Diagnosis and Population Analysis of *Pythium* Species Using AFLP Fingerprinting

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ABSTRACT

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Accurate identification of *Pythium* species, the causal agents of Pythium root rot and damping-off of seedlings, and characterization of populations within the species would greatly assist in selecting and implementing control strategies for these pathogens. Several molecular techniques offer methods for accurate and rapid identification of species, but provide little information about their populations. In this study, amplified fragment length polymorphism (AFLP) fingerprinting was used to characterize plant-pathogenic *Pythium* species and intraspecific populations. Species-diagnostic AFLP fingerprints for *Pythium aphanidermatum*, *P. irregulare*, and *P. ultimum*, and tentative fingerprints for six other species, were identified. Intraspecific distance analyses of *P. aphanidermatum*, *P. ultimum*, and *P. irregulare* revealed distinct patterns of intraspecific variation among the three species. *P. aphanidermatum* showed the smallest mean distance among isolates (15%), followed by *P. ultimum* (37%). *P. irregulare* had the largest mean distance among isolates (64%), which were divided into two populations with great genetic differentiation ($F_{ST} = 0.2$), suggesting the presence of a cryptic species boundary within this species.

Additional keywords: diagnostic fingerprints, genetic marker, population biology

The use of molecular techniques, particularly DNA analyses, has increased the ability of researchers to identify and characterize organisms rapidly, especially microorganisms that have few variable (polymorphic) morphological characters useful for identification. *Pythium* species, the causal agents of Pythium root rot and damping-off of seedlings, are traditionally differentiated on the basis of microscopic characters that include the production and morphology of zoospores and release of zoospores, internal proliferation of zoospores, formation of hyphal swellings, oogonium size, the extent to which the oospore fills the oogonium, oogonium wall ornamentation, and the origin, mode of attachment, and number of antheridia per oogonium (8,26,45). Identification of species that belong to this genus, as well as other members of the Oomycota, is very difficult due to overlapping similarities and intraspecific variation in morphological characteristics (26).

The capacity to accurately identify *Pythium* to species and then characterize populations within the species would greatly assist in selecting and implementing control strategies for this pathogen, particularly in greenhouse crop production where losses occur as a result of Pythium root rot each year. Several *Pythium* species are plant pathogenic on a wide variety of plants, but others are weak pathogens, saprophytes (6,7), or beneficial because of their biological control properties (28,36). There can be considerable variation in pathogenicity among isolates and differences in sensitivity to control chemicals within species (31,32). Although weakly pathogenic species may be managed through manipulation of the fertilizer concentration applied (30) or other cultural practices, an attempt should be made to eliminate highly pathogenic species from the production area. In order to eliminate the pathogen from the production system, its source must be found. One approach to finding it is to compare characteristics of isolates from infected plants with those from potting soil, soil from outside the greenhouse, soil under and between greenhouse benches, the water supply, or new plants brought into the production system.

In recent years, several molecular tools have been used in *Pythium* isolate characterization, including isozyme analysis (33), nucleotide sequence of mitochondrial DNA (26), internal transcribed spacer (ITS) sequences (1,21,31), restriction fragment length polymorphisms (RFLPs) of genomic (17,18) and mitochondrial DNA (27), RFLPs of the ITS regions and

random amplified polymorphic DNA (RAPD) (19,20,29), and reverse dot blot hybridization of ribosomal DNA (24). These techniques have been useful to identify *Pythium* species but are time-consuming, can be costly, and provide little information beyond species identity. If additional information about populations and dynamics of the species is desired, a technique with higher resolution at the population level is required. Amplified fragment length polymorphism (AFLP; 47) is a versatile technique that has been used to perform fingerprinting analyses, mapping, and other genetic studies in a broad range of organisms. This technique has been successfully used in genetic studies of Oomycetes, including species of *Peronospora* (38) and *Phytophthora* (22,43,44,48), and serves as a promising source of information for studies on *Pythium*.

The objectives of this investigation were to (i) generate diagnostic AFLP fingerprints for selected *Pythium* species present in greenhouse crops in Pennsylvania, and (ii) explore the intraspecific variation among isolates of *Pythium aphanidermatum*, *P. irregulare*, and *P. ultimum* associated with agricultural crops using AFLP fingerprinting, in order to establish a base of information for future population studies of these species.

MATERIALS AND METHODS

Isolates studied. Eighty-four isolates from nine plant-pathogenic *Pythium* species from diverse hosts and agricultural systems were used in this study (Table 1). Included were the three species most often associated with greenhouse floral crops in Pennsylvania: *P. aphanidermatum*, *P. irregulare*, and *P. ultimum* (31), and six less commonly found species: *P. dissotocum*, *P. heterothallicum*, *P. myriotylum*, *P. torulosum*, *P. spinosum*, and *P. vexans*. Isolates of *Phytophthora* species were included as external reference groups (outgroups). The isolates chosen for this study were collected by G. W. Moorman from Pennsylvania greenhouses, provided by colleagues, or acquired from the American Type Culture Collection or CAB International. All the isolates were morphologically identified using Van Der Plaats-Niterink's keys to species (45). Most of the isolates came from the United States, but some international isolates were included to increase the genetic diversity of the sample. Fifty-five isolates were tested for resistance to metalaxyl (resistant isolates were defined as those whose growth on

