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Phytophthora foliorum sp. nov., a new species causing leaf blight of azalea

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ABSTRACT

A previously unknown *Phytophthora* was recovered more than 60 times from evergreen hybrid azalea leaves collected during surveys for the sudden oak death pathogen *Phytophthora ramorum* in California and Tennessee. The novel *Phytophthora* was discovered when genomic DNA from this species cross-reacted with the ITS-based diagnostic PCR primers used to screen plants for the presence of *P. ramorum*. This species had caducous, semi-papillate sporangia, was homothallic with both paragynous and amphigynous antheridia, and was pathogenic on both wounded and intact azalea leaves. Nuclear and mitochondrial sequence data indicate that this species is related to, but distinct from, *P. ramorum*. AFLP analysis indicates that the isolates of this species have limited genotypic diversity and share no markers with *P. ramorum*. This paper presents the formal description of *P. foliorum* as a new species and underscores the need for caution when relying solely on DNA-based diagnostic tools.

Introduction

Commonly called water moulds, more than 60 *Phytophthora* species have currently been described and are classified in the kingdom *Chromista* (Yoon *et al.* 2002; Erwin & Ribeiro 1996). Most *Phytophthora* species are considered plant pathogens (Cooke *et al.* 2000; Erwin & Ribeiro 1996). *P. ramorum*, a recently described species (Werres *et al.* 2001), has been found to cause oak death in 14 counties of coastal forest in California and in a single county in southern Oregon in the USA. The disease has been detected in nurseries and garden centres in the western USA (Washington, Oregon and California), British Columbia, Canada, as well as in Europe (Hansen *et al.* 2005). *P. ramorum* is found on several genera of woody nursery stock in Europe (Tooley *et al.* 2004). In response to the concern that *P. ramorum* will move on nursery stock and infect the red oak woodlands of the eastern USA, the United States Department of Agriculture-Animal and Plant Health Protection Service–Plant Protection and Quarantine (USDA-APHIS-PPQ) funded a nationwide survey of nursery stock in 2004 and 2005. New species of *Phytophthora* have been described as a result of this survey and forest survey efforts (Hansen *et al.* 2003; Jung *et al.* 2003). The USDA mandated that a single ITS-based nested PCR test be used the detection of *P. ramorum* in the US national survey. *P. hibernalis*, a close relative of *P. ramorum*, has been previously shown to yield false-positives in this

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P. ramorum detection assay (Blomquist *et al.* 2005). As a result of intensive survey efforts in California and Tennessee in 2004 and 2005, a previously undescribed *Phytophthora* species with distinctly different morphology from *P. ramorum* was identified. This previously undescribed *Phytophthora* species produced a false-positive in the *P. ramorum* detection assay. This paper presents the morphological description of *P. foliorum* as a new pathogen of azalea, the phylogenetic relationship to other *Phytophthora* species based on nuclear and mitochondrial DNA sequences, and the intraspecific variation within the species *P. foliorum* as assessed by AFLP.

Materials and methods

Cultures

All isolates used in this study are listed in Table 1. Cultures were obtained from leaf samples submitted during the national survey for the sudden oak death pathogen *Phytophthora ramorum*. Leaf tissue from the edge of foliar lesions was plated on corn meal agar, (CMA; Sigma, St Louis, MO) amended with PARP (25 ppm pimaricin, 100 ppm ampicillin, 25 ppm rifampicin and 25 ppm pentachloronitrobenzene) (Erwin & Ribeiro 1996). Cultures were subsequently hyphal-tipped to ensure single isolates. Hyphal-tipping was accomplished by growing each culture on water agar and then subculturing a single strand from the expanding margin of the culture. Cultures were incubated in the dark at room temperature for 7–10 d before microscopic examination. Cultures were maintained on CMA-PARP, CMA and V8 agar (V8) and were stored long-term at room temperature at 18 °C as colonized agar discs of V8 and Rye A medium (Caten & Jinks 1968; Erwin & Ribeiro 1996) in sterile water containing two autoclaved hempseeds. Colony morphology, growth rate, and oospore morphology were determined by culturing isolates on CMA, V8 and half-strength potato dextrose (PDA) agar. Cardinal growth temperatures were determined using CMA plates inoculated with a 5-mm diam agar plug and incubated in the dark at: 4, 10, 15, 18, 21, 24, and 26 °C ($n = 3$). Isolates of *P. cactorum*, *P. ramorum*, and *P. hibernalis* were also used in temperature growth rate assays for comparison. For oospore production, CMA and V8 plates were placed in the dark at 18 °C for 7–10 d. Oospores were measured using a Nikon Eclipse (E600 Series) microscope. Sporangia were produced by transferring actively growing agar plugs of mycelium to Petri plates containing soil extract water (150 g soil in 1 l deionized water mixed with a stirring bar for 30 min, decanted, and run through a 20 μm filter) and incubated at room temperature overnight both in the light and dark. A representative isolate, LT192, has been selected as holotype and deposited in the American Type Culture Collection (ATCC), and an isotype is included the *Phytophthora* species collection at the University of California, Riverside.

Table 1 – *Phytophthora* isolates used in this study

Isolate	County, State	Host
P. foliorum		
1283844	Alameda, CA	*Rhododendron* sp. (azalea) 'Duc de Rohan'
1301442	San Joaquin, CA	*Rhododendron* sp. (azalea) 'Sherwood Red'
1367244	Orange, CA	*Rhododendron* sp. (azalea) 'Red Ruffle'
1307997	Sonoma, CA	*Rhododendron* sp. (azalea)
1330048	San Mateo, CA	*Rhododendron* sp. (azalea) 'Rose Glow'
1314489	Los Angeles, CA	*Rhododendron* sp. (azalea) 'Phoenicia'
1307658	Butte, CA	*Rhododendron* sp. (azalea) 'Imperial Princess'
1267257	Ventura, CA	*Rhododendron* sp. (azalea) 'Fielder's White'
1313743	Sacramento, CA	*Rhododendron* sp. (azalea) 'Brilliant'
LT192[a]	Shelby, TN	*Rhododendron* sp. (azalea) 'Pink Ruffles'
LT1261	Shelby, TN	*Rhododendron* sp. (azalea) 'Pink Ruffles'
P. cactorum		
1314491	Los Angeles, CA	*Pyracantha koidzumii* 'Victory'
1342723	Santa Cruz, CA	*Rhamnus californica*
P. ramorum		
1289753	Santa Cruz, CA	*Rhododendron* sp. (rhododendron)
1341211	Sacramento, CA	*Rhododendron* sp. (rhododendron) 'Minnetonka'
P. hibernalis		
1330912	Solano, CA	Camellia 'Ice Follies'

a Isolate used in pathogenicity experiments.

Plant inoculations

Initial pathogenicity tests were done to complete Koch's postulates using nursery stock azalea plants 'Pink Ruffles' (courtesy of M. Windham) and *Phytophthora foliorum* isolate 192. Before inoculations, plants were grown in a greenhouse under ambient lighting in 1 gallon pots containing soil-less potting media (Redi-Gro, Sacramento, CA). Inoculation experiments were conducted in a growth chamber maintained at approximately 22 °C with a 12 h dark/light cycle. Seven millimetre plugs of actively growing mycelium, grown for 7 d on V8 at 22 °C, were adhered by placing the plugs on the under sides of both wounded ($n = 4$) and intact ($n = 4$) newly unfurled leaves. The plants were misted with water and placed into sealed plastic bags for 48 h. Plants were removed from the plastic bags after 48 h and symptom development was monitored daily for two weeks.

