ABSTRACT

THOMAS, ANNA. Biology, Epidemiology and Population Genomics of *Pseudoperonospora cubensis*, the Causal Agent of Cucurbit Downy Mildew. (Under the direction of Dr. Peter S. Ojiambo and Dr. Ignazio Carbone).

Cucurbit Downy Mildew (CDM) caused by the obligate oomycete, *Pseudoperonospora cubensis*, is economically the most important disease of cucurbits worldwide. During the past two decades, a resurgence of CDM has occurred around the world resulting in severe disease epidemics. In the United States, resurgence of the disease occurred in 2004 and resulted in significant crop losses. Introduction of a new lineage, a new pathotype or new genetic recombinants of the pathogen have been suggested as potential reasons for this resurgence. The potential for sexual reproduction, the current pathotype structure of *P. cubensis* in the United States, and the role different cucurbit host types and geography play in the shaping the genetic structure of *P. cubensis* were investigated.

Pairing assays involving known mating type tester strains and unknown isolates revealed, for the first time, the presence of both A1 and A2 mating types in the United States. Mating type was found to be significantly ($P < 0.0001$) associated with host type with isolates collected from cucumber being primarily of the A1 mating type, while those from squash and watermelon being of the A2 mating type. Similarly, a significant ($P = 0.0287$) association of mating type with geography was also observed. Isolates collected from northern tier states of Michigan, New Jersey, New York and Ohio were all A1, while isolates belonging to either the A1 or A2 mating type were present in equal proportions in southern-tier states of Alabama, Florida, Georgia, North Carolina, South Carolina and Texas. Plasmolysis test showed that about 40% of the oospores produced were viable and this suggests that oospores may play a role in the epidemiology of CDM in the United States.

Compatibility assays were conducted using 22 isolates and fifteen host differentials to classify the isolates into pathotypes. Based on the compatibility with the differential host set, five pathotypes (1, 3, 4, 5 and 6) were identified among the 22 isolates. Pathotypes 1 and 3 had not been previously described in the United States and isolates of these two new pathotypes were also compatible with Poinsett 76, a cultivar of *C. sativus* known to be resistant to CDM prior to 2004. Virulence within the pathogen population was expressed based on virulence factors, virulence phenotypes and virulence complexity. The number of virulence factors ranged from 2 to 8 indicating a complex virulence structure with 77% of the isolates having 5 to 8 virulence factors. Thirteen virulence phenotypes were identified and the mean number of virulence factors per isolate and mean number of virulence factors per virulence phenotype was 5.05 and 5.23, respectively, indicating that complex isolates and phenotypes contributed equally to the complex virulence structure of *P. cubensis*. Gleason and Shannon indices of diversity were 3.88 and 2.32, respectively and indicated a diverse virulence structure of *P. cubensis* population within the United States.

Comparative whole genomic analysis of nine isolates collected from diverse cucurbit hosts revealed the presence of two distinct evolutionary lineages. In addition, phylogenetic analysis showed that *P. cubensis* and its sister species, *P. humuli*, shared a recent common ancestor. A reconstruction of ancestral recombination suggested a hybrid origin of lineage II of *P. cubensis*, with *P. humuli* and lineage I of *P. cubensis* as putative parents. Subsequently, the role of different cucurbit host types in shaping the structure of *P. cubensis* population was investigated using 93 isolates with the double digest Restriction Associated DNA Sequencing technology. Many fixed polymorphisms throughout the genome separated lineage II that included isolates mainly from *Cucumis* spp. from lineage I that comprised isolates from

Cucurbita spp. and *Citrullus lanatus*. A distinct genetic cluster within lineage I associated mainly with *C. lanatus* was also identified. The association of lineage II with new pathotypes and the lack of genetic differentiation among lineage II isolates suggest that lineage II might have been responsible for the resurgence of CDM in the United States. A strong association of mating types with lineages was also observed with all A1 mating type isolates belonging to lineage II and all A2 mating type isolates belonging to lineage I, irrespective of the host of origin. However, the structuring of the pathogen population by geography was not evident and hence, the direction of seasonal migration of *P. cubensis* could not be inferred.

Biology, Epidemiology and Population Genomics of *Pseudoperonospora cubensis*, the Causal Agent of Cucurbit Downy Mildew

by
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A Dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Plant Pathology

Raleigh, North Carolina

2016

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DEDICATION

To my family

BIOGRAPHY

Anna Thomas was born and raised in a small village named Marady in the coastal state of Kerala, India. After finishing her high school, she joined the University of Agricultural Sciences, Bangalore, and studied agriculture. Having been brought up by parents who loved farming, Anna soon realized her passion for agriculture. She thoroughly enjoyed school and towards the end of her undergraduate studies developed a strong interest in Plant Pathology. Anna joined the Department of Plant Pathology at University of Georgia for her Masters under the guidance of Drs. Katherine Stevenson and David B. Langston, Jr. and worked on fungicide resistance management of the gummy stem blight pathogen, *Didymella bryoniae*. Upon completion of her Master's program in 2011, Anna joined the Department of Plant Pathology at North Carolina State University in fall 2011 to study the population biology of *Pseudoperonospora cubensis* under the direction of Dr. Peter Ojiambo and Dr. Ignazio Carbone. Her project focused mainly on understanding the possible causes for the resurgence of the disease in the United States in an effort to device better disease management strategies.

ACKNOWLEDGMENTS

I am extremely thankful to many people who have contributed to the completion of my doctoral program. I would like to express my sincere gratitude to my co-advisors Drs. Peter Ojiambo and Ignazio Carbone for their guidance, support and kindness during my graduate education. I also would like to thank my advisory committee members, Dr. David Shew and Dr. Lina Quesada-Ocampo, for their valuable insights and assistance they availed to me during my graduate work. The past and present members of the Ojiambo lab, Lucky Mehra, Wendy Britton, Katie Neufeld, Barbara Stevens, Taylor Ghiloni, Mary Lewis, Mike Adams, Thomas Keever, and Jane Marian Luis, have been great co-workers and friends and would like to thank them for their friendship. I also would like to thank Rakhi Singh for her guidance and help in learning the molecular techniques. I also would like to thank all the collaborators of CDM ipmPIPE program for sending me infected samples without which my project would not have been possible: Drs. Amanda Gevens, David Langston, Anthony Keinath, Steve Rideout, Kate Everts, Andy Wyendant, Margaret McGrath, Mary Hausbeck, Melanie Ivey, Thomas Isakeit, Kenneth Seebold, Dan Egel, Mohammed Babadoost, Sally Miller, Beth Gugino, Steve Bost, Edward Sikora, Shouan Zhang, Gary Vallad, Elaine Roddy, Mahfuzur Rahman, Nathan Kleczewski, Bhabesh Dutta and Emily Pfeufer. Thanks are also due to Drs. Gerald Holmes and Anthony Keinath for very helpful discussions on cucurbit downy mildew. The faculty, staff and fellow graduate students in the department have always been a pleasure to interact with and I would like to thank them all for making my time at NCSU a very memorable one. For their constant encouragement and support, I would like to thank my dear parents and siblings. And I am indebted to my friends Sara Thomas and Nadia Chacko for being there at the other end of the phone whenever I needed them. Last but not

the least, I would like to thank my husband Eldhose Kilithattil for his everlasting patience, love and support and our little Sarah for brightening my days with her sweetest smile.

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Literature Review

1.0 Importance of cucurbits

The family Cucurbitaceae is fairly large consisting of two well-defined subfamilies, Cucurbitoideae and Zanonioideae and includes 118 genera and 825 species (Lebeda et al. 2007). Members of Cucurbitaceae are primarily frost-sensitive annual vines (Jeffrey 1990). Cucurbit species in the subfamily Zanonioideae are cultivated mainly in Asian countries, primarily for medicinal purposes. However, most members of the subfamily Cucurbitoideae have a widespread distribution globally and are the major food crops within the family (Robinson and Decker-Walters 1930). Economically important members of the family including cucumber, squashes, melons, pumpkin, gourd vegetables etc. form the major food crops of the family. Cucurbit vegetables can be consumed raw or cooked and the seeds act as an important source of protein in Africa, Asia and Latin American countries. Leaves and flowers of some cucurbits are consumed in many parts of the world and are a rich source of vitamins and minerals (Zitter et al. 1998).

The major cucurbit crops, namely cucumber, squash and watermelon have centers of origins in different continents. Cucumbers are considered to have their center of origin in Asia and may have descended from wild *Cucumis sativus* var. *hardwickii*, which is found in the foothills of Nepal and Northern India (Whitaker and Davis 1962; Harlan 1975). Cucumber later got introduced to different parts of the world and is now distributed throughout most temperate and tropical climates and is the fourth most widely grown vegetable crop behind tomato, cabbage and onion (Tatlioglu 1993). Squashes (*Cucurbita*

spp.) on the other hand originated in North and South American continents and later were introduced into Europe, then to other parts of the world (Sanjur et al. 2002) and now also have a global distribution. All *Citrullus* spp. including watermelon originated in Africa with its cultivation later expanding to the Middle East and Asia and later into the New World countries (Robinson and Decker-Walters 1930).

In the United States, cucurbits form an important group of vegetable crops and are valued at $1.4 billion annually (Anonymous 2016). Cucurbits account for more than 10% of the total value of vegetables, melons, potatoes and sweet potatoes produced nationally. The most widely grown cucurbits in the US are cucumber, cantaloupe, pumpkin, butternut squash, acorn squash and watermelon. These cucurbits collectively are produced on a total area of more than 380,000 acres that is concentrated mainly in the eastern states and in California, Arizona and Texas (Anonymous 2016). The states of Florida, Georgia and North Carolina are among the major producers of cucumber, squash and melon in the nation and these states account for more than 33% of the total value of the crop produced in the country (Anonymous 2013). Cucurbits are susceptible to a number of diseases such as fusarium wilt, phytophthora crown and root rot, anthracnose, gummy stem blight, powdery mildew, downy mildew etc. Of these diseases, cucurbit downy mildew is economically the most important disease of cucurbits worldwide.

1.1 Cucurbit downy mildew

Cucurbit Downy Mildew (CDM) caused by the obligate biotrophic oomycete, *Pseudoperonospora cubensis*, is the most important limiting factor in cucurbit production worldwide (Lebeda and Cohen 2011). The disease has been reported from more than 70

countries and affects approximately 60 species of cucurbits (Lebeda 1992). The pathogen is widely distributed in temperate and semi-arid regions and has a wide host range that includes both wild and cultivated cucurbits. The most widely grown cucurbits in the US including cucumber, cantaloupe, pumpkin, squash and watermelon are highly susceptible to the disease.

Cucurbit downy mildew is primarily a foliar disease and the symptoms of the disease vary with the host type affected. In case of cucumber and squash, symptoms are characterized by the appearance of angular pale yellow oily lesions on the adaxial leaf surface restricted by veins. Chlorotic lesions may expand and coalesce as the infection progresses over time. Symptoms in case of cantaloupe and watermelon are slightly different from those of cucumber and squash and in this case, symptomatic lesions are not vein bound, they are either circular or irregular in shape and they tend to turn necrotic rapidly. The disease can progress rapidly in the field under favorable environmental conditions (i.e., warm and moist weather), and can result in rapid necrosis and complete crop failure in the absence of adequate control measures (Cohen 1981; Holmes et al. 2004; Palti and Cohen 1980). Heavy necrosis of leaf tissues exposes fruits to damage by the sun, affects fruit development and causes secondary rot which results in a reduction in the quality and quantity of the produce (Keinath et al. 2007).

1.2 History and re-surgence of cucurbit downy mildew

Cucurbit downy mildew was first reported on a non-cultivated cucurbit in Cuba and was described by Berkeley and Curtis in 1868 (Berkeley and Curtis 1868). The disease was not reported again until 1889 when it was detected in Japan and the United States in the same

year (Stewart 1897). In the United States, CDM was first reported in New Jersey on greenhouse grown cucumber and later on field grown squash, pumpkin and cucumbers (Halstead 1889). Cucurbit downy mildew in Europe was first reported in 1899 on cucumber and squash in England (Massee 1899) and subsequently, several outbreaks of CDM were reported from different parts of Europe. The disease had also been reported from other parts of the world including Russia, Brazil, Dutch East Africa, Japan and India and was believed to have a worldwide distribution by 1920 (Foster 1920).

Since its first report in 1889, CDM had occurred every year in the United States, became a serious problem and established itself as the most destructive of all cucumber diseases by late 1890's (Foster 1920). Disease management efforts were soon initiated including the development of genetically resistant cultivars and effective fungicides. Cucumber breeding efforts by W. Barnes led to the development of several cultivars with high levels of resistance to CDM (Barnes 1948; Barnes and Epps 1954). The resistance available in the cultivars was derived from the Plant Introduction (PI) 197087 originally collected in India (Wehner and Shetty 1997). Wide deployment of effectively resistant cucumber cultivars in the 1960's made CDM a minor concern and rarely required the use of fungicides thereafter for the management of CDM in the United States (Criswell et al. 2010). However, CDM continued to be a problem in some of the Asian countries (Israel, Japan and India) whereas in Europe it was present only in the Mediterranean regions (Lebeda and Cohen 2011).

In the second half of 1980's, CDM started to spread all over Europe on cucumber and established itself as the most important yield limiting factor for cucurbit cultivation in Europe (Lebeda and Cohen 2011). Similarly, in the United States, CDM resurged in 2004 breaking

down the effective host resistance in cucumber that lasted for more than 40 years (Holmes et al. 2006). Severe epidemics were reported in the eastern US in 2004 and 2005 where the disease had appeared earlier than usual during the peak growing season. The disease led to complete crop failure in many fields and an estimated crop loss of 40% was reported in case of cucumber alone, causing an estimated loss of $20 million (Holmes et al. 2006). The disease now occurs annually in the United States and poses a significant threat to cucurbit production nationally.

1.3 Biology of *Pseudoperonospora cubensis*

Pseudoperonospora cubensis is an obligate oomycete that cannot survive independent of its host except as oospores (Lebeda and Cohen 2011). The primary mode of reproduction for *P. cubensis* is through the production of asexual spores, sporangia. The pathogen typically sporulates on the underside of symptomatic leaves through the production of abundant amount of sporangia (light grey to deep-purple in color) that are borne on sporangiophores. Sporangia are ovoid to elliptical in shape and measure 14 to 25 × 20 to 40 µm (Palti 1974) and at maturity they are light grey to deep purple in color (Thomas 1996). Wind-borne sporangia after deposition on the host leaf surface typically germinates indirectly through the production of 5 to 15 biflagellate zoospores measuring 8 to 12 µm in size (Palti and Cohen 1980). Contact with moisture is required for germination and release of zoospores. Zoospores, once released actively swim towards stomatal aperture where they settle and encyst (Cohen 1981). The encysted zoospores then germinate by the formation of a germ tube, produce an appressorium and subsequently a penetration hyphae develop and penetrate the stomata of the leaf tissue. Direct penetration of leaf epidermis by hyphae occurs

rarely. Hyaline ceonocytic hyphae soon after penetration invades the mesophyll and palisade tissues and produces clavate-branched haustoria to obtain nutrients. Following infection and colonization, sporulation occurs on the lower surface of the leaves under high humid conditions.

Pseudoperonospora cubensis is heterothallic and requires two opposite mating types, referred to as A1 and A2, for sexual reproduction to occur (Cohen and Rubin 2012). Like in other oomycetes, sexual reproduction in *P. cubensis* results in the formation of oospores and these oospores can enable the pathogen to survive under unfavorable environmental conditions. Formation of oospores under field conditions is rare and has been reported in a few cases. Reports on oospore formation have been made from Israel (Cohen et al. 2003), India (Singh and Sokhi, 1989), Russia (Rostovzew, 1903), China (Zhang et al. 2006) and Austria (Bedlan 1989). Recently isolates belonging to A1 and A2 mating types were described in Israel in 2010 (Cohen and Rubin 2012). A1 mating type isolates have been reported to be associated mainly with cucumber and melon, while A2 mating type isolates have been recovered mostly from squash, pumpkin or butternut gourd (Cohen et al. 2015). Since the first characterization of opposite mating types within *P. cubensis*, reports on the presence of opposite mating types have been made from several other countries including Ukraine, Russia, Kenya, Angola, Vietnam, India, France, Germany (Cohen et al. 2015) and China (Cohen et al. 2013). The significance of oospores in CDM disease cycle is not well understood. However, a recent study from China reported that spring infection of potted cucumber plants by *P. cubensis* coincided with the germination of oospores (Zhang et al. 2012) suggesting that oospore may potentially serve as an initial inoculum source for early

infection of cucurbits. However, systematic studies need to be conducted to establish the role of oospores on the survival of *P. cubensis* and epidemiology of CDM.

1.4 Ecology and epidemiology of cucurbit downy mildew

Pseudoperonospora cubensis is an obligate pathogen and requires a living host plant for its survival and reproduction (Savory et al. 2011). The pathogen is transmitted primarily through the production of airborne sporangia that are transported from various inoculum sources. The primary infectious propagules of *P. cubensis* are the asexual sporangia that are also responsible for the repeated cycles of disease during the growing season. Low humidity and dry leaf surface are optimal for the dispersal of sporangia. Sporangia produced on the infected foliage are actively liberated into the air current following a reduction in the relative humidity, which results in a hygroscopic twisting movement of sporangiophores, a process that releases the sporangia (Lange et al. 1989). Changes in the relative humidity occur early to mid- morning and hence, concentrations of sporangia are higher during this time of the day (Cohen and Rotem 1971; Neufeld et al. 2013). Sporangia deposited on susceptible host tissue require the conducive environmental conditions for their germination and infection of the host plant. Germination of sporangia can occur between 5 to 28°C but is conducive at 20°C and longer leaf wetness duration (Arauz et al. 2010; Cohen 1981; Cohen 1977; Neufeld and Ojiambo 2012). The optimum temperature for infection is 15°C with a leaf wetness duration of at least 2 h. In absence of moisture no infection will occur even when temperature is optimum. Incubation period varies from 4 to 12 days depending on environmental conditions and inoculum load (Lebeda and Cohen 2011). Pathogen sporulates under favorable conditions in 5 to 7 days and a new infection cycle can be initiated every 7 to 14 days

depending on the environmental conditions on susceptible hosts (Cohen 1981). The disease is polycyclic and the infection cycle repeats resulting in release of abundant sporangia throughout the growing season that can be dispersed over long distances (Ojiambo and Holmes, 2011).

The source of primary inoculum for cucurbit downy mildew has been a major focus of research in the past. In the United States, the lack of survival structures (i.e., oospores) under field conditions coupled with the sensitivity of cucurbits to frost strongly suggests that the pathogen population can survive only as active mycelium in cultivated or wild cucurbit species in frost-free regions (Bains and Jhooty 1976) in southern Florida and along the Gulf of Mexico below the 30° N latitude. Hence, it is widely believed that the disease spread progresses from overwintering sources in the south to northern states above the 30°N latitude as the host become available and temperature become conducive for infection (Nusbaum 1944). Theoretical evidence for such long distance dispersal of *P. cubensis* spores from the southern US to the northern latitudes in the United States has been provided through a series of studies (Holmes et al. 2004, Ojiambo and Holmes 2011; Ojiambo et al. 2015). It has been suggested that sporangia from any source can be dispersed up to a distance of 1,000 km (Ojiambo and Holmes 2011). Detached sporangia are susceptible to UV irradiance and survive longer during cloudy and overcast days as compared to clear and sunny days and can survive up to 48 h (Cohen and Rotem 1971; Kanetis et al. 2010). Spore trapping studies conducted in Florida, North Carolina and Israel have shown that t dispersal of sporangia is at its peak in the morning and continues until late in the evening (Schenck 1968, Cohen and Rotem 1971; Neufeld et al. 2013). Sporangia dispersed late in the evening have a better

chance of survival and thus, likely to infect the host early in the morning when the dew accumulates.

1.5 Disease management

Resistance to cucurbit downy mildew bred into the commercially available cultivars originally derived from the accession PI197087 was one of the success stories in the field of plant breeding (Criswell et al. 2010). The resistance based on the recessive *dm-1* gene introduced in the late 1950's lasted for more than 40 years and enabled cucurbit growers to maximize their yield of with minimal application of fungicides. This long-standing resistance that was in place for almost four decades was eventually overcome when CDM resurged in the United States in 2004 (Holmes et al. 2006). Currently, cucurbit cultivars with commercially acceptable levels of resistance to CDM are not available. Hence, the growers rely heavily on fungicides for managing the disease. In the presence of the disease, an aggressive spray program (fungicide application every 5-7 days in cucumber and every 7-10 days in case of other cucurbits) is usually recommended and this significantly increases the cost of cucurbit production (Hausbeck 2009). In addition, frequent fungicide applications for the control of CDM significantly increases the cost of production and increases the risk for the development of fungicide resistance by *P. cubensis* and non-target effects of increased fungicide applications on the environment. The high evolutionary potential of *P. cubensis* due to its mixed mating system along with airborne dispersal of inoculum further increases the risk for the development of fungicide resistance by the pathogen. A large number of chemical groups have been labelled for use against CDM and these includes phenyl amides, carbamates, cyano-acetamide oximes, benzamide, copper, chlorothalonil, phosphonates,

hymexazol, fentins, dimethomorph, fluazinam, phthalimides and strobilurins but the selection of effective chemicals can be quite challenging (Gisi 2002; Keinath 2016; Lebeda and Cohen 2011).

Resistance in case of *P. cubensis* to phenyl amide-based product mefenoxam was reported shortly after its release to market (Lebeda and Schwinn 1994). Resistance has also been reported against some of the most widely used chemistries such as strobilurins, mandipropamid, phosphonates, propamocarb, phthalimides, and dimethomorph (Lebeda and Cohen 2011). Reduced field efficacy to some other fungicides labelled for the control of CDM has also been reported. For example, fluopicolide, which was considered very effective in combating CDM during 2008 (Ojiambo et al. 2010), has been shown to have reduced efficacy under field conditions in Georgia, North Carolina and Michigan (Adams and Quesada-Ocampo 2014; Adams et al. 2014; Hausbeck and Linderman 2014; Langston and Sanders 2013) and resistance to fluopicolide under in vitro conditions also have been confirmed (Anna Thomas et al. unpublished). Reduced efficacy of dimethomorph and cymoxanil have been reported in the Czech Republic (Pavelková et al. 2014). Increasing the longevity of fungicides in the absence of host resistance is a priority and care must be taken to reduce the selection pressure exerted on the pathogen.

Prediction of disease outbreaks through close monitoring of occurrence and movement of pathogen may help growers make informed decisions about the timing of fungicide application which might help reduce the number of sprays and thus reduce the cost of production. In the United States, the Cucurbit Downy Mildew ipmPIPE forecasting system was established following the resurgence of CDM to help growers time their first spray application based on the risk of disease development in their area of operation (Ojiambo et al.

2011). Integration of cultural practices that take into account the biology and ecology of the pathogen may also help prevent/delay the initiation of disease. For example, reducing the leaf wetness through drip irrigation may help delay the infection. Early planting and decreased plant density also may help reduce risk of infection (Palti and Cohen 1980) by making the microclimate in the field less conducive for infection by the pathogen. Thus, in the absence of effective host resistance, an integrated management strategy with a combination of CDM alert systems, cultural practices and fungicide applications is necessary for sustainable production of cucurbits in the United States (Holmes et al. 2015).

1.6 Physiological specialization

Host specificity within *P. cubensis* was first recognized in Japan where two biological forms of *P. cubensis* were reported whereby one specialized on *Cucumis* spp. and while the other specialized on *C. moschata* in 1941 (Iwata 1941). A similar observation was later made in the United States in 1952 based on the differential affinity of pathogen population for *Cucumis* spp. and *C. lanatus* (Hughes and Van Haltern 1952). Isolates that were more specialized on *C. lanatus* and/or *C. moschata* were shown to be able to cause infections on *Cucumis* spp. but the ones specialized on *Cucumis* spp. caused only mild symptoms and sparse sporangia production on *C. lanatus* (Hughes and Van Haltern 1952) and *C. moschata* (Iwata 1941). Later, Palti (1974) analyzed the pattern of divergence of *P. cubensis* on its hosts who observed that *P. cubensis* was common throughout the world on *Cucumis* spp. (cucumber and melon), less frequent on *Cucurbita* spp. (absent in Europe and certain parts of Asia) and is more restricted in its distribution on *Citrullus*, *Luffa* and *Lagenaria* spp. Palti

(1974) attributed this divergence in virulence pattern exhibited by *P. cubensis* to the existence of different physiological races in various countries.

In an effort to better characterize the pathogenic variability within *P. cubensis*, the pathogen population has been divided into pathotypes based on patterns of virulence or avirulence to a series of differentials in specific host genera. Thomas et al. (1987) identified the presence of five different pathotypes based on the differential response of the pathogen population from Japan, Israel and United States exhibited at the time on *Cucumis sativus, C. melo* var. *reticulatus, C. melo* var. *conomon, C. melo* var. *acidulous, Citrullus lanatus, Cucurbita maxima, C. pepo, C. moschata, Benincasa hispida, Luffa acutangula, L. cylindrica, Momordicha charanatia and Lagenaria siceraria.* Isolates capable of infecting *Cucumis sativus* and *C. melo* var. *reticulatus* were considered as pathotype 1, while those capable of infecting *C. melo* var. *conomon* in addition to the former hosts were considered as pathotype 2. Isolates capable of infecting all the *Cucumis* spp. were considered as pathotype 3. All isolates capable of infecting *C. lanatus* in addition to the *Cucumis* spp. were considered as pathotype 4 and all those that were capable of infecting *Cucurbita* spp. in addition to *Cucumis* spp. and *C. lanatus* were considered pathotype 5. Isolates from Japan belonged to either pathotype 1, 2 or 3; isolates from Israel belonged to pathotype 3 and the isolates obtained from the United States belonged to pathotype 4 or 5 (Thomas et al. 1987). This pathoytpe structure has changed since the study of Thomas et al. (1987) with reports of pathotype 5 in Italy in 2003 (Cappelli et al. 2003) and isolates capable of causing infections on *Cucurbita* spp. being reported in Czech Republic in 2009 (Lebeda et al. 2012). New pathotypes also have been described recently. For example, Cohen et al. (2003) described pathotype 6 that is as capable of causing infection on *Cucumis* spp. and *Cucurbita* spp. but

not *C. lanatus* was described in Israel. Some surveys (Cohen et al. 2015) have also indicated the presence of additional pathotypes (pathotype 7, 8, 9, 10) may be present in China, Vietnam, Russia and India, respectively (Cohen et al. 2015). However, no experimental data has been provided to support the existence of these new pathotypes.

1.7 Pathogen diversity

The cucurbit downy mildew pathogen possess a high degree of pathogenic variation and this is evident from the differential response of individual isolates on different cucurbit host types. Based on the compatibility patterns with differential hosts in specific host genera, *P. cubensis* n has been divided into pathotypes. Due to the challenges associated with assays involving obligate pathogens, the pathotype classification studies have understandably been based on a limited number of isolates. The first attempt to genetically characterize the pathogen population was by Choi et al. (2005). Phylogenetic analysis using the ITS rDNA region of *P. cubensis* and *P. humuli* population showed no difference between and among the two genera and subsequently concluded that *P. humuli* should be considered a taxonomic synonym of *P. cubensis* (Choi et al. 2005). However, subsequent studies (Sarris et al. 2009 and Mitchell et al. 2012) challenged the conclusion by Choi et al. (2005) and suggested that the low variation observed among *P. cubensis* population was probably due to the choice of the genetic marker used. Studies conducted by Sarris et al. (2009) and Mitchell et al. (2012) using AFLP fingerprinting and multilocus genotyping found variations among *P. cubensis* population. Both studies found that the European and North American *P. cubensis* isolates formed distinct clusters when compared to *P. humuli*. However, *P. humuli* isolates collected from Asia grouped into the same cluster with *P. cubensis* isolates collected from Asia.

In the study by Sarris et al. (2009), isolates of *P. cubensis* were also found to cluster based on their geographical location. Populations of *P. cubensis* in central and western Europe (France and the Czech Republic) were found to be genetically distinct from the south eastern European population (Sarris et al. 2009). A subsequent study by Polat et al. (2014) using SRAP and ISSR markers and *P. cubensis* populations from Turkey, Czech Republic and Israel identified three distinct genetic clusters according to geographic location (Polat et al. 2014). Further, isolates collected from Turkey, Czech Republic showed a more uniform genetic background compared isolates from Israel that seemed to be highly diverse. The authors attributed the high genetic diversity in Israel to sexual recombination or migration (Polat et al. 2014). In addition, isolates with same genetic back-ground collected in Turkey and Czech Republic originated from *C. sativus*, whereas *P. cubensis* population collected from Israel originated from a diverse group of host types representing *Cucumis* spp., *Cucurbita* spp. and *Luffa* spp. Thus, it appears that the diversity of host types may have contributed to differences observed in addition to geographical location as suggested by the authors.

Runge et al. (2011) conducted a multilocus phylogenetic analysis of *P. cubensis* using *cox2*, *ypt1* and nrITS sequence data and provided evidence for the presence of two distinct clades within *P. cubensis*. That study showed that isolates collected after the resurgence of CDM in Europe and the United States grouped together with isolates from East Asia. Runge et al. (2011) suggested that the recent resurgence of CDM in the Europe and the United States was probably due to the introduction of isolates from East Asia into Europe and then to the United States. However, only one isolate from the US was included in the study and thus, robust conclusions could not be drawn on the resurgence of CDM in the United States.

In the United States, existence of distinct biological forms of *P. cubensis* on *Cucumis* spp. and *Citrullus lanatus* was experimentally proven by Hughes and Van Haltern (1952). Existence of different pathotypes in the United States was first documented in the study conducted by Thomas et al. (1998). Later, after the resurgence of CDM in the US in 2004, efforts were made to characterize the virulence pattern of *P. cubensis* as *P. cubensis* seemed to have broadened its host range (Colucci, 2008). The study by Colucci (2008) examined the compatibility of 32 isolates with 12 host species belonging to 6 genera of *Cucumis*, *Cucurbita*, *Citrullus*, *Luffa*, *Lagenaria* and *Benincasa* showed 32 different pattern of virulence. However, that study did not establish the pathotypes and estimates of virulence diversity of *P. cubensis* population in the United States. Subsequently, Quesada et al. (2012) genetically characterized the population of *P. cubensis* using microsatellite markers and found six distinct genetic clusters among the global population of *P. cubensis*. Isolates collected on cucumber was found to genetically similar when compared to isolates collected from other cucurbit host types (Quesada-Ocampo et al. 2012).

1.8 Rationale and justification

The resurgence of CDM has led to a need to identify the possible causes for the resurgence of the disease in the United States. Several hypotheses have been suggested to explain resurgence of CDM around the world. Introduction of a new pathotype or a new lineage or even a new species of the pathogen has been suggested as the potential reasons for the resurgence of CDM in the United States (Colucci 2008; Runge et al. 2011). Establishing the factors behind this resurgence can facilitate the development of better control methods for the disease and screening of genotypes for disease resistance. The resurgence of the disease

may be linked to factors that contribute to the increased virulence among *P. cubensis* population such as the possibility of sexual reproduction in the United States. Sexual recombination is one of the important factors that contributes to the diversity within populations of plant pathogens. Cohen et al. (2012) reported that *P. cubensis* is heterothallic requiring two mating types A1 and A2 to form oospores. They observed that the A2 mating type was consistently associated with the genera *Cucurbita* and A1 being associated with the genera *Cucumis*. The population of *P. cubensis* in the United States has pathotypes capable of infecting both *Cucurbita* and *Cucumis* spp. However, no studies have been undertaken to establish the occurrence and distribution of mating types of *P. cubensis* in the United States.

Host range studies of *P. cubensis* are also important to detect any shifts in the pathotype structure that was documented in 1987 by Thomas et al. (1987). According to the study conducted in 1987, isolates in the United States belonged to pathotypes 4 and 5 and were not compatible with resistant cucumber varieties. After the resurgence of CDM in 2004, an increased affinity towards *Cucumis* spp. has been reported (Holmes et al. 2006) and hence it is important to establish the host range of current *P. cubensis* in the United States to determine the prevailing virulence patterns and shifts in the pathogen population, possibly due to new pathotypes since the resurgence in 2004. A temporal delay in causing infection on certain *Cucurbita* spp. compared to infections on *Cucumis* spp. have been observed in the mid-Atlantic and northern states (Colucci 2008; Ojiambo and Holmes, 2011). This could suggest the existence of different forms of pathogen population specialized on different cucurbit species in the United States. It is possible that a new variant and/or pathotype of the pathogen with a mating type compatible with that of the pre-epidemic population was introduced in the United States in 2004. Recombination through sexual reproduction provides

P. cubensis a greater advantage in the evolutionary arms race given the pathogen also reproduces asexually and has a large population size (McDonald and Linde, 2002). This characteristic of the pathogen may enable *P. cubensis* to rapidly overcome resistance in available host genotypes.

Pseudoperonospora cubensis is known to have a wide host range and host specialization among isolates is known to exist among the population and subsequently the population has been divided into pathotypes based on the differential ability of the pathogen to cause infection on different species. However, no systematic study has been conducted to explain the underlying genetic reason for the host specialization that this pathogen exhibits. One hypothesis for the difference in virulence in different pathotypes is attributed to difference in effector content in each isolate. A detailed study to understand the effector proteins involved in the process of infection was carried out by Savory et al (2012) and they identified 271 candidate effectors in *P. cubensis* genome. Detailed study examining the different genomic regions of isolates collected from diverse host types is important in understanding the genetic basis of host specialization within *P. cubensis*.

A study by Quesada-Ocampo (2012) using multilocus genotyping found that *P. cubensis* isolates collected on cucumber had a different cluster composition as compared to those obtained from non-cucumber host types. More recently, Summer et al. (2015) making use of a genotyping by sequencing approach observed distinct clustering of isolates collected from *Cucumis* spp. from those collected on non-*Cucumis* spp. (Summers et al. 2015). Genetic clustering of *P. cubensis* based on geography has also been reported in Europe and Asia (Sarris et al. 2009, Polat et al. 2014). However, systematic studies on the association between *P. cubensis* and cucurbit host types and their geographic location in the United States has not

been conducted. Such studies could facilitate our understanding of the role these factors play in the seasonal temporal progress of CDM across the eastern United States.

It is widely believed that the cucurbit downy mildew inoculum responsible for disease outbreak in the continental US originates annually from below the 30°N latitude primarily in southern Florida where cucurbits are grown year around. However, reports of isolated disease outbreaks (< 5%) inconsistent with the seasonal spread from southern United States was made in 2007 and 2009 suggesting that other independent sources of inoculum may be also responsible for initial outbreaks of the disease in the United States (Ojiambo et al. 2015; Ojiambo and Holmes 2011). Empirical evidence suggests that disease outbreaks above the 30°N latitude are due to overwintering sources of downy mildew in southern Florida (Ojiambo and Holmes 2011). In that study, the extent of spatial association of disease outbreak was ~1,000 km and outbreaks in the Mid-Atlantic States were due to inoculum originating directly from southern FL. The outbreaks in the Great Lake region was suggested to be caused by the inoculum originating from North Carolina/South Carolina/Georgia border or other undocumented sources in Canada rather than overwintering sources in the south (Ojiambo and Kang 2011). Identification of differences and similarities in the genetic makeup of isolates collected from different locations along the theoretical trajectory of *P. cubensis* could provide more direct evidence for the role of overwintering sources of in the south to disease outbreaks in northern latitudes in the continental United States. Knowledge on the initial sources of inoculum and mapping the movement of inoculum has implications in disease monitoring, forecasting and formulation of management decisions.

Based on the above considerations, the overall goal of this dissertation was to understand the population biology and ecology of *P. cubensis* in an effort to establish a

working framework for effective management of CDM. The specific objectives of this dissertation are to:

1. Determine the occurrence and distribution of opposite mating types of *P. cubensis* in the United States (Chapter 1).
2. Establish the virulence structure within the of *P. cubensis* population in the United States (Chapter 2).
3. Conduct comparative genomic analysis to gain insights into the resurgence of cucurbit downy mildew in the United States (Chapter 3)
4. Establish the role of cucurbit host types in shaping *P. cubensis* population structure and the role of overwintering inoculum in southern Florida in causing infections in the northern states (Chapter 4).

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CHAPTER 2

Occurrence and distribution of mating types of *Pseudoperonospora cubensis* in the United States

Submitted to *Phytopathology*:

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ABSTRACT

During the past two decades, a resurgence of cucurbit downy mildew has occurred around the world resulting in severe disease epidemics. In the United States, resurgence of the disease occurred in 2004 and several hypotheses including introduction of a new genetic recombinant or pathotype of the pathogen have been suggested as potential causes for this resurgence. Occurrence and distribution of mating types of *P. cubensis* in the United States were investigated using 40 isolates collected from cucurbits across eleven states from 2005 to 2013. Pairing of unknown isolates with known mating-type tester strains on detached leaves of cantaloupe or cucumber resulted in oospore formation 8 to 10 days after inoculation. Isolates differed in their ability to form oospores across all co-inoculation pairings, with oospore numbers ranging from 280 to 1,000 oospores/cm^2 of leaf tissue. Oospores were hyaline to golden-yellow, spherical and approximately 36 μm in diameter. Of the 40 isolates tested, 24 were found to be of the A1 mating type, while 16 were of the A2 mating type. Mating type was significantly ($P < 0.0001$) associated with host type whereby all isolates collected from cucumber were of the A1 mating type, while isolates from squash and watermelon were of the A2 mating type. Similarly, mating type was significantly ($P = 0.0287$) associated with geographical region where isolates from northern tier states of Michigan, New Jersey, New York and Ohio were all A1, while isolates belonging to either A1 or A2 mating type were present in equal proportions in southern-tier states of Alabama, Florida, Georgia, North Carolina, South Carolina and Texas. Viability assays showed that oospores were viable and on average, about 40% of the oospores produced were viable as determined by the plasmolysis method. This study showed that A1 and A2 mating types of *P. cubensis* are present and the pathogen could potentially reproduce sexually in cucurbits

within the United States. In addition, the production of viable oospores reported in this study suggests that oospores could also have an important role in the biology of *P. cubensis* and could potentially influence the epidemiology of cucurbit downy mildew in the United States.

INTRODUCTION

Pseudoperonospora cubensis is an obligate, biotrophic oomycete that causes cucurbit downy mildew (CDM), a disease that is widely recognized as the most important foliar disease of cucurbits worldwide (Lebeda and Cohen 2011). The pathogen is widely distributed in temperate and semi-arid regions and has a wide host range that includes both wild and cultivated cucurbits (Lebeda 1992). Approximately 60 species of cucurbits have been reported as hosts of *P. cubensis* and the most economically important host crops include cucumber, cantaloupe, watermelon, pumpkin and squashes (Lebeda 1992). In the United States, cucurbits are grown primarily in the eastern half of the country and in California and a total of 383,630 acres were harvested in 2015 with a value of $1.4 billion (Anonymous 2016). Due to the availability of resistant cultivars deployed in the 1960s, downy mildew on cucumber was of minor concern in the United States requiring only very few fungicide applications to control the disease. This long-standing source of resistance in cucumber was the Plant Introduction (PI) accession PI 197087 (Barnes and Epps 1954). However, resistance from PI197087 was overcome after 40 years and a resurgence of CDM was observed in 2004 leading to severe disease epidemics in the eastern United States in the 2004 and 2005 growing seasons with significant or total crop loss in many fields (Holmes et al. 2006). Around this time period, new aggressive pathotypes capable of attacking *Cucurbita* species were also reported in Israel (Cohen et al. 2003), and parts of Europe (Cappelli et al. 2003;

Lebeda and Urban 2004). Since 2004, CDM now occurs annually in the United States and application of fungicides is required to avoid significant crop losses.

There have been considerable efforts to establish possible causes for the resurgence of CDM in the United States (Holmes et al. 2015; Ojiambo et al. 2015; Quesada-Ocampo et al. 2012) and other parts of the world (Cohen et al. 2015; Kitner et al. 2015; Lebeda et al. 2013). It has been suggested that the resurgence of CDM in the United States could have been due to introduction of a new lineage, pathotype or even a new species of the pathogen (Colucci 2008; Runge et al. 2011). It has also hypothesized that cryptic lineages within *P. cubensis* might be able to hybridize resulting in an emergence of hypervirulent strains (Runge et al. 2011). New genetic recombinants as a result of sexual reproduction has also been suspected as a possible cause for the resurgence in Israel (Cohen and Rubin 2012). Studies conducted in Israel have shown that *P. cubensis* is heterothallic and has two mating types, A1 and A2 (Cohen and Rubin 2012). Thus, sexual reproduction can occur when mycelia of both mating types grow together in close proximity on the same leaf. The potential for sexual recombination has two important implications for *P. cubensis*-CDM pathosystem. First, sexual reproduction involves production of thick-walled oospores that can survive in the soil and remain viable for ten months and still cause infection (Zhang et al. 2012). Formation of oospores can create opportunities for *P. cubensis* to overwinter in areas where cucurbits are unable to survive due to harsh winters. In such areas, oospores can survive in the soil or plant debris and thereby serve as an initial source of inoculum for early onset of disease epidemics. Secondly, new genetic combinations from sexual reproduction can create opportunities for the emergence of unusually fit or aggressive offsprings. *Pseudoperonospora cubensis* reproduces asexually via aerially-borne sporangia and sexual reproduction will greatly

increase its evolutionary potential and the ability to overcome host resistance (McDonald and Linde, 2002).

Oospore production in *P. cubensis* is rare and reports on oospore formation have mainly been from Asia and Europe (Bains et al. 1977; Beldan 1989; Hiura and Kawada 1933; Palti and Cohen 1980). In the United States, the quest for oospores as evidence for sexual reproduction can be traced back to 1889, when the pathogen was first reported in the country (Halstead 1889). Halstead (1889) did not observe formation of oospores and the search efforts were initiated again by Foster (1920) following the first report on oospore formation by Rostovzew (1903) in Russia. Doran (1932) continued the search for oospores in the United States in infected leaves of cucumber inoculated with leaf debris and soil suspensions from where cucurbits were grown. Doran (1932) also failed to recover oospores in his study. Vigorous efforts for the search of oospores in the United States were initiated again following the resurgence of the disease in 2004 but none of these efforts were successful in recovering oospores in nature or generating oospores under laboratory conditions (Cohen et al. 2015; Holmes et al. 2015).

In the United States, *P. cubensis* is widely thought to survive in areas that experience mild winters along the Gulf of Mexico and in southern Florida and Texas. Annual introduction of initial inoculum from these overwintering sources assumes that oospores are not present in cucurbit fields in more northern latitudes. In a study on the spatio-temporal aspects of CDM using reports on disease outbreaks, up to 5% of reported disease cases were found to be inconsistent with the annual northward advance of the epidemic wave front in the eastern United States (Ojiambo and Holmes 2011). While this inconsistency in disease spread was very low, it may suggest the existence of other potential but undocumented

sources of initial inoculum that maybe influencing initiation of epidemics. Such potential sources of initial inoculum could include oospores that have not yet been detected in cucurbit fields or greenhouses. The potential impact of sexual reproduction and oospores in the epidemiology of CDM and diversity of the pathogen motivated our investigation of this aspect of the biology of *P. cubensis* within the pathogen population in the United States. The specific objectives of this study were to: i) establish the existence and distribution of A1 and A2 mating types of *P. cubensis* in the United States, ii) determine the production of oospores in co-inoculation of isolates of the two mating types in the United States and iii) determine the viability of oospores and their infectivity on different cucurbit host types. Preliminary findings on some aspects of this present work have been published elsewhere (Thomas et al. 2013).

MATERIALS AND METHODS

Collection and maintenance of isolates. A total of 40 single lesion isolates obtained from field samples collected from different cucurbit host types including cucumber, cantaloupe, watermelon, giant pumpkin, butternut squash and acorn squash were used in this study. These isolates were collected from cucurbit hosts in California and ten other states in the eastern United States (Table 2.1). Most of these isolates were collected between 2004 and 2014. In addition, an isolate of *P. cubensis*, Pc1982, isolated from cantaloupe in South Carolina in 1982 was included in the study. Isolate Pc1982 was previously stored at -80°C and was maintained on cantaloupe by periodic re-inoculation of detached leaves.

Field samples of infected cucurbit leaves were collected through the CDM ipmPIPE disease monitoring network and transported to the Plant Pathology laboratory at North

Carolina State University in Raleigh. Samples were carefully examined and leaves of samples with discrete single lesions were subsequently maintained on their respective hosts as single lesion isolates. Samples without discrete single lesions were processed further to generate single lesion isolates. Briefly, sporangia were washed off the leaves using a Preval sprayer (Complete Unit 267; Precision Valve Corporation, Yonkers, NY) and used to inoculate respective whole host plants at concentrations of about 1×10^4 sporangia per ml. Inoculated host plants were bagged with a polythene bag and placed in a growth chamber at 18°C under dark condition for 24 h. Plants were un-bagged after 24 h and, were incubated at 21/18°C day/night regime with a 12/12-h light/day photoperiod. Plants were examined daily for the development of symptoms and when discrete single lesions became apparent, plants were bagged again and were kept under dark conditions for 24 h to stimulate sporangia production. Individual lesions were subsequently cut out carefully and used to inoculate respective host plants to multiply sporangia of each isolate. All the isolates were maintained in the laboratory by propagation on their respective host plants using detached leaves until they were ready for use in the subsequent experiments. To propagate the isolates, the abaxial side of the first or second leaves from respective host plants were placed on sterile moist paper towel in clear acrylic boxes and the adaxial side was inoculated with a sporangial suspension at about 2×10^4 sporangia per ml and incubated in a growth chamber at 21/18°C day/night with a 12/12-h light/day photoperiod.

