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Molecular phylogeny and taxonomy of the genus *Pythium*

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The phylogeny of 116 species and varieties of *Pythium* was studied using parsimony and phenetic analysis of the ITS region of the nuclear ribosomal DNA. The D1, D2 and D3 regions of the adjacent large subunit nuclear ribosomal DNA of half the *Pythium* strains were also sequenced and gave a phylogeny congruent with the ITS data. All the 40 presently available ex-type strains were included in this study, as well as 20 sequences of recently described species from GenBank. Species for which no ex-type strains were available were represented by either authentic strains (6), strains used in the 1981 monograph of the genus by van der Plaats-Niterink (33), or strains selected on morphological criteria (17). Parsimony analysis generated two major clades representing the *Pythium* species with filamentous or globose sporangia. A small clade of species with contiguous sporangia was found in between the two main clades. A total number of 11 smaller clades was recognized, which often correlated with host-type or substrate and in several cases with a subset of morphological characters. Many characters used in species descriptions, such as antheridium position, did not correlate with phylogeny. A comparison of the ex-type and representative strains with all ITS sequences of *Pythium* in GenBank revealed limited infraspecific variation with the exception of *P. rostratum*, *P. irregulare*, *P. heterothallicum*, and *P. ultimum*. The total number of species examined was 116 (including 60 ex-type strains). Twenty-six species had ITS sequences identical or nearly identical to formerly described species, suggesting possible conspecificity. The importance of comparing ITS sequences of putative new species to the now available ITS database in order to avoid unwarranted new species names being introduced.

INTRODUCTION

The monograph by van der Plaats Niterink (1981) and the keys of Dick (1990) are the most recent tools available to identify most described species of the genus *Pythium*. The historical lack of consensus on the most important morphological characteristics for identification (Matsumoto *et al.* 1999), the high variability within the most important structures and considerable overlap among species, and the absence of diagnostic morphological structures for many isolates or species, all contribute to potential errors in identification, especially for those lacking many years of experience working with this genus.

Few regions have been used in DNA sequence-based phylogenetic studies of *Oomycetes*. The nuclear rDNA is the main region that has been used in phylogenetic studies of genera and species within the *Saprolegniomycetidae*, including some species of the genus *Pythium*. Dick *et al.* (1999) sequenced the complete small nuclear ribosomal subunit of 14 species of *Oomycetes*, including *P. monospermum* and *P. undulatum*, which they named *Phytophthora undulata*. Briard *et al.* (1995) sequenced the D2 domain of the large nuclear ribosomal subunit (LSU) of 14 species of *Pythium* and *Phytophthora* in a study on the evolution of some *Pythiaceae*. The D1 and part of the D2 region of LSU was sequenced in phylogenetic studies of *Oomycetes* for close to 50 species that included *Pythium middletonii*, *P. undulatum*, also named *Phytophthora undulata*, and an unknown *Pythium* sp. (Riethmüller, Weiß & Oberwinkler 1999), for over 20 species including *P. aphanidermatum* (Petersen & Rosendahl 2000), for a study focusing more on *Peronosporales* which included *P. monospermum*, *P. vanterpooli*, *P. aphanidermatum* and two unknown *Pythium* species (Riethmüller

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et al. 2002), and in a study of *Saprolegniaceae* focusing on *Achlya* species in which *P. aquatile* was included (Leclerc, Guillot & Deville 2000). These studies generally demonstrated that *Pythium* was phylogenetically isolated and distant from other genera of *Oomycetes*. In a study based on partial sequencing of the mitochondrial cytochrome oxydase II gene that included 15 genera of *Oomycetes*, including *P. ultimum*, it was demonstrated that *Pythiales* were monophyletic (Hudspeth, Nadler & Hudspeth 2000). Using the same region, Martin (2000) studied the phylogeny of 24 *Pythium* species.

The internal transcribed spacer has been used extensively for systematics studies in mycology, primarily because the PCR primers developed by White *et al*. (1990) universally amplified a highly variable region through all taxa, including Oomycetes. The advantages and limitations of the ITS region for phylogeny were reviewed recently (Bruns 2001). Matsumoto *et al*. (1999) performed a phylogenetic analysis of 30 *Pythium* species using the ITS region. Schurko *et al*. (2003b) used the ITS region to study the genetic variation and phylogeny of *P. insidiosum* and its closely related species. Moorman *et al*. (2002) sequenced the ITS of close to 20 *Pythium* species to characterize the flora in greenhouse flower crops. Lévesque, Harlton & de Cock (1998) and Wang, Wang & White (2003) sequenced the ITS-1 region of three and 30 species, respectively, to develop species specific oligonucleotides. In a phylogenetic study of *Phytophthora* species, Cooke *et al*. (2000) included seven *Pythium* species. Numerous other ITS sequences have been deposited in GenBank from unpublished work or for the description of new species (e.g. Paul *et al*. 1998). These studies showed that the ITS region varied from 750–1050 bp, much longer than the usual 300–700 bp of the Eumycota. The longer length presents more technical challenges for direct sequencing of PCR products, but also provides more characters for analysis. Variability seems appropriate for studies at the species level as demonstrated by the above studies.

The objective of this paper was to study the phylogeny of all known species for which isolates were available, especially isolates used in the original species description or in the monograph by van der Plaats-Niterink (1981). The second objective was to compare all these reference isolates with the complete collection of ITS sequences in GenBank to facilitate identification of *Pythium* species in the future.

MATERIALS AND METHODS

Isolates examined

Table 1 describes the 102 isolates used in this study, mainly the ex-type cultures from the original descriptions or the authentic strains described in van der Plaats-Niterink (1981).

DNA extraction

Mycelium from 5–14 d old liquid cultures (pea broth, de Cock *et al*. 1992) was harvested by vacuum filtration and the DNA was extracted following the protocols of Möller *et al*. (1992).

DNA amplification

Some isolates were sequenced using the protocol of Lévesque *et al*. (1998). Universal eukaryotic primers UN-UP18S42 (5′-CGTAACAAGGTTTCCGTAGGTGAAC-3′) with UN-LO28S576B (5′-CTCCTTGGTCCGTGTTTCAAGACG-3′) or UN-LO28S22 (5′-GTTTCTTTTCCTCCGCTTATTGATATG) were used to prepare the sequencing template by amplifying the internal transcribed spacer (ITS) regions and the 5.8S gene of nuclear ribosomal DNA (Bakkeren, Kronstad & Lévesque 2000). The UN-UP18S42 and UN-LO28S576B pair amplified the ITS region and part of the D1 region of the LSU rDNA. DNA was amplified by polymerase chain reaction (PCR) in volumes of 25 μl with 2 units of AmpliTaq polymerase (Applied Biosystems, Foster City, CA), 0.8 μM of each primer and 1.5 μM $MgCl_2$. Samples were placed in a DNA GENE AMP 2400 (Applied Biosystems) thermal cycler at 95 ° for 3 min for DNA denaturation, after which 31 cycles were performed as follows: 45 s at 95 °, 30 s at 68 °, and 1 min at 72 ° with a final 10 min extension at 72 °. PCR product was purified using a QiaQuick kit (Qiagen, Chatsworth, CA) and quantified by visual comparison to a low DNA mass ladder (Gibco BRL, Gaithersburg, MD) following electrophoresis on a 1.75% agarose gel stained with ethidium bromide (0.25 μg ml^{-1}).

Sequencing. Purified template DNA (30–50 ng) was sequenced according to manufacturer protocol (Big Dye Terminator, Applied Biosystems) with the exception that the sequencing mix was diluted 1:3 with sequencing buffer (200 mM TrisHCl@pH 9.0; 5 mM $MgCl_2$) before use. The ITS-1 region was sequenced using a universal eukaryote forward primer (UN-UP18S42) and a reverse primer (OOM-LO5.8S47B = 5′-CGCATTACGTATCGCAGTTCGCAG-3′), while the ITS-2 region was sequenced with a forward primer (OOM-UP5.8S01 = 5′-CAACTTTCAGCAGTGGATGTCT-3′) and a reverse primer (PY-LO28S22 = 5′-GTTTCTTTTCCTCCGCTTATTAATATG-3′) (Mazzola *et al*. 2002). These primers perfectly match the corresponding rDNA sequences of *Pythium* species. Reactions were placed in a DNA GENEAMP 2400 thermal cycler (Applied Biosystems) for 25 cycles of 15 s at 95 ° and 4.5 min at 60 °. Extension products were purified by ethanol precipitation (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems) and resuspended in Template Suppression Reagent before being transferred into an Applied Biosystems Prism Genetic Analyzer (model 310) for electrophoresis and analysis. LSU sequencing

was done with the same DNA preps through a collaborative project with Applied Biosystems using proprietary primers, some of which are available in the MicroSeq® D2 LSU rDNA Fungal Sequencing Kit (Applied Biosystems).

Sequence analysis

Sequences were edited using Sequence Navigator version 1.01 or Autoassembler 2.1.1 (Applied Biosystems). Pileup (GCG, Madison, WI, Version 10) or MegAlign (DNASTAR, Madison, WI; version 5.06), were used to perform final alignments for analysis. Analyses of the ITS region or LSU rDNA was performed with PAUP version 4.0b10 software (Swofford 2001) using the heuristic search with random stepwise additions. Gaps were treated as missing data. A bootstrap 50% majority-rule consensus tree was generated using 1000 replications. Using the taxa for which both the ITS region and the LSU were sequenced, the Wilcoxon signed ranked test was applied to test for the congruence between the trees given by both data sets (Johnson & Soltis 1998) and to determine the appropriateness of a combined analysis. This was done with PAUP and the 'describe tree option'. The maximum parsimony tree was compared against a strict consensus tree at minimum 70% bootstrap values to test for the main differences.

The sequences from this study were compared to data in GenBank. In order to make sure that all relevant *Pythium* sequences in GenBank were downloaded, two search strategies were used in Oct. 2003. BLAST searches using some widely different sequences from this study were performed, and Entrez searches with keywords for genus and sequence definition fields were done. The search gave 309 ITS sequences, some of which encompassed the entire region (ITS-1, 5.8S and ITS-2), but many were only partial (mostly ITS-1 but a few were ITS-2 only). An alignment using MegAlign and a UPGMA analysis using PAUP with all the ITS sequences from GenBank and those from this study was performed. As an additional step to match closely related sequences, an internal BLAST database was created with all ITS sequences from this study and from GenBank. All sequences were searched by BLAST against each others. Using the results from the internal BLAST searches and the large UPGMA analysis, the ITS sequences were divided into groups corresponding to the phylogenetic clades. Alignment and UPGMA analyses with the sequences from GenBank and from this study were performed for each group of sequences that represented each phylogenetic clade. In several cases we used a long sequence as representative of other GenBank number(s) when a short partial sequence was 100% homologous to a longer one. For the LSU rDNA data, nine partial D1 and D2 sequences of *Pythium* were found in GenBank. They were aligned with all the sequences from this study using Megalign and analyzed by UPGMA.

RESULTS AND DISCUSSION

The ITS region including the 5.8S gene was between 750 and 1050 bp whereas the length of the alignment was 1664. The partial LSU amplified was between 1447 and 1469 in length. The phylogenetic analyses of the ITS region and the LSU divided the *Pythium* strains representing the different species in the genus into 11 clades which were consistent between the analyses for the two rDNA regions (Figs 1–2), the former giving higher bootstrap values with the small clusters and the latter giving higher bootstrap values for the larger clades. A combined analysis for the strains that had both ITS and LSU data was performed because the Wilcoxon signed-ranked test comparing the ITS and LSU data revealed no significant difference between these trees (p-value = 0.16). The combined analysis gave high bootstrap values for most clades (Fig. 3). The analyses revealed the existence of 11 major clades, designed A–K on Figs 1–3, and corresponding with the analyses of all available sequences for each of these clades in Fig. 5A–K.

There were 309 ITS sequences of *Pythium* in GenBank that could be unambiguously fitted into one of the 11 clades from this study, with the exception of two (GI 11120579 and GI 11120584) which are discussed below (Fig. 5). There were nine LSU sequences in GenBank that aligned with ones from this study (Fig. 6).

The number of morphological characters suitable to identify and describe *Pythium* species is relatively limited. As Matsumoto *et al*. (1999) and Martin (2000), we found that in *Pythium* the ornamentation of oogonia and heterothallism could be acquired or lost easily through evolution (Fig. 4). These characters traditionally used in species separation as a first step in taxonomic keys did not correlate with the major clades. The shape of sporangia correlated well with the major clades and some other characters concurred with the smaller clusters (examples discussed below). No correlation was evident with geographic distribution. Most (if not all) common species seem to be distributed worldwide, though it is not known if this ubiquity is natural or due to movement of plant and soil by human activities. Species of restricted occurrence are often rare or newly discovered.

