AN ABSTRACT OF THE THESIS OF

Melanie N. Mitchell for the degree of Master of Science in Botany and Plant Pathology presented on June 2, 2010.

Title: Addressing the Relationship Between *Pseudoperonospora cubensis* and *P. humuli* using Phylogenetic Analyses and Host Specificity Assays.

Abstract approved:

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The two most economically important plant pathogens in the *Pseudoperonospora* (Peronosporaceae) genus are *P. cubensis*, causal agent of cucurbit downy mildew, and *P. humuli*, causal agent of hop downy mildew. These organisms have been shown to be very closely related phylogenetically and morphologically. In 2005, researchers in Korea proposed that based on morphological similarities and internal transcribed spacer of nuclear ribosomal DNA (ITS nrDNA) sequence data, *P. humuli* should be reduced taxonomically to a synonym of *P. cubensis*. As this taxonomic change has implications for identification, management, and regulation, the current study further explores this issue using multigenic analyses and host specificity experiments.

Multigenic sequence analyses were conducted considering five loci for 21 isolates of *P. cubensis* and 14 isolates of *P. humuli*. The five loci used in the analysis were the ITS, β-tubulin gene, cytochrome *c* oxidase II gene (*cox2*), cytochrome *c*

oxidase I gene (*cox1*), and the spacer between *cox2* and *cox1*. Additionally, the cytochrome *c* oxidase genes and spacer were combined for analysis as the *cox* cluster, and all five loci were concatenated for a robust analysis using Bayesian and maximum likelihood inference. Although the topology and statistical support for the topology for each locus differed, there was a consistent separation of a majority of the *P. humuli* isolates and the *P. cubensis* isolates. The primary exceptions were an isolate of *P. humuli* from Korea on *Humulus japonicus* and an isolate of *P. cubensis* from North Carolina on acorn squash.

Two reportedly universally susceptible hosts of *P. cubensis* (cucumber cv. Straight 8 and cantaloupe cv. Ananes Yokneam) were inoculated with four isolates of *P. humuli* from the western U.S. Two highly susceptible hosts of *P. humuli* (cvs. Nugget and Pacific Gem) were inoculated with eight isolates of *P. cubensis* from the eastern U.S. *P. cubensis* frequently infected the hop cultivars but at low rates (77% of replicate plants, typically with fewer than a thousand sporangia per plant) while *P. humuli* produced only one sporangiophore during the course of the studies (3% of replicate plants). Thus, there is evidence that biologically relevant characteristics exist that differentiate the two organisms with implications for the detection and management of both that may be concealed by the reduction of *P. humuli* to a taxonomic synonym of *P. cubensis*.

Addressing the Relationship Between *Pseudoperonospora cubensis* and *P. humuli* using Phylogenetic Analyses and Host Specificity Assays

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Melanie N. Mitchell, Author

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Rationale for Research

Within the genus *Pseudoperonospora* (Peronosporaceae), the most economically important species are *P. cubensis* (Berk. & M.A. Curtis) Rostovzev (causal agent of cucurbit downy mildew) and *P. humuli* (Miyabe & Takah.) G.W. Wilson (causal agent of hop downy mildew). These species have been found to be sister to each other relative to other *Pseudoperonospora* species (Choi et al. 2005, Riethmüller et al. 2002, Voglmayr 2003), and Choi et al. (2005) suggested that *P. cubensis* and *P. humuli* were synonymous based on morphometric characters and internal transcribed spacer of nuclear ribosomal DNA (ITS nrDNA) sequence data. As this reduction from two species into one has implications for identification, management, and regulation, further investigation of this claim is required. For the purpose of this thesis, *P. cubensis* and *P. humuli* will be referred to as different species.

Hop Plant and Production

The cultivated hop plant (*Humulus lupulus* L.) is a dioecious plant in the family Cannabaceae. There are two other species of *Humulus*, *H. japonicus* Siebold & Zucc., and *H. yannanensis* Hu. It is hypothesized that the center of origin for the genus is China, as all three species are natively found there (Neve 1991). While *H. lupulus* is

indigenous throughout the northern hemisphere between about 35° and 70°N, the other two species are indigenous to China and Japan (*H. japonicus*) or solely to China (*H. yannanensis*; Neve 1991). *H. japonicus* is primarily an annual species, although there are suggestions that it is occasionally able to survive for more than a single season (Neve 1991). Very little is known about *H. yannanensis* except that it occurs at high altitudes in southern China at latitudes of approximately 25°N and is a perennial (Neve 1991).

H. lupulus is a perennial climbing plant, using hooked hairs on bines to twine in a clockwise direction around a support. The economically important features of the hop plant are the resins and essential oils found in lupulin glands which give beer its bitterness, aroma, and distinctive flavor (Miyabe & Takahashi 1906). These are found in the highest concentrations in the female inflorescence, or cone. Hop cones, also known as strobiles, or "hops," are produced in greatest quantity on lateral branches, with flowering occurring in late June or early July in the Pacific Northwestern United States (U.S.).

Currently, commercial hop production in the U.S. is mainly limited to Idaho, Oregon, and Washington. The combined hop growing acreage in the Pacific Northwest was 16,089 hectares in 2009, producing 42,945 metric tons of hops, which corresponds to about 30% of the world production in both acreage and production (George 2010). Hop cones are an international trading commodity: each region of the world tends to specialize in certain cultivars, thus trade is necessary to attain a particular taste and bitterness for a specific beer.

The cultivation of hop plants dates back to the 700's CE (Miyabe & Takahashi 1906). Hop plants were first grown in Germany but spread throughout Europe and are now produced on every continent except Antarctica (Barth et al. 1994). In the U.S., hop production began on the east coast soon after the first European settlers arrived (Tomlan 1992). Crops in the eastern and Midwestern U.S. were first attacked by downy mildew, caused by *Pseudoperonospora humuli*, and then by powdery mildew, caused by *Podosphaera macularis* (Wallr.) U. Braun & S. Takam, resulting in the virtual elimination of commercial U.S. hop production in these regions. Hop downy mildew also was responsible for largely confining hop cultivation to the regions of the Pacific Northwest that are relatively arid during most of the growing season (Skotland 1961).

Cucurbit Crops and Production

The gourd family, Curcurbitaceae, is one of the more important and widespread plant families that provide food and fiber for humans (Sitterly 1972). Although cucurbits are not as important as the cereals and legumes at a global scale, they are significant in the tropics, subtropics, and milder portions of temperate zones (Sitterly 1972, Whitaker & Davis 1962). In addition to being a source of carbohydrates, some cucurbits are used as decoration, pottery, baskets, insulation, oil filters, and in ethnopharmacology (Schultes 1990, Sitterly 1972, Whitaker & Davis 1962).

Cucurbitaceae is composed of about 90 genera and about 750 species with both annuals and perennials, although about 6 genera and 12 annual species are cultivated by man (Sitterly 1972). These include cucumber, muskmelon, and gherkin (*Cucumis* L.); watermelon (*Citrullus* Forssk.); squashes, marrow, pumpkin, and figleaf gourd (*Cucurbita* L.); dish-rag and sponge gourds (*Luffa* Mill.); white-flowered gourd (*Lagenaria* Ser.); and chayote (*Sechium* P. Br.). All cucurbits are frost-sensitive, meaning that the whole plant dies after the growing season in non-tropical regions (Robinson & Decker-Walters 1997, Sitterly 1972, Whitaker & Davis 1962).

Many species of cucurbits are suspected to have originated from tropical or subtropical regions of either Africa or southeastern Asia, specifically India, Indo-Malaysia, and southern China. The major exception to this trend is the genus *Cucurbita*, which arose in Mexico and South America (Kalloo & Bergh 1993, Robinson & Decker-Walters 1997, Sanjur et al. 2002). In the genus *Cucumis*, there are about 30 species which are geographically segregated into a large (approximately 20 spp.) group originating in Africa with a basic chromosome number of x=12 (including *C. melo* L., melon) and a small (approximately 10 spp.) group originating in southeast Asia with a basic chromosome number of x=7 (including *C. sativus* L., cucumber; Kalloo & Bergh 1993, Robinson & Decker-Walters 1993). *C. sativus* is believed to be indigenous to an area south and east of the Himalayas, including Nepal, India, and southern China (Kalloo & Bergh 1993, Robinson & Decker-Walters 1993).

Downy Mildews

The downy mildews are members of the oomycete family Peronosporaceae in the kingdom Chromista (Bisby et al. 2009). This family includes 21 genera, 20 genera with species that are parasitic on plants, which comprise one of the largest groups of fungi-like organisms that parasitize flowering plants (Bisby et al. 2009, Palti & Kenneth, 1981). Most of the angiosperms affected are herbaceous dicotyledons, although a few monocotyledons and woody dicotyledons are parasitized by species of Peronosporaceae.

Important diseases of crops caused by Peronosporaceae include downy mildews of hop (causal agent *Pseudoperonospora humuli*), cucurbits (causal agent *Pseudoperonospora cubensis*), lettuce (causal agent *Bremia lactucae* Regel), brassicas (causal agent *Hyaloperonospora parasitica* (Pers.) Constant.), onion (causal agent *Peronospora destructor* (Berk.) Casp. ex Berk.), and grape (causal agent *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni) as well as blue mold of tobacco (causal agent *Peronospora tabacina* D.B. Adam). Also tentatively included in the Peronosporaceae is the genus *Phytophthora* De Bary which contains several very destructive plant pathogens (Cooke et al. 2000, Göker et al. 2003). Some of the more well known diseases caused by *Phytophthora* species include late blight of potato and tomato (causal agent *P. infestans* (Mont.) De Bary), root rot of soybean (causal agent *P. sojae* Kaufm. & Gerd.), and sudden oak death (causal agent *P. ramorum* Werres, De Cock & Man in't Veld). *Phytophthora* is the most extensively studied oomycete genus and includes the model pathosystem involving *P. brassicae* Cock & Man in’t Veld and *Arabidopsis thaliana* (L.) Heynh. (Kamoun 2003).

Hop Downy Mildew

Biology

Hop downy mildew is caused by the oomycete *P. humuli*, an obligate biotroph with a limited host range. *Pseudoperonospora humuli* reproduces both asexually and sexually. The asexual spores are zoosporangia, also called sporangia, which dehisce to become wind- or splash-dispersed. Sporangia germinate only indirectly to produce zoospores, which are the infective agents. Zoospores penetrate the hop with germ tubes through open stomata, leading to local or systemic colonization of the plant. Oospores, the sexual propagule, germinate indirectly via zoospores released from sporangia (Bressman & Nichols 1933). The role of oospores in the disease cycle has yet to be clearly established and it is uncertain whether this organism is homothallic or heterothallic. The pathogen overwinters either as mycelium in a perennially diseased hop rhizome or as oospores that produce sporangia followed by zoospores that infect shoots and leaves as they emerge from the soil (Miyabe & Takahashi 1906, Royle & Kremheller 1981). Overwintering in alternate hosts is not likely, although *P. humuli* has been found to cause limited infection in certain species of the Urticales (Rosales s.l.), which contains the Cannabaceae family. In host range studies with artificial inoculation, *P. humuli* has shown the ability to infect *Urtica*, *Cannabis*, and *Celtis* species (Hoerner 1940, Salmon & Ware 1928, Salmon & Ware 1929). However, the infections of *Urtica*, *Cannabis*, and *Celtis* species were accompanied by

hypersensitive reactions at infection sites and sporulation was relatively sparse compared to *P. humuli* on hop (Hoerner 1940).

History

Hop downy mildew was first reported on hop in Japan during 1905. In the U.S., downy mildew was found on wild hop plants in Wisconsin in 1909. During the 1920's, hop downy mildew caused severe damage throughout the hop production areas of Europe and North America. The rapidity of the spread and the degree of damage raised suspicions about the origin of the pathogen. If the pathogen was indigenous to Europe or North America, such an epidemic would not be expected. Often, when a pathogen is introduced into a new area or to a host with which it has not coevolved, a severe outbreak occurs. An excellent example of this phenomenon is the near destruction of North American stands of American chestnut (*Castanea dentata* (Marsh.) Borkh.) by the introduced ascomycete *Cryphonectria parasitica* (Murrill) Barr (Burdon 1987). It is still unknown whether *P. humuli* is native to Europe or introduced from Asia (Miyabe & Takahashi 1906). While the first record of downy mildew in America was on wild hop plants in Wisconsin in 1909, the disease was not found on cultivated hop plants until 1928 when downy mildew was found in Sardis, British Columbia. The disease later was observed in western Washington and the Willamette Valley of Oregon in 1929. Since that time, downy mildew has become established in the Pacific Northwest (Skotland & Romanko 1964).

Symptoms and Impact

The first evidence of downy mildew typically is the appearance of infected shoots known as "primary basal spikes" in the spring due to systemic infection of rhizomes. Infected shoots have shortened internodes and downward-curling leaves that are pale green to yellow and have a silvery upper surface. The primary basal spikes, once formed, cease growth and may die if not removed from the plant (Miyabe & Takahashi 1906). Sporangia can be produced in abundance on the underside of leaves on primary spikes, and initiate secondary infection of surrounding healthy leaves and shoots. Both healthy shoots and basal spikes are found on the same rhizome, and even the same node (Coley-Smith 1962, Ware 1926). Generally, the chief impact of primary basal spikes is as a source of inoculum for secondary infections of healthy shoots and cones.

Systemic infections of the rhizome occur either by zoospores washing down into the soil or by the growth of *P. humuli* down the plant into the crown. Growth of the pathogen down bines can originate from secondary infections of basal shoots in the spring, or by zoospores infecting the base of bines any time during the growing season (Coley-Smith 1965, Royle & Kremheller 1981). Disease of the rootstock is associated with crown rot, feeble shoot growth during ensuing years, or death of plants in highly susceptible cultivars (Royle & Kremheller 1981). In some cultivars, yield reduction of up to 28% can result from systemic crown infections (Coley-Smith 1962). Rootstock infection and subsequent plant death is a major economic concern in Washington State, where cultivars susceptible to crown rot are widely planted (Skotland 1961).

Infections on leaves create angular chlorotic lesions that are constrained by veins, but can coalesce when the disease is severe (Royle & Kremheller 1981). When healthy basal shoots are infected, "secondary basal spikes" result, which can be distinguished from primary basal spikes by the presence of lower internodes of normal length with at least one pair of healthy leaves (Royle & Kremheller 1981, Skotland & Romanko 1964). If downy mildew is not controlled and if the weather is favorable, the pathogen can spread up the plant. Infection of lateral shoots results in "lateral spikes," which are stunted and may not produce cones. This can gravely impact yield as the majority of cones are produced on lateral branches (Neve 1991, Royle & Kremheller 1981). The tips of the main bines also can be diseased, resulting in "terminal spikes," which cease growing and fall away from the strings (Neve 1991, Royle & Kremheller 1981, Skotland 1961). Yield impact by terminal spikes can be minimized by training lateral shoots to replace the infected bines, although this increases production costs. The major importance of downy mildew infections during the vegetative stages of growth is as a source of inoculum that can infect the cone (Neve 1991, Royle & Kremheller 1981, Skotland 1961).

One of the largest and most common impacts of downy mildew on yield, especially in Europe with a wetter environment during the growing season, is infection of the cone (Royle & Kremheller 1981). When the flower is young or in the "burr" stage when infected, the young cone hardens, turns brown, and falls off the plant (Royle & Kremheller 1981). If the cone is more developed before being attacked by downy mildew, the affected bracts and bracteoles turn reddish-brown (Neve 1991,

Royle & Kremheller 1981, Skotland 1961). The bracteoles are commonly more severely discolored than the bracts (Neve 1991, Royle & Kremheller 1981). Often, only a proportion of bracts and bracteoles are infected on a cone and the cone develops a brown and green variegated appearance. The entire cone can become discolored if all of the bracts and bracteoles are affected. Discolored hops reduce the quality and value of the crop, and may result in the entire harvest being rejected by a brewer (Royle & Kremheller 1981).

Epidemiology

Infections by *P. humuli* are contingent upon the presence of liquid water on the plant surface, since the germination of sporangia and zoospore release only occurs in the presence of free water. A single sporangium releases 4 to 8 zoospores. This process requires wetness from 1 hour at 20° to 22°C to 10 hours at 2°C (Royle & Kremheller 1981). Zoospores move in water to encyst singly on open stomata under lighted conditions. Stomata are located by chemical and physical stimuli. Zoospores are attracted to products of photosynthesis, which leak out of stomata, and to the topography of prominently open stomata (Royle & Kremheller 1981). In the dark, zoospores settle randomly over the leaf surface, with relatively few (<25%) locating stomata (Royle & Kremheller 1981). As the germ tubes of zoospores can only penetrate the hop through stomata, the infection of leaves occurs on the lower surface which has more stomata than the upper surface. The emergence, growth, and entry of zoospore germ tubes require water on the plant surface.

Leaf infection requires a minimum of 1.5 hours of wetness between the temperatures of 5° and 29°C (Royle & Kremheller 1981). A longer minimum wet period (3 to 6 hours) and a more restricted temperature range (8° to 23°C) are necessary for shoot infection (Royle & Kremheller 1981). As zoospores are more likely to locate, encyst, and penetrate stomata in the light, daytime rains or dew are more likely to produce outbreaks of downy mildew than wetness during the night.

Once inside the plant, the rate of *P. humuli* colonization of hop is primarily governed by temperature. Susceptible cultivars develop leaf lesions in 3 to 10 days depending on the temperature, with disease developing between 7° and 28°C (Royle & Kremheller 1981). Spikes need 7 to 22 days at temperatures in a more limited range (9° to 20°C) to develop (Royle & Kremheller 1981). Symptoms of leaf disease often precede sporulation, but under favorable conditions they may occur nearly simultaneously. Similarly, infected shoots tend to develop symptoms of spikes before sporangia are produced. Spikes may not produce sporangia at all, or they may take a long time if the conditions are unfavorable for sporulation (Royle & Kremheller 1981).

Spores are produced through stomata on a diurnal cycle. Both sporangiophores and sporangia form and mature during one night. The most important external factors for sporulation are water vapor pressure and temperature (Johnson & Skotland 1985, Royle & Kremheller 1981). During the morning until 9 or 10 am, sporangia are released into the air in hourly increasing amounts (Royle & Kremheller 1981). This release corresponds to declining relative humidity of the air in the morning. After that

time, the concentration of sporangia in the air decreases until the supply runs out and relatively few are detected at night. The optimal range for sporulation is about 16° to 30°C (Royle & Kremheller 1981). Temperature is favorable for sporulation and infection during most of the season in Europe, but may be limiting during the spring, particularly in the western U.S. Sporulation is best between 95% and 100% relative humidity (Royle & Kremheller 1981). In Washington State, Johnson & Skotland (1985) found that when nightly minimum temperatures were above 5°C, sporulation typically occurred when mean ambient relative humidity between 8 pm and 6 am was above 71%.

Oospore

The role of the sexual spore of *Pseudoperonospora humuli* in the disease cycle has not yet been established conclusively. The sexual reproductive strategy of *P. humuli* is unknown, but it is believed to be homothallic as most of the members of the Peronosporomycetes are homothallic and oospores are routinely formed (Dick 2001). Although oospores are produced in large numbers in infected shoots, leaf lesions, and particularly cones, there is contradictory evidence of whether they are able to cause disease in the field. Some researchers have successfully germinated oospores in the laboratory (Arens 1929, Bressman & Nichols 1933, Chee & Klein 1998, Magie 1942, Parker 2007). This supports the hypothesis that oospores potentially can provide a source of primary inoculum. Also, oospores are found in the pith of dormant buds, which could protect the oospore until bud break, at which time the oospore may

germinate (Parker 2007). However, there is little direct evidence of oospores infecting shoots or leaves in either artificial or natural settings. The importance of oosporic inoculum is presumed based largely on circumstantial evidence, particularly the abundance of oospores in diseased tissues and the ability of those spores to germinate *in vitro*.

Other researchers dismiss the role of the oospore in hop downy mildew epidemiology. Royle and Kremheller (1981) indicate that part of this may be due to repeated failures on the part of some researchers to induce germination. Another circumstantial indicator that the oospore may not be important in the disease cycle is that in both the United Kingdom and Washington State severely-infected fields have been replanted with no disease occurring on the new plants (Coley-Smith 1965, Skotland 1961). The researchers concluded that in those areas there were no surviving oospores in the soil that were able to infect the new planting. Coley-Smith (1962) found that primary basal spikes did not form from potted plants or healthy cuttings of the bine bases (strap cuttings) inoculated with oospores or with field soil. Primary spikes did form on diseased "strap cuttings" under the same conditions, but it is uncertain whether oospores produced the spikes. Coley-Smith (1962) also was able to generate primary basal spikes in potted plants in the absence of oosporic inoculum by inoculating hop plants with sporangia in the autumn.

Skotland (1961) performed a search for oospores in the Yakima Valley during the period from 1956 to 1960. Oospores were only found one time, in a basal spike collected in May 1957. Skotland (1961) concluded that this indicates that although

oospores are produced in the hop growing areas of Washington, oospores are not commonly produced and likely are not an important source of inoculum in that area. The alternative overwintering mechanism then would be that the primary spikes are caused by systemic mycelium in the crown. Several researchers in England have found overwintering mycelium in hop crowns, bolstering the current view that primary basal spikes are produced by such mycelium in dormant buds in some areas (Coley-Smith 1960, Salmon & Ware 1928, Skotland 1961, Ware 1929).

