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Microsatellite markers identify three lineages of *Phytophthora ramorum* in US nurseries, yet single lineages in US forest and European nursery populations

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Abstract

Analysis of 12 polymorphic simple sequence repeats identified in the genome sequence of *Phytophthora ramorum*, causal agent of 'sudden oak death', revealed genotypic diversity to be significantly higher in nurseries (91% of total) than in forests (18% of total). Our analysis identified only two closely related genotypes in US forests, while the genetic structure of populations from European nurseries was of intermediate complexity, including multiple, closely related genotypes. Multilocus analysis determined populations in US forests reproduce clonally and are likely descendants of a single introduced individual. The 151 isolates analysed clustered in three clades. US forest and European nursery isolates clustered into two distinct clades, while one isolate from a US nursery belonged to a third novel clade. The combined microsatellite, sequencing and morphological analyses suggest the three clades represent distinct evolutionary lineages. All three clades were identified in some US nurseries, emphasizing the role of commercial plant trade in the movement of this pathogen.

Keywords: exotic microbe, oomycete, population genetics, SSR, sudden oak death

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Introduction

Phytophthora ramorum S. Werres & A.W.A.M. de Cock, causal agent of 'sudden oak death' (SOD) (Rizzo *et al*. 2002), is considered the latest in a series of emergent forest pathogens, significantly impacting coastal forest ecosystems in North America (Rizzo & Garbelotto 2003). Mortality of tanoaks (*Lithocarpus densiflora*) and coast live oaks (*Quercus agrifolia*) due to SOD has reached epidemic levels in California, and has resulted in the local disappearance of adult populations of tanoaks. The severity of the disease and ongoing expansion of infested areas are characteristics reminiscent of introduced pathogens (Rizzo *et al*. 2005). The pathogen also causes twig blight of *Rhododendron*, *Viburnum*, *Camellia*, *Kalmia*, *Pieris*, *Vaccinium* and other important nursery plant species (Davidson *et al*. 2003). The putative exotic nature of *P. ramorum* has prompted a range of strict national and international regulations aimed at preventing further spread of the pathogen (USDA-APHIS 2002).

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Phytophthora ramorum is heterothallic, requiring interaction of opposite mating types (A1 and A2) for sexual recombination. Heterothallism is a mechanism that favours outcrossing and implies the sympatric coexistence of both mating types in areas where the organism is native. When heterothallic *Phytophthora* spp. are introduced outside of their natural range, usually only single mating types can be identified within the population (Förster *et al*. 2000). Initial mating studies with *P. ramorum* determined that the A1 mating type was only found in Europe (EU), while the A2 mating type was limited to the United States (US), with the exception of a single A2 EU isolate collected from an infected nursery plant in Belgium in 2002 (Werres & De Merlier 2003). In spring 2003, the first A1 US isolates were identified in northern Oregon (Hansen *et al*. 2003), and since then both mating types have been discovered in Oregon and Washington nurseries (Ivors & Garbelotto, unpublished).

Recent studies have emphasized the differences between EU and US isolates and supported the exotic nature of this pathogen in both continents. Analysis of amplified fragment length polymorphism (AFLP) banding patterns

determined that EU and US isolates clustered into separate clades, each displaying low levels of genetic variation, hypothesized to be caused by mitotic recombination and/or mutation (Ivors *et al*. 2004). Single nucleotide polymorphisms (SNPs) have been identified in the mitochondrial cytochrome *c* oxidase subunit I (*cox* I) (Kroon *et al*. 2004), the β-tubulin (Bilodeau *et al*. 2004), and the cellulose binding elicitor lectin (CBEL) (Bilodeau *et al*. 2004) genes, distinguishing *P. ramorum* isolates collected in Europe from those collected in the United States. As well, EU and US isolates exhibit significant differences in growth rate and aggressiveness (Brasier 2003; Werres & Kamiski 2005), and vary in colony morphology (D. Hüberli, personal communication).

In populations with low genetic diversity, it is not possible to estimate the importance of sexual reproduction with multilocus sequence analysis or DNA fingerprints alone (Hoegger *et al*. 2000). Codominant markers such as microsatellites, also known as simple sequence repeats (SSRs), have improved the ability to detect cryptic outcrossing in fungi, and may provide a better understanding of the overall genetic structure of clonal species like *P. ramorum*. Microsatellites have been used to investigate the genetic structure and reproductive biology of numerous plant pathogens (Tenzer *et al*. 1999; Urena-Padilla *et al*. 2002; Atallah *et al*. 2004), including *Phytophthora* (Dobrowolski *et al*. 2002, 2003). Recently, microsatellites specific for *P. ramorum* confirmed the distinction between European and North American isolates, but failed to detect variation within each grouping (Prospero *et al*. 2004).

The complete genome sequence of *P. ramorum* was recently generated (www.jgi.doe.gov) and provided a novel opportunity to identify and evaluate potential SSR markers for investigating the population structure of *P. ramorum*. As part of an effort to gain a better understanding of the origin of new *P. ramorum* infestations, as well as the pathogen's reproductive biology and genetic variation, we developed and optimized 12 microsatellite markers from whole genome sequence data and used them in conjunction with mtDNA *cox* I sequences to characterize 71 isolates from the United States and 80 isolates from nine different European countries. The selection of isolates was broader than that of any previous studies, in an attempt to characterize any genetic diversity previously undetected, and it allowed for a first comparative analysis among isolates obtained from three distinct provenances, namely US forests, US nurseries, and European nurseries.

Materials and methods

Isolates and DNA extraction

Isolates used in this study (Table 1) were chosen to represent the known range of geographical locations and hosts of this pathogen. Seventy-one *Phytophthora ramorum* isolates from the United States (from 12 counties in California, two in Oregon and one in Washington) and 80 from nine countries within Europe, along with two *Phytophthora lateralis* and two *Phytophthora hibernalis* isolates were subjected to microsatellite analysis. Isolates were grown in pea broth on a rotary shaker at RT for approximately 7 days. Genomic DNA was isolated from 20 mg of lyophilized mycelium using the PUREGENE DNA isolation kit and protocol (Gentra) and eluted in 50 μL ultra-pure water. DNA extracts were stored at –20 °C.

