Phylogenetic History of *Phytophthora cryptogea* and *P. drechsleri* Isolates from Floriculture Crops in North Carolina Greenhouses

H. A. Olson, I. Carbone, and D. M. Benson

First, second, and third authors: Department of Plant Pathology, North Carolina State University, Raleigh 27695.

Current address of first author: Virginia Tech, Hampton Roads AREC, Virginia Beach 23455.

Accepted for publication 14 June 2011.

ABSTRACT

Olson, H. A., Carbone, I., and Benson, D. M. 2011. Phylogenetic history of *Phytophthora cryptogea* and *P. drechsleri* isolates from floriculture crops in North Carolina greenhouses. *Phytopathology* 101:1373-1384.

The evolutionary history of *Phytophthora cryptogea* and *P. drechsleri* isolates previously collected from floriculture crops in North Carolina commercial greenhouses was explored with coalescent- and parsimony-based analyses. Initially, 68 isolates representing 13 location–host groups were sequenced at multiple loci. Sequences of all isolates within a group were identical. A subset of isolates were selected, cloned to resolve heterozygous sites, and analyzed with SNAP Workbench. The internal transcribed spacer (ITS) region of the ribosomal DNA and cytochrome oxidase II gene genealogies were congruent and indicated that *P.*

cryptogea and *P. drechsleri* are sister species diverged from a common ancestor with no evidence of gene flow. In contrast, genealogies inferred from β -tubulin (*β -tub*) and translation elongation factor 1 α (*EF-1 α*) genes were in conflict with these loci. Coalescent analysis based on a nonrecombining partition in *β -tub* and *EF-1 α* showed an initial (older) split between *P. cryptogea* and *P. drechsleri*, with a later (recent) event separating the remaining *P. cryptogea* haplotypes from *P. drechsleri*. A parsimony-based minimal ancestral recombination graph inferred recombination between *P. cryptogea* and *P. drechsleri* isolates in the ITS region and *β -tub*, suggesting genetic exchange between species. Also, putative recombination between A1 and A2 mating types of *P. cryptogea* suggests that sexual reproduction has occurred in the history of these *P. cryptogea* isolates.

Multiple species of *Phytophthora* are known to impact floriculture production, including *Phytophthora cryptogea* and *P. drechsleri*. Since the initial work with *P. cryptogea* on ornamentals by Tompkins et al. in the 1930s (45,46), this species has been reported on >40 floriculture plant species (13). Similarly, *P. drechsleri* has been reported on >25 floriculture plant species (14), including multiple hosts which overlap with *P. cryptogea*. *P. cryptogea* was first described by Pethybridge and Lafferty in 1919 with an isolate causing foot rot of tomato in Ireland (40) and, $\approx$ 10 years later, Tucker described *P. drechsleri* from an isolate of *Phytophthora* that caused rot of potato in Idaho (47). Tucker used growth at 35°C as a physiological characteristic to distinguish *P. drechsleri* (growth at 35°C) from *P. cryptogea* (no growth at 35°C). Because of highly overlapping morphological characteristics, differentiating *P. cryptogea* and *P. drechsleri* is considered one of the most difficult taxonomic problems in the genus *Phytophthora* (12). Several research groups have proposed merging the two species because of these morphological identification difficulties (8,17), whereas others believe the species status of each is supported based on the growth at 35°C (22,23).

The development of tools to analyze DNA sequence variation has allowed researchers to begin to discern the genetic relatedness of *Phytophthora* spp. This is particularly useful where morphological and biological characters are ambiguous. Phylogenetic analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) of species of *Phytophthora* placed *P.*

cryptogea and *P. drechsleri* in clade 8a and supported their species status (9). Similarly, Martin and Tooley (33) utilized the cytochrome oxidase I and II regions of the mitochondrial DNA to infer that *P. cryptogea* and *P. drechsleri* were distinct but closely related species. More recent multilocus phylogenetic gene trees support the inferences from these original single-locus studies (3,28,42). For these studies, one or two isolates of *P. cryptogea* and *P. drechsleri* were used to represent the species. Recently, Mostowfizadeh-Ghahamfarsa et al. (35) evaluated a worldwide collection of isolates of *P. cryptogea* and *P. drechsleri* and found that *P. cryptogea* and *P. drechsleri* are distinct species and are genetically variable.

During a 2007–2008 survey of commercial greenhouse facilities in North Carolina, *P. cryptogea* was collected from *Evolvulus glomeratus* (blue daze), *Senecio cineraria* (dusty miller), *Gerbera jamesonii* (gerbera daisy), and *Verbena $\times$ hybrida* (verbena) at five greenhouse locations, and *P. drechsleri* was isolated from *Fuchsia $\times$ hybrida* (fuchsia) and gerbera daisy at two locations (37). During a previous survey in North Carolina, *P. drechsleri* was collected from gerbera daisy and dusty miller at two locations (26). In both surveys, all isolates of *P. drechsleri* exhibited a high level of resistance to mefenoxam, the active ingredient of a fungicide commonly used in the management of *Phytophthora* spp. in floriculture production (26,37). In contrast, all isolates of *P. cryptogea* were sensitive to mefenoxam.

The objective of this study was to characterize the ancestral relationships and evolutionary processes of the isolates of *P. cryptogea* and *P. drechsleri* collected from floriculture crops in North Carolina commercial greenhouse facilities. Specifically, coalescent- and parsimony-based methods were used to explore the evolutionary history of *P. cryptogea* and *P. drechsleri* for evidence of gene flow, which could have important management implications for these species in the floriculture industry.

Corresponding author: H. A. Olson; E-mail address: heather.a.olson@gmail.com

*The e-Xtra logo stands for “electronic extra” and indicates that the online version contains three supplemental figures.

doi:10.1094/PHYTO-11-10-0302

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MATERIALS AND METHODS

Isolates and DNA extraction. Isolates of *P. cryptogea* and *P. drechsleri* were obtained from commercial greenhouse facilities

in North Carolina during surveys conducted in 2001–2002 (26) and 2007–2008 (37) (Table 1). These isolates previously had been characterized for mating type, mefenoxam sensitivity, and growth at 35°C (26,37). In addition, reference isolates of *P. drechsleri*

TABLE 1. Year, location, and host of origin of isolates of *Phytophthora cryptogea* and *P. drechsleri* collected from North Carolina greenhouse facilities initially sequenced