Chee et al. (2006) used random amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF) markers to assess genetic diversity of isolates of *P. humuli* in the Pacific Northwest and found that there is much greater genetic diversity in the Oregon populations, while the Washington populations were practically clonal. Chee et al. (2006) speculated that this indicates that sexual recombination may be occurring in Oregon but not in Washington. The authors hypothesized that the dry, hot summers of the Yakima Valley prevent secondary spread of *P. humuli*, thus limiting oospore development. This would agree with Skotland's (1961) conclusion that oosporic inoculum plays an insignificant part of the disease cycle in the Yakima Valley. A limitation of the study by Chee et al. (2006) was that DNA was extracted from inoculum which was recovered from infected leaves from the field and subsequently analyzed with non-specific RAPD and DAF methods. Although the researchers did exclude those spikes that were obviously contaminated with secondary organisms or were degraded, potentially organisms other than *P. humuli* were present on the spikes that could have been co-amplified by the non-

specific primers (Chee et al. 2006). Thus, the results may reflect the genetic diversity of a community of organisms rather than within *P. humuli*.

Some of the controversy about the role of the oospore in causing disease may be due to the oospore being of greater importance in some areas than in others, such as in the Pacific Northwest. Chee and Klein (1998) found that laboratory oospore production in cultivars Nugget and Fuggle only occurs between 6 and 12°C. They also found that oospores were produced under reduced osmotic potential and were only found in necrotic lesions. The authors propose that these conditions are likely to occur in the fall, which is when oospores are found most abundantly in the field in temperate climates (Chee & Klein 1998). However, it is not known whether the pathogen responds to natural conditions in the same way as the laboratory conditions used for this study, nor how host genotype affects oospore production. Further study on other factors that may affect oospore production and on what natural conditions promote the process, such as conditioning periods similar to several species of *Phytophthora*, is needed (Ann & Ko 1988, Banihashemi & Mitchell 1976, El-Hamalawi & Erwin 1986, Hord & Ristaino 1991).

Cucurbit Downy Mildew

Pseudoperonospora cubensis is a widespread pathogen of cucurbit crops. It has been found on at least 49 wild and cultivated species in 70 countries (Choi et al. 2005, Cohen 1981, Palti & Cohen 1980, Renfro & Bhat 1981). Cucurbit downy mildew affects 9 of the 12 cultivated cucurbit species, the most important of which are

Cucumis sativus, *Cucumis melo*, *Cucurbita* spp., and *Citrullus vulgaris* L. (Choi et al. 2005, Cohen 1981). The pathogen infects plants both in the field and protected cultivation in tropical areas around the world, as well as some semi-arid and temperate regions. Downy mildew of cucurbits was first found in Cuba in 1868 (Berkely & Curtis 1868), and then reported in Japan about 20 years later (Kurosawa 1927, Palti & Cohen 1980). Occurrence of *P. cubensis* in central Europe has been increasing since 1984, resulting in reduced yield in many cucumber production areas (Lebeda 1991).

In the U.S., cucurbit downy mildew on cucumber was controlled for about 20 years through genetic resistance to the disease. However, in 2004, there was a severe outbreak of cucurbit downy mildew in the eastern U.S. (Colucci et al. 2006, Holmes et al. 2006). Cucumber growing areas in North Carolina, Delaware, Maryland, and Virginia were devastated. It was believed to be due to a new pathotype of *P. cubensis* that was more aggressive than previous strains in the area (Colucci 2008, Colucci et al. 2006, Holmes et al. 2006). Voglmayr et al. (2009) also reported *P. cubensis* causing downy mildew on *Impatiens irvingii* (Balsaminaceae) in Cameroon, as confirmed by sequencing the ITS region and finding only one nucleotide change in the ITS2 region from *P. cubensis* sequences AY608616, AY608617, AY608619 and DQ409815 from Choi et al. (2005) and Choi and Shin (2008). The authors did not report whether the pathogen was inoculated onto cucurbit hosts, as further confirmation.

Pathotypes

There is evidence of the existence of a number of pathotypes of *P. cubensis* (Cohen 1981, Palti & Cohen 1980). Thomas (1986) reported five pathotypes of *P. cubensis* based on compatibility tests with specific hosts. Of the five pathotypes found, two were found only in Japan, two only in the U.S., and one in both Japan and Israel. Shetty et al. (2002) found that there are distinct pathotypes of the pathogen in Asia, Poland, and the U.S. In addition to the different host ranges for the various pathotypes, there are some morphological and virulence differences in the appearance of *P. cubensis* depending on the host species and/or environmental conditions (Choi et al. 2005, Palti 1974, Palti & Cohen 1980, Waterhouse & Brothers 1981). Lebeda and Gadasová (2002) proposed a set of 12 cucurbit hosts as a differential set to elucidate pathotypes of *P. cubensis*. With this set of differentials, they were able to distinguish 13 pathotypes from 22 isolates of *P. cubensis* from Europe. Using the same differential set, Colucci (2008) determined the virulence of 32 isolates of *P. cubensis* from the U.S. and found 32 different patterns, suggesting that each isolate was of a different pathotype.

Epidemiology

Spring infections are initiated from zoosporangia from an overwintering host or oospores (Palti & Cohen 1980). Leaf wetness is required for nearly every stage of the infection process, as with other downy mildews. Moisture is required for germination of sporangia and penetration of the germ tube into the host stomata. Favorable environmental conditions for infection include rain, dew, and overhead

irrigation on days with temperatures between 16° and 22°C (Palti & Cohen 1980, Sherf & MacNab 1986).

Germination of the sporangium is dependent on temperature and the presence of oxygen in the layer of water on a leaf. One zoospore will encyst per leaf stoma. The germ tube then enters the substomatal chamber of cucurbit leaves, but not those of petioles, stems, or hypocotyls (Cohen 1981). The minimum wetting period required for successful infection is 2 hours. After entering a stoma, mycelium develops intercellularly with intracellular haustoria (Sitterly 1972). Sporangiophores arise in groups from stomata. Sporulation occurs during period of high relative humidity, but can be inhibited in the presence of free water on the leaf surface during the terminal phase of sporulation (Cohen 1981).

The optimum temperature for downy mildew development on cucurbits in a saturated atmosphere is 15°C (Palti & Cohen 1980). Between the temperatures of 5° and 30°C, sporulation in a saturated atmosphere will occur in 6 to 12 hours, depending on temperature (Palti & Cohen 1980). The maximum temperature for infection by *P. cubensis* is at or below 30°C, regardless of other biotic and abiotic factors (Palti & Cohen 1980). If the temperature exceeds 40°C, the pathogen in infected leaves may be killed (Cohen 1981).

Light has been shown to inhibit the production of sporangiophores and sporulation; thus maturation of sporangia occurs during the night. Peak sporangia dispersal typically occurs between 8:30 and 10:00 am, depending on the host crop (Cohen 1981). The quantity and quality of illumination affects the epidemiology of

cucurbit downy mildew significantly. In general, any factor that enhances photosynthesis in the time before the onset of dew will result in increased sporulation (Cohen 1981). Palti and Cohen (1980) summarize the effect of light as having five outcomes, depending on temperature and moisture conditions. First, sporulation is stimulated by extension of light periods preceding a wet period. Second, when leaves are dry, dark periods shorten the length of required wetness (in the dark) for sporulation. Third, sporangia on sporulating leaves lose viability in dry, hot sunlight. Fourth, weak light reduces the sporulation potential of leaves due to necrosis of lesions at temperatures between 20° and 30°C, but not at 15°C. Finally, after the initial stage, lesion development is stronger at short photoperiods (4-10 hours), but sporulation potential of a lesion at 16-20 hour photoperiods is much greater than that at 8-hour photoperiods. Palti and Cohen (1980) propose that the complexity seen in this system may be due to the disease existing in both temperate and tropical regions. Since day length, humidity, and temperatures are different in temperate areas as compared to tropical areas, the pathogen may have different optimal temperature requirements for sporulation depending on the region.

Overseasoning

Pseudoperonospora cubensis, like *P. humuli*, is an obligate parasite living on a host that only has aerial tissue available for part of the year, as most Cucurbitaceous hosts are frost-sensitive annuals. Thus the pathogen is unable to overseason in living host tissue in most cucurbit hosts in contrast to *P. humuli* that can overwinter in hop

crowns. A perennial cucurbit widely distributed in Europe and the Mediterranean region, *Bryonia dioica* Jacq. (or Cretan bryony), has been successfully artificially infected with *P. cubensis* (Runge & Thines 2009). Although there are no records of *P. cubensis* naturally occurring on *B. dioica*, the plant is a potential perennial host in temperate Europe. Without such a perennial host, the only way for the pathogen to survive during the off-season is as oospores, in hosts living in a favorable climate, or in crops grown in greenhouses. Inoculum from these sources is assumed to be windblown into new areas in the spring (Cohen 1981).

Long distance dispersal of inoculum is suspected to be the source of *P. cubensis* infections along the eastern seaboard of the U.S. and some disease outbreaks in Israel (Cohen 1981). *Pseudoperonospora cubensis* is able to overwinter in the sub-tropical Florida climate on cultivated crops and/or wild or feral hosts (Palti & Cohen 1980). Inoculum is thought to be carried northward during the growing season on prevailing winds and other atmospheric phenomena. The Cucurbit Downy Mildew Forecast model uses the National Oceanic and Atmospheric Administration HYSPLIT model and information about the airborne survival of sporangia to predict ascent, transport, and deposition of sporangia to predict risks of downy mildew outbreaks due to long range dispersal (Anonymous 2008, Main et al. 2001).

Oospores of *P. cubensis* appear to be rare. Oospores have occasionally been reported in Japan, India, Italy, Russia, and China (Cohen 1981, Palti & Cohen 1980). However, it is generally accepted that oospores are not a significant source of primary inoculum, principally due to lack of evidence to the contrary (Cohen 1981, Palti &

Cohen 1980). Cohen (1981) states that the "… only circumstantial evidence to indicate the involvement of oospores in epidemic outbreaks came from North China."

Species Concepts in Downy Mildew

The identity of a species is of great importance in plant pathology, in terms of disease diagnosis, treatment, and regulatory issues, nationally and internationally. Quarantine and plant hygiene laws are especially sensitive to the correct identification of a species (Hall 1996, Rossman & Palm-Hernández 2008).

The downy mildews have been a difficult group for determining what defines a species. Morphometric techniques, measurements of the size, shape, color, etc. of morphological features, have been historically used to distinguish and identify a taxon in many areas of biology. For example, oomycetes were traditionally considered to belong to the kingdom Fungi primarily because oomycetes have filamentous thalli. Morphometric techniques that are often used for other taxa tend to be uninformative in the downy mildews (Cooke et al. 2000, Göker et al. 2003, Hudspeth et al. 2000, Peterson & Rosendahl 2000). This is mainly due to the few visible characters, namely characteristics of the sporangiophore, sporangia, oospore, and antheridia, as well as direct or indirect sporangium germination (Hall 1996, Waterhouse & Brothers 1981). Although some characteristics are consistent within a species, and can be distinctive, many characters can vary widely depending on the host and the environmental conditions (Hall 1996). Sexual structures are only potentially informative if they are

present, which is problematic if the pathogen is heterothallic and lacking the other mating type or if host effects prevent the formation of sexual structures (Hall 1996).

To overcome these problems with morphology, Gäumann (1918) proposed a biological species concept for downy mildews that was based on host specialization (Hall 1996, Thines et al. 2009). Gäumann (1918) assumed that a species of Peronosporaceae would be specific to a single host genus or even species. This concept encouraged species circumscription based on the presence of the pathogen on a host with little consideration of morphometric features (Hall 1996). Gäumann (1918) did not perform cross-infection experiments to confirm his strict assumption about host range (Hall 1996, Thines et al. 2009). This concept is also problematic when multiple species of downy mildew are parasitic on a single host species (Thines et al. 2009, Voglmayr et al. 2009).

The eco-physio-phenetic concept is essentially the intersection between the biological species and morphometric concepts. In this concept the criterion for the delimitation of a species is dependent upon the host specificity and the morphology of the conidiophores and conidia or sporangia (Hall 1996). Ecophysiologically, one or a few species are confined to a single host genus, which is a similar, but perhaps wider view of the biological species concept (Hall 1996). This concept, although appearing as the middle ground, suffers from many of the same problems as the morphometric and biological species concept.

As a result of these problems, and with the advent of molecular techniques, phylogenetic analyses of the downy mildews have been used to attempt to tease apart

species that are monophyletic. According to Hall (1996), "species are defined as clusters of organisms diagnosably different from other clusters and within which there are parental networks of ancestry and descent." The determination of whether a cluster, or clade, is diagnostically different from other clusters relies on statistical probabilities and models to reconstruct phylogenies. How different a clade has to be to represent a species is not clear, and likely depends on the locus, number of loci, and characters used as well as the sample size and how well the sample represents the species as a whole. The representation of organisms outside the taxa of interest as outgroups may also affect the resultant phylogram topology and must be carefully considered. Phylogenetic techniques do not reflect host range information, which is vitally important for obligate parasites, such as the downy mildews. Additionally, circumscribing species solely through molecular techniques would require specialized and relatively expensive equipment and training in order to obtain the sequence data that would be necessary for identifying an organism. Practically, this would make disease diagnosis and regulation significantly more difficult and costly than including other criteria in delineating a species, such as morphology and/or host range.

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

P. cubensis and *P. humuli* have been shown to be closely related based on morphological features (Constantinescu 2000, Fraymouth 1956, Waterhouse & Brothers 1981) and molecular evidence (Göker et al. 2003, Riethmüller et al. 2002, Voglmayr 2003). Choi et al. (2005) examined the taxonomic relationship between the

two pathogens using both morphological and molecular methods. The authors evaluated various morphological features of the two *Pseudoperonospora* species, including symptoms produced on their respective hosts as well as dimensions and characteristics of sporangiophores and sporangia. The study looked at the phylogenetic relationships based on ITS nrDNA (internal transcribed spacer of nuclear ribosomal DNA) sequence data of *Pseudoperonospora* and other closely related genera, and concluded that *P. humuli* should be reduced to a taxonomic synonym of *P. cubensis*.

It has been shown that *P. cubensis* can exhibit different morphology on different hosts and/or in different environmental conditions (Choi et al. 2005, Palti 1974, Palti & Cohen 1980, Waterhouse & Brothers 1981). Thus, the slight differences between downy mildew pathogens could be attributed to the same mechanism if the two really are the same species. The morphological descriptions of both pathogens given by Choi et al. (2005) are somewhat different from previous studies. In general, the ranges for sporangiophore length and trunk width, and sporangial dimensions given in Choi et al. (2005) are broader than those in Palti (1974) and Miyabe & Takahashi (1906). For example, Choi et al. (2005) reported the length of the sporangiophores for *P. cubensis* was 120-480 μm, while the Palti (1974) gave the range as 180-400 μm. Descriptions not involving measurements were also different between Choi et al. (2005) and those in the literature cited. For example, Choi et al. (2005) described the shape of the ultimate brachlets of the sporangiophore for *P. humuli* as being "straight to substraight," while the description by Miyabe & Takahashi (1906) for the same feature was "straight, slightly arcuate or sometimes

reflexed." Choi et al. (2005) stated that these differences between the morphology of *P. humuli* and *P. cubensis* are analogous to the range of values documented on different hosts infected with *P. cubensis*.

Choi et al. (2005) also examined the phylogenetic relationship between *Pseudoperonospora* using the ITS region. The ITS region of both species was 802 base pairs using primers DC6 and ITS4. The authors used both Bayesian inference (MCMC) and maximum parsimony (MP) to analyze the phylogenetic relationships. They found that "*P. cubensis* and *P. humuli* formed a well-supported group with high posterior probability (100% in MCMC and MP trees)." No subcluster within the *P. cubensis-P. humuli* clade was supported with more than 50% support from either Bayesian or maximum parsimony analyses, however. Within the *P. cubensis-P. humuli* clade, three isolates of *P. cubensis* from Korea from three different host plants were clustered as well as two other *P. cubensis* isolates from China and Austria. A subcluster of *P. humuli* isolates, two from Austria and two from Korea (including one from *H. japonicus*) was also found, separating the isolates from Europe and Asia. Four isolates of *P. cubensis* and two isolates of *P. humuli* from *H. japonicus* were not clustered with each other or with any other isolates. The ITS sequences from the two pathogens were "…identical, or more highly conserved (more than 99.5% homology)," indicating that they likely belong to the same species. In fact, the sequences of *P. cubensis* and *P. humuli* were more similar than sequences between *P. cubensis* on different hosts.

Other analyses (Göker et al. 2009, Sarris et al. 2009) have included the dataset from Choi et al. (2005) with other sequences that were deposited on the National Center for Biotechnology Information (NCBI) GenBank database after 2005 and have found more support for the clusters within the *P. cubensis*-*P. humuli* clade. Sarris et al. (2009) inferred a phylogeny of *P. cubensis* and *P. humuli* from ITS sequences using the neighbor-joining method with the isolates of Choi et al. (2005) as well as 22 isolates of *P. cubensis* from the Czech Republic and Greece and one *P. humuli* isolate from the Czech Republic. Sarris et al. (2009) found that the isolates of *P. cubensis* from Europe ("European subcluster") formed a moderately supported clade (64% of 1000 bootstrap replicates) separate from the Asian *P. cubensis* (and the two *P. humuli* on *H. japonicus*) isolates from Choi et al. (2005). The three *P. cubensis* isolates from Korea that were clustered in the analysis by Choi et al. (2005) were also clustered in the neighbor-joining analysis by Sarris et al. (2009) with the same support as the European subcluster. The cluster containing the above isolates was moderately supported (67%) and designated as the "*P. cubensis* clade" by Sarris et al. (2009). The *P. humuli* isolates, excluding the two from Korea on *H. japonicus* mentioned above, formed two moderately supported sister clades (66% and 67% respectively) of the two remaining isolates from Korea and of the four European isolates, including two used by Choi et al. (2005). These two clades were designated the "*P. humuli* cluster" by Sarris et al. (2009).

Göker et al. (2009) inferred the phylogenic relations of the same dataset as Sarris et al. (2009) with two additional *P. cubensis* isolates from Asia using RAxML

(Stamatakis 2006, Stamatakis et al. 2008) and maximum parsimony (MP). The same relationships were found among the subclusters of Sarris et al. (2009). The two additional Asian *P. cubensis* isolates, from China and Taiwan, clustered with the "European subcluster," but the subcluster lacked bootstrap support separating it from the "Asian subcluster." The three *P. cubensis* isolates from Korea that were clustered in Choi et al. (2005) and Sarris et al. (2009) were also clustered with high support (82% ML, 88% MP) in the work by Göker et al. (2009). The "*P. humuli* cluster" did not have more than 50% bootstrap support from either analysis, which is in line with the other two studies. However, like the result of Sarris et al. (2009), two subclusters were resolved with strong bootstrap support. The analysis by Göker et al. (2009) found higher bootstrap support for the sister clades of *P. humuli* from Europe (84% ML, 65% MP) and from Asia (86% ML, 72% MP).

Choi et al. (2005) did not investigate host specificity among the isolates of *P. cubensis* and *P. humuli*. They cite work of other authors showing that *P. humuli* can infect *Urtica*, *Cannabis*, and *Celtis* species (Choi et al. 2005, Hoerner 1940, Salmon & Ware 1928, Salmon & Ware 1929). There is no record of *P. cubensis* successfully infecting wild or cultivated hop, or of infection of cucurbits by *P. humuli*. Hoerner (1940) reported that "… all attempts to infect available hosts of *Pseudoperonspora cubensis* [with *P. humuli*] … were unsuccessful." It would be informative to have cross-inoculations performed with the pathogens and their respective hosts to further explore whether the downy mildews of cucurbits and hop are caused by the same species in accordance with the biological species concept of Gäumann (1918) and the

eco-physio-phenetic concept, in conjunction with examining these pathogens on a genetic level.

DNA Fingerprint Techniques

Genetic information has been found to be a convenient and accurate method to elucidate relationships among different organisms. The genome is informative at many taxonomic levels. Highly conserved regions tend to be more informative for organisms that are more evolutionarily distant, while less conserved regions tend to be more informative for organisms that are more closely related (Hughes et al. 2006, Small et al. 1999). Coding regions tend to have highly conserved nucleotide sequences, as mutations that cause lack of function tend to be selected against. Non-coding regions are more likely have variability since there tends to not be a selection pressure associated with mutations in such regions.

Molecular techniques have been used recently to resolve phylogenetic relationships within various taxonomic levels of the Peronosporales (Cooke et al. 2000, Göker et al. 2003, Göker et al. 2007, Hudspeth et al. 2000, Hudspeth et al. 2003, Riethmüller et al. 2002, Voglmayr 2003). Most of this work has focused on loci in nuclear ribosomal DNA (nrDNA) such as the non-coding internal transcribed spacer (ITS), and the coding large subunit (LSU nrDNA) and small subunit (SSU nrDNA) (Braiser et al. 2004, Cooke et al. 2000, Göker et al. 2003, Göker et al. 2007, Martin & Tooley 2003a, Riethmüller et al. 2002, Voglmayr 2003, Voglmayr et al. 2004). One advantage of these markers is that there are multiple copies present in the genome.

Other nuclear loci that have been studied in the Peronsporales include β-tubulin (*β-tub*) and translation elongation factor 1 alpha (*EF-1α*) (Göker et al. 2007, Kroon et al. 2004). Researchers have also examined mitochondrial DNA (mtDNA) for NADH dehydrogenase subunit 1 (*nadh1*), and the cytochrome *c* oxidase subunit I (*cox1)* and subunit II (*cox2)* (Göker et al. 2007, Hudspeth et al. 2000, Hudspeth et al. 2003, Kroon et al. 2004, Martin & Tooley 2003a, Martin & Tooley 2003b).

