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Reconstruction of the Sudden Oak Death epidemic in California through microsatellite analysis of the pathogen *Phytophthora ramorum*

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Abstract

The genetic structure of the clonally reproducing Sudden Oak Death (SOD) pathogen in California was investigated using seven variable microsatellites. A total of 35 multilocus genotypes were identified among 292 samples representative of populations from 14 forest sites and of the nursery trade. AMOVA indicated significant genetic variability both within (44.34%) and among populations (55.66%). Spatial autocorrelation analyses indicated that Moran's index of similarity reached a minimum of 0.1 at 350 m, increased to 0.4 at 1500 m and then decreased to zero at 10 km. These results suggest a bimodal pattern of spread, with medium range dispersal (1500–10 000 m) putatively attributed to the presence of strong winds. Lack of genetic structure was identified for three groups of populations. One group notably included the nurseries' population and two forest populations, both linked to early reports of the pathogen. A neighbour-joining analysis based on pairwise Φ_{ST} values indicated that the clade inclusive of the nurseries' populations is basal to all California populations. A network analysis identified three common genotypes as the likely founders of the California infestation and proposes a stepwise model for local evolution of novel genotypes. This was supported by the identification in the same locations of novel genotypes and of their 1- or 2-step parents. We hypothesize that the few undifferentiated population groups indicate historical human spread of the pathogen, while the general presence of genetically structured populations indicates that new infestations are currently generated by rare medium or long-range natural movement of the pathogen, followed by local generation of new genotypes.

Keywords: genetic structure, microsatellite, network analysis, *Phytophthora ramorum*, spatial autocorrelation, Sudden Oak Death

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Introduction

The oomycete *Phytophthora ramorum* (Straminopiles: Peronosporales) (Werres *et al*. 2001) is the causal agent of Sudden Oak Death (SOD) (Rizzo & Garbelotto 2003), a disease characterized by lethal trunk lesions that affect tanoak (*Lithocarpus densiflora*), coast live oak (*Quercus agrifolia*), Californian black oak (*Quercus kelloggii*), and Canyon live oak (*Quercus chrysolepis*). SOD was first reported in California in 1994 and *P. ramorum* was first identified as the causal agent of SOD in 2000 (Garbelotto & Rizzo 2005). *P. ramorum* also causes a disease characterized mostly by leaf blight and/or branch dieback in over 100 species of both wild and ornamental plants, including California bay laurel (*Umbellularia cailfornica*), California redwood (*Sequoia sempervirens*), Douglas-fir (*Pseudotsuga menziesii*), *Camellia* and *Rhododrendron* species (Davidson *et al*. 2003). For an updated list of confirmed host plants as of 2007, please consult the US Federal Register (USDA-APHIS 2007).

Infestations by *P. ramorum* are found in Pacific coastal forests, generally less than 30 km inland. Its distribution, from Monterey County in central California to as far north as Curry County, Oregon, is characteristically discontinuous

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(Rizzo *et al*. 2005). *P. ramorum* has also been found in numerous nurseries in several European countries. Indeed, it was originally described as a pathogen of *Rhododendron* and *Viburnum* species in Germany and The Netherlands in 1994 (Werres *et al*. 2001). Importantly, *P. ramorum* has also been sporadically identified in nurseries throughout the USA and Canada as the result of the movement of infested ornamental plants (Rizzo *et al*. 2002; Hansen *et al*. 2003; Ivors *et al*. 2004; Olarte & Garbelotto 2005). In Californian forests, the most commonly infected tree is bay laurel (Garbelotto *et al*. 2003; Davidson *et al*. 2005). Sporangia production on its leaves is prolific and bay laurel is therefore the main source of aerial infectious inoculum. In contrast, sporulation on oak wood is extremely rare and so oak species may be considered as a noncontagious dead-end host in the *P. ramorum* life cycle (Davidson *et al*. 2005). A secondary, but significant, source of inoculum is represented by tanoak twigs (Maloney *et al*. 2005), as exemplified by the Oregon infestation, where bay laurels tend to be either infrequent or do not appear to be infected (Goheen *et al*. 2002a, b).

As a heterothallic Oomycete, *P. ramorum* is able to reproduce asexually (clonally) in the presence of conducive wet and warm environmental conditions (Davidson *et al*. 2005). However, sexual reproduction is only possible when individuals with different mating types interact (Brasier & Kirk 2004). Mating type A1 occurs commonly in Europe, whereas only mating type A2 has been identified in Pacific forests (Brasier & Kirk 2004). Hence, it has been hypothesized that *P. ramorum* propagates in an exclusively clonal manner in California forests. Although isolates belonging to both mating types have been found in the same nurseries in western states of the USA (Hansen *et al*. 2003; Ivors *et al*. 2004; Grunwald *et al*. 2008), regular inspections and quarantines appear so far to have been effective, and no A1 genotypes have yet emerged in the wild. Studies employing mitochondrial DNA sequence variation (Kroon *et al*. 2004), amplified fragment length polymorphism (AFLP) markers (Ivors *et al*. 2006) and microsatellites (Prospero *et al*. 2004, 2007) have indicated that individuals characterized by the A1 and A2 mating types belong to two evolutionarily distinct lineages, each characterized by limited within-lineage genetic variability. A further microsatellite study (Ivors *et al*. 2006) has indicated the presence of a third distinct lineage with the A2 mating type in some nurseries in the USA, and has formally confirmed the clonal nature of pathogen populations in forests in the USA. These results support the idea that *P. ramorum* is an exotic introduction and suggests that the entire forest population of the USA may have been founded by one or very few genotypes. Although the origin of the pathogen remains unknown, the current genetic structure of the pathogen in North America may be best explained as a result of multiple introductions into nursery populations, where all three lineages are present, followed by the introduction of individuals belonging to a single lineage from the nurseries into the wild (Ivors *et al*. 2006). The genome of *P. ramorum* has been recently sequenced (Tyler *et al*. 2006; www.jgi.doe.gov): rates of heterozygosity throughout the genome clearly indicate that the most common clonal genotype in California (Pr102) derives from a sexually reproducing population. The higher-than-expected heterozygosity at several microsatellite loci has been suggested to imply that clearly distinct lineages of *P. ramorum* have occasionally mated in the past in the area of origin, creating heterozygotic genotypes that then undergo abundant asexual reproduction (Ivors *et al*. 2006), as reported for other *Phytophthora* species (Forster *et al*. 1994).

Microsatellites are codominant markers which typically display a high level of variability and are therefore frequently used to investigate the genetic structure of populations. However, the identification of variable microsatellite loci in organisms that reproduce clonally is still relatively rare. In the case of *P. ramorum*, 12 microsatellite markers have been developed by Ivors *et al*. (2006) and 10 by Prospero *et al*. (2007), but only a few of these markers showed significant variation within the NA1 lineage present in Pacific forests. Ivors *et al*. (2006) found almost no variation within forest populations of the USA, while Prospero *et al*. (2007) were able to identify several distinct genotypes. The latter study analyzed the genetic structure of pathogen populations collected between 2001 and 2004 from sites located in a single forest in southwestern Oregon (Curry County) and from Oregon nurseries. Results indicated lack of gene flow between forest and nursery populations in Oregon and indicated low genetic diversity in forests with a common and widespread multilocus genotype (MG) that persisted over the four years, while minor novel genotypes appeared each year. It should be highlighted that repeated eradication efforts maintained pathogen populations at a minimum level in the study areas and therefore the results are likely not to be representative of population dynamics in an unmanaged situation.

