Restriction Fragment Length Polymorphisms in Enzymatically Amplified Ribosomal DNAs of Three Heterothallic *Pythium* Species

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ABSTRACT

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Three heterothallic *Pythium* species, *P. heterothallicum, P. splendens,* and *P. sylvaticum,* were studied by examining restriction fragment length polymorphisms in polymerase chain reaction-amplified nuclear and mitochondrial ribosomal DNAs. Two different sizes of the internal transcribed spacer (ITS) from the nuclear rDNA were observed. The ITS for *P. heterothallicum* and *P. splendens* was about 850 base pair (bp) long, and the ITS for *P. sylvaticum* was about 1,020 bp long. Each length variant showed distinct banding patterns after restriction enzyme digestions. In addition, digestion with three enzymes differentiated *P. heterothallicum* and *P. splendens*. Restriction enzyme digestions of the nuclear small subunit rDNA and an 800-bp DNA from the 5′ end of the nuclear large subunit rDNA also showed species-specific banding patterns. In contrast, a 1,000-bp DNA from the mitochondrial large subunit rDNA did not show as much interspecific variation as did the nuclear rDNA. Little intraspecific variation was observed with nuclear rDNA. The species-specific banding patterns could be used to identify heterothallic *Pythium* species in the absence of mating reactions. The restriction sites in the ITS region were determined, and the maps were aligned to previously published data for five homothallic species. In phylogenetic analysis based on the variable restriction sites in the ITS region, the heterothallic species did not form a monophyletic group, suggesting that heterothallism does not represent a distinct species lineage in *Pythium*.

Additional keywords: convergent evolution, phylogeny.

The genus *Pythium* includes many important plant pathogens that typically have wide host ranges. In ecological and epidemiological studies of *Pythium* diseases, disease-causing agents must be identified to the species level. Identification of *Pythium* species can be difficult, however, because the morphological features by which species are distinguished may be affected by the growth media used, temperature regimes, age of cultures, and intraspecific variability (17). Several plant pathogenic *Pythium* species could not be reliably distinguished based on protein electrophoresis and isozyme analysis due to intraspecific variation (7,9). In addition, it is sometimes difficult to obtain all the asexual and sexual reproductive structures needed to identify an isolate. Heterothallism further complicates the identification process. Heterothallism in the genus *Pythium* was first described by Campbell and Hendrix in 1967 (5). Since then, seven *Pythium* species have been found

to have a tendency to be heterothallic (40) and at least five of them are important plant pathogens (12,20,35,40). The only definite means of separating these species is by mating reactions (17). Identification of heterothallic *Pythium* species requires pairing with characterized tester strains for both antheridial and oogonial mating types. Furthermore, because production of sexual reproductive structures is influenced by environmental conditions (10,16,36), pairing of compatible strains may not always result in production of sexual spores (J. W. Hoy, *personal communication*). This may result in an inability to identify certain species or even misidentification of some species.

One approach to efficiently and reliably identify heterothallic species is to use molecular markers that are characteristic for each species. This approach has been used in studying several plant pathogens, including *Pythium* species (1,8,15,22,25, 30,31,34). Goodwin et al (15) selected cloned genomic DNA probes specifically hybridizing to *Phytophthora parasitica* and used the probes to detect the pathogen in soil and infected plant tissues. Based on DNA sequences in the internal transcribed spacer (ITS) region, Nazar et al (34) designed species-specific PCR primers to detect and differentiate *Verticillium albo-atrum* and *V. dahliae*. Restriction banding patterns of mitochondrial DNA were used to identify asexual isolates of *P. ultimum* (27). Using mitochondrial DNA of *P. oligandrum* and *P. sylvaticum*, Martin (30) identified probes with various levels of specificity for the two species. Some probes reacted with DNA from several species whereas others hybridized to the DNA of particular isolates (30).