A recent trend in exploring the phylogeny of the fungi and fungal-like organisms is to use multiple molecular markers to get better accuracy. Rokas and Carroll (2005) examined genomic data of 14 species of yeast and concluded that using more genes results in more accurate phylogenetic relationships than increasing taxon number in yeast. Blair et al. (2008) inferred the phylogeny of 82 *Phytophthora* species using nuclear loci coding for 28S nrDNA, 60S ribosomal protein L10, β-tubulin, translation elongation factor 1 alpha, enolase, heat shock protein 90, and TigA gene fusion protein. When these seven loci were concatenated, it was found that the resolution of relationships between *Phytophthora* species was much improved over previous analyses that relied solely on the ITS or that included fewer loci. Martin and Tooley (2003b) found that including *cox*2 sequence data with ITS data improved resolution of several *Phytophthora* species. Göker et al. (2007) used ITS, LSU nrDNA, *cox2*, and *β-tub* to explore the relationships among the Peronosporales and concluded that compared to earlier studies, their "…multi-gene approach clearly resulted in greater resolution of the phylogenetic relationships of downy mildews." Kroon et al. (2004) looked at the relationships among the *Phytophthora* with *cox1*,

nadh1, *EF-1α*, and *β-tub*. They found that the phylogenies based on nuclear DNA and mitochondrial DNA had some incongruence, indicative of different evolutionary histories, such as sexual hybridization between related or unrelated species followed by rapid evolution (Kroon et al. 2004). Martin and Tooley (2003a, 2003b) found that there was heterogeneity between the data sets from ITS and *cox2* analyses of *Phytophthora* species. Also, some species were grouped differently depending on the genomic locus. Martin and Tooley (2003a, 2003b) suggest that the divergence could be due to "…differing rates of evolutionary divergence or incorrect assumptions about alignment of the ITS sequences." In the genus *Pythium*, several species have nearly identical ITS sequences and in *Phytophthora* several species are poorly resolved using the ITS, indicating that this may not be the ideal locus for distinguishing oomycete species, at least for certain genera (Lévesque & De Cock 2004, Martin & Tooley 2003a).

In many of the studies of Peronosporales phylogeny, the genus *Pseudoperonospora* is represented by only one or two species if the genus is included at all. Having both *P. cubensis* and *P. humuli* included in a study is fairly uncommon. Due to the extent of previous phylogenetic analyses, often only one or two isolates were used to represent a species, thus ignoring variation within a species but instead focusing on variation above the species level. Multigenic analyses of the phylogeny of *P. cubensis* and *P. humuli* are absent from the literature.

Voglmayr (2003) investigated the phylogeny of the *Peronsporaceae* using the ITS region and included two *P. humuli* isolates and one of *P. cubensis*. The *P. humuli*

isolates were well supported (93% bootstrap support, 100% posterior probability) as a clade separate from the single *P. cubensis* representative. Choi et al. (2005) also used the ITS locus to explore the relationship between nine isolates each of *P. humuli* and *P. cubensis* as well as other downy mildew pathogens. In agreement with other studies, *P. humuli* and *P. cubensis* formed a monophyletic clade separate from the other genera. Further studies of the ITS region of the two pathogens have suggested a more complex relationship between isolates of *P. humuli* and *P. cubensis* from different hosts and different continents (Göker et al. 2009, Sarris et al. 2009). In general, the isolates of *P. cubensis* are sister to most of the isolates of *P. humuli* and within each cluster are subclusters separating European isolates and Asian isolates. The introduction of sequence data from American isolates of each species may further clarify relationships of these pathogens, and in light of the discovery of genetic incongruence by Lévesque and De Cock (2004) and Martin and Tooley (2003a, 2003b), examining other regions of the genome may provide more resolution between *P. cubensis* and *P. humuli*.

Thesis Goal

The goal of this thesis was to examine the relationship between *Pseudoperonospora humuli* and *P. cubensis* using polyphasic characterization to further understanding of the relationship of these pathogens.

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CHAPTER 2: METHODS AND MATERIALS

Plant Material. Plants of the downy mildew susceptible hop cultivars Nugget (Haunold et al. 1984) and Pacific Gem were propagated clonally from softwood cuttings and maintained in a greenhouse free of downy mildews. Plants of susceptible cucumber cultivar Straight 8 (Shetty et al. 2002) and cantaloupe cultivar Ananes Yokneam (Lebeda & Widrlechner 2003) were planted from certified organic seed and grown in the same greenhouse as the hop plants. The greenhouse was maintained at 20 to 25°C with a 14 hour photoperiod. Hop plants were grown in Sunshine Mix number 1 (SunGro Horticulture, Bellevue, WA) in 440-cm^3 pots for approximately 14 days for use in host specificity experiments (described below). Some hop plants were repotted into 648-cm^3 pots for an additional 14 days for maintenance of *P. humuli* isolates. Cucurbit plants were grown in the same soil in 440-cm^3 pots for approximately 4 to 6 weeks. Plants were watered regularly and supplied with Champion 17-17-17 (N-P_2O_5-K_2O) fertilizer with micronutrients (McConkey's, Portland, OR) at each irrigation to promote succulent growth.

Isolates. Isolates of *P. humuli* were collected during 2006 to 2009 from Oregon (8 isolates) and Washington (3 isolates) (Table 1). From diseased hop shoots systemically infected with *P. humuli* from infected plants in the field, monosporangial isolates were attained as described by Gent et al. (2008). Monosporangial isolates were maintained and bulked for DNA extraction of sporangia on hop cv. Nugget using

a droplet inoculation procedure on detached leaves (from 2006 to 2008) as described by Gent et al. (2008), or a spray inoculation procedure for whole plants (during 2009). For the spray inoculation, sporangia were dislodged from sporulating leaves using a pressurized narrow stream of sterile 18-ohm water (MilliQ water; Millipore, Billerica, MA) produced by a Preval Complete Spray Unit (Precision Valve Corporation, Yonkers, NY). The inoculum for maintenance of cultures was adjusted to at least $5x10^4$ sporangia ml^{-1} using a hemacytometer and was sprayed to runoff onto the underside of leaves of hop cv. Nugget. One inoculated plant (in a 440-cm^3 pot) was placed into a prepared 90 ounce (2661.6 cm^3) Pasta Keeper (29.21 cm x 11.43 cm x 11.43 cm; SNAPWARE, Mira Loma, CA) or three inoculated plants (in a 648-cm^3 pot) were placed into a prepared 2.5 gallon (11012.2 cm^3) barrel container (35.56 cm x 20.96 cm x 20.96 cm; SNAPWARE). The containers were prepared by having sterile 18-ohm water sprayed onto the inside walls and inside of the lid to increase humidity. A moistened paper towel folded twice into a square was placed at the bottom of the Pasta Keeper container to aid in increasing the humidity. The inoculated plants were kept overnight in closed plastic containers. The following morning, the plants were removed from the containers and allowed to air dry for 24 hours before being replaced into the dried containers and put into a growth chamber for 6 to 8 days. Isolates were maintained at 20°C with a 12 hour light photoperiod provided by fluorescent lights (approximately 300 $\mu mol/m^2/s$). After 6 days of incubation, sporulation was induced by spraying the inside walls and lid of the container with deionized water and closing

the container overnight. Additionally, two DNA samples of *P. humuli* were received from the Czech Republic and one DNA sample was received from Korea (Table 1). *P. cubensis* isolates from the eastern United States were received from a collaborator that were collected during 2005 to 2009 and included isolates from North Carolina (9 isolates), New Jersey (1 isolate), Michigan (2 isolates), and Ohio (1 isolate) (Table 1). In 2009, isolates from California (6 isolates) and western Oregon (2 isolates) also were received from collaborators (Table 1). Isolates from infected leaves were maintained on cucumber cv. Straight 8 grown from certified organic seed using a spray inoculation procedure on whole plants, same as for *P. humuli*, except the inoculum was adjusted to approximately $6.5x10^3$ spores ml^{-1}. Isolates of *P. cubensis* were not monosporangial due to technical difficulties in attaining a monosporangial isolate of the pathogen. After inoculation, cucumber plants were put into prepared closed plastic containers (either 1 plant in a Pasta Keeper or 2 to 3 plants in a barrel container) as described previously and were put into a dark humid chamber (at least 98% humidity, 21°C) for 24 hours. After 24 hours, the plants were removed from the plastic containers, and placed into trays holding at least 1 liter of deionized water in a growth chamber at 21°C day/18°C night with a 12 hour photoperiod. To induce sporulation after 6 days, the inside walls and lid of the container were sprayed with deionized water and the container was closed and placed into a dark humid chamber overnight. Each isolate was maintained in a separate chamber to avoid cross-contamination. Additionally, herbarium samples of *P. cubensis* and *P. celtidis* were received from South Korea (Table 1).

DNA Extraction. DNA was extracted from sporangial suspensions using a cetyl trimethylammonium bromide (CTAB) procedure modified from Chee et al. (2006) or MoBio Ultra Clean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions with modifications. The modified CTAB extraction was conducted as follows. Sporangial suspensions were centrifuged at 10,600 x *g* for 3 minutes and then resuspended in 100 µl 1x Tris-EDTA (TE). Polyvinyl pyrrolidone (PVP; 0.021 g), CTAB extraction buffer (900 µl; 100 mM Tris-HCl pH 7.5, 1.4 M NaCl, 20 mM EDTA, and 5.49 mM CTAB), and 100 µl of the sporangial suspension in TE were added to a FastPrep Lysing Matrix A Tube (MP Biomedicals, Solon, OH) and put into a FastPrep instrument (Bio 101, Vista, CA), which was run five times at level 6 for 45 sec, placing the tubes on ice for 2 min between each run. Then, 750 µl of the solution was transferred to a 1.5-ml microfuge tube to which was added 7.5 µl β-mercaptoethanol, 22.5 µl 20 mg ml^{-1} proteinase K, and 7.5 µl RNase A, followed by incubation at 65°C for 30 min. After the solution was mixed with 750 µl of 24:1 chloroform/isoamyl alcohol it was centrifuged at 10,600 x *g* for 10 min. Up to 650 µl of the aqueous phase was then transferred to a 1.5-ml microfuge tube and the cholorform/isoamyl alcohol addition and centrifugation steps were repeated. Up to 500 µl of the aqueous phase was transferred to a 1.5-ml microfuge tube and nucleic acids were precipitated by addition of an equal volume of cold isopropanol. The DNA was pelleted by centrifugation at 10,600 x *g* for 20 min at 4°C. The pellet was subsequently rinsed twice with 70% ethanol, dried in a fume hood

on a hot plate at 35°C, and then resuspended in 50µl of 10mM Tris-HCl pH 7.5 and stored at -20°C.

The modifications to the MoBio Ultra Clean Soil DNA Isolation Kit were as follows. To solution S1, 0.140 g of PVP was added to improve the fidelity of PCR amplification following extraction (Gent et al. 2009). After the addition of S1, 200 µl of inhibitor removal solution (IRS; MoBio) was added to the tube with the bead lysis solution. For higher recovery of DNA, the tube with the bead lysis solution, sporangial suspension, and solutions S1 and IRS was boiled for 2 minutes before the first centrifugation. DNA was stored in Tris-EDTA buffer (S5 from MoBio or 10 mM Tris and 1 mM EDTA, pH 8.0) at -20°C.

Polymerase Chain Reaction Amplification, Cloning, and Sequencing. The internal transcribed spacer of nuclear ribosomal DNA (ITS) region, β-tubulin gene (*β-tub*), and the cytochrome *c* oxidase (*cox*) cluster (partitioned into *cox2*, *cox2-cox1* spacer, and *cox1*) were amplified with the primers in Table 2. PCR reactions were carried out in a total volume of 25 µl containing 12 µl PCR-grade water, 10 µl Hot Master Mix (5 PRIME, Gaithersberg, MD), 0.75 µl (0.5 µM) of each forward and reverse primer, 0.5 µl acetylnitrile (50% by volume), and 1 µl template. The amplification program consisted of an initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 20 s, annealing temperature specific for each primer pair (Table 2) for 30 s, and extension at 65°C for 1 min with a final extension at 70°C for 10 min. DNA fragments were electrophoresed in a 1% Tris-acetate EDTA gel.

Ethidium bromide (0.5 μg ml^{-1}) was added to each gel, and the DNA fragments were visualized over a UV transilluminator.

Monosporangial isolates of *P. humuli* were directly sequenced from the PCR product. To produce more template for downstream uses, PCR reaction volumes were doubled when amplicons were cloned. To obtain a single haplotype from non-monosporangial isolates which may be representing multiple individuals, the PCR products for each locus were cleaned with Amicon or Microcon centrifugal filters (Millipore) and ligated into p-GEM T-Easy vector (Promega Corporation, Madison, WI) and cloned in *Escherichia coli* strain DH5α in accordance with the manufacturer's instructions. The insert and a portion of the vector were amplified using plasmid primers M13F and M13R. DNA fragments were electophoresed and visualized as above for confirmation before sequencing. Amplicons from PCR or clones were sequenced bidirectionally by the Center for Genome Research and Biocomputing (Oregon State University, Corvallis, OR).

Sequence Alignment and Phylogenic Analysis. Sequences were aligned in BioEdit version 7.0.9.0 (Ibis Therapeutics, Carlsbad, CA) initially using CLUSTAL W (Thompson et al. 1994) under default settings followed by manual adjustments as needed. Bidirectional sequences were reconciled to produce a consensus sequence. Sequences of *Phytophthora infestans* were downloaded from the National Center for Biotechnology Information (NCBI) GenBank to provide an outgroup to the *Pseudoperonospora* species for all phylogenetic analyses (Table 1). To provide an

additional outgroup more closely related to the *P. cubensis* and *P. humuli*, *P. celtidis* was included in all the analyses except *β-tub* and a concatenated analysis (described below) due to poor amplification of the *β-tub* locus. The *P. urticae* sequence of *β-tub* was downloaded from the NCBI GenBank as an outgroup for that locus (Table 1). From each of the consensus sequences, the primer regions that were not contained within another amplicon were excluded from further analysis to eliminate artifacts due to primer sequence.

To obtain an appropriate model of site substitution for use in likelihood and Bayesian searches for each locus and the concatenated data set, MrModeltest 2.3 (Nylander 2004) for Bayesian analysis was used in conjunction with PAUP* version 4.0b10 (Swofford 2003). Models were chosen based on the best model according to the Akaike information criterion (Table 3). To confirm that data from different loci could be concatenated for the analysis, an incongruence length difference (ILD) test was performed on the suitability of analyzing the *cox* cluster as a locus, and combining ITS, *β-tub*, and *cox* cluster using PAUP* version 4.0b10. Phylogenic analyses were performed using MrBayes version 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) for Bayesian analyses and RAxML version 7.2.5 (Stamatakis 2006, Stamatakis et al. 2008) for maximum likelihood analysis. In the Bayesian analysis, three heated (temperature = 0.2) and one cold simultaneous Markov chains were run for three million generations, saving a tree every 100^{th} generation. Among these, the first 7500 trees were ignored. A 50% majority rule consensus of the remaining trees was constructed by MrBayes version 3.1.2 to obtain estimates for the

posterior probabilities of groups**.** To test the reproducibility of results, the analyses were repeated three times, starting with random trees and default parameter values. Phylogenetic analyses were conducted using RAxML version 7.2.5 under default parameters for the nucleotide substitution model with a rapid bootstrapping (1000 replicates) via the CIPRES web portal (Miller et al. 2009). Additionally, the option to print branch lengths (-k) and to perform a rapid bootstrap analysis and search for the best-scoring ML tree in one single program run (-f a) were selected. PAUP* version 4.0b10 was used to create a 50% consensus tree from the files with the 1000 bootstrap trees from RAxML version 7.2.5. For each locus and for the concatenated dataset, the distance tree from the Bayesian analysis was used as the template to reconcile the trees from each analysis to form a consensus tree with 50% majority rule for the trees inferred by both programs.

An additional phylogenetic analysis was performed incorporating ITS sequences used in this study with all available *P. cubensis* and *P. humuli* sequences in the NCBI database to provide a larger dataset including isolates from Europe and Asia (Table 4). *P. celtidis*, *P. urticae*, and *P. cannabina* ITS sequences were included as additional outgroups. Three sequences from China were 709 base pairs (bp) long, so the other sequences were shortened accordingly for the phylogenetic analysis. Phylogeny was inferred by RAxML version 7.2.5 (same conditions as above) and MrBayes version 3.1.2 using the general time reversible nucleotide model with gamma (GTR+G) rate distribution (as found by MrModeltest version 2.3). The 50% consensus trees were reconciled using the tree inferred by Bayesian analysis as the template. Trees were

rooted using TreeView version 1.6.6 (Page 1996) with *Phytophthora infestans* as the outgroup.

Host Range Study. *P. humuli* isolates from Oregon (3 isolates) and Washington (1 isolate) and *P. cubensis* isolates from North Carolina (5 isolates), Michigan (2 isolates), and Ohio (1 isolate) were used for a host range study conducted during 2008 to 2010 (Table 1). For each experiment, *P. humuli* was spray-inoculated as described above onto 3 cucurbit (cucumber or cantaloupe) plants at a concentration of $5x10^3$ spores ml^{-1} and *P. cubensis* was spray-inoculated onto 3 hop plants (cultivar Nugget or Pacific Gem) at a concentration of $5x10^3$ spores ml^{-1}. Cucurbit and hop plants were trimmed to 3 nodes per plant (nodes 3 to 7, depending on plant age and leaf quality) before inoculation. Most of the experiments used the cucumber cv. Straight 8 and the hop cultivar cv. Nugget. A positive control and negative (water only) control plant was included for each *Pseudoperonospora* species in every run. For example, in each run testing *P. humuli* on cucumber cv. Straight 8, there were three cucumber plants inoculated with an isolate of *P. humuli*, one positive control cucumber plant inoculated with an isolate of *P. cubensis*, and one negative control cucumber plant. All the plants were grown in 440-cm^3 pots and put into Pasta Keeper containers. The plants were incubated as described above for *P. cubensis* on cucumber. All but three of the experiments were conducted at least twice for each isolate and host combination. Additionally, the experiments were repeated on the hop cultivar Pacific Gem and the melon (cantaloupe) cultivar Ananes Yokneam, which are regarded as universally

susceptible to *P. humuli* and *P. cubensis*, respectively (Lebeda & Widrlechner 2003). The hop cultivar was changed from Nugget to Pacific Gem because cv. Pacific Gem is more susceptible to downy mildew than cv. Nugget. The cucurbit species was altered to the cantaloupe cv. Ananes Yokneam to verify the host specificity of *P. humuli* observed on cucumber (described below) was consistent on another cucurbit host. The experiments were rated on the 7th and 14th day post-inoculaton by stereomicroscopic examination of the abaxial surface of each leaf. Each leaf was rated for a hypersensitive response (localized water-soaking, chlorosis, and necrosis) and sporulation. The rating on the seventh day was done without harming the plant, while the 14^{th} day rating was destructive. For 13 out of 16 experiments that had sporulation, sporangiophores and sporangia were counted or estimated if too numerous to identify individual structures. Positive and negative controls also were rated for each pathogen. To confirm that the infection was produced by the organism inoculated (i.e., *P. cubensis* on hop leaves) sporangia were collected from two experiments (*P. cubensis* isolates CDM-255 and CDM-276 on hop) and analyzed by the same molecular methods as described above, focusing on *cox2*. The *cox2* locus was chosen due to the relative easy amplification and the presence of four well-conserved single nucleotide polymorphisms (SNPs) that differentiate *P. humuli* and *P. cubensis* among the isolates used in this study with only three exceptions. The exceptions are isolates SMK 19582, *P. humuli* on *Humulus japonicus* from Korea with 3 SNPs, only 2 of which are found in most *P. cubensis*; CDM-241, *P. cubensis* on squash from North Carolina with 1 SNP found in most *P. humuli*; and CDM-248, *P. cubensis* on acorn squash from North

Carolina with 3 SNPs found in most *P. humuli*. None of these isolates were alive when the host specificity experiments were being performed, nor would it be plausible that the DNA from the three aberrant isolates would contaminate the DNA from the host specificity experiments due to the time of processing the respective samples.

To further confirm that the infection was produced by the organism inoculated, sporangia of *P. cubensis* produced on hop were tested on hop and cucumber to confirm that the pathogen had a similar pathogenicity pattern as *P. cubensis* when inoculated on cucumber. For this experiment, *P. cubensis* (CDM-255) from cucumber cv. Straight 8 was spray inoculated onto 4 hop plants (cv. Pacific Gem) plants in 440-cm^3 pots at $5x10^5$ sporangia ml^{-1} in the manner described above. The four plants were placed into a prepared barrel container and were incubated in the manner of *P. cubensis*. On the seventh day, the inoculum from the hop plants was collected, combined, and quantified. From this inoculum, 4 hop plants (cv. Pacific Gem) and 2 cucumber plants (cv. Straight 8) were spray inoculated at $5x10^3$ sporangia ml^{-1}. The plants were treated as the first 4 hop plants. On the seventh day, the inocula from the hop and cucumber plants were collected, quantified, and DNA was extracted for confirmation that the pathogen infecting these plants was *P. cubensis*.

