Genetic and Pathogenic Relatedness of *Pseudoperonospora cubensis* and *P. humuli*

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ABSTRACT

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The most economically important plant pathogens in the genus *Pseudoperonospora* (family *Peronosporaceae*) are *Pseudoperonospora cubensis* and *P. humuli*, causal agents of downy mildew on cucurbits and hop, respectively. Recently, *P. humuli* was reduced to a taxonomic synonym of *P. cubensis* based on internal transcribed spacer (ITS) sequence data and morphological characteristics. Nomenclature has many practical implications for pathogen identification and regulatory considerations; therefore, further clarification of the genetic and pathogenic relatedness of these organisms is needed. Phylogenetic analyses were conducted considering two nuclear and three mitochondrial loci for 21 isolates of *P. cubensis* and 14 isolates of *P. humuli*, and all published ITS sequences of the pathogens in GenBank. There was a consistent separation of the majority of the *P. humuli* isolates and the *P. cubensis* isolates in nuclear, mitochondrial, and ITS phylogenetic analyses, with the exception of isolates of *P. humuli* from *Humulus japonicus* from Korea. The *P. cubensis* isolates appeared to contain the *P. humuli* cluster, which may indicate that *P. humuli* descended from *P. cubensis*. Host-specificity experiments were conducted with two reportedly universally susceptible hosts of *P. cubensis* and two hop cultivars highly susceptible to *P. humuli*. *P. cubensis* consistently infected the hop cultivars at very low rates, and sporangiophores invariably emerged from necrotic or chlorotic hypersensitive-like lesions. Only a single sporangiophore of *P. humuli* was observed on a cucurbit plant during the course of the studies. Together, molecular data and host specificity indicate that there are biologically relevant characteristics that differentiate *P. cubensis* and *P. humuli* that may be obfuscated if *P. humuli* were reduced to a taxonomic synonym of *P. cubensis*. Thus, we recommend retaining the two species names *P. cubensis* and *P. humuli* until the species boundaries can be resolved unambiguously.

Downy mildews are members of the family *Peronosporaceae* (Oomycota, Oomycetes, Peronosporales), which comprise one of the largest groups of fungi-like organisms that parasitize flowering plants (42). Within the family *Peronosporaceae* are six species of *Pseudoperonospora* Rostovzev. Economically important species of the genus include *Pseudoperonospora cubensis* (Berk. & M. A. Curtis) Rostovzev (the type species) and *P. humuli* (Miyabe & Takah.) G. W. Wilson. *P. cubensis*, causal agent of cucurbit downy mildew, has been found on at least 49 wild and cultivated species of cucurbits in 70 countries (6,7,41). Cucurbit downy mildew affects 9 of the 12 cultivated cucurbit species (6,7,60). *P. cubensis* infects plants in both the field and protected cultivation in tropical areas around the world, as well as in some semiarid and temperate regions. *P. humuli*, the causal agent of hop downy mildew, is found mainly in hop-production regions throughout the northern hemisphere (37). The commercial host of *P. humuli* is the hop plant (*Humulus lupulus* L.), a dioecious plant in the family *Cannabaceae*.

The downy mildews have been a difficult group for determining what defines a species. Morphometric techniques, which include measurements of the size, shape, color, and so on of anatomical features, have been commonly used to distinguish and identify a species in many areas of biology. These techniques can be uninformative for downy mildew pathogens (11,18,44). This is mainly due to the few visible characteristics, because nearly all but the reproductive structures are within plant tissue and many characteristics can vary widely depending on the host matrix and the environmental conditions (22,51). Because downy mildew pathogens are obligate biotrophs, the traditional biological species concept based on mating compatibility is difficult to test experimentally.

To overcome these problems with morphology, Gäumann (13) proposed a biological species concept for downy mildews based on host specialization (22,59). Gäumann (13) proposed that a species of the family *Peronosporaceae* would be specific to a single host genus or even species. However, few cross-infection experiments were performed to confirm his strict assumption about host specificity, sometimes leading to artificial splitting of some species with polyphagous host ranges (13,22,59). This concept is also problematic when multiple species of downy mildew are parasitic on a single host species (59,64).

The ecophysiophenetic concept is essentially the intersection between the biological and morphometric species concepts (22). In this concept, the criterion for the delimitation of a species is dependent upon host specificity (to host genus or family) and morphology of the conidiophores and conidia or sporangiophores and sporangia (22). Although this concept appears as the middle ground, it suffers from many of the same problems as the morphometric and biological species concepts.

In response to these problems, and with the advent of molecular techniques, phylogenetic analyses of the downy mildews have been used to infer reproductive isolation and delineate species that are monophyletic. According to Hall (22), "species are

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defined as clusters of organisms diagnosably different from other clusters and within which there are parental networks of ancestry and descent." The determination of whether a cluster, or clade, is diagnostically different from other clusters relies on statistical probabilities and models to reconstruct phylogenies. However, how different a clade has to be to represent a species is not clear and is influenced by the locus, number of loci, and characteristics used as well as the sample size and how well the sample represents the species as a whole. For example, in the Oomycete genus *Phytophthora, Phytophthora infestans*, *P. mirabilis*, and *P. ipomoea* are mainly distinguished through host range studies while they are almost identical for internal transcribed spacer (ITS) sequences (12,19).

Pseudoperonospora cubensis and *P. humuli* have been found to be sister taxa nested within other *Pseudoperonospora* spp. in phylogenetic analyses of molecular data (6,45,62,63). Choi et al. (6) suggested that *P. cubensis* and *P. humuli* were synonymous based on overlapping dimensions of certain morphometric characteristics and ITS sequence data of nine isolates of each pathogen. Runge et al. (49) challenged the proposed synonymy based on a multigenetic analysis of a larger collection of *P. cubensis* isolates from east Asia, Europe, and the Americas. They included a modest sampling of *P. humuli* (six isolates) in their analysis, none of which were from North America.

P. cubensis can exhibit different morphology on different hosts and in different environmental conditions (6,41,51). Thus, the slight differences between the morphology of the downy mildew pathogens could be attributed to the same phenomenon if the two really are the same species. It is interesting to note that the ranges for sporangiophore length and trunk width and sporangial dimensions given in Choi et al. (6) are broader than those reported by Palti (40) and Miyabe and Takahashi (36).

In the genera *Pythium* and *Phytophthora*, several species have identical or nearly identical ITS sequences, indicating that this may not be the ideal locus for distinguishing oomycete species (27,30,33). Analysis of multiple genetic regions is clearly required to distinguish closely related taxa (2) such as *P. cubensis* and *P. humuli*. Currently, a multigenic, phylogenetic analysis of *P. cubensis* and *P. humuli* is absent and the conclusions based solely on ITS analysis might not be adequate.

Previous studies (6,49) did not report on host specificity among isolates of *P. cubensis* and *P. humuli*, which is a critical characteristic for regulatory consideration of downy mildews. *P. humuli* may infect *Urtica*, *Cannabis*, and *Celtis* spp. at a low level following artificial inoculation (24,52). There is no record of *P. cubensis* successfully infecting wild or cultivated hop, or of infection of cucurbits by *P. humuli*. Hoerner (24) reported that "all attempts to infect available hosts of *Pseudoperonospora cubensis* [with *P. humuli*] … were unsuccessful." The proposed reduction from two pathogenic species into one has implications for pathogen identification, disease management, and regulatory considerations; therefore, a critical evaluation of this proposal is required. To this end, our objectives were to examine the relationship between *P. cubensis* and *P. humuli* in terms of (i) host ranges and (ii) a multigenic, phylogenetic analysis based on a diverse collection of isolates from both taxa.

MATERIALS AND METHODS

Plant material. Plants of the downy-mildew-susceptible hop 'Nugget' (23) and 'Pacific Gem' were propagated from softwood cuttings and maintained in a greenhouse free of downy mildews. Plants of the susceptible cucumber 'Straight 8' (54) and cantaloupe 'Ananes Yokneam' (29,60) were grown from seed in the same greenhouse. The greenhouse was maintained at 20 to 25°C with a 14-h photoperiod. Hop plants were grown in Sunshine Mix number 1 (SunGro Horticulture, Bellevue, WA) in 440-cm^3 pots for ≈14 days for use in host-specificity experiments (described below). Some hop plants were repotted into 648-cm^3 pots for an additional 14 days for maintenance of *P. humuli* isolates. Cucurbit plants were grown in the same soil in 440-cm^3 pots for ≈4 to 6 weeks. Plants were watered regularly and supplied with Champion 17-17-17 (N-P_2O_5-K_2O) fertilizer with micronutrients (McConkey's, Portland, OR) at each irrigation to promote succulent growth.

Isolates. Isolates of *P. humuli* were collected during 2006 to 2009 from Oregon (eight isolates) and Washington (three isolates) (Table 1). Monosporangial field isolates were attained from symptomatic hop shoots infected with *P. humuli* as described by Gent et al. (15). Isolates were maintained and increased for DNA extraction from sporangia on Nugget hop using a droplet inoculation procedure on detached leaves (2006 to 2008) as described by Gent et al. (15) or a spray inoculation procedure for whole plants (during 2009). Additionally, two DNA samples of *P. humuli* were received from the Czech Republic and one DNA sample was received from Korea (Table 1).

For the spray inoculation, sporangia were dislodged from sporulating leaves using a pressurized narrow stream of sterile 18-ohm water (MilliQ water; Millipore, Billerica, MA) produced by a Preval Complete Spray Unit (Precision Valve Corporation, Yonkers, NY). The inoculum for maintenance of cultures was adjusted to at least 5×10^4 sporangia ml^{-1} using a hemacytometer and was sprayed to runoff onto the underside of leaves of Nugget hop. One inoculated plant (in a 440-cm^3 pot) was placed into a prepared 90-ounce (2,661.6-cm^3) plastic container (Pasta Keeper, 29.21 by 11.43 by 11.43 cm; Snapware, Mira Loma, CA) or three inoculated plants (in a 648-cm^3 pot) were placed into a prepared 2.5-gallon (11,012.2-cm^3) plastic barrel container (35.56 by 20.96 by 20.96 cm; Snapware). The containers were prepared by having sterile 18-ohm water sprayed onto the inside walls and inside of the lid to increase humidity. A moistened paper towel folded twice into a square was placed at the bottom of the container to aid in increasing the humidity. The inoculated plants were kept overnight in the containers with the lids closed. The following morning, the plants were removed from the containers and allowed to air dry for 24 h before being replaced into the dried containers and then placed into a growth chamber for 6 to 8 days. Isolates were maintained on host plants at 20°C with a 12-h light photoperiod provided by fluorescent lights (≈300 μmol/m^2/s). After 6 days of incubation, sporulation was induced by spraying the inside walls and lid of the container with deionized water and closing the container overnight.

