5S Ribosomal RNA Gene Spacers as Species-Specific Probes for Eight Species of *Pythium*

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ABSTRACT

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Some species of *Pythium* have tandem arrays of 5S genes unlinked to the ribosomal DNA (rDNA) repeat unit. The gene spacers from 8 such species (*P. intermedium*, *P. macrosporum*, *P. sylvaticum*, *P. ultimum*, *P. okanogenense*, *P. anandrum*, *P. acanthicum*, and *P. mastophorum*) were amplified and used as probes against the genomic DNA of 92 species of *Pythium*. Separate probes were prepared from *P. ultimum* var. *ultimum* and *P. ultimum* var. *sporangiiferum*. All nine of the probes were species or variety specific. Six of the probes also were tested against three or more isolates of the same species and recognized all of them, except in the case of two *P. ultimum* var. *sporangiiferum* isolates, which were recognized by the *P. ultimum* var. *ultimum* probe. Four of the probes also were species specific when amplified DNA was used as the target for hybridization. These results show that the 5S rRNA gene spacer sequence diverged rapidly after speciation and may be useful in defining species boundaries in the genus *Pythium*.

Additional keywords: amplification, tandem gene arrays.

Pythium is an oomycete genus with more than 200 described species, of which approximately 130 are recognized currently (5). Species identification of isolates is often difficult because they may lack the reproductive structures needed for diagnosis or because of characters that are highly variable within species or that overlap between species. Species-specific molecular probes are a powerful means of detecting *Pythium* in soil and plant samples, species name assignment of new isolates, and confirmation of dubious name assignments of isolates in culture collections. Such probes also can be used to clarify boundaries within groups of easily confused species and might serve as objective criteria for justifying the delimitation of new species. DNA probes for several species of *Pythium* have been reported. Martin (9) derived probes for *P. oligandrum* and *P. sylvaticum* from restriction fragments of mitochondrial DNA; Lévesque et al. (7) used parts of the internal transcribed spacers of the ribosomal DNA (rDNA) to make a probe specific for *P. ultimum*, and Matthew et al. (10) found a repeated sequence in a genomic library of *P. irregulare* that recognizes *P. irregulare* and *P. spinosum* isolates.

The method developed in this study depends on the amplification of the 5S rRNA spacer region with primers complementary to conserved sequences at the ends of tandemly repeated 5S rRNA genes. We previously have shown that species of *Pythium* with globose zoosporangia generally have their 5S rRNA genes arranged in tandem arrays (2). Gene-to-gene amplification has been done previously to gain access to the 5S spacer in plants (1,4,12) and for genome fingerprinting of plants (6). The value of this approach is that the target sequence for the primers is comparable in all organisms and that the probe is generated from genomic DNA by the same rapid and simple method for all species. A similar approach was used to develop a species-specific probe for the protozoan *Eimeria tenella* (13). The approach is limited to species having tandem repeats of 5S genes, which exclude the species of *Pythium* (generally those with filamentous zoosporangia) that lack such repeats.

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MATERIALS AND METHODS

Cultivation of strains. Isolates of *Pythium* species were obtained from the CBS (Centraalbureau voor Schimmelcultures, Baarn) collection in the Netherlands and from the Aquatic Phycomycete Culture Collection in Reading, England (Table 1). Cultures were first subcultured on cornmeal agar (occasionally on V8 or V8-sea water agar) at 25°C. After 2 to 3 days (slow-growing species up to 7 days), the colonies were transferred to pea-broth medium (filtered decantation of 200 g of frozen peas boiled for 20 min in 1 liter of water to which 5 g of glucose was added) in petri dishes and incubated at 25°C for 2 to 3 days (slow-growing species up to 10 days). Mycelia were harvested by vacuum-filtration, washed twice with distilled water, and frozen at –20°C. Frozen mycelia were freeze-dried overnight, and dry mycelia were stored at –20°C when not used immediately.

Isolation of DNA. DNA was extracted from all the isolates according to the method of Möller et al. (11) with some modifications. Freeze-dried mycelium (30 mg) was extracted in 1 ml of TES buffer (100 mM Tris, pH 8.0, 10 mM EDTA, and 2% sodium dodecyl sulfate [SDS]) with 100 to 200 mg of proteinase K at 55°C for 30 min with occasional gentle mixing. NaCl (280 µl, 5 M) and 138 µl of cetyltrimethylammonium bromide/NaCl (10%/0.7 M) were added, and the mixture was incubated for 10 min at 65°C. The lysed suspension was centrifuged (16,000 × *g*) for 10 min at 4°C to remove cell debris. Supernatant (1 ml) was extracted

