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# Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes

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***Abstract:*** The phylogenetic relationships of 51 isolates representing 27 species of *Phytophthora* were assessed by sequence alignment of 568 bp of the mitochondrially encoded cytochrome oxidase II gene. A total of 1299 bp of the cytochrome oxidase I gene also were examined for a subset of 13 species. The *cox* II gene trees constructed by a heuristic search, based on maximum parsimony for a bootstrap 50% majority-rule consensus tree, revealed 18 species grouping into seven clades and nine species unaffiliated with a specific clade. The phylogenetic relationships among species observed on *cox* II gene trees did not exhibit consistent similarities in groupings for morphology, pathogenicity, host range or temperature optima. The topology of *cox* I gene trees, constructed by a heuristic search based on maximum parsimony for a bootstrap 50% majority-rule consensus tree for 13 species of *Phytophthora*, revealed 10 species grouping into three clades and three species unaffiliated with a specific clade. The groupings in general agreed with what was observed in the *cox* II tree. Species relationships observed for the *cox* II gene tree were in agreement with those based on ITS regions, with several notable exceptions. Some of these differences were noted in species in which the same isolates were used for both ITS and *cox* II analysis, suggesting either a differential rate of evolutionary divergence for these two regions or incorrect assumptions about alignment of ITS sequences. Analysis of combined data sets of ITS and *cox* II sequences generated a tree that did not differ substantially from analysis of ITS data alone, however, the results of a partition homogeneity test suggest that combining data sets may not be valid.

***Key words:*** *cox* I, *cox* II, ITS phylogeny

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## INTRODUCTION

*Phytophthora* is a complex genus containing about 60 described species that occupy a variety of terrestrial and aquatic habitats (Erwin and Ribeiro 1996). Although oomycetes share many morphological features in common with fungi, evolutionarily they are more closely related to chromophyte algae and plants than to eumycotan fungi (Förster et al 1990a, Knoll 1992, Baldauf and Palmer 1993, Wainright et al 1993, Bhattacharya and Stickel 1994, Leipe et al 1994, 1996, Silberman et al 1996, Weerakoon et al 1998).

Historically speaking, a range of morphological and physiological criteria has been used to classify members of this genus (Stamps et al 1990, Waterhouse 1963), including sporangial structure, antheridial form, host specificity and breeding system (homothallic or heterothallic). However, in light of growing evidence from molecular and ecological studies, it has become apparent that taxonomic groupings, based solely on morphological criteria, might be artificial (Brasier 1991, Brasier and Hansen 1992). The congruence between taxonomic inference derived from molecular data and that based on classical morphological taxonomy is a topic of interest in current studies. Molecular tools used in phylogenetic studies of oomycetes have included analysis of large and small subunit ribosomal RNA genes (rRNA; van de Peer et al 1996, Förster et al 1990a), mitochondrial DNA (Förster et al 1988, Förster and Coffey 1993), and sequence analysis of the internal transcribed spacer (ITS) regions of the rRNA genes (Förster et al 2000, Cooke and Duncan 1997, Crawford et al 1996). Recently, Cooke et al (2000) reported an ITS-based molecular phylogeny, which included 50 *Phytophthora* species, and is the most comprehensive to date. It was re-emphasized by Cooke et al (2000) that the six taxonomic groupings of Waterhouse (1963) do not represent natural assemblages because individual ITS clades contained taxa from multiple Waterhouse groups. Similarly, Förster et al (2000), based on ITS1 sequence analysis, concluded that the morphological characters used in *Phytophthora* taxonomy are of limited value in deducing phylogenetic relationships because they exhibit convergent evolution. Data using additional molecular markers are needed for comparison with ITS-based data, particularly in corroborating and/or clarifying the taxonomy of

groups that cannot be resolved based on ITS data. For example, species such as *P. infestans*, *P. mirabilis*, and *P. phaseoli* are poorly resolved with ITS data while *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi* are not differentiated at all.

The cytochrome c oxidase subunit II (*cox* II) gene has been used previously in studies of Peronosporomycete phylogeny (Hudspeth et al 2000) and phylogenetic studies of 24 *Pythium* species (Martin 2000). The *cox* II gene is mitochondrially encoded and, in contrast to the ITS region, represents a coding region rather than intergenic region. Analysis of *cox* II grouped *Pythium* species into three major clades that were reflective of sporangial morphology (Martin 2000). The objective of this investigation was to characterize the evolutionary relationship among *Phytophthora* species representing different morphological groupings in the genus (Brasier 1983, 1991), with gene sequences from *cox* I and *cox* II genes. The relationship between specific morphological features and phylogeny will be examined in an attempt to identify features that can be used for deducing evolutionary groupings among species. Last, the *cox* gene phylogeny will be compared with the ITS phylogeny to clarify the relationships among species poorly resolved by ITS data and to evaluate congruence of phylogenetic results obtained with mitochondrial gene sequences compared to nuclear encoded spacer sequences.

## MATERIALS AND METHODS

Phytophthora *cultures*.—The cultures used in this study are listed in TABLE I. Isolates of *Phytophthora arecae*, *P. cryptogea*, *P. drechsleri*, *P. erythroseptica*, *P. lateralis*, *P. megasperma*, and *P. syringae* included in this study (TABLE I) also were used in the ITS rDNA phylogenetic study of Cooke et al (2001). Cultures were grown on Rye A medium (Caten and Jinks 1968) at 20 C in darkness and maintained in liquid nitrogen for long-term storage (Tooley 1988).

*DNA amplification and sequence analysis*.—Genomic DNA was extracted by the method of Goodwin et al (1992) or by a boiling miniprep procedure (Martin and Semer 1997). DNA concentrations were determined spectrophotometrically and/or by quantitation on agarose gels stained with ethidium bromide in comparison with commercially obtained standards.

Templates of the *cox* I and II gene cluster were amplified for sequencing by PCR using primers FM 75 (dCCTTGGCAATTAGGATTTCAAGAT) or FM 82 (dTTGGCAATTAGGTTTTCAAGATCC) and FM 77 (dCACCAATAAAGAATAACCAAAAATG) (FIG. 1). While these primers worked well for nearly all isolates examined, FM 77 did not work well for amplification of *P. capsici*, *P. colocasiae* or *P. citricola* (all clustered on Clade 2 in FIG. 2). However, primer FM 83 (dCTCCAATAAAAAATAACCAAAAATG) and FM 75 did amplify DNA from these species. Amplification reactions were done in 50 μL and contained about 50 ng of DNA, 0.5 μM final concentration of each primer, 5 μL of 10× buffer, 100 μM of each dNTP, 2 mM $MgCl_2$, and 3 units of Taq polymerase (Promega, Madison, Wisconsin). Amplifications were done with an Eppendorf (Westbury, NY) Mastercycler Gradient Thermalcycler with these run parameters: one cycle at 95 C for 2 min; 35 cycles of 1 min annealing at 56 C, 2 min extension at 72 C, and 1 min denaturation at 94 C; followed by one extension cycle at 72 C for 10 min. Before purifying the templates for sequencing, all amplifications were separated on a 1.5% agarose gel to check template concentration and purity.

Sequencing templates were purified by using a Millipore (Bedford, MA) Ultrafree MC filter or a Quiagen (Valencia, CA) QIAquick PCR Purification kit following the manufacturer's instructions. After the last centrifugation the solution containing the DNA was transferred to a fresh 500 μL tube, sterile water was added to bring the volume to 200 μL, and 4 μL of 10 M $NH_4OAc$ was added before extraction with an equal volume of chloroform:isoamyl alcohol (24:1). The supernatant was adjusted to 2.0 M $NH_4OAc$, and the DNA was precipitated with 100% ethanol at −20 C. After centrifugation, the DNA pellet was rinsed with 70% ethanol and dried before 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 at Gainesville, using ABI 373a automated sequencers (Applied Biosystems, Foster City, CA). Templates were sequenced in both directions with the primers used for amplification, as well as primers FM78 (dACAAATTTCACTACATTGTCC), FM79 (dGGACAATGTAGTGAAATTTGT) and FM 80 (dAATATCTTTATGATTTGTTGAAA) for *cox* II gene sequencing and FM 84 (dTTTAATTTTTAGTGCTTTTGC), FM 85 (dAACTTGACTAATAATACCAAA), and FM 50 (dGTTTACTGTTGGTTTAGATG) for sequencing the *cox* I gene (FIG. 1). Primers were synthesized by Invitrogen Corp. (Carlsbad, CA).

