Development of a Multiplex Assay for Genus- and Species-Specific Detection of *Phytophthora* Based on Differences in Mitochondrial Gene Order

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Accepted for publication 28 January 2014.

ABSTRACT

Bilodeau, G. J., Martin, F. N., Coffey, M. D., and Blomquist, C. L. 2014. Development of a multiplex assay for genus- and species-specific detection of *Phytophthora* based on differences in mitochondrial gene order. Phytopathology 104:733-748.

A molecular diagnostic assay for *Phytophthora* spp. that is specific, sensitive, has both genus- and species-specific detection capabilities multiplexed, and can be used to systematically develop markers for detection of a wide range of species would facilitate research and regulatory efforts. To address this need, a marker system was developed based on the high copy sequences of the mitochondrial DNA utilizing gene orders that were highly conserved in the genus *Phytophthora* but different in the related genus *Pythium* and plants to reduce the importance of highly controlled annealing temperatures for specificity. An amplification primer pair designed from conserved regions of the *atp9* and *nad9* genes produced an amplicon of ≈340 bp specific for the *Phytophthora* spp. tested. The TaqMan probe for the genus-specific *Phytophthora* test was designed from a conserved portion of the *atp9* gene whereas variable intergenic spacer sequences were used for designing the species-specific TaqMan probes. Specific probes were developed for 13 species and the *P. citricola* species complex. *In silico* analysis suggests that species-specific probes could be developed for at least 70 additional described and provisional species; the use of locked nucleic acids in TaqMan probes should expand this list. A second locus spanning three tRNAs (*trnM-trnP-trnM*) was also evaluated for genus-specific detection capabilities. At 206 bp, it was not as useful for systematic development of a broad range of species-specific probes as the larger 340-bp amplicon. All markers were validated against a test panel that included 87 *Phytophthora* spp., 14 provisional *Phytophthora* spp., 29 *Pythium* spp., 1 *Phytopythium* sp., and 39 plant species. Species-specific probes were validated further against a range of geographically diverse isolates to ensure uniformity of detection at an intraspecific level, as well as with other species having high levels of sequence similarity to ensure specificity. Both diagnostic assays were also validated against 130 environmental samples from a range of hosts. The only limitation observed was that primers for the 340 bp *atp9-nad9* locus did not amplify *Phytophthora bisheria* or *P. frigida*. The identification of species present in a sample can be determined without the need for culturing by sequencing the genus-specific amplicon and comparing that with a reference sequence database of known *Phytophthora* spp.

Additional keywords: real-time PCR.

Species in the genus *Phytophthora* have had a significant impact on production of economic crop plants and the health of native plant communities. The importance of members of the genus as plant pathogens infecting a wide range of host species is shown by the >11,500 entries in the Systematic Mycology and Microbiology Lab website listing fungal-host reports (http://nt.ars-grin.gov/fungaldatabases/fungushost/FungusHost.cfm). This number is likely to increase in the future, as the number of newly discovered and described species continues to increase. From 2000 to 2007, the number of described species in this genus essentially doubled (9) and, whereas the recent taxonomic updates of the genus by Kroon et al. (27) lists 105 described species, a later review noted 117 species (32). However, these listings are already out of date because there have been a number of new species described as well as provisional species named since these manuscripts were published.

Although the increased availability of sequence data for confirming species identification and conducting phylogenetic analysis has contributed to this increase in species description, perhaps equally important is the increased number of field surveys in forest and commercial nursery environments that have resulted in the identification of new species. An example of this in the United States can be seen with the quarantine species *Phytophthora ramorum*, which was initially found in the forest ecosystem of central coastal California but has since spread to other parts of coastal California and southern Oregon (16,38). *P. ramorum* has been found in the nursery industry and been transported on infected nursery stock from west coast to east coast as well as between states within the eastern and western United States (15,18). Significant effort has been put into surveys to determine the extent of pathogen spread and the inspection of plant shipments to prevent introductions into new locations. This has provided a much better picture of other *Phytophthora* spp. present in the various ecosystems investigated and provided isolates of previously undescribed species. Surveys for *P. ramorum, P. kernoviae*, and *P. alni,* which have caused significant disruptions to natural ecosystem in Europe (9–11), have also resulted

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*The *e*-Xtra logo stands for "electronic extra" and indicates that the online version contains three supplementary tables and sequence alignments of the two loci used for marker design.

http://dx.doi.org/10.1094/PHYTO-09-13-0263-R

in the isolation and description of previously unknown species in Europe.

Given the role of international trade in moving plant material around the world, the consequence of the introduction of invasive species on native ecosystems, and the regulatory needs surrounding the plant trade industry to prevent movement of quarantine species, having a robust molecular diagnostic capability for detection of *Phytophthora* spp. is essential. An ideal marker system would be highly sensitive, so that the pathogen could be detected when present in low amounts; be capable of detecting the pathogen at both a species- and genus-specific level; and allow for the systematic development of species-specific markers. Of particular importance is the availability of a robust genus-specific detection capability that can be run concurrently with species-specific detection. This would allow for a broader analysis of any species that may be present in a sample or ecosystem rather than just be able to determine whether an individual species is present. With the availability of a sequence database of the target locus for the genus, it would be possible to characterize the species community without the need for culturing.

A variety of markers have been designed for conventional and real-time polymerase chain reaction (PCR) assays for *Phytophthora* spp. (32). Although the design of some of these has focused on a particular species using a unique random clone, an amplicon from a random amplified polymorphic DNA assay, or a unique sequence differences in a specific gene, several loci that are conserved within a species but variable between them have been used for the systematic development of multiple species-specific diagnostic markers for conventional and real-time PCRs. The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) has been commonly used for this purpose, as have introns of the *Ras*-related *Ypt1* gene and the spacer region between the *cox1* and *cox2* mitochondrially encoded genes (32). Although a genus-specific detection capability for real-time PCR has been reported for the ITS-based diagnostic assay for *P. ramorum* (26) it is not fully genus specific because several *Pythium* spp. were also detected. Likewise, although there is a genus-specific detection capability using the spacer region between the *cox1* and *cox2* genes, this is dependent on control of the annealing temperature for specificity and is not suitable for real-time PCR diagnostic techniques (34). A genus-specific detection capability has been described for the *Ras*-related *Ypt1* gene but this is with conventional PCR and the ≈470-bp amplicon may not be suitable for real-time PCR (40,41). A real-time PCR probe for *Phytophthora* spp. has been developed by Bilodeau et al. (5) using the β-tubulin region but has not been fully validated against a range of *Pythium* spp.

When developing molecular diagnostic techniques for pathogen detection, it is important to keep in mind that the assay must be reproducible and accurate as well as capable of high-throughput sample processing, with different operators using different equipment in different locations. For example, a variety of thermal cyclers may be used in different facilities and, if a narrow window for annealing temperature is necessary for accurate results, then additional revalidation of thermocyclers to account for variation in temperature calibration and ramping speed among machines may be required. Amplification conditions may also need to be monitored consistently as a means for ensuring the accuracy of results. This requirement increases the amount of work needed to complete the assays but also the potential for unnecessary false-positive and false-negative results and problems with reproducibility of results between different diagnostic facilities. The capability of high-throughput sample processing is also required for some diagnostic labs; therefore, the ability to obtain accurate results with an assay using either a 96-well plate or single tubes is essential.

In an effort to improve the diagnostic capability for *Phytophthora* spp., this study approached the design of a new marker system with several goals in mind: (i) a desire to limit the importance of precisely controlled annealing temperature for maintaining specificity by selecting primer annealing sites that were adjacent in *Phytophthora* spp. but separated in *Pythium* spp. and plants, (ii) a robust genus-specific detection capability was needed that could be multiplexed with the species-specific marker, (iii) a desire to identify a locus that would allow for the systematic development of species-specific markers for a wide range of species, (iv) the target sequence needed to be a high copy number to provide the greatest sensitivity of detection, and (v) the diagnostic marker system needed to include a plant amplicon to serve as a positive control. Due to its high copy number, the mitochondrial genome was selected for designing this new marker system. Having the sequenced mitochondrial genomes for 20 *Phytophthora* and 14 *Pythium* spp. (F. Martin, *unpublished data*) provided the opportunity to identify gene orders that were highly conserved in the genus *Phytophthora* but different in the genus *Pythium* and plants and that could be used for the design of genus-specific amplification primers. Rather than have an amplicon used for genus-specific detection and a second amplicon for species-specific detection, the genus-specific primers were designed so that the amplicon included a highly conserved region that could be used for annealing a genus-specific TaqMan probe as well as variable intergenic spacer sequences for design of species-specific TaqMan probes. Sequencing these regions from a wide range of species allowed us to evaluate the feasibility of this location for the design of species-specific TaqMan probes. This report describes the design of two marker systems and validation of their specificity with culture and environmental samples.

MATERIALS AND METHODS

***Phytophthora*, *Pythium*, *Phytopythium*, and plant species used.** The 667 isolates used in this investigation represented 87 valid and 14 provisional *Phytophthora* spp. (95 of these were used for the core plate used to evaluate all primers and probes) and were from the World *Phytophthora* Genetic Resource Collection at the University of California, Riverside (Table 1). Cultures were grown and DNA extracted as previously described (8). Some additional isolates of *P. megakarya* and *P. palmivora* recovered from cacao in Ghana were also included in the analysis (DNA samples kindly provided by F. Govers). Twenty-nine *Pythium* spp. were used to confirm the specificity of the assay (Table 2); information on their culture and DNA extraction can be found in Martin (30). A range of plant species were also used to evaluate the specificity (Table 2). DNAs were extracted as previously noted (34) or were part of the environmental sample evaluation and were extracted using the United States Department of Agriculture–Animal and Plant Health Inspection Service (USDA-APHIS)-approved DNA extraction protocol (46). To verify the presence of amplifiable *Pythium* DNA, real-time PCR with SYBR Green was performed using universal primers ITS1 and ITS4 (47). PCR was performed using SYBR Green PCR Master Mix 1× (Applied Biosystems, Carlsbad, CA) primers ITS1 and ITS4 at 0.4 μM. PCR analysis was performed in a reaction volume of 25 μl on the ICycler instrument (IQ4; Bio-Rad Laboratories, Hercules, CA). Amplification conditions were one cycle of activation at 95°C for 8.5 min; followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s; finishing with a final cycle of 72°C for 10 min. Melt-curve analysis started at 60°C and increased 0.5°C every two cycles 70 times, with a final incubation at 4°C. To confirm the suitability of plant DNA for PCR amplification, the TaqMan internal plant control probe and primers were used, as described below.

Identification of mitochondrial DNA regions for marker development. Mitochondrial genome data for *P. infestans, P. ramorum* (33), *P. sojae*, and 17 other *Phytophthora* spp. and 14 *Pythium* spp. are part of an ongoing mitochondrial genome

sequencing project (F. Martin, *unpublished data*) which provided us the opportunity to use comparative genomic approaches to identify regions of the genome that might be useful for developing diagnostic markers for *Phytophthora* spp. Plant mitochondrial sequences used for this studies were obtained from GenBank. Gene order differences that were conserved among *Phytophthora* spp. but different in *Pythium* spp. and plants were identified. Three regions were the focus of this investigation: *trnM-trnP-trnM*, *atp9-nad9*, and *nad11-secY.* In *Pythium* spp., the gene order *trnM-trnP* was conserved but the last *trnM* was located >10 kb away and in the opposite orientation, *atp9* and *nad9* were located 18 to 30 kb apart, and *nad11* and *secY* were at least 15 kb apart. This may be observed using comparative genomics of mitochondrial genomes deposited in GenBank (AY894827, AY894828, AY894835, DQ832717, DQ832718, and NC002387 for *Phytophthora* spp. and NC014280 for *Pythium ultimum*).