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Table 1. Isolates analyzed in this investigation

Code	Collection designation	Species	ITS sequence ^a	Metalaxyl ^b	Host/substrate	Year	Origin
A01	aphanidermatum TN	<i>Pythium aphanidermatum</i>	AF454357	S	— ^c	—	U.S.
A02	P02	<i>P. aphanidermatum</i>	* ^d	—	<i>Euphorbia pulcherrima</i>	1986	PA
A13	P35	<i>P. aphanidermatum</i>	*	R	<i>E. pulcherrima</i>	1998	PA
A27	P39A	<i>P. aphanidermatum</i>	*	—	Turf ^e	1994	PA
A41	45041-99	<i>P. aphanidermatum</i>	—	S	<i>E. pulcherrima</i>	1999	PA
A42	45044-99	<i>P. aphanidermatum</i>	*	S	<i>E. pulcherrima</i>	1999	PA
A49	71009-98	<i>P. aphanidermatum</i>	*	R	<i>E. pulcherrima</i>	1999	PA
A60	100439R	<i>P. aphanidermatum</i>	AF452149	S	<i>Carica papaya</i>	—	Tanzania
A61	141749R	<i>P. aphanidermatum</i>	AF452146	S	<i>Atropa belladonna</i>	—	India
A62	340458	<i>P. aphanidermatum</i>	AF452148	S	<i>Carica papaya</i>	—	Malaysia
A63	346952	<i>P. aphanidermatum</i>	AF452151	S	<i>Cucumis melo</i>	1991	Israel
A64	363669R	<i>P. aphanidermatum</i>	AF452150	S	<i>Pisum sativum</i>	—	Taiwan
A65	370995R	<i>P. aphanidermatum</i>	*	S	<i>Nicotiana tabacum</i>	—	Venezuela
A66	58847R	<i>P. aphanidermatum</i>	*	S	<i>Nicotiana tabacum</i>	—	Zambia
A68	10-1E	<i>P. aphanidermatum</i>	—	R	<i>E. pulcherrima</i>	2000	PA
A69	10-2E	<i>P. aphanidermatum</i>	—	R	<i>E. pulcherrima</i>	2000	PA
A70	10-3E	<i>P. aphanidermatum</i>	—	R	<i>E. pulcherrima</i>	2000	PA
A71	12-1E	<i>P. aphanidermatum</i>	—	R	<i>E. pulcherrima</i>	2000	PA
A72	12-2E	<i>P. aphanidermatum</i>	—	R	<i>E. pulcherrima</i>	2000	PA
A73	12-3E	<i>P. aphanidermatum</i>	—	R	<i>E. pulcherrima</i>	2000	PA
A74	13-1E	<i>P. aphanidermatum</i>	—	S	<i>E. pulcherrima</i>	2000	PA
A75	13-2E	<i>P. aphanidermatum</i>	—	S	<i>E. pulcherrima</i>	2000	PA
A76	13-3E	<i>P. aphanidermatum</i>	—	S	<i>E. pulcherrima</i>	2000	PA
R01	irregulare TN	<i>P. irregulare</i>	*	S	—	—	US
R16	P40	<i>P. irregulare</i>	AF382820	R	<i>Zinnia</i>	1999	PA
R24	2015	<i>P. irregulare</i>	*	S	—	1999	PA
R33	4-1980	<i>P. irregulare</i>	*	S	—	1980	PA
R38	42144-99	<i>P. irregulare</i>	*	S	<i>Pelargonium</i>	1999	PA
R52	72076-96	<i>P. irregulare</i>	AF452142	S	<i>E. pulcherrima</i>	—	PA
R54	80171-91	<i>P. irregulare</i>	AF454364	—	<i>Pelargonium</i>	—	PA
R59	81774-98	<i>P. irregulare</i>	*	S	<i>E. pulcherrima</i>	—	PA
R62	325393	<i>P. irregulare</i>	—	S	<i>Anigozantus</i>	—	Australia
R71	13-12	<i>P. irregulare</i>	—	S	<i>Pelargonium</i>	—	NY
R72	13-13	<i>P. irregulare</i>	*	—	<i>Pelargonium</i>	—	NY
R78	13-23	<i>P. irregulare</i>	—	S	<i>Pelargonium</i>	—	AL
R80	13-25	<i>P. irregulare</i>	—	—	<i>Pelargonium</i>	—	AL
R82	13-30	<i>P. irregulare</i>	—	S	<i>Pelargonium</i>	—	OK
R86	13-38	<i>P. irregulare</i>	*	—	<i>Pelargonium</i>	—	CA
R90	13-47	<i>P. irregulare</i>	—	—	<i>Pelargonium</i>	—	MI
R91	13-48	<i>P. irregulare</i>	—	—	<i>Pelargonium</i>	—	MI
R93	13-56	<i>P. irregulare</i>	*	R	<i>Pelargonium</i>	—	FL
R94	13-57	<i>P. irregulare</i>	*	S	<i>Pelargonium</i>	—	FL
R95	13-58	<i>P. irregulare</i>	*	—	<i>Pelargonium</i>	—	FL
U01	ATCC32231	<i>P. ultimum</i>	AF452160	S	<i>Phaseolus</i>	—	US
U10	3044	<i>P. ultimum</i>	—	S	<i>Pelargonium</i>	2000	PA
U11	31036-96	<i>P. ultimum</i>	—	S	<i>Pelargonium</i>	1996	PA
U12	3BWA	<i>P. ultimum</i>	*	S	—	1986	PA
U16	81051-96	<i>Phytophthora erythroseptica</i>	*	S	<i>Solanum tuberosum</i>	1996	PA
U18	308273	<i>P. ultimum</i>	*	S	Soil	1973	UK
U21	325953	<i>Phytophthora capsici</i>	*	S	<i>Pisum</i>	—	—
U23	342645	<i>P. ultimum</i>	AF452161	S	<i>Passiflora edulis</i>	—	Zimbabwe
U25	353927	<i>Phytophthora cinnamomi</i>	*	—	<i>Carica papaya</i>	1992	—
U28	369358R	<i>P. ultimum</i>	*	S	<i>Pisum</i>	—	Canada
U29	13-27	<i>P. ultimum</i>	—	S	<i>Pelargonium</i>	—	TX
U30	13-28	<i>P. ultimum</i>	*	S	<i>Pelargonium</i>	—	IN
U31	13-35	<i>P. ultimum</i>	*	S	<i>Pelargonium</i>	—	AL
U32	13-36	<i>P. ultimum</i>	—	S	<i>Pelargonium</i>	—	AL
U33	13-42	<i>P. ultimum</i>	*	S	<i>Pelargonium</i>	—	US
U48	01-1E	<i>P. dissotocum</i>	*	S	Soil	2000	PA
U49	01-3E	<i>P. dissotocum</i>	*	S	Soil	2000	PA
SPP08	73057-96	<i>P. dissotocum</i>	*	R	<i>Pelargonium</i>	1996	PA
SPP09	22017-97	<i>P. dissotocum</i>	*	—	<i>Pelargonium peltatum</i>	1997	PA
SPP10	62092-99	<i>P. dissotocum</i>	*	S	<i>Petunia</i>	1999	PA
SPP11	360198 R	<i>P. dissotocum</i>	AF452154	—	<i>Pyracantha</i>	—	Spain
SPP15	22019-96	<i>P. heterothallicum</i>	*	R	<i>Pelargonium</i>	1996	PA
SPP16	285	<i>P. heterothallicum</i>	AF452162	—	<i>Saccharum</i>	—	LA

