Development of Rapid Isothermal Amplification Assays for Detection of *Phytophthora* spp. in Plant Tissue

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ABSTRACT

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Several isothermal amplification techniques recently have been developed that are tolerant of inhibitors present in many plant extracts, which can reduce the need for obtaining purified DNA for running diagnostic assays. One such commercially available technique that has similarities with real-time polymerase chain reaction (PCR) for designing primers and a labeled probe is recombinase polymerase amplification (RPA). This technology was used to develop two simple and rapid approaches for detection of *Phytophthora* spp.: one genus-specific assay multiplexed with a plant internal control and the other species-specific assays for *Phytophthora ramorum* and *P. kernoviae*. All assays were tested for sensitivity (ranging from 3 ng to 1 fg of DNA) and specificity using DNA extracted from more than 136 *Phytophthora* taxa, 21 *Pythium* spp., 1 *Phytopythium* sp., and a wide range of plant species. The lower limit of linear detection using purified DNA was 200 to 300 fg of DNA in all pathogen RPA assays. Six different extraction buffers were tested for use during plant tissue maceration and the assays were validated in the field by collecting 222 symptomatic plant samples from over 50 different hosts. Only 56 samples were culture positive for *Phytophthora* spp. whereas 91 were positive using the *Phytophthora* genus-specific RPA test and a TaqMan real-time PCR assay. A technique for the generation of sequencing templates from positive RPA amplifications to confirm species identification was also developed. These RPA assays have added benefits over traditional technologies because they are rapid (results can be obtained in as little as 15 min), do not require DNA extraction or extensive training to complete, use less expensive portable equipment than PCR-based assays, and are significantly more specific than current immunologically based methods. This should provide a rapid, field-deployable capability for pathogen detection that will facilitate point-of-sample collection processing, thereby reducing the time necessary for accurate diagnostics and making management decisions.

Additional keywords: sudden oak death.

The genus *Phytophthora* contains some of the most economically devastating plant pathogens on a wide range of host plants, including agricultural crops (14) and forest trees (3,32). Diseases caused by *Phytophthora* spp. include root, stem, crown, and fruit rots and are sometimes difficult to distinguish from other plant pathogens based on symptoms alone (23). In recent years, significantly more species diversity has been identified in the genus *Phytophthora* as molecular data have differentiated taxonomically distinct groups (7,24) and new species have been identified in aquatic ecosystems (16,20,26). Certain Phytophthora diseases are particularly important, such as sudden oak death caused by *Phytophthora ramorum*, a quarantine pathogen which has had a devastating effect on forest ecosystems in several coastal communities, primarily in California and in parts of continental Europe and the United Kingdom (32). This pathogen was also introduced into the nursery production systems of these countries, which has aided in its distribution. Additionally, in 2003, during surveys for *P. ramorum*, a new species later identified as *P. kernoviae* was isolated in the United Kingdom from *Rhododendron* spp. showing leaf and stem necrosis in a woodland area near a commercial nursery. This species could pose a new threat to trees and woodlands in the United States (8).

Currently, *Phytophthora* spp. are detected in a variety of ways, including traditional isolation on selective media, baiting, immunodetection assays, and conventional polymerase chain reaction (PCR) and real-time PCR assays (18,23). Enzyme-linked immunosorbent assay (ELISA) is commonly used to detect *Phytophthora* at a genus-specific level utilizing a generic *Phytophthora* antigen. This technique has successfully detected *Phytophthora* spp. on a variety of plant material and in diverse environments such as irrigation water and soil (2,23) but it is important to note that background detection of some *Pythium* spp. may occur as well (1,22). Species-specific *Phytophthora* PCR markers have been developed that target several nuclear loci (23), including the internal transcribed spacer (15,17,34), ras-related protein *Ypt1* (28,29), β-tubulin and elicitin (4,6), and mitochondrial loci like the *cox1-2* spacer region (25). A few genus-specific PCR based marker systems have also been developed (4,13,21,25,28); however, some have shown cross reactivity with nontarget members of the subclass Peronosporomycetidae, particularly *Pythium* spp. (4,21), or are not suitable for real-time PCR (13,25,28).

Most of the aforementioned detection techniques require a significant time investment for DNA extraction and running the diagnostic assay in the lab or incubation periods to grow the pathogen on media. The ability to identify a *Phytophthora* sp. without the need for a DNA extraction in a short period of time with minimal laboratory equipment would be an important asset to field and laboratory diagnosticians. Isothermal amplification, or the ability to amplify DNA without the aid of a thermocycling

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*The *e*-Xtra logo stands for "electronic extra" and indicates that the online version contains two supplemental figures and two supplemental sequence alignment files.

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apparatus, has the potential to deliver this detection capability. Isothermal amplification reactions incubate at a fixed temperature and are generally more tolerant of inhibitors that reduce the amplification efficiency of PCR (11). The most popular technique is loop-mediated-isothermal amplification (LAMP), and several species-specific assays for *Phytophthora* have been developed using LAMP, including *P. ramorum, P. kernoviae, P. melonis*, and *P. sojae* (9,12,30). Although LAMP technology can be effective, assays can be difficult to design and many of the current assays that have been developed utilize a lateral flow device for detection, which dramatically increases the overall cost. Other techniques such as helicase-dependent amplification (Biohelix Corp., Beverly, MA) utilize two primers and a probe but the technology is proprietary and only a handful of assays are currently available for various foodborne pathogens (33). Recombinase polymerase amplification (RPA) (TwistDx Ltd., Cambridge, UK) is another isothermal technology and kits are available in several formats for development of user-designed assays. The kits are supplied with a lyophilized pellet containing reagents; therefore, all that is needed to prepare an amplification is addition of supplied hydration buffer and user-supplied primers, probe, and 1 µl of sample. To initiate amplification, magnesium acetate is added and mixed and the tube placed in a data collection unit held at 39°C. To ensure a more even distribution of RPA amplicons during amplification and maximum sensitivity, the manufacturer recommends removing the tube after 4 min and mixing again prior to replacement into the data collection unit. The TwistAmp exo kit utilizes two primers 30 to 35 bp long and a labeled probe (46 to 52 bp) to generate an amplicon under 500 bp. RPA is similar to TaqMan real-time PCR in that it offers multiplexed real-time fluorescent DNA detection with FAM- and TAM-labeled probes (although the signal for TAM is much lower than FAM) (27), usually within 5 to 25 min.

Bilodeau et al. (5) recently reported on a systematic approach for development of genus and species-specific TaqMan assays for *Phytophthora* utilizing mitochondrial gene-order differences between *Phytophthora* spp. and similar organisms (i.e., *Pythium* and plant species) to enhance specificity. Highly conserved primers amplified a spacer region and flanking coding regions between the *atp9-nad9* genes that contained a conserved annealing site for a genus-specific TaqMan probe and variable spacer sequences for development of species-specific TaqMan probes. A second locus, *trnM-trnP-trnM*, was also a highly conserved gene order and was highly specific for detection of *Phytophthora* spp. at a genus level using the same approach as with the *atp9-nad9* locus. Based on the specificity and sensitivity of the TaqMan real-time PCR assays that were developed and sequence polymorphisms observed, these loci were chosen as targets for development of an RPA diagnostic assay. The goal of this study was to (i) develop an RPA *Phytophthora* genus-specific detection assay; (ii) identify compatible crude tissue extraction buffers for the RPA system; (iii) develop a systematic approach to construct species-specific RPA markers, particularly for *P. ramorum* and *P. kernoviae*; (iv) develop a method to confirm the identification of the species present in a positive RPA method by DNA sequencing; and (v) develop a rapid, field-portable diagnostic assay that could be used directly at the point of sample collection.