Amplification and sequencing. For sequencing of the *trnM-trnP-trnM* locus, amplification primers were selected from the flanking genes (*lrn* and *rpl14*); amplification primers for the two other loci were in the indicated genes (Table 3). All amplifications were performed using ≈10 ng of template DNA, 0.4 mM forward and reverse primers, 2 mM $MgCl_2$, 200 μM dNTP, 1× amplification buffer, and 1 unit of AmpliTaq (Applied Biosystems) in a volume of 25 μl. Templates were amplified in an ABI 9600 thermal cycler with the following cycling conditions: (i) for *TrnM-TrnM*, one interval of 95°C for 3 min; 35 cycles of 95°C for 1 min, 1 min of annealing at 61°C, and extension at 72°C for 2 min; and one interval of 72°C for 5 min followed by a 4°C hold; (ii) for *atp9-nad9*, one interval of 95°C for 3 min; 40 cycles of 95°C for 1 min, 1 min of annealing at 61°C, and extension at 72°C for 1 min; and one interval of 72°C for 10 min followed by a 4°C hold; and (iii) for *nad11-secY*, 1 interval of 95°C for 3 min; 40 cycles of 95°C for 30 s, 45 s of annealing at 51°C, and extension at 72°C for 1 min; and one interval of 72°C for 5 min followed by a 4°C hold. After confirming template amplification by running samples on an agarose gel, sequencing templates were treated with ExoSap (USB, Cleveland) according to the manufacturer's instructions and sent to the Penn State Genomics Core Facility of the Huck Institute for Life Sciences (University Park, PA) for sequencing with the amplification primers. Each template was sequenced in both directions to generate a consensus sequence based on complementary strands. Sequencher 4.7 (Gene Codes, Ann Arbor, MI) was used to generate consensus sequences while DS Gene v. 2.5 (Accelrys, San Diego, CA) was used for making alignments.

Marker development. Sequence alignments were examined for highly conserved regions for design of amplification primers and a genus-specific TaqMan probe. Intergenic spacer sequences were also examined for GC content and whether the level of sequence divergence was appropriate for development of species-specific TaqMan probes. The IDT SciTools OligoAnalyser 3.1 (Integrated DNA Technologies Inc., Coralville, IA) was used for primer design. Specific primers were designed so that the nucleotides unique to the target were at the 3′ end position of the primer and a TaqMan probe (20) could be designed in the middle of the amplicon when possible. For TaqMan probe design, the following parameters (6) were used, with some exceptions because of the high A-T content of the these mitochondrial region: melting temperature (T_m) of the TaqMan probe was selected to be 10°C higher (when possible; if not, then at least 3 to 5°C degree higher than the amplification primers) and 15 to 36 bp in length, with the total number of Gs or Cs in the last five nucleotides at the 3′ end of the probe not exceeding two. Mismatching nucleotides responsible for species specificity were positioned as close as possible to the middle of the probe rather than at the ends while avoiding positions with secondary structure (14). The 5′ end of the *atp9-nad9* TaqMan probe for the genus *Phytophthora* was labeled with FAM (fluorescein), and other probes tested with CAL Fluor Red, Hex, or Cy5 (Table 3). The *trnM-trnM Phytophthora* genus-specific probe was labeled with Hex. The 3′ end of all probes was labeled with the Black Hole Quencher-1 (BHQ-1; Biosearch Technologies, Inc., Novato, CA) or BHQ-2, depending on the TaqMan assay. The amplification master mix contained the genus-specific primer pair at 0.5 μM (some primers had a 5′ flap to improve the real-time PCR signal) (1). Depending on the assay, the TaqMan probe concentration varied; it was 0.05 μM for *atp9-nad9 Phytophthora* genus-specific, *Phytophthora cactorum, P. cambivora, P. fragariae*, and *P. syringae* species-specific probes; 0.1 μM for *trnM-trnM Phytophthora* genus-specific, *P. alni, P. cinnamomi, P. citricola* complex*, P. kernoviae, P. lacustris, P. nicotianae, P. palmivora, P. pseudosyringae*, and *P. rubi* species-specific probes; or 0.025 μM for the *P. ramorum* species-specific probe. Other components of the amplification mix included addition of 6 mM $MgCl_2$ and Real Master Mix without Rox (5 Prime; Fisher Scientific Company, LLC, Waltham, MA), with PCR cycling conditions set at 95°C for 2 min, 50 cycles at 95°C for 15 s, and the appropriate annealing temperature for each species-specific TaqMan probe (62°C for the *trnM-trnM* genus-specific assay and the temperatures indicated in Table 4 for the remaining markers) for 1 min 30 s, in a reaction volume of 25 μl on the ICycler instrument. The primers for the plant internal control were present at 0.0125 μM and the probe, which was labeled with CAL Fluor Red and BHQ-2, at 0.01 μM.

Testing of the marker systems for specificity and sensitivity. Two loci, the *trnM-trnM* and *atp9-nad9* regions (Fig. 1), were tested for genus specificity on a core of 87 described and 14 provisional *Phytophthora* spp. (101 total) (Table 1), 29 *Pythium* spp. (Table 2), and 39 plant species (Table 2). The intergenic spacer regions of the *atp9-nad9* locus was chosen for design of the species-specific TaqMan probes because this region contained a higher level of sequence divergence than the *trnM-trnM* locus. Fourteen species-specific TaqMan probes (Table 3) were tested with the core *Phytophthora* sp. plate (Table 1) for specificity. Probes were tested on multiple isolates of the species for which they were designed and other species that had similar sequences at probe annealing sites. The *P. ramorum* and *P. kernoviae* species-specific probes were tested on a panel of additional species with 256 isolates of *Phytophthora* (94 species, with 1 to 6 isolates/species). To determine the limit of sensitivity for genus- and species-specific detection, standard curves using a serial dilution from 1 ng to 10 fg of stock DNA of the target species were evaluated.

Evaluation on field samples. Fields samples were received as a blind test for evaluation with the two *Phytophthora* genus-specific TaqMan assays and the *atp9-nad9* species-specific assay. All samples were run in multiplex with the plant internal control to confirm the ability to amplify the DNA sample.

California Department of Food and Agriculture field samples. Ninety-nine plant samples submitted to the California Department of Food and Agriculture (CDFA) from different locations in Californian had DNA extracted using the standard USDA-APHIS procedure for *P. ramorum* (46). All samples were tested with both the *atp9-nad9* and *trnM-trnM Phytophthora* genus-specific markers. The *atp-9-nad9* assay was multiplexed with *P. ramorum* and *P. kernoviae* species-specific probes, with the following species-specific probes tested individually: *P. syringae, P. cambivora, P. citricola* group, and *P. pseudosyringa*e. To confirm the species that were present, the genus-specific amplicon was sequenced for all samples that were positive for a *Phytophthora* sp. and the sequence compared with our sequence database by BLAST analysis using BioEdit ver. 7.0.9.0 (19).

Big Sur. Field samples were collected on 2 June 2010 at Pfeiffer Big Sur State Park and Andrew Molera State Park, Monterey County, CA. Leaf pieces with lesions and nonsymptomatic leaves were cut with a number 3 cork borer, with half of the leaf disk plated on pimaricin–ampicillin–rifampicin-pentachloronitro-

TABLE 1. *Phytophthora*, *Pythium*, and *Phytopythium* spp. isolate numbers and origins included in this investigation

Species	Isolate number[a]	Country
Phytophthora spp.		
alni	***P10564,*** *P11193, P11318, P16202****,* P10563, P10565, P10566, P10567, P10568, P10569, P16203*, P16206**	France, Poland, Netherlands, Hungary, Sweden
andina	***P13660***	Ecuador
asparagi n.i.[b]	***P10690,*** *P10693, P10707*	New Zealand
austrocedrae	***P15132,*** *P16040*	Argentina
bisheria	***P7191,*** *P11311, P10117*	Netherlands, United States
boehmeriae	*P1257, P1378,* ***P6950,*** *P3963, P3964, P3968, P3969, P7460, P7472, P7790, P13823, P3967, P3970*	Papua New Guinea, Argentina, Taiwan
botryosa	*P1044, P3425, P6944,* ***P6945***	Malaysia, Vietnam
brassicae	***P3273,*** *P10155*	Netherlands
cactorum	*P0714, P10365, P11184,* P3138, P3139, P3405, P6186, P6187, P6472, P0715, P10193, P10194, P10195, P10371, P10372, P10373, P10374, P10770, P10773, P10774, P10775, P11095, P11096, P11272, P11281, P11293, P11317, P11322, P1235, P1615, P1721, P1724, P1725, P3468, P3482, P3730, P6681, P8349, P0472, P1258, P1354, P15078, P15079, P15138, P15142, P15290, P15296, P15687, P3132, P3219, P6224, P6486, P6625, P6677, P6690, P6838	Netherlands, Argentina, Poland, Germany, France, United States, United Kingdom, Zimbabwe, Japan, New-Zealand, South Africa, French Polynesia, India, Taiwan, Australia, Canada
cajani	***P3105***	India
cambivora	***P0592,*** *P1431*, P1432, P3465, P3671, P7140, P6359, P6358, P6360, P10196, P10197, P1995, P1996, P11155, P11556	United States, Australia, Japan, United Kingdom, Poland, Germany
sp. *canalensis*	***P10456***	United States
capsici	***P10386,*** *P1319, P3375, P3605, P6522*	United States, Italy
captiosa	***P10719,*** *P10720*	New Zealand
castaneae (*P. katsurae n.i.*)	*P10187, P6921*	Japan, United States
cinnamomi	*P2100, P2121, P2160, P2301, P3232,* ***P6305,*** P3664, P3665, P10933, P3237, P6492, P6493, P11558, P10781, P15837, P6304, P15821, P15822, P15824, P2183, P3656, P3657, P3658, P3659, P3660, P3662, P11307, P11312, P11320, P15881, P15887, P10162, P10140, P2159, P2370, P2371, P2425, P15314, P15332, P15347, P15348, P15349, P15378, P15883, P2284, P15838, P15839, P6379, P10203, P11596, P11600, P2096, P2138, P2288, P2399, P2424, P2428, P2475, P2400, P2110	United States, Madagascar, South Africa, China, Indonesia, Australia, Germany, Japan, Netherlands, Papua New Guinea, Poland, Portugal, Puerto Rico, Spain, Switzerland, Taiwan, West Sumatra
cinnamomi var. *robiniae*	***P16351,*** P16350	China
citricola	*P0716,* P0845, P1579, P10782	Australia, Taiwan, United States
citricola clade E[c]	P10338, P10366, P6624	Argentina, Ireland, Taiwan
citrophthora	***P10341,*** *P10368, P1212,* P0318	United States, United Kingdom (England), Argentina, Brazil, Australia
clandestina	***P3942,*** *P3943*	Australia
colocasiae	***P6317,*** *P6290, P6102*	Indonesia, India
cryptogea	*P10705,* ***P1088,*** *P11822, P16165, P1739, P3103, P3700*	New Zealand, United States, Colombia, Ecuador
sp. *cuyabensis*	***P8213,*** *P8218*	Ecuador
drechsleri	***P10331,*** *P1087, P11638*	United States
erythroseptica	***P0340,*** *P10382, P1693*	Tasmania, United States, Ireland
europea	P10324, P10325, P10326	France, Germany
fallax	*P10722, P10723,* ***P10725***	New Zealand
foliorum	***P10969,*** *P10971*	United States
fragariae	*P11808, P1435,* ***P3820,*** *P6406,* P10737, P10739, P10743, P10746, P10749, P10752, P10948, P11200, P11804, P11806, P3570, P3821, P11805, P3570, P6368	United States, United Kingdom (England, Scotland), France, Canada, Poland
frigida	***P16051,*** *P16053,* ***P16054,*** *P16059*	South Africa
glovera	*P10618,* ***P10619***	Brazil
gonapodyides	***P7050,*** P7186, P6993, P6988, P6135, P7189, P7002, P6137, P7188, P6986, P6992, P6765, P6998, P6989, P6985, P6990, P7187, P6996, P7000, P7171, P6999, P7006, P6872	United Kingdom (England), New Zealand, United States
hedraiandra	***P11678,*** P11052, P11060, P11061, P11093	Italy, Spain, United States
heveae	*P0578, P1000,* ***P3428,*** *P10167, P8240*	Malaysia, Guatemala, Malaysia, Ecuador
hibernalis	***P3822,*** *P7298, P0647*	Australia, United States
humicola	***P3826,*** *P6701*	Taiwan
idaei	***P6767***	United Kingdom (Scotland)
ilicis	***P3939,*** *P6098, P6099, P6860*	Canada, United States, United Kingdom (England)
infestans	***P10650,*** *P12022, P13198, P15168, P15938, P1594,* P10110, P12038	Mexico, Russia, Ecuador, Netherlands, United Kingdom (England), United States
insolita	*P6703,* ***P6195***	Taiwan
inundata	***P8478,*** *P8479, P8619*	United Kingdom (England), Iran
ipomoeae	***P10225,*** *P10226, P10227,* P10145, P10150	Mexico, United States

(*continued on next page*)

[a] Additional information on isolates (hosts) may be obtained at the website for the World *Phytophthora* Genetic Resource Collection at the University of California, Riverside (available online : http://phytophthora.ucr.edu/databasemain.html); isolates starting with the letter "P" are from this collection. Isolates of all species used for the core plate are in bold face. All isolate numbers in italic were tested with the *P. ramorum* and *P. kernoviae*, ATP9-NAD9 *Phytophthora* genus and TrnM *Phytophthora* genus probes. Asterisks: * = subspecies *alni* (type), ** = subspecies *uniformis* (type), and *** = subspecies *multiformis* (type). Isolates starting with a GH are from Ghana and DNA was provided by F. Govers.