(continued on next page)

^a GenBank identification number of ITS-1, 5.8 S, and ITS-2 sequences.

^b Metalaxyl sensitivity: S = sensitive, ED50 < 100 µg/ml; R = resistant, ED50 > 100 µg/ml.

^c Information not available.

^d Known ITS sequence and verified identity.

^e Substrate unknown.

Table 1. (continued from previous page)

Code	Collection designation	Species	ITS sequence ^a	Metalaxyl ^b	Host/substrate	Year	Origin
SPP17	272	<i>P. heterothallicum</i>	—	—	<i>Saccharum</i>	—	LA
SPP19	61024-97	<i>P. myriotylum</i>	*	S	<i>Dianthus</i>	1997	PA
SPP20	30 Pythium	<i>P. myriotylum</i>	AF452156	S	<i>Pelargonium</i>	1998	PA
SPP21	6052-80	<i>P. myriotylum</i>	—	—	—	1980	PA
SPP22	14-4	<i>P. spinosum</i>	*	—	<i>Saccharum</i>	—	Australia
SPP23	1499	<i>P. spinosum</i>	AF452144	—	<i>Panax ginseng</i>	—	Korea
SPP24	1027	<i>P. spinosum</i>	*	—	<i>Saccharum</i>	—	LA
SPP26	86-43	<i>P. spinosum</i>	—	—	—	—	NY
SPP28	69 Pythium	<i>P. myriotylum</i>	—	—	Soil	2000	PA
SPP32	18-5	<i>P. torulosum</i>	AF452155	—	—	—	NC
SPP33	18-8	<i>P. torulosum</i>	*	—	—	—	NC
SPP35	69788-88	<i>P. torulosum</i>	*	—	<i>Saintpaulia</i>	1988	PA
SPP39	80936-95	<i>P. vexans</i>	AF452137	—	<i>Juniperus</i>	1995	PA
SPP40	81708-98	<i>P. vexans</i>	AF452136	—	<i>Pseudotsuga menziesii</i>	1998	PA
SPP41	61183-82	<i>P. vexans</i>	*	—	—	1982	PA
C01	810060-95	<i>Phytophthora cinnamomi</i>	—	—	—	—	PA
C02	22297-91	<i>Phytophthora citricola</i>	—	—	—	—	PA
C03	<i>P. cryptogea</i>	<i>Phytophthora cryptogea</i>	*	—	—	—	PA

corn meal agar containing 100 µg metalaxyl/ml was not slowed by 50% compared with growth in the absence of metalaxyl; Table 1). Mycelia and/or DNA of the isolates in this study are present in the collection maintained by G. W. Moorman at the Pennsylvania State University.

