# Genetic and Pathogenic Variation in *Phytophthora cactorum* Affecting Fruit and Nut Crops in California

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**ABSTRACT**

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Isolates of *Phytophthora cactorum* and 15 other species of *Phytophthora* were characterized according to their genomic DNA, pathogenicity, and sensitivity to mefenoxam. Amplified fragment length polymorphism (AFLP) analysis was completed for 132 isolates of *P. cactorum* (30 from almond, 86 from strawberry, 5 from walnut, and 11 from other hosts) and 22 isolates of 15 other *Phytophthora* spp. from various hosts. All 16 *Phytophthora* spp. were distinguishable by unique AFLP banding patterns. Cluster analysis of the AFLP data revealed high coefficients of genetic similarity (>0.9) among all California isolates of *P. cactorum*. Analysis of molecular variance indicated that, among all 132 isolates of *P. cactorum*, 30.8 and 24.5% of the AFLP variation was associated with hosts and geographical sources of isolates, respectively, whereas 15.0% of the variation was associated with isolate niche (i.e., an aerial plant part, portion of the root system, or soil). Among the 86 isolates of *P. cactorum* from strawberry, characterization by source in the production system (i.e., fruiting field or plant nursery) did not account for a significant proportion of the variation (0.6%, $P = 0.204$). In pathogenicity tests on strawberry plants (cv. Diamante) in a greenhouse, isolates of *P. cactorum* from hosts other than strawberry and an isolate from a strawberry fruit caused only negligible amounts of disease, but isolates from strawberry root systems were highly aggressive. On excised shoot segments of almond (cv. Drake), all isolates of *P. cactorum* originally from almond were pathogenic, and 8 of 17 isolates of the pathogen from other hosts caused significantly less disease than the almond isolates. All 132 isolates of *P. cactorum* were sensitive to mefenoxam at 1 ppm. Populations of *P. cactorum* in California apparently are mefenoxam sensitive and exhibit host specificity with relatively minor variation in genomic DNA. The genetic variation observed in *P. cactorum* included significant geographical and host origin components, which has implications for disease management approaches.

Additional keywords: AMOVA, fixation index

*Phytophthora cactorum* (Lebert & Cohn) J. Schröt. is widely distributed in temperate regions of the world and causes economic loss on many diverse hosts (18). In California, major agricultural hosts of the pathogen include almond, apple, peach, strawberry, and walnut (8,9,38). *P. cactorum* incites crown and root rots on all of these crops and scion cankers on almond (9). Although *P. fragariae* var. *fragariae* and *P. citricola* also have caused disease on California strawberries (11,25), *P. cactorum* is the most prevalent among the three species on the crop.

Variation in *P. cactorum* has been assessed using characteristics of morphology, pathogenicity, isozymes, and DNA (12–14,17,18,20,36,39). Although little morphological variation is known among isolates of *P. cactorum* (34), there are several reports of host specificity in the pathogen (21,22,35,44,45). Previous characterizations of isozymes (39), mitochondrial (mt) DNA restriction fragment length polymorphisms (20), random amplified polymorphic DNA (13,36), microsatellites of mtDNA (14), and internal transcribed spacer sequences (12,13,49) have revealed relatively little genetic variation in collections of *P. cactorum* from various hosts. Random amplified microsatellite (RAMS) markers failed to consistently distinguish birch isolates of *P. cactorum* from strawberry isolates of the pathogen, though the two populations exhibited host specificity (21,22,35). The RAMS markers did distinguish most isolates of *P. cactorum* on strawberry in North America from those associated with the host in Europe (21). It was reported that amplified fragment length polymorphism (AFLP) patterns from strawberry crown rot isolates of *P. cactorum* were distinct from those of strawberry leather rot isolates or isolates of the pathogen from other hosts (16). In another study, AFLP variation was greater among isolates of *P. cactorum* from strawberry than among isolates of the pathogen from other hosts (24).

Genetic and pathogenic variation in populations of *P. cactorum* in California have not been explored extensively, but they may have important disease management implications. For example, ongoing strawberry breeding efforts at the University of California (UC) include evaluations of genetic resistance to *P. cactorum*, in which it is important to adequately represent diversity of the pathogen. The California strawberry industry is functionally and geographically divided into a fruit-production sector (occurring mainly in coastal valleys and plains) and a nursery sector (occurring mainly in the Central Valley and northern high-elevation mountain valleys), and it is unknown whether populations of *P. cactorum* vary between these sectors. Mefenoxam, the active isomeric form of metalaxyl (an older fungicide), is used widely for control of crown rot caused by *P. cactorum* on strawberry, but it is unknown whether California populations of the pathogen are developing resistance to the fungicide. Resistance to mefenoxam was reported in *P. cactorum* from South Carolina (26). In almond, relatively recent and severe outbreaks of perennial Phytophthora cankers have been caused by *P. cactorum* and *P. citricola* (10), and genetic characterization of the pathogen populations may provide additional insight into factors contributing to the disease.

AFLP genotyping is one of the most reliable and reproducible DNA fingerprinting methods (15,27,48). It has been used to detect genetic variation among asexual progeny of *P. infestans* (1), identify a putative neutral marker linked to the A2 mating type in *P. infestans* (30), construct a linkage map of *P. infestans* (47), investigate the genetic structure of *P. capsici* (32) and

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*P. infestans* (41), and provide evidence for natural hybrids between *P. nicotianae* and *P. cactorum* (6).

Herein, we report on use of AFLP genotyping, pathogenicity assays, and mefenoxam sensitivity assays to characterize populations of *P. cactorum* from California. Comparative assessment of AFLP data from *P. cactorum* and additional species of *Phytophthora* is included. Our specific objectives were to (i) examine genetic and pathogenic variation among California populations of *P. cactorum*, with emphasis given to almond and strawberry isolates; (ii) compare the genetic variation among *P. cactorum* and other species of *Phytophthora*; and (iii) evaluate sensitivity to mefenoxam among California populations of *P. cactorum*. Part of this work was reported previously (4).

## MATERIALS AND METHODS

**Collection of *Phytophthora* spp.** In all, 132 isolates of *P. cactorum* were used, including 30 from almond, 86 from strawberry, 5 from walnut, and 11 from other hosts (Table 1). Of these isolates, 77% were cultured directly from host tissue and 23% were isolated from soil around roots of diseased hosts by using a pear baiting method (18). Approximately 25% of the strawberry isolates were from nurseries and the rest came from fruit-production fields. In addition to *P. cactorum*, 22 isolates of 15 other *Phytophthora* spp. from 11 host plants were included (Table 2). All isolates were obtained from single-zoospore colonies, identified morphologically (43), and stored at 14ºC under sterile mineral oil in vials containing V8 juice agar (V8JA).