MATERIALS AND METHODS

***Phytophthora*, *Pythium*, *Phytopythium*, and plant species used.** In total, 155 *Phytophthora* isolates were used in this study, representing 136 taxa. These isolates included three different subspecies of *P. alni* (*alni*, *multiformis* and *uniformis*), three phylogenetic groups of *P. cryptogea* (GI, GII, and sp. *kelmania* GIII), six phylogenetically distinct species (sp. aff. *brassicae*-1, sp. aff. *brassicae*-2, sp. aff. *colocasiae*-1, sp. aff. *erythroseptica*, sp. aff. *siskiyouensis*, *cinnamomi* var. *robiniae*, and *citricola* clade E) and two naturally occurring hybrids (*P.* × *pelgrandis* and *P.* × *serendipita*) (19,24). All *Phytophthora* isolates were obtained and are available from the World *Phytophthora* Genetic Resource Collection at the University of California, Riverside (http://phytophthora.ucr.edu/databasemain.html). In order to check for specificity, 21 *Pythium* spp., 1 *Phytopythium* sp., and 6 plant species were tested initially (Table 1). Additional information about the *Pythium* and *Phytopythium* isolates can be found in Martin et al. (25).

Sequence alignments of mitochondrial loci used for targets were constructed using the software program Geneious (v.4.7.6; Biomatters Ltd., Auckland, New Zealand), and included the *trnM-trnP-trnM*, and *atp9-nad9* regions present in *Phytophthora* spp. and the *cox1* region in plants (for development of a plant internal control). The total number of sequences used to develop the marker system was 174 (representing approximately 120 *Phytophthora* taxonomic entities) for *trnM-trnP-trnM*, 633 (including multiple isolates of the same species to assess intraspecific variation in the target) for *atp9-nad9*, and 28 plant taxa for the *cox1* region, which is highly conserved across a broad range of plant species. Additional information on how these sequences were obtained can be found in Bilodeau et al. (5). Primers were primarily designed manually based on alignments; however, melting temperature and GC% were calculated using the OligoAnalyzer Tool 3.1 (Integrated DNA Technologies, Inc., Coralville, IA).

Various DNAs were obtained from the World Phytophthora Collection at a concentration of 10 ng/µl. This concentration was verified periodically using a Nanodrop (Thermo Fisher Scientific, Waltham, MA) instrument. However, for all standard curve plots and limit of detection information provided in this study, a Qubit Florometer and the Quant-iT dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA) were utilized following the manufacturer's instructions, using 10 µl of DNA at 1 ng/µl (based on initial NanoDrop values).

Marker design for RPA. In order to develop a *Phytophthora* genus-specific assay using the TwistAmp exo kit (TwistDx), the *trnM-trnP-trnM* region of the mitochondrial genome was chosen because of its length; in addition, this gene order is highly conserved in all *Phytophthora* spp. but not present in plants or *Pythium* or *Phytopythium* spp. (5). Six forward and six reverse primers were designed (between 25 and 35 bp) to test various combinations and lengths and TrnM-F and TrnM-R were chosen for the greatest sensitivity and specificity. A probe for this assay was also developed, labeled with a FAM dye and Black Hole Quencher 1, and placed near the forward primer in the first *trnM* (TWGM-P1) (Fig. 1A; Table 2). These primers and probes were designed by following the recommendations of the TwistAmp exo kit manufacturer.

For the *Phytophthora* species-specific assays using the TwistAmp exo kit (*P. kernoviae* and *P. ramorum*), the *atp9-nad9* region in the mitochondrial genome was chosen because of the level of interspecific sequence polymorphisms observed and the fact that this gene order is not present in plants or *Pythium* or *Phytopythium* spp. (5). Two forward primers were tested and the most sensitive and specific one became the common forward primer (Atp9-F). Furthermore, a genus-specific probe labeled with a FAM dye and Black Hole Quencher 1 (Atp9-P) was also designed in the *atp9* gene near the forward primer, following the recommendations of the kit manufacturer. Species-specific reverse primers were placed in the *atp9-nad9* spacer region (Atp9Nad9-Pkern-R and Atp9Nad9-Pram-R) (Fig. 1B; Table 2).

For a plant internal control to multiplex with the *Phytophthora* genus-specific assay to check the quality of the DNA in crude tissue extractions, the *cox1* gene was chosen because it was used previously as an internal control in *Phytophthora* PCR assays (5,25,31) and has been shown to work with a broad range of plant species. Various combinations of two forward and two reverse primers were tested and Cox1-IPC-F and Cox1-IPC-R were

chosen for the greatest sensitivity and specificity. A probe for this assay was also developed, and labeled with a TAMRA dye and Black Hole Quencher 2 (Cox1-IPC-P) (Fig. 1C; Table 2). All primers and probes used in this study were obtained from Integrated DNA Technologies, Inc. and Biosearch Technologies, Inc. (Petaluma, CA), respectively.

RPA and TaqMan amplification. For all RPA assays, DNA was pipetted into separate eight-well strip tubes (200 µl in volume), master mix was added and mixed, then transferred to an eight-well strip of the TwistAmp exo kit containing lyophilized reagents and magnesium acetate was applied to the cap. Reactions were centrifuged briefly to uniformly initiate the reaction with the added Mg^{+2}, mixed by inversion to ensure even distribution of Mg^{+2}, briefly centrifuged again, and placed in the Twista (TwistDx) detection device. All assays were carried out at 39°C for a period of 30 min and fluorometric data were collected every 20 s, per the manufacturer's recommendations. To evenly distribute the amplicons throughout the RPA reaction during amplification, data collection was paused after 4 min and the tubes were removed, mixed by inversion, centrifuged, and placed back into the Twista device for continued data collection, causing a loss of a minute of data. A sample was judged as positive or negative during a range of 6 to 25 min using the slope validation evaluation of the Twista Studio software (version 2.06.06), with the following slope validation parameters: four time points or periods collected and an overall slope (in mV/min) of 30 and 12 for the *Phytophthora* and plant internal control RPA assays, respectively. The time point at which an amplification was classified as positive is referred to as the onset of amplification. This was calculated by subtracting the preagitation step of 5 min from the onset of amplification; then, the log of this value was calculated. This value was termed the log of the onset of amplification (OT) and was then was plotted against the log of concentration.

A 50-µl reaction of the multiplexed RPA assay for *Phytophthora* genus specificity and the plant internal control contained the following reagents (primers and probes were all at an initial concentration of 10 µM): 0.1 µl of TrnM-F, 2.9 µl of TRNM-R, 1.25 µl of Cox1-IPC-F and Cox1-IPC-R, 0.6 µl of TrnM-P and Cox1-IPC-P, 29.5 µl of rehydration buffer, 10.3 µl of DNase-free water, 1 µl of template, and 2.5 µl of 280 mM magnesium acetate.

For the species-specific assays (*P. ramorum* and *P. kernoviae*), a 50-µl reaction contained the following reagents (primers and probes were all at an initial concentration of 10 µM): 29.5 µl of rehydration buffer, 11.9 µl of DNase-free water, 1 µl of template, 2.5 µl of 280 mM magnesium acetate, and 0.6 µl of Atp9-F and 3.6 µl of Atp9Nad9-Pram-R for *P. ramorum* or 0.2 µl of Atp9-F and 4.0 µl of Atp9Nad9-Pkern-R for *P. kernoviae*.

