Taxonomic and Phylogenetic Analyses of Ten *Pythium* Species Using Isozyme Polymorphisms

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ABSTRACT

Chen, W., Schneider, R. W., and Hoy, J. W. 1992. Taxonomic and phylogenetic analyses of ten *Pythium* species using isozyme polymorphisms. Phytopathology 82:1234-1244.

Isozyme analysis of 13 enzymes was used to compare 204 isolates of the following 10 *Pythium* species from diverse geographic locations: *P. aphanidermatum, P. arrhenomanes, P. deliense, P. graminicola, P. heterothallicum, P. irregulare, P. myriotylum, P. spinosum, P. torulosum,* and *P. ultimum*. Morphologically distinct species could be differentiated by banding patterns. However, morphologically similar species, such as *P. aphanidermatum* and *P. deliense* or *P. arrhenomanes* and *P. graminicola,* showed similar or identical banding patterns. Cluster and principal components analyses showed that isolates within morphological species generally clustered together, but considerable variation existed within certain species. Intraspecific variation could not be attributed to the host or habitat from which the isolates were originally obtained. With some species, intraspecific clusters and geographic origin were closely correlated. It was concluded that *Pythium* species could not be characterized by isozyme banding patterns. These results are interpreted in light of current theories in population genetics and evolutionary biology with regard to life histories and host-parasite interactions.

The genus *Pythium* contains species with life histories that range from saprotrophic, facultative parasites with extensive host ranges to highly pathogenic species with limited host ranges (34,78). *Pythium* species are capable of causing decline syndromes known as feeder root necrosis (34,67), cryptic disease (37), subclinical infection (74), and replant diseases (34) in numerous crops. We have been investigating the effects of several *Pythium* species on sugarcane (*Saccharum officinarum* L.) and rice (*Oryza sativa* L.) in which multiple species can be isolated from plant roots. In sugarcane *P. arrhenomanes* Drechs. is the one highly pathogenic species (36), whereas variability in pathogenicity within *Pythium* species occurs in rice (67; W. Chen, R. W. Schneider, and J. W. Hoy, *unpublished data*). It is often difficult to identify *Pythium* isolates to species on the basis of morphological traits, and pathogenic isolates within species cannot be distinguished. Therefore, we initiated a study to determine whether biochemical analyses would be useful for conducting pathological and ecological studies of *Pythium* species (14–16).

Protein electrophoresis and isozyme analysis have been widely used in genetic and systematic studies of fungi (5,43,51,68,75). Species-specific isozyme banding patterns were recognized for a number of *Phytophthora* species (58), and protein electrophoresis (14), isozyme analysis (19), and isoelectric focusing profiles (2) also were reported to be useful in differentiating *Pythium* species. Although variations within species were observed, it was concluded that protein patterns could be used to differentiate species (2,19).

Allele frequencies within populations are useful for the development of evolutionary theory; however, these data are often difficult to obtain. Cheverud (17) showed that phenotypic correlations between characters may be estimated with greater precision and are simpler to obtain. He concluded that characters such as enzyme electrophoretic phenotypes (EPs) may be substituted for their genetic equivalents in evolutionary models provided that sample size is adequate. Phylogenies based on EPs have gained widespread acceptance, and developments in mathematical and statistical analyses of character data have allowed their use in inferring life history traits (23,32,75).

Enzyme polymorphisms, or isozyme frequency data, are representative of genotypic diversity and have been used for identifying factors that affect genetic variation in populations and species (39,57). With regard to plant pathogens, lack of sexual reproduction (11,12,77), environmental homogeneity (72), host specificity (42,50), and founder effects (11) have all been associated with low electrophoretic variability, whereas high electrophoretic variability has been associated with an outcrossing plant pathogen (24) and geographic isolation (4,9). The latter is thought to be a major factor in genetic divergence and speciation in saprophytic and pathogenic fungi (9). The spatial scale for geographic isolation is quite variable with distances ranging from tens of meters (21) to intercontinental (9).

We previously reported comparisons between banding patterns of total soluble proteins and isozymes for taxonomic and systematic analyses in which we concluded that isozymes provide more reliable data (14). The objectives of the present research were to compare several *Pythium* species by isozyme analysis in an attempt to develop a rapid electrophoretic technique for routine identification, to examine interspecific and intraspecific variation, and to further our understanding of the evolutionary biology of this genus by the application of theories of population genetics. A preliminary account has been published (16).

MATERIALS AND METHODS

Collection, maintenance of cultures, and morphology. A total of 204 isolates representing 10 *Pythium* species was obtained from surface-sterilized roots of rice and sugarcane from Louisiana and from other hosts and soils (Table 1). Isolates were identified or confirmed to species according to the descriptions and key of

TABLE 1. Isolate number, host/habitat, geographic origin, and electrophoretic phenotype (EP) of *Pythium* isolates used in this study