TABLE 1. Isolates of *Pythium* species used in this study

Pythium species	Culture[a]	Status[b]	MT[c]	Ref. no.[d]	Origin[e]
acanthicum	CBS 227.94			1	France
	CBS 284.31	TYPE		2	U.S.
	CBS 431.68			3	Neth.
acanthophoron	CBS 337.29	AU		4	U.S.
acrogynum	CBS 549.88			5	China
adhaerens	CBS 520.74			6	Neth.
anandrum	CBS 285.31	TYPE		7	?
angustatum	CBS 522.74	PN		8	Neth.
aphanidermatum	CBS 216.46			9	?
apleroticum	CBS 772.81			10	Neth.
aquatile	CBS 215.80	NEOTYPE		11	U.K.
arrhenomanes	CBS 324.62	TYPE		12	U.S.
ascophallon	APCC 4004a			13	Russia
boreale	CBS 551.88			14	China
buismaniae	CBS 288.31	TYPE		15	Neth.
capillosum	CBS 222.94	AU		16	France
catenulatum	CBS 842.68	PN	a	17	U.S.
	CBS 843.68	PN	b	18	U.S.
chamaehyphon	CBS 259.30	TYPE		19	?
chondricola	CBS 203.85	TYPE		20	Neth.
coloratum	CBS 154.64	TYPE		21	Aust.
conidiophorum	CBS 223.88			22	U.K.
cylindrosporum	CBS 218.94	TYPE		23	Ger.
deliense	CBS 314.33	NEOTYPE		24	?
diclinum	CBS 664.79	NEOTYPE		25	Neth.
dimorphum	CBS 406.72	TYPE		26	U.S.
dissimile	CBS 155.64	TYPE		27	Aust.
dissotocum	CBS 166.68	PN		28	U.S.
P. sp. (*P. drechsleri*)	CBS 221.94	TYPE		29	Alger.
echinulatum	CBS 281.64	PN		30	Aust.
erinaceus	CBS 505.80	TYPE		31	N.Z.
flevoense	CBS 234.72	TYPE	f	32	Neth.
	CBS 236.72	PN	m	33	Neth.
folliculosum	CBS 220.94	TYPE		34	Switz.
graminicola	CBS 327.62	NEOTYPE		35	Jam.
grandisporangium	CBS 286.79	TYPE		36	U.S.
helicandrum	CBS 393.54	AU		37	U.S.
helicoides	CBS 286.31	PN		38	U.S.
heterothallicum	CBS 451.67	TYPE	f	39	Can.
	CBS 450.67	TYPE	m	40	Can.
hydnosporum	CBS 253.60	PN		41	Ger.
hypogynum	CBS 692.79			42	Can.
inflatum	CBS 168.68	PN		43	U.S.
insidiosum	CBS 574.85	TYPE		44	C.R.
intermedium	CBS 136.87		?	45	Nor.
	CBS 221.68	PN	+	46	Neth.
	CBS 222.68		–	47	Neth.
	CBS 223.68		+	48	Neth.
	CBS 266.38	PN	–	49	Neth.
	CBS 268.38		–	50	Neth.
	CBS 380.34		–	51	?
	adc 94.24		?	52	Neth.
irregulare	CBS 250.28	NEOTYPE		53	Neth.
iwayamae	CBS 156.64	PN		54	Aust.
	CBS 697.83			55	Japan
kunmingense	CBS 550.88	TYPE		56	China
lutarium	CBS 222.88	TYPE		57	U.K.
macrosporum	CBS 574.80	TYPE	+	58	Neth.
	CBS 575.80	TYPE	–	59	Neth.
	CBS 579.80		–	60	Can.
mamillatum	CBS 251.28	PN		61	Neth.
marinum	CBS 312.93			62	U.S.
marsipium	CBS 773.81			63	Neth.
mastophorum	CBS 375.72	PN		64	U.K.
middletonii	CBS 528.74	PN		65	Neth.
minus	CBS 226.88	TYPE		66	U.K.
monospermum	CBS 158.73	NEOTYPE		67	U.K.
multisporum	CBS 470.50	TYPE		68	U.S.
myriotylum	CBS 254.70	NEOTYPE		69	Israel
nagae	APCC 4321c			70	U.K.
oedochilum	CBS 292.37	AU		71	U.S.
	CBS 738.94			72	S. Af.
okanoganense	CBS 315.81	TYPE		73	U.S.
oligandrum	CBS 382.34	PN		74	U.K.
oligandrum	CBS 530.74			75	Neth.
orthogonon	CBS 376.72	TYPE		76	Leb.
ostracodes	CBS 768.73	PN		77	Spain
pachycaule	APCC 4117a			78	U.K.
paddicum	CBS 698.83			79	Japan
paroecandrum	CBS 157.64	PN		80	Aust.
	CBS 203.79			81	Neth.
parvum	CBS 225.88	TYPE		82	U.K.
periilum	CBS 169.68	PN		83	U.S.
periplocum	CBS 289.31	TYPE		84	U.S.
pleroticum	CBS 776.81			85	Neth.
polymastum	CBS 811.70	PN		86	Neth.
porphyrae	CBS 369.79	PN		87	Japan
prolatum	CBS 845.68	TYPE		88	U.S.
pyrilobum	CBS 158.64	TYPE		89	Aust.
radiosum	CBS 217.94	TYPE		90	France
rostratum	CBS 533.74	NEOTYPE		91	Neth.
salpingophorum	CBS 471.50	PN		92	Ger.
scleroteichum	CBS 294.37	AU		93	U.S.
spinosum	CBS 275.67	PN		94	Neth.
splendens	CBS 266.69	PN	+	95	Belg.
	CBS 462.48	PN	–	96	U.S.
sulcatum	CBS 603.73	TYPE		97	U.S.
sylvaticum	CBS 226.68		m	98	Neth.
	CBS 228.68		m	99	Neth.
	CBS 230.68		f	100	Neth.
	CBS 232.68		f	101	Neth.
	CBS 233.68		f	102	Neth.
	CBS 452.67	TYPE	m	103	U.S.
	CBS 453.67	TYPE	f	104	U.S.
	CBS 633.67		m	105	U.K.
	CBS 720.94		m	106	Can.
	CBS 721.94		m	107	Can.
	CBS 722.94		m	108	Can.
	CBS 723.94		?	109	Can.
	adc 94.11		?	110	
	adc 94.12		?	111	
	adc 94.15		?	112	
	adc 94.16		?	113	
tardicrescens	APCC 4215a			114	Can.
torulosum	CBS 316.33	PN		115	Neth.
tracheiphilum	CBS 323.65	TYPE		116	Italy
tumidum	CBS 223.94	TYPE		117	France
ultimum var. *sporangiiferum*	CBS 111.65			118	Leb.
	CBS 114.79			119	Spain
	CBS 171.68			120	?
	CBS 219.65	TYPE		121	U.S.
ultimum var. *ultimum*	CBS 114.19			122	?
	CBS 249.28			123	Neth.
	CBS 264.38			124	Neth.
	CBS 291.31			125	?
	CBS 296.37			126	U.K.
	CBS 305.35			127	Neth.
	CBS 378.34			128	?
	CBS 398.51	NEOTYPE		129	Neth.
	CBS 488.86			130	Pol.
	CBS 489.86			131	Pol.
	CBS 490.86			132	Pol.
	CBS 491.86			133	Pol.
	CBS 725.94			134	Can.
	CBS 726.94			135	Can.
	CBS 728.94			136	Can.
	CBS 729.94			137	Can.
	CBS 730.94			138	Can.
	CBS 656.68			139	Neth.
	adc 94.06			140	Nor.
uncinulatum	CBS 518.77	TYPE		141	Neth.
undulatum	CBS 157.69	NEOTYPE		142	U.S.
vanterpoolii	CBS 295.37	TYPE		143	U.K.
vexans	CBS 119.80	PN		144	Iran
violae	CBS 159.64	PN		145	Aust.
volutum	CBS 699.83			146	Japan
zingiberis	CBS 216.82			147	Japan

