		M	E	C	3	3	4	3	**Operator:** Huang Liping	**Dispatch:** 16.04.07	**PE:** Gillian Carmichael
	Journal Name				Manuscript No.				**Proofreader:** Chen Xiaoming	**No. of Pages:** 16	**Copy-editor:** Apple Rosales

Molecular Ecology (2007) doi: 10.1111/j.1365-294X.2007.03343.x

Population dynamics of the sudden oak death pathogen *Phytophthora ramorum* in Oregon from 2001 to 2004

S. PROSPERO,* E. M. HANSEN,* N. J. GRÜNWALD*† and L. M. WINTON‡

**Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331-2902, USA, †Horticultural Crops Research Laboratory, USDA ARS, Corvallis, OR 97330, USA, ‡Subarctic Agricultural Research Unit, USDA ARS, Fairbanks, AK 99775, USA*

Abstract

***Phytophthora ramorum* (Oomycetes) is an emerging plant pathogen in forests in southwestern Oregon (Curry County). Moreover, since 2003 it has been repeatedly isolated from plants in Oregon nurseries. In this study, we analysed the genetic diversity of the *P. ramorum* population in Oregon from 2001 to 2004 by using microsatellites. A total of 323 isolates (272 from the infested forest; 51 from nurseries) were screened at 10 loci. The overall *P. ramorum* population in Oregon is characterized by low genetic diversity and has all the hallmarks of an introduced organism. All isolates within the A2 mating type belonged to the same clonal lineage and no recombinant genotypes were found. The forest population (24 genotypes) was dominated by a single multilocus genotype which persisted over years, indicating that eradication efforts in the forest have not completely eliminated inoculum sources. In contrast, genotypic evidence suggests that eradication was effective in nurseries. In 2003 and 2004, a total of 11 genotypes were found in the nurseries (one belonged to the European lineage of *P. ramorum*) but no genotype was recovered in both sampling years. Significant differentiation and low gene flow were detected between nursery and forest populations. Only two nursery genotypes were also found in the forest, and then at low frequency. Thus, the nursery infestation is not caused by the genotypes observed in Curry County, but likely resulted through introduction of novel genotypes from nurseries out-of-state. This highlights the continued importance of sanitation and quarantine in nurseries to prevent further introduction and spread of *P. ramorum*.**

Keywords: epidemiology, eradication, forest, genetic diversity, microsatellites, nurseries, oomycetes

Received 23 November 2006; revision accepted 12 March 2007

Introduction

Species of the genus *Phytophthora* (Oomycetes) include some of the most devastating pathogens of woody plants, having a significant ecological and economic impact both on plantation crops and in natural ecosystems (Erwin & Ribeiro 1996). In recent years, new *Phytophthora* species have been increasingly associated with tree decline and mortality in forest ecosystems. To mention just a few, *Phytophthora quercina* Jung causes root rot of European oaks (Jung *et al*. 1999), *Phytophthora alni* Brasier & Kirk kills riparian alders in Europe (Brasier *et al*. 2004), and *Phytophthora ramorum* Werres, De Cock & Man in't Veld (Werres *et al*. 2001) is an invasive pathogen in native forests in the western United States.

Extensive mortality caused by *P. ramorum* was first observed in 1995 in coastal forests of California (Garbelotto *et al*. 2001). To date, the disease called sudden oak death is widespread in 14 California counties and is also present in a small area of southwestern Oregon (Rizzo *et al*. 2005). The pathogen, which was first isolated in 1993 from rhododendrons in European nurseries (Werres *et al*. 2001), affects a broad range of hosts (www.suddenoakdeath.org/) especially in the families Ericaceae (e.g. species of *Rhododendron* and *Vaccinium*) and Fagaceae [e.g. species of *Quercus*; tanoak,

Correspondence: Simone Prospero, INRA, UMR1202 Biodiversité Gènes et Communautés, Equipe de Pathologie Forestière, 71 Avenue Edouard Bourleaux, BP 81, 33883 Villenave d'Ornon, France. Fax: 33 (0)5 57 12 26 21; E-mail: sprosper@bordeaux.inra.fr

Lithocarpus densiflorus (Hook. & Arn) Nutt.] (Hansen *et al*. 2005). *Phytophthora ramorum* infects aerial parts of the plants and symptoms include lethal bole cankers (e.g. on oaks), shoot dieback, or leaf blight (Hansen *et al*. 2002). The recovery of viable spores of *P. ramorum* in forest soil and in stream water indicates that both can act as reservoirs for *P. ramorum* inoculum (Davidson *et al*. 2005; Hansen & Sutton 2005). Streams may also be important pathways for the long-distance dispersal of the pathogen. However, it is still unknown if spores in stream water can infect plants in nature (Davidson & Shaw 2003; Rizzo *et al*. 2005).

In Oregon forests, *P. ramorum*, which was first detected in 2001, is restricted to an area of about 24 km^2 in the southwestern corner of the state (Curry County) where it is killing tanoak and causing a dieback of Pacific rhododendron (*Rhododendron macrophyllum* G. Don) and evergreen huckleberry (*Vaccinium ovatum* Pursh). Because of the relatively small size of the infestation, eradication of the pathogen by cutting and burning of all potential hosts within a disease patch is being attempted (Goheen *et al*. 2002; Goheen *et al*. 2006). Nevertheless, new infections are detected every year, indicating that inoculum sources are still present. Since 2003, *P. ramorum* has also been repeatedly isolated from infected plants in Oregon horticultural nurseries; quarantine and eradication efforts continue in these nurseries (Osterbauer *et al*. 2003; Osterbauer *et al*. 2004).

Phytophthora ramorum is a diploid organism and being heterothallic it requires interaction of two mating types (A1 and A2) for sexual reproduction (Werres *et al*. 2001). Sexual reproduction results in the formation of oospores that to date have only been documented under laboratory conditions (Werres *et al*. 2001; Brasier & Kirk 2004). Oospores could represent an additional source of primary inoculum for *P. ramorum* as they are long-lived resting structures. Additionally, they could increase the genetic variation in the *P. ramorum* population. Presently, all forest isolates from North America have been of the A2 mating type, whereas European isolates were of the A1 mating type (Garbelotto 2004). Previous population genetic analyses based on amplified fragment length polymorphisms (AFLP) and microsatellites have revealed a low level of genetic variation in the *P. ramorum* population in California, with a higher diversity in nurseries than in forests (Ivors *et al*. 2004; Ivors *et al*. 2006). In this study, we analysed the population structure of *P. ramorum* in Oregon from 2001 to 2004 by using microsatellite markers. Specifically, we addressed the following objectives: (i) considering that *P. ramorum* is being actively eradicated both in forest and nurseries, we evaluated the success of the eradication efforts in the two different environments; (ii) based on the population structure, we made inferences about the origins of the forest and nursery populations; (iii) as nurseries can represent a pathogen's reservoir that could potentially migrate toward the forest, we investigated if gene flow took place between forest and nurseries in the 2003–2004 period; and (iv) given the importance of sexual recombination as an additional source for genetic diversity, we analysed evidence of sexual reproduction in the Oregon population of *P. ramorum*.

Materials and methods

Infested forest

The infested forest area is located in the Siskiyou Mountains in Curry County, on the southwest coast near the town of Brookings (42.07°N, –124.27°W) (Fig. 1a). This area, which is part of the Klamath Mountain Range, is geologically, topographically, and climatically complex (Franklin & Dyrness 1988). As a consequence, forests consist of a mosaic of different vegetation types, including redwood [*Sequoia sempervirens* (Lamb. ex D. Don) Endl.] forests mixed with Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] and mixed-evergreen (*Pseudotsuga*-sclerophyll) forests. Past landscape disturbances (e.g. flooding and wind) and human activities (e.g. fire and logging) have also strongly influenced forest structure and composition, increasing the heterogeneity of plant communities (Rizzo *et al*. 2005). Currently, the dominant tree species in the infested area are tanoak and Douglas-fir, and common understorey species include salal (*Gaultheria shallon* Pursh), evergreen huckleberry, Pacific rhododendron, Oregon grape (*Berberis nervosa* Pursh), and manzanita (*Arctostaphylos* spp.) (Zanzot 2004). *Phytophthora ramorum* was first detected in 2001 in nine scattered locations (each of 0.2- to 4.5-ha size) within a 21-km^2 area (Rizzo *et al*. 2005; Goheen *et al*. 2006). Since then, new infected trees have been detected every year. Most new infections occur close (< 100 m) to previously infected trees, whereas long-distance spread (> 1 km) is only occasionally observed (Rizzo *et al*. 2005).

Sampling strategy

Each year, at least two aerial surveys to locate trees that recently died are conducted by the Oregon Department of Forestry (Goheen *et al*. 2006). During these flights, the approximate locations of the dead trees are recorded on a digital map using global positioning system (GPS). All trees detected are subsequently inspected on the ground to determine the cause of death. In addition, susceptible plants growing within and adjacent to known infested sites are also periodically examined for symptoms of *P. ramorum*. Symptomatic plant tissues (i.e. leaves, bark, and twigs) are collected for *P. ramorum* isolation. To determine the presence of *P. ramorum* in the soil, samples (0.5 dm^3) are periodically taken within the original eradication sites in close proximity of stumps of infected trees that were cut and burnt. The presence of *P. ramorum* is also monitored in

