Genetic Diversity in Populations of *Phytophthora citricola* Associated with Horticultural Crops in California

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ABSTRACT

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California populations of the plant pathogen *Phytophthora citricola* were examined for amplified fragment length polymorphism (AFLP), pathogenicity on almond, and sensitivity to mefenoxam. The characterizations of AFLP variation and mefenoxam sensitivity were based on 86 isolates (44 from almond, 11 from avocado, 3 from strawberry, 18 from walnut, and 10 from six other hosts). Cluster analysis of the AFLP data using the unweighted pair group method indicated a high level of genetic diversity among the isolates, and four main clusters were identified—one dominated by isolates from almond, another including all isolates from avocado, and two including isolates from several hosts other than avocado. Analysis of molecular variance revealed that 38.4 and 24.9% of the AFLP variation were associated with host and geographical factors, respectively. Of 24 isolates, including those from almond, avocado, strawberry, and walnut, 22 were aggressive on almond shoots; there was no evidence of host specificity. All but 1 of the 86 isolates grew at different rates on V8 juice medium amended with mefenoxam at 1 ppm, indicating partial tolerance to the fungicide. Isolates of *P. citricola* from California populations are genetically diverse, and much of the variation is associated with host and geography. These populations are all potentially pathogenic on almond and tolerant to mefenoxam.

Additional keyword: AMOVA

Phytophthora citricola Sawada is an important soilborne pathogen worldwide and causes diseases on many host plants (1,14,19,21,29,31,39,42,46,47). On most hosts, *P. citricola* incites root, crown, and stem or trunk rots initiated belowground, but it also causes twig blights, fruit rots, and scion cankers resulting from aerial infections (4,9,26,27). In California, *P. citricola* occurs on hosts in coastal as well as interior regions. It is most important as an economic pathogen on almond, avocado, and walnut, but also infects many other hosts in the state, including citrus, hops, several oak species, Monterey Pine, olive, raspberry, strawberry, sweet cherry, and various container-grown ornamentals (4,6,11,32,35,36,40,43,50).

Diversity in *P. citricola* has been reported in terms of morphological traits (13,49,50), total protein profiles (12), isozyme patterns (20,37), mitochondrial and nuclear DNA restriction sites (18), and

DNA sequence differences in ribosomal internal transcribed spacers (ITS) 1 and 2 (8,10,17). Oudemans et al. (37) suggested that *P. citricola* consisted of several subgroups, some of which may be regarded as separate biological taxa. Although amplified fragment length polymorphism (AFLP) genotyping has not been applied extensively to *P. citricola*, the method is one of the most comprehensive and reproducible DNA fingerprinting approaches (24,48).

Genetic and pathogenic characterizations of *P. citricola* may provide important insights into the epidemiology and management of the diseases it causes. For example, although *P. citricola* was not previously known as a widespread pathogen on almond in California, within the last 10 years it has contributed to high incidences of lethal perennial cankers in several San Joaquin Valley orchards (4). It is unknown whether or not these outbreaks are due to novel, highly aggressive strains of the pathogen or to strains endemic to the Central Valley; genetic fingerprinting could help examine these possibilities. Also, for evaluations of genetic resistance to *P. citricola* in rootstocks, adequate representation of the genetic diversity of the pathogen in screening programs would be facilitated by genetic analysis of the pathogen's populations.

Some of the diseases caused by *P. citricola* have been managed, in part, by appli-

cations of the systemic acylalanine fungicide, metalaxyl (mefenoxam is the active isomer). However, there have been no recent extensive evaluations of resistance to the compound in California populations of the pathogen. Tolerance to metalaxyl was reported previously for *P. citricola* in California (7,16,25).

A collection of *P. citricola* from numerous locations and hosts in California has been accumulated over the last 30 years and maintained by the United States Department of Agriculture–Agricultural Research Service at the University of California, Davis (G. T. Browne and S. M. Mircetich, unpublished). Herein, we report on use of this collection for AFLP genotyping, pathogenicity assays, and mefenoxam sensitivity assays. Our objectives were to examine genetic and pathogenic variation in isolates of *P. citricola* from California, with emphasis on isolates from almond, avocado, and walnut, and to evaluate their sensitivity to mefenoxam.

