Selection of DNA Probes Useful for Isolate Identification of Two *Pythium* spp.

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ABSTRACT

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DNA fragments from regions of the mitochondrial chromosome of *Pythium oligandrum* and *P. sylvaticum* showing intra- and interspecific variation provided probes with varying levels of isolate and species specificity. In this genus, the mitochondrial DNA (mtDNA) is most commonly a circular molecule composed primarily of an inverted repeat region separated by small and large single-copy unique regions. The small unique region was the most variable portion of the genome for *P. oligandrum*. A 0.1-kb clone containing sequences entirely from within the inverted repeat region adjacent to the small unique region hybridized strongly to DNA of 10 isolates of *P. oligandrum* from geographically diverse areas and three of 27 other *Pythium* spp. A clone that spanned the single-copy, small unique region and also contained some sequences from the inverted repeat region hybridized strongly to *P. oligandrum* and *P. acanthicum*. Several clones containing sequences entirely from within the single-copy small unique region were specific for those isolates of *P. oligandrum* that shared the same restriction map of this region. For *P. sylvaticum*, a probe with a significant level of isolate specificity was constructed from a portion of the mtDNA associated with an insertion-deletion event in the inverted repeat region.

Investigations on the field ecology and epidemiology of diseases caused by *Pythium* spp. are often hindered by similarities in growth morphologies of different species on agar media. This complicates investigations in which multiple species are present in the same field, making it necessary to isolate individual colonies and identify them to species level. Presently, there are more than 120 described species in the genus *Pythium* (3); many of which may differ by minor morphological features. Identification of isolates requires some expertise in taxonomy, may be complicated by variation in morphological features among isolates, and is a time-consuming task for those cases in which similar species may be present in the same field. While morphological features may provide species identification, they will not allow for the differentiation of specific isolates within a species population, a requirement for studies on population genetics.

One tool that would be helpful for conducting this research is a DNA probe that could identify the particular isolate or species under investigation. This approach has been used for several fungal pathogens; Henson (7) used a random mitochondrial DNA (mtDNA) clone from *Gaeumannomyces graminis* var. *tritici* for restriction fragment length polymorphism (RFLP) analysis of different isolates and varieties of *G. graminis*. This mtDNA clone also demonstrated some level of genus specificity; it hybridized primarily to DNA from *Gaeumannomyces* spp. with weak background hybridization to DNA from *Phialophora* spp. and *Neurospora crassa*. Species-specific probes also have been selected for detection of the fungal pathogens *Phytophthora parasitica* (4,5), *Phytophthora citrophthora* (6), and *Phoma tracheiphila* (21) by screening a genomic DNA clone library until the requisite probe was identified. Specific probes also have been constructed for isolates of several *Pythium* spp. using cloned DNA sequences from the circular mitochondrial plasmids these isolates contained (17).

The selection of specific probes could be simplified by identification and directed cloning of specific portions of the genome that contain the necessary variation. S. B. Lee and J. W. Taylor (*personal communication*) used this approach to select probes specific for several species of *Phytophthora*. Sequence analysis of the transcribed spacer region between the coding regions for the 17S and 5.8S ribosomal RNA (rRNA) revealed regions that were variable among species but were conserved among isolates of the same species. Twenty base pair oligonucleotide probes constructed from this region were species-specific for *Phytophthora megakarya* Brasier et Griffin, *P. palmivora* (Butler) Butler, *P. capsici* Leonian, *P. citrophthora* (R. et E. Smith) Leonian, and *P. cinnamomi* Rands.

For the genus *Pythium*, portions of the mitochondrial chromosome may provide a similar opportunity for selection of specific probes. The mitochondrial genome ranges in size from 60 to 73 kb (16,19,20) and has a predominantly circular physical orientation, however, several species have been identified in which the orientation is linear (16). A major portion of the genome (71–83%) is made up of a repeated sequence arranged in an inverted fashion which, for circular molecules, is separated by two single-copy unique regions. These single-copy sequences are referred to as either small or large unique regions depending on their relative sizes (16,19,20). The mitochondrial genome for individual species appears to be stable; when mtDNA is digested with restriction enzymes and size fractionated by agarose electrophoresis, there is a high degree of conservation of restriction profiles (15,18). However, the converse is true when different species are compared; even morphologically similar species may have few bands in common.