Testing various crude extraction buffers. Several extraction buffers were tested for their effectiveness to extract DNA from plant tissue samples for RPA assays. Five ELISA grinding buffers were tested, including two from Agdia, Inc. (Elkhart, IN), GEB2 (ACC 00130) and GEB3 (ACC 00360); and a standard ELISA grinding buffer (containing 100 ml of 10× phosphate-buffered saline buffer, 2 g of bovine serum albumen, 20 g of PVP-40, and 0.5 ml of Tween 20, brought to a final volume of 1 liter) at various pH levels (8.0, 9.5, and 11.0). Additionally, a polyethylene glycol (PEG) buffer (containing 60 g of PEG 200 with 0.93 ml of 2 M KOH and 39 ml of water at pH 13.5) was also tested (10). All six buffers were tested by taking 0.5 g of fresh *Fragaria* × *ananassa* crown tissue and 5 ml of extraction buffer and macerating the tissue in a plastic mesh bag (ACC 00930; Agdia, Inc.) with a tissue homogenizer tool (ACC 00900; Agdia,

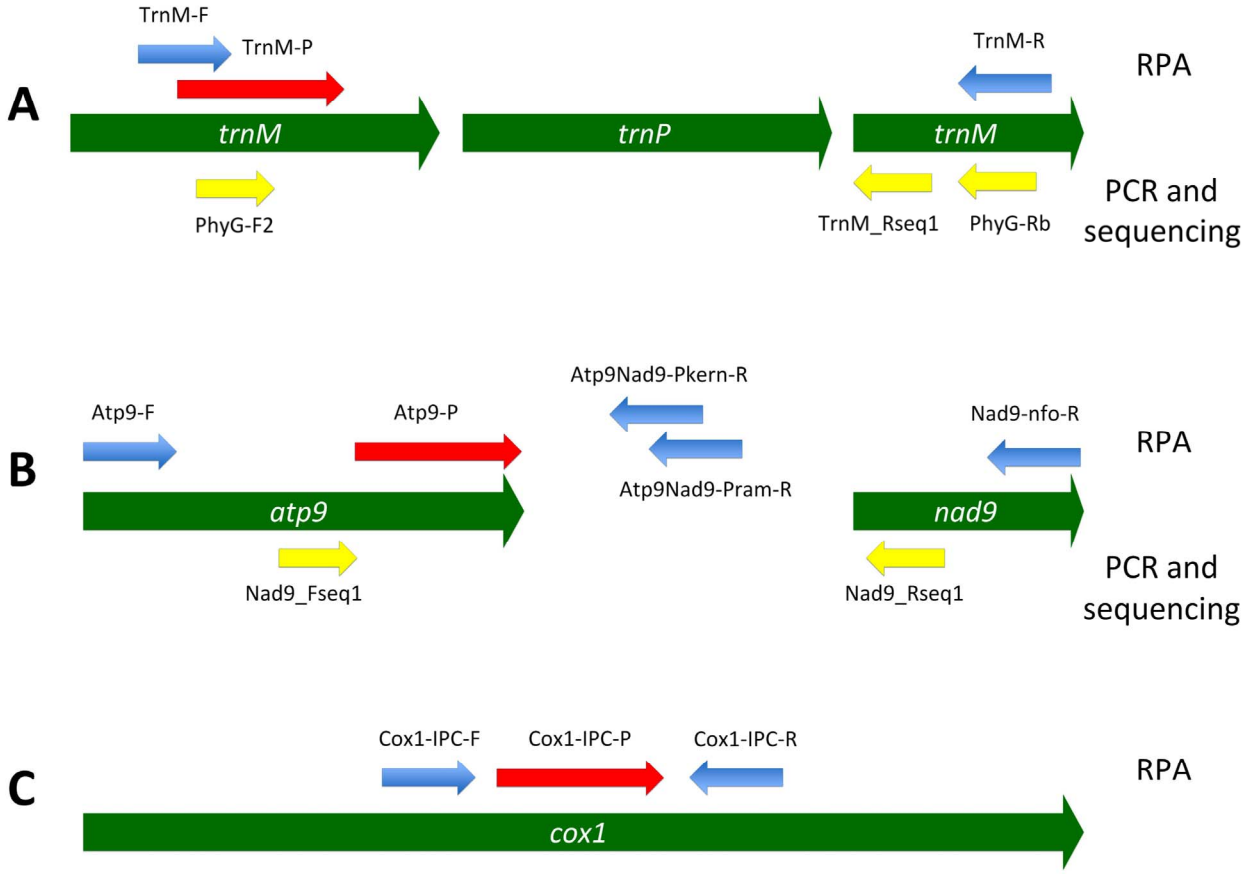

Fig. 1. Mitochondrial loci used in this study for **A,** the genus-specific detection of *Phytophthora*; **B,** the *Phytophthora* spp.-specific assays (*Phytophthora kernoviae* and *P. ramorum*); and **C,** the plant internal control. Also denoted is the location of primers and probes used in recombinase polymerase amplification (RPA) detection and the location of nested polymerase chain reaction (PCR) and sequencing primers used in the confirmation of a positive product. Refer to Table 2 for the nomenclature of these primers and probes.

TABLE 1. List of *Phytophthora*, *Pythium*, *Phytopythium*, and plant species included in this investigation, their isolate numbers, and origin of recovery