In the present study, we investigate the population genetic structure of *P. ramorum* in California using microsatellite markers that have previously been shown to be variable within the NA1 lineage of *P. ramorum* (Ivors *et al*. 2006; Prospero *et al*. 2007). Inferences regarding the origin of the epidemic are made by comparing the allelic composition of the nursery population with that of forest populations. Inferences regarding the spread of the pathogen were made based upon the degree of genetic differentiation among the 14 forest populations, and on spatial autocorrelation analyses of genetic similarity among pathogen genotypes with geographical distance. To elucidate the evolutionary dynamics of the introduced pathogen, and in particular to study the extent to which *P. ramorum* populations are becoming spatially and temporally structured, we investigated the presence of genetic structure between old, intermediate and new infestations. We performed a genotype network analysis

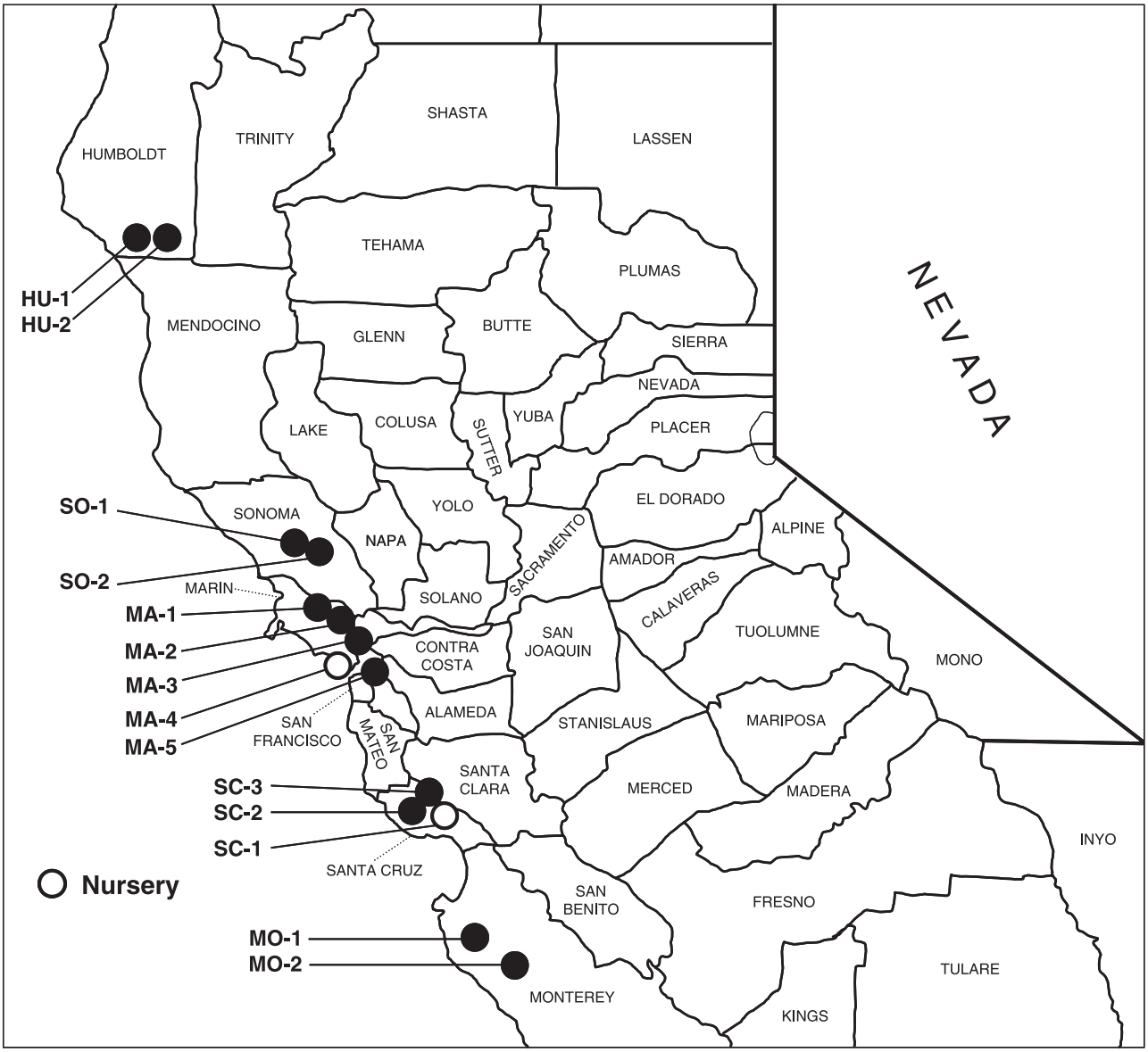

Fig. 1 Map of the *Phytophthora ramorum* localities sampled in California. Open circles indicate those populations that were genetically indistinguishable from the nurseries' population.

to elucidate the evolutionary relationships among individuals. Finally, in order to differentiate between evolutionary changes associated with range expansion, and changes achieved through genotypic replacement in older infestations, we compared the genetic structure of two populations, coming from the same site but sampled three years apart.

Materials and methods

Phytophthora ramorum *collection*

Of the 292 isolates of *Phytophthora ramorum* examined in this study, the majority (277) were collected from 14 forest stands in five Californian counties (Humboldt, Sonoma, Marin, Santa Cruz and Monterey) for an extensive coverage of the current range of the distribution of *P. ramorum* (Fig. 1). Additionally, 15 isolates were collected from nurseries in 12 States in 2004, and are taken as representative of the nursery population in the USA. Isolates in all nurseries were traced to ornamental plants (*Camellia* spp., *Rhododendron* spp., and *Viburnum* spp.) coming from California and Oregon. Due to the fact that plants are constantly sold and moved to and from individual nurseries, we felt a broad sampling would be more representative than sampling one individual nursery in California.

