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Isozyme characterization of sexual and asexual *Phytophthora infestans* populations

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ABSTRACT: We assayed mycelium of *Phytophthora infestans* for activity of 38 enzymes using starch gel electrophoresis. Activity was detected for 17 enzymes and 24 loci were resolved. The isozyme diversity found in *P. infestans* sampled from an asexual population (representing the United States, Canada, and Europe) was compared to that found in isolates from Mexico, where the sexual stage of the fungus exists. Both populations were monomorphic and identical at 11 enzyme loci. We obtained information for only 4 of 13 polymorphic loci due to inadequate resolution of the other 9 loci. The sexual and asexual populations were polymorphic at the *Gpi-1* (glucosephosphate isomerase) and *Pep* (peptidase) loci, while the sexual population alone was polymorphic at the *Me* (malic enzyme) and *Xdh* (xanthine dehydrogenase) loci. Observed banding patterns were consistent with the hypothesis that *P. infestans* has a diploid vegetative stage. Fifteen genotypic classes existed in the sexual population, while four classes existed in the asexual population. At the *Gpi-1* and *Pep* loci, genotypic distributions in the sexual population were consistent with Hardy-Weinberg equilibrium, while those in the asexual population were not. This suggests the occurrence of random mating in the Mexican population of *P. infestans*.

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PHYTOPHTHORA INFESTANS (Mont.) de Bary (class Oomycetes) is the fungus that causes potato late blight, perhaps the most destructive disease of the potato (*Solanum tuberosum* L.). There are two different mating types of *P. infestans*, designated A1 and A2[5]. In the United States, Canada, and Europe, only a single mating type (A1) has been found until very recently[10]; thus, reproduction is almost exclusively asexual. In Mexico, which is thought to be the center of origin of the *Solanum*:*P. infestans* pathosystem, both mating types are found[7] and the potential for sexual recombination exists. However, the actual frequency of sexual recombination in *P. infestans* and its role relative to asexual mechanisms[11,12] in maintaining genetic variation is unknown.

Pathogenic diversity in *P. infestans* is expressed in terms of specific virulence on host genotypes containing single dominant genes for resistance[2]. Mexican *P. infestans* isolates show greater pathogenic diversity than do isolates from the United States, Canada, or Europe[16]. Because it is feared that sexual recombination has given rise to such pathogenic diversity, quarantine regulations restrict the importation of potatoes and cultures of *P. infestans* from Mexico.

The high level of pathogenic diversity observed for Mexican *P. infestans* isolates could be independent of sexual recombination, however. Instead, it could be caused by the strong selection for specific virulence exerted by the wide array of host genotypes and species found in Mexico and not in the United States, Canada, or Europe[16].

To better elucidate the role of sexual recombination in *P. infestans* our goal was to assess genetic variation at loci at which selection is less intense and less variable between populations than at loci for specific virulence.

Isozyme analysis presumably offers a relatively independent and selectively neutral means of identifying population levels of genetic variation[13]. Expression of alleles coding for isozymes is generally not strongly dependent on the environment, nor is selection at such loci likely to be as intense and population-specific as selection that occurs at loci for specific virulence.

If information from isozyme loci does provide unbiased estimates of population genetic variation, then the effects of sexual recombination in a population may be detected from isozyme data. Because much is known about the genetic basis of commonly observed iso-

zyme phenotypes[13], isozyme analysis often allows the assessment of genotypic as well as phenotypic frequencies in populations. If sexual recombination occurs frequently in a population, a wider range of genotypes may be observed relative to an asexual population. If random mating should occur, genotype frequencies may conform to Hardy-Weinberg expectations[9].

Isozyme analysis also could provide valuable genetic markers for use in future studies on *P. infestans*. The lack of such markers has plagued *Phytophthora* geneticists for many years[24]. Isozyme markers would be useful since many individuals may be assayed on a single gel, since heterozygotes may be distinguished from homozygotes, and since isozyme banding patterns are generally not strongly affected by environmental factors. Markers used in previous studies on *P. infestans* have included drug resistance[22] and specific virulence[6,19], both of which are laborious to assess and may yield inconsistent results.

