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RESEARCH ARTICLE

Mitochondrial genome sequences and comparative genomics of *Phytophthora ramorum* and *P. sojae*

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Abstract The sequences of the mitochondrial genomes of the oomycetes *Phytophthora ramorum* and *P. sojae* were determined during the course of complete nuclear genome sequencing (Tyler et al., Science, 313:1261,2006). Both mitochondrial genomes are circular mapping, with sizes of 39,314 bp for *P. ramorum* and 42,977 bp for *P. sojae*. Each contains a total of 37 recognizable protein-encoding genes, 26 or 25 tRNAs (*P. ramorum* and *P. sojae*, respectively) specifying 19 amino acids, six more open reading frames (ORFs) that are conserved, presumably due to functional constraint, across *Phytophthora* species (*P. sojae*, *P. ramorum*, and *P. infestans*), six ORFs that are unique for *P. sojae* and one that is unique for *P. ramorum*. Non-coding regions comprise about 11.5 and 18.4% of the genomes of *P. ramorum* and *P. sojae*, respectively. Relative to *P. sojae*, there is an inverted repeat of 1,150 bp in *P. ramorum* that includes an unassigned unique ORF, a tRNA gene, and adjacent non-coding sequences, but otherwise the gene order in both species is identical. Comparisons of these genomes with published sequences of the *P. infestans* mitochondrial genome reveals a number of similarities, but the gene order in *P. infestans* differed in two adjacent locations due to inversions and specific regions of the genomes exhibited greater divergence than others. For example, the breakpoints for the inversions observed in *P. infestans* corresponded to regions of high sequence divergence in comparisons between *P. ramorum* and *P. sojae* and the location of a hypervariable microsatellite sequence (eight repeats of 24 bp) in the *P. sojae* genome corresponds to a site of major length variation in *P. infestans*. Although the overwhelming majority of each genome is conserved (81–92%), there are a number of genes that evolve more rapidly than others. Some of these rapidly evolving genes appear specific to *Phytophthora*, arose recently, and future evaluation of their function and the effects of their loss could prove fruitful for understanding the phylogeny of these devastating plant pathogens.

Keywords Inverted repeat · Mitochondrial microsatellite · *Phytophthora infestans*

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Introduction

The genus *Phytophthora* has a wide geographic distribution throughout the world and contains more than 70 species, many of which cause important plant diseases (Erwin and Ribiero 1996). While members of the genus, along with other oomycetes, share morphological similarities with eumycotian fungi, these features have arisen independently, as oomycetes are phylogenetically more closely related to heterokont algae within the kingdom Stramenopiles (Förster et al. 1990; Knoll 1992; Baldauf and Palmer 1993; Wainright et al. 1993; Bhattacharya and Stickel 1994; Weerakoon et al. 1998; Dick 2001). A recent phylogeny of the stramenopiles utilizing multiple mitochondrial genes clarified the relationship between oomycetes and the sister clade of heterokont algae (including brown algae in the Phaeophyceae, diatoms in the Bacillariophyceae, and the Chrysophyceae; Oudot-Le Sec et al. 2006). Within the oomycetes there are two major subclasses, the Peronosporomycetidae (which includes the genus *Phytophthora*) and the Saprolegniomycetidae. Oomycetes differ from eumycotan fungi in features such as being diploid throughout their life cycle and forming motile, biflagellate spores called zoospores that are capable of swimming in water.

The mitochondrial genomes in *Phytophthora* have been reported to be circular mapping and range in size from ~37.0 to 45.3 kb (Paquin et al. 1997; Avila-Adame et al. 2006; McNabb and Klassen 1988; Förster et al. 1987; Shumard-Hudspeth and Hudspeth 1990). These have been commonly used in RFLP studies for identification of isolates and to help clarify the taxonomic placement of particular species (Förster et al. 1988, 1989; reviewed in Erwin and Ribeiro 1996). Mitochondrial gene sequences also have been used to infer phylogenetic relationships among species of the genus as well (Martin and Tooley 2003a, b; Kroon et al. 2004).

The only species of *Phytophthora* for which complete mitochondrial genome sequences are available is *P. infestans*, the causal agent of potato late blight. This has been determined for four separate haplotypes; the first sequenced was later termed Ib (Paquin et al. 1997), with the others being Ia, IIa, and IIb (Avila-Adame et al. 2006). A total of 68 coding regions were identified in these genomes, inferred to encode mitochondrial respiratory chain proteins, subunits of the mitoribosome, ribosomal RNAs, tRNAs, and unassigned open reading frames (ORFs). With the exception of some of the unassigned ORFs, this same set of coding regions were also found in the related oomycete *Saprolegnia ferax* (subclass Saprolegniomycetidae; Grayburn et al. 2004). Intraspecific variation among the *P. infestans* haplotypes includes both single nucleotide substitutions dispersed throughout the genome as well as length variations caused by insertions/deletions that occurred primarily in two locations (Avila-Adame et al. 2006).

Interest in the genus *Phytophthora* has increased lately due to the serious impact several species are having as plant pathogens. *Phytophthora ramorum* is a recently described species that initially was found to be responsible for diseases of nursery crops in Germany and the Netherlands (Werres et al. 2001). This pathogen has been discovered in other European countries and more recently has become a problem in field ecosystems (Brasier et al. 2005). While this species also is a problem in some nursery production crops in North America, a far bigger impact has been its role as the cause of sudden oak death, a disease that has killed large numbers of trees and shrubs in natural ecosystems in central coastal California (Rizzo et al. 2002; Davidson et al. 2003). This is a highly regulated pathogen with stringent quarantine restrictions in place in North America and Europe in an effort to halt its spread. *P. sojae* is widely spread in soybean (*Glycine max*) production areas of North and South America, Asia and Australia and causes serious crop production losses due to root and stem rot (Erwin and Ribiero 1996). There is a continuing effort in soybean breeding programs to develop resistant germplasm as a means for controlling the disease.

Complete draft sequences for the nuclear genomes of *P. ramorum* and *P. sojae* have been recently determined (Tyler et al. 2006). As part of this sequencing project, the complete mitochondrial genomes were also assembled. The objective of this study is to annotate and describe these mitochondrial genomes and compare them to the mitochondrial genomes of *P. infestans* and other oomycetes.