Table 1 Sample localities, number of Isolates and duration of infestation by *Phytophthora ramorum* in Californian forests

Code	County	Locality*	Longitude	Latitude	*n*	Collection year	Approx. duration of infestation†
HU-1	Humboldt	Redway 1	–123.8333	40.1196	19	2005	< 5 years
HU-2	Humboldt	Redway 2	–123.8337	40.2583	14	2005	< 5 years
SO-1a	Sonoma	Jack London SP	–122.4709	39.2950	13	2002	> 10 years
SO-1b	Sonoma	Jack London SP	–122.5521	38.3537	25	2005	> 10 years
MA-1	Marin	Tomales Bay SP	–122.8723	38.1152	9	2005	< 5 years
MA-2	Marin	Angel Island SP	–122.4391	37.8589	18	2005	> 5 < 10 years
MA-3	Marin	China Camp SP	–122.4610	38.0057	23	2005	> 10 years
MA-4	Marin	Golden Gate NRA	–122.6678	37.9419	24	2005	> 5 < 10 years
MA-5	Marin	Samuel. P. Taylor SP	–122.7361	38.0296	24	2005	> 10 years
SC-1	Santa Cruz	Bean Creek	–122.0074	37.0831	24	2005	> 10 years
SC-2	Santa Cruz	Felton	–122.0262	36.9720	24	2005	> 5 < 10 years
SC-3	Santa Cruz	Harry Cowell SP	–122.0727	37.0438	16	2002	> 5 < 10 years
MO-1	Monterey	Deetjens	–121.6702	36.1588	22	2005	> 5 < 10 years
MO-2	Monterey	Los Padres NF	–121.4399	35.91524	21	2005	< 5 years

*SP, State Park; NF, National Forest; NRA, National Recreational Area.
†Duration of infestation as estimated at sampling year.

In spring 2002, isolates were collected from in and around Henry Cowell State Park (Santa Cruz County, site SC-3) and Jack London State Park (Sonoma County, site SO-1a). These early collections were obtained by surveying a number of different locations and making isolations from symptomatic plants including oaks (*Quercus* spp.), tanoaks (*Lithocarpus densiflorus*) and bay laurel (*Umbellularia californica*). All other sites were sampled in 2005, using the following approach: individual forest stands were sampled intensively by collecting symptomatic bay or tanoak leaves along three transects. The minimum distance between sampled trees was 15 m, to minimize repeated sampling of the same clonal genotype. A total of 45 trees were sampled in each forest, and up to 24 isolates per site were kept and used for this study. With the exception of the two sites in Humboldt County, the GPS coordinates of each tree sampled were determined using a GEOEXPLORER 3 (Trimble).

Although sites were chosen carefully and strategically to broadly cover the geographical zone of SOD infestation in 2005 (Fig. 1), and to represent the temporal progression of the disease (Table 1), it should be noted that some intermediately aged sites are 5 km or less from old infestation sites. Several notable features characterize some of the study sites. First, Bean Creek (SC-1) in Santa Cruz County is in the immediate vicinity of an ornamental plant nursery that was confirmed as heavily infested by *P. ramorum* in 2001. Second, the Bolinas Ridge site in the Golden Gate National Recreation Area (Marin County, MA-4) is about 5 km from the site where SOD was first observed in California. Third, Angel Island (MA-2) is in the San Francisco Bay but less than 3 km from the mainland. All sites, with the exception of the more remote Plaskett Canyon (MO-2) are in areas best described as lying at the urban–wildland interface, whereby homes and landscaped gardens are intertwined with forest stands, and numerous plant nurseries are present throughout the region. One site, Jack London State Park, was sampled twice — in 2002 (SO-1a) and in 2005 (SO-1b). The sampling in and around Harry Cowell SP site in 2002 (SC-3) abutted the Bean Creek (SC-1) site sampled in 2005.

P. ramorum *isolation and DNA extraction*

Several small pieces (approximately 2×2 mm) of the symptomatic portion of sampled leaves were plated on selective Primaricin-Ampicillin-Rifampicin-pentachloronitrobenzene agar (PARP) (Erwin & Ribeiro 1996), incubated at room temperature in the dark for three weeks and transferred into pea-broth liquid medium at room temperature for seven days. Mycelia were harvested by filtering on Whatman paper and lyophilized for 24 h. DNA was extracted using the protocol described by Ivors *et al*. (2006).

Microsatellite analysis

Although a selection of at least 22 *P. ramorum* microsatellites is available, the majority show no variation within the NA1 lineage of *P. ramorum* that is present in Pacific forests of the USA. We selected seven microsatellite loci that were previously identified as polymorphic with the NA1 lineage: PrMS39a, PrMS39b, PrMS43a, PrMS43b, PrMS45 (Prospero *et al*. 2007) and locus 18 and locus 64 (Ivors *et al*. 2006).

PrMS39a, PrMS39b and PrMS45 were amplified using one set of primers and the following polymerase chain reaction (PCR) conditions: initial denaturation of 2 min at

92 °C, followed by 30 cycles of 92 °C for 30 s, 52 °C for 30 s, 65 °C for 30 s and by a final step of 5 min at 65 °C. PrMS43a and PrMS43b were amplified using one set of primers and the following PCR program: initial denaturation of 2 min at 92 °C, followed by 35 cycles of 92 °C for 30 s, 52 °C and 72 °C for 1 min and by a final step of 45 min at 72 °C. Locus 18 and locus 64 were amplified using the following PCR program: initial denaturation of 2 min at 95 °C, followed by 35 cycles of 94 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s and a final extension step of 10 min at 72 °C. All amplification reactions were performed in a final volume of 10 μL using 10 ng of template DNA, 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.2 μM of each primer and 1 U Taq polymerase. The PCR products were sized on an ABI377 (Applied Biosystems) using Rox 500 as the size marker. Amplifications were conducted in 96 well plates, and negative and positive controls (isolates of known genotype) were included in each plate. Each isolate was genotyped twice. For statistical analysis, allele-fragment length was substituted with the number of repeats corresponding to the allele size.

Evolutionary relationships among the multilocus genotypes

In order to assess the possible evolutionary relationships among the individual MGs, a matrix of genotype distances was calculated using the simple, general and robust method of Bruvo *et al*. (2004), taking repeat number into account. A minimum spanning network was then calculated from this matrix using the program MINSPNET (Excoffier & Smouse 1994). The network was visualized using GRAPHVIZ (www.graphviz.org).

Analysis of molecular variance

Analysis of molecular variance (AMOVA) (Excoffier *et al*. 1992), using the ARLEQUIN software (http://cmpg.unibe.ch/software/arlequin3) (Excoffier & Schneider 2005), was employed to estimate the proportion of genetic variation within and among the samples when defined in two ways: (i) spatially (i.e. among the 14 Californian forest populations); and (ii) temporally (i.e. among the five 'old' sites — sampled after *c*. 10 years infestation, the four 'new' sites — sampled after less than five years infestation, and the five 'intermediate' sites — sampled after 5–10 years infestation) (Table 1). Analyses were repeated including and excluding the nursery population, as appropriate. Examination of the MGs (see Table S1, Supplementary material) reveals that each locus evolves simply: with either only one allele varying and the other allele remaining fixed, or with both alleles varying in a concerted (always homozygous) manner. From the point of view of molecular variation then, one allele at each locus is effectively redundant. The data were therefore reduced to a haploid state. This permits a maximally efficient analysis of these data by allowing Φ_{ST} to incorporate the 'evolutionary' distance between all loci simultaneously ('haplotype-like') rather than being calculated as an average across loci. Pairwise estimates of Φ_{ST} among all samples (including the nursery population) were also calculated using the ARLEQUIN software. The statistical significance of the Φ_{ST} estimates and the AMOVA tests was tested with 10 000 randomization replicates per comparison.