[a] CBS = accession numbers of strains obtained from Centraalbureau voor Schimmelcultures, Baarn, Netherlands; APCC = accession numbers of strains obtained from the Aquatic Phycomycete Culture Collection, Reading, England; and adc = strains not maintained.

[b] TYPE = strain from which the type material was derived; AU = authentic strain, identified by the author of the species; PN = strain used by van der Plaats-Niterink (15) for description of the species of the genus *Pythium*; NEOTYPE = strain designated as neotype (15) because all type material is missing; and ? = uncertain or unknown.

[c] MT = mating type; a, b, or + = opposite MT; f = female; and m = male.

[d] Ref. no. = number by which the isolate is referred to in text and figure captions.

[e] Neth. = Netherlands; ? = unknown; Aust. = Australia; Ger. = Germany; Alger. = Algeria; Switz. = Switzerland; Jam. = Jamaica; Can. = Canada; C.R. = Costa Rica; Nor. = Norway; S. Af. = South Africa; Leb. = Lebanon; Belg. = Belgium; and Pol. = Poland.

with an equal volume of chloroform/isoamyl alcohol (24:1) for 30 min on ice. The mixture was centrifuged (16,000 × *g*) for 10 min at 4°C, and the supernatant was extracted by adding 450 µl of 5 M ammonium acetate and incubating for at least 30 min on ice. The mixture was centrifuged again (16,000 × *g*) for 10 min at 4°C. DNA was precipitated from 1,300 µl of the aqueous phase by addition of 715 µl of isopropanol. The solution was centrifuged (16,000 × *g*) for 15 min, the DNA pellet was washed for 15 min with 1 ml of 70% ethanol at room temperature, and the pellet was dried and resuspended in 100 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0).

Amplification of DNA. Polymerase chain reaction (PCR) was done in a volume of 50 µl with the following reagents: 5 µl of 10× *Taq* DNA polymerase reaction buffer (Promega, Madison, WI), 3 µl of 25 mM $MgCl_2$ (final concentration 1.5 mM), 4 µl of dNTP mixture (final concentration of each nucleotide 200 µM), 1 µl (32 pmol) of each of the relevant oligonucleotide primers, 0.25 µl of *Taq* DNA polymerase (1.25 units) (Promega), 2 µl of template DNA solution, and 33.5 µl of ultrapure water. Pairs of primers were used: SL (dAGCCTAAGATGGCCGTCGAC), SR (dGAAGCCCGGGTGCTGTCTAC), N_2 (dTAGACGGCCATCTTAGGC), Y (dTCGCAGAGCGAACGGGAT), and Y_3 (dGTAGACAGCACCCGGACTTC) (Fig. 1). The DNA template solution consisted of 20 ng of a crude nucleic acid extract per microliter. The amplification was performed in a programmable thermal controller (PTC-100, MJ Research, Watertown, MA), using the step cycle program, including denaturation at 93°C for 1 min, annealing at 50°C for 1 min, and polymerization at 72°C for 2 min with N_2 and Y, 1 min with SL and SR, or 0.5 min with N_2 and Y_3. The PCR cycle was repeated 30 times. The amplification products were subjected to electrophoresis on 1.5% agarose gels, stained with ethidium bromide (10 ng of gel per milliliter), and viewed under UV light.

