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A Molecular Phylogeny of *Phytophthora* and Related Oomycetes

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Cooke, D. E. L., Drenth, A., Duncan, J. M., Wagels, G., and Brasier, C. M. 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genetics and Biology* 30, 17–32. Phylogenetic relationships among 50 *Phytophthora* species and between *Phytophthora* and other oomycetes were examined on the basis of the ITS sequences of genomic rDNA. *Phytophthora* grouped with *Pythium, Peronospora,* and *Halophytophthora,* distant from genera in the Saprolegniales. *Albugo* was intermediate between these two groups. Unlike *Pythium, Phytophthora* was essentially monophyletic, all but three species forming a cluster of eight clades. Two clades contained only species with nonpapillate sporangia. The other six clades included either papillate and semipapillate, or semipapillate and nonpapillate types, transcending traditional morphological groupings, which are evidently not natural assemblages. *Peronospora* was related to *P. megakarya* and *P. palmivora* and appears to be derived from a *Phytophthora* that has both lost the ability to produce zoospores and become an obligate biotroph. Three other Phytophthoras located some distance from the main *Phytophthora–Peronospora* cluster probably represent one or more additional genera.

Index Descriptors: evolution; rDNA; internal transcribed spacers; ITS; *Pythium; Peronospora.*

Phytophthora is a major genus of plant pathogens within the diploid, alga-like oomycete fungi. Currently, the genus is assigned to the Order Pythiales and Phylum Oomycota within the group of heterokont, biflagellate organisms that comprise the Kingdom Chromista (Cavalier-Smith, 1986; Hawksworth *et al.*, 1995). The Oomycota, therefore, although behaviorally similar are biologically distinct from the other main fungal groups which comprise the Kingdom Fungi (Corliss, 1994; Cavalier-Smith, 1998).

The natural relationship of *Phytophthora* to the other oomycetes has yet to be demonstrated. However, the proposal of Gaümann (1952) that they occupy an intermediate position between the more "primitive" saprotrophic, aquatic, zoosporangium-producing genera such as *Saprolegnia, Achlya,* and *Dictyuchus* and the more "advanced" obligate conidial plant pathogens, such as *Albugo, Peronospora,* and *Bremia,* has been widely accepted. This perceived intermediate evolutionary position has led to uncertainty over their taxonomic classification. Waterhouse (1973), acknowledging the uncertainty, accepted the consensus that the Peronosporales comprised the Albuginaceae (white blister rusts such as *Albugo*), Peronosporaceae (true downy mildews such as *Peronospora*), and Pythiaceae (including *Pythium* and *Phytophthora*). More recently, Dick *et al.* (1984) have proposed that the Pythiaceae be elevated to the Order Pythiales, separate from the Peronosporales. The proposed order consists of one fam-

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ily, the Pythiaceae, with 10 genera including *Pythium*, *Phytophthora*, and *Halophytophthora* (Dick, 1990). On grounds of sexual mechanism and conidial dispersal, however, Brasier and Hansen (1992) suggested that *Phytophthora* might be more closely related to *Peronospora* and *Bremia*, while *Pythium* species have more in common with *Achlya*. *Pythium* (a genus of c. 120 species; Martin, 1995) and *Phytophthora* have long been thought to share a common ancestor. Because of its more primitive sporangial development including external rather than internal differentiation of zoospores and its less specialized parasitism, *Pythium* is generally viewed as ancestral to *Phytophthora* (Tucker, 1931; Gaümann, 1952).

There are 60 described *Phytophthora* species (Erwin and Ribeiro, 1996). Most are primary invaders of healthy plant tissue with limited saprotrophic ability. Many are responsible for serious diseases of economically important crops (Gregory, 1983), and some cause extensive damage to natural plant communities (e.g., Zentmyer, 1983; Brasier, 1992a; Wills, 1993). The economically important species are usually well described and relatively well represented in culture collections, but for others there often exist only a few cultures or the original herbarium material. The morphological properties and pathology of many of the described species are summarized in Erwin and Ribeiro (1996). In the traditional taxonomy, Phytophthoras were discriminated mainly on the structure of the sporangium (nonpapillate, semipapillate, or papillate), the form of the antheridium (amphigynous or paragynous), and on whether the taxon is inbreeding (homothallic) or outbreeding with A1 and A2 sexual incompatibility or mating types (heterothallic) (Tucker, 1931; Waterhouse, 1963). Heterothallic taxa are exclusively amphigynous while homothallic taxa may be either amphigynous or paragynous or, in some cases, have antheridia of both types. Waterhouse (1963) assigned *Phytophthora* taxa to six morphological groups which have provided the framework for a number of traditional identification keys (e.g., Stamps *et al.*, 1990).

Whether Waterhouse's morphological groups represent natural relationships in the genus has remained unclear. In light of Gaümann's hypothesis (Gaümann, 1952), Brasier (1983) suggested a range of primitive or advanced developmental characteristics, breeding systems, and nutritional strategies for Phytophthoras and proposed two main phylogenetic lines. One was toward a lifestyle as soil-inhabiting, root-infecting parasites with noncaducous sporangia, represented at its "peak" by the heterothallic *P. cinnamomi*, the other toward a lifestyle of aerial dispersal and infection involving caducous papillate sporangia, represented at the "peak" by the heterothallic *P. palmivora*. Since the "A1–A2" system of heterothallism occurred in both suggested lineages, it was proposed that either this system of incompatibility and the associated system of amphigyny had evolved more than once in the genus or the system of heterothallism was ancestral to the two lineages. Neither Gaümann's (1952) nor Brasier's (1983) proposals have been rigorously tested. Other phylogenetic issues include the relationship to *Phytophthora* of several marine *Phytophthora*-like species currently assigned to a sister genus, *Halophytophthora* (Ho and Jong, 1990), and of *Pythium undulatum*, transferred from *Pythium* to *Phytophthora* by Dick (1989) on account of a *Phytophthora*-like DNA polymorphism and other "*Phytophthora*-like" characters.

Since 1980, population–genetic studies based increasingly on molecular methods have improved our ability to both identify fungal species units and determine their natural relationships (Brasier, 1997). Despite this, species units in *Phytophthora* have remained poorly defined, because of its somewhat intractable breeding system and the plasticity of its morphological and cultural characters (Brasier, 1992b). In both *Phytophthora* and *Pythium*, however, phylogenetic analysis of protein patterns, isozymes, and RFLPs of mitochondrial and nuclear DNA has successfully resolved issues within groups of species (e.g., Mills *et al.*, 1991; Hansen *et al.*, 1986; Oudemans and Coffey, 1991a,b; Förster and Coffey, 1993). The combined use of population and molecular criteria has also demonstrated that some traditional *Phytophthora* morphospecies, such as *P. megasperma*, comprise multiple species units (e.g., Hansen *et al.*, 1986; Förster *et al.*, 1989; Förster and Coffey, 1993) and that traditional taxonomic concepts in *Phytophthora* may bear little relationship to the evolutionary structure of the genus (Brasier, 1991; Hansen, 1991; Brasier and Hansen, 1992). In one case, morphologically distinct Phytophthoras have been shown to be part of a hybrid complex (Brasier *et al.*, 1999).