Some population comparisons had very low Φ_{ST} estimates that were not statistically significant. The samples were therefore recursively clustered into meta-samples, based on Φ_{ST} (Roewer *et al*. 2005), with the pair of populations or clusters that yielded the minimum Φ_{ST} being pooled at each round, until no further insignificant clustering (i.e. minimum Φ_{ST} $P > 0.05$) was possible. A neighbour-joining (NJ) tree was calculated using PHYLIP (Felsenstein 2005) to display the genetic relationships among the final set of significantly differentiated sample populations and meta-populations.

Jack London SP was sampled twice, once in 2002 (SO-1a), and once in 2005 (SO-1b). Furthermore, the 2005 Bean Creek sampling (SC-1) was in very close proximity to the 2002 Santa Cruz sampling in and around Harry Cowell SP (SC-3). In order to test for temporal changes occurring at the same location, pairwise Φ_{ST} values were therefore calculated for these two pairs. Additionally, the frequencies of the MGs in the two populations for each of the two sample-pairs were compared by Monte Carlo Markov Chain (MCMC) contingency table analysis (Raymond & Rousset 1995) using the software RxC (20 batches, 2500 permutations per batch, and 1000 step burn-in).

Spatial autocorrelation analysis and Mantel tests

The spatial genetic structure of the data was assessed in three ways. First, the matrix of pairwise geographical distances between the average longitude and latitude for each of the 14 spatially defined sites was computed. The correlation between this matrix and the matrix of pairwise Φ_{ST} estimates was tested using a Mantel test (Mantel & Valand 1970) with the software ZT (Bonnet & Van De Peer 2002). Second, the pairwise genetic distances ($\Phi_{ST}/1 - \Phi_{ST}$) were regressed on the natural logarithm of the pairwise geographical distances. Third, spatial autocorrelation analysis of the microsatellite loci was carried out using Moran's *I*-index (Sokal & Oden 1978) to measure the correlation between allelic repeat numbers at given geographical intervals. For eight localities (SO-1b, MA-2, MA-1, MA-2, MA-3, MA-4, MA-5, SC-1, MO-2), the exact GPS latitude and longitude coordinates for each individual sample tree were known. These were therefore converted into NAD1986 UTM coordinates using ARCVIEW 9 (ESRI) and autocorrelation evaluated over 16 approximately equally

frequent distance classes using SPAGEDI (Hardy & Vekemans 2002). The statistical significance of the Moran's *I*-values was assessed using 20 000 permutations of localities.

Results

Variability and genetic diversity

Microsatellites PrMS39a and PrMS45 were invariant. Locus 18 was variable in four individuals and locus 64 was variable only in one individual. Locus 18 and locus 64 were previously described as being the only two variable microsatellites out of a set of 12 examined in Californian forest populations by Ivors *et al*. (2006). In that study, the forest isolates included four different MGs: (i) US1, the most common, characterized by genotype combination 220/278 (locus 18) and 342/374 (locus 64); (ii) US2 with genotypes 220/272 (locus 18) and 338/374 (locus 64); (iii) US3 with genotypes 220/278 (locus 18) and 338/374 (locus 64); and (iv) US4 with genotypes 222/— (locus 18) and 340/356 (locus 64). Sizes of alleles found in this study for the above loci matched sizes measured by Ivors *et al*. (2006) either by capillary electrophoresis or by direct sequencing. All the isolates examined in the present study belong to MG US1, with the exception of four isolates: three (MG03, MG33, MG34) belong to MG US2 and one (MG35) to MG US4. The remaining three microsatellites, PrMS39b, PrMS43a and PrMS43b exhibited four and five alleles, respectively, as reported by Prospero *et al*. (2007). Gene diversity values H_E, corrected for sample size (Nei 1978), for the four microsatellites that were polymorphic in the California forest populations were: PrMS39b, $H_E = 0.2614$; MS43a, $H_E = 0.431$; PrMS43b, $H_E = 0.7147$; and PrMS18, $H_E = 0.5137$.

The combination of these variable loci identified a total of 35 MGs. Eleven (32%) of the MGs (MG01, MG02, MG03, MG06, MG11, MG23, MG29, MG30, MG32, MG33, MG34, MG35) were represented by a single isolate and five (14%) (MG22, MG24, MG25, MG26, MG31) were detected only in two isolates. The most frequent MGs were MG15 (21.50% of isolates), MG14 (18.77% of isolates) and MG13 (10.58% of isolates), followed by MG09 (5.80% of isolates), MG05 (6.16% of isolates), MG17 (4.78% of isolates) and MG27 (4.78% of isolates). Among the genotypes present more than once in the entire sample collection, three were found only in one locality: MG07 (six isolates in MA-1, Marin), MG26 (two isolates in MA-2, Marin) and MG22 (two isolates in MA-5, Marin) (see Table S1, Supplementary material). With the exception of MG36, the other five MGs found in the nurseries were found also in the forest and in each of the localities known to be among the first to be infested in California (Table 2). The three most common MGs (MG13, MG14 and MG15) differed only by a single tetra-repeat at PrMS43b. These three MGs were present in all the localities except the recently infested sites HU-1, HU-2 and MA-1 (Table 2).

PrMS39b, PrMS43a, PrMS43b and PrMS45 were genotyped both in the present study and the Oregon study by Prospero *et al*. (2007). The MGs defined by these four loci differ greatly between the two regions. Of the 24 MGs identified in the forests in Oregon, only nine were found in the Californian forests. PrOR1, the most common MG found in Oregon (179 forest isolates; ~64.62%) corresponds to MG30 and was found in just a single isolate from MA-3 (Marin County, China Camp SP). The three most common MGs found in California were MG15 (21.50%), MG14 (18.77%) and MG13 (10.58%). MG15 and MG14 correspond to PrOR29 and PrOR20. These MGs were only found in nurseries (13 and three isolates, respectively). MG13 was not present in Oregon at all.

Evolutionary relationships of the multilocus genotypes

The minimum spanning network in Fig. 2 reveals a few centrally located and frequent MGs with rare or singleton MGs on the extremities of the network and genotypes of intermediate frequency between these extremes. The network clearly fits the general pattern that might be expected if one (or a few very closely related) initial clonally expanding propagules were allowed to grow and give rise to occasional new genotypes by mutation over time. Inspection of Fig. 2 in conjunction with Table 2 indicates that the common MGs (e.g. 15, 14 and 13) were indeed spread widely among the sampling localities. This comparison also indicates that for a given MG from a particular sampling location, the other MGs in that location appeared likely to form part of cluster of immediate (1-step) neighbours — in other words, MGs at a particular location are likely to be locally evolved from one or few founders. For example, out of 12 singleton MGs, 10 (83%) had at least one immediate neighbour in the same sample population (the remaining two were found with a two-step neighbour or potential 'grandparent'). Accepting that the network is of finite size, we tested this formally. For each MG (node), we calculated the proportion of its immediate network neighbours that co-occurred with that MG in each population, *k*, in which that MG was found. This proportion was averaged across the *k* and finally across nodes to yield *S*, the average number of 1-step neighbours per node per population. We tested the significance of *S* against its null distribution, given the network, using MCMC 'checkerboard' permutation of the binary MG vs. population presence–absence matrix, holding row and column totals constant (Stone & Roberts 1990; Artzy-Randrup & Stone 2005). The Markov Chain was sampled 1 000 000 times at 500-step intervals following a burn-in of 100 000 steps. When considering either only the singleton MGs, or all MGs, *S* was highly significant — MGs were found in the same population as their evolutionary neighbours more often than expected by chance (singleton MGs only, $S = 0.7500$, $S_{null} = 0.1861$, $P < 1 \times 10^{-6}$; all MGs, $S = 0.5861$, $S_{null} = 0.2323$, $P < 1 \times 10^{-6}$).