[b] Initials *n.i.* = *noma invalidum*; a well-characterized species but the name is not valid.

[c] *P. citricola* clade E refers to the classification of Jung and Burgess (25).

[d] Not determined.

TABLE 1. (*continued from preceding page*)

Species	Isolate number[a]	Country
iranica	***P3882***	Iran
sp. *kelmania*	***P10613,*** *P1810, P10614*	United States
kernoviae	***P10681,*** *P10956, P10958, P10957, P10671*	New Zealand, United Kingdom (England)
lacustris	*P10337,* P10283, P10284	United Kingdom
sp. *lacrimae*	***P15880***	Netherlands
sp. *lagoariana*	*P8220,* ***P8223***	Ecuador
lateralis	*P3361,* P1728, P3888	United States
macrochlamydospora	***P10267,*** *P8017*	Australia
meadii	***P6128***	India
medicaginis	*P0127,* ***P10683,*** *P7029*	Australia, United States
megakarya	*P1664, P1672,* ***P8516,*** GH-AR 06, GH-AR 08, GH-AR 15, GH-AR 16, GH-AR 18, GH-BAR 17, GH-BAR 21, GH-BAR 26, GH-BAR 28, GH-VR 04, GH-VR 09, GH-VR 10, GH-VR 13, GH-WR 21, GH-WR 47, GH-WR 51, GH-WR 56, GH-WR 60, 327, 328	Nigeria, Cameroon, Sao Tome, Ghana
megasperma	*P10340, P1679,* ***P3136,*** *P3600, P6957*	Australia, Japan, United States
melonis	***P3609,*** *P6870,* ***P1475,*** *P1748*	Japan, China
mengei	***P127*****,** P1275	United States
mexicana	P0646	Mexico
mirabilis	*P10231,* ***P3005,*** *P3010,* P3007	Mexico
multivesiculata	***P10670***	New Zealand
multivora	***P7902*****,** P10300, P10458, P10977, P11094, P11569, P11832, P1817	South Africa, Spain, United States
sp. *napoensis*	*P8225, P8221,* ***P8222***	Ecuador
nemorosa	***P10288,*** *P16352*	United States
nicotianae	***P10297,*** *P10381, P6915, P7146 ,* P1325, P10381, P6915, P7146, P3458, P10802, P10297, P6915, P1494, P7665, P7387, P7330, P1751, P1753, P1752, P7346, P6113, P7522, P0700, P3815, P1577, P6115, P1955, P7622, P16870, P16883, P1083, P1452, P16824, P3813, P1350, P1495, P0583, P1333, P1334, P1335, P0582, P16823, P3234, P6832, P3549, P3456, P7449, P3461, P3118, P7561	United States, China, Germany, Mexico, Venezuela, Japan, Australia, Ponape, South Africa, Spain, Greece, Pakistan, India, United Kingdom, Philippines
sp. *niederhauserii*	*P10279,* ***P10617,*** *P10616, P10976, P16237,* P16384, P7377	Hungary, United States, Netherlands
sp. *novaeguineae*	***P3389,*** *P1256*	Papua New Guinea
sp. *ohioensis*	***P16050***	United States
palmivora	*P6390,* ***P0255,*** *P0113,* P0376, P0633, P0739, P10212, P10213, P10272, P10296, P10336, P10366, P10420, P10422, P10423, P10425, P10769, P10817, P10818, P11005, P11007, P11009, P11010, P11011, P11012, P11013, P11026, P11099, P11175, P1182, P11851, P15825, P16385, P3249, P3502, P6213, P6218, P6375, P7090, P7537, P7551, P8690, P8702, P8766, GH-WR 61, GH-WR 38, GH-ER 18, GH-CR 15, GH-BAR 13, GH-BAR 12, GH-AR 22, 329,* ***P10213***	Indonesia, Costa Rica, United States, American Samoa, Argentina, Ghana, Guam, Indonesia, Malaysia, Philippines, Windward Island
parsiana	***P21282,*** *P21281*	Iran, United States
parvispora	***P8495,*** *P7154,*P8494, P6378, P2404	Germany, Israel, Taiwan
sp. *personii*	***P11555***	United States
sp. *PgChlamyo*	P10669, P6133, P6983, P6997, P6134, P6138	New Zealand, United Kingdom, United States
phaseoli	*P6609, P10150,* ***P10145***	United States
pini	*P0767,* P10204, P10762, P10763, P10764, P10765, P11154, P1632, P1801, P1806	Canada, Poland, United States
pinifolia	***P16100***	Chile
pistaciae	***P6197,*** *P6196*	Iran
plurivora	*P1805*, P0316, P0768, P10185, P10189, P10623, P10627, P10679, P11058, P11100, P11386, P11425, P11426, P11427, P11500, P11833, P11834, P15137, P3543, P6810, P7491	New Zealand, Poland, Slovenia, United Kingdom, United States
polonica	***P15005***	Poland
porri	*P6207, P7899,* ***P10728,*** *P7518*	Switzerland, Denmark, France, Netherlands
primulae	*P10220,* ***P10333***	Germany
pseudosyringae	***P10443,*** *P16355,* P10437, P10444, P16354	Germany, United States
pseudotsugae	***P10339,*** *P10218*	United States
psychrophila	*P10434,* ***P10433***	France, Germany
quercetorum	***P15555***	United States
quercina	***P10334,*** *P10441*	Germany, Serbia
quininea	***P3247,*** *P8488*	Peru
ramorum	*P10102,* ***P10301,*** *P10084, P10090, P10130, P11047, P11122, P11333, P10343, P11051*	Germany, Netherlands, United States, Poland, Belgium
richardiae	***P6875,*** *P3876*	United States
rubi	*P3316,* ***P3289*****,** P6404, P15596	United States, United Kingdom (Scotland), Germany
sansomea	***P3163***	United States
siskiyouensis	***P15122,*** *P15123, P16301*	United States
sojae	*P10704,* ***P3114,*** *P6497, P7061, P0405*	New Zealand, United States, Canada
sp. *sulawesiensis*	***P6306***	Indonesia
syringae	***P10330,*** *P10332, P2004,* P6903, P6901, P11836, P11835, P15090, P15093, P15092, P15094, P7018, P6208, P3013, P3014, P3016, P3015, P3012	Germany, United Kingdom (Scotland), Australia, Switzerland
tentaculata	*P10363,* ***P8497***	Argentina, Germany

(*continued on next page*)

TABLE 1. (*continued from preceding page*)

Species	Isolate number[a]	Country
sp. *thermophilum*	***P10457***	United States
trifolii	***P1462***	United States
tropicalis	***P10329***	United States
uliginosa	***P10328,*** *P10413*	Germany, Poland
vignae	***P3019,*** *P7471*	Australia
Outgroup species		
Pythium sp.	*P8209, P8201*	Ecuador
Pythium sylvaticum	***P15580***	Canada
Phytopythium vexans	***P3980***	n.d.[d]

benzene agar V8 medium (24) for selective isolation of *Phytophthora* spp. and half used for DNA extraction using the USDA-APHIS protocol (46) with the Qiagen DNeasy Plant MiniKit (Qiagen, Valencia, CA). Cultures were checked after a few days to determine whether a *Phytophthora* sp. or *P. ramorum* were growing and with species identification done by conventional morphological classification. DNA extractions were diluted 1/10 in sterile water and tested with the *atp9-nad9 Phytophthora* genus-specific probe and *P. ramorum* and *P. kernoviae* species-specific probes. Results were also independently validated in a PCR hybridization array system (13).

Oregon raspberry: (P. rubi). DNA was extracted from a total of 8 raspberry roots using the previously described USDA-APHIS protocol (46). The DNA was diluted 1/10 and 1/50 and assayed using the *atp9-nad9 Phytophthora* genus-specific assay with *P. rubi* and *P. fra*gariae species-specific probes. Two DNA samples previously extracted from infected raspberry were also included in the assay.

U.K. samples: (P. kernoviae). DNA from leaves naturally and artificially inoculated with *P. kernoviae* were provided by Jennifer Tomlinson (Food and Environment Research Agency, Sand Hutton, York, UK) and tested in the Martin laboratory using the *atp9-nad9 Phytophthora* genus-specific assay and the *P. ramorum* and *P. kernoviae* species-specific probes.

RESULTS

Mitochondrial gene order differences in *Phytophthora* versus *Pythium* and plants. Three conserved mitochondrial gene order differences were observed when the organization of the

TABLE 2. *Pythium* and plant species tested with the *Phytophthora* genus-specific markers and the plant internal control[a]

Species DNA	Sample number	Phy Genus ATP9 probe	Phy Genus TrnM probe[b]	Plant probe	ITS SYBR Green[c]
Pythium[d]					
Pythium oligandrum	81-10	N/A	N/A	N/A	30.4
P. splendens	85-3	N/A	N/A	N/A	26.6
P. spinosum	79-4	N/A	N/A	N/A	27.4
P. ultimum (HS isolate)	23-1	N/A	N/A	N/A	24.9
P. graminicola	1986-1	N/A	N/A	N/A	29.1
P. catenulatum	1986-8	N/A	N/A	N/A	26.7
P. mamillatum	1986-37	N/A	N/A	N/A	33
P. sylvaticum	1987-73	N/A	N/A	N/A	30.4
P. paroecandrum	1987-60	N/A	N/A	N/A	26.1
P. sylvaticum	1987-14	N/A	N/A	N/A	27.6
P. sulcatum	1987-98	N/A	N/A	N/A	26.6
P. nunn	1987-58	N/A	N/A	N/A	28.6
P. myriotylum	1987-134	N/A	N/A	N/A	25.9
P. ultimum	1987-69	N/A	N/A	N/A	26.8
P. irregulare	1987-68	N/A	N/A	N/A	24.6
P. ultimum (HS isolate)	1987-78	N/A	N/A	N/A	27.5
P. ultimum	1987-65	N/A	N/A	N/A	26.8
P. ultimum	1987-92	N/A	N/A	N/A	24.4
P. ultimum spor	1993-25	N/A	N/A	N/A	26
P. aristosporum	1994-15	N/A	N/A	N/A	21.5
P. volutum	1995-105	N/A	N/A	N/A	32.5
P. coloratum	1999-20	N/A	N/A	N/A	25.2
P. dissimile	1999-23	N/A	N/A	N/A	35.7
P. aristosporum	1999-19	N/A	N/A	N/A	32.6
P. vanterpoolii	1999-31	N/A	N/A	N/A	29.7
P. pyrilobum	1999-28	N/A	N/A	N/A	26.4
P. acanthicum	A-6	N/A	N/A	N/A	33.4
P. deliense	1989-1a	N/A	N/A	N/A	24.9
P. arrhenomanes	1991-12	N/A	N/A	N/A	26.1
P. irregulare	1991-15	N/A	N/A	N/A	26.3
Plant species					
Cucumus sativa[e]	…	N/A	N/A	23.3	16.7
Citrullus lantanus[e]	…	N/A	N/A	21.1	12.9