Detailed restriction maps of 11 geographically separated isolates of *P. oligandrum* Drechs. indicate that the most variable portion of the mitochondrial genome for this species was the single-copy small unique region (16). Mutations in this area were due primarily to insertion or deletion events, which caused variation in size as well as additional unique restriction sites. In contrast to the small unique region, the inverted repeat regions were relatively invariant. Insertion or deletion events were not detected in the large unique or inverted repeat regions, and the size of the mitochondrial genome, with the exception of the *Pst*I fragment spanning the small unique region, was equal for all isolates investigated (16). Similar results were obtained for *P. irregulare* Buisman and *P. ultimum* Trow; however, comparison of two isolates of *P. sylvaticum* W. A. Campbell & J. W. Hendrix showed an insertion-deletion event occurring in the inverted repeat region (F. N. Martin, *unpublished*).

If these insertion events are composed of unique sequences, it is a feature of the evolutionary stability of the genome that may be advantageous for the selection of isolate-specific DNA probes. To evaluate the use of these regions for probe construction,

a better understanding of intraspecific mtDNA variation is needed. The objective of this research was to more thoroughly examine the small unique region of *P. oligandrum* mtDNA to clarify intraspecific variation and to identify regions that are conserved and unique among isolates of the species. This information can then be used for constructing probes to be evaluated for isolate specificity and screened against other members of the genus for species specificity. Because the variation for one isolate of *P. sylvaticum* occurs in a different portion of the genome as compared to *P. oligandrum*, investigations were conducted with this species as well.

MATERIALS AND METHODS

Fungal isolates. All isolates of *Pythium* spp. in this study were used in a previous investigation (18) and were classified in accordance with the key of Van der Plaats-Niterink (24). Detailed restriction maps of the mitochondrial chromosome for the isolates of *P. oligandrum* have been published (16), and a consensus map is included in Figure 1. Isolates 1987-73 and 1987-14 of *P. sylvaticum* are antheridial and oogonial mating types, respectively, and differ by a *Hin*dIII polymorphism in mtDNA (14).

Construction of restriction maps and probes. Mitochondrial DNA was purified by cesium chloride plus bisbenzimide ultracentrifugation of total DNA as described (18). The mtDNA of *P. oligandrum* was digested with *Pst*I to excise the region spanning the small unique region of the mitochondrial chromosome (Fig. 1), and the DNA fragments were separated by horizontal agarose electrophoresis. The fragment containing the small unique region was retrieved from the gel (23) and ligated into a dephosphorylated *Pst*I cloning site of the vector pBluescript (Stratagene; La Jolla, CA). Insert ligation, transformation of *Escherichia coli* (DH5 α), and plasmid mini-prep isolation were performed by standard procedures (22). Restriction mapping of the inserts and electrophoretic separation of DNA fragments were as previously reported (16). For separation of DNA fragments less than 2 kb in size, electrophoresis was in 2–4% Nusieve GTG agarose (FMC Bioproducts; Rockland, ME) with 0.5× TBE (1× TBE = 89 mM Tris-HCl, 89 mM boric acid, 2.0 mM EDTA, pH 8.0). Restriction maps for the mtDNA of *P. sylvaticum* were constructed by single and double digests of mtDNA (16).

Selection of DNA fragments for use as probes was based on comparison of restriction maps for different isolates of the same species. Specific fragments were recovered from agarose gels and subcloned into pBluescript. The *Alu*I and *Taq*I fragments of *P. oligandrum* were cloned into the *Eco*RV and *Cla*I cloning sites, respectively, of pBluescript.

DNA extraction and dot-blot analysis. Fungal cultures were grown and total genomic DNA isolated as described (18), except that cesium chloride plus bisbenzimide ultracentrifugation was not done. Concentrations and the purity of the total DNA were determined spectrophotometrically (22). For dot blots, 3.0 μg of total DNA was denatured with an equal volume of 0.5 N NaOH for 10 min before chilling on ice. The NaOH concentration was adjusted to 0.125 N by the addition of 1× SSC (0.01875 M NaCl, 0.00187 M Na citrate) to a final concentration of 0.125× SSC. The DNA was transferred to a nylon membrane (Gene Screen Plus; DuPont, NEN Products, Boston, MA) by using a dot-blot manifold. After being transferred, the membranes were immersed in 0.4 N NaOH for 60 s and then in 0.2 M Tris, pH 7.5, plus 2× SSC for 1–2 min. The DNA was fixed to the membrane by UV crosslinking (Stratalinker, Stratagene; La Jolla, CA).