**Extraction of DNA.** Mycelia were grown for 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). The cultures were incubated for 4 days at 20 to 22ºC. Total genomic DNA was extracted from the mycelia using the method of Lee and Taylor (33). As a control, sterile V8JA disks incubated in clarified V8JB were extracted likewise.

**Table 1.** Backgrounds of isolates of *Phytophthora cactorum* used in this study

| Host plant | Location in California | No. of isolates | Isolates[a] |
|---|---|---|---|
| Almond | Central Interior | 30 | gb392, gb393, gb398, gb592[b,c], gb622, gb626[c], gb687, gb689, gb1040, gb1093, gb1094, gb1095, gb1098[c], gb1100, gb1217, gb1218, gb1223[c], gb1225, gb1363, gb2047[b,c], gb2058, gb2675, gb2676, gb2678[c], gb2730, gb2743, gb2759[b,c], gb2944, gb2957, gb3297[c] |
| Apple | Central Interior | 2 | sm3472[c], sm3493 |
| | Central Coast | 2 | sm3360[b,c], sm3399 |
| | Outside of CA | 2 | sm3302, sm3488 |
| Apricot | Central Coast | 1 | sm3468[b,c] |
| Live Oak | Northern Interior | 1 | sm3339[c] |
| Pear | Northern Interior | 1 | gb4025 |
| Safflower | Northern Interior | 1 | sm3387 |
| Strawberry | Central Coast | 24 | gb2562[b,c], gb2623, gb2626, gb2629, gb2990a[b,c], gb2993, sm3366, sm3367[b,c], sm3379, gb3656[b,c], gb3657, gb3669, gb3670, gb3671, gb3672, gb3673, gb3674, gb3675, gb3676, gb2010-S, sk02-01, sk02-03, sk02-04, skTri1-3-5 |
| | Northern Interior | 15 | gb2450, gb2867, gb2896[b,c], gb2930, gb3289, sm3351, gb3458, gb3567, gb3570, gb3645, gb3646, gb3647, gb3648, gb4028[b,c], gb4029 |
| | South Coast | 46 | gb1750, gb1751, gb2462B, gb2463Crn, gb2463Rts, gb2464, gb2466B, gb2466C, gb2466J, gb2466R, gb2466U, gb2467, gb2475, gb2477B, gb2477C, gb2478, gb2479D, gb2480B, gb2481, gb2486Crn, gb2486Rts, gb2487Crn, gb2487Rts, gb2490Crn, gb2490Rts[b,c], gb2491, gb2492, gb2493, gb2495C, gb3422, gb3423, gb3429, gb3430, gb3431, gb3439, gb3440, gb3445, gb3446, gb3447, gb3450, gb3454, gb3456, gb3696, gb3700[b,c], gb3701, gb3729 |
| | Outside of California | 1 | gb2978 |
| Toyon | Central Interior | 1 | sm3348 |
| Walnut | Central Interior | 3 | sm3337[c], sm3380[c], sm3471[c] |
| | Northern Interior | 2 | sm3398[b,c], sm3405[c] |

[a] Letters 'gb' and 'sm' indicate isolates from G. T. Browne and S. M. Mircetich, respectively, United States Department of Agriculture–Agricultural Research Service, University of California, Davis; 'sk' indicates isolates originally from S. T. Koike, University of California Cooperative Extension, Salinas.
[b] Isolates used for pathogenicity tests on strawberry plants.
[c] Isolates used for pathogenicity tests on almond shoots.

**Table 2.** Isolates used in amplified fragment length polymorphism analysis for comparison among species of *Phytophthora*

| *Phytophthora* sp. (abbreviation) | Host | Isolates[a] |
|---|---|---|
| *P. cactorum* (Pcac) | Apple (Ae), Almond (Ad), Strawberry (Sy), Walnut (Wt) | gb2047, gb2562, gb3337, gb3360 |
| *P. cambivora* (Pcam) | Almond, Oak (Ok) | gb2452, sm3346 |
| *P. capsici* (Pcap) | Tomato (To) | gb3300 |
| *P. cinnamomi* (Pcin) | Walnut, Grape (Ge) | gb647, gb940r |
| *P. citricola* (Pcit) | Almond, Strawberry | gb614, sm3466 |
| *P. citrophthora* (Pctr) | Citrus (Cs) | sm3477 |
| *P. cryptogea* (Pcry) | Apple, Carrot (Ct) | gb343, sm14-2-5 |
| *P. drechsleri* (Pdre) | Almond | gb2065 |
| *P. fragariae* (Pfra) | Raspberry (Ry) | gb3295 |
| *P. infestans* (Pinf) | Tomato (To) | R1-US6 |
| *P. lateralis* (Plat) | Unknown (NA) | sm3338 |
| *P. megasperma* (Pmeg) | Almond, Strawberry | gb2658, gb718 |
| *P. nemarosa* (Pnem) | Oak | P-43 |
| *P. parasitica* (Ppar) | Almond, Walnut | gb1970, gb3273 |
| *P. ramorum* (Pram) | Oak | Pr-104sz |
| *P. syringae* (Psyr) | Almond | gb2007, sm3357 |

[a] Letters 'gb' and 'sm' indicate isolates from G. T. Browne and S. M. Mircetich, respectively, United States Department of Agriculture–Agricultural Research Service, University of California, Davis; isolate R1-US6 was provided by R. M. Bostock, University of California, Davis; and Pr-104sz and P-43 were from D. M. Rizzo, University of California, Davis.

**AFLP analysis.** AFLP fingerprinting was performed using the AFLP Analysis System II kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions, with one 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, about 250 ng of total DNA per isolate was digested completely using restriction enzymes *Eco*R1and *Mse*1 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). The PCR steps included 20 cycles of 94ºC for 30 s, 56ºC for 60 s, and 72ºC for 60 s, with a final step of cooling to 4ºC. 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 94ºC for 30 s, 65ºC for 30 s (annealing temperature was lowered 0.7ºC for each cycle), and 72ºC for 60 s, followed by 23 cycles at 94ºC for 30 s, 56ºC for 30 s, and 72ºC for 60 s, with a final extension for 7 min and cooling to 4ºC. The PCR products were stored at 4ºC until they were electrophoresed.

In selective amplifications, primers for the *Eco*R1 adapter contained two selective nucleotides (E-A + 1 and E-T + 1) and those for the *Mse*1 adapter contained three selective nucleotides (M-CA + 1 and M-CT + 1). After prescreening of 64 primer pairs (the possible combinations of 8 *Eco*R1 and 8 *Mse*1 primers), 12 primer pairs were selected (E-AA/M-CAA, E-AA/M-CAC, E-AC/M-CTC, E-AG/M-CTA, E-AT/M-CAT, E-AT/M-CTT, E-TA/M-CAG, E-TA/M-CTG, E-TC/M-CAG, E-TC/M-CTG, E-TG/M-CTC, and E-TG/M-CTT). These primer pairs provided consistent amplification of DNA fragments when used repeatedly in successive runs on test isolates. DNA samples from *Arabidopsis* and tomato, which were supplied in the AFLP kit, were used as positive controls for selective amplification. An extraction from V8JA served as a negative control.

