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AFLPs detect low genetic diversity for *Phytophthora nemorosa* and *P. pseudosyringae* in the US and Europe

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ABSTRACT

In California and Oregon, two recently described oomycete forest pathogens, *Phytophthora nemorosa* and *P. pseudosyringae*, overlap in their host and geographic ranges with the virulent *P. ramorum*, causal agent of "sudden oak death." Epidemiological observations, namely broader geographic distribution and lack of landscape-level mortality, led to the hypothesis they are native to this region, whereas multiple lines of evidence indicate *P. ramorum* is exotic to North America. We used AFLP analysis to measure genetic variability in the homothallic *P. nemorosa* and *P. pseudosyringae* and to evaluate the hypothesis of endemism. We analysed 39 *P. nemorosa* and 48 *P. pseudosyringae* isolates (29 American and 19 European) from throughout their geographic and host ranges. In the US, both *P. nemorosa* and *P. pseudosyringae* have a dominant AFLP clone with several closely related variants. There is no evidence that genetic diversity is partitioned by host or location in *P. nemorosa*, but the US *P. pseudosyringae* clonal lineage is largely nested within a more genetically variable European group. Though the absence of highly variable sampled source populations does not allow us to determine whether each species is native or introduced in the western US with certainty, the results are most consistent with the hypothesis that both are introduced — *P. pseudosyringae* perhaps from Europe. Invasive *Phytophthora* species are increasingly being implicated in emergent forest diseases, highlighting the need to identify and characterize both native and previously unknown introduced forest *Phytophthoras*.

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Introduction

Although biotic surveys of ecosystems damaged by invasive pathogens often uncover previously undescribed microbes, the shortage of comprehensive baseline data on endemic microbial community composition makes it extremely challenging to determine whether newly identified microbes are native or introduced (Desprez-Loustau *et al.* 2007). The limited knowledge of the impact of invasive microbes on endemic microbes highlights the importance of recognizing and monitoring previously unknown native species (Rizzo 2005). Conversely, it is important to identify invasive microbes that cause less visible symptoms and might otherwise go undetected in order to interrupt unknown pathways of introduction.

Long known in agriculture for causing destructive crop diseases, such as the Irish potato blight, invasive oomycetes of

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the genus *Phytophthora* are receiving increasing attention worldwide as emergent forest pathogens (Erwin & Ribeiro 1996; Hansen 2008). *P. cinnamomi* is destroying Australian jarrah forest and many other forest and crop trees internationally (reviewed in Hardham 2005); *P. ramorum* causes widespread mortality of oaks and tanoaks in California and Oregon; *P. lateralis* causes a lethal disease of American Port Orford cedars; the *P. alni* species complex is responsible for the dieback of European alders; and multiple *Phytophthora* species are contributing to European oak decline, European beech dieback and to outbreaks of chestnut ink disease (Balci & Halmschlager 2003; Biocca *et al.* 1994; Brasier *et al.* 2004; Gibbs *et al.* 2003; Hansen *et al.* 2000; Jönsson *et al.* 2005; Jung 2006; Jung & Blaschke 2004; Jung *et al.* 1999; Jung *et al.* 2000; Rizzo *et al.* 2002; Tucker & Milbrath 1942; Vettraino *et al.* 2001; Vettraino *et al.* 2005). The causal agents of most of these diseases are hypothesized to be introduced, but confirmation has been provided in only a few cases, as locating microbial source populations is difficult and information on endemic *Phytophthoras* is sparse. Often, limited genetic and phenotypic variability among populations of these emergent pathogens is presumed to be the result of a recent bottleneck and used as evidence to support their exotic origin (Cooke *et al.* 2005; Hansen *et al.* 2000; Ivors *et al.* 2004; Zentmyer 1988). Environmental surveys in conjunction with these emerging diseases have isolated many previously unidentified forest *Phytophthora* species and, due to the scarcity of baseline data, it is often extremely difficult to determine whether these too are native or introduced (Balci *et al.* 2007; Belbahri *et al.* 2006; Brasier *et al.* 2003; Brasier *et al.* 2004; Brasier *et al.* 2005; Cooke *et al.* 2007; Davidson *et al.* 2002; Hansen & Delatour 1999; Jung *et al.* 1999; Jung *et al.* 2000; Jung *et al.* 2002; Jung & Nechwatal 2008; Reeser *et al.* 2007; Vettraino *et al.* 2002).

