ELSEVIER

Available online at www.sciencedirect.com
ScienceDirect

Fungal Genetics and Biology 45 (2008) 266–277

FUNGAL GENETICS AND BIOLOGY

www.elsevier.com/locate/yfgbi

A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences

Jaime E. Blair [a,*], Michael D. Coffey [b,*], Sook-Young Park [a], David M. Geiser [a], Seogchan Kang [a]

[a] *Department of Plant Pathology, The Pennsylvania State University, University Park, PA 16802, USA*
[b] *Department of Plant Pathology and Microbiology, University of California, Riverside, CA 92521, USA*

Received 23 April 2007; accepted 15 October 2007
Available online 23 October 2007

Abstract

Phytophthora species are devastating plant pathogens in both agricultural and natural environments. Due to their significant economic and environmental impact, there has been increasing interest in *Phytophthora* genetics and genomics, culminating in the recent release of three complete genome sequences (*P. ramorum*, *P. sojae*, and *P. infestans*). In this study, genome and other large sequence databases were used to identify over 225 potential genetic markers for phylogenetic analyses. Here, we present a genus-wide phylogeny for 82 *Phytophthora* species using seven of the most informative loci (approximately 8700 nucleotide sites). Our results support the division of the genus into 10 well-supported clades. The relationships among these clades were rigorously evaluated using a number of phylogenetic methods. This is the most comprehensive study of *Phytophthora* relationships to date, and many newly discovered species have been included. A more resolved phylogeny of *Phytophthora* species will allow for better interpretations of the overall evolutionary history of the genus.
© 2007 Elsevier Inc. All rights reserved.

Keywords: *Phytophthora ramorum*; *Phytophthora sojae*; *Phytophthora infestans*; Oomycetes; Genomes; Phylogeny; Molecular markers

1. Introduction

The genus *Phytophthora* contains a large diversity of devastating plant pathogens which occur in both natural and agricultural settings (Erwin and Ribeiro, 1996; Judelson and Blanco, 2005). Many species of *Phytophthora* are able to infect a broad range of hosts. For example, over 2000 plant species are thought to be susceptible to infection by *P. cinnamomi* in Australia, where this pathogen has severely altered native plant communities since its accidental introduction in the 1920s (Hardham, 2005). Other species show narrow host ranges; *P. sojae* has caused upwards of $2 billion (US) in agricultural losses on its primary host, soybean (Tyler, 2007). Emerging species (e.g., *P. ramorum*, Rizzo and Garbelotto, 2003) are inflicting immeasurable damage on forest ecosystems over extremely short timescales. In addition, the rapidly expanding global commodity trade will likely accelerate the introduction and establishment of invasive species. Due to their significant environmental and economic importance, there has been increasing interest in the molecular genetics and genomics of *Phytophthora* species (Govers and Gijzen, 2006; Kamoun, 2003), as well as in archiving phenotypic and genotypic data from across the genus (e.g., http://www.PhytophthoraDB.org, S.K. unpublished data).

While *Phytophthora* and related genera exhibit morphological features analogous to pathogens in Kingdom Fungi, they in fact reside in Kingdom Stramenopila with diatoms

* Corresponding authors. Present address: Department of Biology, Amherst College, 139 McGuire Life Sciences Building, Amherst, MA 01002, USA. Fax: +1 413 542 7955 (J.E. Blair).
E-mail addresses: jblair@amherst.edu (J.E. Blair), coffey@ucr.edu (M.D. Coffey).

1087-1845/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2007.10.010

and brown algae (Dick, 2001; Förster et al., 1990; Gunderson et al., 1987; Sogin and Silberman, 1998). They are currently classified in the Peronosporomycetes within the Oomycota, although phylogenetic relationships within this group remain in question (Dick, 1990; Dick et al., 1999; Hudspeth et al., 2000; Peterson and Rosendahl, 2000; Riethmüller et al., 2002; Riethmüller et al., 1999). Unlike most members of Kingdom Fungi, oomycetes are predominantly diploid during their life cycles, producing a transient haploid phase prior to fertilization (Dick, 1990). *Phytophthora* species have traditionally been divided into six morphological groups based on features of the sporangium, antheridium, and reproductive behavior, although these characteristics are sometimes ambiguous (Newhook et al., 1978; Stamps et al., 1990; Waterhouse, 1963).

Previous molecular studies have explored the relationships among *Phytophthora* species using one or a few genetic loci, predominantly the ITS region of the nuclear ribosomal DNA (Cooke et al., 2000; Crawford et al., 1996; Förster et al., 2000; Lee and Taylor, 1992) and cytochrome oxidase I and II of the mitochondrion (Martin and Tooley, 2003a). More recent studies have used multiple loci from both the nuclear and mitochondrial genomes (Donahoo et al., 2006; Ivors et al., 2004; Kroon et al., 2004; Martin and Tooley, 2003b; Villa et al., 2006). While these studies have been successful in establishing a number of well-supported clades within the genus, they have been unable to resolve the deeper evolutionary relationships among the clades. In addition, some newly described *Phytophthora* species have been placed in an unresolved, basal group that appears to be outside the main radiation of the genus (Belbahri et al., 2006; Brasier et al., 2005; Dick et al., 2006). Establishing a well-resolved phylogeny of the genus *Phytophthora* is important not only for validating diagnostic methods of species identification (e.g., Kong et al., 2004; Lees et al., 2006; Martin et al., 2004; Schena et al., 2006; Tooley et al., 2006), but also for interpreting the evolutionary history of various genetic traits of interest, such as pathogenicity factors (Jiang et al., 2006; Liu et al., 2005; Shan et al., 2004; Whisson et al., 2004), transposable elements (Ah Fong and Judelson, 2004; Judelson, 2002), and mating types (Cvitanich et al., 2006; Qi et al., 2005).

The goal of this study was to utilize the available genome sequence data for *P. ramorum* and *P. sojae* (Tyler et al., 2006), along with the large numbers of expressed sequence tags (ESTs) from *P. infestans* (Randall et al., 2005; Win et al., 2006), *P. nicotianae* (Shan and Hardham, 2004), and others, to identify phylogenetically informative molecular markers. Loci were sought that would be informative across the genus and/or within clades or species complexes. Approximately 40 million bases (Mb) of genome sequence were analyzed, from which over 225 potential markers were identified. A subset of these markers was evaluated through PCR and sequence analyses. Here, we present a genus-wide phylogeny for 82 *Phytophthora* species using seven of the most informative loci, totaling approximately 8.7 kilobases (kb) of sequence data. Our results suggested the presence of 10 well-supported clades within the genus. We also addressed the relationships among the clades using an array of phylogenetic methods. The phylogenetic relationships recovered here are contrasted with those suggested by previous studies.

2. Materials and methods

2.1. Isolate selection and culturing

A total of 234 isolates from 82 species of *Phytophthora* and 2 species of *Pythium* were analyzed in this study. Ten new species currently being described were included, and their provisional names are used here. Most isolates (218) are maintained at the World *Phytophthora* Genetic Resources Collection (WPGRC, http://phytophthora.ucr.edu) at the University of California, Riverside, where accessions are preserved cryogenically under liquid nitrogen. Working cultures were maintained on either clarified or non-clarified V8 agar (Ribeiro, 1978) or ryeseed B agar (*P. infestans* and *P.* sp. "*andina*", Caten and Jinks, 1968). At all stages of growth, cultures were checked for bacterial contamination by incubation for 24 h in Luria broth. For DNA extraction, actively growing cultures were produced in either clarified (1:2) V8 broth or pea broth (for *P. infestans* and *P.* sp. "*andina*"), and harvested after 4–10 days. Approximately 200 mg of mycelium was rinsed with ultrapure water, placed in a 1.7 ml microcentrifuge tube and frozen by immersion in liquid nitrogen. DNA was extracted from frozen tissue using the FastDNA kit and FastPrep FP 120 instrument (MP Biomedicals Inc., Irvine, CA) according to the manufacturer's instructions, with modifications using 1 ml of CLS-VF cell lysis solution and omitting the PPS protein precipitation solution. DNA concentration was determined using a 260/280 ratio with a Beckman DU 64 UV spectrophotometer (Beckman Coulter Inc., Fullerton, CA). All DNA samples were stored in ultrapure water at −86 °C, and are available through the WPGRC DNA Bank upon request (to M.D.C.).