Identification and development of SSR markers

Coverage of the complete genome of *P. ramorum* isolate Pr102 is 7X, and is estimated at 65 Mb in size with over 1 million reads assembled into 2576 scaffolds. At the time of our SSR project in October 2003, the sequence consisted of 445 030 FASTA reads at 4X. Computer software developed within Plant Research International (PRI) was used for SSR mining and primer design. This software comprised a compilation of five different modules, generating a data set of unique sets of polymerase chain reaction (PCR) primers for the amplification of microsatellite sequences. Primers were designed using the following criteria: min. T_m = 60 °C, GC content = 50%, primer size 18–21 bp, PCR product size between 100 and 350 bp, and GC clamp = 1. For each Candidate loci, five different forward and reverse primer combinations were developed. One-hundred and two (102) unique primer pairs (Illumina) were selected from all microsatellite loci identified by the program to screen for polymorphism within a panel of eight isolates of US and EU origin expected to be variable based on previous AFLP (Ivors *et al*. 2004) and mating types tests. Amplification reactions consisted of 10 ng template DNA, 200 μM dNTPs, 1 U *Taq* DNA polymerase (Roche), 1.5 mM $MgCl_2$, and 0.2 μM of each primer in a 20-μL reaction volume. Amplifications were run in a PTC200 thermocycler (MJ Research), with initial denaturation at 95 °C for 2 min, followed by 35 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. Samples containing 1 μL de-ionized formamide loading buffer and 1–2 μL of PCR product from successful amplifications were then denatured at 92 °C for 3 min, subjected to polyacrylamide gel electrophoresis and silver stained using standardized procedures (Ausubel *et al*. 1999) and the Silver Sequence DNA sequencing system (Promega).

Genetic analysis of microsatellite loci

A subset of loci showing polymorphism from the initial screening of eight *P. ramorum* isolates were selected for microsatellite analysis of 151 total US and EU isolates using fluorescently labelled primers. The forward primers of 12

Table 1 Isolates of *Phytophthora* species used in this study

Isolate number*,†	Host	Origin	Genotype
Phytophthora ramorum (USA)			
Pr01[DR], CBS110534	*Quercus agrifolia*	Marin Co., CA	US1
Pr03[DR], CBS110535	*Lithocarpus densiflora*	Marin Co., CA	US1
Pr06[DR], ATCC MYA-2435	*Q. agrifolia*	Marin Co., CA	US1
Pr52[DR], CBS110537, ATCC MYA-2436	*Rhododendron* sp.[N]	Santa Cruz Co., CA	US1
Pr62[DR]	*Q. agrifolia*	San Mateo Co., CA	US1
Pr65[DR], CBS110538, ATCC MYA-2437	*Q. parvula*[N]	Santa Cruz Co., CA	US1
Pr67[DR]	Soil	Sonoma Co., CA	US1
Pr70[DR], CBS110539	*Vaccinium ovatum*	Marin Co., CA	US1
Pr71[DR], CBS110540	*Q. agrifolia*	Sonoma Co., CA	US1
Pr72[DR]	*Rhododendron* sp.[N]	Santa Cruz Co., CA	US1
Pr82[DR]	*V. ovatum*	Marin Co., CA	US1
Pr84[DR]	Soil	Marin Co., CA	US1
Pr86[DR], CBS110541, ATCC MYA-2440	*Arbutus menziessi*	Marin Co., CA	US1
Pr87[DR]	*A. menziessi*	Marin Co., CA	US1
Pr97[DR], CBS110955	*Q. agrifolia*	Napa Co., CA	US2
Pr98[DR]	*Umbellularia californica*	Napa Co., CA	US1
Pr102[DR], ATCC MYA-2949	*Q. agrifolia*	Marin Co., CA	US1
Pr106[DR], CBS110956	*U. californica*	Sonoma Co., CA	US1
Pr110[DR], CBS110542	*U. californica*	Marin Co., CA	US1
Pr114[DR]	*U. californica*	Marin Co., CA	US1
Pr120[DR]	*L. densiflora*	Mendocino Co., CA	US1
Pr136[DR], ATCC MYA-2441	*Aesculus californica*	Marin Co., CA	US1
Pr137[DR]	*A. californica*	Marin Co., CA	US1
Pr145[DR]	*L. densiflora*	Santa Clara Co., CA	US1
Pr153[DR]	*U. californica*	Solono Co., CA	US1
Pr155[DR]	*L. densiflora*	Santa Clara Co., CA	US1
Pr160[DR]	*Q. agrifolia*	Napa Co., CA	US1
Pr161[DR]	*Q. agrifolia*	Napa Co., CA	US1
Pr168[DR]	*L. densiflora*	Mendocino Co., CA	US1
Pr173[DR]	*L. densiflora*	Santa Cruz Co., CA	US1
Pr196[DR]	*Heteromeles arbutifolia*	Marin Co., CA	US1
Pr218[DR]	*Rhamnus cathartica*	Sonoma Co., CA	US1
Pr219[DR]	*R. cathartica*	Sonoma Co., CA	US1
Pr220[DR]	*U. californica*	Mendocino Co., CA	US1
Pr223[DR]	*U. californica*	Humbolt Co., CA	US1
Pr236[DR]	*Pseudotsuga menziesii*	Sonoma Co., CA	US1
Pr237[DR]	*Trientalis latifolia*	Monterey Co., CA	US1
Pr240[DR]	*U. californica*	Sonoma Co., CA	US1
Pr340[DR]	*Lonicera hispidula*	Monterey Co., CA	US1
Pr341[DR]	*Acer macrophyllum*	Monterey Co., CA	US1
Pr345[DR], PrJL3.5.3[DR], CBS110544	*S. sempervirens*	Sonoma Co., CA	US1
Pr346[DR]	*S. sempervirens*	Santa Cruz Co., CA	US1
Pr351[DR]	*U. californica*	Sonoma Co., CA	US1
Pr354[DR]	*L. densiflora*	Marin Co., CA	US1
Pr367[DR]	*U. californica*	Sonoma Co., CA	US1
Pr375[DR]	*V. ovatum*	Marin Co., CA	US1
Pr379[DR]	*H. arbutifolia*	Marin Co., CA	US1
Pr387[DR]	*Q. chrysolepis*	Marin Co., CA	US1
Pr389[DR]	*Corylus cornuta*	Marin Co., CA	US1
PDR1282257[CB]	*Camellia sasanqua* var. Bonanza[N]	Stanislaus Co., CA	US1
PDR184965[CB]	*U. californica*	Contra Costa Co., CA	US1
Pr-OR1004.1[EH]	*V. ovatum*	Curry Co., OR	US1
Pr-OR2018.1[EH]	*L. densiflora*	Curry Co., OR	US1
Pr-OR2109[EH]	Unknown	Curry Co., OR	US1
Pr-OR2181[EH]	*Rhamnus purshiana*	Curry Co., OR	US1
Pr-OR4144.1[EH]	*L. densiflora*	Curry Co., OR	US1