Table 2 Multilocus genotype (MG) occurrence and counts by sampling locality and duration of infestation

				Old						Intermediate						**New**				
	n Σ=292	%	Nurseries (15)	SO-1 (13)	SO-2 (25)	MA-3 (23)	MA-5 (24)	SC-1 (24)	All old (109)	MA-2 (19)	MA-4 (24)	SC-2 (24)	SC-3 (16)	MO-1 (22)	All int (105)	HU-1 (19)	HU-2 (14)	MA-1 (9)	MO-2 (21)	All new (63)
MG01	1	0.34														1				**1**
MG02	1	0.34									1				**1**					
MG03	1	0.34																	1	**1**
MG04	4	1.37		1					**1**								3			**3**
MG05	18	6.16			4				**4**							6	8			**14**
MG06	1	0.34																1		**1**
MG07	5	1.71																5		**5**
MG08	6	2.05		1	1				**2**							1	1	1	1	**4**
MG09	19	6.51		1	2			1	**4**			1		2	**3**	11			1	**12**
MG10	10	3.42	1		1			2	**3**		2		3		**5**		1			**1**
MG11	1	0.34								1					**1**					
MG12	6	2.05								2		3			**5**				1	**1**
MG13	31	10.62		1		2			**3**			2		12	**14**				14	**14**
MG14	55	18.84	1	2	6	6	10	5	**29**	5	5	9	1	4	**24**				1	**1**
MG15	64	21.92	10	4	4		3	16	**27**		16	2	8	1	**27**					
MG16	4	1.37			1				**1**				3		**3**					
MG17	14	4.79								9		5			**14**					
MG18	4	1.37								2				1	**3**				1	**1**
MG19	4	1.37			2		1		**3**					1	**1**					
MG20	7	2.40	1		2		4		**6**											
MG21	1	0.34											1		**1**					
MG22	2	0.68					2		**2**											
MG23	1	0.34															1			**1**
MG24	2	0.68		1		1			**2**											
MG25	2	0.68		1		1			**2**											
MG26	2	0.68				2			**2**											
MG27	14	4.79			1	8	4		**13**			1			**1**					
MG28	3	1.03	1			2			**2**											
MG29	1	0.34										1			**1**					
MG30	1	0.34				1			**1**											
MG31	3	1.03		1	1				**2**									1		**1**
MG32	1	0.34												1	**1**					
MG33	1	0.34																1		**1**
MG34	1	0.34																	1	**1**
MG35	1	0.34	1																	
*R**			0.37	0.61	0.41	0.32	0.22	0.13	**0.17**	0.22	0.13	0.30	0.28	0.29	**0.22**	0.16	0.30	0.37	0.35	**0.18**

*Clonal genotype diversity $R = (G - 1)/(N - 1)$, where G is the number of multilocus genotypes identified in the sample and n is the number of isolates.

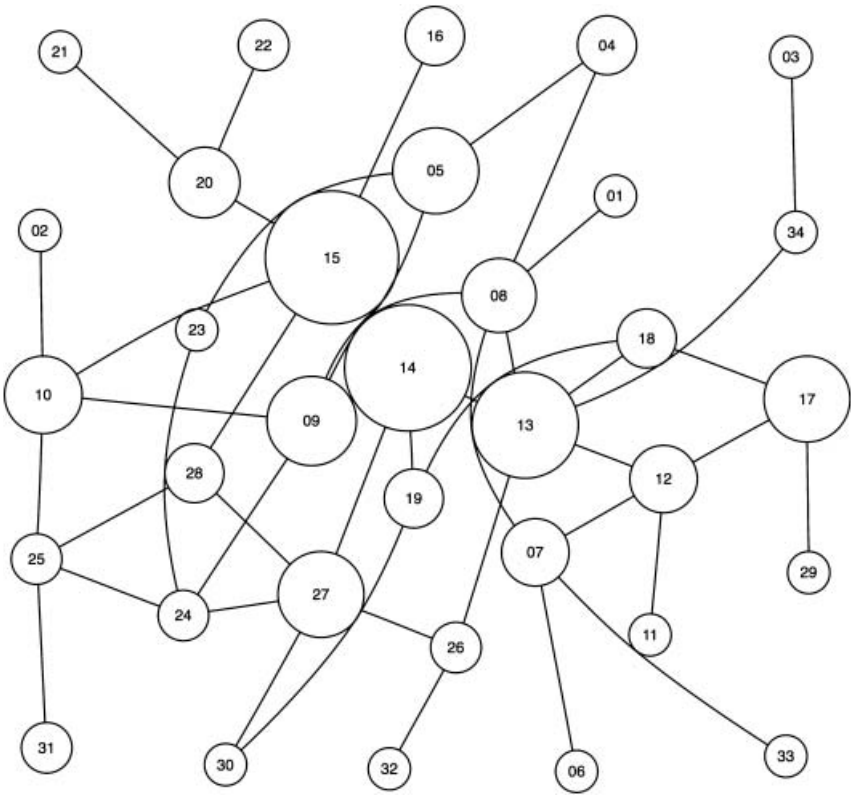

Fig. 2 Minimum spanning network among the 34 multilocus microsatellite genotypes detected in the Californian forest populations.

Table 3 AMOVA results for the14 *Phytophthora ramorum* populations in the Californian forest

Source of variation	d.f.	Sum of squares	Variance components	% of variation	Φ_{ST}†
Among all populations*	13	276.884	1.0629	55.6	0.5566
Within all populations*	257	217.610	0.8467	44.34	
Among old populations	4	19.620	0.1970	18.77	0.1877
Within old populations	100	85.256	0.8526	81.23	
Among new populations	3	59.301	1.2406	60.91	0.6091
Within new populations	59	46.985	0.7636	39.09	
Among intermediate populations	4	98.864	1.1662	57.24	0.5724
Within intermediate populations	98	85.369	1.6120	44.31	
Among old and new populations	1	80.963	1.0120	44.31	0.4431
Among old and intermediate populations	1	19.227	0.1714	10.88	0.1088
Among new and intermediate populations	1	55.752	0.6905	28.05	0.2805

*Excludes nursery population, inclusion makes little difference (Φ_{ST} = 0.5631).
†For all among group comparisons Φ_{ST} was highly significant ($P < 0.00001$).