An additional 10 isolates were obtained from the Pennsylvania Department of Agriculture and six isolates from West Virginia University. Working cultures were maintained on either clarified or non-clarified V8 agar (Ribeiro, 1978) at 15–20 °C. For DNA extraction, a small piece of colonized agar was transferred to 50 ml sterile 10% V8 broth and incubated at room temperature for 3–7 days at 100 rpm. Mycelium was then collected, rinsed, and a small portion (10–20 mg) was lyophilized. Each sample was ground using a sterile pestle in 500 μl of extraction buffer (100 mM Tris–HCl, 10 mM EDTA, 1 M KCl, pH 8), and incubated at 75 °C for 15 min. Some samples were additionally treated with 300 μl phenol/chloroform (1:1). The aqueous phase was collected after centrifugation for 5 min (16,000*g*), and DNA was precipitated using 300 μl isopropanol. Samples were centrifuged for 10 min (16,000*g*) to pellet the DNA, which was then washed with 70% ethanol, dried, and resuspended in 100 μl TE buffer

(1× of 10 mM Tris–HCl, 1 mM EDTA, pH 8). All DNA samples were stored at −20 °C, and are available upon request (to S.K.).

2.2. Genome analysis and marker selection

Complete genome scaffolds for *P. ramorum* were visualized using the Department of Energy Joint Genome Institute Genome Browser (http://genome.jgi-psf.org/ramorum1/ramorum1.home.html). Ninety scaffolds ranging in size from 1.24 Mb to 192 kb were analyzed, totaling approximately 40 Mb of genome sequence. Individual gene models within each scaffold were considered as potential markers if they demonstrated the following criteria: regions of high similarity with the corresponding *P. sojae* gene model as represented by the VISTA browser (for optimal primer design), regions of lower similarity or predicted introns (for variable characters), similarity to open reading frames in other eukaryotes, and the presence of corresponding EST sequences from *P. sojae* (gene models represent real gene products). A total of 229 regions equivalent to putative genes were considered potentially informative. The corresponding nucleotide sequences for each of the gene models were downloaded from the *P. ramorum* and *P. sojae* genome projects (Tyler et al., 2006), and homologous sequences from other eukaryotes were obtained from the National Center for Biotechnology Information (NCBI) database using BLAST (Altschul et al., 1997). If regions of high conservation could be identified from aligned sequences, primers were designed to amplify a product approximately 1 kb in length. Web-based primer design tools (Primer3, Rozen and Skaletsky, 2000) were used to estimate the optimal annealing temperature and to check for potential secondary structure within each primer sequence. Primers were designed for 72 potential markers, of which 27 were tested by PCR. Primer melting temperature and G–C content were estimated assuming 2.5 mM $MgCl_2$ using the on-line resource NetPrimer (Premier Biosoft International, Palo Alto, CA; http://www.premierbiosoft.com/netprimer/index.html).

2.3. DNA amplification and sequencing

Standard PCR conditions were applicable for most markers. Reaction mixtures were prepared using ~5 ng template DNA, 200 μM dNTPs, 1 U *Taq* DNA Polymerase (AmpliTaq; Applied Biosystems, Foster City, CA), 2.5 mM $MgCl_2$, and 1 μM of each primer in a reaction volume of 20 μl. Some samples amplified well only with the use of a high-fidelity *Taq* DNA Polymerase (Platinum *Taq*; Invitrogen, Carlsbad, CA). In addition, a PCR optimization system was used for certain templates that could not be amplified under standard PCR conditions (Fail-Safe PCR System; EpiCentre Biotechnologies, Madison, WI). Amplifications were performed with a MJ Research PTC-100 thermal cycler (Waltham, MA) using the following cycling protocol: an initial denaturing step of 94 °C for 2 min; 35 cycles of 94 °C for 30 s, the locus-specific annealing temperature for 30 s, 72 °C extension (68 °C for high-fidelity *Taq*) for 60 s (2 min for amplicons over 1 kb); final extension of 5 min at 72 °C (68 °C for high-fidelity *Taq*). A touchdown PCR program was used when the standard amplification protocol was unsuccessful: 10 cycles of 94 °C for 30 s, 65 °C for 30 s (reduced by one degree each cycle to 56 °C), 72 °C extension for 2 min; 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C extension for 2 min; final extension of 5 min at 72 °C. All PCR products were evaluated for successful amplification using gel electrophoresis.

PCR products were then prepared for sequencing using an enzymatic purification system following the manufacturer's instructions (ExoSAP-IT; USB Corporation, Cleveland, OH). Cycle sequencing reactions were performed at the Pennsylvania State University's Huck Institute Nucleic Acid Facility using the BigDye system (version 3.1 dye terminators; Applied Biosystems, Foster City, CA) and run on an ABI 3730XL DNA Analyzer, using the ABI Data Collection Program (version 2.0) and ABI Sequencing Analysis software (version 5.1.1). ABI trace files were analyzed using Sequencher version 4.6 (GeneCodes, Ann Arbor, MI); heterozygous or ambiguous sites were labeled using the IUPAC code and consensus sequences were exported for phylogenetic analysis. Sequence data have been deposited in GenBank under Accession Nos. EU079474–EU080906 (see Supplemental Table 1 for individual accession numbers) and are also available from the *Phytophthora* Database (http://www.PhytophthoraDB.org).

2.4. Phylogenetic analyses

Sequences were aligned using ClustalX under default settings (Thompson et al., 1997). Alignments were visually inspected and edited manually for small (single nucleotide or codon) indels. Preliminary phylogenetic trees were reconstructed for each marker using neighbor-joining with a Kimura two parameter nucleotide substitution model as implemented in MEGA version 3.1 (Kumar et al., 2004). Seven markers (Table 1) were chosen for more rigorous analysis as taxonomic representation across the genus was complete, or nearly so, for these loci. Individual datasets were analyzed using Modeltest version 3.7 (Posada and Crandall, 1998), and the general time reversible nucleotide substitution model with gamma-distributed rate variation and a proportion of invariable sites received the highest score based on the hierarchical likelihood ratio test and the Akaike Information Criterion (corrected AIC and Bayesian Information Criterion results were identical). Neighbor-joining trees were then reconstructed for each dataset using locus-specific model parameters and maximum likelihood distances in PAUP* version 4.0b10 (Swofford, 1998). Bootstrap analyses were performed on each dataset using neighbor-joining with 1000 replicates. Individual gene datasets and neighbor-joining topologies have been deposited in TreeBase (Accession No. SN3532;

Table 1
Primers used in this study for DNA amplification and sequencing

Locus	Primer name	Primer sequence (5′–3′)	Genomic transcript[a]	Primer coordinates[b]	Ta[c]	Reference
28S Ribosomal DNA	LROR-O	ACCCGCTGAACTYAAGC	X75631[d]	25–41	53	Moncalvo et al. (1995)
	LSUFint[e]	CKTTGACGAAATGGAGCGAT		798–817		This study
	LSURint[e]	TTTCCACACCCTAACACTTGC		880–860		This study
	LR6-O	CGCCAGACGAGCTTACC		1366–1350		Riethmüller et al. (2002)
60S Ribosomal protein L10	60SL10_for	GCTAAGTGTTACCGTTTCCAG	72,378	16–36	53	This study
	60SL10_rev	ACTTCTTGGAGCCCAGCAC		512–493		This study
Beta-tubulin	Btub_F1	GCCAAGTTCTGGGAGGTCATC	72,114	52–72	60	This study
	Btub_F1A	GCCAAGTTCTGGGARGTSAT		52–71		This study
	Btub_F2[e]	CGGTAACAACTGGGCCAAGG		291–310		Kroon et al. (2004)
	Btub_R2[e]	GATCCACTCAACGAAGTACG		1035–1016		This study
	Btub_R1	CCTGGTACTGCTGGTACTCAG		1278–1259		Kroon et al. (2004)
	Btub_R1A	CCTGGTACTGCTGGTAYTCMGA		1278–1258		This study
Elongation factor 1 alpha	EF1A_FL	GGTCACCTGATCTACAAGTGC	71,250	73-93	60	This study
	EF1A_for	TCACGATCGACATTGCCCTG		212–231		Kroon et al. (2004)
	EF1A_R2[e]	TTSACCGACACGTTCTTGAC		917–898		This study
	EF1A_rev	ACGGCTCGAGGATGACCATG		1183–1164		Kroon et al. (2004)
	EF1A_RL	CCTTCTTGTTCACCGACTTG		1297–1278		This study
Enolase	Enl_for	CTTTGACTCGCGTGGCAAC	72,298	36–54	60	This study
	Enl_FY	CAACCCSACCGTYGAGGT		51–68		This study
	Enl_rev	CCTCCTCAATACGMAGAAGC		1288–1269		This study
Heat shock protein 90	HSP90_F1	GCTGGACACGGACAAGAACC	71,510	171–190	62	This study
	HSP90_F1int[e]	CAAGGTGATCCCGGACAAGGC		198–218		This study
	HSP90_F3[e]	ACGCCTCGTTCTACAAGTCG		848–867		This study
	HSP90_F2[e]	ATGGACAACTGCGAGGAGC		1039–1057		This study
	HSP90_R1[e]	ACACCCTTGACRAACGACAG		1091–1072		This study
	HSP90_R2	CGTGTCGTACAGCAGCCAGA		1935–1916		This study
TigA gene fusion protein	Tig_for	TTCGTGGGCGGYAACTGG	71,061	25–42	64[f]	This study
	Tig_FY	TCGTGGGCGGYAAYTGGAA		26-44		This study
	Tig_F2[e]	GCCTACATCACGGAGCARA		424–442		This study
	G3PDH_for[e]	TCGCYATCAACGGMTTCGG		779–797		This study
	Tig_rev[e]	CCGAAKCCGTTGATRGCGA		797–779		This study
	G3PDH_rev	GCCCCACTCRTTGTCRTACCAC		1719–1698		This study