Table 1 *Continued*

Isolate number*,†	Host	Origin	Genotype
Pr-OR4169.1[EH]	Unknown	Curry Co., OR	US1
Pr-OR4173.2[EH]	*V. ovatum*	Curry Co., OR	US1
Pr-OR5467[EH]	*U. californica*	Curry Co., OR	US1
Pr-3-74-1[EH]	*Pieris* sp.[N]	Clackamas Co., OR	EU1
Pr-3-74-2[EH]	*Viburnum bodnantense* 'Dawn'[N]	Clackamas Co., OR	EU1
Pr-WA1741[AW]	*Rhododendron* sp. var. Jean Marie[N]	King Co., WA	US1
Pr-WA1743[AW]	*Rhododendron* sp. var. Jean Marie[N]	King Co., WA	EU1
Pr-WA1747[AW]	*Rhododendron* sp. var. Jean Marie[N]	King Co., WA	EU1
Pr-WA1748[AW]	*Rhododendron* sp. var. Jean Marie[N]	King Co., WA	US1
Pr-WA1772[AW]	*Viburnum plicatum* var. Mariesii[N]	King Co., WA	EU1
Pr-WA1820[AW]	*Camellia japonica*[N]	King Co., WA	US1
Pr-WA1838[AW]	*Rhododendron* sp. var. Grace Seabrook[N]	King Co., WA	US1
Pr-WA1839[AW]	*Rhododendron* sp. var. Bariton[N]	King Co., WA	US3
Pr-WA2017[AW]	*Camellia japonica*[N]	King Co., WA	US1
Pr-WA0692[AW]	*Rhododendron* sp. var. Capistrano[N]	King Co., WA	US4
P. ramorum (EU)‡			
233[PB], BBA9/95[SW], CBS101553 (TYPE)	*Rhododendron catawbiense*[N]	Germany	EU1
235[PB], PD93/56[HG]	*Rhododendron* sp.[N]	The Netherlands	EU1
238[PB], PD98/6285[HG]	*Rhododendron* sp.[N]	The Netherlands	EU1
240[PB], PD98/5233[HG]	*Viburnum* sp.[N]	The Netherlands	EU1
472[PB], BBA13/99-1[SW], CBS109279	*Rhododendron* sp.[N]	Germany	EU1
474[PB], BBA16/99[SW], CBS109278	*V. bodnantense*[N]	Germany	EU1
497[PB], PD20018722[HG]	*Rhododendron* sp.[N]	The Netherlands	EU1
500[PB], RH/122/98[LO]	*Rhododendron* sp.[N]	Poland	EU1
501[PB], RH/2/00[LO]	*Rhododendron* sp.[N]	Poland	EU1
502[PB], RH/6/00[LO]	*Rhododendron* sp.[N]	Poland	EU1
508 [PB], PD20023399-2[HG], CBS110901	*V. bodnantense*[N]	Belgium	EU1
509[PB], Phyram1[EM], BBA4/02-1[SW]	*R. catawbiense* var. Grandiflora[N]	Mallorca, Spain	EU1
511[PB], Phyram3[EM], BBA4/02-4[SW]	*R. catawbiense*[N]	Mallorca, Spain	EU1
514[PB], BBA15/01-11[SW]	*Viburnum* sp.[N]	Germany	EU2
515[PB], BBA15/01-18[SW]	*V. bodnantense* var. Dawn[N]	Germany	EU1
517[PB], 2[AS]	*Rhododendron* sp. var. Roseum Elegans[N]	The Netherlands	EU3
519[PB], 4 [AS]	*Rhododendron* sp. var. Tarantella[N]	The Netherlands	EU1
520[PB], 5[AS]	*V. tinus* var. Eve Price[N]	Scotland	EU1
521[PB], 6[AS]	*V. tinus* var. Eve Price[N]	England	EU4
524[PB], 9[AS]	*V. tinus* var. French White[N]	England	EU1
525[PB], 10[AS]	*V. tinus* var. Eve Price[N]	Netherlands	EU1
528[PB], 13[AS]	*R. catawbiense* var. Grandiflora[N]	England	EU1
532[PB], 18[AS]	*V. bodnantense*[N]	Germany	EU1
533[PB], 19[AS]	*R. catawbiense* var. Grandiflora[N]	Mallorca, Spain	EU1
535[PB], 21[AS]	*V. bodnantense* var. Dawn[N]	England	EU1
536[PB], 22[AS]	*V. tinus* var. Bewleys Variegatus[N]	France	EU1
538[PB], CSL1527[KH]	*V. bodnantense*[N]	Germany	EU1
539[PB], CSL1560[KH]	*V. tinus*[N]	Dorset, England	EU1
540[PB], CSL1604[KH]	*R. catawbiense* var. Grandiflora[N]	Cheshire, England	EU1
541[PB], CSL1612[KH]	*Rhododendron* sp.[N]	Guernsey, UK	EU1
542[PB], CSL1614[KH]	*V. farreri*[N]	Cambridgeshire, England	EU4
543[PB], CSL1622[KH]	*V. bodnantense*[N]	Surrey, England	EU1
544[PB], CSL1623[KH]	*V. plicatum*[N]	North Yorkshire, England	EU1
545[PB], CSL1653[KH]	*Rhododendron* sp.[N]	Staffordshire, England	EU1
546[PB], Phy50[EM]	*V. tinus*[N]	Mallorca, Spain	EU1
547[PB], Phy51[EM]	*R. catawbiense*[N]	Mallorca, Spain	EU1
549[PB], PD20011060[HG]	*Viburnum* sp.[N]	The Netherlands	EU1
550[PB], 4201[KuH]	*Rhododendron* sp.[N]	Oostakker, Belgium	EU1
551[PB], 4467[KuH]	*Rhododendron* sp. var. Ponticum variegatus[N]	Lochristi, Belgium	EU1
552[PB], 1478[KuH]	*Viburnum* sp.[N]	Oosterzele, Belgium	EU1
553[PB], 4295a[KuH]	*V. bodnantense* var. Dawn[N]	Kortemark, Belgium	EU1

