The Genetic Structure of *Pseudoperonospora cubensis* Populations

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Abstract

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Pseudoperonospora cubensis is a destructive foliar pathogen of economically important cucurbitaceous crops in the United States and worldwide. In this study, we investigated the genetic structure of 465 *P. cubensis* isolates from three continents, 13 countries, 19 states of the United States, and five host species using five nuclear and two mitochondrial loci. Bayesian clustering resolved six genetic clusters and suggested some population structure by geographic origin and host, because some clusters occurred more or less frequently in particular categories. All of the genetic clusters were present in the sampling from North America and Europe. Differences in cluster occurrence

were observed by country and state. Isolates from cucumber had different cluster composition and lower genetic diversity than isolates from other cucurbits. Because genetic structuring was detected, isolates that represent the genetic variation in *P. cubensis* should be used when developing diagnostic tools, fungicides, and resistant host varieties. Although this study provides an initial map of global population structure of *P. cubensis*, future genotyping of isolates could reveal population structure within specific geographic regions, across a wider range of hosts, or during different time points during the growing season.

Pseudoperonospora cubensis (Berk. & M.A. Curtis) Rostovzev is the causal agent of cucurbit downy mildew, a destructive foliar disease reported in more than 70 countries (24,58). *P. cubensis* causes disease on approximately 20 genera of cucurbits (approximately 60 species) including cucumber (*Cucumis sativus*), cantaloupe (*C. melo*), pumpkin (*Cucurbita maxima*), watermelon (*Citrullus lanatus*), squash (*Cucurbita pepo*), gourd (*C. moschata*), and several wild Cucurbitaceae species (29,41,51,62). There may exist two distinct subspecies or species that parasitize cucurbits within the *P. cubensis* species complex (50) (*P. cubensis* sensu lato, which is referred to as *P. cubensis* in this article), and different pathotypes and physiological races in *P. cubensis* have been previously described (24,55).

Cucurbit downy mildew has been a major pathogen in Europe since the mid-1980s (27), and severe disease pressure results in a reduction in quantity and quality of cucurbit fruit production (26). In the United States (U.S.), host resistance successfully limited downy mildew of cucumber for many years. Since 2004, resistant cucumber varieties are no longer sufficient to control *P. cubensis* in the U.S., and severe economic losses result if fungicides are not applied frequently (21). High costs associated with fungicide use and fungicide-resistant populations of *P. cubensis* that have been documented in several regions challenge the profitability of the industry (34,36,46,53,63,64,67).

Because *P. cubensis* is an obligate parasite, its survival is dependent on the availability of a susceptible host (8). The pathogen overwinters (3) in areas with mild winter temperatures such as the southern U.S. and in greenhouses that produce cucurbits year-

round (61). Sporangia are primarily wind dispersed within and between fields (61), and *P. cubensis* spreads northward each growing season on northerly winds (14,20,65). In the U.S., it is currently thought that *P. cubensis* overwinters in southern Florida, which provides a source of inoculum for states as far north as North Carolina. It has been hypothesized that the Great Lakes and mid-Atlantic states receive inoculum through dispersal of sporangia from North Carolina and surrounding states or from greenhouses that produce cucumber year-round in neighboring regions (40).

Populations of *P. cubensis* have been previously characterized for pathogenicity, virulence and fungicide resistance (25,28), and populations have been studied using traditional phylogenetic methods and a single nuclear marker (nuclear internal transcribed spacer [nrITS]) in the Czech Republic, Greece, the Netherlands, and France, where this pathogen causes significant losses (54). Such investigations provided some information about local genetic diversity of *P. cubensis*. No genetic structure was noted by pathogenicity or virulence but isolates showed structure by geographic origin (54). It is currently unknown whether *P. cubensis* populations are structured by geography across continental scales or within the U.S., although Runge et al. (50) observed some spatiotemporal structure in their phylogenetic investigation.

Bayesian clustering has recently been used to visualize overall patterns of genetic structure in other oomycete (18,44) and fungal (5,16) pathogens. In this study, we used Bayesian clustering and genetic diversity and differentiation estimates to examine the genetic structure of worldwide *P. cubensis* sensu lato populations. Specifically, we sought to establish whether *P. cubensis* worldwide populations show significant population structure and genetic differentiation when grouped by geography and host of origin. We also examined population structure by year for isolates collected in the U.S. This study provides an initial map of the global population structure of *P. cubensis*.

Materials and Methods

Isolate selection and maintenance. In total, 465 *P. cubensis* isolates originating from diverse hosts throughout the world were obtained from colleagues or selected from the collection main-

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*The e-Xtra logo stands for “electronic extra” and indicates that a Supplementary table is available online.

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tained in the laboratory of Dr. Hausbeck at Michigan State University (MSU) (Supplementary Table S1). Samples included sporangial suspensions and individual leaf lesions that were used for DNA extractions. To obtain sporangial suspensions, *P. cubensis* sporangia were removed from leaves by pipetting sterile distilled water onto lesions on the leaf, dislodging sporangia using an ethanol-disinfested bacterial loop, and using a pipette to transfer the spore suspension from the leaf into a 2.2-ml sterile microcentrifuge tube. When sporulation was not present on the leaf, lesions that displayed symptoms typical of downy mildew were excised with a sterile scalpel and placed into a 2.2-ml sterile microcentrifuge tube.

DNA extraction. Contents of microcentrifuge tubes with sporangia suspensions or leaf lesions were transferred to a mortar and ground with a pestle in liquid nitrogen. All implements were previously sterilized. The DNeasy Plant Minikit (Qiagen) was used to extract genomic DNA according to the manufacturer's instructions. DNA was quantified using the NanoDrop ND 1000 spectrophotometer and NanoDrop 2.4.7c software (NanoDrop Technologies Inc.). DNA integrity was analyzed by electrophoresis in 1% (wt/vol) agarose gel in 0.5× Tris-borate-EDTA buffer (32), and stained with ethidium bromide (5 µg/ml) for visualization.

Primer design, DNA amplification, and sequencing. GenBank was used to identify genomic regions as sources of single nucleotide polymorphism markers (Table 1). Selected regions were previously used in oomycete coalescence, genetic diversity, and phylogenetic studies (7,13,17,42). Oomycete candidate genes were compared with the preliminary data of the *P. cubensis* genome

project (courtesy B. Day, MSU) using BLAST (33). Sequences from GenBank and the *P. cubensis* genome were used as input data in Primer3 (49) for primer design using default settings. Two regions of the mitochondrial genome (subunit 1 and 5 of the NADH dehydrogenase gene [*Nad1* and *Nad5*, respectively] and five nuclear genes (*β-tubulin*, *glucose phosphate isomerase* [*gpi*], nrITS, large ribosomal subunit [nrLSU], and *TigA* gene fusion protein [*TigA*]) were amplified by polymerase chain reaction (PCR). After collecting preliminary sequence data, additional nested primers were designed for *TigA* to increase sequencing quality. The forward primer for nrITS (12) and both nrLSU primers (35,47) were obtained from previously published literature. A 25-µl-volume PCR was executed using 1 µl of DNA at 5 ng/µl, 5 µl of 5× PCR reaction buffer (Invitrogen), 1.25 µl of 25 mM MgCl₂ (Invitrogen), 0.5 µl of 10 mM dNTP mix (Invitrogen), 1 µl of each 10 µM primer (MSU Macromolecular Structure Facility), 0.7 µl of Platinum Taq DNA polymerase (Invitrogen), and 14.6 µl of sterile water. A Mastercycler thermal cycler (Eppendorf) was used to run the PCR as follows: 3 min of denaturation at 94°C; followed by 45 cycles at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 60 s; with a final extension step of 10 min at 72°C. PCR products were analyzed by electrophoresis in 1% (wt/vol) agarose gel in 0.5× Tris-borate-EDTA buffer (32), stained with ethidium bromide (5 µg/ml) for visualization, and compared with a 100-bp ladder (Invitrogen). Controls without *P. cubensis* DNA were included.

