Population Dynamics of Aerial and Terrestrial Populations of *Phytophthora ramorum* in a California Forest Under Different Climatic Conditions

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ABSTRACT

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Limited information is available on how soil and leaf populations of the sudden oak death pathogen, *Phytophthora ramorum*, may differ in their response to changing weather conditions, and their corresponding role in initiating the next disease cycle after unfavorable weather conditions. We sampled and cultured from 425 trees in six sites, three times at the end of a 3-year-long drought and twice during a wet year that followed. Soil was also sampled twice with similar frequency and design used for sampling leaves. Ten microsatellites were used for genetic analyses on cultures from successful isolations. Results demonstrated that incidence of leaf infection tripled at the onset of the first wet period in 3 years in spring 2010, while that of soil populations remained unchanged. Migration of genotypes among sites was low and spatially limited under dry periods but intensity and range of migration of genotypes significantly increased for leaf populations during wet periods. Only leaf genotypes persisted significantly between years, and genotypes present in different substrates distributed differently in soil and leaves. We conclude that epidemics start rapidly at the onset of favorable climatic conditions through highly transmissible leaf genotypes, and that soil populations are transient and may be less epidemiologically relevant than previously thought.

Additional keywords: genetic diversity, genetic structure, multilocus genotypes.

The success of an invasive exotic pathogen may be determined by the ecological amplitude of the organism; that is, a combination of the range of hosts available, aggressiveness on each host (38,63), release from natural enemies and competitors (45), and reproductive rate and dispersal potential of infectious propagules in a new environment (56,68,85). Further complexity is encountered when dealing with pathogens that may completely change their lifestyle in different hosts or habitats; for example, when plant pathogens switch from a saprotrophic to a pathogenic state depending on the substrate (60), a host species jump occurs (84), or zoonotic diseases transition from transmissive to dead-end hosts (43,59).

Although only recently discovered in temperate forests (5,34, 36,67), aerial *Phytophthora* spp. (order: Peronosporales; kingdom: Stramenopila) represent a unique opportunity to study the epidemiology of infectious forest pathogens deeply affected by environmental conditions such as temperature and rainfall (19). These organisms are capable of colonizing completely different substrates, including leaves, branches, trunks, soil, and water (27). The epidemiology of these aerial *Phytophthora* spp. is largely unresolved and the role played by each substrate in their population dynamics is unknown. To date, the majority of research on aerial *Phytophthora* spp. has focused on *Phytophthora ramorum*, the causal agent of sudden oak death in North America, sudden larch death in Great Britain, and ramorum blight in nurseries worldwide (27,32); and *P. infestans*, the pathogen causing potato blight (31,73).

Studies of *P. ramorum* have focused on understanding the ecological and environmental parameters that facilitate infection (1,15,41,79), describing the effects of the disease on individual plant species or plant communities (11,48,57), and reconstructing the evolutionary and recent history of both wild and nursery populations of the organism (29,42,54,55). However, limited information has been generated on how climatic patterns, both within and between years, affect the active size ("active" here is meant loosely as the portion of the population that sporulates and, thus, is infectious), diversity, and migration potential of *P. ramorum* genotypes. Although some studies have shown how virulence and sporulation are traits greatly affected by the plant hosts colonized by *P. ramorum* (40,44), little information has ever been provided for this or any other mixed-substrate (e.g., soil-air-water) plant pathogen on how populations from different substrates may interact and differently contribute to the epidemiology and to the microevolution of a species. The genetic structure in years characterized by limiting unfavorable environmental conditions, the change experienced by such populations as conditions become favorable, and the epidemiology of populations from different substrates are elements essential to the understanding of infectious diseases, including those caused by plant pathogens.

Although there are over 100 species of plants that are either natural hosts of *P. ramorum* (3,14,26,35,78) or have been shown to be susceptible in lab trials, the leaves of California bay laurel (*Umbellularia californica*) provide the most prolific substrate for sporulation of the pathogen in California forests, with limited to no effect on the overall health of the host (11,15,17). *P. ramorum* populations infecting bay laurel leaves are strongly affected by seasonal temperatures and rainfall in California (1,15,17,40). The

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Californian climate is typically Mediterranean, with generally moderate temperatures year round. The winter and spring months are characterized by mild temperatures and rain, which are followed by long periods of dry weather during the summer months (Fig. 1). In the years preceding this study (2007 to 2009), the San Francisco Bay Area experienced an atypical drought period in which the usual spring rains were much reduced. This drought was broken in 2010, with a return to wetter spring conditions. This drought-nondrought climatic transition, as well as the relative fluctuations of climate within each year, are both the focus of this study. *P. ramorum* moves through the landscape primarily via airborne sporangia produced on leaves and twigs of infectious hosts, including but not limited to bay laurel (15, 26,52), while thick walled chlamydospores, also produced on leaves or twigs, are suspected to be the main source for soil inoculum (22). It is unknown whether soil represents a reservoir of inoculum for this pathogen, relevant to its survival during the unfavorable dry summer months or during extended drought periods. It has been postulated that leaves may be the substrate where *P. ramorum* oversummers (23) but direct evidence on this aspect of the disease is still lacking.

Most plant pathogens require precise environmental conditions for transmission to occur and, consequently, the density of pathogen populations and disease severity fluctuate significantly (50, 53,62). Although this dependence of dispersal on environmental conditions implicitly suggests that unfavorable conditions will lead to a bottleneck in pathogen populations, in the case of *P. ramorum*, it is pivotal to understand whether both soil and leaves are effective reservoirs of inoculum necessary to start the next disease cycle, whether survival occurs everywhere or only in refugial sites, and whether all genotypes respond equally to the onset of warmer and wetter conditions that are more favorable for the pathogen.

Additionally, when a pathogen colonizes different substrates, it is important to determine whether some genotypes may show adaptation to a specific substrate and whether the effect of the substrate on pathogen fitness differs across substrates. Although this issue of distinct populations of the same pathogen in different hosts and substrates has been widely studied for parasitic or zoonotic diseases (2,4,39,69), it has only rarely been investigated in plant pathogens (51,66,86).

Specific goals of this study included to (i) determine how disease incidence, size, and genotypic composition of populations of the pathogen in bay laurel leaves are affected by the onset of favorable rainy conditions after a persistent drought; (ii) compare the genotypic diversity, migration, presence, and evenness of individual foliar genotypes during the transition between a drought and a wet year; (iii) determine whether soil and leaf populations sampled at the same time in the dry spring of 2009 and the wet spring of 2010 represent genetically isolated groups displaying differential adaptation to the two substrates and comparable spatial aggregation of alleles in the presence of different climatic conditions; and, finally, (iv) determine whether populations from both substrates may be a significant source of inoculum for next year's disease cycle.

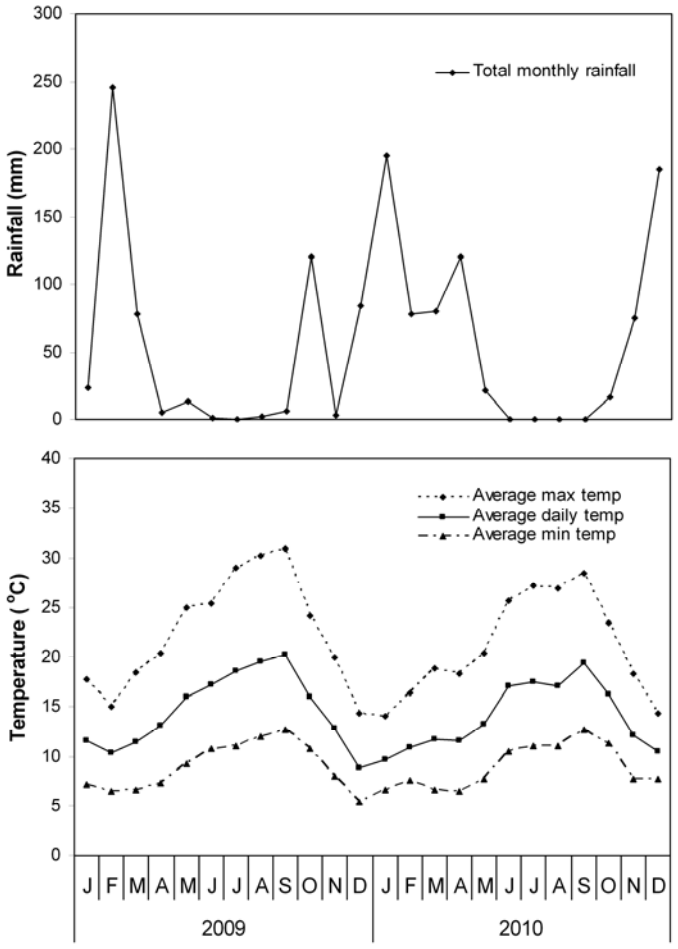

Fig. 1. Monthly climate data for the San Francisco Public Utility Commission watershed study area, taken from the Remote Automated Weather Station weather station at Pulgas: (i) total rainfall (mm) and (ii) air temperature; daily average and average monthly minimum and maximum.