Table 1 *Continued*

Isolate number*,†	Host	Origin	Genotype
554[PB], 2 N256[NS]	*Rhododendron* sp. var. Cynthia[N]	Vergetot, France	EU1
555[PB], 2 N386[NS]	*V. bodnantense*[N]	Suce-sur-Erdre, France	EU5
556[PB], 2 N389[NS]	*V. tinus*[N]	Guillan, France	EU3
557[PB], 2 N624[NS]	*Rhododendron* sp.[N]	Lanton, France	EU1
558[PB], 2 N1043[NS]	*Rhododendron* sp.[N]	Breix, France	EU1
559[PB], 2R33/1[NS]	*Rhododendron* sp. var. Yakushimanum[N]	Champenoux, France	EU1
560[PB], V144/2002[CO]	*Rhododendron* sp.[N]	Sweden	EU1
561[PB], V166/2002[CO]	*Rhododendron* sp.[N]	Sweden	EU1
562[PB], CRA2338[DD], BBA26/02[SW], CBS110901	*V. botnantense*[N]	Belgium	EU1
571[PB], CSL2023125[KH]	*R. ericaceae* var. Cheer[N]	North Yorkshire, England	EU4
572 [PB], BBA9/3[SW], CBS101552	Recycling water[N]	Germany	EU1
573[PB], CSL2022739[KH]	*V. tinus* var. Gwenllian[N]	West Sussex, England	EU1
574[PB], CSL2022479[KH]	*V. tinus* var. French White[N]	Worcestershire, England	EU1
577[PB], CSL2022689[KH]	*Rhododendron* sp.[N]	Channel Islands, UK	EU6
580[PB], CSL2022232[KH]	*V. tinus* var. Eve Prince[N]	England	EU1
581[PB], CSL2022290[KH]	*R. catawbiense* var. Grandiflora[N]	England	EU1
584[PB], CSL2022690[KH]	*Rhododendron* sp.[N]	Channel Islands, UK	EU1
585[PB], PD20018722[HG]	*Rhododendron* sp.[N]	the Netherlands	EU1
586[PB]	Nursery mulch[N]	England	EU1
594[PB], CSL2023117[KH]	*R. ponticum* var. Variegatum[N]	East Yorkshire, England	EU1
595[PB], CSL2022193[KH]	*V. bodnantense* var. Dawn[N]	The Netherlands	EU4
596[PB]	Nursery mulch[N]	England	EU1
597[PB], PD20021281[HG]	*Rhododendron* sp.[N]	The Netherlands	EU1
599[PB], CSL2022159[KH]	*V. tinus* var. Gwenllian[N]	England	EU1
629[PB], PD20024319[HG]	*V. bodnantense* var. Dawn[N]	The Netherlands	EU1
630[PB], PD20023659[HG]	*R. catawbiense* var. Grandiflora[N]	The Netherlands	EU1
631[PB], PD20022991[HG]	*Rhododendron* sp. var. Roseum Elegans[N]	The Netherlands	EU1
632[PB], PD20026168[HG]	*V. bodnantense* var. Dawn[N]	The Netherlands	EU1
635[PB], PD20024377[HG]	*V. bodnantense* var. Dawn[N]	The Netherlands	EU1
660[PB], BBA15/01-29d[SW]	*R. ferrugineum* × *hirsutum*[N]	Germany	EU1
675[PB], BBA15/01-5b[SW]	*V. fragans*[N]	Germany	EU1
676[PB], BBA15/01-39[SW]	*V. plicatum*[N]	Germany	EU1
677[PB], BBA23/01[SW]	*V. tinus*[N]	Germany	EU1
678[PB], BBA15/01-8a[SW]	*Viburnum* sp.[N]	Germany	EU1
679[PB], BBA15/01-11a[SW]	*Viburnum* sp.[N]	Germany	EU2
680[PB], BBA15/01-14[SW]	*Viburnum* sp.[N]	Germany	EU7
681[PB], BBA15/01-38a[SW]	*Viburnum* sp.[N]	Germany	EU1
682[PB], BBA19/02[SW]	*Viburnum* sp.[N]	Germany	EU1
690[PB], PD5548[HG]	*Q. rubra*	The Netherlands	EU1
Phytophthora lateralis			
PL27[MG]	*Taxus brevifolia*	Del Norte Co., CA	N/A
PL33[MG]	*Chamaecyparis lawsoniana*	Del Norte Co., CA	N/A
Phytophthora hibernalis			
338[PT], 1894[DR], ATCC56353	*Citrus sinensus*	Australia	N/A
379[PT], 1895[DR], ATCC64708	*Aquilegia vulgaris*	New Zealand	N/A

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†Each isolate was derived from a separate plant, i.e. specific host samples were not processed more than once for isolation.
‡Due to US quarantine regulations, European isolates were collected, grown and extracted in Peter Bonants's laboratory at Plant Research International, Wageningen, the Netherlands.
[N]Indicates *P. ramorum* isolate from nursery.

Table 2 Sequence of 12 primer pairs used to amplify polymorphic loci of *Phytophthora ramorum*

ID	Locus*	Repeat motif†	Primer sequence (5′ to 3′)‡
18	AC0336	$(AC)_{39}$	F: [FAM] TGCCATCACAACACAAATCC R: TGTGCTATCTTTCCTGAACGG
29	AGC0010	$(AGC)_{6}$	F: [HEX] TTCCTGTGTCTACGACTGCG R: TCTGCTGTTCAGTTTGCTGC
33	AT0142	$(AT)_{17}$	F: [HEX] CCAACAATGACCCAGTGGAG R: GATGTCAATTTGAGGGGCAC
63	CT0409	$(CT)_{15}$	F: [FAM] ACACGTACACGTAGGGCTCC R: GCTATTGCAGTGACGTGTGC
64	CT0005	$(CT)_{16}$	F: [FAM] GCGCTAAGAAAGACACTCCG R: CAACATGTAGCCATTGCAGG
65	CT0570	$(CT)_{19}$	F: [HEX] GCAACAACAGCAACAGCATC R: GTTCTTCGACGTGTGTGTGG
79	GT0302	$(GT)_{11}$	F: [FAM] CGTGCGAGAATGAGAGTGG R: TTTCTCCTTCTGCCCTACCC
82	GT0462	$(GT)_{14}$	F: [HEX] CCACGTCATTGGGTGACTTC R: CGTACAAGTCACGACTCCCC
97	TGC0032	$(TGC)_{12}$	F: [FAM] ACGTCTTCTTGGAGTGGTGG R: TCTTGGACTTGGCTGACCTC
104	AC0104	$(AC)_{6}$	F: [HEX] ACAAGGTCCGTTTCGTTGAG R: CAACGAGTTCGATCGGTAGG
278	CT0278	$(CT)_{7}$	F: [FAM] TGGAGAATTTCCTGTCGGAG R: TGAAGGTGCTATCAGGGTCC
562	CT0562	$(CT)_{8}$	F: [FAM] ACGCTCTGCAGTCACCATC R: CCGCACTTCCGTATCTCAGT

*Locus ID created by the database.
†Repeat motif and the number of times repeated in the 4X draft genome sequence of *P. ramorum* isolate Pr102.
‡Fluorophores (FAM or HEX) used for labelling each forward primer are specified within primer sequence.

informative primer pairs were labelled with either FAM or HEX (Table 2), and resulting amplification products were sized by capillary electrophoresis on an automated ABI 3100 using the molecular standard GeneScan-500 ROX and GENESCAN 3.1.2 software (Applied Biosystems). Extracts of two isolates each of the related species *P. lateralis* and *P. hibernalis* (Table 1) were also amplified with the 12 primer pairs to determine if cross-species amplification occurred under the described conditions.