P. nemorosa and *P. pseudosyringae* were first identified in the western US while sampling for *P. ramorum,* the causal agent of a complex of plant diseases including 'sudden oak death', and the two were initially grouped under the name '*P. ilicis*-like' (Davidson *et al.* 2002; Hansen *et al.* 2003; Murphy & Rizzo 2006; Rizzo *et al.* 2002; Rizzo *et al.* 2005; Werres *et al.* 2001; Wickland *et al.* 2008). Molecular phylogenetic analyses from a variety of nuclear and mitochondrial loci show the homothallic *P. nemorosa* and *P. pseudosyringae* are closely related but distinct taxa situated in a clade with *P. ilicis* and *P. psychrophila;* both are rather distantly related to *P. ramorum* (Blair *et al.* 2008; Ivors *et al.* 2004; Martin & Tooley 2003; Schena & Cooke 2006). All three of these species can be aerially dispersed and have overlapping host and geographic ranges, and although they appear to have different environmental limits, combinations of the three species can be found on the same sites and even on the same naturally infected host (Hansen *et al.* 2003; Jensen *et al.* 2006; Murphy & Rizzo 2006; Wickland & Rizzo 2006; Wickland *et al.* 2008). Both *P. nemorosa* and *P. pseudosyringae* alone cause symptoms similar to those of *P. ramorum* on shared hosts, including foliar lesions of California bay laurel trees, *Umbellularia californica* (Hook. & Arn.) Nutt., which have been shown to be key hosts in *P. ramorum* sporulation and disease spread (Davidson *et al.* 2005; Hansen *et al.* 2003; Jensen *et al.* 2006; Reeser *et al.* 2008; Wickland *et al.* 2008). However, mortality associated with *P. nemorosa* and *P. pseudosyringae* appears much more limited than the landscape-scale mortality due to *P. ramorum* (Hansen *et al.* 2003; Wickland & Rizzo 2006). Outside of nurseries, North American *P. ramorum* is currently confined to counties along the central California coast and a single location in Oregon, but the distributions of both *P. nemorosa* and *P. pseudosyringae* in this region are wider. The range of *P. pseudosyringae* begins farther south in San Luis Obispo County, and both species are isolated continuously from Monterey County in California through southern Oregon and disjunctly from interior sites in California's Sierra Nevada Mountains (Wickland *et al.* 2008). *P. nemorosa* is currently only known to occur in California and Oregon, but *P. pseudosyringae* has also been isolated from streams in the eastern US, and it is associated with declining oaks, beeches, and alders in Europe, where it has been described as a root and stem pathogen (Diana *et al.* 2006; Hwang *et al.* 2007; Jung *et al.* 2003). When compared with *P. ramorum*, the broader geographic distribution and reduced virulence of *P. nemorosa* and *P. pseudosyringae* led to the hypothesis that they were native to the western US (Garbelotto & Rizzo 2005; Hansen *et al.* 2003).

The aim of this study is to provide an assessment of the genetic structure of *P. nemorosa* from western North America and of *P. pseudosyringae* from western North America and Europe using AFLP genetic markers. Analysis of genetic diversity and the relationship between genetic and spatial data should assist in addressing whether these two recently described forest *Phytophthora* species are native to the western US. This study will also provide one of the first analyses of the genetic structure of homothallic *Phytophthoras* in forest ecosystems [see Cooke *et al.* (2005) for the first such study].

Materials and methods

Isolates and DNA extraction

Thirty-nine *Phytophthora nemorosa* isolates from California and Oregon and 48 *P. pseudosyringae* isolates from California, Germany, and Italy were analysed. All isolates were collected between 1997 and 2004. Although most North American isolates were collected along a latitudinal gradient in forests of the Pacific Coast mountain range, one *P. nemorosa* and two *P. pseudosyringae* isolates came from forests in Mariposa County, on the slopes of the Sierra Nevada mountain range. The two mountain ranges are separated by a significant extent of lowlands, currently and historically characterized by vegetation assemblages distinct from those found in the mountains. The known US geographic range for these two *Phytophthora* species is thus constituted by two disjunct areas, each represented by one of the two mountain ranges sampled in this study. The selected isolates represented most of the known host and geographic ranges at the time the study was conducted, though expanded ranges for both have subsequently been identified. *Phytophthora nemorosa* has currently only been isolated in California and Oregon, while *P. pseudosyringae* is also present in Europe; hence, 19 *P. pseudosyringae* isolates from four sites in Europe were also included in this study for comparison. One isolate of the closely related *P. ilicis* was included as an outgroup. Isolate details are listed in Table 1. *P. nemorosa* and *P. pseudosyringae* samples were treated together and randomized irrespective of species during the extraction and AFLP processes.

