Investigating the Evolutionary Relationship of *Pseudoperonospora cubensis* and *P. humuli* Through Phylogenetic and Host Range Analyses

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Downy mildew on hop, caused by *Pseudoperonospora humuli*, and downy mildew on cucurbits, caused by *P. cubensis,* are two distinct pathogens. Growers protect these crops against the mildew by planting resistant cultivars and supplementing with fungicides and cultural techniques. The disease on cucurbits has been managed by planting moderately resistant cultivars and didn't require much fungicide applications until a recent resurgence of *P. cubensis* in 1984 in Europe and 2004 in the U.S. This reemergence of cucurbit downy mildew caused an estimated loss of ~$20 million and 40% loss in cucumber yields for growers in the Eastern United States alone. Recent research has indicated that there may have been a genetic bifurcation among cucurbit downy mildew species responsible for the recent epidemics. It has been suggested that cucurbit downy mildew has arisen from hop downy mildew via a host jump from Japanese hop, *Humulus japonicus,* due to *P. humuli* on *H. japonicus* from Korea being placed basal to the *P. humuli – P. cubensis* phylogenetic complex. Phylogenetic analyses were conducted utilizing 59 *P. cubensis* isolates from 6 countries and 31 *P. humuli* isolates from 7 countries by Bayesian Inference, Maximum Likelihood and

Minimum Evolution methods based on two nuclear (internal transcribed spacer and *ypt1*) and one mitochondrial (cytochrome c oxidase II) loci. The resulting phylogenies support the conjecture that the downy mildew species responsible for the recent epidemic on cucurbits form phylogenetic clusters that are separated from the pre-epidemic cucurbit pathogen. However, the ancestry of the *P. cubensis – P. humuli* complex cannot be resolved from the phylogenetic analyses employed in this study. Host range experiments on *H. japonicus* were conducted using 6 *P. humuli* isolates from the U.S., 2 *P. humuli* isolates from Japan and 1 *P. cubensis* isolate from the U.S.. Only a single sporangiophore could be observed in the course of the study and therefore indicates that a host barrier between the downy mildew pathogens found on *H. japonicus*, *H. lupulus*, and cucurbit species. This finding also suggests that *H. japonicus* may be a source of host resistance genes for introgression into common hop.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Introduction

Downy mildew diseases are economically destructive to numerous plant species and often downy mildews are managed with intensive chemical inputs. Proper pathogen identification is a critical aspect for risk assessment, regulatory measures such as quarantine, and implementing appropriate control measures. In previous research the cucurbit downy mildew pathogen, *Pseudoperonospora cubensis*, and the closely related pathogen of hop, *P. humuli*, have been shown to be sister taxa in phylogenetic analyses (Choi et al. 2005, Voglmayr 2003). These organisms form two well-resolved genetic clades with the exception of isolates from *Humulus japonicus* from Korea (Mitchell et al. 2011, Runge et al. 2011, Sarris et al. 2009). The emerging body of knowledge indicates that *P. humuli* and *P. cubensis* are morphologically and genetically similar, but possess measurable physiological and genetic differences. Further investigations of the species-population boundary, pathogenicity to *H. japonicus* and other hosts in Asia, and a more intensive sampling of Asian isolates of *P. cubensis* and *P. humuli* are needed to determine the true evolutionary history of this group. This thesis project will aim to analyze a broader collection of isolates from the U.S. and Asia to better define the species-population boundaries of *P. humuli* and *P. cubensis* through molecular phylogenetic studies and host specificity experiments.

The Downy Mildews

Downy mildews and white blister rusts belong to the class Oomycetes (Peronosporomycetes), and comprise a group of greater than 1000 species of plant pathogens (Kirk et al., 2001). Many of the most economically damaging diseases of

plants are caused by downy mildew pathogens, with notable examples including downy mildew of hop and cucurbits (caused by *Pseudoperonospora* spp.), grapevine and sunflower (*Plasmopara*), lettuce (*Bremia*), spinach (*Peronospora*) and sorghum (*Sclerospora*) (Mathews 1981, Runge and Thines 2011). Downy mildew pathogens generally are believed to be obligate biotrophs and fully dependent on living host cells for vegetative growth and reproduction (Göker et al. 2007). These pathogens tend to have extremely high reproductive capacity and are characterized by explosive epidemics when rigorous control measures are not employed (Palti and Rotem 1981). Thus, downy mildew diseases often are managed by regular application of pesticides. Gisi (2002) estimated that 17% of the world fungicide market is directed towards controlling downy mildew diseases.

The downy mildews are closely related to *Phytophthora*, which contain other Peronosporomycete pathogens that cause root and foliar rots of plants. Plant pathogens within the genus *Phytophthora* are responsible for catastrophic events such as the sudden oak death disease in North America (*Phytophthora ramorum*) first reported in 1995 and the 1845 Irish Potato Famine (*Ph. infestans*) (Thines 2009, Ristaino 2002). *Phytophthora infestans* is the most studied oomycete, and is the casual agent of the reemergent disease late blight of potato and tomato (Kamoun 2003). Phytophthora and downy mildews were recently shown to be phylogenetically and morphologically connected by "bridging taxa" pathogenic on certain grass species and therefore normal paradigms used to distinguish between the two genera show variance (Thines 2009).

The Genus Pseudoperonospora

Pseudoperonospora humuli

Within the genus *Pseudoperonospora* there are six widely recognized species, with *P. humuli* and *P. cubensis* being the most economically important pathogens (Runge et al. 2011). *Pseudoperonospora humuli* was first reported on cultivated hop (*Humulus lupulus*) in Japan in 1905 (Miyabe and Takahashi 1906).The center of origin of *P. humuli* is unknown, but has been postulated to be East Asiapossibly Japan (Neve, 1991). The pathogen is thought to be capable of attacking three species within the genus *Humulus*: *H. lupulus* (common hop), *H. japonicus* (Japanese hop) and *H. yunnanensis*. Neve (1991) hypothesized that the host genus originated in China since all three species exist there.

The disease was originally classified under the genus *Peronoplasmopara*, exhibiting characteristics similar to both the genus *Peronospora* and genus *Plasmopara* (Miyabe et al. 1906). The spore wall in *Peronospora* is non-poroid (lacking a lid-like apex on the asexual spore stage), uniformly thick and germination can only occur directly via a germ tube, whereas *Plasmopara* spores have poroid walls and germinate by motile zoospores (Palti and Cohen 1980). The genus *Pseudoperonospora* has true sporangia (the asexual spore) with a poroid apex and germinate by zoospores, similar to *Plasmopara* sporangia, differentiating in the ways the spore bearing structures (sporophores) branch and terminate (Palti and Cohen 1981). Miyabe and Takahashi (1906) reported that in 1901, Berlese created a new subgenus *Peronoplasmopara* in the genus *Plasmopara*, placing *Peronospora cubensis* and *Peronospora celtidis* under it, thus defining the range of the subgenus. According to Miyabe and Takahashi (1906), S.

Rostovzev founded the new genus *Pseudoperonospora* during his study of *Peronospora cubensis* in 1903, but did not place any specific species under the genus. Miyabe and Takahashi (1906) characterized the hop disease under the descriptive characters of *Peronoplasmopara* given by Berlese's description of *Peronospora cubensis* and *Peronospora celtidis*, rather than the indefinite descriptive characters of *Pseudoperonospora* as presented by Rostovzev since he did not place any specific species under the genus, thus naming the pathogen *Peronoplasmopara humuli*. Eight years later, Wilson (1914) renamed the pathogen to *Pseudoperonospora humuli* by comparing Japanese specimens to *Pseudoperonospora celtidis* var. *humuli* collected from wild hops in Wisconsin. Wilson (1914) cited too great of a similarity of morphometric characters between specimens to mistake the Japanese downy mildew species for anything other than genus *Pseudoperonospora*.

Hop Downy Mildew Epidemiology and Management

P. humuli perennates in infected hop crowns as systemic mycelia and potentially also as dormant, sexual spores (oospores) in soil or plant debris (Royle and Kremheller 1981). The infection process by downy mildew pathogens is dependent on liquid water, and in the case of the hop downy mildew pathogen this free water is typically associated with daytime rain, or less frequently extended periods of dew (Gent and Ocamb 2009; Royle and Kremheller 1981). The pathogen is capable of infecting the entire hop plant, causing localized disease on leaves, flowers and cones, as well as systemic infection of shoots and the root system (Royle and Kremheller 1981).

The primary infection of hop plants occur in early spring within the shoots (hop bines), causing stunted growth with characteristic yellowish, down-curled and brittle

leaves (Royle and Kremheller 1981). Selected shoots are hand trained onto strings in spring providing close proximity for the disease to spread to neighboring leaves and to healthy shoots. The disease can cause direct losses in cone yield if the trained bines and cone-bearing lateral branches become infected, which arrests development of these shoots and prevents cone formation (Royle and Kremheller 1981). Infection in the cones can cause browning, often accompanied with sporulation on the underside of the bracts and bracteoles. Consequently, the disease diminishes the amount of bittering alpha-acids produced from cones and their brewing quality, thus adversely affecting producers and the brewing industry (Royle and Kremheller 1981, Danenhower et al. 2008). The disease in susceptible cultivars can also cause chronic weak growth during subsequent seasons and eventual plant death (Gent et al. 2010).

Leaf infection requires a minimum wet period of 1.5 hours, whereas shoot infection requires a longer minimum wet period of approximately 3 hours at optimum temperatures (Royle and Kremheller 1981). Sporangia germinate indirectly, by zoospores, during wetness lasting 1 hour at 20-22°C to 10 hours at 2°C (Royle and Kremheller 1981). The amount of infection is dependent on the frequency in which zoospores encounter the stomata of the hop leaves, which is the infection course for the pathogen (Royle and Kremheller 1981). The zoospores encyst and penetrate stomata by means of germ tubes, with the zoospore distribution highest in the light where the stomata are open, rather than in the dark where the stomata are mostly closed (Royle and Kremheller 1981). According to Royle and Kremheller (1981), symptoms (chlorotic lesions) on susceptible leaves develop in 3-10 days The sporangia-bearing sporangiophores emerge from stomata on the underside of leaves and produce new

sporangia in a diurnally rhythmic process (Royle and Thomas, 1972). Sexual oospores also are produced in diseased tissue, particularly diseased cones, although the role of oosporic inoculum in the disease cycle is unclear.

Hop downy mildew is managed by regular application of fungicides. Historically, hop growers have used a routine set of applications (Royle and Kremheller 1981). However, recently, Gent et al. (2010) showed that hop downy mildew can be managed effectively with fewer fungicide applications if applied concurrently with the predicted appearance of disease or detection of airborne inoculum (Gent et al. 2009). The disease is also managed with planting resistant cultivars, such as Fuggle, when resistant cultivars are available with the organoleptic characteristics demanded by brewers (Royle and Kremheller 1981). Cultural practices such as hand removal of diseased shoots and spring pruning of basal foliage using chemical desiccants also help prevent the spread of inoculum (Royle and Kremheller 1981).

Pseudoperonospora cubensis

Cucurbit downy mildew was first described from a herbarium specimen collected in Cuba in 1868 (Berkely and Curtis 1868). *P. cubensis* is now known to occur on all continents where cucurbit plants are cultivated—mainly in warm, temperate climates in regions such as the Middle East, America, Europe, Japan, Australia and South Africa (Cohen 1981, Lebeda and Cohen 2011). *Pseudoperonospora cubensis* affects nine of the 12 cultivated Curcurbitaceae (Cohen 1981). *P. cubensis* has been reported on at least 49 spp. in 70 different countries for the genus *Cucumis* alone (Cohen 1981), although the host range of individual isolates varies. According to Cohen (1981), the most important cultivated cucurbits affected by the disease are: *Cucumis sativus* L.

(cucumber), *Cucumis melo* L. (cantaloupe), *Cucurbita* spp. (pumpkin, squash and gourd) and *Citrullus vulgaris* L. (watermelon). The greatest prevalence of the disease occurs on *Cucumis* species (Lebeda and Cohen 2011). *P. cubensis* also has been reported to attack the ornamental plant *Impatiens irvingii* (Voglmayr et al. 2009).

Many cucurbit hosts of *P. cubensis* are suspected to have originated from either Africa or southeastern and southern China (Mitchell 2010). Kirkbride (1993) recognizes about 30 wild species of *Cucumis* native to Africa, with only *C. sativus* L. and *C. melo* L. as natural hosts of *P. cubensis* (Palti and Cohen 1980). *Cucurbita* spp. arose in South America and exhibit the greatest taxonomic diversity in Mexico (Sanjur et al. 2002, Cutler and Whitaker 1961); Palti and Cohen (1980) report only three species of *Cucurbita* as natural hosts of *P. cubensis*: *C. maxima* (squash), *C. moshata* (butternut squash) and *C. pepo* (pumpkin and other gourds). In the genus *Citrullus*, two of the four taxonomically valid species are natural hosts of *P. cubensis*: *C. colocynthis* (bitter cucumber) and *C. lanatus* (watermelon) (Palti and Cohen 1980).

Cucurbit Downy Mildew Epidemiology and Management

Pseudoperonospora cubensis attacks the leaves of cucurbitaceous plants (Cohen 1981). Morphological symptoms differ among cucurbit species, such as leaf lesions on cucumber and *Luffa* spp. that are localized and restricted by leaf veins, in comparison to cantaloupe and watermelon leaf lesions that exhibit more circular and irregular lesions that are not restricted by leaf veins (Lebeda and Cohen 2011).