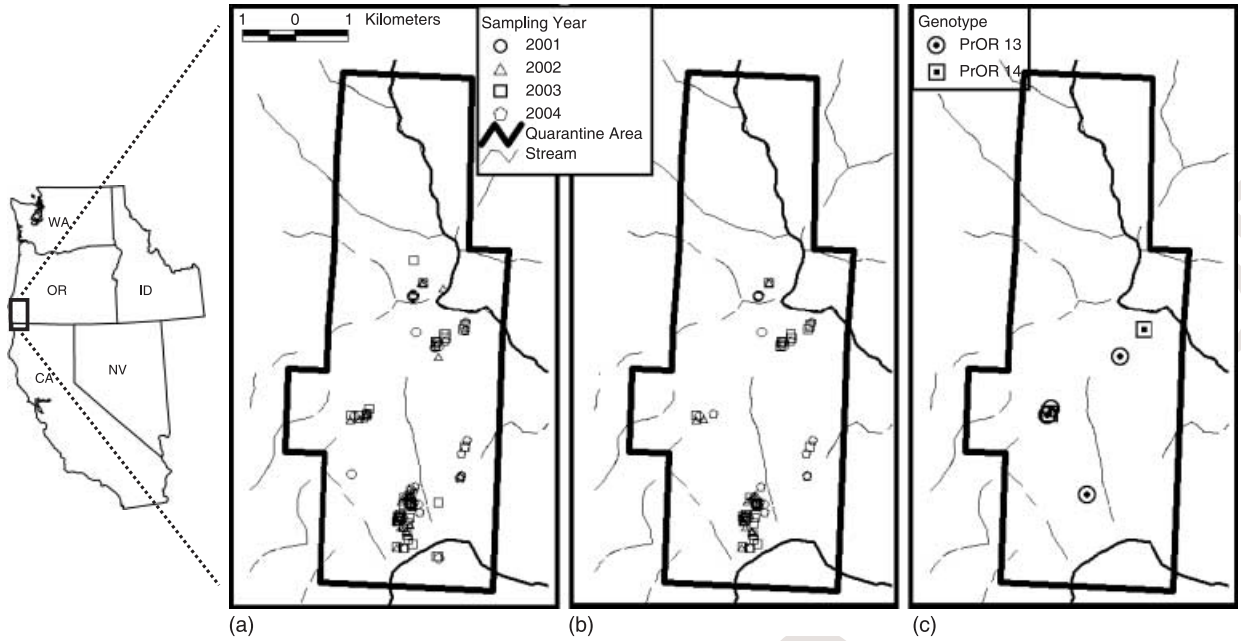

Fig. 1 Spatial distribution of multilocus genotypes of *Phytophthora ramorum* in the infested forest in Curry County, Oregon. (a) Location of the investigated forest population (different symbols refer to different sampling years), (b) distribution of the dominant genotype PrOR1, and (c) distribution of rare genotypes, for example PrOR13 and PrOR14.

all 11 streams draining the original eradication sites, as well as in 35 streams not directly associated with infested sites. Rhododendron and tanoak leaves floating in mesh bags are used as baits and are collected every 2 weeks.

Starting in 2003, horticultural nurseries in Oregon have been routinely inspected by the Oregon Department of Agriculture (ODA) and samples of symptomatic plants are collected for *P. ramorum* isolation (Osterbauer *et al*. 2003).

Isolation and identification of Phytophthora ramorum

Small pieces (*c*. 2 mm × 2 mm) of symptomatic leaves or bark collected in the infested forest in Curry County were placed onto *Phytophthora* selective CARP medium (Hansen *et al*. 2005). Leaves were only plated in the laboratory, whereas bark samples taken from the margin of lesions were plated both in the laboratory and in the field. *Phytophthora ramorum* was retrieved from soil samples that were flooded with distilled water by baiting with fresh foliage segments (*c*. 2 cm length) of Port-Orford-cedar [*Chamaecyparis lawsoniana* (A. Murr.) Parl.], entire leaves of Pacific rhododendron, or whole pear fruits. After 4–6 days, leaf baits and tissue from developing lesions on the pears were plated on CARP medium amended with 30 mg benlate and 25 mg hymexazol. The plates containing plant tissues (i.e. leaves, bark, or pear) were incubated at room temperature for 10 days and checked every 2–3 days under a microscope. The production of deciduous sporangia and chlamydospores was used for identification of *P. ramorum* (Werres *et al*. 2001). Growing colonies of *P. ramorum* were transferred onto corn meal agar (Hansen *et al*. 2005) and contaminated cultures were subcultured on CARP.

Given current quarantine regulations, *P. ramorum* is eradicated wherever it is found in Oregon nurseries, making systematic sampling impossible. To isolate the pathogen, symptomatic leaves collected in nurseries were plated on *Phytophthora* selective PARP medium by the ODA (Osterbauer *et al*. 2004) and kindly provided to us by Nancy Osterbauer.

Genomic DNA was extracted from *P. ramorum* cultures using a cetyltrimethyl ammonium bromide (CTAB)–chloroform-isopropanol extraction protocol as described by Winton & Hansen (2001).

Characteristics of the Phytophthora ramorum *isolates*

A total of 323 isolates of *P. ramorum* collected from 2001 to 2004 were analysed in this study; 272 were recovered from the infested forest (Curry County) and 51 were obtained from nurseries (Table 1). Seven isolates from Curry County were previously analysed in the study of Ivors *et al*. (2006). About 80% of the forest isolates were collected from infected trees or shrubs. Only one isolate was sampled per tree or shrub and, given ongoing eradication practices, any previously sampled, infected tree or shrub could not be resampled in subsequent years. The most frequent host species was tanoak (85% of the isolates), followed by Pacific rhododendron and evergreen huckleberry. Thirty

Table 1 Number of *Phytophthora ramorum* isolates recovered from the infested forest (Curry County, Oregon) and Oregon nurseries between 2001 and 2004

Origin	2001	2002	2003	2004	Total
1) Forest	48	50	127	47	272
Trees/Shrubs	42	44	102	25	213
Streams	0	2	15	13	30
Soil	6	4	10	9	29
2) Nurseries*	0	0	20	31	51
Total	48	50	147	78	323

*Nursery isolates were kindly provided by Nancy Osterbauer, Oregon Department of Agriculture (ODA).

isolates were obtained from baits in seven streams and 29 isolates were obtained from soil samples. The 51 nursery isolates originated from infected plants (i.e. *Camellia* sp., *Rhododendron* sp., *Viburnum* sp., and *Pieris* sp.) grown in 15 nurseries (i.e. retail nurseries, greenhouse, and wholesale growers).

Microsatellite isolation and amplification

The isolates of *P. ramorum* were screened for alleles at 10 microsatellite loci (Table 2). The dinucleotide (Pr9C3) and trinucleotide (PrMS6, PrMS9, PrMS13, PrMS21, and PrMS27) repeat loci were previously isolated from an enriched genomic library as described by Prospero *et al*. (2004). Four additional tetranucleotide repeat loci (PrMS39, PrMS42, PrMS43, and PrMS45) were identified by screening the genome of *P. ramorum* (available online at: http://genome.jgi-psf.org/) for common tetranucleotide repeats [e.g. $(AAAG)_6$, $(GAGT)_6$, and $(GATA)_6$]. For each locus, the specific forward and reverse polymerase chain reaction (PCR) primers were designed using the program PRIMER 3 (Whitehead Institute for Biomedical Research) to have a T_m between 57 °C and 63 °C. In each primer pair, the forward primer was labelled at the 5′-end with a fluorescent dye (i.e. 6-FAM-blue or HEX-green) for detection on a capillary sequencer (Table 2).

All microsatellite loci, with the exception of locus PrMS43, were amplified using a PCR programme of 1 cycle of 92 °C for 2 min, followed by 30 cycles of 92 °C for 30 s,

Table 2 Characteristics of analysed microsatellite loci of *Phytophthora ramorum*

Locus	Repeat motif*	Forward (F) and reverse (R) primer sequence (5′–3′)	Fluorescent label	GenBank Accession no.	Alleles†	
					N	Size (bp)
Pr9C3	$(CA)_{15}$	F: TCACACGAAGCAGCAACTCT R: AGCGGCACTACGGAATACAT	FAM	AY644773	2	216, 226
PrMS6	$(CGA)_8$	F: AATCGATCTCTCGGCTTTGA R: TATAGCCCCAGCTGCAACA	FAM	AY644775	2	165, 168
PrMS9	$(TCG)_9$	F: TTCACAGCGAGCTGAACATT R: GCCCGTCAAGAATAGTTTGG	FAM	AY644776	2	148, 176
PrMS13	$(TTG)_6$	F: GTGCATGCGTATGTGAGTCC R: CACCACTTATCACGCACCAC	HEX	AY644777	2	172, 187
PrMS21	$(TCG)_7$	F: ACTGTCGGTGGAGGAAATTG R: AAGTCCACCACCATCCTCTG	FAM	AY644778	1	247
PrMS27	$(CAC)_8$	F: GTACTCGGTCGAGCCCTTCT R: ACGGAAACCACAGAGTCGTC	HEX	AY644779	1	213
PrMS39a‡	$(GA)_{11}$	F: GCACGGCCAGAGATTGATAG R: ATCTGCCGACGTGAAGAAGT	HEX	DQ530436	1	129
PrMS39b‡	$(GA)_4(GATA)_{33}$	F: GCACGGCCAGAGATTGATAG R: ATCTGCCGACGTGAAGAAGT	HEX	DQ530437	4	242–254
PrMS42	$(GTGA)_4$(...)$(GTGA)_8$	F: AGTGCGTGAGTGAGTGATGG R: TGCTCCCCATTTTCTCTGTT	FAM	DQ530438	4?§	132–248
PrMS43a¶	$(CAGA)_{71}$	F: AAATATGCAAAAAGGCAGGA R: CCGCGTAACCTAGTCTGCTC	FAM	DQ641493	6	357–385
PrMS43b¶	$(CAGA)_{75}$(...)$(CAGA)_{17}$	F: AAATATGCAAAAAGGCAGGA R: CCGCGTAACCTAGTCTGCTC	FAM	DQ641494	7	466–506
PrMS45	$(TCCG)_{11}$	F: CGTGCTGCATCTGGTGTAGT R: GAAAGTCCGGATTTGCGTTA	FAM	DQ530439	2	167, 187

*Refers to the dominant *Phytophthora ramorum* multilocus genotype in Oregon (PrOR1); †only alleles of the North American multilocus genotypes (A2 mating type) are shown; ‡initially considered a single locus (PrMS39), see Results for further details; §see Results for further details; ¶initially considered a single locus (PrMS43), see Results for further details.

52 °C for 30 s, 65 °C for 30 s, and 1 cycle of 65 °C for 5 min. Fluorescent multiplex PCRs were performed in 10-μL volumes with the following final concentrations: 1× Red-*Taq* PCR Reaction Buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; Sigma), 200 μM dNTPs, 0.3 μM mix of primer pairs (Mix 1: 2 μM PrMS9, 1.6 μM PrMS21 and 1.2 μM PrMS27; Mix2: 1 μM Pr9C3, 1 μM PrMS6 and 0.4 μM PrMS13; PrMix3: 1.2 μM PrMS39, 1 μM PrMS42 and 1 μM PrMS45), 0.05 U Red*Taq* DNA polymerase (Sigma), and 0.5 μL (20–40 ng) DNA template. Amplification of the locus PrMS43 with the above-mentioned PCR programme and mix failed. Thus, this locus was amplified separately using the following PCR programme: 1 cycle of 92 °C for 2 min, 35 cycles of 92 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, and 1 cycle of 72 °C for 45 min. The final concentrations of the reaction mixture (10-μL volume) were 1× Red*Taq* PCR Reaction Buffer, 400 μM dNTPs, 0.3 μM forward and reverse primers, 0.1 U Red*Taq* DNA polymerase, and 0.6 μL DNA template.