P. cubensis isolates from the United States were received from other researchers during 2005 to 2009 and included isolates from California (6), North Carolina (9), New Jersey (1), Oregon (2), Michigan (2), and Ohio (1) (Table 1). Some of these isolates were previously characterized to pathotype (10) and were found to be unique pathotypes based on their reaction on the differential hosts described by Lebeda and Widrlechner (29). Isolates from infected cucurbits leaves were maintained on Straight 8 cucumber using a spray inoculation procedure on whole plants, the same as for *P. humuli*, except that the inoculum was adjusted to $\approx 6.5 \times 10^3$ spores ml^{-1}. Isolates of *P. cubensis* were not monosporangial due to difficulties in attaining a monosporangial isolate of the pathogen. After inoculation, cucumber plants were put into prepared closed plastic containers (either one plant in a Pasta Keeper or two to three plants in a barrel container) as described previously and were put into a dark, humid chamber (at least 98% humidity, 21°C) for 24 h. After 24 h, the plants were removed from the plastic containers and placed into trays holding at least 1 liter of deionized water in a growth chamber at 21 and 18°C (day and night, respectively) with a 12-h photoperiod. To induce sporulation after 6 days, the inside walls and lid of the container were sprayed with deionized water and the container was closed and placed into a dark humid chamber overnight. Each isolate was maintained in a separate chamber to avoid cross-contami-

nation. Additionally, herbarium samples of *P. cubensis* and *P. celtidis* were received from South Korea (Table 1).

DNA extraction. DNA was extracted from sporangial suspensions using a cetyl trimethylammonium bromide (CTAB) procedure modified from Chee et al. (5) or a MoBio Ultra Clean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions, with modifications. The modified CTAB extraction was conducted as follows. Sporangial suspensions were centrifuged at 10,600 × *g* for 3 min and then resuspended in 100 µl of 1× Tris-EDTA (TE). Polyvinyl pyrrolidone (PVP) (0.021 g), CTAB extraction buffer (900 µl; 100 mM Tris-HCl [pH 7.5], 1.4 M NaCl, 20 mM EDTA, and 5.49 mM CTAB), and 100 µl of the sporangial suspension in TE were added to a FastPrep Lysing Matrix A Tube (MP Biomedicals, Solon, OH) and put into a FastPrep instrument (Bio 101, Vista, CA), which was run five times at level 6 for 45 s; tubes were placed on ice for 2 min between each run. Then, 750 µl of the solution was transferred to a 1.5-ml microfuge tube to which 7.5 µl of β-mercaptoethanol, 22.5 µl of proteinase K at 20 mg ml^{-1}, and 7.5 µl of RNase A at 10 mg/ml was added, followed by incubation at 65°C for 30 min. After the solution was mixed with 750 µl of 24:1 chloroform/isoamyl alcohol, it was centrifuged at 10,600 × *g* for 10 min. Up to 650 µl of the aqueous phase was then transferred to a 1.5-ml microfuge tube and the chloroform/isoamyl alcohol addition and centrifugation steps were repeated. Up to 500 µl of the aqueous phase was transferred to a 1.5-ml microfuge tube and nucleic acids were precipitated by addition of an equal volume of cold isopropanol. The DNA was pelleted by centrifugation at 10,600 × *g* for 20 min at 4°C. The pellet was subsequently rinsed twice with 70% ethanol, dried in a fume hood on a hot plate at 35°C, and then resuspended in 50 µl of 10 mM Tris-HCl, pH 7.5, and stored at –20°C.

The modifications to the MoBio Ultra Clean Soil DNA Isolation Kit were as follows. To solution S1, 0.140 g of PVP was added to improve the fidelity of polymerase chain reaction (PCR) amplification following extraction (14). After the addition of S1, 200 µl of inhibitor removal solution (IRS) (MoBio Laboratories) was added to the tube with the bead lysis solution. For higher recovery of DNA, the tube with the bead lysis solution, sporangial suspension, and solutions S1 and IRS was boiled for 2 min before

TABLE 1. Identity and origin of organisms used in this study, including notes on isolates used in different host-specificity studies

Organism	Isolate	Host	Collection location	Year	GenBank accession number[a]		
					ITS	β-Tubulin	*cox* Cluster
Pseudoperonospora cubensis	CDM-237	*Citrullus lanatus*	United States, New Jersey	2007	JF304653	JF304687	JF414537
P. cubensis	CDM-241	*Cucurbita* sp.	United States, North Carolina	2007	JF304654	JF304688	JF414538
P. cubensis	CDM-246	*Momordica charantia*	United States, North Carolina	2007	JF304655	JF304689	JF414539
P. cubensis	CDM-247	*Cucurbita* sp.	United States, New Jersey	2007	JF304656	JF304690	JF414540
P. cubensis	CDM-248	*Cucurbita pepo*	United States, North Carolina	Unknown	JF304657	JF304691	JF414541
P. cubensis[b]	CDM-251	*Cucumis sativus*	United States, Michigan	2007	JF304658	JF304692	JF414542
P. cubensis[b,c]	CDM-252	*C. sativus*	United States, Ohio	2007	JF304659	JF304693	JF414543
P. cubensis[b]	CDM-253	*C. sativus*	United States, North Carolina	2007	JF304660	JF304694	JF414544
P. cubensis[c]	CDM-254	*C. sativus*	United States, North Carolina	2006	JF304661	JF304695	JF414545
P. cubensis[b,c]	CDM-255	*C. sativus*	United States, Michigan	2005	JF304662	JF304696	JF414546
P. cubensis	CDM-266	*C. sativus*	United States, California	2009	JF304663	JF304697	JF414547
P. cubensis	CDM-268	*C. sativus*	United States, California	2009	JF304664	JF304698	JF414548
P. cubensis	CDM-269	*C. sativus*	United States, California	2009	JF304665	JF304699	JF414549
P. cubensis	CDM-272	*C. sativus*	United States, California	2009	JF304666	JF304700	JF414550
P. cubensis	CDM-273	*C. sativus*	United States, California	2009	JF304667	JF304701	JF414551
P. cubensis	CDM-274	*C. sativus*	United States, California	2009	JF304668	JF304702	JF414552
P. cubensis[c]	CDM-275	*Cucurbita pepo*	United States, North Carolina	2005	JF304669	JF304703	JF414553
P. cubensis[c]	CDM-276	*C. pepo*	United States, North Carolina	2006	JF304670	JF304704	JF414554
P. cubensis[c]	CDM-277	*C. moschata*	United States, North Carolina	2008	JF304671	JF304705	JF414555
P. cubensis	CDM-278	*Cucumis sativus*	United States, Oregon	2009	JF304672	JF304706	JF414556
P. cubensis	CDM-279	*C. sativus*	United States, Oregon	2009	JF304673	JF304707	JF414557
P. cubensis[c]	CDM-282	*C. melo*	United States, South Carolina	2010	…	…	…
P. humuli	HDM-094	*Humulus lupulus*	United States, Washington	2006	JF304674	JF304708	JF414558
P. humuli	HDM-103	*H. lupulus*	United States, Washington	2006	JF304675	JF304709	JF414559
P. humuli	HDM-110	*H. lupulus*	United States, Oregon	2006	JF304676	JF304710	JF414560
P. humuli	HDM-140	*H. lupulus*	United States, Oregon	2006	JF304677	JF304711	JF414561
P. humuli	HDM-158	*H. lupulus*	United States, Oregon	2007	JF304678	JF304712	JF414562
P. humuli	HDM-170	*H. lupulus*	United States, Oregon	2007	JF304679	JF304713	JF414563
P. humuli	HDM-171	*H. lupulus*	United States, Oregon	2007	JF304680	JF304714	JF414564
P. humuli[d,e]	HDM-224	*H. lupulus*	United States, Oregon	2008	JF304681	JF304715	JF414566
P. humuli[d,e]	HDM-247	*H. lupulus*	United States, Washington	2008	JF304682	JF304716	JF414567
P. humuli[d]	HDM-254	*H. lupulus*	United States, Oregon	2008	JF304683	JF304717	JF414568
P. humuli[d]	HDM-257	*H. lupulus*	United States, Oregon	2008	JF304684	JF304718	JF414569
P. humuli	HDM-263	*H. lupulus*	Czech Republic, Chrastany	Unknown	JF304685	JF304719	JF414570
P. humuli	HDM-266	*H. lupulus*	Czech Republic, Kolesovice	Unknown	JF304686	JF304720	JF414571
P. humuli	SMK19582	*H. japonicus*	Korea, Pyongchang	2003	JF314769	JF304721	JF414565
P. celtidis	SMK17780	*Celtis sinensis*	Korea, Dongduchon	2000	JF314768	…	JF414536
P. urticae[f]	HV713	*Urtica dioica*	Austria, Oberösterreich	Unknown	…	DQ361163	…
Phytophthora infestans[f]	P106050	*Solanum tuberosum*	Mexico	Unknown	…	EU079633.1	…
P. infestans[f]	INF-PO	*S. tuberosum*	Italy	Unknown	AJ854292	…	…
P. infestans[f]	Genome[g]	*S. tuberosum*	Unknown	Unknown	…	…	U17009.2

[a] ITS = internal transcribed spacer and *cox* = cytochrome *c* oxidase.
[b] *P. cubensis* isolate used in host-specificity experiments on hop Nugget.
[c] *P. cubensis* isolate used in host-specificity experiments on hop Pacific Gem. Note that *P. cubensis* isolate CDM-282 was utilized in host range studies, but not in phylogenetic studies.
[d] *P. humuli* isolate used in host-specificity experiments on cucumber Straight 8.
[e] *P. humuli* isolate used in host-specificity experiments on cantaloupe Ananes Yokneam.
[f] Sequences from National Center for Biotechnology Information GenBank.
[g] Complete mitochondrion genome.

the first centrifugation. DNA was stored in TE buffer (S5 from MoBio or 10 mM Tris and 1 mM EDTA, pH 8.0) at –20°C.