Mating type determination and oospore production. The mating type of individual isolates was determined by pairing, in equal proportions, sporangia of an isolate with sporangia of a tester strain with a known mating type as described by Cohen and Rubin (2012). In this study, two tester strains were used: an A2 isolate originally isolated from

butternut squash in Israel and an A1 isolate (A11), which was originally isolated from cucumber in North Carolina in 2012. Co-inoculation of these two tester strains resulted in oospore production. The two tester strains were also maintained on the respective hosts throughout the entire study as described above.

To determine the mating type of each isolate, sporangial suspension (2×10^4 spores/ml) of the tester and unknown isolate were mixed together in equal proportion (1:1 v/v) and the resultant suspension was used to inoculate detached first true leaves of cantaloupe (cv. Ananas) or cucumber (cv. Straight Eight) plants. Cantaloupe and cucumber have been reported as favorable hosts to support production of oospores (Cohen and Rubin 2012). Plants were grown in the greenhouse periodically to ensure a steady supply of the first true leaves that were used in the assay. The adaxial side of the detached leaves was placed on moist paper towels in clear acrylic boxes and the abaxial side of the leaf was spot inoculated on at least 20 different spots with 10 µl of the sporangial mixture. Inoculated leaves were then incubated in a growth chamber at 21°C under 12 h/12-h light/dark cycle. At 7 to 10 days post inoculation, leaf discs measuring 11 mm diameter were cut from infected leaves and clarified for 24 h in ethyl alcohol-acetic acid solution (3:1 v/v). Clarified leaf discs were washed three times in de-ionized water and then examined under a compound microscope (×100 magnification) for the presence of oospores. Unknown isolates were designated as either A1 or A2 if oospores were produced when co-inoculated with the A2 or A1 tester strain, respectively. Appropriate crosses were also conducted as internal controls for oospore production using specific isolate × tester combinations.

Viability of oospores. The standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (Sutherland and Cohen 1983) was used to

determine the viability of oospores produced from crosses conducted in this study. Oospore viability was also evaluated using the plasmolysis method (Jiang and Erwin 1990) since the MTT test has been reported to be unstable and inconsistent with high rates of false positives (Etxeberria et al. 2011; Ribeiro et al. 1971). In both tests, an oospore suspension to evaluate viability was prepared by homogenizing leaves inoculated with both mating types of *P. cubensis* in 10 ml (per leaf) of sterile distilled water. To ensure consistency in the observed results, assays of oospore viability were repeated at least once.

Viability of oospores using the MTT test was determined as described by Sutherland and Cohen (1983). Briefly, 500 µl of the prepared oospore suspension was mixed with an equal volume of 0.1% MTT stain (Sigma-Aldrich). The suspension containing a mixture of MTT and oospores was then incubated at 35°C for 48 h. Thereafter, a droplet of the incubated suspension was dispensed on a glass slide with a cover slip and were examined under a compound microscope (×200 magnification) for color reaction. A total of 100 oospores were sequentially examined for color reaction as follows: oospores that stained red to rose are considered to be viable dormant, blue are viable activated (pre-germination phase), and black and unstained oospores are considered non-viable (Sutherland and Cohen 1983).

To assess oospore viability using the plasmolysis method, 50 µl of oospore suspension was first centrifuged for 1 min at 13,000 rpm. The supernatant was discarded and the resultant pellet was re-suspended in 50 µl of 4 M NaCl solution and the mixture incubated at room temperature for 45 min. A total of 100 oospores were then observed microscopically for plasmolysis and classified as either viable (i.e., plasmolized) or non-viable (i.e., non-plasmolized). Oospores that are viable have their cytoplasm contracted to

form a ball-like structure, while non-viable oospores do not plasmolize since they lack the differential permeability of their cellular membranes (Jiang and Erwin 1990).

Infectivity of oospores. Oospore suspension used to inoculate cucurbit hosts to determine the ability of oospores to cause infection was prepared as described above. Vegetative structures of *P. cubensis* were differentially killed by exposing the homogenized suspension to two cycles of drying and wetting as described by Rubin and Cohen (2006). Briefly, 5 ml aliquots of the homogenized inoculum suspension were pipetted into 9-cm Petri dishes, then dishes without leads were in the incubator at 26ºC for 12 h to allow the liquid homogenate to dry up. Thereafter, 5 ml of water was added to each dish, and the homogenized suspensions were re-suspended in the water with the aid of a clean spatula and dried again for 12 h. The dry homogenates were finally re-suspended in 3 ml of sterile distilled water and used to spot-inoculate adaxial sides of detached leaves (20 µl per spot) of five cucurbit host types as described above. Inoculated leaves were incubated in a growth chamber at 21°C under a 12/12-h light/dark cycle for 21 days for formation of sporulating lesions.

Mating type and host specificity. A set of six randomly selected *P. cubensis* isolates belonging to A1 and A2 mating types (three isolates each) originally isolated from diverse cucurbit host types from six different states (Table 2.4) were tested for their compatibility on a set of eight differential host types. Plants of the host differential set that included, *Cucumis sativus, C. melo reticulatus, C. melo conomon, Citrullus lanatus*, *Cucurbita* spp. (acorn squash and butternut squash), *Luffa cylindrica* and *Lagenaria siceraria*, were grown in the greenhouse to the third true-leaf stage as described above. For each host, first and second true leaves were placed with the adaxial side on moist paper towels in clear acrylic boxes and

spray inoculated with a sporangial suspension of an A1 or A2 isolate at a concentration of 5 × 10^3 sporangia/ml. Inoculated leaves were then incubated in a growth chamber at 21/18°C under 12/12-h light/dark cycle. Disease severity and the corresponding level of sporulation for the inoculated leaves were assessed 7 days after inoculation. Disease severity was assessed visually on a per leaf basis as the leaf area (%) infected. To assess sporulation, leaf discs (9-mm diameter) were randomly cut from infected leaves and shaken in an Eppendorf tube (two discs per leaf) containing 0.5 ml of 50% ethanol to dislodge sporangia. The concentration of sporangia in the resultant suspension was then determined using hemocytometer counts. Compatibility reactions were recorded based on sporulation and disease severity as described by Cohen et al. (2013) and Thomas et al. (1987) as follows: compatible (+): ~5 × 10^3 sporangia/ml and disease severity ≥50%, and incompatible (–): either low compatibility with ~3 × 10^3 sporangia/ml and <50% disease severity or no sporulation or infection. Pathotype designation for each isolates was based on compatible or incompatible reaction using a scheme proposed by Thomas et al. (1987) and later expanded by Cohen et al. (2013, 2015).

Data analysis. Associations between the frequency of the two mating types of *P. cubensis* and their original host of isolation and location of the host within the United States, were examined using contingency tables and the corresponding number of isolates. First, A1 or A2 mating type isolates were grouped based on presence or absence in the eleven states and presence or absence in northern- or southern-tier states. Northern-tier states comprised of Michigan, New Jersey, New York and Ohio, southern-tier states included Alabama, Florida, Georgia, North Carolina, South Carolina, and Texas (Ojiambo and Holmes 2011). Secondly, A1 and A2 mating type isolates were grouped based on whether they were isolated from

acorn squash, butternut squash, cantaloupe, cucumber, giant pumpkin or watermelon. Finally, A1 and A2 mating type isolates were grouped based on whether they were isolated from cucumber or other cucurbits (i.e., acorn and butternut squash, cantaloupe, giant pumpkin and watermelon). Statistical differences in the distribution of A1 and A2 mating type isolates within these three categories were tested based on Fisher's exact test using PROC FREQ in SAS (version 9.4; SAS Institute, Cary, NC).

RESULTS

Mating type and mating type associations. Pairing of *P. cubensis* isolates with unknown mating type with either the A1 or A2 tester strain led to production of oospores in the combinations examined. Unknown isolates were either of the A1 or A2 mating type and no self-fertile isolates were observed among isolates collected in this study (Table 2.1). Out of a total of 40 isolates tested, 24 isolates (60%) were of the A1 mating type, while 16 isolates (40%) were of the A2 mating type. A1 mating type isolates were all isolated from cucumber except 2013B17 which was isolated from cantaloupe, and 2013C3 and 08C1 that were isolated from giant pumpkin (Table 2.1). Further, A1 isolates were also recovered from host samples collected in ten of the eleven states that were part of this study (Table 2.1). Unlike the A1 isolates, isolates belonging to the A2 mating type were isolated mainly from *Cucurbita* species and *Citrullus lanatus* but not *C. sativus* (Table 2.1). Isolate Pc1982 collected from cantaloupe in 1987 belonged to the A2 mating type. The A2 mating type isolates were also confined to host samples collected only in Alabama, Florida, Georgia, North Carolina, South Carolina, and Texas (Table 2.1). Both A1 and A2 mating types of *P.*

cubensis were not isolated from samples of the same cucurbit host except for cantaloupe (Table 2.1).

The distribution of isolates belonging to the A1 and A2 mating types within and among states and within and among cucurbit hosts differed within this study (Table 2.2). For example, the distribution of A1 and A2 isolates was not significantly different within states ($P = 0.6178$). However, significant differences ($P = 0.0287$) in the distribution of the two mating types were observed when states from which samples were collected were classified as either in the northern- or southern-tier state. Isolates belonging to A1 and A2 mating types were present in equal proportions in southern-tier states, while all isolates in northern-tier states were A1 (Fig. 2.1B). Significant differences in the distribution of A1 and A2 isolates were also observed within ($P < 0.0001$) and between ($P < 0.0001$) cucurbit host type (Table 2.2). Within host types, A1 isolates were exclusively associated with cucumber and giant pumpkin (only two isolates), while A2 isolates were exclusively associated with acorn squash, butternut squash and watermelon (Fig. 2.1A). Although both A1 and A2 isolates were associated with cantaloupe, the majority of the isolates on cantaloupe were of the A2 mating type (Fig. 2.1A). When isolate distribution on cucumber was compared to that on other host types, isolates obtained from cucumber were exclusively of the A1 mating type, while the majority of isolates (84%) that were obtained from other cucurbits were of the A2 mating type (Fig. 2.1B).

Oospore production. Co-inoculation of unknown isolates of *P. cubensis* with the A1 or A2 tester strain on detached cantaloupe or cucumber leaves led to production of oospores in pairings involving unknown isolates with the A1 or A2 tester strain. Oospores were produced in detached leaves between 8 and 10 days after pairing unknown isolates with the

tester strain. The number of oospores produced was highly variable and ranged from about 280 oospores/cm^2 of leaf tissue, for a less prolific cross between A11×2013F1 to about 1,000 oospores/cm^2 of leaf tissue, for a highly prolific cross between A11× D3 (Table 2.3). Oospores were hyaline to golden yellow in color and spherical in shape with thick cell walls composed of 2 to 3 layers (Fig. 2.2). Oospores that were hyaline in color were observed in test crosses conducted on detached cucumber leaves, while golden-yellow oospores were observed when crosses were performed on cantaloupe leaves. The size of oospores was variable and ranged between 24.4 μm from the cross between 2013A7 and D3 to 44.6 μm from the cross between A11 and 2013F1 (Table 2.3). The mean diameter of oospores across all crosses evaluated in this study ranged from 33.9 to 38.9 μm.

Oospore viability. The percentage of oospores that were viable varied among crosses and the method used to assay oospore viability. For example, the most viable oospores were obtained from a cross between 2013A17 and D3 (96.5%) based on the MTT test, while the cross between A11 and D3 produced the most viable oospores (59.3%) using the plasmolysis method (Table 2.3). In addition, viability based on the MTT test was consistently higher in every cross (range 67.2 to 96.5%) than viability estimates based on the plasmolysis method (range 33.5 to 59.3%). On average, oospore viability as estimated by the MTT test was ~1 to 2.5 times higher than viability estimates based on the plasmolysis method.

Oospore infectivity. Inoculation of cucurbit host types with oospores produced from the crosses evaluated in the study did not result in sporulating lesions on any of the inoculated cucurbit host type even after 21 days after inoculation. After 21 days, leaves of cucurbit host types inoculated with oosporic inoculum started to disintegrate and daily observations for sporulation and formation of sporangia on the lesions were terminated.

Mating type compatibility reaction. Isolates belonging to A1 and A2 mating types differed in their degree of compatibility with the host differential set used in the study (Table 2.5). A1 isolates were all compatible with *C. sativus*, *C. melo* var. *reticulatus* and *C. melo* var. *conomon* except 2013B17 which was not compatible with *C. melo* var. *conomon* (Table 2.5). Further, all A1 isolates were also compatible with *Lagenaria siceraria* except 2013B17. Based on their compatibility scheme, A1 isolates were designated as belonging to pathotype 1 (2013B17) and pathotype 3 (2013A18 and 2013A19). Unlike the A1 isolates, isolates belonging to the A2 mating type were all compatible with *C. sativus, C. melo, Citrullus lanatus, and Cucurbita* spp. except 2013E1 that was incompatible with *Cucurbita* spp. (Table 2.5). Based on their compatibility scheme, isolates belonging to the A2 mating type were designated as belonging to pathotype 4 (2013E1) and pathotype 5 (2013D6 and 2013F2) (Table 2.5).

DISCUSSION

This study examined the occurrence and distribution of mating types of *P. cubensis* and the potential of sexual reproduction within the pathogen population in the United States. It was determined that A1 and A2 mating types are present among isolates in the United States. Pairing of isolates belonging to the different mating types resulted in the production of viable oospores indicating that the pathogen could sexually reproduce in the United States. As such, *P. cubensis* may now have the potential to overwinter in the field and oospores could serve as an initial source of inoculum in the continental United States. Both mating types were also found to be associated with specific cucurbit host types. This study represents the first documentation of the A1 and A2 mating types of *P. cubensis* and oospore

production in the United States and provides a working framework to examine the role of oospore in the biology and epidemiology of cucurbit downy mildew in the United States.

Pseudoperonospora cubensis is heterothallic (Cohen and Rubin 2012) and the presence of A1 and A2 mating types and production of oospores is the evidence for the possible existence of a sexually reproducing population. Earlier attempts to detect mating types and oospores of *P. cubensis* in the United States were not successful (Cohen et al. 2015). This unsuccessful search could partly be explained by limited information, at that time, on the mating system in *P. cubensis* and conditions needed for oospore production. In the present study, both mating types were detected among isolates of *P. cubensis* collected across the United States. The A1 isolates were primarily associated with cucumber, while the A2 isolates were associated with other cucurbits. Host preference within *P. cubensis* has also been reported in Israel (Cohen et al. 2013) where A1 isolates showed preference to *Cucumis* species, while A2 isolates showed preference to *Cucurbita* species. In this study, the two isolates collected from giant pumpkin (*C. maxima*) belonged to the A1 mating type, while all isolates from *C. maxima* in the study by Cohen et al (2013) were of the A2 mating type. In-vitro studies indicate that although both A1 and A2 mating type isolates can infect *C. maxima*, A2 isolates sporulate more on *C. maxima* than A1 isolates (Anna Thomas et al. unpublished). The mechanism controlling host preference in different mating types of *P. cubensis* is currently unknown. Lebeda et al. (2014) analyzed *P. cubensis* isolates from some European countries and grouped them based on whether they were collected prior (clade I) or after (clade II) the change in the virulence in the pathogen population in 2009. It was hypothesized that the A1 mating type was similar to isolates in clade II that had preference to *Cucumis* species, while the A2 mating type was similar to isolates in clade I that preferred

other cucurbit hosts (Lebeda et al. 2015). Understanding how these clades are associated with different host types may also provide insights into the potential mechanism that control host preference within *P. cubensis*.

In this study, only the A1 mating type was found in northern tier states of New Jersey, New York, Ohio and Michigan, while both A1 and A2 mating types were present in southern states from Florida to North Carolina. While this may suggest a possible association of mating type with geographical region, the association between the A1 mating type and north-tier states was based on a limited number of isolates and did not include isolates from hosts from which only A2 isolates were obtained in the south. Thus, a large sample of isolates from northern-tier states especially from other host types such as acorn and butternut squash and watermelon would provide more robust conclusions. Since the resurgence of CDM in United States was associated with severe losses on cucumber, it is likely that virulent strains of the A1 mating type of *P. cubensis* may have been responsible for the breakdown of the long-standing source of resistance in cucumber from PI 197087. New pathotypes of *P. cubensis* that are aggressive and cause severe epidemics on *Cucurbita* spp. and *C. lanatus* have been reported in Czech Republic since 2009 (Kitner et al. 2015). These new pathotypes were associated with an increase in virulence and most of the isolates belonging to these new pathotypes also contain signatures of sexual recombination (Kitner et al. 2015). Thus, it is possible that a recombinant F1 heterozygote of the A1 mating type that was fit and able to survive and rapidly spread across the eastern states over a relatively short period of time, may have been responsible for the resurgence of CDM in the United States.

Oospores production varied among pairings of *P. cubensis* parental isolates where some crosses were prolific and resulted in a high density of oospores. Variation in oospore

production can be explained by isolate-specific and combination-specific effects that are analogous to general combining ability and specific combining ability, respectively, reported in genetic studies involving plant and animal breeding (Griffing 1956). Here, isolate-specific effects reflect the average performance of an isolate in parental pairings, while combination-specific effects reflect cases in which certain pairings do relatively better or worse than would be expected on the basis of the average performance of the parent involved. Similar patterns in oospore production have also been reported in *P. cubensis* isolates in Israel (Cohen et al. 2013) and isolates of *P. infestans* from Mexico (Flier et al. 2001) and the UK (Pittis and Shattock 1994). Oospores produced in this study were hyaline to golden-yellow and with a diameter ranging from 24.4 to 44.6 μm. The size of oospores in the present study is similar to that reported in Japan (Hiura and Kawada 1933) and Israel (Cohen et al. 2013). However, oospores in the present study were much larger than those reported in India, whose diameter ranged from 13.5 to 19.8 μm (Bains et al. 1977). Variation in host substrates used for the production of oospores has been suggested as a plausible explanation for differences in oospore size (Cohen et al. 2013). Cucumber was the substrate used in the present study and in studies by Cohen et al. (2013) and Hiura and Kawada (1933) and these three studies had a similar oospore size. However, *Melothria maderaspatana* was the host substrate in the study by Bains et al. (1977) where oospores were comparatively smaller in size. The oospores in the study of Bains et al (1977) were also observed in intact leaves in the field and not detached leaves which may also influence oospore size.

The use of germination as an absolute measure of oospore viability is complicated for oomycetes due to dormancy of the oospore. Thus, techniques such as the MTT test and the plasmolysis method have been developed to assess oospore viability to determine their

potential role in the biology and epidemiology of plant diseases. In this study, estimates of oospore viability were consistently higher based on the MTT test than the plasmolysis method. Similar observations on high estimates of oospore viability by the MTT test compared to the plasmolysis method have also been reported in other oomycetes (Etxeberria et al. 2011; Flier et al. 2001; Pittis and Shattock 1994). Despite its wide use, the subjectivity in color readings, inconsistency and existence of false positives has led researchers to conclude that the MTT test is unreliable. However, the plasmolysis method has been reported to be reliable in assessing oospore viability in other oomycetes (e.g., Etxeberria et al. 2011; Kunjeti et al. 2016). The plasmolysis method is based on the principle that a cell membrane of a viable cell has differential permeability and the latter is lost for a dead cell. A very high correlation has also been observed between the ability of oospore to plasmolyze and to germinate after deplasmolysis, further indicating the reliability of the plasmolysis method (Jiang and Erwin 1990). Thus, we can conclude that the percentage of viable of oospores of *P. cubensis* in the present study was about 40%.

The presence of A1 and A2 mating types and production of viable oospores by *P. cubensis* isolates has two important implications on the biology and epidemiology of CDM in the United States. Epidemics of CDM in states that experience winter temperatures below freezing have historically been thought to rely on aerial transport of sporangia from overwintering sources in southern Florida. Oospore production and viability suggests that *P. cubensis* could overwinter in the continental United States and oospores could potentially serve as an initial source of inoculum in states where winter temperatures are below freezing. Since oospores can survive for ten months and still be infective (Zhang et al. 2012), they could potentially be a source of local initial inoculum in the continental United States, from

northern Florida to the north east and Great Lakes region, where the duration between the end of the production period and the next cucurbit season is about 10 months. Since our findings were based on in-vitro studies, we are unable to directly relate oospore production to their ability to cause infection under field conditions. Thus, additional field studies are needed to establish oospore production under natural conditions in the United States. These field studies should involve sampling necrotic tissues of cantaloupe towards the end of the season since both mating types prefer cantaloupe. In addition, sampling fields with cantaloupe planted next to cucumber is likely to increase the detection of oospore under natural field conditions. Sexual recombination can result in new combinations of genes resulting in an offspring with adaptation and virulence traits that are different from those of the parents. Recent molecular studies on *P. cubensis* isolates in Czech Republic (Kitner et al. 2015) before and after the shift in virulence of the pathogen population occurring there in 2009 showed that about 67% of isolates sampled after 2009 had two heterozygous positions in their *nrITS* sequence, suggestive of sexual reproduction. More direct evidence for the implications of sexual recombination in *P. cubensis* was provided by Cohen et al. (2011) where recombinant progenies exhibited altered sensitivity to metalaxyl and a broadened host range compared to their parents.

Studies conducted by Cohen et al. (2011) showed that *P. cubensis* oospores only formed in presence of continuous moisture on leaves but the role of intermittent moisture on oospore production, germination and infection of cucurbits is yet to be established. Zhang et al. (2012) reported that oospores were able to survive under natural conditions with temperatures of about -20°C and were able to cause infection on inoculated cucumber plants. In this study, inoculation of host types with oosporic inoculum from our crosses did not result

infection. Lack of infection from oosporic inoculum generated from in-vitro crosses has also been reported in *P. infestans* (Mayton et al. 2000). In the only other study where infection by oosporic inoculum of *P. cubensis* from in-vitro crosses has been reported (Cohen et al. 2013), the frequency of oosporic infection was < 0.5% with most of the oospores from the crosses resulting in no infection. This erratic or lack of infection could be related to the dormancy of oospores. In other oomycetes, germination and infection of oospores from in-vitro crosses has been observed only when oospores are incubated in the soil for several months at certain temperatures (Mayton et al. 2000) or using specialized media (Rubin and Cohen 2006). However, factors that influence in-vitro germination and infection of oospores of *P. cubensis* are still not well understood. In addition, although oospores of *P. cubensis* can overwinter in nature (e.g., Zhang et al. 2012), systematic studies are still needed to establish whether they can result in infection under natural conditions. Such studies addressing oospore infection will need to deal with the challenge of ascertaining that oospores in infected leaves are responsible for observed infection given that vegetative hyphae of isolates of the mating types used to produce oospores are still present in the leaf, and are also capable of causing infection.

A compatibility assay based on six isolates showed that A1 isolates belonged to pathotype 1 and 3, while A2 isolates belonged to pathotype 4 and 5. Unlike pathotype 4 and 5 reported earlier by Thomas et al. (1987), pathotype 1 and 3 isolates had not been previously reported in the United States. All A2 isolates belonged to pathotype 4 and 5 as was the isolate Pc1982 collected from cantaloupe in 1982. While this may suggest an association between pathotype and mating type in *P. cubensis*, an additional study using more isolates is needed to generate robust conclusions. A host range study in Michigan (Cespedes-Sanchez et al.

2015) reported that *P. cubensis* was capable of infecting only on *Cucumis* species and *L. siceraria*. Since we observed that A1 isolates were capable of causing infection only on *Cucumis* species and *L. siceraria*, it is likely that isolates in Michigan were of the A1 mating type. Since pathotype 3 had not previously been reported in the United States and that it was known to be present only in East Asia and Israel, it is plausible that migration of the A1 mating type of *P. cubensis*, possibly from Israel or East Asia, could have contributed to the resurgence of CDM the United States. Anthropogenic activities have been suggested as potential avenues for the introduction of new pathotypes that may have resulted in the resurgence of CDM in United States and Europe (Runge et al. 2011). The CDM pathogen can be seedborne (Cohen et al. 2014) and global seed trade and exchange of germplasm for resistance breeding could provide opportunities for the migration of strains of *P. cubensis* into new habitats. Regardless of the cause of the resurgence of CDM, *P. cubensis* in the United States now has the potential for sexual reproduction and the ability to generate diversity within its population. Asexual reproduction in *P. cubensis* will allow for a rapid dissemination of recombinant progenies of the pathogen. This mixed mode of reproduction can facilitate the rapid evolution of the pathogen (McDonald and Linde 2002) leading to a high risk for developing fungicide resistance and less durability of host resistance genes already deployed for the control of cucurbit downy mildew.

ACKNOWLEDGEMENTS

This study was supported by Hatch Funds from North Carolina Agricultural Experiment Station for Project Number NC02251 and NC02432. We thank collaborators on the CDM ipmPIPE project for their assistance in collecting field samples used in this study and Taylor

Ghiloni and Barbara Stevens for assistance with laboratory and greenhouse aspects of this work.

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Table 2. 1. Source and description of *Pseudoperonospora cubensis* isolates collected from cucurbits to determine the mating type structure of the population in the eastern United States

Isolate	Year	State	County	Host	Mating type
Pc1982	1987	South Carolina	Charleston	Cantaloupe	A2
05A1	2005	Michigan	Oceana	Cucumber	A1
05A2	2005	Michigan	Oceana	Cucumber	A1
05F1	2005	North Carolina	Johnston	Acorn squash	A2
07B1	2007	North Carolina	Sampson	Cantaloupe	A2
08A1	2008	California	Salinas	Cucumber	A1
08F1	2008	Georgia	Tift	Acorn squash	A2
A12	2008	Florida	St. Johns	Cucumber	A1
08D1	2008	North Carolina	Johnston	Butternut squash	A2
08C1	2008	New York	Suffolk	Giant Pumpkin	A1
D3	2012	South Carolina	Charleston	Butternut squash	A2
A11	2012	North Carolina	Johnston	Cucumber	A1
2013A1	2013	Florida	Miami-Dade	Cucumber	A1
2013A2	2013	Florida	Collier	Cucumber	A1
2013A3	2013	Georgia	Worth	Cucumber	A1
2013A4	2013	Florida	Hillsborough	Cucumber	A1
2013A5	2013	North Carolina	Wayne	Cucumber	A1
2013A7	2013	Georgia	Sparks	Cucumber	A1
2013A9	2013	North Carolina	Johnston	Cucumber	A1
2013A10	2013	South Carolina	Charleston	Cucumber	A1
2013A11	2013	South Carolina	Charleston	Cucumber	A1
2013A14	2013	North Carolina	Hertford	Cucumber	A1
2013A15	2013	Alabama	Macon	Cucumber	A1
2013A16	2013	North Carolina	Edgecombe	Cucumber	A1
2013A17	2013	North Carolina	Wake	Cucumber	A1
2013A18	2013	New Jersey	Salem	Cucumber	A1
2013A19	2013	Ohio	Sandusky	Cucumber	A1
2013A20	2013	Ohio	Medina	Cucumber	A1
2013C3	2013	North Carolina	Johnston	Giant Pumpkin	A1
2013B17	2013	New York	Ontario	Cantaloupe	A1
2013B1	2013	Florida	Hillsborough	Cantaloupe	A2
2013E1	2013	Florida	Collier	Watermelon	A2
2013E2	2013	Texas	Hidalgo	Watermelon	A2
2013F1	2013	Florida	Collier	Acorn squash	A2
2013F2	2013	South Carolina	Charleston	Acorn squash	A2
2013D1	2013	Florida	Miami-Dade	Butternut squash	A2
2013D2	2013	Florida	Hillsborough	Butternut squash	A2
2013D3	2013	South Carolina	Charleston	Butternut squash	A2
2013D4	2013	North Carolina	Johnston	Butternut squash	A2
2013D6	2013	Alabama	Escambia	Butternut squash	A2

Table 2.2. Statistics of Fisher's exact test for the differences in the frequency distribution of A1 and A2 mating types of *Pseudoperonospora cubensis* among states and cucurbit host type

Level	df	χ^2	Prob < χ^2	Prob < P^a
State	10	9.7180	0.4646	0.6178
North vs. southern states	1	5.9348	0.0148	0.0287
Host type	5	36.8750	0.0001	0.0001
Cucumber vs. other cucurbits	1	29.4737	0.0001	0.0001

[a] Refers to the table probability as calculated by Fisher's exact test.

Table 2.3. Number, size and viability of oospores produced on detached leaves of cantaloupe or cucumber in selected crosses between isolates belonging to different mating types of *Pseudoperonospora cubensis*

Cross (A1 × A2)	Host substrate	Oospore density (number/cm^2)[a]	Oospore diameter (µm)		Oospore viability (%)[b]	
			Range	Mean	MTT	Plasmolysis
A11 × D3	Cantaloupe	1004	30.6-41.9	37.1	76.5	59.3
A11 × 2013F1	Cucumber	562	28.0-44.6	36.4	60.1	33.4
08A1 × D3	Cantaloupe	401	30.3-42.6	35.9	82.7	41.5
2013A7 × D3	Cucumber	883	24.4-43.3	33.9	96.5	37.5
2013A2 × D3	Cantaloupe	642	31.2-42.3	38.3	81.8	33.5
2013A15 × D3	Cantaloupe	281	31.6-43.1	38.9	88.6	37.3
2013A10 × D3	Cucumber	602	27.7-40.7	34.5	73.1	44.1
2013A17 × D3	Cantaloupe	481	29.2-41.1	34.9	67.2	37.4
Mean	--	607	33.9-38.9	36.2	72.5	40.5
SE[c]	--	85	--	0.6	4.1	2.9

[a] Density of oospores was assessed 8 to 10 days after inoculating specific host substrates with inoculum of isolates of the pathogen belonging to different mating types.

[b] MTT denotes the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test.

[c] SE denotes the standard error of the mean.

Table 2.4. Compatibility reaction on a set of cucurbit host differentials following inoculation with a selection of six isolates belonging to different mating types of *Pseudoperonospora cubensis*

	Compatibility reaction by mating type [a]					
	A1			A2		
Differential host[b]	2013B17	2013A19	2013A18	2013E1	2013F2	2013D6
Cucumis sativus	+ (48.3)	+ (12.3)	+ (25.0)	+ (7.3)	+ (9.5)	+ (26.6)
C. melo reticulatus	+ (6.1)	+ (5.5)	+ (9.2)	+ (23.0)	+ (5.3)	+ (16.1)
C. melo conomon	– (3.0)	+ (6.9)	+ (13.2)	+ (5.1)	+ (7.8)	+ (25.0)
Citrullus lanatus	– (0.0)	– (1.1)	– (0.5)	+ (8.4)	+ (9.2)	+ (3.6)
Cucurbita spp.	– (0.0)	– (2.5)	– (0.0)	– (1.4)	+ (28.1)	+ (25.6)
Luffa cylindrical	– (0.6)	– (3.6)	– (4.2)	– (3.7)	– (1.2)	– (0.3)
Lagenaria siceraria	– (3.2)	+ (6.7)	+ (10.6)	– (3.2)	– (1.2)	+ (4.0)
Designated pathotype[c]	1	3	3	4	5	5

[a] Compatibility reactions are defined as follows: '+' = highly compatible with a sporulation rate of ~ 5×10^3 sporangia/cm^2 of inoculated leaf disc and disease severity ≥ 50%; '–' = either low compatibility with a sporulation rate of ~ 3×10^3 sporangia/cm^2 of inoculated leaf disc and disease severity <50%, very low compatibility with very sparse sporulation or incompatible reaction with no evidence of sporulation. Values in parenthesis are counts of sporangia ($\times 10^3$) per cm^2 of inoculated leaf disc.

[b] *Cucurbita* spp. include *Cucurbita pepo* (acorn squash) and *Cucurbita moschata* (butternut squash).

[c] Pathotype designation is based on the pattern of compatibility reaction between an isolate and the host differential set based on the scheme originally developed by Thomas et al. (1987) and later expanded by Cohen et al. (2013).

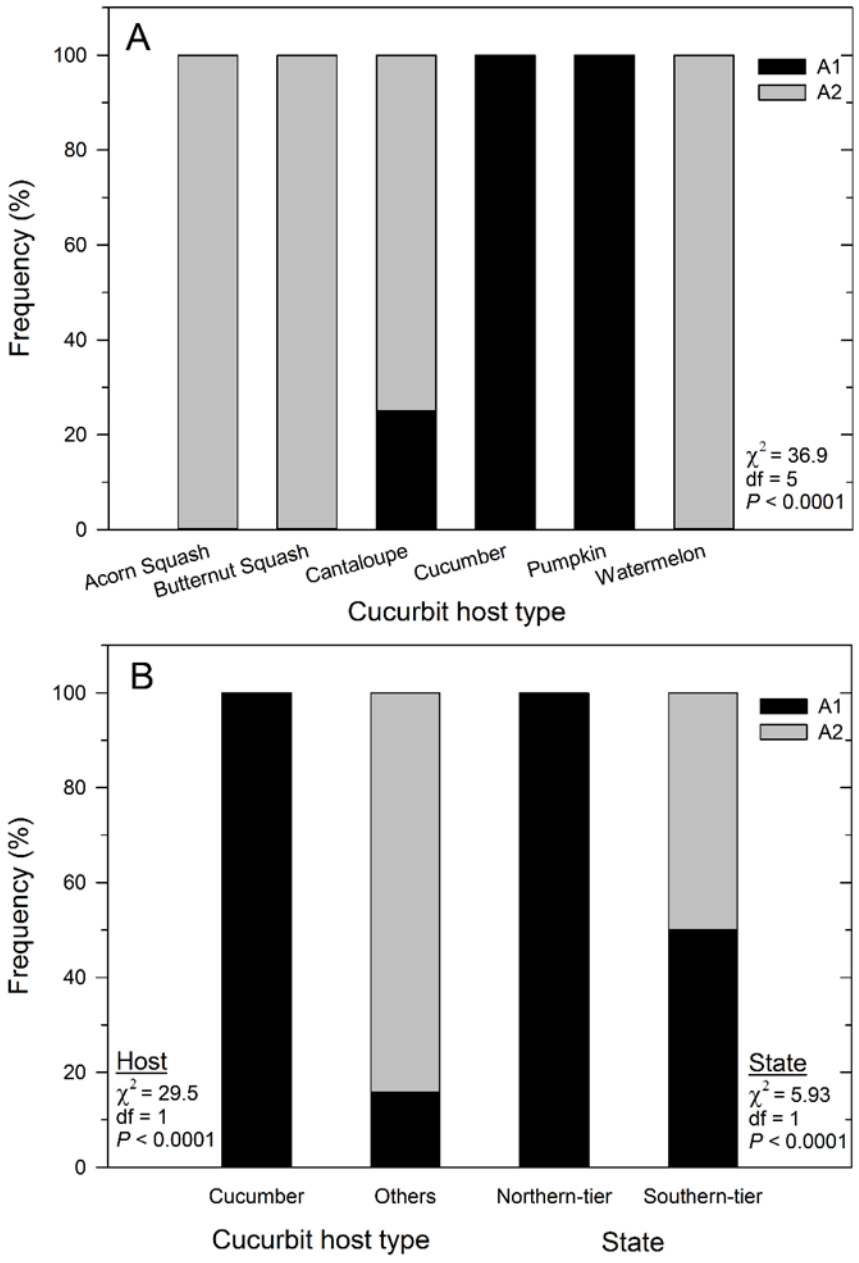

Figure 2.1. Frequency distribution of A1 and A2 mating types of *Pseudoperonospora cubensis* based on isolates of the pathogen collected from all cucurbit host types (**A**), cucumber versus other cucurbits (**B**), and northern- and southern-tier states (**B**) across the eastern United States. Host type denote as 'Others' refers to acorn squash, butternut squash, cantaloupe, pumpkin and watermelon.

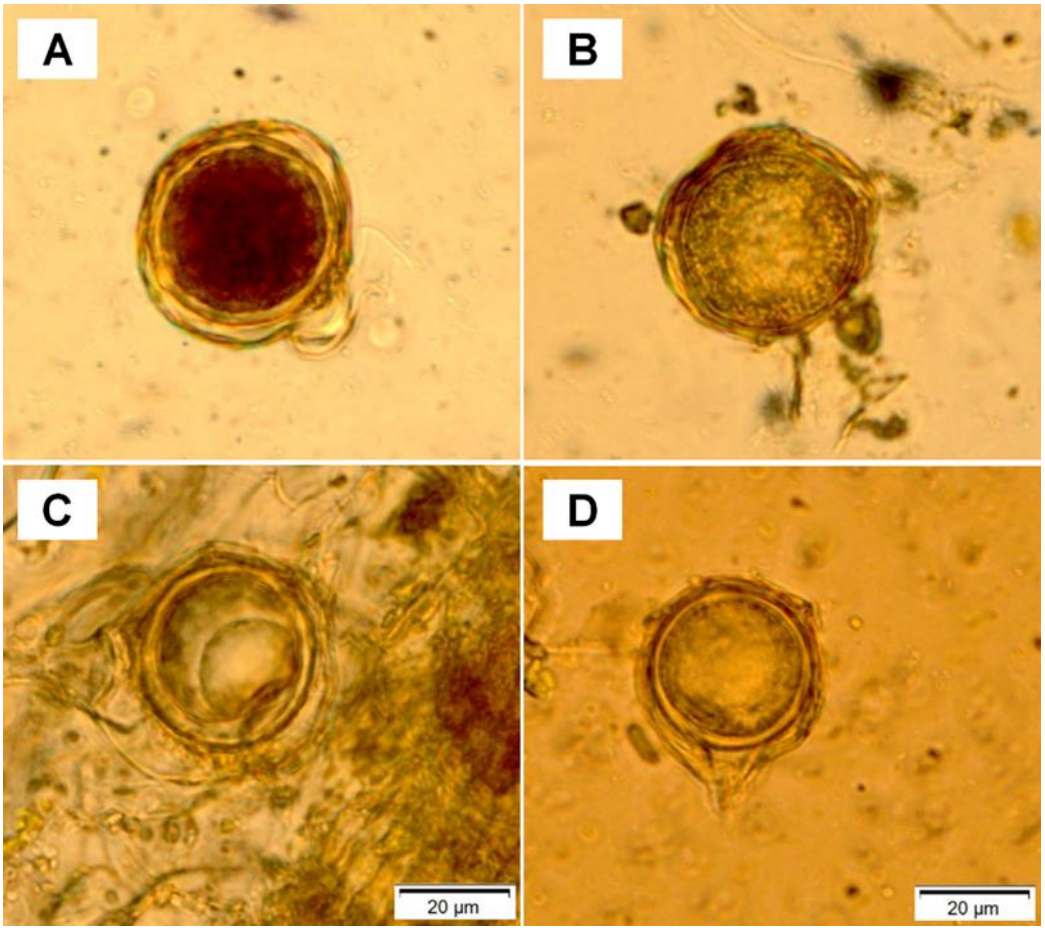

Figure 2.2. Viability tests of oospores produced by crossing A1 and A2 mating types of *Pseudoperonospora cubensis*. Oospores were stained with 0.1% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution and resulting in viable oospores being stained with a bright to deep-red color (**A**), while non-stained oospores (**B**) are non-viable. Viability was also determined using the plasmolysis method, where plasmolysis (**C**) indicate a viable oospore, while unplasmolyzed (**D**) indicate non-viable oospore.

CHAPTER 3

Virulence structure within the population of *Pseudoperonospora cubensis* in the United States

Submitted to *Phytopathology*:

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ABSTRACT

Cucurbit downy mildew (CDM) caused by the obligate oomycete *Pseudoperonospora cubensis*, has resurged around the world in the past two decades. A new pathotype or genetic recombinant of *P. cubensis* have been suggested as possible reasons for the resurgence of CDM in the United States in 2004. A total of 22 isolates collected between 2004 and 2014 mainly in the eastern United States were tested for their compatibility with a set of fifteen cucurbit host types. The virulence structure within these isolates was evaluated on a set of twelve differential genotypes from eight genera. All isolates were highly compatible with the susceptible cultivar of *Cucumis sativus*, while the least compatibility was observed with *Luffa cylindrica* and *Momordica charantia*. Based on the compatibility with the differential host set, five pathotypes (1, 3, 4, 5 and 6) were identified among the 22 isolates. Pathotypes 1 and 3 had not been previously described in the United States and isolates of these two new pathotypes were also compatible with Poinsett 76, a cultivar of *C. sativus* known to be resistant to CDM prior to 2004. Virulence within the pathogen population was expressed based on virulence factors, virulence phenotypes and virulence complexity. The number of virulence factors ranged from 2 to 8 indicating a complex virulence structure with 77% of the isolates having 5 to 8 virulence factors. Thirteen virulence phenotypes were identified and the mean number of virulence factors per isolate and mean number of virulence factors per virulence phenotype was 5.05 and 5.23, respectively, indicating that complex isolates and phenotypes contributed equally to the complex virulence structure of *P. cubensis*. Gleason and Shannon indices of diversity were 3.88 and 2.32, respectively, indicating a diverse virulence structure of *P. cubensis* within the United States population. The diverse virulence and high virulence complexity within the pathogen population indicate that host resistance

alone in available cucurbit cultivars will not be effective to control CDM. An integrated approach involving a combination of fungicide application and introduction of cultivars with new resistance genes will be required for effective management of CDM.

INTRODUCTION

Cucurbitaceae is a fairly large family and includes more than 118 genera and 825 species with members distributed widely throughout the tropics and warm temperate regions of the world (Lebeda et al. 2007). In the United States, cucurbits are an important group of vegetable crops and account for about 10% of the total value of vegetables produced nationally (Anonymous 2016). The most widely grown cucurbits include cucumber, cantaloupe, pumpkin, butternut squash, acorn squash and watermelon. In 2014, cucurbits were produced on approximately 390,000 acres concentrated mainly in the eastern half of the United States and in Arizona, California and Texas and were valued at $1.5 billion (Anonymous 2016). Florida, Georgia and North Carolina are among the major producers of cucumber, squash and melon accounting for more than 32% of the total value of the crop produced in the United States (Anonymous 2016).

Cucurbit downy mildew (CDM) caused by the obligate oomycete *Pseudoperonospora cubensis* is perhaps the most economically important limiting factor in cucurbit production worldwide (Lebeda and Cohen 2011; Ojiambo et al. 2015). This is particularly the case since the resurgence of CDM in many parts of the world during the last decade has resulted in severe epidemics and crop losses (Cohen et al. 2015; Holmes et al. 2015). In the United States, the disease was only of minor concern on cucumber prior to 2004 and required very little application of fungicides due to resistant cultivars deployed in 1960s

using the resistance source Plant Introduction (PI) 197087 (Barnes and Epps 1954). However, this long-standing resistance that was effective for about 40 years was overcome resulting in a resurgence of the disease in the United States in 2004 (Holmes et al., 2006). Resurgence of CDM was also reported in Israel in 2002 (Cohen et al. 2003) and in Europe in 2009 (Lebeda et al. 2011). In the United States, resurgence of CDM resulted in severe epidemics in the eastern part of the country in 2004 and 2005 resulting in significant crop losses (Holmes et al., 2006). In the United States, CDM now occurs annually, posing a significant threat to cucurbit production (Holmes et al. 2015).

Resurgence of CDM has dramatically affected production of cucurbits and deployment of disease management options worldwide (e.g., Holmes et al. 2015). Thus, considerable efforts have been initiated to determine and understand possible causes for the resurgence of CDM in Europe (Lebeda et al. 2013), Israel (Cohen et al. 2015) and the United States (Holmes et al. 2015; Ojiambo et al. 2015), to devise effective disease management strategies. Anthropogenic introduction of new pathotypes of *P. cubensis*, possibly from Asia, has been suggested as a plausible explanation for the resurgence of CDM around the world (Runge et al. 2011). In *P. cubensis*, pathotypes are physiological forms of the pathogen that differ in host specificity on the level of genus, species or subspecies of various Cucurbitaceae (Lebeda and Widrlechner 2003). Like in other oomycetes, the interaction between *P. cubensis* and cucurbits is discontinuous and displays a compatible or incompatible pattern. This compatible or incompatible reaction with a set of differential host species is the basis for classifying pathotypes of *P. cubensis*. Thomas et al. (1987) first identified pathotypes of *P. cubensis* based on a differential set composed of *Cucumis sativus*, *C. melo* var. *reticulatus*, *C. melo* var. *conomon*, *C. melo* var. *acidullus*, *Citrullus lanatus* and *Cucurbita pepo*. Isolates

capable of infecting *Cucumis sativus* and *C. melo* var. *reticulatus* were considered as pathotype 1, while those capable of infecting *C. melo* var. *conomon* in addition to the former hosts were considered as pathotype 2. Isolates capable of infecting all the *Cucumis* spp. were considered as pathotype 3. All isolates capable of infecting *Citrullus lanatus* in addition to the *Cucumis* spp. were considered as pathotype 4 and all those that were capable of infecting *Cucurbita* spp. in addition to *Cucumis* spp. and *Citrullus lanatus* were considered pathotype 5. Isolates from Japan belonged to pathotype 1, 2 or 3, while those from the United States belonged to pathotype 4 or 5 (Thomas et al. 1987). The host differential set of Thomas et al. (1987) composed of differentials from three genera has since been expanded to twelve differentials from six genera that include *Benincasa*, *Luffa* and *Lagenaria* (Lebeda and Widrlechner 2003). Recently, the latter host differential set has been expanded to fifteen genotypes to include hosts from two additional genera, *Momordica* and *Trichosanthes* (Cohen et al. 2015).