(*continued on next page*)

[a] N/A = no amplification and NT = not tested.
[b] For the TrnM assay, the baseline was 6 to 15 cycles and threshold at 10.
[c] Amplification using the internal transcribed spacer (ITS)1 and ITS4 primers (47).
[d] *Pythium* isolate DNA concentration used was <1 ng/µl; *Pythium* isolate information may be found in Martin (30). *Pythium ultimum* hyphal swelling (HS) isolate, no oospores produced.
[e] Plant DNA concentration was 0.5 to 16 ng/µl.
[f] DNA for plant species from field sample.

mitochondrial genome of *Phytophthora* spp. was compared with *Pythium* spp. and plants. The *trnM-trnP-trnM* region located between the rRNA-large subunit (*rnl*) and ribosomal protein L14 (*rpl14*) spanned a region of ≈258 bp (size varied among species) containing the three indicated tRNAs (Fig. 1; Table 3). Because of the level of conservation of the tRNA coding sequences, it was possible to design highly conserved forward and reverse primers from the *trnM* coding regions (amplicon ≈206 bp) with a genus-specific probe designed from the 3′ end of the first *trnM* coding region. In total, >170 isolates representing a wide range of species in the genus were sequenced and, although there was enough interspecific sequence variation in the coding and spacer sequences to accommodate the design of 38 species-specific probes, this region was not variable enough to use for systematically designing markers for a wide range of *Phytophthora* spp. and was used subsequently as a genus-specific marker only.

The *atp9-nad9* gene order was highly conserved in *Phytophthora* spp. and the presence of highly conserved sequences allowed for the design of amplification primers in the flanking regions of each gene and a *Phytophthora* genus-specific probe in the 3′ end of the *atp9* gene (Fig. 1; Table 3). Amplicons were ≈340 bp (size varied among species) and contained 110 bp of the 3′ end of the *atp9* gene and 85 bp of the *nad9* gene, with the remaining sequences representing intergenic spacer. This region was sequenced for >720 isolates representing 91 valid and 30 provisional *Phytophthora* spp. In total, 84 species-specific probes for *Phytophthora* were designed from this locus (70 in silico) (Supplementary sequence alignment files).

The *nad11-secY* gene order was also highly conserved in *Phytophthora* spp. but the sequence divergence in these genes precluded the development of conserved primers and probe for genus-specific detection in the coding regions. In the spacer region between these two genes, there is a *trnL-trnL* tRNA cluster that is suitable for designing a genus-specific marker (amplicon ≈172 bp) and, although there was enough sequence divergence for designing species-specific TaqMan probes for ≈65 species, the same *trnL-trnL* gene order was present in the *Pythium* mitochondrial genome and, consequently, a problem with primer specificity leading to background amplification of some *Pythium* spp. Additional work with this locus was not pursued.

Evaluation of specificity of genus-specific amplification. The t*rnM-trnM* and *atp9-nad9* genus-specific primers and probe did not amplify (verified by running amplification on agarose gel) or have any background detection of *Pythium* or plant species when tested by real-time PCR (Table 2). When evaluated against a wide range of *Phytophthora* spp. (256 isolates representing 87 valid and 14 provisional *Phytophthora* spp.; culture accessions in italics in Table 1), the t*rnM-trnM* assay detected all species and isolates (data not shown) while the *atp9-nad9* marker also detected all the same isolates and species with the exception of *P. bisheria* and *P. frigida* (Table 4).

Specificity of species-specific TaqMan probes. In total, 14 species-specific probes were tested in multiplex with the genus-specific TaqMan probe for specificity against the core plate of *Phytophthora* spp.; all probes were highly specific and identified only the intended species (Table 4). The probe for the *P. citricola*

TABLE 2. (*continued from preceding page*)

Species DNA	Sample number	Phy Genus ATP9 probe	Phy Genus TrnM probe[b]	Plant probe	ITS SYBR Green[c]
Sequois sempervirens[e]	…	N/A	N/A	33.4	N/A
Prunus sp.[e]	…	N/A	N/A	30.2	21.6
Quercus agrifolia[e]	…	N/A	N/A	20.5	15.6
Citrus sp.[e]	…	N/A	N/A	18.3	14.7
Fragaria x ananassa[e]	…	N/A	N/A	25.1	20.5
Ipomoea batatas[e]	…	N/A	N/A	20.6	N/A
Lithocarpus densiflorus[f]	…	N/A	N/A	23.5	NT
Umbellularia californica[f]	…	N/A	N/A	24.5	NT
Acer sp.[f]	…	N/A	N/A	23.3	NT
Poplar sp.[f]	…	N/A	N/A	22.8	NT
Rubus sp.[f]	…	N/A	N/A	…	NT
Rhododendron sp.[f]	…	N/A	N/A	17.5	NT
Mahonia aquifolium[f]	…	N/A	N/A	18.4	NT
Magnolia grandiflora[f]	…	N/A	N/A	16.7	NT
Xylosma sp.[f]	…	N/A	N/A	18.4	NT
Pieris japonica[f]	…	N/A	N/A	14.7	NT
Osmanthus fragrans[f]	…	N/A	N/A	16.3	NT
Osmanthus heterophyllus[f]	…	N/A	N/A	16.8	NT
Heteromeles arbutifolia[f]	…	N/A	N/A	19.1	NT
Rhamnus californica[f]	…	N/A	N/A	20.0	NT
Kalmia sp.[f]	…	N/A	N/A	20.0	NT
Laurus nobilis[f]	…	N/A	N/A	16.5	NT
Psidium cattleianum[f]	…	N/A	N/A	21.3	NT
Loropetalum chinensis[f]	…	N/A	N/A	19.1	NT
Arbutus unedo[f]	…	N/A	N/A	33.2	NT
Photinia fraseri[f]	…	N/A	N/A	15.7	NT
Schefflera actinophylla[f]	…	N/A	N/A	16.3	NT
Viburnum tinus[f]	…	N/A	N/A	18.1	NT
Sapium sebiferum[f]	…	N/A	N/A	18.8	NT
Camellia japonica[f]	…	N/A	N/A	19.5	NT
Olea sp.[f]	…	N/A	N/A	17.3	NT
Mahonia aquifolium[f]	…	N/A	N/A	19.1	NT
Ficus microcarpa[f]	…	N/A	N/A	16.1	NT
Rhus integrifolia[f]	…	N/A	N/A	28.7	NT
Osmanthus fragrans[f]	…	N/A	N/A	15.3	NT
Tristania sp.[f]	…	N/A	N/A	19.7	NT
Frangula californica[f]	…	N/A	N/A	18.5	NT
Controls					
Phytophthora ramorum P10301	…	22.2	23.2	N/A	21.1
P. palmivora P0255	…	22.9	21	N/A	21
H_2O	…	N/A	N/A	N/A	N/A

complex detected *P. citricola, P. citricola* clade E, *P. multivora, P. pini*, and *P. plurivora*. Further testing of the *P. ramorum* and *P. kernoviae* species-specific TaqMan probes was done with a wide range of *Phytophthora* spp. (256 isolates representing 87 valid and 14 provisional *Phytophthora* spp.; culture accessions in italics in Table 1) and, in all cases, species specificity was observed. To confirm that the plant internal control would not influence the sensitivity or accuracy of detection of these species, the plant

TABLE 3. Primers and probes tested in this study

Primer-probe name	Sequence 5′–3′[a]	Modification	Target	Notes	Standard curve equation[b]	E (%)[c]	R^2
Sequencing primers							
Nad9-F	TACAACAAGAATTAATGAGAAC		ATP9-NAD9 spacer	…	…	…	…
Nad9-R	GTTAAAATTTGTACTACTAACAT		ATP9-NAD9 spacer	…	…	…	…
Lrn-F	CTGAAAGCATCTAAGTAAGA		lrn-TrnM region	…	…	…	…
TrnM-R	GAACCTACATCTTCAGATTA		lrn-TrnM region	…	…	…	…
PhyG_ATP9_nested_F	TTYTGTTTAATGATGGCTTT		ATP9-NAD9 spacer	Nested primer	…	…	…
Real-time PCR primers[d]							
Genus primers, probe							
ATP9-NAD9 region	…	…	…	…	$y = -3.24x + 18.79$	103	0.9939
PhyG_ATP9_2FTail	**AATAAATCATAA**CCTTCTTTACAA CAAGAATTAATG	…	*Phytophthora* sp.	5′ Flap	…	…	…
PhyG-R6_Tail	**AATAAATCATAA**ATACATAATTCA TTTTTATA	…	*Phytophthora* sp.	5′ Flap	…	…	…
ATP9_PhyG2_probeR	AAAGCCATCATTAAACARAATAAA GC	Fam/BHQ1	*Phytophthora* sp.	Probe	…	…	…
TrnM region			*Phytophthora* sp.	…	NA	…	…
PhyG-F2	CGTGGGAATCATAATCCT	…	*Phytophthora* sp.	…	…	…	…
PhyG-Rb	CAGATTATGAGCCTGATAAG	…	*Phytophthora* sp.	…	…	…	…
TrnM_PhyG_probe2	ATRTTGTAGGTTCAARTCCTAYCAT CAT	Hex/BHQ1	*Phytophthora* sp.	Probe	…	…	…
Species-specific probes							
PfraVf_nad9sp_TaqMan2	ATCTCGTAATAGATATATATGTATA TTTAATACGT	Hex/BHQ1	*Phytophthora fragariae*	Probe	$y = -3.21x + 19.52$	105	0.9985
Pcact_nad9sp_probe2	TTACATGTTATATAATTATTAACAC TATTTATAAAA	Quasar670/BHQ2	*P. cactorum*	Probe	$y = -3.14x + 20.94$	108	0.9953
Pker_nad9sp_1Fb	TTTATATTATTCACAGATTTATTAA TTTTTTTCTA	Quasar670/BHQ2	*P. kernoviae*	Probe	$y = -3.11x + 21.91$	110	0.9982
Pram_nad9sp_1F	ACGTTACGTCTAGACTTGTATTATG CATTG	Hex/BHQ1	*P. ramorum*	Probe	$y = -3.21x + 21.56$	105	0.9727
Palni_nad9sp_probe1	AATAGATATATACGTATATTTAAC GCATAATTAGC	Quasar670/BHQ2	*P. alni*	Probe	$y = -2.996x + 22.006$	116	0.9952
Ppalm_nad9sp_probe2	TATAATTACTTAGRCYTGAGTATTT AAATTGAAA	Quasar670/BHQ2	*P. palmivora*	Probe	$y = -3.252x + 19.657$	103	0.996
Psyr_nad9sp_probe1	TACTTTTARCTAAATGTTAACTATT TTTCTAA	Quasar670/BHQ2	*P. syringae*	Probe	$y = -2.952x + 21.067$	118	0.9949
Pcambi_nad9sp_probe1	ATCCTATAATAGGTATATATGTAC ATTTAATGCA	Hex/BHQ1	*P. cambivora*	Probe	$y = -3.34x + 22.973$	99	0.9917
Pcinn_nad9sp_probe1	AAGAAATATTTAGTTTATTAATATA TAATATAACT	Quasar670/BHQ2	*P. cinnamomi*	Probe	$y = -3.063x + 20.498$	112	0.9824
PfraVrubi_Atp9_TaqMan1	ATATATACGTGTATTTAATGCATAA TCAGCTA	Quasar670/BHQ2	*P. rubi*	Probe	$y = -3.1631x + 17.334$	107	0.993
Pcit_nad9sp_T1F	AATAATAGTTTATTTTTTTGATATA TAAATATTTAT	CALFluorRed 610/BHQ2	*P. citricola group*	Probe	$y = -3.043x + 22.058$	113	0.9936
Pnicot_ATP9_Probe1	ATGTTATATCATTATTTTTTATTAT ATATACAAAT	Quasar670/BHQ2	*P. nicotianae*	Probe	NA	…	…
Ppssyr_ATP9_Probe	TTAGATATGTAAGTACTTATAGTGT TTATATT	Quasar670/BHQ2	*P. pseudosyringae*	Probe	$y = -3.362x + 20.457$	98	0.9918
Pgona_nad9sp_probe2	ATAATACACGTATACTTAAACCCTT TTTAGTA	Quasar670/BHQ2	*P. lacustris*	Probe, Salix-soil	$y = -3.546x + 17.936$	91	0.9984
Plant primers and probe							
FMPl2b	GCGTGGACCTGGAATGACTA	…	Plant	…	…	…	…
FMPl3b	AGGTTGTATTAAAGTTTCGATCG	…	Plant	…	…	…	…
Plant CAL-Red probe	CTTTTATTATCACTTCCGGTACTGG CAGG	CALFluorRed 610/BHQ2	Plant	Probe	NA	…	…
ITS universal primers[f]							
ITS1[g]	TCCGTAGGTGAACCTGCGG	…	Universal	SYBR Green	…	…	…
ITS4[g]	TCCTCCGCTTATTGATATGC	…	Universal	SYBR Green	…	…	…