Isolate	Species	Year	Location	Host scientific name
P1088/ATCC46721 ^a	<i>P. cryptogea</i>	1935	California	<i>Callistephus chinensis</i>
NC108	<i>P. cryptogea</i>	2007	B	<i>Gerbera jamesonii</i>
NC110	<i>P. cryptogea</i>	2007	B	<i>G. jamesonii</i>
NC208	<i>P. cryptogea</i>	2008	C	<i>Evolvulus glomeratus</i>
NC594	<i>P. cryptogea</i>	2008	E	<i>G. jamesonii</i>
NC597	<i>P. cryptogea</i>	2008	E	<i>G. jamesonii</i>
NC1105	<i>P. cryptogea</i>	2008	I	<i>Verbena × hybrida</i>
NC1145	<i>P. cryptogea</i>	2008	J	<i>Senecio cineraria</i>
NC1146	<i>P. cryptogea</i>	2008	J	<i>S. cineraria</i>
NC1147	<i>P. cryptogea</i>	2008	J	<i>S. cineraria</i>
NC1148	<i>P. cryptogea</i>	2008	J	<i>S. cineraria</i>
NC1149	<i>P. cryptogea</i>	2008	J	<i>S. cineraria</i>
NC1150	<i>P. cryptogea</i>	2008	J	<i>S. cineraria</i>
NC1151	<i>P. cryptogea</i>	2008	J	<i>S. cineraria</i>
NC1152	<i>P. cryptogea</i>	2008	J	<i>S. cineraria</i>
NC1153	<i>P. cryptogea</i>	2008	J	<i>S. cineraria</i>
P1087/ATCC46724 ^a	<i>P. drechsleri</i>	1936	California	<i>Beta vulgaris</i>
NC83	<i>P. drechsleri</i>	2007	B	<i>G. jamesonii</i>
NC86	<i>P. drechsleri</i>	2007	B	<i>G. jamesonii</i>
NC88	<i>P. drechsleri</i>	2007	B	<i>G. jamesonii</i>
NC91	<i>P. drechsleri</i>	2007	B	<i>G. jamesonii</i>
NC95	<i>P. drechsleri</i>	2007	B	<i>G. jamesonii</i>
NC96	<i>P. drechsleri</i>	2007	B	<i>G. jamesonii</i>
NC99	<i>P. drechsleri</i>	2007	B	<i>G. jamesonii</i>
NC103	<i>P. drechsleri</i>	2007	B	<i>G. jamesonii</i>
NC109	<i>P. drechsleri</i>	2007	B	<i>G. jamesonii</i>
NC114	<i>P. drechsleri</i>	2007	B	<i>G. jamesonii</i>
NC947	<i>P. drechsleri</i>	2008	G	<i>G. jamesonii</i>
NC948	<i>P. drechsleri</i>	2008	G	<i>G. jamesonii</i>
NC955	<i>P. drechsleri</i>	2008	G	<i>G. jamesonii</i>
NC957	<i>P. drechsleri</i>	2008	G	<i>Fuchsia × hybrida</i>
NC961	<i>P. drechsleri</i>	2008	G	<i>Fuchsia × hybrida</i>
NC966	<i>P. drechsleri</i>	2008	G	<i>G. jamesonii</i>
CRGB1	<i>P. drechsleri</i>	2002	B	<i>G. jamesonii</i>
CRGB6	<i>P. drechsleri</i>	2002	B	<i>G. jamesonii</i>
CRGB8	<i>P. drechsleri</i>	2002	B	<i>G. jamesonii</i>
CRGB11	<i>P. drechsleri</i>	2002	B	<i>G. jamesonii</i>
CRGB42	<i>P. drechsleri</i>	2002	B	<i>G. jamesonii</i>
CRGB51	<i>P. drechsleri</i>	2002	B	<i>G. jamesonii</i>
FDM2	<i>P. drechsleri</i>	2001	A	<i>S. cineraria</i>
FDM4	<i>P. drechsleri</i>	2001	A	<i>S. cineraria</i>
FDM5	<i>P. drechsleri</i>	2001	A	<i>S. cineraria</i>
FDM6	<i>P. drechsleri</i>	2001	A	<i>S. cineraria</i>
FDM8	<i>P. drechsleri</i>	2001	A	<i>S. cineraria</i>
FDM10	<i>P. drechsleri</i>	2001	A	<i>S. cineraria</i>
FDM11	<i>P. drechsleri</i>	2001	A	<i>S. cineraria</i>
FDM13	<i>P. drechsleri</i>	2001	A	<i>S. cineraria</i>
FDM14	<i>P. drechsleri</i>	2001	A	<i>S. cineraria</i>
FDM15	<i>P. drechsleri</i>	2001	A	<i>S. cineraria</i>
FWDM1	<i>P. drechsleri</i>	2002	A	<i>S. cineraria</i>
FWDM16	<i>P. drechsleri</i>	2002	A	<i>S. cineraria</i>
FWDM18	<i>P. drechsleri</i>	2002	A	<i>S. cineraria</i>
FWDM19	<i>P. drechsleri</i>	2002	A	<i>S. cineraria</i>
FWDM20	<i>P. drechsleri</i>	2002	A	<i>S. cineraria</i>
FWDM23	<i>P. drechsleri</i>	2002	A	<i>S. cineraria</i>
FWDM24	<i>P. drechsleri</i>	2002	A	<i>S. cineraria</i>
FWDM25	<i>P. drechsleri</i>	2002	A	<i>S. cineraria</i>
FWDM26	<i>P. drechsleri</i>	2002	A	<i>S. cineraria</i>
FWGB1	<i>P. drechsleri</i>	2002	A	<i>G. jamesonii</i>
FWGB2	<i>P. drechsleri</i>	2002	A	<i>G. jamesonii</i>
FWGB3	<i>P. drechsleri</i>	2002	A	<i>G. jamesonii</i>
FWGB4	<i>P. drechsleri</i>	2002	A	<i>G. jamesonii</i>
FWGB5	<i>P. drechsleri</i>	2002	A	<i>G. jamesonii</i>
FWGB6	<i>P. drechsleri</i>	2002	A	<i>G. jamesonii</i>
FWGB7	<i>P. drechsleri</i>	2002	A	<i>G. jamesonii</i>
FWGB9	<i>P. drechsleri</i>	2002	A	<i>G. jamesonii</i>
FWGB10	<i>P. drechsleri</i>	2002	A	<i>G. jamesonii</i>
FWGB11	<i>P. drechsleri</i>	2002	A	<i>G. jamesonii</i>

^a Isolate obtained from World Oomycete Genetic Resource Collection; ATCC = American Type Culture Collection (44,46).

and *P. cryptogea* were obtained from the World Oomycete Genetic Resource Collection (Table 1). For *P. drechsleri*, isolate P1087 (ATCC46724) isolated from sugar beet by Tompkins et al. (44) was chosen because Mostowfizadeh-Ghalamfarsa et al. (35) used it as the type isolate for *P. drechsleri*, and *P. cryptogea* isolate P1088 (ATCC46721) collected from China aster by Tompkins et al. (46) was chosen for reference because Blair et al. (3) used P1088 to represent *P. cryptogea* in a multilocus *Phytophthora* phylogeny. Each isolate was grown in pea broth for 5 to 7 days at 20 to 25°C. Three 7-mm agar disks of an isolate were transferred to 25 ml of pea broth in a 250-ml flask. Pea broth was prepared by autoclaving 120 g of frozen green peas in 500 ml of deionized water for 5 min. After cooling, the solution was filtered through several layers of cheesecloth to remove the peas. Deionized water was added to bring the total volume to 1 liter. Aliquots (25-ml) of pea broth were placed into 250-ml flasks, which were subsequently autoclaved for 30 min. Mycelia mats were harvested by vacuum filtration, placed in a 1.5-ml microcentrifuge tube, and stored at -20°C.

