

Population genetics of worldwide and California isolates of *P. ramorum* using microsatellites

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Introduction

- *Phytophthora ramorum*, oomycete
- In U.S. and E.U., twig blight on economically important nursery plant spp. (e.g. *Rhododendron*, *Viburnum*, *Camellia*, *Kamia*, *Pieris* and *Vaccinium*)
- Sudden Oak Death (SOD), canker of tanoak (*Lithocarpus densiflora*), coast live oak (*Quercus agrifolia*), California black oak (*Q. kelloggii*) and Shreve's oak (*Q. parvula* var. *shrevei*) in CA and OR
- Heterothallic (A1 and A2 mating types)
- E.U. (A1 and 1 A2) and U.S.(A2),





Redwood



Maple



Bleeding oak



Tanoak



Rhododendron



Douglas-fir

Above ground symptoms



Bay laurel



Toyon



Madrone



Lesion stops at soil line

Rhododendron



Camellia japonica



Camellia sasanqua



Introduction

- Microsatellite, simple sequence repeats (SSR) codominant markers
- High variability
- Distinguish individuals in clonal populations
- Detect potential outcrossing
- Used in other plant-pathogen systems, genetic structure and reproductive biology (e.g. *Colletrichum* spp., *P. cinnamomi*, *Sclerotinia sclerotiorum* and *Venturia inaequalis*)

Materials and methods

- Isolates grown in liquid pea broth (7 days)
- gDNA extraction, PUREGENE DNA isolation kit
- Microsatellite, 12 informative primer pairs, labeled forward primers (HEX or FAM)
 - PCR, ABI 3100, GeneScan-500 ROX and GeneScan 3.1.2 software
- Sequencing, 12 unlabeled primer pairs
 - PCR, TOPO-TA cloning kit, 15 colonies, T7F and M13R, ABI 3100, Sequencher 4.1.2 software and PAUP v.4b2

ID	Locus*	Repeat motif†	Primer sequence (5' to 3')‡
18	AC0336	(AC) ₃₉	F: [FAM] TGCCATCACAACACAAATCC R: TGTGCTATCTTTCCCTGAACGG
29	AGC0010	(AGC) ₆	F: [HEX] TTCCTGTGTCTACGACTGCG R: TCTGCTGTTTCAGTTTGTCTGC
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] CCACGTCAATGGGTGACTTC R: CGTACAAGTCAOACTCCCC
97	TGC0032	(TGC) ₁₂	F: [FAM] ACGTCTTCTTGGAGTGGTGG R: TCTTGGACTTGGCTGACCTC
104	AC0104	(AC) ₆	F: [HEX] ACAAGGTCCGTTTTCGTTGAG R: CAACGAGTTCGATCGGTAGG
278	CT0278	(CT) ₇	F: [FAM] TGGAGAATTTCTCTGTCCGGAG R: TGAAGGTGCTATCAGGGTCC
562	CT0562	(CT) ₈	F: [FAM] ACGCTCTGCAGTCAACCATC R: CCGCACTTCCGTATCTCAGT