PCR amplification, cloning, and sequencing. Two nuclear loci and one mitochondrial locus were sequenced for phylogenetic analysis. The ITS of the nuclear ribosomal DNA (nrDNA) region, the nuclear β-tubulin gene (β-*tub*), and the mitochondrial cytochrome *c* oxidase (*cox*) cluster (partitioned into *cox2*, *cox2-cox1* spacer, and *cox1*) were amplified with the primers specified in Table 2. PCR reactions were carried out in a total volume of 25 µl containing 12 µl of PCR-grade water, 10 µl of Hot Master Mix (5 PRIME, Gaithersburg, MD), 0.75 µl (0.5 µM) of each forward and reverse primer, 0.5 µl of acetonitrile (50% by volume), and 1 µl of template. The amplification program consisted of an initial denaturation at 94°C for 2 min; followed by 40 cycles of denaturation at 94°C for 20 s, annealing temperature specific for each primer pair (Table 2) for 30 s, and extension at 65°C for 1 min; with a final extension at 70°C for 10 min. DNA fragments were electrophoresed in a 1% Tris-acetate EDTA gel. Ethidium bromide (0.5 µg ml^{-1}) was added to each gel, and the DNA fragments were visualized over a UV transilluminator.

Monosporangial isolates of *P. humuli* were directly sequenced in both directions from the PCR product. To obtain a single haplotype from non-monosporangial isolates, which could represent multiple individuals, the PCR products for each locus were cleaned as follows: amplicons were cleaned using the Amicon or Microcon centrifugal filters (Millipore), ligated into p-GEM T-Easy vector (Promega Corporation, Madison, WI), and cloned in *Escherichia coli* strain DH5α in accordance with the manufacturer's instructions. The insert and a portion of the vector were amplified using plasmid primers M13F and M13R. DNA fragments were electrophoresed and visualized as described above for confirmation before sequencing. Amplicons from PCR or clones were sequenced bidirectionally by the Center for Genome Research and Biocomputing on an ABI Prism 3730 Genetic Analyzer (Oregon State University, Corvallis). Typically, a single clone was chosen for each combination of host and locus to prevent the potential sequencing of multiple individuals in the non-monosporangial isolates. Most clones were sequenced one time (bidirectionally), unless there was ambiguity in the sequence, in which case sequencing was repeated until the sequence could be determined conclusively.

Sequence alignment and phylogenic analysis. Sequences were aligned in BioEdit (version 7.0.9.0; Ibis Therapeutics, Carlsbad, CA) initially using ClustalW (61) under default settings followed by manual adjustments as needed. Complete sequences of *Phytophthora infestans* was available for all loci used in this study, and these sequences were downloaded from the National Center for Biotechnology Information (NCBI) GenBank to provide an outgroup to the *Pseudoperonospora* spp. for phylogenetic analyses (Table 1). Additionally, *Pseudoperonospora urticae* and *P. celtidis* sequence data were included as outgroups more closely related to *P. cubensis* and *P. humuli* for the nuclear (ITS and β-*tub*) and mitochondrial (*cox* cluster) analyses, respectively. The *P. urticae* sequences of ITS and β-*tub* were downloaded from the NCBI GenBank (Table 1).

MrModeltest 2.3 (38) was used to obtain an appropriate model of nucleotide substitutions. Models were chosen based on the Akaike information criterion. The best models for the nuclear and mitochondrial data sets were the Hasegawa, Kishino, and Yano model with gamma-distributed substitution rate (HKY+G) and the general time-reversible model with a proportion of invariant nucleotide sites (GTR+I), respectively. Incongruence length difference (ILD) tests were performed on the suitability of analyzing the *cox* cluster as a locus and for concatenating the nuclear loci for analysis using PAUP* version 4.0b10 (58). Phylogenic analyses were performed using MrBayes version 3.1.2 (25,47) for Bayesian analyses and RAxML version 7.2.5 (56,57) for maximum likelihood analysis. In the Bayesian analysis, three heated (temperature = 0.2) and one cold simultaneous Markov chains were run for three million generations, saving a tree every 100 generations. Among these, the first 7,500 trees were ignored. A 50% majority rule consensus of the remaining trees was constructed by MrBayes version 3.1.2 to obtain estimates for the posterior probabilities of groups**.** To test the reproducibility of our results, the analyses were repeated three times, starting with random trees and default parameter values.

Phylogenetic analyses were conducted using RAxML version 7.2.5 under default parameters for the nucleotide substitution model with a rapid bootstrapping (1,000 replicates) via the CIPRES web portal. Additionally, the option to print branch lengths (-k) and to perform a rapid bootstrap analysis and search for the best-scoring maximum likelihood (ML) tree in one single program run (-f a) were selected. PAUP* version 4.0b10 was used to create a 50% consensus tree from the files with the 1,000 bootstrap trees from RAxML version 7.2.5. For each locus and for the concatenated data set, the distance tree from the Bayesian

TABLE 2. Polymerase chain reaction and sequencing primers used in this study for different sequenced loci

Primer	Sense	Sequence (5′–3′)	Locus[a]	Annealing temperature (°C)	Source
ITS1	Forward	TCCGTAGGTGAACCTGCGG	ITS	51	65
ITS4	Reverse	GCATATCAATAAGCGGAGGA	ITS	51	65
HDM07	Forward	AGAATTGACTGCGAGTCC	ITS	58.4	14
HDM04	Reverse	AGCCACACAACACATAGT	ITS	58.4	14
bTub136-OW	Forward	CGCATCAAYGTRTACTACAAYG	β-*Tub*	47	17
bTub1024R-OW	Reverse	CGAAGTACGAGTTCTTGTTC	β-*Tub*	47	17
bTubMM254-276R	Reverse	GTGATCTGGAAACCCTGCA	β-*Tub*	60	This study
bTubMM202-220F	Forward	ATTGACTCGGTGCTTGACG	β-*Tub*	60	This study
bTubMM558-576R	Reverse	GTGATACCAGACATGGCG	β-*Tub*	60	This study
bTubna492F	Forward	CATTTGCTTCCGCACACTTA	β-*Tub*	60	This study
FM35	Forward	CAGAACCTTGGCAATTAGG	*cox2*	56	32
FM36	Reverse	CAAATTTCACTACATTGTCC	*cox2*	56	32
FMPh-8b	Forward	AAAAGAGAAGGTGTTTTTTATGGA	*cox2-cox1* spacer	56	34
FMPh-10b	Reverse	GCAAAAGCACTAAAAATTAAATATAA	*cox2-cox1* spacer	56	34
FM84	Forward	TTTAATTTTTAGTGCTTTTGC	*cox1*	56	33
FM85	Reverse	AACTTGACTAATAATACCAAA	*cox1*	56	33
FM85RC[b]	Reverse	TTTGGTATTATTAGTCAAGTT	*cox1*	56	33
FM77	Reverse	CACCAATAAAGAATAACCAAAAATG	*cox1*	56	33
FM83	Reverse	CTCCAATAAAAAATAACCAAAAATG	*cox1*	56	33
M13F	Forward	TGTAAAACGACGGCCAGT	Plasmid	50	…
M13R	Reverse	CAGGAAACAGCTATGACC	Plasmid	50	…

[a] ITS = internal transcribed spacer, β-*Tub* = β-tubulin, and *cox* = cytochrome *c* oxidase.
[b] FM85RC is the reverse compliment of FM85 (33).

analysis was used as the template to reconcile the trees from each analysis to form a consensus tree with 50% majority rule for the trees inferred by both programs.

An additional phylogenetic analysis was performed incorporating ITS sequences obtained in this study with all available published *P. cubensis* and *P. humuli* sequences in the NCBI database to provide a larger data set, including isolates from Europe and Asia (Table 3). *P. celtidis*, *P. urticae*, and *P. cannabina* ITS sequences were included as additional outgroups. Three sequences from China were 709 bp long; therefore, the other sequences were shortened accordingly for the phylogenetic analysis. Phylogeny was inferred by RAxML version 7.2.5 (same conditions as above) and MrBayes version 3.1.2 using the general time-reversible nucleotide model with gamma distributed substitution rate (GTR+G) found by MrModeltest version 2.3. The 50% consensus trees were reconciled using the tree inferred by Bayesian analysis as the template. Trees were presented using TreeView version 1.6.6 (39) with *Phytophthora infestans* as the outgroup.

Host range study. *Pseudoperonospora humuli* isolates from Oregon (three isolates) and Washington (one isolate) and *P. cubensis* isolates from North Carolina (five isolates), Michigan (two isolates), Ohio (one isolate), and South Carolina (one isolate) were used for a host range study conducted during 2008 to 2010 (Table 1). The pathotype of three of the *P. cubensis* isolates was determined previously (10), each being considered a unique pathotype. During a given inoculation experiment, *P. humuli* was spray inoculated as described above onto three cucumber plants (Straight 8) at a concentration of 5×10^3 spores ml^{-1} while *P. cubensis* was spray inoculated onto three hop plants (Nugget) at a concentration of 5×10^3 spores ml^{-1}. Cucumber and hop plants were trimmed to three nodes per plant (nodes 3 to 7, depending on plant age and leaf quality). A positive control and negative (water-only) control plant was included for each *Pseudoperonospora* spp. in every experiment. All the plants were grown in 440-cm^3 pots and put into Pasta Keeper containers. The plants were incubated as described above for *P. cubensis* on cucumber. All but three of the pathogen strains were evaluated at least twice for both host combinations.