ExoSAP-IT (Affymetrix, Inc.) was used according to the manufacturer's protocol to purify PCR products, which were subse-

Table 1. Mitochondrial and nuclear genes (subunit 1 [*Nad1*] and 5 [*Nad5*] of the NADH dehydrogenase gene, *β-tubulin*, glucose phosphate isomerase [*gpi*], nuclear ribosomal internal transcribed spacer [nrITS], large nuclear ribosomal subunit [nrLSU], and *TigA* gene fusion protein [*TigA*]) analyzed in *Pseudoperonospora cubensis*

Target DNA	Primer	Primer sequence	Annealing temperature (°C)	Sequenced fragment (bp)	Analyzed region (bp)	Source
Mitochondrial						
<i>Nad1</i>	Nad1-F	5'TGGACGGTAATCCCTTTAGGA3'	59	499	425	DQ361222
	Nad2-R	5'ATGGTGCTAACCACCT3'	59	...	...	...
<i>Nad5</i>	Nad5-F	5'TGCAGCAACAATGGTAACAG3'	58	320	255	AY423338
	Nad5-R	5'CTTGCTCATCACCCATTGC3'	58	...	...	...
Nuclear						
<i>β-tubulin</i>	BTub-F	5'GTCGAAAATGCCGATGAAGT3'	60	419	350	DQ361162
	BTub-R	5'TTCAGCATTTGCTCGTCAAC3'	60	...	...	...
<i>gpi</i>	GPI-F	5'TGGTAAGCGTGTAAGCATGG3'	59	430	365	Draft genome
	GPI-R	5'GTTGATGCCCCAGATAGAGC3'	59	...	...	...
nrITS	ITS-F	5'GAGGGACTTTTGGGTAATCA3'	59	700	620	12
	ITS-R	5'TCCTCCGCTTATTGATATGC3'	59	...	...	Draft genome
nrLSU	LSU-F	5'CGCCAGACGAGCTTACC3'	59	800	500	47
	LSU-R	5'ACCCGCTGAACCTTAAGC3'	59	...	...	35
<i>TigA</i>	TigA-F	5'GACATTGACGGATTCTCTCGT3'	59	511	400	Draft genome
	TigA-R	5'CTCGTACAGCTCGTGGTTCA3'	59	...	...	...
	TigA-R	5'CGTTCACAACGATGTGCTTT3'	60	298	200	Sequenced product

Table 2. Diversity estimates, neutrality tests, and recombination for target mitochondrial and nuclear genes in *Pseudoperonospora cubensis*

Target DNA ^d	Diversity estimates ^a						Recombination estimates ^b		Neutrality tests ^c		
	s (%)	h	Hd	π	θw	k	R	RM	Tajima's D	Fu and Li's D	Fu and Li's F
Mitochondrial											
<i>Nad1</i>	2 (0.5)	4	0.48	0.001	0.3	0.56	0	0	1.08	0.59	0.89
<i>Nad5</i>	2 (0.8)	3	0.34	0.001	0.3	0.34	0	0	0.19	0.59	0.55
Nuclear											
<i>β-tubulin</i>	5 (1.4)	9	0.34	0.001	0.68	0.5	0.40	1	-0.42	0.87	0.51
<i>gpi</i>	2 (0.5)	3	0.04	0	0.27	0.04	0	0	-0.95	0.56	0.07
nrITS	13 (2.1)	19	0.31	0.001	1.75	0.57	0.001	3	-1.45	0.59	-0.25
nrLSU	19 (2.7)	41	0.19	0.001	2.56	0.83	0.001	8	-1.57	0.1	-0.08
<i>TigA</i>	7 (1.8)	15	0.60	0.004	0.94	0.8	4.2	2	-0.28	1.02	0.67

^a Abbreviations: s, number of polymorphisms; h, number of haplotypes per gene; Hd, haplotype diversity; π, nucleotide diversity; θw, Watterson's theta estimator per gene from sequence; k, average number of nucleotide differences.

^b R, recombination parameter and RM, minimum number of recombination events.

^c For Fu and Li's D and Fu and Li's F, none of the neutrality tests were significant at 0.05.

^d Subunit 1 (*Nad1*) and 5 (*Nad5*) of the NADH dehydrogenase gene, *β-tubulin*, glucose phosphate isomerase (*gpi*), nuclear ribosomal internal transcribed spacer (nrITS), large nuclear ribosomal subunit (nrLSU), and *TigA* gene fusion protein (*TigA*).

Fig. 1. Genetic structure of *Pseudoperonospora cubensis* isolates by geographic origin. Geographic origin: **A**, North America; **B**, Europe and Asia. Each isolate is represented by a thin bar, which is partitioned into colored segments each representing the individual's proportionate genetic membership in a given Kth cluster. Cluster colors correspond to red = 1, purple = 2, yellow = 3, green = 4, blue = 5, and turquoise = 6.

quently sent to the Michigan State University Research Technology Support Facility (East Lansing) for sequencing. An ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) was used to complete two cycle-sequencing reactions (1 μ l of purified PCR product, 3 μ l of primer, and 8 μ l of sterile water) directly from the clean PCR products. Haplotypes were resolved according to the methods outlined by Quesada-Ocampo et al. (44).

Sequence analysis. Lasergene SeqMan Pro (version 8.0; DNASTAR Inc.) was used for manual editing of base calls and for sequence alignment. FASTA files were imported into MacClade (31) and converted to NEXUS files for subsequent analyses. Sequences from mitochondrial and nuclear loci were analyzed individually and collapsed into unique single-locus haplotypes using DnaSPv5 (30). Haplotypes found for each gene were deposited in GenBank under accession numbers HQ636479 to HQ636573. Haplotype sequences were compared with sequence data from the *P. cubensis* genome project (courtesy B. Day, MSU) by using nucleotide BLAST (1,2) to determine that data corresponded to the *P. cubensis* target genes.

