Isozyme Variability Among Isolates of *Phytophthora megasperma*

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ABSTRACT

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Isozyme analysis was used to study over 300 field isolates of the species *Phytophthora*. Interspecific diversity for isozyme banding was identified among the four species assayed, *P. megasperma, P. cactorum, P. cryptogea*, and *P. parasitica* var. *nicotianae*. Intraspecific isozyme diversity was present among isolates of *P. megasperma* that were recovered from 10 host plant species and from many geographical origins; these isolates represented many morphological types and included the three formae speciales of *P. megasperma* (f. sp. *glycinea*, f. sp. *medicaginis*, and f. sp. *trifolii*). Isozyme analysis separated *P. megasperma* isolates into at least six intraspecific groups: the three *P. megasperma* formae speciales—*glycinea, medicaginis*, and *trifolii*—and the apple/apricot/cherry, Douglas fir, and broad host-range groups. These groups parallel those previously reported in the literature. The broad host-range group was further divided into at least three subgroups by isozyme analysis. All of the 224 isolates of *P. m.* f. sp. *glycinea* and 41 of the 45 isolates of *P. m.* f. sp. *medicaginis* had identical isozyme banding patterns within their respective formae speciales. Isolates of *P. m. glycinea* and *P. m. medicaginis* baited from the same geographical sites had no intra-formae speciales variant or intermediate banding patterns. This, and evidence previously reported, lead us to conclude that the two formae speciales, *P. m. medicaginis* and *P. m. glycinea*, are not naturally intermating populations and that they exist as two biological species distinct from other *P. megasperma* isolates.

Phytophthora megasperma Drechs. is a taxon of oomycetous fungi whose members are destructive pathogens of many hosts; these include alfalfa, arrowleaf clover, chickpea, deciduous fruit trees, Douglas fir, and soybean. This species is homothallic and has internally proliferating, nonpapillate sporangia, and antheridia that occur predominantly in the paragynous position (44). Atypical heterothallic isolates with predominantly amphygynous antheridia also occur, as well as isolates that do not produce sexual structures (2,40). Because the members of this species have diverse characters (20), the taxonomy of this species has been the subject of intense scrutiny. Several taxonomic subdivisions based on either oogonial size (35) or host specialization (27,39) have been proposed, but neither of these criteria is adequate because many isolates of *P. megasperma* have either intermediate or variable oogonial size (19), overlapping host ranges (23,31,45), or both (15). Genetic diversity among isolates of *P. megasperma* is further demonstrated by variations in numerous physiological and biochemical properties, as revealed by the analysis of total proteins, nitrate utilization, sensitivity to fungicides, chromosome numbers, DNA content, restriction fragment length polymorphisms of mitochondrial DNA (mtDNA), and mtDNA size (4,11,12,15–18,20,22,23,31).

Isozyme analysis is another means of identifying genetic diversity among field isolates. In many phyla, including the fungi, isozyme analysis has been used for several purposes: to clarify taxonomic relationships among organisms that are presumably closely related but that are difficult or impossible to distinguish morphologically (3,7,29,32,33), to study phylogenetic relationships among organisms (5,30,32,34), and to study the genetics of organisms within the same biological species (1,5,8,29,34,36,42). Isozymic techniques for isolate characterization offer several advantages over techniques for total protein visualization. Isozyme banding patterns are less complex than total protein patterns and are easier to differentiate and interpret. Also, isozyme analyses provide information about protein function, whereas other electrophoretic techniques only separate proteins on the basis of physical properties.

The need for the application of additional biochemical methods such as isozyme analysis to clarify taxonomic relationships within the problematically defined *Phytophthora* species, e.g., *P. megasperma*, has been suggested (6,31). The objective of this study was to look for interspecific differences and intraspecific diversity among *P. megasperma* isolates from various hosts and geographical locations using isozyme analysis, and to compare our findings to those derived by other methods (4,11,12,15–18, 20,22,23,31). The more than 300 field isolates used in these studies represent: four *Phytophthora* species (*P. megasperma, P. cactorum* (Leb. & Cohn) Schroet., *P. cryptogea* Pethyb. & Laff., *P. parasitica* var. *nicotianae* (Breda de Haan) Tucker); three formae speciales of *P. megasperma* (f. sp. *glycinea* Kuan & Erwin (from soybean), f. sp. *medicaginis* Kuan & Erwin (from alfalfa), and f. sp. *trifolii* Pratt (from arrowleaf clover)]; 17 races of *P. m. glycinea*; and many morphological types of *P. megasperma* isolated from 10 host plant species and from many geographical origins. Additionally, we report the first isolation of *P. m. medicaginis* and *P. m. glycinea* from the same site. A portion of this study has appeared in an abstract (14).

MATERIALS AND METHODS

Isolates. The *Phytophthora* isolates obtained from other researchers are listed in Tables 1–3. *P. m. glycinea* race designations given in Table 3 were provided by the scientists who supplied the isolates. The 150 Wisconsin isolates of *P. m. glycinea* (Table 3) were recovered from two Racine County sites by direct or indirect isolation methods and were not typed to race. Direct isolations from soybeans grown in fields infected by *P. m. glycinea* were performed as described by Tooley et al (43). Indirect isolation of *P. m. glycinea* was accomplished by the soil-baiting technique described by Canaday and Schmitthenner (9). Six *Pythium* spp. isolates recovered by the indirect isolation method were kept for

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isozyme comparison to *Phytophthora* spp. isolates. In another group of isolations, both *P. m. medicaginis* and *P. m. glycinea* isolates were recovered from soil collected from each of three other Racine County sites. These three sites were chosen because they had histories of soybean damage by *P. m. glycinea* and had been managed in an alfalfa-soybean-corn crop rotation system. All three sites were in soybean cultivation when the soil samples were collected.

