Ecology and Population Biology

Population Structure of *Phytophthora infestans* in the Toluca Valley Region of Central Mexico

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ABSTRACT

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We tested the hypothesis that the population of *Phytophthora infestans* in the Toluca valley region is genetically differentiated according to habitat. Isolates were sampled in three habitats from (i) wild *Solanum* spp. (WILD), (ii) land-race varieties in low-input production systems (RURAL), and (iii) modern cultivars in high-input agriculture (VALLEY). Isolates were sampled in 1988-89 (n = 179) and in 1997-98 (n = 389). In both sampling periods, the greatest genetic diversity was observed in RURAL and VALLEY habitats. Based on the *Glucose-6-phosphate isomerase* and *Peptidase* allozymes, the subpopulations from the three habitats were significantly differentiated in both sampling periods. In contrast to allozyme data for 1997-98, no differences were found among the three subpopulations for sensitivity to metalaxyl. Two groups of isolates identical for allozyme and mating type were further investigated by restriction fragment length polymorphism fingerprinting; 65% of one group and 85% of another group were demonstrated to be unique. The genetic diversity data and the chronology of disease occurrence during the season are consistent with the hypothesis that populations of *P. infestans* on wild *Solanum* populations are derived from populations on cultivated potatoes in the central highlands of Mexico near Toluca.

Additional keywords: evenness, fungicide resistance, genotypic diversity, potato late blight, rarefaction, richness.

Much evidence has accumulated that the central highlands of Mexico, including the Toluca valley, are a center of diversity of *Phytophthora infestans* (Mont.) de Bary, the causal organism of potato late blight. Genetic diversity for virulence (24,28,40,41), allozyme (9,39) and restriction fragment length polymorphism (RFLP) loci (9) is higher in central Mexico than elsewhere in the world, and mating types can be found in a 1:1 ratio (9). Despite this general knowledge, the population structure of *P. infestans* within the Toluca valley is not well understood.

The Toluca valley is home to several wild *Solanum* spp. The valley (2,600-m elevation) is surrounded by mountains and the volcano Nevado de Toluca (Fig. 1) to the southwest. The volcano reaches an altitude of 4,690 m. Potatoes (*S. tuberosum*) are grown at altitudes up to 3,500 m, whereas wild species of *Solanum* have been found at the edges of pine and *Abies* forests at 3,800 m (12). Several *Solanum* spp. are endemic to the Toluca valley: *S. demissum* Lindt. is by far the most abundant species of *Solanum*, followed by *S. verrucosum* Schlechtd., *S. iopetalum* (Bitt.) Hawkes, *S. brachycarpum* Correll, *S.* × *edinense* Berth. subsp. *salamanii* (Hawkes) Hawkes, and *S. stoloniferum* Schlechtd. et Bché. (32). All of these species can be infected by *P. infestans*, and an extensive survey between 1982 and 1986 by Rivera-Peña established that approximately 10% of these plants were infected in a given year (30). Levels of rate-reducing resistance and the composition of R-genes vary among specific genotypes of *S. demissum* (19,22,31), and these characteristics are also expected to vary among genotypes for the other wild *Solanum* spp. *S.* × *edinense* is actually a hybrid between *S. demissum* and *S. tuberosum* (12).

Solanum spp. occur in three distinctly different types of habitats in the central highlands. The first habitat consists of patches of wild *Solanum* spp. (hereafter referred to as WILD) in areas of subsistence farming or uncultivated areas. The second habitat is on low-input, rural farms (hereafter RURAL). These farms produce mostly land-race ('criolla') potato (*S. tuberosum*) varieties for additional income or subsistence. Individual fields average 0.2 to 3 ha in size, and the potato varieties range from moderately resistant to highly susceptible to late blight. Some of these varieties harbor R-genes conferring resistance to *P. infestans* (S. P. Fernández-Pavía and N. J. Grünwald, *unpublished data*). Applications of agrochemicals vary from field to field, ranging from an application of fungicide one to two times per season to once per week. Whereas previously the WILD habitat was also found in the valley, nowadays the WILD habitat is found co-occurring with the RURAL habitat on the slopes of the volcano Nevado de Toluca (Fig. 1). The third habitat consists of large, commercial seed-tuber production farms that grow cultivars susceptible to potato late blight (cv. Alpha has no R-genes and cv. Atlantic has R1). The farming practices of these operations are intensive, applying pesticides two to three times per week, and the fields are large, ranging from 2 to 10 ha. In our study, these farms occurred in the valley southeast of the city of Toluca (hereafter VALLEY).

Different production systems and genetic variation in the wild *Solanum* spp. might be expected to exert detectable selection on the Toluca valley population of *P. infestans* (Table 1). Most known R-genes originated from *S. demissum* (27), but levels of rate-reducing resistance and R-gene composition vary among plants

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(1,19,22,31) and presumably among populations. Consequently, one might predict greater genetic diversity in populations of *P. infestans* growing on *S. demissum* (WILD) than in populations from cultivated potatoes (VALLEY or RURAL), which are relatively homogeneous with respect to R-genes. Fungicide usage may be an additional force of selection. Fungicide usage is most intense in the central valley, and no fungicides are used on wild species of *Solanum.* One might expect to find a higher proportion of the subpopulation in the VALLEY habitat to be resistant to metalaxyl relative to subpopulations from wild species of *Solanum.* Thus, we expect to find differences between VALLEY, RURAL, and WILD subpopulations in terms of genetic structure and frequency distribution of metalaxyl resistance, unless migration rates are high among *P. infestans* populations from these three habitats.

We tested three hypotheses with regard to understanding the population structure of *P. infestans* in the Toluca valley region: (i) the population of *P. infestans* in the Toluca valley is genetically differentiated according to habitat, (ii) genetic differentiation of the Toluca valley population is similar over two sampling periods separated by almost a decade (1988-89 and 1997-98), and (iii) the