DNA purification. Isolates were grown in 20% V8 broth shake cultures for 3 to 10 days at 110 rpm. The mycelium was collected on filter paper using a Buchner funnel, rinsed with water, and macerated using liquid nitrogen. DNA was extracted using DNAzol ES plant genomic DNA isolation reagent (MRC, Inc., Cincinnati, OH) according to the manufacturer's instructions. The quality of the DNA was assessed using agarose gel electrophoresis by comparing the samples with a High DNA Mass Ladder (Invitrogen Life Technologies, Carlsbad, CA), and by measuring the nucleic acid/protein absorbance ratio using a spectrophotometer.

Sequence analysis. The ITS sequences were obtained for at least eight isolates of *P. aphanidermatum*, *P. irregulare*, and *P. ultimum* and at least two isolates of other species as described by Moorman et al. (31). All the ITS sequences were compared with sequences submitted to GenBank using BLAST to verify the identity of isolates to species level.

AFLP analysis. The AFLP protocol employed was a modification of that described by Vos et al. (47), using primers with one and two additional nucleotides in the preselective and selective amplifications, respectively. Genomic DNA was double-digested, and DNA fragments were ligated to adapters (*Eco*RI: 5'-CTC-GTAGACTGCGTACC/AATTGGTACGC-AGTC-3'; *Mse*I: 5'-TACTCAGGACTCAT/GACGATGAGTCCTGAG-3') using *Eco*RI and *Mse*I digestion enzymes and T4 ligase in a single step restriction-ligation reaction, at 37°C for 8 h, in a volume of 10 µl

(T4 DNA ligase buffer 1.1 µl; 0.5 M NaCl 1.1 µl; *Mse*I 0.05 µl; *Eco*RI 0.05 µl; T4 DNA ligase 0.03 µl; BSA [1 mg/ml] 0.55 µl; *Mse*I adapter solution [50 µM of each primer] 1.0 µl; *Eco*RI adapter solution [5 µM of each primer] 1 µl; DNA template [~100 ng/µl] 1.0 µl; polymerase chain reaction (PCR) water 4.1 µl). Restriction/ligation products (R/L) were diluted up to 200 µl in TE-0.1 (20 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Preselective amplification was performed using primers *Eco*RI+A (5'-GACTGCGTACCAATTCA-3') and *Mse*I+C primers (5'-ATGAGT-CCTGAGTAAC-3') in 20-µl PCR reactions (R/L 4.0 µl; *Eco*RI preselective primer [10 µM] 0.5 µl; *Mse*I preselective primer [10 µM] 0.5 µl; *Taq* DNA polymerase [5 U/µl] 0.08 µl; 10× *Taq* buffer 2 µl; 10× dNTPs [2 mM each] 2 µl; PCR water 10.92 µl), with the following cycle profile: 2 min at 72°C, followed by 25 cycles of a 20-s DNA denaturation step at 94°C, a 30-s annealing step at 56°C, and a 2-min extension step at 72°C. The PCR products were diluted up to 200 µl with TE-0.1. The selective amplification was performed using the same *Mse*I preselective primer and a fluorescently labeled *Eco*RI+AG primer (*Eco*RI Sel3-JOE: 5'-GACTGCGTACCAATTCAAG-JOE-3') with the following cycling profile: 2 min at 94°C followed by 9 cycles of a 20-s denaturing step at 94°C, a 30-s annealing step-wise step at 65°C reducing 1°C every cycle down to 57°C, and a 2-min extension step at 72°C, followed by 20 cycles of a 20-s denaturing step at 94°C, a 30-s annealing step at 56°C, and a 2-min extension step at 72°C. Aliquots of 0.8 µl of the final products were run in polyacrylamide gels and read using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA), and analyzed using the GeneScan analysis software ver. 3.7 (Applied Biosystems) and visual evaluation of gel images and spectrograms to produce one

AFLP band profile (AFLP fingerprint) per isolate.

AFLP fingerprinting analysis. The individual AFLP fingerprints were integrated into a single data matrix, aligning bands by their size in base pairs (bp). The data matrix was transformed into binary data by scoring 1 for the presence of an AFLP band and 0 for its absence using Excel (Microsoft Corporation). Jaccard's genetic similarities among isolates were calculated from the binary data, and neighbor-joining (NJ) cluster analysis was performed using NTSYS (Exeter Software, Setauket, NY). Results of these analyses were represented as a dendrogram to determine if all the isolates of each morphological species grouped together and to detect any misidentified isolates. Isolates of *Phytophthora* were included as outgroups (Table 1).

The binary databases were also analyzed to establish the frequency of AFLP bands (each band was considered as one allele) in each species. Bands present in all of the isolates of a species with small sample ($n < 20$), in 22 out of 23 in *P. aphanidermatum*, and in 19 out of 20 in *P. irregulare* isolates (16), were considered monomorphic (fixed alleles). The particular combination of monomorphic bands in a species constitutes a diagnostic fingerprint for that species that can be used for identification purposes. For one isolate to be identified as belonging to a *Pythium* species, its AFLP fingerprint has to include all of the bands of that species diagnostic fingerprint. The obtained fingerprints were used to identify 48 isolates with blind labels. These included isolates from the species under study and isolates from other species. Identification was verified using ITS sequences and morphology when information was available.