Materials and methods

Strains sequenced

Mitochondrial sequences were obtained from *P. ramorum* strain Pr-102 (isolated form California) and *P. sojae* strain P6497 (isolated from Mississippi). Sequences were obtained from GenBank for the mitochondrial genome of the oomycete *S. ferax* (AY534144) and for each haplotype of the mitochondrial genome of *P. infestans*: Ia (AY894835), Ib (NC_002387), IIa (AY898627), and IIb (AY898628).

Sequencing and contig assembly

For each of these two species, total DNA preparations were 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. An additional library was created in a fosmid vector. End sequences were determined for a large number of randomly selected clones from each of these libraries, then assembled using JAZZ (Aparicio et al. 2002) to form a complete draft whole-genome shotgun assembly of these nuclear genomes. Detailed protocols are available at <http://www.jgi.doe.gov/sequencing> and this process and the results are further described in the report of the complete nuclear genome sequences (Tyler et al. 2006). Although no effort was expended to target the mitochondrial genomes, even a small contamination by mtDNA in these preparations, coupled with the high ratio of these sequences compared to any portion of the nuclear genomes, guarantees that any whole-genome shotgun sequencing projects will include many sequencing reads from clones of mtDNA. In this case, the assembly of *P. sojae* mtDNA included a total of 26,052 successful sequencing reads totaling 17,490,898 nucleotides for ~407-fold coverage. The assembly of *P. ramorum* mtDNA included a total of 3,718 successful sequencing reads totaling 2,622,145 nucleotides for ~67-fold coverage.

Annotation and comparative genomics

Annotation of coding regions and prediction of ORFs was done with DS Gene v1.5 (Accelrys, San Diego, CA, USA) using the universal genetic code. Identification of protein- and rRNA-encoding genes was done by comparison with sequences reported for *P. infestans* (Paquin et al. 1997; NC_002387) and BLAST analysis to other sequences in GenBank. Genes for tRNAs were found using tRNAscan SE v1.1 (Lowe and Eddy 1997; http://www.genetics.wustl.edu/eddy/tRNAscan-SE/). Pairwise 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.

Results

Genome size and organization

The mitochondrial genomes for both species map in a circular orientation and range in size from 39,314 bp for *P. ramorum* (Fig. 1; GenBank DQ 832718) to 42,977 bp for *P. sojae* (Fig. 2; GenBank DQ 832717) with a GC content of 22.0 and 21.7%, respectively. This compares to 37,957 bp for the Ib haplotype (Paquin et al. 1997) and 37,992, 39,870, and 39,840 bp for the Ia, IIa, and IIb haplotypes of *P. infestans*, respectively (Avila-Adame et al. 2006). The 37 protein- and rRNA-encoding genes of known function in *P. infestans* mtDNA are also present in *P. sojae* and *P. ramorum*, and none contain introns. This set comprises 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 (Fig. 3). ATG is the start codon for all genes and TAA is the stop codon for all genes except *nad11*, for which it is TGA. The *nad11* gene also ends with TGA in *P. infestans* (Paquin et al. 1997), but it is TAA in *S. ferax* (Grayburn et al. 2004).

The tRNA-scan search in *P. sojae* mtDNA identified a set of 25 tRNAs specifying 19 amino acids that are homologous to those reported for *P. infestans* (Paquin et al. 1997), including two copies each of the tRNAs for *trnG* (anticodons gcc and ucc), *trnL* (uaa, uag), *trnR* (ucu, gcg), *trnS* (gcu, uga), and *trnM* (cau). There is an additional tRNA gene with an anticodon of cau, but we interpret this as *trnI* because of its similarity to an homologous tRNA gene of *P. infestans* and *S. ferax* (Paquin et al. 1997; Grayburn et al. 2004) wherein the first anticodon nucleotide is post-transcriptionally modified to lysidine to allow translation of the AUA codon for isoleucine (Gray et al. 1998) (as opposed to the ATG codon for methionine). The *trnT* gene could not be identified in these genomes. *P. ramorum* has this same tRNA gene set plus an additional copy of *trnR*(*ucu*) adjacent to *cob* relative to *P. sojae* due to this tRNA being encoded in the inverted repeat (discussed below).

Some variability in gene sizes was observed, in the 3′ end of *cob*, *rps3*, *rps13*, and *ymf16* and in other regions for *rpl5*, *rps11*, and *rps19* (Table S1). Size variation was noted also for *rps7* due to the position of the start codon. In *P. ramorum* the start codon could be inferred to be in the same relative position as in *P. infestans* (base 15,676, giving a coding region 477 bp long) but in *P. sojae* there is no start codon in this position and one 45 bp downstream was inferred (both *P. ramorum* and *P. infestans* have a potential start codon at this position as well). Length variation was also observed in comparison with *S. ferax*, where the gene is 531 bp in size (Grayburn et al. 2004).

There are five predicted open reading frames (ORF; *orf32*, *orf79*, *orf100*, *orf142*, and *orf217*) previously described in *P. infestans* (Paquin et al. 1997) as well as an additional ORF for this species (*orf64*)