Ribosomal DNAs of eukaryotes are arranged in tandem repeats in specific chromosomes and are subject to concerted evolution, a tendency for such repeated sequences to become homogeneous, first among gene copies within genomes and then among individuals within populations (11,13,19). Use of such molecules should increase resolving power for identifying discrete genetic units (e. g., populations or species) (11). Ribosomal DNA consists of transcribed and nontranscribed regions, and there are noncoding sequences (transcribed spacers) in the transcribed region. Different regions of the rDNA evolve at different rates (11,19,41). Analyzing the different regions provides various levels of sensitivity for systematic studies at different taxonomic levels. For *Pythium* species analyzed previously, length variations of PCR-amplified ITS regions and restriction digestion profiles were characteristic of five homothallic *Pythium* species (8).

In studies of *Pythium* populations from forest nursery samples, five isolates did not form any sexual spores in single-isolate cultures. Mating these cultures with characterized strains of *P. sylvaticum* Campbell & Hendrix resulted in insufficient numbers of oospores for species identification. The objectives of this study are to examine the variation in ribosomal DNAs of three heterothallic *Pythium* species and to identify species-specific variations for potential applications in identification of the heterothallic species (6).

MATERIALS AND METHODS

Fungal isolates and culture maintenance. Eighteen isolates of three heterothallic *Pythium* species, *P. heterothallicum* Campbell & Hendrix, *P. splendens* Braun, and *P. sylvaticum,* are listed in Table 1 along with their original hosts or habitat and geographic origins. Type cultures of *P. heterothallicum* and characterized opposite mating types of the other two species were included in this study. Cultures were maintained in half-strength cornmeal agar (CMA) slants and in sterile water vials.

Cultural conditions and DNA isolation. Single-isolate cultures were grown in a liquid medium (10 g of sucrose, 1 g of yeast extract per 1,000 ml of distilled water) in 250-ml flasks containing 100 ml of medium. Actively growing colonies on CMA were transferred from the edge of the colonies into the liquid medium and incubated at room temperature (22–26 C). After a 5-day incubation, mycelium was filtered through two layers of cheesecloth, blotted dry with paper towels, and frozen immediately at −80 C. The mycelium was kept at −80 C for 1 h to 7 days before DNA isolation. For DNA isolation, the frozen mycelium was ground to a fine powder in liquid nitrogen with a mortar and pestle. Total genomic DNA was isolated from the mycelium powder using a technique described previously (8).

Polymerase chain reaction and restriction enzyme digestion. PCR primers used to amplify the nuclear small-subunit rDNA (SrDNA) and the ITS region were as described before (8). The primers ML1 and ML4 were used to amplify a portion of the mitochondrial large-subunit ribosomal DNA (mt-LrDNA) (41). PCR primers F63 and R635 (26) were used to amplify a DNA from the 5′ end of the nuclear large-subunit rDNA (LrDNA). PCR reactions were performed in 50- or 100-μl volumes containing 0.8 μM each of the primers; 500 μM each of the four deoxynucleotides, dATP, dCTP, dGTP, and dTTP, in a PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl, and 0.1% Triton X-100); 1.25 units (for 50-μl reactions) or 2.5 units (for 100-μl reactions) of *Taq* DNA polymerase (Promega, Madison, WI). The reactions were overlaid with mineral oil to prevent evaporation. Temperature parameters of the PCR were 93 C for DNA denaturation, 3 min for the first cycle and 1 min for subsequent cycles, 50 C for 1 min for primer annealing, and 72 C for 1 min for primer extension. The total number of cycles was 30, plus a final extension at 72 C for 10 min. The temperature parameters were

TABLE 1. Isolate number, original host/habitat, and geographic locations of *Pythium* species used in this study