Hybridization studies. Clones containing the desired insert sequences were labeled with [^{32}P]-dCTP by nick translation (Bethesda Research Laboratories; Bethesda, MD) and were denatured before use by treatment with 0.15 vol of 2 N NaOH for 10 min followed by the addition of the same volume of 1 M Tris, pH 7.4. Dot blots were prehybridized in 50% formamide, 1% sodium lauryl sulfate, and 1 M NaCl for at least 30 min before adding heat-denatured, salmon sperm DNA (100 μg/ml) and the denatured probe. Hybridizations were conducted at 42 C overnight, after which the blots were washed twice in 2× SSC at room temperature, twice in 2× SSC plus 1% sodium dodecyl sulfate for 30 min at 65 C, and twice at room temperature in 0.1× SSC. Autoradiography was done with Kodak X-OMAT AR5 film and DuPont Cronex Hi-Plus intensifying screens at −80 C.

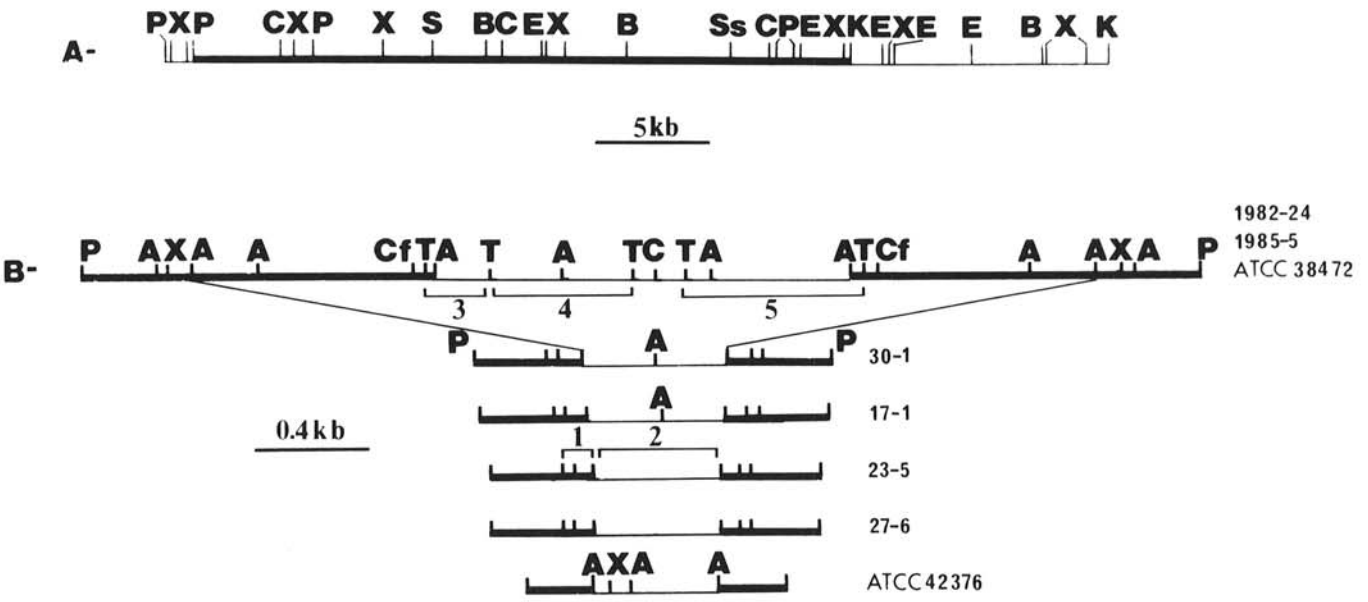

Fig. 1. A, Consensus linearized restriction map of the mitochondrial chromosome of *Pythium oligandrum* (isolate 23-5). The thick line represents one arm of the inverted repeat region, and the left and right flanking thin lines span the small and large unique regions, respectively. **B,** More detailed restriction analysis of the *Pst*I fragment spanning the small unique region and showing the variation present in isolates of *P. oligandrum*. Thick lines represent portions of the inverted repeat regions, and the thin lines span what appears to be single-copy small unique regions. Portions of the inverted repeat regions marked by vertical lines that do not have restriction enzyme abbreviations have the same restriction sites as indicated in the top map. The labeled fragments enclosed with thin bracket lines correspond to the clones (listed in Table 1) used as probes of the dot blots. Restriction enzyme abbreviations are: A = *Alu*I, B = *Bgl*II, C = *Cla*I, Cf = *Cfo*I, E = *Eco*RI, K = *Kpn*I, P = *Pst*I, S = *Sal*I, Ss = *Sst*II, T = *Taq*I, and X = *Xba*I.