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, CA), 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 (bp) 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. cactorum* on strawberry.** Fourteen isolates of *P. cactorum*, including eight from strawberry, three from almond, and one each from apple, apricot, and walnut, were evaluated for pathogenicity and aggressiveness on potted plants of strawberry cv. Diamante in two greenhouse experiments. In each test, UC potting mix (2) was infested artificially with inocula grown on V8 juice–oat seed–vermiculite substrate (V8JOV) as described previously (8), with slight modifications. The substrate was distributed to 0.9-liter jars, each receiving 700 $cm^3$ of coarse horticultural vermiculite (Therm-O-Rock West Inc., Chandler, AZ), 40 $cm^3$ of oat seed, and 480 ml of diluted V8JB (V8 juice:water, 1:5, vol/vol; 0.2% $CaCO_3$). The jars were sealed with lids that had openings plugged by polyether foam. The filled jars were autoclaved on three successive days at 120ºC for 60 min, allowed to cool, and inoculated with mycelium of the pathogen. After incubation for 1 month at 20 to 22ºC, the inoculated V8JOV was used to infest UC potting mix; 100 $cm^3$ of the infested substrate was added per liter of potting mix. The potting mix and inoculum were mixed thoroughly. Noninfested potting mix was prepared in the same way using sterile V8JOV.

Strawberry plants were obtained from a commercial nursery that had stored them conventionally at –2ºC after digging. The plants were stored at 4ºC for a few days before planting in 1-liter pots filled with either the artificially infested or noninfested UC potting mix; one plant was used per pot. Inoculum treatments, each involving potting mix infestation with a different isolate of *P. cactorum* or the control, were arranged in four randomized complete blocks. There were six plants per inoculum treatment in each block. The plants were watered daily and fertilized weekly with 11N-7P-26K (Grow More, Gardena, CA). Disease development was favored by weekly flooding of the potting mix; beginning 4 weeks after planting and at weekly intervals thereafter, the potting mix in each pot was flooded for 24 h by adding water to the potting mix surface after placing the pots in bowls that were slightly larger than the pots. Pathogenicity of the isolates was assessed 12 weeks after planting, when disease ratings were assigned using a scale based on size and health of the plant canopies, where 0 = full-sized, healthy plant; 1 = approximately 25% reduction in canopy diameter (compared with the control); 2 = approximately 50% reduction in canopy diameter; 3 = approximately 75% reduction in canopy diameter, with external rotting of petioles and leaves; 4 = >75% reduction in canopy diameter, with >80% of the canopy showing necrotic symptoms; and 5 = death of the plant. Also, shoot and root fresh weights were measured after the roots were washed free from potting mix. The percentage of each root system exhibiting necrosis (dark discoloration) was estimated visually. Each root crown (stem) was split longitudinally and the percentage of the exposed crown area that was necrotic was estimated visually using the following scale: 0 = 0%, 1= 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100%. For each inoculum treatment, 10 root segments (1 cm long, 1 to 2 mm in diameter) from one randomly chosen plant in each block were cultured on a semiselective medium (PARP) (28) that was modified to contain corn meal agar (17 g/liter; BBL, Kansas City, MO), pimaricin (10 mg/liter), ampicillin (250 mg/liter), rifampicin (10 mg/liter), and pentachloronitrobenzene (25 mg/liter). If the selected plants exhibited necrosis in the root crowns, 10 pieces of the necrotic tissue (each approximately 2 by 2 by 5 mm) also were cultured on the medium. The culture plates were incubated for 1 week at 18ºC before incidence of isolation of *P. cactorum* was determined by microscopic observation. The pathogenicity experiment was conducted once in spring 2004 (ambient temperature 15 to 25ºC) and once in summer 2004 (20 to 35ºC).

**Pathogenicity of *P. cactorum* on almond.** Twenty-five isolates of *P. cactorum*, including all of those tested for pathogenicity on strawberry, were tested for pathogenicity on segments of almond shoots. Each experiment included eight isolates from almond, eight from strawberry, five from walnut, two from apple, and one each from apricot and live oak (Table 1). Almond shoot segments (1 to 2 years old, 20 cm long, 5 to 15 mm in diameter) were cut from cv. Drake, wounded by using a cork borer to remove a 6-mm-diameter disk of bark from the center of each shoot segment (37), and inoculated by placing a 4-mm-diameter disk of V8JA covered by mycelium of an isolate of *P. cactorum* into the wound, with the mycelium facing the wound. Control shoots were wounded and inoculated with sterile V8JA. After inoculation, all wounds were covered with electrical tape. The shoots were incubated at 20 to 22ºC 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 to mefenoxam.** All 132 isolates of *P. cactorum* were tested for sensitivity to mefenoxam. Isolate 259 of *Pythium irregularae* (completely resistant to metalaxyl) and isolate 266 of *P. ultimum* (sensitive to metalaxyl), both provided by R. M. Davis, University of California, Davis, were used as standards for comparison. For all isolates, a 4-mm-diameter disk covered with 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 using 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 22ºC by measuring the resulting colony diameters and calculating the percentage of radial growth suppression resulting from mefenoxam amendment relative to the nonamended controls. The experiment was conducted three times, each with one mefenoxam-amended and one nonamended plate per isolate.

**Data analyses.** Binary code of 1 or 0 was used to indicate the presence or absence, respectively, of AFLP bands from 75 to 350 bp, and a rectangular data matrix of AFLP bands × isolates was prepared. Genetic relationship among isolates was computed using the Dice coefficient of similarity in SimQual module of NTSYSpc (version 2.11S software; 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. The AFLP data were subjected also to analysis of molecular variance (AMOVA) using Arlequin software (version 2.000 42). 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 adapts squared Euclidean distances between pairs of haplotypes to compute variance components (19). Variance was partitioned into components among and within isolate groupings based on (i) host (almond, strawberry, walnut, or other hosts); (ii) niche (an aerial plant part, root system, or soil); (iii) geographical area (Central Coast, South Coast, Central Interior, or Northern Interior of California, or outside of California); and (iv) for strawberry isolates only, agricultural production system (plant nursery or fruiting field). Variance components and the 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 (ANOVA) using PROC ANOVA (SAS software release 8.02; SAS Institute, Raleigh, NC). Treatment means were separated according to least significant differences (LSD) at an $\alpha$ probability level of 0.05.

