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Phylogenetic relationships of *Phytophthora ramorum*, *P. nemorosa*, and *P. pseudosyringae*, three species recovered from areas in California with sudden oak death

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Sudden oak death has been an emerging disease problem in coastal California and has caused significant losses in forest ecosystems in some regions of the state. The causal agent of this disease has been described as *Phytophthora ramorum* with two other less aggressive species, *P. nemorosa* and *P. pseudosyringae*, recovered from some symptomatic plants. The phylogenetic relationship of these species with other members of the genus was examined by sequence alignment of 667 bp of the mitochondrially-encoded cytochrome oxidase II gene and the nuclear encoded rDNA internal transcribed spacer region. *P. ramorum* was most closely related to *P. hibernalis* and *P. lateralis* in trees from both regions, although the specific relationship among species differed depending on the tree. In the *cox* II tree these species were on a single clade with *P. lateralis* basal to a group containing *P. ramorum* and *P. hibernalis*. On the maximum parsimony ITS tree *P. ramorum* was most closely affiliated with *P. lateralis* and in the same clade as *P. hibernalis*, but with maximum likelihood analysis *P. ramorum* was basal to a grouping of *P. hibernalis* and *P. lateralis*. While bootstrap support was strong for the grouping of these species together, it was not for determining the relationship among them. In contrast to the *cox* II tree, the clade containing these three species grouped with *P. cryptogea*, *P. drechsleri*, *P. erythroseptica*, and *P. syringae* in the ITS tree. Since the same isolates of these species were used for both the *cox* II and ITS sequence analysis, this difference in species grouping suggests either a differential rate of evolutionary divergence for these two regions, incorrect assumptions about alignment of ITS sequences, or different evolutionary histories of the regions under study. Analysis of combined *cox* II and ITS data sets gave trees where the relationships among these species were the same as for the ITS tree alone, although the results of the partition homogeneity test ($P = 0.072$) suggest caution should be used in interpretation of this data. All analyses supported a close relationship between *P. ilicis*, *P. nemorosa* and *P. pseudosyringae*, although the analysis did not clarify the evolutionary relationships among these three species. Interestingly, these three species had a unique 6 bp deletion in the *cox* II gene just before the termination codon. While there was some similarity in phylogenetic grouping of these species and morphological characteristics, this was not consistent across all comparisons in the genus. Data would suggest that *P. ramorum*, *P. nemorosa* and *P. pseudosyringae* are phylogenetically distinct new species and not the result of interspecific hybridization.

INTRODUCTION

Sudden oak death (SOD) is a disease that is currently having a significant impact in forest ecosystems of central coastal areas of California. Not only does this disease cause mortality of mature oak trees (*Quercus agrifolia*, *Q. kelloggi*, and *Q. parvula* var. *shrevei*), but also is capable of causing losses of a number of under story plants as well (Rizzo *et al.* 2002a). *Phytophthora ramorum* has been recognized as the causal agent of the disease (Werres *et al.* 2001, Rizzo *et al.* 2002a) and since it's initial observation in the mid-1990s has spread to 12 counties along the central and northern coastal region of California (Anon. 2003). It also has been identified in a localized area in southern Oregon (Goheen *et al.* 2002), where attempts at eradication by the state include clear cutting and burning in the infested areas (Kanaskie *et al.* 2002). Aside from its impact on disruption of the forest ecosystem and the practical problems associated with disease management and removal of dead trees, this pathogen has had a significant regulatory impact on the state as well. Currently there are quarantine restrictions at both the federal (Anon. 2002) and state level (Anon. 2003) affecting movement of 22 different plant species outside of infested counties. This pathogen was initially described in Europe, where it is a pathogen of ornamental plants (Werres *et al.* 2001, Moralejo & Werres 2002, Orlikowski & Szkuta 2002).

In addition to *P. ramorum*, the recently described species *P. nemorosa* and *P. pseudosyringae* also have

Table 1. Isolates of *Phytophthora* spp. used in this investigation and GenBank accession numbers for *cox* II and rDNA ITS sequences.

Species	Group	Isolate Number[a]	Host	Origin	GenBank accession nos.
P. arecae	II	[b]IMI 348342	*Cocos nucifera*	Indonesia	AY129176
P. boehmeriae	II	325^{PT}, P1257^{MC}	*Boehmeria nivia*	Papua New Guinea	AY129177
P. cactorum	I	311^{PT}	*Pseudotsuga menziesii*	Washington	AY129178
		385^{PT}, NY568^{WW}	*Malus sylvestris*	New York	AY129179
		SB2079^{GB}	*Fragaria* × *ananassa*	California	AY129180
P. capsici	II	302^{PT}(A-1)	*Capsicum annuum*	Florida	AY129181
P. cinnamomi	VI	Cn-2^{DJM} (A-2 mating type)	*Vaccinium* spp.	Florida	AY129182
P. citricola	III	Cr-4^{DJM}	*Cornus* spp.	Florida	AY129183
		SB2084	*Fragaria* × *ananassa*	California	AY129184
P. colocasiae	IV	345^{PT}, ATCC 56193, P1696^{MC}	*Colocasia esculenta*	China	AY129185
		346^{PT}, P3773^{MC}	*Colocasia esculenta*	Indonesia	AY129186
		347^{PT}, ATCC 52233, P1179^{MC}	*Colocasia esculenta*	India	AY129187
P. cryptogea	VI	[b]IMI 045168	*Lycopersicon esculentum*	New Zealand	AY129188
P. drechsleri	VI	301^{PT}, 6503^{DS}	*Capsicum* spp.	Mexico	AY129189
		[b]ATCC 46724 (ex-type)	*Beta vulgaris*	USA	AY129190
P. erythroseptica	VI	[b]366^{PT}, ATCC 36302	*Solanum tuberosum*	Ohio	AY129191
		387^{PT}, NY513^{WW}	*S. tuberosum*	California	AY129192
		388^{PT}, NY559^{WW}, IMI34684	*S. tuberosum*	Ireland	AY129193
P. fragariae fragariae	V	394^{PT}, ATCC 13973	*Fragaria* × *ananassa*	Maryland	AY129194
		398^{PT}	*F.* × *ananassa*	Oregon	AY129195
P. fragariae rubi	V	397^{PT}	*Rubus* spp.	Australia	AY129196
P. gonapodyides	IV	393^{PT}, NY353^{WW}	*Malus sylvestris*	New York	AY129197
P. heveae	II	Hv-2^{DJM}	*Theobroma cacao*	Brazil	AY129198
P. hibernalis	IV	338^{PT}, ATCC 56353, P3822^{MC}	*Citrus sinensus*	Australia	AY129199, AY369369[d]
		379^{PT}, ATCC 64708, CBS 522.77	*Aquilegia vulgaris*	New Zealand	AY129201, AY369370[d]
		380^{PT}, ATCC 60352, CBS 270.31	*Citrus sinensus*	Portugal	AY129200, AY369375[d]
P. ilicis	IV	343^{PT}, P6099^{MC}, 771^{PH}	*Ilex aquifolium*	Oregon	AY129202
		344^{PT}, ATCC 56615, P3939^{MC}	*I. aquifolium*	Canada	AY129203
P. infestans	IV	176^{PT}, 915^{KD} (A-2)	*Solanum tuberosum*	Pennsylvania	AY129204
		180^{PT}, WW-IX^{KD}(A-1)	*S. tuberosum*	Washington	AY129205
		580^{PT}	*S. demissum*	Mexico	AY129206
		[c]West Virginia 4		West Virginia	NC002387
P. lateralis	V	[b]IMI 040503 (ex-type)	*Chamaecyparis lawsoniana*	USA	AT129207
		452^{PT}, 365^{EH}	*C. lawsoniana*	California	AY369360
		455^{PT}, T4P3^{EH}	*C. lawsoniana*	Medford, OR	AY369361
P. megakarya	II	327^{PT}, P132^{CB}	*Theobroma cacao*	Nigeria	AY129208
		328^{PT}, P184^{CB}	*T. cacao*	Cameroon	AY129209
P. megasperma	V	309^{PT}, 336^{PH}	*Pseudotsuga menziesii*	Washington	AY129210
		335^{PT}, 63^{PH}, 261S-1^{WW}	*Prunus* spp.	California 2	AY129212
		[b]IMI 133317	*Malus sylvestris*	Australia	AY129211
		[c]695T		California 1	L04457
P. mirabilis	IV	340^{PT}, ATCC 64070, P3007^{MC}	*Mirabilis jalapa*	Mexico 1	AY129213
		342^{PT}, ATCC 64073, P3010^{MC}	*M. jalapa*	Mexico 2	AY129214
P. nemorosa	IV	P-13^{EH} 482^{PT} ex-type	*Lithocarpus densiflorus*	California	AY429504
		2052.1^{EH} 483^{PT}	*L. densiflorus*	Oregon	AY429505
P. nicotianae	II	Pn-17^{DJM} (A-1)	*Citrus* spp.	Florida	AY129215
		332^{PT}	*Nicotiana tabacum*	Australia	AY129216
P. palmivora	II	329^{PT}, P131^{CB}	*Theobroma cacao*	Nigeria	AY129217
		Pl-5^{DJM}, P626^{UCR}	*T. cacao*	Brazil	AY129218
		Pl-10^{DJM}	*T. cacao*	Costa Rica	AY129219
		Pl-14^{DJM}	*Citrus* sp.	Florida	AY129220
P. phaseoli	IV	330^{PT}	*Phaseolus lunatus*	Maryland	AY129221
P. pseudosyringae	III	PSEU16^{TJ} 484^{PT}, NFV-BU97-15	*Fagus sylvatica*	Germany	AY429506
		P96^{EH} 485^{PT}	*Umbellularia californica*	Contra Costa Co., CA	AY429507
		470^{PT}, P193907B^{CBL}	*Manzanita* spp.	Royal Oaks, CA	AY369357, AY369371[d]