MATERIALS AND METHODS

Isolates of *P. citricola*. The study included 86 isolates of *P. citricola* from different regions in California. In all, 44 of the isolates were from almond, 11 from avocado, 3 from strawberry, 18 from walnut, and 10 from six other plant species (Table 1). Of these isolates, 88% originally were cultured directly from host tissue, and the remaining were isolated from soil surrounding roots of diseased hosts by using a pear bait method (14). All 86 isolates were subcultured from single-zoospore colonies, identified morphologically (44), and stored at 14°C under sterile mineral oil in vials containing V8 juice agar (V8JA).

Extraction of DNA from *P. citricola*. Mycelia were grown for DNA extraction by transferring 10 4-mm-diameter disks of V8JA covered with actively growing mycelium to 10 ml of sterile, clarified V8 juice broth (V8JB) (200 ml of V8 juice, 2 g of CaCO₃, and 800 ml of deionized water). The cultures were incubated for 4 days at 20 to 22°C. Total genomic DNA was extracted from the mycelia using the method of Lee and Taylor (30). As a control, sterile V8JA disks incubated in clarified V8JB were subjected to the DNA extraction procedure.

AFLP analysis of *P. citricola*. AFLP fingerprinting was performed using the

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AFLP Analysis System II kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions with the following modification: the fluorescent dye, ChromaTide tetramethylrhodamine-6-dUTP (TMR; Molecular Probes, Eugene, OR), was used in the polymerase chain reaction (PCR) mix instead of radioactively labeled primers to facilitate detection using an ABI 377 DNA sequencer. Briefly, approximately 250 ng of total DNA per isolate was digested completely using restriction enzymes *EcoRI* and *MseI* before adapters were ligated to restriction fragments using T4 DNA ligase. A 10-fold dilution of the ligated DNA was subjected to nonselective preamplification using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA). Primers for nonselective preamplification were *MseI*-adapter primer containing one selective nucleotide (M+C) and *EcoRI*-adapter primer containing no selective nucleotide (E+0). The PCR steps included 20 cycles of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s, with a final step of cooling to 4°C. Preamplified PCR products were diluted 1:50 with Tris-EDTA buffer and subjected to selective AFLP amplification. Selective amplification was achieved with 14 cycles of 94°C for 30 s, 65°C for 30 s (annealing temperature was lowered 0.7°C for each cycle), and 72°C for 60 s; followed by 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s; with a final extension for 7 min and cooling to 4°C. All PCR products were stored at 4°C if the gel was not run immediately after the amplification.

In selective amplifications, primers for the *EcoRI* adapter contained two selective nucleotides (E-A + 1 and E-T + 1) and those for *MseI* adapter contained three selective nucleotides (M-CA + 1 and M-

CT + 1). After prescreening of 64 primer pairs (the possible combinations of 8 *EcoRI* and 8 *MseI* primers), 12 primer pairs were selected (E-AA/M-CAC, E-AA/M-CTA, E-AT/M-CAT, E-AG/M-CTC, E-AC/M-CAA, E-AC/M-CAC, E-TA/M-CTA, E-TA/M-CTC, E-TG/M-CAG, E-TG/M-CTG, E-TC/M-CTT, and E-TC/M-CTC). These primer pairs provided consistent amplification of DNA fragments when used repeatedly in successive runs on test isolates. For each selective PCR amplification, DNA samples of *Arabidopsis* and tomato, which were supplied in the AFLP kit, were used as positive controls, and the extraction from V8JA served as a negative control.

Samples of the PCR products (2 µl) were mixed with 1 µl of standard molecular weight markers (GeneScan-500 ROX; Applied Biosystems), absorbed in 96-lane porous membrane combs (The Gel Company, San Francisco), loaded on 5% polyacrylamide gels, and subjected to analysis on an ABI 377 DNA sequencer. Bands of the fluorescing DNA were tracked and extracted using GeneScan software (version 3.1; Applied Biosystems). The base pair length of each DNA band was determined using Genotyper software (version 2.5; Applied Biosystems). Subsequently, all bands from 75 to 350 bp were scored and labeled manually according to their size in base pairs and the primer pairs that amplified them.