Isolate	Host/habitat	Origin	EP
P. aphanidermatum			
907	Sugarcane	Florida	aph-1
349	Poinsettia	Ohio	aph-2
1448	Spinach	Yamagata, Japan	aph-3
1449	Spinach	Yamagata, Japan	aph-4
1450	Buckwheat f.[a]	Yamagata, Japan	aph-4
1451	Cucumber f.	Yamagata, Japan	aph-4
1452	Tomato f.	Iwate, Japan	aph-4
1453	Cucumber	Okinawa, Japan	aph-3
1454	Tomato	Okinawa, Japan	aph-4
1455	Cabbage f.	Okinawa, Japan	aph-4
1456	Cucumber f.	Okinawa, Japan	aph-4
1457	Eggplant f.	Okinawa, Japan	aph-5
1458	Eggplant f.	Okinawa, Japan	aph-6
1459	Cucumber	Kumamoto, Japan	aph-6
1460	Cabbage f.	Fukuoka, Japan	aph-3
1461	Watermelon f.	Fukuoka, Japan	aph-7
1462	Watermelon f.	Fukuoka, Japan	aph-5
1463	Cabbage f.	Kagoshima, Japan	aph-8
1464	Cucumber f.	Miyazaki, Japan	aph-4
1465	Cucumber f.	Miyazaki, Japan	aph-6
1466	Corn f.	Kumamoyo, Japan	aph-4
1467	Soybean	Ibaraki, Japan	aph-4
1468	Cucumber	Mie, Japan	aph-7
1469	Cucumber	Mie, Japan	aph-4
1470	Chrysanthemum f.	Mie, Japan	aph-4
1471	Onion f.	Osaka, Japan	aph-4
1472	Welsh onion f.	Nara, Japan	aph-4
1473	Strawberry f.	Nara, Japan	aph-7
1474	Pea f.	Wakayama, Japan	aph-7
1475	Lettuce f.	Ehime, Japan	aph-7
1476	Nursery f.	Kagawa, Japan	aph-4
1477	Soybean f.	Tokushima, Japan	aph-7
1502	Red pepper	Korea	aph-6
1522	Papaya	Hawaii	aph-9
24-1	Turf	Kentucky	aph-5
24-2	Turf	Indiana	aph-10
24-3	Rye	Pennsylvania	aph-10
24-5	Cucumber	North Carolina	aph-5
24-6	Turf	Illinois	aph-5
24-7	Turf	Illinois	aph-5
24-8	Turf	Illinois	aph-5
25-1	Sugarbeet	Arizona	aph-10
25-2	Spinach	Canada	aph-11
25-3	Potato	Arizona	aph-10
25-4	Soil	Mexico	aph-10
25-5	Potato	Arizona	aph-10
P. arrhenomanes			
141	Sugarcane	Louisiana	arr-1
147	Sugarcane	Louisiana	arr-1
153	Sugarcane	Louisiana	arr-1
156	Sugarcane	Louisiana	arr-1
198	Sugarcane	Louisiana	arr-2
212	Sugarcane	Louisiana	arr-2
230	Sugarcane	Louisiana	arr-2
909	Wheat	Oklahoma	arr-2
12-1	Corn	Georgia	arr-2
12-3	Corn	Georgia	arr-2
12-4	Corn	Georgia	arr-2
14-1	Sugarcane	Australia	arr-2
1031	Sugarcane	Louisiana	arr-2
12531	Corn	ATCC[b]	arr-2
1521	Bermuda grass	Hawaii	arr-3
19-1	Wheat	Oklahoma	arr-4
19-2	Wheat	Oklahoma	arr-4
19-3	Wheat	Oklahoma	arr-4
P. deliense			
20-1	Sugarbeet	Texas	del-1
25-6	Sugarbeet	Arizona	del-2
P. graminicola			
13-6	Wheat	Arizona	(arr-2)[c]
18-4	Turf	North Carolina	gra-1
34999	Bentgrass	ATCC	gra-9
28458	Sugarcane	Puerto Rico (ATCC)	gra-2
1419	Soybean f.	Miyazaki, Japan	gra-3
1420	Soybean f.	Miyazaki, Japan	gra-4
1421	Sweetpotato	Kagoshima, Japan	gra-5
1422	Corn f.	Kumamoto, Japan	gra-6
1423	Corn f.	Kumamoto, Japan	gra-7
1424	Corn f.	Kumamoto, Japan	gra-3
1425	Corn f.	Kumamoto, Japan	gra-3
1426	Soybean f.	Miyazaki, Japan	gra-8
1523	Bermuda grass	Hawaii	gra-10
1524	Bermuda grass	Hawaii	gra-10
1525	Bermuda grass	Hawaii	gra-10
P. heterothallicum			
272	Sugarcane	Louisiana	het-1
285	Sugarcane	Louisiana	het-1
11-1	Sugarcane	Louisiana	het-1
16-13	Soil	ATCC 18197	het-1
16-14	Soil	ATCC 18198	het-1
P. irregulare			
6-1	Sugarcane	Louisiana	irr-1
6-2	Sugarcane	Louisiana	irr-1
7-4	Sugarcane	Louisiana	irr-1
900	Sugarcane	Florida	irr-1
908	Wheat	Oklahoma	irr-1
1032	Sugarcane	Louisiana	irr-1
1037	Sugarcane	Louisiana	irr-1
1043	Sugarcane	Louisiana	irr-1
1046	Sugarcane	Louisiana	irr-2
1371	Rice	Louisiana	irr-2
1377	Wheat	Georgia	irr-1
1378	Wheat	Georgia	irr-2
1379	Wheat	Georgia	irr-1
26-11	Sugarcane	Louisiana	(spi-2)
26-12	Sugarcane	Louisiana	irr-1
26-17	Sugarcane	Louisiana	irr-1
83-6	Alfalfa	California	irr-1
86-10	Alfalfa	California	irr-2
86-14	Alfalfa	California	irr-1
87-9	Alfalfa	California	irr-2
AR12-8	Wheat	Washington	irr-1
33	Geranium	Ohio	irr-1
18-3	Apple	New York	irr-5
11120	Unknown	ATCC	irr-1
16970	Corn	ATCC	irr-2
10951	Unknown	ATCC	irr-1
1427	Peanut f.	Aomori, Japan	irr-3
1428	Peanut f.	Aomori, Japan	irr-3
1429	Peanut f.	Aomori, Japan	irr-3
1430	Garden soil	Iwate, Japan	irr-3
1431	Garden soil	Iwate, Japan	irr-3
1432	Watermelon f.	Kagoshima, Japan	irr-3
1433	Sweetpotato	Kagoshima, Japan	irr-3
1434	Watermelon f.	Kumamoto, Japan	irr-4
1435	Sweetpotato f.	Kagoshima, Japan	irr-4
1436	Sweetpotato f.	Kagoshima, Japan	irr-4
1437	Sweetpotato f.	Kagoshima, Japan	irr-4
1438	Cabbage f.	Kanagawa, Japan	irr-4
1439	Grassland	Hokkaido, Japan	irr-4
1440	Broad bean f.	Chiba, Japan	irr-4
1441	Melon	Chiba, Japan	irr-4
1442	Melon f.	Chiba, Japan	irr-4
1443	Melon f.	Chiba, Japan	irr-4
1444	Chrysanthemum	Mie, Japan	irr-4
1445	Radish f.	Wakayama, Japan	irr-4
1446	Citrus f.	Kagawa, Japan	irr-4
1447	Citrus f.	Kagawa, Japan	irr-4
1498	Ginseng	Korea	irr-6
1501	Ginseng	Korea	irr-6
1514	Alfalfa	California	irr-7

P. graminicola (continued on next page)

[a] Indicates that isolate was obtained from field soil in which the indicated host was currently being grown.
[b] Isolate numbers correspond to American Type Culture Collection designations.
[c] Values in parentheses indicate that EPs are identical with those in other species.