Table 1 – *Phytophthora* isolates used in this study

Isolate identification[a]	Host	Collection location	Collector[b]	Collection date
Phytophthora ilicis				
4175A	*Ilex aquifolium*	Azalea park, Brookings, Curry Co., OR	*Everett Hansen*	22 Jan 2002
P. nemorosa				
1050	*Lithocarpus densiflora*	Curry Co. OR	*Everett Hansen*	31 Jul 2002
2052.2	*L. densiflora*	Curry Co. OR	*Everett Hansen*	10 Oct 2002
2055.2	*L. densiflora*	Curry Co. OR	*Everett Hansen*	11 Oct 2001
2059.2	*L. densiflora*	Curry Co. OR	*Everett Hansen*	18 Oct 2001
2059.4	*L. densiflora*	Curry Co. OR	*Everett Hansen*	18 Oct 2001
2134.1	*L. densiflora*	Curry Co. OR	*Everett Hansen*	30 Apr 2002
2140.1	*L. densiflora*	Curry Co. OR	*Everett Hansen*	1 May 2002
2140.2	*L. densiflora*	Curry Co. OR	*Everett Hansen*	1 May 2002
2146.1	*L. densiflora*	Curry Co. OR	*Everett Hansen*	1 May 2003
2155.3	*L. densiflora*	Curry Co. OR	*Everett Hansen*	15 May 2002
2156.1	*Umbellularia californica*	Curry Co. OR	*Everett Hansen*	15 May 2002
2162.1	*L. densiflora*	Curry Co. OR	*Everett Hansen*	20 May 2002
2201	*L. densiflora*	Curry Co. OR	*Everett Hansen*	14 Aug 2002
2204	*L. densiflora*	Curry Co. OR	*Everett Hansen*	28 Aug 2002
2500	*L. densiflora*	Curry Co. OR	*Everett Hansen*	27 Jul 2002
2501	*L. densiflora*	Curry Co. OR	*Everett Hansen*	27 Jul 2002
2502	*L. densiflora*	Curry Co. OR	*Everett Hansen*	27 Jul 2003
2510	*L. densiflora*	Curry Co. OR	*Everett Hansen*	1 Aug 2002
2512	*L. densiflora*	Curry Co. OR	*Everett Hansen*	1 Aug 2002
4083.1	*L. densiflora*	Curry Co. OR	*Everett Hansen*	6 Aug 2001
5104	*U. californica*	Curry Co. OR	*Everett Hansen*	10 Dec 2001
HC T4 B14	*U. californica*	Henry Cowell SP, Santa Cruz Co., CA	*Patricia Maloney*	1 Mar 2002
HC T7 B23	*U. californica*	Henry Cowell SP, Santa Cruz Co., CA	*Patricia Maloney*	1 Mar 2002
MW T5 B71	*U. californica*	Muir Woods, Marin Co., CA	*Patricia Maloney*	1 Apr 2002
P-7[c]	*L. densiflorus*	Stewart Point Road, Sonoma Co., CA	D.M.R.	8 Nov 2000
P-13[d]	*L. densiflorus*	Panther Gap, Humboldt Co., CA	D.M.R.	13 Dec 2000
P-16[e]	*U. californica*	Mt. Madonna, Santa Clara Co., CA	D.M.R.	22 Aug 2001
P-37	*L. densiflorus*	Fickle Hill, Eureka, Humboldt Co., CA	D.M.R.	24 Oct 2001
P-38	*L. densiflorus*	Fickle Hill, Eureka, Humboldt Co., CA	D.M.R.	6 Nov 2001
P-44	*U. californica*	Rockefeller Forest, Humboldt Co., CA	D.M.R.	12 Dec 2001
P-51	*U. californica*	Tilden, Contra Costa Co., CA	D.M.R.	4 Mar 2002
P-52	*U. californica*	Big Creek Reserve, Monterey Co., CA	D.M.R.	19 Mar 2002
P-59[f]	*U. californica*	Fickle Hill, Humboldt Co., CA	D.M.R.	8 May 2002
P-61	*U. californica*	Muir Woods, Marin Co., CA	D.M.R.	15 Apr 2002
P-91	*U. californica*	Yosemite Valley, Mariposa Co., CA	D.M.R.	20 Jun 2002
P-106	*U. californica*	Samuel P. Taylor, Marin Co., CA	D.M.R.	17 Jan 2002
P-113	*U. californica*	Samuel P. Taylor, Marin Co., CA	D.M.R.	30 Jul 2002
P-114	*U. californica*	Samuel P. Taylor, Marin Co., CA	D.M.R.	30 Jul 2002
P-115	*U. californica*	Samuel P. Taylor, Marin Co., CA	D.M.R.	30 Jul 2002
P. pseudosyringae				
P-39	*Quercus agrifolia*	Wildcat Canyon, Contra Costa Co., CA	D.M.R.	30 Nov 2001
P-40	*Q. agrifolia*	Wildcat Canyon, Contra Costa Co., CA	D.M.R.	30 Nov 2001
P-41	*U. californica*	Wildcat Canyon, Contra Costa Co., CA	D.M.R.	30 Nov 2001
P-45	*U. californica*	Hoog Park, Novato, Marin Co., CA	D.M.R.	6 Feb 2002
P-50	*U. californica*	Franklin Canyon Road, Contra Costa Co., CA	D.M.R.	26 Feb 2002
P-54	*Q. agrifolia*	Santa Lucia Preserve, Monterey Co., CA	D.M.R.	16 Apr 2002
P-55	*Q. agrifolia*	Santa Lucia Preserve, Monterey Co., CA	D.M.R.	16 Apr 2002
P-56	*Q. agrifolia*	Santa Lucia Preserve, Monterey Co., CA	D.M.R.	16 Apr 2002
P-62	*U. californica*	Meyers Grade, Sonoma Co., CA	D.M.R.	29 May 2002
P-69	*Q. agrifolia*	Santa Lucia Preserve, Monterey Co., CA	D.M.R.	11 Jun 2002
P-70	*Q. agrifolia*	Santa Lucia Preserve, Monterey Co., CA	D.M.R.	11 Jun 2002
P-72	*Q. agrifolia*	Santa Lucia Preserve, Monterey Co., CA	D.M.R.	11 Jun 2002
P-73	*Q. agrifolia*	Santa Lucia Preserve, Monterey Co., CA	D.M.R.	11 Jun 2002
P-76[g]	*U. californica*	Humboldt Co., CA	D.M.R.	2 Jun 2002
P-80	*U. californica*	Warm Spring Dam, Napa Co., CA	*Cheryl Blomquist*	16 Jun 2002
P-81	*U. californica*	City of Napa, Napa Co., CA	*Cheryl Blomquist*	16 Jun 2002
P-83[h]	*U. californica*	Napa Co., CA	*Cheryl Blomquist*	16 Jun 2002
P-84	*U. californica*	Calistoga, Napa Co., CA	*Cheryl Blomquist*	16 Jun 2002
P-85	*U. californica*	Yountville, Napa Co., CA	*Cheryl Blomquist*	16 Jun 2002

Table 1 – (*continued*)