Additionally, host range studies were conducted on Pacific Gem hop and Ananes Yokneam melon (cantaloupe), which are regarded as universally susceptible to *P. humuli* and *P. cubensis*, respectively (29). In the Pacific northwestern United States, Pacific Gem plants are killed by downy mildew because of their

TABLE 3. Identity and origin of all available *Pseudoperonospora* spp. internal transcribed spacer sequences retrieved from the National Center for Biotechnology Information GenBank database

Organism	Isolate	Host	Origin	GenBank accession no.
Pseudoperonospora cannabina	MZM71018	*Cannabis sativa*	Latvia	AY608612
P. celtidis	SMK17780	*Celtis sinensis*	Korea	AY608613
P. cubensis	HV222	*Cucumis sativus*	Austria	AY198306
P. cubensis	HV2279	*Impatiens irvingii*	Cameroon	EU660054
P. cubensis	JinShan	*C. sativus*	China	DQ025515
P. cubensis	MinHang	*C. sativus*	China	DQ025516
P. cubensis	PuDong	*C. sativus*	China	DQ025517
P. cubensis	Not available	*C. sativus*	China	AY744946
P. cubensis	JM_12/00	*C. sativus*	Czech Republic	EU876600
P. cubensis	JM_39/01	Unknown	Czech Republic	EU876601
P. cubensis	Leb_4/95	*C. sativus*	Czech Republic	EU876604
P. cubensis	OL_1/88	*C. sativus*	Czech Republic	EU876599
P. cubensis	OL_26/01a	Unknown	Czech Republic	EU876598
P. cubensis	SC_75/01	Unknown	Czech Republic	EU876603
P. cubensis	ZL_35/01	Unknown	Czech Republic	EU876602
P. cubensis	WE_3/00	*C. sativus*	France	EU876597
P. cubensis	CEC_2811	*C. sativus*	Greece	EU876592
P. cubensis	CEC_2812	*C. sativus*	Greece	EU876591
P. cubensis	CEC_2813	*C. sativus*	Greece	EU876590
P. cubensis	CEC_2814	*C. sativus*	Greece	EU876589
P. cubensis	CEC_2815	*C. sativus*	Greece	EU876588
P. cubensis	CEC_2816	*C. sativus*	Greece	EU876587
P. cubensis	CEC_2818	*C. sativus*	Greece	EU876586
P. cubensis	CEC_2819	*C. sativus*	Greece	EU876585
P. cubensis	CEC_2820	*C. sativus*	Greece	EU876584
P. cubensis	HIE_2409	*C. sativus*	Greece	EU876596
P. cubensis	HIE_2410	*C. sativus*	Greece	EU876595
P. cubensis	HIE_2412	*C. sativus*	Greece	EU876594
P. cubensis	HIE_2413	*C. sativus*	Greece	EU876593
P. cubensis	SMK11284	*C. melo*	Korea	AY608614
P. cubensis	SMK12174	*C. sativus*	Korea	AY608616
P. cubensis	SMK13288	*Cucurbita moschata*	Korea	AY608619
P. cubensis	SMK14235	*Citrullus vulgaris*	Korea	AY608618
P. cubensis	SMK15170	*Cucumis melo*	Korea	AY608615
P. cubensis	SMK18951	*C. sativus*	Korea	AY608617
P. cubensis	SMK19205	*Cucurbita moschata*	Korea	AY608620
P. cubensis	SMK21327	*Lagenaria siceraria*	Korea	DQ409815
P. cubensis	D2	*Trichosanthes cucumerina*	Malaysia	GU233293
P. cubensis	Not available	Unknown	Taiwan	EF050035
P. humuli	HV136	*Humulus lupulus*	Austria	AY198304
P. humuli	HV148	*H. lupulus*	Austria	AY198305
P. humuli	Not available	*H. lupulus*	Czech Republic	AF448225
P. humuli	SMK11608	*H. japonicus*	Korea	AY608621
P. humuli	SMK11675	*H. lupulus*	Korea	AY608624
P. humuli	SMK18856	*H. japonicus*	Korea	AY608622
P. humuli	SMK19582	*H. japonicus*	Korea	AY608623
P. urticae	HV715	*Urtica dioica*	Korea	AY198307

extreme susceptibility to the disease. The cucurbit species was changed to Ananes Yokneam cantaloupe to verify that the host specificity of *P. humuli* observed on cucumber (described below) was consistent on another cucurbit host.

The experiments were rated 7 and 14 days postinoculation by stereomicroscopic examination of the abaxial surface of each leaf. Each leaf was rated for a hypersensitive response (HR) (localized water soaking, chlorosis, and necrosis) and sporulation. The rating on the 7th day was done without harming the plant, whereas the day-14 rating was destructive. For 15 of 18 isolate–host combinations in which sporulation was observed, sporangiophores and sporangia were counted or were estimated if individual spore structures were too numerous to identify.

To confirm that the infection was produced by the organism inoculated (i.e., *P. cubensis* on hop leaves), sporangia were collected from studies on two strains (*P. cubensis* isolates CDM-255 and CDM-276 on hop) and identified using the *cox2* locus. *Cox2* was chosen due to the relative ease in amplification and the presence of four well-conserved single-nucleotide polymorphisms (SNPs) that differentiate *P. humuli* and *P. cubensis* among the isolates used in this study with only three exceptions. The exceptions are isolates SMK19582 (*P. humuli* from *H. japonicus* in Korea with three SNPs, only two of which are found in most *P. cubensis*); CDM-241 (*P. cubensis* from squash in North Carolina, with one SNP found in most *P. humuli*); and CDM-248 (*P. cubensis* from acorn squash in North Carolina, with three SNPs found in most *P. humuli*). None of these isolates were alive when the host-specificity experiments were performed, nor would it be plausible that the DNA from the three aberrant isolates would contaminate the DNA from the host-specificity experiments because the host range studies and DNA extractions were not conducted at the same time.

To further confirm that the infection was produced by the organism inoculated onto hop and cucumber, sporangia of *P. cubensis* produced on hop were tested on hop and cucumber to confirm that the pathogen had a similar pathogenicity pattern as *P. cubensis* when inoculated on cucumber. For this experiment, *P. cubensis* (CDM-255) from Straight 8 cucumber was spray inocu-

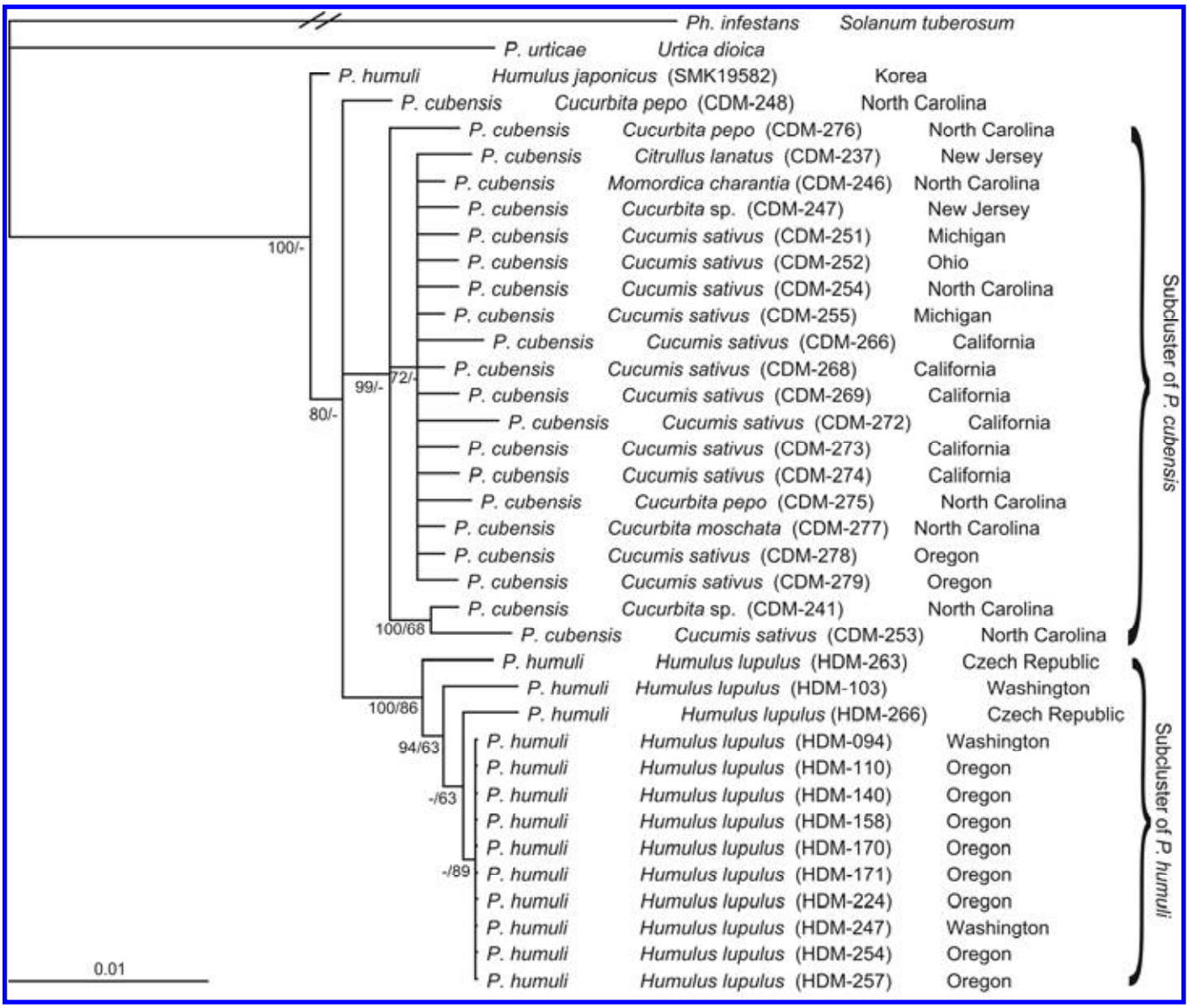

Fig. 1. A 50% consensus tree of Bayesian and maximum likelihood analyses of an alignment of the internal transcribed spacer and β-tubulin loci for *Pseudoperonospora cubensis* and *P. humuli*. Genetic distances were computed according to the Hasegawa, Kishino, and Yano model, additionally assuming a gamma-distributed substitution rate. Tree topology was rooted with *Phytophthora infestans*. Numbers on branches are posterior probabilities (3×10^6 generations) followed by bootstrap support values (1,000 replicates). Dashes indicate support <50%. The expected number of nucleotide substitutions between taxa is represented by branch length and the scale bar equals the expected number of nucleotide substitutions per site. The interrupted root branch was scaled to one-quarter its length.

lated onto four hop plants (Pacific Gem) plants in 440-cm^3 pots at 5×10^5 sporangia ml^{-1} in the manner described above. The four plants were placed into a prepared barrel container and were incubated in the manner of *P. cubensis*. On the seventh day, the inoculum from the hop plants was collected, combined, and quantified. From this inoculum, four hop plants (Pacific Gem) and two cucumber plants (Straight 8) were spray inoculated at 5×10^3 sporangia ml^{-1}. The plants were treated in the same way as the first four hop plants. On the seventh day, the inocula from the hop and cucumber plants were collected and quantified, and DNA was extracted for confirmation that the pathogen infecting these plants was *P. cubensis*.

