RESEARCH ARTICLE

Mitochondrial haplotype determination in the oomycete plant pathogen *Phytophthora ramorum*

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Abstract The mitochondrial genome of an isolate of *Phytophthora ramorum* from Europe (EU) was sequenced and compared to the previously published genome sequence of an isolate from California (NA). The EU mitochondrial genome had the identical gene order and encoded for the same suite of genes as the NA mitochondrial genome, but had 13 single nucleotide polymorphisms (SNPs) and at 39,494 bp was 180 bp longer. This length difference was due to an increase in the size of the spacer region between the *nad*5 and *nad*6 genes caused by a chimeric region containing duplication of the spacer sequence and additional sequences from the flanking genes. Recombination between the 1,150 bp-inverted repeats (IR) generated orientational isomers where the gene order was reversed between the IR. A total of seven primer pairs were developed for amplification of regions where the SNPs were located and two other regions where additional SNPs were encountered when a larger number of isolates were examined. Sequence data for a total of 5,743 bp for 40 isolates collected from a range of geographic areas was compared and 28 loci were found to be polymorphic. The combination of these polymorphisms revealed a total of 4 mitochondrial haplotypes; the traditional EU (haplotype I), the traditional NA (haplotype IIa), the third nuclear lineage of the pathogen recovered from a nursery in Washington State (haplotype III) and a new haplotype representing a subgroup of NA isolates from an Oregon forest (haplotype IIb). Phylogenetic analysis using the sequences generated from the haplotype analysis supported a high affinity for haplotypes IIa and IIb, both of which were distinct from haplotype I, with haplotype I basal to these and haplotype III representing the ancestral state.

Introduction

The oomycete plant pathogen *Phytophthora ramorum* was described in 2001 from cultures recovered from *Rhododendron* and *Viburnum* spp. in Germany and the Netherlands, and subsequently has spread to a number of nursery production areas in Europe (Werres et al. 2001). Concomitant to this description the pathogen was also recovered from declining trees in forest ecosystems in California and was determined to be the causative agent of a disease that has since became known as sudden oak death (Rizzo et al. 2002). The pathogen subsequently has been found in nursery production systems in North America and quarantine restrictions have been enacted to prevent its spread (Anonymous 2002; California Department of Food And Agriculture 2003; Canadian Food Inspection Agency 2003). While it was initially thought that the presence of the pathogen in both Europe (EU) and North America (NA) was due to movement from one location to the other, AFLP and microsatellite analysis confirmed that the populations were distinct and clonally reproducing (Ivors et al. 2004, 2006). *Phytophthora ramorum* is heterothallic and one noticeable difference between the EU and NA populations is in mating type, with the EU population being A1 while the isolates found in the NA population are A2, although there are reports of isolates with A2 mating types in Belgium and A1 mating types in North American nurseries

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that share the same nuclear background of other EU isolates (Brasier 2003; Werres and Merlier 2003; Ivors et al. 2004, 2006; Hansen et al. 2003). In addition to the NA and EU populations (NA1 and EU1, respectively), microsatellite analysis and sequence analysis of the mitochondrially encoded *cox1* gene revealed a third lineage of the pathogen (NA2) present in Washington State nurseries (Ivors et al. 2006).

Given the heterothallic mating system and the general geographic separation of the opposite mating types it is important that regulatory efforts are designed and monitored in a manner to prevent the introduction of opposite mating types into the same locations as this could lead to sexual recombination and the generation of new genotypes. One molecular marker system capable of identifying the EU1 and NA1 genotypes is based on a single nucleotide polymorphism (SNP) in the mitochondrially encoded *cox1* gene that separates isolates into one of two mitochondrial haplotypes (Kroon et al. 2004). There is a consistent correlation between the PCR-RFLP results and the geographic populations of the isolates to the point where both the A1 EU1 isolates and the A2 isolates recovered in Belgium can be differentiated from NA1 A2 mating type isolates. However, the NA2 lineage of *P. ramorum* recovered from nursery plants in Washington State cannot be differentiated from the NA1 lineages by this marker but can by sequence differences in the *cox1* gene (Ivors et al. 2006). Having additional tools that could easily identify these lineages would simplify the regulatory efforts to manage this destructive pathogen.

The mitochondrial genome has been sequenced for an isolate of *P. ramorum* from California (Martin et al. 2007) but with the exception of the previously mentioned SNPs in the *cox*1 gene the level of intraspecific polymorphism in the genome and how many mitochondrial haplotypes are present in this species is not known. The only species of *Phytophthora* for which mitochondrial genome sequences from multiple isolates are available is *P. infestans*, the causal agent of potato late blight. This has been determined for four separate haplotypes; the first sequenced was Ib (Paquin et al. 1997), with Ia, IIa, and IIb sequenced more recently (Avila-Adame et al. 2005). Intraspecific variation among the *P. infestans* haplotypes includes both SNPs dispersed throughout the genome as well as length variations caused by insertions/deletions that occurred primarily in two locations (Avila-Adame et al. 2005). While the mitochondrial genomes of these two species essentially encode the same suite of genes (there are some differences in the presence of specific ORFs), there are two inversions in *P. infestans* relative to *P. ramorum* that reverses gene order and the *P. ramorum* genome has a 1,150 bp inverted repeat (IR) (Martin et al. 2007). It is unclear if intraspecific variation in the mitochondrial genome of *P. ramorum* occurs in a similar fashion as *P. infestans*, but the presence of the IR in *P. ramorum* may contribute to novel types of intraspecific variation relative to *P. infestans* in that recombination between the two arms of the IR in other oomycetes can generate isomeric forms of the genome (Hudspeth et al. 1983; Boyd et al. 1984; Grayburn et al. 2004). Clarification of the mutations responsible for intraspecific polymorphisms in the mitochondrial genome of *P. ramorum* would provide a framework for understanding the processes contributing to genome evolution in the genus as well as facilitate development of markers for mitochondrial haplotype determinations.

The objective of this study was to sequence the mitochondrial genome of an isolate of *P. ramorum* that was representative of the EU1 population and compare it to the genomic sequence from a previously sequenced NA1 isolate (DQ 832718) in an effort to clarify the extent and types of mutation events contributing to genome evolution within the species. The appropriate primers for PCR amplification and sequence analysis of polymorphic regions were developed to examine these regions from a broader geographic collection of isolates to evaluate if the observed results are representative of the species as a whole. This sequence data was also used to classify mitochondrial haplotypes for *P. ramorum* and clarify phylogenetic relationships among haplotypes within this species in an effort to better understand the different introductions of pathogen populations and their current distribution.