Pythium species	Isolate number	Host/ habitat	Geographic location
P. heterothallicum	ATCC18197	Soil	Canada
	ATCC18198	Soil	Canada
	272	Sugarcane	Louisiana
	285	Sugarcane	Louisiana
	11-1	Sugarcane	Louisiana
P. splendens	168	Soil	Hawaii
	169	Soil	Hawaii
	199	Unknown	Ohio
P. sylvaticum	CAL-1	Unknown	Canada
	CAL-2	Unknown	Canada
	13-2	Lettuce	England
	13-3	Lettuce	England
	299	Sugarcane	Louisiana
	54	Soil	Illinois
	55	Soil	Illinois
	56	Scotch pine	Illinois
	59	Soil	Illinois
	60	Scotch pine	Illinois

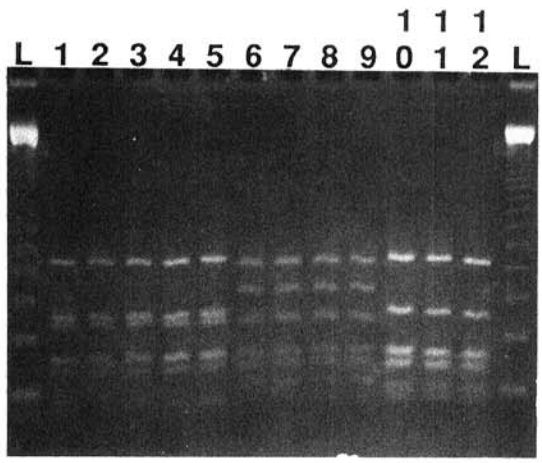

Fig. 1. Agarose gel showing PCR-amplified nuclear small subunit ribosomal DNA digested with restriction enzyme *Taq*I. Lane L, BRL 123-bp DNA ladder; lanes 1, 2, 3, 4, and 5, isolates 13-2, 13-3, 55, 60, and 299, respectively, of *Pythium sylvaticum*; lanes 6, 7, 8, and 9, isolates 18197, 18198, 272, and 285, respectively, of *P. heterothallicum*; lanes 10, 11, and 12, isolates 168, 169, and 199, respectively, of *P. splendens*.

controlled by using a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT). Either positive displacement or filtered pipette tips were used in setting up PCR to prevent cross contamination. A negative control was included in each set of PCR reactions. The efficacy of amplification was checked by running 3 or 5 μl of the reaction solution through a mini-agarose gel, staining with ethidium bromide, and visualizing under ultraviolet (UV) light (37).

PCR products were either purified with ethanol precipitation (8) or used directly for restriction enzyme digestions. Restriction enzymes (GIBCO BRL, Gaithersburg, MD) were used according to the manufacturer's instructions, except when digesting unpurified PCR products, the buffer concentration was adjusted accordingly. The enzyme-digested DNA was run through a 2% composite agarose gel composed of 1% NuSeive GTG agarose (FMC BioProducts, Rockland, ME) and 1% regular agarose, stained with ethidium bromide, and visualized and photographed under UV light (37).

Because very little intraspecific variation was observed, one isolate of each species was selected for restriction site mapping. The restriction sites in the ITS region were determined for isolates 13-3, 168, and 18198 for *P. sylvaticum*, *P. splendens*, and *P. heterothallicum,* respectively. Restriction sites were determined by double digestions and further confirmed by digesting PCR fragments amplified with internal primers as described before (8). The restriction sites in the ITS region were aligned with previously published data (8).

Phylogenetic analysis. The aligned restriction maps were compared to the previously published restriction sites in the ITS region of five homothallic *Pythium* species (8). The presence or absence of a variable restriction site was coded with 1 or 0, respectively, to form a data matrix. The *Cfo*I sites were excluded from the analysis because the *Cfo*I sites were not mapped in the homothallic species. The computer program PAUP version 3.0 (39) was used for the phylogenetic analysis as described before (8).

RESULTS

Nuclear small subunit rDNA. PCR primers NS1 and NS8 amplified nearly the entire SrDNA (8,41). The nuclear SrDNA was about 1,800 bp long for all three heterothallic *Pythium* species. No restriction fragment length polymorphisms were observed after digestion of the SrDNA with enzymes *Cfo*I, *Eco*RI, *Mbo*I, and *Msp*I (gels not shown). Digestion with enzyme *Taq*I, however, revealed three banding patterns corresponding to the three species (Fig. 1). No intraspecific variation was observed.

