Molecular Detection of *Phytophthora ramorum*, the Causal Agent of Sudden Oak Death in California, and Two Additional Species Commonly Recovered from Diseased Plant Material

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ABSTRACT

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Sudden oak death is a disease currently devastating forest ecosystems in several coastal areas of California. The pathogen causing this is *Phytophthora ramorum*, although species such as *P. nemorosa* and *P. pseudosyringae* often are recovered from symptomatic plants as well. A molecular marker system was developed based on mitochondrial sequences of the *cox*I and II genes for detection of *Phytophthora* spp. in general, and *P. ramorum*, *P. nemorosa*, and *P. pseudosyringae* in particular. The first-round multiplex amplification contained two primer pairs, one for amplification of plant sequences to serve as an internal control to ensure that extracted DNA was of sufficient quality to allow for polymerase chain reaction (PCR) amplification and the other specific for amplification of sequences from *Phytophthora* spp. The plant primers amplified the desired amplicon size in the 29 plant species tested and did not interfere with amplification by the *Phytophthora* genus-specific primer pair. Using DNA from purified cultures, the *Phytophthora* genus-specific primer pair amplified a fragment diagnostic for the genus from all 45 *Phytophthora* spp. evaluated, although the efficiency of amplification was lower for *P. lateralis* and *P. sojae* than for the other species. The genus-specific primer pair did not amplify sequences from the 30 *Pythium* spp. tested or from 29 plant species, although occasional faint bands were observed for several additional plant species. With the exception of one plant species,

the resulting amplicons were smaller than the *Phytophthora* genus-specific amplicon. The products of the first-round amplification were diluted and amplified with primer pairs nested within the genus-specific amplicon that were specific for either *P. ramorum*, *P. nemorosa*, or *P. pseudosyringae*. These species-specific primers amplified the target sequence from all isolates of the pathogens under evaluation; for *P. ramorum*, this included 24 isolates from California, Germany, and the Netherlands. Using purified pathogen DNA, the limit of detection for *P. ramorum* using this marker system was ≈2.0 fg of total DNA. However, when this DNA was spiked with DNA from healthy plant tissue extracted with a commercial miniprep procedure, the sensitivity of detection was reduced by 100- to 1,000-fold, depending on the plant species. This marker system was validated with DNA extracted from naturally infected plant samples collected from the field by comparing the sequence of the *Phytophthora* genus-specific amplicon, morphological identification of cultures recovered from the same lesions and, for *P. ramorum*, amplification with a previously published rDNA internal transcribed spacer species-specific primer pair. Results were compared and validated with three different brands of thermal cyclers in two different laboratories to provide information about how the described PCR assay performs under different laboratory conditions. The specificity of the *Phytophthora* genus-specific primers suggests that they will have utility for pathogen detection in other *Phytophthora* pathosystems.

Additional keyword: mitochondrial DNA.

Phytophthora ramorum (Werres, de Cock and Man in 't Veld) causes sudden oak death (SOD), a disease which has killed thousands of oak trees along the California coast (16,35) and also has been detected in southern Oregon (18). This pathogen was first described in Europe causing twig blight on *Rhododendron* and *Viburnum* spp. (40) before being identified as the pathogen species responsible for widespread death of oaks in California (35). The disease currently has spread to 12 counties in the state and has a broad host range of at least 11 plant families and 28 plant

species (16,23,34). 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 (38) and state level (7) affecting movement of specific plant species outside of infested counties.

In addition to *P. ramorum*, two recently described species, *P. nemorosa* (22) and *P. pseudosyringae* (25), also can be recovered from symptomatic tissue from a range of different hosts in the forest ecosystem. Both species initially were referred to as a *P. ilicis*-like species due to their morphological similarity prior to publication of the species description (22,35). The foliar disease symptoms caused by these pathogens are indistinguishable from those caused by *P. ramorum*, and the host range and geographic distribution is similar. *P. nemorosa* commonly is recovered from leaf spots and is associated with individual lethal trunk cankers, but not with large tree stand mortality as is *P. ramorum* (22). Thus far, *P. pseudosyringae* has been observed primarily as a leaf and twig pathogen in California (C. Blomquist, *unpublished data*), which is in contrast to the description as a root and collar rot

pathogen of trees in Europe in the original species description (25). These two additional species can be distinguished in culture from *P. ramorum* in part by the fact they are homothallic and do not produce chlamydospores (22,25), whereas *P. ramorum* is heterothallic and produces chlamydospores (40, 41). Although morphologically similar, *P. pseudosyringae* can be differentiated from *P. nemorosa* by the catenulate hyphal swellings produced in water culture and by having a higher temperature growth optimum (22,25).

Due to the wide host range of *P. ramorum* and its potential for spread to new areas of the United States, quarantine regulations have been developed by the U.S. Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS) to restrict the potential spread of *P. ramorum* from known infested areas (specific counties in California and Oregon) on a variety of host species (38). To clarify the current distribution of the pathogen, field surveys have been organized in states that have potential hosts of the pathogen and environmental conditions favorable for disease development (J. Jones, *personal communication*). In addition, Canada has placed restrictions on importation of reported *P. ramorum* host species (8). The development of accurate and rapid diagnostic methods for *P. ramorum* will be key for providing the tools needed for detecting the pathogen and determining its potential for becoming established in new regions. Due to the presence of other *Phytophthora* spp. that cause similar symptoms on diseased plants, symptom expression alone will not be adequate for accurate diagnosis of this pathogen. If isolated in

pure culture, *P. ramorum* can be identified by its unique morphological characteristics; however, due to morphological similarities of other the *Phytophthora* spp. that may be recovered from symptomatic tissue, identification of these species by morphology alone may require special training. Furthermore, successful culturing of *P. ramorum* seems dependent on environmental conditions where the samples were collected, host response, and the presence of competing organisms in the plant tissue, all of which may inhibit culturing of the pathogen (35; C. Blomquist, *unpublished data*). A strong need exists for additional tools that will allow for rapid and accurate identification of pathogens from symptomatic tissue that are independent of environmental conditions and allows for processing of large sample sizes.

Methods based on DNA sequence information hold much promise in *P. ramorum* detection; however, if used for field diagnosis, they must be able to clearly distinguish *P. ramorum* from several other *Phytophthora* spp. that may be encountered, including *P. nemorosa*, *P. pseudosyringae*, *P. cinnamomi*, *P. syringae*, *P. hevea*, *P. cactorum*, *P. citricola*, and others (10; C. Blomquist, *unpublished data*). Thus, a high research priority has been the development of a polymerase chain reaction (PCR)-based detection method for *P. ramorum*. PCR-based methods have proven to be among the most rapid, specific, and sensitive methods available for detection of *Phytophthora* spp. (1,3,13,20,36,37,42). A molecular detection system already has been described for *P. ramorum* based on the internal transcribed spacer (ITS) region of the ribosomal DNA (17). In this method, one set of species-

TABLE 1. Isolates of *Phytophthora* spp. used in this investigation

Species	Group[a]	Isolate number[b]	Host	Origin
Phytophthora boehmeriae	II	325[PT], P1257[MC]	*Boehmeria nivia*	Papua New Guinea
P. cactorum	I	SB2079[GB]	*Fragaria × ananassa*	California
P. capsici	II	Cp-25[DJM] (A-2)	*Citrullus lantatus*	Florida
P. cinnamomi	VI	Cn-2[DJM] (A-2 mating type)	*Vaccinium* spp.	Florida
P. citricola	III	SB2078[GB]	*Fragaria × ananassa*	California
P. citrophthora	II	Ct-1[DJM]	*Theobroma cacao*	Brazil
P. clandestine	I	IMI287317[DC]	*Trifolium subterranean*	Australia
P. colocasiae	IV	346[PT], P3773[MC]	*Colocasia esculenta*	Indonesia
P. cryptogea	VI	IMI045168	*Lycopersicon esculentum*	New Zealand
P. drechsleri	VI	ATCC 46724 (Type)	*Beta vulgaris*	United States
P. erythroseptica	VI	367[PT]	*Solanum tuberosum*	New York
P. fragariae fragariae	V	395[PT], ATCC 13974	*Fragaria × ananassa*	New York
P. gonapodyides	VI	393[PT], NY353[WW]	*Malus sylvestris*	New York
	…	P245[DC], IMI389725	*Salix mastsudana*	United Kingdom
	…	P501[DC], IMI389729	*Ilex* roots	United Kingdom
P. heveae	II	Hv-2[DJM]	*Theobroma cacao*	Brazil
P. hibernalis	IV	337[PT], ATCC 32995, P0647[MC]	*Citrus sinensus*	California
P. humicola	V	IMI302303[DC]	Soil from citrus	Taiwan
P. idaei	I	IDA3[DC] (Type)	*Rubus idaeus*	Scotland
P. ilicis	IV	343[PT], P6100[MC], 802[PH]	*Ilex aquifolium*	Oregon
P. inflata	III	IMI342898[DC]	*Syringa* sp.	
P. infestans	IV	127[PT], ATCC 48723	*Solanum tuberosum*	New York
P. iranica	I	IMI158964[DC]	*S. melongera*	Iran
P. katsurae	II	IMI360596[DC]	*Cocos nucifera*	Ivory Coast
P. lateralis	V	IMI040503 (Type)	*Chamaecyparis*	United States
P. medii	II	IMI129185[DC]	*Hevea brasiliensis*	India
P. megasperma	V	IMI133317	*M. sylvestris*	Australia
P. megakarya	II	328[PT], P184[CB]	*T. cacao*	Cameroon
P. melonis	VI	IMI325917[DC]	*Cucumis* sp.	China
P. mirabilis	IV	340[PT], ATCC 64070, P3007[MC]	*Mirabilis jalapa*	Mexico
P. nemorosa	IV	P-13[EH], 482[PT] Type	*Lithocarpus densiflorus*	California
	…	2052.1[EH], 483[PT]	*L. densiflorus*	Oregon
P. nicotianae	II	331[PT]	*Nicotiana tabacum*	North Carolina
P. palmivora	II	Pl-5[DJM], P626[UCR]	*T. cacao*	Brazil
P. phaseoli	V	352[PT], ATCC 60171, CBS 556.88	*Phaseolus lunatus*	Unknown
	…	373[PT]	*P. lunatus*	Delaware