[a] Reference transcripts from the *Phytophthora ramorum* genome project v1.1 (Joint Genome Institute, http://genome.jgi-psf.org/Phyra1_1/Phyra1_1.home.html).
[b] Primer coordinates with reference to the ATG start codon (positions 1–3) of the reference transcript.
[c] Annealing temperature used in template amplification.
[d] Reference transcript from *P. megasperma* (NCBI GenBank).
[e] Primers used for sequencing only.
[f] Template amplifications also performed using touchdown PCR (see Section 2).

http://www.treebase.org) and are available upon request (to J.E.B).

For the concatenated analysis, individual gene datasets were limited to one representative isolate for each species (Table 2). The general time reversible nucleotide substitution model with gamma-distributed rate variation and a proportion of invariable sites was selected based on Modeltest as described above (see Supplemental Table 2 for all estimated model parameters). The concatenated dataset was analyzed under maximum likelihood with GARLI version 0.951 (Zwickl, 2007); this program uses a heuristic genetic algorithm, rather than strict hill climbing, to simultaneously estimate the model parameters, topology, and branch lengths that maximize the likelihood (Zwickl, 2006). The model parameters generated by GARLI were then fixed for a bootstrap analysis using 1000 replicates. Maximum parsimony analysis was conducted using PAUP* version 4.0b10 (Swofford, 1998) with missing data and indels coded separately; 1000 bootstrap datasets were analyzed with each replicate using a heuristic search with random sequence addition, tree-bisection-reconnection (TBR) branch swapping, and allowing multiple trees ('multrees' options). Five hundred bootstrap replicates were also generated using SeqBoot and analyzed with DNAPars in the PHYLIP package (Felsenstein, 2005); support values were similar to those generated by PAUP*.

Bayesian analysis was performed with MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). Two analyses

Table 2
Isolate information for those species shown in Fig. 1

Clade[a]	*Phytophthora* species	Isolate details					Number of isolates sequenced	Waterhouse group
		Isolate identification		Isolate origins				
		Local[b]	International[c]	Host	Country	Date		
1	*P. nicotianae*	P6303		*Grammatophyllum* sp.	Indonesia	1989	9	II
1a	*P. cactorum*	P0714	ATCC10091 CBS231.30	*Syringa vulgaris*	The Netherlands	1930[d]	3	I
	P. hedraiandra	P11056		*Rhododendron* sp.	USA	2006[e]	3	I
	P. idaei (T)[f]	P6767		*Rubus idaeus*	UK	1989[e]	1	I
	P. pseudotsugae (T)[f]	P10339	IMI331662	*Pseudotsuga menziesii*	USA	2003[e]	2	I
1b	*P. clandestina*	P3942	ATCC58715 CBS349.86	*Trifolium subterraneum*	Australia	1986[d]	1	I
	P. iranica (T)[f]	P3882	ATCC60237 CBS374.72 IMI158964	*Solanum melongena*	Iran	1969	1	I
	P. tentaculata	P8497		*Chrysanthemum leucanthemum*	Germany	1994[e]	2	I
1c	*P.* sp. "*andina*" (T)[f]	P13365		*Solanum brevifolium*	Ecuador	2001	2	IV
	P. infestans	P10650		*Solanum tuberosum*	Mexico	2004[e]	3	IV
	P. ipomoeae	P10225		*Ipomoea longipedunculata*	Mexico	2003[e]	3	IV
	P. mirabilis	P3005	ATCC64068	*Mirabilis jalapa*	Mexico		2	IV
	P. phaseoli	P10145		*Phaseolus lunatus*	USA	2003[e]	5	IV
2	*P. bisheria* (T)[f]	P10117		*Fragaria* sp.	USA	1999	2	III
	P. citricola	P7902		*Pinus radiata*	USA	1993	7	III
	P. multivesiculata (T)[f]	P10410	CBS545.96	*Cymbidium* sp.	The Netherlands	1995	3	IV
2a	*P. botryosa*	P6945	IMI130422	*Hevea brasiliensis*	Malaysia	1986	1	II
	P. citrophthora	P6310		*Theobroma cacao*	Indonesia	1989	1	II
	P. colocasiae	P6317		*Colocasia esculenta*	Indonesia	1989	2	IV
	P. inflata	P10341	IMI342898	*Syringa* sp.	UK	1990	2	III
	P. meadii	P6128	ICRI-240	*Elettaria cardamomum*	India	1989[e]	2	II
2b	*P. capsici*	P0253	ATCC46012	*Theobroma cacao*	Mexico	1964	8	II
	P. sp. "*glovera*"	P10619		*Nicotiana* sp.	Brazil		2	na
	P. mexicana (T)[f]	P0646	ATCC46731 CBS554.88 IMI092550	*Solanum lycopersicum*	Mexico	1988[d]	1	II
	P. tropicalis (T)[f]	P10329	CBS434.91	*Macadamia integrifolia*	USA (Hawaii)	1975	3	II
3	*P. ilicis*	P3939	ATCC56615	*Ilex* sp.	Canada	1988[e]	2	IV
	P. nemorosa	P10288		*Lithocarpus densiflorus*	USA	2003[e]	2	IV
	P. pseudosyringae	P10437		*Quercus robur*	Germany	1997	3	III
	P. psychrophila (T)[f]	P10433		*Quercus robur*	Germany	1995	2	IV
4	*P. arecae*	P10213	ATCC-MYA4039	*Citrus* sp.	USA	2003[e]	3	II
	P. megakarya	P8516		*Theobroma cacao*	Sao Tome		3	II
	P. palmivora	P0255	ATCC26200	*Theobroma cacao*	Costa Rica	1964	3	II
	P. sp. "*quercetorum*"	MD9-2	ATCC-MYA4086	*Quercus rubra* rhizosphere	USA	2004	1	I
	P. quercina (T)[f]	P10334	CBS782.95	*Quercus robur*	Germany	1995	2	I
5	*P. heveae*	P10167	ATCC16701 IMI131373	soil	USA	2003[e]	3	II
	P. katsurae	P10187		*Castanea crenata*	Japan	1970	3	II
6	*P.* sp. "*asparagi*"	P10690		*Asparagus officalis*	New Zealand	1986	1	V
	P. gonapodyides	P10337		*Salix matsudana*	UK	1972	4	VI
	P. humicola (T)[f]	P3826	ATCC52179 CBS200.81 IMI302303	citrus grove soil	Taiwan	1977	2	V
	P. inundata	P8478	IMI389750	*Aesculus hippocastanum*	UK	1970	2	VI
	P. megasperma	P3136		*Brassica napus* var. *napus*	Australia	1985[e]	2	V
	P. sp. "*personii*"	P11555		*Nicotiana tabacum*	USA	2006[e]	2	na
7a	*P. alni*	P10568		*Alnus glutinosa*	Hungary	1999	3	VI
	P. cambivora	P0592	ATCC46719	*Abies procera*	USA		3	VI
	P. europaea (T)[f]	P10324	CBS109049	*Quercus* rhizosphere	France	1998	3	V
	P. fragariae var. *fragariae*	P3821	ATCC36057	*Fragaria* sp.	UK	1969	2	V

Table 2 (*continued*)

