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Short Communication

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Development of New Oomycete Taxon-specific Mitochondrial Cytochrome *b* Region Primers for Use in Phylogenetic and Phylogeographic Studies

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Abstract

Here, we describe the development of an oomycete-specific primer pair for amplification of the cytochrome *b* region in plant pathogenic species that span the order Peronosporales (*Phytophthora* spp., downy mildews). Because of the high number of variable sites at both inter- and intra-specific levels this marker provides a powerful tool for population genetics and phylogenetic studies in this taxa. We also demonstrate its potential compared with other oomycete-specific mitochondrial markers currently available.

Introduction

Mitochondrial DNA markers provide an attractive tool for inferring phylogeny and evolutionary history of closely related organisms because of their rapid evolutionary rates, their lack or scarcity of meiotic recombination, and, in most cases, the strict uniparental mode of inheritance (Avise, 2000). The cytochrome oxidase (subunits 1 and 2), the NADH dehydrogenase and the cytochrome *b* (cyt*b*) genes have proven to be powerful tools suitable for phylogenetic analysis in Fungi and Oomycota (Hudspeth et al., 2003; Wattier et al., 2003; Choi et al., 2006; Schena and Cooke, 2006; Tsui et al., 2008). In particular, the cyt*b* region is known to harbour extensive intra-specific variation allowing for comprehensive molecular identification at fine taxonomical levels such as within a species-complex (Yokoyama et al., 2001; Biswas et al., 2003) and also for population genetic studies (Chen et al., 2007). However, the use of cytochrome *b* for oomycetes is hampered by the lack of an established specific PCR protocol able to target a broad range of genera. Although the oomycetes have been previously classified into the Kingdom Fungi based on morphological traits, more recent molecular and biochemical analyses have separated them from fungal organisms (Chesnick et al., 1996). The oomycetes belong to stramenopiles, a remarkably diverse yet monophyletic group, that also includes planktonic diatoms and brown algae. Plant pathogenic oomycetes are economically damaging to several crop and ornamental plants, as well as forest trees (Lamour et al., 2007). The availability of universal markers such as cytochrome *b* designed specifically for cross utility in oomycetes would be an useful additional tool to address ecological and evolutionary processes. For example, such markers could be useful in studies of inter-specific hybridation (Brasier et al., 2004), introduction and colonization history (Gomez-Alpizar et al., 2007; Delmotte et al., 2008), cryptic speciation (Goker et al., 2007) and adaptation to selective pressures resulting from host-plant resistance genes or chemical treatments (Gisi and Sierotzki, 2008; Sacristan and Garcia-Arenal, 2008).

In this context, our aim was to develop specific cytochrome *b* primers useful at both inter-and intra-species levels in a variety of oomycete taxa. The primers were designed against mitochondrial genome sequence data available for *Phytophthora infestans* (NC002387), *Phytophthora ramorum* (EU427470), *Phytophthora sojae* (NC009385) (Tyler et al., 2006) and *Hyaloperonospora parasitica* (http://genome.wustl.edu), along with the already released cyt*b* sequences of *Plasmopara viticola* (DQ459464) (Chen et al., 2007). These were chosen in order to maximize the potential of designing a primer pair that could successfully amplify the entire Peronosporales order. The resulting fragment contains polymorphisms of use in molecular identification and phylogeny, together with unprecedented levels of within species nucleotide variability.

Materials and Methods

Design of primers

Primers were designed using a ClustalX (Thompson et al., 1997) alignment of the complete cyt*b* gene sequence and its flanking region of the five plant

pathogenic oomycetes described above (Fig. 1). The forward primer was designed in the conserved region of subunit 9 of the NADH dehydrogenase (nad9) gene. The reverse primer was designed within the cyt*b* gene since synteny of the cyt*b* and the following gene was not conserved across Peronosporales. The sequences of the two defined primers were: cob_oo_F 5′-TCW GAA ATT TGT GCW GTW GAT TAT AT-3′ and cob_oo_R 5′-CCA ATA ACA AAY TTT AAA AAT MGG TC-3′ where degenerate positions are represented by the following codes: M = A/C; W = A/T; Y = C/T.