Limited sequence analysis of the large and small subunits of the ribosomal RNA genes (rDNA) has confirmed *Phytophthora* as a chromist (van de Peer *et al.*, 1996; Förster *et al.*, 1990) and its relatedness to *Pythium* (Briard *et al.*, 1995). The internal transcribed spacer (ITS) regions of rDNA have proven particularly useful for separation of fungal taxa at the species to genus level, because the rate of accumulation of mutations in these regions often approximates to the rate of speciation (Bruns *et al.*, 1991; Lee and Taylor, 1992). Sequence analysis of ITS regions has confirmed its value in distinguishing morphologically similar *Phytophthora* species and some preliminary phy-

logenies of species have been compiled (Förster *et al.*, 1995; Crawford *et al.*, 1996; Cooke and Duncan, 1997). So far, none of this work has produced a unified and coherent phylogeny of the genus or examined its relatedness to other oomycetes.

In this study, we present a ITS-based phylogenetic analysis of 50 described taxa, of *Phytophthora,* a diverse group of *Pythium* species and representatives of the Saprolegniales and Peronosporales. One objective was to investigate how the main lineages within *Phytophthora* correspond to the species groups defined by traditional taxonomy. This has been approached through a wider study of the rDNA ITS regions of over 234 *Phytophthora* isolates, some of the detail of which will be addressed in subsequent publications. Another objective was to examine outstanding evolutionary issues within *Phytophthora,* such as the evolution of its breeding systems and ecological strategies (c.f. Brasier, 1983; Brasier and Hansen, 1992; Irwin *et al.*, 1997; Drenth and Goodwin, 1999) by comparing the ITS phylogeny with the biological properties of the taxa. A third objective was to investigate the position of *Phytophthora* in relation to other oomycete genera, in particular *Pythium* and *Peronospora.* In addition, the ITS information yielded will strengthen and extend current PCR-based diagnostics, detection, and identification of *Phytophthora* species (Bonants *et al.*, 1997; Cooke and Duncan, 1997).

MATERIALS AND METHODS

Organisms and culture conditions. The ITS regions of 234 *Phytophthora* isolates have been sequenced in this study (Table 1). Details of the 50 *Phytophthora* isolates selected as representative of each taxon are listed in Table 1 together with nine species from other genera of the Oomycota. The isolates were sourced from the culture collections of the authors and CABI Biosciences (Egham, UK). Three of the species shown were not held by the authors. In these cases, DNA, sporangia, or, in the case of *P. multivesiculata,* unpublished sequence data were kindly provided by colleagues (see Table 1). *Albugo candida* was collected on fresh plant material by the first author. Further details of isolates in Table 1 are available upon request. Most culturable isolates were stored on oatmeal agar at 5°C; some tropical species were stored at 15°C. Routine stock cultures for research studies were grown on French bean agar at 20°C at the Scottish Crop Research Institute (SCRI) or on V8 medium at 25°C at the Cooperative Research Centre for Tropical Plant Pathology (CRCTPP).

rDNA amplification and sequencing. Isolates were grown in 20 ml still culture of a sucrose/asparagine/mineral salts broth containing 30 μg ml^{-1} β-sitosterol (Elliott *et al.*, 1966) at SCRI and unclarified V8 medium at CRCTPP. After vacuum filtration, the mycelium was freeze-dried for extended storage at −20°C. DNA was extracted from mycelium or, in the case of biotrophs, from plant material using a Puregene DNA extraction kit, Flowgen (Lichfield, England) at SCRI and using a method described in Drenth and Govers (1993) at CRCTPP.

DNA was amplified using the universal primers ITS6 and ITS4 at SCRI (White *et al.*, 1990) (Table 2) and primers TW81 and AB28 (Crawford *et al.*, 1996) at CRCTPP. Primer ITS6 is similar to ITS5 (White *et al.*, 1990), but modified according to the *P. megasperma* 18S rDNA sequence (Förster *et al.*, 1990) to allow more efficient amplification in these taxa (Cooke and Duncan, 1997). For DNA from biotrophs, a seminested PCR using DC6 (Table 2) and ITS4 in the first round and ITS6 and ITS4 in the second was adopted. In combination with ITS4, DC6 selectively amplifies the ITS regions of members of the orders Peronosporales and Pythiales (Bonants *et al.*, 1997).

All PCRs consisted of 1 cycle of 95°C for 2 min; 30 cycles of 95°C for 20 s, 55°C for 25 s, 72°C for 50 s; and a final cycle of 72°C for 10 min at SCRI and 1 cycle at 94°C for 5 min; 35°C cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 2 min, and a final cycle of 72°C for 10 min at CRCTPP. Amplification products were purified through Wizard Prep columns (Promega, Southampton, England) and after electrophoresis on agarose, their concentrations were estimated by comparison with λ DNA (Pharmacia Biotech, St. Albans, England). Direct sequencing of PCR products was initiated using primers ITS6 and ITS4 at SCRI and primers S1 and S6 (Crawford *et al.*, 1996) in a dye-terminator cycle-sequencing reaction (FS sequencing kit, Applied Biosystems, Warrington, UK) and run on an ABI373 automated sequencer (Applied Biosystems). In a few cases, additional sequencing reactions using the 5.8S gene-based primers ITS7 and its reverse complement ITS8 (Table 2) were run to clarify polymorphic ITS types. As with ITS6, these primers were modified from ITS2 and ITS3, respectively (White *et al.*, 1990), on the basis of the *P. megasperma* 5.85 gene sequence (van der Auwera *et al.*, 1994).

Phylogenetic analysis. Sequences of rDNA of more than 234 isolates across the genus were determined and trimmed to remove all 18S and 28S sequences according

TABLE 1
Isolates of *Phythphthora* Included in the Study, Their Designations, and Origins