Clade[a]	*Phytophthora* species	Isolate details					Number of isolates sequenced	Waterhouse group
		Isolate identification		Isolate origins				
		Local[b]	International[c]	Host	Country	Date		
	P. uliginosa	P10328	CBS109055	*Quercus robur* rhizosphere	Germany	1998	2	V
7b	*P. cajani*	P3105	ATCC44388	*Cajanus cajan*	India		1	VI
	P. cinnamomi	P2159	ATCC46678 IMI157799	*Vitis vinifera*	South Africa	1971	4	VI
	P. cinnamomi var. *parvispora*	P8495		*Beaucamea* sp.	Germany	1994[e]	2	VI
	P. melonis	P10994		*Trichosanthes dioica*	India		2	VI
	P. sp. "*niederhauserii*"	P10617		*Thuja occidentalis*	USA	2001	2	na
	P. pistaciae	P6197	ATCC62268	*Pistacia vera*	Iran		2	V
	P. sinensis (T)[f]	P1475	ATCC46538 CBS557.88	*Cucumis sativus*	China	1988	1	VI
	P. sojae	P3114		*Glycine max*	USA		2	V
	P. vignae	P3019		*Vigna unguiculata*	Australia		2	VI
8a	*P. cryptogea*	P1088	ATCC46721 CBS290.35	*Aster* sp.	USA	1935[d]	3	VI
	P. drechsleri	P10331		*Gerbera jamesonii*	USA	2003	3	VI
	P. erythroseptica	P1699	ATCC36302 CBS956.87	*Solanum tuberosum*	USA	1987[d]	3	VI
	P. sp. "*kelmania*"	P10613		*Abes fraseri*	USA	2002	2	na
	P. medicaginis	P10683	ATCC32997	*Medicago sativa*	USA		2	V
	P. richardiae	P10811		*Zantedeschia aethiopica*	Japan	1989	9	VI
	P. sp. "*sansomea*"	P3163	CBS117692	*Silene latifolia* subsp. alba	USA		1	V
	P. trifolii	P7010		*Trifolium* sp.	USA		2	V
8b	*P. brassicae*	P10414	CBS113350	*Brassica oleraceae*	The Netherlands	1994	5	IV
	P. porri	P10728		*Daucus carota*	France	2004	3	III
	P. primulae	P10333	CBS620.97	*Primula acaulis*	Germany	1997	3	III
	P. syringae	P10330	CBS110161	*Rhododendron* sp.	Germany	1995	2	III
8c	*P. foliorum*	P10969		*Rhododendron* sp.	USA	2005	4	III
	P. hibernalis	P3822	ATCC56353 CBS114104 IMI134760	*Citrus sinensis*	Australia	1958	3	IV
	P. lateralis	P3888		*Chamaecyparis lawsoniana*	USA	1988[5]	2	V
	P. ramorum	P10301	CBS101329	*Rhododendron* sp.	The Netherlands	1998	9	IV
9	*P. captiosa* (T)[f]	P10719		*Eucalyptus saligna*	New Zealand	1992	2	VI
	P. sp. "*cuyabensis*"	P8213		rainforest soil	Ecuador		3	V
	P. fallax	P10725		*Eucalyptus fastigata*	New Zealand	2004	2	VI
	P. insolita (T)[f]	P6195	ATCC38789 CBS691.79 IMI288805	soil	Taiwan	1979	2	V
	P. sp. "*lagoariana*"	P8223		rainforest soil	Ecuador		2	na
	P. macrochlamydospora	P10267		*Glycine max*	Australia	1994	3	VI
	P. polonica	P15005		*Alnus glutinosa* rhizosphere	Poland	2006[e]	3	V
	P. quininea	P3247	ATCC56964 CBS406.48	*Cinchona officinalis*	Peru	1948[d]	2	V
10	*P. boehmeriae* (T)[f]	P6950	CBS291.29 IMI180614	*Boehmeria nivea*	Taiwan	1929[d]	1	II
	P. kernoviae	P10681		*Annona cherimola*	New Zealand	2002	5	II
Outgroups								
	Pythium undulatum	P10342	IMI337230	*Larix* sp.	UK	1989	1	
	Pythium vexans	P3980	ATCC12194 CBS340.49 IMI32044	na	na	1989[e]	1	

na, not available.
[a] Molecular clade as shown in Fig. 1.
[b] Local identification numbers from the World *Phytophthora* Genetic Resource Collection (P) and West Virginia University (MD).
[c] International identification abbreviations: ATCC, American Type Culture Collection, USA; CBS, Centraalbureau fur Schimmelcultures, The Netherlands; ICRI, Indian Cardamom Research Institute, India; IMI, CABI Biosciences, UK.
[d] Date culture was obtained by CBS.
[e] Date culture was obtained by the World *Phytophthora* Genetic Resource Collection.
[f] Type isolate of the species (T).

were run simultaneously for two million generations with three heated chains (temperature = 0.2) and one cold chain. Flat Dirichlet priors were used for the nucleotide base frequencies and the six rate parameters of the general time reversible model. Uniform priors between 0 and 1 were used for the gamma shape parameter and the proportion of invariable sites. Trace files generated after each run were evaluated using Tracer version 1.3 (Rambaut and Drummond); all parameters displayed appropriate levels of mixing and convergence after the burn-in period. The majority-rule consensus tree was calculated after removing the first 200,000 generations from each run as burn-in. A second Bayesian analysis was also performed with BEAST version 1.4.5 (Drummond and Rambaut, 2006) using the uncorrelated lognormal relaxed clock method. Uniform prior distributions were used for model parameters, with starting values based on empirical data. The maximum clade credibility topology was determined after five million generations with the first 500,000 generations discarded as burn-in.

Alternative topological hypotheses were tested using the Kishino–Hasegawa (Kishino and Hasegawa, 1989) and Shimodaira–Hasegawa (Shimodaira and Hasegawa, 1999) tests with 10,000 replicates as implemented in PAML version 3.14 (Yang, 1997). The maximum likelihood score was calculated for each topology using the general time reversible substitution model with the gamma shape parameter set to 0.75.

3. Results and discussion

Previous studies have primarily focused on the generation and analysis of data from the ITS region of the ribosomal DNA (e.g., Cooke et al., 2000; Förster et al., 2000; Villa et al., 2006). While this locus is easily amplified and contains a large number of variable sites, the quality of the multiple sequence alignment of ITS data quickly degrades as evolutionary distance increases (for recent review, see Feliner and Rossello, 2007). Our goal was to utilize the available genome sequence data for *Phytophthora* to identify molecular markers that would be informative at different phylogenetic levels, as well as those that could be unambiguously aligned across the entire genus. Over 1600 sequences were generated from 27 potential markers. Taxonomic representation was not evenly distributed as some markers could not be amplified from a range of species. Therefore, seven loci (Table 1) that were successfully amplified and sequenced across the genus were chosen for more comprehensive analysis, as described above. These loci included portions of seven protein-coding genes and the 5′ portion of the 28S ribosomal DNA. These genes do not contain introns, and are conserved throughout eukaryotes. The proportion of informative sites, and therefore the amount of phylogenetic signal present in each locus, was variable (see Supplemental Table 3 for bootstrap support values). The tigA locus contains two genes, triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase, which are fused into a single transcriptional unit (Unkles et al., 1997). This gene fusion product has also been found in other stramenopiles (Liaud et al., 2000).

The 28S ribosomal DNA locus was consistently amplified across all isolates used in this study, and provided good resolution of species relationships within clades. This locus has been used in other studies of Peronosporomycete and Oomycete phylogeny (e.g., Peterson and Rosendahl, 2000; Riethmüller et al., 2002; Voglmayr et al., 2004), and is an appropriate candidate for comparisons across genera and families due to its high level of sequence conservation. The 60S ribosomal protein L10 locus was also consistently amplified across the genus; however, this locus provided very little phylogenetic resolution among species due to its short length (496 bp) and thus limited number of variable characters.

Of the protein-coding loci analyzed in this study, the beta-tubulin locus provided the highest level of phylogenetic signal across the genus. A larger portion of the beta-tubulin gene was used here (1226 bp) as compared to other studies (658–989 bp, Kroon et al., 2004; Villa et al., 2006). Amplifications were successful for most isolates, however, modified primer sequences with degenerate bases were used for some *Phytophthora* species and the *Pythium* outgroups (Table 1). The enolase and tigA loci also required the use of degenerate forward primers for some isolates, and were difficult to amplify across the genus. The enolase locus could not be sequenced for *P. quininea*, *P. pistaciae*, or the *Pythium* outgroups despite numerous attempts, and was coded as missing data for these taxa in the phylogenetic analyses; a sequence from *Apodachlya brachynema* (AY430415, Harper and Keeling, 2004) was used as the outgroup for the neighbor-joining analysis of this locus. Despite these difficulties, the enolase and tigA loci contained a higher percentage of informative sites and showed good phylogenetic resolution within most clades.

The heat shock protein 90 and elongation factor 1 alpha loci contained moderate levels of phylogenetic signal, and were able to resolve relationships within most clades. Some species of *Phytophthora* posses single codon indels in their heat shock protein 90 gene, which could be informative characters for species identification and diagnostics. Sequences generated from the elongation factor 1 alpha locus contained a significant proportion of heterozygous sites (i.e., sites containing more than one peak in the electropherograms), suggesting that this gene may be present in more than one copy in some or all *Phytophthora* species; additional studies are needed. A larger amplicon (1224 bp) of the elongation factor 1 alpha locus was obtained for some species using different primers (Table 1) than those used in previous studies (971 bp, Donahoo et al., 2006; Kroon et al., 2004).