RESULTS

Restriction map of the small unique region of *P. oligandrum*. Depending on the isolate, the mtDNA fragment spanning the small unique region and flanked by *Pst*I restriction sites varied in size from 0.98 to 3.92 kb and in restriction sites (Fig. 1). The *Pst*I fragments from isolates 1982-24, ATCC38472, and 1985-5 had identical sizes (3.92 kb) and restriction maps, as did isolates 23-5 and 27-6 (1.2 kb). Variation in size and restriction sites was observed for the fragment from isolates 17-1 (1.22 kb), 30-1 (1.25 kb), and ATCC42376 (0.98 kb). Based on the similarity in restriction maps for the terminal regions of some *Pst*I fragments, it appeared that this fragment contained part of the mitochondrial inverted repeat region, as indicated by the bold lines in Figure 1. Sequences from the inverted repeat region may extend farther into what is represented as the small unique region, however, adequate restriction sites to evaluate this have not been identified. Weak hybridization was observed between the 0.23- and 0.62-kb *Taq*I fragments (clones 3 and 5, respectively) for isolates 1982-24, 1985-5, and ATCC38472 (data not shown), indicating that the inverted repeat region does not extend much past the indicated *Alu*I restriction sites for these isolates. Restriction sites for *Hae*III, *Msp*I, *Rsa*I, and *Tha*I were not present in these *Pst*I fragments.

Probe specificity. The specificities of the selected mtDNA clones were partially dependent on the region of the chromosome of *P. oligandrum* from which they were recovered. The 0.10- and 0.47-kb *Alu*I fragments (clones 1 and 2, respectively) hybridized strongly to DNA of all isolates of *P. oligandrum*, while the 0.23-, 0.50-, and 0.62-kb *Taq*I fragments (clones 3, 4, and 5, respectively) from isolate 1982-24 were specific for isolates of *P. oligandrum*, which shared a similar mtDNA restriction map (ATCC38472 and 1985-5; Table 1).

When tested for specificity against other *Pythium* spp., clone 1 hybridized strongly to DNA from isolates of *P. graminicola*, *P. aphanidermatum*, and *P. acanthicum* and weakly to *P. myriotylum*, *P. catenulatum*, and *P. violae*. The weak levels of hybridization in these trials were very faint and detectable only after prolonged exposure (generally 24–48 h; see Fig. 2 for a visual comparison of the different levels of hybridization). The internal 0.47-kb *Alu*I fragment (clone 2) hybridized strongly to the DNA from the isolate of *P. acanthicum* and weakly with *P. violae*. In addition to these species, the entire *Pst*I fragment from isolate 23-5 hybridized strongly to DNA from *P. iwayamai* and *P. nunn*; moderately to *P. torulosum*; and weakly to *P. sylvaticum*, *P. vexans*, *P. paroecandrum*, *P. periplocum*, and *P. mamillatum* (data not shown). The 0.23-kb fragment (clone 3) also hybridized to *P. myriotylum*, *P. ultimum*, and *P. violae*, while the 0.50- and 0.62-kb fragments (clones 4 and 5, respectively) hybridized weakly to *P. graminicola*, *P. violae*, *P. vexans*, *P. nunn*, and *P. paroecandrum*. The degree of hybridization of clones 4 and 5 with these species was low and visible only after prolonged exposure, generally an additional 48 h.

TABLE 1. Hybridization of DNA fragments from variable regions of the mitochondrial genome of *Pythium oligandrum* and *P. sylvaticum* to dot blots of total DNA from other *Pythium* spp.