PCR products were screened for successful amplification on 1.2% agarose gels and subsequently sized on an ABI PRISM 3100 DNA sequencer (PE Applied Biosystems) using the internal size standard ROX-500 (PE Applied Biosystems). Results were analysed using GENESCAN and GENOTYPER software packages (PE Applied Biosystems). Isolates giving ambiguous results were re-analysed.

Sequencing of polymorphic loci

Polymorphism among Oregon isolates of *P. ramorum* was only observed for the loci PrMS39, PrMS43, and PrMS45 not previously described in Prospero *et al*. (2004). To verify that the differences in allele sizes were real, PCR products resulting from the amplification of loci PrMS39 and PrMS43 for four isolates belonging to different genotypes were cloned and sequenced according to Prospero *et al*. (2004).

At locus PrMS45, most isolates were heterozygous, except for seven isolates showing only one allele. For 10 isolates, PCR amplification of the locus PrMS43b did not produce any amplicon. To verify that the nonamplification of alleles at these two loci was not due to low quality and/or quantity of the template DNA, we re-extracted genomic DNA from pure cultures of the problematic isolates and subsequently genotyped them.

Population genetic analyses

For genetic analyses, *P. ramorum* populations were defined by year (2001–2004) and environment (forest vs. nurseries). For the total population and for the 2003 and 2004 populations, analyses were performed with and without the isolates recovered from the nurseries. Only forest isolates were available in 2001 and 2002. Since the European multilocus genotype PrOR33 (Prospero *et al*. 2004) detected in three nurseries does not belong to the North American population of *P. ramorum* (i.e. recently introduced from Europe, Hansen *et al*. 2003), it was excluded from the genetic diversity analyses.

Genetic variation within and among populations was estimated by analysing genotypic diversity and gene diversity (Milgroom 1996). Genotypic diversity was characterized by three indices describing richness, diversity, and evenness. Given the differences in sample sizes, genotypic richness (NG_{Exp}, i.e. number of expected genotypes in a population of $N = 47$) was calculated using rarefaction curves (Grünwald *et al*. 2003). Genotypic diversity was quantified with Stoddart and Taylor's index $G = 1/\Sigma_i p_i^2$, where p_i = observed frequency of i^{th} genotype, and genotypic evenness (i.e. distribution of the genotypes within a sample) was estimated with the index $E_5 = (1/\lambda - 1)/(e^{H'} - 1)$, where λ corresponds to Simpson's index (Simpson 1949) and H' to Shannon-Wiener's index (Shannon & Weaver 1949). Support for differences in the G and E_5 values among populations was tested with bootstrapping, which was conducted using 2000 resamples at a confidence level of 90%. For each population, mean value and standard confidence intervals with bias correction were calculated using the SAS macro <jack-boot.sas> (Grünwald *et al*. 2003).

Gene diversity was characterized by polymorphism, allelic diversity, observed and expected heterozygosities, Wright's *F*-statistics (fixation index and differentiation coefficient), and genetic distance. Incidence of polymorphic loci (P_L) and mean number of observed alleles per locus (A_O) were calculated using the program POPGENE version 1.31 (Yeh & Boyle 1999; www.ualberta.ca/~fyeh/). Observed heterozygosity (H_O) and unbiased expected heterozygosity (H_E & Nei 1978) were determined with Tools for Population Genetic Analyses (TFPGA) version 1.3 (Miller 1997). Fixation index (F_{IS}) was calculated for each population as $F_{IS} = 1 - H_O/H_E$. Weir & Cockerham (1984) coefficient of differentiation θ (equivalent to Wright's F_{ST}) was calculated with TFPGA. Confidence intervals for θ were generated by bootstrapping over loci (1000 replications, 95% confidence level). The hypothesis of nondifferentiation among populations was tested by comparing the observed θ value with the value calculated for data sets in which alleles were resampled without replacement (1000 randomizations) using the program MULTILOCUS version 1.3 (Agapow & Burt 2001; www.agapow.net/software/multilocus). Nei's (1972) genetic unbiased distances among populations were calculated as implemented in TFPGA. The resulting distance matrix was used to construct a phenogram based on the unweighted pair-group method of averages (UPGMA) algorithm in TFPGA. Statistical support for phenogram branches was obtained using 1000 bootstrapped samples of the data set. An exact test using a Monte Carlo approach (10 batches, 2000 permutations per batch, and 1000 dememorization steps) was performed on pairwise

combinations of populations to determine if genetic differences were significant, as described by Raymond & Rousset (1995). Analyses were conducted with all isolates of a population and clone-corrected data sets (i.e. only one representative of each multilocus genotype). To reveal genetic relationships among the detected *P. ramorum* genotypes, an unrooted neighbour-joining tree based on Nei's (1972) genetic distance was constructed as implemented in the program POPULATIONS 1.2.28 (Langella 2002; www.cnrs-gif.fr/pge/bioinfo/populations/). Statistical support for the tree was obtained by conducting 1000 bootstraps. The program TREEVIEW version 1.6.6 (Page 1996) was used to visualize the tree. Gene flow between forest and nursery *P. ramorum* populations was estimated by calculating the effective migration rate $N_m = 0.25(1 - F_{ST})/F_{ST}$ with the program POPGENE.

The hypothesis of asexual (i.e. clonal) reproduction of *P. ramorum* in Oregon was tested using the parsimony tree length permutation test (PTLPT) and the index of association (I_A) statistics. The PTLPT test, which was conducted with PAUP* version 4.0b10 (Swofford 1998), compares the length of the most parsimonious tree obtained from the data set to the lengths of 1000 trees estimated from randomized data sets (Maynard Smith *et al*. 1993). The I_A index implemented in the program MULTILOCUS is computed by comparing the variance of the distances between all pairs of individuals in the data set with the variance expected without linkage disequilibrium (Agapow & Burt 2001). For each population, the I_A values expected under completely random mating were generated with 1000 randomizations (Burt *et al*. 1996). Analyses were conducted with all isolates of a population and clone-corrected data sets, and with or without including the European multilocus genotype PrOR33. The presence/absence of multilocus genotypes sharing alleles of both the North American and the European lineage of *P. ramorum* (i.e. recombinant genotypes) was also used as evidence for sexual/clonal reproduction.

Results

Sequencing and amplification of microsatellite loci

Sequence analyses revealed that primer sets PrMS39 and PrMS43 amplified microsatellite loci. Differences in size of alleles among genotypes were due to a different number of repeat units in the microsatellite. Given the significant size differences (> 100 bp) between the two amplicons produced by each primer set, we were not sure if these amplicons represented alleles at a single locus or alleles at two independent loci. Screening the sequenced genome of *Phytophthora ramorum* with these two primer sets showed that the two amplicons for each primer set were located in different scaffolds (PrMS39: scaffolds 531 and 436, PrMS43: scaffolds 512 and 151). Excluding a few point mutations and short insertions, a large portion (> 200 bp) of the sequence upstream and downstream from the microsatellite was the same in both scaffolds (data not shown). Thus, it is likely that this region is duplicated in the genome of *P. ramorum*. Therefore, we considered the two amplicons of each primer set to represent alleles of two different loci (i.e. each primer set amplifies two loci). Locus PrMS39a was invariant and was thus not included in further analyses.

In each *P. ramorum* isolate, PCR amplification of locus PrMS42 produced four amplicons that were not variable among isolates. Therefore, this locus was not used for population analyses. At locus PrMS45, six out of seven isolates that were homozygous at a first genotyping were also homozygous at a second genotyping following PCR amplification with newly extracted DNA. One isolate was heterozygous as were most isolates at this locus. At locus PrMS43b, 10 isolates that did not show any amplicon for the first microsatellite reaction were also negative for a second reaction with independent DNA extraction.

Population structure

A total of 33 multilocus genotypes were identified from 2001 to 2004; 22 genotypes were only recovered from the infested forest (Curry County), 9 genotypes were only detected in nurseries, and 2 genotypes (PrOR19 and PrOR25) were observed both in the infested forest and in nurseries (Fig. 2a–d).

In all four sampling years, the forest population was dominated by a single multilocus genotype (PrOR1) at frequencies of 59.6–70.9% for 2001–2004 (Fig. 2a–d). All other genotypes, except for genotypes PrOR4 (7.7%) and PrOR8 (5.5%), occurred at less than 5% frequency. Four genotypes (PrOR1, PrOR4, PrOR6, and PrOR8) were found in all four sampling years, whereas 12 genotypes were only detected in one particular year (Table 3). Considering all four sampling years, the dominant genotype PrOR1 was found in all main mortality centres, whereas the rare genotypes had a sporadic distribution (e.g. Fig. 1b, c). The three most common forest genotypes (PrOR1, PrOR4, and PrOR8) were isolated from all three sources of samples (i.e. infected trees or shrubs, soil, and stream). Three genotypes (PrOR16 in 2003, PrOR24 and PrOR25 in 2004) were only found in soil samples and genotype PrOR17 was recovered only from a stream in 2003. A total of six genotypes were recovered from seven streams draining infested sites. The dominant genotype PrOR1 was found in six of these streams. Disregarding PrOR1, in only two cases was the same genotype detected in the same stream in two different sampling years (PrOR14 in stream WA9 and PrOR4 in stream WA12).