Pathogenicity of *P. citricola* on almond. Twenty-four isolates of *P. citricola* were tested for pathogenicity on excised segments of almond shoots. Each experiment included eight isolates from almond, five from avocado, three from strawberry, and eight from walnut (Table 1). The 1- to 2-year-old almond shoot segments (20 cm long and 5 to 15 mm in diameter) were cut

from the cultivar Drake, wounded by using a cork borer to remove a 6-mm-diameter disk of bark midway along each segment's length (34), and inoculated by placing a 4-mm-diameter disk of V8JA covered by mycelium of an isolate of *P. citricola* into the wound (mycelium facing the wound surface). Control shoots were wounded and inoculated with sterile V8JA. After inoculation, all wounds were wrapped with electrical tape. The shoots were incubated at 20 to 22°C on supports above a free water surface in ventilated incubation chambers that were sealed in polyethylene bags. There was one shoot per inoculum treatment in each of 10 randomized complete blocks (incubation chambers). Seven days after inoculation, a sharp knife was used to remove the epidermis and outer bark from each shoot segment to reveal the margins of necrotic and discolored bark tissue. The total length of necrosis and discoloration extending from the point of inoculation was measured. The experiment was conducted twice.

Sensitivity of *P. citricola* to mefenoxam. All 86 isolates of *P. citricola* were tested for sensitivity to mefenoxam. Isolate 267 of *Pythium irregulare* (completely resistant to metalaxyl) and isolate 254 of *P. sulcatum* (partially resistant to metalaxyl), both provided by R. M. Davis, University of California, Davis, and isolate sm3398 of *Phytophthora cactorum* (sensitive to mefenoxam) were used as standards for comparison. The isolate that was completely resistant to metalaxyl grew normally (without any reduction in growth) in the presence of mefenoxam at 1 to 100 ppm. In contrast, the isolate that was partially resistant to metalaxyl exhibited up to 50% growth reduction, whereas the sensitive isolate did not grow in the presence of mefenoxam. For all isolates, a 4-mm-

Table 1. Backgrounds of the 86 isolates of *Phytophthora citricola* characterized in this study

Host plant	Location in California	No. of isolates	Isolates ^a
Almond	Central Interior	44	gb614 ^b , gb695, gb739, gb801 ^b , gb802, gb811, gb812 ^b , gb819, gb821, gb822, gb1119, gb1205, gb1206, gb1207, gb1219, gb1227, gb1229, gb1230, gb1232, gb1235 ^b , gb1237, gb1243, gb1261, gb1322, gb1436, gb1925, gb1934, gb1964, gb1971, gb2002, gb2003, gb2008 ^b , gb2012, gb2063 ^b , gb2066, gb2067, gb2222, gb2224 ^c , gb2226, gb2734, gb2758 ^b , gb2767, gb2982, sm3416
Apricot	Central Interior	1	sm3342
Avocado	South Coast	11	tc-gb3588, tc-gb3590 ^b , M213, M214, M215 ^b , M216 ^b , M218, M219 ^b , M220, M265 ^b , M266
Cherry	Central Interior	2	sm3491, sm3492
Hops	Northern Interior	2	sm3343, sm3392
Lilac	Northern Interior	2	sm3341, sm3361
Olive	Northern Interior	1	sm3487
	South Coast	1	gb3156
Raspberry	Central Coast	1	sm3350
Strawberry	Central Coast	2	skTri2.3.7 ^b , skTri2.3.9 ^b
	South Coast	1	sm3466 ^b
Walnut	Central Interior	6	gb1281 ^b , sm3474 ^b , sm3475, sm3495, sm3496, gb3974
	Northern Interior	10	gb572, gb579, gb582 ^b , gb1494, gb1572 ^b , gb1592 ^b , gb2062 ^b , sm3390 ^b , sm3410, sm3494
	South Coast	1	gb2461 ^b
	Outside of California	1	sm3304

^a Abbreviations: 'gb' and 'sm' indicate isolates from G. T. Browne and S. M. Mircetich, respectively, United States Department of Agriculture-Agricultural Research Service, CPGRU, University of California, Davis; 'sk' and 'tc-gb' indicate isolates originally from S. T. Koike, University of California Cooperative Extension, Salinas and C. T. Chao, University of California, Riverside, respectively; and 'M' indicates isolates from J. A. Menge, University of California, Riverside.

