Characterization of the Hyphal Swelling Group of *Pythium*: DNA Polymorphisms and Cultural and Morphological Characteristics

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ABSTRACT

Kageyama, K., Uchino, H., and Hyakumachi, M. 1998. Characterization of the hyphal swelling group of *Pythium*: DNA polymorphisms and cultural and morphological characteristics. Plant Dis. 82:218-222.

The hyphal swelling (HS) group of *Pythium* species and *P. ultimum* were studied for cultural and morphological characteristics, restriction fragment length polymorphisms of the amplified internal transcribed spacer (ITS) region in nuclear rDNA, and random amplified polymorphic DNA (RAPD) analysis of genome DNA. The shape of sporangia was spherical to subspherical or lemoniform and averaged 18.1–23.0 μm . All isolates could grow at 5 to 35°C, and the rate at the optimal temperature, 30°C, was 29–34 mm/24 h. The size of the ITS region amplified by polymerase chain reaction and the banding patterns after digestion with the restriction enzymes showed no variation between the HS group and *P. ultimum*. No difference in banding patterns was shown between the HS group and *P. ultimum* by RAPD analysis with each of three primers. Isolates examined were from Japan, and results should be confirmed from other regions.

Most *Pythium* species are homothallic with only seven species having been designated as heterothallic (18). However, many isolates do not produce sexual structures when paired with other isolates. These asexual isolates have been divided into several groups based on their cultural characteristics and the morphology of their hyphal swellings or sporangia (18). The isolates belonging to the hyphal swelling (HS) group have spherical hyphal swellings that germinate only by producing germ tubes—they produce no zoospores. These isolates are important pathogens that have been implicated in the damping-off or root rot of kidney bean (*Phaseolus vulgaris* L.) (6,11,17) and soybean (*Glycine max* (L.) Merr) (1,7) in the United States and Japan, as well as sugar beet (*Beta vulgaris* L.) (16) in the United Kingdom.

Saunders and Hancock (19) and Francis et al. (5) have demonstrated that the isolates of the HS group mate with self-fertile isolates of *P. ultimum* to produce oospores. Martin (12) found that the restriction patterns of the mitochondrial DNA, the morphology of hyphal swellings, and the pathogenicity to sugar beet among isolates of *P. ultimum* were identical to those in several isolates of the HS group. Therefore, the HS group of *Pythium* is believed to include of asexual isolates of *P. ultimum*.

A molecular technique may be useful for determining the relatedness between the isolates within a species and for distinguishing between species. Species-specific variation has been reported in the internal transcribed spacer (ITS) regions of the rDNA in a number of fungi (22). Thus, examination of the restriction fragment length polymorphisms (RFLPs) of the amplified ITS region by polymerase chain reaction (PCR) might be a powerful technique for the identification of fungi if the type cultures were available. In several species of *Pythium*, this technique has been used both to identify the species and to determine the phylogenetic relationships (2–4). Random amplified polymorphic DNA (RAPD) analysis is used to randomly amplify the genomic DNA segments by PCR with single oligonucleotide primers (21,23). This technique has been used widely for the identification of pathotype, race, forma specialis, and species of plant pathogenic fungi, including *Pseudocercospora herpotrichoides* (Fron) Deighton (14), *Bipolaris maydis* Shoemaker (15), *Fusarium oxysporum* Schltd. f. sp. *ciceris* (Padwick) Matuo and Sato (8), and *Trichoderma* species (24). Therefore, this technique may be applicable to investigating the relatedness of *Pythium* species.

The objective of this study was to characterize isolates of *P. ultimum* and the HS group of *Pythium* species on the basis of DNA polymorphisms and their cultural and morphological characteristics.