Phytophthora species	Isolate details: Isolate numbers: International[a]	Isolate numbers: Local[b]	Isolate numbers: GenBank[c]	Origins: Host	Origins: Country	Origins: Date	Number of isolates sequenced
P. arecae	IMI348342	UQ2820	AF266781	*Cocos nucifera*	Indonesia	1991	1
P. botryosa	IMI136915 CBS581.69	BOT1	AF266784	*Hevea brasiliensis* leaf	Malaysia	1966	1
P. cactorum	IMI296524	CAC2	AF266772	*Rubus idaeus*	Wales	1985	12
P. cajani		P536	AF266765	*Cajanus cajani*	India		1
P. cambivora	IMI296831	CAM1	AF266763	*Rubus idaeus*	Scotland	1985	6
P. capsici	IMI352321	UQ2819	AF266787	*Piper nigrum*	India	1989	7
P. cinnamomi		UQ881	AF266764	*Syzygium aromaticum*	Malaysia		17
P. citricola	IMI031372	CIT2 R96	AF266788	*Rubus idaeus*	Ireland	1986	11
P. citrophthora	IMI332632		AF266785	*Actinidia chinensis*	Chile	1989	9
P. clandestina[a]	IMI287317	CLA2 R193	AJ131989	*Trifolium subterranea*	Australia	1985	2
P. colocasiae	IMI368918		AF266786	*Colocasia esculenta*	Malaysia	1995	1
P. cryptogea	IMI045168	CRY3 P16	AF266796	*Lycopersicon esculentum*	New Zealand	1951	27
P. drechsleri (T)[d]	ATCC46724 CBS292.35	DRE5 P538	AF266798	*Beta vulgaris*	U.S.A.	1935	7
P. erythroseptica	ATCC36302 CBS956.87	ERY1 R143	AF266797	*Solanum tuberosum*	U.S.A.		4
P. fragariae v. *rubi*		CH132	AF266761	*Rubus idaeus*	Sweden		5
P. fragariae v. *fragariae*	IMI330736	FVF12	AF266762	*Fragaria* x *ananassa*	Scotland	1979	3
P. gonapodyides		P245	AF266793	*Salix matsudana*	UK	1972	11
Phytophthora sp "O" group		P246b	AF266791	*Salix matsudana*	UK	1972	4
P. heveae (T)[d]	IMI180616 CBS296.29		AF266770	*Hevea brasiliensis*	Malaysia (CBS)	1929	1
P. humicola	IMI302303 ATCC52179 CBS200.81		AF266792	citrus orchard soil *via* citrus bait	Taiwan	1981 (CBS)	1
P. idaei (T)[d]		IDA3	AF266773	*Rubus idaeus*	Scotland	1986	4
P. ilicis[a]		ILI1	AJ131990	*Ilex aquilifolium*	UK		1
P. infestans	IMI66006	UQ2808	AF266779	*Solanum tuberosum*	The Netherlands		4
P. inflata	IMI342898		AF266789	*Syringa*			2
P. insolita (T)[d]	IMI288805 ATCC38789 CBS691.79		AF271222	Soil	Taiwan	1979 (CBS)	1
P. iranica[a]	IMI158964 CBS374.72		AJ131987	*Solanum melongena*	Iran	1969	1
P. katsurae	IMI360596		AF266771	*Cocos nucifera*	Ivory Coast	1993	2
P. lateralis (T)[d]	IMI040503 ATCC11261 CBS168.42		AF266804	*Chamaecyparis lawsoniana*	U.S.A.	1942 (CBS)	1
P. macrochlamydospora		UQ205		*Glycine max*	Australia	1976	7
P. meadii[f]	IMI129185			*Hevea brasiliensis*	India	1968	0
P. medicaginis		UQ125	AF266799	*Medicago sativa*	Australia	1987	3
P. megakarya	IMI337104	UQ2822	AF266782	*Theobroma cacao*	Ghana	1990	2
P. megasperma	IMI133317	MEG23	AF266794	*Malus sylvestris*	Australia	1968	18

TABLE 1—*Continued*

Phytophthora species	Isolate details: Isolate numbers: International[a]	Isolate numbers: Local[b]	Isolate numbers: GenBank[c]	Origins: Host	Origins: Country	Origins: Date	Number of isolates sequenced
P. sp (asparagus)		UQ2141	AF266795	*Asparagus officinalis*			3
P. melonis	IMI325917		AF266767	*Cucumis* sp.	China	1988	1
P. mirabilis (T)[e]	ATCC64130 CBS678.85		AF266777	*Mirabilis jalapa*	Mexico		3
P. multivesiculata[e]	CBS545.96		AF266790	*Cymbidium*	The Netherlands	1995	1
P. nicotianae		UQ848	AF266776		Australia		6
P. palmivora		UQ1294	AF266780	*Theobroma cacao*	Papua New Guinea	1994	15
P. phaseoli[e]	ATCC60171 CBS556.88		AF266778	*Phaseolus lunatus*			1
P. porri[e]	CBS782.97		AF266801	*Brassica chinensis*	The Netherlands	1994	2
P. primulae	CBS620.97		AF266802	*Primula acaulis*	Germany	1997	1
P. pseudotsugae (T)[d]	IMI331662	PSE1 R209	AF266774	*Pseudotsuga menziesii*	U.S.A.		1
P. quercina (T)[d,g]		QUE4	AJ131986	*Quercus robur*	Germany	1995	6
P. richardiae	IMI340618		AF271221	*Zantedeschia* (root)	The Netherlands		1
P. sinensis (T)[d]	ATCC46538 CBS557.88		AF266768	*Cucumis sativa*	China		1
P. sojae		UQ1200	AF266769	*Glycine max*	Australia	1994	6
P. syringae	IMI296829	SYR1 R19	AF266803	*Rubus idaeus*	Scotland	1985	3
P. tentaculata	CBS552.96		AF266775	*Chrysanthemum leucanth.*	Germany	1992	1
P. trifolii		UQ2143	AF266800	*Trifolium*			2
P. vignae		UQ136	AF266766	*Vigna sinensis*	Australia	1988	2
Other oomycetes							
Albugo candida		CAN1	AF271231	*Capsella bursa-pastoris*	Wales	1997	1
Halophytophthora batemanensis	IMI327602	BAT1	AF271223	*Avicennia* sp.	Australia	1982	1
Peronospora sparsa[e]	IMI344695		AF266783	*Rosa* sp.	England		4
Pythium aphanidermatum		UQ2071	AF271227	Sugarcane soil	Australia	1992	1
Pythium dissotocum		UQ2623	AF271228	Root rot	Australia	1988	1
Pythium graminicola		UQ604	AF271229	Root rot	Australia	1993	3
Pythium irregulare		UQ2622	AF271226	Root rot	Australia		5
Pythium undulatum	IMI337230		AF271230	*Larix* sp.	Scotland	1989	1
Pythium ultimum		UQ1496	AF271225	*Euphorbia pulcherrima*	U.S.A.		1
Pythium vexans		UQ2074	AF271224	Sugarcane soil	Australia	1992	1

[a] International designations: ATCC, American Type Culture Collection, U.S.A.; IMI, CABI Bioscience (International Mycological Institute), United Kingdom; CBS, Centraalbureau fur Schimmelcultures, The Netherlands.

[b] Numbers given in collections held by authors of this paper: all designations beginning UQ, University of Queensland, Australia; all beginning with P, Forest Research, UK; all others, Scottish Crop Research Institute.

[c] Accession numbers for GenBank database.

[d] Type isolate of the species.

[e] DNA of *P. mirabilis*, *P. phaseoli*, and *P. porri* kindly provided by Sophien Kamoun, University of Ohio. The ITS sequence of *P. multivesiculata* provided by Peter Bonants, IPO-DLO, Wageningen. Frozen sporangia of *Pe. sparsa* provided by Richard Shattock, University of Wales, Bangor.

[f] Restriction digests of the ITS region (Cooke and Duncan, 1997) matched that of *P. botryosa.*

[g] Submitted as part of a separate study (Cooke *et al.*, 1999).

TABLE 2
PCR Primers Designed for This Study, Their Sequences, and Location within the Genomic Ribosomal RNA Gene Repeat (rDNA)

Primer	Sense	Primer sequence (5′ → 3′)	Location in rDNA
DC6	Forward	GAGGGACTTTTGGGTAATCA	18S gene
ITS6[a]	Forward	GAAGGTGAAGTCGTAACAAGG	18S gene
ITS7	Reverse	AGCGTTCTTCATCGATGTGC	5.8S gene
ITS8	Forward	GCACATCGATGAAGAACGCT	5.8S gene

[a] Primers ITS6, ITS7, and ITS8 are versions of the "universal" ITS primers ITS5, ITS2, and ITS3, respectively, of White *et al.* (1990) modified by D. E. L. Cooke to improve the amplification of rDNA from oomycetes.

to sequence boundaries defined by Lee and Taylor (1992). These trimmed sequences, along with ITS sequences of related species in the database GenBank, were included in a preliminary analysis (data not shown). From this analysis single isolates representative of each taxon were selected for further detailed phylogenetic analyses (Table 1). In the case of *Pythium*, the selected taxa represented much of the diversity within the genus, as revealed by a comparison of our ITS1 sequences with those previously published (Grosjean, 1992).