Martin (2000) examined the phylogenetic relationships of 24 species of *Pythium* based on the partial sequencing of the mitochondrial cytochrome oxidase II gene. The phylogeny based on ITS sequences in the present paper agrees well with the phylogeny based on coxII sequences. The clusters I, II, IIIA, IIIB, IIIC and IIIC in the paper by Martin (2000) correspond to the clusters E–G, I, D, B, C and A in the present paper, respectively.

The basal branching in our gene trees of *Pythium* species separated the *P. vexans* group (clade K; Figs 1–4). The common ancestor therefore most likely had globose sporangia. There has been some debate

Table 1. Isolates used in this study, their status, host or substrate and geographic origin, CBS accession number, and other relevant reference numbers.

Clade	CBS no.	Status[a]	Species name	Host/substrate	Locality	Other collections[b]	GenBank accession no.
A	CBS 520.74	–	*P. adhaerens*	soil	The Netherlands		AY598619[c]
A	CBS 369.79	PN	*P. porphyrae*	*Porphyra yezoensis*	Japan	IFO 30347	AY598673[d]
A	CBS 203.85	T	*P. chondricola*	*Chondrus crispus*	The Netherlands		AY598620[c]
A	CBS 314.33	A/T	*P. deliense*	*Nicotiana tabacum*	Sumatra	ATCC 12280	AY598674[d]
A	CBS 118.80	PN	*P. aphanidermatum*	unknown	unknown		AY598622[c]
A	CBS 158.73	PN	*P. monospermum.*	unknown	unknown		AY598621[c]
B1	CBS 522.74	PN	*P. angustatum*	soil	The Netherlands		AY598623[c]
B1	CBS 842.68	PN	*P. catenulatum*	turf grass	SC, USA		AY598675[d]
B1	CBS 316.33	PN	*P. torulosum*	grass roots	The Netherlands	BR 489	AY598624[c]
B1	CBS 220.94	T	*P. folliculosum*	soil	Switzerland		AY598676[d]
B1	CBS 223.88	–	*P. conidiophorum*	soil	UK		AY598629[c]
B1	CBS 471.50	PN	*P. salpingophorum*	*Lupinus angustifolius*	Germany	BR 669	AY598630[c]
B1	CBS 323.65	T	*P. tracheiphilum*	*Lactuca sativa*	Italy		AY598677[d]
B1	CBS 254.70	PN	*P. myriotylum*	*Arachis hypogaea*	Israel		AY598678[d]
B1	CBS 216.82	–	*P. zingiberis*	*Zingiber mioga*	Japan		AY598679[d]
B1	CBS 294.37	A	*P. scleroteichum*	*Ipomoea batatas*	USA		AY598680[d]
B1	CBS 155.64	T	*P. dissimile*	*Pinus radiata* seedlings	S. Australia	ATCC 16515	AY598681[d]
B1	CBS 603.73	T	*P. sulcatum*	*Daucus carota*	WI, USA	ATCC 24736, BR 652	AY598682[d]
B1	CBS 327.62	PN	*P. graminicola*	*Saccharum officinarum*	Jamaica	IMI 91329, BR 170	AY598625[c]
B1	CBS 169.68	PN	*P. periilum*	soil	FL, USA	IMI 120405	AY598683[d]
B1	CBS 168.68	PN	*P. inflatum*	*Saccharum officinarum*	LA, USA	IMI 120417	AY598626[c]
B1	CBS 100530	T	*P. plurisporium*	*Agrostis palustris*	NC, USA	ATCC 200024	AY598684[d]
B1	CBS 295.37	T	*P. vanterpoolii*	*Triticum sativum*	UK	BR 488	AY598685[d]
B1	CBS 699.83	–	*P. volutum*	snow rot of *Triticum* and *Hordeum*	Japan	IMI 280171	AY598686[d]
B1	CBS 263.38	T	*P. aristosporum*	*Triticum aestivum*	Canada	ATCC 11101, BR 136	AY598627[c]
B1	CBS 324.62	*1	*P. arrhenomanes*	*Zea mays*	WI, USA	IMI 96342, ATCC 12531	AY598628[c]
B1/2	CBS 158.64	T	*P. pyrilobum*	*Pinus radiata*	S. Australia	ATCC 16516, BR 472	AY598636[c]
B2	CBS 772.81	–	*P. apleroticum*	*Nymphoïdes peltata*	The Netherlands		AY598631[c]
B2	CBS 215.80	PN	*P. aquatile*	unknown	unknown	BR 654	AY598632[c]
B2	CBS 227.88	T	*P. pachycaule*	soil	UK	IMI 308330	AY598687[d]
B2	CBS 154.64	T	*P. coloratum*	nursery soil	S. Australia	BR 677	AY598633[c]
B2	CBS 222.88	T	*P. lutarium*	soil	UK	IMI 308163	AY598688[d]
B2	CBS 750.96	–	*P. marinum*	soil	UK	IMI 308171	AY598689[d]
B2	CBS 166.68	PN	*P. dissotocum*	wheat roots	OH, USA	IMI 120408	AY598634[c]
B2	CBS 664.79	PN	*P. diclinum*	*Beta vulgaris*	The Netherlands		AY598690[d]
B2	CBS 222.94	A	*P. capillosum*	cultivated soil	France		AY598635[c]
B2	CBS 234.72	T	*P. flevoense*	soil	The Netherlands	IMI 176045	AY598691[d]
C	CBS 286.79	T	*P. grandisporangium*	*Distichilis spicata*	FL, USA	ATCC 28295	AY598692[d]
C	CBS 211.85	*2	*P. grandisporangium*	decaying leaf of *Zostera marina*	The Netherlands		AY598716[d]
C	CBS 574.85	T	*P. insidiosum*	horse	Costa Rica	ATCC 58643	AY598637[c]
D	CBS 377.34	PNb	*P. acanthicum*	*Solanum tuberosum*	Sweden	BR 228	AY598617[c]
D	CBS 289.31	T	*P. periplocum*	*Citrullus vulgaris*	USA		AY598670[d]
D	CBS 552.88	A	*P. amasculinum*	soil vegetable garden	China	ATCC 52092	AY598671[d]
D	CBS 253.60	PN	*P. hydnosporum*	unknown	Germany		AY598672[d]
D	CBS 382.34	PN	*P. oligandrum*	*Viola* sp.	UK		AY598618[c]
E1	CBS 549.88	A	*P. acrogynum*	soil under *Spinaceae oleraceae*	China		AY598638[c]
E1	CBS 234.94	–	*P. hypogynum*	soil	France		AY598693[d]
E1	CBS 281.64	PN	*P. echinulatum*	soil forest nursery	S. Australia		AY598639[c]
E1	CBS 505.80	T	*P. erinaceus*	wheatfield soil	New Zealand		AY598694[d]
E1	CBS 217.94	T	*P. radiosum*	cultivated soil	France		AY598695[d]
E1	CBS 533.74	PN	*P. rostratum*	soil	The Netherlands	BR 649	AY598696[d]
E2	CBS 528.74	PN	*P. middletonii*	soil	The Netherlands		AY598640[c]
E2	CBS 470.50	T	*P. multisporum*	soil	IL, USA		AY598641[c]
E2	CBS 225.88	T	*P. parvum*	soil	UK		AY598697[d]
E2	CBS 776.81	–	*P. pleroticum*	*Nymphoides peltata*	The Netherlands		AY598642[c]
E2	CBS 226.88	T	*P. minus*	soil	UK	IMI 308176	AY598698[d]

Table 1. (*Cont.*)

Clade	CBS no.	Status[a]	Species name	Host/substrate	Locality	Other collections[b]	GenBank accession no.
E2	CBS 773.81	–	*P. marsipium*	*Nymphoides peltata*	The Netherlands		AY598699[d]
F	CBS 550.88	T	*P. kunmingense*	soil under *Vicia faba*	China		AY598700[d]
F	CBS 275.67	PN	*P. spinosum*	compost	The Netherlands		AY598701[d]
F	CBS 218.94	T	*P. cylindrosporum*	soil	Germany		AY598643[c]
F	CBS 250.28	PN	*P. irregulare*	*Phaseolus vulgaris*	The Netherlands	BR 486	AY598702[d]
F	CBS 251.28	PN	*P. mamillatum*	*Beta vulgaris*	The Netherlands	BR 648	AY598703[d]
F	CBS 157.64	PN	*P. paroecandrum*	soil	Australia	BR 601	AY598644[c]
F	CBS 453.67	T	*P. sylvaticum*	soil	GA, USA	ATCC 18196	AY598645[c]
F	CBS 752.96	–	*P. debaryanum*	*Tulipa* sp.	UK	IMI 173340 ATCC 48115	AY598704[d]
F	CBS 574.80	T	*P. macrosporum*	flower bulb	The Netherlands	BR 492	AY598646[c]
F	CBS 266.38	PN	*P. intermedium*	*Agrostis stolonifera*	The Netherlands	BR 487	AY598647[c]
F	CBS 132.37	*2	*Brevilegnia macrospora*	*Viola tricolor*	The Netherlands	ATCC 11270, IMI 346214	AY598717[d]
G	CBS 779.96	–	*P. nagaii*	soil	UK	IMI 308183	AY598705[d]
G	CBS 159.64	PN	*P. violae*	soil	Australia	BR 322	AY598706[d]
G	CBS 178.76	PNb, *2	*P. violae*	*Daucus carota*	The Netherlands		AY598715[d]
G	CBS 156.64	PN	*P. iwayamai*	soil in pine plantation	S. Australia		AY598648[c]
G	CBS 698.83	–	*P. paddicum*	snow rot of *Triticum* and *Hordeum*	Japan	IMI 280172	AY598707[d]
G	CBS 315.81	T	*P. okanoganense*	*Triticum sativum*	USA	ATCC 38595	AY598649[c]
G	CBS 701.83	*2	*P. okanoganense*	root of *Triticum* and *Hordeum*	Japan	IMI 280168	AY598718[d]
H	CBS 285.31	A/T	*P. anandrum*	*Rheum rhaponticum*	USA		AY598650[c]
H	CBS 845.68	T	*P. prolatum*	*Rhododendron* sp.	GA, USA		AY598652[c]
H	CBS 393.54	A	*P. helicandrum*	*Rumex acetosella*	USA		AY598653[c]
H	CBS 406.72	T	*P. dimorphum*	*Pinus taeda*, roots	LA, USA	ATCC 22843	AY598651[c]
H	CBS 157.69	PN	*P. undulatum*	soil under *Pinus* sp.	AL, USA		AY598708[d]
I	CBS 450.67	T	*P. heterothallicum*	soil under *Sambucus*	AB, Canada	BR 491	AY598654[c]
I	CBS 462.48	PN	*P. splendens*	unknown	unknown		AY598655[c]
I	CBS 219.65	T	*P. ultimum* var. *sporangiiferum*	*Chenopodium album*	MD, USA	ATCC 13647, BR 650	AY598656[c]
I	CBS 398.51	PN	*P. ultimum* var. *ultimum*	*Lepidium sativum*	The Netherlands	BR 748	AY598657[c]
J	CBS 808.96	T	*P. nunn*	soil	CO, USA	IMI 324024	AY598709[d]
J	CBS 376.72	T	*P. orthogonon*	*Zea mays*	Lebanon	IMI 14351	AY598710[d]
J	CBS 337.29	A/T	*P. acanthophoron*	*Ananas sativus*	Hawaii		AY598711[d]
J	CBS 674.85	–	*P. perplexum*	*Vicia faba*	The Netherlands		AY598658[c]
J	CBS 288.31	T	*P. buismaniae*	*Linum usitatissimum*	The Netherlands		AY598659[c]
J	CBS 811.70	PN	*P. polymastum*	*Lactuca sativa*	The Netherlands		AY598660[c]
J	CBS 518.77	T	*P. uncinulatum*	*Lactuca sativa*	The Netherlands		AY598712[d]
J	CBS 375.72	PN	*P. mastophorum*	*Apium graveolens*	UK	IMI 133677	AY598661[c]
K	CBS 551.88	–	*P. boreale*	soil under *Brassica caulorapa*	China		AY598662[c]
K	CBS 768.73	PN	*P. ostracodes*	soil	Ibiza		AY598663[c]
K	CBS 292.37	A	*P. oedochilum*	–	USA		AY598664[c]
K	CBS 259.30	A/T	*P. chamaehyphon*	*Carica papaya*	Hawaii		AY598666[c]
K	CBS 286.31	A/T	*P. helicoides*	*Phaseolus vulgaris*	USA		AY598665[c]
K	CBS 748.96	–	*P. cucurbitacearum*	indet. host	Australia	IMI 333340	AY598667[c]
K	CBS 119.80	PN	*P. vexans*	soil	Iran	BR 484	AY598713[d]
K	CBS 261.30	PN	*P. indigoferae*	*Cucumis sativus*	India		AY598714[d]
outgroup	CBS 188.85	–	*Phytophthora avicenniae*	*Avicennia marina*	NSW, Australia	ATCC 64709	AY598668[c]
outgroup	CBS 680.84	T	*Phytophthora polymorphica*	leaf of *Eucalyptus* sp.	NSW, Australia	ATCC 56966	AY598669[c]

[a] Abbreviations and codes: T, ex-type strain; A, authentic strain, identified by the author of the species; A/T, authentic strain, probably used for original description; PN, strain used for description in the monograph of van der Plaats-Niterink (1981); PNb, strain examined in the monograph of the genus *Pythium* by van der Plaats-Niterink (1981) but not the strain for the description (1981); *1, ex-type strain according to van der Plaats-Niterink (1981), no confirmation in any of the collection databases; *2, Included in analyses in Fig. 5 only.