(*continued on next page*)

[a] Waterhouse morphological group (Waterhouse 1963).

[b] CB = Clive Brasier, DC = DNA supplied by David Cooke, MC = Michael Coffey, KD = Ken Deahl, PH = Phil Hamm (E. Hansen), DJM = Dave Mitchell, DS = Dave Shaw, PT = Paul Tooley, UCR = University of California at Riverside, SW = Sabine Werres, WW = Wayne Wilcox, DR = Dave Rizzo, CDFA = Cheryl Blomquist, California Department of Food and Agriculture, and GB = Greg Browne.

[c] Species groupings of Brasier et al. (5).

specific PCR primers was designed to amplify a 687-bp fragment from the ITS1, 5.8S, and ITS2 regions of *P. ramorum*, while a second nonspecific pair amplifies a 291-bp portion of the ITS2 region nested within the first amplicon. This method has been used for preliminary identification of new hosts of *P. ramorum* (16,17,35); the first set of primers has been used as a single-round amplification procedure in at least one European laboratory (27) and is one of the procedures selected by USDA-APHIS for conducting the pathogen survey in a number of states in 2003 (28). Another molecular detection system designed to identify and detect *P. lateralis* (42) also has been modified for use in detecting *P. ramorum* (28) and a detection system based on sequence variation of the β-tubulin gene is under development (2).

One complicating factor in diagnosis of foliar pathogens from forest ecosystems and nursery settings is that there are several *Phytophthora* spp. that can cause symptoms similar to those caused by *P. ramorum*. Although it is important to accurately identify *P. ramorum* in field samples, from a diagnostician's stand point it also would be helpful to know if other *Phytophthora* spp. are responsible for the observed disease symptoms. This would not only assist in development of disease management strategies but also help clarify the disease problems of the various hosts under study, including the possible identification of new pathogens. Furthermore, with any molecular diagnostic system it also is important to have an internal control to validate that the DNA extraction procedure produced template DNA of sufficient quality

to allow DNA amplification to take place, thereby reducing the potential for false negatives (42).

The objective of this study was to develop a mitochondrially based PCR molecular marker system that was specific for *Phytophthora* spp. and could be used to detect a range of species. The mitochondrial genome was chosen for a target due to its high copy number and identification of a specific region of sequence heterogeneity among species that was bordered by highly conserved sequences. The *Phytophthora* genus-specific primer pair was developed from conserved sequences in the 3′ end of the *cox*II gene and the 5′ end of the *cox*I gene and amplified the spacer region between these two genes. Sequence variation was observed in the spacer region that allowed for the development of nested species-specific primer pairs for identification of *P. ramorum*, *P. nemorosa*, or *P. pseudosyringae*. To facilitate the use of this marker system for processing field samples, a primer pair for amplification of plant sequences was developed to use in the first-round amplification to serve as a positive control to ensure the quality of DNA extraction.

MATERIALS AND METHODS

Cultures and DNA extraction. Cultures of *Phytophthora* spp. used in this study are listed in Table 1 and have been described previously (32,33). Cultures were grown on Rye A medium (9) at 20°C in darkness and genomic DNA was extracted by the method

TABLE 1. (*Continued from preceding page*)

Species	Group[a]	Isolate number[b]	Host	Origin
P. phaseoli	…	403[PT]	*P. lunatus*	Delaware
	…	406[PT] (Race D)	*P. lunatus*	Maryland
P. porri	III	CBS 782.97[DC]	*Brassica chinensis*	The Netherlands
P. primulae	III	CBS 620.97[DC]	*Primula acaulis*	Germany
P. pseudosyringae	IV	PSEU16[TJ], 484[PT], NFV-BU97-15	*Fagus sylvatica*	Germany
	…	P96[EH], 485[PT]	*Umbellularia californica*	Contra Costa Co., CA
	…	470[PT], P193907A[CDFA]	*Manzanita* sp.	Royal Oaks, CA
	…	471[PT], 1168699[CDFA]	*U. californica*	Napa, CA
	…	472[PT], 1168676[CDFA]	*U. californica*	Calistoga, CA
	…	473[PT], P110361[CDFA]	*U. californica*	Yountville, CA
P. pseudotsugae	I	308[PT], H270[PH]	*Pseudotsugae menziesii*	Oregon
P. quercina	V	IMI340618[DC]	*Quercus robur*	Germany
P. ramorum	IV	Prn-1, PD93/844[SW]	*Rhododendron* sp.	The Netherlands
	…	Prn-2, PD94/844[SW]	*Rhododendron* sp.	The Netherlands
	…	Prn-3, PD98/8/6743[SW]	*Rhododendron* sp.	The Netherlands
	…	Prn-4, PD98/8/6285[SW]	*Rhododendron* sp.	The Netherlands
	…	Prn-5, PD98/8/2627[SW]	*Rhododendron* sp.	The Netherlands
	…	Prn-6, PD98/8/5233[SW]	*Viburnum* sp.	The Netherlands
	…	Prg-1, BBA 69082[SW]	*Rhododendron* sp.	Germany
	…	Prg-2, BBA 9/95[SW], CBS 101553 (Type)	*Rhododendron catawbiense*	Germany
	…	Prg-3, BBA 14/98-a[SW]	*R. catawbiense*	Germany
	…	Prg-4, BBA 12/98[SW]	*R. catawbiense*	Germany
	…	Prg-5, BBA 13/99-1[SW]	*R. catawbiense*	Germany
	…	Prg-6, BBA 16/99[SW]	*Viburnum bodnantense*	Germany
	…	Prg-7, BBA 9/3[SW]	Water	Germany
	…	Prg-8, BBA 104[SW]	Water	Germany
	…	288[MG]	*Rhododendron* sp.	California
	…	73101[CDFA]	*L. densiflorus*	California
	…	044519[CDFA]	*U. californica*	California
	…	044522[CDFA]	*L. densiflorus*	California
	…	P072648[CDFA]	*Q. agrifolia*	California
	…	201C[DR]	*Rhododendron* sp.	California
	…	0217[DR]	*Rhododendron* sp.	California
	…	Coen[MG]	*Rhododendron* sp.	California
	…	013[DR]	*L. densiflorus*	California
	…	016[DR]	*Q. agrifolia*	California
P. richardiae	VI	ATCC 46538[DC]	*Zantedeschia* sp. root	The Netherlands
P. sojae	V	312[PT], ATCC 48068	*Glycine max*	Wisconsin
P. syringae	III	IMI296829	*Rubus idaeus*	Scotland
P. tentaculata	I	CBS 552.96[DC]	*Chrysanthemum leucanth.*	Germany
Phytophthora sp. "O" group[c]	…	P246[DC], IMI389751	*Salix* roots	United Kingdom
P. taxon Pgchlamydo[c]	…	P510[DC], IMI389731	*Pseudotsugae* roots	Canada
P. taxon Raspberry[c]	…	P896[DC], IMI389744	Soil	Tasmania

of Goodwin et al. (19) or a boiling miniprep procedure (31). Cultures of *Pythium* spp. were from the culture collection of F. Martin with classifications based on the keys of Van der Plaats-Niterink (39) and DNA extraction done by a miniprep procedure (30). Plant DNA was extracted using a miniprep procedure developed for *Pythium* spp. (30) or using lyophilized tissue ground with liquid nitrogen and extracted with a FastPrep DNA extraction kit from Qbiogene (Carlsbad, CA). DNA concentrations were determined spectrophotometrically or by quantitation on agarose gels stained with ethidium bromide in comparison with commercially obtained standards. Extracted DNA for 19 of the cultures examined was kindly provided by D. Cooke (Table 1).