In the detailed analyses, preliminary sequence alignments, using CLUSTAL W (Thompson *et al.*, 1994), were adjusted after visual examination and analyzed by both distance-based and maximum-likelihood methods in PHYLIP (Felsenstein, 1993). The transition/transversion parameter was estimated using the PUZZLE program (Strimmer and von Haeseler, 1996) and used in PHYLIP DNADIST (Felsenstein, 1993) and PHYLIP DNAML (Felsenstein and Churchill, 1996) programs. The DNADIST program option to allow for among-site rate heterogeneity was used (Jin-Nei option) again using PUZZLE to estimate the appropriate parameter (coefficient of variation) (Yang, 1996). The distance matrices produced by PHYLIP DNADIST were used as input to the PHYLIP NEIGHBOR program to construct neighbor-joining phylogenetic trees. The robustness of both trees was tested using DNADIST and NEIGHBOR in 500 bootstrap trials. Trees were drawn using TREEVIEW (Page, 1996).

Statistical testing of phylogenetic constraint trees was carried out using the Hasegawa, Kishino, and Templeton test (Kishino and Hasegawa, 1989) in the PHYLIP DNAML maximum-likelihood program.

RESULTS

DNA Amplification and Sequence Analysis

PCR always resulted in a single band of c. 900 bp whether with primers ITS6 and ITS4 (SCRI) or with primers TW81 and AB28 (CRCTPP). Primers DC6 and ITS4 resulted in a single 1300-bp product from plants infected with *Peronospora* or *Albugo.* Despite the fact that the PCR products were sequenced directly (i.e., a pooled PCR product rather than a single clone), very few point mutations or length polymorphisms were observed. The lengths of the ITS1, 5.8S, and ITS2 fragments varied from 752 bp (*P. capsici*) to 915 bp (*Halophytophthora batemanensis*). The greatest variations in length and sequence were observed in the ITS1 region which had a mean similarity of 75.5% compared with that of 84.8% recorded for the ITS2 region. The length of the 5.8S subunit was constant in all Phytophthoras examined, though there were a few, single-base substitutions. Sequencing of the same isolates at SCRI and CRCTPP gave identical results in all cases.

Phylogenetic Analysis

From the preliminary analysis of the 234 ITS sequences two new phylograms were constructed. The first included a diverse range of genera and species within the oomycetes (Fig. 1). However, the ITS1 regions of widely divergent genera such as *Phytophthora* and *Achyla* could not be aligned accurately. This analysis was therefore based only on the more conserved 5.8S and ITS2 sequences. Together these regions comprised 722 base pairs in the alignment, of which 7.2% were constant. The second phylogram was based on the complete ITS1, 5.8S, and ITS2 sequences. This was confined to all but three of the *Phytophthora* species and to only a single species of *Peronospora, Pe. sparsa* (Fig. 2), which, in a separate study, was shown to be representative of 10 other *Peronospora* taxa (data not shown). In this case, the aligned sequence represented 889 aligned base pairs, of which 48.5% were constant. All the sequences and alignments have been submitted to GenBank and TreeBASE (http://www.herbaria.harvard.edu/treebase/console.html), respectively.

Structure of the Oomycete Tree

The taxa in the oomycete tree (Fig. 1) fall broadly into two groups. One contains members of the Saprolegniales

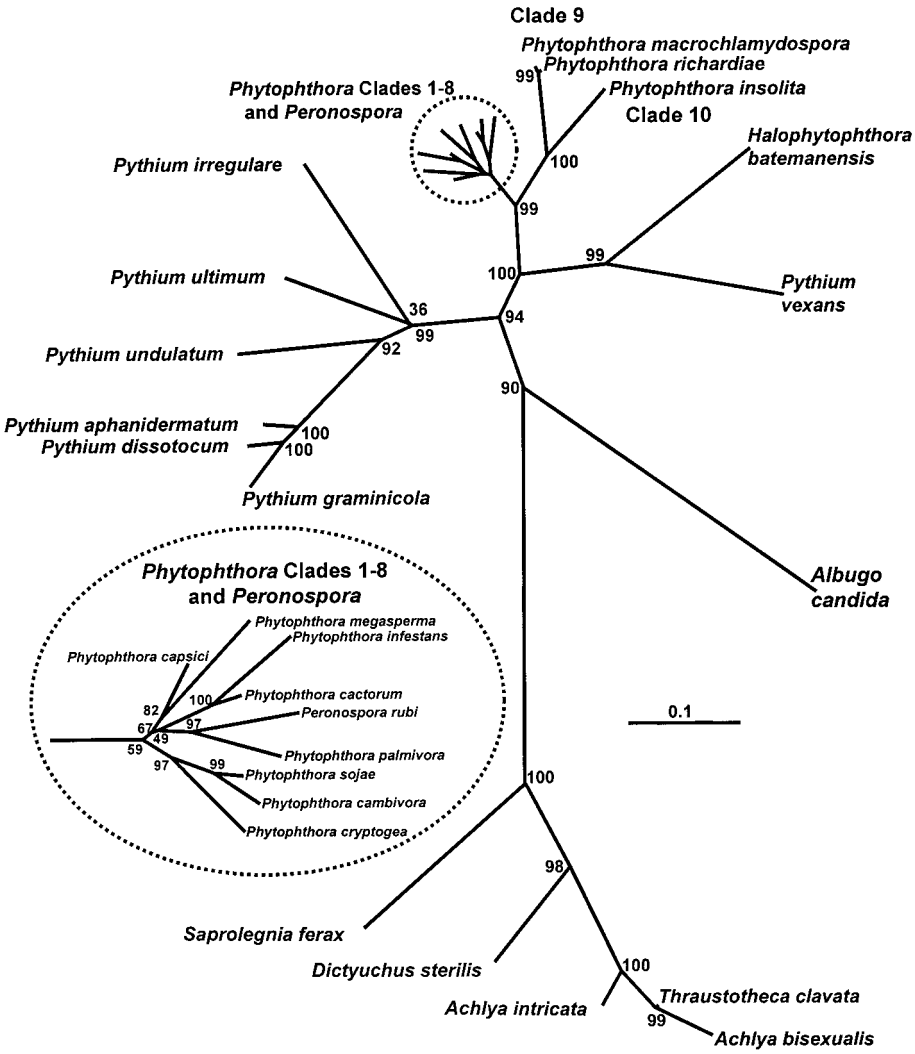

FIG. 1. Phylogram of genera and species of the Saprolegniales, Pythiales, and Peronosporales (Oomycota) obtained by DNA distance-based analysis of the combined 5.8S subunit and ITS2 regions of the genomic ribosomal RNA tandem gene repeat. DNA sequences of *Achyla intricata, Dictyuchus sterile, Saprolegnia ferax* and *Thraustotheca clavata* were from Daugherty *et al.* (1998) and *Achlya bisexualis* from Crawford *et al.* (1996). The numbers at the branch points indicate the percentages of bootstrap values (based on 500 bootstraps). The inset shows the details of the relationships among eight *Phytophthora* species and a *Peronospora* species.