Materials and methods

Strains sequenced

Cultures of CBS101553 (type culture) were grown on ½ strength V-8 broth (Ayers and Lumsden 1975) for several days at 20ºC prior to placing in a blender cup and homogenizing for a few seconds to disrupt the cell walls and pouring the homogenate into fresh medium. After several days the cultures were harvested and the liquid blotted out with paper towels prior to freezing in liquid nitrogen. Total DNA was extracted and the mitochondrial DNA purified by cesium chloride:bisbenzimide ultracentrifugation using standard techniques (Martin and Kistler 1990). This genomic sequence was compared with the previously published genomic mitochondrial sequences for the NA1 *P. ramorum* strain Pr-102 (DQ 832718; (Martin et al. 2007). Additional isolates of *P. ramorum* included in the analysis of individual loci are listed in Table 1. Isolates that were genotyped in previously published AFLP (Ivors et al. 2004) or microsatellite studies (Ivors et al. 2006; Prospero et al. 2007) are footnoted.

Table 1 Isolates of *Phytophthora ramorum* used in this study and their mitochondrial haplotype

Isolate #[a]	Host	Origin	Mt Haplotype
Prn-1[PT], PD93/844[sw]	*Rhododendron* sp.	Netherlands	I
Prn-2 [PT], PD94/844[sw,c]	*Rhododendron* sp.	Netherlands	I
Prn-3 [PT], PD98/8/6743[sw,c]	*Rhododendron* sp.	Netherlands	I
Prn-4 [PT], PD98/8/6285[sw,c,d]	*Rhododendron* sp.	Netherlands	I
Prn-5 [PT], PD98/8/2627[sw]	*Rhododendron* sp.	Netherlands	I
Prg-1 [PT], BBA 69082[sw,c]	*Rhododendron* sp.	Germany	I
Prg-2 [PT], BBA 9/95[sw], CBS101553 (Type)[c,d]	*Rhododendron catawbiense*	Germany	I
Prg-3 [PT], BBA 14/98-a[sw]	*Rhododendron catawbiense*	Germany	I
Prg-4 [PT], BBA 12/98[sw,c]	*Rhododendron catawbiense*	Germany	I
Prg-6 [PT], BBA 16/99[sw,d]	*Viburnum bodnantense*	Germany	I
P10318[MC], CBS110545	*Rhododendron* sp.	Poland	I
P10343 [MC], CBS 110901[d]	*Viburnum bodnantense*	Belgium	I
P10322 [MC], CBS 110548	*Rhododendron* sp.	France	I
P10546 [MC], CSL 20316495	*Aesculus hippocastrum*	England	I
D12A[NG]	Nursery	Oregon	I
288[MG]	*Rhododendron* sp.	California	IIa
73101[CDFA]	*Lithocarpus densiflorus*	California	IIa
044519[CDFA]	*Umbellularia californica*	California	IIa
044522[CDFA]	*Lithocarpus densiflorus*	California	IIa
P072648 [CDFA]	*Quercus agrifolia*	California	IIa
201C[DR,c]	*Rhododendron* sp.	California	IIa
0-217, Pr-52[DR,c,d]	*Rhododendron* sp.	California	IIa
Coen[MG]	*Rhododendron* sp.	California	IIa
0-13, Pr-5[DR,c]	*Lithocarpus densiflorus*	California	IIa
P10321 [MC], CBS 110543[c]	*Lithocarpus densiflorus*	Oregon	IIa
P10637 [MC]	*Rhododendron* sp.	Washington State	IIa
Pr-102 (DQ 832718)[c,d]	*Quercus agrifolia*	California	IIa
883[NG]	Nursery	Florida	IIa
04-189-B5[NG,e]	*Viburnum bodnantense*	Oregon	IIa
04-207-Q[NG,e]	*Pieris japonica*	Oregon	IIa
wsda2519.3[NG]	Nursery	Washington	IIa
wsda4164[NG]	Nursery	Washington	IIa
wsda4165[NG]	Nursery	Washington	IIa
1020.1[NG,e]	*Lithocarpus densiflorus*	Oregon	IIa
WA15.3-080403[NG]	Stream	Oregon	IIa
4361[NG,e]	*Lithocarpus densiflorus*	Oregon	IIa
4301.1[NG,e]	*Lithocarpus densiflorus*	Oregon	IIa
wsda3765[NG]	Nursery	Washington	III
RHCC1 [NG,MG,b]	*Rhododendron* sp.	Sacramento, CA	III
2027.1[NG,e]	*Lithocarpus densiflorus*	Oregon	IIb
1033.1[NG,e]	*Lithocarpus densiflorus*	Oregon	IIb
2092[NG,e]	*Lithocarpus densiflorus*	Oregon	IIb

[a] Isolates or DNA were obtained from *CDFA* Cheryl Blomquist, California Department of Food and Agriculture, *MC* Michael Coffey, *NG* Nik Grunwald, *MG* Mateo Garbelotto, *EH* Everett Hansen, *KL* Kurt Lamour, *DR* Dave Rizzo, *PT* Paul Tooley, *SW* Sabine Werres

[b] This isolate was recovered from plants shipped from a nursery in Washington State where the third lineage of Ivors et al. (2006) was recovered

[c] Same isolates used in the AFLP study of Ivors et al. (2004)

[d] Same isolates used in the microsatellite study of Ivors et al. (2006)

[e] Same isolates used in the microsatellite study of Prospero et al. (2007)

Sequencing and contig assembly

At the Joint Genome Institute Production Genomics Facility (Walnut Creek, CA) the mitochondrial DNA preparation was randomly sheared using a Hydroshear device (Genomic Solutions, Ann Arbor, MI, USA), in separate aliquots, to fragments averaging either about 3 kb or about 8 kb. These were gel purified and enzymatically repaired to blunt ends, then cloned into plasmids to generate two genomic libraries. End sequences were determined for a large number of randomly selected clones from these libraries, then assembled using Consed (Gordon 2004; Gordon et al. 1998) with final confirmation using Sequencher ver. 4.7 (Gene Codes, Ann Arbor, MI, USA) to form a consensus sequence. Detailed protocols are available at http://www.jgi.doe.gov/sequencing. The assembly of *P. ramorum* mtDNA included a total of 958 successful sequencing reads averaging 643 bp in length and totaling 615,532 nucleotides for approximately 15.6-fold sequencing coverage.