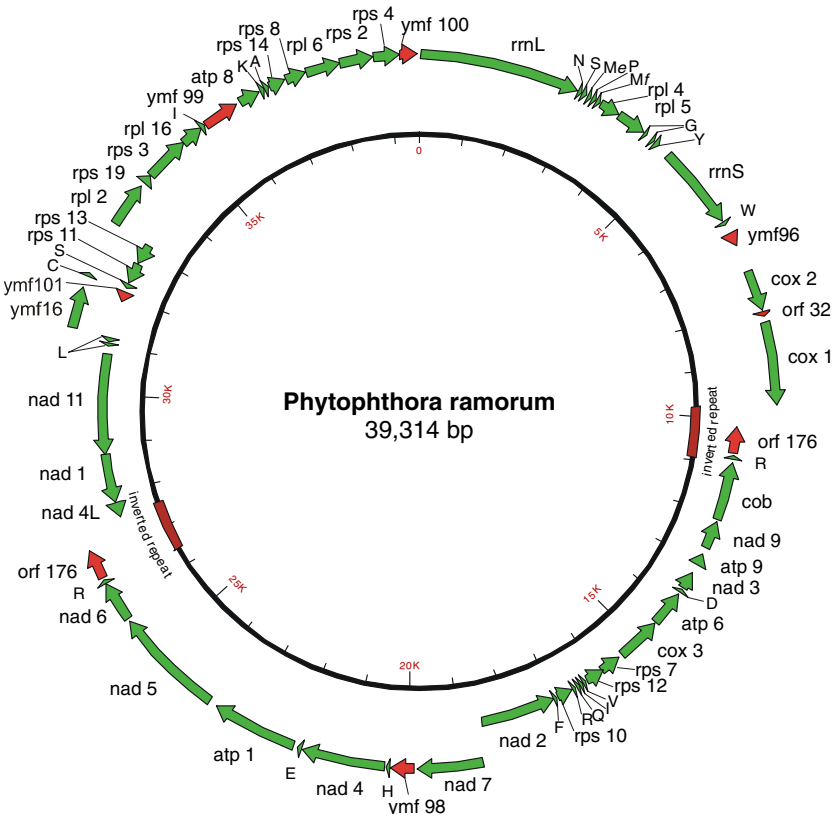

Fig. 1 Mitochondrial gene map for *Phytophthora ramorum*. *Arrows* indicate transcriptional orientation, *clockwise* for the outer row and *counterclockwise* for the inner row, with *green* representing coding regions and *red* representing other unidentified ORFs. Genes for transfer RNAs are designated by the one-letter code for the corresponding amino acid; *Me* and *Mf* indicate the genes for the methionine transfer RNAs that play a role in elongation and initiation with formylmethionine, respectively. The position of the *inverted repeat* is indicated on the inner ring. The designation of "*ymf*" indicates unassigned ORFs that are conserved in the genus whereas "*ORF*" indicates unassigned ORFs of this number of predicted amino acids without obvious homologs

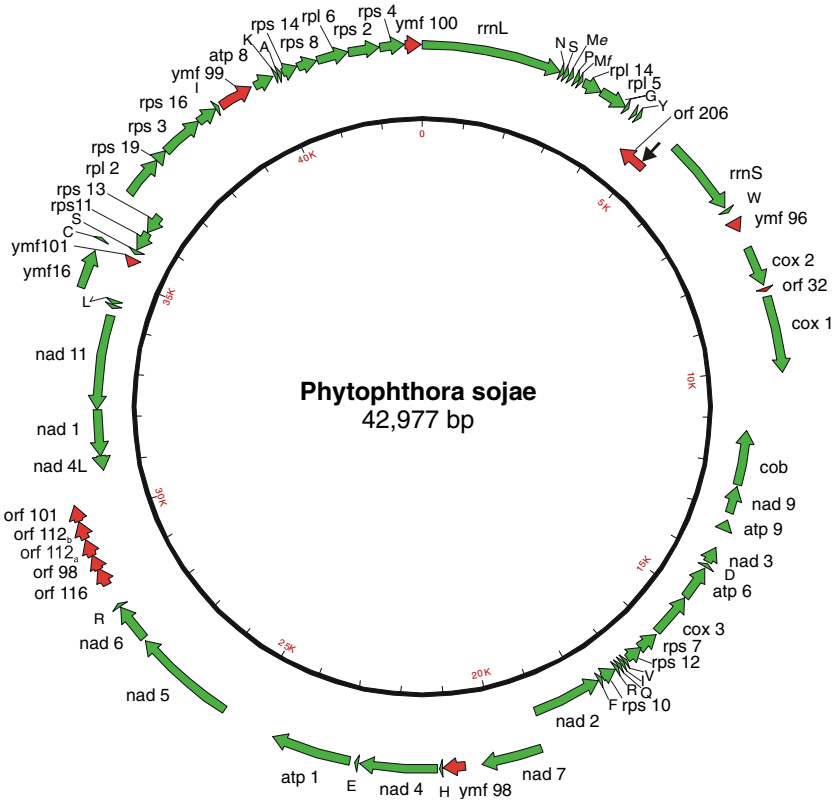

Fig. 2 Mitochondrial gene map for *Phytophthora sojae*. *Genes* are indicated as in Fig. 1. The *arrow* indicates the site of the hypervariable microasatellite within the 5′ end of *orf206*

noted by Grayburn et al. (2004) that appear to have homologues in *P. ramorum* and *P. sojae*. Due to small differences in predicted amino acid lengths for *orf64*, *orf79*, *orf100*, *orf142*, and *orf217* these ORFs will be referred to by their *ymf* designation (*orf64* = *ymf101*, *orf79* = *ymf96*, *orf100* = *ymf100*, *orf142* = *ymf98*, and