(*Achyla, Dictyuchus, Saprolegnia, Thraustotheca*) (Hawksworth *et al.*, 1995), the other genera usually assigned to the Pythiales and Peronosporales. *Albugo* is distantly related to all other taxa, although rooted closer to the Pythiales than to the Saprolegniales. The sequence obtained for *Albugo candida* precisely matched that of three other *A. candida* isolates from wild crucifers (Jacobsen *et al.*, 1998).

Pythium is polyphyletic and more diverse than *Phytophthora.* Six of the seven species examined in this study, including *Py. undulatum*, form a single divergent clade, while *Pythium vexans* groups with the marine species *Halophytophthora batemanensis.*

Structure of the Phytophthora Tree

Phytophthora is paraphyletic and divisible into two groups (Figs. 1 and 2). However, all but 3 of the 50 taxa examined fall within a cluster comprising approximately eight clades, numbered 1–8 in Fig. 2. Also lying within this main *Phytophthora* cluster was *Peronospora* (clade 4, Fig. 2). *P. macrochlamydospora, P. richardiae,* and *P. insolita*

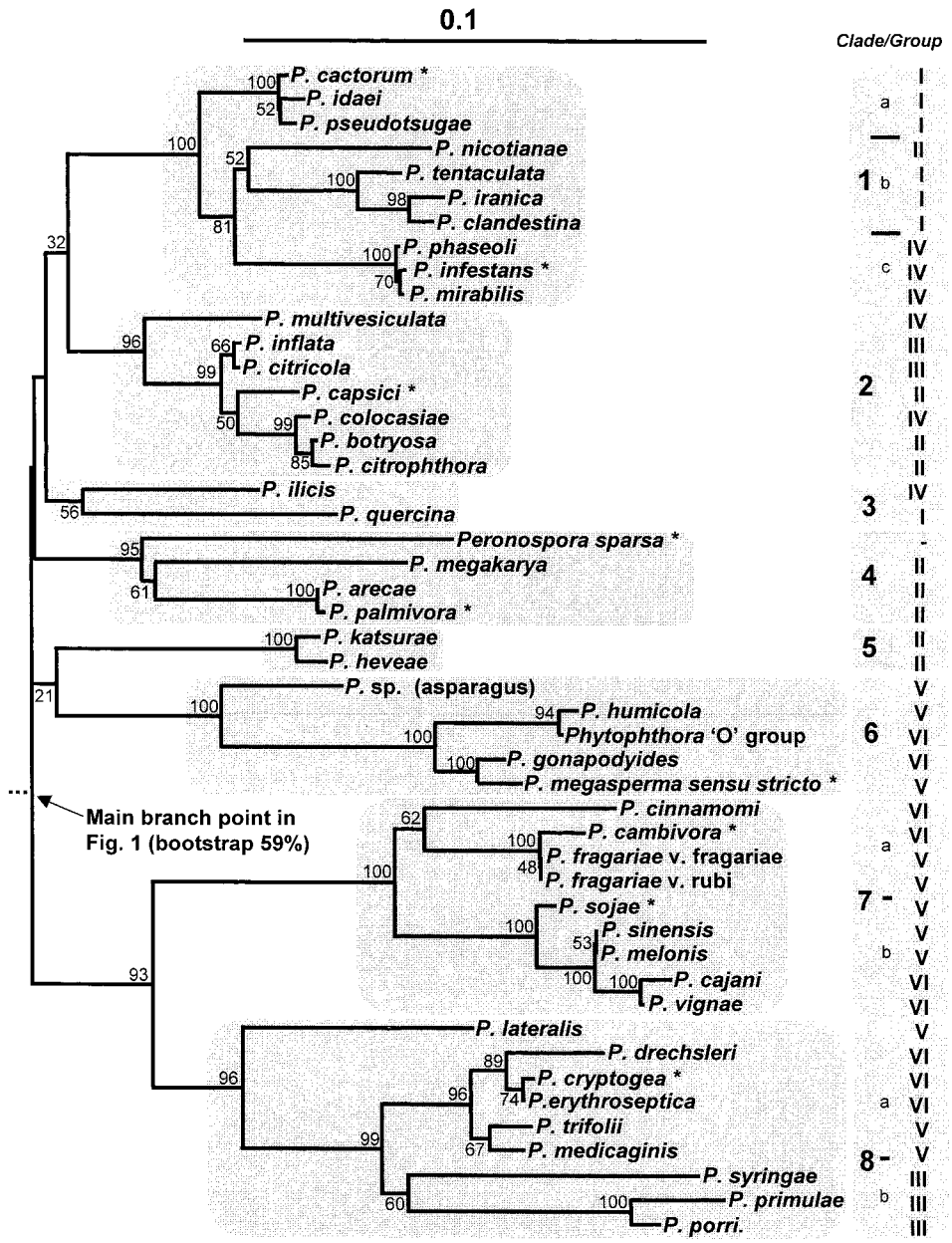

FIG. 2. Detailed phylogram of 47 *Phytophthora* taxa and 1 *Peronospora* species, chosen as representatives of *Phytophthora* clades 1–8 (see text). The phylogram was constructed after DNA distance-based analysis of the combined ITS1, 5.8S subunit, and ITS2 regions of the genomic ribosomal RNA tandem gene repeat. The numbers at the branch points indicate the percentages of bootstrap values (based on 500 bootstraps). Taxa marked with an asterisk are those included in Fig. 1.

are separate from this main cluster though still its nearest relatives (clades 9 and 10, Fig. 1).

The main *Phytophthora* cluster is characterized by a series of relatively short branches radiating from the nexus of clades 1–6. These six clades form one part of a major division of the cluster, while the more clearly defined clades 7 and 8 form the other (Figs. 2 and 3). Apart from the short branches at the bases of clades 1–6, bootstrap values were generally strong for all major branch points (Fig. 2).

Of the 29 *Phytophthora* taxa in clades 1–6, the majority, 24, have papillate or semipapillate sporangia. The other 5

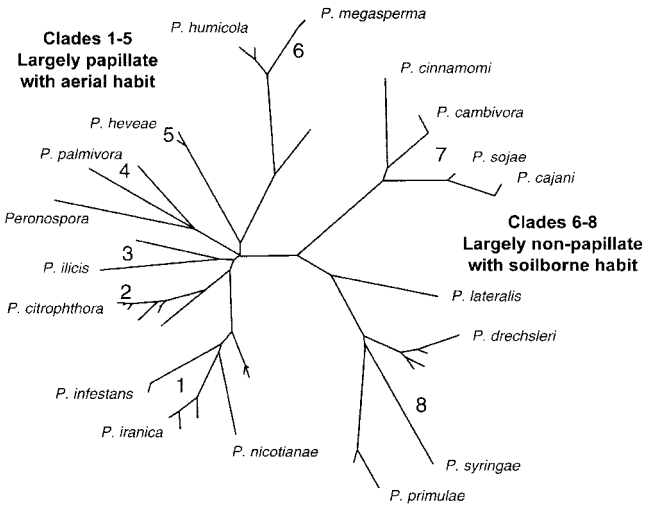

FIG. 3. Radial version of Fig. 2 showing the broad evolutionary trends among clades 1–8 of the main *Phytophthora–Peronospora* cluster and selected individual taxa at the clade termini.

taxa, all nonpapillate, are confined to clade 6. Of the 18 taxa in clades 7 and 8, 15 are nonpapillate; the remaining 3, *P. syringae, P. primulae,* and *P. porri* (clade 8b), are semipapillate.