PCR-RFLP and AFLP

Nested PCR amplification was performed as per Davidson *et al.* (2003) and 10 μl of the final amplicon from nested PCR was digested with *Hae*III (New England Biolabs, Ipswich, MA) and the restriction profiles were resolved on a 3 % NuSieve gel

(Cambrex, Rockland, ME). ITS amplicons (two *Phytophthora foliorum* and one *P. ramorum*) were amplified as described above, and 10 µl from each reaction was digested separately with *Alu*I (NEB) and *Msp*I (Promega, Madison, WI) according to the manufactures instructions. Ten microlitres of the digested ITS amplicons were resolved on a 3 % NuSieve gel (Cambrex, Rockland, ME) run at 20 V for 12 h. Genomic DNA from 11 isolates of *P. foliorum* and one isolate of *P. ramorum* was assayed for AFLP markers using *Eco*RI, *Mse*I restriction enzymes, adapters, and primers as described by Vos *et al.* (1995). Selective amplifications were done using Eco-AC, Mse-CCC primers, and labelled in separate reactions as described by Habera *et al.* (2004). Fluorescently labelled products were resolved on a Beckman-Coulter CEQ8000 capillary genetic analysis device (Fullerton, CA, USA) and the fragment profiles edited manually to determine the number of mono- and polymorphic AFLP markers.

DNA amplification and sequencing

Mycelium was grown in V8-PARP broth, lyophilized, and genomic DNA was extracted using Qiagen's DNeasy Plant Mini-kit (Valencia, CA). Table 2 lists the primers used for amplification and sequencing. PCR reactions and cycling parameters for the ITS, β-tubulin, translation elongation factor 1α (EF-1α), and cytochrome oxidase (cox) are as described by Cooke *et al.* (2000), Kroon *et al.* (2004), and Martin and Tooley (2003a,b), respectively. PCR products were resolved on 1 % agarose gels to verify a single product, cleaned using Qiagen's Qiaquick PCR purification kit, and submitted to the sequencing core facility at the University of Tennessee.

Phylogenetic analysis

Nucleotide sequences for all other *Phytophthora* species in this study are listed in Table 3. Sequences from 54 *Phytophthora* species used in the study of Kroon *et al.* were used in the comparison of the β-tubulin and EF-1α genes. These included 49 distinct *Phytophthora* species with multiple isolates of *P. infestans*, *P. mirabilis*, and *P. fragariae* with *Pythium aphanidermatum* treated as an outgroup (Kroon *et al.* 2004). For phylogenetic analysis of the coxII sequences from the same 64 taxa used by Martin and Tooley (2003b) were included. These were comprised 30 *Phytophthora* species with multiple taxa represented for several species (Table 2). The ITS sequences used in the publication of Cooke *et al.* (2000), plus additional sequences representing other more recently described species, were downloaded from GenBank and are listed in Table 3.

Parsimony analysis

We conducted independent phylogenetic analysis of concatenated nuclear, ITS and coxII nucleotides using the parsimony criterion implemented in PAUP 4.0b10 (Swofford 2002). All characters were treated as unordered. Trees were constructed by a heuristic search with tree bisection–reconnection (TBR) branch swapping in a random stepwise addition of taxa repeated 1000 times. Maxtrees was set to increase incrementally. Node support was evaluated by nonparametric BS resampling (Felsentein 1985). BS scores were calculated from 1000 replicates, with each replicate consisting of three searches starting with a tree built by stepwise addition using the simple addition sequence.

ML analysis

Phylogenetic trees were also estimated using the ML criterion implemented in PAUP 4.0b10 (Swofford 2002). Nucleotide substitution models for each molecular data set were selected using Modeltest 3.06 (Posada & Crandall 1998). Once a model was selected for a given molecular data set, we used this model and its parameter estimates to search for an optimal ML tree via heuristic searches of tree space using TBR branch swapping in a random stepwise addition of taxa repeated 15 times.

Bayesian analysis

Bayesian MCMC phylogenetic analysis was conducted using MrBayes 2.01 (Huelsenbeck *et al.* 2001) using the models and parameters suggested for each data matrix by Modeltest. Each Markov chain in the Bayesian search was started from a random tree and run for 1×106 cycles, sampling every 1000th cycle from the chain. Four chains were run

Table 2 – Primers used for amplification and sequencing

Target	Primer name	Sequence	Reference
ITS	ITS4	5′-TCCTCCGCTTATTGATATGC-3′	Cooke *et al.* 2000
	ITS6	5′-GAAGGTGAAGTCGTAACAAGG-3′	
Beta-tubulin (β-*tub*)	TubuF2	5′-ACGGCTCGAGGATGACCATG-3′[a]	Kroon *et al.* 2004
	TubuR1	5′-CCTGGTACTGCTGGTACTCAG-3′[a]	
Translation elongation	ElongF1	5′-TCACGATCGACATTGCCCTG-3′[a]	
factor 1 alpha (*EF-1α*)	ElongR1	5′-ACGGCTCGAGGATGACCATG-3′[a]	
Cytochrome oxidase	FM75	5′-CCTTGGCAATTAGGATTTCAAGAT-3′[a]	Martin & Tooley 2003a
(*cox*) I and II	FM77	5′-CACCAATAAAGAATAACCAAAAATG-3′	
	FM78	5′-ACAAATTTCACTACATTGTCC-3′[a]	
	FM79	5′-GGACAATGTAGTGAAATTTGT-3′[a]	
	FM80	5′-AATATCTTTATGATTTGTTGAAA-3′[a]	

a Primers used in sequencing reactions.

Table 3 – *Phytophthora* species sequence accession numbers from GenBank for isolates used in the phylogenetic analyses