Genetic diversity. The intraspecific genetic variation of *P. aphanidermatum* ($n = 23$), *P. irregulare* ($n = 20$), and *P. ultimum*

($n = 12$) was analyzed. Genetic similarities (S) among isolates of each species were calculated as described above and were used to calculate genetic distances ($D = 1 - S$). Relatedness among isolates was analyzed using the unweighted pair-group method with arithmetic means (UPGMA) cluster analysis. The results were represented as rooted trees using *Pythium irregulare* or *Phytophthora cryptogea* as outgroups. The genetic differentiation (differences in allele frequencies among populations) within *P. irregulare* was ana-

lyzed using the F_{ST} statistic estimates (F_{ST} value of 0 to 0.05 = low, 0.05 to 0.15 = moderate, 0.15 to 0.25 = high, >0.25 = very high genetic differentiation; 49) in Microsoft Excel 2000. The null allele frequencies and average heterozygosities were estimated as recommended by Lynch and Milligan (25).

RESULTS

AFLP fingerprinting. A total of 399 fragments in a range of 50 to 499 nucleotides (nt) were generated for nine *Pythium*

and three *Phytophthora* species using one selective primer combination. Included in the interspecies analyses were 255 polymorphic AFLP bands (each representing a locus). The NJ analysis of all the isolates in the sample distributed most of them into nine groups (Fig. 1) based on their genetic similarities. Each group includes isolates of the same species and appears as a branch in the Figure 1 dendrogram. The analysis also showed that five isolates had been misidentified as *P. ultimum* based on morphological characters (Fig. 1). Two of them grouped with *P. dissotocum* isolates and the other three with *Phytophthora* isolates. Comparison of sequences of their ITS regions with those in GenBank identified them as *Phytophthora erythroseptica* (U16), *Phytophthora cinnamomi* (U25), *Phytophthora capsici* (U21), and *Pythium dissotocum* (U48 and U49). Phylogenetic relationships among *Pythium* and *Phytophthora* species were not studied and were not considered in the construction of the multiple-species tree.

Monomorphic AFLP band patterns were identified within each species. The diagnostic fingerprints of *Pythium aphanidermatum*, *P. irregulare*, and *P. ultimum* were obtained and are described in Table 2. Fingerprints for the other six *Pythium* species were also obtained but are reported here as tentative because of their small sample sizes (Table 2). Some individual AFLP bands were shared among fingerprints; however, each diagnostic fingerprint was unique and species specific. Analysis of 48 blind samples using diagnostic and tentative species fingerprints resulted in correct identification of 29 isolates of the nine species under study, without false positives among the remaining 19 isolates that belonged to other *Pythium* species (data not shown).

Genetic diversity. *P. aphanidermatum*. Of 64 bands analyzed in this species, 41 were unique to single isolates, and 23 were polymorphic among isolates. The sample of *P. aphanidermatum* was geographically diverse, including one isolate from South America, two from Africa, four from Asia, and 16 from North America (Table 1). Distance analysis showed that the *P. aphanidermatum* sample had a mean distance among isolates of 15%. The UPGMA dendrogram of this species (Fig. 2) has a main cluster that includes 21 isolates, including most of the U.S. isolates and those from Tanzania, Israel, India, Venezuela, and Zambia. The genetic distances among isolates of this cluster are small to moderate (mean = 6%, maximum = 28%) compared with the other two species. These isolates share 20 out of 23 (87%) bands and are subdivided into four groups: all isolates in group AP-I have the same 23 bands, isolates in group AP-II lack a 467-nt band, isolates in group AP-III lack a 315-nt band, and isolates in group AP-IV lack a 74-nt band. Isolate A41 lacks both a

Fig. 1. Neighbor-joining tree reconstructed from Jaccard's similarities among isolates, based on amplified fragment length polymorphisms (AFLP) from 87 isolates of nine *Pythium* species, illustrating grouping of isolates by species. Phylogenetic relationships are not represented.

74- and a 467-nt band. Isolate A61 lacks three bands found in other isolates. Group AP-IV consists of isolates from outside the United States. Groups AP-I, AP-II, and AP-III contain mostly U.S. isolates, with the exception of isolate A62 from Malaysia. Nine U.S. isolates (A68 to A76; Table 1) came from Pennsylvania greenhouse-grown poinsettias (*Euphorbia pulcherrima*). Of these, six isolates in group AP-I (A68 to A73) were resistant to metalaxyl, while the three isolates in group AP-III (A74 to A76) were metalaxyl sensitive and lacked the 315-nt band found in the resistant ones.

The two isolates that stayed out of the main branch were isolated from poinsettia (A02, Pennsylvania, USA) and pea (A64, Taiwan). Isolates A02 and A64 are responsible for most of the genetic variability found in the sample, as together they introduce 41 unique bands (62.5%) of the 64 bands analyzed. Isolate A02 lacks 3 of the bands present in the main branch and has 16 additional bands, while isolate A64 lacks 8 bands and has 25 additional bands. All the ITS sequences analyzed from this species were identical.