DNA sequence variability, neutrality tests, and recombination. Polymorphism, neutrality, and recombination analysis were carried

out on individual loci for all samples and for isolates grouped in host (*Cucumis sativus* or other host), geographic (continent, country, United States or other, and U.S. state), and temporal (U.S. isolates only: 1982, 2004, 2007, 2008, and 2009) categories using the program DnaSPv5. Only categories with at least eight individuals were included for these analyses following the methods of previous studies (18,44). Using DnaSPv5, sequence diversity estimates and statistics, including the number of segregating sites (*s*) and haplotypes (*h*), haplotype diversity (37), Tajima's π (59), the average number of pairwise nucleotide differences (*k*) (59), and Watterson's theta (θ_w) per sequence (66) were calculated. Fu and Li's *D* and *F*, and Tajima's *D* were used to assess whether sequence variation in each gene for all isolates deviated from neutrality (15,60); and the recombination parameter (*R*) per gene (22) and minimum number of recombination events (*RM*) (23) were also estimated for each gene and all isolates.

Population subdivision analysis. The model-based Bayesian clustering algorithm implemented in Structure 2.3X was used to assess population subdivision (43). Following preliminary analyses, the values for burnin, chain replication, and lambda were chosen to be 300,000, 100,000, and 0.8, respectively. The optimal number of populations (*K*) was determined by comparing posterior

Fig. 2. Genetic structure of *Pseudoperonospora cubensis* isolates grouped by geographic categories: **A**, continent; **B**, country; and **C**, region. Geographic origin abbreviations: U.S.A. = United States of America, CA = California, DE = Delaware, FL = Florida, GA = Georgia, IA = Iowa, IN = Indiana, LA = Louisiana, MI = Michigan, NC = North Carolina, NY = New York, OH = Ohio, SC = South Carolina, TX = Texas, VA = Virginia, and WI = Wisconsin. Each isolate is represented by a thin bar, which is partitioned into colored segments each representing the individual's proportionate genetic membership in a given Kth cluster. Cluster colors correspond to red = 1, purple = 2, yellow = 3, green = 4, blue = 5, and turquoise = 6. A white space separates individual subcategories.

distribution likelihoods among three independent runs of $K = 1$ to $K = 40$ using the established parameters. Data included all loci individually coded by haplotype and were analyzed under the admixture model with correlated allele frequencies and without previous population information. To visualize the distribution of clusters in predefined categories, population structure figures with prior population information, obtained from the defined geographic, host, and year categories and sorted by the proportional membership (Q) of an isolate in a cluster, were generated using the Population Sorting Tool, a graphic editing program created in R (45) (J. J. Morrice, unpublished data). Genetic differentiation indexes (Wright's F_{ST}) were calculated for isolates grouped by geographic region, host, and year categories using DnaSPv5. Genetic differentiation was interpreted to be low, moderate, or high according to the standards in Hartl and Clark (19). Statistical significance was determined for each index by running 5,000 permutations ($\alpha = 0.05$).

Results

In total, 465 *P. cubensis* isolates originating from diverse hosts (*C. sativus*, *C. melo*, *Cucurbita pepo*, *C. maxima*, and *C. moschata*)

and geographic regions (three continents, 13 countries, and 19 U.S. states) were genotyped for two mitochondrial and five nuclear regions (Table 1). All genes were polymorphic but nuclear genes were more variable than mitochondrial genes, as indicated by diversity estimates (Table 2). In all, 50 polymorphisms were detected, and nuclear genes presented low values of R and RM. Fu and Li's D and F and Tajima's D indicated no significant deviation in neutrality for any of the genes. No unique haplotypes were found in the mitochondrial genes. Unique single-locus haplotypes occurred in all nuclear genes except *gpi*, and their frequency ranged between 13% (*TigA*) and 40% (nrLSU) (data not shown). In total, there were 322 multilocus genotypes within the sample of 465 isolates. Isolates that shared a multilocus genotype were found in all three continents, across multiple hosts, and across multiple years, suggesting that some genotypes are widespread and persistent.

Bayesian clustering revealed significant population structure by geographic region (Figs. 1 and 2) and host (Fig. 3), where some clusters occurred more frequently in some categories than others. However, the analysis showed no direct correspondence between

Fig. 3. Genetic structure of *Pseudoperonospora cubensis* isolates grouped by predefined host and year categories: **A**, *Cucumis sativus* or other; **B**, hosts (U.S. state); and **C**, year. Each isolate is represented by a thin bar, which is partitioned into colored segments each representing the individual's proportionate genetic membership in a given Kth cluster. Cluster colors correspond to red = 1, purple = 2, yellow = 3, green = 4, blue = 5, and turquoise = 6. A white space separates individual subcategories.

groupings of *P. cubensis* isolates in the predefined host and geography categories and inferred genetic clusters. The highest likelihood scores were observed when the number of clusters was set to six ($K = 6$, $\ln P = -2,877$; $K = 5$, $\ln P = -2,987$; and $K = 7$, $\ln P = -2,940$), and individual ancestry coefficients were highly consistent across replicate runs. The bar plots indicated that approximately half of the isolates were assigned predominantly to clusters 1, 2, 3, 4, 5, or 6 and the other half were assigned partial membership in clusters 2, 3, and 4 (referred to as mixed isolates in this article, based on genetic clustering).

***P. cubensis* population structure by geography.** Population structure was detected when data were analyzed using geographic categories as prior population information (Figs. 1 and 2). F_{ST} values indicated low differentiation between U.S. and non-U.S. isolates (Table 3), and diversity estimates were comparable between isolates from both categories (Table 4). Isolates belonging to each of the clusters were present in the sampling from North America and Europe (Figs. 1 and 2A). Isolates belonging predominantly to cluster 1 occurred more frequently in Europe (69% of cluster 1 isolates), whereas clusters 2, 3, and 5 were more frequently found in North America (100, 97, and 85% of isolates in each cluster, respectively). F_{ST} estimates indicated moderate differentiation between isolates from Western Asia and isolates from Europe and North America (Table 3). Diversity estimates were higher for iso-

lates from Europe and North America than those obtained from Western Asia (Table 4).