RESULTS

Phylogenic analysis. We obtained sequence data for 21 *P. cubensis*, 14 *P. humuli*, 1 *P. celtidis* (*cox* cluster) or *P. urticae* (ITS and β-*tub*), and 1 (per analysis) *Phytophthora infestans* isolates. Overall, the phylogenetic analyses included 809 bp of the ITS, consisting of the complete ITS region (ITS1, 5.8S nrDNA, and ITS2), 696 bp of β-*tub*, and 2,067 bp of the *cox* cluster (673 bp of partial *cox2*, 204 bp of *cox2-cox1* spacer, and 1,190 bp of partial *cox1*). The nuclear data set, consisting of the ITS and β-*tub*, contained 1,555 bp, of which there were 41 parsimony-informative characteristics, 205 that were parsimony uninformative, and 1,309 that were constant. For the *cox* cluster, there were 56 parsimony-informative sites, 284 that were parsimony uninformative, and 1,727 that were constant. For some isolates, the entire ITS, β-*tub*, and *cox1* loci did not amplify; therefore, alternate primers were used (ITS or *cox1*) or designed (β-*tub*) to amplify overlapping smaller fragments, which were then aligned to obtain a sequence for the whole region (Table 2). The *cox2* gene and the *cox2-cox1* spacer amplified well for all isolates used in this study.

The ILD test showed no significant difference between the loci within the *cox* cluster ($P = 0.774$) or between ITS and β-*tub* ($P = 0.951$), indicating that the loci within these data sets were congruent and could be analyzed with single-nucleotide substitution models. Additionally, the relatively conserved topology

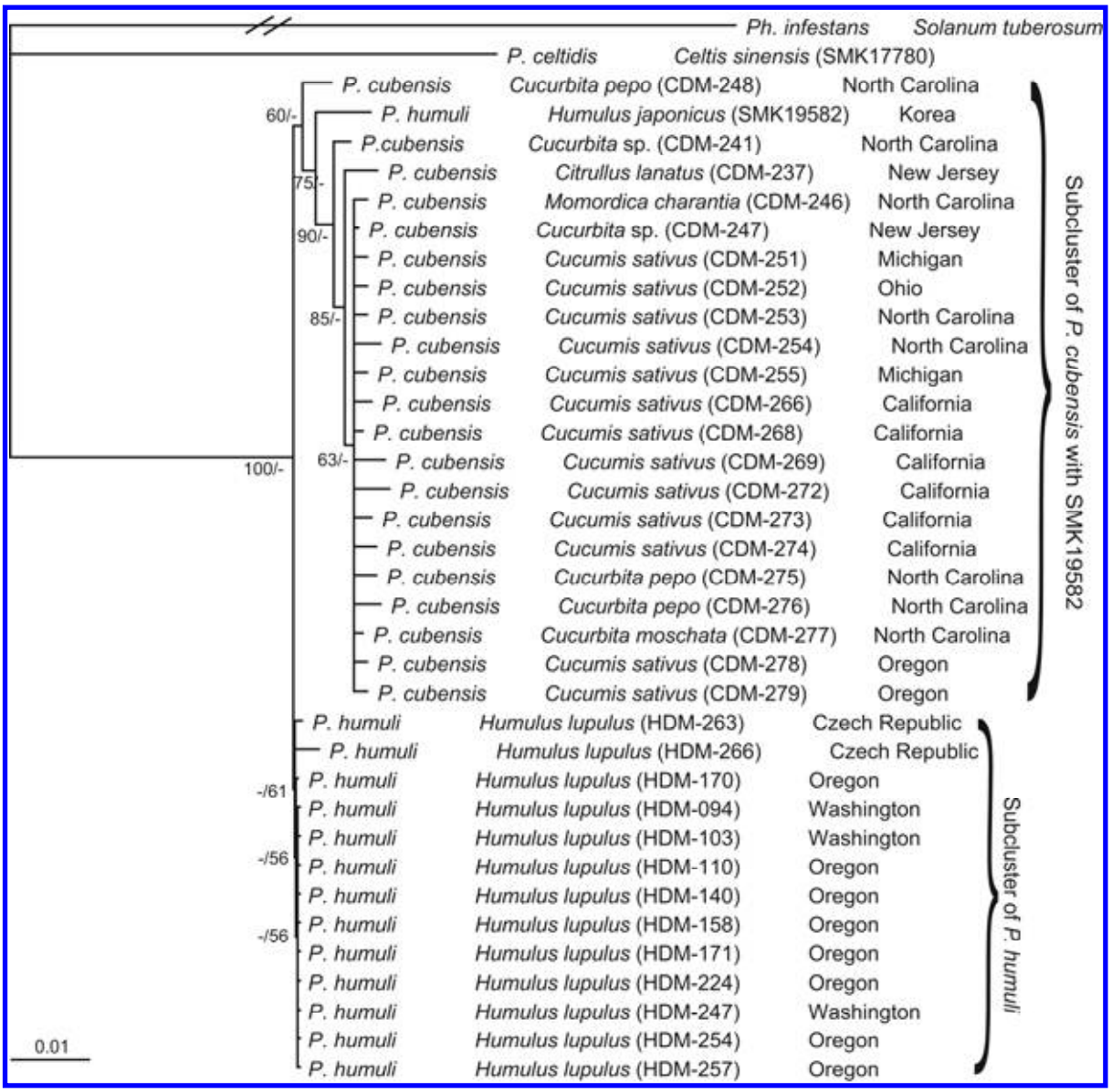

Fig. 2. A 50% consensus tree of Bayesian and maximum likelihood analyses of an alignment of the cytochrome *c* oxidase (*cox*) cluster region (partial *cox2*, *cox2-cox1* spacer, and partial *cox1*) for *Pseudoperonospora cubensis* and *P. humuli*. Genetic distances were computed according to the general time-reversible model, additionally assuming a proportion of invariant nucleotide sites. The tree was rooted with *Phytophthora infestans*. Numbers on branches are posterior probabilities (3×10^6 generations) followed by bootstrap support values (1,000 replicates). Dashes indicate support <50%. The expected number of nucleotide substitutions between taxa is represented by branch length and the scale bar equals the expected number of nucleotide substitutions per site. The interrupted root branch length was scaled to one-half its length.

(and statistical support) of the trees inferred by the individual loci suggested that concatenation of the loci into nuclear and mitochondrial data sets was appropriate. The phylogenetic relationships between *Pseudoperonospora cubensis* and *P. humuli* inferred from Bayesian analysis and heuristic ML analysis (performed by RAxML) of the aligned nucleotide sequences are shown for the nuclear loci (Fig. 1), *cox* cluster (Fig. 2), and ITS nrDNA sequence data downloaded from GenBank (Fig. 3). All four Bayesian analyses resulted in the same tree topology with almost identical posterior probability (PP) values for the analysis of the *cox* cluster and nuclear loci. The topology differed slightly for two of the four Bayesian analyses of the ITS nrDNA sequence data downloaded from GenBank in that the cluster of *P. cubensis* isolates from this study, Europe, and some from Asia was not resolved. Also, the GenBank sequence for one isolate of *P. humuli* on *H. japonicus* (SMK19582) clustered basally to the *P. humuli*–*P. cubensis* isolates. The PP values were nearly identical for the unchanged clusters.

The trees inferred by nuclear loci agreed on the separation of most of the *P. humuli* isolates from the *P. cubensis* isolates. A cluster of all the *P. humuli* isolates, with the exception of the *H. japonicus* from Korea (SMK19582), was well supported by both