While the individual loci were able to resolve relationships among closely related species, they were limited in their ability to address the phylogeny of the entire genus.

The alignments of the seven loci were therefore concatenated and analyzed with a number of phylogenetic methods. Maximum likelihood (ML), maximum parsimony (MP), and Bayesian analyses (BA) all produced the same topology (Fig. 1), with the genus divided into 10 well-supported clades separated by short internal branches. Relationships among the clades received moderate to high bootstrap support (41–100%) in the maximum likelihood and maximum parsimony analyses. Bayesian analyses produced highly significant posterior probabilities for all nodes (>0.96). While the maximum clade credibility topology generated by BEAST was identical to the consensus tree from MrBayes, the chain did not show sufficient mixing of model parameters even after five million generations; the posterior probabilities generated by MrBayes are therefore presented, as percentages, in Fig. 1. Alternative topological hypotheses of the relationships among the clades were also tested, and the topology shown in Fig. 1 possessed the highest likelihood and RELL bootstrap values (Table 3). However, a closer relationship between Clades 7 and 8 could not be significantly rejected. In general, the topology presented in Fig. 1 was robust to significant alterations of the relationships among the clades.

Clades 1 through 8 correspond to those previously suggested by Cooke et al. (2000) and supported, in general, by Kroon et al. (2004). Our results support three divisions within Clade 1 (a, b, and c); the position of *P. nicotianae* remained ambiguous although there was moderate support for its position basal to subclades 1b and 1c (ML 96%, MP 60%, BA 1.0). As shown previously, *P. hedraiandra* was closely related to *P. cactorum* within Clade 1a (de Cock and Levesque, 2004). *P. tentaculata* was significantly supported in Clade 1b, and not closely related to *P. multivesiculata* as suggested by Kroon et al. (2004). Clade 1c was identical to that of Kroon et al. (2004), although the relationships among the five taxa were not resolved. We identified two well-supported divisions within Clade 2 (a and b); *P. citricola* was consistently found basal to these two subclades, but the positions of *P. multivesiculata* and the new species *P. bisheria* (Abad et al., 2007) relative to the rest of the clade were not resolved. The new species *P.* sp. "*glovera*" was found within Clade 2b.

We have used the presence of *P. ilicis* to identify Clade 3; this group also included *P. psychrophila* and *P. pseudosyringae* as suggested previously (Jung et al., 2003), and *P. nemorosa*. *P. quercina*, originally placed in Clade 3, was instead found to be closely related to Clade 4, although bootstrap support was moderate for this relationship. The monophyly of the remaining members of Clade 4, including the new species *P.* sp. "*quercetorum*", was very strongly supported, and it is possible that further analyses may reveal a different affinity for *P. quercina*. *P. katsurae* and *P. heveae* were strongly supported as the two members of Clade 5. Within Clade 6, *P. inundata* was closely related to *P. humicola* as suggested previously (Brasier et al., 2003). The relationship between *P. megasperma* and *P. gonapodyides* was unresolved as the MP analysis

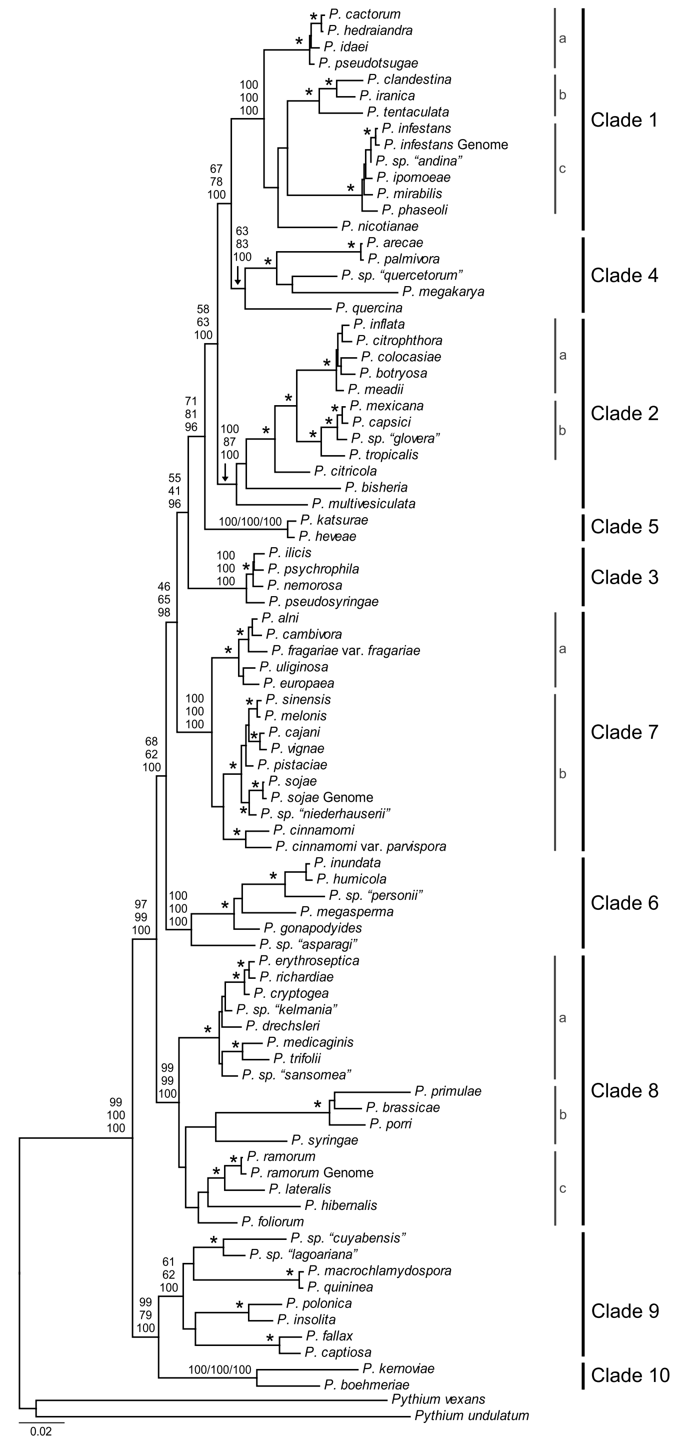

Fig. 1. A genus-wide phylogeny for *Phytophthora* using seven nuclear loci (~8700 nucleotides). Maximum likelihood branch lengths are shown. Numbers on nodes represent bootstrap support values for maximum likelihood (top) and maximum parsimony (middle), and Bayesian posterior probabilities presented as percentages (bottom). Nodes within clades receiving unambiguous (100%) support in all three analyses are marked with an asterisk (*). Scale bar indicates number of substitutions per site.

grouped these taxa together (57% bootstrap support), while the other analyses produced the topology shown in Fig. 1 (ML 65%, BA 0.99). The new species *P.* sp. "*personii*" was also found in Clade 6; *P.* sp. "*asparagi*" most likely

Table 3
Alternative topological hypotheses testing under maximum likelihood

Clade topology	ln *L*	Difference	Standard error	pRELL[a]	pKH[b]	pSH[c]
((((((((1,4),2),5),3),7),6),8),(9,10));	−84552.735	0.000	0.000	0.905	na	na
(((((((1,4),2),5),3),6),(7,8)),(9,10));	−84585.746	33.011	23.969	0.078	0.084	0.408
((((((((2,4),1),5),3),7),8),6),(9,10));	−84593.053	40.318	18.993	0.013	0.017	0.308
(((((((1,4),5),(2,3)),7),6),8),(9,10));	−84612.692	59.958	22.116	0.003	0.003	0.143
((((((1,4),2),5),3),(6,7,8)),(9,10));	−84623.655	70.920	20.731	0.000	0.000	0.052
(((((((((1,4),2),5),3),10),7),6),8),9);	−84821.275	268.540	36.713	0.000	0.000	0.000
(((((((1,4),2),5),3),(6,7,9)),8),10);	−84830.260	277.525	40.699	0.000	0.000	0.000

na, not applicable.
[a] *p*-Value for RELL bootstrapping (Yang, 1997).
[b] *p*-Value for Kishino–Hasegawa test (Kishino and Hasegawa, 1989).
[c] *p*-Value for Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999).

corresponds to the species previously referred to as *P.* sp. (asparagus) in the analysis of Cooke et al. (2000).