When isolates were grouped by country, mixed isolates were present in the samples collected from all countries except Turkey (Figs. 1 and 2 B). Isolates from cluster 1 were more frequently sampled in the Czech Republic, while isolates from clusters 2, 3, and 5 were mostly found in the U.S.. Isolates from cluster 6 were mainly sampled in Canada, the Czech Republic, and the U.S.. Italy only presented isolates from cluster 5, except for one mixed isolate (Figs. 1 and 2B). Countries (France, Germany, the Netherlands, and Norway) in Western Europe showed similar cluster composition, as did Greece and Italy. Spain showed similar cluster composition to countries in Western Europe, Greece, and Italy. Turkey and the Czech Republic displayed similar cluster composition. Israel showed similar cluster composition to the Czech Republic but not to Greece or Italy (Figs. 1 and 2B), and F_{ST} values detected low genetic differentiation between Israel and the Czech Republic but high differentiation among Israel, Greece, and Italy. F_{ST} values indicated high genetic differentiation between isolates from Italy and other countries (Table 3). F_{ST} values established high genetic differentiation between isolates from Greece and Italy but lower than differentiation between the Czech Republic and Greece or Italy (Table 3). Differentiation estimates also indicated high differentiation between isolates from Greece and

Table 3. Genetic differentiation estimates of mitochondrial and nuclear genes for *Pseudoperonospora cubensis* isolates grouped by continent, country, and country group

Category ^a	F_{ST}^b															
	Continent								Country							
	Asia		Europe		Canada		Czech Rep.		Greece		Israel		Italy		Country group	
	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N
Continent																
Europe	0.08*	0.05*	...	...	...	...	...	...	...	...	...	...	...	...	...	...
North America	0.16*	0.05*	0.01	0.06*	...	...	...	...	...	...	...	...	...	...	...	...
Country																
Czech Republic	...	...	...	...	0.01	0.12*	...	...	...	...	...	...	...	...	...	...
Greece	...	...	...	...	0.27*	0.12*	0.22*	0.2*	...	...	...	...	...	...	...	...
Israel	...	...	...	...	0	0.05*	0.01	0.1*	0.24*	0.16*	...	...	...	...	...	...
Italy	...	...	...	...	0.72*	0.18*	0.68*	0.21*	0.34*	0.08*	0.72*	0.22*	...	...	...	...
United States	...	...	...	...	0.19*	0.04*	0.14*	0.11*	0	0.06*	0.17*	0.07*	0.43*	0.13*	...	...
Country group																
Other	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.04*	0.03*

^a Average values for mitochondrial (M) and nuclear genes (N) are presented; * = significant at 0.05.

^b Categories with less than eight isolates were excluded from analyses and are not shown.

Table 4. Diversity estimates of mitochondrial and nuclear genes for *Pseudoperonospora cubensis* isolates grouped by continent, country, and country group

Category ^b	Isolates ^c	Diversity estimates ^a							
		Hd		π		θ_w		k	
		M	N	M	N	M	N	M	N
Continent									
Asia	11	0.16	0.32	0.001	0.001	0.17	1.26	0.16	0.84
Europe	95	0.37	0.44	0.002	0.001	0.29	1.20	0.40	1.14
North America	359	0.43	0.25	0.001	0.001	0.31	1.09	0.47	0.35
Country									
Canada	29	0.21	0.30	0.002	0.001	0.13	0.52	0.21	0.40
Czech Republic	56	0.27	0.48	0	0.001	0.33	1.21	0.28	1.46
Greece	14	0.47	0.31	0	0.001	0.31	0.26	0.47	0.35
Israel	10	0.18	0.25	0.002	0.001	0.18	0.68	0.18	0.54
Italy	8	0.34	0.26	0.001	0.001	0.39	0.96	0.34	0.72
United States	327	0.43	0.24	0	0.001	0.31	0.82	0.47	0.33
Country group									
United States	327	0.43	0.24	0.001	0.001	0.31	0.82	0.47	0.33
Other	138	0.36	0.40	0.001	0.002	0.27	1.29	0.39	1.01

^a Average values for mitochondrial (M) and nuclear (N) genes are presented. Hd, haplotype diversity; π , nucleotide diversity; θ_w , Watterson's theta estimator per gene from sequence; k, average number of nucleotide differences.

^b Categories with less than eight isolates were excluded from analyses and are not shown.

^c Number of isolates.

from other countries, except for the U.S.. Moderate to low differentiation was detected among isolates from the Czech Republic, Israel, Canada, and the U.S.. Diversity estimates were higher for isolates from the Czech Republic, Greece, and Italy, while isolates from Canada, Israel, and the U.S. presented lower values (Table 4).

When isolates were grouped by U.S. state (Figs. 1A and 3B), it was observed that the pattern of cluster occurrence was similar in Georgia, New York, South Carolina, and Texas; North Carolina, Indiana, and Florida; California, Delaware, Kentucky, Maryland, Michigan, Ohio, Virginia, and Wisconsin; and Louisiana, Massachusetts, and Mississippi. A greater number of clusters were detected in the sample from Iowa, and the pattern of cluster occur-

rence was unique (Figs. 1A and 3B). However, F_{ST} values indicated low differentiation between isolates from Michigan, Ohio, Wisconsin, Iowa, and California. Similarly, low differentiation was observed between isolates from North Carolina, South Carolina, Georgia, Indiana, and Texas. Isolates from New York, Delaware, Virginia, and Florida also showed low differentiation among each other. Isolate composition from Louisiana had very high differentiation compared with all other states (Table 5) and these isolates also presented the lowest diversity estimates of any state (Table 6). Diversity estimates were high for isolates from Georgia, Iowa, and North Carolina, followed by moderate to high estimates obtained for isolates from Indiana (Table 6). Isolates from Delaware, Michigan, New York, Ohio, South Carolina, Virginia, and Wisconsin

Table 5. Genetic differentiation estimates for *Pseudoperonospora cubensis* isolates grouped by U.S. state^a

State ^c	F_{ST}^b													
	CA		DE		FL		GA		IA		IN		LA	
	M	N	M	N	M	N	M	N	M	N	M	N	M	N
DE	0	0.05*	...	...	...	...	...	...	...	...	...	...	...	...
FL	0.07*	0.04*	0.02	0.02	...	...	...	...	...	...	...	...	...	...
GA	0.56*	0.09*	0.43*	0.05*	0.36*	0.04*	...	...	...	...	...	...	...	...
IA	0.03	0.09*	0	0.15*	0.03	0.13*	0.37*	0.16*	...	...	...	...	...	...
IN	0.15*	0.04*	0.03	0.01	0	0.02	0.2*	0.006	0.04	0.13*	...	...	...	...
LA	0.97*	0.09*	0.89*	0.11*	0.81*	0.07*	0.29*	0.07*	0.85*	0.06*	0.68*	0.06*	...	...
MI	0	0.02	0	0.05*	0.08*	0.04*	0.55*	0.09*	0.01	0.1*	0.14*	0.06*	0.97*	0.08*
NC	0.42*	0.06*	0.28*	0.08*	0.21*	0.08*	0	0.08*	0.23*	0.05*	0.07	0.05*	0.42*	0.06*
NY	0.18*	0.04*	0.01	0	0.04	0.01	0.23*	0.04*	0.02	0.14*	0	0.01	0.75*	0.09*
OH	0	0	0.02	0.03*	0.06*	0.03*	0.5*	0.02	0.05	0.11*	0.14*	0.04*	0.95*	0.09*
SC	0.29*	0.08*	0.13*	0.01	0.1*	0.01	0.11*	0.03	0.09*	0.16*	0	0.01	0.58*	0.1*
TX	0.56*	0.11*	0.41*	0.05*	0.36*	0.05*	0	0.02	0.35*	0.18*	0.19*	0.02	0.28*	0.1*
VA	0.03	0.12*	0.07*	0.1*	0.05*	0.15*	0.51*	0.12*	0.1*	0.23*	0.13*	0.09*	0.91*	0.21*
WI	0	0.02*	0	0.06*	0.12*	0.03	0.59*	0.1*	0.03	0.12*	0.18*	0.05*	1*	0.12*
	MI		NC		NY		OH		SC		TX		VA	
	M	N	M	N	M	N	M	N	M	N	M	N	M	N
NC	0.4*	0.09*	...	...	...	...	...	...	...	...	...	...	...	...
NY	0.17*	0.04*	0.13*	0.08*	...	...	...	...	...	...	...	...	...	...
OH	0.02	0.01	0.4*	0.09*	0.19*	0.02*	...	...	...	...	...	...	...	...
SC	0.28*	0.07*	0.02	0.09*	0	0.01	0.19*	0.02*	...	...	...	...	...	...
TX	0.55*	0.1*	0	0.11*	0.22*	0.03*	0	0.01	0.2*	0.02*	...	...	...	...
VA	0.08*	0.13*	0.37*	0.18*	0.2*	0.09*	0.2*	0.03*	0.29*	0.06*	0	0.11*	...	...
WI	0	0.02	0.4*	0.11*	0.21*	0.02*	0.2*	0.09*	0.55*	0.09*	0.04	0	0.1*	0.1*