In contrast to the infested forest, the nursery population of *P. ramorum* was not dominated by a single multilocus genotype (Fig. 2c, d). The most common genotype (PrOR29)

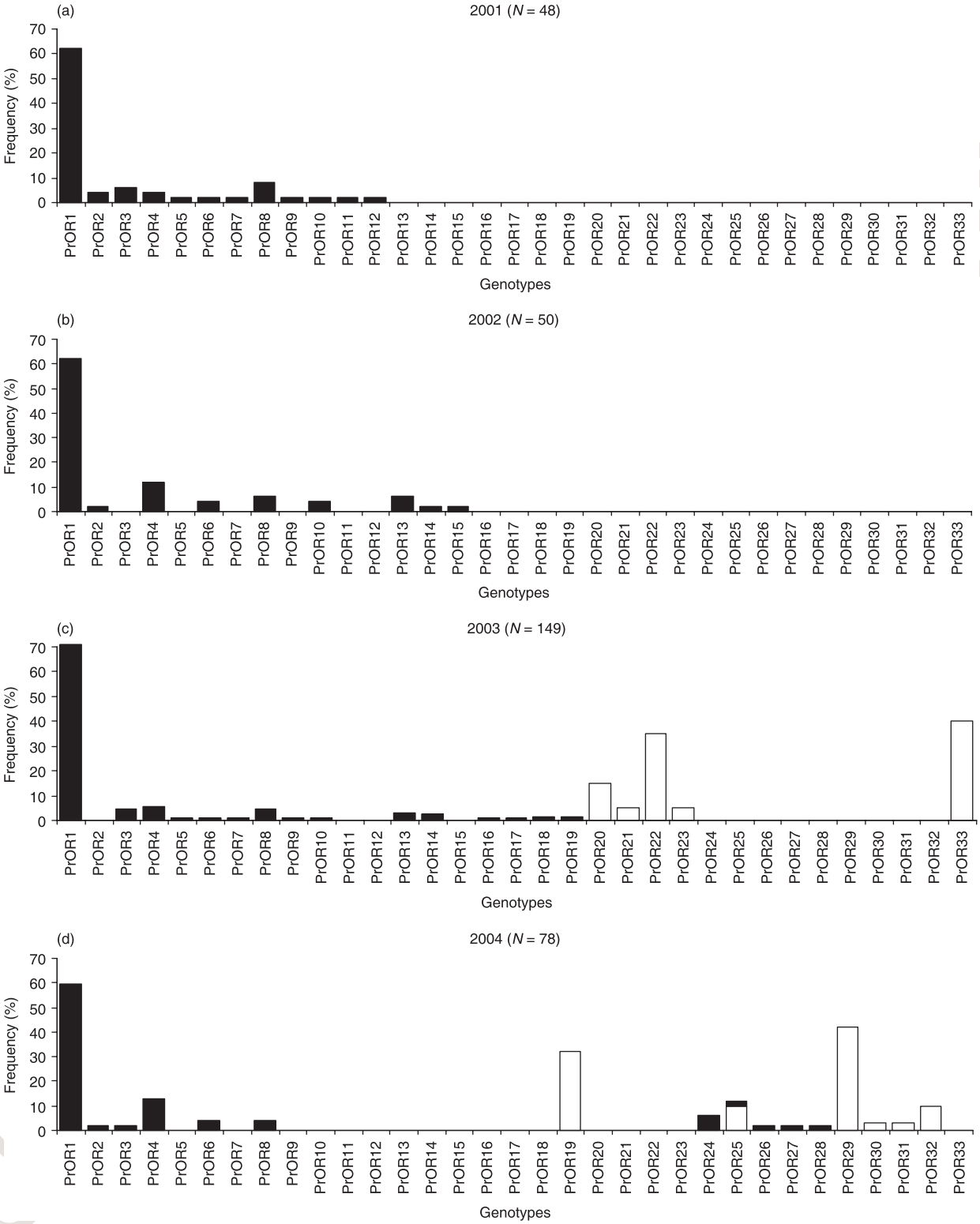

Fig. 2 Incidence of *Phytophthora ramorum* multilocus genotypes detected in 2001 (a), 2002 (b), 2003 (c), and 2004 (d) in Oregon. Black bars indicate multilocus genotypes detected in the infested forest (i.e. from infected trees or shrubs, soil samples, or stream baits); white bars refer to multilocus genotypes detected in the nurseries. Frequencies of forest and nursery multilocus genotypes were calculated independently.

Table 3 Recovery of *Phytophthora ramorum* multilocus genotypes (MG) from infected trees or shrubs, soil, and streams at the infested forest (Curry County, Oregon) from 2001 to 2004

		Recovery from*			
MG	Isolates (*N*)	2001†	2002	2003	2004
PrOR1	179	F, S	F, S, W (WA12)	F, S, W (WA4, WA12)	F, S, W (WA1, WA9, WA12, WA16, WA27)
PrOR2	4	F	F		F
PrOR3	10	F		F, W (WA1)	W (WA12)
PrOR4	21	F	F	F, S, W (WA9, WA12)	F, W (WA1, WA12)
PrOR5	2	F		F	
PrOR6	6	F	F	F	F
PrOR7	2	F		F	
PrOR8	15	F, S	F	F, S, W (WA12)	F, W (WA1)
PrOR9	2	S		F	
PrOR10	4	F	F	F	
PrOR11	1	F			
PrOR12	1	F			
PrOR13	7		F	F	
PrOR14	4		W (WA9)	F, W (WA9)	
PrOR15	1		F		
PrOR16	1			S	
PrOR17	1			W (WA15)	
PrOR18	2			F	
PrOR19	2			F	
PrOR24	3				S
PrOR25	1				S
PrOR26	1				F
PrOR27	1				F
PrOR28	1				F

*F, infected trees or shrubs; S, soil; and W, streams (name of the stream); †in 2001 no stream monitoring was performed.

comprised 25.5% of the isolates and was found in 6 out of 15 nurseries. Each of the other 10 genotypes had a frequency of 2–20%. None of the nursery genotypes was recovered both in 2003 and in 2004. In 11 nurseries, only a single genotype was detected, whereas four nurseries were characterized by the presence of multiple genotypes (one nursery with four genotypes and three nurseries with two genotypes). The genotype PrOR33, only recovered from the nurseries, belonged to the European lineage of *P. ramorum* (Prospero *et al*. 2004) and was of the A1 mating type (Hansen *et al*. 2003). At five loci (PrMS6, PrMS9, PrMS13, PrMS21, and PrMS45), this genotype shared an allele with North American genotypes; at four loci (Pr9C3, PrMS27, PrMS39b, and PrMS43a), it had different alleles; and at the locus PrMS43b, no amplicon was produced (Table 4). PrOR33 was found in three nurseries, in one of which it was sampled together with isolates belonging to a North American genotype and compatible mating type. However, none of the genotypes identified in the infested forest or in nurseries had allele patterns that suggested a recombination between PrOR33 and a North American genotype (Table 4).

Genotypic diversity 2001–2004

The 48 isolates collected in 2001 from the infested forest belonged to 12 multilocus genotypes (Table 5). From 2002 to 2004 a total of 12 additional genotypes were detected at the infested forest site. The highest incidence of new genotypes was observed in 2004, with five new genotypes out of 11 detected. The expected number of genotypes in a sample of $N = 47$ forest isolates (i.e. the smallest sample size of the populations being compared) ranged from 9 (2002 and 2003) to 12 (2001). Including nursery isolates in the analysis increased the number of new genotypes detected from 2002 to 2004 to 21 and the number of expected genotypes to 12 in 2003 and to 13 in 2004 (Table 5). Genotypic diversity estimated with the Stoddart and Taylor index was very similar for forest populations of *P. ramorum* from all 4 years ($G = 1.93$–2.61). The 90% confidence intervals of the individual populations calculated with bootstrapping overlapped, indicating no significant differences in the G values among populations. The same situation was observed for the evenness index E_5. Including the nursery isolates in the 2003 and 2004 populations of *P. ramorum*,

Table 4 Allele sizes at 10 microsatellite loci analysed of the 33 multilocus genotypes (MG) of *Phytophthora ramorum* detected in Oregon from 2001 to 2004

MG	Recovery*	Isolates (*N*)	Microsatellite loci									
			Pr9C3	PrMS6	PrMS9	PrMS13	PrMS21	PrMS27	PrMS39b	PrMS43a	PrMS43b	PrMS45
PrOR1	F	179	216/226	165/168	148/176	172/187	247/247	213/213	250/250	377/377	486/486	167/187
PrOR2	F	4	216/226	165/168	148/176	172/187	247/247	213/213	250/250	377/377	486/486	167/167
PrOR3	F	10	216/226	165/168	148/176	172/187	247/247	213/213	250/250	377/377	490/490	167/187
PrOR4	F	21	216/226	165/168	148/176	172/187	247/247	213/213	250/250	373/373	486/486	167/187
PrOR5	F	2	216/226	165/168	148/176	172/187	247/247	213/213	250/250	377/377	478/478	167/187
PrOR6	F	6	216/226	165/168	148/176	172/187	247/247	213/213	250/250	377/377	482/482	167/187
PrOR7	F	2	216/226	165/168	148/176	172/187	247/247	213/213	250/250	377/377	494/494	167/187
PrOR8	F	15	216/226	165/168	148/176	172/187	247/247	213/213	250/250	381/381	486/486	167/187
PrOR9	F	2	216/226	165/168	148/176	172/187	247/247	213/213	250/250	381/381	490/490	167/187
PrOR10	F	4	216/226	165/168	148/176	172/187	247/247	213/213	254/254	377/377	486/486	167/187
PrOR11	F	1	216/226	165/168	148/176	172/187	247/247	213/213	250/250	377/377	506/506	167/187
PrOR12	F	1	216/226	165/168	148/176	172/187	247/247	213/213	250/250	377/377	466/466	167/187
PrOR13	F	7	216/226	165/168	148/176	172/187	247/247	213/213	250/250	373/373	482/482	167/187
PrOR14	F	4	216/226	165/168	148/176	172/187	247/247	213/213	250/250	373/373	490/490	167/187
PrOR15	F	1	216/226	165/168	148/176	172/187	247/247	213/213	250/250	381/381	—/—†	167/187
PrOR16	F	1	216/226	165/168	148/176	172/187	247/247	213/213	250/250	357/357	486/486	167/187
PrOR17	F	1	216/226	165/168	148/176	172/187	247/247	213/213	250/250	381/381	506/506	167/187
PrOR18	F	2	216/226	165/168	148/176	172/187	247/247	213/213	250/250	385/385	486/486	167/187
PrOR19	F, N	12	216/226	165/168	148/176	172/187	247/247	213/213	246/246	377/377	490/490	167/187
PrOR20	N	3	216/226	165/168	148/176	172/187	247/247	213/213	246/246	373/373	486/486	167/187
PrOR21	N	1	216/226	165/168	148/176	172/187	247/247	213/213	246/246	377/377	—/—	167/187
PrOR22	N	7	216/226	165/168	148/176	172/187	247/247	213/213	246/246	373/373	—/—	167/187
PrOR23	N	1	216/226	165/168	148/176	172/187	247/247	213/213	242/242	373/373	—/—	167/187
PrOR24	F	3	216/226	165/168	148/176	172/187	247/247	213/213	246/246	377/377	486/486	167/187
PrOR25	F, N	4	216/226	165/168	148/176	172/187	247/247	213/213	250/250	369/369	486/486	167/187
PrOR26	F	1	216/226	165/168	148/176	172/187	247/247	213/213	250/250	373/373	478/478	167/187
PrOR27	F	1	216/226	165/168	148/176	172/187	247/247	213/213	250/250	373/373	482/482	167/167
PrOR28	F	1	216/226	165/168	148/176	172/187	247/247	213/213	250/250	377/377	482/482	167/167
PrOR29	N	13	216/226	165/168	148/176	172/187	247/247	213/213	246/246	373/373	490/490	167/187
PrOR30	N	1	216/226	165/168	148/176	172/187	247/247	213/213	246/246	369/369	490/490	167/187
PrOR31	N	1	216/226	165/168	148/176	172/187	247/247	213/213	246/246	357/357	490/490	167/187
PrOR32	N	3	216/226	165/168	148/176	172/187	247/247	213/213	242/242	373/373	486/486	167/187
PrOR33‡	N	8	218/224	165/165	148/170	187/187	247/256	216/216	136/140	146/146	—/—	163/187