Annotation and comparative genomics

Annotation of coding regions and prediction of ORFs was done with Accelrys Gene v2.5 (Accelrys, San Diego, CA, USA) using the universal genetic code. Identification of protein- and rRNA-encoding genes was done by comparison with sequences previously reported for the mitochondrial genome of *P. ramorum* (Martin et al. 2007; DQ 832718) and BLAST analysis to other sequences in GenBank. Genes for tRNAs were confirmed using tRNAscan SE v1.1 (Lowe and Eddy 2007; http://www.genetics.wustl.edu/eddy/tRNAscan-SE/). Pair wise comparisons among genomes were made using mVISTA (Mayor et al. 2000; Frazer et al. 2004; http://genome.lbl.gov/vista/servers.shtml). Sequences were aligned using LAGAN (Brudno et al. 2003) within mVISTA.

Orientational isomers

Two combinations of double digests with specific restriction enzymes were used to determine if recombination has occurred between the two arms of the inverted repeat, which would result in a mixed population of mitochondrial genomes with the region between the IR in opposite orientations ("head-to-head" and "head-to-tail" isomers) as has been observed for the oomycetes *Achlya* spp. (Hudspeth et al. 1983; Boyd et al. 1984) and *Saprolegnia ferax* (Grayburn et al. 2004). The combinations of restriction enzymes were selected based on the DNA sequence data for their ability to cut infrequently, with one cutting in the area in between the two IRs and the other external to this region. Digestion with restriction enzymes *Pvu*II + *Sal*I (cut at base 21,372 and 2,274 + 38,202, respectively) and *Kpn*I + *Sac*I (cut at base 18,548 + 24,950 and 2,243 + 8,599, respec-

tively) was done in accordance with the manufacturer's instructions (New England Biolabs, Beverly, MA, USA). Digests were separated on a 0.7% agarose gel and stained with ethidium bromide for band visualization.

Mitochondrial haplotype determination

Based on the SNPs observed in comparisons between mitochondrial genomes for CBS101553 from Germany and Pr-102 from California (DQ 832718), additional primers were designed for amplification and sequencing of the regions containing the 13 SNPs and several other regions of the genome from a range of isolates of *P. ramorum* (Table 2, Fig. 1). Template amplification was done with the indicated annealing temperatures and $MgCl_2$ concentrations with 1-min extension times at 72°C. Sequencing templates were treated with ExoSap (USB, Cleveland, OH, USA) in accordance with the manufacturer's instructions and sent to the Nucleic Acid Sequencing Facility at the Penn State University (University Park, Pennsylvania) for sequencing with the amplification primers unless otherwise noted (Table 2). Sequencher 4.7 was used to edit the sequences and generate consensus sequences.

RFLP analysis for haplotype determination

In an effort to differentiate mitochondrial haplotypes III and IIb by RFLP analysis the sequences of the variable regions were examined to determine if the SNP associated with the different haplotypes altered restriction sites. Haplotype III could be differentiated from the other haplotypes due to the base change at base 7,868 in DQ 832718 (in the spacer between the *cox1* and *cox2* genes) generating an additional *Nla*III site. To amplify this region for RFLP analysis the *Phytophthora* genus specific primer pair Phy-8b and Phy-10b was used with previously reported amplification conditions (Martin et al. 2004). Digestion with *Nla*III was done in accordance with the manufacturer's instructions (New England Biolabs).

A portion of the *cox1* gene was amplified and sequenced using primers FM50 and FM38 (Table 2), which amplify part of the same region as primers PrnestF and PrnestR (Kroon et al. 2004) that have been used to differentiate the EU1 haplotype (I) from the NA1 haplotype (IIa) by RFLP analysis with *Apo*I. Sequence analysis of this region was done for all isolates listed in Table 1 to evaluate if additional SNPs were present and if restriction sites of other restriction enzymes could differentiate all mitochondrial haplotypes.

Phylogenetic analysis

A total of 5,743 bp of *P. ramorum* sequences were used in the alignment used for phylogenetic analysis; 915 bp of the *cox2* gene + spacer region, 463 bp of the *cox1* gene, 744 bp

Table 2 Primers used for amplification and sequencing in this investigation

Marker	Amplification primers	Position in mt genome[a]	Amplicon size (bp)	MgCl$_2$ Concentration	Annealing temperature (°C)
Prv-1	Prv1-F CAATTGATGTATCTATTCATAC	3939–3960	885	3	62
	Prv1-R AAACTACCAACTACCATAATC	4803–4823			
Prv-8	Prv-8 F GTTCTCATTAATTCTTGTTGA	12673–12693	367	4	52
	Prv8-R TTTATATTTGGTGTAGTGATG	13019–13039			
Prv-9	Prv9-F CTATTAACATTTAAGATAGTCCA	13661–13683	563	4	52
	Prv9-R GAACAATTTGAAATTTTACCTA	14202–14223			
Prv-11	Prv11-F CTTCAGCTTCAGGTAAATC	27946–27964	346	4	52
	Prv11-R GATTTGGTATGTTTTTTACTG	28270–28291			
Prv-13	Prv13b-F CCTGAATATAGTAATCATTCTG	33816–33817	1,295	3	59
	Prv13-R TAATGAAATTCCACTAATTTCA	35089–35110			
Prv-14	Prv14-F GATTATAACAGATACTCTTTC	36904–36924	1,590	3	57
	Prv14-R AAAAATTTTACGTAATAAATACAT	38470–38493			
ymf16	SecY-F TCTATCGTGTTTACCAATTTC	30861–30881	946	3	59
	SecY-R TAACAAATGGATCTTCTTTAAAA	31784–31806			
cox2 + spacer[b]	FM 35 CAGAACCTTGGCAATTAGG	7110–7128	1,041	4	54
	Phy10b GCAAAAGCACTAAAAATTAAATATAA	8125–8150			
cox1[b]	FM50 - GTTTACTGTTGGTTTAGATG	8952–8971	463	1	50
	FM38 - AGCATCAGGAAAATCAGG	9397–9414			
Sequencing primers					
Prv-13	Prv13fd AATTGGAATTCTAAATGGATTG	34,008–34,029			
	Prv13br TGAAGTTACAGGTGTATCTG	34,986–35,005			
	Prv13c-r AAAAAATTCTTGAAGCATATTATC	34,488–3,4511			
Prv-14	Prv14b-f AATAAAACGTTTATCAAAACCAG	37,117–37,139			
	Prv14b-r ATAATATAATCATGTCGACTAC	38,024–38,045			
Cox2 + spacer	FM82 TTGGCAATTAGGTTTTCAAGATCC	7117–7140			
	FM80 AATATCTTTATGATTTGTTGAAA	8097–8117			