Sequence determination

Amplification of all 12 polymorphic microsatellite loci was performed with unlabelled primers using the amplification protocol above. PCR products were cloned into plasmids and transformed into *Escherichia coli* using the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. Fifteen positive colonies from each reaction were amplified using T7 forward and M13 reverse primers (Sambrook *et al*. 1989), sequenced on an ABI 3100, and aligned using the multi-alignment program SEQUENCHER 4.1.2. Generated sequences of all 12 loci for US isolate Pr218 and EU isolate 500 have been deposited in GenBank (Table 3). The predicted sequence from the genome sequence of isolate Pr102 was aligned as described above and compared with sequences of cloned alleles.

Phylogenetic and multilocus analyses

To determine phylogenetic placement of the newly discovered clade of *P. ramorum*, templates of the ITS and *cox* I gene were sequenced. ITS amplification was performed with primers ITS1 and ITS4 (White *et al*. 1990) as previously described (Bonants *et al*. 1997). The *cox* I and II gene cluster was amplified using primers FM 35 (5′-CAGAACCTTGGC-AATTAGG-3′) and FM 55 (5′-GGCATACCAGCTAAAC-CTAA-3′) as previously described (Martin 2000), yielding fragments over 2 kb. Smaller fragments from the *cox* I gene were sequenced using FM 35 and FM 55 as internal primers. Alignments were produced using SEQUENCHER 4.1.2 and phylogenetic analyses were performed with parsimony, distance or branch and bound methods using the default settings in the software PAUP version 4.0b10 (Swofford 2002). Robustness of the topology of the phylogram was evaluated by bootstrap analyses (1000 replicates) of the data set.

A neighbour-joining (NJ) tree was generated using allele frequencies for each unique multilocus genotype and software programs CONSENSE version 1.0, GENDIST version 1.0,

Table 3 Allele sizes and frequencies of the 12 microsatellite loci analysed in this study, including GenBank Accession nos of allele sequences for US isolate Pr218 and EU isolate 500

Locus 18				Locus 29				Locus 33				Locus 63				Locus 64				Locus 65			
Length*	Length†	Freq.	Gen Bank	Length*	Length†	Freq.	GenBank	Length*	Length†	Freq.	GenBank	Length*	Length†	Freq.	GenBank	Length*	Length†	Freq.	GenBank	Length*	Length†	Freq.	GenBank
218	218	0.282	DQ103275	325	327	0.636	DQ103279	315	316	0.497	DQ103284	155	157	0.218	DQ103287	338	342	0.215	DQ103290	220		0.003	
220	220	0.216	DQ103277	337	342	0.364	DQ103280	323	324	0.282	DQ103283	157		0.282		340		0.003		222		0.003	
222		0.003						325		0.003		159	161	0.003	DQ103285	346	350	0.282	DQ103292	234	234	0.215	DQ103298
264	264	0.276	DQ103278					327		0.003		163	163	0.215	DQ103286	356		0.003		236	235	0.282	DQ103297
266		0.007						337		0.215	DQ103282	165	165	0.282	DQ103289	374	370	0.215	DQ103291	244	244	0.282	DQ103299
272		0.003														388	389	0.007	DQ103293	252	249	0.215	DQ103296
278	278	0.213	DQ103274													392	394	0.262	DQ103294				
																394		0.013					

Locus 79				Locus 82				Locus 97				Locus 104				Locus 278				Locus 562			
Length*	Length†	Freq.	Gen Bank	Length*	Length†	Freq.	GenBank	Length*	Length†	Freq.	GenBank	Length*	Length†	Freq.	GenBank	Length*	Length†	Freq.	GenBank	Length*	Length†	Freq.	GenBank
224	222	0.633	DQ103301	106		0.002		300	303	0.700	DQ103318	220	220	0.216	DQ10331	240	235	0.691	DQ103316	334	336	0.285	DQ103308
254		0.004		108		0.002		312	312	0.300	DQ103310	222	222	0.502	DQ103312	242	238	0.304	DQ103315	338	338	0.215	DQ103319
256	254	0.363	DQ103303	110	109	0.144	DQ103304					226	226	0.282	DQ103313	250				340	342	0.219	DQ103317
				112		0.330														345	350	0.281	DQ103320
				114		0.146																	
				130	127	0.188	DQ103305																
				136		0.007																	
				140		0.179																	
				144		0.002																	

*Size of microsatellite amplicon based on results from GENESCAN Analysis 3.1.2 software.

†Size of microsatellite amplicon based on results from actual sequence analysis. Due to poor size calling by the ROX-500 standard in the size ranges of 200–250 bp, some fragment sizes vary between GENESCAN and sequence analysis.

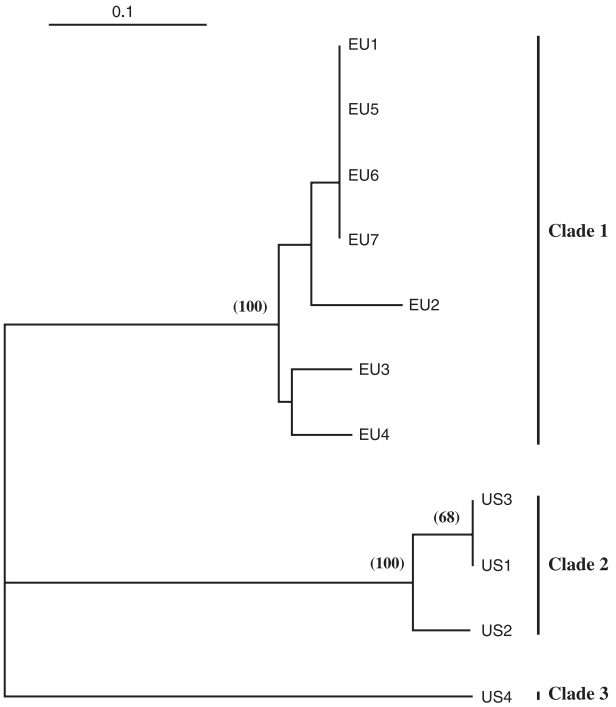

Fig. 1 Neighbour-joining phylogram based on allele frequencies at 10 microsatellite loci inferred from GENESCAN Analysis 3.1.2 software results. Numbers in parentheses are jackknife values over 50, derived from a jackknife consensus tree based on 1000 replications. Genotypes appearing as equal were differentiated from one another by the two excluded loci.