*Data analysis*.—Overlapping sequences from each sequencing primer and the sequences from opposite strands were aligned with the computer program Omiga 1.1 (Accelrys Inc, Manassas, VA). If any regions of sequence ambiguity were observed, the original output files of complementary strands from the ABI 373a sequencer were compared for correction and the sequencing reactions were repeated if necessary. A total of 568 bp was used for phylogenetic analysis with the *cox* II gene, which included bases 94 to 661 of the gene. For *cox* I, 1299 bp was used, which represented bases 1 to 1299 of the gene. Sequence alignments and phylogenetic analyses were done with PAUP ver. 4.0b8. Decay indices were determined with AutoDecay (v 4.0) of Torsten Eriksson (1999; http:/www.bergianska.se/personal/TorstenE/). Phylogenetic relationships among *Phytophthora* spp., using DNA sequence data, were inferred by maximum-parsimony (MP) analysis with a heuristic tree search and Neighbor-joining (NJ) tree reconstruction, using the Kimura 2-K parameter correction method. Heuristic searches were performed with MULPARS on, steepest-descent option off, simple addition of sequences and TBR branch swapping. Maximum-likelihood (ML) anal-

ysis with a heuristic tree search was done with the number of substitution types set at 2, transition/transversion ratio empirically determined from the data; a molecular clock was not enforced with the balance of the settings corresponding to the HKY85 model. To determine support for the various clades of the trees, the analysis was bootstrapped with the number of replicates indicated in the figure legends. The outgroup for the analysis was *Saparomyces elongates;* sequence data for *Peronophythora litchii* and *Lagenidium giganteum* were included as well (GenBank AF086698, AF086697, and AF086700, respectively; Hudspeth et al 2000). Data for an isolate of *Pythium aphanidermatum* from Mexico (1987-61) also was included in the analysis, using *cox* II data previously reported (Martin 2000; GenBank AF196579) and *cox* I data obtained in this study. Phylogenetic inferences based on ITS and 5.8S rDNA sequence data were based on the results of Cooke et al (2000) with the alignments retrieved from TreeBASE (accession M751). The same region for *Pythium aphanidermatum* (GenBank AF271227) was included in the analysis as an outgroup. The relative alignment for the *Phytophthora* sequences remained the same with a few modifications to improve alignment and with gaps introduced across all species of this genus to accommodate the alignment with sequences of *Pythium aphanidermatum.* DNA sequence data obtained in this study have been deposited in GenBank (TABLE I), and the results of this analysis have been deposited in TreeBASE (S774).

## RESULTS

*Sequence analysis.*—Gaps were not observed in the *cox* II or *cox* I sequence alignments, and sequences generally were conserved well within a species but were divergent among species. Sequence distance measurements within a species, using the Kimura 2-parameter correction method, ranged from 0 to 4.9%. Examination of multiple isolates of five species (*P. citricola*, *P. colocasiae*, *P. fragariae*, *P. hibernalis* and *P. mirabilis*) revealed no intraspecific variation in the gene sequence. However, intraspecific variation was observed in nine other species that were examined. In six of the species (*P. cactorum*, *P. drechsleri*, *P. erythroseptica*, *P. ilicis*, *P. infestans* and *P. nicotianae*), variation was low among isolates (<0.5%), but higher rates were observed among isolates of *P. palmivora* (0 to 1.1%), *P. megakarya* (3.4%) and *P. megasperma* (0 to 4.9%). Sequence divergence between *Phytophthora* spp. and *Pythium aphanidermatum* ranged from 9.6 to 16.5%. The estimated transition/transversion ratio for maximum-likelihood analysis of the *cox* II gene was determined to be 0.606, with nucleotide frequencies for A, C, G and T of 0.318, 0.110, 0.167 and 0.405, respectively. For *cox* I, this was determined to be 0.602, 0.280, 0.121, 0.174, and 0.424, respectively.

*Phylogenetic relationships based on* cox *II DNA sequences.*—The topology of *cox* II gene trees, constructed by a heuristic search based on maximum parsimony for a bootstrap 50% majority-rule consensus tree, revealed 18 species grouping into seven clades and nine species unaffiliated with specific clades (FIG. 2). Species grouping did not always reflect morphological groupings (TABLE II). For example, Clade 2 contained species in morphological groups II (*P. capsici*), III (*P. citricola*) and IV (*P. colocasiae*). Likewise, species within a specific morphological group did not always cluster together. Clade 1 contained species in morphological Group VI (*P. drechsleri*, *P. erythroseptica* and *P. cryptogea*), but members of this morphological group also were present on Clade 5 (*P. gonapodyides*) and not associated with a specific clade (*P. cinnamomi*). Species of morphological Group II were found on clades 2 and 7 and not associated with specific clades (*P. nicotianae*, *P. boehmeriae*, *P. megakarya* and *P. heveae*). Species of morphological Group IV were found on clades 2, 4 and not associated with specific clades (*P. hibernalis* and *P. ilicis*). Species of morphological Group V were found on clades 5, 6 and not associated with specific clades (*P. lateralis*). The tree was identical when this analysis was rerun with *cox* II sequence data of *Pythium aphanidermatum* added, with *P. aphanidermatum* clustering with *Lagenidium giganteum* (data not shown). Another genus included for outgroup comparison (*Peronophythora litchii*) clustered with *P. palmivora* and *P. arecae* on Clade 7, although the bootstrap support was not strong (56%).

With several exceptions, all isolates of a single species grouped together. In Clade 1, the isolate of *P. erythroseptica* from Ireland grouped with the *P. cryptogea* isolate rather than with the other *P. erythroseptica* isolates. The significance of this is unclear, because the morphological features of these isolates supported the species classification. More divergence was observed for *P. megasperma* than for the other species examined (Clade 5), with the isolates from Washington different from the Australia and California 1 isolates and the California 2 isolate was the most divergent. *Phytophthora gonapodyides* also grouped among isolates of *P. megasperma* in this clade and were more similar to other isolates of *P. megasperma* than the California 2 isolate. Likewise, *Phytophthora arecae* grouped among isolates of *Phytophthora palmivora* and was more similar to other isolates of *P. palmivora* than the isolate from Brazil.

With a few minor differences, the relationships among species in the MP tree (FIG. 2) were the same as observed with maximum-likelihood or neighbor-joining analysis (data not shown). The only differences observed were the grouping of *P. colocasiae*, relative to other species in Clade 2 (this species grouped with *P. capsici* in the MP [58% bootstrap]

TABLE I. Isolates of *Phytophthora* spp. used in this investigation and GenBank accession numbers for *cox* I and II sequences