[b] Abbreviations: IFO, Institute for Fermentation, Osaka; ATCC, American Type Culture Collection, Manassas; IMI, CABI Bioscience, Egham; BR, culture numbers from the Canadian Collection of Fungal Cultures (CCFC), Ottawa.

[c] ITS-1, 5.8S and ITS-2; and D1, D2 and D3 of the LSU rDNA.

[d] ITS-1, 5.8S and ITS-2.

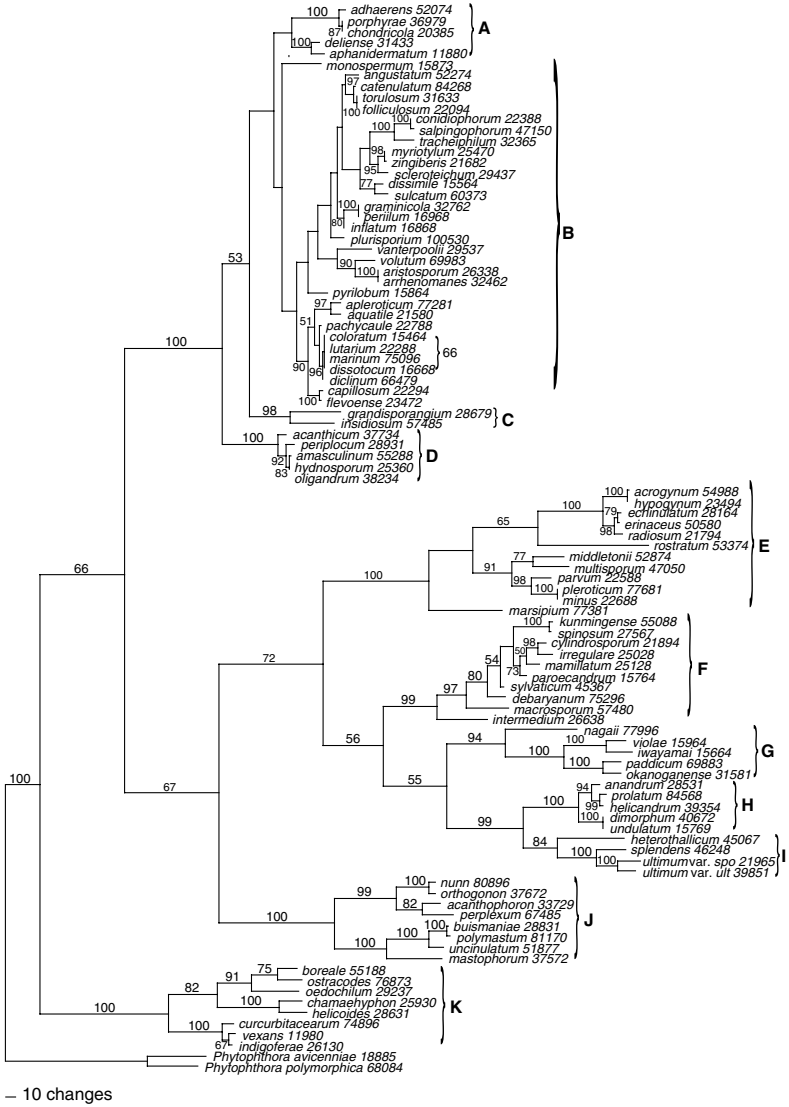

Fig. 1. Phylogeny of *Pythium* species based on ITS (1 and 2) and the 5.8S gene of nuclear rDNA. One of the 40 equally parsimonious trees of a heuristic search is shown. Numbers within the tree represent the bootstrap values (100 replications) and branches that had less than 50 % support are greyed out and show no bootstrap value. Length = 4350, CI = 0.445, RCI = 0.356, and RI = 0.800. Numbers beside species names are the CBS numbers without decimal point.

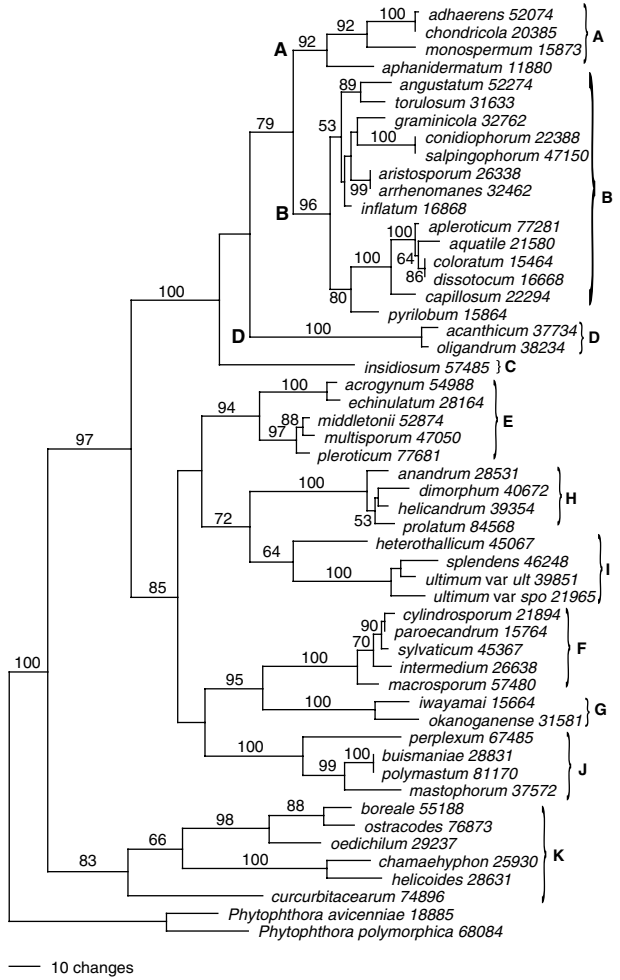

Fig. 2. Phylogeny of *Pythium* species based on the D1 to D3 regions of the nuclear large ribosomal subunit. One of the 104 equally parsimonious trees of a heuristic search is shown. Numbers within the tree represent the bootstrap values (1000 replications) and branches that had less than 50 % support are greyed out and show no bootstrap value. Length = 1286, CI = 0.461, RCI = 0.367, and RI = 0.797. Numbers beside species names are the CBS numbers without decimal point.

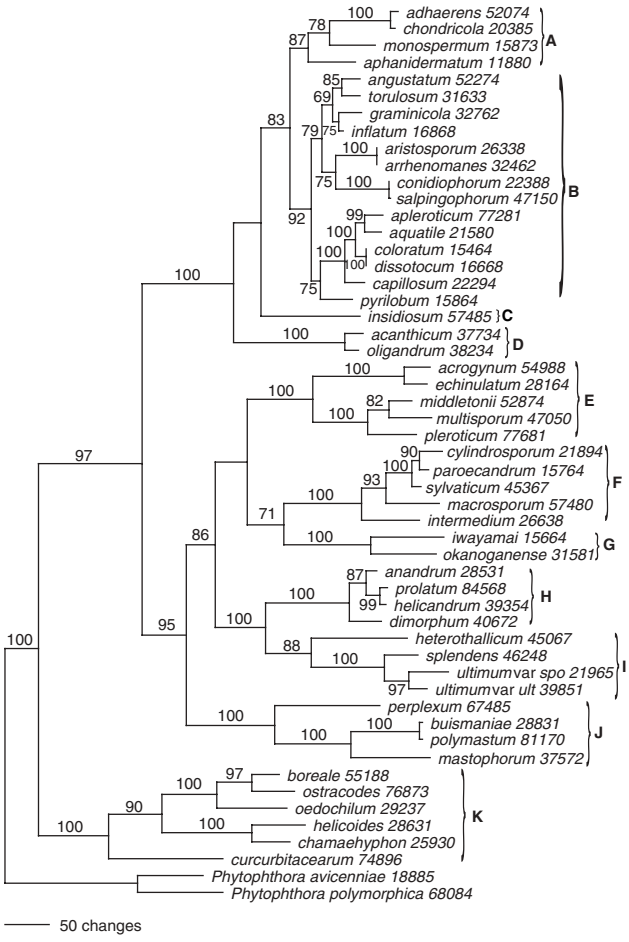

Fig. 3. Phylogeny of *Pythium* species based on the ITS region and the D1 to D3 regions of the nuclear large ribosomal subunit. The one tree produced by the heuristic search is shown. Numbers within the tree represent the bootstrap values (1000 replications) and branches that had less than 50% support are greyed out and show no bootstrap value. Length = 4546, CI = 0.502, RCI = 0.383, and RI = 0.764. Numbers beside species names are the CBS numbers without decimal point.

over whether or not the species belonging to clade K are appropriately classified in *Pythium* (Briard *et al.* 1995, Panabières *et al.* 1997, Dick 2001), and there are some similarities between these species and those of *Phytophthora*, such as the presence of elicitin genes (Panabières *et al.* 1997). The marine species *P. avicenniae* and *P. polymorphica* were chosen as outgroup instead of more common *Phytophthora* species because they form a clade between all *Pythium* species and the mostly non-marine *Phytophthora* species (data not shown). Our study was not designed to resolve the taxonomic status of clade K but in an analysis of the ITS or LSU data with all available *Pythium* and *Phytophthora* species, *Pythium* becomes polyphyletic because of clade K. Therefore, all the genera closely related to *Pythium* and *Phytophthora*, and a large sample of *Pythium* and *Phytophthora* species, should be included in a new study designed to solve the generic status of clade K.

The next and probably most significant evolutionary divergence separated the species with filamentous sporangia from the ones with globose sporangia (Figs 1–4). One smaller clade within the filamentous group includes several different types of sporangia although all are of the contiguous type (see section on clade D). If one considers that hyphal swellings could be a form of globose sporangium when zoospores have never been observed, the separation between globose and filamentous sporangia has only very few exceptions, which are discussed below (Fig. 4).

Clade A

This clade is heterogeneous, consisting of two small and quite different clusters (Fig. 4). The cluster with *Pythium adhaerens*, *P. porphyrae*, and *P. chondricola* is characterized by filamentous non-inflated sporangia, slow growth (5 mm d^{-1}) and 1–4 diclinous antheridia. They all originate from algae, with *P. adhaerens* also isolated from dicotyledons. *P. porphyrae* has only been found in Japan, and *P. chondricola* only in The Netherlands. *P. deliense* and *P.* aphanidermatum, the species in the other cluster, have filamentous inflated sporangia, fast growth (30 mm d^{-1} or more), and 1–2 monoclinous, often intercalary antheridia. Both affect dicotyledons in warmer regions. From the ITS analysis it is unclear whether or not *P. monospermum* belongs to this clade, but the LSU and the combined analyses put it unequivocally into clade A.

There was congruence between the strains from clade A and most of the ITS sequences in GenBank (Fig. 5A). However, several of these GenBank accessions are from a greenhouse survey compared to our database before submission (GI 179808-, series deposited by Moorman *et al.* 2002). *P. aphanidermatum* and *P. deliense* are close but clearly separated by RAPD analysis (Herrero & Klemsdal 1998). Two strains of *P. aphanidermatum* from GenBank are slightly different from the representative strain and have some sequence similarity to the representative strain of *P. deliense*; both are from as yet unpublished studies and are likely to be from Asia. These strains should be included in any study comparing *P. deliense* and *P. aphanidermatum*. *P. porphyrae* and *P. chondricola* are 100% identical in their ITS sequences (Fig. 1, Table 2). *P. porphyrae* and *P. chondricola* both have filamentous, non-inflated sporangia, similar sexual structures, and both come from marine substrates. The main differences are the considerably lower cardinal temperatures for growth, larger oogonia and aplerotic oospores in *P. chondricola* (de Cock 1986).