Our results support two divisions within Clade 7 (a and b). *P. alni* was closely associated with *P. cambivora* and *P. fragariae* as previously suggested (Brasier et al., 2004); *P. uliginosa* and *P. europaea* were also placed in Clade 7a (Jung et al., 2002). Although not included in this study, *P. fragariae* var. *rubi*, which has recently been redescribed as *P. rubi* (Man in't Veld, 2007), is most likely a member of Clade 7a. Clade 7b contains a variety of species, including *P. pistaciae* (Mirabolfathy et al., 2001) and the new species *P.* sp. "*niederhauserii*". *P. cinnamomi* was also placed in Clade 7b with high support (ML 96%, MP 88%, BA 1.0). Our data revealed considerable genetic distance between *P. cinnamomi* and *P. cinnamomi* var. *parvispora*, suggesting little to no gene flow between these two lineages; like *P. rubi*, this variety could potentially be elevated to species status upon further analysis.

Three divisions within Clade 8 were supported here; Clades 8a and 8b corresponded to Cooke et al. (2000) while our Clade 8c encompassed the 8c and 8d clades of Kroon et al. (2004). A noteworthy difference between our results and those of previous studies was the position of *P. richardiae*; this species was placed in Clade 9 of Cooke et al. (2000) and Clade 8e of Kroon et al. (2004). Our sequence data from six isolates significantly supported the placement of *P. richardiae* within Clade 8a, closely related to *P. erythroseptica* and *P. cryptogea* as suggested previously (Förster et al., 2000). Data from two isolates identified as *P. richardiae*, which correspond to those used in previous studies (Cooke et al., 2000; Kroon et al., 2004), were nearly identical to sequences from *P. macrochlamydospora*; these isolates may need to be reevaluated and the position of *P. richardiae* addressed with additional accessions. The new species *P.* sp. "*kelmania*" and *P.* sp. "*sansomea*" were also placed in Clade 8a. The grouping of *P. syringae* with the long branched *P. primulae*, *P.brassicae*, and *P. porri* as Clade 8b was highly supported (ML 99%, MP 98%, BA 1.0). A recently described species, *P.austrocedrae*, was found to be closely related to *P. syringae* in an analysis of ITS data (Greslebin et al., 2007); this species is most likely a member of Clade 8b. *P. ramorum* was placed in Clade 8c, closely related to *P. lateralis* as shown previously (Werres et al., 2001). *P. hibernalis* and *P. foliorum* were also included in Clade 8c with moderate bootstrap support (ML 88%, MP 61%, BA 1.0); the affinity of these species with *P. ramorum* has been noted elsewhere (Donahoo et al., 2006).

The remaining *Phytophthora* species were found in a well-supported group basal to the other eight clades. Clade 9 encompassed Clades 9 and 10 *sensu* Cooke et al. (2000) by including *P. macrochlamydospora* and *P. insolita*. A majority of the species found in Clade 9 have only recently been described, including *P. polonica* (Belbahri et al., 2006), *P. captiosa* and *P. fallax* (Dick et al., 2006), and two new species, *P.* sp. "*cuyabensis*" and *P.* sp. "*lagoariana*", discovered in the Cuyabeno Reserve, Ecuador (M.D.C. unpublished data). The monophyly of this clade was only moderately supported by the maximum likelihood and maximum parsimony analyses; Bayesian analyses supported the topology shown in Fig. 1 with very high support (>0.98). *P. kernoviae* was closely related to *P. boehmeriae* as previously suggested (Brasier et al., 2005). These species were placed in a separate clade (Clade 10) as they are classified morphologically in group two according to Waterhouse (1963); all members of Clade 9 are classified in Waterhouse (1963) groups five and six. Alternative positions for these two basal clades, such as grouping Clade 9 with Clades 6 and 7, or an association of Clade 10 with Clades 1 through 5, were significantly rejected (Table 3). The robustness of these clades will be further assessed as additional basal *Phytophthora* species are discovered.

Previous studies have demonstrated that the original species groups suggested for classification purposes by Waterhouse (1963) do not represent natural divisions or clades (Cooke et al., 2000; Förster et al., 2000; Kroon et al., 2004; Martin and Tooley, 2003a). Our data indicate that most clades, however, are comprised of species from the same Waterhouse group (Table 2). Two exceptions include Clades 2a and 8c, which contain species with different sporangial structures. *P. inflata* and *P. colocasiae* possess semi-papillate sporangia, unlike the other papillate members of Clade 2a. *P. lateralis*, which is closely related

to *P. ramorum* in Clade 8c, possesses non-papillate sporangia, while the other members are semi-papillate. It has been noted that morphological differences among species are often small and overlapping (Brasier, 1991; Erwin and Ribeiro, 1996; Waterhouse et al., 1983). Some traits may exist as a continuum of phenotypes, with species differences exaggerated by the original descriptions of type cultures. However, morphological distinctions, such as Waterhouse groupings, may remain useful for species comparisons in a phylogenetic framework.

Clades 4, 6, 7, and 8 contain both homothallic and heterothallic species with amphigynous and/or paragynous antheridia. Heterothallic species always exhibit amphigynous attachment of the antheridium to the oogonium, and are heterozygous for a reciprocal chromosomal translocation in a region suggested to contain the mating type locus (Sansome, 1987). This complexity led Cooke et al. (2000) to suggest that heterothallism and its associated amphigyny was the ancestral condition of the main *Phytophthora* radiation. However, using a parsimony approach, Kroon et al. (2004) suggested that homothallism was the ancestral condition, with as many as eight independent transitions to heterothallism. Our results may be more congruent with those of Kroon et al. (2004), as species within the basal Clades 9 and 10 have all been described as homothallic. However, these data must be interpreted carefully, as it is evident that sexual characteristics have repeatedly been gained or lost along different *Phytophthora* lineages during the evolution of the genus. Interpretations about the evolutionary polarity of hetero- versus homothallism can be confounded by incorrect assumptions about sexuality and taxon sampling (Geiser et al., 1998; Paoletti et al., 2005; Yun et al., 1999). The continued discovery of *Phytophthora* species may further alter our interpretations of the evolutionary history of sexuality within the genus.

4. Conclusions

The phylogeny for the genus *Phytophthora* presented here represents a significant advance over previous studies as almost all currently described and some newly discovered species have been included. In addition, our data from seven nuclear loci provide a robust phylogenetic framework for interpreting the evolutionary history of the genus. A more thorough understanding of the relationships among *Phytophthora* species allows for better validation of diagnostic methods (e.g., Kong et al., 2004; Lees et al., 2006; Martin et al., 2004; Schena et al., 2006; Tooley et al., 2006), which are becoming increasingly important for quarantine issues and disease monitoring (Kang et al., 2006). The molecular markers described here also provide the *Phytophthora* community with suitable loci for species identification, population- and clade-level phylogenetic analyses, and targets for future multi-locus sequence typing (MLST) diagnostic methods.

Acknowledgments

The authors acknowledge Seong Hwan Kim from the Pennsylvania Department of Agriculture, Yilmaz Balci from West Virginia University, Gloria Abad from USDA-APHIS-PPQ-PHP-PSPI-MDL, David Cooke from the Scottish Crop Research Institute, and Tomasz Oszako from the Polish Forest Research Institute for kindly providing cultures. We thank Jeniffer Yanez, Jean Juba, Michele Mansfield, Masoomeh Peiman, Thomas Vu, Julie Huss, Jaeger Davis, Sappho Chow, Peggy Ju, Carrie Tran, Shirley Tu, and Erik Haw for laboratory assistance, Bongsoo Park for assistance submitting sequence data to GenBank, and Ashley Price, Sheila Plock, and Deborah Grove at the Nucleic Acid Facility at Penn State for their excellence in generating sequence data. This study was supported by the USDA-NRI Plant Biosecurity Program (award number 2005-35605-15393). The WPGRC is funded in part by the UC/GRCP Annual Grants Program in Support of Imperiled Living Collections of Genetic Resources.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2007.10.010.

References

Abad, Z.G., Abad, J.A., Coffey, M.D., Oudemans, P.V., Man in't Veld, W.A., de Gruyter, H., Cunnington, J., Louws, F.J., 2007. *Phytophthora bisheria* sp. nov., a new species identified in isolates from the Rosaceous raspberry, rose, and strawberry in three continents. Mycologia, in press.

Ah Fong, A.M.V., Judelson, H., 2004. The *hAT*-like DNA transposon *DodoPi* resides in a cluster of retro- and DNA transposons in the stramenopile *Phytophthora infestans*. Mol. Gen. Genomics 271, 577–585.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Belbahri, L., Moralejo, E., Calmin, G., Oszako, T., Garcia, J.A., Descals, E., Lefort, F., 2006. *Phytophthora polonica*, a new species isolated from declining *Alnus glutinosa* stands in Poland. FEMS Microbiol. Lett. 261, 165–174.