Table 1. (*Cont.*)

Species	Group	Isolate Number[a]	Host	Origin	GenBank accession nos.
		471^{PT}, 1168699^{CBL}	*Umbellularia californica*	Napa, CA	AY369358, AY369372[d]
		472^{PT}, 1168676^{CBL}	*U. californica*	Calistoga, CA	AY369373[d]
		473^{PT}, P110361^{CBL}	*U. californica*	Yountville, CA	AY369359, AY369374[d]
P. pseudotsugae	I	308^{PT}, H270^{PH}	*Pseudotsuga menziesii*	Oregon	AY129222
P. ramorum	IV	CBS101553, BBA 9/95^{SW}, Prg-2^{PT} (ex-type)	*Rhododendron catawbiense*	Germany	AY369365
		016^{DR}	*Quercus agrifolia*	Marin County, CA	AY369362
		013^{DR}	*Lithocarpus densiflorus*	Marin County, CA	AY369363
		Coen^{TT}	*Rhododendron* sp.	Santa Cruz, CA	AY369364
		0217^{DR}	*Rhododendron* sp. cv Gomer	Santa Cruz, CA	AY369368[d]
P. sojae	V	312^{PT}, ATCC 48068	*Glycine max*	Wisconsin	AY129223
P. syringae	III	442^{PT}, [b]IMI 296829	*Rubus idaeus*	Scotland	AY129224
		468^{PT}, Kalmia-1^{RL}	*Kalmia latifolia*	Oregon	AY369366
		469^{PT}, Kalmia-2^{RL}	*Kalmia latifolia*	Oregon	AY369367

[a] CB, Clive Brasier; CBL, Cheryl Blomquist; MC, Mike Coffey; PH, Phil Hamm; EH, E. Hansen; DJM, Dave Mitchell; DS, Dave Shaw; PT, Paul Tooley; UCR, University of California at Riverside; WW, Wayne Wilcox; DR, Dave Rizzo; TT, T. Tidwell; TJ, T. Jung; RL, Robert Linderman; and SW, Sabine Werres.

[b] Isolates included in the rDNA ITS analysis of Cooke *et al.* (2000).

[c] Sequences obtained from GenBank.

[d] Sequence of the ITS region of the rDNA.

been recovered from symptomatic tissue collected in California and Oregon (Rizzo *et al.* 2002a, Hansen *et al.* 2003, C. Blomquist, pers. comm.). The disease symptoms caused by these pathogens are indistinguishable from those caused by *P. ramorum*, and the three pathogens share a similar host range and geographic range of recovery. *P. nemorosa* is commonly recovered from leaf spots and twig cankers, but in general is not associated with lethal trunk cankers on mature trees. In cases where it has caused lethal cankers, *P. nemorosa* is usually associated with single killed trees rather than patches of dead trees that can be observed with infection by *P. ramorum* (Hansen *et al.* 2003). Thus far, *P. pseudosyringae* has been observed primarily as a leaf and twig pathogen (C. Blomquist, pers. comm.), which is in contrast to its description as a root and collar rot pathogen of trees in the original species account (Jung *et al.* 2003).

The recent identification of these pathogens associated with diseased *Quercus* spp. and other plant species in the coastal California forest ecosystem raises the question of where these pathogens came from and how are they related to other previously described species in the genus. The genus *Phytophthora* contains approximately 67 described species that occupy a variety of terrestrial and aquatic ecological habitats and are capable of causing disease symptoms ranging from foliar blight to root and crown rot (Erwin & Ribeiro 1996). Morphological criteria such as sporangial structure, antheridial attachment, chlamydospore formation and breeding system have been used for species classification (Waterhouse 1963, Stamps *et al.* 1990). However, phylogenetic relationships do not always parallel morphological similarity (Brasier 1991, Brasier & Hansen 1992, Cooke *et al.* 2000, Förster, Cummings & Coffee 2000, Martin & Tooley 2003). In the most complete phylogenetic examination of the genus thus far, Cooke *et al.* (2000) reported an ITS-based molecular phylogeny confirming that the six morphological groupings of Waterhouse (1963) are not reflective of phylogenetic relationships. Similar conclusions were drawn by Förster *et al.* (2000) using sequence analysis of the ITS-1 region and by Martin & Tooley (2003) using the cytochrome c oxidase subunit II (*cox* II) and subunit I gene.