^a CA = California, DE = Delaware, FL = Florida, GA = Georgia, IA = Iowa, IN = Indiana, LA = Louisiana, MI = Michigan, NC = North Carolina, NY = New York, OH = Ohio, SC = South Carolina, TX = Texas, VA = Virginia, and WI = Wisconsin.

^b Average values for mitochondrial (M) and nuclear genes (N) are presented; * = significant at 0.05.

^c Categories with less than eight isolates were excluded from analyses and are not shown.

Table 6. Diversity estimates of mitochondrial and nuclear genes for *Pseudoperonospora cubensis* isolates grouped by U.S. state

Category ^b	Isolates ^c	Diversity estimates ^a							
		Hd		π		θ_w		k	
		M	N	M	N	M	N	M	N
California	15	0.07	0.15	0	0.001	0.15	0.10	0.07	0.15
Delaware	10	0.20	0.17	0.001	0.001	0.35	0.11	0.20	0.17
Florida	38	0.35	0.24	0.001	0.001	0.36	0.45	0.37	0.32
Georgia	21	0.58	0.27	0.002	0.001	0.42	0.28	0.65	0.39
Iowa	12	0.30	0.34	0.001	0.001	0.50	0.48	0.32	0.45
Indiana	18	0.49	0.27	0.002	0.001	0.44	0.39	0.54	0.36
Louisiana	9	0	0.31	0	0.001	0	0.35	0	0.36
Michigan	54	0.06	0.21	0	0.001	0.33	0.53	0.06	0.32
NC	23	0.62	0.27	0.002	0.001	0.41	0.32	0.72	0.34
New York	25	0.38	0.17	0.001	0.001	0.27	0.18	0.38	0.21
Ohio	27	0.10	0.14	0	0.001	0.13	0.22	0.10	0.19
South Carolina	27	0.51	0.20	0.002	0.001	0.39	0.22	0.56	0.27
Texas	14	0.13	0.52	0	0.003	0.31	0.25	0.13	0.52
Virginia	11	0.20	0.52	0.001	0.003	0.35	0.28	0.20	0.52
Wisconsin	8	0.46	0.58	0.001	0.004	0.48	0.82	0.50	0.84

^a Average values for mitochondrial (M) and nuclear (N) genes are presented. Hd, haplotype diversity; π , nucleotide diversity; θ_w , Watterson's theta estimator per gene from sequence; k, average number of nucleotide differences.

^b Categories with less than eight isolates were excluded from analyses and are not shown.

^c Number of isolates.

presented moderate values for diversity estimates, whereas isolates from California and Texas showed moderate to low values (Table 6).

Cluster occurrence for isolates from Ontario, Canada, was similar to what was observed for isolates from California, Delaware, Kentucky, Maryland, Michigan, Ohio, Virginia, and Wisconsin (Fig. 1A). When Ontario was analyzed for genetic differentiation with individual U.S. states, F_{ST} values indicated that isolates from Ohio had low differentiation with Ontario (Table 7). Isolates from Michigan, New York, Wisconsin, and Virginia presented low to moderate differentiation with Ontario isolates. Moderate to high differentiation was observed between Ontario isolates and isolates from other states. Diversity estimates for Ontario isolates were lower than those obtained for isolates from Georgia, Iowa, Indiana, North Carolina, and South Carolina; comparable with isolates from

Florida, Delaware, and New York; and higher than isolates from California, Louisiana, Michigan, Ohio, and Texas (Table 4).

***P. cubensis* population structure by host of origin.** Population structure was revealed when isolates were organized by whether they originated from cucumber or another cucurbit host (Fig. 3A). Isolates from clusters 1, 2, 3, 4, and 6, as well as mixed isolates, were mostly sampled in *Cucumis sativus* (88, 77, 81, 86, 85, and 91% of isolates in those clusters, respectively, were sampled from *C. sativus*), whereas 85% of cluster 5 isolates were sampled from other hosts (Table 8; Fig. 3A). F_{ST} values showed moderate to high differentiation between isolates from *C. sativus* and other hosts (Table 9), and diversity estimates were higher for isolates from non-cucumber hosts (Table 10).

When we further subdivided samples from each state by host (*C. sativus* or other), isolates from *C. sativus* were mostly mixed isolates, regardless of the state of origin (Fig. 3B). Most of the isolates retrieved from non-cucumber hosts belonged to cluster 5, except for isolates sampled from Iowa, Michigan, and Ohio. Most of the non-cucumber samples from Iowa belonged to cluster 1, samples from Michigan were mostly of the mixed type, and samples from Ohio showed a diversity of cluster occurrence. Mixed isolates were also found in non-cucumber isolates from Florida (Fig. 3B).

F_{ST} values showed little to moderate differentiation between isolates from cucumber from Michigan and isolates from cucumber from other states (Table 11). Isolates from Florida cucumber showed low to moderate differentiation with isolates retrieved from cucumber in Indiana, New York, South Carolina, Virginia, and Wisconsin; moderate differentiation with Michigan cucumber isolates; and moderate to great differentiation with cucumber isolates from North Carolina and Ohio (Table 11). Moderate to high differentiation was observed for isolates retrieved from cucumber in California, Delaware, Florida, Indiana, Michigan, North Carolina, New York, Ohio, South Carolina, Virginia, and Wisconsin; and isolates retrieved from non-cucumber hosts in Florida, Georgia, Louisiana, North Carolina, South Carolina, and Texas. Non-cucumber isolates retrieved from Michigan showed low to moderate differentiation with cucumber isolates from other states, except for cucumber isolates from North Carolina, which showed moderate to great differentiation with Michigan cucumber isolates (Table

Table 7. Genetic differentiation estimates of mitochondrial and nuclear genes for *Pseudoperonospora cubensis* isolates from Ontario, Canada with isolates from U.S. states

Category ^b	F_{ST} ^a	
	M	N
California	0.05*	0.04
Delaware	0.09*	0.04*
Florida	0.05*	0.05*
Georgia	0.5*	0.07*
Iowa	0.12*	0.1*
Indiana	0.13*	0.04*
Louisiana	0.9*	0.13*
Michigan	0.09*	0.04
North Carolina	0.37*	0.07*
New York	0.19*	0.04
Ohio	0.02	0.04
South Carolina	0.28*	0.07*
Texas	0.52*	0.09*
Virginia	0	0.09*
Wisconsin	0.12*	0.04

^a Average values for mitochondrial (left column) and nuclear genes (right column) are presented; * = significant at 0.05.