P. cubensis infects frost-sensitive plants and thus must survive the off-season in hosts living in warmer climates or greenhouses, on perennial weed hosts, or perhaps as dormant oospores. From one of these sources of overwintering inoculum, infection of

cucurbit leaves begins with free moisture present on the leaf to promote zoospore release from sporangia (Cohen 1981). Rainwater is often the best source of moisture because the rainfall will provide wetness at higher temperatures and will extend the wetness period of dew (Palti and Cohen 1980). Periods of wetness are crucial for zoospore release and penetration of the germ tubes into the host; the minimum wetting period is approximately 2 hours and the optimum temperature for germination is 10-20°C (Palti and Cohen 1980, Cohen 1977, 1981). Zoospores encyst singly on stomatal openings and germinate via the germ tube (Cohen 1981). High humidity (>90%) is required for sporulation, regardless of light or dark incubation, and occurs roughly 4-5 days after inoculation under optimal conditions (Palti and Cohen 1980, Lebeda and Cohen 2011). Environmental conditions preceding the onset of the wetting period, especially factors that enhance photosynthesis of the host will increase spore production (Cohen 1981). Light inhibits the production of sporangiophores and sporangia production, therefore, sporulation occurs at night; an increased light period and intensity, in addition to raised temperatures will favor sporulation in the dark (Cohen 1981). Sporulation potential has been shown to be higher on young leaves and symptoms develop more rapidly on young leaves than on older leaves (Cohen 1981).

Disease management relies mostly on host resistance supplemented with chemical control measures when warranted. The United States began to breed for downy mildew resistance in cucurbits in the 1930's and 1940's (Palti and Cohen 1980). A highly resistant cultivar 'Poinsett' was released in 1966 and this source of host resistance has been bred into most popular cucumber cultivars to limit the need for

fungicides (Holmes et al. 2006). Other management techniques include wide spaced planting to minimize pathogen spread and crop rotation (Palti and Rotem 1981).

There has been a resurgence of *P. cubensis* on cucumber in North America and Europe (Lebeda and Cohen 2011). Since 2004, severe outbreaks in the U.S. have been found on highly susceptible cultivars up to two months earlier than in previous years and the disease has caused moderate infection in previously resistant cultivars (Holmes et al. 2006). Growers from Eastern United States estimated a loss of ~$20 million and 40% of their cucumber yield due to the reemergence of cucurbit downy mildew (Holmes et al. 2006). Resistant cultivars must now be supplemented with fungicide applications in order to effectively manage the disease. Holmes et al. (2006) attributes these sudden outbreaks to a more virulent *P. cubensis* pathogen, a distinctive pathotype, or a different species entirely, although the cause of the re-emergence of cucurbit downy mildew remains speculative. The sexual, overwintering oospore of *P. cubensis* is rarely found (Cohen 1981; Palti and Cohen 1980). However, Cohen et al. (2011) recently reported that strains of *P. cubensis* associated with recent outbreaks of cucurbit downy mildew produce fertile oospores, which could provide an additional source of overwintering inoculum of the pathogen.

Evolutionary Relationship of *P. cubensis* and *P. humuli*

In 2005, Choi et al. (2005) showed that *Pseudoperonospora humuli* and *Pseudoperonospora cubensis* share a high level of sequence similarity in the internal transcribed spacer region (ITS) of the nuclear ribosomal DNA (rDNA) and a similar morphology (Choi et al. 2005). Choi et al. (2005) conducted Bayesian inference and

maximum parsimony sequence analyses for the complete internal transcribed spacer region (ITS) for 18 isolates of *P. cubensis* and *P. humuli*, collected mostly from Asia. The authors found that all *P. cubensis* and *P. humuli* isolates clustered together in a monophyletic clade and showed 100% support for both phylogenetic analyses. As a result, Choi et al. (2005) reduced *P. humuli* to a taxonomic synonym of *P. cubensis*.

Astudy by Sarris et al. (2009) refuted this proposal based on a broader analysis of ITS sequence data. The authors analyzed 22 *P. cubensis* isolates, including all previously deposited ITS-I and ITS-II rDNA sequences from the National Center for Biotechnology Information (NCBI) GenBank database for *P. cubensis* in addition to the *P. humuli* (6 isolates) sequence data from Choi et al. (2005). The analysis supported two distinct clades for *P. cubensis* and *P. humuli*. There was no variability found within the ITS-I region, but ITS-II sequences were grouped into two sub-clusters: European isolates and Asian isolates (Sarris et al. 2009). Furthermore, two Korean *P. humuli* isolates on *Humulus japonicus* (SMK19582 and SMK11608, included in this study) were sub-clustered among the Asian *P. cubensis* isolates. The results of this study imply potential genetic bifurcation between the Korean *Pseudoperonospora* isolates and those outside of Korea.

In a more recent study, Runge et al. (2011) reported a possible cryptic species within the *Pseudoperonospora* lineage. The authors conducted a multigenetic phylogenetic analysis (ITS, *cox*II, *ypt1*) for *P. cubensis* and *P. humuli* species, resulting in three monophyletic clades consisting of they referred to as clade 1 (*P. cubensis* isolates from North America and Europe collected before the reemergence of downy mildew), clade 2 (pre- 1984 European epidemic *Pseudoperonospora* spp. isolates from

Japan and Korea on cucurbits), and clade 3 (*P. humuli* strains from *Humulus lupulus*) (Runge et al. 2011). In addition to these three clades, a cryptic *Pseudoperonospora* sp. on *Humulus japonicus* from South Korea was found to occupy a basal position to the *P. cubensis* cluster according to an unpublished tree, providing evidence that *Pseudoperonospora cubensis* arose via a host jump from hop, possibly *H. japonicus* (Runge et al. 2011). Runge et al. (2011) also noted that clade 2 included recent epidemic strains of *Pseudoperonospora* from the Czech Republic, Germany and the USA. Since these strains group with the pre-2004 epidemic strains from East Asia, the authors proposed that the recent cucurbit downy mildew epidemic strains are associated with a cryptic species and suggested that the species is indigenous to East Asia (Runge et al. 2011). The cryptic species is identified as such, rather than a highly virulent pathogen as a result of spontaneous mutation, from supporting data that the recent epidemics are being caused by a new lineage "with a high degree of adaptability and virulence plasticity" (Runge et al. 2011). They suggested that this cryptic species may have recently escaped from Asia and is the cause of the re-emergence of cucurbit downy mildew in North America and Europe. Runge et al. (2011) refrained from renaming the cryptic species as a new taxon and insisted on a broader phylogenetic evaluation based on multiple loci before assessing the actual identity of the species or sub-species.

In a parallel study, Mitchell et al. (2011) conducted phylogenetic analyses of *P. cubensis* and *P. humuli* isolates using two nuclear (internal transcribed spacer and β-tubulin) and three mitochondrial loci (cytochrome c oxidase I, II and the cytochrome oxidase c I/II spacer). These analyses expanded the quantity of *Pseudoperonospora sp.*

sequences analyzed to include new isolates mainly from the United States (21 *P. cubensis isolates* and 14 *P. humuli* isolates), together with all *Pseudoperonospora* ITS sequences published in the NCBI GenBank. The results of this study further reinforce that *Pseudoperonospora* pathogens on hop and cucurbits are distinctly lineages, with the exception of *P. humuli* isolates collected from *H. japonicus* in Korea, where one *H. japonicus* isolate clustered within the *P. cubensis* clade containing Korean isolates and the second *H. japonicus* isolate was basal to the *P. cubensis – P. humuli* clade (Mitchell et al. 2011). Mitchell et al. (2011) suggested that the grouping of *P. humuli* on *H. japonicus* with *P. cubensis* could indicate that *P. humuli* may have descended from *P. cubensis;* however, Runge et al. (2012) refuted this statement, noting that the trees indicate a polytomy for *P. cubensis* placement in relation to *P. humuli*.

Further investigation into the genetic structure of *P. cubensis* populations found that Bayesian clustering using five genetic markers resolved six genetic clusters among 465 isolates from 13 countries, demonstrating significant population structure by host and geographic region (Quesada-Ocampo et al. 2012). Quesada-Ocampo et al. (2012) also found that all six genetic clusters were present in all continents, however, three clusters were found more frequently in North America and a separate cluster predominates in Europe. Both North America and Europe demonstrated high genetic variation and indicate potential difficulty in breeding resistant cucurbit cultivars in the near future.

Host Range of *P. cubensis* and *P. humuli*

In addition to molecular studies, Mitchell et al. (2011) conducted host specificity studies to further evaluate the host range and evolutionary relationship of *Pseudoperonospora*

humuli and *Pseudoperonospora cubensis*. Cross-inoculations between U.S. *P. cubensis* and *P. humuli* on highly susceptible natural hosts of each pathogen resulted in a consistently low rate of infection for *P. cubensis* on hop (48/61 whole plant replicates with some level of sporulation) and only a single sporangiophore of *P. humuli* observed on a cucurbit host (1/33 whole plant replicates) (Mitchell et al. 2011). Mitchell et al. (2011) concluded that the host specificity data implies that specific physiological characteristics define each organism, thus advocating maintenance of the two species names until the species relationship can be defined precisely.

Runge et al. (2012) conducted independent host specificity experiments using two strains of *P. humuli* from Germany and one strain of *P. cubensis* from the Czech Republic. Inoculations were conducted on wild cucumber (*C. sativus*), the perennial climbing vine *Bryonia dioica*, and common hop. Interestingly, the results of this host range experiment greatly differ from those in Mitchell et al. (2011). Runge et al. (2012) found that *P. humuli* infected cucumber in 7 of 25 trials and *B. dioica* in 16 of 25 trials, and *P. cubensis* infected hop in 6 of 25 trials. The authors noted that sporulation of *P. cubensis* on *H. lupulus* consisted of a few sporangiophores or group of sporangiophores and *P. humuli* sporulation occurred in isolated groups on the entire lower surface of the leaf. Although the *P. cubensis* infection rates are similar to those seen in Mitchell et al. (2011), there is a much greater rate of infection with *P. humuli* observed on cucumber using strains from Germany. The authors attribute this difference in results to the type of host strain and host resistance variability.

Purpose of Study

The purpose of this study is to construct phylogenies using molecular markers with high resolution (ITS, *coxII*, *yptI*) from abroad collection of U.S., European and Asian isolates, and to determine the pathogenicity of U.S. and Asian *P. humuli* and *P. cubensis* isolates on the host *H. japonicus.* These investigations aim to provide evidence for the possible host jump from *P. humuli* to *P. cubensis* via *Humulus japonicus*, as well as to better resolve the evolutionary boundaries for Asian and non-Asian *Pseudoperonospora* species.

CHAPTER 2: MATERIALS AND METHODS

Plant Material and Isolate Maintenance

Downy mildew susceptible hop plant of cultivars Nugget and Pacific Gem, *Humulus japonicus* (accession CHUM1022 (U.S.), CHUM798 (China)), and cucumber cultivar Straight 8 were maintained in a greenhouse according to the protocol in Mitchell et al. (2011). *P. humuli* isolates were collected from Czech Republic, Japan and United States (Oregon, New York and Washington) during 2006-2012 (Table 1). Monosporangial isolates were obtained from diseased hop shoots collected from commercial hop yards and the isolates were maintained and increased on hop using a droplet inoculation procedure on detached leaves (Gent et al., 2008) or a spray inoculation procedure for whole plants (Mitchell et al., 2011).

P. cubensis isolates from the United States (California, New Jersey, North Carolina, Ohio, Oregon, Michigan, and South Carolina) (Table 1) were received from other researchers during 2005-2011. Live isolates were maintained on cucumber as described by Mitchell et al. (2011).

Korean herbarium *P. humuli* (1) and *P. cubensis* (7) isolates were received from the National Fungal Collection and DNA samples (2) of *P. humuli* from Czech Republic were received from other researchers for analysis.

DNA Extraction, Cloning, PCR Amplification, Sequencing

DNA was extracted from sporangial suspensions using the MoBio Ultra Clean Soil DNA Isolation Kit with modifications as described by Mitchell et al. (2011) and PowerSoil DNA Isolation Kit with no modifications. The ITS of nuclear ribosomal region, Ras-related gene *ypt1* and mitochondrial locus *coxII* were amplified using

primers specified in Table 2. These loci provide varying resolution for phylogenetic inference. ITS is a highly conserved region among Peronosporales and readily utilized in phylogenetic investigation (Choi et al. 2005, Sarris et al. 2009, Mitchell et al. 2011, Runge et al. 2011, Quesada-Ocampo et al. 2012). The *coxII* locus was chosen due to the relative ease of amplification and the presence of four well-conserved single nucleotide polymorphisms (SNPs) that differentiate *P. humuli* and *P. cubensis* and a more rapid mutational rate than ITS (Mitchell et al. 2011)**.** The nuclear locus *ypt1* has been shown to provide high phylogenetic resolution for species within the Peronosporaceae family (Runge et al. 2011, Schröder et al. 2011).

PCR reactions were carried out according to Mitchell et al. (2011) with the following modifications: MangoTaq Polymerase (Bioline, Luckenwalde, Germany) or TaKaRa Ex Taq DNA polymerase (Clontech Laboratories, Mountain View, CA) was used when Hot Master Mix (5 Prime, Gaithersburg, MD) was unsuccessful, including PCR for all ypt1 amplifications. The amplification programs used for ITS and *coxII* were in accordance with Mitchell et al. (2011) and the amplification program for *ypt1* was carried out as follows: denaturation at 94˚C for 2 min; followed by 42 cycles of denaturation at 94˚C for 1 min, annealing temperature at 56˚C for 1 min, and extension at 72˚C for 1 min; and a final extension at 72 ˚C for 5 min.

