

## 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* (*cytb*) 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 *cytb* 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 *cytb* 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 *cytb* 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 *cytb* gene since synteny of the *cytb* 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 MgCl<sub>2</sub>, 150 µM of each dNTP, 4 pmol of each primer and 0.3 U Silverstar *Taq* DNA polymerase (Eurogentec, Liège, 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*-*cytb* fragment as a phylogenetic tool, PCR products were

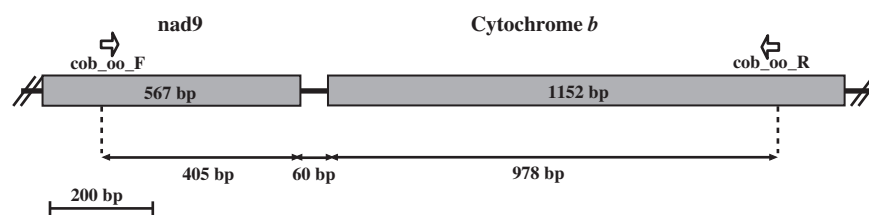


Fig. 1 Schematic representation of gene organization of the mitochondrial fragment amplified in this study based on the sequence observed in *Phytophthora infestans* (NC002387). The open reading frames of genes are indicated by gray boxes. The bold line between genes represents the intergenic region. 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

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

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

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 *cytb* 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 PHYLIP 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 *cytb* 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 *cytb* (Fig. 1). No intron sequence was found in *cytb* gene of the oomycete species sequenced in this study. We will later refer to this fragment as the *nad9-cytb* region.

### Phylogenetic properties of cytochrome *b*

The alignment of the *nad9-cytb* 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 *cytb* (16.4%).

The maximum-likelihood tree obtained using the *nad9-cytb* 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-cytb* 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