Species	Isolate number[a]	Host species	Country
Phytophthora spp.			
sp. aff. *brassicae-1*	P10728	*Daucus carota*	France
sp. aff. *brassicae-2*	P6207	*Allium cepa*	Switzerland
sp. aff. *colocasiae-1*	P10341	*Syringa* sp.	England
sp. aff. *erythroseptica*	P10811	*Zantedeschia aethiopica*	Japan
sp. aff. *siskiyouensis*	P1200	*Theobroma cacao*	Brazil
sp. *brasilensis*	P0630	*Theobroma cacao*	Brazil
sp. *cuyabensis*	P8213	…	Ecuador
sp. *hungarica*	P10281	…	Hungary
sp. *kelmania* (*P. cryptogea* GIII)	P10613	*Abes fraseri*	United States
sp. *erwinii*	P3132	*Banksia integrifolia*	Australia
sp. *napoensis*	P8221	…	Ecuador
sp. *ohioensis*	P16050	…	United States
sp. *personii*	P11555	*Nicotiana tabacum*	United States
sp. *PgChlamydo*	P10669	*Idesia polycarpa*	New Zealand
sp. *sulawesiensis*	P6306	*Syzygium aromaticum*	Indonesia
sp. *thermophium*	P10457	…	United States
alni subsp. *alni*	P16203	*Alnus glutinosa*	The Netherlands
alni subsp. *multiformis*	P16202	…	…
alni subsp. *uniformis*	P16206	*Alnus* sp.	Sweden
alticola	P16948, P19861	…	…
amnicola	P19862	…	…
andina	P13365	*Solanum brevifolium*	Ecuador
asiatica	P19977	…	…
asparagi	P10690	*Asparagus officinalis*	New Zealand
austrocedrae	P15132	*Austrocedrus chilensis*	Argentina
bahamensis	P3930	…	Bahamas
bisheria	P10117	*Fragaria* sp.	United States
boehmeriae	P6950	*Boehmeriae nivea*	Taiwan
botryosa	P3425	*Hevea brasiliensis*	Malaysia
brassicae	P10414	*Brassica oleraceae*	The Netherlands
cactorum	P0714	*Syringa vulgaris*	The Netherlands
cajani	P3105	*Cajanus cajani*	India
cambivora	P0592	*Abies procera*	United States
canalensis	P10456	…	United States
capensis	P1819	*Curtisia dentata*	South Africa
capsici	P3605	*Capsicum annuum*	United States
captiosa	P10719	*Eucalyptus saligna*	New Zealand
cichorii	P19844	…	…
cinnamomi	P2110	*Cinnamomum burmannii*	Indonesia
cinnamomi var. *robiniae*	P16350	*Robinia pseudoacacia*	China
citricola	P0716, P6880	*Citrus sinensis, Fragaria* × *ananassa*	Taiwan, Bulgaria
citricola clade E[b]	P1321	*Rubus* sp.	United States
citrophthora	P6310, P0318	*Theobroma cacao, Citrus*	Indonesia, Australia
clandestina	P3942	*Trifolium subterraneum*	Australia
colocasiae	P6317	*Colocasia esculenta*	Indonesia
cryptogea GI	P1088, P7788	*Callistephus chinensis, Daucus carota*	United States, United Kingdom
cryptogea GII	P3103	*Solanum marginatum*	Ecuador
dauci	P19845	…	…
drechsleri	P10331	*Gerbera jamesonii*	United States
elongata	P19597	…	…
erythroseptica	P1699	*Solanum tuberosum*	United States
europaea	P10324	*Quercus rhizosphere*	France
fallax	P10725	*Eucalyptus fastigata*	New Zealand
fluvialis	P19584	…	…
foliorum	P10969	*Rhododendron* sp.	United States
fragariae	P3821	*Fragaria* × *ananassa*	United Kingdom
frigida	P16059	*Eucalyptus* sp.	South Africa
gallica	P16826	…	…
gemini	P15880	*Zostera marina*	The Netherlands
gibbosa	P19586, P19587	…	…
glovera	P10619	*Nicotiana tabacum*	Brazil
gonapodyides	P6135	*Ilex* sp.	United Kingdom
gregata	P19588	…	…
hedraiandra	P11056	*Rhododendron* sp.	United States
heterospora	P19902, P19903	…	…
heveae	P3428, P1000, P0578	*Hevea brasiliensis, Persea americana, Theobroma cacao*	Malaysia, Guatemala, Malaysia
hibernalis	P3822	*Citrus sinensis*	Australia

(*continued on next page*)

[a] All isolate numbers were tested with the *P. ramorum* and *P. kernoviae* ATP9-NAD9 spacer and *Phytophthora* genus-specific tRNA-M-tRNA-M probes. Additional information on isolates (hosts) may be obtained at the website for the World Phytophthora Genetic Resource Collection at the University of California, Riverside (http://phytophthora.ucr.edu/databasemain.html).

[b] *P. citricola* clade E refers to the classification of Jung and Burgess (19).

[c] Background information on *Pythium* isolates used in this study can be found in Martin et al. (25).

TABLE 1. (*continued from preceding page*)

Species	Isolate number[a]	Host species	Country
himasilva	P19820	…	…
humicola	P3826	…	Taiwan
hydropathica	P16857	…	United States
idaei	P6767	*Rubus idaeus*	United Kingdom
ilicis	P3939	*Ilex* sp.	Canada
infestans	P10650	*Solanum tuberosum*	Mexico
insolita	P6195	…	Taiwan
inundata	P8478	*Aesculus hippocastanum*	United Kingdom
ipomoeae	P10225	*Ipomoea longipedunculata*	Mexico
iranica	P3882	*Solanum melongena*	Iran
irrigata	P16861	…	United States
katsurae	P10187, P1372, P15169	*Castanea crenata, Cocos nucifera, Agathis australis*	Japan, United States, New Zealand
kernoviae	P10681	*Annona cherimola*	New Zealand
lactucae	P19872, P19875	…	…
lacustris	P10337	*Salix matsudana*	United Kingdom
lagoariana	P8217	…	Ecuador
lateralis	P3888	*Chamaecyparis lawsoniana*	United States
litoralis	P19590, P19591	…	…
macrochlamydospora	P10267	*Glycine max*	Australia
meadii	P6128, P6262	*Elettaria cardamomum, Hevea brasiliensis*	India, India
medicaginis	P10683	*Medicago sativa*	United States
megakarya	P8516	*Theobroma cacao*	Sao Tome
megasperma	P1679, P3136	*Malus sylvestris, Brassica napus* var.. *napus*	United States, Australia
melonis	P10994	*Trichosanthes dioica*	India
mengei	P1273, P0911	*Persea americana, Persea americana*	United States, United States
mexicana	P0646	*Solanum lycopersicum*	Mexico
mirabilis	P3005	*Mirabilis jalapa*	Mexico
multivesiculata	P10410	*Cymbidium*	The Netherlands
multivora	P1821, P7902, P19594	*Ocotea bullata, Pinus radiata*	South Africa, United States
nemorosa	P10288	*Lithocarpus densiflorus*	United States
nicotianae	P1452	*Citrus* sp.	United States
novaeguine	P3389	*Auracaria*	New Guinea
sp. *niederhauserii*	P10617	*Thuja occidentalis*	United States
pachypleura	P19986, P19987, P19988	…	…
palmivora	P0255	*Theobroma cacao*	Costa Rica
parsiana	P15164	*Ficus carica*	Iran
parvispora	P8495	*Beaucamea* sp.	Germany
phaseoli	P10145	*Phaseolus lunatus*	United States
pini	P0767, P10204	*Syringa* sp., *Rhododendron* sp.	Canada, United States
pinifolia	P16100	*Pinus radiata*	Chile
pistaciae	P6197	*Pistacia vera*	Iran
plurivora	P10679	*Juglans regia* L.	New Zealand
polonica	P15005, P19522	…	Poland
porri	P7518	*Allium porrum*	The Netherlands
primulae	P10333	*Primula acaulis*	Germany
pseudosyringae	P10437	*Quercus robur*	Germany
pseudotsugae	P10339	*Psendotsuga menziesii*	United States
psychrophila	P10433	*Quercus robur*	Germany
quercetorum	P15555	*Quercus rubra rhizosphere*	United States
quercina	P10334	*Quercus robur*	Germany
quininea	P3247	*Cinchona officinalis*	Peru
ramorum	P10301	*Rhododendron* sp.	The Netherlands
richardiae	P6875	*Zantedeschia aethiopica*	United States
riparia	P19799	…	…
rosacearum	P3315	*Prunus persica*	United States
rubi	P3289	*Rubus* sp.	United States
sansomeana	P3163	*Silene latifolia* subsp. *alba*	United States
sinensis	P1475	…	…
siskiyouensis	P15122	Seasonal tributary	United States
sojae	P3114	*Glycine max*	United States
syringae	P10330	*Rhododendron* sp.	Germany
tentaculata	P8497	*Chrysanthemum leucanthemum*	Germany
trifolii	P7010	*Trifolium* sp.	United States
tropicalis	P10329	*Macadamia integrifolia*	United States
uliginosa	P10413	Rhizosphere of *Quercus robur*	Poland
vignae	P3019	*Vigna unguiculata*	Australia
virginiana	P19828, P19829	…	…
× *pelgrandis*	P19984	…	…
× *serendipita*	P19985	…	…
Pythium and *Phytopythium* spp.[c]		…	…
Pythium sp.	1989-19	…	…
Pythium acanthicum	A-6	…	…
P. aphanidermatum	1991-5	…	…
P. aristosporum	1999-19	…	…

(*continued on next page*)

Inc.). Then, 1 µl from each bag and 1 µl of *P. cactorum* DNA (P0714) at 0.3 ng/µl was loaded into the multiplexed RPA assay described above. The buffer with the earliest onset of amplification for both the pathogen and the plant was considered the optimal buffer for tissue maceration.