Isolate identification[a]	Host	Collection location	Collector[b]	Collection date
P-86	*U. californica*	Santa Rosa, Sonoma Co., CA	*Cheryl Blomquist*	16 Jun 2002
P-87	*U. californica*	Bothe State Park, Napa Co., CA	*Cheryl Blomquist*	16 Jun 2002
P-88	*U. californica*	Elkhorn Slough, Monterey Co., CA	*Cheryl Blomquist*	16 Jun 2002
P-90	*U. californica*	Yosemite Valley, Mariposa Co., CA	*D.M.R.*	20 Jun 2002
P-92[i]	*U. californica*	Yosemite Valley, Mariposa Co., CA	*D.M.R.*	20 Jun 2002
P-93	*U. californica*	Lafayette, Contra Costa Co., CA	*Cheryl Blomquist*	16 Jun 2002
P-94	*U. californica*	Lafayette, Contra Costa Co., CA	*Cheryl Blomquist*	16 Jun 2002
P-96	*U. californica*	Wildcat Canyon, Contra Costa Co., CA	*Cheryl Blomquist*	16 Jun 2002
P-97	*U. californica*	Wildcat Canyon, Contra Costa Co., CA	*Cheryl Blomquist*	16 Jun 2002
P-118	*U. californica*	Templeton, San Luis Obispo Co., CA	*D.M.R.*	29 Aug 2002
Bu97-15	*Fagus sylvatica* (bark)	Göttingen, Germany	*Günter Hartmann*	winter 1997
PSEU3	*Q. robur*	rhizosphere soil near Gerolzhofen, Northern Bavaria, Germany	*Thomas Jung*	Nov 1997
FC 1A	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Aug 2003
FC 1B	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Aug 2003
FC 1C	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Aug 2003
FC 1D	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Aug 2003
FC 1F	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Aug 2003
FC 2C	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Nov 2004
FC 2D	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Nov 2004
FC 2E	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Nov 2004
FC 2F	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Nov 2004
FC NN A	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Nov 2004
FC NN B	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Nov 2004
FC NN C	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Nov 2004
FC NN D	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Nov 2004
Faggio 1[j]	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Nov 2004
Faggio 2	*F. sylvatica* (bark)	Abruzzo National Park, Italy	S.O.C.	Nov 2004
ML1[k]	*F. sylvatica* (bark)	Lazio (Latium), Italy	S.O.C.	2001
ML2	*F. sylvatica* (bark)	Lazio (Latium), Italy	S.O.C.	2001

a All isolate identifiers are those of the original collectors, to the best of our knowledge.
b Collectors: Cheryl Blomquist, California Department of Food & Agriculture; Everett Hansen, Oregon State University; Günter Hartmann, Lower Saxony Forest Research Station; Thomas Jung, Phytophthora Research and Consultancy; Patricia Maloney, UC Davis.
c Deposited in the World Phytophthora Genetic Resource Collection Database at UC Riverside (WPGRCD), accession number: P10288.
d Species type culture. Deposited in the Oregon State University Mycological Herbarium, OSC # 104381 and ATCC Accession # MYA-2948.
e Deposited WPGRCD, accession number: P10289.
f Deposited WPGRCD, accession number: P16352.
g Deposited WPGRCD, accession number: P16353.
h Deposited WPGRCD, accession number: P16354.
i Deposited WPGRCD, accession number: P16355.
j Deposited in the International Mycological Institute (IMI), accession number: IMI 391716.
k Deposited IMI, accession number: IMI 390500.

Isolates were grown in pea broth (120 g peas l^{-1}) and incubated as stationary cultures for 7–10 d at 16 °C. Total genomic DNA was extracted from 10–20 mg freeze-dried mycelium using the Puregene DNA extraction kit (Gentra, Minneapolis, MN), following manufacturer's instructions for fungal DNA extraction. DNA extracts were stored at −20 °C.

AFLP analysis

AFLPs are informative molecular markers to address a study system and study questions like these (Bonin *et al.* 2007). AFLP reactions were performed as in Vos *et al.* (1995) using the AFLP core reagent kit (Invitrogen, Carlsbad, CA) with modifications and thermocycler protocols described in Ivors *et al.* (2004). Ligation products were diluted 1:10 before primary amplification. A total of 18 selective-base primer pairs were screened for each species, and six informative primer pairs for *P. nemorosa* and four for *P. pseudosyringae* were chosen, respectively. Ten isolates of each species were run in duplicate during the screening process to ensure reproducibility of selected markers. Selective-base primer pairs chosen for *P. nemorosa* were: E00-GC/M00-A, E00-GC/M00-G, E00-GC/M00-TG, E00-TA/M00-AG, E00-TA/M00-CC, and E00-TA/M00-TG. Primer pairs for *P. pseudosyringae* were: E00-AC/M00-AC, E00-GC/M00-A, E00-GC/M00-G, and E00-GC/M00-AC. As there was only partial overlap between the informative primer pairs for each species, two isolates of *P. nemorosa* were included in the analysis of the full *P. pseudosyringae* dataset and two *P. pseudosyringae* isolates were included in the full *P. nemorosa* analysis. AFLP reaction products were sized on an ABI 3100 genetic analyser using the GeneScan-500 (ROX) molecular weight standard (ABI, Foster City, CA). Fragment profiles were analysed with GeneScan software (v. 3.1.2; ABI), and only unambiguous fragments over a threshold intensity of 100 were scored. All bands

were confirmed by a visual check, and a binary data matrix of presence (1) or absence (0) of bands of each size by isolate was generated. The AFLP procedure was replicated from the initial restriction digest step for all study strains.