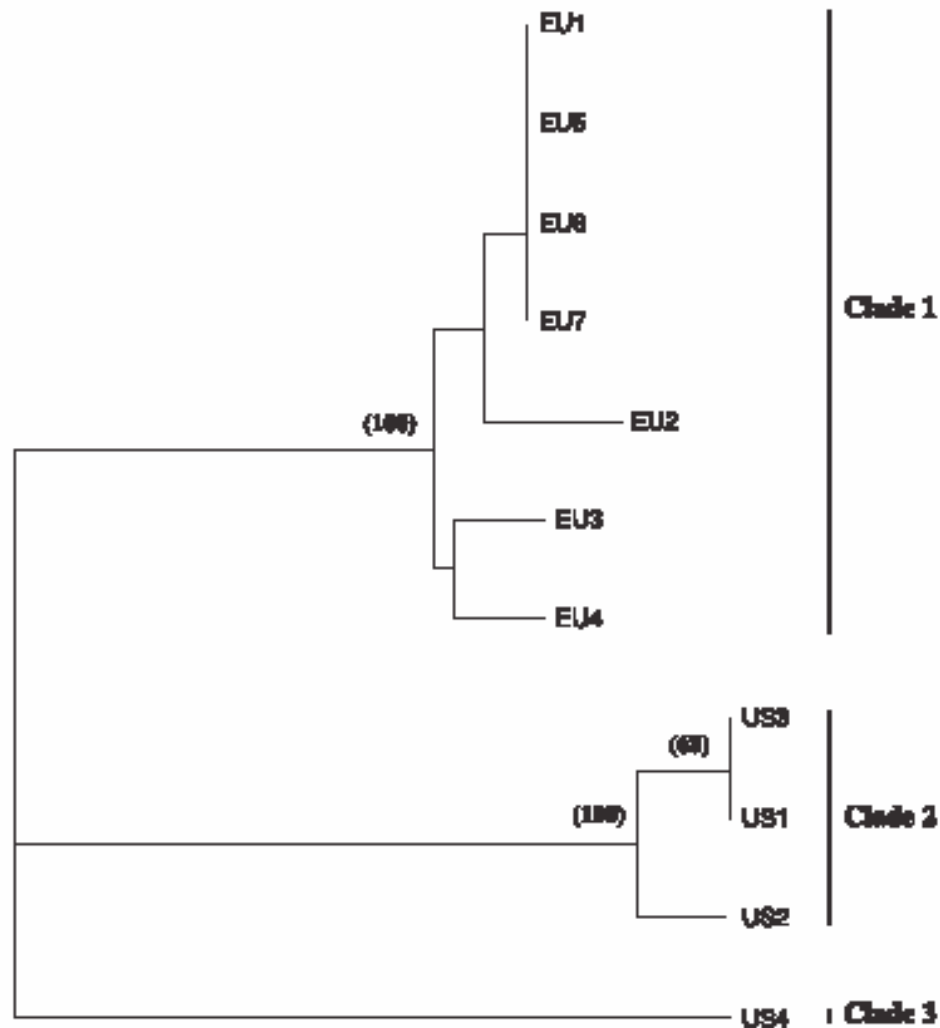
*Locus ID created by the database.

†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.

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

0.1



Results (Microsatellite)

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.

Results (Microsatellite)

Genotype	Isolate(s)	Locus ^a											
		18	29	33	63	64	65	79	82	97	104	278	562
US1	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	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	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	Pr-WA0692 ^N	222/-	-/337	325/327	155/157	340/356	220/222	254/256	106/108/110/112	NA ^b	222/-	242/250	334/340
EU1	Clone (69 EU isolates)	218/264	325/337	315/323	155/163	346/392	236/244	224/256	112/130/140	300/-	222/226	240/-	334/345
	Pr-WA1743 ^N												
	Pr-WA1747 ^N												
	Pr-WA1772 ^N												
	Pr-3-74-1 ^N												
	Pr-3-74-2 ^N												
EU2	514 ^N	218/266	325/337	315/323	155/163	346/392	236/244	224/256	112/130/136	300/-	222/226	240/-	334/345
	679 ^N												
EU3	517 ^N	218/264	325/337	315/323	155/163	346/388	236/244	224/256	112/130/140	300/-	222/226	240/-	334/345
	556 ^N												
EU4	521 ^N	218/264	325/337	315/323	155/163	346/394	236/244	224/256	112/130/140	300/-	222/226	240/-	334/345
	542 ^N												
	571 ^N												
	595 ^N												
EU5	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	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	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
New	WSDA3403	220/-	340/-	327/-	155/-	340/356	220/-	253/-	103/106	-/-	220/222	240/248	334/340
	WSDA3765	220/-	340/-	327/-	-/-	340/356	220/-	253/-	103/106	-/-	220/222	240/248	334/340
	Davis001	220/-	340/-	-/-	155/-	340/356	220/-	253/-	103/106	-/-	220/222	240/248	334/340
	Davis003	220/-	340/-	327/-	155/-	340/356	220/-	253/-	103/106	-/-	220/222	240/248	334/340
	Davis005	220/-	340/-	327/-	155/-	340/356	220/-	253/-	103/106	-/-	220/222	240/248	334/340
	Total no. alleles	7	2	5	5	8	6	3	9	2	3	3	4