TABLE 1. Continued from preceding page

Isolate	Host/habitat	Origin	EP
1515	Alfalfa	California	irr-7
1516	Alfalfa	California	irr-7
1517	Alfalfa	California	irr-7
1518	Alfalfa	California	irr-7
1519	Alfalfa	California	irr-7
19-4	Wheat	Oklahoma	irr-8
19-5	Wheat	Oklahoma	irr-8
19-6	Wheat	Oklahoma	irr-8
21-7	Rapeseed	Georgia	irr-2
23-1	Soybean	Kentucky	irr-6
23-2	Soybean	Kentucky	irr-6
23-3	Soybean	Kentucky	irr-6
P. myriotylum			
P884R	Rice	Louisiana	myr-2
14-3	Sugarcane	Australia	myr-1
14-4	Sugarcane	Australia	myr-1
36440	Strawberry	ATCC	myr-1
21-2	Snap bean	Georgia	myr-3
21-3	Snap bean	Georgia	myr-4
21-5	Cowpea	Georgia	myr-6
25-7	Snap bean	Arizona	myr-5
P. spinosum			
1045	Sugarcane	Louisiana	spi-1
1027	Sugarcane	Louisiana	spi-2
872	Rice	Louisiana	spi-1
26-1	Sugarcane	Louisiana	spi-1
26-2	Sugarcane	Louisiana	spi-1
26-3	Sugarcane	Louisiana	spi-1
26-4	Sugarcane	Louisiana	spi-2
26-5	Sugarcane	Louisiana	spi-1
26-7	Sugarcane	Louisiana	spi-2
26-8	Sugarcane	Louisiana	spi-1
26-9	Sugarcane	Louisiana	spi-2
26-10	Sugarcane	Louisiana	spi-2
26-23	Sugarcane	Louisiana	spi-2
1499	Ginseng	Korea	spi-3
1500	Ginseng	Korea	spi-3
P. torulosum			
7-2	Soil	Louisiana	tor-1
7-7	Soil	Louisiana	tor-1
18-5	Unknown	North Carolina	tor-2
18-8	Unknown	North Carolina	tor-2
P. ultimum			
906	Sugarcane	Florida	ult-1
211	Poinsettia	Ohio	ult-1
12-92	Wheat	Washington	ult-10
18-1	Unknown	North Carolina	ult-2
18-2	Cotton	North Carolina	ult-2
1405	Watermelon f.	Kagoshima, Japan	ult-3
1406	Cabbage	Mie, Japan	ult-4
1409	Chrysanthemum f.	Mie, Japan	ult-4
1410	Buckwheat	Yamagata, Japan	ult-4
1411	Pea f.	Wakayama, Japan	ult-4
1412	Eggplant f.	Kagoshima, Japan	ult-4
1413	Sweetpotato f.	Kagoshima, Japan	ult-4
1414	Sweetpotato f.	Kagoshima, Japan	ult-4
1415	Lettuce f.	Ehime, Japan	ult-4
1418	Radish f.	Kagowa, Japan	ult-4
1497	Ginseng	Korea	ult-5
1520	Protea	Hawaii	ult-6
20-2	Sugarbeet f.	Texas	ult-7
20-3	Sugarbeet f.	Texas	ult-8
21-4	Cowpea	Georgia	ult-11
21-6	Tomato	Georgia	ult-12
23-4	Soybean	Kentucky	ult-5
23-5	Soybean	Kentucky	ult-9
23-6	Soybean	Kentucky	ult-9
23-7	Soybean	Kentucky	ult-9
23-8	Soybean	Kentucky	ult-9
23-9	Soybean	Kentucky	ult-9
23-10	Soybean	Kentucky	ult-9
23-11	Soybean	Kentucky	ult-9

van der Plaats-Niterink (78). Cultures were maintained on V8 agar (200 ml of V8 juice, 2 g of $CaCO_3$, 17 g of agar, and 800 ml of water) plugs in sterile water at room temperature or on Schmitthenner agar (66) slants at 12 C. This liquid medium contains the following ingredients per liter of distilled water: 5 g of sucrose, 0.54 g of L-asparagine, 0.15 g of KH_2PO_4, 0.15 g of K_2HPO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 80 mg of $CaCl_2 \cdot 2H_2O$, 10 mg of ascorbic acid, 2 mg of thiamine·HCl, 4.4 mg of $ZnSO_4 \cdot 7H_2O$, 1 mg of $FeSO_4 \cdot 7H_2O$, and 0.7 mg of $MnCl_2 \cdot 4H_2O$. The medium was solidified with 17 g of agar per liter.

To enhance the development of sexual reproductive structures, cultures were grown on V8 agar (200 ml of V8 juice plus 17 g of agar per liter) and then transferred to cleared V8 agar amended with 20 mg/L of β-sitosterol. After 1 wk of incubation at 24 C, microscopic observations of antheridia, oogonia, and oospores were made directly on culture plates. Morphological measurements were recorded for 20 randomly selected oospores for each isolate. Sporangia and zoospore production were observed on hyphae extending into filter-sterilized soil extracts from V8 agar disks.

Cultural conditions and protein extraction. Single-isolate cultures for isozyme analysis were grown in 1.5% water agar for 48 h. Agar disks were taken from the actively growing colonies and transferred to Schmitthenner's liquid medium. Four agar plugs (4 mm in diameter) per isolate were inoculated into 100 ml of the medium (two flasks per isolate) and incubated for 5 days as shake cultures (100 rpm) at room temperature. For extraction, mycelial mats were collected by filtration through Whatman No. 1 filter paper, washed with chilled (4 C), sterile, distilled water, and frozen at −70 C. The frozen mycelial mats were then lyophilized, ground to a fine powder with a chilled (−20 C) mortar and pestle, and transferred to centrifuge tubes. Extraction buffer (0.05 M Tris, pH 7.1) was added at a rate of 1 ml/100 mg of mycelium, and the tubes were placed on ice and vortexed every 10 min for 40 min. The extraction mixture was then centrifuged at 16,000 *g* for 40 min at <4 C. The supernatant, which was used for electrophoresis, was stored in 20-μl aliquots in microcentrifuge tubes at −20 C, and each aliquot was used only once. All samples were used within 3 mo after extraction.

Electrophoresis and enzyme staining. Horizontal starch gels (12%, w/v) were prepared with Starch-Hydrolysed (Connaught Laboratories, Willowdale, Ontario, Canada) in appropriate gel buffers (62). Protein extracts (13 μl) were absorbed onto sample wicks (4 × 12 mm) made from Whatman No. 3 filter paper. Single paper wicks with protein extracts were placed vertically into sample slots, which were 50 mm from the anode of the gel. Ice packs (Polar Pack, Mid-Lands Chemical Co., Omaha, NE) were placed on the gels during electrophoresis. An electric current was imposed across the gel at a constant amperage (Table 2). After electrophoresis, gels were sliced into 1.5-mm-thick slabs that were used for individual enzyme-staining procedures. Two isolates of *P. arrhenomanes* were included in every gel as standards for comparisons. Each isolate was assayed at least twice either in the same gel or in separate gels. A total of 29 enzymes was initially tested for resolution and consistency in four electrophoretic buffer systems. Previously published staining procedures were followed (31,51,70,76). Thirteen resolvable enzymes were selected for further study.

Data analysis. A computer program, NTSYS-PC version 1.60 (Exeter Publishing Co., Setauket, NY) (63), was used for analysis

of the isozyme data. The presence or absence, 1 and 0, respectively, of a particular enzyme band was recorded. The relative intensity of bands was ignored. A matrix of simple matching coefficients (S_{sm}) for each pair of isolates was constructed using a similarity program (SIMQUAL) within NTSYS-PC by the formula $S_{sm} = (a + b)/n$, where a = the number of isozyme bands common to the pair of isolates, b = the number of isozyme bands absent in the pair of isolates but present in at least one isolate, and n = the total number of isozyme bands. A phenetic tree was generated from the matrix of similarity coefficients by the unweighted pair-group method with arithmetic average (UPGMA).

A Q-type principal components analysis of the original data matrix was performed according to the method of Sneath and Sokal (71). Product-moment correlation coefficients among variables were calculated on the basis of the standardized data. Eigenvectors were extracted from the correlation matrix, and the standardized data were projected onto the eigenvectors (63). Percentage of variance was used to decide the number of principal components to be included in the projection (25). No rotation of the coefficient was performed, and no attempts were made to name the factors.

RESULTS

Isozyme analyses. Thirteen enzymes produced distinct and consistent banding patterns (Table 2). Seventy-seven discrete bands were resolved with these 13 enzymes. Isolates were grouped by EPs (Table 1), and 59 distinct EPs were distinguished (Table 3). The method of Nygaard et al (58) was used to produce diagrammatic representations of all EPs (Fig. 1). This figure can be used by other investigators to classify additional isolates and relate them to our results.