Phytophthora species	Isolate	GenBank accession numbers			
		ITS	β-tubulin	EF-1α	coxII
P. alni subsp. *multiformis*	[d]	AF139368			
P. arecae	IMI 348342[a,c]	AF266781			AY129176
	CBS 148.88[b]		AY564049	AY564105	
P. bisheria	P1.33[d]	AF408625			
P. botryose	IMI 136915[a]	AF266784			
	IMI 136916[b]		AY564051	AY564107	
P. brassicae	CBS 178.87	AF380147			
P. cactorum	IMI 296524[a]	AF266772			
	P6183[b]		AY564052	AY564108	
	311[c]				AY129178
	385[c]				AY129179
	SB2079[c]				AY129180
P. cajani	P536[a]	AF266765			
P. cambivora	IMI 296831[a]	AF266763			
P. capsici	IMI 352321[a]	AF266787			
	302[PT]				AY129181
P. cinnamomi	UQ881[a]	AF266764			
	RADACI B[b]		AY564054	AY564110	
var. *parvispora*		AY302184			
	Cn-2[DJM]				AY129182
P. citricola	IMI 031072[a]	AF266788			
	P1817[b]		AY564055	AY564111	
	Cr-4[DJM]				AY129183
	SB2084				AY129184
P. citrophthora	IMI 332632[a]	AF266785			
	CBS 274.33[b]		AY564056	AY564112	
P. clandestina	IMI 287317[a,b]	AJ131989	AY564057	AY564113	
P. colocasiae	IMI 368918[a,b]	AF266786	AY564058	AY564114	
	ATCC 56193[c]				AY129185
	P3773[MC]				AY129186
	ATCC 52233[c]				AY129187
P. cryptogea	IMI 045168[a]	AF266796			AY129188
	HR1/ss/pp/99[b]		AY564059	AY564115	
P. drechsleri	ATCC 46724[a,b,c]	AF266798	AY564060	AY564116	AY129190
	301[PT]				AY129189
P. erythroseptica	ATCC 36302[a]	AF266797			AY129191
	ATCC 46725[b]		AY564061	AY564117	
	388[PT c]				AY129193
P. europaea	OSU-2 AE2[d]	AF449491			
P. fragariae var. *rubi*	CH132[a]	AF266761			
P. fragariae var. *rubi* I	FVR67[b]		AY564064	AY564120	
P. fragariae var. *rubi* II	FVR30[b]		AY564065	AY564121	
P. fragariae var. *rubi*	397[PT c]			AY129196	
P. fragariae var. *fragariae*	IMI 330736[a]	AF266762			
P. fragariae var. *fragariae* II	NS4[b]		AY564063	AY564119	
P. fragariae var. *fragariae*	394[PT c]				AY129194
P. fragariae var. *fragariae*	398[PT c]				AY129195
P. gonapodyides	P245[y]	AF266793	AY564066	AY564122	
P. gonapodyides	393[PT c]				AY129197
Phytophthora sp. "O" group	P246b[a]	AF266791			
P. hedraiandra	CBS 111725[d]	AY707987			
P. heveae (T)	IMI 180616[a,b]	AF266770	AY564067	AY564123	
P. hibernalis	ATCC 64708[b,c]	AY564068	AY564124		AY129201
	ATCC 56353[c]				AY129199
	ATCC 60352[c]				AY129200
	380[c]	AY369375			
P. humicola	IMI 302303 [a,b]	AF266792	AY564069	AY564125	
P. idaei (T)	IDA3[a]	AF266773			
	IMI 313727[b]		AY564070	AY564126	

Table 3 (*continued*)

Phytophthora Isolate	Isolate	GenBank Accession numbers			
		ITS	*β-tubulin*	*EF-1α*	*cox II*
P. ilicis	ILI 1[a]	AJ131990			
	PD91/595[b]		AY564071	AY564127	
	343[PT]				AY129202
	ATCC 56615[c]				AY129203
P. infestans	IMI 66006[a]	AF266779			
	176[PT]				AY129204
	180[PT]				AY129205
	580[PT]				AY129206
P. infestans Ia	Pic99186[b]		AY564035	AY564093	
P. infestans IIa	Dr98004[b]		AY564036	AY564094	
P. ipomoeae	Pic99165[d]	AY770742			
P. inflata	IMI 342898[a,b]	AF266789	AY564072	AY564128	
P. insolita (T)	IMI 288805[a,b]	AF271222	AY564073	AY564129	
P. inundata	P246b[d]	AF266791			
P. iranica	IMI 158964[a,b]	AJ131987	AY564074	AY564130	
P. katsurae	IMI 360596[a]	AF266771			
	IMI 325914[b]		AY564075	AY564131	
P. lateralis (T)	IMI 040503[a,b,c]	AF266804	AY564076	AY564132	AT129207
	452[PT]				AY369360
	455[PT]				AY369361
P. meadii	IMI 129185[b]		AY564077	AY564133	
P. medicaginis	UQ125[a]	AF266799			
P. megakarya	IMI 337104[a]	AF266782			
	IMI 337098[b]		AY564078	AY564134	
	327[PT]				AY129208
	328[PT]				AY129209
P. megasperma	IMI 133317[a,b]	AF266794	AY564079	AY564135	
P. sp. (on asparagus)	UQ2141[a]	AF266795			
P. melonis	IMI 325917[a]	AF266767			
P. mirabilis (T)	ATCC 64130[a]	AF266777			
	ATCC 64070[c]				AY129213
	ATCC 64073[c]				AY129214
P. mirabilis I	Pic99129[b]		AY564038	AY564095	
P. mirabilis II	P3001[b]		AY564039	AY564096	
P. mirabilis IV	G4-4[b]		AY564041	AY564098	
P. mirabilis V	G15-4[b]		AY564042	AY564099	
P. multivesiculata	CBS 545.96[a,b]	AF266790	AY564080	AY564136	
P. nicotianae	UQ848[a]	AF266776			
	P582[b]		AY564081	AY564137	
	Pn-17[DJM c]				AY129215
	322[PT]				AY129216
P. niederhauseii	PPIL.01.5112[d]	AY550916			
P. nemorosa	482[PT]	AY332654			AY429504
	483[PT]				AY429505
P. pistaciae	PIS15[d]	AF403506			
P. palmivora	UQ1249[a]	AF266780			
	CBS 236.30[b]		AY564082	AY564138	
	329[PT]				AY129217
	Pl-5[DJM]				AY129218
	Pl-10[DJM c]				AY129219
	Pl-14[DJM c]				AY129220
P. phaseoli	ATCC 60171[a,b]	AF266778	AY564044	AY564101	
	330[PT]				AY129221
P. porri	CBS 782.97[a]	AF266801			
P. primulae	CBS 620.97[a]	AF266802			
P. pseudosyringae	484[PT c]				AY429506
	485[PT]				AY429507
	470[PT]				AY369357
	471[PT]				AY369358
	473[PT]	AY369374			AY369359
P. pseudotsugae	IMI 331662[a,b]	AF266774	AY564084	AY564140	
P. pseudotsugae	308[PT c]				AY129222

(*continued on next page*)

Table 3 (continued)

Phytophthora Isolate	Isolate	GenBank Accession numbers			
		ITS	β-tubulin	EF-1α	cox II
P. psychrophila	IFB-PSY 2[d]	AF449494			
P. quercina (T)	QUE4[a]	AJ131986			
P. richardiae	IMI 340618[a]	AF271221			
	CBS 240.30[b]		AY564086	AY564142	
P. ramorum	PD93/51[b]		AY564092	AY564149	
	Prg-2^{PT} [c]				AY369365
	016^{DR} [c]				AY369362
	013^{DR} [c]				AY369363
	Coen^{TT} [c]				AY369364
	20315247	AY616757			
P. sinensis (T)	ATCC 46538[a]	AF266768			
	P1475[b]		AY564087	AY564143	
P. sojae	UQ1200[a]	AF266769			
	P6497[b]		AY564047	AY564104	
	ATCC 48068[b]				AY129223
P. syringae	IMI 296829[a]	AF266803			
	IMI 045169[b]		AY564088	AY564144	
	442^{PT} [c]				AY129224
	468^{PT} [c]				AY369366
	469^{PT} [c]				AY369367
P. sp. (on spathiphyllum)	[c]		AY564091	AY564147	
P. tentaculata	CBS 552.96[a]	AF266775			
P. trifolii	UQ2143[a]	AF266800			
P. tropicalis	H352[d]	AY208125			
	AN97/86[b]		AY564046	AY564103	
P. uliginosa	IFB-ULI 1[d]	AF449495			
P. vignae	UQ136[a]	AF266766			
	CBS 241.73[b]		AY564090	AY564146	
Pythium aphanidermatum			AY564048[b]		

a Isolate number and accession obtained from Cooke *et al.* (2000).
b Isolate number and accession obtained from Kroon *et al.* (2004).
c Isolate number and accession obtained from Martin & Tooley (2003b).
d Isolate number and accession obtained directly from GenBank.
(T), ex-type culture.

simultaneously, three 'hot' and one 'cold'. Each simulation was run twice. We used the default settings for the priors on the rate matrix (0–100), branch lengths (0–10), and proportion of invariant sites (0–1). Stationarity (of the sum of the natural log of the likelihoods of the trees in each of the four chains weighted according to the temperatures of the chains) was evaluated by monitoring likelihood values graphically. The initial 100 trees in each run were discarded as 'burn-in'. The remaining trees were used to construct majority rule consensus trees. Bayesian PPs for each clade were derived from trees remaining after the discarding the burn-in samples. For ease of visual comparison to BS values, we present these probabilities as whole numbers ranging from 0–100. PPs greater than or equal to 95 % are generally regarded as strong support for a clade's existence (Wilcox *et al.* 2002).