Testing of RPA markers for sensitivity and specificity. To test the sensitivity of the multiplexed RPA assay, purified DNA of *P. cactorum* (P0714), *P. cinnamomi* (P2110), and *P. ramorum* (Prg-2) was loaded separately into a reaction at a concentration ranging from 3 ng to 1 fg (based on Qubit Florometer values).

TABLE 1. (*continued from preceding page*)

Species	Isolate number[a]	Host species	Country
P. arrhenomanes	1991-9	…	…
P. catenulatum	1986-8	…	…
P. graminicola	1992-13	…	…
P. insidiosum	58643	…	…
P. irregulare	1987-97	…	…
P. myriotylum	1987-134	…	…
P. nunn	1987-58	…	…
P. oligandrum	C3	…	…
P. paroecandrum	1987-60	…	…
P. pyrilobum	1999-28	…	…
P. rostratum	1986-14	…	…
P. spinosum	79-4	…	…
P. splendens	85-3	…	…
P. sulcatum	1987-98	…	…
P. sylvaticum	1991-23	…	…
P. ultimum (HS isolate)	23-1	…	…
P. ultimum	1987-92	…	…
P. ultimum	P2006	Pea, cucumber	United States
P. undulatum	P10342	…	…
P. vanterpoolii	1999-31	…	…
Phytopythium vexans	P8419, P3980	*Solanum tuberosum*	Canada
Plant species	…	…	…
Citrus sp.	…	…	United States
Fragaria × *ananassa*	…	…	United States
Umbellularia californica	…	…	United States
Persea americana	…	…	United States
Rhododendron sp.	…	…	United States
Rubus sp.	…	…	United States

TABLE 2. Primers and probes used in this study for genus-specific *Phytophthora* detection, plant internal control, and species-specific *Phytophthora ramorum* and *P. kernoviae* detection with the TwistAmp exo and nfo kits (TwistDx Ltd., Cambridge, UK)[a]

Primers, probes	Sequence (5′–3′)	Length (bp)	GC (%)	Target
Primers				
Phytophthora genus-specific				
TrnM-F	ATGTAGTTTAATGGTAGAGCGTGGGAATC	29	41.4	tRNA-M
TrnM-R	GAACCTACATCTTCAGATTATGAGCCTGATAAG	33	39.4	tRNA-M
Atp9-F	CCTTCTTTACAACAAGAATTAATGAGAACCGCTAT	35	34.3	ATP9
Nad9-nfo-R	GTAGAAATATTAATACATAATTCATTTTTRTA	32	14.1	NAD9
Plant internal control				
Cox1-IPC-F	CATGCGTGGACCTGGAATGACTATGCATAGA	31	48.4	COXI
Cox1-IPC-R	GGTTGTATTAAAGTTTCGATCGGTTAATAACA	32	31.3	COXI
Phytophthora spp.-specific				
Atp9Nad9-Pram-R	CAATGCATAATACAAGTCTAGACGTAACGT	30	36.7	*P. ramorum* ATP9-NAD9 spacer
Atp9Nad9-Pkern-R	ATATTACAAGATATATATTTATATTATTCACAG	33	15.2	*P. kernoviae* ATP9-NAD9 spacer
Nested PCR and sequencing				
PhyG-F2	CGTGGGAATCATAATCCT	18	44.4	tRNA-M
PhyG-Rb	CAGATTATGAGCCTGATAAG	20	40.0	tRNA-M
TrnM_Rseq1	ACCTATTACTCTATCTCATC	20	35.0	tRNA-M
Nad9 _Fseq1	GCATTAACTGAAGCAATTGC	20	40.0	ATP9
Nad9_Rseq1	GTAAAATTTGTAATAAATATTGACT	25	16.0	NAD9
Probes				
Phytophthora genus				
TrnM-P	TAGAGCGTGGGAATCATAATCCTAATGTTG [FAM-dT] A [THF] G [BHQ1-dT] TCAAATCCTACCATCAT [3′-C3SPACER]	51	37.3	tRNA-M
Plant internal control				
Cox1-IPC-P	GGTCCGTTCTAGTGACAGCATTCCYACTTTTATTA [TAM-dT] C [THF] C [BHQ2-dT] YCCGGTACTGGC [3′-C3SPACER]	51	49.0	COXI
P. ramorum, *P. kernoviae*, and nfo genus-specific				
Atp9-P	TTGCTTTATTYTGTTTAATGATGGCWTTY [FAM-dT] T [THF] A [BHQ1-dT] YTTATTTGCTTTTT [3′-C3SPACER]	47	22.3	ATP9

[a] All primers in this study were obtained from Integrated DNA Technologies and all probes were obtained from Biosearch Technologies. Guanine-cytosine content (%) of each primer or probe is denoted by GC.

Standard curve plots of the log of concentration and the OT were constructed based on two replicate reactions.

To compare the sensitivity of RPA technology to more conventional TaqMan real-time PCR, a serial dilution of purified DNA (2 ng to 0.2 fg) from *P. ramorum* (Prg-2) was amplified with the *Phytophthora* genus-specific *trnM-trnP-trnM* and *P. ramorum* species-specific *atp9-nad9* TaqMan assays developed by Bilodeau et al. (5). The same serial dilution was also loaded into the multiplexed *Phytophthora* genus-specific and *P. ramorum* species-specific RPA assays described above using two replicate reactions per experiment. The log of concentration was plotted against cycle threshold (Ct) values and OT values for TaqMan and RPA assays, respectively.

In order to compare the effect of plant DNA on amplification efficiency and sensitivity, *Umbellularia californica P. ramorum*-free leaf tissue DNA was extracted using a DNeasy Plant Mini kit (Qiagen, Venlo, The Netherlands) following the manufacturer's recommendations and the Animal and Plant Health Inspection Service (APHIS) protocol guidelines for *P. ramorum* detection (32). Then, 2 µl of this extract (73 ng/µl) was loaded into each tube of the *P. ramorum* serial dilution and run in the *atp9-nad9* TaqMan assay of Bilodeau et al. (5). The effect of plant material on RPA was also investigated for the *Phytophthora* genus-specific RPA assay in pathosystems such as *P. cactorum–Fragaria* × *ananassa* crown tissue, *P. cinnamomi–Persea americana* root tissue, and *Phytophthora ramorum–U. californica* leaf tissue. Crude tissue macerations were taken from disease-free plants of the aforementioned pathosystems as previously described and 1 µl of this, along with 1 µl of a serial dilution of culture-purified DNA from the corresponding *Phytophthora* spp. (3 ng to 0.3 fg), was loaded into the RPA assay and the log of concentration was plotted against OT values from two replicate reactions.

To test for specificity, purified DNA from each of the isolates listed in Table 1 (1 ng/µl) was tested in the multiplexed RPA assay for *Phytophthora* genus-specific and plant internal control detection. To test specificity in the *Phytophthora* species-specific RPA assays (*P. ramorum* and *P. kernoviae*), a bulked segregate analysis was used to save reagents. In other words, in these assays, template volumes were 5 µl (1 µl from each of five species bulked) and, if cross reactivity was detected, the assay was repeated with each species individually.

