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Phylogenetic relationships of *Pythium* species based on ITS and 5.8S sequences of the ribosomal DNA

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The sequences of ITS regions in 30 species and two groups of the genus *Pythium* were resolved. In the phylogenetic trees, the species were generally divided into two clusters, referred to here as the F and S groups. The species in the two groups correspond in terms of their sporangial morphology, with the F group being filamentous/lobulate and the S group being spherical. Genetic divergence within the F group was lower than that within the S group. Other morphological characteristics such as oogonial structure and sexual nature appeared to be unrelated to the groupings in these trees. An alignment analysis revealed common sequences to all the species and arrangements specific to each F or S group. It was found that the ITS region was a good target in designing species-specific primers for the identification and detection of *Pythium* species. In the tree based on 5.8S rDNA sequences, oomycetes are distantly related to other fungi but separated from algae in Chromista.

Key Words——Chromista; internal transcribed spacer; phylogeny; *Pythium*; 5.8S rDNA.

Pythium is a large genus of the class Oomycetes including more than 80 species (van der Plaats-Niterink, 1981), some of which are important plant pathogens with worldwide distribution. The taxonomy of this genus is mainly based on the morphology of reproductive structures such as the oogonium, oospore, antheridium, and sporangium. Since Matthews (1931) and Sideris (1932) proposed the first keys for *Pythium* spp., several keys have been published as new species have been described. Researchers providing systematic taxonomy in the genus are Middleton (1943), Waterhouse (1967), and van der Plaats-Niterink (1981). Hendrix and Papa (1974) introduced the concept of 'species complexes' in relation to taxonomy of the genus *Pythium*. This is an arrangement in which the species of *Pythium* are lumped into 15 species groups based on their morphological characteristics. In the taxonomic keys for *Pythium*, the size of each structure and common morphological characteristics are taken as criteria, including: (1) the presence of sexual reproductive structures – homothallic or heterothallic; (2) the sporangial morphology-spherical, filamentous or lobulate; (3) the oogonial wall character-smooth or ornamental; (4) the oospore character-plerotic or aplerotic; and (5) the antheridial character-monoclinous or diclinous. However, each author regarded different characteristics as more important. For example, Middleton (1943) used the sporangial morphology at the first branch, whereas Waterhouse (1967) frequently utilized the reproductive structure size in the separation of species. Van der Plaats-Niterink (1981) regarded the presence of reproductive structures and the oogonial wall character as more important criteria. These differences in the interpretation of the taxonomic value of each character have resulted in a confusing taxonomic system for the *Pythium* species. It is therefore necessary to determine which morphological characteristic is the most phylogenetically important.

Recently, the use of molecular biological methods has become popular in determining the taxonomy of microorganisms, as these methods provide a great deal of information regarding the genetic background of species. In particular, comparative studies of the nucleotide sequences of ribosomal DNA (rDNA) genes provide a means of analyzing phylogenetic relationships over a wide range of taxonomic levels (Berbee et al., 1995; Okada et al., 1997; Cantrell and Hanlin, 1997; Ko et al., 1997). Coding regions consisting of large subunit rDNA (LrDNA), small subunit rDNA (SrDNA), and 5.8S rDNA evolve relatively slowly and are used for studying distantly related organisms from kingdoms to genera. Non-coding regions consisting of internal transcribed spacers (ITS) and intergenic spacers (IGS) evolve more rapidly and can be useful for comparisons at the species or population level. Furthermore, the development of the polymerase chain reaction (PCR) with universal oligonucleotide primers for fungi (White et al., 1990) has allowed the easy amplification and rapid analysis of these regions. In recent studies, the taxonomic placement of species in *Aspergillus* (Peterson, 1995) and *Sarcoscypha* (Harrington and Potter, 1997) and genera in Trichocomaceae (Berbee et al., 1995) have been reexamined based

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on analyses of the sequences of coding regions and non-coding regions of nuclear rDNA. Nakasone (1996), and Norman and Egger (1996) have reported species that have common morphological characteristics but belong to different genera are phylogenetically closely related by rDNA analysis. In the genus *Pythium*, a correlation was found between groups of species defined by molecular data for 28S rDNA and groups defined on the basis of their sporangial morphology (Briard et al., 1995). Wang and White (1997) confirmed this finding based on ITS-RFLP. However, because length polymorphism is found in the ITS regions of the genus *Pythium*, making distinctions based on similar RFLP patterns is very difficult. It is, therefore, necessary to examine the sequences for a correct analysis of the ITS regions.

Oomycetes included in the genus *Pythium* have recently been separated from other fungi and repositioned in the Kingdom Chromista together with hyphochytrimycetes and labyrinthulomycetes, which were for many years believed to be true fungi (Cavalier-Smith, 1981, 1986). This kingdom is primarily defined by two ultrastructural features: the presence of tubular, tripartite hairs (mastigonemes) on the surface of the flagellum, and the presence of a chloroplast endoplasmic reticulum. The kingdom includes Cryptophyta, Haptophyta, and Heterokontophyta, to which Chrysophyceae, Raphidophyceae, Bacillariophyceae and some other algae belong. Oomycetes are characterized by the presence of tubular cristae in mitochondria, specific ultrastructural features of zoospores, the formation of oospores resulting from the touch of gametes, diploid vegetative hyphae, and constitutive elements of the cell wall. These characteristics are different from those of other fungi and therefore make oomycetes appear to be more closely related to algae than "true" fungi (Chytridiomycetes, Zygomycetes, Ascomycetes, Basidiomycetes, Deuteromycetes). This new taxonomic system is supported by sequence analysis of the small subunit rRNAs (Gunderson et al., 1987; Förster et al., 1990), and it seems to reflect both physiological and biochemical characteristics (Cavalier-Smith, 1989).