Isolation of *P. megasperma* with alfalfa or soybean baits. Nalgene beakers (250-ml capacity) were filled with 50 ml of sterilized vermiculite over which 100 ml of infested soil was placed. The soil was thoroughly saturated with water and any excess removed. Fifty seeds of *Phytophthora*-susceptible alfalfa (*Medicago sativa* L. 'Saranac') were planted, covered with about 1 cm of sterilized vermiculite, and watered daily to keep the vermiculite surface moist but not wet. Beakers were incubated at ambient temperature (about 22 C) and window light. After seedling emergence, water was added to keep the vermiculite and lower hypocotyls submerged. Alfalfa seedlings began to damp-off 2–3 days after flooding. Diseased seedlings were removed, rinsed, surface-disinfested in 0.5% sodium hypochlorite for 10 sec, rinsed in sterilized distilled water, allowed to air-dry, and

TABLE 1. *Phytophthora* species and isolate sources, codes, hosts, and geographical origins

Phytophthora species	Abbreviation	Source[a]	Isolate code	Host	Geographical origin[b]
P. megasperma f. sp. *glycinea*	Pmg	Grau	1-16[c]	Soybean	WI
P. m. medicaginis	Pmm	Miller	5b4[c]	Alfalfa	MN
P. m. trifolii	Pmt	Pratt	102	Clover	MS
P. megasperma	Pm1	Hamm	C-17	Douglas fir	OR
			336	Douglas fir	WA
P. megasperma	Pm2	Hamm	B3A	Douglas fir	OR
			345	Douglas fir	OR
P. cactorum	Pcac	Mitchell	Pc	Apple	WI
		Jeffers	NY188	Apple	NY
			NY193	Strawberry	OH
			NY195[d]	Apple	Quebec, Canada
			NY285	Nonagricultural soil	NY
P. cactorum	Pcac	Parke	Pc-g-1[d]	Ginseng	WI
P. cryptogea	Pcry	Hamm	317	Douglas fir	OR
P. parasitica var. *nicotianae*	Ppn	Apple	1452[c]	Tobacco	KY

[a]Scientist who provided isolate.
[b]State in the United States unless otherwise indicated.
[c]Isolate also discussed in Förster et al (16–18).
[d]Isolate also discussed in Bielenin et al (4) and Pc-g-1 was named as NY 230 (4).

TABLE 2. *Phytophthora megasperma* isolate source, code, geographical origin, host, total protein pattern group, and isozyme pattern group designation

Original source	Isolate code	Geographical origin[a]	Host	Total soluble protein group[b]	Isozyme pattern group[c]
Lavollette	75-35	IN	Soybean	SOY	I
	5-58	IN	Soybean	SOY	I
Grau	1-16	WI	Soybean	SOY[d]	I
Mircetich	19-2-1	CA	Apple	AC	II
Christen	WI	WA	Alfalfa	ALF	III
Hamm	S1	OR	Alfalfa	ALF	III
	PC3	OR	Alfalfa	ALF	III
Miller	5b4[e]	MN	Alfalfa	ALF	III
Pratt	113	MS	Clover	CLO	IV
Hamm	B3A	OR	Douglas fir	DF	V
Stack	75	NY	White cockle	DF	V
Mircetich	6-1-9(K1)	CA	Cherry	BHR	VI
	19-2-1	CA	Grape soil[f]	BHR	VI
	24-3-8	CA	Kiwi	BHR	VI
Wilcox	275C-1	CA	Cherry	BHR	VI
Brasier	P128[g]	Gr. Britain	Brassica	BHR	VII
Hamm	510	OR	Alfalfa	BHR	VII
Jeffers	NY029	NY	Apple	BHR	VIII
	NY055	NY	Apple	BHR	VIII
	NY088	NY	Apple	BHR	VIII
Hamm	336	WA	Douglas fir	BHR	IX

[a]State in the United States unless otherwise indicated.
[b]Protein group based on total soluble electrophoretic proteins (SOY = soybean, AC = apple/apricot/cherry, ALF = alfalfa, CLO = clover, DF = Douglas fir, BHR = broad host range) as determined by Hansen et al (20).
[c]Isozyme pattern group designation determined using malate dehydrogenase, phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, diaphorase, and aconitase (isozyme patterns are shown in Fig. 3).
[d]E. M. Hansen, *personal communication.*
[e]Miller 5b4 is a single zoospore subculture of Maxwell 5b tested in Hansen et al (20).
[f]Isolate baited from a grape vineyard soil.
[g]Brasier, P128 = CMI 56348.

plated onto PAR medium amended with the fungicide PCNB (25). Sporangia were observed on the tissue after 2 days, at which time hyphal tips were transferred to PAR medium without PCNB. Eight cycles of alfalfa recropping in the same soil samples were required before *P. m. medicaginis* was consistently recovered. Prior to this cycle, seedlings were overwhelmingly colonized by *Pythium* spp., and *Phytophthora* could not be isolated.

Another subset of soil samples was used for the isolation of *P. m. glycinea*. The *Phytophthora*-susceptible soybean (*Glycine max* L. (Merr.) 'McCall,' no race-specific resistance genes identified) was planted twice. Because *Phytophthora* was isolated infrequently, soybean cotyledons were used as baits. Soybean seeds were germinated for 3 days in sterilized, dampened, and rolled paper towels. The cotyledons were removed, surface-disinfested, and aseptically punctured several times. The soil surface was slightly flooded, and wounded cotyledons were immediately floated over the soil surface. Two to 3 days later, cotyledons with red-brown lesions associated with the wound sites were rinsed, surface-disinfested, sectioned into quarters through the lesion, and plated on PAR medium with PCNB. Subsequently, hyphae were transferred to PAR medium without PCNB and *P. m. glycinea* was efficiently recovered.