Fig 1 – Lesions produced on the leaves of azalea cv. 'Pink Ruffles' 3 d following wounded (A) and not wounded (B) inoculations with *Phytophthora foliorum*.

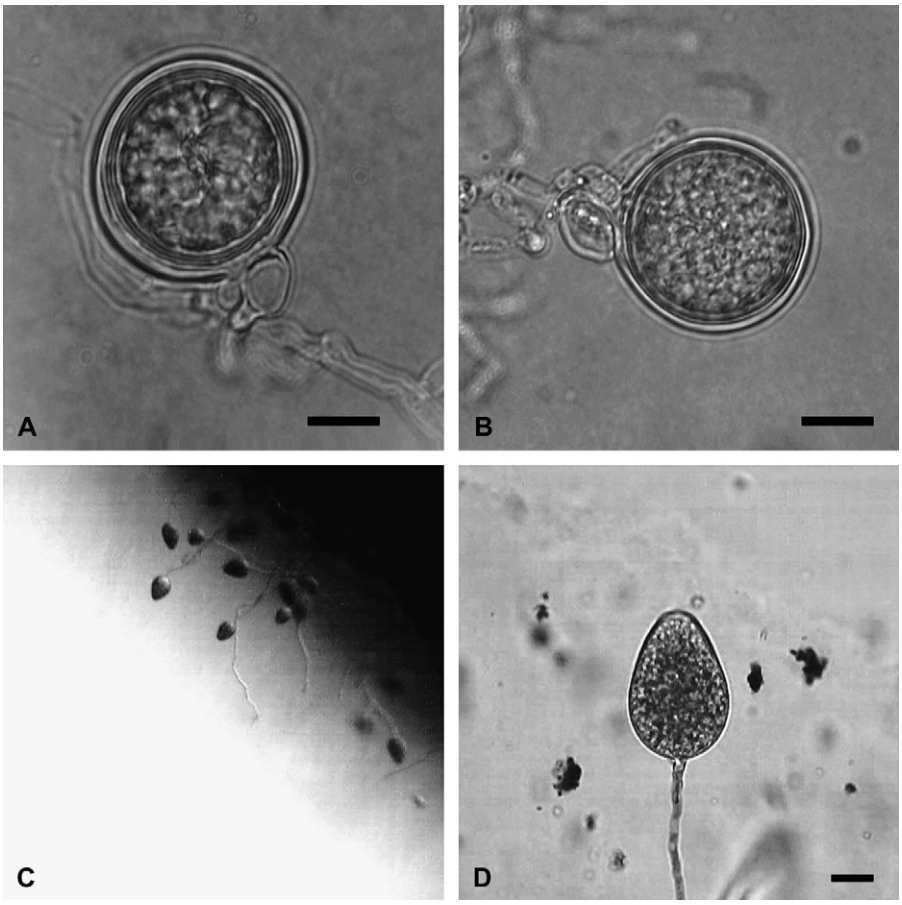

Fig 2 – Oospores produced by isolates of *Phytophthora foliorum*. Both paragynous (top left) and amphigynous (top right) antheridial attachments were observed. Sporangia of *Phytophthora foliorum* born terminally on the sporangiophore C, and are ovoid, semi-papillate, with a short pedicle D. Bar = 10 μm for A, B, D.

Results

Isolates and pathogenicity

More than 60 isolates of *Phytophthora foliorum* were recovered from leaf samples of azalea collected in California and Tennessee during *P. ramorum* surveys in the summer of 2004 and winter of 2005. *P. foliorum* was isolated from the margin of brown leaf spots and healthy tissue. Inoculation experiments to complete Koch's postulates with 'Pink Ruffles' and *P. foliorum* isolate 192 resulted in lesions similar to those seen on naturally-infected plants. Artificially-wounded leaves developed lesions at a faster rate than non-wounded leaves (Fig 1). *P. foliorum* was isolated from the margins of expanding lesions on both wounded and not wounded leaves after 14 d.

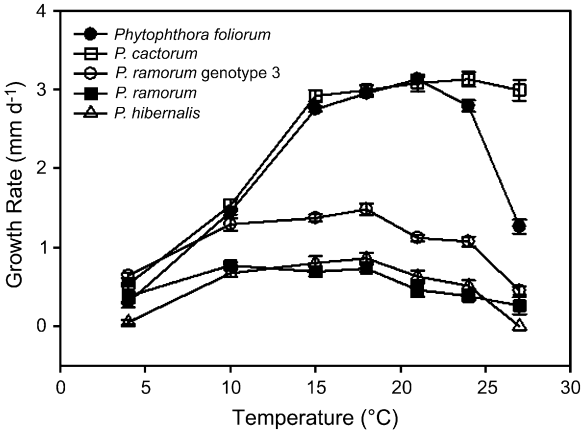

Fig 3 – Growth rates of *Phytophthora* species frequently recovered from nursery samples during sudden oak death sampling. Isolates were grown on CMA in the dark ($n = 3$).

Oospores were produced in culture after 7 d on CMA-PARP, CMA, and V8-PARP and V8 agar plates. Oospore width ranged from 28.2–38.2 μM and averaged 33 μM ($n = 120$). Antheridia were generally paragynous and attached to the oogonia next to the oogonial stalk (Fig 2). Sporangia were not produced on any of the media tested. Sporangia proved to be difficult to stimulate and were only observed in soil extract water. The sporangia are borne terminally on the sporangiophore, and are caducous, ovoid, and semi-papillate (Fig 2). *P. foliorum* was able to grow at temperatures ranging from 4–28 °C (Fig 3). Isolates exhibited a stressed growth response of sectoring on all media at temperatures greater than 24 °C. Growth on V8 and CMA was appressed whereas on PDA, growth was aerial and cottony (Fig 4). The optimum temperature for *P. foliorum* growth in culture is 21–22 °C. At this temperature isolates grew ~3 mm d^{-1}. *P. foliorum* resembled *P. cactorum* in culture, but in culture *P. cactorum* is distinguished from *P. foliorum* by