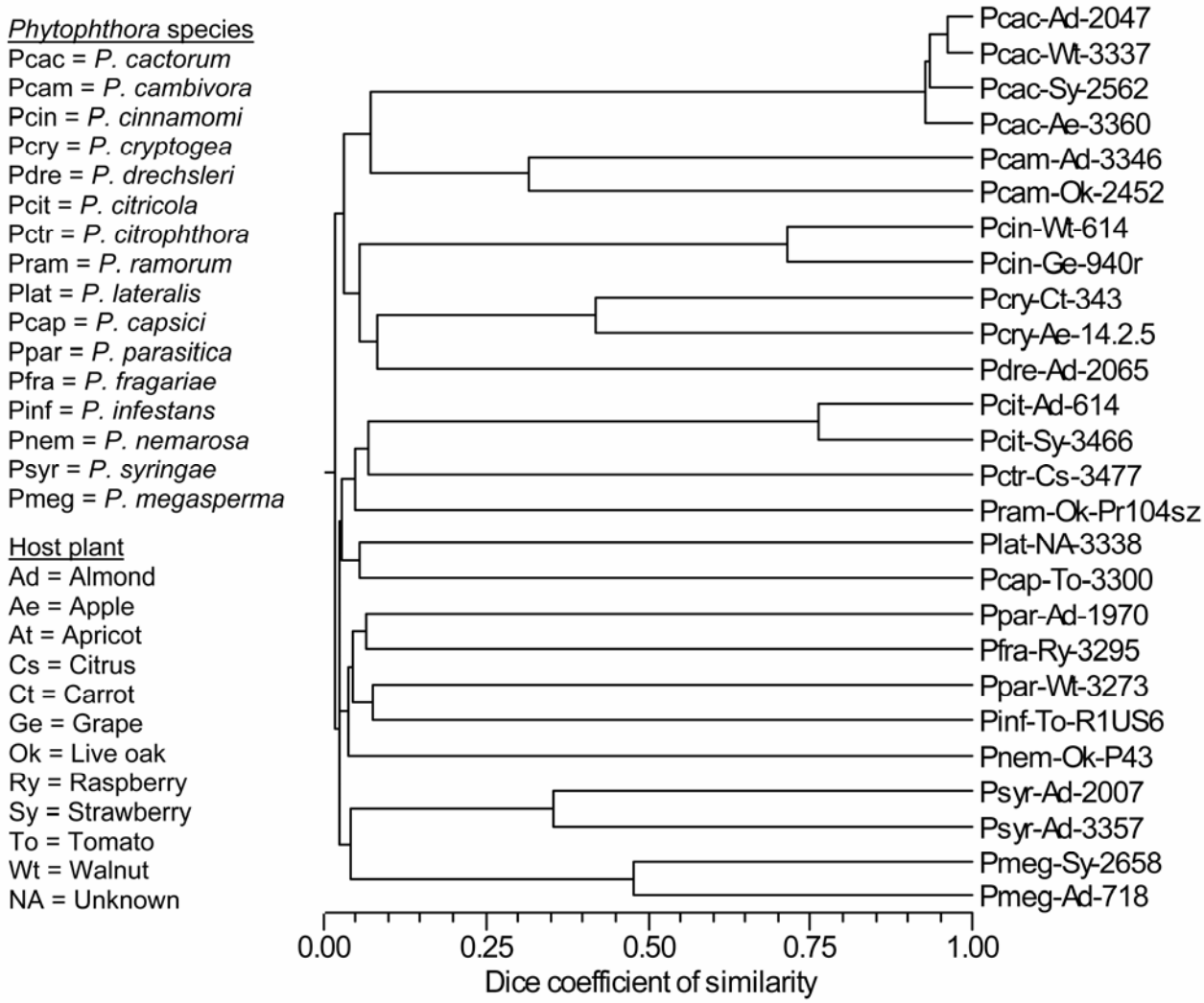


**Fig. 1.** Dendrogram depicting the genetic relatedness among 16 *Phytophthora* spp., based on an unweighted pair group method with arithmetic mean cluster analysis of DNA amplified fragment length polymorphisms generated from polymerase chain reaction with 12 sets of primer pairs. The first four letters of isolate labels indicate species of *Phytophthora* according to morphology, and the next two letters indicate source hosts (Table 2).

## RESULTS

**Genetic diversity among species of *Phytophthora*.** The AFLP analysis among four isolates of *Phytophthora cactorum* and 22 isolates of 15 other *Phytophthora* spp. revealed a large number of distinct and reproducible species-specific DNA fragments. Among all isolates, the 12 primer pairs yielded a total of 808 polymorphic DNA fragments within the scored size range of 75 to 350 bp. On average, the 26 isolates produced 67 fragments per primer pair; the minimum was 38 fragments for primer pair E-AC/M-CTC and the maximum was 89 fragments for primer pair E-TG/M-CTC. Depending on the isolate, the 12 primer pairs produced a total of 35 to 102 distinct DNA fragments per isolate; the average was 64 fragments per isolate.

The UPGMA analysis resolved different species of *Phytophthora* into clusters that diverged from one another at Dice similarity coefficients of <0.10 (Fig. 1). The isolates of *P. cactorum* were clustered tightly and shared coefficients of similarity of >0.90, whereas isolates within other species were less similar, sharing coefficients of similarity from 0.05 to 0.76 (Fig. 1).

**Genetic similarity among isolates of *P. cactorum*.** The AFLP analysis using the 12 primer pairs among all 132 isolates of *P. cactorum* revealed a high degree of similarity within the species. The isolates produced a total of 84 DNA fragments in the scored size range of 75 to 350 bp, and 60% of the bands were monomorphic. In all, 5 to 11 distinct DNA fragments were produced per primer pair combination. Primer pairs E-TA/M-CTG and E-AA/M-CAC produced 44% of the polymorphic bands in *P. cactorum* while the other 10 primer pairs produced the remaining polymorphism. Depending on the isolate, the 12 primer pairs produced a total of 55 to 70 distinct DNA fragments per isolate; the average was 61 fragments per isolate.

The UPGMA analysis indicated a high level of genetic similarity among the 25 isolates of *P. cactorum* used in pathogenicity assays (coefficients of similarity of >0.90), and there was no clear relationship among cluster members, isolate hosts, and geographical sources of isolates (Fig. 2). Similarly, UPGMA of all 132 isolates of *P. cactorum* clustered them into closely related subgroups that shared coefficients of similarity of ≥0.95, and clustering was not clearly related to isolate hosts or geographical locations in California (*data not shown*).