^b Isolates used for pathogenicity tests on almond shoots.

diameter disk covered by mycelium was removed from the margin of a 4- to 5-day-old V8JA culture and placed in the center of a 90-mm-diameter petri plate containing 10 ml of V8JA amended with mefenoxam at 1 ppm (active ingredient in Ridomil Gold; Syngenta Crop Protection, Inc., Greensboro, NC). Control inoculations

were conducted identically, but with nonamended V8JA. Sensitivity to mefenoxam was assessed after 5 days of incubation at 20 to 22°C by measuring the resulting colony diameters and calculating the percentage of radial growth reduction due to mefenoxam amendment relative to the nonamended controls. The same meth-

ods and standards were used to assay sensitivity to mefenoxam at 1, 10, 25, or 100 ppm in the 24 isolates of *P. citricola* examined for pathogenicity on almond. The experiments were conducted three times, each with one culture plate per mefenoxam concentration (and the nonamended control) per isolate.

Data analyses. Binary code of 1 or 0 was used to indicate the presence or absence, respectively, of the AFLP bands from 75 to 350 bp, and a rectangular data matrix of AFLP bands $\times$ isolates was prepared. The genetic relationship among isolates was computed using the Dice coefficient of similarity in SimQual module of NTSYSpc software (version 2.11S; Exeter Software, New York), and the resulting similarity matrix was subjected to cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) algorithm in the SAHN module of NTSYSpc. Bootstrap values were computed for UPGMA clustering using PAUP* software (version 4.0b10; Sinauer Associates, Inc., Sunderland, MA). The AFLP data also were subjected to analysis of molecular variance (AMOVA) using Arlequin version 2.000 software (41). Hierarchical partitioning of molecular variation within and among various isolate groupings was performed by considering AFLP banding patterns of isolates as haplotypes with an unknown gametic phase. The AMOVA module uses squared Euclidean distances between pairs of haplotypes to compute variance components (15). Variance was partitioned into components among and within isolate groupings based on the following parameters: (i) host (almond, avocado, strawberry, walnut, or other hosts), (ii) niche (an aerial plant part, root system, or soil), and (iii) geographical region (Central Coast, South Coast, Central Interior, or Northern Interior of California; or Outside of California). The variance components and fixation index (F_{ST}) were calculated for each grouping with 16,000 permutations.

Disease and growth data from the pathogenicity and mefenoxam experiments were subjected to analysis of variance using PROC ANOVA (SAS software release 8.02; SAS Institute, Raleigh, NC). Treatment means were separated according to least significant differences at an α probability level of 0.05.

RESULTS

Genetic variation and cluster analysis of AFLP data of *P. citricola*. AFLP analysis using the 12 primer pairs among all 86 isolates of *P. citricola* revealed a high degree of genetic dissimilarity in the collection. Among the isolates, 263 polymorphic DNA fragments were produced in the size range of 75 to 350 bp. In all, 7 to 32, with an average of 22, distinct DNA fragments were produced per primer pair. The 12 primer pairs produced a total of 28 to 75

Fig. 1. Dendrogram of genetic relationship among 86 isolates of *Phytophthora citricola*, based on an unweighted pair group method with arithmetic mean cluster analysis of DNA amplified fragment length polymorphisms generated from polymerase chain reaction with 12 primer pairs. Numbers at nodes represent bootstrap values from 1,000 replications, and bootstrap values $>55\%$ are indicated. The first two letters of isolate labels indicate the source host and the next two letters indicate the source region.

distinct DNA fragments per isolate; the average was 50 fragments per isolate.

The cluster analysis using UPGMA with all 86 isolates indicated high levels of genetic diversity both among and within isolate clusters, and some of the clusters were dominated by isolates from certain hosts in certain geographical regions (Fig. 1). At Dice coefficients of similarity (DCS) of approximately 0.21 to 0.50, four main clusters were resolved, arbitrarily designated Alm, Avo, Mix I, and Mix II (Fig. 1). The Alm cluster contained only eight isolates from almond with shared DCS values of 0.55 to 0.72. Cluster Mix I contained 58 isolates representing all hosts but avocado; its isolates represented all geographical regions and shared DCS values from 0.54 to 1.0. One of the major subclusters within Mix I consisted almost exclusively of 24 isolates from almond; it contained only