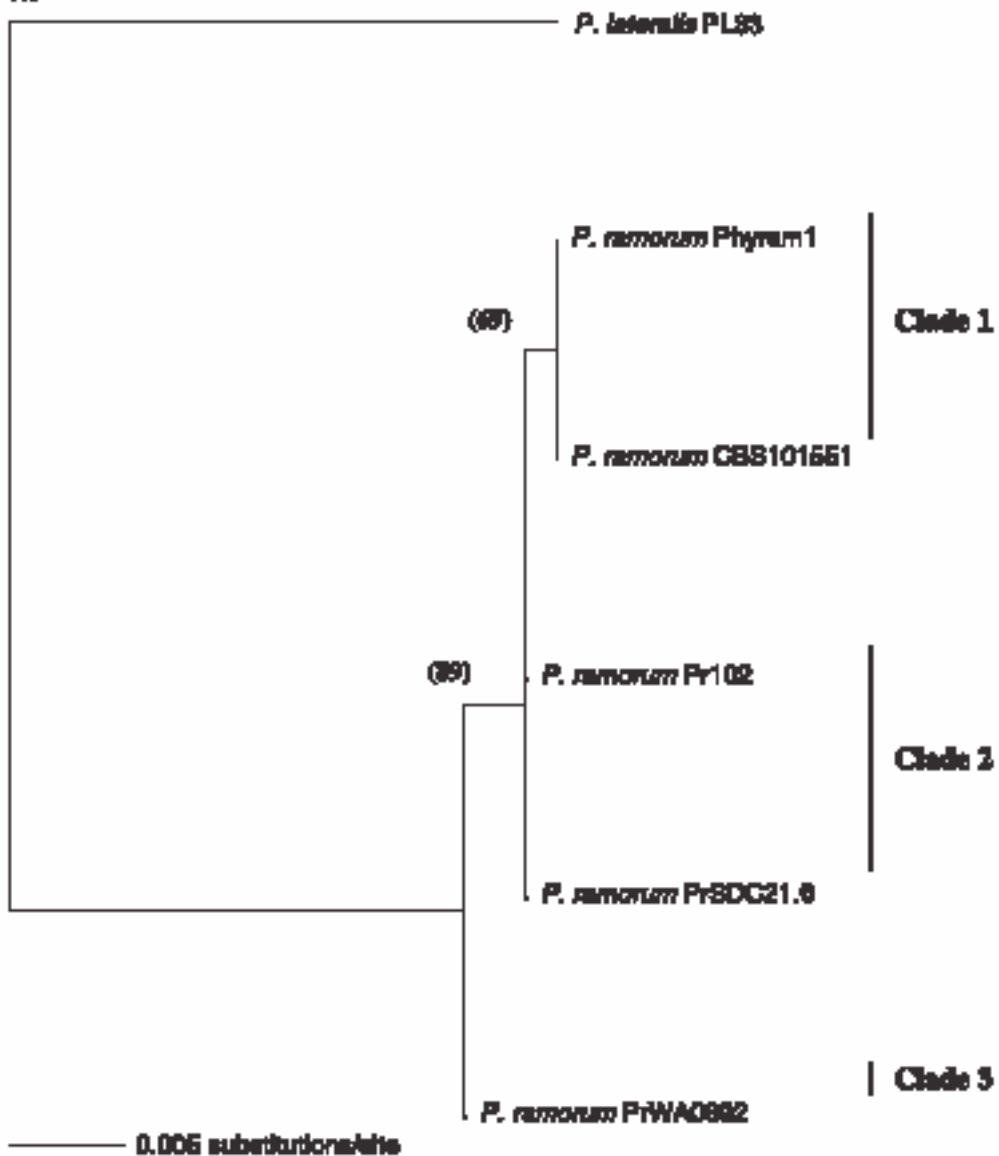
^a Dash (-) indicates loss of an allele or homozygosity with the remaining allele.

^b NA indicates non-amplification of this isolate at this locus with selected microsatellite primers

^N Indicates *P. ramorum* isolate from nursery.