Cluster analysis. The value for cophenetic correlation for the phenetic tree (Fig. 2) was 0.89. To reduce the size of the phenetic tree, isolates that showed identical banding patterns for the 13 enzymes are represented by a single branch with one isolate. Although there was considerable variation, isolates within species generally clustered together (Fig. 2). *P. torulosum* Coker & F. Patterson, *P. myriotylum* Drechs., *P. ultimum* Trow, and *P. heterothallicum* W. A. Campbell & J. A. Hendrix clustered independently and were distinct from other species. However, isolates of *P. irregulare* Buisman and *P. spinosum* Sawada formed a tight, single cluster. *P. aphanidermatum* (Edson) Fitzp. clustered with *P. deliense* Meurs (Fig. 2). Seven of the eight Japanese isolates of *P. graminicola* Subramanian were separated from the other *P. graminicola* isolates that clustered with isolates of *P. arrhenomanes*.

TABLE 2. Enzymes with detectable activity, enzyme abbreviations, enzyme commission number, and buffer systems used to study *Pythium* species

Enzyme	Abbreviation	EC number	Buffer system
Aspartate aminotransferase	AAT	2.6.1.1	A[a]
Aconitase	ACON	4.2.1.3	B[b]
Fumarase	FUM	4.2.1.2	A
Glucose-6-phosphate dehydrogenase	G6PDH	1.1.1.49	B
Glucose phosphate isomerase	GPI	5.3.1.9	B
Isocitrate dehydrogenase	IDH	1.1.1.42	B
Lactate dehydrogenase	LDH	1.1.1.27	B
Malate dehydrogenase	MDH	1.1.1.37	A
Malic enzyme	ME	1.1.1.40	A
Mannose phosphate isomerase	MPI	5.3.1.8	B
6-Phosphogluconate dehydrogenase	6PGD	1.1.1.44	A
Phosphoglucomutase	PGM	2.7.5.1	A
Superoxide dismutase	SOD	1.15.1.1	A

[a] Electrode buffer = 68 mM Tris, 37 mM citric acid, pH 6.3, as described by Micales et al (12); gel buffer = 6 mM Tris and 3 mM critic acid, pH 6.7.

[b] Electrode buffer = 1.37 M Tris, 0.314 M citric acid, pH 8.1, 1:3 dilution for cathode and 1:4 dilution for anode (24); gel buffer = 74 mM Tris and 9 mM citric acid, pH 8.4.

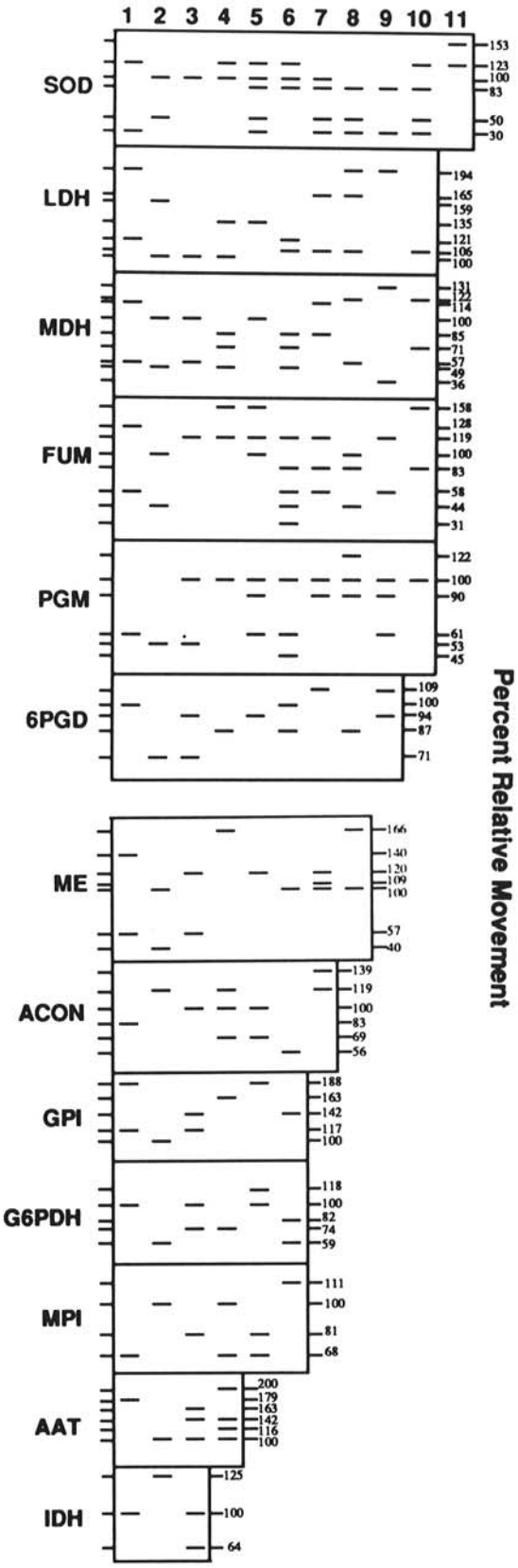

Fig. 1. Isozyme banding patterns for 59 electrophoretic phenotypes of 10 *Pythium* species represented as the percent movement relative to the most common, anodally migrating band (designated as 100). The enzymes are superoxide dismutase (SOD), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), fumarase (FUM), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PGD), malic enzyme (ME), aconitase (ACON), glucose phosphate isomerase (GPI), glucose-6-phosphate dehydrogenase (G6PDH), mannose phosphate isomerase (MPI), aspartate aminotransferase (AAT), and isocitrate dehydrogenase (IDH).

TABLE 3. Electrophoretic phenotype (EP) designations for enzyme polymorphisms of 10 *Pythium* species

