Available online at www.sciencedirect.com
SCIENCE DIRECT®
Fungal Genetics and Biology 41 (2004) 766–782
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences

L.P.N.M. Kroon,[a,*] F.T. Bakker,[b] G.B.M. van den Bosch,[a] P.J.M. Bonants,[a] and W.G. Flier[a]

[a] *Plant Research International, P.O.Box 16, 6700 AA, Wageningen, The Netherlands*
[b] *Nationaal Herbarium Nederland, Wageningen University branch, Gen. Foulkesweg 37, 6703 BL, Wageningen, The Netherlands*

Received 21 November 2003; accepted 30 March 2004

Abstract

A molecular phylogenetic analysis of the genus *Phytophthora* was performed, 113 isolates from 48 *Phytophthora* species were included in this analysis. Phylogenetic analyses were performed on regions of mitochondrial (cytochrome *c* oxidase subunit 1; NADH dehydrogenase subunit 1) and nuclear gene sequences (translation elongation factor 1α; β-tubulin) and comparisons made to test for incongruence between the mitochondrial and nuclear data sets. The genus *Phytophthora* was confirmed to be monophyletic. In addition, results confirm that the classical taxonomic grouping as described by [Waterhouse (1963)] does not reflect true phylogenetic relations. *Phytophthora* species were redistributed into 8 clades, providing a more accurate representation of phylogenetic relationships within the genus *Phytophthora*. The evolution and transition of morphological, pathogenic, and reproductive traits was inferred from the cladogram generated in this study. Mating system was inferred to be a homoplasious trait, with at least eight independent transitions from homothallism to heterothallism observed.
© 2004 Elsevier Inc. All rights reserved.

Index Descriptors: *Phytophthora* phylogeny; Translation elongation factor 1α; β-Tubulin; Cytochrome *c* oxidase; NADH dehydrogenase; *Pythium*; *Achlya*; *Pylaiella*; *Ophiocytium*; *Fragilaria*

1. Introduction

The genus *Phytophthora* consists of more than 70 species and is classified within the diploid, algae-like Oomycetes in the Stramenopile clade of the Chromista (Cavalier-Smith, 1986; Dick, 1995; Yoon et al., 2002). The genus harbors devastating plant pathogens that have a large impact on agriculture (e.g., *Phytophthora infestans*, potato late blight), arbiculture (e.g., *Phytophthora ramorum*, sudden oak death), and whole ecosystems (e.g., *Phytophthora cinnamomi* in Australia).

Recently, Riethmüller et al. (2002) clarified the phylogenetic relationships of the Peronosporomycetidae on the basis of analysis of nuclear large subunit ribosomal DNA (nLSU rDNA) sequences. The Peronosporomycetidae represent one of the three subclasses within the Oomycetes, in which the genus *Phytophthora* is classified. Their results challenged the longstanding opinion that *Phytophthora* and *Pythium* are sister genera. Riethmüller et al. (2002) concluded that the genus *Phytophthora* is nested within one of the (pseudo)*Peronospora* clades, as are the genera *Peronophythora*, *Bremia*, and *Plasmopara*. Sister group to this assemblage is a clade comprising the genera *Pythium* and *Lagenidium*.

Recent molecular analysis (Cooke et al., 2000; Martin and Tooley, 2003) has substantially increased our understanding of the phylogenetic relationships between *Phytophthora* species. Both studies, however, were based on either sequence information of single DNA-regions [rDNA internal transcribed spacers (ITS)] or genes [cytochrome oxidase II (*Cox*II)], or covered only a limited subset of *Phytophthora* species (for *Cox*I). Our phylogenetic study is based on five sets of sequence data from the nuclear and mitochondrial genome for a wide range

* Corresponding author. Fax: +31-317-418094.
E-mail address: laurens.kroon@wur.nl (L.P.N.M. Kroon).

1087-1845/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2004.03.007

of *Phytophthora* species with an emphasis on the phylogenetic position of *P. infestans* and related species. The value of conduction phylogenetic analysis on multiple genes has been shown recently in *Fusarium* (O'Donnel et al., 1998) and yeast phylogenies (Rokas et al., 2003) and is now a well-established practice in, for instance, the angiosperms (Davis and Chase, 2004).

Timing of proliferation of *P. infestans* and related species, such as *Phytophthora mirabilis*, *Phytophthora ipomoeae*, and *Phytophthora phaseoli*, can be important from a phytopathological point of view. DNA sequence divergence rates may serve as a measurement for the level of isolation between these species and may be indicative for the possibility of gene flow between *P. infestans* and closely related species (Goodwin et al., 1999). Such gene flow can have a major impact on pathogenicity as interspecies crosses have resulted in progeny with host ranges broader than either parental species (Man in 't Veld et al., 1998).

The principal aim of our study was to examine, in detail, the phylogenetic relationships within the genus *Phytophthora*. Because of their different mechanisms of inheritance, a comparison of phylogenies based on nuclear and mitochondrial gene sequences was fundamental to this study. Nuclear genes are inherited from both the maternal and paternal line, but mitochondrial genes are exclusively transmitted through the maternal line (Whittaker et al., 1994). The hypothesis will be tested that sequence information originating from both nuclear and mitochondrial DNA can be pooled for phylogenetic analysis. The second objective is to test the validity of *Phytophthora* sub-clades proposed in earlier studies (Cooke et al., 2000) and to establish the position of *P. infestans* and closely related species within the *Phytophthora* clade. The third objective is to explore patterns of evolution of sexual traits (homothallic vs. heterothallic mating system, antheridial attachment), morphological characteristics (presence or absence of papillae), and ecological niche preference (soil or foliage).

2. Materials and methods

2.1. Selection of isolates

Isolates used in this study were selected from 45 *Phytophthora* species (Table 1), with an emphasis on *P. infestans* and the related taxa *P. mirabilis* and *P. ipomoeae* (Flier et al., 2002). All isolates were classified according to Erwin and Ribeiro (1996). A *Phytophthora* isolate, of which the taxonomic identity has not yet been determined, isolated by the Dutch Plant Protection Service from *Spathiphyllum* spp. (unpublished data), was also included in this study, as was an isolate of putative hybrid origin, isolated from alder trees in the Netherlands (Brasier et al., 1999). These isolates will be referred to as *P.* sp. Spathiphyllum and *P.* hybrid-Dutch variant, respectively. In addition, a group of isolates from the Andean Highlands in Ecuador was included. This group will be referred to as *Phytophthora andina*, a putative new species described by Adler et al. (2004).

Outgroups were selected on the basis of a phylogenetic study by Riethmüller et al. (2002). *Pythium aphanidermatum* and the following Stramenopile representatives were used as outgroups in our analysis: *Fragilaria striatula* (GenBank Accession Nos. AB020224), *Ophiocytium majus* (AB000210), *Pylaiella littoralis* (Z72500), *Pythium ultimum* (AF218256), and *Achlya klebsiana* (J05597), each covering a single gene sequence in the data set.

Isolates of *P. infestans* were chosen from a wide range of host plant species and geographic locations. For *P. infestans*, *P. mirabilis*, and *P. ipomoeae*, peptidase (Pep) and glucose-6-phosphate isomerase (Gpi) allozyme patterns (Goodwin et al., 1995) were used as an additional selection criterion in order to maximize the likely diversity amongst the selected isolates. For *P. infestans* (37 isolates), *P. mirabilis* (15), and *P. ipomoeae* (4), isolates were pooled into groups with close to 100% sequence identity, and from each of these groups, one representative isolate was selected for subsequent phylogenetic analysis. This resulted in four groups of *P. infestans* isolates, corresponding to the four *P. infestans* haplotypes (Ia, Ib, IIa, and IIb) (Griffith and Shaw, 1998), five groups of *P. mirabilis* isolates (which we will refer to as types I–V), and one group of *P. ipomoeae* isolates.

To facilitate comparison between the data presented in this paper and the analysis of the ITS-region (ITS 1 and 2 flanking the 5.8S rDNA subunit) by Cooke et al. (2000), we have used the same isolates when available (Table 1) and adopted the ITS clade nomenclature whenever applicable.

2.2. DNA extraction

Isolates were grown for 10–14 days at 20 °C in pea broth. This medium was prepared by autoclaving 120 g of frozen peas in 1 L of tap water (20 min at 121 °C), filtering through cheesecloth and re-autoclaving (Flier et al., 2003). The mycelium was harvested, lyophilized, and total DNA extracted using the Puregene kit (Gentra/Biozyme, Landgraaf, The Netherlands) according to the manufacturer's instructions. DNA pellets were dissolved in 100 μl TE [10 mM Tris–HCl (pH 8.0), 1 mM EDTA] and stored at −20 °C.

2.3. Primer design

Primers were developed using published DNA sequences for the genus *Phytophthora*. The last nucleotide

Table 1
Isolates of *Phytophthora* used in this study, their designations, origins and year of collection