The *cox* II region is mitochondrially encoded, and in contrast to the ITS region, represents a coding region rather than intergenic region. This gene has been used to evaluate peronosporomycete phylogeny (Hudspeth, Nadler & Hudspeth 2000) as well as phylogenetic groupings of *Pythium* (Martin 2000) and *Phytophthora* species (Martin & Tooley 2003). Analysis of 568 bp of the *cox* II gene for 27 species of the genus *Phytophthora* revealed seven major clades with nine species unaffiliated with a specific clade. Results for a smaller number of species and isolates for the *cox* I gene tree were similar. The *cox* II gene tree paralleled the ITS tree of Cooke *et al.* (2000) in many respects with a major exception observed for species relationships with the *P. cryptogea*, *P. drechsleri*, and *P. erythroseptica* clade (these species grouped together in both analyses). In the ITS tree *P. lateralis* and *P. syringae* were basal to this clade while in the *cox* II gene tree they were unaffiliated with these species. Since the same isolates were used in both analyses, these results suggested that either assumptions about ITS alignment were incorrect or there are different rates of evolutionary divergence for these two regions. The results of a partition homogeneity test suggested that the *cox* II and ITS data sets could not be concatenated for combined analysis.

The phylogenetic placement of *P. ramorum* within the genus *Phytophthora* is of great interest due to the recent emergence of this species as an ornamental pathogen in Europe (Werres *et al.* 2001, Moralejo & Werres 2002, Orlikowski & Szkuta 2002) and a devastating forest pathogen in California with a wide host range (Rizzo *et al.* 2002a, b, Garbellotto *et al.* 2003). There is speculation that *P. ramorum* could have arisen in California as a natural hybrid or variant of an existing *Phytophthora* species, or been introduced from another part of the world such as Asia. That *P. ramorum* attacks both trees as well as ornamental plant species makes attempts to track its origins more complex. Large numbers of rhododendrons and other *P. ramorum* host species are imported into the USA each year from Asia and other parts of the world, making trade a strong potential source of introduction. Knowledge of the genetic relationships between *P. ramorum* and other *Phytophthora* species could thus shed light on its potential origin.

In the original species description, Werres *et al.* (2001) reported that *P. ramorum* was most closely related to *P. lateralis* based on sequence analysis of the ITS region of the rDNA; these species differed by three and eight nucleotides in the ITS1 and ITS2 regions, respectively. In phylograms based on both ITS1 and ITS2 regions, *P. ramorum* and *P. lateralis* were also associated with *P. cryptogea* and *P. drechsleri*. These species relationships were confirmed for California isolates of *P. ramorum* by Rizzo *et al.* (2002a). Likewise, Hansen *et al.* (2003) and Jung *et al.* (2003) reported a close relationship of *P. nemorosa* and *P. pseudosyringae*, respectively, with *P. ilicis* based on ITS trees. The objective of this current investigation was to characterize the evolutionary relationship among *P. ramorum*, *P. nemorosa* and *P. pseudosyringae* with other species in the genus using *cox* II gene sequences and the nuclear encoded ITS region of the rDNA. These results will be compared with morphological and etiological observations in an effort to better understand the potential origin of these species and their evolutionary relationship with other species in the genus.

MATERIALS AND METHODS

Phytophthora *cultures*

Cultures used in this study are listed in Table 1. Isolates of *Phytophthora arecae*, *P. cryptogea*, *P. drechsleri*, *P. erythroseptica*, *P. lateralis*, *P. megasperma*, and *P. syringae* included in this study were also used in the ITS rDNA phylogenetic study of Cooke *et al.* (2000). Isolate P-13 is an ex-type culture for *P. nemorosa* (Hansen *et al.* 2003), while isolate PSEU16 is from the species description of *P. pseudosyringae* by Jung *et al.* (2003). Cultures were grown on Rye A medium (Caten & Jinks 1968) at 20 °C in darkness and maintained in liquid nitrogen for long-term storage (Tooley 1988). Pathogenicity was assessed on detached leaves of English holly (*Ilex aquifolium*) as described by Davidson *et al.* (2002) and Buddenhagen & Young (1957).

For morphological analysis of *P. nemorosa* and *P. pseudosyringae*, isolates were grown on 10 and 15% clarified, carbonated V8-juice agar made by adding 1 g $CaCO_3$ per 100 ml V-8 juice (Campbell Soup, Camden, NJ), centrifuging at 7000 rpm for 10 min, filtering the supernatant through Whatman no. 4 filter paper, bringing to 1 l and amending with 30 μg β-sitosterol ml^{-1}. For studies of sporangial morphology and to observe hyphal swellings, 6 mm diameter plugs of 10% clarified V-8 agar colonized by the pathogens were placed into non-sterile stream water (filtered through 5 μm pore size nuclepore polycarbonate filters) (Whatman, Clifton, NJ) in the dark at 18 °. For observation of sexual structures, grass blades were collected, boiled for 10 min, placed in sterile water in 60 mm-diameter Petri dishes, seeded with small agar plugs and observed over a several week period (Martin 1992). For temperature studies, 6-mm diam agar plugs were removed from colony margins and used to seed 60 mm diam Petri dishes of 10% clarified V-8 juice agar (7 ml per plate). Plates were placed on a thermogradient plate (an aluminum plate with a hot water bath at one end and a cooling bath at the other) at 2 ° intervals from 10–28 ° in darkness. Temperatures at the agar surface were monitored using a Type J thermocouple probe connected to a Minitrend model V5 paperless recorder (Honeywell, Hampshire, UK).

DNA amplification and sequence analysis

Genomic DNA was extracted by the method of Goodwin *et al.* (1992) or a boiling miniprep procedure (Martin & Semer 1997). DNA concentrations were determined spectrophotometrically and/or by quantitation on agarose gels stained with ethidium bromide in comparison with commercially obtained standards. Templates of the *cox* II gene were amplified for sequencing by PCR using previously described methods (Martin & Tooley 2003). Templates for rDNA ITS sequencing were amplified using primers ITS 1 and 4 and standard conditions for amplification (White *et al.* 1990).

Sequencing of the *cox* II gene was done by the DNA Sequencing Laboratory of the Interdisciplinary Center for Biotechnological Research of the University of Florida, Gainesville, using ABI 373a automated sequencers (Applied Biosystems, Foster City, CA). Templates were sequenced in both directions with the primers previously described (Martin & Tooley 2003). Sequencing in both directions of the rDNA ITS region for *P. ramorum*, *P. hibernalis*, and *P. pseudosyringae* was performed using amplification primers with an Applied Biosystems model 310 sequencer and BigDye cycle sequencing kit at the USDA-ARS Foreign Disease-Weed Science Research Unit, Fort Detrick, MD. Primers were synthesized by Invitrogen (Carlsbad, CA).

Data analysis

Overlapping sequences from each sequencing primer and the sequences from opposite strands were aligned using the computer program Omiga 1.1 (Accelrys, Manassas, VA). If any regions of sequence ambiguity were observed, the original output files of complementary strands from the ABI 373a sequencer were compared for correction and the sequencing reactions were repeated if necessary. 667 bp was used for phylogenetic analysis with the *cox* II gene, which included bases 94 to 761 of the gene. Phylogenetic analyses were done with PAUP ver. 4.0b10. Phylogenetic relationships among *Phytophthora* spp. using DNA sequence data was inferred by maximum parsimony (MP) analysis with a heuristic tree search. Heuristic searches were performed with MULPARS on, steepest decent option off, random addition of sequences (1000 replicates) and TBR branch swapping. To determine support for the various clades of the trees, the analysis described above were bootstrapped with 1000 replicates with the same conditions noted above used with the exception that there were 10 replicates for the random addition of samples. Maximum likelihood (ML) analysis with a heuristic tree search was done with the number of substitution types set at 2, transition/transversion ratio empirically determined from the data, a molecular clock was not enforced with the balance of the settings corresponding to the HKY85 model. Data for an isolate of *Pythium aphanidermatum* from Mexico (1987-61) and *P. ultimum* from Florida (110-2) also was included in the analysis using *cox* II data previously reported (Martin 2000; GenBank accession nos. AF196579 and AF196629, respectively). Phylogenetic inferences based on ITS and 5.8S rDNA sequence data were based on the results of Cooke *et al.* (2000) with the alignments retrieved from TreeBASE (accession no. M751). Additional sequence data for *P. ramorum*, *P. hibernalis*, *P. nemorosa* and *P. pseudosyringae* were determined in this study. The same region for *Pythium aphanidermatum* (GenBank accession no. AF271227) was included in the analysis as an outgroup. The relative alignment for the *Phytophthora* sequences remained the same with a few modifications to improve alignment and with gaps introduced across all species of this genus to accommodate the alignment with sequences of *Pythium aphanidermatum*. DNA sequence data obtained in this study has been deposited in GenBank and the results of this analysis have been deposited in TreeBASE (accession no. 5985 SN1570).

