaxa*). Chapter III exemplifies the usefulness of the DNA barcoding approach for the discovery of cryptic species, and highlights the potential for larval morphological characters to offer synapomorphies for above-species level groups (e.g.,

genera) identified by molecular phylogenies. This chapter, comprising a formal description of four species, the revision of another species, and the designation of a new nemertean genus, was published in *Zoological Science* (Hiebert and Maslakova 2015a). Chapters IV and V demonstrate the utility of DNA barcoding in identifying larval stages as well as in uncovering hidden diversity. Chapter IV is a comprehensive identification guide for nemertean larvae collected in southern Oregon. It is currently available online (www.nemerteanlarvalid.com) and the associated manuscript (coauthored with S Maslakova) will be submitted to *ZooKeys*. Chapter V, revealing the identity of a mysterious larval form that was described over 100 years earlier, was published in *Invertebrate Biology* (Hiebert et al. 2013). Chapter VI, a description of larval development in two nemertean species from Oregon, was published in *Biological Bulletin* (Hiebert and Maslakova 2015c). Finally, Chapter VII demonstrates how DNA barcoding helps to place larvae into a phylogenetic context and to address questions about the evolution of larval forms (in preparation for the *Journal of Zoological Systematics and Evolutionary Research)*.

Bridge to Chapter II

In Chapter I, we describe what was previously known about nemertean adult and larval fauna in the Oregonian Biogeographic Province. We introduce our project that began as an exercise in DNA barcoding for larval identification and became an updated assessment of diversity. In a region that was previously believed to be well described with respect to the phylum Nemertea, we uncovered a significant amount of new diversity by targeting both adult and larval stages. We suggest that this methodology would similarly uncover new diversity for other phyla with bi-phasic life histories in any part of the world. In Chapter II, we present an updated synopsis of nemertean fauna within the Oregonian Biogeographic Province, and briefly describe the new species we detected during the period of 2008–2015. This synopsis includes information on the total number of species we've observed (as larvae, adults or in both life-history stages) and adult morphological characters that can be used for morphological identification. (For larval identification

guide with characters of larval morphology, see Chapter IV). The use of DNA sequence data for species delimitation is discussed following an analysis of over 900 sequences. These data provide the most comprehensive evaluation of intra- and interspecific divergence values for nemertean species to date and offer basis for DNA-based species delimitation in subsequent Chapters III and IV.

CHAPTER II
AN ASSESSMENT OF NEMERTEAN DIVERSITY

The second chapter of this dissertation will be submitted to *Zootaxa* and is co-authored with S Maslakova. This assessment of nemertean diversity is a project that began when S Maslakova arrived at OIMB in 2008. Thus, she contributed with many early observations, collections, and associated molecular analyses, and editing this manuscript. During my time at OIMB (2011–2015), I contributed to this manuscript by collecting specimens, making observations, carrying out most of the molecular benchwork and analyses, and submitting all finalized sequence data to GenBank. I drafted the manuscript and prepared all figures.

INTRODUCTION

DNA barcoding as a tool for uncovering cryptic diversity

The majority of marine eukaryotic diversity remains undescribed (Mora et al. 2011; Appeltans et al. 2012). As species are going extinct before they can be described (Novacek and Cleland 2001; Bellwood et al. 2004; Barber and Boyce 2006), documenting this diversity should be a priority. However, precise estimates of undescribed diversity vary greatly (Mora et al. 2011; Appeltans et al. 2012) and establishing the size of the task at hand is paramount. Here, we show that much better estimates of species-level diversity can be achieved by sampling and connecting life-history stages using DNA sequence data. Although the use of 'DNA barcoding' (Hebert et al. 2003) for species identification and delimitation has been debated (Sites and Marshall 2003; Blaxter 2004; DeSalle et al. 2005; Rubinoff et al. 2006), there is considerable evidence that DNA sequence data can reveal previously cryptic species (e.g., Hebert et al. 2004; Fukami et al. 2004; Bickford et al. 2006; Hyde et al. 2008; Schulze et al. 2012; Hiebert and Maslakova 2015a) and hidden diversity (e.g., Barber and Boyce 2006).

Nemertean life history and diversity

The Nemertea comprises a small phylum of soft-bodied worms characterized by an eversible proboscis housed in a fluid-filled rhynchocoel that is encircled anteriorly by the brain. There are 1,285 species currently known and described worldwide (Kajihara et al. 2008), but estimates of the real phylum-level diversity range from 1,985–2,685 species (Appeltans et al. 2012). Nemerteans are almost exclusively marine (save for a few fresh-water and terrestrial species) where they occur in all habitats from high intertidal zones to the deep-sea benthos, and one small group adopted a holopelagic existence. Most benthic nemerteans exhibit a biphasic life-history with planktonic larvae of various types and pelagic durations. Adults either broadcast spawn gametes that are fertilized in the water column or lay mucous cocoons from which microscopic free-swimming larvae emerge. Each broadcast spawning adult has the capacity to produce thousands to millions of planktonic larvae (Young et al. 2002). Thus, the chances of encountering some species (e.g., subtidal or rare) are often greater among plankton samples, as larvae, than within the sediment. Yet because species descriptions and keys are based largely on adult morphology and the development of few species is known, few nemertean larvae can be currently identified (e.g., Johnson 2001). In this study we used traditional embryology (when reproductive adults of both sexes were available) and DNA barcoding to connect nemertean adults and larvae (see also Chapter IV) and, in doing so, discovered many new and cryptic species.

The nemertean fauna of the northeast Pacific (NEP) has been studied and characterized by several prominent biologists (Stimpson 1857; Coe 1895, 1901, 1904, 1905, 1940, 1943; Griffin 1898; Corrêa 1964; Roe et al. 2007) and is considered among the best known in the world. However, like many early diversity assessments, previous nemertean assessments are based on few collecting trips to a handful of sites (collection sites mostly include locations in Alaska and California, and very few in Oregon or Washington). The Oregon Institute of Marine Biology (OIMB) is situated on the southern Oregon coast at the mouth of one of the largest estuaries (Coos Bay) on the West coast of North America,

offering unprecedented access to a diversity of habitats from mudflats to protected coves to open coast rocky shore. The most recent estimate of nemertean diversity in NEP is by Roe et al. (2007), which covers a region from Central California to Oregon within the Oregonian Biogeographic Province (OBP), and comprises 65 intertidal species. Since 2008, we have carried out regular sampling of nemertean adults and planktonic larvae (mostly in southern Oregon), and connected the two faunas using DNA sequence data. Here, we provide an updated synopsis of nemertean fauna and report many undescribed species, cryptic species complexes, and previously described species (genera and families) that are new for NEP region. It is noteworthy that this synopsis is derived from sampling intertidally in a small region within the OBP, which is nested within the NEP (see Chapter I). Thus, our findings can only hint toward the real intertidal diversity in these larger biogeographic regions. For example, there are many species known only to Alaska or southern California and a number of described and undescribed pelagic and subtidal species reported from this region, which are not included in this assessment. Finally, by barcoding nearly all specimens encountered, including many closely related species, we are able to provide new estimates of intra- and interspecific divergence values for 16S and COI gene regions for nemertean species.

Species delimitation in nemerteans

The best practice for species delimitation is an integrative approach that combines all available evidence including morphological, geographic, and habitat characters, phylogenetic evidence (e.g., reciprocal monophyly, haplotype networks), and methods based on DNA sequence divergence, e.g., presence of a distinct 'barcode gap' between the maximum intraspecific divergence and the minimum interspecific divergence values (e.g., Meyer and Paulay 2005; Barber and Boyce 2006; Chen et al. 2010; Mahon et al. 2010; Bucklin et al. 2011; Kvist et al. 2014; Leasi and Norenburg 2014). We illustrate this integrative approach in Chapter III (Hiebert and Maslakova 2015a). Although 'DNA barcoding' alone is not sufficient for species delimitation it can be used as the first step to flag potentially new and cryptic species. However, the usefulness of this approach

depends on the accuracy of estimates of intra- and interspecific divergence values for 'barcoding gene regions' (e.g., COI and 16S) for a particular taxon.

Intraspecific divergence values – Phylogenetic inference from sequence divergence values can be accomplished with a variety of methods (see Van der Peer 2009). The observed portion (or percentage) of differences between two sequences, with no correction for multiple substitution events, is called the uncorrected p-distance. Prior estimates of intraspecific divergence values, as p-distances, for nemertean species for the COI gene region include those by Kvist et al. (2014) and Leasi and Norenburg (2014). Kvist et al. (2014) reported uncorrected p-distances, from a dataset including 137 putative species spanning all Nemertea, to range 0.0–20.87% (average = 1.14%), with 88% of intraspecific comparisons showing divergence of less than 2%. Leasi and Norenburg (2014) observed genetic distances within putative species in three nemertean genera (*Cephalothrix*, *Ototyphlonemertes*, and *Tetrastemma*, respectively) to be 0.15–2.13% (mean = 0.60%), 0.17–6.59% (mean = 1.37%), 0.17–1.86% (mean = 0.69%). Chen et al. (2010) calculated COI sequence divergence using the K2P evolutionary model, which corrects for multiple substitution events at two different rates (transitions and transversions), and found mean intraspecific values to be less than 1.5% for the genus *Cephalothrix* (minimum 0.126%). In many cases, these maximum (and average) values are likely inflated because they are based on Genbank sequences (where species are sometimes misidentified and cryptic species are often listed under the same name). Below we attempt to offer improved estimates by calculating divergence values for species identified and delimited by us using an integrative approach.

Interspecific divergence values – Previous research suggests that the average interspecific divergence between congeneric nemertean species for the 16S rDNA gene region is 4.8% (uncorrected p-distance, Mahon et al. 2009). However, average values of interspecific divergence are not especially useful for species delimitation because closely related species certainly exhibit smaller divergences. Paulay et al. (2005) argued that minimal

interspecific values should be used instead. For the COI gene region, interspecific values calculated by Kvist et al. (2014) were 0.10–40.13% (average = 19.61%), with 99.6% of interspecific comparisons yielding genetic distances of over 10%. Likewise, Leasi and Norenburg (2014) determined those between species within the genera *Cephalothrix*, *Ototyphlonemertes*, and *Tetrastemma* to be 1.06–36.36% (mean = 15.85%), 9.80–22.47% (mean = 17.17%), and 1.67–20.59% (mean = 14.69%), respectively. Interspecific values for *Cephalothrix*, calculated as K2P distances by Chen et al. (2010), were greater than 4.3% (maximum 32.0%). Interspecific divergences from congeneric species can also offer inflated values, when non-monophyletic genera are included in the analysis. A large fraction of described nemertean species belong to the genera that have been shown to be non-monophyletic, e.g., the so-called 'mega-genera' *Lineus, Cerebratulus, Micrura, Tetrastemma* and *Amphiporus* (Sundberg and Saur 1998; Schwartz and Norenburg 2001; Sundberg et al. 2001; Strand and Sundberg 2005; Schwartz 2009), thus prior estimates of interspecific divergences, which included species from these mega-genera, are almost certainly inflated (e.g., Kvist et al. 2014). Below we offer new estimates of average and minimum interspecific divergence values for COI and 16S gene regions in nemerteans, using congeneric species within well-defined monophyletic genera.

METHODS

Specimen collection

Adult and larval nemerteans were collected over the period of several years (2008–2015, collecting permit numbers: 18512, 18586, 18664, 19353, CF-14081). Most adult nemerteans were collected by hand or with the aid of a shovel in rocky intertidal or estuarine mudflat habitats within 10 miles of the OIMB (43.344˚N, 124.328˚W) and examined at the OIMB in Charleston, OR. Additional specimens were collected from other sites on the West Coast of North America (including northern California, northern Oregon, Washington and Alaska). Live worms were kept in 150 ml glass dishes submerged in a sea table with running seawater at ambient sea temperature (9–12˚C). Worms were photographed using a Leica DFC400 digital camera mounted to a Leica

MZ10F dissecting microscope and accompanying software (Leica Application Suite V3.6). Tissue samples from all examined specimens were preserved for histology, DNA extraction or both, cataloged and kept at OIMB until further processing.

Larvae were collected using a 0.5 m diameter 153 µm plankton net (SeaGear) in Coos Bay, from the Charleston marina docks or by boat in the Charleston Channel. Plankton tows were conducted sporadically throughout the year in 2008–2011, and 2013–2015 and regularly (at least weekly) during 2011–2013. All nemertean larvae were isolated by hand, photographed and cryopreserved (-80 ˚C) live in a small volume (< 10 µl) of filtered sea water (FSW, 0.45 µm). Larvae collected at early developmental stages were sometimes maintained in bowls of FSW at ambient sea temperature (e.g., 9–12˚C) and planktotrophic species were fed *Rhodomonas lens* (Pascher and Ruttner, CCMP739) for up to 10 weeks to observe morphological characteristics present at later stages in development or after metamorphosis. The identity of all larvae was confirmed using DNA sequence data as described below.

Additional nemertean larvae were recently collected and identified with DNA sequence data by students in OIMB's Marine Molecular Biology course Fall 2015 taught by Maslakova (N Moss and K Plummer, unpublished). Sequences of the species *Paranemertes* sp. 3, *Cephalothrix* cf. *spiralis* (larva), *Tubulanus* sp. 3, and *Lineus* sp. 3 (for GenBank accession numbers, see Appendices A–C) were included in phylogenetic analyses and our overall assessment of diversity, but were not included in calculations of genetic distance (see below).

DNA extraction, PCR amplification, and sequence editing

DNA extraction and subsequent molecular work on adult and larval tissue samples was carried out at the OIMB. Two small (2 x 2 mm) pieces of tissue were preserved from each adult individual (one cryopreserved and kept at -80˚C, and another immersed in 80% EtOH and kept at -20˚C). DNA extraction from adult tissue was carried out using a

DNEasy Blood and Tissue Kit (Qiagen) or Wizard SV Genomic DNA Purification System (Promega). Tissue lysis occurred in a solution of Nuclei Lysis solution, 0.5 M EDTA and proteinase K (20 mg ml-1) at 56˚C for ~ 8–12 hours. PCR-quality DNA was obtained from whole larvae using Chelex matrix (InstaGene, BioRad) with initial incubation at 56˚C for 30 min followed by an 8 min incubation at 98˚C. We amplified two 'barcoding' regions of mitochondrial genes 16S ribosomal DNA (~460 bp, 16S), cytochrome c oxidase subunit I (658 bp, COI). PCR amplification was carried out with previously publisehd primers: 16SARL [5' CGCCTGTTTATCAAAAACAT 3'] and 16S BRH [5' CCGGTCTGAACTCAGATCACGT 3'] (Palumbi et al. 1991) for 16S; LCO 1490 [5' GGTCAACAAATCATAAAGATATTGG 3'] and HCO 2198 [5' TAAACTTCAGGGTGACCAAAAAATCA 3'] (Folmer et al. 1994) for COI. Occasionally, higher quality amplification was achieved by pairing nemertean-specific reverse primers for the 16S and COI gene regions (16SKr [5' AATAGATAGAAACCAACCTGGC 3'], COIDr [5' GAGAAATAATACCAAAACCAGG 3'] (Norenburg, unpublished)) with corresponding universal forward primers (16SARL and LCO1490, respectively). PCR thermocycling was carried out in a 20 µl reaction using 1–8 µls of unquantified DNA template, 1U using GoTaq DNA Polymerase (Promega), and XuM of each primer with the following parameters: 95˚C initial denaturation for 2 min, 35 cycles of 95˚C for 40 s, 45-55˚C for 40 s and a 60 s extension at 72˚C. Following the last cycle there was an additional 2 minutes at 72˚C for final extension, after which products were stored at 4˚C. PCR products were purified using Wizard SV Gel and PCR Cleanup kit (Promega) and sequenced (Sequetech Inc, Mountain View, CA) in both directions using forward and reverse primers to maximize sequence length and accuracy. Sequences were trimmed to remove primers, assembled in contigs, proofread for quality using Geneious v.7.0.6 or Codon Code Aligner v. 3.7.1 (Codon Code Corp, MA) and deposited in Genbank (see Appendices A–C for accession numbers).

Sequence alignment and phylogenetic analysis

Our molecular analysis is based on 558 16S and 463 COI sequences, each from a unique individual collected in the NEP, by us, in addition to relevant sequences from GenBank (for accession numbers see Appendices A–D). We carried out phylogenetic analyses to compare our sequences with each other and to those obtained from GenBank, and determine the number and identity of species. Nemertean sequences were sorted by order-level taxon (Hoplonemertea, Palaeonemertea and Pilidiophora) and sequences were aligned using ClustalW as implemented by Geneious v 7.0.6 (Biomatters Ltd) with gap opening and extension costs set to default (15 and 6.66, respectively). The length of alignments were 697, 658, and 697 bp for COI and 590, 619, and 516 bp for 16S for palaeo-, pilidio-, and hoplonemerteans, respectively. Bayesian phylogenetic analyses were conducted in MrBayes v 3.2.1 (Ronquist et al. 2012) where evolutionary model parameters for each taxon and gene region were determined using jModel-Test v 2.1 (Posada 2008). Markov chain Monte Carlo (MCMC) parameters were set to default (four chains run for 1,000,000 generations, sampling every 1000 generations with first 25% discarded as burn-in). Maximum Likelihood Phylogenetic analyses on individual datasets (to maximize species representation) were carried out using PhyML v. 3.0 (Guindon et al., 2010) and clade support was estimated using 1,000 bootstrap replicates (Felsenstein 1985), using the same evolutionary models as our Bayesian analyses. For palaeonemertean and hoplonemertean sequences, the General Time Reversible (GTR) evolutionary model was determined the ideal evolutionary model for 16S and COI gene regions. For the Pilidiophora, the GTR was also the best model for the 16S gene region, while Timura-Nei (TN93) was the best model for pilidiophoran COI sequence data. Gene tree topologies were viewed in FigTree v 1.3.1 (Rambaut 2009) or Geneious (Biomatters Ltd).

Species delimitation and barcoding gap detection

Sequence divergence values were calculated as uncorrected p-distances (and converted to percentage) from pairwise sequence alignments using Geneious. We used an integrative

approach to determine species boundaries combining morphological characters, sequence divergence, and phylogenetic information (e.g., reciprocal monophyly, statistical parsimony; see also Chapter III). We considered two individuals as belonging to different species if they exhibited 1) morphological disparity, 2) sequence divergences near or above those of closely related and well-defined species, 3) congruence across various phylogenetic analyses and gene regions (i.e. reciprocal monophyly). We also compared our results with those obtained by Automatic Barcode Gap Discovery analysis (ABGD, Puillandre et al. 2012) using default parameters (P_{min} 0.001, P_{max} 0.1, Steps 10, Gap width 0.05–1.5 and JC69 distances). ABGD analysis was carried out using the same alignments as in our phylogenetic analyses above.

RESULTS & DISCUSSION

Molecular analyses

Sixty-five intertidal nemertean species are currently reported from central California to Oregon (Roe et al. 2007). Our assessment finds up to 113 species (including species whose presence we have not been able to confirm directly), 25 of which are represented in our analyses by larvae only, 29 by adults only and 37 by both adult and larval stages. We lack sequence data for 22 species reported to occur in the region (Table 2.1). Furthermore, we have yet to find some species that are reported to occur in the region and, while we have DNA sequence data from these species (black "X" in Table 2.1), their presence remains unconfirmed (e.g., *Oerstedia dorsalis, Lineus ruber*). Our assessment defines species based on an integrative approach, including phylogenetic analysis, intra- and interspecific divergence values, and morphology (see below). Occasionally, the number of putative species suggested by the two gene regions (16S and COI) differs because 16S is more conserved than COI, (e.g., *Paranemertes peregrina*, *P*. sp. 1 and sp. 2) and we discuss discrepancies in the associated text.

Phylogenetic analysis – Both 16S and COI phylogenetic analyses find 19 palaeonemertean species (red and black text, Fig. 2.1). The addition of palaeonemertean

Table 2.1. Nemertean diversity by species, divided into order level taxa, with new diversity shown in bold text. Species for which we have adult and/or larval sequence data are indicated with an "X"; red X's are adults and/or larval samples that we have collected, while black X's are sequences provided by other researchers.

		adult	larva
Palaeonemertea			
Tubulanus Renier, 1804	*T. polymorphus* Renier, 1804	X	X
	T. sexlineatus Renier, 1804	X	X
	T. cingulatus Griffin, 1898		
	T. capistratus Coe, 1904		
	T. pellucidus Coe, 1901	X	
	***Tubulanus* sp. 1**		X
	***Tubulanus* sp. 2**		X
	***Tubulanus* sp. 3**		X
Carinoma Oudemans, 1885	*C. mutabilis* Griffin, 1898	X	X
	***C. hamanako* Kajihara et al., 2011**	X	X
	***C.* sp. "white"**	X	X
	***C.* sp. "yellowback"**	X	X
	***C.* sp. 5**		X
Carinina Hubrecht, 1885	***C.* sp. "chocolate"**	X	X
Cephalothrix Wijnhoff, 1910	*C. major* Coe, 1930	X	
	C. spiralis Coe, 1930	X	X
	C. cf. *spiralis*	X	X
	***Cephalothrix* sp. 1**		X
	***Cephalothrix* sp. 2**		X
	***Cephalothrix* sp. 3**		X
	***Cephalothrix* sp. 4**		X
Carinomella Coe, 1905	***C. lactea* Coe, 1905**		
Pilidiophora			
Hubrechtella Bergendal, 1902	***H. juliae* Chernyshev, 2003**	X	X

Heteronemertea			
	Lineidae gen. sp. “large eggs”	X	
	Heteronemertea gen. sp. 1		X
	Heteronemertea gen. sp. 2		X
	Heteronemertea gen. sp. 3		X
	Heteronemertea gen. sp. 5		X
	Heteronemertea gen. sp. 4		X
	Heteronemertea gen. sp. 6		X
Cerebratulus Renier, 1804	*C. albifrons* Coe, 1901	X	X
	C. californiensis Coe, 1905	X	X
	C. herculeus Coe, 1901	X	
	C. longiceps Coe, 1901	X	X
	C. cf. *marginatus* Renier, 1804	X	X
	C. montgomeryi Coe, 1901	X	
	C. occidentalis Coe, 1901		
	***C.* sp. “Sunset Bay”**	X	X
	***C.* sp. “pink proboscis”**	X	X
	***C.* sp. “spade head”**	X	X
	***Cerebratulus* sp. 1**		X
	***Cerebratulus* sp. 2**		X
***Maculaura* Hiebert and Maslakova, 2015a**	***M. alaskensis* Hiebert and Maslakova, 2015a**	X	X
	***M. aquilonia* Hiebert and Maslakova, 2015a**	X	X
	***M. cerebrosa* Hiebert and Maslakova, 2015a**	X	X
	***M. magna* Hiebert and Maslakova, 2015a**	X	X
	***M. oregonensis* Hiebert and Maslakova, 2015a**	X	
Micrura Ehrenberg, 1831	*M. coei* Coe, 1905		
	M. olivaris Coe, 1905		
	M. verrilli Coe, 1901	X	
	M. wilsoni Coe, 1904	X	X
	***M.* sp. “not coei”**	X	
	***M.* sp. “dark”**	X	X
	***M.* sp. “albocephala”**	X	X
	***M.* sp. 4**		X
	***M.* sp. 3**		X

Lineus Sowerby, 1806	*L. bilineatus* Renier, 1804	X	
	L. flavescens Coe, 1904	X	X
	L. pictifrons Coe, 1904		
	L. rubescens Coe, 1904	X	
	L. ruber Müller, 1774	X	
	L. torquatus Coe, 1901	X	
	L. viridis Müller, 1774	X	
	***L.* sp. "red"**	X	X
	***L.* sp. "crescent"**	X	X
	***Lineus* sp. 1**		X
	***Lineus* sp. 2**		X
Euborlasia Vaillant, 1890	*E. nigrocincta* Coe, 1940		
Ramphogordius Rathke, 1843	*R. sanguineus* Rathke, 1843	X	
Zygeupolia Thompson, 1900	*Z. rubens* Coe, 1895	X	
Baseodiscus Diesing, 1850	*B. punnetti* Coe, 1904	X	
Riserius Norenburg, 1993	*R. pugetensis* Norenburg, 1993	X	
	***Riserius* sp. "no eyes"**		X
	***Riserius* sp. "eyes"**		X
Hoplonemertea			
Carcinonemertes Coe, 1902	*C. errans* Wickham, 1978		X
	C. epialti Coe, 1902		
Emplectonema Stimpson, 1857	*E. buergeri* Coe, 1901	X	
	***Emplectonema* sp. 1**	X	X
Paranemertes Coe, 1901	*P. californica* Coe, 1904	X	X
	P. peregrina Coe, 1901	X	
	***P.* sp. 1**	X	X
	***P.* sp. 2**		X
	P. sanjuanensis Stricker, 1982	X	
Nemertopsis Bürger, 1895	*N. gracilis* Coe, 1904		
Oerstedia Quatrefages, 1846	*O. dorsalis* Abilgaard, 1806	X	

Ototyphlonemertes Diesing, 1863	*O. americana* Gerner, 1969		
	***Ototyphlonemertes* sp. 1**		X
Pantinonemertes Moore and Gibson, 1981	*P. californiensis* Gibson, Moore and Crandall, 1982	X	X
Malacobdella Blainville, 1827	*M. grossa* Müller, 1774	X	
	M. macomae Kozloff, 1991		
	M. minuta Coe, 1945		
	M. siliquae Kozloff, 1991	X	X
Tetrastemma Ehrenberg, 1828	*T. albidum* Coe, 1905	X	
	T. candidum Müller, 1774	X	
	T. nigrifrons Coe, 1904		
	T. quadrilineatum Coe, 1904		
	T. reticulatum Coe, 1904		
	T. signifer Coe, 1904		
	***T. bilineatum* Coe, 1904**	X	X
	***Tetrastemma* sp. 1**	X	
Poseidonemertes Kirsteuer, 1967	*P. collaris* Roe and Wickham, 1984	X	X
Gurjanovella Ushakov, 1926	***G. littoralis* Ushakov, 1926**	X	X
Zygonemertes Montgomeryi, 1897	*Z. albida* Coe, 1901		
	Z. thalassina Coe, 1901		
	***Zygonemertes* sp. 1**	X	X
Nipponnemertes Friedrich, 1968	*N. bimaculatus* Coe, 1901	X	X
Amphiporus Coe, 1940	*A. angulatus* Müller, 1774	X	
	A. cruentatus Verrill, 1879	X	
	A. flavescens Coe, 1905		
	A. formidabilis Griffin, 1898	X	
	A. imparispinosus Griffin, 1898	X	
	A. rubellus Coe, 1905		
	A. similis Coe, 1905		

species for which we lack sequence data, brings the estimated total number of palaeonemertean species in the area to 20–22 (Table 2.1). Our phylogenetic analyses finds 42 and 47 pilidiophoran species, for COI and 16S gene regions, respectively (Fig. 2.2). With five additional pilidiophoran species without available sequence data, the total pilidiophoran diversity is 49–52 species (Table 2.1). Finally, our 16S analysis suggests 21 hoplonemertean species, while our COI analysis suggests 25 species (Fig. 2.3). Including additional 14 species for which we lack sequence data, we suggest that there are at least 35–39 hoplonemertean species (Table 2.1).

Intraspecific divergence – Our divergence estimates come from species we believe to be well identified and high quality sequence data. We base our estimates on species for which we have two or more sequences: 59 species (16S) and 52 species (COI), and 401 individual sequences for COI (61 palaeonemertean, 273 pilidiophoran, and 67 hoplonemertean) and 497 for 16S (63 palaeonemertean, 384 pilidiophoran, and 50 hoplonemertean) (Appendices A–G). Intraspecific divergence values for the 16S gene region range from 0.0–3.5%, while COI intraspecific divergence values range from 0.0–11.3% (Table 2.2). However, most intraspecific divergence values species we delimited are < 2% for both gene regions (black horizontal bars, Fig. 2.4) and the average intraspecific divergence values (among palaeo-, hoplo-, and pilidiophorans) are 0.3–0.4% for the 16S gene region and 1.3–1.9% for COI (Table 2.2). Species contributing to the upper end of intraspecific range values may represent closely related cryptic species, or species in the process of further speciation. These cases are discussed in detail for each order-level taxon below. These intraspecific divergence values are similar to those previously reported for nemertean species (Chen et al. 2010; Kvist et al. 2014; Leasi and Norenburg 2014).

Interspecific divergence – To get a better estimate of minimal interspecific divergences for nemerteans we compared divergence values in three reasonably diverse but well-supported genera for each order-level taxon including palaeonemerteans *Tubulanus*,

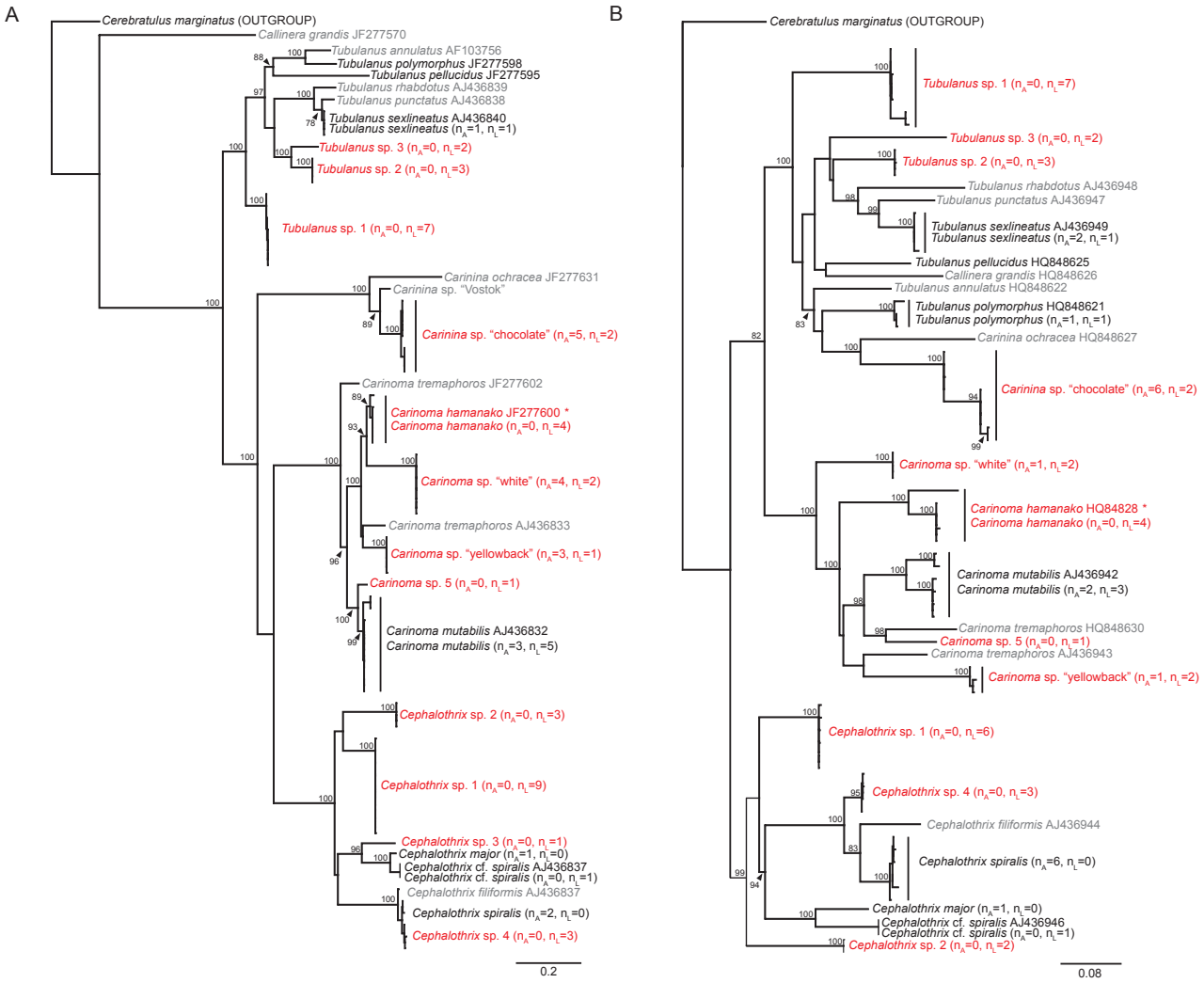

Figure 2.1. 16S (A) and COI (B) maximum likelihood phylogenies for the Palaeonemertea. Bayesian posterior probabilities (>70) are indicated above nodes. Species known or believed to occur in southern Oregon prior to this study (Roe et al. 2007) are shown in black text and new diversity in red text; note species previously described from other geographic regions are indicated with an asterisk. Species included from outside the NEP are shown in grey text.

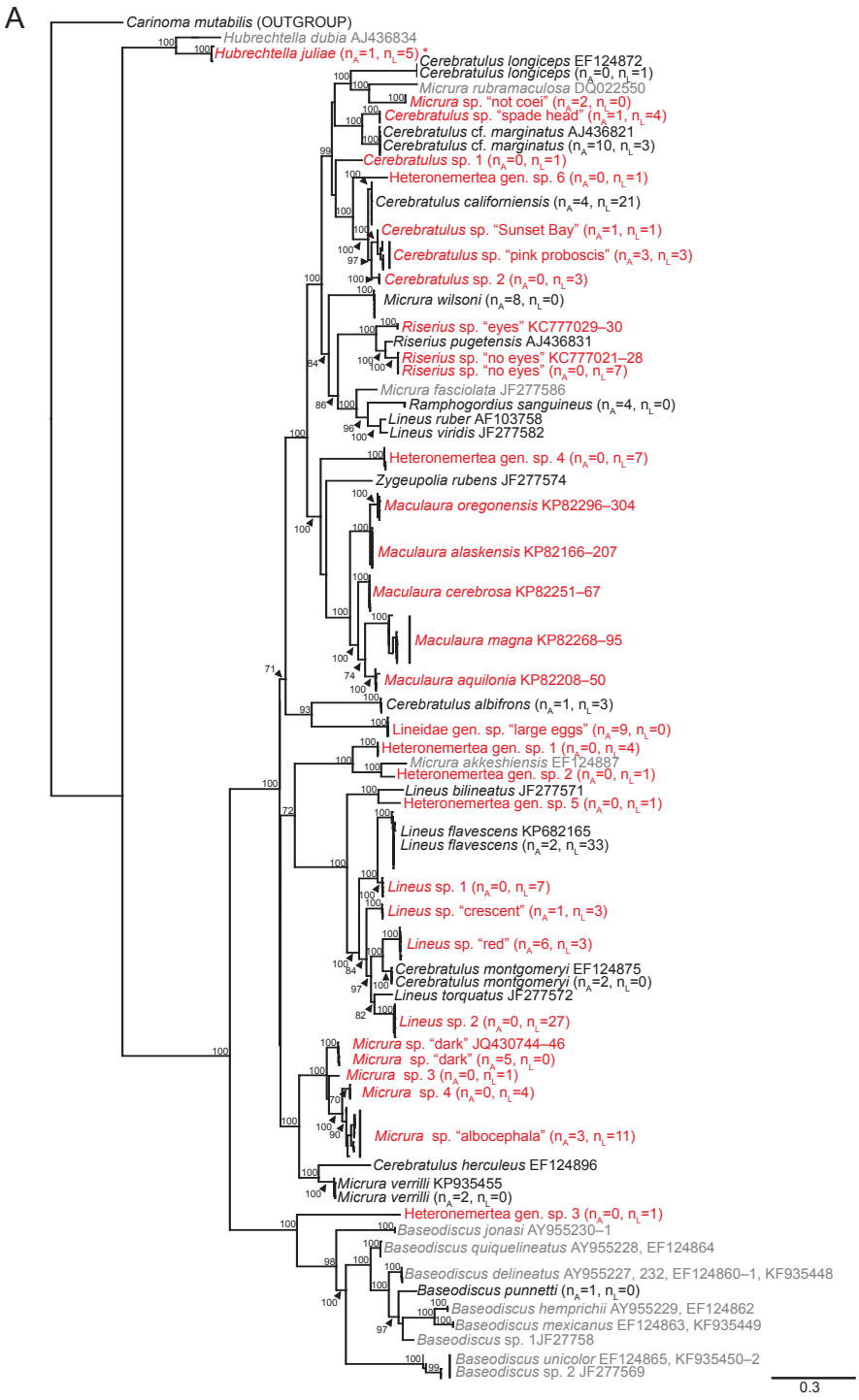

Figure 2.2A. 16S maximum likelihood phylogenies for the Pilidiophora. Bayesian posterior probabilities (>70) are indicated above nodes. Species known or believed to occur in southern Oregon prior to this study (Roe et al. 2007) are shown in black text and new diversity in blue text; note species previously described from other geographic regions are indicated with an asterisk. Species included from outside the NE Pacific are shown in grey text.

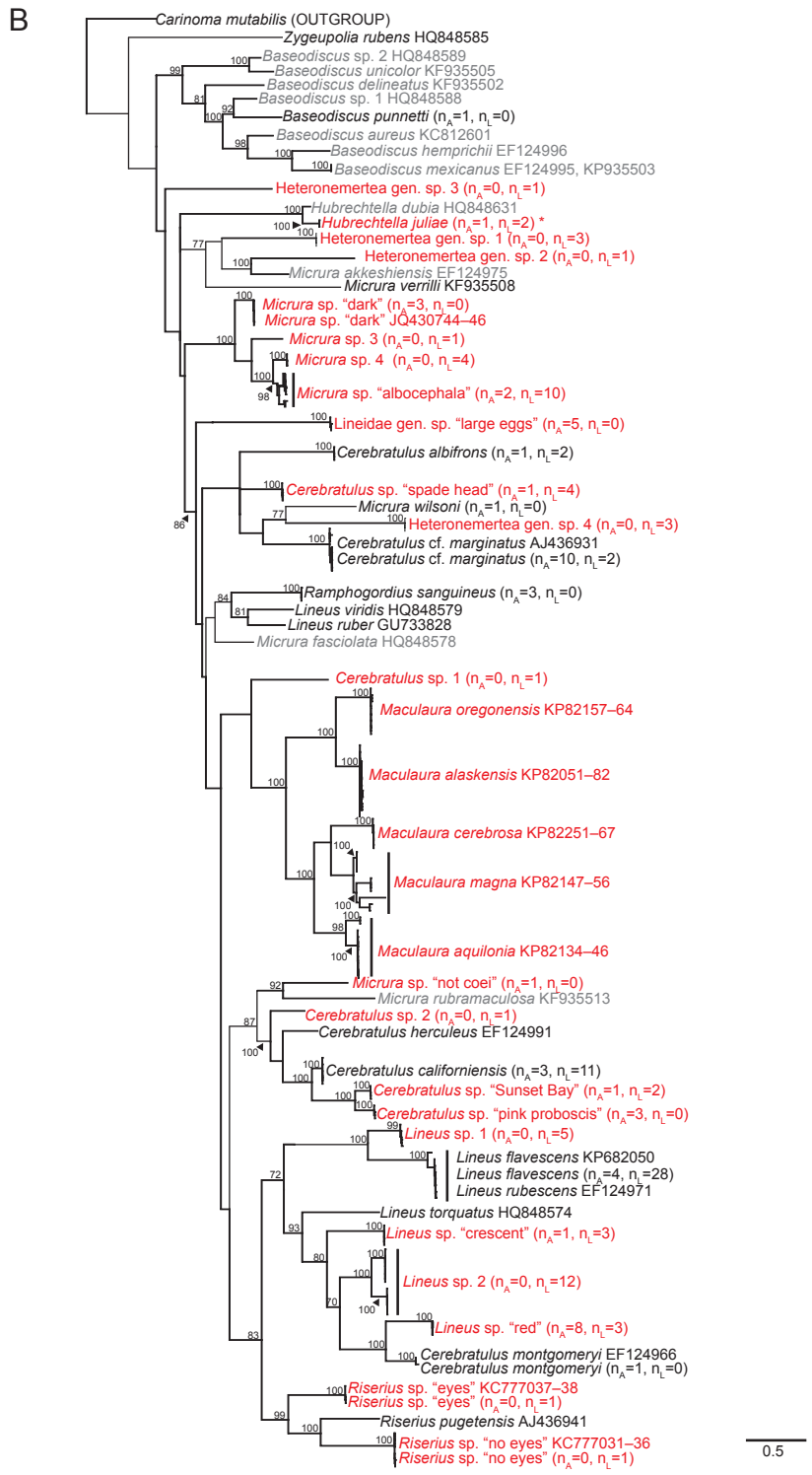

Figure 2.2B. COI maximum likelihood phylogenies for the Pilidiophora. Bayesian posterior probabilities (>70) are indicated above nodes. Species known or believed to occur in southern Oregon prior to this study (Roe et al. 2007) are shown in black text and new diversity in blue text; note species previously described from other geographic regions are indicated with an asterisk. Species included from outside the NE Pacific are shown in grey text.

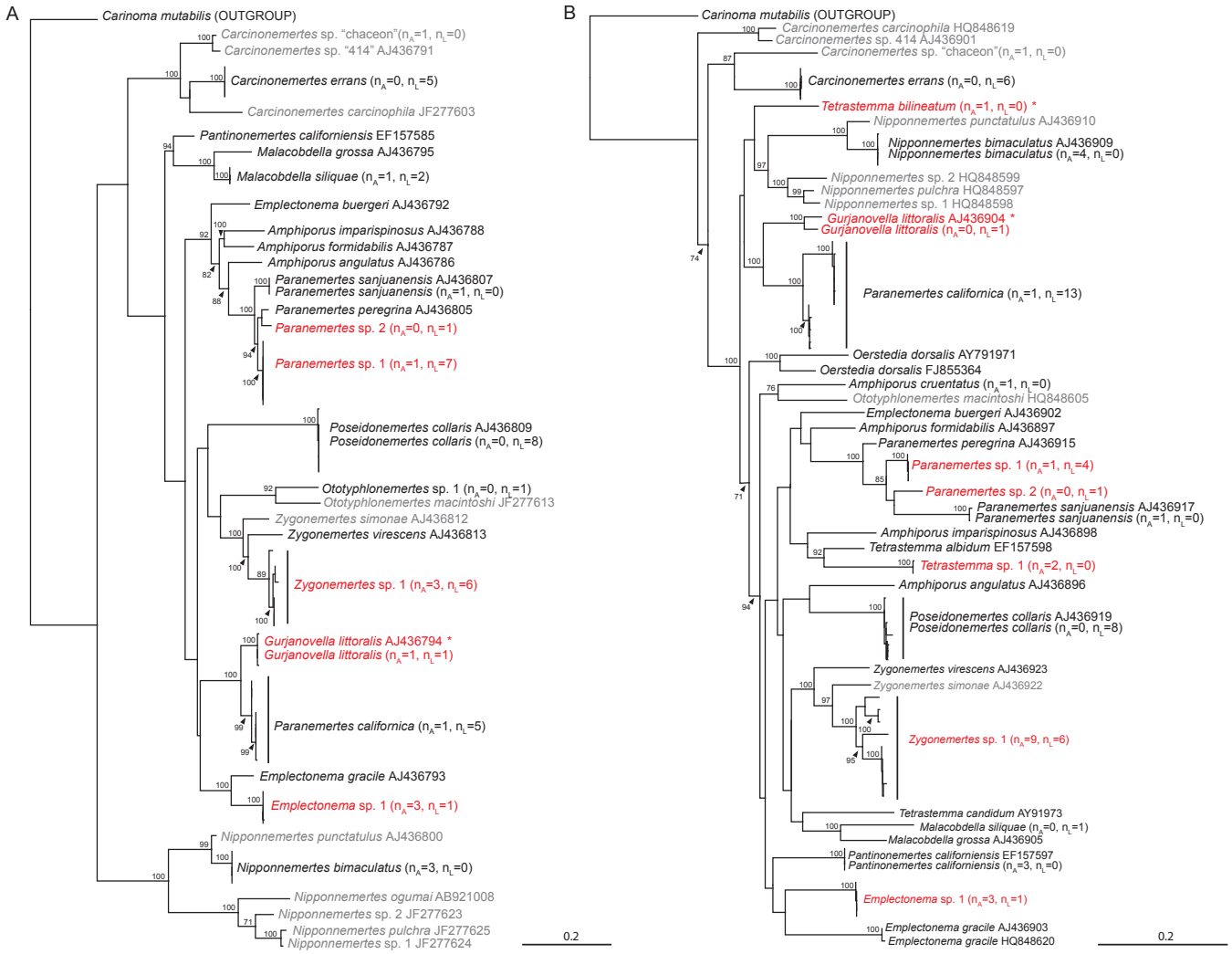

Figure 2.3. 16S (A) and COI (B) maximum likelihood phylogenies for the Hoplonemertea. Bayesian posterior probabilities (>70) are indicated above nodes. Species known or believed to occur in southern Oregon prior to this study (Roe et al. 2007) are shown in black text and new diversity in blue text; note species previously described from other geographic regions are indicated with an asterisk. Species included from outside the NE Pacific are shown in grey text.

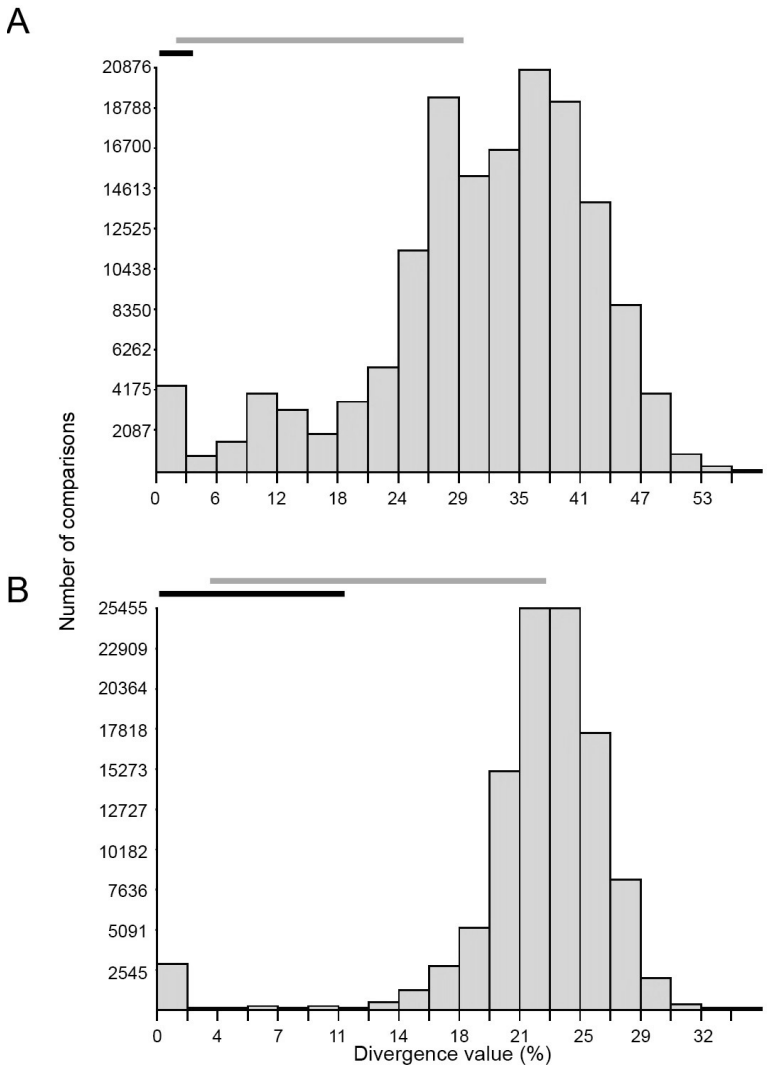

Figure 2.4. Results from ABGD software for 16S (A) and COI (B) sequence data for entire nemertean dataset. Histograms show divergence values (% divergence) for all species compared. Horizontal bars indicate intraspecific range (lower black bar) and interspecific range (upper grey bar) determined in this study. Note bimodal distribution for COI sequence data (B). Note that x and y axes are not consistent throughout.

Carinoma and *Carinina*, heteronemerteans *Baseodiscus*, *Riserius*, and *Maculaura*, and hoplonemerteans *Nipponnemertes*, *Zygonemertes* and *Carcinonemertes*. Although our COI phylogenetic analyses did not support the monophyly of some of these genera (e.g., *Tubulanus*, *Carcinonemertes*), they appear well-supported on our 16S phylogenies, molecular phylogenetic analyses done by others (Sundberg and Hylbom 1994; Sundberg and Saur 1998; Strand et al. 2005; Thollesson and Norenburg 2003; Andrade et al. 2012;

Table 2.2. Intra- and interspecific average values shown as percent divergence (upper number) and ranges (lower numbers) for 16S and COI sequence data.

	16S intraspecific	COI intraspecific		16S interspecific	COI interspecific
Palaeonemertea	0.4	1.9			
	(0.0 – 2.9)	(0.0 – 11.3)			
			Tubulanus	3.7 – 29.6	9.9 – 19.6
			Carinoma	4.3 – 16.4	11.7 – 18.0
			Carinina	7.7 – 17.7	16.4 – 18.3
Pilidiophora	0.3	1.3			
	(0.0 – 3.2)	(0.0 – 11.0)			
			Baseodiscus	2.9 – 26.5	9.0 – 19.6
			Riserius	6.7 – 12.1	15.6 – 21.1
			Maculaura	3.1 – 13.1	11.8 – 18.9
Hoplonemertea	0.4	1.5			
	(0.0 – 3.5)	(0.0 – 8.9)			
			Nipponnemertes	1.6 – 24.6	4.1 – 15.6
			Zygonemertes	9.0 – 11.2	10.5 – 15.3
			Carcinonemertes	2.2 – 14.1	4.9 – 16.5

Kvist et al. 2014; Hiebert and Maslakova 2015a) and possess clear morphological synapomorphies. Furthermore, we only used the well-supported species pairs to determine minimum interspecific divergence values (e.g., *T. punctatus* and *T. sexlineatus* (Fig. 2.1), *M. oregonensis* and *M. alaskensis* (Fig. 2.2), *N. punctatulus* and *N. bimaculatus* (Fig. 2.3)).

The minimum interspecific divergence for *Tubulanus* species was 3.7% (16S) and 9.9% (COI), *Carinoma* 4.3% (16S) and 11.7% (COI), *Carinina* 7.7% (16S) and 16.4% (COI). Among evaluated pilidiophoran genera, minimum interspecific divergences were 2.9%

(16S) and 9.0% (COI) for *Baseodiscus* species, 6.7% (16S) and 15.6% (COI) for *Riserius* species and 3.1% (16S) and 11.8% (COI) for *Maculaura* species. Minimum interspecific divergence values for hoplonemertean species was 1.6% (16S) and 4.1% (COI) for *Nipponnemertes* species, 9.0% (16S) and 10.5% (COI) for *Zygonemertes* species and 2.2% (16S) and 4.9% (COI) for *Carcinonemertes* species (Table 2.2). The comparatively low minimum interspecific divergence for the 16S gene region in *Nipponnemertes* is due to the species *N. pulchra* and *N.* sp 1. Likewise *Carcinonemertes carcinophila* and *C.* sp. 414 show very little divergence for the 16S gene region. Both pairs exhibit higher divergence for the COI gene region and it is currently unclear whether these are the same or different species. Of course, it is possible that species pairs (analyzed by us) are not true sister species – i.e., their sister species may not have been sampled yet, but we think these estimates better approximate the minimum interspecific divergence values for nemertean species for these gene regions, than previously published reports (e.g., Kvist et al. 2014), which likely included conspecific sequences incorrectly attributed to different species and thus suggested artificially low minimum interspecific values (0.1% for COI). Even better minimum interspecific estimates may be achieved by sampling additional well-supported species pairs, that are not necessarily from monophyletic genera. For example, divergence values between *Lineus ruber* and *L. viridis* are 6.3 and 12.7% and between *Amphiporus imparispinosus* and *A. formidabilis* are 12.6 and 13.0% for 16S and COI, respectively (see Appendices E–G).

Barcoding gap – The ABGD analysis found a clear barcoding gap for COI gene region including all sequences (between 2–12% divergence, Fig. 2.4B). However, this does not correspond to what we found with our additional delimitation methods (a combination of morphology, divergence values, and reciprocal monophyly, described above), where no clear gap was observed (grey and black horizontal bars, Fig. 2.4B). A barcoding gap was less definite for 16S sequence data using the ABGD analysis, due to few species with especially high intraspecific divergences (e.g., *Micrura* sp. “albocephala”, *Maculaura magna*, Appendix F) and a few with especially low interspecific divergences (e.g.,

Paranemertes californica and *Gurjanovella littoralis*, Appendix G). For the 16S gene region, the ABGD analysis was similar to our species delimitation (see below) and found ~112 species at a cutoff value of 4–6% (i.e., intraspecific variation less than 4%, interspecific divergence greater than 6%). For COI sequence data, ABGD tended to over-split our delimited taxa (see below) and found ~130 species with intraspecific variation less than 1.3% and interspecific divergence greater than 6%. Prior estimates of the barcoding gap among nemertean sequences using ABGD analyses found intraspecific variation to be approximately 2%, while interspecific divergences were ~8% for the COI gene region (Kvist et al. 2014).

DNA-based species delimitation – Our species delimitation estimates, based on a combination of the criteria described above, found 103–115 nemertean species. Our results suggest that nemertean species exhibit maximum intraspecific divergences of < 3.5% (16S) and < 11.3% (COI) and a minimum divergence between closely related congeners of 1.6% (16S) and 4.1% (COI). Although most species showed an intraspecific divergence of less than 2% for both gene regions, our results show some overlap between intra- and interspecific divergence ranges (see Fig. 2.4 and Table 2.2). Thus, our results suggest that DNA sequence divergences alone are not sufficient for delineating nemertean species. The following descriptions are based on our integrative approach to species delimitation.

Systematics

Illustrating all or even the most common nemertean species encountered by us is beyond the scope of this manuscript. Instead, we provide brief descriptions for all species and illustrate at least some of the newly discovered species.

Palaeonemertea

Nine intertidal and subtidal palaeonemertean species are currently reported from central California to Oregon (Roe et al. 2007). Our data suggest that there are 20–22 palaeonemertean species in this region (Table 2.1). Our phylogenetic analyses were based on alignments that were 527 bp (16S) and 658 (COI) in length comprising 78 (16S) and 74 (COI) sequences from adult and larval specimens (for GenBank accession numbers Appendices A, D). Most of these specimens are NEP species, but some species from outside the NEP region were also included to help with identification of our samples (Fig. 2.1, grey text). We lack sequence data for three palaeonemertean species reported from the area: *Tubulanus cingulatus*, *T. capistratus* and *Carinomella lactea* (Table 2.1). Two species found by us as adults and/or larvae are described but previously unknown to the NEP: *Hubrechtella juliae* (Chernyshev 2003) and *Carinoma hamanako* (Kajihara et al. 2011) (asterisks Fig. 2.1). Eight species on our molecular phylogeny are represented by larval specimens, which we have yet to encounter as adults (Tables 2.1), but all can be identified, based on molecular data and morphology, to various palaeonemertean genera (described below).

The results of the ABGD analysis, which includes all species used in our phylogenetic analysis, agree with our number of hypothesized species for the 16S gene region (Fig. 2.1A), but slightly overestimate the number of species suggested by COI phylogeny (Fig. 2.1B). Specifically, ABGD analysis for the COI gene region over-split the species *Carinoma mutabilis* and *Carinoma hamanako* but lumped *Carinoma* sp. 5 and *Carinoma mutabilis* as well as *Cephalothrix major* and *Cephalothrix spiralis*.

All divergence values for palaeonemertean species are listed in Appendix E. Average intraspecific sequence divergence for palaeonemertean species is 0.4% for the 16S gene region and 1.9% for COI (Table 2.2). The interspecific divergence range as determined from the genera *Tubulanus*, *Carinoma* and *Carinina* is 3.7–29.6% for the 16S gene region and 9.9–19.6% for the COI gene region (Table 2.2). The intraspecific divergence range

overlaps the interspecific range for the COI gene region only, due to the high intraspecific divergence values in *Carinoma mutabilis, Carinoma hamanako* and *Carinina* sp. "chocolate" (Appendix E). The largest intraspecific divergence is seen between two GenBank sequences of *Carinoma tremaphoros* (Appendix E), both collected from Florida, USA (Thollesson and Norenburg 2003; Andrade et al. 2012). These two sequences very likely represent cryptic species (see Fig. 2.1), and were not included in our intraspecific divergence value estimates. Incidentally, additional evidence of cryptic species of *Carinoma tremaphoros* in Florida comes from embryological observations by S. Maslakova (unpublished). This is an example of how intraspecific divergence values can be overestimated based on sequence data alone.

***Carinina* Hubrecht, 1885**

The genus *Carinina* has not been previously reported from the west coast of North America.

***Carinina* sp. "chocolate" (Fig. 2.5)**

Individuals medium sized, several centimeters in length and up to 2 mm in width. Body reddish-brown to dark chocolate-brown; lighter around the head margins; slightly lighter ventrally than dorsally; rounded anteriorly and dorso-ventrally flattened posteriorly; midgut region with irregular transverse constrictions, somewhat paler in color than the background; a ring of differentiated epidermis present in middle of foregut region; head flattened dorso-ventrally, rounded anteriorly or somewhat heart-shaped (i.e., pointed at center); eyes absent; cephalic furrows short and shaped like rounded flaps; cerebral organs present; oocytes (120–140 μm in diameter with tight chorion) easily visible through the body wall of reproductive females. Planktonic larvae were collected in January 2013 and September 2015, and ripe females were observed in May. Adults are relatively rare, with several individuals collected on one or two collecting trips from 2008–2015; occurs in low intertidal mudflats. A combination of morphological characters and phylogenetic evidence (Fig. 2.1) place this species within the genus

Carinina, however it does not fit any description of a known species within the genus, and thus likely represents a species new to science.

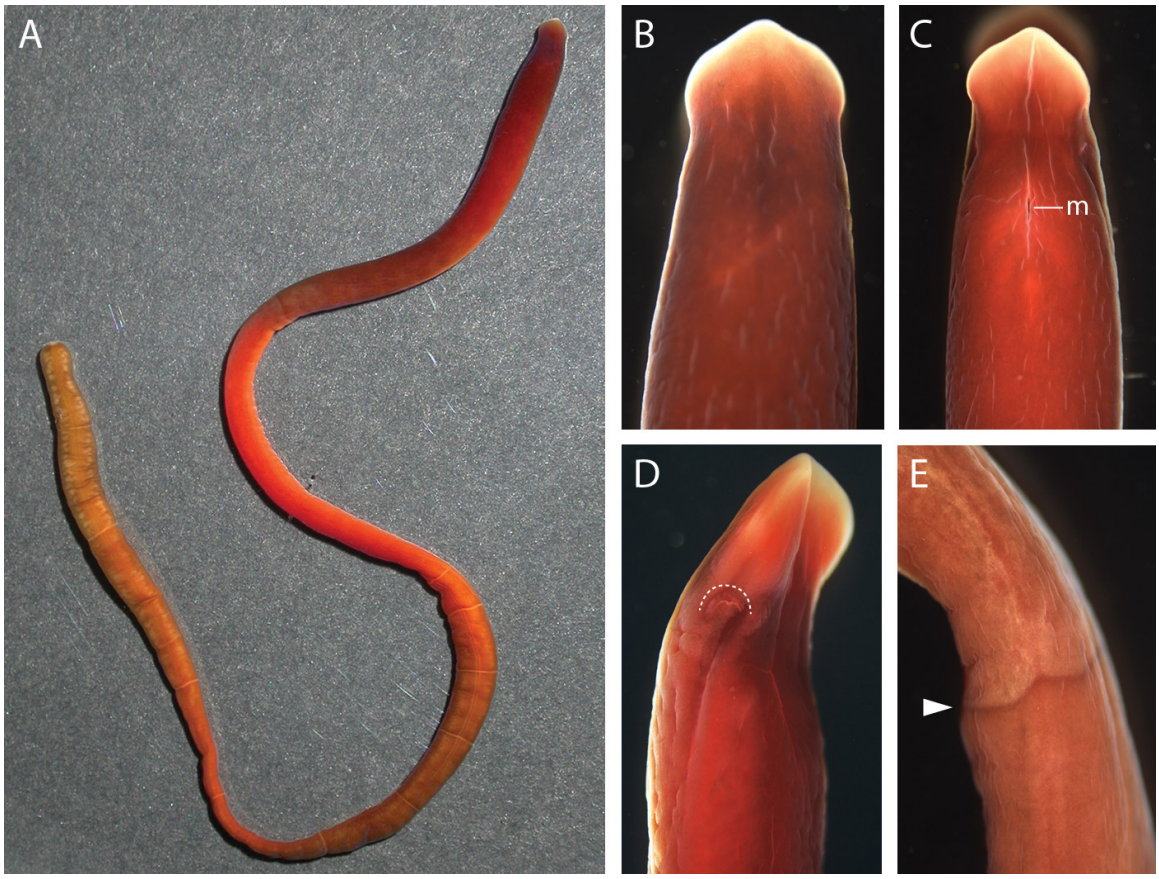

Figure 2.5. The undescribed palaeonemertean species, *Carinina* sp. "chocolate". Photographs from an individual relaxed in $MgCl_2$. (A) Entire specimen with anterior at upper right, (B–D) close-up of anterior dorsal (B), ventral (C), and lateral (D) views; note mouth (m) and rounded shape of cephalic furrows (dashed line, D). (E) Middle of foregut of the same individual; note ring of differentiated epidermis (arrowhead).

Carinoma Oudemans, 1885

Previous accounts report a single species of *Carinoma, C. mutabilis,* from the west coast of North America (Griffin 1898; Coe 1905; Corrêa 1964; Roe et al. 2007). Here we provide evidence for four additional species, including one previously described from Japan, and three undescribed species.

***Carinoma mutabilis* Griffin, 1898**

(Griffin 1898; Corrêa 1964; Roe et al. 2007) Individuals several cm in length and 1–3 mm in width. Body pale white to cream anteriorly, sometimes semi-transparent; proboscis easily visible through anterior body wall; conspicuous yellowish brown intestinal region dorsally with abrupt transition to more pale ventrum, most boldly colored in mid-body and posterior, although not as boldly colored as *C.* sp. "yellowback"; head shape is ovate to broadly rounded, somewhat pointed or triangular in gliding, and body gradually tapers to anterior point; body rounded in cross-section anteriorly, and dorso-ventrally flattened posteriorly, margins not sharp but sides curve ventrally; cephalic furrows, cerebral organs and eyes absent. Adults are not common, but many individuals collected from 2008–2015; occurs in intertidal mudflats and sandflats. Wild-caught larvae collected in February. *Carinoma mutabilis* can be differentiated from the, superficially similar species, *Carinomella lactea* and *Tubulanus pellucidus*, by its lack of sensory organs (lateral or cerebral) (Roe et al. 2007). Furthermore, *C. mutabilis* has more conspicuous intestinal diverticula and a broad, dorso-ventrally flattened posterior (Coe 1905). The type region is British Columbia, Canada and the known range extends to Mexico. This species was previously believed to be the only *Carinoma* species on the west coast of North America, however, we have found five *Carinoma* species on the southern Oregon coast alone (see below). Griffin (1898) described two variants of *C. mutabilis* (*C. mutabilis argillina* and *C. mutabilis vasculosa*) based on size and characters of internal anatomy. Whether these correspond to two of the species we describe here is unknown.

***Carinoma hamanako* Kajihara et al., 2011**

(Kajihara et al., 2011) Body size is 8 cm in length and 1 mm in width. White to semi-transparent anterior and cream, yellowish-white or peach posteriorly; anterior dorsoventrally flattened, foregut region round and posterior flattened in cross-section; cephalic slits and/or furrows absent; eyes absent (Kajihara et al 2011). Larvae identified as belonging to this species were collected by us in May, July and October in Coos Bay,

OR. We have yet to encounter the adults of this species. Type locality is Lake Hamana, on the Pacific coast of Honshu, Japan. This is the first record of this species on the west coast of North America.

***Carinoma* sp. "white"**

Individuals small, not more than a few centimeters in length. Body pale white to cream; semitransparent anteriorly, coiled proboscis is sometimes visible through the anterior body wall; head shape is ovate to broadly rounded, when gliding head shape is triangular and body gradually tapers to anterior point; body rounded in cross-section anteriorly, but flattens posteriorly; cephalic furrows, cerebral organs and eyes absent. Adults are relatively rare, with several individuals collected on one or two collecting trips from 2008–2015; occurs in intertidal mudflats and sandflats. Larvae collected in February. Adults were collected near Seaside, OR and Charleston, OR.

***Carinoma* sp. "yellowback"**

Individuals several centimeters in length and 1–3 mm in width; morphologically similar to *C. mutabilis,* but darker and more striking in color, especially dorsally. Body pale white to cream ventrally; conspicuous yellow-orange dorsum with abrupt transition from pale ventrum, most boldly colored in mid-body and posterior; semitransparent anteriorly and white, coiled proboscis is sometimes visible through the body wall; head shape is ovate to broadly rounded, when gliding head shape is triangular and body gradually tapers to anterior point; body anteriorly round in cross-section, but flattens posteriorly, margins not sharp but sides curve ventrally; cephalic furrows, cerebral organs and eyes absent. Adults are not common, but many individuals collected from 2008–2015; occurs in intertidal mudflats and sandflats. Larvae collected in March. Range outside of southern Oregon is currently unknown.

***Carinoma* sp. 5. (Fig. 4.31 in Chapter IV)**

A single larva of this species was collected in October 2012. Sequence divergence and phylogenetic data suggests that it represents a new species of *Carinoma* (Fig. 2.1).

***Carinomella* Coe, 1905**

***Carinomella lactea* Coe, 1905**

(Coe 1905; Roe et al. 2007) Reported distribution includes both Atlantic (Florida) and Pacific coasts of North America. On the West coast of North America ranges from central to southern California, including the type region (Gibson 1995; Roe et al. 2007). Individuals are superficially similar to *Carinoma* species (e.g., *C. mutabilis*), but lacks conspicuous intestinal diverticula and has a more narrow posterior (Coe 1905). We have not observed this species in southern Oregon and sequence data are not available for this species.

***Cephalothrix* Oersted, 1843**

Cephalothrix species are morphologically uniform and difficult to differentiate. Recent DNA barcoding studies of the genus based on COI sequences suggest that the number of undescribed and cryptic species greatly exceeds that of known species worldwide (Chen et al. 2010; Leasi and Norenburg 2014). Two *Cephalothrix* species are currently reported from NEP: *Cephalothrix spiralis* and *C. major* (Roe et al. 2007). In addition to these two, whose presence we confirm based on collections of adult specimens, we provide evidence for the presence of four other species in southern Oregon (*Cephalothrix* sp. 1, *Cephalothrix* sp. 2, *Cephalothrix* sp. 3, and *Cephalothrix* sp. 4), which we only find as planktonic larvae (see Chapter IV). Their sequences do not match those of any known *Cephalothrix* species. Furthermore, one of the previously published sequences of *C. spiralis* (AJ436837) from San Juan Island, WA appears to represent yet another cryptic species in the area. Interestingly, two additional species (including *C. simula* and an undescribed species) are reported from San Diego, CA by Leasi and Norenburg (2014).

This brings the known number of *Cephalothrix* species on the west coast of North America to nine, and six of those we find within the OBP (Table 2.1).

***Cephalothrix major* Coe, 1930**

(Coe 1930 (=*Procephalothrix major*); Roe et al. 2007 (=*Procephalothrix major*))
Body long (up to 1 m in length) and thin (2–5 mm); color pale yellow to greenish-yellow, flesh-toned, semitransparent, or pinkish (especially head region); no variation in color dorsally to ventrally; body cylindrical in cross-section throughout; head narrow and pointed anteriorly; pre-oral region very long; gametes visible through the body wall in ripe individuals. Tends to occur in knotted masses of several individuals, but does not contract into a uniform corkscrew shape (as does *C. spiralis*, below). Differentiating *C. major* and *C. spiralis* requires examination of nephridia (Coe 1930; Roe et al. 2007). Intertidal and subtidal in sediments and under rocks in mudflats and sandflats; sometimes in surf zone (Roe et al. 2007). NEP distribution includes southern Oregon to Mexico (Gibson 1995; Roe et al. 2007). We collected a single adult of this species from southern Oregon, and our DNA sequences group with those from other *C. major* specimens previously reported by Chen et al. (2010) from Oregon (Fig. 2.6, Appendix D).

***Cephalothrix spiralis* Coe, 1930**

(Coe 1930 (=*Procephalothrix spiralis*); Corrêa 1964 (=*Procephalothrix spiralis*); Roe et al. 2007 (=*Procephalothrix spiralis*)) Individuals shorter than *C. major,* up to 15 cm in length and thin (2–3 mm); tend to occur in knotted masses of several individuals; worm contracts into a uniform corkscrew shape (Plate 86G, Roe et al. 2007). Body color pale

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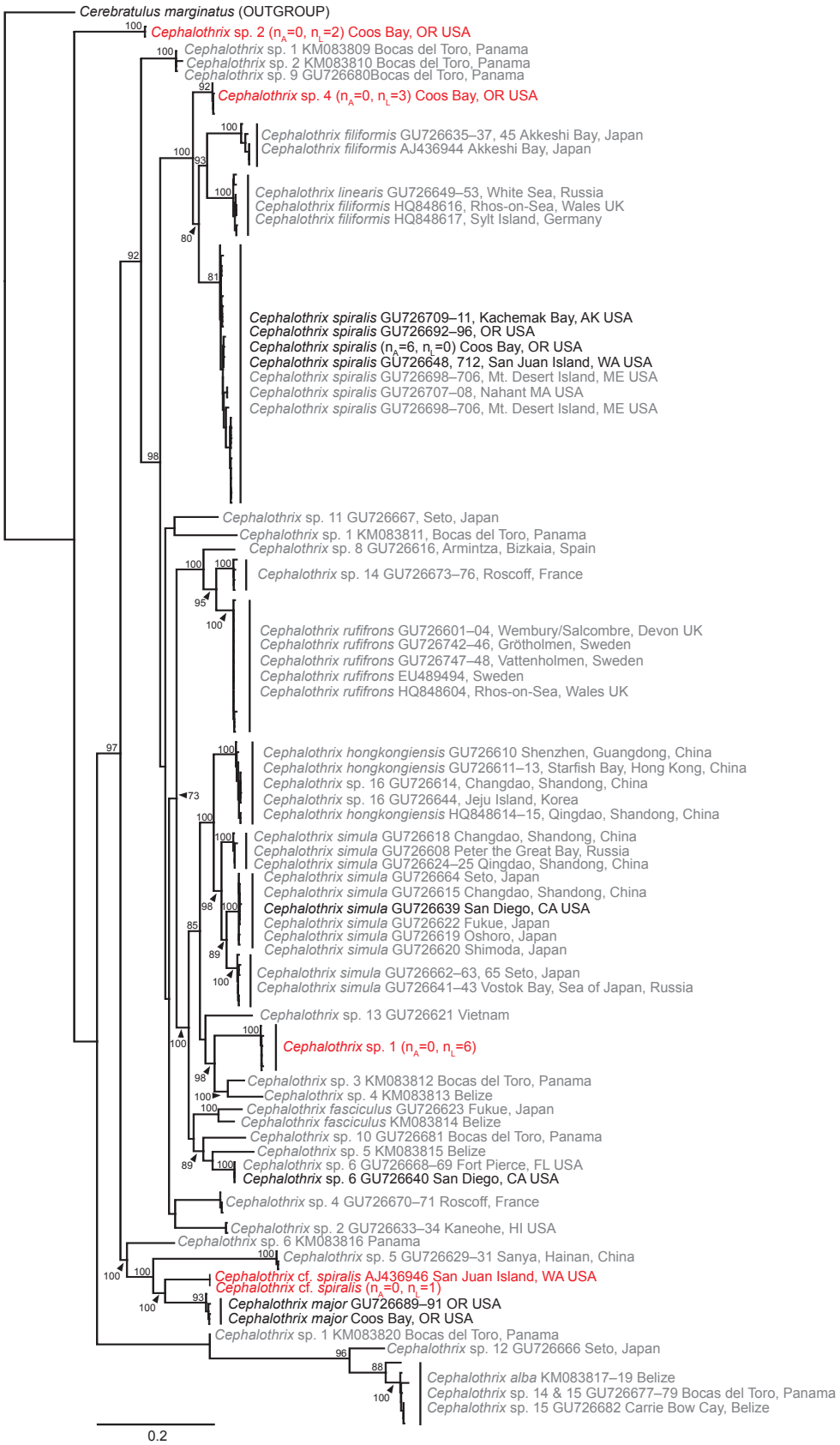
Figure 2.6 (next page). COI maximum likelihood phylogeny for the palaeonemertean genus *Cephalothrix,* including all available sequence data (this study and GenBank sequences, for accession numbers see Appendix D). Bayesian posterior probabilities (>70) are indicated above nodes. Species known or believed to occur in the NE Pacific prior to this study (Roe et al. 2007; Leasi and Norenburg 2014) are shown in black text and species reported here for the first time in red text. Species from outside the NEP are shown in grey text.


Cerebratulus marginatus (OUTGROUP)
Cephalothrix sp. 2 (n_A=0, n_L=2) Coos Bay, OR USA
Cephalothrix sp. 1 KM083809 Bocas del Toro, Panama
Cephalothrix sp. 2 KM083810 Bocas del Toro, Panama
Cephalothrix sp. 9 GU726680Bocas del Toro, Panama
Cephalothrix sp. 4 (n_A=0, n_L=3) Coos Bay, OR USA
Cephalothrix filiformis GU726635–37, 45 Akkeshi Bay, Japan
Cephalothrix filiformis AJ436944 Akkeshi Bay, Japan
Cephalothrix linearis GU726649–53, White Sea, Russia
Cephalothrix filiformis HQ848616, Rhos-on-Sea, Wales UK
Cephalothrix filiformis HQ848617, Sylt Island, Germany
Cephalothrix spiralis GU726709–11, Kachemak Bay, AK USA
Cephalothrix spiralis GU726692–96, OR USA
Cephalothrix spiralis (n_A=6, n_L=0) Coos Bay, OR USA
Cephalothrix spiralis GU726648, 712, San Juan Island, WA USA
Cephalothrix spiralis GU726698–706, Mt. Desert Island, ME USA
Cephalothrix spiralis GU726707–08, Nahant MA USA
Cephalothrix spiralis GU726698–706, Mt. Desert Island, ME USA
Cephalothrix sp. 11 GU726667, Seto, Japan
Cephalothrix sp. 1 KM083811, Bocas del Toro, Panama
Cephalothrix sp. 8 GU726616, Armintza, Bizkaia, Spain
Cephalothrix sp. 14 GU726673–76, Roscoff, France
Cephalothrix rufifrons GU726601–04, Wembury/Salcombre, Devon UK
Cephalothrix rufifrons GU726742–46, Grötholmen, Sweden
Cephalothrix rufifrons GU726747–48, Vattenholmen, Sweden
Cephalothrix rufifrons EU489494, Sweden
Cephalothrix rufifrons HQ848604, Rhos-on-Sea, Wales UK
Cephalothrix hongkongiensis GU726610 Shenzhen, Guangdong, China
Cephalothrix hongkongiensis GU726611–13, Starfish Bay, Hong Kong, China
Cephalothrix sp. 16 GU726614, Changdao, Shandong, China
Cephalothrix sp. 16 GU726644, Jeju Island, Korea
Cephalothrix hongkongiensis HQ848614–15, Qingdao, Shandong, China
Cephalothrix simula GU726618 Changdao, Shandong, China
Cephalothrix simula GU726608 Peter the Great Bay, Russia
Cephalothrix simula GU726624–25 Qingdao, Shandong, China
Cephalothrix simula GU726664 Seto, Japan
Cephalothrix simula GU726615 Changdao, Shandong, China
Cephalothrix simula GU726639 San Diego, CA USA
Cephalothrix simula GU726622 Fukue, Japan
Cephalothrix simula GU726619 Oshoro, Japan
Cephalothrix simula GU726620 Shimoda, Japan
Cephalothrix simula GU726662–63, 65 Seto, Japan
Cephalothrix simula GU726641–43 Vostok Bay, Sea of Japan, Russia
Cephalothrix sp. 13 GU726621 Vietnam
Cephalothrix sp. 1 (n_A=0, n_L=6)
Cephalothrix sp. 3 KM083812 Bocas del Toro, Panama
Cephalothrix sp. 4 KM083813 Belize
Cephalothrix fasciculus GU726623 Fukue, Japan
Cephalothrix fasciculus KM083814 Belize
Cephalothrix sp. 10 GU726681 Bocas del Toro, Panama
Cephalothrix sp. 5 KM083815 Belize
Cephalothrix sp. 6 GU726668–69 Fort Pierce, FL USA
Cephalothrix sp. 6 GU726640 San Diego, CA USA
Cephalothrix sp. 4 GU726670–71 Roscoff, France
Cephalothrix sp. 2 GU726633–34 Kaneohe, HI USA
Cephalothrix sp. 6 KM083816 Panama
Cephalothrix sp. 5 GU726629–31 Sanya, Hainan, China
Cephalothrix cf. *spiralis* AJ436946 San Juan Island, WA USA
Cephalothrix cf. *spiralis* (n_A=0, n_L=1)
Cephalothrix major GU726689–91 OR USA
Cephalothrix major Coos Bay, OR USA
Cephalothrix sp. 1 KM083820 Bocas del Toro, Panama
Cephalothrix sp. 12 GU726666 Seto, Japan
Cephalothrix alba KM083817–19 Belize
Cephalothrix sp. 14 & 15 GU726677–79 Bocas del Toro, Panama
Cephalothrix sp. 15 GU726682 Carrie Bow Cay, Belize
0.2

white, flesh-toned, semitransparent; no variation in color dorsally to ventrally; body cylindrical in cross-section throughout; head narrow and pointed anteriorly; pre-oral region very long; gametes visible through the body wall in ripe individuals. Reproductive specimens have been observed in Charleston, OR in Winter-early Spring. Gametes can be dissected from the males and females and fertilized in the lab, and embryos and early larval stages are easily cultured in the laboratory (Maslakova, unpublished). However, we have yet to find the larvae of this species in the plankton. Differentiating *C. major* and *C. spiralis* requires examination of nephridia (Coe 1930; Roe et al. 2007). Intertidal and subtidal (up to 20 m) in sediments and under rocks, in mudflats and associated with anoxic sediments (Gibson 1995); more common encountered in Coos Bay than *C. major*. Distribution includes Alaska to southern California as well as, the type region, in the north Atlantic (Gibson 1995; Roe et al. 2007, Chen et al. 2010, Leasi and Norenburg 2014). A common intertidal species in southern Oregon.

Cephalothrix* cf. *spiralis

This species, represented in our analyses by GenBank sequences AJ436837 (16S) and AJ436946 (COI) was collected and identified as *C. spiralis* by J Norenburg from San Juan Island, WA (Thollesson and Norenburg 2003). Although morphologically indistinguishable from *C. spiralis,* sequence data suggests it is clearly a different species (Figs. 2.6). We have not encountered adults of this species in Oregon. A single larva identified as belonging to this species was collected by Kara Robbins in Coos Bay in May 2015.

***Cephalothrix* sp. 1, 2, 3, and 4**

These four *Cephalothrix* species we currently find as planktonic larvae. We have not found them as benthic adults and they do not match any known *Cephalothrix* species for which we have sequence data (Fig. 2.4, Table 2.1). For descriptions of these larvae, see Chapter IV (Figs. 4.33–36 in Chapter IV).

***Tubulanus* Renier, 1804**

Five species of *Tubulanus* are reported to occur in the region from central California to Oregon (Roe et al. 2007): *T. capistratus, T. cingulatus, T. pellucidus, T. polymorphus* and *T. sexlineatus*. We have only encountered the adults of the latter two species in the region, where they are common. In addition, we encountered three distinct species of *Tubulanus* larvae in the plankton. As we lack sequence data from *T. cingulatus* and *T. capistratus*, it is possible that two of those larval species correspond to *T. cingulatus* and *T. capistratus*, but at least one (and possibly all three) represent new species of *Tubulanus* for the NEP. Thus the total number of species of *Tubulanus* in the region is 7–9. Furthermore, recent work (J von Döhren, D Krämer, T Bartolomaeus, unpublished) suggests that the NEP *T. polymorphus* is a distinct species from the *T. polymorphus* from Europe (the type region).

***Tubulanus capistratus* Coe, 1901**

(Coe 1901 (=*Carinella capistrata*); Roe et al. 2007) Described from specimens collected in Alaska; reported distribution from Alaska to California (Roe et al. 2007) and Japan (Coe 1944; Gibson 1995). We have yet to observe this species in southern Oregon and no sequence data are currently available for this species.

***Tubulanus cingulatus* Coe, 1904**

(Coe 1904 (=*Carinella cingulata*); Corrêa 1964; Roe et al. 2007) Described from specimens collected by dredge in Monterey Bay, California (Ref); reported distribution from Alaska (Gibson 1995) to California (Bolinas and San Diego, Roe et al. 2007). We have yet to observe this species in southern Oregon and no sequence data are available for this species.

***Tubulanus pellucidus* Coe, 1895**

(Coe 1895 (=*Carinella pellucida*); Corrêa 1961; Roe et al. 2007) Reported distribution includes both Atlantic (type region, New England to Florida) and Pacific coasts of North America. NE Pacific range currently includes California, from San Francisco to San

Diego (Gibson 1995; Roe et al. 2007). We have not encountered this species in our sampling area. Sequence data in our analyses is from specimens collected in North Carolina, USA (Andrade et al. 2012).

Tubulanus polymorphus **Renier, 1804**

(Renier 1804 (=*Carinella polymorpha*); Griffin 1898 (=*Carinella rubra*); Coe 1901(=*Carinella speciosa*); Coe 1905 (=*Carinella rubra*); Corrêa 1964; Roe et al. 2007) Individuals reported at lengths to 3 m are extremely stretchy and often occur under rocks and amongst algae in parchment tubes, intertidal and subtidal. Bold bright orange body color is uniform throughout; head shape rounded, wider than body, and orbicular with gradual and conspicuous constriction at transition between head and body; body rounded in cross-section, long and thin; eyes absent; cephalic furrows, cerebral organs and lateral sensory organs present. Reproductive individuals are found in summer months (Stricker 1987; Coe 1905, Maslakova pers. obs.). Large bright orange opaque eggs develop into short-lived uniformly ciliated larvae (Stricker 1987). A single larva determined to belong to this species by sequence data was collected, by us, from Coos Bay, Oregon in February. The type locality of *T. polymorphus* is in the Mediterranean. Although this species has an apparent wide geographic distribution within the northern hemisphere, morphological and molecular data strongly suggest different Pacific and Atlantic species, with the Pacific species *T. ruber* (Griffin, 1898) and the Atlantic *T. polymorphus* (Renier, 1804) (J von Döhren, D Krämer, T Bartolomaeus, unpublished). Current reported distribution includes the west coast of North America, from the Aleutian Islands to Monterey, California as well as northern Europe (Roe et al. 2007).

Tubulanus sexlineatus **Griffin, 1898**

(Griffin 1898 (=*Carinella sexlineata*); Coe 1901 (=*Carinella dinema*), 1940; Corrêa 1964; Roe et al. 2007) Individuals can be rather long (although not as long as *T. polymorphus,* above) and have been reported over 1 m, however are typically much shorter s (e.g., 25 cm in length and 2–3 mm in width). Black to brown body color with

conspicuous white transverse lines connected by 5–6 white longitudinal lines running the body length, a pattern that continues to anteriormost tip. Head rounded, wider than body, and orbicular with gradual and conspicuous constriction at transition between head and body; body equally rounded throughout, long and thin; eyes absent; cephalic furrows, cerebral organs and lateral sensory organs present. Reproductive females have been observed spawning in April. Oocytes are pale pink and 105 μm in diameter. Wild-caught larvae were collected in March and April and identified with DNA sequence data. Intertidal and subtidal, in parchment tubes under rocks and amongst algae. Described from specimens collected by dredge in Puget Sound, Washington and Alaska; known distribution from Alaska to southern California (Gibson 1995; Roe et al. 2007).

***Tubulanus* sp. 1, 2, and 3**

These three *Tubulanus* species we currently find as planktonic larvae. We have not found them as benthic adults and they do not match any known *Tubulanus* species for which we have sequence data (Fig. 2.4, Table 2.1). For descriptions of these larvae, see Chapter IV (Figs. 4.39–41 in Chapter IV).

Pilidiophora

Twenty-three pilidiophoran species are currently reported from central California to Oregon (Roe et al. 2007). Our data suggest that there are likely at least 52 species in this region, based on sampling in southern Oregon alone (Table 2.1). Our pilidiophoran phylogenetic analyses were based on alignments that were 619 bp (16S) and 658 (COI) in length comprising 408 (16S) and 298 (COI) sequences from adult and larval specimens (for GenBank accession numbers see Appendix B). Most of these specimens are NEP species, but species from outside the NEP were also included (Fig. 2.2, grey text) to help with identification of our sequences. We lack sequence data for five pilidiophoran species reported from central California to Oregon (Roe et al. 2007): *Cerebratulus occidentalis*, *Micrura coei*, *M. olivaris*, *Lineus pictifrons*, and *Euborlasia nigrocincta* (Table 2.1).

Fourteen pilidiophoran species are represented by larval specimens only, and we have yet to encounter adults (the so-called 'orphan larvae'). One of these, *Hubrechtella juliae*, has not previously been reported from the NEP (asterisk, Fig. 2.2). Eight of these orphan larvae have been assigned, based on molecular data and morphology, to the pilidiophoran genera described below. The larvae of the remaining six species cannot be identified to genus level. Three of these species are modified pilidia (Heteronemertea gen. sp. 1, 2 and 3, see Figs. 4.21–4.23 in Chapter IV) with lecithotrophic development. This type of development does not characterize a particular clade of species, but appears to have evolved secondarily many times within the Pilidiophora (Schwartz 2009; Maslakova and Hiebert 2014; see also Chapter VII). The three remaining species have been encountered once (Heteronemertea gen. sp. 5 and sp. 6) or many times (Heteronemertea gen. sp. 4), but do not group closely with any heteronemertean clade or genus (Fig. 2.2; see Chapter VII).

The ABGD analysis, which includes all species used in our phylogenetic analysis, finds a barcoding gap for the COI gene region from ~ 0.2–1.3%, but a less definite gap is present for the 16S gene region (~ 0.3–1.8%). These analyses overestimated the number of pilidiophoran species, as determined by us, for both the 16S and COI gene regions. Specifically, the ABGD 16S analysis over-split the species *Lineus flavescens, Maculaura magna, Micrura* sp. "albocephala", and lumped the species *Cerebratulus* sp. "Sunset Bay" with *Cerebratulus* sp. "pink proboscis". The COI analysis over-split the same species as 16S in addition to *Cerebratulus* cf. *marginatus*, *Lineus* sp. 2 and *Maculaura aquilonia*.

Pilidiophoran divergence values are shown in Appendix F. Average intraspecific sequence divergence for pilidiophoran species is 0.3% for the 16S gene region and 1.3% for COI (Table 2.2). The interspecific divergence range for each gene region (determined from the genera *Baseodiscus*, *Riserius* and *Maculaura*) is 2.9–26.5% (16S) and 9.0–

21.1% (COI) (Table 2.2). The upper end of intraspecific divergence values overlaps the minimum interspecific divergence values for both gene regions due to few 'problematic' species (e.g., *Maculaura magna*, *Lineus* sp. 1, *Lineus* sp. 2 and *Micrura* sp. "albocephala"). These species exhibit high intraspecific divergence values, while, at the same time, showing strong reciprocal monophyly, morphological uniformity, and support from multiple gene regions.

***Hubrechtella* Bergendal, 1902**

***Hubrechtella juliae* Chernyshev, 2003**

(Chernyshev 2003) Size is 10–14 mm in length and 0.5–1.0 mm in width. Body color pale white and semitransparent; head shape orbicular; anterior rounded and bulbous posterior; pink or yellow color of intestine is sometimes visible through the body wall; short transverse cephalic furrows present; caudal cirrus and eyes absent. Subtidally in silt and sandy habitats (Chernyshev 2003). We have yet to find the adults of this species, but their larvae have been encountered frequently in southern Oregon. Larvae collected Oct–Feb. This is the first record of a hubrechtid species on the west coast of North America; with known distribution including the Sea of Japan, Russia and NE (southern Oregon) Pacific.

***Baseodiscus* Diesing, 1850**

***Baseodiscus punnetti* Coe, 1904**

(Coe 1904 (=*Taeniosoma punnetti*); Roe et al. 2007) Pale pink to magenta or red body color, color is more pale ventrally; head shape orbicular; conspicuous constriction between head and body marked with oblique cephalic furrows; secondary cephalic furrows present; longitudinal cephalic slits absent; pale color present at head margins is narrow at anterior tip and widens just anterior to constriction between head and body; many ocelli (80–120, total) are present and visible within the pale region of the head; head can withdraw almost completely into body; body is large, fleshy and wrinkly; rounded anteriorly and slightly flattened posteriorly, in cross-section; caudal cirrus

absent. Dark brown or black pigment was noted anteriorly and dorsally by Coe (1904), but this has not been observed by us. Described from specimens collected by dredge in southern California (Coe 1904); known distribution from Monterey Bay, California to Mexico (Gibson 1995; Roe et al. 2007); occurs intertidally and subtidally among algae and their holdfasts and rocks. We have yet to observe this species in southern Oregon, but have examined individuals collected subtidally in Santa Barbara, California.

***Cerebratulus* Renier, 1804**

Note that this 'mega-genus' is almost certainly non-monophyletic (Sundberg and Saur 1998; Schwartz and Norenburg 2001; Sundberg et al. 2001; Strand and Sundberg 2005; Schwartz 2009; also see Fig. 2.2), and is in desperate need of revision. Ideally, only the species shown to be closely related to the type species, *C. marginatus* Renier, 1804 should remain within *Cerebratulus*, while others should be transferred to other existing or new genera as appropriate. Such a revision, however, is outside the scope of this manuscript. Therefore, we refer to species by their current names, and provisionally identify unknown samples as "*Cerebratulus*" if they group within monophyletic clades comprising *Cerebratulus* sequences, otherwise fit the rather vague morphological definition of *Cerebratulus,* or exhibit larval morphology similar to other species identified as *Cerebratulus.*

***Cerebratulus albifrons* Coe, 1901**

(Coe 1901; Corrêa 1964; Roe et al. 2007) Individuals may be several centimeters in length (15–30 cm, Coe 1901; Roe et al. 2007). Body color dark black to grey, brown or purple; anterior half of head white; head shape ovate; body, in cross-section, rounded anteriorly and dramatically flattened posteriorly; caudal cirrus present; body margins sharp and pale; longitudinal cephalic slits present; eyeless (see Plate IV Fig. 3, Coe 1901). Body morphology and coloring very similar to *Micrura* sp. "albocephala". Intertidal and subtidal, under rocks and within mud. Planktotrophic pilidium larvae have

been collected in July, other reproductive characters unknown. NEP distribution from Alaska to San Diego, California; rare in southern Oregon.

***Cerebratulus californiensis* Coe, 1905 species complex**

Cerebratulus californiensis

(Coe, 1905; Roe et al. 2007) Individuals, 10–15 cm in length and 3–5 mm in width, are not as large as *C.* cf. *marginatus;* fragment easily. A variety of colors morphs were used originally to describe the species *C. californiensis* (Coe 1905; Coe 1940). However, based on our analyses, the description of *C. californiensis* contains at least three cryptic species. The species to which we refer as *C. californiensis* is uniformly flesh-toned, pinkish or yellowish; the second species is olive green in color with bright pink proboscis (*C*. sp. "pink proboscis", Fig. 2.7C); the third is pale white or transparent, with lateral blood vessels conspicuous through the body wall, and with dark reddish brown head (*C.* sp. "Sunset Bay", Fig. 2.7A–B). Head shape of *C. californiensis* is ovate to triangular and tapers to a point anteriorly; posterior-most region of the head is narrower than body; body (in cross-section) equally flattened dorso-ventrally throughout with very sharp margins; longitudinal cephalic slits present, flaring while swimming; eyeless, caudal cirrus present. Larvae collected from January to February. Burrows in sand and mud, intertidally and subtidally. Range from Washington to Mexico (Gibson 1995; Roe et al. 2007). Members of the *C. californiensis* species complex can be differentiated from the commonly co-occurring species, *C*. cf. *marginatus* (see description below) as the latter species has a head shape that becomes wider than the adjacent body, while *C. californiensis* and allies have head that is relatively small and narrower than adjacent body.

***Cerebratulus* sp. "Sunset Bay" (Fig. 2.7A–B)**

Resembles *C. californiensis* in size and body shape. Body color is pale white or transparent, and lateral blood vessels are conspicuous through the body wall; dark reddish brown pigment is conspicuous anteriorly; head shape triangular and tapers to a point;

body, in cross-section, is dorso-ventrally oval anteriorly and flattening posteriorly, with sharp margins; conspicuous longitudinal cephalic slits present, flaring when swimming; small caudal cirrus present; eyeless. Larvae collected in October; other reproductive characters unknown. Currently only known from Sunset Bay, Cape Arago in Charleston, Oregon.

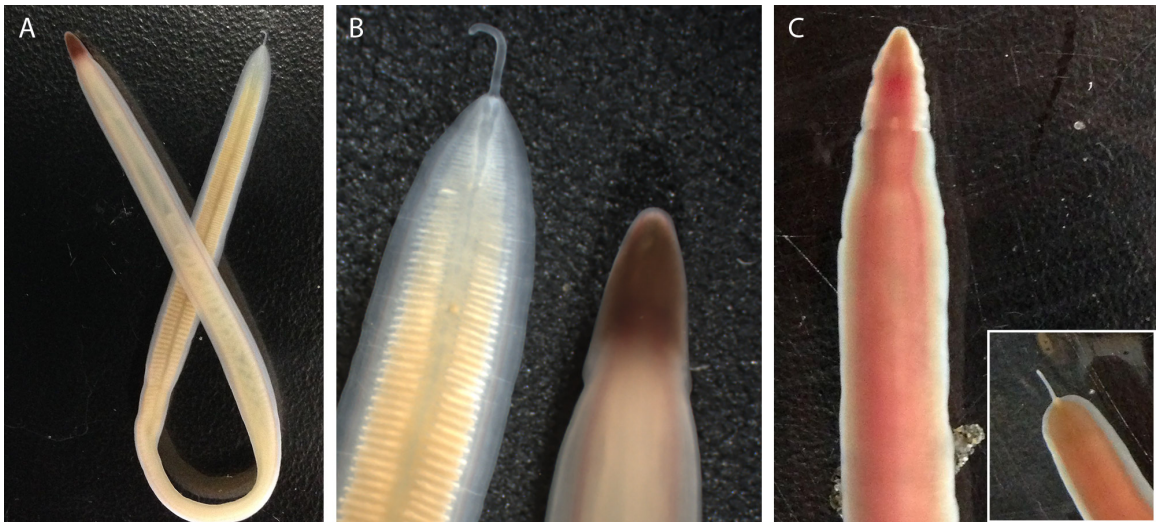


Figure 2.7. Pilidiophoran species *Cerebratulus* sp. "Sunset Bay" (A–B) and *C*. sp. "pink proboscis" (C). *Cerebratulus* sp. "Sunset Bay", individual relaxed in $MgCl_2$ (A) and close-up of anterior (right) and posterior (left) of same individual; note brown anterior (B). (C) *Cerebratulus* sp. "pink proboscis" anterior and posterior (inset) of individual relaxed in $MgCl_2$.

***Cerebratulus* sp. "pink proboscis" (Fig. 2.7C)**

Resembles *C. californiensis* in size and body shape. Olive green to grayish in color; proboscis is readily everted and bright pink; head shape triangular and tapers to a point; body, in cross-section, is dorso-ventrally oval anteriorly and flattening posteriorly, with sharp margins; conspicuous longitudinal cephalic slits present, flaring when swimming; small caudal cirrus present; eyeless. Head shape narrows more dramatically than the closely related species *C*. sp. "Sunset Bay" or *C. californiensis*. Larvae collected in summer months; other reproductive characters unknown. Known from San Juan Island, Washington and southern Oregon.

***Cerebratulus herculeus* Coe, 1901**

(Coe 1901; Roe et al. 2007) Originally described from specimens collected in Alaska (Coe 1901), reported distribution from Alaska to southern California (Gibson 1995; Roe et al. 2007), however, we have yet to observe this species in southern Oregon. We have sequence data from a specimen collected in San Juan Island, Washington (Schwartz and Norenburg, unpublished).

***Cerebratulus longiceps* Coe, 1901**

(Coe 1901; Corrêa 1964; Roe et al. 2007) Originally described from specimens collected in Alaska (Coe 1901) , reported distribution from Alaska to California (Gibson 1995; Roe et al. 2007), however, we have yet to observe this species in southern Oregon. We have sequence data from a specimen collected in San Juan Island, Washington (Schwartz and Norenburg, unpublished). A single larva of *C. longiceps* was collected in Jan 2014 (see Fig. 4.24E in Chapter IV) and the lecithotrophic larval form (pilidium nielseni, Maslakova and von Dassow 2012) is with two transverse ciliated bands, as seen in *Micrura* sp. "dark", *M.* sp. "albocephala", M. sp. 3, and M. sp. 4 (Hunt and Maslakova, unpublished).

***Cerebratulus montgomeryi* Coe, 1901**

(Coe 1901; Corrêa 1964; Roe et al. 2007) Individuals large in size, up to 1–2 m in length and 10 mm width. Body color deep to bright red; anterior tip of head white (not extending nearly as far posterior as in *C. albifrons*); no dorso-ventral difference in body color; head shape ovate; anterior body shape, in cross-section, rounded with round margins and narrow, becoming wider and more flattened posteriorly; longitudinal cephalic slits present; eyeless; caudal cirrus absent (see Plate VI Fig. 1, Coe 1901, also Maslakova pers. observations). Reproductive individuals encountered in summer months (Coe 1901), but we have yet to observe larvae in plankton samples. Intertidal and subtidal to 400 m depths (Gibson 1995). Described from specimens collected in Alaska and Puget Sound, Washington, known distribution from Alaska to Monterey Bay,

California (Gibson 1995; Roe et al. 2007). We have collected this species in San Juan Island, WA but yet to observe it in southern OR. It is noteworthy that this species lacks a caudal cirrus and groups within a well-supported clade of *Lineus* species (e.g., *Lineus flavescens*) on our molecular phylogenies (see Fig. 2.2). Thus, it is most likely not a true member of the genus *Cerebratulus*.

***Cerebratulus occidentalis* Coe, 1901**

(Coe 1901; Corrêa 1964; Roe et al. 2007) Distribution from Alaska (type region) to San Francisco Bay, California (Coe 1905; Gibson 1995; Roe et al. 2007). Coe's original description of external morphology is somewhat similar to that of *Lineus* sp. "red" (Hiebert and Maslakova 2015b; see Fig. 6.1C in Chapter VI), however, subtle differences (e.g., reproductive timing and dorso-ventral body color) suggests they may not be the same species. Caudal cirrus absent. We have not encountered individuals that ultimately correspond to the description, and no sequence data are available for this species.

***Cerebratulus* cf. *marginatus* Renier, 1804**

Individuals can be very large, up to 1 m in length (Roe et al. 2007) and 7–10 mm in width. Brown to dark brown or grey body color with no dorso-ventral difference in color; conspicuous longitudinal cephalic slits present, flaring when swimming; head shape ovate and widening to posterior reaches of cephalic slits, where posterior region of head is wider than body, but not as wide and spade-shaped as the morphologically similar species, *C.* sp. "spade head". Body shape, in cross-section, rounded anteriorly, with round margins; posterior body is flattened dorso-ventrally but with margins less sharp than *C. californiensis*; eyeless; caudal cirrus present; fragmentation occurs easily. Intertidal and subtidal, burrows in sand and mud. Reproductive individuals have been collected in the spring and summer, with gametes easily visible through the body wall; planktotrophic pilidium larvae collected in May and July. The apparent widespread distribution of this species is the result of extensive synonymies (Gibson 1995). The type

locality is Naples, Italy and, most likely *C. marginatus* Renier, 1804 is not the same species that we find in the NEP (no sequence data are available for specimens from the Mediterranean). Instead, we have a morphologically similar species, that we provisionally call *C.* cf. *marginatus;* its range confirmed by sequence data extends from San Juan Island, Washington to southern Oregon.

***Cerebratulus* sp. "spade head"**

Resembles *C.* cf. *marginatus* in body size and shape. Body color dark to light pinkish-brown; head shape ovate and prominently rounded posteriorly before constricting at the head-body transition; widest region of head is wider than in the morphologically similar species, *Cerebratulus* cf. *marginatus*; rounded anteriorly and flattening posteriorly, with sharp margins; conspicuous longitudinal cephalic slits present, flaring when swimming; eyeless; prominent caudal cirrus. Intertidal in sandflats; uncommon. Larvae were collected in autumn; other reproductive characters and range outside of southern Oregon unknown.

***Cerebratulus* sp. 1 and 2**

These two species we currently find as larvae, but not as adults. *Cerebratulus* sp. 1 is represented by a single larva collected in Jan 2013 and *Cerebratulus* sp. 2 by several larvae collected in Oct 2012 and 2014 (Table 2.1, Fig. 2.2, Appendix B).

***Euborlasia* Vaillant, 1890**

***Euborlasia nigrocincta* Coe, 1940**

(Coe 1940; Friedrich 1970; Roe et al. 2007) Described from specimens collected in Monterey Bay and San Diego, California; known distribution from Monterey Bay, California to Mexico (Gibson 1995; Roe et al. 2007) and northwest Pacific (Kajihara and Nishi 2013). We have yet to observe this species in southern Oregon. No sequence data are available for this species.

Lineidae gen. sp. "large eggs" (Fig. 2.8)

Average size (gliding) is 5 cm in length and 2 mm in width. Light to dark brown. Head shape is ovate with inconspicuous constriction between head and body; longitudinal cephalic slits present; posterior gradually tapers to a small bump-like cirrus; eyeless. Individuals are short and stubby, reminiscent in shape and color of shed needles of the Douglas Fir tree (*Pseudotsuga menziesii*) (Fig. 2.8A), when contracted; not markedly flattened posteriorly. A common heteronemertean in the rocky intertidal, under rocks and amongst *Phyllospadix* spp. root masses. Co-occurs with *Micrura* sp. "dark" and *M.* sp. "albocephala", *Lineus* sp. "red" and *Maculaura cerebrosa.* Reproductive individuals are found in winter months; oocytes are approximately 500 µm in diameter (Fig. 2.8B, inset). The development of this species is not yet known, but is presumably, lecithotrophic, and possibly encapsulated (see Maslakova and Hiebert 2014; see also Chapter VII). Range currently unknown outside of southern Oregon.

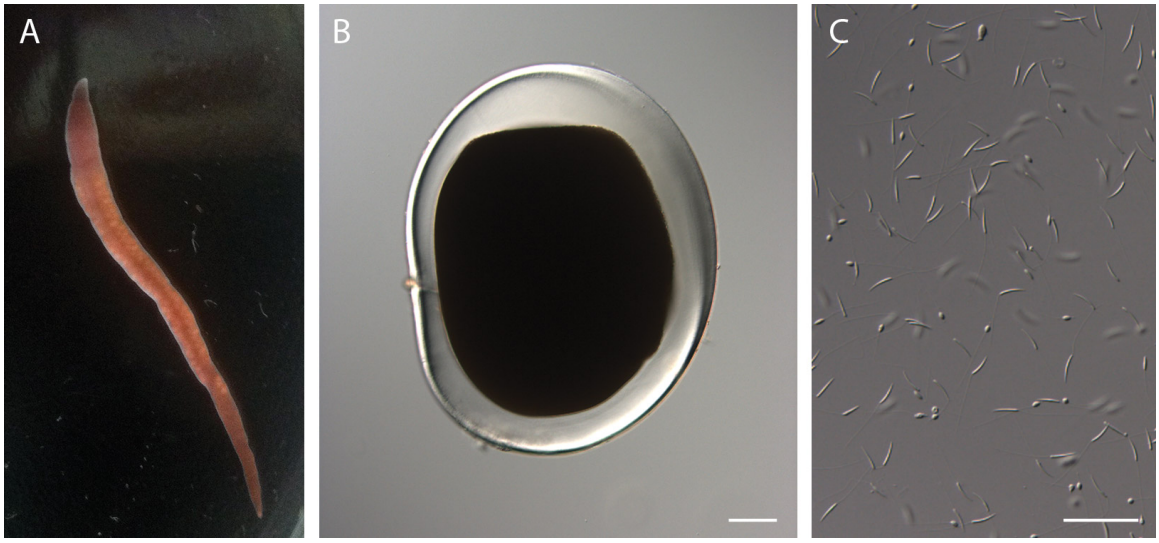


Figure 2.8. The undescribed pilidiophoran species, Lineidae gen. sp. "large eggs" adult (A), dissected oocyte (B), and sperm (C). Scale bars 100 µm (B) and 25 µm (C).

***Lineus* Sowerby, 1806**

Similar to the genus *Cerebratulus*, *Lineus* is a nemertean 'mega-genus' that is non-monophyletic (Sundberg and Saur 1998; Schwartz and Norenburg 2001; Sundberg et al. 2001; Schwartz 2009; also see Fig. 2.2) and in need of revision. Only those species

shown to be closely related to the type species, *L. longissimus* Gunnerus, 1770 should remain within *Lineus*, while others should be transferred to other existing or new genera as appropriate. As a revision of this genus is beyond the scope of this manuscript, we refer to species using current names. Currently undescribed species (larvae or adults) are referred to as "*Lineus*" if they form monophyletic clades with, fit the description of, or exhibit larval morphology similar to other *Lineus* species. However, their names do not denote them being true members of this genus.

***Lineus* cf. *bilineatus* Renier, 1804**

(Renier 1804 (=*Cerebratulus bilineatus*); McIntosh 1873; Roe et al. 2007) Type locality in the Mediterranean, thus possibly not the same species as in NE Pacific. Known range along the west coast of North America from Alaska to San Diego, California (Roe et al. 2007) and has not yet been observed by us in southern Oregon. We have, however, observed color varieties of *L. flavescens* (see below, Fig. 2.9) that resemble descriptions of *L. bilineatus*, but molecular data firmly identifies them as *L. flavescens*.

***Lineus flavescens* Coe, 1904 (Fig. 2.9)**

(Coe 1904; Roe et al. 2007) Individuals small, 8–120 mm in length (Roe et al. 2007) and 1–2 mm in width. Head shape is obtuse; ventral body color is more pale than dorsal; body shape, in cross-section, is slightly compressed dorso-ventrally, but not flattened and equal throughout; two elongated eyes anteriorly resemble commas; longitudinal cephalic slits present; caudal cirrus absent. Two color varieties found: one with bluish or purplish anterior, dark olive green posterior and short, rectangular white patch anteriorly, between two laterally elongated pigmented areas (putative ocelli) (Fig. 2.9B); the second, uniformly pale greenish or yellowish with pink brain visible through the body wall and an anterior white patch between two putative ocelli; the white patch extends as a conspicuous longitudinal mid-dorsal yellow line, posteriorly (Fig. 2.9A). Adults occur intertidally and subtidally (to depths of 100 m or more, Gibson 1995) under rocks, amongst algae and within sand and mud. Juveniles have been collected with a plankton

net and planktotrophic pilidium larvae were collected frequently from December to February. Oocytes are 75 µm in diameter and have one or several reddish patches. Range from southern Oregon to southern California (Roe et al. 2007).