MATERIALS AND METHODS

Habitat or plot selection. Six study plots were established in San Mateo County within the 9,300-ha watershed (latitude: 37.519539°, longitude: –122.368952°) controlled by the San Francisco Public Utility Commission (SFPUC). Plots were equally divided between the Crystal Springs and Pilarcitos drainages, at 95 to 320 m in elevation; Pilarcitos is, on average, higher in elevation than Crystal Springs and is the only drainage to include tanoaks (*Notholithocarpus densiflorus*). The entire watershed is closed to the public and relatively undisturbed, and *P. ramorum* has been present at least since 2001 (D. M. Rizzo, *personal communication*). Sufficient time has passed for the pervasive infestation of all appropriate habitats in the area selected for this study, thus eliminating possible effects of infestation age on the analysis. Plots were separated by at least 2 km, a distance gap only occasionally bridged by the pathogen (27), and equally distributed in two drainages. Each plot consisted of six 100-m transects, 10 m wide, emanating at 60° from the plot center. Each bay laurel stem was tagged to enable repeated sampling of the same trees. Stems were defined as major branches separating from the main stem below breast height, measuring >1 cm in diameter. Local climate data were acquired from the Remote Automated Weather Station (RAWS) at Pulgas (latitude: 37.47500°, longitude:–122.29810°), SFPUC data archives, and local temperature collected in each plot using HOBO data loggers (Onset Computer Corporation, MA).

Leaf sampling. Bay laurel trees were surveyed every 10 m along the six transects in each plot; six symptomatic leaves were sampled from the lower canopy of each tree. Leaves were classified as symptomatic if they displayed lesions characteristic of *P. ramorum* infection (i.e., a black or brown irregularly shaped lesion at the tip and or edges of leaves, often with a pixelated appearance and with edges characterized by a dark border line or a yellow halo). Younger leaves were sampled if possible, regardless of size, with the aim of sampling current viable pathogen infections. Sampling of symptomatic leaves occurred up to three times per year: "Early" sampling in late winter or early spring (February to March), when conditions were not yet ideal for pathogen sporulation and infection (15,41); "Peak" sampling in

early June, when sporulation and infection levels are generally highest; and "Late" sampling in the hot and dry fall (September), when the pathogen is the least active and least infectious. Leaves were sampled three times in 2009 (Early, Peak, and Late) and twice in 2010 (Early and Peak). Rainfall levels in 2009 and 2010 were ≈50 and 110%, respectively, of the 30-year average (California Department of Water Resources 2010), with almost no rainfall in spring 2009 and with higher than average spring rainfall in 2010. The monthly aridity index (AI) (16) was calculated as $AI_M = P/(T + 10)$, where P is the precipitation (millimeters) and T is the mean air temperature (°C). This index provides a unified term of reference to define whether overall weather conditions were unfavorable or favorable to pathogen dispersal; index values are low when aridity is high.

The edges of lesions from the six leaves collected from each surveyed tree were plated in PARP selective medium (pimaricin at 400 µl/liter, ampicillin at 250 mg/liter, rifampicin at 10 mg/liter, and pentachloronitrobenzene at 25 mg/liter) (82). Plates were incubated in the dark at 18°C for 5 to 7 days. *P. ramorum* growth was identified from mycelial morphology and then subcultured onto clean PARP medium to eliminate contaminants.

Soil sampling and baiting. Soil was sampled concurrently with leaf collections for two consecutive years during the two peak sampling efforts of 2009 and 2010. Soil (≈500 g) was taken from three points around the base of each tree or branch from which leaves were sampled and pooled together. Surface duff was removed and collection was from the surface to ≈20 cm in depth. Samples were stored at 10°C for a maximum of 48 h, before being mixed thoroughly and flooded with dH_2O until the soil was completely covered. Ten leaf discs of uninfected *Rhododendron* var. Cunningham's White were placed in mesh bags and floated on the surface of the water. Baited soil was incubated in the dark at 18°C for 5 to 7 days. Following incubation, leaf discs were plated into PARP + hymexozol at 25 mg/liter (PARP+H) selective medium (82), with discs completely submerged into agar. Plates were incubated in the dark at 18°C for 1 to 4 days: growth usually occurred from leaf discs within 1 to 3 days. *P. ramorum* colonies were selected based on microscopic morphology and subcultured onto clean PARP medium.

DNA extraction. Mycelial isolates maintained on PARP were inoculated into 12% pea broth (120 g of pea per liter of dH_2O, autoclaved for 20 min at 121°C, 1.05 kg/cm^2, strained, and reautoclaved under the same conditions) liquid culture and grown for 1 week at room temperature, followed by lyophilization. Lyophilized mycelium was ground using 5 mm glass beads and a bead amalgamator. DNA was extracted by addition of 200 µl of 0.5 M NaOH to ≈10ng of crushed mycelium, mixing thoroughly, followed by dilution of 5 µl of this in 495 µl of 10 mM Tris-HCl, pH 8.0 (61,83).

Microsatellite data generation. *P. ramorum* DNA was amplified using six sets of primers amplifying a total of 10 microsatellite loci: Ms18 and 64 (42); Ms39, 43, and 45 (64); and MsILVO145 (81) (Supplementary Table 1). Polymerase chain reaction (PCR) reactions (10 µl) were set up with final concentrations for each reaction as follows: 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM F primer (labeled with HEX or FAM), 0.2 µM R primer, and GoFlexiTaq (Promega Corp.) at 1 U/µl. Thermal cycling programs varied for different primers and were taken from Ivors et al. (42), Prospero et al. (64), and Vercauteren et al. (81).

A 3730 ABI Sequencer was used for fragment analysis using the LIZ 500 size standard (Applied Biosystems) and analyzed using Peakscanner v1 (ABI Biosystems). Fragment sizes were converted to numbers of microsatellite motif repeats for downstream analysis. Multilocus genotypes (MLGs) were assigned to each isolate using Gimlet v1.1.3 (80).

Genetic diversity indices and minimum spanning networks. The following indices of diversity were calculated: (i) clonal genotype diversity, $R = (G - 1)/(N - 1)$, where G is the number of MLGs present in a sample and N is the sample size (54); (ii) Stoddart and Taylor's index, $G = 1/\Sigma p_i^2$ where p_i is the frequency of the ith MLG (76); (iii) evenness index, $E_5 = (1/\lambda) - 1/e^{H'} - 1$, where λ = Simpson's index of diversity and H' = Shannon-Wiener diversity index (33,49,71); E_5 tends toward 0 as a single genotype becomes more dominant and, when $E_5 = 1$, all genotypes are equally represented; and (iv) allelic richness (A_r) was calculated using rarefaction to account for unequal sample sizes of populations using ADZE v1.0 software (77). ADZE v1.0 was also used to produce genotype accumulation curves, for each sampling time for both leaf and soil.