In this study, we examined sequences of ITS regions including 5.8S rDNA to deal with three issues concerning *Pythium* taxonomy: (i) to clarify the relationship between sporangial morphology and phylogenetic evolution; (ii) to assess the potential of ITS regions in developing of species-specific PCR primers or DNA probes for use in identification and detection; (iii) to review the position of the oomycetes by sequence analysis of the 5.8S rDNA coding regions with some algae and other fungi.

Materials and Methods

Isolates Thirty species and two groups of *Pythium* used in this study are listed in Table 1. All isolates were maintained on cornmeal agar (CMA) at 5°C.

DNA extraction Three agar plugs were removed from the growing margin of 2-d-old cultures on CMA with a 1-cm cork borer and transferred to a flask containing 50 ml of 20% V8-juice broth containing 2.5 g/l $CaCO_3$. After incubation for 4–7 d at 25°C, mycelial mats were collected on filter paper, washed with sterile distilled water, and frozen at −80°C. The total genomic DNA was extracted according to the procedure of Lee and Taylor (1990). The frozen mycelia were ground to a fine powder with a pre-frozen mortar and pestle. Mycelial powder was suspended in 600 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM EDTA, 3% SDS, 1% 2-mercaptoethanol) and heated at 65°C for 1 h. After centrifugation at 10,000 *g* for 5 min, the aqueous phase was withdrawn. One volume of aqueous phase was mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), and after centrifugation (10,000 *g* for 15 min), the aqueous phase was withdrawn. The DNA was extracted again with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). DNA in the resulting solution was precipitated by adding 2 volumes of 99.9% ethanol in the presence of 3 M sodium acetic acid (0.1 vol.) and centrifuging at 10,000 *g* for 2 min. The precipitated DNA was rinsed with 70% ethanol, centrifuged at 10,000 *g* for 2 min, and after drying up, dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA).

Sequencing of rDNA-ITS region Primers ITS1 (5′-GTAGTCATATGCTTGTCTC-3′) and ITS4 (5′-CTTCCGTCAATTCCTTTAAG-3′), which have been described by White et al. (1990), were used to amplify the nuclear rDNA region of internal transcribed spacers (ITS), including the 5.8S gene. A total volume of 50 μl of reaction mixture contained 1 μM primers, 1.25 units of *Taq* DNA polymerase (Takara Shuzo Co. Ltd., Shiga, Japan), 0.2 mM dNTP mixture, 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), and 200 ng of DNA template. The reaction was carried out with a DNA Thermal Cycler (Perkin-Elmer, Applied Biosystems, Norwalk, CT). The temperature cycling parameters were programmed for one cycle of 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and one cycle of 10 min at 72°C. PCR products were electrophoresed in 1.2% Agarose LO3 (Takara Shuzo) gel in TAE buffer (40 mM Tris-HCl, pH 7.5, 19 mM glacial acetic acid, 2 mM EDTA), and then stained with ethidium bromide. After amplification was confirmed, agarose gel containing the targeted bands was cut out. ITS regions were extracted from the agarose gel using TE-saturated phenol and phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). A Big Dye™ Terminater Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) was used for the sequence reaction according to the manufacture's instructions. The reactions were carried out with a Gene Amp PCR system 2400 (Perkin-Elmer). Electrophoresis of the sequencing products was carried out with an ABI 377 DNA sequencer (Perkin-Elmer) or an ABI 310 DNA sequencer (Perkin-Elmer).

Phylogenetic analysis Except for *Pythium* spp., the sequence data were drawn from the EMBL databank. All sequences were first aligned using the multiple sequence alignment program CLUSTAL W ver. 1.60 (Thompson et al., 1994), and the alignment was optimized. Alignment

Table 1. *Pythium* species isolates used in this study.

Species	Isolate	Host	Origin	Accession no.[a]
P. aphanidermatum	TOc159	Carrot	Gifu, Japan	AJ233438
P. arrhenomanes	GUP6	Sugarcane	Kagoshima, Japan	AJ233439
P. catenulatum	MK4-10-2S	Zoysia grass	Gifu, Japan	Y18164
P. chamaehyphon	MS6-10-8V		Gifu, Japan	AJ233440
P. coloratum	TM321	Carrot	Gifu, Japan	AJ233441
P. deliense	MAFF305568	Pumpkin	Okinawa, Japan	AJ233442
P. dissotocum	MAFF305576		Chiba, Japan	AJ233443
P. graminicola	TMF	Sugar beet	Kagoshima, Japan	AJ233444
P. hydnosporum	MAFF305861		Fukuoka, Japan	AJ233445
P. inflatum	MAFF305863		Fukuoka, Japan	AJ233446
P. intermedium	MAFF305570		Hokkaido, Japan	AJ233447
P. irregulare	MAFF305572		Kanagawa, Japan	AJ233448
P. middletonii	MS2-10-8V	Zoysia grass	Gifu, Japan	AJ233449
P. myriotylum	BeAT1	Kidney bean	Hokkaido, Japan	AJ233450
P. nunn	ATCC20693			AJ233451
P. orthogonon	DS2-6-9D	Zoysia grass	Gifu, Japan	AJ233452
P. paroecandrum	CBS157.64		Australia	AJ233453
P. periilum	S2-8-1S	Zoysia grass	Gifu, Japan	AJ233454
P. periplocum	DK1-5-1S	Zoysia grass	Gifu, Japan	AJ233455
P. rostratum	DS5-7-1S	Bent grass	Gifu, Japan	AJ233456
P. spinosum	OD231	Carrot	Gifu, Japan	AJ233457
P. sulcatum	CTMa7	Carrot	Gifu, Japan	AJ233458
P. sylvaticum	OM121	Carrot	Gifu, Japan	AJ233459
P. torulosum	60-2	Zoysia grass	Hyogo, Japan	AJ233460
P. ultimum	Py 79	Sugar beet	Hokkaido, Japan	D86515
P. vanterpoolii	DK1-6-3D	Zoysia grass	Gifu, Japan	AJ233461
P. vexans	MAFF305905		Kochi, Japan	AJ233462
P. violae	OPy 6	Garden pansy	Hyogo, Japan	AJ233463
P. volutum	IFO31926			AJ233464
P. zingiberum	UOP389	Ginger	Wakayama, Japan	AJ233465
Group G	Py 37	Sugar beet	Hokkaido, Japan	Y18165
Group HS	Py 55	Sugar beet	Hokkaido, Japan	AJ233466

