Amplified Fragment Length Polymorphism Analysis and Internal Transcribed Spacer and *coxII* Sequences Reveal a Species Boundary Within *Pythium irregulare*

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ABSTRACT

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Pythium irregulare is a plant-pathogenic oomycete that causes significant damage to a variety of crops, including ornamentals and vegetables. Morphological as well as molecular studies have reported high levels of genetic diversity within *P. irregulare* sensu lato which has raised the question as to whether it is a single species or is actually a complex of morphologically similar (cryptic) species. In this study, we used amplified fragment length polymorphism (AFLP) fingerprinting and DNA sequence analysis of the internal transcribed spacer (ITS) region of the ribosomal genes (ITS region) and a portion of the mitochondrial cytochrome oxidase II gene and the spacer region between *coxI* and *coxII* to characterize 68 isolates of *P. irregulare* from the United States. The ITS

sequence of a *P. irregulare* neotype at the CBS collection as well as ITS and *coxII* sequences for *P. irregulare*, *P. spinosum*, and *P. sylvaticum* from previous studies were included in our analysis. Cluster analysis identified a 19-isolate group (IR-II) that separated itself from the rest of the sample (IR-I). Population structure and sequence analyses supported the distinction of IR-I and IR-II and identified IR-II as *P. irregulare* sensu stricto. IR-I was designated *Pythium* sp. clade IR-I. Two insertion/deletion mutations and nine nucleotide substitutions in the ITS region and three in the sequence of *coxII* and the adjacent spacer region separated the two species. Additionally, they differed significantly ($P > 0.01$) in the frequency of 182 (77%) AFLP alleles. Gene flow results suggested that *P. irregulare* sensu stricto and *Pythium* sp. clade IR-I are cryptic species capable of exchanging favorable alleles ($N_m = 0.72$).

Additional keywords: cryptic speciation, damping-off, diagnostic fingerprints, genetic marker, *Pythium* root rot.

Pythium irregulare Buisman (kingdom Stramenopila, phylum Oomycota) is a ubiquitous plant-pathogenic species with multiple and diverse hosts. It causes pre- and postemergence damping-off of seedlings, root rots, and blackleg of several crops including *Triticum*, *Solanum*, *Pisum*, *Citrus*, *Saccharum*, *Rhododendron*, *Chrysanthemum*, and *Pelargonium* (9,26). In the United States, there is an increased concern about these diseases due to recently detected resistance to mefenoxam (enantiomer of metalaxyl) and propamocarb (1,26,27) in *P. irregulare*.

Management of plant diseases requires accurate and timely identification of pathogens. Fungicide treatment is often recommended when *Pythium* propagules are found in diseased plant material, regardless of the species. More accurate diagnosis of species could reduce wasteful use of resources because, while some *Pythium* species are aggressive, others are weak plant pathogens, and others are pathogens of fungi and can actually be beneficial (5). Fungicide applied to control all species is wasteful and may leave the crop more vulnerable to disease. Furthermore, resistance to mefenoxam or propamocarb fungicides has been detected in several *Pythium* species (26,27). Accurate knowledge of the pathogen present in a crop will allow growers to implement disease management strategies that effectively use the resources available to them.

Pythium species are difficult to identify because they have few variable morphological characters. *P. irregulare* sensu lato (s.l. = in the broad sense), in particular, represents a challenge because

of its variable oogonial morphology (36,37) ranging from smooth to having blunt conical or finger-like projections of variable length. Ornamented and smooth-spherical oogonia can be found in the same isolate. Oogonia can be globose to irregular, 16 to 21 μ m diameter, intercalary, or terminal. Antheridia originate at some distance from the oogonial stalk, but there are members of the species with sessile or hypogynous antheridia as well (36).

To facilitate identification, a variety of biochemical and molecular biology methods (3,13,20,25,39) have been adapted to *Pythium* species. These studies have also revealed that *P. irregulare* s.l. has high levels of intraspecific genetic diversity. Every study has identified two or more protein, DNA fragment patterns, or DNA sequences for this morphological species. Protein analyses allowed differentiation among several *Pythium* species; however, their effectiveness in separating closely related species was not good. Serological profiling using mono- and polyclonal antibodies (39) produced two markedly different patterns of reactivity for *P. irregulare*, but could not effectively distinguish between *P. irregulare* and *P. sylvaticum*. A study of 10 *Pythium* species using polymorphisms in 13 isozymes reported that 62 *P. irregulare* isolates divided into three clusters that tightly overlapped with two *P. spinosum* clusters, leading to the conclusion that these two species were indistinguishable based on isozyme polymorphisms (7). Barr et al. (4) compared the isozyme-based genetic diversity (11 isozyme loci), morphological characters, and growth rate of 125 *P. irregulare* isolates from diverse locations and hosts around the world and distinguished 33 multilocus genotypes that formed two taxonomically distinct groups. DNA-based methods have easily distinguished *P. irregulare* s.l. from other species. Intraspecific genetic variation has been detected using DNA sequencing of the ribosomal DNA internal transcribed spacers (ITS

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region) and the cytochrome oxidase II gene (*coxII*), and DNA fingerprinting (random amplified polymorphic DNA [RAPD] and amplified fragment length polymorphism [AFLP]) analyses (3,13, 25). Two independent phylogenetic studies (19,22) on the genus *Pythium* identified two clades in *P. irregulare*, based on DNA sequences of nuclear and mitochondrial genes. Matsumoto et al. (24) analyzed 47 isolates of *P. irregulare* from diverse geographical origins and hosts, using morphological characteristics of the oogonia and oospore, ITS sequences, ITS restriction fragment length polymorphism (RFLP), and RAPD analysis using seven arbitrary primers. They observed four divisions within *P. irregulare* (I to IV) supported by RAPD and ITS sequence analysis. Garzón et al. (13) identified diagnostic-AFLP fingerprints for nine *Pythium* species and assessed the genetic diversity of *P. aphanidermatum*, *P. ultimum*, and *P. irregulare*. Two main groups were identified within a sample of 20 *P. irregulare* isolates mostly from the United States, each having its own distinctive AFLP fingerprint.