[a] Nucleotide position for the mitochondrial genome of *P. ramorum* (DQ 832718; Martin et al. 2007)

[b] FM35 was previously reported in Martin (2000), FM50 in Martin and Tooley (2003) and Phy10b in Martin et al. (2004)

of *ymf16*, 641 bp of Prv-1, 354 bp of Prv-8, 536 bp of Prv-9, 340 bp of Prv-11, 916 bp of Prv-13, and 834 bp of Prv-14. For the purpose of rooting the tree the same regions were sequenced in an isolate of *P. hibernalis* (isolate P10655) with the exception of Prv-1 and the *cox1* and *cox2* spacer region (due to length variation in these noncoding regions making it difficult to accurately align the sequences) with the total size of the aligned sequences 4,892 bp (including gaps for *P. hibernalis*). Sequence alignments were optimized using MacClade ver. 4.02 (Sinaur Associates, Sunderland, MA, USA), and PAUP ver. 4.0b10 (Sinaur Associates) was used for phylogenetic analyses. Phylogenetic relationships were inferred by maximum likelihood analysis with heuristic searches performed with MULPARS on, steepest decent option off, random addition of sequences (10 replicates) and TBR branch swapping. To determine support for the various clades of the trees, the analysis described above was bootstrapped with 1,000 replicates with the same conditions noted above. Analysis using maximum parsimony and distance criteria was evaluated as well. DNA sequence data obtained in this study

have been deposited in GenBank (Table 3) and the results of the phylogenetic analysis have been deposited in Tree-BASE (S2051).

Results

Mitochondrial genome of *P. ramorum* strain CBS101553

The mitochondrial genome of *P. ramorum* strain CBS101553 from Europe (GenBank accession EU427470) maps circular in orientation and is 39,494 bp in size (180 bp larger than DQ 832718). It encodes the same set of genes in the same order as Pr-102, the strain from California (DQ 832718); 37 recognizable protein- and rRNA-encoding genes without introns (18 respiratory chain proteins, 16 ribosomal proteins, the rRNAs for the large and small ribosomal subunits, and an import protein (*ymf16*) of the *sec*Y-independent pathway), 8 putative open reading frames, and 26 tRNAs encoding 19 amino acids. As in DQ 832718 there was an inverted repeat 1,150 bp in size. Start
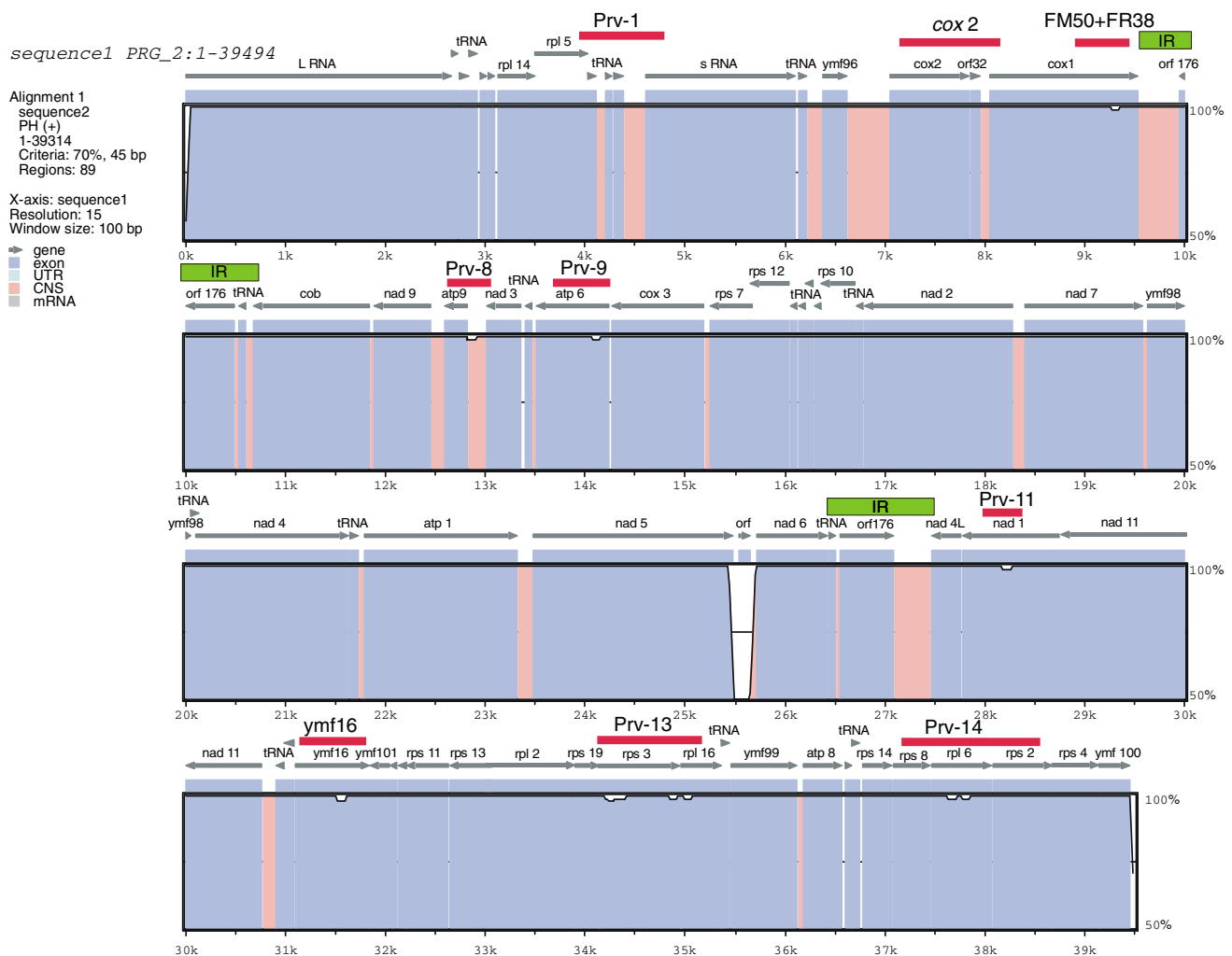
Fig. 1 VISTA plot comparison between the mitochondrial genome of *Phytophthora ramorum* isolate CBS101553 from Europe (reference sequence, EU427470) and isolate P-102 (DQ 832718) from California. Coding regions are denoted by the *blue color* and the *labeled arrows* above the plot indicating the direction of transcription while noncoding regions are denoted by the *pink color*. The *height* of the *peaks* indicates the percent identity of sequences for a 45 bp moving window. The *green box* marked "IR" is the region of the inverted repeat in *P. ramorum* while the *labeled red lines* represent the PCR amplified regions used for additional sequence analysis

and stop codons were also the same as previously reported (Martin et al. 2007).