Isolates were cultured on V8 agar (200 ml of V-8 juice, 2.5 g of $CaCO_3$, 15 g of agar, and 800 ml of distilled water) before pathogenicity testing and were maintained on PAR medium or cornmeal agar (Difco, Detroit, MI) slant vials at 12 C. These isolates of *P. megasperma* were tested for pathogenicity to soybean and alfalfa to determine whether they were *P. m. glycinea* or *P. m. medicaginis*. Pathogenicity to soybean was determined with the hypocotyl-inoculation test as modified by Tooley et al (43) with cultivars McCall or NK1492 (no race-specific resistance genes identified). Isolates were tested for pathogenicity to *Phytophthora*-susceptible alfalfa cultivars by one of two methods. Pathogenicity to alfalfa (cultivar Vernal) was tested with the postemergence damping-off seedling test (24) and pathogenicity to alfalfa seedlings (cultivar Saranac) by a more rapid method used originally for testing pathogenicity of *Aphanomyces euteiches* to alfalfa seedlings (13). All pathogenicity tests were conducted in a growth chamber at 24 C with a 12-hr photoperiod. After 2 wk, isolates were scored as pathogenic to alfalfa if damping-off occurred or pathogenic to soybean if, after 5 days, soybean hypocotyls had collapsed due to a progressing lesion. Uninoculated alfalfa and wounded, uninoculated soybeans were controls in all tests.

TABLE 3. Geographical origin, race, and number of isolates of *Phytophthora megasperma* f. sp. *glycinea* and *P. megasperma* f. sp. *medicaginis* examined for isozyme diversity with the AC buffer system

Formae speciales and races	Geographical origin[a] and number of isolates	Number of isolates
f. sp. *glycinea*		
race 1	KS (1), MD (1), MS (10), OH (1), WI (2)	15
race 2	IN (1), MS (2)	3
race 3	NE (1), WI (4)	5
race 4	KS (1), MS (1), WI (2)	4
race 5	IN (1), MS (1), WI (1)	3
race 6	KS (1)	1
race 7	IN (1), OH (1), WI (3)	5
race 8	IN (1), OH (1), WI (1)	3
race 9	IN (1), OH (1), WI (4)	6
race 10	IN (1), MS (1)	2
race 11	Ontario, Canada (2)	2
race 12	IN (1)	1
race 13	IN (1), MS (1)	2
race 14	IN (1), MS (1)	2
race 15	IN (1)	1
race 17	MS (1)	1
race 19	WI (1)	1
Not typed to race	MD (2), WI (150)	152
Total		209
f. sp. *medicaginis*	CA (9), OH (2), MN (1), MS (1), NY (10), WI (5), Australia (2)	30

[a]State in the United States unless otherwise indicated, and number of isolates indicated in parenthesis.

Isolates of *P. m. glycinea* were characterized to race by determining their reaction on selected soybean genotypes after wound-inoculating the hypocotyls of soybean cultivars (43) with the following race-specific resistance genes: Rps_1 (cv. AP 200), Rps_{1c} (cv. Corsoy 79), and Rps_6 (cv. LL 1771). These genes confer resistance to races 1 and 2, races 1–3 and 6–9, and races 1–4, respectively (38). The races 1, 3, and 4 were expected to be most prevalent (43, and C. R. Grau, *personal communication*). Isolates of *P. m. glycinea* were categorized as race 1 or 2 (indistinguishable by these cultivars), 3, 4, 6–9, or as belonging to some other race (i.e., race 5 or greater than 9). All pathogenicity and race-typing tests were repeated once.

Isozyme analysis by gel electrophoresis. Actively growing mycelial plugs cut from colonies growing on V8 agar were transferred to 125-ml Erlenmeyer flasks containing 25 ml of clarified V8 juice broth medium (200 ml of Campbell's V-8 juice, 800 ml of distilled water, and 2.5 g of $CaCO_3$, clarified by low-speed centrifugation). Cultures were grown for 5–7 days at ambient temperature (about 22 C) and light. Mycelium was collected on No. 1 Whatman filter paper by vacuum filtration and ground in distilled water or in 10X RW gel buffer (37). The procedure for horizontal starch gel electrophoresis described by O'Malley et al (37) was followed, except that the hydrolyzed potato starch was obtained from Connaught Labs, Inc. (Willowdale, Ontario, Canada) or from Sigma Chemical Co. (St. Louis, MO). Results were usually recorded as full-scale line drawings on grid paper, and less frequently were photographically or photostatically reproduced. Each isozyme band was given a designation that represented the enzyme abbreviation and the percent migration relative to the most common anodally moving isozyme band in the experiment (e.g., MDH 100 = the most common anodal isozyme band of malate dehydrogenase). Extracts from 20–30 isolates were run on a gel, and extracts from *P. m. medicaginis* 5b4 and *P. m. glycinea* 1-16 were included on each gel as standards.