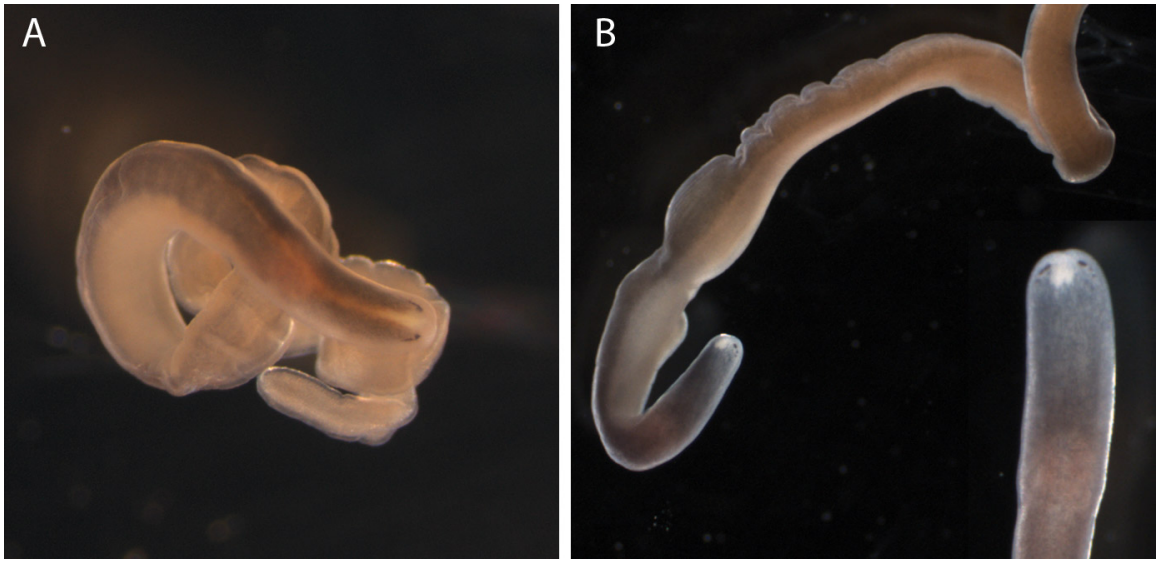


Figure 2.9. Two color varieties of the pilidiophoran species, *Lineus flavescens*. (A) Individual with olive green color dorsally, yellow streak anteriorly, and pink brain visible through the body wall. (B) Individual with blue and violet anterior and white anteriormost patch (inset).

***Lineus pictifrons* Coe, 1904**

(Coe 1904; Corrêa 1964; Roe et al. 2007) Known range from Washington to Mexico (Coe 1904; Gibson 1995; Roe et al. 2007) but has not yet been observed, by us, in southern Oregon. No sequence data are available for this species.

***Lineus ruber* Müller, 1774**

(Müller 1774 (=*Fasciola rubra*); Corrêa 1964; Roe et al. 2007) Apparent widespread distribution may be due to extensive synonymies (see Gibson 1995). NEP distribution from Alaska to Monterey, California is reported (Roe et al. 2007). However, this species has not been observed in southern Oregon, by us, or in central California, Puget Sound or Alaska (Roe et al. 2007). *Lineus ruber* is morphologically similar to a closely related

species, *Ramphogordius sanguineus* (see Roe et al. 2007), whose presence in the area we confirm based on our collections in Oregon, and samples obtained by colleagues from California. Sequences in our analysis are from specimens collected in Sweden and the UK (Sundberg and Saur 1998; Chen et al. 2010).

***Lineus rubescens* Coe, 1904**

(Coe 1904, 1905; Roe et al. 2007) Known range in California from San Francisco to San Diego (Coe 1904; Gibson 1995; Roe et al. 2007), but has not yet been observed, by us, in southern Oregon. Available COI sequence data for this species (EF124971) are identical to our *L. flavescens* sequences (Fig. 2.2B). Coe's descriptions (1904, 1905) of *L. rubescens* describe a worm that is much different in color (e.g., pink or rosy to bright pinkish red) than our specimens of *L. flavescens* (see Plate 3, Fig. 33, Coe 1905). Whether *L. rubescens* and *L. flavescens* are two color morphs of the same species as suggested by this sequence, remains to be determined.

***Lineus torquatus* Coe, 1901**

(Coe 1901; Roe et al. 2007) Distribution includes much of the north Pacific with northeast range from Alaska to northern California (Gibson 1995; Roe et al. 2007). We have yet to observe this species in southern Oregon, but have sequence data from specimens collected in Japan (Andrade et al. 2012).

***Lineus viridis* Müller, 1774**

(Verrill 1879 (=*Fasciola viridis*); Roe et al. 2007) Widespread distribution, with extensive synonymies (Gibson 1995), in the northern hemisphere, where it co-occurs with the closely related, but different species, *L. ruber* (Rogers 1992). NEP distribution only noted in Alaska (Coe 1901; Roe et al. 2007) and this species has not been observed in southern Oregon, by us, or in central California, Puget Sound or Alaska (Roe et al. 2007). Morphologically similar to the closely related species *Ramphogordius sanguineus* (see

Roe et al. 2007). Sequences in our analysis are from specimens collected in Germany (Andrade et al. 2012).

***Lineus* sp. "crescent"**

Body color dark to light brown; pink brain visible through the body wall; white half-moon shape at anterior tip, not unlike a fingernail; body rounded in cross-section anteriorly and only slightly flattened posteriorly, without sharp margins; longitudinal cephalic slits present; eyeless; no caudal cirrus. Single observed individual was several centimeters in length and 1 mm in width and was collected from the rocky intertidal, amongst *Phyllospadix* spp. root masses; rare. Larvae collected in winter months; other reproductive characters and range outside of southern Oregon unknown.

***Lineus* sp. "red" (see Fig. 6.1C in Chapter VI)**

Body color somewhat variable, including deep red brown to yellow ochre; head shape rectangular or slightly rounded; head is only slightly marked from body; anterior body rounded in cross-section, dorso-ventrally flattened and wrinkled posteriorly; longitudinal cephalic slits present; pink to red brain is sometimes visible through the anterior body wall; eyeless; caudal cirrus absent. Average size is 6–10 cm in length and 2 mm in width. Intertidal sandflats; common. Also found under rocks in gravel and among root masses of *Phyllospadix* spp. Reproductive in winter months and proceeds via a planktotrophic pilidium larva (Hiebert and Maslakova 2015b; Chapter VI). Range currently unknown outside of southern Oregon.

***Lineus* sp. 1, 2, and Heteronemertea gen. sp. 5**

At least three *Lineus* species are represented by larvae for which we have yet to find adults. Two of these species we find as larvae in the plankton frequently and we are confident that the adults live nearby – *Lineus* sp. 1 and 2. It is possible that *Lineus* sp. 2 is actually two closely related species based on our COI phylogeny (Fig. 2.2B) and genetic divergence data (2% divergence between two groups for 16S and 8.5% for COI,

see Appendix F). The third species in the genus *Lineus*, we have only encountered once (Heteronemertea gen. sp. 5) (Table 2.1, Fig. 2.2).

***Maculaura* Hiebert and Maslakova, 2015a**

***Maculaura alaskensis* Hiebert and Maslakova, 2015a (Fig. 2.10A; Chapter III)**

(Coe 1901 (=*Micrura alaskensis*, in part); Roe et al. 2007) Body color pale pink, salmon or milky flesh-toned; head shape rectangular; eyeless; rounded anteriorly and flattens posteriorly, in cross section; longitudinal cephalic slits present; caudal cirrus present and begins with abrupt transition from posterior-most body. Average size is 3–4 cm in length and 1–2 mm in width. Morphologically similar to *M. aquilonia* and *M. cerebrosa* (Fig. 2.10A). Intertidal in sandflats; common. Reproduction proceeds via planktotrophic pilidium larva (Maslakova 2010; Hiebert and Maslakova, 2105a; Chapter III). Oocytes are without chorion and 75 µm in diameter and sperm head length is 5 µm. Known range is San Juan Island, WA to southern Oregon.

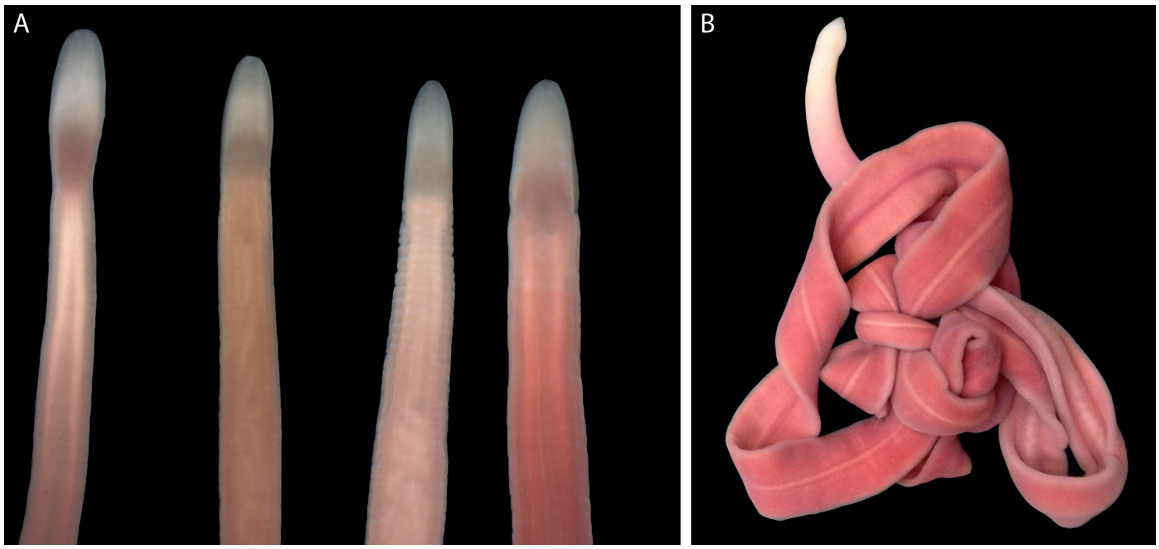


Figure 2.10. Pilidiophoran species in the genus *Maculaura.* Anterior of individuals relaxed in $MgCl_2$ including *Maculaura cerebrosa, M. aquilonia, M. alaskensis, M. oregonensis* (A, left to right). (B) *Maculaura magna*, entire specimen with anterior at upper left.

***Maculaura aquilonia* Hiebert and Maslakova, 2015a (Fig. 2.10A; Chapter III)**

Body color pale pink, salmon or milky flesh-toned; a brownish hue is apparent anteriorly in freshly collected specimens; head shape rectangular; eyeless; rounded anteriorly and flattens posteriorly, in cross section; longitudinal cephalic slits present; caudal cirrus present and begins with abrupt transition from posterior-most body. Average size is 3–4 cm in length and 1–2 mm in width. Morphologically similar to *M. alaskensis* and *M. cerebrosa* (Fig. 2.10A). Intertidal in mudflats; less common than *M. alaskensis* and *M. cerebrosa*. Reproduction proceeds via planktotrophic pilidium larva (Hiebert and Maslakova 2015a; Chapter III). Oocytes are without chorion and 90–100 μm in diameter and sperm head length is 7.5 μm. Known range includes the northeast Pacific from Alaska to southern Oregon, and Sea of Okhotsk.

***Maculaura cerebrosa* Hiebert and Maslakova, 2015a (Fig. 2.10A; Chapter III)**

Body color pale to dark pink, salmon or milky flesh-toned; conspicuous pink pigment of the brain is visible through the body wall anteriorly; head shape rectangular; eyeless; rounded anteriorly and flattens posteriorly, in cross section; longitudinal cephalic slits present; caudal cirrus present and begins with gradual transition from posterior-most body. Average size is 5–10 cm in length and 2–4 mm in width. Morphologically similar to *M. alaskensis* and *M. aquilonia* (Fig. 2.10A). Intertidal under rocks and amongst shell hash and gravel, among root masses of *Phyllospadix* sp.; common. Reproduction proceeds via planktotrophic pilidium larva (Hiebert and Maslakova 2015a; Chapter III). Oocytes are with chorion and 95 μm in diameter and sperm head length is 10 μm. Known range includes southern Oregon to Crescent City, California.

***Maculaura magna* Hiebert and Maslakova, 2015a (Fig. 2.10B; Chapter III)**

Body color pale white anteriorly and dusty rose to dark pink posteriorly; head shape ovate; eyeless; rounded anteriorly and flattens posteriorly (in cross section), without distinct margins; longitudinal cephalic slits present; caudal cirrus present. The largest *Maculaura* species; average size is 20–30 cm in length and 3–4 mm in width. Intertidal

in sandflats; not uncommon. Reproduction proceeds via planktotrophic pilidium larva (Hiebert and Maslakova 2015a; Chapter III). Oocytes are with chorion and 125 μm in diameter and sperm head length is 15 μm. Known distribution currently limited to southern Oregon.

***Maculaura oregonensis* Hiebert and Maslakova, 2015a (Fig. 2.10A; Chapter III)**

Body color pale white anteriorly and dark pink to red posteriorly; head shape ovate; eyeless; rounded anteriorly and flattens posteriorly (in cross section), without distinct margins; longitudinal cephalic slits present; caudal cirrus present. Average size is 8–15 cm in length and 3–5 mm in width. Intertidal in sandflats; rarest *Maculaura* species. Reproduction currently unknown. Oocytes morphology unknown and sperm head length is 7–8 μm (Hiebert and Maslakova 2015a; Chapter III). Known distribution currently limited to southern Oregon.

***Micrura* Ehrenberg, 1831**

As is the case for *Cerebratulus* and *Lineus* (described above), *Micrura* is a nemertean 'mega-genus', is almost certainly non-monophyletic (Sundberg and Saur 1998; Schwartz and Norenburg 2001; Sundberg et al. 2001; Strand and Sundberg 2005; Schwartz 2009; also see Fig. 2.2), and is in need of revision. Only the species shown to be closely related to the type species, *M. fasciolata Ehrenberg, 1828* should remain within *Micrura*, while others should be transferred to other existing or new genera as appropriate. Although a revision of this entire genus is outside the scope of this manuscript, we revised and transferred five species, previously fitting the description of *Micrura alaskensis*, to a newly designated genus *Maculaura* (Hiebert and Maslakova 2015a, above). For other *Micrura* species, however, we refer to species by current names, and provisionally identify unknown samples as "*Micrura*" if they group within monophyletic clades comprising *Micrura* sequences, otherwise fit the rather vague morphological definition of *Micrura,* or exhibit larval morphology similar to other species identified as *Micrura.*

***Micrura coei* Coe, 1905**

(Coe 1905 (=*Micrura pardalis*); Gibson 1995 (renamed); Roe et al. 2007) Known range from Monterey Bay to Mexico (Gibson 1995; Roe et al. 2007), has not been observed by us in southern Oregon. No sequence data are available for this species.

***Micrura* sp. "not coei" (Fig. 2.11)**

Body color pale cream ventrally with densely arranged longitudinal dark stripes of irregular length dorsally (in larger individuals); gradual but conspicuous transition between dorsal and ventral body color; pink brain is visible through the body wall anteriorly; large (350–400 μm) pink oocytes (Fig. 2.11A, inset) also visible through body wall, in females, posteriorly; prominent caudal cirrus present; head shape is rectangular with numerous (10–18) ocelli running laterally along each cephalic slit (Fig. 2.11B, arrowheads); longitudinal cephalic slits present; body equally rounded in cross-section throughout. Individuals are 10–25 mm in length and 1–2 mm in width. Movement occurs by shifting body weight laterally from side to side. Individuals co-occur in rocky intertidal and amongst *Phyllospadix* spp. root masses with *Micrura* sp. "dark", *M.* sp. "albocephala" and Lineidae gen. sp. "large eggs", but is the least common of these species. Reproductive individuals (females only) collected in winter months. The range of this species is currently not known outside of southern Oregon. The morphology of this species is similar to the description of *Micrura coei* (Coe 1905) including longitudinal dorsal striping and antero-lateral ocelli. However, Coe's descriptions in 1905 and 1940 indicate reproductive females in late summer which had bright yellow eggs. Furthermore, the development in *M. coei* proceeded as pilidium larvae (Coe 1940). Although we have not observed development in *Micrura* sp. "not coei", planktotrophic pilidium larvae typically develop from eggs that are much smaller (approximately 75–160 μm in diameter, Stricker 1987; Schwartz 2009; Maslakova 2010) and the egg size of this species is strongly suggestive of lecithotrophy (see Maslakova and Hiebert 2014).

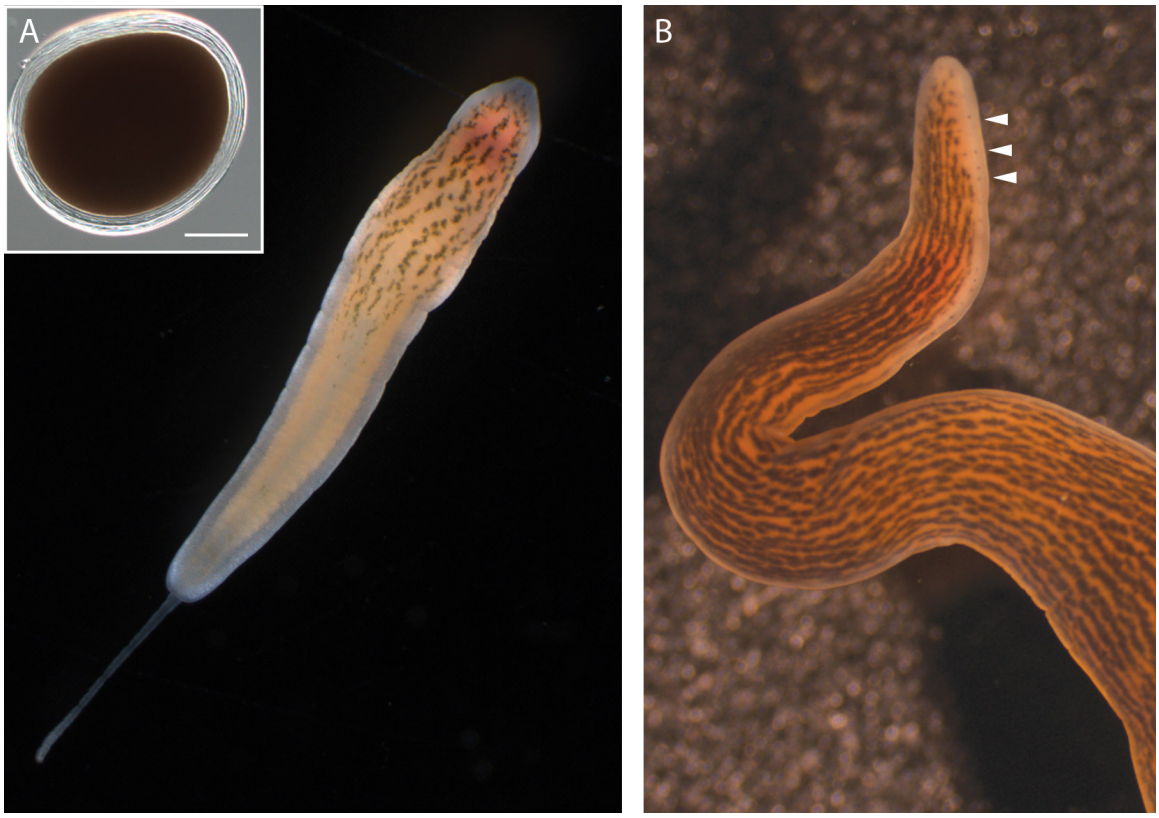


Figure 2.11. The undescribed pilidiophoran species, *Micrura* sp. "not coei"; young individual (A) and close up of anterior in larger individual (B); note lateral ocelli (arrowheads, B). Dissected oocytes are approximately 300 µm in diameter (inset, A). Scale bar 100 µm (A).

***Micrura olivaris* Coe, 1905**

(Coe 1905) Known only from California (Coe 1905; Gibson 1995; Roe et al. 2007) and has not yet been observed by us in southern Oregon. No sequence data are available for this species.

***Micrura verrilli* Coe, 1901**

(Coe 1901; Corrêa 1964; Roe et al. 2007) A brightly-colored heteronemertean that has purple pigment dorsally, broken by white transverse bands; tip of the head is triangular in shape and distinctly orange in color; regenerating posterior also orange; ventrum is pale white; caudal cirrus present; longitudinal cephalic slits present; serial gonopores visible dorso-laterally on either side as white dots on the purple background; body uniformly rounded throughout. Individuals commonly a few centimeters in length and 2–3 mm in

width; Coe (1901) noted largest individual observed 30 cm in length and 6 mm in width. Occurs in the rocky intertidal and subtidal, under rocks. Reproduction in summer months (Coe 2901, Schwartz 2009, Maslakova, pers. obs). Large (200 μm) oocytes are white with a bright reddish-purple spot (Schwartz, 2009; Maslakova pers. obs.). Coe suggests planktotrophic pilidial development, while Schwartz (2009) observed lecithotrophic larvae; we have not collected the larvae of this species in southern Oregon. Range along the west coast of North America, Alaska to Monterey Bay, California (Roe et al. 2007). We collected this species in San Juan Island, WA and southern Oregon.

***Micrura wilsoni* Coe, 1904 (see Fig. 6.1B in Chapter VI)**

(Coe 1904 (=*Lineus wilsoni*); Corrêa 1964; Roe et al. 2007) Body color dark-brown to grey-black with faint transverse constrictions of lighter color running entire length; anteriormost margin of head white; head shape rectangular; no difference between dorsal and ventral body color; body (in cross-section) rounded anteriorly and slightly flattened posteriorly, posterior body coils and wrinkles like a ribbon; longitudinal cephalic slits present; eyeless; caudal cirrus present. Individuals up to 15 cm in length and 3–4 mm in width, widest at mid-body. Not uncommon intertidal and subtidally on the open coast, under rocks and boulders. Reproductive in summer; development is via a planktotrophic pilidium larva (Hiebert and Maslakova 2015b; see also Chapter VI). Known range includes Alaska to Mexico (Roe et al. 2007); we have collected this species in San Juan Island, WA and southern Oregon.

***Ramphogordius* Rathke, 1843**

***Ramphogordius sanguineus* Rathke, 1799**

(Rathke 1799 (=*Planaria sanguinea*), Coe 1940 (=*Lineus vegetus*); Riser 1994 (=*Myoisophagos sanguineus*), 1998; Roe et al. 2007) Body color reddish-brown anteriorly, greenish-brown posteriorly; head shape rectangular; transition from head to body gradual; faint transverse constrictions running body length; longitudinal cephalic slits present; single row of 2–8 ocelli along the cephalic slit on either side of head; body

uniformly rounded throughout (in cross-section); caudal cirrus absent (see Fig. 1–2, Caplins and Turbeville 2001). Intertidal, under rocks often in clusters of several individuals. Reproduction reportedly asexual by fragmentation, but individuals with gametes have been observed in March. Distribution in the northern hemisphere includes the northeast and northwest Atlantic (type locality) and NEP from Washington to California (Gibson 1995; Roe et al. 2007). Morphologically similar to the closely related species, *L. ruber* and *L. viridis,* but coils into a spiral rather than contracting by becoming short and wide,when disturbed (see Roe et al. 2007).

***Riserius* Norenburg, 1993**

***Riserius pugetensis* Norenburg, 1993**

(Norenburg 1993) Pale white or cream body color; head-shape rectangular but rounded; pre-oral end significantly long; deep cephalic pits present and anterior to transverse V-furrow; caudal cirrus absent; body equally rounded in cross-section; epidermis sticky; eyeless. Individuals are 15 mm in length and up to 0.2 mm in width. Occurs subtidally and interstitially within the top 5–10 cm of sediment. Reproductive specimens were collected in late summer (Norenburg 1993). Distribution outside Puget Sound, Washington is currently unknown.

***Riserius* sp. "no eyes" (see Fig. 5.5, 5.6, 5.9 in Chapter V)**

Juvenile morphology resembles that of *R. pugetensis* (Hiebert et al. 2013; Chapter V). Individuals readily ingest the larvae and juveniles of the hoplonemertean, *Carcinonemertes errans* (Hiebert et al. 2013). Larvae were collected intermittently year-round with most found in July–August (see Fig. 4.19 in Chapter IV). Adults have yet to be found. Range outside of southern Oregon currently unknown.

***Riserius* sp. "eyes" (see Fig. 4.20 in Chapter IV)**

We have identified the larvae of two currently undescribed species (*Riserius* sp. "no eyes" and "eyes") in the genus *Riserius* (Hiebert et al. 2013; see also Chapter V) in

southern Oregon. *Riserius* sp. “eyes” is a species currently represented by a few laval specimens. we have yet to find adults or raise juveniles from wild-caught larvae.

“Trochonemertes”

***Micrura* sp. “dark” (Fig. 2.12A)**

A reddish-brown worm with darkest pigment anteriorly and an ovate head; longitudinal cephalic slits present; body rounded anteriorly and flattened posteriorly, in cross-section; eyeless; with prominent caudal cirrus. Individuals commonly 10–15 cm in length and 2–3 mm in width. Movement is with distinct, sharp peristaltic waves. Most common in rocky intertidal zones on the open coast, particularly among root masses of *Phyllospadix* spp. Reproductive in winter months and development proceeds via a lecithotrophic, trochophore-like larva (Maslakova and von Dassow 2012; Maslakova and Hiebert 2014; Hunt and Maslakova, unpublished). Range currently unknown outside of southern Oregon.

Figure 2.12. The undescribed pilidiophoran species, (A) *Micrura* sp. “dark”; note two peristaltic waves at anterior end and caudal cirrus at posterior and (B) *M.* sp. “albocephala” with white head and dark grey to black body.

***Micrura* sp. "albocephala" (Fig. 2.12B)**

Dark ash to black body color with distinct white anterior and ovate head-shape; longitudinal cephalic slits present; body rounded anteriorly, flattening slightly posteriorly; eyeless; caudal cirrus present. A medium-sized heteronemertean, commonly several cm in length and 2–3 mm in width. Resembles *Cerebratulus albifrons*. Co-occurs with *Micrura* sp. "dark", amongst *Phyllospadix* spp. root masses in the rocky intertidal, but less common, also found in sandflats. Reproductive in winter months and development proceeds via a lecithotrophic, superficially trochophore-like larva (Maslakova and Hiebert 2014; Hunt and Maslakova, unpublished). Range currently unknown outside of southern Oregon.

***Micrura* sp. 3 and 4 (see Figs. 4.24B, D in Chapter IV)**

There are likely at least two additional species (*Micrura* sp. 3 and sp. 4) that form a clade with *Micrura* sp. "dark" and *M.* sp. "albocephala" (above) (Table 2.1, Fig. 2.2). Morphological and molecular data suggest that this clade represents a new heteronemertean genus, "Trochonemertes", a name that references the trochophore-like larva observed in all members (Maslakova and Hiebert 2014; Hunt and Maslakova, unpublished; see Fig. 4.24 in Chapter IV; see Chapter VII).

***Zygeupolia* Thompson, 1900**

***Zygeupolia rubens* Coe, 1895**

(Coe 1895 (=*Valencinia rubens*), 1940; Roe et al. 2007) Distribution from Monterey Bay, California to Mexico (Gibson 1995; Roe et al. 2007). We have yet to observe this species in southern Oregon.

Hoplonemertea

Thirty-four intertidal hoplonemertea species are currently reported from central California to Oregon (Roe et al. 2007). Our observations and data suggest that there may be as many as 39 species (Table 2.1) in southern Oregon alone. Our hoplonemertean

phylogenetic analyses were based on alignments that were 512 bp (16S) and 658 bp (COI) in length comprising 75 (16S) and 94 (COI) sequences from adult and larval specimens (for GenBank accession numbers see Appendix C). Most of these specimens are NE Pacific species, but species from outside the NE Pacific are also included (Fig. 2.3, grey text). We lack sequence data for 14 hoplonemertean species reported from central California to Oregon (Roe et al. 2007) (see Table 2.1).

Over the course of our work we encountered eleven species (red "X" Table 2.1) that are described and previously reported from central California to Oregon (Roe et al. 2007). DNA sequence data suggests that at least two of the common local species (*Emplectonema gracile* and *Zygonemertes virescens*) previously identified as described species with wide geographic distribution, in fact, represent cryptic species, distinct from those reported from the type region (North Atlantic). Two species are described but previously unknown to the southern Oregon coast (*Tetrastemma bilineatum*, *Gurjanovella littoralis*, asterisk, Fig. 2.3), and three species are represented by orphan larvae (*Paranemertes* sp. 2, *Ototyphlonemertes* sp. 1 Table 2.1, Fig. 2.3) that either belong to a new species, or a previously reported species for which we currently lack sequence data (14 total species, see Table 2.1).

ABGD analysis, which includes all species used in our phylogenetic analysis, finds barcoding gaps from ~0.2–1.5% and ~0.2–1.4%, for COI and 16S gene regions, respectively. These analyses confirm species determined by us and indicated in our phylogeny for 16S and COI sequence data (Fig. 2.3). However, ABGD analysis over-split the species we delimited: *Zygonemertes* sp. 1 and *Gurjanovella littoralis* (COI only).

All hoplonemertean divergence values are shown in Appendix G. Average intraspecific sequence divergence for hoplonemertean species is 0.4% for the 16S gene region and 1.5% for COI (Table 2.2). The interspecific divergence range, as determined from the well-supported genera *Nipponnemertes*, *Zygonemertes* and *Carcinonemertes*, is 1.6–

24.6% for the 16S gene region and 4.1–16.5% for the COI gene region (Table 2.2). The relatively low minimum interspecific divergence for the 16S gene region is due to the species *N. pulchra* and *N.* sp 1 as well as *C. carcinophila* and *C.* sp. 414 (Fig. 2.3, Appendix G), and it is unknown whether these pairs may represent closely related but different species, or divergent populations of the same species. The relatively high upper value of intraspecific divergence for hoplonemertean species is due to *Paranemertes peregrina*, *P. californica*, *Gurjanovella littoralis* and *Zygonemertes* sp. 1. *Paranemertes peregrina* and *P. californica* have higher divergence values for COI sequence data, with 0.9 and 0.6% for 16S and 3.6 and 3.1% for COI, respectively (Fig. 2.3, Appendix G). Based on recent data (N Moss and K Plummer, unpublished), which was not included in our genetic divergence calculations, at least *P. peregrina* and potentially *P. californica* comprise cryptic species complexes (see descriptions below). Only one larval sequence was obtained for *Gurjanovella littoralis* and, although we found high divergence between this sequence and a reference sequence from GenBank (4.7%) for COI data, the 16S divergence was only 0.3% (Appendix G).

***Amphiporus* Coe, 1940**

Note that this 'mega-genus' is almost certainly non-monophyletic (Sundberg et al. 2001; Schwartz 2009; also see Fig. 2.3), and is in desperate need of revision. Ideally, only the species shown to be closely related to the type species, *A. lactifloreus* Johnston, 1828 should remain within the genus, while others should be transferred to other existing or new genera. Such a revision, however, is outside the scope of this manuscript. Therefore, we refer to species by their current names, but in many cases, this generic designation is likely incorrect.

***Amphiporus angulatus* Müller, 1774**

(Coe 1901; Gibson and Crandall 1989; Roe et al. 2007) Body dark purplish-brown dorsally, creamy-white ventrally; head shape orbicular and slightly pointed anteriorly; anterior color consistent with dorsal body color; white angular lateral patches extend

transversely, forming an anterior purplish-brown triangle (see Fig. 46 in Coe 1905); transverse V-shaped neck furrow present and posterior to cerebral ganglia; numerous ocelli present in two patches on each side (four total), one along antero-lateral margin and the second group dorsally, within the white angular patches; stylets smooth without sculpture, basis and stylet of equal length; two pouches of accessory stylets, each with 5–7 reserve stylets. Individuals large and fleshy, up to 20 cm in length and 10 mm in width. Reproductive season, based on gametes observed by Coe in Alaska (1901) is late summer. Intertidal and subtidal, under stones and amongst sand and cobble. Range from Alaska to Point Conception, California; rare south of Puget Sound, Washington; apparent circumpolar distribution includes Greenland (type locality), NW Atlantic and NW Pacific (Japan) (Gibson 1995; Roe et al. 2007). We have collected specimens in Alaska, but have yet to find this species in southern Oregon.

***Amphiporus cruentatus* Verrill, 1879**

(Verrill 1879; Coe 1905 (=*Amphiporus leptacanthus* and *A. cruentatus,* synonymized by Coe in 1943, but Gibson and Crandall 1989, listed them as two species in need of investigation (Gibson 1995)); Corrêa 1964; Roe et al. 2007) Body color transparent pale white or yellow; red blood vessels serpentine and conspicuous through the body wall; longitudinal white streak mid-dorsally (not present in all individuals); gametes visible through body wall; head narrow and anteriorly pointed with gradual transition from head to body; ocelli present in two lateral rows of 5–10 ocelli each and can extend beyond anterior cephalic grooves; transverse V-shaped neck furrow present posterior to cerebral ganglia; stylet with long (70–100 μm) and slender basis (see Plate 90JJ, Roe et al. 2007); two pouches of accessory stylets, with 2–4 stylets each; body uniformly cylindrical throughout and tapers at anterior and posterior ends. Individuals up to 25 mm in length and 2–3 mm in width. Ripe female specimen collected in March in southern Oregon. Intertidal to subtidal; amongst algae and algal holdfasts (in southern Oregon co-occurs with *Micrura* sp. “dark” and Lineidae gen. sp. “large eggs”); uncommon. Range from

Puget Sound, Washington to San Diego, California and western Atlantic coast from New England to Florida (Gibson 1995; Roe et al. 2007).

***Amphiporus flavescens* Coe, 1905**

(Coe 1901; Roe et al. 2007) Distribution reported from British Columbia, Canada to Monterey Bay, California (Roe et al. 2007) and Mexico (Gibson 1995). We have have yet to collect this species in southern Oregon, and no sequence data are available.

***Amphiporus formidabilis* Griffin, 1898**

(Griffin 1898; Coe 1904; Corrêa 1964; Roe et al. 2007) Body pinkish-white ; pink brain visible through the body wall anteriorly; large individuals with two grey longitudinal stripes on dorsal side of head and anterior body, the stripes gradually becoming more diffuse posteriorly; head shape orbicular; numerous ocelli in four groups with up to 50 ocelli each (see Plate 90BB, Roe et al. 2007); shallow transverse neck V-furrow present and posterior to ocelli and cerebral ganglia; stylet shorter than basis (see Plate 90CC, DD, Roe et al. 2007), with 6–12 accessory pouches, each with 2–3 stylets; Morphologically similar to the congener, *A. imparispinosus* (below), but larger, darker anteriorly, and with more accessory stylet pouches and ocelli. Individuals up to 30 cm in length and 3–4 mm in width. Reproductive individuals observed in Winter months in Washington and southern Oregon (Maslakova pers. obs.). Oocytes are large and dark green. Intertidal and subtidal, occurs with algae and mussels and under rocks; very common. Reported distribution in the north Pacific including Honshu and Kyushu, Japan, Alaska to Monterey Bay, California; type region Puget Sound, Washington (Gibson 1995; Roe et al. 2007).

***Amphiporus imparispinosus* Griffin, 1898**

(Griffin 1898; Coe 1901 (=*Amphiporus leuciodus*); Roe et al. 2007) Body pinkish-white; pink to brownish brain visible through the body wall anteriorly; head shape orbicular; numerous ocelli in four groups with up to 15 ocelli each (see Plate 90W, Roe et al. 2007);

shallow transverse neck V-furrow present and posterior to ocelli; stylet shorter than basis (see Plate 90X, Y, Roe et al. 2007); 2–3 accessory stylet pouches. Morphologically similar to the congener, *A. formidabilis* (above), but smaller and with fewer accessory stylet pouches and ocelli. Individuals up to 5 cm in length and 2–3 mm in width. Intertidal and subtidal, occurs with algae and mussels and under rocks; common. Reported distribution in the north Pacific including the coast of Siberia (Corrêa 1964; Gibson 1995), and Alaska to Mexico; type region Puget Sound, Washington (Gibson 1995; Roe et al. 2007).

***Amphiporus rubellus* Coe, 1905**

(Coe 1905; Roe et al. 2007) Known only from southern California (Gibson 1995; Roe et al. 2007). We have have yet to collect this species in southern Oregon and no sequence data are available. The generic designation is likely incorrect (Gibson and Crandall 1989; Roe et al. 2007).

***Amphiporus similis* Coe, 1905**

(Coe 1905; Roe et al. 2007) Synonymized with *A. imparispinosus* by some authors (Coe 1940, 1943; Gibson and Crandall 1989); unique to *A. similis* is the presence of two pairs of cephalic grooves and *A. imparispinosus* has one (Roe et al. 2007). Originally described from central California (Roe et al. 2007); distribution includes the west coast of North America from Puget Sound, Washington to Mexico (Gibson 1995). We have have yet to collect this species in southern Oregon and no sequence data are available.

***Carcinonemertes* Coe, 1902**

***Carcinonemertes errans* Wickham, 1978**

(Wickham 1978; Roe et al. 2007) Body color orange to reddish or rose pink, sometimes brownish; pink brain is easily visible through the body wall, anteriorly; no difference in color dorsally to ventrally; cerebral organs transparent and visible through body wall; two black ocelli present; body equally rounded throughout; short and stout; head not

demarcated from the body; stylet 11 µm in length and basis 35 µm in length; no accessory stylets. Individuals are small, up to 6 mm in length and 0.5 mm in width. Obligate symbiosis with the Dungeness Crab, *Cancer magister*, where individuals occur near joints and under abdominal flap of male and female crabs. The life cycle of *C. errans* is tightly coupled with that of *C. magister* as the hoplonemertean ingests eggs, grows to adulthood and reproduces on the crab host (Kuris 1993). *Carcinonemertes errans* can significantly reduce host crab egg clutch size and crabs may seek parasite refuge in less saline waters (Dunn 2011). Wild-caught *C. errans* larvae (see Fig. 4.42 in Chapter IV) occur October–May and are recognizable from juveniles or adults by the presence of two pairs of ocelli, the posterior pair of ocelli are immediately in front of cerebral ganglia and very close together, appearing as a single mid-dorsal ocellus. Juvenile and larval *C. errans* are readily ingested by another nemertean, the heteronemertean *Riserius* sp. "no eyes" (Hiebert et al. 2013; see Chapter V). Range includes Alaska to Monterey Bay, California; type locality Bodega Bay, California (Wickham 1978; Roe et al. 2007). *Carcinonemertes errans* shares its range with the morphologically similar congener, *C. epialti* (below) and characters that differentiate the two species include the presence of epidermal spots and a longer stylet in *C. errans*; *C. epialti* individuals are often found ensheathed, but *C. errans* are not (Wickham 1978). Furthermore, *Carcinonemertes errans* is believed to be host specific, while *C. epialti* is not. However, recent molecular work among individuals in the genus *Carcinonemertes* suggests that *C. errans* and *C. epialti* may not be distinct species after all and may instead be a single and non-host specific egg predator (J. Norenburg, personal communication, in Dunn 2011).

***Carcinonemertes epialti* Coe, 1902**

(Coe 1902; Roe et al. 2007) Reported distribution includes the Pacific coast of North America (Gibson 1995; Roe et al. 2007). Occurs on the legs and abdomen of crab species (e.g., *Pugettia producta, Hemigrapsus oregonensis, H. nudus, Pachygrapsus crassipes*), often within a sheath (Wickham 1978), and feeds on their developing embryos

(Roe et al. 2007). *Carcinonemertes epialti* shares a range with *C. errans*, the latter species is believed to differ from *C. epialti* in being host-specific but recent molecular work among individuals in the genus *Carcinonemertes* suggests otherwise (see. *C. errans*, above).

***Zygonemertes* Montgomery, 1897**

***Zygonemertes albida* Coe, 1901**

(Coe 1901; Roe et al. 2007) Distribution from British Columbia, Canada to Monterey Bay, California (Roe et al. 2007*)* and Mexico (Gibson 1995). We have yet to collect this species in southern Oregon and no sequence data are available.

***Zygonemertes thalassina* Coe, 1901**

(Coe 1901; Roe et al. 2007) Distribution from, the type region, Alaska to Point Reyes, California (Roe et al. 2007). We have yet to collect this species in southern Oregon and no sequence data are available.

***Zygonemertes virescens* Verrill, 1879**

(Verrill 1879 (*=Amphiporus virescens)*; Coe 1905; Corrêa 1964; Roe et al. 2007) Reported distribution of *Zygonemertes virescens* is from Alaska to Mexico, with type locality in the northwestern Atlantic. Molecular analysis of our specimens, which were collected in southern Oregon, and Atlantic specimens suggests cryptic speciation rather than widespread distribution for *Z. virescens* (Fig. 2.3, Appendix G). Thus, we refer to southern Oregon specimens that are morphologically similar to *Z. virescens* as a distinct species, *Zygonemertes* sp. 1 (below). We have no evidence that true '*Z. virescens*' occurs in southern Oregon.

***Zygonemertes* sp. 1 (Fig. 2.13A–B)**

Pale green to bluish green, with underlying hues of yellow; pink brain visible through body wall anteriorly; head shape ovate but angular, with short cephalic furrows at widest

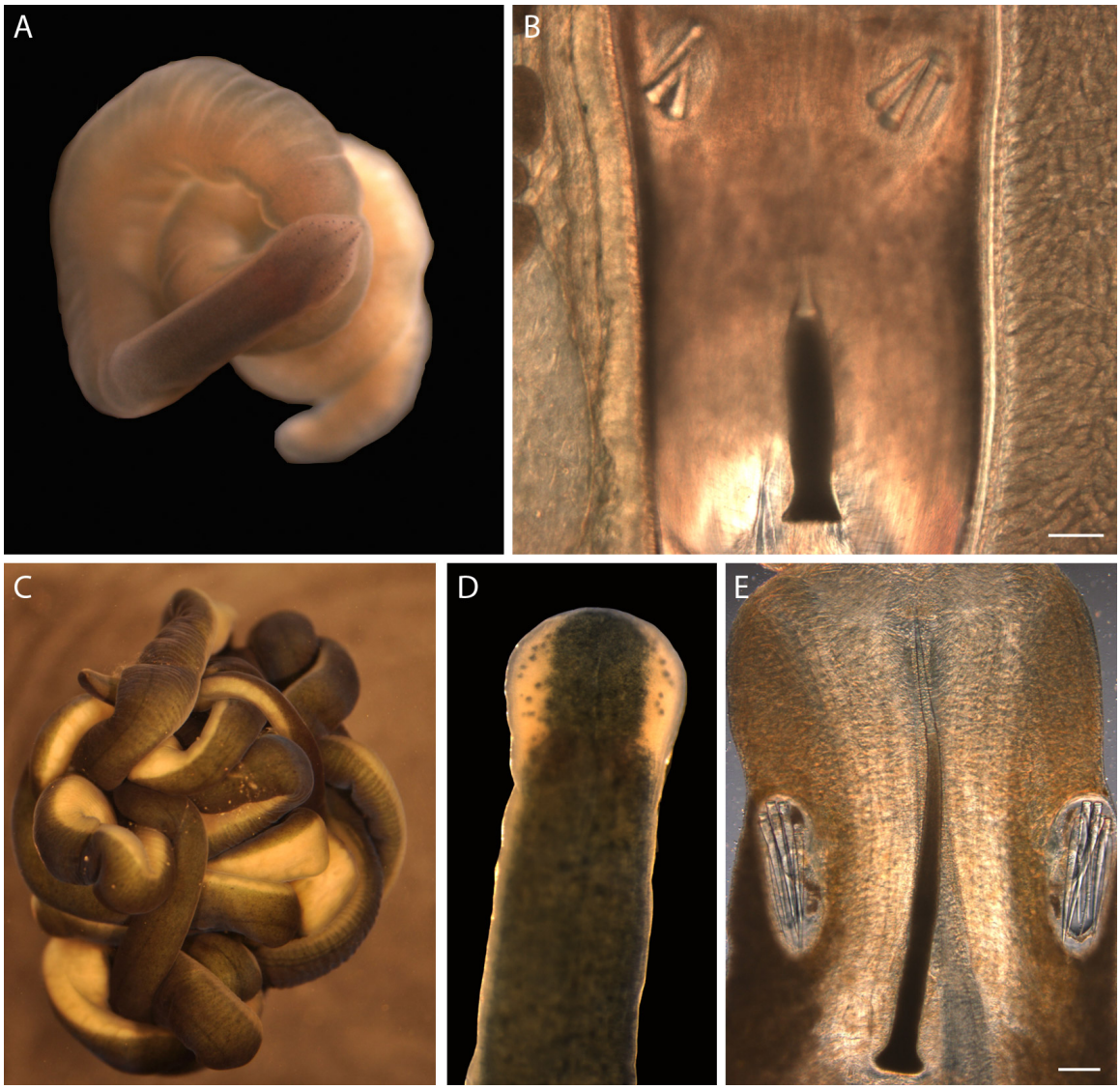


Figure 2.13. Hoplonemertean species *Zygonemertes* sp. 1 (A–B) and *Emplectonema* sp. 1 (C–E). *Zygonemertes* sp. 1 individual (A) and central stylet with two accessory stylet pouches (B). Several *Emplectonema* sp. 1 individuals entangled (C), anterior of individual relaxed in $MgCl_2$ (D), and central stylet with two accessory stylet pouches (E). Scale 100 μm (B, E).

point; head meets body with transverse V-shaped furrow; body equally rounded and somewhat dorso-ventrally flattened in cross section; numerous ocelli originate at anterior tip of head and extend laterally along lateral margins; stylet smooth, 1/3 as long as basis; 2–3 accessory stylets per pouch (Fig. 2.13B). Size 10–15 cm in length and 2–3 mm in width. Intertidal and subtidal; under rocks and amongst algae; common. Ripe individuals were observed in October and larvae were collected in winter months.

Morphologically similar to the description of *Z. thalassina* except with 2–3 accessory stylets instead of 4–5 (Roe et al. 2007). *Zygonemertes* sp. 1 fits the description of *Z. virescens* Verrill, 1879, described from the northwest Atlantic coast (=*Amphiporus virescens*), however, our molecular data suggests that specimens from southern Oregon are different from those on the Atlantic coast (e.g., Florida, Appendix C). Supposed range of *Z. virescens* includes British Columbia, Canada to Mexico as well as Atlantic coasts. The range of *Zygonemertes* sp. 1 is currently unknown outside of southern Oregon, but we hypothesize that reports of *Z. virescens* in this region are actually *Zygonemertes* sp. 1.

***Emplectonema* Stimpson, 1857**

***Emplectonema buergeri* Coe, 1901**

(Griffin 1898 (=*Emplectonema violaceum*); Coe 1901; Roe et al. 2007) Reported distribution from Alaska to Monterey Bay, California (Roe et al. 2007), Bering Sea (Pribilof Islands) and Japan (Gibson 1995). We have observed this species in Friday Harbor, WA, but have yet to encounter it in southern Oregon.

***Emplectonema gracile* Johnston, 1837**

(Johnston 1837 (=*Nemertes gracilis*), Coe 1901; Corrêa 1955; Roe et al. 2007) Reported distribution from Alaska to Mexico and on the Atlantic coast (South Carolina, Turbeville 2011), but type locality in northern Europe and molecular analysis between specimens collected in southern Oregon and Europe suggests cryptic speciation rather than widespread distribution. Instead, in southern Oregon, we find *Emplectonema* sp. 1 (below), a morphologically similar, but molecularly distinct species from *E. gracile*. We have no evidence that true '*E. gracile*' occurs in southern Oregon.

***Emplectonema* sp. 1 (Fig. 2.13C–E)**

Dark green dorsally, pale white to yellow ventrally with conspicuous and abrupt dorso-ventral transition; head shape ovate with pale yellow color laterally; in ripe individuals,

gametes are visible through the ventral body wall; numerous ocelli (Fig. 2.13D); body equally rounded throughout in cross section; caudal cirrus absent; stylet long, slender and curved; basis 2–3 times longer than stylet; two accessory stylet pouches, each with 5–7 slender stylets (Fig. 2.13E). Long slender worms approximately 10 cm in length and 2 mm in width. Intertidal; under and on rocks among barnacles, amongst algae and common within mussel hummocks; several individuals often entangled together (Fig. 2.13C). Larvae collected in October; reproductive individuals observed in Winter months in Oregon (Maslakova pers. obs.). Morphologically, *Emplectonema* sp. 1 fits the description of *Emplectonema gracile* Johnston, 1837, a species with a wide distribution including west coast of North America, northern Europe (type region) and the Mediterranean. Our molecular analysis suggests cryptic speciation rather than a widespread distribution and we hypothesize that *Emplectonema* sp. 1, found in the NEP is a different species than *E. gracile* from other geographic regions (e.g., United Kingdom).

***Gurjanovella* Uschakov, 1926**

***Gurjanovella littoralis* Uschakov, 1926**

(Uschakov 1926; Chernyshev 1998)

Gurjanovella littoralis is a species described from the White Sea in northwestern Russia and has not been previously reported in the NEP (Roe et al. 2007). We have collected a single adult individual at False Bay, San Juan Island, WA and have encountered a larva of this species in southern Oregon in March 2013 (see Fig. 4.48 in Chapter IV).

***Malacobdella* Blainville, 1827**

Species of this genus are obligate commensals of bivalve mollusks. These nemerteans reside inside the mantle cavity of host clams and are attached to the host by a posterior sucker.

***Malacobdella grossa* Müller, 1774**

(Müller 1774; Corrêa 1964; Roe et al. 2007) Type region is the NE Atlantic; distribution is widespread and includes the NW Atlantic and NE Pacific (Puget Sound, Washington to California) coasts (Gibson 1995; Roe et al. 2007). Occurs in the mantle cavity of clams, but specific hosts require further investigation (Roe et al. 2007). Sequence data from specimens collected in the NW Pacific and western Atlantic (Appendix C, Thollesson and Norenburg 2003) are included in our analysis, but we have not observed this species in southern Oregon.

***Malacobdella macomae* Kozloff, 1991**

(Kozloff 1991; Roe et al. 2007) Species described from specimens collected in California and Coos Bay, Oregon (Kozloff 1991); distribution Oregon to central California (Roe et al. 2007). Occurs in the mantle cavity of clams, *Macoma secta* and *M. nasuta.* Despite examining local clam species, we have have yet to collect this species and no sequence data are currently available.

***Malacobdella minuta* Coe, 1945**

(Coe 1945; Roe et al. 2007) Species described from a single specimen collected in California (Coe 1945). Distribution speculated in southern and central California based on host (*Yoldia cooperi*) range (Roe et al. 2007). We have have yet to collect this species in southern Oregon and no sequence data are currently available.

***Malacobdella siliquae* Kozloff, 1991**

(Kozloff 1991; Roe et al. 2007) Body white, semi-transparent, short and broad; undulating intestine (15–16 undulations) and gametes easily visible through body wall; testes white; oocytes pink; posterior sucker present; stylet absent; ocelli absent; anterior spatulate. Individuals up to 42 mm in length and 5 mm in width. Occurs in the mantle cavity of the razor clam, *Siliqua patula*, from Washington (type region) to Oregon (Kozloff 1991). We have encountered adults of this species inside mantle cavity of

Siliqua patula near Charleston, OR and collected planktonic larvae in February in Coos Bay, OR.

***Nemertopsis* Bürger, 1895**

***Nemertopsis gracilis* Coe, 1904**

(Coe 1904; Sun and Dong 1998; Roe et al. 2007) Reported distribution from Puget Sound, Washington to Mexico (Gibson 1995; Roe et al. 2007); type region in California (Coe 1904), however, we have not observed this species in southern Oregon and no sequence data are currently available.

***Nipponnemertes* Friedrich, 1968**

***Nipponnemertes bimaculatus* Coe, 1901**

(Coe 1901(=*Amphiporus bimaculatus*); Gibson and Crandall 1989; Roe et al. 2007 (=*Amphiporus bimaculatus*)) Body color dark orange to red dorsally and pale pink ventrally; in ripe individuals, greenish oocytes are visible through the ventral body wall of females, and whitish, pinkish or yellowish testes are visible; between the gut diverticula of males. Head pale white with two triangular patches of dark brown dorsally; pale longitudinal mid-dorsal line present anteriorly; head somewhat triangular in shape; four groups of 5–16 ocelli in pale head region as well as two groups of 2–5 ocelli posterior to anterior cephalic grooves; anterior cephalic grooves with secondary furrows; transverse V-shaped furrow present; stylet is long (e.g., 120 μm in 100 mm long individual, Coe 1901) and slender and about twice as long as basis (see Plate 89O, Roe et al. 2007); with 2–4 accessory stylet pouches; proboscis readily everted and pink; body dorso-ventrally flattened in cross-section throughout, but margins are not sharp; individuals readily swim when placed in fresh seawater, although not as well as *Cerebratulus* cf. *marginatus* or *C. californiensis*. Individuals 5–15 cm in length and 4–6 mm in width. Reproductive individuals collected in May; in one instance, reproductive individuals were observed washed ashore in large numbers in a small cove in southern Oregon and were spawning on the surface of the sand out of water. Intertidal and

subtidal, under rocks and amongst algae, within kelp holdfasts subtidally. Range from Alaska to Mexico (Gibson 1995; Roe et al. 2007).

Oerstedia **Quatrefages, 1846**

Oerstedia dorsalis **Abilgaard, 1806**

(=*Tetrastemma dorsalis, T. dorale, Oerstedia dorsale, Planaria dorsalis* Abildgaard 1806; Bürger 1895; Envall and Sundberg 1993; Roe et al. 2007) Wide distribution reported (but cryptic speciation is likely, see Sundberg et al. 2009a) including, the NEP from Washington to Mexico, the N Atlantic (North American and European coasts); type locality is in northern Europe (Gibson 1995; Roe et al. 2007). We have not observed this species in southern Oregon and have sequence data from Sweden (Strand and Sundberg 2005; Sundberg et al. 2009a).

Ototyphlonemertes **Diesing, 1863**

Ototyphlonemertes species are mesopsammic and easily overlooked. They are recognizable by the presence of a pair of statocysts in the posterior region of ventral cerebral ganglia (Envall and Norenburg 2011). Along the NEP, *O. spiralis* was originally described from San Diego, California, and *O. americana* was originally described from San Juan Island and Puget Sound, Washington (Coe 1940; Gerner 1969), but has since been collected just south of San Francisco, California (Roe et al. 2007). Although only *O. americana* is reported for the most recent intertidal guide from central California to Oregon (Roe et al. 2007), the presence of other *Ototyphlonemertes* species in this region is likely. In fact, adults of what may be *O. macintoshi* (Norenburg pers. comm.) were collected by SA Maslakova intertidally near Coos Bay, OR, but confirmed identity awaits sequence data (currently unavailable).

Ototyphlonemertes americana **Gerner, 1969**

(Gerner 1969; Roe et al. 2007) Distribution from Puget Sound, Washington south to San Francisco, California (Roe et al. 2007) and the Galapagos Islands (Gibson 1995). No

sequence data are available for *O. americana* and we have yet to observe adults of this species in southern Oregon.

***Ototyphlonemertes* sp. 1**

We found one *Ototyphlonemertes* species (*O*. sp. 1) as advanced larvae in the plankton in Coos Bay (see Fig. 4.52 in Chapter IV). These may be the larvae of *O. americana* because their statocysts contain a polystatolith with multiple (more than 12) granules (Roe et al. 2007), but this identification requires confirmation by sequence data, which are not currently available.

***Pantinonemertes* Moore and Gibson, 1982**

***Pantinonemertes californiensis* Gibson, Moore and Crandall, 1982**

(Gibson, Moore and Crandall 1982; Roe et al. 2007) Body color varies from grayish green, light tan to bluish; color darkest dorsally, particularly at midline; more pale ventrally; when ripe posterior body regions are pink (females) or whitish-grey (males), due to the color of gametes; two pairs of cephalic grooves, anterior pair shallow and reduced, posterior pair meet in shallow V-shaped furrow; head shape slightly bilobed or heart-shaped, anteriorly (see Fig. 1, Gibson, Moore and Crandall 1982); two groups of ocelli, one anterior and one posterior; anterior groups contain 5–100 ocelli, posterior groups with fewer (up to 50) (see Fig. 1, Gibson, Moore and Crandall 1982); stylet ~125 μm in length; basis equal in width throughout and at least twice stylet length; 2 accessory pouches, with 1–5 stylets each; body nearly equal diameter throughout, slightly flattened posteriorly. Individuals up to 2–45 cm in length and 2–3 mm in width. High intertidal and supra-littoral, occurs under rocks amongst barnacles and algae (e.g., *Fucus* sp.); common in patches. Range from Puget Sound, Washington to southern California (Gibson 1995; Roe et al. 2007); type region Tomales Bay, California (Gibson, Moore and Crandall 1982). Reproduction takes place in June–July (Roe 1993) and development via a lecithotrophic planuliform larva has been described (Hiebert et al. 2010). The generic

designation is likely incorrect (Gibson 1995;; Roe et al. 2007, Maslakova and Norenburg 2008).

***Paranemertes* Coe, 1901**

***Paranemertes californica* Coe, 1904**

(Coe 1904; Roe et al. 2007) Orange and pale yellow to pinkish flesh color; almost translucent; reddish brain (anteriorly) and grayish dark green intestine (posteriorly) are visible through the body wall; ventrum only slightly paler than dorsum, if at all; two pairs of cephalic grooves, anterior pair deep and conspicuous, posterior pair meet mid-dorsally in shallow V-shaped furrow; head shape pointed anteriorly and rounded, sometimes wider than body, and retractable into anterior groove; four clusters of 2–3 ocelli: anterior pair near the anterior tip of head, posterior pair immediately in front of cerebral ganglia (see Plate 89H, Roe et al. 2007); body nearly equal diameter throughout, slightly flattened posteriorly; central stylet slender and 1/2 basis length, with longitudinal striation at base (see Plate 89K, Roe et al. 2007); 2–6 accessory stylet pouches, with 2–3 stylets each. Individuals up to 45 cm in length and 4–5 mm in width. Intertidal and subtidal, burrowing in sand and mud. Recently spawned specimens were reported by Coe (1904) in July, and wild caught larvae, were collected by us in January and February. Range from southern Oregon to Mexico; type region southern California (Coe 1904). Our molecular analysis of COI sequence data suggests two cryptic species are present in southern Oregon. Divergence values between the two putative species groups are 5.1% for the COI gene region, but only 0.8% for 16S (Fig. 2.3). We conservatively call them all *P. californica*, but further investigation will likely reveal at least two species (see also Chapter IV).

***Paranemertes peregrina* Coe, 1901**

(Coe 1901; Corrêa 1964; Roe et al. 2007) Body deep brown to purplish-brown dorsally; pale cream to yellow ventrally (see Fig. 1, Maslakova and von Döhren 2009); pale marks dorsolaterally over groups of ocelli; two pairs of cephalic grooves, anterior pair rather

inconspicuous, posterior pair meet in shallow V-shaped furrow (see Plate 89A, Roe et al. 2007); head shape pointed anteriorly and somewhat spatulate, sometimes wider than body; body nearly equal diameter throughout, slightly flattened posteriorly; ocelli in two groups, each group with 5–12 ocelli. Stylet morphology, and color suggest multiple cryptic species; one with central stylet bearing spiral sculpture (see Plate 89B, Roe et al. 2007), two accessory stylet pouches and brownish purple body color reported from California to Juneau, Alaska (Coe 1905); the other with smooth stylet (see Plate 89C, Roe et al. 2007), four accessory stylet pouches and body color that is more purple reported from Alaska (Coe 1901,Roe et al. 2007, Maslakova pers. obs.). Individuals up to 25 cm in length and 2–4 mm in width. Intertidal and subtidal, burrowing in sand and mud; common; frequently observed on sediment surface immediately after falling tide; co-occurs with *P. sanjuanensis,* but is more common. Reproductive individuals observed in March and April, April and November, and June and November (Edmonds rocky area, Garrison Bay, Snug Harbor, Washington, Roe 1976, 1979). Oocytes are 250 μm in diameter, yellowish or pinkish and surrounded by a chorion (Maslakova and von Döhren 2009); lecithotrophic larvae settle after ten days (Maslakova and von Döhren 2009). Range from the type region in Alaska (Coe 1901) to Mexico. DNA sequence data (16S and COI gene regions) suggests three cryptic species in the NEP region: *P. peregrina*, *P.* sp. 1, and *P.* sp. 2 (Fig. 2.3, Table 2.1). Sequence data from *Paranemertes peregrina* collected from San Juan Island, Washington (AJ436805 and AJ436915, Thollesson and Norenburg) exhibits a sequence divergence of 3.5% (16S) and 9.8% (COI) from *P.* sp. 2, a species we have currently only collected as a single larval specimen collected in Feb 2014 (N Moss and K Plummer, unpublished). Meanwhile, divergences between the species we collect as both adults and larvae in southern Oregon, which matches the above description of *P. peregrina*, we refer to as *P.* sp. 1. Divergence values between *P. peregrina* and *P.* sp. 1 are 2.3% (16S) and 8.9% (COI) (see below).

***Paranemertes* sp. 1**

Individuals up to 25 cm in length and 2–4 mm in width and morphologically similar to *Paranemertes peregrina*. Further investigation is need to determine if *P.* sp. 1 is one of two cryptic species based on color and stylet morphology reported by Coe (1905) and Roe et al. (2007). Body deep brown to purple dorsally; pale cream ventrally; pale marks dorsolaterally over groups of ocelli; two pairs of cephalic grooves, anterior pair rather inconspicuous, posterior pair meet in shallow V-shaped furrow; head shape pointed anteriorly and somewhat spatulate, sometimes wider than body; body nearly equal diameter throughout, slightly flattened posteriorly; ocelli in two groups, with up to 12 ocelli in each group. Adults are common in many habitats, particularly mudflats, in and around Coos Bay, Oregon. Early stage cleavage embryos and lecithotrophic larvae collected in Jan and Feb 2013 in Coos Bay (see Fig. 4.44 in Chapter IV). Distribution outside of southern Oregon unknown and the range of all of species within this species complex (see above) awaits further investigation.

***Paranemertes* sp. 2**

A single larval specimen was collected in Feb 2014 in Coos Bay by T Hiebert and DNA sequence analysis (N Moss and K Plummer, unpublished) reveals it is likely one of three cryptic species (see *P. peregrina*, above). We have yet to collect adults of this putative species.

***Paranemertes sanjuanensis* Stricker, 1982**

(Stricker 1982; Roe et al. 2007) Body color yellowish, beige, cream or flesh toned; with small, brown epidermal pigment spots; color similar dorsally and ventrally; red brain visible through anterior body wall; two pairs of cephalic grooves, anterior pair deep and conspicuous, posterior pair meet in shallow V-shaped furrow; head shape pointed anteriorly and widening, to sometimes wider than body, mid-way to a posterior furrow; body circular in cross-section, slightly dorso-ventrally flattened in posterior; 18–35 ocelli present in four groups along head margin that extend inward; central stylet 90 μm in

length, with spiral striations (see Fig. 25, Stricker 1982) and five accessory pouches, 2–3 stylets each; basis slender, 86 μm in length. Individuals up to 11 cm in length and 2–3 mm in width. Intertidal and subtidal, burrowing in sand and mud; common; frequently observed on sediment surface immediately after falling tide; co-occurs with *P. peregrina*, but is less common. Reproductive females observed in November (San Juan Island, Washington, Stricker 1982), however wild-caught larvae were not collected in southern Oregon. Type locality is San Juan Island, Washington (Stricker 1982), known range from Puget Sound, Washington to southern Oregon (Gibson 1995) and potentially as far south as Bodega Harbor (Roe et al. 2007).

Poseidonemertes **Kirsteuer, 1967**

Poseidonemertes collaris **Roe and Wickham, 1984**

(Roe and Wickham 1984) Cream, pink to peach or orange; occasionally with small white or brown epidermal pigment spots; grey, green and/or yellow intestinal diverticula can be seen through posterior body wall; two cephalic groove present, one anterior (most conspicuous) and one posterior to the brain; head shape pointed and retractable into anterior most body, forming a collar (see Fig. 2 in Roe and Wickham 1984); one pair of ocelli near anteriormost tip; cylindrical central stylet is 87.5 μm in length with basis 119.4 μm in length; basis with dark pigment anteriorly; two accessory stylet pouches, with 0–4 stylets each (see Fig. 10 in Roe and Wickham 1984); body short and stout; individuals 20 mm in length, 0.7–2 mm in width. Intertidal in soft sediment, sand and mud; burrows in surface sediments; common. Reproductive in late summer, oocytes are 92 x 112 μm, white or pink and covered in mucus membrane; larvae have eyespots (after 3 days), are fast swimmers, planktonic, common and collected locally from November to May. Distribution from southern Oregon to, the type locality, Bodega Harbor, California (Roe and Wickham 1984; Roe et al. 2007).

***Tetrastemma* Ehrenberg, 1831**

***Tetrastemma albidum* Coe, 1905**

(Coe 1905 (=*Prosorhochmus albidus*); Roe et al. 2007) Distribution reported from Monterey Bay, California to Mexico (Gibson 1995; Roe et al. 2007). We have yet to collect this species in southern Oregon, but have acquired COI sequence data from southern California (Maslakova and Norenburg 2008).

***Tetrastemma candidum* Müller, 1774**

(Coe 1905 (=*Fasciola candida*); Roe et al. 2007) Type region in NE Atlantic coast; widespread distribution includes the NE Pacific, from Alaska to Mexico, the Mediterranean and South Africa (Gibson 1995; Roe et al. 2007). We have have yet to collect this species in southern Oregon, but have acquired COI sequence data from individuals collected in Wales, UK (Strand and Sundberg 2005).

***Tetrastemma nigrifrons* Coe, 1904**

(Coe 1904; Corrêa 1964; Roe et al. 2007) Type locality in central California; distribution includes Washington to Costa Rica, Sea of Japan (Gibson 1995; Roe et al. 2007) and NW Pacific: Akkeshi Bay, Hokkaido, Japan (Maslakova pers. obs.). We have encountered this species in Friday Harbor, WA, but have yet to collect this species in southern Oregon and we currently lack sequence data.

***Tetrastemma quadrilineatum* Coe, 1904**

(Coe 1904; Roe et al. 2007) Distribution from Monterey Bay to Mexico (Gibson 1995; Roe et al. 2007). We have yet to collect this species in southern Oregon and no sequence data are available.

***Tetrastemma reticulatum* Coe, 1904**

(Coe 1904; Roe et al. 2007) Distribution in southern California and, potentially north to Monterey Bay (Gibson 1995; Roe et al. 2007). We have yet to collect this species in southern Oregon and no sequence data are available.

***Tetrastemma signifer* Coe, 1904**

(Coe 1904; Roe et al. 2007) Distribution in from Monterey Bay to San Diego, California (Gibson 1995; Roe et al. 2007). We have yet to collect this species in southern Oregon and no sequence data are available.

***Tetrastemma bilineatum* Coe, 1904 (Fig. 2.14A)**

(Coe 1904) Pale cream to flesh color, with two deep brown longitudinal stripes dorsally; body equally rounded in cross-section throughout; anterior and posterior ends gradually taper to a point; head shape triangular to ovate; four ocelli just outside and sometimes directly beneath brown stripes, arranged in two pairs, anterior and posterior to one another; slight constriction and transverse furrow where head meets body. Individuals small and range from 5–10 mm in length. Intertidal; under rocks and amongst *Phyllospadix* spp. root masses; common. Oocytes are 325 µm in diameter, surrounded by a chorion and yellow; females brood them in parchment tube surrounding their body in the spring (Fig. 2.14A). This species is not in current intertidal guides, but was described by Coe in 1904 from San Diego, California; the range of this species now extends northward to southern Oregon.

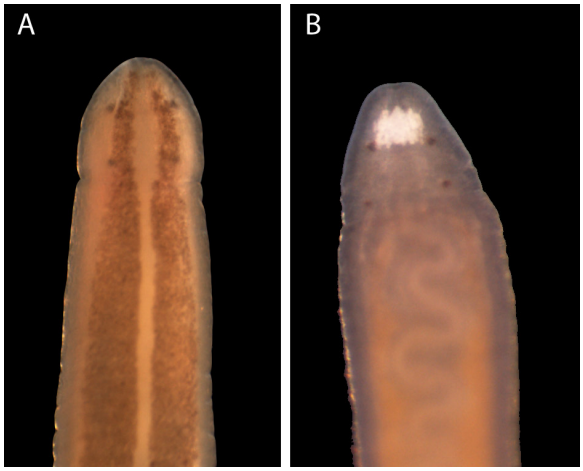


Figure 2.14. Hoplonemertean species in the genus *Tetrastemma*. (A) *Tetrastemma bilineatum*, anterior of individual relaxed in $MgCl_2$. (B) *Tetrastemma* sp. 1, anterior of relaxed individual.

***Tetrastemma* sp. 1 (Fig. 2.14B)**

Yellowish-pink, salmon, flesh colored and semitransparent; distinct white patch anteriorly and between anterior-most eyes; no variation between dorsal and ventral surfaces; coiled proboscis can be seen through body wall anteriorly; body equally rounded in cross-section throughout; anterior and posterior ends gradually taper to a point; head shape triangular to ovate; four ocelli arranged in two pairs anterior and posterior one another; slight constriction and transverse furrow where head meets body. Individuals small and range from 5–10 mm in length. Intertidal; under rocks and near the base of *Phyllospadix* spp. plants; common. Specimens most easily collected by submerging *Phyllospadix* spp. in bucket of seawater and *Tetrastemma* species crawl to water line. Reproductive biology and range outside of southern Oregon is currently unknown.

BRIDGE TO CHAPTER III

Chapter II served to update what is known of NE Pacific nemertean fauna, which includes many undescribed species. Describing all of the new diversity found since 2008 is outside the scope of this dissertation. Thus, we chose to focus formal species descriptions on the *Micrura alaskensis* species complex. This monophyletic complex comprises five closely related species, all of which partially fit descriptions of one member – *Micrura alaskensis*. In Chapter III, we designate a new heteronemertean genus for this complex of species, revise the description of *M. alaskensis,* and describe the remaining four species using an integrative approach.

CHAPTER III

INTEGRATIVE TAXONOMY OF THE *MICRURA ALASKENSIS* COE, 1901 SPECIES COMPLEX (NEMERTEA; HETERONEMERTEA), WITH DESCRIPTIONS OF A NEW GENUS *MACULAURA* GEN. NOV. AND FOUR NEW SPECIES FROM THE NE PACIFIC

This chapter of my dissertation was published in *Zoological Science* (2015, 32(6): 615-637) in the special proceedings of the 8th International Conference on Nemertean Biology, which took place in Qingdao, China in June 2014. It is co-authored with SA Maslakova, who discovered undescribed members of the *Micrura alaskensis* species complex in 2008. She noted morphological characters to differentiate species and carried out molecular work and associated analysis in the early stages of this project. SA Maslakova also aided in the production of embryonic cultures of *Maculaura aquilonia*. I contributed by collecting many specimens in OR and Juneau, AK from 2011–2015 and carried out the DNA extraction and gene region amplification from those individuals. I followed the development of two species (*M. cerebrosa* and *M. aquilonia*), carried out histological preservation, sectioning and staining for 1–2 individuals per species, compiled DNA sequences, edited and aligned them, and carried out associated phylogenetic analyses including maximum likelihood and statistical parsimony (using TCS networks). In preparing the manuscript, I compiled data, formatted images, wrote the first draft and submitted sequence data to GenBank and voucher material to NMNH.

INTRODUCTION

Micrura alaskensis Coe, 1901 is an eye-less pink worm with longitudinal cephalic slits and caudal cirrus. It is one of the most common intertidal nemerteans found in sand flats and under rocks along the Pacific coast of North America, with reported northeastern Pacific occurrence from Alaska to Southern California (Corrêa, 1964; Gibson, 1995; Roe et al., 2007). It was first described by Coe (1901) from several locations in Alaska including Glacier Bay, Sitka, Yakutat, and Prince William Sound. Coe (1905) re-

described *Micrura alaskensis* and adjusted the range southward to San Pedro, California. Coe (1940) subsequently synonymized *Micrura griffini* Coe, 1905 with *Micrura alaskensis* and reported *Micrura alaskensis* from Alaska to southern California and Mexico. At the same time, Japanese authors (Yamaoka, 1940; Iwata, 1954) reported the presence of this species in Akkeshi, Japan (Kajihara, 2007). In recent years, *Micrura alaskensis* has become a model for studies of fertilization (e.g. Stricker and Smythe, 2000, 2001, 2003; Stricker et al., 2001, 2013), larval function (von Dassow and Maslakova, 2013; von Dassow et al., 2013), and development (e.g. Maslakova, 2010; Bird et al., 2014; Swider et al., 2014; Maslakova and von Dassow, 2014; Hiebert and Maslakova, 2015) due to its abundance, accessibility, and amenability to embryological work. This species has been used in molecular phylogenies of the phylum (Thollesson and Norenburg, 2003), and its developmental transcriptome has been sequenced and assembled (Meyer et al., in prep.; Hiebert and Maslakova, unpublished). Here, we show that worms that fit the description of *Micura alaskensis* and occur intertidally in southern Oregon possess subtle morphological and not-so-subtle reproductive differences, and form at least five genetically distinct lineages. In other words, *Micrura alaskensis* is clearly not one but several closely related cryptic species. Furthermore, our preliminary experiments on cross-fertilization between some of these forms support their biological species status. Coe's original description (Coe, 1901) and revisions thereafter (Coe, 1904, 1905, 1940) clearly combine characters (e.g. body color, habitat) from several of these species. Type material does not exist, which makes it impossible to be certain which of the five species served as the basis for the original species description (Coe, 1901). It seems apparent that subsequent revisions incorporated more than one species (Coe, 1905, 1940). In order to improve nomenclatural stability and preclude species misidentification we re-describe *Micrura alaskensis* and designate a neotype, retaining the original specific name for the lineage that is the subject of many recent studies, and describe the other four as new species, based on new material from Alaska, Washington, Oregon, California, and Russia.

The genus *Micrura,* which currently contains ~12% of all described heteronemertean species (Gibson, 1995), is poorly defined and certainly non-monophyletic (Sundberg and Saur, 1998; Thollesson and Norenburg, 2003; Schwartz, 2009; Andrade et al., 2012; Kvist et al., 2014). As is the case with other nemertean mega-genera, such as *Cerebratulus* and *Lineus,* membership in *Micrura* is based on combinations of non-unique characters of internal anatomy, e.g. proboscis muscle crosses, presence/absence of caudal cirrus, etc. (Schwartz, 2009). The only reasonable solution to this taxonomic problem is to redefine the genus *Micrura* to include only those species that are closely related to the type species, *Micrura fasciolata* Ehrenberg, 1828, and to move all other species to other (new or existing) genera, as appropriate. Although *Micrura alaskensis* fits the rather vague diagnosis of the genus *Micrura* (Ehrenberg, 1828; McIntosh, 1873–1874; Bürger, 1895), it is only distantly related to the type species for this genus in molecular phylogenies (Schwartz, 2009; Hiebert and Maslakova, in prep). Incidentally, uncorrected sequence divergence values between members of the *Micrura alaskensis* species complex and *Micrura fasciolata* are approximately 20% for 16S and 18% for COI sequence data, nearing or exceeding maximum interspecific divergence values for congeneric nemertean species in well-supported monophyletic genera e.g. *Carinoma*, *Riserius*, *Nipponnemertes* (Hiebert and Maslakova, in prep). These five species of the *Micrura alaskensis* complex constitute a monophyletic group based on molecular phylogenies of the Pilidiophora (Hiebert and Maslakova, in prep), and share characters of adult anatomy and larval morphology, supporting the new genus proposed here, *Maculaura* gen. nov.

MATERIALS AND METHODS

Material examined

We collected hundreds of adult individuals in the NE Pacific that fit the broad description of *Micrura alaskensis* Coe, 1901 that has emerged over the last hundred years. Collection sites ranged from Juneau, AK to Crescent City, CA (Fig. 3.1) (collecting-permit numbers: 18512, 18586, 19353, CF-14081). Maps showing geographic locations of all collection

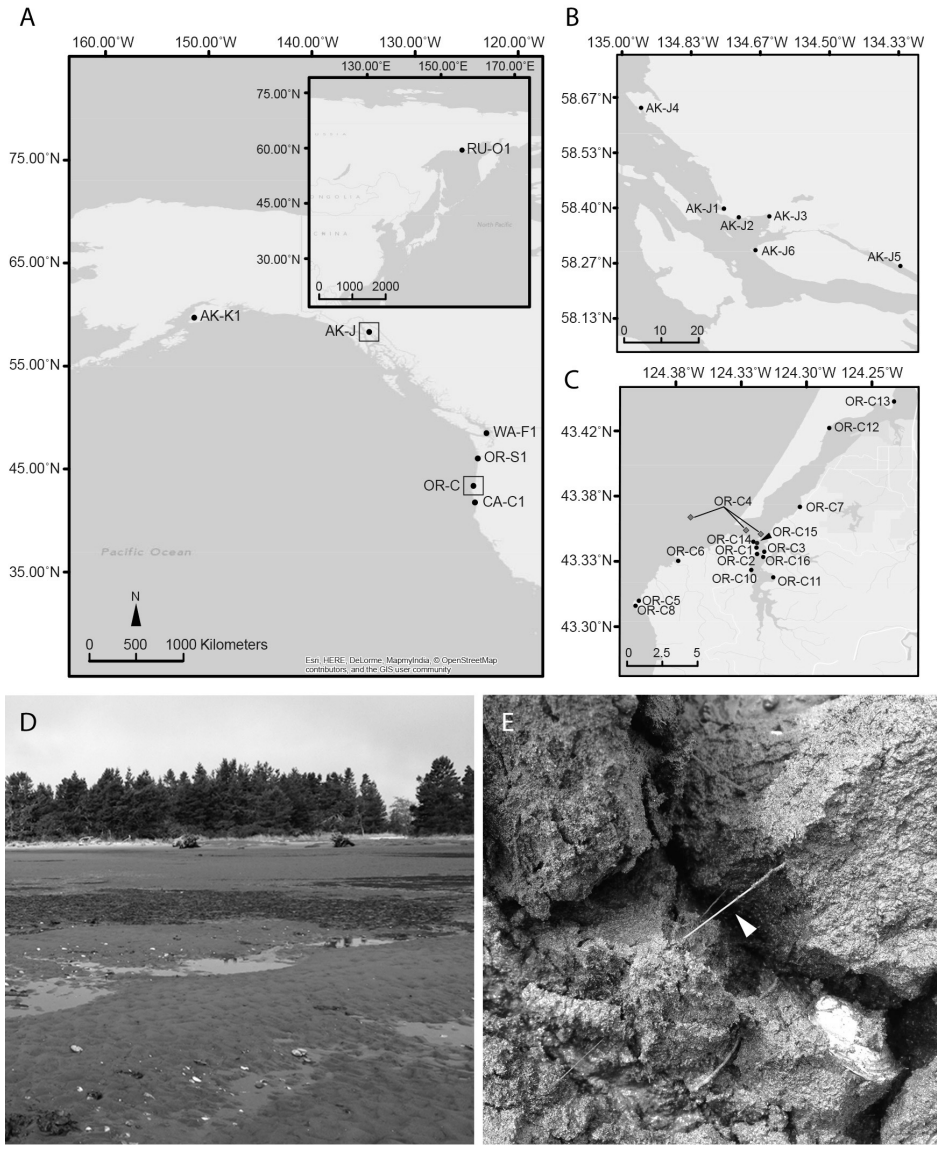


Figure 3.1 (A–C) Collection sites for members of the "*Micrura alaskensis*" species complex used in this study; **(A)** sampling sites along the shoreline of the northwestern United States in Alaska (AK), Washington (WA), Oregon (OR), and California (CA), and the Sea of Okhotsk in eastern Russia (RU, inset); **(B)** multiple collection sites in Juneau, AK; **(C)** sampling sites in Coos Bay, OR (regions outlined by boxes on A); locations where adult specimens were collected are indicated with closed circles, larval collection sites are shown as diamonds. **(D)** Typical habitat of *Maculaura* spp. is silty sand or mud in protected coves or bays; shown here is North Spit (OR-C13), near Charleston, OR. **(E)** An individual of *Maculaura alaskensis* comb. nov. stretched between mud clods.

sites were generated using ArcGIS v. 10.2. One adult preserved for molecular analysis was collected in the Sea of Okhotsk near the city of Magadan, Russia and kindly provided by Dr. Alexei V. Chernyshev (Institute of Marine Biology, Far East Branch of the Russian Academy of Sciences). The descriptions herein are based on examination of over 100 specimens (including those used for histology and molecular analysis). See the species descriptions below for detailed information on locations and habitats of each species (Table 3.1). Type and voucher material is deposited at the National Museum of Natural History, Smithsonian Institution, in Washington, D.C., USA (NMNH, see species descriptions for lot numbers) (Table 3.1). Additional material is kept at the Oregon Institute of Marine Biology, in Charleston, OR, USA (OIMB).

Thirty specimens collected near Juneau, AK were examined externally and preserved for molecular analysis at the University of Alaska, Southeast (UAS). All other specimens were examined at the OIMB. Live worms were kept in 150-ml glass dishes submerged in a sea table with running seawater at ambient sea temperature. Adults were photographed using a Leica DFC400 digital camera mounted to a Leica MZ10F dissecting microscope and accompanying software (Leica Application Suite v. 3.6) at OIMB.

Twenty-six pilidium larvae were collected using a plankton net (SeaGear, 0.5-m diameter with 153-µm mesh) in Coos Bay, from the Charleston marina docks or by boat in the Charleston Channel (OR-C4, Fig. 3.1C). Larvae were photographed individually using the same camera and software as above and an Olympus BX51 compound microscope equipped with differential interference contrast optics. For photography, larvae were gently trapped between glass slide and cover slip supported by small clay feet. Young wild-caught larvae were reared in the lab on a diet of the unicellular cryptophyte alga *Rhodomonas lens* (Pascher and Ruttner, CCMP739) in bowls of filtered seawater (FSW, 0.45 µm) for up to 10 weeks and periodically photographed. Larval identity was confirmed using DNA sequence data as described below.

Table 3.1. Collection information and associated GenBank and USNM numbers. Individual abbreviations correspond to those in Fig. 3.1 and Fig. 3.12. Larval specimens are indicated with bold text. The total number of sequences (n) used in phylogenetic analyses are shown for each species; superscripts: [1] holotype, [2] paratype, [3] topogenetype, [4] hologenetype, [5] neotype.

					Accession Number	
Abbreviation	**Collection location**	**coordinate**	**Collector(s)**	**NMNH Number**	**16S**	**COI**
Maculaura alaskensis						
OR-C1-A04	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	S. Maslakova		KP682166	-
OR-C1-C04	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	S. Maslakova		KP682167	-
OR-C1-D04	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	S. Maslakova		KP682168	-
OR-C1-E03	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	S. Maslakova		KP682169	-
OR-C1-E04	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	S. Maslakova		KP682170	-
OR-C1-E5A6	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert		KP682171	-
OR-C1-E5A7	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert		KP682172	-
OR-C1-E5A8	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert		KP682173	-
OR-C1-E5A9	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert		KP682174	-
OR-C1-F03	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	S. Maslakova		KP682175	-
OR-C1-F04	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	S. Maslakova		KP682176	-
OR-C1-G03	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	S. Maslakova		KP682177	-
OR-C1-M13	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert	1282107	KP682178	KP682051
OR-C1-M14	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert	1282106 [5]	KP682179	KP682052
OR-C1-M15	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert	1282108	KP682180	KP682053
OR-C1-M16	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert	1282109	KP682181	KP682054
OR-C1-M17	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert		KP682182	KP682055 [3]
OR-C1-M18	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert		KP682183	KP682056
OR-C1-M19	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert		KP682184	KP682057
OR-C1-MMB8	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert		-	KP682058
OR-C1-MMB9	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert		-	KP682059
OR-C10-M29	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert	1282110	-	KP682062
OR-C10-M30	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert	1282111	-	KP682063
OR-C10-M31	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert		KP682189	KP682064
OR-C10-M33	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert		KP682190	KP682065
OR-C10-M34	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert		KP682191	KP682066
OR-C10-M35	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert		KP682192	KP682067
OR-C12-M23	North Spit Boat Ramp, North Bend OR	43.4168˚N, 124.2755˚W	T. Hiebert		KP682193	KP682068

Abbreviation	Collection location	coordinate	Collector(s)	NMNH Number	Accession Number	
					16S	COI
OR-C12-M22	North Spit Boat Ramp, North Bend OR	43.4168˚N, 124.2755˚W	T. Hiebert		KP682194	-
OR-C12-M24	North Spit Boat Ramp, North Bend OR	43.4168˚N, 124.2755˚W	T. Hiebert		KP682195	KP682069
OR-C15-M5	Outer Boat Basin, Charleston OR	43.3445˚N, 124.3215˚W	T. Hiebert		KP682196	KP682070
OR-C2-103	High Tide Mudflat, Charleston OR	43.3379˚N, 124.3247˚W	T. Hiebert & S. Maslakova		-	KP682060
OR-C2-104	High Tide Mudflat, Charleston OR	43.3379˚N, 124.3247˚W	T. Hiebert & S. Maslakova		KP682185	KP682061
OR-C4-E4G8	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682186**	**-**
OR-C7-66	Domehouse Mudflat, Charleston OR	43.3691˚N, 124.2981˚W	S. Maslakova		KP682187	-
OR-C8-E2H6	Middle Cove Cape Arago, Charleston OR	43.3033˚N, 124.4017˚W	T. Hiebert & S. Maslakova		KP682188	-
OR-S1-E3B7	Necanicum River, Gearhart OR	46.0159˚N, 123.9202˚W	T. Hiebert		KP682197	KP682071
OR-S1-E3B8	Necanicum River, Gearhart OR	46.0159˚N, 123.9202˚W	T. Hiebert		KP682198	KP682072
OR-S1-E3B9	Necanicum River, Gearhart OR	46.0159˚N, 123.9202˚W	T. Hiebert		KP682199	KP682073
WA-F1-M11	False Bay, Friday Harbor WA	48.4855˚N, 123.0699˚W	S. Maslakova		KP682200	KP682075
WA-F1-M12	False Bay, Friday Harbor WA	48.4855˚N, 123.0699˚W	S. Maslakova		KP682201	KP682076
WA-F1-M13	False Bay, Friday Harbor WA	48.4855˚N, 123.0699˚W	S. Maslakova		KP682202	KP682077
WA-F1-M14	False Bay, Friday Harbor WA	48.4855˚N, 123.0699˚W	S. Maslakova		KP682203	KP682078
WA-F1-M15	False Bay, Friday Harbor WA	48.4855˚N, 123.0699˚W	S. Maslakova		KP682204	KP682079
WA-F1-M16	False Bay, Friday Harbor WA	48.4855˚N, 123.0699˚W	S. Maslakova		-	KP682080
WA-F1-M18	False Bay, Friday Harbor WA	48.4855˚N, 123.0699˚W	S. Maslakova		KP682205	KP682081
WA-F1-M19	False Bay, Friday Harbor WA	48.4855˚N, 123.0699˚W	S. Maslakova		KP682206	KP682082
WA-F1-M20	False Bay, Friday Harbor WA	48.4855˚N, 123.0699˚W	S. Maslakova		KP682207	KP682074
					n = 42	n = 32
Maculaura aquilonia						
AK-J1-E4B7	Lena Beach, Juneau AK	58.3952˚N, 134.7512˚W	L. Hiebert		KP682208	KP682084
AK-J1-E4B8	Lena Beach, Juneau AK	58.3952˚N, 134.7512˚W	L. Hiebert		-	KP682085
AK-J1-E4B9	Lena Beach, Juneau AK	58.3952˚N, 134.7512˚W	L. Hiebert		KP682209	KP682086
AK-J1-E4C1	Lena Beach, Juneau AK	58.3952˚N, 134.7512˚W	L. Hiebert		KP682210	KP682087
AK-J1-E4C2	Lena Beach, Juneau AK	58.3952˚N, 134.7512˚W	L. Hiebert		KP682211	KP682088

Abbreviation	Collection location	coordinate	Collector(s)	NMNH Number	Accession Number	
					16S	COI
AK-J1-J1	Lena Beach, Juneau AK	58.3952˚N, 134.7512˚W	T. Hiebert	1282113 [2]	KP682212	-
AK-J1-J5	Lena Beach, Juneau AK	58.3952˚N, 134.7512˚W	T. Hiebert		KP682213	KP682089
AK-J2-J10	Auke Bay, Juneau AK	58.3777˚N, 134.7239˚W	T. Hiebert		KP682214	KP682090
AK-J2-J11	Auke Bay, Juneau AK	58.3777˚N, 134.7239˚W	T. Hiebert		KP682215	KP682091
AK-J2-J12	Auke Bay, Juneau AK	58.3777˚N, 134.7239˚W	T. Hiebert		KP682216	KP682092
AK-J3-J15	Auke Creek, Juneau AK	58.3806˚N, 134.6433˚W	T. Hiebert		KP682217	KP682093
AK-J3-J16	Auke Creek, Juneau AK	58.3806˚N, 134.6433˚W	T. Hiebert		KP682218	KP682094
AK-J3-J17	Auke Creek, Juneau AK	58.3806˚N, 134.6433˚W	T. Hiebert		KP682219	-
AK-J3-J18	Auke Creek, Juneau AK	58.3806˚N, 134.6433˚W	T. Hiebert		KP682220	KP682095
AK-J3-J19	Auke Creek, Juneau AK	58.3806˚N, 134.6433˚W	T. Hiebert		KP682221	KP682096
AK-J3-J20	Auke Creek, Juneau AK	58.3806˚N, 134.6433˚W	T. Hiebert		KP682222	KP682097
AK-J3-J22	Auke Creek, Juneau AK	58.3806˚N, 134.6433˚W	T. Hiebert		KP682223	KP682098
AK-J3-J23	Auke Creek, Juneau AK	58.3806˚N, 134.6433˚W	T. Hiebert		KP682224	KP682099
AK-J4-J36	Bridget Cove, Juneau AK	58.6358˚N, 134.9462˚W	T. Hiebert		KP682225	KP682100
AK-J5-J45	Sheep Creek, Juneau AK	58.2608˚N, 134.3256˚W	T. Hiebert		KP682226	KP682101
AK-J5-J46	Sheep Creek, Juneau AK	58.2608˚N, 134.3256˚W	T. Hiebert		KP682227	KP682102
AK-J6-J48	Outer Point Douglas, Juneau AK	58.3004˚N, 134.6779˚W	T. Hiebert		KP682228	KP682103
AK-J6-J49	Outer Point Douglas, Juneau AK	58.3004˚N, 134.6779˚W	T. Hiebert		KP682229	KP682104
AK-J6-J50	Outer Point Douglas, Juneau AK	58.3004˚N, 134.6779˚W	T. Hiebert		KP682230	KP682105
AK-J6-J51	Outer Point Douglas, Juneau AK	58.3004˚N, 134.6779˚W	T. Hiebert		KP682231	KP682106
AK-J6-J52	Outer Point Douglas, Juneau AK	58.3004˚N, 134.6779˚W	T. Hiebert		KP682232	KP682107
AK-J6-J53	Outer Point Douglas, Juneau AK	58.3004˚N, 134.6779˚W	T. Hiebert		KP682233	KP682108
AK-J6-J54	Outer Point Douglas, Juneau AK	58.3004˚N, 134.6779˚W	T. Hiebert		KP682234	KP682109
AK-J6-J55	Outer Point Douglas, Juneau AK	58.3004˚N, 134.6779˚W	T. Hiebert	1282114 [2]	KP682235	KP682110
AK-J6-J56	Outer Point Douglas, Juneau AK	58.3004˚N, 134.6779˚W	T. Hiebert	1282115 [2]	KP682236	KP682111
AK-J6-J57	Outer Point Douglas, Juneau AK	58.3004˚N, 134.6779˚W	T. Hiebert		KP682237	KP682112
AK-J6-J58	Outer Point Douglas, Juneau AK	58.3004˚N, 134.6779˚W	T. Hiebert		KP682238	KP682113
AK-J6-J59	Outer Point Douglas, Juneau AK	58.3004˚N, 134.6779˚W	T. Hiebert		KP682239	KP682114
AK-J6-J60	Outer Point Douglas, Juneau AK	58.3004˚N, 134.6779˚W	T. Hiebert	1282116 [2]	KP682240	KP682115
OR-C10-E2G2	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert & S. Maslakova		KP682244	KP682126

Abbreviation	Collection location	coordinate	Collector(s)	NMNH Number	Accession Number	
					16S	COI
OR-C10-E2G3	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert & S. Maslakova		KP682245	KP682127
OR-C10-E3A3	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert & S. Maslakova		KP682246	KP682128
OR-C10-M32	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert	1282117 [2]	KP682247	KP682129
OR-C10-M37	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert	1282118	KP682248	KP682130 [3]
OR-C12-M20	North Spit Boat Ramp, North Bend OR	43.4168˚N, 124.2755˚W	T. Hiebert		KP682249	KP682131
OR-C2-102	High Tide Mudflat, Charleston OR	43.3379˚N, 124.3247˚W	T. Hiebert & S. Maslakova		KP682241	KP682122
OR-C4-205	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**G. von Dassow**		**KP682243**	**-**
OR-C4-82	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**S. Maslakova**		**KP682242**	**KP682124**
RU-O1	Sea of Okhotsk, Magadan RUSSIA	59.5620˚N, 150.7444˚W	A. Chernyshev		KP682250	KP682133
AK-K1-A2	Kachemak Bay, AK	59.4677˚N, 151.5565˚W	S. Maslakova & J. Norenburg		-	KP682116
AK-K1-C1	Kachemak Bay, AK	59.4677˚N, 151.5565˚W	S. Maslakova & J. Norenburg		-	KP682117
AK-K1-C2	Kachemak Bay, AK	59.4677˚N, 151.5565˚W	S. Maslakova & J. Norenburg		-	KP682118
AK-K1-D1	Kachemak Bay, AK	59.4677˚N, 151.5565˚W	S. Maslakova & J. Norenburg		-	KP682119
AK-K1-E1	Kachemak Bay, AK	59.4677˚N, 151.5565˚W	S. Maslakova & J. Norenburg		-	KP682120
AK-K1-F4	Kachemak Bay, AK	59.4677˚N, 151.5565˚W	S. Maslakova & J. Norenburg		-	KP682121
AK-K1-G1	Kachemak Bay, AK	59.4677˚N, 151.5565˚W	S. Maslakova & J. Norenburg		-	KP682083
OR-C16-M2	Qualman Mudlfat, Charleston OR	43.3382˚N, 124.3206˚W	T. Hiebert	1282112 [1]	-	KP682132
OR-C4-213	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**G. von Dassow**		**-**	**KP682125**
OR-C4-81	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**L. Hiebert**		**-**	**KP682123**
					n = 43	n = 51
Maculaura cerebrosa						

Abbreviation	Collection location	coordinate	Collector(s)	NMNH Number	Accession Number	
					16S	COI
CA-C1-E1A8	Crescent City, CA	41.7362˚N, 124.1744˚W	G. Paulay		KP682251	KP682134
OR-C10-M36	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert		KP682259	KP682138
OR-C13-M11	North Spit, North Bend OR	43.4366˚N, 124.2338˚W	T. Hiebert	1282120 [2]	KP682260	KP682139
OR-C13-M12	North Spit, North Bend OR	43.4366˚N, 124.2338˚W	T. Hiebert	1282121 [2]	KP682261	KP682140
OR-C14-M10	Inner Boat Basin, Charleston OR	43.3465˚N, 124.3272˚W	T. Hiebert		KP682263	KP682142
OR-C14-M9	Inner Boat Basin, Charleston OR	43.3465˚N, 124.3272˚W	T. Hiebert		KP682262	KP682141
OR-C15-M3	Outer Boat Basin, Charleston OR	43.3445˚N, 124.3215˚W	T. Hiebert	1282119 [1]	KP682264	KP682143
OR-C15-M4	Outer Boat Basin, Charleston OR	43.3445˚N, 124.3215˚W	T. Hiebert		KP682265	KP682144
OR-C15-M6	Outer Boat Basin, Charleston OR	43.3445˚N, 124.3215˚W	T. Hiebert	1282122 [2]	KP682266	KP682145
OR-C15-M7	Outer Boat Basin, Charleston OR	43.3445˚N, 124.3215˚W	T. Hiebert	1282124	KP682267	KP682146 [3]
OR-C2-107	High Tide Mudflat, Charleston OR	43.3379˚N, 124.3247˚W	T. Hiebert & S. Maslakova	1282123 [2]	KP682252	-
OR-C4-196	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682253**	**-**
OR-C6-2008	Outer Boat Basin, Charleston OR	43.3445˚N, 124.3215˚W	S. Maslakova		KP682256	-
OR-C4-210	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682254**	**-**
OR-C4-211	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682255**	**KP682135**
OR-C5-173	North Cove Cape Arago, Charleston OR	43.3096˚N, 124.3991˚W	S. Maslakova		KP682257	KP682136
OR-C6-30	Sunset Bay, Charleston OR	43.3347˚N, 124.3756˚W	S. Maslakova		KP682258	KP682137
					n = 17	n = 13
Maculaura magna						
OR-C1-M8	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert	1282125 [1]	KP682268	KP682147 [4]
OR-C2-105	High Tide Mudflat, Charleston OR	43.3379˚N, 124.3247˚W	T. Hiebert & S. Maslakova		KP682270	KP682148
OR-C2-13	High Tide Mudflat, Charleston OR	43.3379˚N, 124.3247˚W	S. Maslakova		KP682269	-
OR-C3-93	Fisherman's Grotto Mudflat, Charleston OR	43.3419˚N, 124.3193˚W	T. Hiebert & S. Maslakova		KP682271	KP682150
OR-C3-95	Fisherman's Grotto Mudflat, Charleston OR	43.3419˚N, 124.3193˚W	T. Hiebert & S. Maslakova		KP682272	-

Abbreviation	Collection location	coordinate	Collector(s)	NMNH Number	Accession Number	
					16S	COI
OR-C3-96	Fisherman's Grotto Mudflat, Charleston OR	43.3419˚N, 124.3193˚W	T. Hiebert & S. Maslakova		KP682273	KP682151
OR-C4-112	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682274**	**KP682152**
OR-C4-131	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682275**	**-**
OR-C4-177	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682276**	**-**
OR-C4-E3A6	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682277**	**-**
OR-C4-E3H4	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682278**	**-**
OR-C4-E3H6	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682279**	**-**
OR-C4-E3I1	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682280**	**-**
OR-C4-E3I3	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682281**	**-**
OR-C4-E3I5	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682282**	**KP682153**
OR-C4-LWB7	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682283**	**-**
OR-C4-LWB8	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682284**	**-**
OR-C4-LWB9	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682285**	**-**
OR-C4-LWC1	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682286**	**-**
OR-C4-LWC2	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682287**	**-**
OR-C4-LWC7	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682288**	**-**
OR-C4-LWC8	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682289**	**-**
OR-C4-LWD7	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682290**	**-**
OR-C4-LWE6	**Outer Boat Basin, Charleston OR**	**43.3445˚N, 124.3215˚W**	**T. Hiebert**		**KP682291**	**-**
OR-C5-162	North Cove Cape Arago, Charleston OR	43.3096˚N, 124.3991˚W	S. Maslakova		KP682292	-
OR-C5-163	North Cove Cape Arago, Charleston OR	43.3096˚N, 124.3991˚W	S. Maslakova		KP682293	KP682154
OR-C6-174	Sunset Bay, Charleston OR	43.3347˚N, 124.3756˚W	S. Maslakova		KP682294	KP682155
OR-C6-175	Sunset Bay, Charleston OR	43.3347˚N, 124.3756˚W	S. Maslakova		KP682295	KP682156
OR-C2-159	High Tide Mudflat, Charleston OR	43.3379˚N, 124.3247˚W	T. Hiebert & S. Maslakova		-	KP682149
OR-C3-M42	Fisherman's Grotto Mudflat, Charleston OR	43.3419˚N, 124.3193˚W	T. Hiebert	1282126 [2]	-	-
OR-C1-M43	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert & S. Maslakova	1282127 [2]	-	-

Abbreviation	Collection location	coordinate	Collector(s)	NMNH Number	Accession Number	
					16S	COI
					n = 28	n = 10
Maculaura oregonensis						
OR-C10-M25	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert	1282128 [1]	KP682299	KP682159 [4]
OR-C10-M26	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert	1282129 [2]	KP682300	KP682160
OR-C11-E2I6	Brown's Cove, Charleston OR	43.3234˚N, 124.3144˚W	T. Hiebert & S. Maslakova		KP682301	KP682163
OR-C13-E5B3	North Spit, North Bend OR	43.4366˚N, 124.2338˚W	T. Hiebert		KP682302	-
OR-C13-MMB12	North Spit, North Bend OR	43.4366˚N, 124.2338˚W	T. Hiebert		KP682303	-
OR-C13-MMB13	North Spit, North Bend OR	43.4366˚N, 124.2338˚W	T. Hiebert		KP682304	KP682164
OR-C3-94	Fisherman's Grotto Mudflat, Charleston OR	43.3419˚N, 124.3193˚W	T. Hiebert & S. Maslakova		KP682298	-
OR-C10-M27	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert		-	KP682161
OR-C10-M28	Collver Cove, Charleston OR	43.3272˚N, 124.3263˚W	T. Hiebert		-	KP682162
OR-C1-E4A2	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert		KP682297	KP682158
OR-C1-E3G6	Portside Mudflat, Charleston OR	43.3428˚N, 124.3218˚W	T. Hiebert		KP682296	KP682157
					n = 9	n = 8

Embryonic cultures

To characterize development, embryonic cultures were established in the lab when gravid males and females of each species were available, and larvae were reared to metamorphosis as previously described by Maslakova (2010). To further confirm the species status of the five lineages, we carried out cross-fertilization experiments between different forms when reproductive adults of more than one form were available. Cross-fertilization was initially attempted with the same sperm concentrations (~1/1000) for embryonic culturing of con-specifics but was increased over time to ensure that sperm concentration was not a limiting factor and to promote hybridization.

Molecular analysis

Tissue from 128 adults was preserved for molecular analysis. Two small (2 × 2 mm) pieces of tissue were preserved from each individual (one cryopreserved and kept at −80°C, and the other immersed in 80% EtOH and kept at −20°C). DNA extraction from adult tissue was carried out using a DNeasy Blood and Tissue Kit (Qiagen) or Wizard SV Genomic DNA Purification System (Promega). Tissue lysis occurred in a Nuclei Lysis solution composed of 0.5 M EDTA and proteinase K (20 mg/ml) at 56°C for 6–12 hr. Twenty-six larvae were cryopreserved whole in a small volume (< 10 μl) of FSW at −80°C after being photographed. PCR-quality DNA was obtained from larvae using Chelex matrix (InstaGene, BioRad) with initial incubation at 56°C for 30 min followed by a short (8 min) incubation at 98°C.

"Barcoding" regions of two mitochondrial genes were amplified: 16S rRNA (460–537 bp, 16S) and cytochrome *c* oxidase subunit I (658–698 bp, COI). PCR amplification was carried out with universal primers: 16SARL [5′ CGCCTGTTTATCAAAAACAT 3′] and 16S BRH [5′ CCGGTCTGAACTCAGATCACGT 3′] (Palumbi et al., 1991); LCO 1490 [5′ GGTCAACAAATCATAAAGATATTGG 3′] and HCO 2198 [5′ TAAACTTCAGGGTGACCAAAAAATCA 3′] (Folmer et al., 1994). Occasionally, higher quality amplification was achieved by pairing nemertean-specific reverse primers

(16SKR [5′ AATAGATAGAAACCAACCTGGC 3′], COIDr [5′ GAGAAATAATACCAAAACCAGG 3′] (Norenburg, unpublished)) with corresponding universal forward primers. PCR thermocycling was carried out using 1–8 µl of DNA extract in a 20-µl reaction volume with the following parameters: 95°C initial denaturation for 2 min, 35 cycles of 95°C for 40 s, 45–55°C for 40 s and a 60-s extension at 72°C. Following the last cycle there was an additional 2 min at 72°C for final extension after which products were stored at 4°C. PCR products were purified using Wizard SV Gel and PCR Cleanup kit (Promega) and sequenced (Sequetech Inc, Mountain View, CA) in both directions using forward and reverse primers to maximize sequence length and accuracy. COI sequence data from specimens collected near the Kasitsna Bay Laboratory (NOAA) in Kachemak Bay, AK (AK-K1, Fig. 3.1A) were provided by Dr. Jon L. Norenburg (Smithsonian Institution). Sequences were trimmed to remove primers, assembled in contigs, proofread for quality using Geneious v. 7.0.6, and deposited in GenBank (accession numbers KP682050–KP682304).

Histology

Adults were relaxed in a 1:1 mixture of $MgCl_2$ and FSW for 30–60 min and preserved for histology in 10% buffered formalin for at least 24 hr, then post-fixed in Hollande-Bouin's Fixative (Electron Microscopy Sciences, Hatfield, PA) for 24–72 hr, rinsed in 70% EtOH until all traces of Bouin's were removed, as assessed by the color of solution (solution exchanged at least once daily for 10 days) and stored in 70% EtOH until processed. Specimens were dehydrated through an EtOH series, cleared with several washes in xylene and embedded in paraffin (56°C, melting point). Slides with serial sections of 7–8 µm thickness were stained with Crandall's polychrome method—a combination of the Mallory, Gomori, Koneff and Gurr-McConail techniques where time in red stain and counter stain were slightly modified to 3 and 4 min, respectively—and mounted using Permount (Electron Microscopy Sciences, Hatfield, PA). Sections were imaged using an Olympus BX51 compound microscope, Leica DFC400 digital camera and Leica Application Suite v. 3.6 software.

Alignment, phylogenetic analysis, haplotype networks, and species delimitation

Our phylogenetic, statistical parsimony, and species delimitation analyses were performed on 139 (16S) and 114 (COI) sequences, which were trimmed to the same length to minimize missing data (440 bp for 16S and 512 for COI). Sequences were aligned using ClustalW (gap opening and extension costs set to default parameters, 15 and 6.66, respectively) as implemented in Geneious v. 7.0.6 (Biomatters Ltd). Sequence divergence values were calculated as uncorrected *p*-distances (and converted to percentage) from pairwise sequence alignments in Geneious v. 7.0.6. Maximum likelihood phylogenetic analysis was carried out including 139 (16S) and 114 (COI) sequences using PhyML v. 3.0 (Guindon et al., 2010) with TN93 (Tamura and Nei, 1993) substitution model and default parameters. Clade support was estimated using 1000 bootstrap replicates (Felsenstein, 1985). Bayesian phylogenetic analyses were conducted in MrBayes v. 3.2.1 (Ronquist et al., 2012) where evolutionary model parameters for each gene region were TN93 (Tamura and Nei, 1993) selected by jModel-Test v. 2.1 (Posada, 2008) as the best-fit model. Four chains were run for 1,000,000 generations, sampling trees every 1000 generations, excluding the first 25% discarded as burn-in. Gene tree topologies were viewed in FigTree v. 1.3.1 (Rambaut, 2009) or Geneious v. 7.0.6. Sequences from another member of the family Lineidae, *Lineus flavescens* Coe, 1904, were used as an outgroup to root the trees. This individual (*L. flavescens*) was collected from along the South Slough estuary in Charleston, OR (OR-C10, Fig. 3.1C, GenBank accession numbers KP682165 (16S) and KP682050 (COI)). Haplotype networks were generated with TCS v. 1.21 (Clement et al., 2000) based on 95% confidence intervals using 136 (16S) and 115 (COI) sequences. Two additional DNA taxonomy methods were used on the same 16S and COI sequences, including a Bayesian implementation of the Poisson Tree Processes model (bPTP, Zhang et al., 2013) and the Automatic Barcode Gap Discovery (ABGD, Puillandre et al., 2012) method. ABGD analysis was carried out using default parameters (P-min 0.001, P-max 0.1, steps 10, gap width 1.5). Default parameters were also used for bPTP (thinning 100, burn-in 0.1, seed 123), which was run on a

maximum likelihood tree (PhyML), with 500,000 MCMC generations 500,000 with convergence checked for each analysis (Zhang et al., 2013; Leasi and Norenburg, 2014).