**Hierarchical partitioning of genetic variation in *P. cactorum*.** When the 132 isolates of *P. cactorum* were sorted into four host groups (almond, strawberry, walnut, and other hosts), AMOVA and the associated $F_{ST}$ indicated that 30.8% of the variance in AFLP band incidence was attributable to host grouping ($P$ = 0.000) (Table 3). Similarly, 15.0% ($P$ = 0.000) and 24.5% ($P$ = 0.000) of variance were attributable to niche (aerial plant part, root system, or soil) and geographical area (Central Coast, South Coast, Central Interior, Northern Interior of California, or Outside of California), respectively. In contrast, among the 86 isolates of *P. cactorum* from strawberry, sector of production

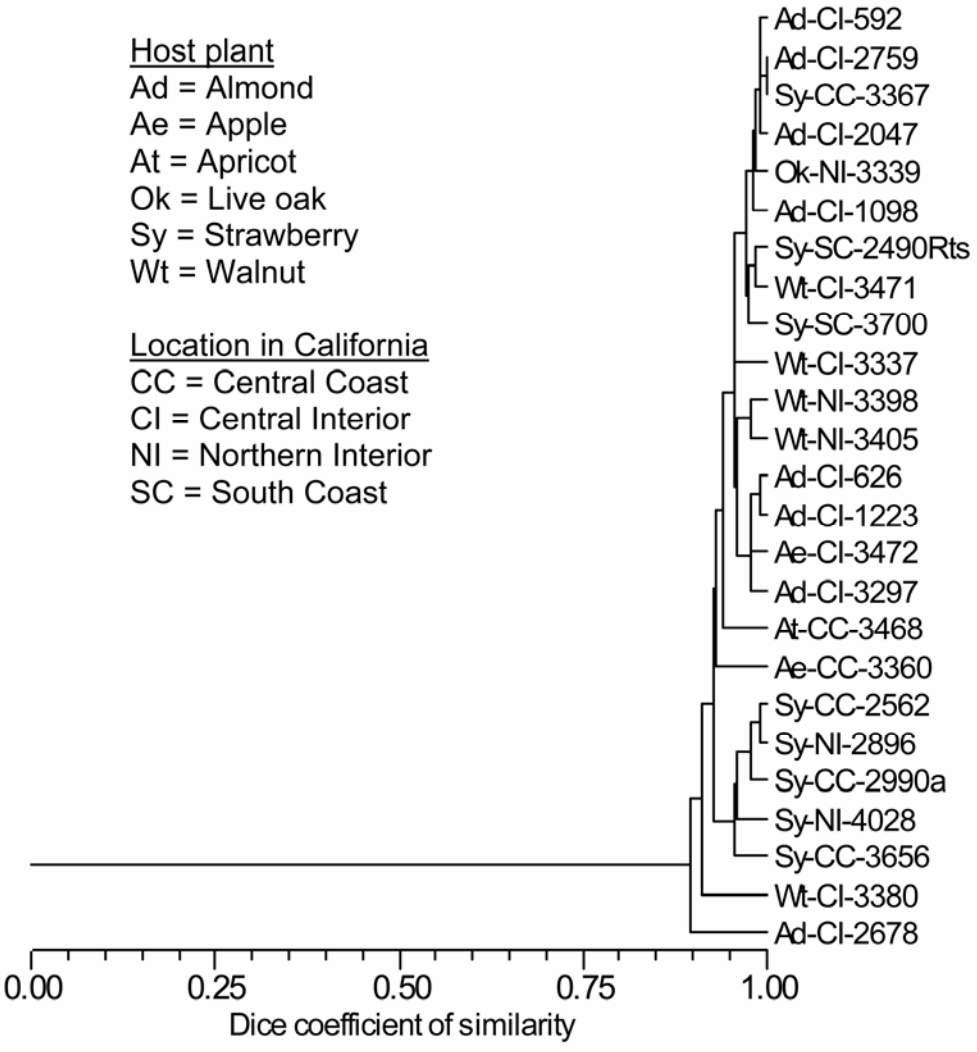


**Fig. 2.** Dendrogram displaying the genetic similarity among 25 isolates of *Phytophthora cactorum*, based on an unweighted pair group method with arithmetic mean cluster analysis of DNA amplified fragment length polymorphisms generated from polymerase chain reaction with 12 sets of primer pairs. The first two letters of isolate labels indicate source hosts (Table 2) and the next two letters indicate the geographical locations.

(nursery or fruiting field) did not account for significant AFLP variation ($P = 0.204$).

The $F_{ST}$ values facilitated pairwise genetic comparisons between groups of isolates of *P. cactorum* (Table 4). All groups based on host differed significantly from one another ($P \leq 0.027$; Table 4). Among niche categories, the aerial plant part group differed significantly from the root system group ($P = 0.000$), but it did not differ significantly from the soil group ($P = 0.114$). Also, the root system group varied significantly from the soil group ($P = 0.000$). Among geographical categories, the isolate groups from the Central Interior and from Outside of California differed significantly from each other as well as from the Northern Interior and South Coast groups ($P = 0.000$), but there was no significant variation between Northern Interior and South Coast groupings ($P = 0.923$). Similarly, the Central Coast group differed significantly from the Central Interior and the Outside groups ($P = 0.000$), but it did not vary from the Northern Interior or South Coast group ($P > 0.05$).

**Pathogenicity of *P. cactorum* on strawberry.** With all parameters used to assess pathogenicity on strawberry, there was significant experiment–inoculum treatment interaction ($P = 0.03$ to $<0.0001$); therefore, the results are presented separately by experiment (Table 5). Strawberry isolates of *P. cactorum*, except for sm3367, induced high disease ratings (means 2.2 to 3.1 in the spring experiment and 3.1 to 3.5 in the summer experiment), whereas inoculation with sm3367; isolates from almond, apple, apricot, or walnut; and the control resulted in negligible disease ratings in both experiments (means 0.0 to 0.2). In both experiments, potting mix infestation with any of the strawberry isolates except sm3367 resulted in lower root and top fresh weights associated with moderate to severe root and crown rot compared with the noninoculated control treatment, which resulted in no disease. The aggressive isolates from strawberry generally caused greater severity of root and crown rot in experiment 2 than in experiment 1. In both experiments, plants inoculated with isolate sm3367 or the isolates from non-strawberry hosts remained relatively healthy, with little or no reductions in shoot and root weight and negligible amounts of root and crown rot. At the end of both experiments, *P. cactorum* was isolated from 50 to 100% of root pieces from plants exposed to the aggressive isolates on strawberry and from 0 to 30% of root pieces from plants exposed to nonaggressive isolates. The pathogen was not isolated from roots cultured from the control plants.