Genetic distances were calculated according to Bruvo et al. (7) in GenoDive 2.0b10 (58) using the stepwise mutation model. When calculating Bruvo distances, mutation is exponentially related to step length; thus, this metric has been deemed ideal for the comparison of closely related individuals (55). Bruvo distances were also used to produce a minimum spanning network (MSN) to depict the relationship and interconnectedness of genotypes, using MINSPNET (21) and visualized using Graphviz v2.28 (www.graphviz.org). To examine the origin of the high genotypic diversity observed in the soil, we generated a MSN for the more diverse 2010, in which MLGs were identified as present in (i) leaf only, (ii) both soil and leaf, and (iii) soil only (Fig. 2). The most likely progenitor of each soil-only genotype was then selected as the closest MLG toward the center of the MSN, as described by Mascheretti et al. (55). The number of progenitors of soil-only MLGs in each of the three possible classes was computed to understand the possible origin of the genotypic diversity detected in soil in 2010. For MLGs present both in soil and leaves, the contribution of each substrate was assigned based on the frequencies in Figure 2.

Analyses of genetic structure and spatial autocorrelation. Populations were tested for genetic structure using Arlequin v3.5.1.2 (20). Standard analyses of molecular variance (AMOVAs) were performed on an external distance matrix of Bruvo distances among MLGs, and 10,000 permutations were performed. AMOVAs

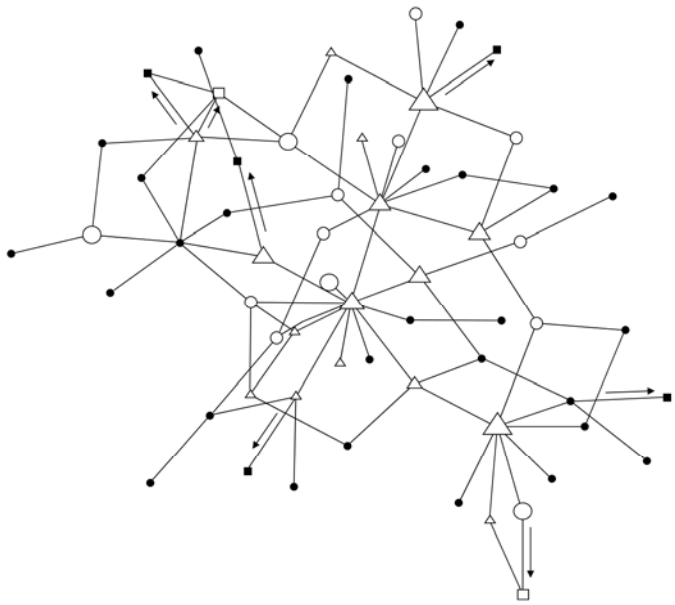

Fig. 2. Minimum spanning network for all multilocus genotypes (MLGs) detected in 2010. Each node represents a unique MLG. Node shape indicates the substrate from which an MLG was isolated: circle = leaf, square = soil, triangle = both soil and leaf. Nodes are shaded black for singleton MLGs (those represented by only 1 individual) and scaled proportionally to the number of individuals represented with that MLG: 1 (black) = singletons and 2 = 2 to 5, 3 = 5 to 10, 4 = 11 to 20, 5 = 21 to 30, and 6 = 31 to 40 individuals. Arrows mark the most likely progenitors of soil-only MLGs.

were employed to (i) study temporal genetic differentiation of populations collected at different times, (ii) study spatial genetic structure of populations collected at the same time from different sites, and (iii) infer the presence or absence of significant migration between foliar and soil populations collected from the same site.

Spagedi v1.3 (37) was used to determine presence of significant clustering of alleles using spatial autocorrelation independently for soil and leaf samples. Data were analyzed at the individual level and spatial coordinates for each tree along transects were supplied in universal transverse mercator (UTM) format (WGS84). Moran's index of genetic similarity, I (74), was used to correlate allelic repeats with spatial distance classes. In total, 20,000 permutations were used for significance testing of Moran's I.

Statistical analyses. Pearson's χ^2, Fisher's exact, and Spearman's rank correlation coefficient tests were performed in R (65) to test the significance of differences between soil and leaf isolation success among sampling times and to compare the abundance of MLGs between leaf and soil substrates and sampling times. Spearman's rank correlation coefficient test and Fisher's Exact test were used to analyze persistence of MLGs between years by comparing the rankings of MLGs within substrates between years.

The leaf data were also grouped by whether the AI in the plot for 15 and 30 days prior each sampling time was above or below the average AI calculated for the whole duration of the study. Using JMP software (version 10.0.0; SAS Institute Inc., Cary, NC), a Kruskal Wallace nonparametric analysis of variance (ANOVA) was used to compare the isolation success (%), diversity (G) and evenness (E_5) between samples above and below average aridity.

RESULTS

Climatic conditions. Weather data (Fig. 1) for the study area showed a cyclic fluctuation in air temperature throughout the year. The lowest average daily temperatures recorded each year were in February 2009 (10.3°C) and January 2010 (9.7°C), and highest daily average was in September both years (2009: 20.2°C and 2010: 19.4°C). The average daily minimum temperature was in December each year (2009: 5.4°C and 2010: 6.6°C) and average daily maximum was in September (2009: 30.9°C and 2010: 28.4°C). The highest individual temperature recorded on any day was in August 2010 at 42.8°C, and the lowest in December 2009 (–1.6°C). Monthly total rainfall peaked each year in the winter months; precisely, in February 2009 (246 mm) and in January 2010 (195 mm). Monthly rainfall in 2009 peaked in February (246 mm) and decreased in the following months to virtually nothing between April and September (0 to 12.7 mm). In 2010, the period of rain was more prolonged, peaking in January 2010 (195 mm), with rainfall remaining >20 mm until May (21 to 120 mm), before reducing to nothing. There was an absence of rain in both years during the summer months, with rain returning in the fall–winter (Fig. 1).

Sampling success and genetic diversity. In total, 425 trees were surveyed five times. From these, 1,665 symptomatic leaf samples were collected cumulatively in five sampling times, and 610 soil samples were obtained in the two Peak sampling times. Results by sampling time for soil and leaf sampling are summarized in Table 1. Isolation success from leaves ranged from 10% in the very dry fall/Late season of 2009 to 49% in the rainy and warm late spring/Peak season of 2010 (Fig. 1); incidence was found to be significantly different among sampling times (χ^2 = 146.320, df = 4, $P < 0.0001$). Soil isolation success was slightly higher in 2009 (24%) than in 2010 (19%) but this difference was not significant.

For foliar populations, diversity (expressed by G) was relatively stable during 2009, at 8.715 to 9.377. G increased in early 2010 (10.249), and reached its highest value in Peak 2010 (14.687). R decreased overall in 2009, dipping lowest in Peak 2009 (0.333) and increasing slightly in Late 2009 (0.378). In 2010, R had an inverse pattern to G, decreasing from Early 2010 (0.393) to its lowest overall value in Peak 2010 (0.282) (Table 1; Fig. 3). E_5 was greatest in Late 2009, decreased in Early 2010, and reached its lowest value in Peak 2010. Genetic structuring, measured by Fst, increased through 2009 (0.152 to 0.244) and peaked in Early 2010 (0.321), before decreasing in Peak 2010 (0.242) (Table 1; Fig. 3).

Changes in soil diversity indices were less marked than those recorded for foliar populations. The R, G, and A_r diversity indices were higher in soil in 2010 than in 2009 (2009: R = 0.268, G = 9.063, A_r = 2.77; 2010: R = 0.386, G = 12.27, A_r = 3.316) (Table 1; Fig. 3); however, fixation index (Fst, genetic differences among populations) remained relatively unchanged between years (2009: 0.216 and 2010: 0.202) while the E_5 of genotypes showed a slight increase (2009: 0.653 and 2010: 0.726), indicating lack of dominance of any particular soil genotype across sites.

Isolation success from leaves was significantly greater when the AI in the 15 days prior to the sampling period was below average (i.e., wetter; for example, Peak 2010) (Kruskall Wallace nonparametric ANOVA: P = 0.0297). G and E_5 compared between the two groups were approaching significance at the 0.05 level (G: P = 0.0825; E_5: P = 0.0825). When calculations were performed using weather data from a 30-day period prior to sampling, there was no significant difference in isolation success (sampling month AI: 0.2089 and month prior AI: P = 0.3778), G (sampling month AI: P = 0.527 and month prior AI: P = 0.5277), or E_5 (sampling month AI: 0.4807 and month prior AI: P = 0.5277).