DNA was extracted from mycelia with the PUREGENE DNA Isolation Kit for 10 to 20 mg of fungal tissue (Qiagen, Valencia, CA) with a protocol optimized for *Phytophthora* spp. Frozen mycelial tissue was ground in a 1.5-ml microcentrifuge tube using a disposable pestle for 15 to 20 s. To lyse cells, 600 µl of the cell lysis solution was added, and the cells were ground with the pestle. Once the solution was homogenized, 3 µl of Proteinase K was added and tubes were vortexed on high for 20 s. Samples were incubated at 55°C overnight. Tubes were centrifuged at 5,000 × *g* for 5 min. The resulting supernatant was pipetted into new 1.5-ml microcentrifuge tubes, and 3 µl of RNase A solution was added. Samples were incubated at 37°C for 25 min and then cooled to 20 to 25°C. To remove proteins, 200 µl of protein precipitation solution were added, and samples were vortexed on high speed for 20 s. Tubes were incubated on ice for 20 min. The samples were centrifuged at 18,000 × *g* for 3 min and then returned to ice. The clear portion of the supernatant was trans-

ferred to new 1.5-ml microcentrifuge tubes containing 600 µl of 100% isopropanol. The tubes were gently inverted 30 times to mix and, subsequently, centrifuged at 18,000 × *g* for 3 min. The supernatant was discarded, and the resulting DNA pellets were retained in the microcentrifuge tubes. To wash the DNA, 400 µl of 70% ethanol was pipetted down the side of the tubes. The samples were centrifuged at 18,000 × *g* for 3 min. The ethanol was poured off, and the DNA pellets were retained in the microcentrifuge tubes. Samples were incubated at 55°C to evaporate any remaining ethanol. The DNA was rehydrated by adding 50 µl of nuclease-free water and incubating at 55°C for 1 h. Extracted DNA was stored at -20°C.

Polymerase chain reaction and DNA sequencing. Four loci were chosen for genetic characterization: the ITS region (ITS1, 5.8S, and ITS2) of the rDNA; the translation elongation factor 1α gene (*EF-1α*); the β-tubulin gene (*β-tub*); and the cytochrome oxidase II and internal spacer (*coxII*) region of the mitochondrial DNA. The polymerase chain reaction (PCR) primers, reaction mixtures, and cycling conditions are summarized in Table 2. Amplification products were confirmed by gel electrophoresis (1% agarose; 95 V for 75 min), purified with the Wizard SV Gel and PCR Clean-up System (Promega Corp., Madison, WI), sequenced using the BigDye Terminator 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), and analyzed on an Applied Biosystems 3730xl Genetic Analyzer at the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT).

Cloning of PCR products. Initially, 52 isolates of *P. drechsleri* and 16 isolates of *P. cryptogea* were sequenced at all loci (Table 1). All isolates within a group were found to have identical sequences based on visual inspection. For this work, a group is defined as isolates of a species collected on the same date from the same host species occurring within a growing block at a location. Visual inspection of the chromatograms revealed the presence of heterozygous sites in the ITS, *β-tub*, and *EF-1α* sequences of isolates of both species. Therefore, representative

TABLE 2. Polymerase chain reaction primer sequences, reaction mixtures, and cycling conditions for the internal transcribed spacer (ITS) region, cytochrome oxidase subunit II and spacer (*coxII*) region, β-tubulin (*β-tub*), and elongation factor 1α (*EF-1α*) loci

Locus	Primers (reference)	Reaction mixtures in 25-µl volumes	Cycling conditions ^a
ITS	ITS4 5' TCCTCCGCTTATTGATATGC (9) ITS6 5' GAAGGTGAAGTCGTAACAAGC (9)	5 µl of 5× Green GoTaq buffer ^b	94°C 3 min
		1.5 µl of 25 mM MgCl ₂	30 cycles of
		0.5 µl of 40 mM dNTP mix	94°C 30 s
		0.25 µl of 50 µM ITS4 primer	55°C 30 s
		0.25 µl of 50 µM of ITS6 primer	72°C 1 min
		0.25 µl of GoTaq Flexi DNA polymerase ^b	72°C 10 min
		16.25 µl of nuclease-free sterile water	...
<i>coxII</i>	FM35 5' CAGAACCTTGGCAATTAGG (32) FMPhy-10b 5' GCAAAAGCACTAAAAATTAATATAA (34)	5 µl of 5× Green GoTaq buffer	94°C 4 min
		3 µl of 25 mM MgCl ₂	35 cycles of
		0.5 µl of 40 mM dNTP mix	94°C 30 s
		0.5 µl of 50 µM FM35 primer	59°C 30 s
		0.5 µl of µM FMPhy-10b primer	72°C 90 s
		0.5 µl of GoTaq Flexi DNA polymerase	72°C 10 min
		14 µl of nuclease-free sterile water	...
<i>β-tub</i>	BTubF1 5' GCCAAGTCTCTGGGAGGTCATC (39) BTubR1 5' CCTGGTACTGCTGGTACTCAG (28)	5 µl of 5× Green GoTaq buffer	94°C 2 min
		1.5 µl of 25 mM MgCl ₂	35 cycles of
		0.5 µl of 40 mM dNTP mix	94°C 30 s
		0.5 µl of 50 µM BTubF1 primer	60°C 30 s
		0.5 µl of µM BTubR1 primer	72°C 1 min
		0.25 µl of GoTaq Flexi DNA polymerase	72°C 10 min
		15.75 µl of nuclease-free sterile water	...
<i>EF-1α</i>	EF1AF 5' TCACGATCGACATTGCCCTG (28) EF1AR 5' ACGGCTCGAGGATGACCATG (28)	5 µl of 5× Green GoTaq buffer	94°C 2 min
		1.5 µl of 25 mM MgCl ₂	35 cycles of
		0.5 µl of 40 mM dNTP mix	94°C 30 s
		0.25 µl of 50 µM EF1AF primer	60°C 30 s
		0.25 µl of 50 µM of EF1AR primer	72°C 1 min
		0.25 µl of GoTaq Flexi DNA polymerase	72°C 10 min
		16.25 µl of nuclease-free sterile water	...

^a Cycling conducted on an PTC-100 Peltier thermal cycler (MJ Research, Waltham, MA).

^b Green GoTaq buffer and GoTaq Flexi DNA polymerase from Promega Corp. (Madison, WI).

isolates for each group of isolates of *P. cryptogea* and *P. drechsleri* (Table 3) were chosen for direct cloning of PCR templates to determine alternate alleles for isolates having two or more heterozygous sites. Cloning was completed with the p-GEM T Vector System (Promega Corp.). Plasmids were purified with the UltraClean Standard Mini Plasmid Prep Kit (MO BIO Laboratories, Carlsbad, CA), and 4 to 10 clones per individual were sequenced with the universal M13 primers. Sequences for each locus were aligned, trimmed, and edited manually with Sequencher 4.9 (Genecodes, Ann Arbor, MI). Edited sequences included in analyses were deposited in GenBank (ITS, accession numbers HQ455558 to HQ455646; *coxII*, accession numbers HQ455741 to HQ455765; *β-tub*, accession numbers HQ455647 to HQ455690; and *EF-1α*: accession numbers HQ455691 to HQ455740).

Tests of compatibility, neutrality, and population subdivision. Sequence alignments for each locus were analyzed with components of SNAP Workbench (41). Initially, the sequence alignments were collapsed with phenotype (species) into unique haplotypes with SNAP Map (1) with insertions and deletions (indels) and infinite sites violations excluded. The resulting sequence alignment for each locus was examined for site incompatibilities with SNAP Clade and Matrix (5). When incompatibilities were identified, SNAP CladeEx (5) was used to find the largest nonrecombining partitions. For loci with multiple nonrecombining partitions, each partition was subjected to all of the later analyses separately, and the results compared to determine whether the inferences changed based on the partition. Departures from neutrality were tested by examining the sequences for an excess of rare alleles using Tajima's D (43), Fu and Li's D* and F* (16), and Fu's Fs (15). Each locus was evaluated initially for population subdivision at the species level with the nonparametric tests Hudson's K_{ST} (25) and Hudson's nearest neighbor statistic (S_{nn}) (24). To generate the migration matrix for later coalescent analysis, the asymmetrical migration rates between *P. cryptogea* and *P. drechsleri* were estimated with the isolation with migration (IM) model (21), which allows for divergence with or without gene flow and shared common ancestry. For the IM program, two independent 6-h runs with a burn-in time of 10 million were

carried out. Because IM is less robust for analyses of data from single loci, each locus was tested for equilibrium migration versus shared ancestral polymorphisms between *P. cryptogea* and *P. drechsleri* with the migration with division (MDIV) model (36). Specifically, the time of divergence (T) and the equilibrium migration parameter (M) were estimated with MDIV and compared with the estimates from IM for agreement. The length of the Markov chain was set to 20 million and the burn-in time was 5 million for the MDIV analyses.