**Pathogenicity of *P. cactorum* on almond.** Isolates of *P. cactorum* differed significantly ($P < 0.0001$) in the amount of bark necrosis that they caused on almond shoots (Fig. 3). Canker lengths were not affected significantly by experiment or experiment–inoculum treatment interaction ($P = 0.18$ and 0.98, respectively); therefore, the data were combined from the two experiments before presentation. No ne-

**Table 3.** Variation among and within population groups of *Phytophthora cactorum* based on hierarchical analysis of molecular variance

| | Observed partition | | | |
|---|---|---|---|---|
| **Sources of variation** | **Variance** | **Total (%)** | **$F_{ST}$** | ***P*[a]** |
| Host[b] | | | | |
| Among groups | 1.376 | 30.79 | 0.308 | 0.000 |
| Within groups | 3.093 | 69.21 | | |
| Niche[c] | | | | |
| Among groups | 0.609 | 14.97 | 0.150 | 0.000 |
| Within groups | 3.460 | 85.03 | | |
| Geographical area[d] | | | | |
| Among groups | 1.049 | 24.47 | 0.245 | 0.000 |
| Within groups | 3.238 | 75.53 | | |
| Strawberry production system[e] | | | | |
| Among groups | 0.017 | 0.55 | 0.006 | 0.204 |
| Within groups | 3.156 | 99.45 | | |

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

[b] 132 isolates were sorted into four groups based on host (almond, strawberry, walnut, and other hosts) from which isolates of *P. cactorum* were obtained.

[c] 132 isolates were sorted into three groups based on isolation source: aerial (stems, branches, or fruit), roots (root system, including crown and roots), and soil.

[d] 132 isolates were sorted into five groups based on geographical area in California or elsewhere from which infected and infested plant and soil samples were collected (i.e., Central Interior, Central Coast, Northern Interior, South Coast, and Outside of California).

[e] 86 isolates were sorted into two groups (commercial field and nursery) from which isolates of *P. cactorum* were obtained. For groupings in this category, only the isolates from strawberry were considered.

**Table 4.** Pairwise genetic differentiation among isolates of *Phytophthora cactorum* based on $F_{ST}$ values[a]

| | **Host[b]** | | | | **Niche[c]** | | | **Geographical area[d]** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Ad (30)** | **Sy (86)** | **Wt (5)** | **Others (11)** | **Aerial (26)** | **Roots (78)** | **Soil (28)** | **CI (36)** | **CC (27)** | **NI (20)** | **SC (46)** | **OU (3)** |
| Ad | … | 0.000 | 0.004 | 0.027 | 0.387 | 0.000 | 0.000 | 0.998 | 0.000 | 0.000 | 0.000 | 0.000 |
| Sy | 0.356** | … | 0.000 | 0.000 | 0.000 | 0.966 | 0.000 | 0.000 | 0.377 | 0.980 | 0.815 | 0.000 |
| Wt | 0.233** | 0.335** | … | 0.012 | 0.014 | 0.000 | 0.148 | 0.012 | 0.000 | 0.000 | 0.000 | 0.004 |
| Others | 0.068* | 0.226** | 0.160* | … | 0.582 | 0.000 | 0.448 | 0.093 | 0.000 | 0.002 | 0.000 | 0.022 |
| Aerial | 0.000 | 0.235** | 0.160* | –0.015 | … | 0.000 | 0.114 | 0.497 | 0.000 | 0.000 | 0.000 | 0.000 |
| Roots | 0.324** | –0.008 | 0.310** | 0.197** | 0.203** | … | 0.000 | 0.000 | 0.112 | 0.970 | 0.996 | 0.000 |
| Soil | 0.091** | 0.159** | 0.061 | 0.000 | 0.025 | 0.139** | … | 0.002 | 0.000 | 0.002 | 0.002 | 0.000 |
| CI | –0.026 | 0.335** | 0.169** | 0.036 | –0.005 | 0.303** | 0.068** | … | 0.000 | 0.000 | 0.000 | 0.000 |
| CC | 0.375** | 0.001 | 0.318** | 0.223** | 0.238** | 0.014 | 0.134** | 0.349** | … | 0.410 | 0.059 | 0.000 |
| NI | 0.338** | –0.022 | 0.299** | 0.172** | 0.185** | –0.022 | 0.111** | 0.306** | –0.002 | … | 0.923 | 0.000 |
| SC | 0.322** | –0.007 | 0.304** | 0.189** | 0.194** | –0.014 | 0.127** | 0.297** | 0.023 | –0.022 | … | 0.000 |
| OU | 0.316** | 0.374** | 0.362** | 0.143* | 0.201** | 0.352** | 0.260** | 0.286** | 0.379** | 0.318** | 0.359** | … |

[a] Numbers below the diagonal are fixation index ($F_{ST}$) values, and the numbers above the diagonal are probabilities of having more extreme $F_{ST}$ values than observed by chance alone; ** and * indicate significant differences at $P = 0.05$ and 0.01, respectively. Numbers of isolates within each group are indicated in parentheses.

[b] Based on host (almond [Ad], strawberry [Sy], walnut [Wt], and others [apple, apricot, live oak, pear, safflower, and toyon]), 132 isolates of *P. cactorum* were sorted into four groups.

[c] Based on niche, 132 isolates of *P. cactorum* were sorted into three groups: Aerial (stems, branches, and fruit), Roots (root system, including crown and roots), and Soil.

[d] Based on geographical areas from which samples were collected, 132 isolates of *P. cactorum* were sorted into five groups: CI (Central Interior), CC (Central Coast), NI (Northern Interior), SC (South Coast), and OU (outside of California).

crosis or discoloration resulted from control inoculations. All eight isolates from almond caused cankers (mean length of necrosis 67 mm, range of means 40 to 89 mm), and five of them caused significantly more disease than the other three. Approximately half of the 17 isolates of *P. cactorum* from other hosts caused similar amounts of brownish discoloration as the less-aggressive isolates from almond, but the other non-almond isolates were less aggressive than any isolates from almond (mean length of discoloration 27 mm, range of means 11 to 48 mm). It was observed that the necrosis caused by the almond isolates permeated all layers of bark for the entire length of cankers, whereas the necrosis caused by the less-aggressive isolates from other hosts typically was limited to internal layers of the bark and the cambium. *P. cactorum* was re-isolated from the streaks in the cambium as well as from the necrotic bark.

**Sensitivity of *P. cactorum* to mefenoxam.** All 132 isolates of *P. cactorum* were highly sensitive to mefenoxam. On V8JA without mefenoxam, radial growth of the mycelium averaged 64 mm in 5 days (range of means 37 to 86 mm). On V8JA amended with mefenoxam at 1 ppm, 110 of the isolates failed to grow at all, 19 isolates grew 95 to 99% less than the controls on nonamended V8JA, and the remaining three isolates (gb3450, sm3367, and gb3729) grew 81 to 90% less than the controls. Growth of the metalaxyl-insensitive control, *Pythium irregulare* isolate 259, was not inhibited by mefenoxam at 1 ppm, whereas that of the metalaxyl-sensitive control, *P. ultimum* isolate 266, was completely suppressed.