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Table 1. Identity and origin of organisms used in this study with isolates used in host specificity study noted

Organism	Strain	Host	Location	Year	GenBank accession no.
Pseudoperonospora cubensis	CDM-237	*Citrullus lanatus*	USA, New Jersey	2007	
P. cubensis	CDM-241	*Cucurbita* sp.	USA, North Carolina	2007	
P. cubensis	CDM-246	*Momordica charantia*	USA, North Carolina	2007	
P. cubensis	CDM-247	*Cucurbita* sp.	USA, New Jersey	2007	
P. cubensis	CDM-248	*Cucurbita pepo*	USA, North Carolina	Unknown	
P. cubensis[c]	CDM-251	*Cucumis sativus*	USA, Michigan	2007	
P. cubensis[cd]	CDM-252	*Cucumis sativus*	USA, Ohio	2007	
P. cubensis[c]	CDM-253	*Cucumis sativus*	USA, North Carolina	2007	
P. cubensis[d]	CDM-254	*Cucumis sativus*	USA, North Carolina	2006	
P. cubensis[cd]	CDM-255	*Cucumis sativus*	USA, Michigan	2005	
P. cubensis	CDM-266	*Cucumis sativus*	USA, California	2009	
P. cubensis	CDM-268	*Cucumis sativus*	USA, California	2009	
P. cubensis	CDM-269	*Cucumis sativus*	USA, California	2009	
P. cubensis	CDM-272	*Cucumis sativus*	USA, California	2009	
P. cubensis	CDM-273	*Cucumis sativus*	USA, California	2009	
P. cubensis	CDM-274	*Cucumis sativus*	USA, California	2009	
P. cubensis[d]	CDM-275	*Cucurbita pepo*	USA, North Carolina	2005	
P. cubensis[d]	CDM-276	*Cucurbita pepo*	USA, North Carolina	2006	
P. cubensis[d]	CDM-277	*Cucurbita moschata*	USA, North Carolina	2008	
P. cubensis	CDM-278	*Cucumis sativus*	USA, Oregon	2009	
P. cubensis	CDM-279	*Cucumis sativus*	USA, Oregon	2009	
P. humuli	HDM-094	*Humulus lupulus*	USA, Washington	2006	
P. humuli	HDM-103	*H. lupulus*	USA, Washington	2006	
P. humuli	HDM-110	*H. lupulus*	USA, Oregon	2006	
P. humuli	HDM-158	*H. lupulus*	USA, Oregon	2007	

Table 1 (continued)

P. humuli	HDM-170	*H. lupulus*	USA, Oregon	2007	
P. humuli	HDM-171	*H. lupulus*	USA, Oregon	2007	
P. humuli[ab]	HDM-224	*H. lupulus*	USA, Oregon	2008	
P. humuli[ab]	HDM-247	*H. lupulus*	USA, Washington	2008	
P. humuli[a]	HDM-254	*H. lupulus*	USA, Oregon	2008	
P. humuli[a]	HDM-257	*H. lupulus*	USA, Oregon	2008	
P. humuli	HDM-263	*H. lupulus*	Czech Republic, Chrastany	Unknown	
P. humuli	HDM-266	*H. lupulus*	Czech Republic, Kolesovice	Unknown	
P. humuli	SMK19582	*H. japonicus*	Korea, Pyongchang	2003	
P. celtidis	SMK17780	*Celtis sinensis* Pers.	Korea, Dongduchon	2000	
P. urticae[e]	HV713	*Urtica dioica* L.	Austria, Oberösterreich	Unknown	DQ361163
Phytophthora infestans[e]	P106050	*Solanum tuberosum* L.	Mexico	Unknown	EU079633.1
Ph. infestans[e]	INF-PO	*S. tuberosum*	Italy	Unknown	AJ854292
Ph. infestans[e]	Complete mitochondrian genome	*S. tuberosum*	Unknown	Unknown	U17009.2

[a] *P. humuli* isolate used in host specificity experiments on cucumber cv. Straight 8.
[b] *P. humuli* isolate used in host specificity experiments on cantaloupe cv. Ananes Yokneam.
[c] *P. cubensis* isolate used in host specificity experiments on hop cv. Nugget.
[d] *P. cubensis* isolate used in host specificity experiments on hop cv. Pacific Gem.
[e] Sequences from NCBI GenBank.

Table 2. PCR and sequencing primers used in this study

Primer	Sense	Primer 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
HDM07	Forward	AGAATTGACTGCGAGTCC	ITS	58.4	Gent et al. 2009
HDM04	Reverse	AGCCACACAACACATAGT	ITS	58.4	Gent et al. 2009
bTub136-OW	Forward	CGCATCAAYGTRTACTACAAYG	*β-Tub*	47	Göker et al. 2007
bTub1024R-OW	Reverse	CGAAGTACGAGTTCTTGTTC	*β-Tub*	47	Göker et al. 2007
bTubMM254-276R	Reverse	GTGATCTGGAAACCCTGCA	*β-Tub*	60	This study
bTubMM202-220F	Forward	ATTGACTCGGTGCTTGACG	*β-Tub*	60	This study
bTubMM558-576R	Reverse	GTGATACCAGACATGGCG	*β-Tub*	60	This study
bTubna492F	Forward	CATTTGCTTCCGCACACTTA	*β-Tub*	60	This study
FM35	Forward	CAGAACCTTGGCAATTAGG	*cox2*	56	Martin 2000
FM36	Reverse	CAAATTTCACTACATTGTCC	*cox2*	56	Martin 2000
FMPh-8b	Forward	AAAAGAGAAGGTGTTTTTTATGGA	*cox2-cox1* Spacer	56	Martin et al. 2004
FMPh-10b	Reverse	GCAAAAGCACTAAAAATTAAATATAA	*cox2-cox1* Spacer	56	Martin et al. 2004
FM84	Forward	TTTAATTTTTAGTGCTTTTGC	*cox1*	56	Martin & Tooley 2003
FM85	Reverse	AACTTGACTAATAATACCAAA	*cox1*	56	Martin & Tooley 2003
FM85RC	Reverse	TTTGGTATTATTAGTCAAGTT	*cox1*	56	Martin & Tooley 2003[a]
FM77	Reverse	CACCAATAAAGAATAACCAAAAATG	*cox1*	56	Martin & Tooley 2003
FM83	Reverse	CTCCAATAAAAAATAACCAAAAATG	*cox1*	56	Martin & Tooley 2003
M13F	Forward	TGTAAAACGACGGCCAGT	Plasmid	50	
M13R	Reverse	CAGGAAACAGCTATGACC	Plasmid	50	

[a] FM85RC is the reverse compliment of FM85 by Martin & Tooley, 2003.

Table 3. Nucleotide models with best AIC scores by MrModeltest for Bayesian analyses for ITS region; partial *β-Tubulin* gene; *cox* cluster, consisting of *cox2*, *cox2-cox1* spacer, and *cox1*; and concatenated ITS, *β-Tubulin*, and *cox* cluster

Locus	Model chosen by MrModeltest[a]
ITS	GTR+G
β-Tubulin	GTR+G
cox cluster	GTR+I
cox2	GTR+G
cox2-cox1 spacer	GTR+I
cox1	GTR+G
Concatenated	GTR+G

[a] Model abbreviations: GTR: general time reversible (Rodriguez et al., 1990), HKY: Hasegawa-Kishino-Yano 1985; G: gamma-distributed rate distribution, I: proportion of invariable sites.

Table 4. Identity and origin of all available *Pseudoperonospora* ITS sequences retrieved from National Center for Biotechnology Information (NCBI) GenBank database

Organism	Strain	Host	Location	GenBank accession no.
P. cannabina	MZM71018	*Cannabis sativa*	Latvia	AY608612
P. celtidis	SMK17780	*Celtis sinensis*	Korea	AY608613
P. cubensis	HV222	*Cucumis sativus*	Austria	AY198306
P. cubensis	HV2279	*Impatiens irvingii*	Cameroon	EU660054
P. cubensis	JinShan	*Cucumis sativus*	China	DQ025515
P. cubensis	MinHang	*Cucumis sativus*	China	DQ025516
P. cubensis	PuDong	*Cucumis sativus*	China	DQ025517
P. cubensis	n/a	*Cucumis sativus*	China	AY744946
P. cubensis	JM_12/00	Cucumis sativus	Czech Republic	EU876600
P. cubensis	JM_39/01	Unknown	Czech Republic	EU876601
P. cubensis	Leb_4/95	*Cucumis sativus*	Czech Republic	EU876604
P. cubensis	OL_1/88	*Cucumis sativus*	Czech Republic	EU876599
P. cubensis	OL_26/01a	Unknown	Czech Republic	EU876598
P. cubensis	SC_75/01	Unknown	Czech Republic	EU876603
P. cubensis	ZL_35/01	Unknown	Czech Republic	EU876602
P. cubensis	WE_3/00	*Cucumis sativus*	France	EU876597
P. cubensis	CEC_2811	*Cucumis sativus*	Greece	EU876592
P. cubensis	CEC_2812	*Cucumis sativus*	Greece	EU876591
P. cubensis	CEC_2813	*Cucumis sativus*	Greece	EU876590
P. cubensis	CEC_2814	*Cucumis sativus*	Greece	EU876589
P. cubensis	CEC_2815	*Cucumis sativus*	Greece	EU876588
P. cubensis	CEC_2816	*Cucumis sativus*	Greece	EU876587
P. cubensis	CEC_2818	*Cucumis sativus*	Greece	EU876586

P. cubensis	CEC_2819	*Cucumis sativus*	Greece	EU876585
Table 4 (Continued)				
P. cubensis	CEC_2820	*Cucumis sativus*	Greece	EU876584
P. cubensis	HIE_2409	*Cucumis sativus*	Greece	EU876596
P. cubensis	HIE_2410	*Cucumis sativus*	Greece	EU876595
P. cubensis	HIE_2412	*Cucumis sativus*	Greece	EU876594
P. cubensis	HIE_2413	*Cucumis sativus*	Greece	EU876593
P. cubensis	SMK11284	*Cucumis melo*	Korea	AY608614
P. cubensis	SMK12174	*Cucumis sativus*	Korea	AY608616
P. cubensis	SMK13288	*Cucurbita moschata*	Korea	AY608619
P. cubensis	SMK14235	*Citrullus vulgaris*	Korea	AY608618
P. cubensis	SMK15170	*Cucumis melo*	Korea	AY608615
P. cubensis	SMK18951	*Cucumis sativus*	Korea	AY608617
P. cubensis	SMK19205	*Cucurbita moschata*	Korea	AY608620
P. cubensis	SMK21327	*Lagenaria siceraria*	Korea	DQ409815
P. cubensis	D2	*Trichosanthes cucumerina*	Malaysia	GU233293
P. cubensis	n/a	Unknown	Taiwan	EF050035
P. humuli	HV136	*H. lupulus*	Austria	AY198304
P. humuli	HV148	*H. lupulus*	Austria	AY198305
P. humuli	n/a	*H. lupulus*	Czech Republic	AF448225
P. humuli	SMK11608	*H. japonicus*	Korea	AY608621
P. humuli	SMK11675	*H. lupulus*	Korea	AY608624
P. humuli	SMK18856	*H. japonicus*	Korea	AY608622
P. humuli	SMK19582	*H. japonicus*	Korea	AY608623
P. humuli	UASWS0294	*Rhododendron* sp.	Poland	EF126356
P. urticae	HV715	*Urtica dioica*	Korea	AY198307

CHAPTER 3: RESULTS

Phylogenic Analysis. Sequence data for 21 *P. cubensis*, 14 *P. humuli*, one *P. celtidis* (ITS and *cox*) or *P. urticae* (*β-tub*), and one (per locus) *Phytophthora infestans* isolates that were included for phylogenetic analysis included 809 bp of the ITS, including a partial 18S nrDNA, complete ITS region (ITS1, 5.8S nrDNA, and ITS2) and partial 28S nrDNA; 696 bp of partial *β-tub*; and 2067 bp *cox* cluster (673 bp partial *cox2*, 204 bp *cox2-cox1* spacer, 1190 bp partial *cox1*). The concatenated dataset, which did not include sequence data from *P. celtidis* or *P. urticae* due to missing loci, contained 3771 bp. Out of 3771 characters, there were 39 that were parsimony-informative, 486 that were parsimony-uninformative, and 3246 that were constant. In the individual locus analyses, there were 18 parsimony-informative characters in the ITS region, 22 in *β-tub*, and 56 in the *cox* cluster (19 in *cox2*, 8 in the *cox2-cox1* spacer, and 29 in *cox1*). For some isolates the entire ITS, *β-tub* and *cox1* loci did not amplify, so alternate primers were used (ITS, *cox1*) or designed (*β-tub*) to amplify smaller fragments, which were then aligned and combined to produce the whole locus (Table 2). The *cox2* gene and the *cox2-cox1* spacer amplified well for all isolates used in this study.

The ILD test showed no significant difference between the loci within the *cox* cluster ($P = 0.774$) or between ITS, *β-tub*, and *cox* cluster ($P = 0.708$), indicating that the concatenated regions were congruent and could be analyzed with a single nucleotide substitution model. Additionally, the relatively conserved topology of the

trees inferred by the individual loci suggested that concatenation of the loci was appropriate. The phylogenetic relationships between *P. cubensis* and *P. humuli* were inferred from Bayesian analysis and heuristic maximum likelihood (ML) analysis (performed by RAxML) of the aligned nucleotide sequences are shown below for ITS nrDNA (Fig. 1), *β-tub* (Fig. 2), *cox2* (Fig. 3), *cox2-cox1* spacer (Fig. 4), *cox1* (Fig. 5), and *cox* cluster (Fig. 6) individually and concatenated (Fig. 7) as well as for ITS nrDNA from sequences downloaded from GenBank (Fig. 8). All four Bayesian analyses resulted in the same tree topology with almost identical posterior probability (PP) values for all of the analyses.

The trees inferred by Bayesian and ML analyses of the ITS region both strongly supported a clade of all the *P. humuli* isolates except the isolate from Korea on *H. japonicus* (SMK19852; 100% PP, 97% bootstrap support (BS); Fig. 1). Both analyses also supported the monophyly of the *P. cubensis-P. humuli* clade with 59% PP and 93% BS. Three isolates of *P. cubensis* from watermelon (CDM-237) and acorn squash (CDM-248 and CDM-276) subtended clades of *P. humuli* and *P. cubensis*.

The analyses of *β-tub* also both supported a clade of most of the *P. humuli* isolates, except SMK 19582 and an isolate of *P. humuli* from the Czech Republic (HDM-263; 78% PP, 57% BS; Fig. 2). The Bayesian analysis gave 81% PP support to a clade with most of the *P. cubensis* isolates sister to the *P. humuli* cluster. The only exception was an isolate from squash (CDM-248), which subtended the two clades.

The *cox2* locus was not very well resolved in either analysis. Only one common clade was inferred by both Bayesian and ML analyses (Fig. 3), which included all but two of the *P. cubensis* isolates (52% PP, 61% BS).

The *cox2-cox1* spacer, in contrast, provided clades that were well resolved by both analyses (Fig. 4). The monophyly of the *P. cubensis-P. humuli* clade was strongly supported (99% PP, 93% BS). A clade containing most of the *P. cubensis* isolates, except isolates from watermelon and squash (CDM-237, 241, 248), as well as SMK19582 was also supported (80% PP, 80% BS).

The trees inferred from the *cox1* gene resolved a monophyletic *P. cubensis-P. humuli* clade in both analyses (97% PP, 55% BS; Fig. 5). Only the Bayesian analysis was able to resolve a clade of the *P. humuli* isolates, in addition to CDM-248 (88% PP).

The consensus trees of the *cox* cluster inferred by Bayesian and ML analyses were either able to resolve the *P. cubensis* isolates, with which the Korean isolate was clustered, or the non-Korean *P. humuli* isolates, respectively (Fig. 6). The separation of either cluster from the other group was only moderately supported (60% PP for the primarily *P. cubensis* containing clade, 61% BS for the *P. humuli* cluster).

The trees inferred from the concatenated data set agreed on the separation of the *P. humuli* isolates from most of the *P. cubensis* isolates (Fig. 7). The Korean *P. humuli* isolate (SMK 19582) and an isolate from acorn squash (CDM-248) were included in the weakly supported cluster (62% PP, 55% BS). There was strong

support, however, for a clade composed of the remaining *P. humuli* isolates (100% PP; 91% BS) separate from the aforementioned isolates.

The trees inferred by ML and Bayesian analysis of the ITS region that included all *Pseudoperonospora* sequences in GenBank had similar topology (Fig. 8). Both analyses strongly supported a clade of *P. cubensis* and *P. humuli* subtended by the other *Pseudoperonospora* species (73% PP, 80% BS). Moderate to strong support was found for a clade of all but two *P. humuli* isolates (98% PP, 74% BS). Two subclusters were moderately to strongly supported within the *P. humuli* cluster (81-100% PP, 75-90% BS) separating U.S. and European isolates from those originating from Korea. Within the *P. cubensis* isolates, a cluster of three isolates from Korea was strongly supported by both analyses (100% PP, 87% BS) as well as a moderately supported cluster of two isolates from this study (CDM-253 and CDM-276; 87% PP, 72% BS).

Host Range Study. *P. cubensis* isolates inoculated to hop plants sporulated on 43 of 56 replicate plants (77%; Table 5). Isolates of *P. humuli* inoculated to cucurbit plants sporulated on 1 out of 33 replicate plants (3%; Table 5). The positive controls of both pathogens were always characterized by profuse sporulation but no hypersensitive-like (HR-like) symptoms; no sporulation or HR-like responses were ever observed in any of the negative controls. In all experiments, the inoculated leaves showed scattered HR-like lesions when inoculated with the reciprocal pathogen (Figs. 9, 10). When sporulation was observed, the sporangiophore(s) always emerged from the center or the inside edge of a chlorotic or, more commonly, a necrotic lesion (Fig. 10).

Typically fewer than half of the HR-like lesions contained sporangiophores when hop plants were inoculated with *P. cubensis* (Table 5). The proportion of lesions with sporangiophores was greater with cv. Pacific Gem than with cv. Nugget.

Differences in virulence were seen both with *P. cubensis* on the two hop cultivars and with *P. humuli* on the two universally susceptible species of cucurbits. *P. cubensis* sporulated more profusely on cv. Pacific Gem than cv. Nugget (Table 5), and more HR-like lesions were seen on cv. Pacific Gem. This observation is limited as only one isolate of *P. cubensis* (CDM-252) was inoculated onto both of the hop cultivars, but the same pattern was seen comparing isolates inoculated onto one cultivar with those inoculated onto the other host. For the cucurbit species, cantaloupe cv. Ananes Yokneam had more necrosis whether infected with *P. cubensis* or *P. humuli* as compared with cucumber cv. Straight 8. When inoculated with *P. humuli*, cantaloupe had more HR-like lesions than cucumber. The single cucurbit plant on which *P. humuli* sporulated was a cantaloupe and there was one sporangiophore bearing four sporangia emerging from near the center of a necrotic HR-like lesion on the 7-day rating. Unfortunately, the leaf with the sporangiophore withered before the 14^{th} day rating, preventing photographic documentation and further examination of the leaf.

Inoculum harvested from hop infected with *P. cubensis* verified that the pathogen contained SNPs in the *cox2* region that corresponded with all but two *P. cubensis* isolates (CDM-241 and CDM-248). Interestingly, there were one to two SNPs unique to each of the *P. cubensis* isolates from host specificity experiments.

However, upon direct (i.e., without cloning the amplicon) resequencing these unique SNPs did not persist.

In reinoculation experiments to further verify that the sporulation on hop plants was indeed *P. cubensis*, the first set of four 'Pacific Gem' hop plants yielded a total of 3.7×10^4 sporangia per ml in about 8 ml inoculum (about 7.4×10^4 sporangia per plant). The same isolate inoculated onto two cucumber 'Straight 8' plants yielded a total of 2.78×10^5 sporangia ml^{-1} in more than 30 ml total inoculum (at least 4.2×10^6 sporangia per plant). The hop plants displayed similar symptoms to those seen in the host specificity experiments, but had more HR lesions, presumably due to the higher concentration of sporangia used for inoculation. The second set of four 'Pacific Gem' plants had similar symptoms as seen in the host specificity experiments (Fig. 11) and yielded a total of about 1.0×10^4 sporangia ml^{-1} in about 20 ml inoculum (about 5×10^4 sporangia per plant). The two cucumber 'Straight 8' plants inoculated with inoculum from the first set of hop plants yielded a total of 3.1×10^5 sporangia ml^{-1} in about 29 ml of inoculum (about 4.5×10^6 sporangia per plant). The cucumber exhibited typical disease symptoms for cucumber plants inoculated with *P. cubensis* (Fig. 11). The sporulation, while abundant, was not as profuse as many of the positive control cucumbers in the host specificity experiments, perhaps due to the inoculum coming from a poor host.