^b Categories with less than eight isolates were excluded from analyses and are not shown.

Table 8. Genetic clusters of isolates organized by whether they were retrieved from cucumber or another cucurbit host

Host	Number of isolates in predominate genetic cluster (%) ^a								Total
	1	2	3	4	5	6	Mixed	Other	
Cucumber	42 (88)	10 (77)	29 (81)	6 (86)	15 (15)	40 (85)	182 (91)	10 (83)	334
Other hosts	6 (12)	3 (23)	7 (19)	1 (14)	87 (85)	7 (15)	18 (9)	2 (17)	131

^a Each isolate was assigned to the *K*th genetic cluster to which the isolate predominately belonged based on results of Bayesian clustering. Mixed isolates were assigned partial membership in clusters 2, 3, and 4. An isolate was assumed to belong equally to two or more genetic clusters if its membership was not >0.1 (proportion membership) higher in one genetic cluster than another; these isolates are designated "other."

Table 9. Genetic differentiation estimates of mitochondrial and nuclear genes for *Pseudoperonospora cubensis* isolates grouped by host

Category ^b	F_{ST} ^a											
	Host group		Host type		Host species							
	<i>Cucumis</i> spp.		Cucumber		<i>Cucumis melo</i>		<i>C. sativus</i>		<i>Cucurbita maxima</i>		<i>C. moschata</i>	
	M	N	M	N	M	N	M	N	M	N	M	N
Host group												
<i>Cucurbita</i> spp.	0.52*	0.07*	...	...	...	...	...	...	...	...	...	...
Host type												
Other	...	...	0.48*	0.06*	...	...	...	...	...	...	...	...
Host species												
<i>Cucumis sativus</i>	...	...	...	...	0.31*	0.05*	...	...	...	...	...	...
<i>Cucurbita maxima</i>	...	...	...	...	0.03	0.07*	0.43*	0.05*	...	...	...	...
<i>C. moschata</i>	...	...	...	...	0.43*	0.15*	0.85*	0.23*	0.21*	0.21*	...	...
<i>C. pepo</i>	...	...	...	...	0.14*	0.04*	0.64*	0.09*	0.02	0.05*	0.14*	0.07*

^a Average values for mitochondrial (M) and nuclear genes (N) are presented. *, significant at 0.05.

^b Categories with less than eight isolates were excluded from analyses and are not shown.

11). Michigan cucumber isolates showed moderate to great differentiation with non-cucumber isolates collected from other states (Table 11). When isolates collected from non-cucumber hosts were compared, moderate to high differentiation was observed between states, except for South Carolina and Texas, where low differentiation was suggested (Table 12). As noted above, diversity estimates tend to be higher for isolates from non-cucumber hosts than isolates from cucumber hosts. The highest diversity estimates were for non-cucumber isolates from Florida and North Carolina (Table 13).

***P. cubensis* temporal population structure.** No clear structure was identified when isolates were grouped by year (Fig. 3C). For the 2007 to 2009 samples from the U.S., it was observed that

clusters were similarly represented across the 3 years of sampling. Most samples from before 2004 were obtained from Europe, except for one sample from North Carolina that was obtained in 1982, which was a mixed isolate.

Discussion

This study investigated the genetic structure of *P. cubensis* populations from a worldwide collection. When Bayesian clustering was used to identify population subdivision, six genetic clusters were detected. Approximately half of the isolates belonged predominantly to one of five genetic clusters and the other half were mixed isolates (assigned approximately equal membership in clus-

Table 10. Diversity estimates of mitochondrial and nuclear genes for *Pseudoperonospora cubensis* isolates grouped by host

Category ^b	Isolates ^c	Diversity estimates ^a							
		Hd		π		θ_w		k	
		M	N	M	N	M	N	M	N
Host group									
<i>Cucumis</i> spp.	391	0.28	0.28	0.001	0.001	0.23	1.27	0.30	0.53
<i>Cucurbita</i> spp.	74	0.45	0.32	0.002	0.001	0.41	1.11	0.51	0.56
Host									
<i>Cucumis sativus</i>		0.19	0.28	0.001	0.001	0.24	1.24	0.20	0.54
Other	131	0.54	0.31	0.002	0.002	0.37	1.17	0.60	0.50
Host species									
<i>C. melo</i>	57	0.56	0.29	0.002	0.002	0.33	0.45	0.62	0.40
<i>C. sativus</i>	334	0.19	0.30	0.001	0.002	0.24	1.24	0.20	0.58
<i>Cucurbita maxima</i>	24	0.61	0.33	0.002	0.001	0.54	0.41	0.75	0.44
<i>C. moschata</i>	12	0.15	0.12	0	0	0.17	0.11	0.15	0.14
<i>C. pepo</i>	38	0.40	0.32	0.001	0.002	0.36	1.18	0.43	0.68

^a Average values for mitochondrial (M) and nuclear (N) genes are presented. Hd, haplotype diversity; π , nucleotide diversity; θ_w , Watterson's theta estimator per gene from sequence; k, average number of nucleotide differences.

^b Categories with less than eight isolates were excluded from analyses and are not shown.

^c Number of isolates.

Table 11. Genetic differentiation estimates of mitochondrial and nuclear genes for *Pseudoperonospora cubensis* isolates collected from cucumber (column) and non-cucumber (row) hosts grouped by U.S. state of origin