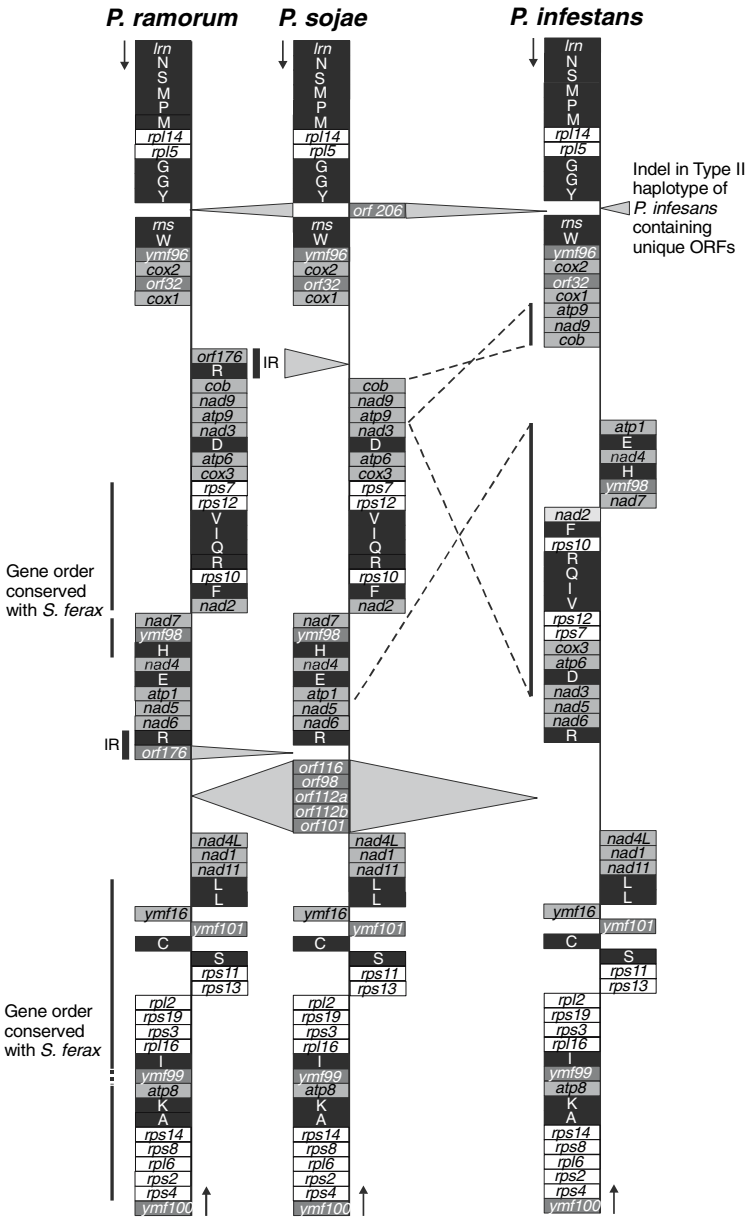

Fig. 3 Comparison of the mitochondrial gene order for *Phytophthora ramorum*, *Phytophthora sojae* and *Phytophthora infestans* (haplotype Ib; Paquin et al. 1997; Grayburn et al. 2004). This is a linear representation of the gene order in the circular mapping genomes and does not take into account spacing between coding regions. *White blocks* represent ribosomal protein encoding genes; *light gray blocks* with *black* lettering represent genes coding for proteins involved in respiratory chains; *dark gray blocks* with *white* lettering represent open reading frames; and *black blocks* represent RNA genes (rRNA and tRNA). The inverted repeat in *P. ramorum* is indicated by the *thick vertical line* labeled IR. The *thick black vertical lines* to the left of *P. infestans* and the *dashed lines* to *P. sojae* represent the two inversions in *P. infestans* relative to the other two species. Regions of gene order conservation with *Saprolegnia ferax* (Grayburn et al. 2004) are indicated on the left. The *dashed line* at *ymf99* indicates that in *S. ferax* this region is represented by *orf273*, which has little sequence conservation with *ymf99*

orf217 = *ymf99*). Comparative analysis of the DNA sequence differences between *P. sojae*, *P. ramorum*, *P. infestans*, and, where there is a clear homolog, *S. ferax*, suggests that these ORFs are functional (Table 1). In all cases where comparisons could be made, there is a lower frequency of point substitutions that would result in amino acid changes (changes at non-synonymous sites; dN), than at synonymous sites (dS). Moreover, in most cases the ratio of non-synonymous to synonymous difference (dN : dS) is significantly lower than 1, as expected from protein-coding sequence and not as expected for pseudogenes (Table 1). The case is less clear for *ymf96* (*orf79*) because analysis of synonymous and non-synonymous sites was challenged by the accumulation of small insertions and deletions. However, divergence at synonymous sites is very high (dS in Table 1), and so the existence of recognizable homologues of *ymf96* in *P. sojae*, *P. ramorum* and *P. infestans* suggests that even this ORF is under some functional constraint.

Phytophthora sojae and *P. ramorum* mtDNAs contain six and one predicted ORFs, respectively, longer than 100 bp, that are not present in the other species. These range in size from 297 to 621 bp and are named after their amino acid length (excluding the termination codon). The six additional ORFs in *P. sojae* are present in two locations: *orf206* is between *trnY*(*gua*) and *rrnS* and the remaining five (*orf116*, *orf98*, *orf112*$_a$, *orf112*$_b$, and *orf101*) are clustered together between *nad6* and *nad4L* (Fig. 2). The 3′ end of *orf116* overlaps

Table 1 DNA sequence conservation of open reading frames (*ORF*) shared among *Phytophthora infestans*, *P. ramorum*, *P. sojae*, and *Saprolegnia ferax*

		bp	*Phytophthora ramorum*		*Phytophthora sojae*		*Saprolegnia ferax*[b]	
			DNA divergence[c] (%)	dN, dS, ratio[d]	DNA divergence (%)	dN, dS ratio[d]	DNA divergence (%)	dN, dS ratio[d]
orf32	*Phytophthora infestans*[a]	99	15.2	0.12, 0.37, 0.33*	11.1	0.08, 0.28, 0.27*	–	–
	Phytophthora ramorum	99	–	–	8.1	0.07, 0.15, 0.46	–	–
	Phytophthora sojae	99	–	–	–	–	–	–
ymf101	*Phytophthora infestans*	204[b]	7.4	0.07, 0.14, 0.52[e]	5.8	0.05, 0.09, 0.59[e]	32.3	0.39, 1.3, 0.30[e]**
	Phytophthora ramorum	198	–	–	4.8	0.06, 0.08, 0.73	30.2	0.35, 1.7, 0.20[e]**
	Phytophthora sojae	198	–	–	–	–	30.2	0.37, 1.0, 0.37[e]**
	Saprolegnia ferax (*orf64*)	195	–	–	–	–	–	–
ymf96	*Phytophthora infestans* (*orf79*)	240	34.5	NA	37.1	NA	–	–
	Phytophthora ramorum	243	–	–	42.8	NA	–	–
	Phytophthora sojae	243	–	–	–	–	–	–
ymf100	*Phytophthora infestans* (*orf100*)	303	11.9	0.09, 0.68, 0.13**	10.2	0.06, 0.87, 0.07**	–	–
	Phytophthora ramorum	309	–	–	9.9	0.07, 1.1, 0.06**	–	–
	Phytophthora sojae	303	–	–	–	–	–	–
ymf98	*Phytophthora infestans* (*orf142*)	429	10.6	0.08, 0.84, 0.10**	10.1	0.08, 0.37, 0.21**	29.4	0.32, 2.5, 0.13[e]**
	Phytophthora ramorum	420	–	–	10.6	0.07, 1.1, 0.07**	26.0	0.31, 1.8, 0.17[e]**
	Phytophthora sojae	429	–	–	–	–	28.2	0.32, 2.3, 0.14[e]**
	Saprolegnia ferax (*orf143*)	432	–	–	–	–	–	–
ymf99	*Phytophthora infestans* (*orf217*)	654	11.8	0.09, 0.73, 0.12**[d]	8.8	0.06, 0.56, 0.11**	–	–
	Phytophthora ramorum	660	–	–	9.7	0.07, 0.82, 0.08**[d]	–	–
	Phytophthora sojae	678	–	–	–	–	–	–
cox2	*Phytophthora Infestans*	774	5.7	0.01, 1.0, 0.01**	6.5	0.01, 1.0, 0.01**	20.3	0.15, 3.3, 0.04**
	Phytophthora ramorum	777	–	–	5.7	0.01, 0.69, 0.02**	20.8	0.15, 3.3, 0.04**
	Phytophthora sojae	777	–	–	–	–	20.8	0.15, 3.3, 0.04**
	Saprolegnia ferax	759	–	–	–	–	–	–