Phytophthora species	Isolate numbers		Origins		
	International	Local	Host	Country	Year
P. infestans haplotype Ia	—	Pic99186	*Solanum stoliniferum*	Mexico	1999
P. infestans haplotype Ib	—	West Virginia 4	*Solanum tuberosum*	USA	—
P. infestans haplotype IIa	—	Dr98004	*Solanum tuberosum*	The Netherlands	1998
P. infestans haplotype IIb	—	Can4	—	Canada	—
P. mirabilis type I	—	Pic99129	*Mirabilis jalapa*	Mexico	1999
P. mirabilis type II	—	P3001	*Mirabilis jalapa*	Mexico	1984
P. mirabilis type III	—	Pic99145	*Mirabilis jalapa*	Mexico	1999
P. mirabilis type IV	—	G4-4	*Mirabilis jalapa*	Mexico	1998
P. mirabilis type V	—	G15-4	*Mirabilis jalapa*	Mexico	1998
P. ipomoeae	—	Pic99165	*Ipomoea longipedunculata*	Mexico	1999
P. phaseoli [a]	ATCC60171	CBS556.88	*Phaseolus lunatus*	—	—
P. andina	—	EC3421	*Solanum muricatum*	Ecuador	2001
P. arecae	—	CBS148.88	*Chamaedorea seifrizzi* × *erumpens*	USA	—
P. boehmeriae	—	CBS291.29	*Boehmeria nivea*	Japan	—
P. botryosa	IMI136916	CBS533.92	*Hevea brasiliensis*	Thailand	—
P. cactorum	—	P6183	*Rubus idaeus*	USA	—
P. hybrid-Dutch variant	—	PD92/1471	*Alnus cordata*	The Netherlands	1992
P. tropicalis	AN97/86	PD97/11132	*Rosa* spp.	The Netherlands	1997
P. cinnamomi	RADICI B	10A6	*Persea americana*	—	—
P. citricola	—	P1817	*Medicago sativa*	South Africa	—
P. citrophthora	CBS274.33	PD94/353	*Citrus limonium*	Cyprus	—
P. clandestina [a]	IMI287317	R193	*Trifolium subterranea*	Australia	1985
P. colocasiae [a]	IMI368918	—	*Colocasia esculenta*	Malaysia	1995
P. cryptogea	—	HR1/ss/pp/99	*Lycopersicon esculentum*	UK	—
P. drechsleri [a]	—	CBS292.35	*Beta vulgaris var. altissima*	USA	1935
P. erythroseptica	ATCC46725	CBS951.87	*Solanum tuberosum*	Australia	—
P. fragariae var. *fragariae* I	—	A2	*Fragaria* × *ananassa*	—	—
P. fragariae var. *fragariae* II	—	NS4	*Fragaria* × *ananassa*	—	—
P. fragariae var. *rubi* I	—	FVR67	*Rubus idaeus*	—	—
P. fragariae var. *rubi* II	—	FVR30	*Rubus idaeus*	Scotland	—
P. gonapodyides [a]	—	P245	*Salix matsudana*	UK	1972
P. heveae [a]	IMI180616	CBS296.29	*Hevea brasiliensis*	Malaysia	1929
P. hibernalis	ATCC64708	CBS522.77	*Aquilegia vulgaris*	New Zealand	—
P. humicola [a]	IMI302303	—	citrus orchard soil *via* citrus bait	Taiwan	1981
P. idaei	IMI313727	R66	—	UK	—
P. ilicis	—	PD91/595	Ilex aquifolium	The Netherlands	1991
P. inflata [a]	IMI342898	—	Syringa	UK	1990
P. insolita [a]	IMI288805	—	soil	Taiwan	—
P. iranica [a]	IMI158964	CBS374.72	*Solanum melongena*	Iran	1969
P. katsurae	IMI325914	CBS587.05	soil	Taiwan	1979
P. lateralis [a]	IMI040503	CBS168.42	*Chamaecyparis lawsoniana*	USA	—
P. meadii [a]	IMI129185	CBS219.88	*Hevea brasiliensis*	India	1968
P. megakarya	IMI337098	—	*Theobroma cacao*	Equatorial Guinea	—
P. megasperma [a]	IMI133317	MEG23	*Malus sylvestris*	Australia	1968
P. multivesiculata [a]	CBS545.96	PD95/8679	Cymbidium	The Netherlands	1995
P. nicotianae	—	P582	*Nicotiana tabacum*	USA	—
P. palmivora	—	CBS236.30	*Cocos nucifera*	India	—
P. brassicae	—	CBS179.87	*Brassica oleracea*	The Netherlands	1987
P. pseudotsugae [a]	IMI331662	PSE1	*Pseudotsuga menziesii*	USA	—
P. quininea	—	CBS407.48	*Cinchona officinalis*	Peru	—
P. richardiae	—	CBS240.30	*Zantedeschia aethiopica*	USA	—
P. sinensis	—	P1475	—	—	—
P. sojae	—	P6497	*Glycine max*	—	—
P. syringae	IMI045169	CBS364.52	*Prunus armeniaca*	New Zealand	—
P. tentaculata [a]	—	CBS552.96	*Chrysanthemum leucanthemum*	Germany	—
P. vignae	—	CBS241.73	*Vigna sinensis*	Australia	1992
P. sp. Spathiphyllum	—	—	*Spathiphyllum* spp.	—	—
P. ramorum US-type	—	USA 0.13	Quercus agrifolia	USA	—
P. ramorum European-type	—	PD93/51	*Rhododendron catawbiense*	The Netherlands	1993

[a] These isolates were used in the ITS-based analysis by Cooke et al. (2000).

of the primers (at the 3′ position, where amplification starts) was chosen to be located at the last or first base of a triplet, and was either a G or C. Forward and reverse primers were constructed to amplify a product of 900–1100 basepairs (bp), in coding regions. For each DNA region, 2–4 primers were developed and tested in pairs. Primer pairs that amplified the target sequences best in all species were selected and used for sequencing (Table 2). Primer regions were not included in the sequence alignments.

For the translation elongation factor 1 alpha gene (*EF*-1α), the *P. infestans* mRNA described by Van 't Klooster et al. (2000) was used as a basis for primer selection (Accession No. AJ249839). Primers ELONGF1 and ELONGR1 amplified a 972-bp central fragment of the gene. No introns were present in this region. For β-tubulin (β-*tub*) primer selection, the *P. cinnamomi* mRNA Accession No. U22050 was used (Weerakoon et al., 1998). Amplification with TUBUF2 and TUBUR1 yielded a fragment of 989 bp, with no introns present.

For all mitochondrial regions analyzed in this study, the complete *P. infestans* mitochondrial DNA-sequence U17009 (Paquin et al., 1997) was used as a template for primer design. For the cytochrome *c* oxidase subunit 1 gene (*cox*1), primers COXF4N and COXR4N amplified a region of 972 bp. From a subset of isolates (*P. infestans*, *P. mirabilis*, *P. ipomoeae*, *P. phaseoli*, *P. andina*, and *Phytophthora tropicalis*) *Cox*1 was also amplified with primers used in *P. infestans* haplotyping (Griffith and Shaw, 1998). Primers F4 and R4 amplified a region of 964 bp (referred to as P4), with a 719-bp overlap with the COXF4N/COXR4N fragment. This resulted in an extension of 191 bp of sequence information for this subset of isolates, of which a region of 148 bp consists of non-coding spacer DNA. The NADH dehydrogenase subunit 1 gene (*nadh*1) is present in reverse orientation, and the primers used to amplify it were NADHF1 and NADHR1, which yielded a fragment of 897 bp of coding sequence. For part of the ATP synthase F1 subunit α (*atp*1) gene, the glutamic acid tRNA (trnE), and part of the NADH dehydrogenase subunit 4 (*nad*4) gene (in total referred to as P2), primers F2 and R2 amplified a region of 1070 bp. Only a subset of isolates (*P. infestans*, *P. mirabilis*, *P. ipomoeae*, *P. phaseoli*, *P. andina*, *P. tropicalis*, and *Phytophthora sojae*) was used for amplification with these primers, designed for haplotyping *P. infestans* isolates (Griffith and Shaw, 1998). Two non-coding spacers (bp14,102–14,181 and bp14,254–14,282 bp) separated the three coding regions in this fragment.

2.4. DNA amplification and sequencing

Amplifications were performed in a PTC200 thermocycler (MJ Research, Waltham, MA, USA). The thermocycle sequence was as follows: an initial denaturation at 94 °C for 2 min; 35 cycles consisting of denaturation at 94 °C for 30 s (60 s for P2 and P4), annealing for 30 s, and extension at 72 °C for 60 s; a final extension at 72 °C for 10 min. Annealing temperatures were 60 °C for *EF*-1α and β-*tub*, 52 °C for *cox*1, 53 °C for *nadh*1, and 62 °C for P2 and P4. The reaction mix consisted of 10–20 ng of template DNA, 200 μM dNTPs, 1 U *Taq* DNA polymerase (Roche, Indianapolis, USA), 1.5 mM $MgCl_2$, and 25 ng of each primer in a reaction volume of 25 μl. For mtDNA gene amplification, the $MgCl_2$ concentration was raised to 3.5 mM. Successful amplification was confirmed by gel electrophoresis.

Table 2
Primers used in this study

Target DNA	Primer	Primer sequence	Reference[a]	Primer orientation[b]	T_m[c]	GC[d]	Size[e]
Translation elongation factor 1α	ELONGF1	5′ TCACGATCGACATTGCCCTG 3′	AJ249839	180–199	69.5	55.0	972
	ELONGR1	5′ ACGGCTCGAGGATGACCATG 3′	U22050	1132–1151	69.7	60.0	
β-Tubulin	TUBUF2	5′ CGGTAACAACTGGGCCAAGG 3′		570–589	68.5	60.0	989
	TUBUR1	5′ CCTGGTACTGCTGGTACTCAG 3′		1538–1558	61.3	57.1	
Cytochrome *c* oxidase subunit 1	COXF4N	5′ GTATTTCTTCTTTATTAGGTGC 3′	U17009	9126–9147	53.2	31.8	972
	COXR4N	5′ CGTGAACTAATGTTACATATAC 3′	U17009	10,076–10,097	50.6	31.8	
P4	F4	5′ TGGTCATCCAGAGGTTTATGTT 3′		9379–9400	62.1	40.9	964
	R4	5′ CCGATACCGATACCAGCACCAA 3′		10,321–10,342	69.6	54.5	
NADH dehydrogenase subunit 1	NADHF1	5′ CTGTGGCTTATTTTACTTTAG 3′	U17009	27,278–27,298	52.7	33.3	897
		5′ CAGCAGTATACAAAAACCAAC 3′					
	NADHR1	5′ CAGCAGTATACAAAAACCAAC 3′		26,402–26,422	56.3	38.1	
P2	F2	5′ TTCCCTTTGTCCTCTACCGAT 3′	U17009	13,613–13,633	63.5	47.6	1070
	R2	5′ TTACGGCGGTTTAGCACATACA 3′		14,661–14,682	66.0	45.5	

[a] Reference to the GenBank accession containing the DNA sequence, on which the primer is based.
[b] Reference to the location of the primer within the original DNA sequence.
[c] Melting temperature of the primer.
[d] GC-content of the primer.
[e] Average amplicon length.