[a] EMBL, GenBank and DDBJ Nucleotide Database.

gaps were treated as missing data and ambiguous positions were excluded from the analysis. Nucleotide variation of these sequences was analyzed by the maximum-parsimony method of the Phylogenetic Analysis Using Parsimony (PAUP) program 3.1.1 (Swofford, 1993). The bootstrap analysis was implemented using 100 replicates of heuristic searches to determine the confidence levels of the inferred phylogenies (Felsenstein, 1988). In the analysis of the ITS regions, the sequence data of *Achlya bisexualis* (Accession no. L41389) and *Phytophthora megasperma* (Accession no. X75632) were used as an outgroup for comparisons.

Results

Pythium species

Sequence alignment The exact lengths of the ITS and 5.8S rDNA regions were resolved by sequence analysis (Table 2). ITS1 lengths varied from 171 bp to 303 bp, and ITS2 lengths varied from 393 bp to 520 bp. The length of the 5.8S rDNA region was 159 bp in all of the *Pythium* species. The ITS regions including 5.8S rDNA tended to be shorter in the species having filamentous and lobulate sporangial morphology (F group) and longer in those having spherical sporangial morphology (S group) (Table 2). More sequence variation was evident in the ITS1 regions, and dozens of gaps were interspersed mosaically in the F group. On the other hand, for the ITS2 regions, we could align most of the sequences other than *P. nunn*, *P. vexans*, and *P. rostratum*, which had peculiar sequences. The nucleotide sequence data have been registered in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers shown in Table 1.

Genetic divergence In all the species used in this study, interspecific genetic divergence varied 0.0–29.0% with a mean distance of 11.4% in ITS1 and 0.0–29.1% with a

Table 2. Relationship of morphological characters (from van der Plaats-Niterink, 1981) with lengths of ITS region of *Pythium*.