P. irregulare. Nineteen of the isolates analyzed came from the United States (7 from Pennsylvania and 12 from other states), and one came from Australia (Table 1). Thirteen were isolated from *Pelargonium*; the other seven came from diverse hosts. One isolate of *Phytophthora cryptogea* was used as the outgroup.

Of the 103 bands analyzed, 65 were shared among isolates and 38 were unique to single isolates. The results of the UPGMA cluster analysis are represented in

Fig. 2. *Pythium aphanidermatum* unweighted pair-group method with arithmetic means (UPGMA) tree constructed from amplified fragment length polymorphism (AFLP) data. Scale reflects Jaccard's distances among isolates.

Table 2. Species fingerprints for nine *Pythium* species generated with one amplified fragment length polymorphism (AFLP) selective primer combination plus the individual fingerprints for *P. irregulare* clades I and II^a

<i>P. dissotocum</i> ^c	<i>P. heterothallicum</i> ^c	<i>P. myriotylum</i> ^c	<i>P. spinosum</i> ^c	<i>P. torulosum</i> ^c	<i>P. vexans</i> ^c	<i>P. aphanidermatum</i> ^b	<i>P. ultimum</i> ^b	<i>P. irregulare</i> ^b		
								Clade I	Clade II	
51 ^d	50	60	62	61	92	55	54	78	78	78
71	88	75	69	77	106	116	64	128	128	96
109	93	84	78	121	134	126	153	131	131	128
121	105	105	95	128	187	130	163	197	173	131
128	152	116	99	144	192	143	211	470	186	164
207	167	129	105	164	198	153	234		197	166
289	246	153	111	177	236	167	252		209	197
306	275	180	116	180	254	176	265		299	298
	324	183	128	184	271	186	304		434	311
	326	208	158	200	287	213	325		470	319
	331	210	188	210	334	226	328			327
	344	222	197	244	349	248	373			366
	374	238	199	256	377	251	466			462
	378	257	222	327	382	263				470
	464	264	224	353	419	345				
		282	267	492	423	373				
		289	287		455	380				
		291	293		459	406				
		365	297		467	426				
		419	311							
		444	316							
		454	466							

^a Presence of the complete fingerprint is needed for a positive species diagnosis. Allele frequencies in large populations have yet to be determined.

^b Species-diagnostic fingerprints.

^c Tentative species fingerprint.

^d Values indicate fragment size in base pairs (+/- 1 bp).

Figure 3. The *P. irregulare* sample was subdivided into two groups of 10 isolates, IR-I and IR-II, not correlated with geographic distribution, host, or metalaxyl sensitivity. Group IR-II included an internal cluster with seven isolates from *Pelargonium*.

The mean distance within the sample was 64%, with a minimum distance of 8% and a maximum of 89%. Within group IR-I, the mean distance was 54%, with a minimum of 15% and a maximum of 72%; in group IR-II the distance values were 42, 8, and 69%, respectively. The 0.2 fixation index (F_{ST}) value, estimated for 54 loci, indicates that there is a high level of genetic divergence between the two groups of *P. irregulare* (49). Tentative diagnostic AFLP fingerprints for *P. irregulare* groups IR-I and IR-II contain 10 and 14 monomorphic bands, respectively (Table 2). Analysis of the ITS sequences supports the division of *P. irregulare* into two closely related groups (data not shown).

P. ultimum. Twenty-six bands present in single isolates and 41 shared among isolates were analyzed. Distance analysis reported a mean distance of 37%, with a maximum of 71% and minimum of 0% for two isolates that had identical fingerprints

(U12 and U18). The UPGMA tree shows some internal divisions (Fig. 4). The two groups of interest are UL-I (*Pelargonium*, U.S.) and UL-II (diverse hosts and locations). The small number of isolates in each of these groups did not allow us to perform F_{ST} analysis. All isolates of this species were sensitive to metalaxyl.

The topology of each species tree was different from the topology of the interspecies dendrogram. This is caused by the presence of AFLP bands from unrelated isolates that have the same size even if they represent different loci (homoplasy), which introduces noise that affects the fine resolution of the analysis when differences among isolates are small. The resolution among species, however, was not affected.

DISCUSSION

To provide a simple and accurate method to identify *Pythium* species as an alternative to morphological characterization, the diagnostic AFLP fingerprints of *P. aphanidermatum*, *P. ultimum*, and *P. irregulare* were described. Fingerprints of *P. dissotocum*, *P. heterothallicum*, *P. myriotylum*, *P. torulosum*, *P. spinosum*, and *P. vexans* were reported as tentative due to the small number of isolates of these spe-

cies in our sample. The utility of these fingerprints was proved by the successful identification of 29 isolates of the described species out of 48 blind samples, without misidentification of isolates from other species. However, as variation of allele frequencies in larger samples has yet to be determined, these fingerprints should be used with caution. AFLP fingerprinting also showed that five isolates were originally misidentified as *P. ultimum* based on morphological characters, when they belonged to *Pythium dissotocum*, *Phytophthora erythroseptica*, *Phytophthora capsici* and *Phytophthora cinnamomi*.