*F, infested forest (Curry County); *N*, nursery; †no amplicons produced by PCR amplification; ‡multilocus genotype belonging to the European lineage of *Phytophthora ramorum* (A1 mating type).

caused an increase of the *G* value both in 2003 and 2004, and of the E_5 value in 2004 (Table 5). However, significant differences were only observed in the genotypic diversity between the population in 2004 ($G = 5.64$) and the other three annual populations and in the genotypic evenness between the population in 2004 ($E_5 = 0.52$) and the two populations in 2003.

Gene diversity 2001–2004

The mean number of alleles observed per locus in the single populations was very similar and ranged from 2.0 ± 0.7 (2002) to 2.7 ± 1.7 (2003, forest and nurseries) (Table 5). Eight loci were polymorphic but only the tetranucleotide repeat loci (i.e. PrMS39b, PrMS43a, PrMS43b, and PrMS45) showed differences among North American genotypes (excluding PrOR33, European clonal lineage). At loci PrMS39b, PrMS43a, and PrMS43b the frequency distributions for single alleles in the forest and nursery populations were different (e.g. at locus PrMS39b, allele 250 was dominant in the forest population and allele 246 was prevalent in the nursery population) (Table 4). The unrooted neighbour-joining tree revealed that forest and nursery genotypes did not group separately, but the bootstrap supports for the nodes were generally very low (< 50%) (Fig. 3). The observed heterozygosity (H_O) varied between 0.494 (2004, forest)

Table 5 Diversity of annual populations (i.e. 2001, 2002, 2003, and 2004) and of the total (i.e. 2001–2004) population of *Phytophthora ramorum* in Oregon forest and nursery environments

			2003		2004		2001–2004	
Statistics*	2001	2002	*F*†	*F+N*‡	*F*	*F+N*	*F*	*F+N*
Sample size (*N*)	48	50	127	149	47	78	272	323
NG_{Obs}	12	9	15	19(+1)§	11	16	24	32(+1)§
NG_{Exp}	12	9	9	12	11	13	4	5
NG_{New}	—	3	4	9	5	9	—	—
G	2.43	2.42	1.93	2.61	2.61	5.64	2.22	3.12
	(1.54–3.31)	(1.60–3.24)	(1.32–2.54)	(1.61–3.61)	(1.69–3.52)	(3.99–7.23)	(1.44–3.00)	(1.77–4.48)
E_5	0.34	0.43	0.28	0.27	0.39	0.52	0.23	0.20
	(0.26–0.43)	(0.33–0.53)	(0.21–0.35)	(0.19–0.35)	(0.30–0.49)	(0.41–0.64)	(0.16–0.31)	(0.12–0.28)
P_L	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
A_O	2.4 ± 1.7	2.0 ± 0.7	2.6 ± 1.6	2.7 ± 1.7	2.2 ± 1.0	2.4 ± 1.3	2.8 ± 2.0	2.9 ± 2.1
H_O	0.496	0.498	0.500	0.500	0.494	0.496	0.498	0.498
H_E	0.316	0.326	0.318	0.340	0.327	0.411	0.320	0.358
F_{IS}	−0.569	−0.528	−0.572	−0.471	−0.511	−0.207	−0.556	−0.396

NG_{Obs}, number of observed multilocus genotypes; NG_{Exp}, number of expected multilocus genotypes in a sample of $N = 47$ (i.e. smallest population being compared); NG_{New}, number of new multilocus genotypes (i.e. not recovered before the specific sampling year); *G*, Stoddart and Taylor's index (90% confidence interval); E_5, index of evenness (90% confidence interval); P_L, incidence of polymorphic loci; A_O, mean number of observed alleles per locus (± standard deviation); H_O, observed heterozygosity; H_E, unbiased expected heterozygosity (Nei 1978); F_{IS}, fixation index; *the European multilocus genotype PrOR33 was not included in the calculation of NG_{Exp}, *G*, E_5, P_L, A_O, H_O, H_E, and F_{IS}; †*F*, isolates recovered from the infested forest (Curry County, Oregon); ‡*F+N*, isolates recovered from the infested forest and from the nurseries; §the European multilocus genotype PrOR33 detected in three nurseries is indicated as (+1).

Table 6 Estimation of Weir & Cockerham's (1984) coefficient of differentiation θ among different Oregon populations of *Phytophthora ramorum*

Populations*	θ value	95% confidence interval†	*P* value‡
Forest vs. nurseries	0.316	0.0662–0.5605	< 0.001
Forest and nurseries vs. year§	0.0283	0.0001–0.0698	< 0.001
Nurseries 2003 vs. nurseries 2004	0.160	−0.0043–0.3815	< 0.001
Forest vs. year	0.0006	−0.0002–0.0020	0.302

*The multilocus genotype PrOR33 was not included in the analyses; †confidence intervals for θ values were generated by bootstrapping over loci (1000 replications) using TFPGA; ‡*P* values < 0.05 indicate a significant differentiation among populations; §all collected isolates from forest and nurseries at each sampling year included, that is 2001 and 2002 forest, 2003 and 2004 forest and nurseries.

and 0.5 (populations in 2003) and in all populations it was higher than the expected heterozygosity, giving a negative fixation index. The highest unbiased expected heterozygosity ($H_E = 0.411$) was observed in the 2004 population (forest and nurseries), whereas the lowest ($H_E = 0.316$) was observed in the first population sampled in 2001.

Forest and nursery populations were significantly ($P < 0.001$) differentiated ($\theta = 0.316$, Table 6) and gene flow between the two populations was low ($N_m = 1.8$; clone-corrected $N_m = 3.1$). Significant differentiation was also detected among the four annual *P. ramorum* populations when forest and nursery isolates were combined ($\theta = 0.0283$), as well as between the nursery populations in 2003 and in 2004 ($\theta = 0.160$). In contrast, randomization tests indicated no significant differentiation ($\theta = 0.0006$, $P = 0.302$) among the four annual forest populations of *P. ramorum* (Table 6).

The tree based on Nei's unbiased genetic distance showed that the four annual forest populations of *P. ramorum* were genetically very close (0.003 Nei's genetic distance, Fig. 4a). The initial population in 2001 was more closely related to the population in 2003, and the population in 2002 to the population in 2004. Based on exact tests, differentiation was significant ($P = 0.032$) only between the 2001 and the 2004 forest populations using non-clone-corrected data sets (Table 7). A considerably larger genetic distance was

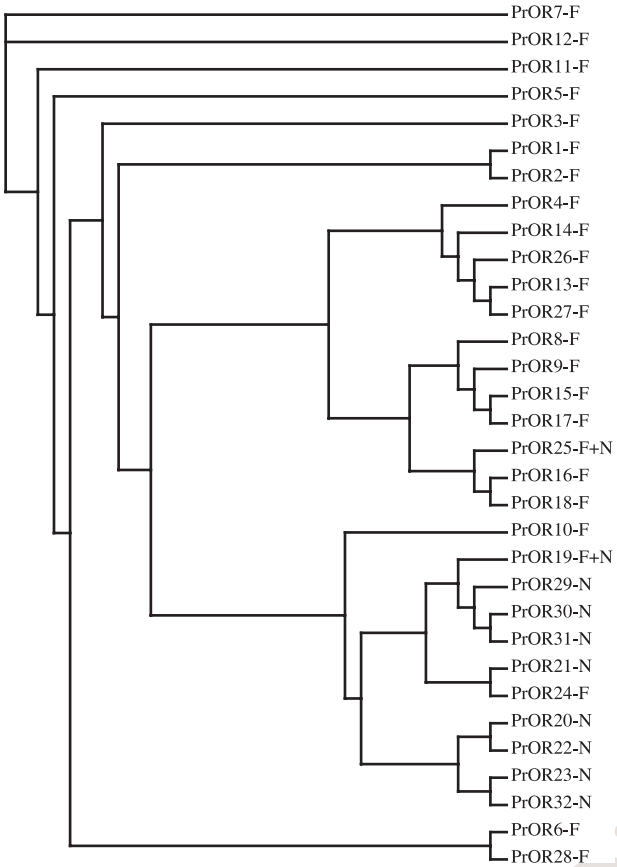

Fig. 3 Unrooted neighbour-joining tree based on Nei's (1972) genetic distance of the *Phytophthora ramorum* multilocus genotypes detected in Oregon from 2001 to 2004. Statistical support for the tree was obtained by conducting 1000 bootstraps and only values above 50% are shown. The European multilocus genotype PrOR33 was excluded from the analyses. F, multilocus genotypes recovered from the infested forest (Curry Co.); N, multilocus genotypes recovered from nurseries; F+N, multilocus genotypes recovered from the infested forest and from nurseries.

observed between the forest population and the nursery population (0.235 Nei's genetic distance, Fig. 4c). In 74% of the bootstrapped samples, a node also appeared separating the 2003 from the 2004 nursery populations. Exact tests based on non-clone-corrected data sets showed a highly significant ($P < 0.001$) differentiation among all three populations (nurseries 2003, nurseries 2004, and forest 2001–2004) (Table 8). When using clone-corrected data sets, differentiation was significant ($P = 0.0034$) only between the forest population and the nursery population in 2004. Comparative trees derived from clone-corrected data sets showed qualitatively similar results (Fig. 4b, d).