Distribution of genotypes by substrate and time. Genotype accumulation curves for the mean number of distinct alleles indicate that, despite unequal numbers of isolates obtained from each sampling time and different substrates, the intense field sampling effort was sufficient to capture a good representation of the diversity present in both substrates in the overall dataset (Fig. 4) and in the two drainages considered independently (data not shown). The sample size where each population can be assessed evenly (g = 76) falls in the plateau of each accumulation curve (Fig. 4). The rarefied A_r index for Soil Peak and Leaf Peak are

TABLE 1. Sampling and isolation summary, monthly aridity index (AI_M) and gene diversity indices for soil and leaf samples[a]

Sampling time	AI_M	Trees surveyed	Symptom incidence	Sampled	Isolations	Isolation success (%)	MLGs (year)	MLGs (season)	G	R	E_5	A_r	Fst
Soil and leaf totals	…	…	…	2,275	523	23	84	…	…	…	…	…	…
Leaf Early 2009	2.64	425	0.69	295	56	19	35	25	8.805	0.429	0.638	2.875	0.152
Leaf Peak 2009	0.22	425	0.93	394	63	16	…	22	8.715	0.333	0.632	2.709	0.208
Leaf Late 2009	2.22	425	0.91	388	39	10	…	15	9.377	0.378	0.798	2.6	0.244
Leaf Early 2010	44.17	425	0.79	334	57	17	55	23	10.249	0.393	0.654	2.831	0.321
Leaf Peak 2010	0	425	0.83	354	173	49	…	49	14.687	0.282	0.593	3.075	0.242
Soil 2009	…	…	…	302	72	24	20	...	9.063	0.268	0.653	2.77	0.216
Soil 2010	…	…	…	308	59	19	23	...	12.277	0.386	0.726	3.136	0.202

[a] MLGs = multilocus genotypes detected, G = Stoddart and Taylor's index, R = clonal genotype diversity, E_5 = evenness index, A_r = allelic richness with rarefaction; Fst values among sites calculated by analysis of molecular variance (P = 0.0001).

very similar when compared within each year; 2009 Peak Leaf and Soil are both ≈2.7, and increased equally in 2010 for both Leaf Peak and Soil Peak to 3.07 and 3.13, respectively (Table 1; Fig. 5).

In total, 33 and 20 MLGs were identified from leaves and soil, respectively, in 2009; that number increased to 55 and 23, respectively, in 2010 (Table 1; Supplementary Table 2). Many of the MLGs identified were singletons (i.e., distinct MLGs represented by only a single isolate. In leaves, singleton MLGs represented 43% of all leaf MLGs in 2009 and 57% in 2010. However, in terms of individuals, singletons represented only a small proportion of the population (2009: 9% and 2010: 14%). In soil, the same pattern was seen; 35% of soil MLGs were singletons in 2009 and 48% in 2010, representing 10 and 19% of individuals, respectively.

A number of MLGs were found in both soil and leaves each year: 13 in 2009 and 15 in 2010. When the abundances (as a per-

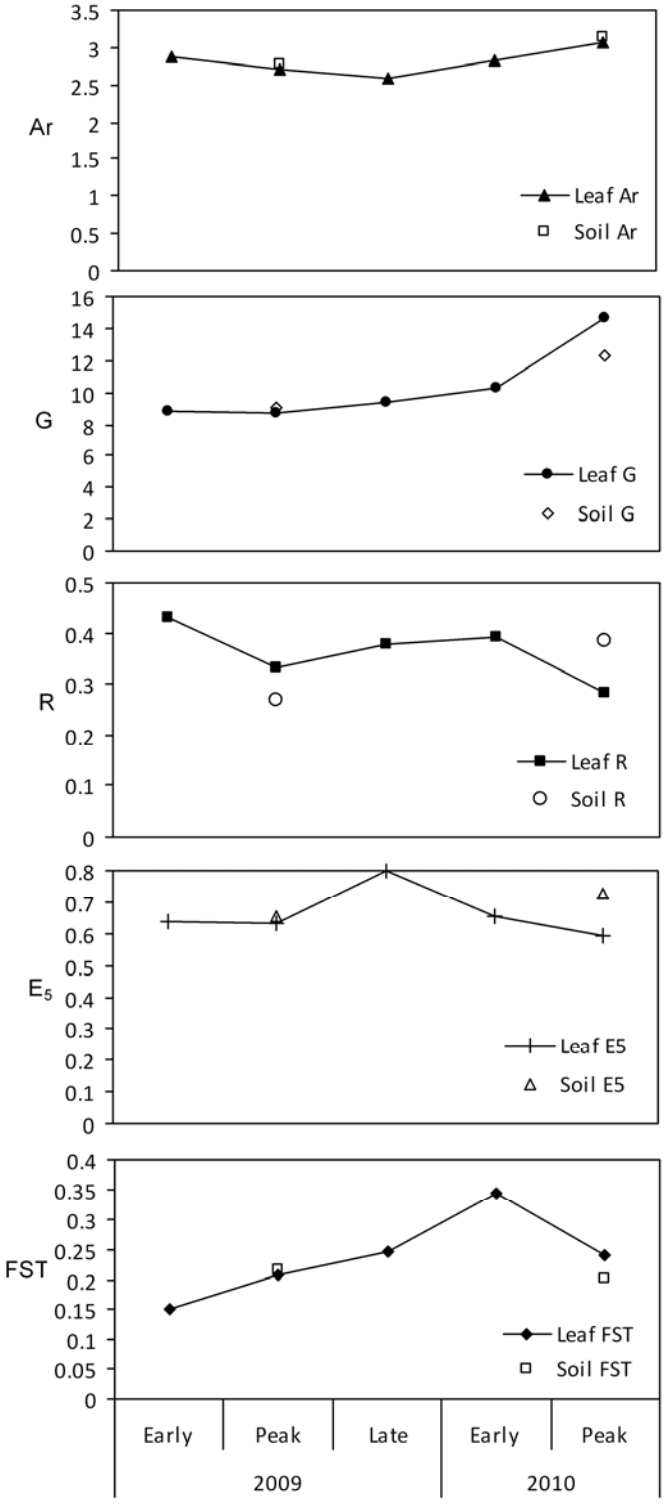

Fig. 3. Genetic diversity indices compared between soil and leaf populations for each sampling time (2009: Early, Peak, and Late; 2010: Early and Peak): (i) Allelic richness (*Ar*); (ii) Stoddart and Taylor's index (*G*); (iii) clonal genotypic diversity (R); (iv) evenness index (E_5); (v) genetic structure (Fst) of individual populations partitioned by plot calculated by analysis of molecular variance.

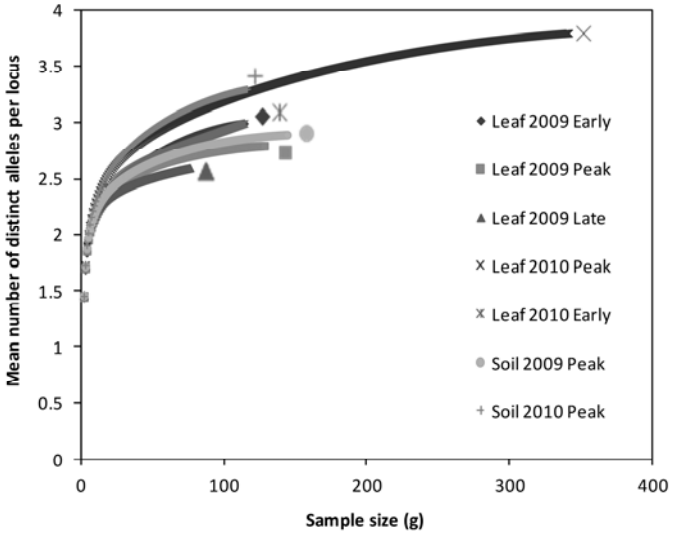

Fig. 4. Genotype accumulation curves calculated using rarefaction for the mean number of distinct alleles per locus for soil and leaf population at each sampling time.