Species	Isolate	Hybridization with clone[a] 1	2	3	4	5	6
P. oligandrum	ATCC38472	+++	+++	+++	+++	+++	–
	1985-5	+++	++	+++	+++	+++	–
	1982-24	+++	++	+++	+++	+++	–
	ATCC42376	+++	++	–	–	–	–
	17-1	+++	+++	–	–	–	–
	23-5	+++	+++	–	–	–	–
	27-6	+++	+++	–	–	–	–
	30-1	+++	++	–	–	–	–
	CMI96345	+++	++	–	–	–	–
	C-3	+++	++	–	–	–	–
P. graminicola	1986-1	+++	–	–	+	+	–
P. arrhenomanes	1987-110	–	–	–	–	–	–
P. aristosporum	1987-70	–	–	–	–	–	–
P. aphanidermatum	43-5	+++	–	–	–	–	–
P. myriotylum	1987-63	+	–	++	–	–	–
P. torulosum	1986-19	–	–	–	–	–	–
P. vanterpoollii	1986-20	–	–	–	–	–	–
P. pyrilobum	1987-86	–	–	–	–	–	–
P. volutum	1987-67	–	–	–	–	–	–
P. sulcatum	1987-98	–	–	–	–	–	–
P. catenulatum	1986-8	+	–	–	–	–	–
P. heterothallicum	1986-24	–	–	–	–	–	–
P. splendens	1985-3	–	–	–	–	–	–
P. sylvaticum	1987-73	–	–	–	–	–	–
P. ultimum	110-2	–	–	+++	–	–	–
P. violae	1986-38	+	+	++	+	+	–
P. pulchrum	1986-17	–	–	–	–	–	–
P. rostratum	1986-14	–	–	–	–	–	–
P. iwayamai	1986-13	–	–	–	–	–	–
P. vexans	1986-2	–	–	–	+	+	–
P. nunn	1987-58	–	–	–	+	+	+++
P. paroecandrum	1986-60	–	–	–	+	+	–
P. irregulare	1987-97	–	–	–	–	–	–
P. periplocum	1986-26	–	–	–	–	–	+
P. acanthicum	A-6	+++	+++	–	–	–	–
P. mamillatum	1986-37	–	–	–	–	–	–
P. spinosum	79-4	–	–	–	–	–	–

[a]See Figures 1 and 3 for explanation of clones. Dot blots contained 3.0 μg of total DNA per well. The presence of a (–) indicates a lack of hybridization and a (+) indicates hybridization, with + = weak; ++ = moderate; and +++ = strong. See Figure 2 for visual comparisons. Weak hybridizations were visible only after prolonged exposure of the X-ray film to the blots (24-48 h).

Restriction map of mtDNA of *P. sylvaticum* and probe construction. The mitochondrial chromosome of isolate 1987-73 of *P. sylvaticum* is a circular molecule approximately 62.3 kb in size with an inverted repeat region of 19.0 kb and small and large unique regions of 5.7 and 18.6 kb, respectively (Fig. 3); the sizes of the unique regions are estimates pending more detailed restriction analysis to determine the exact extent of the inverted repeat region. The restriction map for isolate 1987-14 was similar except for insertion events adjacent to the *Sal*I restriction site that increased the size of the inverted repeat by 2.4 kb; an additional 1.7 kb was present to the left and 0.6 kb to the right of the *Sal*I site. Variation in this region for *Cla*I, *Eco*RI, and *Bgl*II restriction sites also was observed in comparison to isolate 1987-73. The 4.5-kb *Eco*RI fragment of isolate 1987-14, which contains the insertion event to the left of the *Sal*I site, was cloned, and restriction digests were tested for hybridization with mtDNA