Overall, microsatellite loci did not show evidence for significant linkage disequilibria. The PTLPT supported the hypothesis of clonality only in four populations when non-clone-corrected data sets were used (Table 9). Following clone correction, clonal reproduction was rejected in all populations. For most Oregon populations of *P. ramorum* (forest; forest and nurseries), the index of association (I_A) values observed were not significantly different from those expected under completely random mating (Table 9), indicating no significant linkage disequilibrium. The same tests conducted with the inclusion of a representative of the European genotype (PrOR33) in the data sets showed significant ($P < 0.001$) linkage disequilibrium in all populations, both using non-clone-corrected and clone-corrected data sets.

Table 7 Pairwise probabilities for *Phytophthora ramorum* population differentiation in the infested forest (Curry County, Oregon) calculated with an exact test using a Monte Carlo approach (10 batches, 2000 permutations per batch, and 1000 dememorization steps). Above diagonal, tests based on non-clone-corrected data sets; below diagonal, tests based on clone-corrected data sets

Population	2001	2002	2003	2004
2001	—	0.1143	0.8189	0.0320
2002	0.9666	—	0.2522	0.4305
2003	0.9937	0.9984	—	0.3601
2004	0.7803	0.9837	0.9508	—

Table 8 Pairwise probabilities for *Phytophthora ramorum* population differentiation between infested forest (Curry County, Oregon) and nurseries (2003 and 2004) calculated with an exact test using a Monte Carlo approach (10 batches, 2000 permutations per batch, and 1000 dememorization steps). Above diagonal, tests based on non-clone-corrected data sets; below diagonal, tests based on clone-corrected data sets

Population	Forest	Nursery 03	Nursery 04
Forest	—	0.0009	0.0001
Nursery 03	0.0828	—	0.0099
Nursery 04	0.0034	0.935	—

Discussion

Our study indicates that *Phytophthora ramorum* eradication at the infested forest site in Curry County, Oregon, is not completely successful, while eradication in nursery environments is effective. The Curry County population of *P. ramorum* is composed of 24 multilocus genotypes. One genotype is very frequent (66% of the isolates) and 23 genotypes have a frequency of 1–8%. The dominant genotype was recovered in all main disease centres from infected trees or shrubs and from the soil. In addition, it was also found in an increasing number of streams. The rare genotypes were more sporadically distributed in the infested forest.

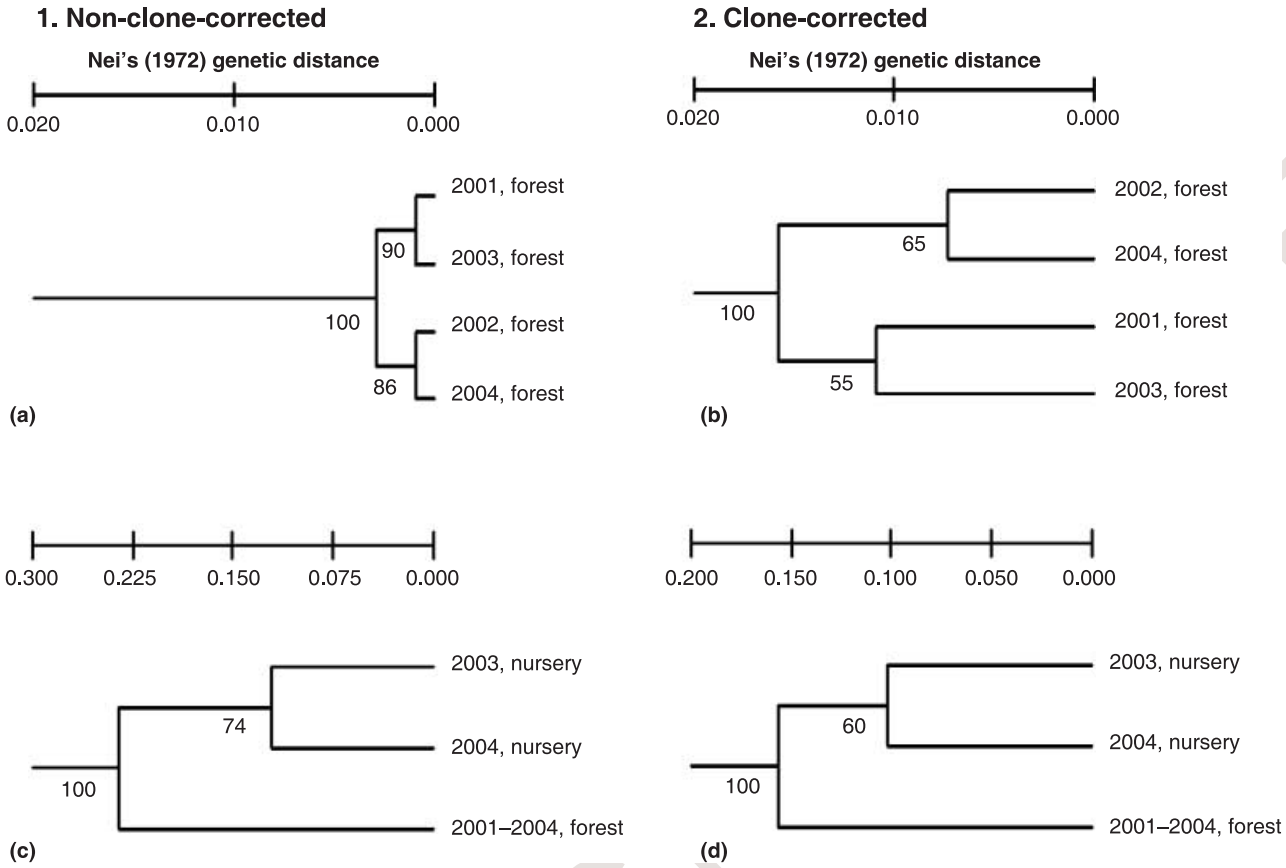

Fig. 4 Phenogram constructed using the unweighted pair-group method of averages (UPGMA) algorithm based on the Nei's (1972) unbiased genetic distance of populations. Statistical support for branches was obtained using 1000 bootstrapped samples of the data set. The European multilocus genotype PrOR33 was excluded from the analyses. (a) and (b) only isolates recovered from the infested forest (Curry County, Oregon) were included in the analysis, (c) and (d) all isolates (i.e. infested forest and nurseries) were included in the analysis.

However, as with the dominant genotype, they were not only isolated from infected plants but they were also detected in soil and in streams. Thus, systematic soil and stream sampling as well as sampling from infected plants is necessary for an accurate survey of the dynamics of the *P. ramorum* populations. The recovery of a specific genotype at different sampling years indicates the persistence of inoculum sources in the forest. Rapid destruction of all potential hosts growing around a symptomatic plant clearly reduces the inoculum levels and the spread of the pathogen but has not completely prevented new infections (Rizzo *et al*. 2005). Eradication efforts of *P. ramorum* were successful in Oregon nurseries. In 2003 and 2004 a total of 11 genotypes were found in the nurseries but the same genotypes were not detected in both years. This suggests effective eradication of nursery infestations in 2003, followed by new introductions from different sources in 2004, as supported by ODA 'traceback' records (N. Osterbauers, personal communication). Application of control measures (i.e. prevention and sanitation practices) in nurseries is simpler than in the forest. For example, plants can be inspected more closely and more often for *P. ramorum* symptoms and all suspicious plant material can be rapidly destroyed. In the forest, control measures at the individual tree level (e.g. early detection or chemical treatment) are often not realistic and management strategies must be applied to a landscape scale (Rizzo *et al*. 2005).

The overall *P. ramorum* population in Oregon is characterized by low genetic diversity. Polymorphism among isolates was only revealed for the four tetranucleotide repeat microsatellites. Therefore, it could be speculated that the low diversity is also due to the markers chosen. However, similar results were previously reported by Ivors *et al*. for microsatellites (2006) or AFLP analyses (2004). In their microsatellite study, most of the variation among *P. ramorum* isolates within the United States population was based on differences detected at two microsatellite loci out of 12 loci considered. The authors also analysed seven forest isolates from Curry County, Oregon, and concluded that these isolates belonged to the same genotype. Genotyping

Table 9 Tests for random mating within single annual populations and the total population of *Phytophthora ramorum* in Oregon

		PTLPT§				Index of association (I_A)¶			
		NA genotypes††		NA and PrOR33‡‡		NA genotypes††		NA and PrOR33‡‡	
Population†	Isolates‡ (*N*)	All isolates	Clone corr.§§	All isolates	Clone corr.§§	All isolates	Clone corr.§§	All isolates	Clone corr.§§
2001	48 (12)	21	21	34	34	–0.14	–0.52	1.68***	2.15***
2002	50 (9)	14	14	26	26	0.07	–0.33	1.72***	2.69***
2003, *F*	127 (15)	28	28	41	41	0.25**	–0.35	1.06***	1.82***
2003, *F+N*	139 (19)	36	36	49	49	0.22**	–0.45	0.81***	1.42***
2004, *F*	47 (11)	18	18	31	31	0.02	–0.37	1.62***	2.16***
2004, *F+N*	78 (16)	28***	28	41***	41	0.51***	–0.05	1.04***	1.54***
Total, *F*	272 (24)	43	43	56	56	0.10*	–0.32	0.50***	1.29***
Total, *F+N*	315 (32)	59***	59	72***	72	0.39***	–0.24	0.60***	0.92***

†*F*, isolates recovered from the infested forest (Curry County); *F+N*, isolates recovered from the infested forest and from nurseries; ‡number of isolates used in the analyses; (clone-corrected, one isolate representing each multilocus genotype). For analyses including the European multilocus genotype PrOR33, eight additional isolates belonging to this genotype have to be added. §Parsimony tree length permutation test (PTLPT). The significance of the test was determined by comparing the length of the most parsimonious tree obtained from the data set to the lengths of 1000 trees estimated from artificially recombined data sets (Burt *et al*. 1996). ***$P < 0.001$ supporting the hypothesis of clonal reproduction in the specific population; ¶index of association (I_A) statistic (Agapow & Burt 2001). The significance of I_A was tested by comparing the observed value to that expected under the null hypothesis of completely random mating (1000 randomizations). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ††only the North American multilocus genotypes were included in the calculations; ‡‡North American multilocus genotypes and European multilocus genotype PrOR33 were included in the calculations; §§clone-corrected, only one representative of each multilocus genotype included in the calculations.

with our microsatellite markers, however, showed that the seven isolates belonged to three different multilocus genotypes. Thus, the low genetic diversity that we detected is probably real and not a consequence of a lack of power of our microsatellites. Based on our results, tetranucleotide repeat microsatellites seem to be more polymorphic than tri- and dinucleotide repeat microsatellites. Reports in the literature about the influence of the length of the repeat motif on the mutation rate at microsatellite loci are conflicting. Some studies have shown that dinucleotide repeat loci are highly polymorphic (e.g. Vigouroux *et al*. 2002), whereas other studies suggest a higher mutation rate for tetranucleotide loci (e.g. Weber & Wong 1993). A potential drawback of tetranucleotide repeat loci is that they can be difficult to amplify and tend to produce strong stutter bands (e.g. PrMS43a and PrMS43b) as previously observed by other authors (Walsh *et al*. 1996).