The ITS-based tree (Fig. 2) was compared to one in which each of the 47 taxa was constrained to its respective Waterhouse group. *Pe. sparsa* was excluded from this analysis. The log likelihoods of these two trees (−6311.9 and −7607.7, respectively) were compared using the Hasegawa, Kishino, and Templeton test (Kishino and Hasegawa, 1989). The difference in log likelihoods was highly significant (difference of 1295.8; standard deviation of difference 86.9; $P < 0.001$). In a similar manner the complete ITS tree of all 48 taxa (Fig. 2) was compared to one in which *Pe. sparsa* was constrained as an outlier to the main group rather than within clade 4. When the log likelihoods of these two trees (−6517.3 and −6533.9, respectively) were compared by the Hasegawa, Kishino, and Templeton test, the difference was again significant (difference of 16.6; standard deviation of difference 7.5; $P < 0.05$).

DISCUSSION

This analysis provides the first comprehensive ITS-based phylogeny of *Phytophthora* and one in which both intrageneric and intergeneric relationships of Phytophthoras are assessed. Aspects of Gaümann's hypotheses (Gaümann, 1952) regarding evolution of the oomycetes as a whole are tested for the first time using molecular evidence.

Evolutionary and Taxonomic Significance of the Oomycete Tree

The majority of the *Phytophthora* taxa formed a recently evolved monophyletic group (clades 1–8, Figs. 1 and 2). Their nearest neighbors were three other *Phytophthora* species, *P. macrochlamydospora, P. richardiae,* and *P. insolita,* which represented a distinct lineage (clades 9 and 10, Fig. 1). The morphogenetic and behavioral properties of the latter taxa need to be reexamined to determine whether they should be retained within *Phytophthora* or, as seems likely, reassigned to one or more new genera.

In the preliminary analysis of 234 ITS sequences, 10 different *Peronospora* species (17 isolates/sequences) were examined. These formed a monophyletic group (results not shown) within the *Phytophthora/Peronospora* cluster illustrated in the inset to Fig. 1. A representative species, *Pe. sparsa,* the sequence of which matched that of Lindquist, Koponen, and Valkonen (1998), was selected for more detailed analyses (Figs. 1–3). The relationship revealed (Fig. 2) between *Peronospora* and *Phytophthora* supports Gaümann's (1952) view that the biotrophic downy mildews evolved relatively recently from a hemibiotrophic or necrotrophic *Phytophthora* ancestor. The nearest relatives to *Peronospora* were *Phytophthora palmivora* and *P. megakarya,* tropical species exhibiting evolutionarily advanced characters of caducous, papillate sporangia and an adaption to an aerial habitat (Brasier, 1983). The position of *Peronospora* within the main *Phytophthora* cluster was further supported by the significant difference between the tree in Fig. 2 and one in which *Peronospora* was constrained as an outlier of the main *Phytophthora* cluster. Other studies (A. P. Rehmany, personal communication, and D. E. L. Cooke and J. M. Duncan, data not shown) indicate that *Bremia* also roots within this *Phytophthora/Peronospora* cluster (Fig. 1).

Peronospora and *Bremia* therefore appear to be obligate biotrophic *Phytophthora* lineages that have lost the ability to produce zoospores. Indeed, sporangia of many aerially dispersed Phytophthoras (e.g., *P. palmivora*) can germinate both indirectly via zoospores and, like those of *Peronospora,* directly as a conidium. Total loss of the ability to produce zoospores may require only a simple developmental change (Brasier and Hansen, 1992). The

TABLE 3
Key Properties of Taxa within *Phytophthora* Clades 1–8

		Character and group																		
		Sporangium				Reproductive behavior						Optimum °C			Pathogenicity					
Clade	Species	Nonpapillate	Semipapillate	Papillate	Caducous	Homothallic	Heterothallic	Metaphase ring	Amphigynous	Paragynous	Paragynous + amphi	Low temperature <22°C	Moderate temperature 22–27°C	High temperature >28°C	Single host	Multiple hosts	Legume hosts only	Woody perennial hosts	Aerial	Waterhouse group
1a	*P. cactorum*			P	C	Ho				P			M			Mu		W	Ae	I
	P. idaei			P		Ho				P	PA	L			S			W		I
	P. pseudotsugae			P		Ho				P	PA		M		S			W		I
1b	*P. nicotianae*			P	C		He	R	A					H		Mu		W	Ae	II
	P. tentaculata			P	C	Ho				P	PA	L				Mu				I
	P. iranica			P		Ho				P	PA		M			Mu				I
	P. clandestina			P	C	Ho				P	PA		M			Mu	Le			I
1c	*P. phaseoli*		S		C	Ho			A			L				Mu			Ae	IV
	P. infestans		S		C		He	R	A			L				Mu			Ae	IV
	P. mirabilis		S		C		He		A			ND			S				Ae	IV
2	*P. multivesiculata*	NP/S?				Ho			A											IV
	P. inflata		S			Ho				P			M			Mu		W		III
	P. citricola		S			Ho				P			M			Mu		W	Ae	III
	P. capsici			P	C		He	R	A					H		Mu		W	Ae	II
	P. colocasiae		S		C		He		A					H		Mu		W	Ae	IV
	P. botryosa			P	C		He	R	A				M		S			W	Ae	II
	P. citrophthora			P			[a]						M			Mu		W		II
3	*P. ilicis*		S		C	Ho			A			L				Mu		W	Ae	IV
	P. quercina			P		Ho				P		L			S	—		W		I
4	*P. megakarya*			P	C		He	R	A				M		S			W	Ae	II
	P. arecae			P	C		He		A					H		Mu				II
	P. palmivora			P	C		He	R	A					H		Mu		W	Ae	II
	Peronospora sparsa	Conidial			C		He	R		P				H		Mu		W	Ae	
5	*P. katsurae*			P		Ho		R					M			Mu		W	Ae	II
	P. heveae			P	C	Ho		R					M			Mu		W	Ae	II
6	*P.* sp. (asparagus)	N				Ho				P	PA				S?					V
	P. megasperma	N				Ho				P	PA					Mu		W		V
	P. gonapodyides	N					[a]									Mu		W		VI
	P. humicola	N				Ho				P	PA				ND					V
7a	*P. cinnamomi*	N					He	R	A				M			Mu		W		VI
	P. cambivora	N					He	R	A				M			Mu		W		VI
	P. fragariae v. *fragariae*	N				Ho				P	PA	L			S			W		V
	P. fragariae v. *rubi*	N				Ho				P	PA	L			S			W		V
7b	*P. sojae*	N				Ho				P	PA		M		S		Le			V
	P. sinensis	N					He		A					H	S					V
	P. melonis	N					He		A					H	S					V
	P. cajani	N				Ho			A						S		Le			VI
	P. vignae	N				Ho			A					H		Mu	Le			VI
8a	*P. lateralis*	N				Ho						L				Mu		W		V
	P. drechsleri	N					He	R	A					H		Mu		W		VI
	P. cryptogea	N					He	R	A				M			Mu		W		VI
	P. erythroseptica	N				Ho			A				M			Mu	Le			VI
	P. trifolii	N				Ho				P	PA		M			Mu	Le			V
	P. medicaginis	N				Ho					PA		M			Mu	Le			V