Brasier, C.M., 1991. Current questions in *Phytophthora* systematics: the role of the population approach. In: Lucas, J.A., Shattock, R.C., Shaw, D.S., Cooke, L.R. (Eds.), *Phytophthora*. Cambridge University Press, New York, pp. 104–128.

Brasier, C.M., Beales, P.A., Kirk, S.A., Denman, S., Rose, J., 2005. *Phytophthora kernoviae* sp. nov., an invasive pathogen causing bleeding stem lesions on forest trees and foliar necrosis of ornamentals in the UK. Mycol. Res. 109, 853–859.

Brasier, C.M., Kirk, S.A., Delcan, J., Cooke, D.E.L., Jung, T., Man in't Veld, W.A., 2004. *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. Mycol. Res. 108, 1172–1184.

Brasier, C.M., Sanchez-Hernandez, E., Kirk, S.A., 2003. *Phytophthora inundata* sp. nov., a part heterothallic pathogen of trees and shrubs in wet or flooded soils. Mycol. Res. 107, 477–484.

Caten, C.E., Jinks, J.L., 1968. Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural variation. Can. J. Bot. 45, 329–348.

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.

Crawford, A.R., Bassam, B.J., Drenth, A., Maclean, D.J., Irwin, J.A.G., 1996. Evolutionary relationships among *Phytophthora* species deduced from rDNA sequence analysis. Mycol. Res. 100, 437–443.

Cvitanich, C., Salcido, M., Judelson, H., 2006. Concerted evolution of a tandemly arrayed family of mating-specific genes in *Phytophthora* analyzed through inter- and intraspecific comparisons. Mol. Gen. Genomics 275, 169–184.

de Cock, A.W.A.M., Levesque, C.A., 2004. New species of *Pythium* and *Phytophthora*. Stud. Mycol. 50, 481–487.

Dick, M.A., Dobbie, K., Cooke, D.E.L., Brasier, C.M., 2006. *Phytophthora captiosa* sp. nov. and *P. fallax* sp. nov. causing crown dieback of *Eucalyptus* in New Zealand. Mycol. Res. 110, 393–404.

Dick, M.W., 1990. Phylum oomycota. In: Margulis, L., Corliss, J.O., Melkonian, M., Chapman, D.J. (Eds.), Handbook of Protoctista. Jones and Bartlett Publishers, Boston, pp. 661–685.

Dick, M.W., 2001. The Peronosporomycetes. In: McLaughlin, D., McLaughlin, E., Lemke, P. (Eds.), The Mycota. Springer-Verlag, New York, pp. 39–72.

Dick, M.W., Vick, M.C., Gibbings, J.G., Hedderson, T.A., Lopez Lastra, C.C., 1999. 18S rDNA for species of *Leptolegnia* and other Peronosporomycetes: justification for the subclass taxa Saprolegniomycetidae and Peronosporomycetidae and division of the Saprolegniaceae *sensu lato* into the Leptolegniaceae and Saprolegniaceae. Mycol. Res. 103, 1119–1125.

Donahoo, R., Blomquist, C.L., Thomas, S.L., Moulton, J.K., Cooke, D.E.L., Lamour, K.H., 2006. *Phytophthora foliorum* sp. nov., a new species causing leaf blight of azalea. Mycol. Res. 110, 1309–1322.

Drummond, A.J., Rambaut, A., 2006. BEAST version 1.4. Available from: <http://beast.bio.ed.ac.uk/>.

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

Feliner, G.N., Rossello, J.A., 2007. Better the devil you know? Guidelines for insightful utilization of nrDNA ITS in species-level evolutionary studies in plants. Mol. Phylogenet. Evol. 44, 911–919.

Felsenstein, J., 2005. PHYLIP version 3.6. Available from: <http://evolution.gs.washington.edu/phylip.html/>.

Förster, H., Coffey, M.D., Elwood, H.J., Sogin, M.L., 1990. Sequence analysis of the small subunit ribosomal RNAs of three zoosporic fungi and implications for fungal evolution. Mycologia 82, 306–312.

Förster, H., Cummings, M.P., Coffey, M.D., 2000. Phylogenetic relationships of *Phytophthora* species based on ITS1 DNA sequence analysis with emphasis on Waterhouse groups V and VI. Mycol. Res. 104, 1055–1061.

Geiser, D.M., Frisvad, J.C., Taylor, J.W., 1998. Evolutionary relationships in *Aspergillus* section *Fumigati* from partial beta-tubulin and hydrophobin DNA sequences. Mycologia 90, 831–845.

Govers, F., Gijzen, M., 2006. *Phytophthora* Genomics: the plant destroyers' genome decoded. Mol. Plant-Microbe Int. 19, 1295–1301.

Greslebin, A.G., Hansen, E.M., Sutton, W., 2007. *Phytophthora austrocedrae* sp. nov., a new species associated with *Austrocedrus chilensis* mortality in Patagonia (Argentina). Mycol. Res. 111, 308–316.

Gunderson, J.H., Elwood, H., Ingold, A., Kindle, K., Sogin, M.L., 1987. Phylogenetic relationships between chlorophytes, chrysophytes, and oomycetes. Proc. Natl. Acad. Sci. USA 84, 5823–5827.

Hardham, A.R., 2005. *Phytophthora cinnamomi*. Mol. Plant Pathol. 6, 589–604.

Harper, J.T., Keeling, P.J., 2004. Lateral gene transfer and the complex distribution of insertions in eukaryotic enolase. Gene 340, 227–235.

Hudspeth, D.S.S., Nadler, S.A., Hudspeth, M.E.S., 2000. A *COX2* molecular phylogeny of the Peronosporomycetes. Mycologia 92, 674–684.

Ivors, K.L., Hayden, K.J., Bonants, P.J.M., Rizzo, D.M., Garbelotto, M., 2004. AFLP and phylogenetic analyses of North American and European populations of *Phytophthora ramorum*. Mycol. Res. 108, 378–392.

Jiang, R.H.Y., Tyler, B.M., Whisson, S.C., Hardham, A.R., Govers, F., 2006. Ancient origin of elicitin gene clusters in *Phytophthora* genomes. Mol. Biol. Evol. 23, 338–351.

Judelson, H.S., 2002. Sequence variation and genomic amplification of a family of *Gypsy*-like elements in the Oomycete genus *Phytophthora*. Mol. Biol. Evol. 19, 1313–1322.

Judelson, H.S., Blanco, F.A., 2005. The spores of *Phytophthora*: weapons of the plant destroyer. Nat. Rev. Microbiol. 3, 47–58.

Jung, T., Hansen, E.M., Winton, L., Oswald, W.F., Delatour, C., 2002. Three new species of *Phytophthora* from European oak forests. Mycol. Res. 106, 397–411.

Jung, T., Nechwatal, J., Cooke, D.E.L., Hartmann, G., Blaschke, M., Oswald, W.F., Duncan, J.M., Delatour, C., 2003. *Phytophthora pseudosyringae* sp. nov., a new species causing root and collar rot of deciduous tree species in Europe. Mycol. Res. 107, 772–789.

Kamoun, S., 2003. Molecular genetics of pathogenic Oomycetes. Eukaryot. Cell 2, 191–199.

Kang, S., Blair, J.E., Geiser, D.M., Khang, C.-H., Park, S.-Y., Gahegan, M., O'Donnell, K., Luster, D.G., Kim, S.H., Ivors, K.L., et al., 2006. Plant pathogen culture collections: it takes a village to preserve these resources vital to the advancement of agricultural security and plant pathology. Phytopathology 96, 920–925.

Kishino, H., Hasegawa, M., 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in hominoidea. J. Mol. Evol. 29, 170–179.

Kong, P., Hong, C.X., Tooley, P.W., Ivors, K., Garbelotto, M., Richardson, P.A., 2004. Rapid identification of *Phytophthora ramorum* using PCR-SSCP analysis of ribosomal DNA ITS-1. Lett. Appl. Microbiol. 38, 433–439.

Kroon, L.P.N.M., Bakker, F.T., van den Bosch, G.B.M., Bonants, P.J.M., Flier, W.G., 2004. Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. Fungal Genet. Biol. 41, 766–782.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief. Bioinform. 5, 150–163.

Lee, S.B., Taylor, J.W., 1992. Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. Mol. Biol. Evol. 9, 636–653.

Lees, A.K., Wattier, R., Shaw, D.S., Sullivan, L., Williams, N.A., Cooke, D.E.L., 2006. Novel microsatellite markers for the analysis of *Phytophthora infestans* populations. Plant Pathol. 55, 311–319.