PCR products were purified on Sephadex plates (Multiscreen HV, Millipore, Bedford, USA) to remove excess primers and nucleotides and sequenced with the corresponding primers using the BigDye sequencing kit (Applied Biosystems, Foster City, USA) on an ABI3700 DNA Analyzer (Applied Biosystems). The trace files were transferred to the SeqMan 5.0 module of DNASTAR (DNASTAR, Madison, WI, USA). As the sequence results originated from PCR products, heterozygous sites were observed and labeled according to the IUPAC coding system. Forward and reverse sequences were linked in Editseq 5.0 (DNASTAR) and aligned using the ClustalW algorithm provided in the MegAlign module (DNASTAR). Due to length variation in the spacer regions of the P2 and P4 amplicons, some manual adjustment of the alignment of gaps was necessary.

2.5. Phylogenetic analysis

Sequence data for five coding mtDNA regions (*cox*1, *nadh*1, *atp*1, trnE, and *nad*4) and two nuclear encoded genes (β-*tub* and *EF*-1α) were compared for 62 accessions. In this study, an accession was defined as an isolate or group of isolates covered by a single entry in our data matrix (Table 1). For *P. infestans* (4 accessions, representing 44 sequenced isolates), *P. mirabilis* (5 accessions, representing 19 sequenced isolates), *Phytophthora fragariae* (4 accessions, representing 4 sequenced isolates), and *P. ramorum* (2 accessions, representing 5 sequenced isolates), species are represented by more than one type. For each accession, sequence entries from a single representative isolate were used. Not all regions were sequenced for all accessions; data coverage is listed in Table 3. Phylogenetic analysis was performed using PAUP* version 4.0b10 (Swofford, 2002) running on a G4 Power Macintosh computer.

We conducted separate phylogenetic analyses for individual genes, the combined mtDNA, combined nDNA, and combined mt + nDNA data sets. Jackknife analysis was carried out to assess data structure and to identify significantly supported clades. In addition, between-data set congruence was tested for by comparing jackknife topologies, which was done by visual inspection. Jackknife analysis (10,000 replicates) was carried out using PAUP* with settings so as to emulate the Parsimony Jackknifer (Farris et al., 1996), i.e., percentage of characters deleted in each replicate = 37, 'fast' stepwise addition and "Jac" resampling method used. Subsequent parsimony search was performed using a heuristic search, which involved TBR branch swapping, MULTREES 'on,' and 'collapse branches when maximum length is zero.' Starting trees were either generated by 500 cycles of random addition sequence (RAS) holding 3 trees at each step, or by swapping on sub-optimal trees generated from 100 RAS with no swapping, MULTREES 'off' and hence keeping one tree from each replicate, even if not optimal over all replicates.

2.6. Association between functional traits and phylogeny

The *Phytophthora* species included in this study were analyzed for selected traits. The following 'characters' were traced using MacClade 4 (Maddison and Maddison, 2000): mating system (two states; homothallic or heterothallic); presence and shape of papillae (three states; non-papillate, semi-papillate, or papillate); and type of antheridia (two states; amphigynous or paragynous). Character states were obtained from literature reports and are listed in Table 4 (Adler et al., 2004; Aragaki and Uchida, 2001; Erwin and Ribeiro, 1996; Flier et al., 2002; Ilieva et al., 1998; Man in 't Veld et al., 2002; Werres et al., 2001).

3. Results

3.1. Phylogenetic analysis of nDNA and mtDNA data sets

The combined nuclear DNA data set comprised 1874 characters for 61 accessions, which included 58 *Phytophthora* accessions, two *Pythium* accessions, and *A. klebsiana* as an outgroup. For *EF*-1α 57 accessions were represented, for β-*tub* 60, and for the two genes combined 61. The combined nuclear DNA data set contained 411 potentially phylogenetically informative characters.

The combined mitochondrial DNA data set comprised 2952 characters for 63 accessions, including 59 *Phytophthora* accessions, *P. aphanidermatum*, *F. striatula* (Fragilariophyceae), *O. majus* (Xantophyceae), and *P. littoralis* (Phaeophyceae); the latter three were used as outgroup. For the P2 region, 14 accessions were represented, for *cox*1 63, for *nadh*1 59, and for all mtDNA regions combined 63. The combined mtDNA data set contained 511 characters that were potentially phylogenetically informative.

Jackknife analyses of the separate mitochondrial and separate nuclear gene data sets resulted in largely congruent topologies (data not shown). Only *Phytophthora meadii*, *Phytophthora heveae*, and *Phytophthora citricola* were located on unexpected branches based on *cox*1, *Phytophthora katsurae* and *Phytophthora hibernalis* based on *nadh*1, and *Phytophthora insolita* in the analysis of the *EF*-1α region. However, as omitting these six taxa from the combined nuclear and mitochondrial data set did not alter the resulting overall tree topology (data not shown), we opted to combine the mitochondrial genes and the nuclear genes into two data sets and to include the six taxa *P. meadii*, *P. heveae*, *P. citricola*, *P. katsurae*, *P. hibernalis*, and *P. insolita* in our analysis.

Table 3
Data coverage for accessions included in the analysis

Phytophthora species	nDNA genes		mtDNA genes		
	β-tubulin	*EF*-1α	*cox*1	*nadh*1	P2
P. infestans haplotype Ia	AY564035	AY564093	AY564150	AY563977	AY564209
P. infestans haplotype Ib	ND[a]	ND	U17009	U17009	U17009
P. infestans haplotype IIa	AY564036	AY564094	AY564151	AY563978	AY564210
P. infestans haplotype IIb	AY564037	ND	AY564152	AY563979	AY564211
P. mirabilis type I	AY564038	AY564095	AY564153	AY563980	AY564212
P. mirabilis type II	AY564039	AY564096	AY564154	AY563981	AY564213
P. mirabilis type III	AY564040	AY564097	AY564155	AY563982	AY564214
P. mirabilis type IV	AY564041	AY564098	AY564156	AY563983	AY564215
P. mirabilis type V	AY564042	AY564099	AY564157	AY563984	AY564216
P. ipomoeae	AY564043	AY564100	AY564158	AY563985	AY564217
P. phaseoli	AY564044	AY564101	AY564159	AY563986	AY564218
P. andina	AY564045	AY564102	AY564160	AY563987	AY564219
P. arecae	AY564049	AY564105	AY564164	AY563991	ND
P. boehmeriae	AY564050	AY564106	AY564165	AY563992	ND
P. botryosa	AY564051	AY564107	AY564166	AY563993	ND
P. cactorum	AY564052	AY564108	AY564167	AY563994	ND
P. hybrid-Dutch variant	AY564053	AY564109	AY564168	AY563995	ND
P. tropicalis	AY564046	AY564103	AY564161	AY563988	AY564220
P. cinnamomi	AY564054	AY564110	AY564169	AY563996	ND
P. citricola	AY564055	AY564111	AY564170	AY563997	ND
P. citrophthora	AY564056	AY564112	AY564171	AY563998	ND
P. clandestina	AY564057	AY564113	AY564172	AY563999	ND
P. colocasiae	AY564058	AY564114	AY564173	AY564000	ND
P. cryptogea	AY564059	AY564115	AY564174	AY564001	ND
P. drechsleri	AY564060	AY564116	AY564175	AY564002	ND
P. erythroseptica	AY564061	AY564117	AY564176	AY564003	ND
P. fragariae var. *fragariae* I	AY564062	AY564118	AY564177	AY564004	ND
P. fragariae var. *fragariae* II	AY564063	AY564119	AY564178	AY564005	ND
P. fragariae var. *rubi* I	AY564064	AY564120	AY564179	AY564006	ND
P. fragariae var. *rubi* II	AY564065	AY564121	AY564180	AY564007	ND
P. gonapodyides	AY564066	AY564122	AY564181	AY564008	ND
P. heveae	AY564067	AY564123	AY564182	AY564009	ND
P. hibernalis	AY564068	AY564124	AY564183	AY564010	ND
P. humicola	AY564069	AY564125	AY564184	AY564011	ND
P. idaei	AY564070	AY564126	AY564185	AY564012	ND
P. ilicis	AY564071	AY564127	AY564186	AY564013	ND
P. inflata	AY564072	AY564128	AY564187	AY564014	ND
P. insolita	AY564073	AY564129	AY564188	AY564015	ND
P. iranica	AY564074	AY564130	AY564189	AY564016	ND
P. katsurae	AY564075	AY564131	AY564190	AY564017	ND
P. lateralis	AY564076	AY564132	AY564191	AY564018	ND
P. meadii	AY564077	AY564133	AY564192	AY564019	ND
P. megakarya	AY564078	AY564134	AY564193	AY564020	ND
P. megasperma	AY564079	AY564135	AY564194	AY564021	ND
P. multivesiculata	AY564080	AY564136	AY564195	AY564022	ND
P. nicotianae	AY564081	AY564137	AY564196	AY564023	ND
P. palmivora	AY564082	AY564138	AY564197	AY564024	ND
P. brassicae	AY564083	AY564139	AY564198	AY564025	ND
P. pseudotsugae	AY564084	AY564140	AY564199	AY564026	ND
P. quininea	AY564085	AY564141	AY564200	AY564027	ND
P. richardiae	AY564086	AY564142	AY564201	AY564028	ND
P. sinensis	AY564087	AY564143	AY564202	AY564029	ND
P. sojae	AY564047	AY564104	AY564162	AY563989	AY564221
P. syringae	AY564088	AY564144	AY564203	AY564030	ND
P. tentaculata	AY564089	AY564145	AY564204	AY564031	ND
P. vignae	AY564090	AY564146	AY564205	AY564032	ND
P. sp. Spathiphyllum	AY564091	AY564147	AY564206	AY564033	ND
P. ramorum US-type	ND	AY564148	AY564207	ND	ND
P. ramorum European-type	AY564092	AY564149	AY564208	AY564034	ND
P. aphanidermatum	AY564048	ND	AY564163	AY563990	ND

[a] ND, not determined.