| Species | | Group isolate number[a] | Host | Origin | GenBank |
|---|---|---|---|---|---|
| *Phytophthora arecae* | II | [b]IMI 348342 | *Cocos nucifera* | Indonesia | AY129176 |
| *Phytophthora boehmeriae* | II | 325[PT], P1257[MC] | *Boehmeria nivia* | Papua New Guinea | AY129177 |
| *Phytophthora cactorum* | I | 311[PT] | *Pseudotsuga menziesii* | Washington | AY129178 |
| | | 385[PT], NY568[WW] | *Malus sylvestris* | New York | AY129179, AY129174[d] |
| | | SB2079[GB] | *Fragaria* × *ananassa* | California | AY129180 |
| *Phytophthora capsici* | II | 302[PT] (A-1) | *Capsicum annuum* | Florida | AY129181, AY129166[d] |
| *Phytophthora cinnamomi* | VI | Cn-2[DJM] (A-2 mating type) | *Vaccinium* spp. | Florida | AY129182 |
| *Phytophthora citricola* | III | Cr-4[DJM] | *Cornus* spp. | Florida | AY129183 |
| | | SB2084 | *Fragaria* × *ananassa* | California | AY129184 |
| *Phytophthora colocasiae* | IV | 345[PT], ATCC 56193, P1696[MC] | *Colocasia esculenta* | China | AY129185, AY129173[d] |
| | | 346[PT], P3773[MC] | *Colocasia esculenta* | Indonesia | AY129186 |
| | | 347[PT], ATCC 52233, P1179[MC] | *Colocasia esculenta* | India | AY129187 |
| *Phytophthora cryptogea* | VI | [b]IMI 045168 | *Lycopersicon esculentum* | New Zealand | AY129188 |
| *Phytophthora drechsleri* | VI | 301[PT], 6503[DS] | *Capsicum* spp. | Mexico | AY129189 |
| | | [b]ATCC 46724 (Type) | *Beta vulgaris* | USA | AY129190 |
| *Phytophthora erythroseptica* | VI | [b]366[PT], ATCC 36302 | *Solanum tuberosum* | Ohio | AY129191 |
| | | 387[PT], NY513[WW] | *Solanum tuberosum* | California | AY129192 |
| | | 388[PT], NY559[WW], IMI34684 | *Solanum tuberosum* | Ireland | AY129193 |
| *Phytophthora fragariae fragariae* | V | 394[PT], ATCC 13973 | *Fragaria* × *ananassa* | Maryland | AY129194 |
| | | 398[PT] | *Fragaria* × *ananassa* | Oregon | AY129195 |
| *Phytophthora fragariae rubi* | V | 397[PT] | *Rubus* spp. | Australia | AY129196 |
| *Phytophthora gonapodyides* | IV | 393[PT], NY353[WW] | *Malus sylvestris* | New York | AY129197, AY129175[d] |
| *Phytophthora heveae* | II | Hv-2[DJM] | *Theobroma cacao* | Brazil | AY129198 |
| *Phytophthora hibernalis* | IV | 338[PT], ATCC 56353, P3822[MC] | *Citrus sinensus* | Australia | AY129199, AY129170[d] |
| | | 379[PT], ATCC 64708, CBS 522.77 | *Aquilegia vulgaris* | New Zealand | AY129201 |
| | | 380[PT], ATCC 60352, CBS 270.31 | *Citrus sinensus* | Portugal | AY129200 |
| *Phytophthora ilicis* | IV | 343[PT], P6099[MC], 771[PH] | *Ilex aquifolium* | Oregon | AY129202 |
| | | 344[PT], ATCC 56615, P3939[MC] | *Ilex aquifolium* | Canada | AY129203, AY129172[d] |
| *Phytophthora infestans* | IV | 176[PT], 915[KD] (A-2) | *Solanum tuberosum* | Pennsylvania | AY129204 |
| | | 180[PT], WW-IX[KD] (A-1) | *Solanum tuberosum* | Washington | AY129205 |
| | | 580[PT] | *Solanum demissum* | Mexico | AY129206 |
| | | [c]West Virginia 4 | | West Virginia | NC002387 |
| *Phytophthora lateralis* | V | [b]IMI 040503 (Type) | *Chamaecyparis* | USA | AT129207 |
| *Phytophthora megakarya* | II | 327[PT], P132[CB] | *Theobroma cacao* | Nigeria | AY129208 |
| | | 328[PT], P184[CB] | *Theobroma cacao* | Cameroon | AY129209 |
| *Phytophthora megasperma* | V | 309[PT], 336[PH] | *Pseudotsuga menziesii* | Washington | AY129210 |
| | | 335[PT], 63[PH], 261S-1[WW] | *Prunus* spp. | California 2 | AY129212 |
| | | [b]IMI 133317 | *Malus* sylvestris | Australia | AY129211 |
| | | [c]695T | | California 1 | L04457 |
| *Phytophthora mirabilis* | IV | 340[PT], ATCC 64070, P3007[MC] | *Mirabilis jalapa* | Mexico 1 | AY129213, AY129171[d] |
| | | 342[PT], ATCC 64073, P3010[MC] | *Mirabilis jalapa* | Mexico 2 | AY129214 |

TABLE I. Continued

| Species | | Group isolate number[a] | Host | Origin | GenBank |
|---|---|---|---|---|---|
| *Phytophthora nicotianae* | II | Pn-17$^{DJM}$ (A-1) | *Citrus* spp. | Florida | AY129215 |
| | | 332$^{PT}$ | *Nicotiana tabacum* | Australia | AY129216, AY129169[d] |
| *Phytophthora palmivora* | II | 329$^{PT}$, P131$^{CB}$ | *Theobroma cacao* | Nigeria | AY129217 |
| | | Pl-5$^{DJM}$, P626$^{UCR}$ | *Theobroma cacao* | Brazil | AY129218 |
| | | Pl-10$^{DJM}$ | *Theobroma cacao* | Costa Rica | AY129219 |
| | | Pl-14$^{DJM}$ | *Citrus* spp. | Florida | AY129220 |
| *Phytophthora phaseoli* | IV | 330$^{PT}$ | *Phaseolus lunatus* | Maryland | AY129221, AY129168[d] |
| *Phytophthora pseudotsugae* | I | 308$^{PT}$, H270$^{PH}$ | *Pseudotsuga menziesii* | Oregon | AY129222, AY129167[d] |
| *Phytophthora sojae* | V | 312$^{PT}$, ATCC 48068 | *Glycine max* | Wisconsin | AY129223 |
| *Phytophthora syringae* | III | [b]IMI 296829 | *Rubus idaeus* | Scotland | AY129224 |

[a] CB = Clive Brasier, MC = Mike Coffey, KD = Ken Deahl, PH = Phil Hamm (E. Hanson), DJM = Dave Mitchell, DS = Dave Shaw, PT = Paul Tooley, UCR = University of California at Riverside, WW = Wayne Wilcox.

[b] Isolates included in the rDNA ITS analysis of Cooke et al (2000).

[c] Sequences obtained from GenBank.

[d] *cox* I sequences.

and NJ tree, while in the ML tree it was on the same clade, but independent), and *P. mirabilis*, relative to other species on Clade 4 (this species grouped with *P. phaseoli* in the MP [58% bootstrap] and NJ tree, while in the ML tree it was on the same clade, but independent). The grouping of *P. megakarya* also was different for the NJ tree; it clustered as a separate branch with the isolates of Clade 7 (but with only 52% bootstrap support).

The topology of the shortest tree generated by a heuristic search based on maximum parsimony was similar to the majority-rule consensus tree, with the exception that more ancestral relationships among species were observed (FIG. 3). While many of the relationships among species observed on individual clades in the majority-rule consensus tree (FIG. 2) were maintained in the shortest tree (FIG. 3), rather than the clades unaffiliated with other species observed in FIG. 2, many of these clades were grouped together in FIG. 3. For example, the species groupings observed on clades 2, 5 and 6 in FIG. 2 were conserved in FIG. 3, but all three groupings were on the same clade on the shortest tree. Similar results were observed for clades 3 and 4 of FIG. 2 as well as *P. megakarya* and Clade 7. One difference, compared with the majority rule consensus tree, was that on the shortest tree (FIG. 3) Clade 1 was intermediate between *L. giganteum* and *P. aphanidermatum* and other species included in the analysis. In addition to the tree in FIG. 3, three other trees with a length of 599 were obtained and differed from FIG. 3 in that, in one tree, *P. syringae* grouped with species on Clade 1 (basal to the other species); *P. nicotianae* was intermediate between *L. giganteum* and *P. aphanidermatum* (one tree); the clade containing *P. hibernalis* and *P. lateralis* grouped with *P. ilicis* (two trees); and the positions of *P. sojae* and *P. cinnamomi* were reversed (one tree).

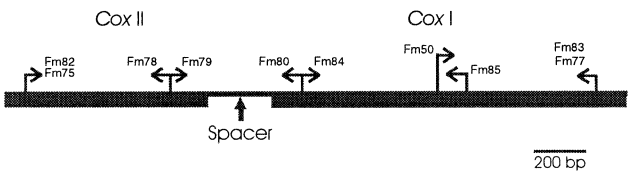


FIG. 1. Organization of the mitochondrially encoded cox I and II gene cluster in *Phytophthora* and location of the PCR primers that were used for template amplification and sequencing reactions.