Liaud, M.-F., Lichtl, C., Apt, K., Martin, W., Cerff, R., 2000. Compartment-specific isoforms of TPI and GAPDH are imported into diatom mitochondria as a fusion protein: evidence in favor of a mitochondrial origin of the eukaryotic glycolytic pathway. Mol. Biol. Evol. 17, 213–223.

Liu, Z., Bos, J.I.B., Armstrong, M., Whisson, S.C., da Cunha, L., Torto-Alalibo, T., Win, J., Avrova, A.O., Wright, F., Birch, P.R.J., et al., 2005. Patterns of diversifying selection in the phytotoxin-like *scr74* gene family of *Phytophthora infestans*. Mol. Biol. Evol. 22, 659–672.

Man in't Veld, W.A., 2007. Gene flow analysis demonstrates that *Phytophthora fragariae* var. *rubi* constitutes a distinct species, *Phytophthora rubi* comb.nov. Mycologia 99, 222–226.

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

Martin, F.N., Tooley, P.W., 2003b. Phylogenetic relationships of *Phytophthora ramorum*, *P. nemorosa*, and *P. pseudosyringae*, three species recovered from areas in California with sudden oak death. Mycol. Res. 107, 1379–1391.

Martin, F.N., Tooley, P.W., Blomquist, C., 2004. Molecular detection of *Phytophthora ramorum*, the causal agent of sudden oak death in California, and two additional species commonly recovered from diseased plant material. Phytopathology 94, 621–631.

Mirabolfathy, M., Cooke, D.E.L., Duncan, J.M., Williams, N.A., Ershad, D., Alizadeh, A., 2001. *Phytophthora pistaciae* sp. nov. and *P. melonis*: the principal causes of pistachio gummosis in Iran. Mycol. Res. 105, 1166–1175.

Moncalvo, J.-M., Wang, H.-H., Hseu, R.-S., 1995. Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. Mycologia 87, 223–238.

Newhook, F.J., Waterhouse, G.M., Stamps, D.J., 1978. Tabular key to the species of *Phytophthora* de Bary. Mycol. Papers No. 143.

Paoletti, M., Rydholm, C., Schwier, E.U., Anderson, M.J., Szakacs, G., Lutzoni, F., Debeaupuis, J.-P., Latge, J.-P., Denning, D.W., Dyer, P.S., 2005. Evidence for sexuality in the opportunistic fungal pathogen *Aspergillus fumigatus*. Curr. Biol. 15, 1242–1248.

Peterson, A.B., Rosendahl, S., 2000. Phylogeny of the Peronosporomycetes (Oomycota) based on partial sequences of the large ribosomal subunit (LSU rDNA). Mycol. Res. 104, 1295–1303.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Qi, J., Asano, T., Jinno, M., Matsui, K., Atsumi, K., Sakagami, Y., Ojika, M., 2005. Characterization of a *Phytophthora* Mating Hormone. Science 309, 1828.

Rambaut, A., Drummond, A.J., 2004. Tracer version 1.3. Available from: <http://tree.bio.ed.ac.uk/software/tracer/>.

Randall, T.A., Dwyer, R.A., Huitema, E., Beyer, K., Cvitanich, C., Kelkar, H., Ah Fong, A.M.V., Gates, K., Roberts, S., Yatzkan, E., et al., 2005. Large-scale gene discovery in the Oomycete *Phytophthora infestans* reveals likely components of phytopathogenicity shared with true Fungi. Mol. Plant-Microbe Int. 18, 229–243.

Ribeiro, O.K., 1978. A Source Book of the Genus *Phytophthora*. J. Cramer, Vaduz, Liechtenstein.

Riethmüller, A., Voglmayr, H., Goker, 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.

Riethmüller, A., Weiss, M., Oberwinkler, F., 1999. Phylogenetic studies of Saprolegniomycetidae and related groups based on nuclear large subunit ribosomal DNA sequences. Can. J. Bot. 77, 1790–1800.

Rizzo, D.M., Garbelotto, M., 2003. Sudden oak death: endangering California and Oregon forest ecosystems. Front. Ecol. Environ. 1, 197–204.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Rozen, S., Skaletsky, H.J., 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S., Misener, S. (Eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 365–386.

Sansome, E.R., 1987. Fungal chromosomes as observed with the light microscope. In: Rayner, A.D.M., Brasier, C.M., Moore, D. (Eds.), Evolutionary Biology of the Fungi. Cambridge University Press, Cambridge, pp. 97–113.

Schena, L., Hughes, K.J.D., Cooke, D.E.L., 2006. Detection and quantification of *Phytophthora ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* in symptomatic leaves by multiplex real-time PCR. Mol. Plant Pathol. 7, 365–379.

Shan, W., Cao, M., Leung, D., Tyler, B.M., 2004. The *avr1b* locus of *Phytophthora sojae* encodes an elicitor and a regulator required for avirulence on soybean plants carrying resistance gene *rps1b*. Mol. Plant-Microbe Int. 17, 394–403.

Shan, W., Hardham, A.R., 2004. Construction of a bacterial artificial chromosome library, determination of genome size, and characterization of an *Hsp70* gene family in *Phytophthora nicotianae*. Fungal Genet. Biol. 41, 369–380.

Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114–1116.

Sogin, M.L., Silberman, J.D., 1998. Evolution of the protists and protistan parasites from the perspective of molecular systematics. Int. J. Parasitol. 28, 11–20.

Stamps, D.J., Waterhouse, G.M., Newhook, F.J., Hall, G.S., 1990. Revised tabular key to the species *Phytophthora*. Mycol. Papers No. 162.

Swofford, D.L., 1998. PAUP*4.0: Phylogenetic Analysis Using Parsimony. Sinauer Associates, Sunderland, MA.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Tooley, P.W., Martin, F.N., Carras, M.M., Frederick, R.D., 2006. Real-time fluorescent polymerase chain reaction detection of *Phytophthora ramorum* and *Phytophthora pseudosyringae* using mitochondrial gene regions. Phytopathology 96, 336–345.

Tyler, B.M., 2007. *Phytophthora sojae*: root rot pathogen of soybean and model oomycete. Mol. Plant Pathol. 8, 1–8.

Tyler, B.M., Tripathy, S., Zhang, X., Dehal, P., Jiang, R.H.Y., Aerts, A., Arredondo, F.D., Baxter, L., Bensasson, D., Beynon, J.L., et al., 2006. *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. Science 313, 1261–1266.

Unkles, S.E., Logsdon, J.M.K.R., Kinghorn, J.R., Duncan, J.M., 1997. The *tigA* gene is a transcriptional fusion of glycolytic genes encoding Triose-Phosphate Isomerase and Glyceraldehyde-3-Phosphate Dehydrogenase in Oomycota. J. Bacteriol. 179, 6816–6823.

Villa, N.O., Kageyama, K., Asano, T., Suga, H., 2006. Phylogenetic relationships of *Pythium* and *Phytophthora* species based on ITS rDNA, cytochrome oxidase II and beta-tubulin sequences. Mycologia 98, 410–422.

Voglmayr, H., Riethmüller, A., Goker, M., Weiss, M., Oberwinkler, F., 2004. Phylogenetic relationships of *Plasmopara*, *Bremia* and other genera of downy mildew pathogens with pyriform hausteria based on Bayesian analysis of partial LSU rDNA sequence data. Mycol. Res. 108, 1011–1024.

Waterhouse, G.M., 1963. Key to the species of *Phytophthora* de Bary. Mycol. Papers No. 92.

Waterhouse, G.M., Newhook, F.J., Stamps, D.J., 1983. Present criteria for classification of *Phytophthora*. In: Erwin, D.C., Bartnicki-Garcia, S., Tsao, P.H. (Eds.), *Phytophthora*: Its Biology, Taxonomy Ecology, and Pathology. APS Press, St. Paul, MN, pp. 139–147.

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.

Whisson, S.C., Basnayake, S., Maclean, D.J., Irwin, J.A.G., Drenth, A., 2004. *Phytophthora sojae* avirulence genes *Avr4* and *Avr6* are located in a 24 kb, recombination-rich region of genomic DNA. Fungal Genet. Biol. 41, 62–74.

Win, J., Kanneganti, T.-D., Torto-Alalibo, T., Kamoun, S., 2006. Computational and comparative analyses of 150 full-length cDNA sequences from the oomycete plant pathogen *Phytophthora infestans*. Fungal Genet. Biol. 43, 20–33.

Yang, Z., 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. Bioinformatics 13, 555–556.

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

Zwickl, D.J., 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation, The University of Texas, Austin.

Zwickl, D.J., 2007. GARLI version 0.95. Available from: <http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html/>.