one additional isolate from raspberry. An additional subcluster within Mix I contained all of the 17 isolates from walnut as well as 16 isolates representing all hosts except avocado. The cluster Mix II consisted of nine isolates from four hosts (six from almond and one each from hops, strawberry, and walnut). Collectively, Mix II isolates originated from Central Interior and Northern Interior regions, the Central Coast, and one location Outside of California. Mix II isolates shared coefficients of similarity of 0.50 to 0.67. The cluster Avo included only the 11 isolates from avocado (all from the South Coast), and they shared DCS values of 0.48 to 0.83.

Hierarchical partitioning of genetic variation in *P. citricola*. When the 86 isolates of *P. citricola* were sorted into five host groups (almond, avocado, strawberry, walnut, and other hosts), AMOVA and the

associated fixation index (F_{ST}) indicated that 38.4% of the variance in AFLP band incidence was attributable to host groupings ($P = 0.000$; Table 2). Similarly, 24.9 and 5.5% of variance was attributable to geographical area (Central Coast, South Coast, Central Interior, or Northern Interior of California; or Outside of California) and niche (an aerial plant part, root system, or soil), respectively ($P = 0.000$ to 0.003).

F_{ST} values facilitated pairwise genetic comparisons between groups of isolates (Table 3). Nearly all groups sorted by host differed significantly from one another ($P = 0.000$ to 0.018); the exceptions were that isolates from strawberry did not differ significantly from isolates in the almond or others groups ($P = 0.073$ to 0.199; Table 3). Among geographical categories, groups from the Central Interior, Northern Interior, and South Coast of California differed significantly from each other ($P \leq 0.002$). Similarly, the Central Coast group differed significantly from the South Coast group ($P = 0.036$), but there was no significant differentiation between Central Coast and Central Interior groupings ($P = 0.688$) or between Central Coast and Northern Interior groupings ($P = 0.095$). The Outside group did not differ significantly from the Central Interior, Central Coast, or the Northern Interior groups ($P = 0.998$), but it did differ significantly from the South Coast group ($P = 0.000$). Among niche categories, the aerial plant part group differed significantly from the root and soil groups ($P = 0.008$ and 0.006, respectively), but the root system group did not differ significantly from the soil group ($P = 0.708$).

Pathogenicity of *P. citricola* on almond. Canker lengths were not affected significantly by experiment or experiment–

Table 2. Hierarchical partitioning of variation among and within population groups of *Phytophthora citricola* based on analysis of molecular variance in 86 isolates of the pathogen

Source of variation	Observed partition		F_{ST}	P^a
	Variance	Total (%)		
Host ^b				
Among groups	10.919	38.42	0.384	0.000
Within groups	17.503	61.58	...	...
Geographical region ^c				
Among groups	6.992	24.90	0.249	0.000
Within groups	21.091	75.10	...	...
Niche ^d				
Among groups	1.407	5.52	0.055	0.003
Within groups	24.069	94.48	...	...

^a Probability of obtaining more extreme random variance component and fixation index (F_{ST}) than the observed values by chance alone.

^b Isolates were grouped into five host categories (almond, avocado, strawberry, walnut, and other hosts).

^c Isolates were grouped into five geographical region categories: Central Interior, Central Coast, Northern Interior, South Coast, and Outside California.

^d Isolates were grouped into four niche categories—aerial plant parts, root system, and soil—from which cultures of *P. citricola* were isolated.

Table 3. Pairwise genetic differentiation among groupings of 86 isolates of *Phytophthora citricola* based on analysis of molecular variance and associated fixation index (F_{ST}) values^a