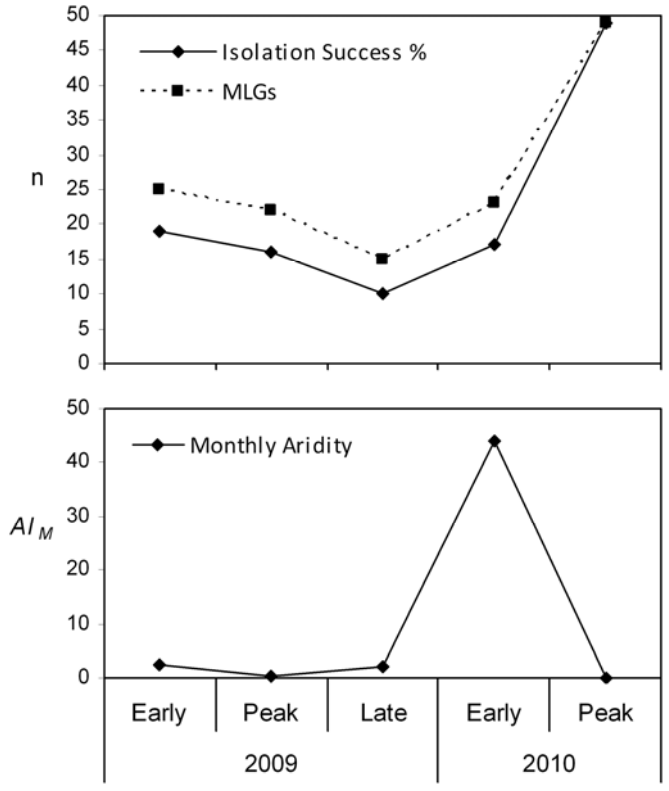

Fig. 5. Success of isolation (%) of *Phytophthora ramorum* from leaf samples, number of multilocus genotypes (MLGs) detected, and the monthly aridity for each sampling time (2009: Early, Peak, and Late; 2010: Early and Peak).

centage of the whole population in each substrate) of the MLGs present in both soil and leaves were compared, no significant difference was observed in 2009 (χ^2 = 10.5222, df = 12, P = 0.5702) but a significant difference was detected between the two substrates in 2010 (χ^2 = 27.0098, df = 14, P = 0.0192) (Fig. 6).

Temporal turn-over of genotypes in leaves and soil. In total, 63 MLGs were found in leaf populations during the study. Of these, 27 were isolated in both 2009 and 2010 (42.8% of MLGs). When the relative abundance of each leaf MLG in each year was ranked, there was significant correlation between years (Spearman's rank: ρ = 0.76, n = 27, P = 0.0000), indicating a persistent foliar population between years. In soil, 35 MLGs were found during the study (total for 2009 and 2010 together). Only eight of these MLGs (22.8% of all MLGs) were found in both years. In contrast to the leaf populations, the overall rank of these persistent soil MLGs varied between years (Spearman's rank: ρ = –0.18542, n = 8, P = 0.704). The relative abundances of certain soil MLGs changed dramatically between years. For example, MLG 66 was present in very low numbers in 2009 (n = 2, 2% of all isolates) but was the most abundant MLG found in soil in 2010 (n = 10, 12%). Similarly, although in the reverse direction, MLG43 was most common in soil in 2009 (n = 19, 26% of all isolates) but its abundance was reduced to only 3 individuals in 2010, reduced 20% from 2009 to just 5% of all isolates (Table 2). This same pattern was seen occasionally in individual leaf MLGs (e.g., MLG66 in leaf populations had 3 individuals in 2009, increasing to 27 in 2010) but, generally, the most abundant leaf MLGs in 2009 remained the most abundant in 2010.

Of 64 trees that yielded viable leaf isolations in 2009, 51 (80%) also yielded viable cultures in 2010 and 16 (25%) yielded the same MLGs both years. Of 72 viable soil isolations from 2009, only 13 (18%) were successful again in 2010, and the same MLG was recovered from the same location in only 2 (3%) soil samples in consecutive years. A Fisher's exact test determined that persistence of identical MLGs was significantly higher in leaves than in soil (two-tailed P value < 0.0001).

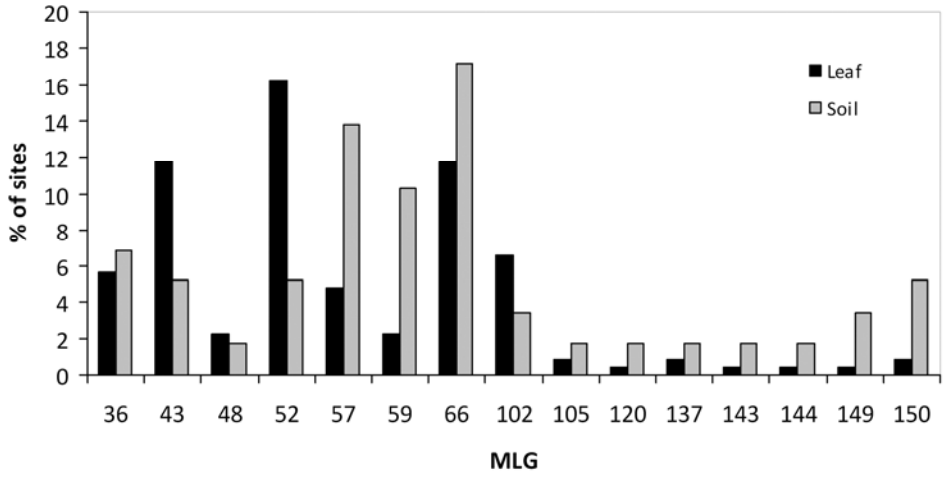

Fig. 6. Comparison of the relative abundance of the overlapping multilocus genotypes (MLGs) that were isolated from both soil and leaf samples in 2010. Values are the percentage of the whole population that each MLG represents in either soil or leaves.

TABLE 2. Incidence of multilocus genotypes (MLGs) found in both years in leaf and soil populations

Leaf				Soil			
MLG	2009	2010	+ or – Percent 2009	MLG	2009	2010	+ or – Percent 2009
43	35	27	–10.2	43	19	3	–21.2
52	24	37	1.1	57	6	8	5.5
57	15	11	–4.6	36	6	4	–1.4
102	13	15	–1.6	52	6	3	–3.2
59	11	5	–4.7	102	4	2	–2.1
48	8	5	–2.8	59	3	6	6.2
118	8	8	–1.5	48	3	1	–2.4
132	6	3	–2.5	66	2	10	14.5
106	4	6	0.1	…	…	…	…
36	3	13	3.8	…	…	…	…
66	3	27	10.0	…	…	…	…
62	2	5	0.9	…	…	…	…
149	2	1	–0.8	…	…	…	…
19	2	7	1.8	…	…	…	…
70	2	2	–0.4	…	…	…	…
26	2	1	–0.8	…	…	…	…
97	1	2	0.2	…	…	…	…
9	1	1	–0.2	…	…	…	…
137	1	2	0.2	…	…	…	…
56	1	7	2.4	…	…	…	…
11	1	2	0.2	…	…	…	…
68	1	2	0.2	…	…	…	…
94	1	2	0.2	…	…	…	…
37	1	1	–0.2	…	…	…	…
40	1	1	–0.2	…	…	…	…
61	1	1	–0.2	…	…	…	…
153	1	1	–0.2	…	…	…	…

An AMOVA analysis was performed in which all leaf isolates obtained at the same time within a year (Early, Peak, and Late) represented a population, independent of site of origin but hierarchically grouped in the year according to the time when sampling was performed. In leaf populations, there was no significant genetic structuring between 2009 and 2010 samplings (gentic differences between groups [Fct] = 0.00434, $P = 0.11603$) or among sample times within each year (genetic differences among populations within groups [Fsc] = 0.00176, $P = 0.27663$). Conversely, AMOVA analysis of soil samples revealed significant structure between 2009 and 2010 (Fst = 0.04654, $P \leq 0.001$).