[a] NA = not available.
[b] Sequences in bold at the start of the amplification primers are the 5′ flaps, as described by Afonina et al. (1) and Arif et al. (2).
[c] Efficiency.
[d] PCR = polymerase chain reaction.
[e] Tooley et al. (45).
[f] Internal transcribed spacer (ITS) universal primers used with SYBR Green.
[g] White et al. (47).

TABLE 4. *Phytophthora* spp. core plate tested with the *atp9-nad9 Phytophthora* genus and target species probes

	Cycle threshold (C_t) value at each annealing temperature (°C)[b]														
	Phytophthora genus	*P. alni*	*P. cactorum*	*P. cambivora*	*P. cinnamommi*	*P. citricola* group	*P. fragariae*	*P. lacustris*	*P. kernoviae*	*P. nicotianae*	*P. palmivora*	*P. pseudosyringae*	*P. ramorum*	*P. rubi*	*P. syringae*
Target probes tested[a]	57	60	57	60	57	60	57	57	57	53	57	57	57	60	60
Phytophthora spp.[c]															
alni[d]	22.8	23.9	–	–	–	–	–	–	–	–	–	–	–	–	–
andina	20.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
asparagi n.i.[d]	21.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
austrocedrae[d]	21.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
bisheria[d]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
boehmeriae[d]	20.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
botryosa[d]	21.4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
brassicae[d]	21.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
cactorum[d]	22.7	–	21.9	–	–	–	–	–	–	–	–	–	–	–	–
cajani	24.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
cambivora[d]	25.4	–	–	26.9	–	–	–	–	–	–	–	–	–	–	–
sp. *canalensis*	22.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
capsici[d]	21.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
captiosa[d]	29	–	–	–	–	–	–	–	–	–	–	–	–	–	–
castaneae[d]	21.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
cinnamomi[d]	22.7	–	–	–	22	–	–	–	–	–	–	–	–	–	–
cinnamomi var. *robiniae*[d]	19.7	–	–	–	–	–	–	–	–	–	–	–	–	–	–
citricola complex[d]	20.2	–	–	–	–	20.9	–	–	–	–	–	–	–	–	–
citrophthora[d]	21.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
clandestina[d]	22.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
colocasiae[d]	28.7	–	–	–	–	–	–	–	–	–	–	–	–	–	–
cryptogea[d]	21.7	–	–	–	–	–	–	–	–	–	–	–	–	–	–
cuyabensis[d]	19.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
drechsleri[d]	24	–	–	–	–	–	–	–	–	–	–	–	–	–	–
erythroseptica[d]	22.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
fallax[d]	32.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–
foliorum[d]	22.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
fragariae[d]	19.4	–	–	–	–	–	18.9	–	–	–	–	–	–	–	–
frigida	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
glovera[d]	22.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
gonapodyides[c,d]	25.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
hedraiandra[d]	22.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
heveae[d]	21.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
hibernalis[d]	22.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
humicola[d]	21.8	–	–	–	–	–	–	–	–	–	–	–	–	–	–
idaei	23.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ilicis[d]	25.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
infestans[d]	23.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
insolita[d]	26.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
inundata[d]	22.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ipomoeae[d]	21.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
iranica	23.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
kelmania[d]	22.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–
kernoviae[d]	23.3	–	–	–	–	–	–	–	23	–	–	–	–	–	–
lacrimae[d]	20.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
lagoariana[d]	22.8	–	–	–	–	–	–	–	–	–	–	–	–	–	–
macrochlamydospora[d]	23.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
meadii	21.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
medicaginis[d]	30.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
megakarya[d]	22.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
megasperma[d]	20.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
melonis[d]	21.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
mengei	21.4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
mirabilis[d]	21.4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
multivesiculata	22	–	–	–	–	–	–	–	–	–	–	–	–	–	–

(*continued on next page*)

[a] All baselines were set up at 8 to 16 cycles, excepted for *P. fragariae* (6 to 18); *P. pseudosyringae* and *P. ramorum* were "auto" set up. The threshold was set at 50 for all assays excepted for *P. cactorum* (C_t = 25), *P. kernoviae* (C_t = 100), and *P. palmivora* (C_t = 75).

[b] The DNA preparation could be amplified with primers for other loci but – indicates that these species were not detected by this genus-specific primer and probe combination.

[c] Not tested with the core plate but tested with other isolates: *P. lateralis* P3361; *P. lacustris* P10337, P10283, and P10284; *P. mexicana* P0646; *Pythium* sp. P8209 and P8201; and *P. megasperma* P1679.

[d] Tested again with all isolates of this specific species and closely related species with the same results for specificity.

TABLE 4. (*continued from preceding page*)

	Cycle threshold (C_t) value at each annealing temperature (°C)[b]														
	Phytophthora genus	*P. alni*	*P. cactorum*	*P. cambivora*	*P. cinnamomni*	*P. citricola* group	*P. fragariae*	*P. lacustris*	*P. kernoviae*	*P. nicotianae*	*P. palmivora*	*P. pseudosyringae*	*P. ramorum*	*P. rubi*	*P. syringae*
Target probes tested[a]	57	60	57	60	57	60	57	57	57	53	57	57	57	60	60
napoensis[d]	24	–	–	–	–	–	–	–	–	–	–	–	–	–	–
nemorosa[d]	29.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
nicotianae[d]	27.7	–	–	–	–	–	–	–	–	22.6	–	–	–	–	–
niederhauserii[d]	21.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
novaeguineae[d]	18.8	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ohioensis	22.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
palmivora[d]	20.2	–	–	–	–	–	–	–	–	–	21	–	–	–	–
parsiana[d]	22.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
parvispora[d]	26.7	–	–	–	–	–	–	–	–	–	–	–	–	–	–
personii	22	–	–	–	–	–	–	–	–	–	–	–	–	–	–
phaseoli[d]	23.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
pinifolia	21.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
pistaciae[d]	20.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
polonica	32.7	–	–	–	–	–	–	–	–	–	–	–	–	–	–
porri[d]	29	–	–	–	–	–	–	–	–	–	–	–	–	–	–
primulae[d]	21.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
pseudosyringae[d]	22.4	–	–	–	–	–	–	–	–	–	–	21.8	–	–	–
pseudotsugae[d]	22	–	–	–	–	–	–	–	–	–	–	–	–	–	–
psychrophila[d]	21.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
quercetorum[d]	22.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
quercina[d]	24.4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
quininea[d]	23.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ramorum[d]	22.3	–	–	–	–	–	–	–	–	–	–	–	25	–	–
richardiae[d]	22.4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rubi[d]	21.2	–	–	–	–	–	–	–	–	–	–	–	–	20.2	–
sansomea	19.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
sinensis[d]	23.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–
siskiyouensis[d]	20.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
sojae[d]	24.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
sulawesiensis	21.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
syringae[d]	22.8	–	–	–	–	–	–	–	–	–	–	–	–	–	23.7
tentaculata[d]	35.7	–	–	–	–	–	–	–	–	–	–	–	–	–	–
thermophilum	19.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
trifolii	22.7	–	–	–	–	–	–	–	–	–	–	–	–	–	–
tropicalis	27.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
uliginosa[d]	20.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
vignae[d]	22.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pythium sylvaticum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Phytopythium vexans	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

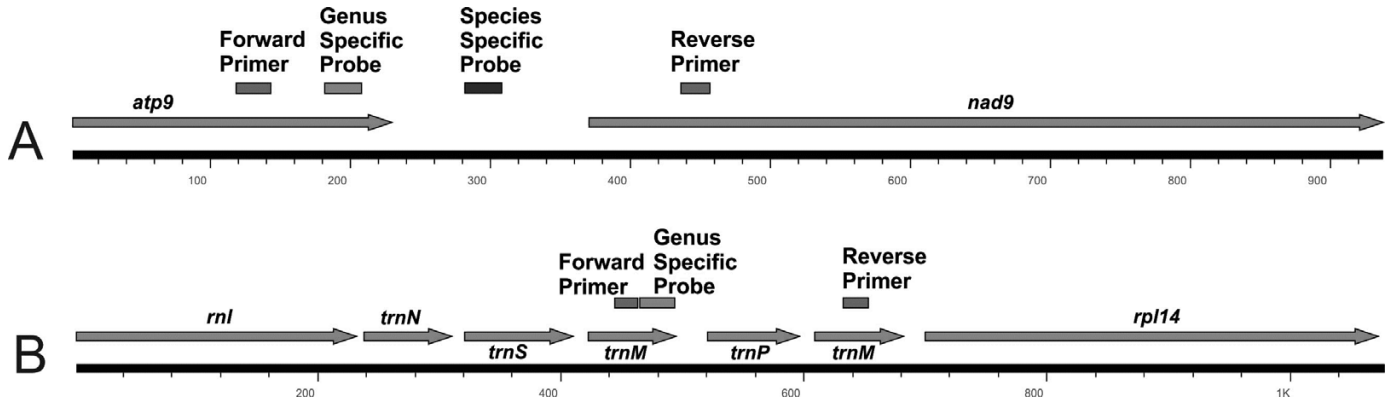

Fig. 1. *Phytophthora* genus-specific primer and probe location on mitochondrial genome: **A,** *atp9-nad9* region and **B,** *rnl-rpl14* region.

amplification primers and TaqMan probe were included in these assays and had no effect on results. When tested against additional isolates of the species the probe was designed to detect, as well as other species with sequence similarity at the probe annealing site, the 12 remaining species-specific TaqMan probes were all species specific (Table 5). This includes the probe specific for the hybrid species *P. alni*, which detected all three subspecies (*P. alni* subspp. *alni*, *multiformis*, and *uniformis*).