A total of 30 Peronosporale species including 25 *Phytophthora* species infecting diverse plant hosts, four species of downy mildews (*Plasmopara*, *Bremia*, *Peronospora*) and one species of Pythiales (*Pythium intermedium*) were used as template to test our primers (Table 1). For one isolate of each species, DNA was extracted according to the modified cetyltrimethylammonium bromide method described previously in Delmotte et al. (2006). PCR reactions were carried out in a final volume of 15 µl containing 50 ng of genomic DNA, 2 mM of MgCl2, 150 µM of each dNTP, 4 pmol of each primer and 0.3 U Silverstar T*aq* DNA polymerase (Eurogentec, Liége, Belgium) in reaction buffer. Reactions were performed with the following program: an initial denaturation step of 3 min at 94°C, followed by 40 cycles of 30 s denaturation at 94°C, 30 s annealing at 53°C, 90 s elongation at 72°C and a final elongation step of 7 min at 72°C.

Evaluation of cytochrome *b* polymorphism

To evaluate the resolving power of the nad9-cyt*b* fragment as a phylogenetic tool, PCR products were

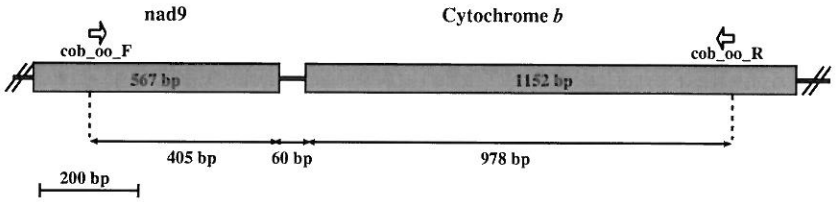

Fig. 1 Schematic representation of gene organization of the mitochondrial fragment amplified in this study based on the sequence observed in *Phytophthora infestans* (NC002387). The open reading frames of genes are indicated by gray boxes. The bold line between genes represents the intergenic region. Arrows above the fragment indicate the primer pair cob_oo_F/cob_oo_R that has been used to amplify this region. Oomycetes belonging to the order Peronosporales share the same organization in this region

Table 1
The 31 species of phytopathogenic oomycetes successfully amplified using the primer pair designed for this study. A single fragment of the mitochondrial nad9-cytochrome *b* region was generated in each case. Where fragments were sequenced the relevant GenBank accession number is given. Those fragments visualized by agarose gel electrophoresis only are denoted by Na

Order	Genus	Species/subspecies	Region size (bp)	GenBank Accession no.
Peronosporales	*Phytophthora*	*Phytophthora alni* subsp. *alni*	1422	FJ810084
		Phytophthora alni sp. multiformis	1428	FJ810085
		Phytophthora alni sp. *uniformis*	1400	Na
		Phytophthora cactorum	1400	Na
		Phytophthora cambivora	1428	FJ810086
		Phytophthora cinnamomi	1428	FJ810088
		Phytophthora citricola	1400	Na
		Phytophthora citrophthora	1436	FJ810091
		Phytophthora cryptogea	1400	Na
		Phytophthora europaea	1400	Na
		Phytophthora fragariae var. *fragariae*	1400	Na
		Phytophthora fragariae var. *rubi*	1428	FJ810087
		Phytophthora gonapodyides	1430	FJ810093
		Phytophthora humicola	1424	FJ810094
		Phytophthora ilicis	1400	Na
		Phytophthora infestans	1443	FJ810095
		Phytophthora inundata	1430	FJ810090
		Phytophthora lateralis	1400	Na
		Phytophthora megasperma	1400	Na
		Phytophthora nicotianae var. *parasitica*	1430	FJ810089
		Phytophthora palmivora	1430	FJ810092
		Phytophthora parasitica	1400	Na
		Phytophthora pseudosyringae	1400	Na
		Phytophthora psychrophila	1400	Na
		Phytophthora quercina	1400	Na
		Phytophthora ramorum	1400	Na
	Plasmopara	*Plasmopara halstedii*	1443	FJ810096
		Plasmopara viticola	1446	DQ459464
	Peronospora	*Peronospora pisi*	1400	Na
	Bremia	*Bremia lactucae*	1420	FJ810099
Pythiales	*Pythium*	*Pythium intermedium*	1400 <	Na

