Genetic and Morphological Diversity of Temperate and Tropical Isolates of *Phytophthora capsici*

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ABSTRACT

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Phytophthora capsici is a diverse species causing disease on a broad range of both temperate and tropical plants. In this study, we used cultural characteristics, amplified fragment length polymorphism (AFLP), and DNA sequence analyses of the ribosomal internal transcribed spacer (ITS) region and mitochondrial cytochrome oxidase II (*cox II*) genes to characterize temperate and tropical isolates from a wide range of host species. All but one temperate isolate grew at 35°C, while all tropical isolates did not. All but two tropical isolates formed chlamydospores, while temperate isolates did not. There was strong bootstrap support for separation of temperate and tropical isolates using AFLP analysis; however, the temperate isolates appeared as a subgroup within the observed variation of the tropical isolates. The majority of temperate isolates clustered within a single clade with low variation regardless of host or geographical origin, while the tropical isolates were more variable and grouped into three distinct clades. Two clades of tropical isolates grouped together and were affiliated closely with the temperate isolates, while the third tropical clade was more distantly related. Phylogenetic analysis of the ITS regions resulted in similar groupings and variation within and

between the temperate and tropical isolates as with the AFLP results. Sequence divergence among isolates and clades was low, with more variation within the tropical isolates than within the temperate isolates. Analysis of other species revealed shorter branch lengths separating temperate and tropical isolates than were observed in comparisons among other phylogenetically closely related species in the genus. Analysis of *cox II* sequence data was less clear. Although the temperate and tropical isolates grouped together apart from other species, there was no bootstrap support for separating these isolates. Restriction fragment length polymorphism (RFLP) analysis of the ITS regions separated the temperate and tropical isolates, as in the AFLP and ITS phylogenetic analyses. However, RFLP analysis of the *cox I* and *II* gene cluster did not distinguish between temperate and tropical isolates. The differences in grouping of isolates in these two RFLP studies should be helpful in identifying isolate subgroups. Our data do not fully clarify whether or not temperate and tropical isolates should be separated into different species. The available worldwide data are incomplete and the full range of variation in the species is not yet known. We suggest refraining from using the epithet *P. tropicalis* until more data are available.

Additional keywords: phylogeny.

Phytophthora capsici Leonian was first described in 1922 on *Capsicum annuum* L. (chili pepper) in New Mexico (29). Originally, this pathogen was considered to be host specific (44), but since has been reported as a devastating pathogen on bell pepper, squash, pumpkin, tomato, and other temperate solanaceous and cucurbit hosts worldwide (13,28,40). This fungal-like pathogen also has been reported to cause disease on a variety of tropical hosts, including cacao, rubber, macadamia, papaya, black pepper, and others (13). *P. capsici* causes root and crown rots, as well as stem, leaf, and fruit lesions (40). The pathogen is heterothallic and requires both A1 and A2 compatibility types for sexual reproduction. Both compatibility types are common in the same fields, and have been found within the same plant (25,39). *P. capsici* forms papillate sporangia that are borne on long pedicels and are caducous (13).

Chlamydospores are rare in cultures of isolates from solanaceous or cucurbit hosts, but are reported to be abundant in cultures of isolates from black pepper, cacao, and macadamia (1,13,39,44,45).

Previously, all isolates of *Phytophthora* from cacao were classified as *P. palmivora* (Butl.) Butler (7). However in 1976, isolates of *P. palmivora* from cacao were placed into one of four morphological groups (MF1, MF2, MF3, and MF4) (17). After further study, the MF4 form was considered to be *P. capsici* Leonian (13, 43,48). MF4 isolates from cacao, black pepper, and other tropical crops, some of which formed chlamydospores, were characterized as *P. capsici* in an amended description of the species (43).

It was suggested that isolates that are pathogenic to tropical crops, avirulent to pepper, and that produce chlamydospores are distinct from *P. capsici* (3,46). Oudemans and Coffey (37), using isozyme analysis, separated isolates of *P. capsici* into three subgroups: CAP1, CAP2, and CAP3. Isolates determined to be distinct from *P. capsici* fell within both CAP2 and CAP3 (46). An expanded isozyme analysis by Mchau and Coffey (36) using 15 enzymes separated 113 isolates of *P. capsici* into two subgroups, CapA and CapB, but each subgroup contained isolates from a wide range of hosts and geographical locations, and members of each group varied in morphology. *P. capsici* was broadly redefined based on additional isozyme data and the wide range of morphological attributes found in a worldwide collection (36). Nevertheless, there have been continued reports that there exists considerable diversity in host-specific pathogenicity (4,39,46) and genetic variability among and within populations of *P. capsici* (26).

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In a study to re-examine the species, Aragaki and Uchida (4) compared morphological attributes of over 100 isolates of *P. capsici* with deciduous, long-pedicellate sporangia. The isolates were separable into two groups: *P. capsici* and a proposed new species, *P. tropicalis* sp. nov. The proposed new species would include those isolates that morphologically differ from *P. capsici* by having narrow sporangia (less than 26 µm in diameter), a sporangial length-to-breadth ratio of 1.8 or more, predominantly tapered sporangial bases, production of chlamydospores, poor or no growth at 35°C, and weak or no pathogenicity on pepper. In a molecular sequence study with a limited number of isolates, Zhang et al. (49) reported that the internal transcribed spacer (ITS) ITS1 and ITS2 rDNA regions (with the 18S, 5.8S, and 28S subgroups removed) of *P. capsici* and isolates referred to as *P. tropicalis* were the same size but had 21 nucleotide substitutions between the two groups. At some sites, only one of the four *P. tropicalis* isolates differed from the two *P. capsici* isolates, while at other sites, all four *P. tropicalis* isolates differed from *P. capsici*. These differences, however, were fewer than the differences between the *P. capsici*/*P. tropicalis* group and either *P. palmivora* or *P. nicotianae*, and fewer than the differences between *P. palmivora* and *P. nicotianae*. Isolates of *P. tropicalis* were genetically more variable than isolates of *P. capsici*. Zhang et al. (49) concluded that their results support the separation of *P. tropicalis* as a distinct species. However, are the differences reported within this group of isolates, as opposed to the differences among other species, enough to support a new species given that what was studied was only a small number of isolates and a small region of the genome? Kong et al. (24) also reported a separation of isolates of *P. capsici* and *P. tropicalis* using single-strand-conformation polymorphisms (SSCP) of the ITS1 rDNA region. However, only four isolates of *P. capsici* and one isolate of *P. tropicalis* were included in the study. The variation within and between the species was not presented, and it is not known whether or not a continuum or a delimitation of responses exists among this group of isolates using this methodology.