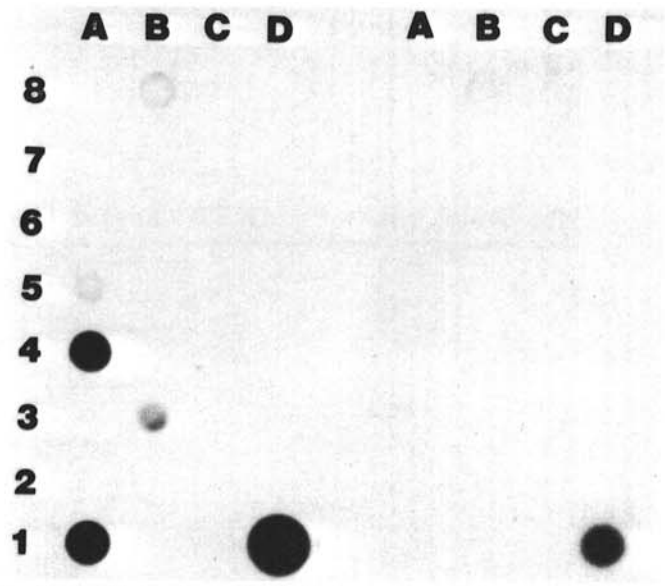

Fig. 2. Dot blots of total DNA (3.0 μg per well) from *Pythium* spp., listed in Table 1, probed with radiolabeled clone 1 (left panel) or clone 2 (right panel) from isolate 23-5 of *Pythium oligandrum*. Hybridizations are to DNA from the following species: (left panel) 1A, *P. graminicola*; 4A, *P. aphanidermatum*; 5A, *P. myriotylum*; 3B, *P. catenulatum*; 8B, *P. violae*; 1D, *P. acanthicum*; (right panel) 8B, *P. violae*; and 1D, *P. acanthicum*. Isolates of *P. oligandrum* are not included but have a similar intensity of hybridization as observed for *P. acanthicum*.

from isolate 1987-73 by Southern analysis (Fig. 4). There was a lack of hybridization between 1987-73 mtDNA and the 1.7-kb *Eco*RI plus *Cla*I fragment of the clone, indicating that this portion of the genome for isolate 1987-14 was composed of unique sequences. This was supported by the lack of hybridization of mtDNA from isolate 1987-73 and the 1.8-kb *Cla*I plus *Bgl*II fragment of the mtDNA of isolate 1987-14 (Fig. 4; lane 4). A deletion mutant of the cloned 4.5-kb *Eco*RI fragment was constructed; it retained the 2.06-kb *Bgl*II plus *Eco*RI portion of the insert as indicated in Figure 3 (clone 6). This contains the 1.7-kb *Eco*RI plus *Cla*I fragment evaluated above, and did not hybridize to mtDNA from isolate 1987-73. This subclone (clone 6) was evaluated for species specificity by hybridization of labeled DNA with dot blots of DNA from different *Pythium* species. Strong and weak hybridizations with DNA of *P. nunn* and *P. periplocum*, respectively, were observed (Table 1).

DISCUSSION

With the exception of the *Pst*I fragment spanning the small unique single-copy region, the mtDNA restriction maps for isolates of *P. oligandrum* were invariant in size and had little variation in restriction sites (16). Detailed restriction maps of the variable *Pst*I fragment indicate that size variation among isolates is due not only to insertion-deletion events in the single-copy small unique region as previously reported (16), but also to the amount of inverted repeat sequences extending into this region. For half the isolates (30-1, 17-1, 23-5, and 27-6), the inverted repeat sequences occupied the terminal 0.4 kb of the *Pst*I clone, for one isolate (ATCC42376) it was 0.25 kb, and for the others (1982-24, 1985-5, and ATCC38472) it was 1.2 kb. For the isolates falling in these first two categories, the boundary between the inverted repeat and small unique regions was not precisely known because of the lack of informative restriction sites in the central portions of the clones. DNA sequence analysis is presently in progress and should clarify this (F. N. Martin, *unpublished*). The only isolates for which the small unique single-copy region had been clearly identified were 1982-24, 1985-5, and ATCC38472. Only the middle 1.4 kb of these 3.98-kb *Pst*I clones were unique single-copy sequences, as evidenced by the poor hybridization between clones 3 and 5.

The specificities of the selected mtDNA clones were partially dependent on the region of the mitochondrial chromosome of *P. oligandrum* from which they were recovered. Probes constructed entirely from single-copy sequences (clones 4 and 5) hybridized strongly only to those isolates that had a similar restriction map of the small unique region. A clone from isolate 23-5, spanning the small unique region and possibly containing some sequences from the inverted repeat (clone 2), hybridized to all isolates of *P. oligandrum* and the morphologically similar species *P. acanthicum*. Based on the high level of conservation of restriction sites in rDNA, this species also appears to be closely related to *P. oligandrum* (F. N. Martin, *unpublished*). Increasing the amount of inverted repeat sequences in the probes reduced species specificity. A 0.1-kb clone (clone 1) from the inverted repeat region adjacent to the above mentioned 0.47-kb clone hybridized to *P. oligandrum*, *P. acanthicum*, and two additional species, while the entire 1.2-kb *Pst*I fragment, containing an additional 0.25 kb of inverted repeat sequences and encompassing clones 1 and 2, hybridized strongly to two more species. This indicates that the farther away inverted repeat sequences are from the single-copy small unique region, the more highly conserved they are among species. Therefore, construction of a probe specific for *P. oligandrum* and not hybridizing to *P. acanthicum* may only require reducing the amount of inverted repeat sequences in clone 2. This has not been attempted in this investigation because of the lack of restriction sites in this portion of the clone. However, DNA sequence analysis of this region is in progress and may identify regions from which species-specific oligonucleotide probes may be constructed.