Hybridization. Probes were labeled with digoxigenin-11-dUTP by the random primed method according to the supplier's instructions (Boehringer GmbH, Mannheim, Germany). The whole PCR product or one DNA band eluted from the agarose gel by the freeze-squeeze method (14) was used as the template for the labeling reaction. Dot blots were prepared by spotting 1 µl of denatured (heated at 100°C for 5 min) genomic or amplified DNA target on a Hybond-N membrane (Amersham Corp., Arlington Heights, IL). DNA was fixed to the membrane by cross-linking with UV light for 4 min. Membranes were incubated at 65°C for 1 h in 15 ml of hybridization solution (1% SDS and 1 M NaCl) and hybridized with constant agitation overnight at 65°C with SL-SR probes or at 55°C with the 5S gene probe added to the hybridization solution. Probe concentrations were from 5 to 10 ng/ml. After hybridization, filters were washed twice, 5 min per wash, in 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0) and 0.1% SDS at room temperature, then twice for 20 min in 0.1× SSC and 0.1% SDS at 68 or 55°C for the gene probe.

Chemiluminescent detection was performed with anti-digoxigenin (DIG) antibody and CDP-Star substrate (Boehringer) according to the manufacturer's instructions as follows. After posthybridization washes, membranes were washed briefly in buffer 1 (100 mM maleic acid and 150 mM NaCl, pH 7.5) and incubated in buffer 2 (1% blocking reagent in buffer 1) for 1 h with gentle agitation. Anti–DIG-alkaline phosphatase was added to fresh buffer 2 to achieve a dilution of 1:10,000, followed by incubation with gentle agitation for 30 min. Membranes were washed twice for 15 min in buffer 1 and incubated in buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$) for 2 min. Membranes were placed on plastic sheets, and 100 µl of a 1:200 dilution of a 25 mM solution of CDP-Star was spread over each 15 cm^2 of membrane. Membranes were sealed in the plastic sheets and exposed to Kodak X-Omat x-ray film (Eastman Kodak Co., Rochester, NY) for 1 to 5 min to record chemiluminescence.

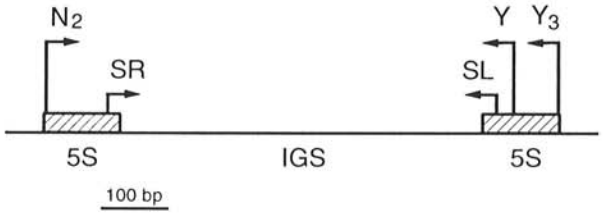

Fig. 1. *Pythium* 5S ribosomal RNA tandem genes showing positions of primers used for amplification. The gene to the left shows downstream primers, and the gene to the right shows upstream primers. Sites complementary to all primers occur on both genes. IGS = intergenic spacer.

RESULTS

Probe construction. Amplification of genomic DNA from different species of *Pythium* with primers SL and SR produced 0.3- to 1.0-kb fragments (Fig. 2). Each of these amplifications was performed at least twice to yield reproducible profiles. In many cases, more than one fragment size was produced, although there was usually a major fragment near 0.5 kb. In a number of cases, the second most prominent band in the profile was about twice the size of the main band near 0.5 kb and, thus, is likely to be the dimer produced by amplification across two spacer regions (Fig. 2, lanes 5, 9, and 10). Other additional bands were presumably due to the presence of more than one kind of 5S rRNA gene array, differing in the length of the spacer region. For all of the species in this study, except *P. anandrum* (lane 2), *P. ultimum* var. *sporangiiferum* (lane 7), and *P. mastophorum* (lane 8), the products shown in Figure 2 were labeled and used as probes without further purification; for the latter three species, the most prominent band was eluted from the gel and labeled separately. Purification and labeling of the 0.45-kb band for *P. anandrum*, the 0.3-kb band for *P. ultimum* var. *sporangiiferum*, and the 0.5-kb band for *P. mastophorum* greatly improved the specificity of these probes.

The other six probes consisted of all of the amplicons produced by SL-SR amplification in each case. To determine the identity of the weak amplicons, the most abundant amplicon was purified from each gel profile, labeled, and used to probe the Southern blot of its own profile at high stringency. In all cases, all bands visible on the gel were recognized by the probe (data not shown). The presence of primers SL and SR in both probe and target might account for this, but the experiment is like the experiment shown in Figure 3, where the presence of common primer sequences in probe and target did not lead to nonspecific background hybridization at high

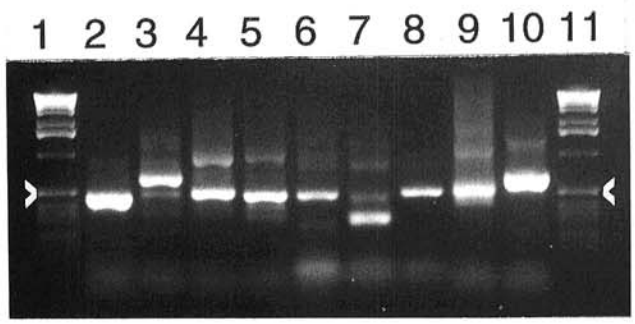

Fig. 2. Polymerase chain reaction products of primer pair SL-SR amplification used for the preparation of *Pythium* species-specific probes. Lanes 1 and 11, BRL 1-kb ladder (marked bands [arrows] are at 0.5 kb, and bands immediately above are 1.0, 1.6, 2.0, and 3.1 kb); lane 2, *P. anandrum* (CBS 385.31); lane 3, *P. intermedium* (CBS 266.38); lane 4, *P. macrosporum* (CBS 574.80); lane 5, *P. sylvaticum* (CBS 452.67); lane 6, *P. ultimum* var. *ultimum* (CBS 398.51); lane 7, *P. ultimum* var. *sporangiiferum* (CBS 219.65); lane 8, *P. mastophorum* (CBS 375.72); lane 9, *P. okanoganense* (CBS 315.81); and lane 10, *P. acanthicum* (CBS 284.31).

stringency. The probes created for these experiments obviously could be used as species-specific probes instead of the mixtures of strong and weak amplicons that were actually used, but as long as the mixed probes are truly species specific, they may be preferable because they are easier to prepare.