TABLE 3—*Continued*

Clade	Species	Character and group: Sporangium: Nonpapillate	Sporangium: Semipapillate	Sporangium: Papillate	Sporangium: Caducous	Reproductive behavior: Homothallic	Reproductive behavior: Heterothallic	Reproductive behavior: Metaphase ring	Reproductive behavior: Amphigynous	Reproductive behavior: Paragynous	Reproductive behavior: Paragynous + amphi	Optimum: Low temperature <22°C	Optimum: Moderate temperature 22–27°C	Optimum: High temperature >28°C	Pathogenicity: Single host	Pathogenicity: Multiple hosts	Pathogenicity: Legume hosts only	Pathogenicity: Woody perennial hosts	Pathogenicity: Aerial	Waterhouse group
8b	*P. syringae*		S			Ho				P	PA	L				Mu		W		III
	P. porri		S			Ho					PA		M			Mu				III
	P. primulae		S			Ho				P	PA	L			S					III
9	*P. insolita*	N				Ho			—	—	—			H	ND					V
10	*P. richardiae*	N				Ho			A		PA		M		S	Mu				VI
	P. macrochlamydospora		S			[a]							M		S		Le			III or IV

[a] Oospores never observed.

main *Phytophthora/Peronospora* cluster, therefore, encompasses organisms that range in ecological strategy from aquatic necrotrophs with saprotrophic ability, such as *P. gonapodyides,* to aerial obligate biotrophs, such as *Peronospora.* This represents much of the ecological range of the oomycetes as a whole.

Gaümann's (1952) hypothesis that *Phytophthora* evolved via a *Pythium*-like ancestor is supported by this study and that of Briard *et al.* (1995). The nature of the putative ancestral organism is unclear, but on the present evidence it is unlikely to have been *Saprolegnia*-like. This study also shows that the Peronosporales, as defined by Waterhouse (1973) and Dick (1990), are polyphyletic: *Albugo* is distantly related to *Phytophthora, Pythium,* and *Peronospora.* Gaümann's (1952) view of *Albugo* as an "evolutionary sideline, the ancestral types of which are unknown," is therefore confirmed.

The creation of the new genus *Halophytophthora* (Ho and Jong, 1990) to reclassify a group of marine species previously assigned to *Phytophthora* is supported by its position in the tree (Fig. 1). The isolate of *Halophytophtora batemanensis* examined was unrelated to *Phytophthora* and most Pythiums, sharing a recent common ancestor with *Py. vexans.* In another ITS-based study, Förster *et al.* (1995) showed that *H. exoprolifera* was more closely related to *P. insolita* than to other Phytophthoras. Its morphology, saprotrophic habit, and relatedness to *Py. vexans* suggest that *Halophytophthora* is more akin to a marine form of *Pythium* than to a *Phytophthora.*

The *Pythium* species examined in this study were selected to represent much of the diversity within the genus, based on a previous study of 40 species in which ITS1 types broadly corresponded with sporangial structure (Grosjean, 1992). Six of the *Pythium* species, including *Py. undulatum,* formed a single group. As observed in 18S analysis (Briard *et al.,* 1995), the divergence between *Pythium* species was much greater than that between the Phytophthoras. *Pythium vexans* grouped in a separate clade together with *Halophytophthora* (Fig. 1). Thus, *Pythium* also is polyphyletic and may warrant subdivision into several genera.

Dick (1989) transferred *Pythium undulatum* into the genus *Phytophthora* (as *Phytophthora undulata*), a move not supported by this analysis in which it grouped closely with *Pythium* (Fig. 1). In a separate analysis of the ITS1 sequences of many *Pythium* species (selected from Grosjean, 1992), *Py. undulatum* was most closely related to *Py. anandrum* and *Py. helicandrum* (Duncan and Cooke, unpublished).

The molecular phylogeny suggests that revisions in the taxonomy are necessary. Probably the taxonomic treatment that best fits the molecular phylogeny is one that places the genera *Phytophthora, Halophytophthora,* and *Pythium* and at least two genera of downy mildews in a

single Order, i.e., the Peronosporales (c.f. Waterhouse, 1973). This contrasts with the proposed grouping of *Phytophthora* and *Pythium* in the Pythiales, separate from *Peronospora* and *Albugo* in the Peronosporales (Dick, 1990). However, Fig. 1 is based on an analysis of combined 5.8S and ITS2 sequences, probably at the limits of resolution at this taxonomic scale. Therefore, further studies with the 18S and/or 28S subunits of rDNA, or other gene sequences, are needed to confirm the detail of the relationship.

Evolutionary and Taxonomic Significance of the Phytophthora Tree

The ITS phylogeny provides an opportunity to evaluate major evolutionary and taxonomic issues in the genus by comparing the *Phytophthora* tree (Fig. 2) with the known morphogenetic and behavioral properties of the individual taxa. Key criteria of the taxa examined, with the taxa grouped by clade, are summarized in Table 3. Some of the criteria should be treated with a degree of caution; for example, it may not be known whether migrant species that are now globally distributed with multiple hosts had single or multiple hosts in their centers of origin.

Status of Waterhouse taxonomic groups and the type species. The grouping of all *Phytophthora* taxa in one major clade, with the vast majority of species forming a closely related group (Figs. 1–3), demonstrates that the traditional concept of the genus represents a good natural assemblage. Equally, however, a significant feature of the main *Phytophthora* tree, confirmed by constraint analysis, is that Waterhouse's (1963) six taxonomic groups do not represent natural assemblages (Table 3 and Fig. 2). For example, clades 1a–1c comprise taxa from Groups I, II, and IV. This possibility was certainly recognized by Waterhouse herself, who emphasized that the groups were constructed solely as a taxonomic tool (Waterhouse, 1963). The groups will undoubtedly remain of value in the identification of traditional *Phytophthora* morphospecies, at least until molecular identification methods are more widely applied.

The classical "type species" of *Phytophthora* is *P. infestans* (Fig. 2, clade 1). In a genus exhibiting an enormous range of properties (Table 3), the properties of *P. infestans* cannot adequately represent the genus as a whole. Indeed the concept of a type species for *Phytophthora* is now surely as redundant as that of the "type isolate" (c.f. Leonian, 1934; Brasier, 1992b; Hansen, 1991).

Emergence of soil-borne and aerial lineages. In the main *Phytophthora* tree there is good evidence for separate lines of evolution toward "caducous-papillate-aerial" and "nonpapillate soil-borne" lifestyles (Brasier, 1983). This is not manifest as a simple broad division of the genus (Fig. 2). Nonetheless, the two "extreme" sporangial types that represent the two lifestyles, papillate and nonpapillate, never occur together in the same clade (Fig. 3). Taxa with semipapillate (intermediate) sporangia, on the other hand, are associated with either lineage. Most probably these are morphological variants of the papillate and nonpapillate types. The main exception to a simple split is clade 6. This comprises exclusively soil-borne, nonpapillate species, yet in the ITS phylogeny they are more closely related to the papillate taxa of clades 1–5 than they are to the other nonpapillate taxa in clades 7 and 8. Further molecular analyses are needed to establish the precise relationship of clade 6 to the other clades.