Finally, isozyme analysis could provide confirming evidence for diploidy in *P. infestans*. Cytological and genetic analyses[20,24,25] indicate that vegetative mycelium of *Phytophthora* is diploid. Isozyme analysis could provide additional evidence for diploidy by revealing whether observed banding patterns conform to those seen in diploid organisms.

Our objectives were thus to characterize isozyme systems useful for studies on *P. infestans*, and to compare levels of isozyme variation in *P. infestans* populations reproducing sexually in Mexico with those reproducing asexually in the United States, Canada, and Europe. Furthermore, we wished to determine whether the effects of sexual recombination potentially occurring in Mexico are detectable in terms of isozyme variation.

Materials and Methods

Manipulations with living isolates. Isolates of *P. infestans* were obtained from Mexico (sexual population, Table I) and from the United States, Canada, Ireland, and Wales (asexual population, Table II). Many isolates from the asexual population were provided to us by other workers. We collected Mexican isolates during early September 1983 and August 1984 as follows: small tissue pieces from single blight lesions on potato foliage or from infected tubers were surface-disinfested in 1 percent sodium hypochlorite, blotted on a paper towel, and plated on a selective agar medium containing the following ingredients (per liter): 200 ml V8 juice, 2.8 g calcium carbonate, 2ml pimaricin (25 mg/ml), 67mg Terrachlor (75 percent pentachloronitrobenzene), 100mg Benlate (50 percent benomyl), 200mg vancomycin, 500mg ampicillin, and 20g agar.

The mating type of each isolate was determined by pairing it with isolates known to be of A1 and A2 mating type. Pairings were made on V8-lima bean agar[17] in 9-cm-diameter petri dishes at 18°C in darkness. A no. 2 cork borer plug from the colony margin of each isolate was paired with a plug from a colony of isolate 101 (A1 mating type) and isolate 126 (A2 mating type) in separate petri dishes. After 3-4 weeks, numerous sexual spores (oospores) were visible for one of the two pairings in a distinct band where the two thalli coalesced. If oospores were formed in the A1 pairing, the unknown isolate was designated A2; if oospores were formed in the A2 pairing, the isolate was designated A1.

To determine whether individual isolates consisted of mixtures of genotypes, we compared isozyme patterns of several single-zoospore cultures with those of their parent isolates. Since zoospores are uninucleate and asexual, cultures growing from single zoospores should represent genetically pure lines. Thus, if our original isolates were not mixtures of genotypes, banding patterns of single-zoospore cultures should be identical to those of the parent isolates.

To obtain single-zoospore cultures, we grew four parent isolates (nos. 101, 108, 115, and 127, identified in Table II) on V8-lima bean agar in 9-cm-diameter petri dishes at 18°C in darkness to induce sporangial formation. Sporangia were washed from the plates with 15 ml of sterile distilled water and filtered through four layers of sterile cheesecloth to remove mycelial fragments. Sporangial suspensions were placed at 4°C for 3 hours and then warmed to room temperature to induce zoospore differentiation and release. Zoospore suspensions were diluted to 5000 zoospores per milliliter and 0.75 ml were plated on half-strength Difco lima bean agar (Difco Laboratories, Detroit, MI) in 9-cm-diameter petri dishes, then spread with a flamed bent glass rod. After about 18 hours, germinating zoospore cysts were transferred to a concentrated V8-juice agar medium containing 14 g calcium carbonate and 20 g of agar per liter of V8 juice and incubated at 18°C in darkness. Colonies developing after about 2 weeks were transferred to V8-juice agar for further use.

Starch gel electrophoresis. Mycelial mats growing in V8-juice or V8-lima bean broth[17] were vacuum-dried on Whatman no. 1 filter paper and transferred to 12 x 75 mm test tubes. Several drops of extraction buffer (tris-HCl, 0.05 M, pH 7.1) were added to the samples (about 0.15 g of vacuum-dried mycelium per sample), which were ground with a glass rod and centrifuged at 1000 RPM for 5 minutes in a Sorvall model GLC-1 centrifuge. Samples were plated on 14 percent starch gels according to the methods of May et al.[15]. Staining procedures were those of Allendorf et al.[1] and Shaw and Prasad[23]. We use the system of genic nomenclature of Allendorf et al.[1].