Table 4
Properties of taxa within *Phytophthora* clades 1-8 as identified in this study [and correlating as much as possible with clade numbering by Cooke et al. (2000)]

Clade	Species	Group[a]	Antheridia[b]	Papillae[c]	Mating[d]	Niche[e]
1a	*P. cactorum*	I	P	P	Ho	Fol/soil
1a	*P. idaei*	I	P	P	Ho	Soil
1a	*P. pseudotsugae*	I	P	P	Ho	Soil
1b	*P. clandestina*	I	AP	P	Ho	Soil
1b	*P. iranica*	I	P	P	Ho	Soil
1c	*P. infestans*	IV	A	S	He	Foliage
1c	*P. andina*	—	A	S	He	Foliage
1c	*P. ipomoeae*	—	A	S	Ho	Foliage
1c	*P. mirabilis*	IV	A	S	He	Foliage
1c	*P. phaseoli*	IV	A	S	Ho	Foliage
1d	*P. nicotianae*	II	A	P	He	Fol/soil
2a	*P. citrophthora*	II	A	P	He	Soil
2a	*P. colocasiae*	IV	A	S	He	Foliage
2a	*P. meadii*	II	A	P	He/Ho	Foliage
2a	*P. tropicalis*	—	A	P	He	Foliage
2a	*P. citricola*	III	P	S	Ho	Soil
2a	*P. inflata*	III	P	S	Ho	Soil
2b	*P. multivesiculata*	—	A	S	Ho	Foliage
2b	*P. tentaculata*	I	P	P	Ho	Foliage
3	*P. ilicis*	IV	A	S	Ho	Foliage
4	*P. botryosa*	II	A	P	He	Foliage
4	*P. palmivora*	II	A	P	He	Foliage
4	*P. arecae*	II	A	P	He	Foliage
4	*P. megakarya*	II	A	P	He	Foliage
5	*P. heveae*	II	A	P	Ho	Foliage
5	*P. katsurae*	VI	A	P	Ho	Soil
6	*P. humicola*	V	P	N	Ho	Soil
6	*P. gonapodyides*	VI	A	N	He	Soil
6	*P. megasperma*	V	P	N	Ho	Soil
7a	*P. fragariae* var. *fragariae*	V	A	N	Ho	Soil
7a	*P. fragariae* var. *rubi*	V	A	N	Ho	Soil
7a	*P.* hybrid-Dutch variant	—	AP	N	Ho	Soil
7b	*P. sojae*	V	P	N	Ho	Soil
7b	*P. sp.* Spathiphyllum	—	ND[f]	ND	ND	ND
7b	*P. sinensis*	V	A	N	Ho	Foliage
7b	*P. vignae*	VI	A	N	Ho	Soil
7c	*P. cinnamomi*	VI	A	N	He	Soil
8a	*P. cryptogea*	VI	A	N	He	Soil
8a	*P. erythroseptica*	VI	A	N	Ho	Soil
8a	*P. drechsleri*	VI	A	N	He	Soil
8b	*P. syringae*	III	P	S	Ho	Soil
8b	*P. brassicae*	—	A	S	Ho	Soil
8c	*P. hibernalis*	IV	A	S	Ho	Fol/soil
8d	*P. lateralis*	V	P	N	Ho	Soil
8d	*P. ramorum* European-type	—	A	S	He	Foliage
8d	*P. ramorum* US-type	—	A	S	He	Foliage
8e	*P. quininea*	V	P	N	Ho	Soil
8e	*P. richardiae*	VI	A	N	Ho	Soil
8f	*P. boehmeriae*	II	A	P	Ho	Fol/soil
8f	*P. insolita*	V	ND	N	Ho	Soil

[a] According to Waterhouse (1963).
[b] P, paragynous attachment of the antheridium to the oogonium; A, amphigynous attachment.
[c] P, papillate sporangia; S, semi-papillate sporangia; and N, non-papillate sporangia.
[d] Ho, homothallic species; He, heterothallic species.
[e] Niche, in which the pathogen is commonly found.
[f] ND, not determined.

Analyses of these combined mitochondrial and combined nuclear data sets were carried out to assess congruence between the resultant phylogenetic trees. Jackknife consensus trees from the combined nDNA and combined mtDNA analyses are shown in Figs. 1A and B. The nDNA based jackknife topology contains at least four main (sub)clades (1, 2a, 7a, and 7b) that also occur in the mtDNA based topology. Incongruence

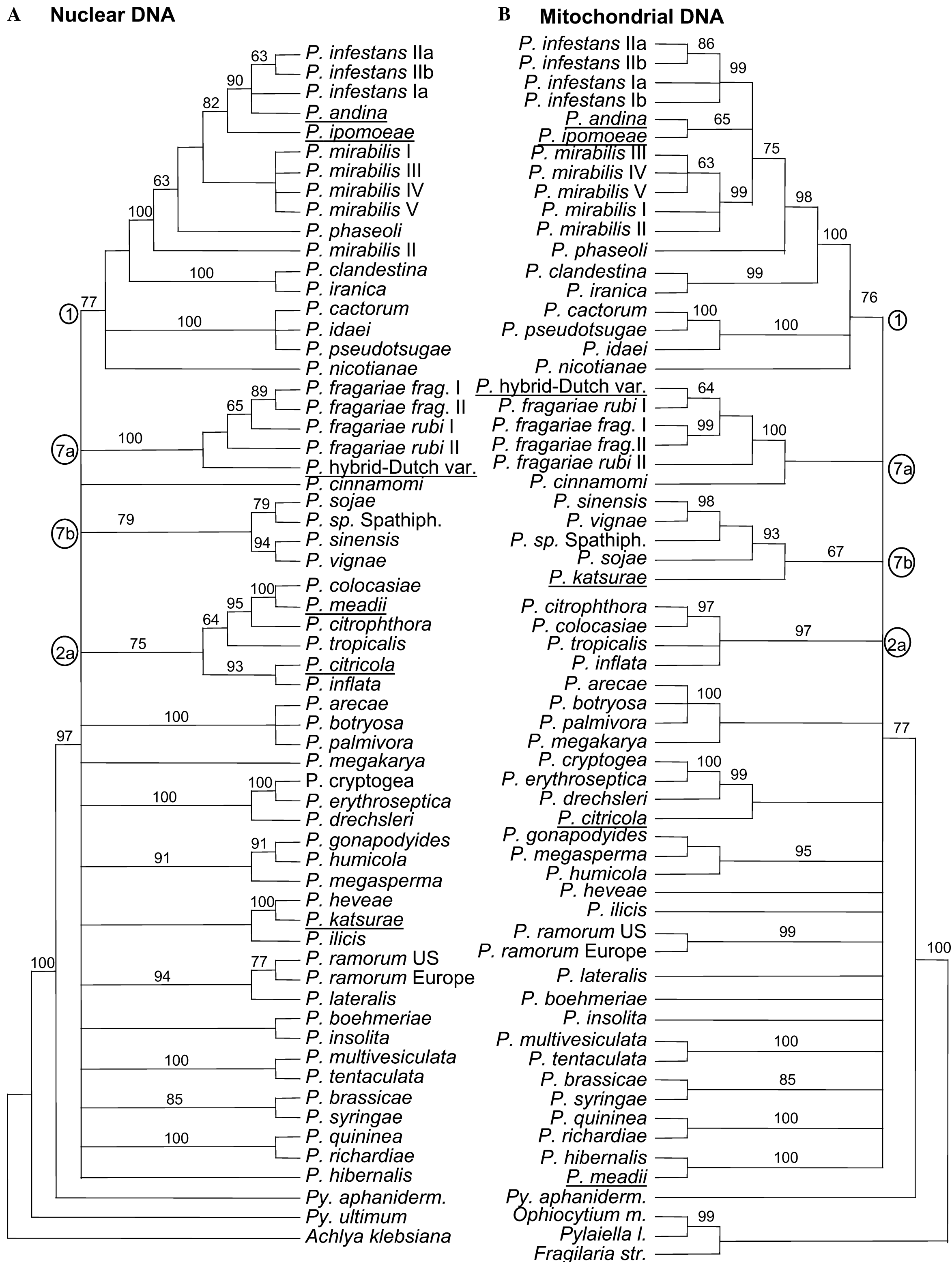

Fig. 1. Nuclear and mitochondrial DNA sequence data compared: jackknife consensus trees (10,000 replicates) of combined nuclear encoded genes (*β-tubulin* + *EF*-1α; A) and combined mtDNA encoded genes and spacer sequences (B). Numbers on branches indicate jackknife frequencies, underlined names indicate incongruently placed taxa at >63% jackknife support level. Outgroup taxa *P. ultimum* and *A. klebsiana* were not available for the mtDNA data set, whereas *Ophiocytium*, *Pylaiella*, and *Fragilaria* were not included in the nuclear data set. Numbers indicate the largest supported (sub)clades.

between the phylogenetic placement in the mitochondrial and nuclear sequence based trees was evident in six species (underlined in Fig. 1) at the 63% jackknife level. A poor resolution is evident at the basal nodes of both the nuclear and mitochondrial trees. The heuristic search for the nDNA data set yielded 27 most parsimonious trees (MPT) of 1957 steps long which were distributed on a single tree island (CI = 0.38, RI = 0.70). The strict consensus tree topology of these 27 MPTs contains 32 nodes with >63% jackknife support (Fig. 2A).

The mtDNA based jackknife topology contains less clades with >63% jackknife support as compared to the nuclear DNA based jackknife topology (12 vs. 14 supported (sub)clades). Heuristic search of the combined mtDNA data set yielded 22 most parsimonious trees of 2572 steps long, distributed on a single tree island (CI = 0.44, RI = 0.63). The strict consensus tree topology of these 22 MPTs contains 32 nodes with >63% jackknife support (Fig. 2B).

3.2. Combined data sets

The pooled nDNA and mtDNA sequences were analyzed using the same jackknife and heuristic search settings as described above. The combined nuclear + mtDNA data set comprised 4826 characters for 63 accessions, including 59 *Phytophthora* accessions, *P. aphanidermatum*, *F. striatula*, *O. majus*, and *P. littoralis*. The latter three were used as outgroup. *A. klebsiana* and *P. ultimum* were excluded to avoid potential long branch-attraction artifacts.