RESULTS

Taxonomy

PILIDIOPHORA Thollesson and Norenburg, 2003

Class **HETERONEMERTEA**

Family **LINEIDAE** McIntosh, 1874

Genus ***Maculaura*** gen. nov.

Type species. *Micrura alaskensis* Coe, 1901, fixed by original designation.

Etymology. The generic name is feminine in gender, neologized by combining the first part of the Latin *maculosus* (having spots, spotted) and *aura* (air, breeze) in reference to the characteristic spotted pigmentation of the thin veil-like amnion surrounding the juvenile inside the pilidium larva, a suspected synapomorphy of this genus (*pilidium maculosum* morphotype, Fig. 3.2A).

Diagnosis. Body wall of typical heteronemertean composition with outer and inner longitudinal muscle layers separated by middle circular layer; middle circular muscle layer thickens in posterior esophageal region; outer dermis markedly glandular; sub-epithelial foregut glands—referred to as "accessory buccal glands" by Coe (1901)—associated with buccal cavity and extending into ventral outer longitudinal musculature; proboscis unbranched and unarmed, with four muscle layers including outer and inner (endothelial) circular, longitudinal and, sometimes inconspicuous, diagonal muscle layer —i.e., the musculature is "modified heterotype" according to Chernyshev (2015); two proboscis muscle crosses present although sometimes thin; outer longitudinal musculature in proboscis absent or present as two muscle strands outside main proboscis nerves; rhynchocoel with outer circular and inner longitudinal muscle layers, not

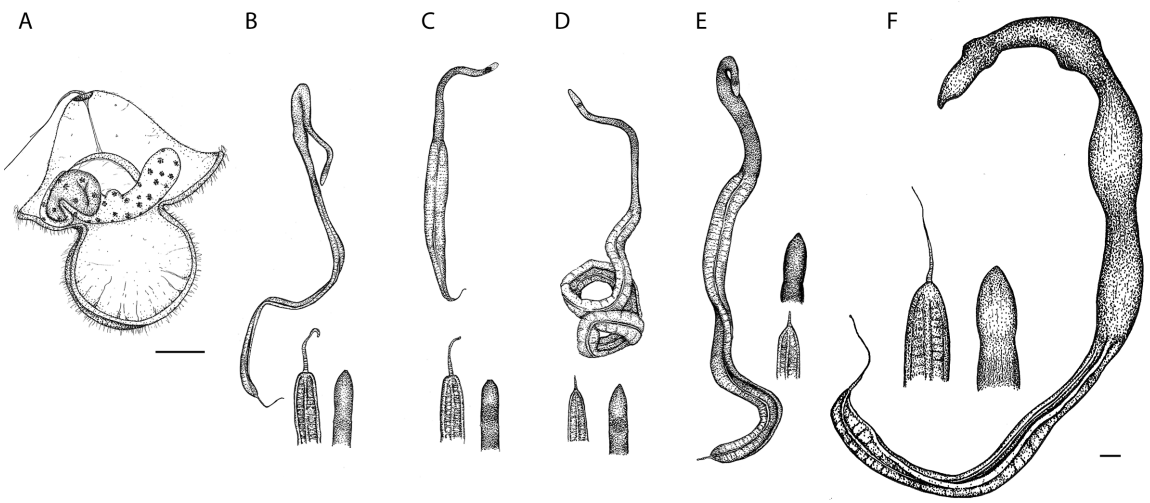


Figure 3.2. Comparison of five species in *Maculaura* gen. nov. **(A)** The *pilidium maculosum* larval morphotype. **(B–F)** External appearance of adults: **(B)** *Maculaura alaskensis* (Coe, 1901) comb. nov., **(C)** *Maculaura aquilonia* sp. nov., **(D)** *Maculaura cerebrosa* sp. nov., **(E)** *Maculaura oregonensis* sp. nov., **(F)** *Maculaura magna* sp. nov. Scale bars: 100 μm (A) and 1 mm (B–F).

consistently interwoven with body wall musculature; dorsal ganglia separate posteriorly into upper and lower neuropile; neurochord cells absent; inner and outer neurilemma present; external color of live worms pale to dark pink, sometimes white anteriorly; lateral cephalic slits relatively shallow compared to those in other lineids (A. V. Chernyshev, pers. comm.); ocelli lacking at all stages of development as well as in adults; head shape ovate to rectangular, with obtuse apex (Fig. 3.2B–F), not distinctly marked from the rest of body (i.e. posterior margins of lateral slits linear), except when contracted; body oval in cross-section in foregut region, dorso-ventrally flattened in midgut region; lateral body margins not sharp; worms glide, but do not swim; caudal cirrus present and regenerated easily if lost; gonochoric, with gonads serially arranged and alternating with intestinal diverticula; fertilization occurs externally in water column (i.e. broadcast spawning); oocytes 75–125 μm, with or without chorion; sperm morphology modified or primitive with headpiece size 5–15 μm long (Fig. 3.3); with planktotrophic pilidium larva of "*gyrans*" type with conspicuous reddish, black, or brown pigment spots decorating the juvenile amnion (*pilidium maculosum).*

Composition. This genus contains five species: *Maculaura alaskensis* comb. nov., *Maculaura aquilonia* sp. nov., *Maculaura cerebrosa* sp. nov., *Maculaura magna* sp. nov., and *Maculaura oregonensis* sp. nov. (Fig. 3.2B–F).

Geographic distribution. The geographic distribution of this genus, as confirmed by DNA sequence data, includes the NE Pacific: Alaska (Juneau and Kachemak Bay), Washington (False Bay, San Juan Island), Oregon (Seaside, Coos Bay, Charleston), and California (Crescent City); and the NW Pacific (the Sea of Okhotsk, Russia) (Fig. 3.1A). Additional records, awaiting confirmation by DNA sequence data, include various locations in Alaska: Glacier Bay, Sitka, Yakutat, Prince William Sound (Coe, 1901), British Columbia (Coe, 1940), southern California to Ensenada, Mexico (Coe, 1940), and Hokkaido, Japan (Yamaoka, 1940; Iwata, 1954; Gibson, 1995; Roe et al., 2007; Kajihara, 2007). Numerous larvae that were confirmed to belong to this genus have been collected from Coos Bay, OR. Larvae of this morphotype have also been found in plankton near Bamfield, B.C., Canada (Lacalli, 2005).

Maculaura alaskensis (Coe, 1901) comb. nov.

(Figs. 3.2B, 3.3A–B, 3.4A–B, 3.5, 3.6)

Micrura alaskensis Coe, 1901, in part.

Etymology. The specific epithet is an adjective (*-ensis*, *-ense*), referring to the geographic origin of specimens originally described by Coe (1901).

Type material. Morphological types do not exist. We hereby designate a series of transverse histological sections (18 slides USNM# 1282106) and associated ethanol-preserved material from an individual collected from a mudflat in Charleston, OR by T. Hiebert (Table 3.1) as the neotype, according to Article 75.3 of the Code (ICZN, 1999). Neotype is deposited at the NMNH. We further designate a partial COI sequence from a

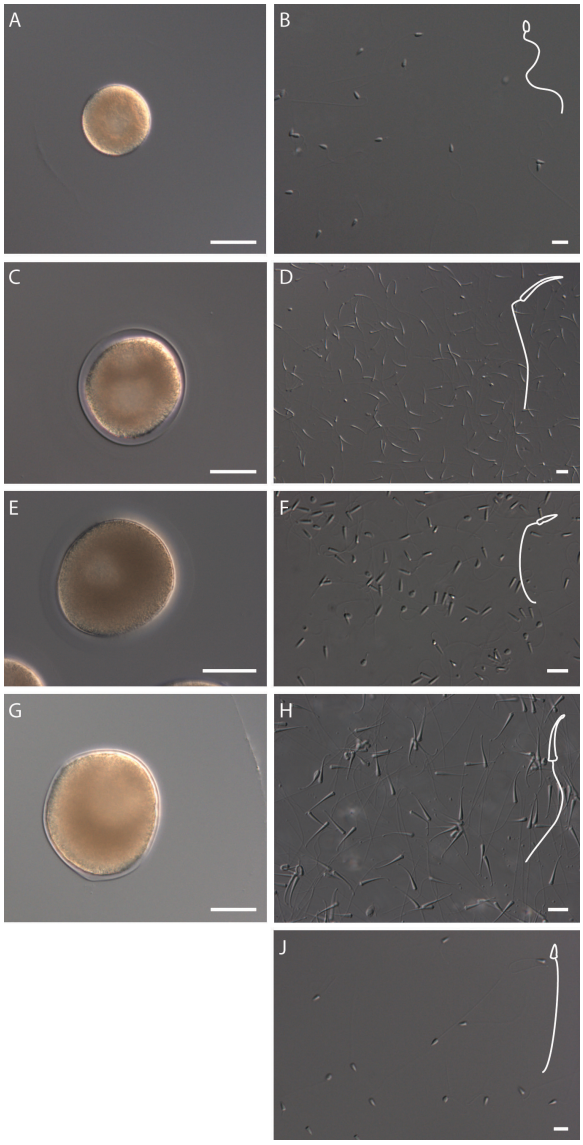


Figure 3.3. Primary oocytes and sperm dissected from the members in *Maculaura* gen. nov. **(A, B)** *Maculaura alaskensis* comb. nov., **(C, D)** *Maculaura cerebrosa* sp. nov., **(E, F)** *Maculaura aquilonia* sp. nov., **(G, H)** *Maculaura magna* sp. nov., **(J)** *Maculaura oregonensis* sp. nov. Scale bars: 50 µm (A, C, E, G), 10 µm (B, D, F, H, J).

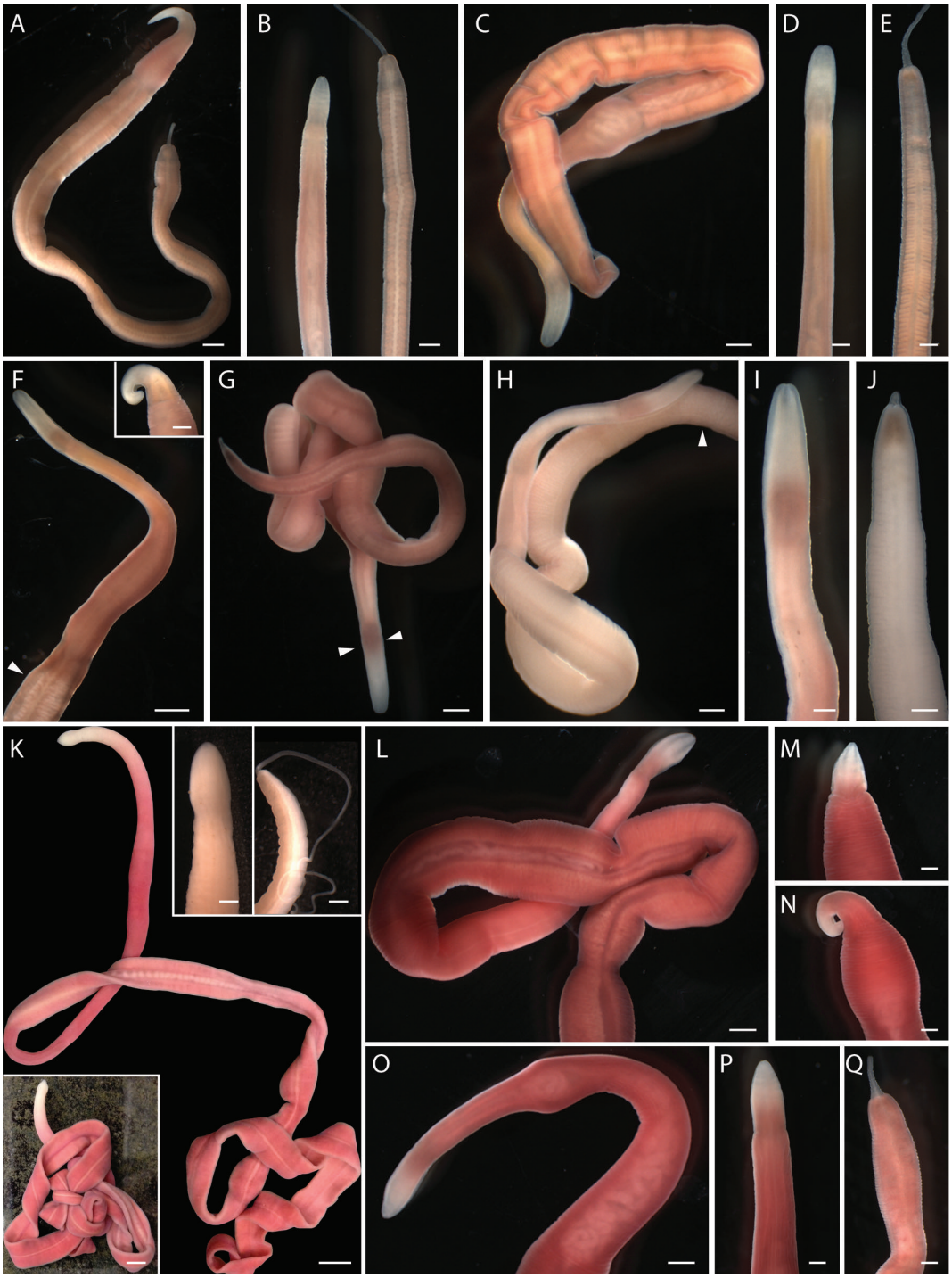
different individual collected from the same location as a topogenetype (GenBank accession number KP682055, Table 3.1).

Material examined. Forty-seven adult individuals (collected from False Bay, Friday Harbor, WA as well as locations in or near Gearhart and Charleston, OR) and one wild-caught larva were examined and their identification confirmed by DNA sequence data (see Table 3.1 for GenBank accession numbers). These included serial transverse histological sections of two individuals (36 slides USNM# 1282107 and 18 slides, neotype, USNM# 1282106) , and four whole specimens preserved for histology (USNM#s 1282108–1282111); ethanol-preserved tissue from all six specimens is deposited at the NMNH (Table 3.1). Ethanol-preserved tissue and/ or extracted DNA from remaining individuals are held at the OIMB.

Diagnosis. *Maculaura alaskensis* comb. nov. differs from *Maculaura magna* sp. nov. and *Maculaura oregonensis* sp. nov. by its smaller size, narrower body, and not as pink body color. It most resembles *Maculaura aquilonia* sp. nov. and *Maculaura cerebrosa* sp. nov. in body shape, color,

and size. *Maculaura alaskensis* differs from *Maculaura cerebrosa* by having cerebral ganglia of the same general hue as the body (as opposed to having a distinctly pink brain) and a relatively longer caudal cirrus with an abrupt (as opposed to gradual) transition from posterior body (compare Fig. 3.4B with 3.4J). Differentiating *Maculaura alaskensis* from *Maculaura aquilonia* is challenging and best achieved with freshly collected specimens, as colors can fade in the lab over time. Whereas *Maculaura alaskensis* is pale anteriorly, *Maculaura aquilonia* can have a brownish region near the brain (Fig. 3.4F). The easiest way to tell apart *Maculaura alaskensis* from *Maculaura aquilonia* and the other three species is by comparing their eggs and sperm (Fig. 3.3). At 75 µm in diameter, *Maculaura alaskensis* eggs are the smallest in the genus (*Maculaura oregonensis* egg size is not known), and they lack a chorion. *Maculaura alaskensis* sperm is primitive with a short (5 µm) headpiece, similar to that in *Maculaura oregonensis*, but distinctly different from the sperm in the other three species, which have variously elongated headpieces.

Figure 3.4 (next page). External appearance of live adults in *Maculaura* gen. nov. **(A, B)** *Maculaura alaskensis* (Coe, 1901) comb. nov.: **(A)** entire body of non-type specimen; **(B)** anterior and posterior ends of same individual as (A), relaxed in $MgCl_2$. **(C–F)** *Maculaura aquilonia* sp. nov., two different specimens: **(C)** paratype, entire body; **(D)** magnification of head, same individual as (C), relaxed in $MgCl_2$; **(E)** magnification of tail, same individual as (C), relaxed in $MgCl_2$; **(F)** reproductive male (topogenetype specimen) with testes visible through the body wall (arrowhead), with magnification of head in upper-right inset. **(G–J)** *Maculaura cerebrosa* sp. nov.: **(G)** non-type specimen, showing the distinctly pink brain (indicated by arrowheads); **(H)** reproductive female (non-type specimen) with ovaries (indicated by arrowhead) visible through the body wall; **(I)** head of a relaxed (topogenetype) individual; **(J)** tail, same individual as (I). **(K)** *Maculaura magna* sp. nov., body of holotype and close-up of anterior and posterior of the same individual after relaxation in $MgCl_2$ (upper right insets); the background has been removed using Adobe Photoshop to emphasize the body color; bottom left inset shows the same individual on original background. **(L–Q)** *Maculaura oregonensis* sp. nov.: **(L–O)** body and anterior of holotype; note coiled proboscis visible through the anterior body wall (O); **(P–Q)** anterior and posterior of relaxed individual. Scale bars: 5.0 mm (K and left inset), 1.0 mm (A, C, F, G, H, K, L, O), and 0.5 mm (B, D, E, F inset, I, J, K insets, M, N, P, Q). Topogenetypes are associated with specimens pictured in F, I, J.


A
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Habitat, type locality, and distribution. The known range of this species confirmed by DNA sequences extends from False Bay, San Juan Island, Washington to southern Oregon (WA-F1 to OR-C, Fig. 3.1A), where it is common. Individuals are commonly encountered in the top 10–15 cm of silty, relatively small-grained sand and mud from mid- to low intertidal (e.g. Fig. 3.1D) in protected bays and estuaries. Although patchy in distribution, several individuals can be found in one shovel-load, stretched like threads between clods and clumps of sand (Fig. 3.1E). It is quite possible that this species occurs further north and south along the Pacific coast of North America (including Alaska), but none of the individuals from outside Oregon and Washington, that we have sequenced, belong to this species. Coe (1901) did not specify a type locality, but based his description on specimens from a variety of locations in Alaska. Later, Coe (1904, 1940) revised the species range to include southern California and Mexico. The type locality of *Maculaura alaskensis* comb. nov. is now regarded to be Charleston, OR, as the place of origin of the neotype becomes the type locality of the nominal species-group taxon, according to Article 76.3 of the Code (ICZN, 1999).

Description. *External appearance.* Largest specimens examined by us were 5 cm in length and 2–3 mm in width, with average length 3–4 cm and width 1–2 mm (Figs. 3.2B, 3.4A, B), while gliding. Living worms are, generally, a uniform pinkish or flesh-color. Body is rounded in the foregut region and dorso-ventrally flattened in the midgut region. Sexually mature individuals can appear pale yellow to white due to the color of gametes visible through the body wall between intestinal diverticula. The ventral surface is only slightly lighter than dorsal, if at all. The tip of the head is narrow and rounded, and head is not prominently demarcated from body when worm is gliding. Relatively shallow horizontal cephalic slits extend from anterior tip of the head to about the anterior margin of the mouth. Ocelli are lacking. Mouth is slit-like and elongated. The caudal cirrus is abruptly demarcated from the posterior end of body, and tends to be relatively long and thin compared to that in several other members of the genus (e.g. compare Figs. 3.2B with 3.2D, E, and 3.4B with 3.4J). Movement is without distinct peristalsis (Fig. 3.4A).

Worms fragment during collection, especially when sexually mature, and posterior end regenerates routinely; anterior regeneration has not been observed. To avoid fragmentation worms should be collected with clumps of sand or mud, and cleaned in the laboratory.

Body wall. The epidermis is ciliated and of uniform thickness, situated on top of a thin dermis; the latter term used here in reference to the thin layer of extracellular matrix underlying the epidermis (Fig. 3.5E). Gland cells, staining red and blue, are interspersed within the cutis (subepidermal glandular region between the dermis and outer longitudinal musculature, OLM) (Fig. 3.5A–D). The transition from cutis to the OLM is gradual and visible only by the presence of gland cells that are confined to the cutis anteriorly and extend into the OLM immediately anterior and posterior to the mouth (compare Fig. 3.5A with 3.5C, E). The OLM is slightly thinner than the inner longitudinal muscle layer (ILM, e.g. Fig. 3.5E), but the two layers are of equal thickness in the intestinal region where the circular musculature (CM) is thickest (Fig. 3.5F). The thick esophageal circular muscle layer (1/2 as thick as body wall CM reported by Coe (1901) was not observed.

Proboscis and rhynchocoel. The rhynchocoel opening is slightly subterminal (ventral); proboscis is long and coiled. The rhynchocoel musculature under the vascular plug is not interwoven with body wall musculature. The proboscis consists of four distinct muscle

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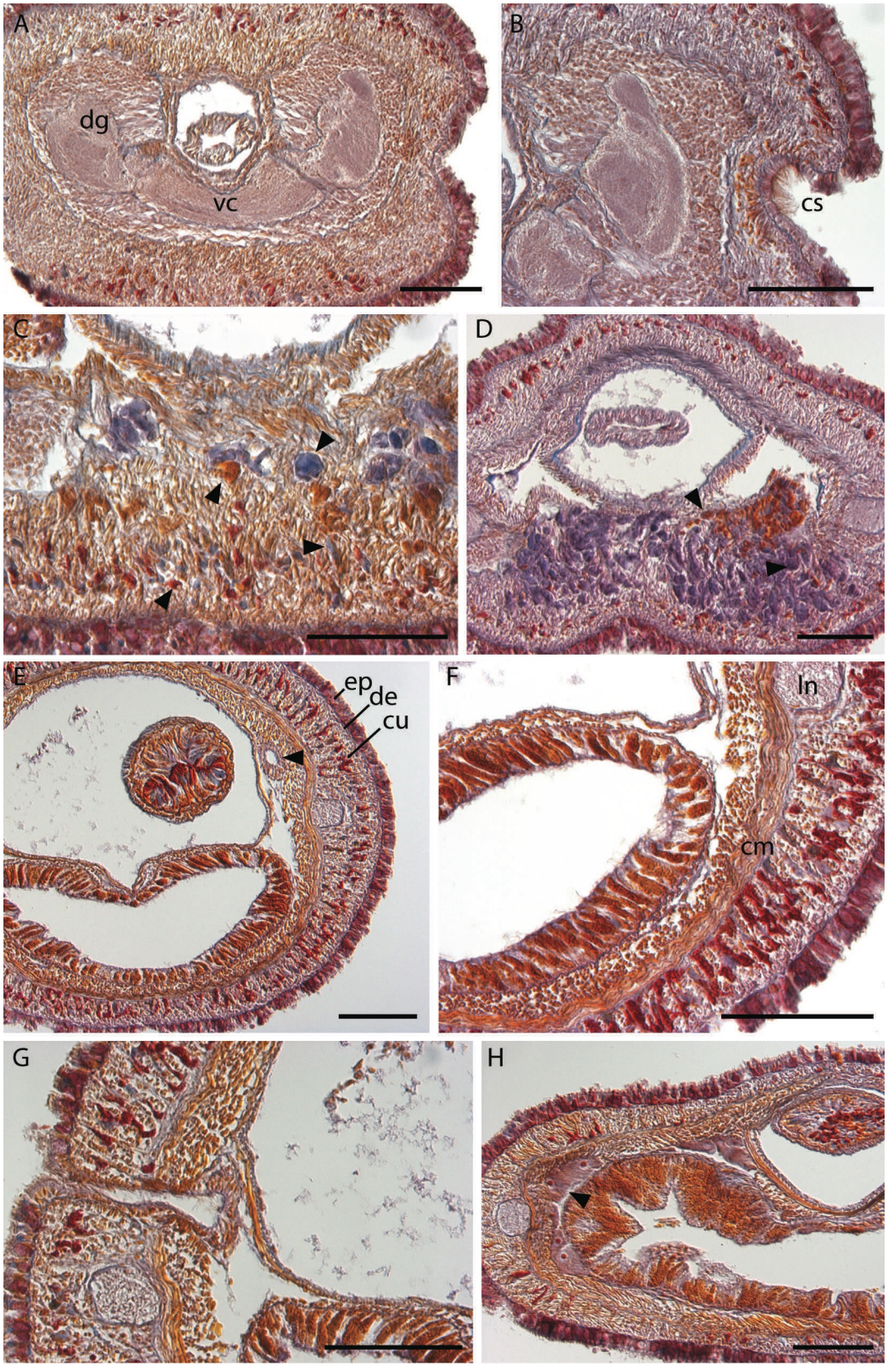
Figure 3.5 (next page). *Maculaura alaskensis* (Coe, 1901) comb. nov., USNM# 1282106 (neotype specimen), photomicrographs of transverse sections: **(A)** brain; **(B)** left cephalic slit; **(C, D)** gland cells in ventral longitudinal musculature anterior to mouth opening; note four distinct gland cell types, two in the cutis and two foregut (arrowheads, C); **(E)** nephridial collecting tubule (arrowhead) in intestinal region; **(F)** body-wall inner circular muscle layer in intestinal region; **(G)** nephridiocanal; **(H)** ovary; note several oocytes and nuclei (arrowhead). Abbreviations: **cm**, circular musculature; **cu**, cutis; **de**, dermis; **dg**, dorsal ganglion; **ep**, epidermis; **ln**, lateral nerve; **vc**, ventral commissure. Scale bars: 100 µm.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layers including inner (endothelial) circular, longitudinal, diagonal, and outer circular; two thin muscle crosses were observed in confocal sections (A. Chernyshev, personal communication). The proboscis epithelium sits atop a thin layer of extracellular matrix; glandular ridge present and with red-staining gland cells (Fig. 3.5E, H).

Digestive system. The mouth is situated ventrally. The opening is a short (80 µm), thin slit that begins immediately posterior to the cephalic slits. Just anterior to the mouth opening, gland cells that open into the foregut ("accessory buccal glands", following Coe's terminology), become apparent ventrally (Fig. 3.5D) and remain prominent throughout the anterior esophageal region. At least two types of gland cells are associated with foregut epithelium, one staining orange-red and the other staining purple, and, at times, their bodies may extend into the OLM (Fig. 3.5D). The foregut is densely ciliated, folded and packed with gland cells. The transition from foregut to intestine is gradual. Intestinal diverticula are not branched. Short, unbranched hindgut opens via a ventral anus anterior to the caudal cirrus.

Excretory system. Relatively large nephridia are found 5 mm from the anterior tip (Fig. 3.5E) and extend as canals before opening to the outside via two dorso-lateral nephridiopores, one on each side (Fig. 3.5G) near the transition between foregut and intestine.

Vascular system. Two conspicuous lateral blood vessels flank the rhynchocoel, and a dorsal blood vessel is situated within the ventral wall of the rhynchocoel for the length of the foregut. The mid-dorsal blood vessel enters the rhynchocoel near the brain and forms a single ventral vascular plug. Two lateral cephalic blood lacunae are connected anteriorly via an anastomosing lacuna and, at the level of the brain commissures, surround the ventral rhynchocoel. These blood lacunae are, at times, connected with the blood sinuses surrounding the foregut and become distinct vessels posteriorly. The dorsal blood vessel originates from the commissure between the two lateral vessels just

posterior to the ventral brain commissure and is easily observed ventral to the rhynchocoel wall in the intestinal region.

Nervous system. Dorsal and ventral cerebral ganglia are connected via dorsal and ventral commissures, respectively, surrounding the anterior rhynchocoel. The brain is relatively large, and the ventral commissure (Fig. 3.5A) is nearly twice as thick as the dorsal commissure. The proboscis has two lateral nerves clearly visible anteriorly, which arise from—and enter the proboscis anterior to—the ventral brain commissure. Lateral nerve chords are situated just outside the CM (Fig. 3.5F) and consist of a fibrous core and ganglionic region, which are surrounded by blue-staining inner and outer neurilemma, respectively. Two esophageal nerves originate from the inner margin of ventral cerebral lobes at the level of the cerebral organs and are apparent lateral and ventral of the main lateral nerves.

Sense organs. Paired cerebral organs lie just posterior to ventral brain commissure and their canals open into lateral cephalic slits (Fig. 3.5B). The epithelium of each cerebral organ canal is densely ciliated with underlying conspicuous gland and nerve cells staining orange and fuchsia, respectively (Fig. 3.5B). Each cerebral organ is connected to the dorsal cerebral ganglion via a cerebral organ nerve. Three apical sense organs were observed in confocal sections (A. Chernyshev, personal communication), but were not observed with histological sections.

Reproduction and development. Reproductive females and males have been collected March–September in OR and WA, with ripest individuals found in summer months. Gonads are arranged laterally between intestinal diverticula. Ovaries contain dozens of oocytes (Fig. 3.5H). Once dissected into seawater and rounded up, oocytes are 75 μm in diameter and without a chorion (Fig. 3.3A, Maslakova, 2010). Sperm head pieces (Fig. 3.3B, Maslakova, 2010) are 5-μm long, cone-shaped i.e. not modified (Stricker and Folsom, 1998). The wild-caught larva, identified as belonging to this species by DNA

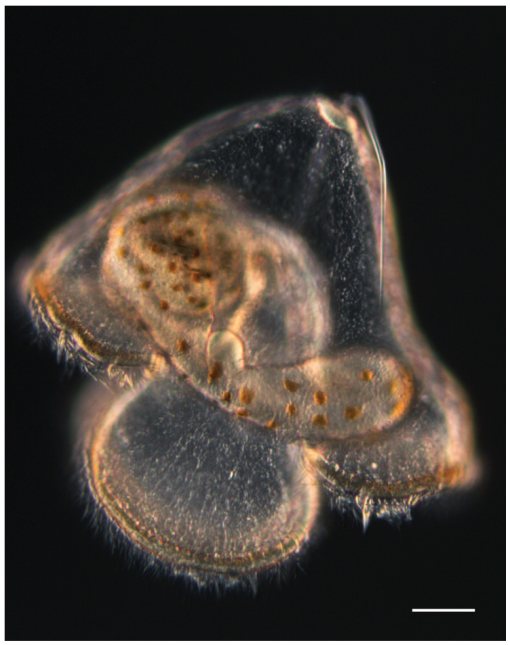

Figure 3.6. Larva of *Maculaura alaskensis* (Coe, 1901) comb. nov., wild-caught from plankton sample taken 13 October 2013 from Coos Bay (diamonds, Fig. 1C) and identified using DNA sequence data. Scale 100 µm.

sequence data, was collected from Coos Bay plankton in October (Fig. 3.1C). When reared in the lab, first and second cleavage occurs at 2 and 3 hours after fertilization, respectively, at 11–14°C and larvae begin feeding on *Rhodomonas lens* at 3 days (Maslakova, 2010). They have three pairs of imaginal discs as early as 14 days, and the discs fuse to form a complete juvenile worm by as early as 28 days (Maslakova, 2010). Metamorphosis has been observed in lab culture as early as 35 days after fertilization. Prior to metamorphosis larvae are approximately 500 µm tall and wide (Maslakova, 2010). The larva exhibits the characteristic *pilidium maculosum* morphotype (Fig. 3.6), where the amnion surrounding the juvenile worm is pigmented with a polka-dot pattern consisting of red, black, and maroon pigment spots (Maslakova, 2010).

Maculaura aquilonia sp. nov.

(Figs. 3.2C, 3.3E–F, 3.4C–F, 3.7A–D, 3.8, 3.9)

Etymology. This specific epithet is a Latin adjective (*aquilonius*, *-a*, *-um*; "northerly" or "northern"), in reference to the geographic range of this species, reaching the northernmost latitudes for this genus.

Type material. Type material is deposited at the NMNH and includes serial transverse sections of the holotype (male, 20 slides USNM# 1282112) and one paratype (18 slides USNM# 1282113) as well as additional ethanol-preserved tissue. Additional paratypes

include four un-sectioned paratypes preserved for histology (USNM#s 1282114–1282117) and associated ethanol-preserved tissue (Table 3.1). We designate a partial COI sequence from an individual collected from Charleston, OR as a topogenetype (GenBank accession number KP682130, Table 3.1); ethanol-preserved tissue from this individual is also deposited at NMNH (USNM# 1282118).

Material examined. Forty-three adult individuals, including holotype and paratypes, and four wild-caught larvae were examined and their identification confirmed by DNA sequence data. These individuals were collected from locations near Juneau, AK, and Charleston, OR, USA, as well as from the Sea of Okhotsk near Magadan, Russia (Table 3.1). COI sequence data from seven individuals collected in Kachemak Bay, AK, by S. Maslakova and J. Norenburg were supplied by J. Norenburg (Smithsonian Institute) from archived specimens (Table 3.1). Ethanol-preserved tissue and/or extracted DNA from remaining individuals are held at the OIMB.

Diagnosis. *Maculaura aquilonia* differs from *Maculaura magna* and *Maculaura oregonensis* by its smaller size, narrower body, and not as pink body color. It is similar to *Maculaura alaskensis* and *Maculaura cerebrosa* in body shape, color, and size. *Maculaura aquilonia* differs from *Maculaura cerebrosa* by having cerebral ganglia of the same general hue as the body (Fig. 3.4C, D) (as opposed to having a distinctly pink brain, Fig. 3.4G, I) and a relatively longer caudal cirrus with an abrupt (Figs. 3.2C, 3.4E) (as opposed to gradual, Figs. 3.2D, 3.4J) transition from posterior body. *Maculaura aquilonia* can be differentiated from *Maculaura alaskensis* by the presence of a subtle brownish region near the brain, which is best observed in freshly collected specimens (Fig. 3.4C, D, F). We observed this color in the majority of freshly collected specimens, but not all, and it seemed to fade over time in the lab. The most accurate way to differentiate *Maculaura aquilonia* from *Maculaura alaskensis* and the other three species is by comparing their eggs and sperm (Fig. 3.3). *Maculaura alaskensis* eggs are the smallest in the genus (*Maculaura oregonensis* egg size is not known), and they lack a

chorion (Fig. 3.3A). The eggs of *Maculaura aquilonia* also lack a chorion, but are larger, 90–100 µm in diameter (Fig. 3.3E). *Maculaura alaskensis* sperm is primitive with a short (5 µm) headpiece (Fig. 3.3B). *Maculaura aquilonia* and *Maculaura oregonensis* sperm are indistinguishable, they both have slightly elongated but not curved headpieces 7–8 µm in length (Fig. 3.3F, J). In comparison, the sperm headpieces of *Maculaura cerebrosa* and *Maculaura magna* are longer (10–15 µm) and slightly curved. *Maculaura aquilonia* can also be differentiated from the latter two species by body color: it is not as pink (compare Fig. 3.4C with 3.4L–Q).

Habitat, type locality, and distribution. Type locality is in Juneau, AK (58.3952°N, 134.7512°W) (AK-J1, Fig. 3.1B). This species exhibits the largest confirmed range for any species in the genus *Maculaura*, including eastern Russia, Alaska, and southern Oregon (RU-O1, AK-K1 to OR-C, Fig. 3.1A). This species is common in silty sand and mud from mid- to low intertidal, sometimes even occurring in black anoxic mud. However, in Juneau, AK, it is found under rocks and within fine mud, where it appears to be the most common nemertean species. In the southern portion of its range, this species is less common than morphologically similar *Maculaura alaskensis* and was most abundant at one mudflat along the South Slough estuary near Charleston, OR (OR-C10, Fig. 3.1C).

Description. *External features.* Largest specimens were 5 cm in length and 2–3 mm in width, with average individuals on the order of 3–4 cm and 1–2 mm, in gliding (Fig. 3.4C–F). Head is off-white and body color is pale yellowish pink to brownish ochre. Posterior can be very pale yellow to off-white in reproductive individuals where gametes are seen through the body wall (Fig. 3.4F). Body color is the same dorsally and ventrally. Lightly colored brownish pigment near the brain can be seen through the anterior body wall in freshly collected specimens (Fig. 3.4F). The posterior-most region of the body transitions abruptly to the caudal cirrus (Fig. 3.4E). Movement is without distinct peristalsis and is led with the head which often turns dramatically to one side forming a

hook or ‘u-shape’ (Fig. 3.4C, F inset). Fragmentation occurs during collection, especially in sexually mature individuals; posterior end regenerates routinely. Individuals can be rather short and stout when ripe with gametes. External morphology is very similar and sometimes un-differentiable from *Maculaura alaskensis* (compare Fig. 3.4A with 3.4C).

Internal features. Internal anatomy as in *Maculaura alaskensis* (see Fig. 3.7A–D).

Reproduction and development. Sexes are separate. Gonads are regularly arranged between intestinal diverticula (Fig. 3.4F). Reproductive individuals have been collected in March 2013. Gametes from both sexes are released when ripe through serially arranged dorsolateral gonopores. Dissected primary oocytes are 90–100 μm in diameter and lack a chorion (Fig. 3.3E). Sperm headpiece is cone-shaped, 7.5 μm in length (n = 10, Fig. 3.3F). Wild-caught larvae identified as belonging to this species using DNA sequence data were collected from plankton in Coos Bay in April and May in 2009 and 2012 (Fig. 3.1C). The amnion surrounding the juvenile worm inside the larva is less pigmented in this species as compared to the others in this genus (Fig. 3.8). However, we observed pigment spots in the amnion of lab-reared larvae during and immediately

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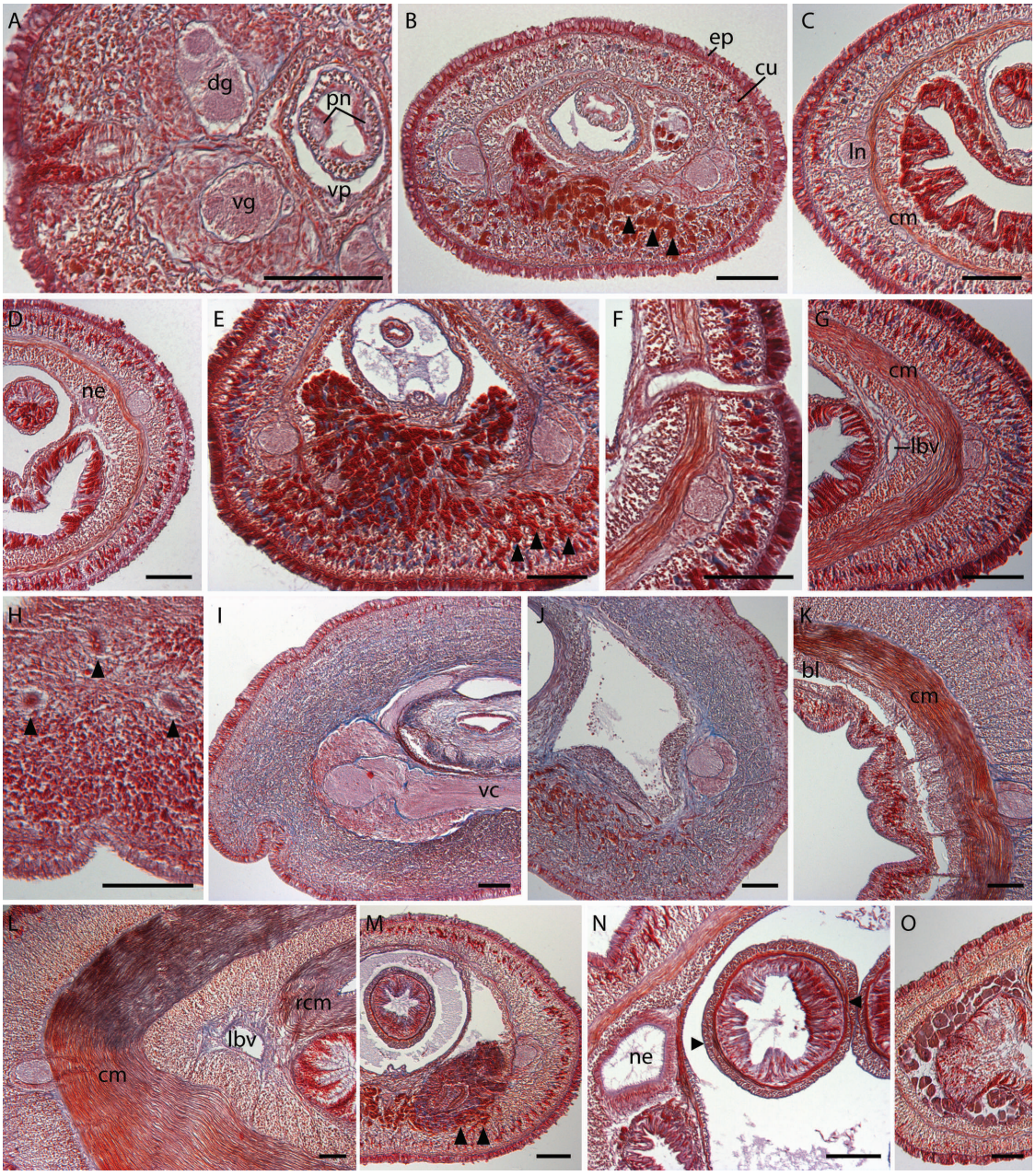
Figure 3.7 (next page). Internal anatomy of *Maculaura* gen. nov. **(A–D)** *Maculaura aquilonia* sp. nov., USNM# 1282112 (holotype): **(A)** right cerebral organ and cerebral ganglia, proboscis, and rhynchocoel; **(B)** anterior to mouth opening, showing ventral gland cells (arrowheads); **(C)** foregut region; **(D)** intestinal region. **(E–G)** *Maculaura cerebrosa* sp. nov., USNM# 1282119 (holotype): **(E)** anterior to mouth opening, showing ventral gland cells (arrowheads); **(F)** left dorsolateral nephridiocanal and pore; **(G)** posterior intestinal region. **(H–L)** *Maculaura magna* sp. nov., USNM# 1282125 (holotype): **(H)** apical sense organs (arrowheads); **(I)** ventral cerebral commissure; **(J)** anterior to mouth opening; **(K)** posterior to mouth opening; **(L)** mid intestinal region, showing extremely thick circular musculature. **(M–O)** *Maculaura oregonensis* sp. nov., USNM# 1282128 (holotype): **(M)** anterior to mouth opening, showing ventral gland cells (arrowheads); **(N)** intestinal region, showing two proboscis muscle crosses (arrowheads); **(O)** ovary. Abbreviations: **bl**, blood lacunae; **cm**, circular musculature; **cu**, cutis; **dg**, dorsal ganglion; **ep**, epidermis; **lbv**, lateral blood vessel; **ln**, lateral nerve; **ne**, nephridium; **pn**, proboscis nerves; **rcm**, rhynchocoel circular musculature; **vc**, ventral commissure; **vg**, ventral ganglion; **vp**, vascular plug. Scale bars: 100 μm.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following metamorphosis, when the amnion collapses and is swallowed by the juvenile (Fig. 3.9). When reared in the lab at 12°C, first and second cleavage occur at approximately 2 and 3 hours after fertilization, respectively, and larvae begin feeding on *Rhodomonas lens* at 2 days (Fig. 3.8A). Pilidia have three pairs of imaginal discs by 2.5 weeks and the trunk and cerebral organ discs fuse by 42 days (Fig. 3.8C). The larvae reach advanced-proboscis stage by 81 days (Fig. 3.8D), and metamorphosis was observed as early as 95 days. Metamorphically competent larvae are approximately 550 µm tall (Figs. 3.8E, F). Metamorphosis is catastrophic, as in other pilidia, and the juvenile nemertean ingests the larval body (Fig. 3.9).

Maculaura cerebrosa sp. nov.

(Figs. 3.2D, 3.3C–D, 3.4G–J, 3.7E–G, 3.10)

Etymology. The specific name is a compound unorthodox adjective (*cerebrosus*, *-a*, *-um*) rather freely formed by fusing two Latin words (*cerebrum* = brain, *roseus* = pink), in reference to the pinkish brain.

Type material. Serial transverse sections of the holotype (ripe male, 37 slides USNM# 1282119), four paratypes (USNM#s 1282120–1282123) and associated ethanol-preserved tissue are deposited at the NMNH (Table 3.1). We designate a partial COI sequence from an individual collected in Charleston, OR as a topogenetype (GenBank accession number KP682146, Table 3.1) and ethanol-preserved tissue from this individual is also deposited at NMNH (USNM# 1282124).

Material examined. Fourteen adult individuals, including holotype and paratypes, and three wild-caught larvae were examined and their identification confirmed by DNA sequence data. These individuals were collected from locations near Charleston, OR, and Crescent City, CA (Table 3.1). Ethanol-preserved tissue and/or extracted DNA from remaining individuals are held at the OIMB.

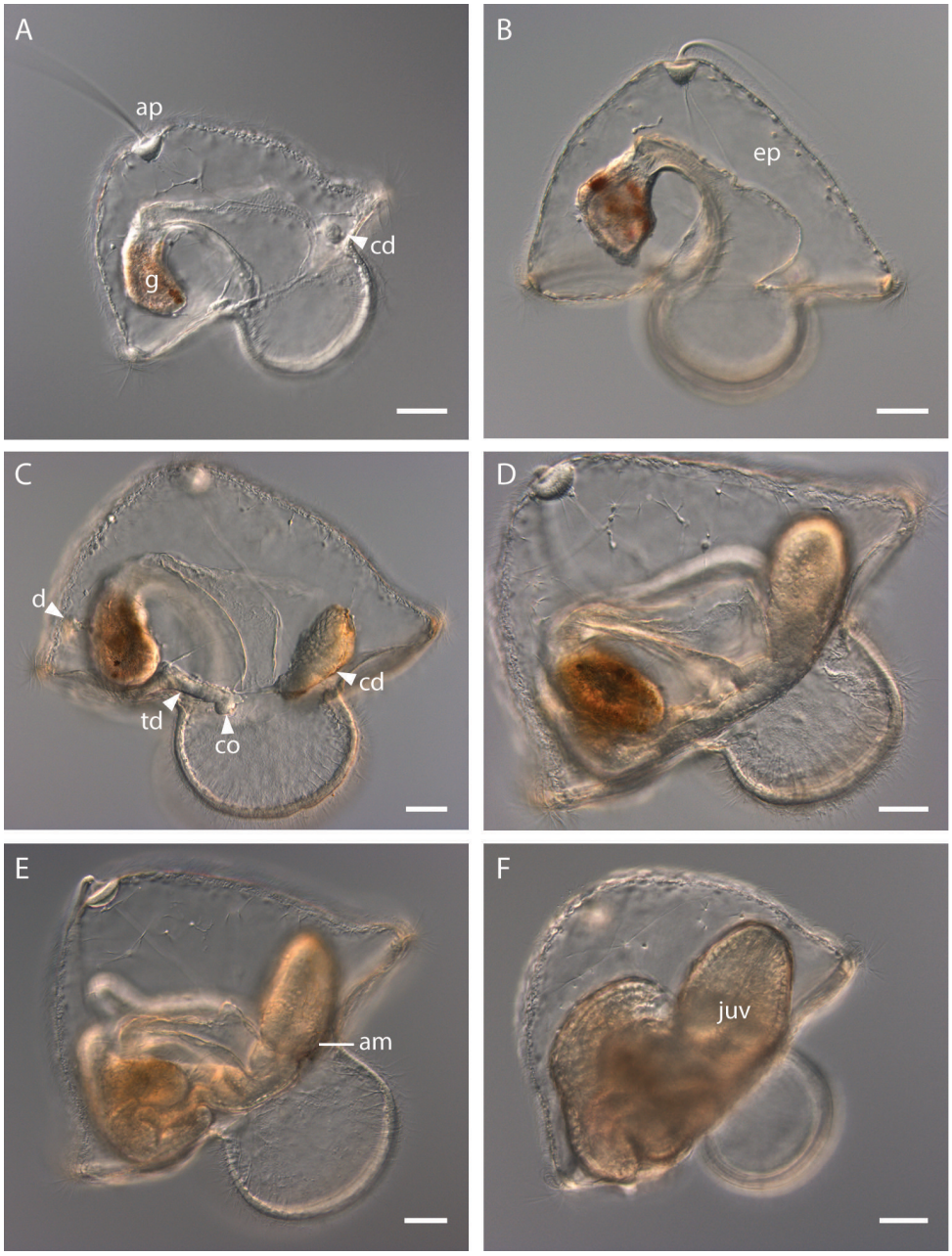

Figure 3.8. Development in *Maculaura aquilonia* sp. nov. **(A)** Two-day-old larva with cephalic disc in focus and gut positioned at left. **(B)** An 11-day-old larva. **(C)** Forty-two-day old larva with trunk discs and cerebral organ discs fused; the unpaired dorsal rudiment is also visible. **(D–F)** Larvae with juvenile; **(D)** 81-day old larva; **(E)** 91-day old larva; **(F)** 95-day old larva. Abbreviations: **am**, amnion; **ap**, apical tuft; **cd**, cephalic disc; **co**, cerebral organ disc; **d**, dorsal disc (unpaired); **ep**, episphere; **g**, gut; **juv**, juvenile; **td**, trunk disc. Scale bars 100 μm.

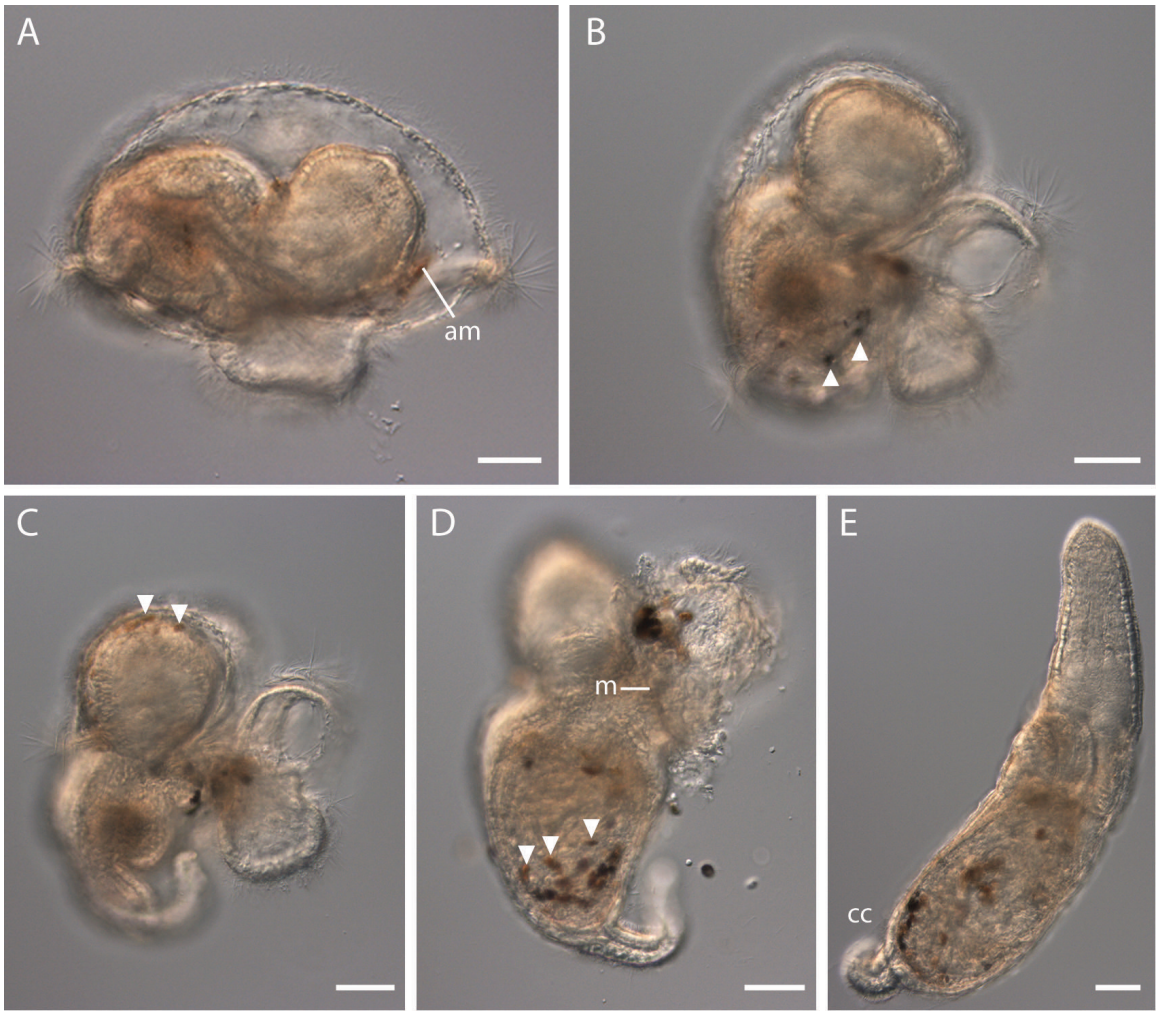

Figure 3.9. Metamorphosis in *Maculaura aquilonia* sp. nov. **(A–E)** Pigment spots on the amnion (am) become apparent as the amnion disappears into the juvenile mouth (m) and collapses within the gut (arrowheads, B–D); (E) newly metamorphosed juvenile with caudal cirrus (cc). Scale bars: 100 µm.

Diagnosis. *Maculaura cerebrosa* differs from *Maculaura magna* and *Maculaura oregonensis* by its smaller size, narrower body, and not as pink body color. It is morphologically similar to *Maculaura alaskensis* and *Maculaura aquilonia* in body shape, color, and size. *Maculaura cerebrosa* is distinguishable from the latter two species by having a distinctly pink brain, which is visible through the body wall, and a short caudal cirrus with gradual (as opposed to a relatively longer caudal cirrus with an abrupt) transition from posterior body (Figs. 3.2D, 3.4G–J). In addition to the conspicuous pink

brain, *Maculaura cerebrosa* can be differentiated from *Maculaura alaskensis* and *Maculaura aquilonia* by comparing their eggs and sperm (Fig. 3.3). The eggs of *Maculaura cerebrosa* and *Maculaura magna* are both surrounded by egg chorions (the eggs of *Maculaura oregonensis* have not been observed), but the egg diameter is distinctly different: 95 μm in *Maculaura cerebrosa* and 125 μm in *Maculaura magna* (compare Fig. 3.3C with 3.3G). The sperm headpieces in both species are elongated, but are 10 μm long in *Maculaura cerebrosa* and 15 μm long in *Maculaura magna* (Fig. 3.3D and 3.3H). The remaining species (for which we know gamete morphology) have eggs without distinct chorions, and "primitive" (not elongated) sperm (see Fig. 3.3).

Habitat, type locality, and distribution. The type locality is a mudflat near the outer Charleston Boat Basin in Charleston, OR (43.3445°N, 124.3215°W) (OR-C15, Fig. 3.1C). The known range of this species confirmed by DNA sequences extends from southern Oregon to northern California (OR-C to CA-C1, Fig. 3.1A), although a larger range is expected. This species can co-occur with other members in this genus, particularly *Maculaura alaskensis;* however, *Maculaura cerebrosa* is more common under rocks in mid-intertidal gravel and shell hash rather than sand. Individuals are often found intertwined under small rocks along the edges of mudflats (as in Coos Bay, OR or Crescent City, CA) or on the open coast in shell hash and amongst the roots of *Phyllospadix* sp. (e.g. in Sunset Bay, Middle Cove, South Cove, and North Cove at Cape Arago, OR).

Description. *External features.* Overall body and head shape as in *Maculaura alaskensis* and *Maculaura aquilonia*, but reaching greater lengths than either species. Largest specimens are 10 cm in length and 3–4 mm in width, with average individuals on the order of 5 cm long and 2 mm wide (Fig. 3.4G–J), while gliding. Head is narrow anteriorly and not prominently demarcated from body when worm is gliding (Fig. 3.2D). Head shape changes dramatically when contracted, at which time the head tip can be rather pointed. Body is generally a pale pink, rounded anteriorly and dorso-ventrally

flattened posteriorly. The posterior of reproductive individuals can be pale pink to yellow, and gametes are visible through the body wall (Fig. 3.4H). Ventral surface of body slightly lighter colored than dorsal. The most notable exterior feature in this species is a conspicuous pink or rose pigment of the brain (Fig. 3.4G–I). Caudal cirrus is distinct from those of the other species in the genus as it is rather short and gradually tapers from posterior of body instead of transitioning abruptly, e.g. as in *Maculaura alaskensis* and *Maculaura aquilonia* (cf. Fig. 3.4B, E, J). Movement is without distinct peristalsis and is led with the head, which can contract and taper dramatically. Individuals often seek out and attempt to burrow under objects in glass bowls (e.g. rulers, rocks). Fragmentation occurs during collection, especially in sexually mature individuals, and posterior end regenerates routinely as in *Maculaura alaskensis* and *Maculaura aquilonia*.

Internal features. Internal anatomy as in *Maculaura alaskensis*. (see Fig. 3.7E–G).

Reproduction and development. Sexes are separate. Reproductive females and males have been collected March through October, with sexually mature individuals mostly found in spring months, slightly earlier than is seen in *Maculaura alaskensis*. Gametes are arranged serially between intestinal diverticula, as in other *Maculaura* species. Dissected oocytes are 95 μm in diameter (n = 10) and surrounded by a chorion (Fig. 3.3C). Sperm headpiece is shaped like the blade of an agricultural scythe and is 10 μm in length (Fig. 3.3D).

When reared in the lab, first and second cleavage occurs at roughly 2 and 3 hours post fertilization (at 12°C), respectively, and larvae begin feeding on *Rhodomonas lens* at 2–3 days. The cephalic discs and trunk discs develop at approximately one week (Fig. 3.10A) and polka-dot pigment characteristic of the *pilidium maculosum* morphotype is apparent on the cephalic discs by 14 days (arrowhead, Fig. 3.10B). Larvae have all three pairs of imaginal discs as early as 18 days (Fig. 3.10C) and reach the torus stage (Maslakova, 2010) as early as 25 days (Fig. 3.10D). In a single cohort (fertilized in March 2014),

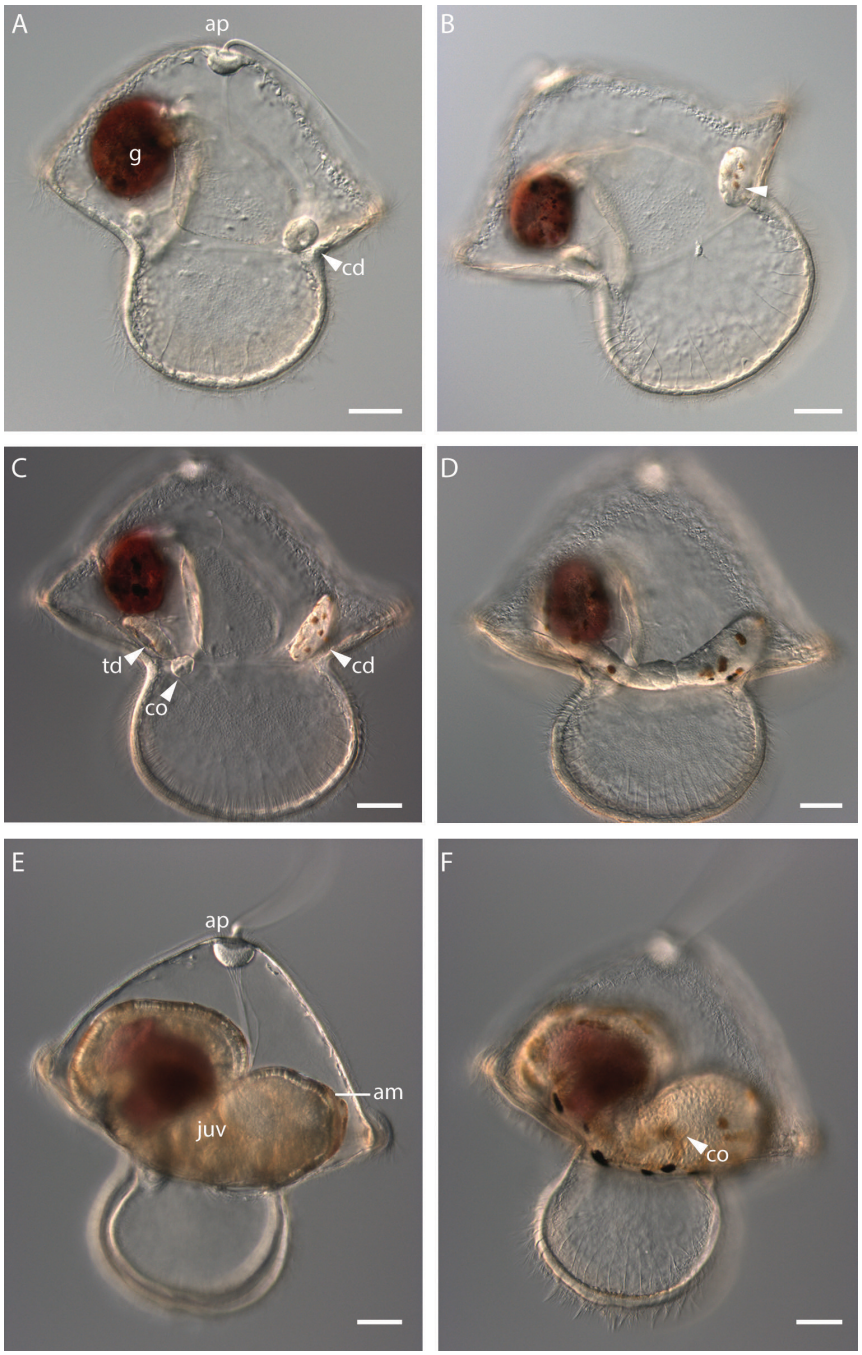

Figure 3.10. Development in *Maculaura cerebrosa* sp. nov. **(A)** A nine-day-old larva with cephalic discs in focus and gut positioned at left. **(B)** Polka-dot pigment spots on cephalic discs present at 14 days (arrowhead). **(C)** An 18-day-old larva. **(D)** Twenty-five-day old larva with fused discs. **(E, F)** The juvenile nemertean surrounded by a pigmented amnion (in focus in F) and competent to metamorphose at approximately 45 days; note cerebral organs (F). Abbreviations: **cd**, cephalic disc; **co**, cerebral organ disc; **g**, gut; **juv**, juvenile; **td**, trunk disc. Scale bars: 100 µm.

metamorphosis was observed after approximately 45 days post-fertilization. Metamorphically competent larvae are approximately 500 μm from apical tuft to apex of lateral lappet (Fig. 3.10E). Metamorphosis is catastrophic, as in other pilidia, and the emerging juvenile devours the larval body. The amnion surrounding the juvenile worm is decorated with red, black, and maroon pigment spots (Fig. 3.10F). Wild-caught larvae, confirmed to belong to this species by DNA sequencing, were collected from plankton in Coos Bay in August.

Maculaura magna sp. nov.

(Figs. 3.2F, 3.3G–H, 3.4K, 3.7H–L, 3.11)

Etymology. This specific name is a Latin adjective (*magnus*, *-a*, *-um*; "great" or "large") in reference to the large size of this species, reaching sizes greater than any other known member of this genus.

Type material. Type material is deposited at NMNH and includes serial transverse sections of the holotype (56 slides USNM# 1282125) and two un-sectioned paratypes preserved for histology and their associated ethanol-preserved tissue (USNM#s 1282126–1282127) (Table 3.1). We designate a partial COI sequence derived from the holotype as a hologenetype (GenBank accession number KP682147, Table 3.1).

Material examined. Thirteen adult individuals, including the holotype and paratypes, and 18 wild-caught larvae were examined and in most cases their identification was confirmed by DNA sequence data. All individuals were collected from locations near Charleston, OR (Table 3.1). Two paratypes lack good quality DNA sequence data, but were confidently identified by morphology alone (USNM#s 1282126–1282127). Ethanol-preserved tissue and/or extracted DNA from remaining individuals are held at the OIMB.

Diagnosis. *Maculaura magna* is the largest species in the genus (Fig. 3.2B–F). It differs from *Maculaura alaskensis*, *Maculaura aquilonia*, and *Maculaura cerebrosa* by a pinkish-red body color (Fig. 3.4K). Internally the cutis of *Maculaura magna* has fewer gland cells than in other members of this genus (Fig. 3.7I). *Maculaura magna* is morphologically most similar to *Maculaura oregonensis* because both species have pink body color. However, *Maculaura* is overall larger and its body is more of a dusty rose color compared to *Maculaura oregonensis*, which is brighter pink or reddish (compare Fig. 3.4K with 3.4L–Q). The most accurate way to differentiate species in this genus is by comparing their eggs and sperm (Fig. 3.3). The eggs of *Maculaura magna* are large (125 μm in diameter), surrounded by an egg chorion and sperm headpieces are elongated and 15 μm in length (Fig. 3.3G–H). The only other species that has gametes of similar morphology is *Maculaura cerebrosa*; however, the gametes of *Maculaura cerebrosa* are smaller than those of *Maculaura magna*. The eggs of *Maculaura cerebrosa* are 95 μm and sperm headpieces are 10 μm in length (compare Fig. 3.3G–H with 3.3C–D).

Habitat, type locality, and distribution. The type locality is a mudflat in Charleston, OR (43.3428°N, 124.3218°W) (OR-C1, Fig. 3.1C). This species is currently only known from southern OR where it is common in sand (e.g. Fig. 3.1D) and mud from mid to low intertidal. Single individuals are usually found, not occurring in groups, and they can be burrowed quite deep (to 0.75 m). We have found specimens in a variety of sandflats along the shores of Coos Bay, north to Empire, as well as along South Slough estuary near the Charleston Marina. Several individuals have been collected from a sandy beach at North Cove near Cape Arago, OR. However, at these locations it is not nearly as common as *Maculaura alaskensis* or *Maculaura cerebrosa*.

Description. *External features.* Resembles species of *Cerebratulus* in being rather large, broad, and dorso-ventrally flattened. Largest specimens are up to 30 cm in length and 1 cm in width, and average individuals are approximately 20 cm in length and 3–4 mm wide (Fig. 3.4K). Head is pale white and body can be rather dark pink (Fig. 3.4K bottom

left inset), with a gradual transition in color from the anterior to posterior. The foregut region is rounded in cross-section, while the mid-body region is flattened dorso-ventrally (Fig. 3.2F), but rounded in individuals packed with gametes. Dorsal side can be somewhat darker than the ventral side in some individuals, with a sharp lateral transition between the dorsal and ventral color. Head can be pointed and change shape dramatically when contracted (Fig. 3.2F). The posterior region of the body ends abruptly with caudal cirrus (Figs. 3.2F inset, 3.4K upper right inset). Movement is with gentle peristalsis and is led with the head, which can curve from side to side. Fragmentation occurs frequently during collection in the field, due to the large size of this species. The posterior end regenerates easily, but slower than in smaller species (*Maculaura alaskensis*, *Maculaura aquilonia*, and *Maculaura cerebrosa*), and anterior regeneration has not been observed. Regenerated region is typically lighter in color than adjacent (non-regenerated) body.

Internal features. Internal anatomy similar to *Maculaura alaskensis* (see Fig. 3.7I–L). Three relatively large apical sense organs are clearly visible just anterior to the proboscis pore in histological sections (Fig. 3.7H). Mouth is ventral and quite long (up to 1 mm). Transition from foregut to intestinal region is met with a dramatic thickening of the circular musculature (Fig. 3.7L). The proboscis musculature exhibits two distinct crosses extending from the circular muscle to the proboscis endothelium. The diagonal proboscis muscle layer is thin and less conspicuous in *Maculaura magna* than in the other four species. The proboscis also contains two outer longitudinal muscle strands outside the main proboscis nerves, which were not observed in the other four species. The nephridial canals are larger in this species than in other *Maculaura* species, as is, perhaps, fitting, since it is a larger species.

Reproduction and development. Sexes are separate. Ripe females were collected in June, and a reproductive male was collected in January 2012. Gametes are arranged laterally between intestinal diverticula. Dissected oocytes are 125 μm in diameter (n = 10), surrounded by a chorion (Fig. 3.3G). Sperm headpiece is elongated, 15 μm in length (Fig.

3.3H). Wild-caught larvae, confirmed to belong to this species by DNA sequence data, were collected from plankton in Coos Bay March through December and exhibit the *pilidium maculosum* morphotype (Fig. 3.11). The larval episphere is haystack-shaped and tall with relatively short lateral lappets (Fig. 3.11B, C).

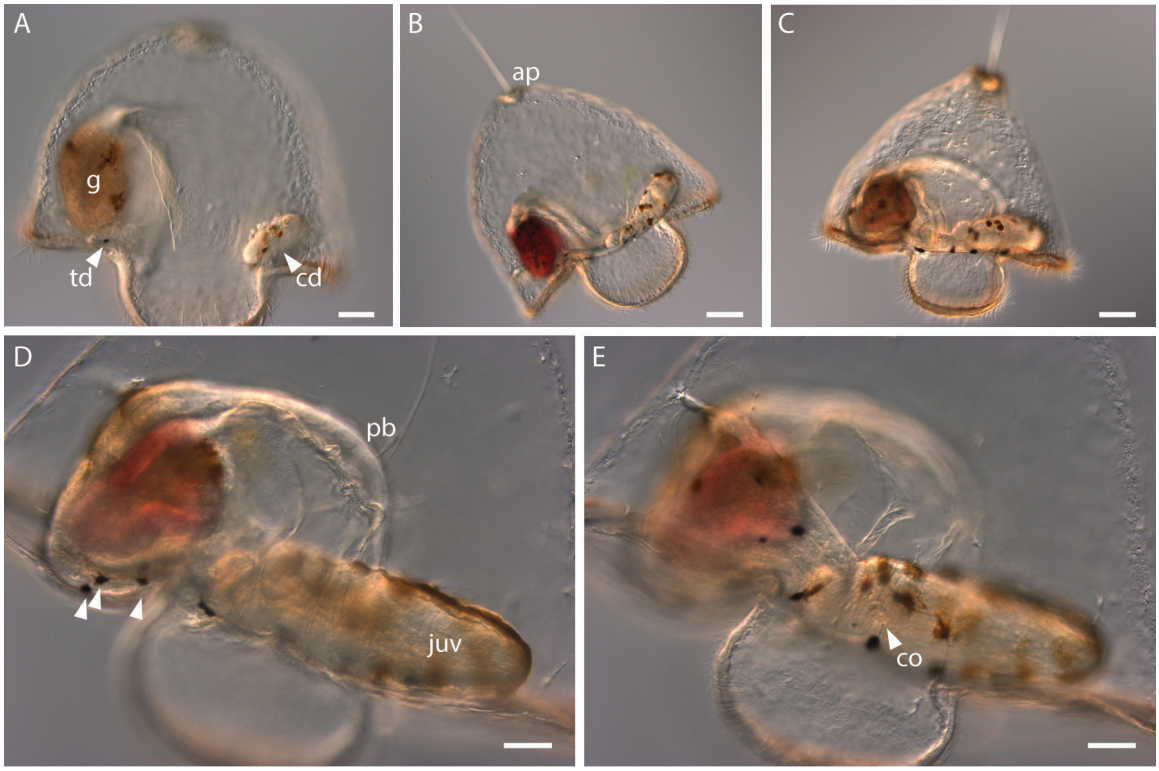

Figure 3.11. Wild-caught larvae of *Maculaura magna* sp. nov. **(A)** Larva collected 2 July 2013; note polka-dot pigment spots on cephalic discs. **(B)** Larva collected 10 June 2013 with fused discs and apical tuft just out of focal plane. **(C–E)** Larva collected 1 July 2013 with advanced juvenile, proboscis rudiment, and pigment spots on amnion (D, arrowheads); a different focal plane shows the cerebral organ (E, arrowhead). Abbreviations: **ap**, apical tuft; **cd**, cephalic discs; **co**, cerebral organ; **g**, gut; **juv**, juvenile; **pb**, proboscis rudiment; **td**, trunk disc. Scale bars: 100 µm.

Maculaura oregonensis sp. nov.

(Figs. 3.2E, 3.3J, 3.4L–Q, 3.7M–O)

Etymology. The specific name is an adjective (*-ensis*, *-ense*), referring to the type locality and currently known distribution of this species.

Type material. Serial transverse sections of the holotype (female, 39 slides USNM# 1282128), one paratype preserved for histology (USNM# 1282129) and associated ethanol-preserved tissue are deposited at NMNH (Table 3.1). We designate a partial COI sequence from the holotype as a hologenetype (GenBank accession number KP682159, Table 3.1).

Material examined. Eleven adult individuals, (all collected from locations near Charleston, OR) including the holotype and paratype were examined and their identification confirmed by DNA sequence data (Table 3.1). Ethanol-preserved tissue and/or extracted DNA from remaining individuals are held at the OIMB.

Diagnosis. *Maculaura oregonensis* differs from *Maculaura alaskensis*, *Maculaura aquilonia*, and *Maculaura cerebrosa* by its larger size, wider body, and pink body color. Internally, *Maculaura oregonensis* differs from these three species further, in that more red and fewer blue staining gland cells exist in the cutis (compare Fig. 3.7E with 3.7M). External color in *Maculaura oregonensis* most closely resembles *Maculaura magna*; however, the latter species is significantly larger than *Maculaura oregonensis* and the body color differs slightly between the two species. *Maculaura oregonensis* is bright pink or reddish in color while *Maculaura magna* exhibits more of a dusty rose body color (compare Fig. 3.4K with 3.4L–Q). The sperm headpiece is shorter in *Maculaura oregonensis* compared to that in *Maculaura cerebrosa* and *Maculaura magna*. The sperm of *Maculaura oregonensis* has similar morphology to *Maculaura alaskensis* but is slightly longer (compare Fig. 3.3B with 3.3J). *Maculaura oregonensis* and *Maculaura*

aquilonia sperm are indistinguishable; they both have slightly elongated headpieces (although not as long as *Maculaura cerebrosa* or *Maculaura magna*) that are 7–8 μm in length. Instead, these species can be differentiated by body color alone, in that *Maculaura oregonensis* is significantly darker pink (compare Fig. 3.4C with 3.4L–Q). At present we lack information on size of oocytes and presence of the chorion in *Maculaura oregonensis.*

Habitat, type locality, and distribution. The type locality is a mudflat in Charleston, OR (43.3272°N, 124.3263°W) (OR-C10, Fig. 3.1C). This species is, at present, only found in southern Oregon where it is relatively rare. Individuals can be found in sand and mud from mid- to low intertidal (e.g. Fig. 3.1D). Several specimens were collected in north Coos Bay, near the McCullough Bridge, and few individuals were collected with other species of this genus at a variety of mudflats along the South Slough estuary (Fig. 3.1C). In one instance, several individuals were observed surrounding the hoplonemertean, *Paranemertes peregrina*.

Description. *External features*. Largest specimens were 15 cm in length and 5 mm in width, while gliding, with average individuals about 8–10 cm long and 3 mm wide (Fig. 3.4L–Q). Head is pale white and body color is dark pink. The dorsal side is the same color as ventral side. The body is rounded in cross-section anteriorly and dorso-ventrally flattened posteriorly. Head narrows to a point while worm is gliding (Fig. 3.4L–O). The proboscis is paler than the background color of the body and can easily be seen through the body wall (Fig. 3.4O). Brain is pink and shows through the body wall, as a somewhat darker pink at the transition from the pale color of the head to the bright pink of the body. Caudal cirrus present, somewhat intermediate in shape between that of *Maculaura cerebrosa* and the other species in the genus (Figs. 3.2E, 3.4Q). Movement is with gentle peristalsis, as in *Maculaura magna*, and is led with the head, which can curl dramatically (Fig. 3.4N). *Maculaura oregonensis* is often seen retracting its head within its body (Fig.

3.4M). Fragmentation occurs during collection and posterior end regenerates routinely, but anterior regeneration has not been observed.

Internal features. Internal anatomy as in *Maculaura alaskensis* (see Fig. 3.7M–O). The two proboscis muscle crosses are most conspicuous in this species and the nephridia are comparatively larger than in *Maculaura alaskensis*, *Maculaura aquilonia* and *Maculaura cerebrosa*, but smaller than *in Maculaura magna* (Fig. 3.7N).

Reproduction and development. Sexes are separate. The gametes of one ripe male were observed on 20 May 2014 (Fig. 3.3J). Sperm headpiece is approximately 7–8 μm (n = 10) in length. Reproductive females were observed in the Summer 2014, but the size of the oocytes was not recorded. Each ovary of the holotype contained approximately 35 oocytes (Fig. 3.7O).

Phylogenetic analysis, haplotype networks, and species delimitation

Bayesian (not shown) and ML analyses (Fig. 3.12) of the 16S and COI datasets each resulted in five well-supported monophyletic clades, corresponding to the five species described here (Fig. 3.12). Consistently between different analyses, *Maculaura alaskensis* and *Maculaura oregonensis* were sister species; however, the relationships between the other three species differed depending on the gene region (compare Fig. 3.12A with 3.12B). The average uncorrected intraspecific and interspecific percent divergence values are reported in Table 3.2. Four of the five species have intraspecific divergence values of < 1% for both 16S and COI gene regions (Table 3.2). *Maculaura magna* is an exception; it exhibits the largest intraspecific variation at 1.1% and 7.1% for

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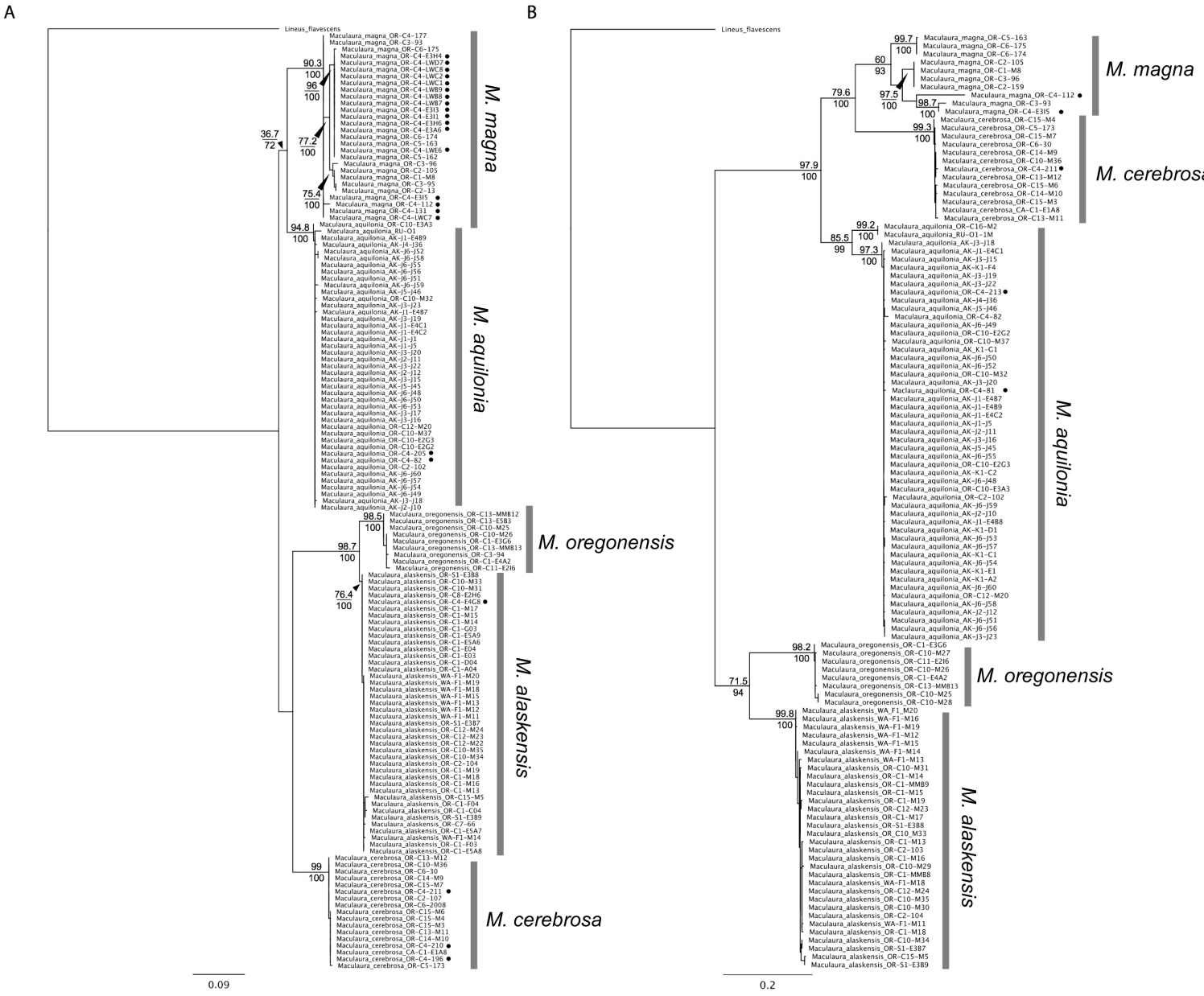
Figure 3.12 (next page). (A) 16S and **(B)** COI maximum likelihood phylogenies for the genus *Maculaura* gen. nov. Bootstrap support value (> 70%) in maximum likelihood analysis (above node) and Bayesian posterior probabilities (below node) are indicated for each clade. Individual collection locations are shown and correspond to those in Fig. 3.1A–C. Larval sequences are indicated with closed circles.


A
Lineus_flavescens
Maculaura_magna_OR-C4-177
Maculaura_magna_OR-C3-93
Maculaura_magna_OR-C6-175
Maculaura_magna_OR-C4-E3H4
Maculaura_magna_OR-C4-LWD7
Maculaura_magna_OR-C4-LWC8
Maculaura_magna_OR-C4-LWC2
Maculaura_magna_OR-C4-LWC1
Maculaura_magna_OR-C4-LWB9
Maculaura_magna_OR-C4-LWB8
Maculaura_magna_OR-C4-LWB7
Maculaura_magna_OR-C4-E3I3
Maculaura_magna_OR-C4-E3I1
Maculaura_magna_OR-C4-E3H6
Maculaura_magna_OR-C4-E3A6
Maculaura_magna_OR-C6-174
Maculaura_magna_OR-C5-163
Maculaura_magna_OR-C4-LWE6
Maculaura_magna_OR-C5-162
Maculaura_magna_OR-C3-96
Maculaura_magna_OR-C2-105
Maculaura_magna_OR-C1-M8
Maculaura_magna_OR-C3-95
Maculaura_magna_OR-C2-13
Maculaura_magna_OR-C4-E3I5
Maculaura_magna_OR-C4-112
Maculaura_magna_OR-C4-131
Maculaura_magna_OR-C4-LWC7
M. magna
Maculaura_aquilonia_OR-C10-E3A3
Maculaura_aquilonia_RU-O1
Maculaura_aquilonia_AK-J1-E4B9
Maculaura_aquilonia_AK-J4-J36
Maculaura_aquilonia_AK-J6-J52
Maculaura_aquilonia_AK-J6-J58
Maculaura_aquilonia_AK-J6-J55
Maculaura_aquilonia_AK-J6-J56
Maculaura_aquilonia_AK-J6-J51
Maculaura_aquilonia_AK-J6-J59
Maculaura_aquilonia_AK-J5-J46
Maculaura_aquilonia_OR-C10-M32
Maculaura_aquilonia_AK-J3-J23
Maculaura_aquilonia_AK-J1-E4B7
Maculaura_aquilonia_AK-J3-J19
Maculaura_aquilonia_AK-J1-E4C1
Maculaura_aquilonia_AK-J1-E4C2
Maculaura_aquilonia_AK-J1-J1
Maculaura_aquilonia_AK-J1-J5
Maculaura_aquilonia_AK-J3-J20
Maculaura_aquilonia_AK-J2-J11
Maculaura_aquilonia_AK-J3-J22
Maculaura_aquilonia_AK-J2-J12
Maculaura_aquilonia_AK-J3-J15
Maculaura_aquilonia_AK-J5-J45
Maculaura_aquilonia_AK-J6-J48
Maculaura_aquilonia_AK-J6-J50
Maculaura_aquilonia_AK-J6-J53
Maculaura_aquilonia_AK-J3-J17
Maculaura_aquilonia_AK-J3-J16
Maculaura_aquilonia_OR-C12-M20
Maculaura_aquilonia_OR-C10-M37
Maculaura_aquilonia_OR-C10-E2G3
Maculaura_aquilonia_OR-C10-E2G2
Maculaura_aquilonia_OR-C4-205
Maculaura_aquilonia_OR-C4-82
Maculaura_aquilonia_OR-C2-102
Maculaura_aquilonia_AK-J6-J60
Maculaura_aquilonia_AK-J6-J57
Maculaura_aquilonia_AK-J6-J54
Maculaura_aquilonia_AK-J6-J49
Maculaura_aquilonia_AK-J3-J18
Maculaura_aquilonia_AK-J2-J10
M. aquilonia
Maculaura_oregonensis_OR-C13-MMB12
Maculaura_oregonensis_OR-C13-E5B3
Maculaura_oregonensis_OR-C10-M25
Maculaura_oregonensis_OR-C10-M26
Maculaura_oregonensis_OR-C1-E3G6
Maculaura_oregonensis_OR-C13-MMB13
Maculaura_oregonensis_OR-C3-94
Maculaura_oregonensis_OR-C1-E4A2
Maculaura_oregonensis_OR-C11-E2I6
M. oregonensis
Maculaura_alaskensis_OR-S1-E3B8
Maculaura_alaskensis_OR-C10-M33
Maculaura_alaskensis_OR-C10-M31
Maculaura_alaskensis_OR-C8-E2H6
Maculaura_alaskensis_OR-C4-E4G8
Maculaura_alaskensis_OR-C1-M17
Maculaura_alaskensis_OR-C1-M15
Maculaura_alaskensis_OR-C1-M14
Maculaura_alaskensis_OR-C1-G03
Maculaura_alaskensis_OR-C1-E5A9
Maculaura_alaskensis_OR-C1-E5A6
Maculaura_alaskensis_OR-C1-E04
Maculaura_alaskensis_OR-C1-E03
Maculaura_alaskensis_OR-C1-D04
Maculaura_alaskensis_OR-C1-A04
Maculaura_alaskensis_WA-F1-M20
Maculaura_alaskensis_WA-F1-M19
Maculaura_alaskensis_WA-F1-M18
Maculaura_alaskensis_WA-F1-M15
Maculaura_alaskensis_WA-F1-M13
Maculaura_alaskensis_WA-F1-M12
Maculaura_alaskensis_WA-F1-M11
Maculaura_alaskensis_OR-S1-E3B7
Maculaura_alaskensis_OR-C12-M24
Maculaura_alaskensis_OR-C12-M23
Maculaura_alaskensis_OR-C12-M22
Maculaura_alaskensis_OR-C10-M35
Maculaura_alaskensis_OR-C10-M34
Maculaura_alaskensis_OR-C2-104
Maculaura_alaskensis_OR-C1-M19
Maculaura_alaskensis_OR-C1-M18
Maculaura_alaskensis_OR-C1-M16
Maculaura_alaskensis_OR-C1-M13
Maculaura_alaskensis_OR-C15-M5
Maculaura_alaskensis_OR-C1-F04
Maculaura_alaskensis_OR-C1-C04
Maculaura_alaskensis_OR-S1-E3B9
Maculaura_alaskensis_OR-C7-66
Maculaura_alaskensis_OR-C1-E5A7
Maculaura_alaskensis_WA-F1-M14
Maculaura_alaskensis_OR-C1-F03
Maculaura_alaskensis_OR-C1-E5A8
M. alaskensis
Maculaura_cerebrosa_OR-C13-M12
Maculaura_cerebrosa_OR-C10-M36
Maculaura_cerebrosa_OR-C6-30
Maculaura_cerebrosa_OR-C14-M9
Maculaura_cerebrosa_OR-C15-M7
Maculaura_cerebrosa_OR-C4-211
Maculaura_cerebrosa_OR-C2-107
Maculaura_cerebrosa_OR-C6-2008
Maculaura_cerebrosa_OR-C15-M6
Maculaura_cerebrosa_OR-C15-M4
Maculaura_cerebrosa_OR-C15-M3
Maculaura_cerebrosa_OR-C13-M11
Maculaura_cerebrosa_OR-C14-M10
Maculaura_cerebrosa_OR-C4-210
Maculaura_cerebrosa_CA-C1-E1A8
Maculaura_cerebrosa_OR-C4-196
Maculaura_cerebrosa_OR-C5-173
M. cerebrosa
0.09
B
Lineus_flavescens
Maculaura_magna_OR-C5-163
Maculaura_magna_OR-C6-175
Maculaura_magna_OR-C6-174
Maculaura_magna_OR-C2-105
Maculaura_magna_OR-C1-M8
Maculaura_magna_OR-C3-96
Maculaura_magna_OR-C2-159
Maculaura_magna_OR-C4-112
Maculaura_magna_OR-C3-93
Maculaura_magna_OR-C4-E3I5
M. magna
Maculaura_cerebrosa_OR-C15-M4
Maculaura_cerebrosa_OR-C5-173
Maculaura_cerebrosa_OR-C15-M7
Maculaura_cerebrosa_OR-C6-30
Maculaura_cerebrosa_OR-C14-M9
Maculaura_cerebrosa_OR-C10-M36
Maculaura_cerebrosa_OR-C4-211
Maculaura_cerebrosa_OR-C13-M12
Maculaura_cerebrosa_OR-C15-M6
Maculaura_cerebrosa_OR-C14-M10
Maculaura_cerebrosa_OR-C15-M3
Maculaura_cerebrosa_CA-C1-E1A8
Maculaura_cerebrosa_OR-C13-M11
M. cerebrosa
Maculaura_aquilonia_OR-C16-M2
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Maculaura_aquilonia_AK-J1-E4C1
Maculaura_aquilonia_AK-J3-J15
Maculaura_aquilonia_AK-K1-F4
Maculaura_aquilonia_AK-J3-J19
Maculaura_aquilonia_AK-J3-J22
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Maculaura_aquilonia_AK-J4-J36
Maculaura_aquilonia_AK-J5-J46
Maculaura_aquilonia_OR-C4-82
Maculaura_aquilonia_AK-J6-J49
Maculaura_aquilonia_OR-C10-E2G2
Maculaura_aquilonia_OR-C10-M37
Maculaura_aquilonia_AK_K1-C1
Maculaura_aquilonia_AK-J6-J50
Maculaura_aquilonia_AK-J6-J52
Maculaura_aquilonia_OR-C10-M32
Maculaura_aquilonia_AK-J3-J20
Maclaura_aquilonia_OR-C4-81
Maculaura_aquilonia_AK-J1-E4B7
Maculaura_aquilonia_AK-J1-E4B9
Maculaura_aquilonia_AK-J1-E4C2
Maculaura_aquilonia_AK-J1-J5
Maculaura_aquilonia_AK-J2-J11
Maculaura_aquilonia_AK-J3-J16
Maculaura_aquilonia_AK-J5-J45
Maculaura_aquilonia_AK-J6-J55
Maculaura_aquilonia_OR-C10-E2G3
Maculaura_aquilonia_AK-K1-C2
Maculaura_aquilonia_AK-J6-J48
Maculaura_aquilonia_OR-C10-E3A3
Maculaura_aquilonia_OR-C2-102
Maculaura_aquilonia_AK-J6-J59
Maculaura_aquilonia_AK-J2-J10
Maculaura_aquilonia_AK-J1-E4B8
Maculaura_aquilonia_AK-K1-D1
Maculaura_aquilonia_AK-J6-J53
Maculaura_aquilonia_AK-J6-J57
Maculaura_aquilonia_AK-K1-C1
Maculaura_aquilonia_AK-J6-J54
Maculaura_aquilonia_AK-K1-E1
Maculaura_aquilonia_AK-K1-A2
Maculaura_aquilonia_AK-J6-J60
Maculaura_aquilonia_OR-C12-M20
Maculaura_aquilonia_AK-J6-J58
Maculaura_aquilonia_AK-J2-J12
Maculaura_aquilonia_AK-J6-J51
Maculaura_aquilonia_AK-J6-J56
Maculaura_aquilonia_AK-J3-J23
M. aquilonia
Maculaura_oregonensis_OR-C1-E3G6
Maculaura_oregonensis_OR-C10-M27
Maculaura_oregonensis_OR-C11-E2I6
Maculaura_oregonensis_OR-C10-M26
Maculaura_oregonensis_OR-C1-E4A2
Maculaura_oregonensis_OR-C13-MMB13
Maculaura_oregonensis_OR-C10-M25
Maculaura_oregonensis_OR-C10-M28
M. oregonensis
Maculaura_alaskensis_WA_F1_M20
Maculaura_alaskensis_WA-F1-M16
Maculaura_alaskensis_WA-F1-M19
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Maculaura_alaskensis_WA-F1-M15
Maculaura_alaskensis_WA-F1-M14
Maculaura_alaskensis_WA-F1-M13
Maculaura_alaskensis_OR-C10-M31
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Maculaura_alaskensis_OR-C1-M15
Maculaura_alaskensis_OR-C1-M19
Maculaura_alaskensis_OR-C12-M23
Maculaura_alaskensis_OR-C1-M17
Maculaura_alaskensis_OR-S1-E3B8
Maculaura_alaskensis_OR_C10_M33
Maculaura_alaskensis_OR-C1-M13
Maculaura_alaskensis_OR-C2-103
Maculaura_alaskensis_OR-C1-M16
Maculaura_alaskensis_OR-C10-M29
Maculaura_alaskensis_OR-C1-MMB8
Maculaura_alaskensis_WA-F1-M18
Maculaura_alaskensis_OR-C12-M24
Maculaura_alaskensis_OR-C10-M35
Maculaura_alaskensis_OR-C10-M30
Maculaura_alaskensis_OR-C2-104
Maculaura_alaskensis_WA-F1-M11
Maculaura_alaskensis_OR-C1-M18
Maculaura_alaskensis_OR-C10-M34
Maculaura_alaskensis_OR-S1-E3B7
Maculaura_alaskensis_OR-C15-M5
Maculaura_alaskensis_OR-S1-E3B9
M. alaskensis
0.2

Table 3.2. Inter- and intraspecific variation shown as uncorrected *p*-distances for 16S rDNA and COI (bold text) gene regions.

	M. alaskensis	*M. aquilonia*	*M. cerebrosa*	*M. magna*	*M. oregonensis*
Maculaura alaskensis	0.2	10.9	11.2	10.5	4.0
	0.7	**17.9**	**17.4**	**17.0**	**14.3**
Maculaura aquilonia		0.1	8.8	8.0	12.2
		0.3	**14.8**	**13.9**	**16.3**
Maculaura cerebrosa			0.1	10.2	11.7
			0.6	**12.9**	**18.0**
Maculaura magna				1.1	11.0
				7.1	**16.3**
Maculaura oregonensis					0.4
					0.2

the 16S and COI gene regions, respectively. A sufficient barcoding gap (Meyer and Paulay, 2005) exists between species, as the interspecific divergence between the two most closely related species, *Maculaura oregonensis* and *Maculaura alaskensis*, is 4.0% and 14.3% for the 16S and COI gene regions, respectively.

A similar barcoding gap was detected using ABGD: between 5% and 6% (16S) and between 3% and 7–9% (COI). For the 16S gene region, the four species *Maculaura alaskensis*, *Maculaura aquilonia*, *Maculaura cerebrosa*, and *Maculaura oregonensis* were corroborated in ABGD using a cut-off value of 1.3%; however, *Maculaura magna,* the species that exhibits the greatest degree of sequence divergence, was divided into five groups (not shown). With a cut-off value from 2.2–6.0%, ABGD reveals four species *Maculaura magna*, *Maculaura cerebrosa*, *Maculaura aquilonia*, and a species composed of both *Maculaura alaskensis* and *Maculaura oregonensis* (Table 3.3). ABGD analysis of COI data (using cut off values from 1.7% to 10%) consistently found nine taxa (Table 3.3). *Maculaura alaskensis*, *Maculaura cerebrosa*, and *Maculaura oregonensis* were consistent with our previous delimitation; however, *Maculaura magna* was partitioned into four species and *Maculaura aquilonia* into two.

TCS networks were generated from the same alignments using a 95% confidence interval (Hart and Sunday, 2007) (Table 3.3). While analysis of 16S data revealed five networks that correspond to the five species described above, analysis of COI data revealed additional networks within *Maculaura magna* (3 total) and *Maculaura aquilonia* (2 total). Four of the five species show little intraspecific divergence with 10 or fewer haplotypes each (Fig. 3.13A–E). Haplotype networks for *Maculaura cerebrosa* reveal three haplotypes, separated by one nucleotide change (Fig. 3.13C) and both *Maculaura alaskensis* (Fig. 3.13A) and *Maculaura aquilonia* (Fig. 3.13B) have 10 haplotypes each. *Maculaura oregonensis* has four haplotypes, separated by 1–5 nucleotide changes (Fig. 3.13D) and *Maculaura magna* has eight 16S haplotypes separated by the largest number of nucleotide differences observed in these five species (Fig. 3.13E).

TCS analysis of COI data reveals a single haplotype network for each of three species (*Maculaura alaskensis*, *Maculaura cerebrosa*, and *Maculaura oregonensis*) (Fig. 3.13F–H), while *Maculaura aquilonia* consists of two haplotype networks (Fig. 3.13I) and *Maculaura magna*, exhibiting the greatest amount of genetic variation, comprises three haplotype networks (Fig. 3.13J) that cannot be connected using a 95% confidence interval. *Maculaura alaskensis* comprises 15 haplotypes (Fig. 3.13F); *Maculaura cerebrosa*, eight haplotypes (Fig. 3.13G); and *Maculaura oregonensis*, five haplotypes (Fig. 3.13H). *Maculaura aquilonia* is divided into two networks including 20 haplotypes; one network with one haplotype (from individuals found in both eastern and western Pacific) and the other with 19 (Fig. 3.13I). The three haplotype networks for *Maculaura magna* include one network with a single haplotype, and two networks with two haplotypes each, one separated by one nucleotide and the other separated by seven nucleotide changes (Fig. 3.13J).

Table 3.3. Comparison between different species delimitation methods. The five species described here are indicated in the top row; the number of taxa (i.e. species) suggested by each method are shown in the table. The total number of species in the genus *Maculaura* gen. nov. suggested by each method is shown at far right.

		Maculaura alaskensis	*Maculaura oregonensis*	*Maculaura aquilonia*	*Maculaura cerebosa*	*Maculaura magna*	total
morphology							
	adult	I	I	I	I	I	5
	gamete	I	I	I	I	I	5
16S							
	reciprocal monophyly	I	I	I	I	I	5
	statistical parsimony (TCS)	I	I	I	I	I	5
	ABGD	I		I	I	I	4
	bPTP	I	I	I	I	I I I I I	9
COI							
	reciprocal monophyly	I	I	I	I	I	5
	statistical parsimony (TCS)	I	I	I I	I	I I I	8
	ABGD	I	I	I I	I	I I I I	9
	bPTP	I	I	I I	I	I I I I I	10

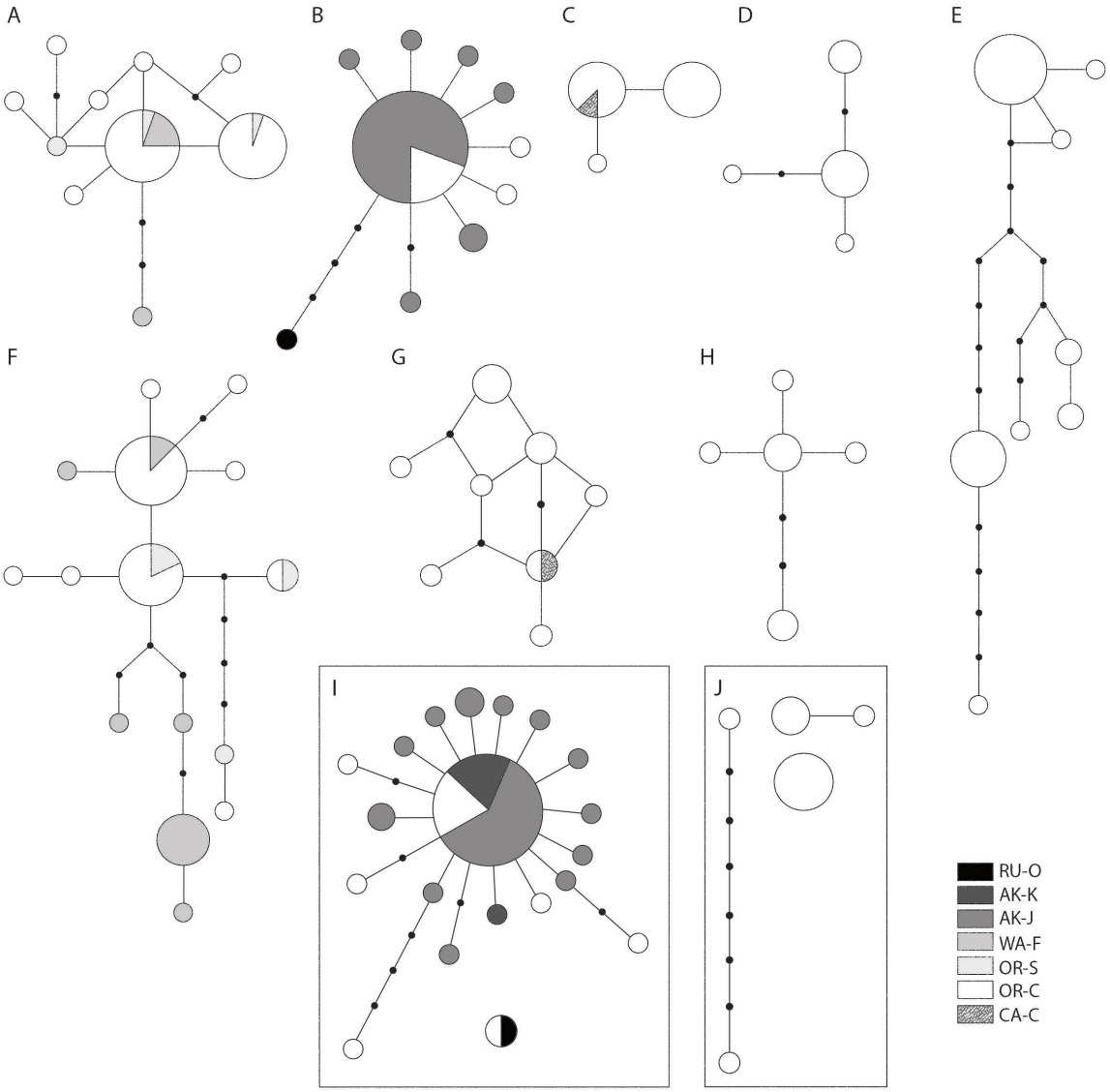


Figure 3.13. (A–E) 16S rDNA and **(F–J)** COI haplotype networks for **(A, F)** *Maculaura alaskensis* comb. nov., **(B, I)** *Maculaura aquilonia* sp. nov.*,* **(C, G)** *Maculaura cerebrosa* sp. nov.*,* **(D, H)** *Maculaura oregonensis* sp. nov.*,* **(E, J)** *Maculaura magna* sp. nov. Sample sites are shown for each haplotype. *Maculaura aquilonia* sp. nov. and *Maculaura magna* sp. nov. haplotypes did not group into a single network for the COI gene region and haplotypes associated with these species are surrounded by boxes (I, J).

Nine species were revealed with the bPTP analysis using 16S and ten species using COI sequence data (Table 3.3). Analyses of 16S and COI sequences from *Maculaura alaskensis*, *Maculaura cerebrosa*, and *Maculaura oregonensis* grouped them into one species each. *Maculaura magna* was partitioned into five groups in both analyses. *Maculaura aquilonia* was a single species according to 16S data, but was split into two species in COI analysis.

Cross-fertilization experiments

Based on our observations, only three *Maculaura* species have somewhat overlapping reproductive timing, *Maculaura alaskensis*, *Maculaura aquilonia*, and *Maculaura cerebrosa*. Reciprocal crosses were attempted between a single male and female of each *Maculaura alaskensis* and *Maculaura cerebrosa* on 8 July 2013 (one replicate each). Although sperm appeared to be attracted to the eggs, no cleavage occurred in either reciprocal cross. Control crosses, however, developed normally for both species. An additional cross (one replicate) was attempted between a single male *Maculaura aquilonia* and female *Maculaura cerebrosa* on 25 February 2014. This cross also resulted in no cleavage. Due to the lack of availability of both sexes during this time, control crosses were not attempted for *Maculaura aquilonia* and *Maculaura cerebrosa*; however, conspecific fertilization and larval culturing has been successful in the laboratory for each of these species at other times (see Figs. 3.8–10).

DISCUSSION

Integrative taxonomy of the "*Micrura alaskensis*" species complex

Although most species descriptions are limited to adult morphology, this information typically is not sufficient to differentiate between closely related or cryptic species (e.g. Manchenko and Kulikova, 1996; Hebert et al., 2004; Strand and Sundberg, 2005; Lavoué et al., 2010; Schulze et al., 2012). This, indeed, is the case for the "*Micrura alaskensis*" species complex. Often, cryptic species can be distinguished from each other by using additional kinds of data such as DNA sequences, gamete morphology, or other

reproductive characters, an approach called integrative taxonomy (e.g. Chen et al., 2010; Puillandre et al., 2014; Welton et al., 2014). Here we present evidence from adult morphology, partial sequences of 16S and COI , gamete morphology, and interbreeding experiments to show that "*Micrura alaskensis*" is not one but five different species.

Some of these five species can be distinguished from each other based on external appearance of live adults using characters such as shape, size, and color of body, and shape and size of the caudal cirrus. Other species are difficult to distinguish based on adult morphology alone. However, they can be easily differentiated using gamete morphology. While we lack information on the egg size of *Maculaura oregonensis*, the other four species can be differentiated by characteristics of the eggs (size and presence of chorion), with further support from sperm morphology. Interestingly, the presence of the chorion in the eggs of *Maculaura cerebrosa* and *Maculaura magna* correlates with modified sperm morphology (scimitar or spear-shaped sperm head). It is possible that a modified sperm head assists in penetrating the egg chorion in these species. Because changes in gamete morphology or other reproductive characteristics can form a barrier to fertilization, such changes are among the first differences one would expect to observe in recently diverged species (e.g. Landry et al., 2003). Indeed, our preliminary cross-breeding experiments (*Maculaura alaskensis* × *Maculaura cerebrosa*; *Maculaura cerebrosa* × *Maculaura aquilonia*) provide evidence that at least some of these species are reproductively isolated.