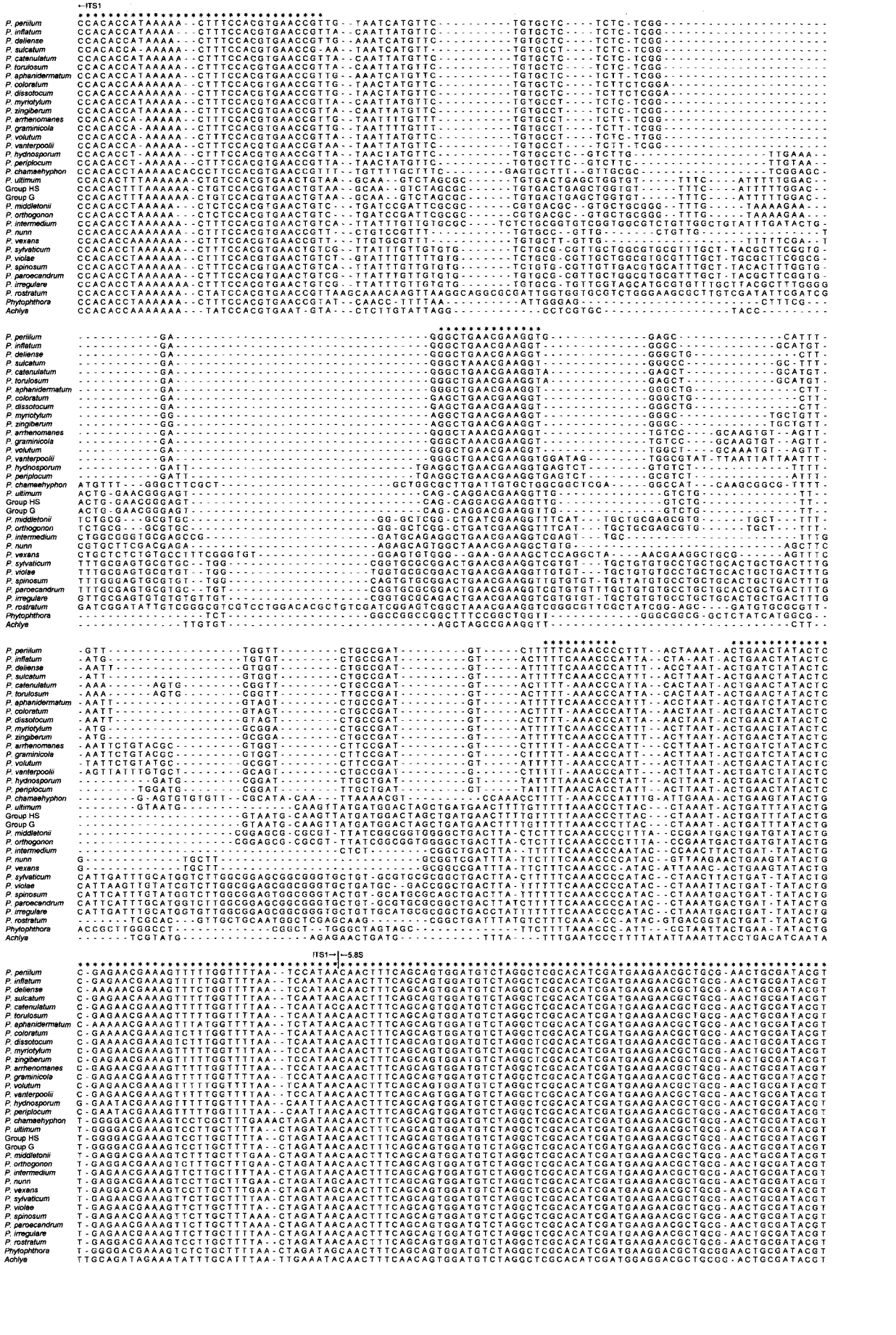
Species	Sexual reproduction[a]	Oogonial wall[b]	Oospore[c]	Antheridia[d]	Sporangia[e]	Lengths (bp)			
						ITS1	5.8S	ITS2	Total
P. hydnosporum	Ho	O	AP	M	—	189	159	417	765
P. periilum	Ho	S	P	MD	F	174	159	435	768
P. inflatum	Ho	S	P	D	F	172	159	440	771
P. deliense	Ho	S	A	M	F	173	159	441	773
P. sulcatum	Ho	S	A	MD	F	171	159	444	774
P. catenulatum	He	S	P	D	F	179	159	437	775
P. torulosum	Ho	S	P	M	F	179	159	437	775
P. aphanidermatum	Ho	S	A	MD	F	174	159	444	777
P. coloratum	Ho	S	A	MD	F	175	159	443	777
P. dissotocum	Ho	S	AP	MD	F	175	159	443	777
P. periplocum	Ho	O	A	D	F	191	159	428	778
P. myriotylum	Ho	S	A	D	F	175	159	445	779
P. zingiberum	Ho	S	A	D	F	175	159	445	779
P. chamaehyphon	Ho	S	A	D	S	240	159	393	792
P. arrhenomanes	Ho	S	P	D	F	187	159	450	796
P. graminicola	Ho	S	A	M	F	187	159	450	796
P. volutum	Ho	S	A	D	F	187	159	455	801
P. vanterpoolii	Ho	S	P	M	F	198	159	453	810
P. ultimum	Ho	S	A	M	(S)	221	159	445	825
Group HS	—	—	—	—	(S)	221	159	445	825
Group G	—	—	—	—	S	221	159	445	825
P. middletonii	Ho	S	P	MD	S	240	159	455	854
P. orthogonon	Ho	S	P	MD	S	240	159	456	855
P. intermedium	He	S	A	D	(S)	227	159	480	866
P. nunn	Ho	S	A	MD	(S)	196	159	519	874
P. vexans	Ho	S	A	M	S	222	159	494	875
P. sylvaticum	He	S	A	D	(S)	290	159	458	907
P. violae	Ho	S	A	M	—	288	159	469	916
P. spinosum	Ho	O	P	M	(S)	292	159	466	917
P. paroecandrum	Ho	S	A	M	S	294	159	478	931
P. irregulare	Ho	O	AP	MD	S	299	159	479	937
P. rostratum	Ho	S	P	M	S	303	159	520	982