Monosporangial *P. humuli* isolates were directly sequenced (bidirectionally) from PCR products. DNA for non-monosporangial isolates, including all *P. cubensis* isolates, were cleaned, cloned and amplified using primers and protocols from Mitchell et al. (2011), with the exception of three isolates (HDM489, HDM494, HDM497) due to difficulty in obtaining a monosporangial live isolate. Cloned PCR products were

sequenced bidirectionally by the Center for Genome Research and Biocomputing (Oregon State University, Corvallis, OR).

Phylogenetic Inference

Minimum Evolution, Bayesian Inference and Maximum Likelihood analyses were conducted on each of the individual loci as well as for a concatenated data set. Non-monosporangial isolates HDM489, HDM494, HDM497 were included in all analyses because they showed no sequence variability at any locus. Herbarium specimens HDM421, SMK11675, SMK11608 SMK17780, SMK18856, SMK18951 were included in the ITS and *coxII* single locus analyses because of their Korean origin, but they could not be included in the concatenated analyses due to incomplete sequence data for all three loci. All sequence data from Runge et al. (2011) were retrieved from Genbank and were included in each analysis. *P. cannabina* is considered to be the most basal species of the genus *Pseudoperonospora* (Choi et al. 2005) and was used to root the phylogenetic trees.

Sequences were aligned in Bioedit (version 7.1.11) using ClustalW (Thompson et al. 1994) multiple alignment tool with manual editing as needed. Minimum Evolution analysis was done with MEGA 5.20 (Tamura et al. 2011) under the Tamura-Nei substitution model and 1,000 bootstrap replicates; all other values were set to default. Trees were obtained in MEGA 5.20. Bayesian Inference was conducted under nucleotide substitution models (Table 3) chosen according to the Akaike Information Criterion by MrModeltest 2.3 (Nylander, J. 2004). MrBayes version 3.2.1 (Huelsenbeck and Ronquist 2001) on XSEDE at Cipres Science Gateway (Miller et al. 2010) was used to run analysis with the following parameters: three heated chains and one cold chain

were run for 10,000,000 generations with a sampling frequency of 1,000; the first 2,500 trees were discarded and the remaining 7,500 were used to obtain a 50% majority rule consensus tree and for inferring posterior probabilities. Maximum likelihood analysis was conducted using RAxML7.4.4 (Stamatakis, et al. 2008) on XSEDE using Cipres Web Portal utilizing 1,000 bootstrap iterations of rapid bootstrap analysis and 'search for best-scoring ML tree in one single program run' and 'print branch lengths' options; all other values were set to default. Paup* version 4.0b10 (Swofford 2003) was used to create a 50% consensus tree from RAxML output. Maximum likelihood and Bayesian trees were formatted in TreeView version 1.6.6 (Page 1996). Each analysis method was repeated three times to ensure reproducibility of results.

Host Range Study

Host range experiments were conducted with a total of 8 *P. humuli* isolates from Oregon (3), New York (3) and Japan (2) and one *P. cubensis* isolate from North Carolina. Each of these isolates was inoculated individually onto each of two accessions of *Humulus japonicus,* one from the United States (Accession: CHUM1022) and one from China (Accession: CHUM798) (Table 1). For a given run of an experiment, each pathogen isolate was spray inoculated according to the protocol used in Mitchell et al. (2011) onto three *H. japonicus* plants at a concentration of 5×10^3 sporangia ml^{-1}. All *P. humuli* isolates from New York and Japan were also spray inoculated onto three cucumber plants. Positive controls on *H. lupulus* (inoculated with *P. humuli*) and *C. sativus* (inoculated with *P. cubensis*) were included for each experiment to ensure susceptibility of the plants and viability of inoculum. Negative controls mock inoculated with water were included for each host plants as a check

against preexisting infection. Each experiment was repeated at least twice. Plants were incubated in growth chambers and sporulation was induced according to conditions used for host range experiments using *P. cubensis* in Mitchell et al. (2011). Growth chambers were chosen at random and cleaned thoroughly between each run in order to prevent potential contamination; positive controls, experimental plants, and negative controls were separated using plastic barriers in a single growth chamber. All plants were rated by stereomicroscopic examination on the 7^{th} (non-destructive examination) and 14^{th} day post-inoculation (destructive examination). Each leaf was examined for hypersensitive response (localized chlorosis and necrosis) and presence of sporulation.

Table 1. Identity and origin of Oomycete isolates used in this study

Pathogen	Host	Isolate Name	Country of Origin	Year Collected	GenBank Accession Number[f]		
					ITS	*coxII*	*ypt1*
P. cannabina	*Cannabis sativa*	NY409[d]	Poland	1958	HM636052	HM635956	HM636004
P. cannabina	*Cannabis sativa*	U181[d]	Latvia	1936	HM636051	HM635955	HM636003
P. celtidis	*Celtis occidentalis*	NY411[d]	USA, PA	1981	HM636045	HM635949	HM635997
P. celtidis	*Celtis sinensis*	D232[d,e]	Korea	2004	HM636050	HM635954	HM636002
P. celtidis	*Celtis sinensis*	SMK17780[a,d]	Korea	2000	JF314768	—	—
P. cubensis	*Citrullus vulgaris*	NY419[d]	USA, LA	1887	HM636014	HM635918	HM635966
P. cubensis	*Citrullus vulgaris*	NY435[d]	USA, FL	1935	HM636013	HM635917	HM635965
P. cubensis	*Cucumis anguria*	NY416[d]	USA, St. Croix	1923	HM636020	HM635924	HM635972
P. cubensis	*Cucumis melo*	D320[d]	Korea	2006	HM636008	HM635912	HM635960
P. cubensis	*Cucumis melo*	NY420[d]	USA, SC	1903	HM636006	HM635910	HM635958
P. cubensis	*Cucumis melo*	NY421[d]	USA, SC	1905	HM636015	HM635919	HM635967
P. cubensis	*Cucumis melo*	NY442[d]	USA, CT	1902	HM636011	HM635915	HM635963
P. cubensis	*Cucumis melo*	U187[d]	USA, ME	1899	HM636010	HM635914	HM635962
P. cubensis	*Cucumis melo*	U189[d]	USA, MA	1910	HM636005	HM635909	HM635957
P. cubensis	*Cucumis sativus*	CDM251	USA, MI	2007	JF304658	JF414542	—
P. cubensis	*Cucumis sativus*	CDM252	USA, OH	2007	JF304659	JF414543	—
P. cubensis	*Cucumis sativus*	CDM253	USA, NC	2007	JF304660	JF414544	—
P. cubensis	*Cucumis sativus*	CDM254	USA, NC	2006	JF304661	JF414545	—
P. cubensis	*Cucumis sativus*	CDM255	USA, MI	2005	JF304662	JF414546	—
P. cubensis	*Cucumis sativus*	CDM266	USA, CA	2009	JF304663	JF414547	—
P. cubensis	*Cucumis sativus*	CDM268	USA, CA	2009	JF304664	JF414548	—
P. cubensis	*Cucumis sativus*	CDM269	USA, CA	2009	JF304665	JF414549	—
P. cubensis	*Cucumis sativus*	CDM272	USA, CA	2009	JF304666	JF414550	—
P. cubensis	*Cucumis sativus*	CDM273	USA, CA	2009	JF304667	JF414551	—
P. cubensis	*Cucumis sativus*	CDM274	USA, CA	2009	JF304668	JF414552	—
P. cubensis	*Cucumis sativus*	CDM278	USA, OR	2009	JF304672	JF414556	—
P. cubensis	*Cucumis sativus*	CDM279	USA, OR	2009	JF304673	JF414557	—
P. cubensis	*Cucumis sativus*	CDM289	Korea	1993	—	—	—
P. cubensis	*Cucumis sativus*	CDM291	Korea	2006	—	—	—
P. cubensis	*Cucumis sativus*	CDM292	Korea	2009	—	—	—
P. cubensis	*Cucumis sativus*	SMK18951[a,d]	Korea	2005	AY608617	—	—
P. cubensis	*Cucumis sativus*	NY423[d]	USA, MA	1892	HM636018	HM635922	HM635970
P. cubensis	*Cucumis sativus*	NY429[d]	USA, NJ	1889	HM636026	HM635930	HM635978
P. cubensis	*Cucumis sativus*	NY446[d]	Germany	1909	HM636007	HM635911	HM635959

Table 1 (continued)

P. cubensis	*Cucumis sativus*	U190[d]	USA, NJ	1889	HM636017	HM635921	HM635969
P. cubensis	*Cucumis sativus*	U191[d]	USA, WI	1921	HM636019	HM635923	HM635971
P. cubensis	*Cucumis sativus*	U195[d]	Czech Republic	1912	HM636009	HM635913	HM635961
P. cubensis	*Cucumis sativus*	U197[d]	Philippines	1919	HM636038	HM635942	HM635990
P. cubensis	*Cucumis sativus*	21226[d,e]	Czech Republic	2007	HM636033	HM635937	HM635985
P. cubensis	*Cucumis sativus*	22238[d,e]	Czech Republic	2007	HM636035	HM635939	HM635987
P. cubensis	*Cucumis sativus*	23231[d,e]	USA, MI	2007	HM636036	HM635940	HM635988
P. cubensis	*Cucumis sativus*	24230[d,e]	Germany	2007	HM636034	HM635938	HM635986
P. cubensis	*Cucumis sativus*	NY425[d,e]	Japan	1924	HM636030	HM635934	HM635982
P. cubensis	*Cucumis sativus*	U196[d,e]	Japan	1904	HM636028	HM635932	HM635980
P. cubensis	*Cucumis sativus*	U201[d,e]	Japan	1934	HM636037	HM635941	HM635989
P. cubensis	*Cucurbita maxima*	NY430[d]	USA, NJ	1905	HM636022	HM635926	HM635974
P. cubensis	*Cucurbita maxima*	NY452[d]	USA, AL	1907	HM636025	HM635929	HM635977
P. cubensis	*Cucumis melo*	CDM282	USA, SC	2010	—	—	—
P. cubensis	*Cucumis melo*	CDM285	Korea	1993	—	—	—
P. cubensis	*Cucumis melo*	CDM287	Korea	1990	—	—	—
P. cubensis	*Cucurbita moschata*	CDM277	USA, NC	2008	JF304671	JF414555	—
P. cubensis	*Cucurbita moschata*	CDM293	Korea	1996	—	—	—
P. cubensis	*Cucurbita moschata*	CDM298	Korea	2005	—	—	—
P. cubensis	*Cucurbita moschata*	D190[d,e]	Korea	2002	HM636021	HM635925	HM635973
P. cubensis	*Cucurbita pepo*	CDM275[c]	USA, NC	2005	JF304669	JF414553	—
P. cubensis	*Cucurbita pepo*	CDM276	USA, NC	2006	JF304670	JF414554	—
P. cubensis	*Cucurbita pepo*	NY427[d]	USA, MA	1943	HM636024	HM635928	HM635976
P. cubensis	*Cucurbita pepo*	NY434[d]	USA, FL	1935	HM636029	HM635933	HM635981
P. cubensis	*Cucurbita pepo*	NY447[d]	USA, FL	1935	HM636027	HM635931	HM635979
P. cubensis	*Cucurbita pepo*	NY440[d,e]	Japan	1890	HM636031	HM635935	HM635983
P. cubensis	*Cucurbita sp.*	CDM247	USA, NJ	2007	JF304656	JF414540	—
P. cubensis	*Lagenaria leucantha*	NY448[d]	USA, FL	1925	HM636016	HM635920	HM635968
P. cubensis	*Lagenaria siceraria*	D265[d,e]	Korea	2005	HM636047	HM635951	HM635999
P. cubensis	*Melothria crassifolia*	NY431[d]	USA, FL	1935	HM636023	HM635927	HM635975
P. cubensis	*Sicyos angulatus*	U192[d]	USA, WI	1920	HM636012	HM635916	HM635964
P. humuli	*H. japonicus*	SMK11608[a,d]	Korea	2005	AY608621	—	—
P. humuli	*H. japonicus*	SMK18856[a,d]	Korea	2005	AY608622	—	—
P. humuli	*H. japonicus*	SMK19582[a,d]	Korea	2003	AY608623		
P. humuli	*H. japonicus*	D149[d,e]	Korea	2003	HM636032	HM635936	HM635984
P. humuli	*H. japonicus*	HDM421[a,b]	Korea	1994	—	—	—
P. humuli	*H. lupulus*	SMK11675[a]	Korea	2005	AY608624	—	—
P. humuli	*H. lupulus*	NY461[d,e]	USA,WI	1909	HM636046	HM635950	HM635998
P. humuli	*H. lupulus*	10124[d]	Germany	2005	HM636042	HM635946	HM635994

Table 1 (continued)