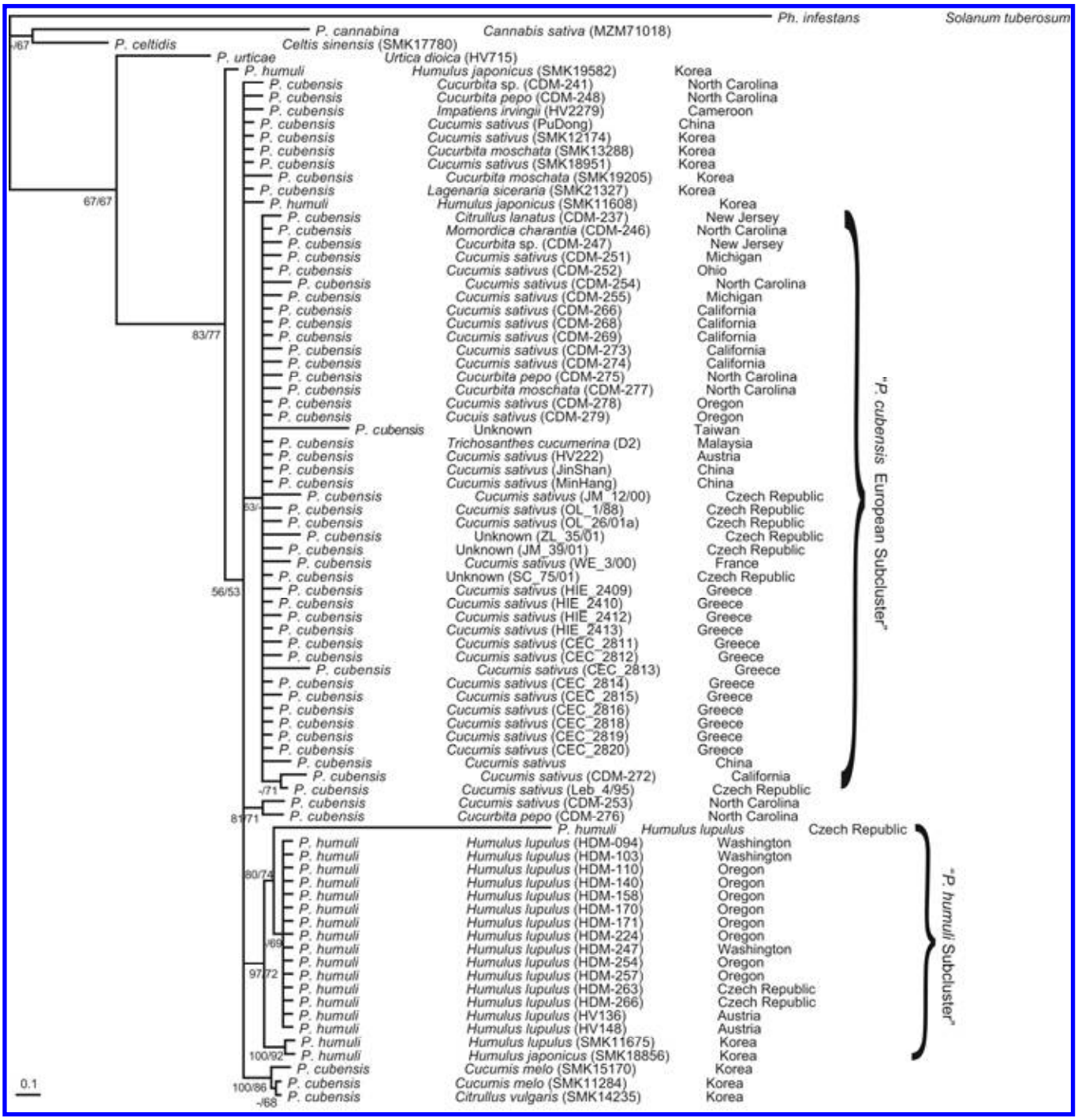

Fig. 3. A 50% consensus tree of Bayesian and maximum likelihood analyses of an alignment of published *Pseudoperonospora* spp. internal transcribed spacer sequences from GenBank. Genetic distances were computed according to the general time-reversible model, additionally assuming a gamma-distributed substitution rate. The tree was rooted with *Phytophthora infestans*. Numbers on branches are posterior probabilities (3×10^6 generations) followed by bootstrap support values (1,000 replicates). Dashes indicate support <50%. The expected number of nucleotide substitutions between taxa is represented by branch length and the scale bar equals the expected number of nucleotide substitutions per site.

the Bayesian and ML analyses (Fig. 1; 100% PP, 86% bootstrap support [BS]). Only the Bayesian analysis resolved the relationships within the *P. cubensis* isolates, with a cluster of all *P. cubensis* isolates except one from *Cucurbita pepo* from North Carolina (CDM-248) supported with 99% PP.

The consensus trees of the *cox* cluster inferred by Bayesian and ML analyses separated the *P. cubensis* isolates from the *P. humuli* isolates, with the exception of the Korean *P. humuli* isolate (Fig. 2). The separation of either cluster from the other group was supported in the Bayesian analysis 100% but not in terms of BS. A cluster of *P. cubensis* isolates exclusive of an isolate on *C. pepo* from North Carolina (CDM-248) and the Korean *P. humuli* isolate had 90% PP.

The trees inferred by ML and Bayesian analysis of the ITS region that included all published *Pseudoperonospora* sequences in GenBank had similar topology (Fig. 3). Both analyses supported a clade of *P. cubensis* and *P. humuli* subtended by the other *Pseudoperonospora* spp. (83% PP, 77% BS). A cluster of all the *P. humuli* isolates except for two isolates from *H. japonicus* from Korea (SMK11608 and SMK19582) was found with both analyses (97% PP, 72% BS). Two subclusters were defined within the *P. humuli* cluster (80 and 100% PP, 74 and 92% BS) separating U.S. and European isolates from those originating from Korea. Within the *P. cubensis* clade, a cluster of three isolates from *Cucumis melo* and *Citrullus vulgaris* from Korea was supported by both analyses (100% PP, 86% BS), including a cluster of two isolates sampled in this study (CDM-253 and CDM-276; 81% PP, 71% BS).

Host range study. *P. cubensis* isolates inoculated on hop plants sporulated on 48 of 61 replicate plants (79%) (Table 4). Isolates of *P. humuli* inoculated onto cucurbit plants sporulated on only 1 of 33 replicate plants (3%) (Table 4), and the sporulation consisted of a single sporangiophore. The positive control plants for both pathogens were always characterized by profuse sporulation but never localized necrosis. No sporulation or localized necrosis was observed in any of the negative control plants. In contrast, the inoculated leaves developed scattered areas of localized necrosis when inoculated with the reciprocal pathogen (Figs. 4 and 5). We consider the localized necrosis to be HR-like if the necrosis was localized to a few cells rather than dispersed and spreading, as typified in the positive controls. When sporulation was observed, the sporangiophores invariably emerged from the center or the inside edge of a chlorotic or, more commonly, necrotic lesion (Fig. 5). Typically, fewer than half of the HR-like lesions contained sporangiophores when hop plants were inoculated with *P. cubensis*. The proportion of lesions with sporangiophores appeared to be greater with Pacific Gem than with Nugget, although no attempt was made to quantify this observation (Table 4).

Differences in virulence were seen both with *P. cubensis* on the two hop cultivars and with *P. humuli* on the two universally susceptible species of cucurbits. *P. cubensis* sporulated relatively more profusely on Pacific Gem than on Nugget (Table 4), and more HR-like lesions were seen on Pacific Gem. However, this observation is limited because only one isolate of *P. cubensis* (CDM-252) was inoculated onto both of the hop cultivars. For the cucurbit species, Ananes Yokneam cantaloupe had more necrosis whether infected with *P. cubensis* or *P. humuli* compared with Straight 8 cucumber. When inoculated with *P. humuli*, cantaloupe had more HR-like lesions than cucumber. The one cucurbit plant on which *P. humuli* sporulated was a cantaloupe in which there was one sporangiophore bearing four sporangia emerging from near the center of a necrotic HR-like lesion on the 7-day rating.

PCR analysis of the inoculum harvested from hop infected with *P. cubensis* verified that the pathogen contained SNPs in the *cox2* region which correspond with all but two *P. cubensis* isolates (CDM-241 and CDM-248; see above). In reinoculation experiments to further verify that the sporulation on hop plants was indeed *P. cubensis*, the first set of four Pacific Gem hop plants yielded $\approx 7.4 \times 10^4$ sporangia/plant. The same isolate inoculated onto two Straight 8 cucumber plants yielded $>4.17 \times 10^6$ sporangia/plant. The hop plants displayed symptoms similar to those seen in the host-specificity experiments but had more HR lesions, presumably due to the higher concentration of sporangia used for inoculation. The second set of Pacific Gem plants had symptoms similar to those seen in the host-specificity experiments (Fig. 6) and yielded $\approx 5 \times 10^4$ sporangia/plant. The two cucumber plants (Straight 8) inoculated with inoculum from the first set of hop plants yielded $\approx 4.5 \times 10^6$ sporangia/plant. The cucumber exhibited typical disease symptoms for cucumber plants inoculated with *P. cubensis* (Fig. 6A and C). However, the sporulation, while abundant, was not as profuse as in many of the positive-control cucumber plants in the host-specificity experiments, perhaps due to the inoculum coming from a poor host.

DISCUSSION

For each of the sequence data sets, the resulting trees consistently divided isolates of *P. cubensis* from isolates of *P. humuli* into separate clades, with the exception of *P. humuli* on *H. japonicus* from Korea (SMK19582 and SMK11608). The nuclear loci and ITS sequence analyses strongly supported a cluster of the majority of *P. humuli* isolates with both the Bayesian and ML analyses. Within the *P. cubensis* clusters, there did not appear to be a significant genetic difference between isolates from the eastern United States and those from the western United States.

Other work that focused on multiple isolates of *P. cubensis* and *P. humuli* primarily reconstructed phylogenies based on ITS sequence. Choi et al. (6) examined the phylogenetic relationship within the genus *Pseudoperonospora* by using ITS sequence data from nine isolates of each pathogen with Bayesian and maximum parsimony (MP) analyses. Sarris et al. (53) inferred a phylogeny of *P. cubensis* and *P. humuli* from ITS sequences using the neighbor-joining method with the isolates of Choi et al. (6) as well as 22 isolates of *P. cubensis* from the Czech Republic and

TABLE 4. Results of host-specificity experiments with *Pseudoperonospora cubensis* on two hop cultivars and *P. humuli* on two cucurbit species

Isolate	Cultivar, species	Proportions of infections[a]	Sporangiophores/plant[b]
P. cubensis			
CDM 251	Nugget	3/6	38.6 ± 28.5
CDM 252	Nugget	9/15	36.5 ± 33.4
CDM 253	Nugget	4/6	0.3 ± 0.5
CDM 255	Nugget	4/6	NF
CDM 252	Pacific Gem	6/6	>600
CDM 254	Pacific Gem	3/3	>900
CDM 275	Pacific Gem	6/6	451.7 ± 380
CDM 276	Pacific Gem	6/6	239.2 ± 183.0
CDM 277	Pacific Gem	2/2	107.0 ± 70.7
CDM 282	Pacific Gem	5/5	>1,909
P. humuli			
HDM 224	Cucumber	0/6	0
HDM 247	Cucumber	0/6	0
HDM 254	Cucumber	0/6	0
HDM 257	Cucumber	0/6	0
HDM 224	Cantaloupe	0/3	0
HDM 247	Cantaloupe	1/6	1

[a] Proportion of successful infections was defined as the number of plant replicates with sporulation out of the total number of plant replicates for that isolate. Positive controls were characterized by profuse sporulation and negative controls were free of sporulation in every run of the experiments. The cucumber cultivar was Straight 8 and the cantaloupe cultivar was Ananes Yokneam.

[b] Number of sporangiophores per plant (± standard error) was calculated only for those replicates for which the number of sporangiophores was enumerable at 14 days postinfection (dpi). NF = sporangiophores were not found at 14 dpi but were found at 7 dpi.

Greece and 1 *P. humuli* isolate from the Czech Republic. Göker et al. (16) inferred the phylogenetic relationships of the same data set as Sarris et al. (53) with two additional *P. cubensis* isolates from Asia using RAxML and MP. In general, the resolution of the tree increased with each study and the topology of the trees remain fairly stable, although the statistical support for branches varied depending on the number of isolates and the type of analysis used (6,16,49,53). The isolates of *P. cubensis* tend to be in a clade divided into three groups. Two of the groups are a subcluster with isolates of *P. cubensis* from Europe and China and a subcluster consisting of isolates from Korea (6,16,49,53). The third group is composed of four *P. cubensis* isolates from Korea and two *P. humuli* isolates on *H. japonicus* from Korea of Choi et al. (6) that are not resolved into a clade but, instead, appear to be basal or adjacent to the two subclades (6,16,53). The *P. humuli* isolates are partitioned into a separate cluster from *P. cubensis* (except the two *P. humuli* isolates from *H. japonicus* clustering with *P. cubensis*) composed of two subclades. The subclades divide the remaining *P. humuli* isolates from Korea clustering together from *P. humuli* isolates from Europe.