NEIGHBOR version 1.0 and SEQBOOT version 1.0 (Metrowerks) within the Phylogeny Inference Package (PHYLIP). Robustness of NJ branches was inferred from jackknife values from a consensus tree generated after 1000 replications (Fig. 1). SEQBOOT version 1.0 computed genetic distance from a set of gene frequencies in different populations using Cavalli-Sforza's chord measure. Loci 82 and 97 were excluded from analysis due to the presence of multiple alleles (locus 82) and nonamplification of the SSR for the US4 genotype (locus 97).

To test the mode of reproduction of US and EU populations, binary multilocus genotype data sets were analysed for linkage disequilibrium using a modified index of association (I_A) equation in the software program MULTILOCUS version 2.2. (Agapow & Burt 2001). Significance of I_A was determined by randomization (1000 times) procedures by comparing the observed value of I_A to that expected under the null hypothesis of complete panmixis on a clone-corrected data set (Burt *et al*. 1996).

Results

Identification of SSR markers and subsequent PCR

The PRI software identified a total of 1334 unique microsatellite repeats. Dinucleotide repeats were the most common, making up 82% of all repeats, with AG/CT being the most abundant. From these 1334 repeats, 102 loci (13%) were selected to screen for polymorphism in a subset of eight total US and EU isolates. Of the 102 microsatellite primer pairs tested, only 62 (60.8%) consistently produced an amplicon. Polymorphisms within the subset of isolates were recorded for 32 (51.6%) primer sets. Twelve of these polymorphic loci were then selected for genotyping the global collection of *Phytophthora ramorum* isolates.

Genetic analysis of microsatellite loci

Microsatellite alleles were determined for 71 isolates from the US (from 12 California, two Oregon, and one Washington counties), as well as 80 isolates from the EU (from nine countries) (Table 1) at the 12 microsatellite loci using primers labelled with FAM or HEX (Table 2). Only one of 12 primer combinations (104) amplified fragments from *Phytophthora lateralis* and *Phytophthora hibernalis* and in neither case did their length match any of those amplified from *P. ramorum*.

Microsatellite allele sizes from all isolates were scored (Table 4). The number of alleles observed at each locus ranged from two to nine, with the largest number of alleles identified at locus 82. Average heterozygosity across the entire collection of isolates was ~83%. Heterozygosity ranged from a low of 0.44 to a high of 1.0, with observed heterozygosity values higher than expected heterozygosity values at every locus (Table 4). Eleven total multilocus genotypes, five in the US and seven in the EU, were identified among the global collection of *P. ramorum*. Common genotypes were collected over the range of different host species (Table 1) and no correlation was demonstrated between host species and genotype. Five isolates from US nurseries were identical to the most common EU genotype (EU1), indicating that both EU and US genotypes exist in US nurseries. A new clade (Clade 3) of *P. ramorum*, represented by the genotype US4, was identified within a US nursery. Excluding this isolate, two loci were found to be polymorphic among isolates from the US (loci 18 and 82), while three loci were found to be polymorphic within EU isolates (loci 18, 64 and 82). Hence, loci 18, 64 and 82 were the most informative, as they showed variation within US or EU populations.

Sequence analysis of polymorphic loci

Sequence data were generated for all 12 microsatellite loci, including nonrepetitive flanking regions (Table 3). Sequence alignments indicated allelic variation almost exclusively due to variation in microsatellite repeat length. Alignment of flanking regions of cloned PCR products for those loci showing more than two alleles also indicated high levels of sequence homology between clones. For all loci, the number of different cloned PCR fragments ranged between

Table 4 All microsatellite multilocus genotypes of *Phytophthora ramorum* recovered and a summary of alleles amplified with fluorescent SSR primers

		Locus†											
Genotype*	Isolate(s)	18	29	33	63	64	65	79	82	97	104	278	562
US1 (41.72%)	Clone (63 US isolates)	220/278	325/–	315/337	159/165	338/374	234/252`	224/–	110/112/114	300/312	220/222	240/242	338/340
US2 (0.66%)	Pr97	220/272	325/–	315/337	159/165	338/374	234/252	224/–	110/112/114	300/312	220/222	240/242	338/340
US3 (0.66%)	Pr-WA1839^{N}	220/278	325/–	315/337	159/165	338/374	234/252	224/–	114	300/312	220/222	240/242	338/340
US4 (0.66%)	Pr-WA0692^{N}	222/–	–/337	325/327	155/157	340/356	220/222	254/256	106/108/110/112	NA‡	222/–	242/250	334/340
EU1 (49.01%)	Clone (69 EU isolates) Pr-WA1743^{N} Pr-WA1747^{N} Pr-WA1772^{N} Pr-3-74-1^{N} Pr-3-74-2^{N}	218/264	325/337	315/323	155/163	346/392	236/244	224/256	112/130/140	300/–	222/226	240/–	334/345
EU2 (1.32%)	514^{N} 679^{N}	218/266	325/337	315/323	155/163	346/392	236/244	224/256	112/130/136	300/–	222/226	240/–	334/345
EU3 (1.32%)	517^{N} 556^{N}	218/264	325/337	315/323	155/163	346/388	236/244	224/256	112/130/140	300/–	222/226	240/–	334/345
EU4 (2.65%)	521^{N} 542^{N} 571^{N} 595^{N}	218/264	325/337	315/323	155/163	346/394	236/244	224/256	112/130/140	300/–	222/226	240/–	334/345
EU5 (0.66%)	555^{N}	218/264	325/337	315/323	155/163	346/392	236/244	224/256	112/130/144	300/–	222/226	240/–	334/345
EU6 (0.66%)	577^{N}	218/264	325/337	315/323	155/163	346/392	236/244	224/256	114/130/140	300/–	222/226	240/–	334/345
EU7 (0.66%)	680^{N}	218/264	325/337	315/323	155/163	346/392	236/244	224/256	112/130/136	300/–	222/226	240/–	334/345
	Total no. alleles	7	2	5	5	8	6	3	9	2	3	3	4
	H_O	0.99	0.56	1.0	1.0	1.0	1.0	0.57	0.99	0.43	0.99	0.44	1.0
	H_E	0.75	0.46	0.63	0.75	0.76	0.75	0.47	0.78	0.42	0.62	0.43	0.75