Clade B, subclade B1

This subclade consists almost entirely of species with filamentous-inflated sporangia and is comprised of clusters with some distinct characters.

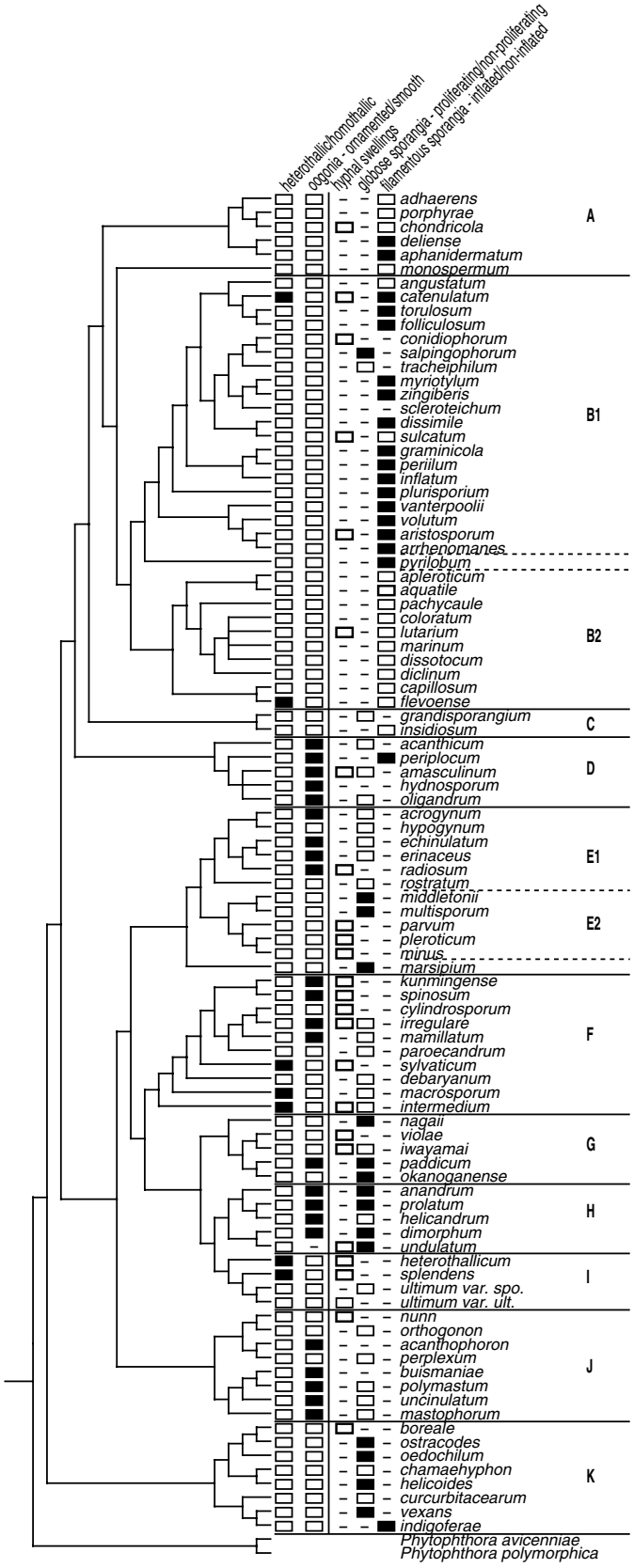

Fig. 4. Phylogenic tree of *Pythium* species based on the internal transcribed spacers (1 and 2) and the 5.8S gene from Fig. 1 correlated with morphological characters. The characters in each of the five columns are coded the following way. *Heterothallism*: heterothallic ■, homothallic □. *Oogonia*: ornamented oogonia ■, smooth oogonia □, no oogonia (-). *Hyphal swellings*: presence of hyphal swelling □, no hyphal swelling (-). *Globose sporangia*: proliferating globose sporangia ■, non-proliferating globose sporangia □, no globose sporangia (-). *Filamentous sporangia*: inflated filamentous sporangia ■, non-inflated filamentous sporangia □, no filamentous sporangia (-).

Cluster B1a

The species belonging to this cluster are *Pythium angustatum*, *P. catenulatum*, *P. torulosum* and *P. folliculosum*. They are species with filamentous inflated sporangia, except for *P. angustatum*, and they have a moderate growth rate (9–15 mm d^{-1}). These species have mostly been isolated from monocotyledons, bryophytes, green algae and soil, occasionally from dicotyledons and conifers. Their distribution is worldwide.

There were several GenBank accessions that belonged to this cluster (middle portion of Fig. 5B), including an LSU sequence deposited as *P. aphanidermatum* (Fig. 6; GI 13309832, Petersen & Rosendahl 2000) which perfectly matched the isolate of *P. torulosum* used for description in van der Plaats-Niterink (1981). However, there was no clear separation or clustering among the GenBank ITS sequences to correlate with *P. catenulatum*, *P. torulosum* and *P. folliculosum*. Indeed the *P. catenulatum* strain in our study seemed out of place, being so close to many *P. torulosum* sequences when compared to three other *P. catenulatum* ITS sequences from GenBank (GI 6468660, Matsumoto *et al.* 1999; GI 28883554 and GI 27448082). The issues of whether these three morphologically similar species are conspecific and whether or not CBS 842.68 is representative of *P. catenulatum* deserve further attention. *P. folliculosum* and *P. torulosum* are morphologically very similar and the only difference seems to be the oogonia, which are larger, sac-like, and may contain more than one oospore in *P. folliculosum*. The morphological difference between *P. catenulatum* and *P. torulosum* seems to be more significant; the former has larger oogonia (mean diam 22.8 *vs* 15 μm) and catenulate hyphal swellings. Though these hyphal swellings are mentioned as forming germ tubes and not zoospores (Matthews 1931, van der Plaats-Niterink 1981), they may be homologous to sporangia, which may have similar shapes. In addition *P. catenulatum* is heterothallic and *P. torulosum* is homothallic. There are enough ITS polymorphisms among the isolates from this and other studies to be able to look at heterozygosity in single oospores from dual cultures and so investigate if they are biological species.

Cluster B1b

The species in this cluster are *Pythium conidiophorum*, *P. salpingophorum*, and *P. tracheiphilum*. *P. conidiophorum* was considered a doubtful species name by van der Plaats-Niterink (1981), and isolates classified under this name were treated as *P. salpingophorum*. The isolate we used was obtained from IMI under the name *P. conidiophorum*. Its morphology was similar to that of *P. salpingophorum*, which is in agreement with the ITS similarity between the two species. This cluster seems out of place in clade B because the species included have globose rather than filamentous sporangia. The main reported differences between *P. salpingophorum* and *P. tracheiphilum* seem to be the presence of oogonia with plerotic oospores, without antheridia in the former species, and with chlamydospores in the latter species; these structures, however, may look very similar. Further study is required to confirm the close relationships between these two uncommon species and others with filamentous sporangia.

There were only two ITS sequences in GenBank that were related to this cluster (bottom of Fig. 5B). The isolate identified as *P. tracheiphilum* (GI 12863077) matched the ex-type sequence and an isolate identified as *P. salpingophorum* (GI 12863074) was very closely linked to the *P. conidiophorum*/*P. salpingophorum* cluster.

Cluster B1c

The species belonging to this cluster are *Pythium myriotylum*, *P. zingiberis*, *P. scleroteichum*, *P. dissimile*, and *P. sulcatum*. Except for *P. sulcatum*, all have filamentous and inflated sporangia, but most other characters are variable. They are mainly isolated from dicotyledons. The ITS region of *P. myriotylum* and *P. zingiberis* differs by 1 bp in ITS-2. Matsumoto *et al.* (1999) found identical ITS sequences for these two species and mentioned that Ichitani & Shinsu (1980) had reported differences in morphology, the number of antheridia, and the characteristics and sizes of oogonia. However, if the original descriptions are compared, the only difference between *P. myriotylum* and *P. zingiberis* seems to be in the oospores, given as aplerotic in the former and plerotic in the latter. The typical clusters of appressoria often formed in *P. myriotylum* have not been described in *P. zingiberis*.

There were many sequences deposited in GenBank that belonged to this cluster, several deposited as *P. myriotylum* and matching the strain described as such by van der Plaats-Niterink (1981) (top of Fig. 5). The two ITS sequences of *P. sulcatum* in GenBank (GI 6468685, Matsumoto *et al.* 1999; and GI 12863086) matched the ex-type sequence. The ITS sequence of *P. contiguanum* (Paul 2000b) was very close to that of the ex-type of *P. dissimile*. *P. contiguanum* was not compared to *P. dissimile* in the original species description, and *P. dissimile* produces what were described as 'complexes of lateral, globose or elongated irregular outgrowths of hyphae, simple or branched' by van der Plaats-Niterink (1981), and showed contiguous sporangia in her illustration (Fig. 26).

Clusters B1d and B1e

The species in these two clusters all have filamentous, inflated sporangia and moderately thick hyphae (up to 6.5 μm). They are mostly isolated from monocotyledons, predominantly grasses. The species from cluster B1d, *P. graminicola*, *P. periilum*, *P. inflatum*, and *P. plurisporium*, have relatively high cardinal temperatures