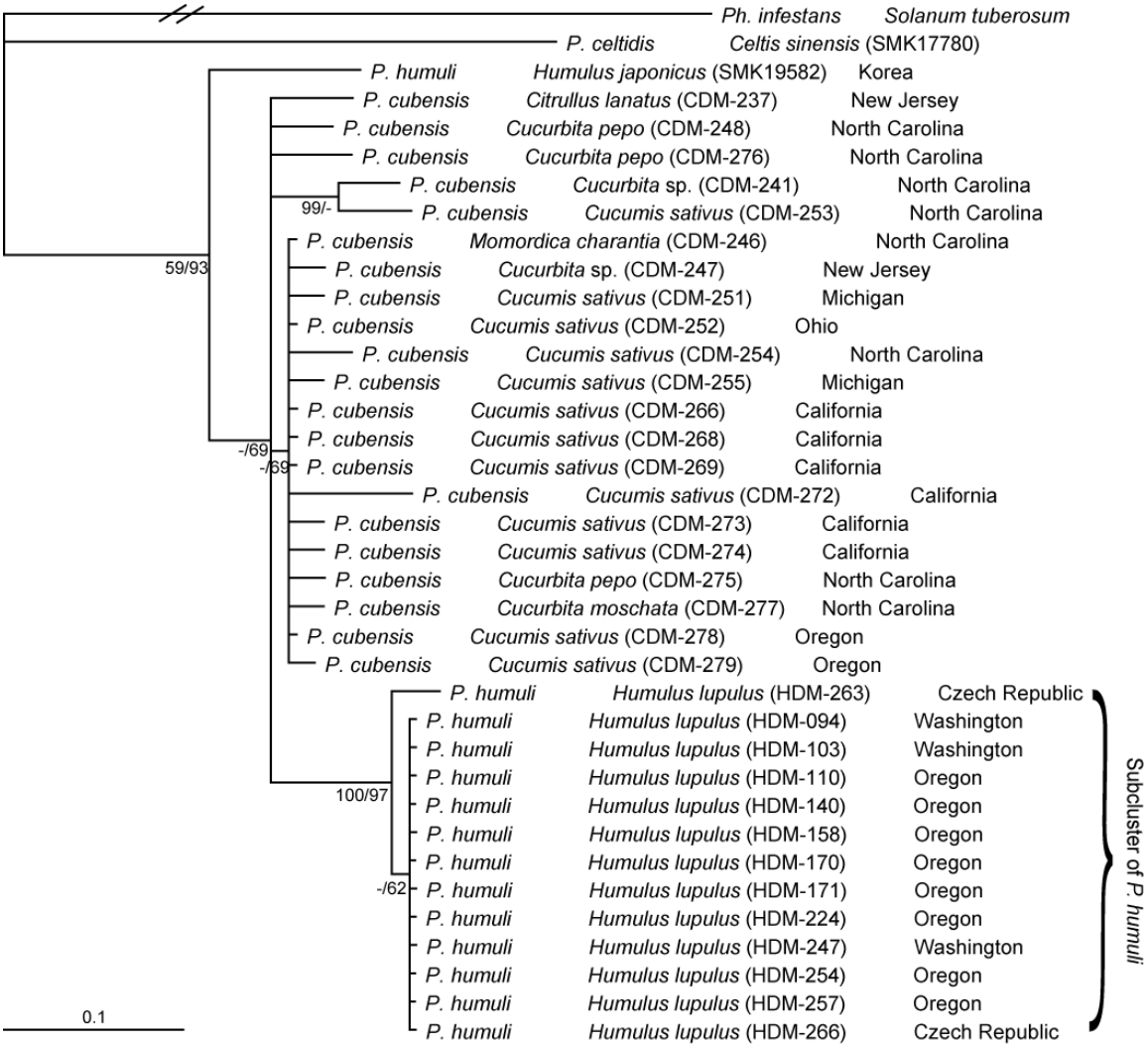

Figure 1. 50% consensus tree of Bayesian and maximum likelihood analyses of an alignment of partial 18S nrDNA, complete ITS region (ITS1, 5.8S nrDNA, and ITS2) and partial 28S nrDNA for *Pseudoperonospora cubensis* and *P. humuli*. Genetic distances were computed according to the general time reversible model, additionally assuming a gamma distributed substitution rate. Tree topology was rooted with *Phytophthora infestans*. Numbers on branches are posterior probability (3×10^6 generations) followed by bootstrap values (1000 replicates). Dashes indicate support lower than 50%. The expected number of nucleotide substitutions between taxa is represented by branch length and the scale bar equals the expected number of nucleotide substitutions per site. Interrupted branch was scaled to a twentieth of the length.

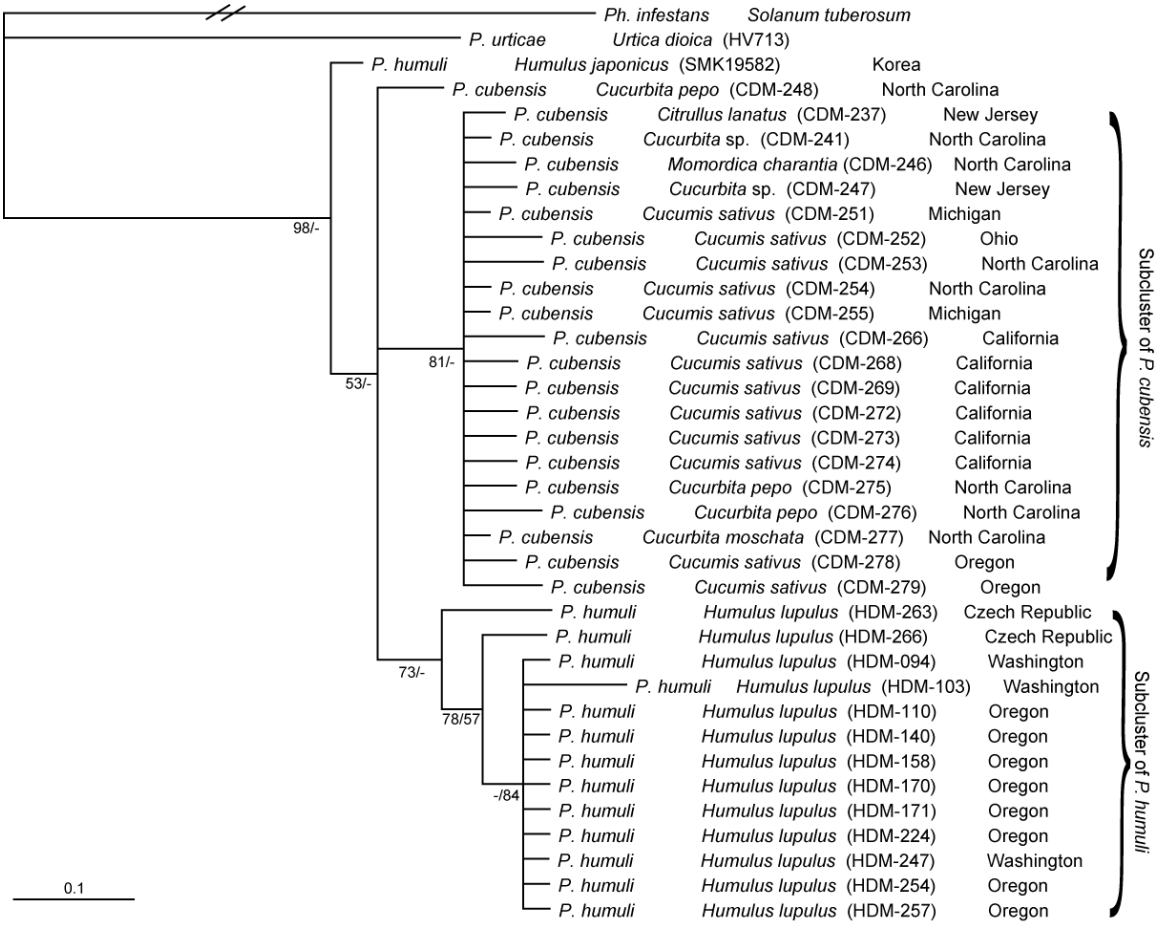

Figure 2. 50% consensus tree of Bayesian and maximum likelihood analyses of an alignment of partial *β-tub* for *Pseudoperonospora cubensis* and *P*. *humuli*. Genetic distances were computed according to the general time reversible model, additionally assuming a gamma distributed substitution rate. Tree topology was rooted with *Phytophthora infestans*. Numbers on branches are posterior probability (3×10^6 generations) followed by bootstrap values (1000 replicates). Dashes indicate support lower than 50%. The expected number of nucleotide substitutions between taxa is represented by branch length and the scale bar equals the expected number of nucleotide substitutions per site. Interrupted branch was scaled to a quarter of the length.

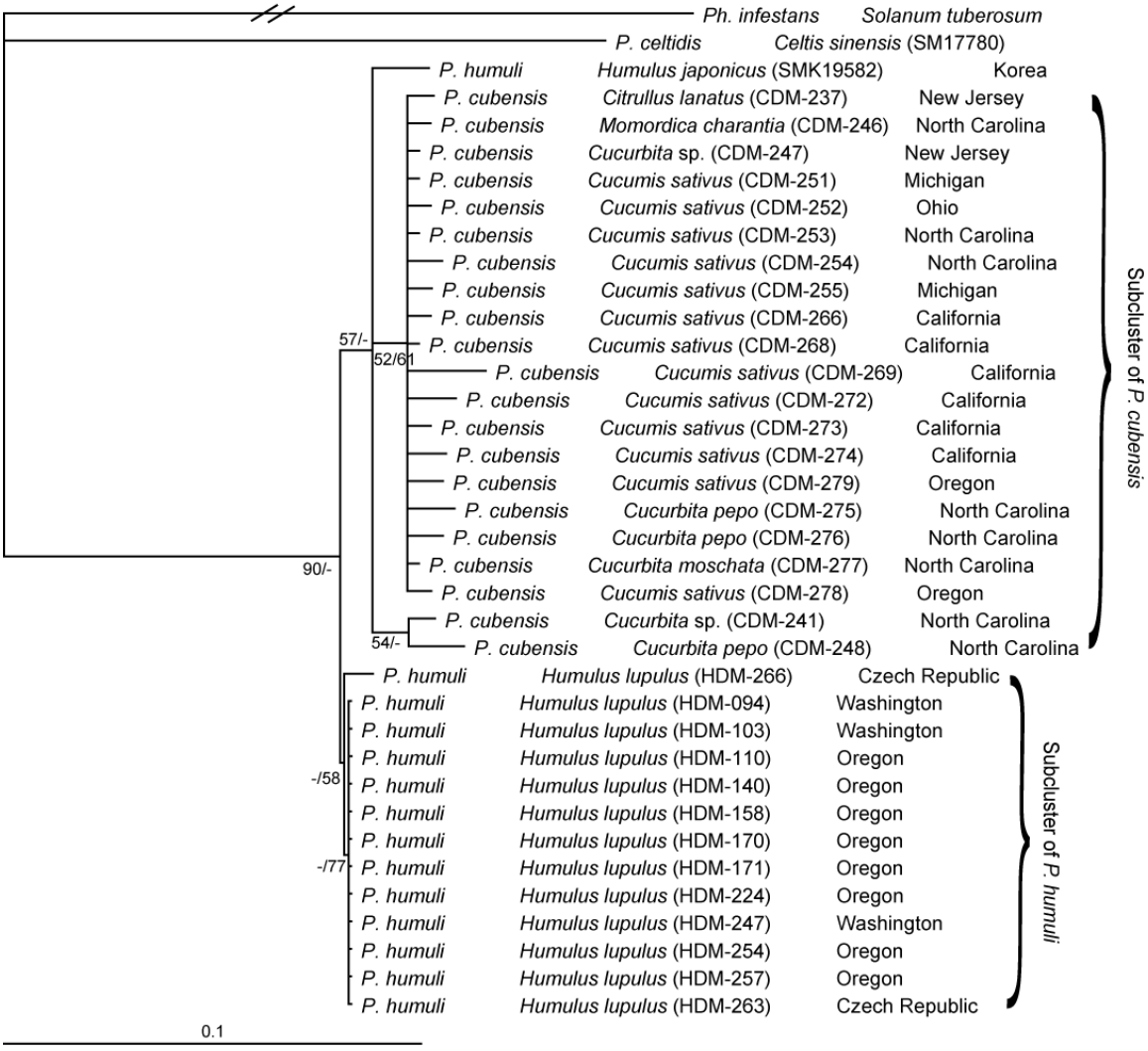

Figure 3. 50% consensus tree of Bayesian and maximum likelihood analyses of an alignment of partial *cox2* for *Pseudoperonospora cubensis* and *P. humuli*. Genetic distances were computed according to the general time reversible model, additionally assuming a gamma distributed substitution rate. Tree topology was rooted with *Phytophthora infestans*. Numbers on branches are posterior probability (3×10^6 generations) followed by bootstrap values (1000 replicates). Dashes indicate support lower than 50%. The expected number of nucleotide substitutions between taxa is represented by branch length and the scale bar equals the expected number of nucleotide substitutions per site. Interrupted branch was scaled to half of the length.

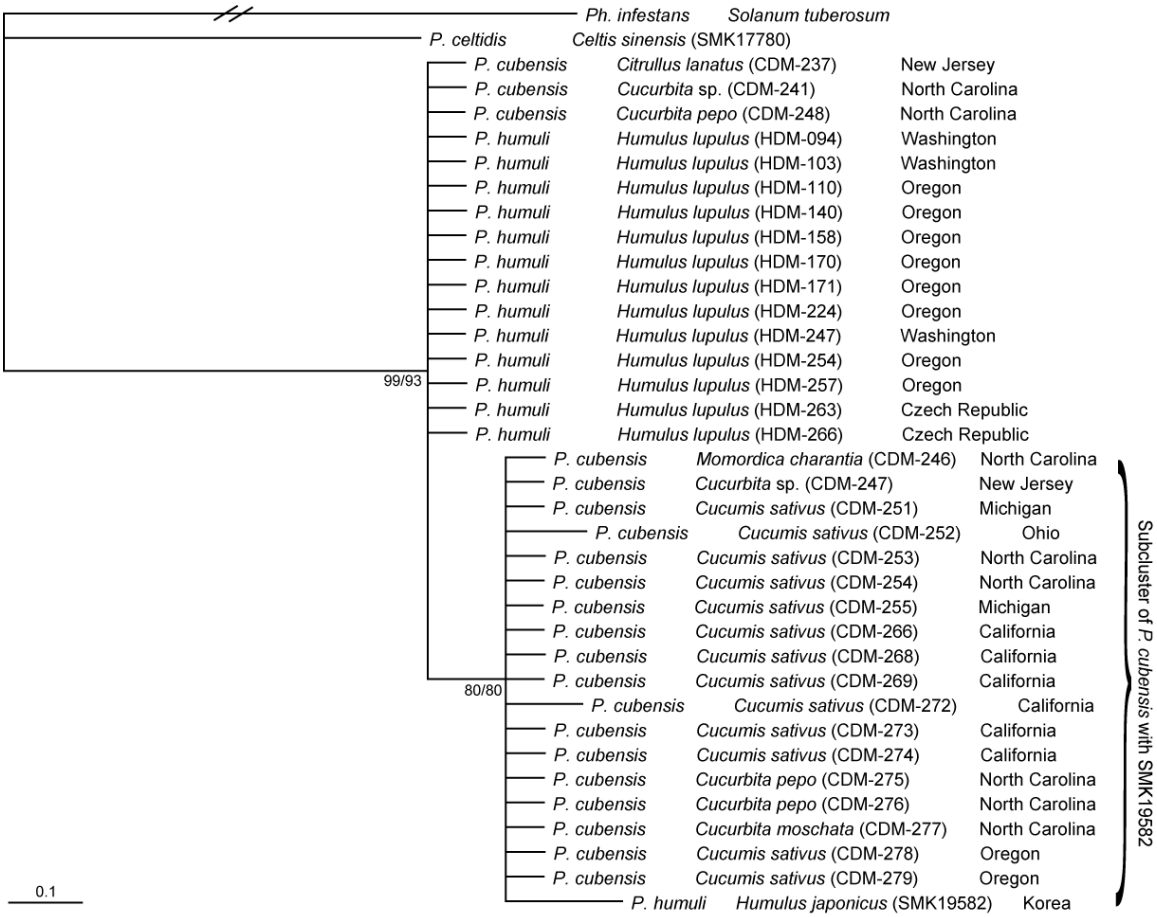

Figure 4. 50% consensus tree of Bayesian and maximum likelihood analyses of an alignment of *cox2-cox1* spacer for *Pseudoperonospora cubensis* and *P. humuli*. Genetic distances were computed according to the general time reversible model, additionally assuming a proportion of invariant nucleotide sites. Tree topology was rooted with *Phytophthora infestans*. Numbers on branches are posterior probability (3 x 10^6 generations) followed by bootstrap values (1000 replicates). Dashes indicate support lower than 50%. The expected number of nucleotide substitutions between taxa is represented by branch length and the scale bar equals the expected number of nucleotide substitutions per site. Interrupted branch was scaled to half of the length.

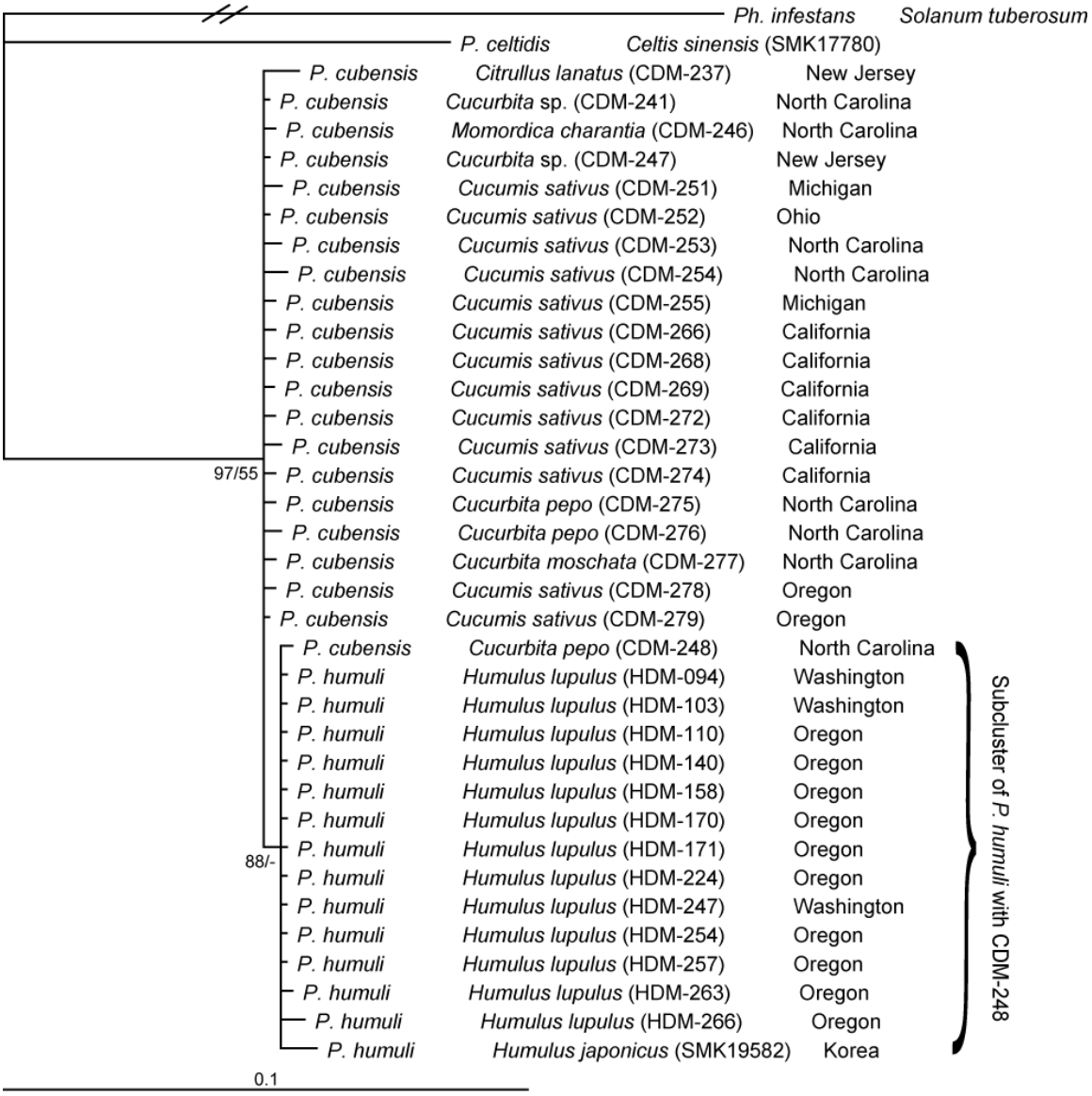

Figure 5. 50% consensus tree of Bayesian and maximum likelihood analyses of an alignment of partial *cox1* for *Pseudoperonospora cubensis* and *P. humuli*. Genetic distances were computed according to the general time reversible model, additionally assuming a gamma distributed substitution rate. Tree topology was rooted with *Phytophthora infestans*. Numbers on branches are posterior probability (3×10^6 generations) followed by bootstrap values (1000 replicates). Dashes indicate support lower than 50%. The expected number of nucleotide substitutions between taxa is represented by branch length and the scale bar equals the expected number of nucleotide substitutions per site. Interrupted branch was scaled to half of the length.