DNA taxonomy methods based on reciprocal monophyly, existence of separate haplotype networks in statistical parsimony analyses, and the presence of a barcoding gap are commonly used as evidence in support of separate species hypotheses (Hebert et al., 2003; Meyer and Paulay, 2005; Mahon et al., 2009; Chen et al., 2010; Bucklin et al., 2011). Here we show that the existence of five distinct lineages, corresponding to the five species described by us, is supported by reciprocal monophyly on both 16S and COI phylogenies (Fig. 3.12). Furthermore, statistical parsimony analysis of 16S sequence data

supports the existence of the same five species (Fig. 3.13). Haplotype network analysis of COI sequence data results in one network for each of the three species, *Maculaura alaskensis*, *Maculaura cerebrosa*, and *Maculaura oregonensis*. *Maculaura aquilonia* and *Maculaura magna* are further split into two and four networks, respectively. This is not surprising because statistical parsimony analyses tends to over-split species compared to other methods of species delimitation (e.g. Jörer et al., 2012). The three species (*Maculaura alaskensis*, *Maculaura cerebrosa*, and *Maculaura oregonensis*) were also supported by the bPTP and ABGD analyses of both data sets. These latter methods tended to over-split the species *Maculaura magna* (both gene regions) and *Maculaura aquilonia* (COI only) (Table 3.3). The same five species are also supported by the presence of a clear barcoding gap (Meyer and Paulay, 2005; Bucklin et al., 2011) between the maximum intra-specific uncorrected sequence divergences (1.1% for 16S and 7.1% for COI) and the minimum interspecific divergences (4.0% for 16S and 12.9% for COI). Species delimitation methods for *Maculaura magna* and, to some extent, *Maculaura aquilonia* suggest that these species may represent multiple species or are in the process of further speciation. In fact, we have noticed subtle differences in morphology (e.g. body color) among specimens of *Maculaura magna*. However, we do not have sufficient information to confidently split species further than we have here. To sum it up, most of the molecular analyses support our designation of five species. If anything, we are being conservative, and it is possible that *Maculaura magna* represents more than one species. Future sampling and studies of more individuals (their morphology, reproductive biology, and DNA sequence data) of this possibly diversifying species are needed to confirm or exclude this possibility.

Micrura griffini *Coe, 1905*

Coe (1905) described *Micrura griffini* based on specimens collected in San Pedro, California. This species resembled *Micrura alaskensis*, but was larger, more reddish in color and lacked accessory buccal glands (Coe, 1905). Despite these differences in morphology, Coe (1940) later synonymized these two species. We have not attempted to

sample *Maculaura* specimens south of Crescent City, California and can only speculate on the identity of *Micrura griffini* as described by Coe (1905). The external morphology (e.g. size, body color), habitat, reproductive season and oocyte size are similar to *Maculaura magna*. The accessory buccal glands are not as prominent in *Maculaura magna* as they are in other *Maculaura* species (e.g. compare Figs. 3.5D, 3.7B, 3.7E with 3.7J), but are not lacking entirely, as Coe indicated for *Micrura griffini*. Several characters are distinctly different between the two species. First, the oocytes of *Micrura griffini* are described as being remarkably clear (Coe, 1905), which is not the case for *Maculaura magna* (Fig. 3.3G). Second, Coe (1905) described a distinctly pink brain region for *Micrura griffini*. We have not observed this in *Maculaura magna*, but only see it in *Maculaura cerebrosa*. Furthermore, the body color of *Micrura griffini* was described as rosy, bright pinkish red or purplish. We have never observed purplish color in *Maculaura magna* specimens. Thus it is possible that *Micrura griffini* represents a distinct species, possibly within *Maculaura* gen. nov. Future efforts must be directed toward obtaining samples from California for morphological and DNA analyses in order to assess the status of *Micrura griffini* Coe 1905.

Maculaura alaskensis *versus* Maculaura aquilonia

Although Coe (1901) originally described *Micrura alaskensis* from Alaska, his later revisions (Coe, 1904, 1905, 1943) expanded the range of this species south to Ensenada, Mexico, and his revised descriptions clearly include characteristics of more than one species. In fact, all the five species described here fit, at least in part, Coe's (1901, 1904, 1905, 1943) descriptions of "*Micrura alaskensis*". Because the type material does not exist, it is not clear which of the five species Coe (1901) originally encountered and described. DNA sequence data from *Micrura alaskensis*-like specimens, collected from two different locations in Alaska by ourselves and colleagues (albeit none of the locations mentioned in Coe's original description), matches the sequences of one of the five species described here (*Maculaura aquilonia*), but, confusingly, not the one that all recent studies refer to as *Micrura alaskensis*. A literature search for "*Micrura alaskensis*" yields at least

29 publications by 26 authors (as of May 2015) ranging from 1987 to 2014 (Stricker, 1987, 2006; Stricker and Folsom, 1998; Stricker and Smythe, 2000, 2001, 2003; Stricker et al., 2001, 2013; Thollesson and Norenburg, 2003; Maslakova and Matz, 2005; Thiel and Junoy, 2006; Schwartz, 2009; Hiebert and Maslakova, 2010; McDonald and Grünbaum, 2010; Maslakova 2010; Deguchi et al., 2011; Hiebert and Maslakova, 2012, 2014; Hiebert et al., 2013; von Dassow and Maslakova, 2013; von Dassow et al., 2013; Bartolomaeus et al., 2014; Bird et al., 2014; Maslakova and von Dassow, 2014; Mulligan et al., 2014; Swider et al., 2014; Maslakova and Hiebert, 2014, Hiebert and Maslakova, 2015). The species used in nearly every study can be linked by collection location, DNA sequence data, or personal observation to the only species of this complex that is currently known to occur in False Bay, San Juan Island, WA (*Maculaura alaskensis*) and also occurs in southern Oregon. It is distinct from the only species of the genus that we have encountered in Alaska.

We could, of course, assume that the species we found in Alaska (*Maculaura aquilonia*) was the same species originally encountered by Coe (1901), and retain the epithet *"alaskensis"* for it. This would be nomenclaturally straightforward. However, we believe that this would create a significant problem for a community of researchers who know "*Micrura alaskensis*" as a different species, and use it for cell biology, developmental biology, and other types of biological research. Importantly, some of these researchers are not systematists, and might find the name change confusing. It would be especially confusing because the name "*alaskensis*" would not be simply synonymized with another, but would apply to a different, closely related, and morphologically similar species.

In order to maintain nomenclatural stability and facilitate future research using these species, we chose to retain the specific epithet "*alaskensis*" for the species from Washington and Oregon used in recent studies cited above (and designated a neotype and a topogenetype from one of these locations), even though it is possible that it is not the

species originally described by Coe (1901) from Alaska. It is possible that this species does occur in Alaska, but we have not come across it yet. Moreover, even if it does not currently occur in Alaska, it is not inconceivable that it may expand its range northward in the future (e.g. Jones et al., 2012; Chust et al., 2014). To sum up, *Maculaura alaskensis* may not occur in Alaska, but it is the species that most researchers know as "*Micrura alaskensis*". On the other hand, *Maculaura aquilonia* is the only member of the genus we have encountered in Alaska so far.

***Support for the genus* Maculaura**

As members of the genus *Maculaura* all partially fit previous descriptions of *Micrura alaskensis* (Coe, 1901, 1904, 1905, 1940, 1943), characters previously considered exclusive to this species may represent synapomorphies for the new genus described here. For example, Coe (1901, 1940) noted peculiar species-specific "accessory buccal glands" (Coe, 1901, plate 13, fig. 1) in "*Micrura alaskensis*". These foregut glands are sub-epithelial, associated with the buccal cavity and their bodies often penetrate into the inner longitudinal musculature or extend beyond the circular musculature and into the ventral outer longitudinal musculature. Such glands occur in all *Maculaura* species, to varying degrees (Figs. 3.5, 3.7). Importantly, these glands are not observed in *Micrura fasciolata* (USNM# 1098168, Hiebert, Maslakova and Norenburg, personal observation), the type species of the genus *Micrura*. Furthermore, none of the nine species that are described (e.g. Riser, 1998) or coded in character matrices (Schwartz, 2009) as having similar glandular morphology to *Maculaura* (*Micrura wilsoni* USNM# 1107414, *Cerebratulus marginatus* USNM# 1098145–46, *Zygeupolia rubens* USNM# 1098190, *Lineus viridis* USNM# 1098162, *Lineus rubescens* USNM# 1098157, *Fragilonemertes rosea* USNM# 170035, *Eousia verticivarius* USNM# 1098166–67, *Micrura formosana* USNM# 1098170–71, *Notospermus geniculatus* USNM#1098180) exhibit the accessory buccal gland cells observed in *Maculaura* spp. (Norenburg, personal communication). These glands were also not observed in two local undescribed lineiform species (*Micrura* sp. "dark" and *Micrura* sp. "not coei", T. Hiebert, personal observation).

Another morphological synapomorphy of the genus *Maculaura* may be the *pilidium maculosum* larval form characterized by the pigment spots on the juvenile amnion (Figs. 3.6, 3.10, 3.11). Larval development is known in four of the five species (Maslakova, 2010; this study). Three of those exhibit typical pigmentation pattern described here, and one species exhibits less prominent pigmentation (*Maculaura aquilonia*; Figs. 3.8, 3.9). To our knowledge this type of pigmentation has not been observed in any other species of pilidiophoran nemertean, whose larva is known. The development in the type species for the genus *Micrura – Micrura fasciolata*, is currently unknown. Lacalli (2005) described larvae of *pilidium maculosum* type from Bamfield Inlet, BC, Canada. Based on the fact that Bamfield is within the geographical range of *Maculaura*, it is very likely that those larvae belong to one of the species described here.

Aside from these morphological characters, the monophyly of *Maculaura* is supported by phylogenetic analyses of COI, 16S rDNA, and 28S rDNA sequence data, and the clade is only distantly related to *Micrura fasciolata* (Hiebert and Maslakova, in prep).

BRIDGE TO CHAPTER IV

In Chapter III, we described the integrative taxonomy of the *Micrura alaskensis* species complex including a revision of *M. alaskensis* and description of four new species. We designated the new heteronemertean genus, *Maculaura*, for this monophyletic clade of five species. The name of this genus is derived from the distinct larval morphology found in four out of five members. In Chapter IV, we transition focus from nemertean taxonomy to larval identification. In the form of an identification guide, we describe the larvae of over 30 nemertean species, which were identified using both traditional embryology (see also Chapters V and VI) and DNA barcoding.

CHAPTER IV

NEMERTEAN LARVAL IDENTIFICATION: A GUIDE TO PLANKTONIC NEMERTEAN LARVAE FROM THE NORTHEAST PACIFIC COAST

This chapter of my dissertation is co-authored with SA Maslakova and will be submitted to *Zookeys*. This identification guide is available online (www.nemerteanlarvalid.com, since 2014). SA Maslakova collected and identified many larvae in the early stages of this project (2008–2011) and I collected and identified larvae for the duration of my dissertation (2011–2015). I maintained our DNA sequence database, compiled data and images into the online larval identification website, produced all figures, and will submit the final manuscript. With over 400 larval sequences, many people have contributed to the identification of nemertean larvae by virtue of enrollment in SA Maslakova's Marine Molecular Biology course at OIMB, for which I served as TA (2012–2013).

INTRODUCTION

By passing seawater through a fine mesh net one can concentrate tiny planktonic organisms, many of which are the larvae of benthic marine invertebrates. Marine invertebrates often exhibit a bi-phasic life-history where benthic adults reproduce by broadcast spawning gametes which are fertilized and develop in the water column as larvae. These larvae are planktonic for hours to months until they metamorphose and settle into the benthos to begin an adult existence. Identifying larvae can provide insight into population connectivity (e.g., Cowen and Sponaugle 2009), species diversity (e.g., Barber and Boyce 2006), and larval evolution (e.g., Strathmann 1985). Yet few larvae can be identified. Most identification guides are based on formal taxonomic works and species descriptions that provide characters to differentiate species. However, these descriptions and guides are usually based on adult morphology, which means that other developmental stages (e.g., larvae), often with dissimilar morphology, cannot be identified. This is because the development of few species is known and equally few larvae have been reared to metamorphosis so that they can be identified by adult

morphology. The phylum Nemertea is no exception. In the most recent identification guide for marine invertebrate larvae in the Oregonian Biogeographic Province, *An Identification Guide to the Larval Invertebrates of the Pacific Northwest* (Shanks 2001), the general developmental mode of 14 species was known, but only the larvae of one (*Micrura alaskensis*) species could accurately be identified by larval morphology alone (Maslakova 2010a). Using DNA sequence data from barcoding gene regions, as well as traditional embryology, we identified the larvae of 36 species and produced this identification guide. We also uncovered new diversity. Nineteen species are what we refer to as "orphan larvae", representing species that we currently only find as larvae in the plankton and have yet to find in their adult form.

Nemertean larvae

Pilidium larvae – The name pilidium comes from the Greek word meaning "cap" because the typical pilidium larva is shaped like a hat with earflaps that are pulled down over the ears. This larval form is the most indirect-developing form within the Nemertea. Characteristics of the pilidial body include a blade-like apical tuft that sits atop a dome-shaped episphere, two lobes (anterior and posterior), and two lateral lappets (left and right) (Fig. 4.1A). Spanning the lobes and lappets is a conspicuous ciliated band. This band, coupled with muscular contractions of the lobes and lappets, aids in larval feeding (von Dassow et al. 2013). Pilidia are recognizable in the plankton by their unique shape, but also by the way in which they swim. Using their ciliated band, they swim with apical tuft first, usually rotating around their anterior-posterior axis. The movement is distinct in that they swim smoothly, almost drifting, and one often recognizes this first before focusing on their hat-like appearance. What makes this developmental mode indirect is that the juvenile worm has a distinct body plan (similar to that of the adult) and develops within the larval body from a series of isolated rudiments (Salensky 1912; Schmidt 1930; Maslakova 2010a; L. Hiebert and Maslakova 2015). Eventually, these rudiments fuse around the larval gut to form a complete worm. The metamorphosis from planktonic

larva to benthic juvenile is dramatic as the juvenile worm breaks free and, typically, ingests its larval body (Cantell 1966; Lacalli 2005; Maslakova 2010a).

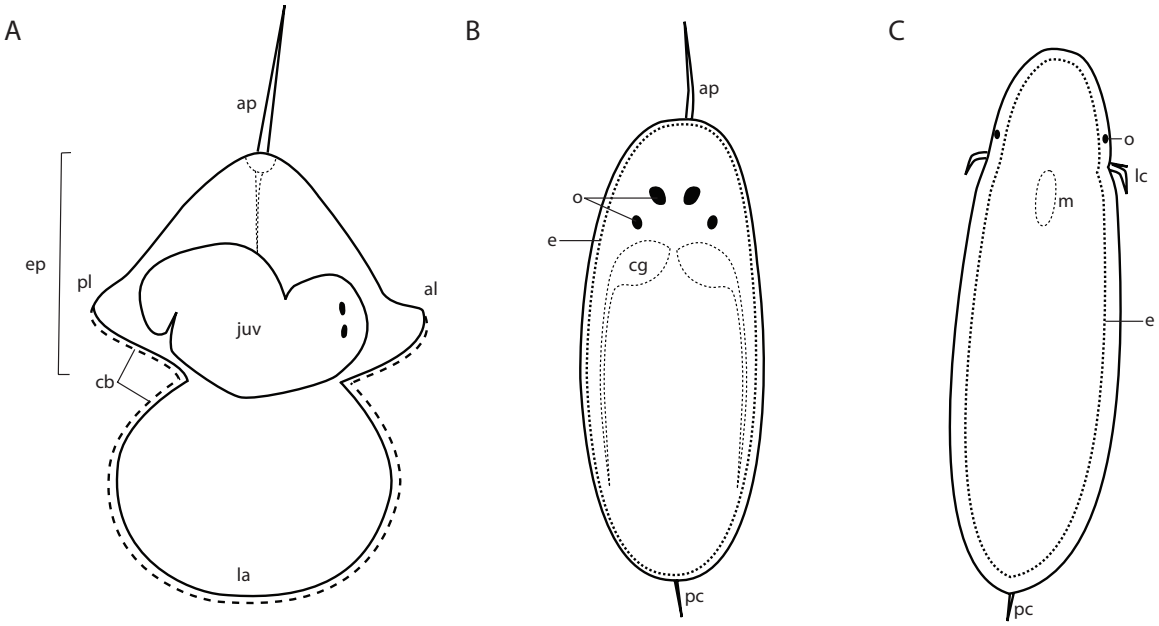


Figure 4.1. Diagrams of generalized nemertean developmental modes. A) Pilidium larva with developing juvenile (juv) inside the larval body. B–C) Planuliform larvae of hoplo- (B) and palaeonemerteans (C). Abbreviations: episphere (ep), anterior lobe (al), posterior lob (pl), apical tuft (ap), ciliated band (cb), ocelli (o), epidermis (e), cerebral ganglion (cg), lateral cirri (lc), posterior cirrus (pc), mouth (m).

Planuliform larvae – Hoplonemertean and palaeonemertean larvae develop more directly. Although they often have a planktonic larval stage, their larvae are uniformly ciliated, resemble the juvenile, and settle into a benthic habitat without overt conspicuous metamorphosis (unlike in the pilidium larva, above). Many hoplonemertean larvae are described to resorb or shed their larval epidermis, but this is a subtle transition (e.g., see Hiebert et al. 2010, Maslakova and von Döhren 2009). Hoplonemertean larvae are characterized by an apical tuft at the anterior and a less prominent posterior cirrus (Fig. 4.1B). When present, their eyes are sub-epidermal and often occur in several pairs. One can often note the proboscis armed with stylets and conspicuous cerebral ganglia in hoplonemertean larvae. Although hoplonemertean larvae are traditionally considered

lecithotrophic (i.e., non-feeding), recent evidence suggests that some species may feed in the plankton (Maslakova and Hiebert 2014). Palaeonemertean larvae exhibit a similar shape to hoplonemertean larvae, but when eyes are present, they are within the epidermis. Some palaeonemertean larvae have prominent lateral cirri while, usually, lacking a prominent apical tuft (Fig. 4.1C). Palaeonemertean larvae tend to swim in a rotating pattern beginning near the bottom of a sorting dish upward where hoplonemertean larvae are found swimming throughout the dish. Palaeonemertean larvae are known to feed in the plankton on large prey (e.g., larvae of other marine invertebrates), and they have a conspicuous mouth.

Pilidial morphotypes

Johannes Müller discovered the pilidium larva in 1847. As it was unrecognizable to him as a nemertean, he assigned the larva the binomen, pilidium gyrans, referring to its hat-like appearance and rotating (i.e., gyrating) swimming behavior (Müller 1847). Since then, many different pilidial morphotypes have been described that vary in shape, pigmentation and juvenile morphology and researchers have assigned these unique morphotypes binomina out of tradition. These names do not replace species names, but provide some descriptive quality for pilidia that are awaiting species-level identification. Whether these morphotypes represent species or groups of closely related species has been debated historically (see Dawydoff 1940), but we believe them to represent and characterize groups of closely related species.

Pilidium gyrans was described by Müller 1847, and subsequently, Bürger (1895) who highlighted the orangish hue along the larval ciliated band. Characters of this larval morphotype, aside from its classic pilidium shape, include a small episphere with respect to the rest of the larval body and a juvenile that develops two small eyes and no caudal cirrus (Fig. 4.2A). A pyramid-like episphere defines the morphotype called pilidium pyramidum (= pilidium pyramidale, Bürger 1895; Dawydoff 1940; Thorson 1946; Chernyshev 2001). Often, these pilidia also have spots on their lobes and lappets (Fig.

4.2B). The amniotic sac surrounding the juvenile nemertean is decorated with polka-dots in the pilidium maculosum morphotype (Fig. 4.2C), which was collected by Lacalli in Bamfield, Canada (2005) and described by T. Hiebert and Maslakova (2015a). The pigment spots are in colors maroon, black and brown and can be seen early in development, surrounding the imaginal discs.

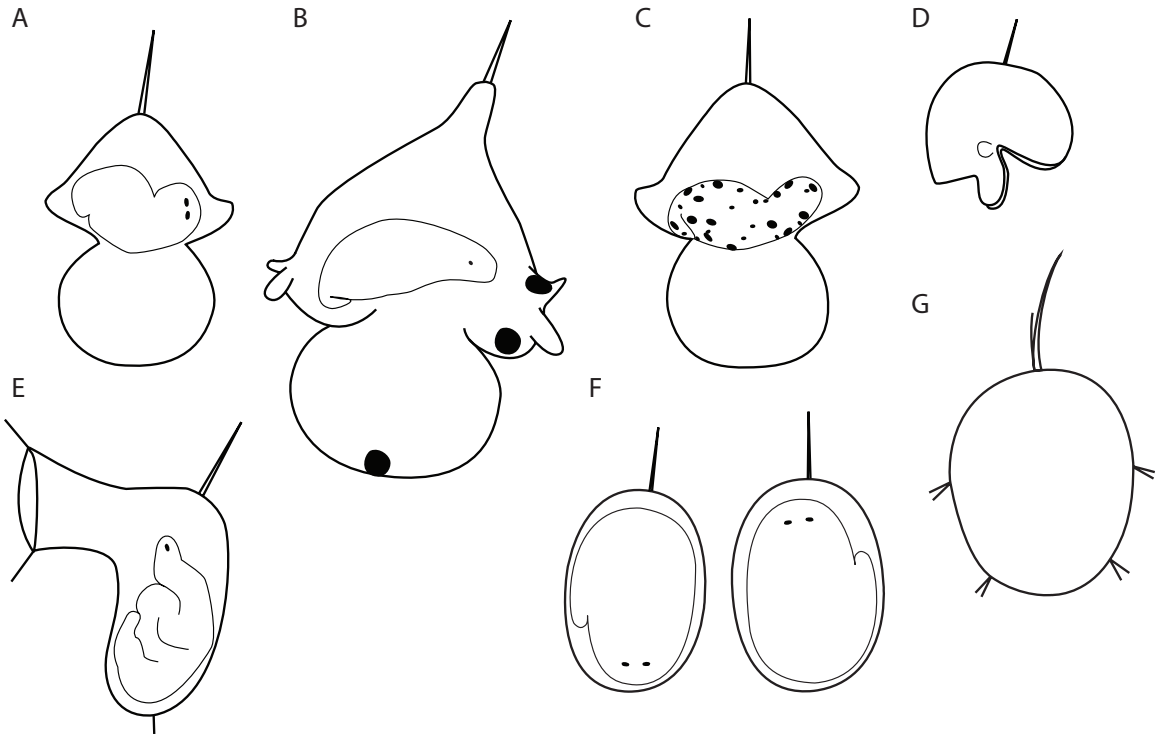


Figure 4.2. Diagrams of pilidial morphotypes. A) pilidium gyrans, B) pilidium pyramidum; note pigment spots on anterior lobe and lappets, C) pilidium maculosum; note pigment spots on juvenile amnion, D) pilidium auriculatum, E) pilidium recurvatum; note juvenile anterior-posterior (AP) axis is parallel to larval AP axis, F) two modified pilidia where AP axis of developing juvenile inside is parallel to (right) or opposite (left) larval AP axis, G) pilidium nielseni; note two transverse ciliated bands.

Pilidium auriculatum, described by Leuckart and Pagenstecher (1858), characterizes a palaeonemertean genus, *Hubrechtella* and is characterized by narrow, side-burn like lappets (Fig. 4.2D) and prominent epidermal cell outlines. These larvae are shaped like a Roman helmet and the juvenile inside is often situated at an oblique angle to the larval apical axis. The pilidium recurvatum morphotype (Fewkes 1883) looks like a sock (Fig.

4.2E). Atop the heel of the sock is an apical tuft and at the toe, a posterior cirrus. Thus, the anterior-posterior axis of the juvenile nemertean is parallel to that of the larval axis. A funnel with stiff marginal cirri opens to the larval esophagus leading to the gut. This larval morphotype belongs to members of the heteronemertean genus, *Riserius* (Hiebert et al. 2013).

The diverse morphology among pilidiophoran larvae has come full circle, as some pilidia more closely resemble direct-developing hoplo- or palaeonemetean larvae than other pilidia. They do, however, still metamorphose catastrophically as other pilidia and one can often see the developing juvenile nemertean inside the larval body. These forms, called modified pilidia, are characterized by an oblong shape, an apical tuft (Fig. 4.2F), and, above all, lecithotrophy. The axis of the developing juvenile can be parallel to (Schwartz and Norenburg 2005; Schwartz 2009) or opposite (Iwata 1958; Maslakova and Hiebert 2014) the larval axis, where the apical tuft marks the larval anterior (Fig. 4.2F). Recently, a new form of modified pilidium, named pilidium nielseni was described by Maslakova and von Dassow (2012). These larvae have two transverse ciliated bands (Fig. 4.2G) and they can be recognized by their unique swimming behavior that is interrupted by brief periods of motionlessness. As in other modified pilidia, the developing juvenile can often be seen within the larval body. Pilidium nielseni larvae have a very prominent and blade-like apical tuft and a posterior cirrus that is either between the ciliated bands or immediately opposite the apical tuft.

METHODS

Collection of specimens

Nemertean adults were collected intertidally from a variety of locations in the NE Pacific (e.g., False Bay, Washington, Crescent City, California, see Appendix A) with several sites on the southern Oregon coast in and around Charleston, OR. Wild-caught nemertean larvae were collected on a regular basis (i.e., weekly or more) from plankton samples gathered off the Charleston Marine docks or in the Charleston Channel in Charleston, OR

from 2008–2014. Samples were obtained with a 0.5-m diameter plankton net with 153 μm mesh size (SeaGear) and stored in 1-gal glass jars. Upon returning to the lab, dense plankton samples were diluted with filtered seawater (0.45 μm filtered, FSW) to an ideal sorting dilution and maintained in a sea table with flow-through seawater such that plankton remained at ambient seawater temperature (11–14˚C) for the duration of larval sorting. Samples were sorted in ~ 20 ml increments in 150 ml glass custard dishes and a dissecting microscope with transmitted light.

Larval rearing

Larvae were reared from artificially fertilized gametes through metamorphosis when gravid conspecific males and females were available (e.g., *Micrura wilsoni*, *Maculaura cerebrosa*, *Lineus* sp. "red", *Nipponnemertes bimaculata*), as described elsewhere (Maslakova 2010a; Hiebert and Maslakova, 2015a, b). As many nemertean larvae acquire more distinctive characteristics in later developmental stages, wild-caught larvae encountered at early stages were often maintained on a diet of the cryptomonad *Rhodomonas lens* in a 150 ml glass custard dish partly submerged in a sea table with flow through seawater to maintain local seawater temperatures between 11–14˚C.

Photomicroscopy

Live larvae were photographed periodically, as development progressed, or immediately prior to cryopreservation for molecular analysis. Images were acquired from larvae which were gently trapped between a microscope slide and coverslip with four small clay feet at each corner. Photographs were obtained using an Olympus BX51 microscope equipped with DIC using a Leica DFC400 camera and accompanying software (Leica Application Suite V3.6).

Molecular analysis

Adult tissue samples (2 x 2 mm cubes) were preserved in 80% EtOH at -20˚C or dry at -80˚C. DNA extraction from adult tissue was carried out using a DNEasy Blood and

Tissue Kit (Qiagen) or Wizard SV Genomic DNA Purification System (Promega). Tissue lysis occurred in a solution of Nuclei Lysis solution, 0.5 M EDTA and proteinase K (20 mg ml-1) at 56˚C for 6–12 hours. Larvae were cryopreserved (-80˚C) in a small volume (< 10µl) of FSW in 1.5 ml microfuge tubes. DNA extraction was carried out using a Chelex-based method (InstaGene, BioRad) with initial incubation at 56˚C for 30 min followed by a short (8 min) incubation at 98˚C. We amplified two barcoding regions of mitochondrial genes: 16S ribosomal DNA (460 bp, 16S) and cytochrome c oxidase subunit I (658 bp, COI). PCR amplification was carried out with universal primers: 16SARL [5‘ CGCCTGTTTATCAAAAACAT 3’] and 16S BRH [5’ CCGGTCTGAACTCAGATCACGT 3’] (Palumbi et al. 1991); LCO 1490 [5’ GGTCAACAAATCATAAAGATATTGG 3’] and HCO 2198 [5’ TAAACTTCAGGGTGACCAAAAAATCA 3’] (Folmer et al. 1994). Higher quality amplification was occasionally achieved by pairing nemertean-specific reverse primers (16SKR [5’ AATAGATAGAAACCAACCTGGC 3’], COIDr [5’ GAGAAATAATACCAAAACCAGG 3’] (Norenburg, unpublished)) with corresponding universal forward primers. PCR thermocycling was carried out using 1–8 µls of DNA extract in a 20 µl reaction with the following parameters: 95˚C initial denaturation for 2 min, 35 cycles of 95˚C for 40 s, 45–55˚C for 40 s and a 60 s extension at 72˚C. Following the last cycle there was an additional 2 minutes at 72˚C for final extension after which products were stored at 4˚C. PCR products were purified using Wizard SV Gel and PCR Cleanup kit (Promega) and sequenced (Sequetech Inc, Mountain View, CA) in both directions using forward and reverse primers to maximize sequence length and accuracy. Sequences were trimmed to remove primers, assembled in contigs, proofread for quality using Geneious v.7.0.6, and deposited in Genbank. Additional reference sequence data was downloaded from GenBank (for accession numbers, see Tables 2.1–2.3 in Chapter II).

Species identification

Larvae were identified using both phylogenetic analyses (for phylogenetic methods, see Chapter II) and sequence divergence values (p-distances). Interspecific divergence values for the 16S gene region range from approximately 4–12%, while intraspecific values range 0–1%. Likewise, for the COI gene region, interspecific values range from 13–18%, while intraspecific range is 0–7% (Mahon et al. 2009; Meyer and Paulay 2005; Kvist et al. 2014; Chapter II, this study). In our hands, larval sequences almost exactly matched adult sequences or differed by less than 1% sequence divergence. The sequence divergence was found to be higher than 1% on only two occasions and we discuss this in the larval description (see *Carinoma hamanako* and *Paranemertes californica*).

RESULTS

The following key and subsequent descriptions include 56 nemertean larvae and is also available online (www.nemerteanlarvalid.com). The larvae of 37 species have been identified and 19 larvae represent species which we currently only find as larvae in the plankton and have yet to find in their adult form (i.e., "orphan larvae").

The 56 total species comprise 28 pilidiophorans, 16 palaeonemerteans, and 12 hoplonemerteans. Eight pilidiophoran larvae match known NE Pacific species and 20 represent new diversity. Of those 20 species, one is a species previously known to a different region (*Hubrechtella juliae*), and nine match species we find as adults that are undescribed and ten are orphan larvae. Four palaeonemertean larvae match known NE Pacific species while 17 represent new diversity, comprising one species known to a different region (*Carinoma hamanako*) and eight orphan larvae. Eight hoplonemertean larvae can be matched to known NE Pacific species and four represent new diversity. One larva matches a species previously unknown to the NE Pacific (*Gurjanovella littoralis*) and only one hoplonemertean species is represented by an orphan larva, and can be identified to genus-level (*Ototyphlonemertes*). The remaining three species we find as adults, but are currently undescribed.

Identification key to the nemertean larvae of the southern Oregon coast

1. Larva vermiform or oblong in shape – **2**

   Larva not vermiform and with elaborate flaps, funnel or vestibule – **12**

2. Larva uniformly ciliated – **3**

   Larva uniformly ciliated, but also equipped with two transverse bands of dense cilia – **"Trochonemertes" (*Micrura* sp. "dark" and allies)**

3. Larval eyes present – **4**

   Larval eyes absent – **7**

4. Larval eyes intra-epidermal – **5**

   Larval eyes sub-epidermal – **10**

5. Single, mid-ventral eye anterior to mouth – **6**

   Eyes paired, each eye single or double – ***Cephalothrix* spp.**

6. Lateral cirri present; few anterior cirri – ***Carinoma* spp.**

   Lateral cirri present; several anterior cirri are claw-like and curled – ***Carinina* sp. "chocolate"**

7. Larva with thick, short lateral cirri – ***Tubulanus* spp.**

   Larva without lateral cirri – **8**

8. Larva with thick, conspicuous epidermis – ***Tubulanus sexlineatus***

   Larva without distinctively thick epidermis – **9**

9. Larva with prominent apical tuft; < 500 μm in length – **modified pilidia (Heteronemertea gen. sp. 1, 2, 3)**
Pale larva without apical tuft; < 500 μm in length; with dorsal longitudinal furrow – ***Malacobdella siliqua***
Orange larva with short apical tuft; > 500 μm in length – ***Tubulanus polymorphus***

10. Larva with two statocysts, each with a polystatolith – ***Ototyphlonemertes*** **spp.**
Larva without statocysts – **11**

11. Larva orange in color; two pairs of eyes present, the posterior pair closely apposed resembling a single eye – ***Carcinonemertes errans***
Larva green in color; two pairs of eyes present in advanced individuals (likely one pair in young larvae), neither pair overlapping – ***Emplectonema*** **sp. 1**
Larval body color not orange or green; two or more pairs of eyes; conspicuous apical tuft present or absent– **other hoplonemertean larvae (*Paranemertes californica*, *Nipponnemertes bimaculata*, *Tetrastemma bilineatum*, *Poseidonemertes collaris*, *Gurjanovella littoralis*, *Paranemertes* sp. 1, *Zygonemertes* sp. 1, *Pantinonemertes californiensis*)**

12. Larval body transparent; shape is sock-like, with large esophageal funnel – ***Riserius*** **spp.**
Larval body transparent, with prominent epidermal cell outlines and nuclei; shape is helmet-like, with narrow lateral lappets – ***Hubrechtella*** **spp.**
Larval body transparent; shape is hat-like, with rounded lateral lappets – **13**

13. Juvenile or rudiments with polka-dot pigment spots on amnion – ***Maculaura*** **spp**.
Amniotic pigment absent; larval lobes and/or lappets with pigment spots or patches – **14**

Amniotic pigment absent; larval lobes and lappets without conspicuous pigmentation – **15**

14. Larval lappets and lobes with pigment spots, lobes scalloped; juvenile with caudal cirrus and two small eyes **– *Cerebratulus californiensis* and allies**
Larval anterior lobe with one pigment patch on either side, lappets not pigmented, lobes not scalloped; juveniles with two eyes and caudal cirrus – ***Micrura wilsoni***

15. Larva with relatively small episphere (equal to in size, or smaller than lappets) that is shaped like a low dome – ***Lineus flavescens* and allies**
Larva with a relatively large episphere that is shaped like a dome or pyramid – **16**

16. Larval lobes large and floppy; juvenile cirrus absent in advanced larvae; juvenile develops at larval center – ***Lineus* sp. "crescent"**
Larval lobes not floppy or disproportionately large; juvenile cirrus present; juvenile develops at larval center or at oblique angle to larval lobes – **17**

17. Larval episphere as wide as or wider than tall – **18**
Larval episphere taller than wide; juvenile develops slightly off larval center (toward the posterior larval lobe) – ***Cerebratulus albifrons***

18. Juvenile develops at larval center; caudal cirrus inconspicuous (best visible in newly metamorphosed individuals) and posterior with cluster of lipid granules – **Heteronemertea gen. sp. 4**
Juvenile develops at an oblique angle to larval anterior and posterior lobes; caudal cirrus conspicuous – ***Cerebratulus* cf. *marginatus* and allies**

Pilidiophora; Heteronemertea

***Cerebratulus albifrons* (Fig. 4.3)**

Larvae collected: Oct 2011; July 2013

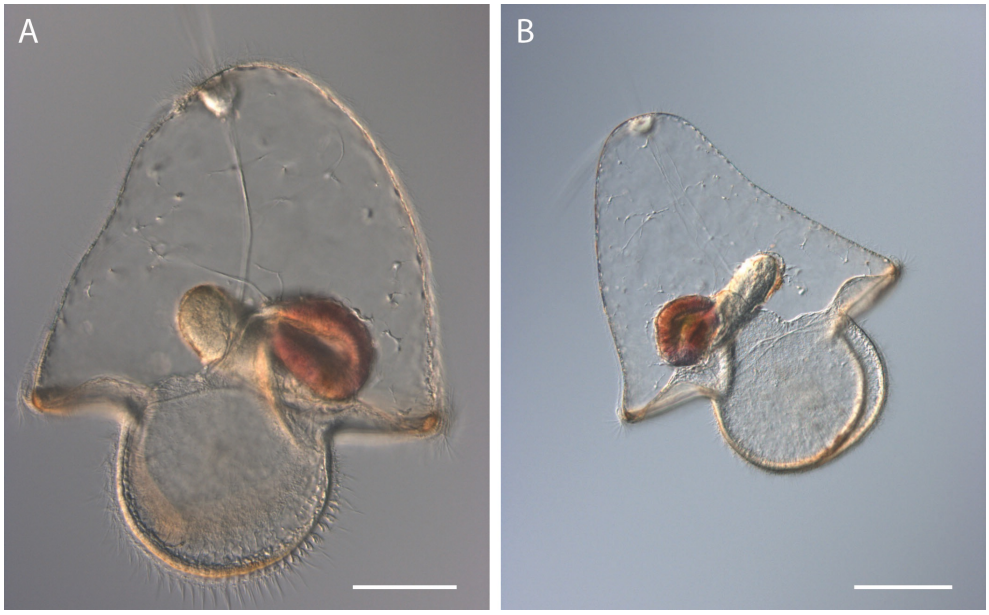


Figure 4.3. Two wild-caught larvae of *Cerebratulus albifrons.* Both larvae were collected in July 2013. Scale bar 100 µm.

Larvae of *Cerebratulus albifrons* exhibit what we refer to as pilidium pyramidum morphology. Their episphere is rather tall (300–500 µm), usually taller than wide, and pyramid-shaped. An additional feature of this species and some other *Cerebratulus* larvae (e.g., *Cerebratulus* sp. "spade head") is that the juvenile anterior-posterior axis is not perpendicular to that of the larval body, but tilted apically. The juvenile is positioned slightly off-center (toward the posterior larval lobe). As far as we know, the combination of these characters with lack of larval body pigment is unique to *C. albifrons* larvae in this region. Many other species whose larvae exhibit the pilidium pyramidum morphotype have spots on their lobes and lappets (e.g., see Lacalli 2005), but this is not the case for *C. albifrons*. We have not yet observed larvae with advanced juveniles, thus the presence of a caudal cirrus (present in adults) and ocelli (common in other species with similar larvae) is suspected, but not yet observed.

***Micrura wilsoni* (Fig. 4.4)**

Larvae not yet collected in plankton

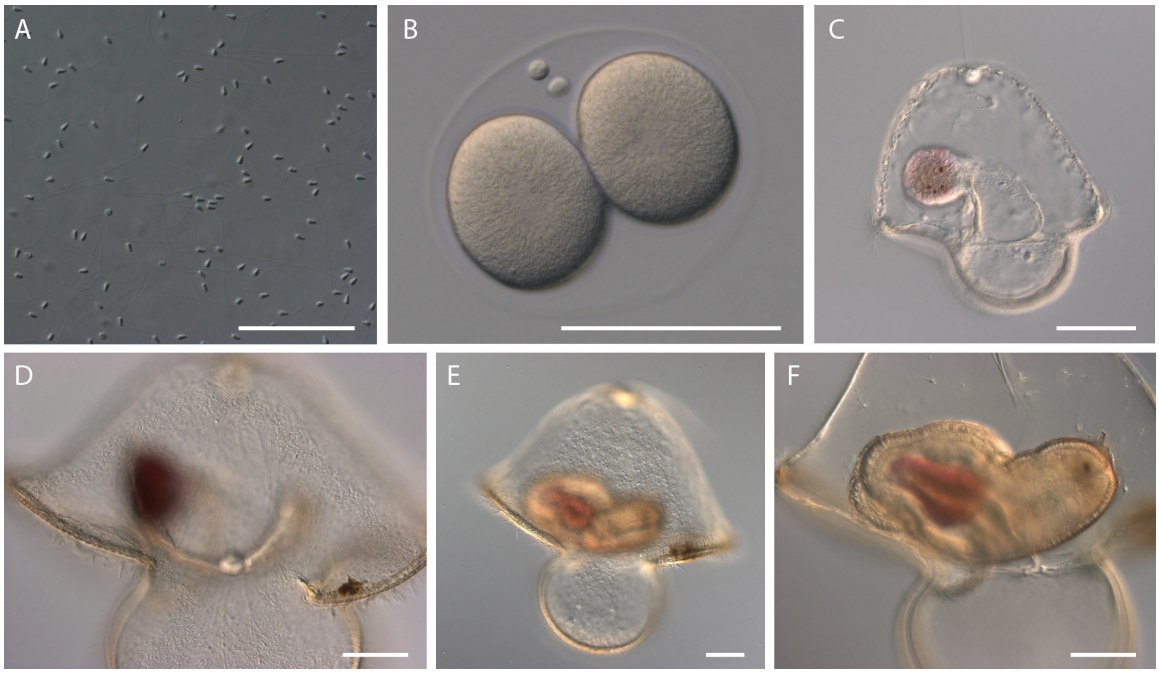


Figure 4.4. Development in *Micrura wilsoni* from gametes fertilized in July 2013. A) Sperm dissected from male, B) two-cell stage with two polar bodies in view at 11 o'clock, C) 5-day old larva; note pink gut from eating *R. lens* cells, D) forty-one day old larva with fused juvenile rudiments and anterior black pigment patch, E–F) 63-day old larva at two magnifications and focal planes to show pigment patch on larval anterior lobe (E) and juvenile with eye and caudal cirrus (F). Scale bars 100 µm (B-F), 50 µm (A).

The development of *Micrura wilsoni* was followed from artificially fertilized gametes (Hiebert and Maslakova, 2015b). Although the adults are not uncommon in rocky intertidal habitats in southern Oregon, we have yet to collect their larvae in a plankton sample. The larvae of *M. wilsoni* are of the pilidium gyrans type, but have characteristic pigment spots on either side of the anterior lobe. The spots are irregular (rather than circular) in shape. Unlike other pilidia with pigment spots (e.g., *Cerebratulus californiensis*), *M. wilsoni* larvae only exhibit these spots on the anterior lobe (and not the lateral lappets or posterior lobe). In advanced larvae, the juvenile inside has two small black eyes and a caudal cirrus. The eyes are lost in adults, but the cirrus remains.

Maculaura

The genus *Maculaura* is characterized by a unique pilidial morphotype which we call pilidium maculosum. Both the genus name and that of the larval morphotype reflect the polka-dot pigment pattern on the amnion inside which the juvenile develops while in the larval body (Hiebert and Maslakova, 2015a). This morphotype appears to be unique to members of this genus. These larvae are not uncommon in Coos Bay plankton. A larva of similar morphology was collected near the mouth of Bamfield Inlet (Lacalli 2005), and adults of the genus *Maculaura* are known to occur from Alaska to at least northern California, and possibly, further south. *Maculaura* currently includes five species, the larvae are known for four of those species, and all look very similar to each other.

***Maculaura alaskensis* (Fig. 4.5)**

Larva collected: Oct 2013

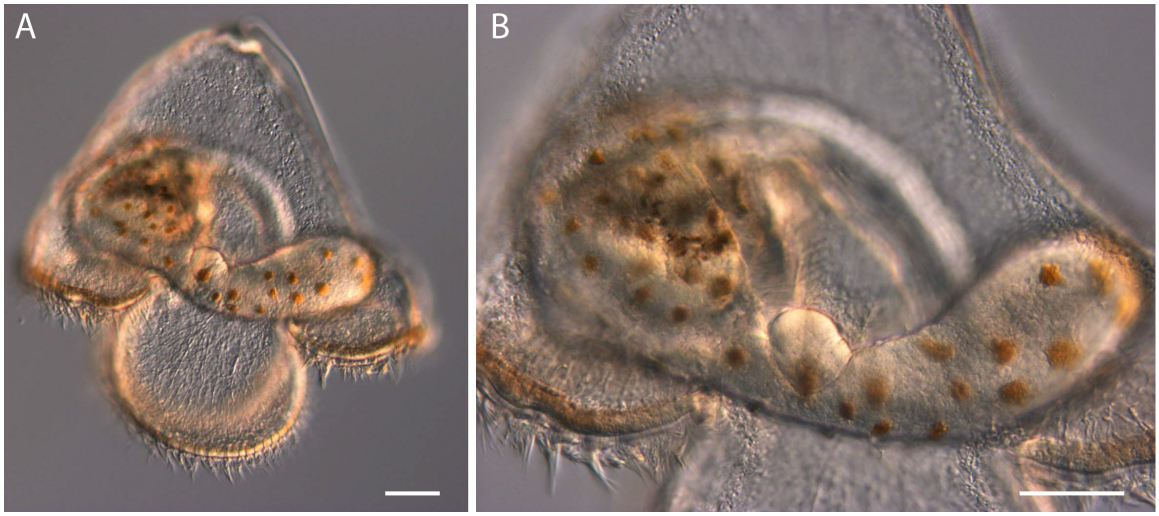


Figure 4.5. Wild-caught larva of *Maculaura alaskensis* collected 13 Oct 2013. Scale 100 µm.

Maculaura alaskensis (=*Micrura alaskensis* Coe 1901, in part) was the first pilidiophoran species to have its development described from fertilization through metamorphosis (Maslakova 2010a). These larvae are easily recognizable due to the pigment spots on the juvenile amnion. Although *M. alaskensis* are very commonly encountered as adults and have served as model systems in many types of research (e.g., Stricker and Smythe, 2000,

2001, 2003; Stricker et al., 2001, von Dassow et al., 2013, Maslakova, 2010a; Bird et al., 2014; Swider et al., 2014; L. Hiebert and Maslakova, 2015), we have only collected and identified one wild-caught larva of this species in October 2013. The larvae of this species are almost indistinguishable from the larvae of *M. cerebrosa*, however, the reproductive timing is slightly different between the two species. In southern Oregon, *M. cerebrosa* are reproductive in the spring and early summer while *M. alaskensis* are reproductive later in the summer.

***Maculaura cerebrosa* (Fig. 4.6)**

Larvae collected: Aug 2012

Figure 4.6. The larvae of *Maculaura cerebrosa*. A) An 18-day old larva reared in the lab from known adults in March 2013 and B) an advanced wild-caught larva collected in August 2013. Scale bars 100 µm.

The development of *Maculaura cerebrosa* is briefly described by Hiebert and Maslakova (2015a). Like other members of this genus, the larvae of *M. cerebrosa* can be recognized by the pigment pattern of the juvenile amnion which is visible early in development. Adult *M. cerebrosa* are common in Charleston, OR, particularly amongst rocks and shell

hash and their larvae have been collected in late summer. Unfortunately, the larvae are indistinguishable from the larvae of *M. alaskensis* and, instead, larvae are best identified by season (*M. cerebrosa* are reproductive in the spring and early summer while *M. alaskensis* are reproductive in later summer months) and/or by rearing from known adults.

***Maculaura aquilonia* (Fig. 4.7)**

Larvae collected: May, April 2012

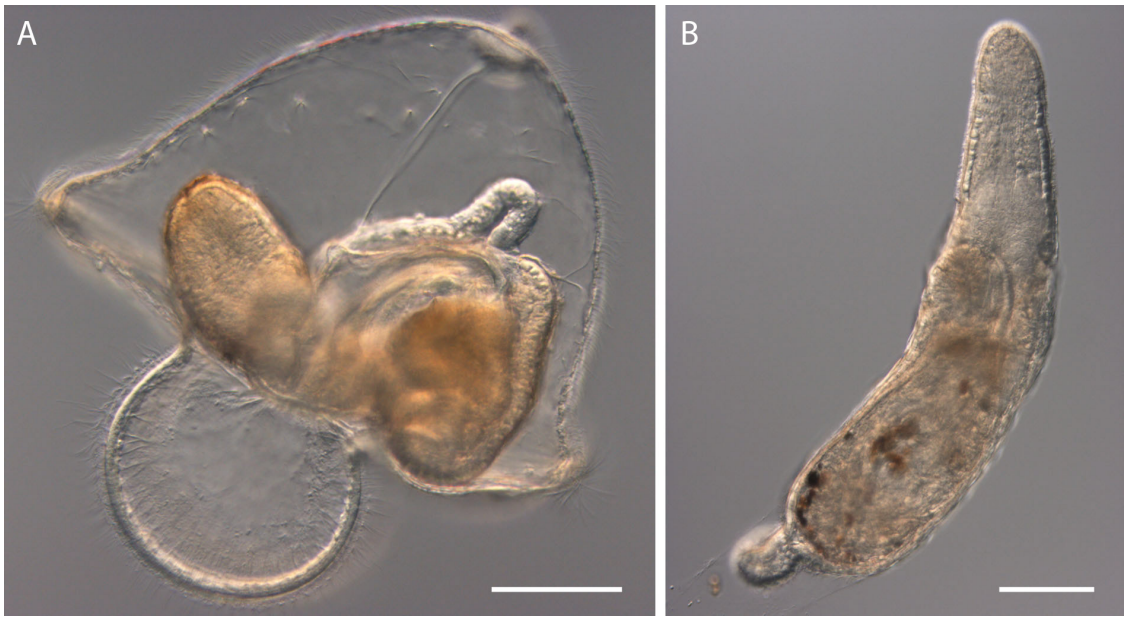


Figure 4.7. Larva (A) and newly metamorphosed juvenile (B) of *Maculaura aquilonia*, which were reared in the lab from known adults in March 2013. Scale bars 100 µm.

The development and metamorphosis of *Maculaura aquilonia* is described by Hiebert and Maslakova (2015a). Although the pigment spots on the juvenile amnion are quite distinct in other members of this genus, they are less conspicuous in *M. aquilonia*. Instead, the polka-dot pigment spots only became visible when the amnion is collapsed within the juvenile gut following metamorphosis. As a result, the larvae superficially resemble other hat-like pilidia. In southern Oregon, reproductive adults are found in March and planktonic larvae have been collected in April and May.

***Maculaura magna* (Fig. 4.8)**

Larvae collected: Nov, Dec 2011; May, June, July 2013

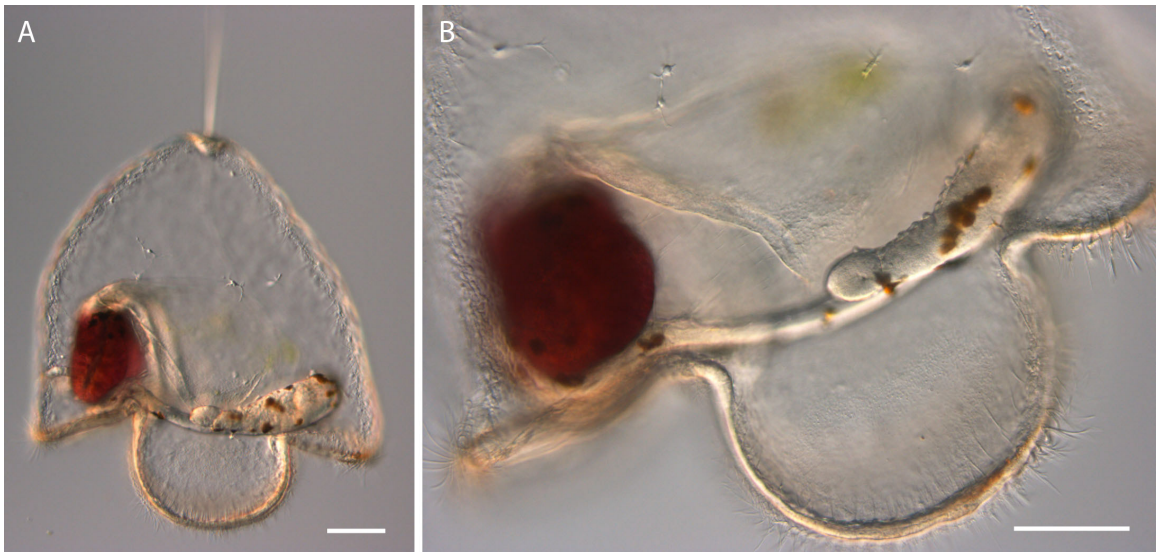


Figure 4.8. Wild-caught larva of *Maculaura magna*, collected from plankton in July 2013. The same larva is depicted at different magnifications to show body and episphere shape (A) and spotted amnion on fused juvenile rudiments (B). Scale bars 100 μm.

Although the complete development of this species has yet to be documented, wild-caught pilidia were very common in the summer of 2013. The larvae of *Maculaura magna* have a conspicuous polka-dot pattern on the juvenile amnion as is seen in *M. alaskensis* and *M. cerebrosa*. Although it can be difficult to differentiate the larvae of these three species, *M. magna* larvae are sometimes recognizable because they have a larger haystack-shaped episphere. Likewise, the lateral lappets appear to be relatively smaller than the rest of the larval body in this species than in the other two species.

***Cerebratulus californiensis* and allies**

Many of the undescribed species we have uncovered in the NE Pacific belong to a closely related groups of species that are currently lumped under the same name. One example is the *Cerebratulus californiensis* species complex. There are two other NE Pacific species that form a species complex (a clade which is well supported on molecular phylogenies as well as morphologically) with *C. californiensis*. All three species share a larval

morphotype characterized by a pyramidal episphere (pilidium pyramidum morphotype, Fig. 2B) and pigment spots on larval lobes and lappets. Interestingly, some other larvae of similar morphotype collected from other regions of the world (e.g., Bay of Panama, Victoria, Australia) and identified with DNA sequence data are apparently related to the group of species we find in the NE Pacific. However, in at least one case, a larva of similar morphology collected from Vostok Bay, Russia was only distantly related (25% (16S) and 17.1% (COI) sequence divergences) to this group (see Chapter VII).

***Cerebratulus californiensis* (Fig. 4.9)**

Larvae collected: Feb 2010; Jan, Feb, March, June, July 2013

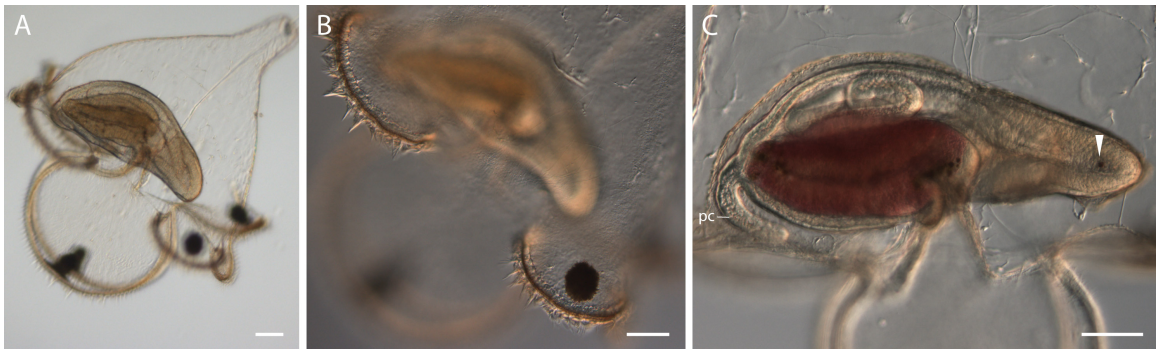


Figure 4.9. Wild-caught *Cerebratulus californiensis* larvae, collected in February 2013. (A–B) Same larva shown at different magnification; note circular pigment spots on lateral lappets (A) and anterior lobe (in focus, B). (C) Different individual from (A), with advanced juvenile; note posterior cirrus (pc) and eye (arrowhead). Scale bars 100 µm.

The larvae of *Cerebratulus californiensis* can be very common in the plankton in Charleston, OR. In 2013, we encountered many individuals in February. They are easily recognizable by the characteristic pyramidal episphere and the circular pigment spots on their lobes and lappets. Although pigment spots are usually confined to one spot per lappet or on either side of anterior and posterior lobes, several smaller spots are also seen. When viewed from the apical tuft down, the anterior (and sometimes posterior) lobes appear scalloped or divided into three sub-lobes. Advanced larvae are very large (up to 1200 µm in total height) and the juvenile inside is long and slender with pointed anterior, two small eyes and a caudal cirrus.

***Cerebratulus* sp. "Sunset Bay" (Fig. 4.10)**

Larvae collected: Oct 2008; Oct 2013

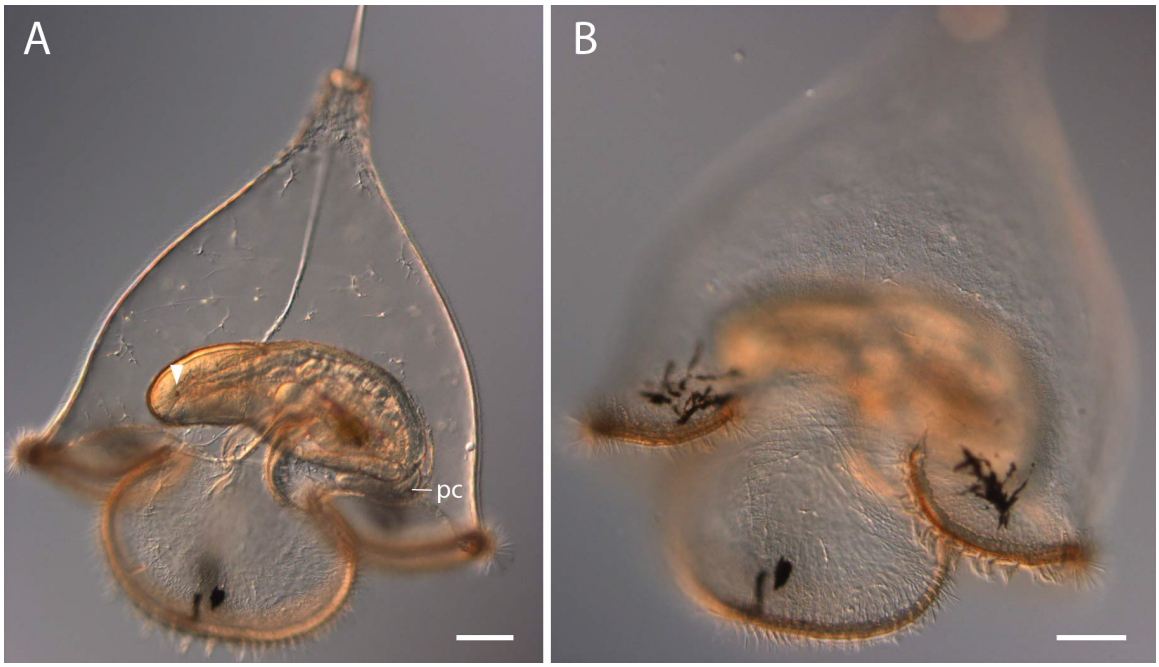


Figure 4.10. Wild-caught larva of *Cerebratulus* sp. "Sunset Bay" collected October 2012. (A) Advanced juvenile has two small eyes (single ocellus indicated with arrowhead) and a posterior cirrus (pc). Larvae have black pigment patches on lateral lappets and both anterior and posterior lobes (in focus, B). Scale bars 100 μm.

This is the larva of one of the undescribed species in the *Cerebratulus californiensis* species complex, that we refer to as *Cerebratulus* sp. "Sunset Bay" in reference to the location in which we once found an adult (Sunset Bay, Cape Arago, Oregon). The larval body resembles that of other members of the *Cerebratulus californiensis* species complex with pyramid-shaped episphere and pigment spots. Advanced juveniles are pointed anteriorly and posteriorly, with two small eyes and a caudal cirrus. A potential difference between this larva and the larvae of other members of this species complex is the pigment pattern on the larval lobes and lappets. Rather than being round, the pigment spots in *Cerebratulus* sp. "Sunset Bay" are irregular amorphously star-shaped patches. We have only found and identified a single larval specimen that belongs to this species, so it is not clear whether the shape of pigment spots is species-specific or varies intra-specifically.

***Cerebratulus* sp. "pink proboscis" (Fig. 4.11)**

Larvae collected: Oct 2008; Oct, July 2013

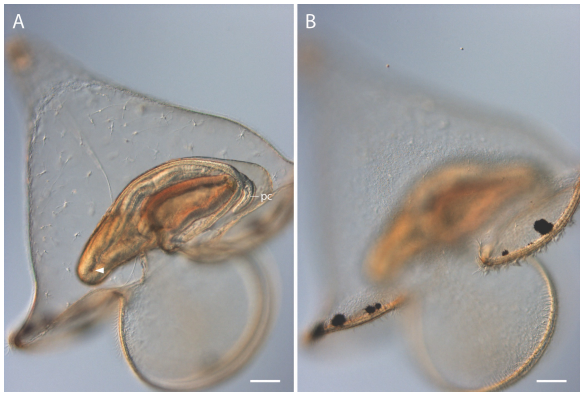


Figure 4.11. Wild-caught larva of *Cerebratulus* sp. "pink proboscis" collected July 2013. (A) Advanced juvenile has two small eyes (single ocellus indicated with arrowhead) and a posterior cirrus (pc). Larvae have several black, irregularly-shaped pigment spots on lateral lappets and both anterior and posterior lobes (in focus, B). Scale bars 100µm.

This is the larva of the second undescribed species in the *Cerebratulus californiensis* species complex and we call this species *Cerebratulus* sp. "pink proboscis". The larvae are not differentiable from those of *C. californiensis,* although they are less common in the plankton. We usually find the larvae of this species in October. They exhibit the same larval morphotype observed for *C. californiensis* and *C.* sp. "Sunset Bay". Although we have only collected and identified three larvae, we have yet to observe the scalloping of the anterior or posterior lobes, which is seen in the larvae of *C. californiensis*. Advanced juveniles have two small eyes, are pointed anteriorly and posteriorly and have a caudal cirrus. The pigment spots develop early, which can be seen in the young larva collected in October 2013.

***Cerebratulus* cf. *marginatus* and allies**

The larvae of *Cerebratulus* cf. *marginatus* and a closely related and undescribed species, *Cerebratulus* sp. "spade head", have broad anterior and posterior lobes and a pyramidal episphere. Although *Cerebratulus* cf. *marginatus* is a common intertidal species and its early development has been described (Coe 1899; Schmidt 1930), we have yet to observe advanced larvae.

***Cerebratulus* cf. *marginatus* (Fig. 4.12)**

Larvae collected: July 2013

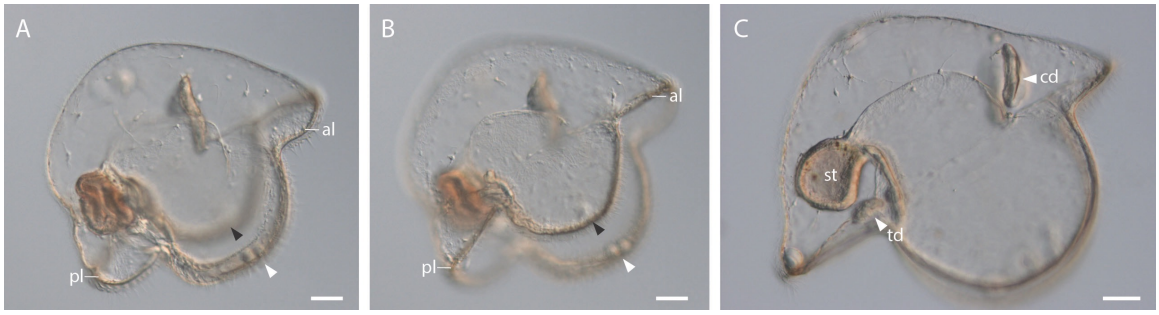


Figure 4.12. Young, wild-caught larva of *Cerebratulus* cf. *marginatus,* collected in July 2013. Three focal planes are shown to highlight larval body shape; note left (white arrowhead) and right (black arrowhead) lappets and lobes (A–B), as well as juvenile rudiments and stomach (in focus, C). Abbreviations: anterior lobe (al), posterior lobe (pl), cephalic disc (cd), trunk disc (td), stomach (st). Scale bars 100 µm.

Cerebratulus cf. *marginatus* is a common heteronemertean species on the southern Oregon coast and larvae can be reared in the lab on a diet of *Rhodomonas lens* from artificially inseminated dissected gametes (although not as easily as those of *Maculaura* spp. or some other species we have attempted to raise). The larvae are found in late spring/early summer. They are recognizable by their tall and wide episphere, lack of pigment, and a juvenile, which we suspect develops at an oblique angle to the larval anterior and posterior lobes. The larvae of *C*. cf. *marginatus* resemble the larvae of another *Cerebratulus* species, currently undescribed – *Cerebratulus* sp. "spade head" (below). However, the reproductive timing is different in the two species where *C*. cf. *marginatus* larvae have been found in May and July where the larvae of *C*. sp. "spade head" are, so far, found only in October. Interestingly, the larvae of *C*. cf. *marginatus* seem to be less common in the plankton than the adults are intertidally.

***Cerebratulus* sp. "spade head" (Fig. 4.13)**

Larvae collected: Oct 2008; Oct 2012

This larva belongs to an undescribed species (closely related to *C*. cf. *marginatus*), which we call *Cerebratulus* sp. "spade head". The larvae and adults of this species look very

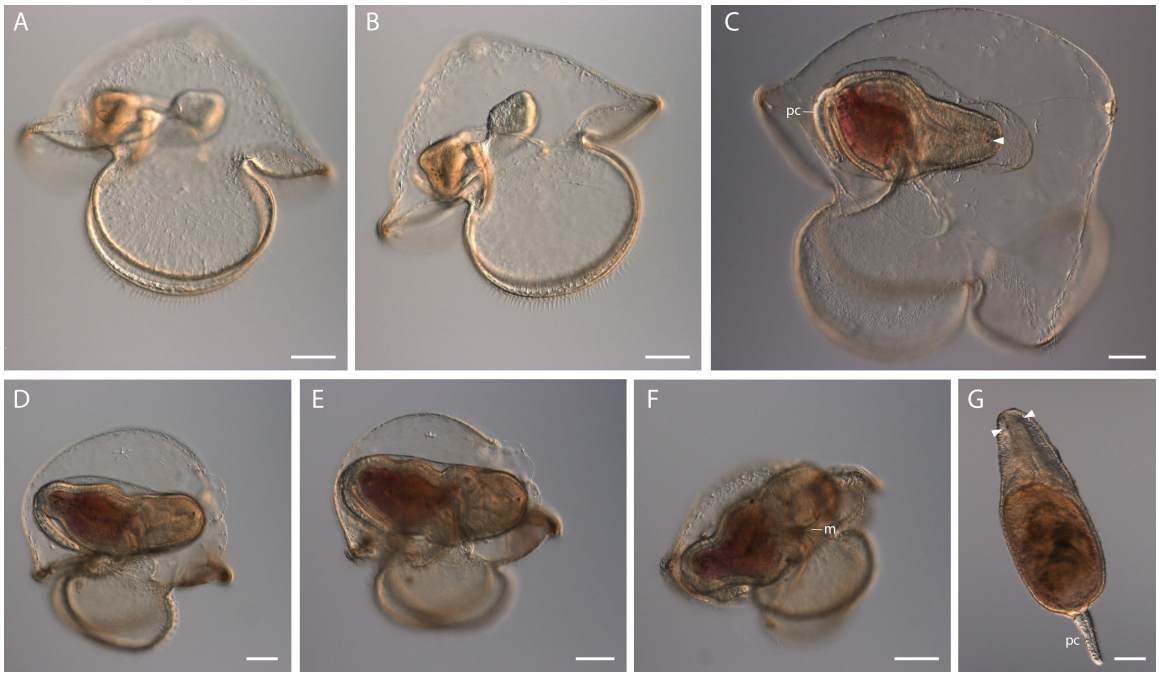


Figure 4.13. Wild-caught larva of *Cerebratulus* sp. "spade head" collected 22 October 2012, with two focal planes shown (A–B). Same larva was imaged (C) and metamorphosed (D–G) on 05 November 2012; note juvenile eyes (indicated with arrowheads) and posterior cirrus (pc) (C, G), ingestion of larval body (D–F), and post-metamorphosis juvenile (G). Abbreviations: mouth (m). Scale bars 100µm.

similar to those of *C*. cf. *marginatus*. The larvae have a wide episphere and large vestibule, the juvenile develops at an oblique angle to the larval anterior and posterior lobes, and advanced juveniles possess two small eyes and a caudal cirrus. So far, the larvae of this species have only been collected in October (the larvae of *C*. cf. *marginatus* are usually found in the spring and summer). The larvae of this species readily eat cultured *Rhodomonas lens* and we maintained a single larva for two weeks at which point the juvenile metamorphosed and ingested its larval body.

Lineus flavescens **and allies**

Lineus flavescens is a species known to be found from central CA to OR, and we have found the adults intertidally. We also discovered several currently undescribed species that are closely related to *L. flavescens*. We commonly find adults of a species we refer to as *Lineus* sp. "red", and we have both collected its larvae in the plankton and cultured

them in the laboratory from fertilization to metamorphosis (Hiebert and Maslakova, 2015). Two other related species we have, so far, only encountered as wild-caught larvae. The larvae of these four species are indistinguishable. They all look like pilidium gyrans, with an episphere that is relatively small when compared to the larval lappets (e.g., Fig. 4.2A).

***Lineus flavescens* (Fig. 4.14)**

Larvae collected: May 2009; Feb 2010; Nov, Dec 2011; Aug, Oct 2012; Jan, Feb, March, July 2013; Oct 2014

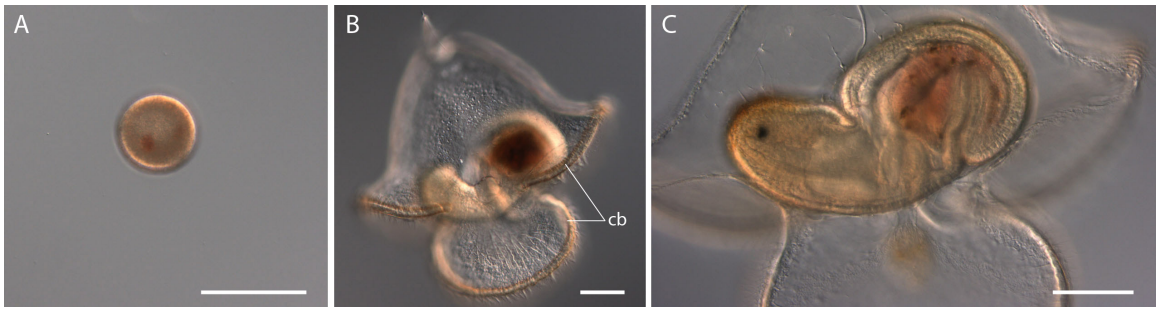


Figure 4.14. Oocytes of *Lineus flavescens* dissected from female in April 2013 (A) and wild-caught larvae collected in January 2013 (B) and October 2012 (C). Note the orange hue near the larval ciliated band (cb, B) and juvenile eye (in focus, C). Scale bars 100 µm.

These larvae exhibit the pilidium gyrans (Bürger, 1895) morphology. They are shaped like a hat with earflaps and sometimes the episphere can be relatively small compared to the lateral lappets. There is often a faint orange hue along the ciliated band in these larvae, and the developing juvenile has two eyes, which can appear reddish-brown or black. They are common in the plankton and easy to rear to metamorphosis on a diet of *Rhodomonas lens*.

***Lineus* sp. 1 (Fig. 4.15)**

Larvae collected: Oct 2008; Oct 2011; Aug, Oct 2012

These larvae belong to an undescribed lineid (fam. Lineidae) species, which we currently only find as larvae in the plankton and have yet to find as adults. Although the larvae are

difficult to differentiate from *L. flavescens*, DNA sequence data strongly suggests that these larvae represent another species (6.6 and 12.9 % divergence for 16S and COI, respectively).

Figure 4.15. Larva of *Lineus* sp. 1, which was collected in October 2012; two focal planes show orange pigment along ciliated band (cb, A) and fully competent juvenile worm (B). Scale 100 µm.

***Lineus* sp. 2 (Fig. 4.16)**

Larvae collected: Jan, Feb 2013

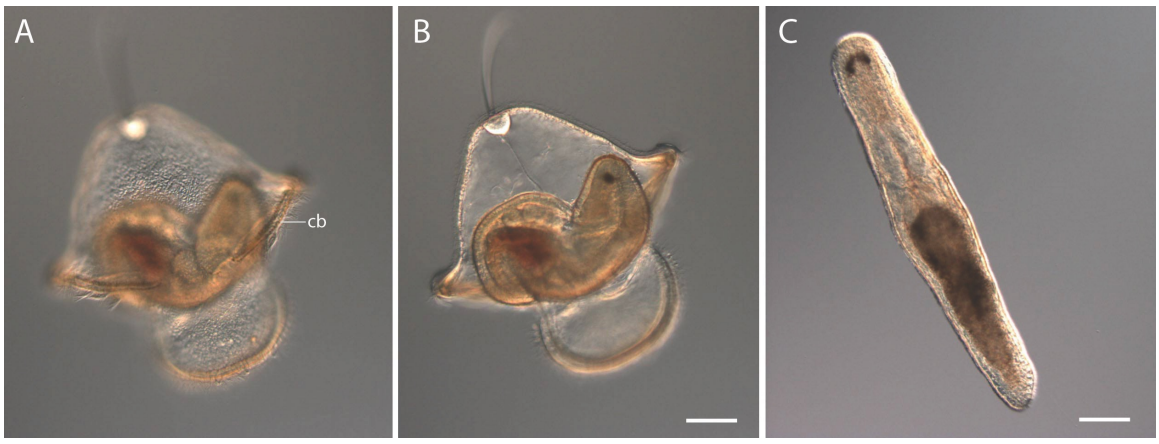


Figure 4.16. Wild-caught *Lineus* sp. 2 larva collected in February 2013 (A–B); two focal planes show orange pigment along ciliated band (cb, A) and fully competent juvenile worm (B). (C) Newly metamorphosed juvenile of *Lineus* sp. 2, which was collected as a larva, also in February 2013. Scale bars 100 µm.

This is the larva of yet another undescribed lineid species, which we currently only find as larvae in the plankton and have yet to find as adults. Although the larvae are difficult to differentiate from *L. flavescens*, DNA sequence data strongly suggests that these larvae represent another species (14.2 and 18.3 % divergence for 16S and COI, respectively). The eyes of the metamorphosed juvenile appear fused. However, this is also seen in other closely related species and does not appear to be a distinguishing character.

***Lineus* sp. "red" (Fig. 4.17)**

Larvae collected: Jan 2013

Figure 4.17. Wild-caught larva of *Lineus* sp. "red", which was collected in January 2013; note stomach is red from eating *R. lens* cells. Scale bar 100 µm.

These are the larvae of a common and undescribed species in the NE Pacific, to which we refer as *Lineus* sp. "red". Adults are found in intertidal sand and mudflats and the larvae have been found in the plankton in January. Ripe adults were collected in February and the development of this species described from fertilization to metamorphosis (Hiebert and Maslakova, 2015b), where development proceeds as described for *Maculaura alaskensis* (Maslakova 2010a). These larvae are virtually indistinguishable from the larvae of *Lineus flavescens* and *Lineus* sp. 1, 2.

***Lineus* sp. "crescent" (Fig. 4.18)**

Larvae collected: Jan, Feb 2013

These larvae are easy to recognize. They are the larvae of *Lineus* sp. "crescent", an undescribed nemertean species which we find amongst *Phyllospadix* sp. root masses in

Figure 4.18. Wild-caught larva of *Lineus* sp. “crescent", collected in February 2013. Note developing juvenile at larval center and large, floppy larval lobes. Scale bar 100 µm.

the rocky intertidal. We only found a single adult specimen and it was a small nemertean, but had a distinctive white patch anteriorly in the shape of a crescent moon, not unlike a fingernail. The larvae of this species are very large and almost as wide (lobe to lobe) as they are tall (apical plate to summit of lappet). Likewise, their anterior and posterior lobes are large, wide and floppy. The height from the lobes to the apical tuft is shorter than in other large pilidia which gives the episphere a wide stance. The juvenile nemertean develops in vicinity of the larval center and is quite small compared to the size of the larval body.

Riserius

The pilidium recurvatum morphotype was described in 1883 by Walter Fewkes from the northwest Atlantic. These larvae have a very distinctive sock-like shape where the esophageal region is a large funnel that can be collapsed toward the larval body while swimming or outstretched at a near right angle to the rest of the larva. Additionally, Fewkes’ larvae had a ring of longer cilia surrounding the “toe” of the sock. A similar type of larva was later described from Güllmarfjord, Sweden by Cantell (1966). Pilidium recurvatum that we find in northeastern Pacific lack the posterior ciliary band, but are otherwise similar in shape to pilidium recurvatum (Hiebert et al. 2013). Such larvae have also been described from the Sea of Japan by Chernyshev (2001) and named pilidium prorecurvatum (Chernyshev et al. 2013). The juvenile nemertean develops within the toe of the sock and is situated parallel to the anterior posterior axis of the larva (as opposed to the hat-like pilidium where larval and juvenile axis are perpendicular). Similar larvae

(called pilidium recurvum) have also been found in the Bay of Nha Trang in Vietman (Dawydoff 1940). Recently, we identified pilidium (pro)recurvatum larvae as belonging to the unusual heteronemertean genus, *Riserius* (Hiebert et al. 2013). This genus is monotypic and its only described species, *Riserius pugetensis* (Norenburg 1993), is interstitial. This genus was previously unknown outside of Puget Sound, WA in the NE Pacific, and we found two types of these larvae in Oregon, that represent two new species in this genus (Hiebert et al. 2013).

***Riserius* sp. "no eyes" (Fig. 4.19)**

Larvae collected: Aug 2012; July, Aug, Sep, Oct 2013

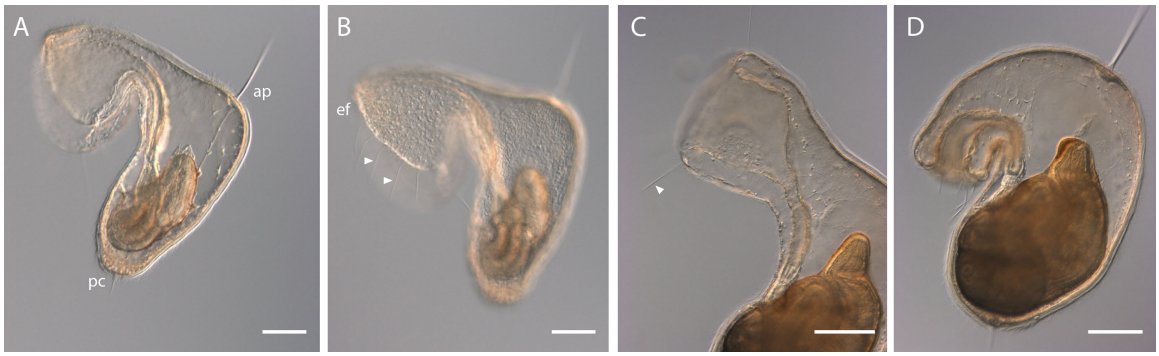


Figure 4.19. Wild-caught pilidium larvae of *Riserius* sp. "no eyes". (A) Larval morphotype that characterized genus; note apical tuft (ap) at larval anterior and posterior cirrus (pc) at posterior. (A–B) Larva collected July 2013, at two focal planes to show developing juvenile (A) and esophageal funnel (ef) margin (B); note stiff cirri on funnel margin (arrowheads, B, C). (C–D) Larva collected August 2012 in two postures: "feeding" (C) and "swimming" (D). Scale bars 100µm.

Riserius sp. "no eyes" and "eyes" are only differentiable in advanced developmental stages, by the presence or absence of eyes in the developing juvenile. Unfortunately, maintaining these larvae in the laboratory is currently a challenge as their diet is unknown. They survive when raw plankton is filtered to 75 µm suggesting that they eat small flagellates (George von Dassow, personal communication). If you are able to obtain larvae at advanced developmental stages, maintaining the juveniles is actually rather straightforward – they will readily ingest larvae, juveniles, and possibly adults of another nemertean, *Carcinonemertes errans* (Hiebert et al. 2013).

***Riserius* sp. "eyes" (Fig. 4.20)**

Larvae collected: Feb, Dec 2013

Figure 4.20. Wild-caught larva of *Riserius* sp. "eyes", collected in December 2013; note small black eyes at juvenile anterior (arrowhead). Scale 100µm.

Advanced developing juveniles have two small black eyes of *Riserius* sp. "eyes". We have not yet reared larvae of the pilidium recurvatum morphotype in the lab (see above). Thus, the timing of the emergence of these presumed ocelli is unknown. The larvae of *Riserius* sp. "eyes" are less common in plankton samples than *Riserius* sp. "no eyes".

modified pilidia

Some pilidiophoran species are known to have lecithotrophic development, in which the resemblance to the hat-like pilidium is lost and, instead, larvae more closely resemble the vermiform morphology of palaeo- or hoplonemertean larvae. A decade ago only three species were known to have such development (*Lineus ruber*, *Lineus viridis* and *Micrura akkeshiensis*), but recently, many more pilidiophorans with lecithotrophic larvae have been discovered (reviewed in Maslakova and Hiebert, 2014). The juvenile develops inside the larval body via imaginal discs, and metamorphosis is catastrophic, as is seen in typical pilidia. The larvae of *Lineus viridis* and *Lineus ruber* are encapsulated and called Desor's and Schmidt's larvae, respectively (Schmidt 1964; Norenburg and Stricker 2002). *Micrura akkeshiensis* produces planuliform modified pilidia called Iwata's larva (Iwata 1958). Several other pilidiophoran species have recently been shown to possess modified development, including *Micrura rubramaculosa* (Schwartz and Norenburg 2005), *Micrura verrilli* and two undescribed species in the genus *Micrura* (Schwartz 2009). Recently, Maslakova and von Dassow (2012) described yet another lecithotrophic pilidium which they named pilidium nielseni (Fig. 4.2G). These modified larvae differ from each other in the orientation of the juvenile antero-posterior (AP) axis with respect to the larval AP axis, and the presence or absence of transverse ciliated bands. It is likely

that such larvae evolved several times within the Pilidiophora (Maslakova and Hiebert, 2014). Below are three additional types of uniformly ciliated modified pilidia (confirmed to belong to different species based on DNA sequences). These three types of modified pilidia we have collected from plankton samples in southern Oregon have an apical tuft marking the larval anterior, and the juvenile antero-posterior axis is opposite to that of the larva (i.e., juvenile posterior coincides with larval anterior, Fig. 4.2F). The juvenile is sometimes conspicuous within the larval body.

Heteronemertea gen. sp. 1 (Fig. 4.21)

Larvae collected: Feb 2010; Jan, Feb 2013

Figure 4.21. Wild-caught modified pilidium of Heteronemertea gen. sp. 1, collected in February 2013. (A–B) Larva at two focal planes; note juvenile posterior projects from left side (arrowhead) and pigmented stomach (st) region at larval anterior. (C–D) Metamorphosed juvenile from larva in A and B; note coiled proboscis (pb) and no caudal cirrus. Scale bars 100 μm.

This modified pilidium was collected in winter months. Although the larva shown above has a conspicuous apical tuft, we did collect another larva that appeared to lack an apical tuft (but revealed to belong to the same species by DNA sequencing). Possibly, the apical tuft was either lost or damaged during collection, or was not visible. Upon metamorphosis, the juvenile nemertean ingested its larval body, as is the case for most pilidiophorans. Note the dark mass (former larval body) within the juvenile gut.

Heteronemertea gen. sp. 2 (Fig. 4.22)

Larvae collected: Jan 2013

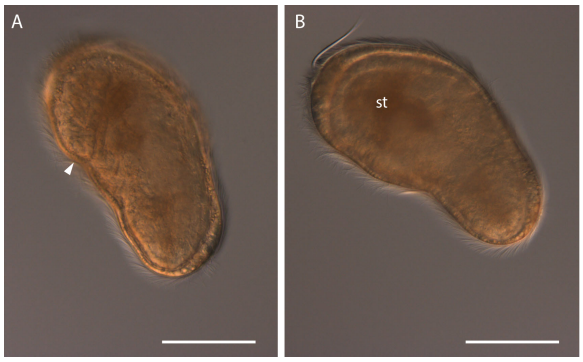


Figure 4.22. Wild-caught modified pilidium of Heteronemertea gen. sp. 2, collected in January 2013. (A–B) Larva at two focal planes; note juvenile posterior projects from left side (arrowhead in A) and pigmented stomach (st) region at larval anterior (st in B). Scale bars 100 µm.

This modified pilidium was collected in January 2013 and is similar to the two others. The larva is uniformly ciliated, has a prominent apical tuft and a curled juvenile inside, positioned with its anterior toward larval posterior.

Heteronemertea gen. sp. 3 (Fig. 4.23)

Larva collected: Dec 2012

This modified pilidium was collected in December 2012 and possessed a blade-like apical tuft but an inconspicuous posterior cirrus. The juvenile axis is opposite of the larval axis, and the posterior tip of the juvenile curved around and toward the juvenile's anterior right side. The larval epidermis was ciliated and contained golden lipid droplets.

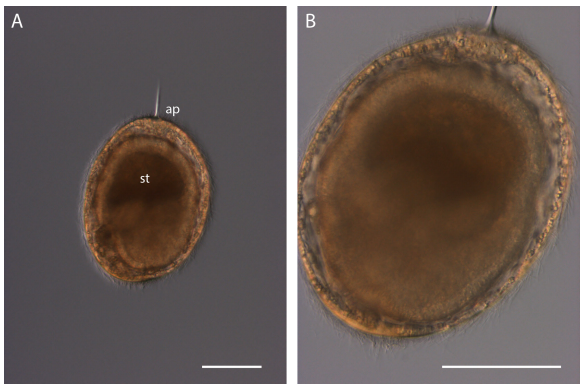


Figure 4.23 (left). Wild-caught modified pilidium of Heteronemertea gen. sp. 3, collected in December 2012. (A–B) Larva at two magnifications; note juvenile stomach (st in A) and posterior are at larval anterior (marked by apical tuft, ap, A). Scale bars 100 µm.

“Trochonemertes"

Maslakova and von Dassow (2012) discovered a new modified pilidial morphotype which they named pilidium nielseni, in honor of a distinguished zoologist and theorist of

animal evolution, Prof. Claus Nielsen. The larva they discovered was completely ciliated, but in addition had a blade like apical tuft, two transverse ciliated bands, and an unpaired ciliary cirrus between the two bands. Although it looked very unlike a typical pilidium larva, a nemertean juvenile was visible inside, and they observed catastrophic metamorphosis and matched this larva to a local and undescribed species, *Micrura* sp. "dark" using DNA sequence data. Since then, we have found that there are actually five species (Fig. 4.24) which produce this type of modified pilidium that superficially resembles the trochophore larva of some annelids (Fig. 4.2G). Hence, we nicknamed this group, which likely represents a new heteronemertean genus (Hunt and Maslakova, unpublished) "Trochonemertes". Not all species are currently differentiable, but some can be identified by their size, the location of their ciliated bands and ciliary cirrus (see descriptions below). They are found in fall and winter months. The pilidium nielseni morphotype can be separated into two groups based on the position of the transverse ciliated bands and ciliary cirrus. There are two species which produce larvae where the anterior-most ciliated band is positioned equatorially and the ciliary cirrus is found between the two ciliary bands (*Micrura* sp. "dark" and *Micrura* sp. 3) and those where the anterior-most ciliated band is positioned more posteriorly, and the cirrus, when visible, is at the larval posterior end (*Micrura* sp. "albocephala", *Micrura* sp. 4, and *Cerebratulus longiceps*).

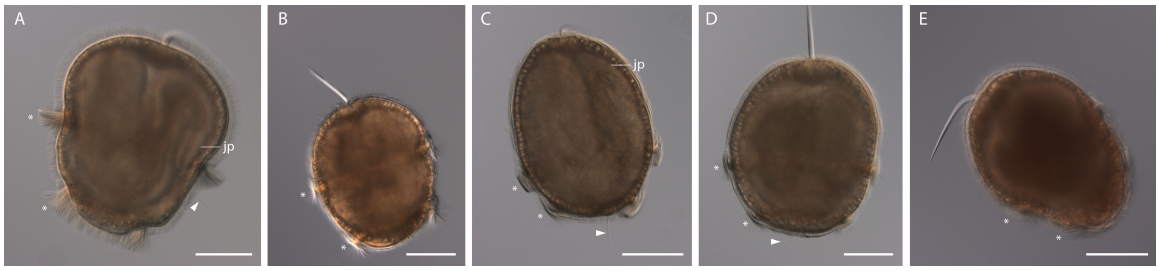


Figure 4.24. Wild-caught larvae with pilidium nielseni morphotype collected in January of 2013 and 2014. A) *Micrura* sp. "dark", B) *Micrura* sp. 3, C) *Micrura* sp. "albocephala", D) *Micrura* sp. 4, E) *Cerebratulus longiceps*. Note transverse ciliated bands (asterisks) and larval cirri (arrowheads) as well as juvenile posterior (jp). Scale bars 100 µm.

***Micrura* sp. "dark" (Fig. 4.24A)**

Larvae collected: Dec 2011

The larvae of *Micrura* sp. "dark" were identified in 2012 (Maslakova and von Dassow, 2012). They have a prominent blade-like apical tuft, and two transverse ciliated bands. The anterior band is equatorial and the posterior band is at the larval posterior. Between the two bands is a stiff ciliary cirrus. The larval epidermis contains numerous spherical lipid granules and a worm can often be observed within the larval body. The swimming behavior of the larvae of this species is particularly notable. The cilia beat regularly, but stop intermittently during which time the larva is suspended and somewhat motionless. The larva also often constricts along the two ciliated bands. Adult *Micrura* sp. "dark" are collected from amongst *Phyllospadix* sp. root masses in the rocky intertidal, and are ripe in fall and winter months (Hunt and Maslakova, unpublished).

***Micrura* sp. 3 (Fig. 4.24B)**

Larva collected: January 2013

We have only found one larva from this species in the plankton in January, and its morphology was similar to *Micrura* sp. "dark". The anterior ciliated band was positioned equatorially, the larva had a prominent apical tuft, but the position of the ciliary cirrus is currently not known. The larvae of this species are certainly more rarely encountered than the larvae of *Micrura* sp. "dark" and we have yet to find their adults. Not surprisingly, the larvae of these two species not only have similar morphology, but group sister to one another on molecular phylogenies (Hiebert and Maslakova, in prep.).

***Micrura* sp. "albocephala" (Fig. 4.24C)**

Larvae collected: Dec 2011; Dec 2012; Jan 2013

Of the species which produce larvae of the pilidium nielseni morphotype where the anterior ciliated band is positioned posterior to the larval equator, and the ciliary cirrus is posterior, *Micrura* sp. "albocephala" is the only one for which we've found corresponding adults. *Micrura* sp. "albocephala" is an undescribed nemertean species found in mudflats

in Charleston, OR. Lecithotrophic larvae usually develop from larger eggs than their planktotrophic relatives, and this species is no exception. The eggs of *Micrura* sp. "albocephala" were collected from a ripe female in December, and they are 200 µm in diameter and are surrounded by both a conspicuous chorion and an egg jelly. The larvae of this species are somewhat smaller than the morphologically similar *Micrura* sp. 4 larvae, but this characteristic has yet to be quantified.

***Micrura* sp. 4 (Fig. 4.24D)**

Larvae collected: Dec 2011; Jan 2013

Like the larvae of *Micrura* sp. "albocephala", these larvae exhibit two transverse ciliated bands which are posterior to the larval equator, and a posterior ciliary cirrus. We have observed the larvae of this species several times in the plankton, however, we have yet to find their adults intertidally. These larvae are, for the most part, indistinguishable from the larvae of *Micrura* sp. "albocephala" although they tend to be a bit larger (but this character has not yet been quantified).

***Cerebratulus longiceps* (Fig. 4.24E)**

Larva collected: Jan 2014

The third species to produce larvae of the pilidium nielseni morphotype with two ciliated bands posterior to the equator is the larva of *Cerebratulus longiceps*. This larva has only been collected once and it was recognized as unique because of its overall red body color, particularly along the ciliated bands. Although all other species which produce the pilidium nielseni morphotype form a well-supported monophyletic clade on molecular phylogenies, this species is distantly related. Thus, this morphotype may have evolved independently in more then one pilidiophoran lineage.

Heteronemertean orphan larvae

Surprisingly, we have encountered several heteronemertean species as larvae in the plankton that we have yet to find as adults and for which we cannot reliably assign to any

heteronemertean genus using our molecular phylogenies. For many of these species, we have found only a single larva at an early developmental stage such that describing the larva in this key is not possible. However, for one heteronemertean orphan larva (Heteronemertea gen. sp. 4, below), we have found many larvae and have documented many developmental stages.

Heteronemertea gen. sp. 4 (Fig. 4.25)

Larvae collected: Sep, Oct 2011; Oct 2014

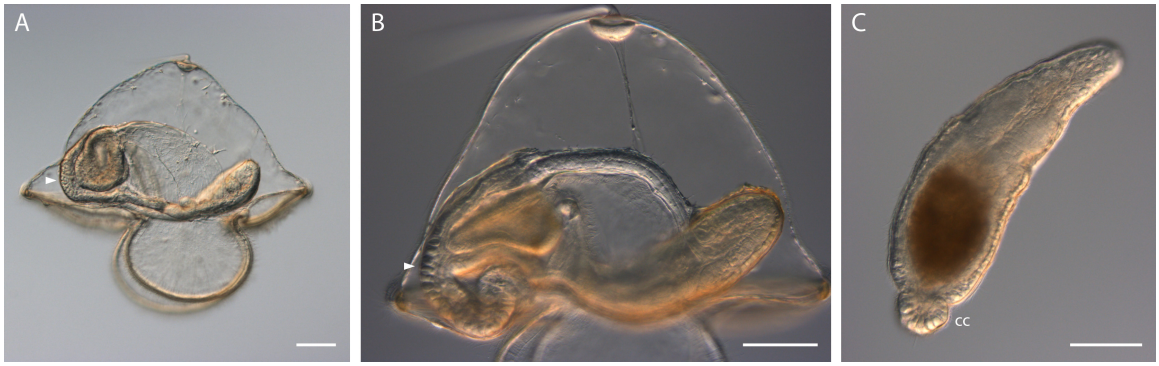


Figure 4.25. Heteronemertea gen. sp. 4 larva collected from plankton in November 2011 (A–B) and raised through metamorphosis (C); note concentration of lipid granules at posterior in developing juvenile (arrowheads, A–B) and short caudal cirrus (cc) in the, newly metamorphosed, juvenile (C). Scale bars 100 µm.

This is an example of an orphan larva (a species which we find as larvae but have yet to find as adults). Furthermore, this orphan larva cannot be assigned easily to any heteronemertean genus or group using molecular phylogenies. This larval type, which we nicknamed "piano-mover", is recognizable because of its broad, robust and strong episphere. An additional feature that we noticed upon close inspection is the presence of a cluster of lipid granules in the juvenile posterior. These larvae were very common in the fall of 2011 and were observed again in October 2014.

Pilidiophora; Fam. Hubrechtiidae

Hubrechtella

Larvae of the genus *Hubrechtella* exhibit the pilidium auriculatum morphotype (Fig. 4.2D). These larvae are characterized by narrow lappets, resembling side-burns (Norenburg and Stricker 2002, Cantell 1969, Maslakova 2010b, Maslakova and Hiebert 2014). Also characteristic are the conspicuous outlines and nuclei of epidermal cells. Wild-caught larvae have been reared in the lab on a diet of *Rhodomonas lens*, and they develop very slowly. The juvenile develops around the larval gut and is at an oblique angle to the anterior-posterior axis of the larval body.

Hubrechtella juliae **(Fig. 4.26)**

Larvae collected: Feb 2010; Dec 2011; Sep 2013; Oct 2014

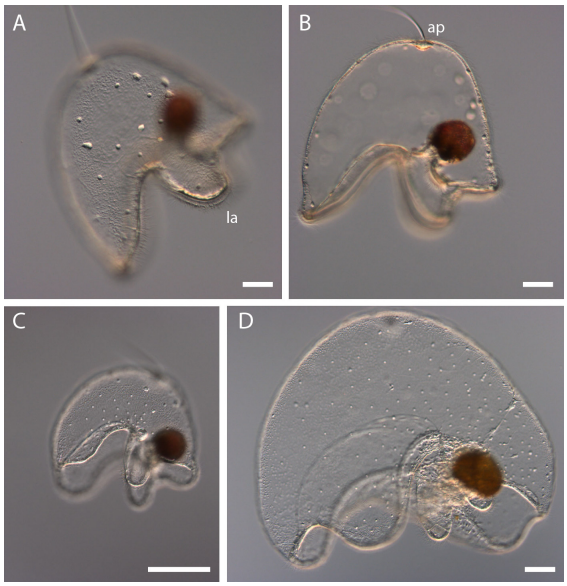


Figure 4.26. Wild-caught larva of *Hubrechtella juliae* collected in November 2012 (A–C) and raised for over two months, at which point the larval episphere became enormous (C) but no juvenile developed. Note epidermal cell outlines and nuclei (in focus, A, C), narrow lateral lappets (la, A), and apical tuft at larval anterior (ap, B). Scale bars 100µm.

Using DNA sequence data we identified these larvae are *Hubrechtella juliae* (described from the Sea of Japan, Chernyshev 2003) from a plankton sample taken in the Charleston marina (Maslakova and Hiebert, 2014). There are no previous records of Hubrechtid nemerteans on the west coast of North America. We have collected a few individuals of this species as larvae in the plankton each year, but we have yet to find the adults. Hubrechtids (Fam. Hubrechtiidae, formerly of the order Palaeonemertea) are interesting because molecular phylogenies place it as the sister clade to the Heteronemertea; together the two clades comprise the Pilidiophora (Thollesson and Norenburg 2014, Andrade

et al. 2014), characterized by the presence of the pilidium larva.

Palaeonemertea

***Carinina* sp. "chocolate" (Fig. 4.27)**

Larvae collected: Jan 2013, Oct 2015

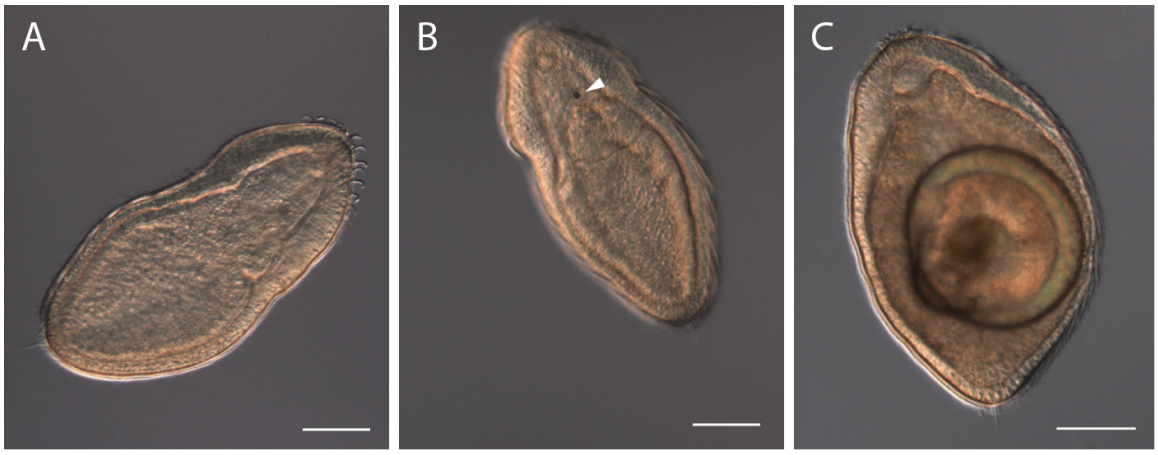


Figure 4.27. Wild-caught larva of *Carinina* sp. "chocolate" collected in January 2013. Note anterior claw-like cirri at 2 o'clock (A), larval eye anterior to the mouth (arrowhead, B). This individual ingested a bivalve veliger larva, that is clearly visible within the palaeonemerteans stomach (in focus, C). Scale bars 100 μm.

This larva belongs to an undescribed species we call *Carinina* sp. "chocolate" due to the deep brown body color in adult individuals which we find intertidally in mud and sand flats in Charleston, OR. Note that this genus has not been previously reported from the West Coast of North America. The larvae of *Carinina* sp. "chocolate" occur in the plankton in winter months. They are characterized by a single mid-ventral intra-epidermal eye which is anterior to the mouth, a feature also present in larvae of the genus *Carinoma*. The larvae of *Carinina* sp. "chocolate" are distinct because of their claw-like anterior cirri along their anteriormost region. They also exhibit a thick epidermal layer and cerebral organ pits (a distinct feature also present in adults) and cirri, laterally. They are bean-shaped, pale yellow to golden in color, lack any marked pigmentation and are covered in small golden epidermal pigment granules. At their posterior they have a small cirrus. Shown here is a *Carinina* sp. "chocolate" larva which has just ingested a bivalve veliger larva — the perfect illustration of macrophagous planktonic feeding by

palaeonemertean larvae. Reproductive adults were observed in May 2012 and released oocytes are approximately 120–140 µm in diameter and lack a chorion.

Carinoma

Larvae of the genus *Carinoma* are recognizable by their single intra-epidermal eye which is ventral and usually just anterior to the mouth (Norenburg and Stricker 2002; Maslakova et al. 2004a, b). Lateral cirri in the anterior body region have also been observed in *Carinoma* larvae (*C. tremaphoros*, Norenburg and Stricker 2002). The early development of *Carinoma tremaphoros* has been described (Maslakova et al. 2004a, 2004b). *Carinoma mutabilis* is the only *Carinoma* species reported between central CA to OR. However, we have found that there are actually five species of *Carinoma* in southern Oregon alone, and we've encountered and identified the larvae of all five.

***Carinoma mutabilis* (Fig. 4.28)**

Larvae collected: Feb, Sep 2013

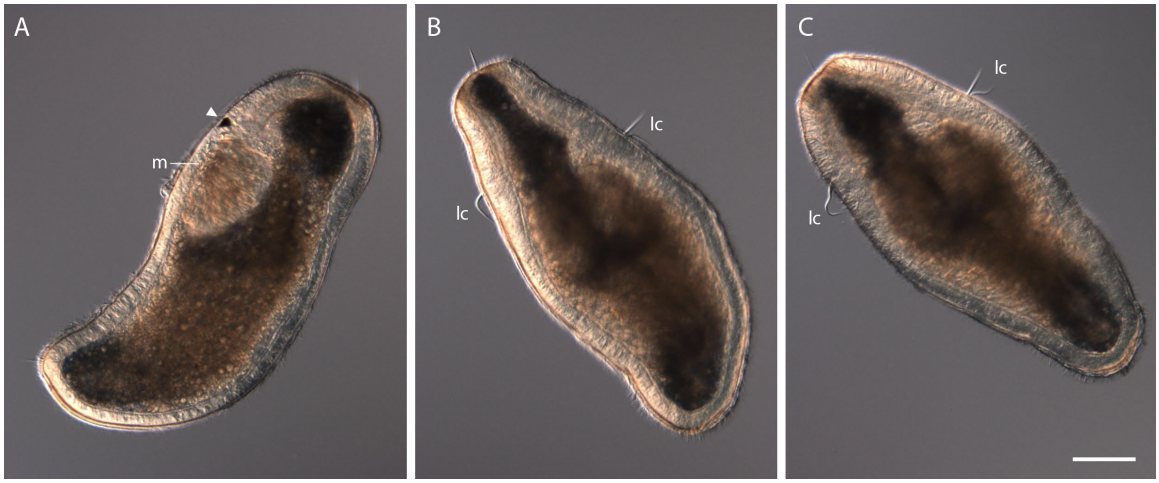


Figure 4.28. Wild-caught larva of *Carinoma mutabilis* collected in February 2013. Views shown include lateral view (A) and dorsal (B–C). Note mid-ventral black eye (arrowhead, A) and mouth (m, A) and lateral cirri that are both stiff and flapping (lc, B–C). Scale bar 100 µm.

The larva of *Carinoma mutabilis* has a small triangular eye. As is true for other *Carinoma* larvae (e.g., *C. tremaphoros*, Norenburg and Stricker 2002), *C. mutabilis* larvae have several lateral cirri in the anterior region of the body. The cirri are not bilaterally symmetrical and some are stiff and extend outward while others flutter like flames. The larval anterior is rather blunt and possesses a small apical tuft, while the posterior tapers, terminating with an inconspicuous posterior cirrus. The larvae of *C. mutabilis* are easy to differentiate from other *Carinoma* larvae by the deep blue color in anterior-most (pre-oral) and posterior-most regions of the gut. The size of palaeonemertean larvae varies considerably as they feed and grow in the plankton. In Charleston, OR, *C. mutabilis* larvae we collected in February 2013 were approximately 500–650 µm in length. In Friday Harbor, WA ripe adults were collected between January and February 2007 (Bartolomaeus et al. 2014) and Coe reported sexually mature individuals in August (California and Puget Sound, 1901; 1905).

***Carinoma hamanako* (Fig. 4.29)**

Larvae collected: Oct 2008; May 2012; Feb, July, Oct 2013

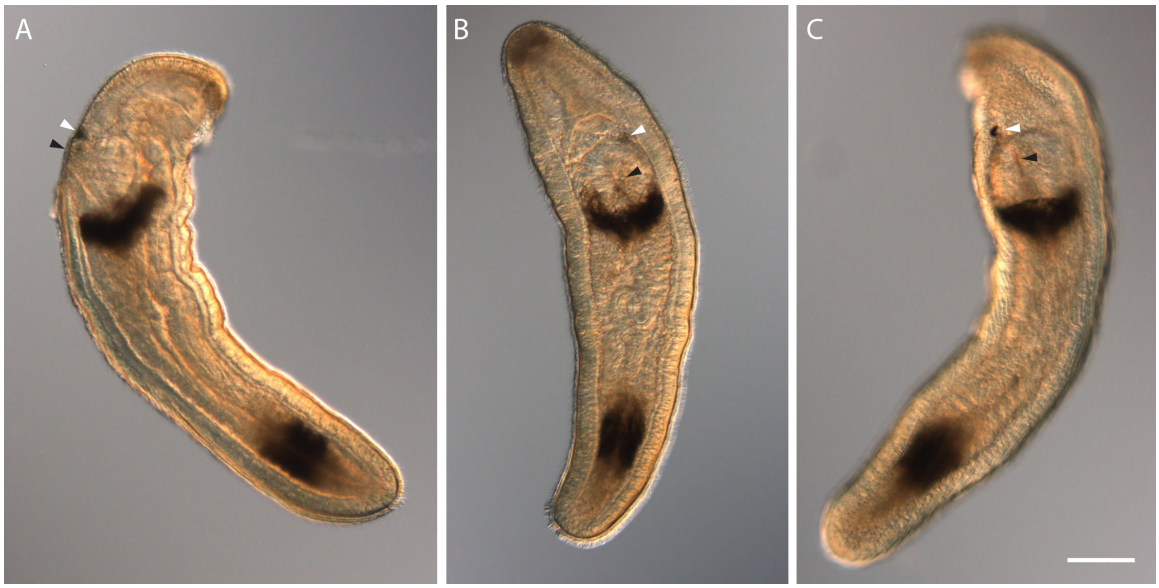


Figure 4.29. Wild-caught larva of *Carinoma hamanako* collected October 2013. Views shown include lateral (A), dorsal (B) and, ventral (C). Note small black eye (white arrowheads) anterior to the mouth (black arrowheads). Scale bar 100 µm.

This is an example of a larva found in plankton samples from southern OR that likely belongs to a species described from another geographic region and not previously reported from the NE Pacific. *Carinoma hamanako* is a species described from Lake Hamana on the Pacific coast of Honshu, Japan (Kajihara et al. 2011). Although, we have yet to find the adults of *C. hamanako* along the NE Pacific coast, we have found what we believe are their larvae in the plankton. The larvae are easily recognizable as *Carinoma* larvae, with a single large and round mid-ventral eye. But, these larvae are distinct from other *Carinoma* larvae in possessing two prominent patches of dark color in the gut, one just posterior to the mouth and the other in the posterior-most region of the gut. They also have a patch of darker color in the epidermis of the anterior tip of the body and a cirrus at the larval posterior. We have collected one young *C. hamanako* larva, which did not yet possess these pigmented areas. Instead, the specimen had an eye and distinct, long lateral cirri in the body region anterior to the mouth. Advanced larvae have a large mouth and proboscis and tend to rotate between the coverslip and microscope slide such that the mouth is down.