Starch from Connaught Labs, Inc., and distilled water extracts of hyphae initially were used with 24 enzymes and two buffer systems to evaluate the isolates listed in Table 1. The buffers tested were the AC (0.04 M citric acid adjusted to pH 6.1 with *N*-(3-aminopropyl)-morpholine and diluted 10-fold for use as the gel buffer [10,37]), and the RW (citrate-lithium-borate buffer, pH 8.5 [37,41]). The histochemical stain modifications made by O'Malley et al (37) for the enzymes were employed. Eleven enzyme stains consistently resolved isozymes of *P. m. medicaginis* and *P. m. glycinea*. These enzymes were acid phosphatase (ACP, EC 3.1.3.2), aconitase (ACO, EC 4.2.1.3), diaphorase (DIA, EC 1.6.4.3), fumarase (FUM, EC 4.2.1.2), glucose-6-phosphate dehydrogenase (G6P, EC 1.1.1.49), isocitrate dehydrogenase (IDH, EC 1.1.1.42), lactate dehydrogenase (LDH, EC 1.1.1.27), malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.40), 6-phosphogluconate dehydrogenase (6PG, EC 1.1.1.44), and phosphoglucose isomerase (PGI, EC 5.3.1.9). There was inconsistent or no resolution of the isozymes of aspartate amino transferase (EC 2.6.1.1), alcohol dehydrogenase (EC 1.1.1.1), fructose-1,6-diphosphatase (EC 3.1.3.11), glutamate dehydrogenase (EC 1.4.1.3), glutamic-pyruvic transaminase (EC 2.6.1.2), hexokinase (EC 2.7.1.1), mannitol dehydrogenase (EC 1.1.1.67), mannose-6-phosphate isomerase (EC 5.3.1.8), sorbitol dehydrogenase (EC 1.1.1.14), superoxide dismutase (EC 1.11.1.1), and xanthine dehydrogenase (EC 1.2.3.2). Therefore, for simplicity, the AC buffer system and the 11 enzymes that reliably stained *P. m. medicaginis* and isozymes of *P. m. glycinea* were used to survey the large number of isolates of *P. m. medicaginis* and *P. m. glycinea* in Table 3.

Simple matching coefficients (SMC) were calculated for each pair of isolates within a set of isolates by the formula:

$$SMC = (a + b)/n,$$

where a = the number of pairs of isozyme bands common to a pair of isolates, b = the number of pairs of isozyme bands absent in both of these isolates but present in at least one isolate of the set, and n = the number of different isozyme bands detected in the set of isolates.

Twenty-one isolates of *P. megasperma* (Table 2) were assayed for their isozyme patterns. These isolates were a subset of those that had been intensively characterized by others (4,16–18,20). Isolates were randomly coded before assay for MDH, PGI, G6P, LDH, DIA, ACP, IDH, and ME isozymes. The RW buffer system was used, as it was found to allow resolution of more LDH isozymes than the AC buffer system. Mycelium was ground in distilled water.

In another series of gels, five isolates of *P. m. medicaginis* and five of *P. m. glycinea* from each of the three Racine County soils were assayed for MDH, ME, PGI, and G6P isozymes. Mycelium was ground in 10X RW gel buffer and electrophoresis conducted with the starch from Sigma Chemical Co., using the RW buffer system.