Internal transcribed spacer. Primers ITS1 and ITS4 amplified the ITS region, including the 5.8 S rDNA located between the nuclear SrDNA and LrDNA. Length variations were detected in this region. The ITS region was about 850 bp long for *P. heterothallicum* and *P. splendens* and about 1,020 bp long for *P. sylvaticum*. Each length variant showed distinct banding patterns after digestion with enzymes *Eco*RI and *Hin*fI (Fig. 2A). Enzyme *Msp*I had one restriction site in the ITS region of *P. sylvaticum* but had no cutting site in that of *P. heterothallicum* and *P. splendens* (gel not shown). In addition, *P. heterothallicum* and *P. splendens* were differentiated from each other by digestions

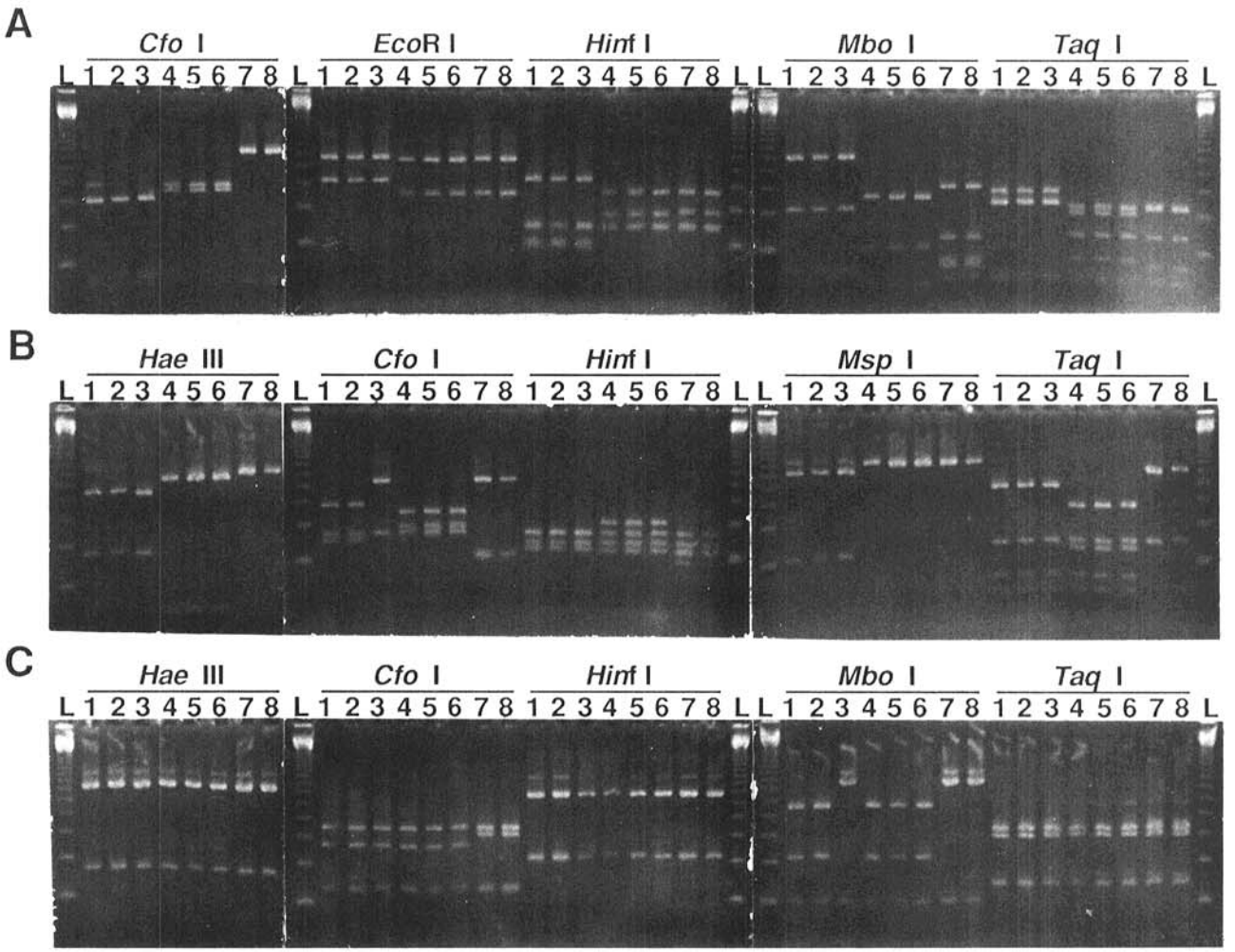

Fig. 2. Agarose gels showing restriction banding patterns of PCR-amplified nuclear and mitochondrial rDNAs. **A,** The internal transcribed spacer of nuclear rDNA; **B,** an 800-bp fragment from the 5′ end of the nuclear large subunit rDNA; and **C,** a 1,000-bp fragment from mitochondrial large subunit rDNA. Lane L, BRL 123-bp DNA ladder; lanes 1, 2, and 3, isolates 13-2, 60, and 299, respectively, of *P. sylvaticum*; lanes 4, 5, and 6, isolates 18197, 18198, and 272, respectively, of *P. heterothallicum*; lanes 7 and 8, isolates 168 and 169, respectively, of *P. splendens*.

with enzymes *Cfo*I, *Mbo*I, and *Taq*I (Fig. 2A). The only intraspecific variation observed in the ITS region was in *P. sylvaticum* after digestion with *Cfo*I (Fig. 2A). Isolate 13-2 was differentiated from the other nine isolates of the same species by the presence of a larger DNA fragment (Fig. 2A). This fragment could be the result of incomplete digestion; however, digestions with excess enzymes did not cut this fragment. It appeared after digestions of three independent PCR products from isolate 13-2, and it did not appear in any of the other nine isolates of the same species. Thus, this fragment most likely resulted from sequence microheterogeneity.