Coalescent analysis of *P. cryptogea* and *P. drechsleri*. A coalescent with subdivision model, which assumes no recombination, was implemented in Genetree version 9.0 (2,19) to infer all possible mutational pathways and approximate the ancestry of isolates of *P. cryptogea* and *P. drechsleri* for each locus. The estimations of θ and asymmetrical migration (M_{PdPc} and M_{PcPd}) parameters obtained from the MDIV and IM analyses, respectively, were used in the likelihood estimations. For each locus, five independent runs with 10 million simulations of the coalescent for each run were performed to estimate the genealogy with the highest root probability. The resulting gene genealogies were examined for convergence. Coalescent-based trees were viewed with Treepic (2,19). To estimate the time to most recent common ancestor in coalescent time units, a maximum likelihood estimate of θ was calculated based on the genealogy with the highest root probability and then coalescent simulations were performed with the estimated θ to determine the ages of mutations on the genealogy.

Minimal ancestral recombination graph. Because recombination was identified in ITS, *β-tub*, and *EF-1α*, the ancestral history of haplotypes of each locus was reconstructed with a parsimony method that assumes mutation and recombination backward in time. Specifically, minimal ancestral recombination graphs (ARGs) were constructed. All graphs were rooted with *P. ramorum* isolate P10301. Sequences for P10301 were obtained from the *Phytophthora* Database (39) (accession numbers PD_00058_ITS, PD_00058_Btub, and PD_00058_EF-1α). For the ITS and *β-tub* loci, the branch and bound search was implemented in Beagle to identify the minimum recombination events within the histories of the isolates (30). For *EF-1α*, a heuristic

TABLE 3. Characteristics of isolates of *Phytophthora cryptogea* and *P. drechsleri* cloned, sequenced, and analyzed

Isolate	Group ^a	Species	Year	Host	Location	Mating type	Growth rate (mm/day) at	
							20°C	35°C
P1088/ATCC46721 ^b	...	<i>P. cryptogea</i>	1935	China aster	California	A1	9.8	0
NC108	Pc1	<i>P. cryptogea</i>	2007	Gerbera daisy	B	A1	15.4	3.1
NC110	Pc1	<i>P. cryptogea</i>	2007	Gerbera daisy	B	A1	14.8	2.8
NC208	Pc2	<i>P. cryptogea</i>	2008	Blue daze	C	A2	13.9	0
NC594	Pc3	<i>P. cryptogea</i>	2008	Gerbera daisy	E	A2	16.4	2.7
NC597	Pc3	<i>P. cryptogea</i>	2008	Gerbera daisy	E	A2	16.1	4.1
NC1105	Pc4	<i>P. cryptogea</i>	2008	Verbena	I	A1	16.1	3.0
NC1146	Pc5	<i>P. cryptogea</i>	2008	Dusty miller	J	A2	12.1	0
NC1151	Pc5	<i>P. cryptogea</i>	2008	Dusty miller	J	A2	12.0	0
P1087/ATCC46724 ^b	...	<i>P. drechsleri</i>	1936	Beet	California	A2	6.3	6.8
NC88	Pd1	<i>P. drechsleri</i>	2007	Gerbera daisy	B	A1	14.6	14.2
NC103	Pd1	<i>P. drechsleri</i>	2007	Gerbera daisy	B	A1	12.5	10.9
NC947	Pd2	<i>P. drechsleri</i>	2008	Gerbera daisy	G	A1	16.7	11.7
NC955	Pd2	<i>P. drechsleri</i>	2008	Gerbera daisy	G	A1	15.8	11.8
NC957	Pd3	<i>P. drechsleri</i>	2008	Fuchsia	G	A1	13.5	11.8
NC961	Pd3	<i>P. drechsleri</i>	2008	Fuchsia	G	A1	14.1	11.4
NC966	Pd4	<i>P. drechsleri</i>	2008	Gerbera daisy	G	A1	12.2	11.6
CRGB6	Pd5	<i>P. drechsleri</i>	2002	Gerbera daisy	B	A1	15.6	12.5
CRGB42	Pd5	<i>P. drechsleri</i>	2002	Gerbera daisy	B	A1	10.0	9.2
FDM5	Pd6	<i>P. drechsleri</i>	2001	Dusty miller	A	A1	17.0	14.5
FDM14	Pd6	<i>P. drechsleri</i>	2001	Dusty miller	A	A1	14.6	14.2
FWDM20	Pd7	<i>P. drechsleri</i>	2002	Dusty miller	A	A1	13.6	13.3
FWDM25	Pd7	<i>P. drechsleri</i>	2002	Dusty miller	A	A1	14.3	14.3
FWGB4	Pd8	<i>P. drechsleri</i>	2002	Gerbera daisy	A	A1	13.3	13.2
FWGB7	Pd8	<i>P. drechsleri</i>	2002	Gerbera daisy	A	A1	10.2	8.5

^a For this work, a group is defined as isolates collected on the same date from the same host species occurring within a growing block at a location.

^b Isolate obtained from World Oomycete Genetic Resource Collection; ATCC = American Type Culture Collection (44,46).

implementation of Beagle was conducted with the program Kward because the exhaustive branch and bound search could not go to completion (30). Five independent Kward simulations were run to ensure that similar ancestral histories were found.

RESULTS

Population genetic analyses. Successful amplification of all loci investigated was obtained for all isolates of *P. cryptogea* and *P. drechsleri*. The total number of sequences of *P. cryptogea* and *P. drechsleri* analyzed varied by locus because of the presence of heterozygosity in some isolates at some loci and not at others (Table 4). A high level of variability, represented by a large number of rare single-nucleotide polymorphisms (SNPs) found in single clone sequences, was found in the ITS repeat array within an individual isolate. Because of this, only phylogenetically informative sites were retained in the sequence alignment and subjected to further analyses. In addition, an analysis of a concatenated data set containing all loci was not possible because of the heterozygosity and the difficulty of resolving the phase of alleles at multiple loci. Initially, 30, 8, 16, and 40 haplotypes were identified for the ITS, coxII, β -tub, and EF-1 α loci, respectively (Supplemental Figure 1). There were 33 variable sites in the ITS and coxII regions, 36 variable sites in β -tub, and 38 variable sites in EF-1 α .

Incompatibilities among pairs of variable sites were identified in the compatibility matrices of all loci. The incompatibilities identified in the coxII locus appear to be mutational hotspots rather than recombination events because mitochondrial DNA generally does not undergo recombination, and clear recombi-

nation breakpoints were not evident in the compatibility matrix (data not shown). All incompatibilities were excluded because these variable positions violate the assumption of an infinite sites model, which is required for subsequent coalescent analysis. SNAP CladeX identified two nonrecombining partitions for the ITS, β -tub, and EF-1 α loci and a single nonrecombining partition for the coxII region. This reduced the number of segregating sites (*s*) included in subsequent analyses (Table 4). Isolates of *P. cryptogea* had higher average numbers of pairwise nucleotide differences (*k*) than isolates of *P. drechsleri* for all loci (Table 4). The highest *k* for *P. cryptogea* and *P. drechsleri* was obtained for β -tub (*k* = 7.44 and 2.24, respectively), and the lowest average number of nucleotide differences for both species was estimated for the ITS region (*P. cryptogea*: *k* = 2.80 and *P. drechsleri*: *k* = 0.44).