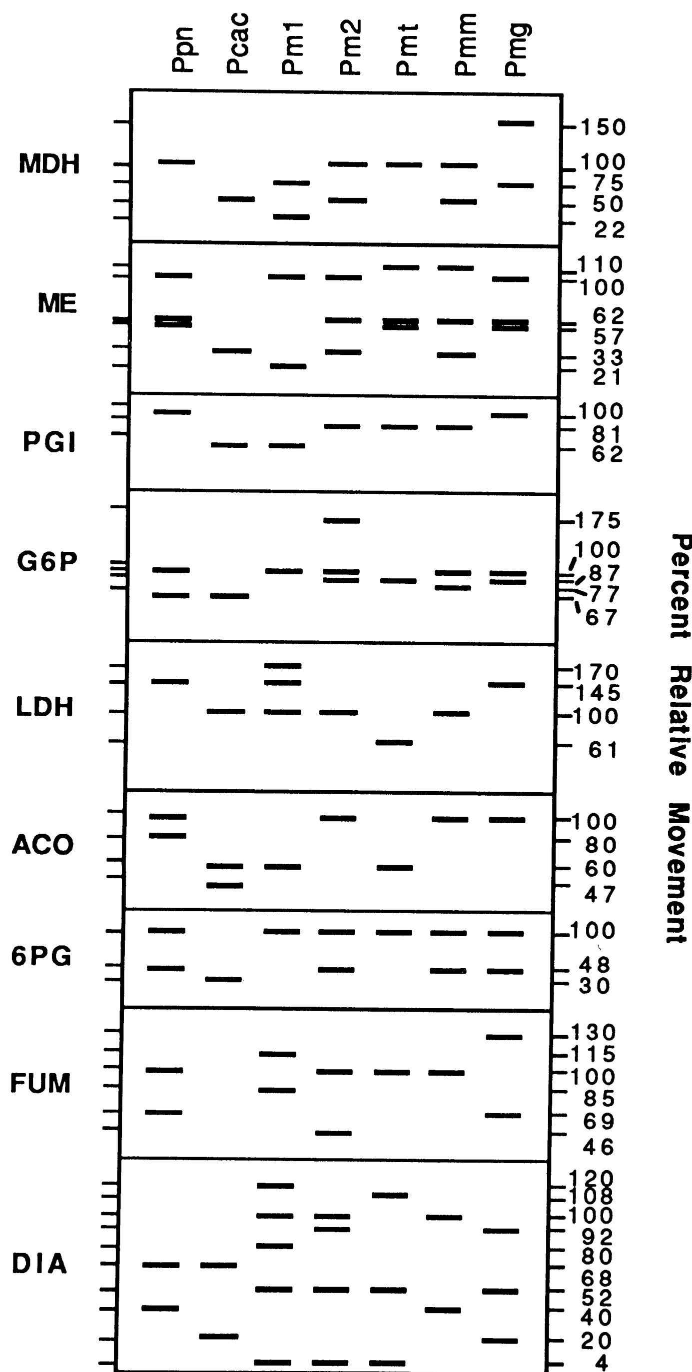

Fig. 1. Isozyme banding patterns represented as the percent movement relative to the most common, anodally migrating band for *Phytophthora parasitica* var. *nicotianae* (Ppn), *P. cactorum* (Pcac), *P. megasperma* isolates C-17 and 336, *P. megasperma* isolates B3A and 345, *P. megasperma* f. sp. *trifolii* (Pmt), *P. megasperma* f. sp. *medicaginis* (Pmm), and *P. megasperma* f. sp. *glycinea* (Pmg). Isozyme banding patterns were determined with the 0.04 M citric acid (pH 6.1) gel buffer system (AC buffer) and are given for the enzymes: malate dehydrogenase (MDH), malic enzyme (ME), phosphoglucose isomerase (PGI), glucose-6-phosphate dehydrogenase (G6P), lactate dehydrogenase (LDH), aconitase (ACO), 6-phosphogluconate dehydrogenase (6PG), fumarase (FUM), and diaphorase (DIA).

RESULTS

Interspecific and intraspecific diversity. *P. parasitica* var. *nicotianae*, *P. cactorum*, and *P. cryptogea* had isozyme patterns distinct from one another and from any of the isolates of *P. megasperma* when tested with the AC (Fig. 1) or RW (Fig. 2) buffer systems. For the *P. megasperma* isolates, unique isozyme patterns (Fig. 1) were associated with each of the three groups of host-specific isolates (*P. m. medicaginis*, *P. m. glycinea*, and *P. m. trifolii*). The four isolates of *P. megasperma* that were not host-specific had two distinct isozyme patterns, with those from *P. megasperma* C-17 and *P. megasperma* 336 (Table 1) in one group and those from *P. megasperma* B3A and *P. megasperma* 345 (Table 1) in another group. Notably, Hansen et al (20) found that isolates *P. megasperma* C-17 and *P. megasperma* 336 had electrophoretic protein patterns of the broad host-range type, while isolates *P. megasperma* B3A and *P. megasperma* 345 had the Douglas fir pattern type.

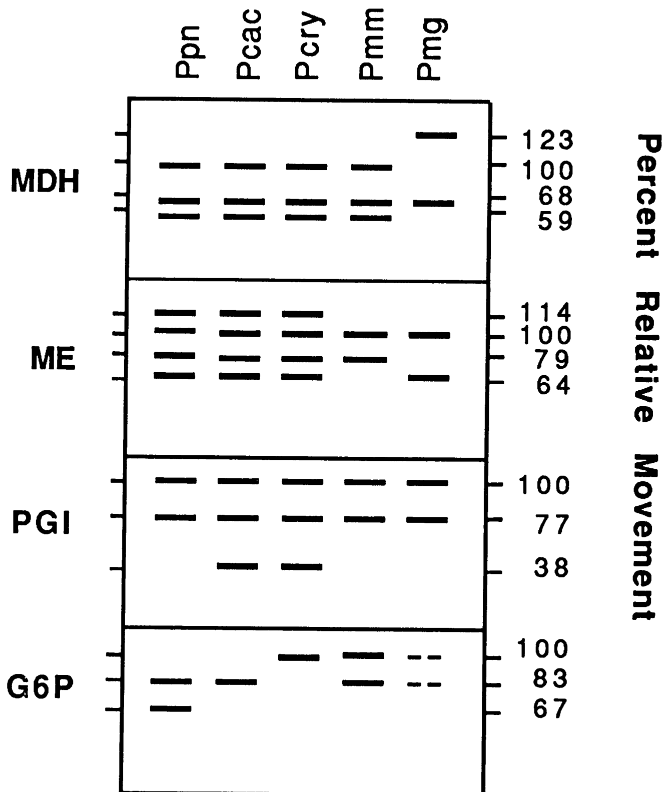

Fig. 2. Isozyme banding patterns represented as the percent movement relative to the most common, anodally migrating band for *Phytophthora parasitica* var. *nicotianae* (Ppn), *P. cactorum* (Pcac), *P. cryptogea* (Pcry), *P. megasperma* f. sp. *medicaginis* (Pmm), and *P. megasperma* f. sp. *glycinea* (Pmg). Isozyme banding patterns were determined with the citrate-lithium-borate (pH 8.5) gel buffer system (RW buffer) and are given for the enzymes: malate dehydrogenase (MDH), malic enzyme (ME), phosphoglucose isomerase (PGI), and glucose-6-phosphate dehydrogenase (G6P). Dashed lines indicate very faintly resolved bands.

