INCOMPATIBILITY AND SPECIATION

WITHIN & BETWEEN SPECIES

by

HANNA MOORE

A THESIS

Presented to the Department of BIOLOGY
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

June 2015

An Abstract of the Thesis of

Hanna Moore for the degree of Bachelor of Science
in the Department of Biology to be taken June 2015

Title: Incompatibility and Speciation Within & Between Species

Approved: _____

Patrick C. Phillips, Ph.D.

The process of speciation is integral to producing and maintaining the great biodiversity of life on Earth; yet the underlying mechanisms of reproductive isolation remain largely a mystery. The widely accepted Bateson-Dobzhansky-Muller model cites the accumulation of intrinsic post-zygotic isolating mechanisms as the barrier to reproduction between two species. However, the characteristics of such accumulated incompatibilities are complex and varied and thus hold the process of speciation from being understood on a mechanistic level. Using four *Caenorhabditis* populations, three geographically separate strains of *C. remanei*, and one strain of closely related *C. latens*, a multiple cross analysis of parental, F1, and F2 fitness levels was conducted to address these details of speciation, particularly when comparing standing within species incompatibility to incompatibility developed between species. Pairing the analysis of phenotypic measures of fitness and genetic incompatibility with a Mendelian Inheritance model presents compelling evidence of deleterious epistatic interaction in the between species crosses as well as within species crosses. These results indicate that markers of incipient speciation are quantifiably present in otherwise compatible populations of the same species.

ii

Acknowledgements

I would like to use this space to thank and acknowledge all those who contributed their skill, knowledge, help, and mental and emotional support to make this thesis a reality. I thank Dr. Patrick Phillips for his mentorship and guidance in designing and carrying out this experiment, as well as his patience and support in helping me to analyze and synthesize my findings. I thank both Dr. Samantha Hopkins and Dr. Peter Wetherwax for their participation on my thesis committee and their general advising and support over the past couple of years. I would like to sincerely thank and acknowledge Valerie Haskins, Chris Hanson, and Addison Kaufman all of whom contributed *many* volunteered hours of their time in the lab helping me transfer, hatch off, and count the 71,335 worms and 277,850 eggs I analyzed (as well as the extras that weren't used). I would like to sincerely thank both Miriam Rigby and Christine O'Connor for contributing their computational skills, time, and knowledge and making all of the data figures and edits possible. I thank John Willis and Janna Fierst for working to help me begin the genomics analysis that didn't quite make it in time to be included. And lastly, but absolutely not least, I thank all of my friends and family who have feigned or expressed real interest in hearing about "my worms" and who have provided their support and encouragement throughout the past three years of data collection and the entire thesis process.

Table of Contents

General Introduction

Earth is home to a staggering amount of diversity. Estimates project that the earth currently houses approximately 8.7 million eukaryotic species (Mora, et al. 2011). If you were to take one second to look at each one of those species (leaving no time to sleep or eat) and keeping in mind that no bacteria are represented, it would take close to 108 days! Yet this projection of the earth's diversity is not a static figure. In the past few years it has become common to hear discussion of threats to diversity—increased development, pollution, and climate change to name a few—with a focus on factors that cause a decrease in diversity. However, the other side of change in diversity, its increase, is less commonly considered. Not all organisms and species were in place at the moment of the earth's formation. The first life on Earth is thought to have been single cellular prokaryotic bacteria; yet, 3.7 billion years later this singular event has led to the generation of millions of new species of prokaryotes and eukaryotes (Freeman 2014). Diversification and speciation occurred—new species were generated.

Speciation

Speciation is the evolutionary process by which a new species is created (Cook 1906). Using the generally accepted, Biological Species Concept understanding of a species, speciation occurs when two populations of organisms are unable to reproduce, and thus are reproductively isolated (Mayr 1942). In studying the incompatibilities between known species, reproductive isolation is found to be caused by the accumulation of genetic and phenotypic incompatibilities (reproductive isolating

mechanisms) that gradually lead to the finalizing, and ultimately isolating, step of speciation (Bateson 1909, Dobzhansky 1937, Muller 1958).

Is the accumulation of incompatibilities predictable or random? Does distance between populations matter in the rate of accumulation of incompatibilities (Orr 1993)? Are there measurable effects from males or females in terms of reproductive success? These questions are difficult to answer because evolution does not have a clear beginning or end and the processes that underlie speciation can take place over a period of hundreds of years—far longer than any scientist's lifetime. However, with the right model, these questions can be addressed within an experimental setting in order to derive conclusions that may still apply generally to the natural environment. Answers to these fundamental questions can lead us to a better understanding of the diversification of life on earth, lending new insight into extinct species' evolution as well as currently diverging populations of species.

Reproductive Isolating Mechanisms (RIMs)

The incompatibilities that are instrumental in reproductive isolation and speciation can be placed in two general categories—pre-zygotic RIMs and post-zygotic RIMs (Mayr 1963, Seehausen, et al. 2014). A zygote is the initial stage of embryonic development in organisms following the fertilization of the egg by sperm. Thus, RIMs are grouped into two classifications: those that prevent the egg and sperm from meeting, and those that arise following fertilization (Mayr 1963).

While pre-zygotic and post-zygotic isolating mechanisms are typically both involved in leading to the final speciation between two groups, generally the process begins with extrinsic, or external environmentally focused factors of post-zygotic

isolation (Mayr 1963). In considering the basic life purpose of organisms: to mate, reproduce, and pass on their genes to future generations, pre-zygotic barriers to reproduction are considered to be the more energy efficient of the two methods (Mayr 1963, Futuyma 2013).

Pre-zygotic isolation can be caused by a variety of factors. Common pre-zygotic factors range from extrinsic factors such as life history patterns of organisms and environmental differences, to intrinsic structural differences and chemical incompatibilities (Seehausen, et al. 2014). Temporal isolation is caused when organisms are isolated by time of reproduction, or even more generally, time of interaction. Individuals from two tree species may flower and fruit at different times of the year and therefore not have the opportunity to cross-pollinate. Alternatively, a nocturnal animal may never interact with, let alone attempt to reproduce with a diurnal animal (an animal active during the day). Similar to temporal isolation is habitat isolation; like the diurnal and nocturnal animals, organisms that live in different habitats will never have the opportunity to attempt reproduction. Less environment-centered pre-zygotic isolating mechanisms include behavioral and structural isolation. Behavioral isolation is commonly observed in animals with mating rituals such as deer, in male combat, or frogs or birds, in their songs. If an organism is not attracted to a mate by the specific mating behavior, they will not mate. Perhaps most obvious of pre-zygotic RIMs is structural isolation: some organisms are physically unable to mate with each other. While pre-zygotic isolating mechanisms are varied in the way they prevent mating, in terms of energy cost, they all prevent an organism from the costs associated with raising an offspring that may not survive or reproduce. Thus, in the context of the organismal

purpose of passing on genes, through pre-zygotic isolating mechanisms, the organism eliminates the chance for wasted energy and is instead able to apply that energy towards successful mating endeavors.

Post-zygotic RIMs are more costly as they rely on the expenditure of energy in growing and raising an inviable or infertile offspring. Post-zygotic mechanisms lead to isolation between interbreeding species after the production of inviable hybrid offspring, infertile hybrid offspring, or generally lower fitness hybrid offspring (Mayr 1963, Baird 2002). Inviable offspring include offspring that do not fully develop but may be terminated at some stage in their development, or offspring that are produced with such deleterious genetic or phenotypic mutations that they are unable to function and survive (Mayr 1963). Infertile hybrid offspring do survive but are unable to produce offspring of their own (Mayr 1963, Baird 2002). In both cases any possibility of a future gene line is terminated directly with the hybrid generation.

Unlike pre-zygotic RIMs, which are caused by well-established ecological, structural, or behavioral barriers to mating, post-zygotic RIMs are most typically caused by intrinsic post-zygotic isolation (Seehausen, et al. 2014). Although the processes underlying intrinsic post-zygotic isolation are not well understood (as there are a myriad of factors that could cause the inviable or infertile offspring) genetic incompatibility, with its ability to affect and disrupt a host of developmental systems, is believed to be the broader cause (Coyne & Orr 2004).

When considering the accumulation of incompatibilities in terms of pre- and post-mating barriers, it is common to construct a linear understanding. It is assumed that an external pre-mating barrier (whether it be distance, time, or some other variable)

4

exists, separating two populations and creating a situation in which there is an accumulation of post-zygotic reproductive isolating mechanisms that then lay the groundwork for reinforcing pre-zygotic isolating mechanisms (Dobzhansky 1963, Mayr 1963). However, it is important to note that each type of RIM is not mutually exclusive nor is it entirely dependent on its counterpart.

Population Divergence and the Accumulation of Incompatibilities

Reproductive isolating mechanisms are not inherent in the genetic structure of organisms; nor is their primary purpose to keep two populations from interbreeding; the latter tends to be a side effect of adaptive or divergent evolution. While there may be pre- or post-zygotic RIMs in place for separated species, for those species that are diverging (becoming increasingly incompatible), reproductive isolation is commonly developed through the aforementioned *accumulation of incompatibilities,* more specifically, the accumulation of intrinsic post-zygotic isolating barriers (Seehausen, et al. 2014). Through the genetic processes of recombination and epistatsis, the Bateson-Dobzhansky-Muller (BDM) model incorporates explanations for both the accumulation of intrinsic post-zygotic isolating barriers as well as the reasons for the subsequent hybrid breakdown (Bateson 1909, Dobzhansky 1937, Muller 1958).

In addressing the separation, or divergence of populations, the allopatric (geographic) model begins with the organisms in one individual population (Figure 1A). The population experiences variation in phenotypes (traits) and genotypes (specific allele combinations) but that variation does not preclude organisms from interbreeding—they are one species (Figure 1A). Due to extrinsic isolating factors: geological processes, environmental processes, range expansion, or migration, the

initial population splits into two (or more) subpopulations (Figure 1B). Initially, these two subpopulations are comprised of the same individuals that the ancestral population was composed of. As the time since original separation increases, multiple generations pass.

As time passes, based on differences in environments or "founder effects", in which the allele frequencies of founding migrant populations set create the mean for traits as the population goes forward, the populations will diverge in both phenotype and genotype, becoming geographically, phenotypically, and genetically distinct (Figure 1C). After the divergence of the population, the new strains have become so uniquely adapted to their new environment that they are no longer capable of interbreeding (Figure 1D).