All PCR products were initially visualized on 2% agarose gel and sizes estimated using a 1 kb size marker (Fermentas, St Leon-Rot, Germany).

sequenced as above (Table 1). The sequences obtained were then aligned using ClustalX under default settings (Thompson et al., 1997) along with the cyt*b* sequences of *P. infestans*, *P. ramorum*, *P. sojae*, *Phytophthora megasperma* (L16863), *P. viticola*, *H. parasitica* and *Saprolegnia ferax* (extracted from GenBank and genome project databases). The alignment was edited manually to ensure that codon alignments were preserved resulting in an aligned region of 1331 nucleotides. A phylogenetic tree constructed using the software PHYMIL 3.0 (Guindon and Gascuel, 2003) in order to determine the relationships between the 14 species sequenced in this study and the further seven stated above (Fig. 2). Bootstrap support for the nodes was calculated over 1000 replicates using the same program.

In order to test the potential of this conserved marker for use in studies of genetic structure and phylogeography, we sequenced 10 isolates of *P. halstedii*, 10 isolates of *P. viticola*, five isolates of *Bremia lactucae* and seven individuals of *P. infestans* coming from diverse geographic origins (France, Morocco) and having different virulence profiles (Table 2). Moreover, for *P. infestans* and *P. viticola* the previously described mitochondrial haplotypes (Avila-Adame et al., 2006; Chen et al., 2007) were included in the analysis (Table 2).

Results and Discussion

Evaluation of primers

The primer pair amplified a single fragment in all the Peronosporales and in the unique *Pythium* species tested (Table 1). The mean size of amplified products was 1431 bp (range 1420–1446 bp) based on 15 sequences (for accession numbers see Table 1). The observed variation between species is due to length differences in the intergenic region between nad9 and cyt*b* genes. Based on the sequence of *P. infestans*, the fragment amplified contains 405 bp of the mitochondrial nad9 gene, a highly polymorphic intergenic region of 60 bp and the first 978 bp of the cyt*b* (Fig. 1). No intron sequence was found in cyt*b* gene of the oomycete species sequenced in this study. We will later refer to this fragment as the nad9-cyt*b* region.

Phylogenetic properties of cytochrome *b*

The alignment of the nad9-cyt*b* region revealed a high proportion of polymorphic sites, with a total of 391 variable positions (29.1%) and 235 parsimony-informative sites (17.5%). Among the parsimony-informative sites, 64 were localized in the 351 bp of the nad9 gene (18.2%), 20 in the 60-bp intergenic region (33.3%) and 151 in the 920 bp of the cyt*b* (16.4%).

The maximum-likelihood tree obtained using the nad9-cyt*b* region presented was in agreement with the clusters observed in previous phylogenetic studies: the obligate biotrophic species (*Hyaloperonospora* and *Bremia* spp.) appeared to form a monophyletic group (bootstrap proportion, BP = 91%) as previously described (Goker et al., 2007). Cooke et al. (2000) had previously divided the *Phytophthora* genus into 10 well-supported clades based on the analysis of the combined ITS1, 5.8S subunit and ITS2 regions of the genomic ribosomal RNA tandem gene repeat. Our