*Phylogenetic relationships based on* cox *I DNA sequences.*—The topology of *cox* I gene trees constructed by a heuristic search, based on maximum parsimony for a bootstrap 50% majority-rule consensus tree for 13 species of *Phytophthora*, revealed 10 species grouping into 3 clades and 3 species unaffiliated with a specific clade (FIG. 4). The groupings in general agreed with what was observed in the *cox* II tree, with the exception that Clade 1 of the *cox* I tree included species from the *cox* II tree that grouped on clades 3 (*P. infestans*, *P. mirabilis* and *P. phaseoli*), 4 (*P. pseudotsugae* and *P. cactorum*) and *P. nicotianae*. *Phytophthora mirabilis* also clustered with *P. infestans* in the *cox* I tree, while it clustered with *P. phaseoli* in the *cox* II tree (although with only 58% bootstrap support). Maximum-likelihood analysis of *cox* I data generated

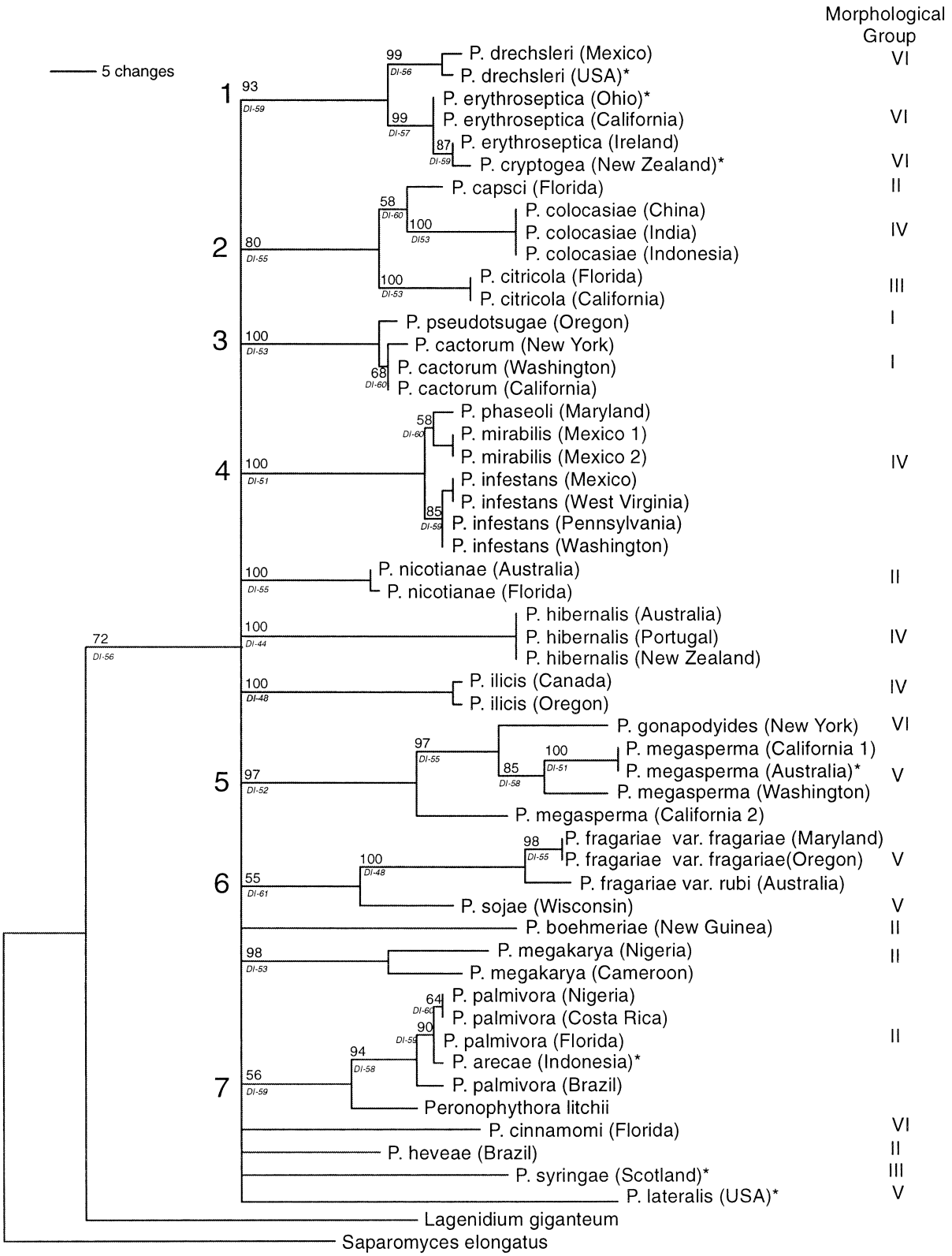


FIG. 2. Phylogenetic relationships among *Phytophthora* species using *cox* II DNA sequence data, based on maximum parsimony inferred by a heuristic tree search. Fifty percent majority-rule consensus tree; the numbers above the nodes are the percentage of the trees from bootstrap analysis (1000 replications) that support the observed topography (values above 60% indicated). Values below the nodes and preceded by DI- are decay indices calculated by AutoDecay (v 4.0). Of the 568 total characters, 378 were constant, 64 were variable and parsimony uninformative, and 126 were parsimony informative. Tree length = 623, consistency index (CI) = 0.438, homoplasy index (HI) = 0.562, retention index = 0.682, rescaled consistency index = 0.299. Isolates marked with an (*) were the same cultures that were used by Cooke at al (2000) to infer rDNA ITS-based phylogenetic trees.

TABLE II. Selected characters of 26 *Phytophthora* species in relation to phylogenetic groupings based on the *cox* II gene

| Clade (FIG. 2) | Species | Group[a] | Papillate[b] | Caducous[c] | Sporangial proliferation | Chlamydospores | Antheridial attachment[d] | Homo- or Heterothallic[e] | Temp. optimum[f] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *P. drechsleri* | VI | N | N | + | + | A | He | H |
| 1 | *P. erythroseptica* | VI | N | N | + | − | A | Ho | M |
| 1 | *P. cryptogea* | VI | N | N | + | − | A | He | M |
| 2 | *P. capsici* | II | P | C | − | +/− | A | He | H |
| 2 | *P. colocasiae* | IV | S | C | − | + | A | He | H |
| 2 | *P. citricola* | III | S | N | − | − | P | Ho | M |
| 3 | *P. pseudotsugae* | I | P | N | − | − | P | Ho | M |
| 3 | *P. cactorum* | I | P | C | − | +/− | P | Ho | M |
| 4 | *P. phaseoli* | IV | S | C | − | − | A | Ho | L |
| 4 | *P. mirabilis* | IV | S | C | − | − | A | He | L |
| 4 | *P. infestans* | IV | S | C | − | − | A | He | L |
| 5 | *P. gonapodyides* | VI | N | N | + | − | A | He | H |
| 5 | *P. megasperma* | V | N | N | + | − | PA | Ho | LM |
| 6 | *P. fragariae* | V | N | N | + | − | PA | Ho | L |
| 6 | *P. fragariae rubi* | V | N | N | + | − | PA | Ho | L |
| 6 | *P. sojae* | V | N | N | + | +/− | PA | Ho | M |
| 7 | *P. palmivora* | II | P | C | − | + | A | He | H |
| 7 | *P. arecae* | II | P | C | − | +/− | A | He | H |
| | *P. nicotianae* | II | P | C | − | + | A | He | H |
| | *P. hibernalis* | IV | S | C | − | − | PA | Ho | L |
| | *P. ilicis* | IV | S | C | − | − | A | He | L |
| | *P. boehmeriae* | II | P | C | − | + | A | Ho | M |
| | *P. megakarya* | II | P | C | − | + | A | He | M |
| | *P. cinnamomi* | VI | N | N | + | + | A | He | M |
| | *P. hevea* | II | P | C | − | − | A | Ho | M |
| | *P. syringae* | III | S | N | − | − | PA | Ho | L |
| | *P. lateralis* | V | N | N | + | + | P | Ho | L |

[a] Waterhouse morphological grouping.
[b] S = semipapillate, P = papillate, N = nonpapillate sporangia.
[c] C = caducous, N = noncaducous sporangium.
[d] A = amphigynous, P = paragynous, PA = both amphigynous and paragynous.
[e] He = heterothallic, Ho = homothallic.
[f] L = low temperature optimum (<22 C), M = moderate temperature optimum (22–27 C), H = high temperature optimum (>28 C).

a tree that was identical to the maximum-parsimony tree, with the exception that *P. fragariae* var. *fragariae* was on a clade intermediate between *Pythium aphanidermatum* and the rest of the *Phytophthora* species examined (62% bootstrap support, data not shown). *Phytophthora ilicis* also clustered on Clade 2, although there was low bootstrap support (59%).