Samples of DNA from plant samples collected from the field exhibiting disease symptoms were processed at the California Department of Food and Agriculture (CDFA). Leaf lesions and stem cankers were plated on PARP (pimaricin, ampicillin, rifampicin, and PCNB) medium for selective isolation of *Phytophthora* spp. (26). Tissue samples also were removed for DNA extraction using the FastPrep extraction kit in accordance with the manufacturer's instructions and 2 μl was used for PCR amplifications. The ITS-based species-specific primers of Garbelotto et al. (17,28) were used to confirm the presence of *P. ramorum*.

Primers and DNA amplification. Plant primers FMPl-2b (dGCGTGGACCTGGAATGACTA) and FMPl-3b (dAGGTTGT-ATTAAAGTTTCGATCG) were constructed from the mitochondrially encoded cytochrome oxidase I gene (base 522 to 664 in *Pisum sativum*, X14409) and generated an amplicon 143 bp in length. *Phytophthora* genus-specific primers FMPh-8b (dAAAA-GAGAAGGTGTTTTTTATGGA) and FMPh-10b (dGCAAAA-GCACTAAAAATTAAATATAA) were derived from mitochondrial sequences of the coxII (base 624 to 657) and *cox*I gene (base 73 to 98), respectively, and amplified a product 457 bp in length for *Phytophthora ramorum*. Sequences of the genus-specific amplicon for *P. ramorum*, *P. nemorosa*, and *P. pseudosyringae* have been deposited in GenBank (AY534689, AY534690, and AY53691, respectively). Species-specific primers for *P. ramorum* (FMPr-1a, dGTATTTAAAATCATAGGTGTAATTTG and FMPr-7, dTGG-TTTTTTTAATTTATATTATCAATG), *P. nemorosa* (FMnem-1, AATAAAATTAATTTTAATATATAATTAG and FMnem-3, dTAT-GTTTAATATCTGTAAATAATAG), and *P. pseudosyringae* (FMPps-1, dCAGTTTCATTAGAAGATTATTTAC and FMPps-2, dAAAATTGTTTGATTTTATTAAGTATC) were nested within

the genus-specific primer pair and derived from spacer sequences between the *cox*II and *cox*I gene and generated amplicon sizes of 134, 100, and 158 bp, respectively. Primers were synthesized by Invitrogen Corporation (Carlsbad, CA). Primer pairs were tested singly in a master mix containing a final concentration of 1 μM for each primer, 100 μM dNTPs, 1× Ampli*Taq* Buffer, and 2 units of Ampli*Taq* (Applied Biosystems, Foster City, CA) in a total volume of 25 μl with varying MgCl$_2$ concentrations and annealing temperatures to optimize the amplification. Purified water (W4502; Sigma-Aldrich, St. Louis) was used in all primer dilutions and amplifications. Glycerol (redistilled; Roche Diagnostics, Indianapolis) was diluted to make a 50% solution and evaluated at different rates as a cosolvent to enhance primer specificity. Unless otherwise indicated, DNA concentrations used were 20 ng for *Phytophthora* spp., approximately 100 ng for *Pythium* spp., and an estimated minimum of 100 ng for plant species. A water blank was included as a negative control in each round of amplifications. In California, amplification was done in an Eppendorf Mastercycler Gradient Thermal cycler (Eppendorf Scientific, Westbury, NY) in a 500-μl Eppendorf thin-walled PCR tube with the Ampli*Taq* added to each prechilled amplification tube prior to placing it into a preheated block of the thermal cycler. The ramping time was set at the fastest setting (3°C/s). In Ft. Detrick, amplifications were done in an Applied Biosystems Model 9700 thermal cycler with a ramping time of 2.3°C/s.

Once amplification parameters for the various primer pairs were determined, multiplex amplifications were evaluated in an effort to simplify the diagnostic procedure using a mixture of purified plant DNA and 20 ng of *Phytophthora* DNA (at 1 μl each). The first round contained the plant primer pair FMPl-2b and FMPl-3b (0.1 μM final concentration each), the *Phytophthora* genus-specific primer pair FMPh-8b + FMPh-10b (1.0 μM final concentration each), 1× Ampli*Taq* reaction buffer, 3 mM MgCl$_2$, 100 μM dNTPs, and 2% glycerol (1 μl of a 50% solution) with 2 units of Ampli*Taq* added to prechilled reaction mix just prior to placing into the preheated block of the thermalcycler. Bovine serum albumin (BSA; New England Biolabs, Beverly, MA; 1:20 dilution of a 100 mg/ml stock solution) was included in the reaction mix at a rate of 1 μl in some experiments to enhance the efficiency of the amplification. The amplification parameters were 95°C for 3 min followed by 40 cycles of 95°C for 1 min, 66°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 5 min. The mix (12 μl) was run on either a 1.5% agarose gel or a 3% Nusieve 3:1 gel with 0.5× Tris-borate EDTA. The product of the first amplification was diluted 1:100 in sterile water and added to a master mix containing the *Phytophthora* genus-specific primer pair (FMPh-8b + FMPh-10b, 0.1 μM final concentration each), the *P. ramorum* species-specific primer pair (FMPr-1a + FMPr-7, 1 μM final concentration), 1× Ampli*Taq* reaction buffer, 3 mM MgCl$_2$, and 100 μM dNTPs. A water blank and a *Phytophthora* control (any *Phytophthora* sp. for the first-round amplification and *P. ramorum* for the second-round amplification) were included in each amplification. If the results for these control amplifications were inconsistent with expectations, the amplifications were repeated. Two units of Ampli*Taq* were added to the prechilled reaction mixture just prior to placing the tubes into the preheated block of the thermal cycler. The amplification parameters were 95°C for 3 min followed by 35 cycles of 95°C for 30 s and 64°C for 30 s, with a final extension of 72°C for 5 min. Amplification of plant samples collected from the field followed this procedure with the exception that 2 μl of the FastPrep DNA extraction was used in the first multiplex amplification and this was diluted 1:25 prior to addition of 1 μl to the second-round multiplex amplification.

In samples from the second round of multiplex amplifications where the *Phytophthora* genus-specific band was amplified but the *P. ramorum*-specific amplicon was not, a second amplification of the 1:25 dilution of the first multiplex reaction was done with
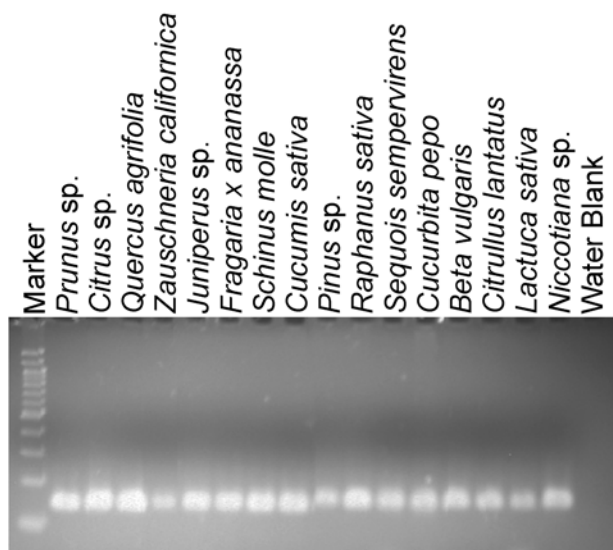
Fig. 1. Amplification of various plant species with the plant primers FMPl-2b + FMPl-3b using an annealing temperature of 66°C with 12 μl of the amplification mixture loaded into each well of a 1.5% agarose gel. The molecular size marker is a 100-bp ladder from New England Biolabs and the size of the plant amplicon is 143 bp.

the *P. nemorosa* or *P. pseudosyringae* primer pair. The master mix was the same as the previously described second-round multiplex amplification with the exception that the only primers present were specific for *P. nemorosa* (FMnem-1 and FMnem-3) or *P. pseudosyringae* (FMPps-1 and FMPps-2) at a final concentration of 1.0 μM each. The amplification cycling parameters were the same as well, except that the annealing temperature was 61 or 65°C, respectively.

The sensitivity of the detection system was evaluated by using a dilution series of purified DNA from *P. ramorum* that had been spectrophotometrically quantified for amplification with the genus-specific primer pair. This was diluted 1:25 and 1 μl was added to a second-round amplification with the nested *P. ramorum* species-specific primer pair. To evaluate the influence of plant DNA extracted with the FastPrep procedure by the CDFA noted above on the sensitivity of detection, parallel samples of the *P. ramorum* dilution series were spiked with 2 μl of DNA from healthy plants prior to amplification with the genus-specific primer pair.

In an effort to identify species that were amplified from infected plant material using the *Phytophthora* genus-specific primer pair, templates were sequenced 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 prepared as previously described (32) and sequenced in both directions, with the same primers used for amplification.