RESULTS

Morphological analysis of Phytophthora nemorosa *and* P. pseudosyringae

The four *Phytophthora pseudosyringae* isolates from California (470, 471, 472 and 473) were compared with the *P. nemorosa* ex-type isolate P-13 (Hansen *et al.* 2003) and isolate PSEU16 of *P. pseudosyringae* (Jung *et al.* 2003). All *P. pseudosyringae* isolates were morphologically indistinguishable from one another, and clearly more similar to *P. pseudosyringae* isolate PSEU16 than to *P. nemorosa*. Paragynous antheridia were observed, sporangial length : breadth ratios were closer to that described for *P. pseudosyringae* (1.61) than for *P. nemorosa* (1.32), catenulate hyphal swellings were present, there was a distinctly petaloid growth morphology on V8 agar, and a temperature optimum of 20 °. Three isolates (470, 472 and 473) infected holly leaves but lesions barely extended beyond the margins of the agar plug used for inoculation. One isolate (471) showed moderate pathogenicity on English holly, producing lesions roughly half the size of those produced by *P. ilicis* isolate 344. The temperature-linear growth response for P-13, the type culture of *P. nemorosa*, indicated a temperature optimum of 18–20 ° with a 72% reduction in growth at 24 °.

Sequence analysis

Intraspecific variation in ITS sequence data was not observed for isolates of *Phytophthora hibernalis* or *P. nemorosa* and *P. nemorosa* had the same sequence as reported for the type (AY332651; Hansen *et al.* 2003). While only a single isolate of *P. ramorum* was sequenced (isolate 0217), the sequence was identical to the ITS I and II sequences deposited in GenBank for the ex-type culture CBS 101553 (AF429767 and AF429773, respectively), three other isolates from Europe (AF429774, AF429768; AF429771, AF429772; AF429769, AF429770), and one isolate from California (AY038050). The ITS sequences for *P. pseudosyringae* isolates 470, 471, 472 and 473 were all identical and the same as reported for the ex-type isolate of *P. pseudosyringae* (AY230190; Jung *et al.* 2003). The estimated transition/transversion ratio for maximum likelihood analysis of the ITS region was determined to be 1.238. Intraspecific sequence variation in the *cox* II gene was not observed for *P. ramorum*, *P. nemorosa* or *P. pseudosyringae*, although limited variation was observed for the additional isolates of *P. syringae* and *P. lateralis* that were examined. A length mutation was observed in the *cox* II gene for *P. ilicis*, *P. nemorosa* and *P. pseudosyringae* relative to all other species examined; there was a 6 bp deletion from base 762–767 immediately prior to the termination codon. The estimated transition/transversion ratio for maximum likelihood analysis of the *cox* II gene was determined to be 0.6723.

Phylogenetic relationships based on cox *II DNA sequences*

A heuristic search of the *cox* II data generated four most parsimonious trees with equal tree statistics; the only variation observed among them were minor differences in the relationship between *Phytophthora arecea*

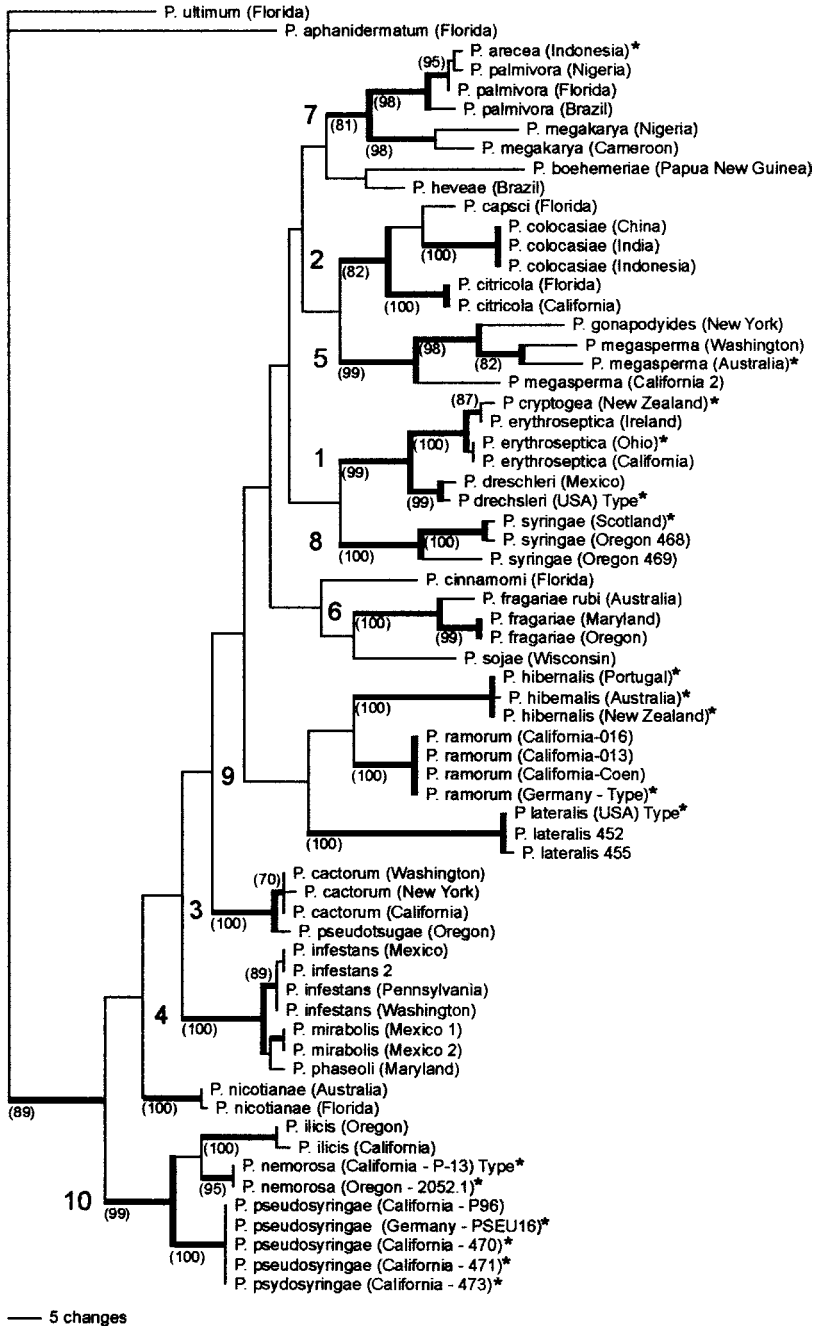

Fig. 1. One of four most parsimonious trees using *cox* II DNA sequence data based on maximum parsimony inferred by a heuristic tree search. Thick branch lines represent clades that are supported by bootstrap analysis (1000 replications) with the numbers in brackets at the nodes the percentage of the trees above 70% that support the observed topography. Numbers to the left of the clades correspond to the clade numbering previously reported (Martin & Tooley 2003). Of the 667 total characters, 464 were constant, 36 were variable and parsimony uninformative and 167 were parsimony informative. Tree length = 638, consistency index = 0.458, homoplasy index = 0.542, retention index = 0.781, rescaled consistency index = 0.357. Isolates marked with an (*) are the same cultures that were used in this submission to infer the ITS-based phylogenetic tree in Fig. 2. 'Type' = ex-type isolates.