Validation of RPA assays using field samples. Samples were collected from several locations throughout California in order to validate the RPA assays with environmental samples and compare them with results from traditional isolation on media and the *Phytophthora* genus-specific *atp9-nad9* TaqMan assay. Samples were collected from a variety of hosts (Table 3). Fresh host tissue (0.5 g) was ground as described above in 5 ml of GEB2 buffer

TABLE 3. Field validation of recombinase polymerase amplification (RPA) assay for *Phytophthora* genus-specific and *Phytophthora ramorum* species-specific detection[a]

Host species	Host common name	Tissue type	Pos	Origin	*Phytophthora* spp. identified[b]	*Phytophthora* genus-specific RPA	*P. ramorum* species-specific RPA[c]	TaqMan PCR	Isolation[d]	Neg[e]
Agave potatorum	Agave	Root	4	Tulare	*nicotianae*	+	nt	+	+	0
Arctostaphylos sp.	Manzanita	Leaf	1	Contra Costa, San Luis Obispo	sp. *PgChlamydo*	+	–	+	NP	1
Aucuba japonica	Gold dust plant	Root	1	Tulare	*citricola* clade E	+	nt	+	+	3
Capsicum sp.	Chili pepper	Root	3	Santa Barbara	*inundata*	+	nt	+	NS	1
Ceanothus sp.	Ceanothus	Leaf	1	Stanislaus	*palmivora*	+	–	+	NP	1
Citrus sp.	Navel orange, grapefruit, Valencia orange, lemon	Fruit	11	Riverside	*citrophthora** and *syringae**	+	nt	+	+	0
Citrus sp.	Grapefruit	Root	1	Riverside	*citrophthora*	+	nt	+	+	15
Diospyros sp.	Persimmons	Root	1	Tulare	*nicotianae*	+	nt	+	NS	1
Fragaria × *ananassa*	Strawberry	Crown	6	Ventura, Santa Barbara	*cactorum**	+	nt	+	+	15
Gardenia jasminoides	Gardenia	Root	11	Tulare	*nicotianae*	+	nt	+	+	0
Hedera sp.	Ivy	Leaf	1	San Luis Obispo	*tropicalis*	+	–	+	NP	0
Laurus nobilis	Bay laurel	Leaf	1	Sacramento	*multivora*	+	–	+	NP	0
Lavandula sp.	Lavender	Root	2	Santa Barbara	*nicotianae*	+	nt	+	+	0
Leucophyllum zygophyllum	Cimmeron	Root	1	Tulare	*nicotianae*	+	nt	+	NS	2
Magnolia grandiflora	Magnolia	Leaf	1	Ventura	*palmivora*	+	–	+	NP	0
Myrtus sp.	Myrtus	Crown	1	Santa Barbara	*nicotianae*	+	–	+	NP	0
Persea americana	Avocado	Root	4	Santa Barbara, Riverside	*cinnamomi* and *citrophthora*	+	nt	+	+	19
Pittosporum tenuifolium	Silver Sheen	Root	1	Santa Barbara	*nicotianae*	+	nt	+	NS	2
Pseudotsuga menziesii	Douglas fir	Leaf	1	El Dorado	*cambivora*	+	–	+	NP	0
Rhamnus californica	Coffee berry	Root	2	Santa Cruz	*cactorum**	+	nt	+	+	0
Rhododendron sp.	Azalea	Leaf	2	Los Angeles, Ventura	*tropicalis*	+	–	+	NP	2
Rhododendron sp.	Rhododendron	Leaf	5	Marin	*ramorum*	+	+	+	+	0
Rhododendron sp.	Azalea fuchsia	Root	1	Tulare	*nicotianae**	+	nt	+	+	2
Rhus integrifolia	Lemonade berry	Root	1	Santa Barbara	*nicotianae*	+	nt	+	+	0
Rosmarinus officinalis	Rosemary	Root	4	Tulare	*nicotianae*	+	nt	+	+	0
Rubus sp.	Raspberry	Crown	8	Santa Cruz	*rubi**	+	nt	+	+	2
Rubus sp.	Raspberry	Root	1	San Benito	*cactorum**	+	nt	+	+	0
Syringa sp.	Lilac	Root	1	Tulare	*nicotianae*	+	nt	+	NS	1
Umbellularia californica	Bay laurel	Leaf	11	Marin, Monterey, Sonoma	*ramorum**	+	+	+	+	23
Viburnum sp.	Viburnum	Leaf	2	Marin	*ramorum*	+	+	+	+	3

[a] Assays were validated with TaqMan polymerase chain reaction (PCR) and traditional isolation on corn meal agar amended with pimaricin, ampicillin, and rifampicin (CMA-PAR) media. Pos = numbers of positive samples, Origin = origin of samples (California county), and Neg = number of negative samples.

[b] Species identifications were obtained by sequencing the genus-specific TaqMan amplicon. Species identification that were also confirmed using the nfo-nested amplification protocol are denoted with an asterisk.

[c] Only leaf samples which tested positive for *Phytophthora* were tested with the species-specific *P. ramorum* test; nt = samples that were not tested.

[d] NP = not possible and NS = No significant *Pythium* spp. present.

[e] Samples tested which tested positive for the internal plant control but negative using the RPA and the TaqMan *Phytophthora* genus-specific tests and a *Phytophthora* sp. was not recovered from the tissue through isolation on CMA-PARP media.

and tested using the multiplexed RPA assay for *Phytophthora* genus-specific and plant internal control detection. DNA was then extracted from this crude extract using the DNeasy Plant Mini kit (Qiagen) starting at step 7 and following the manufacturer's protocols. TaqMan amplification reactions of the *atp9-nad9* locus were then performed as outlined by Bilodeau et al. (5) and amplicons were sequenced with the NAD9-F primer (5′-TACA ACAAGAATTAATGAGAAC-3′) to confirm identification of the species present. For species identification, a local BLAST was performed using the BioEdit Sequence Alignment Editor v7.2.5 (Ibis Biosciences, Carlsbad, CA) against our sequence database for these loci (Supplemental Files 1 and 2). For traditional isolation, five separate 1-cm tissue sections were rinsed in sterile deionized water, blotted dry on sterile paper towels, and placed on corn meal agar amended with pimaricin at 5 µg/ml, ampicillin at 250 µg/ml, and rifampicin at 10 µg/ml (18). Cultures were incubated at 22°C in the dark for 3 to 7 days. Isolates were then transferred to V8 solid agar media (200 ml of V8 juice, 2 g of $CaCO_3$, and 15 g of agar per liter) and identified by morphology.

Technologies to confirm a positive RPA reaction. To confirm a positive detection and determine the species present by sequencing, two different techniques were developed. To verify if a *Phytophthora* sp. was present from a positive *Phytophthora* genus-specific RPA detection (*trnM-trnP-trnM* locus), samples were removed after 25 min of amplification and incubated at 95°C for 5 min to deactivate enzymes present in RPA, and a 10-fold dilution of the products was created. Afterward, a nested PCR reaction was performed containing 1 µl of the dilution from the RPA, 2 mM $MgCl_2$, 400 nM genus-specific PCR primers PhyG-F2 and PhyG-Rb, and standard PCR reagents (5). The amplification protocol included an initial denaturization step at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The reaction was completed by a 10-min extension at 72°C. PCR products were separated on a 1.5% agarose Tris-borate-EDTA gel to confirm amplification. Amplicons were then sequenced with PhyG-F2 and TrnM_Rseq1.