RESULTS

Amplification with plant primers. The plant primers amplified target sequences well with 2 to 4 mM MgCl₂ (less amplification at 1 mM) with an optimum of 2 mM (data not shown). Annealing temperatures as high as 68°C did not inhibit amplification. The plant primers amplified all plant species tested, including the 16 presented in Figure 1 as well as a *Rhododendron* sp., *Aesculus californica*, *Salal* sp., *Acer macrophyllum*, *Arbutus menziesii*, *Umbellularia californica*, *Sambucus* sp., *Pseudotsuga menziesii*, *Rhamnus* sp., *Manzanita* sp., *Heteromeles arbutifolia*, and a *Liriope* sp. (data not shown).
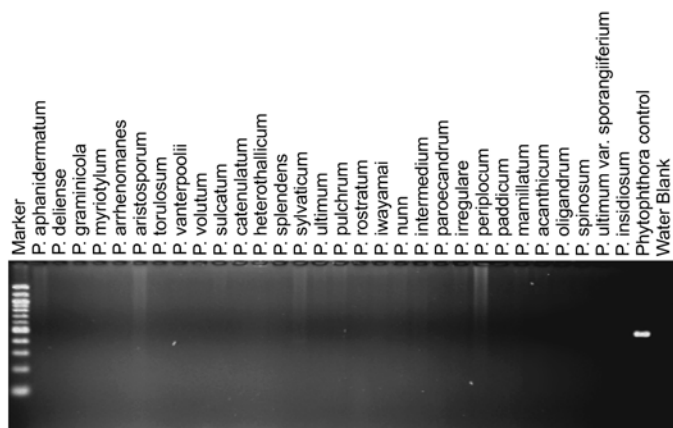
Amplification with *Phytophthora* genus-specific primers. The primers amplified target sequences most efficiently at 3 mM MgCl₂, with less amplification at 4 mM and no amplification at 1 or 2 mM (data not shown). At the stringency tested (66°C annealing temperature and the addition of glycerol at a 2% final concentration), this primer pair amplified all *Phytophthora* spp. tested in Figure 2 with weak amplification of *Phytophthora lateralis* and *P. sojae*. Additional species listed in Table 1 but not included in Figure 2 were amplified as well (data not shown) to
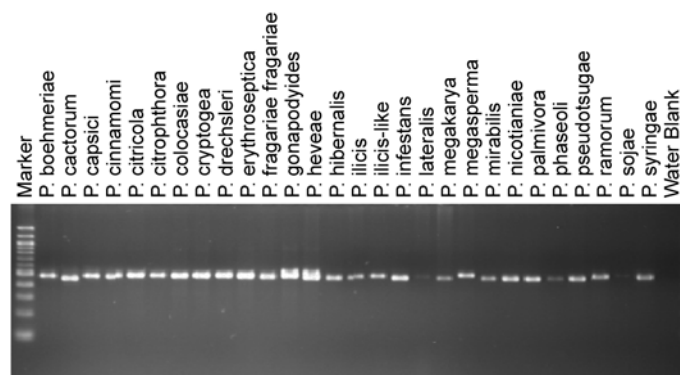
give a total of 45 species that have been tested. Both *P. lateralis* and *P. sojae* were amplified more efficiently if the stringency was reduced by eliminating the glycerol from the reaction mix or lowering the annealing temperature (data not shown). Depending on the amplification, doublet bands nearly the same size were occasionally observed, but these behaved the same as single bands in subsequent analysis (data not shown). None of the *Pythium* spp. tested was amplified with this primer pair (Fig. 3) although, in some amplifications, an occasional faint band of ≈0.4 kb was amplified for the *Pythium sylvaticum* isolate tested (data not shown). Under the amplification conditions used, none of the 29 plant species noted above were amplified by this primer pair (data not shown), although occasional inconsistent amplification of a band was occasionally observed with DNA from *Rhododendron* spp. Eliminating glycerol from the amplification mixture reduced the stringency and allowed amplification of *Prunus* sp., *Citrus* sp., *Quercus agrifolia*, *Juniperus* sp., and *Fragaria × ananassa* (data not shown). All isolates of *Phytophthora ramorum*, *P. nemorosa*, and *P. pseudosyringae* listed in Table 1 were amplified with this primer pair (data not shown).
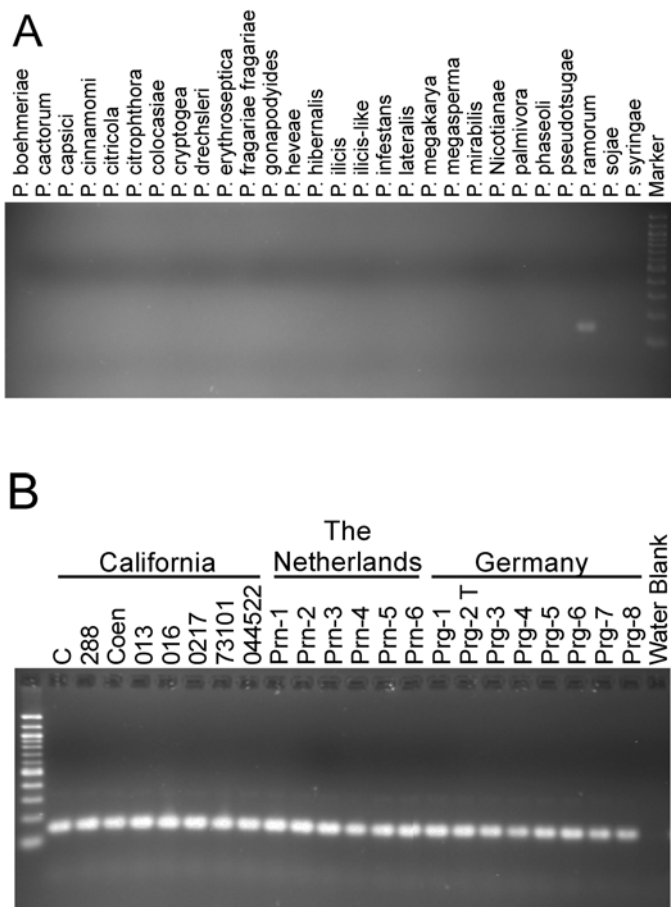
Specificity of *P. ramorum*-, *P. nemorosa*-, and *P. pseudosyringae*-specific primers. The *P. ramorum* species-specific primer pair amplified target sequences most efficiently with a 64°C annealing temp and 2, 3, or 4 mM MgCl₂, with less amplification at 1 mM MgCl₂ (data not shown). When the genus-specific fragment generated by amplification with FMPh-8b + FMPh-10b was used as a template, only *P. ramorum* was amplified (Fig. 4A). This species-specific primer pair amplified all *P. ramorum* isolates tested (including the type culture CBS101553, Fig. 4B), as well as isolates 201C and P072648, but did not amplify *P. nemorosa* (data not shown). The *P. nemorosa* primer pair amplified target sequences most efficiently with a 61°C annealing temperature and 4 mM MgCl₂; no amplification was observed with lower concentrations of MgCl₂ (data not shown). When the genus-specific fragment generated by amplification with FMPh-8b + FMPh-10b was used as a template, only *P. nemorosa* was amplified (Fig. 5). This species-specific primer pair amplified both *P. nemorosa* isolates listed in Table 1, including the type culture PE-13. The *P. pseudosyringae*-specific primer pair amplified target sequences most efficiently with a 65°C annealing temperature and 1 to 4 mM MgCl₂, with 3 mM MgCl₂ subsequently used for assays (data not shown). When the genus-specific fragment generated by amplification with FMPh-8b + FMPh-10b was used as a template, only *P. pseudosyringae* isolates were amplified (Fig. 6). This primer

Fig. 2. Amplification of various *Phytophthora* spp. with the *Phytophthora* genus-specific primers FMPh-8b + FMPh-10b with 2% glycerol in the reaction mixture and an annealing temperature of 66°C with 11 μl of amplification mixture loaded into each well of a 3% NuSieve 3:1 agarose gel. The molecular size marker is a 100-bp ladder from New England Biolabs.

Fig. 3. Test of amplification of various *Pythium* spp. with the *Phytophthora* genus-specific primers FMPh-8b + FMPh-10b with 2% glycerol added to the reaction mixture and an annealing temperature of 66°C. A minimum of 100 ng of *Pythium* total DNA was added to each reaction with 12 μl of the amplification mixture loaded into each well of a 3% NuSieve 3:1 agarose gel. The molecular size marker is a 100-bp ladder from New England Biolabs.

pair amplified the six *P. pseudosyringae* isolates listed in Table 1, including isolate PSEU16, which was one of the cultures included in the species description (25), but did not amplify *P. nemorosa* (data not shown). In addition to the *Phytophthora* spp. presented in Figures 4 through 6, the three sets of species-specific primers did not amplify bands when FMPh-8b + FMPh-10b amplicons from the following species were used as a template DNA: *P. clandestine, P. humicola, P. ideai, P. iranica, P. katsurae, P. medii, P. melonis, P. inflata, P. primulae, P. richardiae, P. porri, P. quercina, P. tentaculata*, or isolates from three of the tentative species groupings described by Brasier et al. (5) (P. taxon Raspberry, P. taxon Pgchlamydo, or *Phytophthora* sp. "O" group) (data not shown).