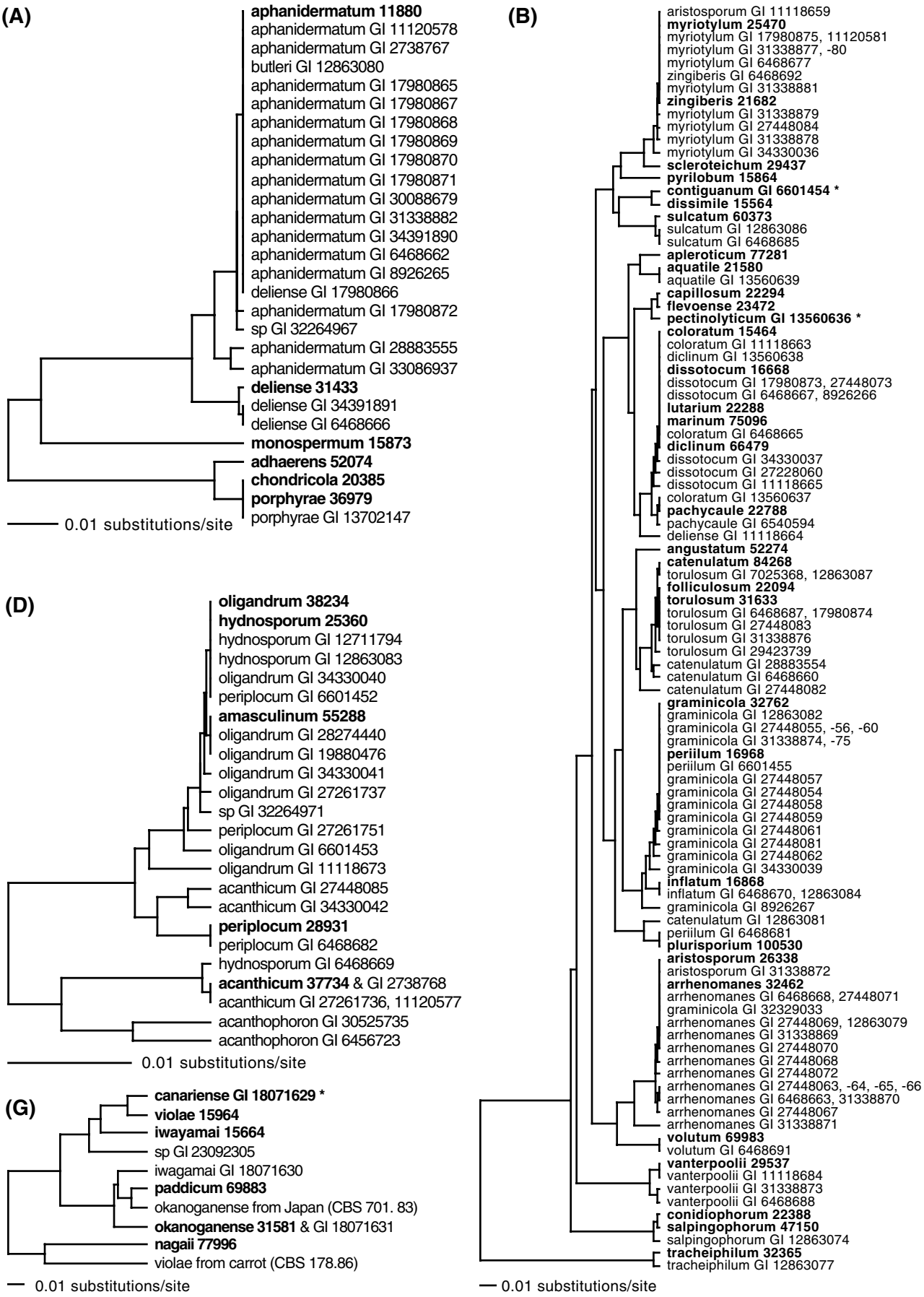
(A)
aphanidermatum 11880
aphanidermatum GI 11120578
aphanidermatum GI 2738767
butleri GI 12863080
aphanidermatum GI 17980865
aphanidermatum GI 17980867
aphanidermatum GI 17980868
aphanidermatum GI 17980869
aphanidermatum GI 17980870
aphanidermatum GI 17980871
aphanidermatum GI 30088679
aphanidermatum GI 31338882
aphanidermatum GI 34391890
aphanidermatum GI 6468662
aphanidermatum GI 8926265
deliense GI 17980866
aphanidermatum GI 17980872
sp GI 32264967
aphanidermatum GI 28883555
aphanidermatum GI 33086937
deliense 31433
deliense GI 34391891
deliense GI 6468666
monospermum 15873
adhaerens 52074
chondricola 20385
porphyrae 36979
porphyrae GI 13702147
0.01 substitutions/site
(B)
aristosporum GI 11118659
myriotylum 25470
myriotylum GI 17980875, 11120581
myriotylum GI 31338877, -80
myriotylum GI 6468677
zingiberis GI 6468692
myriotylum GI 31338881
zingiberis 21682
myriotylum GI 31338879
myriotylum GI 27448084
myriotylum GI 31338878
myriotylum GI 34330036
scleroteichum 29437
pyrilobum 15864
contiguanum GI 6601454 *
dissimile 15564
sulcatum 60373
sulcatum GI 12863086
sulcatum GI 6468685
apleroticum 77281
aquatile 21580
aquatile GI 13560639
capillosum 22294
flevoense 23472
pectinolyticum GI 13560636 *
coloratum 15464
coloratum GI 11118663
diclinum GI 13560638
dissotocum 16668
dissotocum GI 17980873, 27448073
dissotocum GI 6468667, 8926266
lutarium 22288
marinum 75096
coloratum GI 6468665
diclinum 66479
dissotocum GI 34330037
dissotocum GI 27228060
dissotocum GI 11118665
coloratum GI 13560637
pachycaule 22788
pachycaule GI 6540594
deliense GI 11118664
angustatum 52274
catenulatum 84268
torulosum GI 7025368, 12863087
folliculosum 22094
torulosum 31633
torulosum GI 6468687, 17980874
torulosum GI 27448083
torulosum GI 31338876
torulosum GI 29423739
catenulatum GI 28883554
catenulatum GI 6468660
catenulatum GI 27448082
graminicola 32762
graminicola GI 12863082
graminicola GI 27448055, -56, -60
graminicola GI 31338874, -75
periilum 16968
periilum GI 6601455
graminicola GI 27448057
graminicola GI 27448054
graminicola GI 27448058
graminicola GI 27448059
graminicola GI 27448061
graminicola GI 27448081
graminicola GI 27448062
graminicola GI 34330039
inflatum 16868
inflatum GI 6468670, 12863084
graminicola GI 8926267
catenulatum GI 12863081
periilum GI 6468681
plurisporium 100530
aristosporum 26338
aristosporum GI 31338872
arrhenomanes 32462
arrhenomanes GI 6468668, 27448071
graminicola GI 32329033
arrhenomanes GI 27448069, 12863079
arrhenomanes GI 31338869
arrhenomanes GI 27448070
arrhenomanes GI 27448068
arrhenomanes GI 27448072
arrhenomanes GI 27448063, -64, -65, -66
arrhenomanes GI 6468663, 31338870
arrhenomanes GI 27448067
arrhenomanes GI 31338871
volutum 69983
volutum GI 6468691
vanterpoolii 29537
vanterpoolii GI 11118684
vanterpoolii GI 31338873
vanterpoolii GI 6468688
conidiophorum 22388
salpingophorum 47150
salpingophorum GI 12863074
tracheiphilum 32365
tracheiphilum GI 12863077
0.01 substitutions/site
(D)
oligandrum 38234
hydnosporum 25360
hydnosporum GI 12711794
hydnosporum GI 12863083
oligandrum GI 34330040
periplocum GI 6601452
amasculinum 55288
oligandrum GI 28274440
oligandrum GI 19880476
oligandrum GI 34330041
oligandrum GI 27261737
sp GI 32264971
periplocum GI 27261751
oligandrum GI 6601453
oligandrum GI 11118673
acanthicum GI 27448085
acanthicum GI 34330042
periplocum 28931
periplocum GI 6468682
hydnosporum GI 6468669
acanthicum 37734 & GI 2738768
acanthicum GI 27261736, 11120577
acanthophoron GI 30525735
acanthophoron GI 6456723
0.01 substitutions/site
(G)
canariense GI 18071629 *
violae 15964
iwayamai 15664
sp GI 23092305
iwagamai GI 18071630
paddicum 69883
okanoganense from Japan (CBS 701. 83)
okanoganense 31581 & GI 18071631
nagaii 77996
violae from carrot (CBS 178.86)
0.01 substitutions/site

Fig. 5. (*Cont.*)

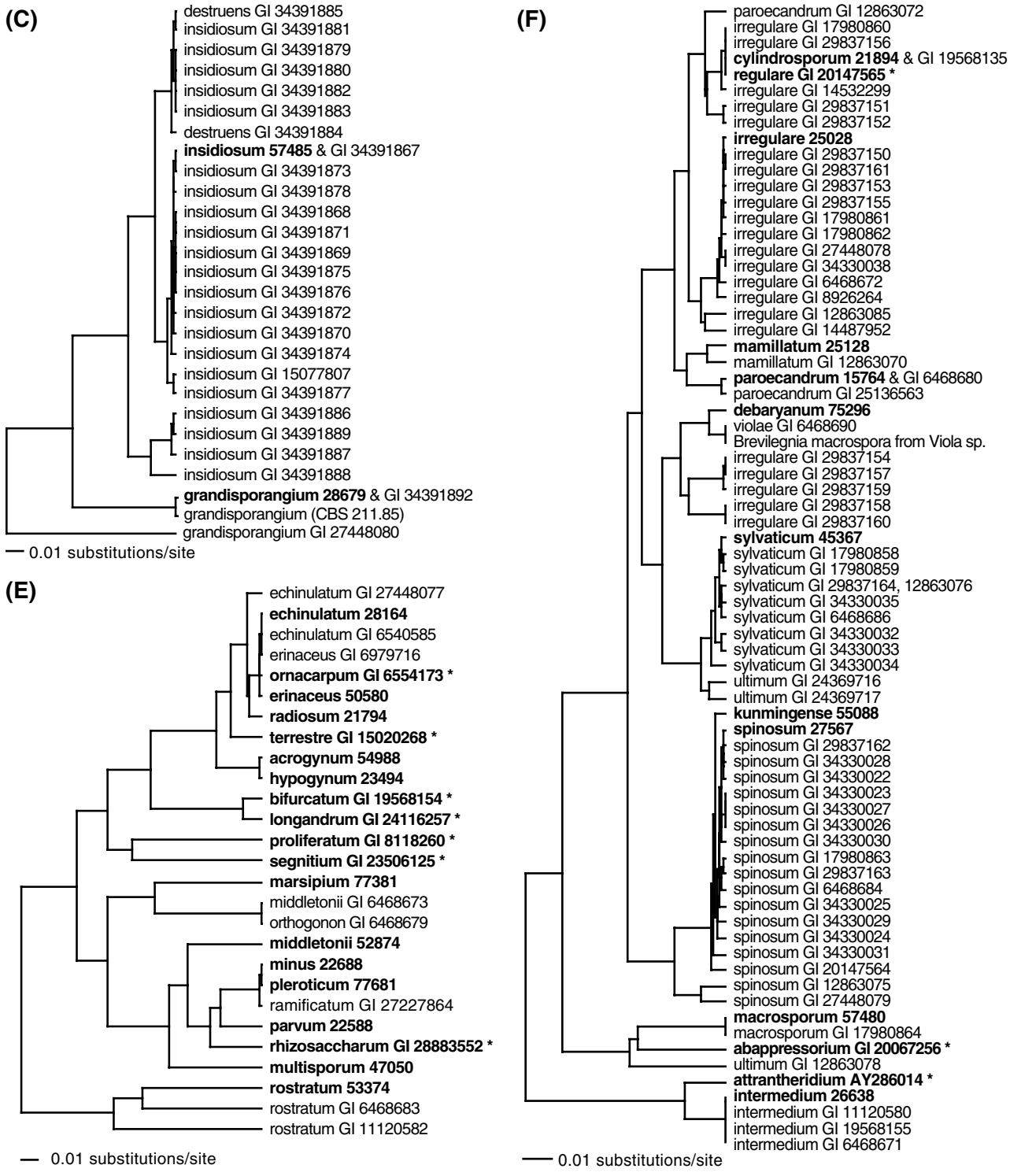

Fig. 5. (*Cont.*)

for growth (optimum *ca* 30 °, maximum *ca* 40 °) and occur worldwide. On the other hand, the species from cluster B1e, *P. vanterpoollii*, *P. volutum*, *P. aristosporum*, and *P. arrhenomanes*, have moderate cardinal temperatures (optimum *ca* 25 °, maximum *ca* 30 °) and occur mainly in the Northern Hemisphere.

There were many GenBank accessions within these clusters (bottom half of Fig. 5B). *P. graminicola* and *P. periilum* have identical ITS sequences. They are morphologically similar species; the main difference being the larger oogonia, and the presence of strictly filamentous elements in the sporangia of *P. periilum*. There was a large *P. graminicola* cluster of GenBank data including the GI 274480- series which was compared to the database of this study before being deposited by Heelan *et al*. in 2003. Two ITS sequences deposited in GenBank as *P. inflatum* (GI 6468670, Matsumoto *et al*. 1999; and GI 12863084) matched the

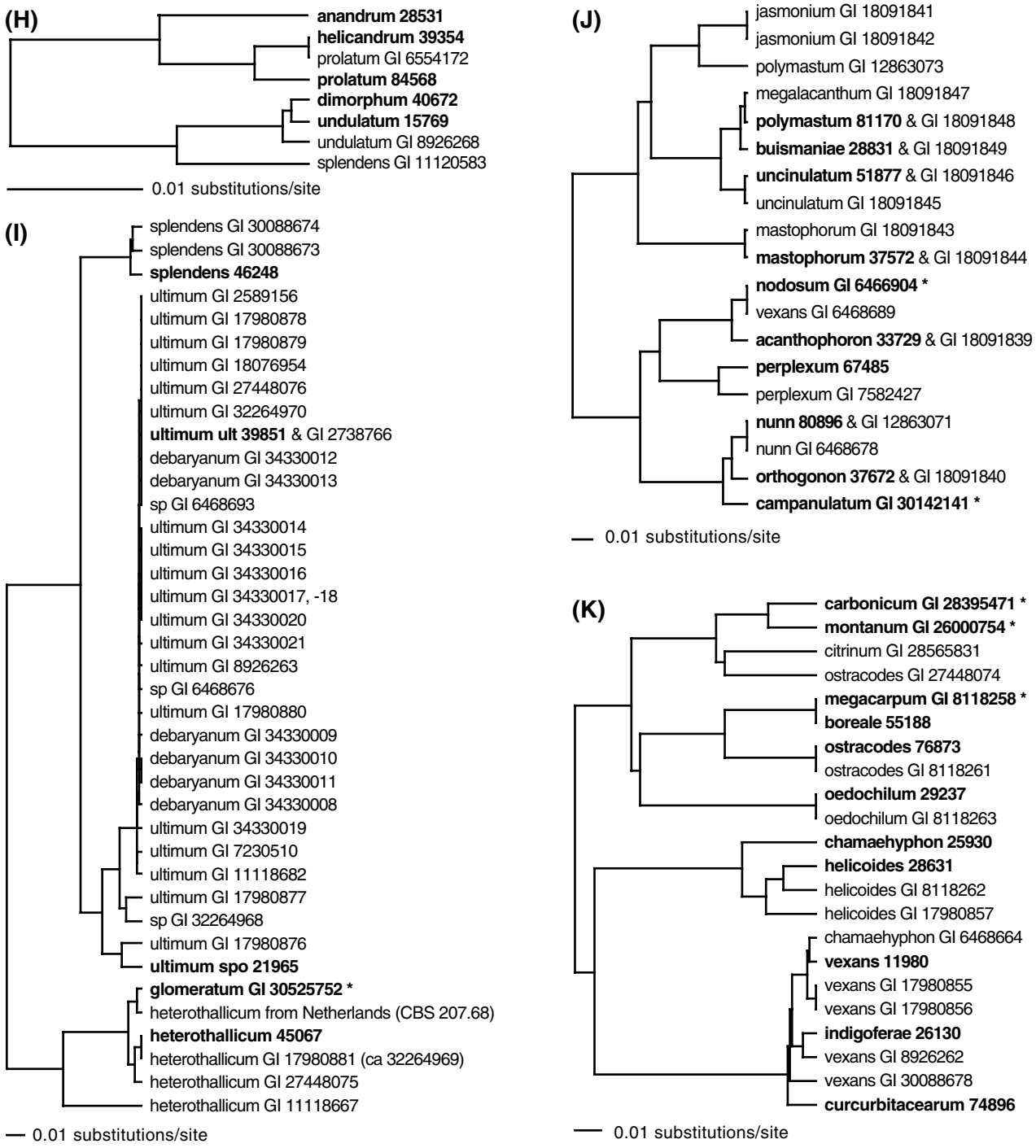

Fig. 5. UPGMA trees for each clade represented by a letter in Figs 1–3 to compare ITS sequences of representative strains (in bold with the CBS numbers without decimal point) against all available entries in Genbank (GI numbers). Recently described ex-type strains with their sequences deposited in Genbank are in bold with an asterisk after the GI number. Some entries with identical sequence or near identical (ca) are represented by two GI numbers or by the extensions of the last digits.

strain of this species described in van der Plaats-Niterink (1981). In GenBank there was an ITS sequence deposited as *P. periilum* (GI 6468681, Matsumoto *et al.* 1999), which was identical to *P. plurisporium* and an isolate deposited as *P. catenulatum* (GI 12863081), which also had a very similar ITS sequence to *P. plurisporium*.