Details on specific isolates included in this analysis may be found in Supplementary Table 1a to k, repectively (*P. ramorum* and *P. kernoviae*, *P. alni*, *P. cactorum* and *P. fragariae*, *P. cambivora* and *P. syringe*, *P. citricola* (group), *P. cinnamomi*, *P. nicotianae*, *P. palmivora*, *P. pseudosyringae*, *P. rubi*, and *P. lacustris*). Some of these additional validations were run in multiplex to mimic how the markers may be used in diagnostic labs. The tests for *P. ramorum* and *P. kernoviae* were run together with the *atp9-nad9 Phytophthora* genus-specific probe. To mimic detection of the pathogen in strawberry, *P. cactorum*, *P. fragariae*, and the *P. citricola* complex probes were multiplexed. To mimic detection of a *Phytophthora* sp. in forest or nurseries samples, probes for *P. syringae* and *P. cambivora* were multiplexed.

Standard curve. To evaluate amplification efficiency and the limit of detection, a standard curve for all genus- and species-specific assays was determined using a dilution series of target DNA [$E = (10^{(-1/\text{slope})} - 1) \times 100$] (Table 2). Amplification efficiency was usually close to or slightly greater than 100%. In general, amplification of the *Phytophthora* genus-specific *atp9-nad9* assay exhibited a linear response until the DNA concentration was <100 fg (10^{-4} dilution), with an amplification efficiency of 103%.

Marker validation with field samples. The two genus-specific amplicons and six of the *atp9-nad9* species-specific probes were evaluated with 99 field samples collected by the CDFA in Sacramento, CA (Table 6). In all, 16 different taxonomic entities were identified (11 described species and five unnamed phylogenetic species) in a total of 58 samples; no *Phytophthora* sp. was detected with either genus-specific marker in 41 of the samples. These results agreed with the findings of the CDFA (based on culturing of the isolate or a positive PCR using the accepted USDA-APHIS *P. ramorum* molecular assay). All probes were confirmed to lack nonspecific background detection with these environmental samples and the species specificity for four of the probes was validated; *P. ramorum* was accurately detected in 11 samples with no false positives for the four *P. hibernalis* and three *P. foliorum* samples; *P. pseudosyringae* was detected in three samples with no false positive for the *P. nemorosa* sample; and *P. syringae* was detected in 11 samples with no false positive for the *P. austrocedrae* sample. Three additional samples tested positive for *P. syringae* but these had a sequence identity of 97% when the sequence of the *atp9-nad9* genus-specific amplicon was compared with known *P. syringae* isolates. The species-specific probe for the *P. citricola* complex detected *P. multivora* in five samples, *P. pini* in one sample, and *P. plurivora* in three samples. The only species-specific probes that were not validated in these environmental samples were for *P. kernoviae* and *P. cambivora* (all samples were negative for these species).

In addition to formally described species, several undescribed phylogenetic species were detected. *P*. sp. *aff. colocasiae* 1 (three samples) is a phylogenetic species closely related to *P. colocasiae* but distinct in phylogenetic analysis (F. Martin, *unpublished data*). Likewise, *P.* sp. *aff. brassicae* 1 (two samples from *Schefflera actinophylla*) is another phylogenetic species closely related to *P. brassicae*. Two species were also detected that did not match any of the other sequences in our database or GenBank (samples 1518453-1 and 1556826) and may represent new, undescribed species.

Analysis of the 18 samples collected from Big Sur with the *atp9-nad9* genus-specific and *P. ramorum* and *P. kernoviae* species-specific probes revealed five samples that were culture positive for *P. ramorum*, all of which were also positive with the genus- and species-specific *P. ramorum* probe (Table 7). One sample that was culture negative had a positive real-time PCR result (sample 6) with a relatively low cycle threshold (C_t) of 26.6. No positive detections were observed for the *P. kernoviae* probe. Two additional samples were tested but, because the plant positive control did not amplify, they were not included in the analysis (data not shown). Of the 10 root samples from *Rubus* spp., 7 of them tested positive for *P. rubi* with both the *trnM-trnM* and *atp9-nad9* genus-specific markers. All of these samples also tested positive with the *atp9-nad9 P. rubi* probe (Table 8). None of these samples tested positive with the *P. fragariae* probe, confirming the species specificity of detection of this probe. Of the five samples extracted from tissue infected with *P. kernoviae*, four tested positive with the *atp9-nad9* genus-specific and *P. kernoviae*-specific probe and one tested positive for *P. ramorum* (data not shown). These results agreed with the collaborator's identifications the samples.

DISCUSSION

Comparative mitochondrial genomics was effective for identifying gene order differences useful for developing genus- and species-specific markers for *Phytophthora*. With this approach, we hoped to improve the specificity of the detection assay and provide a technique that would work under a wider range of amplification conditions with a reduced risk of false positives, the thought being that, even if nonspecific primer annealing occurred due to lower stringency (such as reduced annealing temperatures), the intervening distance between the primers would be too great for amplification to occur under the cycling parameters used. The loci that were the primary focus of this investigation had primer annealing sites that were close enough in *Phytophthora* spp. to be useful for designing a TaqMan real-time PCR assay but distant enough (10 kb+) in the related genus *Pythium* and plant species to prevent amplification.

Three regions exhibiting conserved gene order differences in the genus *Phytophthora* were evaluated for developing primers

TABLE 5. Listing of species and number of isolates on which the *Phytophthora* primers and species-specific TaqMan probes were tested on to fully validate specificity

Species-specific probe[a]	Species (number of isolates) tested[b]
Phytophthora alni	*P. alni* subsp. *alni* (7), subsp. *multiformis* (1), subsp. *uniformis* (2), *P. fragariae* (19), *P. rubi* (4)
P. cactorum	*P. alni* subsp. *alni* (2), subsp. *multiformis* (1), subsp. *uniformis* (1), *P. cactorum* (48), *P. fragariae* (20), *P. rubi* (5), *P. hedrianda* (11)
P. cambivora	*P. cambivora* (15), *P. syringae* (18)
P. cinnamomi	*P. cinnamomi* (60), *P. parvispora* (5), *P. cinnamomi* var. *robinae* (2), *P.* sp. *niederhauseri* (7)
P. citricola group	*P. citricola* (7), *P. citricola* clade E (3), *P. multivora* (8), *P. pini* (4), *P. plurivora* (23), *P. citrophthora* (1), *P. europea* (3), *P. lateralis* (3), *P. mengei* (3), *P. mexicana* (1)
P. fragariae	*P. alni* subsp. *alni* (2), subsp. *multiformis* (1), subsp. *uniformis* (1), *P. cactorum* (53), *P. fragariae* (20), *P. rubi* (5), *P. hedrianda* (8)
P. lacustris	*P. lacustris* (3), *P. gonapodyides* (24), *P.* sp. PgChlamydo (5)
P. nicotianae	*P. nicotianae* (46), *P. infestans* (2), *P. cactorum* (1), *P. ipomoeae* (1), *P. mirabilis* (2), *P. phaseoli* (2)
P. palmivora	*P. palmivora* (52), *P. megakarya* (20)
P. pseudosyringae	*P. pseudosyringae* (4)
P. rubi	*P. alni* subsp. *alni* (7), subsp. *multiformis* (1), subsp. *uniformis* (2), *P. fragariae* (19), *P. rubi* (4)
P. syringae	*P. cambivora* (15), *P. syringae* (18)

[a] Probes for *P. ramorum* and *P. kernoviae* were tested against 231 isolates in italics in Table 1 representing 87 described and 14 provisional *Phytophthora* spp.
[b] Isolates used in this validation were different from the core plate of isolates tested in Table 4 unless only a single representative isolate was available.

and probes for multiplex real-time PCR detection: *atp9-nad9*, *trnM-trnM*, and *nad11-secY*. With the exception of the *nad11-secY* locus, the other loci had highly conserved sequences in the flanking genes for design of amplification primers and exhibited polymorphisms in the intervening spacer sequences useful for design of species-specific TaqMan probes. The *atp9-nad9* region was the most useful region because the amplicon size of ≈340 bp was suitable for TaqMan real-time PCR, a single primer pair was used to generate an amplicon where both a genus and species-specific probe could anneal, and the intergenic spacer region exhibited enough sequence divergence that species-specific probes could be designed for the widest range of species. Unfortunately, the genus-specific primer pair was unable to amplify *P. bisheria* and *P. frigida*. Although the reason for this has not been determined conclusively, preliminary data suggest that there is an unusual gene order difference in these species that separates the two target genes (T. Miles and F. Martin, *unpublished data*).

The second region examined spanned three tRNAs (*trnM-trnP-trnM*) located between the *rnl* and *rpl14* coding regions. A single primer pair generated an amplicon from all species of ≈206 bp that contained annealing sites for a *Phytophthora* genus-specific probe and, for some species, a species-specific probe. Species-specific probes could be designed for approximately half the number of species as the *atp9-nad9* locus due to a lower level of sequence divergence than the 340-bp amplicon. For this reason, work with this locus focused only on developing an additional *Phytophthora* genus-specific detection assay. The *nad11-secY* region was the third region examined and, due to the inability to design truly genus-specific primers, work using this locus was discontinued.

The *atp9-nad9* and *trnM-trnM* markers were validated for genus specificity by testing against 29 *Pythium* spp. and 39 plant species as well as a total of 256 isolates representing 87 described and 14 provisional *Phytophthora* spp. Consistent results were also

TABLE 6. Results for real-time polymerase chain reaction (PCR) assays with environmental samples collected from California and tested with *Phytophthora* genus- and species-specific probes for *Phytophthora ramorum, P. kernoviae, P. syringae*, *P. cambivora*, *P. pseudosyringae*, and the *P. citricola* group

		Cycle threshold (C_t) for each dilution and probe[a]									
			Und	1/10	Undiluted		1/10				
Sample	Host, county	Plant probe	TrnM-PhyG	PhyGenus Atp9	*P. ramorum*	*P. kernoviae*	*P. syringae*	*P. cambivora*	*P. citricola* gr.	*P. pseudosyr.*	Confirmed[b]
1470622-8	*Rhododendron* sp., Sacramento	19.6	29.3	28.3	26	N/A	N/A	N/A	N/A	N/A	*P. ramorum*
1556828	*U. californica,* Alameda	18.3	19.6	21.4	18.9	N/A	N/A	N/A	N/A	N/A	*P. ramorum*[c]
1504926-5C	*U. californica,* Monterey	23.7	31.8	29.7	27.7	N/A	N/A	N/A	N/A	N/A	*P. ramorum*[c]
1504927-4	*U. californica,* Monterey	18.8	20.5	21.2 (und)	21	N/A	N/A	N/A	N/A	N/A	*P. ramorum*[c]
1527809	*U. californica,* San Mateo	18.9	34.2	32.2	29.1	N/A	N/A	N/A	N/A	N/A	*P. ramorum*[c]
1527818	*U. californica,* San Mateo	18.9	33.3 (1/20)	30.7 (und)[b]	30.2	N/A	N/A	N/A	N/A	N/A	*P. ramorum*[c]
1527813	*U. californica,* San Mateo	19	34.4	24.3	28.4	N/A	N/A	N/A	N/A	N/A	*P. ramorum*[c]
1536190	*U. californica,* Napa	17.3	37.1	36.9 (und)** 31.2	31.3	N/A	N/A	N/A	N/A	N/A	*P. ramorum*
1441167	*U. californica,* Santa Cruz	18.4	27.7	25.4	24.8	N/A	29.2	N/A	N/A	N/A	*+C +PCR*[c]
1481745	*U. californica,* Sonoma	19.5	33.6 (1/20)	37.3 (und)** 30.9	41.9 (1/20)** 30.2	N/A	N/A	N/A	N/A	N/A	*+pcr*[c]
1481747	*U. californica,* Sonoma	19.4	44	36.1	29.6	N/A	N/A	N/A	N/A	N/A	*+C +PCR*[c]
1504296-34	*Arbutus* sp., Contra Costa	20.3	28.8 (1/20)	29.8	N/A	N/A	N/A	N/A	N/A	N/A	*P. syringae* like
1543967-8	*Ficus microcarpa,* Orange	16.1	33.4	29.6	N/A	N/A	44.2	N/A	N/A	N/A	*P. syringae*
1368945-23	*Leucothoe fontanesiana,* Humboldt	17.1	24.1	24.5	N/A	N/A	27.3	N/A	N/A	N/A	*P. syringae*[c]
1426059-6	*M. grandiflora,* San Diego	27.9	37.4	28.1	N/A	N/A	32.9	N/A	N/A	N/A	*P. syringae*
1426065-17	*M. grandiflora,* San Diego	18.9	29.5	28	N/A	N/A	32.5	N/A	N/A	N/A	*P. syringae*
1426059-7	*M. grandiflora,* San Diego	18.2	35.3	28.1	N/A	N/A	32.3	N/A	N/A	N/A	*P. syringae* like (97% identity)
1345363-58	*Magnolia grandiflora,* San Diego	18.8	44.2	25.5	N/A	N/A	29.1	N/A	N/A	N/A	*P. syringae*
1345363-53	*Photinia fraseri,* San Diego	15.7	21	23.1	N/A	N/A	26.4	N/A	N/A	N/A	*P. syringae*
1413764-2	*Psidium cattleianum,* Solano	21.3	28.8	29.1	N/A	N/A	30.9	N/A	N/A	N/A	*P. syringae*
1290387-23	*Rhamnus californica,* Butte	20.5	22	23.5	N/A	N/A	26.7	N/A	N/A	N/A	*P. syringae*[c]
1555575	*Rhododendron* sp., Lake	19	28.5	27	N/A	N/A	29.8	N/A	N/A	N/A	*P. syringae*[c]
1458992-31	*Rhododendron* sp., San Luis Obispo	17.5	24.6	25	N/A	N/A	28.3	N/A	N/A	N/A	*P. syringae*[c]
1458992-32	*Rhododendron* sp., San Luis Obispo	17.3	25.4	26	N/A	N/A	29	N/A	N/A	N/A	*P. syringae*[c]
1459346-10	*Sapium sebiferum,* San Luis Obispo	18.8	26.1	25.8	N/A	N/A	28.8	N/A	N/A	N/A	*P. syringae* like[c]