To further confirm that the labeled products had originated from the 5S rRNA spacer, the genomic DNA from each of the eight species also was amplified with primers N_2 and Y (Fig. 1). In each case, a prominent band was produced that was about 130 bp longer than the SL-SR product, which could be predicted from the map (Fig. 1; data not shown). N_2-Y included 120 bp of the first gene, the spacer, and 50 bp of the second gene. SL-SR included 20 bp of the first gene, the spacer, and 20 bp of the second gene.

Hybridization to genomic spot blots. Genomic DNA from 92 species of *Pythium* (including seven mating pairs of heterothallic species and the two varieties of *P. ultimum*) was denatured and applied in subsets to five hybridization membranes in concentrations determined by preliminary hybridizations. The amount of target DNA in each spot was normalized by hybridization with the 5S rRNA gene on the assumption that the number of genes would equal the number of gene spacers. The gene probe was obtained by amplification of *P. irregulare* and *P. torulosum* genomic DNA with primers N_2 and Y_3 and isolation of the 120-bp bands produced (Fig. 1). This product represents amplification from one end of the gene to the other. The amplification also produced the expected 0.6-kb gene-to-gene product.

The two products from the two species were mixed in equal proportions to prepare the 5S gene probe. The two species were chosen because there is a 2-bp difference between the sequences of the 5S genes of *Pythium* species with globose zoosporangia and those with filamentous zoosporangia (2). *P. irregulare* represents the former, and *P. torulosum* the latter. The *P. torulosum* sequence was included because many of the target species were species with filamentous zoosporangia. The results of hybridization with the 5S gene probe are shown in Figure 4A. Perfect normalization of target amounts in each spot is costly in terms of the DNA amounts needed in preliminary tests, so differences in target amounts are still present in the final spot blots, but the specificity of probes shown in this study is high enough to allay fears about unequal target amounts. Spots contain from 0.05 to 1.0 μg of DNA.

When the probe derived from the type culture of *P. sylvaticum* (male) was hybridized against the same target set as that shown in Figure 4A, it recognized only itself (Fig. 4B, spot 103) and the type culture of *P. sylvaticum* (female) (spot 104). The *P. intermedium* probe (CBS 266.38 mating type [MT]–) was derived from an isolate used by van der Plaats-Niterink in her portrayal of the species (15). It recognized only itself (Fig. 4C, spot 49) and *P. intermedium* CBS 221.68 MT+ (spot 46). The *P. macrosporum* probe was derived from the type culture (MT+). It recognized itself (Fig. 4D, spot 58) and *P. macrosporum* MT– (spot 59), but the latter hybridization was much less intense. The *P. okanogenense* probe was derived from the type culture. It recognized only itself (Fig. 4E, spot 73). The *P. anandrum* probe also was derived from the type culture and recognized only itself (Fig. 4F, spot 7). Weak reactions with many spots in the last two columns are thought to be nonspecific reactions, probably due to inadequate posthybridization washes. The probe derived from the type culture of *P. ultimum* var. *ultimum* recognized itself primarily (Fig. 4G, spot 129), but when the blots were overexposed, there were very weak reactions with the type culture of *P. ultimum* var. *sporangiiferum* (spot 121) and the two isolates of *P. splendens* (CBS 462.48 MT–, spot 96, and CBS 266.69 MT+, spot 95). The probe derived from the 0.3-kb band of the amplification profile of the type culture of *P. ultimum* var. *sporangiiferum* hybridized only to itself (Fig. 4H, spot 121). The probe derived from the type culture of *P. Acanthicum* (CBS 284.31) also hybridized only to itself (Fig. 4I, spot 2). Finally, the probe derived from *P. mastophorum* CBS 375.72, which was used by van der Plaats-Niterink (15) to portray the species, hybridized only to itself (Fig. 4J, spot 64).

Intraspecific hybridizations with species-specific probes. Results with the heterothallic pairs above indicate that probes derived from one MT generally recognize the opposite MT within the same species. To find out whether other isolates within the species also are recognized, probes were hybridized against as many isolates as were available from CBS. Negative controls were included with each hybridization.