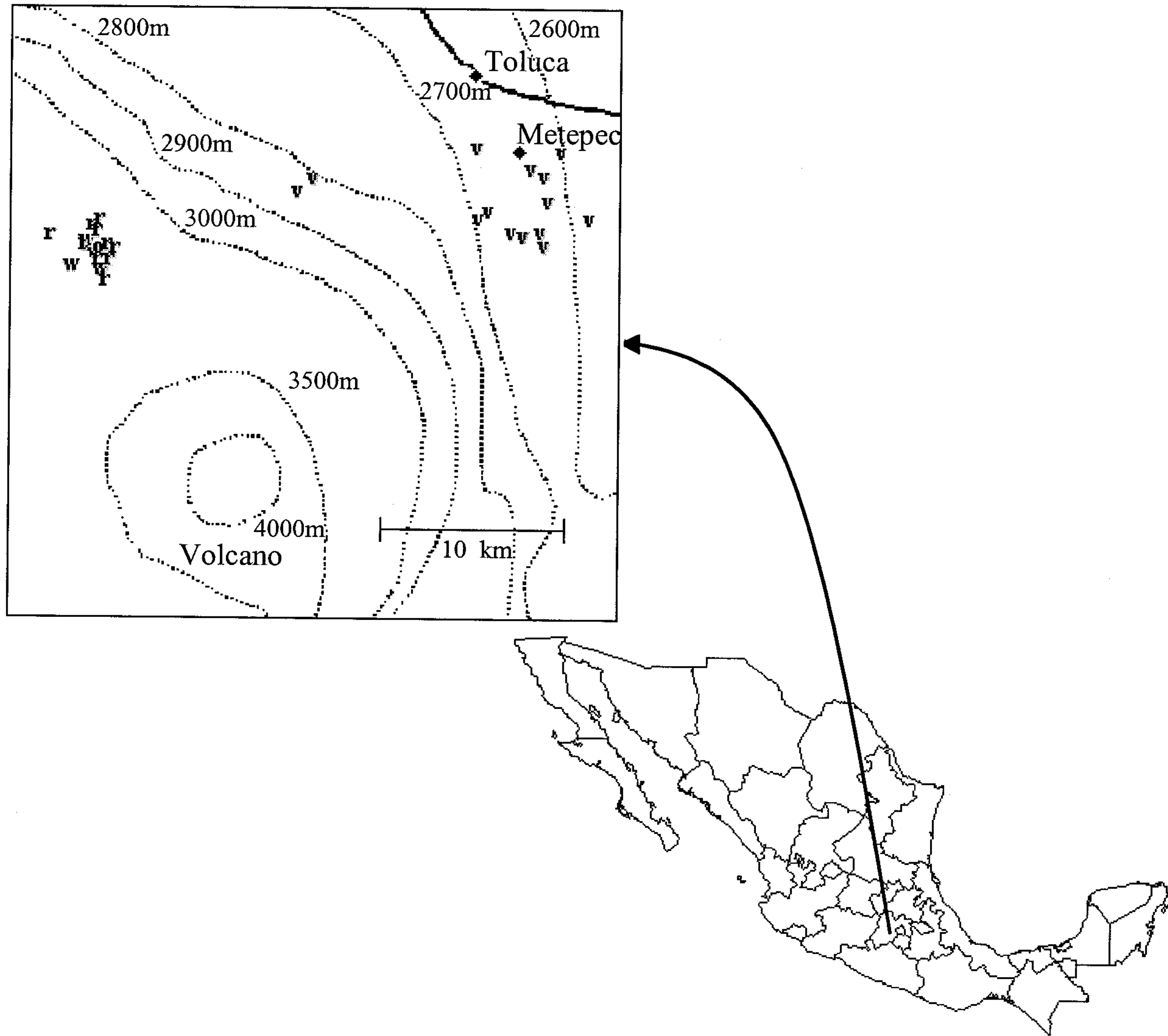

Fig. 1. Sites of collection of *Phytophthora infestans* isolates in Mexico in 1997-98. The insert shows a topographic map of the Toluca valley located in the state of Mexico. Isolates were obtained (n = 398) from three different pathogen-plant habitats including (i) populations of *P. infestans* growing on populations of wild *Solanum* spp. on the slopes of the volcano Nevado de Toluca (3,000 to 3,500 m above sea level) in which no fungicides are applied and all plants are expected to carry R-genes (WILD = w); (ii) on potatoes in rural, low-input potato fields on the slopes of the volcano Toluca (3,000 to 3,500 m above sea level) in which fungicide is applied sporadically and R-genes occur (RURAL = r); and (iii) on potatoes in pesticide-intensive potato fields where fungicides are applied every 2 to 3 days and only susceptible cultivars with no R-genes are grown (VALLEY = v).

TABLE 1. Habitats from which isolates of *Phytophthora infestans* were obtained

Habitat[a]	Host[b]	Fungicide usage	Altitude (m)
WILD	*Solanum demissum*, R-genes present, and low to high rate-reducing resistance	None	2,600–2,800 in 1988-89 or 3,000–3,500 in 1997-98
RURAL	Land-race and modern potato cultivars, some R-genes, and low to moderate rate-reducing resistance	Low to moderate	3,000–3,500
VALLEY	Modern potato cultivars (Alpha and Atlantic), no R-genes or R1 in cv. Atlantic, and low rate-reducing resistance	Intense	2,600–2,900

[a] WILD = wild *Solanum* spp., RURAL = land-race varieties in low-input production systems, and VALLEY = modern cultivars in high-input agriculture.
[b] In the 1988-89 sampling isolates were sampled from several wild *Solanum* spp. including *S. demissum.*

WILD subpopulation would show a lower proportion of metalaxyl-resistant isolates than RURAL or VALLEY subpopulations. Our approach was to determine metalaxyl resistance, mating type, and allozyme patterns at the *Glucose-6-phosphate* (*Gpi*) and *Peptidase* (*Pep*) loci for individual pathogen isolates sampled in the three habitats.

MATERIALS AND METHODS

1997 and 1998 samples. Three hundred fifty-two isolates were collected in 1997 and 30 isolates in 1998 (Table 2). The sampling scheme in 1997 consisted of obtaining 10 to 15 isolates each from at least 10 fields in the valley (VALLEY), from at least 10 fields on the slopes of the volcano (RURAL), and from 10 patches of wild *Solanum* spp. (WILD). Each isolate originated from a randomly selected, single-leaf lesion of either *S. tuberosum* or *S. demissum*. Additional isolates were sampled in 1998 from RURAL and WILD populations to ensure a sufficiently large sample size. WILD isolates were more difficult to sample because they occurred sporadically and only late in the season. Sampling locations and geographic relationships are shown in Figure 1. The VALLEY habitats were approximately 10 to 30 km from the RURAL and WILD habitats (Fig. 1). The WILD and RURAL subpopulations occurred in close proximity on the slopes of the volcano Nevado de Toluca at 3,000 to 3,500 m altitude (Fig. 1). The VALLEY subpopulation occurs at 2,600 to 2,900 m. In the 1988-89 collection, the WILD population was primarily from the valley floor in close proximity to commercial fields, but also from the slopes of the volcano.

1988 and 1989 samples. Isolates were collected in 1988-89 as described previously (23) (Table 3). The metalaxyl resistance data for these isolates have been published (23). Most of the isolates from wild *Solanum* spp. (probably sampled from *S. demissum*, *S. stoloniferum*, and *S.* × *edinense*) were collected in the valley and not on the slopes of the volcano.

Isolation and storage of isolates. Isolates collected in 1997 and 1998 were obtained by placing a portion of a leaf lesion under a slice of potato tuber (≈0.5 cm thick) in a petri dish. Tuber slices were obtained from surface sterilized, healthy tubers of cv. Alpha. Isolation from mycelial tufts on top of the tuber slice was performed 4 to 5 days later by transfer onto selective or regular Rye-A agar (2). Selective Rye-A agar was regular Rye-A agar containing the following antibiotics and fungicides: 20 mg/liter of rifampicin, 50 mg/liter of polymyxin B sulfate, 200 mg/liter of ampicillin, 67 mg/liter of PCNB (pentachloronitrobenzene), and 100 mg/liter of benomyl. For the 1997-98 isolates, replicate cultures of most isolates are stored temporarily by cryogenic storage in liquid nitrogen (38) and under mineral oil at 18°C at the PICTIPAPA culture collection in Toluca, Mexico, and permanently by cryogenic storage in liquid nitrogen (38) at Plant Research International, Wageningen, the Netherlands.

Isolates collected in 1988-89 were isolated as described previously (23). They have been stored at –135°C in the *P. infestans* culture collection at Cornell University.

Mating type/allozyme/RFLP analysis. Mating type was determined by pairing each isolate with known A1 and A2 tester strains on Rye-A agar. Oospores forming in a cross of the unknown paired with the A1 tester strain were of the A2 mating type and vice-versa.