While insertion or deletion events occur primarily in the small unique region for *P. oligandrum*, insertion events took place in the inverted repeat region adjacent to the *Sal*I restriction site for an oogonial isolate of *P. sylvaticum* (1987-14). These sequences were not duplicate copies of other portions of the genome (as evidenced by the lack of homology to 1987-73 mtDNA), but were unique sequences that could be used for identification of isolate 1987-14. While the clone also hybridized to DNA from *P. nunn*, this species has a limited reported distribution and should not prevent the use of this clone. The polymorphism on which probe construction was based was not present in all oogonial isolates; the restriction map for isolate ATCC18196 was the same as for the antheridial isolate 1987-73 (F. N. Martin, *unpublished*).

The lack of single isolate or species specificity for some clones may preclude their use for diagnostic purposes by dot-blot or colony hybridization procedures (4), however, they may still be useful for isolate identification by RFLP analysis. As demonstrated by the restriction maps in Figure 1, variation in size and restriction sites was observed in the vicinity of the small unique region among isolates of *P. oligandrum*. Depending on the combination of the restriction enzyme and probe used, many of these isolates could be differentiated. The restriction maps of the small unique regions of other species differ significantly (19,20; F. N. Martin, *unpublished*); therefore, it is possible that selection of clones spanning the small unique region and containing inverted repeat sequences (e.g., the 1.2-kb *Pst*I fragment from isolate 23-5) may be useful in RFLP analysis of different species in the genus in much the same way as Henson (7) used a random mtDNA clone from *G. g. tritici* for RFLP analysis of different isolates and varieties of *G. graminis*. The selection of probes from the small unique region may be less time-consuming than selection and screening of random clones of genomic DNA, as has been reported for *Fusarium* spp. (9,12,13), *Agaricus* spp. (2,11), or *Armillaria mellea* (1). mtDNA clones also may offer a greater level of specificity for differentiation of species or individual isolates than observed with the nuclear-encoded genes for rRNA (1,8,10).

Thus far, the most detailed evaluation of intraspecific variation of mtDNA in the genus has been conducted with *P. oligandrum* (16), however, preliminary evidence suggests that similar mechanisms (e.g., insertions or deletions in the small unique region) are responsible for variation in *P. irregulare* and *P. ultimum* as well (F. N. Martin, *unpublished*). To identify variable regions for use in probe construction for other *Pythium* spp., generalized restriction maps of mtDNA may be constructed with the restriction enzymes *Sal*I, *Sst*II, and *Bgl*II. McNabb et al (19,20) reported that the relative positions of some of these sites were conserved in *P. torulosum*, *P. diclinum*, *P. irregulare*, and *P.*

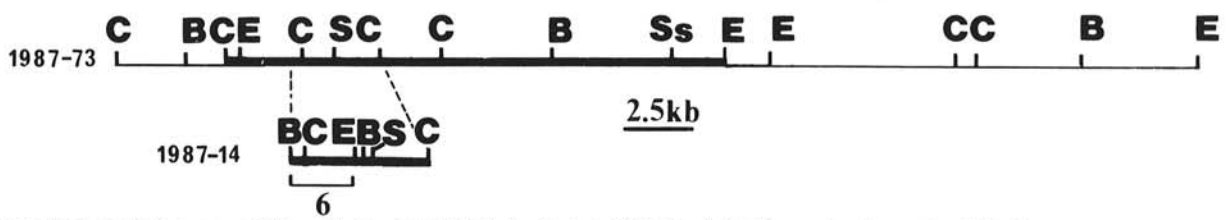

Fig. 3. Linearized restriction map of the mitochondrial DNA for isolate 1987-73 of *Pythium sylvaticum*; the thick line represents one arm of the inverted repeat region, while the left and right flanking thin lines span the small and large unique regions, respectively. The short thick line below the map represents the portion of the chromosome that contains the insertion found in isolate 1987-14 associated with the isolate-specific probe (clone 6). Abbreviations for the restriction enzymes are: B = *Bgl*II, C = *Cla*I, E = *Eco*RI, S = *Sal*I, and Ss = *Sst*II.