MATERIALS AND METHODS

Isolates. The isolates of *Pythium* used in the present study are listed in Table 1. The isolate of *P. aphanidermatum* (Edson)

Fitzp., UOP390, was provided by T. Ichitani, University of Osaka Prefecture, Japan. Other isolates of *Pythium* were identified by the authors, based on the identification key of Plaats-Niterink (18). All isolates were cultured from a single hyphal tip, and the cultures were maintained on cornmeal agar (CMA) at 25°C.

Morphological characteristics and growth temperature. Cultures of *Pythium* isolates were grown on CMA at 25°C in darkness for 1 month, after which the diameters of 25 arbitrarily selected sporangia were measured. Zoospore formation was induced according to Waterhouse (20). The mycelial disks from the growing margin of 2-day-old cultures were placed on CMA in contact with pieces of autoclaved grass blades. After colonization of the fungi, the grass blades were incubated in water at 20 or 25°C for 1 to 7 days. To determine the linear growth rate, cultures were grown on potato carrot agar (PCA) for 2 days, after which agar plugs were taken from the PCA cultures using a 1-cm cork borer and placed on newly prepared PCA plates. The plates were incubated in darkness at various temperatures, ranging from 5 to 35°C at 5°C intervals. Mycelial growth measurements were taken for three replicate plates after a 24 h incubation period.

DNA extraction. For DNA extraction, three agar plugs were removed from the growing margin of 2-day-old cultures on PCA with a 1-cm cork borer and transferred to 20% V8-juice broth. Mycelial mats were collected on filter paper placed in a Buchner funnel after 3 to 4 days of incubation at 25°C and rinsed several times

Fig. 1. Colony appearance of the hyphal swelling group of *Pythium* on potato-dextrose agar.

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Accepted for publication 3 November 1997.

with distilled water. After removing excess water, the mycelial mats were frozen at -80°C for more than 1 day and ground to powder with a sterilized, pre-frozen mortar and pestle. The extraction of DNA was conducted according to the procedure of Lee and Taylor (9), except that the DNA was precipitated with cold ethanol.

Amplification of ITS region using PCR and digestion of PCR products. The ITS region was amplified using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCT-TATTGATATGC-3') according to White et al. (22). A total volume of 100- μl reaction mixture contained 1 μM of each primer, 2.5 units of *rTaq* DNA polymerase (Takara Shuzo Co. Ltd., Shiga, Japan), 0.2 mM dNTP mixture, 1 $\times$ PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM MgCl_2), and 100–300 ng of DNA template. The reaction was conducted using a Perkin-Elmer cetus thermal cycler (model PJ2000), and the amplification condition was an initial denaturation at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min with a final extension at 72°C for 10 min. PCR products were precipitated with two volumes of cold ethanol for purification, and their size was examined by electrophoresis for 60 min in 1.2% agarose LO3 (Takara Shuzo Co. Ltd., Shiga, Japan)

Fig. 2. Sporangia of the hyphal swelling group of *Pythium*. Bar = 10 μm .

Fig. 3. Mycelial growth of the isolates of the hyphal swelling group (Py 12 and Py 56) and *Pythium ultimum* (Py79 and OF321) on potato carrot agar.

gel with 0.5 $\times$ Tris-borate buffer. Gels were stained with ethidium bromide and photographed under ultraviolet light. Restriction enzyme digestion was performed according to the manufacturer's instructions. Restriction fragments were separated by electrophoresis for 60 or 70 min in 3% NuSieve 3:1 agarose (FMC Bioproducts, Philadelphia, PA) gel with Tris-acetate buffer.

RAPD analysis. Three primers, P14 (5'-CCACAGCACG-3'), R1 (5'-GTCCATT-CAGTCGGTGCT-3') and R28 (5'-ATGG-ATCCGC-3'), were used for the RAPD

analysis. A total volume of 25- μl reaction mixture contained 2 μM of primer, 0.625 units of *rTaq* DNA polymerase, 0.2 mM dNTP mixture, 1 $\times$ PCR buffer, and 50 ng of DNA template. The amplification condition was an initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, and extension at 72°C for 3 min with a final extension at 72°C for 10 min. Electrophoresis of the PCR products was performed with 1.5% agarose LO3 for 60 min.