All neutrality tests for all loci of isolates of *P. cryptogea* and *P. drechsleri* were not significant (data not shown) based on examining the sequences for an excess of rare nucleotides. This suggests that the loci have not been under selection and that the populations are at equilibrium. Population subdivision between *P. cryptogea* and *P. drechsleri* was detected with Hudson's *K*_{ST} and Hudson's *S*_{nn} tests. The tests of population subdivision between the species were significant for all loci (Table 5). The estimates of time to divergence (*T*) and the equilibrium migration parameter (*M*) obtained with MDIV (Table 5) were consistent with the tests of population subdivision and indicate divergence of *P. cryptogea* and *P. drechsleri*, with negligible migration between the species. The *T* of the species varied by locus. For the ITS and coxII loci, the *T* estimates suggest a moderate divergence time (*T* = 7.2 and 4.5, respectively); whereas the β -tub and EF-1 α indicate a rela-

TABLE 4. Summary population statistics of DNA polymorphisms in isolates of *Phytophthora cryptogea* and *P. drechsleri* from floriculture crops produced in North Carolina greenhouse facilities based on sequences of the internal transcribed spacer (ITS) and cytochrome oxidase subunit II and spacer (coxII) regions and the β -tubulin (β -tub) and elongation factor 1 α (EF-1 α) genes

Locus	Species	Length (bp) ^a	Sample size ^b	Segregating nucleotide sites	Nucleotide differences ^c	θ ^d
ITS	<i>P. cryptogea</i>	785	50	12	2.80	2.68
	<i>P. drechsleri</i>	785	39	1	0.44	0.24
coxII	<i>P. cryptogea</i>	930	9	16	4.94	5.89
	<i>P. drechsleri</i>	930	15	3	0.72	0.89
β -tub	<i>P. cryptogea</i>	1,113	12	19	7.44	6.29
	<i>P. drechsleri</i>	1,113	32	8	2.24	1.99
EF-1 α	<i>P. cryptogea</i>	891	18	16	4.60	4.65
	<i>P. drechsleri</i>	891	32	7	1.42	1.74

^a Consensus sequence length for alignment containing DNA sequences for *P. cryptogea* and *P. drechsleri*.

^b Number of DNA sequences included in the phylogenetic analyses.

^c Average number of pairwise nucleotide differences.

^d Coalescent-based estimate of θ per locus.

TABLE 5. Test statistics for population subdivision and population parameters based on variation in the internal transcribed spacer (ITS) and cytochrome oxidase II and internal spacer (coxII) regions and β -tubulin (β -tub) and elongation factor 1 α (EF-1 α) genes of isolates of *Phytophthora cryptogea* and *P. drechsleri* collected from floriculture crops produced in North Carolina commercial greenhouses

Locus ^a	Hudson's <i>K</i> _{ST} (<i>P</i> value) ^b	Hudson's <i>S</i> _{nn} (<i>P</i> value) ^c	θ ^d	<i>T</i> ^e	<i>M</i> ^f	<i>M</i> _{PdPc} ^g	<i>M</i> _{PcPd} ^g	TMRCa ^h
ITS	0.54 (<i>P</i> = 0.0000)*	0.99 (<i>P</i> = 0.0000)*	1.46	7.2	<0.01	0.245	0.065	7.68
coxII	0.17 (<i>P</i> = 0.0230)*	0.82 (<i>P</i> = 0.0002)*	3.39	4.5	<0.01	0.275	0.105	5.08
β -tub	0.08 (<i>P</i> = 0.0020)*	0.90 (<i>P</i> = 0.0000)*	4.14	2.0	0.01	0.125	0.145	4.36
EF-1 α	0.09 (<i>P</i> = 0.0010)*	0.96 (<i>P</i> = 0.0000)*	3.20	1.3	0.01	0.205	0.205	3.19

^a Parameter estimates for each locus are for the nonrecombining partitions shown in the Figure 1 gene genealogies.

^b Sequence-based test statistic for population subdivision best with small equal sample sizes and a high rate of mutation (25). An * denotes a significant *P* value (*P* ≤ 0.05), indicating population subdivision between isolates of *P. cryptogea* and *P. drechsleri*.

^c Sequence-based test statistic for population subdivision useful for analyzing variable sample sizes and rates of mutation (24). An * denotes a significant *P* value (*P* ≤ 0.05), indicating population subdivision between isolates of *P. cryptogea* and *P. drechsleri*.

^d Coalescent-based estimate of θ per locus calculated with Genetree (2,19).

^e Time to divergence (*T*) between species estimated from the posterior probability distribution calculated with migration with division (MDIV) (36).

^f Equilibrium migration parameter estimated from the posterior probability distribution calculated with MDIV (36). Probability distributions for the migration parameter estimates are given in Supplementary Figure 2.

^g Migration parameter, *2N_em*, estimated with isolation with migration. *2N_em* is defined as the effective number of migrants exchanged between two populations in each generation, where *N_e* is the effective population size and *m* is the migration rate per generation. *M*_{PdPc} is migration from *P. drechsleri* to *P. cryptogea* and *M*_{PcPd} is migration from *P. cryptogea* to *P. drechsleri*. (36). Probability distributions for the migration parameter estimates are given in Supplementary Figure 3.

^h Coalescent-based estimate of the time to most recent common ancestor (TMRCa) of the sample sequences.

tively short divergence time ($T = 2.0$ and 1.3 , respectively) (Table 5). The asymmetrical migration parameters (M_{PdPc} and M_{PcPd}) estimated with IM were consistent with the equilibrium migration estimates (Table 5) and indicate no evidence of gene flow between floriculture isolates of *P. cryptogea* and *P. drechsleri* obtained from North Carolina greenhouse facilities.

Coalescent analysis. The coalescent simulations inferred a single gene genealogy with the highest root probability for the *coxII* locus and two genealogies with the highest root probability for each nonrecombining partition of the ITS, *β -tub*, and *EF-1 α* loci. First, for ITS, *β -tub*, and *EF-1 α* , the two genealogies for each partition of a locus were compared. For all three loci, the genealogies had similar topologies with only minor differences in the positions of mutations in the deepest branches of the tree. Next, the genealogies for the two partitions for each locus were compared. One tree is shown for each locus (Fig. 1) because only minor differences in isolate distributions were found between genealogies inferred from each nonrecombining partition. For the ITS partitions, NC1105 (*e,h*) of haplotype H10 were switched with NC1105 (*b,i*) of H13. For the *β -tub* partitions, P1088 (*b*) of H2 exchanged with NC208 of H3, and haplotypes H9 and H10 were collapsed into a single haplotype in the other *EF-1 α* partition. The distribution of isolates and clone variants for each locus for the shown genealogies is given in Table 6.

For the ITS locus, 12 haplotypes were inferred based on 25 mutations (Fig. 1). Twelve mutations were fixed between the haplotypes comprised of *P. cryptogea* isolates (H1, H2, H3, H6, H7, H8, H9, H10, H11, and H12) and the haplotypes associated with isolates of *P. drechsleri* (H4 and H5). The genealogy inferred from the *coxII* locus was similar to the ITS region (Fig. 1). In total, 30 mutations were associated with eight haplotypes, with 11 mutations fixed between haplotypes consisting of isolates of *P. cryptogea* (H2, H3, H4, H5, and H6) and haplotypes containing *P. drechsleri* isolates (H1, H7, and H8).