Comparison between the mitochondrial genome of strain CBS101553 (haplotype I) and DQ 832718 (haplotype IIa) revealed 13 single nucleotide polymorphisms (SNPs) distributed throughout the genome with all but one of them (Prv-8) in coding regions (Fig. 1). The base changes in 8 of the SNPs were transversions and represented a mixture of synonomous/nonsynonomous substitutions (Table 4). There also was a 180 bp insertion located between the *nad*5 and *nad*6 genes in the genome of CBS101553 (haplotype I). In DQ 832718 (haplotype IIa) the spacer region between these genes is 66 bp whereas in CBS101553 (haplotype I) it is 246 bp. This size difference is due to a duplication of the sequences in this area with the first 66 bp of the spacer identical to DQ 832718 followed by 108 bp of the 5′ end of

the *nad6* gene plus 6 bp of the terminal sequences of the *nad5* gene, followed a second copy of the 66 bp spacer (Fig. 2). Within this additional sequence is a putative open reading frame that is 114 bp (*orf37*).

Orientational isomers

Summation of the band sizes from double digests of puri-fied mitochondrial DNA revealed sizes larger than the 39.5 kb of the genome (75.1 and 59.4 kb for *Pvu*II + *Sal*I and *Kpn*I + *Sac*I, respectively) (Fig. 3). For *Pvu*II + *Sal*I the 19.1 and 16.7 kb bands correspond to the sequence orienta-tion observed in EU427470 whereas the fainter 22.5 and 13.2 kb bands were reflective of the reverse orientation of the region between the inverted repeats. Likewise, for *Kpn*I + *Sac*I the 9.9 and 16.6 kb band corresponded to the

Table 3 GenBank accession numbers for polymorphic regions of the *Phytophthora ramorum* mitochondrial genome used to determine mitochondrial haplotype

Marker	Mitochondrial haplotype[a]			
	I	IIa	IIb	III
Prv-1	EU427475	EU427476	EU427478	EU427477
Prv-8	EU427479	EU427480	EU427482	EU427481
Prv-9	EU427483	EU427484	EU427486	EU427485
Prv-11	EU427487	EU427488	EU427490	EU427489
Prv-13	EU427491	EU427492	EU427494	EU427493
Prv-14	EU427495	EU427496	EU427498	EU427497
ymf16	EU427499	EU427500	EU427502	EU427501
Cox2 + spacer	EU427471	EU427472	EU427474	EU427473

[a] Mitochondrial haplotype I is from Europe, IIa is the most common haplotype in the USA, haplotype IIb is from Oregon forest isolates, and III represents the NA2 nuclear lineage from Washington State (Ivors et al. 2006)

sequence orientation observed in EU427470 whereas the fainter 3.5 and 23.0 kb were reflective of the reverse orientation of the region between the inverted repeats.

Mitochondrial haplotype determination

While genomic comparisons between the sequenced NA1 (haplotype IIa) and EU1 (haplotype I) mitochondrial genomes revealed 13 SNPs and a 180 bp indel; expanding the analysis to include 40 additional isolates for 8 loci identified a total of 28 SNPs. The grouping of these SNPs revealed a total of 4 mitochondrial haplotypes; the traditional EU1 (haplotype I) and NA1 (haplotype IIa) that had 13 SNPs between them, the NA2 lineage of the pathogen recovered from a nursery in Washington State (haplotype III) that shared some SNPs with both haplotype I and IIa but also had an additional 14 unique SNPs differentiating this haplotype from the others, and a haplotype representing

Table 4 Positions of the single nucleotide polymorphisms observed in the mitochondrial genomes of various isolates of *Phytophthora ramorum*

Marker	SNP[a]	Mitochondrial haplotype[b]				Substitution[c]
		I	IIa	IIb	III	
Prv-1	4,248	G	G	G	A	–
	4,398	G	G	G	A	–
Prv-8	12,859	A	G	G	G	–
	12,912	A	A	A	G	–
Prv-9	14,113	T	G	G	G	S
Prv-11	28,045	T	C	C	C	NS
	28,259	T	T	T	A	S
Prv-13	34,053	T	C	C	T	S
	34,089	C	G	G	G	NS
	34,137	A	A	A	G	NS
	34,175	A	C	C	C	S
	34532	A	A	A	C	S
	34,695	G	T	T	G	NS
	34,847	A	G	G	G	NS
	34,864	C	C	C	T	S
Prv-14	37,297	A	A	T	A	NS
	37494	T	A	A	A	NS
	37,560	T	T	T	C	S
	37,630	A	C	C	A	S
ymf16	31,371	T	A	A	T	S
	31,377	T	G	G	T	NS
cox1	8,980	A	A	A	C	S
	9,312[d]	C	T	T	T	S
	9,327	T	T	T	C	S
	9,373	T	T	T	C	S
Cox2 + spacer	7,294[e]	A	A	A	C	S
	7,868 (spacer)	A	A	A	G	–
	7,963 (spacer)	T	T	T	A	–

[a] SNP, single nucleotide polymorphism at the indicated base position in the mitochondrial genome of *P. ramorum* (DQ 832718)

[b] Mitochondrial haplotype I is from Europe, IIa is the most common haplotype in the USA, haplotype IIb is from Oregon forest isolates, and III represents the third nuclear lineage of Ivors et al. (2006) from Washington State

[c] Substitution type with S, synonymous; NS, nonsynonymous and "–" for noncoding regions

[d] This is the SNP used by Kroon et al. (2004) to differentiate the EU from NA haplotypes by RFLP analysis