Eight isolates of *P. intermedium* were tested, and all were recognized by the probe (Fig. 5A). Two of the isolates had significantly weaker reactions than the others. For *P. sylvaticum*, genomic DNA from 16 isolates was probed, and although all isolates were clearly recognized by the probe, 6 of them had weak reactions (Fig. 5B). This suggests that there may be substantial intraspecific variation within *P. sylvaticum* with respect to the 5S rRNA spacer sequence. As reported above, the *P. macrosporum* MT+ probe did not hybridize strongly to *P. macrosporum* MT–, but when *P. macrosporum* CBS 579.80 MT– was used as a target, hybridization was intense (Fig. 5C). Nineteen isolates of *P. ultimum* var. *ultimum* were tested with the *P. ultimum* var. *ultimum* probe (Fig. 5D). The probe recognized all isolates at high intensity. Of the four isolates of *P. ultimum* var. *sporangiiferum* tested

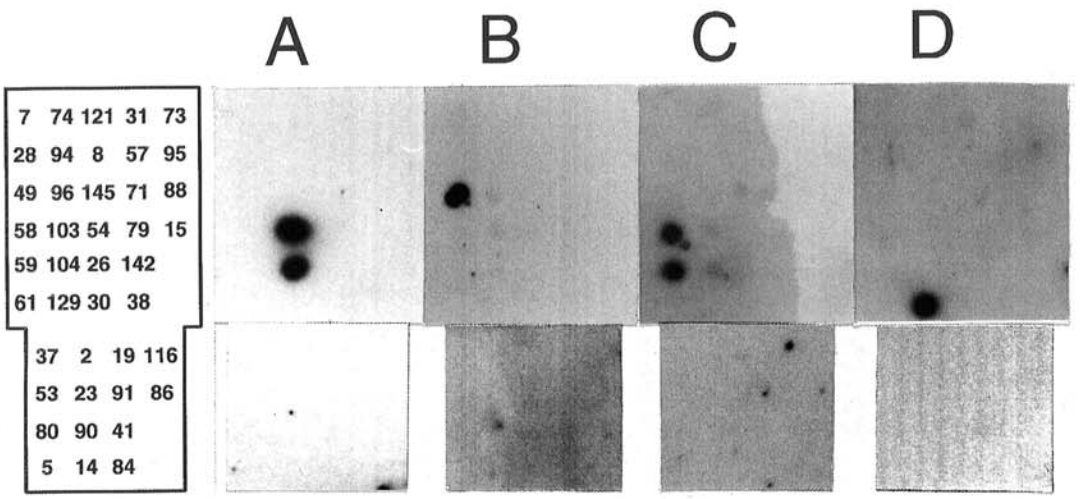

Fig. 3. Hybridization of *Pythium* species-specific probes versus polymerase chain reaction-amplified targets (primer pair N_2-Y). Spot positions are given in the schematic diagram to the left; numbers correspond to reference numbers provided in Table 1. **A,** *P. sylvaticum* CBS 452.67 probe. Reaction with spots 103 and 104. **B,** *P. intermedium* CBS 266.38 probe. Reaction with spots 49, 96, and 103. **C,** *P. macrosporum* CBS 574.80 probe. Reaction with spots 58 and 59. **D.** *P. ultimum* var. *ultimum* CBS 398.51 probe. Reactions with spots 129 and 121.

(Fig. 5E), only two reacted with the *P. ultimum* var. *sporangiiferum* probe (CBS 219.65 and CBS 111.65); the other two reacted with the *P. ultimum* var. *ultimum* probe (CBS 114.79 and CBS 171.68). Finally, two additional isolates of *P. acanthicum* (CBS 227.94 and CBS 431.68) were tested with the *P. acanthicum* probe and were recognized with high intensity. These results indicate that the probes are not simply isolate specific but that they recognize a variety of isolates within the same species.

Probing PCR-amplified targets. To show that it may not be necessary to isolate relatively large amounts of pure genomic DNA to prepare a target for the probes, targets were prepared by amplification of the 5S rRNA gene spacer with primers N_2 and Y (Fig.