	Host ^b					Geographical location ^c					Niche ^d		
	Ad (44)	Sy (3)	Wt (18)	Ao (11)	Others (10)	CI (56)	CC (3)	NI (12)	SC (14)	OU (1)	Aerial (53)	Roots (23)	Soil (10)
Ad	...	0.073	0.000	0.000	0.000	0.507	0.479	0.000	0.000	0.998	0.219	0.000	0.000
Sy	0.112	...	0.016	0.002	0.199	0.162	0.998	0.189	0.037	0.998	0.371	0.450	0.258
Wt	0.302**	0.261*	...	0.000	0.018	0.000	0.008	0.576	0.000	0.000	0.000	0.028	0.134
Ao	0.497**	0.475**	0.688**	...	0.000	0.000	0.002	0.000	0.744	0.998	0.000	0.000	0.000
Others	0.128**	0.0450	0.110*	0.568**	...	0.030	0.254	0.637	0.000	0.000	0.000	0.560	0.907
CI	–0.002	0.0510	0.199**	0.504**	0.0490*	...	0.688	0.002	0.000	0.998	0.130	0.002	0.032
CC	–0.0090	–0.3340	0.337**	0.447**	0.0310	–0.0350	...	0.095	0.036	0.998	0.931	0.414	0.278
NI	0.189**	0.0380	–0.0120	0.585**	–0.0140	0.102**	0.101	...	0.000	0.998	0.000	0.432	0.871
SC	0.355**	0.238**	0.507**	–0.0270	0.372**	0.357**	0.221*	0.393**	...	0.000	0.000	0.000	0.000
OU	0.0440	–0.2870	–0.655**	0.531**	–0.463**	–0.1400	–0.3150	–0.6030	0.212**	...	0.998	0.998	0.998
Aerial	0.0050	0.0060	0.223**	0.367**	0.084**	0.009	0.069	0.123**	0.224**	0.1160	...	0.008	0.006
Roots	0.112**	–0.0220	0.069*	0.432**	–0.0140	–0.050**	–0.025	–0.005	0.255**	–0.523	0.053**	...	0.708
Soil	0.129**	0.0210	0.037	0.562**	–0.0410	0.047*	0.026	–0.035	0.365**	–0.5470	0.084**	–0.0220	...

^a Numbers below the diagonal are F_{ST} values, and the numbers above the diagonal are probabilities (P) of having more extreme F_{ST} values than the observed by chance alone. Single (*) and double (**) asterisks indicate F_{ST} values with $P \leq 0.05$ and 0.01, respectively.

^b The isolates were grouped by source host: Ad = almond, Sy = strawberry, Wt = walnut, Ao = Avocado, and others = apricot, cherry, hops, lilac, olive, and raspberry. The number of isolates in each group is indicated in parentheses.

^c The isolates were grouped by source regions: CI = Central Interior, CC = Central Coast, NI = Northern Interior, SC = South Coast, and OU = Outside of California. The number of isolates in each group is indicated in parentheses.

^d The isolates were grouped by source niche: Aerial = aerial parts; Roots = root system, including crown and roots; and Soil. The number of isolates in each group is indicated in parentheses.

inoculum treatment interaction ($P = 0.21$ and 0.98 , respectively); hence, the data were combined across experiments. All isolates of *P. citricola* were pathogenic (i.e., caused necrosis) on the almond shoots, but isolates M215 from avocado and gb1592 from walnut were less aggressive than the other isolates (Fig. 2). No necrosis resulted from the control inoculations. *P. citricola* was reisolated from necrotic bark on representative twigs inoculated with each isolate.

Sensitivity of *P. citricola* to mefenoxam. On V8JA without mefenoxam, radial growth of the mycelium of all 86 isolates of *P. citricola* averaged 77 mm in 5 days; means ranged from 51 to 85 mm. None of the isolates was completely resistant to mefenoxam compared with the metalaxyl-insensitive control isolate of *Pythium irregulare*. On V8JA amended with mefenoxam at 1 ppm, growth reductions of 51 to 90% occurred in 69 isolates and growth reductions of 91 to 100% occurred in 16 isolates (Fig. 3), and they were classified as having tolerance to mefenoxam. Only isolate gb3156 was completely sensitive and failed to grow on the amended medium. Mefenoxam at 10, 25, and 100 ppm caused little more growth suppression than mefenoxam at 1 ppm (Table 4; Fig. 4). Growth of the metalaxyl-

insensitive control, *P. irregulare* isolate 267, was not inhibited by mefenoxam at any of the concentrations tested, whereas that of the mefenoxam-sensitive control, *Phytophthora cactorum* isolate sm3398, was completely suppressed at all concentrations (Fig. 4). Growth of *Pythium sulcatum* isolate 254, the partially resistant

standard, was inhibited from 35 to 54% at 1 to 100 ppm mefenoxam.