Limit of detection. A faint genus-specific band was observed with total DNA concentrations of 0.2 pg for isolate Prg-2 of *P. ramorum*, below which no amplified products were observed (Fig. 7A). When this was diluted 1:25 and 1 μl was used as target DNA for amplification with the *P. ramorum*-specific primer pair, the limit of detection was 2.0 fg. Similar results were obtained for two other isolates, with the exception that one of the isolates had an amplified species-specific band at 20.0 but not 2.0 fg (data not shown). Spiking the DNA dilution series with 2 μl of plant DNA

extracted at the CDFA (the same rate that would be used for evaluation of field samples) reduced the level of sensitivity by different amounts depending on the plant species (Fig. 7B). For *U. californica*, the sensitivity was reduced by a factor of approximately 100 (from 2 fg to 200 fg); whereas for *Acer macrophylum, Arbutus menziesii*, and *Rhamnus californica*, the sensitivity was reduced by a factor of approximately 1,000, with less amplification observed when spiking with *Acer macrophylum* DNA than the other two plant species.

Multiplex amplification. Multiplex amplifications with a mixture of DNA from *Citrus* sp. and either *P. ramorum* or *P. pseudosyringae* were used to optimize conditions for multiplex amplification. The first-round amplification with the plant and *Phytophthora* genus-specific primers was found to work best with 3 mM MgCl$_2$, 2% glycerol, and an annealing temperature of 66°C (data not shown). A 0.1-μM final concentration for the plant primers worked best; higher concentrations were found to reduce the amplification of the *Phytophthora* genus-specific amplicon due to the smaller size of the plant amplicon and the fact that the plant DNA concentration was much higher than the *Phytophthora* DNA. The second-round multiplex amplification worked best with an annealing temperature of 64°C for *P. ramorum*; addition of glycerol at any concentration inhibited amplification with this primer pair. Addition of the *Phytophthora* genus-specific primer pair at a concentration of 0.1 μM to this second round amplifica-
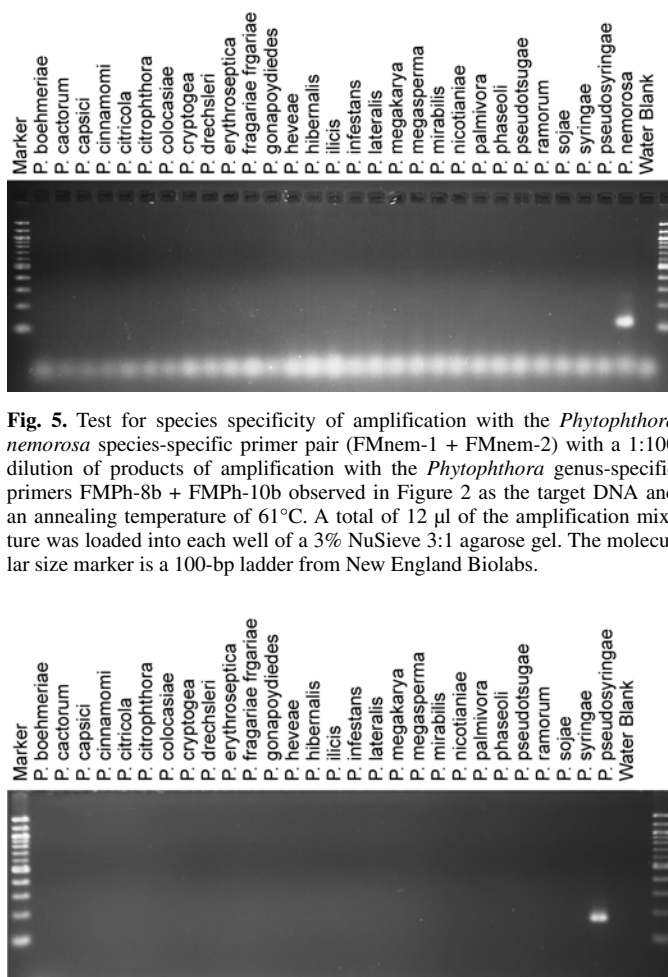
Fig. 5. Test for species specificity of amplification with the *Phytophthora nemorosa* species-specific primer pair (FMnem-1 + FMnem-2) with a 1:100 dilution of products of amplification with the *Phytophthora* genus-specific primers FMPh-8b + FMPh-10b observed in Figure 2 as the target DNA and an annealing temperature of 61°C. A total of 12 μl of the amplification mixture was loaded into each well of a 3% NuSieve 3:1 agarose gel. The molecular size marker is a 100-bp ladder from New England Biolabs.

Fig. 4. A, Test for species specificity of amplification with the *Phytophthora ramorum* species-specific primer pair (FMPr-1a + FMPr-7) with a 1:100 dilution of the first-round amplification with the *Phytophthora* genus-specific primers FMPh-8b + FMPh-10b observed in Figure 2 as the target DNA. The annealing temperature was 64°C. **B,** Amplification of different isolates of *P. ramorum* from California and Europe with the *P. ramorum* species-specific primer pair (FMPr-1a + FMPr-7). The template DNA was a 1:100 dilution of products of the first-round amplification with the *Phytophthora* genus-specific primers FMPh-8b + FMPh-10b using an initial concentration of 20 ng of target DNA for each isolate. A total of 12 μl of the amplification mixture was loaded into each well of a 3% NuSieve 3:1 agarose gel. Isolate Prg-2 T is the type culture for this species. The molecular size marker is a 100-bp ladder from New England Biolabs.

Fig. 6. Test for species specificity of amplification with the *Phytophthora pseudosyringae* species-specific primer pair (FMPsy-1 + FMPsy-2) with a 1:100 dilution of products of amplification with the *Phytophthora* genus-specific primers FMPh-8b + FMPh-10b observed in Figure 2 as the target DNA and an annealing temperature of 65°C. A total of 12 μl of the amplification mixture was loaded into each well of a 3% NuSieve 3:1 agarose gel. The molecular size marker is a 100-bp ladder from New England Biolabs.

tion enhanced the sensitivity of detection at the genus level in cases where the target *Phytophthora* DNA was present in low concentrations; however, higher primer concentrations reduced amplification of the *P. ramorum* diagnostic fragment (data not shown). Combining both the *Phytophthora* genus-specific and *P. ramorum* species-specific primers in a second round multiplex amplification produced an amplification profile that was different from using the primers individually due to generation of additional amplicons between these primer pairs that are approximately 300 bp in size (Fig. 8). Although the inclusion of BSA in the amplification master mix was found to improve the robustness of amplification, and hence the sensitivity of the assay, the use of this component of the master mix was discontinued due to formation of nonspecific background bands in some amplifications.

Amplification of field samples. Samples of extracted DNA from plant material collected from the field and processed at the

CDFA were provided to F. Martin blind and assayed using the mitochondrial-based primer system described above. The plant primers were tested on a total of 84 samples, 74 of which had the appropriate-sized plant fragment amplified, indicating that the procedure used for DNA extraction provided suitable DNA for PCR analysis. Seven of the samples that were not amplified were tested to determine if this was due to the presence of PCR inhibitors by aliquoting samples into amplification mixtures with the *Phytophthora* genus-specific primers containing purified DNA from *P. infestans*; in all cases, the presence of the plant DNA extract inhibited amplification of the *Phytophthora* genus-specific fragment (data not shown). The first-round multiplex amplification with the plant primer pair and the *Phytophthora* genus-specific primer pair generated different-sized bands that were diagnostic of their target sequences and did not exhibit cross reactivity between primer pairs or nonspecific background amplification (Fig. 9A).