In order to assess the degree of genetic similarity between all isolates, a pairwise distance matrix for each species was generated using the Jaccard coefficient of similarity (S_J) (Jaccard 1908), as calculated in LE PROGICIEL R (v.4; Casgrain & Legendre 1999). This coefficient measures the proportion of shared AFLP markers between each pair of isolates while correcting for the dominance of the AFLP data by disregarding shared absence of bands. Distance dendrograms for *P. nemorosa* and *P. pseudosyringae* were then constructed using the FITCH program (Fitch & Margoliash 1967) in PHYLIP (v. 3.67; Felsenstein 2005). *P. ilicis* was the outgroup for each dendrogram. For each species, we used the binary matrix as input and generated 1 K bootstrapped Jaccard distance matrices in PHYLTOOLS (http://www.spg.wau.nl/pv/pub/pt). Distance trees were calculated for all of these matrices using FITCH with the multiple datasets option, and BS consensus values were calculated in the PHYLIP program CONSENSE.

To further investigate intercontinental genetic structure of *P. pseudosyringae* we performed AMOVA (Excoffier *et al.* 1992) using the method of Peakall & Smouse implemented in GENALEX version 6.1 (Peakall & Smouse 2006). Each continent was designated a population. Significance of φ_{ST} was tested with 999 permutations.

Results

Results from both AFLP replications were substantively the same, so only one set of results is presented here.

Phytophthora nemorosa

A total of 251 AFLP bands were scored for *Phytophthora nemorosa*, 19 of which (7.6 %) were polymorphic, and a total of 11 distinct AFLP genotypes among the 38 *P. nemorosa* isolates were identified. Nine of the 11 genotypes were each represented by a single isolate. There was a high degree of genetic similarity among the *P. nemorosa* isolates, with pairwise S_J values ranging from 0.963–1 (0.993 ± 0.00864; mean $\pm$ S.D.) with the largest distance being between the Mariposa County isolate, P-91, and isolate HC T7 B23. A value of 1 indicates 100 % similarity. A frequency histogram of the pairwise S_J data distribution is shown in Fig 1A. Most isolate pairs were 100 % similar. The largest AFLP clone incorporated 28 of the *P. nemorosa* isolates (Fig 2A). The geographically isolated individual from Mariposa County, CA, appears on a very short, basal branch of the dendrogram unsupported by BS analysis, but the branching pattern provides no other evidence that genetic variation is partitioned by source host or by area of origin.

Phytophthora pseudosyringae

A total of 201 AFLP bands were scored for *Phytophthora pseudosyringae*, 37 of which (27.5 %) were polymorphic, and 17 distinct AFLP genotypes among the 48 *P. pseudosyringae* isolates were identified, including eight US genotypes and nine from Europe. No individual genotypes were shared between the US and Europe. Eleven of the 17 genotypes were each represented by a single isolate, while 69 % of the US and 47.4 % of the European genotypes were part of the largest AFLP clone for each respective region. The range of S_J values for the whole *P. pseudosyringae* dataset was 0.912–1 (0.98 ± 0.0166; mean $\pm$ S.D.; Fig 1B). When the data for the two regions are separated, the range of S_J values for pairwise comparisons between US isolates alone was 0.958–1 (0.991 ± 0.0104; mean $\pm$ S.D.) with the largest distance separating isolate P-94 from P-80 and P-83. The range between European isolates alone was 0.912–1 (0.978 ± 0.0203; mean $\pm$ S.D.) with the largest distance between the German isolate, Bu97-15, and the Italian FC 2D (Fig 3). The S_J distribution for the European isolates has fewer clones and a higher amount of genetic dissimilarity when compared with that for the US isolates. In fact, more genetic diversity was observed at a single site in the Italian Abruzzo National Park than throughout the entire US sample set, which covered a range of several hundreds of kilometers. Although no genotypes were shared between the US and Europe, the distance dendrogram for the *P. pseudosyringae* data (Fig 2B) does not show the isolates to be distributed in two separate clades, one for Europe and one for the US. Rather, the US isolates are largely contained in a subclade within the European isolates weakly supported by a BS value of 67, with a few US individuals appearing outside of this subclade. Each of the two Mariposa County isolates has a unique AFLP genotype, though located on very short branches unsupported by BS analysis. In contrast, the European population shows some signs of genetic structure driven by location, as shown by the clustering of two isolates from Latium (Italy) in a supported clade distinct from other European sites. AMOVA yielded a φ_{ST} value of 0.452 ($P < 0.001$): about 55 % of genetic variation was contained within continental populations and 45 % between US and European populations.

Discussion

In this assessment of the genetic structure and diversity of *Phytophthora nemorosa* and *P. pseudosyringae*, results showed extremely high levels of within-species genetic similarity and no strong evidence of partitioning of genetic diversity within North America based upon host or site of provenance, including those from the geographically isolated inland site in Mariposa County. The degree of genetic similarity detected for these two species is comparable with that found in a study of the introduced North American population of heterothallic *P. ramorum*. AFLP analysis indicated that this population, comprised of only one mating type, is dominated by a single clone genotype with a few rare, but highly genetically similar, variants (Ivors *et al.* 2004). Studies of California and Oregon forest populations of *P. ramorum* using SSRs yielded similar results (Ivors *et al.* 2006; Prospero *et al.* 2004; Prospero *et al.* 2007). The mean degrees of genetic similarity within both *P. nemorosa* and *P. pseudosyringae* are slightly lower than that determined through AFLP analysis for the homothallic *P. quercina* in European forests (Cooke *et al.* 2005). The largest genetic distance for US *P. pseudosyringae* population was between coastal isolates, not between coastal and interior.