It is important to know whether morphologically similar isolates of a described species from diverse hosts represent one worldwide population, or if these isolates represent an assemblage of cryptic species occurring with hosts and climate. The genetic differences among isolates of a species are very important to those designing breeding programs for disease resistance. Thus, the characterization of pathogen isolates used as challenge inoculum and their relation to field populations of the species in question is critical. Likewise, in a biological control approach, biological control agents often are highly specific for target populations (6,23). Thus, it is very important to know if isolates from different climates or hosts are similar or different based on genetic, morphologic, or pathogenetic criteria, or if their relationship is based on a combination of any of these criteria. Will disease resistance or control methodology developed for one population be applicable to similar populations?

Here we report the characterization of isolates of *P. capsici* and *P. tropicalis* across numerous host plants, geographical regions, and climates, using morphological and multigene approaches. Amplified restriction fragment length polymorphism (AFLP) analysis was used to assess the genetic variation at the species level. Sequencing data of one nuclear locus, ribosomal DNA internal transcribed spacer regions (ITS) (9), and one mitochondrial locus, cytochrome oxidase II (*cox II*) gene (33), were used to investigate molecular phylogenetic relationships of these isolates with closely related taxa. Restriction fragment length polymorphism (RFLP) analysis of these two loci also was done to provide additional comparison with other members of the genus (11,34). The data were used to determine if subgroups within the species exhibit enough genetic distance to lead us to recognize these subgroups as two different taxa.

Phytophthora cultures. Isolates of *P. capsici* and *P. tropicalis* used in this study are listed in Table 1. Isolates were maintained on 5% clarified V8-juice (CV8) agar medium (5) in the dark at 12 to 14°C and on 0.5-cm² pieces of CV8 agar medium in small glass bottles with sterile distilled water and a sterile hemp seed at room temperature. Radial growth of the isolates was assessed as described by Aragaki and Uchida (4). Radial growth experiments were performed twice with two replications per isolate in each experiment. Data were analyzed using the SAS statistical package (SAS Inst., Cary, NC). Chlamydospore production was assessed using the submerged culture method (42,45).

DNA extraction. Cultures from storage were transferred to fresh CV8 agar medium and grown in the dark at 22 to 24°C for 3 to 5 days. Agar plugs were taken from the colony margins and inoculated into 40 ml of 10% CV8 broth medium in a 250-ml flask. Stationary broth cultures were incubated as above for 7 to 10 days. Mycelial mats were harvested by washing with sterile distilled water, were dried on filter paper with a vacuum, placed in a sterile, disposable centrifuge tube, frozen at -20°C overnight, and then lyophilized. Approximately 20 mg of lyophilized mycelium was transferred to a sterile microcentrifuge tube and ground with a micropestle. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions and then quantified using a Fluoroskan Ascent and Ascent software version 2.4 (Thermo Electron Corp., Waltham, MA) with the PicoGreen dsDNA Quantification Kit (Molecular Probes, Eugene, OR).

AFLP analysis. AFLP analysis was carried out using the AFLP Microbial Fingerprinting Kit in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, CA). The restriction-ligation reaction for each sample was carried out overnight at room temperature in a total volume of 11 µl, with approximately 100 ng of genomic DNA resuspended in 5.5 µl of sterile distilled water and 5.5 µl of an enzyme master mix in accordance with manufacturer's instructions. *EcoRI* and *MseI* enzymes and associated buffers not in the AFLP kit were obtained from New England BioLabs (Beverly, MA).

The restriction-ligation reaction products were diluted 1:19 in 0.1% Tris-EDTA (TE) buffer, and stored at 4°C. Preselective amplification was performed on 4.0 µl of diluted restriction-ligation reaction and 16.0 µl of an AFLP Amplification Core Mix with preselective primers (core sequences) *EcoRI* (5'-GACTGCGTAC-CAATTC-3') and *MseI* (5'-GATGAGTCCTGAGTAA-3'). Preselective amplification was carried out in a Gene Amp polymerase chain reaction (PCR) System 9700 (Applied Biosystems) with the temperature cycling program described in the AFLP protocol. PCR products were diluted 1:20 in 0.1% TE buffer and stored at 4°C. The selective amplification procedure was modified slightly from the AFLP protocol. The selective amplification master mix consisted of 7.5 µl of AFLP Amplification Core mix, 0.75 µl of 1.0 µM *EcoRI* dye-labeled primer, and 0.75 µl of 5.0 µM *MseI* primer. Nine microliters of the selective amplification master mix was mixed with 1.0 µl of diluted preselective amplification products. The selective amplification *EcoRI* primers had an additional two-nucleotide extension at the 3' end (-AG, -AC, -AT, and -AA) and were labeled at the 5' end with a fluorescent WellRED D4-PA dye-labeled phosphoramidite (Invitrogen). The selective amplification *MseI* primer had an additional nucleotide extension on the 3' end (-C). Selective amplifications were carried out using a combination of each modified *EcoRI* primer with *MseI*-C using the temperature cycling program given in the original AFLP protocol. Selective amplification products were diluted 30-fold in Sample Loading Solution (Beckman Coulter, Fullerton, CA), which included Beckman Coulter 400-bp DNA size standards. Fragments were separated and detected using capillary electrophoresis on the CEQ 8000 Genetic Analysis System (Beckman

Coulter, Inc.). Fragment data were analyzed using the CEQ 8000 Fragment Analysis Software (version 6.0) with parameters that recognized peaks exceeding a height threshold of 10% of the height of the second largest peak and a slope threshold of 10. The bin (peak) width was adjusted to 1.0. The automated scoring was examined and manually adjusted if necessary. Samples were scored for each bin as a "1" if a fragment of that size was present, and as a "0" if a fragment of that size was not present. All bins scored were polymorphic and were included in the analysis. Data were imported into the freeware program FreeTree, version 0.9.1.50 (12,18). A similarity matrix was generated using Jaccard's similarity coefficient, and cluster analysis was performed using the unweighted-pair group method with arithmetic mean algorithm (UPGMA) and bootstrap analysis with 1,000 resamplings. Clades with bootstrap values above 70% were considered strongly supported by the data. Trees were visualized using TreeView, version 1.6.6 (38).