Results

We detected activity for 17 enzymes, and 24 loci were resolved. Table III presents these enzyme loci, the buffer systems for best resolution, and whether *P. infestans* was monomorphic or polymorphic at the loci.

All isolates were monomorphic and identical at 11 loci (Table III). Of these, the best resolved were *Ldh*, *Mdh-2*, and *Pgd*.

Table I. Accession numbers and sources of isolates of *Phytophthora infestans* from Mexico (sexual population) used for isozyme analysis

Isolate nos.	Source
501-504*, 506-528	Toluca; international late blight screening trial
529	Atizapan; international late blight screening trial
533-536, 538-539	El Tecolote; commercial potato field
541	Mexicalcingo; commercial potato field
542-546	La Puerta; commercial potato field
547	Raices; commercial potato field
550	Tenango; wild *Solanum* species
551	El Tecolote; wild *Solanum* species
560, 561	Chapingo; *Solanum cardiophyllum*†
562-570	Chapingo; potato research plot
126 (ATCC 32835)‡	unknown source in Mexico

*The dash indicates that all isolates in numerical succession are included; thus, isolates 501, 502, 503, and 504 are indicated by 501-504

†Isolates 560 and 561 were provided by Dr. J. Galindo of Chapingo University

‡American Type Culture Collection accession number

Only 4 of the 13 polymorphic loci (*Gpi-1*, *Pep*, *Me*, and *Xdh*) were resolved adequately to allow unequivocal scoring of all individuals. Diagrams of banding patterns observed at these loci and alleles postulated to correspond to the observed phenotypes are presented in Figure 1. Figure 2 illustrates some representative phenotypes at the *Gpi-1* locus.

The sexual and asexual populations were polymorphic at *Gpi-1* and *Pep* (Figure 1). A single isolate in the sexual population (isolate 551) varied from all other isolates for *Me* and *Xdh*; thus, only the sexual population was polymorphic at these loci.

Table IV presents the genotypic distributions for the *Gpi-1* and *Pep* loci in the sexual and asexual populations. In the sexual population, genotypic distributions at both loci were found not to deviate significantly from those expected under Hardy-Weinberg equilibrium, while those in the asexual population showed significant deviation from Hardy-Weinberg equilibrium (Table IV). The deviation from equilibrium observed for the asexual population was largely due to excesses of heterozygotes at the *Gpi-1* and *Pep* loci (Table IV). Hardy-Weinberg calculations were not performed for data from the *Me* or *Xdh* loci since only a single variant individual existed.

Table V presents the genotypic classes, including mating type, for *P. infestans* isolates in our collection. Sixteen genotypic classes were identified. Individuals from the sexual population were represented in 15 of the classes, while those from the asexual population were represented in four classes.

Table II. Accession numbers and sources of isolates of *Phytophthora infestans* from the United States, Canada, and Europe (asexual population) used for isozyme analysis

Isolate nos.	Source
101 (ATCC 48824)*	R. J. Young, W. Virginia Univ., USA†
102 (ATCC 48716)	Wyoming Co., N.Y., USA
103 (ATCC 48717)	Washington Co., N.Y., USA
104 (ATCC 46301)	Riverhead, N.Y., USA
105 (ATCC 48718)	H.D. Thurston, Cornell Univ., USA†
106 (ATCC 48719)	Avoca, N.Y., USA
107 (ATCC 48825)	R. Zaccharius, Philadelphia, PA, USA†
108 (ATCC 48826)	H.D. Thurston, Cornell Univ., USA†
109 (ATCC 48827)	New Brunswick, Canada (W. H. Hodgson)
110 (ATCC 48828)	New Brunswick, Canada (W. H. Hodgson)
111 (ATCC 48720)	Albany Co., N.Y., USA
112a (ATCC 48721)	Albany Co., N.Y., USA
112b	Albany Co., N.Y., USA
114 (ATCC 48829)	N.Y. State, USA
115 (ATCC 48722)	Wyoming Co., N.Y., USA
118 (ATCC 48830)	H. D. Thurston, Cornell Univ., USA†
119 (ATCC 48831)	North Wales, U.K. (R. C. Shattock)
120 (ATCC 48832)	North Wales, U.K. (R. C. Shattock)
123 (ATCC 48833)	North Wales, U.K. (R. C. Shattock)
127 (ATCC 48723)	Spencerport, N.Y., USA
128 (ATCC 48723)	Steuben Co., N.Y.
130	H. D. Thurston, Cornell Univ., USA†
134 (ATCC 52012)	Portage Co., WI., USA (W. R. Stevenson)
135 (ATCC 52009)	Portage Co., WI., USA (W. R. Stevenson)
136 (ATCC 52010)	Langlade Co., WI., USA (W. R. Stevenson)
137 (ATCC 52011)	Langlade Co., WI., USA (W. R. Stevenson)
138, 139	R. W. Goth, Beltsville, MD, USA†
140	Ireland (M. Coffey)
141	Wales (M. Coffey)
142, 143	California, USA (M. Coffey)
146	North Wales (R. C. Shattock)
147–149	Groton, N.Y., USA (B. P. May)
150, 153–159	Auburn, N.Y., USA
151	North Wales (R. C. Shattock)
152	Erie Co., N.Y., USA (J. Carroll)