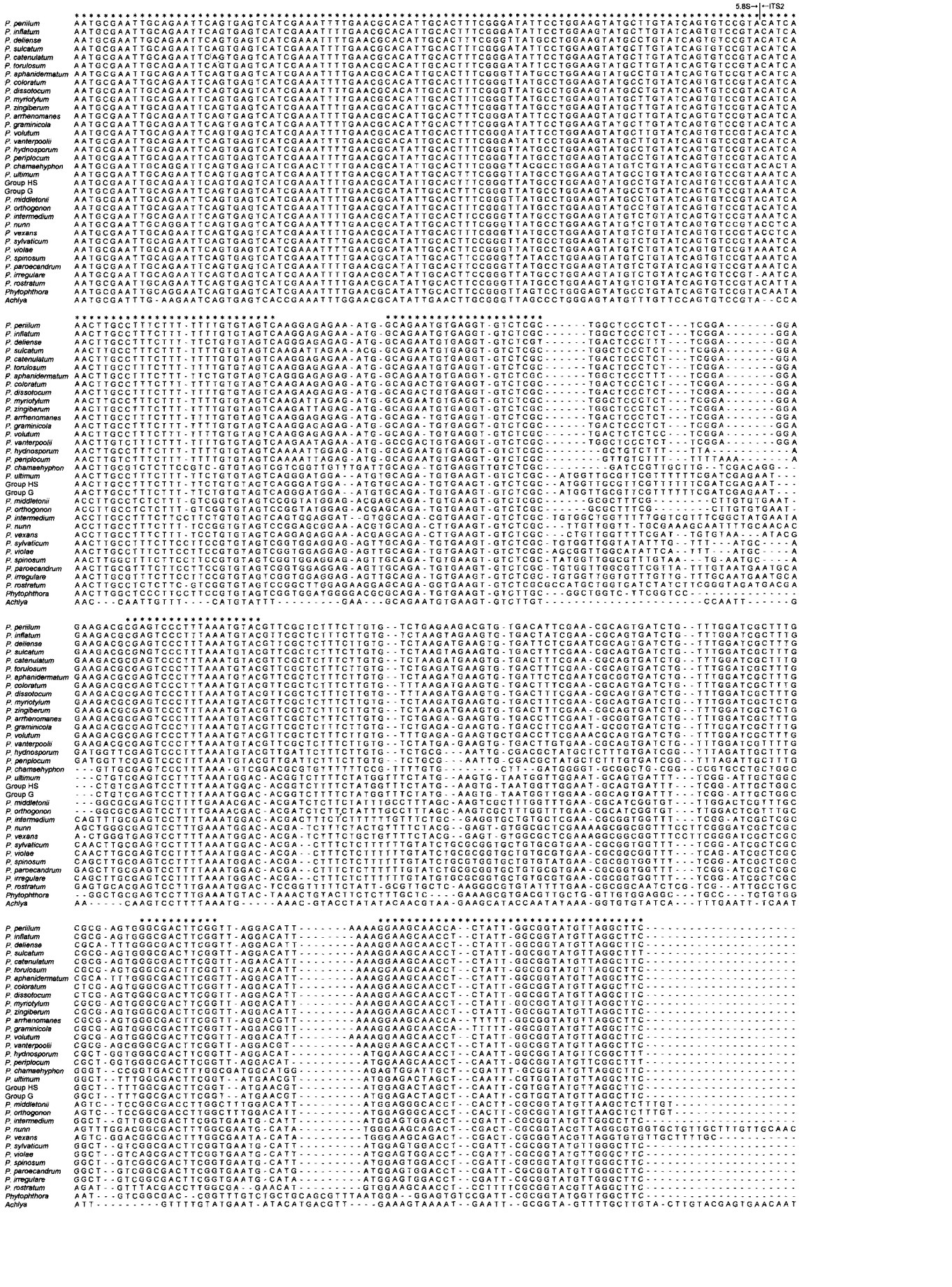
a Ho: homothallic, He: heterothallic, —: reproductive structure not known.
b S: smooth, O: ornamental, —: reproductive structure not known.
c P: plerotic, A: aplerotic, —: reproductive structure not known.
d M: monoclinous, D: diclinous, —: reproductive structure not known.
e F: filamentous and lobulate, S: spherical and subspherical, (S): hyphal swelling, —: sporangia not known.

Table 3. Averages and ranges of percent genetic divergence in ITS1, the 5.8S rDNA, and ITS2 from pairwise comparisons among F and S groups of *Pythium*.

	ITS1	5.8S	ITS2	Entire region
Within F group[a]	3.6[c] (0.0–12.0)[d]	1.1 (0.0–2.5)	1.5 (0.0–5.6)	1.8 (0.0–5.7)
Within S group[b]	11.4 (0.0–22.0)	1.1 (0.0–2.5)	15.7 (0.0–29.1)	8.9 (0.0–14.5)
Between F and S groups	16.1 (10.0–29.0)	2.4 (0.0–5.0)	14.5 (8.3–22.7)	10.2 (7.7–16.5)

Genetic divergence (%) = (number of substitution / total number of sequences) × 100
a Included 16 filamentous/lobulate sporangial species.
b Included 14 spherical sporangial species.
c Average of percent genetic divergence.
d Range of percent genetic divergence.


←ITS1
ITS1→|←5.8S
P. periilum
P. inflatum
P. deliense
P. sulcatum
P. catenulatum
P. torulosum
P. aphanidermatum
P. coloratum
P. dissotocum
P. myriotylum
P. zingiberum
P. arrhenomanes
P. graminicola
P. volutum
P. vanterpoolii
P. hydnosporum
P. periplocum
P. chamaehyphon
P. ultimum
Group HS
Group G
P. middletonii
P. orthogonon
P. intermedium
P. nunn
P. vexans
P. sylvaticum
P. violae
P. spinosum
P. paroecandrum
P. irregulare
P. rostratum
Phytophthora
Achlya


5.8S→
←ITS2
P. perilum
P. inflatum
P. deliense
P. sulcatum
P. catenulatum
P. torulosum
P. aphanidermatum
P. coloratum
P. dissotocum
P. myriotylum
P. zingiberum
P. arrhenomanes
P. graminicola
P. volutum
P. vanterpoolii
P. hydnosporum
P. periplocum
P. chamaehyphon
P. ultimum
Group HS
Group G
P. middletonii
P. orthogonon
P. intermedium
P. nunn
P. vexans
P. sylvaticum
P. violae
P. spinosum
P. paroecandrum
P. irregulare
P. rostratum
Phytophthora
Achlya