Likelihood ratio test shows dN : dS is significantly different from one; two-tailed * $P < 0.05$; ** $P < 0.001$. The likelihood ratio test was implemented using codeml of paml (Yang 1997)

NA Not applicable because alignment had frame shift mutations (insertions or deletions whose length is not a multiple of 3) so estimation of dN : dS is unreliable

[a] Data from Paquin et al. (1997)

[b] Data from Grayburn et al. (2004)

[c] Sequence divergence calculated without corrections for differential rates of nucleotide substitution and gaps were excluded from the analysis

[d] Number of point substitutions per non-synonymous site (dN); number of point substitutions per synonymous site (dS); and ratio of dN : dS were estimated using the method of Yang and Nielsen (2000) as implemented in yn00 of paml 3.14b (Yang 1997, http://abacus.gene.ucl.ac.uk/software/paml.html)

[e] A small difference in stop codon position, so the pairwise alignment was analyzed up to the first stop codon occurrence

the 5′ end of *orf98* by 20 bp and the 3′ end of *orf98* overlaps $orf112_a$ by 17 bp. There is only one unique ORF (*orf176*) longer that 100 bp in *P. ramorum* mtDNA, and this is part of an inverted repeat and is present in two copies in opposite orientation (Fig. 1 and discussed below). The termination codon for all of these unassigned ORFs is TAA with the exception of *orf98* and $orf112_b$ (TAG) and $orf112_a$ (TGA). Both these termination codons are used in different ORFs in haplotype IIa and IIb of *P. infestans* (Avila-Adame et al. 2006). BLAST analysis of sequences in GenBank did not identify any significant sequence similarity among these ORFs or any potential homologs in other organisms (blastn at http://www.ncbi.nlm.nih.gov/blast/ with expect threshold = 0.01).

The coding regions are closely packed in the genome with 70% of the spacer regions (intergenic regions) being less than 30 bp long (Table S2). Overall the spacer regions represent a relatively small percentage of the genome compared to coding regions (11.5 and 18.4% for *P. ramorum* and *P. sojae*, respectively). Genes are divided between the two strands and in general are clustered into five non-overlapping groups alternating between strands (Figs. 1, 2).

Henceforth we refer to positions in the *P. sojae* and *P. ramorum* circular mitochondrial genome maps that are based on a first position chosen so that it would correspond to the first position of the *P. infestans* mitochondrial genome in GenBank. Starting at base 1 the first group is *rrnL* through *cox1* followed by *trnR* through *nad2*, *nad7* through *nad6*, *nad4L* through *trnL*, and *rpl2* through *ymf100*. There are some exceptions to these groupings; most notably transcription of *orf206* in *P. sojae* and *ymf16* through *rps13* for all three species is in an opposite direction than the adjacent coding regions (Figs. 1, 2; Paquin et al. 1997). There are also two examples where the 3′ end of a gene overlaps the 5′ end of the following gene in *P. ramorum* and *P. sojae*; *rps12* overlaps *rps7* by 26 bp and *nad11* overlaps *nad1* by 4 bp. These overlaps were also observed in *P. infestans*, but it was by 3 and 70 bp, respectively (Paquin et al. 1997). If the start codon 45 bp downstream from the reported start of the *rps7* gene was used for *P. infestans* (the same relative position for *P. sojae* and *S. ferax*) the overlap is the same as for *P. ramorum* and *P. sojae* (26 bp). Likewise, there is a 23 bp overlap of *rps12* and *rps7* in *S. ferax* (Grayburn et al. 2004). Unlike *S. ferax* (Grayburn et al. 2004), none of the genes abut in *P. ramorum* and *P. sojae*.

Including all ORFs, 88.5 and 81.6% of the mitochondrial genome for *P. ramorum* and *P. sojae*, respectively, is represented by coding sequences. When ORFs without apparent homologues in other species are excluded, this is reduced to 85.8 and 76.5%, respectively. This compares to values for *P. infestans* mtDNA of approximately 90% when including ORFs and 86% when excluding unique ORFs.

Inverted repeat

One unusual feature of the *P. ramorum* genome is that it contains a duplication of 1,150 bp present in an inverted orientation (hereafter referred to as an inverted repeat, IR) with one copy situated between *cox1* and *cob* (bases 9,540–10,689) and the second in the opposite orientation between *nad6* and *nad4L* (bases 26,173–27,322). This inverted repeat contains *orf176*, which is unique to this species, as well as *trnR*(*ucu*). The first copy of the IR starts 8 bp after the termination codon of *cox1* and the opposite end includes 13 bp of the 3′ end of *cob*. The second copy is identical in sequence but is a mirror image of the first copy and starts with 74 bp of the 3′ end of the *nad6* gene and has 38 bp of the 3′ end of *nad4L*. This copy of the IR is in the same position of the genome as the clustered five unique ORFs of *P. sojae* while the other copy appears to coincide with an inversion breakpoint relative to *P. infestans* (Fig. 3). Comparisons of sequences between *cox1* and *cob* and *nad6* and *nad4L* for *P. ramorum* and *P. sojae* (the region where the IR is found in *P. ramorum*) revealed limited sequence similarity between these two species. Likewise, there is little sequence similarity among species in the region that has a breakpoint in both of these species and *P. infestans* (*nad6–nad4L*).