Us and EU= Excess heterozygosity
WA= Excess homozygosity

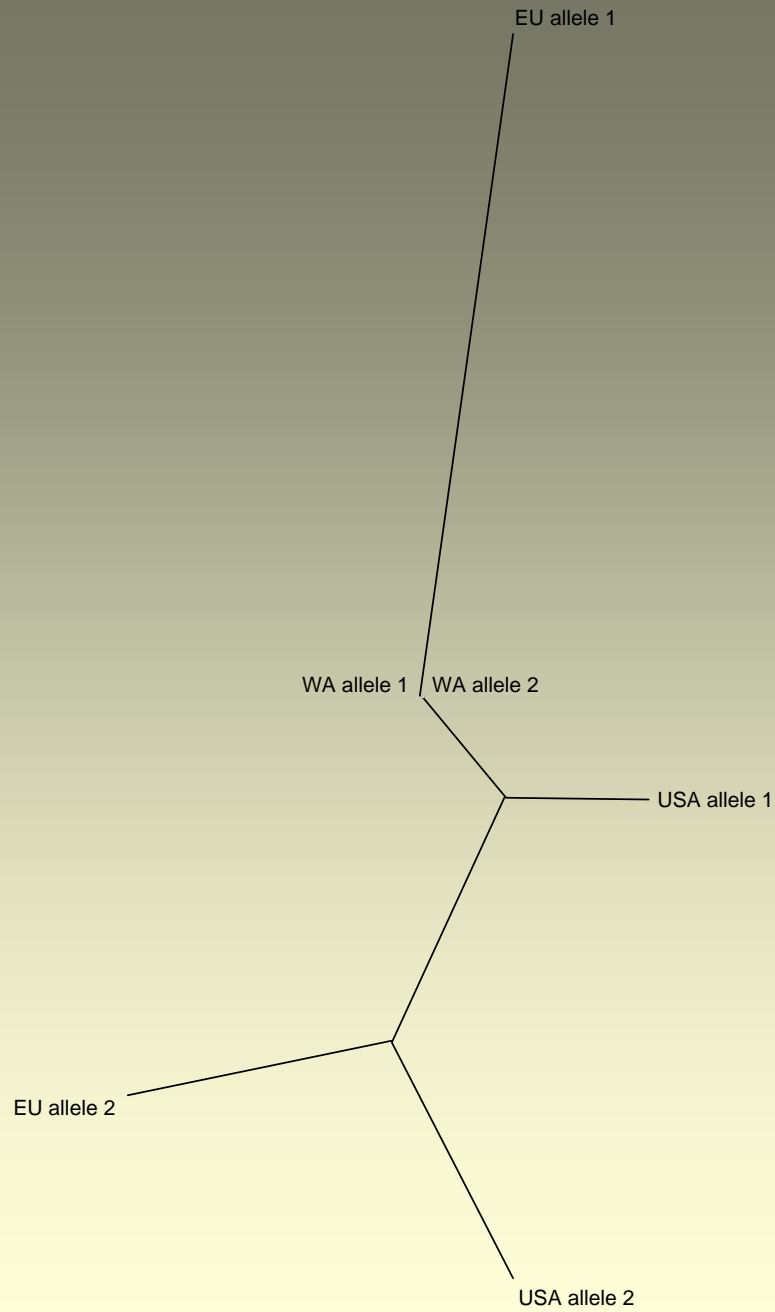
NJ



Results (sequencing)

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

Results (sequencing)



Materials and methods

- 8 mm discs were cut with a sterile cork borer from the colony margins of 11-day-old on V8 juice agar
- Transferred, mycelial side down, to the center of individual 9 cm Petri-plates containing 20 ml of V8A
- Plates were sealed with Parafilm incubated at 20°C in the dark
- After 1 week, plates transferred to 12, 16, 20, 24 or 28 ±1°C in darkness, 4 replicates per isolate-temperature treatment
- Radius measured after 10 days along two lines intersecting at right angles from center of the inoculum disc.

Results (Growth Rates)