The spine of the combined tree again lacked well-supported nodes (Fig. 3A), but the overall number of supported nodes (35) was greater than for the trees based on nDNA (32 nodes) and mtDNA (32 nodes) alone. The heuristic search yielded 6 MPTs of 4459 steps long, again, all situated on one single island (CI = 0.41, RI = 0.66). The differences between these six MPTs were minimal, involving only minor shifts in the placement of *Phytophthora nicotianae* and *P. infestans* type Ib. In the strict consensus tree of the 6 MPTs based on the combined data (Fig. 3A) the six taxa demonstrating incongruence between the nuclear and mtDNA data (Figs. 1 and 2) were grouped broadly in line with the nuclear data. Finally, branch lengths were estimated for the MPT with the highest likelihood (Fig. 3B), on the basis of the 9-parameter model (GTR + I + Γ) in PAUP*. Prior to branch length optimization, outgroups were pruned to avoid error in branch lengths due to long branch-attraction artifacts.

3.3. Main clades

Based upon the strict consensus tree shown in Fig. 3A, and in comparison with the clade nomenclature proposed by Cooke et al. (2000), 8 main clades were identified in the genus *Phytophthora* (Table 4). Clades 1, 2, 7, and 8 were (arbitrarily) divided into sub-clades, as within these clades significantly (jackknife) supported clusters of *Phytophthora* species could be identified.

Phytophthora nicotianae was less closely related to other taxa in clade 1, joining at a basal position, and therefore defined as sub-clade 1d. Character traits also supported this separation (Table 4). In this study, *Phytophthora tentaculata* was consistently shown to be a sister taxa to *Phytophthora multivesiculata* in sub-clade 2b, which differed from the ITS-based study (Cooke et al., 2000) in which *P. tentaculata* was shown to share a common ancestor with clade 1 taxa.

Ambiguity in the relatedness of *P. meadii* and *P. citricola* (Figs. 1 and 2) to other clade 2 taxa resulted from unusually low sequence similarity in the *cox*1 gene compared to all other genes. The consensus tree, however, suggests they share a common ancestor with all clade 2 species. The species *Phytophthora botryosa* was not included in clade 2 (in which it was placed in Cooke's analysis), since it showed high similarity with *Phytophthora arecae* and *Phytophthora palmivora*.

Phytophthora botryosa, *P. arecae*, and *P. palmivora* were closely related and alongside their nearest relative, *Phytophthora megakarya*, were included in clade 4. Despite the lack of jackknife support for including *P. megakarya* in clade 4 in the separate nDNA and mtDNA analyses it was supported by their character states (Table 4) and by the combined phylogenetic analysis (Fig. 3A).

Clade 3, which consisted of *Phytophthora ilicis* and *Phytophthora quercina* in Cooke's analysis, was marked by low bootstrap support and was diverse in character states for the ITS phylogeny. As *P. quercina* was not included in this study and *P. ilicis* was included in no other clade in our combined analysis, clade 3 *sensu* Cooke et al. (2000) was retained.

There is strong support for the close relationship of *P. heveae* and *P. katsurae* in clade 5 of our analysis of nuclear gene sequences, but on the basis of mtDNA data, the sister taxa of *P. katsurae* are the non-papillate species in clade 7 (Figs. 1B and 2B). The composition of clade 6 is identical in both studies, with high jackknife support for nDNA and mtDNA data.

In the well-supported clade 7, *P. cinnamomi* joins at a basal position and has thus been nominated clade 7c, because there was no support for including it in either sub-clade 7a or 7b. *P.* hybrid-Dutch variant from alder trees (Brasier et al., 1999) clusters with *P. fragariae* isolates (clade 7a) and the isolate found on *Spathiphyllum* spp. was most closely related to taxa in sub-clade 7b.

There is considerable diversity amongst the taxa in clade 8 and only some of its sub-clades have significant jackknife support. *Phytophthora cryptogea*, *Phytoph-*

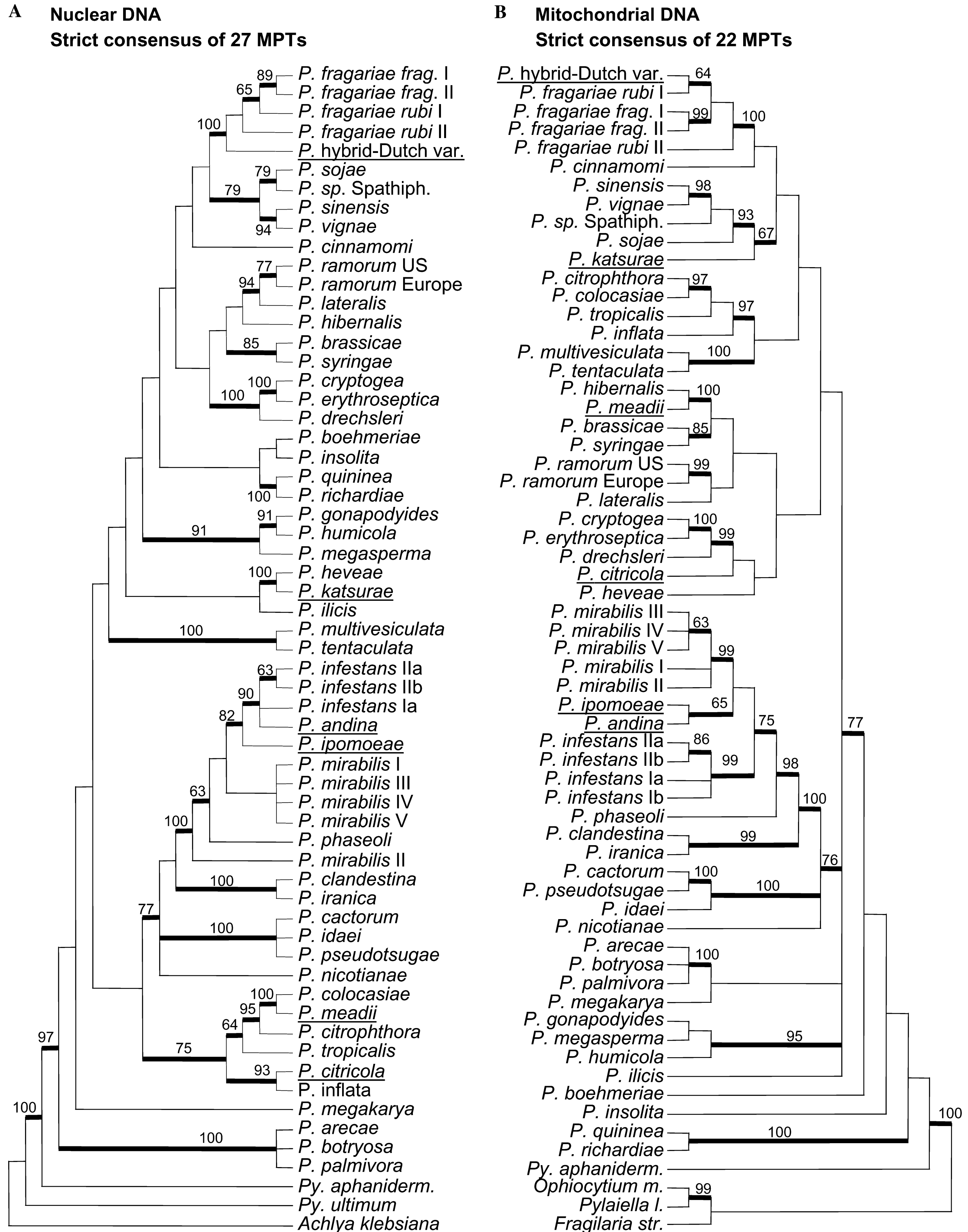

Fig. 2. Nuclear and mitochondrial DNA sequence data compared: strict consensus trees of 27 most parsimonious trees calculated from the combined nuclear DNA data set (A) and of 22 MPTs calculated from the combined mtDNA data set (B). Numbers on branches indicate jackknife frequencies, thick lines indicate significantly supported branches (>63% jackknife) and underlined names indicate incongruently placed taxa at >63% jackknife support level.