The restriction sites in the ITS region were determined and the maps were aligned to the conserved *Eco*RI sites as previously described (8). The length variation occurred primarily in the ITS-1 region between the SrDNA and the 5.8S rDNA (Fig. 3). The ITS-1 region amplified with primers ITS1 and ITS2 (8,41) was about 260 bp long for *P. heterothallicum* and *P. splendens* and about 370 bp long for *P. sylvaticum* (Fig. 3, gel not shown).

Nuclear and mitochondrial large subunit rDNAs. Primers F63 and R635 specifically amplified an 800-bp DNA fragment from the 5′ end of nuclear LrDNA. Amplification of this specific region from *Pythium* species was verified by using primer R635 separately with primers ITS1 and ITS3, which resulted in expected fragment sizes (gels not shown). The amplified product from the LrDNA was about 800 bp long and uniform in length among the 18 isolates used in this study. Restriction digestions with five enzymes revealed species-specific banding patterns except for the digestion with *Msp*I, in which *P. heterothallicum* and *P. sylvaticum* were not differentiated from each other (Fig. 2B). No *Eco*RI site resides in this region (gel not shown). The only intraspecific variation observed in this DNA region of *P. sylvaticum* was after digestion with *Cfo*I. Isolates CAL-1, CAL-2, and 299 of *P. sylvaticum* differed from the other seven isolates of the same species (isolate 299 shown in Fig. 2B). This intraspecific variation could be explained by a gain or loss of one restriction site.