Uncertain Identity – As mentioned in our methods section, matching larvae to adults using DNA sequence data is usually relatively straightforward because the sequences match so closely. In fact, unless mentioned otherwise all larvae identified with sequence data on this website show sequence divergences of <1% from their corresponding adults (for both 16S and COI gene regions). The larvae of *C. hamanako* shown here exhibit a sequence divergence of 1.7% and 8.7% from adult sequences for *C. hamanako*, for the 16S and COI gene regions, respectively. In our experience, interspecific divergence values for the 16S gene region range from approximately 4–12%, while intraspecific values range 0–1%. Likewise, for the COI gene region, interspecific values range from 13–18%, while intraspecific range is 0–7% (see also Mahon et al. 2009; Meyer and Paulay 2005; Kvist et al. 2014). Thus, divergences between these larvae and *C. hamanako* remain under the minimum interspecific sequence divergence for congeneric nemertean species. However, it remains higher than we typically see for species-level

larval identification. We frequently find these larvae in the plankton and we are confident that the adults live nearby. Whether these are the larvae of *C. hamanako* or another closely related *Carinoma* species remains to be seen.

***Carinoma* sp. "white" and *Carinoma* sp. "yellowback"**

There are two different species of *Carinoma* that produce larvae with distinctive orange color, both species are awaiting formal description, *Carinoma* sp. "yellowback" and *Carinoma* sp. "white" (Fig. 4.30). Interestingly, the adults are easy to tell apart, but the larvae are (thus far) indistinguishable. The sequence divergence between these two species is 16.5% (16S) and 17.4% (COI).

Figure 4.30. Wild-caught larvae of *Carinoma* sp. "yellowback" (A), collected March 2013 and *C*. sp. "white" (B), collected February 2013. Both larvae exhibit similar morphology, shown here in lateral view; note black eye anterior to the mouth and orange body color. Scale 100 μm.

***Carinoma* sp. "white" (Fig. 4.30A)**

Larvae collected: Feb 2013

This is an example of a larva that belongs to a currently undescribed species. We find adults intertidally from Charleston to Gearhart, OR and refer to them as *Carinoma* sp. "white". As the name suggests, this species exhibits relatively simple adult morphology and a pale color. The larvae, on the other hand, are recognizable by their bright orange color (due to the color of the gut) and are, so far, indistinguishable from those of *Carinoma* sp. "yellow back". Their mid-ventral eye is rather large and rounded. Besides the orange color, the mid-anterior most region is rather bulbous in proportion to the rest of the body. The larvae have long anterior lateral cirri — one pair dorsally and ventrally across from one another. Most individuals we encountered in February had very large mouths and well developed proboscis. The posterior end tapers in all individuals and is marked with a small cirrus.

***Carinoma* sp. "yellowback" (Fig. 4.30B)**

Larvae collected: March 2013; Oct 2014

The larvae of *Carinoma* sp. "yellowback" are also distinctly orange. We found the larva of this species in plankton samples taken in March 2013 and October 2014. In October, we collected a large specimen (1.5 cm in length) that was actively swimming in the plankton, was orange in color and had a single mid-ventral eye.

***Carinoma* sp. 5 (Fig. 4.31)**

Larvae collected: Oct 2013

This is an example of a larva which belongs to yet another *Carinoma* species, but this one we have yet to find as a benthic adult. Our 16S and COI phylogenies both situate this larva within the genus *Carinoma*, and it exhibits morphological characteristics of *Carinoma* larvae. We have encountered just one larva of this species, which has a small mid-ventral eye, is brown in color and rather pointed at anterior and posterior ends. This specimen has lateral cirri, as is common for larvae of this genus. They are situated

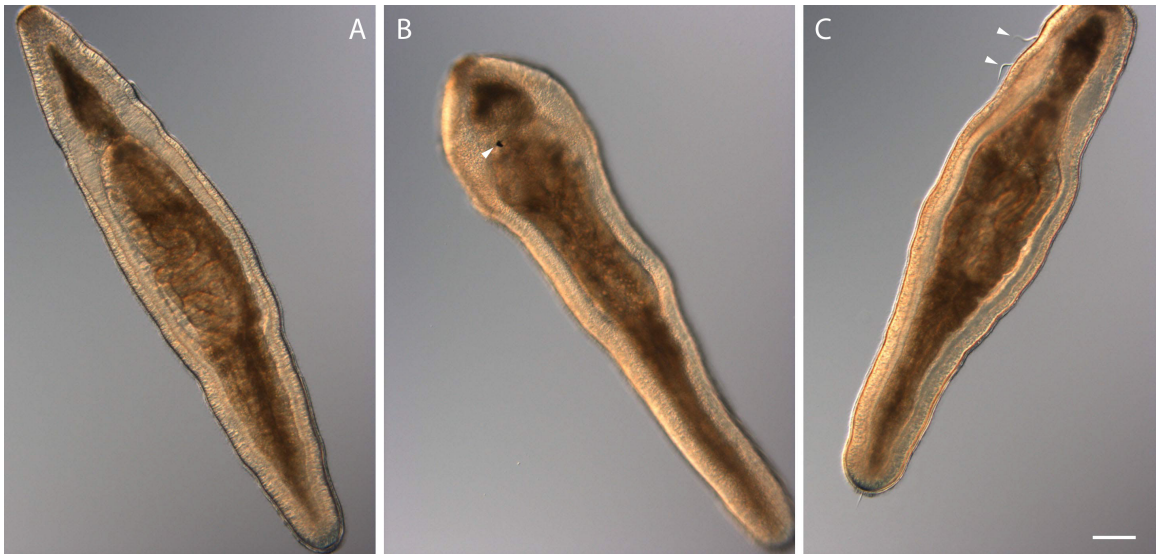


Figure 4.31. Wild-caught larva of the fifth *Carinoma* species found in southern OR, *Carinoma* sp. 5, collected March 2013. Larval anterior and posterior are pointed and coiled proboscis is visible in dorsal view (A), mid-ventral eye is small (arrowhead, B) and lateral cirri are not stiff (arrowheads, C). Scale bar 100 µm.

equatorially and anterior to the mouth and are quite long (as seen in *Carinoma* sp. "white" and "yellow back") and we detected two cirri on the left side. The larval posterior has a distinct yet small cirrus and this specimen was equipped with a very developed and coiled proboscis.

Cephalothrix

There are at least six *Cephalothrix* species in the NE Pacific and three of those are currently only found as larvae. Larvae of the genus *Cephalothrix* have paired intra-epidermal eyes. In some species each eye consists of two small eyes (e.g., *Cephalothrix* sp. 1, *Cephalothrix* sp. 2). *Cephalothrix* larvae often have a small apical tuft, posterior cirrus, and lateral cirri. In our experience, the lateral cirri are symmetrical and are usually anterior to the eyes. The development of *Cephalothrix rufifrons* (Smith 1935) and *C. filiformis* and *similus* (Iwata 1985; Johnson 2001) have been described. Two species in the genus *Cephalothrix* (=*Procephalothrix*) are reported in the NE Pacific (Roe et al. 2007): *C. spirals* and *C. major*. However, we find that there are likely at least six species and four of those we have collected as larvae only.

***Cephalothrix spiralis* (Fig. 4.32)**

Larva collected: May 2015

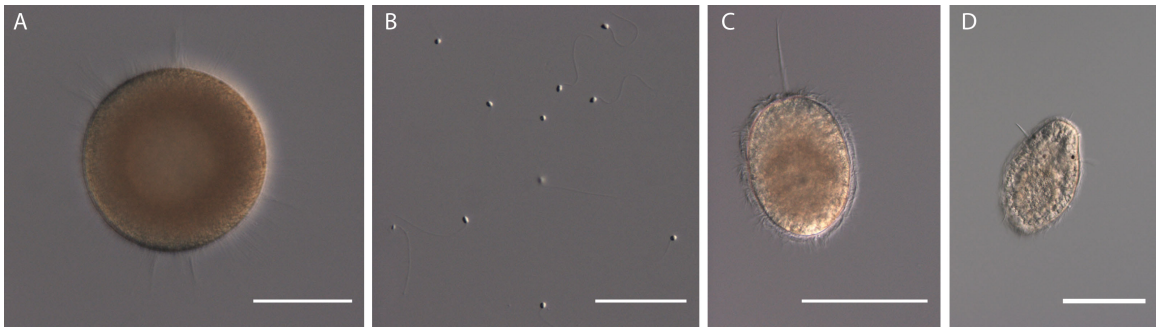


Figure 4.32. Development of *Cephalothrix spiralis*, as reared in the laboratory from known adults. Oocytes (A) and sperm (B) were dissected from ripe adults in March 2013. Larvae of artificially reared *C. spiralis* shown at four (C) and 11 days (D). Scale bars 100 µm.

We have collected the adults of *C. spiralis* on two occasions on the southern Oregon coast (one near Charleston, one near Cape Blanco) and were able to rear larvae from adults in March 2013. Eggs are 95 µm in diameter, have an egg jelly layer, and sperm headpiece is approximately 2–4 µm in length. Brownish orange epidermal granules are apparent in young larvae, and a prominent apical tuft is visible at four days after fertilization. The larvae have eyes as well as several (relatively) long lateral cirri by 11 days and some individuals had thick anterior comb-like cirri. Unfortunately, we are yet to identify the preferred food for *Cephalothrix* larvae and we were not able to rear these larvae beyond 18 days.

Cephalothrix species are notoriously difficult to classify (e.g., Chen et al. 2010) and, when analyzed with available sequence data from around the world, molecular phylogenies of this genus reveal an unresolved taxonomy (Hiebert and Malsakova, manuscript in prep). In particular, two *C. spiralis* specimens from San Juan Island, WA are only distantly related on COI phylogenies (18.5% sequence divergence). Thus, the real identity of the species to which we refer to as *C. spiralis* will require more analysis.

What remains clear is that there are at least 5–6 *Cephalothrix* species in the NE Pacific where three were previously reported (Roe et al. 2007).

***Cephalothrix* sp. 1 (Fig. 4.33)**

Larvae collected: May 2012; Jan, Feb, July, Aug 2013

Figure 4.33. Wild-caught larva of *Cephalothrix* sp. 1 collected November 2013; note epidermal yellow pigment and lateral cirrus anterior to the left eye pair (in detail, inset). Scale bar 100 µm.

This is an example of a *Cephalothrix* species that we currently only find as larvae in the plankton. This species is recognizable by two double eyes and lateral cirri, as in other local *Cephalothrix* larvae, but individuals also have a dark grapefruit pink body and gut color, presumably due to orange epidermal pigment granules. A young individual, collected in August 2013, had long lateral cirri, but another larger individual that we collected in October had truncated cirri.

***Cephalothrix* sp. 2 (Fig. 4.34)**

Larvae collected: Jan, Oct 2013

This species is the most commonly encountered *Cephalothrix* species we collect as larvae from the plankton. We find individuals almost year-round, with most individuals collected in May. We have yet to find the adults of this species but their larvae are easy to recognize by their yellow epidermal granules. They also have two double eyes, which are inconspicuous at times. In young individuals, the eyes are not always doubled. Near the eyes are lateral cirri that are not excessively stiff (as in *Tubulanus* larvae), but do not flap (as in *Carinoma* larvae (e.g., *Carinoma mutabilis*)). The larvae are often quite large (500–750 µm), have a large

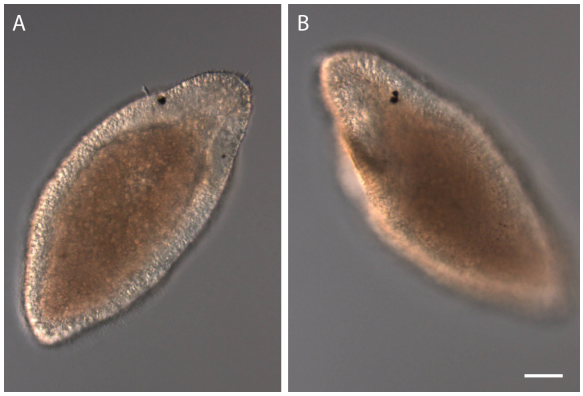


Figure 4.34. Wild-caught larva of an undescribed *Cephalothrix* species, *Cephalothrix* sp. 2 collected in January 2013. Dorsal (A) and lateral (B) views shown; note large mouth opening at 8 o'clock (B). Intra-epidermal eyes are sometimes so close together, they appear as one (compare A to B). Scale bar 100 µm.

mouth, and we have observed planktonic individuals with a very well developed proboscis.

***Cephalothrix* sp. 3 (Fig. 4.35)**

Larva collected: May, Oct 2013; Oct 2015

We only observed this larva once. It is bright blue and easy to spot in plankton samples. Upon closer inspection, it has red pigment between the blue and pale regions ventrally near the mouth. This individual had two eyes which were intra-epidermal, but not doubled. Additionally, there was a general reddish hue anteriorly. We also did not notice any distinct lateral cirri and the mouth was a small circle, unlike the large folded slits we see in other *Cephalothrix* larvae. This larva represents yet another undescribed species for which we have yet to find adults.

Figure 4.35. Wild-caught larva of *Cephalothrix* sp. 3 collected in May 2013. Larva bears distinctly blue body pigment, a red anterior, and pale ventral pigment patches (B) near the mouth (arrowhead, B and C). Scale bars 100 µm.

***Cephalothrix* sp. 4 (Fig. 4.36)**

Larvae collected: Feb, March 2013

Figure 4.36. Wild-caught larva of *Cephalothrix* sp. 4 collected February 2013, note two single eyes and one lateral cirrus, in focus and below left eye. Scale bar 100 µm.

We've found these *Cephalothrix* larvae several times since 2009, usually in early spring. They are characterized by paired single eyes (rather than paired double eyes found in other local *Cephalothrix* larvae), a large slit-like mouth and lateral cirri. They also tend to have orange or brownish epidermal color and lipid droplets within the gut, but these characters are less consistent than others (e.g., single eyes).

Tubulanus

The palaeonemertean genus *Tubulanus* includes five local species (*T. sexlineatus*, *T. polymorphus*, *T. pellucidus*, *T. capistratus* and *T. cingulatus*, Roe et al. 2007). We have personally observed only two species locally (*T. sexlineatus* and *T. polymorphus*) and have sequence data for these, two plus *T. rhabdotus*, *T. punctatus*, *T. pellucidus* and *T. annulatus*. So far we have encountered larvae of four species of *Tubulanus* in the plankton, including those of *T. polymorphus*, *T. sexlineatus* and three others, that do not match any other species of *Tubulanus* sequenced so far by us or others (based on GenBank data). We have found three distinct larval types that are nested within the genus *Tubulanus* on our molecular phylogenies, but currently we cannot know if these larvae belong to undescribed species or if they are the larvae of *Tubulanus* species previously reported from the NE Pacific for which we have yet to obtain sequence data (e.g., *T. capistratus*, *T. cingulatus*). Most *Tubulanus* larvae are recognizable from other palaeonemertean larvae in that they are eyeless, have thick handlebar-like lateral cirri and a relatively thick epidermis. However, *T. polymorphus* larvae are large, short-lived larvae, which are rarely observed in the

plankton and lack the thick lateral cirri observed in other larvae of this genus. Some *Tubulanus* species secrete and inhabit mucous or parchment tubes, inside which both sexes can be found as they deposit and fertilize eggs (Coe 1943).

***Tubulanus polymorphus* (Fig. 4.37)**

Larvae collected: Feb 2013

Figure 4.37. Wild-caught larva of *Tubulanus polymorphus* collected February 2013. Note thin and short apical tuft (1 o'clock) at larval anterior. Scale 100 µm.

The larvae of *Tubulanus polymorphus* are lecithotrophic and short-lived (Stricker 1987). We collected this larva in February 2013 and, at first, did not recognize it as the larva of a nemertean. It was large (550 µm), slow-moving and blimp-like, orangish in color, and had an inconspicuous apical tuft. This larva drifted in the plankton sample, rather than moving with apical tuft forward as is usually the case for other nemerteans. Furthermore, this individual tended to compress itself back and forth in variety of shapes, similar to cnidarian planulae. Sequence data revealed that this is the larva of *T. polymorphus* a common bright orange nemertean in local rocky intertidal areas (e.g., Cape Arago). *Tubulanus polymorphus* is reported to be sexually mature in early (San Juan Island, WA, Stricker 1987) to late summer (Coe 1905, Maslakova pers. obs.) and can produce large numbers of eggs up to 350 µm in diameter (Stricker 1987). The larvae develop quickly and have a short pelagic duration, suggesting that this larva would rarely be encountered in the plankton.

***Tubulanus sexlineatus* (Fig. 4.38)**

Larvae collected: April 2015

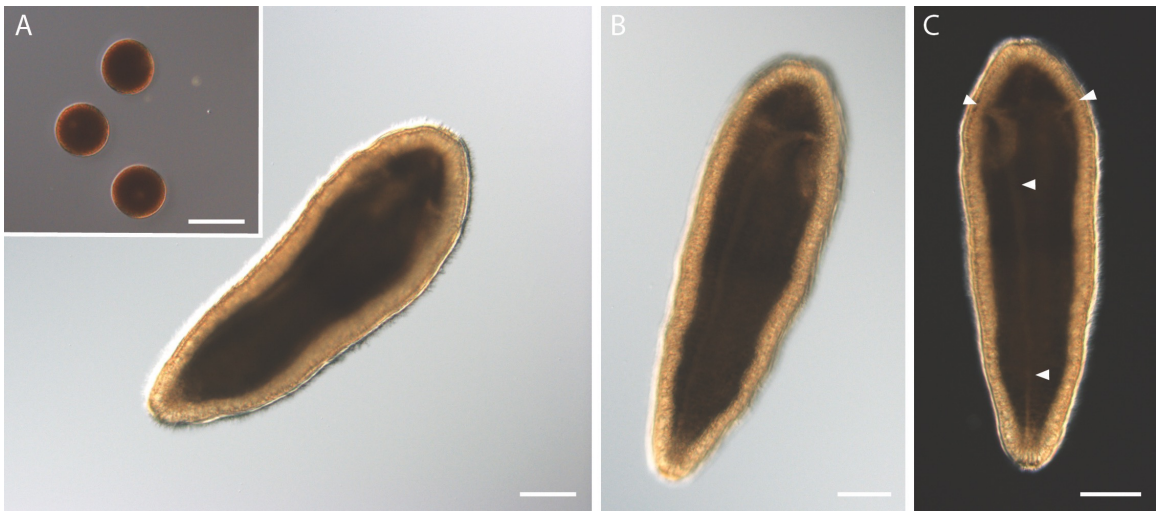


Figure 4.38. Oocytes (A, inset) released from female and wild-caught larva of *Tubulanus sexlineatus*, both ripe female and larva collected April 2015. Note anterior transverse pale line and connects to run longitudinally for the larval body length (arrowheads). Scale 100 µm.

The first time the larvae of *Tubulanus sexlineatus* were observed in plankton samples was April 2015. These larvae are like other *Tubulanus* larvae in that they are eyeless and bear a thick epidermis. However, the photographed *T. sexlineatus* larva did not possess the thick lateral cirri common in other *Tubulanus* larvae. Instead, this larva had a conspicuous and pale transverse anterior line that connected ventrally and ran longitudinally for the length of the body. Ripe females spawn 95 µm in diameter, pale pink oocytes that are connected by a loose jelly such that they remain near, and almost surround the body of the female.

***Tubulanus* sp. 1 (Fig. 4.39)**

Larvae collected: Jan, Oct 2013

This *Tubulanus* larva has very conspicuous and handlebar-like lateral cirri at younger stages, however in later stages, they are less distinct. The larvae lack eyes or apical tuft, and possess a small caudal cirrus. They can compress themselves antero-posteriorly where the body wrinkles resembling segments. Later developmental stages have a very

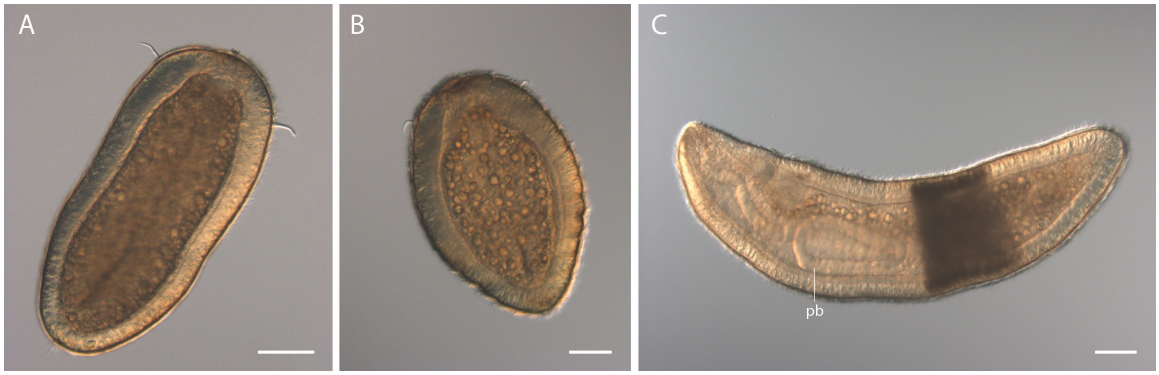


Figure 4.39. Wild-caught larva of *Tubulanus* sp. 1 collected in January (A–B) and October 2013 (C). A–B) Two postures shown include swimming (A) and compressed antero-poseriorly (B). Note short, thick lateral cirri anteriorly (A–B) and coiled proboscis (pb, C), and epidermal pigment band in advanced larvae. Scale 100 µm.

prominent dark brown to black epidermal band toward the larval posterior. The gut region remains filled with golden lipid droplets, which are not seen in other *Tubulanus* larvae we have observed.

***Tubulanus* sp. 2 (Fig. 4.40)**

Larvae collected: Feb, Oct 2013

This larva resembles later developmental stages of *Tubulanus* sp. 1, except that the lateral cirri remain thick at later developmental stages and curve downward in this species. Furthermore, the posterior band or color is not nearly as distinct and sharp as in larvae of *Tubulanus* sp. 1. There is also a stark dark color in the gut region. Larvae belonging to this species have been collected both in February and October 2013, suggesting a reproductive season with

Figure 4.40 (left). Wild-caught larva of *Tubulanus* sp. 2 collected in February 2013. Note thick lateral cirri anteriorly and a light pigment band posteriorly. Scale 100 µm.

several peaks throughout the year, extreme variation from year to year, or very long-lived larvae.

***Tubulanus* sp. 3 (Fig. 4.41)**

Larvae collected: Oct 2013

Figure 4.41. Wild-caught larva of *Tubulanus* sp. 3 collected in October 2013, shown here in dorsal (A–B) and lateral (C) view. Note lateral cirrus (in focus, B) and "pseudosegments" on compressed individual (C). Scale 100 µm.

This larva exhibits a combination of the characters from the *Tubulanus* sp. 1 and sp. 2 larvae discussed above. This species has lateral cirri, dark gut color and compresses its body such that “pseudo-segments" are visible. However, we did not observe any epidermal color patterns in the larva of this species. Having only found and identified one larva from this species so far, we cannot be sure that this is a species-specific character or dependent on developmental stage.

Hoplonemertea

***Carcinonemertes errans* (Fig. 4.42)**

Larvae collected: Oct 2008; Oct 2009; Feb 2010; May 2012; Jan 2013

The larvae of *Carcinonemertes errans* are easily recognizable. Unlike many hoplonemertean larvae, these larvae do not have a prominent blade-like apical tuft at the stage of development which we encounter in plankton. They do, however, have very

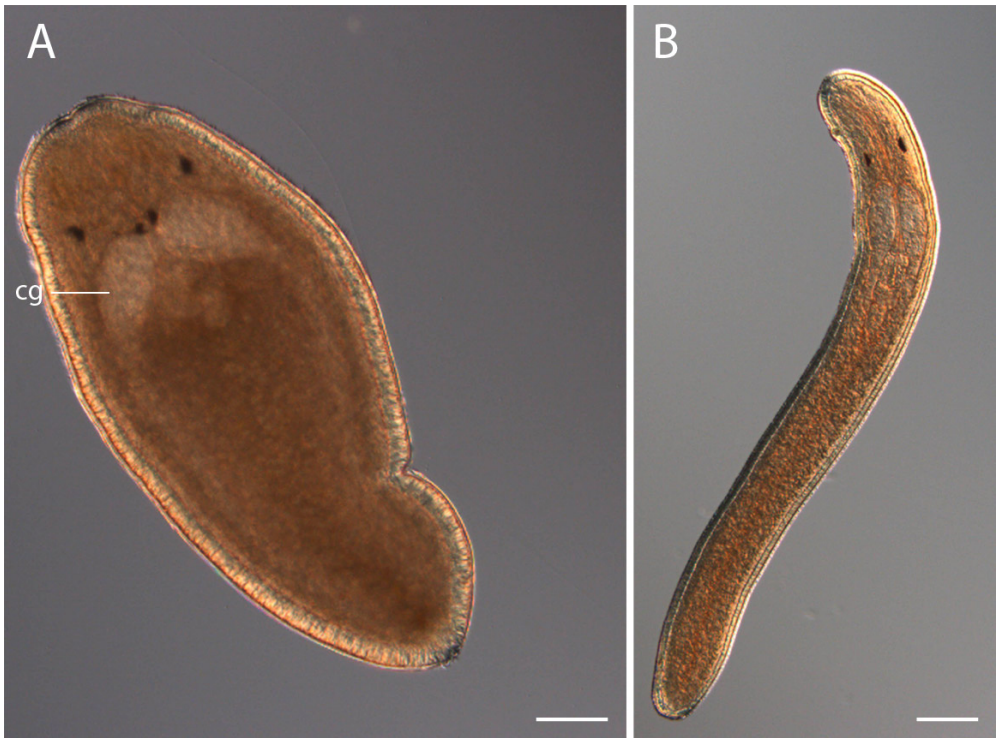


Figure 4.42. A) Wild-caught *Carcinonemertes errans* larva collected in January 2013; note four total eyes and transparent cerebral ganglia (cg). B) Juvenile *C. errans,* which was collected from an adult male Dungeness crab; note only two total eyes. Scale bars 100 µm.

conspicuous cerebral ganglia and two pairs of sub epidermal eyes. The upper and outermost pair is maintained through metamorphosis (concurrent with settlement onto host crab) (Dunn 2011). The innermost pair, which are sometimes so close together they appear as a single ocellus, are lost during metamorphosis and not present in newly settled juveniles. The proboscis and rhynchocoel are very short, the accessory stylet sacks are lacking, central stylet is located immediately posterior to cerebral ganglia. The larvae are fast swimmers and are approximately 500 µm in length. They are a distinct orange to pink color and have a cirrus at the larval posterior.

Carcinonemertes errans juveniles settle and grow into adulthood on the Dungeness crab, Cancer magister, where they make a living as egg predators. Adult *Carcinonemertes* species pierce crab eggs with nail-like stylets on their eversible proboscis and suck out

the contents. Tens of thousands of *C. errans* individuals can be found on a single crab host and they are capable of reducing the crab's egg mass by as much as 60 % (Wickham 1980; Kuris 1993). Interestingly, members of the Maslakova lab discovered that another nemertean species readily attacks and ingests *C. errans* larvae and juveniles. We maintained newly metamorphosed juveniles of the larva pilidium recurvatum (*Riserius* sp.) into adulthood (over 1.5 years) on a diet consisting solely of *C. errans* larvae (wild-caught) and juveniles (collected from male Dungeness crabs) (Hiebert et al. 2013).

***Emplectonema* sp. 1 (Fig. 4.43)**

Larvae collected: Oct 2013

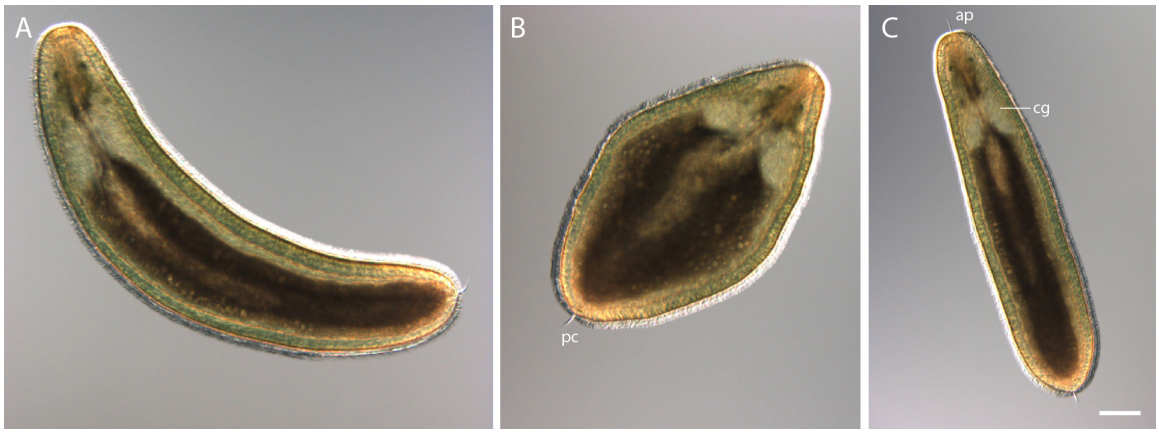


Figure 4.43. Wild-caught larva of *Emplectonema* sp. 1, collected in October 2013 shown here in three different positions. Note green color, cerebral ganglia (cg), apical tuft (ap) and posterior cirrus (pc). Scale 100 µm.

The larvae of *Emplectonema* sp. 1 are easily recognizable as hoplonemertean larvae. They have 2–3 pairs of sub-epidermal eyes[1]. One pair is anterior and conspicuous (Iwata 1960 reports this single pair, only). Additional eyes may be pigment patches and are situated near the cerebral organs, one large pair narrowly spaced and the other smaller pair near the anterior lateral cerebral organ region. The larvae have an apical tuft and posterior cirrus. What makes these larvae easy to recognize as the larvae of *E.* sp. 1 is that

[1] It is likely that the larvae of many hoplonemerteans (e.g., *Emplectonema* sp. 1, *Paranemertes californica, Poseidonemertes collaris, Gurjanovella littoralis, Tetrastemma bilineatum*) start out with just a single pair of eyes, and possibly, develop more pairs before settlement because adult individuals often have many more eyes.

they are green. Adult *E*. sp. 1 are dark green dorsally and pale green ventrally (right) and their larvae exhibit similar coloring. The early development of *E. gracile* from the Akkeshi coast in Japan (which is likely a different species that the one in the NE Pacific) has been described (Iwata 1960), but there is no mention of the brilliant green color we observed.

***Paranemertes* sp. 1 (Fig. 4.44)**

Larvae collected: Jan, Feb 2013

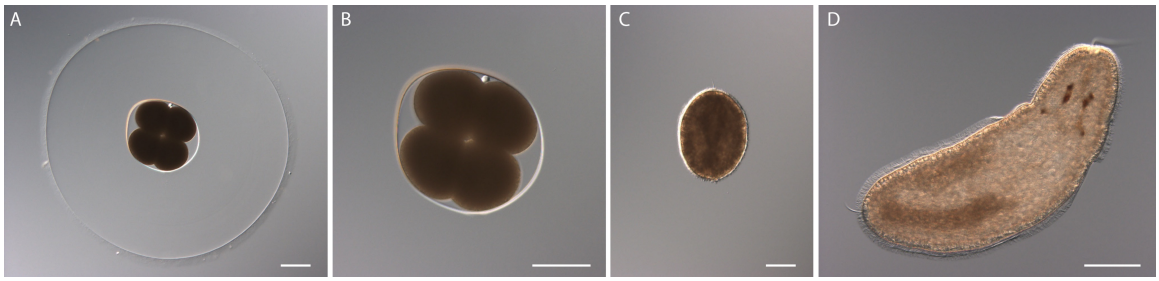


Figure 4.44. A–B) Early cleavage stage embryos of *Paranemertes* sp. 1 collected from plankton in February 2013. Embryos were raised in the laboratory until they developed into ciliated oblong larvae after two days (C), which acquired several pairs of eyes three days later (five-day old larva, D). Scale bars 100 µm.

The larvae of *Paranemertes* sp. 1 and a local *Zygonemertes* sp. 1 are difficult to differentiate. They are 'classic' hoplonemertean larvae with several pairs of eyes (usually three), a conspicuous apical tuft, a posterior cirrus and distinct cerebral ganglia. Sometimes, the larvae of these two species can be recognized by the color of their eyes: *P.* sp. 1 with reddish eyes and *Zygonemertes* sp. 1 with black eyes, but this is not completely consistent. Instead, these species can be differentiated by their early embryos both of which are common in plankton samples in winter months. The development of *Paranemertes* sp. 1 has been described (Maslakova and von Döhren 2009). The embryos of *P.* sp. 1 are 250 µm in size and surrounded by a large egg chorion (especially when compared to the egg chorion of *Zygonemertes* sp. 1). After 2–3 days, a ciliated larva with apical tuft begins swimming, and at six days the larva has three pairs of eyes. The larvae are lecithotrophic and planktonic for as little as 10 days (Maslakova pers. obs.) and up to

8 weeks (Roe 1976; Maslakova and von Döhren 2009), but their embryos are very common in the plankton.

***Zygonemertes* sp. 1 (Fig. 4.45)**

Larvae collected: Dec 2011; Dec 2012; Jan, April, March 2013

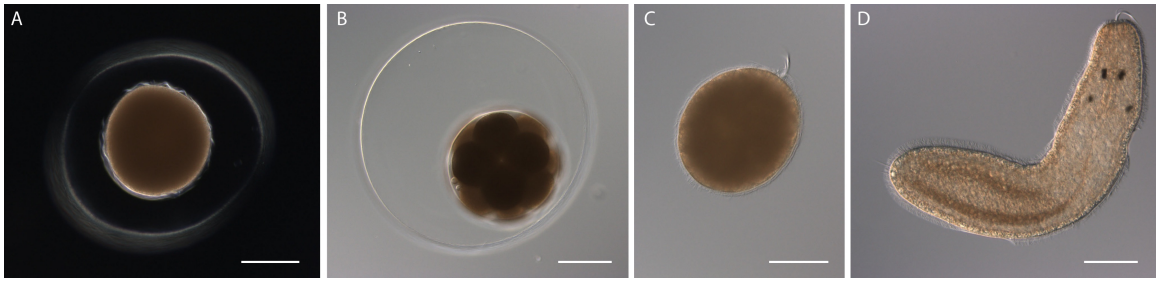


Figure 4.45. A–B) Early cleavage stage embryos of *Zygonemertes* sp. 1 collected from plankton in February 2013. Embryos were raised in the lab for five days. They were ciliated and swimming at day two (C) and had sub-epidermal paired eyes after five days (D). Scale bars 100 µm.

The embryos of *Zygonemertes* sp. 1, on the other hand, are 180 µm in diameter and surrounded by a smaller chorion than those of *P.* sp. 1. Ciliated larvae with an apical tuft begin swimming after two days and develop into larva with three pairs of eyes at five days.

***Paranemertes californica* (Fig. 4.46)**

Larvae collected: May 2012; Jan, Feb, March, June 2013

The larvae of *Paranemertes californica* range in size dramatically. Although hoplonemertean larvae are supposedly non-feeding, recent evidence based on DNA sequence data of gut contents and variations in size of conspecific larvae, suggests that some species may be feeding while in the plankton (Maslakova and Hiebert 2014). Advanced *P. californica* larvae have three pairs of eyes, prominent cerebral ganglia and a distinct head shape with a constriction at the posterior most pair of eyes immediately anterior to the cerebral organs, and corresponding the the position of the anterior cephalic furrows of juveniles and adults. Larger larvae also have a long coiled proboscis (which is

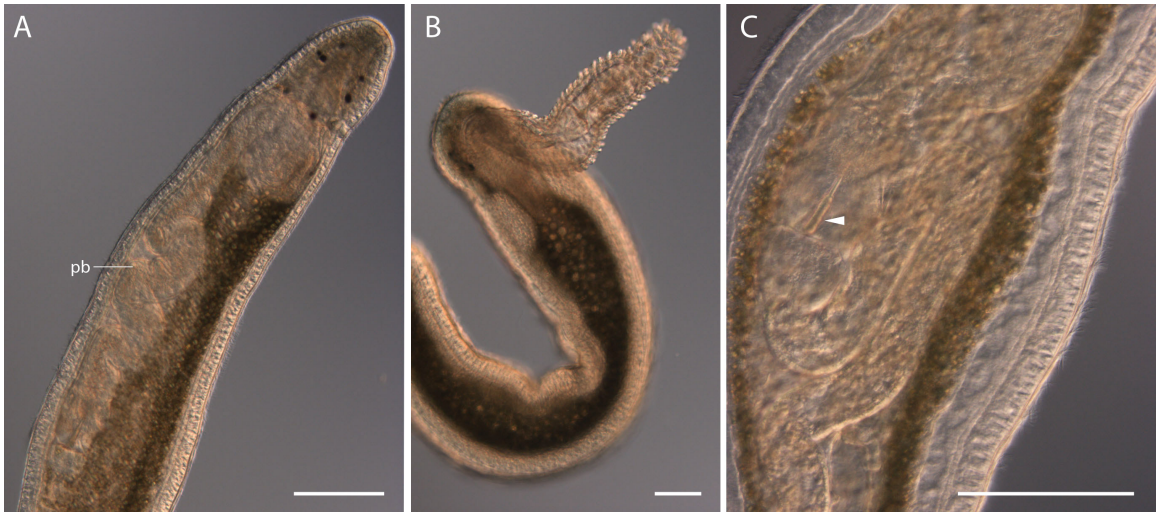


Figure 4.46. Wild-caught larva of *Paranemertes californica* collected in February 2013. Note the three pairs of eyes and coiled proboscis (pb, A), which is everted (B) readily and is equipped with a nail-like stylet (arrowhead, C). Scale bars 100 µm.

everted readily) and a stylet can be observed. They also tend to contort into a variety of positions such that individual paired eyes can be difficult to distinguish.

Identity uncertain – As mentioned in our methods section, matching larvae to adults using DNA sequence data is relatively straightforward because the sequences usually match so closely. In fact, unless mentioned otherwise all larvae identified with sequence data on this website show sequence divergences of <1% from their corresponding adults (for both 16S and COI gene regions). The larvae identified here as *Paranemertes californica* show little divergence for the 16S gene region (0.8%, below). For the COI gene region, however, there are two distinct groups with divergence of 5.9% between them. In our experience, interspecific divergence values for the 16S gene region range from approximately 4–12%, while intraspecific values range 0–1%. Likewise, for the COI gene region, interspecific values range from 13–18%, while intraspecific range is 0–7% (see also Mahon et al. 2009; Meyer and Paulay 2005; Kvist et al. 2014). Thus, divergences between these larvae and *P. californica* remain under the minimum interspecific sequence divergence for congeneric nemertean species. However, it remains higher than we typically see for species-level larval identification. We frequently find

these larvae in the plankton and we are confident that the adults live nearby. Whether these are the larvae of *P. californica* or another closely related species remains to be seen.

***Poseidonemertes collaris* (Fig. 4.47)**

Larvae collected: Nov, Dec 2011; Nov 2012; March, May 2013; Jan 2014

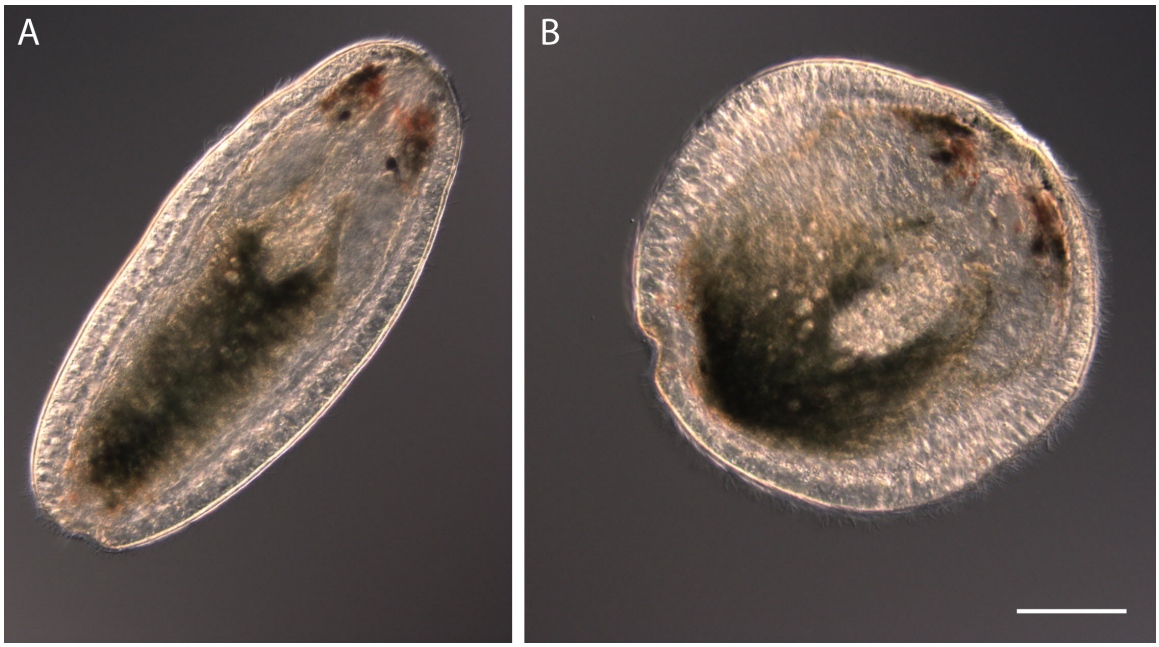


Figure 4.47. Wild-caught larva of *Poseidonemertes collaris* collected in February 2014, shown here in two common postures, including swimming (A) and antero-posterioly compressed (B). Note red pigment streaks anteriorly and two eyes. Scale

The larvae of the common local intertidal hoplonemertean, *Poseidonemertes collaris*, have two eyes and two anterior red streak-like patches, a gut filled with greenish or reddish lipid droplets, and conspicuous cerebral ganglia. At their posterior, they sometimes have a caudal cirrus and a small epidermal indentation. When observed on a microscope slide, these larvae tend to roll into ball-like shapes, which is a behavior also seen in *Gurjanovella littoralis* larvae. Other hoplonemerteans tend to compress themselves antero-posteriorly on a microscope slide (e.g., *Nipponnemertes bimaculata*), but they don't curl into balls like the larvae of *P. collaris* and *G. littoralis*.

***Gurjanovella littoralis* (Fig. 4.48)**

Larvae collected: March 2013

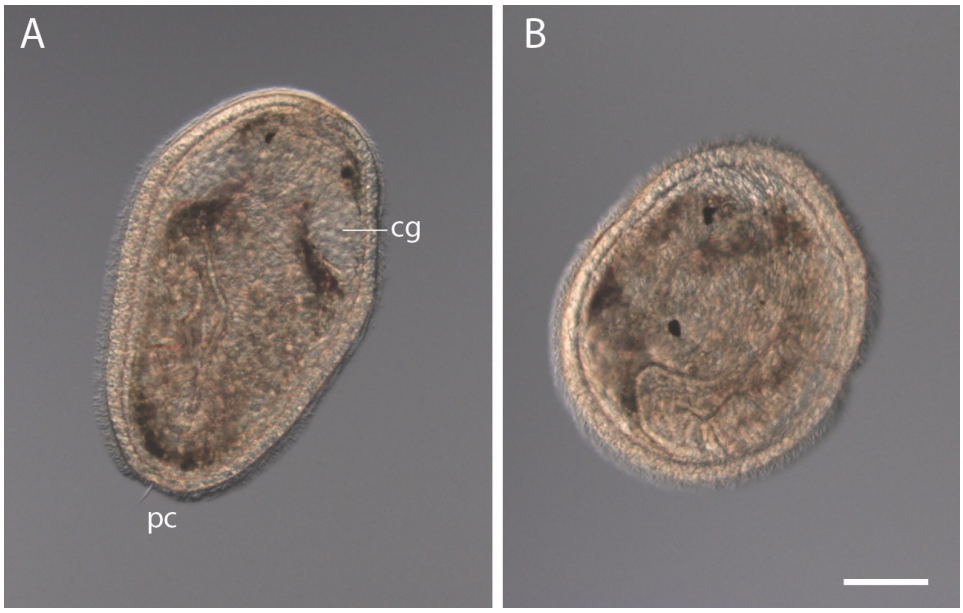


Figure 4.48. Wild-caught larva of *Gurjanovella littoralis* collected in March 2013. A) Swimming posture; note two eyes, under which are cerebral ganglia (cg), and a posterior cirrus (pc). B) Larva is compressed antero-posteriorly and view is anterior, where eyes are in focus. Scale 100 µm.

The larvae of *Gurjanovella littoralis* most closely resemble the larvae of *Posiedonemertes collaris*, in that they possess two larval eyes and a similar behavior when trapped of rolling into a ball. The larvae of *G. littoralis*, however, lack the red anterior streaks that are seen in P. collairs and, at least in the single individual we observed, are more pale in color. They lack a prominent apical tuft but possess a short posterior cirrus and have conspicuous cerebral ganglia. *Gurjanovella littoralis* is a species described from northwestern Russia (Ushakov 1926) and is not reported in the most recent assessment of intertidal nemerteans from central CA to OR (Roe et al. 2007). However, a single adult was collected in False Bay, on San Juan Island, WA and the larvae are present in southern OR plankton. Interestingly, the sequence data from this larva, *G. littoralis* from the White Sea and *G. littoralis* from Friday Harbor were identical for the 16S gene region.

***Nipponnemertes bimaculata* (Fig. 4.49)**

Larvae not yet collected in plankton

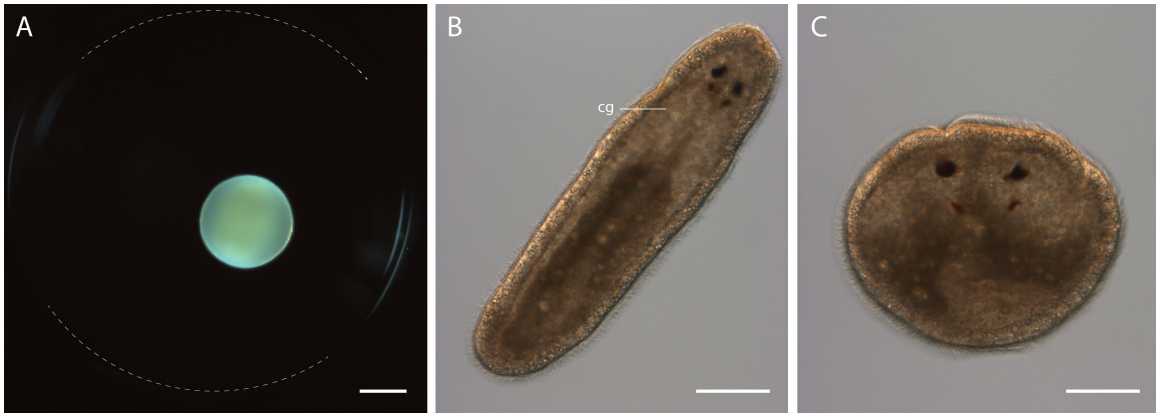


Figure 4.49. Oocytes collected from spawning female *Nipponnemertes bimaculata* in May 2013; note the reflection of the large jelly coat at 3 and 10 o'clock (outline of jelly coat is suggested by dotted line). Larva of *Nipponnemertes bimaculata* raised in the laboratory from artificially fertilized gametes, also in May 2013, shown here in three typical postures. Note two pairs of eyes and cerebral ganglia (cg). Scale 100 µm.

The larvae of *Nipponnemertes bimaculata* resemble those of *P. peregrina* and *Zygonemertes* sp. 1, however, they do not have as prominent an apical tuft. They also exhibit a behavior that is seen in *Poseidonemertes collaris* and *Gurjanovella littoralis*, where they compress themselves in a ball, however, this behavior is more of an antero-posterior compression rather a ball-like shape as in *P. collaris* and *G. littoralis*. Larvae of *Nipponnemertes bimaculata* possess three pairs of eyes (two pair in young larvae) that are reddish-black. The embryos in this species are also distinct, the oocytes are bright green in color and can be seen through the female body wall, are 260 µm in diameter and are surrounded by a large jelly layer. In May 2013, we observed several hundred ripe *N. bimaculata* adults washed ashore and spawning in the rocky intertidal at South Cove (Cape Arago, OR). As this species is known to inhabit macroalgal holdfasts, this suggests that they swarm out of the holdfasts to spawn and somehow accidentally became washed ashore.

***Malacobdella siliquae* (Fig. 4.50)**

Larvae collected: Feb 2013

Figure 4.50. Wild-caught larva of *Malacobdella siliqua* collected in February 2013; note longitudinal groove at upper right (arrowhead) and posterior cirrus (pc) at lower left. Scale 100 µm.

This is the larva of *Malacobdella siliquae*. The adults of this species are found within the mantle cavity of the razor clam, *Siliqua patula* (Kozloff 1991; Roe et al. 2007). The larvae were collected once in February, are very small and show no obvious hoplonemertean larval characteristics – there is no apical tuft, no conspicuous cerebral organs, no proboscis and no eyes. Instead, larvae may be recognized by a long (1/4 body length) mid-ventral groove at the anterior end. They have a rounded anterior and blunt posterior with a posterior cirrus (above). The larvae we collected were young and superficially resembled small palaeonemertean larvae.

Pantinonemertes californiensis

Larvae not yet collected in the plankton.

The larval development (including the shedding of the larval epidermis) has been described for *Pantinonemertes californiensis* (Hiebert et al. 2010). Reproductive adults were observed in July-September in southern Oregon, and March-November in California (Roe 1993). Females have pinkish oocytes approximately 100 µm in diameter and spawned oocytes are surrounded by a conspicuous egg chorion. Planktonic larvae hatch after 30 hours and have a prominent apical tuft, two red eyes and a less conspicuous posterior cirrus after three days (Hiebert et al. 2010). Larvae survive and swim in cultures for several weeks, but did not grow or settle, which may suggest that they require food to develop (Roe, 1993, Maslakova pers. observation). We have yet to collect these larvae from the plankton.

***Tetrastemma bilineatum* (Fig. 4.51)**

Larvae not yet collected in plankton

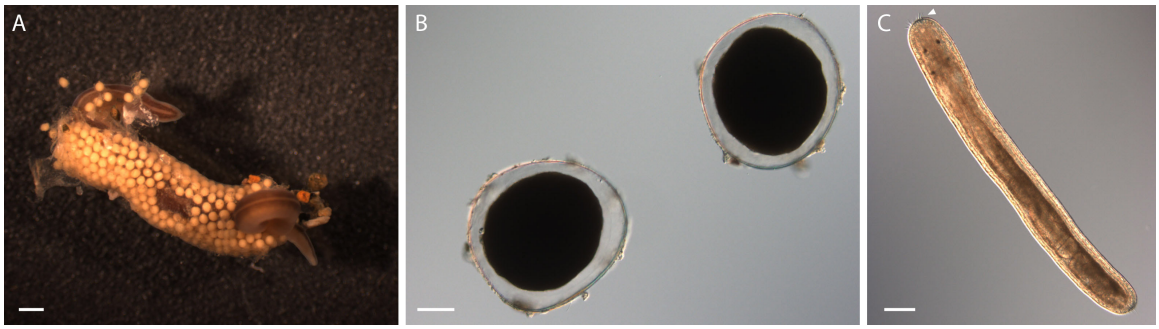


Figure 4.51. Adult *Tetrastemma bilineatum*, collected in March 2013 and surrounded by yellow embryos (A). When collected in March, embryos had polar bodies (9 o'clock on left embryo, B) and after several days developed into hoplonemertean larvae (C). Note hair-like anterior projections (arrowhead) and two pairs of eyes (C). Scale bars 1.0 mm (A) and 100 µm (B–C).

There are six species in the hoplonemertean genus *Tetrastemma* reported from central CA to OR (Roe et al. 2007). Of those, we have sequence data for three species (*T. albidum* and *T. candidum* and *T. bilineatum*). *Tetrastemma bilineatum*, with two distinct longitudinal brown lines, is morphologically differentiable from the other species. The development is known for several *Tetrastemma* (or *Quasitetrastemma*, Chernyshev 2008) species including the encapsulated development of *T. candidum* (Maslakova and Malakhov 1999) and the free-swimming lecithotrophic development of *T. vermiculum* (Lebedinksii 1898 in Chernyshev 2008), *Q. nigrifrons* and *Q. stimpsoni* (Chernyshev 2008). The embryos of *Tetrastemma bilineatum* are yellow and brooded in a parchment-like cocoon which surrounds the adult body. Brooding adults were found under rocks on a sandy beach (North Spit) in March 2013. The ciliated embryos were surrounded by a thick chorion and free-swimming larvae emerged after several days. These seemingly lecithotrophic larvae have two pairs of eyes and a tuft of several ciliary cirri rather than a single blade-like apical tuft at the anterior end.

Ototyphlonemertes

The larvae of the genus *Ototyphlonemertes* are easy to recognize by the presence of statocysts positioned in the posterior region of ventral cerebral ganglia (Envall and Norenburg 2011). *Ototyphlonemertes* are believed to have short-lived larvae dependent on yolk reserves (Norenburg and Stricker 2002; Andrade et al. 2011b) and they are found in near-shore coastal plankton (Chernyshev 2000). The larvae of three *Ototyphlonemertes* species were described from the Sea of Japan (Chernyshev 2000) including *O. martynovi*, *O. aurita* and *O. Norenbugia* sp.

***Ototyphlonemertes* sp. 1 (Fig. 4.52)**

Larvae collected: Dec 2011

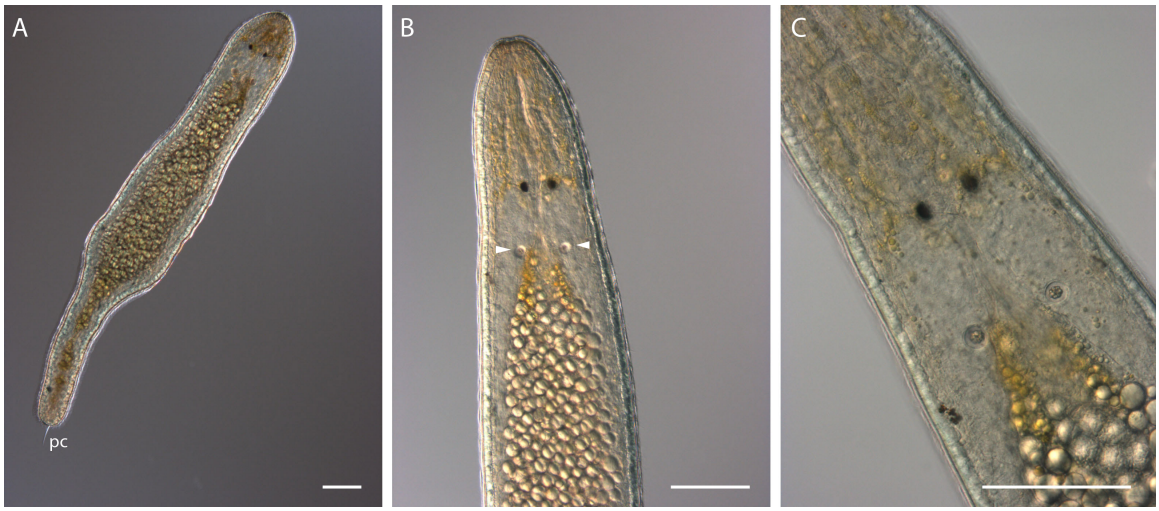


Figure 4.52. Wild-caught larva of *Ototyphlonemertes* sp. 1 collected in October 2013. Note posterior cirrus (pc, A), black eyes and statocysts (arrowheads, B) with polystatoliths inside (C). Scale bars 100 µm.

We found the larvae of an unknown *Ototyphlonemertes* species in plankton samples in Coos Bay. They are very fast swimmers (as has been seen for *O. martynovi*, Chernyshev 2000) and trapping them between a cover slip and microscope slide is not an easy task. They have two black eyes, which are lost in adults. The most distinctive character of these *Ototyphlonemertes* larvae are two spherical statocysts, each containing a polystatolith (a statolith consisting of more than 12 granules). The statolith shape is not

unlike that of the locally reported *O. americana* (Roe et al. 2007). Note, however, that *Ototyphlonemertes* adults are small, mesopsammic, and thus easily overlooked, and there are likely many more species locally, and our larvae could easily belong to an undescribed species (Envall and Norenburg 2011). We have collected the larvae of a single *Ototyphlonemertes* species, their gut is always packed with golden lipid granules and, at their posterior, they have a caudal cirrus. They have a rounded anterior and no prominent apical tuft. The sizes can range dramatically between these larvae, suggesting that they may be feeding in the plankton. Although hoplonemerteans are supposedly non-feeding and lack the ability to feed, recent evidence based on DNA sequence data of gut contents and variations in conspecific larval size, suggests that some species may be feeding while in plankton (Maslakova and Hiebert 2014).

DISCUSSION

We identified the larvae of 36 nemertean species, and four of those are species previously unknown to this region (see below). The most surprising result of this work, however, is that 19 species we collected as larvae cannot be matched to any known species. It is likely that at least some of these larvae represent species that are completely new to science or previously unknown to this area. This is particularly intriguing because the nemertean fauna in the NE Pacific, unlike other regions of the world, is believed to be well characterized (Stimpson 1857; Coe 1899, 1901, 1904, 1905, 1940, 1943; Griffin 1898; Corrêa 1964; Schwartz 2009). It is noteworthy that there are nine nemertean species thought to occur in our biogeographic region (based mostly on reports from adjacent geographic regions, Roe et al. 2007), for which we have yet to find adults and obtain sequence data. Thus, some of our so-called orphan larvae may not be orphans at all, but may be the larvae of species currently not in our reference database. Nevertheless, at least ten larval morphospecies and potentially as many as 19 represent new diversity. Below we discuss this new diversity and suggest reasons for the surprising amount we discovered in a supposedly well characterized region.

Many nemerteans are broadcast spawners, potentially releasing thousands to millions of oocytes during spawning. This suggests that subsequent planktonic larvae should greatly outnumber their benthic adults. We may encounter some species more frequently as larvae in the plankton than as adults because there are simply more larvae and the adults are, at times, more difficult to collect (e.g., subtidal species). For example, we are yet to observe wild-caught larvae of some of the most common intertidal nemertean species in the area (e.g., *Micrura wilsoni*). This emphasizes how DNA barcoding of both adult and larval life-history stages produces more accurate biodiversity assessments than sampling one stage only (see also Barber and Boyce 2006). In one palaeonemertean genus (*Cephalothrix*), we found six species where previous assessments (based on benthic adults only) indicated two; two species we currently only find as adults and the remaining four species we have only found as larvae in the plankton. Although more sampling is required to describe specifically which nemertean species are found on the southern Oregon coast, our attempts to identify larvae have provided us with a better estimate of how many species we should expect to find.

Much of the new diversity we have found is in the form of new and undescribed species, but we have also discovered four species which are well described but previously unreported from this region. We have found larvae that match species previously reported from western Russia (*Gurjanovella littoralis*) and several from the northwest Pacific (e.g., *Hubrechtella juliae, Carinoma hamanako*). How can we confirm these species-level matches over such a wide geographic range?

Current limitations in nemertean taxonomy (e.g., few characters of adult morphology and cryptic diversity) compromise public repositories of DNA sequence data (i.e., a BLAST match to a species should be taken with a grain of salt). Based on sequence analyses of nemertean genera, we have found that individuals by the same name from two different regions are not always the same species (e.g., *Cephalothrix*, Chen et al. 2010; Chapter II, this study). We have also observed naming discrepancies (i.e., cryptic diversity) among

specimens collected from the same geographic region. Two DNA sequences from species identified as *Carinoma tremaphoros*, both collected in Florida (JF277601 and AJ436833), have sequence divergences revealing that they are not the same species at all (12.2% (16S) and 14.7% (COI)). While two DNA sequences that match closely are likely the same species, the name of that species may be less clear. Many of the larvae we have matched to adults are species we find locally and we are confident that they are the larvae of the species in question. The species we find as larvae having been previously reported from the northwest Pacific can be confirmed with larval morphology (*Hubrechtella* and *Carinoma* have distinct larvae) or adults in close proximity. One species, *G. littoralis*, we have also collected as adults in Friday Harbor, WA and the presence of their larvae in southern Oregon confirms them as members of northeast Pacific nemertean fauna.

Questions often arise as to why we see the larvae of species currently only known to the NW Pacific here in the NE Pacific. Coos Bay and other NE Pacific bays are major shipping hubs. Large ships leave Coos Bay loaded with stripped fir trees or wood chips, head across the Pacific, and return with ballast water. Current regulations (Oregon Revised Statute 783.620-640; Murphy et al. 2004) require that ships conduct mid-oceanic ballast exchanges such that species cannot be transported from one bay to another. However, it is known that many species have been transported around the world this way before these regulations were in place (Carlton and Geller 1991; Carlton and Cohen 2007) and nemerteans are no exception. It is unlikely that the larvae we collect are directly derived from ballast water for a several reasons. First, when we find larvae, we usually find them repeatedly and seasonally. Next, in many instances, larvae identified to species from the NW Pacific were too young to have traveled 5,500 miles by boat, a trip taking typically around two weeks by ship. Finally, the likelihood of finding a single larva from a ballast of, at most, 100,000 tons diluted into Coos Bay, the largest Bay in Oregon, is very low. While it is unlikely we would encounter larvae from ballast directly, it is possible that nemertean species were established in Coos Bay by this mechanism.

Without careful observation and documentation, once species are established in a region, one cannot know if they were transported from elsewhere or vise versa. A taxonomic description (i.e., type locality) from the NW Pacific does not imply place of origin and instead, often reflects the first location in which a species was found. Species invasions and populations dynamics are particularly difficult to measure without an appropriate baseline of biodiversity from which to begin. This emphasizes the importance of accurate biodiversity assessments, which we show are best achieved by sampling all life-history stages. The fact that the nemertean fauna in the NE Pacific was believed to be well known was the very motivation for this project of identifying, the less well known, nemertean larvae. By including the often-overlooked larval stage we now know that the nemertean fauna was not as well known as was believed and suggest that the same may be true for other marine invertebrate phyla with bi-phasic life histories. In the face of species invasions and climate change, our understanding of community structure and adaptability is undermined by the fact that the majority of marine eukaryotic diversity remains undescribed (Mora et al. 2011; Appeltans et al. 2012). This emphasizes the need for rapid and accurate assessments of diversity, which are best achieved using the two-pronged approach we describe here.

BRIDGE TO CHAPTER V

Chapter IV is an identification guide for the larvae of nemertean species collected on the southern Oregon coast, in the northeast Pacific. It is also available online (www.nemerteanlarvalid.com). Chapters V and VI are each examples of larval identification using traditional embryology (rather than DNA barcoding, which dominated in Chapter IV), including raising wild-caught larvae through metamorphosis and rearing larvae from identified adults. In Chapter V, we identify the unique pilidium larva called pilidium recurvatum, in part, by the morphology of newly metamorphosed juveniles. We reveal that these larvae belong to the genus *Riserius* as well as the surprising preferred prey choice of metamorphosed juveniles and young adults.

CHAPTER V

THE PECULIAR NEMERTEAN LARVA *PILIDIUM RECURVATUM* BELONGS TO *RISERIUS* SP., A BASAL HETERONEMERTEAN THAT EATS *CARCINONEMERTES ERRANS,* A HOPLONEMERTEAN PARASITE OF DUNGENESS CRAB

This chapter is a co-authored publication which was published in *Invertebrate Biology* (2013, 132:207-225). with authors, G von Dassow, L Hiebert and SA Maslakova. G von Dassow contributed photo- and video-microscopy, collecting and rearing larvae, raising juveniles, observing the first *Carcinonemertes errans* larva within the gut of a *Riserius* juvenile, and final manuscript edits. L Hiebert contributed by virtue of enrollment in SA Malsakova's Marine Molecular Biology course at the OIMB during the fall term of 2008, where she participated in a group project that collected and identified the first *pilidium recurvatum* larva with DNA sequence data. SA Maslakova contributed to this work by organizing the over arching project, photo-microscopy, collecting, rearing and preserving larvae, and preliminarily identified the newly metamorphosed *Riserius* juveniles based on morphology. My contribution to this project involved larval collection, rearing, preservation, and identification by DNA sequence data. Confocal microscopy was carried out by SA Maslakova and myself. I raised several juveniles following metamorphosis for over one year, conducted all sequence editing, alignment, and accompanying phylogenetic analyses as well as contributed sequences to GenBank.

INTRODUCTION

Most nemerteans (phylum Nemertea, ribbon worms) have a biphasic life cycle with a benthic adult and a planktonic larval stage (Norenburg and Stricker 2002). The pilidium is a charismatic long-lived planktotrophic larval form found in one clade of nemerteans called, for that reason, the Pilidiophora (Thollesson and Norenburg 2003; Maslakova 2010a). The pilidium is remarkable for its mode of development (via imaginal discs) and catastrophic metamorphosis in which the emerging juvenile rapidly devours the larval

body (Cantell 1966a; Lacalli 2005; Maslakova 2010b; Maslakova and von Dassow 2012). These telltale larvae are frequently encountered in plankton samples and have many distinct morphotypes (e.g., Dawydoff 1940; Cantell 1969; Lacalli 2005) and yet the species-, genus-, or even family-identity of most of these larvae remains a mystery, because the development of most nemerteans is undescribed (e.g., Johnson 2001).

Originally described in 1847 by the German anatomist and physiologist Johannes Müller, a typical pilidium looks like a transparent deer-stalker cap with ear flaps pulled down (Fig. 5.1A). Although he suspected that it may be the larva of some animal, Müller did not know that pilidium is the larva of a nemertean worm, and he originally assigned it a binomen *pilidium gyrans* (in reference to its hat-like shape and rotating swimming motion) (Müller 1847). Since then many other pilidial morphotypes[1] have been described based on unidentified specimens collected from plankton, and, following Müller's lead, assigned various binomena that reflect their distinct morphology – e.g., *pilidium auriculatum* (Leuckart and Pagenstecher 1858), *pilidium recurvatum* (Fewkes 1883), *pilidium depressum* (Dawydoff 1940).

Pilidium recurvatum is one of the most remarkable pilidial morphotypes. If a typical pilidium looks like a hat, *pilidium recurvatum* looks more like an athletic sock, swimming heel first and toe trailing behind (Fig. 5.1B). *Pilidium recurvatum* was discovered by Walter Fewkes in 1883 in a plankton sample taken off the coast of Rhode Island (Fewkes 1883, Fig. 5.2A) and named in reference to the characteristic curvature of its anterior end. Similar morphotypes have since been reported from other parts of the world: the Bay of Nha Trang, Vietnam (Dawydoff 1940, Fig. 5.2B), Güllmarfjord, Sweden (Cantell 1966a, Fig. 5.2C), the Sea of Japan, Russia (Chernyshev 2001, Fig. 5.2D) and the NE Pacific off Washington and Oregon (Schwartz 2009; Maslakova 2010a). Fewkes (1883) believed *pilidium recurvatum* to be the larva of a heteronemertean from the genus *Lineus* (Fam. Lineidae) and, later, Cantell speculated that this larva may belong to the heteronemertean family Baseodiscidae (1966a) or

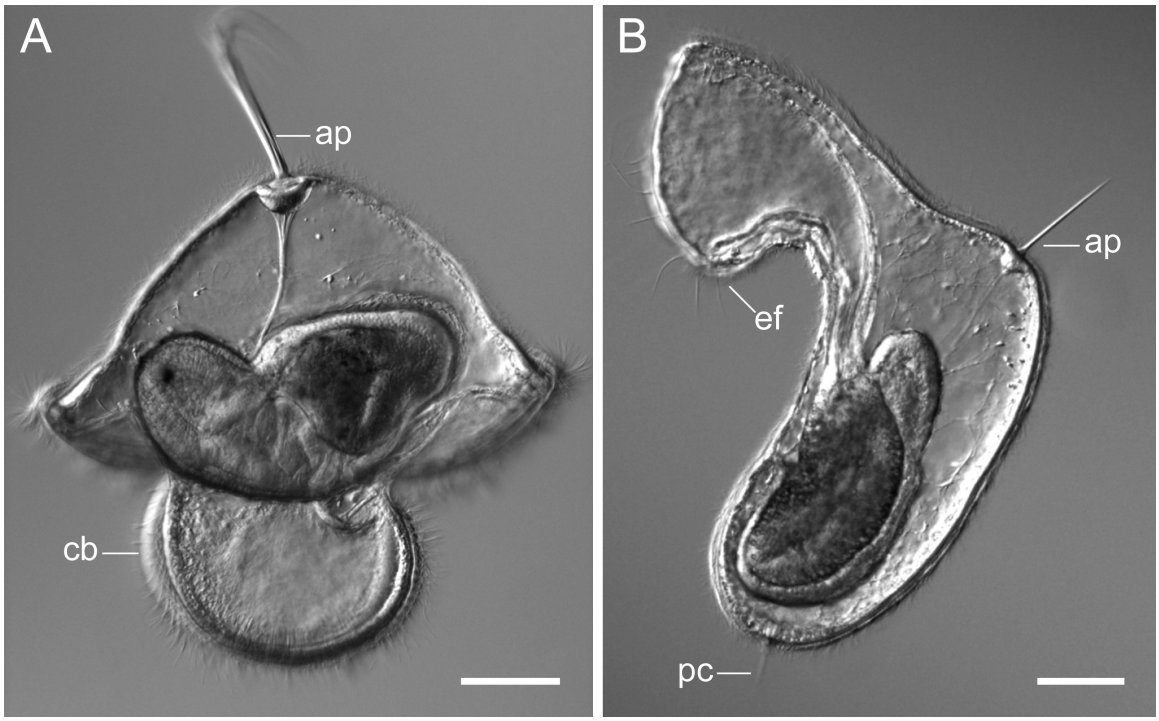


Figure 5.1. Overall morphology of the typical hat-like pilidium larva (A) and *pilidium recurvatum* from Oregon (B). A. A typical pilidium larva from Coos Bay plankton with a prominent apical tuft (ap) at the larval anterior end and ciliated band (cb) spanning the lobes and lappets at the larval posterior end. The anterior-posterior axis of the developing juvenile, is perpendicular to that of the larva (eyes mark the juvenile anterior). B. A sock-like *pilidium recurvatum* larva from Coos Bay plankton. The larval anterior is marked with the apical tuft (ap), and the larval posterior with a small cirrus (pc). The anterior-posterior axis of the developing juvenile inside is parallel to that of the larval body. The prominent larval esophageal funnel (ef) is extended perpendicular to the larval/juvenile anterior-posterior axis, and lacks a prominent ciliated band along its margin. Scale bars 100 μm.

Valenciniidae (more specifically the genus *Oxypolella*) (1966b), but until now the identity of *pilidium recurvatum* remained undetermined.

Because of its distinctive morphology and the orientation of the juvenile anterior-posterior axis with respect to the larval axes, Gösta Jägersten suggested, in his monograph on the Evolution of the Metazoan Life Cycle (1972), that *pilidium recurvatum* may represent an evolutionary intermediate between the juvenile-like planuliform nemertean larva, found in non-pilidiophoran nemerteans and presumed to be ancestral for the phylum (reviewed in Maslakova 2010a), and the typical pilidium.

Evaluating this hypothesis depends on being able to determine the phylogenetic position of *pilidium recurvatum.*

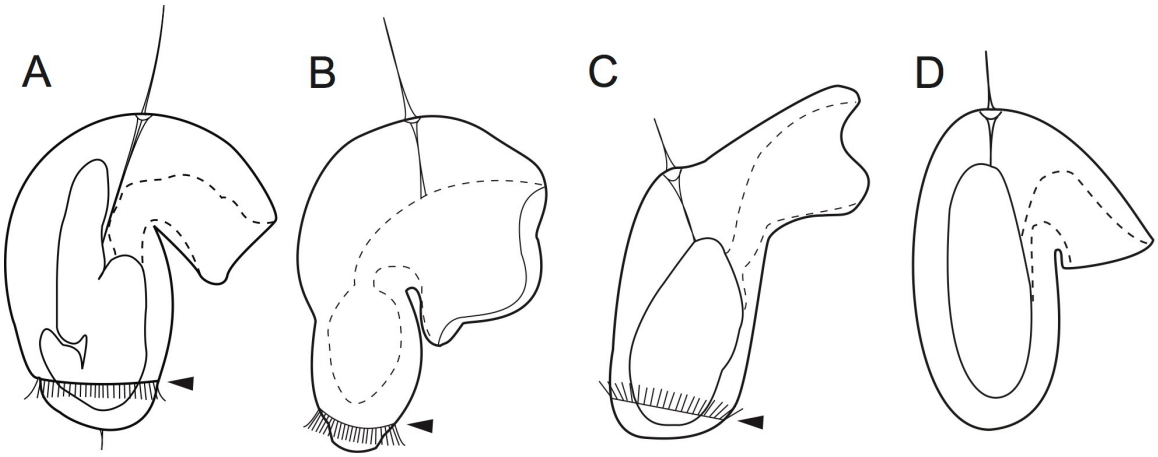


Figure 5.2. Diagrams highlighting the morphology of the previously described *pilidium recurvatum*-like larvae. A. *Pilidium recurvatum* from the northeast Atlantic (after Fewkes 1883); B. *Pilidium incurvatum* from the Bay of Nha Trang, Vietnam (after Dawydoff 1940); C. *Pilidium recurvatum* from Gullmarfjord, Sweden (after Cantell 1966a); D. *Pilidium recurvatum* from the Sea of Japan (after Chernyshev 2001). Note presence of a posterior transverse ciliated band in *pilidium recurvatum* from the northeast Atlantic, Bay of Nha Trang and Gullmarfjord (arrowhead in A, B, C).

Here we use a combination of mtDNA sequence data and juvenile morphology to reveal the identity and phylogenetic position of *pilidium recurvatum* from Oregon: it is the larva of *Riserius*, previously a monotypic genus within the order-level taxon Pilidiophora. We document with video-microscopy the catastrophic metamorphosis of *pilidium recurvatum*, and reveal, for the first time, its morphology by use of confocal microscopy. We also report our surprising discovery of the prey choice of *pilidium recurvatum* juveniles – they preferentially fed on the larvae and juveniles of *Carcinonemertes*, a hoplonemertean predator on crab eggs - which may be relevant to the biology of the Dungeness crab, a commercially harvested species.

METHODS

Collecting and maintaining live larvae

Pilidium recurvatum were collected opportunistically from plankton samples taken from the Charleston Marina docks in Charleston, OR using a plankton net with 153 μm mesh. We found one individual of the *pilidium recurvatum* morphotype on each of October 09 and 30 in 2008, one or two in Spring of 2009, and several individuals (< 10) on October 11 and November 01 in 2011. The largest number of individuals (~50) were encountered in samples from 14-17 August in 2012, and small numbers of these larvae were present in the plankton samples taken from August to October in 2012. All individuals were photographed, and some were preserved for DNA extraction or confocal microscopy. The most developmentally advanced larvae were maintained live in 150 ml bowls with filtered seawater (FSW, 0.45μm) in a sea table with flow-through seawater (9-12˚C) to document metamorphosis and juvenile morphology. We were able to prompt and observe metamorphosis in some of these individuals by compressing them gently between a glass slide and a coverslip supported over the slide using small clay feet.

Larvae of *Micrura alaskensis* COE 1901 used here for comparison to illustrate the morphology of a typical pilidium were reared from eggs fertilized *in vitro* as previously described (Maslakova 2010b). The larva depicted on Fig. 5.1A was collected from plankton in Coos Bay, OR on December 5, 2011 and has been identified by DNA sequence as likely belonging to one of the local *Lineus* spp. (e.g., *L. rubescens* COE 1904, *L. pictifrons* COE 1904, or an undescribed species).

Photo- and videomicroscopy

Live larvae were photographed on an Olympus BX51 microscope equipped with DIC using either a Leica DFC400 or a Grasshopper2 (Point Grey Research) camera. Video recordings of metamorphosis were done on the same microscope using a Grasshopper2 camera and Astro IIDC v. 4.07 imaging program.

DNA extraction and PCR amplification

Individual live larvae were photographed and cryopreserved (-80˚C) in a small drop of sea water. DNA from larval samples was extracted using a chelex-based method (InstaGene, BioRad). To obtain reference sequences from the local species, *Micrura wilsoni* COE 1904*,* we collected adult specimens from Middle Cove, Cape Arago, OR in September 2009 and gathered tissue samples from which we extracted DNA (ethanol-preserved) using DNEasy Blood and Tissue Kit (Qiagen). We amplified two mitochondrial gene regions: ~ 460 bp fragment of the large subunit ribosomal DNA (16S), and a 658 bp "barcoding" region of the cytochrome *c* oxidase subunit I (COI), and, in one case, also a ~ 300 bp region of the nuclear gene encoding histone H3. We used previously published "universal" primers: 16SArL [5‘ CGCCTGTTTATCAAAAACAT 3’] and 16S BrH [5’ CCGGTCTGAACTCAGATCACGT 3’] (Palumbi et al. 1991) for 16S rDNA; LCO 1490 [5’ GGTCAACAAATCATAAAGATATTGG 3’] and HCO 2198 [5’ TAAACTTCAGGGTGACCAAAAAATCA 3’] (Folmer et al. 1994) for COI; and H3NF [5’ ATGGCTCGTACCAAGCAGAC 3’] and H3R [5’ ATATCCTTRGGCATRATRGTGAC 3’] (Clogen et al. 2000) for histone H3. In several instances, nemertean-specific primers designed and kindly provided to us by Dr. Jon Norenburg (Smithsonian Institution) proved to be more successful for COI amplification (especially when used in combination with the Folmer primers): COIDr [5’ GAGAAATAATACCAAAACCAGG 3’] and COILf [5’ TTTCAACAAATCATAAAGATAT 3’]. PCR thermocycling was carried out as follows using 8 µl of undiluted template DNA: 95˚C for 2 min; 35 cycles of 95˚C for 40 s, 45-55˚C (45-52˚C for COI, 52˚C for 16S and H3) for 40 s. 72˚C for 1 min; followed by a 2 min final extension at 72˚C. PCR products were purified using Wizard SV Gel and PCR Cleanup kit (Promega) and sent to Sequetech (Mountain View, CA) to sequence in both forward and reverse directions using PCR primers.

Sequence analysis

Sequences were proofread and trimmed in Codon Code Aligner v. 3.7.1 (Codon Code Corp, MA). To determine the identity of *pilidium recurvatum* we conducted phylogenetic analyses including all of the 16S rDNA sequences obtained from the larval samples, as well as several of the local nemertean species, and GenBank sequences from a broad range of pilidiophoran taxa (see Table 5.1 for accession numbers). All sequences were aligned using ClustalX v. 2.1 (Larkin et al. 2007) with default gap penalties. We conducted a neighbor-joining analysis using ClustalX. Uncorrected pairwise distances were calculated for all larval samples using PAUP* v. 4b1.0 (Swofford 2002). Phylogenetic analysis using parsimony was also carried out in PAUP* using heuristic search (random sequence addition with 1000 replicates, 10 best trees held at each step, and TBR branch-swapping algorithm). Clade support was estimated using 1000 bootstrap replicates (Felsenstein 1985). Phylogenetic analysis using Bayesian inference was carried out using MrBayes v. 3.2.1 (Ronquist et al. 2012) with evolutionary model parameters determined with jModelTest v. 2.1 (Posada 2008) using Akaike and Bayesian Information criteria. Both criteria determined the Tamura-Nei (1993) evolutionary model (gamma distributed with invariant sites) to be most appropriate for our data. Markov chain Monte Carlo (MCMC) was set for 10^6 generations, sampling every 100 generations with the first 25% of trees discarded as burn-in. Tree topologies were viewed using FigTree v. 1.3.1 (Rambaut 2009).

Antibody labeling and confocal microscopy

Ten *pilidium recurvatum* larvae were relaxed in a 1:1 mixture of 0.34 M $MgCl_2$ and filtered seawater (0.45µm, FSW) for approximately 30 minutes prior to fixation. Larvae were preserved in 4% paraformadlehyde (made up in FSW from 20% ultrapure paraformaldehyde, Electron Microscopy Sciences) for 1 hr at room temperature (RT). Fixative was removed by a few quick (<1 min) washes in phosphate buffered saline (PBS, Fisher Scientific). Larval tissues were permeabilized in PBS with 0.1% Triton X-100 and 0.1% Bovine Serum Albumin (PBT+BSA) in three consecutive 10-min

Table 5.1. Classification, accession numbers and references for sequences used in the 16S phylogenetic analysis.

Nemertea, Anopla, Heteronemertea		
Baseodiscus delineatus	EF124860	Schwartz and Norenburg, unpublished
Baseodiscus hemprichii	EF124862	Schwartz and Norenburg, unpublished
Baseodiscus mexicanus	EF124863	Schwartz and Norenburg, unpublished
Baseodiscus quinquelineatus	EF124864	Schwartz and Norenburg, unpublished
Baseodiscus unicolor	EF124865	Schwartz and Norenburg, unpublished
Carinoma mutabilis	AJ436832	Thollesson and Norenburg 2003
Cerebratulus lacteus	JF277575	Andrade et al. 2012
Cerebratulus marginatus	AJ436821	Thollesson and Norenburg 2003
Cerebratulus montgomeryi	EF124875	Schwartz and Norenburg, unpublished
Dushia atra	EF124878	Schwartz and Norenburg, unpublished
Evelineus tigrillus	EF124879	Schwartz and Norenburg, unpublished
Lineus acutifrons	JF277573	Andrade et al. 2012
Lineus alborostratus	AJ436822	Thollesson and Norenburg 2003
Lineus bicolor	AJ436823	Thollesson and Norenburg 2003
Lineus bilineatus	JF277571	Andrade et al. 2012
Lineus longissimus	AJ436825	Thollesson and Norenburg 2003
Lineus torquatus	JF277572	Andrade et al. 2012
Micrura akkeshiensis	EF124887	Schwartz and Norenburg, unpublished
Micrura alaskensis	AJ436827	Thollesson and Norenburg 2003
Micrura callima	EF124889	Schwartz and Norenburg, unpublished
Micrura fasciolata	JF277585	Andrade et al. 2012
Micrura purpurea	JF277577	Andrade et al. 2012
Micrura verrilli	EF124899	Schwartz and Norenburg, unpublished
Micrura wilsoni	-	Maslakova, unpublished
Notospermus geniculatus	AJ436824	Thollesson and Norenburg 2003
Oxypolella alba	AF103767	Sundberg and Saur 1998
Parborlasia corrugatus	AJ436829	Thollesson and Norenburg 2003

washes. To block non-specific labeling, larvae were incubated in 5% Normal Goat Serum in PBT+BSA for 2 h at RT. To reveal the structure of the serotonergic nervous system larvae were incubated in rabbit anti-serotonin (1:500) primary antibody (Immunostar, Cat# 20080) at 4˚C for 48 hours, followed by three 10-min washes in PBT+BSA, and labeling with Alexa Fluor 488 goat anti-rabbit secondary antibody (1:200) for 2 hr at RT. To help visualize the cell nuclei and the muscles, larvae were additionally labeled with Hoechst 33342 (2 μM) and Rhodamine Phalloidin (Sigma, 165 nM in PBT+BSA). Fluorescently labeled larvae were washed in PBS, and imaged in PBS or 90% glycerol in glass coverslip-bottomed Petri dishes (MatTek). Glycerol-mounted larvae were noticeably more clear. Confocal stacks of 0.5-0.75 μm sections were obtained using an Olympus FluoView 1000 confocal system on an Olympus IX81 inverted microscope equipped with UPlanSApo 20x0.85 NA oil lens. Z-projections were reconstructed using ImageJ v. 1.46 (Wayne Rasband, National Institutes of Health, Bethesda, MD) and overlaid and false-colored in Adobe Photoshop (CS3).

For comparative purposes we included confocal images from pilidium larvae of *Micrura alaskensis*. Antibody labeling was carried out as described above, larvae were mounted in Vectashield (Vector Laboratories) and confocal stacks were obtained at the Friday Harbor Laboratories (University of Washington) using BioRad Radiance 2000 laser scanning confocal system mounted on a Nikon Eclipse E800 microscope with a 40X 1.3 N.A. oil lens. Images were processed in ImageJ and Adobe Photoshop as described above.

RESULTS

Larval morphology and behavior

Pilidium recurvatum from Oregon has an elongated transparent body and a large recurved "trunk" or esophageal funnel (Figs. 5.1B, 5.3). The larva is equipped with a prominent apical tuft at the anterior end and a small ciliary cirrus at the posterior end (Fig. 5.3C). The apical plate is connected to the developing juvenile by an apical muscle (Fig. 5.3C), which can be more or less conspicuous depending on the individual (its developmental

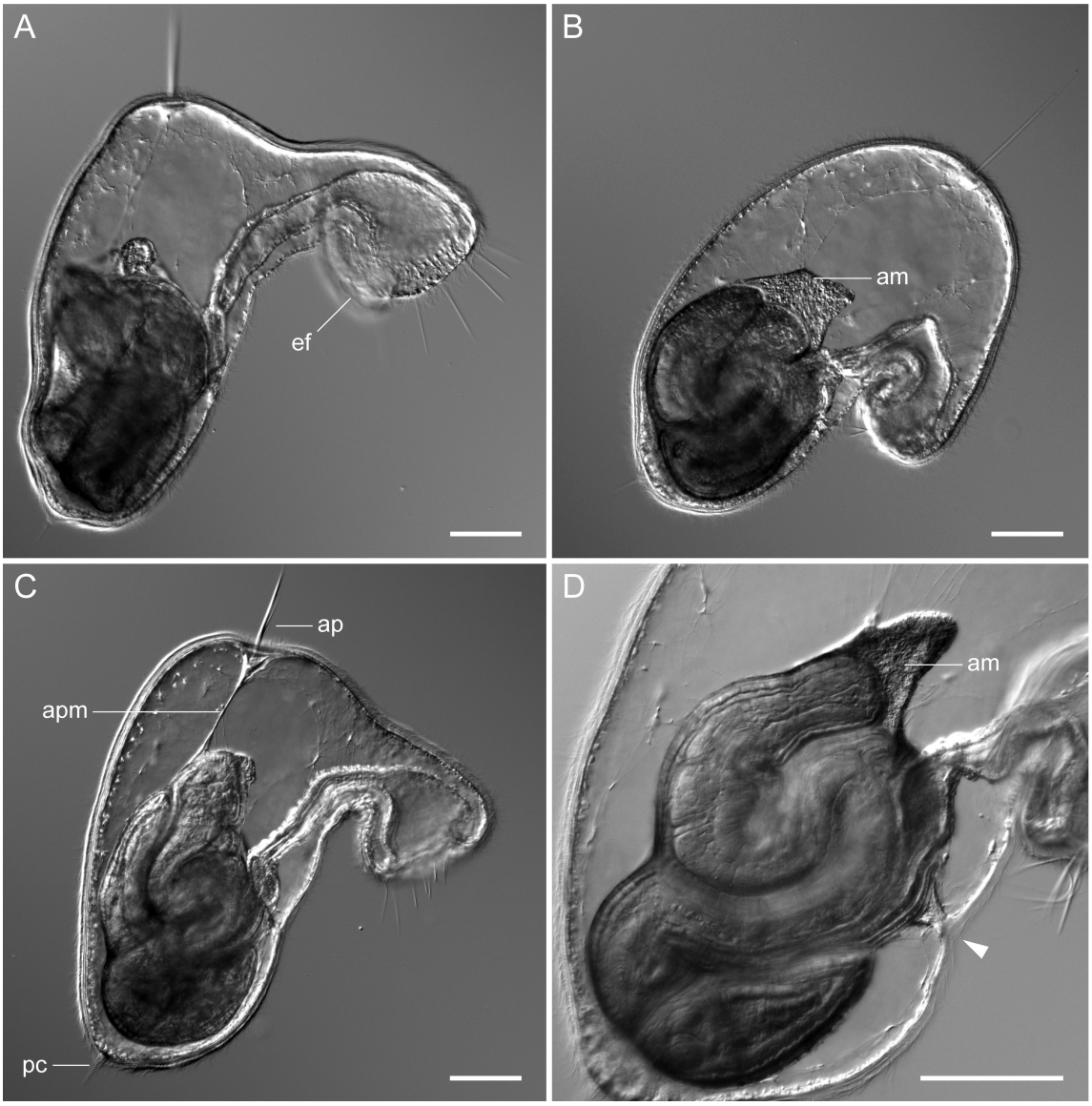
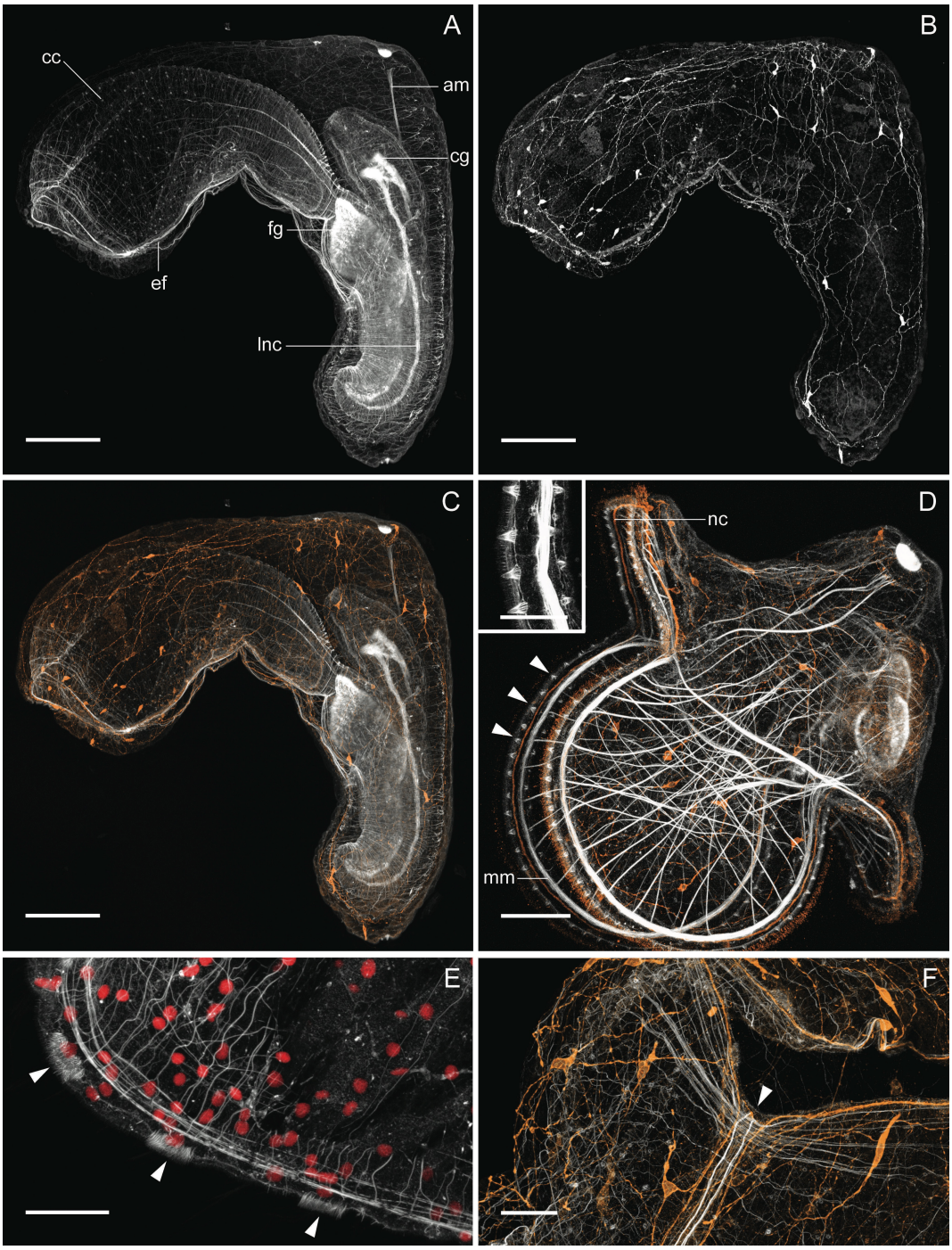


Figure 5.3. Morphology and behavioral postures in *pilidium recurvatum* larvae from Coos Bay, OR. A. 'Feeding posture' in *pilidium recurvatum.* Note several stiff ciliary cirri along the margin of the esophageal funnel (ef). B. The 'swimming posture', in which the esophageal funnel is contracted. Note the coiled juvenile nemertean inside the thin amniotic membrane (am). C. An intermediate posture with esophageal funnel partially outstretched. Note the blade-like anterior apical tuft (ap) and posterior ciliary cirrus (pc). Connecting the apical tuft to the juvenile amnion is an apical muscle (apm). D. The juvenile amnion (am) opens to the outside by the ventral amniotic pore (arrowhead). Scale bars 100 μm.

stage and overall condition). The pilidial body is uniformly ciliated except for the distinct apical tuft, the posterior cirrus, and approximately 20 conspicuous (~ 100 μm long) ciliary cirri arranged along the margin of the esophageal funnel (Fig. 5.3A). *Pilidium recurvatum* larvae from Oregon lack the posterior transverse ciliary band, referred to as the telotroch (compare Figs. 5.1B and 5.2A-C).

Figure 5.4 (next page). Muscular and neural anatomy in *pilidium recurvatum* from Oregon (A-C, E-F), and a typical pilidium (D, inset), as revealed by confocal microscopy. Apical plate is up. A. Confocal z-projection of a *pilidium recurvatum* stained with phalloidin. Note the lack of a prominent muscle band, or the collar cells along the margin of the esophageal funnel (ef). On the other hand, what we think are numerous collar cells (cc), visible here as small dots, are scattered over the inner surface of the funnel. The juvenile foregut (fg), is brightly labeled likely due to the dense lining of microvilli. The juvenile cerebral ganglia (cg), lateral nerve cords (lnc) and apical muscle (am) are also highlighted with phalloidin. B. The same larva as on (A) labeled with anti-5HT antibody showing sub-epidermal serotonergic nerve network. Note the absence of a prominent serotonergic nerve cord along the margin of the esophageal funnel. C. Same larva as on (A) and (B). An overlay of phalloidin (grey) and 5-HT (orange) channels, shows the relative position of muscles and serotonergic neurons and fibers. D. A confocal projection of a conventional pilidium larva *(Micrura alaskensis)*, stained with phalloidin (grey) and anti-5HT antibody (orange). Note the prominent marginal muscle (mm) and nerve cord (nc) which correspond to the marginal ciliated band along the pilidial lobes and lappets. Also note regularly spaced prominent collar cells (arrowheads and inset) arranged along the marginal ciliated band. E. A close up view of the margin of the esophageal funnel in a *pilidium recurvatum* stained with phalloidin (grey) and Hoechst (red). Note the absence of collar cells found in a conventional pilidium (D, inset). Instead, there are somewhat wider spaced dense microvillar fields (arrowheads), each originating from a single cell (as evident from the arrangement of the nuclei). Based on the number and distribution of these microvillar fields, we think that they likely correspond to the marginal cirri (Fig. 5.3A). F. Ventral view of the funnel margin of *pilidium recurvatum* stained with phalloidin (grey) and anti-5HT antibody (orange) showing the ventral notch (arrowhead) and multiple fine muscle fibers and serotonergic nerves along the margin and the ventral groove of the funnel, as opposed to the single prominent muscle and nerve running along the marginal ciliated band in the typical pilidium (D). These morphological differences likely reflect the feeding strategies of the two larval types. Scale bars 100 μm (A, B, C, D), 25 μm (E, F) and 20 μm (D inset).


A
cc
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Neurons in the serotonergic nervous system of *pilidium recurvatum* are distributed within the larval epidermis, and their processes form a fine sub-epidermal network (Fig. 5.4B). Unlike in the typical pilidia (e.g., see Salensky 1912; Maslakova 2010b; Fig. 5.1A, here) there is no distinct ciliary band along the margin of the esophageal funnel of *pilidium recurvatum* (Figs. 5.1B, 5.3A-C). Accordingly, there is no prominent muscle strand (Figs. 5.4A, C, E, F) analogous to the one spanning lobes and lappets of a typical pilidium (Fig. 5.4D), nor a thick serotonergic nerve cord (Figs. 5.4B, C, F) present in a typical pilidium (Fig. 5.4D). Instead there are numerous fine muscle and nerve fibers (not unlike elsewhere in the body) running along the margin of the esophageal funnel (Fig. 5.4A-C, F).

The marginal ciliary band of a typical pilidium possesses several rows of what we refer to as collar cells, each equipped with a single stationary cilium (not shown) supported by a cone of microvilli (Fig. 5.4D, inset). These collar cells, which are spaced out regularly along the margin of lobes and lappets (at a distance of about 8 μm) likely serve to detect food particles (GvD, SM, pers. obs.). The margin of the esophageal funnel in *pilidium recurvatum* lacks these characteristic collar cells (Fig. 5.4E). Instead, the margin of the esophageal funnel bears the long cirri (Fig. 5.3A), each composed of multiple cilia (not shown) that originate from a single cell (judging from the number of associated nuclei on Fig. 5.4E) and are supported by a 10-12 μm wide field of microvilli. These fields of microvilli are separated from each other by a distance of approximately 25-35 μm (Fig. 5.4E). At the same time, we noticed what appear to be collar cells dispersed throughout the inner surface of the esophageal funnel (Fig. 5.4A) of *pilidium recurvatum*. Accordingly, we noticed many single stationary cilia inside the funnel (not shown), similar to those originating from the collar cells in a marginal ciliary band of a typical pilidium.

We observed two distinct postures or behaviors in *pilidium recurvatum* from Oregon, to which we provisionally refer as the “swimming posture” and “feeding posture”. In the

swimming posture, the funnel is contracted and folded back against the larval body (Fig. 5.3B). Larvae in this posture swim apical organ forward while revolving along the antero-posterior axis. In the feeding posture, the funnel is dramatically expanded and positioned perpendicularly to the larval body, or flexed further toward the apical organ (Fig. 5.3A). Larvae in this posture do not swim, but slowly drift in the water column. In this posture the marginal cirri protrude stiffly outward, not unlike the hair-like projections of the Venus flytrap. This uncanny resemblance is further strengthened by the bi-lobed appearance of the *pilidium recurvatum* funnel, as the margin of the funnel bears a distinct ventral notch (Fig. 5.4F). The inner surface of the funnel is ciliated densely, but not entirely uniformly. There is a distinct tract of denser ciliation lining the ventral esophageal groove that originates deep within the funnel and terminates at the ventralnotch. Corresponding to this ciliary tract, there is a concentration of fine serotonergic nerve and muscle fibers running along the ventral esophageal groove (Fig. 5.4F). Although we introduced individuals of *pilidium recurvatum* to a variety of potential food items, including small phytoflagellates (*Rhodomonas* and *Dunaliella*), and larger prey, such as marine invertebrate larvae (molluscan veligers, echinoderm blastulas, bipinnarias and plutei, a variety of hoplonemertean larvae), euglenids, diatoms, and *Noctiluca,* none of these elicited a response. We also tried offering *pilidium recurvatum* natural plankton (excluding items larger than 100 μm), but did not observe any change in gut contents after an overnight trial. We have not yet observed actual feeding, or direct evidence thereof, in these larvae.

Catastrophic metamorphosis

The antero-posterior axis of the developing juvenile inside *pilidium recurvatum* coincides with the larval AP axis (Fig. 5.1B), unlike in a typical pilidium where the axes are approximately perpendicular (Fig. 5.1A). A juvenile approaching metamorphosis is long compared to the larval body and is, typically, coiled inside the amnion (Fig. 5.3), which opens to the outside via a short ciliated canal and a ventral pore (Fig. 5.3D). We observed metamorphosis in numerous individuals, and captured two on film. In all cases

that we observed, the posterior portion of the larval body dramatically contracted at the beginning of metamorphosis (Figs. 5.5A, B). The juvenile then emerged posterior end first by rupturing the ventral larval epidermis posterior to the amniotic pore (Fig. 5.5B). As the juvenile emerged, the amnion collapsed and appeared as a dark brown mass inside the larva (Fig. 5.5D). Once the juvenile head was out, the larval body was drawn into the juvenile mouth, at a considerable distance away from the anterior tip (Figs. 5.5D, E). Metamorphosis was complete within minutes in all but one of the instances that we witnessed and the entire larval body was swallowed by the juvenile worm (Fig. 5.5F). Although we did not witness metamorphosis in all collected individuals, we frequently noted dark brown gut contents in recently metamorphosed juveniles, which suggests that they ingest the larval body (including the amnion) as a matter of course (Fig. 5.5F). The juvenile epidermis was rather sticky and individuals often remained twisted on the bottom of culture dishes in a tight coil for several days after metamorphosis.

Juvenile morphology

Newly metamorphosed juveniles are approximately 1.5 mm and lack a caudal cirrus or lateral cephalic furrows. The other characteristic features of juvenile morphology include 1) an unusually long pre-oral region — about 40-50% of the total juvenile body length immediately after metamorphosis (Fig. 5.5D), 2) a pair of conspicuous cerebral organ pits (Fig. 5.6), and 3) a V-shaped transverse cephalic furrow located immediately posterior to the cerebral organ pits, but anterior to the mouth (Fig. 5.6). We found two distinct *pilidium recurvatum* morphotypes in Coos Bay, OR, easily differentiable by the presence or absence of juvenile eyes, which become apparent in advanced larvae (Fig. 5.7).

DNA sequence analysis

We obtained sequences of 16S and COI gene regions from ten and eight individuals of *pilidium recurvatum*, respectively (GenBank accession numbers KC777021 - KC777038). For the phylogenetic analyses we used all acquired *pilidium recurvatum* sequences, a sequence of *Micrura wilsoni* from Oregon (obtained by us), and a diverse

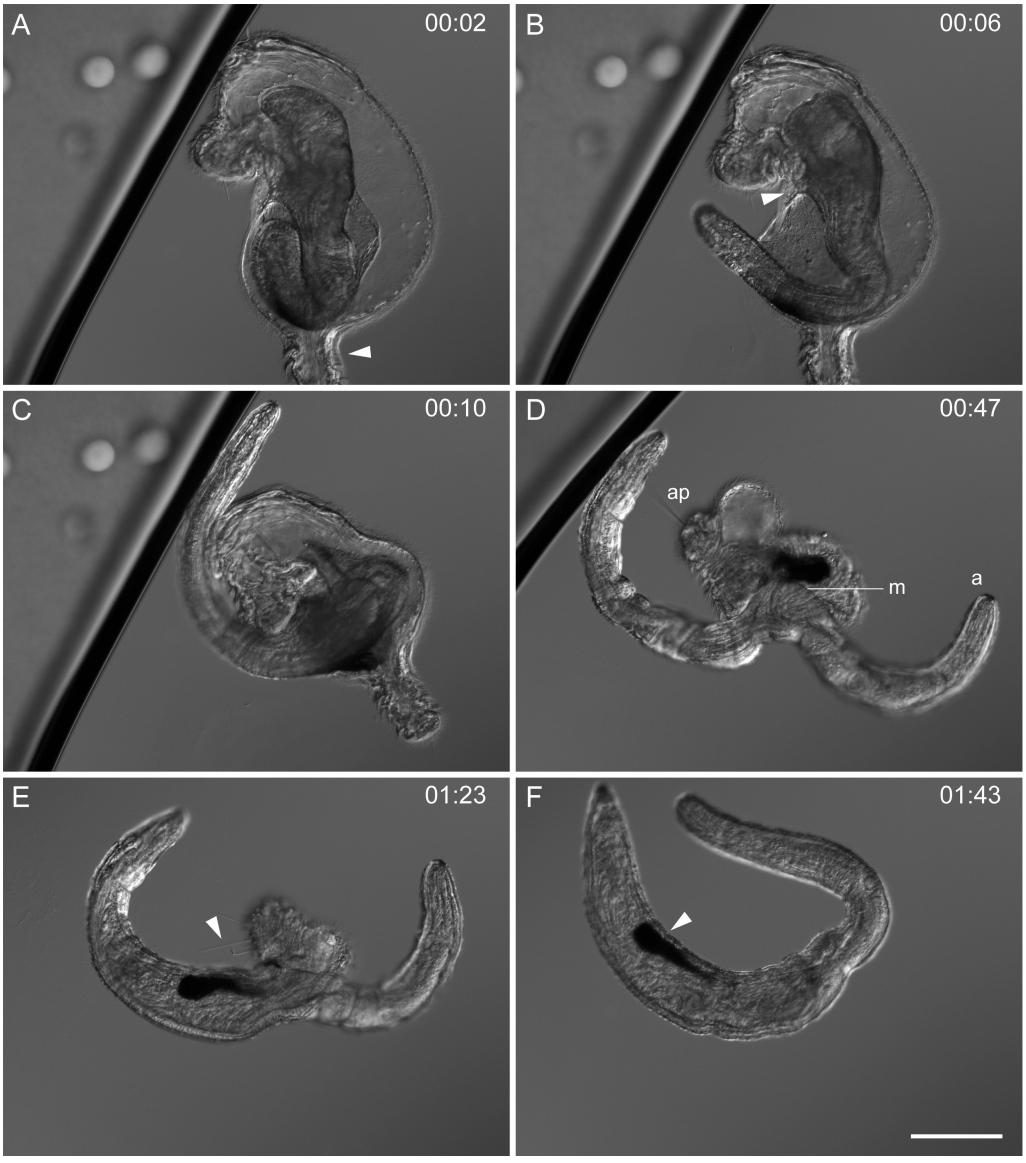

Figure 5.5. Metamorphosis in *pilidium recurvatum.* Time stamp (min:sec) is at upper right. A. Metamorphosis begins with the contraction of the larval posterior (arrowhead). B-C. The juvenile posterior end emerges first by rupturing the ventral larval epidermis posterior to the amniotic pore (arrowhead). D. The anterior end of the juvenile (a) emerges, and the juvenile begins to swallow the larval body, including the dark collapsed amnion. The margin of the mouth (m) and the larval apical tuft (ap) are sharply in focus. Note the characteristically long pre-oral end, and the lack of the juvenile caudal cirrus. E. The stiff ciliary cirri (arrowhead) arranged along the margin of the larval esophageal funnel are among the last structures to be ingested. F. The collapsed amnion (black body, arrowhead) can be seen inside the gut of metamorphosed juveniles. Scale bar 200 μm.