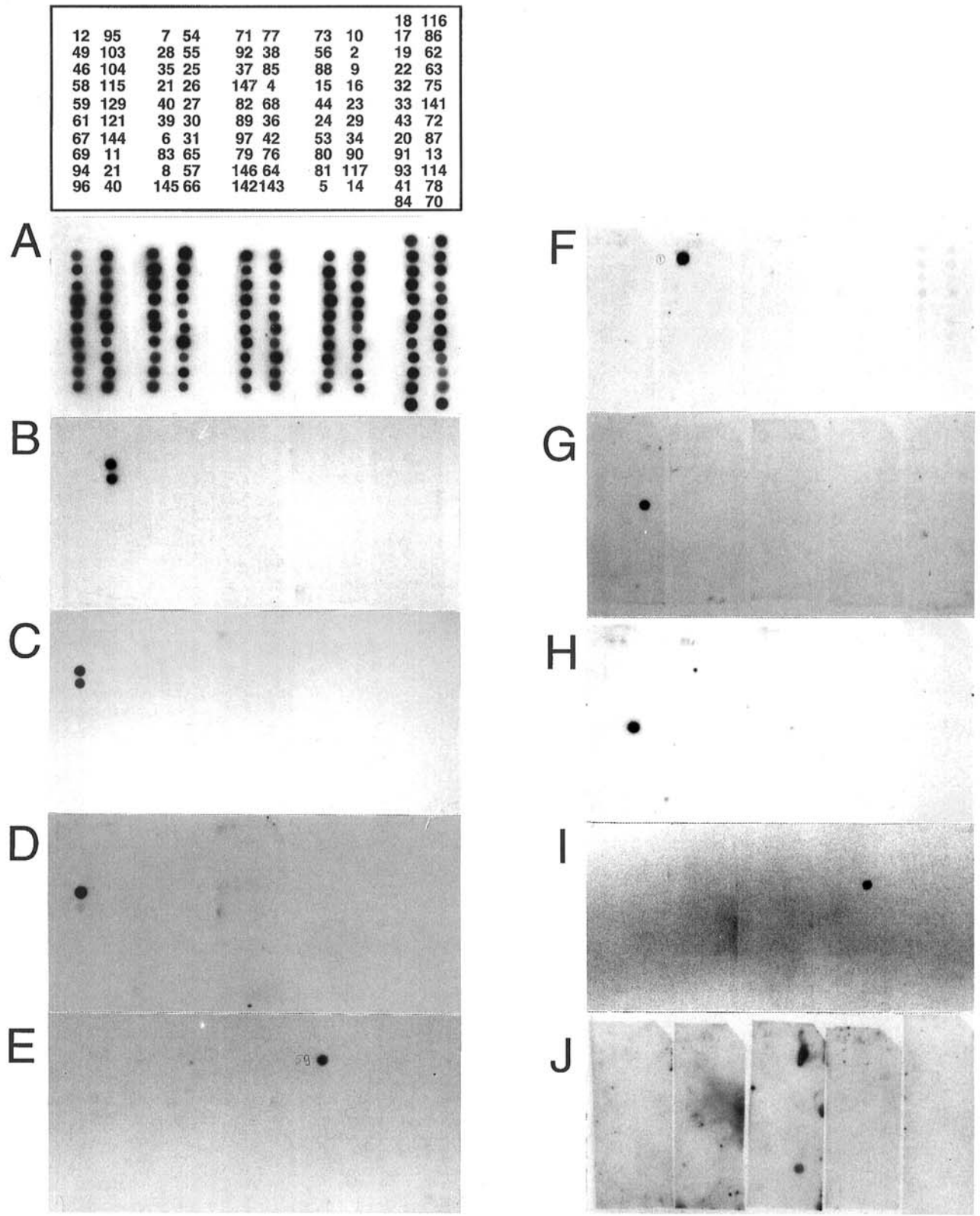

Fig. 4. Hybridization of the *Pythium* 5S ribosomal RNA (rRNA) gene and spacer probes against spot blots of genomic DNA. Positions of spots are given in the schematic diagram (upper left); numbers correspond to reference numbers provided in Table 1. **A,** 5S rRNA gene probe (primer pair N_2.Y_3). **B through J,** primer pair SL-SR products (Fig. 2) used as probes. **B,** *P. sylvaticum*; **C,** *P. intermedium*; **D,** *P. macrosporum*; **E,** *P. okanoganense*; **F,** *P. anandrum*; **G,** *P. ultimum* var. *ultimum*; **H,** *P. ultimum* var. *sporangiiferum*; **I,** *P. acanthicum*; and **J,** *P. mastophorum.*

1) (primers SL and SR could have been used, but independent origins of probe and target increase the confidence of the test). Each probe was tested against all the isolates that could be amplified with N_2 and Y. This set of 38 species included 3 heterothallic pairs and both varieties of *P. ultimum*. Results obtained with four of the probes are included to illustrate the similarities and differences in the genomic blots.

The *P. sylvaticum* probe distinguished the N_2-Y products from itself and from its opposite MT (Fig. 3A) with extreme specificity. The *P. intermedium* probe also was highly specific (Fig. 3B), although there was some background hybridization, which is not likely to be significant (the opposite mating type was not included in the target set). The *P. macrosporum* probe also was highly specific, recognizing itself as well as its opposite MT (Fig. 3C). This is surprising because the opposite MT was not well recognized in the genomic blots. Presumably, PCR amplification increased the relative abundance of a target sequence. Finally, the *P. ultimum* var. *ultimum* probe recognized itself and, weakly, the N_2-Y target from *P. ultimum* var. *sporangiiferum* (Fig. 3D). This agrees with the results of the genomic blots, except that there was no recognition of the *P. splendens* N_2-Y products. This may indicate that the weak reaction of the *P. ultimum* var. *ultimum* probe with genomic DNA of the *P. splendens* was an artifact.