New pathotypes and shifts in virulence within the population of *P. cubensis* have been associated with the resurgence of CDM around the world. For example, Lebeda et al. (2013) reported new pathotypes and substantial increase in virulence of *P. cubensis* following the resurgence of CDM in Czech Republic in 2009. A new pathotype (pathotype 6), that did not infect watermelon but caused severe disease outbreaks on pumpkin and summer squash appeared in Israel in 2002 (Cohen et al. 2003). Similarly, pathotype 5 that was aggressive on squash appeared in Italy in 2002 (Cappelli et al. 2003). Some surveys (Cohen et al., 2015) have indicated the presence of pathotypes 7, 8, 9, and 10 in China, Vietnam, Russia and India, respectively. Systematic studies on the virulence structure within the population of *P. cubensis* are still lacking in the United States. Earlier work by Thomas et

al. (1987), which included only two isolates, indicated the presence of only pathotypes 4 and 5 in the United States. Colucci (2008) later conducted a more comprehensive study on the host range of *P. cubensis* following the 2004 resurgence of CDM and concluded the pathogen population after disease resurgence was very diverse in the United States. However, no pathotypes were identified nor was the level of virulence diversity quantified in that study. In addition, a link between changes in virulence of *P. cubensis* and the resurgence of CDM in the United States has not been established.

More recently, Cespedes-Sanchez et al. (2015) in a field study in Michigan, reported no differences in dominant pathotypes available between 2010 and 2011. However, no specific pathotypes were identified in that study to determine whether they were different from those reported earlier by Thomas et al. (1987). Documentation of prevailing pathotypes is a useful reference for the study by Thomas et al. (1987) when determining whether pathotypes other than pathotypes 4 and 5 are present in the United States. Knowledge on the prevailing pathotypes of *P. cubensis* is also essential for effective deployment of host resistance within a given location or region. In a recent study based on a limited sample size, isolates characterized as pathotypes 1 and 3 were all of the A1 mating type, while those characterized as pathotypes 4 and 5 were of the A2 mating type (Thomas et al. 2016). It would be interesting to determine whether this association between pathotype and mating type holds using a robust set of isolates. Given that host range studies are laborious and time-consuming, genotypic markers associated with mating types (Lebeda et al. 2014) could be used to rapidly determine and narrow down potential pathotypes and hence the host range of *P. cubensis* within a given field. To further elucidate the pathotype and mating type structure of *P. cubensis*, this study focused on the following specific objectives: i) establish the current

host range of *P. cubensis* collected from various cucurbit hosts, ii) determine the prevailing pathotypes of *P. cubensis* within the pathogen population, iii) establish virulence and virulence diversity within the *P. cubensis* population, and iv) characterize the prevalence and distribution of the mating types of *P. cubensis* within the prevailing pathotypes of *P. cubensis* in the United States.

MATERIAL AND METHODS

Isolate collection and maintenance. A total of 22 isolates of *P. cubensis* generated from single lesions were used in this study. The isolates were obtained from different cucurbit host types that included cucumber, cantaloupe, watermelon, pumpkin, butternut squash and acorn squash primarily from the eastern United States (Table 3.1). All isolates were collected from 2005 to 2013 except Pc1982 which was originally isolated from cantaloupe leaves collected in South Carolina in 1982. Pc1982 was previously stored at -80°C and maintained by periodic re-inoculation on detached cantaloupe leaves under controlled conditions.

To generate single lesion isolates, field samples of infected leaves of cucurbits were collected within the CDM ipmPIPE disease monitoring network (Ojiambo et al. 2011) and transported to the Plant Pathology Laboratory at North Carolina State University in Raleigh. Leaves of infected samples were carefully examined and isolates from leaves with discrete lesions were subsequently maintained on their respective hosts in the laboratory as single lesion isolates. Leaf samples without discrete single lesions were further processed to generate single lesion isolates. Sporangia from such lesions were washed off infected leaves using a Preval sprayer (Complete Unit 267; Precision Valve Corporation, Yonkers, NY) and

the suspension used to inoculate respective whole plants at a concentration of about 1×10^4 sporangia per ml. Inoculated plants were bagged with polythene bags and incubated in a growth chamber at 18°C with no light for 24 h. After 24 h, plants were un-bagged and incubated at 21/18°C day/night for disease development with a photoperiod of 12 h. Plants were examined daily for symptoms and when discrete single lesions were visible, plants were bagged again and kept under dark conditions for 24 h to stimulate sporangia production. An individual lesion was subsequently cut out from a leaf and collected sporangia were used to inoculate the original host to multiply sporangia of each isolate. To propagate isolates, first or second leaves were placed on sterile moist paper towel in clear acrylic boxes. Abaxial sides of the leaves were then inoculated with ~ 2×10^4 sporangia per ml and incubated in a growth chamber at 21/19°C day/night and a photoperiod of 12 h. All the isolates were maintained in the laboratory by propagating them on their respective hosts using the detached leaf assay until they were ready for use in subsequent experiments.

Host range and pathotypes. Isolates collected in this study were used to determine the host range of *P. cubensis* based on compatibility reaction with cultivated and wild cucurbit hosts belonging to eight genera: *Cucumis* (*C. sativus*, *C. melo* var. *reticulatus*, and *C. melo* var. *conomon*), *Citrullus* (*C. lanatus*), *Cucurbita* (*C. moschata*, *C. pepo*, and *C. maxima*), *Luffa* (*L. cylindrica*), *Lagenaria* (*L. siceraria*), *Trichosanthes* (*T. cucumerina*), *Benincasa* (*B. hispida*) and *Momordica* (*M. charantia*) (Table 3.2). The differential *C. melo* var. *acidulus* used by Thomas et al. (1987) was not included in this study due to the unavailability of seed. To promote germination, seed of *C. melo* var. *reticulatus, C. melo* var. *conomon, C. lanatus, L. cylindrica, L. siceraria,* were pre-germinated on moist filter papers in glass petri plates in an incubator at 30°C for 5 to 6 days. Seed of *T. cucumerina* and *B.*

hispida were first soaked in warm water for 24 h, while seed of *M. charantia* were soaked in water at room temperature for 24 h prior to the pre-germination treatment described above. For *T. cucumerina*, tips of the seed area where the radicle emerges were also slightly broken off to facilitate emergence of radicles prior to the pre-germination treatment. Seed of *C. sativus* and all pre-germinated seeds were then planted in small plastic cups and placed in the greenhouse at 26°/22°C day/night cycle where all plants were maintained.

Compatibility of isolates with differential hosts was determined using the detached leaf assay. First and second primary leaves from four to five week-old plants were placed with the adaxial side on moist paper towels in clear acrylic boxes and inoculated as described above with 5×10^3 sporangia per ml. A negative control with water-only inoculation was included in the assay. Inoculated leaves were then incubated in a growth chamber at 21/18°C under 12 h/12-h light/dark cycle and monitored for symptoms. Disease severity (percent leaf area infected) and sporulation were assessed at 5 and 7 days after inoculation, respectively. To assess sporulation, two leaf discs measuring 9 mm in diameter were cut from each leaf and vortexed for 20 min in microcentrifuge tubes containing 500 µl of 50% ethanol to dislodge the sporangia (Cohen et al. 2003). Sporangial counts were then determined using a hemocytometer and expressed as sporangia per cm^2 of leaf tissue. Acrylic boxes with inoculated leaves were arranged in the growth chamber in a completely randomized design and the assay was repeated at least once.

Compatibility of *P. cubensis* with cucurbit hosts has been assessed based on: i) sporulation intensity, and ii) disease severity. Here, these two measures were combined (Lebeda et al. 2012; Salati et al. 2010) to provide a comprehensive assessment of compatibility reaction as follows: i) high compatibility = sporulation rate of ~ 5×10^3

sporangia/cm^2 leaf tissue and > 50% disease severity, ii) low compatibility = sporulation rate of ~ 3 × 10^3 sporangia/cm^2 leaf tissue and 30 to 50% disease severity, iii) very low compatibility with sparse sporulation, and iv) incompatible = no evidence of sporulation. To classify isolates into specific pathotypes, compatibility reactions were grouped into two categories as follows: i) compatible (+) = highly compatible reaction as described above, and ii) incompatible (–) = either low compatibility, very low compatibility or an incompatible reaction as described above. Isolates were subsequently classified into pathotypes based on their patterns of compatible and incompatible reaction with the differential host set composed of eight genera (Cohen et al. 2003, 2015; Lebeda and Gadasová, 2002; Thomas et al. 1987).

Mating type determination. The mating type of isolates was determined as described by Cohen and Rubin (2012) by pairing of sporangia of an unknown isolate with sporangia of a tester strain with a known mating type. Here, equal proportions (1:1 v/v) of sporangial suspension (2 × 10^4 spores/ml) of the tester and unknown isolate were mixed together in equal proportion and the resultant suspension was used to inoculate detached first true leaves of cantaloupe (cv. Ananas) or cucumber (cv. Straight Eight) plants. Cantaloupe and cucumber have been reported as favorable hosts for oospore production (Cohen and Rubin 2012). Plants were grown and maintained in the greenhouse as described above to ensure a steady supply of the first true leaves that were used in the assay. The adaxial side of the detached leaves was placed on moist paper towels in clear acrylic boxes, while the abaxial side of the leaf was spot inoculated at least on 20 different spots with 10 µl of the sporangial mixture. Inoculated leaves were then incubated in a growth chamber at 21°C at 50 to 60% relative humidity under 12/12-h light/dark cycle. At 7 to 10 days post inoculation, 11-mm diameter leaf discs were cut from infected leaves and clarified for 24 h in ethyl alcohol-

acetic acid solution (3:1 v/v). Clarified leaf discs were washed three times in de-ionized water and then examined under a compound microscope (×100 magnification) for the presence of oospores. Unknown isolates were designated as either A1 or A2 if oospores were produced in co-inoculations with an A2 or A1 tester strain, respectively.

Data analysis. Sporulation intensity and disease severity data from experimental runs were first tested for homogeneity of variance using GLIMMIX procedure in SAS (version 9.4; SAS Institute, Cary, NC). Homogeneity tests was not significant for both sporulation (P = 0.1045) and disease severity (P = 0.4072) and data from experimental runs were combined for subsequent analysis. The effect of isolate and host on sporulation intensity and disease severity was analyzed using PROC GLIMMIX to determine the degree and extent of heterogeneity in the compatibility of *P. cubensis* with cucurbit hosts.

Virulence was quantified based on: i) number of compatible reactions for an isolate, i.e., virulence factor (v-factor) and ii) number of unique compatibility/incompatibility patterns for all isolates, i.e., virulence phenotype (v-phenotype). In the first set of analysis, virulence complexity of the pathogen population was estimated as described by Andrivon (1994) based on: i) mean number of v-factors per isolate (C_i) and ii) the mean number of virulence factors per v-phenotype (Cp) as:

$$C_i = \sum_j \left(p_j \cdot v_j \right) \quad (1)$$

and

$$C_p = \sum_j \left(v_j \right) / N_p \quad (2)$$

in which p_j is the frequency of the jth v-phenotype in the population with j ranging from 1 to N_p, v_j is the number of v-factors in the v-phenotype j in the population and N_p is the number

of v-phenotypes identified in the population. In the second set of analyses, diversity of v-phenotypes (Lebeda 1982) was estimated using both Gleason (H_G) and Shannon indices (H_S), since the former accounts only for richness, while the latter accounts for both evenness and richness. The indices were calculated as follows:

$$H_G = \frac{N_p - 1}{\ln N_i} \tag{3}$$

$$H_S = -\sum_j \left(p_j \cdot \ln p_j \right) \tag{4}$$

where N_i is number of isolates tested, and p_j and N_p are as defined above. To allow for direct comparison of these diversity estimates with those reported in other studies, relative indices for the Gleason (H_{GR}) and Shannon (H_{SR}) were also calculated as follows:

$$H_{GR} = (N_p - 1) / (N_i - 1) \tag{5}$$

and

$$H_{SR} = H_S / \ln N_i \tag{6}$$

The association of pathotype and mating type (A1 or A2 mating type) of *P. cubensis* within each pathotype was examined using contingency tables based on the frequency of isolates within a pathotype and the number of isolates within the corresponding mating type. Differences in the distribution of A1 and A2 mating type isolates within each pathotype were examined based on Fisher's exact test using PROC FREQ in SAS.

RESULTS

Sporulation intensity and disease severity. Inoculation of the differential host set with isolates collected in this study resulted in different levels of sporulation intensity

ranging from no sporulation to 9.54 × 10^4 sporangia/cm^2 of leaf tissue (Table 3.3). Sporulation intensity differed significantly among isolates (F = 10.37; P < 0.0001), differential hosts (F = 25.39; P < 0.0001), and the interaction between isolates and differential hosts (F = 3.17; P < 0.0001) (Table 3.4). Sporulation intensity across all differential hosts was significantly (P < 0.0001) higher for isolate 2013D6 with a sporulation intensity of 2.09 × 10^4 sporangia/cm^2 than all other isolates tested except Pc1982 and 2013D2, that had an intensity of 1.66 and 1.85 × 10^4 sporangia/cm^2, respectively. Similarly, sporulation intensity across all isolates was significantly (P < 0.0001) higher on CMa that had 2.13 × 10^4 sporangia/cm^2 than all other differential hosts except Cmo and CS1 that had 1.82 and 2.11 × 10^4 sporangia/cm^2, respectively. Inoculation of CMo with isolate 2013D6 resulted in the highest level of sporulation observed among all isolate × differential host combinations. Other isolate × differential combinations that resulted in high levels of sporulation included 2013D2 × CP3 and 2013D6 × CMa with 6.66 and 5.81 × 10^4 sporangia/cm^2, respectively. Disease severity also differed significantly among isolates (F = 7.22; P < 0.0001), differential hosts (F = 30.65; P < 0.0001), and the interaction between isolates and differential hosts (F = 2.57; P < 0.0001) (Table 3.4).

Compatibility reactions and pathotypes. A wide range of compatibility reactions ranging from no compatibility to highly compatible were observed when cucurbit host types were inoculated with isolates collected in this study (Table 3.5). For example, all isolates were highly compatible with CS1, CMr and CMa except 2013A15, 2013F11 and 2013D2 that had a very low compatibility with CMr and 2013A10 that had a very low compatibility with CMa. Similarly, all isolates had either an incompatible or a very low compatible reaction with LC, TC and MC except 2013A20 and Pc1982 that were highly compatible with

TC (Table 3.5). Approximately 50% of the isolates resulted in a highly compatible with CP1, CP2, CP3 and CS2 with the remaining isolates resulting in either an incompatible or a very low compatible reaction with these host types.

There was a wide range of variation in compatibility when sporulation intensity data were grouped into either compatibility (+) or incompatible (–) reactions to establish pathotypes present in the study (Fig. 3.1). For example, the differentials CS1, CMa, CMr and CMc were compatible with >60% of the isolates evaluated in this study. The remaining host differentials had a compatible with 18 to 40% of the isolates evaluated except host differentials LC and MC and TC that had a compatible reaction with <10% of the test isolates (Fig. 3.1). Based on the pattern of compatibility and incompatibility reactions with the differential host set, the isolates were classified into a total of five pathotypes (Table 3.6). Pathotypes 1 and 3 were the most frequent constituting 25 to 30% of the isolates evaluated, while pathotype 4 was the least frequent with <10% of the isolates tested and included Pc1982 (Fig. 3.2A). None of the isolates collected in this study were classified as pathotype 2.

Virulence factors, phenotypes, complexity and diversity. The number of v-factors per isolate ranged from 2 to 8, indicating a complex virulence, with an average of 5.05 v-factors present across all isolates evaluated (Table 3.6). The most commonly occurring v-factors was v-5 that were present in 41% of all the isolates tested (Table 3.6) and isolate Pc1982 collected in 1982 had seven v-factors. A total of thirteen v-phenotypes were described in this study with v-phentoype P2, P3 and P9 being the most frequent, with most of the remaining v-phenotypes including P6 represented by the historical isolate Pc1982, having

only a single isolate (Table 3.6). Most v-phenotypes differed from one another by one or two v-factors.

Virulence complexity as estimated by the mean number of v-factors per isolate (C_i) and mean number of v-factors per v-phenotype (C_p) was 5.05 and 5.23, respectively, for the entire pathogen population studied. Values of C_i and C_p were very similar indicating that about 50% of the v-phenotypes in the population were the complex v-phenotypes. Values for the Gleason (H_G) and Shannon (H_S) indices for the population were 3.88 and 2.32, respectively. Relative indices for the two measures of diversity, H_{GR} and H_{SR} were 0.57 and 0.75, respectively. Given that H_{GR} was slightly higher than 0.5, richness had a large contribution (compared to evenness) to the overall diversity of the *P. cubensis* population in this study.

Association of pathotype with mating type. The distribution of isolates with either A1 or A2 mating type among the five pathotypes differed significantly ($\chi^2 = 20.0$, $P < 0.0001$) for the isolates evaluated in the study (Fig. 3.2B). For example, Pathotypes 1 and 3 were exclusively composed of isolates belonging to the A1 mating type. In contrast, pathotypes 4, 5 and 6 were exclusively composed of isolates of the A2 mating type (Fig. 3.2B).

DISCUSSION

The virulence structure within the *P. cubensis* population was examined in this study to determine the level and variation in the compatibility of the pathogen with cucurbit hosts, identify existing pathotypes and determine the diversity of virulence within the pathogen population in the United States. A high degree of variability in compatibility was observed

between the differential host set and isolates collected from various hosts across the United States. Further, we identified a total of five pathotypes, with three pathotypes being reported for the first time in the United States. Thirteen virulence phenotypes were identified, with some phenotypes and pathotypes being more frequent than others with estimates of virulence diversity and complexity being high across the entire pathogen population. This study provides the first comprehensive assessment and documentation of virulence characteristics of the pathogen and serves as a useful foundation to examine the potential role of changes in the virulence structure of the *P. cubensis* in the 2004 resurgence of CDM in the United States.

The interaction of *P. cubensis* with cucurbits is very specific and results in clear patterns of compatibility or incompatibility (Thomas et al. 1987) that provide a framework to describe the population and virulence structure of the pathogen. While *P. cubensis* is known to have a wide host range and to exhibit high degree of pathogenic variation (Colucci 2008; Lebeda 1992, Lebeda et al. 2012), systematic studies on this aspect of the pathogen in the United States were still lacking. In this study, differences in compatibility of *P. cubensis* with cucurbit host types were observed and all isolates evaluated were compatible with the susceptible cultivar of *C. sativus* as reported in previous studies (e.g., Cohen et al. 2003; Lebeda and Gadasová 2002; Thomas et al. 1987). However, unlike the susceptible cultivar of *C. sativus*, about 50% of the isolates evaluated had either a very low compatibility or were incompatible with the Poinsett 76 cultivar of *C. sativus*. Poinsett 76 was a resistant cultivar developed from PI 197087 in 1950s and contained the *dm-1* gene that had been effective until the resurgence of CDM in 2004 (Criswell et al. 2010; Cohen et al. 2015). *Cucurbita maxima* had the second highest number of compatible reactions where all isolates except

2013A10 resulted in a compatible reaction. In the study by Thomas et al. (1987), isolates of *P. cubensis* that were incompatible with other *Cucurbita* species were also incompatible or lowly compatible with *C. maxima*. The observation by Thomas et al. (1987) is in contrast with results from the present study and studies conducted in the Czech Republic (Lebeda and Gadasová 2002) and Malaysia (Salati et al. 2010) where most of the isolates that were incompatible with other *Cucurbit* species were highly compatible with *C. maxima*. Differences in the pathogenic profile of isolates may explain the high compatibility with *C. maxima* observed in the present study unlike in the study by Thomas et al (1987). As reported in other studies conducted in Israel (Lebeda and Cohen 2011) and the Czech Republic (Lebeda and Widrlechner 2003), *L. cylindrica* and *M. charantia* exhibited the lowest level of compatibility with our test isolates. However, *L. cylindrica* and other *Luffa* species have been reported to be highly compatible with *P. cubensis* isolates in China (Cohen et al. 2015) and India (Jamadar and Desai 1999). Thus, the *P. cubensis* population in the United States or Czech Republic may be composed of a pathotype that is distinctively different from that in the pathogen population in India or China.

Like for many other oomycete plant pathogens, description of pathotypes provides a useful means to characterize and thereby understand the virulence structure of *P. cubensis* within a given population (McDonald and Linde 2002). Colucci (2008) indicated a broader virulence pattern of *P. cubensis* isolates from the United States following the 2004 resurgence of CDM than previously reported by Thomas et al. (1987) but did not describe the pathotypes or their distribution. In the present study, five pathotypes were described from a total of twenty two isolates evaluated. Three of these pathotypes, pathotype 1 [which includes 1(A)], pathotype 3 [which includes 3(B) and 3(C)] and pathotype 6 [which includes

6(A)] had previously not been reported in the United States. Additionally, pathotypes 1 and 3 previously reported to be present only in East Asia and Israel (Cohen et al. 2003; Thomas et al. 1987) were the most predominant within the pathogen population. Pathotype 6 was reported for the first time following the resurgence of CDM in Israel in 2002 (Cohen et al. 2003).

A distinct pattern in the distribution of pathotypes across the United States was also evident in this study. Most of the isolates collected from the northern states (i.e., Michigan, New Jersey, New York and Ohio) were either pathotype 1 or pathotype 3, while isolates collected from the southern states (i.e., Alabama, Florida, Georgia, North Carolina, South Carolina and Texas) were diverse and belonged to all the five pathotypes reported in this study. The diversity of pathotypes in southern states could be due to the proximity of these states to the overwintering sources in the southern Florida and the Gulf of Mexico where a diverse range of cucurbits are cultivated year-round. Overwintering hosts and seasonally cultivated cucurbit species exert different degrees of selection pressure that results in differences in prevailing pathotypes in specific geographic areas (Thomas et al. 1987). A high genetic diversity has also been detected in *P. cubensis* populations in southern states of Georgia and North Carolina (Quesada-Ocampo et al. 2012). A closer examination of the compatibility reactions shows that isolates belonging to pathotypes 1 and 3 were compatible with *C. sativus* cultivar Poinsett 76, while isolates belonging to all other pathotypes exhibited either an incompatible or a very lowly compatible reaction with Poinsett 76 cultivar. Given that Poinsett 76 was a highly resistant cultivar (Criswell et al. 2010) and pathotypes 1 and 3 had not been reported in the United States prior to 2004, the breakdown of resistance in Poinsett 76 and other resistant cucumber cultivars that resulted in the resurgence of CDM in

the United States may have been due to the introduction of an aggressive strain of pathotype 1 or 3.

Pathotypes of *P. cubensis* in the United States have not been characterized following the initial study by Thomas et al. (1987). Thus, the host differential set developed by Thomas et al. (1987) serves a useful baseline for establishing whether new pathotypes are present in the current population of *P. cubensis* in the United States. Unavailability of some differentials (e.g., *C. melo var. acidulous*) used earlier (Thomas et al. 1987) led to some isolates not grouping perfectly within pathotypes described by Thomas et al. (1987). For example, isolate 2013A3 was compatible with *C. sativus* and *C. melo* var *reticulatus* and would be designated as pathotype 1 based on Thomas et al. (1987). However, 2013A3 was compatible with *C. maxima* unlike in the study by Thomas et al. (1987), where pathotype 1 isolates were only lowly incompatible with *C. maxima*. The high sporulation cutoff value used by Thomas et al. (1987) may partly explain differences observed between the two studies. Compatibility of *C. maxima* with several *P. cubensis* isolates based on the sporulation cutoff used in the present study has also been reported elsewhere (e.g., Lebeda and Gadasová 2002; Lebeda and Widrlechner 2003). Thus, rather than assign 2013A3 a new pathotype, that isolate was designated as pathotype 1A, i.e., a variant of pathotype 1 that is compatible with *C. maxima*. This approach was adopted to assign pathotypes to isolates that did not perfectly fit the Thomas et al (1987) model. Studies conducted on *P. cubensis* populations in the Czech Republic (Lebeda and Gadasová, 2002) and Malaysia (Salati et al. 2010) have also reported a lack of perfect fit with the pathotype model proposed by Thomas et al. (1987). Based on the differential set used in the present study, Lebeda and Gadasová (2002) observed that only 1 of their 22 isolates tested corresponded to the pathotype model of

Thomas et al. (1987). This was attributed to the limited taxonomic range of the differential set used by Thomas et al. (1987) making it unable to capture the extent of variability within *P. cubensis* (Lebeda and Widrlechner 2003). Nonetheless, the pathotypes described in the present study serve as a useful working framework for subsequent studies on the structure and variation of pathotypes of *P. cubensis* in the United States.

The level of virulence of all isolates examined in this study was moderate with a mean virulence factor of 5.1 across the entire population. Although virulence of *P. cubensis* isolates in the United States has previously been examined (Colucci 2008), the level of virulence has not quantified and thus, we are unable to compare our findings with those reported by Colucci (2008). Additionally, the level of virulence observed in the present study is comparatively lower than that reported for *P. cubensis* in the Czech Republic where the pathogen population has been reported to have a high level of virulence with a mean virulence factor of 9.0 (Lebeda et al. 2013). In the present study, virulence phenotypes, complexity and diversity were analyzed to determine differences in the *P. cubensis* population in the United States. Thirteen virulence phenotypes were identified and it is likely that v-phenotypes P6, P7, P8, P9 and P10 were present in the United States prior to 2004 since they were identified as pathotypes 4 and 5 described earlier by Thomas et al. (1987). All the other v-phenotypes were not associated with previously described pathotypes and could be considered new virulence phenotypes. In addition, v-phenotypes P1 and P2 (pathotype 1) and P3, P4 and P5 (pathotype 3) were compatible with Poinsett 76 and they may be associated with the resurgence of CDM in 2004. Diversity estimates indicated that virulence diversity within the *P. cubensis* population in the United States is high. Although virulence diversity estimates for *P. cubensis* have not been reported prior to this study, the

normalized estimate for Shannon index of diversity reported here is comparable to that of *P. infestans* (H_{SR} = 0.72) infecting potato and tomato in the United States (Andrivon 1994). This high virulence diversity is consistent with the high pathogenic variability observed in this study. High pathogenic variation of *P. cubensis* has also been reported in the Czech Republic (Lebeda et al. 2013), although specific estimates of virulence diversity were not presented. In addition, the mean virulence complexity (C_i = 5.05 and C_p = 5.23) was high with virulence factors per isolate and virulence factors per v-phenotype equally contributing to virulence complexity of *P. cubensis*. The high pathogenic variability observed in this study verifies the genetic flexibility of *P. cubensis* and its ability to evolve and adapt more rapidly to fungicides and changes in cucurbit hosts (Quesada et al. 2012).

The pathotypes of isolates evaluated in this study were significantly associated with the mating type of the corresponding isolates. Specifically, all pathotype 1 and 3 isolates were of the A1 mating type, while the pathotypes 4, 5 and 6 isolates were of the A2 mating type. Molecular studies conducted on *P. cubensis* in the Czech Republic showed that isolates sampled before the shift in virulence in 2009 exhibited the genotype of the subspecies of Clade II, while isolates sampled after the shift in virulence exhibited a genotype of the subspecies of Clade I (Kitner et al. 2015). It was suggested that genotypes within Clade II, probably indigenous to East Asia, were of the A1 mating type, while genotypes in Clade I were the A2 mating type of *P. cubensis* (Lebeda et al. 2014). Isolates belonging to pathotypes 1 and 3 were also of the A1 mating type and had not been previously reported in the United States. Given that pathotypes 1 and 3 isolates were indigenous in East Asia, it is highly possible that they were introduced from East Asia to the United States via Europe (Runge et al. 2011) through anthropogenic activities. In addition, since only isolates of pathotype 1 and

3 were compatible with Poinsett 76 that was resistant to CDM until 2004, Kitner et al. (2014) provides additional support that strains belonging to pathotypes 1 and 3 may have been responsible for the resurgence of CDM in the United States in 2004. New pathotypes of *P. cubensis* that are aggressive and cause severe epidemics on *Cucurbita* spp. and *C. lanatus* have also been reported since 2009 in the Czech Republic (Kitner et al. 2015). These new pathotypes have been linked with an increase in virulence within the pathogen population in Czech Republic and most of the isolates belonging to these new pathotypes also contain signatures of sexual recombination (Kitner et al. 2015). Although genetic signatures of pathotypes 1 and 3 isolates (A1 isolates) reported in this study were not examined, it is also possible that a recombinant F1 heterozygote of the A1 mating type that was fit and thus, able to survive and rapidly spread across the eastern states may have been responsible for the resurgence of CDM in the United States in 2004.

The high pathogenic variability observed in this study indicates that *P. cubensis* has the genetic plasticity to evolve rapidly. The pathogen has also been shown to have the ability to reproduce sexually (Cohen and Rubin 2012; Thomas et al. 2013; Zhang et al. 2012). The latter characteristic is expected to facilitate the rapid evolution (McDonald and Linde 2002) of *P. cubensis* leading to a high risk for developing resistance to available fungicides and less durability of host resistance genes already deployed to control CDM. Thus, it is unlikely that resistance alone in available cucurbit cultivars will be effective to control CDM. An integrated approach involving a combination of timely application of fungicides, rotation of fungicides with different chemistries and introduction of cultivars with new resistance genes will be required for effective management of CDM.

ACKNOWLEDGEMENTS

This study was supported by Hatch Funds from North Carolina Agricultural Experiment Station for Project Number NC02251 and NC02432. Aleš Lebeda work was supported by project MSM 6198959215 (Ministry of Education, Youth and Sports) and the project QH 71254 (Czech Ministry of Agriculture).We thank collaborators on the CDM ipmPIPE project for their assistance in collecting field samples used in this study and Taylor Ghiloni and Barbara Stevens for assistance with laboratory and greenhouse aspects of this work.

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Table 3.1. Source and description of *Pseudoperonospora cubensis* isolates used to determine the current host range and pathotype structure of pathogen population in the eastern United States

Year	Isolate	Host of origin	Host species	State	County
1982	Pc1982	Cantaloupe	*Cucumis melo*	South Carolina	Charleston
2005	05A1	Cucumber	*Cucumis sativus*	Michigan	Oceana
2008	08A1	Cucumber	*Cucumis sativus*	California	Salinas
2008	08F1	Acorn squash	*Cucurbita pepo*	Georgia	Tift
2008	08C1	Pumpkin	*Cucurbita maxima*	New York	Suffolk
2008	08E1	Watermelon	*Citrullus lanatus*	Texas	Bryan
2012	D3	Butternut squash	*Cucurbita moschata*	South Carolina	Charleston
2012	A11	Cucumber	*Cucumis sativus*	North Carolina	Johnston
2013	2013A3	Cucumber	*Cucumis sativus*	Georgia	Worth
2013	2013A10	Cucumber	*Cucumis sativus*	South Carolina	Charleston
2013	2013A15	Cucumber	*Cucumis sativus*	Alabama	Macon
2013	2013A18	Cucumber	*Cucumis sativus*	New Jersey	Salem
2013	2013A19	Cucumber	*Cucumis sativus*	Ohio	Sandusky
2013	2013A20	Cucumber	*Cucumis sativus*	Ohio	Medina
2013	2013C3	Giant pumpkin	*Cucurbita maxima*	North Carolina	Johnston
2013	2013B17	Cantaloupe	*Cucumis melo*	New York	Ontario
2013	2013E1	Watermelon	*Citrullus lanatus*	Florida	Collier
2013	2013F2	Acorn squash	*Cucurbita pepo*	South Carolina	Charleston
2013	2013F11	Acorn squash	*Cucurbita pepo*	New York	Suffolk
2013	2013D2	Butternut squash	*Cucurbita moschata*	Florida	Hillsborough
2013	2013D4	Butternut squash	*Cucurbita moschata*	North Carolina	Johnston
2013	2013D6	Butternut squash	*Cucurbita moschata*	Alabama	Escambia

Table 3.2. Description and source of cucurbit host types used as differential hosts to determine the compatibility and specificity of *Pseudoperonospora cubensis* isolates in the United States

Host type	Acronym	Common name	Cultivar	Seed supplier
Benincasa hispida	BH	Wax gourd	Lai Jud	Kitazawa Seed
Citrullus lanatus	CL	Watermelon	Mickylee	Hollar Seed
Cucumis sativus	CS1	Cucumber	Straight Eight	Wyatt-Quarles
Cucumis sativus	CS2	Cucumber	Poinsett 76	Clifton Seed
C. melo var. *reticulatus*	CMr	Cantaloupe	Ananas	Willhite Seed
C. melo var. *conomon*	CMc	Pickling melon	Green Striped	Kitazawa Seed
Cucurbita pepo	CP1	Summer squash	Dixie	Seminis
Cucurbita pepo	CP2	Acorn squash	Table Queen	Wyatt-Quarles
Cucurbita pepo	CP3	Pumpkin	Jack-O-Lantern	Burpee
Cucurbita moschata	CMo	Butternut squash	Waltham	Wyatt-Quarles
Cucurbita maxima	CMa	Giant pumpkin	Big Max	Wyatt-Quarles
Lagenaria siceraria	LS	Bottle gourd	Nam Tao Yao	Kitazawa Seed
Luffa cylindrical	LC	Sponge gourd	Short	Kitazawa Seed
Momordica charantia	MC	Bitter gourd	Futo Spindle	Kitazawa Seed
Trichosanthes cucumerina	TC	Snake gourd	Buag Ngu	Kitazawa Seed

Table 3.3. Sporulation of *Pseudoperonospora cubensis* on detached leaves of cucurbit host types following inoculation with isolates collected from different cucurbits in the United States[a]

	Sporulation ($\times 10^3$) on inoculated cucurbit host type[b]														
Isolate	CS1	CMr	CMc	CP1	CP2	CMo	CMa	CL	BH	LC	LS	TC	MC	CP3	CS2
05A1	16.5	5.2	7.3	0.0	0.2	2.9	18.6	1.3	0.0	0.0	19.9	5.0	0.0	0.5	16.6
	(2.2)	(4.8)	(3.9)	(0.0)	(0.2)	(2.9)	(13.6)	(1.3)	(0.0)	(0.0)	(6.8)	(5.0)	(0.0)	(0.5)	(16)
08A1	14.5	6.0	3.1	0.6	0.0	0.0	4.1	0.0	1.2	0.0	13.1	0.5	0.0	0.3	13.7
	(1.7)	(3.2)	(0.1)	(0.6)	(0.0)	(0.0)	(1.6)	(0.0)	(1.2)	(0.0)	(4.7)	(0.1)	(0.0)	(0.3)	(7.2)
2013A18	25.0	9.2	13.2	0.0	0.0	8.1	6.7	0.5	0.0	4.2	10.6	0.0	0.0	0.2	10.9
	(19.0)	(1.1)	(0.7)	(0.0)	(0.0)	(6.2)	(2.0)	(0.2)	(0.0)	(3.3)	(2.5)	(0.0)	(0.0)	(0.2)	(3.7)
2013A19	12.3	5.5	6.9	5.5	0.0	0.0	8.8	1.1	2.5	3.6	6.7	3.1	0.3	2.8	7.2
	(1.7)	(0.1)	(4.7)	(2.3)	(0.0)	(0.0)	(0.9)	(1.1)	(2.1)	(2.3)	(5.1)	(1.2)	(0.3)	(2.8)	(5.6)
2013A10	7.8	15.9	0.0	0.0	0.0	0.0	1.7	0.0	1.2	1.7	0.0	3.6	0.0	2.9	1.4
	(1.9)	(11.6)	(0.0)	(0.0)	(0.0)	(0.0)	(1.1)	(0.0)	(1.2)	(1.7)	(0.0)	(0.2)	(0.0)	(2.9)	(0.5)
2013A3	24.7	7.1	7.0	0.0	0.0	11.5	9.2	0.0	0.0	0.0	0.6	0.0	0.0	0.0	3.3
	(15.9)	(7.1)	(3.9)	(0.0)	(0.0)	(9.7)	(3.1)	(0.0)	(0.0)	(0.0)	(0.3)	(0.0)	(0.0)	(0.0)	(0.8)
A11	10.9	11.5	7.9	1.6	0.0	0.0	21.7	0.4	7.0	0.0	24.3	3.1	0.6	0.0	13.3
	(4.4)	(2.2)	(1.4)	(1.6)	(0.0)	(0.0)	(1.7)	(0.4)	(2.0)	(0.0)	(11.1)	(3.1)	(0.6)	(0.0)	(1.4)
2013A15	48.0	4.8	5.6	5.1	0.0	0.0	12.9	0.3	4.4	0.3	6.9	0.0	0.6	0.6	9.2
	(28.4)	(3.6)	(2.5)	(5.1)	(0.0)	(0.0)	(2.9)	(0.0)	(2.8)	(0.0)	(5.0)	(0.0)	(0.6)	(0.3)	(7.0)
2013A20	20.4	2.5	1.6	0.0	0.3	17.8	29.4	3.3	6.6	0.3	5.3	6.1	0.5	10.5	9.1
	(1.7)	(0.9)	(0.0)	(0.0)	(0.3)	(17.8)	(16.9)	(2.0)	(2.2)	(0.3)	(4.4)	(5.4)	(0.5)	(8.3)	(0.6)
2013B17	48.3	6.1	3.0	0.0	0.0	0.0	14.3	0.0	4.9	0.6	3.2	0.3	0.3	0.0	11.5
	(33.9)	(3.9)	(2.6)	(0.0)	(0.0)	(0.0)	(5.6)	(0.0)	(0.9)	(0.6)	(3.2)	(0.3)	(0.3)	(0.0)	(7.5)
2013C3	28.1	8.4	3.7	2.5	0.0	0.0	7.5	0.0	0.8	0.0	1.7	2.8	0.9	0.0	14.2
	(9.4)	(3.4)	(2.2)	(1.9)	(0.0)	(0.0)	(7.2)	(0.0)	(0.8)	(0.0)	(1.1)	(2.8)	(0.3)	(0.0)	(0.5)
08C1	46.7	6.7	9.0	1.7	0.6	0.4	12.9	0.3	5.0	4.2	4.8	5.6	1.6	4.4	4.5
	(15.4)	(0.1)	(9.0)	(1.7)	(0.6)	(0.4)	(4.8)	(0.3)	(2.8)	(2.6)	(6.4)	(5.6)	(0.3)	(3.7)	(1.1)
2013E1	7.3	23.0	5.1	1.7	1.1	3.0	17.7	8.4	0.6	3.7	3.2	2.8	0.0	9.7	0.0
	(4.5)	(15.2)	(2.0)	(1.7)	(0.5)	(3.0)	(1.4)	(2.8)	(–)	(0.9)	(1.1)	(2.5)	(0.0)	(5.4)	(0.0)
08E1	7.8	3.6	4.1	15.5	5.0	1.2	6.1	17.1	14.1	0.0	9.6	1.9	0.0	6.5	0.6
	(5.05)	(2.0)	(0.6)	(9.5)	(2.5)	(0.9)	(2.3)	(3.7)	(11.2)	(0.0)	(4.3)	(0.6)	(0.0)	(1.5)	(0.6)

Table 3.3. Continued

08F1	6.4	6.4	4.3	26.8	11.6	25.4	26.3	2.7	0.9	0.0	1.7	4.5	1.7	25.7	0.0
	(6.4)	(3.9)	(1.2)	(5.3)	(2.3)	(2.6)	(15.7)	(1.1)	(0.3)	(0.0)	(0.5)	(0.5)	(0.2)	(18.9)	(0.0)
D3	32.9	5.6	4.2	23.6	46.2	39.5	27.6	4.1	2.6	0.3	1.2	2.0	0.0	12.8	16.4
	(14.1)	(3.1)	(0.8)	(12.5)	(26.6)	(2.0)	(6.7)	(0.3)	(2.6)	(0.0)	(0.6)	(0.2)	(0.0)	(3.1)	16.4
2013D4	5.4	10.1	3.9	34.8	19.3	31.7	33.7	4.1	4.8	3.4	2.5	3.7	2.0	24.8	0.0
	(4.5)	(1.7)	(1.4)	(11.4)	(11.2)	(01.1)	(13.1)	(3.1)	(4.8)	(2.5)	(2.5)	(1.9)	(1.1)	(4.2)	(0.0)
2013D6	26.6	16.1	25.0	34.2	16.9	95.4	58.1	3.6	0.0	0.3	4.0	1.4	0.8	30.3	0.3
	(21.4)	(6.0)	(15.0)	(4.6)	(4.4)	(32.4)	(7.5)	(2.0)	(0.0)	(0.3)	(2.7)	(1.4)	(0.8)	(6.0)	(0.3)
2013F2	9.5	3.9	7.8	23.7	32.6	51.3	33.4	9.2	9.5	1.2	1.2	2.2	0.3	27.9	1.1
	(3.3)	(2.0)	(2.8)	(8.1)	(3.9)	(15.8)	(0.9)	(7.3)	(2.6)	(0.3)	(0.6)	(0.9)	(0.0)	(9.8)	(1.1)
2013F11	10.1	0.2	0.3	35.5	41.9	53.1	27.5	0.3	1.9	0.9	4.2	3.4	0.9	47.9	0.0
	(3.9)	(0.1)	(0.3)	(12.7)	(24.7)	(5.6)	(1.9)	(0.0)	(1.9)	(0.9)	(2.3)	(1.2)	(0.3)	(2.9)	(0.0)
2013D2	9.2	0.9	12.2	24.6	28.7	56.2	55.9	0.8	3.7	0.1	14.1	2.3	1.7	66.6	0.0
	(7.3)	(0.6)	(6.2)	(1.8)	(6.6)	(25.0)	(4.1)	(0.5)	(2.2)	(0.1)	(5.9)	(0.5)	(0.5)	(12.9)	(0.0)
Pc1982	45.6	43.4	51.6	0.0	1.9	3.1	36.4	16.1	0.0	1.2	18.9	12.6	3.9	14.1	0.2
	(2.5)	(6.6)	(7.5)	(0.0)	(0.0)	(1.2)	(7.3)	(5.5)	(0.0)	(1.2)	(4.8)	(0.5)	(0.2)	(5.0)	(0.2)

[a] Sporulation values are number of sporangia produced per cm^2 leaf tissue and are means of two to four replications except for 2013E1 on BH that was not replicated. Values shown in parenthesis are standard errors of the mean for sporulation on each host type.

[b] Cucurbit host types are defined based on acronyms described in Table 3.1.

Table 3.4. Summary of analysis of sporulation intensity and disease severity on inoculated host differentials to determine compatibility of *Pseudoperonospora cubensis* with cucurbit host types

			Sporulation		Disease severity	
Source	ndf[a]	ddf[a]	*F*	Pr > *F*	*F*	Pr > *F*
Isolate	21	325	10.37	0.0001	7.22	0.0001
Differential	14	325	25.39	0.0001	30.65	0.0001
Isolate × Differential	280	325	3.17	0.0001	2.57	0.0001

[a] Refers to numerator degrees of freedom (ndf) and denominator degrees of freedom (ddf).

Table 3.5. Compatibility of *Pseudoperonospora cubensis* with a range of cucurbit host types under controlled conditions following inoculation with isolates collected from different cucurbits across the United States

	Cucurbit host type[a]														
Isolate	CS1	CMr	CMc	CP1	CP2	CMo	CMa	CL	BH	LC	LS	TC	MC	CP3	CS2
05A1	+++	+++	+++	±	±	±	+++	±	–	–	+++	±	–	±	+++
08A1	+++	+++	+	±	–	–	+++	–	±	–	+++	±	–	±	+++
2013A18	+++	+++	+++	±	±	±	+++	±	–	±	+++	±	±	±	+++
2013A19	+++	+++	+++	±	–	–	+++	±	±	±	+++	±	±	±	+++
2013A10	+++	+++	±	–	–	–	±	±	±	±	±	±	–	±	±
2013A3	+++	+++	±	–	±	±	+++	–	–	–	±	–	–	±	±
A11	+++	+++	+++	±	–	–	+++	–	+++	–	+++	±	–	–	+++
2013A15	+++	+++	+++	+++	–	–	+++	±	+	±	+++	±	±	±	+++
2013A20	+++	±	±	–	±	+	+++	±	+++	±	+++	+++	±	±	+++
2013B17	+++	+++	+	±	–	±	+++	–	±	±	+	±	–	–	+++
2013C3	+++	+++	+	±	–	–	+++	–	–	–	+	±	±	±	+++
08C1	+++	+++	+	±	±	±	+++	±	+	±	+	±	±	±	+++
2013E1	+++	+++	+++	±	±	±	+++	+++	±	±	±	±	±	+++	±
08E1	+++	+	±	±	±	±	+++	+++	+++	±	+++	±	±	+++	±
08F1	+++	+++	+	+++	+++	+++	+++	±	±	±	±	+	±	+++	–
D3	+++	+++	+++	+++	+++	+++	+++	+	±	±	±	±	±	+++	±
2013D4	+++	+++	+++	+++	+++	+++	+++	+++	±	±	+	±	±	+++	–
2013D6	+++	+++	+++	+++	+++	+++	+++	+	–	±	±	±	±	+++	±
2013F2	+++	+++	+++	+++	+++	+++	+++	+++	+++	±	±	±	–	+++	±
2013F11	+++	±	±	+++	+++	+++	+++	±	±	±	+	+	±	+++	–
2013D2	+++	±	+++	+++	+++	+++	+++	±	±	±	+++	±	±	+++	–
Pc1982	+++	+++	+++	–	±	±	+++	–	±	±	+++	+++	±	+++	±

[a] Cucurbit host types are defined based on acronyms described in Table 3.1.

[b] Compatibility reactions are as follows: +++ = high compatibility with a sporulation rate of ~ 5×10^3 sporangia/cm^2 leaf tissue and disease severity of >50%, + = low compatibility with a sporulation rate of ~ 3×10^3 sporangia/cm^2 leaf tissue and disease severity of 30 to 50%, ± = very low compatibility with sparse sporulation, and – = incompatible with no evidence of sporulation.