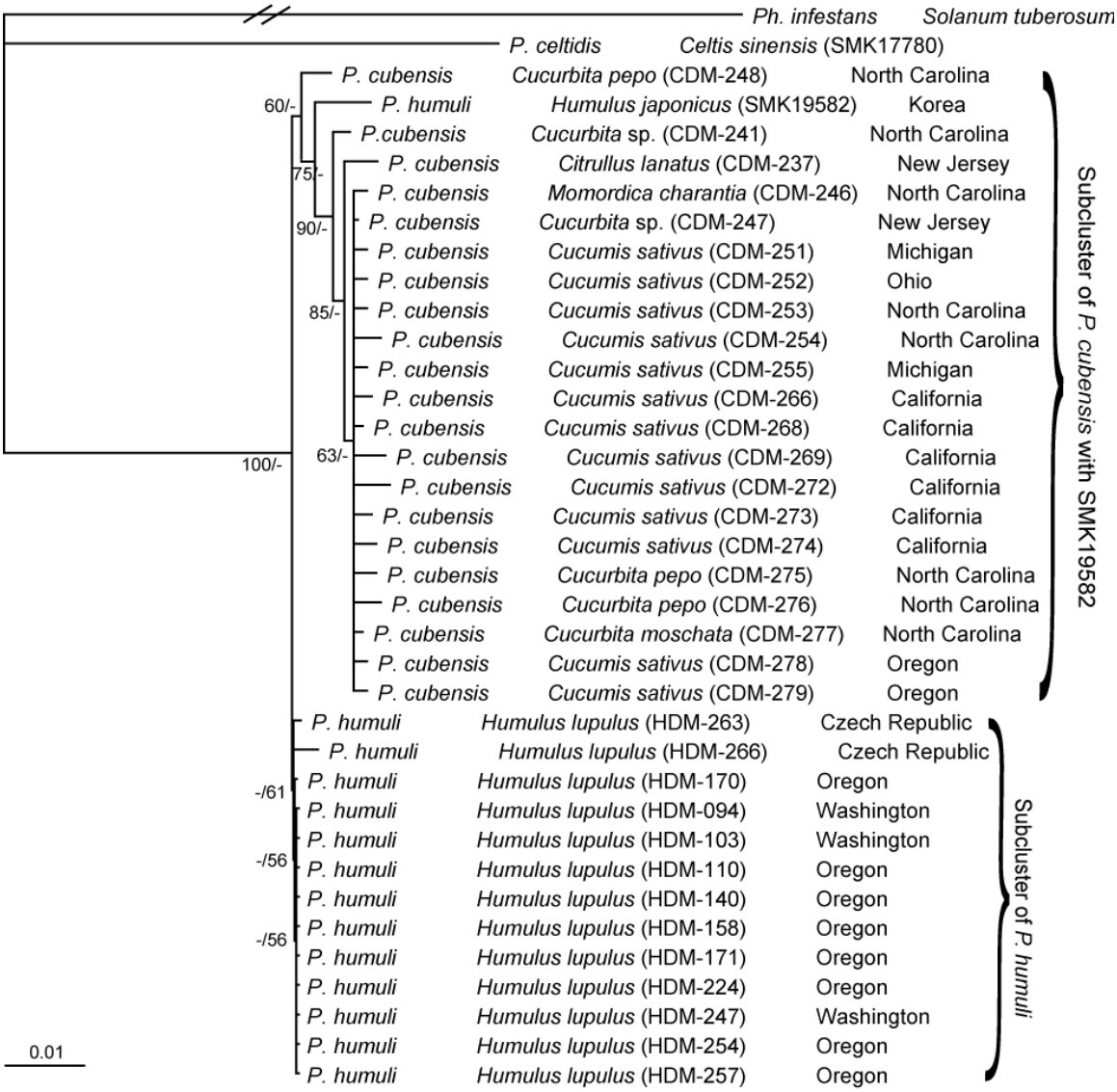

Figure 6. 50% consensus tree of Bayesian and maximum likelihood analyses of an alignment of *cox* cluster (partial *cox2*, *cox2-cox1* spacer, and partial *cox1*) for *Pseudoperonospora cubensis* and *P. humuli*. Genetic distances were computed according to the general time reversible model, additionally assuming a proportion of invariant nucleotide sites. Tree topology was rooted with *Phytophthora infestans*. Numbers on branches are posterior probability (3×10^6 generations) followed by bootstrap values (1000 replicates). Dashes indicate support lower than 50%. The expected number of nucleotide substitutions between taxa is represented by branch length and the scale bar equals the expected number of nucleotide substitutions per site. Interrupted branch was scaled to half of the length.

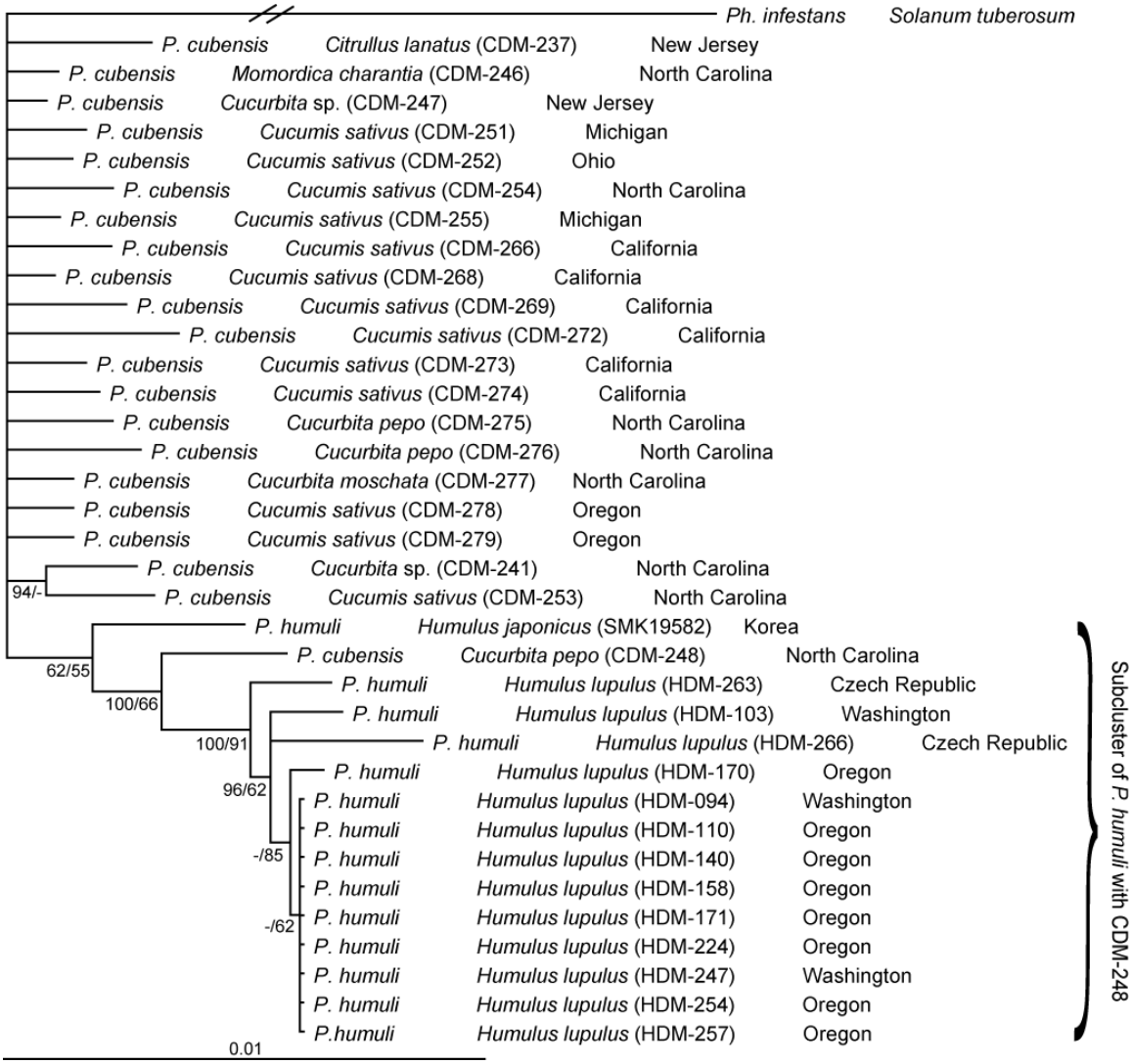

Figure 7. 50% consensus tree of Bayesian and maximum likelihood analyses of an alignment of the concatenated dataset (ITS, *β-tub*, and *cox* cluster) for *Pseudoperonospora cubensis* and *P. humuli*. Genetic distances were computed according to the general time reversible model, additionally assuming a gamma distributed substitution rate. Tree topology was rooted with *Phytophthora infestans*. Numbers on branches are posterior probability (3×10^6 generations) followed by bootstrap values (1000 replicates). Dashes indicate support lower than 50%. The expected number of nucleotide substitutions between taxa is represented by branch length and the scale bar equals the expected number of nucleotide substitutions per site. Interrupted branch was scaled to a tenth of the length.

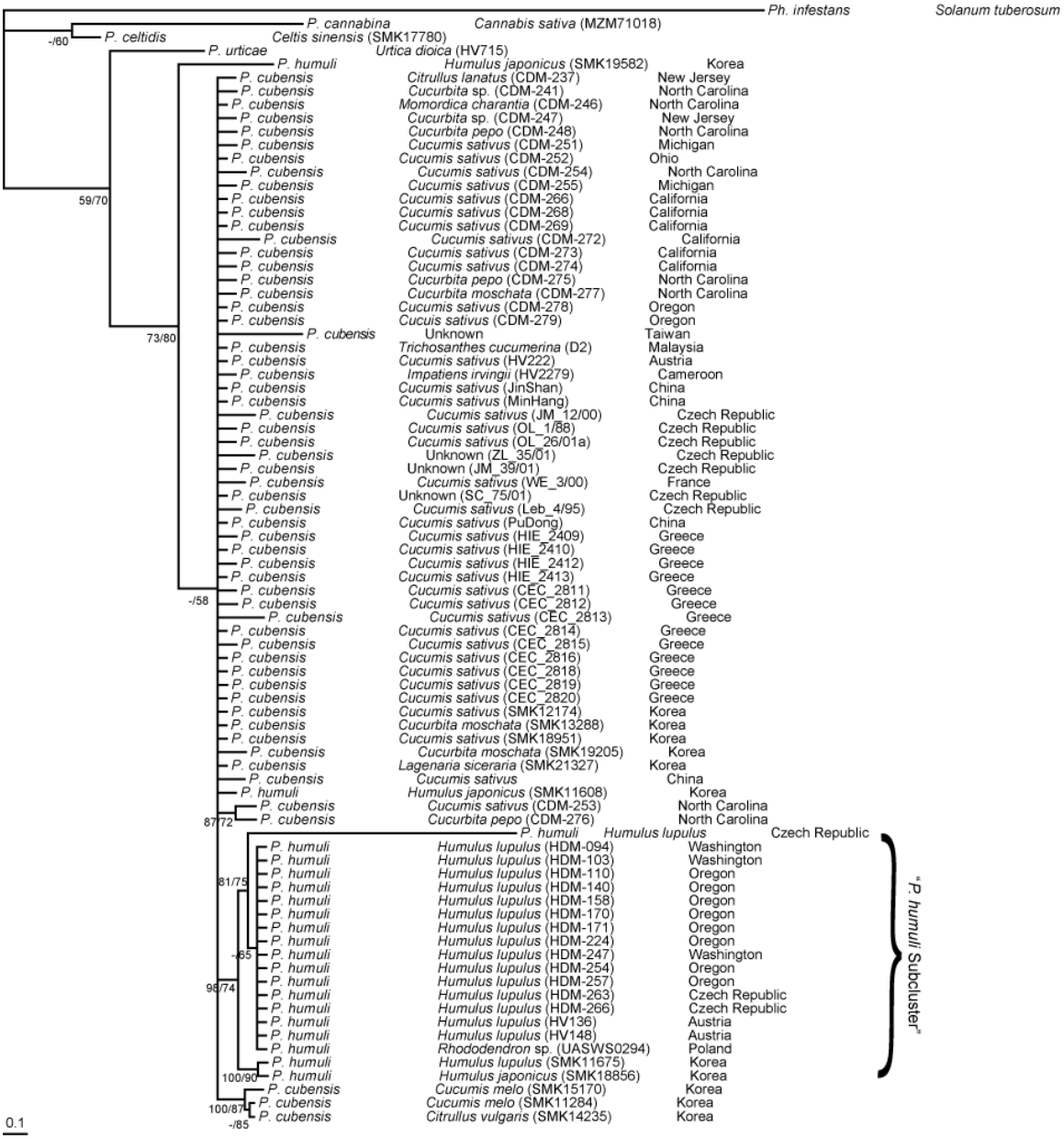

Figure 8. 50% consensus tree of Bayesian and maximum likelihood analyses of an alignment of available *Pseudoperonospora* ITS sequences from GenBank. Genetic distances were computed according to the general time reversible model, additionally assuming a gamma distributed substitution rate. Tree topology was rooted with *Phytophthora infestans*. Numbers on branches are posterior probability (3×10^6 generations) followed by bootstrap values (1000 replicates). Dashes indicate support lower than 50%. The expected number of nucleotide substitutions between taxa is represented by branch length and the scale bar equals the expected number of nucleotide substitutions per site.

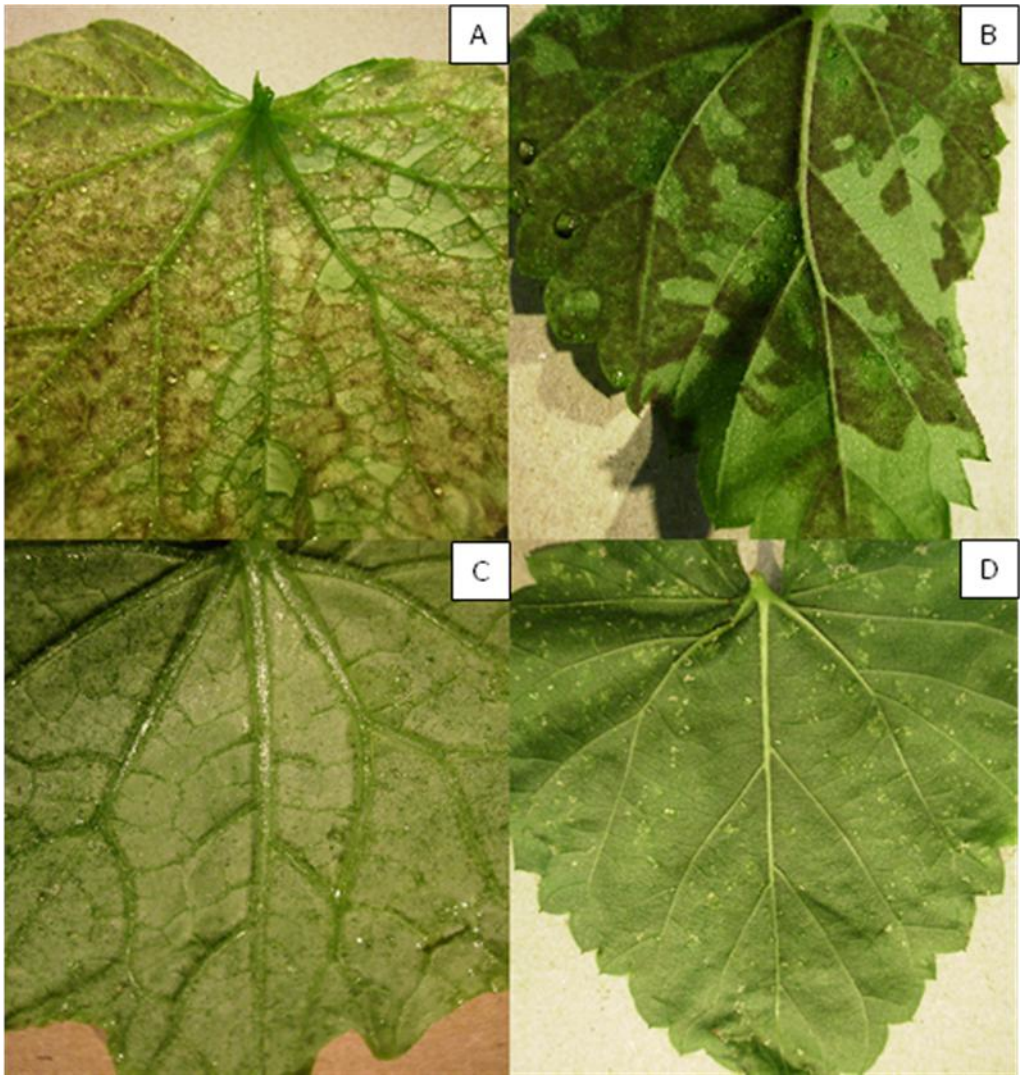

Figure 9. Macroscopic disease signs and symptoms from host specificity experiments with *Pseudoperonospora cubensis* and *P. humuli*. *P. cubensis* inoculated on cucumber cv. Straight 8 (**A**), *P. humuli* inoculated on hop cv. Pacific Gem (**B**), *P. humuli* inoculated on cucumber cv. Straight 8 (**C**), and *P. cubensis* inoculated on hop cv. Pacific Gem (**D**). The positive controls (**A and B**) are characterized by profuse sporulation and few hypersensitive-like lesions, while the opposite is true of the reciprocal inoculations (**C and D**).

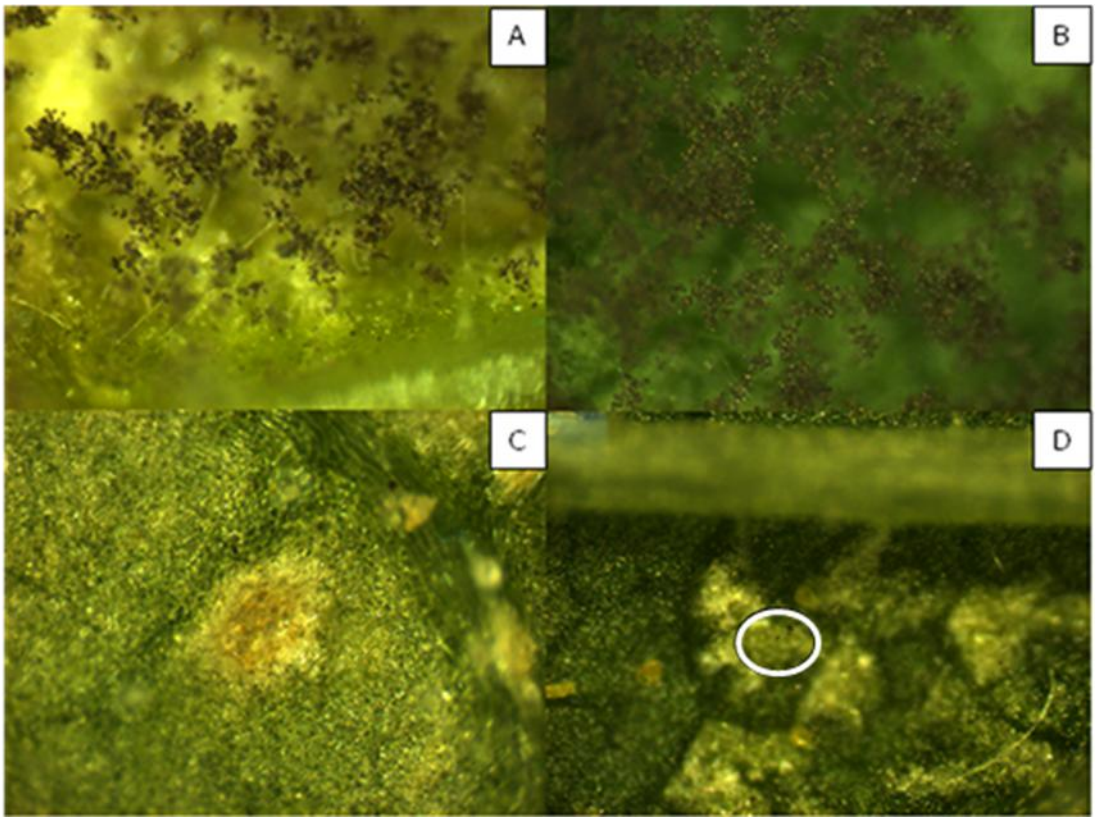

Figure 10. Microscopic disease signs and symptoms from host specificity experiments with *Pseudoperonospora cubensis* and *P. humuli* (50 x magnification). *P. cubensis* inoculated on cucumber cv. Straight 8 (**A**), *P. humuli* inoculated on hop cv. Pacific Gem (**B**), *P. humuli* inoculated on cucumber cv. Straight 8 (**C**), and *P. cubensis* inoculated on hop cv. Pacific Gem (**D**). The positive controls (**A and B**) are characterized by profuse sporulation and few hypersensitive-like lesions, while the opposite is true of reciprocal inoculations (**C and D**). Sporangia can be seen in the circled area of panel **D**.

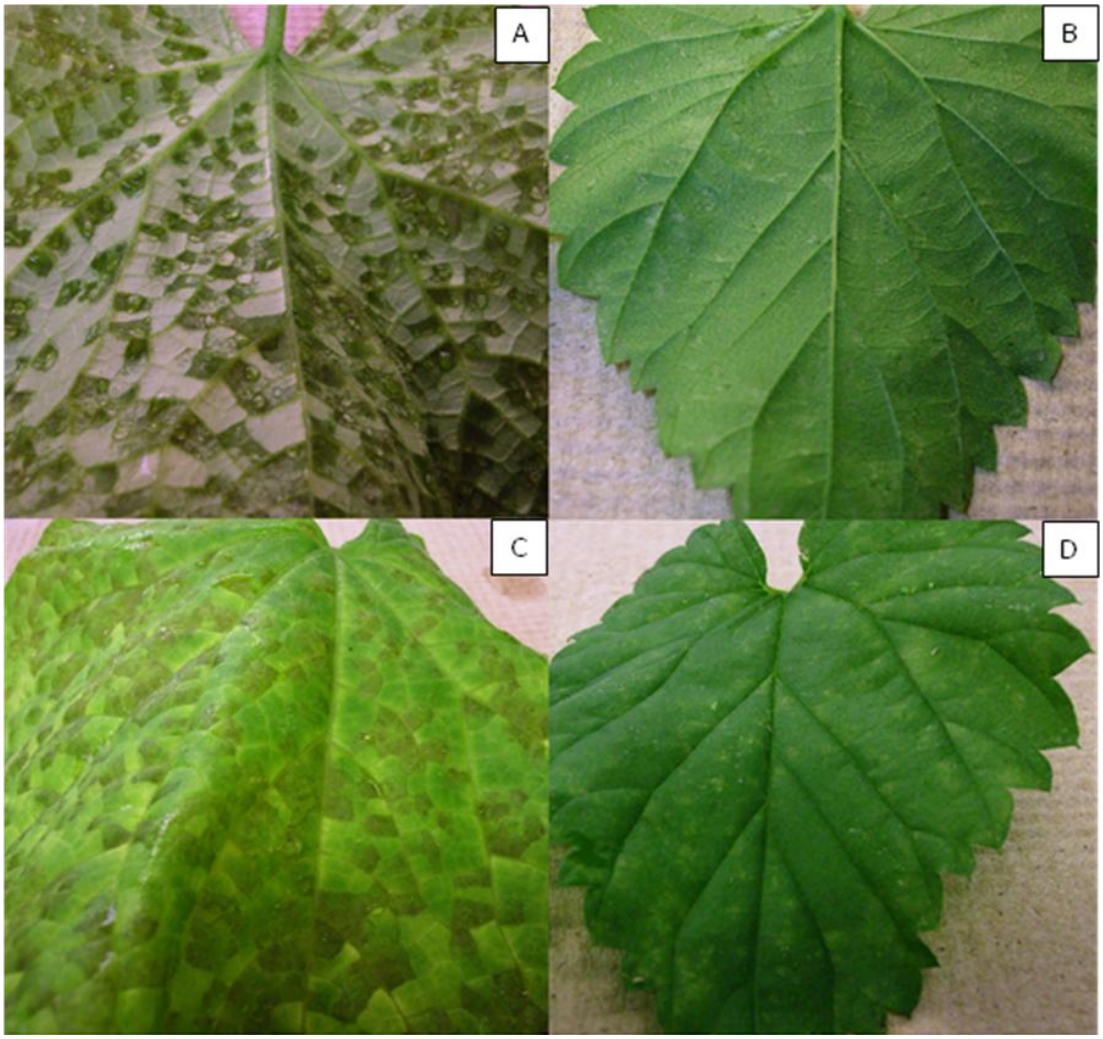

Figure 11. Macroscopic disease signs and symptoms of *Pseudoperonospora cubensis* isolate CDM-255 recovered from 4 hop cv. Pacific Gem plants and then re-inoculated onto cucumber cv. Straight 8 (**A and C**) and hop cv. Pacific Gem (**B and D**). Symptoms in panels **A** and **C** are typical of *P. cubensis* infecting cucumber cv. Straight 8 although sporulation is not as profuse as when the inoculum is harvested from cucumber rather than from hop.