Analysis on combined *cox* II and *cox* I data sets generated trees that essentially were identical to *cox* I trees. Maximum-likelihood and neighbor-joining analysis trees were the same as the maximum-parsimony *cox* I tree presented in FIG. 4 (data not shown). Maximum-parsimony analysis of the combined data generated a tree that was identical to these trees, with the exception that *P. fragariae* var. *fragariae* was on a clade intermediate between *Pythium aphanidermatum* and the rest of the *Phytophthora* species examined (59% bootstrap support, data not shown).

*Comparison of phylogenetic relationships inferred from rDNA ITS sequences.*—ITS sequence data, for species included in this study and used by Cooke et al (2000), was retrieved from TreeBase and the analysis rerun with *Pythium aphanidermatum* as an outgroup. Tree reconstruction using maximum parsimony (MP) revealed four major clades that reflected the grouping of the neighbor-joining (NJ) tree reported by Cooke et al (2000), with the exception that some of the basal nodes of the NJ tree reflecting ancestral relationships were not observed in the MP tree (FIG. 5). For example, species on clades 2 and 3 of the MP tree were on the same clade along with *P. ilicis* in the

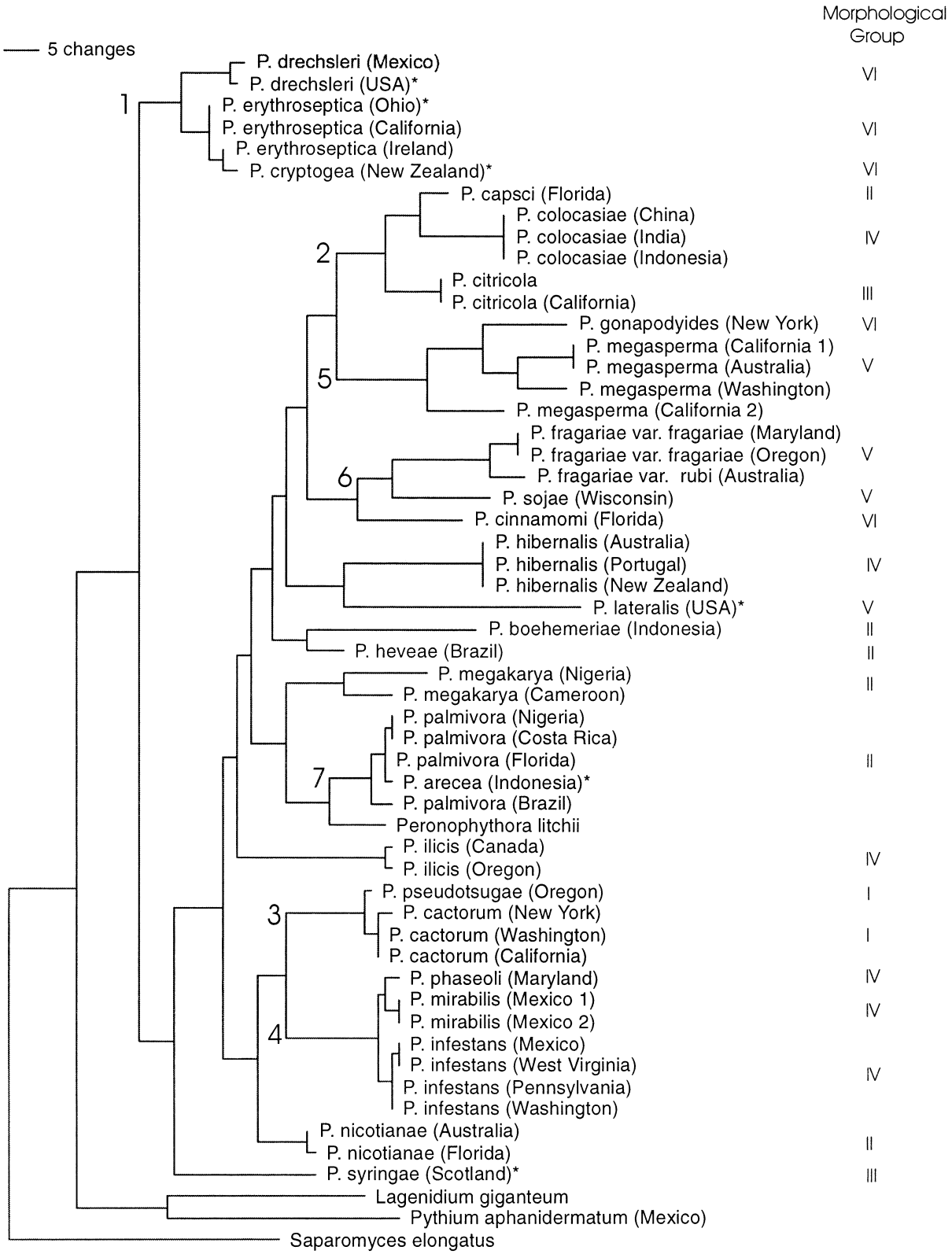


FIG. 3. Phylogenetic relationships among *Phytophthora* species using *cox* II DNA sequence data, based on maximum parsimony inferred by a heuristic tree search. This is one of four trees with the shortest length with the numbers at the nodes corresponding to the node labels in FIG. 2. Tree length = 599, consistency index (CI) = 0.467, homoplasy index (HI) = 0.533, retention index = 0.722. Isolates marked with an (*) were the same cultures that were used by Cooke at al (2000) to infer rDNA ITS-based phylogenetic trees.