DISCUSSION

Using a collection of 86 isolates of *Phytophthora citricola* from 10 important horticultural and ornamental hosts mainly in California, we obtained evidence that

Fig. 3. Sensitivity of 86 isolates of *Phytophthora citricola* to mefenoxam at 1 ppm. Sensitivity was determined by comparing radial growth of mycelium on amended and nonamended V8 juice agar medium cultures in petri dishes 5 days after inoculation; percentages of growth suppression indicate the effect of mefenoxam amendment relative to the nonamended control. For each isolate, there were three replicate cultures per amended and nonamended treatment.

Fig. 2. Pathogenicity of isolates of *Phytophthora citricola* from different hosts on 1- to 2-year-old shoot segments of almond cv. Drake. The shoot pieces were inoculated on wounds and incubated in chambers at $21 \pm 1^\circ\text{C}$ with 100% humidity. Vertical bars are the least significant difference values at an αP level of 0.05. The control shoots had no necrosis or tissue discoloration.

populations of the pathogen are genetically diverse in the state and that much of the differentiation is associated with host and geography. Percentages of AFLP variation that AMOVA associated with hosts (38.4%) and geographical locations (24.9%) are considered to be “very great” and “great” levels of genetic differentiation, respectively, whereas the genetic differentiation associated with niche (5%) is considered to be relatively small (22). Pairwise comparisons, used in conjunction with AMOVA, facilitated additional partitioning of the variance, which revealed genetic differentiation not apparent from the cluster analysis.

It should be considered that, in our AMOVA analyses, host, geography, and niche factors were confounded to varying degrees with one another and with other unknown factors. For example, the geographical locations of hosts were governed largely by growing regions for the hosts, which are affected by climatic requirements of the crops. For example, avocados are largely restricted to coastal regions in the state because of sensitivity to frost, whereas winter chilling required for optimum performance of almond and walnut tends to limit these crops to interior regions of the state.

Despite the high level of genomic diversity found in *P. citricola*, relatively little variation in aggressiveness was detected after inoculation of wounds on excised almond shoots. In these assays, each of the representative isolates was pathogenic and most of them were aggressive, regardless of the original host or geographical origin. Additional research involving more of the source hosts and additional methods of inoculation would be needed for a comprehensive assessment of aggressiveness and host specificity in *P. citricola*. Excised shoot assays involve wounding, which may allow the pathogen to bypass aspects of the natural infection process that potentially are influenced by aggressiveness.

The extent of genetic and pathogenic variation found in *P. citricola* contrast markedly with the extent found previously in California populations of *P. cactorum* (2). In the present study, *P. citricola* had a relatively high level of AFLP fingerprint diversity together with relatively little variation in aggressiveness on almond; whereas, previously, for *P. cactorum*, we found relatively little genetic diversity but significant variation in pathogenicity and aggressiveness on almond. For *P. cactorum*, aggressiveness on almond and strawberry were linked to host specificity. Despite their differences, California populations of both *P. cactorum* and *P. citricola* had significant percentages of their genomic AFLP variation partitioned into components associated with hosts, geographical regions, and niche categories.

Our findings, based on analysis of genomic DNA, confirm previous reports of

diversity in *P. citricola* and highlight the sensitivity of AFLP in resolving variation at the isolate level within such a diverse species. In previous studies, analysis of protein patterns (12), mtDNA (18), and isozymes (37) all revealed high levels of genetic diversity in *P. citricola*. Based on the isozyme examinations, Oudemans et al. (37) distinguished five distinct groups within *P. citricola* and suggested that strains of the pathogen from avocado may be a distinct taxon. Similarly, in our study, four major clusters of isolates could be distinguished, including one from avocado. However, in our study, high levels of genetic diversity and additional clustering occurred within each of the major groups. Interestingly, we found that oogonia and oospores of isolates from avocado were

smaller than those of isolates from other hosts (*data not shown*), which was consistent with previous reports (37,50).