From a phytopathological perspective, the great diversity within *P. irregulare* s.l. represents a diagnostic and management challenge. If the variability can be characterized as population structure, identification of the factors associated with it may provide

useful information for the management of diseases caused by this pathogen. For example, if the population structure in greenhouse crops is determined by geographic origin, then disease management strategies should aim at preventing the introduction of the pathogen into production areas from movement of soil, water, or plants from the facility or its surroundings. If the population structure is associated with host, then control measures should focus on movement of plant material in and out of production areas. However, if the population structure is a result of a speciation process, then each group should be studied as an individual species to avoid misinterpretation of the data. Although intra-specific division of *P. irregulare* s.l. has been reported in the literature repeatedly (4,7,13,19,22), the depth of this division and its repercussion in the management of the pathogen are still unclear.

In this study, we used DNA sequence analysis of the ITS region (including the 5.8S ribosomal RNA gene) of the nuclear ribosomal genes and a partial sequence of the mitochondrial cytochrome oxidase II gene and the adjacent spacer region between *coxI* and *coxII*, and whole genome AFLPs to characterize *P. irregulare* s.l. isolates from diverse hosts and geographic areas within the United States, with and without resistance to mefenoxam.

TABLE 1. Isolates analyzed in this investigation

Collection designation	Species	ITS ^a	cox ^a	Metalaxyl ^b	Host	Year	Origin
P15	<i>P. irregulare</i>	AY907892	— ^c	—	<i>Euphorbia pulcherrima</i>	1995	PA
P25	<i>P. irregulare</i>	—	—	R	<i>Euphorbia pulcherrima</i>	1997	PA
P31	<i>P. irregulare</i>	—	—	R	<i>Chrysanthemum</i>	1998	PA
P37	<i>P. irregulare</i>	—	—	R	<i>Ranunculus</i>	1999	PA
P39	<i>P. irregulare</i>	—	—	S	<i>Pelargonium</i>	1999	PA
P50	<i>P. irregulare</i>	AY907893	AY907918	S	<i>Euphorbia pulcherrima</i>	1999	PA
P54	<i>P. irregulare</i>	—	—	R	<i>Pulmonaria</i>	2000	PA
P55	<i>P. irregulare</i>	—	—	R	<i>Cordyline</i>	2000	PA
P56	<i>P. irregulare</i>	—	—	R	<i>Chrysanthemum</i>	2000	PA
P57	<i>P. irregulare</i>	AY907894	—	R	<i>Bocopa</i>	2000	PA
1103-81	<i>P. irregulare</i>	AY907895	AY907919	S	<i>Crassula</i>	1981	PA
2076-80	<i>P. irregulare</i>	AY907907	AY907932	S	<i>Capsicum annuum</i>	1980	PA
22009-99	<i>P. irregulare</i>	—	—	S	<i>Thymus</i>	1999	PA
22164-98	<i>P. irregulare</i>	—	—	S	<i>Cyclamen</i>	1998	PA
31074-99	<i>P. irregulare</i>	—	—	—	<i>Catharanthus</i>	1989	PA
42130-97	<i>P. irregulare</i>	AY907896	AY907944	S	<i>Pelargonium</i>	1997	PA
42143-99	<i>P. irregulare</i>	AY907897	AY907920	S	<i>Pelargonium</i>	1999	PA
42144-99	<i>P. irregulare</i>	AY907898	AY907925	S	<i>Pelargonium</i>	1999	PA
42145-99	<i>P. irregulare</i>	—	—	S	<i>Pelargonium</i>	1999	PA
45021-97	<i>P. irregulare</i>	—	—	R	<i>Sedum</i>	1997	PA
52066-96	<i>P. irregulare</i>	—	—	S	<i>Pelargonium</i> × <i>hortorum</i>	1996	PA
6028-80	<i>P. irregulare</i>	—	AY907931	—	<i>Capsicum annuum</i>	1980	PA
61186-84	<i>P. irregulare</i>	—	—	—	<i>Malus</i>	1984	PA
63067-99	<i>P. irregulare</i>	—	—	R	<i>Pelargonium</i> × <i>hortorum</i>	1999	PA
63105-99	<i>P. irregulare</i>	—	—	R	<i>Heuchera</i>	1999	PA
63108-98	<i>P. irregulare</i>	—	AY907933	S	<i>Schlumberger</i>	1998	PA
63108-99	<i>P. irregulare</i>	AY907908	AY907934	S	<i>Aquilegia</i>	1999	PA
72076-96	<i>P. irregulare</i>	AF452142	AY907945	S	<i>Euphorbia pulcherrima</i>	1996	PA
73028-97	<i>P. irregulare</i>	AY907909	AY907936	S	<i>Euphorbia pulcherrima</i>	1997	PA
80171-91	<i>P. irregulare</i>	AY907899	AY907921	—	<i>Taxus</i>	1991	PA
81271-97	<i>P. irregulare</i>	—	—	S	<i>Sedum</i>	1997	PA
81758-98	<i>P. irregulare</i>	AY907910	AY907929	S	<i>Ocimum basilicum</i>	1998	PA
81774-98	<i>P. irregulare</i>	AY907900	AY907928	S	<i>Euphorbia pulcherrima</i>	1998	PA
325393	<i>P. irregulare</i>	AY907901	AY907926	S	<i>Anigozanthus</i>	—	Australia
346955	<i>P. irregulare</i>	—	—	—	<i>Gypsophila</i>	1991	Israel
363670 R	<i>P. irregulare</i>	AY907911	AY907930	S	<i>Allium</i>	—	Taiwan
371142 R	<i>P. irregulare</i>	—	—	—	<i>Gerbera</i>	—	Italy
371144 R	<i>P. irregulare</i>	AF452141	—	—	<i>Basilicum</i>	—	Italy
13-1	<i>P. irregulare</i>	—	—	—	<i>Pelargonium</i>	—	MI
13-7	<i>P. irregulare</i>	AY907912	AY907937	S	<i>Pelargonium</i>	—	MI
13-10	<i>P. irregulare</i>	AY907913	AY907940	—	<i>Pelargonium</i>	—	MI