The tree based on ITS sequence data was similar in topology and support values to those of Göker et al. (16). All 14 *P. humuli* isolates from the western United States clustered with those from Europe, separate from a subcluster of *P. humuli* from Korea. The "*P. humuli* cluster" was well supported in these analyses, similar to Runge et al. (49) who used a different set of isolates from Europe and Argentina. All but four of the isolates of *P. cubensis* from the current study clustered with the "European subcluster" of Sarris et al. (53). However, this subclade was only supported in the Bayesian analysis with 63% PP and was not found in two of the four Bayesian analyses. In the analyses of this study, isolates of *P. cubensis* on *Cucumis sativus* from China (PuDong), on *C. sativus* and *Cucurbita* spp. from North Carolina (CDM-241, 248, 253, and 276), and on *Impatiens irvingii* from Cameroon (HV2279) were not included in the "European subcluster."

Our analyses indicate differences both between and within *P. cubensis* and *P. humuli*. There appears to be a genetic divergence between the isolates from Korea and isolates from many other regions of the world in both the *P. cubensis* and *P. humuli* clusters (49). Two of the four *P. humuli* isolates from *H. japonicus* (SMK11608 and SMK19582) cluster with Korean *P. cubensis* isolates. Within the *P. humuli* cluster, the Korean isolates are strongly supported as being separate from both the European and American isolates. These differences could be explained by several factors. One obvious explanation is that the *Pseudoperonospora* sp. on *H. japonicus* is distinct from *P. humuli*. Isolates SMK11608 and SMK19582 are more *P. cubensis*-like and either cluster basally (49) or with *P. cubensis.* Another explanation could be that these observations are sampling artifacts based on two very different isolates. The differences may be due to increased genetic diversity in isolates collected in Korea compared with isolates collected in other locations around the world. Further sampling in Korea and other parts of Asia and molecular markers with greater resolution are needed to clarify

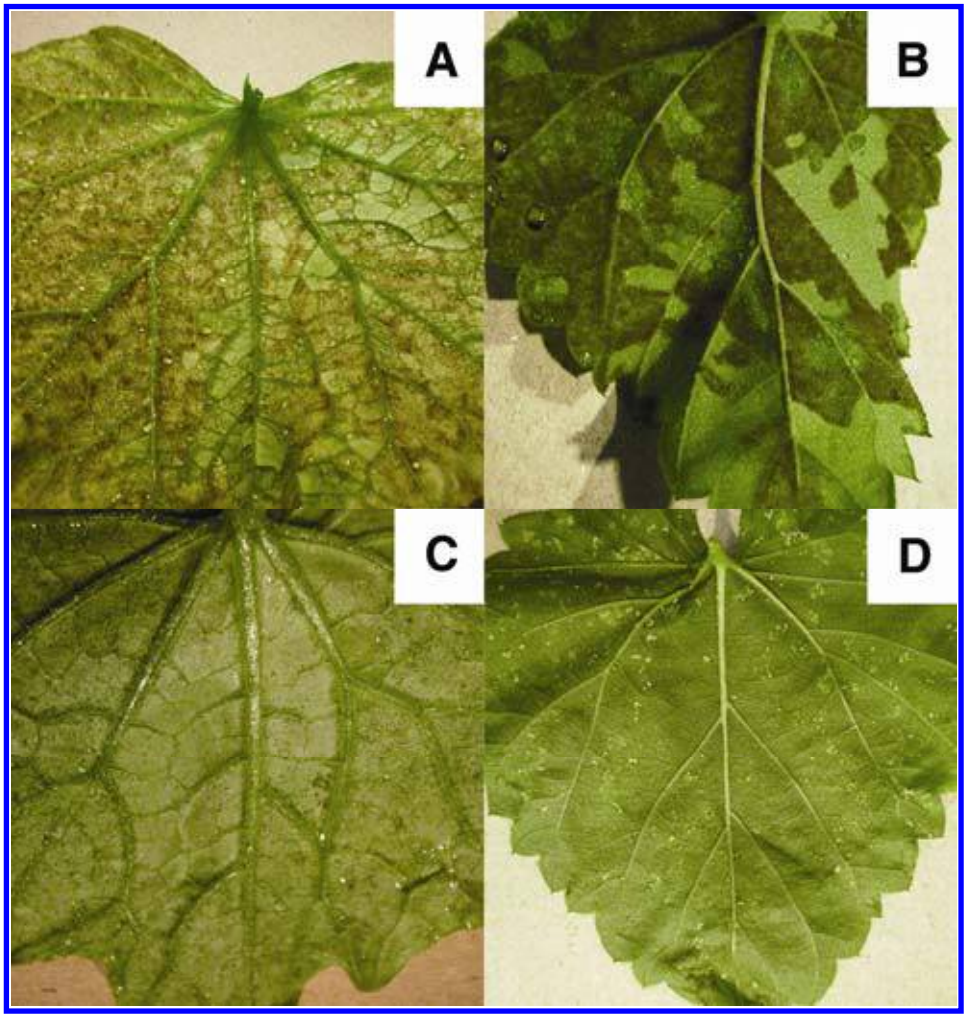

Fig. 4. Macroscopic signs and symptoms from host-specificity experiments with *Pseudoperonospora cubensis* and *P. humuli*. **A,** *P. cubensis* inoculated on cucumber 'Straight 8'; **B,** *P. humuli* inoculated on hop 'Pacific Gem'; **C,** *P. humuli* inoculated on Straight 8; and **D,** *P. cubensis* inoculated on Pacific Gem. **A and B,** Positive controls are characterized by profuse sporulation and few hypersensitive-like lesions, whereas **C and D,** the opposite is true of the reciprocal inoculations.

these observations and the diversity within and among *P. humuli* on *Humulus* spp.

Hop downy mildew was first recorded in Japan in 1905 and it is possible that *P. humuli* originated in Asia (36). It is interesting to note that Asia, especially the areas of India, Indo-Malaysia, and China, is the presumed center of origin for the genus *Humulus* (37) as well as many genera and species of cucurbits, including *C. sativus* and several other *Cucumis* spp. (26,46). *Citrullus* spp., most *Cucumis* spp. (including *C. melo*), and *Cucurbita* spp. arose in Africa (*Citrullus* and *Cucumis* spp.) and South America (*Cucurbita* spp.) and some, but not all, of the *P. cubensis* isolates that were from these hosts (i.e., CDM-241, 248, and 276 as well as SMK11284, 14235, and 15170) did not cluster with the other *P. cubensis* isolates in the phylogenetic analyses, indicating separate origins.

Host-specificity experiments indicate that *P. cubensis* and *P. humuli* are biologically distinct, because *P. cubensis* appears to be able to infect the primary host of *P. humuli*, albeit at very low levels, whereas *P. humuli* was essentially unable to successfully infect two highly susceptible hosts of *P. cubensis*. This indicates that occasional host jumps appear possible, and suggests that a host jump may have occurred recently. Hop plants inoculated with *P. cubensis* sporulated in 79% of the replicates whereas *P. humuli* only sporulated once on a cucurbit host out of 33 replicate plants and, in that instance, only a single sporangiophore was observed. Each host exhibited lesions consistent with an HR when inoculated with the reciprocal pathogen, and sporulation was observed in the center or inner edge of the necrotic, or occasionally chlorotic, lesion. It appears that at least two of the reportedly universal hosts of *P. cubensis* are extremely poor or, more likely, non-hosts of *P. humuli* under natural conditions; while the two hop cultivars tested, both highly susceptible to hop downy mildew, were also poor or non-hosts for *P. cubensis*. The existence of hop production in countries where cucurbit downy mildew occurs but not hop downy mildew (e.g., Australia, South Africa, and New Zealand) also circumstantially supports the idea that, under natural conditions, hop does not host *P. cubensis* at detectable levels. Hoerner (24) unsuccessfully attempted to infect unspecified ("all available") hosts of *P. cubensis* with *P. humuli*. Unfortunately, the experiments were undocumented aside from a passing remark (24); therefore, the conditions and exact organisms are unknown. Host jumps and close species boundaries have been observed for other oomycete pathogens; for example, in clade 1c, *Phytophthora* spp., including *Phytophthora ipomoeae*, *P. mirabilis*, and *P. infestans*, and are likely to be frequent in the downy mildews, as exemplified by the *Hyaloperonospora* genus (21,59).

The ability of *Pseudoperonospora cubensis* to successfully infect and sporulate, albeit at low levels, on the two hop cultivars tested may be due to its polyphagic lifestyle. *P. cubensis* has been