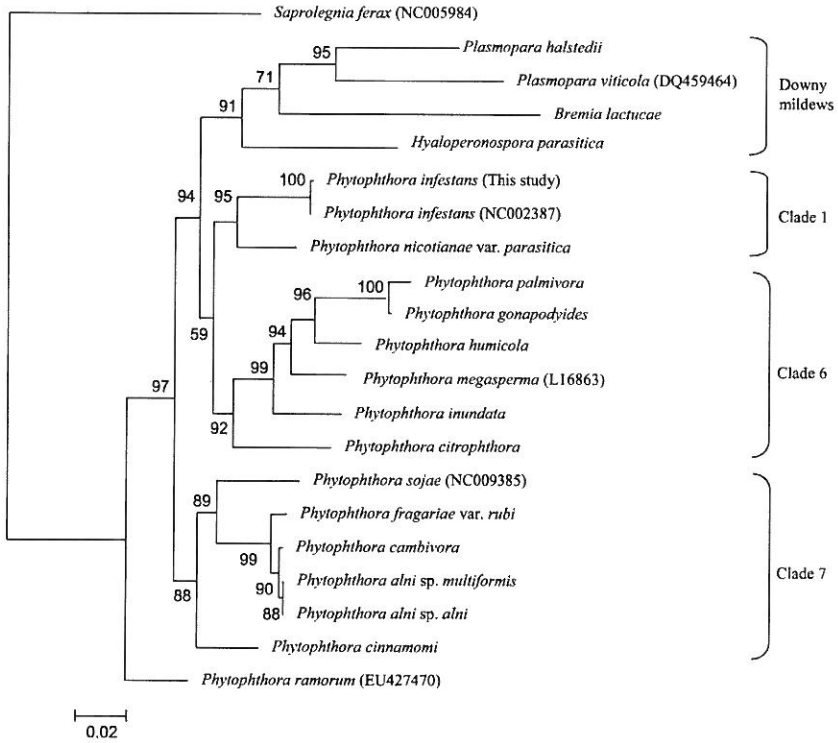

Fig. 2 A genus-wide phylogeny for 19 plant pathogenic oomycete species based on the analysis of the 1331 bp alignment of the mitochondrial nad9-cyt*b* region (Table 1). Sequences with accession numbers into brackets were extracted from GenBank nucleotide database. Numbers at nodes indicate bootstrap support from maximum-likelihood analysis. The scale bar denotes the estimated number of substitutions per site. The tree was rooted with *Saprolegnia ferax*, an oomycete pathogen of a broad range of aquatic animals

Table 2
Intra-specific nucleotide variability of the mitochondrial nad9-cyt*b* region assessed for four phytopathogenic oomycetes species: *Plasmopara viticola*, *Plasmopara halstedii*, *Bremia lactucae* and *Phytophthora infestans*. For each species the number (*n*) of isolates sharing the same haplotype and the position of the variable sites are given

Species	Haplotype name	*n*	Accession no.	Polymorphic sites													
				nad9		Cytochrome *b*											
				3 2 4	3 3 9	4 5 5	6 2 1	6 5 8	7 0 9	7 3 5	8 4 0	8 6 5	9 0 3	1 0 5	1 1 5	1 1 6	1 2 3
Plasmopara halstedii	Haplotype 1	2	FJ810096	T	A	C	T	A	T	T	A	G	A	A	C	C	T
	Haplotype 2	5	FJ810097	.	.	T	.	.	.	.	.	.	.	T	.	.	.
	Haplotype 3	3	FJ810098	C	-	.	.	.	.	.	.	.	.	.	.	.	.
Plasmopara viticola[a]	Haplotype IS	6	DQ459464	T	T	A	G	T	G	C	G	C	G	A	T	A	T
	Haplotype IR	2	DQ459466	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	Haplotype IIS	1	DQ459467	.	.	.	.	C	.	.	.	T	.	.	.	G	C
	Haplotype IIR	1	DQ459469	.	.	.	.	C	.	.	C	T	.	.	.	G	C
Bremia lactucae	Haplotype 1	3	FJ810099	T	A	G	G	T	A	G	C	T	A	T	T	T	T
	Haplotype 2	1	FJ810100	.	.	.	.	.	G	.	.	.	.	.	.	.	.
	Haplotype 3	1	FJ810101	.	.	.	A	.	.	.	.	.	.	.	.	.	.
Phytophthora infestans[b]	Haplotype Ia	6	AY894835	T	A	A	T	A	T	G	A	G	G	C	A	C	T
	Haplotype Ib	1	NC002387	.	.	.	.	.	.	A	.	.	A	.	.	.	.
	Haplotype IIa	1	AY898628	.	.	.	.	.	.	.	.	.	A	.	.	.	.
	Haplotype IIb	1	AY898628	.	.	.	.	.	.	T	.	.	A	.	.	.	.
	Haplotype 5	2	FJ810095	.	.	.	.	.	.	.	.	.	.	.	T	.	.