(Continued on following page)

^a GenBank accession numbers of internal transcribed spacer (ITS) region and *coxII* gene-spacer region sequences.

^b Metalaxyl sensitivity: S = sensitive, 50% effective dose (ED₅₀) < 100 µg/ml; R = resistant, ED₅₀ > 100 µg/ml.

^c — Indicates information not available.

^d Isolates from Matsumoto et al. (24).

^e Partial *coxII*-I gene cluster sequences provided by F. Martin.

Population structure and gene flow analyses among populations were performed to characterize intraspecific divisions and assess the level of separation among them.

MATERIALS AND METHODS

Isolates. Sixty-eight isolates morphologically identified as *P. irregulare* s.l. (36) of known location of origin, host, and/or year of isolation were studied (Table 1). One *P. ultimum* isolate was included as the outgroup for AFLP analysis. These isolates were stored at room temperature at The Pennsylvania State University in pieces of water agar in vials containing sterile tap water. For DNA extraction, isolates were grown on potato dextrose agar for 1 day and transferred to 4-cm-diameter petri dishes with 3 ml of 20% V8 broth, and grown for 3 to 7 days at 23°C in the dark. Mycelial masses were harvested using a Buchner funnel and rinsed with distilled water. Each mycelial mass was then transferred to 2-ml centrifuge tubes and freeze-dried. Dehydrated mycelia were weighed and stored at -20°C until used.

DNA purification. DNA was purified from approximately 100 µg of dehydrated mycelium using Qiagen DNeasy Plant Mini Kits (Qiagen, Valencia CA), according to the manufacturer instructions, with the exception that mycelia were macerated at room temperature in lysis buffer with 1.5-ml microcentrifuge

tubes, micro-pestles, and sterile quartz sand. DNA concentration was estimated using 1.5% agarose gels and 4 µl of High DNA Mass Ladder (Invitrogen Life Technologies, Carlsbad, CA). DNA purity was assessed by measuring the nucleic acid/protein absorbance ratio with a spectrophotometer.

AFLP analysis. Restriction-ligation and preselective reactions were performed as described previously (13). Eight selective *EcoRI/MseI* primer combinations were tested. All primers were obtained from PE Applied Biosystems (Foster City, CA). AFLP fragments were analyzed in an ABI 3100 automated capillarity system, using GeneScan Analysis Software (Applied Biosystems). Three selective primer combinations were selected (*MseI*+C/*EcoRI*+AG-VIC, *MseI*+C/*EcoRI*+CC-FAM, and *MseI*+C/*EcoRI*+CG-VIC) based on the high number of polymorphic markers and the intensity of fragment peaks. Data were imported to Microsoft Excel, and fragments were aligned by size manually or using a program in the SAS system (SAS Institute, Cary, NC) and visually verified to prevent possible errors. Markers with low mean peak amplitude relative to noise were eliminated to avoid ambiguities. Data were transformed into a binary database, scoring presence and absence of markers as 1 or 0, respectively. AFLP markers were assumed to represent one locus each and were treated as such during the analyses. Loci with alleles found in three or fewer isolates were eliminated to reduce homoplasy.

TABLE 1. (Continued from preceding page)