*American Type Culture Collection accession number

†For these isolates, only the personal source is known; the exact geographical source within the United States is unknown

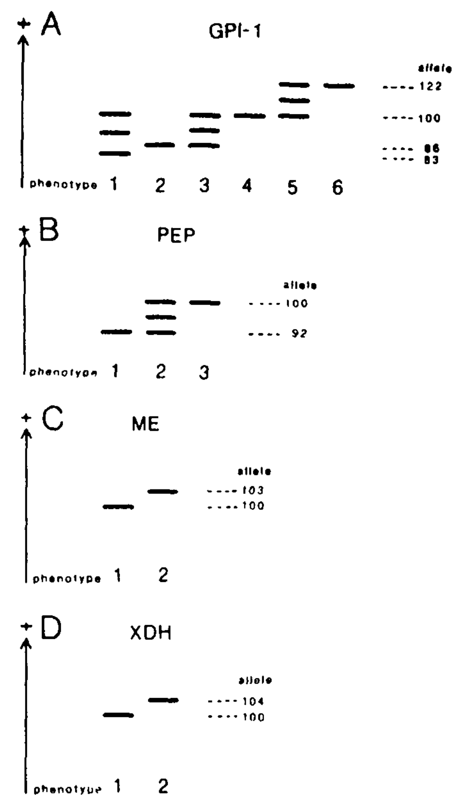

FIGURE 1 Electrophoretic phenotypes with interpretable genetic bases observed for five marker loci used to identify genotypic classes of *Phytophthora infestans*. We use the system of genic nomenclature described by Allendorf et al.[1] Allelic variants are designated according to relative electrophoretic mobiity. One allele (usually the most common) is arbitrarily designated *100*. This unit distance represents the migration distance of the isozyme coded for by this allele. Other alleles are then assigned a numerical value representing their mobility relative to this unit distance.

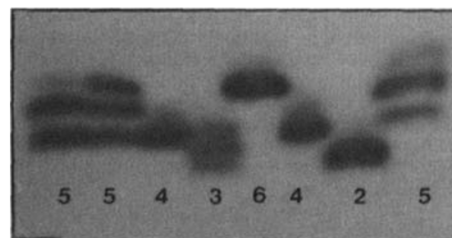

FIGURE 2 Enzymatic activity at the *Gpi-1* locus in vegetative mycelium of *Phytophthora infestans*. Phenotypes = presumed genotypic bases (as in Figure 1): 2 = *86/86*, 3 = *86/100*, 4 = *100/100*, 5 = *100/122*, 6 = *122/122*.