The DNA fragments that constitute each of the bands of the diagnostic fingerprints could also be used to develop probes for real-time PCR methods. Real-time PCR reactions use specific primers to amplify a target DNA sequence, and a labeled probe (short DNA sequence) that produces a color signal only when it is bound to the target sequence. The color signal is detected and can be quantified in real-time (34). For use in real-time PCR, the AFLP bands of interest have to be purified and sequenced. From their sequences, PCR primers and probes can be designed that are specific for the species of interest. Real-time PCR has already been used with excellent results to study oomycete and fungal pathogens (3,37,46) and could be used for diagnostic purposes as well as for quantification of *Pythium* populations in tissue, soil, or water samples.

To effectively design *Pythium* disease management strategies for greenhouse crops, it is desirable to know the origin of inoculum present in facilities. *Pythium* propagules can be introduced into a greenhouse on infected plant tissue, infested potting mixes, water, and even carried by insects (14). Determining which of these is the source of the pathogen causing crop losses is a challenge. Our approach to this problem will be based on characterizing and comparing pathogenic populations isolated from various locations within the greenhouse to those from diseased plants. However, in order to compare the populations of a pathogen, some basic knowledge about the genetic variability of the species is necessary.

The genetic variability of *P. aphanidermatum*, *P. irregulare*, and *P. ultimum* in agricultural systems was studied. These species are those most commonly associated with greenhouse crops in Pennsylvania (31). To analyze their general genetic variability, we chose the AFLP technique, among other available molecular techniques, because it is a versatile method that generates large numbers of reproducible genetic markers and can be used to characterize species and their populations.

Most genetic studies on the genus *Pythium* have focused on *P. ultimum*. Francis and collaborators (11) estimated the levels and distribution of variation in this species,

Fig. 3. *Pythium irregulare* unweighted pair-group method with arithmetic means (UPGMA) tree obtained from amplified fragment length polymorphism (AFLP) data. Two groups (IR-I and IR-II) were identified in this tree that may represent two cryptic species. Group IR-II includes a branch with isolates associated to *Pelargonium*. Scale reflects Jaccard's distances among isolates.

and looked at the roles of sexual and asexual reproduction in generating and maintaining this variation. They reported that *P. ultimum* has high levels of genetic variation, that inheritance of markers was consistent with diploidy, and showed that *P. ultimum* isolates were able to outcross (10). Some *P. ultimum* isolates exhibited mitotic instability and appeared to have more chromosomes or duplicate chromosomes relative to mitotically stable isolates. Thus, the authors suggested that polyploidy, aneuploidy, or heterokaryosis may also contribute to genetic variation (9,11). Nonetheless, they found evidence that the three morphological types present in this species, homothallic (HO), isolates producing hyphal swellings (HS), and zoospore-producers (*P. ultimum* var. *sporangiferum*), were not genetically distinct (9). Our analysis of *P. ultimum* was limited by the number of isolates available. The sample analyzed was small and did not consider the three morphological types. However, in agreement with previous studies, our results reflected a considerable genetic diversity within this species, with a mean genetic distance among isolates of 37% and some internal subdivision. These results suggest that sexual reproduction may play a role in the genetic recombination of this species, although geographic distribution and host movement may be limiting factors. If, as suggested by previous studies, the three morphological types do not represent different populations within *P. ultimum*, including them in the analysis may not result in an increase of the overall genetic diversity of the species. However, analysis of a representative sample of genetic diversity of the species could provide information about the genetic differences that determine the morphological types.

Little is known about the population biology of *P. irregulare*. This pathogen has a broad range of hosts, causing serious economic impact in the floricultural industry by reducing the quality of crops, although it rarely kills the host plant (31). Few genetic studies have analyzed populations of *P. irregulare*. In 1997, Barr et al. (2) performed an isozyme analysis of isolates from a worldwide collection. They found a division of *P. irregulare* into two groups, supported by morphological differences in oogonium size and ornamentation. One of those groups was more closely related to *P. sylvaticum* than to the other *P. irregulare* group. Matsumoto et al. (29) studied 47 *P. irregulare* isolates from diverse hosts, 30 from Japan, and 17 from the United Kingdom, Australia, the United States, Poland, and the Netherlands, using ITS-RFLP and RAPD analyses. Isolates of *P. sylvaticum*, *P. paroecandrum*, *P. spinosum*, and *P. ultimum* were included as external groups. Their analysis divided all the *P. irregulare* isolates into two main groups, each divided into two smaller clusters. The distance

between the two main *P. irregulare* groups was larger than the distance between either of them and *P. sylvaticum*, regardless of their country of origin. Harvey et al. (18) studied a population of 29 *P. irregulare* samples from 12 different locations in Australia isolated from cereal crops (*Hordeum vulgare* and *Triticum aestivum*), medic (*Medicago truncatula*), and sub-clover (*Trifolium subterraneum*). Using RFLP analysis, they found two distinct groups within this species. Most of their genetic diversity was attributed to host species, with a level of genetic differentiation ($G_{ST} = F_{ST}$; 35) between host groups of 0.21. Neither study found a correlation between population division and geographic distribution.