Of the 74 samples where the plant fragment was amplified, 61 samples were tested with the *Phytophthora* genus-specific primer pair. Of these, 32 had a *Phytophthora* genus-specific fragment amplified; 16 of these also had a *P. ramorum* species-specific band amplified with FMPr-1a + FMPr-7 (Table 2). The identity of four of these *P. ramorum*-positive samples was confirmed by sequencing the *Phytophthora* genus-specific amplicon and comparing to sequencing data for *P. ramorum* isolates Prg-2 (type culture), 013, 016, and Coen; all were identical with the exception that one isolate differed at one base (data not shown). For detection of *P. ramorum*, there was perfect agreement for these 16 samples with the analysis performed at the CDFA (culture of *P. ramorum* or positive amplification with the ITS-based marker for *P. ramorum*) (Table 2). The mitochondrial-based markers also identified 16 additional samples that had been infected by another *Phytophthora* sp., 13 of which were confirmed by pathogen recovery from infected tissue. Based on recovery from infected plant tissue and amplification with the species-specific primer pair, five of these samples were identified as a *P. pseudosyringae*. This was confirmed by sequencing the *Phytophthora* genus-specific amplicon for these isolates and comparing to sequencing data for the six isolates of *P. pseudosyringae* listed in Table 1; all isolates were identical with the exception of two that differed at one base (data not shown). *Phytophthora* isolates also were cultured from four tissue samples of *U. californica* and were amplified with *P. nemorosa* species-specific primers (Table 2); sequence analysis of the *Phytophthora* genus-specific amplicon for these samples confirmed they were *P. nemorosa*. Three additional positives for *P. nemorosa* were identified by species-specific amplification and sequence analysis from *U. californica* samples that
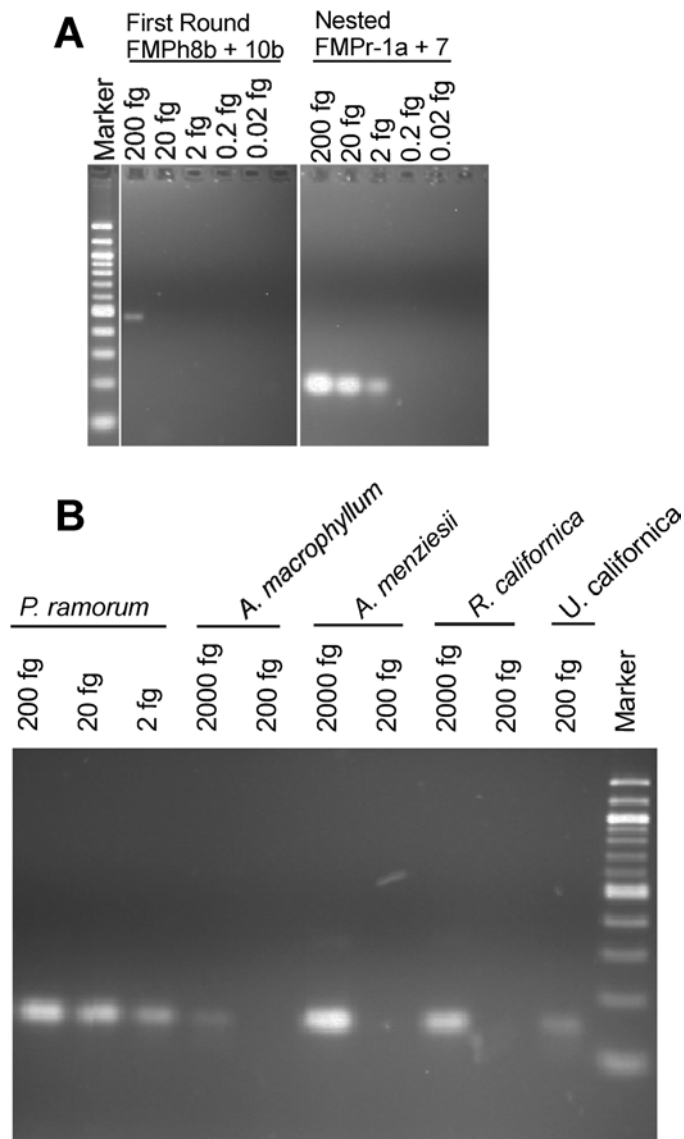
Fig. 7. Limit of detection of *Phytophthora ramorum* using a dilution series of total DNA. **A,** Amplification with the *Phytophthora* genus-specific primers followed by nested amplification with the *P. ramorum* species-specific primer pair using 1 µl of a 1:25 dilution of the first-round amplification with the genus-specific primer pair. **B,** Nested amplification of purified *P. ramorum* DNA with the species-specific primer pair when the first-round amplification with the genus-specific primer pair was spiked with water or DNA from four different plant species. A total of 12 µl of the amplification mixture was loaded into each well of a 3% NuSieve 3:1 agarose gel. The molecular size marker is a 100-bp ladder from New England Biolabs.
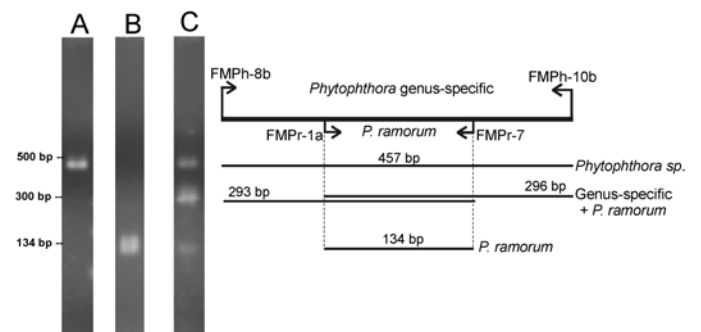
Fig. 8. Amplified products of *Phytophthora ramorum* using **A,** the *Phytophthora* genus-specific primer pair (FMPh-8b + FMPh-10b), **B,** the *P. ramorum* species-specific primer pair (FMPr-1a + FMPr-7), and **C,** multiplex amplification containing 0.1 µM final concentrations of the *Phytophthora* genus-specific primer pair (FMPhy-8b + FMPhy-10b) and 1.0 µM final concentrations of the *P. ramorum* species-specific primer pair (FMPr-1a + FMPr-7). A total of 12 µl of the amplification mixture was loaded into each well of a 3% NuSieve 3:1 agarose gel.

did not yield cultures of a *Phytophthora* spp. when plated on a selective medium (Table 2). Sequence analysis of the *Phytophthora* genus-specific fragment also identified two isolates of *P. syringae* (both on *Rhododendron* spp.) and one unidentified *Phytophthora* sp. (from *Rhamnus californica*). One inconsistency in results between the mitochondrial marker system and pathogen isolations from infected tissue was for the *Manzanita* sp. sample; no *Phytophthora* genus-specific band was amplified but an isolate was cultured from a twig lesion (Table 2; sample 907B in Fig. 9B). Due to the small size of the lesion in this sample, different lesions were used for pathogen recovery and DNA extraction.

Depending on the sample, the first-round amplification with the *Phytophthora* genus-specific primer pair did not always generate a diagnostic amplicon confirming the presence of a *Phytophthora* sp. For example, field samples 302, 773A, and C8 had clear genus-specific bands following the first-round multiplex amplification, whereas samples 301, 303, and 322 had faint diagnostic bands (Fig. 9A). Inclusion of the *Phytophthora* genus-specific primer pair in the second-round amplification with the *P. ramorum* species-specific primer pair enhanced the sensitivity of detection

of *Phytophthora* sp. with visible genus-specific bands observed for a number of samples that were negative following the first round of amplification (Fig. 9B).

Amplifications with different thermalcyclers. The results discussed above were obtained in the laboratory of F. Martin using an Eppendorf Mastercycler Gradient thermal cycler. The experiments presented in Figures 2 and 4, as well as evaluation of the specificity of the genus-specific primer pair against plant DNA, were repeated in the same lab using an MJ PTC-100 (MJ Research, Waltham, MA) with the same results obtained when the annealing temperature was reduced by 1°C for each primer pair. The experiments presented in Figures 2, 4, and 6 also were repeated at Ft. Detrick using an Applied Biosystems model 9700 thermal cycler with similar results obtained using the above-noted amplification parameters for the species-specific primer pairs (data not shown). Although the *Phytophthora* genus-specific primer pair did not amplify bands from *Rhododendron* 'Cunningham's White' or *Lycopersicon esculentum* with a 66°C annealing temperature, background bands were amplified for *Kalmia latifolia* 'Madeline' (≈0.3 kb), *U. californica* (≈0.8 kb), *Glycine max* (≈0.4 kb), and *Solanum tuberosum* (≈0.3 kb), all of which were different in size from the genus-specific diagnostic amplicon. The annealing temperature had to be increased to 67°C to reduce background amplification for these plant DNAs, but occasional faint bands different in size from the *Phytophthora* genus-specific amplicon still were observed for *G. max* and *S. tuberosum* (data not shown). At this annealing temperature, the diagnostic genus-specific band was amplified for *P. ramorum*, but not for *P. capsici*, *P. hevae*, *P. ilicis*, *P. sojae*, and some isolates of *P. pseudosyringae* (data not shown).

DISCUSSION

The described PCR-based mitochondrial DNA detection system utilized a two-step multiplex amplification procedure for determining if a *Phytophthora* sp. was present in symptomatic plant tissue and clarifying if it was *P. ramorum*, *P. nemorosa*, or *P. pseudosyringae*. The inclusion of a primer pair for amplification of plant sequences in the first multiplex amplification provided a positive control to confirm the quality of the DNA extract and eliminate false negatives due to the presence of PCR inhibitors or badly degraded DNA. The *Phytophthora* genus-specific primers amplified diagnostic amplicons from all 45 *Phytophthora* spp. tested and were useful in the assay for determining the presence of additional *Phytophthora* pathogens, such as *P. syringae*, in field samples submitted for analysis. Furthermore, using species-specific primers for detection of *P. ramorum*, *P. nemorosa*, and *P. pseudosyringae* nested within the genus-specific amplicon improved the sensitivity of detection in the second-round amplification. All primers exhibited a high degree of specificity in tests with purified DNA as well as field samples.