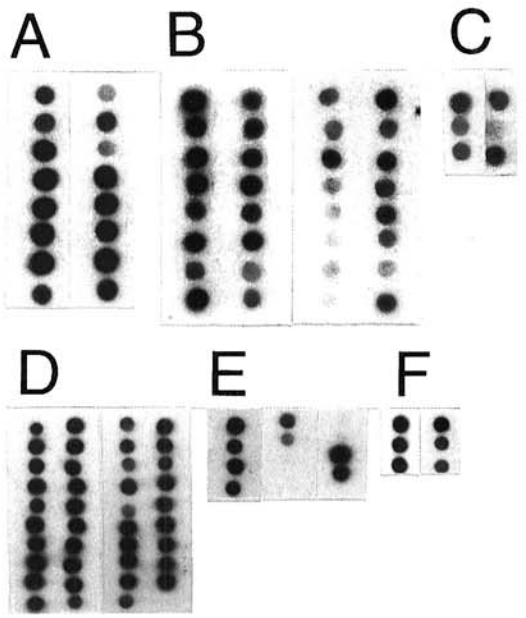

Fig. 5. Hybridization of *Pythium* species-specific probes to intraspecfic targets. **A,** Probes: first column, 5S ribosomal RNA (rRNA) gene; and second column, *P. intermedium* (CBS 266.38). Targets (top to bottom, both columns) (Table 1 provides reference numbers): *P. intermedium* 49, 50, 46, 48, 51, 45, 47, and 52. **B,** Probes: first and second columns, 5S rRNA gene; and third and fourth columns, *P. sylvaticum* CBS 452.67. Targets (top to bottom): first column, *P. sylvaticum* 103, 104, 102, 99, 100, 101, 98, and 105; and second column, 109, 110, 111, 112, 113, 108, 106, and 107. Third and fourth columns, identical to first and second columns, respectively. **C,** Probes: first column, 5S rRNA gene; and second column, *P. macrosporum* CBS 574.80. Targets (top to bottom, both columns): *P. macrosporum* 58, 59, and 60. **D,** Probes: first and second columns, 5S rRNA gene; and third and fourth columns, *P. ultimum* var. *ultimum* CBS 398.51. Targets (top to bottom): first column, *P. ultimum* var. *ultimum* 129, 130, 122, 127, 128, 138, 135, 136, 137, and 123; and second column, *P. ultimum* var. *ultimum* 124, 125, 126, 139, 140, 134, 131, 132, and 133 and *P. mamillatum* 61 (control). Third and fourth columns, identical to first and second columns, respectively. **E,** Probes: first column, 5S rRNA gene; second column, *P. ultimum* var. *sporangiiferum* CBS 219.65; and third column, *P. ultimum* var. *ultimum* CBS 398.51. Targets (top to bottom, all columns): *P. ultimum* var. *sporangiiferum* 121, 118, 119, and 120. **F,** Probes: first column, 5S rRNA gene; and second column, *P. acanthicum* CBS 284.31. Targets (top to bottom, both columns): *P. acanthicum* 2, 3, and 1.

DISCUSSION

Species-specific molecular probes for *Pythium* would greatly simplify species identification, which is often difficult because of the limited number of variable morphological characters. A number of approaches have been tried in the search for *Pythium* species-specific probes. Selected mitochondrial DNA fragments from *P. oligandrum* and *P. sylvaticum* were tried in one study (9) and were useful, but species-specificity was elusive. A probe derived from the amplified internal transcribed spacer (ITS1) of *P. ultimum* was tested against a selection of approximately two-dozen species (7), from which it recognized only *P. ultimum* var. *ultimum*, *P. ultimum* var. *sporangiiferum*, and several strains of *Pythium* group G (15). A repetitive sequence from a genomic library of *P. irregulare* was used to detect *P. irregulare* in soil (10) and was tested for specificity against 12 other species. Only *P. spinosum* was recognized in addition to *P. irregulare*.

Our study of a sample of *Pythium* species shows that species-specific probes can be obtained by amplification of the 5S rRNA intergenic spacer for species in which the 5S genes exist in tandem arrays. At first we expected that the presence of the SL and SR primer sequences in both the probe and the target would lead to a certain level of background hybridization in each test, but this did not happen when conditions of maximum stringency were used. Another concern was that amplification with SL and SR primers sometimes resulted in more than one product. In these cases (*P. anandrum*, *P. ultimum* var. *sporangiiferum*, and *P. mastophorum*), specificity was achieved by separation of the most prominent DNA band in the gel profile. Although specificity was achieved in this way, further analysis of the other products is necessary before conclusions can be drawn about relationships between isolates.

For the nine candidate probes obtained in this study, we showed that each one recognized the genomic DNA of the isolate from which the probe had been derived but not the genomic DNA from more than 90 other *Pythium* species. This finding suggests that 5S rRNA spacers between tandem repeats diverged rapidly after speciation. For six of the probes, several conspecific isolates also were tested and reacted satisfactorily with the relevant probes. In the case of *P. sylvaticum*, the degree of hybridization was highly variable, suggesting intraspecific variation; for *P. intermedium* and *P. ultimum* var. *ultimum*, hybridization was fairly uniform. In the case of *P. macrosporum*, only two additional isolates were available: one reacted weakly, and the other reacted strongly. These results suggest that the 5S spacer sequence is usually sufficiently homogeneous within a species to allow recognition of that species by one probe. Such homogeneity in repeated gene families can be attributed to concerted evolution acting within species (3,8).

The case of *P. ultimum* var. *sporangiiferum* is complex. Of the four isolates used in the study, only two were recognized by the probe derived from the type culture. The other two were recognized by the *P. ultimum* var. *ultimum* probe. These results, although they may be useful for the identification of certain isolates, should not be taken as evidence of the absence of a close relationship between the two varieties of *P. ultimum*, because the *P. ultimum* var. *sporangiiferum* probe was derived from one particular band in the amplification profile. Analysis of the other bands in the profile may uncover common elements in the two varieties.

When the 5S spacers of *Pythium* species with 5S tandem repeats were amplified and used as targets for the probes, the results agreed with those obtained when genomic DNA was the target. For *P. macrosporum*, the isolate that reacted weakly when its genomic DNA was used as a target, reacted strongly when its PCR product was used. This result cannot be attributed to an unusually low abundance of the target in the genomic DNA because the target amount was normalized by hybridization to the 5S gene probe, unless there are many more genes than spacers in this isolate. Further study is required, but the result points to the sensitivity of the PCR approach to the preparation of targets.

Our approach to species-specific probe construction has a number of advantages in common with other PCR-based techniques. Because the primers recognize highly conserved sequences in the 5S rRNA genes, the same primer pairs can be used with distantly related organisms. Also, the target for the probe can be amplified with the same primers with very small amounts of genomic DNA; this may obviate the need to culture isolates before detection. Finally, the use of 5S rRNA spacer probes for all organisms having tandem repeat families, including plants and animals, may lead to a more objective and quantitative criterion for species delimitation.

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