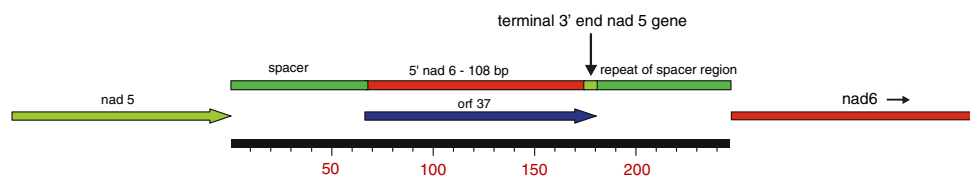[e] Base 234 of the *cox2* gene

Fig. 2 Organization of the spacer region between the *nad5* and *nad6* genes in *Phytophthora ramorum* isolate CBS101553 from Europe (haplotype I) where there was a 180 bp insertion relative to isolate P-102 (DQ 832718) from California
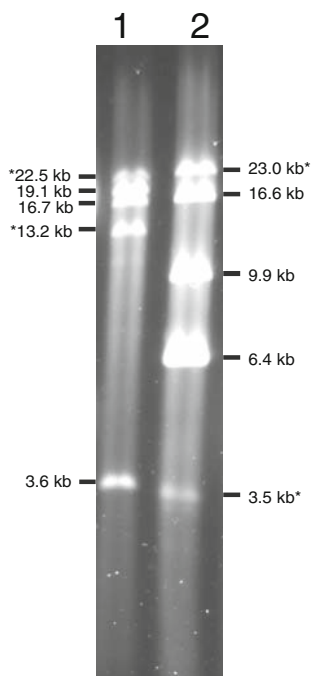

Fig. 3 Double digests of purified mitochondrial DNA that demonstrate the presence of two orientational isomers for the mitochondrial genome of *Phytophthora ramorum* isolate CBS 101553. *Lane 1* is double digests with *Pvu*II + *Sal*I whereas *lane 2* is *Kpn*I + *Sac*I that have been separated on a 0.7% agarose gel. The values on the sides reflect the molecular size of the indicated bands with those marked with an "*" from the isomeric form reflecting an inversion of the region between the inverted repeat relative to EU427470

a subgroup of isolates from an Oregon forest (haplotype IIb) that differed from haplotype IIa at only 1 position in Prv-14 (Table 4). Comparisons between haplotype I and III exhibited the highest number of SNPs with 22, followed by haplotype III and IIb (20 SNPs), and haplotype I and III (19 SNPs).

RFLP analysis to differentiate haplotype III and IIb

Digestion of the genus-specific amplicon generated using prime pair Phy8b and Phy10b with *Nla*III cleaved the 457 bp amplicon at base 55 for all isolates of *P. ramorum* examined. There also was an additional restriction site at the SNP differentiating haplotype III mtDNA from the other haplotypes, which generated a unique restriction pat-

tern for this haplotype (Fig. 4). There are other SNPs that could be useful in RFLP analysis for differentiating haplotypes but were not tested because specificity for *Phytophthora* when amplified from infected tissue had not been validated with these primer pairs. In marker Prv-1 there is an additional *Bsm*FI site (base 4,248 in DQ 832718) and in marker Pv8 there is an additional *Msl*I site (base 12,912 in DQ 832718) in haplotype III relative to the other three haplotypes. Likewise, in haplotype IIa there is a loss of an *Alu*I site in the *cox2* gene (base 7,293, DQ 832718) relative to the other haplotypes. There were no suitable restriction enzymes available for differentiating haplotype IIb from the others, while *Mse*I cut at the SNP of this haplotype this restriction enzyme generated to many small fragments to be useful.

Phylogenetic analysis

Sequence data for 7 loci from the 4 haplotypes of *P. ramorum* and an isolate of *P. hibernalis* were concatenated for a total of 4,892 bp and used in phylogenetic analysis with the tree rooted to the outgroup *P. hibernalis* (321 variable sites, only 10 of which were parsimony informative). Maximum likelihood analysis revealed strong bootstrap support for the close affinity of haplotype IIa and IIb but weaker support (boot strap value of 73%) for the basal grouping of haplotype I to these two haplotypes with haplotype III as the ancestral haplotype (Fig. 5). Similar results were observed when the analysis was repeated using maximum parsimony or distance criteria (data not shown). Removing *P. hibernalis* from the data set and adding sequence data from *cox1–cox2* and Prv-1 spacer regions of *P. ramorum* mitochondrial DNA for a total of 5,743 bp generated a data set that had 28 polymorphic sites, only 5 of which were parsimony informative. Additional phylogenetic analysis with this data set did not further resolve the relationships of haplotype III with the others (data not shown).

Discussion

The sequence of the mitochondrial genome of the type culture of *P. ramorum* from Europe (CBS101553) is identical
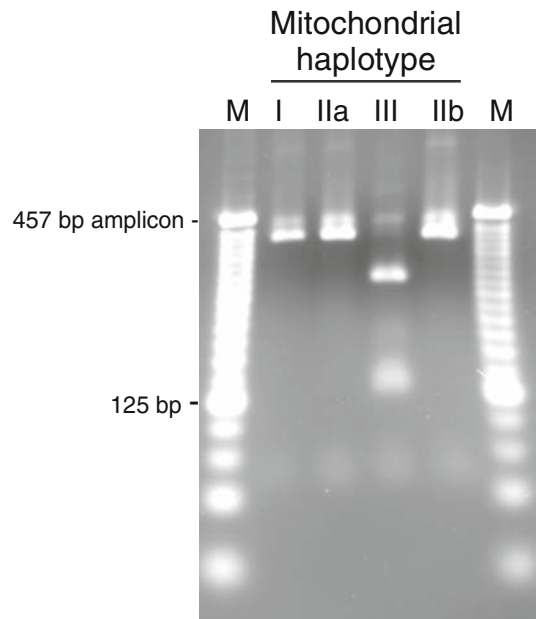
Mitochondrial
haplotype

Fig. 4 *Nla*III digest of the amplicon generated by primers Phy8b and Phy10b for the 4 mitochondrial haplotypes of *Phytophthora ramorum* separated on a 4% Nusieve 3:1 agarose gel. The fainter band at 457 bp in each *lane* is nondigested amplicon. *Lane M* is for the 25 bp ladder molecular size marker