Collection designation	Species	ITS ^a	cox ^a	Metalaxyl ^b	Host	Year	Origin
13-12	<i>P. irregulare</i>	AY907902	AY907942	S	<i>Pelargonium</i>	—	NY
13-13	<i>P. irregulare</i>	AY907914	AY907941	—	<i>Pelargonium</i>	—	NY
13-14	<i>P. irregulare</i>	—	—	—	<i>Pelargonium</i>	—	NY
13-18	<i>P. irregulare</i>	—	—	—	<i>Pelargonium</i>	—	CA
13-21	<i>P. irregulare</i>	—	—	—	<i>Pelargonium</i>	—	CA
13-22	<i>P. irregulare</i>	AY907903	AY907943	R	<i>Pelargonium</i>	—	CA
13-24	<i>P. irregulare</i>	—	—	—	<i>Pelargonium</i>	—	AL
13-29	<i>P. irregulare</i>	AY907915	AY907938	S	<i>Pelargonium</i>	—	OK
13-30	<i>P. irregulare</i>	AY907916	AY907939	S	<i>Pelargonium</i>	—	OK
13-32	<i>P. irregulare</i>	AY907904	AY907922	S	<i>Pelargonium</i>	—	CT
13-33	<i>P. irregulare</i>	—	—	—	<i>Pelargonium</i>	—	CT
13-43	<i>P. irregulare</i>	—	—	—	<i>Pelargonium</i>	—	—
13-44	<i>P. irregulare</i>	—	—	—	<i>Pelargonium</i>	—	NY
13-45	<i>P. irregulare</i>	—	—	—	<i>Pelargonium</i>	—	MI
13-47	<i>P. irregulare</i>	—	—	—	<i>Pelargonium</i>	—	MI
13-48	<i>P. irregulare</i>	—	—	—	<i>Pelargonium</i>	—	MI
13-50	<i>P. irregulare</i>	—	—	—	<i>Pelargonium</i>	—	MI
13-56	<i>P. irregulare</i>	AY907905	AY907923	R	<i>Pelargonium</i>	—	FL
13-57	<i>P. irregulare</i>	—	AY907927	S	<i>Pelargonium</i>	—	FL
13-58	<i>P. irregulare</i>	AY907906	AY907924	—	<i>Pelargonium</i>	—	FL
13-59	<i>P. irregulare</i>	AY907917	AY907935	—	<i>Pelargonium</i>	—	FL
4I	<i>P. irregulare</i>	—	—	R	Soil	2000	PA
5I	<i>P. irregulare</i>	—	—	R	Soil	2000	PA
6-1I	<i>P. irregulare</i>	—	—	R	Soil	2000	PA
8I	<i>P. irregulare</i>	—	—	R	Soil	2000	PA
P62	<i>P. irregulare</i>	—	—	S	Soil	2000	PA
P64	<i>P. irregulare</i>	—	—	S	Soil	2000	PA
ATCC 32231	<i>P. ultimum</i>	AF452160	—	S	<i>Phaseolus</i>	—	USA
CBSnr 733.94	<i>P. irregulare</i>	AY598702	—	—	—	—	—
UOP359 ^d	<i>P. irregulare</i>	AB108005	—	—	—	—	—
MAFF305572 ^d	<i>P. irregulare</i>	AB107999	—	—	—	—	—
EP-2 ^d	<i>P. irregulare</i>	AB107997	—	—	—	—	—
ATCC 42912 ^d	<i>P. irregulare</i>	AB107996	—	—	—	—	—
74-22 ^d	<i>P. irregulare</i>	AB107995	—	—	—	—	—
Point B ^d	<i>P. irregulare</i>	AB108000	—	—	—	—	—
K6-2 ^d	<i>P. irregulare</i>	AB107998	—	—	—	—	—
Py63 ^d	<i>P. irregulare</i>	AB108003	—	—	—	—	—
Py26 ^d	<i>P. irregulare</i>	AB108001	—	—	—	—	—
Py61 ^d	<i>P. irregulare</i>	AB108002	—	—	—	—	—
Py64 ^d	<i>P. irregulare</i>	AB108004	—	—	—	—	—
OM121 ^d	<i>P. sylvaticum</i>	AJ233459	—	—	—	—	—
OD231 ^d	<i>P. spinosum</i>	AJ233457	—	—	—	—	—
ATCC 96201 ^e	<i>P. irregulare</i>	—	AF196602	—	<i>Panax</i>	—	Korea
ATCC 96946 ^e	<i>P. sylvaticum</i>	—	AF196624	—	Soil	—	Korea
79-4 ^e	<i>P. spinosum</i>	—	AF196616	—	Soil	—	FL

The data matrix was deposited in TreeBASE (accession no. S1336-M2350).

Cluster analysis. Two genetic distance methods were used to analyze the sample for internal clusters. First, total distances between individual AFLP genotypes were calculated and trees were constructed by the unweighted pair-group method with arithmetic average (UPGMA) algorithm using PAUP version 4.0b 10 (Sinauer Associates, Inc., Sunderland, MA). Tree branch stability was assessed by bootstrap analysis with 1,000 repetitions. Second, Jaccard's distances were calculated and a neighbor-joining (NJ) phenogram was constructed using NTSYS version 2.02j (Exeter Software, Setauket, NY).

Population subdivision and gene flow analysis. Two hundred thirty-six bands with an observed frequency of less than $1 - (3/N)$ were included in the analysis (21). To determine the genetic differentiation among populations, Nei's gene diversity statistics (28), namely total gene diversity (H_T), genetic diversity within populations (H_S), the proportion of the total diversity among populations (G_{ST}), and the average number of migrants between populations per generation (N_m) (31) were estimated using POPGENE (Exeter Software). POPGENE calculated allele frequencies considering AFLP fragments as dominant markers, assuming each fragment represents a single locus. Hardy-Weinberg (HW; $F_{IS} = 0$) equilibrium was assumed in the absence of empirical F_{IS} values. As reported by Goodwin et al. (16), due to an error in the N_m calculations with diploid dominant data in POPGENE version 1.21, the program used the haploid formula $0.5(1 - G_{ST}/G_{ST})$ to calculate N_m instead of the diploid formula $0.25(1 - G_{ST}/G_{ST})$. Correction of the N_m value was performed manually by dividing the N_m value calculated by POPGENE by 2. N_m values were interpreted according to Porter (30): between species, N_m values greater than or near 1 indicate constant gene flow among populations; values between 0.5 and 1 indicate that gene flow is weak but permits exchange of favorable genes; and values below 0.5 indicate the groups are almost or fully genetically isolated. In addition, null allele frequencies and average heterozygosities were estimated as described by Lynch and Milligan (21). Chi-square analyses were performed to determine differences in allele frequencies among populations.