*Frequency of each genotype within the global collection of isolates is listed in parentheses.
†Dash (–) indicates loss of an allele or homozygosity with the remaining allele.
‡NA indicates nonamplification of this isolate at this locus with selected microsatellite primers.
^{N}Indicates *P. ramorum* isolate from nursery.

two (loci 29 and 82) and six (locus 65) (Table 3). Sequence analysis of cloned PCR products revealed a range of fragment lengths between 109 and 394 bp (Table 3). Additionally, lengths of nonrepetitive flanking regions ranged from 85 to 384 bp, with 1–8 SNPs between different clones from the same isolate (data not shown). The number of repeat units among all loci ranged from $(AT)_1$ to $(CT)_{76}$. Locus 97 contained interruptions in the microsatellite repeat motif (TGC) (data not shown). In a few instances, allelic size variation was also due to insertions or deletions, with size of insertional elements between 6 and 73 bp (data not shown). Indels in the flanking regions did not affect amplification of any of the microsatellite loci; furthermore indels were never solely responsible for variation in allele size, which was determined by either variation of the repeat sequence or variation in the repeat sequence and presence/absence of indels in the flanking region. Ten of the 12 putative loci were detected in the draft of the genome sequence. Putative loci 33 and 63 could not be found, for unknown reasons. Putative locus 82 was found on three distinct scaffolds, and loci 18 and 84 were found on two scaffolds. Seven predicted sequences perfectly matched sequences of cloned alleles from US forest isolate Pr218. Predicted sequence contained an extra SSR at loci 82 and 278, and one nucleotide insertion plus one extra SSR at locus 97.

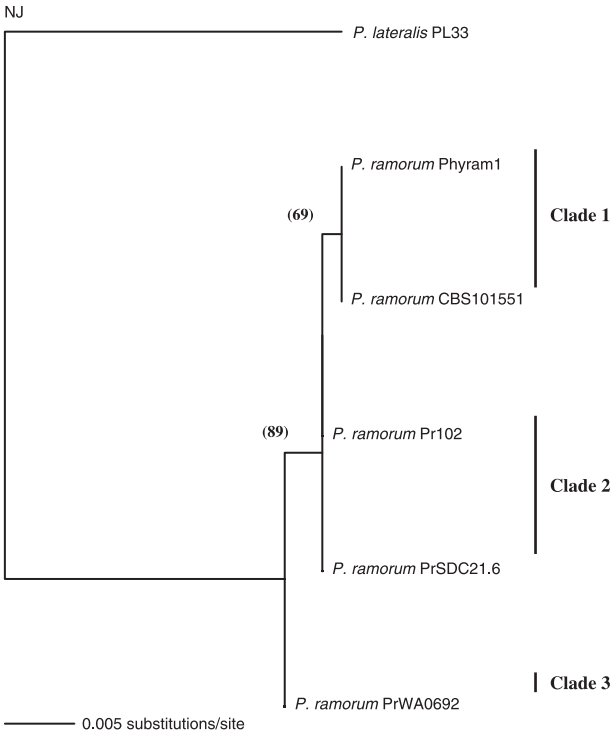

Fig. 2 Neighbour-joining phylogram based on representative *cox* I sequences. Numbers in parentheses are booptstrap support values over 50 derived from a bootstrap consensus tree obtained through 1000 replications.

Phylogenetic and multilocus analysis

The NJ tree, based on microsatellite allelic frequencies, grouped the 11 genotypes into three distinct clades (Fig. 1). Genotype US4 (isolate Pr-WA0692) was placed in a third clade. EU and US clades were differentiated from this third clade with strong (100) jackknife support. Although some isolates appear as identical in the NJ tree, they were not collapsed into common genotypes because their diversity was determined by one of the two loci excluded from the analysis. Clade 1 included seven genotypes found both in EU and US nurseries while Clade 2 included three genotypes found in US wildlands and nurseries. Only one isolate belonging to Clade 3 was included in this study, hence diversity within this clade needs to be addressed by further studies.

All *P. ramorum* isolates shared identical ITS sequences; however, nucleotide variation was observed between US, EU and Pr-WA0692 isolates in the *cox* I region. Parsimony, neighbour-joining, and branch and bound analyses of *cox* I sequences indicated the unique isolate Pr-WA0692 as representing a different mitochondrial clade, ancestral to both EU and US clades, however, more closely related to the US type (Fig. 2). Positioning of this isolate as a separate ancestral clade was supported by bootstrap values of 83, 89 and 88 for the three analyses, respectively. Representative *cox* I sequences were submitted to GenBank and their accession numbers and synonym labels are as follows: PL33 = DQ117981, Phyram1(Pr509) = DQ117982, CBS101551(Pr218) = DQ117983, PrSDC21(Pr233) = DQ117984, Pr102 = DQ117985, PrWA0692 = DQ117986.

Binary data sets of each multilocus genotype observed within the two different populations (US and EU), as well as the entire isolate collection, were analysed to determine the role played by sexual recombination in shaping the population structure of the samples included in this study. The index of association, I_A, has an expected value of zero if there is no association of alleles at unlinked loci as assumed in a randomly mating population; therefore, high within-population levels of linkage disequilibria are expected in largely clonal species. At the species level, the observed I_A value ($I_A = 17.15$) for all *P. ramorum* isolates was significantly higher ($P \leq 0.01$) than the I_A calculated from 1000 artificially recombined data sets. When populations were considered separately, the observed I_A across loci in the US population ($I_A = 28.82$) was significantly higher ($P \leq 0.01$) than the I_A calculated from 1000 artificially recombined data sets. Such results do not confirm recombination events among the global collection of isolates or within the US population. In comparison, the observed I_A across loci in the EU population ($I_A = -0.14$) fell within the distribution for randomized data sets, indicating no significant correlation ($P = 0.53$) of alleles across

loci. Hence, the null hypothesis of recombination within the EU population could not be rejected.