Amplification and sequencing of the ITS regions. The ribosomal DNA region containing the ITS regions ITS1 and 2, and the

5.8S rRNA gene, was amplified with the universal primers ITS6 - (forward) (5'-GAAGGTGAAGTCGTAACAAGG-3') and ITS4 (reverse) (5'-TCCTCCGCTTATTGATATGC-3') (9,10,47). PCR - Reaction mixture contained 1X Platinum PCR Super Mix (Invitrogen), 0.4 µM each of primers ITS6 and ITS4 (Invitrogen), and 1 µl of genomic DNA for a total volume of 50 µl. Amplifications were done in the Gene Amp PCR System 9700 with cycling conditions as follows: 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Reaction products were purified using QIAquick PCR Purification Kit (Qiagen) as per manufacturer's instructions, using 50 µl of that kit's Buffer EB, and then placing the final product in a heating block at 55°C for 3 to 5 min to remove traces of residual ethanol. The PCR products were diluted 1:25 in Buffer EB and then DNA concentration was determined by UV absorbance using a spectrophotometer. Each sample was diluted to 20 ng/µl and the primers diluted to 0.8 µM. The sample DNA was sequenced at the University of Maryland DNA Sequencing Facility, Center for Biosystems Research (College

TABLE 1. Isolates of *Phytophthora capsici* and *P. tropicalis* used in this study

Isolate ^a	Host		Origin	Environment	Source ^b	GenBank - ITS ^c	GenBank – <i>cox II</i>
R198	Bell pepper	<i>Capsicum annuum</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464018	-- ^d
R499	Eggplant	<i>Solanum melongena</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464019	--
R599	Bell pepper	<i>Capsicum annuum</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464020	--
R699	Tomato	<i>Lycopersicon esculentum</i> Mill.	New Jersey	Temperate	S. A. Johnston	DQ464021	DQ469719
R899	Bell pepper	<i>Capsicum annuum</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464022	--
R998	Bell pepper	<i>Capsicum annuum</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464023	--
R1098-1	Bell pepper	<i>Capsicum annuum</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464024	--
R1099	Yellow squash	<i>Cucurbita pepo</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464025	--
R1198	Bell pepper	<i>Capsicum annuum</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464026	DQ469720
R1298	Bell pepper	<i>Capsicum annuum</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464027	DQ469721
219	Bell pepper	<i>Capsicum annuum</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464028	--
220	Eggplant	<i>Solanum melongena</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464029	DQ469722
223	Bell pepper	<i>Capsicum annuum</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464030	--
224	Bell pepper	<i>Capsicum annuum</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464031	--
230	Tomato	<i>Lycopersicon esculentum</i> Mill.	New Jersey	Temperate	S. A. Johnston	DQ464032	--
RA1	Eggplant	<i>Solanum melongena</i> L.	New Jersey	Temperate	S. A. Johnston	--	DQ469724
RA4	Bell pepper	<i>Capsicum annuum</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464033	DQ469723
RA16	Bell pepper	<i>Capsicum annuum</i> L.	New Jersey	Temperate	S. A. Johnston	DQ464034	--
23	Bell pepper	<i>Capsicum annuum</i> L.	North Carolina	Temperate	J. A. Ristaino	DQ464035	--
B2HH4	Bell pepper	<i>Capsicum annuum</i> L.	North Carolina	Temperate	J. A. Ristaino	DQ464036	DQ469725
Cp-1	Cacao	<i>Theobroma cacao</i> L.	Brazil	Tropical	D. J. Mitchell	DQ464037	DQ469726
Cp-2	Black pepper	<i>Piper nigrum</i> L.	Puerto Rico	Tropical	D. J. Mitchell	DQ464038	DQ469727
Cp-16	Bell pepper	<i>Capsicum annuum</i> L.	Florida	Temperate	D. J. Mitchell	DQ464039	--
Cp-17	Bell pepper	<i>Capsicum annuum</i> L.	Florida	temperate	D. J. Mitchell	DQ464040	--
Cp-30	Bell pepper	<i>Capsicum annuum</i> L.	Florida	Temperate	D. J. Mitchell	DQ464041	--
Cp-32	Bell pepper	<i>Capsicum annuum</i> L.	Florida	Temperate	D. J. Mitchell	DQ464042	--
Cp-D1	Lima bean	<i>Phaseolus lunatus</i> L.	Delaware	Temperate	C. Davidson	DQ464043	--
Cp-D2	Lima bean	<i>Phaseolus lunatus</i> L.	Maryland	Temperate	C. Davidson	DQ464044	DQ469728
Cp-D3	Lima bean	<i>Phaseolus lunatus</i> L.	Maryland	Temperate	C. Davidson	DQ464045	DQ469729
Cp-In	Arecanut	<i>Areca catechu</i> L.	India	Tropical	R. Bhat	DQ464046	DQ469730
1E	Cacao	<i>Theobroma cacao</i> L.	Brazil	Tropical	E. D. Luz	DQ464047	--
4E	Cacao	<i>Theobroma cacao</i> L.	Brazil	Tropical	E. D. Luz	DQ464048	--
23E	Cacao	<i>Theobroma cacao</i> L.	Brazil	Tropical	E. D. Luz	DQ464049	--
59Ea	Cacao	<i>Theobroma cacao</i> L.	Brazil	Tropical	E. D. Luz	DQ464050	DQ469731
106E	Cacao	<i>Theobroma cacao</i> L.	Brazil	Tropical	E. D. Luz	--	--
182E	Rubber	<i>Hevea brasiliensis</i> (Willd. ex Adr. Juss.) Müll. Arg.	Brazil	Tropical	E. D. Luz	DQ464051	--
183E	Rubber	<i>Hevea brasiliensis</i> (Willd. ex Adr. Juss.) Müll. Arg.	Brazil	Tropical	E. D. Luz	DQ464052	--
185E	Rubber	<i>Hevea brasiliensis</i> (Willd. ex Adr. Juss.) Müll. Arg.	Brazil	Tropical	E. D. Luz	DQ464053	--
197E	Rubber	<i>Hevea brasiliensis</i> (Willd. ex Adr. Juss.) Müll. Arg.	Brazil	Tropical	E. D. Luz	DQ464054	DQ469732
215E	Cacao	<i>Theobroma cacao</i> L.	Brazil	Tropical	E. D. Luz	DQ464055	DQ469733
CBS 128.23	Chile pepper	<i>Capsicum annuum</i> L.	New Mexico	Temperate	CBS	DQ464056	DQ469734
CBS 434.91	Macadamia	<i>Macadamia integrifolia</i> Maiden & Betche	Hawaii	Tropical	CBS	DQ464057	DQ469735

^a *Phytophthora capsici* (CBS 128.23, IMI 040502) (29) and *P. tropicalis* (CBS 434.91) (4) are the type isolates for their respective species.