The six *Pythium* spp. isolates varied greatly from one another and from *P. m. medicaginis* and *P. m. glycinea* (data not shown). Only one isozyme of IDH was visualized for any of the isolates, and this band was common to all *Pythium* spp. isolates, *P. m. medicaginis* 5b4, and *P. m. glycinea* 1-16. For the two other enzymes (LDH and G6P) assayed, the *Pythium* spp. isolates had no isozymes in common with *P. m. medicaginis* or *P. m. glycinea.*

For isolates of *P. p. nicotiana, P. cactorum,* and *P. m. medicaginis*, more isozymes were resolved for MDH, ME, and PGI in the RW buffer system with the Sigma Chemical Co. starch (Fig. 2), than with the AC system and Connaught Labs, Inc. starch (Fig. 1); whereas for G6P, detectable isozyme numbers were the same but relative positions of the bands were different. Several isozymes stained lightly and inconsistently: the single LDH isozyme of *P. p. nicotiana* and *P. m. trifolii* (Fig. 1); the ME 110 isozyme of *P. m. medicaginis* and *P. m. trifolii* (Fig. 1); the ME 114 and ME 79 isozymes of *P. p. nicotiana* (Fig. 2); and the ME 64 isozyme of *P. m. glycinea* (Fig. 2). Isozymes that consistently appeared broader and more diffuse than the others were MDH 75 of isolates *P. megasperma* 336 and *P. megasperma* C-17 (Fig. 1) and FUM 85 of *P. p. nicotiana* (Fig. 1). No FUM enzyme activity was detected for *P. cactorum* (AC buffer system) (Fig. 1), and only faint G6P activity was detected for *P. m. glycinea* (RW buffer system) (Figs. 2 and 3).

Reproducible isozyme pattern diversity was evident within *P. megasperma* (Figs. 1 and 2); therefore, numerous isolates of *P. megasperma* from many geographical origins and hosts were assayed (Tables 2 and 3). Based on MDH, PGI, G6P, LDH, DIA, and ACP isozyme patterns, each isolate could be described by one of nine different isozyme patterns, designated Groups I–IX (Fig. 3 and Table 2). Group I consisted of all isolates recovered from soybean and included those that Hansen et al (20, and E. M. Hansen, *personal communication*) had designated as having the soybean electrophoretic protein pattern. Likewise, isozyme pattern groups II, III, IV, and V corresponded to the following electrophoretic protein groups of Hansen et al (20): apple/apricot/cherry, alfalfa, clover, and Douglas fir, respectively. Isozyme pattern groups VI–IX consisted of isolates of *P. megasperma* in the broad host-range electrophoretic protein group (20). It should be noted that PGI isozyme bands in the RW buffer system were more numerous when mycelium was ground in 10X RW gel buffer than when ground in distilled water (Figs. 2 and 3,