[a]For *P. viticola*, the isolates analysed in this study fitted to the four European mitochondrial haplotypes that have been previously described by Chen et al. (2007); [b]For *P. infestans*, we added in this analysis the mitochondrial haplotypes (Ia, Ib, IIa, IIb) previously described by Avila-Adame et al. (2006).

maximum-likelihood phylogenetic analysis approach supported three of these *Phytophthora* clades (clades 1, 6 and 7) with high bootstrap support (88–95%). In the same way, the subspecies of *Phytophthora alni* were closely associated with *Phytophthora cambivora* and *Phytophthora fragariae* var. *rubi* as previously suggested (Brasier et al., 2004; Ioos et al., 2006). Despite a high global level of congruence with previous studies, it is worth noting that the position of *P. palmivora* and *P. ramorum* slightly differs from results obtained in previous studies (Cooke et al., 2000; Voglmayr, 2003). However, the *P. palmivora* placement may likely result from the low number of *Phytophthora* species of clade 4 included in the analysis. For *P. ramorum*, we see a typical long-branch artefact which could be because of the rapid evolution of this species within the *Phytophthora* genus and could possibly be resolved by including more *Phytophthora* species in the analysis.

In order to test the power of the new marker compared to already published ones, we performed a review of the primers currently available for the amplification of mitochondrial regions in different oomycete species, especially in the genus *Phytophthora* (Table 3) (Hudspeth et al., 2000; Martin and Tooley, 2003; Wattier et al., 2003; Kroon et al., 2004; Schena and Cooke, 2006). We compare their power as phylogenetic markers by using an *in silico* analysis of published sequences using the software DNASP (Rozas et al., 2003). The derived summary statistics are presented in Table 2b. These were based on data that include five *Phytophthora* species (*P. infestans*, *P. megasperma*, *P. nicotianae*, *P. sojae*, *P. ramorum*). The nad9-cyt*b* region is the largest of the mitochondrial region amplified in oomycetes recorded in the literature so far (Table 3) and our literature review reveals it to be the most useful marker in terms of its cross species utility among the Peronosporales. This region presents the greatest mean number of nucleotide differences (*k*), the highest number of parsimonious informative sites (Par) and the lowest *R* value (transitional pairs/transversional pairs). The level of nucleotide diversity (Pi = 0.069 ± 0.009) indicated an evolutionary rate around the mean for a mitochondrial fragment, which ensures sufficient levels of polymorphism while preserving the quality of the alignment. Therefore, the nad9-cytb fragment described in this study could provide a suitable tool for the rapid identification of Peronosporales species using DNA barcoding method, especially as it has been suggested that where mitochondrial fragments are > 600 bp the accuracy of species recognition is much improved (Benbouza et al., 2006).