Fig. 5 Maximum likelihood tree inferred by a heuristic tree search illustrating the phylogenetic relationship among the different mitochondrial haplotypes of *Phytophthora ramorum*. Values at the nodes of the trees reflect the percentage of bootstrap replicates (1,000 replicates) that supported the observed topography. Analysis based on 4,871 bp of *P. ramorum* sequence data from the *cox2* gene, *cox1* gene, *ymf16*, and Prv-8, -9, -11, -12, -13, and -14 regions with the tree rooted to *P. hibernalis*. The phylogram was expanded horizontally to separate the branches for *P. ramorum* mitochondrial haplotypes such that the branch length for *P. hibernalis* is not reflective of the observed level of sequence divergence

to the previously published genome sequence of isolate Pr-102 from California (DQ 832718; Martin et al. 2007) with the exception of 13 SNPs distributed throughout the genome and a length variation of 180 bp in the space between the *nad*5 and *nad*6 genes. The SNPs were primarily in coding regions and represented a combination of synonymous and nonsynonymous substitutions. The length variation was unusual in that it was caused by a duplication of the spacer sequences and the first 108 bp of the *nad*6 gene that appeared to be inserted in front of the terminal 6 bp of the *nad5* gene. The boundaries of this insertion were examined and revealed that the terminal 7 bp of the *nad5* gene and the first 7 bp of the spacer were identical (AAAATAA; left boundary of the insertion) and the terminal 13 bp of the 108 bp portion of the 5′ end of the *nad6* gene and the 13 bp upstream from the terminal 6 bp of the *nad5* gene were identical (TTTTTTTTTAATA; right boundary of insertion), which may have contributed to the translocation of duplicated sequences into the current position.

Double digests with restriction enzymes supports the presence of orientational isomers of the region in between the IRs due to recombination between the repeated sequences inverting the gene order. This has been previously reported for other oomycetes such as *Achlya ambisexualis* (Hudspeth et al. 1983), *A. klebsiana* (Boyd et al. 1984) and *Saprolegnia ferax* (Grayburn et al. 2004), however, the IR is larger in these species compared to what is
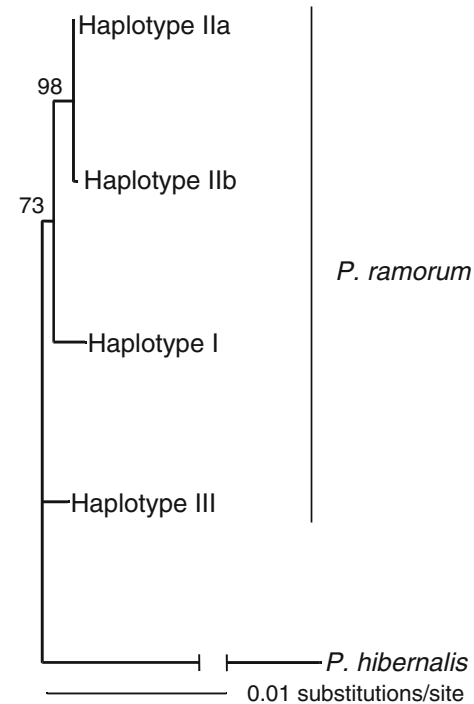
found in *P. ramorum* and also encodes a different group of genes. For example, the IR in *S. ferax* encodes the two ribosomal RNA subunits, four functional genes, 5 tRNAs and represents 37% of the genome size (the IR is 8,618 bp in size); similar results for the size of the IR were also observed for *Achlya ambisexualis* (Hudspeth et al. 1983) and *A. klebsiana* (Boyd et al. 1984). In contrast, for *P. ramorum* the IR encodes only a putative open reading frame (*orf176*) and at 1,150 bp, represents only 5.8% of the genome size. Even though it is comparatively smaller in size it is large enough for a translocation between the two repeat elements to occur, which can be important in genome evolution at an interspecific level as changes in gene order due to inversions is observed among species in the genus (Martin et al. 2007, F. Martin, P. Richardson, unpublished).

The observed level of genome polymorphisms between the EU1 and NA1 mitochondrial genomes (haplotype I and IIa, respectively) was similar to what was reported for comparisons between haplotype Ia and Ib of *P. infestans* (Avila-Adame et al. 2005), which differed by 11 SNPs (8 of

which were in coding regions), 3 single base indels, and a 36 bp indel in the space between *ymf96* and the *cox2* gene (this 36 bp indel was a subrepeat that was present twice in haplotype Ia but three times in haplotype Ib). There was a greater level of polymorphisms observed in comparisons between haplotype I and II of *P. infestans*, with as many as 152 polymorphic sites and a large indel adjacent to the small ribosomal RNA. There was no similarity in the locations of polymorphic bases from intraspecific comparisons for *P. ramorum* and *P. infestans*.

Previous work had identified 3 mitochondrial haplotypes of *P. ramorum* based on sequence differences in the *cox1* gene (Kroon et al. 2004; Ivors et al. 2006). This current study used whole mitochondrial genome comparisons to get a more complete evaluation of intraspecific variation between isolates representing the most commonly encountered EU1 and NA1 populations of the pathogen (haplotypes I and IIa, respectively). The 13 SNPs observed were distributed throughout the genome but could be sequenced by amplification of 7 specific regions. Amplification and sequencing of these regions from an additional 40 isolates representing the geographical distribution of the pathogen identified 9 new SNPs and an additional haplotype (haplotype IIb) represented by three isolates recovered from *Lithocarpus densiflorus* in an Oregon forest. This haplotype differed from haplotype IIa by a single nonsynonymous substitution in the *rps8* gene.

In addition to the seven regions where SNPs were observed in comparisons between haplotype I and IIa mitochondrial genomes, two other regions that were polymorphic in other studies examining *Phytophthora* mitochondrial DNA were selected for additional analysis. The region between the *rpl5* gene and the sRNA (Prv-1) exhibited polymorphisms in intraspecific comparisons among *P. infestans* mitochondrial haplotypes (Avila-Adame et al. 2005) as well as in interspecific comparisons for a range of species (Schena and Cooke 2006). Likewise, preliminary results from several isolates of *P. ramorum* obtained while conducting phylogenetic analysis also identified intraspecific variation in the *cox2* gene and adjacent spacer sequences (F. Martin, unpublished). An additional 6 SNPs were identified in these two regions for the 40 isolates that were examined to bring the total number of SNPs observed in this study to 28. Some of the isolates included in this current study were also part of prior AFLP (Ivors et al. 2004; 11 isolates) or microsatellite studies (Ivors et al. 2006; Prospero et al. 2007; 6 and 8 isolates, respectively, see foot note Table 1); with the exception of haplotype IIb isolates the grouping of the mitochondrial haplotypes corresponded to the grouping obtained with the nuclear markers.