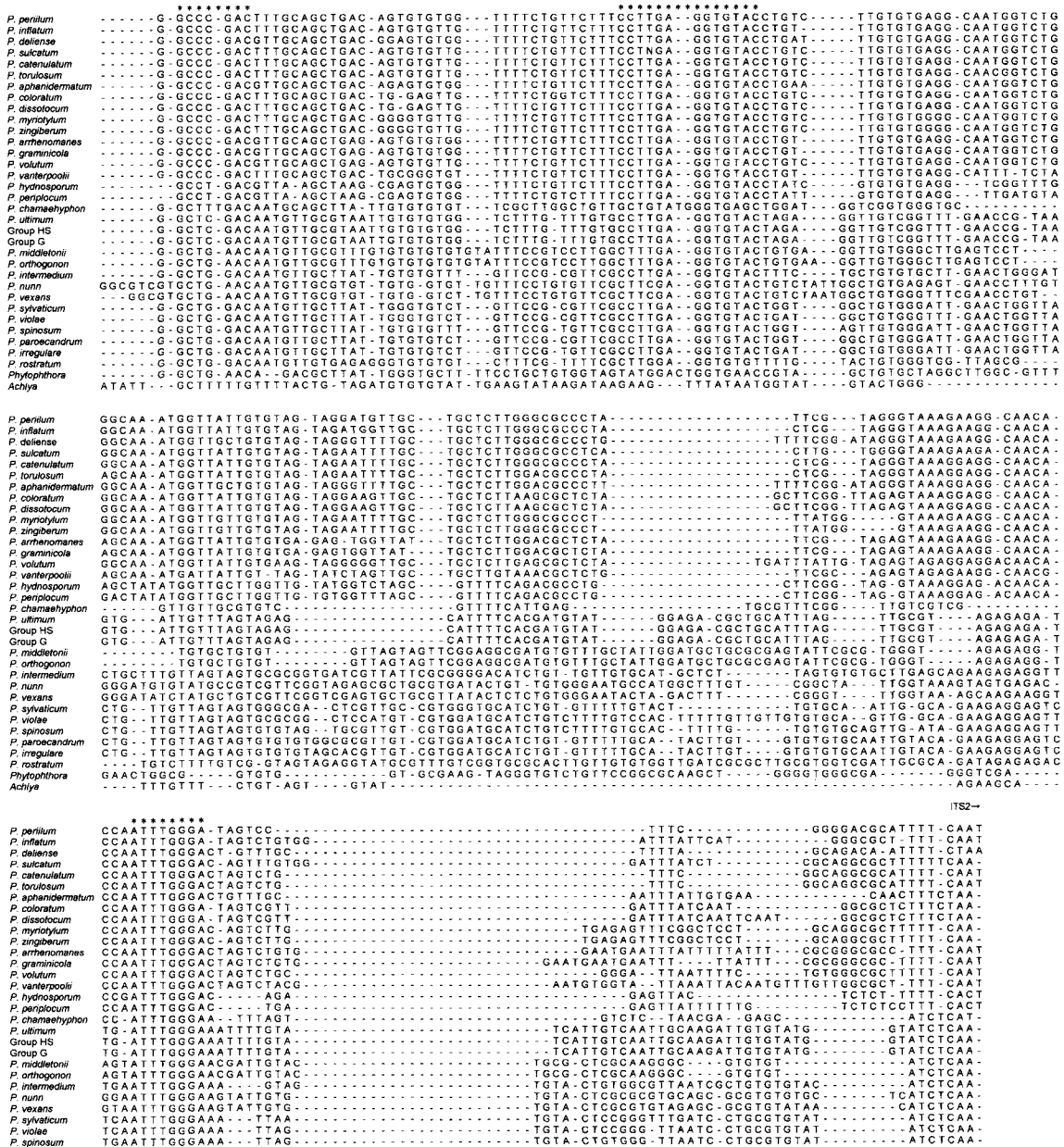


Fig. 1. Aligned DNA sequences of the two internal transcribed spacers and 5.8S rDNA. Asterisks designate the region included in the phylogenetic analysis. Sequence gaps are shown as dashes, and undetermined sequences are shown by N.

mean distance of 10.6% in ITS2. The mean distances were 4.1% in ITS1 and 1.7% in ITS2 within the F group, 9.8% in ITS1 and 13.5% in ITS2 within the S group, and 15.9% in ITS1 and 14.3% in ITS2 between the both groups (Table 3). Within the F group, specifically, the genetic divergence was lower than within the S group and between the S and F groups.

Phylogenetic analysis The data from 34 taxa including two outgroups provided 1092 aligned sites. Of these, 106 sites from ITS1, 153 sites from ITS2, and all 160 sites from 5.8S were used for phylogenetic analysis (Fig. 1). The parsimony analysis of these characters produced one minimum length tree of 235 steps, with consistency index (CI) and retention index (RI) of 0.664 and 0.778, respectively (Fig. 2). In this tree, most of the branches in the bootstrap consensus tree were determined by applying the 50% strict consensus. The F group and *P. hydnosporum* (Mont.) Schroter, which forms no sporangia, formed one cluster, while the S group and *P. violae* Chesters & Hickman, which forms no sporangia, separated into six clusters.

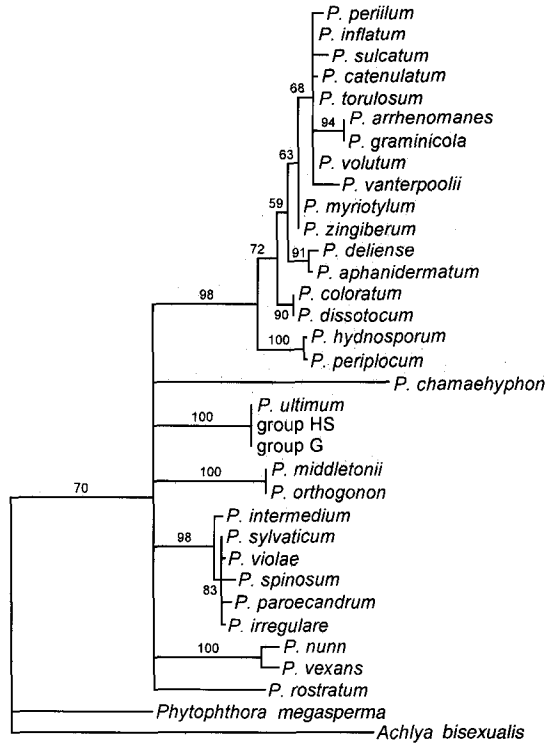


Fig. 2. Consensus tree of *Pythium* species based on the combined ITS and 5.8S rDNA sequences. The consensus tree was generated from maximum parsimony analysis using the heuristic search algorithm of PAUP 3.1.1. Numbers of the branches indicate the bootstrap values resulting from 100 bootstrap replications.