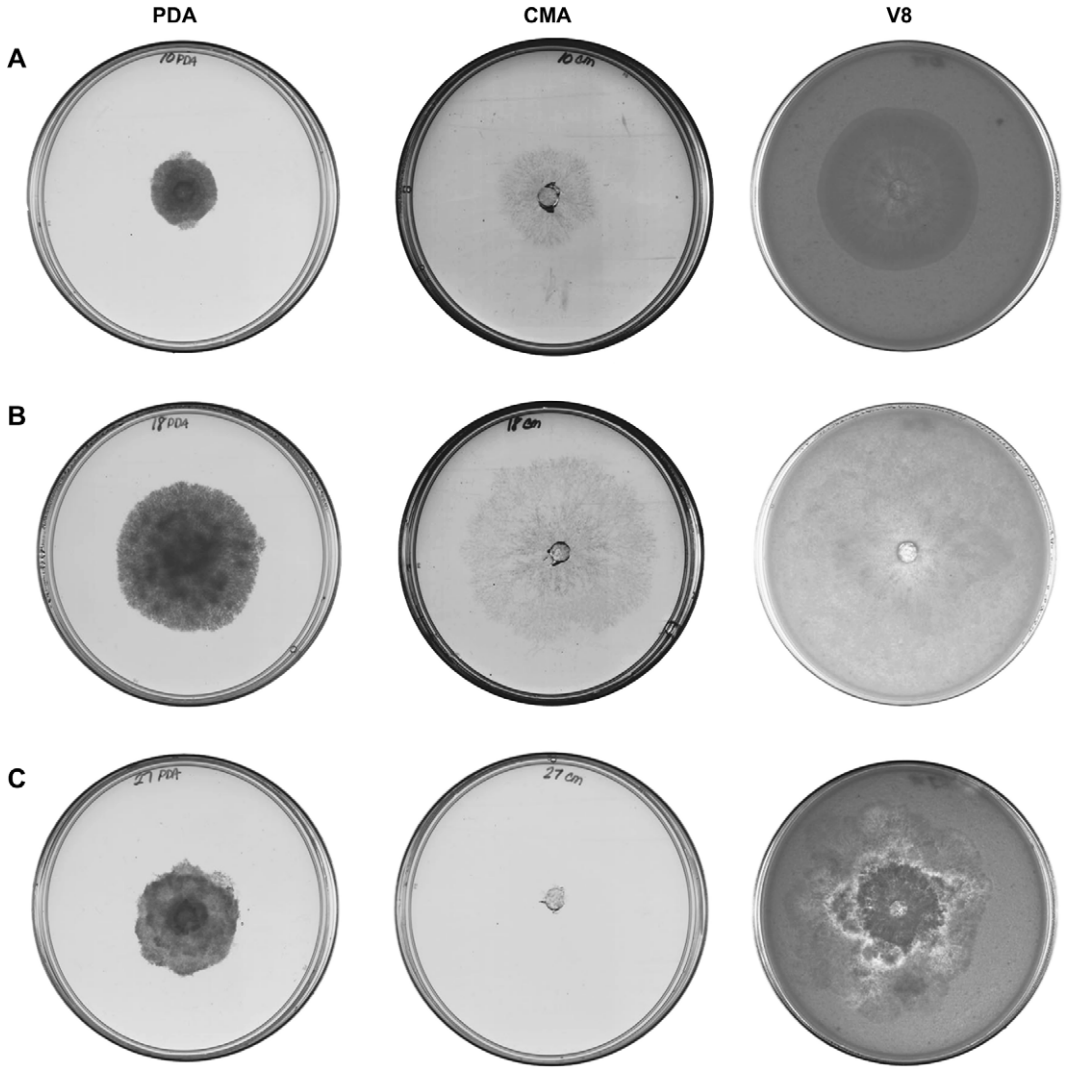

Fig 4 – Growth of *Phytophthora foliorum* on various media at different temperatures. Rows: A = 10 °C; B = 18 °C; C = 27 °C.

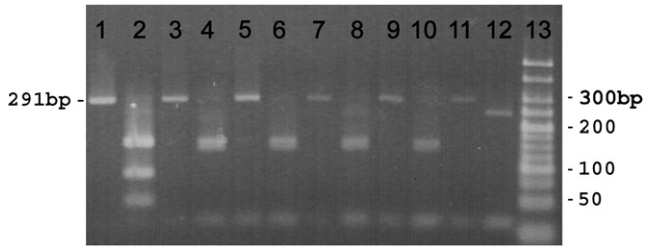

Fig 5 – Amplicons and *Hae*III restriction profiles from the second amplification reaction of the nested PCR protocol for the detection of *Phytophthora ramorum*. Lanes 1 and, 2: DNA from *P. ramorum*-infected *Umbellularia californica*; Lanes 3–10: DNA from *P. foliorum*-infected azaleas; Lanes11,12: DNA from *P. hibernalis*-infected rhododendron. Odd numbered lanes are undigested PCR amplicons. Even numbered lanes are *Hae*III digests of amplicons in the previous lane. Lane 13: size standards.

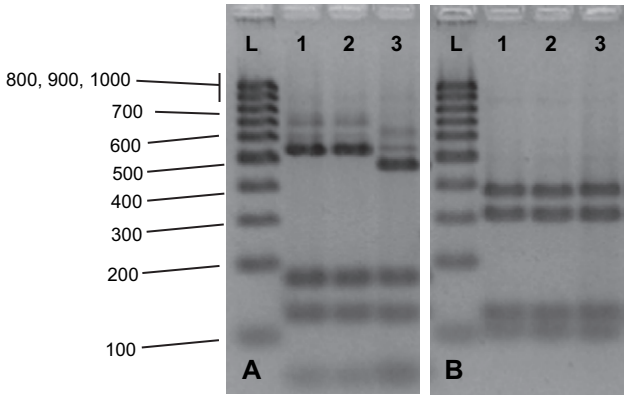

Fig 6 – Restriction digests of the ITS ribosomal DNA region using A, *Alu*1 and B, *Msp*1 for *Phytophthora foliorum* (1, 2) and *P. ramorum* (3). L = 100 bp ladder.

its rapid growth at higher temperatures (i.e., >24 °C) and production of sporangia (Erwin & Ribeiro 1996). *P. foliorum* is morphologically somewhat similar to *P. syringae* in culture, yet is distinguishable by sporangia being cauducous and born terminally, whereas for *P. syringae* sporangia are persistent, and form in succession in a close monochasial sympodium formation (Erwin & Ribeiro 1996). At all temperatures, *P. foliorum* had a greater growth rate than *P. ramorum* and *P. hibernalis*. *P. foliorum* isolates grew faster in total darkness than with a photoperiod. The growth rate of isolates on CMA at 21 °C was found to be reduced by 60 % when isolates were exposed daily to a 12 h photoperiod (data not shown).

Nested PCR and PCR-RFLP

Azaleas and rhododendrons naturally infected with *Phytophthora foliorum* and *P. hibernalis*, respectively, produced amplicons in the second amplification step of the PCR assay that co-migrated with the *P. ramorum* amplicon on Nusieve 3:1 agarose gels (Fig 5). These amplicons tended to be less bright when compared with *P. ramorum* bands and not always reproducible. *Hae*III digestion of the amplicons easily distinguished the three species (Fig 5). Likewise, restriction digests of the ITS amplicons generated from pure culture genomic DNA when cut with *Alu*I resulted in one clearly resolved polymorphic fragment, whereas digestion with *Msp*I resulted in identical restriction profiles (Fig 6).

Genotypic and phylogenetic analysis

AFLP

Thirteen reproducible AFLP markers were generated using the described AFLP primers. No polymorphisms were present among the 11 *Phytophthora foliorum* isolates, suggesting limited genotypic diversity in this new species (Table 1, Fig 7). A comparison of *P. ramorum* with *P. foliorum* using the same primer combination indicated that they have no markers in common (Fig 7).