The results of this study provide no evidence that high incidence of perennial Phytophthora canker disease (PPC) on almond in the last 10 years (4; G. T. Browne *unpublished*) resulted from introduction of exotic or highly aggressive strains of *P. citricola*. In total, 82% of the isolates from trees affected by the disease, including the oldest isolate (sm3416, collected in 1980), were clustered in the Mix I and Mix II groups, along with isolates from other miscellaneous hosts (e.g., apricot, cherry, hops, lilac, olive, strawberry, and walnut). This clustering is consistent with a hypothesis that PPC-causing populations are not new, but rather are similar

Table 4. Sensitivity of 24 isolates of *Phytophthora citricola* to concentrations of mefenoxam in V8 juice agar medium^a

Mefenoxam (ppm)	No. of isolates with indicated percentages of mycelial growth reduction			
	61 to 70%	71 to 80%	81 to 90%	91 to 100%
1	2	12	8	2
10	2	9	11	2
25	2	9	11	2
100	2	4	12	6

^a Sensitivity to mefenoxam was assessed in V8 juice agar medium after 5 days of incubation at 20 to 22°C by measuring the resulting colony diameters and calculating the percentage of radial growth reduction from mefenoxam amendment, relative to the nonamended controls. For each isolate, there were three replicate V8 juice agar cultures per concentration of mefenoxam and nonamended control.

Fig. 4. Effect of various concentrations of mefenoxam on 5-day growth reduction of four isolates of *Phytophthora citricola* (Pcit). Standards were Pyirr = *Pythium irregulare* (completely resistant to metalaxyl/mefenoxam), Pysul = *P. sulcatum* (partially resistant to metalaxyl and mefenoxam), and Pcac = *P. cactorum* (sensitive to mefenoxam). Error bars indicate the standard deviations.

to those endemic and affecting multiple hosts in the Central Valley of California. On the other hand, it is possible that some differentiation has occurred in the populations causing PPC; 18% of the PPC isolates clustered separately from all other isolates, and an additional 54% of PPC isolates occurred in the nearly exclusive subcluster of Mix I that contained 24 PPC isolates and one isolate from raspberry.

The origin of diversity in populations of *P. citricola* in California is unknown. Previous investigations have documented the diversity of *P. citricola* worldwide (37,50) and it seems plausible that multiple introductions of diverse strains of the pathogen may have occurred in California over time on various horticultural and ornamental crops. Though *P. citricola* is homothallic, out-crossing might have occurred among diverse members of its populations to generate recombinant variants. Outcrossing was demonstrated for *P. sojae*, which is also homothallic (3). Additional research would be required to establish whether such outcrossing can occur in *P. citricola*. It is likely that such research could shed light on genes involved in host-pathogen interactions and taxonomic traits among strains from almond, avocado, walnut, and other hosts.

The results of the mefenoxam sensitivity assays indicate that California populations of *P. citricola* have tolerance to the chemical. All but one of the isolates in this study would be considered tolerant to mefenoxam at 1 ppm. Furthermore, a subset of the isolates was tolerant to mefenoxam at 10, 25, and 100 ppm. In an earlier study, all California isolates of *P. cactorum* were sensitive to mefenoxam at 1 ppm (2). The results with *P. citricola* and *P. cactorum* suggest that, in some situations, mefenoxam may be less effective for control of *P. citricola* than for control of *P. cactorum*. Nevertheless, in previous studies, mefenoxam suppressed diseases caused by both pathogens (5,33). The fact that a level of tolerance to mefenoxam was almost universal in our collection of *P. citricola* suggests that the tolerance is a trait associated with the species, although more work with populations from other parts of the world would be required to confirm this. It is unlikely that the tolerance resulted solely from selection pressure due to agricultural use of mefenoxam; several of the isolates were obtained from sources that would not have been exposed to the chemical. Field resistance to mefenoxam has been reported in *P. cactorum* (23) and *P. capsici* (28,38). Also, in vitro resistance to metalaxyl was selected in *P. citricola* (7,16,25) and in *P. cactorum* (45), which implies that field resistance to mefenoxam might evolve due to excessive use of the chemical to manage diseases caused by these pathogens.

The results of our AFLP characterizations suggest some practical applications.

For example, given the genetic diversity in *P. citricola*, evaluations of host genetic resistance should include multiple isolates of the pathogen. Also, some of the AFLP markers may be useful for examinations of genetic inheritance of phenotypic traits in the pathogen or as PCR markers for diagnostic purposes.

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