Intra-specific polymorphism of the cytochrome *b*

We revealed the presence of three to five different haplotypes per species, with a mean number of different haplotypes per taxa of 3.75 (Table 2). From two to five mutational sites appeared per haplotype corresponding to a mean of 2.5 SNPs per kb in this region. The three haplotypes of *P. halstedii* contained a polymorphism in the coding regions of nad9 and cyt*b* genes at four nucleotide positions (three mutations and one insertion-deletion) (Table 2). Two of the three mutational events were synonymous, and one mutation caused a change of a threonin to a methionin in the amino acid sequence of the Cyt*b*. The deletion of one base occurring at the end of the nad9 gene adds an asparagin in the amino acid sequence downstream from the N

Table 3
Summary of the published primers available for the amplification of mitochondrial regions across the Peronosporales order. Summary statistics for each region were computed based on the alignment of five *Phytophthora spp.* nucleotidic sequences that were retrieved from GenBank: *P. infestans*, *P. megasperma*, *P. nicotianae*, *P. sojae* and *P. ramorum*

Mitochondrial-targeted region	Primer name	Primer sequence (5′ → 3′)	Size (bp)	L[a] (bp)	K[b]	Par[c]	R[d]	Pi[e] ± SD	%GC	Accession numbers	References
atp9-nad9[f]	ATPF	TTTATTCTGTTTAATGATGGC	353	274	23	13	0.6	0.085 ± 0.011	0.1	DQ162873; DQ162883; DQ162874; DQ162888; DQ162879	Wattier et al. (2003), Schena and Cooke (2006)
	ATPR	CAGCACAAATTTCAGATAATAC									
cox1	COXF4N	GTATTTCTTCTTTATTAGGTGC	926	836	60	43	0.7	0.072 ± 0.010	0.31	AY564150; AY564194; AY564196; AY564162; AY564208	Kroon et al. (2004)
	COXR4N	CGTGAACTAATGTTACATATAC									
cox2	–	GGCAAATGGGTTTTCAAGATCC	618	567	37	8	0.7	0.064 ± 0.013	0.29	DQ365743; DQ365747; DQ365750; DQ365753; NC009384	Hudspeth et al. (2000)
	–	CCATGATTAATACCACAAATTTCAC-TAC									
cox2-cox1[f]	IgCoxF	AAAAGAGARGGTGTTTTTTAYGGA	433	373	26	14	0.7	0.070 ± 0.010	0.18	DQ162855; DQ162871; DQ162856; DQ162853; DQ162851	Martin and Tooley (2003), Schena and Cooke (2006)
	IgCoxR	GCAAAAGCACTAAAAATTAAATATAA									
nad1	NADHF1	CTGTGGCTTATTTTACTTTAG	855	855	45	47	0.6	0.053 ± 0.005	0.27	AY563977; AY564021; AY564023; AY563989; AY564034	Kroon et al. (2004)
	NAD-HR1	CAGCAGTATACAAAAACCAAC									
trnG-trnY[f]	Mt13F	ACAGTTTTTCGAATTAAAAACAGAA	293	248	18	14	0.9	0.074 ± 0.010	0.31	DQ162890; DQ162903; DQ162891; DQ162898; DQ162913	Schena and Cooke (2006)
	Mt15F	TTGCCAAGGTTAATGTTGAGG									
	Mt3R	GGAGAAAGTAGGATTCGAACCT									
trnY-rns[f]	Mt1F	TGGCTGAGTGGTTAAAGGTG	ND[g]	ND	ND	ND	ND	ND	ND	–	Schena and Cooke (2006)
	Mt2F	TGGCAGACTGTAAATTTGTTGAA									
	Mt5R	TTGCATGTGTTAAGCATACCG									
	Mt6R	CTCACCCGTTCGCTATGTTT									
nad9-cyt*b*[f]	cob_oo_F	TCWGAAATTTGTGCWGTWGATTATAT	1331	1310	91	52	0.5	0.069 ± 0.009	0.25	FJ810095; L16863; FJ810089; NC009385; EU427470	This study
	cob_oo_R	CCAATAACAAAYTTTAAAAATMGGTC									

[a]Number of sites compared after removing gaps and misaligned regions; [b]Average number of nucleotide difference; [c]Number of parsimony informative sites; [d]Transitional pairs/transversional pairs; [e]Nucleotide diversity; [f]The amplified fragment contains the intergenic region between the two quoted genes; [g]Non-determined since only two of the five *Phytophthora* sequences were available.