A. Single Interbreeding population → B. Geographical Separation is introduced → C. Divergence and Fixation of new traits → D. Reproductive Isolation
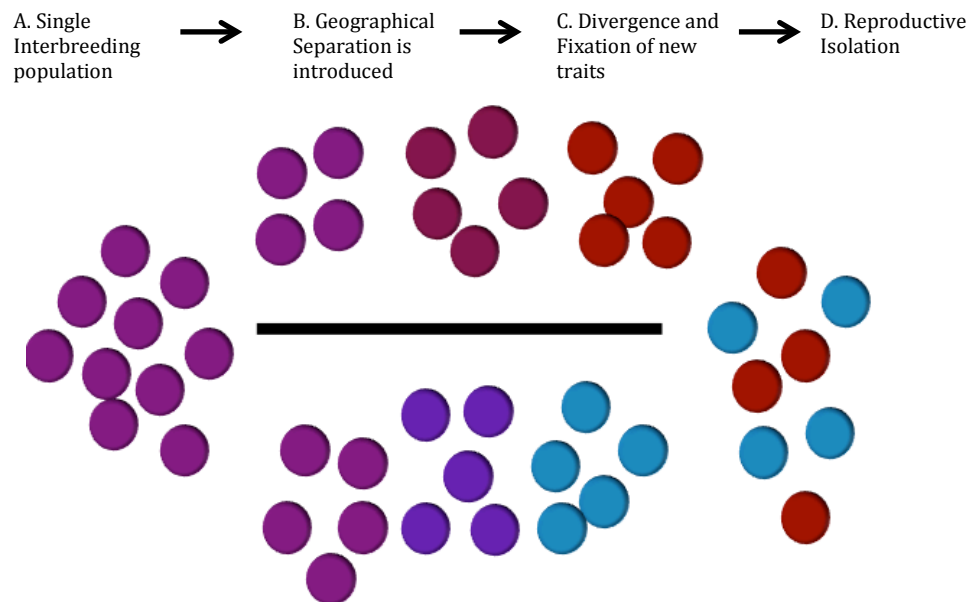
Figure 1. Allopatric effects leading to the build-up of intrinsic post-zygotic isolating barriers.

Incompatibilities are implicit in the growing degrees of differentiation between the subpopulations. While the accumulation of incompatibilities (expressed as differences)

does not cause depressed fitness in their own populations they may cause decreases in compatibility between the populations. Because new phenotypes and genotypes have arisen separately in both populations, they have never had the chance to interact within a common genome and therefore may be antagonistic when the new combinations of alleles are expressed together (Bateson 1909).

Following the allopatric separation of populations in Figure 1D, the BDM model predicts that when considering inbred (populations that are assumed to be genetically identical through generations of crossing within the population) parental strains, the F2 hybrid offspring will be incompatible due to novel allele combinations. In order to understand the interactions of novel allele combinations, it is important to know that every individual has a specific genome (the sum of their individual DNA) that displays specific genes. Each gene, linked to a specific trait or function, has its own specific place on the chromosomes called a locus. At each locus (or the pair of loci), in organisms that have two sets of chromosomes (diploid organisms), there are two alleles (forms of the gene); one on each homologous (pair) chromosome. One allele is contributed from the paternal background from a haploid (one chromosome) sperm and one is contributed from the maternal background from the haploid egg. In forming the haploid gametes (sex cells) that fertilize to produce the offspring, parental genetic material goes through the process of meiosis (the process of creating haploid cells, Figure 2).
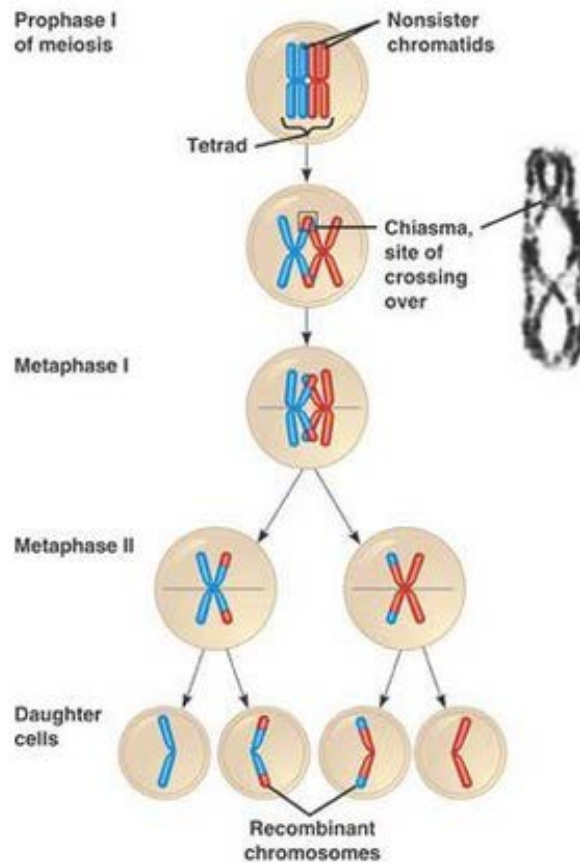
Figure 2. The process of meiosis as the homologous chromosomes undergo crossing-over and consequently produce novel allele combinations (Image source: http://year12biologyatsmc.wikispaces.com/Crossing+Over).

During meiosis, the parent's genetic material undergoes crossing-over and recombination, in which genes between homologous chromosomes are exchanged and re-configured into new arrangements along the chromosomes in order to create greater genetic diversity in the offspring—these processes result in novel allele combinations (Figure 2, Futuyma 2013).
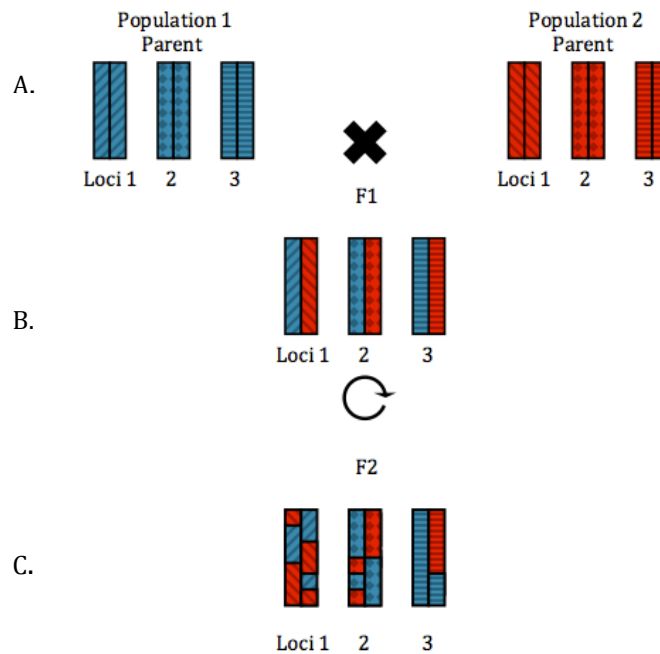
Figure 3. The processes of crossing-over and recombination creating novel allelic interactions between populations of unique evolutionary backgrounds. Epistasis in the F2 generation causes incompatibility between novel allele combinations.

Following hundreds of generations of inbreeding within their respective populations, the blue and red populations from Figure 1 have evolved distinct genomes both from their once ancestral genome, as well as from each other's genomes. As seen in Figure 3A, at loci 1, 2, and 3, the red population and the blue population evolved different allele combinations. Also note that each locus presented is homozygous (display the same alleles) due to the generations of inbreeding. After generations of inbreeding, the individuals within the populations display effectively identical genetic material. In this parental generation only the evolved allele combinations are present, when offspring are produced within the inbred populations, effects of recombination are negligible since the loci are homozygous.

When individuals of the parental population are mated they produce F1 (first generation) offspring (Figure 3B). Each parent contributes only half of their genetic

background, in order to maintain diploidy, thus, each locus on each chromosome of the F1 offspring will be comprised of 50% Population 1 background (blue allele) and 50% Population 2 background (red allele, Figure 3B). At this stage offspring are heterozygous (have different allele combinations) at each of the three loci. However, because the blue alleles and red alleles arose separately, they have not yet had a chance to interact. In producing F1 offspring, recombination occurs in the parental generation and therefore does not create a chance for interaction between the two separately derived backgrounds.

When individuals from the heterozygous F1 population are mated to produce F2 (second generation) hybrid offspring, during meiosis in the F1 parents, the process of crossing-over causes the mixing and recombination of the uniquely derived alleles (Figure 3C). When novel alleles combinations are produced they have the chance to interact epistatically (when one gene may be affected or moderated by the presence of one or more other genes, epistasis). Since the red and blue allele combinations arose separately and have yet to be subjected to natural selection when present in a combinatory manner, there is a high chance that the alleles will interact negatively and may cause vital developmental processes to malfunction, resulting in hybrid inviability (Bateson 1909, Fierst & Hansen 2010). Based on the BDM model, by looking for hybrid inviability or depressed hybrid fitness (specifically in the F2 generation), one can determine the extent to which two populations have become incompatible or speciated.

Implications of the BDM model based on Mendelian Genetics

As illustrated in Figure 3, the genetic incompatibilities upon which the BDM model of speciation relies are not clear until the hybrid generations are produced. In

10

order to gain a deeper understanding of how separately successful allele combinations can be deleterious in hybridization we apply the model of Mendelian inheritance. Based on Mendel's Law of Segregation, it is understood that the alleles for each gene segregate so that each gamete produced only carries one allele for each gene, and thus, due to the Law of Independent Assortment, the genes for different traits can segregate freely during gamete production, leading to a wide range of variation in the offspring (Blumberg 1997). These Mendelian laws parallel the processes occurring in meiosis. As we follow Mendel's laws of inheritance it then becomes possible to understand how incompatibilities are presented due to novel allele combinations. Similar to Figure 3, accumulation of hybrid incompatibilities can be considered using a Mendelian inheritance approach to understand varying levels of fitness in the parental, F1, and F2 generations.

In applying a model of Mendelian inheritance, once again we will begin by considering two inbred, and thus effectively genetically identical but separate parental strains. Again, because populations are inbred, they will display a high degree of homozygosity, in this example, we consider two loci: A and B. Population 1 is $A_1A_1B_1B_1$ and Population 2 is homozygous $A_2A_2B_2B_2$. In this scenario A_1 and B_2 will represent recessive deleterious mutations. Each population displays one of the recessive deleterious mutations. In considering the fitness of the parental generation, it is reasonable to conclude that fitness may be slightly depressed when compared to populations and individuals with higher genetic diversity. The likelihood of depressed fitness is linked to inbred populations having higher degrees of homozygosity. As homozygosity increases in inbred populations, the instances of recessive deleterious

mutations being displayed also increases, and thus, fitness drops in comparison to more heterozygous populations. Following meiosis in the parental generation, haploid gametes are formed. The double homozygous nature of the parental genotype causes the production of four possible gametes, each displaying the same genotype (Figure 4).

$$A_1A_1B_1B_1 \qquad x \qquad A_2A_2B_2B_2$$

$$A_1B_1 \quad A_1B_1 \quad A_1B_1 \quad A_1B_1 \qquad\qquad A_2B_2 \quad A_2B_2 \quad A_2B_2 \quad A_2B_2$$
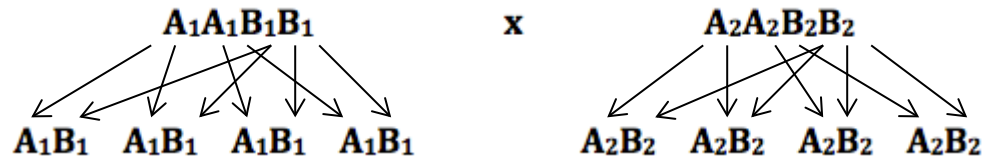
Figure 4. Inbred parental lines and possible genotype combinations. Same genotypes are created regardless of segregation.