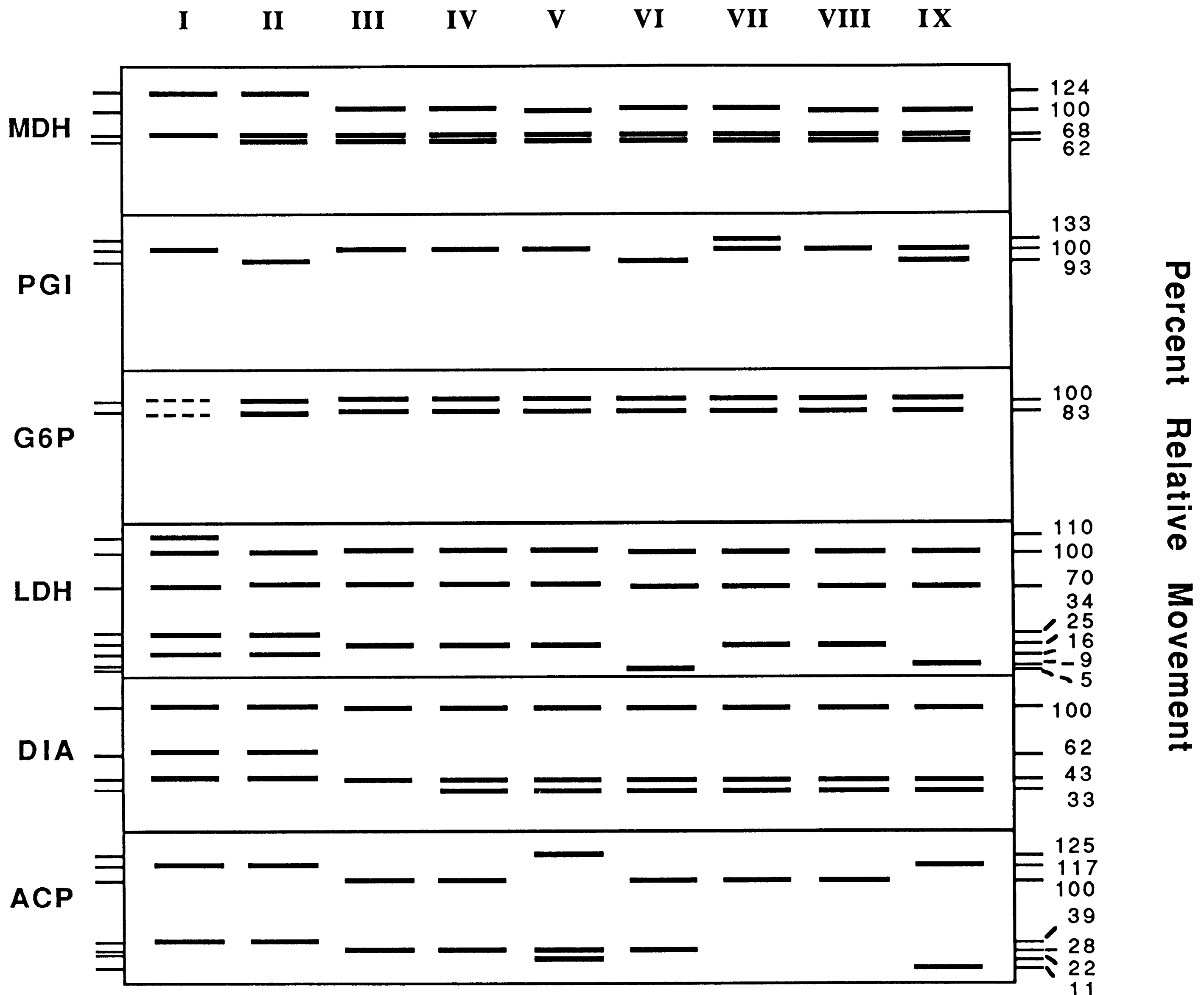

Fig. 3. Nine isozyme banding patterns (I–IX) represented as the percent movement relative to the most common, anodally migrating band for the 21 *Phytophthora megasperma* isolates listed in Table 2. Isozyme banding patterns were determined with the citrate-lithium-borate (pH 8.5) gel buffer system (RW buffer) and are given for the enzymes: malate dehydrogenase (MDH), phosphoglucose isomerase (PGI), glucose-6-phosphate dehydrogenase (G6P), lactate dehydrogenase (LDH), diaphorase (DIA), and acid phosphatase (ACP). Dashed lines indicate faintly resolved bands.

respectively).

Simple matching coefficients (SMCs) were calculated as an estimation of the similarity for inter- and intraspecific pairwise comparisons of selected *Phytophthora* isolates (Tables 4 and 5). The highest SMC among the interspecific comparisons of *P. cactorum, P. cryptogea, P. m. glycinea, P. m. medicaginis*, and *P. p. nicotianae* was between *P. cactorum* and *P. cryptogea* (SMC = 0.80, Table 4), whereas the lowest value was between *P. cryptogea* and *P. m. glycinea* (SMC = 0.30, Table 4). In the intraspecific comparisons among the nine isozyme pattern groups (Table 2) of *P. megasperma* isolates, SMCs of 0.90 or greater were obtained for pairwise comparisons between isolates of *P. m. trifolii* and *P. megasperma* in isozyme protein pattern groups VI, VII, and VIII; between isolates of *P. m. medicaginis* and *P. megasperma* in isozyme protein pattern group VIII; and between isolates of isozyme protein pattern groups VII and VIII (Table 5). An SMC of 0.81 was obtained for the comparison between *P. m. glycinea* and isolates with isozyme protein pattern group II. Other pairwise comparisons had lower SMCs than 0.81. The SMC for the comparison between *P. m. glycinea* and *P. m. medicaginis* was 0.51, one of the lowest SMCs.

When 209 isolates of *P. m. glycinea*, which represented 17 races, and 30 isolates of *P. m. medicaginis* from many geographical origins (Table 3) were assessed for isozymes in 11 enzyme systems (ACP, ACO, DIA, FUM, G6P, IDH, LDH, MDH, ME, 6PG, and PGI), all isolates of *P. m. glycinea* had isozyme patterns identical to that of *P. m. glycinea* 1-16 regardless of race or geographical origin, and 26 of the isozyme patterns of the isolates of *P. m. medicaginis* were identical to that of *P. m. medicaginis* 5b4 (Fig. 1, bands for the enzymes ACP and IDH not shown). However, four isolates of *P. m. medicaginis*, while being identical to *P. m. medicaginis* 5b4 for nine of the 11 enzymes, demonstrated the same variant isozymes for MDH and ME (data not shown). Instead of having the MDH 50 and ME 33 bands (Fig. 1), the four isolates each had more slowly migrating bands, which were designated MDH 7 and ME 6 (not shown). Two of the variant isolates of *P. m. medicaginis*, EP260 and P1316 (formerly Hamm 1130-14 [20]), originated in California and the other two, P127 and P1253, in Australia. *P. m. medicaginis* P127 and EP260 have been maintained in culture since 1955 and 1964, respectively. All isolates of *P. m. medicaginis* had mean oogonial diameters of <45 μm, and all were isolated from alfalfa except for *P. m. medicaginis* P1253. The latter was isolated from chickpea but is highly pathogenic on alfalfa (23). The average SMCs for all pairwise comparisons of the 209 isolates of *P. m. glycinea* and the 30 isolates of *P. m. medicaginis* were 1.0 and 0.99, respectively.

Isozyme patterns for *P. m. medicaginis* and *P. m. glycinea* isolates from the same sites. At least five isolates of *P. m. glycinea* and five of *P. m. medicaginis* were recovered from each of the three field soil samples from Racine County, WI. The most prevalent races of *P. m. glycinea* were races 3 and 4, with the remainder of the isolates belonging to race 1 or 2, or other races. The isolates identified as race 1 or 2 were most probably race 1, because race 2 isolates have not yet been identified in Wisconsin (C. R. Grau, *personal communication*). No other isolates of *P. megasperma* or *Phytophthora* species were recovered from these soils.

Five isolates of *P. m. glycinea* and five of *P. m. medicaginis* from each of the three sites were assessed for MDH, ME, PGI, and G6P isozymes, as these enzymes gave the clearest differentiation between isolates of *P. m. glycinea* and *P. m. medicaginis*. Isozyme banding patterns of the 30 isolates were identical to those of their respective forma specialis standard isolates (i.e., *P. m. glycinea* 1-16 and *P. m. medicaginis* 5b4) and appeared as described in Figure 2.

DISCUSSION

This investigation has illustrated the value of isozyme analysis for differentiating and grouping isolates within the species *P. megasperma* and the potential for its use within the genus. The isozyme banding patterns for *P. cactorum, P. cryptogea, P. megasperma*, and *P. p. nicotianae* easily distinguished these species and thus could be used in their identification.