Allozyme genotypes for *Gpi* (EC 5.3.1.9) and *Pep* (EC 3.4.3.1) were determined. For the 1997-98 samples, cellulose acetate membranes were used as described by Goodwin et al. (8). For the 1988 and 1989 samplings, potato starch gels were used as published by Spielman et al. (36). The mating type and allozyme genotype data for the 1988-89 isolates were acquired 12 to 24 months after sampling.

DNA fingerprinting analysis was performed on two subsets of isolates collected in 1997-98. Each subset was monomorphic for mating type and for *Gpi* and *Pep* genotypes (A2, *100/100*, *100/100*; or A2, *86/100*, *100/100*). The moderately repetitive clone

TABLE 2. Sampling information for 389 isolates collected in 1997 and 1998

Habitat[a]	Habitat subpopulation	Location	No. of isolates	Host
VALLEY	10	Metepec	7	‘Alpha’
VALLEY	11	Metepec	13	‘Alpha’
VALLEY	12	Metepec	13	‘Alpha’
VALLEY	13	Metepec	12	‘Alpha’
VALLEY	14	Metepec	34	‘Alpha’
VALLEY	1	La Silva	11	‘Alpha’
VALLEY	2	La Comunidad	15	‘Alpha’
VALLEY	3	Los Champiñones	14	‘Atlantic’
VALLEY	4	El Cerrito	12	‘Atlantic’
VALLEY	5	La Loma	13	‘Atlantic’
VALLEY	6	Las Minas	12	‘Alpha’
VALLEY	7	El Corral	6	‘Alpha’
VALLEY	8	El Refugio	9	‘Atlantic’
VALLEY	9	La Tolva	7	‘Atlantic’
RURAL	1	Loma Alta	7	‘Criolla’
RURAL	2	Loma Alta	13	‘Criolla’
RURAL	3	Raices	11	‘Criolla’
RURAL	4	Loma Alta	9	‘Criolla’
RURAL	5	Loma Alta	17	‘Alpha’
RURAL	6	Loma Alta	8	‘Criolla’
RURAL	7	Loma Alta	9	‘Marsiana’
RURAL	8	Loma Alta	8	‘Marsiana’
RURAL	9	La Puerta	9	‘Criolla’
RURAL	10	Buena Vista	6	‘Criolla’
RURAL	11	Loma Alta	18	‘Marsiana’
WILD	1	Patch 1	20	*S. demissum*
WILD	2	Patch 3	19	*S. demissum*
WILD	6	Patch 4	15	*S. demissum*
WILD	7	Patch 7	8	*S. demissum*
WILD	8	Patch 9	7	*S. demissum*
WILD	9	Patch 2	11	*S. demissum*
WILD	10	Patch 7	20	*S. demissum*

[a] WILD = wild *Solanum* spp., RURAL = land-race varieties in low-input production systems, and VALLEY = modern cultivars in high-input agriculture.

TABLE 3. Sampling information for 179 isolates collected in 1988 and 1989

Habitat[a]	Habitat subpopulation	Location	No. of isolates	Host
VALLEY	1	Calimaya	13	...
VALLEY	2	Atizapán	14	‘Rosita’
VALLEY	3	Metepec	22	‘Rosita’
VALLEY	4	Metepec	19	‘Alpha’
VALLEY	5	Metepec	9	‘Atzimba’
VALLEY	6	Metepec	9	‘Lopez’
VALLEY	7	S.E. Tenango	7	...
VALLEY	8	N.E. Calimaya	9	...
VALLEY	9	E. Calimaya	4	...
VALLEY	10	Conalep	4	...
VALLEY	11	Lerma	8	...
VALLEY	12	San Felipe del Progreso	6	...
VALLEY	13	Santiago Tianguistenco	14	...
RURAL	1	El Mesón	2	...
RURAL	2	La Puerta	2	...
RURAL	3	Raices	3	...
RURAL	4	Ojo de Agua	7	...
RURAL	5	San Juan Huerta	2	...
WILD	1	El Mesón	1	WILD
WILD	2	San Felipe del Progreso	4	WILD
WILD	3	East of S. Felipe P.	8	WILD
WILD	4	Atizapán	2	WILD
WILD	5	S.N. Coatepec	2	WILD
WILD	6	Metepec	1	WILD
WILD	7	Atizapán	7	WILD

[a] WILD = wild *Solanum* spp., RURAL = land-race varieties in low-input production systems, and VALLEY = modern cultivars in high-input agriculture.

RG-57 was used in Southern analysis for DNA fingerprint (7) using the Renaissance nonradioactive detection kit as described by the manufacturer (New England Nuclear, Boston).

Sensitivity to metalaxyl. Metalaxyl sensitivity for the 1997 and 1998 samplings was determined by growing isolates in vitro. Radial growth of each isolate on Rye-A agar (2) containing 0, 5, or 100 μg ml^{-1} metalaxyl was determined. Technical grade metalaxyl (Novartis, Greensboro, NC) was dissolved in dimethylsulfoxide (DMSO) and each liter of medium was treated with 1 ml of DMSO containing 0, 5, or 100 mg of metalaxyl. Agar plugs (5-mm diameter) containing *P. infestans* were transferred to the center of a petri dish and incubated at 20°C in the dark for 8 days. Mean radial growth was measured in two directions and averaged for each of three replications in the nonamended treatment and two

TABLE 4. Frequencies of multilocus genotypes based on mating type, *Glucose-6-phosphate isomerase* (*Gpi*) allozyme, and *Peptidase* (*Pep*) allozyme patterns of isolates of *Phytophthora infestans* sampled in 1988-89 (n = 179) and 1997-98 (n = 389 excluding and 393 including genotype with five-banded *Gpi* allozyme pattern)