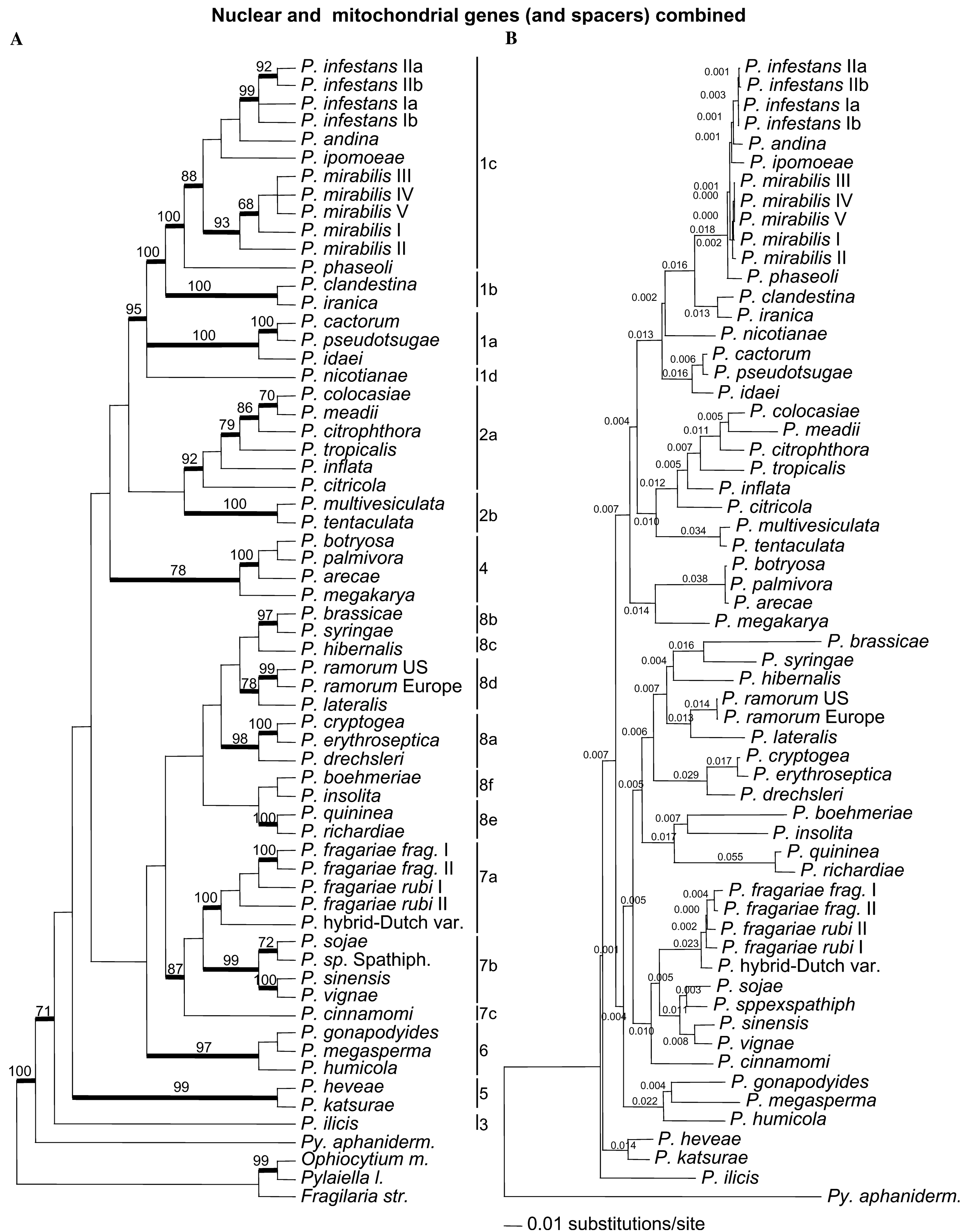

Fig. 3. Nuclear and mitochondrial DNA sequence data combined: strict consensus tree (A) of 6 most parsimonious trees with numbers on branches indicating jackknife frequencies; thick lines indicate significantly supported branches (>63% jackknife). Bars indicate the (sub)clades as described in Table 2; numbers refer to clades as identified in this study [and correlating as much as possible with clade numbering by Cooke et al., 2000]. (B) One of the 6 MPTs (the one with greatest overall likelihood) with non-Pythiacean outgroups pruned, and with branch lengths optimized based on a 9 parameter model (see text); numbers on branches indicate the number of substitutions per site. Note that *P. ultimum* and *A. klebsiana* were excluded to avoid long-branch attraction artifacts.

thora erythroseptica, and *Phytophthora drechsleri* form clade 8a. In clade 8b, the newly described species *Phytophthora brassicae* (Man in 't Veld et al., 2002) is clustered with *Phytophthora syringae*. The sudden oak death pathogen *P. ramorum* (Werres et al., 2001) clusters closely with *Phytophthora lateralis* and these species are included in clade 8d. *Phytophthora quininea* and *Phytophthora richardiae* together form clade 8e, and *Phytophthora boehmeriae* and *P. insolita* form clade 8f; note that the last two sub-clades constitute a basal grade in the analysis of the mtDNA data set (Fig. 2B), however, without support.

3.4. *Phytophthora infestans and closely related species*

The intraspecific variation in the clade 1c taxa, *P. infestans* and *P. mirabilis* was in general lower than the interspecific variation. An exception, however, was the *P. mirabilis* isolate P3001 (*P. mirabilis* II in Fig. 2A) in which the *β-tubulin* gene sequence differed markedly from the other *P. mirabilis* isolates. *P. phaseoli* shares the most distant common ancestor with the rest of the clade (Fig. 3A), based on both nDNA and mtDNA analyses. *P. ipomoeae* and *P. andina* are related to both *P. infestans* and *P. mirabilis*, showing closer sequence similarity to *P. infestans* in the case of nDNA and to *P. mirabilis* in the case of mtDNA (Figs. 1 and 2).

3.5. *Association between functional traits and phylogeny*

Waterhouse (1963) assigned *Phytophthora* species to six groups, based on morphological characteristics. To re-assess the validity of this classification, the combined nDNA + mtDNA strict consensus tree topology was used to explore possible correlations between functional characteristics and phylogeny (Figs. 4A–C). Functional characteristics (syndromes) were expressed as multistate 'characters' and optimized on the cladogram, enabling inference of evolutionary patterns.

4. Discussion

Our analysis provides the first multi-gene based phylogeny of a broad range of *Phytophthora* species. We propose a revision (as presented in Table 4) of the classification of *Phytophthora* species, based on our combined nDNA and mtDNA sequence based phylogeny, as well as on morphological traits and niche preference. The traditional classification, based on morphological traits and growth characteristics, has several limitations. Growth characteristics (like colony morphology and optimal growth temperature) are not unequivocal, and traits depend on the method used for measurement (e.g., the effect of growth media or host tissue on oospore size), or may vary because of ambiguity in trait description by observers (e.g., papillate vs. semi-papillate). The problems associated with assigning isolates to either *P. drechsleri* or *P. cryptogea* exemplify this situation (Erwin and Ribeiro, 1996; Forster et al., 2000). Additionally, growth characteristics and morphological traits are phenotypic, and groups of species sharing similar traits do not necessarily reflect evolutionary relatedness, since they may have evolved independently (convergent evolution).

In our study, we found some incongruence between phylogenies for nuclear and mitochondrial DNA. Sexual hybridization of related (Brasier et al., 1999) and unrelated (Man in 't Veld et al., 1998) species has been reported. Such hybridization events and rapid evolution within daughter species will likely confound phylogenetic analysis and a branched tree may not always be the optimal way to present such phylogenetic relationships. It is only by studying phylogenetic reconstructions based on multiple mitochondrial and nuclear genes that such relationships may be revealed. In case of recurrent hybridization events and nested species radiation, reticulate evolution may provide a more realistic explanation of these relationships (Koch et al., 2003).

The phenomena encountered in the study of *Phytophthora* hybrids in alder (Brasier et al., 1999) are exemplary of how hybridization events might influence phylogenetic inference. In these hybrids, two species are considered to have fused, resulting in tetraploid (allodiploid) offspring. The karyotype of the offspring was, however, not stable, as complete chromosomes were lost in subsequent meiosis, resulting in a heteroploid hybrid swarm (Delcan and Brasier, 2001). Crossing-over events between homeologous chromosomes in meiosis may result in hybrid gene fragments and either parent may contribute its mitochondrial DNA. All of these processes will affect the consistency of phylogenetic analyses over different genes or genomes for species that have been involved in hybridization events (Posada and Crandall, 2002; Sang and Zhong, 2000). These processes may be responsible for the observed incongruity in the classification of *P. katsurae*. The placement of *Phytophthora megasperma* in clade 2b instead of clade 6 for the *β-tubulin* sequence analysis might be another illustration of reticulate evolution. In both nuclear and mitochondrial cladograms, clade 2b (*P. multivesiculata* and *P. tentaculata*) had 100% jackknife support, while in ITS studies (Cooke et al., 2000), using identical isolates, these species were clearly placed in distinct clades. This may be another example of reticulate evolution.

For other sequence results, the situation is more difficult to explain by reticulation alone. For example, *P. meadii* and *P. citricola* appear to share a common ancestor in clade 2a on the basis of *β-tubulin*, *nadh*1, and *EF-1α* regions, however, the *cox*1 data groups *P. meadii* with *P. hibernalis* (clade 8c) and *P. citricola* with clade

A
Fragilaria str.
Ophiocytium m.
Pylaiella l.
Py. aphaniderm.
P. ilicis
P. heveae
P. katsurae
P. humicola
P. gonapodyides
P. megasperma
P. cinnamomi
P. sojae
P. sp. Spathiph.
P. sinensis
P. vignae
P. hybrid-Dutch var.
P. fragariae rubi I
P. fragariae rubi II
P. fragariae frag. I
P. fragariae frag. II
P. boehmeriae
P. insolita
P. quininea
P. richardiae
P. drechsleri
P. cryptogea
P. erythroseptica
P. hibernalis
P. brassicae
P. syringae
P. lateralis
P. ramorum US
P. ramorum Europe
P. megakarya
P. arecae
P. botryosa
P. palmivora
P. multivesiculata
P. tentaculata
P. citricola
P. inflata
P. tropicalis
P. citrophthora
P. colocasiae
P. meadii
P. clandestina
P. iranica
P. phaseoli
P. mirabilisII
P. mirabilis I
P. mirabilis III
P. mirabilis IV
P. mirabilisV
P. ipomoeae
P. andina
P. infestans Ia
P. infestans Ib
P. infestans IIa
P. infestans IIb
P. idaei
P. cactorum
P. pseudotsugae
P. nicotianae
papillae
unordered
nonpapillate
semipapillate
papillate
equivocal

B
sexuality
unordered
homothallic
heterothallic
equivocal

C
antheridium
unordered
amphigyn
paragyn
equivocal

8a taxa. For *nadh*1, *P. hibernalis* clustered with *P. nicotianae*, and *P. katsurae* had 100% homology with the isolate from *Spathiphyllum*. Sansome et al. (1991) postulated that *P. meadii* might be a hybrid species, based on variability and instability in chromosome structure, observed in meiosis. This observation could explain for the inconsistencies in the classification of *P. meadii* in our phylogenetic analysis. A group of *P. meadii* isolates was found to be tetraploid, presumably allopolyploid. Several *Phytophthora* species were mentioned by Sansome et al. as potential parental species for these hybrid isolates: *P. palmivora*, *P. capsici*, *P. botryosa*, and *P. heveae*. Our sequence results indicate that *Phytophthora colocasiae* is a more likely candidate, moreover because this species also occurs on rubber (*Hevea brasiliensis*) in south-east Asia (Ho et al., 1984). Based on homology in the *cox*1 region, *P. hibernalis* could be the other parental species involved in the formation of this hybrid species. Isozyme analysis and DNA fingerprinting experiments may give additional information on the origin of the *P. meadii* hybrids.