(*continued on next page*)

[a] Numbers in parentheses indicate that different amounts of DNA were used in the amplification than indicated in the column heading. A 1/20 refers to using this dilution rather than the 1/10 and und refers to using undiluted DNA. In some cases (**), two C_t values with one of the alternative DNA concentrations previously mentioned are listed; in these examples, two different DNA dilutions were tested. N/A = no amplification and PS = poor sequence data, unable to determine species.

[b] Sequence confirmed. Genus-specific amplicons were sequenced and the results compared against a sequence database generated from DNA from purified cultures of all isolates listed in Table 1.

[c] Species identification was confirmed by culturing the pathogen or a positive amplification with the USDA-APHIS-approved *P. ramorum* molecular diagnostic assay. Cultures were not obtained for all samples.

[d] Based on DNA sequence analysis of the *cox2, nad9, rps10*, and *secY* genes, isolates representing this group are phylogenetically distinct and are closely related to the indicated formally described species (F. Martin, *unpublished data*).

observed between the two different markers when used to evaluate the presence of a *Phytophthora* sp. in the 130 environmental samples evaluated. In an effort to improve the fluorescent signal of the *atp9-nad9* detection and, hence, the sensitivity of the assay, amplification primers were modified with a 5′ flap (tail of AAT AAATCATAA) that did not anneal to target sequences in a similar fashion as reported by Afonina et al. (1) and Arif et al. (2). Using these modified primers improved the amplification efficiency of the reaction, increased the fluorescence signal compared with using unmodified primers, and thereby increased sensitivity of detection.

Due to the level of sequence divergence observed in the spacer region between the *atp9* and *nad9* genes, this region was useful for the systematic design of species-specific TaqMan probes that could be multiplexed with the *atp9-nad9* genus-specific assay. Species-specific probes were validated for 13 species, including the quarantine pathogens *P. ramorum* and *P. kernoviae,* and one species complex. As part of the validation process, specificity was tested against (i) multiple isolates of the target species collected from different geographic regions, (ii) other species that exhibited sequence similarity at the probe annealing site, and (iii) environmental samples. After the work on the specific primer for the *P. citricola* complex was validated in this study, this species complex has been separated into *P. citricola, P. multivora* (42)*, P. plurivora* (25), *P. capensis* (3), *P. pini* (21), and *P. mengei* (22); consequently, the TaqMan probe is specific only for the species complex. The genus-specific amplicon was sequenced for >720 isolates representing 114 species (90 recognized, 17 provisional, and 7 phylogenetically distinct species) (F. Martin, *unpublished data*). *In silico* analysis of this data reveled sequence differences that should support the development of TaqMan probes specific

TABLE 6. (*continued from preceding page*)

		Cycle threshold (C_t) for each dilution and probe[a]									
			Und	1/10	Undiluted		1/10				
Sample	Host, county	Plant probe	TrnM-PhyG	PhyGenus Atp9	*P. ramorum*	*P. kernoviae*	*P. syringae*	*P. cambivora*	*P. citricola* gr.	*P. pseudosyr.*	Confirmed[b]
1353896-26	*L. chinensis,* Los Angeles	20.5	42.4	29.4 (und)	N/A	N/A	N/A	N/A	37.7 (und)	N/A	*P. multivora*
1353896-20	*Loropetalum chinensis,* Los Angeles	19.1	43.3	31	N/A	N/A	N/A	N/A	33.4	N/A	*P. multivora*
1404765-4	*Osmanthus heterophyllus,* Sacramento	16.8	21.8	23	N/A	N/A	N/A	N/A	26.6	N/A	*P. multivora*
1543960-14	*Rhododendron* sp., Orange	28.7	26.5	25.3	N/A	N/A	N/A	N/A	29.7	N/A	*P. multivora*
1503028-1	*U. californica,* Contra Costa	18.1	21.6	22.8	N/A	N/A	30.6	N/A	28.7	N/A	*P. multivora*
1556829	*U. californica,* Alameda	20.4	29.3	34.3	N/A	N/A	N/A	N/A	N/A	N/A	*P. nemorosa*
1470622-10	*Osmanthus fragrans,* Sacramento	16.3	34.6 (1/20)	27.1	N/A	N/A	N/A	N/A	31.8	N/A	*P. pini*
1309588-15	*Rhododendron* sp., Los Angeles	19.2	23.1	23.1	N/A	N/A	N/A	N/A	26	N/A	*P. plurivora*
1543961-25	*Rhododendron* sp., Orange	21.1	28	26.5	N/A	N/A	N/A	N/A	30.1	N/A	*P. plurivora*
1426752-6	*Rhododendron* sp., San Diego	21.1	24.7	24.9	N/A	N/A	N/A	N/A	29.4	45.9	*P. plurivora*
1404765-11	*Laurus nobilis,* Sacramento	16.5	29.9	27.8	N/A	N/A	N/A	N/A	N/A	N/A	*P.* sp. *aff. colocasiae* 1[d]
1470622-9	*Pieris japonica,* Sacramento	14.7	22	23.4	N/A	N/A	N/A	N/A	27.8	N/A	*P.* sp. *aff. colocasiae* 1[d]
1290221-19	Unknown, Amador	19.8	24.6	26.7	N/A	N/A	N/A	N/A	N/A	N/A	*P.* sp. *aff. colocasiae* 1[d]
1537555	*Sequoia sempervirens,* Madera	22.2	27.8	27.6	N/A	N/A	N/A	N/A	N/A	N/A	*P. austrocedrae*
1556835-2	*Umbellularia californica,* Alameda	19.3	22.7	24.9	N/A	N/A	31.9	N/A	N/A	26.8	*P. pseudosyringae*[c]
1504925-1	*U. californica,* Monterey	17.8	22.5	24.5	N/A	N/A	N/A	N/A	N/A	26.6	*P. pseudosyringae*
1504925-19	*U. californica,* Monterey	19.9	43.3	39.3	N/A	N/A	N/A	N/A	N/A	42.1	*P. pseudosyringae*
1543964-18	*Rhododendron* sp., Orange	22.1	31.1 (1/20)	32.9	N/A	N/A	N/A	N/A	N/A	N/A	*P. foliorum*[c]
1543964-19	*Rhododendron* sp., Orange	21	44.7	37.2	N/A	N/A	N/A	N/A	N/A	N/A	*P. foliorum*
1483418-8	*Rhododendron* sp., Stanislaus	20.1	25.1	26.2	N/A	N/A	N/A	N/A	N/A	N/A	*P. foliorum*[c]
1322287-3	*Camellia japonica,* Santa Clara	19.5	36.9	25.8	N/A	N/A	N/A	N/A	N/A	N/A	*P. hibernalis*
1543964-25	*Rhododendron* sp., Orange	20.7	39.7	25.5	N/A	N/A	N/A	N/A	N/A	N/A	*P. hibernalis*
1413767-4	*Rhododendron* sp., Solano	21.3	21.9	22	N/A	N/A	N/A	N/A	N/A	N/A	*P. hibernalis*
1504971	*Xylosma* sp., Marin	18.4	24.5	25.1	N/A	N/A	N/A	N/A	N/A	N/A	*P. hibernalis*
1309587-25	*Schefflera actinophylla,* Los Angeles	16.3	22.8	21.9	N/A	N/A	N/A	N/A	N/A	N/A	*P.* sp. *aff. brassicae*-1 like (93% identity)[d]
1309587-26	*S. actinophylla,* Los Angeles	17.2	34.8	25.9	N/A	N/A	N/A	N/A	N/A	N/A	*P.* sp. *aff. brassicae*-1 like (93% identity)[d]
1470362-41	*Kalmia* sp., Sacramento	20	33.9	27	N/A	N/A	31	N/A	N/A	N/A	*P. tropicalis*[c]
1556826	*Osmanthus fragrans,* Alameda	15.3	21.1	22.1	N/A	N/A	N/A	N/A	27.6	N/A	No homology in database
1518453-1	*Quercus* sp., Merced	18.6	44	32.4	N/A	N/A	N/A	N/A	N/A	N/A	No homology in database
1364175-13	*R. californica,* Santa Clara	19	31.4 (1/20)	31.6	N/A	N/A	N/A	N/A	38.5 (und)	43.1	PS
1364175-12	*Rhamnus californica,* Santa Clara	17.7	47.2	30.6	N/A	N/A	N/A	N/A	N/A	N/A	PS
1422489	*Rhododendron* sp., Alameda	16.1	19.1	21.7	N/A	N/A	25.8	N/A	N/A	N/A	PS-*CDFA* + *P. syringae* culture
1261274-2	*Tristania* sp., Riverside	19.7	46	33.1	N/A	N/A	N/A	N/A	N/A	N/A	PS

for an additional 58 described and 12 provisional species (putative probes in Supplementary Table 2), including some species in the *P. citricola* complex (*P. citricola, P. multivora*, and *P. plurivora*). This means that species-specific markers should be able to be developed for >75% of the species evaluated in this study and, with the use of locked nucleic acids in the probe, the number species-specific probes may increase.

A number of real-time PCR assays have been developed for detection of *Phytophthora* spp., most of which have used primarily SYBR Green or TaqMan technology and either the ITS or *Ypt1* locus as a target for systematically developing primers or probes (32). The mitochondrial assays described herein offer several advantages over those previously available, perhaps most important of which is the genus-specific detection capability. Although a real-time PCR *Phytophthora* genus-specific detection capability has been reported for an ITS-based detection assay, background detection of *Pythium* spp. was noted (26). The use of loci spanning conserved mitochondrial gene-order differences for *Phytophthora* spp. has eliminated this problem for the two diagnostic markers described in this work. Although there were two species that were not amplified by the *atp9-nad9* primer pair, all species were amplified and detected by the *trnM-trnM* detection assay. The ability to confirm whether a *Phytophthora* sp. is present with the use of the genus-specific amplicon rather than just test for a particular species will improve diagnostic capabilities and facilitate a better understanding of the involvement of *Phytophthora* spp. in agricultural and natural ecosystems. With the *atp9-nad9* locus, it will be possible to identify unknown species without culturing by sequencing the amplicon and comparing the data with the sequence database representing >720 isolates of 114 distinct phylogenetic entities (F. Martin, *unpublished data*). When more than one species is present, such as with samples recovered by baiting from streams, cloning may be necessary to generate useful sequence data.