The ex-type sequence of *P. arrhenomanes* matches that of another ex-type, *P. aristosporum*, a species described ten years after *P. arrhenomanes*. *P. arrhenomanes* and *P. aristosporum* are very similar, the main differences being the larger number of antheridia, which are only diclinous, and higher cardinal temperatures in *P. arrhenomanes*. One sequence of *P. aristosporum*

Table 2. *Pythium* species with mutually identical or near identical ITS sequences.

First described species[a]	ITS identical or very close to
P. porphyrae Takah. & Sasaki 1977 – PN	*P. chondricola* De Cock 1986 T
P. myriotylum Drechsler 1930 – PN	*P. zingiberis* M. Takah. 1954
P. dissimile Vaartaja 1965 – T	*P. contiguanum* B. Paul 2000 – T
P. torulosum Coker & P. Patt. 1927 – PN	*P. folliculosum* B. Paul 1991 – T
	P. catenulatum V. D. Matthews 1931
P. graminicola Subraman. 1928 – PN	*P. periilum* Drechsler 1940 PN
P. flevoense Plaäts-Nit. 1972 – T	*P. capillosum* B. Paul 1987 – A
	P. pectinolyticum. B. Paul 2001 – T
P. arrhenomanes Drechsler 1928 – T	*P. aristosporum* Vanterp. 1938 – T
P. salpingophorum Drechsler 1930 – PN	*P. conidiophorum* Jokl 1918[b]
P. dissotocum Drechsler 1930 – PN	*P. coloratum* Vaartaja 1965 – T
	P. lutarium Ali-Shtayeh 1985 – T
	P. marinum Sparrow 1934 – M. Dick
	P. diclinum Tokun. 1935 – PN
P. hydnosporum (Mont.) J. Schröt 1845 – PN	*P. oligandrum* Drechsler 1930 – PN
	P. amasculinum T. F. Yu 1973[b]
P. echinulatum V. D. Matthews 1931 – PN	*P. erinaceus* J. A. Robertson 1977 – T
	P. ornacarpum B. Paul 1999 – T
P. hypogynum Middleton 1943	*P. acrogynum* T. F. Yu 1973 – A[b]
P. pleroticum T. Itô 1944	*P. minus* Ali-Shtayeh 1985 – T
P. cylindrosporum B. Paul 1992 – T	*P. regulare* B. Paul 2003 – T
P. spinosum Sawada 1927 – PN	*P. kunmingens* T. F. Yu 1973 – T[b]
P. undulatum H. E. Petersen 1909 – PN	*P. dimorphum* F. F. Hendrix & W. A. Campb. 1971 – T
P. heterothallicum W. A. Campb. & F. F. Hendrix 1968 – T	*P. glomeratum* B. Paul 2003 – T
P. polymastum Drechsler 1939 – PN	*P. buismaniae* Plaäts-Nit. 1981 – T
P. boreale R. L. Duan 1985	*P. megacarpum* B. Paul 2000 – T

[a] PN, strain used for description in van der Plaats-Niterink (1981); T, ex-type strains.
[b] Doubtful or excluded by van der Plaats-Niterink (1981).

(GI 31338872) and 16 sequences deposited as *P. arrhenomanes* also matched the ex-type sequences from this study, including ten sequences from the GI 274480-series. The sequence of *P. volutum* (GI 6468691) from Matsumoto *et al.* (1999) perfectly matched the sequence with the same name in our study. Although there was up to 2% sequence variation among them, sequences deposited as *P. vanterpooli* in GenBank (GI 6468688, Matsumoto *et al.* 1999; GI 11118684 and GI 31338873) matched the ex-type sequence in our study.

Subclade B2

The species in this subclade are *Pythium apleroticum*, *P. aquatile*, *P. pachycaule*, *P. coloratum*, *P. lutarium*, *P. marinum*, *P. dissotocum*, *P. diclinum*, *P. capillosum*, and *P. flevoense*. This group consists of species with filamentous non-inflated to slightly inflated sporangia, smooth oogonia mostly smaller than 30 μm diam, and a moderate growth rate (mostly 10–20 mm d^{-1}). Except for *P. dissotocum*, which is commonly found in several greenhouse crops such as lettuce and tomato, most species seem to be uncommon. Some species such as *P. aquatile* are probably ubiquitous but underrepresented in collections because they do not cause plant diseases. They have been isolated from soil, algae, or *Pinus* roots and occasionally from dicotyledons. *P. pyrilobum* cannot be placed phylogenetically in either B1 or B2 based on the ITS data (Fig. 1), whereas it is in cluster B2 based on the LSU data (Fig. 2). According to the morphology of its sporangia, it would fit better in the B1 cluster (Fig. 4).

The ITS sequences of *P. coloratum*, *P. lutarium*, *P. marinum* and *P. dissotocum* are identical, and *P. diclinum* differed by 1 bp (Fig. 1). They are morphologically rather similar in sexual structures and they all have filamentous, slightly inflated sporangia, except *P. marinum*. Moreover, *P. marinum* also differs in having exclusively terminal oogonia, and only one antheridium per oogonium. The species was originally isolated from a marine substrate, and the identity of the isolate we used is doubtful as it is not from a marine site. However, the identification could not be verified as the isolate no longer forms sexual structures.

GenBank sequences falling in this cluster included seven identified as *P. dissotocum*, two as *P. coloratum*, and one as *P. diclinum* (top half of Fig. 5B). The species concept in this important group clearly needs to be investigated, ideally in a study with several highly variable genes. *P. pachycaule* is closely related to the group, with more than 97% homology. An ITS-1 sequence of *P. pachycaule* in GenBank (GI 6540594) was almost identical to the ex-type sequence of this species, as was another ITS-1 sequence (GI 13560637, Paul 2001b) that had been identified as *P. coloratum*. An ITS-1 sequence deposited as *P. deliense* (GI 11118664) was very close to *P. pachycaule* and *P. dissotocum*, but had a few base differences at the 5′ end that made it unique. An ITS-1 sequence of *P. aquatile* from GenBank (GI 13560639) was identical to the representative strain of this species,

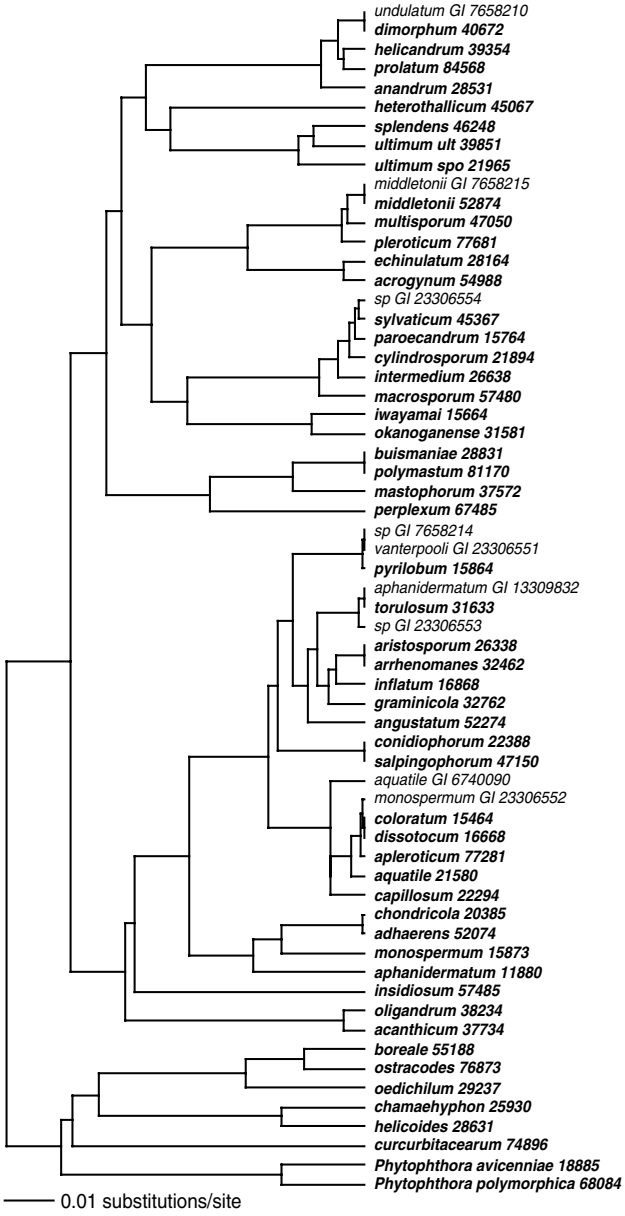

Fig. 6. UPGMA tree to compare the partial D1 and D2 sequences of the large ribosomal subunit from all available entries in Genbank (GI numbers) with sequences from this study (bold with the CBS numbers without decimal point).

whereas an LSU sequence (GI 6740090, Leclerc *et al.* 2000) was not (Fig. 6).

The homology for any of the pairwise comparisons between *P. capillosum*, *P. flevoense* and the new species *P. pectinolyticum* (Paul 2001b) is above 99%, suggesting that these species should be compared and re-evaluated for possible conspecificity. *P. flevoense* and *P. capillosum* were both isolated from soil, have non-inflated filamentous sporangia, one or more diclinous antheridia that are often entwined with the oogonium, thick-walled oospores of similar sizes, and a slow growth (<10 mm d^{-1}). The only difference is the position of the oogonia, terminal in the former and intercalary in the latter. *P. pectinolyticum* shares the characters mentioned above, but forms significantly larger oogonia and mostly lacks antheridia. Finally, for this subclade, two identical LSU sequences in GenBank (GI 23306551, Riethmüller *et al.* 2002; and GI 7658214, Riethmüller *et al.* 1999), identified as *P. vanterpooli* and *Pythium* sp., respectively, had more than 99% homology with the ex-type of *P. pyrilobum*, but our LSU data did not contain the sequence of *P. vanterpooli*.

Clade C

This clade consists of *Pythium grandisporangium* and *P. insidiosum*. *P. grandisporangium* has been isolated only from substrates in marine environments and *P. insidiosum* is a pathogen of mammals from warmer regions. There are few obvious morphological similarities between these two species, and their phylogenetic relatedness is therefore surprising. To confirm this, we sequenced another isolate of *P. grandisporangium* (CBS 211.85) and found that it differed by only 1 bp with the ex-type sequence of *P. grandisporangium*. Most of the ITS sequences in GenBank from clade C (Fig. 5C) come from Schurko *et al.* (2003b). The ITS sequence of one *P. grandisporangium* isolate (GI 27448080) shows less than 70% sequence homology with the ex-type of *P. grandisporangium*.

Clade D

The species that belong to clade D are *Pythium acanthicum*, *P. periplocum*, *P. amasculinum*, *P. hydnosporum*, and *P. oligandrum*. The species in this clade are characterized by fast growth (25–35 mm d^{-1}) and oogonia ornamented with acute spines. Antheridia are (mostly) absent in *P. oligandrum* and *P. amasculinum*, but where they are present, they are entirely attached to the oogonium and are constricted, except in *P. hydnosporum*, where they are hypogynous. Sporangia are contiguous, consisting of (sub-)globose elements that are connected by hyphal segments in *P. acanthicum* and *P. oligandrum*. Sporangia of *P. periplocum* were described by van der Plaats-Niterink (1981) as being 'filamentous inflated', however, this may be a misinterpretation of a contiguous type. Zoospore production has not been observed in *P. amasculinum* and *P. hydnosporum*; however, both those species produce contiguous hyphal swellings that are morphologically similar to the sporangia of the other species. Besides being pathogens of mainly dicotyledons, *P. acanthicum*, *P. periplocum*, and *P. oligandrum* are mycoparasites. These species have been found worldwide, except for *P. amasculinum*, which was isolated only once.