Table III. Designation of loci coding for different enzymes, buffer systems for best resolution, and whether *Phytophthora infestans* was monomorphic (M) or polymorphic (P) at the locus

Enzyme (EC no.)	Abbr.	Locus desig. (if mult.)	Buffer syst.*	M or P
Alkaline phosphatase (3.1.3.1)	AKP		C	P[†]
Diaphorase (1.6.4.3)	DIA	1	C	P[†]
		2	C	M
Fumarase (4.2.1.2)	FUM		C, 4	P[†]
Glucokinase (2.7.1.2)	GK	1	4	M
		2	4	P[†]
Glucosephosphate isomerase (5.3.1.9)	GPI	1	4	P
		2	4	M
B-Glucosidase (3.2.1.21)	B-GLU	1	C, R	P[†]
		2	C	M
Glutathione reductase (1.6.4.2)	GR	1	M	P[†]
		2	M	M
Isocitrate dehydrogenase (1.1.1.42)	IDH		M, C	P[†]
Lactate dehydrogenase (1.1.1.27)	LDH		M	M
Malate dehydrogenase (1.1.1.37)	MDH	1	4, R	P[†]
		2	4, R	M
Malic enzyme (1.1.1.40)	ME		C	P
Mannitol dehydrogenase (1.1.1.67)	MADH		C	M
Mannosephosphate isomerase (5.3.1.8)	MPI		4	P[†]
Peptidase (3.4.3.1)	PEP		M, 2	P
Phosphogluconate dehydrogenase (1.1.1.43)	PGD		4, M	M
Superoxide dismutase (1.15.1.1)	SOD	1	R	M
		2	R	M
Xanthine dehydrogenase (1.2.1.37)	XDH		R	P

*The buffer systems were as follows: C: electrode buffer, 0.04 M citric acid adjusted to pH 6.1 with N-(3-aminopropyl)-morpholine, diluted 1:10 for gel buffer[4]; 4: buffer no. 4 of Selander et al.[21]; M: electrode buffer, 0.18 M tris-0.1 M boric acid-0.004 M NaEDTA, diluted 1:4 for gel buffer[14]; R: described by Ridgeway et al[18]; 2: buffer no. 2 of Selander et al.[21]

†These loci, although polymorphic, were not resolved adequately to allow accurate and unequivocal scoring. At some loci, the number of bands in multiple-banded phenotypes could not be accurately counted, while at other loci, different phenotypes were so nearly identical that unequivocal scoring of all individuals was not possible

Extremely faint or indetectable activity was noted for the following enzymes, all of which were run using buffers C, M, R, and 4 (see Table III): adenylate kinase, aldolase, alcohol dehydrogenase, alphaglycerophosphate dehydrogenase, aspartate aminotransferase, catalase, creatine phosphokinase, esterase, fructose diphosphate, galactosaminidase, glucose-6-phosphate dehydrogenase, glyceraldehyde-3 phosphate dehydrogenase, hy-

Table IV. Genotype distributions at the *GPI-1* and *PEP* loci in sexual and asexual populations of *Phytophthora infestans*

Gpi-1 genotype	Sexual population ($n=50$)	Asexual population ($n=46$)
83/83	0 (0.00)*	—
83/86	0 (0.08)	—
83/100	1 (0.84)	—
83/122	0 (0.07)	—
86/86	1 (0.32)	0 (9.14)
86/100	6 (6.72)	41 (22.73)
86/122	0 (0.56)	—
100/100	36 (35.28)	5 (14.13)
100/122	5 (5.88)	—
122/122	1 (0.24)	—
Chi-square[†]	4.74 ($P=0.58$)	29.72 ($P<0.005$)
***Pep-1* genotype**	**Sexual population ($n=50$)**	**Asexual population ($n=46$)**
92/92	3 (2.2)*	1 (10.52)
92/100	15 (16.59)	42 (22.96)
100/100	32 (31.20)	3 (12.52)
Chi-square[†]	0.46 ($P=0.50$)	31.64 ($P=0.005$)

*Observed numbers are followed in parentheses by numbers expected based on Hardy-Weinberg equilibrium
†Chi-square value for testing deviation from Hardy-Weinberg equilibrium

Table V. Summary of genotypic classes including mating types of isolates of *Phytophthora infestans* in our collection