	Enzymes[a]												
EP	MDH	SOD	ME	IDH	FUM	LDH	G6PDH	AAT	ACON	MPI	6PGD	PGM	GPI
P. aphanidermatum													
aph-1	2[b]	4	5	2	3	7	3	2	3	3	0	4	2
aph-2	5	4	0	2	0	7	5	2	3	3	0	4	2
aph-3	5	4	8	2	5	8	5	2	3	3	4	8	3
aph-4	5	4	8	1	5	7	5	2	3	3	4	8	3
aph-5	5	4	8	1	5	7	5	2	3	3	5	8	3
aph-6	5	4	8	1	5	8	5	2	3	3	4	8	3
aph-7	5	4	8	2	5	7	5	2	3	3	4	8	3
aph-8	5	4	8	2	5	8	5	2	3	3	4	4	3
aph-9	5	4	5	1	5	8	5	2	3	3	4	8	3
aph-10	5	3	5	1	5	8	5	2	3	3	2	8	3
aph-11	5	4	5	3	5	8	5	2	3	3	2	8	3
P. arrhenomanes													
arr-1	1	1	1	1	1	1	1	1	1	0	1	1	1
arr-2	1	1	1	1	1	1	1	1	1	0	0	1	1
arr-3	1	1	1	1	1	1	1	3	1	0	0	0	1
arr-4	8	2	8	2	1	1	2	1	1	0	7	1	1
P. deliense													
del-1	5	3	5	1	5	8	5	2	3	3	1	4	3
del-2	5	3	5	1	5	0	1	2	3	3	1	4	3
P. graminicola													
gra-1	1	1	4	1	1	1	1	1	1	0	0	0	1
gra-2	1	1	1	1	1	1	1	1	1	0	0	0	1
gra-3	4	5	7	1	6	5	1	2	6	1	4	6	1
gra-4	4	8	7	1	6	4	1	2	5	1	4	6	1
gra-5	1	9	4	1	1	4	5	1	5	3	5	6	3
gra-6	4	10	7	1	6	5	1	2	6	1	4	6	1
gra-7	4	7	7	1	6	5	1	2	6	1	4	6	1
gra-8	4	6	7	1	6	5	1	2	6	1	4	6	1
gra-9	1	1	1	1	2	1	1	1	2	1	0	1	1
gra-10	1	1	1	1	6	8	1	3	1	0	0	0	1
P. heterothallicum													
het-1	12	12	5	2	10	12	6	2	7	6	9	11	3
P. irregulare													
irr-1	2	2	2	2	2	2	2	0	2	2	1	3	2
irr-2	2	2	2	2	2	2	2	0	2	2	0	3	2
irr-4	2	2	2	2	2	6	6	0	2	2	1	5	2
irr-5	2	2	2	2	2	6	6	0	2	2	6	5	2
irr-7	2	2	2	2	2	2	6	0	2	2	0	3	2
irr-8	2	2	2	2	2	2	2	0	2	2	4	3	2
irr-9	2	2	2	2	2	2	2	0	2	2	1	5	2
irr-10	2	2	2	2	8	2	2	0	2	2	1	5	2
P. myriotylum													
myr-1	9	9	4	1	4	7	5	2	3	4	4	6	4
myr-2	9	2	4	1	4	3	5	0	3	3	1	3	2
myr-3	9	2	4	1	4	1	5	2	3	0	0	4	2
myr-4	9	2	4	1	4	4	5	0	3	1	1	3	2
myr-5	9	9	4	1	4	7	5	0	3	4	4	6	4
myr-6	9	4	4	1	4	4	5	2	3	1	4	8	3
P. spinosum													
spi-1	2	3	2	2	2	2	2	0	2	2	1	3	2
spi-2	2	3	2	2	2	2	2	0	2	2	0	3	2
spi-3	2	1	2	2	2	2	6	0	2	2	4	3	2
P. torulosum													
tor-1	1	10	5	2	9	11	1	5	0	5	0	10	6
tor-2	1	4	5	2	9	11	1	5	0	5	0	10	6
P. ultimum													
ult-1	3	4	3	1	3	3	3	0	3	1	3	4	2
ult-2	3	4	3	1	3	3	3	0	3	1	2	4	2
ult-3	3	4	3	1	7	3	3	0	3	1	3	7	2
ult-4	3	4	3	3	7	3	3	0	3	1	3	7	2
ult-5	3	4	3	1	9	3	3	0	3	1	4	7	2
ult-6	3	4	3	1	4	3	3	0	3	1	2	7	2
ult-7	3	4	3	1	9	6	3	0	3	1	5	7	2
ult-8	3	4	3	1	8	4	5	0	3	1	1	7	2
ult-9	8	4	3	1	9	3	3	0	3	1	5	7	2
ult-10	3	4	8	1	1	2	6	0	3	1	1	1	2
ult-11	2	4	3	1	2	3	6	2	3	3	4	8	2
ult-12	2	4	3	1	2	3	6	2	3	2	4	8	2

[a] Refer to Table 2 for enzyme abbreviations.
[b] Numerical designations refer to isozyme patterns depicted in Figure 1.

Principal components analysis. The first six principal components accounted for 24.8, 16.8, 10.4, 9.8, 7.5, and 5.2%, respectively, of the variance. Two-dimensional projections of all possible combinations of the first six principal components were plotted, and the plot of the first and second principal components of all 10 species is presented in Figure 3A. Groups of species are plotted separately (Fig. 3B–E) for comparative purposes. The patterns of clustering found in the phenetic tree (Fig. 2) also can be found in the principal components analysis.

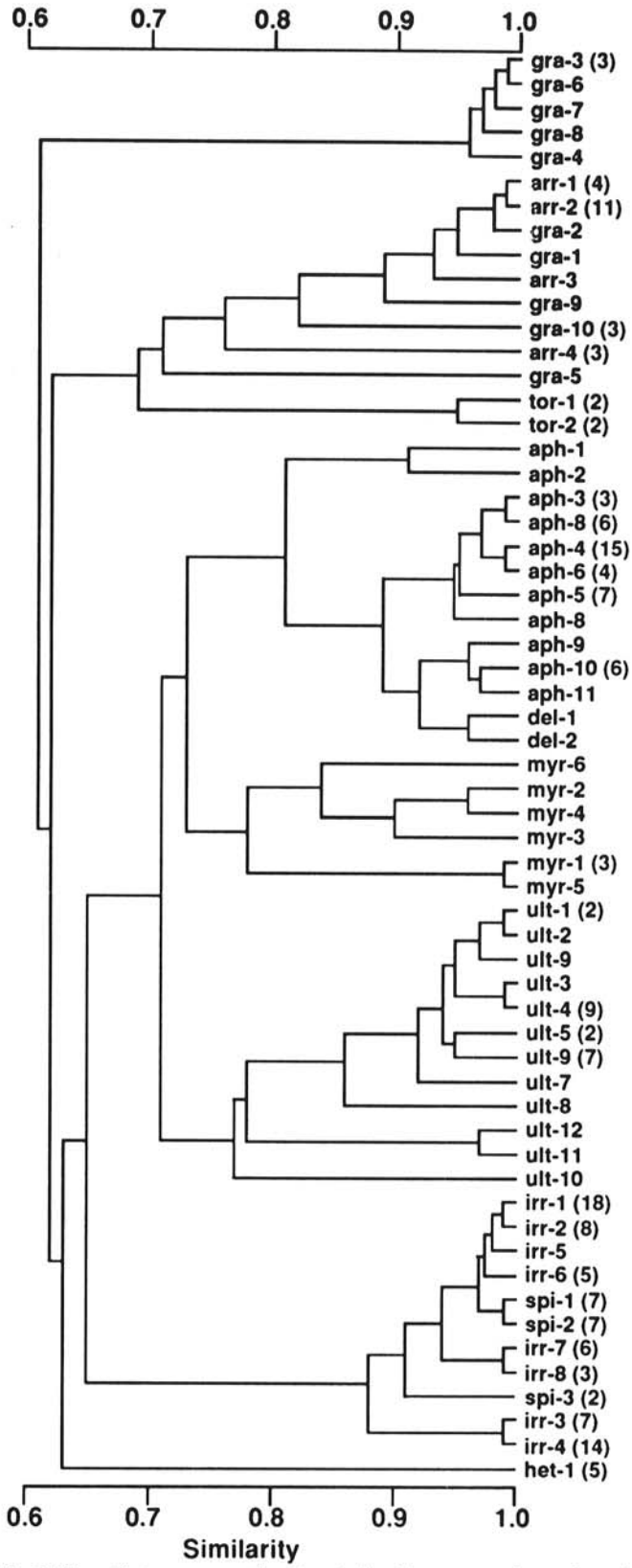

Fig. 2. Phenetic tree representing the relationships among electrophoretic phenotypes (EPs) of 10 *Pythium* species on the basis of banding patterns of 13 enzymes and produced by using the unweighted pair-group method with arithmetic average. Numbers in parentheses after an EP indicate the number of isolates with identical isozyme banding patterns (similarity coefficient = 1.0).