An explanation for the observed inconsistencies in the classification of *P. meadii* and *P. citricola* could be provided by the process of recombination in mitochondrial DNA (Hagelberg, 2003; Lössl et al., 1999; Maynard Smith and Smith, 2002; McVean, 2001; Städler and Delph, 2002). In sexually reproducing organisms, paternal mitochondria are mostly restricted from entering the ovule. There is, however, some indication of "leakage" of paternal mitochondria, which can result in contact between maternal and paternal mtDNA and recombination events between these two mitochondrial types. For *Phytophthora* hybrids, the occurrence of parental leakage might even be more common than in other organisms; if hybrids arise from cell fusion of parental isolates (e.g., fusion of zoospores (Bakonyi et al., 2002; English et al., 1999; Ersek et al., 1995)), it is likely that there are no restrictions for contact and subsequent recombination of mtDNA. Mitochondrial DNA recombination may provide a more satisfying explanation for the observed anomalous mutation patterns as compared with homoplasy or mutation hotspots. In addition, translocated pieces of mtDNA present in the nuclear genome may be mistaken for authentic organellar mtDNA. These sequences are highly similar to the original mtDNA sequences, and may confound the phylogenetic analysis because they have a different phylogenetic history compared with those in the mtDNA (Thalmann et al., 2004).

Some species exhibit such high sequence similarity that the separation into distinct species could be questioned. In this study, data are mainly based on single isolates, so additional sequencing is needed to resolve the presence of species that can be regarded as synonyms. For *P. arecae* and *P. palmivora*, the situation is more clear; our sequencing data confirm the proposition of Oudemans and Coffey (1991), McHau and Coffey (1994), and Martin and Tooley (2003) that *P. arecae* is not a distinct species, but should be regarded as a synonym of *P. palmivora*.

The taxonomic status of *P. botryosa* remains uncertain; this species was placed in clade 2 using ITS sequences (Cooke), but in our study it was placed in clade 4. In both studies, support for the grouping was high, but it should be noted that the isolates of *P. botryosa* used in each study differed. Additional research should clarify the taxonomic status of *P. botryosa*.

Cooke et al. (2000), based on neighbor joining analysis of ITS data, described two additional clades comprising *Phytophthora macrochlamydospora* and *P. richardiae* ('clade 9') and *P. insolita* ('clade 10'). These two clades were placed separately from the other eight *Phytophthora* clades in their analysis, supporting Cooke's theory of *Phytophthora* being paraphyletic. This pattern is also visible in our separate analysis of the mtDNA data set (Fig. 2B). However, in our nDNA analysis (Fig. 2A), as well as in the analysis of the combined data set (Fig. 3A), *P. insolita* and *P. richardiae* are located within the main *Phytophthora* clade. Thus, *P. insolita* and *P. richardiae* are grouped in clade 8 in our study rather than placing them in the small separate clades 9 and 10. Since all species under study form one well-supported clade (Figs. 2A and 3A), we postulate *Phytophthora* to be monophyletic.

The occurrence of two separate lines of evolution, one towards non-papillate species specializing in pathogenesis of root systems and tree trunks, the other towards (semi-) papillate species attacking the foliage of host plants, is clearly visible in the functional trait analysis (Table 3 and Fig. 4A). This observation has been made previously by Brasier (1983) and Cooke et al. (2000). Clades 6, 7, and 8 form a distinct branch in the cladogram, comprising only non- and semi-papillate species, which are predominately soil-borne.

Mating system was inferred to be a homoplasious trait (Fig. 4B), i.e., the observed similarity is based on at least eight independent transitions from homothallism to heterothallism. Homothally was reconstructed on our phylogeny as the ancestral condition. At least three indications of secondary homothallics (Brasier, 1983) are present in our data set: *P. ipomoeae* in clade 1c, the homothallic isolates of *P. meadii* in clade 2, and *P. erythroseptica* in clade 8a. The species *P. phaseoli* in

◀

Fig. 4. (A–C) Strict consensus tree of nuclear + mtDNA sequence data sets combined (the same topology as in Fig. 3) and traced with character syndromes.

clade 1c might be another example of secondary homothallism in the genus *Phytophthora* (Cooke et al., 2000). These results indicate that the switch from homothallism to heterothallism, and vice versa, has occurred quite often in the genus *Phytophthora*. This observation brings up the question of how the underlying genetic mechanism of the switch from homothallism to heterothallism (and vice versa) operates.

Comparisons with well-studied fungal mating systems (Casselton, 2002; Coppin et al., 1997; Metzenberg and Glass, 1990; Pöggeler, 1999; Yun et al., 1999) may be worthwhile in examining the patterns of inheritance and regulation of homo- and heterothallism in *Phytophthora* (Randall et al., 2003).

For antheridial attachment to the oogonium, it can be concluded that amphigyny is likely to be the ancestral state. The transition from amphigynous to paragynous attachment of the antheridium to the oogonium (Fig. 4C) has occurred independently throughout the tree. All heterothallic species have an amphigynous attachment of the antheridium (Brasier, 1983; Cooke et al., 2000).

In this study, we utilize DNA sequences from nuclear and mitochondrial origin as sources of phylogenetic markers to obtain a phylogenetic hypothesis for *Phytophthora* that reconstructs the evolutionary pathways as accurately as possible. Using nuclear and mitochondrial encoded sequences offers the advantage of independently evolving sets of characters in phylogenetic reconstruction. This has resulted in a robust phylogeny that is largely in congruence with the phylogenies described by Cooke et al. (2000) and Martin and Tooley (2003). As we base our phylogenetic tree on a significantly larger data set [900 bp (ITS, Cooke et al., 2000) vs. 1468 bp (*Cox*II & ITS, Martin and Tooley, 2003) vs. 4826 bp (our study)] we consider our phylogenetic tree as best possible estimate of *Phytophthora* phylogeny published thus far. In the light of the discussion on the applicability of ITS-regions in phylogenetic inference in angiosperms (e.g., Álvarez and Wendel, 2003; Bailey et al., 2003), we strove to conduct phylogenetic analysis without the use of ITS-regions. However, the ITS-based topologies of Cooke et al. (2000) and Martin and Tooley (2003) are in line with the results we obtained, indicating that the use of ITS-sequences in phylogenetic inference of the genus *Phytophthora* is appropriate.

For *P. infestans* and the closely related species *P. mirabilis*, *P. ipomoeae*, *P. phaseoli*, and *P. andina*, which comprise clade 1c in our analysis, ITS-sequence similarity was very high (99.9%). The phylogenetic relations between these five species could be further resolved in the analysis of the combined nDNA and mtDNA data set. The position of *P. andina*, placed intermediately between *P. infestans* and *P. mirabilis*, is surprising. This species is found in Ecuador, thousands of miles from the presumed center of origin of *P. infestans*, *P. mirabilis*, and *P. ipomoeae*, the Toluca Valley in central Mexico. The latter three species may have undergone sympatric speciation in this valley (Flier et al., 2002, 2003; Goodwin et al., 1999) and they presumably originate from a single ancestor. The findings of this new intermediate species *P. andina* in Ecuador brings up new questions on the migration of clade 1c species throughout Central and South America during history. Ambiguous gene sequence patterns for nDNA regions in *P. andina* could indicate a hybrid origin of this species, wherein *P. infestans* is one of the parental species.

The data set resulting from this study should serve as a framework for future classifications of *Phytophthora* species. As more sequence data will become available, phylogenetic relationships will become more resolved, and intra-species variation can be measured. Molecular phylogeny will also serve as a validation tool in the process of describing a new species. A third application of this data set exploits sequence polymorphisms for designing rapid diagnostic tests. Based on a sequence difference in the *cox*1 region of *P. ramorum*, an SNP-assay was recently designed to distinguish between European and American isolates of this pathogen, assisting Plant Health authorities in preventing spread and contact of the European and American sub-populations (Kroon et al., 2004).

Acknowledgments

We thank Arthur de Cock, Pierre de Wit, Peter van Baarlen, Mike Coffey, and Rolf Hoekstra for reviewing the manuscript; J.M. Duncan, D.E.L. Cooke, B.M. Tyler, and J. Postma for providing isolates; The Greenomics sequencing facility at Plant Research International for quick and accurate sequencing; and M. de Weerdt for invaluable help with the selection of isolates.

References

Adler, N.E., Erselius, L.J., Chacón, M.G., Flier, W.G., Ordoñez, M.E., Kroon, L.P.N.M., Forbes, G.A., 2004. Genetic diversity of *Phytophthora infestans* sensu lato in Ecuador provides new insight into the origin of this important plant pathogen. Phytopathology 94, 154–162.

Álvarez, I., Wendel, J.F., 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phylogenet. Evol. 29, 417–434.

Aragaki, M., Uchida, J.Y., 2001. Morphological distinctions between *Phytophthora capsici* and *P. tropicalis* sp. nov. Mycologia 93, 137–145.

Bailey, C.D., Carr, T.G., Harris, S.A., Hughes, C.E., 2003. Characterization of angiosperm nrDNA poymorphism, paralogy, and pseudogenes. Mol. Phylogenet. Evol. 29, 435–455.

Bakonyi, J., Laday, M., Ersek, T., 2002. Characterization of parental traits in somatic fusion progeny of *Phytophthora infestans* and *Phytophthora nicotianae*. Acta Phytopathol. Entomol. Hung. 37, 33–46.

Brasier, C.M., 1983. Problems and prospects in *Phytophthora* research. In: Erwin, D.C., Bartnicki-Garcia, S., Tsao, P.H. (Eds.), *Phytophthora*, its Biology, Taxonomy, Ecology and Pathology. Am. Phytopath. Soc., St. Paul, pp. 351–364.

Brasier, C.M., Cooke, D.E.L., Duncan, J.M., 1999. Origin of a new *Phytophthora* pathogen through interspecific hybridization. Proc. Natl. Acad. Sci. USA 96, 5878–5883.

Casselton, L.A., 2002. Mating recognition in fungi. Heredity 88, 142–147.

Cavalier-Smith, T.A., 1986. The kingdom Chromista: origin and systematics. In: Round, F.E., Chapman, D.J. (Eds.), Progress in Phycological Research, vol. 4. Biopress, Bristol, pp. 309–317.

Cooke, D.E.L., Drenth, A., Duncan, J.M., Wagels, G., Brasier, C.M., 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. Fungal Genet. Biol. 30, 17–32.

Coppin, E., Debuchy, R., Arnaise, S., Picard, M., 1997. Mating types and sexual development in filamentous Ascomycetes. Microbiol. Mol. Biol. Rev. 61, 411–428.

Davis, C.C., Chase, M.W., 2004. Elatinaceae are sister to Malpighiaceae; Peridiscaceae belong to Saxifragales. Am. J. Bot. 91, 262–273.