Table 3.6. Pathotype designation, virulence characteristics and mating type of *Pseudoperonospora cubensis* isolates collected from different cucurbits across the United States

	Host differential [a]												Proposed pathotype[b]	Virulence factor[c]	Virulence phenotype[d]	Mating type
Isolate	CS1	CMr	CMc	CL	CP2	CMa	CMo	LS	LC	MC	BH	TC				
2013A10	+	+	–	–	–	–	–	–	–	–	–	–	1	2	P1	A1
2013A3	+	+	–	–	–	+	–	–	–	–	–	–	1(A)	3	P2	A1
2013B17	+	+	–	–	–	+	–	–	–	–	–	–	1(A)	3	P2	A1
2013C3	+	+	–	–	–	+	–	–	–	–	–	–	1(A)	3	P2	A1
08C1	+	+	–	–	–	+	–	–	–	–	–	–	1(A)	3	P2	A1
05A1	+	+	+	–	–	+	–	+	–	–	–	–	3(A)	5	P3	A1
08A1	+	+	+	–	–	+	–	+	–	–	–	–	3(A)	5	P3	A1
2013A18	+	+	+	–	–	+	–	+	–	–	–	–	3(A)	5	P3	A1
2013A19	+	+	+	–	–	+	–	+	–	–	–	–	3(A)	5	P3	A1
2013A15	+	+	+	–	–	+	–	+	–	–	–	–	3(A)	5	P3	A1
A11	+	+	+	–	–	+	–	+	–	–	+	–	3(B)	6	P4	A1
2013A20	+	–	–	–	–	+	–	+	–	–	+	+	3(C)	5	P5	A2
Pc1982	+	+	+	+	–	+	–	+	–	–	–	+	4	7	P6	A2
2013E1	+	+	+	+	–	+	–	–	–	–	–	–	4	5	P7	A2
08E1	+	–	–	+	–	+	–	+	–	–	+	–	4(A)	5	P8	A2
D3	+	+	+	+	+	+	+	–	–	–	–	–	5	7	P9	A2
2013D4	+	+	+	+	+	+	+	–	–	–	–	–	5	7	P9	A2
2013D6	+	+	+	+	+	+	+	–	–	–	–	–	5	7	P9	A2
2013F2	+	+	+	+	+	+	+	–	–	–	+	–	5(A)	8	P10	A2
2013D2	+	–	+	–	+	+	+	+	–	–	–	–	6	6	P11	A2
08F1	+	+	–	–	+	+	+	–	–	–	–	–	6(A)	5	P12	A2
2013F11	+	–	–	–	+	+	+	–	–	–	–	–	6(B)	4	P13	A2

[a] Cucurbit host differentials are defined based on acronyms described in Table 3.1.

[b] Pathotype designation is based on the pattern of compatibility reaction between an isolate and the host differential set, where '+' = highly compatible reaction and '–' = low and very low compatibility or incompatible reaction (see Table 3.4) based on the compatibility scheme originally developed by Thomas et al. (1987) and later expanded by Lebeda and Widrlechner (2003) and

Cohen et al. (2015). Letters in parenthesis refer to a variant of a pathotype that largely conforms to the Thomas et al (1897) model but differs in one or two compatibility reactions.

[c] Refers to the virulence factor of an isolate based on the number of compatible reactions with the host differential set.

[d] Refers to the virulence phenotype of an isolate based on a unique pattern of compatible/incompatible reactions with the differentials

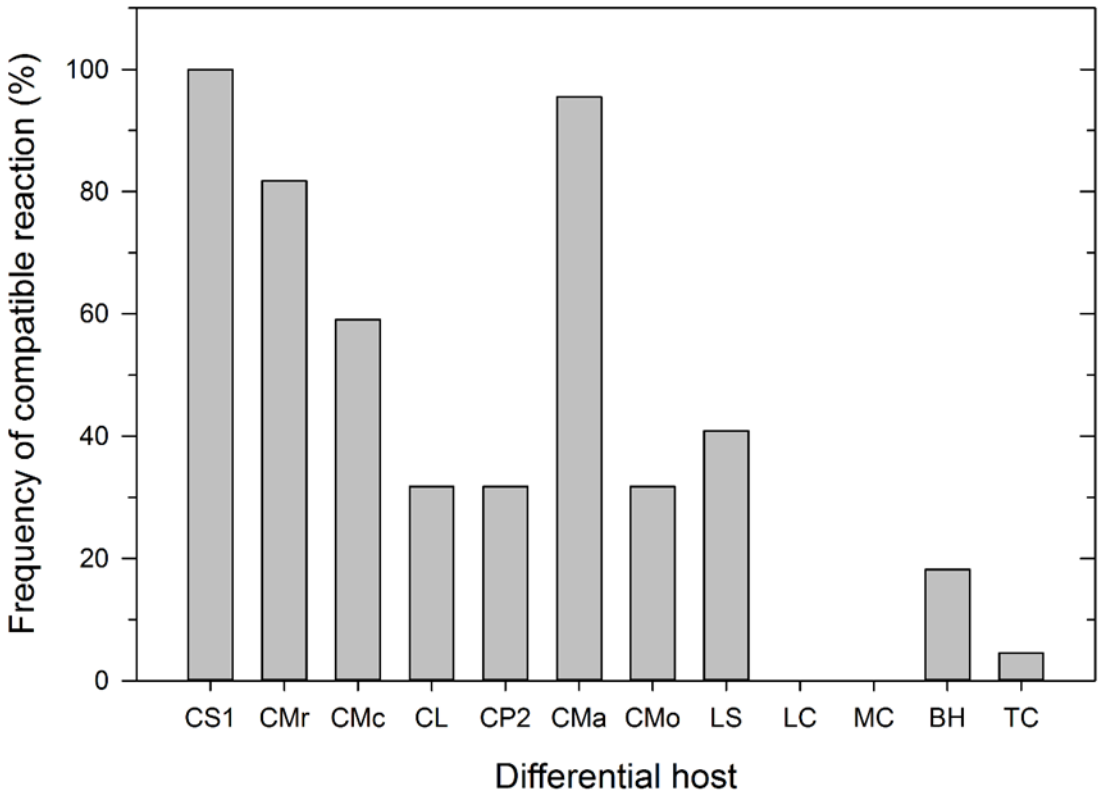

Figure 3.1. Variation in the susceptibility of cucurbit host types used as differentials to establish the virulence structure of *Pseudoperonospora cubensis* population in the United States. Each bar represents the number of isolates with a compatible reaction as a proportion of the total number of isolates evaluated.

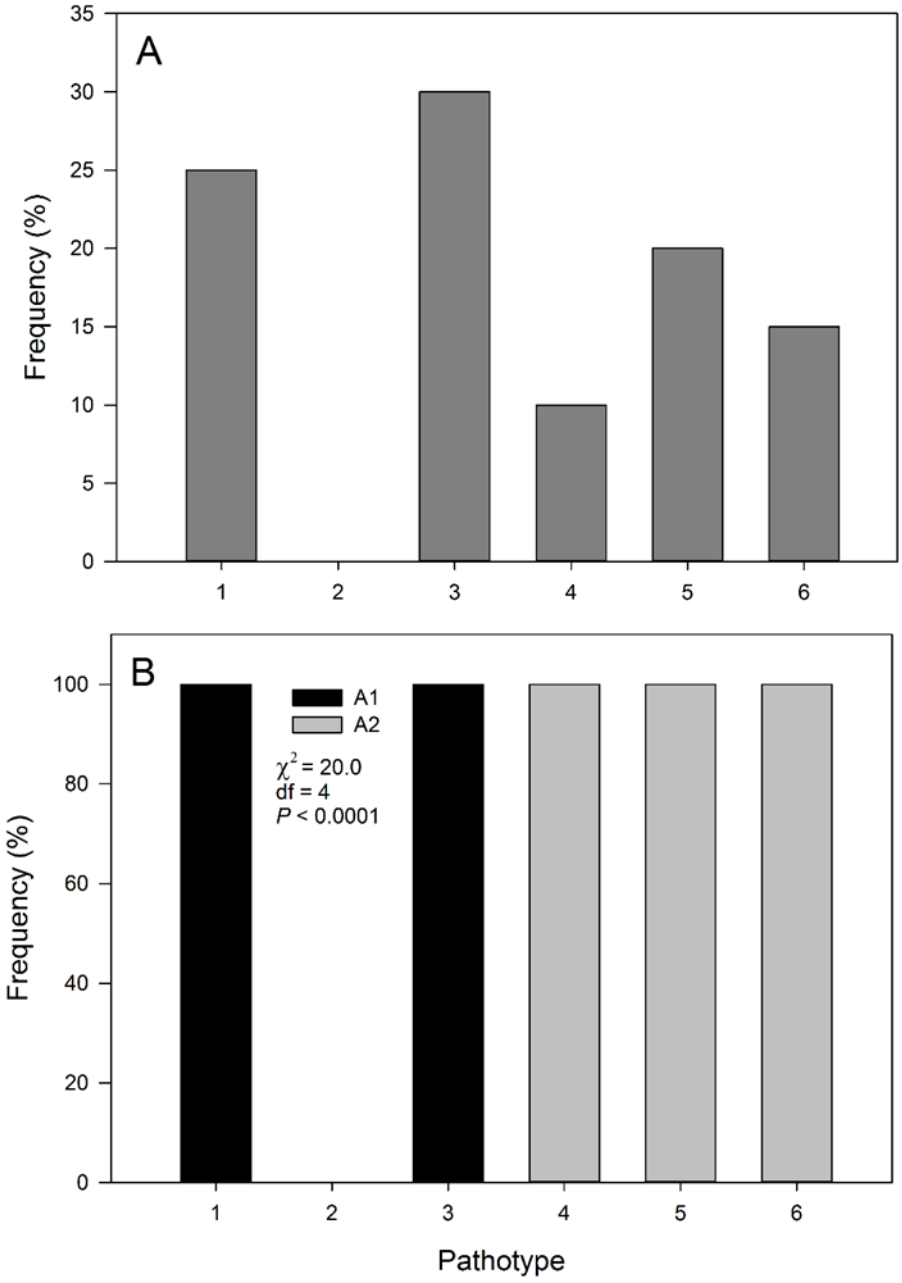

Figure 3.2. Frequency distribution of pathotypes of *Pseudoperonospora cubensis* based on isolates collected from various cucurbit host types in the United States. **A**) Frequency distribution of all *P. cubensis* isolates within each pathotype and **B**) frequency distribution of isolates of the A1 and A2 mating type within each pathotype of *P. cubensis*.

CHAPTER 4

Comparative genomic analysis of *Pseudoperonospora cubensis* provides insights into the resurgence of cucurbit downy mildew in the United States

To be submitted to *Molecular Ecology*:

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Abstract

Pseudoperonospora cubensis, the causal agent of cucurbit downy mildew (CDM), is known to exhibit host specialization and the virulence of the pathogen can be classified into different pathotypes based on compatibility tests with a differential set composed of specific cucurbit host types. However, the genetic basis of host specialization within *P. cubensis* is not yet known. Total genomic DNA extracted from nine isolates of *P. cubensis* collected in 2008 and 2013 from diverse cucurbit host types (*Cucumis sativus, C. melo* var. *reticulatus, Cucurbita maxima, C. moschata, C. pepo* and *Citrullus lanatus*) in the United States, were subjected to whole genome sequencing. Comparative analysis of these nine genomes revealed the presence of two distinct evolutionary lineages (lineage I and II). Many fixed polymorphisms separated one lineage (lineage I) that composed of isolates from *Cucurbita pepo*, *C. moschata* and *Citrullus lanatus* from a second lineage (lineage II) that comprised isolates from *Cucumis* spp. and *Cucurbita maxima*. Phenotypic characterization of the nine isolates revealed that lineage II isolates were of the A1 mating type and belonged to pathotype 1 or 3, pathotypes not known to be present in the United States prior to the resurgence of CDM in 2004. The association of lineage II isolates with the new pathotypes and a lack of genetic diversity among lineage II isolates provide preliminary evidence that lineage II of *P. cubensis* might have been responsible for the resurgence of CDM on cucumber in the United States. Phylogenetic analysis showed that *P. cubensis* and its sister species, *P. humuli*, shared a recent common ancestor. Reconstruction of ancestral recombination indicated a hybrid origin of lineage II of *P. cubensis*, with *P. humuli* and lineage I of *P. cubensis* as putative parents.

Keywords: coalescent analysis, cucurbits, disease resurgence, downy mildew, lineage, phylogenetic analysis

Introduction

Pseudoperonospora cubensis is an obligate biotrophic pathogen and is the causal agent of downy mildew of cucurbits. Cucurbit downy mildew (CDM), is considered as the most economically important disease of cucurbits worldwide. The disease is widely distributed in temperate and semi-arid regions and the pathogen has a wide host range that includes both wild and cultivated cucurbits (Lebeda 1992). Approximately 60 species of cucurbits have been identified as hosts of *P. cubensis* and the most economically important ones include cucumber, cantaloupe, watermelon, pumpkin and squashes (Lebeda 1992). In the United States, downy mildew was primarily a problem only on squashes, watermelon and pumpkin, prior to 2004 with the disease on these cucurbits being effectively managed through application of fungicides. Successful breeding efforts in the 1960's had rendered CDM on cucumber of minor concern requiring very limited application of fungicides due to the availability of resistant cultivars that were widely deployed. However, in 2004, CDM resurged on cucumber overcoming host resistance due to the *dm-1* gene that had been effective for more than 40 years (Criswell et al. 2010). Severe epidemics were reported in the eastern United States in 2004 and 2005, leading to complete crop failure in many fields along the eastern United States (Holmes *et al*. 2006). The disease now occurs annually in the United States and fungicides need to be applied prophylactically to effectively control the disease.

Considerable efforts have been undertaken to establish possible causes of the resurgence of CDM in the United States (Holmes *et al*. 2015; Quesada-Ocampo et al. 2012; Ojiambo *et al*. 2015), in Israel (Cohen *et al*. 2015) and in parts of Europe (Kitner *et al*. 2015; Lebeda *et al*. 2013). It has been hypothesized that the resurgence of CDM in the United States was due to the introduction of a new pathotype or a new cryptic species of the pathogen (Colucci 2008; Runge *et al*. 2011). A new genetic recombinant from sexual reproduction has also been suspected as a possible cause for the resurgence (Cohen & Rubin, 2012; Runge *et al*. 2011). During the past decade, changes in the epidemics of CDM and virulence of the pathogen have been reported around the globe. For example, new pathotypes have been reported in several countries (Cohen *et al.* 2015) and new virulence structure and a change in population dynamics have been reported in the Czech Republic (Lebeda *et al.* 2013, Kitner *et al*. 2015). In addition, both mating types of *P. cubensis* have been observed in Israel, China, the United States and several other countries (Cohen & Rubin, 2012; Cohen *et al*. 2013; Cohen *et al*. 2014; Cohen *et al*. 2015; Thomas *et al.* 2013). The global resurgence of CDM has been reviewed (e.g., Cohen *et al*. 2015; Holmes *et al*. 2015; Ojiambo *et al*. 2015) and understanding the genetic basis for host specialization within *P. cubensis* was identified as an important component in explaining the resurgence of CDM around the globe (Cohen *et al.* 2015).

Unlike most other downy mildew pathogens, *P. cubensis* has a wide host range (Runge *et al*. 2011) and the virulence of the pathogen has been classified into pathotypes based on the differential ability of *P. cubensis* to cause infections on different cucurbit host types. A study examining the physiological specialization of *P. cubensis* using isolates from Japan, Israel and the United States conducted in 1987 identified the existence of five

different pathotypes (Thomas *et al*. 1987) using thirteen differential host types including *Cucumis sativus, C. melo* var. *reticulatus, C. melo* var. *conomon, C. melo* var. *acidulous, Citrullus lanatus, Cucurbita maxima, C. pepo, C. moschata, Benincasa hispida, Luffa acutangula, L. cylindrica, Momordicha charanatia and Lagenaria siceraria*. Isolates capable of infecting *Cucumis sativus* and *C. melo* var. *reticulatus* were classified as pathotype 1, while those capable of infecting *C. melo* var. *conomon* in addition to the former hosts were classified as pathotype 2. Isolates capable of infecting all the *Cucumis* spp. were classified as pathotype 3, while all isolates capable of infecting *C. lanatus* in addition to the *Cucumis* spp. were designated as pathotype 4. All isolates capable of infecting *Cucurbita* spp. in addition to *Cucumis* spp. and *C. lanatus* were classified as pathotype 5. Since then, more pathotypes (up to pathotype 10) have been identified in Asia (Cohen *et al.* 2015). While these studies indicate the presence of a unique level of interaction between *P. cubensis* and cucurbit host type, the genetic basis underlying host specialization within this pathosystem has not been investigated.

Following the resurgence of CDM in 2004, a change in the virulence pattern of *P. cubensis* including increased aggressiveness on cucumber and a widened host range was reported in the United States (Colucci & Holmes 2007). A phylogenetic study conducted in Europe showed that *P. cubensis* is composed of two cryptic species (Runge *et al.* 2011). Clade 1 (cryptic species 1) was shown to be associated with isolates in United States prior to the CDM resurgence and clade 2 (cryptic species 2) was found to be associated with East Asian and European isolates and included one isolate from the United States after the 2004 resurgence. Based on the association of the post-resurgence isolate from the United States with clade 2, it was hypothesized that the resurgence of CDM in United States could have

been due to migration of new strains possibly from East Asia (Runge *et al*. 2011). However, only one isolate from the United States collected after the CDM resurgence was included in the study by Runge *et al*. (2011) and this preclude key insights into population-level processes that may have been initiated and ongoing following the resurgence of CDM in 2004. More recent studies have documented the existence of A1 and A2 mating types of *P. cubensis* around the worldwide (Cohen *et al*. 2015) and in the United States (Thomas *et al*. 2013). In the Czech Republic, the A1 mating type that has a preference to *Cucumis* species was suggested to be associated with the 2009 pre-epidemic isolates, while the A2 that preferred other cucurbit host type was associated with 2009 post-epidemic isolates (Lebeda *et al*. 2014). This study provides some indirect insights into host specialization within *P. cubensis* and its possible association with specific clades of the pathogen.

The evolutionary origin of *P. cubensis* and the relatedness to its sister species, *P. humuli*, has been widely investigated (Choi *et al*. 2005; Göker *et al*. 2009; Kitner, 2015; Mitchell *et al*. 2011; Runge *et al*. 2011; Runge and Thines, 2012). Choi *et al*. (2005) conducted a phylogenetic analysis of *P. cubensis* from cucurbits and *P. humuli* from hops using ITS rDNA region and showed high level of sequence homology between the two species. Based on this observation and the high degree of morphological similarity between the two species, *P. humuli* was reduced to a taxonomic synonym of *P. cubensis*. However, a subsequent study by Sarris *et al*. (2009) based on AFLP fingerprinting and ITS sequence variation in *P. humuli* and a population of *P. cubensis* from *C. sativus* in Europe reported that *P. cubensis* and *P. humuli* formed two distinct clusters. Mitchell *et al*. (2011) examined variation in three nuclear and two mitochondrial loci and reported that *P. humuli* from Europe and the United States formed a distinct clade compared to *P. cubensis* isolated from

herbarium samples from South Korea. In that study (Mitchel *et al*. 2011), one *P. humuli* isolate from Korea clustered with *P. cubensis* isolates from Asia, whereas *P. humuli* isolates from Europe and the United States formed a separate cluster ancestral to *P. cubensis*. Some cohesion between the two species was observed in the cox 2 region of two isolates obtained from *Cucurbita pepo* in North Carolina and a *P. humuli* isolate from South Korea (Mitchel *et al*. 2011), and between *P. humuli* and *P. cubensis* isolates from non-cucumber host types (Kitner *et al*. 2015). Generally, these studies indicate that although *P. cubensis* and *P. humuli* are morphologically and physiologically similar, they exhibit quantifiable physiological and genetic differences that supports that they be retained as two separate species, but more extensive population genetic analysis is needed to determine if these two species are specialized on their respective hosts.

The overall goal of our study was to examine patterns of genome-wide variation underlying host specialization in *P. cubensis* and thus, provide insights into the evolutionary processes that may have driven the resurgence of CDM with increased virulence on cucumber in the United States in 2004. To achieve this goal, we focused this study on three specific objectives: 1) examine genome-wide variation in *P. cubensis* and determine whether it is associated with host specialization, 2) determine whether distinct evolutionary lineages or individual multilocus genotypes are associated with pathotypes and mating types of *P. cubensis*, and 3) investigate the evolutionary relationships between *P. cubensis* and its sister species, *P. humuli.*

Materials and methods

Reference isolates

Nine single lesion isolates from various cucurbit hosts including cucumber, cantaloupe, pumpkin, butternut squash, acorn squash and watermelon obtained from different parts of the United States from 2008 to 2013 were used as reference isolates in this study (Table 4.1). These isolates were maintained in the laboratory by propagating them on their respective host types using the detached leaf assay. Briefly, the abaxial side of detached leaves of 4-week four week old plants were placed on moist paper towels in clear acrylic boxes and spray inoculated with sporangial suspension at a concentration of about 2×10^4 sporangia/ml. Inoculated leaves were incubated in a growth chamber at 21/18°C under 12 h/12 h light/dark cycle and were re-inoculated once sporangia were produced in abundance. The assay was repeated as necessary until the isolates were ready for use in the subsequent experiments. All isolates were characterized with respect to their mating type and pathotype designation.

Mating type determination

The mating type of all the reference isolates was determined by co-inoculating each reference isolate with a known A1 and/ A2 tester strain as described by Cohen and Rubin (2012). The A1 tester strain was originally isolated from cucumber in North Carolina in 2012, while the A2 tester strain was originally isolated from butternut squash in Israel. Equal volumes (1:1) of sporangial suspension (2×10^4 spores/ml) of the tester strain and the reference isolate were mixed together and the mixed suspension was then used to inoculate cantaloupe or cucumber leaves. Cantaloupe and cucumber have been reported as a favorable host substrate that supports maximum production of oospores (Cohen and Rubin, 2012). First leaves of three-week-old cucumber and cantaloupe leaves maintained in the greenhouse were used for inoculations. The abaxial side of detached leaves was placed upside down on moist paper

towels in clear acrylic boxes and the adaxial side was spot inoculated on at least 20 different spots with 10 µl of the sporangial mixture. Care was taken to dispense the inoculum suspension at vein junctions especially for cucumber to ensure maximum infection. Inoculated leaves were incubated in a growth chamber at 21°C at 50 to 60% relative humidity under 12 h/12 h light/dark cycle. At 7 to 10 days post inoculation, leaf discs measuring 11 mm diameter were randomly cut from infected leaves and clarified for 24 h in ethyl alcohol-acetic acid solution (3:1). Clarified leaf discs were washed in three changes of de-ionized water and examined for the presence of oospores using a compound microscope (×100 magnification). If oospores were produced when co-inoculated with an A1 or A2 tester strain, then the unknown isolate was considered to be of the A2 or A1 mating type, respectively.

Pathotype designation

An internationally accepted host differential set (Lebeda & Widrlechner, 2003) that includes *Cucumis sativus, C. melo reticulatus, C. melo acidulous, C. melo conomon, Citrullus lanatus, Cucurbita moschata*, *C. pepo, Luffa cylindrica*, *Lagenaria siceraria, Benincasa hispida* and *Momardicha charantia*, was used to determine the pathotype of all the reference isolates. Poinsett 76, a cucumber cultivar with *dm-1* gene was also included as control to determine reference isolates that were capable of causing disease in previously resistant cucumber cultivars. The compatibility of the reference isolates with the host differential set was determined using the detached leaf assay. First and second primary leaves from four-week-old host differentials placed with the adaxial side on moist paper towels in clear acrylic boxes were spray inoculated with sporangial suspension at a concentration of 5×10^3 sporangia/ml.

Inoculated leaves were then incubated in a growth chamber at 21/18°C under 12 h/12 h light/dark cycle. Disease severity was recorded on a per leaf basis by assessing the percentage of leaf area infected 5 to 7 days after inoculation. Two leaf discs measuring 9 mm in diameter were cut from each leaves at 7 DPI and shaken well in Eppendorf tubes with 500 µl of 50% ethanol to dislodge the sporangia (Cohen *et al*. 2003). Sporangial count was then determined using a hemocytometer and sporangia production was expressed as spores per cm^2 leaf area. A spore concentration of about 5×10^3 spores/cm^2 and a disease severity rating of ≥50 % was considered as compatible (+) reaction and an absence of sporulation or very low sporulation (~ 3×10^3 spores/ml) was considered an incompatible (–) reaction (Thomas *et al*. 1987, Cohen *et al*. 2003; Lebeda & Gadasovà, 2002).

DNA extraction and sequencing

After 7 days post inoculation, leaves inoculated with individual reference isolates were rinsed with sterile distilled water using a Preval sprayer (Complete Unit 267; Precision Valve Corporation, Yonkers, NY). The sporangial suspension was then filtered using sterile mira cloth to remove any plant material. The sporangial suspension was then centrifuged and subjected to DNA isolation using Qiagen DNeasy Plant Mini Kit (Qiagen Corporation, Maryland, USA). Total genomic DNA was quantified using Qubit 2.0 fluorometer (Life Technologies, Grand Island, NY). The DNA quality and integrity of each sample was further checked by amplifying and sequencing β-tubulin, which harbors variation that is species specific (Quesada-Ocambo *et al.* 2012). The sequenced region was searched against the NCBI database using BLASTn and the identity of each isolate was confirmed as *P. cubensis*. At least 1 µg of DNA from each sample was submitted to the Genomic Sciences Laboratory

at North Carolina State University for sequencing, using 150 bp paired-end sequencing on the Illumina HiSeq platform. All nine samples for the reference isolates were multiplexed with nine samples per lane. The watermelon isolate, 2013E1, was also re-sequenced using 300 bp paired-end MiSeq due to low read coverage in the first run. A draft genome assembly of *P. humuli* (Ph) was obtained (Lina M. Quesada-Ocampo).

Comparative whole genome sequence analysis

A publicly available draft genome assembly of *P. cubensis*, MSU-1 (67.9 Mb) originally isolated from cucumber, was used for reference-guided comparative genome analyses. A complete mitochondrial genome assembly (38,553 bp) of *P. cubensis* published recently (Lu *et al*. 2015) was used as the reference for aligning mitochondrial reads generated from our samples.

We developed a pipeline in Mobyle SNAP Workbench (Price & Carbone, 2005; Aylor *et al*. 2006; Monacell & Carbone 2014) for read filtering and genome assembly (Fig. 4.1) of our reference isolates. Raw sequence reads were error-corrected using Quake (Kelley *et al*. 2010) and filtered based on a Phred-quality score >20 using the FASTX-Toolkit (Gordon & Hannon 2010). Filtered read pairs were interleaved into a single FASTQ file using the shuffleSequences.pl script in Velvet version 1.2.10 (Zerbino & Birney 2008). We performed de novo genome assembly using Velvet for a range of kmer values. Redundancies in assembled genomes was reduced by retaining the longest sequence in a cluster of sequences sharing at least 95% sequence similarity using CD-HIT (Li & Godzik 2006). The assembled genome files in FASTA format were converted into BLAST database files using the makedb XML in SNAP Workbench to facilitate genome retrieval by downstream

programs such as BWA. Corrected and filtered reads generated were aligned to the reference genome (MSU-1) (Savory *et al*. 2012) and the reference mitochondrial genome (Lu *et al*. 2015) using BWA (Li & Durbin 2009). Sequence alignment (SAM) files were assembled into cohorts and genotyped using the UnifiedGenotyper variant discovery pipeline in GATK v2.4-9 (DePristo *et al*. 2011). The Variant Call Format (VCF) file output from GATK was then used to conduct population genomic analysis using PLINK (Purcell *et al*. 2007), VCFTOOLS (DePristo *et al*. 2011) and HAPLOVIEW using solid spine algorithm (Barret *et al*. 2004), and to conduct phylogenetic analysis using RAxML (Stamatakis, 2006). Population genomic analyses were based on the ten longest contigs ranging in size from 59 kbp to 106 kbp. Estimates of Tajima's D statistic, allele frequency, linkage disequilibrium (LD), heterozygosity and F_{ST} were based on a sliding window analysis across the contigs. The ancestral recombination graph (ARG) was reconstructed using the branch and bound method implemented in Beagle (Lyngsø *et al*. 2005) and was based on a window of 100 SNPs from a contiguous stretch of DNA on each contig. If there were too many recombination events for branch and bound to go to completion, a heuristic implementation of the Beagle algorithm, KWARG (http://www.stats.ox.ac.uk/~lyngsoe/section26/) was used. Ancestral recombination graphs capture both mutation and recombination in the ancestral history of the sample and provide insights into the putative parents of recombinant haplotypes (Carbone *et al*. 2007; Moore *et al*. 2009).

Coalescent analysis

The ancestral history of *P. cubensis* and *P. humuli* was inferred using Genetree (Griffiths & Tavaré 1994) implemented in Mobyle SNAP workbench. Coalescent analysis was used to

infer rooted gene genealogies showing the relative ages of mutations, haplotypes and clades. Genealogies for segments of the mitochondrial and nuclear genomes were inferred separately. Specifically, nuclear gene genealogies were inferred for each of the ten longest contigs using windows of ~100 SNPs from the start, middle region and end of each contig. All SNPs from the mitochondrial genome (~38 kbp) were used for genealogical analysis. Compatibility among pairs of SNPs was used to determine the largest non-recombining partition of compatible SNPs for each contig using the CladeEx program (Bowden *et al*. 2008). The population mean mutation rate was based on Watterson's θ estimator calculated for each analysis window (Griffiths & Tavaré 1994). The relative probabilities of all rooted genealogies were calculated by performing 1×10^6 simulations of the coalescent assuming panmixis and constant population size, tested a priori using the Tajima's D statistic. Three independent coalescent runs with different starting random number seeds were performed to select the best-rooted gene genealogies for inference of ancestral relationships.

Results

Mating type determination

Out of the nine reference isolates tested, four isolates produced oospores when they were crossed with a known A2 mating type tester isolate and were assigned to the A1 mating type. The remaining five isolates produced oospores only when they were paired with the A1 tester isolate and were assigned to the A2 mating type. Two isolates collected from cucumber (A11 and 08A1), and a single isolate from cantaloupe (2013B17) and pumpkin (2013C) belonged to A1 mating type. Two isolates from butternut squash (D3 and 2013D6), two from acorn

squash (08F1 and 2013F2) and one isolate collected from watermelon (2013E1) were found to belong to the A2 mating type (Table 4.1).

Pathotype designation

All the isolates collected on cucumber, cantaloupe and giant pumpkin were compatible with *Cucumis* spp., *C. maxima* and *L. siceraia* but incompatible with *C. pepo, C. moschata, C. lanatus* or any other host types. Isolate A11 collected from cucumber and 2013F2 collected from acorn squash were found to be compatible with *B. hispida*. Isolate 2013E1 collected on watermelon was compatible with *Cucumis* spp., *C. lanatus* and *C. maxima* but incompatible with any other host types tested, while all isolates collected from squashes were found to be compatible with all *Cucurbita* spp., *Cucumis* spp. and *C. lanatus*. Based on the pattern of compatibility and incompatibility with the host differential set, 2013B17, 2013C3 were designated as pathotype 1, while A11 and 08A1 were designated as pathotype 3 (Table 4.1). Isolate 2013E1 and 08F1 were classified as pathotype 4 and 6, respectively, while the remaining isolates belonged to pathotype 5 (Table 4.1).

Genome assembly

Among the nine reference isolates used in this study, the best genome assembly was for isolate 2013B17 collected from cantaloupe (Table 4.2). Genome assembly for 2013B17 comprised 178,237 contigs, an N50 length of 11.9 kbp and an estimated assembly size of 38.5 Mb. The longest contig for 2013B17 was 159.7 kbp with the ten largest contigs spanning 3.0% of the entire genome. Three out of the nine reference isolates had a low read coverage (<10X); read coverage was higher (>20X) and relatively even across the other

isolates with the highest coverage (26X) for isolate 2013E1 collected from watermelon (Table 4.2)

Population genomics

Although our sample includes only nine isolates of *P. cubensis* much of the molecular sequence variation we examined spans the population-species interface. Computer simulation studies have shown that even a small sample of eight isolates yield accurate estimates of population parameters and processes, such as theta and recombination within sequences, provided that more variable sites are examined and longer sequences are examined when recombination is present (Felsenstein 2006; Pluzhnikov & Donnelly 1996). Recognizing that our sample size is close to this optimal number we examined many linked and unlinked loci for inferences of population processes and parameter estimation. Population genomic analyses were based on 3,356 single nucleotide polymorphisms (SNPs) spanning the ten largest contigs across the nine sequenced genomes. Population parameter estimates were based on a 1 kbp sliding window analysis. Tajima's D value was zero for windows spanning many genomic regions, which is consistent with neutrality. However, there was signature of population size expansion or positive selection (Tajima's $D > 0$) in some regions (Fig. 4.2; Fig. S4.1-4.8). Plots of r^2 revealed distinct LD blocks in 7 out of the 10 contigs, which suggests recent genetic exchange and recombination in *P. cubensis* (Fig. 4.3; Fig. S4.9-4.17). Approximately 37% of the SNPs were heterozygous across both lineages, based on evidence from paired-end reads; among lineage II isolates, 93% of the SNPs were heterozygous.

The ARG indicated extensive recombination among inferred single-locus haplotypes of *P. cubensis* and *P. humuli*. There was evidence of recombination among *P. cubensis* lineage I haplotypes. However, mutation-alone separated haplotypes comprising lineage II, as was observed for 27 out of 29 regions examined. Four out of the ten contigs have provided evidence for a hybrid origin of lineage II. For example, the ARG inferred for a DNA segment on contig 7 indicates that one or more crossovers involving haplotypes H2, H8 and H10 followed by mutation has given rise to the clade that comprises haplotypes H12 and H1 (Fig. 4.10). One putative parental haplotype H2 belongs to *P. cubensis* lineage I, while the other putative parental haplotype H10 represents *P. humuli* and the recombinant haplotypes H12 and H1 comprise *P. cubensis* lineage II isolates. An alternative, but less parsimonious parental haplotype is H8 from *P. cubensis* lineage I; the path from haplotype H8 to the hybrid haplotypes H12 and H1 requires three recombination events instead of one from *P. humuli* haplotype H10 (Fig. 4.10)

Phylogenetic analysis

The mitochondrial phylogeny based on 392 SNPs showed that lineage I of *P. cubensis* shared a recent common ancestor with *P. humuli* (Fig. 4.4). Nuclear phylogenetic analyses based on 3356 SNPs provided evidence of two distinct evolutionary lineages that are associated with specific hosts, mating types and pathotypes (Fig. 4.5; Fig. S4.18-4.26). Lineage II isolates were found to be associated with cucumber, cantaloupe and pumpkin while lineage I isolates were found to be associated with squash and watermelon. Lineages were also found to be associated with mating types and pathotypes. All lineage II isolates belonged to the A1 mating type and pathotypes 1 or 3, while all lineage I isolates belonged to the A2 mating type

and pathotypes 4, 5 or 6. Allelic variation was low ($\pi = 0.005$) among lineage II isolates and high ($\pi = 0.059$) in lineage I.

Coalescent analysis

Mitochondrial gene genealogies were based on 336 SNPs and eleven haplotypes that spanned the largest non-recombining block in the mitochondrial genome of *P. cubensis* and *P. humuli*. Coalescent analysis revealed three distinct evolutionary lineages: *P. cubensis* lineage I, *P. cubensis* lineage II, and *P. humuli* (Fig. 4.6). Although the three lineages are separated by many fixed polymorphisms, lineage I of *P. cubensis* shared a more recent common ancestor with *P. humuli* rather than with its conspecific lineage II. A total of thirty gene genealogies were inferred for the ten longest nuclear contigs. All six gene genealogies inferred for contigs 1 and 2 showed that lineages I and II of *P. cubensis* shared a more recent common ancestor that was distinct from *P. humuli* (Fig. 4.7; Fig. S4.27). This topology was supported in the majority (20/30) of nuclear gene genealogies. In some regions (on contig 3, 4, 6, 9 and 10) mutation rates were low ($\pi = 0.04$) and *P. cubensis* lineages were indiscernible (Fig 4.8; Fig. S4.28), while in other regions, mutation rates were higher ($\pi = 0.27$) and there was evidence of recombination (shared haplotypes) between the two *P. cubensis* lineages (Fig. S4.29). Concordance between the nuclear and mitochondrial gene genealogies was observed for two regions on contigs 3 and 8 (Fig 4.9; Fig. S4.30).

Discussion

Genome-wide variation in *P. cubensis* has provided evidence for the existence of two distinct evolutionary lineages specialized on different cucurbit host types. Lineage I was

associated mainly with squash and watermelon, while ineage II isolates were associated mainly with *Cucumis* spp. Pairing assays of the reference isolates with mating type tester strains showed that all lineage I isolates belonged to mating type A2, while all lineage II isolates belonged to mating type A1. Moreover, lineage I was comprised mainly of isolates belonging to pathotype 4, 5 and 6 whereas lineage II comprised isolates belonging to pathotypes 1and 3. Although evolutionarily distinct, lineages I and II show a history of genetic exchange and recombination with a closely related sibling species, *P. humuli.* Specifically, the evidence of recombination between lineage I of *P. cubensis* and *P. humuli* was supported by a shared common ancestor in mitochondrial gene genealogy and the inference of recombinant haplotypes in nuclear ARGs. This knowledge coupled with the apparent specialization of lineages with host and mating type has implications in the epidemiology and management of CDM.

Previous studies (Kitner *et al*. 2015; Runge *et al*. 2011) reported the existence of two clades within *P. cubensis.* Multilocus phylogenetic analysis using *cox2*, *ypt1* and nrITS sequence data provided evidence of two clades within *P. cubensis* (Runge *et al*. 2011). The two distinct evolutionary lineages identified in the current study, lineage I and II were found to be analogous to clade 1 and 2 (Runge *et al*. 2011), respectively based on a phylogenetic analaysis using sequence data obtained from the NCBI database. In a study using isolates sampled in the Czech Republic before and after 2009, Kitner *et al*. (2015) showed evidence of temporal clustering and hinted to a possible association of clades with different cucurbit host types. A recent study on the genetic variation between *P. cubensis* and *P. humuli* in the United States used principal component analysis and separated *P. cubensis* isolates into two groups, with one group being composed of isolates from *Cucumis* spp., and the other group

comprised of isolates from *Cucurbita* spp. (Summers *et al*. 2015). In the present study, lineage II isolates were also composed primarily of isolates from *Cucumis* spp., while lineage I was composed of isolates from *Cucurbita* spp. and *Citrullus lanatus*. These results are also similar to those reported in the study by Lebeda *et al*. (2014) in which one clade was composed of isolates primarily from *Cucumis* spp., and the other clade was comprised of isolates from a broader host range. The mechanism controlling host preference in *P. cubensis* is still unknown and understanding how these lineages are associated with different host types may provide insights into the potential mechanism(s) that control host preference within the *P. cubensis*-cucurbit pathosystem.

In the present study, phylogenetic analysis showed low allelic variation among lineage II isolates as compared to lineage I isolates. The lower genetic diversity in lineage II that comprised of isolates collected from different hosts across a wide geographic area, is indicative of a recently introduced population, bottleneck or selective sweep within *P. cubensis*. Lineage I comprising isolates belonging to pathotypes 4, 5 and 6, showed higher diversity and pathotype 4 and 5 isolates have previously been in the United States (Thomas *et al*. 1987) prior to the resurgence of CDM in 2004. In the United States, CDM epidemics have always been reported on squashes, pumpkin and watermelon to 2004 (Holmes *et al*. 2006). However, disease outbreaks on cucumber prior to 2004 were very minimal and required little to no fungicide application for disease control. The latter was largely due to the *dm-1* host resistance gene that had been widely deployed in cucumber since 1960s (Criswell et al. 2010). In present study, only isolates within lineage II were highly compatible with Poinsett 76, a cucumber cultivar that has *dm-1* gene. Isolates specialized on *Cucumis* spp. that belong to pathotypes 1 and 3 were previously known to exist only in Asia (Thomas *et al*. 1987). The

low allelic variation coupled with the ability to infect cucumber with the *dm-1* gene suggests that isolates within lineage II may have been responsible for the resurgence of CDM in the United States.

A resurgence of CDM in Europe on cucumber was observed during the second half of 1980s and pathotypes compatible with *Cucurbita* spp. were later reported in Europe from 2003 (Capelli et al. 2003, Lebeda 2012). The resurgence of CDM in Europe was postulated to be mediated through anthropogenic means and that could have led to the migration of lineage II of *P. cubensis* from East Asia to Europe and from Europe to the United States (Runge *et al*. 2011). It plausible that the migration of lineage II from East Asia to Europe and the United States was potentially mediated by the exchange of seed materials as part of a global effort to manage CDM through resistance breeding when it first appeared in Europe (Shetty *et al.* 2002). In a recent study, *P. cubensis* was shown to be seed-borne nature (Cohen *et al*. 2014). Hence, it is possible that seeds harboring the pathogen may have inadvertently introduced isolates within lineage II from East Asia to Europe and the United States. A more detailed population genetic study employing robust SNP markers and a diverse set of isolates sampled from different host types around the world should provide a better understanding of the population structure and migratory pathways of *P. cubensis* on a global scale.

Previous studies (Cohen and Rubin, 2011) have suggested an association between cucurbit host types and mating types of *P. cubensis*. There have also been reports of both mating types being able to infect the same host species suggesting that mating type and virulence might be genetically unlinked (Cohen *et al*. 2015; Falach 2014). In the present study, all lineage II isolates were of the A1 mating type, while all lineage I isolates were of the A2 mating type. However, the association of lineages and cucurbit host type was not very

specific, as both lineage I and lineage II isolates were able to cause infection on susceptible varieties of cucumber, cantaloupe and giant pumpkin. This suggests that the association between host types and mating types may be a reflection of the differences in the lineages of *P. cubensis* rather than differences mediated through host types. In the United States, the distinctiveness between lineages of *P. cubensis* is more evident probably due to a reproductive isolation owing to the absence of lineage II isolates of A1 mating types prior to the resurgence of CDM in 2004. Both mating types of *P. cubensis* were reported recently in the United States (Thomas *et al*. 2013) with a distinct association between mating types and lineages. The distinctiveness between lineages of *P. cubensis* may diminish if recombination becomes more widespread and a process that can result in the formation of hypervirulent strains with a wider host range (Runge *et al*. 2011). Although both mating have been reported in the United States, the extent of sexual recombination among *P. cubensis* field populations is unknown. Coalescent analysis based on SNP variation in the nuclear genome indicated genetic mixing between lineage I and II of *P. cubensis*. This was also evident from LD plots generated for the ten longest contigs. Distinct LD blocks indicative of genetic recombination were present in seven out of ten contigs. Gene genealogies inferred for separate contigs were incongruent which further supports a history of genetic exchange. Recently, Kitner *et al*. (2015) reported that over 60% of *P. cubensis* isolates collected in Czech Republic after 2009 had two heterozygous positions in the nrITS region irrespective of the host of origin which they postulated as an evidence for sexual recombination.

Coalescent-based analysis of SNPs from mitochondrial genomes revealed a close affinity between *P. humuli* and lineage I of *P. cubensis*. With the exception of a few nuclear regions, which supported this grouping, the majority of nuclear gene genealogies examined

supported the monophyly of the two species. One possibility is that *P. humuli* and lineage I of *P. cubensis* initially diverged from a common ancestor (nuclear gene genealogies), and thereafter evolved host specificity on a more recent time scale (mitochondrial gene genealogies), while retaining the ancestral signature of speciation in some genomic regions. Although ancestral recombination graph reconstruction indicated a hybrid origin of *P. cubensis* lineage II isolates with lineage I of *P. cubensis* and *P. humuli* as putative parents, the potential for interspecific hybridization has not yet been investigated. It will be interesting to sample *P. humuli* and *P. cubensis* where both species coexist and examine if there is evidence of ongoing genetic exchange between the species, as has been hypothesized in other studies (Choi *et al*. 2005; Runge & Thines, 2012; Kitner *et al*. 2015). Previous studies have identified two wild cucurbit relatives, *Bryonia dioica* and *Sicyos angulatus* as hosts compatible with both *P. humuli* and *P. cubensis* (Runge & Thines, 2012). Susceptible varieties of hops in the United States have also been shown to be infected by *P. cubensis* (Mitchell *et al*. 2012). There could be more undocumented host types that are compatible with both the *Pseudoperonospora* species and a co-infection of such hosts may facilitate gene flow between the two species. Given the findings from the current study, it is possible that hybridization may have initiated and accelerated the process of host specialization in *P. cubensis*.