Category ^b	F_{ST}^a													
	Florida		Georgia		Louisiana		Michigan		North Carolina		South Carolina		Texas	
	M	N	M	N	M	N	M	N	M	N	M	N	M	N
California	0.32*	0.06*	0.76*	0.11*	0.97*	0.09*	0	0.01	0.64*	0.11*	0.90*	0.20*	0.93*	0.19*
Delaware	0.16	0.02	0.66*	0.08*	0.89*	0.11*	0	0.06*	0.52*	0.09*	0.82*	0.13*	0.84*	0.12*
Florida	0.26*	0.03*	0.70*	0.10*	0.91*	0.11*	0.03	0.06*	0.57*	0.14*	0.85*	0.15*	0.87*	0.13*
Indiana	0.30*	0.06*	0.73*	0.11*	0.94*	0.09*	0.01	0.02	0.60*	0.10*	0.88*	0.19*	0.90*	0.18*
Michigan	0.28*	0.07*	0.79*	0.12*	1.00*	0.07*	0	0.05	0.66*	0.13*	0.93*	0.18*	0.95*	0.16*
North Carolina	0	0.06*	0.36*	0.11*	0.60*	0.05	0.20*	0.07*	0.23*	0.05*	0.54*	0.21*	0.55*	0.20*
New York	0.29*	0.05*	0.75*	0.11*	0.97*	0.10*	0	0.04*	0.62*	0.12*	0.90*	0.16*	0.92*	0.14*
Ohio	0.31*	0.06*	0.74*	0.12*	0.95*	0.11*	0.01	0.03*	0.62*	0.15*	0.89*	0.17*	0.91*	0.16*
South Carolina	0.34*	0.05*	0.79*	0.13*	1.00*	0.11*	0	0.03	0.66*	0.15*	0.93*	0.17*	0.95*	0.16*
Virginia	0.30*	0.11*	0.71*	0.14*	0.92*	0.21*	0.06	0.14*	0.58*	0.19*	0.86*	0.18*	0.88*	0.17*
Wisconsin	0.34*	0.07*	0.79*	0.13*	1.00*	0.12*	0	0.03	0.66*	0.14*	0.93*	0.18*	0.95*	0.16*

^a Average values for mitochondrial (M) and nuclear genes (N) are presented; * = significant at 0.05.

^b Categories with less than eight isolates were excluded from analyses and are not shown.

Table 12. Genetic differentiation estimates of mitochondrial and nuclear genes for *Pseudoperonospora cubensis* isolates from non-cucumber hosts grouped by U.S. state of origin

Category ^b	F_{ST}^a											
	Georgia		Louisiana		Michigan		North Carolina		South Carolina		Texas	
	M	N	M	N	M	N	M	N	M	N	M	N
Florida	0.22*	0	0.46*	0.05	0.27*	0.07*	0.10	0.04*	0.41*	0.05*	0.42*	0.03
Georgia	...	...	0.18	0.08*	0.72*	0.12*	0	0.09*	0.05	0.05*	0.08	0.03*
Louisiana	...	...	...	...	0.94*	0.11*	0.29*	0.10*	0.06	0.17*	0	0.15*
Michigan	...	...	...	...	...	...	0.59*	0.15*	0.87*	0.21*	0.90*	0.19*
North Carolina	...	...	...	...	...	...	...	...	0.16	0.15*	0.21	0.15*
South Carolina	...	...	...	...	...	...	...	...	...	...	0	0.02*

^a Average values for mitochondrial (M) and nuclear genes (N) are presented; * = significant at 0.05.

^b Categories with less than eight isolates were excluded from analyses and are not shown.

ters 2, 3, and 4) according to genetic clustering. There was no direct correspondence between inferred genetic clusters and groupings of isolates by predefined geographic and host categories but some population structure was observed, with some clusters occurring more or less frequently in particular categories. Multilocus genotypes were shared across continental, host, and temporal scales, indicating that some genotypes are widely dispersed and persistent.

When isolates were grouped by geographic regions, all clusters were found in all continents; however, cluster 1 occurred more frequently in Europe, whereas clusters 2, 3, and 5 were more frequently found in North America. F_{ST} values among isolates from North America, Europe, and Western Asia were low to moderate, which could be the result of infrequent human-mediated transport of *P. cubensis*, as also discussed by Runge et al. (50). F_{ST} values are maintained at 0.1 or less, even if only a few individuals per generation migrate, resulting in gene flow between populations (19).

P. cubensis overwinters in southeast Europe (24,27) and Western Asia, as well as in the southern part of North America, where cucurbits are produced year round. Sporangia of *P. cubensis* are wind dispersed and there is evidence of spread up to a thousand kilometers (40). Hence, within regions, limited genetic variation might be expected, and similar cluster composition was observed for countries in Western Europe. Because the pathogen can persist year round in southeast Europe, the high genetic diversity estimated for these countries (Greece and Italy) was expected. The high genetic diversity observed for isolates from the Czech Republic may reflect the more extensive collection of isolates included for this country but might also be influenced by the fact that all but five of these isolates originated from before 2004, in contrast to most other samples. A previous study based on phylogenetic analyses found that isolates from the Czech Republic, the Netherlands, and France were significantly different from isolates from Greece (54), in agreement with our findings.

Within the U.S., *P. cubensis* is hypothesized to spread from south to north along the East Coast (38). Limited genetic variation is expected between neighboring states because *P. cubensis* sporangia may be dispersed up to a thousand kilometers via wind currents (40). Ojiambo and Holmes (40) recently proposed that overwintering sources in southern Florida contribute directly to cucurbit downy mildew outbreaks from Florida up to North

Carolina. Surprisingly, isolates collected from Florida cucumber and non-cucumber hosts were moderately to highly differentiated from isolates from cucumber and non-cucumber hosts in North Carolina. Our study found that isolates from Georgia, North Carolina, Texas, South Carolina, and Indiana are genetically different from Florida, and high genetic diversity was detected in Georgia, Indiana, and North Carolina. These findings suggest that another source of inocula exists in addition to southern Florida, resulting in genetic differences and variability. This source could possibly be Mexico or southern states of the U.S. not included in our sampling. Oospores have not been observed in field samples from the U.S., and it is unclear if oospores play a role in the generation of genetic diversity and in pathogen overwintering in cucurbit crops or weeds. However, there are scattered reports of oospore formation in Europe (6), and abundant oospore formation under laboratory conditions was recently reported from Israel (10). The low genetic differentiation among isolates from Florida, South Carolina, and Virginia is consistent with Florida contributing inocula to states along the East Coast, in agreement with the findings of other studies (38–40). California had low diversity estimates; this may be due to its geographic separation from other states with severe downy mildew outbreaks. Isolates from Louisiana had a different population structure from isolates from other states, likely because of the low genetic and cluster diversity detected there.

It has been suggested that large-scale year-round production of cucumber in greenhouses in Ontario, Canada, may be a source of primary inoculum for field-grown cucumber in the Great Lakes states early in the growing season (40). The results of our study are consistent with this hypothesis, and similarities in cluster composition, low genetic differentiation, and comparable levels of genetic diversity were detected between isolates from the U.S. and Canada. Canadian isolates were obtained from Ontario, a province close to the Great Lakes states, and our analyses revealed high cluster similarity among Ontario, Michigan, and Ohio. F_{ST} values indicate low to moderate genetic differentiation between isolates from Ontario and isolates from Michigan, Ohio, New York, Wisconsin, and Virginia. Moderate genetic differentiation was detected between isolates from Ontario and isolates from Delaware, Florida, Iowa, Indiana, North Carolina, South Carolina, and Texas, whereas high genetic differentiation was observed when Ontario isolates were compared pairwise with isolates from Georgia and Louisiana. Our results do not rule out the possibility that large-scale Ontario

Table 13. Diversity estimates of mitochondrial and nuclear genes for *Pseudoperonospora cubensis* isolates grouped by host (cucumber or other cucurbit host) and geography (U.S. state)