Sequence analysis. Sequences of the ITSs and the 5.8S unit of the ribosomal DNA and partial sequences of the *coxII* gene and the adjacent intergenic spacer (between *coxI* and II) of selected isolates were analyzed. Sequences were generated using an ABI Prism Big Dye version 3.1 (Applied Biosystems) sequencing kit and resolved in an ABI Prism 3100 capillarity sequencing system at the Microbial Properties Research Unit of the National Center for Agricultural Utilization Research (U.S. Department of Agriculture-Agricultural Research Service [USDA-ARS], Peoria). Sequences of the ITS region (ITS1, 5.8S ribosomal RNA gene, and ITS2, complete sequence) of 27 *P. irregulare* s.l. isolates (Table 1) were obtained using primers ITS1 and ITS4 as described in Moorman et al. (26). The selected *P. irregulare* s.l. isolates were distributed along the AFLP tree and were considered representatives of the genetic diversity of the sample. The ITS sequence of a *P. irregulare* neotype at the CBS collection (CBSnr 733.94) was provided by C. A. Lévesque (GenBank accession no. AY598702) and was included in our analysis. Additionally, sequences from nine isolates representing the four *P. irregulare* clades proposed by Matsumoto et al. (24), plus one isolate each of *P. sylvaticum* and one of *P. spinosum* from the same study, were obtained from GenBank (Table 1). The sequences were aligned using Clustal X (35) and manually edited for alignment errors. Sequence analysis was performed with parsimony and distance methods using PAUP version 4.0b 10. Parsimony analysis was performed using a heuristic search with random addition of sequences, saving multiple most parsimonious trees. Genetic distances were estimated using the uncorrected- p measure, and tree reconstruction was performed using the NJ algorithm. ITS sequences of *P. spinosum* and

P. sylvaticum were used as outgroups. Bootstrap analysis with 1,000 repetitions was performed to estimate the support of internal branches. The data matrix was deposited in TreeBASE (accession no. S1336-M2351).

Partial sequences of the cytochrome oxidase (*cox*) II gene and spacer region (513 bp of the 3' end of the *coxII* gene and 141 bp of the adjacent spacer region between the *coxI* and II genes) from 28 isolates of *P. irregulare*, 16 from AFLP defined clade IR-I, and 12 from clade IR-II were obtained from polymerase chain reaction products generated using primers FM 35 (5'-CAGAACC-TTGGAATTAGG-3') and FM 52 (5'-GTTGTGCTAATTCC-ATTCTAA-3') (22), 10 μ M, 1.0 μ l each, in 50- μ l reactions (5 μ l of buffer [0.5 M KCl, 0.1 M Tris-HCl, pH 8.3, and 0.25 mM $MgCl_2$], 5 μ l of 10 $\times$ dNTP mix containing 2 mM each, 0.1 μ l of *Taq* polymerase, and 27.9 μ l of H_2O). Sequences of the forward and reverse strands of the gene were obtained and used in the final alignment. Cycling parameters were those described by Martin (22). *Cox* sequences of two isolates of *P. irregulare* (ATCC 96201 and 1987-68) and one sequence each of *P. spinosum* (79-4) and *P. sylvaticum* (ATCC 96946) were provided by F. Martin (USDA-ARS, Salinas, CA). Sequence analysis was performed as described above for the ITS region with the alignment deposited in TreeBASE (accession no. S1336-M2352).

Fungicide sensitivity. Forty-eight representative isolates were tested for resistance to either metalaxyl or its enantiomer mefenoxam. Isolates were grown on corn meal agar (CMA) amended with the fungicide (100 μ g/ml) and on unamended CMA at 20°C, for 24 h. Isolates whose growth on fungicide-amended CMA was not slowed by 50% compared with growth on unamended agar were designated as fungicide-resistant (26).

RESULTS

Cluster analysis of AFLP data. Three hundred thirty-eight AFLP markers were analyzed. Distance analyses using the UPGMA (Fig. 1) and NJ algorithms generated similar trees (data not included) that differed in some internal branches but agreed in the separation of one 19-isolate cluster from the rest of the sample. This cluster corresponds with previously reported group IR-II (13). The cluster formed by the rest of the sample in the present study contained isolates previously reported as group IR-I. This group was further divided into several subgroups that included two to eight isolates, some with good bootstrap support. Because these groups had small sample sizes, we focused on the analysis of the differences between group IR-II and the rest of the sample. Both IR-I and IR-II included isolates from diverse hosts, geographic origins, and years of isolation (Table 1). IR-I included metalaxyl/mefenoxam-sensitive and -resistant isolates, whereas IR-II included metalaxyl/mefenoxam-sensitive isolates only. Although metalaxyl/mefenoxam sensitivity data were obtained for only 11 isolates in IR-II, the probability of finding 11 sensitive isolates in this subgroup at random (based on a binomial probability), if the 44 isolates tested came from the same population, was very small ($P = 0.005$).

Sequence analysis of the ITS region of ribosomal DNA. rDNA-ITS region sequences of 29 isolates representative of the sample diversity were compared with 11 *P. irregulare*, one *P. sylvaticum*, and one *P. spinosum* sequence (Table 1) from Matsumoto's study (24). The ITS sequence of the *P. irregulare* neotype (CBSnr 733.94; GenBank accession no. AY598702) was also included to identify *P. irregulare* sensu stricto (s.s. = in the strict sense). Parsimony as well as distance analysis using the NJ algorithm revealed two main groups, well-supported by bootstrap (Fig. 2), that corresponded with the groups revealed by AFLP analysis (IR-I and IR-II). One hundred and five parsimony-informative sites were found. Variation in amplicon length was found in both groups with IR-I sequences ranging between 936 and 949 bp, and IR-II sequences ranging between 936 and 938 bp.

A total of two fixed insertion/deletion mutations and nine fixed nucleotide substitutions separated the two groups. All of our sequences clustered with isolates included in clades I and II by Matsumoto et al. (24), IR-II corresponding with their clade I. Inclusion of the ITS sequence of C. A. Lévesque's *P. irregulare* neotype revealed that IR-II and Matsumoto's clade I correspond to *P. irregulare* s.s. From here on, the two groups described in this study will be referred to as *P. irregulare* s.s. and *Pythium* sp. clade IR-I. Isolates from clades III and IV from Matsumoto's study clustered in a completely separate branch, closer to *P. sylvaticum* than to *P. spinosum*, *P. irregulare* s.s., or *Pythium* sp. clade IR-I.