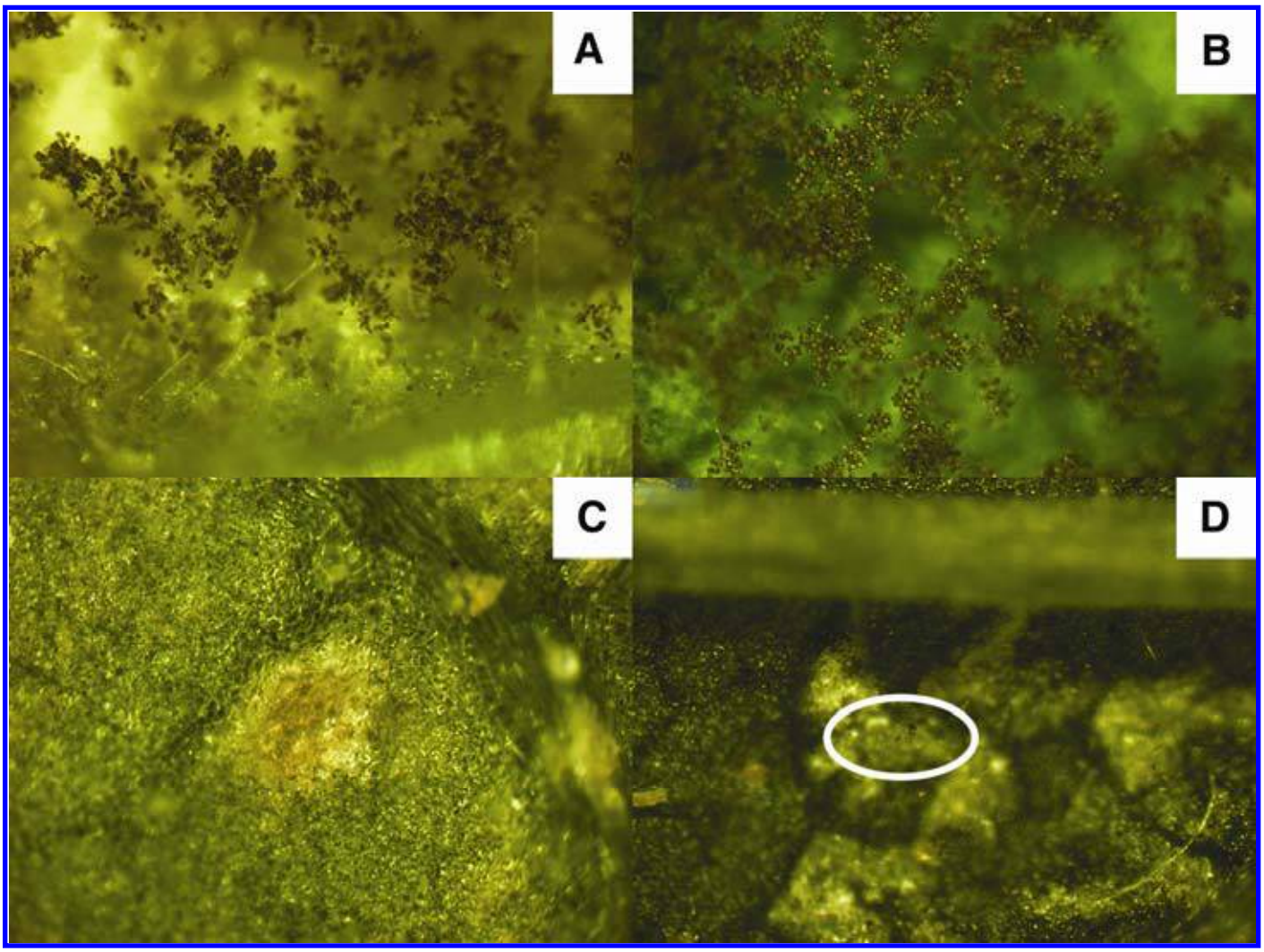

Fig. 5. Microscopic signs and symptoms from host-specificity experiments with *Pseudoperonospora cubensis* and *P. humuli* (×50 magnification). **A,** *P. cubensis* inoculated on cucumber 'Straight 8'; **B,** *P. humuli* inoculated on hop 'Pacific Gem'; **C,** *P. humuli* inoculated on Straight 8; and **D,** *P. cubensis* inoculated on Pacific Gem. **A and B,** Positive controls are characterized by profuse sporulation and few hypersensitive-like lesions, whereas **C and D,** the opposite is true of the reciprocal inoculations. Sporangia can be seen in the circled area of panel **D**.

recorded on at least 49 wild and cultivated species of Cucurbitaceae in 70 countries (6,7,41). Voglmayr et al. (64) reported *P. cubensis* to be causing downy mildew on *I. irvingii* (family *Balsaminaceae*) in Cameroon, although pathogenicity studies were not reported on a cucurbit host with that isolate. We note again that the hop cultivars included in this study are highly susceptible to hop downy mildew and, under natural conditions, can be killed by *P. humuli* if rigorous disease management is not practiced. Thus, reduced level of sporulation of *P. cubensis* on these cultivars suggests that hop is not a primary or preferred host of this pathogen.

Because isolates of both *P. cubensis* and *P. cubensis* from Korea have phylogenetic histories divergent from other isolates from elsewhere in the world, host pathogenicity studies with these isolates, on both *H. lupulus* and *H. japonicus*, are needed to confirm their host range. Although *P. humuli* did not appear to be pathogenic on the reportedly universally susceptible cucumber and cantaloupe hosts utilized in the current study, experimenting with *P. humuli* on the differential set of cucurbit species of Lebeda and Gadasová (28) may reveal a host on which *P. humuli* is better able to colonize.

In addition to the differences found in this study, there are several biologically relevant differences in the life cycles of *P. cubensis* and *P. humuli*. Pathotypes (or race structures) of *P. humuli* have never been demonstrated, whereas there is evidence of the existence of a number of pathotypes of *P. cubensis* (7,10, 28,41,54). Oospores of *P. cubensis* are rarely found (7,41). In contrast, oospores of *P. humuli* can be produced in large numbers in infected shoots, leaf lesions, and cones, although there is contradictory evidence of whether they are able to cause disease in the field (1,3,4,8,9,31,43,48,55). Additionally, while *P. humuli* can overwinter as mycelia in perennating hop crowns and, potentially, as oospores in soil or plant debris (48), *P. cubensis* infects frost-sensitive plants and must survive the off-season in hosts living in warmer climates or greenhouses, on perennial weed hosts, or perhaps as oospores (7,41,50).

In summary, phylogenetic and host-specificity analyses clearly indicate that there are biologically relevant characteristics that differentiate *P. cubensis* and *P. humuli*, with the exception of isolates from *H. japonicus* from Korea. Thus, the proposed synonymy (6) requires further consideration. Aside from two isolates of *P. humuli* on *H. japonicus* from Korea, phylogenetic analyses suggest that *P. humuli* belongs in a cluster separate from *P. cubensis*. These results are consistent with parallel research reported by Runge et al. (49), which also concluded that *P. cubensis* and *P. humuli* should not be considered conspecific. The results of our study suggest that reduction of *P. humuli* to a synonym of *P. cubensis* may be premature, particularly given the quantifiable differences in pathogenicity and genetic lineages between isolates from *Humulus* spp. and cucurbit hosts. Designation of a forma specialis is one means to differentiate important subtaxon differences in physiology (35), such as host range. From a molecular evolutionary perspective, forma specialis designations are not clearly understood and cannot be detected with sequence data. However, such designations are critical for preserving information on host range or preference and legal measures such quarantines.

The current study and emerging body of knowledge (6,16, 49,53) indicate that *P. humuli* and *P. cubensis* are morphologically and genetically very similar but possess measurable physiological

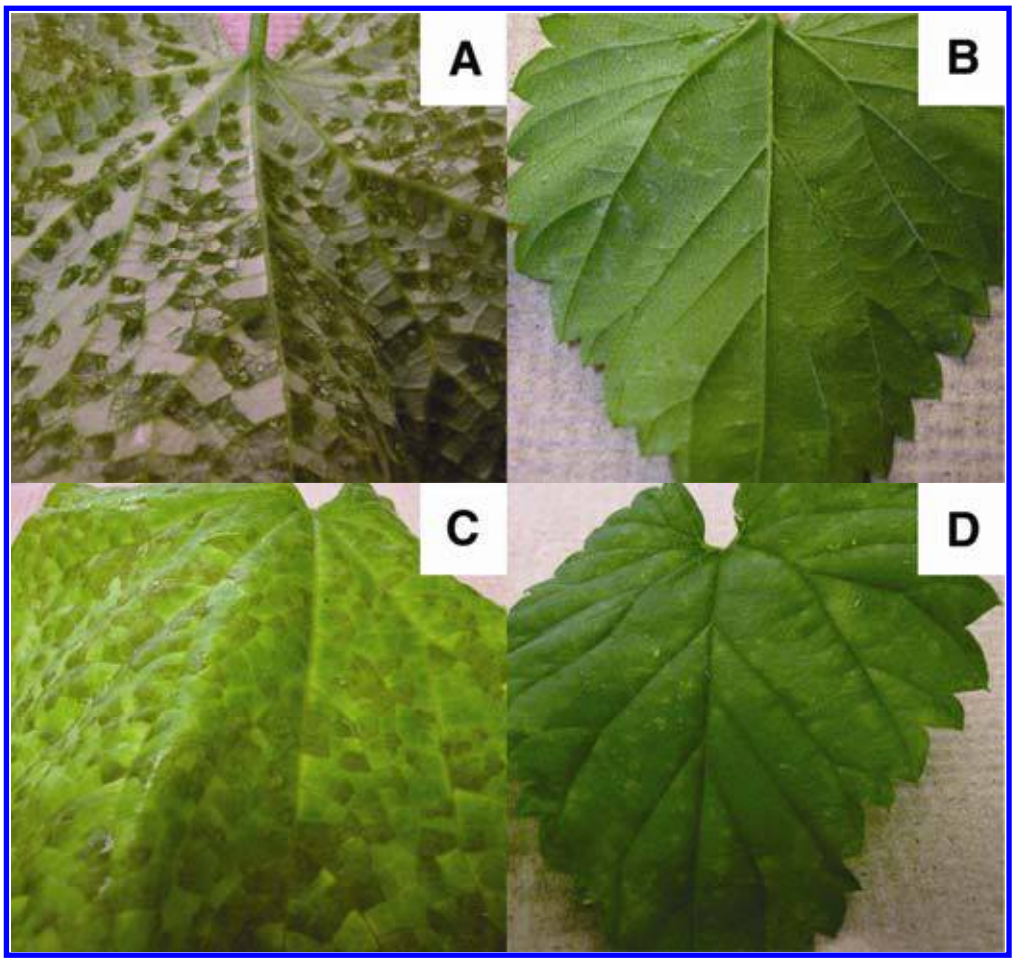

Fig. 6. Macroscopic signs and symptoms of *Pseudoperonospora cubensis* isolate CDM-255 recovered from four hop 'Pacific Gem' plants reinoculated onto **A and C,** cucumber 'Straight 8' and **B and D,** hop 'Pacific Gem'. Symptoms in panels **A** and **C** are typical of *P. cubensis* infecting Straight 8, although sporulation is not as profuse as when the inoculum is harvested from cucumber rather than hop.

and genetic differences. Further investigations of the species-population boundary, host specificity on *H. japonicus* and other hosts in Asia, and a more intensive sampling of Asian isolates of *P. cubensis* and *P. humuli* are needed to determine the true evolutionary history of this group. The true evolutionary history and species boundaries might be resolved by a coalescent analysis, as was done for *Phytophthora ramorum* (20). Until such data is available, we recommend retaining the two species names, *P. cubensis* and *P. humuli.*

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