Chromista

The sequences of the 5.8S rDNA of the oomycetes, *Pythium*, *Phytophthora*, and *Achlya* were compared with sequences of other fungi and algae (Table 4). The parsimony analysis of 161 characters produced one minimum length tree of 199 steps, with CI and RI of 0.493 and 0.470, respectively (Fig. 3). In this tree, oomycetes were located in a different branch from other organisms, which were grouped in a common cluster. Fungi formed one group in this cluster, but *Blastocladiella emersonii* Cantino & Hyatt of chytridiomycetes was not included.

Discussion

Waterhouse (1967) and van der Plaats-Niterink (1981) have regarded the presence of reproductive structures and the character of the oogonial wall as the most important criteria in the taxonomy of the genus *Pythium*. On the other hand, Middleton (1943) has used the sporangial morphology as the determining criterion at the first branch. Briard et al. (1995) and Wang and White (1997) reported a correlation between the groups based on partial sequences of 28S RNA or RFLP analysis of ITS regions and that based on the sporangial morphology. In this study, the relationship between the sequences of the ITS region and sporangial form was examined in detail. In the phylogenetic trees, the F group clustered in one branch at the high bootstrap percentages, while the S group divided into six branches. That is, these clusters based on the sequences of the ITS regions did not match the morphological subgroups of oogonium used in the taxonomies of Waterhouse (1967) and van der Plaats-Niterink (1981) but were related to the morphology of the sporangium. Moreover, the occurrence of homothallic and heterothallic species is not considered to reflect genetic evolution. These results agree with the data of Chen (1992) and Briard et al. (1995) indicating heterothallism in *Pythium* evolved independently. In the same way, characteristics such as oospores being plerotic/aplerotic or the antheridia being monoclinous/diclinous are not informative about evolutionary differentiation. Up to this point, our data support the theory set forth by Middleton (1943), Briard et al. (1995) and Wang and White (1997), which suggests that sporangial form should be considered to be the most important characteristic in the phylogenetic taxonomy of genus *Pythium*. Furthermore, it is interesting that the taxonomic importance of sporangial form has also been shown in *Phytophthora* species (Crawford et al., 1996; Cooke and Duncan, 1997), suggesting that this characteristic may also be indicative of genetic evolution in other genera.

The lengths of the ITS regions including 5.8S rDNA were shorter in the F group (765–810 bp) and longer in the S group (792–982 bp). In addition, the divergence in length was low in the F group (35 bp) and very high in the

Table 4. Database accession number of the species used to compare the sequences of 5.8S rDNA

Species	Accession no.[a]
Achlya bisexualis	L41389
Blastocladiella emersonii	X90410
Chlamydomonas cribrum	AF033281
Cladophora vagabunda	Z38134
Colletotrichum acutatum	X73810
Cortinarius traganus	AF037224
Entrophospora infrequens	U94713
Fusarium oxysporum	X94173
Glomus mosseae	U31996
Gonyaulax spinifera	AF051832
Gracilariopsis tenuifrons	U21246
Hyphochytrium catenoides	X80346
Lentinula edodes	U33080
Monilinia fructicola	AF010501
Nannochloropsis salina	Y07974
Ochromonas danica	Y07976
Pandorina morum	AF053682
Phytophthora megasperma	X75632
Puccinia recondita	L08702
Pyrodinium bahamense	AF051366
Sarcodiotheca gaudichaudii	U30369
Tribonema aequale	Y07978

[a] EMBL, GenBank and DDBJ Nucleotide Database

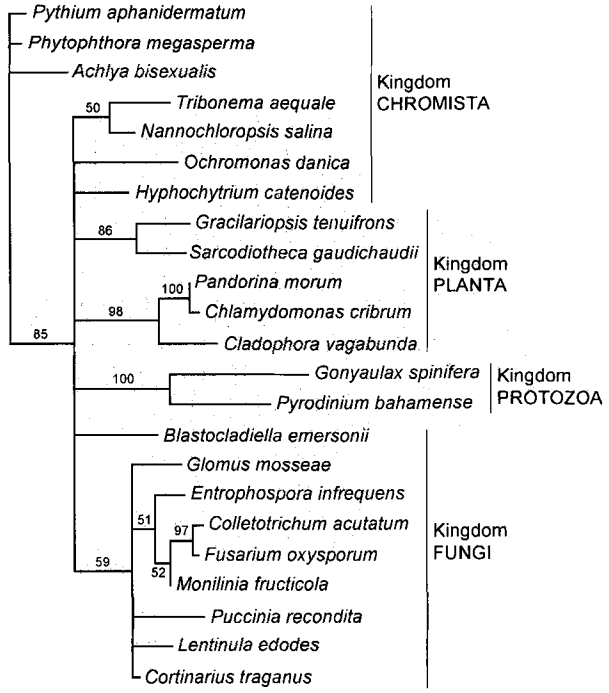


Fig. 3. Consensus tree showing the relationship of oomycetes and other eukaryotes based on the 5.8S rDNA sequences. The consensus tree was generated from maximum parsimony analysis using the heuristic search algorithm of PAUP 3.1.1. Numbers of the branches indicate the bootstrap values resulting from 100 bootstrap replications.