				1988-89[c]							1997-98[c]						
				VALLEY		RURAL		WILD		Overall	VALLEY		RURAL		WILD		Overall
Genotype	Mating type[a]	*Gpi*[b]	*Pep*	n	g_i	n	g_i	n	g_i	g_i	n	g_i	n	g_i	n	g_i	g_i
1	A1	*100/100*	*100/100*	6	0.043	...	...	4	0.160	0.056	15	0.085	18	0.158	3	0.031	0.093
2	A1	*100/100*	*78/100*	…	...	...	...	...	...	...	...	...	1	0.009	...	...	0.003
3	A1	*100/100*	*92/100*	7	0.051	...	...	1	0.040	0.045	7	0.040	1	0.009	4	0.041	0.031
4	A1	*100/100*	*92/92*	...	...	...	...	...	...	...	1	0.006	1	0.009	1	0.010	0.008
5	A1	*100/100*	*96/100*	...	...	...	...	...	...	...	...	...	...	...	1	0.010	0.003
6	A1	*100/111*	*100/100*	2	0.014	...	...	…	...	0.011	...	...	...	...	1	0.010	0.003
7	A1	*100/122*	*100/100*	13	0.094	3	0.188	3	0.120	0.106	20	0.113	4	0.035	11	0.112	0.090
8	A1	*100/122*	*78/100*	...	...	...	...	...	...	...	...	...	1	0.009	...	...	0.003
9	A1	*100/122*	*92/100*	4	0.029	...	...	...	...	0.022	2	0.011	1	0.009	2	0.020	0.013
10	A1	*100/122*	*92/92*	...	...	1	0.063	...	...	0.006	...	...	1	0.009	...	...	0.003
11	A1	*100/122*	*96/100*	...	...	...	...	...	...	...	1	0.006	...	...	...	...	0.003
12	A1	*100/122*	*96/96*	...	...	...	...	...	...	...	2	0.011	...	...	...	...	0.005
13	A1	*122/122*	*100/100*	1	0.007	...	...	...	...	0.006	4	0.023	1	0.009	3	0.031	0.021
14	A1	*122/122*	*92/100*	...	...	...	...	...	...	...	2	0.011	...	...	...	...	0.005
15	A1	*122/122*	*92/92*	1	0.007	...	...	...	...	0.006	...	...	1	0.009	...	...	0.003
16	A1	*122/122*	*96/100*	...	...	...	...	...	...	...	1	0.006	...	...	...	...	0.003
17	A1	*83/122*	*100/100*	...	...	...	...	...	...	...	2	0.011	...	...	...	...	0.005
18	A1	*86/100*	*100/100*	14	0.101	2	0.125	...	...	0.089	14	0.079	11	0.096	7	0.071	0.082
19	A1	*86/100*	*92/100*	1	0.007	...	...	1	0.040	0.011	4	0.023	5	0.044	...	...	0.023
20	A1	*86/100*	*92/92*	2	0.014	...	...	...	...	0.011	1	0.006	...	...	...	...	0.003
21	A1	*86/100*	*96/100*	...	...	...	...	...	...	...	2	0.011	...	...	1	0.010	0.008
22	A1	*86/100*	*96/96*	...	...	...	...	...	...	...	1	0.006	...	...	...	...	0.003
23	A1	*86/122*	*100/100*	20	0.145	1	0.063	1	0.040	0.123	12	0.068	1	0.009	3	0.031	0.041
24	A1	*86/122*	*92/100*	2	0.014	...	...	...	...	0.011	2	0.011	2	0.018	...	...	0.010
25	A1	*86/130*	*100/100*	...	...	...	...	...	...	...	1	0.006	...	...	...	...	0.003
26	A1	*86/86*	*100/100*	6	0.043	...	...	1	0.040	0.039	3	0.017	5	0.044	1	0.010	0.023
27	A1	*86/86*	*92/100*	...	...	...	...	...	...	...	1	0.006	2	0.018	...	...	0.008
28	A1	*86/86*	*92/92*	1	0.007	...	...	...	...	0.006	...	...	...	...	...	...	0.000
29	A2	*100/100*	*100/100*	6	0.043	2	0.125	4	0.160	0.067	13	0.073	13	0.114	25	0.255	0.131
30	A2	*100/100*	*78/100*	...	...	...	...	1	0.040	0.006	...	...	1	0.009	...	...	0.003
31	A2	*100/100*	*92/100*	1	0.007	...	...	...	...	0.006	...	...	6	0.053	6	0.061	0.031
32	A2	*100/100*	*92/92*	1	0.007	...	...	...	...	0.006	...	...	1	0.009	...	...	0.003
33	A2	*100/100*	*96/100*	...	...	...	...	...	...	...	...	...	...	...	7	0.071	0.018
34	A2	*100/100*	*96/96*	1	0.007	...	...	...	...	0.006	...	...	...	...	...	...	0.000
35	A2	*100/111*	*100/100*	...	...	...	...	1	0.040	0.006	...	...	...	...	...	...	0.000
36	A2	*100/111*	*92/100*	1	0.007	...	...	...	...	0.006	...	...	...	...	...	...	0.000
37	A2	*100/122*	*100/100*	9	0.065	...	...	...	...	0.050	15	0.085	8	0.070	7	0.071	0.077
38	A2	*100/122*	*78/100*	...	...	...	...	...	...	...	...	...	1	0.009	...	...	0.003
39	A2	*100/122*	*92/100*	1	0.007	...	...	...	...	0.006	5	0.028	5	0.044	...	...	0.026
40	A2	*100/122*	*92/92*	...	...	...	...	...	...	...	1	0.006	...	...	...	...	0.003
41	A2	*111/122*	*100/100*	...	...	1	0.063	...	...	0.006	...	...	...	...	...	...	0.000
42	A2	*122/122*	*100/100*	4	0.029	...	...	...	...	0.022	1	0.006	2	0.018	...	...	0.008
43	A2	*83/100*	*100/100*	...	...	...	...	...	...	...	...	...	1	0.009	...	...	0.003
44	A2	*86/100*	*100/100*	25	0.181	3	0.188	5	0.200	0.184	28	0.158	9	0.079	1	0.010	0.098
45	A2	*86/100*	*78/100*	...	...	...	...	...	...	...	...	...	2	0.018	...	...	0.005
46	A2	*86/100*	*78/96*	...	...	...	...	...	...	...	1	0.006	...	...	...	...	0.003
47	A2	*86/100*	*92/100*	3	0.022	...	...	3	0.120	0.034	2	0.011	2	0.018	1	0.010	0.013
48	A2	*86/100*	*92/92*	...	...	...	...	...	...	...	2	0.011	...	...	1	0.010	0.008
49	A2	*86/111*	*100/100*	...	...	...	...	...	...	...	1	0.006	...	...	...	...	0.003
50	A2	*86/122*	*100/100*	3	0.022	3	0.188	...	...	0.034	6	0.034	4	0.035	11	0.112	0.054
51	A2	*86/122*	*92/100*	1	0.007	...	...	...	...	0.006	1	0.006	...	...	...	...	0.003
52	A2	*86/122*	*92/92*	1	0.007	...	...	...	...	0.006	...	...	...	...	...	...	0.000
53	A2	*86/86*	*100/100*	1	0.007	...	...	...	...	0.006	2	0.011	3	0.026	1	0.010	0.015
54	A2	*86/86*	*92/100*	...	...	...	...	...	...	...	1	0.006	...	...	...	...	0.003
55	A1	*100/111/122*	*100/100*	...	...	...	...	...	...	...	...	...	1	...	...	...	0.002
56	A2	*100/111/122*	*100/100*	...	...	...	...	...	...	...	1	...	...	...	2	0.020	0.007

[a] The self-fertile isolate was excluded from analysis.

[b] The five-banded *Gpi* genotype was included in the overall frequency analyses, but ignored in the frequency analysis per subpopulation (VALLEY, RURAL, and WILD) and in the genotypic analysis. In the genotypic analysis *P. infestans* is considered to be a diploid organism and *Gpi* a codominant marker.

[c] WILD = wild *Solanum* spp., RURAL = land-race varieties in low-input production systems, and VALLEY = modern cultivars in high-input agriculture. n = number of isolates collected per multilocus genotype. g_i = frequency of ith genotype. ... Indicates genotype not detected in this sample.

replications in the metalaxyl-amended treatments. Radial growth was corrected for plug diameter.

To compare frequency distributions of metalaxyl resistance, growth rates were organized into five classes for relative growth on 5 or 100 µg/ml of metalaxyl. Relative growth is amount of growth on metalaxyl-amended medium relative to growth on DMSO-amended control. The classes were 0 to 19%, 20 to 39%, 40 to 59%, 60 to 79%, and 80 to >100% relative growth. To determine the reproducibility of the method, a subset of 47 isolates was assessed some months after the first assessment, and the results of the two independent assessments were compared. Metalaxyl resistance data for isolates collected in 1988-89 were published previously (23).