To confirm which *Phytophthora* sp. was present after a positive RPA detection, a forward primer (Atp9-F), probe (Atp9-P), and reverse primer (Nad9-nfo-R) were used to amplify the *atp-nad9* locus using the TwistAmp nfo kit (Twistdx). This kit uses chemistry similar to that of the TwistAmp exo kit but does not utilize an exonuclease that degrades the template, and the reaction occurs at a slower rate. The reaction was carried out under the following conditions (primers and probes were all at a concentration of 10 µM): 2.1 µl of Atp9-F, 2.1 µl of Nad9-nfo-R, 0.9 µl of Atp9-P, 29.5 µl of rehydration buffer, 11.9 µl of DNase-free water, 1 µl of template, and 2.5 µl of 280 mM magnesium acetate. Samples were removed after 35 min of amplification and incubated at 95°C for 5 min to deactivate enzymes present in RPA reactions, and a 10-fold dilution of the products was created. A nested PCR reaction was then performed containing 1 µl of the dilution from the RPA, 2 mM $MgCl_2$, 400 nM primers Nad9_Fseq1 and Nad9_Rseq1, and standard PCR reagents. The amplification protocol and PCR product visualization was the same as described above and the amplicon was sequenced with amplification primers.

Detection equipment. Three different devices were tested for their use with the TwistAmp exo kits: the Twista portable real-time fluorimeter (Twistdx), SMART-Dart Bluetooth-enabled device (Diagenetix, Inc., Honolulu, HI), and CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). In order to run RPA assays in the SMART-Dart and CFX96 Real-Time PCR machines, the protocols needed to be modified; therefore, the reaction was incubated at 39°C, data were collected every 20 s for a period of 30 min, and the agitation step 5 min after initiating amplification was removed because of the inability to pause data collection in this unit. For the CFX96 Real-Time PCR Detection System, data were shown in terms of cycles because, whereas the run was developed to take 20 min, the actual run was ≈32 min because of extra time spent during data collection.

RESULTS

Sensitivity of *Phytophthora* RPA assays. Mitochondrial gene order differences between *Phytophthora* and *Pythium* or plant spp. allowed for the development of a highly specific marker system using the RPA technology in the *trnM-trnP-trnM* and *atp9-nad9* regions of *Phytophthora* spp. (Fig. 1). The *Phytophthora* genus-specific (Fig. 2A) and *P. ramorum* (Fig. 2C) species-specific assays were able to detect purified DNA of *P. ramorum* at a concentration of 200 fg, with detection not observed below this threshold (Fig. 2). The onset of amplification for both the *Phytophthora* genus-specific and the *P. ramorum* species-specific RPA assays occurred between 6 and 17 min, depending on initial DNA concentration (Fig. 2B). The lower concentration of 200 fg was detectable but overall FAM fluorescence was low (Fig. 2A and C). Sensitivity and fluorescence measurements of the *P. kernoviae* species-specific assay were similar to results observed with the *P. ramorum* species-specific assay (Fig. 3).

The onset of amplification as it related to the log of DNA concentration was not linear in RPA assays (Fig. 2B). Therefore, the initial 5-min preagitation step was subtracted from the data, and the data were log transformed. This log-transformed value, termed the OT, was plotted against concentration, and the *Phytophthora* genus-specific and the *P. ramorum* and *P. kernoviae* species-specific assays had a linear relationship with concentration, with R^2 values of 0.993, 0.978, and 0.954, respectively (Fig. 2D).

Specificity of *Phytophthora* RPA assays. The specificity of the *Phytophthora* genus and species-specific RPA assays was evaluated on a wide range of *Phytophthora*, *Pythium*, and plant species (Table 1). For the *Phytophthora* genus-specific assay, the onset of amplification for all *Phytophthora* spp. tested was between 6 and 11 min using an initial concentration of 1 ng of DNA. *Pythium* and plant species showed no cross reactivity with the assay. For the *P. ramorum* and *P. kernoviae* species-specific assays, detection occurred at 6.3 and 6 min respectively, and did not cross-react with any other *Phytophthora* spp. at the concentration tested (1 ng DNA). Additionally, no *Pythium* or plant species showed cross reactivity with these species-specific assays.

TaqMan PCR versus RPA assays and the effect of extraction buffers and plant DNA on detection. Extraction buffers tested for their ability to crudely extract DNA from *Fragaria* × *ananassa* crown tissue had an effect on the onset of amplification of the plant internal control and *P. cactorum* detection. Buffer GEB2 and all three ELISA grinding buffers at pH 8.0, 9.5, and 11.0 were able to provide amplifiable *Fragaria* × *ananassa* DNA, and the onset of amplification values were 7.0, 7.0, 7.3, and 8.0 min, respectively. Likewise, these same buffers had limited effect on amplification of *P. cactorum*-purified DNA that was spiked into the crude *Fragaria* × *ananassa* DNA extractions, and the onset of amplification values were 6.3, 6.7, 7.0, and 7.0 min, respectively. Plant and *P. cactorum* templates were not amplified when using the extraction buffer GEB3 and the PEG buffer described above (data not shown). Due to the success and ease of use of the GEB2 buffer, all subsequent crude plant extractions utilized this buffer.

TaqMan PCR and RPA assays had similar limits of detection. The *Phytophthora* genus-specific (targeting *trnM-trnP-trnM*) and *Phytophthora* species-specific (targeting *atp9-nad9*) TaqMan assays were able to detect purified *P. ramorum* DNA at an initial quantity of 200 fg and detection remained linear at increasing concentrations using Ct values (R^2 = 0.997 and 0.996, respectively) (Fig. 4A and B). The *Phytophthora* genus-specific (targeting *trnM-trnP-trnM*) and *Phytophthora* species-specific (targeting

atp9-nad9) RPA assays were also able to detect purified *P. ramorum* DNA at an initial quantity of 200 fg and detection remained linear at increasing concentrations using the OT values (R^2 = 0.980 and 0.974, respectively) (Fig. 4C and D).

Plant extract had a limited effect on both TaqMan and RPA amplification. For TaqMan assays, the standard APHIS-*P. ramorum* DNA extraction protocol for genus- and species-specific detection was tested with healthy *U. californica* samples spiked with a