P. humuli	*H. lupulus*	NY454[d]	Czech Republic	1940	HM636040	HM635944	HM635992
P. humuli	*H. lupulus*	NY455[d]	Germany	1946	HM636041	HM635945	HM635993
P. humuli	*H. lupulus*	NY458[d]	Poland	1957	HM636039	HM635943	HM635991
P. humuli	*H. lupulus*	NY459[d]	Argentina	1980	HM636044	HM635948	HM635996
P. humuli	*H. lupulus*	NY460[d]	Germany	1925	HM636043	HM635947	HM635995
P. humuli	*H. lupulus*	HDM094	USA, WA	2006	JF304674	JF414558	—
P. humuli	*H. lupulus*	HDM110	USA, OR	2006	JF304676	JF414560	—
P. humuli	*H. lupulus*	HDM140	USA, OR	2006	JF304677	JF414561	—
P. humuli	*H. lupulus*	HDM170	USA, OR	2007	JF304679	JF414563	—
P. humuli	*H. lupulus*	HDM224	USA, OR	2008	JF304681	JF414566	—
P. humuli	*H. lupulus*	HDM247	USA, WA	2008	JF304682	JF414567	—
P. humuli	*H. lupulus*	HDM254	USA, OR	2008	JF304683	JF414568	—
P. humuli	*H. lupulus*	HDM257	USA, OR	2008	JF304684	JF414569	—
P. humuli	*H. lupulus*	HDM263[a,b]	Czech Republic	Unknown	JF304685	JF414570	—
P. humuli	*H. lupulus*	HDM266[a,b]	Czech Republic	Unknown	JF304686	JF414571	—
P. humuli	*H. lupulus*	HDM457[c]	USA, OR	2011	—	—	—
P. humuli	*H. lupulus*	HDM471[c]	USA, OR	2011	—	—	—
P. humuli	*H. lupulus*	HDM472[c]	USA, OR	2011	—	—	—
P. humuli	*H. lupulus*	HDM481[c]	USA, NY	2011	—	—	—
P. humuli	*H. lupulus*	HDM482[c]	USA, NY	2011	—	—	—
P. humuli	*H. lupulus*	HDM483[c]	USA, NY	2011	—	—	—
P. humuli	*H. lupulus*	HDM484	Czech Republic	2012	—	—	—
P. humuli	*H. lupulus*	HDM485	Czech Republic	2012	—	—	—
P. humuli	*H. lupulus*	HDM486	Czech Republic	2012	—	—	—
P. humuli	*H. lupulus*	HDM489	Japan	2012	—	—	—
P. humuli	*H. lupulus*	HDM491[c]	Japan	2012	—	—	—
P. humuli	*H. lupulus*	HDM492	Japan	2012	—	—	—
P. humuli	*H. lupulus*	HDM493[c]	Japan	2012	—	—	—
P. humuli	*H. lupulus*	HDM494	Japan	2012	—	—	—
P. humuli	*H. lupulus*	HDM497	Japan	2012	—	—	—
P. humuli	*H. lupulus*	HDM498	Japan	2012	—	—	—
P. urticae	*Urtica dioica*	HUH1111[d]	Italy	2009	HM636048	HM635952	HM636000
P. urticae	*Urtica dioica*	PDD1[d]	Austria	1975	HM636049	HM635953	HM636001

[a] Isolates used in phylogenetic analyses using ITS
[b] Isolates used in phylogenetic analyses using *coxII*
[c] Isolates used in host range experiment on *H. japonicus*
[d] Sequences from NCBI GenBank
[e] Classified by Runge et al. (2011) as *Pseudoperonospora* sp.
[f] GenBank Accession Numbers not available are indicated with a "—''

Table 2. Primers used in PCR and sequencing

Primer	Sense	Sequence (5'-3')	Locus	Annealing Temperature (˚C)	Source
ITS1	Forward	TCCGTAGGTGAACCTGCGG	ITS	51	White et al. 1990
ITS4	Reverse	GCATATCAATAAGCGGAGGA	ITS	51	White et al. 1990
HDM04	Forward	AGCCACACAACACATAGT	ITS	58.4	Gent et al. 2009a
HDM07	Reverse	AGAATTGACTGCGAGTCC	ITS	58.4	Gent et al. 2009a
FM35	Forward	CAGAACCTTGGCAATTAGG	COXII	56	Martin 2000
FM36	Reverse	CAAATTTCACTACATTGTCC	*coxII*	56	Martin 2000
M13F	Forward	TGTAAAACGACGGCCAGT	Plasmid	50	—
M13R	Reverse	CAGGAAACAGCTATGACC	Plasmid	50	—
YPT1	Forward	GACTTTGTGAGTGCCAGTGA	*ypt1*	56	Chen & Roxby 1996
YPT4	Reverse	GGCTGTGAGATCGCTCTTGT	*ypt1*	56	Moorman et al. 2002

Table 3. Nucleotide substitution models used in Bayesian single locus (ITS, *coxII*, *ypt1*) and concatenated analyses

Locus	Nucleotide Substitution Model[a, b]
Concatenated	GTR+I+G
coxII	GTR+I+G
ITS	HKY+G
ypt1	K80

[a] Substitution model chosen by MrModeltest 2.3 according to lowest scoring Akaike Information Criterion

[b] GTR=General Time Reversible; HKY= Hasegawa, Kishino and Yano; K=Kimura; I=Invariant nucleotide sites; G=Gamma-distributed substitution rate

CHAPTER 3: RESULTS

Phylogenetic Analysis

Trees for Bayesian, Maximum likelihood and Minimum Evolution analyses are presented in Figures 1, 2 and 3 respectively. All three phylogenetic analyses for the concatenated dataset consistently produced trees with three distinct clades, but all analyses differed in tree topology. The three clades represented include: clade 1—consisting of pre-epidemic *Pseudoperonospora cubensis* isolates from Japan, collected between 1890-1934 and post-epidemic *P. cubensis* isolates from Czech Republic, Korea and the United States; clade 2—consisting of pre-epidemic *P. cubensis* isolates from Czech Republic, Germany, Philippines, and United States all collected prior to 1943, and a single post-epidemic *P. cubensis* isolate (2006) from Korea (D320); and clade 3—which included all *P. humuli* isolates on *Humulus lupulus*. Support for Clade 1 varied for each analysis method, ranging from low (ME) to moderate (ML) to high (BA), in contrast to the high-maximal support for Clades 2 and 3 in all three analyses. Of the out-groups, *P. urticae* isolates HUH1111 and PDD1 on *Urtica dioica*, formed a monophyletic clade in all analyses with maximal support and *P. celtidis* isolates NY411 (*Celtis occidentalis*) and D232 (*Celtis sinensis*) formed a highly supported monophyletic clade in Bayesian and Maximum Likelihood analyses. However, the topology of the out-groups varied between trees when rooted with *P. cannabina* isolates. A single isolate of *P. humuli* on *Humulus japonicus* (D149) was basal to the *P. cubensis*—*P. humuli* clades with maximal support in Bayesian and Maximum Likelihood methods.

There were three sub-clusters nested within the *P. cubensis* clade 1 that had moderate to high support in trees from all three methods: two Korean *P. cubensis* isolates (CDM285, CDM289) collected in 1993, two United States—North Carolina isolates (CDM253, CDM276) collected in 2007 and 2006, respectively, and three Korean isolates (CDM293, D265 and D190) collected in 1996, 2005 and 2002, respectively. In maximum likelihood and Bayesian analyses 4 of 9 Korean *P. cubensis* isolates (CDM287, CDM293, D190, D265) in this clade were placed basal within the *P. cubensis* post-epidemic cluster. There is one sub-cluster within Clade 2 that has low support in all three trees containing isolates from *Lagenaria leucantha* (NY448) and *Sicyos angulatus* (U192) collected in 1925 and 1920, respectively. In Maximum Likelihood and Minimum Evolution analyses *P. cubensis* isolate U197 from the Philippines (collected in 1918) was placed basal to the rest of the pre-epidemic isolates.

The tree inferred by the Bayesian method (Figure 1) indicated with low support that the pre-epidemic *P. cubensis* isolates (clade 2) and the *P. humuli* isolates (clade 3) sub-cluster together and were sister, with moderate support, to the post-epidemic *P. cubensis* isolates (clade 1). Maximum Likelihood inferred a tree (Figure 2) that placed bifurcated *P. cubensis* sub-clusters (clades 1 and 2) with low support for the bifurcation, sister to the *P. humuli* cluster (clade 3) with moderate support. Minimum Evolution analysis produced a tree that indicated a polytomy but with maximal support for clades 1, 2 and 3.

Single locus analyses for ITS, *coxII* and *ypt1* had varying tree topologies (Appendix). Trees inferred for ITS locus showed *P. cubensis* basal to *P. humuli* in Bayesian (Appendix, Fig.1) and Maximum likelihood (Appendix, Fig. 2) analyses and

P. humuli and *P. cubensis* formed sister clades for Minimum Evolution analysis (Appendix, Fig. 3); 3 of the 5 isolates of *P. humuli* from *H. japonicus* from Korea (HDM421, SMK19582 and SMK 11608) clustered within the *P. cubensis* clade in all three analyses. Bayesian analysis (Appendix, Fig.4) of the *coxII* locus showed a polytomy of the clades 1, 2 and 3 as in the concatenated data set. Bayesian analysis also showed a monophyletic clade of *P. humuli* on *Humulus japonicus* (HDM421, D149) placed basal within clade 1. Sub-clusters of clade 3 *P. humuli* and clade 2 *P. cubensis* isolates were nested within the clade 1 cluster in addition to the monophyletic clade HDM421 and D149 that was placed basal to the clade 2 – clade 3 complex for Maximum Likelihood analysis (Appendix, Fig. 5); the same sub-clusters as seen in Maximum Likelihood analysis formed a polytomy with a monophyletic clade of *P. humuli* on *Humulus japonicus* (HDM421, D149) in Minimum Evolution analysis (Appendix, Fig. 6). Analysis of the *ypt1* placed *P. humuli* and *P. cubensis* as sister clades in all three analyses (Appendix, Figs. 7, 8, 9).

Host Range

The results of the host range experiment produced only a single sporangiophore for *P. humuli* isolate HDM491 (Japan) on *Humulus japonicus* among the 72 plant replicates (Table 4). There was no observed sporulation for *P. cubensis* on *H. japonicus* on 15 plant replicates. Positive controls on *H. lupulus* (*P. humuli*) and *C. sativus* (*P. cubensis*) were always characterized with profuse sporulation and negative controls (water only) on *H. lupulus, H. japonicus* and *C. sativus* were always free of hypersensitive response and sporulation at the 7th day rating. Typical symptoms on positive controls and lack of symptoms on *H. japonicus* are shown in Figure 4.

Figure 1. Phylogenetic tree for concatenated data set using Bayesian inference under the general time reversible (GTR) substitution model assuming gamma-distributed substitution rates and invariant nucleotide sites.

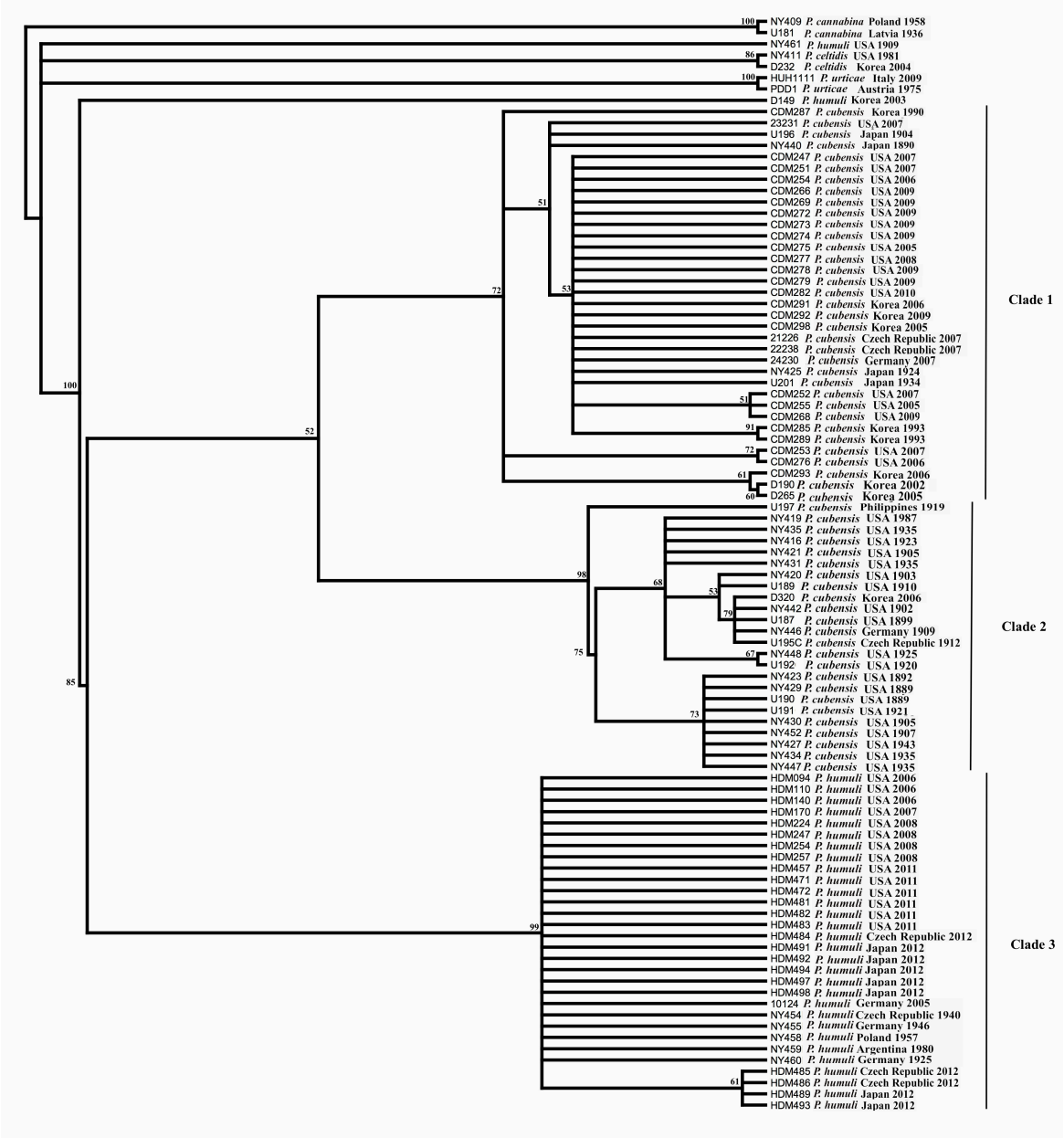


Figure 2. Phylogenetic tree for Maximum Likelihood analysis of concatenated data set using 1,000 bootstrap replicates.