Cytochrome oxidase II mitochondrial gene and intergenic spacer region. Analysis of partial sequences of the *coxII* gene and the intergenic spacer region (651 bp) of 28 *P. irregulare* isolates representative of the sample diversity (*P. irregulare* s.s., *n* = 15; *Pythium* sp. clade IR-I, *n* = 13), plus two sequences of

P. irregulare (ATCC 96201 and 1987-68), and one sequence each of *P. spinosum* (79-4) and *P. sylvaticum* (ATCC 96946) provided by F. Martin, revealed 24 parsimony informative characters. Parsimony analysis generated four most parsimonious trees with a score of 62. Trees obtained by parsimony and NJ (Fig. 3) distance analysis displayed a 14-isolate cluster that branched from the main *P. irregulare* group. This cluster included all the isolates from *P. irregulare* s.s. but one, 13-57, which clustered inside *Pythium* sp. clade IR-I. There were three fixed single nucleotide substitutions between *P. irregulare* s.s. and *Pythium* sp. clade IR-I (in the *coxII* gene at nucleotide positions 509 and in the spacer region at bases 585 and 629). The sequence of isolate ATCC 96201 clustered within *Pythium* sp. clade IR-I, while that of isolate 1987-68 formed an independent branch closer to *P. spinosum* than to *P. irregulare* or *P. sylvaticum*.

AFLP allele frequencies. The allele frequencies of 236 loci were compared. Significant differences in allele frequencies

Fig. 1. Unweighted pair-group method with arithmetic average tree of *Pythium irregulare* depicting *Pythium* sp. clade IR-I and *P. irregulare* sensu stricto and their subpopulations (A to C and D to F, respectively) constructed from amplified fragment length polymorphism data. Numbers over branches represent significant (>70) bootstrap support values (1,000 repetitions). Isolate information is provided: a = reference code (Table 1); b = host; c = state or country of origin; and d = sensitivity to mefenoxam (R = resistant; S = sensitive).

were found between the *Pythium* sp. clade IR-I and *P. irregulare* s.s. The number of polymorphic loci in the *Pythium* sp. clade IR-I was 205 (87%), while in *P. irregulare* s.s., it was 122 (52%). Marker alleles were fixed for one locus and polymorphic at 79 loci in *Pythium* sp. clade IR-I, but were absent in *P. irregulare* s.s. Conversely, alleles at 16 polymorphic loci from *P. irregulare* s.s. were absent in the *Pythium* sp. clade IR-I. The chi-square comparison of allele frequencies indicated that the two clades significantly ($P < 0.01$) differed in the frequencies of 182 (77%) of the alleles. The average heterozygosity of *Pythium* sp. clade IR-I (0.26) was larger than that of *P. irregulare* s.s. (0.17).

Population structure and gene flow. The AFLP gene diversity statistics indicate some genetic differentiation and restricted gene flow in *P. irregulare* s.l. Nei's gene diversity (h) in *P. irregulare* s.s. was 0.15 (standard deviation [SD] = 0.184), and in *Pythium* sp. clade IR-I, it was 0.24 (SD = 0.186). The average gene diversity within subpopulations (H_S) was 0.20 (SD = 0.013), while the gene diversity in the total population (H_T) was 0.26 (SD = 0.022). The coefficient of gene differentiation among subpopulations (G_{ST}) was 0.24. The average Nm over 236 loci was 0.72, indicating that the gene flow between *P. irregulare* s.s. and *Pythium* sp.

clade IR-I is weak but may allow the exchange of favorable genes (30). An F_{IS} value of 1 was used in our calculations to observe the effect of complete inbreeding in the estimation of G_{ST} . The estimate of $G_{ST} = 0.25$ with inbreeding was slightly larger than the estimate under HW equilibrium. Hence, the absence of empirical F_{IS} values does not affect our estimates of differentiation between clades.

Cluster analysis of *P. irregulare* s.s. and *Pythium* sp. clade IR-I from AFLP data (Fig. 1) identified internal groups that were not evident in ITS or *coxI-II* data (Figs. 2 and 3). *Pythium* sp. clade IR-I had three main groups (Fig. 1). Group A included isolates resistant to mefenoxam from diverse hosts and locations, although fungicide sensitivity information was available for only half of the isolates; group B included both mefenoxam-sensitive and -resistant isolates, from diverse hosts and locations; and group C included both mefenoxam-sensitive and -resistant isolates that were collected from diverse hosts in Pennsylvania. *P. irregulare* s.s. contained mefenoxam-sensitive isolates only, clustering in three main groups. Group D included isolates from diverse hosts and locations; group E included isolates from *Pelargonium* sp. from diverse locations; and group F included isolates from diverse hosts from Pennsylvania.

Fig. 2. *Pythium irregulare* neighbor-joining tree from internal transcribed spacer (ITS) sequences. *P. irregulare* sensu stricto (clade IR-II) and *Pythium* sp. clade IR-I are labeled along with clades MI-MIV proposed by Matsumoto et al. (24). Numbers over branches represent significant (≥ 75) bootstrap support values (1,000 repetitions). (M) = isolates from Matsumoto et al. (24); (L) = *P. irregulare* neotype (CBSnr 733.94). Isolate 13-22 has the same ITS sequence as *P. regulare* (AF492018) and *P. cylindrosporum* (AY598643).