Because the frequency of metalaxyl sensitivity in 1997-98 was different from that reported for the 1988-89 collection (23), further tests on different formulations of metalaxyl (or mefenoxam, the active isomer of metalaxyl) were conducted. The different formulations were technical grade metalaxyl, Ridomil-2E (metalaxyl, 25.1%), and Ridomil Gold-4E (mefenoxam, 46.2%) (Novartis Agro, Mexico City). The sensitivities to each of the formulations of a subset of 47 isolates were determined. Technical grade metalaxyl was dissolved in DMSO as described previously with the same concentration of DMSO used for the corresponding control. For metalaxyl and mefenoxam, the active ingredients were already dissolved in a solvent of unknown composition, and distilled water was used as control.

Data analysis. Population structure was studied by analyzing allele frequencies, heterozygosity, genetic distance, and genetic differentiation. A multilocus genotype was constructed for each isolate by combining data for mating type, *Gpi*, and *Pep* loci at each location sampled (6). Except for coefficient of differentiation (G_{ST}) analysis, analyses were conducted on these multilocus genotypes. The A2 mating type was considered to be homozygous and the A1 heterozygous (3,14,15). A chi-square analysis for mating-type frequencies was conducted to detect departure from a 1:1 ratio. Unbiased expected heterozygosities (17,26) were calculated with tools for population genetic analyses (TFPGA) (version 1.3; Department of Biological Sciences, Northern Arizona University, Flagstaff). Cluster analysis of multilocus genotypes was based on allele frequencies observed for the population or each subpopulation. Trees were constructed using the unweighted pair-group method of averages (UPGMA) algorithm from a Rogers' modified genetic distance matrix (42) based on multilocus genotype as defined previously using TFPGA. Statistical support for phenogram branches was obtained using 1,000 bootstrapped samples using TFPGA. Genetic differentiation within the Toluca valley population and among subpopulations was estimated based on genotypes defined by two loci (*Gpi* and *Pep*) using Nei's coefficient of differentiation (G_{ST}) (25,35) using POPGENE (Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, Canada) (43). Exact tests for population differentiation were determined by a Monte Carlo approach (10 batches, 2,000 permutations per batch, and 1,000 dememorization steps) (29) as described in the TFPGA manual.

Multilocus genotypic diversity analysis for the overall population and subpopulations distinguished richness, evenness, and diversity. Genotypic diversity was calculated as Shannon-Wiener's index $H' = -\Sigma_i\, p_i \times \ln(p_i)$ (33) (also known in the plant pathology literature as the Shannon-Weaver index), where p_i is the observed frequency of the ith genotype. The significance of differences between genotypic diversities was calculated for H' using a t test (21) with a Bonferroni correction for multiple comparisons. The evenness index E_5 (20) was calculated as

$$E_5 = \frac{(1/\hat{\lambda})-1}{e^{H'}-1}$$

where $\hat{\lambda}$ corresponds to Simpson's index (34):

$$\hat{\lambda} = \sum_{i=1}^{g} \frac{n_i(n_i-1)}{n(n-1)}$$

and n_i equals the number of observations of the ith genotype and n equals sample size. Genotypic richness expresses the number of expected genotypes in a sample and was estimated using rarefaction curves. Rarefaction curves assume that the number of genotypes, g, expected in a sample of n individuals in a population sampled to a total of N individuals, with number of individuals per genotype (n_i) distributed among each genotype i is

$$E(g_n) = \sum_{i=1}^{g}\left\{1-\left[\frac{(N-n_i/N)}{(N/n)}\right]\right\}$$

$E(g_n)$ computes the expected number of genotypes in a random sample of size n as the sum of the probabilities that each genotype will be included in the sample (20). The algorithm, implemented in the C computer language, calculates $E(g_n)$ for each sample size n = 1 to N and was validated using published data sets (10,13,16, 18,20,21).

Classes of metalaxyl resistance were compared by the nonparametric Wilcoxon-Mann-Whitney test for two groups and Kruskal-Wallis test for three groups, which test the null hypothesis that the distribution of a response variable is the same in multiple independently sampled populations (PROC NPAR1WAY WILCOXON [37]; SAS Institute, Cary, NC). Linear regressions restricting the intercept to be zero (PROC REG with RESTRICT INTERCEPT = 0 option) were used to evaluate different formulations of metalaxyl. This approach redefines the coefficient of determination (R^2) and is preferable to using the NOINT option in PROC REG (5).

RESULTS

Total population. The characteristics of the overall population in each of the two sampling periods (1988-89 versus 1997-98)

TABLE 5. Frequencies of mating type, *Glucose-6-phosphate isomerase* (*Gpi*), and *Peptidase* (*Pep*) alleles by subpopulation (i.e., sampling location and date) (n = 179 and 389, respectively)[a]

			Mating type			*Gpi*[d]						*Pep*			
Date	Location[b]	n[c]	A1	A2	$P > \chi^2$[e]	83	86	100	111	122	130	78	92	96	100
1988-89	VALLEY	138	0.580	0.420	0.061	...	0.319	0.431	0.011	0.239	...	0.000	0.120	0.007	0.873
	RURAL	16	0.438	0.563	0.617	...	0.281	0.406	0.031	0.281	...	0.000	0.063	0.000	0.938
	WILD	25	0.440	0.560	0.549	...	0.240	0.660	0.020	0.080	...	0.020	0.100	0.000	0.880
1997-98	VALLEY	177	0.554	0.446	0.153	0.006	0.260	0.489	0.003	0.240	0.003	0.003	0.105	0.031	0.862
	RURAL	114	0.491	0.509	0.851	0.004	0.246	0.592	0.000	0.158	0.000	0.026	0.140	0.000	0.833
	WILD	98	0.388	0.612	0.026	0.000	0.148	0.643	0.005	0.204	0.000	0.000	0.087	0.046	0.867

[a] Four isolates with five-banded *Gpi* genotype (*100/111/122*) were excluded from this analysis.
[b] WILD = wild *Solanum* spp., RURAL = land-race varieties in low-input production systems, and VALLEY = modern cultivars in high-input agriculture.
[c] n = number of isolates collected.
[d] ... indicates genotype not detected in this sampling.
[e] Probability values for a chi-square analysis for mating-type frequencies to detect departure from a 1:1 ratio.

were quite similar, determined by mating type and allozyme analysis. For both sampling periods, there was much diversity and each population contained many rare genotypes. The 179 isolates sampled in 1988-89 and the 389 isolates sampled in 1997-98 could be classified into 32 and 50 different genotypes, respectively (Table 4). Four isolates showed a five-banded *Gpi* genotype (Table 4). There were unique genotypes in each collection. A total of 15 of 32 genotypes were unique to the 1988-89 collection, and 20 of 48 genotypes (excluding isolates with five-banded *Gpi* allozyme) were unique in the 1997-98 sample. Thus, 8.4% of the isolates collected in 1988-89 and 5.1% of the isolates collected in 1997-98 were unique.

The most common genotype (based on mating type, *Gpi*, and *Pep*) was different in the two sampling periods. The "A2, *86/100*, *100/100*" genotype was the most common genotype in the 1988-89 sample with a frequency of 18.4% (Table 4). The "A2, *100/100*, *100/100*" genotype was most common in the 1997-98 sampling period and showed a frequency of 13.1% (Table 4). In both sampling periods, the *100 Gpi* and *Pep* alleles were most frequent (Table 5). The *83* and *130 Gpi* alleles were rare in the 1997-98 sampling period and were not detected in the 1988-89 sampling. A five-banded genotype was detected only in the 1997-98 sampling period at a low frequency (Table 4). Frequency patterns for each mating type and allozyme allele for the two sampling periods were very similar. Mating-type frequencies did not depart significantly from a 1:1 ratio ($P = 0.204$ for 1988-89; $P = 0.803$ for 1997-98).