Following fertilization of the egg the resulting F1 generation is, in opposition to the parental lines, completely heterozygous: $A_1A_2B_1B_2$. Each locus has half of its genetic material from parental population 1 and half from parental population 2. Since A_1 and B_2 represent recessive deleterious mutations, the heterozygous F1 generation does not display the recessive mutations and will have higher total fitness than either of the homozygous parental lines.

In segregating the F1 genotype into possible gametes, due to crossing over and recombination there are a total of four gametic genotypes (Figure 5). The four possible gametes produced by the F1 hybrids can combine to form nine separate genotypes (Figure 5).

F1 Gametes	A_1B_1	A_1B_2	A_2B_1	A_2B_2
A_1B_1	$A_1A_1B_1B_1$	$A_1A_1B_1B_2$	$A_1A_2B_1B_1$	$A_1A_2B_1B_2$
A_1B_2	$A_1A_1B_1B_2$	$A_1A_1B_2B_2$	$A_1A_2B_1B_2$	$A_1A_2B_2B_2$
A_2B_1	$A_1A_2B_1B_1$	$A_1A_2B_1B_2$	$A_2A_2B_1B_1$	$A_2A_2B_1B_2$
A_2B_2	$A_1A_2B_1B_2$	$A_1A_2B_2B_2$	$A_2A_2B_1B_2$	$A_2A_2B_2B_2$

Grey= Homozygotes. All other crosses are at least partially heterozygous

Figure 5. F1 gametes and the possible F2 genotypes.

As homozygosity reappears in the F2 generation so does the expression of the recessive deleterious mutation. Based on Mendelian inheritance, homozygosity arises in both double homozygotes (grey) as well as in instances of partial homozygosity. The effects of the deleterious mutation caused by A_1 and B_2 can be seen in either genotype where A_1A_1 or B_2B_2 are present. In one case, A1A1 B_2B_2, this causes a double mutant. Due to the reappearance of homozygosity and the case of the recessive mutations, it would be expected that F2 hybrid fitness would be lower than the heterozygous F1 hybrid generation, but higher than the completely homozygous parental genotype. However, the effects of deleterious recessive mutations are not the only possible hybrid incompatibilities.

In adding another layer of possibly deleterious interactions leading to the build-up of incompatibility, we consider epistatic gene-gene interaction. As explained in conjunction with Figure 3, epistatic interactions occur where one (or many more) gene(s) influences another's fitness or function. In terms of total fitness this requires consideration of not just single recessive deleterious mutations, but an entire host of possibly interacting genes.

Returning to the initial inbred parental populations, $A_1A_1B_1B_1$ and $A_2A_2B_2B_2$, we layer epistatic effects on top of the previously outlined recessive deleterious

13

mutations. In this case, in order for A_1 to function B_1 must be present, and for A_2 to function B_2 must be present. Thus, in the inbred parental generation although there is a decrease in fitness due to the presence of the expressed recessive deleterious mutations, the genotype is viable because B_1B_1 is present with A_1A_1 and likewise B_2B_2 is present with A_2A_2, thereby enabling its function epistatically.

In the F1 generation, $A_1A_2B_1B_2$, homozygosity is lost, and with it, the effects of the recessive deleterious mutations. Additionally, although homozygosity is lost, both A_1 and A_2 are enabled to function by the heterozygous pair B_1B_2. Following F1 segregation and F2 recombination there are a variety of possible F2 offspring and interactions (Figure 6).

F1 Gametes	A_1B_1	A_1B_2	A_2B_1	A_2B_2
A_1B_1	$A_1A_1B_1B_1$	$A_1A_1B_1B_2$	$A_1A_2B_1B_1$	$A_1A_2B_1B_2$
A_1B_2	$A_1A_1B_1B_2$	$A_1A_1B_2B_2$	$A_1A_2B_1B_2$	$A_1A_2B_2B_2$
A_2B_1	$A_1A_2B_1B_1$	$A_1A_2B_1B_2$	$A_2A_2B_1B_1$	$A_2A_2B_1B_2$
A_2B_2	$A_1A_2B_1B_2$	$A_1A_2B_2B_2$	$A_2A_2B_1B_2$	$A_2A_2B_2B_2$

Blue= Homozygotes
Purple= Negative epistasis

Figure 6. Possible F2 genotypes and negative interactions in terms of recessive deleterious mutations and epistasis.

When Figure 6 and Figure 5 are compared it is clear that considering negative epistatic effects between just two loci adds a significant amount of negative interaction. Accounting for the breakdown of epistasis following recombination in the F2 sharply increases the probability that there will be negative interactions present in the F2 genotype. In terms of comparative fitness in this scenario if both recessive deleterious mutations and negative epistasis were present and operating, the F2 fitness would be

significantly lower than the F1 (completely compatible) hybrid fitness as well as lower than the inbred parental fitness due to the added negative epistasis.

Up to this point this Mendelian two-locus model has been utilized to consider interactions and incompatibilities present among autosomal cells rather than sex cells. Therefore, in previous consideration, the sex of the allele contributor or receiver has been inconsequential. However, if deleterious mutations or negative epistatic effects arise on either of the sex chromosomes there can be differing outcomes for males as compared to females. Considering the sex chromosomes of females (XX) and males (XY or X in some species) it can be observed that regardless of the model of sex chromosome, XX or XY/X, the female has greater X compensation, and therefore protection in terms of deleterious interactions due to the complementation provided by the second X chromosome. As a female contains two X chromosomes, if there are deleterious effects present on one chromosome but not the other, the more compatible X chromosome will overshadow the deleterious effects. However, in males, where the sex chromosomes are displayed as either a heterogametic pair of XY sex chromosomes or a single X chromosome, there is only the single X chromosome with no potential complementation in the face of deleterious incompatibilities. For this reason, high degrees of incompatibilities, or alternately, incompatibilities centered on the sex chromosomes will produce skewed fitness results with higher female, and more highly depressed male fitness. Extreme cases of this phenomenon result in total hybrid male lethality. This phenomenon is known as Haldane's Rule and is considered one of the markers of intense incompatibility or speciation (Johnson 2008, Orr 1993).

Although simplified, such Mendelian inheritance models can provide powerful analysis in terms of connecting observed depression of fitness with possible interactions (deleterious recessive mutations or a degree of epistasis) based on patterns of appearance and severity of fitness depression.

Quantification of Genetic Incompatibility—Phenotypic Indicators

As previously explained, incompatibility expresses itself through hybrid inviability leading to both post-zygotic and pre-zygotic isolation. However, in order to determine and compare degrees of speciation between different populations, there must be a way to quantify incompatibility.

Survivorship (percent of individuals hatched from eggs), lifetime female fecundity (number of offspring a female produces in her lifetime), and offspring sex-ratios (percent of males in the offspring population) are also useful measures of incompatibility (Cutter, et al. 2006, Cutter, et al. 2012, Dey, et al. 2014). All three of the measures listed can be considered measures of fitness, or an organism's general ability to successfully survive, reproduce, and thrive in its environment. Increases in incompatibilities will be linked to decreases in total organismal and population fitness. By comparing and analyzing these metrics between F1 and F2 populations one can determine decreases in fitness due to F2 breakdown (Figure 3C).

Survivorship compares the number of eggs laid to the number of individuals that fully develop and hatch. Survivorship is expressed as a percentage: a high value expressing relatively high fitness and viability and a low value expressing low viability. When crossing two incompatible inbred populations, it would be expected that the F1 hybrids would display higher percent survival as compared to their inbred parents as

gametic interaction through fertilization has occurred and heterozygosity has returned. However, following crossing-over and recombination when mating F1 hybrids, the F2 individuals would be expected to have a lower percent survivorship than either the F1 or parental populations due to epistatic interaction on top of possible recessive deleterious mutations. By comparing the number of hatched individuals to the number of eggs originally laid, one collects information on the percentage of eggs that were successfully fertilized and developed. Evidence of post-zygotic RIMs is presented by low survivorship percentages indicating explicit incompatibility in gamete development, fertilization or embryonic development.

Female fecundity is a measure of the total viable (hatched) offspring that a female produces in her lifetime. This data is collected by counting the total number of offspring every female in the study population has over her lifetime. High fecundity is indicative of high fitness. Lower fecundity could be caused by a variety of factors: females could produce less offspring due to general lower health, due to the male's general lower health and inability to fertilize the egg, due to a male's decreased mating efficiency, or due to low offspring survivorship. In turn, any of these possibilities could be caused by a number of deleterious mutations or epistatic interactions. Comparing female fecundity side-by-side with the previously discussed survivorship percentages provides a powerful analysis of general fitness.

Skewed offspring sex ratios may be indicative of incompatibilities present on sex chromosomes and can cause post-zygotic RIMs. In a population of obligate out-crossers (organisms that must mate with another individual) of males and females, a healthy population is comprised relatively equally of males and females. In the case of

high genetic incompatibility, or incompatibility arising between sex chromosomes, it is common to see a decrease in the ratio of the heterogametic (male) sex (XY) to homogametic (female) sex (the sex in which only one form of sex chromosome is expressed: XX). A decrease in the male to female sex-ratio not only indicates genetic incompatibilities, it also creates a RIM. By decreasing the number of males available for reproduction, the population is limited in its ability to reproduce and further its lineage. Comparison of these phenotypic indicators of fitness provides both a general sense of the level of incompatibility as well as insight into possible genetic causes for the incompatibilities.

Incompatibilities and Geographical Distance

In keeping with the DBM, there must be an accumulation of incompatibilities during a period of separation due to an external barrier (Dobzhansky-Muller Incompatibilities—DMIs) to cause the eventual event of speciation (Orr 1995, Presgraves, 2010). As predicted by the DBM, DMIs arise and increase in number between separated populations over time. Allen Orr went a step further and proposed a rate for the accumulation of these DMIs. He proposed that incompatibilities arise much faster than linearly with time, and specifically that the probability of speciation between two populations increases at least as fast as the square of the time since separation (Orr, 1995). Incompatibilities arising linearly with time would be a constant relationship between time of separation and novel incompatibility whereas Orr's theory would propose an exponential growth type of relationship. As the time of separation increased the populations would become more and more different, and therefore accumulate more

and more incompatibilities at an increasing rate. While Orr's theory is both appealing and logical, it has yet to be definitively and repeatedly upheld by evidence.

Purpose

It is the purpose of this experiment to expand upon the present knowledge of the processes of speciation. Rather than utilizing only known incompatible, interbreeding species to observe the process, we utilize both geographically distinct strains that are known to successfully interbreed within their same species, as well as a strain of a recently separated species to determine if emerging reproductive isolation within species can be detected. By mating females of each of four strains with males of each of the four strains, and vice versa, possible male or female effects may be seen in the resulting fitness metrics. Carrying out a multigenerational matrix of crosses allows for the comparison of parental, F1, and F2 fitness to determine possible underlying accumulating incompatibilities. By using multiple geographically separated strains of the same species, as well as a geographically and species distinct strain, this experiment will provide data that elucidates the underlying levels of incompatibility within a species and insight into how these incompatibilities may accumulate prior to and following speciation.