Fig. 4. Comparison of the size of polymerase chain reaction-amplified internal transcribed spacer of rDNA between the 16 isolates of *Pythium*. Key: M = size marker from *Syl*I digestion of bacteriophage lambda; HS = hyphal swelling group of *Pythium*, Py 12 and Py 56; ult = *P. ultimum*, Py 79 and OF231; irr = *P. irregulare*, Py 61 and 6K4SSDP; spi = *P. spinosum*, Py 75 and TN254; syl = *P. sylvaticum*, Py 48 and Py 35; aph = *P. aphanidermatum*, UOP390 and TOc159; per = *P. periplocum*, DS1-7-2S and DK4-6-3D; arr = *P. arrhenomanes*, GUP6 and GUP7.

Table 1. Isolates of *Pythium* species used in this experiment

Isolate No.	Host/habitat	Geographic origin
<i>P. aphanidermatum</i>		
Toc159	Carrot field soil	Gifu, Japan
UOP390	Spinach	Osaka, Japan
<i>P. arrhenomanes</i>		
GUP6	Sugarcane	Kagoshima, Japan
GUP7	Sugarcane	Kagoshima, Japan
<i>P. irregulare</i>		
6K4SSDP	Zoysia grass golf course soil	Gifu, Japan
Py61	Sugar beet	Hokkaido, Japan
<i>P. periplocum</i>		
DK4-6-3D	Zoysia grass	Gifu, Japan
DS1-7-2S	Zoysia grass	Gifu, Japan
<i>P. spinosum</i>		
Py75	Sugar beet	Hokkaido, Japan
TN254	Carrot	Gifu, Japan
<i>P. sylvaticum</i>		
Py35 Ho-M	Sugar beet	Hokkaido, Japan
Py48 He-M	Sugar beet	Hokkaido, Japan
<i>P. ultimum</i>		
MK2-2-1V	Zoysia grass	Gifu, Japan
OF231	Carrot field soil	Gifu, Japan
Py74	Sugar beet	Hokkaido, Japan
Py79	Sugar beet	Hokkaido, Japan
Hyphal swelling group		
BeBS2	Kidney bean field soil	Hokkaido, Japan
BeBS5	Kidney bean field soil	Hokkaido, Japan
Py12	Sugar beet	Hokkaido, Japan
Py13	Sugar beet	Hokkaido, Japan
Py30	Sugar beet	Hokkaido, Japan
Py55	Sugar beet	Hokkaido, Japan
Py56	Sugar beet	Hokkaido, Japan
SoyBS13	Soybean field soil	Hokkaido, Japan

RESULTS

Cultural and morphological characteristics and growth rate. There were no differences in the cultural appearance on CMA or PCA between the HS group and *P. ultimum*. Isolates had cottony aerial mycelia and no special colony pattern (Fig. 1).

The shape of sporangia was spherical to subspherical, or lemoniform in all isolates tested (Fig. 2). The sporangia produced no zoospores. The average size was not significantly different (17.4–23.0 μm) among the isolates of the HS group and *P. ultimum* (Table 2).

The radial growth rates on PCA did not differ among isolates of the HS group and *P. ultimum* at any of the temperatures tested (Fig. 3). All isolates were able to grow at 5–35°C. The rate at the optimal temperature, 30°C, was 32–34 mm/24 h.

ITS-RFLP analysis. The length of the PCR products for the isolates of the HS group was the same as that for *P. ultimum* but differed from those of seven other species of *Pythium* (Fig. 4). The length was estimated at approximately 0.92 kb for the HS group and *P. ultimum*; 1.02 kb for *P. irregulare* Buisman, *P. spinosum* Sawada, and *P. sylvaticum* Campbell and Hendrix; and 0.82 kb for *P. aphanidermatum*, *P. arrhenomanes* Drechsler, and *P. periplocum* Drechsler.