## DISCUSSION

We determined that, although California populations of *P. cactorum* have a high degree of genetic similarity detectable by AFLP, isolates within the populations vary greatly in aggressiveness on almond and strawberry. Furthermore, results of our pathogenicity experiments indicate that host specialization exists among almond and strawberry populations of *P. cactorum* in California (Table 5; Fig. 3). Seven of the eight strawberry isolates were highly aggressive on strawberry, whereas none of the three almond isolates caused much disease on strawberry. A lesser converse effect resulted on almond (i.e., five of the eight almond isolates were significantly more aggressive than all eight strawberry

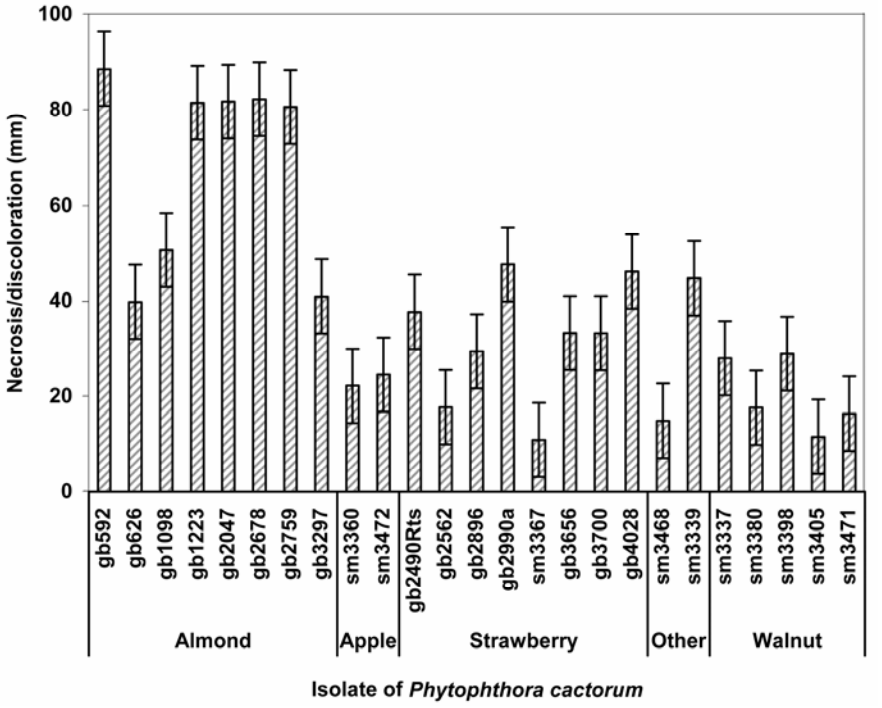


**Fig. 3.** Pathogenicity and aggressiveness of isolates of *Phytophthora cactorum* from different hosts on 1- to 2-year-old shoot segments from almond cv. Drake. The shoot pieces were inoculated on wounds and incubated in chambers at 21 ± 1ºC with 100% humidity. Vertical bars are the least significant difference values at an $\alpha$ *P* level of 0.05. The control shoots had no necrosis or discoloration.

**Table 5.** Pathogenicity and aggressiveness of selected isolates of *Phytophthora cactorum* on strawberry cv. Diamante under greenhouse conditions[a]

| | | | Disease severity[b] | | Shoot fresh wt (g) | | Root fresh wt (g) | | Root rot (%) | | Crown rot[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Original host | Location[d] | Isolate | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
| … | … | Control | 0.0 | 0.1 | 38.7 | 26.0 | 16.4 | 9.1 | 3 | 5 | 0.0 | 0.1 |
| Almond | CI | gb592 | 0.0 | 0.2 | 35.8 | 14.1 | 18.2 | 5.0 | 3 | 5 | 0.0 | 0.0 |
| Almond | CI | gb2047 | 0.1 | 0.2 | 36.6 | 9.8 | 19.6 | 4.5 | 5 | 25 | 0.0 | 0.2 |
| Almond | CI | gb2759 | 0.1 | 0.1 | 31.7 | 13.2 | 13.0 | 5.1 | 5 | 15 | 0.0 | 0.1 |
| Apple | CC | sm3360 | 0.0 | 0.2 | 42.7 | 25.1 | 22.8 | 5.3 | 3 | 5 | 0.0 | 0.0 |
| Apricot | CC | sm3468 | 0.0 | 0.1 | 40.6 | 25.3 | 20.9 | 6.3 | 4 | 4 | 0.0 | 0.1 |
| Strawberry | CC | gb2562 | 2.2 | 3.5 | 10.6 | 2.7 | 8.4 | 3.2 | 56 | 99 | 0.7 | 2.3 |
| Strawberry | CC | gb2990a | 3.0 | 3.3 | 7.7 | 3.0 | 5.6 | 3.2 | 66 | 98 | 0.7 | 2.1 |
| Strawberry | CC | gb3656 | 3.1 | 3.5 | 5.1 | 2.6 | 3.9 | 3.0 | 87 | 100 | 0.9 | 3.0 |
| Strawberry | NI | gb2896 | 2.6 | 3.5 | 7.4 | 2.1 | 5.3 | 3.4 | 78 | 98 | 0.4 | 2.4 |
| Strawberry | CC | sm3367 | 0.1 | 0.1 | 36.0 | 17.6 | 15.2 | 4.9 | 4 | 14 | 0.0 | 0.1 |
| Strawberry | NI | gb4028 | 3.1 | 3.5 | 4.9 | 2.9 | 4.8 | 3.2 | 78 | 98 | 0.8 | 2.5 |
| Strawberry | SC | gb2490Rts | 2.7 | 3.1 | 8.0 | 3.9 | 5.2 | 3.4 | 64 | 89 | 0.2 | 1.9 |
| Strawberry | SC | gb3700 | 2.5 | 3.1 | 8.0 | 3.6 | 5.8 | 3.1 | 69 | 95 | 0.1 | 1.8 |
| Walnut | NI | sm3398 | 0.0 | 0.2 | 33.3 | 20.1 | 19.1 | 5.9 | 2 | 14 | 0.0 | 0.1 |
| $LSD_{(P = 0.05)}$[e] | … | … | 0.5 | 0.5 | 9.2 | 6.2 | 6.0 | 1.3 | 18 | 10 | 0.5 | 0.5 |

[a] Experiment (Exp.) 1 was conducted in spring 2004 and Exp. 2 in summer 2004.

[b] Disease rating scale: 0 = healthy plant, 1= approximately 25% reduction in canopy diameter (compared with the control), 2 = approximately 50% reduction in canopy diameter, 3 = approximately 75% reduction in canopy diameter with external rotting of petioles and leaves, 4 = >75% reduction in canopy diameter with only some green tissue remaining, and 5 = complete death of the plant due to rotting. Disease symptoms were rated 12 weeks after planting strawberry.