^b S. A. Johnston and M. L. Fogg, Rutgers Agricultural Research and Extension Center, Bridgeton, NJ; J. A. Ristaino, North Carolina State University, Raleigh; D. J. Mitchell, University of Florida, Gainesville; C. Davidson, University of Delaware, Newark; R. Bhat, University of California-Davis, Salinas; E. D. Luz, CEPLAC, Itabuna, Brazil; and CBS, Centraalbureau voor Schimmelfcultures, Utrecht, the Netherlands.

^c ITS = internal transcribed spacer.

^d Not done.

Park) with an Applied Biosystems DNA sequencer model 3730 using the amplification primers and BigDye Terminator Sequencing kit (Applied Biosystems, Foster City, CA). Sequences were edited using Biological Sequence Alignment Editor (BioEdit ver. 5.0.9, Tom Hall, Ibis Therapeutics, Carlsbad, CA).

Amplification of the *cox I* and II gene cluster and sequencing of the *cox II* gene. A region spanning the *cox I* and *cox II* genes was amplified by PCR using primers FM75 (5'-CCTTGGCAATTAGGATTTCAAGAT-3') and FM83 (5'-CTCC-AATAAAAAATAACCAAAAATG-3') as previously described (33). Sequencing templates were purified by using a QIAquick PCR Purification kit following the manufacturer's instructions. After the last centrifugation the supernatant was adjusted to 2.0 M NH_4OAc , and then the DNA was precipitated with 100% ethanol at -2°C . Following centrifugation, the DNA pellet was rinsed with 70% ethanol and dried prior to resuspension in sterile TE. All sequencing was done by the DNA Sequencing Laboratory of the Interdisciplinary Center for Biotechnological Research of the University of Florida (Gainesville, FL) using a ABI 373a automated sequencer (Applied Biosystems). Templates were sequenced in both directions with primer FM75 as well as additional internal primers FM78 (5'-ACAAATTTCACTACATTGTCC-3'), FM79 (5'-GGACAATGTAGTGAATTTGT-3') and FM80 (5'-AATA-TCTTTATGATTTGTTGAAA-3'). Primers were synthesized by Invitrogen Corp.

Phylogenetic analysis. A consensus sequence was obtained after alignment of sequences of both strands using the computer program Sequencher ver. 4.1 (Gene Codes, Ann Arbor, MI). A total of 752 bp were used for the ITS analysis and 667 bp were used for the *cox I* and II gene cluster. Sequence alignments for the ITS and *cox II* gene were optimized using MacClade version 4.02 (Sinaur Assoc., Sunderland, MA), and PAUP version 4.0b10 (Sinaur Assoc.) was used for phylogenetic analyses. Phylogenetic relationships among *Phytophthora* spp. using DNA sequence data were inferred by maximum parsimony (MP) analysis with a heuristic tree search. Heuristic searches were performed with MULPARS on, steepest decent option off, random addition of sequences (100 replicates) and TBR branch swapping. To determine support for the various clades of the trees, the analysis described above was bootstrapped with 1,000 replicates with the same conditions noted above, with the exception that there were 10 replicates for the random addition of samples. Data for an isolate of *Pythium aphanidermatum* from Mexico (1987-61), *Pythium ultimum* from Florida (110-2), and *Phytophthora* spp. previously reported (32,33) also were included in the analysis. Phylogenetic inferences based on ITS and 5.8S rDNA sequence data were based on the results of Cooke et al. (9) with the alignments retrieved from TreeBASE (accession M751) and modified as in Martin and Tooley (33) (TreeBASE accession SN 774). DNA sequence data obtained in this study have been deposited in GenBank (Table 1) and the results of the phylogenetic analysis have been deposited in TreeBASE (S1662).

RFLP analysis of *cox I* and II gene cluster and ITS regions. RFLP analysis of the *cox I* and II gene cluster generated by amplification using FM75 and FM83 was conducted as previously described (34). All amplifications were separated on a 1.5% agarose gel to check DNA concentration and purity prior to digestion. The restriction enzymes *AluI*, *MspI*, *RsaI*, and *TaqI* were used in accordance with the manufacturer's instructions (New England BioLabs) and the fragments were separated on a 3% NuSieve 3:1 gel (Cambrex Bio Science, Rockland, ME) in $0.5\times$ TBE buffer (0.045 M Tris-borate, 0.001 M EDTA, pH 8.0) at 45 V for 6 h or until the bromophenol blue dye in the loading buffer had migrated 8 cm from the well. A 100-bp ladder (New England BioLabs) mixed with a nondigested amplicon from *P. infestans* (isolate 580; approximately 2.2 kb based on sequence analysis) provided size markers. The gel was stained in ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) for 30 min, destained in deionized water for 30 min, and photo-

graphed under short wave UV using either Polaroid Type 55 film or an ERDAS imaging system (Kodak, Rochester, NY). RFLP analysis of the ITS region that was amplified with ITS6 and ITS4 was done as reported by Cooke et al. (11). The amplicons were digested with *AluI*, *MspI*, or *TaqI* and separated by agarose gel electrophoresis using the same procedures noted above for the *cox* gene cluster.

Scanned digital images of the Polaroid negatives were imported into the computer program BioNumerics (ver. 2.5, Applied Maths, Sint-Martens-Latem, Belgium). This computer program automatically determines the molecular size of the RFLP bands relative to molecular size standards included on each agarose gel. The images were processed using standard procedures and molecular size determinations of digested bands were done automatically with manual confirmation. To optimize band matching between isolates, the positional tolerance of each band (the maximum shift between two bands that is allowed to consider the bands matching, expressed as a percentage of the length of the gel run) and optimization of the data (this allows for a shift between any two patterns to optimize comparisons of banding patterns, expressed as a percentage of the run length) were determined from the data using the tolerance and optimization options of the program. Positional tolerances of 1.06, 1.0, 1.16, and 1.0% were obtained for *AluI*, *MspI*, *RsaI*, and *TaqI*, respectively. Optimization was determined to be 0 except for *RsaI*, which was set at 0.54%. Bands less than 100 bp in size were excluded from the analysis due to the diffuse nature of the bands when using a 3% NuSieve 3:1 agarose gel. In addition to band size determinations, BioNumerics performed cluster analysis to evaluate the similarity in restriction banding profiles among the various species evaluated. For comparison with other species in the genus, data from a prior analysis of the genus were included in this analysis (34).