While there were some similarities in the mutation events involved in intraspecific genome evolution in both *P. ramorum* and *P. infestans* (single nucleotide polymor-

phisms primarily in coding regions randomly distributed throughout the genome), the apparent duplication and translocation that led to a size difference in the *nad5* and *nad6* spacer region in haplotype I relative to haplotype IIa of *P. ramorum* was not observed in *P. infestans*. Likewise, some of the mutation events leading to intraspecific differences in *P. infestans* were not observed in *P. ramorum* (single base indels, different numbers of subrepeats, larger insertion events upstream from the small ribosomal RNA). Although *P. ramorum* has an inverted repeat (1,150 bp) and *P. infestans* does not, given the location of intraspecific mutation events in *P. ramorum* it does not appear this influenced the generation of intraspecific genome variation other than to generate orientational isomers.

The ability to use a rapid diagnostic procedure for identification of specific mitochondrial haplotypes would be useful for tracking the movement of specific populations of the pathogen as well as a cytoplasmic marker that can be used for determining the maternal parent in the event of outcrossing. Kroon et al. (2004) had reported on a PCR-RFLP technique for differentiation of the common EU1 and NA1 mitochondrial haplotypes (haplotype I and IIa, respectively), but sequence analysis of the *cox1* gene reported in Ivors et al. (2006) and of the amplicon generated by primer pair FM50 and FM38 used in this study revealed this locus could not be used to differentiate haplotypes III and IIb from haplotype IIa. However, there was a SNP in the spacer region between the *cox1* and *cox2* genes that generated a unique *Nla*III restriction site that could differentiate haplotype III from the others (Fig. 4). This region was amplified by Phy-8b and Phy-10b, reported previously as the first round primer pair used in a nested diagnostic assay for *P. ramorum* (Martin et al. 2004). One advantage of this primer pair is that it has been demonstrated to be *Phytophthora* genus specific and not amplify sequences from a range of plant species or the closely related genus *Pythium*, which simplifies the use of this marker system for regulatory purposes because culturing of the pathogen is not needed, amplification can be done directly on field samples. While not experimentally validated, sequence analysis of the *cox1* region for all 4 mitochondrial haplotypes for part of the region amplified by primers PrnestF and PrnestR of Kroon et al. (2004, primers FM50 and FM38 amplify a portion of this same amplicon) revealed an additional *Bfa*I restriction site in haplotype III such that a double digest with *Apo*I and *Bfa*I would cleave the 84 bp amplicon of the Type IIa restriction banding pattern into 65, 13 and 5 bp fragments for haplotype III and into 78 and 5 bp fragments for haplotype IIa and IIb (haplotype I would be 91 and 5 bp).

The phylogenetic analysis of the EU1, NA1 and NA2 Washington State isolates of *P. ramorum* (mitochondrial haplotypes I, IIa, and III, respectively) in Ivors et al. (2006) used 743 bp of the 3′ region of the *cox1* gene with the

results indicating a close affinity between the EU1 and NA1 isolates with the NA2 lineage from Washington State basal to this clade (89% bootstrap support) when *P. lateralis* was used as an outgroup. The *cox1* region amplified with FM50 and FM38 used in the current investigation includes 391 bp of this same region along with an additional 72 bp of the 3′ end of the gene and generated similar results in phylogenetic analysis when *P. hibernalis* was used as an outgroup with the exception that haplotypes I, IIa, and IIb grouped on the same clade with 85% bootstrap support with haplotype III ancestral (data not shown). When an additional 4,408 bp of sequence data from 6 other loci were added to this dataset a tree with a similar topography was observed, but there was lower bootstrap support (73%) for the ancestral relationship of haplotype III to the other haplotypes (Fig. 5). The relationship between haplotype III and I was also different; in the *cox1* phylogram of Ivors et al. (2006) the Washington State NA2 lineage (mitochondrial haplotype III) had a closer affinity to the NA1 lineage (mitochondrial haplotype IIa) whereas in the expanded analysis in this study the EU1 haplotype I was more closely related to the NA2 haplotype III. Addition of 872 bp of spacer sequences from two loci and rerunning the analysis with *P. ramorum* alone did not provide further clarification of the phylogenetic relationship. Since the center of origin of *P. ramorum* has yet to be determined and isolates from this population are not available for study it is unclear what haplotype III represents in the overall phylogeny of the species, but the data supports it being ancestral to the other NA1 and EU1 haplotypes that have been most commonly encountered thus far.

Given the single SNP differentiating mitochondrial haplotype IIa and IIb it is likely that haplotype IIb represents a mutation event rather than a new introduction of the pathogen. This is supported by the nuclear microsatellite groupings of isolates observed in Prospero et al. (2007) where the authors concluded that the populations from Oregon were clonal. Some of the same isolates were included in this current study and mitochondrial haplotype IIb isolates 2092 (multilocus genotype PrOR10) and 1033.1 (multilocus genotype PrOR12) were interspersed in their multilocus genotype groupings with mitochondrial haplotype IIa isolates 1020.1, 4301.1, 4361, 04-207-Q, and 04-189-B5 (multilocus genotype PrOR2, PrOR5, PrOR8, PrOR31, PrOR32, respectively; Nik Grunwald, personal communication).

The results of this study have identified 28 SNPs in comparisons among 42 isolates of *P. ramorum* that represent only 4 mitochondrial haplotypes, with two predominating (haplotype I and IIa) and a restricted distribution for the two others (haplotype III and IIb). This is not surprising given the clonal nature of the populations that have been examined (Ivors et al. 2004, 2006; Prospero et al. 2007) and it is likely that haplotypes I, IIa, and III are reflective of the isolates initially introduced that founded these populations

while haplotype IIb arose from haplotype IIa by a single base mutation. The extent of the distribution and genotypic characterization of haplotype IIb isolates is unknown at this time, but is the subject of further investigation. This study has relied on the results of whole mitochondrial genome sequence comparisons between two geographically diverse isolates to identify polymorphic loci for further analysis, but since 15 of the 28 SNPs were not observed in this comparison it is possible that additional polymorphisms may exist in other isolates at other loci that may identify additional haplotypes. While haplotype determination provides a useful cytoplasmically inherited marker for classification of isolates it is unclear what the biological significance this classification has, but given the clonality of the pathogen populations it will likely be a reflection of the nuclear background of the isolates and provides an additional tool for population studies and regulatory efforts.

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