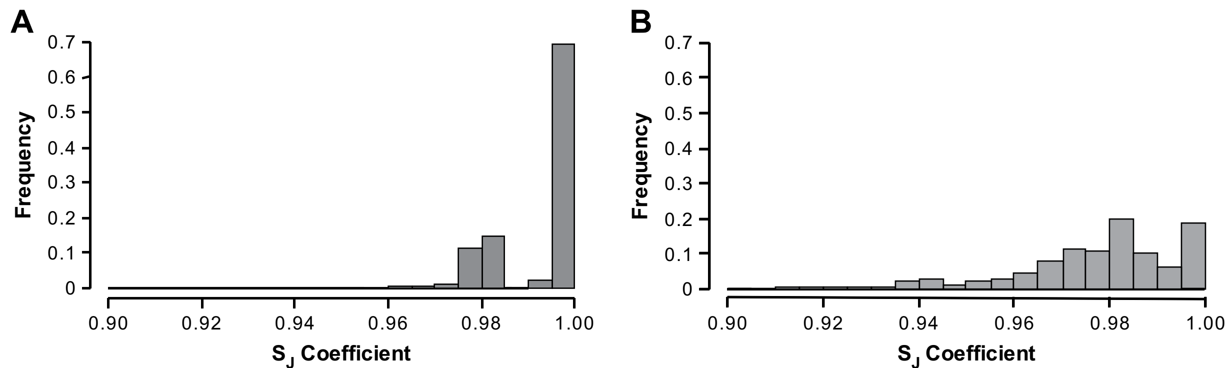

Fig 1 – Distributions of Jaccard coefficients of similarity (S_J) for *Phytophthora nemorosa* and *P. pseudosyringae*. (A) Frequency histogram showing the distribution of all pairwise S_J for the 39 *P. nemorosa* ($n = 741$) isolates. Frequency indicates percent of comparisons with a given S_J value; S_J values of 1 indicate pairs with 100 % similarity. (B) Frequency histogram showing the distribution of all S_J values between each pair of 48 *P. pseudosyringae* isolates ($n = 1128$).

Exotic species often undergo a population bottleneck upon introduction to a new area, whereas native species characteristically exhibit a genetic structure reflective of the presence of geographic or environmental barriers to gene flow. Because *Phytophthora* species are near-obligate pathogens, it has been theorized that all introduced *Phytophthoras* should undergo severe genetic bottlenecks (Goodwin 1997). This has been observed for other invasive agricultural *Phytophthora* species, and the low levels of genetic variation observed in the US for *P. ramorum* and in Europe for *P. quercina* led to the hypothesis both were introduced to those respective areas (Cooke *et al.* 2005; Goodwin *et al.* 1994; Huang *et al.* 2004; Ivors *et al.* 2004; Ivors *et al.* 2006; Prospero *et al.* 2004; Prospero *et al.* 2007). Expectations for genetic diversity and population structure if *P. nemorosa* and *P. pseudosyringae* are endemic, however, are confounded by the fact that both species are homothallic, i.e., can reproduce sexually through selfing. Although some homothallic oomycetes can outbreed (Förster *et al.* 1994; Francis & St. Clair 1993; Whisson *et al.* 1994), widespread selfing may reduce genetic diversity compared with self-incompatible, or heterothallic, species (Goodwin 1997). Moreover, like other homothallic *Phytophthoras,* both *P. nemorosa* and *P. pseudosyringae* can combine selfing and/or outcrossing with clonal propagation via zoospores. To our knowledge, there are no studies of the genetic structure of confirmed-native, homothallic forest *Phytophthoras* to use as a reference; however, the dominant pattern of genetic diversity in endemic clonal plants is a local population comprising multiple clones, whereas widespread clones present in multiple sites are rare (Ellstrand & Roose 1987).

The high degree of genetic similarity in *P. nemorosa* and *P. pseudosyringae* and the lack of genetic structure within their range in western US are most consistent with the hypothesis of relatively recent introductions to the western US. The extremely low levels of genetic variation detected in this study, the wide distribution of AFLP clones, and the lack of population structure are unexpected for endemic organisms, including those with apomictic or selfing reproduction. The almost total lack of differentiation in endemic *Phytophthora* species also seems improbable in light of reports that some *Phytophthoras* that reproduce entirely asexually can accumulate genetic variation, perhaps by mutation or mitotic recombination (Abu-El Samen *et al.* 2003; Dobrowolski *et al.* 2003). A PCR-based diagnostic study of symptomatic and asymptomatic California bay laurel and tanoak leaves collected between 1861 and 1984 and housed at the U.C. Berkeley Jepson Herbarium failed to detect any *Phytophthora* infection, providing a separate line of evidence that *P. nemorosa* and *P. pseudosyringae* are not ancient pathogens of California bay laurel (Monahan *et al.* 2008).