Genome comparisons

With the exception of the IR in *P. ramorum* mtDNA, its gene order is the same as that of *P. sojae*. However, the *P. infestans* mtDNA has two regions that are inverted relative to this arrangement (Fig. 3). One inversion includes *cob, nad9* and *atp9* and the other is immediately adjacent and includes a total of 19 coding regions spanning from *nad3* to *atp1*. The gene order in several regions is the same as that of *S. ferax* (Grayburn et al. 2004), including the linkage of *rps8*, *rpl6*, *rps2*, and *rps4*. Whole genome sequence alignments among the three species revealed a 92% sequence identity when gaps were excluded from the analysis (Table 2). When gaps were included the level of sequence identity ranged from 81 to 92% depending on the species compared.

Genome comparisons using mVISTA provide a graphic representation of the variation among genomes. Using *P. ramorum* as the base sequence,

Table 2 Sequence conservation in whole mitochondrial genome comparisons among *Phytophthora infestans*, *Phytophthora ramorum*, and *Phytophthora sojae*

Genome comparisons[a]	Aligned sequence identity[b]	Total genome sequence identity[c]
Phytophthora ramorum versus *Phytophthora sojae*	92.2% (39,038 bp)	91.5% (*Phytophthora ramorum*) 83.7% (*Phytophthora sojae*)
Phytophthora ramorum versus *Phytophthora infestans*	92.8% (37,153 bp)	87.7% (*Phytophthora ramorum*) 90.8% (*Phytophthora infestans*)
Phytophthora sojae versus *Phytophthora infestans*	92.3% (37,717 bp)	81.3% (*Phytophthora sojae*) 92.0% (*Phytophthora infestans*)

[a] Mitochondrial genome sizes for *Phytophthora ramorum*, *Phytophthora sojae*, and *Phytophthora infestans* are 39,314, 42,975, and 37,957 bp (NC 002387; Paquin et al. 1997), respectively. For the purposes of this comparison the inversions in *Phytophthora infestans* relative to the two other species were reverse complemented to give the same gene order for all species

[b] Sequence identity calculated without corrections for differential rates of nucleotide substitution and gaps in the alignment were excluded from the analysis. The numbers in parentheses reflect the total number of aligned bases for each comparison

[c] Sequence identity calculated without corrections for differential rates of nucleotide substitution and gaps were included in the analysis. Differences in genome size among the species due to indels contributes to these differing values between species

comparison with *P. sojae* reveals that some regions were more variable than others (Fig. 4). Greatest divergence was found in intergenic regions and in some cases the high divergence observed arose because these were not readily alignable. Some of the regions exhibiting the greatest divergence corresponded to the inversion break points in comparisons with the *P. infestans* genome (at the 3′ end of the *cob* gene, between *atp9* and *nad3*, and between *atp1* and *nad5*) or regions of the *P. infestans* genome that exhibited intraspecific length variation due to indels [a larger indel was observed between *trnY* and rns with a smaller indel between *orf79* (*ymf96*) and *cox2*]. However, not all divergent regions corresponded to differences with *P. infestans* in length or gene order (for example, *nad9* to *atp9* and *nad7* to *ymf98*). Vista comparisons including the mitochondrial genome of *P. infestans* (with specific regions reverse complemented to account for the inversions) gave results that did not differ appreciably from those observed in Fig. 4 (data not shown).

Discussion

The size of the mitochondrial genome of *P. ramorum* (39,314 bp) is similar to what had been previously published for *P. infestans* (Paquin et al. 1997; Avila-Adame et al. 2006) whereas that for *P. sojae* was ~8.5% larger (42,977 bp). All these are smaller than the related oomycete *S. ferax*, which has a genome size of 46,930 bp (Grayburn et al. 2004). However, this species has an 8,618 bp inverted repeat and when this is removed, the single copy sequences remaining (38,312 bp) are similar in size to *P. ramorum* and *P. infestans* mitochondrial genomes.

All these genomes encode for a common suite of 35 protein-encoding genes of known function, two rDNAs, and tRNAs encoding for 19 amino acids (Paquin et al. 1997; Avila-Adame et al. 2006; Grayburn et al. 2004). There are six predicted ORFs common to the genus *Phytophthora*, two of which also appear to have homologues in *S. ferax* as well (*ymf101*, *ymf98*; Grayburn et al. 2005). Similar to *P. infestans* (Avila-Adame et al. 2006), there are also ORFs unique for *P. ramorum* (*orf176*) and *P. sojae* (*orf116*, *orf98*, $orf112_a$, $orf112_b$, *orf101*, and *orf206*). All genes in the *P. ramorum* and *P. sojae* mitochondrial genomes lack introns. This lack of introns was also the case for the mitochondrial genomes of other Stramenopiles (*P. infestans*—Paquin et al. 1997; Avila-Adame et al. 2006; *S. ferax*—Grayburn et al. 2004; heterokont brown algae—Oudot-Le Sec et al. 2006).