Hypotheses

Phenotypic indicators of incompatibility between geographically separated populations of *Caenorhabditis remanei* and *Caenorhabditis latens* (see "Model Organism") will display severe incompatibility. Phenotypic indicators of incompatibility between geographically separated populations of *C. remanei* will

display lower levels of incompatibility than that of between species crosses yet similar

markers of incompatibility are expected based on observations in preliminary crosses.

Across all observed incompatible crosses there will be a stronger effect of

incompatibility observed in the male due to the lack of complementation when

considering sex chromosomes.

Model Organism

The soil-dwelling nematodes of the genus *Caenorhabditis* have become

increasingly popular to use as model organisms in the field of evolutionary biology.

Their rise in popularity in the field began with the sequencing of the genome of

Caenorhabditis elegans—the first multi-cellular organism to have a fully sequenced

genome (The *C. elegans* Sequencing Consortium, 1998). Closely related

Caenorhabditis remanei's genome followed soon after.

Figure 7. *C. remanei* female (left) and male (right) mating. The worms are ~1 mm in
length (Image courtesy of Nadine Timmermayer).

This experiment utilizes *C. remanei* (and the closely related *C. latens* from China) as

model organisms for a variety of reasons. The fully sequenced, publically available

genome is a huge benefit in terms of future genetic analysis as analysis can be cross-

referenced to the sequenced genome to determine whether there is evidence for

'speciation' genes (specific loci in which incompatibilities consistently arise across multiple crosses). The wide geographical distribution of *Caenorhabditis* allows for the identification and collection of a variety of geographically dispersed populations (Kiontke & Sudhaus 2006). The high degrees of genetic polymorphism paired with their short lifespans (3-4 days to reach sexual maturity) make it possible to create highly inbred, genetically homogenous lines in a short amount of time. These lines of *C. remanei* and *C. latens* then can be frozen at -80°C and years later can be thawed back to life and utilized in a variety of experimental settings. Their small size (~1 mm, Figure 7) and ability to be frozen for extended periods of time with minimal effects to their fitness, allows for the storage of a wide variety of hundreds of strains and generations of nematodes.

Another benefit to using *C. remanei* and *C. latens* in this experiment is their shared identity as gonochoristic (male or female) obligate out-crossers (Figure 7, Wormbook). The presence of sexually reproducing males and females allows for two sets of crosses to be conducted for each set of strains (between the male and the female, and then between the female and the male) and offspring to be easily sexed. These characteristics facilitate the feasibility of large-scale experiments (both in terms of generation and number of replicates) that are integral for the collection of data on evolutionary trends.

Formal Introduction

Key to understanding the ongoing diversification of life on the earth is an understanding of the process of speciation and the accumulation of incompatibilities between diverging species. Recent studies focused on uncovering the process of speciation in *Mus musculus* and *Caenorhabditis remanei* have utilized phenotypic and genetic approaches side by side in order to gain insight into the underlying mechanisms based upon hybrid Dobzhansky-Muller incompatibilities (Turner, et al. 2014, Dey, et al. 2012, 2014).

Turner, et al.'s 2014 studies of *Mus musculus*, the common house mouse, focused on bringing clarity to the complexity of hybrid incompatibilities. Using genetic analysis and a conditional mapping approach they identified candidate pathways and interactions for loci contributing to commonly observed hybrid sterility (Turner, et al., 2014). While *Mus musculus* presents itself as a model organism for studying the complexities of the pathways causing Dobzhansky-Muller incompatibilities, the nematode *Caenorhabditis remanei* presents itself as an even stronger candidate. With a well-known, simpler genome *C. remanei* not only provides a fully-sequenced and available genome for reference, but also, given its life history characteristics, makes feasible a large-scale, multiple replicate study in order to pinpoint root causes of incompatibility (Dey, et al. 2012, 2014). It has become clear that these modern techniques of analysis are better able to tease apart the complexities of the process of speciation: the 2014 study going as far as to identify gonad morphogenesis as the cause for the hybrid male sterility driving the process (Dey, et al., 2012, 2014).

However, both previous studies examine speciation retroactively using recently speciated populations in their crosses to attempt to uncover the underlying mechanism driving the isolating incompatibility.

Driven by these advances, as well as the lack of information concerning the early stages of speciation or ongoing accumulation of incompatibilities, the focus of this study is to provide a multi-cross, multi-generational analysis of the degree of speciation by hybrid incompatibility between three geographically isolated populations of *C. remanei* and recently speciated *C. latens*. This experimental approach allows for the degrees of incompatibility between crosses to be quantified and compared through the use of phenotypic measures of fitness that can then be verified in future study by genetic analysis aimed at uncovering the causes behind the observed variation in hybrid incompatibility.

Allen Orr theorized that incompatibilities arise and accumulate at a rate that is faster than supposed by a linear model based on their distances of geographical separation (Orr 1995). Although other attempts have been made to address Orr's "snowball theory", they failed to support his theory (Matute, et al. 2010). While a lack of known biogeographic history in *C. remanei* prevents us from examining the validity of Orr's theory, by using three geographically distinct strains of *C. remanei* we can determine the feasibility of the theory by determining whether discernable patterns of incompatibilities arise among within species crosses.

It is hypothesized based upon observations of preliminary crosses that phenotypic markers of incompatibility between geographically separated populations of *Caenorhabditis remanei* and *C. latens* will display varying degrees of accumulation of

incompatibilities. It is hypothesized that a pattern of more highly evident

incompatibility will be observed when males of more highly diverged populations are

used in crosses, thus, reciprocal crosses will be unbalanced in their display of

incompatibility.

Methodology and Experimental Design

Worm Maintenance

We used three strains of *Caenorhabditis remanei* (PX439, PX506 and PX356) and recently identified *C. latens* strain PX534. All strains are hereby referred to by their geographical origin or species name, thus PX439—Ohio, PX356—New York, PX506— Ontario, and PX534—*C. latens* from China. Strains were maintained at 20°C on 100mm Petri plates with NGM-Lite agar media seeded with *Escherichia coli* strain OP50 (Stiernagle 2006). Every 3-4 days worms were transferred to new population plates by chunking in order to prevent overcrowding, contamination, and starvation. Prior to use in crosses the strains were cleaned and age-synchronized using the standard hatch-off procedure of bleach and sodium hydroxide (Stiernagle 2006). Crosses were set up on 35 mm Petri dishes with a central spot of *E. coli* OP50 using a wire pick (Stiernagle 2006).

Phenotypic Data Collection via Crosses

Genetic incompatibility was determined and quantified through the collection of phenotypic data from "pure crosses", F1s, and F2s. In cases in which F1 hybrids were too incompatible to produce F2 hybrids, backcrosses were performed and the resulting offspring were then crossed with their siblings. Data from inter-strain crosses were compared to intra-strain crosses, and data from within species crosses were compared to that of between species crosses. Each cross consisted of twenty replicates with one L4 (sexually mature) virgin female and one L4 male (Figure 8). The worms were left to mate and lay eggs for 24h at 20°C before being transferred together to a new plate. On

replicate plates where the male died, the male was replaced with another male of the same genetic background and generation in order to continue to collect female fecundity and hybrid offspring data.
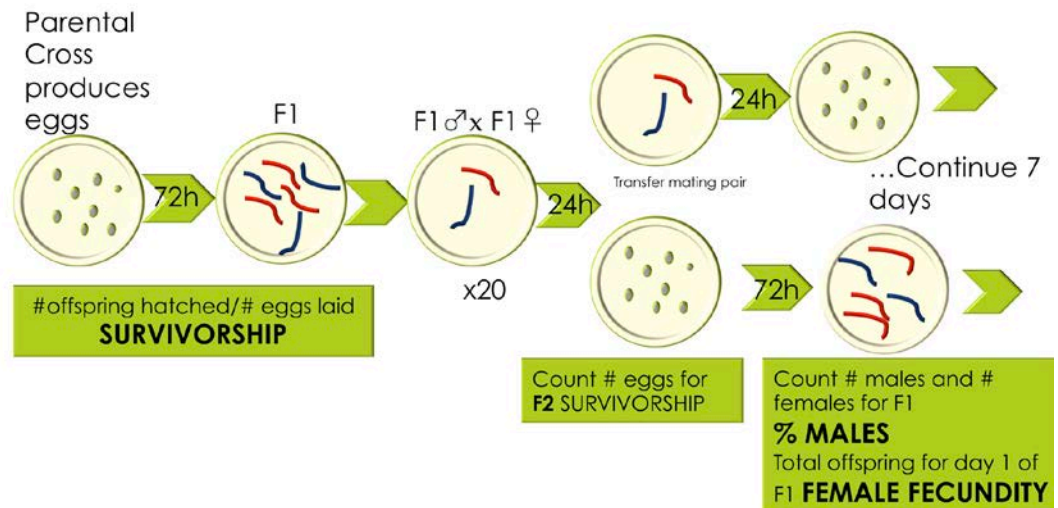

Figure 8. Twenty pair-wise crosses were set up (blue=male, red=female). The mating pair is transferred to a new plate after 24h, the eggs on old plate are counted and the old plate is saved. In 24h the pair is transferred again and eggs are counted again (repeats 7 times) and after 72h the male and female offspring that have hatched from the egg plates are counted.

In the case of female death, the replicate was excluded from further data collection and analysis. The process of transferring continued for five to seven days, at which point at least half of the replicates had been discontinued due to death. At the point of data analysis there were at least five replicates for each cross and up to thirty-five replicates for some crosses.

Following pair transfers, eggs were counted and recorded on each plate using a grid system and a basic hand counter. Plates with eggs were maintained at 20°C for an additional 48h before counting hatched offspring using a dual tally counter to discriminate male and female offspring counts (Figure 8). After the F1 offspring from

the parental cross matured to L4, they were crossed in an identical manner to the parental crosses previously described. Likewise, the F2 offspring were crossed following maturation. Parental, F1, and F2 crosses were conducted in all possible combinations (Figure 9). All egg and offspring counts were utilized to calculate survivability (the percent of individuals that hatched from eggs), total female fecundity (the number of viable offspring a female produced over the course of her life), and sex-ratios (the percent of male or X offspring in the population).

Figure 9. All possible combinations of crosses: parental/pure-crosses, F1s, and F2s.

Results

Across a series of sixteen two-generation crosses, we explored the degree of

hybrid incompatibility due to recessive deleterious effects, epistatic interactions, and X

chromosome effects. As proxies for fitness, we measured the percentage of individuals

that successfully hatched from eggs as a measure of each generation's survivorship,

lifetime female fecundity of each generation based on number of offspring, and each

generation's offspring sex ratios in terms of the percent of male offspring. Each of these

metrics was chosen as an indicator of the degree of hybrid incompatibility using the

data from the parental lines for background comparison of fitness. Measures are

reported in standard box plots with lines indicating the minimum, median, and

maximum as well as outliers. Statistical significance was calculated by a series of one-

way comparisons using non-parametric analysis.