In the genealogy inferred for the *β -tub* locus, 12 haplotypes were inferred from 31 mutations (Fig. 1). Haplotypes H2, H3, H4, H5, H6, and H7 were composed of *P. cryptogea* isolates, and haplotypes H1, H8, H9, H10, H11, and H12 were associated with isolates of *P. drechsleri*. Eleven fixed mutations separated haplotypes H2 and H3, consisting of *P. cryptogea* isolates, from all other haplotypes, and five mutations were fixed between the remaining haplotypes associated with *P. cryptogea* (H4, H5, H6, and H7) and all haplotypes composed of isolates of *P. drechsleri*. Similarly, in the genealogy inferred from *EF-1 α* (Fig. 1), 24 mutations were associated with 15 haplotypes with 7 fixed mutations separating haplotypes H3 and H4 consisting of *P. cryptogea* isolates and a single fixed mutation separating the remaining haplotypes associated with isolates of *P. cryptogea* (H2, H5, H6, H7, H8, and H9) and all haplotypes composed of *P. drechsleri* isolates (H1, H11, H12, H13, H14, and H15).

Minimal ARG of *P. cryptogea* and *P. drechsleri* haplotypes. In total, 14 recombination events were inferred in the history of the ITS locus (Fig. 2). All haplotypes composed of *P. drechsleri* isolates (H12, H13, H14, and H15) have experienced recombination in their history. In contrast, haplotypes H5, H18, H20, H24, and H25 associated with *P. cryptogea* isolates coalesced to a common ancestor without recombination. Recombination events were inferred within the histories of the remaining haplotypes

composed of *P. cryptogea* isolates (H1, H2, H3, H4, H6, H7, H8, H9, H10, H11, H16, H17, H19, H21, H22, H23, and H26). At least two inferred recombination events consisted of isolates of *P. cryptogea* of different mating types (A1 and A2, respectively) (Fig. 2). One putative recombination event occurred between a haplotype comprising *P. drechsleri* A1 isolates (H12) and a haplotype associated with *P. cryptogea* A1 and A2 isolates (H2).

Seven recombination events were inferred in the history of the ARG for the *β -tub* gene (Fig. 3). All haplotypes composed of *P. drechsleri* isolates (H2, H9, H10, H11, H12, H13, and H14) and haplotypes H3, H4, H5, H6, H7, and H8 associated with *P. cryptogea* isolates have experienced recombination. Only haplotype H7, consisting of *P. cryptogea* isolates, coalesced without recombination. Two putative recombination events occurred between haplotypes associated with each species. A recombination event between H2 (*P. drechsleri* A1 isolates) and H8 (*P. cryptogea* A1 and A2 isolates) resulted in H13 and H14 (*P. drechsleri* A1 isolates) (Fig. 3). Likewise, a recombination event between H10 (*P. drechsleri* A1 isolates) and H8 (*P. cryptogea* A1 and A2 isolates) gave rise to H11 (*P. drechsleri* A1 isolates) (Fig. 3).

In all sampled ancestral histories of *EF-1 α* , isolates of *P. cryptogea* and *P. drechsleri* had undergone recombination (data not shown). In total, 34 to 37 recombination events were inferred in the sampled ARGs; however, no clear recent recombination events occurred between *P. cryptogea* and *P. drechsleri*. A single recombination event between A1 and A2 *P. cryptogea* isolates was inferred.

DISCUSSION

The genetic relatedness and ancestry of isolates of *P. cryptogea* and *P. drechsleri* collected from floriculture crops in North Carolina commercial greenhouse facilities was investigated. To accomplish this, two distinct types of analyses were used: (i) maximum likelihood with a coalescent analysis assuming no recombination and population subdivision and (ii) parsimony analysis assuming recombination with an ARG. Overall, the gene genealogies inferred from the coalescent-based analysis support a common ancestry with a recent divergence for the investigated isolates of *P. cryptogea* and *P. drechsleri*. In a study of a collection of 28 isolates of *P. cryptogea* and 14 isolates of *P. drechsleri* of worldwide origin, Mostowfizadeh-Ghalamfarsa et al. (35) found *P. cryptogea* and *P. drechsleri* to be distinct species based on gene phylogenies inferred with a neighbor-joining method. The gene phylogenies for the five individual loci and the combined loci evaluated by Mostowfizadeh-Ghalamfarsa et al. (35) were congruent. In contrast, in our study, different evolutionary histories are evident depending on the locus analyzed. All loci infer common ancestry for isolates of *P. cryptogea* and *P. drechsleri*; however, the level of divergence varies by locus. The coalescent analysis of the ITS and *coxII* regions inferred that *P. cryptogea* and *P. drechsleri* are closely related sister species which clearly have diverged at a constant rate from a common ancestor. Each species forms a monophyletic group within the gene genealogies, with *P. cryptogea* more genetically diverse than *P. drechsleri*. However, the genealogies inferred from the *B-tub* and *EF-1 α* genes were in conflict with the ITS and *coxII* regions. In the *β -tub* and *EF-1 α* genes, there was not a clear divergence of the two

Fig. 1. Coalescent-based gene genealogies showing the distribution of mutations in the internal transcribed spacer region (ITS), the cytochrome oxidase subunit II and spacer (*coxII*) region, and the *β -tubulin* (*β -tub*) and translation elongation factor 1 α (*EF-1 α*) genes from *Phytophthora cryptogea* and *P. drechsleri*. Genealogies were inferred assuming population subdivision by species, with equal population sizes and asymmetrical migration rates estimated with isolation with migration tests. Five independent runs of 10 million simulations of the coalescent were conducted for each locus. Direction of divergence is from the top of the genealogy (past) to the bottom (present). The time scale on the right shows the estimated time to most recent common ancestor in coalescent units of effective population size. Dots along the lines of the tree designate mutations, with 0 indicating mutations in *P. cryptogea* and 1 representing mutations in *P. drechsleri*. Numbers below the tree designate distinct haplotypes, the total frequency each haplotype is found, and the frequency each haplotype is associated with *P. cryptogea* and *P. drechsleri*.

species for our group of isolates, and the β -tub and *EF-1 α* genealogies suggest that *P. cryptogea* and *P. drechsleri* are incipient species that are in the process of diverging. The differences in phylogenetic inferences between loci are likely a result of the differences in the evolutionary histories of the loci. In diploid organisms, such as *Phytophthora* spp., haploid mitochondrial DNA evolves faster than single-copy nuclear genes, such as β -tub and *EF-1 α* , because mitochondrial DNA has a greater rate of nucleotide substitution from a lack of DNA polymerase proof-reading function (20). Therefore, nuclear genes reveal more ancient population processes and mitochondrial regions provide better resolution of more recent population processes. The nuclear β -tub and *EF-1 α* loci suggest that speciation is still underway for our isolates of *P. cryptogea* and *P. drechsleri*, whereas the mitochondrial coxII region inferred clear divergence of the two species with fixed polymorphisms for each species. In addition, the rDNA ITS repeat provided some resolution of more recent population processes, as indicated by the divergence of *P.*

cryptogea and *P. drechsleri*. However, for several isolates of *P. cryptogea* and *P. drechsleri*, it appears that convergent evolution through the processes of gene conversion and unequal crossing over has not had sufficient time to homogenize the rDNA repeat array, as is evident by the level of variability observed between cloned copies of the ITS repeat of an individual (20). The incongruence between gene genealogies highlights the need for loci with differing evolutionary histories to provide multiple levels of resolution when researching the phylogenetic history of a population, particularly with a population that may not have well-defined species boundaries, as is often the case with species of *Phytophthora* (27). In addition, researchers will have to take care when choosing loci for DNA-based methods to differentiate these species because regions of a gene could be more like one species or the other.