RESULTS

Radial growth rates and chlamydospore formation. Radial growth of the isolates and chlamydospore formation are presented in Table 2. All isolates grew at 28°C . With one exception (isolate B2HH4), all isolates originating from a temperate climate grew at 35°C , while those from the tropical climate did not. All but two isolates from the tropical group of isolates formed chlamydospores via the submerged culture method. Conversely, no chlamydospores were observed in isolates within the temperate group.

AFLP analysis. Data from the four primer combinations, *EcoRI*-AG, -AC, -AA, and -AT, each in combination with *MseI*-C, resulted in a total of 540 polymorphic loci, with 140, 162, 115, and 123 polymorphic loci, respectively. Similarities among isolates of *P. capsici* based on AFLP analysis using Jaccard's similarity coefficient and the UPGMA tree building algorithm were supported by bootstrap analysis (Fig. 1). The dendrogram clearly separated the temperate isolates of *P. capsici* from the tropical isolates and from isolates classified as *P. tropicalis* with high bootstrap values (above 90%). Within the temperate group, the majority of the isolates clustered within a single clade (Fig. 1, clade A), however, there was good bootstrap support for the separate grouping of three temperate isolates (isolates 23, 223, and 230). Within the temperate clade A there was not any specific grouping of isolates for host or geographic recovery, although the grouping of two isolates each from bell pepper recovered from Florida and New Jersey was supported by bootstrap analysis.

The tropical isolates grouped into three distinct clades (Fig. 1). Clade B was comprised entirely of isolates recovered from cacao or rubber in Brazil, while clade C contained a mixture of isolates, including the type culture of *P. tropicalis* from macadamia in Hawaii, an isolate from black pepper in Puerto Rico, and an isolate from arecanut in India. Clade D constituted a separate grouping and contained isolates from cacao in Brazil.

TABLE 2. Radial growth of temperate and tropical isolates of *Phytophthora capsici* at 28 and 35°C in darkness, and resulting chlamydospore formation

Isolate	Colony diameter at 48 h (mm)		Chlamydospore formation
	28°C	35°C	
Temperate group			
Pc-W3a	42.0	9.5	– ^c
R699	25.6	16.1	–
R1198	39.6	9.4	–
R1298	38.6	5.4	–
Cp-D2	31.8	23.5	–
Cp-D3	26.5	22.9	–
B2H4	23.2	0.1	–
RA1	40.4	7.9	–
RA4	35.5	15.9	–
220	42.0	13.1	–
Tropical group			
23E	39.6	0	+
23-1E	38.7	0	+
59Ea	36.1	0	+
183E	18.5	0	+
197E	27.1	0	+
215E	34.9	0	+
Cp-1	39.5	0	–
Cp-2	28.4	0	–
Cp-In	31.2	0	+
CBS 434.91 (type)	42.0	0	+
MSD value ^b	1.78	1.57	

^a Colony diameter was measured in millimeters (mm) at the narrowest and widest points and then the two numbers were averaged after subtracting the 6 mm diameter of the agar transfer plug.

^b Waller-Duncan k-ratio *t* test; MSD = minimum significant difference, k=100.

^c Chlamydospore formation after 8 weeks at 16°C following submerged culture method of Uchida and Aragaki (46); + and - indicate formation and no formation of chlamydospores, respectively.

Phylogenetic analysis. Isolate groupings for the ITS data were the same as those observed with the AFLP data, with the temperate isolates forming a single clade and the tropical isolates clustering into three clades, some of which also were supported in bootstrap analysis (Figs. 2 and 3). One noticeable difference was for temperate isolates 23, 230, and 223, which were distantly grouped with the other temperate isolates in the AFLP analysis, but were within the same clade in the ITS analysis (Figs. 2 and 3). Comparisons with sequences of other isolates downloaded from GenBank revealed a clear separation of tropical and temperate isolates and a lack of grouping of isolates from a particular host or geographical location (Fig. 2). The only exception to this was clade D, which had isolates from *Theobroma cacao* recovered from Brazil, Africa, and New Guinea. Sequence divergence between temperate clade A and tropical clade D ranged from approximately 1.0 to 1.6%, but this was greater than sequence divergence within clades. All temperate isolates studied, and those retrieved from GenBank, were grouped in clade A, with a sequence divergence within the clade of only 0.4%. Isolates from different hosts and geographical area were grouped together. In contrast, sequence variation among the three tropical clades was greater, with a 0.7 to 0.8% sequence divergence between tropical clades B and C as well as between clades B and D, and 1.0 to 1.3% sequence divergence between clades D and C. However, sequence divergence between temperate and tropical isolates within the ITS regions was more than in comparisons among other closely related species such as *P. infestans*, *P. mirabilis*, and *P. phaseoli* (from 0.1 to 0.6%) or *P. cactorum* and *P. pseudotsugae* (0.6%).