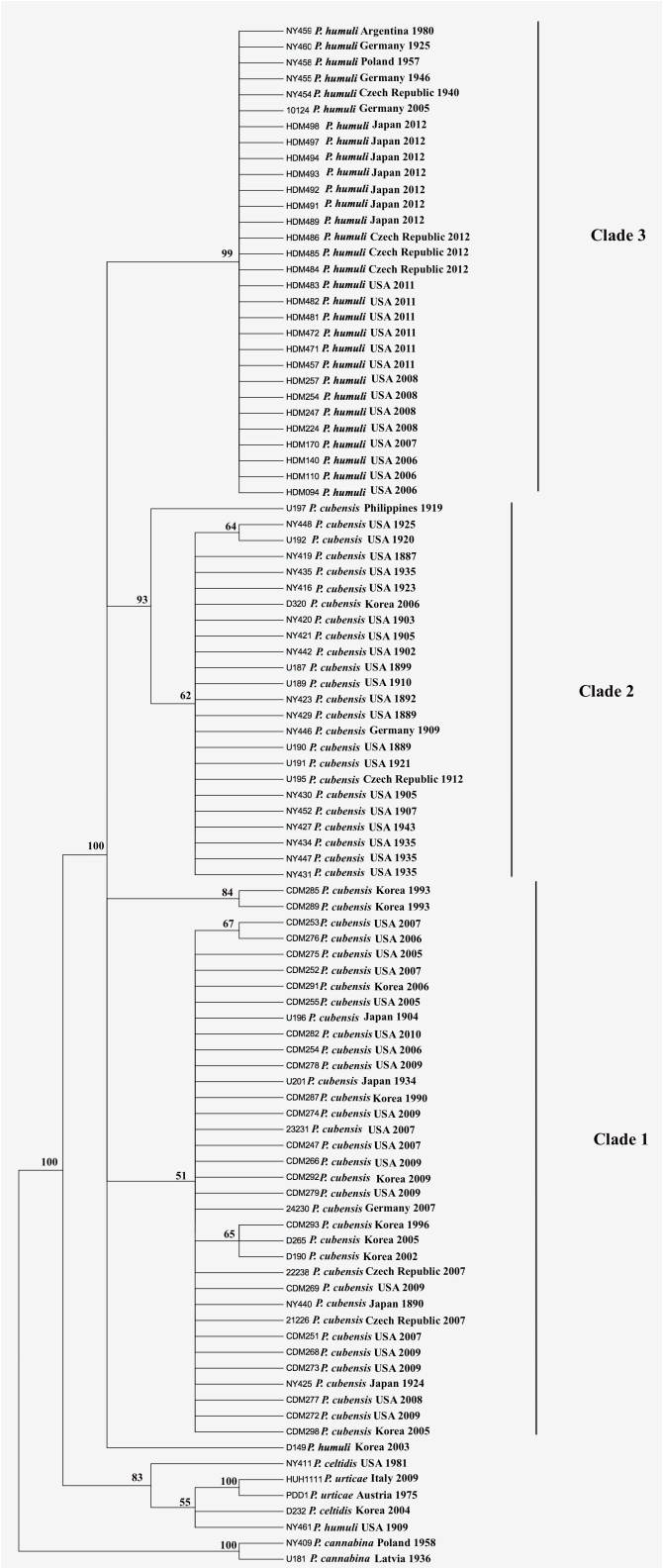


Figure 3. Phylogenetic tree for Minimum Evolution analysis for concatenated data set under Tamura-Nei substitution model and 1,000 bootstrap replicates.

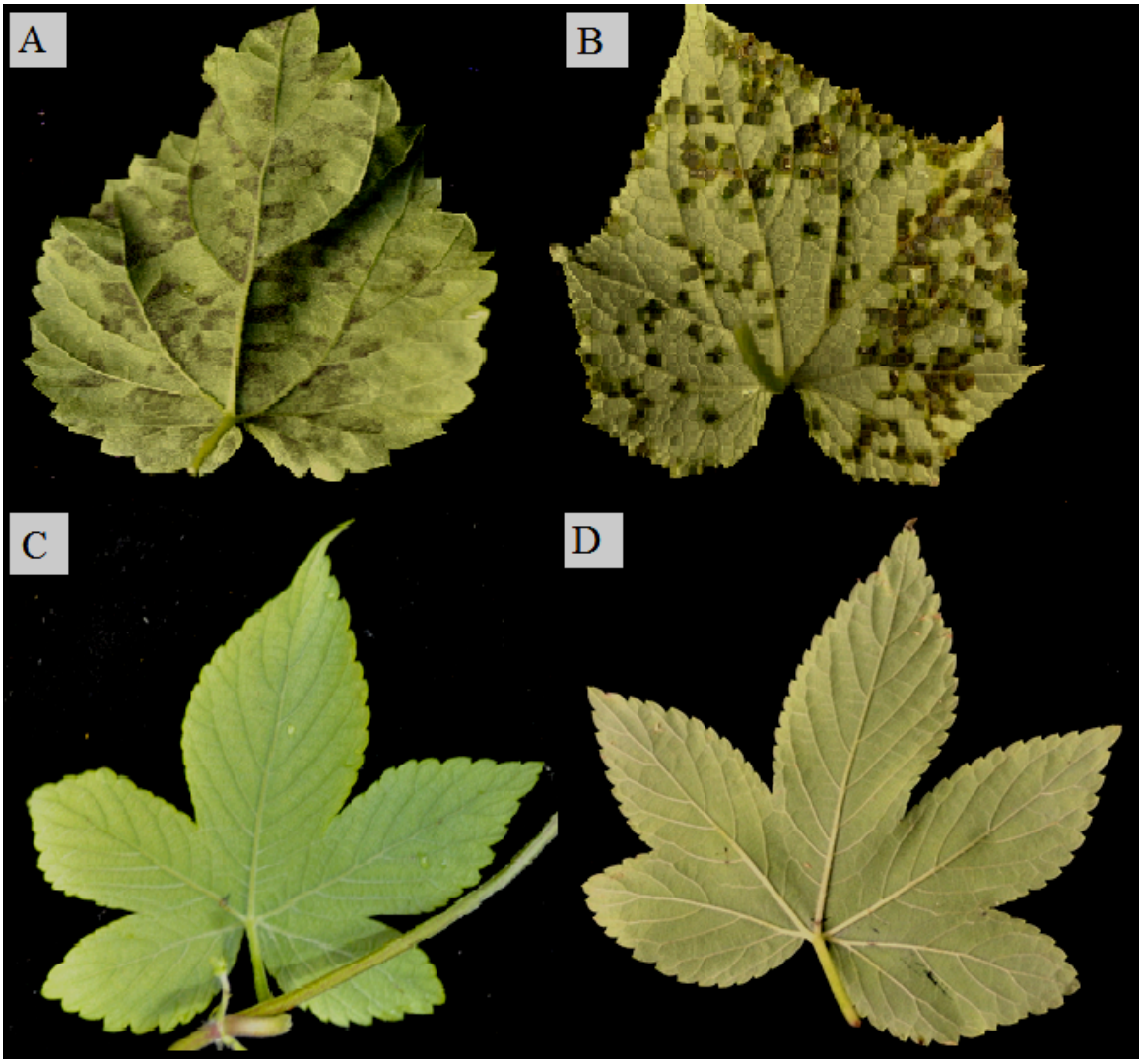


Figure 4. Macroscopic symptoms for positive controls and experimental replicates for host range experiment with *P. humuli* and *P. cubensis* on *H. japonicus*. A) Positive control for *P. humuli* on *H. lupulus*, B) Positive control for *P. cubensis* on *Cucumis Sativus*, C) Representative experimental replicate of *P. humuli* on *H. japonicus* and, D) Representative experimental replicate of *P. cubensis* on *H. japonicus*.

Table 4. Results of host range experiment using *P. cubensis* and *P. humuli* on *Humulus japonicus* and cucumber

Pathogen	Isolate	Origin	Infection rate of *Humulus japonicus*[a]	Infection rate of cucumber[a]
P. cubensis	CDM275[d]	USA, NC	0/15	—
P. humuli	HDM457[b]	USA, OR	0/9	—
P. humuli	HDM471[b]	USA, OR	0/9	—
P. humuli	HDM472[d]	USA, OR	0/12	—
P. humuli	HDM481[d]	USA, NY	0/12	0/6
P. humuli	HDM482[d]	USA, NY	0/12	0/6
P. humuli	HDM483[b]	USA, NY	0/6	0/6
P. humuli	HDM491[c,e]	Japan	1/6	0/6
P. humuli	HDM493[c]	Japan	0/6	0/6

[a] Infection rate is defined as number of individual plant replicates with sporulation per total number of replicates. Positive controls were characterized by profuse sporulation on native host and negative controls on negative host were always free of hypersensitive response (HRS) and sporulation on the 7th day rating.
[b] Inoculated on U.S. *H. japonicus* succession, CHUM1022
[c] Inoculated on Chinese *H. japonicus* succession, CHUM798
[d] Inoculated on both U.S and Chinese *H. japonicus* succession
[e] Single sporangiophore recorded

CHAPTER 4: DISCUSSION

The concatenated trees presented in this study consistently formed three clades in every analysis, corroborating the conjecture that *Pseudoperonospora* spp. responsible for the recent epidemic on cucurbits form phylogenetic clusters that are separate from the pre-epidemic cucurbit pathogen as presented in Runge et al. (2011). Similar to Runge et al. (2011) all pre-epidemic (pre-1984 European, Pre-2004 United States) *P. cubensis* isolates formed a monophyletic clade (clade 2). There were no new *P. cubensis* isolates included in this study that clustered with this pre-epidemic clade as expected because all additional *P. cubensis* isolates from both Asia and the United States were collected after 2005. All *P. humuli* isolates (24 isolates introduced in this study from three continents) formed a monophyletic clade with *P. humuli* isolates from Runge et al. (2011).

In Bayesian and Maximum Likelihood analyses, 4 of the 9 post-epidemic Korean *P. cubensis* isolates from this study were basal within the clade 1 *P. cubensis* post-epidemic cluster. The placement of these Korean *P. cubensis* isolates suggest that they are ancestors to the other post-epidemic isolates within the clade and that they are quite possibly more closely related to the pre-epidemic isolates which would explain a potential geographic origin of the more virulent, post-epidemic *P. cubensis* strain. There are 4 Japanese *P. cubensis* pre-epidemic isolates that were collected prior to 1943 that clustered within the post-epidemic clade 1 *P. cubensis*. These Japanese isolates were collected before 1943, which precedes the release of resistant cucumber cultivars Palmetto and Poinsett in 1948 and 1966, respectively (Holmes et al. 2006). This suggests that the pathogen responsible for the pandemic outbreaks of cucurbit downy mildew included in this study may have originated in Japan and only recently (1984 in Europe, 2004 in U.S.) migrated to other parts of the world. Pre-epidemic clade 2

includes a single post-epidemic Korean *P. cubensis* isolate on *Cucumis melo* collected in 2006. In conjunction with the 4 Korean post-epidemic isolates placing basal within the post-epidemic cluster, as well as the placement of the single post epidemic Korean *P. cubensis* isolate within the pre-epidemic clade, demonstrates that Korea may be the origin of virulent *P. cubensis* strains present in the post-epidemic clade.

Asian *Pseudoperonospora* isolates have been shown to cluster separately from non-Asian *Pseudoperonospora* species (Sarris et al. 2009); of particular interest, Korean *Pseudoperonospora* isolates on *Humulus japonicus* has been shown to sub-cluster within *P. cubensis* clades in a recent phylogenies (Mitchell et al. 2011, Sarris et al. 2009). In order to observe the potential Asian bifurcation presented in Sarris et al. (2009), 7 Japanese *P. humuli* isolates were included in this analysis; however, all *P. humuli* isolates clustered with other *P. humuli* isolates, validating a monophyletic *P. humuli* clade presented in Runge et al. (2011). *P. humuli* isolates that are clustered within this clade are derived from a variety of geographic locations including from Argentina, Czech Republic, Germany, Japan, Poland and the United States indicating that there is not a geographic effect of *P. humuli* on *Humulus lupulus*.

Consistent with previous studies (Mitchell et al. 2011, Runge et al. 2011, Sarris et al. 2009), *P. humuli* on *H. japonicus* from Korea was shown to be basal to the *P. cubensis – P. humuli* complex in all concatenated analyses. Runge et al. (2011) hypothesized that *P. cubensis* arose via a host jump from hop to Cucurbitaceae via *H. japonicus* due to the presence of a monophyletic clade of two *P. humuli* on *H. japonicus* isolates that occupied a basal position to the *P. cubensis* cluster in an unpublished tree. It was not reported whether this relationship was observed from a single locus analysis

of *coxII* or *ypt1*. Results of the concatenated analyses in this study did not indicate any monophyletic clades that included only isolates of *P. humuli* on *H. japonicus* that were basal to only the *P. cubensis* pre-epidemic clade as reported by Runge et al. (2011). A presumably different *P. humuli* isolate (HDM421) on *H. japonicus* from Korea than what was used in Runge et al. (2011) was included in single locus analysis of the ITS and *coxII* locus. Bayesian analysis of the *coxII* locus (Appendix, Fig. 4) showed that a monophyletic clade of *P. humuli* on *Humulus japonicus* (HDM421, D149) placed basal within post-epidemic clade 1 cluster, which is named a 'cryptic species' by Runge et al. (2011). However, this same monophyletic clade was also placed basal to clade 2 *P. cubensis* – clade 3 *P. humuli* complex for Maximum Likelihood analysis (Appendix, Fig. 5) in addition to forming a polytomy with clades 1, 2 and 3 in Minimum Evolution analysis (Appendix, Fig. 6). All three analyses of the ITS locus placed HDM421 and D149 within the *P. cubensis* cluster (Appendix, Figs. 1-3). Analysis of *ypt1* locus using HDM421 was not conducted because sequence data could not be obtained for this locus. Although two *P. humuli* isolates on *H. japonicus* from Korea formed a monophyletic clade in all *coxII* analyses, based on analyses of both the ITS and *coxII* loci, as well as the concatenated analyses presented in this study, there is no evidence of a host jump from hop to Cucurbitaceae via *H. japonicus* as reported by Runge et al. (2011).