Restriction banding patterns were compared among the isolates by digesting the PCR products with four restriction enzymes, *Eco*RI, *Hinf*I, *Mbo*I, and *Taq*I (Fig. 5) (data not shown for *Hinf*I and *Taq*I). Digestion of PCR products with restriction enzymes allowed polymorphisms to be examined, although no difference in the patterns was found between the patterns of the isolates of the HS group and those of *P. ultimum*. The experiment was performed twice with the same results.

To determine the similarity between the isolates of the HS group and *P. ultimum*, a further experiment was conducted using additional restriction enzymes and isolates. The PCR products were digested with 12 restriction enzymes including 4 enzymes that were used in the above experiment. All isolates of the HS group and *P. ul-*

Fig. 5. Restriction banding patterns of polymerase chain reaction-amplified internal transcribed spacer of rDNA after digestion with *Eco*RI and *Mbo*I. Key: M = size marker from *Hinf*I digestion of bacteriophage Φ X174; HS = hyphal swelling group of *Pythium*, Py 12 and Py 56; ult = *P. ultimum*, Py 79 and OF231; irr = *P. irregulare*, Py 61 and 6K4SSDP; spi = *P. spinosum*, Py 75 and TN254; syl = *P. sylvaticum*, Py 48 and Py 35; aph = *P. aphanidermatum*, UOP390 and TOc159; per = *P. periplocum*, DS1-7-2S and DK4-6-3D; arr = *P. arrhenomanes*, GUP6 and GUP7.

Fig. 6. Restriction banding patterns of polymerase chain reaction-amplified internal transcribed spacer of rDNA after digestion with *Taq*I, *Rsa*I, *Hinf*I, and *Ban*III. Key: M = size marker from *Hinf*I digestion of bacteriophage Φ X174; ult = *P. ultimum*, Py 74 and MK2-2-1V; HS = hyphal swelling group of *Pythium*, Py 12, Py 13, Py 55, BeBS2, BeBS5 and SoyBS13.

Table 2. The size of sporangia of *Pythium ultimum* and group HS^a

Species and Isolate	Sporangia (μm)
<i>P. ultimum</i>	
MK2-2-1V	11–25 (17.4 $\pm$ 3.7) ^b
OF231	12–27 (19.8 $\pm$ 4.9)
Py74	11–29 (23.0 $\pm$ 5.2)
Py79	10–32 (18.7 $\pm$ 6.4)
Group HS	
Py12	9–29 (20.0 $\pm$ 5.7)
Py13	9–31 (21.4 $\pm$ 5.7)
Py30	9–24 (19.1 $\pm$ 3.8)
Py55	10–30 (20.8 $\pm$ 5.9)
Py56	12–29 (21.4 $\pm$ 4.4)

^a HS = hyphal swelling. The cultures were grown on corn meal agar for about 1 month.

^b Mean $\pm$ SD.

mum showed the same restriction patterns (Fig. 6). The PCR products had a single site for *Ban*III, *Eco*RI, *Hha*I, *Mbo*I and *Rsa*I; three sites for *Hinf*I and *Taq*I (the fourth band, 70 bp, is not clear in Fig. 6); and no sites for *Hae*III, *Hind*III, *Pst*I, *Sal*I, and *Xba*I (data not shown for *Hha*I, *Hae*III, *Hind*III, *Pst*I, *Sal*I, and *Xba*I). The experiment was performed twice with the same results.

RAPD analysis. To compare the banding patterns for RAPD analysis, three primers and the same isolates of the HS group and the seven species of *Pythium* used in the ITS-RFLP analysis were examined. All banding patterns of the PCR products for each species with each of the three primers were different for the major bands from 300 to 2,000 bp (Fig. 7). In contrast, there was no differ-

ence between the HS group and *P. ultimum* isolates. A further experiment was conducted by using the same primers and additional isolates of the HS group and *P. ultimum* to determine the similarity between them (Fig. 8). No difference in banding patterns was found among the isolates of the HS group and *P. ultimum* for each of the three primers used in PCR. PCR amplification was performed twice with the same results.