Inverted repeat

An unusual feature of the *P. ramorum* mitochondrial genome was the presence of a small (1,150 bp) IR that contained an unassigned ORF and duplicate copies of *trnR*(*ucu*). There is only one other example of an IR in the genus *Phytophthora*. Based on restriction mapping and Southern analysis, Schumard-Hudspeth and Hudspeth (1990) observed a short inverted repeat of 0.5–0.9 kb in size in *P. megasperma* with one copy adjacent to *cox2* and the other in the vicinity of the *cob/atp9* genes (this latter position is similar to that observed for one copy of the IR in *P. ramorum* and coincides with the junction of the inversion seen in *P. infestans* relative to *P. sojae* and *P. ramorum)*. Without further analysis of a greater number of mitochondrial genomes in the genus, it is unclear if the IR arose from duplication of a specific region of the genome or if it reflects a deletion of the large IR found in other genera in the oomycetes (discussed more below). However, given that the single copy of *trnR*(*ucu*) in *P. infestans*

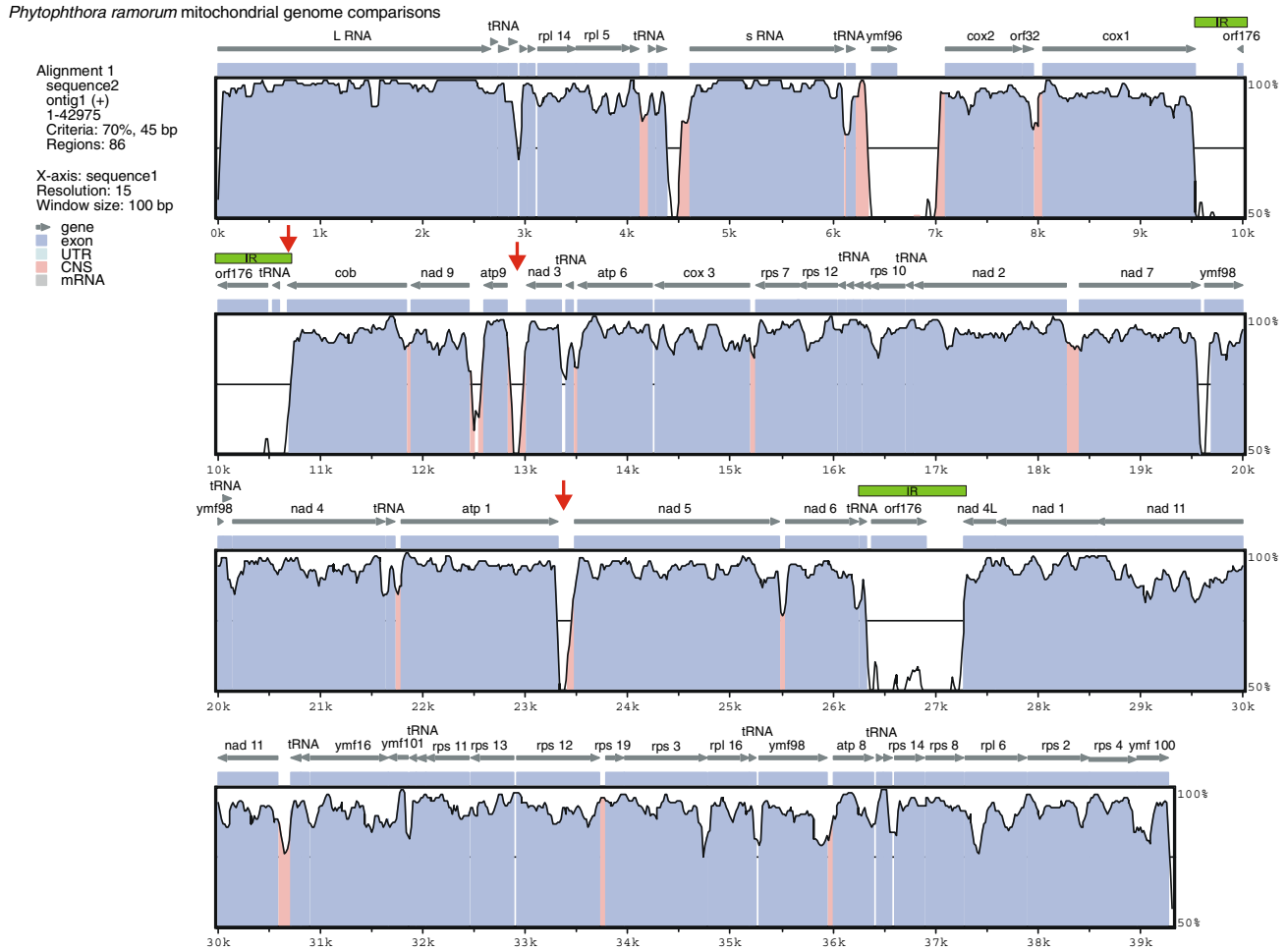

Fig. 4 VISTA plot comparison between the mitochondrial genome of *Phytophthora ramorum* (reference sequence) and *P. sojae*. Coding regions are denoted by the *blue color* and the *labeled arrows* above the *plot* indicating the direction of transcription while non-coding regions are denoted by the *pink 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 *red arrows* denote the ends of the regions that were inverted in the *P. infestans* mitochondrial genome relative to *P. ramorum* and *P. sojae*

and *P. sojae* is present adjacent to the *nad6* gene, and one of the copies of this tRNA gene is in the same position for *P. ramorum*, it is possible that this location reflects the ancestral position, with the other copy of the IR between *cox1* and *cob* being the duplicated copy.

The only other example of a completely sequenced oomycete mitochondrial genome containing an IR is *S. ferax* (subclass Saprolegniomycetidae), which has an IR of 8,618 bp, representing 37% of the genome size and containing genes for four proteins, five tRNAs, and both rRNAs (Grayburn et al. 2004). These coding regions are transcribed from both directions of the mitochondrial genome, whereas the coding regions of each *P. ramorum* IR are on a single strand. Another difference between these genomes is that in *S. ferax*, one edge of both copies of the IR terminates with a partial sequence of a coding region (the 3′ end of *nad5*), whereas in *P. ramorum*, three of the four termini end in a coding region. One end of the IR encodes the terminal 13 bp of the 3′ end of *cob* and the same end of the second copy has 74 bp of the 3′ end of *nad6*. The opposite end either terminates within the spacer region before *cox1* in one copy or has 38 bp of the 3′ end of *nad4L* in the other.