DISCUSSION

Analyses of AFLP fragment data, rDNA-ITS, and the *coxII* gene and adjacent spacer sequences (whole genome, nuclear and mitochondrial DNA, respectively) from 68 isolates from different hosts and locations suggest that *P. irregulare* s.l. has undergone or is currently undergoing speciation, and includes at least two morphologically similar (cryptic) species. Parsimony and distance analysis of fragment and sequence data identified a distinct 19-isolate group that is isolated from the general *P. irregulare* population. The presence of 16 private alleles and absence of 80 AFLP loci, two insertion/deletion mutations and nine nucleotide substitutions in the ITS sequence, and three nucleotide substitutions in the *coxII* gene and adjacent spacer sequence separate this group from the rest of *P. irregulare* s.l. This distinct group includes the neotype of *P. irregulare* (CBSnr 733.94; GenBank accession no. AY598702), and is thus recognized as *P. irregulare* s.s. The re-

maining 49 isolates formed a diverse clade that may or may not include other differentiated groups. Sequence data clearly indicate these isolates do not belong to *P. irregulare* s.s., or the closely related species *P. sylvaticum* and *P. spinosum*. Larger samples are necessary to perform population biology studies in this group. To facilitate the remainder of this discussion, isolates that fit the morphological description of *P. irregulare* s.l. but were not included in *P. irregulare* s.s. by genetic analyses will be referred to as *Pythium* sp. clade IR-I.

Previous studies have reported genetic differences that suggested *P. irregulare* s.l. may be a complex of cryptic species. White et al. (39) performed a serological profiling of 20 *Pythium* species. They included five *P. irregulare* isolates that displayed two very distinct profiles. Both principal component analysis and hierarchical cluster analysis of enzyme-linked immunosorbent assay data separated the two groups as completely independent identities. Barr et al. (4) compared the isozyme-based genetic

Fig. 3. *Pythium irregulare* neighbor-joining tree from *coxII* gene and intergenic spacer (between *coxI* and II genes) sequences. *P. irregulare sensu stricto* and *Pythium* sp. clade IR-I are labeled. Numbers over branches represent significant (≥ 75) bootstrap support values (1,000 repetitions).

diversity of 125 *P. irregulare* isolates from diverse locations and hosts around the world. They distinguished 33 multilocus genotypes defined by 11 isozyme loci that grouped into two taxonomically distinct groups. Matsumoto et al. (24) divided 47 isolates morphologically identified as *P. irregulare* into four groups (I to IV) based on ITS-RFLP analysis and RAPD analysis. In their phylogenetic analysis based on sequences of the ITS region, groups I and II, and III and IV clustered more tightly in the same branch, and the genetic divergence between I and II, and III and IV was higher than between either group and *P. sylvaticum*, suggesting that they may represent two different species. Lévesque and De Cock (19) performed a thorough phylogenetic analysis of the genus *Pythium* based on ITS region sequences. Their study included 24 *P. irregulare* sequences, most of them obtained from GenBank, that formed the same four groups described by Matsumoto et al. (24). Martin (22) performed a phylogenetic analysis of the genus *Pythium* based on sequence analysis of the cytochrome oxidase II gene. This analysis included five isolates morphologically identified as *P. irregulare* s.l., four of which formed a discrete clade, while the remaining one clustered with *P. mamillatum*. A comparison of the groups identified by these studies with our sample was desirable to obtain a broader perspective of the genetic diversity of *P. irregulare* outside the United States. This could be done by including isolates from those studies or at least DNA sequences from some of their isolates in our analysis. We were able to obtain ITS sequences of isolates from the four groups described by Matsumoto et al. (24) that were deposited by the authors in GenBank. Isolates from group I clustered within *P. irregulare* s.s., and isolates from group II clustered within *Pythium* sp. clade IR-I. Together, the two studies include isolates of the two species in the *P. irregulare* complex from 11 countries in America, Europe, Asia, and Australia (namely the United States, Canada, Italy, Netherlands, Germany, UK, Poland, Israel, Taiwan, Japan, and Australia) indicating the two species are globally distributed. Matsumoto et al. (24) found slight morphological and growth rate differences between group I and the other three groups. A morphological study is underway to determine if any characters that differentiate *P. irregulare* s.s. from *Pythium* sp. clade IR-I can be identified. When that study is complete, *Pythium* sp. clade IR-I will be formally described. In our phylogenetic analysis, Matsumoto's groups III and IV clustered more closely to *P. sylvaticum* than to any *P. irregulare* isolate, supporting their suggestion that groups III and IV represent species other than *P. irregulare*. Lévesque and De Cock (19) provided further evidence that supports groups III and IV from Matsumoto et al. (24) as unique entities, as their phylogenetic analysis separated them from *P. irregulare* and clustered them in a monophyletic clade with *P. debaryanum*, *P. violae*, and *P. sylvaticum*. In addition, two *P. irregulare* sequences from the study by Martin (22) were included in our analysis. One of them (ATCC 96201) clustered within *Pythium* sp. clade IR-I, while the other (1987-68) formed an independent branch closer to *P. spinosum* than to *P. irregulare* or *P. sylvaticum*. The fact that in both studies isolate 1987-68 clustered closer to species other than *P. irregulare* suggests it may represent some other species. These observations strongly support the conclusion that the morphological description of *P. irregulare* s.l. is broad, and includes at least four discrete clades that may represent distinct phylogenetic species.

Cryptic species are morphologically indistinguishable groups that show other evidence of separation, in this case, an apparent history of very little or no genetic exchange. Cryptic species have been identified in *Phytophthora* group IV (16) and diverse fungal species such as *Stachybotrys chartarum* (8), *Coccidioides immitis* (6), *Candida albicans* (32,33), and *Aspergillus flavus* (14,33) among others. Our results suggest that *P. irregulare* s.s. and *Pythium* sp. clade IR-I are cryptic species capable of exchanging favorable alleles at a fairly low level ($Nm = 0.72$). Although

P. irregulare s.l. is homothallic and self-fertile, evidence of outcrossing was observed by Harvey et al. (17,18) in populations from a variety of cereal crops, medic (*Medicago truncatula*) and subclover (*Trifolium subterraneum*) in Australia. Evidence for outcrossing in the homothallic species *P. ultimum* and *Phytophthora sojae* has been previously described (10–12,38). Hybridization and limited gene flow could explain isolate 13-57 (*P. irregulare* s.s. according to AFLP and ITS sequence data) sharing its *coxII* gene sequence with the *Pythium* sp. clade IR-I (cytochrome oxidase being one of a superfamily of proteins that act as the terminal enzymes of respiratory chains, hence a protein with an important function).