Much less interspecific variation was observed in the mtLrDNA. The amplified product was about 1,000 bp long for all 18 isolates. No interspecific variation was observed after digestions with enzymes *Hae*III, *Hin*fI, and *Taq*I (Fig. 2C). Digestions with *Cfo*I distinguished *P. splendens* from the other two species but did not differentiate *P. heterothallicum* from *P. sylvaticum* Fig. 2C). Digestion with *Mbo*I resulted in two banding patterns that differentiated *P. heterothallicum* from *P. splendens* (Fig. 2C). Three isolates (CAL-1, CAL-2, and 299) of *P. sylvaticum* showed the banding patterns of *P. splendens*; however, the remaining seven isolates of *P. sylvaticum* had the same banding pattern of *P. heterothallicum* (Fig. 2C, some isolates not shown).

Phylogenetic analysis. Twenty variable restriction sites were used in the phylogenetic analysis. Exhaustive search with Dollo parsimony was employed in the analysis (39). Dollo parsimony is considered appropriate for restriction site data because of the asymmetry in the probability of losing an existing restriction site versus gaining a new site at a particular location (39). Five most parsimonious trees were found (tree length = 25), and one of the five shortest trees is presented (Fig. 4A). This tree is presented because it showed the greatest difference from the consensus tree (Fig. 4B). All of the five most parsimonious trees showed that the heterothallic species were polyphyletic. *P. sylvaticum* was more closely related to *P. irregulare* than to any other heterothallic species (Fig. 4). In the majority rule consensus tree presented in Figure 4B, the percentages of trees supporting the branches are indicated accordingly.

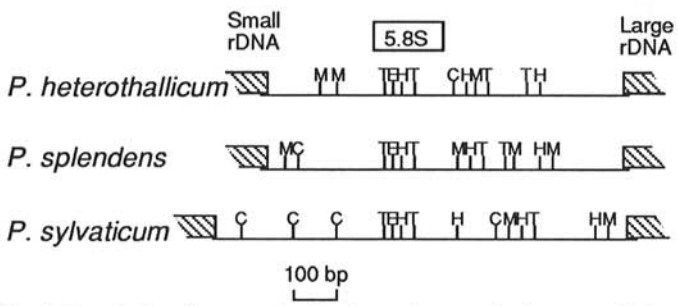

Fig. 3. Restriction site maps in the internal transcribed spacer of the nuclear rDNA of three heterothallic *Pythium* species. The maps were aligned to the conserved *Eco*RI restriction site located in the 5.8S ribosomal DNA. Cutting sites were mapped for restriction endonucleases *Cfo*I (C), *Eco*RI (E), *Hin*fI (H), *Mbo*I (M), and *Taq*I (T). The maps were based on isolates 13-2, 168, and 18198 of *P. sylvaticum, P. splendens,* and *P. heterothallicum*, respectively.

DISCUSSION

In searching for molecular characters for systematics studies, the appropriate molecular markers should resolve at the desired taxonomic level, showing neither excessive variation nor homogeneity at the taxonomic rank of interest (3,24). The different regions of rDNA evolve at different rates. Thus, different regions of rDNA may be appropriate for particular taxonomic level studies. In *Pythium* species, the intergenic region (nontranscribed spacer) is probably too variable for species identifications because variations were observed within single isolate cultures (4,23,28). The internal transcribed spacer of rDNA was shown to be variable between, but largely conserved within, homothallic *Pythium* species (8). Variation corresponded with morphologically delineated species except for two morphologically similar species, *P. arrhenomanes* and *P. graminicola* (8). The study reported here