P. irregulare and *P. spinosum* formed tight, overlapping clusters that were indistinguishable by isozyme analysis (Fig. 3A and C). Several EPs were composed of numerous isolates, and there was clustering by geographic origin (Table 1, Figs. 2 and 3C). All of the Japanese isolates of *P. irregulare* were in two EPs (irr-4 and irr-5) that branched off of the *P. spinosum-P. irregulare* complex (Fig. 2). There was no evidence for host specialization within these two EPs, as isolates were collected from several plant families. The other large EP in *P. irregulare* (irr-1) contained 18 members from throughout North America, again with no evidence of host specialization. The only evidence for host specialization in this species was in irr-8 and irr-9, two multiple member groups from alfalfa in California and wheat in Oklahoma, respectively. However, it is equally likely that these two EPs reflect geographic origin because they were the only isolates received from these two locations. Interestingly, irr-8 contained two isolates from ginseng in Korea and three isolates from soybean in Kentucky. *P. spinosum* showed similar trends in geographic origin with the two largest EPs (spi-1 and spi-2) originating from rice and sugarcane in Louisiana, whereas spi-3 (from ginseng in Korea) was more distant from the other two *P. spinosum* groups (Figs. 2 and 3C).

Isolates of *P. ultimum* were from diverse hosts and geographic regions, yet 83% were tightly clustered (Table 1, Fig. 3A and E). EP ult-4 was composed of nine isolates, all from Japan, but isolated from diverse host plants. Seven isolates from soybean in Kentucky were the sole members of ult-9. Two isolates of *P. ultimum* from Texas, 20-2 and 20-3, did not produce oospores and are the only members of ult-7 and ult-8, respectively. There was intraspecific variation among the isolates from Georgia and Washington.

There was evidence for clustering by geographic origin in *P. aphanidermatum,* although aph-6 contained isolates from Japan, Illinois, North Carolina, and Kentucky (Table 1, Fig. 3D). As with *P. irregulare* and *P. spinosum,* no evidence was shown for clustering by host plant. EPs aph-7 and aph-13, as well as aph-4 and aph-6, appeared in identical clusters on the two-dimensional plots. However, when other dimensions (eigenvectors) were included, these pairs of EPs could be resolved (data not shown). The collection of *P. aphanidermatum* was more diverse than that of *P. spinosum-P. irregulare* (Fig. 3A). Of the 50 isolates of *P. aphanidermatum* included in this study, approximately 87% were clustered in several EPs, although single isolates from Florida and Ohio were phenotypically more diverse (Table 1, Figs. 2 and 3D).

Isolates of *P. myriotylum* clustered independently from but adjacent to the *P. aphanidermatum* cluster (Figs. 2 and 3D). Although the collection for this species was smaller than the others, it contained isolates from diverse locations and hosts, and genetic diversity was evident.

P. graminicola was more diverse than *P. arrhenomanes,* although the two species exhibited greater intraspecific diversity than any of the other species (Figs. 2 and 3B). EP arr-2, the largest in *P. arrhenomanes,* was composed of 11 isolates, including the type species of *P. arrhenomanes,* 12531, one isolate of *P. graminicola,* 13-6, and isolates from sugarcane, wheat, and corn from diverse locations (Table 1). The remainder of the EPs in *P. arrhenomanes* were clustered by both host and geographic origin. EP arr-4, comprising three wheat isolates from Oklahoma, was distant from the other EPs, including arr-2, which contained another wheat isolate from Oklahoma (Figs. 2 and 3B). No EP was dominant in *P. graminicola,* although gra-3, gra-4, gra-6, gra-7, and gra-8, all from Japan, were tightly clustered and distant (similarity value = 0.62) from the remainder of the EPs in this species (Fig. 3B).

The sample sizes and collection sites for *P. heterothallicum* and *P. torulosum* were inadequate for drawing meaningful con-

clusions regarding genetic diversity. However, these species were distinct from the others in that they formed their own major branches on the phenetic tree (Fig. 2).

DISCUSSION

The primary difference between this and previously published reports (2,7,18,19) was the large number of species and isolates from diverse geographic locations included in the present study. A good fit between the phenetic tree, generated by UPGMA cluster analysis, and the original data was indicated by a high value of cophenetic correlation. Some clusters were formed by isolates of morphologically similar species, e.g., *P. aphanidermatum* and *P. deliense* as well as *P. arrhenomanes* and some isolates of *P. graminicola.* Morphologically distinct species formed separate clusters. This validated the results of the clustering analysis (64). In addition, good agreement between the UPGMA cluster analysis and principal components analysis was observed.

In this study, no attempts were made to assign genotypes, i.e., allele frequencies, on the basis of isozyme banding patterns for the following reasons. First, genotype assignments require confirmation by genetic analysis. The only heterothallic species included in this study was *P. heterothallicum,* and techniques for crossing in homothallic *Pythium* species are not currently available. Second, different bands may not necessarily be attributed to allelic variations; instead, they may be caused by posttranslational modification (31) or artifacts attributed to experimental procedures. Finally, the number of bands for a given enzyme in a gel is affected by the buffer system used during electrophoresis. Nygaard et al (58) observed different numbers of bands for malate dehydrogenase and malic enzyme in two buffer systems for several *Phytophthora* species. Micales et al (52) used a buffer system that improved resolution over an earlier study (51) and detected fewer bands of glucose phosphate isomerase and isocitric dehydrogenase for species of *Peronosclerospora.*

A primary objective of this investigation was to determine whether isozyme banding patterns for *Pythium* isolates were species-specific as reported for other fungi (7,19,75). Clare et al (19) used starch gel electrophoresis to study 27 isolates of 11 *Pythium* species. Although variations in protein and isozyme banding