and *P. palmivora*, as well as for *P. phaseoli* and *P. infestans*. *P. ramorum* grouped with *P. hibernalis* (62% bootstrap support, Fig. 1) with this clade affiliated with *P. lateralis*. Likewise, *P. nemorosa* and *P. pseudosyringae* grouped with *P. ilicis* (bootstrap support 99%) although the evolutionary relationship among these species could not be clarified in the bootstrap analysis. The same grouping and similar bootstrap support was observed for these species with maximum likelihood analysis (data not shown). The topology of the 50% majority rule consensus tree based on bootstrap analysis (1000 replicates) of maximum parsimony for the *cox* II gene generated from 667 bp of the *cox* II gene were almost the same as previously

reported for 568 bp of the *cox* II gene (Martin & Tooley 2003) with the difference that *P. megakarya* grouped with *P. palmivora* and *P. arecae* (data not shown). *P. ramorum* grouped with *P. hibernalis* with this clade affiliated with *P. lateralis*, although there was weak bootstrap support for this. The maximum likelihood trees differed from previous reports (Martin & Tooley 2003) in that *P. cinnamomi* and *P. sojae* grouped with *P. fragariae*, although there was low bootstrap support for this (55 and 53%, respectively).

Phylogenetic relationships based on ITS rDNA sequences

A heuristic search of the ITS data generated two most parsimonious trees with only minor differences between them, with *Phytophthora ramorum* grouping with *P. lateralis* and this clade affiliated with *P. hibernalis* (Fig. 2). While bootstrap analysis supported the grouping of these three species in the same clade (100%), it did not clarify which species was most closely related to *P. ramorum* (there was a 51% bootstrap support for the grouping of *P. lateralis* and *P. ramorum* on the same clade). The *P. nemorosa* and *P. pseudosyringae* isolates grouped with *P. ilicis* (bootstrap support 100%) although the evolutionary relationship among these species could not be clarified in the bootstrap analysis. The topology of the 50% majority rule consensus tree based on bootstrap analysis (1000 replicates) of maximum parsimony for the ITS region was the same as previously reported (Martin & Tooley 2003) with *P. hibernalis*, *P. lateralis*, and *P. ramorum* grouping together and affiliated with the *P. cryptogea*, *P. erythroseptica*, *P. drechsleri*, and *P. syringae* clade (data not shown). The maximum likelihood tree was the same as the maximum parsimony tree with the exception that *P. lateralis* grouped with *P. hibernalis* with *P. ramorum* basal to this clade, although there was not strong bootstrap support for this (64%).

Combined data set

Data sets for *cox* II and the ITS region were combined by concatenating sequences for isolates and species to one another. In cases where the isolates were not the same, the assumption was made that all isolates were correctly identified and represented the same species for each data set. If multiple isolates of a single species were represented in the *cox* II data set, the ITS sequence for that species was added to the *cox* II data for each isolate. A partition-homogeneity test with the data for *cox* II and the ITS region was run in PAUP with 1000 replicates using random addition of sequences (10 replicates); the results of this test were low, but not significant ($P = 0.072$). The species groupings in the ML tree using the concatenated data set was similar to the MP tree for the ITS data set alone with the exception of ancestral relationship among the different

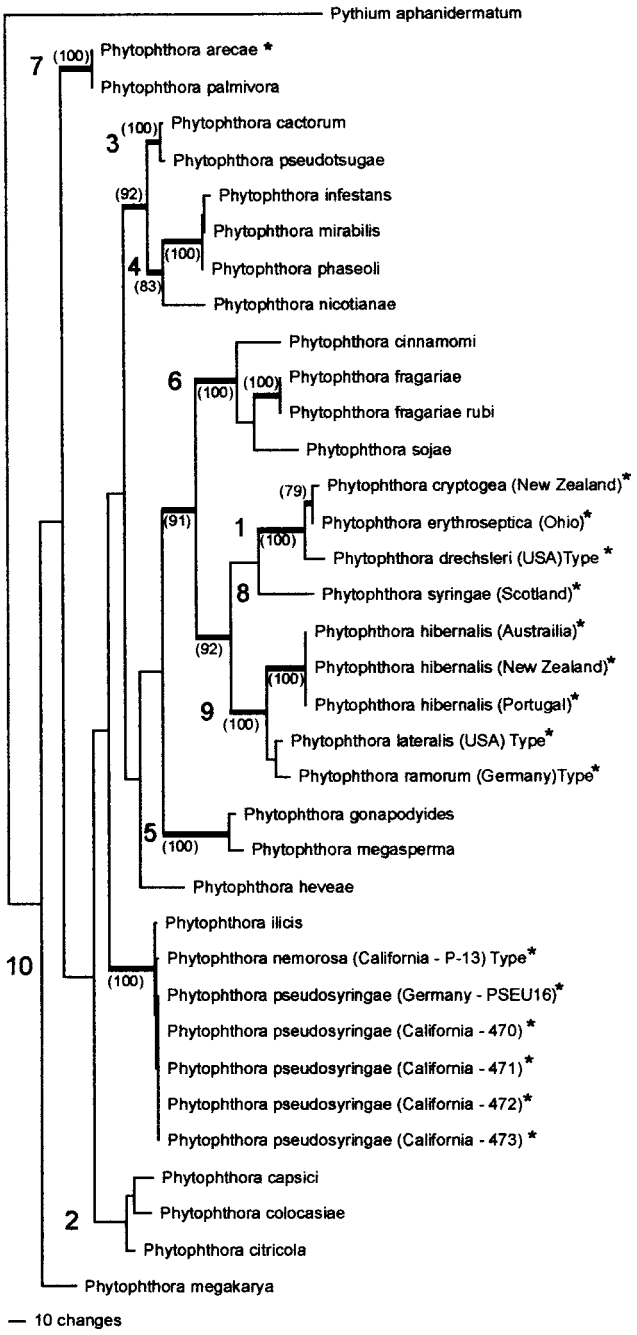

Fig. 2. One of two most parsimonious tree obtained using ITS DNA sequence data based on maximum parsimony inferred by a heuristic tree search. Thick branch lines represent clades that were supported by bootstrap analysis (1000 replications) with the numbers in brackets at the nodes the percentage of the trees above 70% that support the observed topography. Numbers to the left of the clades correspond to the clade numbering in Fig. 1. Of the 900 total characters, 459 were constant, 188 were variable and parsimony uninformative and 253 were parsimony informative. Tree length = 959, consistency index = 0.648, homoplasy index = 0.352, retention index = 0.779. Isolates marked with an (*) are the same cultures that were used in this submission to infer the *cox* II-based phylogenetic tree in Fig. 1. 'Type' = ex-type isolates.