Phylogenetic analysis

Phylogenetic analysis of the concatenated nuclear genes β-tubulin–EF-1α, the coxII mitochondrial gene, and ITS regions shows that *Phytophthora foliorum* is closely related to, but distinct from, *P. ramorum*, *P. lateralis*, and *P. hibernalis* (Figs 8–10). *P. foliorum* is consistently placed towards the base of the clade containing *P. hibernalis*, *P. lateralis* and *P. ramorum*, although its exact position varies slightly depending on the sequences examined. The concatenated β-tubulin–EF-1α sequences suggest that *P. foliorum* shares its most recent common ancestor with *P. hibernalis*, with these two species found basal to the group containing *P. ramorum* and *P. lateralis* (Fig 8). The coxII phylogeny places *P. foliorum* closest to *P. hibernalis* but basal to *P. hibernalis*, *P. lateralis*, and *P. ramorum* (Fig 9). The ML phylogeny based on ITS regions places *P. foliorum* closest to *P. ramorum* yet basal to *P. hibernalis* and *P. lateralis* (Fig 10).

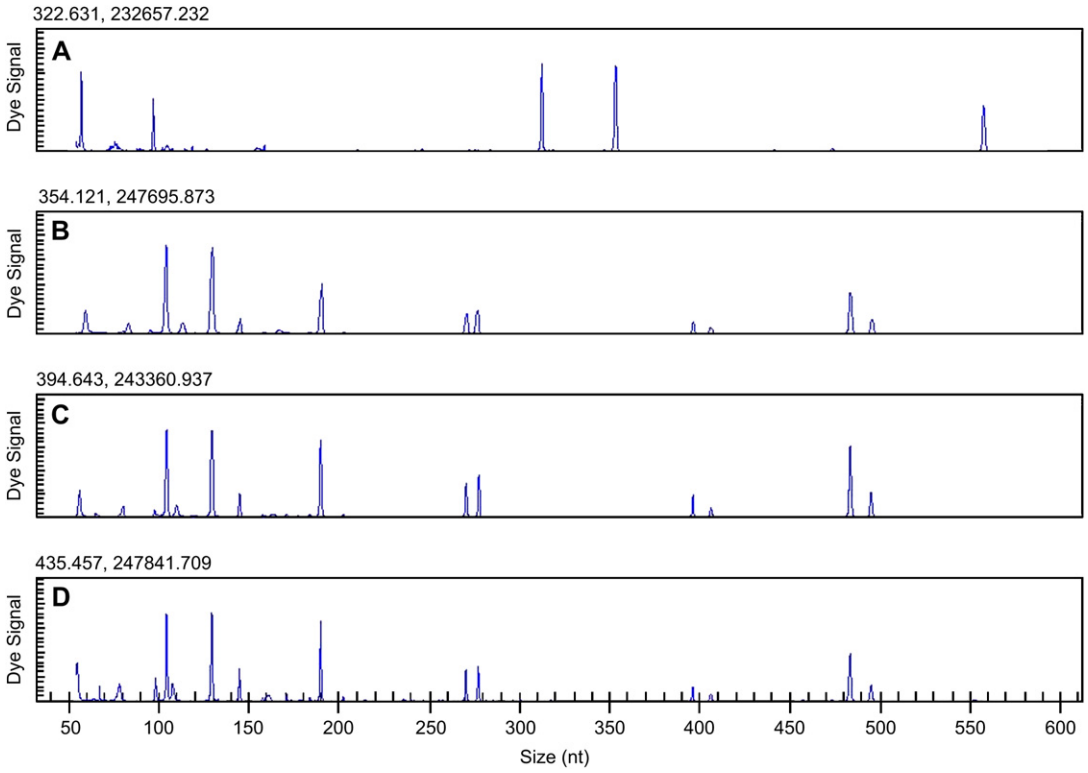

Fig 7 – Electropherograms showing fluorescently labelled AFLP markers for (A) *Phytophthora ramorum*, and (B–D) isolates of *P. foliorum*. Profiles were generated using the selective primer pair E-AC/M-CGC and resolved on a Beckman CEQ 8000 genetic analysis platform. No clearly resolved fragments are shared between the two species.

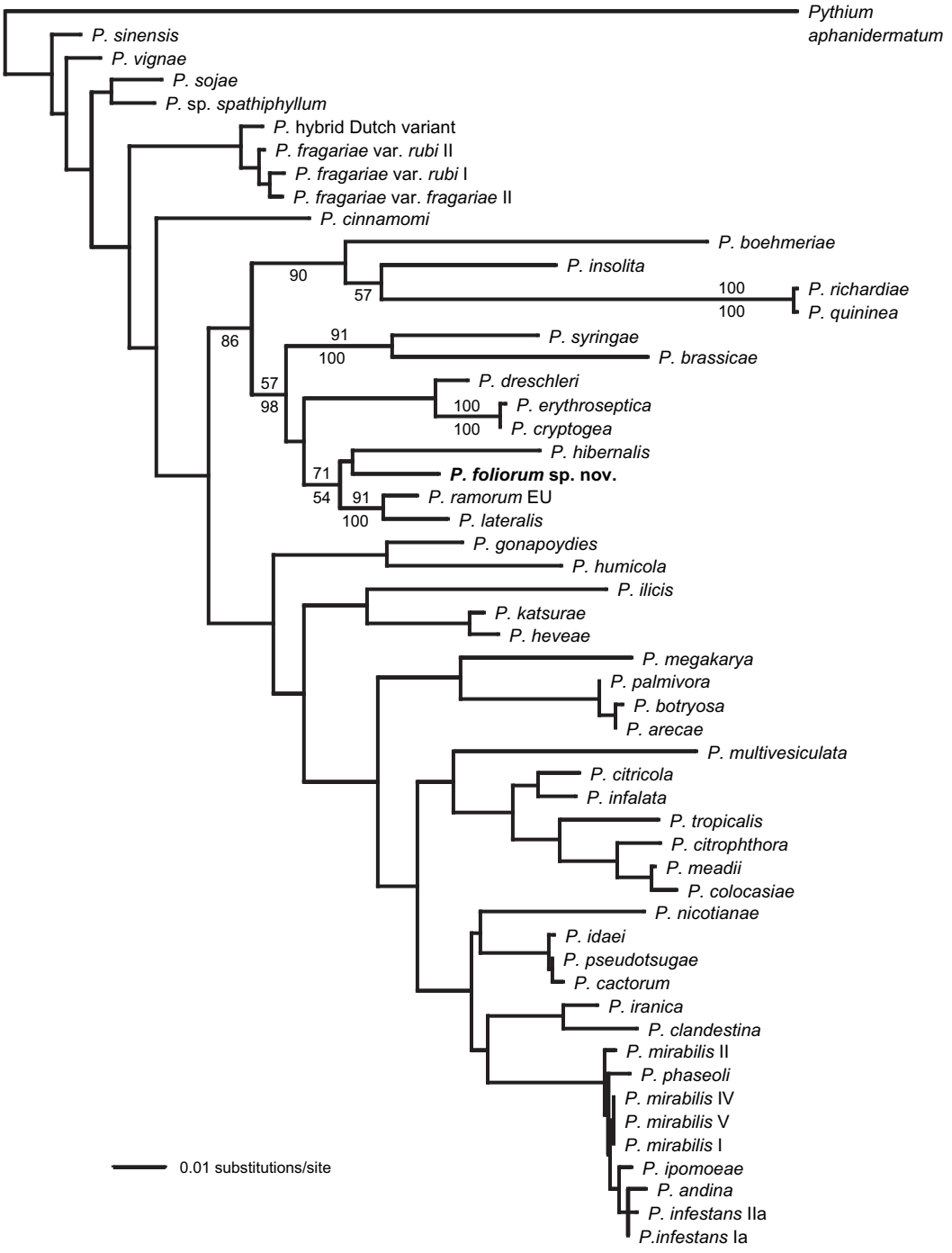

Fig 8 – Phylogenetic tree showing the relationship of *Phytophthora foliorum* within the genus *Phytophthora* based on concatenated β-tubulin–EF-1α sequences. Tree topology based on ML criteria. MP BS scores and Bayesian PPs are shown above and below nodes, respectively, of clades containing the inferred closest relatives of *P. foliorum*.