Our analysis of *P. irregulare* isolates, mostly from floricultural crops from the United States (with the exception of R62 from Australia; Table 1), produced results that were consistent with previous studies on this species. The analyzed sample was divided into two groups (IR-I and IR-II), without correlation with geographic origin, and our estimate of the genetic differentiation among them ($F_{ST} = 0.2$) was similar to that estimated by Harvey et al. (18) for Australian populations. The results obtained from AFLP fingerprinting analysis were supported by analysis of ITS sequences.

Populations of soilborne pathogens can have large differences in allele frequencies when they are established in geographically distant locations due to the lack of genetic exchange among them. An example of this was found by Lamour and Hausbeck in *Phytophthora capsici* populations collected from six farms in four different regions of Michigan between 1998 and 2000 ($F_{ST} = 0.18$ to 0.4 among populations from different locations; 23). However, the genetic differentiation among isolates collected in different years from a single location was not significant ($F_{ST} < 0.05$; 22). Their results indicate that populations of the pathogen were established in the fields, and their genetic differences could be explained by geographic isolation. Apparently, this is not the case of the populations found within *P. irregulare*. Although the studies in Australia (17,18) and Japan (29) considered populations from several locations, and our study in the United States considered isolates from various states, including several isolates from Pennsylvania, the significant differences in allele frequencies ($F_{ST} = 0.2$) between the two populations suggest an interruption of the genetic flux among them that cannot be attributed to geographical separation (e.g., isolates from both populations can be found in the same greenhouse in Pennsylvania, data not shown). Even

Fig. 4. *Pythium ultimum* unweighted pair-group method with arithmetic means (UPGMA) tree obtained from amplified fragment length polymorphism (AFLP) data. Scale reflects Jaccard's distances among isolates.

though the number of isolates analyzed in this study was relatively small, our results are meaningful. The division within *P. irregulare* (population structure) could be more significant than previously recognized.

Species defined by morphological characteristics can be complexes that include two or more phylogenetic species, a phenomenon known as cryptic speciation (4). The results of this investigation suggest the presence of a cryptic species boundary within *P. irregulare*, an aspect that is currently under further study. Cryptic speciation is common in fungi, including *Stachybotrys chartarum* (42), *Coccidioides immitis* (4), and *Aspergillus flavus* (13). Fungal cryptic species differ in host preferences (41) and display metabolic differences (e.g., toxin production; 12) that can have strong implications in terms of pathogenicity and control; therefore, their recognition and characterization is important.

P. aphanidermatum is considered one of the most important pathogens affecting greenhouse vegetable and floricultural crops (15). Although there is abundant information regarding disease incidence, isolate pathogenicity, and control of diseases produced by this species, little is known about its genetics. Herrero and Klemsdal (19) used RAPD analysis to characterize 20 isolates from diverse hosts and geographic origins, mostly from Norway but also from England, Spain, France, Bulgaria, Israel, Sumatra, Hawaii, and the continental United States. Using three primers (OPA-03, OPA-15, OPB-08 from Operon kits A and B), they obtained identical fingerprints for 18 of 20 isolates. Our results show little genetic variation within the species, with no evident differentiation based on host specificity or geographic distribution. Two isolates differed from the others (A02 and A64) in that they displayed more unique bands. This genetic difference could be attributed to geographic isolation (A64, Taiwan) or chronological separation (A02, collected in 1986). Future population studies will require many more AFLP markers to resolve intraspecific variation within *P. aphanidermatum*.

Despite the overall lack of genetic variation, metalaxyl resistance has been detected in several *P. aphanidermatum* populations (5,31,39,40). We found evidence of genetic differentiation between metalaxyl sensitive and metalaxyl resistant isolates in the population of *P. aphanidermatum* from greenhouses in Pennsylvania. Metalaxyl sensitive isolates (A74, A75, and A76) lacked one AFLP fragment (315 nt) present in fungicide resistant isolates (A68, A69, A70, A71, A72, and A73) from this population.

Comparisons among *P. ultimum*, *P. irregulare*, and *P. aphanidermatum* based on AFLP fingerprinting showed that these

species possess distinct patterns of intraspecific variation. We observed that a protocol used to analyze the genetic diversity of one species may not be as informative when applied to another, even if they are closely related; hence the importance of optimizing methods for each of them. The analysis of samples from geographically distant locations and/or from different years can provide a broad view of the genetic composition of a species, providing a reference frame for population analysis. This approach should be considered before drawing conclusions about the genetic diversity of populations from a single or few locations. Clonality can mistakenly be assumed when molecular tools with inadequate resolution are used.

The isolates analyzed in this study came from agricultural systems, mainly greenhouse crops; therefore, our conclusions may not apply to natural populations. To understand the population genetics of *Pythium* species and the epidemiology of the diseases they cause, detailed population biology studies are needed. Current studies focus on the identification of genetic structure within *P. irregulare* and *P. aphanidermatum* and the search for genetic markers of fungicide resistance. This information will provide a basis for more timely advice to growers regarding crop management for disease prevention.

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