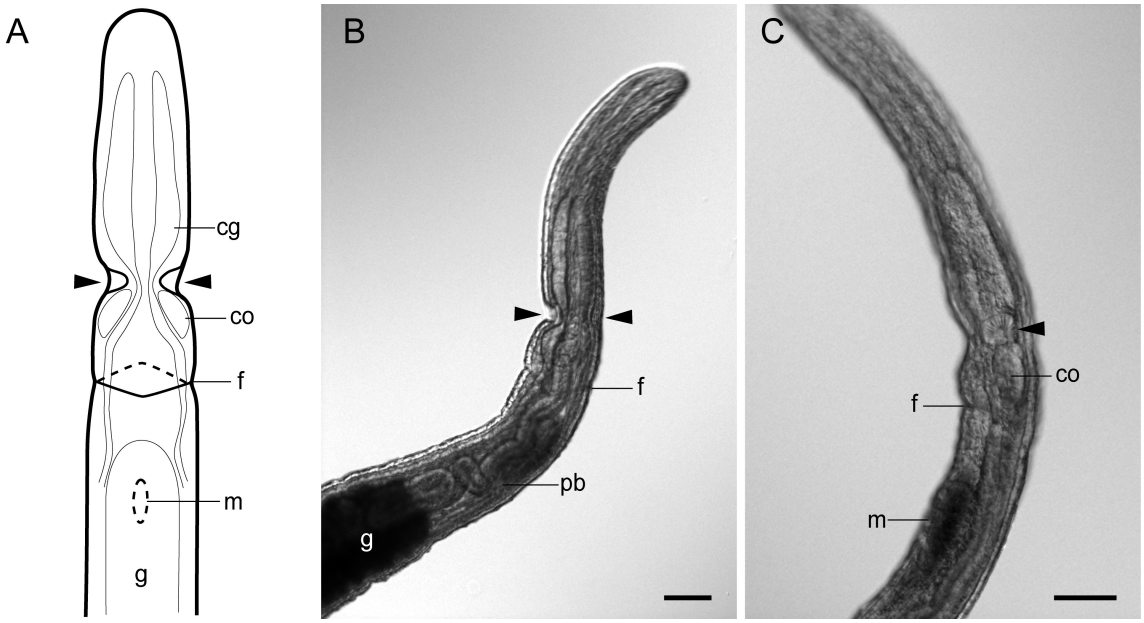

Figure 5.6. Morphology of a newly metamorphosed *pilidium recurvatum* juvenile (i.e., *Riserius* sp.). A. A diagram in dorsal view showing the location of two deep cerebral organ openings (arrowheads) located posterior to the cerebral ganglia (cg) and anterior to the cerebral organs (co) themselves, and the transverse cephalic v-furrow (f) located posterior to the cerebral organs and anterior to the mouth (m). Dorsally, the apex of the "v" points toward the posterior and ventrally - toward the anterior. An unusual characteristic of *Riserius* sp. is the long pre-oral end, as indicated by the position of the mouth and gut (g). B. Juvenile anterior end in dorsal view, showing deep cerebral organ openings (arrowheads) and the v-furrow (f). One can also see the proboscis (pb) and the gut (g). C. Juvenile anterior in lateral view showing one of the cerebral organ openings (arrowhead) which leads to the cerebral organ (co), the v-furrow (f) and the mouth (m).

selection of heteronemertean sequences from GenBank (Table 5.1). The 16S alignment comprised 43 individuals (representing 41 species) and was 571 bp long. Of 571 characters 312 were parsimony informative. Maximum parsimony analysis yielded a strict consensus of three most parsimonious trees (tree length = 2139, consistency index = 0.3406, homoplasy index = 0.6594, Fig. 5.8), which was identical in topology to both the Bayesian and the neighbor-joining trees (not shown). Both types of *pilidium recurvatum* formed a monophyletic clade with *Riserius pugetensis* NORENBURG 1993 with high bootstrap support (BST = 100) and posterior probabilities (PP = 100, Fig. 5.8). The two larval types formed two distinct and well supported clades (*pilidium recurvatum* with

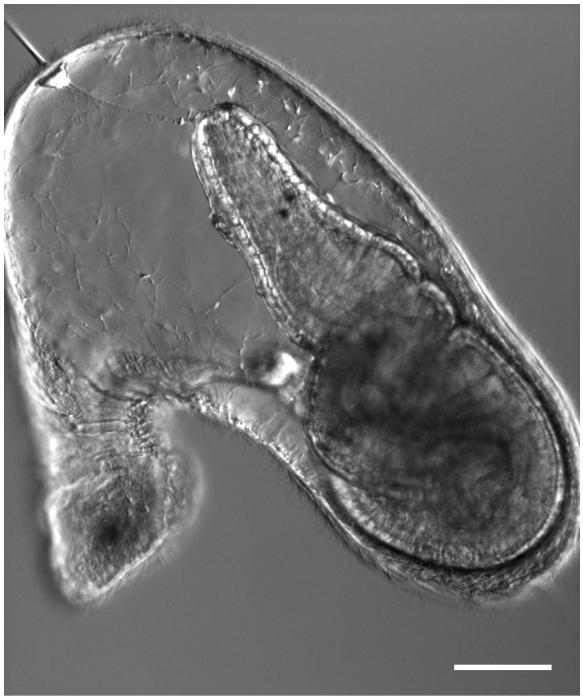

Figure 5.7. The "eyed" *pilidium recurvatum* morphotype encountered in a plankton sample from Coos Bay in November 2011. Note the two eyes in the juvenile head inside the larva. Scale bar 100 μm.

eyes (BST = 100, PP = 100) and *pilidium recurvatum* without eyes (BST = 100, PP = 100)). *R. pugetensis* appeared more closely related to *pilidium recurvatum* without eyes (BST = 99, PP = 89). Our 16S data is reported here because this gene region is known to resolve species level relationships well among nemerteans (e.g., Strand et al. 2005; Schwartz and Norenburg 2005), which is not the case for COI. Average sequence divergences are shown as uncorrected p-distances for 16S and COI gene regions (Table 5.2). Mean divergence distances between the two types of *pilidium recurvatum* were 0.11585 (16S) and 0.18928 (COI). In comparison, mean divergences between *Riserius pugetensis* and *pilidium recurvatum* with eyes were 0.09274 (16S) and 0.17071 (COI); and between *R. pugetensis* and *pilidium recurvatum* without eyes - 0.05974 (16S) and 0.19037 (COI). Sequence divergence observed between individuals of the same morphotype was much smaller (Table 5.2). Mean intraspecific divergences among *pilidium recurvatum* without eyes was 0.00049 (16S, n=8) and 0.00516 (COI, n=6), and among *pilidium recurvatum* with eyes p = 0.0000 (16S, n = 2) and 0.00516 (COI, n = 2).

The surprising diet of Riserius *juveniles*

Recently metamorphosed juveniles of *pilidium recurvatum* without eyes readily preyed on the larvae of the hoplonemertean *Carcinonemertes errans* WICKHAM 1978 collected from the plankton (Fig. 5.9A). We discovered this by accident, because both kinds of

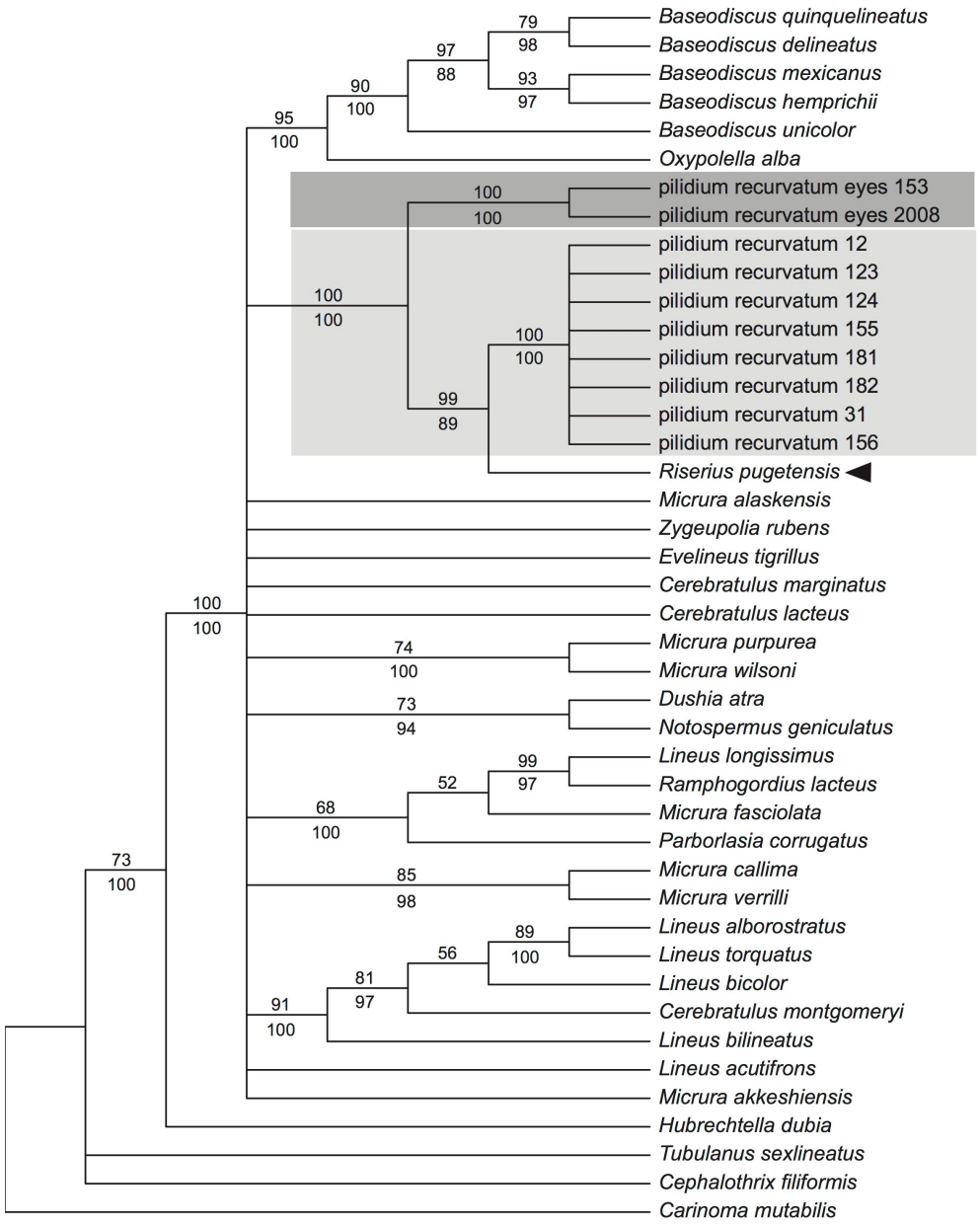

Figure 5.8. A strict consensus of 3 most parsimonious trees resulting from the analysis of 16S rDNA sequence data, including all available *pilidium recurvatum* sequences and a selection of pilidiophoran sequences from GenBank (accession numbers listed in Table 5.1). The topology resulting from the Bayesian analysis was nearly identical. Numbers above branches are bootstrap values (1000 replicates) and numbers below are Bayesian posterior probabilities, which are only shown for clades that were represented in both types of analyses. The two *pilidium recurvatum* morphotypes each form a strongly supported monophyletic clade (grey and light grey boxes), and together they form a well supported monophyletic clade with the only described species within the genus *Riserius* - *R. pugetensis* (arrowhead).

Table 5.2. Average uncorrected p-distances for 16S rDNA (top number) and COI, with divergence ranges included in parenthesis.

	pilidium recurvatum	*pilidium recurvatum* with eyes	*Riserius pugetensis*
pilidium recurvatum	0.0005 (0.000–0.002) 0.005 (0.005–0.011)	0.116 (0.099–0.122) 0.189 (0.203–0.207	0.060 (0.060–0.062) 0.190 (0.189–0.195)
pilidium recurvatum with eyes	– –	0.000 0.003	0.093 (0.087, 0.099) 0.171 (0.169, 0.172)

nemertean larvae shared a bowl in our sea table and one (*pilidium recurvatum*) metamorphosed and ingested the other (*C. errans*) (Fig. 5.9B). We also offered a variety of other potential prey items (e.g., larvae and small adults of other hoplonemerteans, bivalve pediveligers, cyphonautes, echinoid plutei, ophiuroid juveniles, and variety of polychaeate nechtochaetes and juveniles), but without success. We have not attempted to feed the juveniles of *pilidium recurvatum* with eyes.

Figure 5.9 (next page). *Riserius sp.* juveniles feeding on larval and juvenile *Carcinonemertes errans*. A. *Carcinonemertes errans* larva collected from Coos Bay plankton, and identified using DNA sequence data. *C. errans* larvae are easily differentiable from other hoplonemertean larvae because of their unusual arrangement of eyes: two widely-separated anterior, and two closely-positioned posterior immediately in front of the cerebral ganglia (cg). The two posterior eyes are so close together that they often appear as one. B. Larvae like the one depicted on (A) were ingested by newly metamorphosed *Riserius* juveniles; note orange color and the characteristic eye arrangement in the larva of C. errans within the gut of one-month old *Riserius sp*. C. Juvenile *C. errans,* with two eyes rather than four (posterior larval eyes are lost after settlement), which was collected from adult male Dungeness crab. D. Juvenile *C. errans* ingested by a growing juvenile *Riserius*. E. A much grown 6-month-old juvenile of *Riserius* sp. raised on a diet of *C. errans*. Two *C. errans* juveniles (arrowheads) are shown for scale. F. A large *Riserius* raised on a diet of *C. errans* with several dozen *C. errans* juveniles, packed in its gut after a recent meal. Scale bars 100 μm.


A
cg
B
C
D
E
F

The discovery that metamorphosed juveniles of *pilidium recurvatum* feed on *Carcinonemertes errans* was surprising and fortuitous. *Carcinonemertes errans* (Fig. 5.9C) makes a living as a symbiotic egg predator on the Dungeness crab (*Cancer magister* DANA 1852), a commercially important species. We initially supplied the *pilidium recurvatum* juveniles with larval *Carcinonemertes errans* collected by plankton tow, but as these waned in abundance and the *pilidium recurvatum* juveniles grew, we offered them juveniles of *C. errans* (Fig. 5.9D) which we scraped off in large numbers from live male Dungeness crabs purchased at the Fisherman's Wharf market in Charleston, OR. To date we have raised fourteen juveniles of *pilidium recurvatum*, on the diet of *C. errans*. Two of these have been maintained for over a year, and developed oocytes. We found that these juveniles eat and grow better with a thin layer of sand on the bottom of their culture dish. Over half of the metamorphosed juveniles from wild-caught *pilidium recurvatum* readily ate *C. errans* (64%); some of those who did not may have metamorphosed at too small a size, as it was difficult to find *C. errans* for them that were smaller in any dimension, and they exhibited no interest in *C. errans* juveniles that had been chopped into pieces with glass needles. We observed an approximately 20-mm long juvenile of *pilidium recurvatum* ingest several dozen *C. errans* juveniles within a half hour (Fig. 5.9F), at which point its appetite abated. Ingestion usually involves a quick eversion of the proboscis, which is wrapped around *C. errans* and sometimes moves the prey toward the mouth. In other instances, the proboscis is withdrawn, and the heteronemertean engulfs *C. errans* (sometimes several at a time) by pulling its mouth over the prey. Proboscis eversion was nearly always preceded by direct contact between the tip of the head and the body of the prey. Although predation appeared to be aided by the proboscis, it did not apparently disable the prey, which was often swallowed while wriggling. Occasionally *C. errans* juveniles escaped from the heteronemertean predator. Within as little as three months, juveniles of *pilidium recurvatum* exceeded 20 mm in length, more than ten times their length at metamorphosis, on a diet of *C. errans* (Fig. 5.9E). While this manuscript was in revision we found another acceptable prey item — an unidentified species of *Tetrastemma* (Hoplonemertea; Nemertea), collected by us from

the blades of surfgrass in Middle Cove, Cape Arago near Charleston, Oregon. We observed several attacks and feeding by several of our lab-reared *Riserius* sp. individuals on small adults of this species.

DISCUSSION

Larval morphology and behavior

Pilidium recurvatum from Oregon most resemble *pilidium recurvatum* from the Sea of Japan (Chernyshev 2001) in that they lack the posterior transverse ciliary band, also referred to as the telotroch, which characterizes the original *pilidium recurvatum* from Rhode Island (Fewkes 1883), as well as the Swedish (Cantell 1966a) and the Vietnamese forms (Dawydoff 1940).

We noted with interest an important difference in larval morphology between the typical pilidium (e.g., as described in Maslakova 2010a, b) and *pilidium recurvatum* (aside from the obvious difference in the body shape). In a typical pilidium the larval mouth is surrounded with a set of lobes (anterior and posterior) and lappets (typically two lateral) whose margins are spanned by the larval ciliary band. Internally, the ciliary band is supported by a prominent muscle band (Maslakova 2010a; 2010b; Fig. 5.4D, here) and serotonergic nerve (Maslakova 2010a; Fig. 5.4D, here). Absence of the marginal ciliary band in the *pilidium recurvatum* is correlated with the absence of the marginal muscle and serotonergic nerve cord, and the monociliated collar cells. The marginal ciliary band in the typical pilidium larva is involved in capturing food — unicellular algae (von Dassow et al. 2013). Absence of this structure in *pilidium recurvatum* correlates with our observation that *pilidium recurvatum* did not feed on unicellular algae (von Dassow, pers. obs.), such as the cryptomonad *Rhodomonas lens,* which is an excellent food for typical pilidia (Maslakova 2010a; TH, SM, GvD, pers. obs.).

At the same time, we strongly suspect that *pilidium recurvatum* is a planktotroph. Young *pilidium recurvatum* maintained in the lab for several weeks shrank in size over time, presumably from lack of suitable food, and did not proceed to develop imaginal discs. The long ciliary cirri arranged along the margin of *pilidium recurvatum* esophageal funnel (Fig. 5.3A) are unique to this pilidial type, and, we speculate, have something to do with how these larvae detect or capture food. We also suspect that the larval posture in which the esophageal funnel is outstretched (Fig. 5.3A) may be involved in feeding, although we did not succeed in identifying acceptable prey for these larvae, and never observed larval feeding.

None of the previous authors (Fewkes 1883; Dawydoff 1940; Cantell 1966a; Chernyshev 2001) reported the prominent ciliary cirri along the margin of the esophageal funnel, although Cantell (1966a) describes that the "rim of the funnel consists of thickened epithelium with longer cilia". It is interesting to note that Fewkes (1883) also observed the "feeding posture" in one of his *pilidium recurvatum* larvae, but thought that it might be an "individual peculiarity", while Cantell (1966a) depicted a larva in a "feeding posture", but did not comment on it.

Metamorphosis

Similar to *pilidium recurvatum* larvae observed by Fewkes (1883) and Cantell (1966a), larvae from Oregon undergo catastrophic metamorphosis in which the juvenile escapes and devours the larval body. This type of catastrophic metamorphosis has been observed in most other types of pilidium larvae (Cantell 1966b, 1969; Maslakova 2010a, b), with the exception of *pilidium auriculatum*, which belongs to the non-heteronemertean pilidiophoran family Hubrechtidae (Cantell 1966b, 1972).

***Pilidium recurvatum belongs to* Riserius**

Absence of the lateral cephalic furrows and caudal cirrus in the metamorphosed juveniles of *pilidium recurvatum* suggest that they do not belong to the Lineidae, as originally

suggested by Fewkes (1883). Absence of the secondary cephalic furrows suggests that they do not belong to Baseodiscidae, as speculated by Cantell in 1966 (a). Cantell later (1969) suggested the possibility that *pilidium recurvatum* may be the larva of *Oxypolella alba* BERGENDAL 1903*,* however several features in metamorphosed juveniles do not conform to the diagnosis for this genus (e.g., large cerebral organs and long pre-oral lobe) (Cantell 2005).

At the same time, *pilidium recurvatum* juveniles possess a highly unusual combination of morphological features (conspicuous cerebral organ pits, V-shaped transverse cephalic furrow, and long pre-oral end) that is only known in one pilidiophoran — a mesopsammic species from Puget Sound, Washington, USA — *Riserius pugetensis* (Norenburg 1993). The familial affiliation of *Riserius* is uncertain, but molecular phylogenetic analysis suggests that the species is basal within the Heteronemertea (Thollesson and Norenburg 2003).

Analysis of DNA sequence data (16S rDNA and Cytochrome Oxidase I) confirms our morphological identification: the two types of *pilidium recurvatum* larvae from Coos Bay, OR form a clade with *Riserius pugetensis* (Fig. 5.8). Based on the available GenBank 16S sequence data, Mahon et al. (2010) calculated the average interspecific divergence between congeneric nemertean species as ~ 4.8%. However, Meyer and Paulay (2005) argued that minimum interspecific divergences are better suited for species delimitation than the average. Minimum interspecific divergences for pilidiophoran 16S are on the order of 3% (Maslakova, unpubl. data). Either way, the p-distances between the two types of *pilidium recurvatum* from Coos Bay and *R. pugetensis* are too large (e.g., 6-11% for 16S) to consider them conspecific (Table 5.2). As *R. pugetensis* is the only described species of *Riserius*, we speculate that the two types of *pilidium recurvatum* larvae from Oregon represent two new (undescribed) species of *Riserius*.

Pilidium recurvatum is the first instance of an indirect-developing nemertean larva of apparent mesopsammic origin. *Riserius pugetensis* is a mesopsammic species (Norenburg 1993) and, as such, was not expected to have a long-lived planktotrophic larva (Swedmark 1964; Norenburg 1988; Giere 2009). However, numerous small eggs (about 300 eggs 50-60 μm in diameter in one 10-mm long female) reported by Norenburg (1993) suggest planktotrophic development. The largest individuals of *Riserius* sp. described here reached at least 25 mm (in gliding) and we observed oocytes of about 80 μm in diameter, which were released from a small body fragment. Therefore a single *Riserius* sp. female might be reasonably expected to produce at least on the order of 10^3 eggs. Therefore, our observation of a long-lived planktotrophic larva within this genus is not surprising.

Larval evolution

Jägersten (1972) suggested that *pilidium recurvatum* represents an intermediate evolutionary form between planuliform larvae of palaeonemerteans and the conventional hat-like pilidium larva. Because it is likely that the hat-shaped pilidial forms evolved only once, if the *pilidium recurvatum* is shown to belong to a highly derived pilidiophoran taxa, it would be unlikely to represent an ancestral pilidial form. On the other hand, Jägersten's hypothesis would be consistent with taxa characterized by the *pilidium recurvatum* larva being the sister group of pilidiophorans with a hat-shaped pilidium. Our identification of *pilidium recurvatum* as the larva of *Riserius,* which is the sister clade to the rest of the heteronemerteans (Thollesson and Norenburg 2003), would be consistent with Jägersten's hypothesis if *pilidium auriculatum* (Leuckart and Pagenstecher 1858), of the family Hubrechtidae (sister to Heteronemertea + Riserius according to Thollesson and Norenburg 2003, but see Andrade et al. 2012) has evolved separately from hat-like pilidia of heteronemerteans. Jägersten's hypothesis would be supported if *pilidium recurvatum* evolved once, and pilidiophorans with this larval form are paraphyletic with respect to the clade of pilidiophorans with a hat-shaped pilidium.

Distribution and diversity of **Riserius**

Our findings suggest that *Riserius* is a much more diverse and geographically widespread genus than previously appreciated. The two larval types from Oregon clearly represent two new species of *Riserius*. *Riserius* has not been previously reported from Oregon. Based on the similarity of larval morphology, it is possible that the *pilidium recurvatum* from the Sea of Japan (Chernyshev 2001) represents the same species as one of our forms. Trans-Pacific movement of marine species is well documented (e.g., Carlton and Cohen 2007), and we have previously found the larvae of *Hubrechtella juliae* CHERNYSHEV 2004 from the Sea of Japan in Coos Bay, OR (Maslakova 2010b; TH, GvD pers. obs.). Thus, a conservative estimate of *Riserius* diversity in Pacific waters is three species, two of which are so far known only in their larval form as *pilidium recurvatum*: 1) with eyes and without telotroch and 2) with neither eyes nor telotroch, and one so far known only in its adult form - *R. pugetensis*.

The Atlantic and the Vietnamese *pilidium recurvatum* are clearly different from the Pacific forms, because they possess a telotroch. It is possible that the original *pilidium recurvatum* (Fewkes 1883) from Rhode Island is different from the Swedish form (Cantell 1966a) because, unlike Fewkes (1883), Cantell (1966a) did not observe ocelli in the recently metamorphosed juveniles. It seems unlikely that the Vietnamese form (Dawydoff 1940) could be the same species as the Atlantic form(s), based on geography alone. Assuming these larvae are congeneric with those described here, *Riserius* has a worldwide distribution, and contains at least five (but probably many more) species. Indeed, Jon Norenburg (Smithsonian Institution, pers. comm.) has found adult individuals of *Riserius* in sub-littoral mesopsammon off Pacific Panama, southeast Florida, and southeast Brazil.

Riserius *as potential biological control*

Our observation that juveniles of *Riserius* sp. fed on *Carcinonemertes errans,* a symbiotic egg predator of *Cancer magister* (Dungeness crab), suggests that species diversity and

geographic distribution of *Riserius* might be of importance to zoologists and commercial fisherman alike. Carcinonemertidae is a large hoplonemertean family with worldwide distribution (Gibson 1995). It contains three genera and 18 described species (Sadeghian and Santos 2010), all of which specialize as symbiotic egg predators of different decapod crustaceans, many of which are commercially important, e.g., Dungeness crab *Cancer magister*, Red King crab *Paralithodes camtschatica* TILESIUS 1815, Blue crab *Callinectes sapidus* RATHBUN 1896 and American lobster *Homarus americanus* EDWARDS 1837 (Kuris 1993). *Carcinonemertes* infestations can reach an astonishing thousands of individuals per crustacean host (e.g., Wickham 1980), and make a significant impact on the host population. For example, localized high levels of *Carcinonemertes regicides* SHIELDS, WICKHAM & KURIS 1989 infestations and egg predation reduced clutches of Red King crab in the 1980s, which may have led to decreased crab landings and the subsequent closure of commercial fishing in certain Alaskan regions in 1983 (Kuris and Wickham 1987; Shields et al. 1989; Kuris et al. 1991). Also, predation by *C. errans* may have contributed to the collapse and subsequent non-recovery of Dungeness crab fisheries in central California (Wickham 1979, 1986; Kuris and Wickham 1987). However, recent data by Shanks and Roegner (2007) suggest that annual variation in Dungeness crab abundance correlates with abiotic factors, particularly the timing of the spring transition, which may have a more significant affect on the crab abundance than *Carcinonemertes.*.

Carcinonemertes errans larvae are found in the plankton (Dunn 2011; SM, TH, LH, GvD pers. obs), however adults are only found on *Cancer magister* (Wickham 1980). This suggests that if *Riserius* sp. individuals feed on *C. errans* in nature, they too must be found on, or near *C. magister*. *Cancer magister*, as many other crustaceans, exhibit a burying behavior in sand (McGaw 2005). Adults of *Riserius pugetensis,* the only described species in the genus, live interstitially between sand grains (Norenburg 1993), and it is possible that other species of *Riserius* also are mesopsammic. Therefore, our

working hypothesis is that *Riserius* may encounter *Carcinonemertes errans* on crabs that are buried in sand.

At this point we only have observed feeding on *Carcinonemertes* in one species of *Riserius.* Because we also observed feeding by *Riserius sp.* on another hoplonemertean species, it means that, *Riserius* sp. may not be a specialist predator of *Carcinonemertes* in nature. It is not known whether other species also feed on carcinonemertids or whether and how they encounter *Carcinonemertes* in nature. Nevertheless, this observation leads one to wonder about the potential of *Riserius* to control populations of carcinonemertids on commercially and ecologically important crustaceans.

CONCLUDING REMARKS

"It's a shirt. It's a sock. It's a glove. It's a hat. But it has *other* uses. Yes, far beyond that." (Theodor Seuss Geisel 1971, *The Lorax*)

Identifying planktonic larvae of marine invertebrates is important because larvae connect and ensure long-term stability of populations of benthic adults through dispersal and recruitment. Morphological identification of larvae to species is often difficult or impossible because early developmental stages of related species look alike, and the development of very few described species is known. DNA-based identification provides an important alternative. This study highlights additional benefits of DNA barcoding as a tool for identifying marine invertebrate larvae. First, it helps to reveal cryptic and undescribed biodiversity, because some of the species are more likely to be encountered as planktonic larvae than as benthic adults (as is the case with *pilidium recurvatum*). Second, it helps to address questions about larval evolution, as distinct larval morphotypes are placed in the phylogenetic context. And sometimes, the benefits may be entirely unexpected and with far-reaching consequences, such as our serendipitous discovery of the unusual predator-prey interaction between *Riserius* and *Carcinonemertes*

(whose planktonic larvae we also originally identified based on DNA sequence data), and its possible relevance to the biology of commercially important crustaceans.

NOTES

[1] It is not always clear from the literature whether a particular pilidial morphotype represents a species or a group of related or unrelated species (Bürger 1895; Schmidt 1930; Dawydoff 1940). One must be able to identify larvae to species level to determine which is correct. Regardless of whether morphotypes are shown to be representative of species or groups of species, as their identity is revealed, these larvae may properly be referred to by the name of the species or genus to which they belong. Until then it is convenient and appropriate to use morphotype names. In order to differentiate between the proper species names and the larval morphotypes, we do not capitalize morphotype names.

BRIDGE TO CHAPTER VI

In Chapter V, we describe and identify the larva *pilidium recurvatum*. This is a unique larval form that is easily recognizable in plankton samples and can now be identified to the genus level (*Riserius*), based on morphology alone. The identity of this larval form is also a good example of what we can learn about nemertean diversity by investigating both larval and adult stages. We reveal two new species in a, currently monotypic, genus that is previously unknown from central California to Oregon (Roe et al. 2007). To identify this larval form, we used DNA sequence data and morphological characters from the newly metamorphosed juvenile. In Chapter VI, we utilize additional traditional embryological approaches to identify the larvae of two heteronemertean species that we commonly encounter as adults, but rarely see as larvae in the plankton. We describe the development in the two species, *Micrura wilsoni* and *Lineus* sp. “red”, from fertilization through metamorphosis.

CHAPTER VI

LARVAL DEVELOPMENT OF TWO NE PACIFIC PILIDIOPHORAN NEMERTEANS (HETERONEMERTEA; LINEIDAE)

This chapter is co-authored with SA Maslakova and was published in *Biological Bulletin* (2015, *Biological Bulletin* 229(3): 265-275). SA Maslakova provided embryological expertise, demonstrated larval culturing for the undescribed species, *Lineus* sp. "red", and provided necessary edits. I followed the development of *Micrura wilsoni* from start to finish, maintained larval cultures of *Lineus* sp. "red", prepared the manuscript, and served as first author communicating with reviewers and editors at *Biological Bulletin*.

INTRODUCTION

Nemertean worms comprise a lophotrochozoan phylum with about 1275 described species (Kajihara et al., 2008), most of which are marine. As is the case for most benthic marine invertebrates, nemerteans exhibit a biphasic life history with a pelagic larval stage. The two life-history stages can have dramatically dissimilar morphology. The most distinctive nemertean larva is called the pilidium and was first described in 1847 by Johannes Müller, who suspected that it was some sort of marine invertebrate larva but at the time could not make the connection to a nemertean (Müller, 1847). The pilidium is a unique larval form – its morphology is unmistakable and found in no other marine invertebrate. Furthermore, it is observed in only one clade of nemerteans called the Pilidiophora (= Heteronemertea + Hubrechtidae, Thollesson and Norenburg, 2003; Andrade et al., 2014 but see Andrade et al., 2012; Kvist et al., 2014), which comprises about a third of all nemertean species (Kajihara et al., 2008). Unique for pilidial development is the formation of the juvenile body from seven or eight separate rudiments, called imaginal discs (Salensky, 1912; Maslakova, 2010a). Paired discs that arise as invaginations of larval epidermis include the cephalic discs, trunk discs and cerebral organ discs which give rise to the juvenile head, trunk and cerebral organs, respectively. Two unpaired rudiments, which may be mesenchymal in origin, the

proboscis and the dorsal disc, also contribute to the juvenile body (Maslakova, 2010a). Ultimately, these discs fuse to form a juvenile worm that erupts from its larval body in a rapid and catastrophic metamorphosis. In this transition from planktonic to benthic habitat, the juvenile pilidiophoran, typically, ingests its larval body (Cantell, 1966a, 1969; Lacalli, 2005, Maslakova, 2010a; von Döhren, 2011; Maslakova and von Dassow, 2012, T. Hiebert et al., 2013).

Development is known in any detail in only a few species of pilidiophorans because most pilidia require food and take weeks to months to develop through metamorphosis. Maslakova (2010a) published the first description of pilidial development from fertilization to metamorphosis in a common intertidal heteronemertean from the NE Pacific, *Micrura alaskensis* (Coe, 1901). Prior to this study, the most detailed account of pilidial development (Salensky, 1912) was based on wild-caught, unidentified pilidia. Schmidt (1930) published a description of larval development in *Cerebratulus marginatus* from fertilization to the formation of imaginal discs, but did not observe late development or metamorphosis. Partial descriptions of development are available for only a handful of other pilidiophorans (Desor, 1848; Bürger, 1895; Coe, 1899; Wilson, 1900; Salensky, 1912; Nusbaum and Oxner, 1913; Schmidt, 1929, 1932a, 1932b, 1934, 1964; Dawydoff, 1940; Iwata, 1958; Cantell, 1969; Schwartz and Norenburg, 2005; Schwartz 2009, Maslakova, 2010b; von Döhren, 2011; Maslakova and von Dassow, 2012; T. Hiebert et al., 2013; Maslakova and T. Hiebert, 2014; T. Hiebert and Maslakova, 2015). Phylogenetically, all pilidiophorans are expected to produce a pilidium larva (Thollesson and Norenburg, 2003), but the specific larval morphology can be highly variable (e.g., see Leuckart and Pagenstecher, 1858; Dawydoff, 1940; Cantell, 1966a, 1966b; Fewkes, 1883; Lacalli, 2005, Norenburg and Stricker, 2002, Maslakova and T. Hiebert, 2014). Furthermore, whether the larva is feeding or non-feeding cannot be easily anticipated phylogenetically. Lecithotrophic, or modified pilidia, once believed to be rare within the group, likely evolved in several lineages (Schwartz 2009; Maslakova and T. Hiebert 2014). Until more studies are published tracking pilidial development from

fertilization to metamorphosis, one cannot be certain how generally the findings reported for *M. alaskensis* (Maslakova, 2010a) may be applied.

Larval morphology may be phylogenetically relevant for nemerteans, a group where taxonomy is mostly based on characters of adult morphology, which are few, and often not unique. Many traditionally established nemertean genera are non-monophyletic according to molecular phylogenetic analyses, and are in need of revision (e.g., Thollesson and Norenburg, 2003; Andrade et al. 2012). New genera should be defined based on monophyly and, ideally, characterized by morphological (as well as molecular) synapomorphies. Where adult morphology fails to provide such synapomorphies, larval features may provide additional characters that will help to define closely related groups or genera. However, currently the development of few nemertean species is known. Here, we describe the development from fertilization to metamorphosis of two common NE Pacific nemertean species, *Micrura wilsoni* Coe, 1904 and, an undescribed lineiform species which we call *Lineus* sp. "red", because it lacks a caudal cirrus, is reddish-brown in color, and appears to be related to a number of described *Lineus* species, such as *L. bilineatus, L. flavescens*, and *L. torquatus*.

MATERIALS AND METHODS

Micrura wilsoni is a lineiform heteronemertean known from the Pacific coast of North America and reported to occur from Oregon to Mexico (Coe, 1904; Gibson, 1995; Roe et al., 2007, T. Hiebert and Maslakova, in prep.). It resides intertidally and subtidally, in rocky habitats, where it is somewhat common (T. C. Hiebert and S. A. Maslakova, pers. observation), meaning that on any given trip to their known habitat we can find one or a few, but sometimes find none. Reproductive adults of *M. wilsoni* were collected under rocks and from the holes of bivalves (Fam. Pholadidae) boring the rocks, intertidally from Middle Cove and North Cove at Cape Arago near Charleston, OR (Fig. 6.1A–B) in June of 2013. Adults are up to 15 cm in length, dark brown to black with very thin and irregular transverse lines of somewhat lighter color and a characteristic white anterior

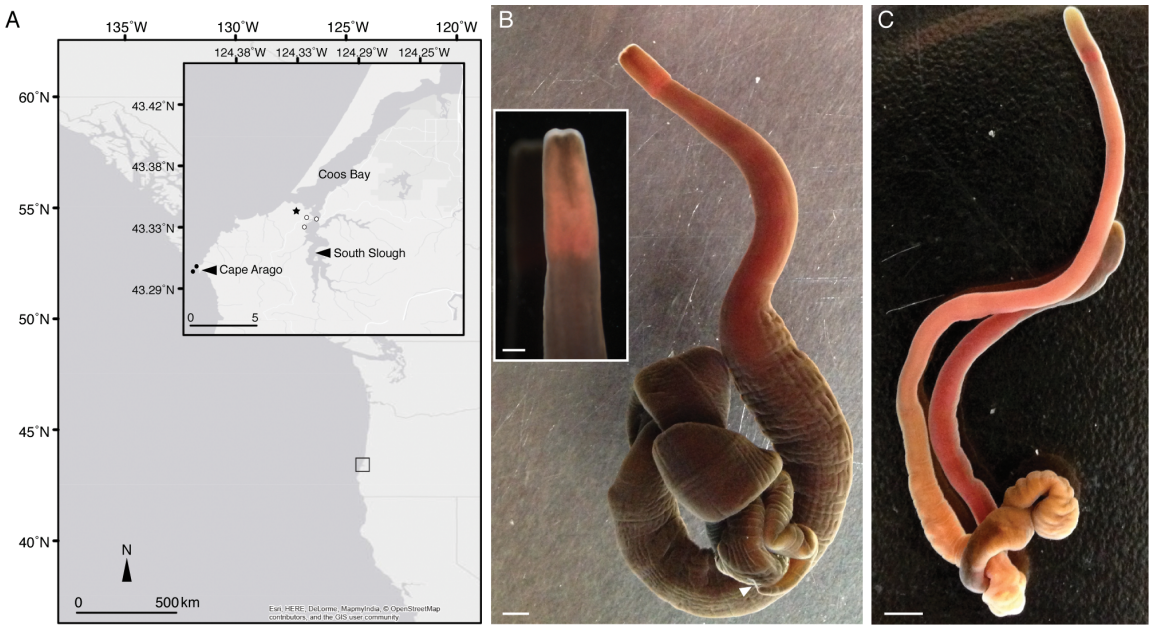

Figure 6.1. Adult morphology and collection sites of *Micrura wilsoni* and *Lineus* sp. "red". (A) *M. wilsoni* (black circles, inset) was collected at Cape Arago, and *Lineus* sp. "red" (white circles, inset) was collected near the confluence of South Slough and Coos Bay estuaries, near Charleston, OR (star, A). (B) Adult morphology of *M. wilsoni;* note distinct whitish anterior margin of the head (also on inset) and caudal cirrus (arrowhead). (C) Adult morphology of *Lineus* sp. "red"; two individuals show light and dark color varieties in this species. Scale bars 1 mm (B, C), 0.5 mm (inset on B).

margin of the head (Roe et al., 2007; Fig. 6.1B). Adults lack ocelli, possess typical lineiform lateral cephalic slits, a ventral mouth and a long caudal cirrus at the posterior end (Fig. 6.1B). The cirrus may be lost during collecting, but readily regenerates if worms are kept in the lab for a few weeks, even without food.

Lineus sp. "red" is an undescribed lineiform species from southern Oregon that groups closely with *Lineus flavescens, L. bilineatus, L. torquatus* and *Cerebratulus montgomeryi* on molecular phylogenies (T. Hiebert and Maslakova, in prep). Adults are fairly common intertidally in mudflats of Coos Bay, OR, as well as among the roots of surfgrass, in silty shell hash and under rocks in coves near Cape Arago, OR. Reproductive adults were collected in February of 2012 from mudflats along the South Slough in Coos Bay, OR (Fig. 6.1A, C). Adults vary in color from dark red or brownish black to ochre (Fig. 6.1C),

with anterior darker than the rest of the body, and are approximately 6–10 cm in length. Head is rectanguloid in shape and only slightly demarcated from the body. Adults lack ocelli, possess typical lineiform lateral cephalic slits, and a ventral mouth. The body is rounded in the foregut region and flattened dorso-ventrally in the midgut region, where it is often transversely creased and coiled like a wrinkled ribbon. A caudal cirrus is absent and, instead, the worm tapers to a blunt end.

Embryological cultures

Primary oocytes and sperm were obtained by dissection. Oocytes had conspicuous germinal vesicles (e.g., Fig. 6.2A) when dissected. After approximately 30 min in filtered sea water (FWS, 0.45µm), oocytes 'rounded up', underwent geminal vesicle breakdown (GVBD) and were fertilized with dilute sperm suspension. Oocytes were fertilized in 150 ml glass culture dishes, in concentrations such that a monolayer formed on the bottom of the culture dish. Dishes were surrounded by flowing seawater at ambient sea temperature (9–12˚C). After 24 hours, larvae were suspended at approximately 1 larva per ml in a 1-gal glass jar. Swimming larvae were maintained at this concentration with constant stirring using plexiglass paddles (Strathmann, 1987). Larvae were fed *Rhodomonas lens* Pascher & Ruttner (CCMP739) at concentrations of approximately 10^4 cells/ml and water was changed every 3 days by reverse filtration. After about two weeks, larval concentration was further reduced to approximately 1 larva per 5 ml. Throughout development individual larvae were removed from culture and photographed using Leica DFC400 digital camera mounted to an Olympus BX51 compound microscope equipped with differential interference contrast (DIC) optics. Larvae were imaged live in a small drop of FWS on a glass slide, covered, and gently trapped with a glass coverslip supported by small clay feet at the corners (Strathmann, 1987).

Confocal microscopy

Ten to 15 *Micrura wilsoni* larvae were relaxed in a 1:1 mixture of FSW and 0.34M $MgCl_2$ for approximately 30 min, and subsequently fixed in 4% paraformaldehyde for 1

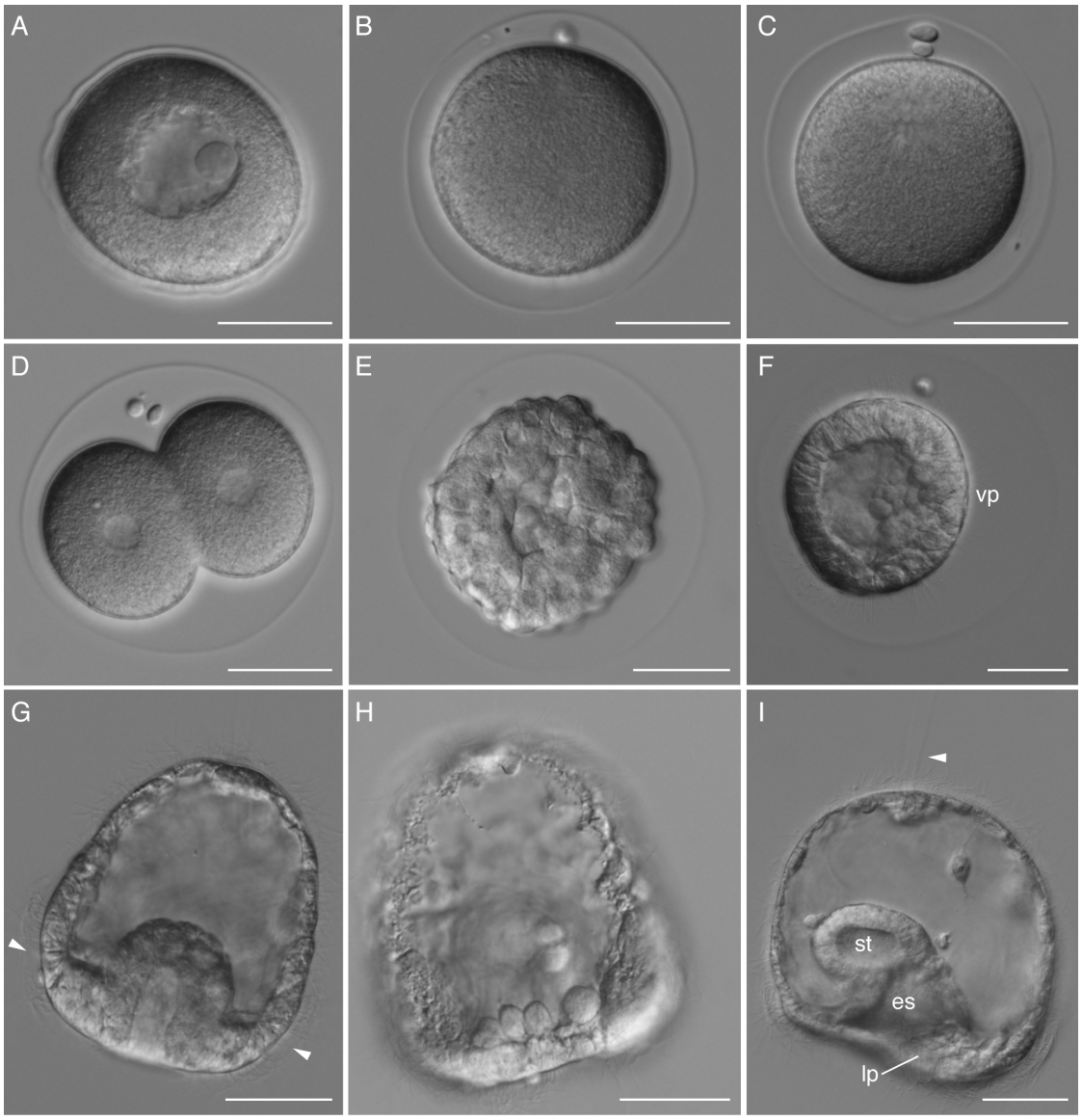

Figure 6.2. Early development in *Micrura wilsoni*. (A) Primary oocyte. (B–C) formation of 1st and 2nd polar body, respectively; note sperm cell at 11 o'clock on (B) and 4 o'clock on (C). (D) First cleavage at 2.5 hrs post fertilization; note cell nuclei. (E-F) Blastulae (17 hrs, E and 26.5 hrs, F; note vegetal plate (vp)) and gastrulae (46 hr (G) and 51 hr (H)); note developing ciliated band (arrowheads on G) and cells inside the blastocoel near the blastopore (in focus, H). (I) Young pilidium larva with apical tuft (arrowhead), stomach (st), (es) and rudimentary lateral lappets (lp). Future juvenile anterior to the right. All scale bars 50 μm.

hour at room temperature. After fixation, larvae were rinsed in several short (<1 min) washes in phosphate buffered saline (PBS, pH 7.4, Fisher Scientific) and permeabilized in PBS with 0.1% Triton X-100 (PBT) in three 10-min washes. To visualize cell nuclei

and musculature, larvae were labeled with Hoechst 33342 (2 μmol L^{-1}) and Rhodamine Phalloidin (Sigma, 165 nmol L^{-1}), respectively. The fluorescently labeled larvae were washed in PBS (three 10-min washes), emersed in 50% glycerol and mounted and imaged in 90% glycerol. Confocal stacks of 0.5–0.75 μm sections were gathered using an Olympus FluoView 1000 confocal system mounted on an Olympus IX81 inverted microscope equipped with UPlanSApo 20X 0.85 NA oil lens. Z-projections were reconstructed using ImageJ v. 1.46 (Wayne Rasband, National Institute of Health, Bethesda, MD). *Lineus* sp. "red" larvae were not preserved for or imaged using confocal microscopy.

RESULTS

Micrura wilsoni *development*

Recently dissected oocytes are approximately 100–110 μm in diameter, relatively transparent, possess a distinct germinal vesicle, and are surrounded by a chorion tightly apposed to the surface (Fig. 6.2A). Sperm is of 'primitive' (Stricker and Folsom, 1998) type with a head piece 4–5 μm in length (Cluster 4–5 in Fig. 11 of von Döhren et al., 2010). The developmental timeline described here is based on observations of a single larval cohort, with seawater temperature at 12˚C (Table 6.1). Following germinal vesicle breakdown in seawater, the egg envelope lifts slightly off the oocyte surface and becomes even more conspicuous following fertilization (Fig. 6.2B). First and second polar bodies are apparent at 1 and 2 hrs post fertilization (PF), respectively (Fig. 6.2B–C). The first cleavage occurs at 2.5 hrs PF. Due to transparency of the eggs, cell nuclei, cleavage spindles and asters are clearly visible with DIC optics (Fig. 6.2B–D). Cleavage is spiral, equal and holoblastic, as is typical of nemerteans. Spherical blastulae, rather than the 'blastosquare' described for *M. alaskensis* (Maslakova, 2010a) form by 17 hrs PF (Fig. 6.2E). Embryos become ciliated by 24 hrs, at which point the blastulae begin to rotate within their chorions (Fig. 6.2F). Invagination of the archenteron is apparent by 46 hrs PF (Fig. 6.2G). Gastrulae hatch from the egg chorion by 46 hrs and swim with a small apical tuft pointing forward, and a densely ciliated region, which represents the nascent ciliated

Table 6.1. Reproductive timelines for the pilidiophoran species *Micrura alaskensis* from Stricker (1987) and Maslakova (2010a), and *M. wilsoni* and *Lineus* sp. "red" (this study).

	Micrura alaskensis [1]	*Micrura alaskensis* [2]	*Micrura wilsoni* [3]	*Lineus* sp. "red" [4]
reproductive season	May-July	July-August	June	Jan-March
temperature	10–12˚C	11˚C	12˚C	10˚C
oocyte diameter	75 *µ*m	75 *µ*m	100–110 *µ*m	90–100 *µ*m
first cleavage	4h	2h 15m	2h 30m	3h 30m
blastula	17h	16h	17h	24h
gastrula	26h	24h	46h	48h
young pilidium	40h	40h	66h	96h
feeding pilidium	62h	66h	72h	120h
cephalic discs	-	7d	9d	10d
trunk discs	-	9d	17d	15d
cerebral organ discs	-	14d	22d	25d
head and trunk stage [2]	-	24d	-	27d
torus stage [2]	-	28d	40d	36d
metamorphosis	-	35d	60d	65d
larval height at three disc stage	-	-	540–545 *µ*m (at 24d)	350–380 *µ*m (at 25–27d)
larval height prior to metamorphosis	-	-	730–735 *µ*m (at 63d)	415–475 *µ*m (at 65d)

[1] Stricker, 1987
[2] Maslakova, 2010a
[3] See Fig. 6.2 and 6.3 of the current study
[4] See Fig. 6.5 and 6.6 of the current study

band, (arrowheads, Fig. 6.2G) encircling the vegetal (posterior) pole. At 51 hrs PF, mesenchymal cells can be seen inside the blastocoel, near the base of the archenteron (Fig. 6.2H). By 66 hrs, the young pilidia have a blind gut differentiated into a funnel-shaped esophagus and a round stomach, and stubby lateral lappets, as well as the anterior and posterior (with respect to the axis of the future juvenile) lobes (Fig. 6.2I). The larval

lobes and lappets are spanned by a primary ciliated band, which is used in larval feeding (von Dassow et al., 2013) and swimming.

The shape of *Micrura wilsoni* pilidia resemble that of other so-called typical pilidia (e.g., unassigned *Lineus* and *Micrura* larvae, Lacalli 2005; *M. alaskensis*, Maslakova 2010a) and are not unlike a deer hunter's cap with earflaps pulled down. Young pilidia begin to feed on *Rhodomonas lens* at 3 days after fertilization (Fig. 6.3A, B). They have a prominent apical organ from which arises the apical tuft, marking the larval apical pole, which is also the larval anterior, based on the direction of swimming (Fig. 6.3A). The two lateral lappets, and the anterior and posterior larval lobes, so named with respect to the future antero-posterior (AP) axis of the juvenile worm inside, are rounded. As in a typical pilidium, the primary larval ciliated band spans the lobes and lappets (Fig. 6.3C) and a stiff larval cirrus is present on the posterior lobe (not shown). Leading into the gut is a funnel-shaped ciliated esophagus with two distinct ciliated ridges along its posterior (again, with respect to future juvenile AP axis) wall (arrowhead, Fig. 6.3A). Lateral lappets are relatively small (e.g., 225 and 280 μm at 24 and 63 days, respectively) compared to the larval episphere (e.g., 320 and 450 μm at 24 and 63 days, respectively) (Figs. 6.3D, G, Table 6.1). After approximately nine days, the first pair of juvenile rudiments begin to invaginate from the larval epidermis (Fig. 6.3C). These invaginations become the cephalic discs (Fig. 6.3C–D), which will give rise to the head of the juvenile worm. Two black pigment patches or chromatophores (Cantell, 1969) – one on either side of the anterior larval lobe develop by 13 days (arrowhead, Fig. 6.3C). After 22 days, all three pairs of imaginal discs are present (Fig. 6.3D). In addition to the cephalic discs, the trunk discs, which give rise to the juvenile trunk, and the cerebral organ discs, which give rise to cerebral organs, can be seen surrounding the larval esophagus. The unpaired proboscis rudiment, described in an unidentified pilidium (Bürger, 1894), *Cerebratulus marginatus* (Schmidt, 1930, 1937), and *M. alaskensis* (Maslakova, 2010a), is also present at this time (Fig. 6.3D, inset). By 40 days, all discs fuse around the larval esophagus (Fig. 6.3E). At this time the unpaired dorsal disc is observed posterior to the the larval gut. The

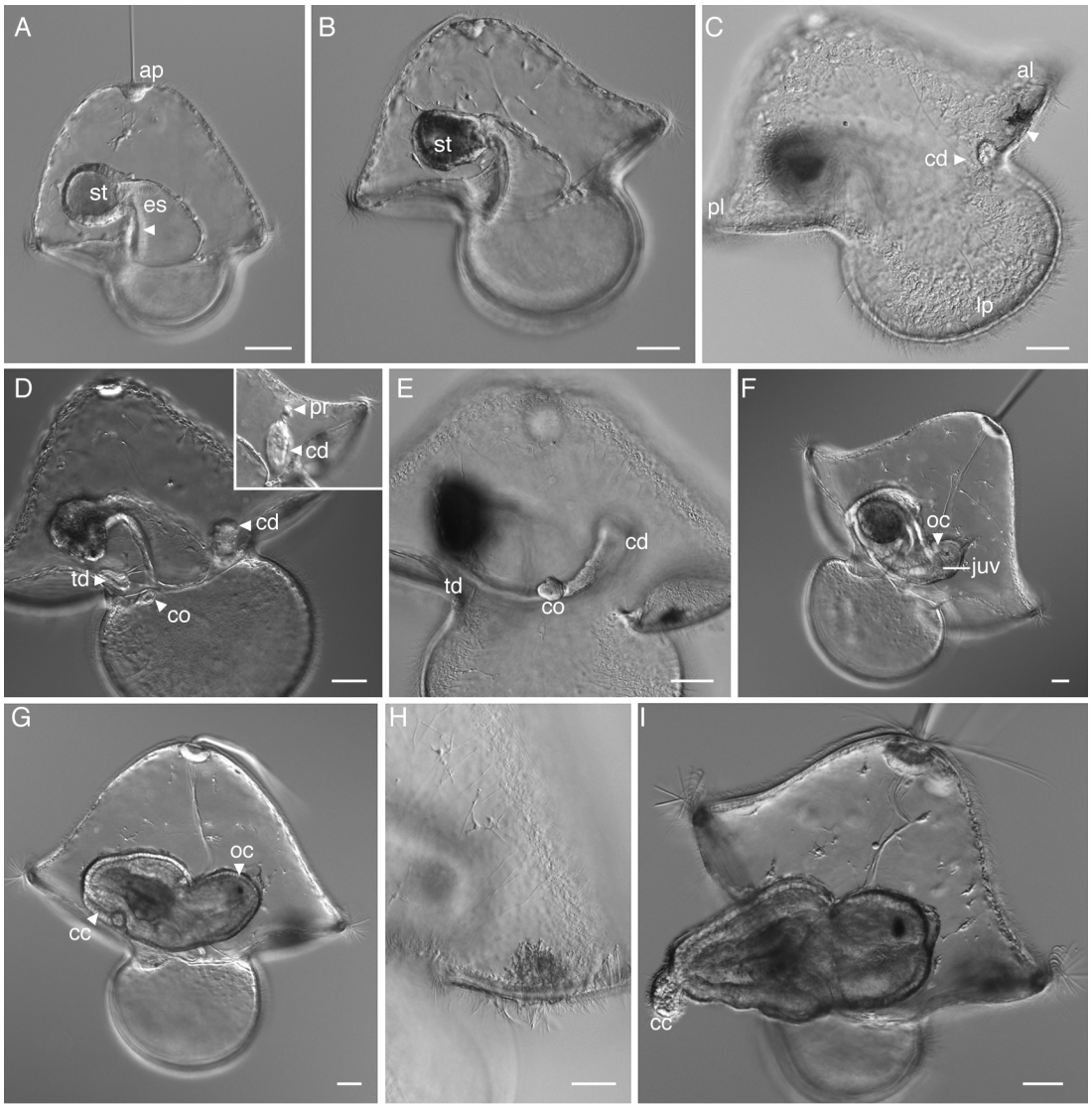

Figure 6.3. Pilidial development in *Micrura wilsoni*. (A) A 5-day old pilidium larva with apical tuft (ap), esophagus (es), and stomach (st); note ciliated ridges along the posterior esophageal wall (arrowhead, A). (B) 9-day old pilidium; stomach (st) is dark because it retains pigment from consumed *Rhodomonas lens* cells. (C) 13-day old pilidium; note one of the cephalic discs (cd) and the pigment spot (arrowhead, C) on the right side of the anterior lobe (al). Posterior lobe (pl), and lappets (lp) remain spotless throughout development. (D) A 24-day-old pilidium with all three pairs of imaginal discs present, the cephalic discs (cd), trunk discs (td) and cerebral organ discs (co) as well as the unpaired proboscis rudiment (pr, inset) from a different individual of the same age. (E) Fused imaginal discs in a 41-day old larva. (F) A hood-stage and (G) pre-metamorphosis-stage larva (staging scheme after Maslakova 2010a) with a developed juvenile (juv, F) inside the larval body; note caudal cirrus (cc, G), and ocelli (oc). (F–G) Pigment patches are present on either side of larval anterior lobe (right patch in focus, H). (I) Beginning of metamorphosis; note juvenile posterior and caudal cirrus (cc) protruding from the ruptured larval body. All scale bars 50 µm.

juvenile is complete by 63 days after fertilization, and possesses a caudal cirrus (Fig. 6.3G), and also, surprisingly, two small patches of pigment, which we assume to be eyes (Fig. 6.3F–G, I), which are lacking in the adult.

The black pigment patches on anterior lobe remain prominent until metamorphosis. These pigment patches appear to be groups of pigment-containing cells that extend into the episphere from the ciliated band, are irregular or stellate in shape (Fig. 6.3C, H), and are only observed on the anterior lobe. However, without additional investigation (e.g., transmission electron microscopy) we cannot be certain whether this pigment is cellular or extracellular. There was variation in the size of this patch among individuals, however the location and presence was consistent within the entire cohort.

Larval musculature of *Micrura wilsoni* is similar to that described for pilidia of *M. alaskensis* (Maslakova, 2010a; von Dassow et al., 2013) with prominent radial muscles in the lobes and lappets, a thick muscle strand along the primary ciliated band, and muscle strands that cross the lappets at their base, which allow the pilidium to constrict the lappets (Fig. 6.4A). Another prominent muscular strand originates at the apical organ, and divides into two strands – each attached to one side of the larval esophagus (arrowheads, Fig. 6.4A). Nuclear (Hoechst) staining (Fig. 6.4B) reveals that cells are arranged most densely in the apical organ, the stomach, the esophageal ciliated ridges, the imaginal discs, and within the primary ciliated band near the pilidial axils – the recesses between the larval lobes and lappets, described by Bird et al. (2014) (asterisks, Fig. 6.4B).

Metamorphosis was observed between 60 and 70 days post fertilization (63-day old larva, Fig. 6.3I). Juvenile *Micrura wilsoni* ruptured and emerged from the larval body caudal cirrus first. Individuals proceeded to back out of their larval body while, at the same time, ingesting it (Fig. 6.3I). We observed metamorphosis several times when individuals were trapped between a microscope slide and coverslip and juvenile worms were often found at the bottom of the culturing jar with evidence of pilidial body within their guts. Thus,

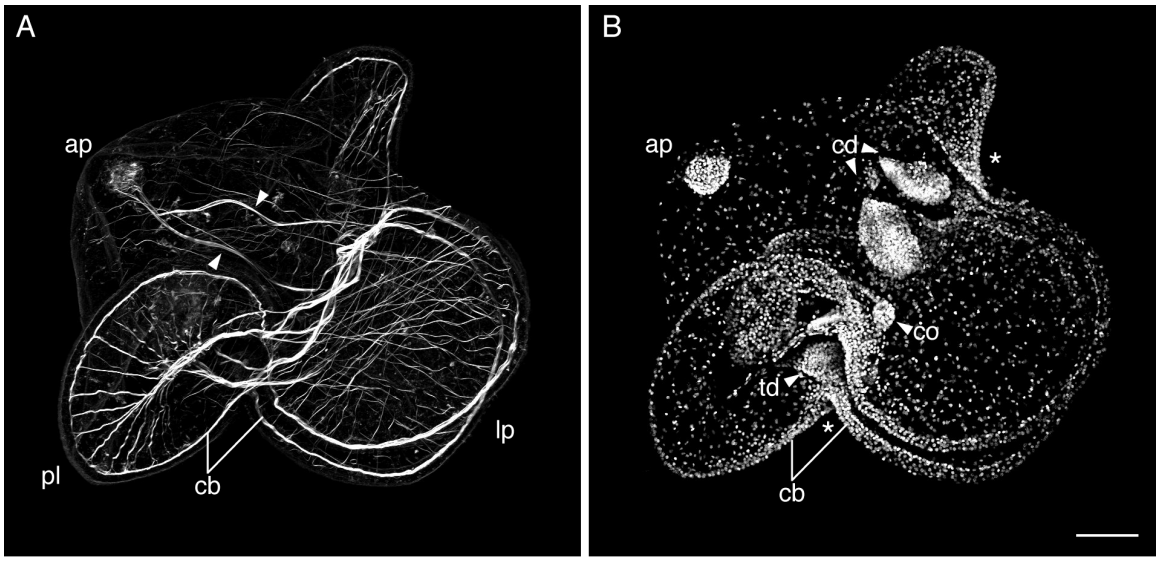

Figure 6.4. Confocal projections of 25-day-old *Micrura wilsoni* pilidium stained with phalloidin (A) and Hoechst (B) to visualize the muscles and cell nuclei, respectively. Apical plate (ap) is at 10 o'clock. Note radiating muscle strands within the posterior lobe (pl) and lateral lappets (lp). A particularly large muscle strand can be observed where larval lappets meet the lobes. An additional muscle can be seen extending from the apical plate and splitting into two strands on either side of the esophagus (arrowheads, A). Another prominent muscle spans the length of the ciliated band (cb, A). Cell nuclei (B) are most densely arranged in apical plate (ap), imaginal discs (two cephalic discs (cd), one trunk disc (td) and one cerebral organ disc (co) are clearly in view) and along the ciliated band (cb), especially near the axils (asterisks, B). Scale bar 50 μm.

we believe that ingestion of the larval body is the rule in this species, as in most pilidiophorans for which metamorphosis has been documented.

Lineus *sp. "red" development*

Dissected oocytes are approximately 90–100 μm in diameter, relatively opaque, (Fig. 6.5B) and are surrounded by a jelly layer (approximately 130 μm in diameter), but lack a chorion (a conspicuous extracellular envelope surrounding the cleavage stages, e.g., see *Micrura wilsoni*, this study). Sperm heads are elongated and slightly curved, i.e., exhibit 'modified' morphology (Stricker and Folsom, 1998), and approximately 10 μm in length (Cluster 3 in Fig. 11 of von Döhren et al., 2010) (Fig. 6.5A–B). At 10˚C, the development of *Lineus* sp. "red" proceeds similar to that described for *M. alaskensis* (Maslakova,

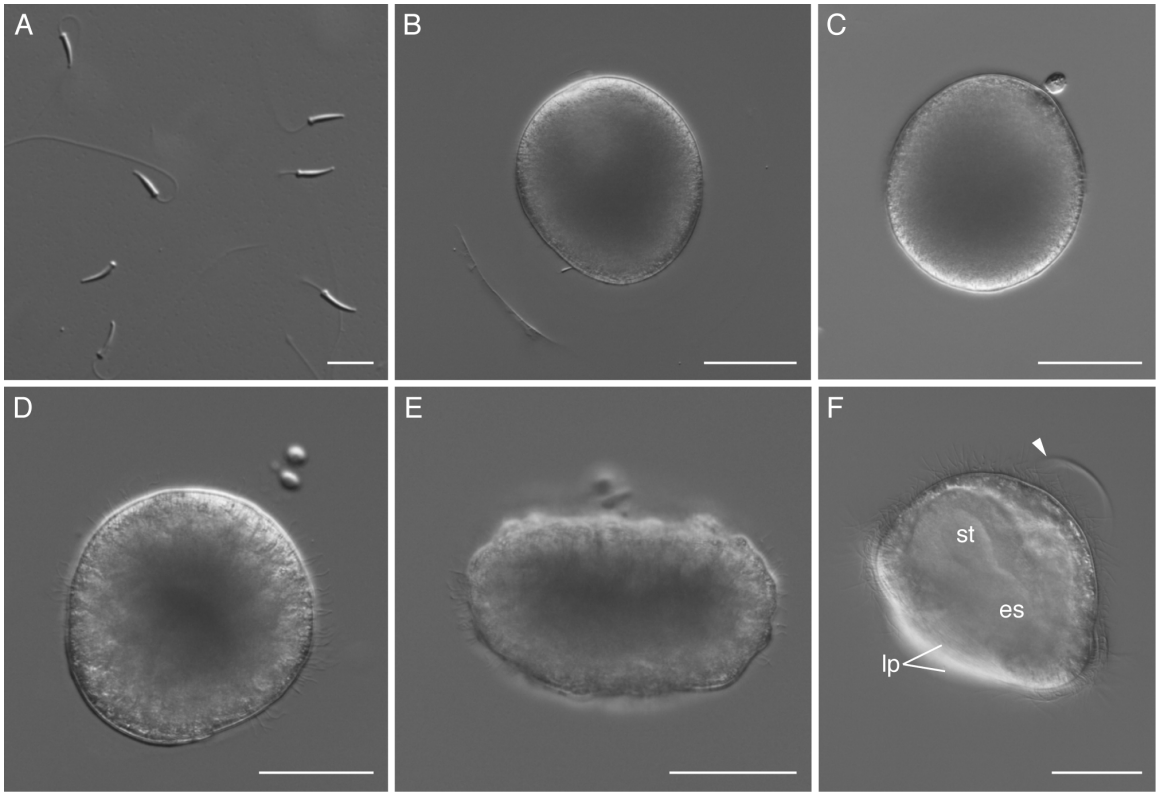

Figure 6.5. Early development in *Lineus sp.* "red". (A) Dissected sperm cells and (B) oocyte after addition of sperm; note the outline of egg jelly and sperm cell at 7 o'clock (B). (C) First polar body formation at 1 hr post fertilization. (D) Blastulae at 24 hrs and (E) 48 hrs; note two polar bodies trapped in the egg jelly. (F) Early pilidium larva at 4 days post fertilization with an apical tuft (arrowhead), esophagus (es), stomach (st) and nascent lateral lappets (lp). Future juvenile anterior to the right. Scale bars 10 µm (A) and 50 µm (B–F).

2010a) and *M. wilsoni* (this study, Table 6.1). We observed development in three cohorts. The first and second polar body formation occurred at 1 and 2 hrs PF, respectively, (Fig. 6.5C) and the first cleavage at 3.5 hrs PF. After 24 hours, ciliated blastulae had thickened vegetal plates (Fig. 6.5D) and were rotating slowly within their egg jelly. Swimming blastulae compressed along the animal-vegetal axis (resembling a bean in a sagittal section) were observed as early as 48 hrs PF (Fig. 6.5E). Young pilidia with an apical tuft, a gut differentiated into an esophagus and a round stomach, and rudimentary lateral lappets developed by 4 days PF (Fig. 6.5F).

Pilidia were first offered food and observed to feed after about one week in culture (Fig. 6.6A). The order of emergence of imaginal discs corresponds to that observed in other pilidia (cephalic discs first, trunk discs next, and cerebral organ discs last). Like for *Micrura wilsoni*, the shape of *Lineus* sp. "red" larvae also resembles that of a typical pilidium. The first two pairs of juvenile rudiments, the cephalic discs and trunk discs, are present by 15 days post fertilization (Fig. 6.6B). The third pair of discs (cerebral organ discs) were observed to fuse with the trunk discs by approximately 25–27 days (arrowhead, Fig. 6.6D). The proboscis rudiment also appears at this time (Fig. 6.6C) and the unpaired dorsal disc was observed at 36 days post fertilization (Fig. 6.6D). Finally, all three pairs of discs fuse around the larval esophagus forming a toroid of juvenile tissue (torus stage, Maslakova, 2010a, table 1) at 36–44 days. Larval lappets and episphere are similar in size (e.g., approximately 160–190 μm each, Figs. 6.6B, D) in most individuals and developmental stages (but see Fig. 6.6E), unlike the body proportions reported for other *Lineus* species (e.g., Lacalli 2005) where larval episphere is relatively small. The anterior lobes of *Lineus* sp. "red" larvae lack pigment spots and the complete juvenile with an anterior pair of pigment patches, that we assume are ocelli, was observed by 53 days PF (Fig. 6.6E). Metamorphosis occurred between 65–90 days PF in the three cohorts under study. Metamorphosis was observed on several occasions, during which the juveniles were seen ingesting the larval body. Often individuals were seen in the bottom of culture dishes with a partially ingested larval body hanging out of the mouth.

In addition to laboratory-reared larvae, we have collected three wild-caught larvae (plankton samples from January 2013), that were confirmed to belong to *Lineus* sp. "red" using DNA sequence data (larva collected 11 Jan 2013, Fig. 6.6F). Often, we were able to raise wild-caught larvae collected at early developmental stages to metamorphosis and their morphology was like that seen in laboratory-reared specimens. *Lineus* sp. "red" juveniles are short and stout and lack a caudal cirrus; they crawl about the bottom of culture dishes, surrounded by thin mucous. Interestingly, a row of 2–3 presumed ocelli is visible on the left and right side along the anterior margin of the juvenile head (Fig.

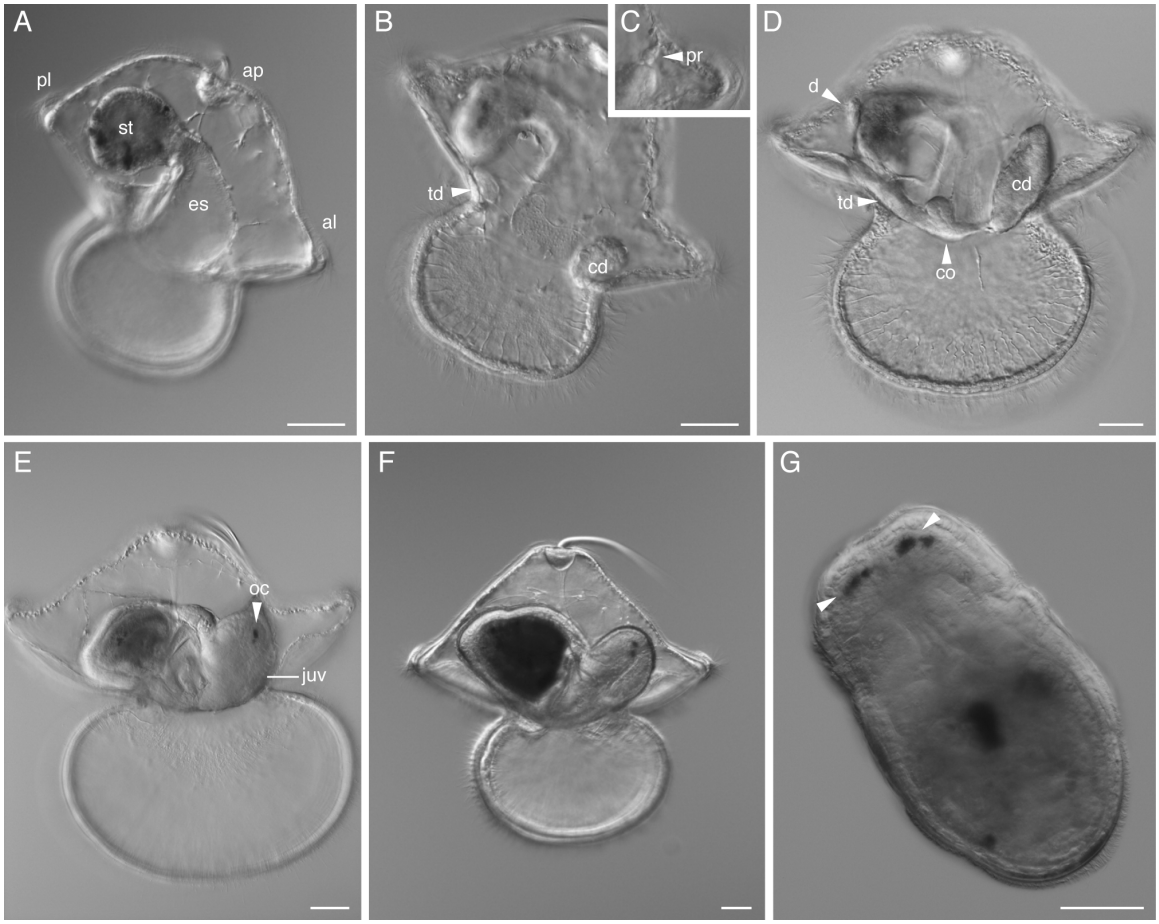

Figure 6.6. Pilidial development in *Lineus* sp. "red". Apical tuft (ap) is up, pilidial anterior lobe (al) and future juvenile anterior to the right, posterior lobe (pl) to the left. (A) A feeding pilidium at 10 days; esophagus (es) leads to the stomach (st), which is dark from ingesting *R. lens* cells. (B) A 20-day-old larva with the first two pairs of imaginal discs, cephalic (cd) and trunk discs (td). (C) The unpaired proboscis rudiment (pr) in 25-day-old larva. (D) The trunk discs (td) fused with the cerebral organ discs (co) and cephalic discs (cd) in a 38-day-old larva; also note unpaired dorsal disc, just out of focus (d). (E) A complete juvenile (juv) within the larval body; note ocelli (oc). (F) A wild-caught larva collected 11 Jan 2013 and identified as *Lineus* sp. "red" using DNA sequence data. (G) Newly metamorphosed juvenile *Lineus* sp. "red" from a laboratory culture; note two rows of ocelli (arrowheads, G) along the anterior margin of the head and the absence of the caudal cirrus. Note dark pigment from the recently ingested larval body within the gut. All scale bars 50 µm.

6.6G). This is noteworthy, because, as is the case in *M. wilsoni*, ocelli appear to be lacking in adults.

DISCUSSION

We describe for the first time the development of *Micrura wilsoni* and *Lineus* sp. "red", two intertidal nemertean species commonly found in southern Oregon. Both species possess typical hat-shaped planktotrophic pilidia and can be reared in the laboratory from fertilized dissected gametes to metamorphosis on a diet of cryptophyte algae (*Rhodomonas lens*), as previously described for *M. alaskensis* (Maslakova, 2010a). The general sequence of developmental events (e.g., the order of appearance of various juvenile rudiments) corresponds to that described for *M. alaskensis* (Table 6.1) and *Cerebratulus marginatus* (Schmidt, 1930). However, the time to metamorphosis for both *M. wilsoni* and *Lineus* sp. "red" was nearly double that reported for *M. alaskensis* (Table 6.1). Developmental rate can vary widely even within a single larval cohort, likely depending on individual feeding rate and, possibly, other factors. Thus, the most developmentally advanced larvae often metamorphose and settle to the bottom of culture jars before this stage is observed in the majority of individuals.

The larvae of *Lineus* sp. "red" are characterized by a juvenile that possesses presumed ocelli and lacks a caudal cirrus, and a larval episphere that is not dramatically larger or smaller than the lappets throughout development. Similar larvae have been observed by Müller (1847), Dawydoff (1940), Thorson (1946), Chernyshev (2001) and Lacalli (2005) but the species-level identities of these larvae are not known. The larvae of *Lineus* sp. "red" exhibit a similar morphology (e.g., presence of juvenile (assumed) ocelli, episphere height smaller than or equal to lappets) to larvae collected by us from plankton in southern Oregon and identified by "DNA barcoding", including those of *Lineus flavescens* (see Fig. 1A in Maslakova, 2010b, this study), and several other closely related species for which we are yet to find the adults (T. Hiebert and Maslakova, in prep). These species form a monophyletic clade with each other as well as *Lineus torquatus* and *Cerebratulus montgomeryi* on molecular phylogenies (T. Hiebert and Maslakova, in prep). Thus, it appears that this particular larval form, where developing juveniles possess two presumed eyes and the larval episphere and lappets are of similar size throughout

development, characterizes a clade of closely related species. At present, the larvae of these species cannot be differentiated from each other using morphology alone.

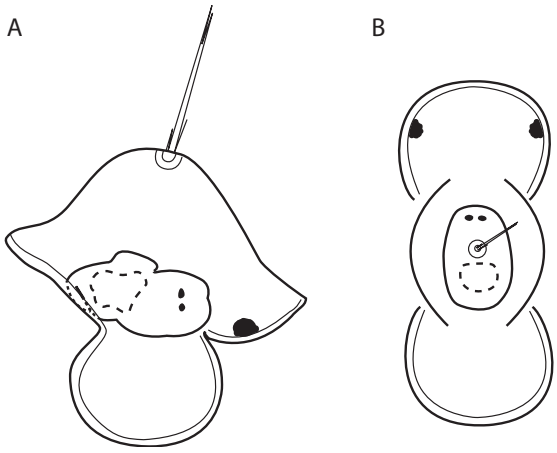

Figure 6.7. The larval morphotype that characterizes *Micrura wilsoni.* (A) Side view with juvenile anterior at right. (B) Apical view with juvenile anterior up.

Conversely, for *Micrura wilsoni,* we reveal a previously unknown, species-specific larval form characterized by the presence of two black pigment spots on the pilidial anterior lobe. To our knowledge, this particular larval morphotype has not been observed in any other nemertean species so far (Fig. 6.7). Although the larval body is shaped like a typical (i.e., hat-like) pilidium, the larvae of *M. wilsoni* have additional characteristics that combine to make a unique morphotype. First, the juvenile nemertean has assumed ocelli and a caudal cirrus. The presence of juvenile eyes and/or a caudal cirrus are not uncommon in other pilidium larvae (e.g., *Cerebratulus californiensis* (Fig. 6.8A–B), *L. flavescens* (Fig. 6.8C), *Lineus* sp. "red", this study), but the larval body in *M. wilsoni* also has pigment spots. Epidermal pigment spots, or chromatophores, occur in other pilidia, but they are usually more circular in shape and found on both larval lobes and lappets (Cantell, 1969; Lacalli, 2005; Schwartz, 2009; Dawydoff, 1940; T. Hiebert and Maslakova, in prep). Typically, there are also more than two spots. In fact, Cantell (1969) found that the number of chromatophores increased with age in *Tenuilineus albocinctus, Lineus bilineatus and M. purpurea*. In *M. wilsoni,* the size of the pigment spots increased over developmental time (compare Fig. 6.3C to 6.3H) and varied among individuals, but the number of spots did not increase with age (i.e., there were always two spots, one on either side of the anterior lobe). The only other locally-collected pilidium with pigment spots is the larva of *Cerebratulus californiensis*

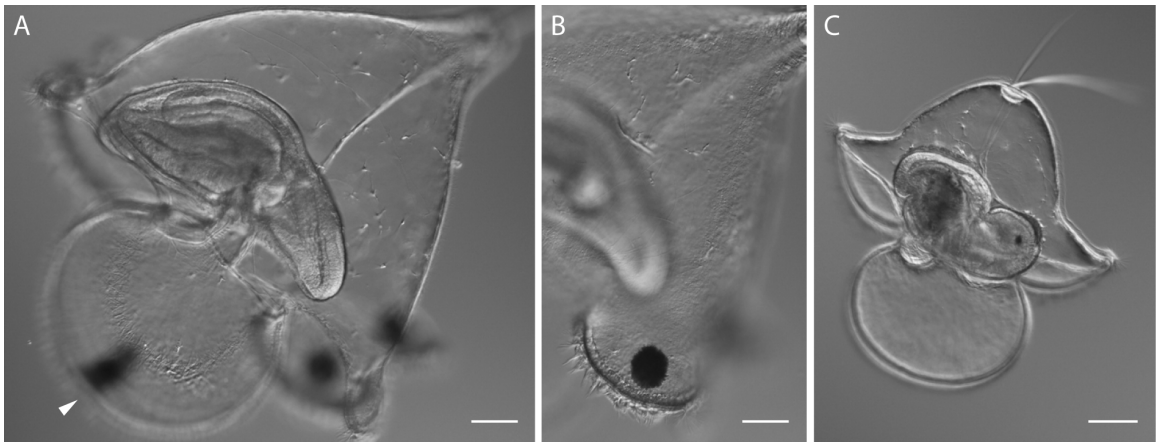

Figure 6.8. The larvaE of *Cerebratulus californiensis* (A–B) and *Lineus flavescens* (C) collected from plankton and identified using DNA sequence data. Apical organ is at upper right. Note large pyramidal episphere in *C. californiensis* and pigment spots on lateral lappets (arrowhead, A) and anterior larval lobe (in focus, B). The larva of *L. flavescens* shares larval characteristics with *Lineus sp.* "red" (compare C and Fig. 6.6F). All scale bars 100 μm.

(Maslakova and T. Hiebert, 2014; T. Hiebert and Maslakova, in prep, Fig. 6.8A–B), but the pigment spots in larvae of this species are present on both the lobes and lappets (Fig. 6.8A–B). These larvae are also characterized by a large pyramidal episphere and larval lobes that are often scalloped into 2–3 sub-lobes (Fig. 6.8A–B), characters not observed in *M. wilsoni* larvae. Thus, the combination of juvenile eyes, caudal cirrus, and the two pigment spots on the anterior lobe appears to be unique to *M. wilsoni*. However, the shape of *M. wilsoni* larvae is not necessarily unique, and the larval dimensions and body proportions most closely resemble other described larvae in this genus. Larvae of the genus *Cerebratulus* tend to have relatively large epispheres. Conversely, *Lineus* larvae often have larger lappets and relatively small epispheres (e.g., Cantell 1969; Lacalli, 2005; T. Hiebert and Maslakova, in prep). The larval body of *Micrura* species falls somewhere in between these two genera. However, concrete comparison of larval dimensions among pilidiophorans is currently lacking and the significance of assigning larval features to these non-monophyletic taxa based on body size and proportion alone requires further investigation. Finally, it is noteworthy that we observed what we believe to be juvenile ocelli in both *M. wilsoni* and *Lineus* sp. "red", which lack eyes as adults.

This is important because it means that the presence of juvenile eyes in a wild-caught pilidium larva does not necessarily indicate that the species possesses eyes as an adult.

BRIDGE TO CHAPTER VII

In Chapter VI, we described the development of two common nemertean species. The larvae of *Micrura wilsoni* are species-specific and, having not previously been observed from plankton samples, can now be identified based on morphology alone. However, the larvae of *Lineus* sp. "red" highlight another important feature of larval identification. These larvae do not exhibit species-specific morphology, but instead appear to characterize a clade of closely related species. In Chapter VII, we focus on this and other larval morphotypes in one clade of nemerteans – the Pilidiophora. We introduce several pilidial morphotypes, including the morphotype produced by *Lineus* sp. "red", that each characterize closely related clades of species. We also introduce larval morphotypes that are non-feeding, which is interesting because most pilidium larvae are feeding. Many of these non-feeding larvae do not characterize clades, but appear to have evolved independently several times. Chapter VII emphasizes the potential of larval characters to provide synapomorphies for new nemertean genera and, at the same time, discusses the evolutionary transitions from feeding to non-feeding pilidia.

CHAPTER VII
DIVERSITY AND EVOLUTION OF PILIDIAL LARVAL DEVELOPMENT (PILIDIOPHORA; NEMERTEA)

This chapter will be submitted to the *Journal of Zoological Systematics and Evolutionary Research* with co-author SA Maslakova. I collected many larvae, carried out molecular identification and phylogenetic analysis, drafted the manuscript, prepared figures and submitted sequences to GenBank. SA Maslakova collected larvae and carried out DNA identification of samples in the early stages of this project, and edited manuscript.

INTRODUCTION

Many marine invertebrates produce planktonic offspring. The parental investment and corresponding developmental modes of these invertebrates span a wide spectrum that can generally be split between larvae that are long-lived and feeding and those that are short-lived and non-feeding (but see intermediate developmental modes, e.g., Emlet 1986; Hart 1996; Allen and Pernet 2007). Larvae that are obligately feeding, or planktotrophic, cannot develop and metamorphose without acquisition of food. As such, they can be morphologically complex, with elaborate structures for capturing planktonic particles. Lecithotrophic, or non-feeding, larvae develop from fertilization to metamorphosis using nutrients provided by the mother, i.e., they develop from large nutrient-rich eggs (Strathmann 1985; Jaeckle 1995). The lack of need to feed obviates the need to have elaborate larval structures for gathering food, resulting in simplified morphology and a more direct development, overall (Strathmann 1978).

Both planktotrophic and lecithotrophic development can be found within marine invertebrate groups, often in very closely related taxa (e.g., *Heliocidaris* spp., Hart et al. 2011), and planktotrophy is generally believed to be the ancestral state in many phyla (Strathmann 1978, 1985). This is due, in part, to the structural complexity of feeding larvae, which as parsimony suggests (but see Strathmann and Eernisse 1994), is unlikely

to evolve many times independently. Vestigial feeding structures have been documented in lecithotrophic larvae (see Emlet 1995), which also supports the "planktotrophy ancestral" hypothesis. For example, the non-feeding metatrochophore larvae of *Schizobranchia insignis* possess a gut and opposed ciliary bands, a mechanism that concentrates phytoplankton cells in feeding larvae of related annelids, but lack a functional digestive system (Pernet 2003). Likewise, the gastropod, *Tritonia hombergii*, retains the ability to collect and ingest phytoplankton cells, but lacks the ability to digest them (Kempf and Todd 1989). It seems unlikely that a sophisticated particle-collecting mechanism would have evolved in the absence of the need to feed or the ability to digest food. Morphological similarity among lecithotrophic larvae found in distantly related groups (e.g., echinoderms, cnidarians) suggests that lecithotrophic larvae may resemble each other due to convergence and functional constraints on the morphology of free-swimming larvae (e.g., Emlet 1994; Wray 1996). The existence of such constraints would also explain similarity between lecithotrophic forms within a phylum as convergence. A clear evolutionary transition from planktotrophy to lecithotrophy is well-supported in some groups, e.g., echinoderms (Emlet 1990; Wray 1996; Smith 1997), polychaetes, gastropods, bivalves, crustaceans (Strathmann 1978, 1985). For example, planktotrophy is ancestral for temnopleurid sea urchins, with a single, clear transition to lecithotrophy in one lineage which contains 11 species (Jeffery et al. 2003). In other cases, lecithotrophic development appears to evolve from planktotrophic ancestors several times independently (e.g., *Patiriella*, Hart et al. 2003; calyptraeid gastropods, Collin 2004). However, the directionality from planktotrophy to lecithotrophy is less clear, and potentially more flexible, in other marine invertebrates (e.g., annelids, molluscs, Strathmann 1978; Salnini-Plawen 1985; Haszprunar et al. 1995; Kupriyanova 2003). In fact, an evolutionary transformation in the opposite direction has been suggested by Haszprunar et al. (1995) for all Bilateria. Collin (2004) produced a phylogeny that suggested three instances of evolution from direct development (with nurse eggs) to feeding larvae in calyptraeid gastropods. Furthermore, some groups have no feeding

larvae (e.g., sponges, hydrozoans, scyphozoans). Thus, it seems unlikely that planktotrophy is plesiomorphic for all marine invertebrate groups.

Nemerteans are lophotrochozoans, with close affinities to brachiopods, annelids and mollusks, as suggested by most recent molecular phylogenies of Metazoa (Dunn et al. 2008; Hejnol et al. 2009; Struck et al. 2014). Nemertean developmental modes vary from encapsulated embryos that develop into crawl-away juveniles and lecithotrophic planktonic larvae to the maximally indirect-developing planktotrophic pilidium larva (Norenburg and Stricker 2002; Maslakova 2010a, Maslakova and Hiebert 2014). Direct development, with lecithotrophic or planktotrophic (macrophagous) planuliform larvae, found in the basal Palaeonemertea and the Hoplonemertea, is thought to be ancestral for the phylum (Maslakova 2010a; Maslakova and Hiebert 2014). One clade of nemerteans, the Pilidiophora, apparently evolved a novel type of maximally-indirect development with a long-lived planktotrophic pilidium larva (Salensky 1912; Schmidt 1930; Maslakova 2010b; Hiebert and Maslakova 2015a). The typical pilidium feeds on unicellular algae which it gathers using a unique system of ciliary bands (von Dassow et al 2013). The juvenile develops inside the pilidium larva from a series of isolated rudiments, called imaginal discs, and erupts from the larval body in catastrophic metamorphosis. While the indirect-developing, planktotrophic pilidium larva is found in members of the Pilidiophora, members of its sister taxon, the Hoplonemertea (Thollesson and Norenburg 2003; Andrade et al. 2014), produce what are thought to be lecithotrophic and, superficially, direct-developing larvae (but see Maslakova and Hiebert 2014). While the development and feeding mode of the hoplonemertean-pilidiophoran ancestral larval form is not known, it is assumed that the planktotrophic pilidium is ancestral to the Pilidiophora (Maslakova and Hiebert 2014). Pilidial morphology varies from the most common hat-like form to those that are more mitten-like or sock-like (Fewkes 1883; Dawydoff 1940; Chernyshev 2001; Lacalli 2005; Maslakova 2010a; Hiebert et al. 2013; Maslakova and Hiebert 2014). It has been suggested that some of these less common forms may represent evolutionary intermediates between the ancestral planuliform-like

larva and the hat-like pilidium larva found in the largest number of pilidiophorans (Jägersten 1972). However, these hypotheses remain untested in the absence of a robust pilidiophoran phylogeny.

Intriguingly, lecithotrophy has also evolved within the Pilidiophora, where larvae superficially resemble their hoplonemertean relatives (see Figs. 7.1, 7.3B–C). Similar to the planktotrophic pilidia these so-called modified pilidia develop via imaginal discs and undergo catastrophic metamorphosis, but they possess greatly simplified morphology and do not feed (Desor 1848; Nusbaum and Oxner, 1913; Iwata 1958; Schwartz and Norenburg 2005; Schwartz 2009; von Döhren 2011; Maslakova and von Dassow 2012; Maslakova and Hiebert 2014). Only a decade ago it was thought that lecithotrophic development was rare among Pilidiophora (found in three species, two of which are closely related), but the most recent estimate, suggests that at least 20 pilidiophoran species exhibit or are expected to have lecithotrophic development (Chernyshev 2011; Maslakova and Hiebert 2014 and references therein, this study). Preliminary evidence suggests multiple instances of evolution of lecithotrophy within the Pilidiophora (Schwartz 2009; Maslakova and Hiebert 2014). The sister relationship of the Fam. Hubrechtiidae (characterized by a planktotrophic pilidium) to the rest of the Pilidiophora (Thollesson and Norenburg 2003; Andrade et al. 2014) also suggests that planktotrophy is ancestral, and that lecithotrophy has evolved secondarily. There is currently no robust phylogeny for the Pilidiophora (comprising approximately 450 described species, Kajihara et al. 2008) and the development of few pilidiophorans is known. We have recently identified the larvae of over 30 heteronemertean species (Hiebert and Maslakova, in prep; see Chapter IV) and, here, we use these data coupled with molecular phylogenies of the group to investigate the evolution of larval form within the Pilidiophora. Specifically, we attempt to determine 1) the ancestral pilidiophoran larval form, 2) the number of instances lecithotrophy has evolved within the Pilidiophora and 3) whether certain other pilidial larval morphotypes resemble each other due to homology or convergence (i.e., characterize pilidiophoran clades).

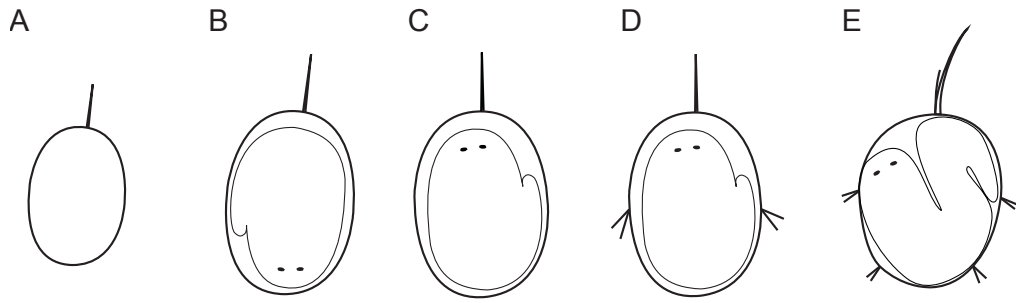

Figure 7.1. A schematic illustrating lecithotrophic larval forms in the Pilidiophora. A) Young lecithotrophic larvae are oblong and uniformly ciliated with an apical tuft at larval anterior (e.g., Heteronemertea gen. sp. 7 and 14). B–C) Juvenile body AP axis can be opposite (B, e.g., Heteronemertea gen. sp. 1, 2, 3, *Micrura akkeshiensis*) or parallel to (C, e.g., *M.* sp. 803, *M. verrilli, Lineus viridis, L. ruber*) the larval body axis (where apical tuft is up). D–E) Lecithotrophic larvae occasionally have one (D, e.g., *M. rubramaculosa*) or two (E, e.g., *Cerebratulus longiceps*, *M.* sp. "dark", *M*. sp. 3, *M*. sp. 4, *M*. sp. "albocephala") transverse and equatorial ciliated bands made of longer cilia, in addition to shorter cilia covering the rest of the body. Note that juvenile eyes are shown to indicate juvenile anterior and are not necessarily present in these species.

METHODS

Wild-caught larvae

Larvae were collected from plankton samples and matched to nemertean adults using DNA sequence data, or reared from known adult species in the lab (see Hiebert et al. 2013; Hiebert and Maslakova 2015b, c). Most nemertean larvae were collected from plankton samples gathered off the Charleston Marina docks or in the Charleston Channel in Charleston, OR from 2008–2015 by T. Hiebert, or other students and faculty at the Oregon Institute of Marine Biology, especially Drs. S. Maslakova, G. von Dassow, R. Emlet and L. Hiebert (see Maslakova and von Dassow 2012; Hiebert et al. 2013; Maslakova and Hiebert 2014; Hiebert and Maslakova 2015c; Chapter IV in this dissertation). Additional nemertean larvae were collected from Vostok Bay (Sea of Japan) by Dr. A.Chernyshev (Fig. 7.2), Victoria (southern Australia) by Dr. R. Emlet (Fig. 7.3), Bocas del Toro (Panama) and Bay of Panama (Panama) by Drs. R. Emlet and S. Maslakova (not shown). Samples collected in Oregon were obtained with a 0.5-m diameter plankton net with 153 μm mesh size (SeaGear) and stored in 1-gal glass jars.

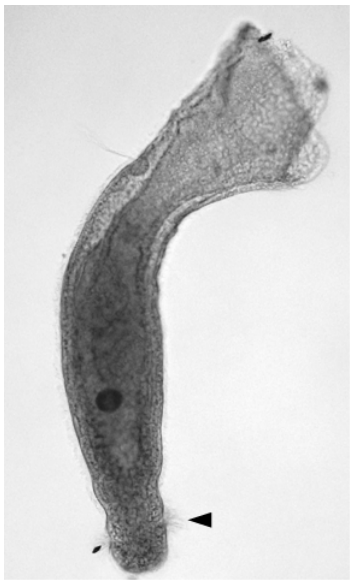

Figure 7.2. Preserved larva of *Riserius* sp. 4 collected from Vostok Bay (Sea of Japan) by A. Chernyshev; note the transverse ciliated band (arrowhead). Larva is approximately 650–700 µm tall (scale unknown). Photo by A.Chernyshev.

Pilidium larvae were sorted from each plankton sample, photographed, and either immediately preserved (cryopreserved at -80˚C in filtered seawater (~0.45 µm, FSW) or at room temperature in 95% EtOH) in 1.5 ml centrifuge tubes or maintained live (up to two weeks) as many nemertean larvae acquire more distinctive characteristics in later developmental stages. Planktotrophic larvae collected at early developmental stages were often maintained on a diet of the cryptomonad *Rhodomonas lens* PASCHER & RUTTNER (CCMP739) in a 150 ml glass custard dishes partly submerged in a sea table with flow through seawater (9–12˚C). Live larvae were photographed after collection and/or periodically, as development progressed, and prior to cryopreservation. Images were acquired from larvae that were in a small drop of FWS on a glass slide, covered, and gently trapped with a glass coverslip supported by small clay feet at the corners (Strathmann 1987). Photographs were obtained using an Olympus BX51 compound microscope equipped with DIC, using a Leica DFC400 camera and accompanying software.

DNA extraction and PCR

DNA extraction was carried out using a Chelex-based method (InstaGene, BioRad) with initial incubation at 56˚C for 30 min followed by a short (8 min) incubation at 98˚C. Larvae preserved in Ethanol were briefly rinsed in nuclease-free water prior to extraction. We amplified regions of two mitochondrial genes, 16S ribosomal DNA (460 bp, 16S), cytochrome c oxidase subunit I (658 bp, COI) and one nuclear gene, 28S ribosomal DNA (~1100 bp, 28S). PCR amplification was carried out with the following "universal" primers: 16SARL [5‘ CGCCTGTTTATCAAAAACAT 3’] and 16S BRH [5’

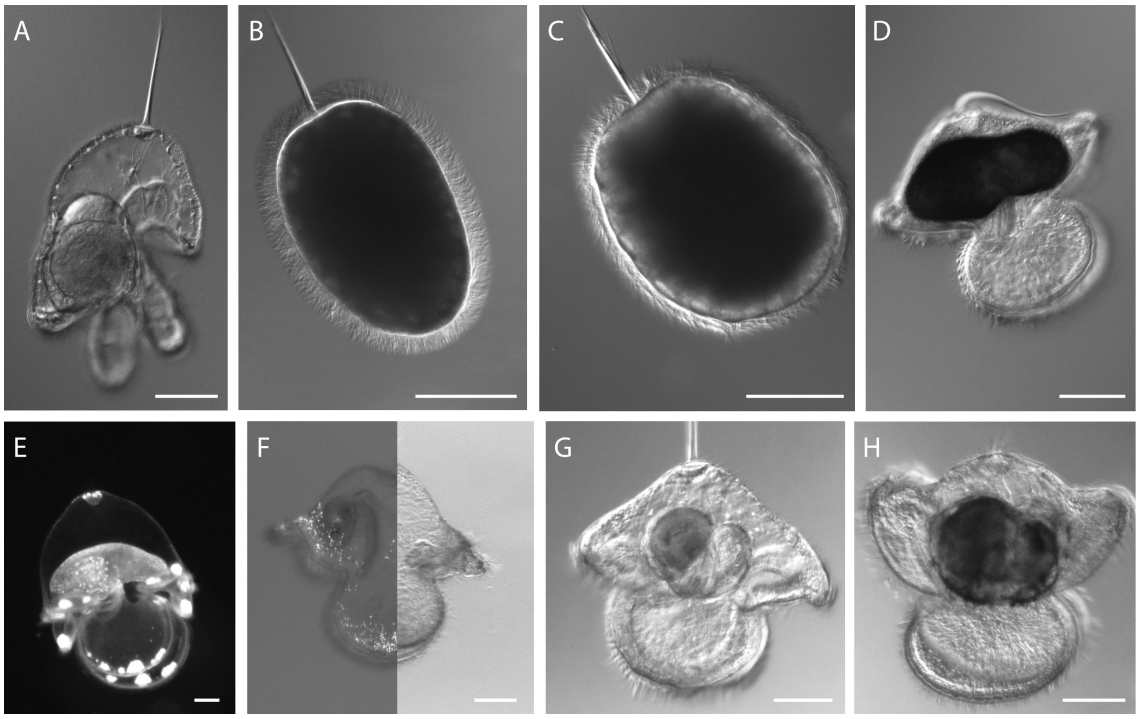

Figure 7.3. Pilidiophoran larvae collected from southern Australia by R. Emlet include *Hubrechtella* sp. (A), Heteronemertea gen. sp. 7 (B), Heteronemertea gen. sp. 14 (C), Heteronemertea gen. sp. 9 (D), Heteronemertea gen. sp. 11 (E), Heteronemertea gen. sp. 15 (F), Heteronemertea gen. sp. 16 (G), Heteronemertea gen. sp. 18 (H). All scale bars 100 µm. Photos by R. Emlet.

CCGGTCTGAACTCAGATCACGT 3'] (Palumbi et al. 1991) for 16S; LCO 1490 [5' GGTCAACAAATCATAAAGATATTGG 3'] and HCO 2198 [5' TAAACTTCAGGGTGACCAAAAAATCA 3'] (Folmer et al. 1994) for COI; and LSU5 [5' ACCCGCTGAAYTTAAGCA 3'] and LSU3 [5' TCCTGAGGGAAACTTCGG 3'] (Littlewood 1994) for 28S. Higher quality amplification was occasionally achieved by pairing nemertean-specific reverse primers (16SKR [5' AATAGATAGAAACCAACCTGGC 3'], COIDr [5' GAGAAATAATACCAAAACCAGG 3'] (Norenburg, unpublished)) with corresponding universal forward primers. PCR thermocycling was carried out using 1–8 µls of DNA extract in a 20 µl reaction with the following parameters: 95˚C initial denaturation for 2 min, 35 cycles of 95˚C for 40 s, 45–55˚C for 40 s and a 60 s extension at 72˚C. Following the last cycle there was an additional 2 minutes at 72˚C for final extension after which products were stored at 4˚C. PCR products were purified using Wizard SV

Gel and PCR Cleanup kit (Promega) and sequenced (Sequetech Inc, Mountain View, CA) in both directions using PCR primers. Sequences were trimmed to remove primers, assembled into contigs, proofread for quality using Geneious v.7.0.6, and deposited in Genbank (Accession #s KU365664–724, Table 7.1).

Phylogenetic analysis

The developmental mode is known for 79 pilidiophoran species, including 32 species whose development was described by others, and 47 whose development was described by us in this or previous work (Table 7.1). Lecithotrophic development is presumed (but not known) for an additional two species with large oocyte diameter, which were included in our analysis (Lineidae gen. sp. "large eggs" and *Micrura* sp. "not coei", see Maslakova and Hiebert 2014). In addition to sequences obtained in this study, phylogenetic analysis included additional pilidiophoran species with known development, whose sequences were available in GenBank (see Table 7.1). COI, 16S and 28S sequences were aligned using ClustalW (gap opening and extension costs set to default parameters, 15 and 6.66, respectively) as implemented in Geneious v 7.0.6. Alignments were trimmed to remove missing data: the 16S alignment trimmed to 558, COI to 657, 28S to 1,097, 16S + 28S to 1,654 and 16S + 28S + COI to 2,310 base pairs. Phylogenetic analyses were carried out on 16S, 28S, COI datasets separately, as well as on concatenated datasets (16S + 28S and 16S + 28S + COI). Phylogenetic analyses included 68, 51, 56, 52, and 47 sequences for 16S, 28S, COI, and concatenated datasets (16S + 28S and 16S + 28S + COI), respectively (see Table 7.1). Appropriate evolutionary models were determined using jModel Test v. 2.1.7 (Posada 2008). The General Time Reversal (GTR) evolutionary model (Tavare 1986) was the best fit model for 16S, 28S and both concatenated datasets, while the Timura-Nei (TN93) (Tamura-Nei, 1993) was selected for the COI gene region. Maximum likelihood phylogenetic analysis was carried using PhyML and default parameters coupled with appropriate evolutionary models for each gene region. Clade support was estimated using 1,000 bootstrap replicates (Felsenstein 1985). Bayesian phylogenetic analyses were conducted in MrBayes v 3.2.1

Table 7.1. Pilidiophoran species with known development, including all samples obtained in this study, as well as those from the literature. GenBank accession numbers provided for all samples with sequence data. Collection information and oocyte diameter (where known) are included.