Analysis of the ITS locus showed that 3 of the 5 *P. humuli* on *H. japonicus* isolates from Korea (HDM421, SMK19582 and SMK 11608) cluster within the *P. cubensis* clade for all three methods, similar to the results of Sarris et al. (2009) indicating that there may be host and/or geographic effects. Sequence data of other loci

(*coxII*, *ypt1*) for these isolates could not be obtained and are therefore not conclusive, consequently these trees are reported in the Appendix (Appendix, Figs. 1-3).

Sequence data from the entirety of Runge et al. (2011) was included in this study, but attempts to reconstruct the trees from their data was unsuccessful using the methods provided in their paper. Topologies of the reconstructed trees using their concatenated data set differed for each method. When sequence data from Runge et al. (2011) was included with isolates from this study, the Maximum Likelihood tree for the concatenated dataset (Figure 2) is the only tree that resembles the same topology as the Minimum Evolution tree reported in Runge et al. (2011). Interestingly, the reported tree in Runge et al. (2011) is cited as the Minimum Evolution topology, however, the bifurcation of *P. cubensis* (clade 1) and *Pseudoperonospora* cryptic species (clade 2) is only supported with Maximum Likelihood analysis, which substantiates the results of the Maximum Likelihood analysis from this study. Similarly, Bayesian analysis of the concatenated data set showed a different topology than what was reported in Runge et al. (2011), however the same arrangement of clades 1, 2 and 3 as a reconstructed Minimum Evolution analysis of Runge et al. (2011). Minimum Evolution analysis produced a polytomy for the concatenated data set, consistent with the reconstructed tree for their data using Bayesian analysis. Clade ancestry differed for reconstructed analyses of sequence data from Runge et al. (2011) and were similar to the trees produced in this study. Therefore, different loci with higher resolution and alternative methods are needed to validate and reconstruct the ancestry of these pathogens.

The purpose of conducting host range experiments on *H. japonicus* was to test the pathogenicity of post-epidemic *P. humuli* and *P. cubensis* pathogens in order to

better understand the possible host jump from hop to Cucurbitaceae via *H. japonicus*. *P. humuli* isolates used for host range experiments originated from the United States and Japan to account for potential differences in host range among geographical locations. The results of the host range experiment on *H. japonicus* had an infection rate of 1/72 plant replicates for *P. humuli*, with the only infection presenting on a single sporangiophore suggesting that there is no geographic host effect. An infection rate of 0/15 was observed for *P. cubensis* on *H. japonicus*.

In addition, a single *P. cubensis* isolate from the U.S. was included, which belongs to the post-epidemic clade I. Previous host range tests using U.S. *P. cubensis* isolate CDM275 on hop yielded 100% infectivity (Mitchell et al. 2011) and this isolate was chosen as representative of the U.S. population. No infection of *H. japonicus* was observed. Unfortunately, live Asian *P. cubensis* isolates could not be obtained for use in this study in order to test for geographic host effect. The near-zero infectivity of both *P. humuli* and *P. cubensis* pathogens shows that *H. japonicus* may be resistant to the pathogens used in this experiment, indicating a possible source of resistance genes. These results suggest that if a host jump of *P. humuli* to *P. cubensis* via *H. japonicus* occurred this host jump is complete.

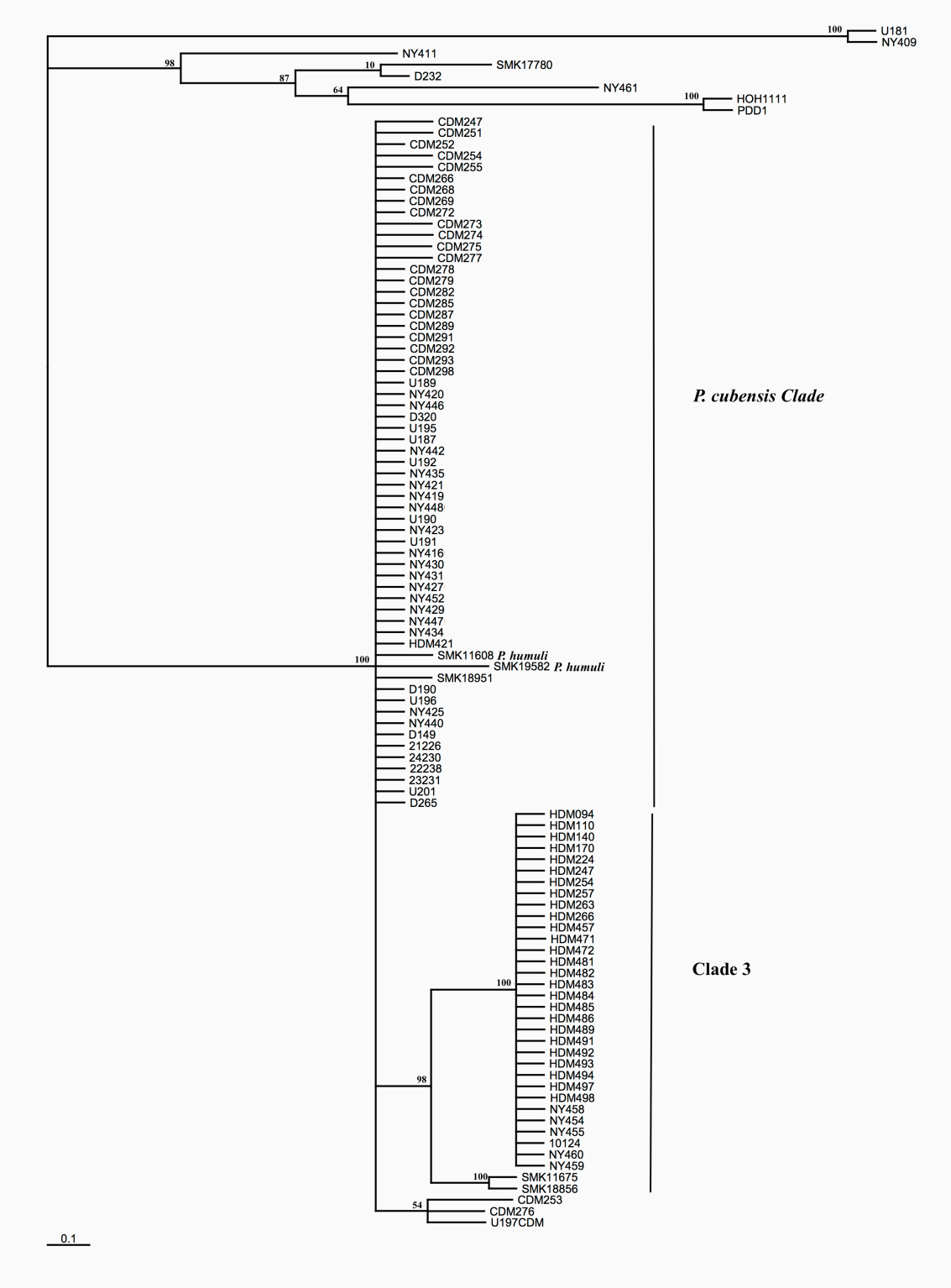
Interestingly, previous host specificity studies have produced different pathogenicity results for *P. humuli* on cucumber (Cucumis sativus)—one study indicated a single sporangiophore in 33 replicate plants for U.S. *P. humuli* (Mitchell et al. 2011) and the second study reported a low rate of infection for 7 of 25 trials using *P. humuli* from Germany (Runge et al. 2012). This suggests potential differential virulence or plant susceptibility for *P. humuli* pathogenicity on cucumber.

Host specificity on common hop yielded similar infectivity in previous studies: 79% infectivity for U.S. *P. cubensis* isolates (Mitchell et al. 2011) and 25% infectivity for Czech Republic *P. cubensis* on hop and 64% for *Bryonia dioica* from the family, Cucurbitaceae (Runge et al. 2012). In light of these infectivity rates, it would seem that the lower rates of European *P. cubensis* infectivity on hop than that with U.S. *P. cubensis* indicates that the European strain in these studies represent a less virulent strain of *P. cubensis* in Europe than in the U.S. because of its divergence from hop as a natural host; this relationship is consistent with the 20 year gap in recent epidemic beginning in Europe in 1984 in comparison to the 2004 epidemic in North America. Asian *P. cubensis* isolates are needed in order to better derive the evolutionary origins of *P. cubensis* via host jump on *H. japonicus* as this would be the most likely pathotype to infect this host. If *H. japonicus* did in fact bridge the evolution of *P. cubensis* from *P. humuli*, a German or other European *P. humuli* isolate should be tested on *H. japonicus* to observe whether there are more virulent strains of *P. humuli* that gave rise to *P. cubensis*.

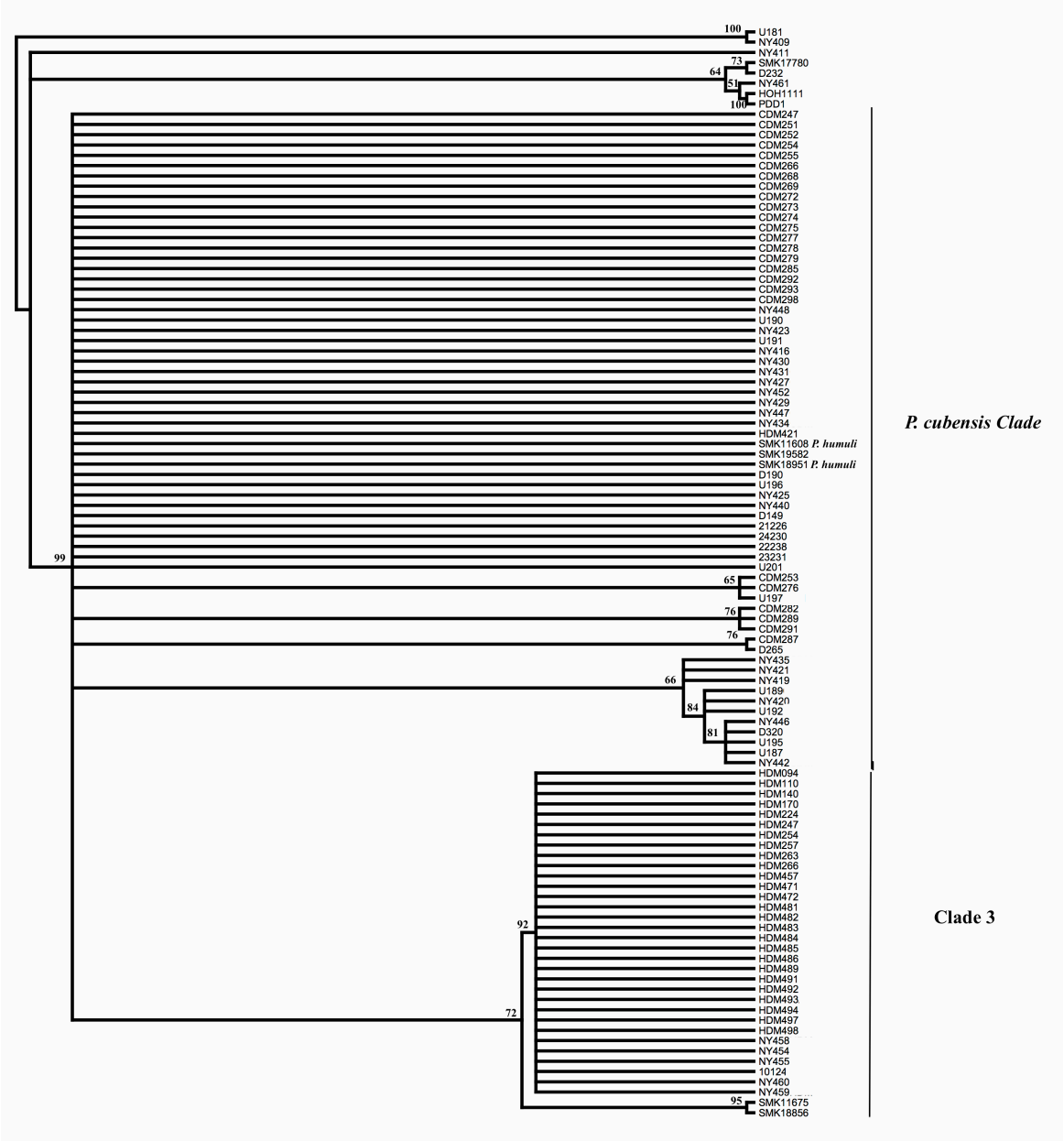
The work done in this study supports the conjecture that *Pseudoperonospora* spp. responsible for recent epidemic on cucurbits form phylogenetic clusters that are separate from the pre-epidemic cucurbit pathogen. Pre-epidemic *P. cubensis* strains from Korea should be included in future analyses to observe if they cluster with post-epidemic isolates similar to the Japanese *P. cubensis* isolates in order to further provide support for the East Asian origin of *P. cubensis*. The ancestry of the *P. cubensis* – *P. humuli* complex cannot be resolved from the phylogenetic analyses employed in this study, therefore, additional methods including coalescent analysis should be used to

understand the evolutionary origins of *Pseudoperonospora* spp. Also, a broader collection of *P. humuli* on *H. japonicus* isolates that can be amplified for multiple loci, as well as pre-epidemic *H. japonicus* isolates from Korea, should be included in future analyses, using higher resolution loci to better resolve the host/geographic effect on ancestry. Strains of *P. cubensis* from Asia are necessary to further clarify the potential host jump from *P. humuli* to *P. cubensis* as this is most likely where it originated.