Genetic comparison between soil and leaf samples. The presence of genetic structure between soil and leaf samples was analyzed using AMOVA. Peak season soil populations were compared with leaf populations obtained at concurrent or earlier samplings. Results showed significant genetic structure (significant Fsc values) between soil and leaf samples in 2010 but not in 2009 (Table 3).

When comparing genetic diversity indices between the two substrates sampled at the same time in Peak time, comparable *G* values were obtained in soil and leaves in dry 2009. In wet 2010, R from leaves (Peak: 0.282) was lower than R from 2009 leaves (Peak: 0.333) and also lower than R from soil (0.386) calculated for the same year (Table 1; Fig. 3).

The MSN analysis showed that four of the soil-only MLGs most likely derived from soil progenitors and the three other soil-only MLGs came from leaves. Soil and leaf genotypes did not segregate in different portions of the network. Singleton MLGs were generally located around the periphery of the network.

Spatial analysis within leaf and soil samples. Examining each substrate separately, genetic structure was identified among different plots both in leaves (variation = 4.7%, Fsc = 0.05436, $P = 0.01287$) and soil (variation = 11%, Fsc = 0.122, $P = 0.000$), whereas drainage was not significantly different for either substrate. When isolates from both substrates were grouped together by plot, there was still overall significant structure both between plots (variation = 16.71%, Fct = 0.16715, $P = 0.0002$) and also between the substrates within those plots (variation = 2.46%, Fsc = 0.02951, $P = 0.0005$). Fst values among leaf populations sampled at the same time from different plots were 0.152 to 0.321 (P value ≤ 0.001) (Table 1; Fig. 3). Fst was lowest (0.15) in the dry and cold winter to early spring sampling of 2009, and gradually increased throughout all successive samplings until winter to early spring 2010, when Fst values peaked at 0.32. A much lower Fst value (0.24) was recorded in the late spring 2010 sampling, only 3 months after the previous 2010 sampling, and in conjunction with the first period of abundant rainfall and warm temperatures in 3 years. When the same analysis was performed on soil samples from both years, the Fst values among plots were found to be highly significant in the analysis in both 2009 and 2010 (Table 1).

For leaf populations, number and frequencies of MLGs present in at least two plots were compared between the Early and Peak 2010 samplings. The number of MLGs in at least two plots increased from five MLGs (representing 21 of 57 isolations) in the 2010 winter to nine MLGs (representing 105 of 171 isolations) in the late 2010 spring (Fisher's exact test, late spring > winter = $P < 0.001$). For eight of the nine MLGs (89% of isolations) present in two or more sites in Peak 2010, a specific MLG had never been detected in at least one of the sites during any of the four earlier samplings.

Spatial autocorrelation analyses. Analysis of how genetic distance correlated with spatial distance found that the general patterns of genetic similarity versus distance were similar for soil and leaves in 2009 (Fig. 7A and C), with significant aggregation of identical alleles until ≈4 m for both soil and leaves. In 2010, aggregation of identical alleles increased for both substrates but at different rates. In fact, significant aggregation was detected ≤12 m for soil and up to just >200 m for leaves (Fig. 7B and D).

DISCUSSION

This is one of the first studies to examine the natural population dynamics for *P. ramorum*, and for a plant pathogen in general, in different substrates (soil and leaves) through time, with sampling encompassing a transition from a drought to a wet year. This study was not designed to determine an exact correlation between climatic parameters and population dynamics but, rather, was focused on identifying shifts in pathogen dynamics at the transition between unfavorable and favorable conditions (i.e., the abundant rainfall between the Early and Peak samplings of 2010 following the 2009 drought). This transition is clearly highlighted by the values of the AI, a composite of rainfall and temperature data, which show a substantial change in conditions in 2010 from

TABLE 3. Composite table of analysis of molecular variance (AMOVA) results from four analyses where soil and leaf populations of *Phytophthora ramorum* are compared[a]

AMOVA analysis, samples compared, and source of variation	df	Sum of squares	Variance component	Variation (%)	Fixation index	*F* value	*P* value
(i) Early leaf 2009 versus soil 2009							
Among plots: a plot is early leaf 2009 pooled with peak soil 2009	3	0.605	0.0096	19.95	Fct	0.1995	0.0191
Between early leaf 2009 and peak soil 2009 by plot	3	0.093	–0.0005	–0.99	Fsc	–0.0124	0.4445
Among individuals within plot from both early leaf 2009 and peak soil 2009	108	4.195	0.0389	81.04	Fst	0.1896	0.0000
Total	114	4.893	–0.0479	…	…	…	…
(ii) Peak leaf 2009 versus soil 2009							
Among plots	3	0.693	0.0092	18.55	Fct	0.1855	0.0180
Between leaf and soil	3	0.179	0.0011	2.23	Fsc	0.0274	0.0679
Among individuals within plot	116	4.575	0.0394	79.22	Fst	0.2078	0.0000
Total	122	5.448	0.0498	…	…	…	…
(iii) Early leaf 2010 versus soil 2010							
Among plots	3	1.048	0.0137	24.14	Fct	0.2414	0.0115
Between leaf and soil	4	0.272	0.0021	3.74	Fsc	0.0493	0.0400
Among individuals within plot	101	4.124	0.0408	72.12	Fst	0.2788	0.0000
Total	108	5.444	0.0566	…	…	…	…
(iv) Peak leaf 2010 versus soil 2010.							
Among plots	3	1.800	0.0114	19.73	Fct	0.1973	0.0083
Between leaf and soil	4	0.367	0.0024	4.21	Fsc	0.0525	0.0009
Among individuals within plot	213	9.330	0.0438	76.05	Fst	0.2395	0.0000
Total	220	11.496	0.0576	…	…	…	…

[a] In the first hierarchical level (Fct), soil and leaf populations for each plot were pooled and plots are compared. In the second hierarchical level (Fsc), soil and leaf populations within each plot were compared with one another. In the third level (Fst), all individuals were compared by plot independent of substrate. Each of the four analyses independently compared an individual population of leaves sampled at a particular time with the soil sampled in the same year.

2009, mirrored by significant changes in isolation success and in values of the several indices calculated for leaf populations. During this climatic transition, all indices of genetic diversity varied in the same direction in the entire dataset and in the two drainages considered independently, indicating that a reliable generalized response had been identified. However, seasonal climatic variations during the drought often triggered differential responses in the two drainages, which can be addressed in future studies.

The ability to culture the pathogen from bay laurel leaves was taken as an indicator of its viability. Although viability may underestimate pathogen presence, due to symptomatic but non-culturable bay laurel leaves potentially harboring dormant *P. ramorum* infections (9), it has been commonly used to study the portion of a pathogen population that responds rapidly to changing weather conditions and that is responsible for outbreaks and new disease cycles (10,13). It is clear that *P. ramorum* can recover extremely rapidly even after a long dry period. Viable infections were low and comparable throughout 2009 and in early 2010 but incidence of viable infections almost tripled in only 3 months as the wet period of prolonged rainfall occurred in spring 2010. This is one of the first quantifications of population recovery of this pathogen after an unfavorable period lasting 3 years.

Viable pathogen populations were larger during 2010, when the more prolonged and abundant rainfall in spring generated conditions conducive to pathogen proliferation. The fast response of the pathogen to shifts in climatic conditions is supported by the significance of analyses using a 15-day period prior to sampling but not a 30-day period. Indeed, isolation success was significantly greater in plots and sampling times that were wetter than average in the 15-day period prior to sampling (i.e., Peak 2010). Concomitantly, overall allelic and genotypic richness were also slightly increased while evenness of genotypes was reduced (i.e., a few highly abundant genotypes dominate), and their migration levels increase more markedly. This correlation between genetic diversity and population size is to be expected in a clonally reproducing species such as *P. ramorum* or *P. cinnamomi*, where new genotypes arise through mutations and somatic recombination events which will happen in direct relationship to population size (18,55).