Species		GenBank Accession # (s)			Collection Information (Reference)	Development (reference)
		16S	COI	28S		
Hubrechtella dubia	-	JF277630	HQ848631	-	Tjärnö, Koster, Sweden (Andrade et al. 2012)	planktotrophic pilidium (Cantell 1969)
Hubrechtella dubia	-	-	-	AJ436889	Fort Pierce, FL USA (Thollesson and Norenburg 2003)	planktotrophic pilidium (Cantell 1969)
Hubrechtella juliae	E1H9	KU197433	KU197756	KU365698	Charleston, OR USA (T. Hiebert, this study)	planktotrophic pilidium (Hiebert and Maslakova, unpublished)
Hubrechtella sp.	SB	KU365679	-	-	Bay of Panama, Panama (R. Emlet, this study)	planktotrophic pilidium (This study, Fig. 7.3A)
Baseodiscus punnetti	E1F1	KU197367	KU197712	KU365681	Santa Barbara, CA USA (L. Friesen, this study)	planktotrophic pilidium (Schwartz 2009)
Cerebratulus albifrons	1	KU197368	KU197713	-	Charleston, OR USA (S. Maslakova, this study)	planktotrophic pilidium (Hiebert and Maslakova, unpublished)
Cerebratulus californiensis	E3G5	KU197390	KU197726	KU365682	Charleston, OR USA (T. Hiebert & S. Maslakova, this study)	planktotrophic pilidium (Coe 1940; pers obs)
Cerebratulus cf. *marginatus*	E3C3	KU197404	KU197738	KU365683	Charleston, OR USA (T. Hiebert, this study)	planktotrophic pilidium (Coe 1899; Hiebert and Maslakova, unpublished)
Cerebratulus lacteus	-	JF277575	HQ848576	HQ856857	Fort Pierce, FL USA (Andrade et al. 2012)	planktotrophic pilidium (Wilson 1898)
Cerebratulus longiceps	5	(unpublished)	-	-	Charleston, OR USA (M. Hunt, unpublished)	lecithotrophic free-swimming larva (Hunt and Maslakova, unpublished)

Species		GenBank Accession # (s)			Collection Information (Reference)	Development (reference)
		16S	COI	28S		
Cerebratulus montgomeryi	-	EF124875	-	EF178489	Canada (Schwartz and Norenburg, unpublished)	planktotrophic pilidium (Coe 1940)
Cerebratulus montgomeryi	71	-	KU197742	-	Cattle Point, San Juan Island, WA USA (S. Malsakova, this study)	planktotrophic pilidium (Coe 1940)
Cerebratulus pantherinus	-	-	-	-	-	planktotrophic pilidium (Schmidt 1931)
Cerebratulus sp. 1	E1I1	KU197412	KU197743	KU365684	Charleston, OR USA (T. Hiebert, this study)	planktotrophic pilidium (Hiebert and Maslakova, unpublished)
Cerebratulus sp. pink proboscis	E3G4	KU197419	KU197747	KU365685	Charleston, OR USA (T. Hiebert, this study)	planktotrophic pilidium (Hiebert and Maslakova, unpublished)
Cerebratulus sp. spade head	E1G5	KU197426	KU197752	KU365686	Charleston, OR USA (T. Hiebert, this study)	planktotrophic pilidium (Hiebert and Maslakova, unpublished)
Cerebratulus sp. Sunset Bay	E4G9	KU197428	KU197755	KU365687	Charleston, OR USA (T. Hiebert, this study)	planktotrophic pilidium (Hiebert and Maslakova, unpublished)
Euborlasia elisabethea	-	-	-	-	-	planktotrophic pilidium (Schmidt 1931)
Heteronemertea gen. sp. 1	E2D3	KU197540	-	KU365688	Charleston, OR USA (T. Hiebert & S. Maslakova, this study)	lecithotrophic free-swimming larva (Maslakova and Hiebert 2014)
Heteronemertea gen. sp. 1	E1H4	-	-	KU365720	Charleston, OR USA (T. Hiebert & S. Maslakova, this study)	lecithotrophic free-swimming larva (Maslakova and Hiebert 2014)

Species		GenBank Accession # (s)			Collection Information (Reference)	Development (reference)
		16S	COI	28S		
Heteronemertea gen. sp. 10	P3	KU365668	KU365722	KU365693	Panama (S. Maslakova, this study)	planktotrophic pilidium (This study)
Heteronemertea gen. sp. 11	RE91	KU365669	-	-	Victoria, Australia (R. Emlet, this study)	planktotrophic pilidium (This study, Fig. 7.3E)
Heteronemertea gen. sp. 12	E5D8	KU365670	-	KU365694	Vostok Bay, Sea of Japan, Russia (A. Chernyshev, this study)	planktotrophic pilidium (This study)
Heteronemertea gen. sp. 13	E5E4_10	KU365671	-	KU365695	Vostok Bay, Sea of Japan, Russia (A. Chernyshev, this study)	planktotrophic pilidium (This study)
Heteronemertea gen. sp. 14	RE262	KU365672	-	KU365696	Victoria, Australia (R. Emlet, this study)	lecithotrophic free-swimming larva (This study, Fig. 7.3C)
Heteronemertea gen. sp. 15	RE37	KU365673	-	-	Victoria, Australia (R. Emlet, this study)	planktotrophic pilidium (This study, Fig. 7.3F)
Heteronemertea gen. sp. 16	RE188	KU365674	-	-	Victoria, Australia (R. Emlet, this study)	planktotrophic pilidium (This study, Fig. 7.3G)
Heteronemertea gen. sp. 17	E5D9	KU365675	KU365723	KU365697	Vostok Bay, Sea of Japan, Russia (A. Chernyshev, this study)	planktotrophic pilidium (This study)
Heteronemertea gen. sp. 18	RE197	KU365676	-	-	Victoria, Australia (R. Emlet, this study)	planktotrophic pilidium (This study, Fig. 7.3H)
Heteronemertea gen. sp. 19	E5D2	KU365677	KU365724	-	Vostok Bay, Sea of Japan, Russia (A. Chernyshev, this study)	planktotrophic pilidium (This study)

Species		GenBank Accession # (s)			Collection Information (Reference)	Development (reference)
		16S	COI	28S		
Heteronemertea gen. sp. 2	E1H6	KU197541	KU197831	-	Charleston, OR USA (T. Hiebert & S. Maslakova, this study)	lecithotrophic free-swimming larva (Maslakova and Hiebert 2014)
Heteronemertea gen. sp. 20	E5D4	KU365678	-	-	Vostok Bay, Sea of Japan, Russia (A. Chernyshev, this study)	planktotrophic pilidium (This study)
Heteronemertea gen. sp. 3	E1G9	KU197542	KU197832	KU365689	Charleston, OR USA (T. Hiebert, this study)	lecithotrophic free-swimming larva (Maslakova and Hiebert 2014)
Heteronemertea gen. sp. 4	119	KU197548	KU197835	KU365690	Charleston, OR USA (T. Hiebert, this study)	planktotrophic pilidium (This study)
Heteronemertea gen. sp. 5	76	KU197543	-	-	Charleston, OR USA (M. Jarvis, this study)	planktotrophic pilidium (This study)
Heteronemertea gen. sp. 6	128	KU365664	-	-	Charleston, OR USA (S. Maslakova, this study)	planktotrophic pilidium (This study)
Heteronemertea gen. sp. 7	RE63	KU365665	-	KU365691	Victoria, Australia (R. Emlet, this study)	lecithotrophic free-swimming larva (This study, Fig. 7.3B)
Heteronemertea gen. sp. 8	P2	KU365666	KU365721	-	Panama (S. Maslakova, this study)	planktotrophic pilidium (This study)
Heteronemertea gen. sp. 8	P1	-	-	KU365692	Panama (S. Maslakova, this study)	planktotrophic pilidium (This study)
Heteronemertea gen. sp. 9	RE304	KU365667	-	-	Victoria, Australia (R. Emlet, this study)	planktotrophic pilidium (This study, Fig. 7.3D)
Lineidae gen. sp. “large eggs”	90	KU197435	-	-	Charleston, OR USA (S. Maslakova, this study)	lecithotrophic development (Hiebert and Maslakova, unpublished)

Species		GenBank Accession # (s)			Collection Information (Reference)	Development (reference)
		16S	COI	28S		
Lineidae gen. sp. “large eggs”	E1B9	-	KU197761	KU365699	Charleston, OR USA (S. Maslakova, this study)	lecithotrophic development (Hiebert and Maslakova, unpublished)
Lineus alborostratus	-	AJ436822	AJ436932	AJ436877	Vostok Bay, Sea of Japan, Russia (Thollesson and Norenburg 2003)	planktotrophic pilidium (Iwata 1960)
Lineus flavescens	-	KP682165	KP862050	-	Charleston, OR USA (Hiebert and Maslakova 2015b)	planktotrophic pilidium (Hiebert and Maslakova, unpublished)
Lineus flavescens	E3A1	-	-	KU365700	Charleston, OR USA (T. Hiebert, this study)	planktotrophic pilidium (Hiebert and Maslakova, unpublished)
Lineus ruber	-	AF103758	-	-	western coast, Sweden (Sundberg and Saur 1998)	adelphophagous encapsulated Schmidt's larva (Desor 1848; Schmidt 1934, Martín-Durán et al. 2015)
Lineus ruber	-	-	GU733828	-	Wales, UK (Chen et al. 2010)	adelphophagous encapsulated Schmidt's larva (Desor 1848; Schmidt 1934, Martín-Durán et al. 2015)
Lineus rubescens	-	-	-	-	-	planktotrophic pilidium (Schwartz 2009)
Lineus sp. “crescent”	E2H3	KU197515	KU197814	KU365703	Charleston, OR USA (T. Hiebert & S. Maslakova, this study)	planktotrophic pilidium (Hiebert and Maslakova, unpublished)
Lineus sp. “red”	E2C8	KU197521	KU197819	KU365704	Charleston, OR USA (T. Hiebert, this study)	planktotrophic pilidium (Hiebert and Maslakova, in press)

Species		GenBank Accession # (s)			Collection Information (Reference)	Development (reference)
		16S	COI	28S		
Lineus sp. 1	E1G3	KU197484	KU197799	KU365701	Charleston, OR USA (T. Hiebert, this study)	planktotrophic pilidium (Hiebert and Maslakova, unpublished)
Lineus sp. 2	E1I8	KU197486	KU197801	KU365702	Charleston, OR USA (R. Emlet, this study)	planktotrophic pilidium (Hiebert and Maslakova, unpublished)
Lineus torquatus	-	JF277572	HQ848574	HQ856856	Akkeshi Bay, Japan (Andrade et al. 2012)	planktotrophic pilidium (Iwata 1957)
Lineus viridis	-	JF277582	HQ848579	HQ856854	Sylt Island, Germany (Andrade et al. 2012)	lecithotrophic encapsulated Desor's larva (Desor 1848, von Döhren 2011)
Lineus viviparous	-	-	-	-	-	viviparous (Isler 1900)
Maculaura alaskensis	E3B7	KP682197	KP682071	KU365705	Gearhart, OR USA (Hiebert and Maslakova 2015b)	planktotrophic pilidium (Maslakova 2010)
Maculaura aquilonia	E2G3	KP682245	KP682127	KU365706	Charleston, OR USA (Hiebert and Maslakova 2015b)	planktotrophic pilidium (Hiebert and Maslakova 2015)
Maculaura cerebrosa	E1A8	KP682251	KP682123	KU365707	Crescent City, CA USA (Hiebert and Maslakova 2015b)	planktotrophic pilidium (Hiebert and Maslakova 2015)
Maculaura magna	E1A2	KP682271	KP682150	KU365708	Charleston, OR USA (Hiebert and Maslakova 2015b)	planktotrophic pilidium (Hiebert and Maslakova 2015)
Maculaura oregonensis	E4A2	KP682297	KP682158	KU365709	Charleston, OR USA (Hiebert and Maslakova 2015b)	planktotrophic pilidium (presumed, Hiebert and Maslakova 2015)
Micrura akkeshiensis	-	EF124887	-	-	Akkeshi, Japan (Schwartz and Norenburg, unpublished)	lecithotrophic modified development (Iwata 1958)

Species		GenBank Accession # (s)			Collection Information (Reference)	Development (reference)
		16S	COI	28S		
Micrura caeca	-	-	-	-	-	planktotrophic pilidium (Verrill 1892)
Micrura kulikovae	-	-	-	-	-	lecithotrophic modified development (Chernyshev 2011)
Micrura leydii	-	-	-	-	-	planktotrophic pilidium (Verrill 1892)
Micrura pardalis	-	-	-	-	-	planktotrophic pilidium (Coe 1940)
Micrura purpurea	-	JF277577	HQ848586	HQ856845	Tjärnö, Skagerak, Sweden (Andrade et al. 2012)	planktotrophic pilidium (Cantell 1969)
Micrura rubramaculosa	-	KF935460	KF935513	KF935349	Bocas del Toro, Panama (Schwartz and Norenburg 2005)	lecithotrophic free-swimming larva (Schwartz and Norenburg 2005)
Micrura sp. “albocephala”	E3A9	KU197574	KU197849	KU365712	Charleston, OR USA (T. Hiebert & S. Maslakova, this study)	lecithotrophic free-swimming larva (Hunt and Maslakova, unpublished)
Micrura sp. “dark”	E2I1	KU197586	KU197858	KU365713	Charleston, OR USA (T. Hiebert & M. Hunt, this study)	lecithotrophic free-swimming larva (Maslakova and von Dassow 2012)
Micrura sp. “not coei”	E4H8	KU197525	KU197826	-	Charleston, OR USA (T. Hiebert & S. Maslakova, this study)	presumed lecithotrophic development (Hiebert and Maslakova, unpublished)
Micrura sp. “not coei”	E5B1_C	-	-	KU365714	Charleston, OR USA (M. Hunt, this study)	presumed lecithotrophic development (Hiebert and Maslakova, unpublished)
Micrura sp. 3	E1H8	KU197563	KU197841	KU365710	Charleston, OR USA (T. Hiebert, this study)	lecithotrophic free-swimming larva (Hunt and Maslakova, unpublished)

Species		GenBank Accession # (s)			Collection Information (Reference)	Development (reference)
		16S	COI	28S		
Micrura sp. 4	E3B2	KU197581	KU197857	KU365711	Charleston, OR USA (T. Hiebert, this study)	lecithotrophic free-swimming larva (Hunt and Maslakova, unpublished)
Micrura sp. 676	-	-	-	EF124946	Carrie Bow Cay, Belize (Schwartz and Norenburg, unpublished)	lecithotrophic free-swimming larva (Schwartz 2009)
Micrura sp. 803	-	EF124895		EF124943	Carrie Bow Cay, Belize (Schwartz and Norenburg, unpublished)	lecithotrophic free-swimming larva (Schwartz 2009)
Micrura verrilli	E3A4	KU197528	-	KU365715	Charleston, OR USA (T. Hiebert & S. Maslakova, this study)	lecithotrophic free-swimming larva (Schwartz 2009)
Micrura verrilli	-	-	KF935508	-	USA (Schwartz and Norenburg, unpublished)	lecithotrophic free-swimming larva (Schwartz 2009)
Micrura wilsoni	E1E9	KU197531	KU197827	-	Charleston, OR USA (L. Hiebert, this study)	planktotrophic pilidium (Hiebert and Maslakova, 2015c)
Micrura wilsoni	MMB25	-	-	KU365716	Charleston, OR USA (T. Hiebert, this study)	planktotrophic pilidium (Hiebert and Maslakova, 2015c)
Parborlasia corrugatus	-	AJ436829	AJ436939	AJ436884	McMurdo Sound, Antarctica (Thollesson and Norenburg 2003)	planktotrophic pilidium (Peck 1983)
Parvicirrus dubius	-	AJ436830	AJ436940	AJ436885	Georgetown, ME USA (Thollesson and Norenburg 2003)	planktotrophic pilidium (Riser 1993)
Ramphogordius sanguineus	E2G7	KU197554	KU197838	KU365717	Charleston, OR USA (T. Hiebert, this study)	planktotrophic pilidium and asexual reproduction (Coe 1931)

Species		GenBank Accession # (s)			Collection Information (Reference)	Development (reference)
		16S	COI	28S		
Ramphogrodius lacteus	-	JF277584	HQ848583	HQ856850	Brittany, France (Andrade et al. 2012)	planktotrophic pilidium (Metschnikoff 1869)
Riserius sp. “eyes”	153	KC777029	KC777038	KU365719	Charleston, OR USA (Hiebert et al. 2013, this study)	planktotrophic pilidium (Hiebert et al. 2013)
Riserius sp. “no eyes”	181	KC777026	KC777034	-	Charleston, OR USA (Hiebert et al. 2013)	planktotrophic pilidium (Hiebert et al. 2013; von Dassow, unpublished)
Riserius sp. 3	E5D1	KU365680	-	-	Vostok Bay, Sea of Japan, Russia (A. Chernyshev, this study)	planktotrophic pilidium (This study)
Riserius sp. 4	E4H6	-	-	KU365718	Vostok Bay, Sea of Japan, Russia (A. Chernyshev, this study)	planktotrophic pilidium (This study, Fig. 7.3)
Tenuilineus albocinctus	-	-	-	EF124959	Belize (Schwartz and Norenburg, unpublished)	planktotrophic pilidium (Cantell 1969)
Tenuilineus bicolor	-	-	-	EF124960	Fort Pierce, FL USA (Schwartz and Norenburg, unpublished)	planktotrophic pilidium (Schwartz 2009)
Tenuilineus bilineatus	-	-	-	-	-	planktotrophic pilidium (Cantell 1969)
Zygeupolia rubens	-	JF277574	HQ848585	HQ856861	Fort Pierce, FL USA (Andrade et al. 2012)	planktotrophic pilidium (Turbeville in Schwartz 2009)

(Ronquist et al. 2012), with the same evolutionary models selected in jModel Test for each gene region or dataset. Markov chain Monte Carlo (MCMC) parameters were set to 1,000,000 generations, 1000 generation sampling with first 25% discarded as burn-in. Gene tree topologies were viewed in Geneious (Biomatters Ltd). Character reconstruction was carried out on the above phylogenies in Mesquite v. 3.03 (Maddison and Maddison 2002) using maximum parsimony. Sequences from all three gene regions were concatenated in Geneious v. 7.0.6. Phylogenetic analysis and character reconstruction was carried out on concatenated datasets as described above. We selected hubrechtid species (*Hubrechtella dubia*, *H. juliae*) as our outgroup based on the sister position of *Hubrechtella* to the Heteronemertea on molecular phylum-level molecular phylogenies (e.g., Thollesson and Norenburg 2003; Andrade et al. 2014).

RESULTS

New reports of lecithotrophic pilidiophoran larvae

Lecithotrophic pilidiophoran larvae differ from each other in patterns of ciliation (uniform vs. possessing one or two transverse ciliary bands), and orientation of the juvenile AP axis with respect to the larval axis (Fig. 7.1). A total of 17 species are currently reported to possess lecithotrophic pilidiophoran larvae: we counted 16 species in a recent review (Maslakova and Hiebert 2014), and one other, *Micrura kulikovae*, is reported by Chernyshev (2011). Here we report an additional three species, based on distinct molecular "barcodes": the larvae of Heteronemertea gen. sp. 7 and 14 collected by R. Emlet in southern Australia, and the larva of *Cerebratulus longiceps* collected and identified by M. Hunt (pers. communication) from Oregon. Australian larvae were collected at early developmental stages (Fig. 7.1A, Figs. 7.3B–C), thus the orientation of the juvenile antero-posterior axis with respected to the larval axis is not known. Our analyses do not always agree on whether distinct morphotypes of lecithotrophic larvae have phylogenetic significance (i.e., certain types appear to characterize clades in some of our analyses, but not others; see Figs. 7.4–7.8).

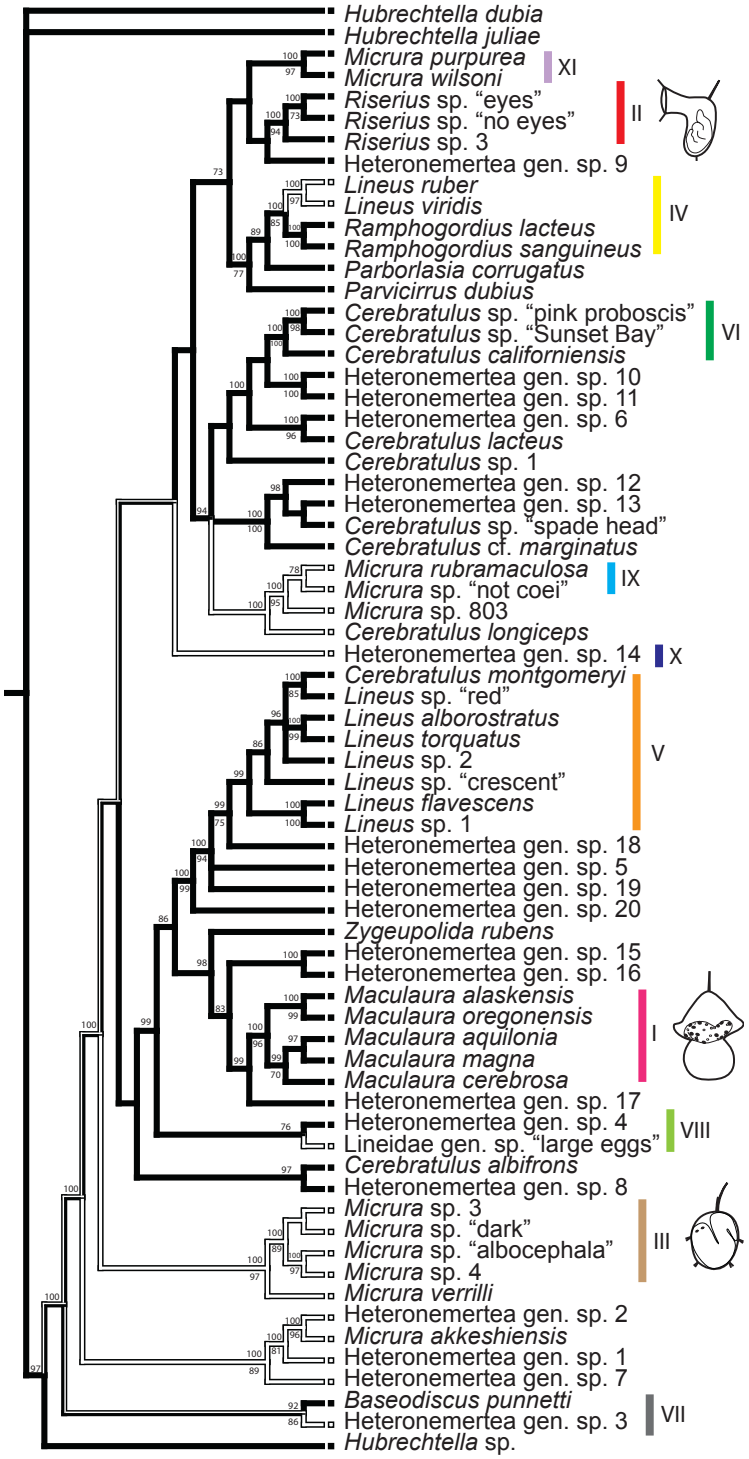

Figure 7.4. 16S Bayesian phylogeny of the Pilidiophora. Lecithotrophic lineages are shown in white and planktotrophic in black. Clade support is indicated by bayesian posterior probabilities (upper number) and bootstrap values (lower number). Clade support values below 70 are not shown. Consistent clades are indicated with roman numerals and colored bars, for ease of comparison between gene regions; larval diagrams that we believe to characterize clades are shown.

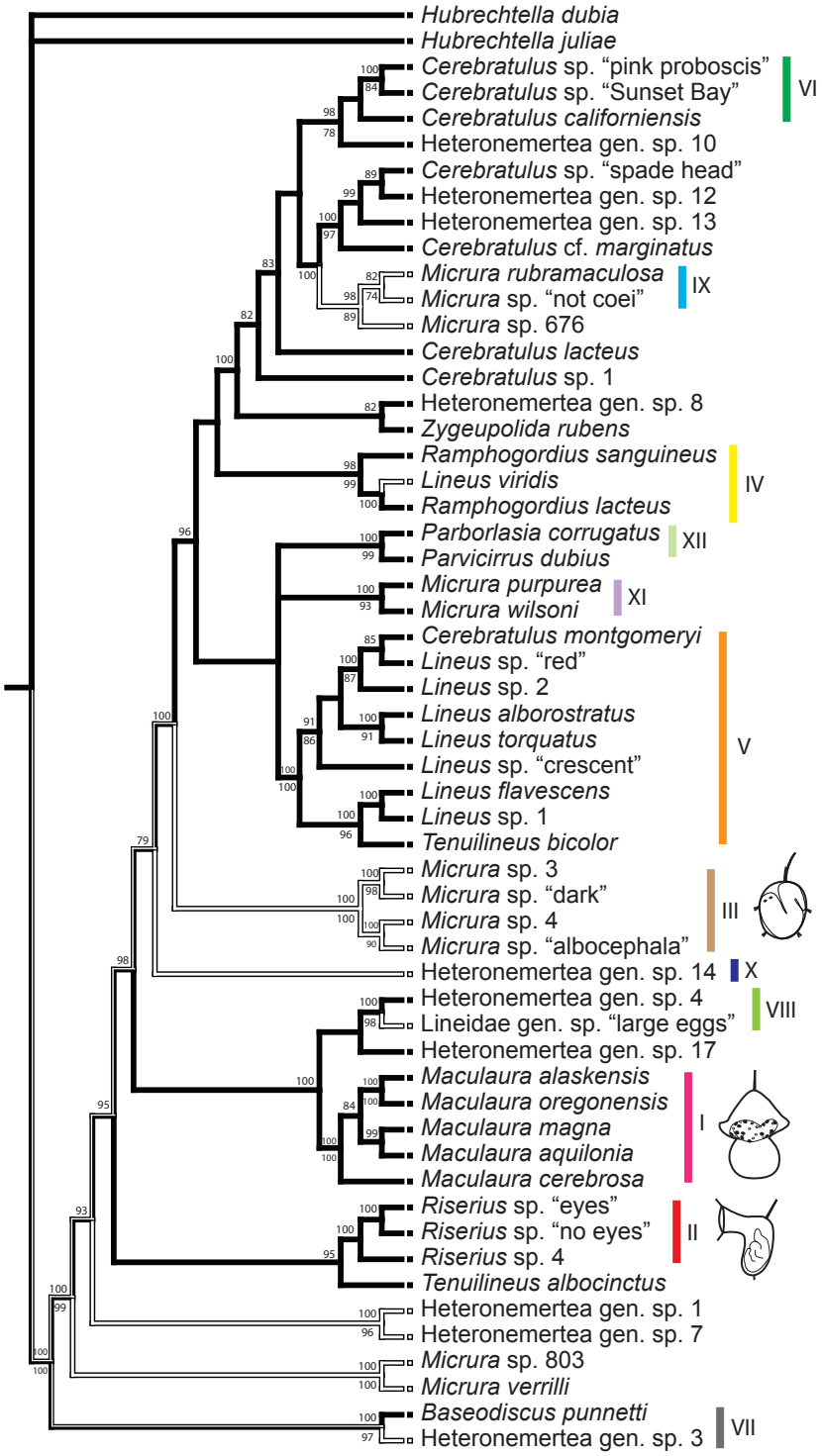

Figure 7.5. 28S Bayesian phylogeny of the Pilidiophora. Lecithotrophic lineages are shown in white and planktotrophic in black. Clade support is indicated by bayesian posterior probabilities (upper number) and bootstrap values (lower number). Clade support values below 70 are not shown. Consistent clades are indicated with roman numerals and colored bars, for ease of comparison between gene regions; larval diagrams that we believe to characterize clades are shown.

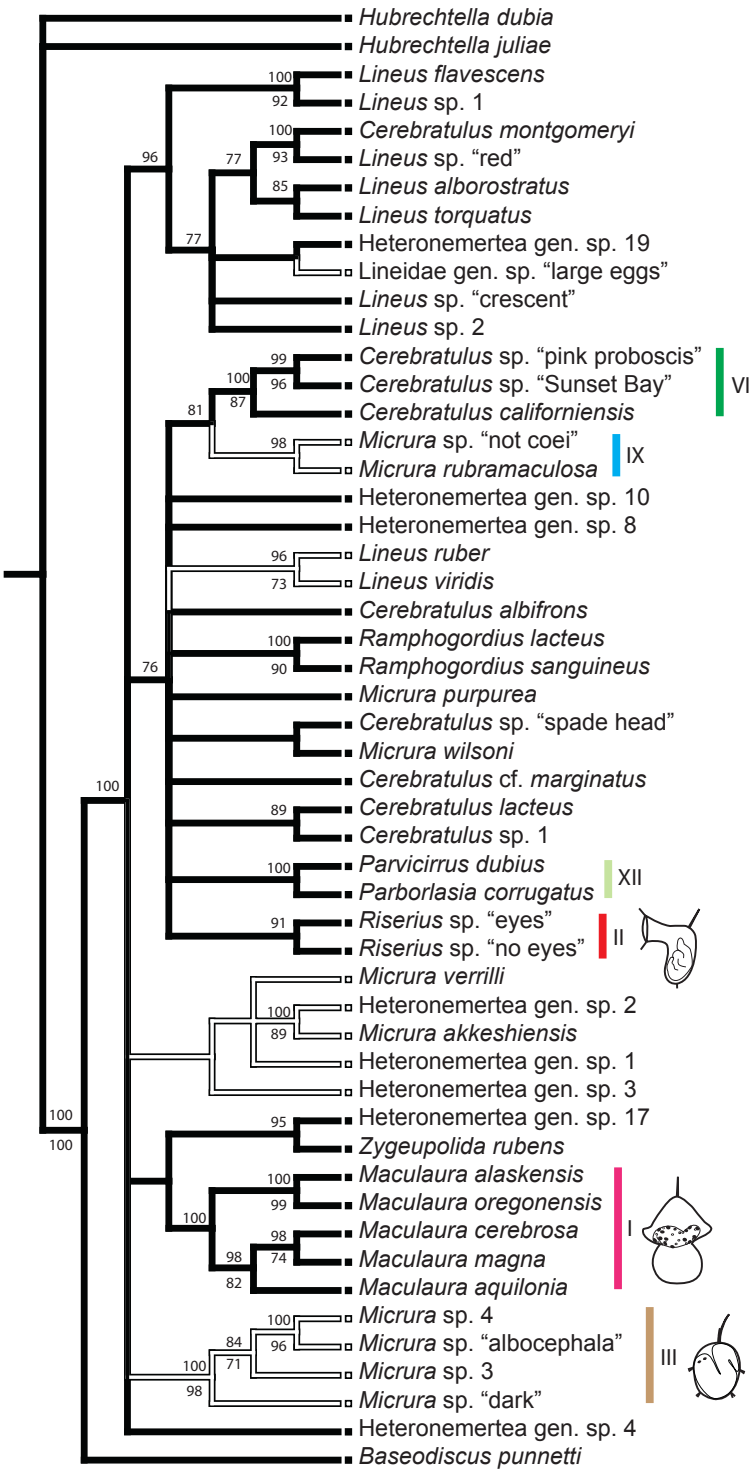

Figure 7.6. COI Bayesian phylogeny of the Pilidiophora. Lecithotrophic lineages are shown in white and planktotrophic in black. Clade support is indicated by bayesian posterior probabilities (upper number) and bootstrap values (lower number). Clade support values below 70 are not shown. Consistent clades are indicated with roman numerals and colored bars, for ease of comparison between gene regions; larval diagrams that we believe to characterize clades are shown.

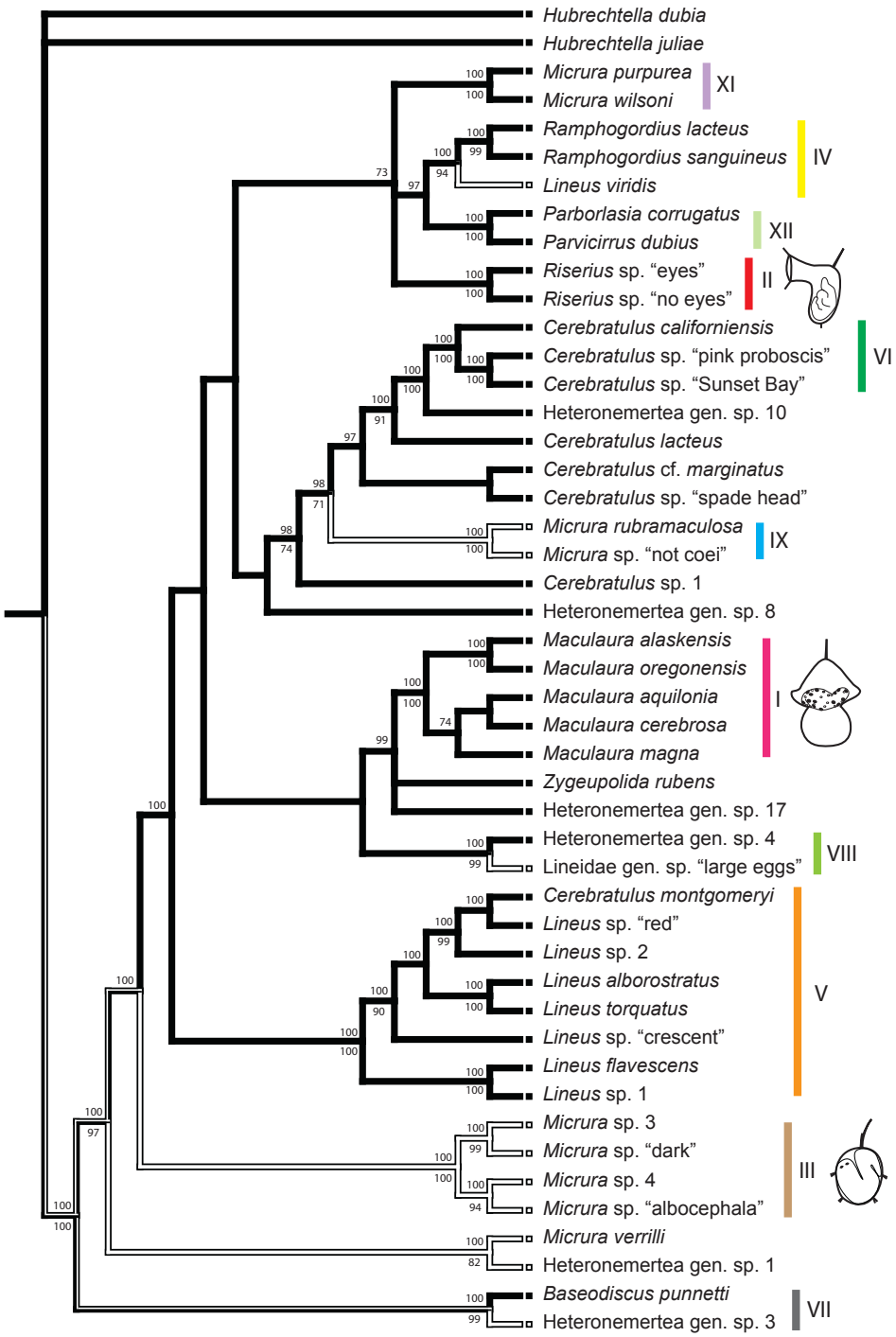

Figure 7.7. Bayesian phylogeny of the Pilidiophora based on concatenated dataset of 16S, 28S and COI gene regions. Lecithotrophic lineages are shown in white and planktotrophic in black. Clade support is indicated by bayesian posterior probabilities (upper number) and bootstrap values (lower number). Clade support values below 70 are not shown. Consistent clades are indicated with roman numerals and colored bars, for ease of comparison between gene regions; larval diagrams that we believe to characterize clades are shown.

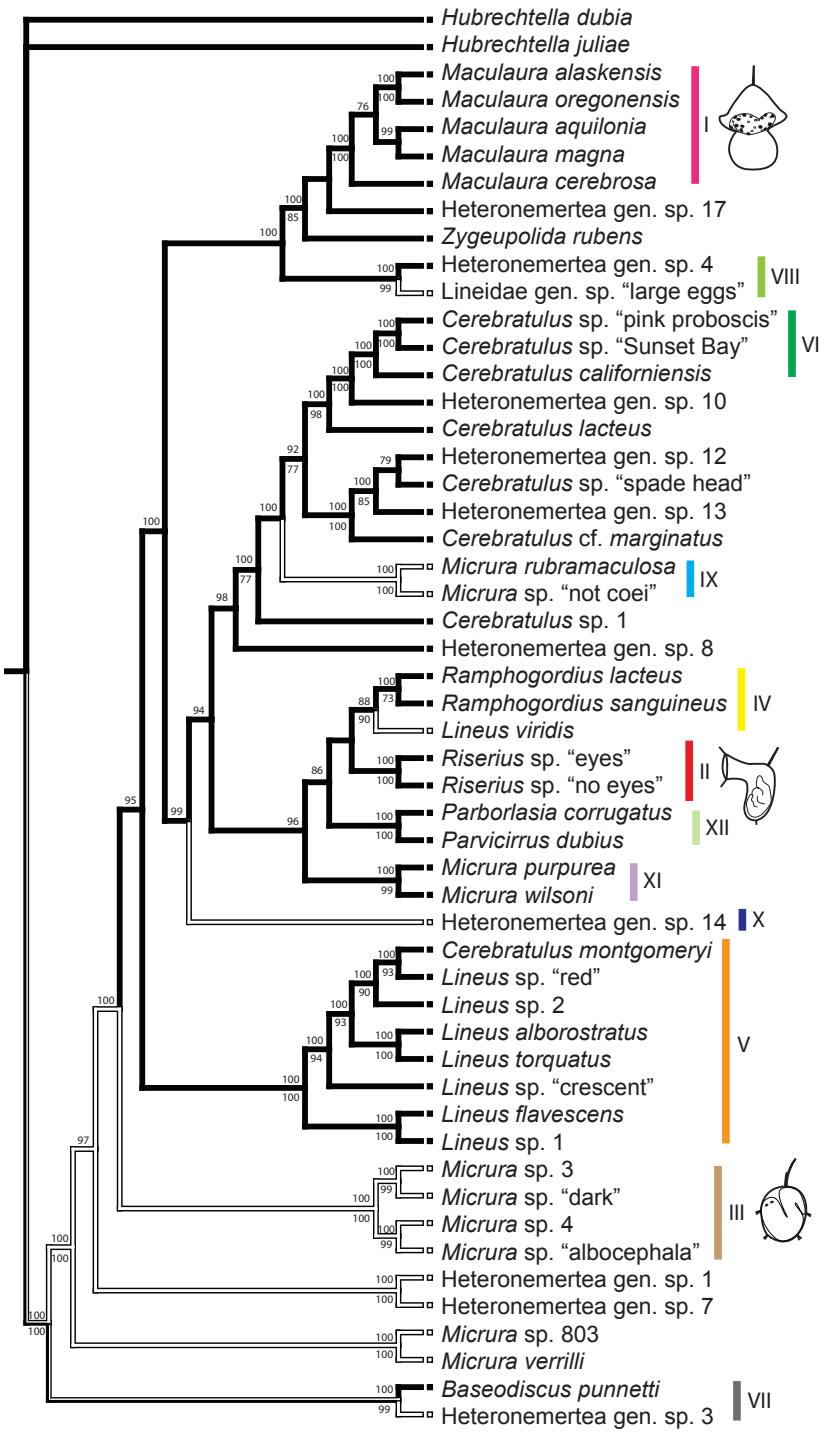

Figure 7.8. Bayesian phylogeny of the Pilidiophora based on concatenated dataset of 16S and 28S gene regions. Lecithotrophic lineages are shown in white and planktotrophic in black. Clade support is indicated by bayesian posterior probabilities (upper number) and bootstrap values (lower number). Clade support values below 70 are not shown. Consistent clades are indicated with roman numerals and colored bars, for ease of comparison between gene regions; larval diagrams that we believe to characterize clades are shown.

Phylogenetic analysis

Bayesian analyses of 16S and 28S gene regions, as well as those of concatenated datasets (16S + 28S and 16S + 28S + COI) produced well-resolved and well-supported topologies, with twelve clades found in phylogenies based on several individual as well as concatenated datasets. Some of these clades (I, II, III) also possess characteristic larval morphotypes (Figs. 7.4, 7.5, 7.7, 7.8, 7.9). The COI dataset produced a less resolved topology (Fig. 7.6), presumably because it is a faster evolving gene region, compared to the 16S and 28S rDNA regions, and thus likely suffers from saturation (i.e., multiple substitutions per site). The differences between topologies resulting from different gene regions can also be partially explained by differences in taxon sampling (i.e., availability of sequence data). As few as six and as many as eight clades include species with known lecithotrophic development (Fig. 7.9).

Clades supported by all or most analyses

Clade I: Maculaura – This clade contains five species (Figs. 7.4-7.8) and is a new pilidiophoran genus (Chapter III, Hiebert and Maslakova 2015b), which is also characterized by a unique larval morphotype, pilidium maculosum (Fig. 2A in Hiebert and Maslakova 2015b).

Clade II: Riserius – Our phylogenies include four different *Riserius* species (Hiebert et al. 2013), represented by larvae. Despite the availability of sequence data we do not include the only described member of the genus, *Riserius pugetensis,* in our analyses because its development has not been described. Two species included in the analysis are from the northeast Pacific (*Riserius* sp. “eyes” and “no eyes”), and have been previously described by us (Chapter V, Hiebert et al. 2013) and two others (*Riserius*. sp. 3 and 4) are from the northwest Pacific (Sea of Japan), and are identified as larvae of *Riserius* here for the first time. All of these larvae have a characteristic sock-like shape with large esophageal funnel and a juvenile that develops parallel to the larval AP axis (Hiebert et al. 2013, Maslakova and Hiebert 2014, Chernyshev et al. 2013). *Riserius* sp. 4 also has a

posterior transverse ciliated band that it is similar to pilidium recurvatum described by Cantell (1966) from Sweden and Fewkes (1883) from Western Atlantic, and pilidium incurvatum described by Dawydoff (1940) from Vietnam (Fig. 7.2). Notably, all four sock-like pilidial species, with and without the posterior ciliated band form well-supported clades on our phylogenies. The position of this group among the Pilidiophora, varies depending on the analysis (Figs. 7.4-7.8), but is never basal.

Clade III: "Trochonemertes" – Four out of the five species that are known to produce the pilidium nielseni morphotype (Maslakova and von Dassow, 2012, Maslakova and Hiebert 2014, Hunt unpublished observation) form a well-supported clade on all phylogenies (Figs. 7.4–7.9). Larvae of these species all possess two transverse ciliated bands, and are lecithotrophic (Fig. 7.1E). The nickname of this clade ("trochonemertes") is based on the superficial resemblance of this nemertean larva to certain trochophore larvae of annelids and mollusks. Remarkably, one of the five species with this type of larva, identified as *Cerebratulus longiceps*, is apparently unrelated to the other four, although we only have data for the 16S gene region (Fig. 7.4).

Clade IV: True Lineus (L. ruber – L. viridis – R. sanguineus) – *Lineus ruber* and *Lineus viridis*, both with encapsulated development (e.g., Desor 1848; Schmidt 1964; von Döhren 2011; Martin-Duran et al. 2015), consistently form a well-supported group with *Ramphogordius lacteus* and *R. sanguineus* (Figs. 7.4–7.9). These species are closely related to the type species of the genus *Lineus*, *Lineus longissimus* according to molecular phylogenies (e.g., Sandberg and Strand 2007), and thus we refer to this clade as the "true *Lineus*". We did not include *L. longissimus* in our analyses despite availability of relevant sequence data in GenBank, because its larval development is not known. The "true *Lineus*" clade includes species known to have both lecithotrophic (*L. ruber* and *L. viridis*) and planktotrophic development (*R. lacteus*). Planktotrophic development, in addition to asexual reproduction, has been suspected for *Ramphogordius sanguineus*, based on the presence of sexual products and small oocyte size (90–100 μm in the synonymized

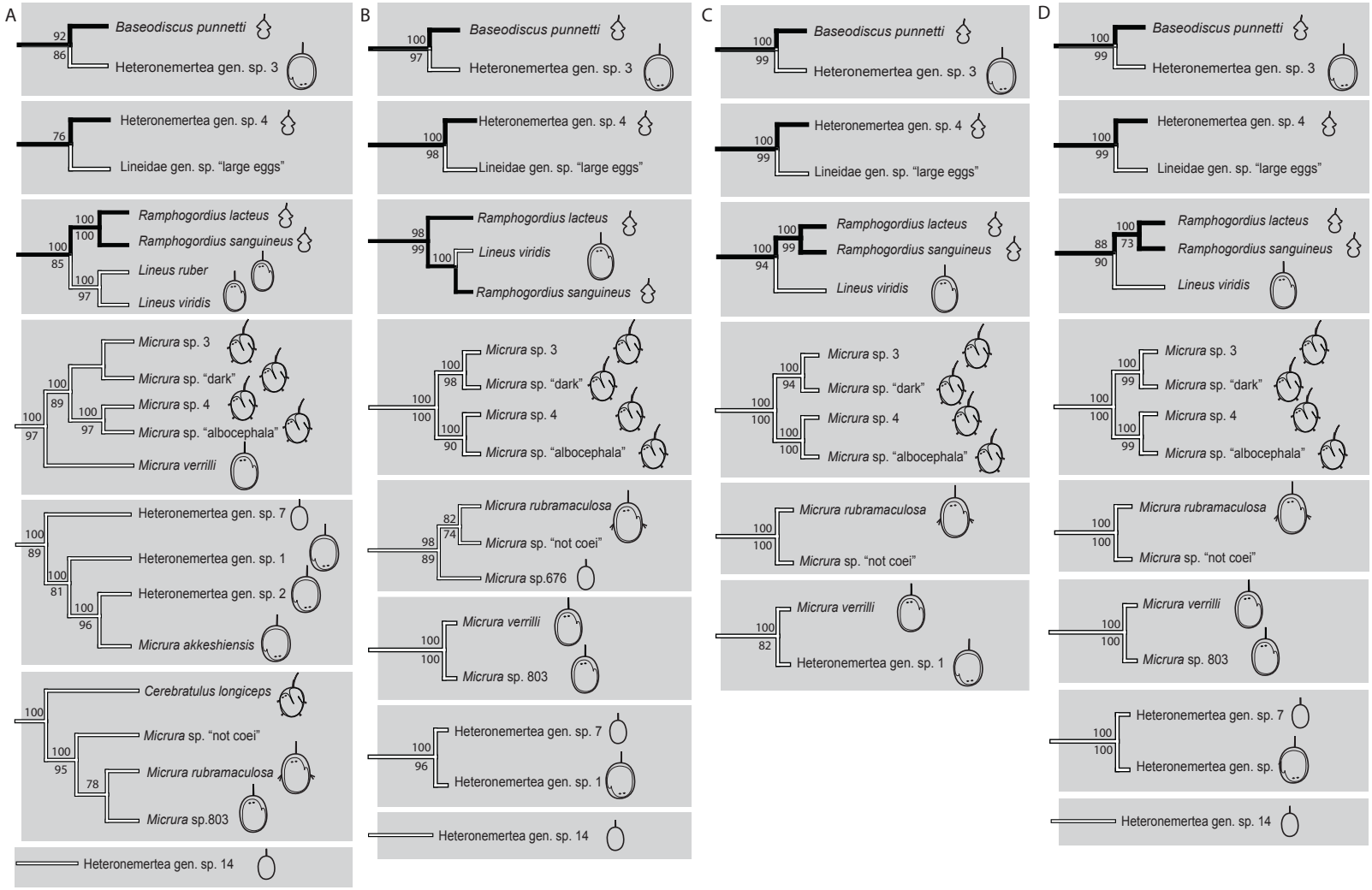

Figure 7.9. Clades including lecithotrophic species found in 16S (A), 28S (B), 16S + 28S + COI (C), and 16S + 28S (D) phylogenies. Clade support is indicated by bayesian posterior probabilities (upper number) and bootstrap values (lower number) for well supported clades (> 70). Lecithotrophic larval forms are shown for each species and correspond to those in Fig. 7.1; note developmental mode is presumed for Lineidae gen. sp. "large eggs" and *Micrura* sp. "not coei" (see text).

species, *L. vegetus,* Coe 1931; Schwartz 2009; pers. observation), but development has not been observed.

Clade V: the "not Lineus*" clade* (C. montgomeryi – L. torquatus – L. flavescens *and allies)* – This lineage mostly includes species currently classified as *Lineus,* or undescribed species which morphologically fit the vague definition of the genus *Lineus*, but does not appear to be closely related to the true *Lineus* (clade IV), thus we refer to it as the "not *Lineus*". This group of species consistently forms a monophyletic clade in 16S, 28S and concatenated analyses (see Figs. 7.4, 7.5, 7.7, 7.8). Larval morphology among members of this group is that of a typical (i.e., hat-shaped) pilidium, the larval lobes and lappets are similar in size to one another, bear no pigment, and the juvenile inside develops two small eyes and lacks a caudal cirrus (see Fig. 8C in Hiebert and Maslakova 2015b). This larval form (described in detail in Chapter VI, Hiebert and Maslakova 2015b) almost certainly occurs in other pilidiophoran species as well (e.g., Müller 1847; Bürger 1895; Dawydoff 1940; Thorson 1946; Chernyshev 2001; Lacalli 2005), and it is currently not known whether they all form a monophyletic clade.

Clade VI: Cerebratulus californiensis *species complex* — this clade includes *C. californiensis* and two undescribed species, C. sp. "Sunset Bay" and C. sp. "pink proboscis". The three species consistently form a well-supported clade (Figs. 7.4–7.8). These species also share a larval morphotype with pigment spots on the lobes and lappets. However, a similar larval morphotype is also seen in Heteronemertea gen. sp. 10, 11 (Fig. 7.3E), and 19, from the Caribbean side of Panama, southern Australia, and the Sea of Japan, respectively. While Heteronemertea gen. sp. 10 groups with clade VI on 16S, 28S and concatenated data, Heteronemertea gen. sp. 19 does not group with clade VI in 16S or COI phylogenies. Meanwhile, Heteronemertea gen. sp. 11 groups with this clade where data is available (16S only, Fig. 7.4).

Clade VII: Baseodiscus punnetti + *Heteronemertea gen. sp. 3* – This clade (Figs. 7.4–7.9), supported by all analyses, is similar to the clade IV (true *Lineus*) in that it combines species with contrasting developmental modes – a typical planktotrophic pilidium in *Baseodiscus punnetti* (Schwartz 2009) and a lecithotrophic bullet-like uniformly ciliated planktonic larva in Heteronemertea gen. sp. 3 (Fig. 4B in Hiebert and Maslakova 2014).

Clade VIII: Lineidae gen. sp. "large eggs" + *Heteronemertea gen. sp. 4* – Like clades II and IV, this clade includes two species with contrasting development (Figs. 7.4–7.9). Lineidae gen. sp. "large eggs" is suspected to have lecithotrophic development, based on large oocyte diameter (approximately 500 μm). This species consistently groups with a species that produces a planktotrophic pilidium (Heteronemertea gen. sp. 4). This relationship is well supported on all phylogenies except 16S (Fig. 7.4) and COI (Fig. 7.6).

Clade IX: Micrura rubramaculosa + Micrura *sp. "not coei"* – This clade (Figs. 7.4–7.9) includes the former species is reported to have lecithotrophic larvae (Schwartz and Norenburg 2005) and the latter suspected to have lecithotrophic development based on oocyte size (>300 μm, Maslakova and Hiebert 2014). The larvae of *M. rubramaculosa* have an equatorial transverse ciliated band and a juvenile with antero-posterior (AP) axis parallel to the larval AP axis (e.g., Fig. 7.1D).

Clade X: Heteronemertea gen. sp. 14 – This species comprises an isolated lecithotrophic lineage on our 16S, 28S and 16S + 28S phylogenies (Figs. 7.4, 7.5, 7.8, 7.9). This species was collected in southern Australia at an early developmental stage. Thus, distinct morphological characters, aside from obvious lecithotrophy (e.g., oblong, opaque, Fig. 7.1A, Fig. 7.3C), were not observed.

Clade XI: Micrura wilsoni + M. purpurea – These two species exhibit planktotrophic pilidial development (Cantell 1969; Hiebert and Maslakova 2015b) and are consistently sister to one another on all phylogenies except COI (Figs. 7.4, 7.5, 7.7, 7.8).

Clade XII: Parborlasia corrugatus + Parvicirrus dubius – As in clade XI (above), these two planktotrophic species (see Peck 1983; Riser 1993) are consistently sister to one another all all phylogenies except our 16S phylogeny (Figs. 7.5, 7.6, 7.7, 7.8).

Less supported but relevant clades in our analyses

Micrura rubramaculosa + Micrura *sp. "not coei" and allies* – On the 16S phylogeny, these two species form a well-supported clade with *Cerebratulus longiceps*, a species whose larvae have not one, but two transverse ciliated bands (e.g., Fig. 1E, Hunt and Maslakova, unpublished). The orientation of the juvenile axis with respect to the larval axis is not known in this species. *Micrura* sp. 676, also with lecithotrophic development (Schwartz 2009), is found in this clade, but data are available for 28S gene only (Fig. 7.5). The relationship of juvenile vs. larval AP axes is not known, and Schwartz (2009) suggests both the presence (page 26) and absence (page 121) of a single transverse ciliary band. Thus, whether the presence of ciliary band(s) is a feature of this group requires further investigation. *Micrura* sp. 803, another undescribed species with lecithotrophic development (Schwartz 2009) groups with the above species on the 16S phylogeny, but is found with a different lecithotrophic group on the 28S phylogeny (Fig. 7.5).

Micrura verrilli – is reported to have lecithotrophic development by Schwartz (2009), which we also confirm based on observations of large oocytes (Maslakova, Hiebert, pers. observations). Surprisingly, Coe (1940) reported planktotrophic pilidium in this species. *Micrura verrilli* consistently groups with other lecithotrophic species in our phylogenies. However, *which* species *M. verrilli* groups with varies. In our 16S + 28S + COI phylogeny, *M. verrilli* groups with Heteronemertea gen. sp. 1 (Fig. 7.7, 7.9). Interestingly, the juvenile develops with its AP axis opposite the larval AP axis in the latter species and parallel to the larval AP axis in the former. However, on 28S and 16S + 28S phylogenies, *M. verrilli* groups with *Micrura* sp. 803. On the 16S phylogeny, *Micrura verrilli* groups with Clade III ("Trochonemertes") described above.

Micrura akkeshiensis – An additional clade, which is well supported on our 16S phylogeny (Fig. 7.4), includes Heteronemertea gen. sp. 1, 2 and *M. akkeshiensis*, all species with lecithotrophic development, where larval and juvenile AP axes are opposite one another (e.g., 1B; see also Fig. 4 Maslakova and Hiebert 2014). This group also includes the lecithotrophic species, Heteronemertea gen. sp. 7 (Figs. 7.3C, 7.4). A lecithotrophic clade including *M. akkeshiensis*, *M. verrilli*, Heteronemertea gen. sp. 1, 2, and 3 is also found on our COI phylogeny, but clade support, beyond the sister grouping of *M. akkeshiensis* and Heteronemertea gen. sp. 2, is weak (Fig. 7.6).

Ancestral larval feeding mode

The formerly palaeonemertean genus, *Hubrechtella*, used as an outgroup in our analyses produces planktotrophic pilidium larvae with unique morphology (Fig. 4.2D) and its sister relationship to the Heteronemertea (Thollesson and Norenburg 2003, Andrade et al. 2014) biases reconstructions of ancestral state toward planktotrophy for the Pilidiophora as a whole. Nevertheless, our analyses show many basal lecithotrophic lineages within the Heteronemertea (e.g., Heteronemertea gen. sp. 1, 3 and 7, *Micrura verrilli*), which results in ambiguous reconstruction of ancestral state for the Heteronemertea: lecithotrophic or planktotrophic (e.g., Figs. 7.7, 7.5, 7.8).

DISCUSSION

The evolution of lecithotrophy in the Pilidiophora

Seventeen pilidiophoran species were known to have lecithotrophic development prior to this study (see Chernyshev 2011; Maslakova and Hiebert 2014). This study brings the number of known pilidiophoran species with lecithotrophic development to 20. Analysis of the concatenated dataset suggests at least three possibilities for the evolution of feeding mode in pilidiophorans, all of which suggest multiple instances of the evolution of lecithotrophy (from four to eight times) within Pilidiophora.

The first scenario is that a lecithotrophic larva is ancestral to the Pilidiophora, and planktotrophy evolved in the hubrechids, *Baseodiscus,* and in the *Lineus-Maculaura-Cerebratulus* clade independently. In this case, lecithotrophy would have later evolved secondarily at least three or four times. This calls for at least three evolutionary appearances of the morphologically complex planktotrophic pilidial body plan, which seems unlikely. The canonical hat-like planktotrophic pilidium is not only widespread among the Pilidiophora, it is also morphologically and functionally complex (e.g., Maslakova 2010b; von Dassow et al. 2013) which makes it unlikely to have evolved convergently.

Another scenario suggests that a planktotrophic larva is ancestral to the Pilidiophora, and lecithotrophy evolved at the base of the Heteronemertean lineage, was lost, and evolved again in 3–5 times in derived lineages (Figs. 7.4–7.8). This situation also requires at least two independent origins of the feeding pilidium (one in hubrechtids, and another in a subset of Heteronemertea).

An alternative possibility suggested by the data is that a planktotrophic pilidium larva is ancestral to the Pilidiophora (and Heteronemertea) and lecithotrophy evolved at least four and as many as eight times independently (Figs. 7.4–7.8). The final scenario is not the most parsimonious if one considers transitions between complex planktotrophic pilidium and morphologically simplified lecithotrophic pilidium to be equally likely in each direction, but it is most parsimonious if one considers loss of complex morphology more likely than independent origins thereof. This particular scenario parallels observations in other marine invertebrate phyla, where lecithotrophic larvae have evolved multiple times independently (e.g., Strathmann 1978; Raff 1987; Wray 1996; Smith 1997). Lecithotrophic pilidia resemble the lecithotrophic larvae of other marine invertebrate taxa (e.g., enchinoderms, cnidarians). This convergence in form among lecithotrophic larvae across phyla suggests a functional significance of this simplified morphology rather than a common phylogenetic origin (Strathmann 1985; Emlet 1994).

Lecithotrophic pilidia come in different kinds – some are uniformly ciliated, others possess one or two transverse ciliary bands in addition to cilia covering the rest of the body (Schwartz and Norenburg 2005; Schwartz 2009; Maslakova and von Dassow 2012, Maslakova and Hiebert 2014, Fig. 7.1E). Lecithotrophic pilidia with transverse ciliary bands superficially resemble the trochophore larvae of annelids, doliolaria larvae of holothuroids, or periclymma larvae of bivalves (Strathmann 1987; Zardus and Morse 1998; Shanks 2001). Our phylogenies suggest that these different morphologies evolved more than once within the Heteronemertea. For example, larvae with two transverse ciliary bands (pilidium nielseni, Maslakova and von Dassow 2012) are found in two unrelated lineages — one includes four known species (clade III, "Trochonemertes"), and the other just a single species, *Cerebratulus longiceps* (Fig. 7.4). Larvae with a single transverse ciliary band are found in 1–2 species, and according to different analysis represent 1–2 independent evolutionary origins. Thus, it appears likely that their evolution is driven by functional constraints (e.g., transverse bands for swimming, Emlet 1991, 1994), as has been shown in other taxa. For example, in echinoderm larvae presence or absence and number of ciliated bands is correlated with larval size (larger larvae lack ciliated bands, Emlet 1994). In case of lecithotrophic pilidia a correlation between size and ciliation is currently unclear and requires a larger sample size. For example, larvae without ciliated bands, Heteronemertea gen. sp. 1, 2, 3 are 350, 275, 275 µm respectively (see Fig. 4 in Maslakova and Hiebert 2014), Heteronemertea gen. sp. 7 and 14 are approximately 225–250 µm (Fig. 7.3B–C), and *M. akkeshiensis* larvae are 300 µm (42 hr post fertilization, Iwata 1958). *Micrura rubramaculosa,* with single ciliated band is 120 µm (see Fig. 1C in Schwartz and Norenburg 2005) and members of the "Trochonemertes" clade, with two ciliated bands, range in size from 250–320 µm (see Fig. 3 in Maslakova and Hiebert 2014). The larva of *Cerebratulus longiceps,* also with two transverse ciliated bands, is approximately 310 µm (Hunt and Maslakova, unpublished). It is noteworthy that our largest lecithotrophic pilidia, uniformly ciliated

and with transverse ciliated bands, are smaller than the echinoderm larvae analyzed by Emlet (1994).

Unconventional planktotrophic pilidia

Although the hat-like shape is common among pilidiophorans, there are several distinctly different forms, e.g., the helmet-like pilidium auriculatum that characterizes *Hubrechtella* (Cantell 1969; Maslakova 2010a; Maslakova and Hiebert 2014), and the sock-like pilidium recurvatum recently linked by us to *Riserius* (see Chapter VI; Hiebert et al. 2013). These forms are substantially different from each other and hat-like forms in morphology, and likely function. The morphology of the conventional hat-like pilidium has recently been explained in terms of its feeding mode and preferred prey (von Dassow et al. 2013), other pilidia likely differ in these aspects, but it is presently not known what and how they eat. Hubrechtid pilidia are also different from the heteronemertean pilidia in that the juvenile of some species do not devour the larval body at metamorphosis, thus one often finds empty post-metamorphic pilidium auriculatum bodies drifting in the plankton (Cantell 1969; Wilson 1882; pers. observation, but see Schwartz 2009).

Here we show that the pilidium incurvatum/recurvatum larva with a transverse ciliary band around the toe compartment of the 'sock' (from the West Pacific), is also related to *Riserius*, thus confirming that sock-like pilidial morphology evolved in a single clade of Heteronemerteans. Jägersten (1972) suggested that pilidium recurvatum/ pilidium incurvatum represented an ancestral pilidial larval form — an evolutionary intermediate between the planuliform larvae of palaeo- and hoplonemerteans and the hat-like pilidium. Thollesson and Norenburg's (2003) phylogeny of Nemertea suggested that it may be a basal lineage within the Heteronemertea, partially supporting Jägersten's hypothesis (but see Sundberg and Strand 2007 and Schwartz 2009 for derived placement of *Riserius*). *Riserius* is never basal in our analyses, suggesting, instead, that it is a derived heteronemertean lineage.

Hubrechtid nemerteans, on the other hand, represent a sister lineage to the rest of the Pilidiophora (Thollesson and Norenburg 2003; Andrade et al. 2014), but whether hat-like pilidia found in many species of Heteronemerteans or the helmet-like pilidium auriculatum of *Hubrechtella* are more similar to the ancestral pilidium remains unclear.

Larval pigmentation

Many types of pilidia possess pigment spots or patches (Dawydoff 1940; Cantell 1969; Lacalli 2005; Schwartz 2009; Maslakova and Hiebert 2014; Hiebert and Maslakova 2015c). Sometimes pigment spots are found on pilidial lobes and lappets, in other species spots cover the juvenile amnion. Schwartz reported a pilidium bearing pigment spots on pilidial lobes, lappets, and the juvenile amnion (P4, Schwartz 2009). Here we show that spots on the juvenile amnion evolved in a single clade (*Maculaura,* Hiebert and Maslakova 2015b), but other types of pigmentation are found in several unrelated lineages. For example, the larvae of *Micrura wilsoni* exhibit larval pigmentation (Hiebert and Maslakova 2015c), but larvae with pigment spots are also found in as many as two other clades (Fig. 7.6). Pilidial pigment spots are not unlike the spots on the velar lobes of gastropod veligers, but whether they are functionally or ecologically significant requires further investigation.

BRIDGE TO CHAPTER VIII

In Chapter VII, we place pilidiophoran larval forms and feeding modes in a phylogenetic context. We show that some distinct pilidial forms characterize nemertean clades and thus could serve as morphological synapomorphies for molecularly defined clades within Nemertea (e.g., provide genus-level characters). Based on our phylogenies, there are many independent origins of lecithotrophy within the Pilidiophora. We suggest that the ancestral feeding mode for the Pilidiophora is planktotrophy. In the final chapter, we conclude this dissertation by summarizing these findings within the Nemertea as well as other marine invertebrate phyla. Finally, we reiterate the importance of a 'life-history approach', from which this dissertation began, for all species with bi-phasic life cycles.

CHAPTER VIII
CONCLUSIONS

Discovering new diversity with a life-history approach

This dissertation demonstrates a powerful tool in diversity assessments for taxa with biphasic life histories. When it comes to marine eukaryotic diversity, the vast majority is undescribed, yet just how many species are undescribed is unknown and estimates vary considerably (e.g., Mora et al. 2011; Appeltans et al. 2012). Here we show that larger numbers of species can be uncovered with surveys utilizing both larval and adult stages. While the focus of our study was the phylum Nemertea in a single biogeographic province within the NEP, this approach is universally applicable to other taxa with multiple life history stages and can be used in any region of the world (see Barber and Boyce 2006). This work follows a number of recent studies that show that DNA sequence data can reveal cryptic species (e.g., Hebert et al. 2004a; Fukami et al. 2004; Bickford et al. 2006; Hyde et al. 2008; Schulze et al. 2012) and previously hidden diversity (e.g., Barber and Boyce 2006). We believe that DNA barcoding larvae is particularly useful for species that are, as adults, difficult to collect (e.g., subtidal) or rare, as each benthic adult is greatly outnumbered by the planktonic larvae they produce (Young et al. 2002).

Updated synopsis of nemerteans in the Oregonian Biogeographic Province

This work improves our understanding of nemertean adult and larval fauna from central California to Oregon. Previous estimates of the nemertean diversity in this area underestimated species numbers by nearly 50%. Where 65 intertidal species were previously reported from central California to Oregon (Roe et al. 2007), our synopsis suggests that 104–113 nemertean species are found in southern Oregon alone. It increases the numbers of known palaeonemerteans in the area from nine to 20–22, pilidiophorans from 23 to 49–52, and hoplonemerteans from 34 to 35–39. As we have primarily sampled for adults intertidally, some of the new species currently represented

by larvae only may be subtidal. We reveal many undescribed species, cryptic species complexes (e.g., *Cephalothrix*, *Carinoma*, *Maculaura* gen. nov.), species with apparent trans-Pacific distribution (*Hubrechtella juliae*, *Carinoma hamanako, Maculaura aquilonia* sp. nov.), and species with more limited distribution then previously thought (e.g., *Emplectonema gracile*, *Zygonemertes virescens*). It is likely that more new nemertean diversity awaits discovery, however, results of our sampling methods show that the rate at which we are encountering new diversity has decreased over the course of this project (Fig. 8.1).

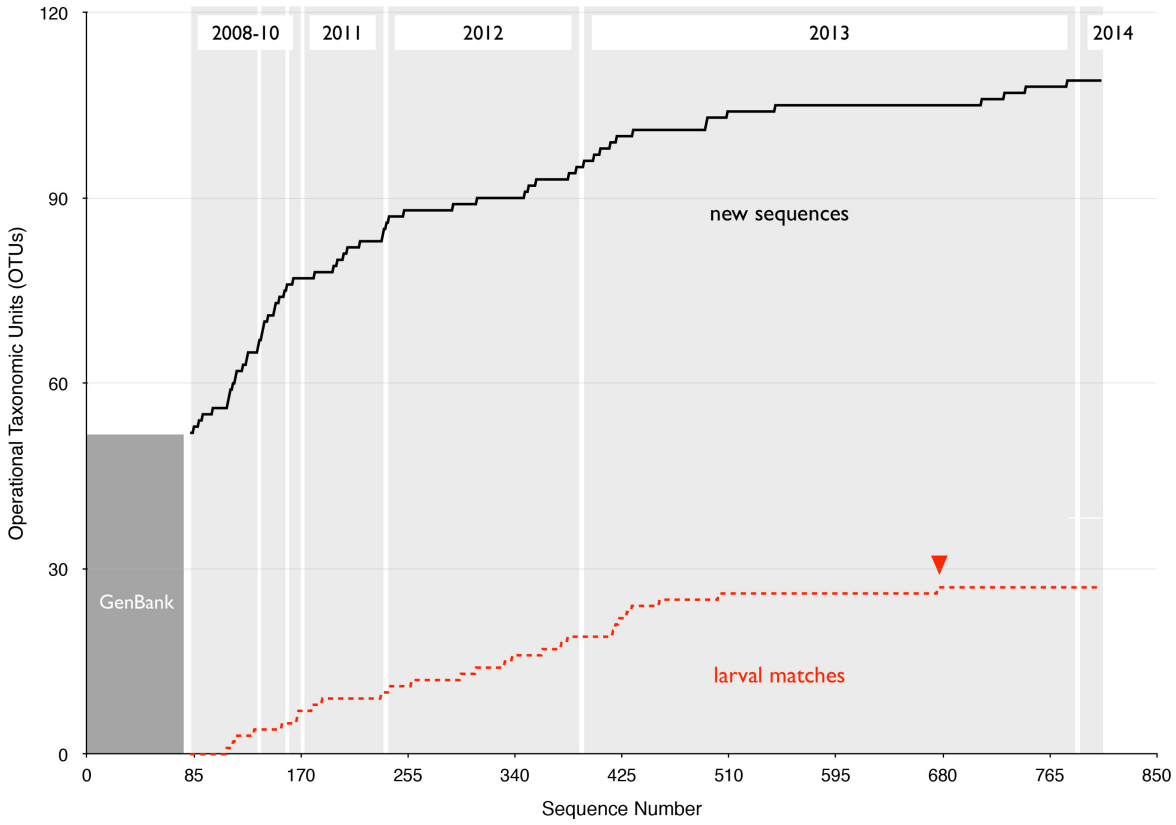

Figure 8.1. The number of operational taxonomic units (i.e., presumed species) per number of total nemertean sequences from 2008 to 2014. GenBank sequences for species reported or believed to be found in the OBP are included as dark grey box. New sequences to our database are shown with a solid black line and larvae that have been identified to species are shown with a dashed red line; red arrowhead indicates most recent larva identified. Light grey boxes highlight the number of sequences added to our database each year.

'DNA barcoding', species delimitation and integrative taxonomy

Adult morphology does not always coincide with speciation (e.g., see deQueroz 2007). Thus, characters of adult morphology are often not sufficient to differentiate between closely related or cryptic species (Bickford et al. 2006). Although the use of DNA sequence data for species identification and delimitation has been debated (Sites and Marshall 2003; Blaxter 2004; DeSalle et al. 2005; Rubinoff et al. 2006), there is considerable evidence that sequence data helps to flag potentially new diversity (e.g., Hebert et al. 2004a; Fukami et al. 2004; Bickford et al. 2006; Hyde et al. 2008; Schulze et al. 2012), prompting further investigations into other characters that may help to distinguish morphologically similar species (e.g., reproductive timing, gamete morphology, or habitat). Ultimately, the best approach to species delimitation is one that integrates all available data (i.e.,"integrative taxonomy") to determine the number and identity of species (e.g., Jörger et al. 2012; Schulzte et al. 2012; Hiebert and Maslakova 2015; Chapter III).

Using an integrative approach to species delimitation, we find that intraspecific divergence values for nemertean species are generally below 2% (for both 16S and COI gene regions), with 98% (16S) of species comparisons exhibiting < 2% intraspecific divergence. Sixty-seven percent of COI species comparisons exhibited < 2% intraspecific divergence and 95% of comparisons had intraspecific divergences < 5%. These results are similar to those previously reported for other other invertebrate and vertebrate groups (e.g., Hebert et al. 2003; Hebert et al. 2004b; Meyer and Paulay 2005). However, the presence of a so-called 'barcoding gap', which has been observed for other taxa (e.g., Hebert et al. 2003; Hebert et al. 2004b; Barrett and Hebert 2005) is less clear for nemerteans. Our data show that nemertean species exhibit a range of intraspecific divergence values from 0.0–3.5% (16S) and 0.0–11.3% (COI). We also find minimum interspecific divergence values (1.6% for 16S and 4.1% for COI) to be within intraspecific ranges. Thus, overlap exists between the maximum intraspecific divergences and minimum interspecific divergences for both gene regions (although

fewer species fall within the region of overlap for COI sequence data.) In most cases sequence data can clearly differentiate nemertean species, but because a small number of species fall within the region of overlap, DNA-based species identification could lead to error.

Using DNA sequence data alone to identify a specimen has, rightfully, been criticized (Sites and Marshall 2003; Blaxter 2004; DeSalle et al. 2005; Rubinoff et al. 2006). Although a universal numerical cut off value for assigning species using sequence data has been suggested (e.g., 2–3% COI, Hebert et al. 2003), this is not accurate in practice. First, different taxa exhibit different rates of evolution for the same gene region. For example, anthozoan mitochondrial DNA is notoriously conserved, and closely related species cannot be distinguished based on COI sequences (Shearer and Coffroth 2008; Bucklin et al. 2010). Second, many taxa do not exhibit a clear barcoding gap (e.g., cowries, Meyer and Paulay 2005; nemerteans, this study). Finally, estimates of intra- vs. interspecific variation depend on accurate species delimitation in the first place, which does not exist for many taxa.

Larval identification provides insight into larval biology and evolution

Since this project began we have identified over 30 nemertean larvae. In doing so, we have improved our understanding of nemertean larval biology and evolution (e.g., Hiebert and Maslakova 2014; Chapter VII). Historically many nemertean larval forms have been documented from plankton samples taken around the world, but their identities were unknown (e.g., Müller 1847; Fewkes 1883; Bürger 1895; Dawydoff 1940; Thorson 1946; Cantell 1966, 1969; Chernyshev 2001; Lacalli 2005). By identifying these larvae with DNA sequence data, this dissertation places these unique larval forms in a phylogenetic context. Our results reveal larval synapomorphies for well-supported clades defined largely by molecular characters (e.g., *Maculaura*, Hiebert and Maslakova 2015, Chapter III). We revealed the genus-level identity of the larva known as pilidium recurvatum, which had been a mystery since its discovery by Fewkes in 1883 (Hiebert et

al. 2013; Chapter V). Furthermore, our analysis showed that this unique sock-like larval form was not ancestral to the typical hat-like pilidium, but is found in a single derived clade (*Riserius,* Chapter VII). At the same time, we revealed many new lecithotrophic lineages in a predominantly planktotrophic nemertean group (Pilidiophora). By identifying new lecithotrophic larvae with DNA sequence data, we suggest that lecithotrophy evolved independently several times within the Pilidiophora, and that planktotrophy is likely ancestral.

Concluding remarks

This dissertation is a comprehensive work describing what was learned about the diversity of a small phylum in a supposedly well-studied region in just eight years. We strongly suspect that more new diversity will be found. We also strongly suspect, using the methods described herein, that similar new diversity would be discovered in other phyla, no matter how well-studied. Most species are described, and thus, defined by adult morphology. We suggest that all aspects of an individual's biology contribute equally to species definitions. What we learned about species diversity came largely from investigating species as larvae and adults equally. Ignoring the often overlooked larval life-history stage in biphasic organisms provides us with a fragmented picture of real species diversity.

APPENDIX A

PALAEONEMERTEAN SPECIMENS AND SEQUENCES

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Callinera grandis	-	JF277570	HQ848626	Andrade et al. 2012	Tjärnö, Koster, Sweden
Carinina ochracea	-	JF277631	HQ848627	Andrade et al. 2012	Tjärnö, Koster, Sweden
Carinina sp. "chocolate"	32	KU197304	KU197653	This study	Charleston, OR USA (S. Maslakova)
Carinina sp. "chocolate"	100	KU197305	KU197654	This study	Charleston, OR USA (S. Maslakova)
Carinina sp. "chocolate"	3_Cc	-	KU197655	This study	Charleston, OR USA (S. Maslakova)
Carinina sp. "chocolate"	E1D9	KU197306	KU197656	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Carinina sp. "chocolate"	E1E1	KU197307	KU197657	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Carinina sp. "chocolate"	E1E2	KU197308	KU197658	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Carinina sp. "chocolate" •	E1I6	KU197309	KU197659	This study	Charleston, OR USA (T. Hiebert)
Carinina sp. "chocolate" •	E3C5	KU197310	KU197660	This study	Charleston, OR USA (T. Hiebert)
Carinina sp. "Vostok"	84	KU197311	-	This study	Vostok Bay, Russia (A. Chernyshev)
Carinoma hamanako	-	JF277600	HQ848628	Andrade et al. 2012	Lake Hamanako, Shizuoka, Honshu, Japan
Carinoma hamanako •	E1I7	-	KU197661	This study	Charleston, OR USA (T. Hiebert)
Carinoma hamanako •	E2D4	-	KU197662	This study	Charleston, OR USA (T. Hiebert)
Carinoma hamanako •	E4B4	-	KU197663	This study	Charleston, OR USA (T. Hiebert)
Carinoma hamanako •	35	KU197312	-	This study	Charleston, OR USA (S. Maslakova)
Carinoma hamanako •	E3H8	KU197313	-	This study	Charleston, OR USA (T. Hiebert)
Carinoma hamanako •	E5A2	KU197314	KU197664	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Carinoma hamanako •	N2	KU197315	-	This study	Charleston, OR USA (T. Hiebert)
Carinoma mutabilis	-	AJ436832	AJ436942	Thollesson & Norenburg 2003	San Juan Island, WA USA
Carinoma mutabilis •	E2D7	-	KU197669	This study	Charleston, OR USA (T. Hiebert)
Carinoma mutabilis	33 *	KU197316	KU197665	This study	Charleston, OR USA (S. Maslakova)
Carinoma mutabilis	109	KU197317	KU197666	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Carinoma mutabilis •	304	KU197318	KU197667	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Carinoma mutabilis •	306	KU197319	-	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Carinoma mutabilis	N21	KU197323	-	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Carinoma mutabilis •	E2B2	KU197322	KU197668	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Carinoma mutabilis •	E2A6	KU197321	-	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Carinoma mutabilis •	E2A2	KU197320	-	This study	Charleston, OR USA (T. Hiebert)
Carinoma tremaphoros	-	JF277602	HQ848630	Andrade et al. 2012	Fort Pierce, FL USA
Carinoma tremaphoros	-	AJ436833	AJ436943	Thollesson & Norenburg 2003	Fort Pierce, FL USA
Carinoma sp. 5 •	E5A1	KU197324	KU197670	This study	Charleston, OR USA (T. Hiebert)
Carinoma sp. “white”	40	KU197325	KU197671	This study	Charleston, OR USA (S. Maslakova)
Carinoma sp. “white” •	E2A1	KU197326	KU197672	This study	Charleston, OR USA (T. Hiebert)
Carinoma sp. “white” •	E2B4	KU197327	KU197673	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Carinoma sp. "white"	N16	KU197328	-	This study	Seaside, OR USA (T. Hiebert)
Carinoma sp. "white"	N17	KU197329	-	This study	Seaside, OR USA (T. Hiebert)
Carinoma sp. "white"	N18	KU197330	-	This study	Seaside, OR USA (T. Hiebert)
Carinoma sp. "yellowback"	34	KU197331	-	This study	Charleston, OR USA (S. Maslakova)
Carinoma sp. "yellowback"	E1E3	KU197332	KU197674	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Carinoma sp. "yellowback" •	E5C7	KU197333	KU197675	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Carinoma sp. "yellowback" •	N40	KU197334	KU197676	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix filiformis	-	AJ436835	AJ436944	Thollesson & Norenburg 2003	Akkeshi Bay, Japan
Cephalothrix spiralis	E1B2	-	KU197677	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cephalothrix spiralis	E1B3	-	KU197678	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cephalothrix spiralis	E1B4	-	KU197679	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cephalothrix spiralis	E1B5	-	KU197680	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cephalothrix spiralis	E2G6	-	KU197686	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cephalothrix spiralis	37	KU197336	-	This study	Cape Blanco, OR USA (S. Maslakova)
Cephalothrix spiralis	E2G5	KU197338	KU197685	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cephalothrix cf. *spiralis*	-	AJ436837	AJ436946	Thollesson & Norenburg 2003	San Juan Island, WA USA

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Cephalothrix cf. *spiralis*	4	KU517439	KU517442	This study	Charleston, OR USA (K. Robbins)
Cephalothrix major	36	KU197340	KU197687	This study	Charleston, OR USA (S. Maslakova)
Cephalothrix sp. 1 •	E2D6	-	KU197691	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix sp. 1 •	E4E5	KU197346	-	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix sp. 1 •	214	KU197341	KU197688	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix sp. 1 •	215	KU197342	KU197689	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix sp. 1 •	216	KU197343	KU197690	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix sp. 1 •	E3C9	KU197344	KU197692	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix sp. 1 •	E4B5	KU197345	KU197693	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix sp. 1 •	N1	KU197347	-	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix sp. 1 •	N5	KU197348	-	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix sp. 1 •	N22	KU197349	-	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix sp. 2 •	33_2	KU197350	-	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix sp. 2 •	E1I5	KU197351	KU197694	This study	Charleston, OR USA (R. Emlet)
Cephalothrix sp. 2 •	E5A4	KU197352	KU197695	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix sp. 3 •	E3A7	KU197353	-	This study	Charleston, OR USA (T. Hiebert)
Cephalothrix sp. 4 •	E2B3	-	KU197681	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cephalothrix sp. 4 •	E2D5	-	KU197682	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Cephalothrix sp. 4 •	E2E9	-	KU197684	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cephalothrix sp. 4 •	9	KU197335	-	This study	Charleston, OR USA (S. Maslakova)
Cephalothrix sp. 4 •	E2D8	KU197337	KU197683	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cephalothrix sp. 4 •	E3H2	KU197339	-	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Tubulanus annulatus	-	-	HQ848622	Andrade et al. 2012	Tjärnö, Koster, Sweden
Tubulanus annulatus	-	AF103756	-	Sundberg and Saur 1998	western coast, Sweden
Tubulanus pellucidus	-	JF277595	HQ848625	Andrade et al. 2012	Pea Island, NC USA
Tubulanus polymorphus	-	JF277598	HQ848621	Andrade et al. 2012	San Juan Island, WA USA
Tubulanus polymorphus •	E2A4	-	KU197696	This study	Charleston, OR USA (R. Emlet)
Tubulanus polymorphus	E2F5	-	KU197697	This study	Charleston, OR USA (T. Hiebert)
Tubulanus punctatus	-	AJ436838	AJ436947	Thollesson & Norenburg 2003	Vostok Bay, Sea of Japan, Russia
Tubulanus rhabdotus	-	AJ436839	AJ436948	Thollesson & Norenburg 2003	Fort Pierce, FL USA
Tubulanus sexlineatus	-	AJ436840	AJ436949	Thollesson & Norenburg 2003	San Juan Island, WA USA
Tubulanus sexlineatus	39	-	KU197698	This study	Charleston, OR USA (S. Maslakova)
Tubulanus sexlineatus	E2F8	-	KU197699	This study	Charleston, OR USA (T. Hiebert)
Tubulanus sexlineatus	B02	KU197354	-	This study	Charleston, OR USA (S. Maslakova)
Tubulanus sexlineatus •	E5D2	KU197355	KU197700	This study	Charleston, OR USA (J. Valley)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Tubulanus sp. 1 •	E1H3	KU197356	KU197701	This study	Charleston, OR USA (T. Hiebert)
Tubulanus sp. 1 •	E4H3	KU197357	KU197702	This study	Charleston, OR USA (T. Hiebert)
Tubulanus sp. 1 •	E5A3	KU197358	KU197703	This study	Charleston, OR USA (T. Hiebert)
Tubulanus sp. 1 •	E5A5	KU197359	KU197704	This study	Charleston, OR USA (T. Hiebert)
Tubulanus sp. 1 •	E5A6	KU197360	KU197705	This study	Charleston, OR USA (T. Hiebert)
Tubulanus sp. 1 •	E5A7	KU197361	KU197706	This study	Charleston, OR USA (T. Hiebert)
Tubulanus sp. 1 •	E5A8	KU197362	KU197707	This study	Charleston, OR USA (T. Hiebert)
Tubulanus sp. 2 •	E2A8	KU197363	KU197708	This study	Charleston, OR USA (T. Hiebert)
Tubulanus sp. 2 •	E3C4	KU197364	KU197709	This study	Charleston, OR USA (T. Hiebert)
Tubulanus sp. 2 •	E4H5	KU197365	KU197710	This study	Charleston, OR USA (T. Hiebert)
Tubulanus sp. 3 •	E4H4	KU197366	KU197711	This study	Charleston, OR USA (T. Hiebert)
Tubulanus sp. 3 •	3	KU517441	KU517445	This study	Charleston, OR USA (T. Hiebert)

• larval sequence

APPENDIX B

PILIDIOPHORAN SPECIMENS AND SEQUENCES

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Hubrechtella dubia	-	AJ436834	-	Thollesson and Norenburg 2003	Fort Pierce, FL USA
Hubrechtella dubia	-	-	HQ848631	Andrade et al. 2012	Tjärnö, Koster, Sweden
Hubrechtella juliae	67	KU197429	-	This study	Sea of Japan (A. Chernyshev)
Hubrechtella juliae •	79	KU197430	-	This study	Charleston, OR USA (M. Jarvis)
Hubrechtella juliae •	127	KU197431	-	This study	Charleston, OR USA (S. Maslakova)
Hubrechtella juliae •	E1H7	KU197432	KU197757	This study	Charleston, OR USA (T. Hiebert)
Hubrechtella juliae •	E1H9	KU197433	KU197756	This study	Charleston, OR USA (T. Hiebert)
Hubrechtella juliae •	E5C3	KU197434	-	This study	Charleston, OR USA (T. Hiebert)
Baseodiscus aureus	-	-	KC812601	Strand et al. 2014	Punta Tumbes, Caleta Canteras, Chile
Baseodiscus delineatus	-	AY955227	-	Strand et al. 2005	Ischia, Italy
Baseodiscus delineatus	-	AY955232	-	Strand et al. 2005	Rottnest Island, Australia
Baseodiscus delineatus	-	KF935448	KF935502	Kvist et al. 2014	Australia
Baseodiscus delineatus	-	EF124860–1	-	Schwartz & Norenburg, unpublished	Carrie Bow Cay, Belize
Baseodiscus jonasi	-	AY955230–1	-	Strand et al. 2005	Guadalcanal, Solomon Islands
Baseodiscus hemprichii	-	AY955229	-	Strand et al. 2005	Hurghada, Egypt
Baseodiscus hemprichii	-	EF124862	-	Schwartz & Norenburg, unpublished	Okinawa, Japan
Baseodiscus hemprichii	-	-	EF124996	Schwartz & Norenburg, unpublished	Queensland, Australia
Baseodiscus mexicanus	-	EF124863	EF124995	Schwartz & Norenburg, unpublished	La Paz, Meixco

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Baseodiscus mexicanus	-	KF935449	KF935503	Kvist et al. 2014	Mexico
Baseodiscus punnetti	E1F1	KU197367	KU197712	This study	Santa Barbara, CA USA (L. Friesen)
Baseodiscus quiquelineatus	-	AY955228	-	Strand et al. 2005	Solomon Islands
Baseodiscus quiquelineatus	-	EF124864	-	Schwartz & Norenburg, unpublished	Queensland, Australia
Baseodiscus unicolor	-	EF124865	-	Schwartz & Norenburg, unpublished	Cat Cay, Belize
Baseodiscus unicolor	-	KF935450–2	KF935505	Kvist et al. 2014	Panama
Baseodiscus sp. 1	-	JF277568	HQ848588	Andrade et al. 2012	Vigo, Pontevedra, Galicia, Spain
Baseodiscus sp. 2	-	JF277569	HQ848589	Andrade et al. 2012	Bocas del Toro, Panama
Cerebratulus albifrons	1	KU197368	KU197713	This study	Charleston, OR USA (S. Maslakova)
Cerebratulus albifrons •	92	KU197369	KU197714	This study	Charleston, OR USA (S. Maslakova)
Cerebratulus albifrons •	LWE2	KU197370	KU197715	This study	Charleston, OR USA (L. Whittier)
Cerebratulus albifrons •	LWE4	KU197371	-	This study	Charleston, OR USA (L. Whittier)
Cerebratulus californiensis	MMB10	-	KU197727	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	MMB69	-	KU197728	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	MMB81	-	KU197729	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis	3	KU197372	-	This study	Charleston, OR USA (S. Maslakova)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Cerebratulus californiensis •	75	KU197373	KU197716	This study	Charleston, OR USA (M. Jarvis)
Cerebratulus californiensis •	77	KU197374	KU197717	This study	Charleston, OR USA (M. Jarvis)
Cerebratulus californiensis	E1D7	KU197375	KU197718	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cerebratulus californiensis •	E1I2	KU197376	KU197719	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	E1I3	KU197377	KU197720	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	E2B6	KU197378	KU197721	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	E2B7	KU197379	KU197722	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	E2D9	KU197380	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	E2E4	KU197381	KU197723	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	E2E7	KU197382	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	E3E1	KU197383	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	E3E7	KU197384	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	E3E8	KU197385	-	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Cerebratulus californiensis •	E3F2	KU197386	KU197724	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	E3F3	KU197387	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	E3F7	KU197388	KU197725	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	E3F8	KU197389	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis	E3G5	KU197390	KU197726	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cerebratulus californiensis •	E3I6	KU197391	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis	E5B1_C	KU197392	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	LWC4	KU197393	-	This study	Charleston, OR USA (L. Whittier)
Cerebratulus californiensis •	MMB63	KU197394	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	MMB75	KU197395	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus californiensis •	MMBP30	KU197396	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus herculeus	-	EF124896	EF124991	Schwartz & Norenburg, unpublished	False Bay, San Juan Island WA, USA
Cerebratulus longiceps	-	EF124872	-	Schwartz & Norenburg, unpublished	USA

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Cerebratulus cf. *marginatus*	-	AJ436821	AJ436931	Thollesson and Norenburg 2003	WA USA
Cerebratulus cf. *marginatus*	4	-	KU197730	This study	Charleston, OR USA (S. Maslakova)
Cerebratulus cf. *marginatus*	160	-	KU197733	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cerebratulus cf. *marginatus* •	212	-	KU197734	This study	Charleston, OR USA (G. von Dassow)
Cerebratulus cf. *marginatus*	E3C1	-	KU197736	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cerebratulus cf. *marginatus*	7	KU197397	-	This study	False Bay, San Juan Island, WA USA (S. Maslakova)
Cerebratulus cf. *marginatus*	70	KU197398	-	This study	False Bay, San Juan Island, WA USA (S. Maslakova)
Cerebratulus cf. *marginatus*	108	KU197399	KU197731	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cerebratulus cf. *marginatus*	110	KU197400	KU197732	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cerebratulus cf. *marginatus* •	C5	KU197401	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus cf. *marginatus*	E1A1	KU197402	KU197735	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Cerebratulus cf. *marginatus*	E3C2	KU197403	KU197737	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus cf. *marginatus*	E3C3 *	KU197404	KU197738	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Cerebratulus cf. *marginatus*	E4A3	KU197405	KU197739	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus cf. *marginatus*	E4A4	KU197406	KU197740	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus cf. *marginatus* •	LWD3	KU197408	-	This study	Charleston, OR USA (L. Whittier)
Cerebratulus cf. *marginatus* •	LWE1	KU197409	KU197741	This study	Charleston, OR USA (L. Whittier)
Cerebratulus montgomeryi	-	EF124875	EF124966	Schwartz & Norenburg, unpublished	Canada
Cerebratulus montgomeryi	12	KU197410	-	This study	Shady Cove, San Juan Island, WA USA (S. Maslakova)
Cerebratulus montgomeryi	71	KU197411	KU197742	This study	Cattle Point, San Juan Island, WA USA (S. Malsakova)
Cerebratulus sp. 1 •	E1I1	KU197412	KU197743	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus sp. 2 •	E1G6	KU197413	KU197744	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus sp. 2 •	E5C1	KU197414	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus sp. 2 •	E5C2	KU197415	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus sp. pink proboscis	2	KU197416	KU197745	This study	False Bay, San Juan Island WA, USA (S. Maslakova)
Cerebratulus sp. pink proboscis •	27	KU197417	-	This study	Charleston, OR USA (S. Maslakova)
Cerebratulus sp. pink proboscis	98	KU197418	KU197746	This study	Charleston, OR USA (S. Maslakova)
Cerebratulus sp. pink proboscis	E3G4	KU197419	KU197747	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Cerebratulus sp. pink proboscis •	E4H1	KU197420	-	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus sp. pink proboscis •	LW35	KU197421	-	This study	Charleston, OR USA (L. Whittier)
Cerebratulus sp. spade head •	26	KU197422	KU197748	This study	Charleston, OR USA (S. Maslakova)
Cerebratulus sp. spade head •	E1A4	KU197423	KU197749	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus sp. spade head •	E1G1	KU197424	KU197750	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus sp. spade head •	E1G2	KU197425	KU197751	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus sp. spade head •	E1G5	KU197426	KU197752	This study	Charleston, OR USA (T. Hiebert)
Cerebratulus sp. Sunset Bay •	22	-	KU197753	This study	Charleston, OR USA (S. Maslakova)
Cerebratulus sp. Sunset Bay	97	KU197427	KU197754	This study	Charleston, OR USA (S. Maslakova)
Cerebratulus sp. Sunset Bay •	E4G9	KU197428	KU197755	This study	Charleston, OR USA (T. Hiebert)
Heteronemertea gen. sp. 1 •	80	KU197537	KU197828	This study	Charleston, OR USA (M. Jarvis)
Heteronemertea gen. sp. 1 •	E1H4	KU197538	KU197829	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Heteronemertea gen. sp. 1 •	E1H5	KU197539	KU197830	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Heteronemertea gen. sp. 1 •	E2D3	KU197540	-	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Heteronemertea gen. sp. 2 •	E1H6	KU197541	KU197831	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Heteronemertea gen. sp. 3 •	E1G9	KU197542	KU197832	This study	Charleston, OR USA (T. Hiebert)
Heteronemertea gen. sp. 5 •	76	KU197543	-	This study	Charleston, OR USA (M. Jarvis)
Heteronemertea gen. sp. 4 •	115	KU197544	KU197833	This study	Charleston, OR USA (G. von Dassow)
Heteronemertea gen. sp. 4 •	116	KU197545	-	This study	Charleston, OR USA (G. von Dassow)
Heteronemertea gen. sp. 4 •	117	KU197546	-	This study	Charleston, OR USA (G. von Dassow)
Heteronemertea gen. sp. 4 •	118	KU197547	KU197834	This study	Charleston, OR USA (T. Hiebert)
Heteronemertea gen. sp. 4 •	119	KU197548	KU197835	This study	Charleston, OR USA (T. Hiebert)
Heteronemertea gen. sp. 4 •	120	KU197549	-	This study	Charleston, OR USA (T. Hiebert)
Heteronemertea gen. sp. 4 •	E5C5	KU197550	-	This study	Charleston, OR USA (T. Hiebert)
Heteronemertea gen. sp. 6 •	128	KU197551	-	This study	Charleston, OR USA (S. Maslakova)
Lineidae gen. sp. “large eggs”	90	KU197435	KU197758	This study	Charleston, OR USA (S. Maslakova)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Lineidae gen. sp. “large eggs”	E1B7	KU197436	KU197759	This study	Charleston, OR USA (S. Maslakova)
Lineidae gen. sp. “large eggs”	E1B8	KU197437	KU197760	This study	Charleston, OR USA (S. Maslakova)
Lineidae gen. sp. “large eggs”	E1B9	KU197438	KU197761	This study	Charleston, OR USA (S. Maslakova)
Lineidae gen. sp. “large eggs”	E1C1	KU197439	KU197762	This study	Charleston, OR USA (S. Maslakova)
Lineidae gen. sp. “large eggs”	E2H1	KU197440	-	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineidae gen. sp. “large eggs”	E2H2	KU197441	-	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineidae gen. sp. “large eggs”	E2I2	KU197442	-	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineidae gen. sp. “large eggs”	E2I3	KU197443	-	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineus bilineatus	-	JF277571	-	Andrade et al. 2012	Kristineberg, Skagerak, Sweden
Lineus flavescens	-	KP682165	KP682050	Hiebert and Maslakova, 2015	Charleston, OR USA
Lineus flavescens	14	-	KU197763	This study	Charleston, OR USA (S. Maslakova)
Lineus flavescens •	141	-	KU197768	This study	Charleston, OR USA (S. Maslakova)
Lineus flavescens •	158	-	KU197770	This study	Port Orford, OR USA (S. Maslakova)
Lineus flavescens •	E1D3	-	KU197773	This study	Charleston, OR USA (L. Hiebert)
Lineus flavescens •	E1D4	-	KU197774	This study	Charleston, OR USA (L. Hiebert)
Lineus flavescens	E2F9	-	KU197783	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Lineus flavescens •	E2I9	-	KU197785	This study	Charleston, OR USA (R. Emlet)
Lineus flavescens	E3A1	-	KU197786	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	MMB20	-	KU197791	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	MMB46	-	KU197792	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	MMB70	-	KU197793	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	MMB115	-	KU197794	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens	15	KU197444	-	This study	Charleston, OR USA (S. Maslakova)
Lineus flavescens •	74	KU197445	KU197764	This study	Charleston, OR USA (S. Maslakova)
Lineus flavescens •	129	KU197446	KU197765	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineus flavescens •	130	KU197447	KU197766	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineus flavescens •	138	KU197448	KU197767	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineus flavescens •	139	KU197449	-	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineus flavescens •	140	KU197450	-	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineus flavescens •	157	KU197451	KU197769	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineus flavescens •	207	KU197452	KU197771	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineus flavescens •	E1D1	KU197453	-	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Lineus flavescens •	E1D2	KU197454	KU197772	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineus flavescens •	E1G4	KU197455	KU197775	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineus flavescens •	E1G7	KU197456	KU197776	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineus flavescens •	E1I9	KU197457	KU197777	This study	Charleston, OR USA (L. Hiebert)
Lineus flavescens •	E2B8	KU197458	KU197778	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	E2C6	KU197459	KU197779	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	E2C7	KU197460	KU197780	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	E2C9	KU197461	KU197781	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	E2D1	KU197462	KU197782	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens	E2G1	KU197463	KU197784	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineus flavescens •	E3A5	KU197464	-	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	E3C6	KU197465	KU197787	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	E3D6	KU197466	-	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	E3D7	KU197467	-	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	E3D9	KU197468	-	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	E3E4	KU197469	-	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	E3E5	KU197470	KU197788	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	E3F4	KU197471	KU197789	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Lineus flavescens •	E3F5	KU197472	KU197790	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	E5C6	KU197473	-	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	LWD2	KU197474	-	This study	Charleston, OR USA (L. Whittier)
Lineus flavescens •	MMB14	KU197475	-	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	MMB64	KU197476	-	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	MMB109	KU197477	-	This study	Charleston, OR USA (T. Hiebert)
Lineus flavescens •	MMBP29	KU197478	-	This study	Charleston, OR USA (T. Hiebert)
Lineus ruber	-	AF103758	-	Sundberg and Saur 1998	western coast, Sweden
Lineus ruber	-	-	GU733828	Chen et al. 2010	Wales, UK
Lineus rubescens	-	-	EF124971	Schwartz & Norenburg, unpublished	Canada
Lineus sp. “crescent” •	E2B5	KU197512	KU197811	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. “crescent” •	E2E2	KU197513	KU197812	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. “crescent” •	E2E3	KU197514	KU197813	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. “crescent”	E2H3	KU197515	KU197814	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Lineus sp. “red”	113	-	KU197817	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. “red”	E2H7	-	KU197820	This study	Charleston, OR USA (L. Hiebert, T. Hiebert & S. Maslakova)
Lineus sp. “red”	MMB5	-	KU197823	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. “red”	MMB6	-	KU197824	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. “red”	MMB12	-	KU197825	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Lineus sp. "red"	31	KU197516	KU197815	This study	Charleston, OR USA (S. Maslakova)
Lineus sp. "red"	65	KU197517	-	This study	Charleston, OR USA (S. Maslakova)
Lineus sp. "red"	91	KU197518	-	This study	Charleston, OR USA (S. Maslakova)
Lineus sp. "red"	101	KU197519	KU197816	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. "red"	E1B6	KU197520	KU197818	This study	Charleston, OR USA (S. Maslakova)
Lineus sp. "red" •	E2C8	KU197521	KU197819	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. "red"	E2G4	KU197522	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. "red" •	E4D7	KU197523	KU197821	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. "red" •	E4D8	KU197524	KU197822	This study	Charleston, OR USA (R. Emlet)
Lineus sp. 1 •	23	KU197479	KU197795	This study	Charleston, OR USA (S. Maslakova)
Lineus sp. 1 •	28	KU197480	KU197796	This study	Charleston, OR USA (S. Maslakova)
Lineus sp. 1 •	121	KU197481	KU197797	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 1 •	122	KU197482	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 1 •	195	KU197483	KU197798	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 1 •	E1G8	KU197485	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 1 •	E1G3	KU197484	KU197799	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	MMB105	-	KU197809	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	MMB128	-	KU197810	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	78	-	KU197800	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	E1I8	KU197486	KU197801	This study	Charleston, OR USA (R. Emlet)
Lineus sp. 2 •	E2B9	KU197487	KU197802	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Lineus sp. 2 •	E2C1	KU197488	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	E2E1	KU197489	KU197803	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	E3C8	KU197490	KU197804	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	E3D2	KU197491	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	E3D8	KU197492	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	E3E3	KU197493	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	E3E9	KU197494	KU197805	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	E3F1	KU197495	KU197806	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	E3F6	KU197496	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	E4E7	KU197497	KU197807	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	E4E8	KU197498	KU197808	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	E4E9	KU197499	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	MMB61	KU197500	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	MMB99	KU197501	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	MMB122	KU197502	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	MMBP17	KU197503	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	MMBP18	KU197504	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	MMBP19	KU197505	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	MMBP20	KU197506	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	MMBP21	KU197507	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	MMBP22	KU197508	-	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Lineus sp. 2 •	MMBP23	KU197509	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	MMBP26	KU197510	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	MMBP28	KU197511	-	This study	Charleston, OR USA (T. Hiebert)
Lineus sp. 2 •	399	KU517440	KU517443	This study	Charleston, OR USA (T. Hiebert)
Lineus torquatus	-	JF277572	HQ848574	Andrade et al. 2012	Akkeshi Bay, Japan
Lineus viridis	-	JF277582	HQ848579	Andrade et al. 2012	Sylt Island, Hordfriesland, Schleswig-Holstein, Germany
Maculaura alaskensis •	-	KP682166–2207	KP682051–2082	Hiebert and Maslakova, 2015	Charleston, OR; Seaside, OR; Friday Harbor San Juan Island, WA USA
Maculaura aquilonia •	-	KP682208–2250	KP682083–2133	Hiebert and Maslakova, 2015	Charleston, OR; Juneau, AK USA; Sea of Okhotsk, Russia
Maculaura cerebrosa •	-	KP682251–2267	KP682134–2146	Hiebert and Maslakova, 2015	Charleston, OR; Crescent City, CA USA
Maculaura magna •	-	KP682268–2295	KP682147–2156	Hiebert and Maslakova, 2015	Charleston, OR USA
Maculaura oregonensis	-	KP682296–2304	KP682157–2164	Hiebert and Maslakova, 2015	Charleston, OR USA
Micrura akkeshiensis	-	EF124887	EF124975	Schwartz & Norenburg, unpublished	Japan
Micrura fasciolata	-	JF277586	HQ848578	Andrade et al. 2012	Tjärnö, Koster, Sweden
Micrura rubramaculosa	-	DQ022550	-	Schwartz and Norenburg 2005	Carrie Bow Cay, Belize
Micrura rubramaculosa	-	-	KF935513	Kvist et al. 2014	Bocas del Toro, Panama
Micrura sp. “albocephala” •	E1H2	KU197569	KU197845	This study	Charleston, OR USA (T. Hiebert)
Micrura sp. “albocephala”	125	KU197564	-	This study	Charleston, OR USA (A. Bird)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Micrura sp. "albocephala" •	142	KU197565	KU197842	This study	Charleston, OR USA (S. Maslakova)
Micrura sp. "albocephala" •	145	KU197566	-	This study	Charleston, OR USA (S. Maslakova)
Micrura sp. "albocephala" •	146	KU197567	KU197843	This study	Charleston, OR USA (S. Maslakova)
Micrura sp. "albocephala"	E1D6	KU197568	KU197844	This study	Charleston, OR USA (T. Hiebert)
Micrura sp. "albocephala" •	E2C2	KU197570	KU197846	This study	Charleston, OR USA (T. Hiebert)
Micrura sp. "albocephala" •	E2C3	KU197571	-	This study	Charleston, OR USA (T. Hiebert)
Micrura sp. "albocephala" •	E2C4	KU197572	KU197847	This study	Charleston, OR USA (T. Hiebert)
Micrura sp. "albocephala" •	E2C5	KU197573	KU197848	This study	Charleston, OR USA (T. Hiebert)
Micrura sp. "albocephala" •	E3A9	KU197574	KU197849	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Micrura sp. "albocephala" •	E3B1	KU197575	KU197850	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Micrura sp. "albocephala" •	E3B3	KU197576	KU197851	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Micrura sp. "albocephala"	E5A9	KU197577	KU197852	This study	Charleston, OR USA (M. Hunt)
Micrura sp. "albocephala"	MMB106	-	KU197853	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Micrura sp. “dark” •	-	JQ430744–746	JQ430741–743	Maslakova and von Dassow 2012	Charleston, OR USA
Micrura sp. “dark”	E2E8	KU197582	KU197859	This study	Charleston, OR USA (T. Hiebert)
Micrura sp. “dark”	E2G9	KU197583	KU197860	This study	Charleston, OR USA (T. Hiebert)
Micrura sp. “dark”	E2H8	KU197584	-	This study	Charleston, OR USA (T. Hiebert & M. Hunt)
Micrura sp. “dark”	E2H9	KU197585	-	This study	Charleston, OR USA (T. Hiebert & M. Hunt)
Micrura sp. “dark”	E2I1	KU197586	KU197858	This study	Charleston, OR USA (T. Hiebert & M. Hunt)
Micrura sp. “not coei”	E4H8	KU197525	KU197826	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Micrura sp. “not coei”	E5B1	KU197526	-	This study	Charleston, OR USA (M. Hunt)
Micrura sp. 3 •	E1H8	KU197563	KU197841	This study	Charleston, OR USA (T. Hiebert)
Micrura sp. 4 •	143	KU197578	KU197854	This study	Charleston, OR USA (S. Maslakova)
Micrura sp. 4 •	144	KU197579	KU197855	This study	Charleston, OR USA (S. Maslakova)
Micrura sp. 4 •	148	KU197580	KU197856	This study	Charleston, OR USA (S. Maslakova)
Micrura sp. 4 •	E3B2	KU197581	KU197857	This study	Charleston, OR USA (T. Hiebert)
Micrura sp. 5 •	5	KU197587	-	This study	Charleston, OR USA (M. Hunt)
Micrura verrilli	-	KF935455	KF935508	Kvist et al. 2014	USA
Micrura verrilli	73	KU197527	-	This study	Cattle Point, San Juan Island, WA USA (S. Malsakova)
Micrura verrilli	E3A4	KU197528	-	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Micrura wilsoni	20	KU197529	-	This study	Charleston, OR USA (S. Maslakova)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Micrura wilsoni	106	KU197530	-	This study	Charleston, OR USA (R. Emlet)
Micrura wilsoni	E1E9	KU197531	KU197827	This study	Charleston, OR USA (L. Hiebert)
Micrura wilsoni	MMB22	KU197532	-	This study	Charleston, OR USA (T. Hiebert)
Micrura wilsoni	MMB23	KU197533	-	This study	Charleston, OR USA (S. Maslakova)
Micrura wilsoni	MMB24	KU197534	-	This study	Charleston, OR USA (T. Hiebert)
Micrura wilsoni	MMB25	KU197535	-	This study	Charleston, OR USA (T. Hiebert)
Micrura wilsoni	MMB26	KU197536	-	This study	Charleston, OR USA (T. Hiebert)
Oxypolellinae gen. sp.	E4H7	KU748595	-	Chernyshev 2016	Vietnam (A. Chernyshev)
Ramphogordius sanguineus	E1A9	KU197552	KU197836	This study	Charleston, OR USA (G. von Dassow)
Ramphogordius sanguineus	E1B1	KU197553	KU197837	This study	Charleston, OR USA (G. von Dassow)
Ramphogordius sanguineus	E2G7	KU197554	KU197838	This study	Charleston, OR USA (T. Hiebert)
Ramphogordius sanguineus	E3I9	KU197555	-	This study	Charleston, OR USA (T. Hiebert)
Riserius pugetensis	-	AJ436831	AJ436941	Thollesson and Norenburg 2003	San Juan Island, WA USA
Riserius sp. “eyes” •	-	KC777029–030	KC777037–038	Hiebert et al. 2013	Charleston, OR USA
Riserius sp. “eyes” •	E4B4_R	-	KU197840	This study	Charleston, OR USA (T. Hiebert)
Riserius sp. “no eyes” •	-	KC777021–028	KC777031–036	Hiebert et al. 2013	Charleston, OR USA
Riserius sp. “no eyes” •	208	KU197556	-	This study	Charleston, OR USA (T. Hiebert)
Riserius sp. “no eyes” •	209	KU197557	-	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Riserius sp. “no eyes” •	E3E6	KU197558	-	This study	Charleston, OR USA (T. Hiebert)
Riserius sp. “no eyes” •	E3H9	KU197559	-	This study	Charleston, OR USA (T. Hiebert)
Riserius sp. “no eyes” •	E3I4	KU197560	KU197839	This study	Charleston, OR USA (T. Hiebert)
Riserius sp. “no eyes” •	LWC3	KU197561	-	This study	Charleston, OR USA (L. Whittier)
Riserius sp. “no eyes” •	LWC9	KU197562	-	This study	Charleston, OR USA (L. Whittier)
Zygeupolia rubens	-	JF277574	-	Andrade et al. 2012	Fort Pierce, FL USA