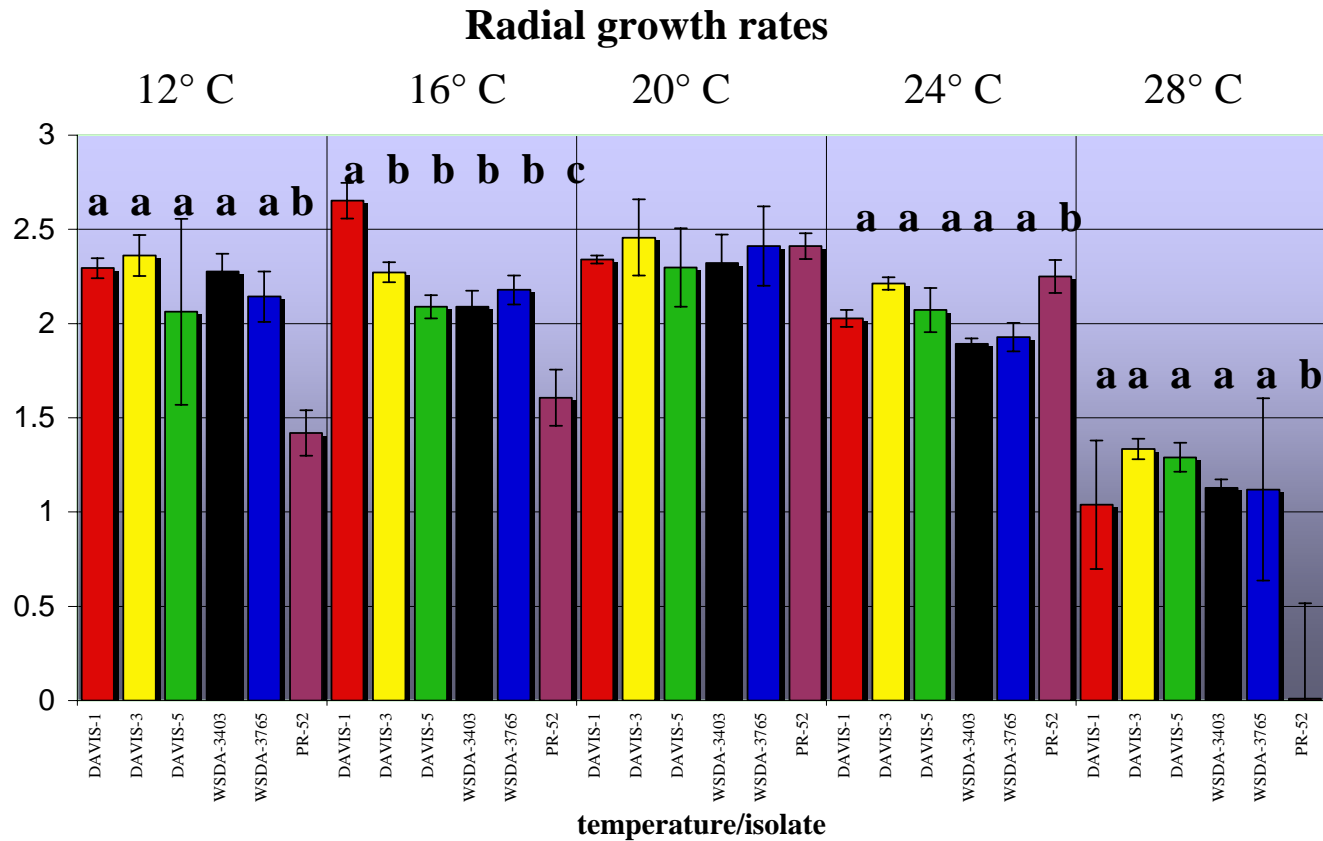


Table 5 Distinctive characteristics of the three proposed lineages of *Phytophthora ramorum*, based on data presented in this study and other unpublished observations. Characters differentiating lineages 1 and 2 are documented by several published studies (see text)

Lineage	Provenance	Microsatellite profile	Mitochondrial <i>cox</i> 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.

Summary

- Three groups of *P. ramorum* exist with distinct combinations of genetic and phenotypic traits
- Sequencing data for both nuclear and mitochondrial loci shows third lineage from WA to be ancestral to both
- While US and EU lineages show excess heterozygosity, indicating mating of two groups followed by clonal reproduction, WA isolates are all A2 and seem not to have mated (abortion rates are also higher)
- Growth studies showed significant differences between nursery isolates and original clone isolate at all temperatures except 20° C
- Genotypic diversity is **91% of total in nurseries vs. 18% of total in forests**. US and WA groups show little genotypic variability

Summary

- Nurseries from US PNW contain all known diversity, three lineages and most genotypes:
- It is likely these nurseries have served as stepping stones for introduction of pathogen
- Pathogen is reproducing clonally in forests

A closer look to US group, using 3 hypervariable microsat loci

- All genotypes are created by accumulation of mutations
- Six genotypes in our nursery sample (limited)
- Most common nursery genotype matches genotype of isolates collected in 2001-2002
- 30+ genotypes in the wild (10 sites, 24 isolates per site) because of huge pathogen population explosion

A closer look to US lineage, using 3 hypervariable microsat loci

- Genotypes in 2005-2006 collection are different from those previously collected
- 8-9 genotypes per sites in old infection centers in Santa Cruz and Marin, 1-4 in areas recently infested
- Each area has dominance of its local genotype: interesting link between Santa Cruz and Mount Tam , Big Sur and Plaskett Canyon, Sonoma and Humboldt
- One genotype is shared among all old infection centers (original genotype), all other are created by adding further mutations at different loci (we can reconstruct network of development of genotypes)

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