These results agree with much work on infectious diseases and pests both of plants and animals (8,28,47). In an epidemic phase, favorable environmental conditions lead not only to increased reproductive rate but also to the dominance of a few genotypes, potentially better adapted to transmission during outbreaks (24). In our study, several MLGs clearly followed this pattern (e.g., MLG66, which went from being relatively rare in 2009 to being dominant in both soil and leaves in 2010). Although post-epidemic chronic conditions are characterized by the presence of relatively few genotypes, lack of transmission and migration of dominant genotypes in unfavorable conditions (24) leads to higher relative local diversity, as reported for Dutch elm disease (70,75).

Soil populations did not display the increased levels of migration and the reduction in genotype evenness between 2009 and 2010 that were observed in leaf populations. It has been suggested that soil populations of *P. ramorum* are derived from foliar infestations (22); consequently, our expectations were that trends in both substrates would have mirrored each other and that source populations (i.e., infectious leaves where most sporulation occurs) would have been genotypically significantly more diverse than sink populations (e.g., soil). The MSN showed that MLGs found in soil and leaves do not segregate in different parts of that network, confirming that populations in the two substrates intermix. Furthermore, based on our MSN analysis, genotypes only found in the soil did not originate exclusively from either soil or leaf progenitor genotypes but were generated in equal numbers from both. Alone, these analyses suggest that leaf and soil populations are overlapping in terms of genotype composition, as would be expected if soil populations were derived from foliar

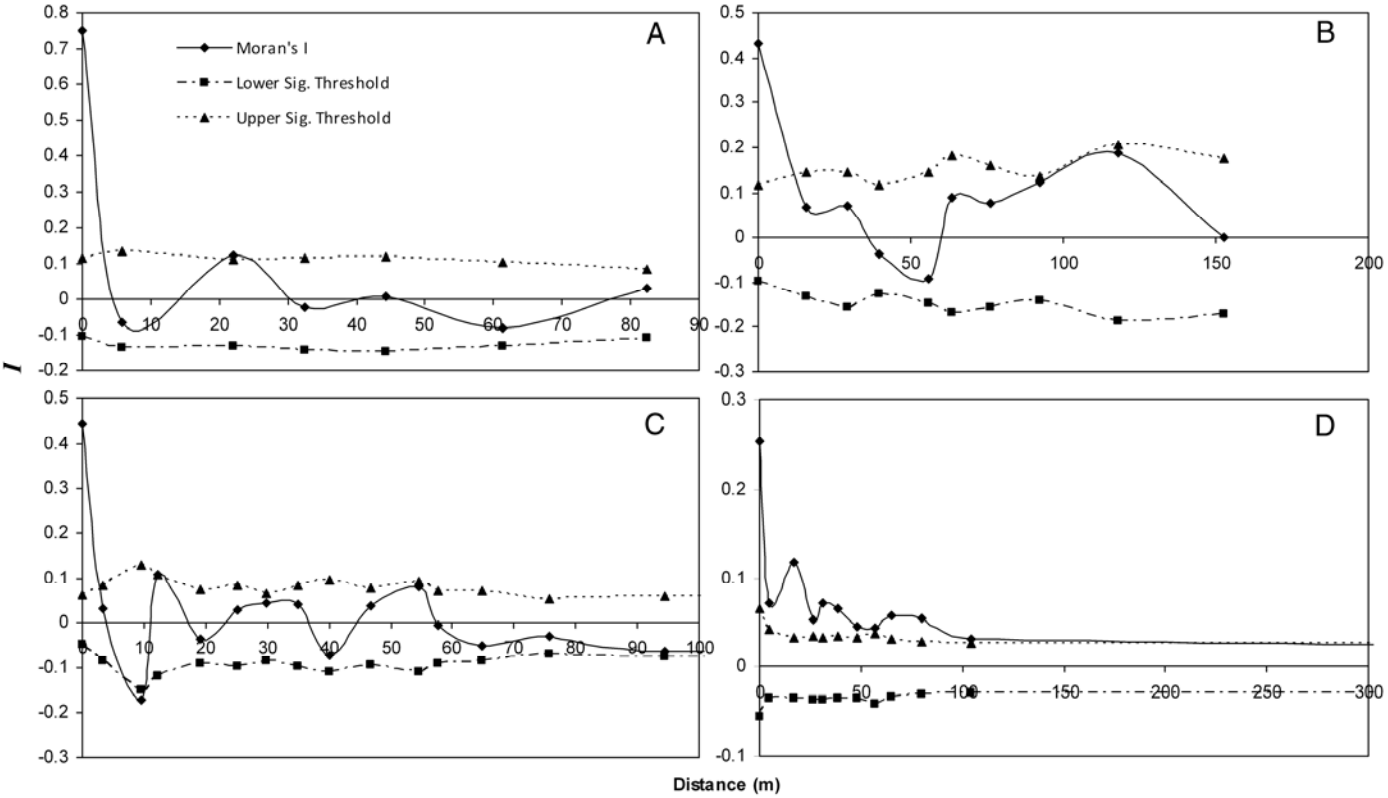

Fig. 7. Genetic similarity, Moran's *I*, plotted against distance (m), with upper and lower significance thresholds plotted: **A,** soil 2009; **B,** soil 2010; **C,** leaf 2009; **D,** leaf 2010.

ones. However, AMOVA identified a small but significant structure between populations in the two substrates, and statistical analyses determined that, whereas frequencies and ranks of genotypes were comparable in dry 2009, that was not the case in wet 2010. We interpret this as the consequence of the dynamics of inoculation of the two different substrates, with infection of leaves being ecologically more stringent and strongly mediated by density of inoculum and ability of the pathogen to rapidly and competitively infect leaves. In contrast, soil inoculation may simply be a function of inoculum density and escape from predation.

A major factor in determining the extent and spread of disease is the volume and spatial distribution of sporangia spreading from infected plants, as well as host susceptibility and weather conditions. *P. infestans* and *P. capsici* are two other species known to produce aerial sporangia. *P. infestans* sporangiophores release windborne sporangia under dry conditions, thus facilitating medium- to long-range dispersal (73). In contrast, sporangial release by *P. capsici* occurs in wet conditions, allowing sporangia to remain turgid (30,31) and resulting in their limited spatial spread. Our data and published literature (15,30,31) suggest that *P. ramorum* is similar to *P. capsici* in terms of dispersal, with sporangial release and distance covered by sporangia being strongly mediated by intensity and length of rain events. Spatial autocorrelation results show convincingly that, in dry conditions, spatial aggregation of identical alleles stops at 4 m from a source, independent of substrate. In wet conditions, that range increases to 12 m for soil populations and >200 m for leaf populations. This short- to medium-range movement may be essential to cross the gaps in forest vegetation that are extremely common in California coastal woodlands. The ability of the pathogen to spread longer distances in environmentally favorable conditions is an additional component that may reduce Fst values by increasing the pathogen's range of migration.

Previous estimates of cumulative multiyear movements for *P. ramorum* ranged between 0 and 350 m, based on genetic (54) and landscape-level (46) observations. This contrasts to *P. infestans*, which is extremely widely dispersed, facilitated both by human transport (across continents) and aerially dispersed over long distances (kilometers) via wind (6,25). *P. ramorum* has also been introduced on multiple occasions into California by human transport but, from these introduction points, it has spread relatively slowly via environmental dispersal (54). Thus, in favorable environmental conditions, *P. ramorum* genotypes will arrive from more distant sources (medium range) but with lower inoculum densities.

Our results also show that, in a natural setting, soil genotypes are capable of spreading either over equal (in a dry year) or much lower (in a wet year) distances than foliar genotypes; thus, soil populations may be less epidemiologically relevant than previously thought with regards to spread of the pathogen within a forest or even between distinct forest stands. Of course, soil has been shown to be infectious (22) and infested soil has been reported past the margins of infested forests, especially along trails used by humans and ungulates (12,15). Thus, there may be a significant epidemiological role for soil infestations in artificial settings or at the edges of forest stands.