The results obtained with the mitochondrial marker system correlated well with the results obtained at the CDFA for pathogen recovery from symptomatic tissue and with PCR amplification for *P. ramorum*, using the rDNA ITS *P. ramorum*-specific primers of Garbelotto et al. (17). For *P. ramorum*, no differences in results were obtained; all samples that scored positive for *P. ramorum* in the CDFA laboratory also were positive with the mitochondrial markers. The results for four of these samples were validated by sequence analysis of the *Phytophthora* genus-specific amplicon and comparison with data from purified cultures of this pathogen. The mitochondrial marker system also identified 16 additional samples infected with other *Phytophthora* spp. that could not be identified with the ITS marker system. With a few explainable exceptions, these results correlated well with pathogen recovery in the CDFA laboratory. One exception was a sample from a *Manzanita* sp. where, due to the small size of the lesions, the tissue samples for DNA extraction were different from the sam-
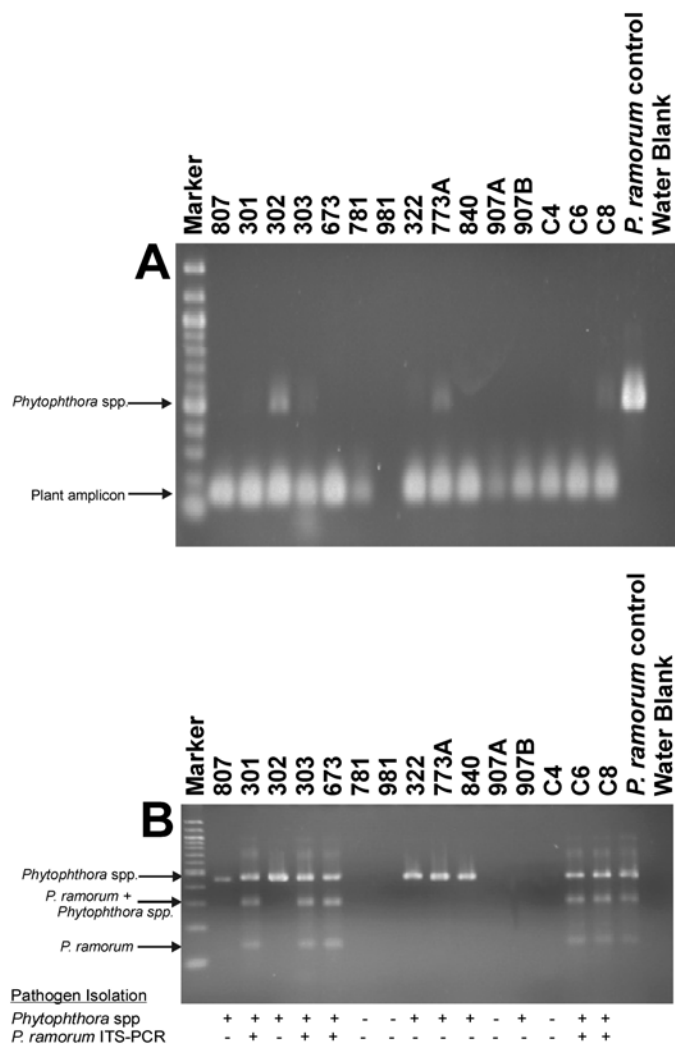
Fig. 9. A, First-round multiplex amplification of field samples with primer pairs FMPl-2b + FMPl-3b and FMPh-8b + FMPh-10b using 2 µl of DNA from the FastPrep extraction. A total of 12 µl of the amplification mixture was added to each well of a 1.5% agarose gel. The molecular size marker is a 100-bp ladder from New England Biolabs. **B,** Second-round multiplex amplification of a 1:25 dilution of the first multiplex amplification using primer pairs FMPh-8b + FMPh-10b (0.1 µM) and FMPr-1a + FMPr-7 (1.0 µM). A total of 12 µl of the amplification mixture was loaded into each well of a 3% NuSieve 3:1 agarose gel. The molecular size marker is a 100-bp ladder from New England Biolabs.

ples plated on selective medium; the lack of amplification of the *Phytophthora* genus-specific amplicon in this sample could be due to another pathogen causing the lesion that was used for DNA extraction. Another exception was amplification of a *Phytophthora* genus-specific amplicon in DNA extracted from symptomatic *U. californica* tissue from which a *Phytophthora* sp. was not cultured. Sequence analysis of the *Phytophthora* genus-specific amplicons confirmed that the band was not an artifact and that they were identical to *P. nemorosa*. Recovery of these pathogens from symptomatic tissue can be inconsistent, with greater success in the wetter winter and spring months and a lower frequency of recovery during the drier summer and fall months (J. Davidson, *personal communication;* C. Blomquist, *unpublished data*). These plant samples were collected on 10 June, which may have contributed to the lack of pathogen isolation.

The *Phytophthora* genus-specific primer pair exhibited a high level of specificity and should be useful as a diagnostic marker for confirming the presence of a *Phytophthora* sp. in diseased tissue. When tested in F. Martin's laboratory with DNA from 29 plant species representing a wide taxonomic range (including reported hosts of *P. ramorum*), *Rhododendron* sp. was the only sample where background amplification was observed. However, this amplification was not consistent across all samples from *Rhododendron* sp. that were evaluated. When the primers were tested with a different thermal cycler at Ft. Detrick, amplification from *Rhododendron* sp. was not observed, but faint background amplifications were observed for *G. max* and *S. tuberosum*, although the amplicon sizes were different in size from the *Phytophthora* diagnostic amplicon. Likewise, the *Phytophthora* genus-specific primer pair exhibited a high level of specificity when evaluated against 30 *Pythium* spp. This may not be important for diagnosis of *Phytophthora* spp. associated with SOD in forest ecosystems because this is a disease of aboveground portions of the plant where *Pythium* spp. would rarely be encountered; however, it could be important for pathogen detection in nursery settings. The annealing temperature and concentration of glycerol are impor-

tant for attaining this specificity. Reduction of annealing temperature by 1°C or elimination of glycerol increased the nonspecific amplification of bands from some plant species, some of which were the same size as the *Phytophthora* diagnostic amplicon. Although the presence of these bands could compromise the use of this marker system for diagnosis of infection by a *Phytophthora* sp. at a genus level, they would not affect detection of *P. ramorum*, *P. nemorosa*, or *P. pseudosyrinigae*, because the species-specific primer pairs did not amplify bands in these samples (data not shown). Including the *Phytophthora* genus-specific primers in the second-round amplification with the species-specific primers enhanced the sensitivity of detection at the genus level, thereby increasing the accuracy of pathogen detection from field samples. However, the concentration of these primers should be lower than the species-specific primers (0.1 µM compared with 1.0 µM for the species-specific primers) to prevent competition in template amplification, reducing the amount of species-specific amplification.

The effectiveness of the marker system was enhanced by using species-specific primers nested within the *Phytophthora* genus-specific amplicon in the second round of amplification. This provided an additional level of primer specificity to prevent nonspecific amplification from plant DNA or other organisms that might be present in infected plant material. It also improved specificity of amplification among *Phytophthora* spp., because occasional faint bands were observed for several species when total DNA rather than the genus-specific amplicon was used as target DNA in amplifications with the *P. ramorum*-specific primer pair (F. N. Martin, *unpublished data*). When the *Phytophthora* genus-specific amplicon was used as target DNA, the species-specific primers were highly specific and did not amplify sequences from any of the 45 other species of the genus tested. This includes the phylogenetically closely related species (33) *P. lateralis* and *P. hibernalis* for the *P. ramorum*-specific primers, as well as *P. ilicis* for the *P. nemorosa*- and *P. pseudosyringae*-specific primer pair. These species-specific primer pairs also amplified target sequences from all isolates of the pathogens evaluated (including