Acknowledgements

This study was supported by Hatch Funds from North Carolina Agricultural Experiment Station for Project Number NC02251 and NC02432. We thank Taylor Ghiloni and Barbara Stevens for assistance with laboratory and greenhouse aspects of this work. Kisurb Choe was

supported by the National Science Foundation's Dimensions of Biodiversity (DoB) program, DEB-1046167, to I. Carbone. This project was also supported by the Agriculture and Food Research Initiative Competitive Grants Program grant no. 2013-68004-20359 from the USDA National Institute of Food and Agriculture (NIFA). Computational work was supported in part by the University of North Carolina General Administration under an award for High Performance Computing (HPC) and Computational Sciences. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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Table 4.1. Source, description and characterization of reference isolates of *Pseudoperonospora cubensis* collected in the eastern United States

Isolate ID	Original host	Year	State	County	Mating type	Pathotype
A11	Cucumber	2012	NC	Johnston	A1	3
08A1	Cucumber	2008	CA	Salinas	A1	3
2013B17	Cantaloupe	2013	NY	Ontario	A1	1
2013C3	Giant Pumpkin	2013	NC	Johnston	A1	1
D3	Butternut Squash	2012	SC	Charleston	A2	5
2013D6	Butternut squash	2013	AL	Escambia	A2	5
2013E1	Watermelon	2013	FL	Collier	A2	4
08F1	Acorn squash	2008	GA	Tift	A2	6
2013F2	Acorn squash	2013	SC	Charleston	A2	5
Ph†	Hop	2013	OR	Marion	-	-

†Refers to *Pseudoperonospora humuli*.

Table 4.2. Assembly statistics for nine sequenced genomes of *Pseudoperonospora cubensis* showing median contig length (N50), maximum contig length, assembly size, total no. of contigs, estimated coverage, and the number of reads used for the assembly.

Isolate ID	Coverage	Assembly size(Mbp)	No. of contigs	Contig N50 (kbp)	Maximum contig length (kbp)	Number of reads used
08A1	9.8X	50.8	264,193	8.1	249.9	20,060,430
2013B17	21.6X	38.5	178,237	11.9	159.7	25,995,955
D3	20.3X	36.6	308,113	10.9	359.3	15,718,982
08F1	5.7X	62.5	456,970	2.1	70.1	15,000,834
2013D6	22.1X	33.9	252,196	9.9	105.7	17,380,705
2013C3	3.6X	126.7	541,035	0.7	62.9	23,922,561
A11	21.8X	33.5	268,921	8.6	86.4	16,939,920
2013F2	22.4X	35.7	201,678	11.7	126.2	21,952,175
2013E1	26.4X	34.8	261,288	9.3	110.7	17,160,523

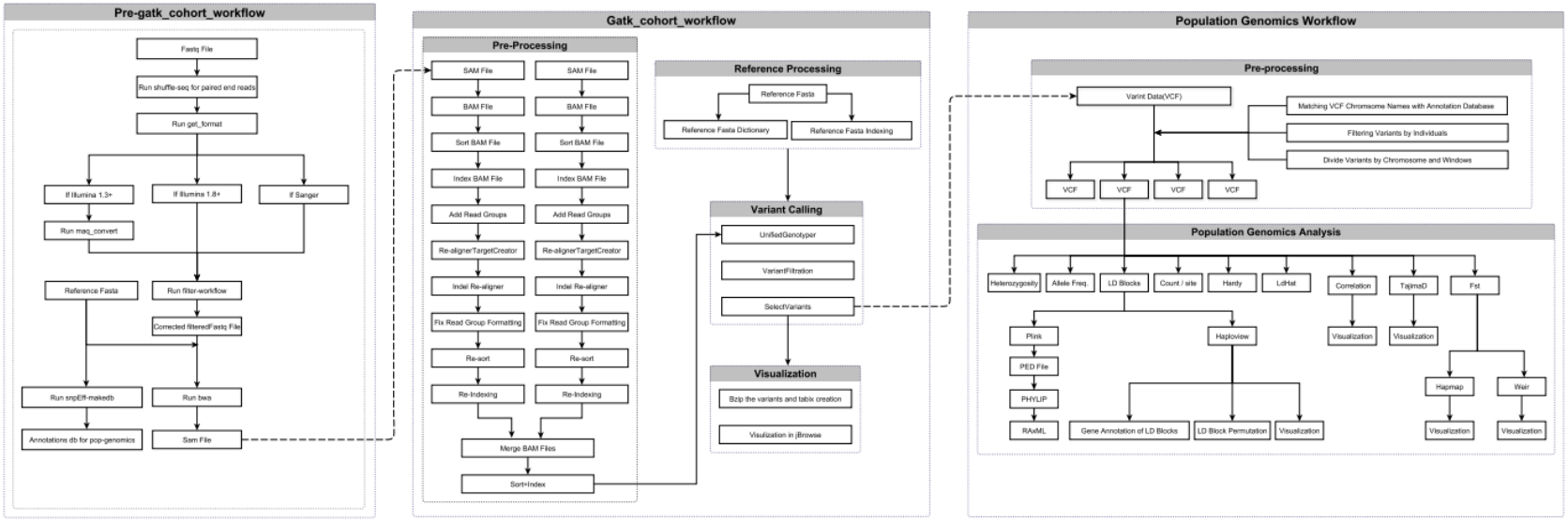

Figure 4.1. A schematic description of the pipeline developed in Mobyle SNAP workbench used for read processing, genome assembly and population genomics of *Pseudoperonospora cubensis* isolates.

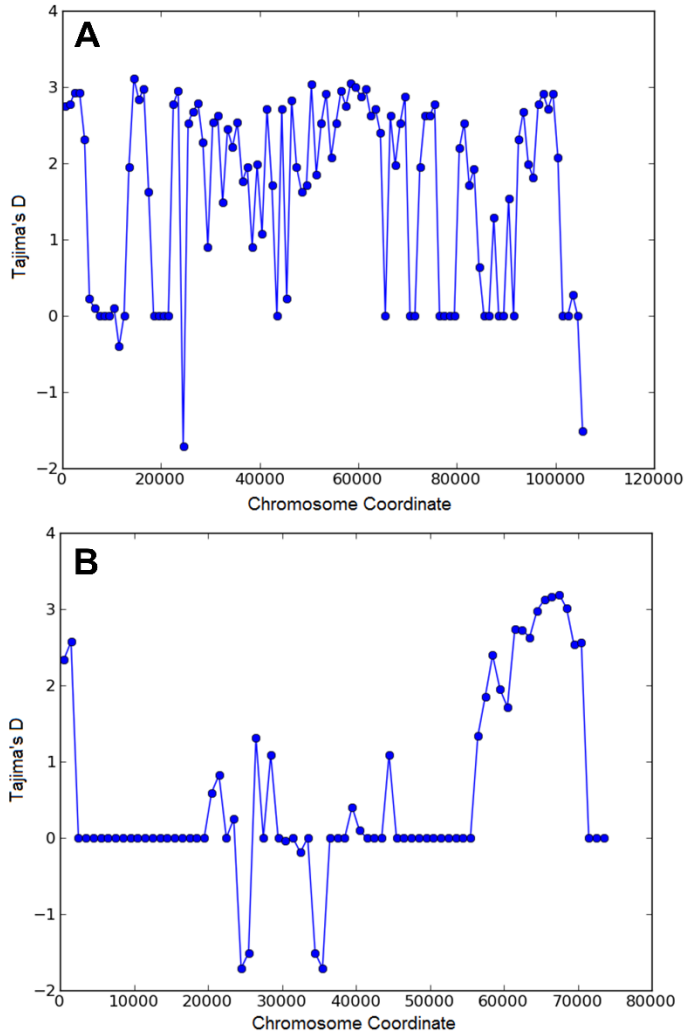

Figure 4.2. Average values of Tajima's D estimated for every 1 kbp across contigs; A) Contig 1 of size 106.0 kbp with 680 SNPs; B) Contig 3 of size 75.3 kbp with 256 SNPs.

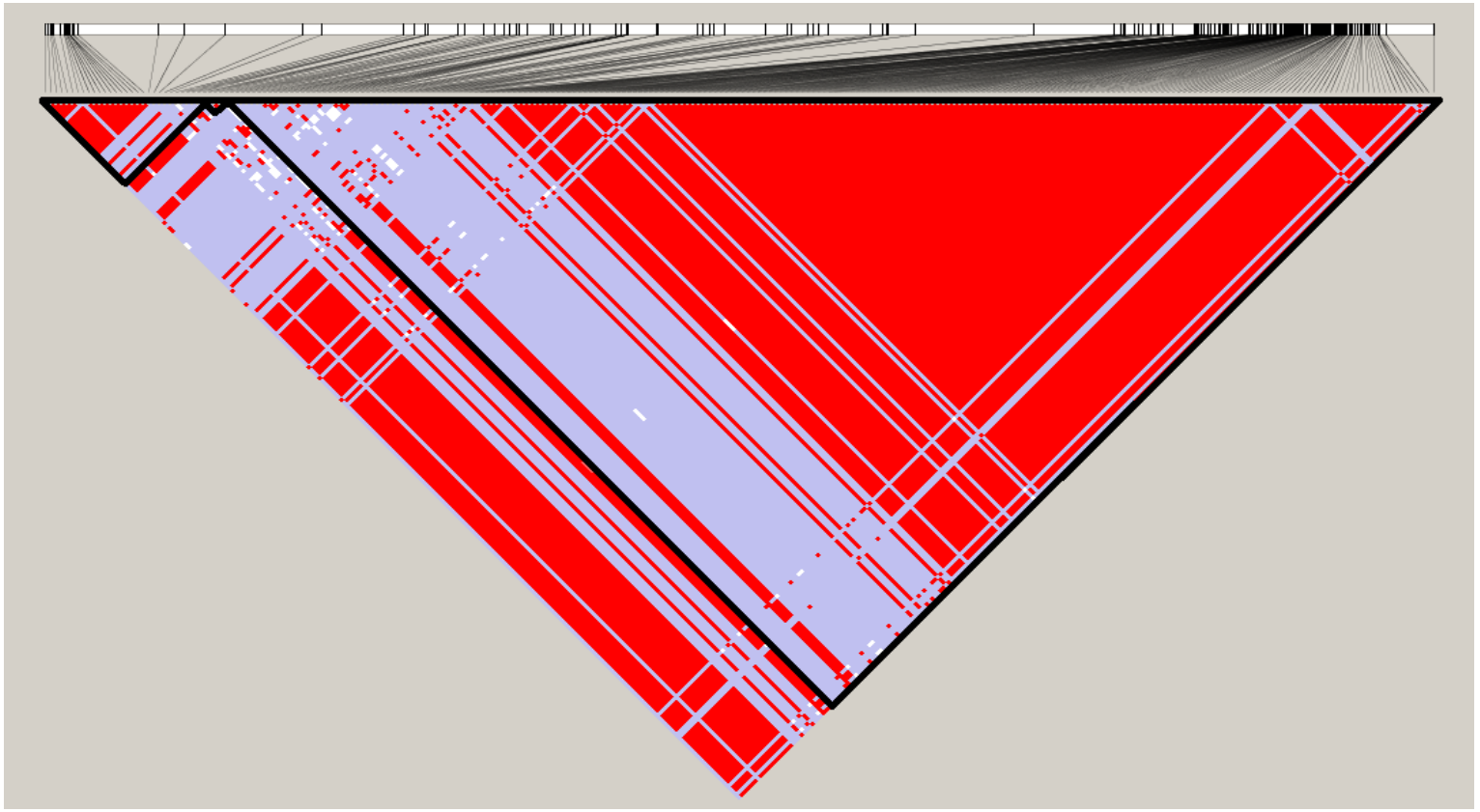

Figure 4.3. Linkage disequilibrium (LD) plot for contig 3 (73.4 kbp; 256 SNPs) generated using solid spine algorithm showing at least two distinct LD blocks. Red indicates a coefficient of determination, $R^2 = 1$ and grey indicates $R^2 < 1$ among sites.

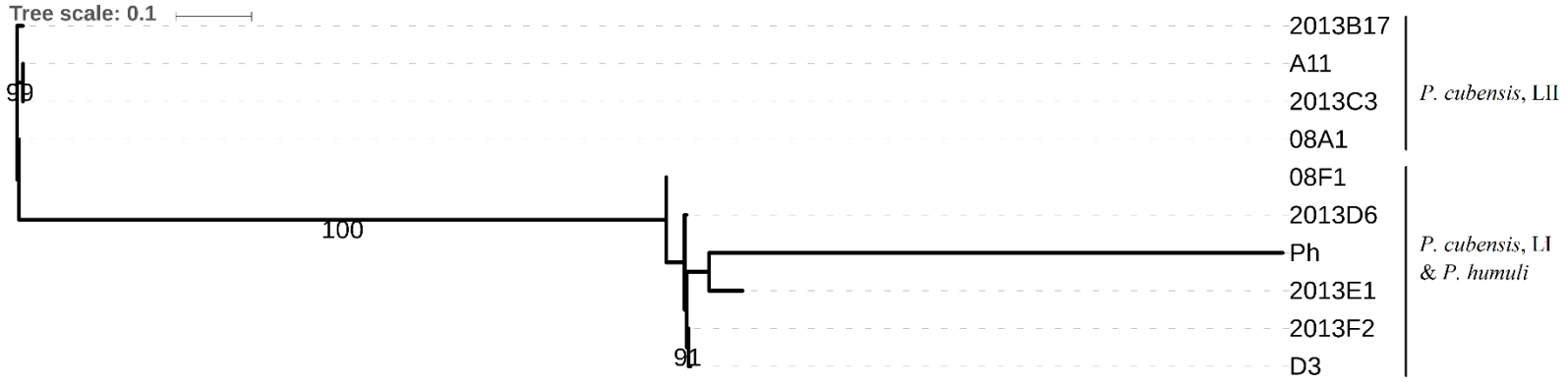

Figure 4.4. Best maximum likelihood phylogenetic tree based on variation in the mitochondrial genome (38.5 kbp, total size; 392 SNPs) for nine isolates of *Pseudoperonospora cubensis* and one isolate of *P. humuli*. *P. cubensis* lineages I and II including *P. humuli* are indicated.

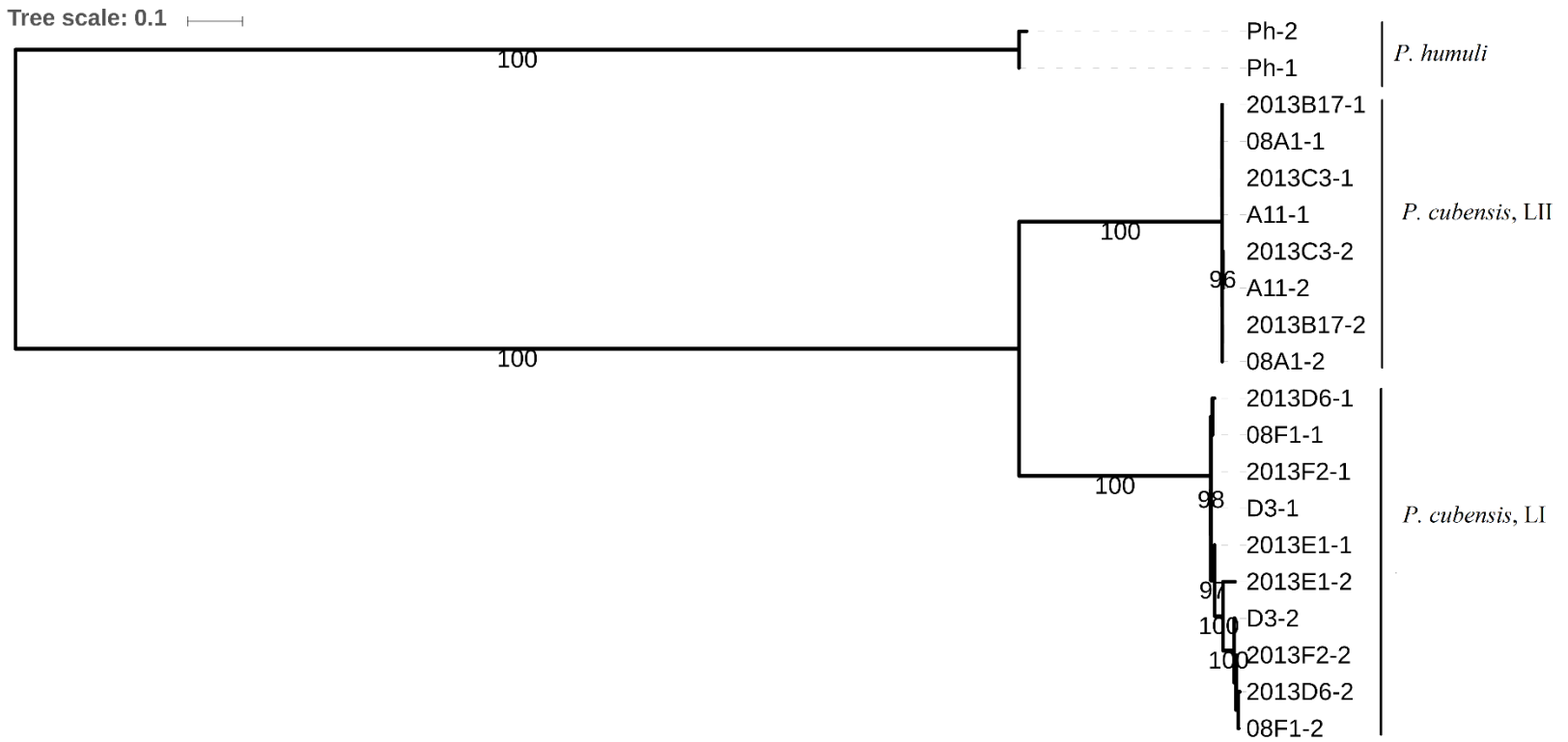

Figure 4.5. Best maximum likelihood phylogenetic tree based on nuclear allelic variation in contig 1 (106 kbp; 680 SNPs) for nine isolates of *Pseudoperonospora cubensis* rooted with *P. humuli*. *P. cubensis* lineages I and II are indicated.

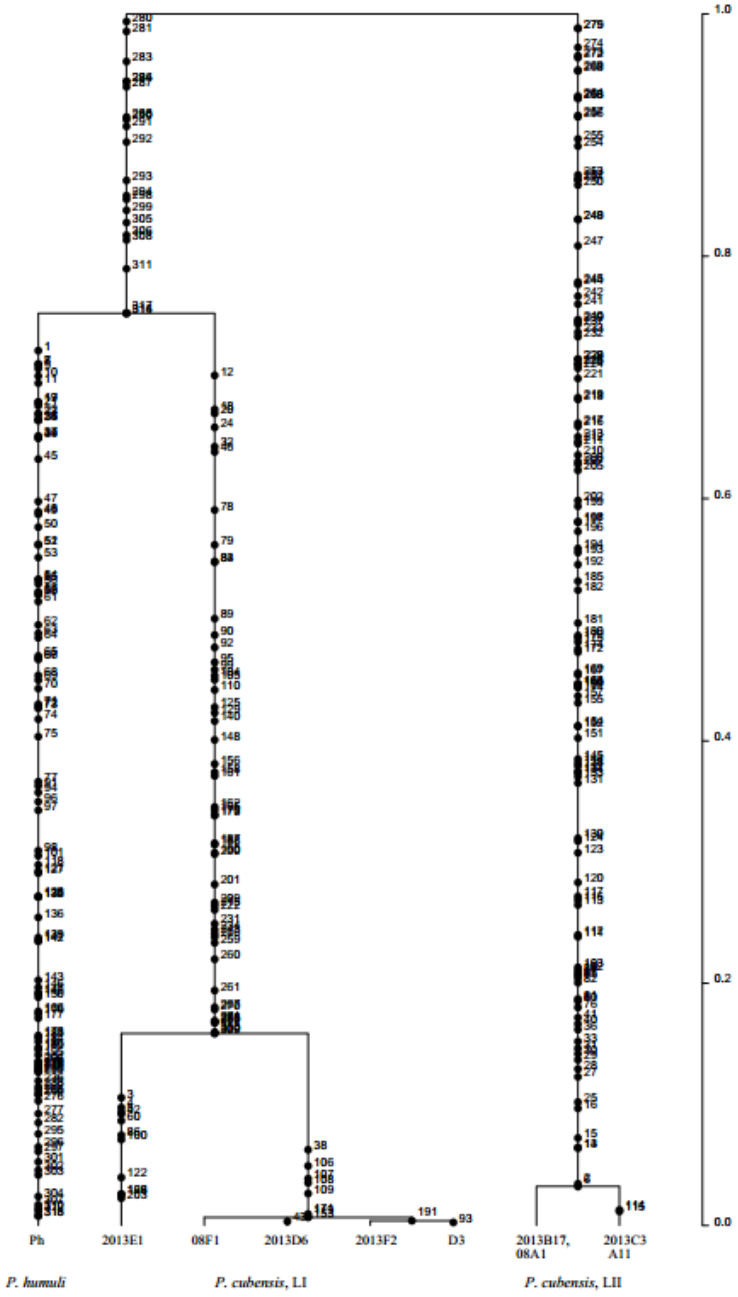

Figure 4.6. Rooted mitochondrial gene genealogy showing the distributions of mutations of *Pseudoperonospora cubensis* and *P. humuli* in the entire mitochondrial genome (38 kb, 366 SNPs). The analysis was run three independent times with a total of 1×10^6 coalescent simulations. There are three distinct evolutionary lineages: *P. cubensis* lineage I (LI), *P. cubensis* lineage II (LII) and *P. humuli*. *P. cubensis* lineage I and *P. humuli* shared a common ancestor in the past. The time scale is in coalescent units and the direction of divergence is from top (past) to the bottom (present).

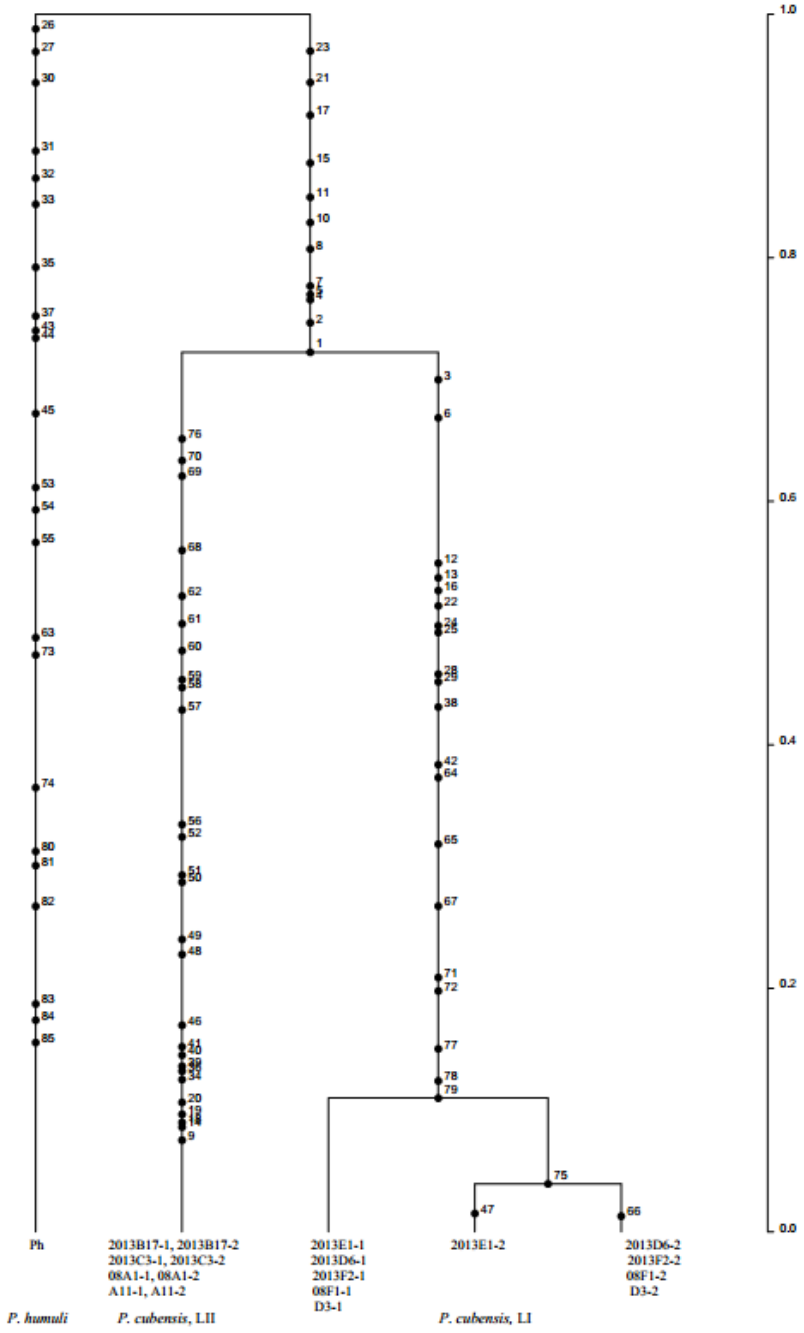

Figure 4.7. Rooted nuclear genealogy showing the distribution of mutations of *Pseudoperonospora cubensis* and *P. humuli* using 100 contiguous SNPs from one end of contig 1. The analysis was performed with a total of 1×10^6 coalescent simulations and the best tree was based on three independent runs simulations. *P. cubensis* lineages I and II have diverged from a common ancestor, which coincides with the speciation event separating *P. humuli* and *P. cubensis*. The time scale is in coalescent units and the direction of divergence is from top (past) to the bottom (present).

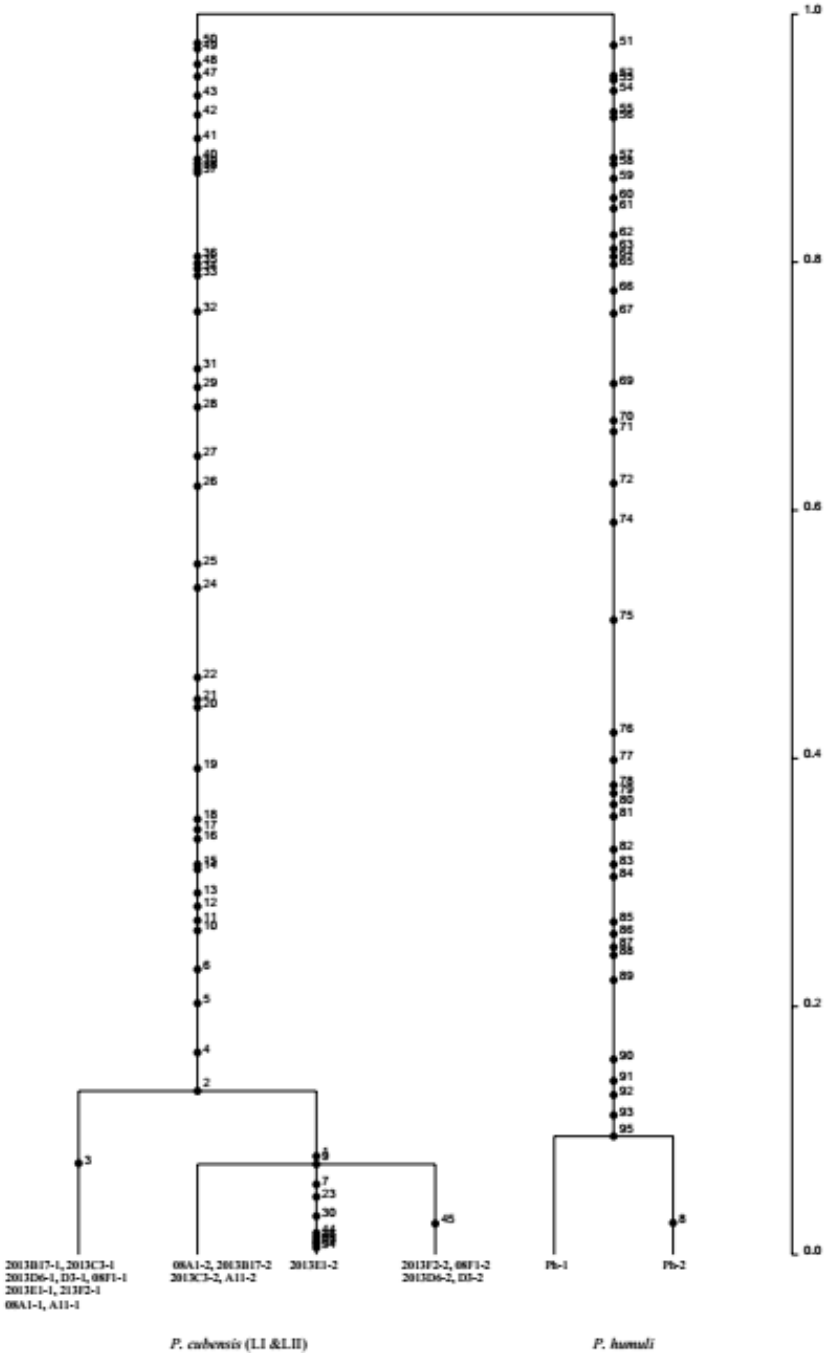

Figure 4.8. Rooted nuclear gene genealogy showing the distribution of mutations of *Pseudoperonospora cubensis* and *P. humuli* using 100 contiguous SNPs from one end of contig 4. The analysis was run with a total of 1×10^6 coalescent simulations and the best tree was based on three independent runs. The genealogy clearly separates the speciation event between *P. humuli* and *P. cubensis* (many fixed polymorphisms) in the distant past (top) from population divergence events (few polymorphisms) within each species on a more recent time scale (bottom). Alleles of *P. cubensis* lineages I and II are shared between isolates and the distinction between the lineages is not apparent at this part of the nuclear genome.

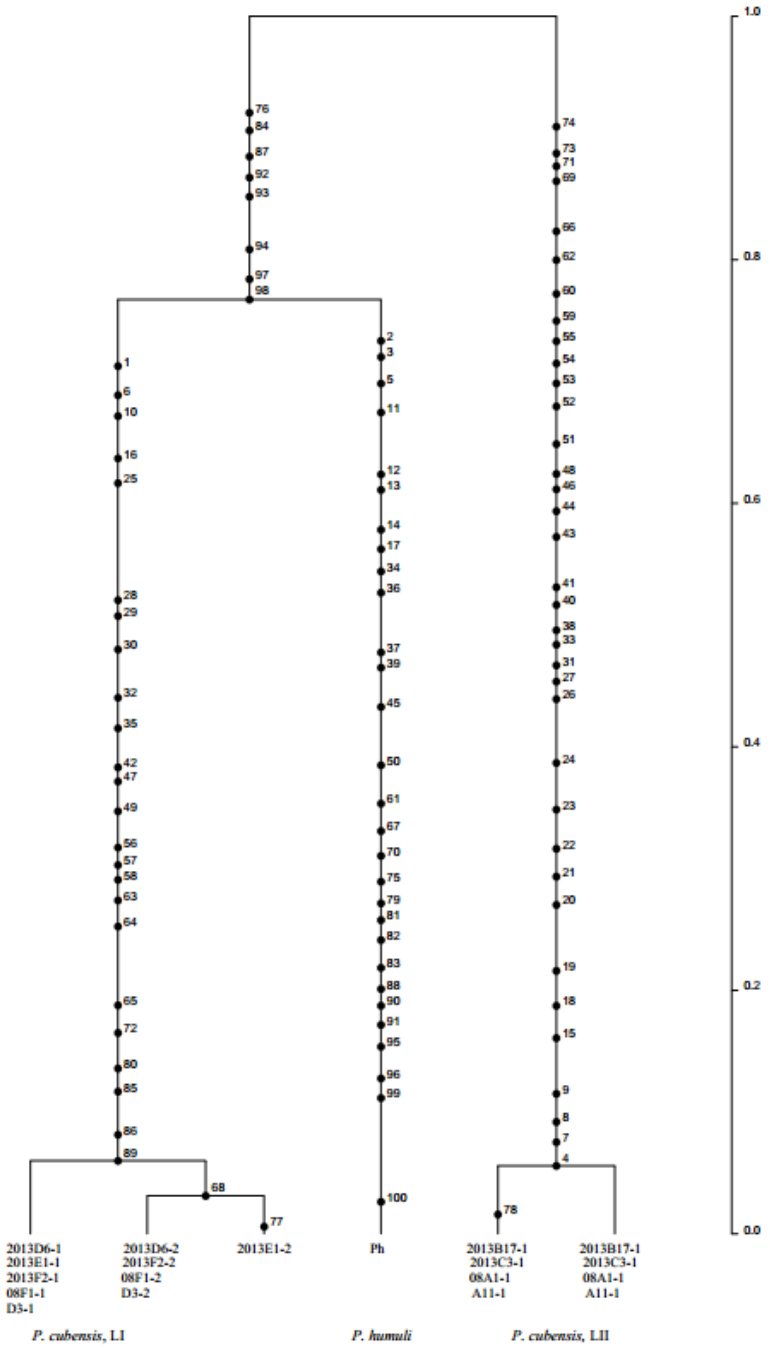

Figure 4.9. Rooted nuclear gene genealogy showing the distribution of mutations of *Pseudoperonospora cubensis* and *P. humuli* based on 100 contiguous SNPs from one end of contig 3. The analysis was run three independent times with a total of 1×10^6 coalescent simulations. There are three distinct evolutionary lineages; *P. cubensis* lineage I and *P. humuli* shared a common ancestor in the past that in turn shared a common ancestor with *P. cubensis* lineage II. The time scale is in coalescent units and the direction of divergence is from top (past) to the bottom (present).

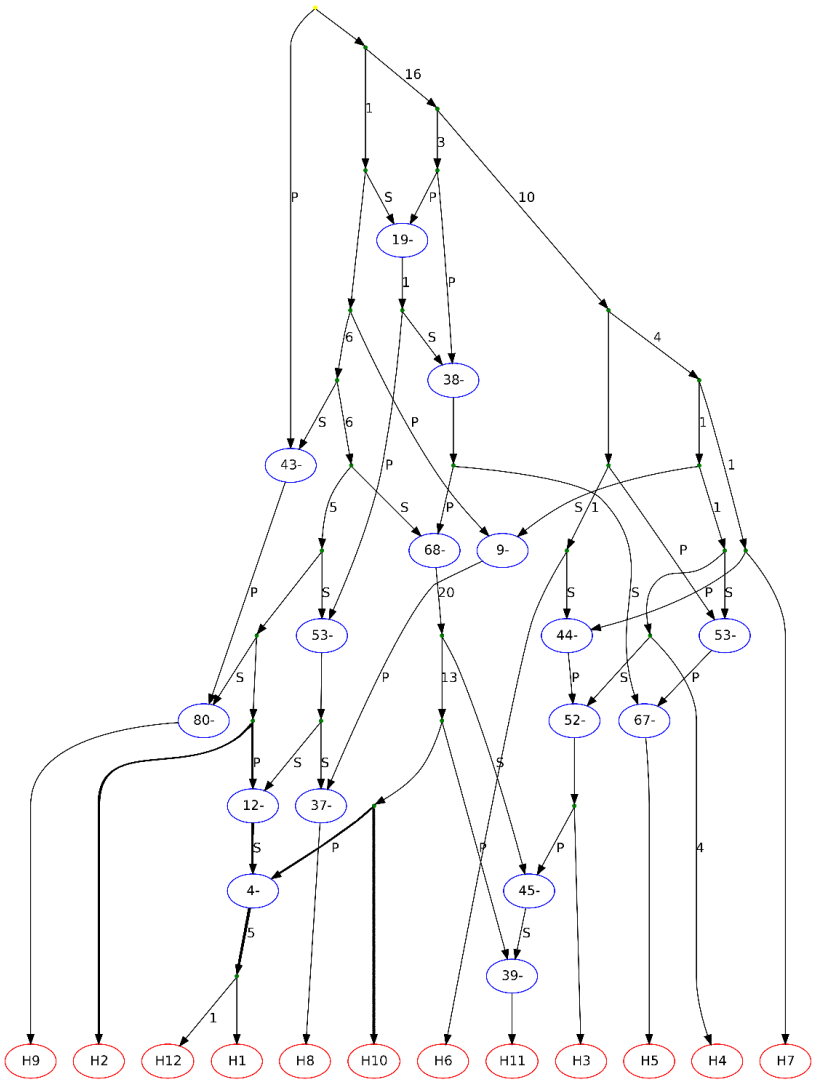

Figure 4.10. Ancestral Recombination Graph (ARG) inferred using the Kwarg heuristic search method for 100 contiguous SNPs spanning the middle segment of nuclear contig 7. The ARG shows one possible reconstruction of mutation and recombination paths giving rise to the sampled haplotypes. The direction of paths is from the top (past) to the bottom (present). The paths leading to the recombination nodes (ovals) are labeled with a P (prefix) or S (suffix), indicating the 5' and 3' segments of the recombinant sequence, respectively, and the numbers displayed along the paths indicates the number of mutation events. The number shown inside the label refers to the variable position immediately to the left of the recombination breakpoint. The ARG shows a putative hybrid origin of *P. cubensis* lineage II (haplotypes H1 and H12) with haplotype H2, comprising isolates belonging to *P. cubensis* lineage I and haplotype H10 (*P. humuli*) as putative parents; the paths leading to putative parents are thickened. The thickened lines represent the minimal number of mutation and recombinant events from parents to recombinant. Haplotype identity is as follows: H1: 08A1-1, 2013B17-1, 2013B17-2, 2013C3-1, A11-1, H2: 08F1-1, 2013D6-1, 2013F2-1: H3 08F1-2, H4: 2013E1-2, H5: 2013D6-2, H6: D3-2, H7: 2013F2-2, H8: 2013E1-1, H9: D3-1, H10: Ph-1, H11: Ph-2, and H12: 08A1-2, 2013C3-2, A11-2.

APPENDICES

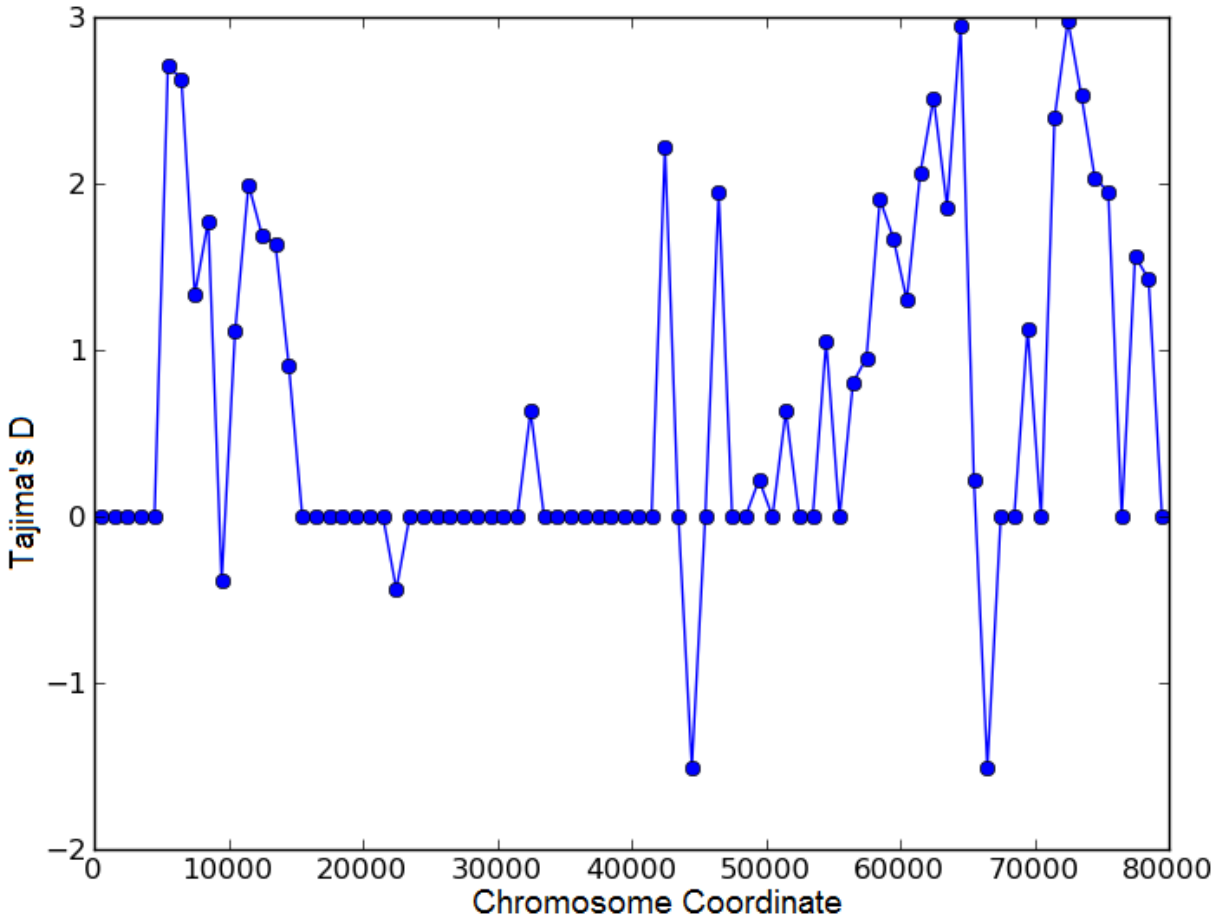

Figure S4.1. Average values of Tajima's D estimated for every 1 kbp across contig 2 of size 80.0 kbp with 243 SNPs.

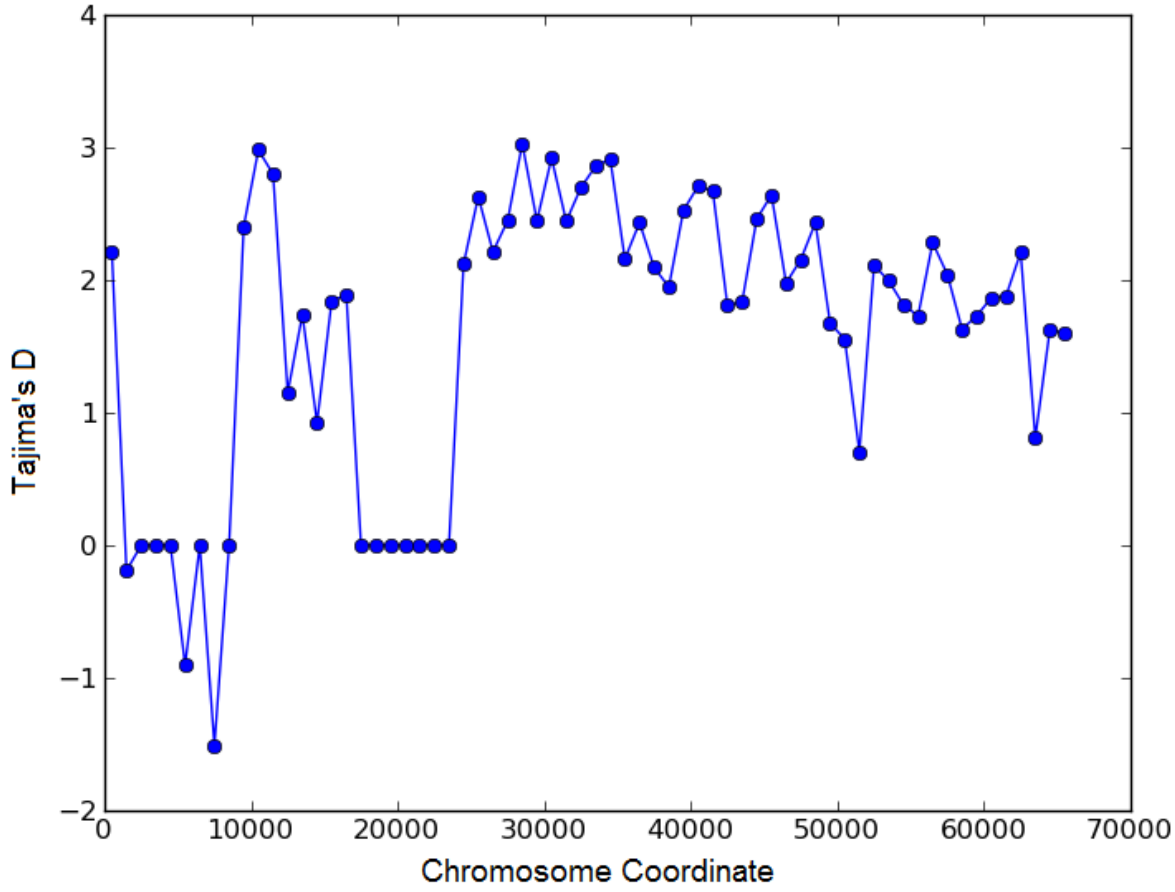

Figure S4.2. Average values of Tajima's D estimated for every 1 kbp across contig 4 of size 65.6 kbp with 607 SNPs.