The same groupings were observed for the analysis of the *cox I* and II gene region although there was no bootstrap support for this separation. The temperate isolates grouped together while the

Fig. 1. Unweighted pair-group method, arithmetic mean cluster analysis showing similarities among isolates of *Phytophthora capsici* based on amplified fragment length polymorphism analysis using Jaccard's similarity coefficient. The numbers at the nodes are the percentage of the trees above 70% that were supported by bootstrap analysis (1,000 replications). Data from four primer combinations were combined for the analysis with a total of 540 polymorphic markers (*Eco*RI-AG, -AC, -AA, and -AT, each in combination with *Mse*I-C with 140, 162, 115, and 123 polymorphic markers, respectively).

tropical isolates were found in three clades, some of which had bootstrap support (Fig. 4). One noticeable difference was observed in clade A, with the *P. capsici* type culture (CBS128.23) and three other isolates grouping together within the clade; this was not observed in the AFLP or ITS analysis. Sequence divergence between temperate and tropical isolates within the *cox* I and II regions studied ranged from 0.15 to 1.0%. The temperate isolates

were very similar, with a sequence divergence of only 0.3% or less, and among the tropical isolates, the sequence divergence ranged from 0.15 to 0.75%. The sequence divergence between temperate (clade A) and tropical (clade C) isolates was similar (0.1 to 0.6%) to the level of variation observed among the tropical isolates. As a comparison, the sequence divergence observed among the *P. infestans*, *P. mirabilis*, and *P. phaseoli* group was 0.6

Fig. 2. One of 120 most parsimonious trees for *Phytophthora* spp. obtained using internal transcribed spacer DNA sequence data based on maximum parsimony inferred by a heuristic tree search. Of the 835 total characters, 624 were constant, 107 were variable and parsimony uninformative, and 104 were parsimony informative. Tree length = 288, consistency index = 0.833, homoplasy index = 0.167, and retention index = 0.894. Data for species other than *P. capsici* and *P. tropicalis* were from alignments in Martin and Tooley (33). Letters to the right of the clades correspond to the clade lettering in Figure 1.

to 0.9%, and there was good bootstrap support to separate these species into distinct groups.

RFLP analysis of PCR amplified products. Cluster analysis of the *cox* I and II gene cluster RFLP data exhibited similarity to the groupings observed in the AFLP, ITS and *cox* II sequence data with some notable exceptions (Fig. 5). While clade D isolates grouped alone on the same clade, isolates from clade B and C had

the same RFLP pattern and were together on the same clade in this analysis. Surprisingly, the type culture for *P. capsici* (a temperate, clade A isolate in prior analysis) was also on this same clade. Isolates from clade A were found on three separate groupings with no apparent association with host or geographic location of recovery. All tropical and temperate isolates grouped together and were separate from other species. RFLP fragment sizes were similar to

Fig. 3. The most parsimonious tree for *Phytophthora* spp. obtained using internal transcribed spacer DNA sequence data based on maximum parsimony inferred by a heuristic tree search. Thick branch lines represent clades that were supported by bootstrap analysis (1,000 replications), with the numbers in brackets at the nodes the percentage of the trees above 70% that support the observed topography. Letters to the right of the clades correspond to the clade lettering in Fig. 1. Of the 906 total characters, 467 were constant, 194 were variable and parsimony uninformative, and 245 were parsimony informative. Tree length = 982, consistency index = 0.632, homoplasy index = 0.368, and retention index = 0.759.

those previously reported for three of the isolate groupings (represented by isolates 302, Cp-30, and 59Ea) (34), but three unique RFLP groupings were observed that differed by the loss of a *TaqI* site (isolates CBS 128.23 and CBS 434.91) or differences in *MspI* restriction sites (isolates CBS 128.23, CBS 434.91 and 220; Table 3).

For the ITS RFLP analysis, the temperate isolates (clade A1, A2, and A3) all grouped in the same major clade separately from the tropical isolates. The groupings of the tropical isolates were reflective of what was observed in the other analysis, with clade B and C more closely affiliated with each other than with clade D (Figs. 1-4). Fragment sizes were similar to what was previously

Fig. 4. One of 19 most parsimonious trees for *Phytophthora* spp. using *cox II* DNA sequence data based on maximum parsimony inferred by a heuristic tree search. Thick branch lines represent clades that are supported by bootstrap analysis (1,000 replications), with the numbers in brackets at the nodes the percentage of the trees above 70% that support the observed topography. Letters to the right of the clades correspond to the clade lettering in Figure 1. Of the 667 total characters, 466 were constant, 37 were variable and parsimony uninformative, and 164 were parsimony informative. Tree length = 617, consistency index = 0.462, homoplasy index = 0.538, and retention index = 0.794. Isolates marked with an asterisk are the same cultures that were used in this submission to infer the internal transcribed spacer-based phylogenetic tree in Fig. 2. Data for species other than *P. capsici* and *P. tropicalis* were from alignments in Martin and Tooley (34).

Fig. 5. Unweighted pair-group method arithmetic mean cluster analysis using Jaccard's similarity coefficients of the *AluI*, *MspI*, *RsaI*, and *TaqI* restriction fragment linked polymorphism restriction profiles of the polymerase chain reaction-amplified product spanning the *cox I* and *II* gene cluster generated using primers FM 75 and FM 77/83. The scale at the top represents percent similarity. Data for species other than *P. capsici* and *P. tropicalis* can be found in Martin and Tooley (34). Letters to the right of the clades correspond to the clade lettering in Figure 1.

reported by Cooke et al. (11) with a few exceptions (Table 4). Isolates in clade C (represented by the type culture of *P. tropicalis* CBS 434.91) had an additional band at approximately 694 bp following *AluI* digestion, while isolates on clade D (represented by isolate 23E) had a unique *AluI* restriction profile.

DISCUSSION

The taxonomic classification of *P. capsici* isolates recovered from hosts in tropical and temperate climates has been an evolving concept (2,4,36,37,43,49). In this current study, an additional 28 temperate and 14 tropical isolates of *P. capsici*/*P. tropicalis* (including the type cultures) were examined using morphological and molecular criteria to evaluate isolates of the species. We asked if the observed differences were enough to warrant the designation of a new species as has been suggested (4), or if these differences represent a level of variability reflective of the species.

Two features reported to correspond with the separation of these species are growth at 35°C (*P. capsici*) and formation of chlamydospores (*P. tropicalis*) (4). Based strictly on these criteria the temperate and tropical isolates examined in this study were separated. All isolates tested were able to grow on agar medium at 28°C, but only the temperate isolates grew at 35°C (Table 2). None of the temperate isolates were able to form chlamydospores using the submerged culture method, but 8 of 10 tropical isolates did. This lack of chlamydospore formation in some tropical isolates has been reported previously (1,43,45).