Genomic fingerprinting. Southern blot analysis of two subsets of isolates belonging to the two most common multilocus genotypes in the 1997-98 sampling period (defined by mating type, *Gpi*, and *Pep*) revealed much diversity within each multilocus genotype. Among 26 individuals with the "A2, *100/100*, *100/100*" multilocus genotype, 17 (65%) had unique RFLP fingerprints, and among 27 individuals with the "A2, *86/100*, *100/100*" multilocus genotype, 23 (85%) had unique RFLP fingerprints. In these two subsets (53 individuals), there were only eight cases in which the same RFLP pattern was observed in more than one isolate. Seven RFLP patterns were each seen in two isolates, whereas one pattern was seen in eight isolates.

Subpopulations associated with different habitats. The pattern of mating-type distribution was similar in both sampling periods (Table 5). Only the WILD subpopulation in the 1997-98 sampling period deviated significantly from a 1:1 ratio for mating-type frequencies (Table 5).

In both sampling periods the greatest diversity occurred in the population from the VALLEY habitat. The VALLEY subpopulation showed the largest number of multilocus genotypes g (Table 6). Multilocus genotypic diversity, H', decreased from VALLEY to WILD subpopulations (Table 6), and was significantly lower in the WILD subpopulation than in the VALLEY subpopulation for both sampling periods (Table 7).

The evenness index, E_5, reflecting whether or not one or a few genotypes dominate a population, was very similar for the three subpopulations in the 1997-98 sampling period in which sample size was large enough to test for evenness (Table 6). Calculating richness on a sample size of $n = 98$ isolates (the largest common sample size of subpopulations to be compared) in a population, we would expect to find 27 distinct genotypes in the VALLEY, and 28 and 21 in RURAL and WILD subpopulations, respectively (Table 6; Fig. 2). Combining information on evenness and richness, it appears that for the 1997-98 sampling period, in which sample size was large, genotypic diversity differs mostly due to changes in richness (28.6% maximal difference for g_{98}) rather than evenness (9.5% maximal difference for E_5) (Table 6).

The Toluca valley population of *P. infestans* was genetically differentiated according to habitat. Unbiased expected heterozygosity was greatest in the VALLEY subpopulations and least in the WILD populations (Table 6). With one exception (*Pep* in the 1988-89 sampling period), heterozygosities at each individual locus (mating type, *Gpi*, and *Pep*) showed the same trend as heterozygosities for all combined loci. The proportion of total genetic diversity due to differentiation (G_{ST}) among sub-subpopulations within subpopulations was moderate to large within all subpopulations. In the 1988-89 collection, it ranged from 0.071 for the VALLEY subpopulation to 0.449 for the WILD subpopulation (Table 6). In the 1997-98 collection it ranged from 0.061 in the VALLEY subpopulation to 0.168 in the WILD subpopulation (Table 6). Overall, differentiation among the VALLEY, RURAL, and WILD subpopulations was moderate with G_{ST} values of 0.189 for the 1988-89 and 0.092 for the 1997-98 sampling periods.

Based on Rogers' genetic distance, the VALLEY and RURAL subpopulations were more closely related to each other than to the WILD subpopulations in both sampling periods, and the VALLEY-RURAL node appeared in 70 and 61% of 1,000 bootstrapped samples (Fig. 3). Based on exact tests, differentiation among the three subpopulations was significant ($P < 0.001$). The VALLEY subpopulation was significantly differentiated from the WILD subpopulation in both sampling periods, whereas RURAL and VALLEY and RURAL and WILD subpopulations were only significantly different in the 1997-98 sampling period (Table 7). The overall genetic differences between VALLEY/RURAL and WILD subpopulations were larger in 1988-89 (0.14 Rogers' genetic distance) than in 1997-98 (0.08 Rogers' genetic distance) (Fig. 3).

Metalaxyl resistance. In contrast to the frequent resistance to metalaxyl in the 1988-89 collection (23), most isolates in the 1997-98 collection were sensitive to metalaxyl (Fig. 4). Frequency distributions of metalaxyl resistance (at both concentrations of metalaxyl) for the three populations were not significantly different based on the Kruskall-Wallis test (Fig. 4B, $\chi^2 = 3.55$, $P = 0.1697$; and Fig. 4C, $\chi^2 = 5.54$, $P = 0.0627$).

Because the frequency distribution of sensitivity to metalaxyl in the 1997-98 collection differed from that in the 1988-89 collection

TABLE 6. Sample size (n), number of observed multilocus genotypes (g), multilocus genotypic diversity (H'), evenness (E_5), richness (g_{16} and g_{98}), unbiased expected heterozygosity (*Het*), and differentiation within the Toluca valley population and among subpopulations for multiple loci (G_{ST}) for two sampling periods (1988-89 and 1997-98) and three subpopulations (VALLEY, RURAL, and WILD)[a]

Date	Location[b]	n[c]	g[d]	H'[e]	E_5[f]	g_{16}[g]	g_{98}[h]	*Het*[i]	G_{ST}[j]
1988-89	VALLEY	138	28	2.8	0.74	10	24	0.432	0.071
	RURAL	16	8	2.0	1.59	8	...	0.391	0.071
	WILD	25	11	2.2	1.23	9	...	0.360	0.449
	Overall	179	32	2.8	0.72	10	24	0.420	0.189
1997-98	VALLEY	177	35	3.0	0.74	11	27	0.429	0.061
	RURAL	114	30	2.9	0.81	11	28	0.408	0.066
	WILD	98	21	2.5	0.78	9	21	0.360	0.168
	Overall	389	48	3.1	0.70	11	27	0.408	0.092

[a] This analysis excludes the five-banded *Gpi* genotype observed in the 1997-98 sampling.
[b] WILD = wild *Solanum* spp., RURAL = land-race varieties in low-input production systems, and VALLEY = modern cultivars in high-input agriculture.
[c] n = number of isolates collected per population or subpopulation.
[d] g = number of multilocus genotypes observed.
[e] H' = Shannon-Wiener diversity index for multilocus genotypes (33).
[f] E_5 = index of evenness for multilocus genotypes (20).
[g] g_{16} = expected number of multilocus genotypes calculated for a sample size of $n = 16$ isolates per population estimated using the rarefaction method.
[h] g_{98} = expected number of multilocus genotypes calculated for a sample size of $n = 98$ isolates per population estimated using the rarefaction method. This index was only calculated for populations with large enough sample sizes. ... indicates it can only be calculated on populations with a sample size $n \geq 98$.
[i] *Het* = unbiased expected heterozygosity based on mating type, *Glucose-6-phosphate isomerase* (*Gpi*) and *Peptidase* (*Pep*) loci (17,26).
[j] G_{ST} = genetic differentiation among populations estimated using Nei's coefficient of differentiation for genotypes defined by *Gpi* and *Pep* loci (25,35).

(23), a portion of the 1997-98 collection was tested again with a subset of 47 isolates from the 1997-98 collection. Correlations between metalaxyl resistance (relative growth of isolates on metalaxyl-amended medium relative to that on medium without metalaxyl) in the two independent experiments on the same isolates were significant at both 5 µg/ml ($r = 0.66$; $P < 0.001$) and 100 µg/ml ($r = 0.58$; $P < 0.001$). Frequency distributions of metalaxyl resistance of the two independent assays were not significantly different based on the Wilcoxon two-sample test ($Z = 1.371$, $P = 0.1703$ at 5 µg/ml; $Z = 0.368$, $P = 0.7132$ at 100 µg/ml).