Male/Female effects

Survivorship

Parental cross data for all three metrics was collected in order to assess differing

contributory effects of the male and female genetic background to the fitness of the

hybrids. Considering parental survivorship, within-species reciprocal crosses displayed

similar levels of fitness (Figure 10). Excluding the cross with Ontario males, which

displayed significantly lower survivorship (Z= -3.8197, p=0.0001), between-species

crosses utilizing *C. latens* females displayed similar levels of survivorship when

compared to *C. remanei* within-species crosses. Between-species crosses utilizing *C.

latens* males showed significantly depressed rates of survivorship from the within-

species crosses as well as the *C. latens* female hybrid crosses (Z=-3.5824 -3.1586, -4.2872, p = 0.0003, 0.0002, <0.0001).

Figure 10. Within-species reciprocal crosses show wide individual variation but consistency in overall fitness trends. Between-species crosses likewise show variation but fitness is higher when *C. latens* females are crossed with *C. remanei* males than in the reciprocal crosses.

Fecundity

Within-species crosses illustrate individual variation in number of offspring/female both in within- and in between-species crosses (Figure 11). There are no significant or consistent patterns found in terms of female fecundity when comparing between strains or between crosses. While a sample of crosses, *C. latens*/*C. latens* compared to *C. latens*/Ontario and Ontario/New York compared to *C. latens*/Ontario,

29

display significantly depressed fitness in relation to each other, these patterns are not

consistent across a wide range (Figure 8, p= <0.0001, 0.0046).

Female parent background

	hio	Ontario	*C. latens*

Fecundity

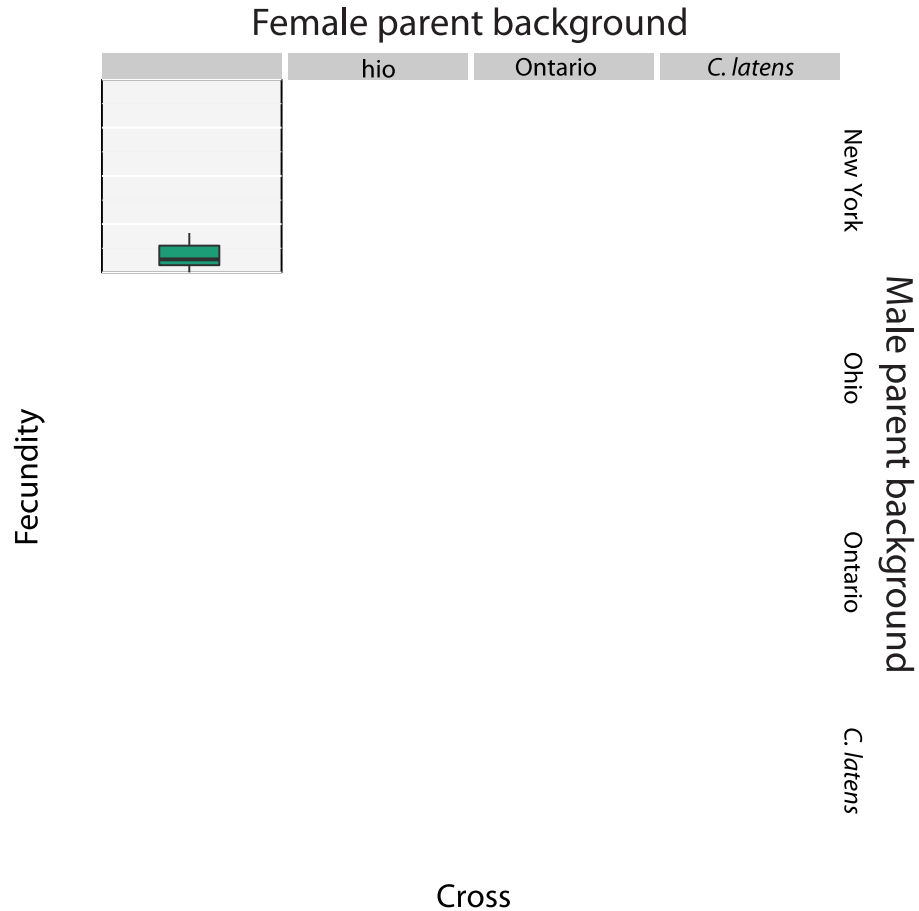Male parent background

New York

Ohio

Ontario

C. latens

Cross

Figure 11. Fecundity within species is consistent across reciprocal crosses in spite of
varying levels of individual variation. Fecundity in between species crosses is higher in
the female *C. latens* crosses than in the male *C. latens* crosses.

Percent Male Offspring

Considering the sex ratios of the parental cross offspring, only the Ohio

male/Ontario cross displayed significantly lower fitness than pure crosses or other

within species crosses (Figure 9, Z= -2.8546, p= 0.0043). When comparing between

species, all of the *C. latens* female hybrid crosses, with the exception of the increased

fitness of the *C. latens* female/Ontario male cross, displayed consistent sex ratios with

the within species crosses (Figure 9, Z= 2.5898, p=0.0096). When comparing the

percent of male offspring obtained in between species crosses utilizing the *C. latens*

male for within species crosses, all hybrid crosses displayed consistently lower fitness

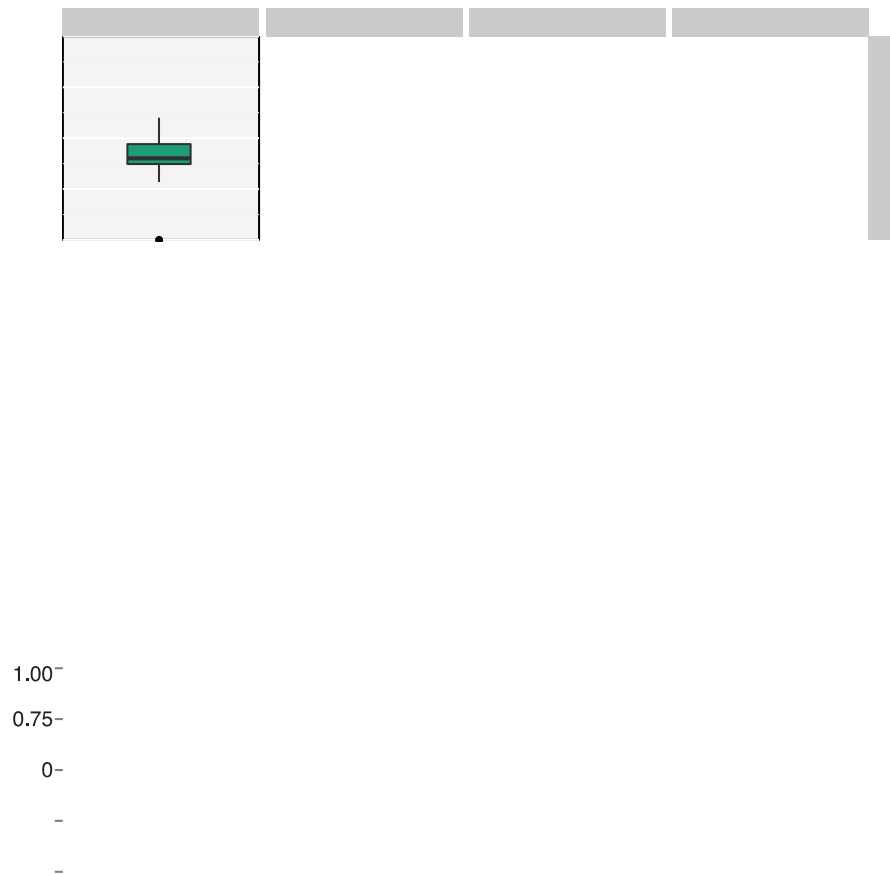(Figure 12, Z= -5.0797, -4.5933, -4.2890, p = <0.0001, <0.0001, <0.0001).

Figure 12. Low variation is seen in pure cross % male offspring. Higher variation exists in hybrid crosses. In within-species crosses reciprocal crosses are consistent. In between-species crosses, crosses with C. latens females and C. remanei males are consistent with fitness of C. latens pure cross. Between-species crossing using male C. latens are depressed in regard to each of the other crosses.
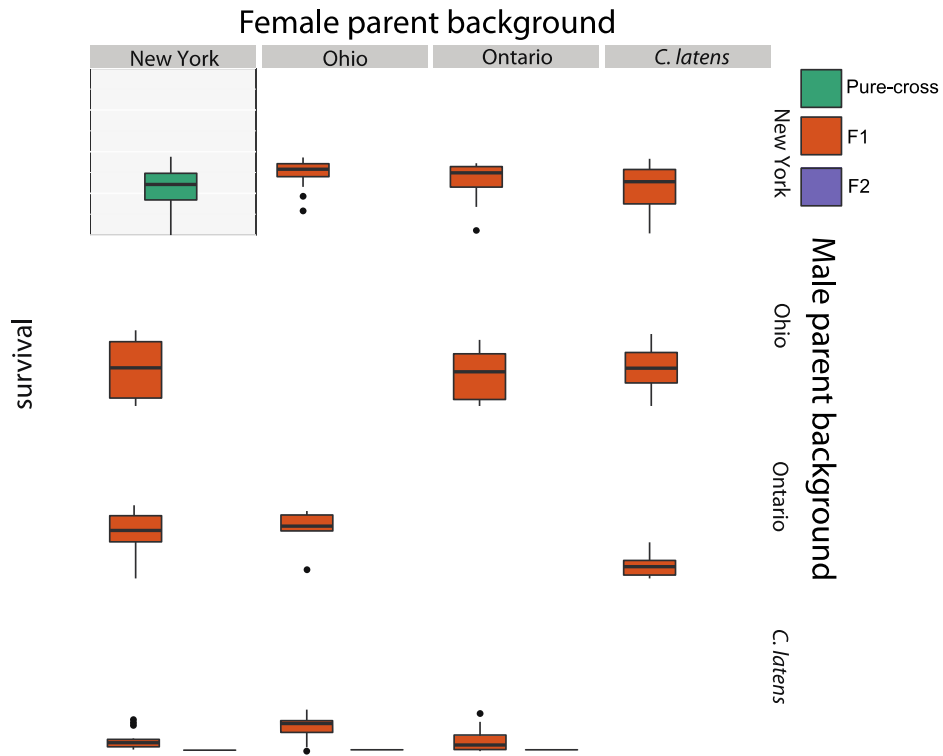
Survivorship

Figure 13. Determining survival of each generation in terms of percent of individuals that hatched from eggs. Survivorship shows higher variation among *C. remanei* crosses and significant incompatibility between *C. remanei* and *C. latens* crosses.

F1

Comparing within-species F1 survivorship to parental pure crosses, the New York male/Ontario female cross displays a significant increase in fitness compared with the Ontario parental pure cross (Figure 13, Z=-2.0274, p=0.0426). Reciprocal crosses indicate consistency and similar levels of fitness in crosses from regardless of parental background (Figure 13).

Between-species F1 survivorship is variable, and is more highly variable when *C. remanei* males are crossed with *C. latens* female (Figure 13). F1 survivorship is

depressed in comparison to the pure parental crosses in the between-species crosses when *C. latens* males are crossed with *C. remanei* females (Figure 13, Z=4.5174, 4.5304, 5.4938, p = <0.0001, <0.0001, <0.0001). In crosses of *C. latens* females with *C. remanei* males, F1 survivorship is depressed when comparing the Ohio and Ontario hybrid F1 survivorship to the *C. latens* pure parental survivorship (Figure 13, Z= 2.4537, 4.9788, p= 0.0110, <0.0001). Additionally, the F1 percent survivorship is significantly depressed when comparing hybrid crosses of *C. latens* males to hybrid crosses utilizing *C. latens* females when considering the New York and Ohio hybrid crosses (Figure 13, Z=-3.3875, -2.4681, p=0.0007, 0.0136).