Inverted repeats in the mitochondrial genome are common in the closely related genus *Pythium* (McNabb et al. 1987; McNabb and Klassen 1988; Martin 1991, 2000) and have also been found in other oomycetes such as *Achlya* spp. (Hudspeth et al. 1983; Boyd et al. 1984; Schumard et al. 1986), *Aplanopsis terrestris*, *Leptolegnia caudate*, and *Sapromyces elongates* (McNabb and Klassen 1988) and *S. ferax* (Grayburn et al. 2004). An IR also has been reported in the hypochytrid *Hypochytrium catenoides* (McNabb et al. 1988;

hypochytrids are Stramenopiles closely related to the oomycetes). In cases where an IR has been described, however, it represents a larger proportion of the genome size (greater than 37% overall, and more than 71% for *Pythium* spp.) and contains the large and small ribosomal RNA coding regions, which is very different from the IR observed in *P. ramorum*. It is believed that the presence of the larger IR in these genomes serves to stabilize the genome with intramolecular recombination occurring between the two arms of the IR leading to concerted evolution of the two copies. Evidence of this recombination has been observed in *Achlya ambisexualis* (Hudspeth et al. 1983), *A. klebsiana* (Boyd et al. 1984), and *S. ferax* (Grayburn et al. 2004) where flip–flop isomerization between the repeats has caused the unique regions to be present in both "head-to-head" and "head-to-tail" orientational isomers in the same isolate. While the potential for recombinational isomers in the isolate of *P. ramorum* sequenced in this study has not been investigated, they have been found in the type isolate (CSB101553 from Germany) following restriction digestion of purified mitochondrial DNA (F. Martin, unpublished).

Genome comparisons

Both *P. ramorum* and *P. sojae* have the same gene order and the differences observed in comparisons with *P. infestans* could be accounted for by two inversions between these two arrangements. Consistent with their similarity of gene order, the former two are the most closely related pair of these three *Phytophthora* species based on comparisons of *cox2* and rDNA ITS data (Martin and Tooley 2003a, b; Cooke et al. 2000), β-tubulin, elongation factor 1-α, *cox1*, and *nadh1* (Kroon et al. 2004). Unfortunately, none of the blocks of gene order shared among the *Phytophthora* species and *S. ferax* allow us to reconstruct whether the inversions occurred in the lineage leading to *P. infestans* versus that leading to *P. sojae* and *P. ramorum*.

Otherwise, though, the gene order in several regions of the *P. sojae*/*P. ramorum* mtDNAs is the same as that of *S. ferax*, including the linkage of *rps8*, *rpl6*, *rps2*, and *rps4* that Grayburn et al. (2004) noted was also conserved in the stramenopile heterokont alga *Chrysodidymus synuroides* (Chrysophyceae; Chesnick et al. 2000). This gene order also is conserved in comparisons with five other related heterokont brown algae in the Phaeophyceae (Oudot-Le Sec et al. 2006), as is the six genes from *rps11* through *rpl16*. There is limited conservation of gene order for other regions of the genomes, precluding the use of gene order to assess broader scale phylogenetic relationships in the Stramenopile kingdom.

Some of the regions where high levels of sequence variability were observed in comparisons between *P. ramorum* and *P. sojae* correspond to the location of differences in genomic organization among species. For example, the terminal regions of the genomic inversions observed with *P. infestans* relative to the other two species corresponds to regions of low sequence similarity between *P. ramorum* and *P. sojae* (Fig. 4, between *cox1-cob*, *atp9-nad3*, and *atp1-nad5*). One of these regions, the area between *cox1* and *cob*, is where one copy of the inverted repeat is located in *P. ramorum*. The other copy is located between *nad6* and *nad4L*, which also was where the five unique ORFs in *P. sojae* are located. Furthermore, the unique *orf206* in *P. sojae* is located between *trnY* and *rrnS*, which was the same location in the genome of *P. infestans* where length mutations associated with the major intraspecific differences in genomic sequences were observed (Avila-Adame et al. 2006). *P. sojae orf206* contains eight repeats of a 24 bp sequence (AAACTGTG(A/G)AGAACAAAATATCAC) near the 5′ end of the ORF (base 5064–4873). This location falls within the 2,262 bp *Hin*dIII fragment of the *P. sojae* mtDNA that Förster et al. (1994) described as being hypervariable. This fragment showed frequent length variations with multiple alleles consistent with the presence of a microsatellite sequence. Variation was observed not only between different genotypes of *P. sojae*, but in three cases also between different clones of the same *P. sojae* isolate that had been cultured in different laboratories. Unequal cross-overs among 8×24 bp repeats or slipped-strand mispairing during replication likely account for the observed hypervariability. Although this microsatellite corresponds in position to a site of intraspecific length variation in *P. infestans*, there is no DNA sequence similarity between the two variable sites, and neither the *P. infestans* nor *P. ramorum* sequence contains tandem repeats.

Another region of the *P. infestans* mitochondrial genome where smaller length mutations were observed among haplotypes is downstream of *orf79* (*ymf96*; indels were 34 and 36 bp in length), which is also a region of sequence variation in comparisons between *P. ramorum* and *P. sojae*. Interestingly, the spacer region in *P. infestans* between *nad3* and *nad5*, which is one juncture of the inversion relative to *P. ramorum* and *P. sojae*, is also variable in haplotype IIb relative to the other three haplotypes (32% sequence similarity). From comparing the genome maps for these three species it is interesting to note that two of the regions variable in interspecific comparisons (between *cox1* and

cob and *nad6* and *nad4L*) correspond to the head-to-head juncture of two clusters of genes transcribed in opposite directions.

The results thus far suggest a high degree of gene order conservation in the genus *Phytophthora* with most of the differences observed explained by two inversions. One reason for this may be the large percentage of the genome that is represented by coding regions leaving few locations that could accommodate rearrangement breakpoints without disrupting a gene. When interspecific variation is observed (rearrangements, unique ORFs, IR) our comparative analysis suggests that these have occurred multiple times independently in the same intergenic regions. These regions may represent hotspots for rearrangement either because rearrangements are more likely for mutational reasons or because they are less likely to disrupt function at these locations. Interestingly, the two main rearrangement hotspots occur in the transitions between groups of genes that are coded for on one strand to a group that is coded on the other. It could be that these genes are coexpressed and maybe expression is disrupted when these groups are broken up, or there could be a mutational role for an RNA intermediate. However, before firm conclusions about genome stability can be drawn additional comparisons among more species are needed to clarify this. This would also clarify the relationship between changes in genome organization and phylogeny in the genus.

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