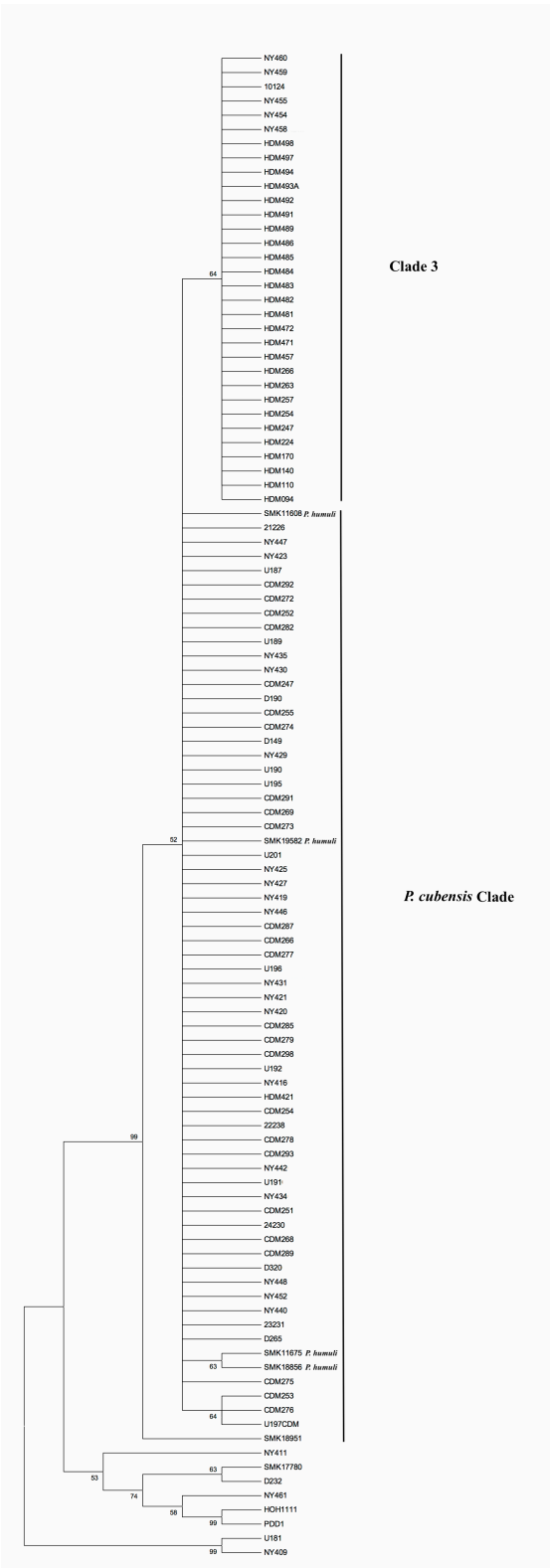
APPENDIX



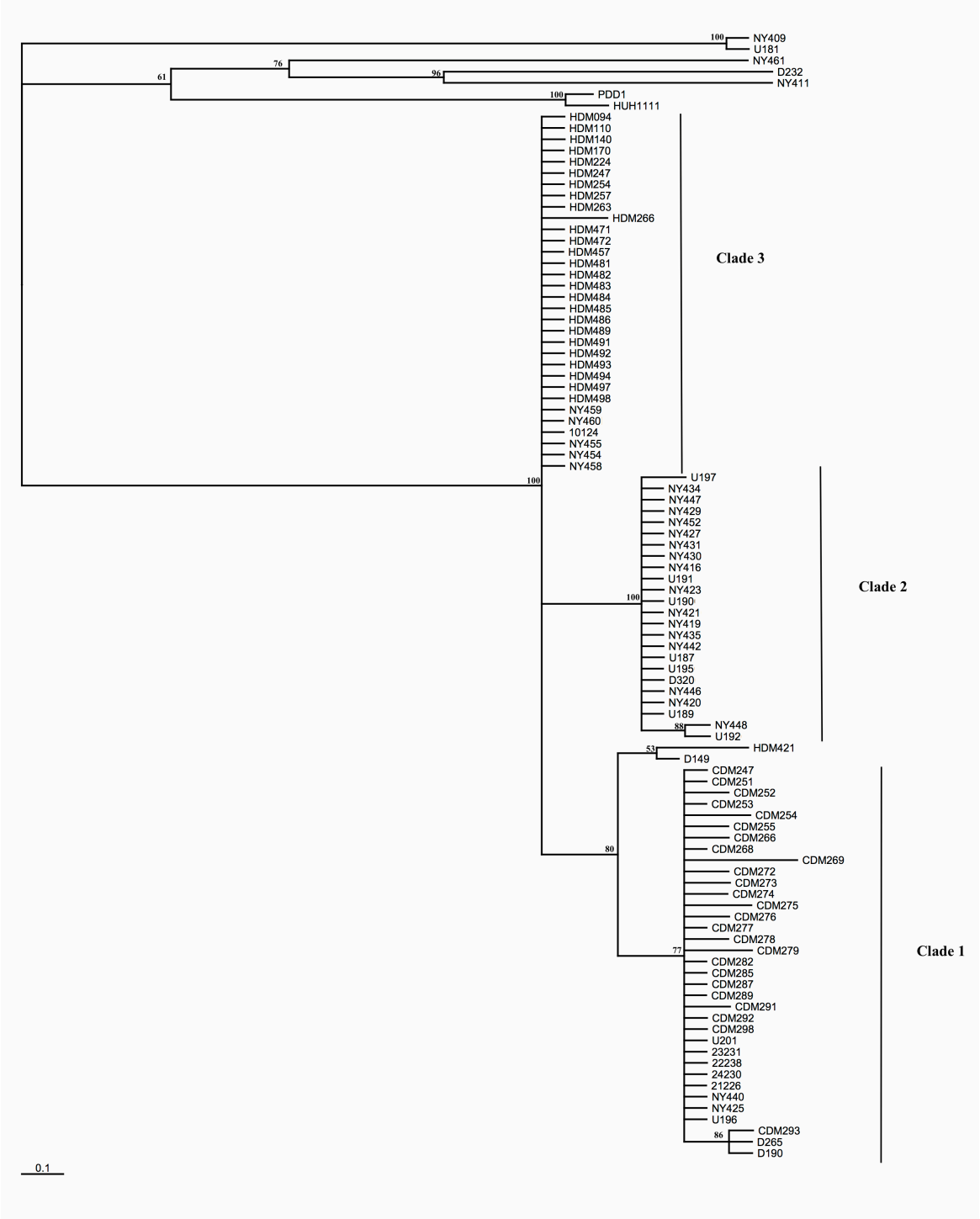
Appendix, Figure 1. Phylogenetic tree for ITS locus using Bayesian inference under the Hasegawa, Kishino and Yano (HKY) substitution model assuming gamma-distributed substitution rates



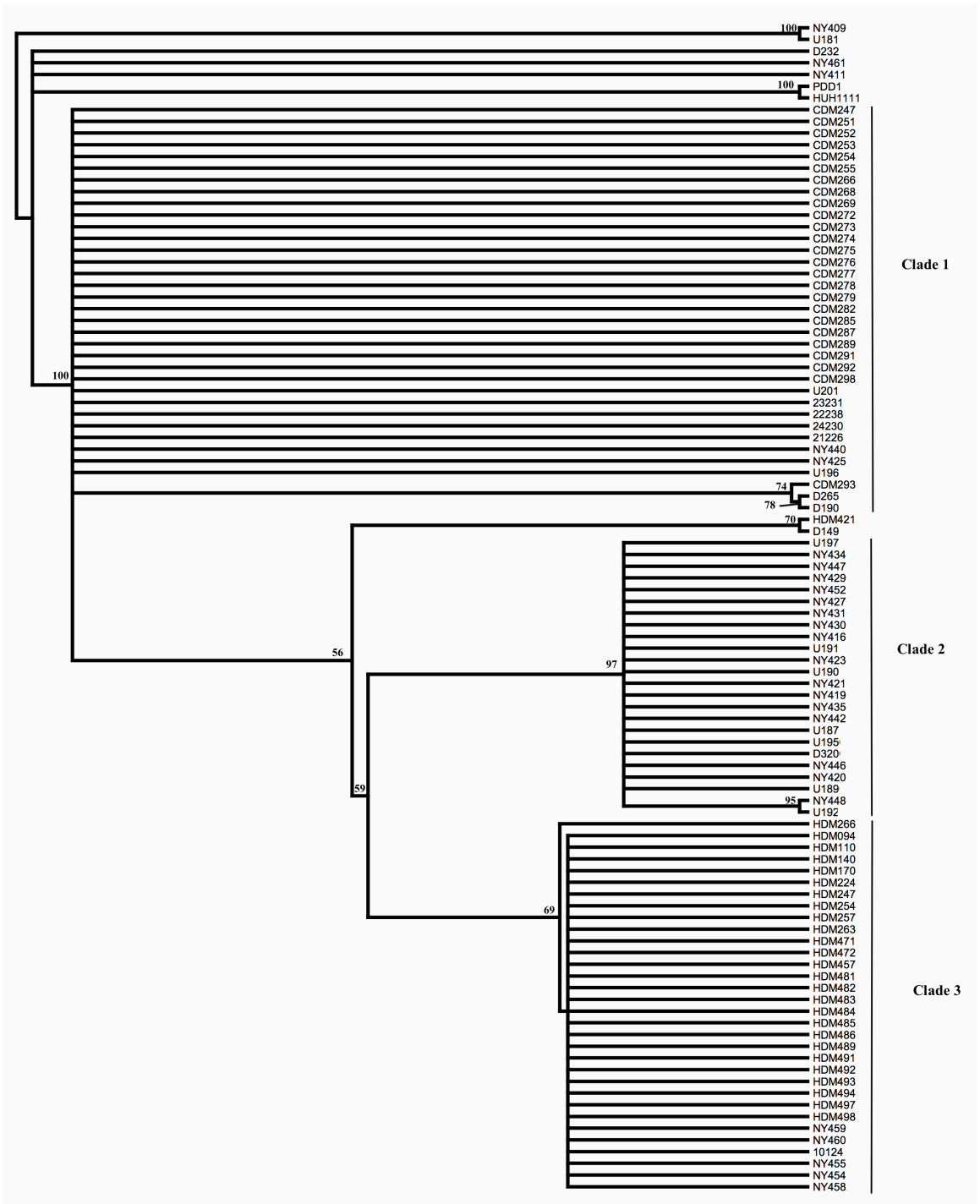
Appendix, Figure 2. Phylogenetic tree for Maximum Likelihood analysis of ITS locus using 1,000 bootstrap replicates.



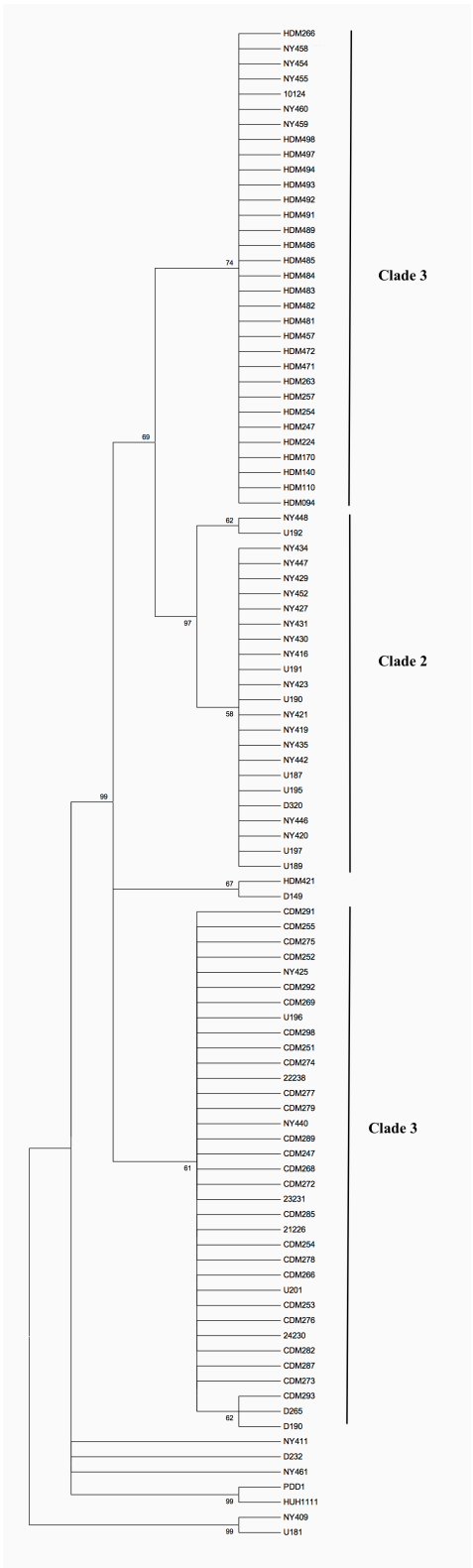
Appendix, Figure 3. Phylogenetic tree for Minimum Evolution of ITS locus under Tamura-Nei substitution model and 1,000 bootstrap replicates