terminal. For *P. infestans*, five different haplotypes were identified corresponding to two synonymous mutations (positions 735, 903) and one non-synonymous mutation (position 1156) which is responsible for the substitution of a serin to a threonin in the aminoacid Cyt*b* sequence. In *B. lactucae*, we observed two independent SNPs at position 621 and 709 that defined three different haplotypes. The first mutation corresponded to non-synonymous change responsible for the substitution of a glutamin to an arginin and the second mutation is silent. For *P.viticola*, we found five mutations that defined four different mitochondrial haplotypes as previously described by Chen et al. (2007).

Conclusions

Although there is increased evidence that plant pathogens exhibit spatially predictable distributions of genetic diversity (Taylor et al., 2006), the application of tools and concepts to study the spatial distribution of genetic variation in oomycetes is a relatively novel endeavour (Beheregaray, 2008). This approach requires the development of specific markers (e.g. simple sequence repeat or single nucleotide polymorphism markers) that are polymorphic at the intra-specific level. However, the development of such markers is time-consuming since specific markers have to be designed for the targeted species and often are not useful in related species. With the advent of complete genome sequencing, the potential for designing new primer sets that target-specific genomic regions and amplify at a broad taxonomic range is increasing as illustrated by this study. The availability of cytochrome *b* primers for a large panel of oomycetes will allow future intra-specific genetic studies that may provide much needed insights into host plant specialization, colonization history and biogeography of these plant-pathogens.

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References

Avila-Adame C, Gomez-Alpizar L, Zismann V, Jones KM, Buell CR, Ristaino JB. (2006) Mitochondrial genome sequences and molecular evolution of the Irish potato famine pathogen, *Phytophthora infestans*. *Curr Genet* **49**:39–46.

Avise JC. (2000) History and conceptual background. In: Phylogeography: The History and Formation of Species. Cambridge, MA, USA, Harvard University Press, pp 447.

Beheregaray LB. (2008) Twenty years of phylogeography: the state of the field and the challenges for the Southern Hemisphere. *Mol Ecol* **17**:3754–3774.

Benbouza H, Jacquemin JM, Baudoin JP, Mergeai G. (2006) Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. *Biotechnol Agron Soc Environ* **10**:77–81.

Biswas SK, Wang L, Yokoyama K, Nishimura K. (2003) Molecular analysis of *Cryptococcus neoformans* mitochondrial cytochrome *b* gene sequences. *J Clin Microbiol* **41**:5572–5576.

Brasier CM, Kirk SA, Delcan J, Cooke DEL, Jung T, Man in't Veld WA. (2004) *Phytophthora alni sp. nov* and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. *Mycol Res* **108**:1172–1184.

Chen WJ, Delmotte F, Richard-Cervera S, Douence L, Greif C, Corio-Costet MF. (2007) At least two origins of fungicide resistance in grapevine downy mildew populations. *Appl Environ Microbiol* **73**:5162–5172.

Chesnick JM, Tuxbury K, Coleman A, Burger G, Lang BF. (1996) Utility of the mitochondrial nad4L gene for algal and protistan phylogenetic analysis. *J Phycol* **32**:452–456.

Choi YJ, Hong SB, Shin HD. (2006) Genetic diversity within the *Albugo candida* complex (Peronosporales, Oomycota) inferred from phylogenetic analysis of ITS rDNA and COX2 mtDNA sequences. *Mol Phylgenet Evol* **40**:400–409.

Cooke DEL, Drenth A, Duncan JM, Wagels G, Brasier CM. (2000) A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genet Biol* **30**:17–32.

Delmotte F, Chen WJ, Richard-Cervera S, Greif C, Papura D, Giresse X, Mondor-Genson G, Corio-Costet MF. (2006) Microsatellite DNA markers for *Plasmopara viticola*, the causal agent of downy mildew of grapes. *Mol Ecol Notes* **6**:379–381.