paddicum; such also has been the case for *P. sylvaticum*, *P. oligandrum* (16), *P. acanthicum*, and *P. ultimum* (F. N. Martin, *unpublished*). An added benefit of using *Sal*I and *Sst*II is that, based on restriction analysis of all the isolates listed in Table 1, they tend to cleave the mtDNA of *Pythium* spp. into only two or three fragments (F. N. Martin, *unpublished*). Therefore, single and double digests with these enzymes may provide a generalized restriction map that could be useful in determining the location of the small unique region and the relative position of insertion or deletion events.

Identification of variable regions of the mitochondrial genome and selection of unique sequences provided an effective approach for development of DNA probes specific for particular isolates of *P. oligandrum* and *P. sylvaticum*. While some isolate and species specificity were observed with the probes constructed from sequences spanning the small unique region of *P. oligandrum*, limited weak background hybridization with DNA from other species also was obtained. However, this was visible only after prolonged exposure of the X-ray film to the blots, and should not prevent the use of these clones for colony hybridizations with techniques as reported by Goodwin et al (4). The presence of this background hybridization and lack of species specificity for some clones may be more of a reflection of the size of the probe and the presence of conserved sequences from the inverted repeat region. DNA sequence analysis of this region of *P. oligandrum* mtDNA and may identify regions that will enhance probe specificity.

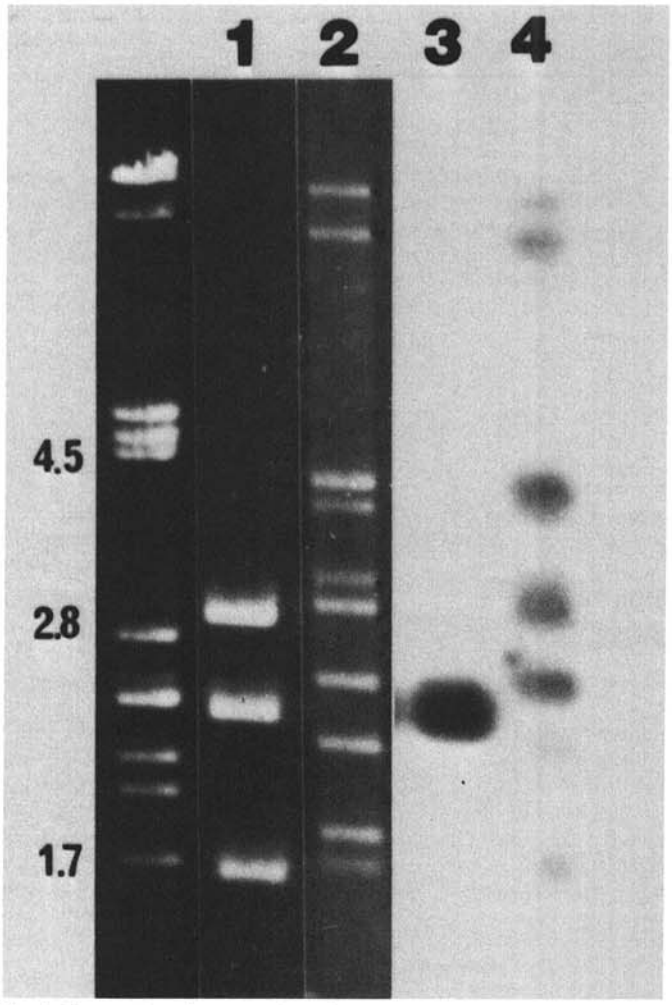

Fig. 4. Electrophoretic separation of the cloned 4.5-kb *Eco*RI fragment from isolate 1987-14 of *Pythium sylvaticum* (lane 1) and mitochondrial DNA (mtDNA) from isolate 1987-14 (lane 2) digested with *Eco*RI plus *Cla*I plus *Bgl*II, and *Cla*I plus *Bgl*II, respectively. The top 3-kb band present in the digest of the clone is linearized vector DNA (pBluescript). Lanes 3 and 4 show an autoradiogram of Southern transfer of lanes 1 and 2, respectively, hybridized with mtDNA of isolate 1987-73. Molecular size markers in the left lane are bacteriophage λ digested with *Pst*I; sizes are in kilobases.

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