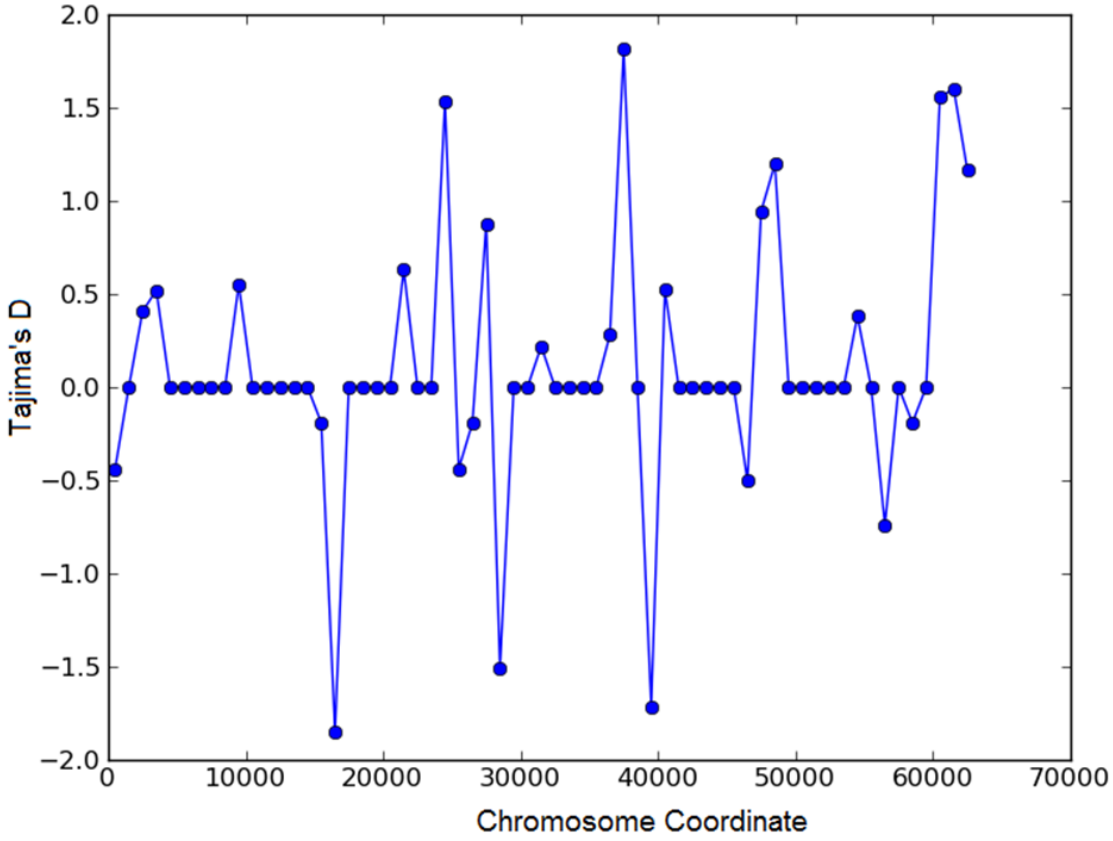

Figure S4.3. Average values of Tajima's D estimated for every 1 kbp across contig 5 of size 64.0 kbp with 135 SNPs.

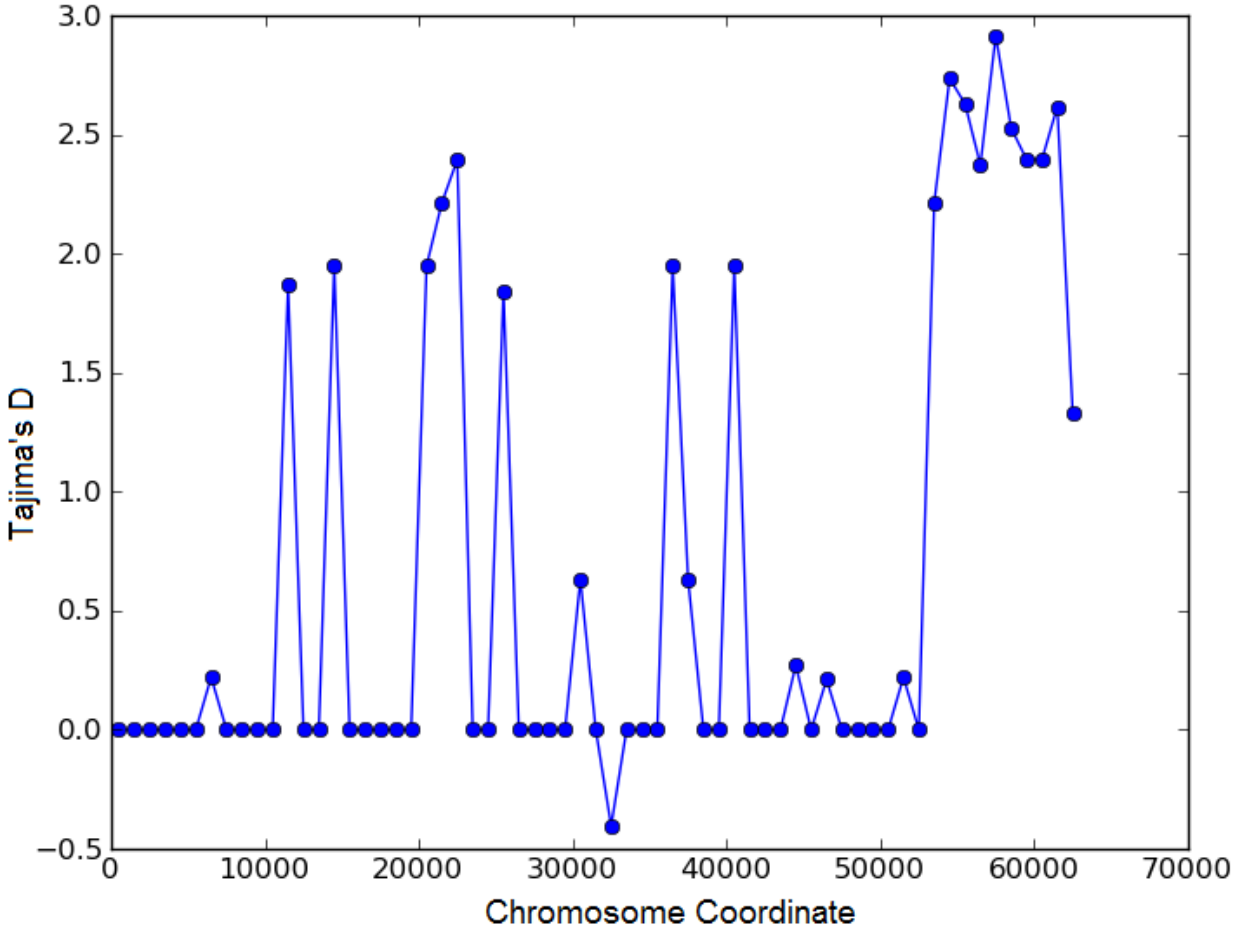

Figure S4.4. Average values of Tajima's D estimated for every 1 kpb across contig 6 of size 62.7 kbp with 127 SNPs.

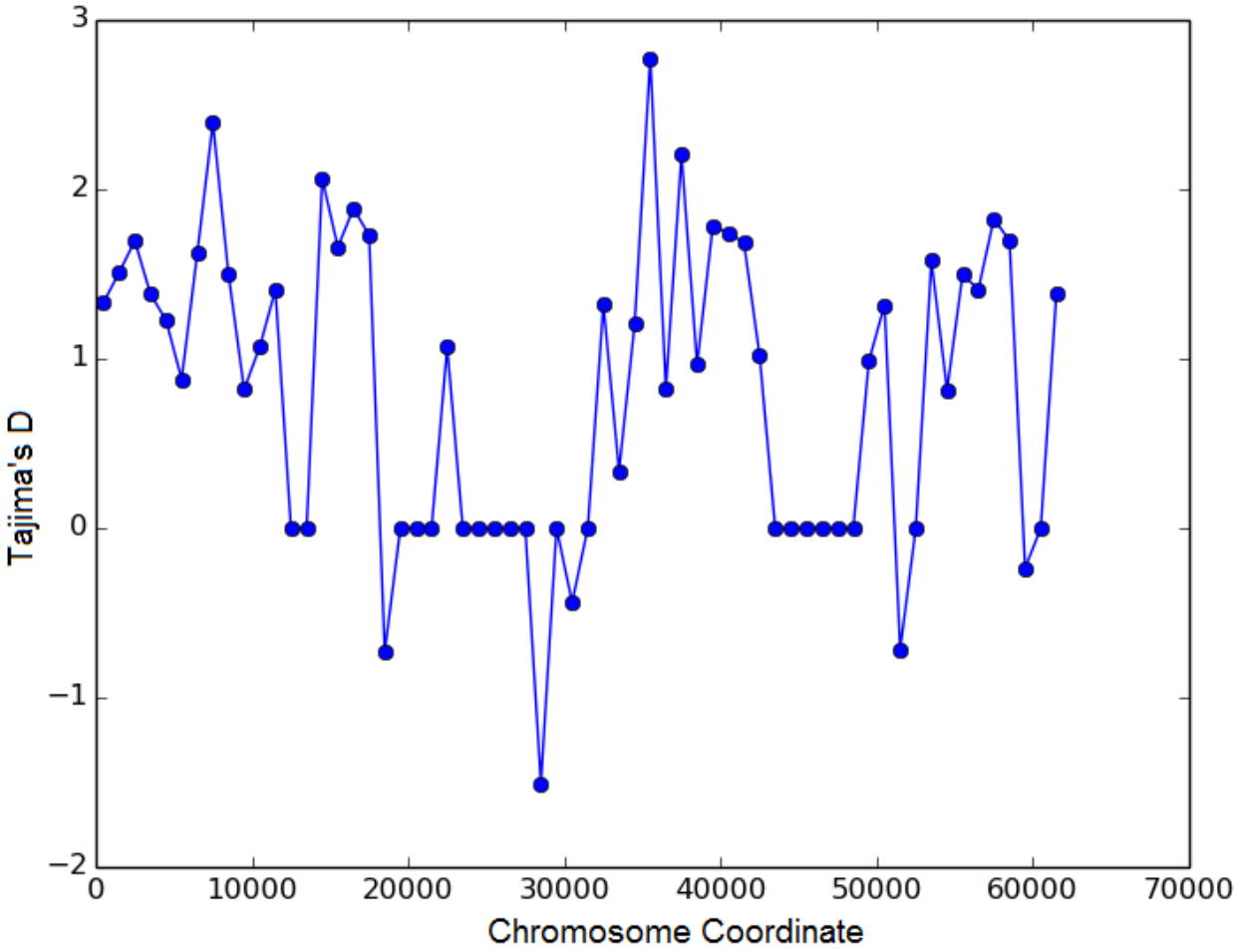

Figure S4.5. Average values of Tajima's D estimated for every 1 kbp across contig 7 of size 61.7 kbp with 307 SNPs.

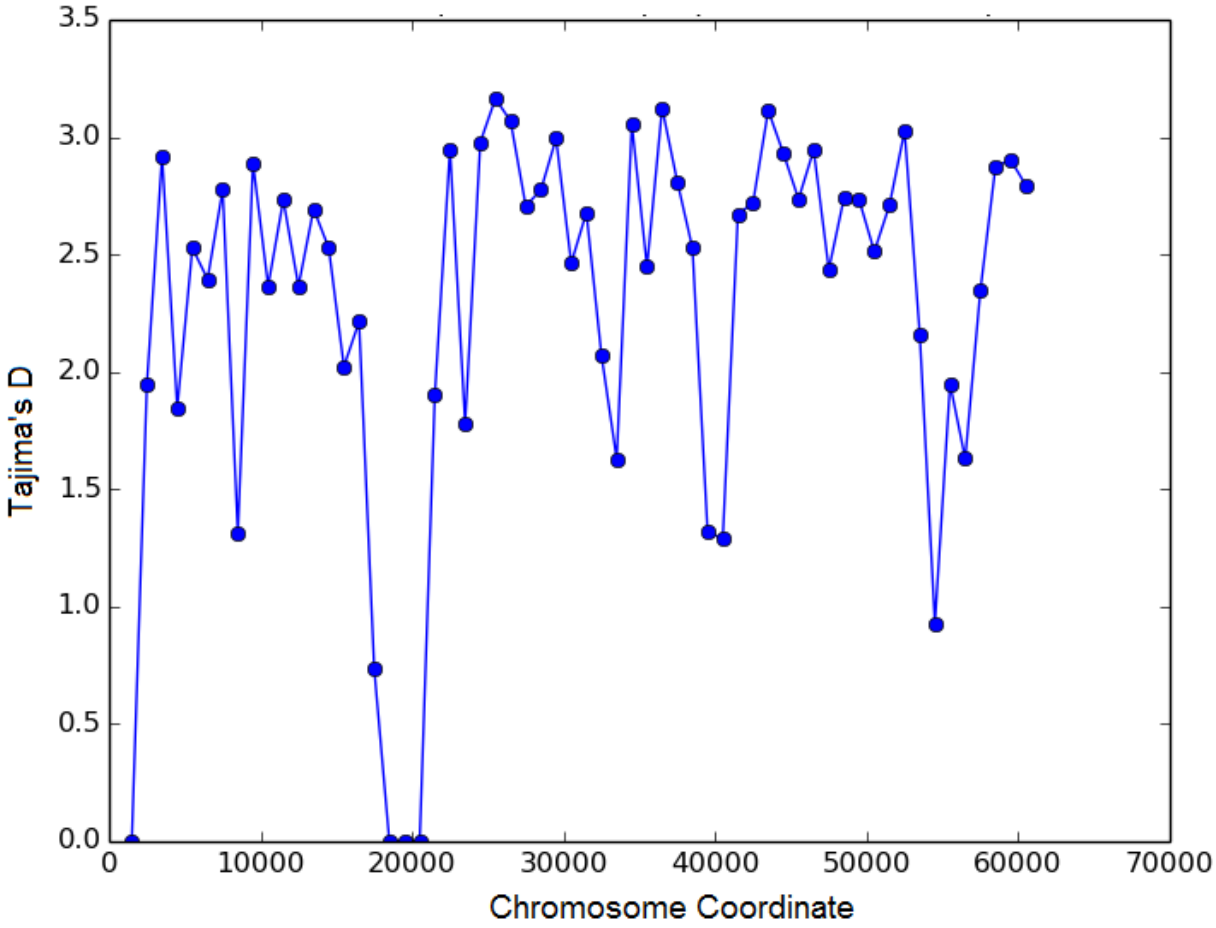

Figure S4.6. Average values of Tajima's D estimated for every 1 kbp across contig 8 of size 60.3 kbp with 715 SNPs.

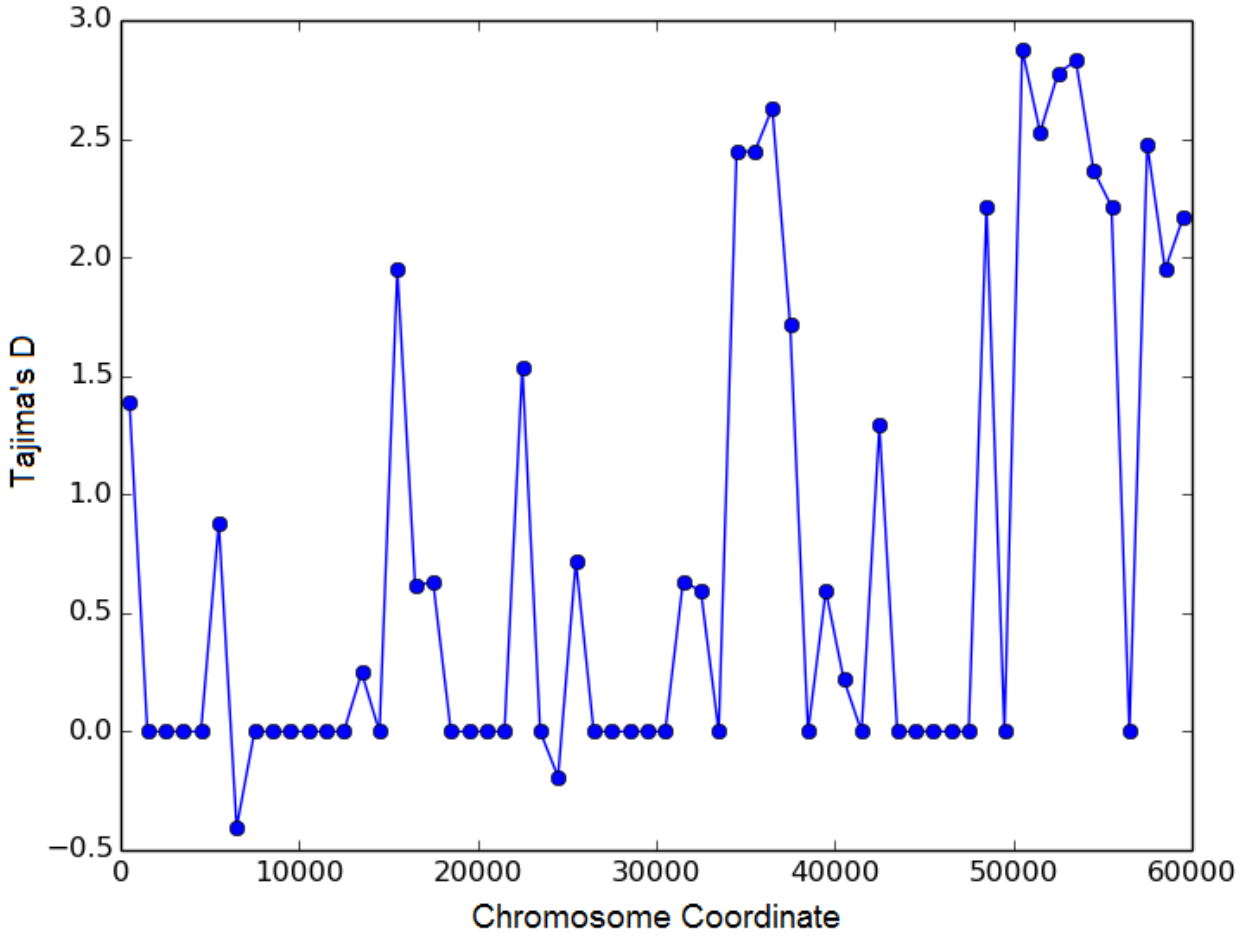

Figure S4.7. Average values of Tajima's D estimated for every 1 kbp across contig 9 of size 59.9 kbp with 149 SNPs.

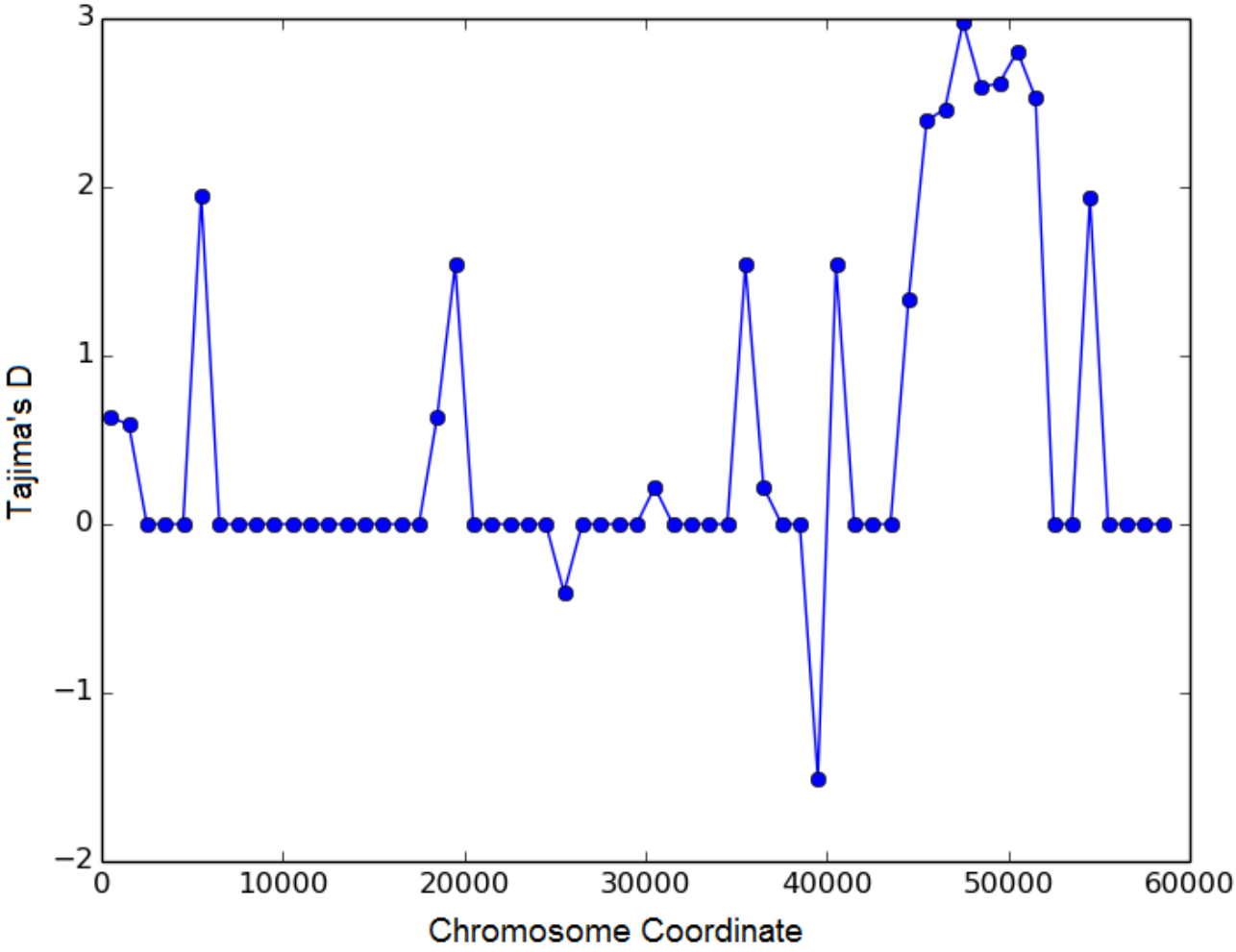

Figure S4.8. Average values of Tajima's D estimated for every 1 kbp across contig 10 of size 59.0 kbp with 137 SNPs.

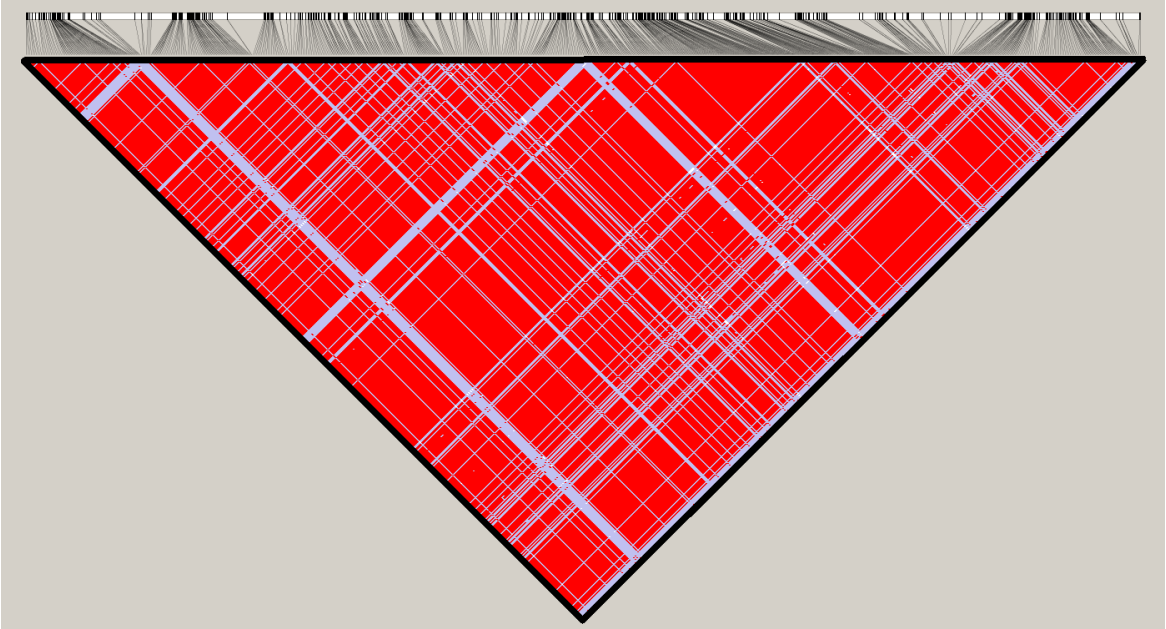

Figure S4.9. LD-plot for contig1 (106 kbp; 680 SNPs) generated using the solid spine algorithm showing one large LD block. Red indicates a coefficient of determination, $R^2 = 1$ and grey indicates $R^2 < 1$ among sites.

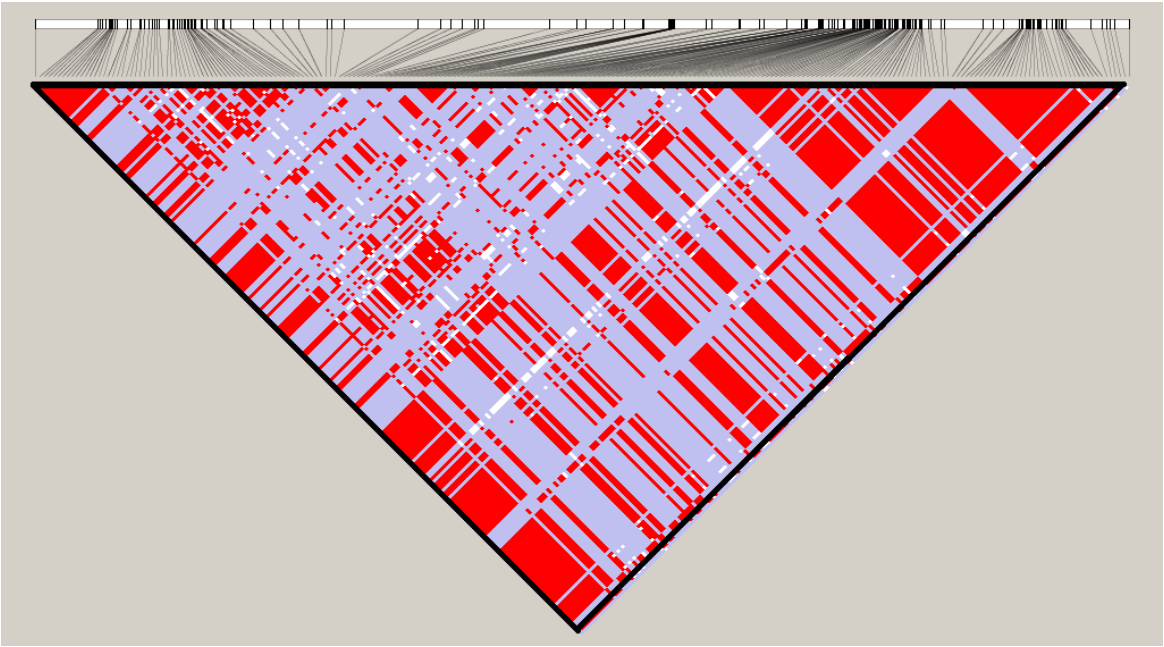

Figure S4.10. LD-plot for contig2 (80.8 kbp; 243 SNPs) generated using the solid spine algorithm showing one large LD block. Red indicates a coefficient of determination, $R^2 = 1$ and grey indicates $R^2 < 1$ among sites.

Appendix K

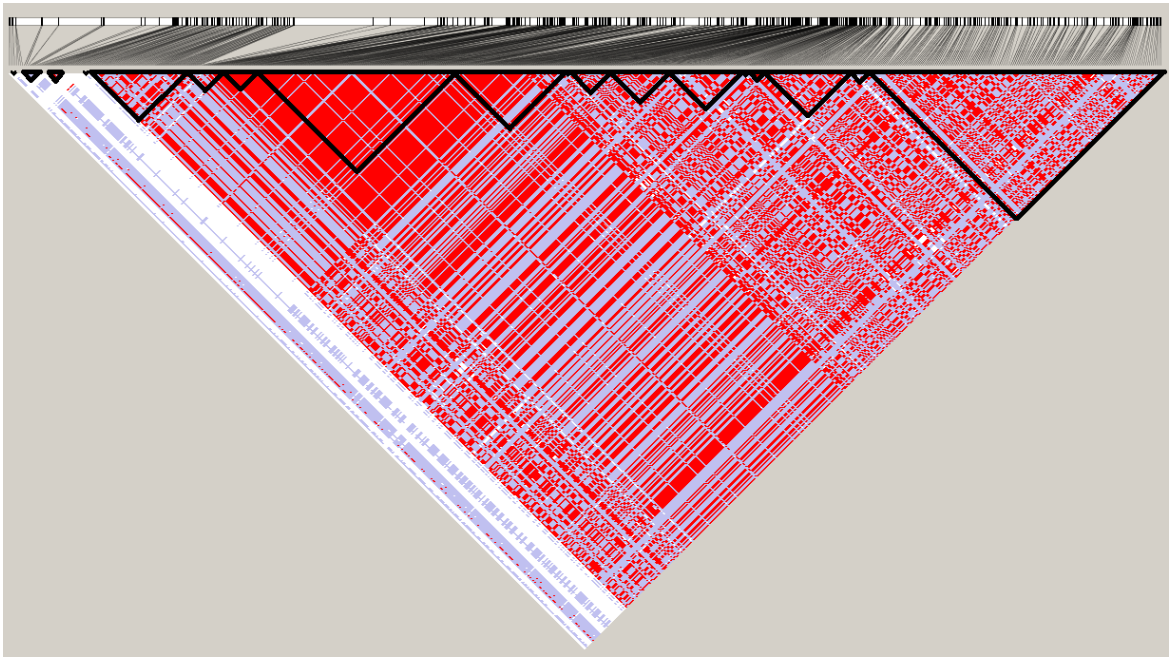

Figure S4.11. LD-plot for contig4 (65.6 kbp; 607 SNPs) generated using the solid spine algorithm showing distinct LD blocks. Red indicates a coefficient of determination, $R^2 = 1$ and grey indicates $R^2 < 1$ among sites.

Appendix L

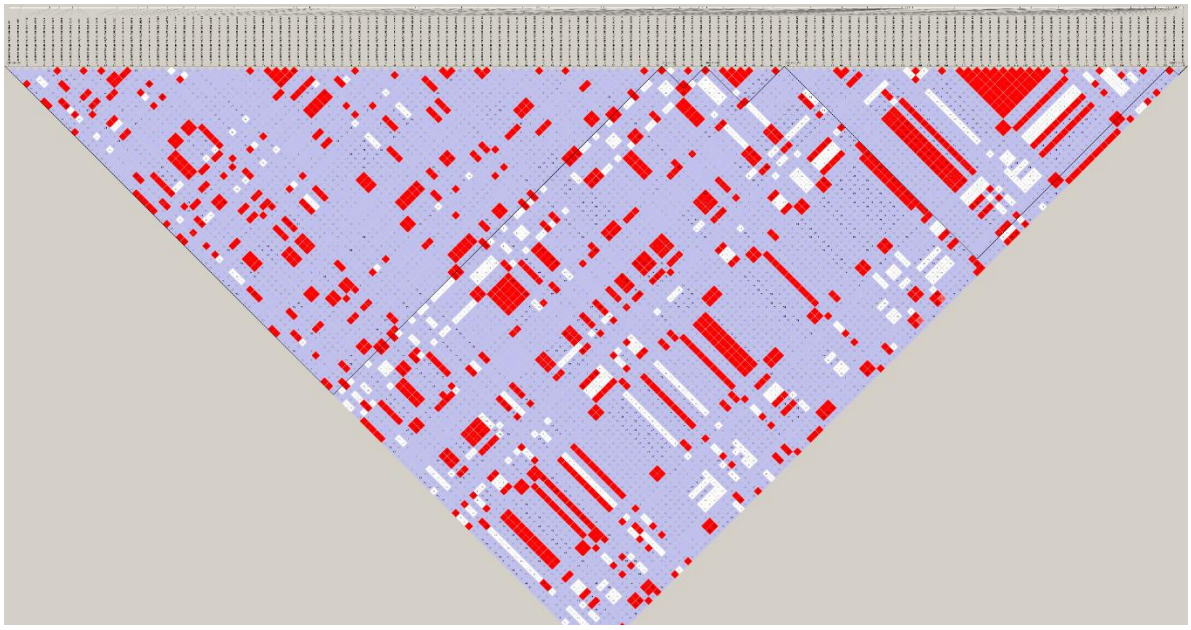

Figure S4.12. LD-plot for contig5 (64 kbp; 135 SNPs) generated using the solid spine algorithm showing distinct LD blocks. Red indicates a coefficient of determination, $R^2 = 1$ and grey indicates $R^2 < 1$ among sites.

Appendix M

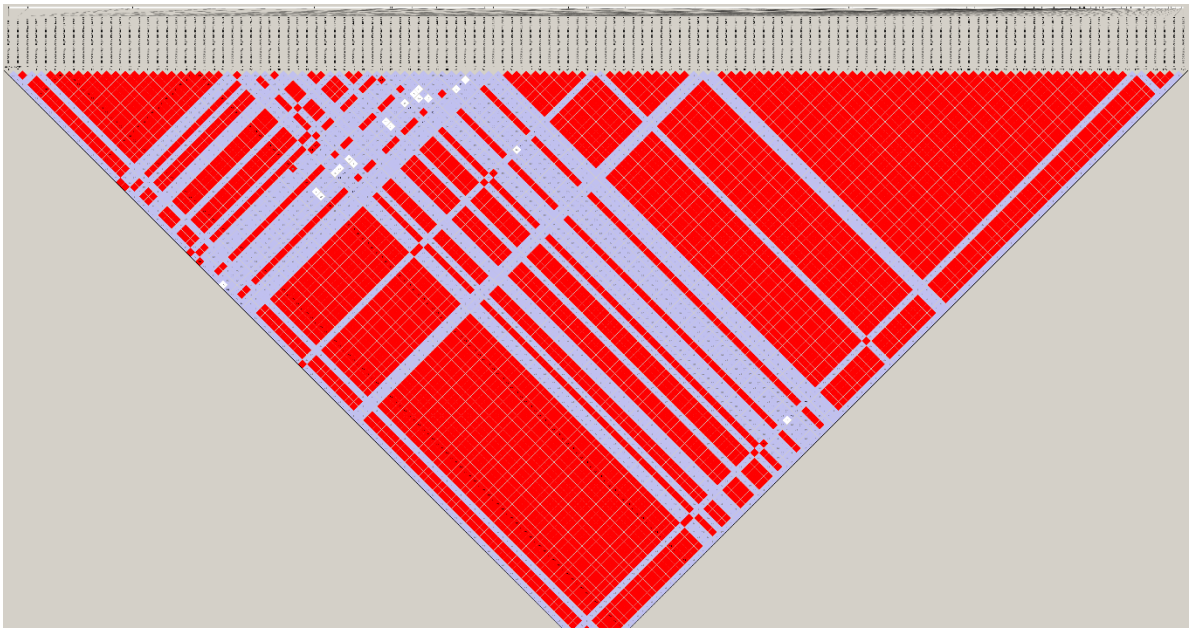

Figure S4.13. LD-plot for contig6 (62.7 kbp; 127 SNPs) generated using the solid spine algorithm showing one large LD block. Red indicates a coefficient of determination, $R^2 = 1$ and grey indicates $R^2 < 1$ among sites.

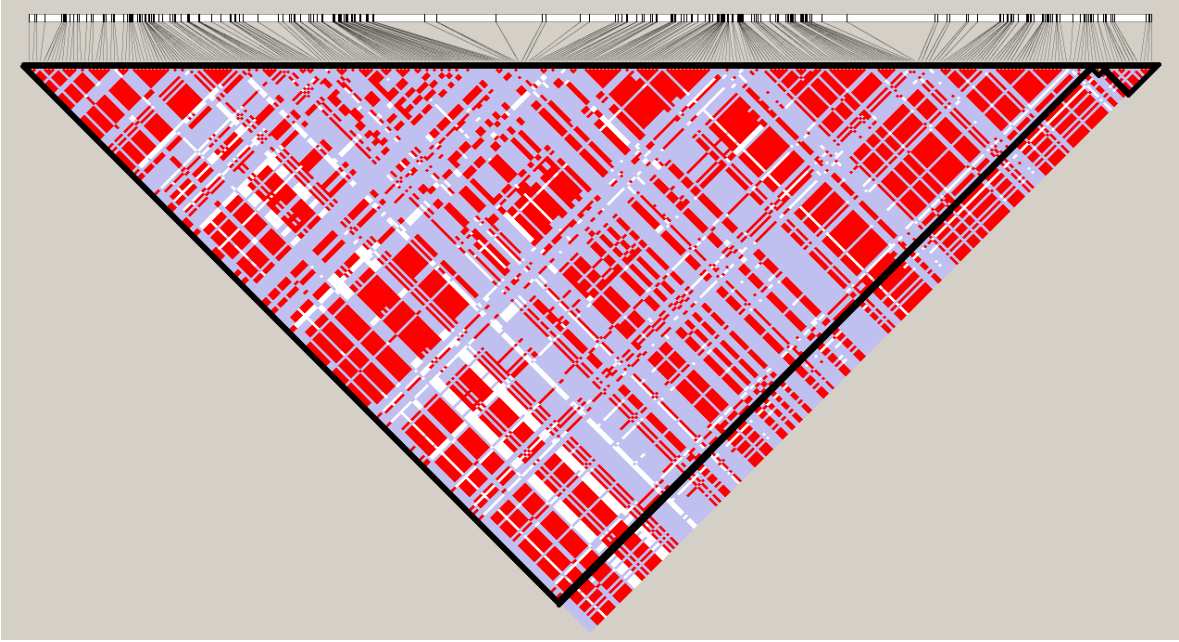

Figure S4.14. LD-plot for contig7 (61.7 kbp; 307 SNPs) generated using the solid spine algorithm showing distinct LD blocks. Red indicates a coefficient of determination, $R^2 = 1$ and grey indicates $R^2 < 1$ among sites.

Appendix O

Figure S4.15. LD-plot for contig8 (60.3 kbp; 715 SNPs) generated using the solid spine algorithm showing distinct LD blocks. Red indicates a coefficient of determination, $R^2 = 1$ and grey indicates $R^2 < 1$ among sites.

Appendix P

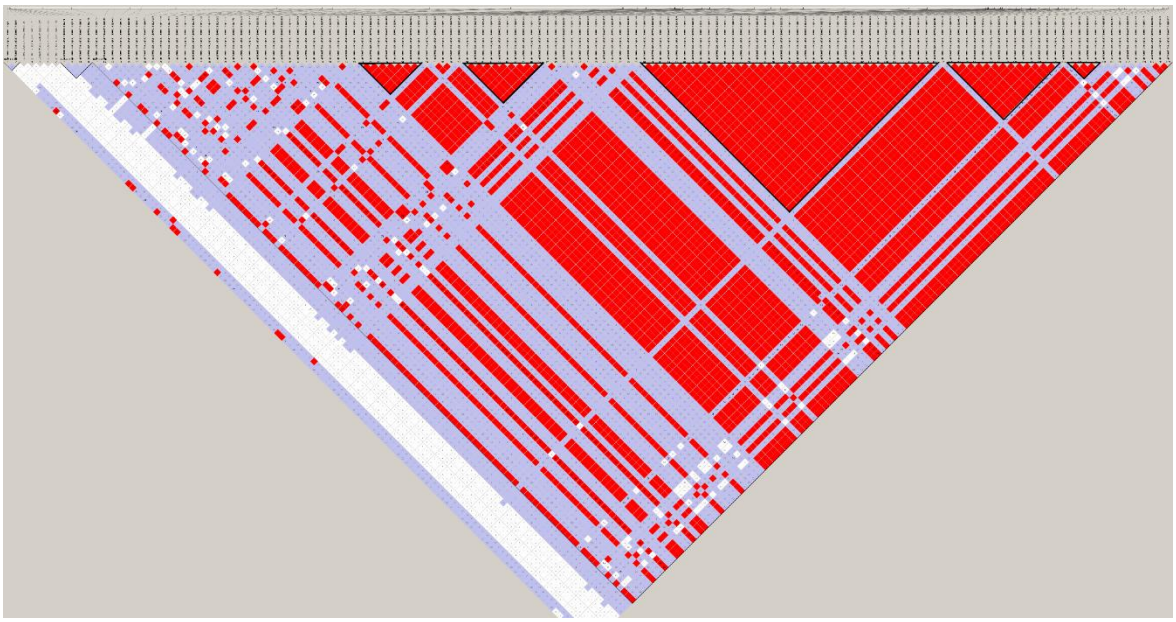

Figure S4.16. LD-plot for contig9 (59.9 kbp; 149 SNPs) generated using the solid spine algorithm showing distinct LD blocks. Red indicates a coefficient of determination, $R^2 = 1$ and grey indicates $R^2 < 1$ among sites.

Appendix Q

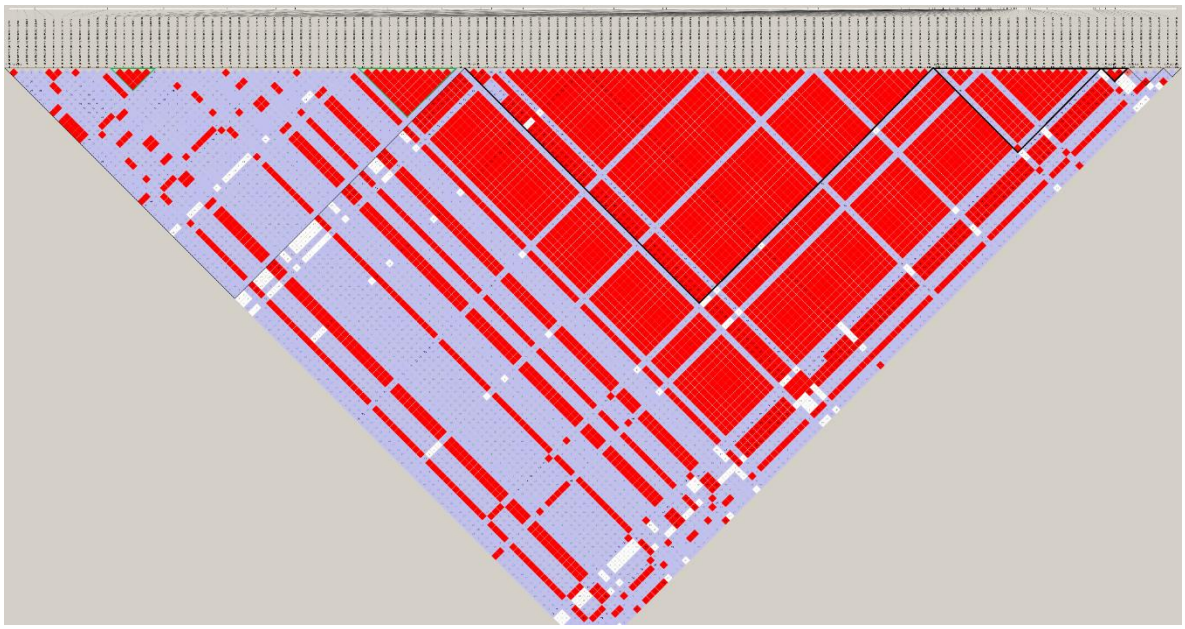

Figure S4.17. LD-plot for contig10 (59 kbp; 137 SNPs) generated using the solid spine algorithm showing distinct LD blocks. Red indicates a coefficient of determination, $R^2 = 1$ and grey indicates $R^2 < 1$ among sites.

Appendix R

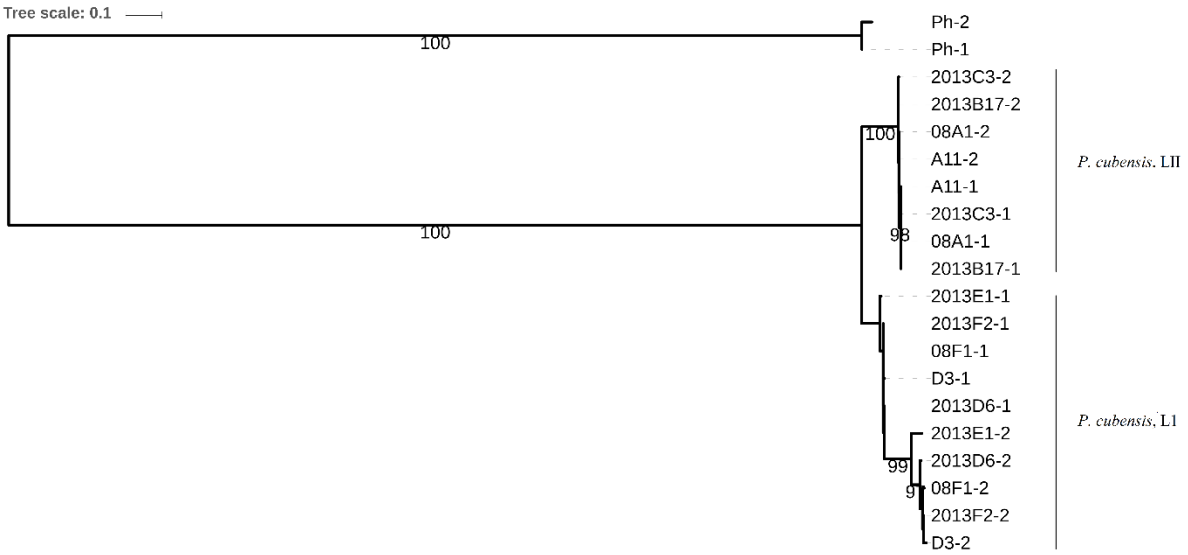

Figure S4.18. Best maximum likelihood phylogenetic tree based on nuclear allelic variation in contig 2 (80.8 kbp; 243 SNPs) for nine isolates of *P. cubensis* rooted with *P. humuli*.