Influence of climate and host specialization on evolution. In the *Phytophthora* tree (Fig. 2 and Table 3) no clear specialization of any clades is evident with regard to temperature growth relationships, i.e., climatic adaptation. However, the evolution of several clades appears to be associated with host specialization. Most taxa in subclade 7b, for example, are pathogens of herbaceous leguminous hosts (c.f. Irwin *et al.*, 1997). In contrast, all the taxa in related subclade 7a attack roots of woody perennial hosts, especially trees and shrubs. Clades 4 and 5, on the other hand, comprise species that commonly attack the aerial parts (stems, fruits, and buds) of tropical woody hosts, in particular Palmae and dicotyledonous trees.

Evolution of heterothallism, amphigyny, and paragyny. Approximately 58% of the *Phytophthora* taxa examined are homothallic (predominantly inbreeding), 38% are heterothallic (potentially outcrossing), and 4% are sexually sterile. Comparison of the *Phytophthora* tree (Fig. 2) with information in Table 3 shows that a majority of clades contain a mixture of homothallic and heterothallic taxa. Hence, the question of whether heterothallism and the associated system of amphigyny (Table 3) is ancestral, or has emerged more than once in the genus (Brasier, 1983), is not yet resolved. Although in the heterothallic species mating type is under simple genetic control (Sansome, 1980; Fabritius and Judelson, 1997), the chromosomal basis of heterothallism is intricate, involving a reciprocal translocation heterozygote upon which the mating type loci are located, which results in a ring of four chromosomes at metaphase I (Sansome, 1980, 1985, 1987). Significantly, along with the A1/A2 incompatibility system, this mechanism is common to all heterothallic Phytophthoras (Table 3) and also to *Peronospora* and *Bremia* (Sansome, 1980; Michelmore and Sansome, 1982).

In view of this intricacy and universality, it seems more likely that the system of heterothallism, together with amphigyny, was ancestral to the radiation of the current clades.

It is generally accepted that paragyny represents an ancestral oomycete character (c.f. *Saprolegnia, Achlya,* and *Pythium*). While heterothallic Phytophthoras are exclusively amphigynous, homothallic taxa are either amphigynous or paragynous, or both (Table 3). The possibility that some homothallic amphigynous species (such as *P. heveae* and *P. phaseoli* in clades 5 and 1c) are secondary homothallics derived from heterothallic ancestors (Brasier, 1983) needs to be investigated with a broader range of molecular markers.

Status of individual Phytophthora taxa. The taxonomic status of many individual taxa is further clarified by this study (Fig. 2). The recently defined species *P. tentaculata* (Kröber and Marwitz, 1993), *P. multivesiculata* (Ilieva *et al.,* 1998), and *P. quercina* (Jung *et al.,* 1999) are all shown to have unique ITS sequences. However, the ITS sequence of *P. citricola* and that of the rarely recorded *P. inflata* were identical. These are morphologically similar species of overlapping host range (e.g., Caroselli and Tucker, 1949; Hall *et al.,* 1992) and are probably conspecific. *P. melonis* and *P. sinensis* also share identical ITS sequences consistent with their previously proposed conspecificity (Ho, 1986; Mills *et al.,* 1991). However, the further proposal that they be synonymized with *P. drechsleri* (clade 8) on morphological and physiological grounds (Ho and Jong, 1986) is not supported by the present data nor is the proposal that *P. cajani* be made a variety or forma specialis of *P. drechsleri* (Pal *et al.,* 1970; Kannayian *et al.,* 1980).

Of the various species units identified within the traditional morphospecies "*P. megasperma*" by Hansen *et al.* (1986), the BHR form (now *P. megasperma sensu stricto*) and the apple–cherry (AC) form (data not shown) fall within clade 6 and, as expected, have distinct ITS sequences, while the SOY, ALF, and CLO forms [now *P. sojae sp. nov., P. medicaginis sp. nov.,* and *P. trifolii sp. nov.;* Hansen and Maxwell (1991)] are located in clades 7 and 8. This confirms once more that *P. megasperma* was a highly polyphyletic assemblage. The more recently defined asparagus type of *P. megasperma* (Förster and Coffey, 1993) also falls within clade 6 and is indicated to be a unique species, as also does the *Phytophthora* sp. "O-group" of Brasier *et al.* (1993) shortly to be named as a new species (C. M. Brasier and E. Sanchez, in preparation). Lastly, many representatives of the much studied (e.g., Mills *et al.,* 1991) *P. drechsleri* and *P. cryptogea* (subclade 8a) were examined here. Although proposed as synonymous by Ho and Jong (1986), in this study they were consistently differentiated as distinct taxa.

Limitations and Practical Application of the ITS Data

Generally, the present ITS tree is in good accord with previous analyses of *Phytophthora* subgroups using other molecular criteria (e.g., Hansen, 1991; Mills *et al.,* 1991; Förster and Coffey, 1993; Oudemans *et al.,* 1994). Nonetheless, the tree must be interpreted with a degree of caution and critical evolutionary or taxonomic issues involving clade branches with weak bootstrap support should be corroborated with other molecular markers. Moreover, since there is no reliable oomycete fossil record and our knowledge of *Phytophthora* biogeography is scant, relating levels of sequence divergence to dates of divergence of taxa is not yet possible. Whether the greater ITS differences between taxa in clades 6–8 relative to those in clades 1–5 (Figs. 2 and 3) are due to their earlier divergence or to unequal rates of evolution between lineages remains to be tested.

The species trees in Figs. 1 and 2 are constructed on the assumption that they are equivalent to the ITS gene trees. However, they may not reveal past progenitors of modern taxa (e.g., a common ancestor for *Pythium* and *Phytophthora*) because relevant taxa are not represented or are extinct. In addition, interspecific hybridization events (leading to a pattern of reticulate evolution) are likely to have been masked by ITS homogenization, a process that is neither predictable nor fully understood (e.g., Dover, 1982; Hillis *et al.,* 1991; Wendel *et al.,* 1995). Therefore, the role of ancient reticulation events in the evolution of *Phytophthora* is unlikely to be revealed by ITS sequence data alone. There are, however, two clear examples of recent interspecific hybridization in the genus. One involves two species in clade 1 (Fig. 2), *P. nicotianae* and *P. cactorum* (Man in't Veld *et al.,* 1998). The other, in which continuing recombination and homogenization of the ITS arrays have been demonstrated, involves *P. cambivora* and a *P. fragariae*-like taxon (Brasier *et al.,* 1999; see clade 7). On this new evidence, interspecific hybridization, along with geographic radiation and host specialization, has probably had a significant role in the evolution of modern *Phytophthora* species.

The ITS sequences published with this study should facilitate the development of further species-specific PCR primers (Lacourt *et al.,* 1996; Bonants *et al.,* 1997) of use in plant health monitoring (especially in forestry and hor-

ticultural crops) and the development and enforcement of quarantine legislation. In several species of considerable economic significance (e.g., *P. palmivora*, *P. fragariae*, and *P. cinnamomi*), the stability of the ITS digest pattern has been confirmed with hundreds of isolates (data not shown). It should again be noted, however, that ITS sequences will not always distinguish between species and that phenotypically unique taxa may sometimes have similar or even identical ITS profiles in the context of hybridization. Both caution and experience will therefore be needed when using ITS-based diagnosis. Due attention must be paid to the wider characteristics of the organism (c.f. Brasier, 1997) where a critical diagnosis is being made.

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