Table 5. Results of host specificity experiments with *Pseudoperonospora cubensis* on hop cultivars and *P. humuli* on cucurbit species

***P. cubensis* Isolate**	**Hop cultivar host**	**Proportion of successful infections[a]**	**Number of sporangiophores per plant ± standard error[b]**	***P. humuli* Isolate**	**Cucurbit host**	**Proportion of successful infections[a]**	**Number of sporangiophores per plant**
CDM 251	Nugget	3/6	38.6 ± 28.5	HDM 224	Cucumber	0/6	0
CDM 252	Nugget	9/15	36.5 ± 33.4	HDM 247	Cucumber	0/6	0
CDM 253	Nugget	4/6	0.3 ± 0.5	HDM 254	Cucumber	0/6	0
CDM 255	Nugget	4/6	[d]	HDM 257	Cucumber	0/6	0
CDM 252	Pacific Gem	6/6	>600	HDM 224	Cantaloupe	0/3	0
CDM 254	Pacific Gem	3/3	>900	HDM 247	Cantaloupe	1/6	1
CDM 275	Pacific Gem	6/6	451.7 ± 380				
CDM 276	Pacific Gem	6/6	239.2 ± 183.0				
CDM 277	Pacific Gem	2/2	107.0 ±70.7				

[a] Proportion of successful infections is defined as the number of plant replicates with sporulation out of the total number of plant replicates for that isolate. Positive controls were always characterized by profuse sporulation and negative controls were always healthy in each run of the each experiment.
[b] The number of sporangiophores per plant and standard error was only calculated for those replicates for which the number of sporangiophores was either counted or estimated at the 14 days post infection (dpi) rating.
[d] Sporagiophores were not found at the 14 dpi rating, but were found at the 7 dpi rating.

CHAPTER 4: DISCUSSION

Although the resolution of any one locus was generally moderate to poor, a majority the isolates of *P. humuli* and *P. cubensis* were separated by the two phylogenetic analyses, occasionally with high support. The phylogenies of the ITS region and *cox2-cox1* spacer yielded the highest statistical support of any of the individual analyses and generally separated *P. cubensis* from *P. humuli* (excluding the isolate from Korea on *H. japonicus*) with high support from both Bayesian and ML analyses. There were several notable exceptions in the genetic resolution of isolates from *Humulus* spp. and cucurbit hosts. Typically, the placement of the isolate of *P. humuli* on *H. japonicus* from Korea was with *P. cubensis* isolates or as subtending the *P. cubensis – P. humuli* clade. An isolate of *P. cubensis* on acorn squash (CDM-248) tended to associate with a clade containing *P. humuli* isolates. Other isolates that were not consistently associated with their respective species include isolates of *P. cubensis* from watermelon (CDM-237), squash (CDM-241), and acorn squash (CDM-276). *P. cubensis* isolates from Oregon and California were always clustered with other isolates of *P. cubensis* from the eastern U.S.

The concatenated analysis provided good resolution of the relations between the isolates of *P. cubensis* and *P. humuli* included in this study. Both analyses agreed about the topology of the non-Korean *P. humuli* isolates and an isolate of *P. cubensis* from acorn squash (CDM-248). The node separating the *P. cubensis* on acorn squash

(CDM-248) from the non-Asian *P. humuli* isolates was given high support by the ML and Bayesian analyses.

Other work that focused on multiple isolates of *P. cubensis* and *P. humuli* primarily reconstruct phylogenies based on the ITS sequence. Choi et al. (2005) examined the phylogenetic relationship within the genus *Pseudoperonospora* by using ITS sequence data. The authors used both Bayesian inference (MCMC) and MP to analyze the phylogenetic relationships. *P. cubensis* and *P. humuli* formed a well-supported clade (100% PP in MCMC and 100% bootstrap support in MP trees). No subcluster within the *P. cubensis-P. humuli* clade was supported with more than 50% support from either Bayesian or MP analyses, however. Within the *P. cubensis-P. humuli* clade, three isolates of *P. cubensis* from Korea from three different host plants (*Citrullus vulgaris*, and two *Cucumis melo* varieties) partitioned into a clade while two other *P. cubensis* isolates from China and Austria were clustered. A subcluster of *P. humuli* isolates, two from Austria and two from Korea (including one from *H. japonicus*) was also found, with the isolates from Europe and Asia separated. Four isolates of *P. cubensis* and two isolates of *P. humuli* from *H. japonicus* were not clustered with each other or with any other isolates.

Other analyses (Göker et al. 2009, Sarris et al. 2009) have included the dataset from Choi et al. (2005) with other sequences that were deposited in the NCBI database after 2005 and have found more support for the clusters within the *P. cubensis-P. humuli* clade. Sarris et al. (2009) inferred a phylogeny of *P. cubensis* and *P. humuli*

from ITS sequences using the neighbor-joining method with the isolates of Choi et al. (2005) as well as 22 isolates of *P. cubensis* from the Czech Republic and Greece and one *P. humuli* isolate from the Czech Republic. Sarris et al. (2009) found that the isolates of *P. cubensis* from Europe ("European subcluster") formed a moderately supported clade (64% of 1000 BS replicates) separate from the Asian *P. cubensis* (and the two *P. humuli* on *H. japonicus*) isolates from Choi et al. (2005), which were designated the "Asian subcluster." The "Asian subcluster" of *P. cubensis* displayed the same topology that was inferred by Choi et al. (2005), with three isolates clustered in the previous analysis forming a clade with 64% bootstrap support. The cluster containing the above isolates was moderately supported (67%) and designated as the "*P. cubensis* clade" by Sarris et al. (2009). The *P. humuli* isolates, excluding the two from Korea on *H. japonicus* mentioned above, formed two moderately supported sister clades (66% and 67% respectively) of the two remaining isolates from Korea and of the four European isolates, including two used by Choi et al. (2005). These two clades were designated the "*P. humuli* cluster" by Sarris et al. (2009).

Göker et al. (2009) inferred the phylogenic relations of the same dataset as Sarris et al. (2009) with two additional *P. cubensis* isolates from Asia using RAxML and maximum parsimony (MP). The same relationships were found among the subclusters of Sarris et al. (2009). The two additional *P. cubensis* isolates from China and Taiwan clustered with the "European subcluster," but the subcluster lacked bootstrap support separating it from the "Asian subcluster." The three *P. cubensis* isolates from Korea that were clustered in the studies of Choi et al. (2005) and Sarris

et al. (2009) were also found to cluster together with high support (82% ML, 88% MP). The "*P. humuli* cluster" did not have more than 50% bootstrap support from either analysis, which is consistent with the other two studies. However, like the result of Sarris et al. (2009), two subclusters were resolved with bootstrap support. Göker et al. (2009) found higher bootstrap support for the sister clades of *P. humuli* from Europe (84% ML, 65% MP) and from Asia (86% ML, 72% MP).

In each of the above studies, the topology of *P. cubensis* and *P. humuli* isolates remains fairly stable, although the statistical support for branches varied depending on the number of isolates and the analysis used (Choi et al. 2005, Göker et al. 2009, Sarris et al. 2009). The isolates of *P. cubensis* tend to be in a clade divided into three groups. Two of the groups are a subclade with isolates of *P. cubensis* from Europe and China and a subclade consisting of isolates from South Korea (Choi et al. 2005, Göker et al. 2009, Sarris et al. 2009). The third group is the four *P. cubensis* isolates and two *P. humuli* isolates of Choi et al. (2005) that are not resolved into a clade, but instead appear to be basal or sister to the two subclades (Choi et al. 2005, Göker et al. 2009, Sarris et al. 2009). The *P. humuli* isolates are partitioned into a separate clade from *P. cubensis* (except the two *P. humuli* isolates from *H. japonicus* clustering with *P. cubensis*) composed of two subclades. The subclades divide the remaining *P. humuli* isolates from Korea clustering together from *P. humuli* isolates from Europe.

An analysis incorporating the isolates used in this study with all available ITS sequences for *P. cubensis* and *P. humuli* from NCBI resulted in similar tree topology

and values as those of Göker et al. (2009). All 14 *P. humuli* isolates from the western U.S. clustered with those from Europe, separate from a subcluster of *P. humuli* from Korea. The "*P. humuli* cluster" was well supported in this analysis, as opposed to the previous studies. In this analysis, no support was found for a European or Asian subcluster of *P. cubensis* isolates.

Analyses of the ITS suggest that there are differences both between and within *P. cubensis* and *P. humuli*. There seems to be a genetic difference between the isolates of Choi et al. (2005) from Korea and isolates from Europe, China, Africa, and the U.S. in both the *P. cubensis* and *P. humuli* clusters. A very obvious example is that of the two *P. humuli* isolates from *H. japonicus* (SMK11608 and SMK19582) that cluster with Korean *P. cubensis* isolates. Within the *P. humuli* cluster, the Korean isolates are strongly supported as being separate from the European and American isolates (Fig. 8, Choi et al. 2005, Göker et al. 2009, Sarris et al. 2009). These differences could be due to more genetic diversity in isolates collected in Korea as compared to those collected in other locations around the world. An investigation into whether the genetic diversity of *P. humuli* and *P. cubensis* truly is greater in Korea and surrounding areas would be illuminating.

The location of the *P. cubensis* isolate on acorn squash (CDM-248) directly subtending the *P. humuli* cluster in the concatenated analysis (as well as the analyses of the ITS region, *cox1*, and *cox2-cox1* spacer) may suggest that *P. humuli* is derived from *P. cubensis*. Analyses of the ITS region also point to this possibility as the *P.*

humuli cluster is consistently subtended by the Korean *P. cubensis* isolates unless the analysis includes another *Pseudoperonospora* species, in which case nearly all the *P. cubensis* isolates subtend the *P. humuli* cluster (Fig. 8; Choi et al. 2005, Göker et al. 2009, Sarris et al. 2009). Hop downy mildew was first recorded in Japan in 1905 and it is possible that *P. humuli* originated in Asia (Miyabe & Takahashi 1906). It is interesting to note that Asia, especially the areas of India, Indo-Malaysia, and China, is the presumed center of origin for *Humulus* (Neve 1991) and many genera and species of cucurbits, including *Cucumis sativus* and other *Cucumis* species. (Kalloo & Bergh 1993, Robinson & Decker-Walters 1997). *Citrullus*, most *Cucumis* species (including *C. melo*) and *Cucurbita* species arose from Africa (*Citrullus* and *Cucumis*) and South America (*Cucurbita*) and some, but not all, of the *P. cubensis* isolates that were from these hosts (i.e., CDM-237, 241, 248, and 276) did not cluster with the other *P. cubensis* isolates in the phylogenetic analyses.

Molecular markers that have better resolution at lower taxonomic levels (i.e., at or below species) should provide better resolution between and among *P. cubensis* and *P. humuli* species. A less conserved region of the genome, such as the intergenic spacer (IGS) that separates tandem repeats of rDNA genes, or microsatellites, may provide this resolution (Chambers & MacAvoy 2000, Gorokhova et al. 2002). However, multiple attempts to amplify the IGS region in our laboratory were unsuccessful, potentially due to the extreme length or repetitiveness of the sequences (Gorokhova et al. 2002). A preliminary microsatellite study with a limited number of isolates of *P. humuli* and *P. cubensis* from the western U.S. appears to indicate that a

number of microsatellite markers may differentiate isolates that originate from *Humulus* and cucurbit hosts. Further sampling, especially from outside the U.S. and in Asia particularly, and development of more sensitive markers is needed before any conclusions on the relatedness of these organisms can be made, however.

The host specificity experiments indicate that *P. cubensis* is better able to infect hop plants than *P. humuli* is able to infect cucurbit plants for the cultivars, conditions, and isolates used in this study. Hop plants inoculated with *P. cubensis* sporulated in 77% of the replicates, with more infection being seen on cv. Pacific Gem, a cultivar more susceptible to hop downy mildew than cv. Nugget. *P. humuli* only sporulated once on a cucurbit host out of 33 replicate plants, and in that instance only a single sporangiophore was observed growing out of a necrotic, HR-like lesion. Each host exhibited lesions consistent with hypersensitive-like responses when inoculated with the reciprocal pathogen. Sporulation always arose from the center or inner edge of the necrotic (occasionally chlorotic) lesion. Other than cultivar, the leaf age and condition often had a large impact on how many lesions contained sporulation, with older leaves tending to have more lesions with sporangiophores as well as more sporagiophores and sporangia per lesion than younger leaves of the same quality (Royle & Thomas 1971). From this study, it appears that at least two of the reportedly universal hosts of *P. cubensis* are extremely poor or, more likely, non-hosts of *P. humuli* under natural conditions, and that at least two hop cultivars highly susceptible to hop downy mildew are also poor hosts of *P. cubensis*. Hoerner (1940) unsuccessfully attempted to infect unspecified (“all available”) hosts of *P. cubensis*

with *P. humuli*. Unfortunately, the experiments were undocumented aside from a passing remark (Hoerner, 1940), so the conditions and exact organisms or isolates are unknown.

As isolates of both *P. cubensis* and *P. cubensis* from Korea have different phylogenetic signals than other isolates from elsewhere in the world, a critical experiment would be to determine whether these isolates vary in terms of pathogenicity on other hosts. Ideally, isolates from many countries on many host plants would be tested to more fully understand the relationships between the pathogens. Although *P. humuli* did not appear to be pathogenic to the reportedly universally susceptible cucumber and cantaloupe hosts utilized in the current study, experimenting with *P. humuli* on the differential set of cucurbit species of Lebeda and Gadasová (2002) may reveal a host on which *P. humuli* is better able to survive than in this study. Similarly, host range experiments involving *P. cubensis* on *H. japonicus* are needed.

The occurrence of sporangiophores within necrotic or chlorotic lesions may indicate that the plant had largely contained the spread of the mycelia, which may starve the biotrophic pathogen over time. However, a limitation of the host specificity experiments reported here was that the plants were observed for only 14 days after inoculation. Perhaps a longer incubation and or different environmental conditions would be more informative about the survival of the pathogen placed on a non-preferred host.

Relatively low numbers of sporangiophores and sporangia may be a result of the pathogen being nutritionally compromised. Microscopy of mycelial penetration of *P. cubensis* into hop leaves and of *P. humuli* into cucurbit leaves would be needed to determine if the plant's HR-like response is truly containing the pathogen. If *P. cubensis* is not contained by the plant's defense response, an exploration into whether *P. cubensis* can mate with *P. humuli in planta* when both pathogens co-infect a hop leaf would be informative. It would also be revealing to examine the extent to which *P. humuli* was able to grow within cucurbit hosts and at what point of the infection process the pathogen is inhibited. If the pathogen is being contained by dead or dying tissue, *in planta* mating between *P. humuli* and *P. cubensis* would be unlikely, conforming to the biological species concept of Gäumann (1918).

The ability of *P. cubensis* to successfully infect and sporulate on the two hop cultivars, albeit at low levels, may be due to its polyphagic lifestyle. *P. cubensis* has been recorded on at least 49 wild and cultivated species of Cucurbitaceae in 70 countries (Choi et al. 2005, Cohen 1981, Palti & Cohen 1980, Renfo & Bhat 1981). Voglmayr et al. (2009) also reported *P. cubensis* causing downy mildew on *Impatiens irvingii* (Balsaminaceae) in Cameroon, although pathogenicity studies were not performed on a cucurbit host with the isolate from *I. irvingii*. An investigation into what aspect of the pathogen (e.g., effectors) enables it to infect such a wide range of host genera would be illuminating. If *P. humuli* is indeed derived from *P. cubensis*, the infection of hop by *P. cubensis* is less unexpected. The relative inability of *P. humuli* to infect the two cucurbit species in this study and that by Hoerner (1940) may be a

result of a bottleneck event that occurred to an individual or population of *P. cubensis* that were able to infect hop. Possibly the event(s) that enabled the pathogen to better utilize hop as a host also prevented it from being as promiscuous as the population of *P. cubensis* on cucurbits.

The *cox2* gene was sequenced from two isolates of *P. cubensis* on hop cv. Pacific Gem plants. One isolate was originally from an acorn squash (CDM-276) and the other was originally from a cucumber (CDM-255). The isolate from cucumber was used in an experiment in which *P. cubensis* from hop cv. Pacific Gem was inoculated onto hop cv. Pacific Gem and cucumber cv. Straight 8. Thus, three sequences were gathered for CDM-255 – *P. cubensis* on hop, *P. cubensis* from hop on hop, and *P. cubensis* from hop on cucumber. The sequences were identical to the majority of the *P. cubensis* (exceptions noted above). However, each sequence had at least one unique SNP, although the SNPs were not found upon resequencing the amplicons without cloning. Thus it is possible that the unique SNPs were an artifact of the cloning process.

Conclusions

Choi et al. (2005) proposed reducing *P. humuli* to a taxonomic synonym of *P. cubensis*, but further examinations of molecular data and host specificity indicate that there are biologically relevant characteristics that differentiate *P. cubensis* and *P. humuli*. Aside from two isolates of *P. humuli* on *H. japonicus* from Korea,

phylogenetic analyses of the ITS region, *β-tub*, and the *cox* cluster individually and concatenated inferred that *P. humuli* belongs in a separate cluster than *P. cubensis*. The *P. humuli* cluster appears to be within the *P. cubensis* isolates, which may indicate that *P. humuli* descended from *P. cubensis.* Host specificity experiments also suggest that *P. cubensis* and *P. humuli* are biologically distinct as *P. cubensis* appears to be able to infect the primary host of *P. humuli*, albeit at low levels, while *P. humuli* was essentially unable to successfully infect at least two highly susceptible hosts of *P. cubensis*. The existence of hop-growing countries (e.g., Australia, South Africa, and New Zealand) that have cucurbit downy mildew but not hop downy mildew indicate, anecdotally, that under natural conditions hop does not support *P. cubensis* at detectable levels. These countries, which have quarantines against *P. humuli* may have their hop production threatened by downy mildew by the change in taxonomy proposed by Choi et al. (2005), that is if *Pseudoperonospora* isolates originating from hop were reduced to a taxonomic synonym of "*P. cubensis.*"

The results of this polyphasic study suggest that *P. humuli* should not be reduced to a synonym of *P. cubensis* but perhaps could be considered a subspecies or *forma specialis* of *P. cubensis* to reflect quantifiable differences between isolates from *Humulus* and cucurbit hosts. A *forma specialis* designation may be the most appropriate following the fourth note of Article 4 of the Vienna Code (McNeill et al. 2006) which stipulates that in the classification of parasitic fungi when authors do not assign a subspecific or varietal value to a taxon "characterized from a physiological standpoint but scarcely or not at all from a morphological standpoint may distinguish

within the species special forms (*formae speciales*) characterized by their adaptation to different hosts." Combining the results of the study of Choi et al. (2005) and this study, it appears that *P. humuli* and *P. cubensis* are morphologically and genetically very similar but are distinct physiologically. Further investigations of the host specificity of a greater sample of both pathogens and with molecular methods with greater resolution are needed to support such a recommendation.

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APPENDIX

Protocol for Maintenance of *Pseudoperonospora humuli* Isolates

Materials

Containment hood

Preval Sprayer 267 (268 is refill)

Petri dish

Whatmann #1 filters

Barrel container from Snapware, hard lid is replaced with filter paper held in place with gasket

Three susceptible hop plants (cv. Nugget or Pacific Gem) in 648-cm^3 pots

Growth chamber

Spray bottle for water

Bag for diseased plant tissue; infected shoots are most appropriate

Cardboard box for putting infected shoot in dark (or dark cupboard)

Hemocytometer and microscope

Procedure

1. Infected shoots are brought in from the field, misted and bagged and placed in the dark overnight to induce sporulation. Alternatively, if an isolate is already being maintained on live plants, prepare the isolate by increasing the humidity of the container.