Discussion

Analyses of genetic variation among isolates of *Phytophthora ramorum* using allelic frequencies at 12 microsatellite loci indicate significant variation between continents, but demonstrate limited variation within both US and EU populations. Genotypic diversity using SSR was more limited than using AFLPs (Ivors *et al*. 2004); however, in both studies, all US forest genotypes are extremely closely related, and higher resolution of genotypes with AFLPs is likely to be due to the much larger number of loci analysed with that method. Most of the variation among isolates within each population was due to SSR differences detected only at 1–3 loci. No correlation was demonstrated between host and genotype, supporting the theory of lack of host specificity, which has also been suggested by previous AFLP (Ivors *et al*. 2004) and inoculation studies (Garbelotto *et al*. 2003). Multilocus analysis rejected the possibility of recombination within wild populations in North America, indicating clonal pathogen reproduction in forests. Rare and very closely related genotypes detected in this study, as well as in a previous study (Ivors *et al*. 2004), are hypothesized to be the result of mitotic or somatic recombination. This mechanism has been shown to be significant in populations of the potato pathogen *Phytophthora infestans* (Goodwin *et al*. 1994), and phenotypic variation identified within clonal lineages of *Phytophthora cinnamomi* have also been explained by mitotic recombination (Dobrowolski *et al*. 2003). Higher than expected heterozygosity across all loci (Table 4) is also consistent with rare outcrossing events among distantly related lineages bearing different mating alleles, followed by significant clonal reproduction, as reported for other *Phytophthora* species (Förster *et al*. 1994).

The data presented in this study indicate a single genotype introduction is responsible for virtually the entire studied forest population, a second genotype in the wild (US2) is closely related to the common one (US1) and found only once. The extremely narrow genetic structure of *P. ramorum* supports the exotic nature of this pathogen in wildlands. Although diversity in the European nursery population is slightly higher, this population structure is most easily explained by the introduction of a few closely related genotypes followed by the creation of new genotypes via mitotic recombination and/or mutation. However, EU isolate 562, from *Viburnum bodnantense* in Belgium, was identified as having the A2 mating type and belonging to the common EU1 multilocus genotype, as previously suggested (Ivors *et al*. 2004; Kroon *et al*. 2004). Although reasons for the rare occurrence of mating type A2 within a population consistently characterized as mating type A1 are unclear, the presence of both mating types in Europe and potential sexual reproduction among closely related individuals may provide an alternative explanation of the genetic structure observed in the European population.

The discovery of the novel genotype US4 in a Washington nursery demonstrates broader genetic diversity than previously reported for this species. SSR analysis of Pr-WA0692 revealed the presence of alleles normally detected in either US (at two loci) or EU populations (at two loci), as well as unique alleles at the other eight loci. The US4 genotype was positioned in a third distinct clade. Such placement is corroborated by the presence of new alleles at many of the loci analysed, and especially by the unique mitochondrial *cox* I sequence of this isolate. It should be noted that although only two isolates per clade were used to build the phylogram, no variation exists among individuals belonging to each clade (Kroon *et al*. 2004). Sequence analyses of the mitochondrial *cox* I region placed US4 ancestral to both the EU (Clade 1) and US (Clade 2) clades. The presence of both EU and US alleles in this isolate can also be explained by ancestral placement of this clade. Interestingly, US4 appears to have a unique combination of phenotypic traits when compared to isolates from the other two clades. The combination of microsatellite, sequence and phenotypic data suggests the three clades may represent three distinct lineages (Table 5).

This discovery of unique alleles at all loci within a single genotype (US4) increases our understanding of the range

Table 5 Distinctive characteristics of the three proposed lineages of *Phytophthora ramorum*, based on data presented in this study and other unpublished observations. Characters differentiating lineages 1 and 2 are documented by several published studies (see text)

Lineage	Provenance	Microsatellite profile	Mitochondrial *cox* I sequence	Growth rate*	Colony type†	Mating type‡
1	EU and US nurseries	Clade 1	Unique (EU)	Fast	Aerial	A1
2	US forests and nurseries	Clade 2	Unique (US)	Slow	Appressed	A2
3	US nurseries	Clade 3	Unique (WA)	Fast	Aerial	A2

*Growth rate on V8 agar *sensu* Brasier (2003).
†Mycelial growth habit on V8 agar at room temperature.
‡A single A2 European nursery isolate (562) is included in lineage 1.

of allelic variation within *P. ramorum* and suggests the genetic structure of current US and EU populations is the result of a strong founder effect and subsequent genetic bottleneck. We believe the EU nursery and the US forest genotypes represent two introduced individuals and their clonal descendants, rather than true populations. Taking into consideration the fact that an unusually large number of heterozygous sites (approximately 200 000) have been identified within the complete genome sequence of *P. ramorum* (Brett Tyler, personal communication), *P. ramorum* should be genetically diverse and sexually active in its place of origin, similar to other *Phytophthora* species like *Phytophthora infestans* (Tooley *et al*. 1985). Based on this interpretation, *P. ramorum* should be viewed as a sexually recombining species in nature, with the currently known genetic structure deeply affected by introduction events and abundant clonal reproduction. The lack of multiple alleles at all analysed loci refutes the hypothesis that *P. ramorum* is a hybrid among different species. Although the presence of more than two alleles at one of the loci could be the result of trisomy, gene duplication or introgression of genes from other *Phytophthora* species, the high homology of flanking regions and the presence of the same amplicon sequence on two scaffolds suggest gene duplication and trisomy, rather than horizontal gene transfer. Multiple alleles and trisomy have been documented in other *Phytophthora* species (Dobrowolski *et al*. 2002; Ospina-Giraldo & Jones 2003; Van der Lee *et al*. 2004).

Most of the microsatellite allelic variation was detected in nursery populations. Ten of 11 *P. ramorum* genotypes were recovered from nurseries while only two closely related genotypes were found in the wild throughout California and Oregon. Higher genotypic diversity in nurseries could be explained by the repeated exchange of pathogen genotypes through the trade of infected plant material, by strong selection pressure selecting new genotypes created through mitotic recombination or mutation, or from both mechanisms. Cultural practices and chemical treatments may be partially responsible for such selection pressure in nurseries. On the other hand, the presence of two and three lineages within the collective populations from Oregon and Washington nurseries, respectively, is unequivocally the result of multiple pathogen introductions presumably through commercial trade of infected plants. The potential role of plant trade in the creation of an 'artificial' panmictic population at the continental level is highlighted by (ii) the observation that rare genotypes were found more than once within Europe, particularly in the UK, where the EU4 genotype was found multiple times in different regions, and (ii) the detection of an EU genotype within Oregon and Washington nurseries.

Recently, we have discovered several different genotypes belonging to third clade of *P. ramorum* reported in this study, indicating their significant presence in US nurseries. These isolates were found in or could be traced back to nurseries in the state of Washington, suggesting valuable information about the origin of this pathogen could be learned by taking a closer look at the provenance of host plants and trade routes of nurseries within this state.

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