TABLE 7. Pairwise probabilities for population differentiation shown above and genotypic diversity shown below the diagonal for both sampling periods[a]

Date	Location[b]	VALLEY	RURAL	WILD
1988-89	VALLEY	...	0.773	0.019
	RURAL	<0.001	...	0.366
	WILD	<0.001	0.004	...
1997-98	VALLEY	...	0.004	<0.001
	RURAL	0.924	...	<0.001
	WILD	0.001	0.006	...

[a] Probability values for the Shannon-Wiener genotypic diversity index were calculated using *t* tests (21). Probability values for population differentiation over three loci (mating type, *Glucose-6-phosphate isomerase*, and *Peptidase*) were calculated using exact tests (29). The Bonferroni correction for these pairwise comparisons for an overall significance value of $\alpha = 0.05$ is $P = \alpha/3 = 0.017$.

[b] WILD = wild *Solanum* spp., RURAL = land-race varieties in low-input production systems, and VALLEY = modern cultivars in high-input agriculture.

Effect of formulation of metalaxyl. Isolates of *P. infestans* responded similarly to the two Ridomil products, metalaxyl and mefenoxam. Correlations between responses to the two commercial formulations of metalaxyl and mefenoxam were highly significant ($P < 0.001$) at 5 µg/ml ($r = 0.91$) and 100 µg/ml ($r = 0.96$) of active ingredient. Similarly, correlations between responses to technical grade and the commercial formulation of metalaxyl were highly significant ($P < 0.001$) at 5 µg/ml ($r = 0.94$) and 100 µg/ml ($r = 0.93$) of metalaxyl. Control treatments with technical grade metalaxyl versus commercial formulations of metalaxyl/mefenoxam consisted of DMSO-amended Rye-A agar versus plain Rye-A, respectively. DMSO-amended Rye-A agar reduced growth of *P. infestans* relative to plain agar by 7%: $y = 0.93\,x$ ($r^2 = 0.99$), where y and x are radial growth (millimeters) on DMSO amended and plain Rye-A agar, respectively.

DISCUSSION

Our observations confirm previous reports (9,39) of high genetic diversity in the population of *P. infestans* found in the Toluca valley region. The overall population maintains approximately equal frequencies of the A1 and A2 mating types and a large diversity of multilocus genotypes even within single fields. Additionally, there was significant diversity among isolates of identical mating type and allozyme pattern. Clearly, the few markers used in this study are insufficient to identify identical individuals. Nonetheless, there was no evidence of dominance by any particular genotype or clonal lineage and, as previously suggested, the population has all the characteristics of a sexually recombining population (9).

Our data support the hypothesis that the Toluca valley population of *P. infestans* is substructured. We found significant substructuring according to host-management system: the WILD, VALLEY, and RURAL subpopulations differed from each other ($G_{ST} = 0.189$ in 1988-89 and 0.092 in 1997-98). Genotypic diversity, richness, and heterozygosity were greatest in the VALLEY subpopulation and were generally smallest in the WILD subpopu-

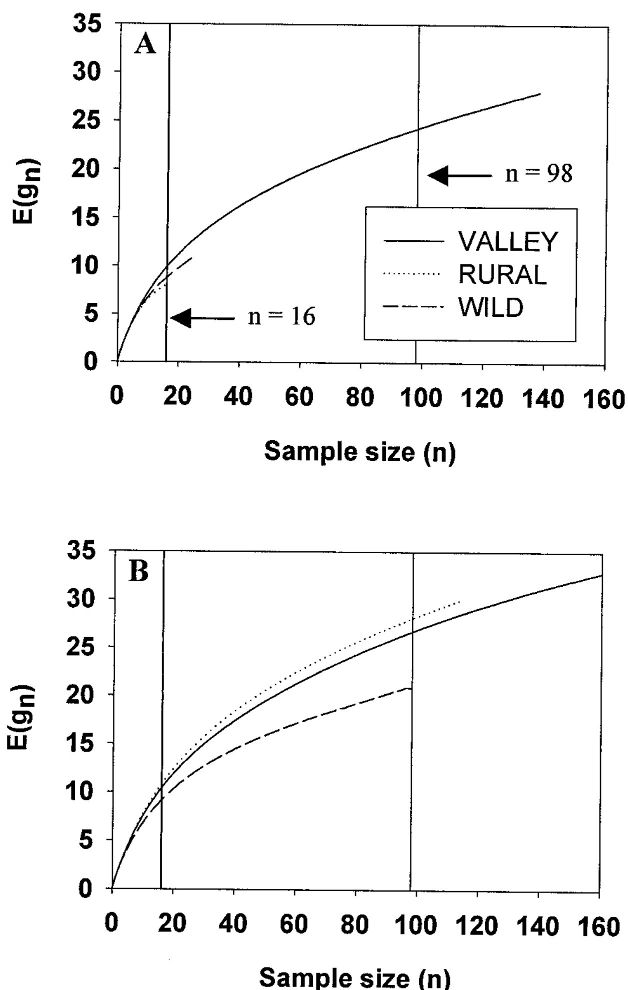

Fig. 2. Expected number of multilocus genotypes estimated with rarefaction curves for three populations of *Phytophthora infestans* within the Toluca valley sampled in **A,** 1988-89 and **B,** 1997-98. The reference lines are drawn at $n = 16$ and $n = 98$.

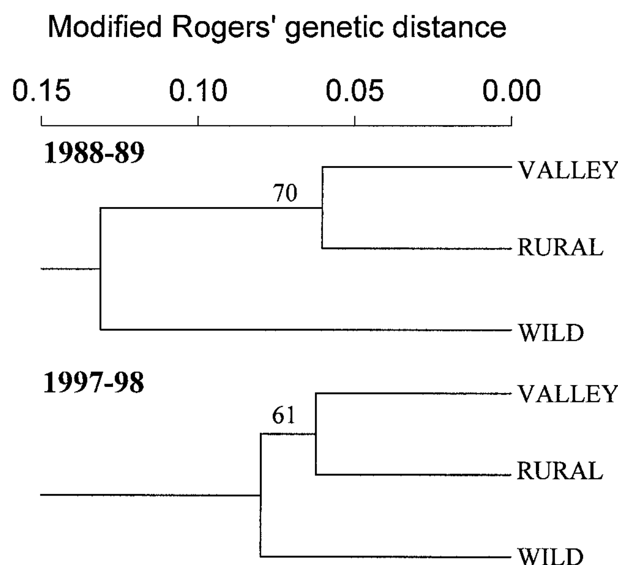

Fig. 3. Unweighted pair-group method with arithmetic average cluster analysis of three subpopulations of *Phytophthora infestans* within the Toluca valley based on the modified Rogers' genetic distance of *Peptidase* and *Glucose-6-phosphate isomerase* allozyme loci sampled in **A,** 1988-89 and **B,** 1997-98. Statistical support for phenogram branches was obtained using 1,000 bootstrapped samples.

lations. There was no predominance by any genotype in any of the host-management systems. Based on the neutral markers used in this study, the WILD subpopulation was more distantly related to VALLEY and RURAL subpopulations than VALLEY and RURAL subpopulations were to each other.