F2

      In within-species crosses, F2 fitness was highly variable both among individuals as well as in comparison to F1 fitness levels (Figure 13). Crosses of New York male with Ohio or Ontario female produced F2 progeny that displayed significantly lower percent survivorship than the F1 generation (Figure 13, Z=2.2089, 3.3735, p=0.0272, 0.0007). In contrast, the F2 hybrid cross of Ohio males with Ontario females observed an increase in fitness compared to the F1 generation (Figure 13, Z=-2.6307, p=0.0239).

      In between-species crosses, F2 fitness was severely depressed in all crosses (Figure 13, Z=3.6584, 3.7431, 4.3076, p=0.0003, 0.0002, <0.0001). The F2 generation of crosses using *C. latens* males was nonexistent due to incompatibility effects discussed in "Percent Male Offspring" (Figure 13).
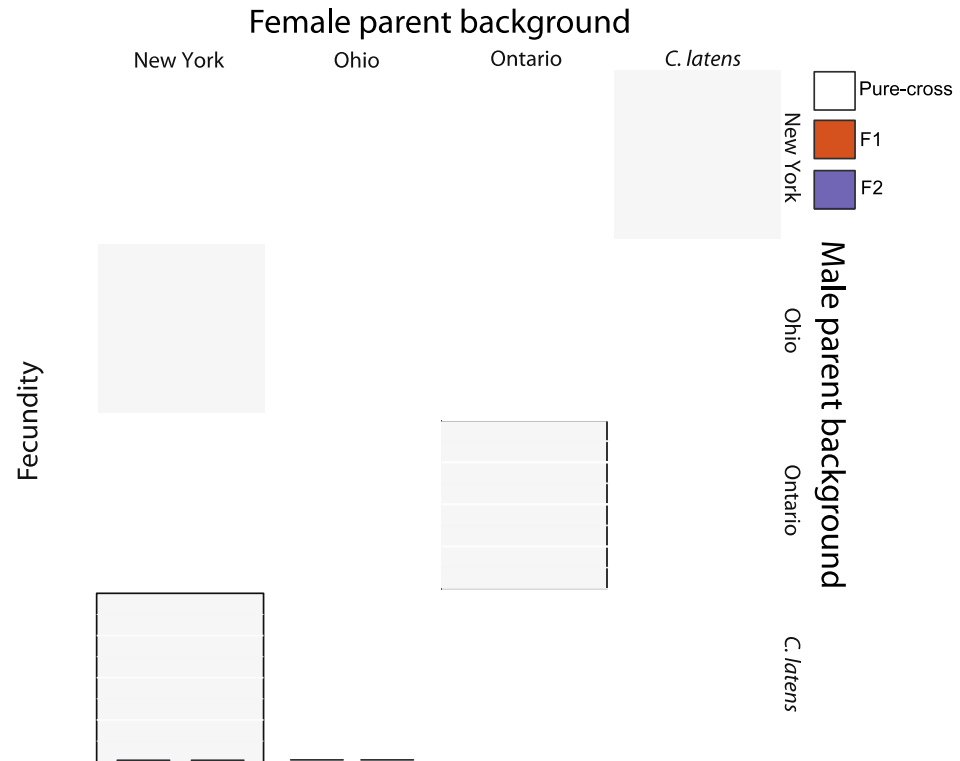
Fecundity

Figure 14. Lifetime fecundity for F1 individuals in within- and between-species crosses.

F1

In within-species crosses F1 fitness varies in its relationship to pure parental crosses. The New York/Ohio cross and its reciprocal display significantly increased fecundity in relation to the New York pure cross (Figure 14, p= <0.0001, 0.0005). Additionally, the Ohio/Ontario cross and its reciprocal each display a significant increase in fitness when compared to both the Ohio and the Ontario pure parental crosses (Figure 14, p=0.0003, 0.0002, <0.0001, <0.0001). The high degree of individual variability obscures any differences in central tendency between the remaining F1

34

crosses and pure parental crosses. There was no significant difference in fitness between crosses of *C. latens* females and *C. remanei* when comparing to pure parental crosses.

F2

When comparing the fitness of F2 crosses to pure parental crosses, variable fitness was observed with no consistent geographic trends. Of the within-species crosses, the F2 hybrid New York/Ohio cross and its reciprocal displayed significantly higher fecundity than the New York and Ohio parental crosses (Figure 14, p=<0.0001, 0.0009, 0.0011). Additionally, the F2 Ontario/Ohio fecundity is markedly higher than the Ontario parental background (Figure 14, p=0.0028). Considering F2 fitness in relation to F1 fitness, F1 fitness was higher between the Ohio/Ontario cross and its reciprocal (Figure 14, p=<0.0001, <0.0001). Individual variability in fecundity was substantial and showed no directional trends that were significant between F1 and F2 fitness.
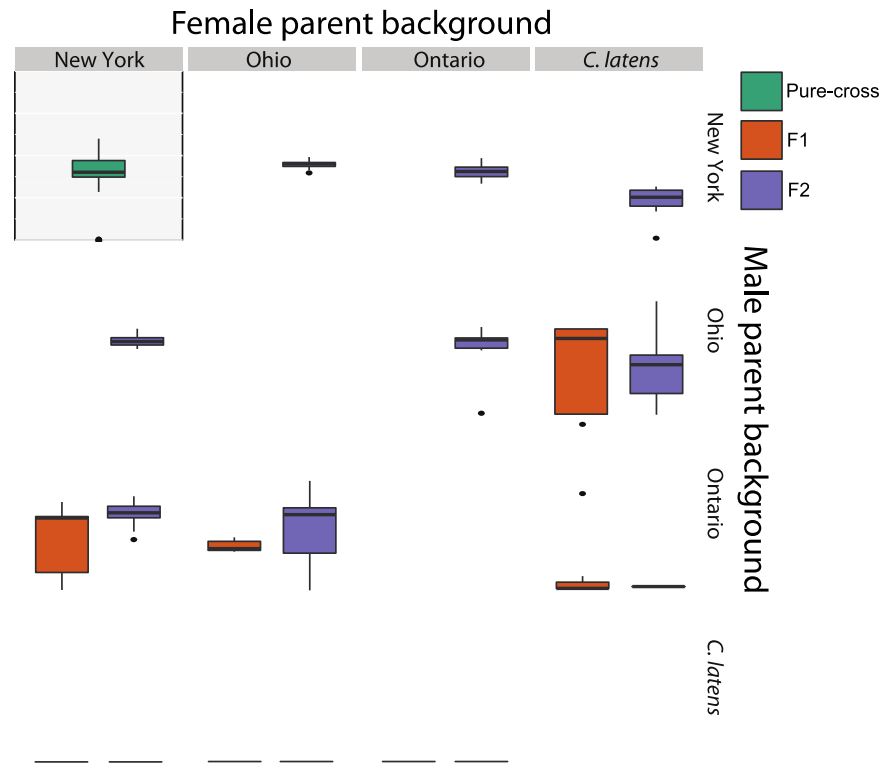
Percent Male Offspring

Figure 15. Variation in compatibility of strains as determined by the percentage of male offspring.

F1

In considering the percent of male offspring produced in parental background pure crosses, only the *C. latens* background produces a higher ratio of male to female offspring as compared to the New York and Ohio backgrounds (Figure 15, Z=2.8621, 2.4155, p=0.0042, 0.0157). In terms of F1 hybrid crosses, New York males crossed with Ontario females produce hybrids that have lower percent male offspring than the New York parental crosses (Figure 15, Z=1.9365, p=0.0528). Additionally, the F1 generations of both Ontario/Ohio crosses produce decreased male offspring sex ratios in comparison to the Ohio background cross (Figure 15, Z= 3.3082, 3.3082, p=0.0009,

0.0009). In addition to displaying a significantly decreased male to female sex ratio in comparison to the parental Ohio population, the Ohio/Ontario cross utilizing the Ohio male displayed significantly decreased male to female sex ratio in comparison to the Ontario/Ohio cross (Figure 15, Z=3.3082, p=0.0009).

In looking at between species crosses, there are no males produced by the F1 generation of *C. latens* males crossed with *C. remanei* females. The total male offspring lethality observed in the F1 generation makes the F2 hybrid generation impossible to set up. However, hybrid F1 crosses between *C. latens* females and New York and Ontario *C. remanei* males displayed significantly decreased male/female offspring sex ratios compared to New York and Ontario pure crosses as well as the China pure cross (Figure 15, Z=2.1308, 2.6306, 3.4043, 2.7303, p=0.0331, 0.0085, 0.0007, 0.0063).

F2

In terms of within species effects, only the F2 hybrid of the New York male /Ontario cross is significantly lower than the parental pure cross of Ontario (Figure 15, Z=2.0270, p=0.0427). The Ohio/Ontario cross has a significantly increased percent male offspring in comparison to its F1 generation (Figure 15, Z=3.3082, p=0.0009). Additionally, the Ohio male/Ontario cross, when compared to its reciprocal cross displayed an increase in percent male offspring (Figure 15, Z=3.3082, p=0.0009). Similar to the pattern in the Ohio/Ontario cross, the Ohio male/New York cross displays significantly increased F2 fitness when compared to the F1 fitness, and displayed a lower fitness in comparison to its reciprocal cross (Figure 15, Z=1.9365, -2.332, p=0.0528, 0.0197).

Examining trends in F2 fitness between species, both *C. latens* female crosses with Ohio and New York displayed significantly decreased fitness in comparison to the Ohio and New York pure crosses (Figure 15, Z=2.2708, 3.9113, p=0.0232, <0.0001). There were no significant trends between F1 and F2 fitness of *C. latens* female hybrid crosses (Figure 15).

Backcross

Figure 16. The results of backcrosses to *C. latens* males.

Backcrosses were conducted between *C. latens* males and F1 *C. latens/C. remanei* hybrid females. Backcross 1 was conducted with the F1 hybrid female and the *C. latens* male, Backcross 2 was conducted using males and females resulting from Backcross 1. Survivorship, fecundity, and percent male offspring were calculated for each (Figure 16). In all cases the Backcross 1 and 2 rescued fitness phenotypes to varying extents (Figure 16).

Discussion

In performing this series of multi-generational, reciprocal cross assays, the ultimate conclusions both strengthen and expand the existing narrative on speciation through accumulation of intrinsic post-zygotic incompatibilities. Data collected through measuring phenotypic indicators (survivorship, female fecundity, and percent of male offspring) of genetic incompatibilities over seven-day assays support the initial hypothesis that incompatibilities would be present at a quantifiable level between the four populations chosen. Additionally, the data support that not only is quantifiable incompatibility observed when considering hybrids of interbreeding species (between-species crosses), but also exists to varying extents when considering crosses made between populations of the same species.