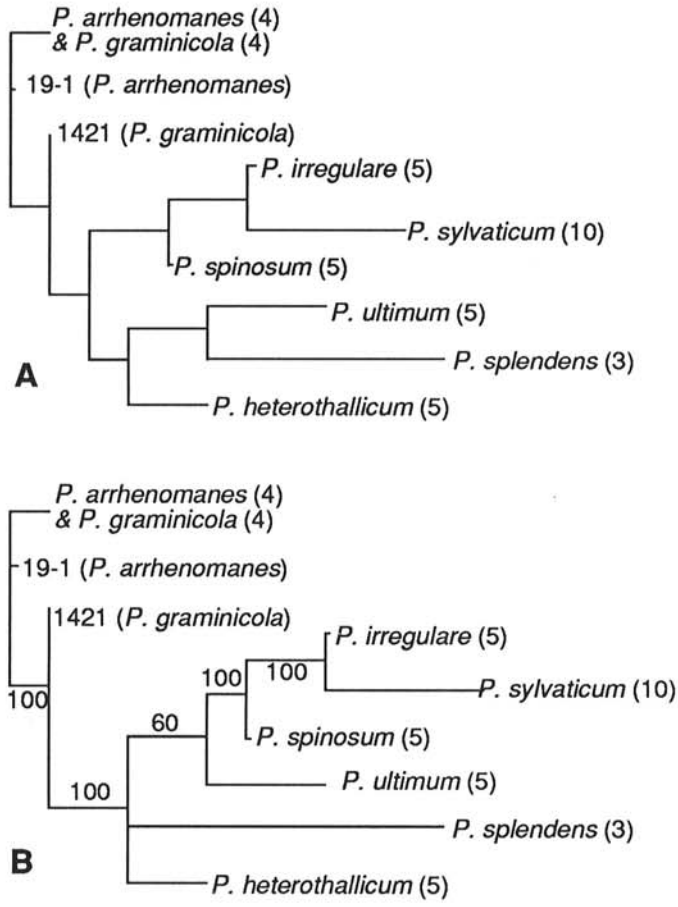

Fig. 4. Unrooted most parsimonious trees showing the relationships of three heterothallic and five homothallic *Pythium* species inferred by parsimony analyses based on variable restriction sites in the internal transcribed spacer of nuclear rDNA. The number in parentheses following each species indicates the number of isolates examined and found to be identical in restriction banding patterns. Vertical distances are for clarity only. The length of horizontal branches is drawn proportional to the number of restriction site changes. **A,** One of the five most parsimonious trees (tree length = 25). **B,** Majority rule consensus tree of the most parsimonious trees. The numbers shown on the major branches indicate the percentages of the most parsimonious trees supporting that particular branch.

extended this observation to three heterothallic species. In addition, it showed that nuclear LrDNA also may provide resolution at the species level. Although variations of SrDNA separated the three heterothallic species in this study, the variability is not at the species level when considered with data from a previous study. For example, *P. sylvaticum* had the same banding pattern of *Taq*I digested-SrDNA as *P. irregulare* and *P. spinosum* (8, and Fig. 1). The information provided by SrDNA comparisons, however, is useful in showing relationships among groups of *Pythium* species. This could be important for the genus *Pythium* because it is a large genus (more than 100 described species) and knowledge of subgeneric structure is needed (2,17).

Mitochondrial DNA is generally considered to be more variable than nuclear DNA due to more frequent length mutations (11,22,25). Nevertheless, in this study and based on data presently available, the 1,000-bp DNA fragment amplified from mt-LrDNA is more conserved than the nuclear LrDNA. The same restriction banding patterns of the mt-LrDNA have been observed in seven homothallic species (W. Chen, *unpublished data*). This is probably not surprising when the unique mitochondrial DNA structure of *Pythium* is considered. Mitochondrial DNAs of Oomycetes in general, and of *Pythium* species in particular, are characterized by the presence of inverted repeats, uniform complexity, and homogeneous populations of molecules (33), although exceptions to homogeneous populations of molecules were reported (18,29). Mt-rDNA is known to reside in the inverted repeats (18,32). Large inverted repeats account for more than 75% of the total mitochondrial genome, and restriction sites in the regions that hybridized strongly to the mt-LrDNA are largely conserved among species (32). It is not clear how such an inverted repeat structure gives stability to the mt-rDNA. In studying the total mitochondrial DNA of *Pythium* species, Martin and Kistler (31) found species-specific banding patterns, which were used as an aid in identifying asexual isolates of *P. ultimum* (27).