APPENDIX C

HOPLONEMERTEAN SPECIMENS AND SEQUENCES

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Amphiporus angulatus	-	AJ436786	AJ436896	Thollesson & Norenburg 2003	Cobscook, ME, USA
Amphiporus cruentatus	E2H4	-	KU197588	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Amphiporus formidabilis	-	AJ436787	AJ436897	Thollesson & Norenburg 2003	San Juan Island, WA USA
Amphiporus imparispinosus	-	AJ436788	AJ436898	Thollesson & Norenburg 2003	San Juan Island, WA USA
Carcinonemertes carcinophila	-	JF277603	HQ848619	Andrade et al. 2012	Beaufort, NC, USA
Carcinonemertes sp. “chaceon"	69	KU197259	KU197595	This study	Gulf of Mexico, USA (G. von Dassow)
Carcinonemertes errans •	154	KU197257	KU197591	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Carcinonemertes errans •	219	-	KU197592	This study	Charleston, OR USA (T. Hiebert)
Carcinonemertes errans •	220	-	KU197593	This study	Charleston, OR USA (T. Hiebert)
Carcinonemertes errans •	43	KU197254	KU197589	This study	Charleston, OR USA (S. Maslakova)
Carcinonemertes errans •	72	KU197255	KU197590	This study	Charleston, OR USA (S. Maslakova)
Carcinonemertes errans •	83	KU197256	-	This study	Charleston, OR USA (M. Jarvis)
Carcinonemertes errans •	E4D6	KU197258	KU197594	This study	Charleston, OR USA (T. Hiebert)
Carcinonemertes sp. 414	-	AJ436791	AJ436901	Thollesson & Norenburg 2003	São Sebastião, Brazil
Emplectonema buergeri	-	AJ436792	AJ436902	Thollesson & Norenburg 2003	San Juan Island, WA USA
Emplectonema gracile	-	AJ436793	AJ436903	Thollesson & Norenburg 2003	Salcombe, UK
Emplectonema sp. 1 •	E4H2	KU197260	KU197596	This study	Charleston, OR USA (T. Hiebert)
Emplectonema sp. 1	E5B5	KU197261	KU197597	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Emplectonema sp. 1	E5B6	KU197262	KU197598	This study	Charleston, OR USA (T. Hiebert)
Emplectonema sp. 1	E5B7	KU197263	KU197599	This study	Charleston, OR USA (T. Hiebert)
Gurjanovella littoralis	88	KU197264	-	This study	False Bay, Friday Harbor, WA USA (S. Maslakova)
Gurjanovella littoralis •	339	KU197265	KU197600	This study	Charleston, OR USA (T. Hiebert)
Gurjanovella littoralis	-	AJ436794	AJ436904	Thollesson & Norenburg 2003	Kandalatsha Bay, White Sea, Russia
Malacobdella grossa	-	AJ436795	AJ436905	Thollesson & Norenburg 2003	VA, USA and White Sea, Russia
Malacobdella siliquae	89	KU197266	-	This study	Charleston, OR USA (S. Maslakova)
Malacobdella siliquae •	E2A5	KU197267	-	This study	Charleston, OR USA (T. Hiebert)
Malacobdella siliquae •	E4B2	KU197268	KU197601	This study	Charleston, OR USA (T. Hiebert)
Nipponnemertes bimaculata	E3G3	-	KU197605	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Nipponnemertes bimaculata	E3F9	KU197269	KU197602	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Nipponnemertes bimaculata	E3G1	KU197270	KU197603	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Nipponnemertes bimaculata	E3G2	KU197271	KU197604	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Nipponnemertes bimaculata	-	-	AJ436909	Thollesson & Norenburg 2003	San Juan Island, WA USA
Nipponnemertes pulchra	-	JF277625	HQ848598	Andrade et al. 2012	Tjärnö, Sweden
Nipponnemertes punctatulus	-	AJ436800	AJ436910	Thollesson & Norenburg 2003	Oshoro, Hokkaido, Japan
Nipponnemertes ogumai	-	AB921008	-	Kajihara et al., in press	Kanagawa, Araihama, Japan

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Nipponnemertes sp. 1	-	JF277624	HQ848598	Andrade et al. 2012	Santa Rosa-Cortes Ridge, California
Nipponnemertes sp. 2	-	JF277623	HQ848599	Andrade et al. 2012	Talcahuano, Biobio, Chile
Oerstedia dorsalis	-	-	AY791971	Strand and Sundberg 2005	Tjärnö, Sweden
Oerstedia dorsalis	-	-	FJ855364	Sundberg et al. 2009	western coast, Sweden
Ototyphlonemertes macintoshi	-	JF277613	HQ848605	Andrade et al. 2012	Praia do Mindelo, Vila do Conde, Portugal
Ototyphlonemertes sp. 1 •	132	KU197272	-	This study	Charleston, OR USA (T. Hiebert)
Pantinonemertes californiensis	-	EF157585	EF157597	Maslakova & Norenburg 2008	USA
Pantinonemertes californiensis	E4A1	-	KU197606	This study	Charleston, OR USA (T. Hiebert & J. Carlton)
Pantinonemertes californiensis	E4A5	-	KU197607	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Paranemertes californica	E1C7	KU197273	KU197614	This study	Charleston, OR USA (S. Maslakova)
Paranemertes californica •	118_Pc	-	KU197610	This study	Charleston, OR USA (T. Hiebert)
Paranemertes californica •	E2A3	-	KU197616	This study	Charleston, OR USA (T. Hiebert)
Paranemertes californica •	E3H3	-	KU197620	This study	Charleston, OR USA (T. Hiebert)
Paranemertes californica •	221	KU197274	KU197612	This study	Charleston, OR USA (T. Hiebert)
Paranemertes californica •	51	-	KU197608	This study	Charleston, OR USA (T. Hiebert)
Paranemertes californica •	104	-	KU197609	This study	Charleston, OR USA (T. Hiebert)
Paranemertes californica •	218	-	KU197611	This study	Charleston, OR USA (T. Hiebert)
Paranemertes californica •	338	-	KU197613	This study	Charleston, OR USA (T. Hiebert)
Paranemertes californica •	E2A7	-	KU197617	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Paranemertes californica •	E1I4	KU197275	KU197615	This study	Charleston, OR USA (T. Hiebert)
Paranemertes californica •	E2E5	KU197276	KU197618	This study	Charleston, OR USA (T. Hiebert)
Paranemertes californica •	E2E6	KU197277	KU197619	This study	Charleston, OR USA (T. Hiebert)
Paranemertes californica •	E4E6	KU197278	KU197621	This study	Charleston, OR USA (R. Emlet)
Paranemertes sp. 1 •	E2A9	-	KU197622	This study	Charleston, OR USA (T. Hiebert)
Paranemertes sp. 1 •	E2D2	KU197279	KU197623	This study	Charleston, OR USA (T. Hiebert)
Paranemertes sp. 1 •	E2I5	KU197280	KU197624	This study	Charleston, OR USA (T. Hiebert)
Paranemertes sp. 1 •	E3B4	KU197281	-	This study	Charleston, OR USA (T. Hiebert)
Paranemertes sp. 1 •	E3D1	KU197282	KU197625	This study	Charleston, OR USA (T. Hiebert)
Paranemertes sp. 1 •	E4E1	KU197283	-	This study	Charleston, OR USA (T. Hiebert)
Paranemertes sp. 1 •	E4F4	KU197284	-	This study	Charleston, OR USA (T. Hiebert)
Paranemertes sp. 1 •	E4F5	KU197285	-	This study	Charleston, OR USA (T. Hiebert)
Paranemertes sp. 1 •	I7			This study	Charleston, OR USA (T. Hiebert)
Paranemertes sp. 2 •	46	KU517438	KU517444	This study	Charleston, OR USA (T. Hiebert)
Paranemertes peregrina	-	AJ436805	AJ436915	Thollesson & Norenburg 2003	San Juan Island, WA USA
Paranemertes sanjuanensis	E2F6	KU197286	KU197626	This study	Charleston, OR USA (T. Hiebert)
Paranemertes sanjuanensis	-	AJ436807	AJ436917	Thollesson & Norenburg 2003	San Juan Island, WA USA
Poseidonemertes collaris •	E1H1	-	KU197633	This study	Charleston, OR USA (T. Hiebert)
Poseidonemertes collaris •	149	-	KU197629	This study	Charleston, OR USA (T. Hiebert)
Poseidonemertes collaris •	E4A9	KU197287	-	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Poseidonemertes collaris •	P133	KU197288	KU197627	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Poseidonemertes collaris •	134	KU197289	KU197628	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Poseidonemertes collaris •	150	KU197290	KU197630	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Poseidonemertes collaris •	151	KU197291	KU197631	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Poseidonemertes collaris •	342	KU197292	KU197632	This study	Charleston, OR USA (T. Hiebert & S. Maslakova)
Poseidonemertes collaris •	E1C8	KU197293	-	This study	Charleston, OR USA (T. Hiebert)
Poseidonemertes collaris •	E5C9	KU197294	KU197634	This study	Charleston, OR USA (T. Hiebert)
Poseidonemertes collaris	-	AJ436809	AJ436919	Thollesson & Norenburg 2003	Bodega Bay, CA USA
Tetrastemma albidum	-	-	EF157598	Maslakova & Norenburg 2008	La Jolla, CA, USA
Tetrastemma bilineatum	E2F3	-	KU197635	This study	Charleston, OR USA (S. Maslakova)
Tetrastemma candidum	-	-	AY791973	Strand & Sundberg 2005	Anglesey, Wales UK
Tetrastemma sp. 1	E2F2	-	KU197636	This study	Charleston, OR USA (S. Maslakova)
Tetrastemma sp. 1	E2I8	-	KU197637	This study	Charleston, OR USA (S. Maslakova)
Zygonemertes sp. 1	E1C3	-	KU197638	This study	Charleston, OR USA (S. Maslakova)
Zygonemertes simonae	-	AJ436812	AJ436922	Thollesson & Norenburg 2003	Fort Pierce, FL, USA
Zygonemertes sp. 1	E2F4	-	KU197639	This study	Charleston, OR USA (S. Maslakova)
Zygonemertes sp. 1	E3D5	-	KU197642	This study	Charleston, OR USA (T. Hiebert)

Species		GenBank Accession # (s)		Study	Collection Information
		16S	COI		
Zygonemertes sp. 1 •	E4E2	-	KU197643	This study	Charleston, OR USA (T. Hiebert)
Zygonemertes sp. 1	E4I3	-	KU197646	This study	Charleston, OR USA (T. Hiebert)
Zygonemertes sp. 1	E4I4	-	KU197647	This study	Charleston, OR USA (T. Hiebert)
Zygonemertes sp. 1	E4I5	-	KU197648	This study	Charleston, OR USA (T. Hiebert)
Zygonemertes sp. 1	E4I7	-	KU197650	This study	Charleston, OR USA (T. Hiebert)
Zygonemertes sp. 1	E4I8	-	KU197651	This study	Charleston, OR USA (T. Hiebert)
Zygonemertes sp. 1 •	E5B3	-	KU197652	This study	Charleston, OR USA (T. Hiebert)
Zygonemertes sp. 1	13	KU197295	-	This study	Charleston, OR USA (T. Hiebert)
Zygonemertes sp. 1 •	135	KU197296	-	This study	Charleston, OR USA (T. Hiebert & S. Malsakova)
Zygonemertes sp. 1 •	136	KU197297	-	This study	Charleston, OR USA (T. Hiebert & S. Malsakova)
Zygonemertes sp. 1 •	E3A8	KU197298	KU197640	This study	Charleston, OR USA (T. Hiebert)
Zygonemertes sp. 1 •	E3C7	KU197299	KU197641	This study	Charleston, OR USA (R. Emlet)
Zygonemertes sp. 1 •	E4E3	KU197300	-	This study	Charleston, OR USA (T. Hiebert)
Zygonemertes sp. 1 •	E4E4	KU197301	KU197644	This study	Charleston, OR USA (T. Hiebert)
Zygonemertes sp. 1	E4I2	KU197302	KU197645	This study	Charleston, OR USA (T. Hiebert)
Zygonemertes sp. 1	E4I6	KU197303	KU197649	This study	Charleston, OR USA (T. Hiebert)
Zygonemertes virescens	-	AJ436813	AJ436923	Thollesson & Norenburg 2003	Fort Pierce, FL, USA

APPENDIX D

CEPHALOTHRIX SEQUENCES AND GENBANK ACCESSION NUMBERS

	COI		
Species	**GenBank Accession #**	**Study**	**Collection Information**
Cephalothrix alba	KM083817	Leasi & Norenburg 2014	Belize
Cephalothrix alba	KM083818	Leasi & Norenburg 2014	Belize
Cephalothrix alba	KM083819	Leasi & Norenburg 2014	Belize
Cephalothrix fasciculus	GU726623	Chen et al. 2010	Fukue, Japan
Cephalothrix fasciculus	KM083814	Leasi & Norenburg 2014	Belize
Cephalothrix filiformis	AJ436944	Thollesson & Norenburg 2003	Akkeshi Bay, Japan
Cephalothrix filiformis	GU726635	Chen et al. 2010	Akkeshi Bay, Japan
Cephalothrix filiformis	GU726636	Chen et al. 2010	Akkeshi Bay, Japan
Cephalothrix filiformis	GU726637	Chen et al. 2010	Akkeshi Bay, Japan
Cephalothrix filiformis	GU726645	Chen et al. 2010	Akkeshi Bay, Japan
Cephalothrix filiformis	HQ848616	Andrade et al. 2012	Rhos-on-Sea, Wales UK
Cephalothrix filiformis	HQ848617	Andrade et al. 2012	Sylt Island, Germany
Cephalothrix hongkongiensis	GU726610	Chen et al. 2010	Shenzhen, Guangdong, China
Cephalothrix hongkongiensis	GU726613	Chen et al. 2010	Starfish Bay, Hong Kong, China
Cephalothrix hongkongiensis	GU726611	Chen et al. 2010	Starfish Bay, Hong Kong, China
Cephalothrix hongkongiensis	GU726612	Chen et al. 2010	Starfish Bay, Hong Kong, China
Cephalothrix hongkongiensis	HQ848614	Andrade et al. 2012	Qingdao, Shandong, China
Cephalothrix hongkongiensis	HQ848615	Andrade et al. 2012	Qingdao, Shandong, China
Cephalothrix linearis	GU726652	Chen et al. 2010	White Sea, Russia
Cephalothrix linearis	GU726653	Chen et al. 2010	White Sea, Russia
Cephalothrix linearis	GU726650	Chen et al. 2010	White Sea, Russia
Cephalothrix linearis	GU726651	Chen et al. 2010	White Sea, Russia
Cephalothrix linearis	GU726649	Chen et al. 2010	White Sea, Russia
Cephalothrix major	GU726689	Chen et al. 2010	OR USA
Cephalothrix major	GU726690	Chen et al. 2010	OR USA
Cephalothrix major	GU726691	Chen et al. 2010	OR USA
Cephalothrix rufifrons	EU489494	Sundberg et al. 2009b	Sweden
Cephalothrix rufifrons	GU726742	Chen et al. 2010	Grötholmen, Sweden
Cephalothrix rufifrons	GU726743	Chen et al. 2010	Grötholmen, Sweden

	COI		
Species	**GenBank Accession #**	**Study**	**Collection Information**
Cephalothrix rufifrons	GU726744	Chen et al. 2010	Grötholmen, Sweden
Cephalothrix rufifrons	GU726745	Chen et al. 2010	Grötholmen, Sweden
Cephalothrix rufifrons	GU726746	Chen et al. 2010	Grötholmen, Sweden
Cephalothrix rufifrons	GU726747	Chen et al. 2010	Vattenholmen, Sweden
Cephalothrix rufifrons	GU726748	Chen et al. 2010	Vattenholmen, Sweden
Cephalothrix rufifrons	GU726601	Chen et al. 2010	Wembury/Salcombre, Devon UK
Cephalothrix rufifrons	HQ848604	Andrade et al. 2012	Rhos-on-Sea, Wales UK
Cephalothrix rufifrons	GU726603	Chen et al. 2010	Wembury/Salcombre, Devon UK
Cephalothrix rufifrons	GU726604	Chen et al. 2010	Wembury/Salcombre, Devon UK
Cephalothrix rufifrons	GU726602	Chen et al. 2010	Wembury/Salcombre, Devon UK
Cephalothrix sp. 1	KM083809	Leasi & Norenburg 2014	Bocas del Toro, Panama
Cephalothrix sp. 1	KM083811	Leasi & Norenburg 2014	Bocas del Toro, Panama
Cephalothrix sp. 1	KM083820	Leasi & Norenburg 2014	Bocas del Toro, Panama
Cephalothrix sp. 10	GU726681	Chen et al. 2010	Bocas del Toro, Panama
Cephalothrix sp. 11	GU726667	Chen et al. 2010	Seto, Japan
Cephalothrix sp. 12	GU726666	Chen et al. 2010	Seto, Japan
Cephalothrix sp. 13	GU726621	Chen et al. 2010	Vietnam
Cephalothrix sp. 14	GU726675	Chen et al. 2010	Bocas del Toro, Panama
Cephalothrix sp. 14	GU726676	Chen et al. 2010	Bocas del Toro, Panama
Cephalothrix sp. 14	GU726674	Chen et al. 2010	Bocas del Toro, Panama
Cephalothrix sp. 14	GU726673	Chen et al. 2010	Bocas del Toro, Panama
Cephalothrix sp. 14	GU726679	Chen et al. 2010	Bocas del Toro, Panama
Cephalothrix sp. 15	GU726682	Chen et al. 2010	Carrie Bow Cay, Belize
Cephalothrix sp. 15	GU726677	Chen et al. 2010	Bocas del Toro, Panama
Cephalothrix sp. 15	GU726678	Chen et al. 2010	Bocas del Toro, Panama
Cephalothrix sp. 16	GU726644	Chen et al. 2010	Jeju Island, Korea
Cephalothrix sp. 16	GU726614	Chen et al. 2010	Changdao, Shandong, China
Cephalothrix sp. 2	KM083810	Leasi & Norenburg 2014	Bocas del Toro, Panama
Cephalothrix sp. 2	GU726633	Chen et al. 2010	Kaneohe, HI USA

	COI		
Species	**GenBank Accession #**	**Study**	**Collection Information**
Cephalothrix sp. 2	GU726634	Chen et al. 2010	Kaneohe, HI USA
Cephalothrix simula	GU726624	Chen et al. 2010	Qingdao, Shandong, China
Cephalothrix simula	GU726625	Chen et al. 2010	Qingdao, Shandong, China
Cephalothrix simula	GU726618	Chen et al. 2010	Changdao, Shandong, China
Cephalothrix simula	GU726608	Chen et al. 2010	Peter the Great Bay, Russia
Cephalothrix simula	GU726615	Chen et al. 2010	Changdao, Shandong, China
Cephalothrix simula	GU726619	Chen et al. 2010	Oshoro, Japan
Cephalothrix simula	GU726664	Chen et al. 2010	Seto, Japan
Cephalothrix simula	GU726622	Chen et al. 2010	Fukue, Japan
Cephalothrix simula	GU726620	Chen et al. 2010	Shimoda, Japan
Cephalothrix simula	GU726641	Chen et al. 2010	Vostok Bay, Sea of Japan, Russia
Cephalothrix simula	GU726665	Chen et al. 2010	Seto, Japan
Cephalothrix simula	GU726643	Chen et al. 2010	Vostok Bay, Sea of Japan, Russia
Cephalothrix simula	GU726662	Chen et al. 2010	Seto, Japan
Cephalothrix simula	GU726642	Chen et al. 2010	Vostok Bay, Sea of Japan, Russia
Cephalothrix simula	GU726663	Chen et al. 2010	Seto, Japan
Cephalothrix simula	KM083812	Leasi & Norenburg 2014	Bocas del Toro, Panama
Cephalothrix sp. 4	KM083813	Leasi & Norenburg 2014	Belize
Cephalothrix sp. 4	GU726671	Chen et al. 2010	Roscoff, France
Cephalothrix sp. 4	GU726672	Chen et al. 2010	Roscoff, France
Cephalothrix sp. 4	GU726670	Chen et al. 2010	Roscoff, France
Cephalothrix sp. 5	KM083815	Leasi & Norenburg 2014	Belize
Cephalothrix sp. 5	GU726629	Chen et al. 2010	Sanya, Hainan, China
Cephalothrix sp. 5	GU726631	Chen et al. 2010	Sanya, Hainan, China
Cephalothrix sp. 5	GU726630	Chen et al. 2010	Sanya, Hainan, China
Cephalothrix sp. 6	KM083816	Leasi & Norenburg 2014	Panama
Cephalothrix sp. 6	GU726640	Chen et al. 2010	San Diego, CA USA

	COI		
Species	**GenBank Accession #**	**Study**	**Collection Information**
Cephalothrix sp. 6	GU726668	Chen et al. 2010	Fort Pierce, FL USA
Cephalothrix sp. 6	GU726669	Chen et al. 2010	Fort Pierce, FL USA
Cephalothrix sp. 6	GU726639	Chen et al. 2010	Fort Pierce, FL USA
Cephalothrix sp. 8	GU726616	Chen et al. 2010	Armintza, Bizkaia, Spain
Cephalothrix sp. 9	GU726680	Chen et al. 2010	Bocas del Toro, Panama
Cephalothrix spiralis	GU726704	Chen et al. 2010	Mt Desert Island, ME USA
Cephalothrix spiralis	GU726708	Chen et al. 2010	Nahant, MA USA
Cephalothrix spiralis	GU726702	Chen et al. 2010	Mt Desert Island, ME USA
Cephalothrix spiralis	GU726700	Chen et al. 2010	Mt Desert Island, ME USA
Cephalothrix spiralis	GU726698	Chen et al. 2010	Mt Desert Island, ME USA
Cephalothrix spiralis	GU726707	Chen et al. 2010	Nahant, MA USA
Cephalothrix spiralis	GU726697	Chen et al. 2010	OR USA
Cephalothrix spiralis	GU726699	Chen et al. 2010	Mt Desert Island, ME USA
Cephalothrix spiralis	GU726703	Chen et al. 2010	Mt Desert Island, ME USA
Cephalothrix spiralis	GU726705	Chen et al. 2010	Mt Desert Island, ME USA
Cephalothrix spiralis	GU726706	Chen et al. 2010	Mt Desert Island, ME USA
Cephalothrix spiralis	GU726701	Chen et al. 2010	Mt Desert Island, ME USA
Cephalothrix spiralis	GU726712	Chen et al. 2010	San Juan Island, WA USA
Cephalothrix spiralis	GU726696	Chen et al. 2010	OR USA
Cephalothrix spiralis	GU726695	Chen et al. 2010	OR USA
Cephalothrix spiralis	GU726711	Chen et al. 2010	Kachemak Bay, AK USA
Cephalothrix spiralis	GU726648	Chen et al. 2010	San Juan Island, WA USA
Cephalothrix spiralis	GU726693	Chen et al. 2010	OR USA
Cephalothrix spiralis	GU726709	Chen et al. 2010	Kachemak Bay, AK USA
Cephalothrix spiralis	GU726692	Chen et al. 2010	OR USA
Cephalothrix spiralis	GU726694	Chen et al. 2010	OR USA
Cephalothrix spiralis	GU726710	Chen et al. 2010	Kachemak Bay, AK USA
Cephalothrix spiralis	AJ436946	Thollesson & Norenburg 2003	San Juan Island, WA USA

APPENDIX E

PALAEONEMERTEAN DIVERGENCE TABLE (%)

	C. m	**C. 5**	**C. h**	**C. y**	**C. t 1**	**C. t 2**	**C. w**	**C. 1**	**Cp. m**	**C. s**	**C. 3**
Carinoma mutabilis (n=9, **n=6**; C. m)	1.0	4.3	11.1	12.5	13.0	10.0	15.8	28.2	28.8	28.6	30.6
	4.1	13.0	14.8	16.3	14.1	13.3	15.2	19.3	20.1	21.0	-
Carinoma sp. 5 (n=1, **n=1**; C. 5)		-	10.1	12.7	12.0	10.0	15.2	27.0	27.0	27.9	28.8
		-	**13.5**	**15.6**	**15.1**	**11.7**	**15.5**	**19.3**	**20.1**	**21.1**	**-**
Carinoma hamanako (n=5, **n=5**; C. h)			0.8	8.8	8.0	11.0	12.1	28.6	29.5	29.3	30.1
			3.7	**18.0**	**15.2**	**15.5**	**14.3**	**20.0**	**19.6**	**20.7**	**-**
Carinoma sp. "yellowback" (n=4, **n=3**; C. y)				0.2	10.0	14.0	16.4	30.6	31.6	30.3	30.2
				0.9	**15.9**	**16.4**	**17.3**	**21.7**	**20.3**	**20.9**	**-**
Carinoma tremaphoros 1 (n=1, **n=1**; C. t)					-	13.0	15.0	29.0	31.0	30.0	31.0
(AJ436833)					**-**	**14.7**	**14.5**	**19.8**	**17.9**	**20.3**	**-**
Carinoma tremaphoros 2 (n=1, **n=1**; C. t)						-	15.0	28.0	30.0	29.0	31.0
(JF277602)						**-**	**14.4**	**19.9**	**19.9**	**21.3**	**-**
Carinoma sp. "white" (n=6, **n=3**; C. w)							0.0	29.4	28.5	29.5	31.6
							0.0	**17.8**	**17.6**	**19.9**	**-**
Cephalothrix sp. 1 (n=9, **n=6**; C. 1)								0.1	20.7	21.2	21.1
								0.3	**15.3**	**15.0**	**-**
Cephalothrix major (n=1, **n=1**; Cp. m)									-	3.7	14.3
									-		**-**
Cephalothrix cf. *spiralis* (n=1, **n=1**; C. s)										-	15.2
										-	**-**
Cephalothrix sp. 3 (n=1; C. 3)											-
											-
Cephalothrix sp. 2 (n=3, **n=2;** C. 2)											
Cephalothrix sp. 4 (n=6, **n=11**; C. 4)											

	C. 2	C. 4	T. a	T. po	T. pu	T. s	T. r	T. 2	T. 3	T. 1	T. pe
Carinoma mutabilis (n=9, **n=6**; C. m)	26.7	29.2	34.6	34.1	32.6	31.2	33.2	33.6	34.5	30.9	35.9
	20.3	22.3	19.5	18.1	20.7	20.6	22.1	18.3	21.9	18.9	19.5
Carinoma sp. 5 (n=1, **n=1**; C. 5)	24.9	29.1	33.8	32.9	31.6	31.1	32.5	31.7	33.4	29.4	35.0
	18.9	**22.3**	**19.7**	**19.7**	**20.1**	**21.5**	**21.9**	**16.9**	**20.8**	**18.1**	**20.0**
Carinoma hamanako (n=5, **n=5**; C. h)	28.2	30.8	34.2	34.4	32.3	31.2	33.6	31.9	32.9	30.4	33.9
	19.1	**21.9**	**20.4**	**19.9**	**20.9**	**19.7**	**21.4**	**18.8**	**21.9**	**19.5**	**21.5**
Carinoma sp. "yellowback" (n=4, **n=3**; C. y)	29.1	30.1	36.9	35.9	34.7	33.6	35.0	34.9	34.7	31.5	35.9
	20.6	**217.0**	**19.6**	**20.3**	**21.3**	**21.4**	**22.6**	**20.3**	**23.5**	**22.0**	**19.7**
Carinoma tremaphoros 1 (n=1, **n=1**; C. t)	27.0	31.0	34.0	34.0	33.0	33.0	34.0	32.0	34.0	30.0	35.0
(AJ436833)	**20.7**	**22.5**	**19.5**	**19.1**	**21.0**	**21.3**	**22.6**	**19.7**	**21.3**	**19.7**	**19.1**
Carinoma tremaphoros 2 (n=1, **n=1**; C. t)	28.0	29.0	36.0	36.0	34.0	32.0	36.0	33.0	33.0	30.0	37.0
(JF277602)	**20.3**	**22.8**	**17.7**	**18.5**	**21.4**	**20.9**	**20.8**	**17.9**	**21.8**	**19.6**	**19.0**
Carinoma sp. "white" (n=6, **n=3**; C. w)	28.8	32.0	32.8	33.1	33.9	32.2	33.7	31.7	33.8	31.1	35.4
	18.8	**19.6**	**16.6**	**15.6**	**20.2**	**20.0**	**20.2**	**16.9**	**21.2**	**18.1**	**18.7**
Cephalothrix sp. 1 (n=9, **n=6**; C. 1)	18.7	19.4	34.5	33.7	33.0	32.0	32.0	30.1	32.1	29.8	36.5
	17.0	**15.4**	**16.4**	**16.5**	**18.3**	**18.0**	**17.8**	**16.4**	**20.6**	**16.4**	**17.5**
Cephalothrix major (n=1, **n=1**; Cp. m)	24.0	26.1	32.7	31.9	31.8	30.6	32.9	29.4	31.2	28.6	33.4
	17.3	**18.8**	**19.0**	**16.6**	**19.1**	**20.0**	**19.0**	**17.9**	**21.2**	**19.0**	**18.4**
Cephalothrix cf. *spiralis* (n=1, **n=1**; C. s)	23.2	26.2	33.4	32.2	31.8	30.7	32.9	30.1	31.9	29.2	34.0
	18.7	**18.7**	**19.9**	**19.0**	**20.7**	**19.6**	**18.9**	**18.8**	**22.1**	**20.6**	**19.3**
Cephalothrix sp. 3 (n=1; C. 3)	23.0	24.3	35.8	35.4	33.4	32.6	34.0	31.1	33.2	32.6	36.5
	-	-	-	-	-	-	-	-	-	-	-
Cephalothrix sp. 2 (n=3, **n=2;** C. 2)	0.0	21.8	35.2	34.7	34.4	34.0	35.0	33.2	34.7	33.1	35.8
	0.2	**18.3**	**18.6**	**17.9**	**19.3**	**18.8**	**20.6**	**17.3**	**20.0**	**19.9**	**20.6**
Cephalothrix sp. 4 (n=6, **n=11**; C. 4)		2.0	35.0	33.4	34.9	33.0	34.0	32.2	34.1	31.4	36.6
		6.1	**18.9**	**20.4**	**22.0**	**21.3**	**22.9**	**21.0**	**22.4**	**20.2**	**20.4**

	C. c	C. v	C. o	C. g
Carinoma mutabilis (n=9, **n=6**; C. m)	32.3	31.2	34.6	35.6
	19.5	-	20.7	20.0
Carinoma sp. 5 (n=1, **n=1**; C. 5)	31.3	30.3	33.3	36.0
	19.9	**-**	**19.0**	**19.9**
Carinoma hamanako (n=5, **n=5**; C. h)	30.4	29.8	32.8	36.8
	21.5	**-**	**21.9**	**22.0**
Carinoma sp. "yellowback" (n=4, **n=3**; C. y)	32.3	32.2	34.8	37.8
	20.5	**-**	**21.7**	**21.8**
Carinoma tremaphoros 1 (n=1, **n=1**; C. t)	32.0	31.0	36.0	37.0
(AJ436833)	**21.6**	**-**	**21.8**	**19.3**
Carinoma tremaphoros 2 (n=1, **n=1**; C. t)	31.0	31.0	35.0	37.0
(JF277602)	**20.2**	**-**	**19.0**	**20.9**
Carinoma sp. "white" (n=6, **n=3**; C. w)	30.4	27.1	34.1	36.5
	18.9	**-**	**20.1**	**21.0**
Cephalothrix sp. 1 (n=9, **n=6**; C. 1)	32.6	31.5	35.0	36.0
	17.1	**-**	**20.3**	**20.4**
Cephalothrix major (n=1, **n=1**; Cp. m)	30.9	30.9	33.8	37.0
	21.1	**-**	**19.2**	**21.3**
Cephalothrix cf. *spiralis* (n=1, **n=1**; C. s)	32.9	31.8	36.0	38.4
	20.1	**-**	**20.5**	**21.2**
Cephalothrix sp. 3 (n=1; C. 3)	35.1	33.7	37.4	38.1
	-	**-**	**-**	**-**
Cephalothrix sp. 2 (n=3, **n=2;** C. 2)	31.4	29.0	34.0	35.7
	18.4	**-**	**20.4**	**22.8**
Cephalothrix sp. 4 (n=6, **n=11**; C. 4)	30.3	28.4	34.5	37.0
	19.8	**-**	**20.5**	**21.8**

	C. 2	C. 4	T. a	T. po	T. pu	T. s	T. r	T. 2	T. 3	T. 1	T. pe
Tubulanus annulatus (n=1, **n=1**; T. a)			-	13.6	24.2	24.0	24.2	19.9	22.7	20.5	22.3
			-	**12.9**	**16.5**	**14.9**	**16.3**	**14.4**	**16.3**	**16.0**	**15.1**
Tubulanus polymorphus (n=1, **n=3**; T. po)				-	22.5	22.0	22.8	19.0	22.9	19.2	23.0
				1.2	**18.4**	**16.5**	**16.4**	**14.7**	**18.6**	**15.1**	**16.4**
Tubulanus punctatus (n=1, **n=1**; T. pu)					-	3.7	9.5	17.3	17.3	20.1	25.4
					-	**10.3**	**15.8**	**13.9**	**16.6**	**17.2**	**16.4**
Tubulanus sexlineatus (n=3, **n=4**; T. s)						0.0	8.3	18.5	17.1	18.7	25.1
						1.0	**15.0**	**12.7**	**16.4**	**17.9**	**17.1**
Tubulanus rhabdotus (n=1, **n=1**; T. r)							-	19.3	18.5	17.7	25.7
							-	**15.3**	**17.4**	**19.5**	**16.9**
Tubulanus sp. 2 (n=3, **n=3**; T. 2)								0.0	11.5	15.7	22.7
								0.1	**14.9**	**15.2**	**15.6**
Tubulanus sp. 3 (n=1, **n=1**; T. 3)									-	18.4	23.4
									-	**17.5**	**18.6**
Tubulanus sp. 1 (n=7, **n=7**; T. 1)										0.2	21.3
										1.3	**17.2**
Tubulanus pellucidus (n=1, **n=1**; T. pe)											-
											-
Carinina sp. "chocolate" (n=7, **n=8**; C. c)											
Carinina sp. "Vostok" (n=1; C. v)											
Carinina ochracea (n=1, **n=1**; C. o)											
Callinera grandis (n=1, **n=1**; C. g)											

	C. c	C. v	C. o	C. g
Tubulanus annulatus (n=1, **n=1**; T. a)	30.1	27.5	35.1	38.7
	16.3	**-**	**16.8**	**17.7**
Tubulanus polymorphus (n=1, **n=3**; T. po)	31.4	27.8	35.4	38.0
	16.3	**-**	**17.5**	**17.5**
Tubulanus punctatus (n=1, **n=1**; T. pu)	29.4	26.8	33.1	38.1
	19.0	**-**	**19.5**	**18.0**
Tubulanus sexlineatus (n=3, **n=4**; T. s)	28.6	26.6	33.3	39.0
	17.7	**-**	**18.9**	**17.5**
Tubulanus rhabdotus (n=1, **n=1**; T. r)	31.7	28.5	35.7	36.8
	18.8	**-**	**20.1**	**18.4**
Tubulanus sp. 2 (n=3, **n=3**; T. 2)	30.2	27.7	33.5	37.7
	16.1	**-**	**16.3**	**15.1**
Tubulanus sp. 3 (n=1, **n=1**; T. 3)	28.3	27.7	34.3	37.3
	19.5	**-**	**18.8**	**19.4**
Tubulanus sp. 1 (n=7, **n=7**; T. 1)	30.0	27.3	33.9	38.0
	18.7	**-**	**19.3**	**18.3**
Tubulanus pellucidus (n=1, **n=1**; T. pe)	30.8	29.4	33.2	43.6
	16.7	**-**	**18.5**	**16.3**
Carinina sp. "chocolate" (n=7, **n=8**; C. c)	0.6	8.0	17.3	39.0
	3.1	**-**	**17.6**	**19.3**
Carinina sp. "Vostok" (n=1; C. v)		-	17.4	38.2
		-	**-**	**-**
Carinina ochracea (n=1, **n=1**; C. o)			-	40.5
			-	**19.1**
Callinera grandis (n=1, **n=1**; C. g)				-
				-

APPENDIX F

PILIDIOPHORAN DIVERGENCE TABLE (%)

	M. f	Z. r	C. c	C. pb	C. SB	C. 2	H. 6	C. m	C. sh	C. 1	M. w	M. sp. nc
Micrura fasciolata (n=1, **n=1**; M. f)	-	28.1	22.7	22.5	22.8	23.3	24.0	20.5	20.3	19.4	22.5	27.1
	-	**17.8**	**17.3**	**18.4**	**18.6**	**18.4**	**-**	**18.2**	**18.1**	**17.8**	**20.1**	**19.1**
Zyeupolia rubens (n=1, **n=1**; Z. r)		-	25.5	27.4	27.5	26.0	25.7	25.7	25.7	25.4	24.4	28.6
		-	**16.2**	**17.0**	**18.5**	**17.4**	**-**	**19.1**	**16.7**	**17.5**	**18.8**	**15.6**
Cerebratulus californiensis (n=25, **n=14**; C. c)			0.3	5.4	5.0	5.1	14.5	18.2	19.1	17.2	18.6	21.7
			0.4	**11.8**	**13.0**	**13.1**	**-**	**18.4**	**17.4**	**17.9**	**19.3**	**14.9**
Cerebratulus sp. "pink proboscis" (n=6, **n=3**; C. pb				0.8	1.8	6.6	15.2	21.8	21.3	19.0	20.5	22.5
				0.5	**10.3**	**16.6**	**-**	**18.1**	**17.8**	**19.3**	**19.4**	**17.0**
Cerebratulus sp. "Sunset Bay" (n=2, **n=3**; C. sb)					0.0	5.4	14.7	21.7	21.5	19.0	19.9	22.2
					0.2	**16.4**	**-**	**19.9**	**19.6**	**19.9**	**20.4**	**17.4**
Cerebratulus sp. 2 (n=3, **n=1**; C. sp. 2)						0.0	14.2	19.1	20.9	17.1	18.3	22.4
						-	**-**	**19.4**	**15.8**	**19.0**	**20.4**	**18.5**
Heteronemertea gen. sp. 6 (n=1; H. 6)							-	19.3	20.3	18.6	19.3	24.6
							-	**-**	**-**	**-**	**-**	**-**
Cerebratulus marginatus (n=14, **n=13**; C. m)								0.0	8.0	15.2	19.7	22.9
								1.0	**15.7**	**16.9**	**17.3**	**18.7**
Cerebratulus sp. "spade head" (n = 5, **n=5**; C. sh)									0.0	15.5	20.6	22.6
									0.2	**18.6**	**15.8**	**16.2**
Cerebratulus sp. 1 (n=1, **n=1**; C. 1)										-	21.1	22.3
										-	**19.6**	**20.4**
Micrura wilsoni (n=8, **n=1**; M. w)											0.0	24.4
											-	**18.1**
Micrura sp. "not coei" (n=2, **n=1**; M. sp. nc)												0.0
												-

	C. l	M. r	R. ne	R. p	R. e	L. r	L. v	R. s	H. 4	C. mo	L. r	L. t
Micrura fasciolata (n=1, **n=1**; M. f)	26.3	25.9	22.0	22.6	23.1	14.2	12.2	17.5	29.8	30.6	30.4	32.8
	-	**16.9**	**22.1**	**21.2**	**18.0**	**16.3**	**16.7**	**17.0**	**22.2**	**17.8**	**20.1**	**18.7**
Zyeupolia rubens (n=1, **n=1**; Z. r)	27.6	28.0	27.1	26.0	25.2	26.9	25.1	27.3	24.5	27.5	27.6	29.3
	-	**15.1**	**21.4**	**20.2**	**19.4**	**17.4**	**15.9**	**18.3**	**20.1**	**17.4**	**19.6**	**18.4**
Cerebratulus californiensis (n=25, **n=14**; C. c)	22.5	21.8	25.3	24.8	23.0	24.4	23.0	23.8	25.3	26.8	27.0	29.6
	-	**15.1**	**20.1**	**20.6**	**18.5**	**16.4**	**17.9**	**17.5**	**19.3**	**18.5**	**19.0**	**18.1**
Cerebratulus sp. "pink proboscis" (n=6, **n=3**; C. pb	24.0	23.9	25.5	24.5	23.0	25.6	23.9	23.7	27.5	26.9	27.0	29.3
	-	**18.1**	**20.9**	**20.4**	**19.9**	**17.2**	**17.9**	**18.2**	**20.5**	**18.2**	**19.6**	**18.5**
Cerebratulus sp. "Sunset Bay" (n=2, **n=3**; C. sb)	24.4	23.5	25.5	24.2	23.0	26.0	24.0	23.9	26.9	26.2	26.0	28.1
	-	**18.8**	**20.6**	**20.6**	**18.9**	**18.4**	**17.8**	**19.4**	**20.5**	**17.1**	**20.4**	**19.9**
Cerebratulus sp. 2 (n=3, **n=1**; C. sp. 2)	23.8	23.6	25.3	24.6	23.7	25.9	24.2	24.3	25.7	25.9	25.9	28.2
	-	**17.3**	**21.7**	**20.8**	**19.8**	**17.5**	**17.6**	**18.7**	**19.2**	**18.4**	**20.6**	**19.5**
Heteronemertea gen. sp. 6 (n=1; H. 6)	23.6	23.4	24.8	25.5	23.5	25.4	24.7	26.4	27.6	25.8	27.9	27.4
	-	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Cerebratulus marginatus (n=14, **n=13**; C. m)	24.1	21.6	24.1	23.5	23.9	22.2	21.7	24.5	27.9	28.4	28.6	30.5
	-	**17.6**	**23.0**	**21.1**	**16.7**	**20.1**	**18.9**	**18.5**	**19.2**	**18.7**	**19.4**	**18.4**
Cerebratulus sp. "spade head" (n = 5, **n=5**; C. sh)	24.5	22.4	24.3	23.1	24.5	21.3	20.7	22.8	28.0	28.3	27.8	30.6
	-	**17.3**	**22.7**	**19.9**	**18.1**	**17.8**	**18.4**	**16.6**	**18.1**	**19.7**	**19.7**	**19.1**
Cerebratulus sp. 1 (n=1, **n=1**; C. 1)	23.7	22.1	22.6	22.9	22.5	22.2	20.7	24.3	26.8	26.0	27.0	30.0
	-	**17.8**	**24.2**	**22.5**	**19.2**	**18.1**	**18.9**	**17.2**	**19.0**	**21.5**	**20.3**	**20.2**
Micrura wilsoni (n=8, **n=1**; M. w)	24.9	26.3	23.0	23.0	22.3	25.0	24.0	25.2	24.0	27.1	28.0	31.3
	-	**20.3**	**24.9**	**21.0**	**18.2**	**17.2**	**18.2**	**19.0**	**18.8**	**18.6**	**20.1**	**20.6**
Micrura sp. "not coei" (n=2, **n=1**; M. sp. nc)	21.9	20.2	26.9	26.8	25.3	27.3	26.0	27.3	29.5	32.0	32.6	33.3
	-	**15.4**	**24.1**	**21.8**	**18.2**	**17.3**	**18.4**	**16.8**	**20.4**	**17.8**	**21.1**	**20.1**

	L. c	L. 2	L. f	L. 1	L. b	H. 5	M. v	C. h	M. d	M. 3	M. 4	M. alb
Micrura fasciolata (n=1, **n=1**; M. f)	30.5	30.9	30.4	29.7	33.1	33.4	29.5	32.7	27.9	27.7	26.6	27.4
	17.8	**17.8**	**19.3**	**18.1**	**-**	**-**	**19.0**	**19.9**	**18.5**	**18.1**	**19.5**	**19.1**
Zyeupolia rubens (n=1, **n=1**; Z. r)	27.3	27.3	28.0	27.3	26.7	28.2	28.4	34.8	27.3	24.7	26.0	24.7
	18.4	**17.2**	**20.1**	**19.3**	**-**	**-**	**17.0**	**17.4**	**16.5**	**16.9**	**15.4**	**16.0**
Cerebratulus californiensis (n=25, **n=14**; C. c)	27.1	29.8	29.3	28.8	29.0	28.6	28.9	33.9	27.5	27.2	27.6	27.2
	18.2	**17.5**	**18.1**	**17.6**	**-**	**-**	**16.5**	**12.0**	**16.8**	**17.6**	**17.6**	**17.4**
Cerebratulus sp. "pink proboscis" (n=6, **n=3**; C. pb	27.2	30.0	29.7	28.9	30.8	30.0	30.0	35.6	29.6	29.4	29.7	29.5
	19.1	**18.5**	**17.4**	**17.9**	**-**	**-**	**19.5**	**17.3**	**18.2**	**18.2**	**19.1**	**18.9**
Cerebratulus sp. "Sunset Bay" (n=2, **n=3**; C. sb)	26.3	29.1	28.6	27.7	30.3	30.0	29.9	35.7	29.5	28.8	29.6	29.2
	20.7	**19.6**	**19.1**	**19.7**	**-**	**-**	**19.1**	**16.2**	**19.6**	**18.5**	**19.1**	**18.5**
Cerebratulus sp. 2 (n=3, **n=1**; C. sp. 2)	25.6	29.0	28.2	27.8	29.1	29.5	29.3	35.2	29.2	28.9	29.0	28.5
	19.8	**18.4**	**20.3**	**19.9**	**-**	**-**	**17.9**	**14.3**	**17.6**	**18.7**	**18.5**	**18.9**
Heteronemertea gen. sp. 6 (n=1; H. 6)	26.7	27.5	28.0	27.8	27.5	28.2	29.7	35.3	25.8	26.1	27.6	27.0
	-	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Cerebratulus marginatus (n=14, **n=13**; C. m)	27.7	27.9	27.9	29.2	29.0	29.3	30.1	33.1	26.2	26.3	27.0	27.3
	19.8	**18.3**	**21.2**	**20.5**	**-**	**-**	**18.0**	**18.3**	**17.5**	**18.8**	**18.9**	**18.3**
Cerebratulus sp. "spade head" (n = 5, **n=5**; C. sh)	28.8	28.1	26.1	26.9	28.9	28.5	30.2	33.2	26.3	25.8	26.4	29.7
	17.7	**18.2**	**20.3**	**18.4**	**-**	**-**	**18.1**	**17.6**	**16.8**	**18.2**	**17.0**	**18.3**
Cerebratulus sp. 1 (n=1, **n=1**; C. 1)	27.2	29.0	27.3	26.7	28.8	26.9	28.0	34.2	25.3	25.8	27.3	27.2
	19.7	**21.0**	**20.8**	**20.5**	**-**	**-**	**19.1**	**18.0**	**17.6**	**19.0**	**17.2**	**17.5**
Micrura wilsoni (n=8, **n=1**; M. w)	29.1	31.6	28.2	26.6	29.6	29.6	28.5	35.4	28.8	28.3	28.9	28.0
	17.9	**17.8**	**20.0**	**20.4**	**-**	**-**	**20.6**	**19.8**	**21.5**	**20.4**	**20.4**	**20.4**
Micrura sp. "not coei" (n=2, **n=1**; M. sp. nc)	30.8	32.9	31.0	31.1	31.1	30.8	32.0	37.2	30.7	30.7	29.8	30.1
	18.6	**17.7**	**20.6**	**19.8**	**-**	**-**	**19.0**	**16.4**	**16.4**	**19.9**	**19.0**	**19.5**

	C. a	L. le	Mi. a	H. 2	M. 1	B. p	B. d	B. a	B. q	B. h	B. j	B. m
Micrura fasciolata (n=1, **n=1**; M. f)	26.0	33.3	34.5	33.8	32.9	36.0	37.2	-	36.6	35.9	38.6	36.0
	21.2	**21.3**	**18.8**	**21.7**	**16.9**	**22.4**	**18.3**	**21.1**	**-**	**20.2**	**-**	**20.2**
Zyeupolia rubens (n=1, **n=1**; Z. r)	28.3	28.9	28.4	26.8	27.2	34.5	34.5	-	33.7	34.7	32.7	34.5
	20.7	**17.4**	**16.9**	**19.2**	**16.5**	**20.3**	**18.0**	**21.1**	**-**	**21.5**	**-**	**19.1**
Cerebratulus californiensis (n=25, **n=14**; C. c)	29.5	31.2	31.5	30.8	28.0	35.2	34.6	-	33.6	35.2	33.5	34.0
	18.0	**18.1**	**17.1**	**19.8**	**16.9**	**19.1**	**18.7**	**18.2**	**-**	**17.8**	**-**	**17.1**
Cerebratulus sp. "pink proboscis" (n=6, **n=3**; C. pb	29.7	31.0	32.7	32.2	29.6	36.4	36.3	-	34.6	35.1	35.0	34.8
	18.9	**19.4**	**20.2**	**20.6**	**17.4**	**20.5**	**18.3**	**19.7**	**-**	**19.6**	**-**	**19.1**
Cerebratulus sp. "Sunset Bay" (n=2, **n=3**; C. sb)	29.1	30.7	31.8	31.5	28.9	36.0	36.0	-	34.9	35.0	34.8	34.3
	20.9	**19.7**	**19.9**	**20.8**	**17.7**	**20.2**	**18.5**	**19.3**	**-**	**18.6**	**-**	**17.5**
Cerebratulus sp. 2 (n=3, **n=1**; C. sp. 2)	28.6	31.8	31.3	31.2	28.5	34.5	34.8	-	34.3	34.7	33.4	33.5
	19.6	**19.2**	**18.8**	**20.3**	**17.9**	**18.4**	**18.3**	**19.6**	**-**	**19.0**	**-**	**19.7**
Heteronemertea gen. sp. 6 (n=1; H. 6)	28.9	29.0	30.1	30.3	28.0	32.4	33.3	-	33.2	34.8	33.3	34.1
	-	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Cerebratulus marginatus (n=14, **n=13**; C. m)	30.1	30.4	29.7	29.4	28.0	34.7	33.0	-	33.3	34.2	34.1	34.1
	17.8	**18.6**	**19.0**	**20.3**	**16.9**	**19.5**	**19.6**	**21.8**	**-**	**19.6**	**-**	**18.5**
Cerebratulus sp. "spade head" (n = 5, **n=5**; C. sh)	28.9	29.6	29.5	28.1	34.4	34.9	33.3	-	33.5	33.6	34.8	34.1
	18.2	**19.2**	**18.8**	**20.0**	**14.6**	**18.7**	**19.1**	**21.9**	**-**	**19.3**	**-**	**19.1**
Cerebratulus sp. 1 (n=1, **n=1**; C. 1)	29.0	29.2	32.6	31.5	28.2	33.8	33.2	-	31.9	32.6	33.4	32.9
	21.0	**19.4**	**19.0**	**21.4**	**18.4**	**21.6**	**19.9**	**24.0**	**-**	**23.6**	**-**	**20.9**
Micrura wilsoni (n=8, **n=1**; M. w)	27.0	31.5	30.0	30.8	29.8	33.6	34.4	-	32.9	34.9	35.0	34.2
	20.6	**18.6**	**20.6**	**22.1**	**18.9**	**20.7**	**20.3**	**21.9**	**-**	**21.6**	**-**	**20.0**
Micrura sp. "not coei" (n=2, **n=1**; M. sp. nc)	31.8	32.7	32.6	31.3	33.0	37.6	36.7	-	35.2	36.0	36.6	37.0
	17.3	**15.5**	**16.0**	**20.3**	**17.3**	**19.3**	**19.0**	**21.5**	**-**	**20.2**	**-**	**18.4**

	B. u	B. 1	B. 2	H. 3	H. j	H. d	Ma. a	Ma. aq	Ma. c	Ma. m	Ma. o
Micrura fasciolata (n=1, **n=1**; M. f)	36.8	37.1	35.3	38.7	39.6	40.9	20.2	19.6	22.3	20.0	20.3
	19.8	**21.8**	**20.5**	**17.7**	**21.8**	**21.9**	**20.2**	**19.3**	**17.8**	**17.5**	**18.0**
Zyeupolia rubens (n=1, **n=1**; Z. r)	33.2	34.6	32.7	31.7	38.1	35.9	17.6	17.4	18.3	18.3	18.9
	19.0	**20.5**	**19.2**	**14.6**	**20.1**	**20.5**	**18.9**	**18.5**	**16.0**	**16.5**	**17.6**
Cerebratulus californiensis (n=25, **n=14**; C. c)	34.9	34.8	32.9	35.7	39.2	39.7	24.3	24.7	24.6	25.6	23.7
	18.3	**18.9**	**18.5**	**16.3**	**19.2**	**19.1**	**19.7**	**19.2**	**18.0**	**18.1**	**16.5**
Cerebratulus sp. "pink proboscis" (n=6, **n=3**; C. pb	35.4	36.3	34.0	37.7	39.6	40.6	25.2	27.2	26.2	27.3	24.6
	19.7	**18.4**	**20.2**	**18.3**	**20.7**	**18.7**	**19.9**	**19.8**	**20.2**	**19.9**	**19.0**
Cerebratulus sp. "Sunset Bay" (n=2, **n=3**; C. sb)	35.4	35.9	33.6	36.9	39.7	40.6	24.9	27.5	26.6	26.9	24.3
	20.8	**20.4**	**21.4**	**20.0**	**20.6**	**19.5**	**20.4**	**20.0**	**19.9**	**19.4**	**18.1**
Cerebratulus sp. 2 (n=3, **n=1**; C. sp. 2)	34.2	34.4	32.7	35.0	38.9	41.0	24.2	25.9	23.8	26.0	24.1
	19.7	**18.4**	**20.2**	**19.7**	**19.8**	**19.2**	**20.5**	**19.4**	**19.1**	**19.2**	**19.0**
Heteronemertea gen. sp. 6 (n=1; H. 6)	33.7	34.0	32.4	36.1	36.0	36.0	22.0	23.4	23.5	21.8	22.4
	-	-	-	-	-	-	-	-	-	-	-
Cerebratulus marginatus (n=14, **n=13**; C. m)	35.5	34.1	33.3	34.5	41.5	40.2	23.6	21.6	22.7	21.2	24.9
	19.7	**20.8**	**20.0**	**18.3**	**22.0**	**21.4**	**20.8**	**18.2**	**17.8**	**16.2**	**21.2**
Cerebratulus sp. "spade head" (n = 5, **n=5**; C. sh)	35.2	34.8	32.4	35.3	41.6	40.4	24.2	22.4	24.2	21.7	25.4
	18.8	**19.5**	**17.6**	**18.5**	**18.7**	**18.4**	**19.8**	**18.0**	**17.2**	**17.1**	**17.2**
Cerebratulus sp. 1 (n=1, **n=1**; C. 1)	33.2	34.8	32.3	34.6	40.3	39.9	24.0	24.8	24.3	22.1	23.7
	20.4	**21.8**	**19.5**	**18.5**	**20.0**	**20.9**	**19.4**	**18.1**	**18.9**	**19.1**	**18.4**
Micrura wilsoni (n=8, **n=1**; M. w)	33.5	35.2	32.4	34.9	40.2	40.7	21.4	23.7	22.0	23.7	23.1
	21.7	**22.1**	**21.3**	**17.8**	**20.3**	**20.5**	**21.0**	**19.3**	**19.5**	**18.7**	**19.0**
Micrura sp. "not coei" (n=2, **n=1**; M. sp. nc)	33.0	38.4	31.9	39.6	42.4	42.1	28.2	29.9	29.1	29.5	29.1
	20.4	**19.3**	**19.0**	**19.4**	**18.3**	**18.3**	**19.5**	**18.7**	**17.5**	**17.6**	**16.9**

	C. l	M. r	R. ne	R. p	R. e	L. r	L. v	R. s	H. 4	C. mo	L. r	L. t
Cerebratulus longiceps (n=1; C. l)	-	24.4	24.0	24.8	23.4	26.2	24.8	27.7	28.4	28.4	30.4	30.7
	-	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Micrura rubramaculosa (n=1, **n=1**; M. r)		-	26.4	25.4	23.6	26.5	25.6	25.5	28.5	30.7	31.8	33.6
		-	**23.5**	**21.5**	**17.5**	**16.6**	**16.9**	**18.1**	**19.7**	**17.9**	**21.0**	**19.2**
Riserius sp. "no eyes" (n=15, **n=7**; R. ne)			0.0	6.7	11.0	21.6	21.7	23.2	26.7	29.2	30.7	32.3
			0.5	**17.8**	**20.6**	**22.2**	**21.3**	**22.5**	**22.3**	**23.6**	**22.4**	**21.9**
Riserius pugetensis (n=1, **n=1**; R. p)				-	10.7	21.3	20.7	22.5	25.2	29.9	31.5	32.7
				-	**16.3**	**21.3**	**19.7**	**20.5**	**22.1**	**17.7**	**23.1**	**20.8**
Riserius sp. "eyes" (n=2, **n=3**; R. sp. e)					0.0	23.4	22.6	24.8	24.6	27.9	29.4	29.6
					0.3	**21.0**	**18.0**	**18.4**	**19.9**	**19.9**	**19.9**	**20.3**
Lineus ruber (n=1, **n=1**; L. r)						-	6.3	16.3	28.1	28.9	30.0	31.8
						-	**12.7**	**15.1**	**20.0**	**18.5**	**19.2**	**19.6**
Lineus viridis (n=1, **n=1**; L. v)							-	15.6	26.7	28.6	28.8	31.3
							-	**16.6**	**20.0**	**19.4**	**20.4**	**20.4**
Ramphogordius sanguineus (n=4, **n=3**; R. s)								0.2	26.1	29.8	30.3	31.5
								0.3	**19.2**	**21.8**	**22.9**	**19.9**
Heteronemertea gen. sp. 4 (n=7, **n=3**; H. 4)									0.2	27.4	30.0	29.1
									0.3	**21.4**	**22.3**	**20.3**
Cerebratulus montgomeryi (n=3, **n=2**; C. mo)										0.3	7.4	9.6
										1.2	**12.4**	**14.7**
Lineus sp. "red" (n=9, **n=11**; L. r)											0.3	12.1
											0.3	**18.0**
Lineus torquatus (n=1, **n=1**; L. t)												-
												-
Lineus sp. "crescent" (n=4, **n=4**; L. c)												
Lineus sp. 2 (n=26, **n=11**; L. 2)												

	L. c	L. 2	L. f	L. 1	L. b	H. 5	M. v	C. h	M. d	M. 3	M. 4	M. alb
Cerebratulus longiceps (n=1; C. l)	31.2	31.6	31.8	30.9	30.1	28.9	33.4	36.7	29.2	29.3	30.9	30.0
	-	-	-	-	-	-	-	-	-	-	-	-
Micrura rubramaculosa (n=1, **n=1**; M. r)	31.0	33.3	31.4	30.9	32.1	31.8	33.7	38.5	29.7	29.9	29.7	30.0
	19.0	**18.2**	**17.7**	**17.2**	-	-	**20.1**	**16.8**	**17.4**	**19.6**	**18.8**	**18.6**
Riserius sp. "no eyes" (n=15, **n=7**; R. ne)	30.9	31.1	32.2	31.0	32.2	30.3	30.4	35.5	27.2	28.1	29.1	27.6
	21.5	**21.8**	**20.6**	**21.9**	-	-	**20.6**	**21.1**	**24.2**	**21.6**	**22.4**	**21.5**
Riserius pugetensis (n=1, **n=1**; R. p)	31.3	32.3	30.4	28.7	31.1	30.7	29.3	34.8	27.9	28.5	28.9	28.0
	20.6	**20.6**	**19.2**	**20.8**	-	-	**20.5**	**21.0**	**22.0**	**20.5**	**20.7**	**20.5**
Riserius sp. "eyes" (n=2, **n=3**; R. sp. e)	28.5	30.2	28.1	26.2	30.7	29.5	29.6	33.7	29.0	28.6	30.0	28.5
	16.2	**18.6**	**18.1**	**20.3**	-	-	**18.7**	**17.4**	**19.6**	**20.1**	**19.8**	**19.5**
Lineus ruber (n=1, **n=1**; L. r)	30.8	30.3	30.8	30.0	31.7	30.0	32.0	36.0	29.0	29.0	28.8	27.5
	18.3	**16.9**	**19.1**	**18.6**	-	-	**19.1**	**19.0**	**18.3**	**19.1**	**18.9**	**18.2**
Lineus viridis (n=1, **n=1**; L. v)	28.4	29.5	29.9	29.2	30.4	30.1	32.0	36.1	28.0	28.2	27.5	27.0
	18.6	**17.2**	**18.5**	**17.2**	-	-	**18.8**	**19.7**	**19.6**	**19.4**	**19.6**	**19.3**
Ramphogordius sanguineus (n=4, **n=3**; R. s)	30.7	31.3	31.7	31.0	33.4	34.1	31.7	38.0	29.8	30.1	29.6	29.9
	19.4	**19.7**	**19.8**	**20.9**	-	-	**20.3**	**19.3**	**16.8**	**19.4**	**18.7**	**19.0**
Heteronemertea gen. sp. 4 (n=7, **n=3**; H. 4)	27.8	29.5	27.5	27.5	37.0	27.5	30.1	34.8	28.5	27.6	27.1	27.3
	21.3	**20.0**	**18.9**	**20.7**	-	-	**19.2**	**19.2**	**18.7**	**21.4**	**20.7**	**21.5**
Cerebratulus montgomeryi (n=3, **n=2**; C. mo)	11.0	11.3	14.4	12.9	16.6	17.2	31.3	36.7	27.1	26.9	27.4	27.5
	15.2	**16.0**	**18.5**	**19.0**	-	-	**18.4**	**17.7**	**18.5**	**19.6**	**19.1**	**19.2**
Lineus sp. "red" (n=9, **n=11**; L. r)	12.0	11.0	15.0	14.7	18.9	17.7	32.5	37.8	28.0	27.7	27.9	27.8
	16.5	**15.7**	**19.6**	**18.7**	-	-	**20.8**	**19.0**	**20.7**	**20.4**	**20.9**	**19.5**
Lineus torquatus (n=1, **n=1**; L. t)	11.3	11.0	14.9	14.1	20.2	19.8	32.8	36.7	27.2	27.4	29.0	28.2
	17.5	**17.7**	**19.4**	**20.1**	-	-	**17.5**	**18.8**	**19.0**	**19.6**	**19.8**	**18.8**
Lineus sp. "crescent" (n=4, **n=4**; L. c)	0.1	10.2	13.9	11.7	18.7	20.6	30.6	35.1	28.8	27.7	28.1	27.9
	0.2	**14.7**	**18.5**	**16.8**	-	-	**18.3**	**18.0**	**19.2**	**18.3**	**17.9**	**17.7**
Lineus sp. 2 (n=26, **n=11**; L. 2)		0.9	14.2	13.0	18.8	18.0	31.0	34.0	28.6	27.8	28.0	28.6

	C. a	L. le	Mi. a	H. 2	M. 1	B. p	B. d	B. a	B. q	B. h	B. j	B. m
Cerebratulus longiceps (n=1; C. l)	31.6	32.6	31.6	31.1	31.5	37.3	37.3	-	35.1	37.3	35.7	37.7
	-	-	-	-	-	-	–	-	-	-	-	-
Micrura rubramaculosa (n=1, **n=1**; M. r)	31.6	32.5	32.7	33.9	30.5	37.3	36.2	-	35.7	36.8	35.4	36.0
	20.0	**17.3**	**16.0**	**19.5**	**17.8**	**20.8**	**19.6**	**22.2**	**-**	**21.1**	**-**	**19.4**
Riserius sp. "no eyes" (n=15, **n=7**; R. ne)	28.0	30.2	32.4	31.3	30.0	34.9	36.4	-	35.8	33.6	35.2	33.6
	24.0	**24.5**	**24.5**	**25.6**	**22.7**	**23.4**	**22.6**	**21.5**	**-**	**21.9**	**-**	**22.2**
Riserius pugetensis (n=1, **n=1**; R. p)	27.3	30.5	31.8	31.7	29.9	34.2	35.1	-	35.1	33.2	35.1	31.5
	23.5	**22.4**	**19.4**	**20.4**	**20.2**	**20.0**	**21.7**	**22.3**	**-**	**21.5**	**-**	**23.8**
Riserius sp. "eyes" (n=2, **n=3**; R. sp. e)	25.5	28.9	31.2	31.2	28.0	34.8	34.9	-	36.1	34.3	35.2	32.7
	21.9	**19.4**	**20.1**	**20.2**	**18.9**	**21.6**	**20.1**	**22.6**	**-**	**21.3**	**-**	**21.1**
Lineus ruber (n=1, **n=1**; L. r)	31.3	32.6	31.2	31.0	30.4	36.7	36.6	-	36.4	35.9	38.6	36.0
	21.0	**17.6**	**18.8**	**21.5**	**17.4**	**19.6**	**19.1**	**20.5**	**-**	**21.1**	**-**	**20.1**
Lineus viridis (n=1, **n=1**; L. v)	31.0	31.4	30.6	30.9	29.1	35.3	35.4	-	34.7	34.7	35.6	35.1
	18.7	**18.7**	**19.1**	**18.7**	**17.0**	**19.1**	**19.3**	**19.4**	**-**	**21.6**	**-**	**22.3**
Ramphogordius sanguineus (n=4, **n=3**; R. s)	30.2	32.9	31.9	32.4	31.2	35.2	36.8	-	36.9	36.8	39.5	35.7
	19.2	**18.5**	**17.9**	**20.3**	**15.7**	**19.6**	**19.4**	**21.5**	**-**	**21.8**	**-**	**18.9**
Heteronemertea gen. sp. 4 (n=7, **n=3**; H. 4)	28.8	28.4	30.6	28.9	27.9	35.8	36.9	-	36.6	37.3	37.6	35.2
	19.3	**19.4**	**21.0**	**23.2**	**19.9**	**19.9**	**18.8**	**20.5**	**-**	**20.7**	**-**	**20.9**
Cerebratulus montgomeryi (n=3, **n=2**; C. mo)	32.7	30.3	27.3	26.3	25.2	31.4	32.6	-	33.5	33.2	35.4	33.1
	19.0	**17.3**	**19.8**	**20.9**	**19.3**	**20.6**	**20.1**	**18.5**	**-**	**19.6**	**-**	**20.2**
Lineus sp. "red" (n=9, **n=11**; L. r)	33.1	30.6	27.3	26.6	26.5	33.1	31.8	-	33.7	33.4	35.3	32.9
	22.8	**17.9**	**22.7**	**23.9**	**20.4**	**22.7**	**22.8**	**21.5**	**-**	**21.6**	**-**	**21.7**
Lineus torquatus (n=1, **n=1**; L. t)	32.5	30.6	29.2	26.5	27.3	33.7	32.2	-	34.7	34.8	35.3	34.5
	22.4	**20.8**	**20.1**	**21.4**	**19.7**	**20.4**	**23.0**	**20.8**	**-**	**19.1**	**-**	**21.2**
Lineus sp. "crescent" (n=4, **n=4**; L. c)	32.4	31.3	30.4	29.6	27.1	34.2	33.7	-	35.1	35.1	34.7	33.7
	21.6	**18.9**	**19.3**	**21.0**	**18.7**	**21.7**	**22.1**	**19.8**	**-**	**20.4**	**-**	**20.3**
Lineus sp. 2 (n=26, **n=11**; L. 2)	31.5	30.0	30.7	28.0	27.7	334.0	31.7	-	34.7	34.3	35.6	34.5

	B. u	B. 1	B. 2	H. 3	H. j	H. d	Ma. a	Ma. aq	Ma. c	Ma. m	Ma. o
Cerebratulus longiceps (n=1; C. l)	34.0	37.6	33.3	39.7	40.9	41.8	27.0	27.0	25.6	27.6	27.4
	-	-	-	-	-	-	-	-	-	-	-
Micrura rubramaculosa (n=1, **n=1**; M. r)	35.3	37.2	32.6	38.1	40.6	40.1	27.0	25.7	26.3	27.3	27.4
	19.0	**20.7**	**19.6**	**17.2**	**19.6**	**20.4**	**19.5**	**20.2**	**18.3**	**18.8**	**17.9**
Riserius sp. "no eyes" (n=15, **n=7**; R. ne)	35.3	35.9	34.0	37.3	38.6	39.1	25.1	26.5	25.6	25.0	25.8
	24.6	**21.8**	**22.0**	**23.5**	**24.6**	**23.2**	**22.7**	**23.3**	**22.7**	**22.4**	**21.4**
Riserius pugetensis (n=1, **n=1**; R. p)	35.9	34.7	34.4	36.5	38.4	40.5	25.0	26.2	26.8	25.5	26.2
	23.3	**21.5**	**22.8**	**20.8**	**23.0**	**22.5**	**23.7**	**22.3**	**23.3**	**23.0**	**22.0**
Riserius sp. "eyes" (n=2, **n=3**; R. sp. e)	35.5	34.4	34.2	35.3	38.3	38.9	22.4	24.0	23.6	24.4	23.5
	19.5	**21.6**	**19.8**	**20.0**	**21.2**	**20.9**	**20.8**	**17.5**	**19.5**	**17.4**	**18.2**
Lineus ruber (n=1, **n=1**; L. r)	37.9	37.4	37.3	37.8	41.4	41.1	26.0	26.4	26.4	25.6	25.9
	18.8	**21.2**	**19.0**	**16.6**	**20.7**	**21.2**	**19.3**	**19.9**	**19.3**	**19.0**	**18.2**
Lineus viridis (n=1, **n=1**; L. v)	37.1	35.3	35.3	36.3	40.7	41.2	25.0	25.2	25.6	25.6	25.0
	19.9	**19.6**	**19.4**	**18.1**	**18.5**	**19.3**	**18.9**	**18.7**	**18.3**	**17.1**	**18.5**
Ramphogordius sanguineus (n=4, **n=3**; R. s)	38.2	36.7	36.0	36.6	41.8	42.2	25.2	26.6	26.0	27.3	25.5
	17.4	**19.9**	**17.9**	**19.5**	**20.4**	**20.8**	**18.9**	**18.9**	**19.8**	**18.7**	**18.9**
Heteronemertea gen. sp. 4 (n=7, **n=3**; H. 4)	36.8	36.6	36.0	35.4	39.3	41.2	22.5	24.1	22.8	23.7	23.2
	21.1	**20.1**	**20.3**	**20.8**	**21.3**	**20.4**	**21.7**	**21.8**	**21.3**	**20.2**	**21.3**
Cerebratulus montgomeryi (n=3, **n=2**; C. mo)	36.5	32.4	36.1	34.7	38.0	38.8	21.7	24.5	23.6	24.2	24.8
	19.5	**20.7**	**19.8**	**17.5**	**23.6**	**21.0**	**21.3**	**18.8**	**18.5**	**16.9**	**17.2**
Lineus sp. "red" (n=9, **n=11**; L. r)	35.4	33.0	34.1	34.4	41.6	40.0	24.7	24.5	25.1	24.6	24.9
	20.0	**23.1**	**20.7**	**17.5**	**23.8**	**24.3**	**20.7**	**17.9**	**20.0**	**17.5**	**19.8**
Lineus torquatus (n=1, **n=1**; L. t)	34.2	33.2	33.7	34.8	39.6	39.3	26.5	25.5	27.1	24.0	25.9
	20.4	**21.9**	**20.1**	**19.8**	**24.4**	**23.1**	**21.3**	**19.3**	**19.3**	**19.4**	**18.8**
Lineus sp. "crescent" (n=4, **n=4**; L. c)	35.5	34.3	34.4	32.4	37.9	32.6	25.1	24.5	26.0	25.1	25.9
	22.7	**20.4**	**21.5**	**17.9**	**22.0**	**21.8**	**21.6**	**19.6**	**18.8**	**17.8**	**17.8**
Lineus sp. 2 (n=26, **n=11**; L. 2)	35.4	34.4	34.2	32.9	38.6	36.4	26.3	24.8	26.0	24.8	26.3

	L. c	L. 2	L. f	L. 1	L. b	H. 5	M. v	C. h	M. d	M. 3	M. 4	M. alb
		4.7	**18.8**	**17.1**	**-**	**-**	**16.5**	**19.4**	**19.1**	**18.2**	**17.8**	**17.8**
Lineus flavescens (n=34, **n=33**; L. f)			0.0	6.9	19.2	19.1	30.0	34.1	28.9	26.7	27.0	26.3
			0.7	**13.7**	**-**	**-**	**19.7**	**21.1**	**20.9**	**21.7**	**21.1**	**20.3**
Lineus sp. 1 (n=7 **n=5**; L. 1)				0.0	20.1	20.3	29.5	34.3	29.0	27.5	28.0	27.4
				10.1	**-**	**-**	**18.7**	**18.5**	**20.3**	**20.5**	**19.3**	**19.0**
Lineus bilineatus (n=1; L. b)					-	12.9	32.4	37.2	30.9	29.1	29.9	29.3
					-	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Heteronemertea gen. sp. 5 (n=1; H. 5)						-	32.7	36.7	30.4	29.9	29.0	29.3
						-	**-**	**-**	**-**	**-**	**-**	**-**
Micrura verrilli (n=3, **n=1**; M. v)							0.5	21.4	24.7	23.8	22.7	23.1
							-	**18.6**	**17.6**	**19.3**	**18.1**	**18.2**
Cerebratulus herculeus (n=1, **n=1**; C. h)								-	27.0	26.6	27.7	28.0
								-	**18.0**	**18.8**	**18.5**	**18.2**
Micrura sp. "dark" (n=8, **n=6**; M. d)									0.0	7.2	10.2	9.5
									0.4	**13.9**	**13.3**	**14.4**
Micrura sp. 3 (n=1, **n=1**; M. 3)										-	8.4	8.4
										-	**12.2**	**13.8**
Micrura sp. 4 (n=4, **n=3**; M. 4)											0.0	4.7
											0.1	**9.2**
Micrura sp. "albocephala "(n=13, **n=11**; M. alb)												2.0
												4.9
Cerebratulus albifrons (n=4, **n=3**; C. a)												
Lineidae sp. "large eggs" (n=9, **n=5**; L. le)												
Micrura akkeshiensis (n=1, **n=1**; Mi. a)												
Heteronemertea gen. sp. 2 (n=1, **n=1**; H. 2)												

	C. a	L. le	Mi. a	H. 2	M. 1	B. p	B. d	B. a	B. q	B. h	B. j	B. m
	21.8	**17.7**	**18.1**	**22.4**	**17.2**	**20.9**	**20.2**	**18.9**	**-**	**18.8**	**-**	**18.1**
Lineus flavescens (n=34, **n=33**; L. f)	30.5	30.2	30.3	29.9	27.6	32.1	31.4	-	33.1	33.0	32.9	32.0
	21.3	**19.8**	**22.1**	**21.9**	**18.4**	**21.8**	**19.4**	**20.7**	**-**	**21.3**	**-**	**22.5**
Lineus sp. 1 (n=7 **n=5**; L. 1)	29.8	30.8	30.4	29.1	28.0	30.9	31.8	-	33.0	31.7	33.3	31.5
	19.9	**20.4**	**20.9**	**22.1**	**19.2**	**19.6**	**19.0**	**20.2**	**-**	**19.9**	**-**	**21.1**
Lineus bilineatus (n=1; L. b)	33.5	31.1	30.3	29.2	27.6	33.3	32.6	-	32.4	34.9	33.1	33.3
	-	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Heteronemertea gen. sp. 5 (n=1; H. 5)	32.0	29.2	30.0	29.5	28.4	34.3	33.8	-	34.2	36.1	33.3	34.8
	-	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Micrura verrilli (n=3, **n=1**; M. v)	27.7	33.5	32.5	30.5	28.7	35.9	36.1	-	36.2	35.7	39.2	35.2
	21.3	**20.1**	**17.4**	**20.0**	**16.3**	**21.4**	**18.7**	**19.0**	**-**	**17.5**	**-**	**18.6**
Cerebratulus herculeus (n=1, **n=1**; C. h)	32.5	35.4	34.8	33.0	32.7	38.7	38.6	-	38.2	40.4	39.8	39.1
	18.5	**18.4**	**17.6**	**20.9**	**18.4**	**19.7**	**19.4**	**19.9**	**-**	**18.6**	**-**	**18.1**
Micrura sp. "dark" (n=8, **n=6**; M. d)	25.9	28.3	29.8	29.1	25.8	32.2	32.7	-	31.3	33.0	34.6	32.8
	19.1	**16.2**	**18.0**	**19.5**	**18.2**	**21.1**	**19.5**	**22.3**	**-**	**19.6**	**-**	**21.0**
Micrura sp. 3 (n=1, **n=1**; M. 3)	26.6	29.5	29.8	28.7	25.3	33.7	32.4	-	32.0	32.8	34.2	32.1
	21.2	**20.1**	**19.0**	**21.5**	**18.4**	**20.2**	**19.6**	**20.5**	**-**	**21.1**	**-**	**19.4**
Micrura sp. 4 (n=4, **n=3**; M. 4)	25.2	28.7	29.3	27.8	25.8	33.6	33.0	-	34.1	32.8	35.1	33.6
	22.0	**18.7**	**17.1**	**19.7**	**17.1**	**21.9**	**19.3**	**22.2**	**-**	**21.0**	**-**	**20.4**
Micrura sp. "albocephala "(n=13, **n=11**; M. alb)	25.8	28.6	29.1	28.7	25.9	33.0	320.0	-	32.3	32.5	33.6	32.4
	21.4	**19.4**	**18.0**	**20.6**	**17.1**	**21.6**	**20.2**	**20.7**	**-**	**21.5**	**-**	**20.7**
Cerebratulus albifrons (n=4, **n=3**; C. a)	0.0	30.3	31.7	32.0	31.3	36.5	35.7	-	36.0	36.6	37.6	36.1
	0.7	**19.6**	**20.3**	**20.7**	**19.2**	**21.9**	**19.4**	**22.3**	**-**	**20.8**	**-**	**23.2**
Lineidae sp. "large eggs" (n=9, **n=5**; L. le)		0.0	30.3	30.2	29.2	37.9	36.7	-	37.0	38.3	36.9	37.2
		0.0	**16.7**	**19.8**	**19.1**	**21.3**	**20.7**	**22.4**	**-**	**21.3**	**-**	**19.9**
Micrura akkeshiensis (n=1, **n=1**; Mi. a)			-	11.3	18.6	36.3	35.6	-	35.4	34.7	32.7	34.5
			-	**14.7**	**17.3**	**20.2**	**20.1**	**21.5**	**-**	**22.2**	**-**	**17.8**
Heteronemertea gen. sp. 2 (n=1, **n=1**; H. 2)				-	16.8	34.0	34.1	-	33.9	35.0	35.3	35.2

	B. u	B. 1	B. 2	H. 3	H. j	H. d	Ma. a	Ma. aq	Ma. c	Ma. m	Ma. o
	20.7	**21.3**	**20.8**	**17.4**	**21.4**	**21.6**	**19.1**	**18.7**	**16.8**	**16.4**	**18.0**
Lineus flavescens (n=34, **n=33**; L. f)	35.4	32.4	34.9	32.2	37.8	38.0	26.0	26.2	26.4	24.8	27.1
	21.6	**20.3**	**21.9**	**19.6**	**21.8**	**20.7**	**19.4**	**19.1**	**20.0**	**21.3**	**17.9**
Lineus sp. 1 (n=7 **n=5**; L. 1)	35.8	31.7	35.2	32.4	39.0	38.2	24.6	25.0	26.2	23.8	26.1
	21.5	**19.1**	**20.1**	**19.1**	**21.0**	**19.9**	**19.6**	**20.0**	**19.9**	**20.5**	**19.4**
Lineus bilineatus (n=1; L. b)	37.3	34.9	35.7	33.3	35.4	33.6	26.8	27.0	27.9	26.3	27.6
	-	-	-	-	-	-	-	-	-	-	-
Heteronemertea gen. sp. 5 (n=1; H. 5)	34.4	36.2	35.2	36.3	40.0	38.4	27.3	28.1	28.4	27.9	27.5
	-	-	-	-	-	-	-	-	-	-	-
Micrura verrilli (n=3, **n=1**; M. v)	34.2	37.0	36.4	37.4	39.1	39.2	28.7	29.7	30.6	30.0	30.0
	19.0	**21.3**	**20.1**	**17.7**	**21.0**	**21.2**	**21.6**	**19.9**	**19.2**	**18.2**	**18.9**
Cerebratulus herculeus (n=1, **n=1**; C. h)	32.6	39.0	38.4	39.5	42.7	42.9	33.5	33.2	33.3	32.6	33.4
	21.1	**19.6**	**19.1**	**18.3**	**21.6**	**20.4**	**20.0**	**20.1**	**17.7**	**19.6**	**18.6**
Micrura sp. "dark" (n=8, **n=6**; M. d)	32.0	33.6	30.8	33.2	38.5	37.7	27.5	25.8	25.9	24.6	27.3
	20.3	**20.2**	**20.4**	**16.8**	**20.0**	**19.3**	**19.4**	**19.5**	**16.9**	**17.9**	**18.7**
Micrura sp. 3 (n=1, **n=1**; M. 3)	32.0	33.8	30.8	33.1	37.5	36.4	26.5	24.5	24.7	24.0	26.5
	21.0	**21.5**	**21.2**	**18.5**	**21.0**	**21.3**	**20.9**	**20.4**	**19.1**	**18.8**	**20.6**
Micrura sp. 4 (n=4, **n=3**; M. 4)	31.1	34.5	30.3	32.6	38.4	38.4	27.6	25.0	25.9	25.4	27.9
	20.2	**20.9**	**20.2**	**17.8**	**20.9**	**20.3**	**21.9**	**20.1**	**18.8**	**18.8**	**20.7**
Micrura sp. "albocephala "(n=13, **n=11**; M. alb)	32.2	33.7	30.9	33.2	38.0	37.5	27.0	24.9	25.4	24.4	26.9
	20.7	**20.4**	**20.4**	**17.4**	**21.3**	**20.5**	**20.7**	**20.0**	**18.8**	**18.8**	**20.5**
Cerebratulus albifrons (n=4, **n=3**; C. a)	33.5	37.1	33.1	36.3	39.0	39.1	28.4	28.6	28.6	29.4	29.9
	22.2	**20.6**	**21.7**	**21.8**	**22.3**	**21.8**	**23.1**	**21.3**	**19.7**	**19.9**	**21.7**
Lineidae sp. "large eggs" (n=9, **n=5**; L. le)	37.4	38.6	36.9	39.1	40.9	39.2	29.1	29.5	29.4	27.2	28.5
	20.5	**22.1**	**21.6**	**17.5**	**20.2**	**20.3**	**20.6**	**20.3**	**18.1**	**17.3**	**17.7**
Micrura akkeshiensis (n=1, **n=1**; Mi. a)	35.0	36.4	34.4	36.0	39.6	39.7	30.0	27.9	29.3	28.8	30.5
	20.6	**20.8**	**20.1**	**17.1**	**19.8**	**20.8**	**20.2**	**21.1**	**19.3**	**18.2**	**18.7**
Heteronemertea gen. sp. 2 (n=1, **n=1**; H. 2)	32.7	35.1	32.9	36.9	39.2	38.7	30.0	28.3	29.7	28.8	30.2

	C. a	L. le	Mi. a	H. 2	M. 1	B. p	B. d	B. a	B. q	B. h	B. j	B. m
				-	**18.4**	**20.6**	**22.8**	**22.1**	**-**	**22.7**	**-**	**19.5**
Heteronemertea gen. sp. 1 (n=4, **n=3**; H. 1)					0.0	35.0	34.7	-	34.6	34.6	35.9	35.4
					0.1	**20.4**	**17.5**	**20.0**	**-**	**18.7**	**-**	**19.7**
Baseodiscus punnetti (n=1, **n=1**; B. p)						-	11.9	-	13.5	15.5	22.1	15.7
						-	**17.6**	**16.2**	**-**	**18.4**	**-**	**17.0**
Baseodiscus delineatus (n=5, **n=1**; B. d)							0.0	-	10.5	15.3	21.8	15.6
							-	**16.7**		**15.6**	**-**	**16.9**
Baseodiscus aureus (**n=1**; B. a)								-	-	-	-	-
								-		**15.4**	**-**	**15.2**
Baseodiscus quiquelineatus (n=2; B. q)									0.5	15.7	21.2	15.7
									-	**-**	**-**	**-**
Baseodiscus hemprichii (n=2, **n=1**; B. h)										0.8	22.4	10.1
										-	**-**	**15.3**
Baseodiscus jonasi (n=2; B. j)											0.0	22.4
											-	**-**
Baseodiscus mexicanus (n=2, **n=2**; B. m)												0.0
												0.0
Baseodiscus unicolor (n=4, **n=1**; B. u)												
Baseodiscus sp. 1 (n=1, **n=1**; B. 1)												
Baseodiscus sp. 2 (n=1, **n=1**; B. 2)												
Heteronemertea gen. sp. 3 (n=1, **n=1**; H. 3)												
Hubrechtella juliae (n=6, **n=2**; H. j)												
Hubrechtella dubia (n=1, **n=1**; H. d)												

	B. u	B. 1	B. 2	H. 3	H. j	H. d	Ma. a	Ma. aq	Ma. c	Ma. m	Ma. o
	22.8	**22.6**	**21.7**	**19.2**	**21.6**	**21.1**	**22.8**	**23.4**	**21.3**	**20.4**	**20.1**
Heteronemertea gen. sp. 1 (n=4, **n=3**; H. 1)	35.6	35.1	34.8	36.3	37.0	38.1	28.5	28.1	28.9	28.1	28.5
	18.9	**20.1**	**18.5**	**17.7**	**19.6**	**18.9**	**18.2**	**18.9**	**17.2**	**16.5**	**17.0**
Baseodiscus punnetti (n=1, **n=1**; B. p)	24.7	9.6	24.6	32.4	39.5	38.5	30.4	30.0	30.1	29.3	32.4
	18.2	**14.5**	**17.7**	**20.0**	**19.0**	**19.5**	**22.0**	**23.0**	**21.0**	**22.0**	**22.0**
Baseodiscus delineatus (n=5, **n=1**; B. d)	24.1	10.3	24.0	31.2	40.1	39.1	31.5	31.1	31.4	30.4	32.1
	18.2	**15.1**	**17.0**	**16.5**	**20.6**	**19.8**	**22.0**	**22.0**	**21.0**	**21.0**	**20.0**
Baseodiscus aureus (**n=1**; B. a)	-	-	-	-	-	-	-	-	-	-	-
	19.6	**15.6**	**18.3**	**19.8**	**23.2**	**21.1**	**23.0**	**23.0**	**21.0**	**22.0**	**22.0**
Baseodiscus quiquelineatus (n=2; B. q)	24.5	14.5	24.7	30.4	37.9	38.2	31.4	31.5	32.3	31.1	32.8
	-	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Baseodiscus hemprichii (n=2, **n=1**; B. h)	24.6	14.9	24.7	29.7	38.1	39.6	32.4	32.5	33.4	32.2	33.8
	18.4	**18.1**	**19.1**	**19.3**	**21.9**	**22.2**	**23.0**	**23.0**	**21.0**	**22.0**	**20.0**
Baseodiscus jonasi (n=2; B. j)	26.0	22.7	26.0	31.2	39.9	41.3	34.0	32.9	30.8	33.2	34.9
	-	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Baseodiscus mexicanus (n=2, **n=2**; B. m)	26.4	15.3	26.4	32.0	37.8	38.4	32.9	32.7	32.8	32.2	33.8
	17.3	**15.5**	**15.8**	**15.8**	**20.8**	**21.1**	**21.0**	**20.0**	**20.0**	**20.0**	**22.0**
Baseodiscus unicolor (n=4, **n=1**; B. u)	4.4	24.0	2.9	27.2	37.5	36.9	33.5	31.2	33.3	34.0	33.8
	-	**17.4**	**9.0**	**17.5**	**21.8**	**22.2**	**21.0**	**19.0**	**21.0**	**19.0**	**20.0**
Baseodiscus sp. 1 (n=1, **n=1**; B. 1)		-	23.8	29.7	39.3	38.9	31.6	30.8	31.8	31.1	32.8
		-	**17.2**	**20.8**	**19.6**	**18.7**	**20.0**	**23.0**	**22.0**	**23.0**	**22.0**
Baseodiscus sp. 2 (n=1, **n=1**; B. 2)			-	26.9	37.7	36.9	34.7	32.6	34.6	35.0	35.2
			-	**18.0**	**20.3**	**20.4**	**20.0**	**19.0**	**19.0**	**19.0**	**19.0**
Heteronemertea gen. sp. 3 (n=1, **n=1**; H. 3)				-	35.0	35.4	34.0	33.2	32.2	33.3	34.0
				-	**20.2**	**21.2**	**21.0**	**18.1**	**18.6**	**17.9**	**18.5**
Hubrechtella juliae (n=6, **n=2**; H. j)					0.4	18.7	37.5	37.1	36.1	37.4	37.1
					0.3	**7.9**	**20.5**	**22.2**	**22.0**	**20.7**	**20.1**
Hubrechtella dubia (n=1, **n=1**; H. d)						-	36.0	36.8	36.3	36.2	36.6

	B. u	B. 1	B. 2	H. 3	H. j	H. d	Ma. a	Ma. aq	Ma. c	Ma. m	Ma. o
						-	**21.0**	**22.9**	**22.6**	**21.0**	**19.4**
Maculaura alaskensis (n=42, **n=32**; Ma. a)							0.2	10.9	11.2	10.5	4.0
							0.7	**17.9**	**17.4**	**17.0**	**14.3**
Maculaura aquilonia (n=43, **n=51**; Ma. aq)								0.1	8.8	8.0	12.2
								0.3	**14.8**	**13.9**	**16.3**
Maculaura cerebrosa (n=17, **n=13**; Ma. c)									0.1	10.2	11.7
									0.6	**12.9**	**18.0**
Maculaura magna (n=28, **n=10**; Ma. m)										1.1	11.0
										7.1	**16.2**
Maculaura oregonensis (n=9, **n=8**; Ma. o)											0.4
											0.2

APPENDIX G

HOPLONEMERTEAN DIVERGENCE TABLE (%)

	C. e	C. c	C. ca	C. 4	N. b	N. o	N. p	N. pn	N. 1	N. 2	O. d	A. a
Carcinonemertes errans (n=5, **n=6**; C. e)	0.2	13.6	13.3	14.1	25.8	29.2	26.8	28.0	30.1	28.3	30.8	24.0
	0.1	**15.6**	**16.4**	**14.5**	**21.2**	**-**	**20.7**	**16.6**	**16.7**	**16.3**	**19.2**	**18.7**
Carcinonemertes sp. "chaceon" (n=1, **n=1**; C. c)		-	13.6	14.1	25.2	27.9	26.3	26.9	26.7	26.7	28.1	25.3
		-	**16.5**	**17.4**	**19.9**	**-**	**19.1**	**18.0**	**17.4**	**17.1**	**18.5**	**18.8**
Carcinonemertes carcinophila (n=1, **n=1**; C. ca)			-	2.2	16.1	27.6	26.8	26.2	26.1	26.5	27.8	27.0
			-	**4.9**	**18.6**	**-**	**17.2**	**16.6**	**16.7**	**15.7**	**17.8**	**19.9**
Carcinonemertes sp. 414 (n=1, **n=1**; C. 4)				-	26.0	27.7	27.3	25.6	26.6	26.8	28.1	27.3
				-	**17.6**	**-**	**16.7**	**15.3**	**16.0**	**15.4**	**17.3**	**18.7**
Nipponnemertes bimaculatus (n=3, **n=5**; A. b)					0.0	24.0	21.3	5.1	22.1	21.1	29.9	27.4
					0.2	**-**	**15.6**	**7.5**	**15.0**	**15.0**	**17.4**	**20.3**
Nipponnemertes ogumai (n=1; N. o)						-	15.4	24.6	17.2	14.7	30.7	28.7
						-	**-**	**-**	**-**	**-**	**-**	**-**
Nipponnemertes pulchra (n=1, **n=1**; N. p)							-	21.1	1.6	8.9	30.1	29.2
							-	**14.5**	**4.1**	**9.0**	**17.7**	**19.3**
Nipponnemertes punctatulus (n=1, **n=1**; N. pn)								-	21.7	20.2	30.9	28.7
								-	**13.9**	**15.1**	**14.9**	**16.7**
Nipponnemertes sp. 1 (n=1, **n=1**; N. 1)									-	9.8	29.4	27.9
									-	**9.5**	**14.6**	**16.6**
Nipponnemertes sp. 2 (n=1, **n=1**; N. 2)										-	27.5	30.4
										-	**16.5**	**18.6**
Oerstedia dorsalis (n=1, **n=2**; O. d)											-	34.1
											-	**18.3**
Amphiporus angulatus (n=1, **n=1**; A. a)												-
												-
Amphiporus formidabilis (n=1, **n=1**; A. f)												

	A. f	A. i	P. p	P. s	E. b	Z. 1	Z. s	Z. v	M. s	M. g	Pa. c	G. l
Carcinonemertes errans (n=5, **n=6**; C. e)	24.7	23.7	23.0	24.6	22.4	23.5	21.1	22.2	21.8	23.7	19.8	21.5
	17.5	**19.3**	**17.9**	**18.8**	**20.7**	**19.4**	**18.6**	**17.5**	**20.3**	**17.0**	**18.7**	**16.2**
Carcinonemertes sp. "chaceon" (n=1, **n=1**; C. c)	25.7	24.4	24.7	24.8	23.2	22.4	20.1	21.8	21.4	20.9	20.0	20.0
	17.0	**19.3**	**18.5**	**18.7**	**18.4**	**16.6**	**17.9**	**17.0**	**19.7**	**17.9**	**17.5**	**15.7**
Carcinonemertes carcinophila (n=1, **n=1**; C. ca)	26.6	25.0	26.0	25.9	24.8	21.9	20.3	21.8	22.2	25.3	21.4	22.3
	18.3	**17.9**	**18.2**	**18.5**	**18.1**	**17.8**	**17.5**	**18.9**	**17.6**	**17.7**	**16.9**	**17.4**
Carcinonemertes sp. 414 (n=1, **n=1**; C. 4)	27.5	25.5	16.6	26.4	25.1	22.1	20.6	21.9	22.6	26.2	22.0	22.2
	18.1	**17.4**	**18.5**	**19.1**	**19.0**	**18.3**	**17.6**	**18.8**	**18.4**	**17.5**	**15.8**	**16.7**
Nipponnemertes bimaculatus (n=3, **n=5**; A. b)	29.9	27.7	28.1	26.7	27.5	27.7	28.7	27.4	25.7	28.0	25.8	26.6
	18.5	**19.3**	**19.2**	**18.4**	**17.7**	**19.3**	**16.8**	**18.5**	**19.0**	**19.2**	**17.9**	**16.0**
Nipponnemertes ogumai (n=1; N. o)	29.3	28.2	27.7	27.5	28.6	28.9	26.6	28.0	27.1	28.9	26.4	26.9
	-	-	-	-	-	-	-	-	-	-	-	-
Nipponnemertes pulchra (n=1, **n=1**; N. p)	28.5	24.2	26.9	26.4	28.2	27.6	24.4	26.6	27.3	28.0	27.8	25.6
	20.1	**17.7**	**19.2**	**19.9**	**17.6**	**19.0**	**15.0**	**18.0**	**18.8**	**17.2**	**18.3**	**16.4**
Nipponnemertes punctatulus (n=1, **n=1**; N. pn)	30.5	27.8	28.2	27.1	27.8	28.8	28.2	27.8	24.4	26.5	25.1	25.0
	13.9	**14.7**	**15.6**	**16.9**	**14.9**	**14.8**	**17.0**	**14.1**	**16.7**	**15.8**	**14.8**	**14.4**
Nipponnemertes sp. 1 (n=1, **n=1**; N. 1)	28.8	24.1	26.9	26.4	27.7	27.8	24.1	27.0	27.9	28.9	27.9	25.9
	15.2	**15.6**	**15.7**	**18.0**	**14.5**	**14.9**	**15.1**	**13.9**	**16.2**	**14.6**	**14.7**	**14.0**
Nipponnemertes sp. 2 (n=1, **n=1**; N. 2)	30.3	27.0	27.9	27.7	28.7	29.0	26.2	27.5	25.6	27.0	26.0	26.1
	13.3	**15.5**	**15.4**	**16.6**	**13.6**	**15.0**	**13.6**	**15.6**	**17.1**	**15.2**	**14.4**	**12.9**
Oerstedia dorsalis (n=1, **n=2**; O. d)	34.9	32.4	34.0	34.1	32.6	33.2	31.6	32.0	29.4	31.3	27.6	29.3
	16.4	**17.8**	**18.1**	**18.9**	**16.1**	**18.4**	**16.6**	**17.1**	**19.4**	**17.7**	**16.4**	**15.0**
Amphiporus angulatus (n=1, **n=1**; A. a)	13.4	13.6	11.7	13.0	13.1	18.2	18.8	19.5	19.2	21.3	17.9	19.2
	16.2	**15.7**	**15.9**	**16.8**	**19.4**	**16.9**	**16.6**	**16.7**	**19.4**	**16.9**	**17.7**	**17.4**
Amphiporus formidabilis (n=1, **n=1**; A. f)	-	12.6	13.1	15.9	13.9	18.2	20.1	20.0	19.2	21.1	18.3	19.6

	Pr. c	E. 1	E. g	Po. c	O. sp.	O. m	T. b	T. 1	T. c	T. a	A. c
Carcinonemertes errans (n=5, **n=6**; C. e)	21.5	21.1	20.1	19.8	22.6	24.4	-	-	-	-	-
	18.0	**18.5**	**21.9**	**18.6**	**-**	**20.7**	**16.0**	**18.1**	**21.1**	**19.3**	**18.0**
Carcinonemertes sp. "chaceon" (n=1, **n=1**; C. c)	20.9	19.8	19.8	21.0	22.3	24.2	-	-	-	-	-
	18.9	**17.4**	**19.2**	**23.6**	**-**	**18.1**	**18.9**	**19.8**	**18.9**	**18.0**	**18.2**
Carcinonemertes carcinophila (n=1, **n=1**; C. ca)	22.1	21.0	21.5	24.0	23.9	25.4	-	-	-	-	-
	15.6	**17.8**	**18.5**	**18.7**	**-**	**19.3**	**16.6**	**18.5**	**22.6**	**18.0**	**16.6**
Carcinonemertes sp. 414 (n=1, **n=1**; C. 4)	22.1	21.3	20.9	24.2	24.3	25.3	-	-	-	-	-
	15.8	**16.9**	**17.7**	**19.6**	**-**	**19.0**	**14.4**	**17.1**	**21.3**	**17.7**	**16.4**
Nipponnemertes bimaculatus (n=3, **n=5**; A. b)	25.7	26.1	26.0	29.5	28.0	26.9	-	-	-	-	-
	16.5	**17.9**	**18.6**	**19.6**	**-**	**17.4**	**15.5**	**20.7**	**18.4**	**15.1**	**16.8**
Nipponnemertes ogumai (n=1; N. o)	25.9	28.6	28.1	27.6	29.8	31.4	-	-	-	-	-
	-	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Nipponnemertes pulchra (n=1, **n=1**; N. p)	25.5	26.3	26.3	28.3	27.5	29.3	-	-	-	-	-
	16.5	**18.9**	**19.0**	**20.7**	**-**	**17.5**	**15.6**	**20.6**	**18.7**	**15.9**	**16.9**
Nipponnemertes punctatulus (n=1, **n=1**; N. pn)	25.3	26.7	25.6	29.8	28.5	29.0	-	-	-	-	-
	14.3	**15.5**	**17.9**	**17.4**	**-**	**16.2**	**13.0**	**15.5**	**16.9**	**13.9**	**15.3**
Nipponnemertes sp. 1 (n=1, **n=1**; N. 1)	25.8	27.4	27.4	28.1	27.8	30.0	-	-	-	-	-
	14.3	**15.1**	**18.2**	**16.1**	**-**	**16.0**	**13.7**	**15.3**	**16.5**	**13.9**	**15.5**
Nipponnemertes sp. 2 (n=1, **n=1**; N. 2)	25.5	28.3	28.3	27.4	30.0	31.5	-	-	-	-	-
	14.3	**13.9**	**18.0**	**15.5**	**-**	**15.9**	**13.6**	**16.6**	**18.0**	**14.1**	**12.7**
Oerstedia dorsalis (n=1, **n=2**; O. d)	29.4	30.2	29.4	33.4	32.2	34.3	-	-	-	-	-
	15.8	**16.9**	**17.2**	**18.0**	**-**	**15.6**	**14.4**	**17.4**	**17.2**	**16.6**	**15.0**
Amphiporus angulatus (n=1, **n=1**; A. a)	18.0	19.0	18.4	21.7	20.4	22.5	-	-	-	-	-
	17.4	**17.8**	**20.4**	**22.9**	**-**	**21.0**	**18.6**	**17.5**	**18.2**	**16.3**	**19.0**
Amphiporus formidabilis (n=1, **n=1**; A. f)	17.4	20.3	19.8	24.0	23.0	24.1	-	-	-	-	-

	A. f	A. i	P. p	P. s	E. b	Z. 1	Z. s	Z. v	M. s	M. g	Pa. c	G. l
	-	**13.0**	**14.1**	**15.4**	**13.1**	**15.4**	**12.7**	**14.6**	**16.7**	**15.2**	**15.2**	**14.0**
Amphiporus imparispinosus (n=1, **n=1**; A. i)		-	12.7	13.3	14.6	17.6	17.5	19.5	17.4	18.4	16.5	17.9
		-	**15.8**	**19.2**	**14.7**	**17.0**	**15.4**	**14.9**	**18.4**	**17.3**	**15.7**	**15.1**
Paranemertes peregrina (n=8, **n=5**; P. p)			0.9	4.8	13.2	17.7	16.1	18.0	18.0	19.4	15.1	16.7
			3.6	**13.2**	**16.2**	**16.7**	**15.5**	**17.9**	**17.3**	**16.3**	**16.5**	**16.6**
Paranemertes sanjuanensis (n=2, **n=2**, P. s)				0.0	13.9	17.3	16.0	17.5	18.0	19.0	16.6	16.9
				0.7	**17.5**	**16.8**	**16.2**	**17.3**	**17.3**	**16.3**	**17.8**	**17.0**
Emplectonema buergeri (n=1, **n=1**; E. b)					-	15.1	16.2	16.5	17.6	19.1	15.2	18.0
					-	**15.3**	**14.1**	**15.2**	**16.8**	**15.5**	**16.2**	**15.0**
Zygonemertes sp. 1 (n=9, **n=15**; Z. 1)						1.2	9.0	10.7	18.8	20.1	16.3	17.5
						3.3	**10.5**	**12.4**	**15.0**	**14.5**	**15.0**	**15.8**
Zygonemertes simonae (n=1, **n=1**; Z. s)							-	11.2	17.1	19.0	16.5	15.2
							-	**12.3**	**16.2**	**14.7**	**15.0**	**15.9**
Zygonemertes virescens (n=1, **n=1**; Z. v)								-	19.7	21.5	17.6	16.7
								-	**16.1**	**14.1**	**15.4**	**16.5**
Malacobdella siliquae (n=3, **n=1**; M. s)									0.0	9.0	12.4	19.0
									-	**12.1**	**17.0**	**18.3**
Malacobdella grossa (n=1, **n=1**; M. g)										-	13.5	19.0
										-	**16.1**	**15.5**
Pantinonemertes californiensis (n=1, **n=3**; Pa. c)											-	15.2
											0.0	**16.1**
Gurjanovella littoralis (n=3, **n=2**; G. l)												0.3
												4.7
Paranemertes californica (n=6, **n=14**; Pr. c)												
Emplectonema sp. 1 (n=4, **n=4**; E. 1)												
Emplectonema gracile (n=1, **n=1**; E. g)												

	Pr. c	E. 1	E. g	Po. c	O. sp.	O. m	T. b	T. 1	T. c	T. a	A. c
	15.8	**14.3**	**18.9**	**17.1**	**-**	**17.2**	**15.9**	**16.1**	**17.6**	**16.2**	**13.3**
Amphiporus imparispinosus (n=1, **n=1**; A. i)	16.0	18.4	17.5	22.3	20.2	21.4	-	-	-	-	-
	15.6	**15.3**	**18.3**	**17.8**	**-**	**16.3**	**16.9**	**17.7**	**19.7**	**17.4**	**13.2**
Paranemertes peregrina (n=8, **n=5**; P. p)	16.3	19.2	16.9	20.6	19.0	21.1	-	-	-	-	-
	15.8	**16.6**	**18.1**	**16.4**	**-**	**17.6**	**16.8**	**18.8**	**19.4**	**15.3**	**17.7**
Paranemertes sanjuanensis (n=2, **n=2**, P. s)	15.9	21.2	18.0	20.6	20.1	21.3	-	-	-	-	-
	17.3	**17.6**	**18.2**	**16.8**	**-**	**18.5**	**16.8**	**19.2**	**19.9**	**15.0**	**17.7**
Emplectonema buergeri (n=1, **n=1**; E. b)	16.4	17.6	17.1	20.7	20.0	23.9	-	-	-	-	-
	16.2	**14.7**	**18.3**	**14.9**	**-**	**15.5**	**17.2**	**16.3**	**18.0**	**13.9**	**15.7**
Zygonemertes sp. 1 (n=9, **n=15**; Z. 1)	17.9	16.8	16.4	22.5	20.9	22.5	-	-	-	-	-
	15.2	**15.6**	**19.4**	**16.0**	**-**	**17.5**	**17.5**	**19.1**	**15.3**	**15.1**	**16.0**
Zygonemertes simonae (n=1, **n=1**; Z. s)	15.4	17.1	16.1	18.9	18.4	19.3					
	15.0	**14.7**	**17.8**	**14.9**	**-**	**16.5**	**14.8**	**16.8**	**18.0**	**12.5**	**14.3**
Zygonemertes virescens (n=1, **n=1**; Z. v)	17.5	17.3	16.6	19.2	20.4	21.6	-	-	-	-	-
	14.8	**17.0**	**19.7**	**16.8**	**-**	**15.5**	**15.4**	**16.7**	**18.0**	**13.9**	**15.9**
Malacobdella siliquae (n=3, **n=1**; M. s)	18.6	18.9	18.0	20.7	20.2	22.7	-	-	-	-	-
	18.2	**19.4**	**18.7**	**17.6**	**-**	**20.3**	**17.5**	**19.8**	**17.3**	**18.6**	**18.6**
Malacobdella grossa (n=1, **n=1**; M. g)	18.7	21.1	19.5	22.6	21.1	24.4	-	-	-	-	-
	16.8	**16.9**	**19.2**	**17.5**	**-**	**18.3**	**17.4**	**18.4**	**16.1**	**15.3**	**16.0**
Pantinonemertes californiensis (n=1, **n=3**; Pa. c)	15.3	17.2	15.6	20.8	21.0	23.2	-	-	-	-	-
	15.2	**15.3**	**17.5**	**16.9**	**-**	**15.5**	**14.8**	**17.9**	**17.8**	**13.3**	**16.1**
Gurjanovella littoralis (n=3, **n=2**; G. l)	4.9	18.5	15.4	20.6	19.2	21.0	-	-	-	-	-
	13.1	**14.9**	**17.8**	**16.6**	**-**	**16.5**	**15.5**	**17.9**	**17.4**	**14.1**	**14.7**
Paranemertes californica (n=6, **n=14**; Pr. c)	0.6	17.3	15.7	21.2	19.4	20.8	-	-	-	-	-
	3.1	**15.3**	**17.4**	**16.2**	**-**	**16.8**	**15.2**	**18.2**	**16.8**	**14.1**	**16.2**
Emplectonema sp. 1 (n=4, **n=4**; E. 1)		0.1	10.0	20.9	22.7	21.8	-	-	-	-	-
		0.1	**16.4**	**18.0**	**-**	**16.0**	**16.7**	**17.1**	**17.6**	**15.3**	**15.8**
Emplectonema gracile (n=1, **n=1**; E. g)			-	21.0	20.8	23.2	-	-	-	-	-

	Pr. c	E. 1	E. g	Po. c	O. sp.	O. m	T. b	T. 1	T. c	T. a	A. c
			-	**19.6**	**-**	**17.7**	**17.5**	**20.2**	**19.3**	**18.0**	**17.1**
Poseidonemertes collaris (n=9, **n=9**; Po. c)				0.2	22.1	23.5	-	-	-	-	-
				0.6	**-**	**19.5**	**17.0**	**17.7**	**18.7**	**17.5**	**15.8**
Ototyphlonemertes sp. 1 (n=1, O. sp.)					-	13.7	-	-	-	-	-
					-	**-**	**-**	**-**	**-**	**-**	**-**
Ototyphlonemertes macintoshi (n=1, **n=1**; O. m)						-	-	-	-	-	-
						-	**15.5**	**18.5**	**17.4**	**14.1**	**14.7**
Tetrastemma bilineatum (**n=1**; T. b)							-	-	-	-	-
							-	**15.2**	**18.4**	**14.0**	**15.3**
Tetrastemma sp. 1 (**n=2**; T. 1)								-	-	-	-
								0.2	**19.6**	**15.2**	**17.4**
Tetrastemma candidum (**n=1**; T. c)									-	-	-
									-	**16.3**	**18.7**
Tetrastemma albidum (**n=1**; T. a)										-	-
										-	**15.9**
Amphiporus cruentatus (**n=1**; A. c)											-
											-

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CHAPTER I

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CHAPTER II

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CHAPTER III

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CHAPTER IV

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CHAPTER VIII

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