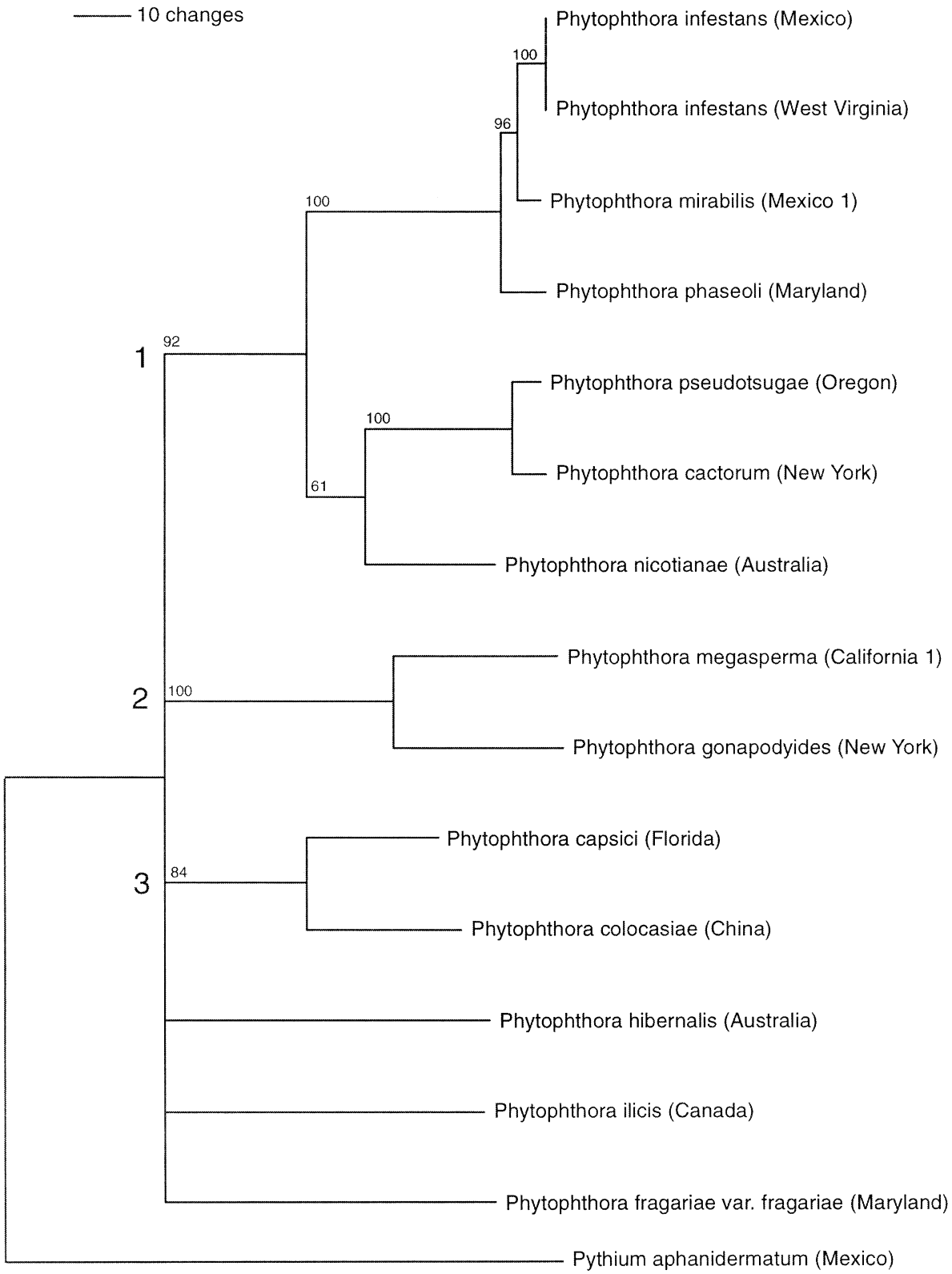


FIG. 4. Phylogenetic relationships among *Phytophthora* species using *cox* I DNA sequence data, based on maximum parsimony inferred by a heuristic tree search. The numbers above the nodes are the percentage of the trees from bootstrap analysis (100 replications) that support the observed topography (values above 60% indicated). Of the 1299 total characters, 1006 were constant, 115 were variable and parsimony uninformative and 178 were parsimony informative. Tree length = 607, consistency index (CI) = 0.634, homoplasy index (HI) = 0.366, retention index = 0.568.

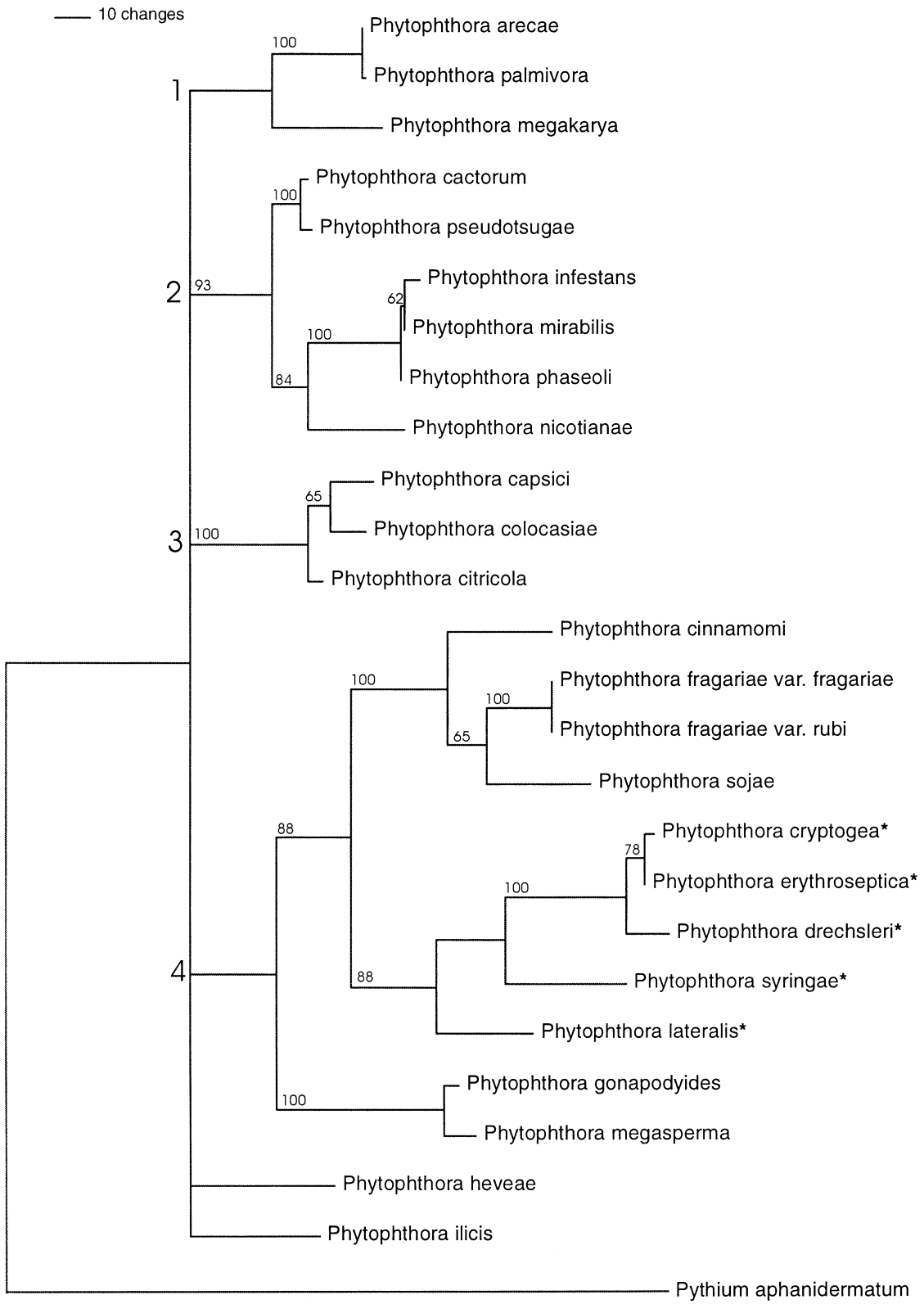


FIG. 5. Phylogenetic relationships among *Phytophthora* species using ITS rDNA sequence alignments of Cooke et al (2000), based on maximum parsimony inferred by a heuristic tree search. The numbers above the nodes are the percentage of the trees from bootstrap analysis (1000 replications) that support the observed topography (values above 60% indicated). Of the 900 total characters, 465 were constant, 204 were variable and parsimony uninformative and 231 were parsimony informative. Tree length = 939, consistency index (CI) = 0.646, homoplasy index (HI) = 0.354, retention index = 0.675. Isolates marked with an (*) were the same cultures that were used in this submission to infer cox II-based phylogenetic trees.

NJ tree of Cooke et al (2000). Likewise, *P. gonapodyides* and *P. megasperma* were grouped with *P. heveae* on the NJ tree of Cooke et al (2000) but were grouped on Clade 4 of the MP tree (FIG. 5), although the bootstrap support was not strong (57%). The relationship between *P. cinnamomi* and *P. sojae* also was different; in the MP tree (FIG. 5) *P. cinnamomi* was ancestral to *P. sojae*, while in the NJ tree of Cooke et al (2000) *P. sojae* was ancestral.

Data sets for *cox* II and the ITS region were combined by concatenating sequences for isolates and species to one another. In cases in which the isolates were not the same, the assumption was made that all isolates were correctly identified and represented the same species for each data set. If multiple isolates of a single species were represented in the *cox* II data set, the ITS sequence for that species was added to the *cox* II data for each isolate. The MP tree that was obtained was the same as was observed for the ITS data set alone, with the exception that *P. gonapodyides* and *P. megasperma* were on Clade 4 of the ITS tree but were on a separate clade (Clade 5) for the combined data set (FIG. 6). A partition-homogeneity test, with the data for *cox* II and the ITS region partitioned, was run in PAUP with 1000 replicates. The results of this test ($P = 0.002$) identified significant heterogeneity among the data sets, suggesting that analysis of combined data sets might not accurately reflect true phylogenetic relationships.