Delmotte F, Giresse X, Richard-Cervera S, M'Baya J, Vear F, Tourvieille J, Walser P, de Labrouhe DT. (2008) Single nucleotide polymorphisms reveal multiple introductions into France of *Plasmopara halstedii*, the plant pathogen causing sunflower downy mildew. *Infect Genet Evol* **8**:534–540.

Gisi U, Sierotzki H. (2008) Fungicide modes of action and resistance in downy mildews. *Eur J Plant Pathol* **122**:157–167.

Goker M, Voglmayr H, Riethmuller A, Oberwinkler F. (2007) How do obligate parasites evolve? A multi-gene phylogenetic analysis of downy mildews. *Fungal Genet Biol* **44**:105–122.

Gomez-Alpizar L, Carbone I, Ristaino JB. (2007) An Andean origin of *Phytophthora infestans* inferred from mitochondrial and nuclear gene genealogies. *Proc Natl Acad Sci USA* **104**:3306–3311.

Guindon S, Gascuel O. (2003) A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**:696–704.

Hudspeth DSS, Nadler SA, Hudspeth MES. (2000) A cox2 molecular phylogeny of the peronosporomycetes. *Mycologia* **92**:674–684.

Hudspeth DSS, Stenger D, Hudspeth MES. (2003) A cox2 Phylogenetic hypothesis for the downy mildews and white rusts. *Fungal Divers* **92**:674–684.

Ioos R, Andrieux A, Marcais B, Frey P. (2006) Genetic characterization of the natural hybrid species *Phytophthora alni* as inferred from nuclear and mitochondrial DNA analyses. *Fungal Genet Biol* **43**:511–529.

Kroon L, Bakker FT, van den Bosch GBM, Bonants PJM, Flier WG. (2004) Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genet Biol* **41**:766–782.

Lamour KH, Win J, Kamoun S. (2007) Oomycete genomics: new insights and future directions. *FEMS Microbiol Lett* **274**:1–8.

Martin FN, Tooley PW. (2003) Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. *Mycologia* **95**:711–727.

Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R. (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19**:2496–2497.

Sacristan S, Garcia-Arenal F. (2008) The evolution of virulence and pathogenicity in plant pathogen populations. *Mol Plant Pathol* **9**:369–384.

Schena L, Cooke DEL. (2006) Assessing the potential of regions of the nuclear and mitochondrial genome to develop a "molecular tool box" for the detection and characterization of *Phytophthora* species. *J Microbiol Methods* **67**:70–85.

Taylor JW, Turner E, Townsend JP, Dettman JR, Jacobson D. (2006) Eukaryotic microbes, species recognition and the geographic limits of species: examples from the kingdom Fungi. *Philos Trans R Soc Lond B Biol Sci* **361**:1947–1963.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. (1997) The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**:4876–4882.

Tsui CKM, Daniel HM, Robert V, Meyer W. (2008) Re-examining the phylogeny of clinically relevant *Candida* species and allied genera based on multigene analyses. *FEMS Yeast Res* **8**: 651–659.

Tyler BM, Tripathy S, Zhang X et al. (2006) *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science* **313**:1261–1266.

Voglmayr H. (2003) Phylogenetic relationships of *Peronospora* and related genera based on nuclear ribosomal ITS sequences. *Mycol Res* **107**:1132–1142.

Wattier RAM, Gathercole LL, Assinder SJ, Gliddon CJ, Deahl KL, Shaw DS, Mills DI. (2003) Sequence variation of intergenic mitochondrial DNA spacers (mtDNA-IGS) of *Phytophthora infestans* (Oomycetes) and related species. *Mol Ecol Notes* **3**:136–138.

Yokoyama K, Wang L, Miyaji M, Nishimura K. (2001) Identification, classification and phylogeny of the *Aspergillus* section *Nigri* inferred from mitochondrial cytochrome b gene. *FEMS Microbiol Lett* **200**:241–246.