The *P. ramorum* population structure has all the hallmarks of an introduced organism. Low population diversity of a pathogen in a specific geographic region is frequently used to support the hypothesis of its non-native character (e.g. *Phytophthora cinnamomi* Rands in South Africa, Linde *et al*. 1997; *Discula destructiva* Redlin, the dogwood anthracnose fungus, in North America, Trigiano *et al*. 1995). Assuming that *P. ramorum* is not native to Oregon, we could suppose that only the dominant genotype PrOR1 was initially introduced in the forest in Curry County. From 2002 to 2004, we annually detected three to five new genotypes, independent of the sample size. Therefore, given the presence of 11 rare genotypes in 2001, when *P. ramorum* was first detected in Oregon, we could roughly estimate that the pathogen had been present in the infested area for several years before 2001. This hypothesis is supported by the retrospective detection of dead tanoak trees on aerial photographs taken in 1997 but not in earlier years (Nelson *et al*. 2005). As an alternative to the hypothesis of introduction of a single genotype, several genotypes could have been simultaneously introduced at the end of the 1990s. The genotype PrOR1 may have been the most abundant among the original genotypes or may have evolved from one of these. As it was best adapted to Curry County conditions, it could rapidly increase its population size and spread in the forest. However, this possibility seems not to be supported by laboratory tests indicating no significant fitness differences among *P. ramorum* genotypes (Hansen *et al*. 2005).

There appears to be little, if any, gene flow between nursery and forest environments in Oregon. Only two genotypes (i.e. PrOR19 and PrOR25) out of 11 found in the nurseries have also been detected in the forest. Both are rare in the forest and neither was detected before 2003. Therefore, it is clear that in Oregon the nursery infestation is not caused by genotypes coming from the *P. ramorum* population in the infested forest in Curry County. This is confirmed by surveys conducted by the Oregon Department

of Agriculture indicating that the infected nursery plants have been introduced from nurseries out-of-state (Parke *et al*. 2003; N. Osterbauer, personal communication). Nursery genotypes of *P. ramorum* migrated from populations located outside Oregon. Spread of genotypes between production facilities via movement of plants was previously reported for *Phytophthora nicotianae* (Breda de Haan) and *Phytophthora drechsleri* Tucker (Lamour *et al*. 2003). The effect of human activity on the structure of populations of *Phytophthora* has been well documented for *Phytophthora infestans* (Mont) de Bary, which evolved in Central Mexico, and where human activity contributed to worldwide migration of novel genotypes (Goodwin 1997; Grünwald & Flier 2005). Some rare *P. ramorum* nursery genotypes might also have evolved locally in the Oregon nurseries through mutation. The two genotypes found both in nurseries and in the infested forest may be the result of independent mutations or a consequence of trading infected nursery plants. The presence of different *P. ramorum* genotypes in the natural forest ecosystem and in nurseries emphasizes the importance of prevention and sanitation practices in nurseries in order to reduce the risk of introducing new genotypes through infected plants and establishing new infections in the wild. Analyses of *P. ramorum* samples from a wider geographic range would provide important information about the source populations that provided migrants found in Oregon nurseries.

Our results show that all Oregon genotypes of *P. ramorum* belong to the same clonal lineage, i.e. are descendants from a single individual. Considering the seven isolates analysed in both studies, this lineage likely corresponds to the US lineage 1 (clade 2) reported by Ivors *et al*. (2006). None of our isolates could be attributed to the second US lineage (clade 3) identified by the same authors. All differences that we detected among genotypes can be explained with a basic model of the microsatellite mutation process (i.e. a stepwise-mutation model or an infinite allele model; Estoup & Cornuet 2001; Balloux & Lugon-Moulin 2002). Similar variation patterns were also observed within clonal lineages of *Phytophthora cinnamomi* in Australia (Dobrowolski *et al*. 2003). Thus, most variation within the Oregon population of *P. ramorum* has probably occurred through mutation, as was previously observed for other *Phytophthora* species (Goodwin 1997). Another source of microsatellite length variation could have been mitotic recombination (Chamnanpunt *et al*. 2001; Dobrowolski *et al*. 2003). Based on our data, however, it is not possible to estimate the contribution of this process to the variation in the *P. ramorum* population analysed. On the contrary, several indicators exclude the occurrence of sexual reproduction in the *P. ramorum* population in Oregon. First, there is low genotypic diversity, with most isolates belonging to a dominant genotype. By comparison, in central Mexico where the heterothallic *P. infestans* is sexually recombining, no dominance of a particular genotype is observed (Grünwald *et al*. 2001). Second, the observed heterozygosity is higher than that expected under Hardy–Weinberg equilibrium, which gives a negative fixation index. In diploid organisms, extreme clonality often results in considerable heterozygote excesses (Birky 1996; Balloux *et al*. 2003). Third, no genotypes with allele patterns indicating sexual recombination between European (A1 mating type) and North American (A2 mating type) isolates were found. All forest and most nursery *P. ramorum* isolates in North America have so far been of the A2 mating type indicating that in this population sexual reproduction does not occur. In populations that are at equilibrium and randomly mating, the two mating types would be expected to be in similar proportions (Milgroom 1996). This is for instance the case in the *P. infestans* population at the centre of origin (Grünwald *et al*. 2001; Grünwald & Flier 2005). Surprisingly, the PTLPT test revealed clonality in only a few Oregon populations of *P. ramorum*. This result could be due to the particular structure of the data sets. Out of 10 loci analysed, only two (PrMS43a and PrMS43b) were very informative and differentiated most genotypes. As suggested by Taylor *et al*. (1999), the presence of highly mutable loci would mask clonality and give the appearance of recombination. As the PTLPT test, the index of association statistics also indicated the presence of significant linkage disequilibrium in only a few Oregon populations of *P. ramorum*. However, adding a single representative of the European genotype PrOR33 to the data sets gave significant results in all populations. A similar situation was previously reported by Dobrowolski *et al*. (2003) for *P. cinnamomi* in Australia. Tests conducted with populations consisting of only one genotype group (i.e. clonal lineage) did not support the hypothesis of clonal reproduction but adding representatives of other genotype groups to the same population data sets indicated linkage disequilibrium. The authors concluded that if only one genotype group is present, tests are not significant because all microsatellite mutations occur within a common genotype, suggesting recombination. Thus, linkage disequilibrium may only be detected if more than one genotype group is present in a population. Although we have found no direct evidence for sexual recombination, with both mating types co-existing in Oregon, sexual reproduction could eventually occur and result in more aggressive genotypes, as has been observed for *P. infestans* in Europe in the last decade (Drenth *et al*. 1994; Fry & Goodwin 1997).

Acknowledgements

We would like to thank Wendy Sutton for technical assistance in the laboratory and Alex Krupkin for helpful advice on microsatellite amplification and electropherogram interpretation. We are grateful to Michael McWilliams for preparing the maps presented in this paper. We also thank the anonymous reviewers for valuable comments on the manuscript.

References

Agapow P-M, Burt A (2001) Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes*, **1**, 101–102.

Balloux F, Lugon-Moulin N (2002) The estimation of population differentiation with microsatellite markers. *Molecular Ecology*, **11**, 155–165.

Balloux F, Lehmann L, de Meeûs T (2003) The population genetics of clonal and partially clonal diploids. *Genetics*, **164**, 1635–1644.

Birky CW (1996) Heterozygosity, heteromorphy, and phylogenetic trees in asexual eukaryotes. *Genetics*, **144**, 427–437.

Brasier CM, Kirk SA (2004) Production of gametangia by *Phytophthora ramorum* in vitro. *Mycological Research*, **108**, 823–827.

Brasier CM, Kirk SA, Delcan J, Cooke DEL, Jung T, Man in't Veld WA (2004) *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. *Mycological Research*, **108**, 1172–1184.

Burt A, Carter DA, Koenig GL, White TJ, Taylor JW (1996) Molecular markers reveal cryptic sex in the human pathogen *Coccidiodes immitis*. *Proceedings of the National Academy of Sciences, USA*, **93**, 770–773.

Chamnanpunt J, Shan WX, Tyler BM (2001) High frequency mitotic gene conversion in genetic hybrids of the oomycete *Phytophthora sojae*. *Proceedings of the National Academy of Sciences, USA*, **98**, 14530–14535.

Davidson JM, Shaw CG (2003) Pathways of movement for *Phytophthora ramorum*, the causal agent of Sudden Oak Death. *Sudden Oak Death Online Symposium*. Available at URL: www.apsnet.org/online/SOD.

Davidson JM, Wickland AC, Patterson H, Falk K, Rizzo DM (2005) Transmission of *Phytophthora ramorum* in mixed-evergreen forests in California. *Phytopathology*, **95**, 587–596.

Dobrowolski MP, Tommerup IC, Shearer BL, O'Brien PA (2003) Three clonal lineages of *Phytophthora cinnamomi*. Australia revealed by microsatellites. *Phytopathology*, **93**, 695–704.

Drenth A, Tas ICQ, Govers S (1994) DNA fingerprinting uncovers a new sexually reproducing population of *Phytophthora infestans* in the Netherlands. *European Journal of Plant Pathology*, **100**, 97–107.

Erwin DC, Ribeiro OK (1996) *Phytophthora Diseases Worldwide*. APS Press, St. Paul, Minnesota.

Estoup A, Cornuet J-M (2001) Microsatellite evolution: inferences from population data. In: *Microsatellites. Evolution and Applications* (eds Goldstein DB, Schlötterer C), pp. 49–65. Oxford University Press, New York.

Franklin JF, Dyrness CT (1988) *Natural Vegetation of Washington and Oregon*, 2nd edn. Oregon State University Press, Corvallis, Oregon.