Parsing the data collected from the three separate fitness metrics allowed us to consider the varied angles from which incompatibility and hybrid breakdown can be observed. Differing levels of incompatibility and hybrid breakdown were observed between strains, between generations, and between reciprocal crosses. The degree and patterns of incompatibility observed provide evidence for high levels of epistasis leading to hybrid breakdown in both the beginning stages of and completed process of speciation.

Effects due to Parental Background

Using the inbred lines of the gonochoristic obligate out-crossing species *C. remanei* and *C. latens* allowed us to analyze parental crosses to determine the contributory effects of males (X in the case of *Caenorhabditis*) and females (XX). It

40

would be expected that if there was dosage compensation (the hypothetical balance of parental genetic material), as predicted by similar experiments, the contributions of the male and female would be equal and reciprocal crosses would show consistent results (Baird 2002). However, if there was not dosage compensation it would be expected that the female (XX) would have a higher contributory factor than the male (X). However, because the male has only a single X chromosome, deleterious mutations are more frequently expressed because there is no complementary chromosome to potentially overshadow homozygous recessive traits. If this is the case, negative X effects would be expected to be stronger in the male direction.

When considering these effects we compared the initial reciprocal crosses within- and between-species crosses to determine if uneven parental contribution occurred causing differential hybrid offspring fitness. Across all three fitness metrics there was insufficient evidence to determine variable fitness in within-species reciprocal hybrids. However, when considering the reciprocal crosses of the between-species crosses, there was consistent evidence for decreased fitness when *C. latens* males were crossed with *C. remanei* females as opposed to when *C. latens* females were crossed with *C. remanei* males. This observation was significant when considering survivorship and percent male offspring. While there is an observable trend when considering female fecundity, individual variation is too great to afford significance.

This finding, of a greater degree of incompatibility and breakdown in one direction of a cross, particularly when considering between-species crosses is supported by a host of studies addressing the dominance theory and involvement of sex chromosomes when considering the accumulation of incompatibility. It is well known

that when considering hybrid breakdown and incompatibility an unevenness due to the dominance of the fitness of the heterogametic sex over the homogametic sex can be observed (Turelli & Orr 1995). As observed when considering hybrids created between *C. latens and C. remanei,* the cross utilizing the male *C. latens* showed consistently lower fitness, thus indicating an incompatibility due to effects from the X chromosome of the *C. remanei* female. These sex chromosome effects have been observed and studied in a host of different organisms (Coyne & Orr 2004, Dey 2012, 2014). Without considering the multi-generational fitness assays we cannot further determine the cause of the uneven fitness of reciprocal between-species crosses.

Within- and Between-Species Incompatibilities

In this section, by comparing fitness levels between generations we determine the degree of hybrid breakdown. We then go further; we compare the degree of hybrid breakdown between crosses and generations to make conclusions about degree of incompatibility within and comparatively across crosses. Although different metrics of fitness are utilized, common themes emerge that connect across fitness metrics as well as with existing research in the field. Each metric's main findings will be summarized before identifying common findings and synthesizing these findings in light of previous research.

Survivorship

Considering the percent survivorship in the parental, F1, and F2 generations, if incompatibilities follow the trends that would be hypothesized by the Mendelian Inheritance model, it would be expected that the F1 generation displayed higher

42

survivorship than inbred parental lines, and that the F2 generation displayed lower survivorship than either the F1 or parental lines. In cases of extreme incompatibility, the F1 survivorship might be lower than the parental lines; this would indicate high levels of epistatic effects.

The survivorship data illustrate generally similar levels of fitness in the within-species crosses over both the F1 and F2 generations. The F1 and F2 fitness is largely consistent with the parental crosses. These results indicate that while there is largely a lack of incompatibility within species, some incompatibility can be found in the exception of the New York male with Ohio or Ontario female which produced F2 progeny that displayed significantly lower percent survivorship than the F1 generation (Figure 13, Z=2.2089, 3.3735, p=0.0272, 0.0007).

When considering the between-species crosses the differential fitness levels displayed in the parental effects graphs carry over. While the F1 hybrids of *C. latens* females and *C. remanei* males display levels of survivorship similar to that of the within-species and pure crosses, the F1 hybrids of the *C. latens* males and *C. remanei* females display depressed survivorship. When considering the F2 hybrid fitness, depressed survivorship is observed in both between-species crosses, although the *C. latens* males and *C. remanei* females display more greatly depressed fitness.

Survivorship data indicates that while there is not a high level of incompatibility present in within-species crosses, there is evidence for some hybrid breakdown as evidenced by depressed F2 survivorship. Previous observations of X-specific effects are upheld as there continues to be uneven levels of fitness when considering the between-

species reciprocal crosses. When considering survivorship data as a whole, there is a large degree of individual variability in fitness.

Female Fecundity

The metric of female fecundity is indicative of incompatibilities connected with mating efficiency and fertilization. It was expected that, should incompatibilities be present, female fecundity would decrease, both due to decreased fertilization success (based upon previous research into gonad morphogenesis) and decreased offspring survival due to developmental defects.

Analyzing the data collected for female fecundity provides the first clear evidence of hybrid vigor in light of inbreeding depression. The female fecundity of the F1 generations of the New York/Ohio cross and its reciprocal, and the Ohio/Ontario cross and its reciprocal, is significantly increased when compared to the pure parental crosses (Figure 14). Unlike the survivorship data, there aren't as many clear patterns that emerge from the female fecundity data. Although significant decreases in F2 fitness are observed in the Ohio/Ontario cross and its reciprocal, there are no other significantly depressed levels of fecundity in within-species crosses. This lack of significance could be due, in part, to the high degrees of individual variation that was once again observed. The high degree of variation could also be due, in part, to the lack of significance when comparing levels of fecundity in between-species crosses to the within-species crosses. Although there is a visual trend in decreased fecundity in the *C. latens*/*remanei* crosses, and more severely decreased fecundity in the hybrid crosses with *C. latens* males, there is a lack of significance.

44

Percent of Male Offspring

Similar to the analysis of uneven parental background contribution, the analysis of the percent of male offspring present from hybrid pairs provides a measure of the degree of hybrid breakdown that can be attributed to X-effects.

Out of the three metrics, the least individual variability was displayed in the percent of male offspring. In terms of general trends, again there was evidence for depressed fitness in some of the F1 and F2 generations of the within-species crosses (New York/Ontario, Ohio/Ontario). Therefore, while it is not nearly as predictable or consistent, there is evidence for some hybrid breakdown in the within species crosses, indicating the beginnings of incipient speciation. When considering the between species crosses, the *C. latens* females crossed with *C. remanei* males again exhibited similar fitness to the within-species crosses (not indicating a large degree of breakdown). However, the F2 generations of these crosses exhibited significant breakdown. In contrast, and in line with the observed X-effects, the *C. latens* male between species crosses displayed total male lethality in the F1 generation. This total male lethality as a result of hypothesized X-effects is an example of Haldane's Rule.

In order to rule out any effects that might be due to the male background (perhaps faulty fertilization or epistatic effects causing breakdowns in development) we performed a backcross with the females produced in the *C. latens* male by *C. remanei* female cross crossed to a *C. latens* male. Any deleterious effects due to the paternal background would be displayed to a higher degree as the resulting offspring were 75% *C. latens* background. However, as observed in Figure 16, previously depressed or nonexistent measures of fitness were totally or partially rescued. These results indicate

45

that the extreme hybrid inviability in the *C. latens* male between-species crosses is due to either X-effects present in the female *C. remanei* background, or else due to some uncontrolled for mating inefficiency on top of deleterious interactions causing depressed male fitness.

Individual Variability

Based on the use of recombinant inbred lines of Caenorhabditis, it was expected that there would be relatively low individual fitness variability based on the assumption that genomes were widely identical within each line. However, in each metric analyzed a high degree of individual variability was observed. As mentioned previously, this individual variation may impede otherwise significant interactions. For this reason, it is integral to perform those crosses that displayed high levels of variability again in order to determine that these observations are repeatable.

However, the high degree of individual variation may in fact be due to *C. remanei*'s remarkable levels of nucleotide diversity as well as differing individual mating success stipulations. Although the populations used are highly inbred, *C. remanei* is known for being one of the most polymorphic species known (Cutter 2006). While generations of inbreeding may come closer to producing populations with effectively identical genomes among all individuals, in species with such high individual nucleotide diversity, even generations of inbreeding may not have fully reconciled the genomes. These remaining polymorphisms could be responsible for the high degree of incompatibility observed. Using genomic analysis, this assertion could be followed to determine if there are high standing levels of genetic polymorphism present between individuals.

Additionally, in a study that compared the fitness success of hybrid offspring of *Drosophila melanogaster* and *simulans* to their parents, it was observed that hybrid offspring displayed high levels of variation in terms of viability (Matute, et al. 2014). Matute and his collaborators determined that the cause of this variability was due to the parental genotype (Matute, et al. 2014). This suggests, as was presented previously, that there must be a standing level of genetic variation upon which the hybrid offspring fitness depends to display such a variation in fitness.

Haldane's Rule and X-effects

Arguably the most significant and consistent finding in this study is in regard to the consistent depressed, and in some cases nonexistent, fitness observed in the between-species *C. latens* male crosses. Based on what we know from countless existing studies of Haldane's rule and sex chromosome effects playing into the Dominance Theory, it is likely that the *C. remanei* X chromosome interacts highly deleteriously with the development of *C. latens* males (Turelli & Orr 1995, Coyne & Orr 2004). In the past five years, *C. latens* has been identified as an outlying species from C. remanei, initially due to patterns of nucleotide divergence (Dey 2012). As the study progress, even in the face of lacking inbred lines, severe hybrid incompatibility was observed to suggest Haldane's Rule (Dey 2014).

In the current study, *C. remanei* females were mated by *C. latens* males and although eggs were produced and laid, fecundity was severely depressed, and no males hatched. The lack of males following egg laying indicates that deleterious effects are connected to integral developmentary pathways—likely interacting with transcription factors driving development (Dey 2014). Existing studies utilized genomic analysis to

47

determine the likelihood of gonad morphogenesis as the main cause of the severely depressed hybrid generations (Dey 2014). Gonad morphogenesis would produce either sterile males, or males that are ineffective at mating.

While gonad morphogenesis may be a contributing factor to the depressed survival of hybrid offspring, males in particular, and depressed fecundity in these highly incompatible between species crosses, we suggest it is not the complete answer. Gonad morphogenesis provides an answer for the depressed levels of males in the between-species crosses, but may not fully explain the decreased fitness in fecundity and survivorship. Gonad morphogenesis fails to take into consideration other aspects of mating efficiency and ability that could cause decreased female fecundity and thus offspring.

The current study was designed to measure phenotypic markers of incompatibility in order to obtain a handle on genetic incompatibility accumulating as intrinsic post-zygotic isolating barriers contributing to the process of speciation. However, this experimental set-up failed to control for or quantify variables such mating behaviors. In terms of mating behavior we suggest that there are two main lines of further inquiry that should be pursued in order to develop a more thorough answer to the questions of uneven between-species hybrid breakdown. Existing research suggests physical variability between individuals in their ability to successfully mate with other individuals as well as the production of certain compounds utilized in the copulatory process, which, if compromised through broken genetic pathways, might cause a decrease in mating success.