However, it is difficult to test the hypothesis of endemism directly, and identifying the origin of introduced forest pathogens can be challenging. Source populations have been confirmed for very few invasive *Phytophthora* species (Hardham 2005; Ristaino 2000). If *P. nemorosa* and *P. pseudosyringae* have been introduced to the western US, it would be expedient to locate their sources. As *P. nemorosa* is only known in the highly clonal western US population, the location of a source remains an open question. For *P. pseudosyringae,* however, we can compare isolates from two continents. Isolates from the US and Europe did not parse cleanly into separate clades according to geographic provenance, as shown for *P. ramorum* (Ivors *et al.* 2004; Ivors *et al.* 2006). Instead, the majority of the US isolates grouped in a clade nested within the European distribution, with a few US isolates outside of this clade. No genotypes were shared between the two continents, and AMOVA detected more differentiation within populations than between US and European provenances of *P. pseudosyringae*. Although the level of genetic variation in both regions is low, it is markedly lower in the US. Finally, there is more genetic diversity among individuals from one site in the Italian Abruzzo National Park than that measured among individuals taken from a range of sites in California. Collectively, these results suggest that the European population may be the source of that in the western US. However, there is no documented evidence indicating the way by which this pathogen or infected plants may have been introduced from Europe to the US. Moreover, our data do not help to determine the history of this species in Europe; genetic variability in our limited sample was low, but higher than in the US, and included some

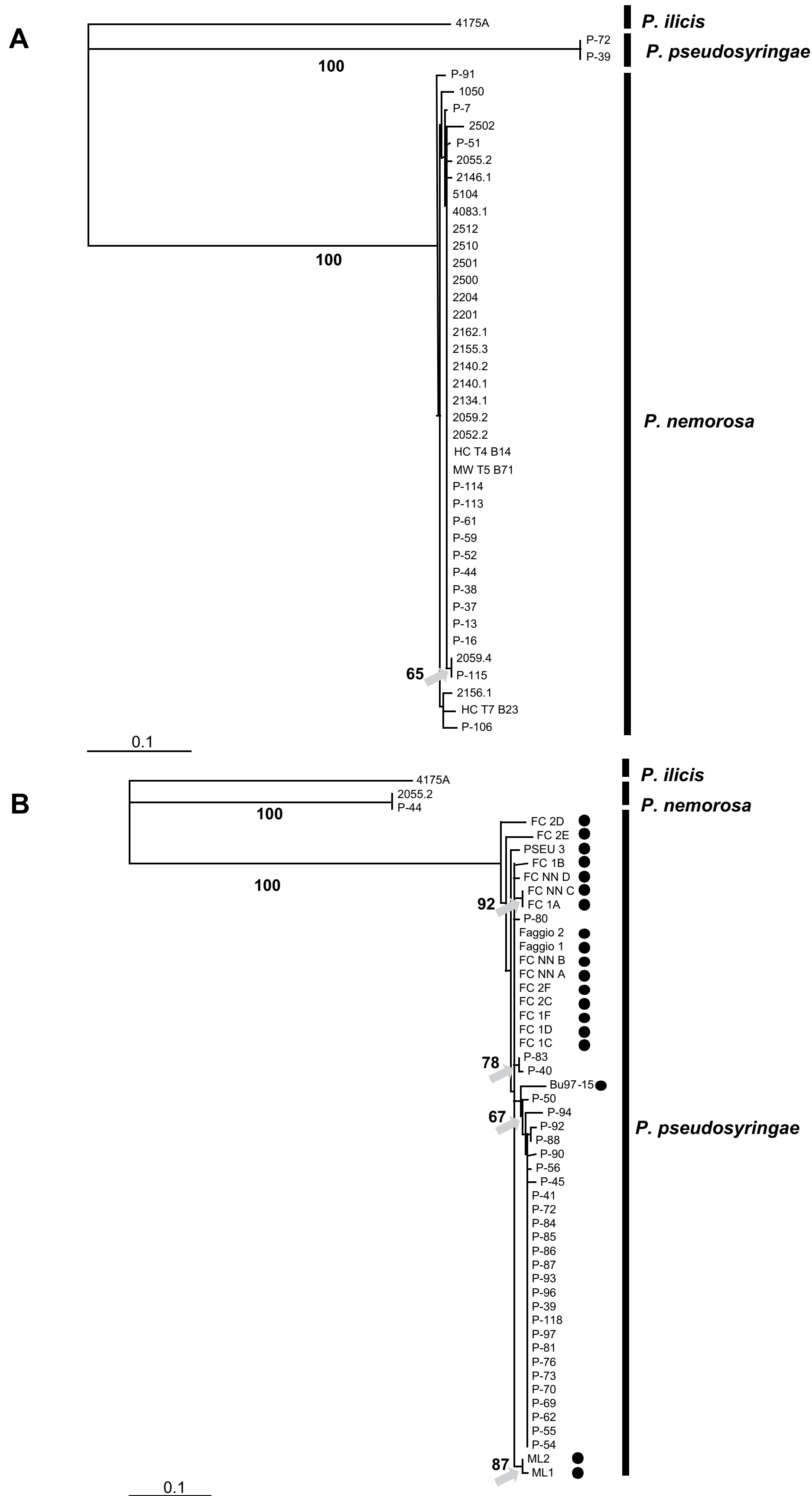

Fig 2 – Dendrograms of AFLP profile distance measures (1- S_J) for *Phytophthora nemorosa* and *P. pseudosyringae*. Groups of isolates with zero branch length are comprised of samples with identical AFLP genotypes. Dendrograms generated in PHYLIP using the FITCH module, and values above 65 from 1 K BS replicates shown. (A) Thirty-nine *P. nemorosa*, one *P. ilicis*, and two *P. pseudosyringae* isolates. (B) Forty-eight *P. pseudosyringae*, one *P. ilicis*, and two *P. nemorosa* isolates. Isolate names marked with filled circles indicate *P. pseudosyringae* individuals originally recovered from Europe.