The small sample size and the fact that isolates from the WILD subpopulation were collected in the valley in 1988-89 limit the comparisons that can be made between the two sampling periods. Within these constraints, we observed a slight shift in the similarities in population structure between the two sampling periods. Richness, evenness, and diversity for the RURAL subpopulation were more similar to the WILD subpopulation in the 1988-89 sampling but became more similar to the VALLEY subpopulation in the 1997-98 sampling. This suggests that the RURAL habitat has become more similar to the VALLEY habitat. One reason for this observation might be that farmers in the RURAL habitat now grow modern cultivars such as Alpha and Atlantic and use fungicides more intensively.

Some isolates in these collections had allozyme alleles previously unreported from central Mexico. The *111 Gpi* allele, previously reported in Mexico only from Los Mochis and Saltillo (9), was observed at low frequencies in both sampling periods. The *90 Gpi* allele, found in 1 of 41 isolates from a 1983 sampling in the Toluca valley (9), was not observed in our sampling. The *78* and *96 Pep* alleles detected in our study have not been reported previously for the Toluca valley (9,39). We did not find any novel alleles that are not currently listed in the global marker database for *P. infestans* (4).

Our data did not support the hypothesis that metalaxyl-resistant isolates occur at a higher frequency in the VALLEY than in the WILD habitat. The distribution of classes of metalaxyl resistance of the VALLEY, RURAL, and WILD subpopulations were not statistically different. Because the wild species are not treated with fungicide, selection for metalaxyl resistance does not occur on these plants. Instead, the occurrence of similar metalaxyl-resistance frequencies in all three systems suggests that either migration among habitats is common or that use of metalaxyl is not common.

Metalaxyl resistance had a very different frequency distribution in 1997-98 than that documented from the 1988-89 collection (23). The proportion of isolates that grew more than 40% of the control on 5 and 100 μg ml^{-1} metalaxyl-amended medium was much lower for isolates collected in 1997-98 (13%) than for isolates collected in 1988-89 (60%). The frequency for 1997-98 is similar to frequencies of metalaxyl resistance observed in central Mexico in the early 1980s (23). It is unlikely that introduction of mefenoxam between the two sampling periods could have resulted in lowering the proportion of isolates sensitive to metalaxyl. We detected no differences in frequency distributions of resistance to either of the two formulations of Ridomil.

Observations on the chronology of epidemics within a field season and geographical relationships among the subpopulations of *P. infestans* are consistent with our observation that the WILD subpopulation is both less heterozygous and less diverse than the RURAL or VALLEY subpopulations. Epidemics within the central valley usually start at the beginning of July when rainfall occurs almost daily (11). However, epidemics of late blight on wild *Solanum* spp. rarely occur before the end of August or beginning of September. In addition to the chronological patterns, the geographic relationship might play a role in structuring populations. Wild *Solanum* spp. occur at the edges of forests, RURAL potato fields, and sometimes at the edge of commercial fields. These species emerge before commercial potatoes emerge and reproduce mostly clonally through tubers.

We therefore hypothesize that the recent subpopulation of *P. infestans* in the WILD habitat is most likely derived from the subpopulations in RURAL or VALLEY habitats. This hypothesis is supported by observations that (i) genetic diversity and heterozygosity are lower in the WILD subpopulation compared with the RURAL/VALLEY subpopulations, and (ii) the frequency distribution of metalaxyl resistance is not different between the WILD and the RURAL/VALLEY subpopulations. It seems likely that the

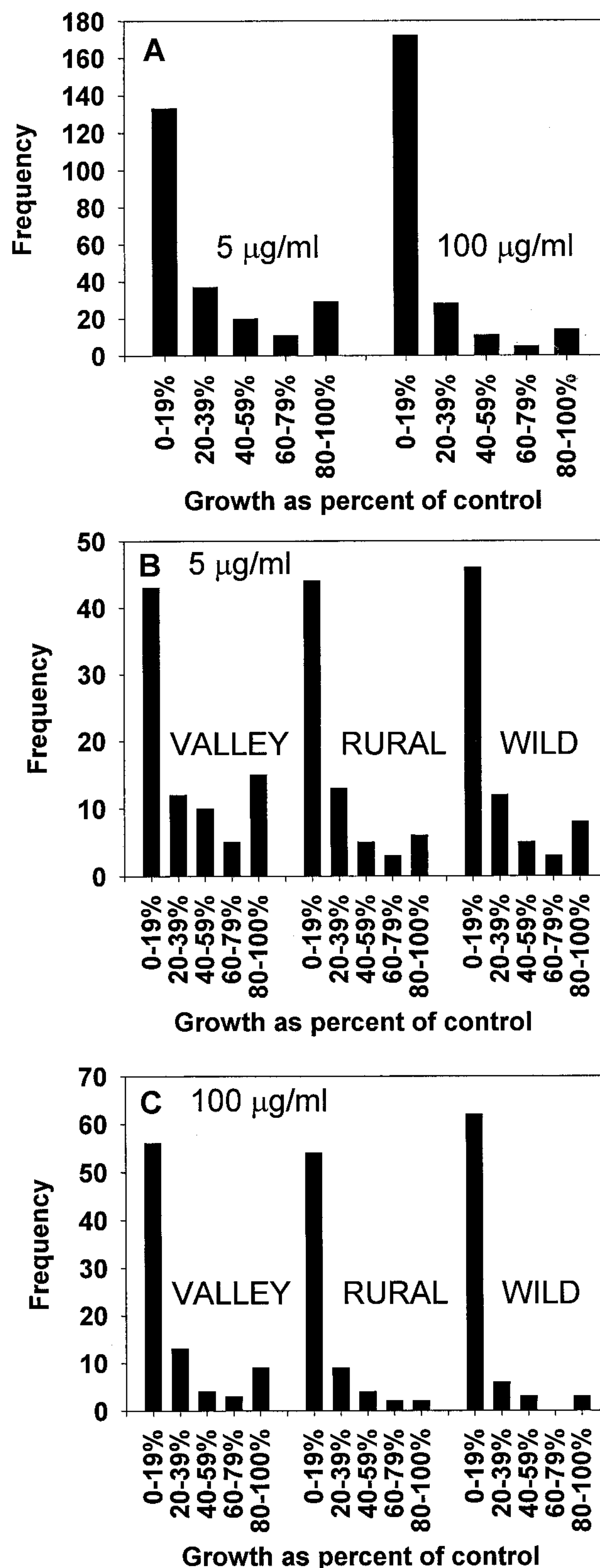

Fig. 4. Relative growth of isolates of *Phytophthora infestans* on Rye-A agar amended with metalaxyl. **A,** Frequency distributions of relative growth of isolates from the Toluca valley population amended with metalaxyl (5 and 100 μg ml^{-1} [n = 230]), and of relative growth of isolates from VALLEY (n = 85), RURAL (n = 71), and WILD (n = 74) subpopulations at **B,** 5 μg of metalaxyl ml^{-1}, and **C,** 100 μg of metalaxyl ml^{-1}.

current situation is different than it was in the 1950s when large-scale potato production was first introduced to the highlands of central Mexico. At that time, wild species were regarded as the source of *P. infestans* that infected commercial potatoes. Decades of intensive potato production could have altered the situation so at the end of the 20th century, populations of *P. infestans* associated with intensive commercial potato production dominate the entire region, including the wild species.

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