Spatial and temporal genetic structure

AMOVA indicated an approximately equal partitioning of genetic variability within and among sampled populations (Table 3), suggesting that individual populations comprised several genotypes, but that genotype composition varied across sites. Consequently, overall Φ_{ST} value among populations was large (Φ_{ST} = 0.5755, $P < 0.0001$; Table 3), indicating that *Phytophthora ramorum* forest populations are genetically structured, with very limited gene flow among them. Genotypic diversity values (R) confirmed the presence of multiple genotypes at all sites, and indicated that diversity across all sites is comparable. The structure detected by AMOVA is thus likely to have been driven largely by differences in the actual genotypes at each site, rather than by significant differences in genotypic diversity.

AMOVA also revealed a notable partitioning of the genetic variability among the three groupings representing differently aged infestations. The Φ_{ST} values between old and new infestations was also large (Φ_{ST} = 0.4605), suggesting that new infestations are characterized by pathogen populations genetically distinct from those present in old infestations.

Populations from intermediately aged infestations displayed significant but lower Φ_{ST} values with respect to either new (Φ_{ST} = 0.2885) or old infestations (Φ_{ST} = 0.1024). When Φ_{ST} values were calculated for old, intermediate and new infestations separately, they were lowest among old infestations (Φ_{ST} = 0. 2008), indicating that old infestations are less structured than either intermediate or new infestations, characterized by Φ_{ST} values of 0.5683 and 0.6090, respectively. These results imply that populations of *P. ramorum* were originally less differentiated, and that differentiation among newly established populations is increasing as the range of the pathogen is expanding.

Despite the overall high level of population differentiation, Φ_{ST} values between some populations were insignificant. When populations where iteratively collapsed based upon minimum Φ_{ST}, three meta-samples emerged, each comprising three populations. The first meta-sample included the nurseries' population, a population in Santa Cruz County (SC-1) neighbouring an infested nursery and a third population in Marin County (MA-4), over 100 km away and adjacent to the site where SOD was first reported. The second meta-sample included the 2002 and 2005 collections from Jack London SP (SO-1a and SO-1b). Finally, the third grouping included two Big Sur populations (MO-1 and MO-2), at 34 km from one another but with intermittent forested patches in between them, and a Santa Cruz infestation (SC-2), separated from the northernmost of the two Big Sur sites (MO-1), by approximately 90 km of agricultural land.

The Φ_{ST}-based NJ tree (Fig. 3) of the populations and meta-samples places the nurseries' population, together with some populations from old and intermediate infestations, in two closely related clades at the bottom of the tree. Populations from three of the four new infestations instead formed a clade of more distantly related populations at the top of the tree. Two intermediate clades could be identified in the center of the NJ tree, containing populations mostly from old and intermediate infestations and from one new infestation. The only new infestation placed in this intermediate position was Plaskett Canyon (MO-2), a population indistinguishable from its closest sampled (34 km) Big Sur neighbour (MO-1), an intermediately old infestation.

The pairwise Φ_{ST} value between the 2002 and the 2005 sampling of Jack London SP was zero (Φ_{ST} = −0.0510, *n.s.*), and the frequency-based MCMC contingency table analysis indicated no significant difference in MG frequency between the two samplings (P = 0.3896). The 2002 and 2005 samplings of neighbouring areas in Santa Cruz County (SC-1 and SC-3) resulted in the moderately significant Φ_{ST} value of 0.1827 (P = 0.0115) and a nonsignificant MCMC-based P-value of 0.0729, indicating only mild genetic differences between the two samplings.

Mantel tests comparing the matrix of genetic distances (Φ_{ST}) with the matrix of geographical distances (using the mean coordinate of each sampling locality) resulted in a mildly statistically significant correlation ($y = 0.2452x + 0.5655$; P = 0.0142, 1-tailed; 10 000 randomizations) characterized by a low correlation coefficient, r = 0.36. The R^2 value resulting from the regression of linearized Φ_{ST} on the logarithm of the distance was also low, R^2 = 0.0266.

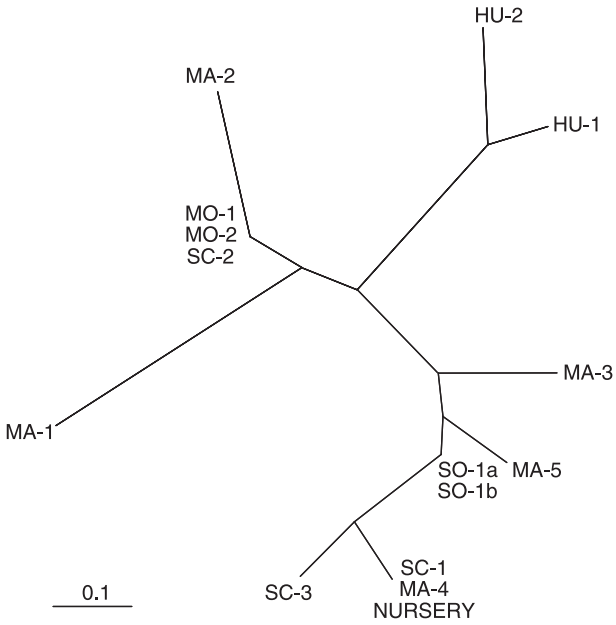

Fig. 3 Neighbour-joining tree illustrating the relationships between statistically significantly differentiated *Phytophthora ramorum* population samples and meta-samples, formed by clustering samples according to minimum Φ_{ST}.

Spatial autocorrelation analyses between Moran's I and geographical distances among all individual samples, were performed using distance intervals ranging between 15 m and 100 km. Excluding negative values of I, significant autocorrelation values were found for all distance intervals between zero and 300 m, all characterized by I decreasing with increasing distance. Interestingly, a significant increase in I was detected at distances of 1000 m followed by a significant decrease in I, approaching zero at 10 km (Table 4 and Fig. 4). In order to verify that the unexpected peak of I at 1000 m was a general phenomenon not exclusively caused by effects of a single population, spatial autocorrelation analyses were run, excluding data from one population at a time. Results of these analyses showed the same pattern of fluctuation in I, independent of populations included in the analyzed dataset.

Discussion

In this study, a total of 35 MGs were detected using six microsatellite loci. It should be highlighted that these MGs are invariant in at least 15 other loci and represent quasi-identical genotypes as determined for genotypes in the Oregon

Table 4 Details of the spatial autocorrelation analysis conducted using 16 geographical distance intervals

Max distance (m)	15	50	75	150	300	400	500	750	1500	10 000	15 000	30 000	60 000	100 000	150 000	300 000
Mean distance (m)	10	32	62	110	212	343	446	609	1003	1984	11 225	20 077	42 387	94 270	126 939	258 972
Number of comparisons	32	120	75	146	203	81	83	48	143	81	253	1414	1632	198	1746	1620
Moran's *I*†	0.521****	0.561****	0.455****	0.248****	0.218****	0.096*	0.136***	0.136*	0.393****	0.241****	0.003 *n.s.*	–0.117****	0.004 *n.s.*	–0.124***	–0.012 *n.s.*	–0.060****

†Statistical significance of Moran's *I*: *n.s.*, not significant; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