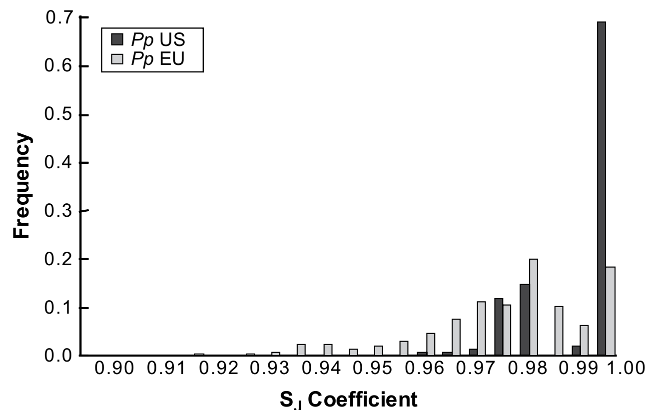

Fig 3 – Intraspecific distributions of Jaccard coefficients of similarity (S_J) within *Phytophthora pseudosyringae* in the US and Europe. Frequency histogram showing the distribution of all pairwise S_J coefficients for the 48 *P. pseudosyringae* isolates: 29 from the US ($n = 406$) and 19 from Europe ($n = 171$) — all S_J between each pair of isolates calculated in LE PROGICIEL R from the AFLP marker binary matrices. Frequency indicates percent of pairwise comparisons with a given S_J value; S_J values of 1 indicate pairs with 100 % similarity in their AFLP genotypes.

evidence of genetic structure linked to location. These results may indicate either that the species is native to Europe, or that it may have been introduced there for a longer time than it has been in the US. Progressive reductions in genetic diversity during nested introductions of *Rubus alceifolious,* a weedy plant capable of self-compatible and clonal reproduction, have been detected using AFLP markers (Amsellem *et al.* 2000). Interestingly, early reports of the morphologically similar *P. syringae* associated with root rot of European beech, *Fagus sylvatica* (e.g. Day 1938), may actually be observations of *P. pseudosyringae*. *Phytophthoras* isolated from beech and identified as *P. syringae* using morphological characteristics have been subsequently identified as *P. pseudosyringae* using their divergent DNA sequences, and beech has not been confirmed as a host of *P. syringae* using molecular evidence (Jung *et al.* 2003). This suggests that *P. pseudosyringae* may have been resident in Europe for at least 70 years.

Although these genetic analyses suggest an exotic origin for the two study *Phytophthoras*, it cannot be categorically ruled out that they are native to the western US. Indeed, although the genetic distances involved are quite small and the montane population is represented by a single individual, the greatest genetic distance observed between *P. nemorosa* isolates was between the interior isolate and a coastal isolate. Selfing and clonal reproduction, accompanied by successful gene flow across their entire disjunct geographic range may result in a similar genetic structure. Furthermore, our knowledge of the biology, ecology, host, and geographic range of these two species is still limited. Sampling new hosts or soilborne populations may uncover further genetic variability.

To further test all aforementioned hypotheses, a population study with sampling reflecting the recent expansion of known host and geographic ranges of *P. pseudosyringae* and *P. nemorosa* is needed. This should include *P. pseudosyringae* from hosts *Lithocarpus densiflorus* in the US and alder, oak and chestnut in Europe, and from sites in Oregon, eastern US streams and additional European locations (Hansen *et al.* 2006; Hwang *et al.* 2007; Jung *et al.* 2003). *P. nemorosa* and *P. pseudosyringae* also have been found in western US nurseries (Anonymous 2006; Yakabe *et al.* 2007), and *P. pseudosyringae* has been recovered in nurseries located in Europe (Hartman *et al.* 2006; Jung 2006; Pintos Varela *et al.* 2007). Given the link between forest and nursery strains for *P. ramorum* (Ivors *et al.* 2006; Prospero *et al.* 2007), genotyping nursery populations could be quite informative in identifying potential sources and transmission pathways of the pathogen. In Europe, there is a large body of evidence linking the spread of *P. pseudosyringae* and other *Phytophthora* spp. to natural forest ecosystems through nursery stock (Hartman *et al.* 2006; Jung 2006; Jung & Blaschke 2004; Pintos Varela *et al.* 2007).

This study was initiated under the assumption that two *Phytophthora* species recently discovered in sympatry with the exotic *P. ramorum* were likely to be native to the western US. This assumption was based on both the broader geographic distribution of these two species and their apparent low virulence. Unexpectedly, the data presented here suggest that these two species may also have been introduced in the western US. Assuming their exotic origin will be confirmed by further studies, these two species provide examples of invasive [*sensu* Richardson *et al.* (2000)] pathogens not causing devastating epidemics. They are also evidence that 'apparently invisible' invasions may occur alongside more obvious ones. Indeed in Germany, *P. pseudosyringae* has been shown to be spread with infected nursery plants together with other more aggressive, and thus more easily observed, *Phytophthora* species, such as *P. cambivora*, *P. citricola*, and *P. quercina*. In spite of this invisibility, these invasions deserve our full attention because they may have undetected effects on the ecology of invaded ecosystems, may later cause emerging disease under varying environmental conditions or after rapid evolutionary change, and because they may share the same invasion routes as destructive exotic pathogens.

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