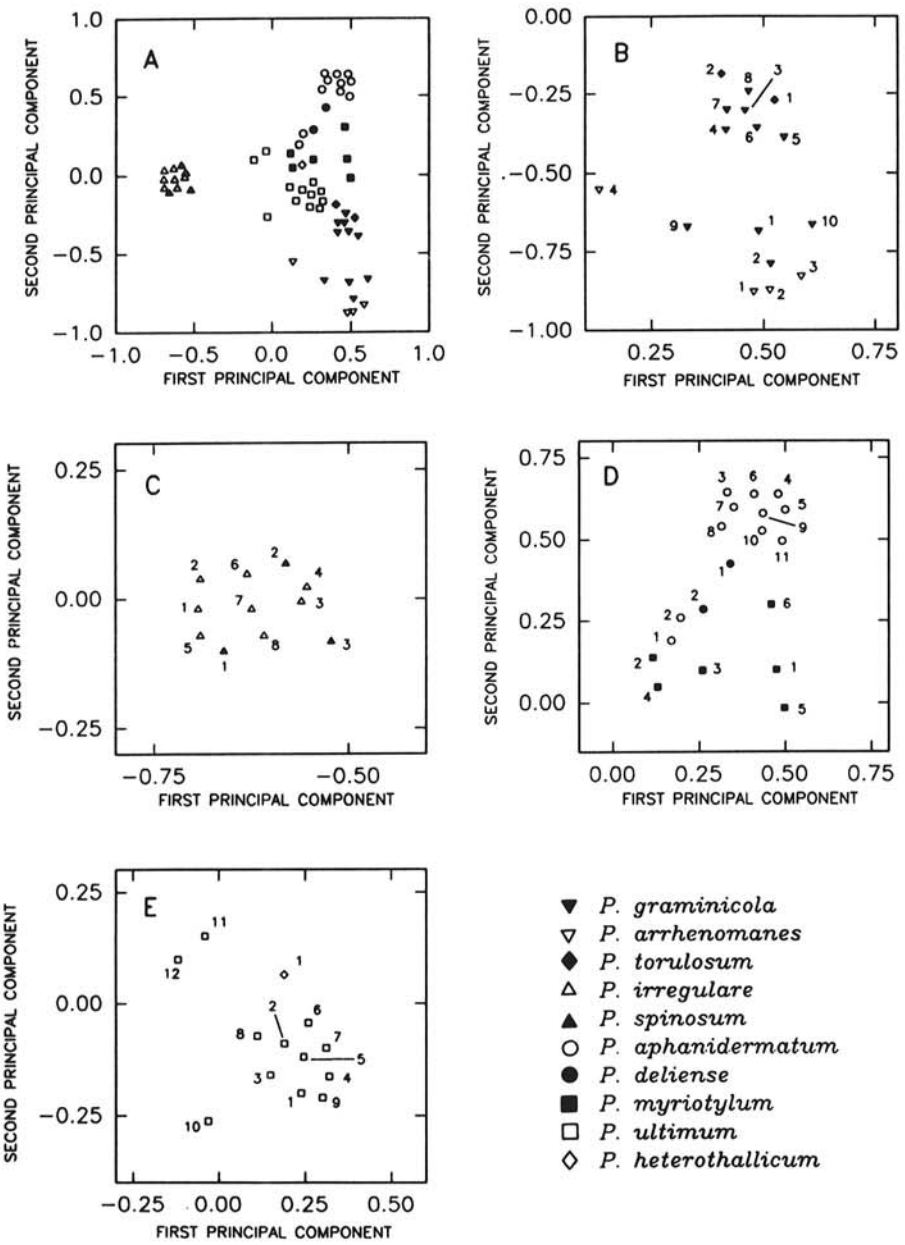

Fig. 3. Plots of the first and second principal components of the banding patterns of 13 enzymes of 10 *Pythium* species. **A,** Composite of all 10 species. **B,** *P. arrhenomanes, P. graminicola,* and *P. torulosum.* **C,** *P. irregulare* and *P. spinosum.* **D,** *P. aphanidermatum, P. deliense,* and *P. myriotylum.* **E,** *P. ultimum* and *P. heterothallicum.* The axis values in **B–E** are identical with those of **A,** with the exception of being expanded for clarity. Numbers adjacent to the symbols indicate electrophoretic phenotypes.

patterns were observed among isolates within *Pythium* species, they concluded that protein and isozyme banding patterns could be used for identification (19). In addition, eight *Pythium* species, represented by a total of 14 isolates, were distinguished by protein banding patterns in isoelectric focusing gels (2). However, our investigation with 204 isolates, from diverse geographic locations representing 10 *Pythium* species, did not confirm the earlier results. Instead, isozyme banding patterns varied considerably among isolates within some morphological species, and banding patterns for two distinct species, *P. irregulare* and *P. spinosum,* were very similar. As a result, it does not seem feasible to use isozyme banding patterns for the conclusive identification of *Pythium* species.

Variations in isozyme banding patterns exist among morphologically distant species. For example, *P. ultimum, P. irregulare,* and *P. graminicola* could be distinguished on the basis of the 13 enzymes used in this study, and, for isolates from one location, they may be separated with fewer enzymes. However, the morphological differences among these species are adequate for identification, and isozyme assays are unnecessary.

The isozyme analyses used in this study did not distinguish some species that are morphologically similar, such as *P. aphanidermatum* and *P. deliense* or *P. arrhenomanes* and *P. graminicola.* Apparently, these 13 enzymes do not reflect the minor morphological differences used to separate these species. Our results are in contrast to the findings of Clare et al (19) and Adaskaveg et al (2) in which *P. aphanidermatum* and *P. deliense* were clearly separable. This may be a result of the small sample sizes for *P. deliense* in our study as well as in those of the aforementioned authors. It is apparent from our results that there are isozyme overlaps between species. Thus, in a small sample, if one unknowingly chooses isolates from the shared portion of the respective clusters, biochemical traits would not be informative. Discrepancies also may be caused by the ambiguity in delineation of narrowly defined species. For instance, *P. deliense* differs from *P. aphanidermatum* in having oogonial stalks that bend towards the antheridium and slender antheridia (78). These subtle differences are not easily recognized and cause confusion among mycologists. Isozyme comparisons did not provide support for maintaining *P. aphanidermatum* and *P. deliense* as separate species. However, additional comparisons are needed with confirmed isolates.

P. spinosum produces highly ornamented oogonia, and *P. irregulare* produces variable numbers of oogonia with a few protuberances (78). Mycologists generally consider these to be easily separable, distinct species. However, Hendrix and Papa (35) considered them to be a single species complex along with *P. acanthicum* Drechs. and *P. mamillatum* Meurs. In fact, the morphological traits used to separate *P. spinosum* and *P. irregulare* are variable and show considerable overlap. Our results showed that *P. irregulare* and *P. spinosum* cannot be differentiated on the basis of isozyme profiles and indicated that these two species are more closely related than was previously thought.

Morphological features used to separate *P. arrhenomanes* and *P. graminicola* are size of oogonia and number of antheridia per oogonium, with *P. arrhenomanes* having larger oogonia and more antheridia per oogonium (78). Isolates within species vary in size of oogonia and in number of antheridia per oogonium and form a continuum. The ranges of the continua overlap; many isolates fall in the overlapping range and may be placed in either species. Intraspecific variations and overlapping of species clusters also were apparent in the isozyme analysis. Four isolates each of *P. arrhenomanes* and *P. graminicola* could not be distinguished by restriction mapping of the internally transcribed spacer region and the small subunit of ribosomal DNA (15). However, single isolates of each species were the only isolates of five *Pythium* species to show intraspecific variation (15). In contrast, a low level of similarity was detected between mitochondrial DNA of single isolates of *P. arrhenomanes* and *P. graminicola* (46). Further study of this species complex is needed to determine the causes of the high level of variability in many traits and whether the maintenance of distinct species is warranted.

The inability of isozyme banding patterns to distinguish *Pythium* species was largely attributed to variation in patterns among isolates within morphological species. *P. irregulare* contained the largest number of isolates with a wide geographic distribution, yet only a small amount of variation was observed within this species. *P. aphanidermatum* and *P. graminicola* exhibited higher degrees of variation, with *P. graminicola* showing more intraspecific variability than interspecific variability between *P. arrhenomanes* and other *Pythium* species. Similar differences in isozyme variation within and among species were reported for *Phytophthora* by Oudemans et al (59).

Shahzad et al (69) recently described a procedure based on calculated morphological ratios of oogonia and oospores that increased the precision for separating *Pythium* species. Although they included only three isolates for each species, their approach, in combination with biochemical and molecular analyses, may provide conclusive evidence for maintaining or modifying the taxonomic schemes currently in use for this species.