Taxonomy

Phytophthora foliorum Donahoo & Lamour, **sp. nov.**

Etym.: foliorum refers to pathogenicity on leaves.

Species homothallica, oosporas in cultura procerans; oogoniis in medio 33 μm; antheridias paragynis. Sporangiis semipapillatis, in medio 51 × 34 μm. Sporangiis saepe deciduis, cum pedicellulo brevi (<5–20 μm). Temperaturae optima 18–22 °C. Incrementum diurnum radiale 3 mm in agaro V8 ad 20 °C.

Typus: **USA**: *Tennessee*: isol. ex Azalea 'Pink Ruffles' in nursery, May 2004, *K. Lamour* 192 (ATCC MYA-3638 — holotypus).

Species homothallic; oospores abundantly produced in culture. Oogonia not ornamented, 37 μm average diam (32–43 μm range). *Oospores* plerotic, spherical, 33 μm average diam (range 28–38 μm). *Antheridia* mostly paragynous and usually attached to the oogonia next to the oogonial

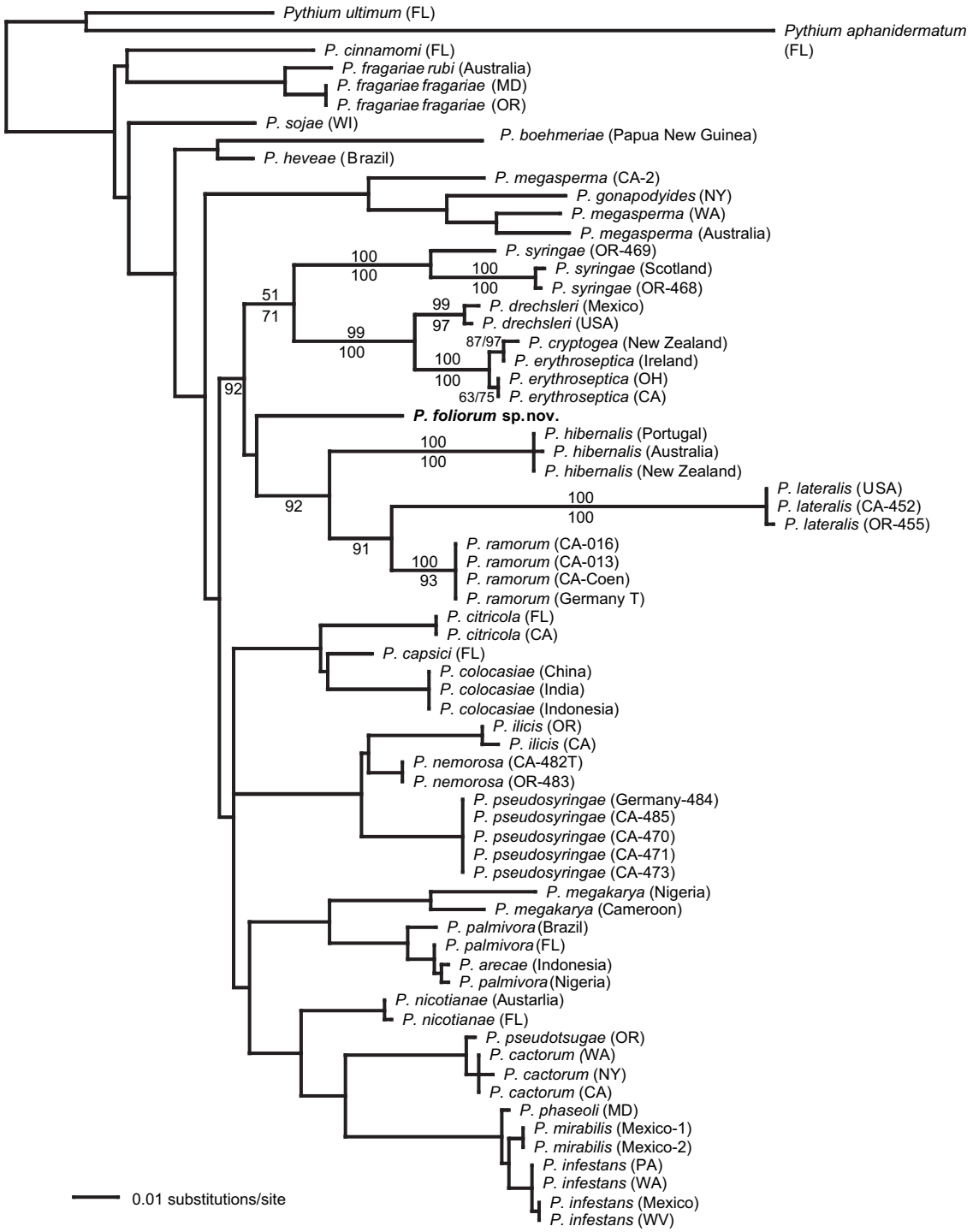

Fig 9 – Phylogenetic tree showing the relationship of *Phytophthora foliorum* within the genus *Phytophthora* based on coxII sequences. Tree topology based on ML criteria. MP BS scores and Bayesian PPs are shown above and below nodes, respectively, of clades containing the inferred closest relatives of *P. foliorum*.

stalk. Growth on CMA between 3–28 °C. *Sporangia* are semi-papillateand are on average 51 × 34 μm. Sporangia are deciduous with short pedicels (<5–20 μm). Optimum growth at 18–22 °C at a rate of 3 mm d^{-1} on V8 agar. No chlamydospores produced. Sporangia were only produced in soil extract water and rarely, if ever, are produced in culture.