TABLE 7. Results of real-time polymerase chain reaction assays for samples collected from Big Sur, California using the *Phytophthora* genus, *P. ramorum* and *P. kernoviae* species-specific and plant-positive control TaqMan assays

			P. ramorum		Cycle threshold[b]			
Isolate[a]	Host, species	Symptoms	Culture PARP-V8	Dilution	Phy-Genus	*P. ramorum*	*P. kernoviae*	Plant
BS1	Tan oak	Leaf spots	–	1/10	N/A	N/A	N/A	23.5
BS 2	Bay laurel	Leaf spots	+	1/10	30.4	29.6	N/A	24.5
BS 3	Bay laurel	Leaf spots	+	1/10	31.2	30.1	N/A	26.5
BS 4	Maple	Leaf spots	–	1/10	N/A	N/A	N/A	23.3
BS 5	Bay laurel	Leaf spots	+	1/10	26.8	26.7	N/A	24.4
BS 6	Bay laurel	Leaf spots	–	1/10	26.8	26.6	N/A	22.7
BS 7	Live oak	Leaf spots	–	1/10	N/A	N/A	N/A	23
BS 10	Redwood	Red leaves, leaf spots	–	1/10	N/A	N/A	N/A	33.4
BS 11	Bay laurel	Leaf spots	+	1/10	30	29.5	N/A	24.8
BS 12	Tan oak	Leaf spots	–	1/10	N/A	N/A	N/A	20.2
BS 13	Tan oak	Leaf spots	–	1/10	N/A	N/A	N/A	20.5
BS 14	Maple	No symptoms	–	1/10	N/A	N/A	N/A	22.1
BS 15	Bay laurel	No symptoms	–	1/10	N/A	N/A	N/A	21.8
BS 16	Live oak	Few leaf spots	–	1/10	N/A	N/A	N/A	22.9
BS 17	Bay laurel	Leaf spots	+	1/10	25.3	24.6	N/A	23.7
BS 18	Poplar	Leaf spots	–	1/10	N/A	N/A	N/A	22.8
BS 19	Live oak	Few leaf spots	–	1/10	N/A	N/A	N/A	22.7
BS 20	Live oak	Leaf spots	–	1/10	N/A	N/A	N/A	22.1
Controls								
P10130	*P. ramorum*	…	0.39 ng/µl	10^{-1}	23.7	23.4	N/A	N/A
P10130	*P. ramorum*	…	0.039000 ng/µl	10^{-2}	28.1	28	N/A	N/A
P10130	*P. ramorum*	…	0.003900 ng/µl	10^{-3}	36	32.3	N/A	N/A
P10130	*P. ramorum*	…	0.000390 ng/µl	10^{-4}	N/A	N/A	N/A	N/A
P10130	*P. ramorum*	…	0.000039 ng/µl	10^{-5}	N/A	N/A	N/A	N/A
P10130	*P. ramorum*	…	0.000004 ng/µl	10^{-6}	N/A	N/A	N/A	N/A
1571	*P. kernoviae*	…	1 ng/µl	10^{0}	21.7	N/A	23.2	N/A
H_2O	…	…	…	…	N/A	N/A	N/A	N/A

[a] Location of the samples BS1–BS13 were collected in Pfeiffer Big Sur State Park and BS14 to BS20 in Andrew Molera State Park, Big Sur, CA.
[b] N/A = no amplification.

TABLE 8. Results of real-time polymerase chain reaction assays for root samples collected from *Rubus* sp. in Oregon using the *Phytophthora* genus, *Phytophthora rubi*, and *P. fragariae* species-specific and plant-positive control TaqMan assays

	Cycle threshold				
Samples (dilution)	TrnM-PhyG	ATP9-PhyGenus	*P. rubi*	*P. fragariae*	Plant assay
Raspberry root DNA (dil. 1/50)–r1DNA	26.9	29.8	29.6	N/A	22.9
Raspberry root DNA (dil. 1/50)–r2DNA	26.8	31.9	30.4	N/A	22.7
Raspberry root DNA (dil. 1/50)–r3DNA	27.1	32.1	30.7	N/A	26.6
Raspberry root DNA (dil. 1/50)–r4DNA	N/A	N/A	N/A	N/A	24.3
Raspberry root DNA (dil. 1/50)–r5DNA	26.9	30.9	29.6	N/A	24.5
Raspberry root DNA (dil. 1/50)–r6DNA	25.3	29.1	28.1	N/A	24
Raspberry root DNA (dil. 1/50)–r7DNA	24.2	28.2	28	N/A	22.8
Raspberry root DNA (dil. 1/50)–r8DNA	N/A	N/A	N/A	N/A	23.1
Raspberry root DNA (dil. 1/50)–DNA number 1	N/A	N/A	N/A	N/A	25.6
Raspberry root DNA (dil. 1/50)–DNA number 2	28.9	34.4	30.4	N/A	32.6
P. rubi isolate (1/10)	–[a]	23.1	22.3	N/A	N/A
P. fragariae var. *frag*. (1/100)	–	25.9	N/A	25.5	N/A
H_2O	N/A	N/A	N/A	N/A	N/A

[a] – Indicates that samples were not tested.

Like the ITS region, the mitochondrial genome is high copy number, thereby improving the sensitivity of detection. However, unlike the nuclear genome, it is uniparentally inherited; thus, caution should be exercised when using these mitochondrial markers to detect hybrid species because the mitochondrial background of a hybrid isolate may differ depending on which species is the maternal parent (7,17,29,35). Therefore, the assay may not provide an accurate detection of hybrids unless coupled with a nuclear marker assay. A species-specific TaqMan probe was developed for one hybrid species, *P. alni*, and found to be specific for all three subspecies (*P. alni* subspp. *alni*, *multiformis*, and *uniformis*) even though *cox1* and *nadh1* gene sequences were different for *P. alni* subsp. *multiformis* and *P. alni* subsp. *uniformis* (*P. alni* subsp. *alni* had sequences that grouped with either of these subspecies) (23). Sequence alignment of the *Phytophthora* genus-specific amplicon for multiple isolates of this species revealed a highly conserved region used for the TaqMan probe annealing site (Supplementary alignment file). The parental lineages of this species has yet to be identified (23); therefore, it is unknown whether these would cause a nonspecific background detection when using the *P. alni*-specific TaqMan probe.

The appropriateness of using this marker system for pathogen quantification has yet to be fully explored. It needs to be experimentally determined whether there are different amounts of mitochondria present depending on the age and condition of the lesion, which would influence the linear relationship between C_t and level of pathogen colonization. Likewise, if the markers are going to be used for pathogen quantification in the soil, the linear relationship between propagule densities and C_t must be characterized. An internal control has been developed with the *atp9-nad9* amplification primers spanning unique internal sequences using the same approach as the internal control for a *Verticillium dahliae* soil quantification assay (4) to evaluate the suitability of this marker system for soil quantification (G. J. Bilodeau, *unpublished data*). Although the ITS region has been used as a target for real-time PCR quantification assays, the suitability of this locus for this purpose may also be compromised due to variation in copy number. Although variation in copy number of the rDNA repeat has been observed in several Eumycotan fungi (4,12,28,36,37,39) and there is evidence to suggest this occurs with the related genera *Pythium* (31) and *Phytopythium* (44), it has not been experimentally verified for *Phytophthora*.

The multiplexed assay includes a plant positive control, confirming whether the extracted DNA is amplifiable thereby reducing the chance of false negatives. The C_t of the plant amplification can also give some idea of whether PCR inhibitors are affecting amplification efficiency and reducing the sensitivity of pathogen detection. Due to the high amount of plant relative to pathogen DNA in most samples, if the C_t for plant amplification is higher than expected, a sample dilution or additional DNA purification steps may be necessary to be able to accurately detect whether the pathogen is present.

Isolating a *Phytophthora* sp. from some environmental samples can be difficult, sometimes requiring multiple attempts at different times of year to obtain a viable isolate. One approach diagnosticians use to evaluate whether a *Phytophthora* sp. is present is an enzyme-linked immunosorbent assay (ELISA)-based test. This has the advantage of giving reliable results year round, even if the tissue has some decay. However, the ELISA-based tests are not entirely genus specific—they can react with some *Pythium* spp. (32)—therefore, positive results must be scrutinized carefully. It is always useful and sometimes necessary to know what *Phytophthora* sp. is causing damage on a particular host even when a live culture is unobtainable. For example, overseas shipment of raspberry mother plants must be tested for *P. rubi* before phytosanitary certification can be obtained for export. If an ELISA test for a *Phytophthora* sp. is positive for one of these plants, a genus-specific PCR test, such as the ones described in this article, can then be used to determine the species infecting the plant by sequencing the amplicon. A species-specific PCR test can also be used to quickly eliminate *P. rubi* from the list of possibilities. Sequencing is now often less expensive and always faster for preliminary identification of a *Phytophthora* isolate, especially if it is an unfamiliar species.

The multiplexed diagnostic assays described herein provide a useful assay for detection and identification of *Phytophthora* spp. in environmental samples. The genus-specific detection capability will significantly increase diagnostic capabilities over current real-time PCR assays by providing a broader view of the involvement of this genus in causing disease. It will also provide a useful tool for evaluating the presence of this genus in natural ecosystems in the absence of disease symptoms (e.g., stream baiting assays) and, with the extensive sequence database of the *atp9-nad9* locus available, will facilitate species identification by BLAST analysis without the need for culturing. The screening completed to date indicates genus level specificity when tested against *P. vexans* and a range of plant and *Pythium* spp.; however, additional validation against other oomycetes would be advisable when conducting ecological studies to ensure specificity. Although species-specific detection capabilities were demonstrated for 13 species and one species complex, *in silico* analysis suggests that species-specific probes can be developed for ≈75% of the species for which sequence data was collected. Given this level of sequence divergence and the available sequence database for the genus, this locus should be useful for the systematic development of markers for newly described species. The design of the marker system with a single pair of amplification primers and different TaqMan probes for genus- and species-specific detection reduces the potential of affecting amplification efficiency when multiple loci are amplified simultaneously. It also simplifies development of multiplex assays because labeling dyes on the TaqMan probe can be adjusted to provide the detection capabilities needed. The *atp9-nad9* and *trnM-trnM* diagnostic assays both support high-throughput sample processing and will provide sequence data that will be useful for development of other molecular diagnostic assays such as macro (13) or micro arrays (43) for pathogen detection. The sequence alignments used for designing species-specific probes may be found in the supplementary material, and *atp9-nad9* sequences for additional isolates may be found on the *Phytophthora* Database (http://www.phytophthoradb.org) and GenBank.

ACKNOWLEDGMENTS

This project was supported by Agriculture and Food Research Initiative Competitive Grant number 2007-55605-17835 from the USDA National Institute of Food and Agriculture and the USDA Forest Service, Pacific Southwest Research Station, Sudden Oak Death-*Phytophthora ramorum* Research Program Fiscal Year 2010 funds. We thank N. Grunwald (USDA Agricultural Research Service [ARS], Corvallis, OR), J. Gigot (Washington State University), and V. Fieland (Oregon State University) for providing DNA for *P. rubi* assay; all the team from Dr. Martin lab's, USDA-ARS, Salinas, CA; and J. Tomlinson, FERA, UK, for providing *P. kernoviae*- and *P. ramorum*-infected plant DNA for validation of the *P. kernoviae* assay.

NOTE ADDED IN PROOF

For additional information about phylogenetic species and sequence analysis of the *atp9-nad9* locus, see *Fungal Genetics and Biology* 66:19-32.

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