Category ^b	Isolates ^c	Diversity estimates ^a							
		Hd		π		θ_w		k	
		M	N	M	N	M	N	M	N
California cucumber	30	0.07	0.15	0	0.001	0.15	0.10	0.07	0.15
Delaware cucumber	20	0.20	0.17	0.001	0.001	0.35	0.11	0.20	0.17
Florida cucumber	58	0.20	0.21	0.001	0.001	0.26	0.26	0.20	0.26
Florida other	18	0.61	0.31	0.002	0.002	0.55	0.58	0.67	0.48
Georgia other	32	0.43	0.29	0.001	0.002	0.45	0.30	0.45	0.42
Indiana cucumber	24	0.15	0.18	0	0.001	0.17	0.21	0.15	0.20
Louisiana other	18	0	0.31	0	0.003	0	0.35	0	0.36
Michigan cucumber	60	0	0.20	0	0.001	0	0.43	0	0.36
Michigan other	48	0.12	0.16	0	0.001	0.40	0.23	0.13	0.20
North Carolina cucumber	26	0.53	0.21	0.002	0.001	0.48	0.16	0.56	0.26
North Carolina other	20	0.60	0.32	0.002	0.001	0.53	0.39	0.69	0.41
New York cucumber	38	0.05	0.13	0	0.001	0.14	0.14	0.05	0.14
Ohio cucumber	46	0.12	0.12	0	0.001	0.14	0.18	0.12	0.17
South Carolina cucumber	34	0	0.15	0	0.001	0	0.15	0	0.18
South Carolina other	20	0.18	0.16	0	0.001	0.18	0.23	0.18	0.22
Texas other	18	0.11	0.28	0	0.001	0.18	0.29	0.18	0.38
Virginia cucumber	22	0.22	0.24	0.001	0.001	0.17	0.16	0.22	0.31
Wisconsin cucumber	16	0	0.11	0	0.001	0	0.06	0	0.11

^a Average values for mitochondrial (M) and nuclear (N) genes are presented. Hd, haplotype diversity; π , nucleotide diversity; θ_w , Watterson's theta estimator per gene from sequence; k, average number of nucleotide differences.

^b Categories with less than eight isolates were excluded from analyses and are not shown.

^c Number of isolates.

greenhouses could be serving as a source of inoculum to some states in the Great Lakes region in addition to the inoculum received from states in the southern U.S. (40). Nonetheless, further studies that test for migration events of *P. cubensis* from Canada to the U.S. are needed to clarify the relative contributions of inocula to the Great Lakes states from possible sources in Ontario compared with possible sources in the southern U.S..

When isolates retrieved from *C. sativus* were compared with isolates from other cucurbit hosts, isolates from cluster 5 were noticeably more common in non-cucumber hosts. It is possible that isolates from different *P. cubensis* clusters are not equally adapted to the various cucurbit hosts and this could result in isolates from a specific cluster such as cluster 5 occurring more frequently in non-cucumber hosts. In Israel, *Cucurbita* spp. were not hosts of *P. cubensis* until 2002 (9), when sporangia belonging to a pathotype capable of infecting *Cucurbita* spp. emigrated from southern or central Europe (24). More detailed *P. cubensis* population studies at the centers of origin or diversity of cucurbit hosts may clarify the differences observed in cluster occurrence in diverse hosts. Notably, in our study, diversity estimates were higher for isolates collected from non-cucumber hosts than from cucumber.

Our results indicated that mixed isolates were more frequently associated with *C. sativus* than with other cucurbit hosts. Recently (mid-2000s), resistance to *P. cubensis* introduced into cucumber became ineffective as a sole control mechanism for cucumber downy mildew in the U.S.. The presence of a new pathotype or physiological race in the U.S. has been proposed as an explanation for the breaking of resistance in cucumber by *P. cubensis* in approximately 2004 (11,55,56). In addition, new pathotypes with an expanded host range have also been described in central Europe (25). A recent study (50) suggests that a new lineage of *Pseudoperonospora* might be responsible for current epidemics in the U.S.. Because mixed isolates were commonly associated with cucumber in different years of sampling in the U.S. (2007 to 2009), it is possible that the new pathotype, physiological race, species, or subspecies infecting cucumber is associated with mixed isolates. The historical sample from North Carolina (year 1982) was a mixed isolate, indicating that this type of isolate has been present in the U.S. since as early as 1982. However, because we only had one U.S. sample obtained before approximately 2004, further studies, including herbarium specimens, are needed to confirm whether mixed isolates were common in the U.S. before approximately 2004.

This study provides an initial map of the population structure of *P. cubensis*. More clusters likely exist in *P. cubensis* because apparent structure may be underestimated when using sparse sampling and few markers (48). Previous studies have shown that, for clustering to be robust, less than 40 markers and as few as 7 markers (43) can be used if they are highly informative; however, using at least 50 markers is preferred (48). Future studies with uniform global sampling and hundreds of markers could determine whether additional genetic clusters are present in *P. cubensis*. The *P. cubensis* genome (57), will provide an invaluable source of markers for robust population genetics studies. Nevertheless, the approach used in this study could be applied to other plant pathogens when using a large number of markers is not feasible due to high costs or scarce genomic resources, as long as markers used are informative. Continued genotyping of *P. cubensis* populations will be necessary to determine the existence of new clusters and to monitor the diversification of clusters detected in this study. A recent study showed that there is some limited infectivity of *P. cubensis* on hop and *P. humuli* on cucumber. In a multilocus phylogeny, Runge et al. (52) showed that *P. humuli* is separated and basal to *P. cubensis*, because they found that two lineages parasitic to hop species branched before the split of the two *Pseudoperonospora* lineages infecting Cucurbitaceae spp. This scenario suggested that an evolutionarily recent host jump from hops took place, with limited cross-infectivity potential present, because Runge and Thines (52) could infect hop with *P. cubensis* and cucumber with *P. humuli*, and confirm the result using molecular identification of emerging

sporangiophores. Population structure analyses including *P. humuli* samples will be key to determine whether gene flow between these sister species exists or whether *P. humuli* populations contributed to the genetic variation observed in *P. cubensis*.

Breeding programs should include isolates that represent the genetic diversity of the pathogen in resistance screenings, given the high genetic variation found in this study and the previous finding that at least two distinct phylogenetic lineages are parasitic to Cucurbitaceae spp. (52). Isolates obtained from cucumber are commonly used in host resistance screenings but this study suggests that inclusion of isolates from *C. melo* and *Cucurbita* spp. that show a different genetic composition and high genetic diversity is necessary to capture the genetic variation of *P. cubensis*. In addition, regions with high genetic diversity such as the U.S. and Europe should be of special concern because *P. cubensis* populations with high levels of genetic variation are likely to adapt more rapidly to resistant hosts and fungicides than populations with little genetic variation. Gene pyramiding within a resistant variety or planting several resistant varieties in a field will be key to prevent or slow pathogen adaptation and future breaking of host resistance (4) by *P. cubensis* populations.

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