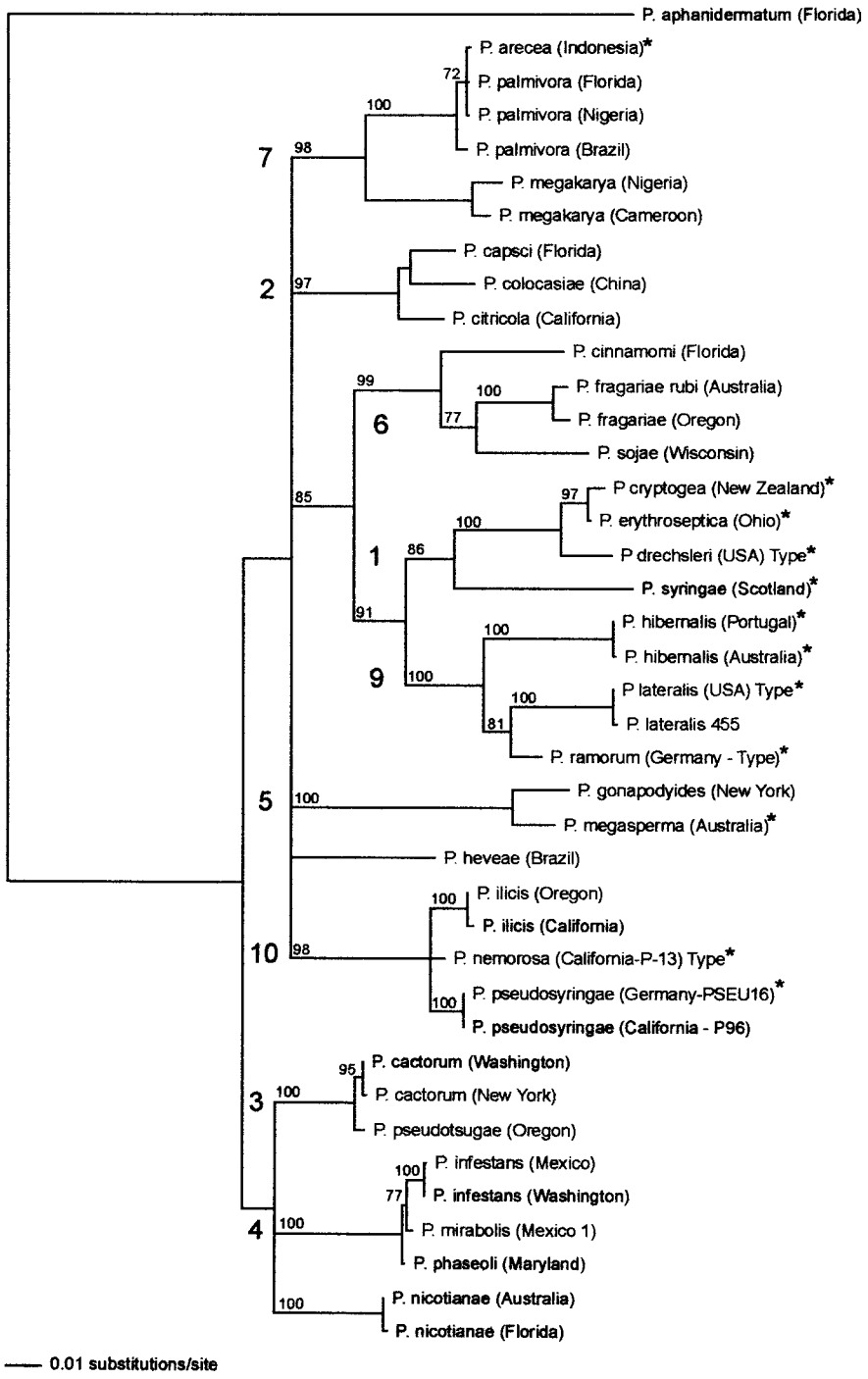

Fig. 3. Maximum likelihood phylogenetic tree obtained using concatenated *cox* II and ITS DNA sequence data inferred by a heuristic tree search ($-\ln L = 16\,359$). The numbers at the nodes are the percentage of the trees above 70% that were supported by bootstrap analysis (100 replications). Numbers to the left of the clades correspond to the clade numbering in Fig. 1. Isolates marked with an (*) are the same cultures that were used for both *cox* II and ITS analysis. 'Type' = ex-type isolates.

clades (Fig. 3). There was strong bootstrap support for grouping *Phytophthora lateralis*, *P. ramorum*, and *P. hibernalis* on the same clade (100%) and for grouping *P. ramorum* with *P. lateralis* (81%). The MP tree for the concatenated data set exhibited a similar grouping of the numbered clades but with different ancestral relationships (Fig. 4). Bootstrap analysis supported these groupings, but was low (65%) for the grouping of *P. lateralis* and *P. ramorum* on the same clade.

DISCUSSION

The *cox* II and ITS sequence data support a close relationship among *Phytophthora ramorum*, *P. lateralis*, and *P. hibernalis*. However, the specific relationship among these species differs depending on the molecular region examined. For the mitochondrially encoded *cox* II gene *P. ramorum* grouped with *P. hibernalis*, but the bootstrap support for this relationship was not