The ITS sequences from GenBank related to clade D were separated into five clusters (Fig. 5D). One contained sequences identical or very closely related to the *P. oligandrum*/*amasculinum*/*hydnosporum* from this study, a group that would need to be evaluated further for possible conspecificity. Sequences within this cluster

all belonged to these three species, except for two sequences of *P. periplocum* (GI 27261751; and GI 6601452, Paul 2000a). *P. hydnosporum* and *P. oligandrum* are similar in their relatively high cardinal temperatures for growth, and spiny oogonia. According to van der Plaats-Niterink (1981), the main difference is in the absence of sporangia and the more slender and pointed oogonial spines in the former species. *P. amasculinum* differs mainly in the plerotic oospores and the complete absence of antheridia (van der Plaats-Niterink 1981). Closely related to this cluster is *P. periplocum* from this study, which had an identical sequence to the isolate of this species from Matsumoto *et al*. (1999). In between the *P. periplocum* and the *P. oligandrum*/*amasculinum*/*hydnosporum* clusters were two isolates identified as *P. acanthicum* (GI 17980855, Moorman *et al*. 2002; and GI 34330042) which may be a new species if not part of the diversity within *P. periplocum*. The ITS sequence of *P. acanthicum* CBS 377.84 from this study was identical to two sequences in GenBank (GI 27261736 and GI 11120577). The ITS sequence from an isolate identified as *P. hydnosporum* (GI 6468669, Matsumoto *et al*. 1999) was almost identical to these *P. acanthicum* sequences. Two isolates labeled as *P. acanthophoron* (GI 6456723 and GI 30525736), however, were different from the representative strain which is in clade J, and could be a new species.

Clade E

Clade E is split into two subclades which could be accorded clade status and letters of their own. However, we decided to treat E1 and E2 as subclades because *Pythium marsipium* is linked to these two subclades but is not in either one of them (Fig. 4). Subclade E1 contains *P. acrogynum*, *P. hypogynum*, *P. echinulatum*, *P. erinaceus*, *P. radiosum*, and *P. rostratum* (Fig. 4). This group is characterized by ovoid to elongate, non-proliferating sporangia; mostly a single, hypogynous antheridium; plerotic oospores, and slow growth (7–12 mm d^{-1}). The species of this subclade have been mostly isolated from soil. *P. echinulatum* and *P. rostratum* are distributed worldwide, but the other species have been found only once.

P. middletonii, *P. multisporum*, *P. parvum*, *P. pleroticum* and *P. minus* occur in subclade E2 (Fig. 4). Sporangia were never observed in half of these species but globose hyphal swellings are present, while proliferating sporangia have been seen in the other species. The oogonia are smooth and of various sizes. These species have been mostly isolated from water, debris, or soil. *P. middletonii* occurs worldwide, but the other species have been isolated only once or rarely.

GenBank contained several ITS sequences from new species recently described that fall into cluster E (Fig. 5E). *P. ornacarpum* (Paul 1999) differs from *P. echinulatum* and *P. erinaceus* by 1 bp. Morphology and growth characteristics of these three species are very similar. Indeed, the only distinguishing features are the absence of zoospore production (which may be a matter of culture condition), antheridial stalks, which occasionally surround the oogonium in *P. ornacarpum*, and the absence of hypogynous antheridia in *P. erinaceus*. The possible conspecificity of these species, including the closely related *P. terrestre* (Paul 2002d) and *P. radiosum*, should be examined. A study of all the strains with sequences deposited in GenBank would be a good start. *P. proliferatum* (Paul 2002a) and *P. segnitium* (Paul 2002c) are genetically distant from each other and are also distant from any of the species from this study (Fig. 5E). The two new species *P. bifurcatum* (Paul 2003a) and *P. longandrum* (Paul 2001a) have 97.7% ITS homology and are genetically distant from any other species. *P. rhizosaccharum* (Singh *et al*. 2003) has only a 92% homology with *P. parvum*. *P. minus* and *P. pleroticum* from this study had identical ITS sequences and differed by one base in the ITS-2 from a sequence deposited as *P. ramificatum* (GI 27227864). *P. pleroticum* resembles *P. minus* in some respects, however, they differ significantly with regard to the size of the oogonia and hyphal swellings, the size ranges only having a small overlap. *P. acrogynum* and *P. hypogynum* from this study had 99% ITS homology. *P. acrogynum* was considered as a synonym of *P. hypogynum* by van der Plaats-Niterink (1981). The difference between the two species is in the presence of papillae on the oogonia and the larger, more irregular antheridia in *P. acrogynum*. There is wide genetic variation in *P. rostratum* isolates, supporting the view that this species is heterogeneous and needs to be morphologically revised. The isolates identified as *P. orthogonon* (GI 6468679, Matsumoto *et al*. 1999) and *P. middletoni* (GI 6468673) are identical, do not match any of the isolates from this study, and probably represent a new species (Fig. 5E). An LSU sequence deposited as *P. middletonii* (GI 7658215, Riethmüller *et al*. 1999) was identical to the sequence of the same species from this study (Fig. 6).

Clade F

This clade consists of important plant pathogens with a worldwide distribution, except for *Pythium kunmingense* and *P. cylindrosporum*, which have been rarely isolated. Most species do not or rarely produce zoospores. They produce either globose, non-proliferating sporangia or globose hyphal swellings (only *P. irregulare* develops both) and have fast growth (often more than 25 mm d^{-1}) and moderate cardinal temperatures (mostly 5–25–(30–35) °).

P. kunmingense, *P. spinosum*, *P. cylindrosporum*, *P. irregulare*, *P. mamillatum*, and *P. paroecandrum* form a subclade that is not strongly supported by the bootstrap value (Fig. 1). However, all these species, with the exception of *P. paroecandrum*, have oogonia with a varying number of blunt spines, mostly of a digitate shape, and different from the predominantly acute ornamentation on oogonia of the other ornamented species. All of the remaining species in clade F develop

smooth oogonia, and all except *P. debaryanum* are heterothallic (Fig. 4).

There are many ITS sequences in GenBank related to clade F (Fig. 5F). Most of the clusters contain at least one of the sequences from this study. There is heterogeneity in the *P. irregulare* group as expected from an extensive study on isozymes (Barr, Warwick & Désaulniers 1997) or with RAPD and rDNA (Matsumoto *et al*. 2000). The top cluster in Fig. 5F contains the ex-type strain of *P. cylindrosporum* even though most species within it are identified as *P. irregulare*. The ITS sequence of the new *P. regulare* (Masih & Paul 2003) is identical to *P. cylindrosporum* and isolates from this cluster should be studied to see if they fit the *P. cylindrosporum* description and are indeed different from *P. irregulare*. The morphology or molecular characteristics of *P. regulare* and *P. cylindrosporum* were not compared in the new species description (Masih & Paul 2003). The similarity in ITS sequence between the two species is surprising in view of the many morphological differences (e.g. in *P. regulare* the hyphal swellings and oogonia are larger, the oogonia are ornamented, and growth is slower). This cluster contained the sequences labelled as group II in Matsumoto *et al*. (2000). The next cluster contains 12 isolates identified as *P. irregulare* and contains the representative strain of this species. It included the sequences of group I in Matsumoto *et al*. (2000). Further down are two small groups of *P. irregulare* sequenced by Matsumoto *et al*. (2000) which represents their group III (GI 29837154, -57, -59) and group IV (GI 29837158, -60). Matsumoto *et al*. (2000) did observe morphological differences between some of these groups and our data support the fact that groups III and IV are indeed unique. The ITS sequences of *P. mamillatum* (GI 12863070) and *P. paroecandrum* (GI 25136563) matched the isolates described in van der Plaats-Niterink (1981), respectively. The ITS of the *P. paroecandrum* from this study had already been sequenced (GI 6468680, Matsumoto *et al*. 1999). Eleven isolates matched the ex-type strain of *P. sylvaticum*, and were all identified as such except for two named as *P. ultimum* (GI 24369716-7). *P. debaryanum* from this study (CBS 752.96) was close to a *P. violae* sequence (GI 6468690, Matsumoto *et al*. 1999; see under clade G for more details on this *P. violae* isolate). This group of strains needs to be compared with species from clade F to see if it is a new species, or if the name *P. debaryanum* should be used for this unique cluster. Seventeen ITS sequences deposited in GenBank as *P. spinosum* matched the isolate described in van der Plaats-Niterink (1981), although the ex-type of *P. kunmingense* is also closely related to these. *P. kunmingense* was differentiated from *P. spinosum* by its 'more regular ornamentation and larger numbers of finger-like protuberances on the oogonia' (van der Plaats-Niterink 1981). An ITS sequence deposited in GenBank as *P. macrosporum* perfectly matched that from the ex-type of this species. *P. abapressorium* (Paulitz, Adams & Mazzola 2003) has only 87% homology with the most closely related *P. macrosporum* from this study. A sequence deposited as *P. ultimum* (GI 12863078) is somewhat related to *P. macrosporum* and *P. abapressorium* but might be an isolate from a new species. Three sequences deposited in GenBank as *P. intermedium* perfectly matched the sequence from the isolate described in van der Plaats-Niterink (1981).

Clade G

Pythium nagaii, *P. violae*, *P. iwayamai*, *P. paddicum* and *P. okanoganense* belonged to this clade. Species of clade G have been isolated mainly from monocotyledons in Asia and the USA. *P. iwayamai* and *P. okanoganense* are known as snow mould fungi. Most species have ovoid, internally proliferating sporangia and smooth oogonia. The ornamentation on the oogonia of *P. paddicum* is unique, and not comparable to that of any other *Pythium* species. All other characters are variable.

There were very few GenBank sequences belonging to clade G. Two sequences from the CBS collection were added: CBS 178.86 isolated from carrot and identified as *P. violae*, and CBS 701.83 identified as *P. okanoganense*. Unlike most of the other clades where smaller clusters with identical sequences formed, all sequences in this clade were different (Fig. 5G). The closest match (ITS homology 95%) was between the newly described *P. canariense* (Paul 2002b) and *P. violae* from soil described in van der Plaats-Niterink (1981). However, this latter isolate did not match an isolate obtained from carrot and morphologically identified as *P. violae*, leaving some questions regarding the identity of the species commonly found on carrots. CBS 132.37, isolated from *Viola tricolor*, was sequenced to try to find a match from an isolate from *V. tricolor*, the species from which the type of the species was isolated. This strain is the ex-type strain of *Brevilegnia macrospora*, but is actually a *Pythium* with the morphology of *P. violae*. The ITS of this isolate was identical to *P. violae* (GI 6468690, Matsumoto *et al*. 1999), also from *Viola* sp. but located in clade F. A partial ITS-1 sequence identified as *P. violae* (GI 11120584) was very different from any other *Pythium* species from this study and from GenBank, but is somewhat related to clade F based on a short alignment (<60% homology, not included in analysis). The morphological concept of *P. violae* evidently needs revision; CBS 159.64, chosen by van der Plaats Niterink (1981) as representative of the species, is probably with hindsight an unfortunate choice. Morphological characters for identification and species boundaries of isolates belonging to clade G need an in depth investigation.

Clade H

The species in this clade were *Pythium anandrum*, *P. prolatum*, *P. helicandrum*, *P. dimorphum*, and

P. undulatum. Morphologically the group is very distinct, being characterized by very large, ovoid to elongate, internally proliferating sporangia and oogonia, ornamented with acute spines. Analyses of the ITS or LSU data that include *Phytophthora* species show that this clade is within *Pythium* (data not shown). Cardinal temperatures are moderate (approx. 5–25–35 °C for all species) and growth is rather fast (20–30 mm d^{-1}). *P. prolatum*, *P. helicandrum* and *P. dimorphum* have been isolated only a few times, but always in the USA and mainly from soil. The more common species *P. anandrum* and *P. undulatum* seem to have a worldwide distribution and have also been isolated from soil. *P. undulatum* has mostly been found in aquatic environments, whereas *P. anandrum* has been isolated from several dicotyledons.