Population size and genetic structure were both greatly affected by yearly climatic variation and, to a smaller degree, by seasonal variations. This raises the issue of whether the bottlenecks experienced during the dry season or during a drought may lead to a significant change in the microevolutionary trajectory of the pathogen in the SFPUC watershed. In other words, was there a significant shift in genotypic composition, in particular following the 2007 to 2009 drought? Additionally, were both soil and leaf populations reservoirs providing pathogen genotypes that started the 2010 outbreak? AMOVAs indicated a very minimal change in the genetic structure of foliar populations, supported by a strong correlation between the rankings of MLGs found in consecutive years (i.e., the few most dominant MLGs found in leaves in 2009 were also dominant in 2010). Conversely, soil populations displayed a significant difference between the 2009 and 2010 samplings. Furthermore, instances where isolations from the same trees or area of soil in consecutive years yielded the same genotype were much more frequent in leaves than in soil, suggesting that infection persists more readily in leaves between years. Carryover of identical genotypes between years may ensure an overall stable genetic composition of populations on leaves. In the case of soil, the low frequency of samples yielding the same genotype indicates that the soil population may not be as genotypically stable through time as that of leaves, and that pathogen survival is less than a year, meaning that that soil was reinoculated during the 2010 rainy season. Other studies of survival of *P. ramorum* in forest soil have indicated that viability of the pathogen was <1 year, and that survival was negatively correlated with temperature and organic richness of the top layer (22). Although this apparent turnover in soil could be thought to be an artifact of insufficient sampling from a much more diverse pool of genotypes, our rarefaction analysis indicates that our soil-sampling efforts were sufficient for the average allele frequencies to be comparable and to capture the extent of diversity. It should be noted that survival in soil may be longer than recorded in this study if two consecutive wet years were to be considered. However, two wet years are a relatively infrequent event in California; thus, we believe that the results presented here identify a general pattern of persistence that differs significantly between leaves and soil. We conclude that oversummering of the pathogen occurs in leaves and not in soil; thus, next year's disease cycle is started by genotypes that survive in bay laurel leaves, while soil genotypes may be epidemiologically less relevant in the natural disease cycle.

The six study plots were located 2 to 7.3 km apart, much closer than distances between plots in other previous studies in California (54,55). To ensure good spatial and ecological coverage, study plots were equally distributed within two different drainages. Although AMOVA detected no significant effect of drainage, there were significant genetic differences among the populations found at each plot. This is consistent with limited migration of the pathogen among even closely spaced sites, leading to strong founder effects (54,55). It has been estimated that three founding genotypes are responsible for most California infestations (54,55); hence, it is likely that a few founding genotypes started the infestations within the SFPUC watershed, giving rise to the observed differences between plots, simply because of stochastic effects, potentially amplified by the different rate of successful isolation among plots. However, when plots within a drainage are grouped and drainages were compared, the increased sample size and spatial coverage resulted in an obvious increase in the chances of capturing all or most of the founding genotypes and their progeny, thus eliminating the source of the genetic differences observed at the individual plot level. This interpretation points to a local diversity that is generated not by a successful process of local diversification but, rather, by a lack of equilibrium among sites, due to limited levels of migration between spatially discrete infestations.

The reasons why the lowest Fst value was obtained in the winter or Early season of 2009 (the first sampling of this study) are not clear; however, this result may be the legacy of the extremely favorable weather conditions of 2005 and 2006, when a major outbreak occurred. Fst values increased at each sampling time following that first sampling time until early 2010. This is an interesting result, showing the cumulative effects of persisting environmentally unfavorable conditions. In unfavorable conditions, genotypes that emerge locally are less likely to be challenged by successful and fast-spreading genotypes coming from other sites within the watershed. The first onset of favorable environmental conditions in the late spring or Peak season of 2010 resulted in a significant drop in Fst (Fig. 3). We surmise that this lower Fst value may be the result of (a) increased repro-

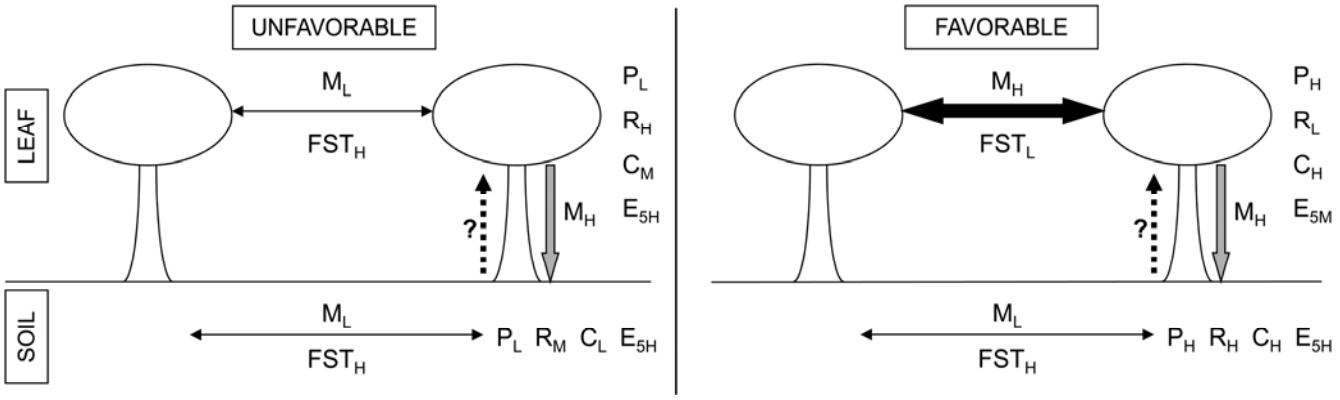

Fig. 8. Schematic summary of the interaction between leaf and soil populations in contrasting environmental conditions, with respect to the effect on population size, diversity, genetic structure, migration, and competition. P, population size; R, local genotypic diversity; C, competition; M, migration; E_5, evenness. Subscripts represent relative levels of each process taking place: L, low; M, medium; H, high.

ductive rate and (b) enhanced movement of successful genotypes among sites. Increased number of genotypes and tripled infection incidence in the wetter 2010 both support the validity of hypothesis a. The validity of hypothesis b is supported by the significant increase in the presence of the same genotype in two or more sites. The fact that these genotypes were missing from at least one of the sites in 89% of the cases suggests that they are not originating locally but are migrating among sites (although obviously not just the six sites included in this study).

Results from this study can be unified into a schematic summary or model for the interaction between soil and leaf populations under contrasting environmental conditions (Fig. 8). During an unfavorable year, population sizes are small both in soil and on leaves, due to repression of sporulation and transmission caused by the lack of favorable environmental conditions (15,41). Infected leaves are a source of inoculum for both uninfected leaves and soil; hence, a depression in sporulation will result in small population size (P_L) in both substrates. Similarly, these unfavorable conditions reduce both the amount and distance of migration (M_L), leading to only moderate levels of competition between local and migrant genotypes (C_M) and resulting in high local genetic diversity (R_H), greater evenness (E_{5H}), and high genetic structure between sites (Fst_H) for both soil and leaves. In a rainy and warm year, favorable conditions facilitate an increase in both population size (P_H) and migration or transmission between foliar hosts (M_H). In favorable conditions, high population size (P_H) and high migration (M_H and Fst_L) lead to a high level of competition for space and resources (C_H), resulting in the dominance of the more fit genotypes and in low genotypic diversity in leaves (R_L) and less evenness (E_{5M}), where transmission ability will be directly correlated with success of infection and establishment. A larger population in leaves during favorable conditions will also result in a more diverse soil population (P_H and R_H) but lack of long-distance soil-to-soil movement (M_L) will result in high soil diversity and high evenness both within (R_H and E_{5H}) and among (Fst_H) sites. Although possible (22), it is unknown whether soil genotypes do actually infect leaves in the canopies of bay laurels.

We conclude that the epidemiology of sudden oak death in California may be significantly altered by identifying what ecological factors characterize foliar refugial sites during droughts, and by attempting to reduce disease incidence on bay laurel leaves in these refugial sites during a dry year, when natural survival of the pathogen is lowest.

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