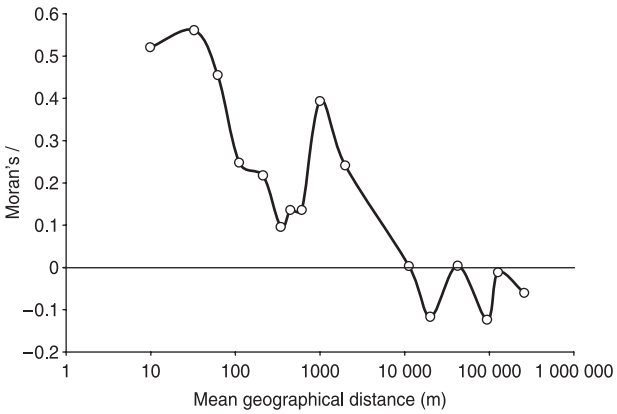

Fig. 4 Spatial autocorrelation analysis of genetic and geographical distance in *Phytophthora ramorum*. Moran's *I*-index, averaged over loci, was calculated from the repeat number at each of four variable microsatellite loci.

infestation (Prospero *et al*. 2007). Because the microsatellite loci selected for this study are highly variable, our results may underestimate the genetic diversity of the studied populations, due to the presence of back-mutations resulting in MGs that are apparently identical to one another but in reality are separated by two or more mutational steps. While a few MGs were dominant and widely distributed, the majority of them were less abundant and usually limited to one or few sites. The dominant and widely distributed MGs are also at the center of the minimum spanning network and thus they are the most likely founder genotypes of SOD, while the less-broadly distributed MGs, usually derived from the founder genotypes in the minimum spanning network may have evolved locally during the epidemic phase following the introduction. All but four isolates belonged to the widespread NA1 genotype described by Ivors *et al.* (2006). On average, 6.36 MGs were found per forest site and 6.0 MGs occurred in the nursery population. Mean genetic diversity *R* was relatively similar in new, old and intermediately aged infestations. It should be noted that in spite of the different durations of infestation, all sites were sampled as a result of a confirmed disease outbreak characterized by an abundance of symptoms. We hypothesize that maximum genotypic diversity is attained during such an outbreak phase, in correlation with maximum population size. During the unfavourable summer and fall months, populations of the pathogen crash to endemic levels and individual genotypes need to recolonize infested sites every late winter and spring. These seasonal variations offer an advantage to already established genotypes during the recolonization process and may also cause the extinction of rare genotypes through drift. The cyclic seasonal nature of the epidemic is thus likely to maintain genotypic diversity at a level proportional to the current size of the population. Microsatellites are neutral markers and do not imply any

diversity in fitness among MGs; however, the presence of one or two dominant genotypes per site may be linked to the ability of such individuals to out-compete novel genotypes. Confirmation of this hypothesis awaits further research.

AMOVA indicated the presence of extensive variation both within and among populations, and high Φ_{ST} values indicated strong structuring of *Phytophthora ramorum* populations. The AMOVA results can be explained by the fact that multiple MGs were present at each site, but the composition of the MGs varied among sites. Unequal frequencies of the MGs indicates that gene flow was limited among our study sites; this was true both for new infestations, where the pathogen range is still in expansion and populations are not in equilibrium, and old infestations, where a longer time may have brought populations closer to equilibrium. Range expansion is occurring despite limited pathogen migration levels, and new infestations are reported often tens of km from previously reported infestations. This pattern may best be explained by rare migration events, by one or a few genotypes, into new unexploited areas, followed by local population build-up and emergence of novel MGs.

To confirm the presence, and to estimate the rate of local diversification events, we ran AMOVAs separately among groups of sites differentiated by the age of the SOD infestation. Φ_{ST} values among old infestations were markedly lower than those among intermediate or new infestations, suggesting that older sites may have been characterized by more gene flow among them, less genetic drift, lower mutation rates, smaller population sizes, or, finally, may have been exposed to different selection pressure than recently established populations. This apparent pattern can be explained by multiple introductions of the same genotypes into different sites early-on in the SOD epidemic. The Φ_{ST} value between old and intermediate infestations was significant but low, whereas it increased significantly when comparing intermediate and new populations of the pathogen. This result suggests that further range expansion of the pathogen is accompanied by a more marked differentiation process, caused by infrequent between-site migration followed by the creation of new local genotypes.

The lack of structure among some populations was exemplified by some Φ_{ST} values approaching zero and indicates the presence of connectivity among some sites. We found a lack of genetic structure among the nursery population, the Bean Creek site (Santa Cruz County, SC-1) and the intermediately aged infestation in the Golden-Gate National Recreational Area (NRA) (MA-4; located less than 5 km from the first reported case of SOD). The latter two forest sites are over 100 km apart and on opposite sides of the San Francisco Bay, hence natural movement of the pathogen at a frequency capable of equalizing allelic frequencies between them is virtually impossible. Instead, we suggest that these sites represent two of the areas where the pathogen first escaped the nursery environment. Direct pathogen escapes from at least one nursery reported as highly infested and immediately adjacent to the Santa Cruz site, combined with the sale of infected plants in the general vicinity of both sites, were the mechanisms that probably ensured gene flow between the nurseries and the forest. This resulted not only in comparable MG frequencies but in the beginning of the SOD epidemic in the region. Finally, the lack of structure between the Felton site in Santa Cruz (SC-2) and the two Big Sur sites (MO-1, MO-2) can be explained by a combination of human-induced transport, presumably through infected plants from Santa Cruz, to the central Big Sur area surrounding Pfeiffer Big Sur State Park, the first site where SOD was reported in the region. We further hypothesize that this introduction may have been followed by the natural spread of the pathogen from the central Big Sur area southward to the Deetjen's site, and then more recently to the Plaskett Canyon site in Los Padres National Forest (NF). Although we cannot exclude further human introductions from being responsible for pathogen spread in the Big Sur area, genotypic frequencies are almost identical in the two study sites; and although located in canyons at considerable distance, the two sites are linked by a series of discrete forests, all within 5 km from one another.

The cases of gene flow cited above are particularly striking in light of the results of the spatial autocorrelation, Mantel, and regression analyses which indicated a low correlation between genetic similarity and distance among populations and individuals over moderate distances. At a smaller spatial scale, though, we found an inverse correlation between Moran's *I*-index of genetic similarity and distance, suggestive of a patch structure in the populations of this pathogen (Sokal 1983). Moran's *I*-values decreased from 0.55 at 15 m to 0.1 at 350 m. Surprisingly, Moran's *I* then climbed to a secondary peak of 0.4, corresponding to a distance of 1000 m, then decreased to 0.2 at 2000 m and finally approached zero at 10 000 m. In the absence of direct measurements of propagule movement, spatial autocorrelation data provides a valuable proxy to infer the potential for spread of the pathogen (Diniz-Filho & Telles 2002), bearing in mind that these estimates should be likened to predictions of likelihood of movement and need to be interpreted in relation to levels of inoculum load. In general, higher potential spread is linked to higher inoculum loads. The data provided by all the spatial analyses indicates that at distances over approximately 10 km there is no isolation by distance (Sokal *et al*. 1997); this result can be explained by the movement of genotypes over that distance being rare. Davidson *et al*. (2005) reported that aerial sporangia could not be caught further than 10 m from a discrete source. Our data confirms only limited spread even at distances of 10 m (Moran's $I = 0.55$). Decreasing Moran's *I* with distance indicates that the efficiency of spread also decreases with distance and