Fig. 7. Random amplified polymorphic DNA analysis of the isolates of *Pythium* species using the primers P14, R1, and R28. Key: M = size marker from a 100-bp DNA ladder (Gibco BRL); HS = hyphal swelling group of *Pythium*, Py 12 and Py 56; ult = *P. ultimum*, Py 79 and OF231; irr = *P. irregulare*, Py 61 and 6K4SSDP; spi = *P. spinosum*, Py 75 and TN254; syl = *P. sylvaticum*, Py 48 and Py 35; aph = *P. aphanidermatum*, UOP390 and TOc159; per = *P. periplocum*, DS1-7-2S and DK4-6-3D; arr = *P. arrhenomanes*, GUP6 and GUP7.

Fig. 8. Random amplified polymorphic DNA analysis of the isolates of *Pythium ultimum* and hyphal swelling group using the primers P14, R1, and R28. Key: M = size marker from a 100-bp DNA ladder (Gibco BRL); ult = *P. ultimum*, Py 74 and MK2-2-1V; HS = hyphal swelling group of *Pythium*, Py 13, Py 55, BeBS2, BeBS5, and SoyBS13.

DISCUSSION

The isolates of the HS group used in the present study were characterized by having a fast growth rate (32–34 mm) and high optimal growth temperature (30°C) on PCA and showed no special colony pattern and a cottony aerial mycelium on CMA and PCA. O'Sullivan and Kavanagh (16) recovered isolates having spherical hyphal swellings and no sexual organs from sugar beets and divided them into two groups. One group consisted of the isolates that showed a faint rosette pattern on PCA and in which the optimum growth temperature was 25°C (20.8 mm) on PCA. The isolates of the other group appeared to be similar to those used in the present study. Martin (12) also found diversity in the HS group using mitochondrial DNA restriction patterns. Thus, the present study may not represent the diversity in HS-group isolates. Given this potential complexity, it is important that the characteristics of isolates are clarified.

The ITS region of rDNA can be reproducibly amplified with universal primers, and has shown interspecific variations among *Pythium* species (2–4). Thus, the data on the RFLP of this region have been comparable among different researchers and can be used for identification. In fact, the banding patterns of the ITS region digested with the four enzymes were identical to those previously reported for *P. ultimum*, as well as for the other *Pythium* species, with the exception of the Py 61 isolate of *P. irregulare* (2–4,10). Although *P. sylvaticum* was the only heterothallic species used in the present study, the restriction fragment patterns of the ITS region in other heterothallic species, such as *P. heterothallicum* and *P. splendens*, were different from those of *P. ultimum* and the HS group (2). RAPD analysis using each of the three primers also revealed inter-specific variations in *Pythium* spp., except for *P. irregulare*. While the two molecular methods showed interspecific variations, there were no differences in the banding patterns among the HS group and *P. ultimum* by ITS-RFLP or RAPD analysis. The results using RFLP of the mitochondrial DNA have confirmed the finding that the HS group is closely related to *P. ultimum* (12). Additional isolates from the HS group need to be examined from other regions to confirm these results.

Two isolates of *P. irregulare*, 6K4SSDP and Py 61, were found to have different restriction patterns. The percentage of oogonia with spines was lower and the number of spines per oogonium was less in the isolate Py 61 than in 6K4SSDP, although both isolates are identified as *P. irregulare* according to the keys of Plaats-Niterink (18). Further investigation is necessary to understand the variations within *P. irregulare* (13).

ACKNOWLEDGMENTS

We thank T. Ichitani for providing the isolates of *P. aphanidermatum* and the Hokkaido Kitami Agricultural Experiment Station for providing kidney and soybean materials from their experimental monoculture fields.

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