In biology, sexual dimorphism, a phenomenon of morphological characters that split a species into two sexual groups (21), is considered to be an advanced reproductive system that enhances genetic recombinations. In the phylogenetic analysis based on the restriction sites in the ITS region, the heterothallic species showed polyphyletic relationships. This suggests that heterothallism in *Pythium* evolved independently (convergent or parallel evolution). There is an associated caveat in this inference; the analysis is based on the assumption that the differences in restriction sites were caused by point mutations. The variation in restriction sites in the ITS region could be due to insertion/deletion events because length variations were observed in this region. An argument for the phylogenetic analysis, however, is that the length variation occurred primarily in the ITS-1 region, whereas most of the informative restriction sites resided in the ITS-2 region (Fig. 3). The phylogenetic relationships of the eight *Pythium* species based on restriction sites in the ITS region are consistent with variations in the SrDNA. The phylogenetic grouping (Fig. 4) also correlates with a morphological character, the number of antheridia per oogonium. *P. irregulare, P. spinosum, P. sylvaticum,* and *P. ultimum* have fewer (one to three) antheridia per oogonium, whereas *P. arrhenomanes, P. graminicola, P. heterothallicum,* and *P. splendens* have multiple (up to eight or more) antheridia per oogonium (40). The meaning of such correlation is not understood. Nevertheless, the phylogenetic relationships of the *Pythium* species were based on the limited data set presently available and should be further verified by independent character sets such as restriction sites of other genes or nucleotide sequences in future studies. It is interesting to note that Belkhiri and Dick (2), using eight DNA characters including G + C contents and hyperchromicities of mitochondrial and nuclear DNAs, also found that *P. heterothallicum* was clustered with *P. rostratum* (a homothallic species) and was distantly related to two other heterothallic species, *P. sylvaticum* and *P. intermedium*.

Morphology and, in the case of heterothallic species, mating reactions continue to form the bases for identification of *Pythium* species. When heterothallic *Pythium* species are encountered in field studies of plant diseases, conclusive identification requires mating with tester strains (5,17,19,35,40). This is a time-consuming process that is not applicable to large numbers of isolates. Furthermore, the mating process is influenced by the physiological state of the cultures and by environmental conditions (10,16,36). Thus, matings between compatible strains may not always give positive reactions.

The results presented in this report demonstrate that three heterothallic *Pythium* species can be easily and reliably differentiated and identified by using molecular markers, which eliminates the need for mating reactions because DNA can be isolated from single-isolate cultures. The sample sizes examined were small, and intraspecific variation within worldwide collection still needs to be assessed. For *P. arrhenomanes*, the intraspecific variation is very low. Thirty-three of 36 isolates from a worldwide collection showed identical restriction banding patterns in the ITS, LrDNA, and a portion of mt-LrDNA regions. The remaining three isolates that were from a single location differed in four of 11 DNA-enzyme combinations (W. Chen and J. W. Hoy, *unpublished data*). Also the applicability of a particular DNA region to identification of other *Pythium* species needs to be experimentally determined. The identification of reliable, easy-to-use molecular markers will greatly facilitate ecological and epidemiological studies of heterothallic species by enabling rapid and accurate species identification.

In general, identification of *Pythium* species by morphology is difficult because so many species are delineated on minor and quantitative differences, and it is difficult to obtain all reproductive structures. Currently, isolates that do not produce reproductive structures are placed in lettered groups (40). The use of molecular markers may eventually provide a speed and degree of certainty for identification of all *Pythium* species that is not possible with traditional methods of identification.

Ribosomal DNAs are repeated hundreds of times. Species-specific variations in the ITS region of rDNA provide a good opportunity for developing specific probes or PCR primers for species identification and detection. Such primers and probes based on repetitive DNA sequences provide greater sensitivity than those based on single-copy sequences (15,34). Combining PCR with specific primers will further enhance sensitivity (14,38,34). There are many potential applications for species-specific molecular markers in plant pathology. Unambiguous and reliable identification of pathogens is critical for disease diagnosis and for ecological and epidemiological studies. Methods that can detect genetic materials of pathogens in infected plants will provide a powerful diagnostic tool.

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