## DISCUSSION

Comparison of 568 bp of the mitochondrially encoded *cox* II gene provided good resolution of the species that were examined in this study. Sequence divergence was low for intraspecific comparisons (4.9%; with the exception of *P. megasperma* it was below 3.4%) but was high enough for interspecific comparisons to provide separation of species (up to 12.2%). For the most part, the consensus tree from 1299 bp of the *cox* I gene (FIG. 4) exhibited the same relationships among species as observed with the *cox* II gene tree (FIG. 2). The one exception was that clades 3 and 4 of the consensus *cox* II tree, along with *P. nicotianae*, were on the same clade in the *cox* I tree (Clade 1). However, this was the same grouping that was observed for the most-parsimonious *cox* II tree (FIG. 3). The transition/transversion ratio (0.606) for *cox* II was similar to what was reported by Sachay et al (1993). There was good congruence between DNA sequence trees inferred by maximum-likelihood, maximum-parsimony and NJ tree reconstruction with only minimal differences in relationships among species observed.

In terms of intraspecific conservation of sequences, no variation was observed for five of 14 species in which multiple isolates were examined, with less than 0.5% sequence divergence for an additional six species. In the case of *P. cactorum*, isolates from strawberry and fir were the same, while the isolate from apple was slightly different. There are reports of some level of host specialization and grouping of isolates into different nuclear DNA RFLP groups in this species (Hantula et al 2000), although a greater number of isolates from different geographic regions must be examined to determine whether this is important phylogenetically. It is interesting to note that there was no correlation for geographic origin or host in clustering of the four *P. palmivora* isolates examined in this study; an isolate from citrus in Florida clustered among isolates from *Theobroma cacao* in Nigeria, Costa Rica and Brazil.

The grouping of the isolate of *P. arecae* among isolates of *P. palmivora* in all *cox* II gene trees indicates that these two species might be conspecific. This observation is consistent with the conclusions of Oudemans and Coffey (1991) and Mchau and Coffey (1994) based on isozyme analysis, as well as Förster et al (1990b) based on mtDNA RFLP analysis. An affiliation between these two species also was observed in the ITS tree of Cooke et al (2000; their isolate of *P. arecae* also was used in this study), but only single isolates of each species were included in the analysis, so conclusions about conspecificity could not be drawn. Morphologically these two species share many features; the differences that are observed might reflect the range of variation inherent in the species.

There are several species for which additional investigations are needed to clarify species concepts. Four isolates of *P. megasperma* were assayed and, while they clustered together on Clade 5 (FIG. 2), they exhibited the greatest intraspecific variation observed in this study (sequence divergence as high as 4.9%). Although this sample size is not large enough to draw conclusions, no correlation between host and geographic origin of isolates was observed. Based on mtDNA RFLP analysis, the *P. megasperma* group is highly divergent and can be divided into six groups (Förster and Coffey 1993; nine groups were reported, but three subsequently have been renamed as distinct species (Erwin and Ribeiro 1996)). Additional studies are needed to determine if the groupings on the *cox* II tree reflect different species and how this relates to the groupings observed by Förster and Coffey (1993). Our analysis, as well as that of Cooke et al (2000), showed that *P. gonapodyides* and *P. megasperma* grouped together. Whether these two species are conspecific or if the relationship among these species on Clade 5 reflects the variation among isolates clas-

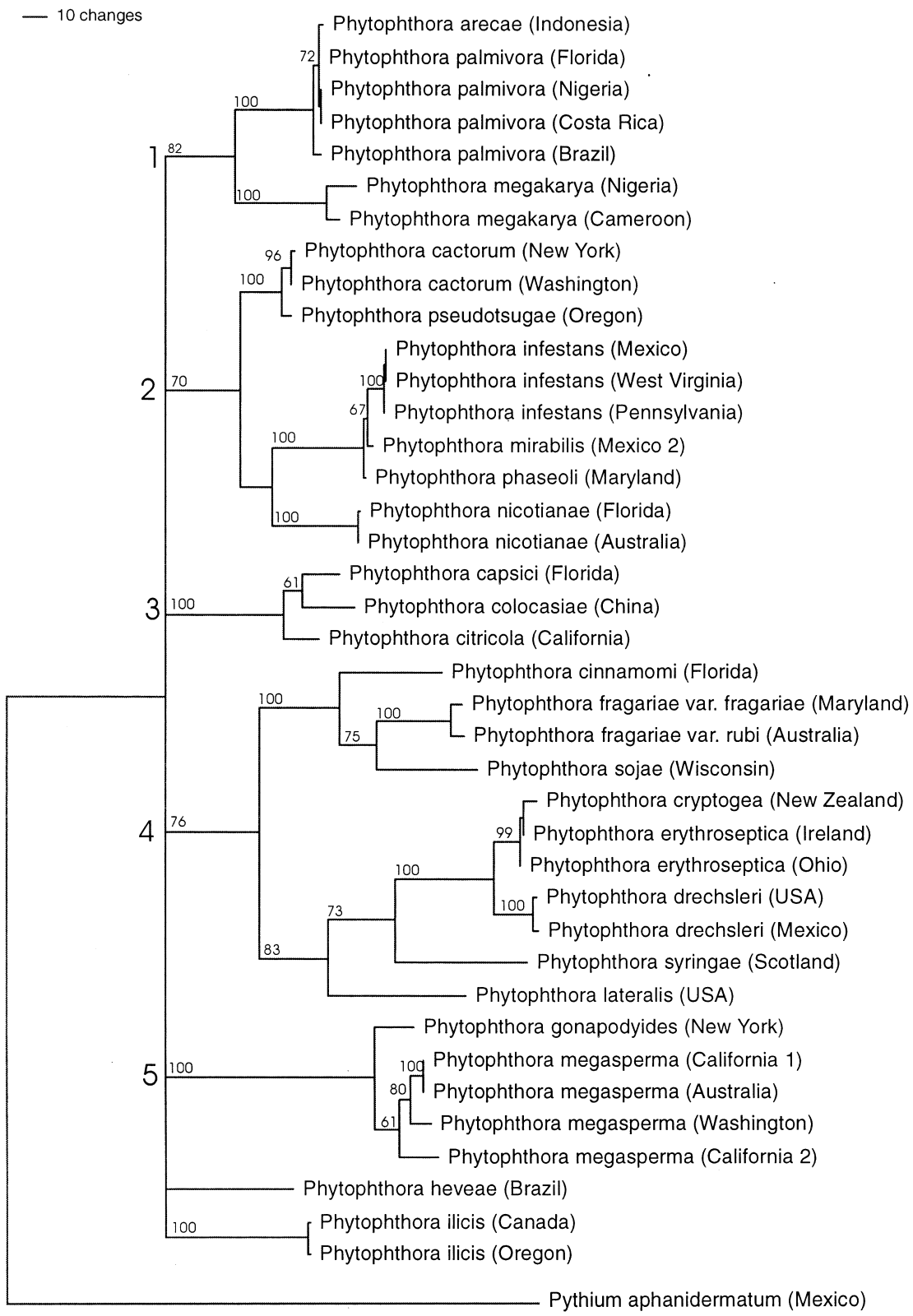


FIG. 6. Phylogenetic relationships among *Phytophthora* species using ITS rDNA sequence alignments of Cooke et al (2000) combined with *cox* II sequence data, based on maximum parsimony inferred by a heuristic tree search. The numbers above the nodes are the percentage of the trees from bootstrap analysis (1000 replications) that support the observed topography (values above 60% indicated). Of the 1468 total characters, 871 were constant, 223 were variable and parsimony uninformative and 374 were parsimony informative. Tree length = 1438, consistency index (CI) = 0.585, homoplasy index (HI) = 0.415, retention index = 0.765.

sified as *P. megasperma* remains to be determined. Morphologically, *P. gonapodyides* and *P. megasperma* share many features, however, they also exhibit distinct differences (*P. gonapodyides* is heterothallic with amphigynous antheridia while *P. megasperma* is homothallic with paragynous antheridia). Based on morphology and protein-banding patterns, *P. gonapodyides* has been linked to *P. drechsleri* and *P. cryptogea* (Brasier 1991, Hansen et al 1988), but it doesn't group with these species in the ITS-based tree (Cooke et al 2000) or in our analysis of *cox* II. While on the same clade, the isolates of *P. megakarya* from Nigeria and Cameroon exhibit a greater sequence divergence than in most other species examined (the only exception being *P. megasperma*). From mtDNA RFLP analysis Förster et al (1990b) also obtained variability among isolates from these two regions, with less than 60% similarity in banding pattern observed.