Discussion

This is the first report of *Phytophthora foliorum* sp. nov., a pathogen of azalea. *P. foliorum* belongs to group III of the Waterhouse classification based on morphological characteristics (Waterhouse 1963; Erwin & Ribeiro 1996). Morphologically, *P. foliorum*

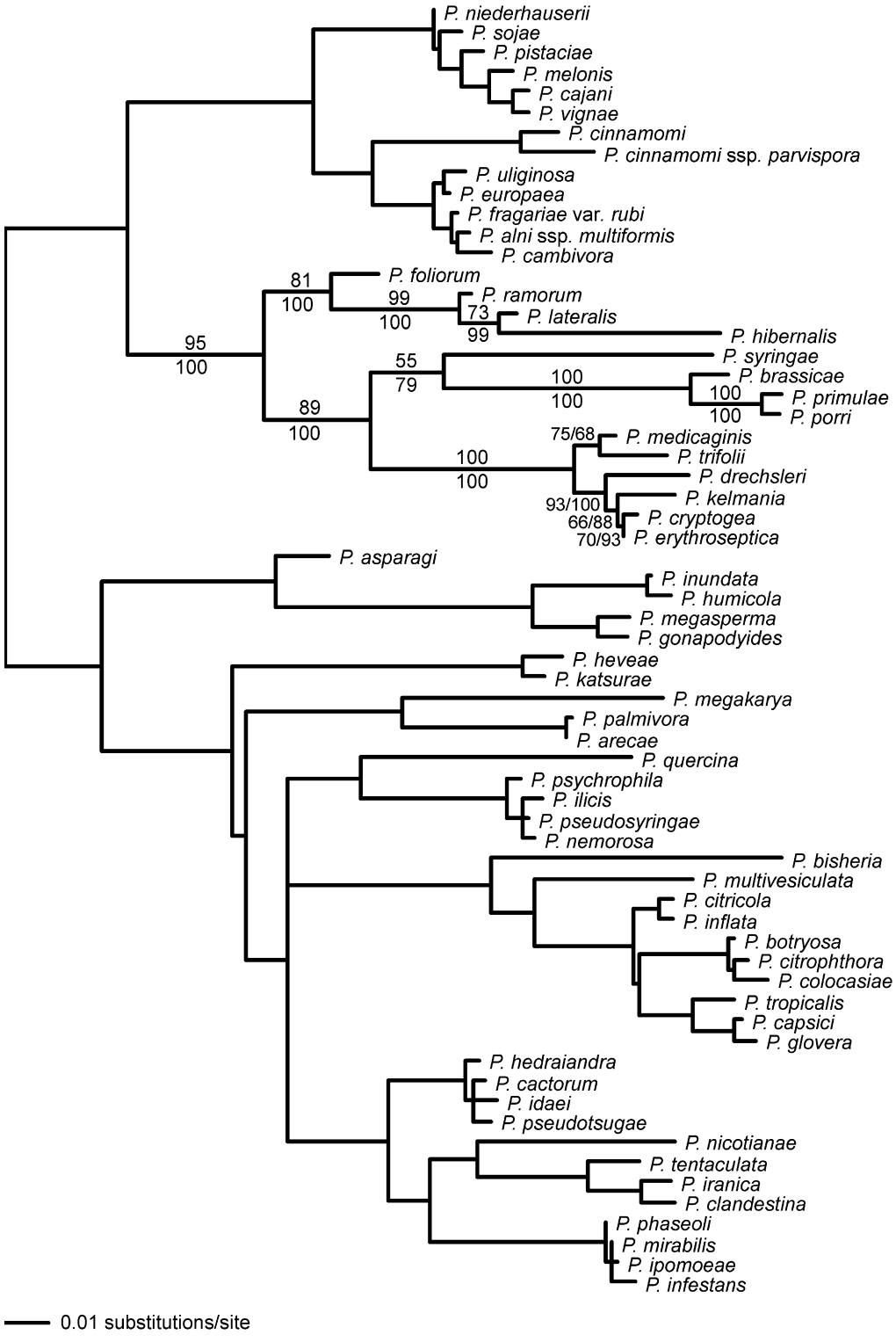

Fig 10 – Phylogenetic tree showing the relationship of *Phytophthora foliorum* within the genus *Phytophthora* based on ITS sequences. Tree topology based on ML criteria. MP BS scores and Bayesian PPs are shown above and below nodes, respectively, of clades containing the inferred closest relatives of *P. foliorum*.

is distinct from its sister taxa (*P. ramorum*, *P. lateralis*, and *P. hibernalis*). *P. foliorum* differs from *P. ramorum* in that it is homothallic and rarely if ever produces sporangia in culture. *P. foliorum* differs from *P. lateralis* in that it has semi-papillate sporangia. Unlike *P. lateralis* and *P. ramorum*, *P. foliorum* has not been found to produce chlamydospores. *P. foliorum* was discovered simultaneously in California and Tennessee during state and national surveys to detect the sudden oak death pathogen *P. ramorum*. Koch's postulates were completed to confirm pathogenicity on azalea. To date there has not been significant azalea mortality

attributed to *P. foliorum* and this species has only been found causing leaf spot symptoms on azalea.

P. ramorum is a pathogen that warrants quarantine of infected plants. A nested PCR assay designed to amplify a unique portion of the *P. ramorum* ITS is one of the assays that has been used to screen plant material. Alignment of the outer and inner *P. ramorum* nested PCR primers with the *P. foliorum* ITS sequence indicates that the corresponding sequences differ by one to two bases for each of the primers in the first and second rounds of PCR. Cross-reactivity of this newly-described species illustrates one of the risks in using DNA-based diagnostics as the sole means of detecting a specific pathogen. Short of subsequent restriction digest on the resulting nested amplicon the current *P. ramorum* nested PCR detection assay can lead to false-positives and the unnecessary destruction of nursery stock thought to be infested with *P. ramorum*. Ideally, cultures should be examined to corroborate DNA-based diagnostic finding with morphological characteristics.

AFLP genotyping indicated that the isolates of *P.* foliorum recovered thus far in the USA have limited genotypic variability (lack of polymorphic fragments). These results are expected with a homothallic species. In comparison with *P. ramorum*, *P. foliorum* generated more markers overall (11 *versus* four), all of which are unique to *P. foliorum*.

Inconsistencies in phylogenies have been found within the genus *Phytophthora* depending on the molecular region, and the analysis method used (Kroon *et al.* 2004; Martin & Tooley 2003a,b). To place *P. foliorum* in a current phylogenetic context, we reconstructed the molecular phylogenies of Kroon *et al.*, Martin & Tooley, and Cooke *et al.* with the inclusion of *P. foliorum* (Kroon *et al.* 2004; Martin & Tooley 2003a,b; Cooke *et al.* 2000). In all three phylogenies, similar topologies were observed revealing that *P. foliorum* shares a common ancestor with *P. ramorum*, *P. hibernalis* and *P. lateralis*. While the topology of the concatenated β-tubulin–EF-1α nuclear genes is similar to the coxII and ITS trees, BS support and PP clade credibility are lacking for *P. foliorum's* placement in relation to *P. hibernalis* (Fig 8). Similarly, lack of BS support was observed throughout the clade containing *P. foliorum* in the coxII phylogeny, and in the case of *P. foliorum* PPs were also low (Fig 9). The ITS phylogeny places *P. foliorum* basal to the clade containing the previously mentioned sister taxa (*P. ramorum*, *P. lateralis*, and *P. hibernalis*) with reasonable support (Fig 10). In total, these results show that *P. foliorum* is a unique species and unlikely a hybrid of two currently known species. All of the species closely related to *P. foliorum*, with exception of *P. ramorum*, are homothallic supporting the conclusion reached by other investigators that homo *versus* heterothallism is not a useful characteristic for inferring evolutionary relatedness.

The origin of *P. foliorum* is not known. At this juncture the threat it may pose to the horticultural industry or natural ecosystems is unclear and further research is needed to assess potential impacts. In particular, the origin, host range, and survivability in natural ecosystems needs to be further elucidated.

Acknowledgements

We thank Frank Martin for excellent advice and use of his data for phylogenetic analysis and Mark Windham for providing plants for inoculation tests. We would like to acknowledge Ledare Finley and Marinell Soriano for their excellent technical assistance and Anni Self from the Tennessee Department of Agriculture for managing the collection and pre-screening of nursery samples in Tennessee.

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