S group (190 bp). In the sequences of the ITS regions, the F group also had less variation than the S group. An alignment analysis revealed common sequences in all the species and specific arrangements for each group. The group-specific sequences were scattered over the ITS regions and contributed to the divergence between the F and S groups. The divergence within each group mainly resulted from an insertion, deletion, and/or substitution in the specific sequences.

Comparison of our sequence data with published restriction maps of *P. arrhenomanes* Drechsler, *P. graminicola* Subramaniam, *P. irregulare*, *P. spinosum*, *P. ultimum*, and *P. sylvaticum* (Chen, 1992; Chen et al., 1992a; Chen and Hoy, 1993) revealed that the sequences matched in most species except for some short fragments that cannot be detected by RFLP analysis. The possibility of intraspecific variation in *P. graminicola* exists, as the sequence of our isolate has one additional restriction site or lacks one restriction site for each enzyme in comparison with the published data. We also compared our sequences with the RFLP analysis reported by Wang and White (1997). This comparison revealed a close match in 18 of 24 species, when allowance was made for short fragments and differences in length that could not be detected by RFLP analysis. For the remaining 6 species, comparison of the lengths of the restriction fragments in detail suggested a few intraspecific variations in *P. graminicola*, *P. sulcatum* Pratt & Mitchell, and *P. volutum* Vanterpool & Truscott because each difference included only one or two restriction sites. In *P. rostratum*, *P. vexans*, and *P. violae*, the restriction patterns were extremely different from those of our isolates, suggesting that the differences might go beyond intraspecific variation. Since these species have indistinct morphological characteristics, the isolates could be incorrectly identified. In the species whose morphological taxonomic criteria are indistinct, or in species that have similar morphology to others, DNA analysis such as RFLP or sequences of ITS regions as well as characterization of morphological structure is necessary for correct identification.

Biochemical and immunological analyses have also been applied to taxonomic as well as a morphologic study of the genus *Pythium*. Biochemical analyses of isozymes and soluble proteins have been reported to be useful to recognize morphologically distinct species, but not to differentiate morphologically similar species such as *P. graminicola* and *P. arrhenomanes*, *P. aphanidermatum* (Edson) Fitzp. and *P. deliense* Meurs, *P. irregulare* and *P. spinosum*, *P. paroecandrum* and *P. ultimum* (Chen et al., 1991; Chen et al., 1992b; White et al., 1994). In our study, the above species can be distinguished on the basis of an analysis of ITS sequences, even though they are closely related. Other species with similar morphology, *P. coloratum* Vaartaja and *P. dissotocum* Drechsler, and *P. middletonii* and *P. orthogonon*, have only one differentiating sequence in the ITS sequences. Concerning species without reproductive structures, group G has been reported to have the same RFLP pattern in its mt-DNA as *P. ultimum* (Huang et al., 1992). Group G has only one distinct sequence from *P. ultimum* in the ITS2 region. To summarize the above, species-specific variation was detected in both ITS1 and ITS2 in most of *Pythium* species, although some species appear to have the same or very similar sequences. Therefore, ITS regions are useful for the development of species-specific PCR primers or DNA probes for use in identification and detection.

In *P. myriotylum* Drechsler and *P. zingiberum* Takahashi, the sequences match completely. The species have many common morphological characteristics and grow at the same rates. *P. zingiberum* was originally isolated in Japan from ginger (*Zingiber officinale* Rosc.; Takahashi, 1954). The morphology and growth rate of this species was compared with those of morphologically similar species such as *P. graminicola*, *P. arrhenomanes*, and *P. aphanidermatum* by the author and later researchers. Only one report compares *P. zingiberum* directly with *P. myriotylum*, stating that they differ in morphology and number of antheridia, and the characters and size of oogonia (Ichitani and Shinsu, 1980). Outside of Japan, *P. zingiberum* has only been reported in Korea. Every isolation of this species has been done from ginger or mioga (*Zingiber mioga* Rosc.), but it has been reported to be pathogenic to cucumber, tomato, spinach, and potato (Katsura and Tanioka, 1967). The results of this analysis suggest that *P. myriotylum* and *P. zingiberum* are related phylogenetically.

Oomycetes have been classified as true fungi in view

of the similarity of morphologic, nutritional, and ecological features. However, in addition to analyses of the physiological and biochemical characteristics of oomycetes, a recent sequence analysis of the 18S rDNA has provided additional evidence that oomycetes should be repositioned in Kingdom Chromista (Cavalier-Smith, 1981, 1986, 1992). In this study, the phylogenetic analysis based on the sequences of the 5.8S rDNA region confirmed that oomycetes are distantly related to other fungi, Chytridiomycetes, Zygomycetes, Ascomycetes, Basidiomycetes, and Deuteromycetes; but we were unable to demonstrate that oomycetes are closely related to particular algae, from which they differ in terms of their 18S rDNA. In future, molecular and cytological research should be carried out that targets a common ancestor of oomycetes and alga in Chromista. In addition, it is necessary to define the logical group for "fungi" beyond the kingdom to avoid confusion regarding the use of this classification in the field of biology and plant pathology.

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