The *P. megasperma* intraspecific groups distinguished by isozyme analysis parallel those established in the literature, which are based on morphological (4,20,26), physiological (viz., pathogenicity and growth, 15,20,27,39), and biochemical data (4,15–18). Isozyme analysis separated isolates of *P. megasperma* into at least six intraspecific groups: the *P. megasperma* formae speciales—*glycinea, medicaginis*, and *trifolii*, and the apple/apricot/cherry, Douglas-fir, and broad host-range groups (terminology for the later three groups is based on Hansen et al [20]) (Figs. 1 and 3). The isolates of the broad host-range group had four isozyme banding patterns (patterns VI–IX, Fig. 3 and Table 2), and the SMCs indicated that isozyme banding pattern groups VII and VIII were very similar to each other yet

TABLE 4. Interspecific simple matching coefficients for *Phytophthora* species

	Pmm[a]	Pmg	Ppn	Pcac	Pcry
Pmm	···	40/79[b]	39/58	35/58	6/10
Pmg	0.51[c]	···	38/58	26/58	3/10
Ppn	0.67	0.66	···	34/58	6/10
Pcac	0.60	0.45	0.59	···	8/10
Pcry	0.60	0.30	0.60	0.80	···

[a]Pmm = *P. megasperma* f. sp. *medicaginis* 5b4, Pmg = *P. megasperma* f. sp. *glycinea* 1-16, Ppn = *P. parasitica* var. *nicotianae*, Pcac = *P. cactorum*, Pcry = *P. cryptogea*.
[b]Above the diagonal: ratio of number of common pairs of isozyme bands present or absent to number of different isozyme bands detected in this set of isolates.
[c]Below the diagonal: simple matching coefficients.

TABLE 5. Intraspecific simple matching coefficients for *Phytophthora megasperma*

	I[a]	II	III	IV	V	VI	VII	VIII	IX
I(SOY)[b]	···	17/21[c]	40/79	35/69	38/69	6/21	8/21	9/21	33/69
II(AC)	0.81[d]	···	9/21	8/21	7/21	10/21	8/21	9/21	11/21
III(ALF)	0.51	0.43	···	53/69	54/69	16/21	18/21	19/21	35/69
IV(CLO)	0.52	0.38	0.77	···	50/69	20/21	19/21	20/21	60/69
V(DF)	0.55	0.33	0.78	0.72	···	14/21	16/21	17/21	36/69
VI(BHR)	0.29	0.48	0.77	0.95	0.67	···	16/21	15/21	14/21
VII(BHR)	0.38	0.38	0.86	0.90	0.76	0.76	···	20/21	14/21
VIII(BHR)	0.43	0.43	0.90	0.95	0.81	0.71	0.95	···	15/21
IX(BHR)	0.48	0.53	0.51	0.58	0.52	0.67	0.67	0.71	···

[a]Isozyme banding pattern groups (see Table 2, Fig. 3): I = *P. megasperma* f. sp. *glycinea*, II = *P. megasperma*, III = *P. megasperma* f. sp. *glycinea*, IV = *P. m. trifolii*, V–IX = *P. megasperma*.
[b]Total electrophoretic protein group as determined by Hansen et al (20): SOY = soybean, AC = apple/apricot/cherry, ALF = alfalfa, CLO = clover, DF = Douglas fir, BHR = broad host range.
[c]Above the diagonal: ratio of number of common pairs of isozyme bands present or absent to number of different isozyme bands detected in this set of isolates.
[d]Below the diagonal: simple matching coefficients.

distinct from isozyme banding pattern groups VI and IX. Förster et al (18) found that the isolates of *P. megasperma* in the isozyme banding pattern groups VI–VIII were in separate groups based on an analysis of differences in restriction fragment length polymorphisms for their mtDNA. Therefore, isozyme analysis elucidated an apparently meaningful diversity within *P. megasperma*.

All of the 224 isolates of *P. m. glycinea* and 41 of the 45 isolates of *P. m. medicaginis* had identical isozyme banding patterns within their respective formae speciales. The average SMCs of 1.0 and 0.99 for the isolates of *P. m. glycinea* and *P. m. medicaginis*, respectively, and an SMC of 0.51 for the comparison between isolates of *P. m. glycinea* and *P. m. medicaginis* are consistent with these two formae speciales being placed in separate taxons. Within the populations of *P. m. glycinea* and *P. m. medicaginis* baited from the same soil sample, there were no variant or intermediate isozyme banding patterns. This evidence, along with the characteristics reported by the other methods (4,11,12, 15–18,20,22,23,27,39), strongly supports the contention that *P. m. medicaginis* and *P. m. glycinea* are not naturally intermating populations and that they exist as two biological species distinct from other isolates of *P. megasperma*. By accepting this conclusion, the many differences that have been reported between *P. m. medicaginis* and *P. m. glycinea* would be phylogenetically based and taxonomically recognized. For example, research has shown that *P. m. medicaginis* and *P. m. glycinea* can be differentiated according to: in vitro colony growth habit (20) and temperature response (21); oogonium diameter-sporangium length ratios (21); host ranges (20,23,27); nuclear DNA content and karyotypes (20); mtDNA size and arrangements (16–18); sensitivity to metalaxyl and phosphorous acid (11,12); and metabolism of nitrate (22). Additionally, protoplasts of *P. m. medicaginis* and *P. m. glycinea* are unable to form viable fusion products with each other (28).

While nuclear and mitochondrial DNA analyses may offer finer resolution of isolate differences and phylogenetic relationships (16–18), they are time-consuming and expensive relative to isozyme techniques. Isozyme analysis is a useful technique that can be used to assay a large number of isolates more rapidly than DNA or pathogenicity-testing techniques. Also, isozymes can be used as markers in genetic studies (8,29,33,42).

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