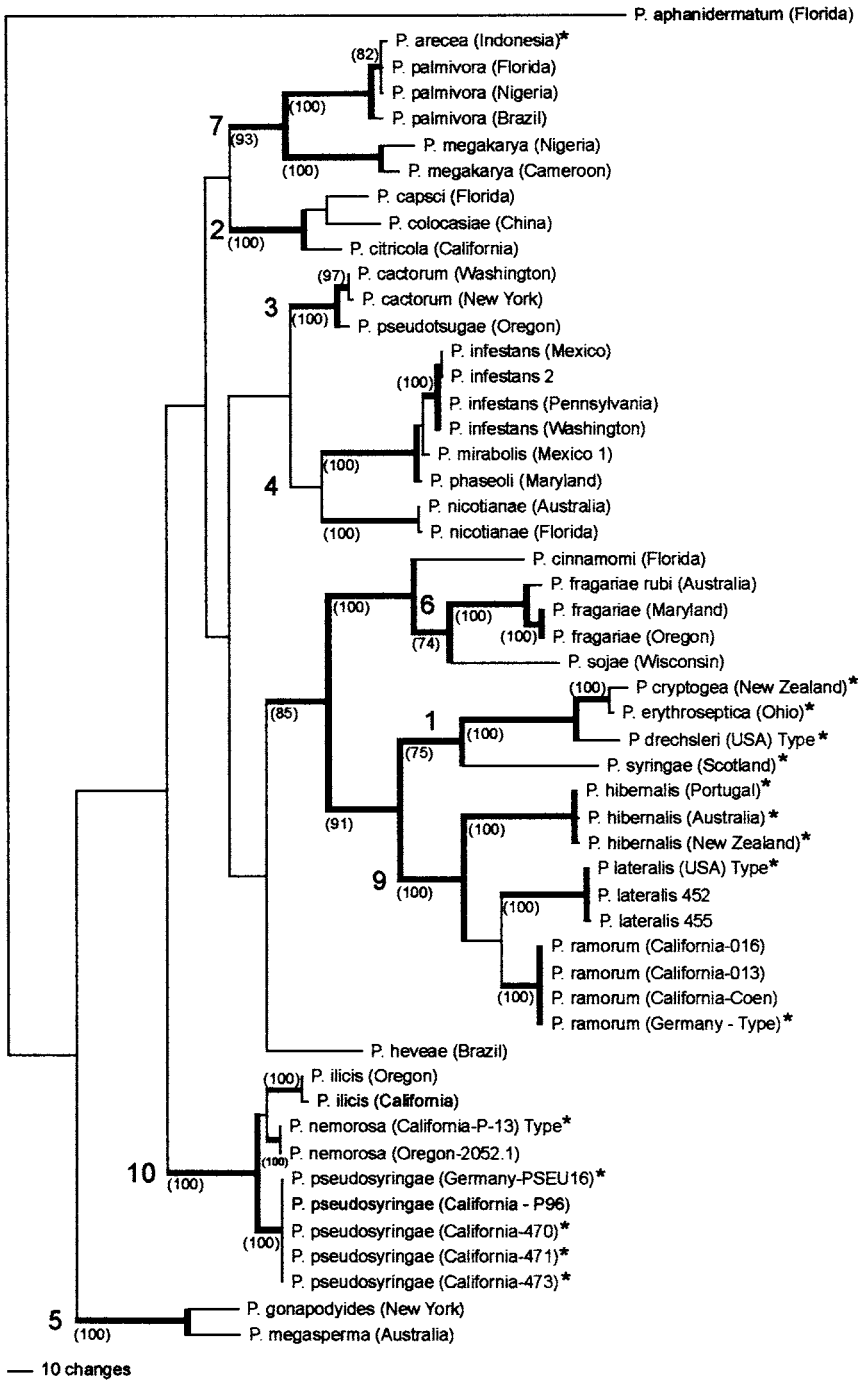

Fig. 4. One of 18 most parsimonious trees obtained using concatenated *cox* II and ITS DNA sequence data based on maximum parsimony inferred by a heuristic tree search. Thick branch lines represent clades that were supported by bootstrap analysis (1000 replications) with the numbers in brackets at the nodes the percentage of the trees above 70% that support the observed topography. Numbers to the left of the clades correspond to the clade numbering in Fig. 1. Of the 1567 total characters, 935 were constant, 208 were variable and parsimony uninformative and 424 were parsimony informative. Tree length = 1522, consistency index = 0.587, homoplasy index = 0.413, retention index = 0.822. Isolates marked with an (*) are the same cultures that were used for both *cox* II and ITS analysis. 'Type' = ex-type isolates.

strong. In contrast, in the ITS tree *P. ramorum* grouped with *P. lateralis* in the most parsimonious tree while *P. hibernalis* grouped with *P. lateralis* in the same clade as *P. ramorum* with maximum likelihood analysis. This close relationship between *P. ramorum* and *P. lateralis* was reported previously based on ITS sequence analysis (Werres *et al.* 2000, Rizzo *et al.* 2002a), however, *P. hibernalis* was not included in these studies. Analysis of the combined data sets yielded species groupings similar to the ITS analysis, with fairly strong bootstrap support in the maximum likelihood tree for grouping *P. ramorum* with *P. lateralis* (82%). One difference in this analysis compared to previous analysis of combined datasets (Martin & Tooley 2003)

was that the partition-homogeneity test was significant ($P=0.002$) in the previous report while in the current analysis it was low but not significant ($P=0.072$). This could be due to the current analysis excluding some of the outgroups used in the previous analysis as well as including an additional 99 bp of *cox* II gene sequences. Given the above results and the low value for the partition-homogeneity test, additional analysis of other gene regions will be needed to clarify the phylogenetic relationships among these three species.

Similar results were also observed for *P. ilicis*, *P. nemorosa*, and *P. pseudosyringae*; there was strong bootstrap support for grouping these species together but the analysis was not able to clarify the relationships among them. The close affinity of *P. nemorosa* and *P. pseudosyringae* with *P. ilicis* using ITS data had been previously described (Hansen *et al*. 2003, Jung *et al*. 2003), this current study confirms these results using the mitochondrially encoded *cox* II gene. An additional molecular characteristic that is shared among *P. ilicis*, *P. nemorosa*, and *P. pseudosyringae* and differentiates them from the rest of the species in the genus examined thus far is a 6 bp deletion in the *cox* II gene just before the termination codon.

To a limited degree, morphological and etiological comparisons for *P. ramorum*, *P. lateralis*, and *P. hibernalis* paralleled phylogenetic groupings. For example, both *P. ramorum* and *P. hibernalis* are in Waterhouse morphological group IV and have semi-papillate, caducous sporangia, which is consistent with species where aerial dispersal of inoculum is an important part of the pathogen's life-cycle (both these species are primarily pathogens of aerial portions of plants; Erwin & Ribeiro 1996, Werres *et al*. 2001). In contrast, *P. lateralis* is in morphological group V and has non-papillate, non-caducous sporangia that is consistent with species that are root infecting pathogens and do not rely on aerial dispersal of inoculum. While *P. hibernalis* does not produce chalmydospores, both *P. lateralis* (lateral on hyphae) and *P. ramorum* (intercalary, terminal, occasionally lateral) do (Erwin & Ribeiro 1996, Werres *et al*. 2001). Although *P. hibernalis* and *P. lateralis* are homothallic, *P. ramorum* is heterothallic (Werres & Zielke 2003). Linear growth of all three species is favoured by cooler temperatures with an optimum of 20 ° for *P. lateralis* (Erwin & Ribeiro 1996) and *P. ramorum* (Werres *et al*. 2001) and 15 ° for *P. hibernalis* (Erwin & Ribeiro 1996). Diseases caused by *P. hibernalis* and *P. lateralis* are also favoured by cooler temperatures (Erwin & Ribeiro 1996), while the range of recovery of *P. ramorum* in California is in the cooler coastal regions of central and northern California (Rizzo *et al*. 2002a). Host ranges of these pathogens differ, however, with *P. hibernalis* primarily a pathogen of citrus and *P. lateralis* on Port Orford cedar (*Chamaecyparis lawsoniana*) while *P. ramorum* has a broader host range of at least 11 plant families and 18 plant species in California (Garbelotto *et al*. 2003) and several ornamentals in Europe (Werres *et al*. 2001, Moralejo & Werres 2002, Orlikowski & Szkuta 2002).