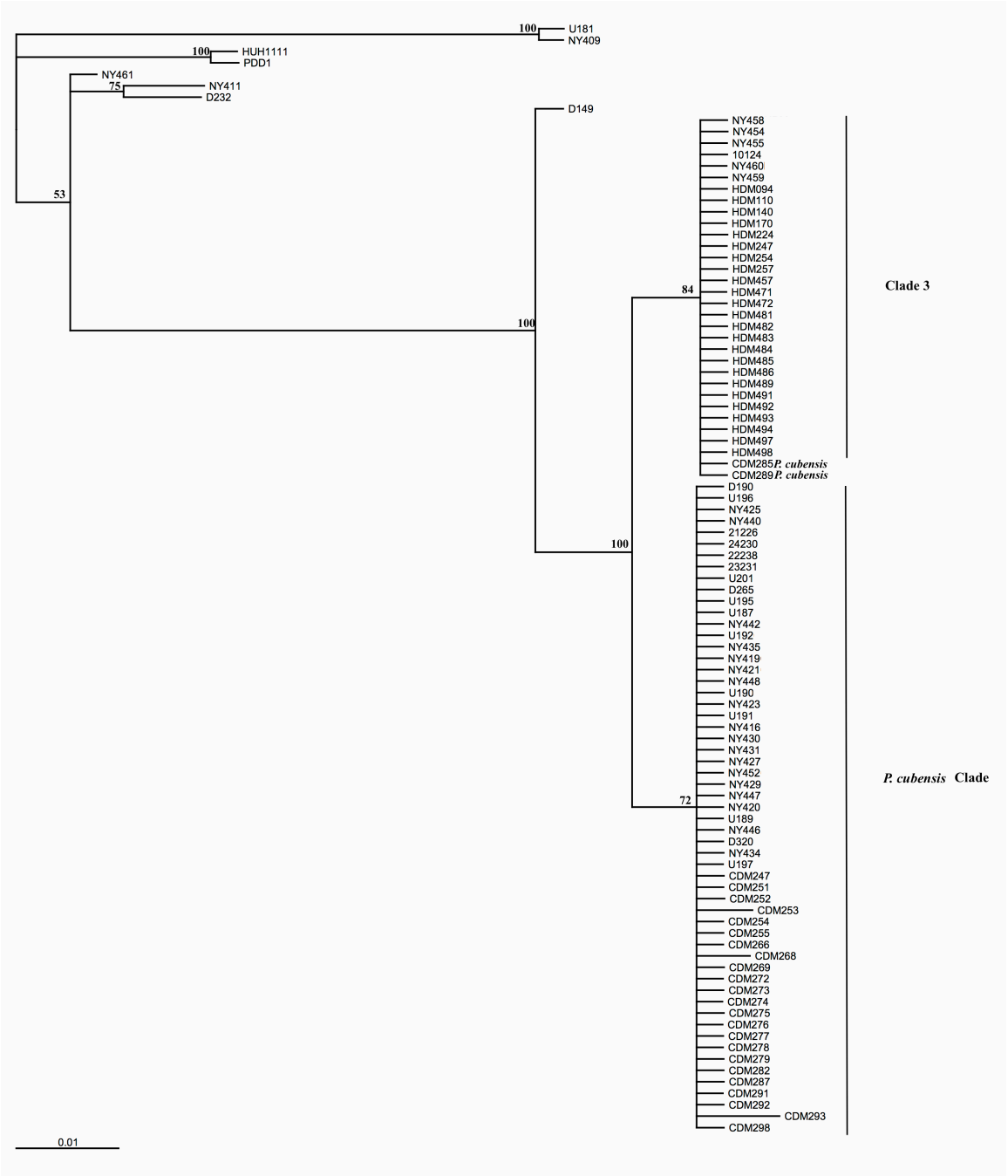
Appendix, Figure 4. Phylogenetic tree for *coxII* locus using Bayesian inference under the general time reversible (GTR) substitution model assuming gamma-distributed substitution rates and invariant nucleotide sites.



Appendix, Figure 5. Phylogenetic tree for Maximum Likelihood analysis of *coxII* locus using 1,000 bootstrap replicates.

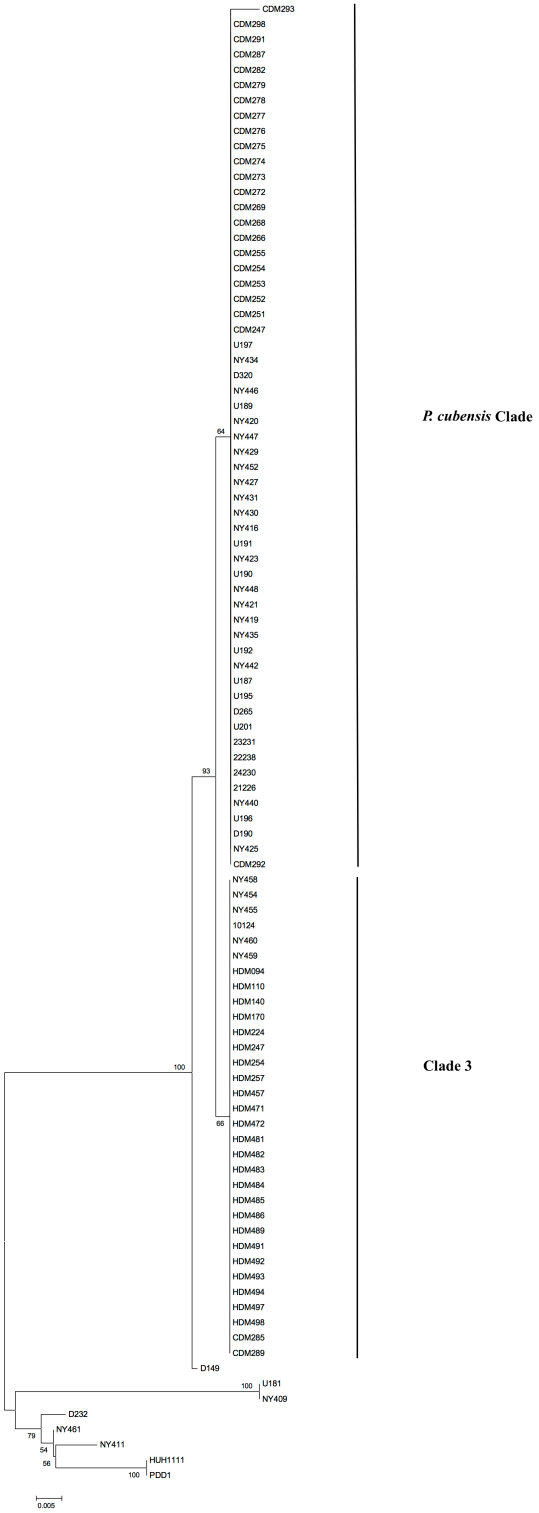


Appendix, Figure 6. Phylogenetic tree for Minimum Evolution of *coxII* locus under Tamura-Nei substitution model and 1,000 bootstrap replicates



Appendix, Figure 7. Phylogenetic tree for *ypt1* locus using Bayesian inference under the Kimura (K80) substitution model

Appendix, Figure 8. Phylogenetic tree for Maximum Likelihood analysis of *ypt1* locus using 1,000 bootstrap replicates.



Appendix, Figure 9. Phylogenetic tree for Minimum Evolution of *ypt1* locus under Tamura-Nei substitution model and 1,000 bootstrap replicates

GLOSSARY

Basal foliage: A basal leaf is one that grows from the lowest part of the stem. Basal, in general, refers to the base of a structure.

Bayesian Inference: Inferences of phylogeny are based upon the posterior probabilities of phylogenetic trees derived from a computational program that uses Bayes theorem. Advantages over other methods of phylogenetic inference include: easy interpretation of results, ability to incorporate prior information (if available) and some computational advantages.

Bifurcation: Splitting of a main body into two parts.

Bines: The climbing "vine" of the hop plant, which climbs by its shoots growing in a helix around a support using stiff hairs to aid in climbing.

Bioedit: A free sequence alignment editor.

Bract: A petal-like structure on the hop cone. The bract is always sterile (never bears a seed at its base).

Bracteole: A petal-like structure on the hop cone. The bracteole is capable of bearing a seed at its base.

Chlorosis: A condition in which leaves produce insufficient chlorophyll. As chlorophyll is responsible for the green color of leaves; chlorotic leaves are pale, yellow, or yellow-white.

Clade: A group consisting of an organism and all its descendants. In phylogeny, a clade is a single branch.

Coalescent analysis: Coalescent analysis is a population genetic analysis tool that reconstructs the genealogical history of a population sample by building a tree going backward in time by pairwise coalescing of samples sharing a common ancestor until all samples coalesce to the most common recent ancestor for the overall sample.

Cox Cluster: Consists of *coxI*, *coxII*, and the spacer between *coxI* and *coxII*.

Cross-inoculation: The process of using an inoculum specific to a particular host species to infect a different host species.

Cultivar: A plant or group of plants selected for desirable characteristics that can be maintained by propagation.

Deionized water: Water that has had its mineral ions removed, such as cations from sodium, calcium, iron, copper and anions such as chloride and bromide.

Desiccant: A chemical used to remove plant foliage.

Diurnal: A pattern that occurs daily.

Dormant: Having normal physical functions suspended or slowed down for a period of time.

Fungicides: Chemical compounds or biological organisms used to kill or inhibit fungi or fungal spores.

Genealogical: Study of species relatedness using DNA analysis.

Genome: The entirety of an organism's hereditary information encoded in DNA.

Germinate: The process in which a plant or fungus emerges from a seed or spore, respectively, and begins growth.

Hemocytometer: A device originally designed for the counting of blood cells. It is also used to count other types of cells and particles, such as spores.

Herbarium specimen: A collection of preserved plant specimens. These specimens may be whole plants or plant parts: these will usually be in a dried form.

Humid chamber: Chamber that controls the amount of humidity presented to the chamber contents.

Hypersensitive response (HR): A mechanism used by plants to prevent the spread of infection by microbial pathogens. The HR is characterized by the rapid death of cells in the local region surrounding an infection. The HR serves to restrict the growth and spread of pathogens to other parts of the plant.

Inoculation: Refers to the communication of a disease to a living organism by transferring its causative agent into the organism.

Inoculum: The microorganism used in an inoculation.

Internal Transcribed Spacer (ITS): Piece of non-functional, nuclear RNA situated between structural ribosomal RNAs.

Lesion: Any abnormal tissue found on or in an organism, usually damaged by disease.

Lineage: A sequence of species that form a line of descent, each new species is the direct result of speciation from an immediate ancestral species. Determined by molecular systematics.

Locus (plural loci): The specific location of a gene or DNA sequence on a chromosome.

Monosporangial: Consisting of a single sporangium.

Morphology: The form and structure of organisms, which includes aspects of the outward appearance (shape, structure, color, pattern) as well as the form and structure of the internal parts.

Mycelium (plural mycelia): The vegetative part of a fungus, consisting of a mass of branching, thread-like hyphae.

NCBI Genbank: The National Center for Biotechnology Information databank for genomic information.

Necrosis: The premature death of cells and living tissue. Necrosis is caused by factors external to the cell or tissue, such as infection, toxins, or trauma.

Nucleotide: Molecules that, when joined together, make up the structural units of RNA and DNA.

Obligate biotroph: An organism that extracts nutrients only from living plant tissue and cannot grow apart from their hosts.

Oospore: A thick-walled sexual spore that develops in oomycete organisms.

Organoleptic: Pertaining to the sensory properties of a particular food or chemical, the taste, color, odor and feel.

Overwinter: Where a plant pathogen survives the winter, during which its normal crop host species is not growing, by transferring to an alternative host, living freely in the soil or surviving on plant refuse.

Parsimony: A non-parametric statistical method commonly used in computational phylogenetics for estimating phylogenies. Under maximum parsimony, the preferred phylogenetic tree is the tree that requires the least evolutionary change to explain some observed data.

Pathotype: An infrasubspecific classification of a pathogen distinguished from others of the species by its pathogenicity on a specific host(s).

Perennial: A plant that lives for at least 2 years without needing to replant.

Photoperiod: The duration of an organism's daily exposure to light.

Phylogenetic Analysis: The use of the structure of molecules to gain information on an organism's evolutionary relationships. The result of a molecular phylogenetic analysis is expressed in a phylogenetic tree.

Phylogeny: The connections between all groups of organisms as understood by ancestor/descendant relationships in phylogenetic trees.

Plasmid: A DNA molecule that is separate from, and can replicate independently of, the chromosomal DNA.

Polymerase Chain Reaction (PCR): A scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Posterior probability: The probability that a particular hypothesis is true given some observed evidence, such as molecular sequence analysis.

RAxML Analysis: A fast implementation of maximum-likelihood (ML) phylogeny estimation that operates on both nucleotide and protein sequence alignments.

rDNA: Ribosomal DNA.

Sister taxa: Any taxa derived from a common ancestral node.

Sporangium (plural sporangia): An enclosure in which spores are formed.

Sporangiophore: A branch structure bearing sporangia.

Spore: A reproductive structure that is adapted for dispersal and surviving for extended periods of time in unfavorable conditions.

Sporulation: The process of spore formation.

Stoma (plural stomata): A pore, found in the leaf and stem epidermis in a plant that is used for gas exchange.

Systemic: A disease that infects the plant as a whole.

Taxon (plural taxa): A group of (one or more) organisms, which a taxonomist adjudges to be a unit.

Taxonomy: The practice of classification and nomenclature for all organisms.

Zoospore: A motile asexual spore that uses a flagellum for locomotion. Fungi use a zoospore to propagate themselves.

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