In the AFLP analysis there also was a distinction between temperate and tropical isolates, and this separation was supported by bootstrap analysis (Fig. 1). Most of the temperate isolates grouped together in the same clade, while the tropical isolates appeared to represent an assemblage of distinct genetic lineages. Groupings of isolates within clades appeared to be independent of host and geographical origin. Bootstrap analysis supported placing 25 of 28 of the temperate isolates in one clade, with the remaining three isolates closely related. The tropical isolates, on the other hand, were

TABLE 3. Size, in number of base pairs (bp), of restriction fragments for the *cox I* and *II* gene cluster amplified with primers FM75 and FM77/83 from *Phytophthora* spp. and digested with the indicated restriction enzyme

<i>Phytophthora</i> sp.	Isolate # ^a	<i>AluI</i>	<i>MspI</i>	<i>RsaI</i>	<i>TaqI</i>
<i>P. capsici</i>	302 ^b	697; 451; 227; 203; 179; 132	Uncut	712; 550; 332; 300; 226; 182	1,573; 743
	Cp-30 ^b	697; 451; 227; 203; 179; 132	1,415; 901	712; 550; 332; 300; 226; 182	1,573; 743
	59Ea ^b	720; 451; 227; 203; 179; 132	1,140; 1,080	712; 550; 332; 300; 226; 182	1,187; 834
	CBS 128.23	697; 451; 227; 203; 179; 132	Uncut	712; 550; 332; 300; 226; 182	Uncut
<i>P. tropicalis</i>	CBS 434.91	697; 451; 227; 203; 179; 132	1,462; 711	712; 550; 332; 300; 226; 182	Uncut
	220	697; 451; 227; 203; 179; 132	1,170 (2x)	712; 550; 332; 300; 226; 182	1,573; 743

^a Amplified products were digested with the indicated restriction enzyme and separated in a 3% NuSieve 3:1 agarose gel. Fragment sizes were determined and the database managed using the computer program BioNumerics (ver. 2.5). While fragment sizes are reported to the base pair by BioNumerics, this level of accuracy is artificial and not supported by the agarose electrophoresis method used for estimation. Doublet bands and fragment sizes smaller than 100 bp are not reported or included in the cluster analysis.

^b Same sizes as reported in Martin and Tooley (34).

TABLE 4. Size, in number of base pairs, of restriction fragments for *Phytophthora* spp. amplified with primers ITS1 and ITS4 and digested with the indicated restriction enzyme

<i>Phytophthora</i> sp.	Clade	Isolate # ^a	<i>AluI</i>	<i>MspI</i>	<i>TaqI</i>
<i>P. capsici</i>	A1	RA1	155; 172; 535 ^b	145; 204; 221; 292 ^b	46; 59; 90; 101; 148; 194; 263 ^c
	A2	CBS 128.23	155; 172; 535 ^b	145; 204; 221; 292; 349 ^c	46; 59; 90; 101; 148; 194; 263 ^c
	A3	Cp-17	155; 172; 535 ^b	145; 204; 221; 292 ^b	46; 59; 90; 101; 148; 263 ^b
<i>P. tropicalis</i>	C	CBS 434.91	155; 172; 535; 694	221; 292; 349 ^d	59; 90; 100; 149; 194; 263 ^d
	B	185E	155; 172; 535 ^{b,c,d}	221; 292; 349 ^d	59; 90; 100; 149; 194; 263 ^d
	D	23E	183; 694	221; 292; 349 ^d	59; 90; 100; 149; 194; 263 ^d

^a Amplified products were digested with the indicated restriction enzyme and separated in a 3% agarose gel. While fragment sizes are reported to the base pair, this level of accuracy is artificial and is not supported by the agarose electrophoresis method used for estimation. These sizes are included for purposes of comparison with the results of Cooke et al. (11).

^b Results are the same as reported by Cooke et al. (11) for *P. capsici*.

^c Results are the same as reported by Cooke et al. (11) for *P. capsici* combination.

^d Results are the same as reported by Cooke et al. (11) for *P. capsici* variant.

separated into three clades, all strongly supported by bootstrap analysis. Clades B and C (which included the type culture for *P. tropicalis*) grouped together and were affiliated more closely with the temperate isolates in clade A than with other tropical isolates in clade D (comprised of Brazilian isolates from *T. cacao*), which were more distantly related. A similar grouping of isolates recovered from *T. cacao* in Brazil also was reported in isozyme analyses (36,37).

Analysis of ITS DNA sequence data of the species revealed similar groupings and variation within and among clades as did the AFLP analysis (Fig. 2). However, sequence divergence among isolates and clades was relatively low. More sequence variation was present within the three clades of tropical isolates than within the temperate clade. Clade D isolates, recovered from *T. cacao* from different regions of the world, grouped separately from other tropical isolates in both analyses and may be indicative of a sub-population. Silvar et al. (41), using random amplified polymorphic DNA analysis, found little variation among Spanish isolates of *P. capsici*, and more variation among predominately tropical isolates. This is similar to our findings of low variation among temperate, North American isolates, and greater variation among tropical isolates. In our study when the phylogenetic analyses were rerun to include more species of the genus, similar groupings were observed, with the branch lengths separating temperate and tropical isolates shorter than the branch lengths separating most other species (Fig. 3). The temperate and tropical isolates were related more to each other than to closely related *P. colocasiae*. In this analysis, however, there were groups of recognized species (*P. infestans*, *P. mirabilis*, and *P. phaseoli* or *P. cryptogea*

and *P. erythroseptica*) that exhibited less sequence divergence than observed between the temperate and tropical groups. This data set, then, presents evidence that could be used to both support and not support the separation of temperate and tropical isolates of *P. capsici*. Thus, decisions on speciation based on results of ITS sequence analysis alone may not be appropriate in this situation. The inclusion of the *cox II* DNA sequence data did not resolve the issue further, as the separation of the temperate and tropical isolates was even less clear (Fig. 4). While there was bootstrap support for separating these isolates from other species, there was no support for separating the temperate and tropical isolates.

In a similar analysis with another species of *Phytophthora*, Ivors et al. (21) found clear differences between isolates of *P. ramorum* from the United States and Europe using AFLP analysis, but phylogenetic analyses revealed no differences in the DNA sequences of three different genes (ITS, *cox II*, and *nad 5*). Even though isolates from different populations within the species can be differentiated using AFLP analysis, all are considered to be of the same species. Like *P. ramorum*, *P. capsici* may consist of different populations within the species.