Phylogenetic analysis of the ITS and *cox* II data indicate a relationship between *P. nemorosa*, *P. pseudosyringae* and *P. ilicis* that parallels the morphological similarity among these species (Hansen *et al*. 2003, Jung *et al*. 2003). They all produce caducous semi-papillate sporangia and are homothallic, but *P. ilicis* and *P. nemorosa* have amphigynous antheridia while *P. pseudosyringae* has predominantly paragynous antheridia. Chlamydospores are rarely formed by *P. ilicis* and are not produced by *P. nemorosa* or *P. pseudosyringae*. Morphologically, *P. nemorosa* is distinguished from *P. ilicis* by having larger oogonia and oospores and a faintly petaloid rather than strongly patterned colony morphology on PDA. Both species cause foliar disease and are favoured by cooler temperatures. While *P. ilicis* has an optimum linear growth at 20 ° *P. nemorosa* has a reported optimum of 15 ° with little growth above 20 ° (Hansen *et al*. 2003), although in this current study the type culture had an optimum growth rate at 18–20 ° with a 72 % reduction in growth at 24 °. However, these species differ in host range, with *P. ilicis* causing a leaf and twig blight on English holly (*Ilex aquifolium*) in North America while *P. nemorosa* does not. *P. nemorosa* has been recovered from necrotic leaves of *Umbellularia californica* (California bay), *Arctostaphylos* sp. (manzanita) and *Sequoia sempervirens* (coastal redwood) and from cankers on *Lithocarpus densiflora* (tan oak) and *Quercus agrifolia* (coastal live oak) from areas of California and Oregon (Rizzo *et al*. 2002a, Hansen *et al*. 2003). Like *P. nemorosa*, *P. pseudosyringae* also has larger oogonia and oospores than *P. ilicis*, but does have a temperature growth optimum similar to *P. ilicis* at 20 °. It also has catenulate hyphal swellings in water culture that differentiates it from the other two species. Jung *et al*. (2003) reported variation in pathogenicity of *P. pseudosyringae* to English holly with isolates from oak and some from beech highly pathogenic while isolates from alder and one from beech almost non-pathogenic. Isolates of *P. pseudosyringae* we studied also showed variable pathogenicity to English holly with some barely pathogenic and one isolate (471) moderately pathogenic. *P. nemorosa*, in contrast, is non-pathogenic on English holly (Davidson *et al*. 2002, Hansen *et al*. 2003). In Europe, *P. pseudosyringae* has been recovered as a soil pathogen causing root and collar rot of deciduous trees and not as a foliar pathogen as observed in California, although this may be because of the paucity of data about *P. pseudosyringae* due to its recent description rather than a reflection of the etiology of diseases caused by this species. Additional field isolations in Europe and California are needed to clarify this.

The origin of these species and how they became established in California has yet to be clarified. Theoretically it is possible they may have arisen from an

interspecific hybridization, but the ITS and *cox* II data does not support this. If an interspecific hybridization had occurred heterozygosity of the ITS region would be expected with two different forms of the ITS region present (Brasier, Cooke & Duncan 1999, Bonants *et al.* 2000), and this was not observed. This observation was consistent for all isolates of *P. ramorum* from California and Europe examined (Werres *et al.* 2001, Rizzo *et al.* 2002a, this study) as well as for isolates of *P. nemorosa* from California (Hansen *et al.* 2003) and *P. pseudosyringae* from Europe and California (Jung *et al.* 2003, this study). The only exception was for two of 13 isolates of *P. pseudosyringae* from Europe that had double peaks for C and T at base 156 in the ITS 1 region (Jung *et al.* 2003). While this type of observation is indicative of heterozygosity, and hence possibly an interspecific hybrid, since it was only a single base with the rest of the sequences invariant this seems unlikely. Furthermore, intraspecific variation in the mitochondrially encoded *cox* II gene was not observed in European and California isolates of *P. ramorum* and *P. pseudosyringae* or between the California and Oregon isolates of *P. nemorosa*. These sequences also were distinct from the other 27 species in the genus that have been studied. However, since this sampling represents approximately half of the described species in the genus it is less than definitive and should be viewed only in the context of being supportive of the ITS conclusions. Therefore, it appears that *P. ramorum*, *P. nemorosa* and *P. pseudosyringae* are phylogenetically distinct species.

It also is possible that these pathogens could have been introduced into California and Oregon from another location, but due to their recent description knowledge of their distribution in other regions of the world is lacking. The only other reported locations where *P. ramorum* was present when SOD was described in California were Germany and The Netherlands (Werres *et al.* 2001, Rizzo *et al.* 2002a). However, based on AFLP analysis (Ivors *et al.* 2002, Garbelotto *et al.* 2003) and mating type (A1 in Europe and A2 in California; de Gruyter *et al.* 2002, Werres & Zielke 2003) the California population is not the same as the European. While *P. pseudosyringae* has been reported from Europe and California, studies evaluating the diversity among these populations are needed before conclusions about possible movement from one location to another can be made. In contrast to *P. ramorum* and *P. pseudosyringae*, *P. nemorosa* has been recovered from California and Oregon, but has not been observed from other locations (Hansen *et al.* 2003). This would argue against introduction of this species into California, although surveys conducted to date have not been extensive enough to exclude this possibility. In view of the association of *P. nemorosa* with individual infected trees compared to the broader levels of infection observed for *P. ramorum*, Hansen *et al.* (2003) suggested that *P. nemorosa* was an endemic pathogen rather than an introduced pest.

These pathogens could have been present and causing low levels of disease in California previously but not identified since they are primarily pathogens in a forest ecosystem and are somewhat out of the public eye. Many of the symptoms they cause are similar to other pathogens encountered in these locations and the standard isolation techniques (selective media or surface disinfectation with sodium hypochlorite) would not be conducive to the recovery of *Phytophthora* spp. Furthermore, pathogen isolation using the proper techniques can sometimes be challenging as pathogen recovery can be difficult outside of the wetter winter season or if the tissue has dried out during transport to the laboratory. In view of the cool, wet conditions favourable for disease development for these species (Rizzo *et al.* 2002a, Jung *et al.* 2003), the heavier rainfall associated with *el nino* weather patterns encountered in coastal California in the mid 1990s may have contributed to the greater distribution of this pathogen in the central coastal California forest ecosystems during this same time period, which in turn would enhance public awareness of this disease problem. Further clarification of the origin of these pathogens will have to await more detailed analysis of population structures from different regions of the world and clarification if *P. ramorum* has a sexual phase in nature.

The 50 % majority rule consensus MP trees obtained using 667 bp of the *cox* II gene was similar to that observed with 568 bp of this gene; the only exception was the grouping of *P. megakarya*, which was on the same clade as *P. palmivora* in the tree using 667 bp of the gene rather than an independent clade that was previously reported (Martin & Tooley 2003). As observed in the previous analysis, there is a lack of congruence in the 50 % majority rule consensus trees generated by bootstrap analysis between the *cox* II and ITS trees for the relationship of *P. lateralis* and *P. syringae* with the clade containing *P. cryptogea*, *P. drechsleri*, and *P. erythroseptica*. In the ITS trees *P. lateralis* and *P. syringae* are on basal nodes of the same clade with these other species while in the *cox* II tree they were independent and not affiliated with each other. Similar results were observed for the most parsimonious trees, with the ITS trees having the same species grouping while *P. lateralis* was separate from the other four species in the *cox* II analysis.

Since the same isolates for some of these species were used for constructing both these trees, this lack of congruence may be indicative of incorrect assumptions about ITS sequence alignment or reflective of different rates of evolutionary divergence for this region. As noted previously, one advantage of using the *cox* II gene is that it is a coding region and does not have length mutations that can complicate sequence alignment (Martin & Tooley 2003); in contrast, the ITS region for the species used in this investigation varies from 752 to 832 bp. One advantage the ITS region does have over the cytoplasmically inherited *cox* II gene is

that it can reflect intra- and interspecific hybridization events whereas the mitochondrial *cox* II gene is uniparentally inherited (Förster & Coffey 1990). Since hybridization between species has been reported in the genus (Brasier, Cooke & Duncan 1999, English *et al.* 1999, Bonants *et al.* 2000, Gu & Ko 2000, Delcan & Brasier 2001) this is an important consideration and a potential drawback of relying strictly on mitochondrial sequences for phylogenetic analysis.

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