reaches a minimum at 350 m. This value has been reported as the size of a wood patch that correlates with maximum infection levels by *P. ramorum* (Meentemeyer *et al*. 2004). Based on our spatial autocorrelation results, and on the fact that every year, multiple infection events occur on multiple hosts, it is likely than in a single season, a patch of forest this size may be efficiently colonized by *P. ramorum*, as reported for other organisms displaying a similar spatial autocorrelation profile (Sokal 1983). The increase in Moran's *I* as distance increases from 400 to 1000 m is a peculiar result that is not commonly observed in the case of other airborne plant pathogens (Aylor 1999). Nonetheless, the relatively high Moran's *I*-values observed in the 1–3 km range are consistent with estimations of spread based on the distances of new *P. ramorum* infections from discrete inoculum sources in Oregon (Prospero *et al*. 2006). Sporangia of *P. ramorum* are rather large structures, measuring over 50 μm in length (Werres *et al*. 2001), and their movement in the air differs significantly from that of airborne fungi characterized by much smaller spores (Aylor 1999). *P. ramorum* may also be different from other Oomycete species, in that it is not clear whether airborne sporangia can be transported in dry form or not. Oomycete species capable of long distance movement normally produce sporangia that can dry before being airborne, for example *P. infestans* (Sunseri & Johnson 2002) and *Peronospora tabacina* (Aylor & Taylor 1982), while species characterized by the movement of turgid sporangia have much smaller spread ranges, for example *Phytophthora capsici* (Liu *et al*. 2007). We suggest that the secondary peak of Moran's *I* observed at 1 km may represent movement of sporangia in the presence of strong winds and rain. The ability of *P. ramorum* to be airborne for a km or more would also explain how the pathogen easily moves from one patch of forest to another: often, in fact, adjacent patches are much further than 350 m apart. The constant decrease in spatial autocorrelation values from 1 to 10 km, and the slightly negative values recorded after that threshold, can be explained either by limited gene flow incapable of counteracting genetic differentiation due to genetic drift, or by negative selection on migrants (Dunham *et al*. 2006). We believe the first to be the only likely explanation to explain the relationship between old and new infestations, while both explanations could justify the relationship between two established infestations.

Lack of genetic structure between two populations indicates gene flow between them but does not indicate the direction of such flow. High levels of gene flow were detected between the nurseries' population and two distant wild populations, suggesting these two wild populations originated from nursery plants. While it could be argued that nurseries received genotypes of *P. ramorum* from the wild, the NJ analysis based on Φ_{ST} values indicates otherwise. The NJ tree, in fact, placed the nurseries' population at one end of the tree, and populations from sites documented to have been infested only recently at the other end. Because it is illogical to imagine older populations derived from new ones, we interpret this result as indicative that the Californian forest infestation of genotypes belonging to the NA1 lineage originated from the nurseries' population and is now evolving into populations that have significantly differentiated from the founder population. The fact that all three known lineages are present in nurseries in the USA (Ivors *et al*. 2006), but that the NA1 lineage is the one most commonly reported, is also in agreement with our 'nursery escape' hypothesis.

Φ_{ST} values generated by the AMOVA analysis indicated that new infestations are significantly different from intermediate and old infestations. This result shows that as the pathogen expands its range and moves into new sites, it evolves into populations that are different in composition from the founder populations. This can be explained by the limited ability of the pathogen to move at distances over 10 km and by local generation of new genotypes. It is also possible that in the future, with the passing of time, gene flow directed back into the founder population may diminish the level of among-population genetic structure. In contrast with differences detected between new and old infestations, when the same site (Jack London SP, Sonoma County) was sampled twice three years apart, no difference between the two samplings was found. This result indicates that established populations do not change easily in time. In a study of Oregon populations, it was reported that the most significant genotypes re-occurred year after year, but that rare genotypes disappeared while new ones surfaced with time (Prospero *et al*. 2007). An intense eradication program has been in place since the Oregon infestation was discovered, and this effort has significantly reduced the local *P. ramorum* population on a yearly basis. In the presence of this human-induced bottleneck, it is reasonable to assume that some of the least abundant genotypes may go locally extinct. We believe that these events may be rare in some California sites extremely favourable to SOD, while it is plausible that sites characterized by climatic conditions less favourable to the pathogen may lead to comparable results in natural conditions.

It has been estimated that millions of bay laurel trees may have been infected by *P. ramorum* in the course of the SOD epidemic in California (Rizzo & Garbelotto 2003). The high infection rate and the abundant sporulation of the pathogen on this host (Davidson *et al*. 2005) have resulted in large population sizes of this pathogen. In the absence of sexual reproduction, new genotypes are generated by mutations or somatic recombination (Dobrowolski *et al*. 2002). With a constant rate of mutation, population size will determine the number of new genotypes. The network analysis identified three genotypes that were common and the most likely source of all other genotypes. Stepwise mutations appear to accumulate and to have created

genotypes that are more distantly related to these founder genotypes. Eleven derived genotypes were detected in single sites: these genotypes were at least two mutational steps away from the putative founder genotypes. In each case, putative 'parental' genotypes that were one or, at most, two (two cases) mutational steps away from these distal genotypes and intermediate between the distal and parental individuals, were found in the same location. The existence of local evolutionary processes leading to new MGs was formally demonstrated through MCMC simulation. Hence, new genotypes are primarily generated via a local stepwise evolutionary process. Oregon populations only marginally overlapped with California ones and mostly through MGs also shared by the nursery trade; this result indicates forest infestations in the two states have largely diverged since the introduction events due to lack of gene flow between them.

In summary, this study provides evidence of a historical link between nursery and wild populations of the pathogen and identifies at least two sites in which the SOD pathogen may have been introduced in California. One of these two sites is adjacent to the site where SOD was first reported, and the other is near the nursery were the first US nursery infestation was described. Interestingly, when the nursery was first sampled, its owner reported he had been observing SOD symptoms for quite some time (M. Garbelotto, personal communication). The spatial autocorrelation analysis indicates that the pathogen has a rather limited spread rate; nonetheless, we were able to detect significant gene flow between distant sites. We interpret this as the result of human movement of the pathogen, most likely through the movement of infected plants. Finally, we show here that while the population at one site has remained unchanged in three years, new infestations are genetically distinct from old ones. Such differentiation is explained not only by the fact that long-range movement of the pathogen is a rare event, but also by the local generation of new genotypes through a stepwise mutation process. Whilst we have no evidence that these genotypes may in general differ in virulence or fitness from old ones, the dominance of a single genotype in the Big Sur (MO-1 and MO-2) area may be the result of increased fitness and warrants further research.

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Supplementary material

The following supplementary material is available for this article:

Table S1 Allele size (size is indicated in bp of the PCR amplification products) of the multilocus genotypes (MGs) identified in the 292 *Phytophthora ramorum* isolates

This material is available as part of the online article from:
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