Phylogenetic relationships between species that vary by the genomic region studied can be a good indication of past recombination events (38). Recombination between two close species is

TABLE 6. Distribution of haplotypes and isolates based on coalescent analysis of the internal transcribed spacer (ITS) and cytochrome oxidase II and internal spacer (coxII) regions and the β -tubulin (β -tub) and elongation factor 1 α (*EF-1 α*) genes of *Phytophthora cryptogea* and *P. drechsleri*

Locus	Haplotype (frequency)	Isolates (clone sequence variant)
ITS	1 (3)	<i>P. cryptogea</i> P1088, NC208 (b,c)
	2 (2)	<i>P. cryptogea</i> NC208 (f,g)
	3 (7)	<i>P. cryptogea</i> NC594 (b,f-h), NC597 (a,b,g)
	4 (12)	<i>P. drechsleri</i> NC88 (d,f,h), NC103 (a,d), CRGB6 (e-g), CRGB42 (a,e,h,j)
	5 (27)	<i>P. drechsleri</i> P1087, NC88 (a,c,e), NC103 (b,c,e,f), NC947, NC955, NC957, NC961, NC966, CRGB (a-d,h,l), CRGB42 (d), FDM5, FDM14, FWDM20, FWDM25, FWGB4, FWGB7
	6 (2)	<i>P. cryptogea</i> NC110 (d,l)
	7 (4)	<i>P. cryptogea</i> NC108 (e,j), NC594 (a,c)
	8 (4)	<i>P. cryptogea</i> NC108 (a,b), NC110 (a,c)
	9 (2)	<i>P. cryptogea</i> NC594 (d), NC597 (d)
	10 (5)	<i>P. cryptogea</i> NC1105 (a,c,e,f,h)
	11 (1)	<i>P. cryptogea</i> NC108 (f)
	12 (10)	<i>P. cryptogea</i> NC208 (a,e,h), NC1146 (a-d), NC1151 (a-c)
	13 (10)	<i>P. cryptogea</i> NC108 (g), NC110 (f,h), NC594 (e), NC597 (c,e), NC1105 (b,d,g,i)
coxII	1 (10)	<i>P. drechsleri</i> NC947, NC955, NC957, NC961, FDM5, FDM14, FWDM20, FWDM25, FWGB4, FWGB7
	2 (2)	<i>P. cryptogea</i> NC108, NC110
	3 (1)	<i>P. cryptogea</i> P1088
	4 (3)	<i>P. cryptogea</i> NC208, NC1146, NC1151
	5 (2)	<i>P. cryptogea</i> NC594, NC597
	6 (1)	<i>P. cryptogea</i> NC1105
	7 (4)	<i>P. drechsleri</i> NC88, NC103, CRGB6, CRGB42
	8 (1)	<i>P. drechsleri</i> P1087
β -tub	1 (13)	<i>P. drechsleri</i> NC88 (b), NC103 (b), NC947 (b), NC955 (b), NC961 (b), NC966 (b), CRGB6 (b), CRGB42 (b), FDM5 (b), FDM14 (b), FWGB4 (b), FWGB7 (b)
	2 (2)	<i>P. cryptogea</i> P1088 (a), NC208
	3 (1)	<i>P. cryptogea</i> P1088 (b)
	4 (2)	<i>P. cryptogea</i> NC108 (b), NC110 (b)
	5 (2)	<i>P. cryptogea</i> NC108 (a), NC110 (a)
	6 (2)	<i>P. cryptogea</i> NC1146, NC1151
	7 (3)	<i>P. cryptogea</i> NC594, NC597, NC1105
	8 (2)	<i>P. drechsleri</i> P1087 (a,b)
	9 (9)	<i>P. drechsleri</i> NC947 (a), NC955 (a), NC957 (a), NC961 (a), NC966 (a), FDM5 (a), FDM14 (a), FWGB4 (a), FWGB7 (a)
	10 (2)	<i>P. drechsleri</i> FWDM20 (a), FWDM25 (a)
	11 (4)	<i>P. drechsleri</i> NC88 (a), NC103 (a), CRGB6 (a), CRGB42 (a)
	12 (2)	<i>P. drechsleri</i> FWDM20 (b), FWDM25 (b)
<i>EF-1α</i>	1 (15)	<i>P. drechsleri</i> P1087 (a), NC947 (b), NC955 (b), NC957 (b), NC961 (a,b), CRGB6 (b), CRGB42 (b), FDM5 (a), FDM14 (a), FWDM20 (a), FWGB4 (a), FWGB7 (b)
	2 (1)	<i>P. cryptogea</i> NC208 (b)
	3 (2)	<i>P. cryptogea</i> P1088 (b), NC208 (a)
	4 (1)	<i>P. cryptogea</i> P1088 (a)
	5 (2)	<i>P. cryptogea</i> NC1146 (b), NC1151 (b)
	6 (2)	<i>P. cryptogea</i> NC1146 (a), NC1151 (a)
	7 (4)	<i>P. cryptogea</i> NC108 (a), NC110 (a), NC594 (b), NC597 (b)
	8 (2)	<i>P. cryptogea</i> NC1105 (a,b)
	9 (2)	<i>P. cryptogea</i> NC108 (b), NC110 (b)
	10 (2)	<i>P. cryptogea</i> NC594 (a), NC597 (a)
	11 (4)	<i>P. drechsleri</i> NC947 (a), NC955 (a), NC966 (a,b)
	12 (2)	<i>P. drechsleri</i> NC88 (a), NC103 (a)
	13 (3)	<i>P. drechsleri</i> P1087 (b), FWDM20 (b), FWDM25 (b)
	14 (4)	<i>P. drechsleri</i> FDM5 (b), FWDM25 (a), FWGB4 (b), FWGB7 (a)
	15 (4)	<i>P. drechsleri</i> NC88 (b), NC103 (b), CRGB6 (a), CRGB42 (a)

known to complicate phylogenetic analyses by reducing fixed DNA sequence differences between the species (38). The coalescent-based analyses inferred the breakup of monophyly of *P. cryptogea* in the β -*tub* and *EF-1 α* loci and grouping of some isolates of *P. cryptogea* more closely with haplotypes of *P. drechsleri* than haplotypes of *P. cryptogea*. However, because the coalescent-based model used for the analysis assumes no recombination, the resulting gene genealogies failed to infer the full extent of the relationship between *P. cryptogea* and *P. drechsleri* that is revealed in the ITS and β -*tub* ARGs because it takes recombination into account when reconstructing the evolutionary history of populations. The inferred ARGs of the ITS region and β -*tub* gene suggest that genetic exchange has occurred within the phylogenetic history of the isolates of *P. cryptogea* and *P. drechsleri* collected from North Carolina greenhouses. Specifically, one recombination event between haplotypes associated with isolates of *P. cryptogea* and *P. drechsleri* was inferred in the ITS ARG,

and two inferred recombination events occurred between the species in the β -*tub* ARG. Recombination between the two species explains the lack of clear divergence in the nuclear gene genealogies. Using similar methodology to analyze *P. ramorum* lineages, Goss et al. (18) found phylogenetic conflict between loci which was explained by ancient recombination events between lineages. Genetic exchange between species of *Phytophthora* in agricultural and natural systems is not without precedent (11). The new species, *P. alni*, was identified as a hybrid of *P. cambivora* and a *P. fragariae*-like species (6,7). Similarly, a hybrid of *P. nicotianae* and *P. cactorum* was found on ornamental plants in greenhouses in The Netherlands (4,31).