While not intended to be used to support phylogenetic relationships between temperate and tropical isolates, RFLP analysis of the mitochondrially encoded *cox I* and *II* gene cluster and nuclear encoded ITS can be used to help identify isolates into specific subgroups. Restriction profile analysis of the *cox* gene cluster separated the *P. capsici* isolates from other species, but did not distinguish between temperate and tropical isolates (Fig. 5). A group of temperate isolates was clustered with tropical isolates, and the type isolate of *P. capsici*, a temperate isolate, was clus-

Fig. 6. Unweighted pair-group method arithmetic mean cluster analysis of *Phytophthora* spp. using Jaccard's similarity coefficients of the *AluI*, *MspI*, and *TaqI* restriction fragment linked polymorphism restriction profiles of the internal transcribed spacer region of rDNA region amplified using primers ITS1 and ITS4. Letters to the right of the clades correspond to the clade lettering in Figure 1.

tered with tropical isolates (clade ABC). Analysis of restriction profiles of the ITS region of the rDNA separated the temperate and tropical isolates in groupings similar to AFLP and ITS phylogenetic analysis (Fig. 6). The same three clades were observed within the tropical group, but the temperate group of isolates was grouped into three subclades with no association with regard to host or geographic origin. Based on the band sizes observed, these restriction profiles may allow for groups within species identification when compared to data from the genus as a whole (11). This statement also may be true of the SSCP analysis (24). An interesting observation from the ITS RFLP data is that the summation of the band sizes was greater than the amplicon size, which indicates heterozygosity for rDNA. This is not surprising given that *P. capsici* is heterothallic and agrees with the findings of Lamour and Hausbeck (27) in Michigan for *P. capsici*, and with data reported by Martin (31) for *Pythium sylvaticum* outcrosses. In view of the differences in grouping of isolates in the *cox* I and II gene cluster compared with the ITS RFLP analysis, combining these techniques should provide an additional tool for identification of isolate subgroups. One possible reason for the lack of correlation between the *cox* I and II gene cluster and ITS RFLP analysis is that mitochondria are uniparentally inherited from the maternal parent in sexual crosses in the genus *Phytophthora* (14). Given that *P. capsici* is heterothallic and that outcrossing has occurred in the isolates examined in this study (as evidenced by the heterozygosity of the ITS), new combinations of the ITS region could be encountered in progeny with a single *cox* I and II RFLP pattern.

Many species of *Phytophthora* have multiple hosts, and *P. capsici* is no exception (13). However, variation in pathogenicity has been reported within the species depending on the host from which a particular isolate was recovered (39). Uchida and Aragaki (46) reported that isolates of *P. capsici* from macadamia were not pathogenic on pepper, and that all tropical isolates of *P. capsici* tested later were uniformly avirulent on pepper (4). In preliminary tests with the isolates used in this report, both temperate and tropical isolates exhibited a range in virulence when inoculated onto cacao pods, with either group just as likely to cause severe or little symptom development (30). Temperate isolates also caused a range in symptoms when inoculated onto pepper fruits, but tropical isolates caused little or no symptoms. These preliminary results coincide with those of Aragaki and Uchida (4), but based on the variation present in this species and others (22), pathogenicity on a particular host may not be a good indication of species status (13). These results do raise an interesting question. Clearly the temperate isolates still carry the genetic ability to infect cacao, and presumably other tropical crops (although this has yet to be tested). The tropical isolates, on the other hand, appear to have limited genetic ability to infect pepper, a crop to which a tropical population may not have been exposed to in recent time.

Our data, taken as a whole, suggest that the temperate isolates may be members of a population resulting from the introduction of members of a parent tropical population into a new environment. When a species is introduced into a new environment, the small, original population is called a founder population (35). These populations typically go through a biological bottleneck where only a fraction of the individuals survive, e.g., those that could infect solanaceous plants, cucurbit plants, or both, in a temperate environment. Thus, these populations are less diverse genetically than the parent population. Our AFLP and ITS sequence data clearly indicate that the temperate population is less diverse genetically than the tropical population, and that the temperate population is encompassed within the variation present in the parent tropical population. These results are characteristic of the founder effect (35), and suggest that the temperate population was introduced, and has become an invasive population in temperate climates. The results of Silvar et al. (41) for Spanish isolates of

P. capsici are an indication that the introduction of the tropical population of the species into a temperate climate may have occurred several times. The relationships among North American and European isolates of *P. capsici* would be important to know to test this hypothesis. Similar arguments were made regarding the introduction of *P. ramorum* into North America and Europe from an unknown source (21).

These two populations of *P. capsici*, however, may not yet be sufficiently isolated to justify separate species status. The low level of sequence variation between the two populations in the mitochondrial *cox* II sequence data indicates that the process may not be far enough along genetically, even though there appear to be some morphological and pathological differences. Similarly with *P. ramorum*, AFLP and DNA sequence data supports different populations of the same species (21). Brasier and Hansen (8) offer selection and reproductive isolation as two aspects of the speciation process in *Phytophthora* spp. that can be inferred from present evidence. With plant pathogens, the host plant can act as a genetic sieve that continues to select and separate populations. These gradual changes may not be observable at one point in time, and we would be hard pressed to determine if this is occurring within the *P. capsici*/*P. tropicalis* group without more information gathered over time. However, if this were found to be true through further research, then the temperate population of the species may have or may be evolving from the tropical population in terms of host specificity, morphological adaptations, and genetic composition.

The presented morphological and molecular data and the previously reported and referenced pathogenicity data (4,22,30) may point in this direction, but will this process lead to reproductive isolation between temperate and tropical isolates? Morphological differences may only be an adaptation to the environment, and only represent different morphospecies (16,19). Studies have shown that some morphospecies are composed of several biological species, phylogenetic species, or both (19). Only studies looking at crosses between and among populations of these two groups will answer the question as to whether the temperate and tropical isolates are conspecific or reflective of different recognized species.

A clearer picture of what level of sequence variation is reflective of variation within a species compared to between closely related species also is needed for the genus as a whole before the data can be used to draw conclusions about species classification of these isolates. Different regions of the genome also need to be sequenced and included in the phylogenetic analysis, and then compared with morphological and pathogenicity data from a wide variety of isolates before enough evidence can be gathered to firmly support the species designation of these isolates. Other authors have reported isolating an organism they called *P. tropicalis* based on available data (15,20). We submit that the available data are incomplete, and that the full range of variation in the species has not yet been examined. Isolates identified as *P. capsici* or *P. tropicalis* may represent different subgroups in a broad continuum of variation that is presently called *P. capsici*. We, therefore, recommend refraining from using the epithet *P. tropicalis* for isolates more related to the tropical population of *P. capsici* until more data are available.

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