TABLE 2. Symptomatic plant samples collected from the field and subsamples divided for plating on selective medium for pathogen isolation or processed for DNA extraction and diagnosis with molecular markers

| Host | CDFA analysis[a] | | | Mitochondrial marker system[b] | | | | | |
	No. of samples	Pathogen	Culture isolation	*P. ramorum* ITS amp.	Genus-specific	*P. ramorum*-specific	*P. pseudo-syringae*-specific	*P. nemorosa*-specific	Sequence confirmation[c]
Umbellularia californica	14	*Phytophthora ramorum*	12	14	14	14	0	ND[d]	4-*P. ramorum*
	9	*P. ilicis*-like[e]	9	0	5	0	5	ND	5-*P. pseudosyringae*
	…	…	…	0	4	0	0	4	4-*P. nemorosa*
	6	None	0	0	3	0	0	3	3-*P. nemorosa*
Rhododendron sp.	6	None	0	0	2	0	0	ND	1-*P. syringae*
	2	*P. ramorum*	2	2	2	2	0	ND	…
	1	*P. syringae*	1	0	1	0	ND	ND	1-*P. syringae*
Aesculus californica	3	ND	ND	0	0	0	ND	ND	…
Acer macrophyllum	6	ND	ND	0	0	0	1-0, 5-ND	ND	…
Arbutus menziesii	2	ND	ND	0	0	0	ND	ND	…
Sequoia sempervirens	3	None	0	0	0	0	ND	ND	…
Sambucus sp.	1	None	0	0	0	0	ND	ND	…
Salal sp.	1	ND	ND	0	0	0	ND	ND	…
Pseudotsuga menziesii	1	ND	ND	0	0	0	ND	ND	…
Heteromeles arbutifolia	2	None	0	0	0	0	ND	ND	…
Rhamnus californica	1	None	0	0	0	0	ND	ND	…
	1	None	0	0	0	0	ND	ND	…
	1	*Phytophthora* sp.	1	0	1	0	0	ND	…
Manzanita sp.	1	*P. ilicis*-like[e]	1	0	0	0	0	ND	…

[a] Plant samples from the field were processed at the California Department of Food and Agriculture (CDFA) by plating on selective medium and confirming species identification based on morphological criteria and amplification of extracted DNA with the *Phytophthora ramorum*-specific internal transcribed spacer) (ITS primers of Garbelotto et al. (17). Not all samples were cultured for pathogen isolation.

[b] Extracted DNA was amplified using the mitochondrial based *Phytophthora* genus-specific, *P. ramorum*, *P. nemorosa*, and *P. pseudosyringae* species-specific primer pairs.

[c] The *Phytophthora* genus-specific fragment was sequenced and compared to sequence data from known cultures listed in Table 1 to confirm isolate identification.

[d] ND = not done.

[e] *P. ilicis*-like refers to either *P. nemorosa* or *P. pseudosyringae* as noted in Rizzo et al. (35); further classification based on morphology was not done due to the lack of a formal description of the species when the work was done.

the type cultures of *P. ramorum* and *P. nemorosa*). The fact that the species-specific primers were nested within the *Phytophthora* genus-specific amplicon also improved the sensitivity of this detection system by nature of a second-round amplification.

The mitochondrial genome is present in multiple copies per cell, thereby improving the sensitivity of the detection system. Using purified pathogen DNA, the detection limit observed with a single round of amplification with the *Phytophthora* genus-specific primer pair for three isolates of *P. ramorum* was 200 fg of DNA; however, when diluted 1:25 and 1 μl used as target DNA for amplification with the *P. ramorum*-specific primer pair, the limit of detection was 2.0 fg for two of the isolates and 20.0 fg for the third. These values compare favorably with detection limits observed with nuclear encoded molecular markers for detection of other *Phytophthora* spp. Tooley et al. (37) observed detection limits of 1 to 10 pg DNA with *P. infestans* and ITS primers. However, *P. infestans* primers based on families of highly repeated DNA showed 100 times greater sensitivity (10 fg of DNA) compared with ITS based primers (24). Ersek et al. (13) observed detection limits between 20 and 200 pg of DNA for *P. parasitica* and approximately 10 ng of DNA for *P. citrophthora*-specific primers constructed from cloned genomic regions, whereas Schubert et al. (36) reported detection limits of 2 pg for *P. citricola* and 4 pg for *P. quercina* based on primers constructed from cloned random amplified polymorphic DNA bands and 100 pg with *P. cambivora* using primers constructed from the ITS region. Grote et al. (20) developed primer pairs for a nested amplification of *P. nicotianae* and obtained a sensitivity of 2.5 pg following a single round of amplification, but this was increased by 1,000-fold (to 2.5 fg) following a second round of amplification using a pair of nested primers. With the *P. ramorum*-specific primers, nested amplification increased sensitivity by a factor of 100 (200.0 fg for a single amplification and 2.0 fg for a nested amplification).

Adding DNA from healthy plant material in an amount equivalent to what would be encountered when processing field samples reduced the sensitivity of *P. ramorum* detection by 100- to a 1,000-fold depending on the plant species. Addition of DNA from *U. californica* extracted with the Fast Prep DNA extraction kit resulted in an ≈100-fold reduction in sensitivity, whereas DNA from three other plant species reduced the sensitivity by 100- to 1,000-fold. Seven samples of extracted plant DNA also were obtained that completely inhibited PCR amplification, indicating that the procedure did not remove all inhibitors. Grote et al. (20) also observed that adding DNA from a healthy plant influenced the sensitivity of amplification for *P. nicotianae*-specific primers when using purified pathogen DNA, reducing the limit of detection from 2.5 pg and 2.5 fg for single and nested PCR, respectively, to 60 pg and 60 fg, respectively, when the samples were spiked with plant DNA. A similar effect of host DNA on reducing the sensitivity of detection of *Phytophthora* spp. also was reported by Schubert et al. (36); however, in this example, it appeared that there was a differential response of plant species on amplification. Sensitivity was decreased by a factor of 10 when 100 ng of oak DNA was added to a dilution series of purified pathogen DNA, but no reduction in sensitivity was observed when an equivalent amount of DNA from beech was added. These results highlight the need for optimization of extraction methods for isolating DNA from infected plant material to ensure the full sensitivity of the marker system can be obtained. Clearly, the extraction method used in this current investigation needs to be further optimized to take advantage of the level of sensitivity of the described mitochondrial marker system.

The marker system was developed and validated using an Eppendorf Mastercycler thermal cycler in F. Martin's laboratory. When tested at the same location with an MJ Research PTC-100 using the same amplification parameters, poor or lack of amplification of target sequences was observed. However, reducing the annealing temperatures by 1°C provided the same results that

were observed with the Eppendorf Mastercycler. Possible reasons for this include different temperature calibration of the heating block as well as different ramping intervals for the two thermal cyclers (the Eppendorf Mastercycler was 3°C/s, whereas the MJ Research was 1.4°C/s). Some of the experiments also were repeated at Ft. Detrick using an Applied Biosystems model 9700 thermal cycler (ramping interval of 2.3°C/s) with the same results; however, with the *Phytophthora* genus-specific primer pair, the annealing temperature had to be increased by 1°C to reduce background amplification of some plant sequences that were not tested in Salinas. However, by increasing the annealing temperature, several *Phytophthora* spp. no longer were amplified. These results highlight the need for validation of the marker system and the PCR thermal cycler before it can be used as a diagnostic tool. This would include testing amplification parameters and primer specificity with DNA extracted from healthy plants that will be included in the survey, purified cultures of the pathogen or pathogens in question, and from infected plant tissue where the presence of the pathogen has been confirmed by plating on selective medium. For assays of root and crown samples, it also would be advisable to confirm that the *Phytophthora* genus-specific primers did not amplify *Pythium* spp. that may be present.

One potential complication in using this mitochondrial-based marker system for identification of pathogens at the species level is that interspecific hybrids of *Phytophthora* spp. have been identified (4,6,11,12,21,29). The mitochondrial genome is uniparentally inherited (14); therefore, the hybrids would have a single mitochondrial genotype of one of the parents. Depending on which species functioned as the maternal parent and contributed the mitochondria, the use of a species-specific primer pair may amplify a diagnostic band indicating the presence of a particular species when, in fact, the isolate represented a species hybrid. This was observed in natural hybrids of *P. nicotianae* and *P. cactorum*, all of which had the mitochondrial DNA restriction fragment length polymorphism of *P. nicotianae* (29). In the case of *P. ramorum*, *P. nemorosa*, and *P. pseudosyringae*, data from the nuclear-encoded rDNA ITS region and the mitochondrially encoded *cox*II gene indicate that these species are phylogenetically distinct and not hybrids (33); therefore, these concerns would not be relevant for these species.

In summary, the PCR-based detection system using the described mitochondrial encoded markers will provide a useful tool for detection of *P. ramorum* and two other species commonly recovered from symptomatic tissue in California and Oregon. One advantage that this marker system has over others that utilize nuclear encoded genes is the emphasis on detecting the presence of *Phytophthora* spp. in the sample with the first round of amplification rather than focusing on detection of a specific species, thereby improving the diagnostician's ability to determine whether additional pathogens from this genus are present. The system may be used alone or in combination with one of the other PCR-based detection methods now in use for *P. ramorum* to provide a cytoplasmic marker to complement the nuclear-based markers that have been described (2,17,42). Work currently is in progress on the development of the mitochondrial marker system into a fluorescent real-time PCR assay (15) to increase ease of application and result in more rapid diagnosis of sudden oak death. Given the specificity of the *Phytophthora* genus-specific primers, the wide range of *Phytophthora* spp. that they can amplify, and the sequence heterogeneity found in the *cox*I and II spacer region, these primers should have utility for pathogen detection of other *Phytophthora* pathosystems as well.

ACKNOWLEDGMENTS

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