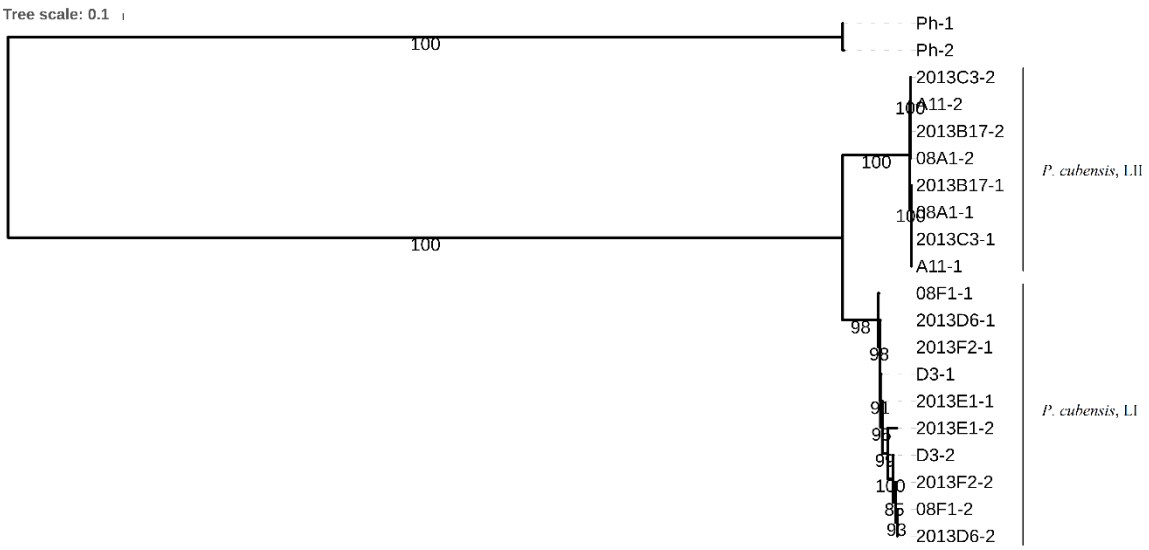

Figure S4.19. Best maximum likelihood phylogenetic tree based on nuclear allelic variation in contig 3 (75.3 kbp; 256 SNPs) for nine isolates of *P. cubensis* rooted with *P. humuli*.

Appendix T

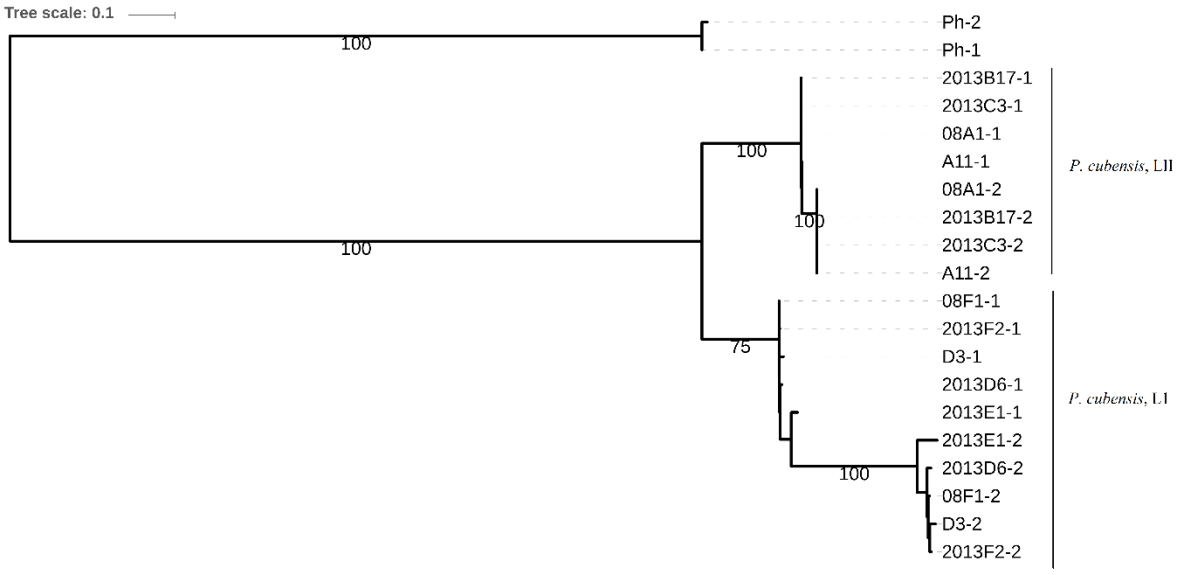

Figure S4.20. Best maximum likelihood phylogenetic tree based on nuclear allelic variation in contig 4 (65.6 kbp; 607 SNPs) for nine isolates of *P. cubensis* rooted with *P. humuli*.

Appendix U

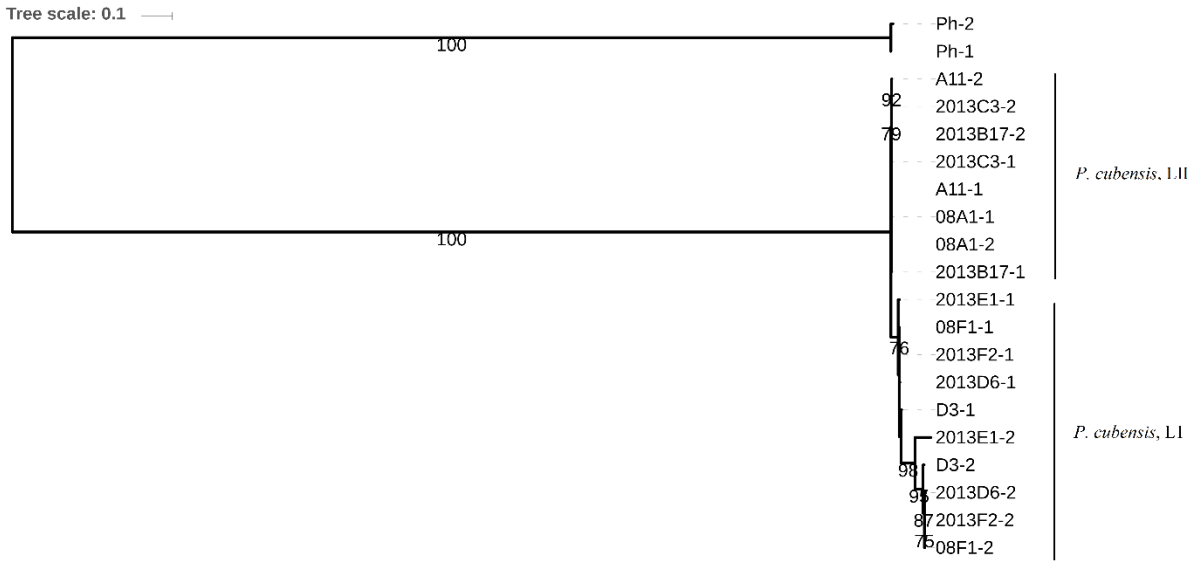

Figure S4.21. Best maximum likelihood phylogenetic tree based on nuclear allelic variation in contig 5 (64 kbp; 135 SNPs) for nine isolates of *P. cubensis* rooted with *P. humuli*.

Appendix V

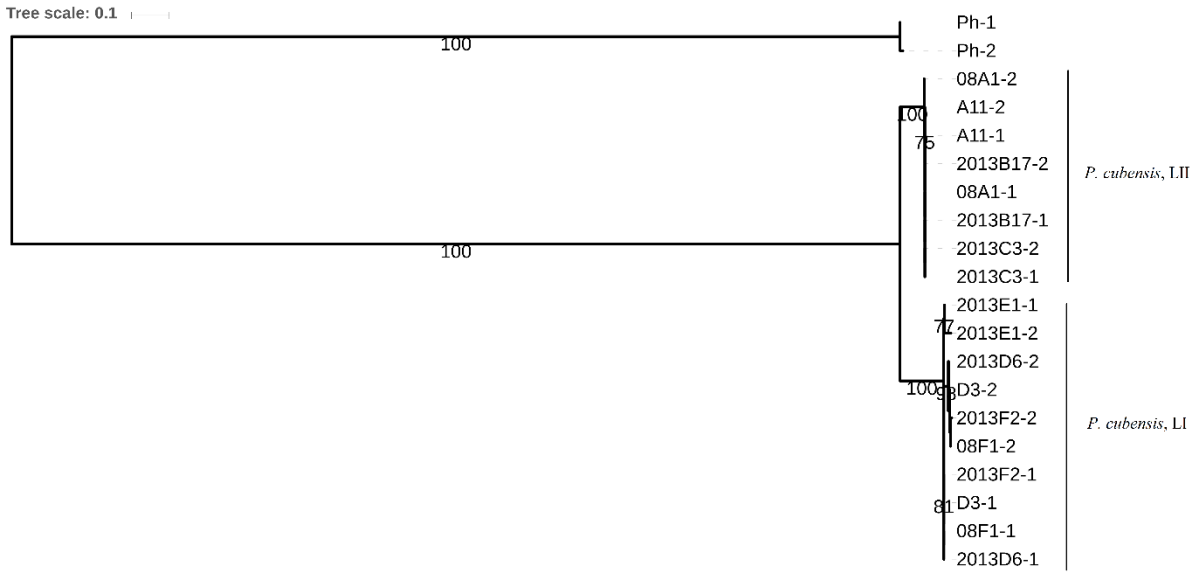

Figure S4.22. Best maximum likelihood phylogenetic tree based on nuclear allelic variation in contig 6 (62.7 kbp; 127 SNPs) for nine isolates of *P. cubensis* rooted with *P. humuli*.

Appendix W

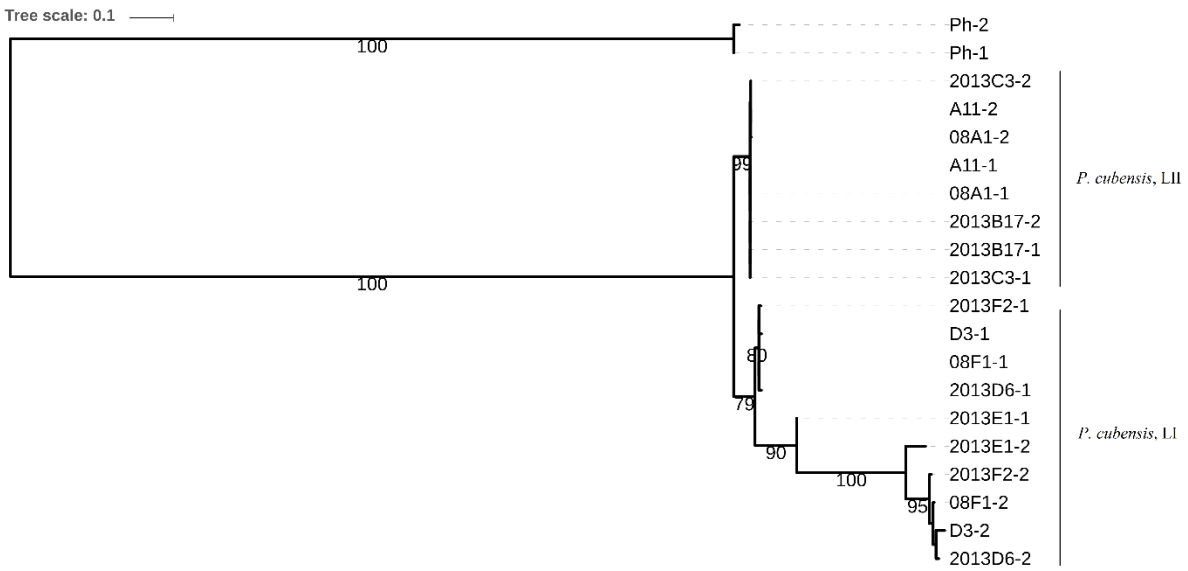

Figure S4.23. Best maximum likelihood phylogenetic tree based on nuclear allelic variation in contig 7 (61.7 kbp; 307 SNPs) for nine isolates of *P. cubensis* rooted with *P. humuli*.

Appendix X

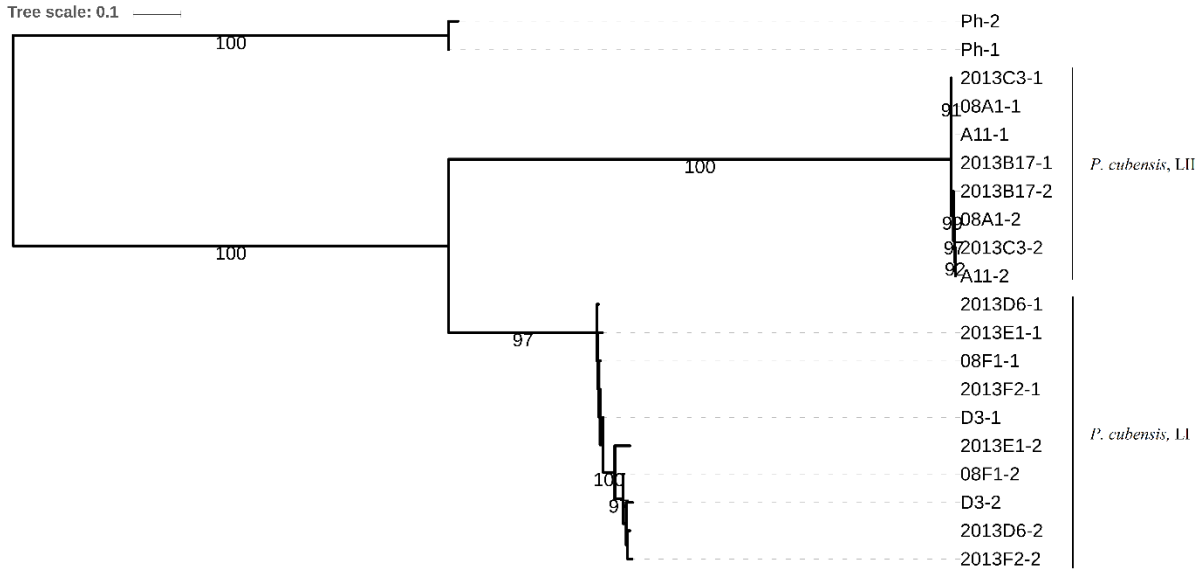

Figure S4.24. Best maximum likelihood phylogenetic tree based on nuclear allelic variation in contig 8 (60.3 kbp; 715 SNPs) for nine isolates of *P. cubensis* rooted with *P. humuli*.

Appendix Y

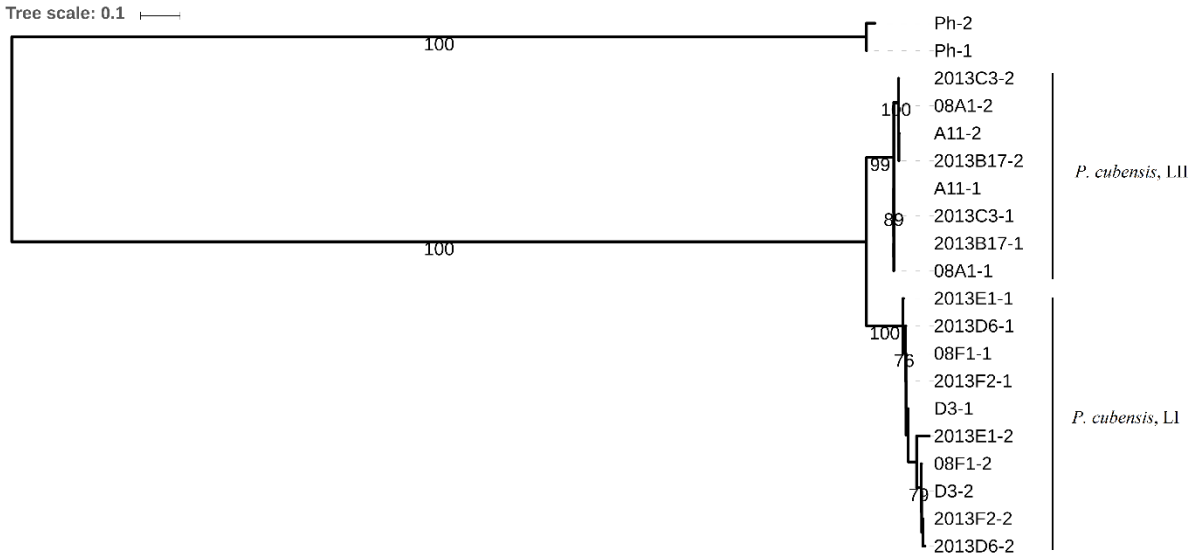

Figure S4.25. Best maximum likelihood phylogenetic tree based on nuclear allelic variation in contig 9 (59.9 kbp; 149 SNPs) for nine isolates of *P. cubensis* rooted with *P. humuli*.

Appendix Z

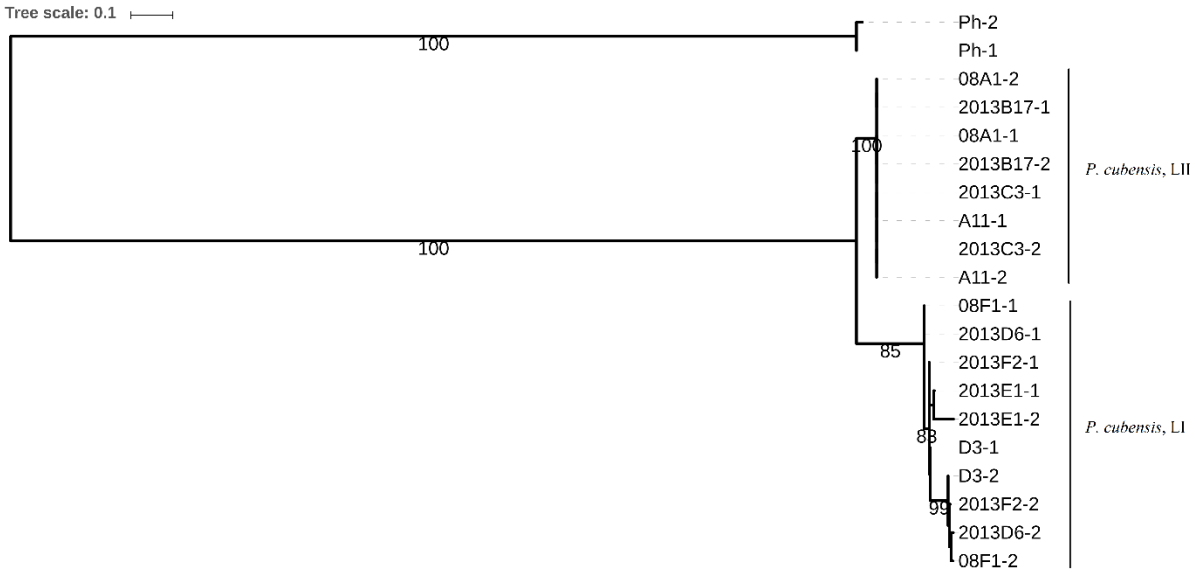

Figure S4.26. Best maximum likelihood phylogenetic tree based on nuclear allelic variation in contig 9 (59.9 kbp; 149 SNPs) for nine isolates of *P. cubensis* rooted with *P. humuli*.

Appendix AA

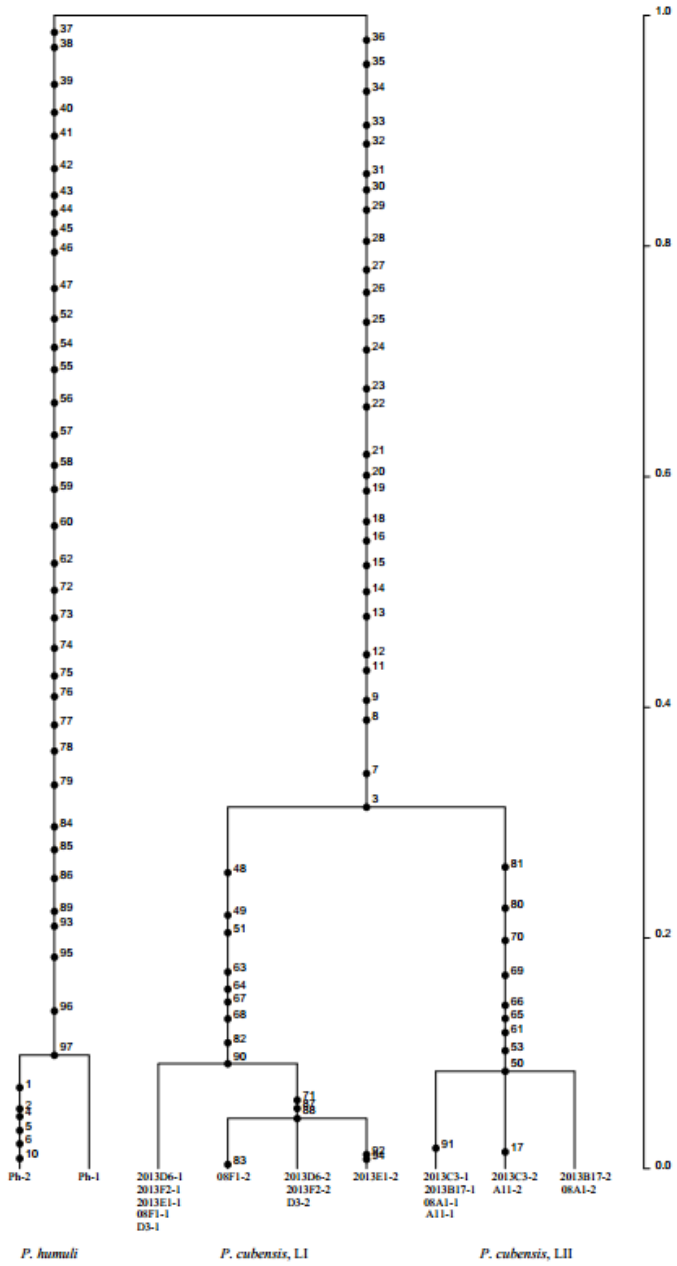

Figure S4.27. Rooted nuclear genealogy showing the distribution of mutations of *Pseudoperonospora cubensis* and *P. humuli* using 100 contiguous SNPs from one end of contig 2. The analysis was performed with a total of 1×10^6 coalescent simulations and the best tree was based on three independent runs. *P. cubensis* lineages I and II have diverged from a common ancestor, which coincides with the speciation event separating *P. humuli* and *P. cubensis*. The time scale is in coalescent units and the direction of divergence is from top (past) to the bottom (present).

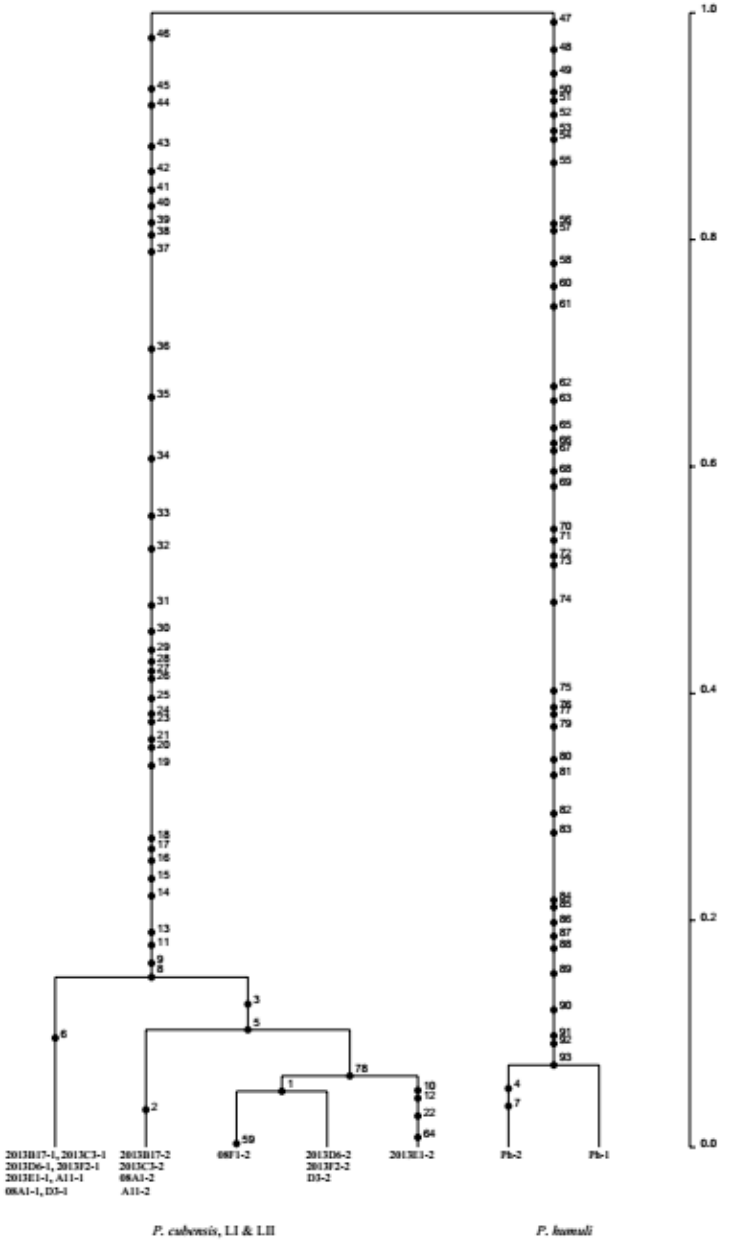

Figure S4.28. Rooted nuclear genealogy showing the distribution of mutations of *Pseudoperonospora cubensis* and *P. humuli* using 100 contiguous SNPs from one end of contig 9. The analysis was run with a total of 1×10^6 coalescent simulations and the best tree was based on three independent runs. The genealogy clearly separates the speciation event between *P. humuli* and *P. cubensis* (many fixed polymorphisms) in the distant past (top) from population divergence events (few polymorphisms) within each species on a more recent time scale (bottom). Alleles of *P. cubensis* lineages I and II are shared between isolates and the distinction between the lineages is not apparent at this part of the nuclear genome.

Appendix AC

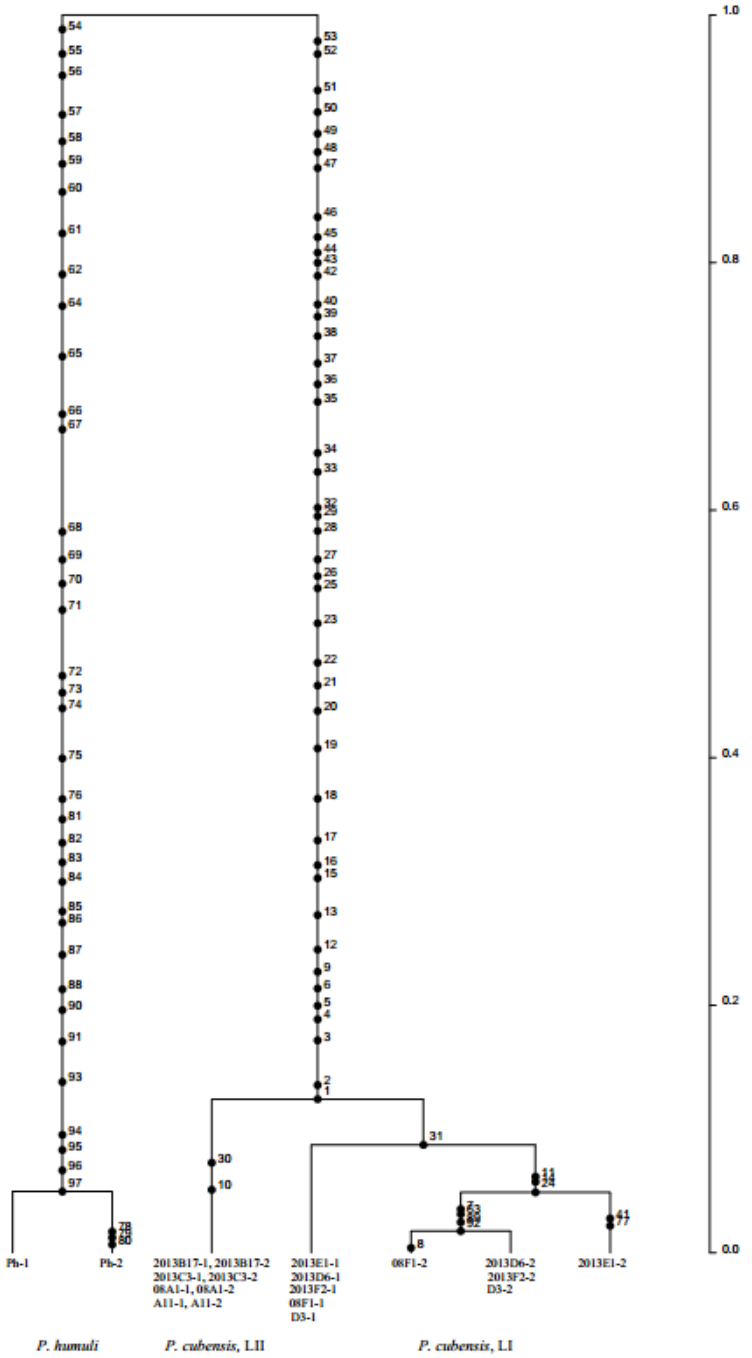

Figure S4.29. Coalescent genealogy showing the distribution of mutations of *Pseudoperonospora cubensis* and *P. humuli* using the first 100 SNPs of contig 10. The analysis was run with a total of 1×10^6 coalescent simulations and the best tree was based on three independent runs. Lineage I and lineage II of *P. cubensis* seemed to have diverged from a common ancestor recently with very little distinction between lineages (3 SNPs). The time scale is in coalescent units and the direction of divergence is from top to the bottom.

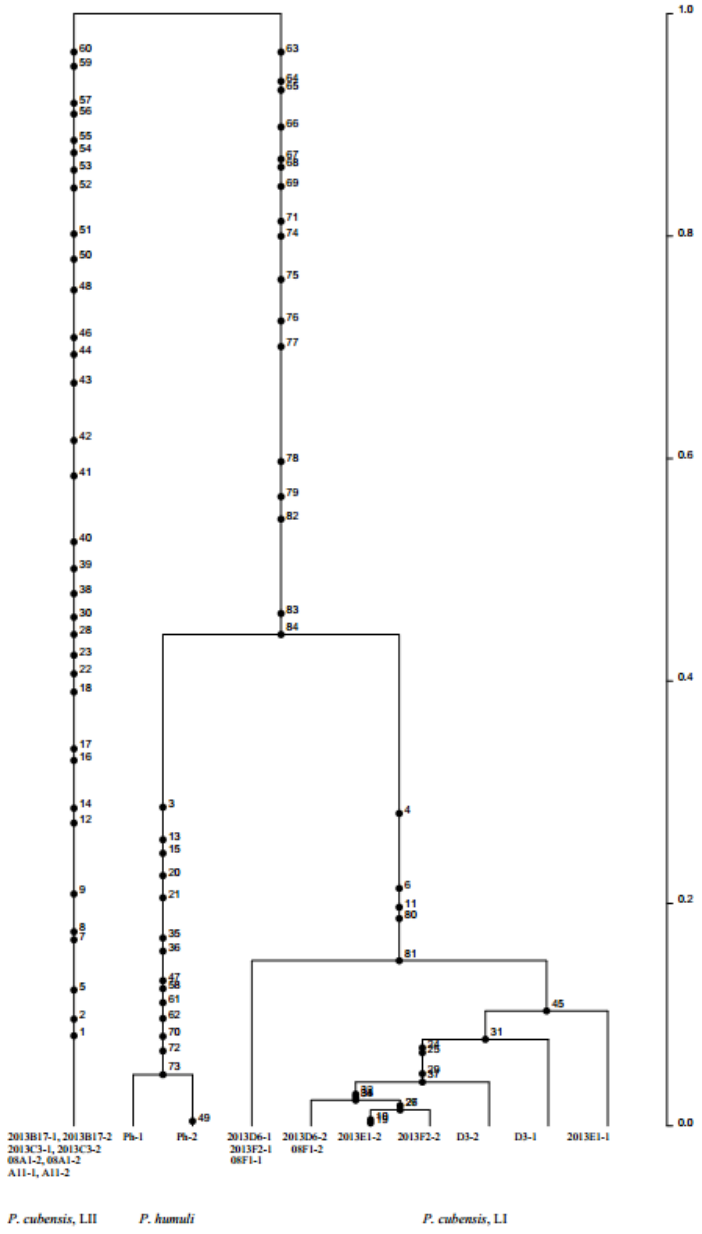

Figure S4.30. Rooted nuclear gene genealogy showing the distribution of mutations of *Pseudoperonospora cubensis* and *P. humuli* based on 100 contiguous SNPs from the middle segment of contig 8. The analysis was run three independent times with a total of 1×10^6 coalescent simulations. There are three distinct evolutionary lineages; *P. cubensis* lineage I and *P. humuli* shared a common ancestor in the past that in turn shared a common ancestor with *P. cubensis* lineage II. The time scale is in coalescent units and the direction of divergence is from top (past) to the bottom (present).

CHAPTER 5

Population genetic structure of *Pseudoperonospora cubensis* as influenced by cucurbit host types and geography in the United States

To be submitted to *Phytopathology*

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ABSTRACT

A global resurgence of cucurbit downy mildew caused by the obligate oomycete pathogen, *Pseudoperonospora cubensis* was observed in the last two decades. In the United States, resurgence of cucurbit downy mildew occurred in 2004 and caused extensive yield losses and still remains to be the most important yield-limiting factor for cucurbit production. The population genetic structure of *P. cubensis* was examined using 93 isolates collected on diverse host types from different locations along eastern United States based on the double digest Restriction Associated DNA Sequencing (ddRADSeq) technology. Results obtained from phylogenetic and STRUCTURE analyses showed that the pathogen population in the United States is composed of two host specialized lineages and a significant subdivision of the pathogen population was detected bewteen lineage I and lineage II isolates (F_{ST} = 0.72). Lineage II was found to be specialized on *Cucumis sativus* and lineage I was specialized on *Cucurbita* spp. and *Citrullus lanatus*. In addition, the presence of a distinct genetic cluster within lineage I, associated primarily with *C. lanatus*, was detected. The two lineages were also associated with mating types of *P. cubensis* with lineage II and I being associated with the A1 and A2 mating type, respectively. However, STRUCTURE analysis did not detect a significant subdivision of the pathogen population based on geography. This study contributes to the knowledge on the current status of the population genetic structure of *P. cubensis* in the United States. Information generated from this study may further aid in the development of diagnostic tools and more precise management strategies for downy mildew on different cucurbit host types.

INTRODUCTION

Cucurbit downy mildew (CDM), the most destructive disease of cucurbits caused by the obligate oomycete pathogen, *Pseudoperonospora cubensis* resurged in the United States in 2004 overcoming the host resistance in cucumber that had been effective for more than 40 years. The resurgence of CDM resulted in extensive yield losses along the eastern United States in 2004 and 2005. The disease still remains the most important limiting factor for cucurbit production (Holmes et al. 2015). Similar resurgence of CDM have been observed in several countries in the last two decades (Cohen et al. 2015). In Europe, CDM resurged in the 1980s resulting in severe epidemics on cucumber (Lebeda and Cohen 2011). Pathogen populations with wider host range were reported in Israel and Italy in the early 2000s and subsequently from other European countries as well (Cappelli et al. 2003; Cohen et al. 2003; Lebeda et al. 2013; Cohen et al. 2015). This global resurgence of CDM has been attributed to changes in the population structure of *P. cubensis* possibly due to an expansion in geography and host range because of migration or sexual recombination (Cohen et al. 2015, Runge et al. 2011).

The downy mildew pathogen, *P. cubensis* has a wide host range (Runge et al. 2011) and is known to exhibit a high degree of host specialization (Doran, 1932; Hughes and Van Haltern 1952; Palti 1974; Palti and Cohen 1980; Thomas et al. 1987). Host specificity exhibited by different isolates of *P. cubensis* was probably first recognized in Japan in 1941 (Iwata, 1941) where the existence of two biological forms of *P. cubensis* were reported with one specialized on *Cucumis* spp. and another on *Cucurbita moschata*. A similar situation was later observed in the United States based on the differential affinity of pathogen population for *Cucumis* spp. and *C. lanatus* in 1952 (Hughes and Van Haltern 1952). Later, Palti (1974)

analyzed the global pattern of divergence of *P. cubensis* on its crop hosts. It was observed that *P. cubensis* was common throughout the world on *Cucumis* spp. (cucumber and melon), less frequent on *Cucurbita* spp. (absent in Europe and certain parts of Asia) and was more restricted in its distribution on *Citrullus* spp., *Luffa* spp. and *Lagenaria* spp. (Palti 1974). This was attributed to the divergence in virulence pattern exhibited by *P. cubensis* as a result of different physiological races in various countries. Since then, the virulence in the pathogen population has been expressed based on pathotypes defined based on patterns of compatibility or incompatibility with a series of differential host types. Thomas et al. (1987) identified five pathotypes based on the differential response of the pathogen population from Japan, Israel and the United States exhibited, at the time, on *Cucumis* spp., *Cucurbita* spp., and *Citrullus lanatus*. Population genetic studies also have suggested host specificity as a potential factor influencing the genetic architecture of *P. cubensis* (Quesada-Ocampo et al. 2012; Kitner et al. 2015). Recently, comparative genomic analysis of nine *P. cubensis* isolates collected from diverse host types identified the presence of two host specialized lineages in the United States with lineage II specializing on *Cucumis* spp. and lineage I on squashes and watermelon (Thomas et al. 2014). Another recent study using 20 *P. cubensis* isolates based on genotyping by sequencing and RNA-Seq analysis also detected two genetic clusters; one associated with *Cucumis* spp. and another with squashes (Summers et al. 2015). These studies suggested that host specificity is an important factor governing pathogen diversity. Thus, the current study aims to examine the role of host dynamics in determining the genetic structure of *P. cubensis* population using a robust set of isolates collected from diverse cucurbit host types in the United States.

The role of geography in shaping the genetic structure of *P. cubensis* population also need to be examined since population differentiation on a spatial scale may provide insights into the potential sources of inoculum for CDM outbreaks in different regions of the United States. The source of primary inoculum for CDM has been a major focus of research in the recent past (Ojiambo and Holmes, 2012; Ojiambo et al. 2015). The pathogen is an obligate parasite and can only survive in areas that experience mild winters that allows host plants to survive (Savory et al. 2012). Although the production of viable oospores under in vitro conditions was recently reported in the United States, their role in the epidemiology of CDM is currently unknown. In addition, although a perennial wild cucurbit host has been identified in the United States (Wallace et al. 2015), the role of such host types in the overwintering of *P. cubensis* is yet to be established. The pathogen is widely believed to overwinter below the 30°N latitude primarily in southern Florida where cucurbits are grown year around (Ojiambo et al. 2015). Aerial dispersal of sporangia is the primary mode of dissemination and spores can be dispersed over long distances. Empirical evidence for long distance dispersal of *P. cubensis* from southern United States to the northern latitudes in the United States has been provided based on patterns of disease outbreak (Holmes et al. 2006; Ojiambo and Holmes, 2011). It was also determined that the extent of spatial association of disease outbreak was ~1,000 km and outbreaks in the Mid-Atlantic States were associated with inoculum originating directly from southern FL. The outbreaks in the Great Lake region was suggested to be caused by the inoculum originating from North Carolina/South Carolina/Georgia border or other undocumented sources in Canada rather than overwintering sources in the south (Ojiambo and Kang 2013). However, a more definitive association of inoculum from

overwintering sources in the south to disease outbreaks in northern latitudes has yet to be determined.

Knowledge on the initial sources of inoculum and the role different host types may play in selection of one genotype/lineage over the others has implications in disease monitoring, forecasting and formulation of management decisions. Identification of differences and similarities in the genetic makeup of isolates collected from different locations along the trajectories of *P. cubensis* may help identify the initial sources of inoculum for different parts of the country. The overall goal of this study was to help understand the dispersal of *P. cubensis* in the continental United States and the role different host types and geographical locations may play in facilitating the dissemination of genotypes of the pathogen across the eastern United States. The specific objectives were to: i) Determine the genetic variation among *P. cubensis* population in the United States, ii) Investigate the association between different host types and genetic clusters of *P. cubensis*, ii) Examine the extent of genetic differentiation between isolates of *P. cubensis* collected from the different geographical regions in the United States.

MATERIAL AND METHODS

Isolates. A total of 93 single lesion isolates collected from seven cucurbit host types (cucumber, cantaloupe, watermelon, pumpkin, butternut squash, acorn squash and summer squash) were used in this study. The isolates were sampled from three main geographical regions of the United States: 1) southern region that included the states of FL, GA, AL, LA, TX, SC, NC and TN, 2) northeast region included the states of VA, WV, MD, DE, NJ, NY, MA, KY, PA, RI and CT, and, 3) Great Lakes region included the states of MI, OH, WI, IN

and IL. Attempts to collect isolates from the western coast were not successful due to the low incidence of CDM in that region. Summary of the isolates collected from different host types in the three geographical regions of the country is provided in Table 5.1.

Isolates used in the study were collected from both sentinel plots and commercial fields across the country whenever a new report of CDM was made on the cucurbit downy mildew monitoring website (Ojiambo et al. 2011). Infected leaf samples from the disease monitoring network were shipped overnight to North Carolina State University, where the samples were further processed. Leaves of infected samples were carefully examined and isolates from leaves with discrete lesions were subsequently maintained on their respective hosts in the laboratory as single lesion isolates. Leaf samples without discrete single lesions were further processed to generate single lesion isolates. Sporangia from such lesions were washed off infected leaves using a Preval sprayer (Complete Unit 267; Precision Valve Corporation, Yonkers, NY) and the suspension was used to inoculate original host plants at a concentration of about 1×10^4 sporangia per ml. Inoculated plants were bagged with polythene bags and incubated in a growth chamber at 18°C with no light for 24 h. At the end of 24 h, plants were un-bagged and incubated in a growth chamber at 21/18°C day/night for disease development with a photoperiod of 12 h. Plants were examined daily for symptoms and when discrete single lesions were visible, plants were bagged again and kept under dark conditions for 24 h to stimulate sporangia production. An individual lesion was subsequently cut out from a leaf and collected sporangia were used to inoculate the original host to multiply sporangia of each isolate. To propagate isolates, first or second leaves from 4 weeks old plants were placed on sterile moist paper towel in clear acrylic boxes. Abaxial sides of the leaves were then inoculated with ~ 2×10^4 sporangia per ml and incubated in a growth

chamber at 21/19°C day/night and a photoperiod of 12 h. All the isolates were maintained in the lab by propagating them on the respective host types through detached leaf assays. After multiplying sporangia, 6 to 8 detached leaves placed on moist paper towel in acrylic containers of approximate size 30 cm×19 cm×10 cm were spray inoculated on the adaxial side using a preval sprayer at a concentration of 1 to 3 $\times 10^4$ sporangia per ml. The leaves were then incubated at 21/18°C light and dark cycle of 12 hours each. Inoculated leaves were observed daily for sporangial production and the leaves showing signs of heavy sporulation were rinsed with sterile water using a preval sprayer. Sporangial suspension collected in 50 ml centrifuge tubes was then centrifuged and subjected to DNA isolation using a Qiagen DNeasy Plant Mini Kit (Qiagen Corporation, Maryland, USA) with slight modifications.

Reduced-representation library construction and illumina sequencing. Library construction for double digest Restriction Associated DNA sequencing (ddRADSeq) was done at the Genomic Science Laboratory at North Carolina State University (Raleigh, NC). Briefly, 200 ng of genomic DNA extracted from each of the ninety-three isolates were digested with two restriction enzymes, MluC1 and Msp I, NEB Buffer 4 and water for 3 hrs at 37°C. The digested DNA products were the cleaned with 1.5x volume Ampure beads followed by ligation of individual strains with unique indexing adapters. A combinatorial indexing scheme was used to label each individual. Total samples were divided into two sub-libraries of 48 and 45 strains each. Each of the strains in the sub-libraries had a unique barcode as part of the adapter ligated to the MspI cut site and a universal barcode is ligated to the MluC1 cut sites. Sample that have both MluC1 and MspI cut sites will only be sequenced. Adapter ligated DNA fragments from sub-libraries were cleaned with 1.5x Ampure bead solution. The fragments were then subjected to size selection to select those

that ranged from 350-450 bp in length using a Pippin-Prep platform (Sage Science, Beverly, MA, USA). The size selected DNA fragment then used as a template is subjected to PCR amplification using an indexed primer and a universal primer. Amplification step adds an index onto each sub-library so that they can be pooled in subsequent steps. Each sub-library was amplified in eight different runs to avoid PCR bias. The quality and concentration of the library was then assessed using Bioanalyzer Trace Analysis. The pooled library was then sequenced in a single lane of the Illumina MiSeq platform to obtain 300 bp paired-end reads.

Sequence data processing and single nucleotide variant detection. All the sequence data processing steps were performed using the Mobyle SNAP Workbench housed at North Carolina State University (Price and Carbone 2005; Aylor et al. 2006; Monacell and Carbone 2014). The raw sequence reads were first examined for the barcode and intact RAD cut sites and were then demultiplexed using the process_radtags program (Bolger et al. 2014). The program then filtered and corrected the reads by dropping those that had a Phred quality score below 10. Demultiplexed reads were then filtered again using Trimmomatic program where it trimmed the adapters, reads with Phred score below 15, leading and trailing low quality bases (below quality 3) and discarded reads less than 36 bases in length. The corrected and filtered paired reads thus obtained were then mapped to the reference genome, MSU-1 (Savory et al. 2012) using the Burrows-Wheeler Alignment Tool version 3.0 (Li and Durbin 2010). Reference-guided alignment files (SAM files) were then subjected to variant (SNP) discovery using Haplotype caller in GATK version 3.5 (DePristo et al. 2011) for a cohort of 48 strains at a time. SNPs inferred among cohorts of 48 sequenced isolates in a Variant Call Format (VCF) files were merged and was then used to conduct population genomic analysis using PLINK (Purcell et al. 2007), VCFTOOLS (DePristo et al. 2011) and

phylogenetic analysis using RAxML (Stamatakis 2006). The SNPs obtained from GATK were subjected to filtering with minor allele frequency filtering set at 0.1 and Max missing set at 0.9 to increase the accuracy of SNP calls across the strains. The values for minor allele frequency and Max filtering were chosen after validating the accuracy of SNP calls for the first 10 contigs on the genome browser created for *P. cubensis*. PLINK PED file obtained after the population genomic analysis was first converted into PHYLIP format and the sequences were then collapsed into haplotypes using the haplotype block workflow program implemented on the Mobyle SNAP Workbench. Compatibility among pairs of SNPs was used to determine the largest non-recombining partition of compatible SNPs for each contig using the CladeEx program (Bowden et al. 2008).