Isolate nos.	Mating type	Genotype*			
		Gpi-1	*Pep*	*Me*	*Xdh*
561	A1	*83/100*	*92/100*	*103/103*	*104/104*
101–109, 112b, 114, 115, 119, 120, 123, 127, 128, 130, 134–141, 147–159	A1	*86/100*	*92/100*	*100/100*	*100/100*
110, 111, 112a, 529, 541, 543, 547	A1	*86/100*	*100/100*	*100/100*	*100/100*
142, 512, 528	A1	*100/100*	*92/92*	*100/100*	*100/100*
108, 118, 143, 146, 509, 527, 534	A1	*100/100*	*92/100*	*100/100*	*100/100*
506, 507, 515, 517, 535, 538, 542, 545, 546	A1	*100/100*	*100/100*	*100/100*	*100/100*
560	A1	*100/122*	*92/92*	*100/100*	*100/100*
565	A1	*100/122*	*92/100*	*100/100*	*100/100*
544, 568	A1	*100/122*	*100/100*	*100/100*	*100/100*
533	A1	*122/122*	*92/100*	*100/100*	*100/100*
550	A2	*86/86*	*100/100*	*100/100*	*100/100*
126	A2	*86/100*	*92/100*	*100/100*	*100/100*
519	A2	*86/100*	*100/100*	*100/100*	*100/100*
502, 508, 511, 514, 526, 564, 566, 567	A2	*100/100*	*92/100*	*100/100*	*100/100*
501, 504, 510, 513, 518, 520, 521, 522, 523, 524, 525, 536, 539, 551	A2	*100/100*	*100/100*	*100/100*	*100/100*
503	A2	*100/122*	*100/100*	*100/100*	*100/100*

*Allelic designations are as indicated in Figure 1

droxybutyric dehydrogenase, leucine aminopeptidase, octanol dehydrogenase, nucleoside phosphorylase, peroxidase, pyruvic kinase, sorbitol dehydrogenase, shikimic dehydrogenase, and triosephosphate isomerase.

Poorly resolved polymorphic loci (see Table III) also were run with buffer no. 2 of Selander et al.[21] and with a pH 6.5 histidine buffer[3]. We also altered the duration of electrophoresis. None of these refinements significantly improved the resolution of these loci.

Isozyme banding patterns of single-zoospore cultures were compared with those of their parental isolates to insure that our original isolates were not mixtures of genotypes. We tested 10 single-zoospore cultures derived from parental isolate 108 (postulated genotype *100/100* at *Gpi-1* and *92/100* at *Pep*) and 4, 11, and 26 single-zoospore cultures derived, respectively, from parental isolates 101, 115, and 127 (all of postulated genotype *86/100* at *Gpi-1* and *92/100* at *Pep*). Banding patterns of single-zoospore cultures were identical to those of the parental isolates.

Discussion

Sexual and asexual populations of *P. infestans* were identical at 11 monomorphic enzyme loci, but differed at four polymorphic loci. At the polymorphic loci, more genotypes were observed in the sexual than the asexual population. This greater variability in the sexual population parallels that observed for specific virulence[16].

Genotypic proportions at the *Gpi-1* and *Pep* loci in the sexual population did not deviate significantly from those expected under Hardy-Weinberg equilibrium, while those in the asexual population did. These results indicate the recombination may be occurring at high rates in the sexual population. They further suggest that random mating may be occurring in the sexual population. If recombination is occurring in the asexual population, its impact was not noticeable at the loci we examined.

We were unable, even with two additional buffer systems, to obtain information at nine additional polymorphic loci because of inadequate resolution (see Table III). Perhaps with additional refinement, these loci will become useful.

The isozyme markers we have identified will be useful in future genetic studies. Our genetic interpretations of the phenotypes observed at the *Gpi-1* and *Pep* loci are consistent with the dimeric subunit structures of these enzymes[8]. As such, the isozyme patterns we observed are those of a diploid organism. These results are consistent with evidence for diploidy in *Phytophthora* from cytological and genetic studies[20,24,25].

Genetic analyses that utilize isozyme markers are required to determine the mode of inheritance of these markers. We recently found (Shattock, Tooley, and Fry, unpub. results) that the *Gpi-1* banding patterns of F_1 progeny from several different crosses between parents differing in *Gpi-1* phenotypes were consistent with monogenic, disomic inheritance. We are now obtaining F_2 and backcross progeny from the above crosses to verify disomic inheritance at the *Gpi-1* locus.

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