Work with the model organism Drosophila melanogaster suggests that individual males may have variable mating success depending on the partner that they mate (Reinhart 2015). Additionally, individuals may not always mate with the individual that they would have the highest reproductive success with (Reinhart 2015). This huge amount of variability in mating ability could lend an explanation to the high degrees of individual variation among crosses, but also provide a partial answer to decreased fitness in between species crosses. Based on experimental observations, it was seen that when mated with *C. remanei* females, *C. latens* males were less interested or quick to pursue the female. This observed, perceived lack of interest could be due to a similar effect as that observed by Reinhart, in that males in between species crosses were less likely to be mated with females whom they could mate successfully. However, the perceived lack of male interest could also be due to a defect in chemical production.

Multiple studies have followed the trail of pheromones and other chemical compounds as they relate to nematode mating behavior. When comparing *C. elegans* and *C. briggsae* gonochoristic versus hermaphroditic mating efficiency, not surprisingly, males that were mated to gonochoristic females rather than hermaphrodites were more successful in their mating and reproductive endeavors (Garcia 2007). When the particular mating behavior was studied, it was determined that the males produce a compound that immobilizes the female and causes the vulval slit to widen so that the sperm and copulatory plug can be more easily inserted, however, this compound does not work on hermaphroditic individuals, thus decreasing the male's mating success (Garcia 2007). A similar effect could be occurring in the between-species males and

females, in that the males are producing a similar compound that is linked to their reproductive success, only to have it deemed useless when confronted with a female of a different species. Along the same vein, it has been determined that nematodes utilize ascaroside, or more generally, pheromone/chemical signaling in order to navigate their world and find other individuals (Braendle 2012, Schroeder 2012). However, similar to the high degrees of nucleotide diversity, ascaroside signaling is found to be differential due to ecological niches (Braendle 2012, Schroeder 2012). Therefore, similar to comparing levels of nucleotide diversity to determine early diverging populations and species, ascarosides may also be used to determine incipient differences between populations, which, through lack of successful mating with separate populations, may become speciated. This line of reasoning based on behavioral and chemical cues influencing mating success requires follow-up in order to negate its presence from the current study. Until such a time, these effects may be considered as possible contributory effects to the intense hybrid breakdown in between-species crosses.

Conclusions

The study carried out provides a range of comparable degrees of incompatibilities both within species and between species. The observed trends suggest that there are quantifiable levels of genetic incompatibility present in crosses conducted within the same species. Additionally, we found that this observed incompatibility is similar to, but less severe than the level of genetic incompatibility found between species. However, the repeated observation of genetic incompatibility in within species crosses is likely evidence of the beginning stages of incipient speciation.

While lacking biogeographic history of *C. remanei* the relevance of Orr's snowball theory cannot be determined based on the collected data. However, based on the observed comparative degrees of accumulation of incompatibility, Orr's theory cannot be excluded from consideration as it would appear that more distantly geographically spread crosses (between-species crosses) display much higher incompatibility than in more geographically constrained population comparisons (within-species crosses). In total, this work paves the way for a host of future work. In determining the cause of the total male lethality in between species crosses, hybrid male mating inefficiency needs to be addressed through follow-up experimentation. Genomic analysis presents itself as an obvious next step in working to identify possible regions of incompatibility within the genome. Gaining genomic insight could provide a depth of understanding as to the range of incompatibility observed on the phenotypic level to determine if particular regions of the genome are targeted consistently during the process of speciation.

Unlike previous experiments in the field of evolutionary biology, this experiment quantified speciation not only between separated species, but also within interbreeding populations of the same species. The findings presented indicate the ongoing nature of evolution and speciation by providing evidence that the presence of intrinsic post-zygotic isolating mechanisms is not constrained to reproductively isolated species. The data, which suggest high rates of epistatic interaction (as well as possible X effects or behavioral incompatibility in terms of between species crosses) support the previously stated conclusion that speciation is an ongoing process that can be observed to some extent, arguably, between all populations. The analysis and conclusions provided lead to a deeper understanding of the ongoing process of speciation and help to elucidate it as an intrinsic force in driving the ongoing diversification of life on earth.

Glossary

Allele: a form of a gene. Diploid organisms (organisms with two sets of chromosomes) inherit one allele for each gene from their mother and one from their father.

Divergence: the initial separation of a population that contributes to separating evolution and possible speciation.

Epistasis/Epistatic Interaction: commonly known as gene-gene interaction. Epistasis describes the phenomena of nearby genes controlling or having an influence on the expression of the gene of interest. Epistatic genes are typically linked due to their chromosomal proximity, but can also be linked by their interaction through similar processes.

Fecundity: the number of viable offspring a female has over the course of her life. Note that fecundity does not consider the number of eggs laid but rather the number of successfully hatched offspring.

Genotypes: the combination of alleles (derived from the organism's parents) at any given point of a chromosome. Different combinations of alleles, on a general level, correspond to differing phenotypes.

Gonochoristic: Male and female comprised populations.

Loci/Locus: position of a gene along a chromosome

L4: a life stage categorization of nematode. Eggs hatch and progress from L1 (newly hatched and impossible to determine the sex using a microscope) to sexually mature and virgin L4s.

Obligate out-crossers: Organisms that must mate with another individual, ie. females mating with males rather than a hermaphrodite self-fertilizing.

Phenotypes: refers to the set of traits expressed by any individual organism. Phenotypes can also be considered in terms of their frequency across the population.

Population: group of individuals that interbreed (members of the same species) and coexist in space (live in a community and share resources).

Sex ratios: the percent of males in an offspring population.

Survivability: the percent of individuals that hatched from eggs laid.

Bibliography

Baird, S.E. 2002. Haldane's Rule by Sexual Transformation in *Caenorhabditis*. *Genetics* 161: 1349-1353.

Bateson, W., 1909 Heredity and variation in modern lights, pp. 85–101 in *Darwin and Modern Science*, edited by A. C. Seward. Cambridge University Press, Cambridge, MA.

Blumberg, Roger B. (ed.) *MendelWeb*. (http://www.mendelweb.org/, Edition 97.1 1997)

Braendle, Christian. 2012. Pheromones: Evolving Language of Chemical Communication in Nematodes. *Current Biology* 22 (9): R294-R296.

Charlesworth, B., J. A. Coyne and N. H. Barton. 1987. The relative rates of evolution of sex chromosomes and autosomes. *Am. Nat*. 130: 113–146.

Choe, Andrea, et al. 2012. Ascaroside Signaling Is Widely Conserved among Nematodes. *Current Biology* 22 (9): 772-780.

Cook O. F. (1906). "Factors of species-formation". *Science* 23 (587): 506–507.

Coyne, J.A. and Orr, H.A. 2004. Speciation. Sinauer Associates, Sunderland, MA.

Cutter, A.D., Baird, S.E., and Charlesworth, D. 2006. High nucleotide polymorphism and the decay of linkage disequilibrium in wild populations of *C. remanei*. *Genetics* 174: 901-913.

Cutter, A.D., et al. 2012. Global Population Genetic Structure of *Caenorhabditis remanei* Reveals Incipient Speciation. *Genetics* 191: 1257-1269.

Darwin, Charles. 1859. *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life*. London, UK: John Murray.

Demuth, Jeffery P. & Michael J. Wade. 2014. On the Theoretical and Empirical Framework for Studying Genetic Interactions within and among Species. *The American Naturalist* 165 (5).

Dey, Alivia, et al. 2014. Gonad morphogenesis defects drive hybrid male sterility in asymmetric breakdown of *Caenorhabditis* nematodes. *Evolution and Development* 20(20): 1-11.

Dobzhansky, T., 1937 *Genetics and the Origin of Species*. Columbia University Press, New York.

Fierst, Janna L. & Thomas F. Hansen. 2010. Genetic Architecture and Postzygotic Reproductive Evolution of Bateson-Dobzhansky-Muller Incompatibilities in a Polygenic Model. *Evolution* 64 (3): 675-693.

Freeman, Scott. *Biological Science*. Ed. 5. Benjamin Cummings. 2013.

Futuyma, Douglas J. *Evolution*. Ed. 3. Sinauer Associates, Inc. 2013.

Garcia, L. Rene, et al. 2007. Diversity in Mating Behavior of Hermaphroditic and Male-Female Caenorhabditis Nematodes. *Genetics* 107: 1761-1771.

Hansson, B. 2013. Speciation genetics. *Heredity* 110: 407-408.

Johnson, N. A. 2008. Haldane's Rule: the Heterogametic Sex. *Nature Education*.

Kiontke K, Sudhaus W. Ecology of Caenorhabditis species. 2006 Jan 9. In: WormBook: The Online Review of C. elegans Biology [Internet]. Pasadena (CA): WormBook; 2005-. Available from: http://www.ncbi.nlm.nih.gov/books/NBK19723/

Matute, Daniel R., et al. 2010. A Test of the Snowball Theory for the Rate of Evolution of Hybrid Incompatibilities. *Science* 329: 1518-1521.

Matute, Daniel R,., et al. 2014. Variable post-zygotic isolation in *Drosophila melanogaster*/ *D. simulans* hybrids. *Journal of Evolutionary Biology* 27: 1691-1705.

Mayr, Ernst. 1942. Systematics and the Origin of Species. Columbia University Press.

Mayr, Ernst. 1963. Animal Species and Evolution. Cambridge: Belknap Press of Harvard University Press.

Mora C, Tittensor DP, Adl S, Simpson AGB, Worm B (2011) How Many Species Are There on Earth and in the Ocean? *PLoS Biol* 9(8): e1001127. doi: 10.1371/journal.pbio.1001127.

Muller, H. J. (1958). Evolution by mutation. *Bull. Amer. Math. Soc.* 64 (4): 137–160.

Orr, H.A. 1993. A mathematical model of Haldane's Rule. *Evolution* 47: 1606-1611.

Orr, H.A. 1995. The population genetics of speciation: the evolution of hybrid incompatibilities. *Genetics* 139: 1805-1813.

Pannell, John R. 2012. Speciation Genetics: Reinforcement by Shades and Hues. *Current Biology* 22 (9): R299-R302.

Presgraves, Daven C. 2010. Speciation Genetics: Search for the Missing Snowball. *Current Biology* 20 (24): R1073-R1074.

Reinhart, Michael, et al. 2015. Characterizing Male–Female Interactions Using Natural Genetic Variation in Drosophila melanogaster *J. Hered*. 106: 67-79.

Seehausen, Ole, et al. 2014. Genomics and the origin of species. *Nature Reviews Genetics* 15: 176-192.

Stiernagle, T. 2006. Maintenance of *C. elegans, WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.101.1, http://www.wormbook.org.

The *C. elegans* Sequencing Consortium. 1998. Genome Sequence of the Nematode *C. elegans*: A Platform for Investigating Biology. *Science* 282: 2012-2018.

Turner, Leslie M., et al. 2014. Genomic Networks of Hybrid Sterility. *PLOS Genetics* 10 (2): 1-23.

Turrelli, M. & H.A. Orr. 1995. The dominance theory of Haldane's Rule. *Genetics* 140 (1): 389-402.

Wu, Chung-I & Chau-Ti Ting. 2004. Genes and Speciation. *Nature Reviews* 5: 114-122.