Clade H had only three additional ITS sequences from GenBank. Sequence GI 8926268 (Cooke *et al.* 2000) helped create a small *P. undulatum* cluster which also included the ex-type of *P. dimorphum*. *P. undulatum* and *P. dimorphum* sequences from this study had 99% ITS homology and are two species that have thick-walled chlamydospores, a rare character in *Pythium* spp. Moreover, they have the same large, papillate sporangia. The only difference is that oogonia have been observed in *P. dimorphum* and not in *P. undulatum*. Since no oogonia are produced by *P. dimorphum* CBS 406.72, this does not seem to be a stable character. An LSU sequence deposited as *P. undulatum* (GI 7658210, Riethmüller *et al.* 1999) exactly matched the *P. dimorphum* from this study (Fig. 6). However, one isolate identified as *P. dimorphon* in GenBank (GI 11120579) did not match anything in our database or any other *Pythium* species in GenBank and could not be included in Fig. 5; the sequence seems to be closer to *Mortierella* spp. An isolate identified as *P. prolatum* (GI 6554172, Paul, Galland & Masih 1999) did not match the ex-type strain in this study, but perfectly matched *P. helicandrum* from van der Plaats-Niterink (1981). An isolate identified as *P. splendens* (GI 11120583) probably belongs to a new species, given its wide genetic distance from any other known species.

Clade I

The taxa belonging to this clade are *Pythium heterothallicum*, *P. splendens*, *P. ultimum* var. *ultimum*, and *P. ultimum* var. *sporangiiferum*. There are no obvious common morphological characters or origin for this clade. *P. ultimum* var. *sporangiiferum* is rare, while the other species have a worldwide distribution. Little is known about the pathogenicity of *P. heterothallicum*, but the other species are mainly pathogenic to dicotyledons. *P. ultimum* var. *sporangiiferum* is the only species developing sporangia and zoospores; the other three instead have hyphal swellings of various sizes. Both varieties of *P. ultimum* are homothallic, while the other two species are heterothallic.

Once the GenBank ITS sequences were added, Clade I was broken down into three main clusters, each representing one of three species from this study, i.e. *P. splendens*, *P. ultimum*, and *P. heterothallicum*. Twenty-seven isolates identified as *P. ultimum* or *P. debaryanum* were nearly identical in their ITS sequences. There were two small subclusters each of two isolates that warrants closer examination to see if these represent a new species. One of these contained the ex-type strain of *P. ultimum* var. *sporangiiferum*, but it is unlikely that morphological characters correlate consistently to this genetic grouping (Francis, Gehlen & St Clair 1994, Barr, Warwick & Désaulniers 1996). *P. heterothallicum* had some genetic variation; the isolate for GI 11118667, though related, might represent a distinct species. *P. glomeratum* (Paul 2003c) was within this cluster and was almost identical in ITS sequence to CBS 207.68 described by van der Plaats-Niterink (1981). Two isolates of P. splendens (GI 30088673-4) very closely matched the strain from this study.

Clade J

This clade contains *Pythium nunn*, *P. orthogonon*, *P. acanthophoron*, *P. perplexum*, *P. buismaniae*, *P. polymastum*, *P. uncinulatum*, and *P. mastophorum*. All species in this clade are uncommon, some of them only known from the original isolation. Two subclades can be recognized. The species in the first subclade, *P. nunn*, *P. orthogonon*, *P. acanthophoron*, and *P. perplexum*, seem to have no distinct characters in common. The second subclade comprises *P. buismaniae*, *P. polymastum*, *P. uncinulatum*, and *P. mastophorum* and is morphologicaly distinct from other *Pythium* spp. All species have large oogonia (with conical spines often placed laterally on short side branches), diclinous antheridia, thick-walled oospores, large globose sporangia (except *P. buismaniae*, which has no sporangia), and have moderate cardinal temperatures (5–25–30 °C).

In clade J, the GenBank sequences of *P. uncinulatum* (GI 18091845), *P. mastophorum* (GI 18091843), and *P. perplexum* (GI 7582427, Galland & Paul 2001) matched the corresponding taxa from this study. An isolate of *P. megalacanthum* (GI 18091847), a species for which the only type material is a slide from de Bary, was almost identical to *P. polymastum* and *P. buismaniae* from this study. Surprisingly, van der Plaats-Niterink (1981) did not compare her new species *P. buismaniae* with *P. polymastum*, although they are morphologically very similar; the only differences are the absence of sporangia and the lower growth rate in *P. buismaniae*. The isolate of *P. megalacanthum* (GI 18091847) may have been misidentified: the original strain of de Bary (1881) produced proliferating sporangia, monoclinous antheridia, and much smaller oogonia, which does not suggest a close relationship to *P. buismaniae* and *P. polymastum*. The ITS-1 sequence of *P. jasmonium* (nom. inval.) differed by at least 15% from any *P. polymastum* sequences in GenBank or from this study.

The *P. vexans* from Matsumoto *et al.* (1999) perfectly matched the sequence from the new species *P. nodosum* (Paul *et al.* 1998). Both species have large bell-shaped antheridia, as does another species in this cluster, *P. perplexum* (see under Cluster K below for more on *P. vexans*). There was a 10% difference between the ITS-1 sequences of *P. nodosum* and *P. acanthophoron* from this study. *P. nunn* (GI 6468678, Matsumoto *et al.* 1999) was identical to the ex-type strain of this study. The ITS region of the new species *P. campanulatum* (Mathew, Singh & Paul 2003) differed by 6% from the *P. orthogonon* ex-type from this study.

Clade K

The species in this clade are *Pythium boreale*, *P. ostracodes*, *P. oedochilum*, *P. chamaehyphon*, *P. helicoides*, *P. cucurbitacearum*, *P. vexans*, and *P. indigoferae*. A common character in this group is the ovoid shape of the sporangia, which often have a papilla and proliferate internally in most species. *P. boreale* does not produce zoospores but has hyphal swellings instead of sporangia. *P. indigoferae* is described as having filamentous inflated sporangia and does not seem to fit in this group. The isolate used, CBS 261.30, no longer sporulates (van der Plaats-Niterink 1981), so its identity could not be verified morphologically. Another character, shared by all species, is the high optimum and maximum temperature for growth (approx. 30 ° and 35–40 ° respectively).

The clade is divided into two subclades. The first, consisting of *P. boreale*, *P. ostracodes*, *P. oedochilum*, *P. chamaehyphon*, and *P. helicoides*, is distinguished by large, smooth oogonia, thick-walled oospores, and mostly 1–2 antheridia, which are elongate and laterally applied to the oogonium. In the second, the isolate of *P. indigoferae* is questionable as explained above, but the other two species, *P. cucurbitacearum* and *P. vexans* have a very similar morphology, of which the bell-shaped, broadly apical attached antheridia is the most striking feature.

P. vexans is a common species from clade K (Fig. 5K), and was well-represented in GenBank with four sequenced isolates (GI 8926262, Cooke *et al.* 2000; GI 30088678; and GI 17980855-6, Moorman *et al.* 2002). Two of these isolates (GI 17980855-6) and *P. chamaehyphon* (GI 64688664, Matsumoto *et al.* 1999) formed a tight cluster with less than 1% sequence divergence around the isolate of *P. vexans* used by van der Plaats-Niterink (1981). The two other *P. vexans* isolates were more closely related to the *P. indigoferae* and *P. cucurbitacearum* from this study. There were two GenBank sequences of *P. helicoides* (GI 17980857, Moorman *et al.* 2002; and GI 8118262, Paul 2000a) for which the closest match was the ex-type of *P. helicoides* from this study but the ITS homology was only slightly above 90%. On the other hand, the sequences of *P. ostracodes* and *P. oedochilum* (GI 8118261, -3, Paul 2000a) both matched the corresponding isolates used by van der Plaats-Niterink (1981). The ITS-1 sequence of *P. megacarpum* (GI 8118258, Paul 2000a) matched the sequence of the strain of *P. boreale* in this study. Despite a number of similarities, some significant differences between these two species seems to be present in the antheridia, which are mainly sessile, clavate and tip-attached in *P. boreale* and stalked, elongate, and lengthwise applied to the oogonia in *P. megacarpum*. Two new species, *P. carbonicum* (Paul 2003b) and *P. montanum* (Nechwatal & Osswald 2003), clustered together but had more than 20% sequence divergence between each other. *P. citrinum* (GI 28565831, nom. inval.) and an isolate identified as *P. ostracodes* (GI 27448074) were not similar to anything else in clade K.

ITS sequences and species boundaries

In several species, the ITS sequence of the representative strain was identical or nearly identical to that of other species clustered with it (Table 2). As discussed by Bruns (2001), homology of ITS sequences does not prove conspecificity: very closely related but distinct species may have similar or identical ITS sequences, for example some *Fusarium* species (O'Donnell *et al.* 1998), *Neofabraea perennans*/*malacorticis* (de Jong *et al.* 2000) and *Cylindrocarpon* species (Seifert *et al.* 2003). The probability that 100% homology in the ITS region means conspecificity is higher in *Pythium* than in ascomycetes, however, because the ITS-1 and ITS-2 spacers are altogether approximately twice the length, providing more chances for polymorphisms. *P. aphanidermatum* and *P. deliense* represent one of the best demonstrations of speciation between two very closely related species. The spacers differ by only 3% and yet the species exhibit differences in several morphological characters that are slightly but consistently different between the two species (Herrero & Klemsdal 1998). *P. attrantheridium* is a recently recognized species described on isolates from different areas (Allain-Boulé *et al.* 2004). It had only 5% ITS divergence with *P. intermedium*, but was morphologically distinct and could not be mated with *P. intermedium*. However, based on ITS sequences alone, no definite conclusion about conspecificity can be drawn at this point. A wider range of isolates should be examined with regard to a number of independent characters to set species boundaries. Multigene analyses to demonstrate phylogenetic species (Taylor *et al.* 2000) could be used with very closely related species.

In contrast, the comparison with GenBank sequences revealed a wide variation within several species. Three strains of *P. rostratum* clustered together, but the depth of the branches show relatively large genetic distance (Fig. 5E). Five strains of *P. heterothallicum* are genetically similar to each other, but one is attached with a long branch (Fig. 5I). The ex-type strain of *P. ultimum* var. *sporangiiferum* is in the same cluster with all *P. ultimum* isolates, however, together with GI 17980876, it is separate from the other strains

(Fig. 5I). The ITS-heterogeneity within *P. insidiosum* (Fig. 5C) was studied in more detail by Schurko *et al.* (2003b) and compared to heterogeneity in the intergenic spacer (Schurko *et al.* 2003a); they found a correlation between genetic clustering of the isolates and their geographical origin, and concluded that *P. insidiosum* might comprise more than one species. Other species with many sequences found in GenBank exhibited considerably less variation: *P. aphanidermatum* (Fig. 5A), *P. dissotocum* (Fig. 5B), *P. graminicola* (Fig. 5B), *P. arrhenomanes* (Fig. 5B), *P. irregulare* (Fig. 5F), and *P. spinosum* (Fig. 5F). In view of this, it is likely that strains at the end of long branches, as in *P. rostratum*, *P. heterothallicum* and *P. ultimum*, represent distinct species. We have detected some probable examples of sequencing/editing errors, the most noticeable examples being cases where a strain was the only one in the entire genus to have a unique base in a highly conserved region. It is likely that other errors were more inconspicuous and artificially increased infraspecific variation in the analyses of Fig. 5.

CONCLUSION

The phylogeny of *Pythium*, as based on ITS sequences, reveals a divergence of globose and filamentous sporangium types. The globose type is likely to be ancestral as both outgroup species and the species in the outmost *Pythium* cluster develop globose, often internally proliferating sporangia. The contiguous type of sporangia, as developed by species in clade D is in between the filamentous and the globose group and appears to be an intermediate form. Heterothallism and oogonium ornamentation are clearly polyphyletic. The latter is present in six locations within the clades of species with globose or contiguous sporangia. In two of these groups (clades F and G), the shape of the ornamentation is distinct; perhaps a more detailed morphological study would reveal differences among the other groups as well. Smaller clusters appear to be related to host or substrate to some degree. Several clusters are characterized by a subset of morphological characters, but most morphological characters do not correlate with the evolutionary pattern, and may be only useful for species identification.

Kroon *et al.* (2004) have produced a multigene phylogeny of *Phytophthora* species with mitochondrial and nuclear genes which was largely congruent with the ITS phylogeny from Cooke *et al.* (2000) and the *Cox*I and *Cox*II phylogeny from Martin & Tooley (2003). Similarly, the conclusions from our ITS and LSU phylogeny should be verified with additional DNA regions.

Six of 20 recently described species have (nearly) identical ITS sequences with existing species and may well be synonyms (Table 2). It is recommended that the ITS database from this study be used for comparison of putative new species: it will reveal their taxonomic position and narrow down the existing species for comparison. This should reduce the number of unwarranted descriptions of new species.

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