[c] The full length of each plant crown (stem) was cut in half longitudinally and assigned a score based on the percentage of the exposed crown area that was necrotic: 0 = 0%, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100%.

[d] Locations in California: CI = Central Interior, CC = Central Coast, NI = Northern Interior, and SC = South Coast.

[e] LSD = least significant difference.

isolates on almond). Isolates gb592 from almond and gb2562 from strawberry interacted differentially with almond and strawberry; isolate gb592 was aggressive on almond, but nonaggressive on strawberry; and isolate gb2562 was weakly aggressive on almond, but highly aggressive on strawberry. The host specialization that we observed is consistent with previous reports involving additional hosts and isolates of *P. cactorum* from other parts of the world (3,21,22,29,35,44,45). For example, Kennedy and Duncan reported that strawberry isolates of *P. cactorum* caused strawberry crown rot but did not rot apple twigs, and apple isolates did not attack strawberry plants but did rot apple twigs (29). Similarly, isolates of *P. cactorum* from walnut were aggressive on walnut, but did not cause disease on apple seedlings (3,) and isolates from birch were aggressive on birch but did not cause disease on strawberry seedlings (22,35).

It is possible that factors in addition to host specialization influenced aggressiveness of our isolates of *P. cactorum*. Isolate sm3367, originally from a strawberry fruit, presented an atypical case because it caused negligible amounts of disease on strawberry. Leather rot of strawberry fruits usually is not an important disease in California (25), and fruit isolates were not well represented in our study. It is uncertain whether the isolate's niche (i.e., a strawberry fruit), time in culture (approximately 30 years), or other factors were responsible for its consistent lack of aggressiveness on strawberry plants. In the almond and strawberry pathogenicity assays, it is possible that the lack of aggressiveness of isolates from apple, apricot, and walnut resulted wholly or in part from time in culture (20 to 30 years) rather than from a lack of specialization for the inoculated hosts. Nevertheless, at least on almond, host specialization was not confounded to a high degree with potential deterioration of aggressiveness due to aging in culture; in our experiments, almond isolates in culture for 4 to 10 years were more aggressive on almond than strawberry isolates in culture for 2 to 6 years. In addition, isolate gb2047 was similarly aggressive on almond in experiments reported here and in a previous experiment 6 years earlier (10). A rigorous assessment of effects of long-term storage at 14ºC on aggressiveness of *P. cactorum* would require additional research.

Although we observed significant experiment–inoculum treatment interaction in strawberry pathogenicity assays, there were no fundamental changes in pathogenicity and aggressiveness between the two experiments among isolates of *P. cactorum*. The differences in disease severity and plant growth between the two experiments may have resulted, at least in part, from corresponding differences in ambient greenhouse temperatures.

We found evidence that isolates can grow or reproduce on a host on which they do not cause much disease. For example, *P. cactorum* was isolated from roots of strawberry plants exposed to the nonaggressive isolates. Also, non-almond isolates that induced relatively little necrosis on almond shoot segments were cultured from necrotic bark surrounding the point of inoculation as well as from the distal (leading) edge of necrotic streaks in the cambium tissue beyond the necrotic bark. Epidemiologically, these results suggest that aggressiveness on a host is not required for geographical dispersal of *P. cactorum* on that host (i.e., in connection with nursery shipments).

The high degree of genetic similarity indicated by cluster analysis of the AFLP data from California isolates of *P. cactorum* is consistent with previous reports that focused mainly on populations of the pathogen from other areas (22,39). In our study, UPGMA of AFLP data did not cluster isolates consistently according to host or geographical location. Similarly, in a European report, UPGMA of RAMS markers did not consistently cluster isolates of *P. cactorum* from Europe and the eastern United States according to host, even though isolates exhibited host specificity (21,22,35). In a report from the eastern United States, most isolates of *P. cactorum* from the northwestern and eastern parts of the country were highly similar, and UPGMA did not cluster them consistently by host (24). Interestingly, Huntula et al. (21) could not distinguish crown and fruit isolates of *P. cactorum* from strawberry using cluster analysis of RAMS markers, but Eikemo et al. (16) did so using cluster analysis of AFLP data.

Although we found relatively little AFLP variation among populations of *P. cactorum* in California, AMOVA permitted significant and informative partitioning of the variance. Host association accounted for approximately 31% of total AFLP variance, which is considered to be a high proportion of genetic differentiation (23). The pairwise genetic comparison of fixation indices ($F_{ST}$ values) indicated significant ($P < 0.027$) AFLP differences among the four host groups (almond, strawberry, walnut, and other hosts), which supports the concept of host adaptation within populations of *P. cactorum*. Similarly, the geographical grouping accounted for 24% of the variance. Geographical and host categories were confounded because all isolates of *P. cactorum* from almond were from the Central Interior of California, and the majority of strawberry isolates were from the Central Coast and South Coast groups (location of fruiting fields) and the Northern Interior group (location of nurseries).

Our data provide no evidence for genetic or pathogenic differentiation between populations of *P. cactorum* from the strawberry nurseries and fruiting fields, even though these sectors of strawberry production are geographically and environmentally distinct. The lack of differentiation between the nursery and fruiting field populations neither establishes that nursery plants are a dominant source of the pathogen in the latter nor eliminates this possibility. In contrast, strawberry transplants infected with *P. cactorum* were implicated in outbreaks of Phytophthora crown rot in the southeastern United States (24).

The AFLP variation within the isolate groupings could be due to random mutation or out-crossing among isolates. It has been shown that out-crossing occurs in the homothallic oomycete *P. sojae* (5), and there is no reason to doubt that this occurs in populations of *P. cactorum*. More experiments are needed to verify the role of recombinants in creating pathogenic variation in *P. cactorum*.

Our results have practical implications. For example, the variation in aggressiveness and virulence among isolates of *P. cactorum* suggests the importance of careful isolate selection for use in evaluations of genetic resistance to the pathogen in cultivars or rootstocks. For such screens, the likelihood of virulence of the pathogen on a host may be maximized by selecting isolates from the same host tissues being evaluated for resistance, and it may be important to test multiple isolates for aggressiveness. The universal sensitivity of all tested California isolates of *P. cactorum* to mefenoxam suggests continuing utility of the fungicide in California (7), but resistance to mefenoxam (and metalaxyl) has been reported elsewhere in *P. cactorum* (26,46) and *P. capsici* (31,40). Therefore, it is important to use integrated management strategies for prevention of Phytophthora crown rot and avoid overuse of mefenoxam. Finally, some of the AFLP bands that were amplified universally from *P. cactorum* DNA and not from the other species of *Phytophthora* may be useful for development of PCR primers to detect *P. cactorum* in soil or plants.


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