Aside from the taxonomic implications of our results, our data provide information relative to genetic diversity and evolutionary biology of *Pythium* species. Four major factors are thought to contribute to genetic diversity: population size, mutation, migration, and selection (6,55,65). We assume that selection is the dominant factor in the genus *Pythium* for the following reasons. Sexual reproduction may result in new recombinants and determines the rate and extent by which new mutations and recombinants are propagated and disseminated in a population. However, in an infinitely large population (greater than 10^4), the role of sexual reproduction is diminished because of the contribution of linkage disequilibrium, which results in a low but continuous source of genetic diversity in a clonal population (47). We assume that rates of mutation are equivalent within the genus, and migration should be an insignificant contributor to diversity. The possible role of different ploidy levels as a contributing factor to genetic diversity (79) among *Pythium* species remains an open question (35). Dick (22) provided evidence for autopolyploidy in the genus and suggested that the organism is capable of maintaining a high degree of heterozygosity, i.e., genetic diversity, in a clonal population. Mechanisms of genetic recombination notwithstanding, the balance between mutation and selection determines the degree of genetic diversity (55). Therefore, we are interested in the role of selection and life history traits as factors responsible for differences in genetic diversity among *Pythium* species.

Butlin (13) and Maynard Smith (48) described the use of phylogenies and population genetics theory for gaining insight into life histories and ecological forces. Parasitism is thought to require greater specialization than saprotrophy (44), particularly in the case of host specificity, and is usually associated with a narrower genetic base (33,41). Exposure of geographically isolated populations to nonpreferred hosts or a variable array of hosts would result in new selection pressures and added genetic diversity (13,50,60). This process would lead ultimately to fragmented or new species. Populations also may diverge in response to selection pressures that are not dependent on host species.

Selection and fixation, processes that lead to diminished genetic diversity, are known to occur in highly specialized host-parasite systems in which the host is the dominant selection force (8,42,80). Alexander et al (3) showed how rapidly this process can occur with their finding that virulence changes and genetic fixation occurred within a population of *Uromyces appendiculatus* (Pers.:Pers.) Unger within the lifetime of a single host individual. Thus, a high degree of genetic diversity in *Pythium* species suggests a variable environment and excludes a specific host-parasite relationship as the primary selection pressure. Cooke and Whipps (20) proposed that diversity-limiting factors operate successively and differentially at various times during the life history of a pathogen. They described the concept of nutritional evolution, progressing from saprotrophy to necrotrophy to biotrophy, in which an organism may spend different portions of its life history in any or all of these categories, each with its own set of selection pressures. The genus *Pythium* exhibits a high level of niche diversity (34). Some species, such as *P. ultimum* and *P. aphani-*

dermatum, are highly competitive colonizers of plant debris as well as being pathogenic on a wide range of plants (27,45,73). In contrast, *P. arrhenomanes* is not readily isolated from soil and is closely associated with a specific plant family, the Graminaceae (34,35). Clearly, the relative contributions of saprophytic, parasitic, and pathogenic life history traits differ among *Pythium* species. As a result, comparisons of differences in genetic diversity among geographically isolated populations within the genus can be informative.

We interpret our results within this theoretical framework. The low level of genetic diversity in *P. irregulare* and *P. spinosum* suggests that these two species have been subjected to selection pressures that are consistent with host specificity. Yet, these species are associated with diverse plant families (78). It is possible that host selection pressures operate at the parasitic and pathogenic levels. Perhaps *P. irregulare* and *P. spinosum* are facultative parasites that are constrained by specific requirements for host exudates or insoluble materials, such as growth factors, amino acids, or specific carbohydrates, that are available in immature root tissues of many plants, but they are generally incapable of overcoming the defense mechanisms of intact, mature plant roots. This would serve as a primary selective force in the evolutionary development of these species. In this regard, Nelson (56) showed that specific compounds in root and seed exudates are required for activation and germination of *Pythium* propagules. Furthermore, Deacon and his colleagues (38,40) demonstrated clear differences in nutritional requirements between mycoparasitic and plant pathogenic *Pythium* species. They reported that several compounds in root exudates elicited one or more stages of the prepenetration sequence of events in *P. aphanidermatum* but that only glutamic acid and aspartic acid elicited all stages.

It seems logical that genetic diversity would be greater in more advanced pathogenic *Pythium* species such as *P. graminicola* and *P. arrhenomanes.* These species have developed more specific relationships with a wide array of graminaceous hosts, in some cases to the cultivar level (1,26,61,66). Such a scenario is equivalent to the frequency-dependent selection with minority advantage of Nei (55), in which a newly arisen, more fit variant would predominate in a specific niche, i.e., host. An ecological selection mechanism that could function to maintain such a population structure within the genus *Pythium* was described by Mitchell and Deacon (54). They showed that root exudates have differential effects on zoospores of compatible (*P. graminicola* and *P. arrhenomanes*) versus incompatible (*P. aphanidermatum* and *P. ultimum*) species and suggested that this is a result of a long evolutionary association of the former two with graminaceous host plants.

With the exception of isolate 1421 from sweetpotato, we provide evidence for clustering by geographic origin in the Japanese isolates of *P. graminicola.* This may be explained by the fact that cropping systems and the array of graminaceous hosts would vary among locales, and long-term exposure to different host populations would provide selection pressures that lead to genetic diversity among populations in different regions. The evidence for clustering by host is inconclusive in this species since all of the isolates from some locations are from the same host. Most isolates of *P. arrhenomanes* show a high degree of similarity, although they are from diverse locations in North America, Puerto Rico, Hawaii, and Australia.

The Japanese collections of *P. aphanidermatum* and *P. ultimum* were less diverse than those from various locations in North America. Host of origin did not appear to affect clustering; however, we did not conduct comparative pathogenicity tests on different hosts. McCarter and Littrell (49) did not find evidence for host specificity but demonstrated that isolates of *P. aphanidermatum* varied markedly in pathogenicity on susceptible hosts. Another well-documented attribute of *P. ultimum* and *P. aphanidermatum* is their ability to rapidly colonize fresh plant debris and to produce large amounts of inoculum for subsequent infection cycles (27,45,73). It is likely that these two species are approaching a dynamic equilibrium in genetic diversity because of the opposing selection pressures exerted by life histories that include both saprophytic and parasitic phases.

Assuming that the genus evolved from a common ancestor, evolutionary theory predicts that we would not expect to find a wide range of intra- and interspecific genetic diversity (53) unless each species has a different mode of pathogenesis or occupies different edaphic niches. Taken all together, clustering by geographic origin, as opposed to clustering by host, suggests that this genus is undergoing allopatric speciation rather than sympatric, host-dominated speciation as proposed for *Phytophthora* (28–30). The degree of genetic diversity is probably a function of the relative importance of saprophytism versus parasitism in the life history of each species.

As stated by Budd and Mishler (10) and Maynard Smith (48), it is important to consider species as lineages and to look for life history traits, selection forces, and genetic mechanisms that are causing them to remain coherent or fragmented over time and space. Previously, relationships among *Pythium* species could be based only on differences in asexual and sexual reproductive structures. Now analyses of isozymes and other biochemical traits are providing information that will allow us to infer lineages with greater accuracy in the genus *Pythium.* Future research should be designed to examine patterns of evolution and to discover the forces that cause these patterns, including life history traits, mechanisms of genetic recombination, and mutation, selection, and gene flow.

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