The grouping observed for the consensus *cox* II gene sequence tree is in general agreement with the conclusions of Cooke et al (2000) from their analysis of rDNA ITS, however, there were some notable exceptions. The isolates of *P. syringae*, *P. lateralis*, *P. drechsleri* (USA), *P. erythroseptica* (Ohio) and *P. cryptogea* (New Zealand) in this study were the same that were used for the ITS study of Cooke et al (2000). All five were on the same branch of Clade 4 in the ITS tree (FIG. 5) but were on different clades in the consensus *cox* II tree (FIG. 2); *P. syringae* and *P. lateralis* were unaffiliated with any specific clade and did not group with *P. drechsleri*, *P. erythroseptica* or *P. cryptogea*. *Phytophthora cinnamomi* grouped with *P. fragariae* on the ITS tree but was unaffiliated with a specific clade in the consensus *cox* II tree. Similar differences were observed for Clade 2 of the ITS tree (represented by clades 3 and 4 in the consensus *cox* II tree) and Clade 1 of the ITS tree (represented by Clade 7 of the consensus *cox* II tree, with *P. megakarya* clustering separately).

Comparison of the most-parsimonious *cox* II tree with the shortest length (FIG. 3) and the ITS tree (FIG. 5) revealed overall similarities in species groupings, but differences were observed. The species groupings observed on clades 2 and 3 of the ITS tree were the same for the *cox* II tree, but relationships among the clades exhibited some differences. For example, clades 2 and 5 on the *cox* II tree grouped together but were separated in the ITS tree. Furthermore, *P. drechsleri*, *P. erythroseptica*, and *P. cryptogea* were ancestral to other *Phytophthora* species in the shortest branch length *cox* II tree (which was not observed in the ITS tree) and these species did not group with *P. syringae* or *P. lateralis* (which was observed in the ITS tree). Thus, these differences in species groupings in the *cox* II and ITS sequence trees reflect differences in phylogenetic resolution of the two regions, either through differing rates of evolutionary divergence or incorrect assumptions about alignment of the ITS sequences.

Concatenation of the *cox* II sequences and the ITS data of Cooke et al (2000) and running phylogenetic analysis generated a tree that did not differ significantly from the tree derived from ITS data alone. However, because a partition-homogeneity test with 1000 replicates indicated there was significant heterogeneity between the two data sets ($P = 0.002$), analysis of combined data sets might not be valid. Possible reasons for conservation of the ITS tree topography after analysis of concatenated sequences might be the greater number of phylogenetically informative sites present in the ITS data set (231 vs 126 for the *cox* II data) and the greater level of sequence divergence observed for the ITS data (17.4% vs 12.2% for the *cox* II data).

The phylogenetic relationships among species on these *cox* II gene trees did not exhibit consistent similarities in groupings for morphology, sexual features or temperature optima (TABLE II). However, some associations were observed among species groupings on the *cox* II gene tree and Waterhouse groupings (FIG. 2). For example, all species in clades 1, 3, 4, 6, and 7 of FIG. 2 were members of a single Waterhouse group with similar sporangial papillation characteristics (groups VI, I, IV, V and II, respectively), but other members of Waterhouse groups II–VI were on separate branches and not included in the aforementioned clades. Förster et al (2000) used the ITS I region for phylogenetic analysis, which did not include the 5.8S and ITS 2 region that Cooke et al (2000) included. They found that species of groups V and VI, which are differentiated by the presence of amphigynous or paragynous antheridia, respectively, were interspersed in the ITS1 tree. Species with papillate and semipapillate sporangia (groups I–IV) clustered together, and this cluster was distinct from those of species with nonpapillate sporangia. There was no congruence among the mode of antheridial attachment, sporangial caduacity or homo- or heterothallic habit and the ITS1 grouping. Using a smaller number of species in the analysis, Crawford et al (1996) and Cooke and Duncan (1997) found that phylogenetic groupings based on ITS sequences generally agreed with morphological groupings, especially those based on sporangial morphology. In contrast, using a wider range of species, Cooke et al (2000) found that their ITS data did not support morphological subgroups. Briard et al (1995), who analyzed part of the D2 domain of the large subunit (28S) ribosomal rRNA, came to the same conclusion with their analysis of 15 *Phytophthora* species. In light

of these results and our findings with *cox* gene phylogenies, it seems clear that at best only partial correlations between molecular and morphological characters might exist, and that morphology alone should not be used to infer phylogeny in *Phytophthora* sensu lato.

Cytochrome oxidase II gene sequences have been used for inferring phylogenetic relationships among members of the Peronosporomycetes (Hudspeth et al 2000) and the genus *Pythium* (Martin 2000). One advantage to using this gene in phylogenetic analysis at the genus level is that length mutations have not been observed, which simplifies sequence alignment. In contrast, alignment of ITS sequences can be more complicated due to the presence of length mutations in different regions of the ITS. For example, with the *Phytophthora* species examined in FIG. 5, the length of the ITS region ranged from 752 (*P. capsici*) to 832 bp (*P. fragariae* var. *rubi*). Following introduction of gaps for alignment purposes, there were 900 bp total, of which 465 characters were constant, 204 were variable and parsimony uninformative, and 231 were parsimony informative. One complicating factor in constructing alignments of sequences with this level of length variation is identifying alignments that accurately reflect phylogenetic divergence among species (e.g., determining correct boundaries of gaps and proper alignment of variable regions). Variation in alignment has been shown to affect subsequent phylogenetic clustering much more significantly than the particular algorithm (parsimony, distance, etc.) used in tree construction (Morrison and Ellis 1997). Another possible complication for using the ITS region for phylogenetic purposes is that multiple, nonorthologous ITS sequences have been found in some fungi (Fatehi and Bridge 1998, O'Donnell et al 1998), along with the occurrence of multiple and divergent ITS sequences within single spores of certain species (Pringle et al 2000), including the closely related genus *Pythium* (Martin 1990). Furthermore, ITS regions might not fully distinguish groups of species that are known to be biologically distinct. For example, *P. infestans*, *P. mirabilis*, and *P. phaseoli* (Cooke et al 2000, Goodwin et al 1999) and *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi* (Cooke et al 2000) are poorly resolved in ITS phylogenies (FIG. 5) yet more clearly differentiated in *cox* II phylogenies (FIG. 2). However, ITS has the advantage of being a nuclear region and sequence data will reflect genetic and evolutionary forces such as intra- and interspecific hybridization not reflected in patterns of evolution of cytoplasmic genes such as *cox* II. Given that interspecific hybridization has been reported in the genus *Phytophthora* (Brasier et al 1999, English et al 1999, Bonants et al 2000, Gu and Ko 2000, Delcan and Brasier 2001) this is an important consideration and a potential drawback in relying strictly on mitochondrial sequences (which are believed to be uniparentally inherited; Förster and Coffey 1990).

Clearly, additional analysis of *cox* II gene sequences with a greater number of species having a broader representation of the morphological variation present in the genus are needed to clarify the phylogeny of the genus. Likewise, inclusion of additional species that are more restricted in their host range, are nonpathogenic, or are representative of differing ecological habitats, is needed. Given the lack of congruence for some species between *cox* II and ITS data, comparison of these cox II gene trees with DNA sequence analysis from other regions of the nuclear genome using the same isolates for both analyses would be important as well for correct determinations of phylogenetic relationships in the genus.

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