It should be considered, nonetheless, that our estimate of Nm is likely to be an overestimate because of homoplasious AFLP markers, which occur where nonhomologous bands of the same size are scored as the same allele. According to Porter (30), in comparing species “when $Nm > \sim 1$ then the gene flow is likely to be an important factor promoting genetic similarity between groups; when $\sim 1 > Nm > \sim 0.5$ then gene flow is weak but probably present at levels strong enough to at least permit rapid exchange of favorable genes; when $\sim 0.5 > Nm > 0$ then the groups are almost or fully genetically isolated, and the gene flow is likely to be unimportant relative to genetic drift, overestimation notwithstanding.” They affirm, nonetheless, that no test can show that a complete boundary exists. It is not clear what factors may have determined the separation of *P. irregulare* s.s. from *Pythium* sp. clade IR-I. Reproductive isolation of the two species is apparent by the fixation of numerous neutral markers in *P. irregulare* s.s. This group had significantly reduced heterozygosity and gene diversity when compared with *Pythium* sp. clade IR-I. Although host, metalaxyl/mefenoxam use and geographic origin do not seem to be involved, it is feasible that geographic isolation took place during a period of time that allowed the differentiation of *P. irregulare* s.s., followed by extensive dispersion of the new species. This scenario is not strange in agricultural systems where cultural practices impose strong selective pressures on pathogen populations.

Harvey et al. (17,18) compared the genetic diversity among populations of *P. irregulare* s.l. from different hosts and locations in Australia using co-dominant RFLP markers. Their first study (17) identified some population differentiation among allopatric populations from cereals and concluded that genetic drift and geographic isolation could be responsible for their genetic differences. They estimated a mean genetic differentiation (G_{ST}) of 0.54 for 29 loci across seven populations. In a second study (18), some genetic differentiation among populations from different hosts was found ($G_{ST} = 0.21$) without clear evidence of host specificity. They observed, however, that populations from fields where hosts were grown in annual rotation displayed more genetic diversity than populations from long-term medic pastures, which had been regenerating annually for many years. Unfortunately, due to differences in the sampling scale and analytic methods between their research and the present study, comparisons of genetic diversity parameters were difficult. They collected samples exclusively from Australia and compared populations defined by location and host, paying more attention to the differences among those populations than to the overall genetic differentiation within the species. The value they estimated for the mean genetic differentiation (G_{ST}) in their first study is approximately double the amount estimated in the present study, which could be attributed to the use of co-dominant markers and a large sample size. Co-dominant markers are more informative than dominant markers (21) because they allow the identification of alternative alleles, as well as heterozygote genotypes, which can be compensated for when working with dominant markers by increasing the sample size and the number of loci analyzed. In the present study, those factors were considered. Nonetheless, it is possible that our G_{ST} may be an underestimation of the actual genetic differentiation between

the two species in *P. irregulare* s.l. caused by the limited information provided by AFLP dominant markers.

The degree of potential sexual fertility between the two groups described here remains unknown, but the high level of genetic divergence between them is consistent with a historical lack of interbreeding. Further genetic analyses, including hybridization studies will provide useful information about the interaction between these species and the nature of their genetic barriers.

An interesting observation was the absence of metalaxyl/mefenoxam resistance in the sample of *P. irregulare* s.s. isolates tested. It is feasible that the low genetic variability in *P. irregulare* s.s. reduces its potential for developing fungicide-resistant genotypes. Mefenoxam/metalaxyl resistance has been detected in *P. cylindrosporum*, *P. dissotocum*, *P. heterothallicum*, *P. splendens*, *P. ultimum*, and *P. aphanidermatum* (26), as well as in other oomycete species, such as *Phytophthora infestans* (15), *Phytophthora erythroseptica* (34), and *Plasmopara halstedii* (2).

Recently, Masih and Paul (23) described *P. regulare* as a new species closely related to *P. irregulare*, based on a single isolate from the Canary Islands with 100% of its oogonia bearing blunt or digitate spines. The ITS sequence from that isolate (GenBank accession no. AF492018) grouped within *Pythium* sp. clade IR-I and is not different from some other *P. irregulare* sequences previously reported in GenBank (data not shown). Preliminary morphological data from *Pythium* sp. clade IR-I show oogonia with variable numbers of spines, some carrying none (data not shown), which fits the description of *P. irregulare* but not of *P. regulare*. Because *P. regulare* was defined by one morphological character of a single isolate and its ITS sequence is identical to those of some *Pythium* sp. clade IR-I isolates (i.e., isolates 13-22 [GenBank accession no. AY907903] and 42143-99 [GenBank accession no. AY907897] among others), the validity of this species is questioned. The ITS region sequence of the ex-type isolate of *P. cylindrosporum* (29) (CBS 218.94, GenBank accession no. AY598643) is also identical to the sequences of *P. regulare* (19) and *Pythium* sp. clade IR-I; morphologically, however, this species is remarkably different from either of them, with characteristic oogonia that measure up to 200 µm. As remarked by Lévesque and De Cock (19), the similarities between the ITS sequences of these species are surprising considering their morphological differences. Hence, morphological as well as molecular comparisons between these species are necessary to clarify their identities and phylogeny.

Further analyses are needed to fully characterize *P. irregulare* s.l. To achieve a better understanding of the population biology of each of the species within the complex, larger samples, collected over a period of time, at selected locations and facilities need to be analyzed.

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