Fry WE, Goodwin SB (1997) Re-emergence of potato and tomato late blight in the United States. *Plant Disease*, **81**, 1349–1357.

Garbelotto (2004) Sudden oak death: a tale of two continents. *Outlooks on pest management*, **April**, 85–89.

Garbelotto M, Svihra P, Rizzo DM (2001) Sudden oak death syndrome fells three oak species. *California Agriculture*, **55**, 9–19.

Goheen EM, Hansen EM, Kanaskie A, McWilliams MG, Osterbauer N, Sutton W (2002) Eradication of sudden oak death in Oregon. *Phytopathology*, **92**, S30.

Goheen EM, Kanaskie A, McWilliams M, Hansen E, Sutton W, Osterbauer N (2006) Surveying and monitoring Sudden Oak Death in southwest Oregon forests. In: *Proceedings of the Sudden Oak Death Second Science Symposium: the State of Our Knowledge* (tech. coord. Frankel SJ, Shea PJ, Haverty MI), pp. 413–415. Gen. Tech. Rep. PSW-GTR-196. Pacific Southwest Research Station, Forest Service US Department of Agriculture, Albany, California.

Goodwin SB (1997) The population genetics of *Phytophthora*. *Phytopathology*, **87**, 462–473.

Grünwald NJ, Flier WG (2005) The biology of *Phytophthora infestans* at its center of origin. *Annual Reviews of Phytopathology*, **43**, 171–190.

Grünwald NJ, Flier WG, Sturbaum AK *et al*. (2001) Population structure of *Phytophthora infestans* in the Toluca valley region of central Mexico. *Phytopathology*, **91**, 882–890.

Grünwald NJ, Goodwin SB, Milgroom MG, Fry WE (2003) Analysis of genotypic diversity data for population of microorganisms. *Phytopathology*, **93**, 738–746.

Hansen EM, Sutton W (2005) *Persistence of Phytophthora ramorum after eradication efforts in Oregon tanoak forests*. Sudden Oak Death Science Symposium, December 15–18, 2002, Monterey, California.

Hansen EM, Sutton W, Parke J, Linderman R (2002) Phytophthora ramorum *and Oregon forest trees—one pathogen, three diseases*. Sudden Oak Death Science Symposium, December 15–18, 2002, Monterey, California.

Hansen EM, Reeser PW, Sutton W, Winton L, Osterbauer N (2003) First report of A1 mating type of *Phytophthora ramorum* in North America. *Plant Disease*, **87**, 1267.

Hansen EM, Parke JL, Sutton W (2005) Susceptibility of Oregon forest trees and shrubs to *Phytophthora ramorum*: a comparison of artificial inoculation and natural infection. *Plant Disease*, **89**, 63–70.

Ivors KL, Hayden KJ, Bonants PJM, Rizzo DM, Garbelotto M (2004) AFLP and phylogenetic analyses of North American and European populations of *Phytophthora ramorum*. *Mycological Research*, **108**, 378–392.

Ivors KL, Garbelotto M, De Vries I *et al*. (2006) Microsatellite markers identify three lineages of *Phytophthora ramorum* in US nurseries, yet single lineages in US forest and European nursery populations. *Molecular Ecology*, **15**, 1493–1505.

Jung T, Cooke DEL, Blaschke H, Duncan JM, Osswald W (1999) *Phytophthora quercina* sp. nov., causing root rot of European oaks. *Mycological Research*, **103**, 785–798.

Lamour KH, Daughtrey ML, Benson DM, Hwang J, Hausbeck MK (2003) Etiology of *Phytophthora drechsleri* and *P. nicotianae* (=*P. parasitica*) diseases affecting floriculture crops. *Plant Disease*, **87**, 854–858.

Langella O (2002) POPULATIONS *1.2.28. Logiciel de génétique des populations*. Available at URL: http://www.cnrs-gif.fr/pge/bioinfo/populations.

Linde C, Drenth A, Kemp GHJ, Wingfield MJ, von Broembsen SL (1997) Population structure of *Phytophthora cinnamomi* in South Africa. *Phytopathology*, **87**, 822–827.

Maynard Smith J, Smith NH, O'Rourke M, Spratt BG (1993) How clonal are bacteria? *Proceedings of the National Academy of Sciences, USA*, **90**, 4384–4388.

Milgroom MG (1996) Recombination and the multilocus structure of fungal populations. *Annual Reviews of Phytopathology*, **34**, 457–477.

Miller MP (1997) *Tools for Population Genetic Analyses (TFPGA) 1.3: A Windows Program for the Analysis of Allozyme and Molecular Population Genetic Data*. Computer software distributed by author.

Nei M (1972) Genetic distance between populations. *American Naturalist*, **106**, 283–292.

Nei M (1978) Estimation of average heterozygosities and genetic distance from a small numbers of individuals. *Genetics*, **89**, 583–590.

Nelson A, Overhulser D, Kanaskie A, McWilliams M, Sprengel K (2005) *Forest Health Highlights in Oregon—2004*. Oregon Department of Forestry and USDA Forest Service, March 2005, Portland, Oregon.

Osterbauer NK, Rehms L, Hedberg J (2003) Official survey of Oregon agricultural commodities for *Phytophthora ramorum*. *Phytopathology*, **93**, S68 (Abstract).

Osterbauer NK, Griesbach JA, Hedberg J (2004) Surveying for and eradicating *Phytophthora ramorum* in agricultural commodities. Online. *Plant Health Progress*, doi: 10.1094/PHP-2004-0309-02-RS.

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*, **12**, 357–358.

Parke J, Pscheidt JU, Linderman R (2003) *Phytophthora ramorum*, a guide for Oregon nurseries. *Oregon State University Extension Service*.

Prospero S, Black JA, Winton LM (2004) Isolation and characterization of microsatellite markers in *Phytophthora ramorum*, the causal agent of sudden oak death. *Molecular Ecology Notes*, **4**, 672–674.

Raymond ML, Rousset F (1995) An exact test for population differentiation. *Evolution*, **49**, 1280–1283.

Rizzo DM, Garbelotto M, Hansen EM (2005) *Phytophthora ramorum*: integrative research and management of an emerging pathogen in California and Oregon forests. *Annual Reviews of Phytopathology*, **43**, 309–345.

Shannon CE, Weaver W (1949) *The Mathematical Theory of Communication*. University of Illinois Press, Urbana, Illinois.

Simpson EH (1949) Measurement of diversity. *Nature*, **163**, 688.

Swofford DL (1998) PAUP*. *Phylogenetic Analysis Using Parsimony (*and Other Methods)*, version 4. Sinauer & Associates, Sunderland, Massachusetts.

Taylor JW, Geiser DM, Burt A, Koufopanou V (1999) The evolutionary biology and population genetics underlying fungal strain typing. *Clinical Microbiology Reviews*, **12**, 126–146.

Trigiano RN, Caetano-Anollés G, Bassam BJ, Windham MT (1995) DNA amplification fingerprinting provides evidence that *Discula destructiva*, the cause of dogwood anthracnose in North America, is an introduced pathogen. *Mycologia*, **87**, 490–500.

Vigouroux Y, Jaqueth JS, Matsuoka Y *et al*. (2002) Rate and pattern of mutation at microsatellite loci in maize. *Molecular Biology and Evolution*, **19**, 1251–1260.

Walsh PS, Fildes NJ, Reynolds R (1996) Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. *Nucleic Acids Research*, **24**, 2807–2812.

Weber JL, Wong C (1993) Mutation of human short tandem repeats. *Human Molecular Genetics*, **2**, 1123–1128.

Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

Werres S, Marwitz R, Man In't Veld WA *et al*. (2001) *Phytophthora ramorum* sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. *Mycological Research*, **105**, 1155–1165.

Winton LM, Hansen EM (2001) Molecular diagnosis of *Phytophthora lateralis* in trees, water, and foliage baits using multiplex polymerase chain reaction. *Forest Pathology*, **31**, 275–283.

Yeh FC, Boyle T (1999) POPGENE: *Microsoft Window-Based Freeware for Population Genetic Analysis*, version 1.31; Available at URL: http://www.ualberta.ca/~fyeh/download.htm. University of Alberta, Canada.

Zanzot JW (2004) Potential susceptibility of tanoak associated and rare ericaceous plant species of southwestern Oregon to *Phytophthora ramorum*. Master Thesis, Oregon State University, Corvallis, Oregon.

Author Query Form

Journal: Molecular Ecology

Article: mec_3343.fm

Dear Author,

During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper's edge. Please remember that illegible mark-ups may delay publication.

Many thanks for your assistance.

No.	Query	Remarks
1	Please expand CARP here.	
2	Please expand PARP here.	
3	The URL for the program populations is not accessible. Please check and supply the correct URL.	
4	The URL for Davidson & Shaw 2003 is not accessible. Please check and supply the correct URL.	
5	The URL for the program populations in Langella 2002 is not accessible. Please check and supply the correct URL.	
6	Please supply the URL for the program cited in Miller 1997.	
7	Please supply complete reference details for Parke et al 2003.	
8	Please supply a paragraph about the research interests of the authors.	

MARKED PROOF

Please correct and return this set

Please use the proof correction marks shown below for all alterations and corrections. If you wish to return your proof by fax you should ensure that all amendments are written clearly in dark ink and are made well within the page margins.

Instruction to printer	*Textual mark*	*Marginal mark*
Leave unchanged	··· under matter to remain	New matter followed by or or
Insert in text the matter indicated in the margin		
Delete	through single character, rule or underline or through all characters to be deleted	or
Substitute character or substitute part of one or more word(s)	through letter or through characters	new character or new characters
Change to italics	under matter to be changed	
Change to capitals	under matter to be changed	
Change to small capitals	under matter to be changed	
Change to bold type	under matter to be changed	
Change to bold italic	under matter to be changed	
Change to lower case	Encircle matter to be changed	
Change italic to upright type	(As above)	
Change bold to non-bold type	(As above)	
Insert 'superior' character	through character or where required	or under character e.g. or
Insert 'inferior' character	(As above)	over character e.g.
Insert full stop	(As above)	
Insert comma	(As above)	
Insert single quotation marks	(As above)	or and/or or
Insert double quotation marks	(As above)	or and/or or
Insert hyphen	(As above)	
Start new paragraph		
No new paragraph		
Transpose		
Close up	linking characters	
Insert or substitute space between characters or words	through character or where required	
Reduce space between characters or words	between characters or words affected	