In the ITS and β -*tub* ARGs, the inferred recombination events occurred between haplotypes of *P. drechsleri* and *P. cryptogea* consisting of isolates of A1 and A2 mating types, respectively. This suggests that sexual reproduction has occurred between *P. cryptogea* and *P. drechsleri*. Experimental crosses between *P.*

Fig. 2. Minimal ancestral recombination graph (ARG) inferred with the branch and bound search implemented in Beagle (30), which shows possible mutation and recombination paths giving rise to the sampled haplotypes of *Phytophthora cryptogea* and *P. drechsleri* for the internal transcribed spacer region. The graph was rooted by specifying haplotype H1 (*P. ramorum*) as the outgroup. Ovals in the interior of the ARG represent recombination nodes, where the number in the oval specifies the variable position in the sequence immediately to the left of the recombination break. Branches leading to the recombination nodes are labeled with a P (prefix) or S (suffix), which denote the 5' and 3' segments, respectively, of the recombinant sequence. Numbers along a branch indicate the number of polymorphisms for a lineage to coalesce. Thickened branches lead from the recombinant haplotypes (H19, H21, and H26) to the putative parental haplotypes (H2, H18; H2, H20; and H2, H12, respectively).

drechsleri isolates and *P. cryptogea* isolates produced oospores (data not shown) but it is unknown whether the progeny are hybrids or the result of induced selfing. Additional studies evaluating sexual reproduction between *P. cryptogea* and *P. drechsleri* and the characteristics of any resulting progeny are needed because understanding the potential for outcrossing could be critical for disease management. Sexual reproduction between *P. cryptogea* and *P. drechsleri* could result in the development of

more fit genotypes and potentially allow the introgression of mefenoxam resistance from *P. drechsleri* to *P. cryptogea*, a species for which mefenoxam resistance has not yet been reported.

In most species of *Phytophthora*, including *P. cryptogea*, the importance and occurrence of sexual reproduction in agricultural and natural settings is unknown. The A1 and A2 mating types of *P. cryptogea* were collected in North Carolina during the 2007 and 2008 survey of greenhouse facilities (37). The A1 and A2

Fig. 3. Minimal ancestral recombination graph (ARG) inferred with the branch and bound search implemented in Beagle (30), which shows possible mutation and recombination paths giving rise to the sampled haplotypes of *Phytophthora cryptogea* and *P. drechsleri* for the β -tubulin gene. The graph was rooted by specifying haplotype H1 (*P. ramorum*) as the outgroup. Ovals in the interior of the ARG represent recombination nodes, where the number in the oval specifies the variable position in the sequence immediately to the left of the recombination break. Branches leading to the recombination nodes are labeled with a P (prefix) or S (suffix), which denote the 5' and 3' segments, respectively, of the recombinant sequence. Numbers along a branch indicate the number of polymorphisms for a lineage to coalesce. Thickened branches lead from the recombinants (H11 and H13, H14) to the putative parental haplotypes (H8, H10 and H2, H8, respectively).

isolates of *P. cryptogea* shared haplotypes in the gene genealogies inferred from the ITS, β -tub, and *EF-1 α* loci. In addition, recombination between haplotypes composed of isolates of *P. cryptogea* of A1 and A2 mating types were inferred in the ITS and *EF-1 α* ARGs. This suggests that sexual reproduction has occurred in the evolutionary history of these isolates of *P. cryptogea*, and the variability observed between cloned copies of the ITS repeat of an individual is likely a result of outcrossing. Mostowfizadeh-Ghalamfarsa et al. (35) identified three *P. cryptogea* subgroups with evidence of genetic exchange between two lineages to form an intermediate lineage.

In the floriculture industry, it is common for plant material to move around the United States and even from off-shore into the United States (10). This movement of plant material allows the movement of new pathogens and new genetic isolates of existing pathogens into production areas. By identifying unique genotypes of a species of *Phytophthora*, the origin of inoculum and pathway of movement of *Phytophthora* spp. within a commodity can potentially be detected. The genealogy of the *coxII* locus identified a possible greenhouse location where *P. drechsleri* may have become established onsite. Isolates CRGB6 and CRGB42 of *P. drechsleri* collected from gerbera daisy at location B in 2002 collapsed into a unique haplotype (H7) with isolates NC88 and NC103 collected from gerbera daisy at the same location in 2007 (37). These isolates also had clone variants that grouped together in the ITS, β -tub, and *EF-1 α* genealogies. The grower reported having a yearly problem with *Phytophthora* root rot on gerbera daisy plants. Similarly, Lamour et al. (29) identified a clonal lineage of *P. nicotianae* that persisted in field-produced snapdragons from the 2000–01 growing season. In addition, Lamour et al. (29) identified infested verbena plants shipped between greenhouse facilities as the source of inoculum of *P. nicotianae* at two greenhouse facilities by developing amplified fragment length polymorphism profiles to identify clonal lineages of *P. nicotianae*. Developing additional information on genotypes of *P. cryptogea* and *P. drechsleri* present in the floriculture industry will be important for improving disease management strategies.

Considering all of the inferences together, it appears likely that divergence between *P. cryptogea* and *P. drechsleri* is recent and that speciation is still in progress for these species. This is in contrast to the study by Mostowfizadeh-Ghalamfarsa et al. (35), which found no evidence of gene flow and concluded that these were well-diverged species. The study by Mostowfizadeh-Ghalamfarsa et al. (35) did not resolve heterozygous sites within the analyzed DNA sequences. Cloning and determining alternate alleles for heterozygous sites in diploid organisms is needed for conducting the most robust analyses possible and was instrumental in detecting the recombination events and conflicts between the gene genealogies in the history of the isolates of *P. cryptogea* and *P. drechsleri* in our study. However, it is possible that our population of isolates has a unique history of recombination between these two species. The study by Mostowfizadeh-Ghalamfarsa et al. (35) used isolates of *P. cryptogea* and *P. drechsleri* from diverse hosts and origins, whereas the present study of these species used only isolates of these species from greenhouse floriculture crops. The greenhouse environment provides a unique opportunity for these two species to interact because *P. cryptogea* and *P. drechsleri* have overlapping floriculture host ranges. In addition, plant material is moved around the industry, providing a chance of introduction of both species to a single facility. In fact, *P. cryptogea* and *P. drechsleri* were found together in a block of gerbera daisies in a greenhouse in North Carolina (37). Additional studies with diverse isolates where alternate alleles are determined for heterozygous sites are needed to determine whether the recombination identified in our study has occurred in the evolutionary history of other isolates of *P. cryptogea* and *P. drechsleri* or is unique to isolates from this production system.

ACKNOWLEDGMENTS

Funding for this research comes, in part, from the Fred C. Gloeckner Foundation, Harrison, NY; the United States Department of Agriculture–Agricultural Research Service Floriculture and Nursery Crops Research Initiative; and the North Carolina Agricultural Research Service, Raleigh. We thank M. Greene, K. L. Ivors, and K. C. Parker for technical assistance.

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