Population statistics, tests of neutrality and recombination analysis. Population diversity estimates, including the number of segregating sites, *s*, population mean mutation rate, θ_w (Watterson 1975) and haplotype diversity, Hd were estimated using DnaSP version 5.10 (Librado and Rozas, 2009). The minimum no. of recombination events were also estimated using DnaSP. Tests of neutrality including Tajima's *D* (Tajima 1989, Fu and Li's *D** and *F** (Fu and Li 1993) and Fu's F_S (Fu 1997) was conducted based on the non-recombining region from each sequence using DnaSP.

Population subdivision analysis. Wright's F_{ST}, a measure of the proportion of the total genetic variance contained in a subpopulation relative to the total genetic variance was estimated to detect evidence for population subdivision in *P. cubensis*. Values for F_{ST} can range from 0 to 1.0 and values greater than 0.05 would indicate a degree of differentiation among populations (Wright 1951). Population differentiation was further explored using SNAP Map to generate sequence files for Seqtomatrix, which then converted sequence file to

a distance matrix. The test, PermtestK then provided Hudson's estimates of K_S, K_T, K_{ST}, and S_{nn} and were used to determine the subdivision in populations (Hudson 2000, Hudson et al. 1992). A value of S_{nn} statistic close to 1 indicates high population subdivision and a value less than or equal to 0.5 indicates panmixia (Hudson 2000). A model-based Bayesian clustering approach using STRUCTURE v. 2.3.4 was used to assess patterns of genetic structure among the sample isolates (Pritchard et al. 2000). Three cycles of structure run each with a burn-in period of 300,000 and 500,000 permutations, were performed for K between 1 and 12. The optimum number of genetic clusters (K) was determined based on the posterior probabilities of the range of K values and the Evanno ΔK method using STRUCTURE HARVESTOR v. 0.6.93 (Earl and VonHoldt, 2012).

RESULTS

Sequence analysis. The average number of raw reads per individual was 250,000, and ranged from 24,638 to 400,622 reads. Filtering using Trimmomatic removed on an average 2.14% of the reads. More than 5000 RAD loci of size 350-450 bp were generated for the entire genome and a part of which was used in the population genomic analyses. After filtering, a total of 670 SNPs inferred from the reads that aligned to the largest 100 contigs of the reference genome using Genome Analysis ToolKit (GATK) were used for the population genomic analysis. A total of 186 alleles inferred from the 93 isolates were collapsed into 85 haplotypes and had a haplotype diversity of 97.6%. In total, 123 segregating sites were identified using SNAP MAP and the population mean mutation rate (θ_w) was estimated to be 21.21 using DnaSP (Table 5.2).

Neutrality and Population subdivision. Inferences on population processes were made based on tests of neutrality that assume constant population size, no recombination and no migration. Evidence for recombination was detected and thus, non-recombining partitions were used to conduct the tests of neutrality. Values for Tajima's *D*, Fu and Li's *D** and *F** were significant and positive and Fu's F_S value was also positive (Table 5.2) and suggested a balancing selection. Positive values of Tajima's *D* and Fu's F_S is indicative of intermediate frequency alleles, which are expected to occur under balancing selection or because of population subdivision. Maximum likelihood analysis conducted using RAxML showed divergence into two distinct evolutionary lineages, lineage I and lineage II (Fig. 5.1). A significant value ($P = 0.000$) for Hudson's test statistics ($K_S = 23.35$, $K_T = 50.76$, $K_{ST} = 0.54$) and Wright's F_{ST} ($F_{ST} > 0.05$) also provided evidence for subdivision among the population (Table 3). The level of genetic divergence between the two sub-populations (lineage I and II) was very great with $F_{ST} = 0.72$. STRUCTURE analysis also revealed clustering of composite samples into two groups (Fig. 5.4). Subsequently, the population was divided into two groups/lineages and the diversity estimates and neutrality tests were conducted for each lineage separately so that any genetic differentiation caused as a result of geographical separation is not confounded by having isolates from different host groups that may belong to the two lineages. (Table 5.2). Neutrality test statistics for lineage II suggested a non-neutral population growth and possibly a balancing selection. However, majority of the observed variation was between the alleles within the lineage (Fig. 5.2). Subpopulation within lineage I appears to be evolving in a neutral manner as indicated by non-significant values for Fu and Li's *D** and a marginally significant Tajima's *D* value ($P < 0.05$).

Population structure by host and geography. Any population structure within lineage II based on the host type they infected was not detected. Lineage II was composed of isolates collected mainly from cucumber, although few isolates collected on cantaloupe (*n* = 6) and pumpkin (*n* = 2) also belonged to lineage II. Lineage I comprised of isolates collected primarily on squashes, watermelon and pumpkin. Few isolates collected on cantaloupe (*n* = 6) and cucumber (*n* = 2) also belonged to lineage I (Fig. 5.5). Limited structure by host type was evident within lineage I (Fig. 5.3). A moderate genetic differentiation was detected between isolates collected on watermelon and those collected from squashes and pumpkin based on F_{ST} values and was also evident based on STRUCTURE analysis (Fig. 5.6) (Table 5.4). Population structure by geography was not evident in any of the analysis conducted (Table 5.3) except in case of lineage II population between south and northeast United States (S_{nn} = 0.674, *P* = 0.008). However, estimate of S_{nn} based on the non-recombining block suggested no genetic differentiation between south and northeastern population. No temporal structure was observed for isolates within individual lineages. The pre-epidemic isolate used in the current study (ind_1982) clustered together with isolates from watermelon in lineage I (Fig. 5.1).

DISCUSSION

This study examined the genetic variation among *P. cubensis* isolates in the United States using a reduced-representation library sequencing technique, double digest Restriction Associated DNA Sequencing technology (ddRADSeq) and provided inferences on the role of cucurbit host types and geography in shaping the population structure of *P. cubensis* in the United States. The pathogen population collected all across the eastern United States formed

two distinct genetic clusters and is in agreement with the previous studies that suggested the existence of two genetic clusters or lineages within *P. cubensis* (Thomas et al. 2014; Summers et al. 2015). A strict host specificity could not be inferred for the two lineages, however, a host specialization in a broader sense was observed. Isolates of *P. cubensis* were found to be largely uniform within each lineage and hence the direction of migration/dispersal of pathogen propagules could not be inferred from the present study.

Distinct genetic clusters among *P. cubensis* populations in the United States identified in the present study is in agreement with previous studies (Naegele et al. 2016; Quesada-Ocampo et al. 2012; Summers et al. 2015;). Quesada-Ocampo et al. (2012) investigated the genetic structure of *P. cubensis* population collected worldwide using five nuclear and two mitochondrial loci. They identified six genetic clusters among 465 isolates collected from five cucurbit host types (*Cucumis sativus, Cucumis melo, Cucurbita pepo, C. maxima, C. moschata*) and observed low genetic diversity among cucumber isolates as compared to isolates from other host types. Summers et al. (2015) examined the variation among 20 *P. cubensis* isolates collected from *Cucumis sativus, Cucumis melo, Cucurbita pepo* and *Cucurbita maxima* using Genotyping by Sequencing (GBS) and RNA-Seq analysis and identified two clusters among *P. cubensis* population with the distinction of squash isolates from cucumber, pumpkin and cantaloupe isolates. This was in agreement with our earlier report on the presence of two distinct evolutionarily lineages (lineage I and II) in the United States based on the whole genome sequence data from nine isolates collected on six cucurbits (*Cucumis sativus, Cucumis melo, Cucurbita pepo, C. maxima, C. moschata* and *Citrullus lanatus*) (Thomas et al. 2014). Lineage II comprised of isolates collected from cucumber, cantaloupe and giant pumpkin, whereas lineage I comprised of isolates collected from squash

and watermelon. Many previous studies have grouped isolates collected on pumpkin along with squash isolates based on pathotype and mating types (e.g., Cohen et al. 2012; Cohen et al. 2013,). Isolates collected on pumpkin have generally been shown to belong to pathotype 5 or 6 along with squash isolates and the A2mating type (Cohen et al. 2012; Cohen et al. 2015). However, studies by Thomas et al. (2014) and Summers et al. (2015) showed a distinction of pumpkin isolate from squash isolates. Further studies are needed to establish the basis of grouping of isolates from pumpkin with those from cucumber in lineage II.

Phylogenetic analysis, F_{ST} estimates, Hudson's statistics and STRUCTURE analysis provided strong support for this subdivision of the pathogen population into two lineages. Lineage II was composed primarily of isolates collected from cucumber and contained a few isolates collected from pumpkin and cantaloupe. Lineage I comprised mainly of isolates collected from squash, pumpkin and watermelon and also contained a few cucumber and cantaloupe isolates. The pre-epidemic isolate used in the present study grouped together with lineage I isolates. Within lineage I, a moderate genetic differentiation between isolates collected from watermelon and butternut squash, acorn squash and pumpkin was detected based on F_{ST} values and STRUCTURE analysis. This observation supports the classification of isolates capable of infecting watermelon but not squashes into a distinct pathotype, i.e., pathotype 4 (Thomas et al. 1987). The distribution of cantaloupe isolates was almost equal among lineage I and II. While lineage II was composed primarily of isolates from cucumber, it also contained a few from pumpkin. Similarly, while lineage I was composed primarily of isolates from squash, watermelon and pumpkin, it also contained a few isolates from cucumber. Hence, a strict host specificity for lineage I and lineage II isolates could not be directly inferred. However, a host specialization was clearly present with lineage II isolates

specialized on cucumber and lineage I specialized on squash, pumpkin and watermelon. A majority of pumpkin isolates showed genetic similarity with squash isolates. However, lineage II also did contain two isolates collected on pumpkin. These two pumpkin isolates may have originated from *C. maxima* (giant pumpkin) and while the other isolates in lineage I may have been collected from either *C. maxima* or *C. pepo* ssp. *pepo* (Jack-O-lantern type pumpkin). Evidence for compatibility of isolates belonging to pathotype 1and 3 (highly compatible with *Cucumis* sativus and *C. melo reticulatus*) on *Cucurbita maxima* but not on *C. pepo* ssp. *pepo* have been presented previously (Thomas et al. 1987; Anna Thomas et al. unpublished). A subset of lineage II isolates (n = 12) from the present study were tested for their compatibility with a set of host differentials to determine their pathotypes and they all belonged to either pathotypes 1 or 3 (Anna Thomas et al. unpublished).

Previous studies have suggested an association between cucurbit host types and mating types of *P. cubensis* where isolates collected from cucumber belonged to A1 mating type, while those collected from squash, watermelon and pumpkin belonged to A2 mating type (e.g., Cohen et al. 2012, 2013, 2015, Thomas et al. unpublished). An exception to this generalized observation was presented, where 3.3 % of isolates collected from cucumber exhibited an A2 mating type and similarly, 2.4 % of the isolates collected on squash belonged to A1 mating type (Cohen et al. 2013). This association has led to the suggestion that mating type and virulence may be genetically unlinked (Cohen et al. 2015). However, a better understanding of the genetic basis of distribution of different mating types on different host type is needed for a better understanding of this association. A subset of the isolates used in the current study (n = 36) was tested for their mating type and out of the 36 isolates, 15 belonged to A1 mating type and 21 belonged to A2 mating type. Interestingly, all A1

mating type isolates irrespective of the host of origin (cucumber, cantaloupe, pumpkin) belonged to lineage II and all the A2 mating types irrespective of the host of origin (cucumber, cantaloupe, pumpkin, squash, watermelon) belonged to lineage I. Thus, mating type of *P. cubensis* appears to be more strongly associated with the evolutionary lineage rather than the host from which the isolate originally obtained. Such associations between lineages and mating types have also been reported in other pathosytems (O'Hanlon et al. 2016).

The existence of two distinct genetic clusters within *P. cubensis* population reported in the present study is in agreement with previous studies conducted in Europe (Runge et al. 2011; Kitner et al. 2015). Clade 1 and 2 identified by Runge et al. (2011) contained herbarium samples of *P. cubensis* predominantly from the United States and *P. cubensis* isolates from East Asia, respectively. Kitner et al. (2015) also observed that all isolates collected before 2009 (collected mainly from cucumber) in the Czech Republic belonged to clade II and majority of the isolates sampled after 2009 that were associated with non-cucumber host types belonged to clade I. One *P. cubensis* isolate collected on cucumber in 2007 from the United States grouped with isolates in Clade 2 (Runge et al. et al. 2011). Lineage I and II identified in the present study is analogous with clade 1 and 2, respectively, as described by Runge et al. (2011).

The role of geography in shaping the genetic structure of *P. cubensis* has been previously examined at both the regional and global scale (Cespedes-Sanchez et al. 2015; Naegele et al. 2016; Polat et al. 2014; Quesada-Ocampo et al. 2012; Wallace et al. 2016). Polat et al. (2014) investigated the genetic variation among isolates collected from Turkey, Czech Republic and Israel using Sequence Related Amplified Polymorphism (SRAP) and

Inter-Simple Sequence Repeat (ISSR) markers and identified three clusters according to geographic locations. However, isolates collected from Turkey, Czech Republic showed a more uniform genetic background compared to isolates collected from Israel that seemed to be highly diverse compared to the other two clusters. It was evident from the that study that isolates with same genetic back ground collected in Turkey and Czech Republic originated from *C. sativus* and formed a distinct cluster as opposed to the *P. cubensis* population collected from Israel that originated from a diverse group of host types representing *Cucumis* spp., *Cucurbita* spp. and *Luffa* spp. Thus, it is possible that the existence of two host specialized lineages may have contributed largely to the differences observed between *P. cubensis* population in Israel compared to those in Turkey and the Czech Republic (Polat et al. 2014).

In the United States, outbreaks of CDM infection are believed to occur as a result of a contagion process and factors occurring on a spatial scale of up to 1000 km contribute to the spatial spread causing an aggregated pattern for initial outbreaks (Ojiambo and Holmes, 2010). Under such a scenario of dispersal, limited genetic variability would be expected between adjacent regions which would support our inability to detect genetic clustering by geographic region in the present study. However, our results are not consistent with those reported by Quesada-Ocampo et al. (2012), wherein a moderate to high genetic differentiation between isolates collected from Florida and North Carolina collected on cucumber and non-cucumber host types was observed. However, based on our findings on the subdivision of the population into two lineages, it is possible that the genetic differentiation reported by Quesada-Ocampo et al. (2012) might have been confounded because non-cucumber host group and cucumber host group may have had isolates that

belonged to two distinct genetic lineages in unequal proportions resulting in an exacerbation of actual genetic differentiation. A recent study conducted using isolates collected from Michigan and Canada (Naegele et al. 2016) identified genetic structure by geography within *P. cubensis* population when examined using nine simple sequence repeat markers. However, another study conducted in North Carolina using ten simple sequence repeat markers failed to detect a genetic structure within *P. cubensis* population by geography when isolates collected from the same host types from different locations were compared (Wallace et al. 2016). The genetic structure by geography observed in case of the study in Michigan (Naegele et al. 2016) could have been due to the inclusion of isolates collected on different host types that may have contained isolates belonging to both lineages.

In the current study, the source of inoculum for the seasonal epidemics of CDM in the United States could not be directly inferred. The population was largely genetically uniform within each lienage across the eastern United States. This could partly be due to the annual extinction and re-colonization events in states above the 30 degree latitude where winter temperature do not allow the pathogen to survive on the host in the field. In such situations, epidemics are expected to be re-initiated annually from a genetic pool of *P. cubensis* from the same source population in southern Florida and as hosts become available, the pathogen reproduces asexually and disseminate aerially through wind-borne sporangia to greater distances (Ojiambo and Holmes, 2011). The large population size may aid in the generation of variation due to random mutations during the season, but the absence of any opportunities to overwinter either as an oospore or on a perennial host may limit the ability to generate variations at a local scale. Identification and development of markers polymorphic to individuals belonging to lineages separately might help capture more variation present across

individual lineages and may be of value to consider for future studies examining genetic structure of *P. cubensis* population in the United States.

In conclusion, results from the present study strongly support the existence of two distinct host specialized lineages in the United States and a distinct genetic group within lineage I that is specialized on watermelon. The distinct lineages must be taken into account while breeding for host resistance and fungicide efficacy assays. The current population of *P. cubensis* appears to be uniform across the United States within each lineage. The present study also provides support that the lineage potentially responsible for the resurgence of CDM in the US is of the opposite mating type as compared to the older population. The distinctiveness between the two lineages may diminish with time if gene flow occurs between lineages (Thomas et al. Unpublished) particualry if the pathogen is able to sexually reproduce in nature, an outcome of which will be the generation of novel genetic variations that would facilitate the ability of *P. cubensis* to overcome host resistance and develop resistance to fungicides currently used to manage CDM.

ACKNOWLEDGEMENTS

This study was supported by Hatch Funds from North Carolina Agricultural Experiment Station for Project Number NC02251 and NC02432. We thank collaborators on CDM ipmPIPE project for collecting field samples used in this study and Barbara Stevens and Tanner Schwarz for their assistance with laboratory and greenhouse aspects of this work.

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Table 5.1. Source and description of *Pseudoperonospora cubensis* isolates collected from different cucurbits to assess genetic variation among the pathogen population in the United States.

Strain_ID	Year	State	Region[a]	Host	Mating type
A11	2012	North Carolina	S	Cucumber	A1
2013A1	2013	Florida	S	Cucumber	A1
2013A2	2013	Florida	S	Cucumber	A1
2013A3	2013	Georgia	S	Cucumber	A1
2013A5	2013	North Carolina	S	Cucumber	A1
2013A10	2013	South Carolina	S	Cucumber	A1
06A1	2006	North Carolina	S	Cucumber	nt
2013A13	2013	Alabama	S	Cucumber	nt
2013A4_a	2013	Florida	S	Cucumber	nt
2013A4_b	2013	Florida	S	Cucumber	nt
2013A4_c	2013	Florida	S	Cucumber	nt
2013A80	2013	Louisiana	S	Cucumber	nt
2014A1	2014	Florida	S	Cucumber	A2
Ind_1982	1982	South Carolina	S	Cantaloupe	A2
07B1	2007	North Carolina	S	Cantaloupe	A2
2013B24	2013	North Carolina	S	Cantaloupe	A1
2014B1	2014	Florida	S	Cantaloupe	nt
2014B2	2013	South Carolina	S	Cantaloupe	nt
2014B4	2014	North Carolina	S	cantaloupe	nt
2014C11	2014	North Carolina	S	Pumpkin	nt
2013C3	2013	North Carolina	S	Pumpkin	A1
2013C19	2013	North Carolina	S	Pumpkin	nt
2014C14	2014	North Carolina	S	Pumpkin	nt
2014C15	2014	North Carolina	S	Pumpkin	nt
2014C2	2014	North Carolina	S	Pumpkin	nt
2014C3	2014	North Carolina	S	Pumpkin	nt
2014D1	2014	Florida	S	Butternut squash	nt
D3	2012	South Carolina	S	Butternut squash	A2
2013D1	2013	Florida	S	Butternut squash	A2
2013D2	2013	Florida	S	Butternut squash	A2
2013D3_1	2013	South Carolina	S	Butternut squash	A2
2013D4	2013	North Carolina	S	Butternut squash	A2
2013D6	2013	Alabama	S	Butternut squash	A2
2013D18	2013	North Carolina	S	Butternut squash	nt
2013D3_2	2013	South Carolina	S	Butternut squash	nt
2013D3_3	2013	South Carolina	S	Butternut squash	nt

Table 5.1. Continued

2013_E12	2013	South Carolina	S	Watermelon	nt
2013_E7	2013	Georgia	S	Watermelon	nt
08_E1	2008	Texas	S	Watermelon	nt
2013_E1	2013	Florida	S	Watermelon	A2
2014_E2	2014	Texas	S	Watermelon	nt
05F1	2005	North Carolina	S	Acorn squash	A2
08F1	2008	Georgia	S	Acorn squash	A2
2013F1	2013	Florida	S	Acorn squash	A2
2013F2	2013	South Carolina	S	Acorn squash	A2
2013F12	2013	South Carolina	S	Acorn squash	nt
2013F15	2013	North Carolina	S	Acorn squash	nt
2013F16	2013	Louisiana	S	Acorn squash	nt
2013F5	2013	North Carolina	S	Acorn squash	nt
2014G1	2014	Tennessee	S	Summer squash	nt
2014G2	2014	Tennessee	S	Summer squash	nt
2013A18	2013	New Jersey	NE	Cucumber	A1
2013A53	2013	New York	NE	Cucumber	nt
2013A58	2013	Rhode Island	NE	Cucumber	nt
2013A60	2013	New York	NE	Cucumber	nt
2013A37	2013	Kentucky	NE	Cucumber	nt
2013A66	2013	New York	NE	Cucumber	nt
2014A15	2014	New York	NE	Cucumber	nt
2014B10	2014	New Jersey	NE	Cantaloupe	nt
2013B17	2013	New York	NE	Cantaloupe	A1
2013B12	2013	New York	NE	Cantaloupe	nt
2013B8	2013	Pennsylvania	NE	Cantaloupe	nt
2014C4	2014	Massachusetts	NE	Pumpkin	nt
2014C13	2014	Maryland	NE	Pumpkin	nt
08C1	2008	New York	NE	Pumpkin	A1
2013C14	2013	Virginia	NE	Pumpkin	A2
2013C18	2013	New York	NE	Pumpkin	A2
2013D12	2013	Maryland	NE	Butternut squash	nt
2013D13	2013	Virginia	NE	Butternut squash	nt
2013D16	2013	New York	NE	Butternut squash	nt
2014_E6	2014	Maryland	NE	Watermelon	nt
2013F11	2013	New York	NE	Acorn squash	nt
2013F7	2013	Massachusetts	NE	Acorn squash	A2
2013A19	2013	Ohio	GL	Cucumber	A1
2013A20	2013	Ohio	GL	Cucumber	A1

Table 5.1. Continued

2013A23	2013	Michigan	GL	Cucumber	nt
2013A65	2013	Ohio	GL	Cucumber	nt
2013A68	2013	Michigan	GL	Cucumber	nt
2013A70	2013	Indiana	GL	Cucumber	nt
2013A72	2013	Wisconsin	GL	Cucumber	A2
2013B10	2013	Ohio	GL	Cantaloupe	A1
2013B15	2013	Wisconsin	GL	Cantaloupe	A2
2013C20	2013	Illinois	GL	Pumpkin	A2
2014C10	2014	Ohio	GL	Pumpkin	nt
2014C12	2014	Ohio	GL	Pumpkin	nt
2013D15	2013	Wisconsin	GL	Butternut squash	nt
2013D17	2013	Indiana	GL	Butternut squash	A2
2013D19	2013	Wisconsin	GL	Butternut squash	nt
2014D4	2014	Indiana	GL	Butternut squash	nt
2013F10	2013	Ohio	GL	Acorn squash	nt
2014F4	2014	Indiana	GL	Acorn squash	nt
2014G4	2014	Ohio	GL	Summer squash	nt
08A1	2008	California	CA	Cucumber	A1

[a]:S: Southern includes states of FL, GA, AL, LA, TX, SC, NC and TN; NE: Northeast includes states of VA, WV, MD, NJ, NY, MA, KY, PA, RI and CT; GL: Great Lakes includes the states of MI, OH, WI, IN and IL.

Table 5.2. Summary on population statistics and neutrality tests for whole population and for lineage I and lineage II of *Pseudoperonospora cubensis* population in the United States.

	Population statistics							Tests of neutrality			
Population	*l*	*n*	*s*	π	Rm	H_d	θ_w	Tajima's *D* statistic	Fu and Li's *D** statistic	Fu and Li's *F** statistic	Fu's F_s statistic
All	123	186	123	0.412	25	0.976	21.21	4.53***	2.55**	4.13**	59.42
Lineage I	183	118	88	0.183	30	0.992	16.47	2.51*	-0.64^{ns}	0.79^{ns}	13.45
Lineage II	293	68	56	0.082	14	0.982	11.69	4.35***	1.68**	3.21**	17.19

l, Consensus sequence length excluding sites with gaps; *n*, sample size; *s*, segregating nucleotide sites; π, average number of nucleotide differences per site; Hd, Haplotype diversity; Rm, minimum number of recombination events; θ, Population mean mutation rate or Watterson's estimate of theta: defined as $\theta = s/(1 + ½ + 1/3 + \ldots + 1/n\text{-}1)$, where *s* is the number of segregating sites and *n* is the sample size. ^{ns}, not significant (P >0.01); *0.05 < P < 0.1; **0.01 < P < 0.05; ***0.001 <P < 0.01.

Table 5.3. Summary of subdivision test statistics K_S, K_T, K_{ST}, and S_{nn} and Wright's index F_{ST} between lineages and geographical region for each lineage of *Pseudoperonospora cubensis* population in the United States.

	Statistic[b]						
Population[a]	K_S	K_T	K_{ST}	*P* value	S_{nn}	*P* value	F_{ST}
All (LI = 118, LII = 68)							
LI vs. LII	23.353	50.765	0.540	0.000	1.000	0.000	0.721
Lineage I (S = 70, NE = 22, GL = 24)							
S vs. NE	67.558	67.009	-0.008	0.913	0.597	0.774	-0.026
S vs. GL	81.344	80.823	-0.006	0.735	0.674	0.106	-0.022
NE vs. GL	73.454	72.231	-0.017	0.852	0.413	0.828	-0.034
Lineage II (S = 30, NE= 22, GL = 14)							
S vs. NE	35.189	34.863	-0.009	0.571	0.674	0.008	-0.027
S vs. GL	36.563	35.864	-0.019	0.968	0.520	0.695	-0.046
NE vs. GL	35.878	35.093	-0.022	0.899	0.500	0.555	-0.047

LI = Lineage I; LII = lineage II; S = southern, NE = northeast, and GL = Great Lakes region of the United States.

[b] K_S: Average no. of differences between sequences within population; K_T: Average no. of differences between sequences in the total population; $K_{ST} = 1- (K_S/K_T)$; S_{nn}: nearest-neighbor statistic; F_{ST}: Wright's fixation index

Table 5.4. Genetic differentiation of isolates collected from different host types within lineage I of *Pseudoperonospora cubensis* based on Wright's F_{ST}.

Host type	Butternut squash	Watermelon	Acorn squash	Pumpkin
Butternut squash	....			
Watermelon	**0.104**	....		
Acorn squash	-0.022	**0.088**	....	
Pumpkin	-0.025	**0.051**	-0.030	....
Cantaloupe	-0.008	-0.015	-0.021	-0.019

Numbers in bold indicate moderate genetic differentiation (0.05-0.15) according to Hartl and Clark(1997).

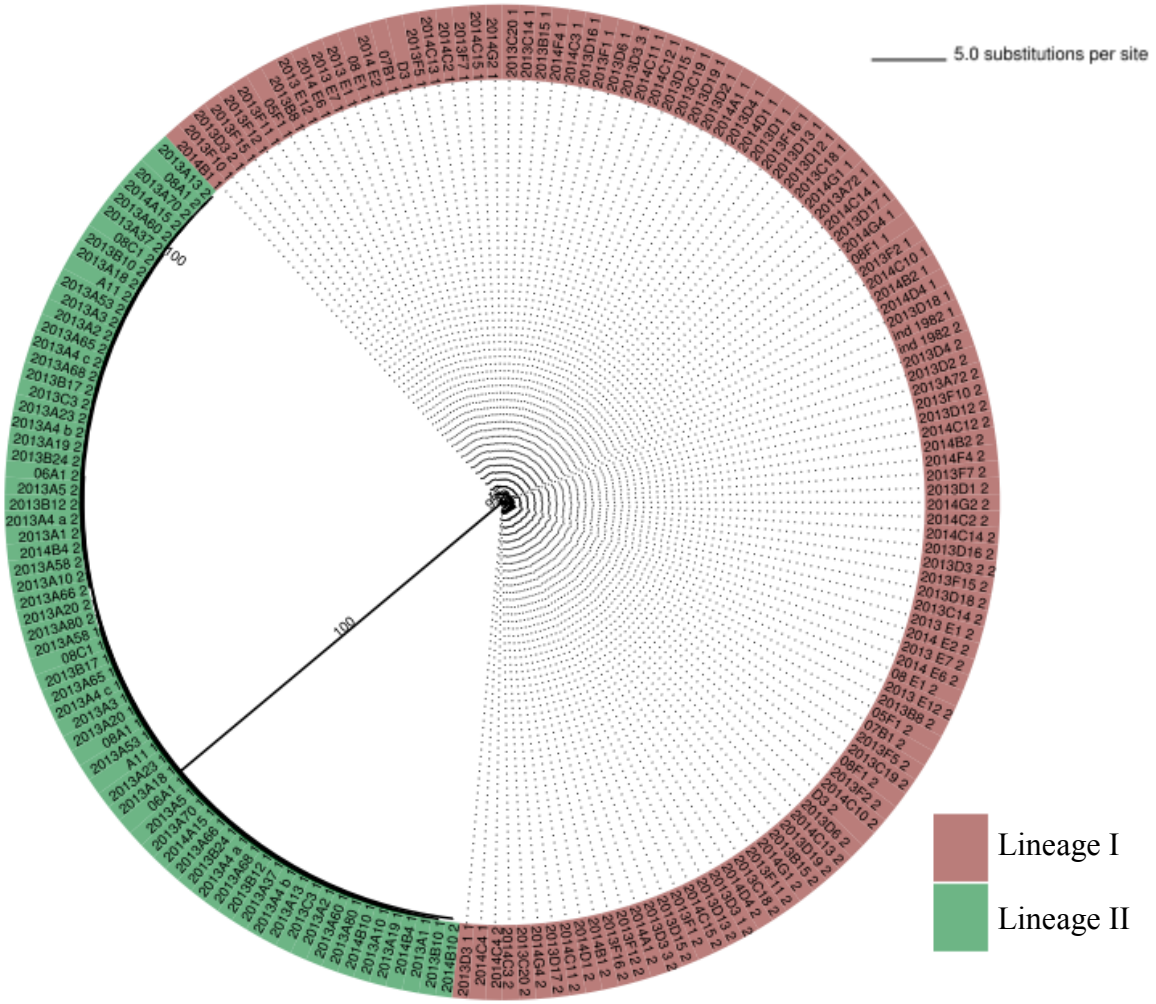

Figure 5.1. Best maximum likelihood phylogenetic tree based on allelic variation across first 100 contigs (670 SNPs) for 93 isolates of *Pseudoperonospora cubensis*. Branch tips highlighted in brown represents lineage I while the one highlighted in green represents lineage II.

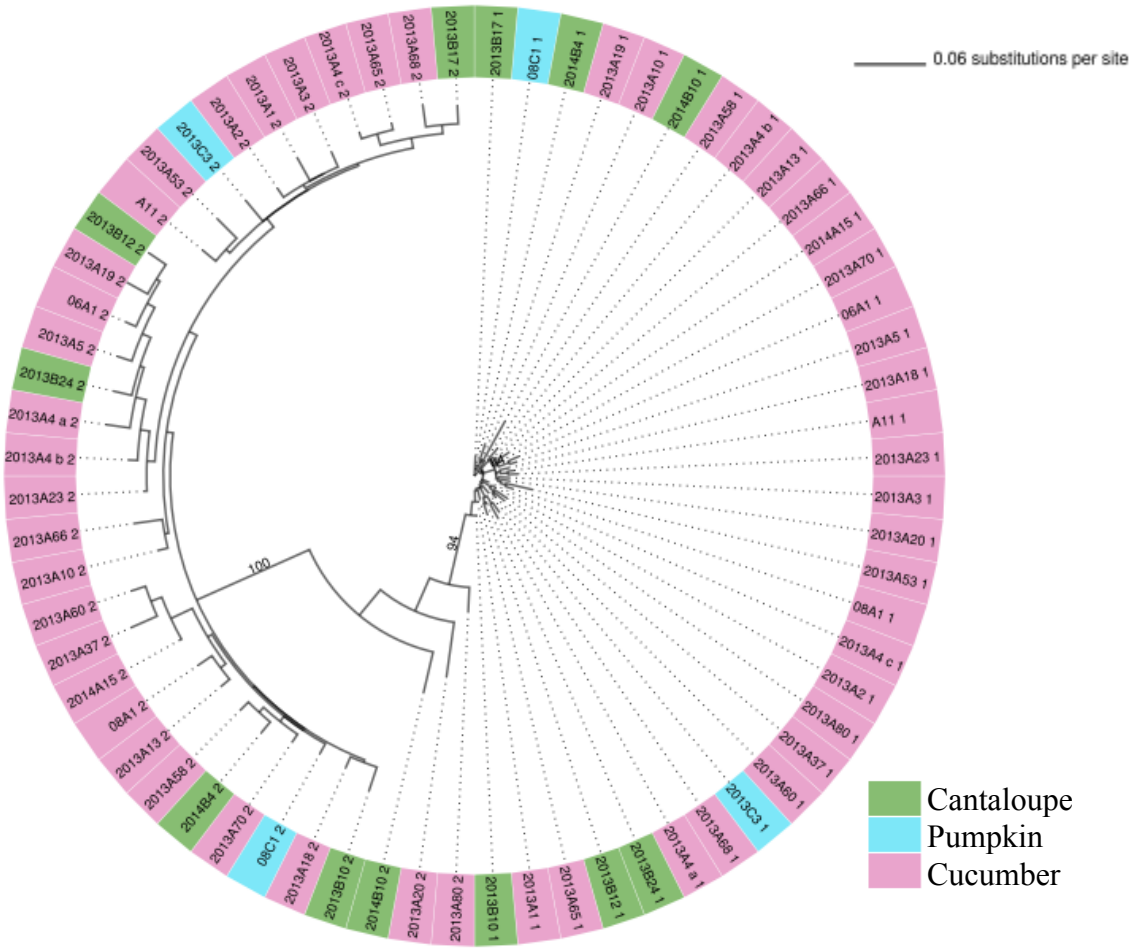

Figure 5.2. Best maximum likelihood phylogenetic tree based on allelic variation across first 100 contigs for 34 isolates of *Pseudoperonospora cubensis* that constitute lineage II.

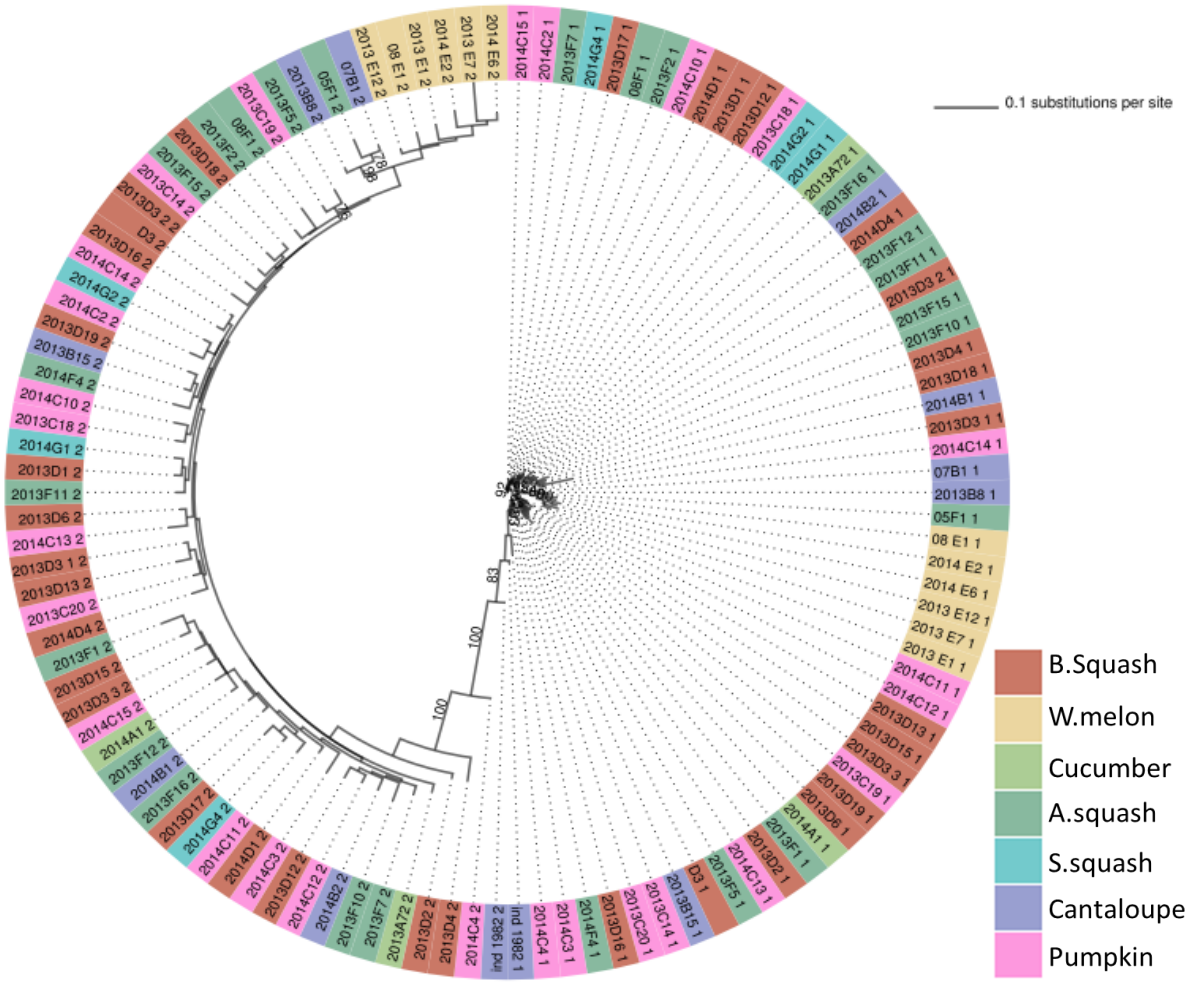

Figure 5.3. Best maximum likelihood phylogenetic tree based on allelic variation across first 100 contigs for 59 isolates of *Pseudoperonospora cubensis* that constitutes lineage I. Legend: 'B.Squash' refers to butternut squash, 'W.melon' refers to watermelon, 'A.squash' refers to acorn squash, and 'S.squash' refers to summer squash.

Figure 5.4. Genetic structure of *Pseudoperonospora cubensis* isolates (*n* = 93) grouped by lineages.

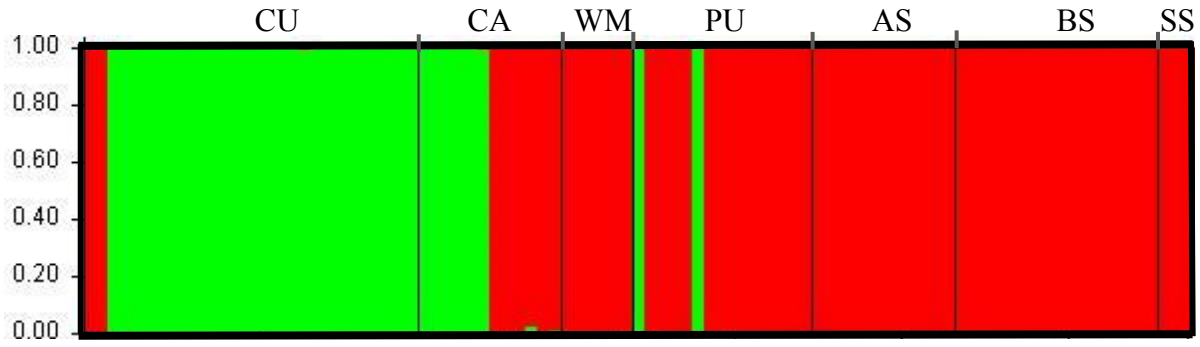

Figure 5.5. Genetic structure of *Pseudoperonospora cubensis* isolates (*n* = 93) when grouped by host of origin. CU: cucumber, CA: cantaloupe, WM: watermelon, PU: pumpkin, AS: acorn squash, BS: butternut squash, SS: summer squash.

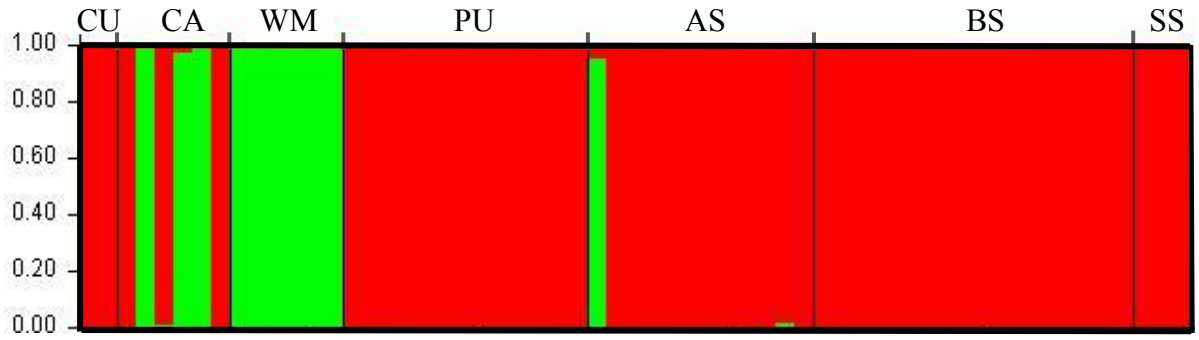

Figure 5.6. Genetic structure of *Pseudoperonospora cubensis* isolates (*n* = 59) within lineage I when grouped by host of origin. CU: cucumber, CA: cantaloupe, WM: watermelon, PU: pumpkin, AS: acorn squash, BS: butternut squash, SS: summer squash.

CHAPTER 6

Conclusions

The research reported in this dissertation provides insights into the possible causes of the resurgence of cucurbit downy mildew (CDM) in the United States in 2004. The disease is caused by the oomycete, *Pseudoperonospora cubensis*, and several hypotheses including the introduction of a new pathotype, a new lineage or a new genetic recombinant of the pathogen, were explored in this project. The findings generated from this research provided a comprehensive and evidence-based explanation for the resurgence of CDM in the United States. In addition, this study sheds light on the role that cucurbit host types and geographic location of disease epidemics play in shaping the genetic structure of *P. cubensis* population in the United States.

The study reported here, for the first time, documents the presence of opposite mating types of *P. cubensis* in the United States. Pairing of *P. cubensis* isolates with known mating type testers obtained from Israel revealed the presence of A1 and A2 mating types in almost equal proportions in the United States. An association of mating types with host was evident in our study, whereby A1 mating type isolates were found primarily on cucumber, while the A2 mating type isolates were associated primarily with squashes and watermelons. About one third of the oospores produced under in-vitro conditions were viable suggesting that they could serve as potential sources of initial inoculum in eastern United States from northern Florida to the Northeast and Great Lakes regions.

Investigations of the pathogenic variation among *P. cubensis* population using a set of 22 isolates based on a host compatibility assay with fifteen cucurbit host types revealed a

high degree of pathogenic variability among the pathogen population and led to the identification of five pathotypes (1, 3, 4, 5 and 6) in the United States. Of these pathotypes, pathotypes 1, 3 and 6 were previously unknown to be present in the United States and were documented for the first time in this study. Only isolates belonging to pathotypes 1 and 3 were compatible with Poinsett 76, a cucumber cultivar known to be resistant to CDM prior to 2004. This finding suggested that the isolates of pathotype 1 or 3 might have been responsible for the resurgence of CDM in the United States in 2004.

In the current study, we identified the presence of two distinct evolutionary lineages among the *P. cubensis* population in the United States based on a comparative whole genomic analysis of nine isolates collected from diverse host types in the United States. Many fixed polymorphisms separated one lineage that included isolates from acorn squash, butternut squash and watermelon from a second lineage that comprised of isolates from *Cucumis* spp. and pumpkin. In addition, phylogenetic analysis showed that *P. cubensis* and its sister species, *P. humuli*, shared a recent common ancestor. A reconstruction of ancestral recombination suggested a hybrid origin of lineage II of *P. cubensis*, with *P. humuli* and lineage I of *P. cubensis* as putative parents.

The presence of two lineages among *P. cubensis* population across the United States was validated using a robust set of isolates collected on diverse host types. Lineage II that comprised primarily of isolates from cucumber also contained a few pumpkin and cantaloupe isolates. Lineage I was composed mainly of isolates collected on acorn squash, butternut squash, pumpkin and watermelon but also contained a few isolates from cantaloupe and cucumber. These results indicate that while the lineages of *P. cubensis* appear to be host specialized, they are not host specific. In addition, an association of lineage II with A1

mating type and pathotypes 1 and 3 and lineage I with A2 mating type and pathotypes 4, 5 and 6 was also observed. A distinct cluster within lineage I that was associated with isolates collected mainly from watermelon was identified and this further helps to explain the classification of isolates capable of infecting watermelon but not squashes into a separate group, pathotype 4. The pre-epidemic isolate used in this study belonged to lineage I, A2 mating type and pathotype 4. The association of lineage II isolates with the new pathotypes (pathotypes 1 and 3), their specialization on cucumber and lower genetic diversity as compared to lineage I indicates that lineage II might have been responsible for the resurgence of CDM in the United States. The population of *P. cubensis* within each lineage was largely uniform across the United States and no genetic structure by geography could be identified. Thus, a strict inference on the source of initial inoculum for seasonal epidemics in the United States could not be drawn. Oospore production suggests that *P. cubensis* can undergo sexual recombination, a process that may help the pathogen to evolve rapidly and overcome host resistance and fungicides that are currently used for CDM management. The findings presented in this study also provides a framework for the development of diagnostic tools that may aid in the detection of lineages of *P. cubensis* which may help growers to better target management efforts for effective management of CDM.