2. A single leaf is removed from the infected shoot or all the leaves are removed from live plants already maintained. A Preval Sprayer is used to wash the spores off the leaf into a Petri dish in a containment hood.

3. Spores are counted using a hemocytometer and adjusted to $1x10^4$ spores per ml in 30 ml for bulk inoculation. Also perform a monosporangial isolation (see protocol for obtaining a monosporangial *P. humuli* isolate) for new isolates.

4. Spores are sprayed onto abaxial side of leaves of live hop plants.
5. Plants are placed in barrel container that has been misted with water.
6. Plants are placed in a greenhouse to get natural, indirect light for 16 to 24 hours. Make sure plants are not in direct sunlight or they will overheat in the tubes.
7. Plants are taken out of the barrel container and leaves are allowed to dry completely (6-8 hours).
8. Plants are placed back into the dried barrel container and placed in a growth chamber set at 13 degrees C, 12 hour light, 12 hour dark cycle for 4 to 6 days. Plants will respire and form their own moisture in the barrel container in Oregon. If they do not, mist the plants on the last incubation night.
9. Harvest spores using a Preval Sprayer and re-inoculate new plants.

Protocol for Obtaining a Monosporangial *P. humuli* Isolate

Materials

Infected plant material with sporulating lesions

MilliQ water

10 µl micropipette

Dissecting microscope

Healthy susceptible hop plants (cv. Nugget or Pacific Gem)

Procedure

1. Flood a sporulating lesion of a plant infected with *P. humuli* with MilliQ water using a micropipette. Use no more than 100 µL and rinse only a few times, as isolating a single spores is increasingly difficult with more spores.
2. Place liquid sample with sporangia onto an opaque object like a weigh boat.
3. Flood sample with MilliQ water to dilute sample in order to better isolate a single sporangium.
4. Using a dissecting microscope and a 10 µL micropipette, aspirate a single spore. Try to aspirate the spore with the least amount of water possible, no more than 5 µL.
5. Place spore into a droplet on a leaf at least 4 nodes from the growing tip of a hop plant. Repeat 5 to 10 times per leaf. Make sure to place the droplets far enough apart on the leaf to ensure the colonies will not converge.
6. Incubate the plants as above.

Protocol for Maintenance of *Pseudoperonospora cubensis* Isolates

Modified from Colucci, S. J. 2008. Host Range, Fungicide Resistance and Management of *Pseudoperonospora cubensis*, Causal Agent of Cucurbit Downy Mildew. MS Thesis. North Carolina State University, Raleigh and personal communication.

Materials:

Containment hood

Envirocide or 70% ethanol

Preval Sprayer 267 (268 is refill)

Petri dish

Whatmann #1 filters

Barrel container or Pasta Keeper tube from Snapware, moist filter paper is held in place with gasket under lid of Pretzel or moist folded paper towel is placed at base of Pasta tube

One to three cucurbit plants (cucumber 'Straight 8' or another susceptible cultivar)

Growth chambers set to "dark humid chamber" or "incubation chamber" settings

Humidifier

Spray bottle for water

Hemocytometer and microscope

<u>Program for dark humid chamber</u>

12:00 pm 21.0°C 99%RH

L1: 000

Program for incubation chamber

6:00 am 21.0°C 0%RH

L1:100

6:00 pm 18.0°C 0%RH

L1:000

Procedure:

1. Infected leaves are brought in from the field or otherwise obtained, misted and bagged and placed in the dark overnight to induce sporulation.
2. A Preval Sprayer is used to wash the spores off the leaves into a Petri dish in a containment hood.
3. Inoculum can be stored on ice in Falcon tubes.
4. Spores are counted using a hemocytometer and adjusted to $6.5x10^3$ spores per ml. 30 to 50 ml total inoculum is sufficient for one to three plants.
5. Spores are sprayed onto abaxial side of leaves of live plants.
6. Plants are placed in barrel containers or Pasta Keeper tubes that have been misted with water.
7. Plants are placed in dark humid chamber (with a humidifier at the bottom of the growth chamber set to turn on one out of every 4 hours).
8. Plants are taken out of the tubes and placed into standing water (at least ½ inch) in incubation chamber. Each isolate should have its own growth chamber to avoid contamination.
9. If more isolates are needed than number of growth chambers available, let plant leaves dry in growth chambers for 1 day, then place back into dry tubes with lid open. Make sure that a filter paper is secured under the lid, to avoid contamination.
10. On the 6^{th} day post infection, mist the container for each isolate and place plants back into containers and put containers into humid dark chamber.

11. On the 7th day, harvest spores using a Preval Sprayer and re-inoculate new plants.

Protocol for Cryopreservation of *P. cubensis* and *P. humuli* Isolates at -80°C: Whole Leaves

From Colucci, S. J. 2008. Host Range, Fungicide Resistance and Management of *Pseudoperonospora cubensis*, Causal Agent of Cucurbit Downy Mildew. Master's Thesis. North Carolina State University.

Materials

Infected plant material

Containment hood, if necessary

Plastic sandwich bag or plastic Petri dishes (100 x 15 mm) and Parafilm

-20°C freezer

-80°C freezer

Procedure

1. 6 days post infection, place infected plants into dark humid chamber (21°C, 99% RH) for 24 hours.
2. 7 dpi, remove prolifically sporulating leaves and allow to dry slightly at room temperature for about 10 min.
3. Leaves are put into labeled plastic sandwich bags or labeled plastic Petri dishes which are then wrapped in Parafilm.
4. Bags or Petri dishes are placed in a -20°C freezer for 24 hours.
5. Cultures are moved to -80°C freezer for long term storage (6-12 months).

6. To revive isolates, remove cultures from -80°C and allow to thaw at room temperature.
7. Harvest sporangia and inoculate healthy plant as normal.

Protocol for Cryopreservation of *P. cubensis* and *P. humuli* Isolates at -80°C: With a Cryoprotectant

Modified from Dahmen, H., Staub, T., and Schwinn, F. J**.** 1983. Technique for long-term preservation of phytopathogenic fungi in liquid nitrogen. Phytopathology 73:241-246.

Materials Needed

Infected plant material

Containment hood

Preval Sprayer 267 (268 is refill)

Petri dishes

Falcon tube (size depends on volume of inoculum)

Ice bucket

Hemacytometer and microscope (if desired)

Microcentrifuge (if desired)

2 ml Eppendorf microfuge tubes or cryogenic storage vials

DMSO

Vortexer

-20°C freezer

-80°C freezer

Procedure

1. 6 days post infection, place infected plants into dark humid chamber (21°C, 99% RH) for 24 hours.
2. A Preval Sprayer is used to wash the spores off the leaves into a Petri dish in a containment hood.
3. Store inoculum on ice in Falcon tubes.
4. If desired, quantify sporangia concentration with hemacytometer for records.
5. If inoculum concentration is less than desired, allow the inoculum to settle on ice or centrifuge at about 1000 rpm for about 3 minutes and remove the appropriate amount of water (i.e., double the concentration by halving the total volume).
6. Add 850 µl inoculum to each of at least 4 labeled 2 ml Eppendorf microfuge tubes or cryogenic storage vials.
7. Add 150 µl DMSO, then vortex to homogenize solution.
8. Place tubes into -20°C freezer for 24 hours.
9. Place tubes into -80°C freezer for long term storage.
10. To revive isolates thaw tubes on ice, then centrifuge at 10000 rpm for 2 minutes, discard supernatant.
11. Add 1 ml sterile MilliQ water, centrifuge at 10000 rpm for 2 minutes, discard supernatant.
12. Repeat step 11.
13. Add 1 ml sterile MilliQ water and add to about 10ml sterile MilliQ water in Preval Sprayer and inoculate healthy plant tissue.

Protocol for CTAB Extraction of *P. humuli* and *P. cubensis* DNA from Sporangia

Modified from Chee, H. Y., Nelson, M. E., Grove, G. G., Eastwell, K. C., Kenny, S. T., and Klein, R. E. 2006. Population biology of *Pseudoperonospora humuli* in Oregon and Washington. Plant Dis. 90:1283-1286.

Materials

Four 1.5 ml microcentrifuge tubes

70% Ethanol (EtOH), cold

Polyvinyl pyrrolidone (PVP)

10mM Tris-HCl pH 7.5

Hot plate with boiling water

Isopropanol, cold

CTAB working stock extraction buffer [see CTAB Extraction Solution Recipes]

Sterile MilliQ water

20mg/ml Proteinase K [see CTAB Extraction Solution Recipes]

pH meter

98% β-mercaptoethanol

P. cubensis or *P. humuli* spore suspension

RNase A

Ice bath

24:1 Chloroform: isoamyl alcohol ($CHCl_3$:IAA)

DNA Fast Prep tube for grinding

Procedure:

1. Place 0.021g PVP in a Fast Prep tube.
2. Suspend spores in 100 µl of 1 x TE. Add 900 µl of CTAB extraction buffer to Fast Prep tube and place 100 µl spore suspension in with the CTAB buffer.
3. Use Fast Prep machine to burst open the spores. Run the machine @ level 6 for 45 sec. Ice between each beat. Repeat the step 4 times.
4. Pipette 750 µl into a clean tube.
5. In chemical hood, add 7.5 µl β-mercaptoethanol to make a 1% solution, 22.5 µl 20 mg/ml Proteinase K to make a 0.3 mg/ml solution, 7.5 µl RNase A to make a 1% solution.
6. Incubate for 30 min. @ 65°C.
7. In chemical hood, add 750 µl of 24:1 CHCl3:IAA. Mix well.
8. Centrifuge for 10 min. @ 14k.
9. Transfer top aqueous phase (up to 650 µl) to new tube.
10. Repeat steps 7 & 8.

11. Transfer top aqueous phase (up to 500 µl) to new tube.
12. Add equal parts of cold isopropanol to the tube and invert several times until the solution is completely mixed.
13. Centrifuge for 20 min. @ 14k and 4°C.
14. Gently pour off the supernatant.
15. Rinse with 70% EtOH.
16. Centrifuge for 20 min. @ 14k and 4°C.
17. Gently pour off the 70% EtOH and dry in a fume hood on a hot plate that will keep the sample at 35°C.
18. When the tubes are completely dry [note* very important that the EtOH is completely dry, but not overdry] resuspend in 50 µl of 10 mM Tris-HCl pH 7.5.
19. Store in -20°C freezer.

CTAB Extraction Solution Recipes

For 100 ml of the CTAB working stock extraction buffer:

10 ml 1M Tris-HCl pH 7.5

28 ml 5M NaCl

4 ml 0.5 EDTA pH 8.0

2 g CTAB

*Mix on a stir plate and bring up to 100 ml with sterile MilliQ water. Remake the CTAB buffer after a month.

CTAB Stock Solutions:

100 ml of 1M Tris pH 7.5

Add 15.76 g Tris to 80 ml of sterile MilliQ water, then pH to 7.5 using a pH meter. Bring to final volume of 100 ml. Autoclave.

100 ml of 5N NaCl

Add 29.2 g NaCl to 80 ml of sterile MilliQ water, then heat on a stir plate and bring up to 100 ml. Autoclave.

100 ml of 0.5M EDTA pH 8.0

Add 18.6 g EDTA to 80 ml of sterile MilliQ water, then pH to 8.0 using a pH meter. Bring final volume to 100 ml. Autoclave

10 ml of Proteinase K Solution

1 ml of 20mM CaCl

5 ml of 50% glycerol

100 µl of 10mM Tris·HCl pH 7.5

Proteinase K Stock Solutions

20 ml of 20mM CaCl

Add 5.88 g CaCl into 10 ml of sterile MilliQ water. Bring up to 20 ml total volume.

10 ml of 10mM Tris-HCl pH 7.5

Add 100 µl of Tris-HCl to 9.9 ml of sterile MilliQ water and check pH.

MoBio Extraction Protocol

Modified from MoBio Laboratories, Inc. Ultra Clean ™ Soil DNA Isolation Kit (Catalog # 12800-100) Instruction Manual. Version 03252005.

Materials:

MoBio Laboratories, Inc. Ultra Clean ™ Soil DNA Isolation Kit

Polyvinyl pyrrolidone (PVP)

Ice bath

Vortex

Hot plate with boiling water

Before starting

Label 1 Bead Solution Tube, 3 catch tubes, and 1 filter tube for each extraction.

Start water boiling.

Procedure

1. To the 2 ml Bead Solution tubes provided, add 60 µl of solution S1 into which 0.140 g PVP has been added (0.002 g/tube).
2. Add 200 µl IRS solution.

3. Add spore solution.
4. Secure tubes on vortex. Vortex at top speed for 10 minutes.
5. Boil tubes for two minutes.
6. Centrifuge for 30 seconds at 10,000 x g.
7. Transfer 400 to 450 µl of the supernatant to a clean catch tube.
8. Add 250 µl of Solution S2. Mix by inversion.
9. Incubate at 4°C for 5 minutes. Ice bucket is best.
10. Centrifuge the tubes for 1 minute at 10,000 x g.
11. Avoiding pellet, transfer up to 650 µl of solution to clean catch tube.
12. Add 1.3 ml of Solution S3. Mix by inversion.
13. Load 700 µl onto the spin filter.
14. Centrifuge for 1 minute at 10,000 x g.
15. Discard flow through and add 700 µl to spin filter.
16. Centrifuge for 1 minute at 10,000 x g.
17. Discard flow through and add remainder of solution to spin filter.
18. Centrifuge for 1 minute at 10,000 x g.
19. Discard flow through and add 300 µl of Solution S4.
20. Centrifuge for 30 seconds at 10,000 x g.
21. Discard flow through.
22. Centrifuge for 1 minute at 10,000 x g with dry filter.
23. Carefully transfer filter to new catch tube. Avoid all solution S4.
24. Add 50 µl of Solution S5.

25. Centrifuge for 30 seconds at 10,000 x g.

26. Discard filter.

27. Store labeled DNA at -20°C.

Ligation Protocol

From Promega pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual, Revised 3/03. Part# TM042.

1. Use 0.2ml microcentrifuge tubes to set up ligation.
2. Vortex 2X Rapid Ligation Buffer vigorously before each use.
3. Centrifuge 2X Rapid Ligation buffer and pGEM T-easy Vector tube very briefly to collect contents in the bottom of tube.
4. Mix the reactions by pipetting.
5. Incubate the reaction 1 hour at room temperature, then overnight at 4°C.

Master Mix	Per Reaction	Per Half Rxn	Per Quarter Rxn
2X Rapid Ligation Buffer	5.0 µl	2.5 µl	1.25 µl
pGEM T-Easy Vector	1.0 µl	0.5 µl	0.25 µl
T4 DNA Ligase	1.0 µl	0.5 µl	0.25 µl
PCR product	3.0 µl*	1.5 µl*	0.75 µl*
DI water to final volume of	10.0 µl	5.0 µl	2.50 µl

*Max volume, molar ratio of PCR product:vector may require optimization. Molar ratio of PCR product:vector can vary from 1:3 to 3:1. The vector is approximately 3 kb and is supplied at 50ng/µl.

*2X Rapid Ligation Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by making single use aliquots of the buffer.

Transformation Protocol

Modified from Promega instructions, Revised 3/03. Part# TM042.

Materials

Ice bath

Thermocycler

Shaking Incubator

Ligation reaction

JM109 High Efficiency Competent Cells

0.2 ml microcentrifuge tubes

SOC medium

LB/ampicillin/IPTG/X-Gal agar plates

Before starting

1. Prepare 2 LB/ampicillin/IPTG/X-Gal plates for each reaction. Equilibrate to room temperature before plating (step 8 below).
2. Aliquot 475 µl SOC into 1.5 ml microcentrifuge tubes and equilibrate to 37°C. Use the hood for this step to avoid contamination of the stock SOC solution.

3. Work on ice except where noted.

Procedure

1. Remove tube(s) of JM109 High Efficiency Competent Cells from the -80°C freezer and place immediately on ice for 5 minutes. Mix by gently flicking as competent cells are fragile.
2. Carefully aliquot 25 µl of cells into sterile 0.2 ml microcentrifuge tubes on ice.
3. Add 2 µl of each ligation reaction the sterile 0.2 ml microcentrifuge tube in step 2.
4. Gently flick and place on ice for 20 minutes.
5. Heat-shock cells for 45-50 seconds in the thermocycler at exactly 42°C.
6. Immediately return tubes to ice for 2 minutes.
7. Add whole transformation mixture to SOC medium and incubate 1.5 hours at 37°C with shaking (150 to 225 rpm).
8. Plate 100 µl of each transformation culture onto duplicate LB/Amp/IPTG/X-Gal plates. Incubate overnight (16-24 hours) at 37°C. Wipe condensation off lid with Kimwipe, wrap in Parafilm and store at 4°C. Blue color will continue to develop.
9. Perform a colony PCR to check for insert.

Transformation Recipes

IPTG stock solution (100 mM)

Dissolve 1.2 g IPTG into 50 ml final volume of MilliQ water. Filter-sterilize and store at 4°C.

X-Gal (2 ml)

Dissolve 100 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside in 2 ml N,N'-dimethylformamide. Cover with aluminum foil and store at -20°C.

LB medium (per liter)

10 g tryptone

5 g yeast extract

5 g NaCl

Dissolve in 1 L MilliQ water. Adjust pH to 7.0 with NaOH. Autoclave to sterilize.

LB plates with ampicillin

Add 15 g Bacto agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50°C before adding ampicillin to a final concentration of 100 μg/ml. Pour 30-35 ml of medium into 85 mm Petri dishes. Let the agar harden. Store at 4°C for up to 1 month or at room temperature for up to 1 week.

LB plates with ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above; spread 100 μl of 100mM IPTG and 20 μl of 50mg/ml X-Gal over the surface of an LB-ampicillin plate and allow it to absorb for 30 minutes at 37°C prior to use.

Protocol for Preparation of Plasmid DNA by Alkaline Lysis with SDS: Minipreparation

Modified from: Sambrook, Joseph and Russell, David W. 2001. Molecular Cloning: A Laboratory Manual. 3rd Ed. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York. Pages 1.32-1.34

Materials

Buffers and Solutions

Alkaline Lysis Solution I (ice-cold)

Alkaline Lysis Solution II (Solution II should be freshly prepared and used at room temperature)

Alkaline Lysis Solution III (ice-cold)

Antibiotic for plasmid selection

95% and 70% ethanol

TE (pH 8.0) containing 20 μg/ml RNase A

LB, YT, or Terrific Broth

Ice bucket

1.5 ml microcentrifuge tubes

Centrifuge

Vortex

Procedure

Preparation of Cells

1. Inoculate 5 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37°C with vigorous shaking.

   To ensure that the culture is adequately aerated:

   - The volume of the culture tube should be at least four times greater than the volume of the bacterial culture.
   - The tube should be loosely capped.

2. Pour 1.5 ml of the culture into a microcentrifuge tube. Centrifuge at maximum speed for 30 seconds at 4°C in a microfuge. Repeat until all of culture has been pelleted.
3. When centrifugation is complete, remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

Lysis of Cells

4. Resuspend the bacterial pellet in 100 µl of ice-cold Alkaline Lysis Solution I by vigorous vortexing. Making sure that the bacterial pellet is completely dispersed in Alkaline Lysis Solution I.
5. Add 200 µl freshly prepared Alkaline Lysis Solution II to each bacterial suspension. Close the tube tightly, and mix the contents by inverting the tube rapidly five times, making sure the entire surface of the tube come in contact with Alkaline Lysis Solution II. *Do not vortex!!* Store the tube on ice.
6. Add 150 µl of Alkaline Lysis Solution III. Close the tube and disperse Alkaline Lysis Solution III through the viscous bacterial lysate by inverting the tube several times. The solution will turn white and cloudy. Store the tube on ice for 3-5 minutes.
7. Centrifuge the bacterial lysate at maximum speed for 5 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh tube. If necessary, tubes can be stored overnight in refrigerator at this step.

Recovery of Plasmid DNA

8. Precipitate nucleic acids from the supernatant by adding 2 volumes of 95% ethanol at room temperature. Mix the solution by vortexing and then allow the mixture to stand for 2 minutes at room temperature.
9. Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge.

10. Remove the supernatant by gentle aspiration as described in Step 3 above. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use a Kimwipe or disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.
11. Add 1 ml of 70% ethanol to the pellet. Recover the DNA by centrifugation at maximum speed for 2 minutes at 4°C in a microfuge.
12. Again remove all of the supernatant by gentle aspiration as described in Step 3 or pour off gently.
13. Remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5-10 minutes).
14. Dissolve the nucleic acids in 50 µl of TE (pH 8.0) containing 20 µg/ml DNase-free RNase A (pancreatic RNase). Vortex the solution gently for a few seconds. Store the labeled DNA solution at -20°C.

Plasmid Minipreparation Extraction/Lysis Solution Recipes

From: Sambrook, Joseph and Russell, David W. 2001. Molecular Cloning: A Laboratory Manual. 3rd Ed. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York. Page A1.16.

Alkaline Lysis Solution I (Plasmid Preparation)

50 mM glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Prepare Alkaline Lysis Solution I from standard stocks in batches of ~100 ml, autoclave for 15 minutes at 15 psi on liquid cycle, and store at 4°C.

Alkaline Lysis Solution II (Plasmid Preparation)

0.2 N NaOH (freshly diluted from a 10 N stock)

1% (w/v) SDS

Prepare Solution II fresh and use at room temperature

Alkaline Lysis Solution III (Plasmid Preparation)

5 M potassium acetate	60.0 ml
Glacial acetic acid	11.5 ml
Water	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at 4°C and transfer it to an ice bucket just before use.