Delcan, J., Brasier, C.M., 2001. Oospore viability and variation in zoospore and hyphal tip derivatives of the hybrid alder *Phytophthoras*. Forest Pathol. 31, 65–83.

Dick, M.W., 1995. Sexual reproduction in the Peronosporomycetes (chromistan fungi). Can. J. Bot. 73 (Suppl. 1), 5712–5724.

English, J.T., Laday, M., Bakonyi, J., Schoelz, J.E., Ersek, T., 1999. Phenotypic and molecular characterization of species hybrids derived from induced fusion of zoospores of *Phytophthora capsici* and *Phytophthora nicotianae*. Mycol. Res. 103, 1003–1008.

Ersek, T., English, J.T., Schoelz, J.E., 1995. Creation of species hybrids of *Phytophthora* with modified host ranges by zoospore fusion. Phytopathology 85, 1343–1347.

Erwin, D.C., Ribeiro, O.K., 1996. *Phytophthora* Diseases Worldwide. APS Press, Am. Phytopathol. Soc., St. Paul.

Farris, J.S., Albert, V.A., Kallersjo, M., Lipscomb, D., Kluge, A.G., 1996. Parsimony jackknifing outperforms neighbor-joining. Cladistics 12, 99–124.

Flier, W.G., Grünwald, N.J., Kroon, L.P.N.M., Van den Bosch, G.B.M., Garay-Serrano, E., Lozoya-Saldaña, H., Bonants, P.J.M., Turkensteen, L.J., 2002. *Phytophthora ipomoeae* sp. nov., a new homothallic species causing leaf blight on *Ipomoea longipedunculata* in the Toluca Valley of central Mexico. Mycol. Res. 106, 848–856.

Flier, W.G., Grünwald, N.J., Kroon, L.P.N.M., Sturbaum, A.K., Van den Bosch, G.B.M., Garay-Serrano, E., Lozoya-Saldaña, H., Fry, W.E., Turkensteen, L.J., 2003. The population structure of *Phytophthora infestans* from the Toluca Valley of central Mexico suggests genetic differentiation between populations from cultivated potato and wild *Solanum* spp. Phytopathology 93, 382–390.

Forster, H., Cummings, M.P., Coffey, M.D., 2000. Phylogenetic relationships of *Phytophthora* species based on ribosomal ITS I DNA sequence analysis with emphasis on Waterhouse groups V and VI. Mycol. Res. 104, 1055–1061.

Goodwin, S.B., Schneider, R.E., Fry, W.E., 1995. Use of cellulose-acetate electrophoresis for rapid identification of allozyme genotypes of *Phytophthora infestans*. Plant Dis. 79, 1181–1185.

Goodwin, S.B., Legard, D.E., Smart, C.D., Levy, M., Fry, W.E., 1999. Gene flow analysis of molecular markers confirms that *Phytophthora mirabilis* and *P. infestans* are separate species. Mycologia 91, 796–810.

Griffith, G.W., Shaw, D.S., 1998. Polymorphisms in *Phytophthora infestans*: four mitochondrial haplotypes are detected after PCR amplification of DNA from pure cultures or from host lesions. Appl. Environ. Microbiol. 64, 4007–4014.

Hagelberg, E., 2003. Recombination or mutation rate heterogeneity? Implications for mitochondrial eve. Trends Genet. 19, 84–90.

Ho, H.H., Liang, Z.R., Zhuang, W.Y., Yu, Y.N., 1984. *Phytophthora* spp. from rubber tree plantations in Yunnan province of China. Mycopathologia 86, 121–124.

Ilieva, E., Man in 't Veld, W.A., Veenbaas-Rijks, W., Pieters, R., 1998. *Phytophthora multivesiculata*, a new species causing rot in *Cymbidium*. Eur. J. Plant Pathol. 104, 677–684.

Koch, M.A., Dobes, C., Mitchell Olds, T., 2003. Multiple hybrid formation in natural populations: Concerted evolution of the internal transcribed spacer of nuclear ribosomal DNA (ITS) in North American *Arabis divaricarpa* (Brassicaceae). Mol. Biol. Evol. 20, 338–350.

Kroon, L.P.N.M., Verstappen, E.C.P., Kox, L.F.F., Flier, W.G., Bonants, P.J.M., 2004. A rapid diagnostic test to distinguish between American and European populations of *Phytophthora ramorum*. Phytopathology 94, 613–620.

Lössl, A., Adler, N., Horn, R., Frei, U., Wenzel, G., 1999. Chondriome type characterization of potato: mt $\alpha, \beta, \gamma, \delta, \varepsilon$ and novel plastid-mitochondrial configurations in somatic hybrids. Theor. Appl. Genet. 98, 1–10.

Maddison, W.P., Maddison, D.R., 2000. MacClade. Sinauer, Sunderland, MA.

Man in 't Veld, W.A., Veenbaas-Rijks, W.J., Ilieva, E., De Cock, A.W.A.M., Bonants, P.J.M., Pieters, R., 1998. Natural hybrids of *Phytophthora nicotianae* and *Phytophthora cactorum* demonstrated by isozyme analysis and random amplified polymorphic DNA. Phytopathology 88, 922–929.

Man in 't Veld, W.A., De Cock, A.W.A.M., Ilieva, E., Levesque, C.A., 2002. Gene flow analysis of *Phytophthora porri* reveals a new species: *Phytophthora brassicae* sp. nov. Eur. J. Plant Pathol. 108, 51–62.

Martin, F.N., Tooley, P.W., 2003. Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. Mycologia 95, 269–284.

Maynard Smith, J., Smith, N.H., 2002. Recombination in animal mitochondrial DNA. Mol. Biol. Evol. 19, 2330–2332.

McHau, G.R.A., Coffey, M.D., 1994. Isozyme diversity in *Phytophthora palmivora*: evidence for a southeast Asian centre of origin. Mycol. Res. 98, 1035–1043.

McVean, G.A.T., 2001. What do patterns of genetic variability reveal about mitochondrial recombination? Heredity 87, 613–620.

Metzenberg, R.L., Glass, L.N., 1990. Mating type and mating strategies in *Neurospora*. Bioessays 12, 53–59.

O'Donnel, K., Kistler, H.C., Cigelnik, E., Ploetz, R.C., 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proc. Natl. Acad. Sci. USA 95, 2044–2049.

Oudemans, P., Coffey, M.D., 1991. A revised systematics of twelve papillate *Phytophthora* spp. based on isozyme analysis. Mycol. Res. 95, 1025–1046.

Paquin, B., Laforesst, M.-J., Forget, L., Roewer, I., Wang, Z., Longcore, J., Lang, B.F., 1997. The fungal mitochondrial genome project: evolution of fungal mitochondrial genomes and their gene expression. Curr. Genet. 31, 380–395.

Pöggeler, S., 1999. Phylogenetic relationships between mating-type sequences from homothallic and heterothallic ascomycetes. Curr. Genet. 36, 222–231.

Posada, D., Crandall, K.A., 2002. The effect of recombination on the accuracy of phylogeny estimation. J. Mol. Evol. 54, 396–402.

Randall, T.A., Ah Fong, A., Judelson, H.S., 2003. Chromosomal heteromorphism and an apparent translocation detected using a BAC contig spanning the mating type locus of *Phytophthora infestans*. Fungal Genet. Biol. 38, 75–84.

Riethmüller, A., Voglmayr, H., Goeker, M., Weiss, M., Oberwinkler, F., 2002. Phylogenetic relationships of the downy mildews (Peronosporales) and related groups based on nuclear large subunit ribosomal DNA sequences. Mycologia 94, 834–849.

Rokas, A., Williams, B.L., King, N., Carroll, S.B., 2003. Genome-scale approaches to resolving incongruence in molecular phylogenies. Nature 425, 798–804.

Sang, T., Zhong, Y., 2000. Testing hybridization hypotheses based on incongruent gene trees. Syst. Biol. 49, 422–434.

Sansome, E., Brasier, C.M., Hamm, P.B., 1991. *Phytophthora meadii* may be a species hybrid. Mycol. Res. 95, 273–277.

Städler, T., Delph, L.F., 2002. Ancient mitochondrial haplotypes and evidence for intragenic recombination in a gynodioecious plant. Proc. Natl. Acad. Sci. USA 99, 11730–11735.

Swofford, D.L., 2002. PAUP. Phylogenetic Analysis using Parsimony. Sinauer Associates, Sunderland, MA.

Thalmann, O., Hebler, J., Poinar, H.N., Pääbo, S., Vigilant, L., 2004. Unreliable mtDNA data due to nuclear insertions: a cautionary tale from analysis of humans and other great apes. Mol. Ecol. 13, 321–335.

Van 't Klooster, J.W., Van den Berg-Velthuis, G., van West, P., Govers, F., 2000. tef1, a *Phytophthora infestans* gene encoding translation elongation factor 1a. Gene 249, 145–151.

Waterhouse, G.M., 1963. Key to the species *Phytophthora* de Bary. Mycology paper No 92, 22 pp. CMI Kew, UK.

Weerakoon, N.D., Roberts, J.K., Lehnen, L.P.J., Wilkinson, J.M., Marshall, J.S., Hardham, A.R., 1998. Isolation and characterization of the single beta-tubulin gene in *Phytophthora cinnamomi*. Mycologia 90, 85–95.

Werres, S., Marwitz, R., Man in 't Veld, W.A., De Cock, A.W.A.M., Bonants, P.J.M., De Weerdt, M., Themann, K., Ilieva, E., Baayen, R.P., 2001. *Phytophthora ramorum* sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. Mycol. Res. 105, 1155–1165.

Whittaker, S.L., Assinder, S.J., Shaw, D.S., 1994. Inheritance of mitochondrial DNA in *Phytophthora infestans*. Mycol. Res. 98, 569–575.

Yoon, H.S., Hackett, J.D., Pinto, G., Bhattacharya, D., 2002. The single, ancient origin of chromist plastids. Proc. Natl. Acad. Sci. USA 99, 15507–15512.

Yun, S.-H., Berbee, M.L., Yoder, O.C., Gillian Turgeon, B., 1999. Evolution of the fungal self-fertile reproductive life style from self-sterile ancestors. Proc. Natl. Acad. Sci. USA 96, 5592–5597.