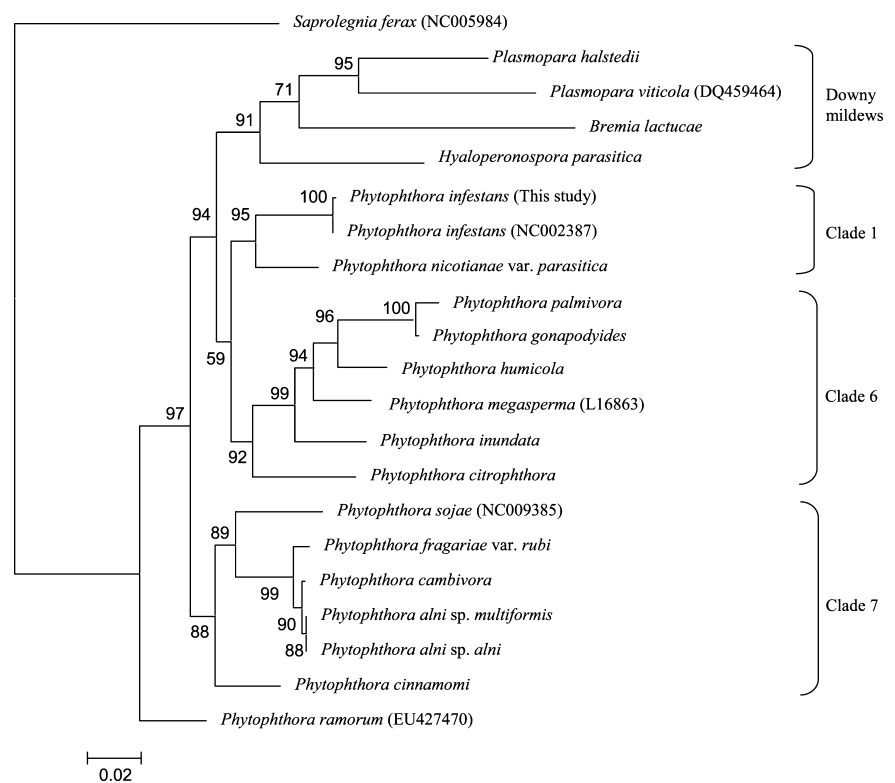


Table 2

Intra-specific nucleotide variability of the mitochondrial nad9-cytb 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	<i>n</i>	Accession no.	Polymorphic sites													
				nad9					Cytochrome <i>b</i>								
				3	3	4	6	6	7	7	8	8	9	1	1	1	1
				2	3	5	2	5	0	3	4	6	0	5	5	9	4
				4	9	5	1	8	9	5	0	5	3	5	6	2	3
<i>Plasmopara halstedii</i>	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	.	.	.	.	.	.	.	.	.	.	.	.	.
<i>Plasmopara viticola</i> <sup>a</sup>	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
	Haplotype IIR	1	DQ459469	.	.	.	.	C	.	.	C	T	.	.	.	.	G
<i>Bremia lactucae</i>	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	.	.	.	.	.	.	.	.	.	.
<i>Phytophthora infestans</i> <sup>b</sup>	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	.	.

<sup>a</sup>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); <sup>b</sup>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-cytb 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 ( $\Pi = 0.069 \pm 0.009$ ) 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 cytb 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 threonine to a methionine in the amino acid sequence of the Cytb. The deletion of one base occurring at the end of the nad9 gene adds an asparagine 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. nucleotide 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 <sup>a</sup> (bp)	K <sup>b</sup>	Par <sup>c</sup>	R <sup>d</sup>	Pi <sup>e</sup> ± SD	%GC	Accession numbers	References
atp9-nad9 <sup>f</sup>	ATPF	TTTATTCGTGTTAATGATGGC	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)
cox1	ATPR COXF4N	CAGCACAAAATTCAGATAATAC GTATTCCTCTTTATTAGGTGC	926	836	60	43	0.7	0.072 ± 0.010	0.31	AY564150; AY564194; AY564196; AY564162; AY564208	Kroon et al. (2004)
cox2	COXR4N –	CGTGAACATAATGTTACATATAC GGCAAAATGGGTTTCAAGATCC –	618	567	37	8	0.7	0.064 ± 0.013	0.29	DQ365743; DQ365747; DQ365750; DQ365753; NC009384	Hudspeth et al. (2000)
cox2-cox1 <sup>f</sup>	IgCoxF	CCATGATTAATACCACAAAATTCAC-TAC AAAAAGARGGTGTTTTTAYGGA	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)
nad1	IgCoxR NADHF1	GCAAAAAGCACTAAAAAATTAATAATAA CTGTGGCTATTTACTTTAG	855	855	45	47	0.6	0.053 ± 0.005	0.27	AY563977; AY564021; AY564023; AY563989; AY564034	Kroon et al. (2004)
trnG-trnY <sup>f</sup>	NAD-HR1 Mt13F	CAGCAGTATACAAAAAACCAC ACAGTTTTTCGAAATTAACAAACAGAA	293	248	18	14	0.9	0.074 ± 0.010	0.31	DQ162890; DQ162903; DQ162891; DQ162898; DQ162913	Schena and Cooke (2006)
trnY-rns <sup>f</sup>	Mt15F Mt13R Mt1F Mt2F Mt5R Mt6R	TTGCCAAGGTTAATGTTGAGG GGAGAAAAGTAGGATTCGAAACCT TGGCTGAGTGGTTAAAGGTG TGGCAGACTGTAATAATTTGTTGAA TTGCATGTGTTAAGCATACCC CTCACCCGTTCCGTATGTTT	ND <sup>g</sup>	ND	ND	ND	ND	ND	ND	–	Schena and Cooke (2006)
nad9-cytb <sup>f</sup>	cob_oo_F cob_oo_R	TCWGAAATTTGTGCWGIWGATTATAT CCAATAACAAAAYTTAAAAATMGGTC	1331	1310	91	52	0.5	0.069 ± 0.009	0.25	FJ810095; L16863; FJ810089; NC009385; EU427470	This study

<sup>a</sup>Number of sites compared after removing gaps and misaligned regions; <sup>b</sup>Average number of nucleotide difference; <sup>c</sup>Number of parsimony informative sites; <sup>d</sup>Transitional pairs/transversional pairs; <sup>e</sup>Nucleotide diversity; <sup>f</sup>The amplified fragment contains the intergenic region between the two quoted genes; <sup>g</sup>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 *Cytb* 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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