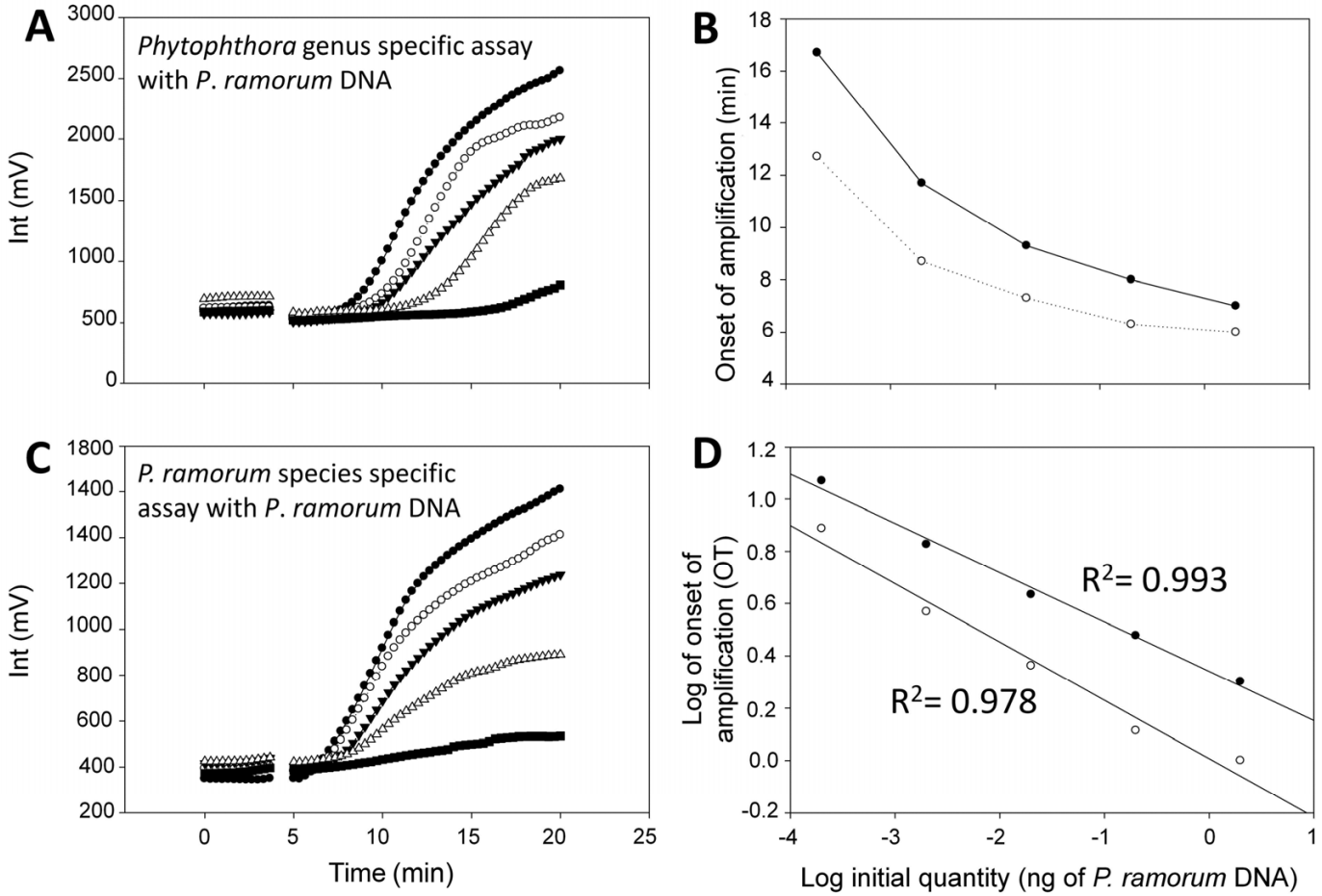

Fig. 2. Serial dilution of purified DNA of *Phytophthora ramorum* and standard curve plots of concentration using two different recombinase polymerase amplification (RPA) assays. **A,** *Phytophthora* genus-specific assay and **C,** *P. ramorum* species-specific assay, ranging from 2 ng to 200 fg. **B,** Log of the initial DNA quantity of *P. ramorum* against the onset of amplification, where the *Phytophthora* genus-specific assay and *P. ramorum* species-specific assays are denoted by closed and open circles, respectively. **D,** Log of the initial DNA quantity of *P. ramorum* against the log of the onset of amplification minus the agitation step (OT), where the *Phytophthora* genus-specific assay and *P. ramorum* species-specific assay are denoted by closed and open circles, respectively.

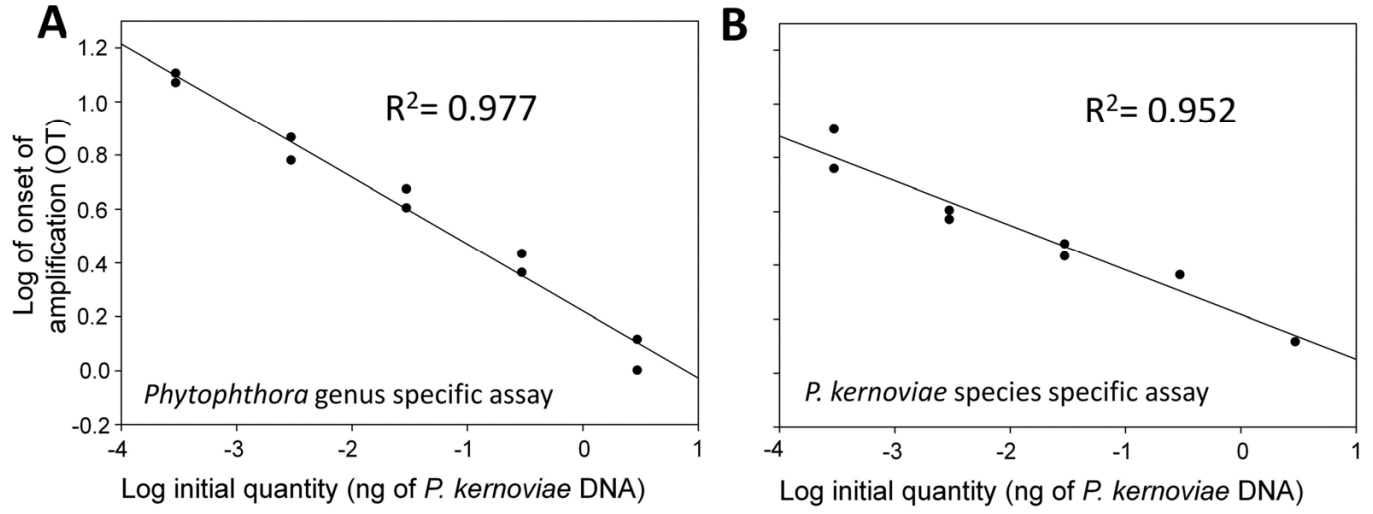

Fig. 3. Serial dilution of purified DNA of *Phytophthora kernoviae* and standard curve plots of the log of initial DNA concentration and the log of the onset of amplification using two different recombinase polymerase amplification assays. **A,** *Phytophthora* genus-specific assay and **B,** *P. kernoviae* species-specific assay, ranging from 3 ng to 300 fg of purified *P. kernoviae* DNA.

serial dilution of purified DNA of *P. ramorum*. The addition of plant DNA to the pathogen dilution series had an effect on *P. ramorum* detection, increasing the Ct value between 1 and 2 across the various dilutions (Fig. 4A and B). However, *P. ramorum* detection still occurred at all concentrations in both the genus- and species-specific assays and remained linear (R^2 = 0.974 and 0.995, respectively), albeit with a shifted time frame. Similarly, for RPA assays, the crude DNA extractions from healthy *U. californica* samples spiked with a serial dilution of purified DNA of *P. ramorum* were tested with the *Phytophthora* genus- and *P. ramorum* species-specific RPA assays. Addition of plant DNA to the *P. ramorum* dilution series had a limited effect on pathogen detection, delaying the onset of amplification across the dilutions, with a greater affect observed for the lowest concentrations (Fig. 4C and D). However, *P. ramorum* detection still occurred at all concentrations in both the genus- and species-specific assays and remained linear (R^2 = 0.978 and 0.986, respectively), albeit with a shifted time frame. A similar effect of the plant extract was observed with the *Phytophthora* genus-specific assay using *P. cactorum* and *P. cinnamomi* spiked into crude plant extracts of *Fragaria* × *ananassa* crown tissue and *Persea americana* root tissue, respectively (Fig. 5). However, the sensitivity of *Phytophthora cinnamomi* detection was reduced to 3 pg with the addition of macerated *Persea americana* root tissue.

Field validation of RPA assays on a variety of plant samples. RPA assays were validated using traditional culturing and conventional DNA extraction followed by TaqMan detection and sequencing. Of the 222 symptomatic plant samples (roots, leaves, fruit, or crowns), 91 samples tested positive with the *Phytophthora* genus-specific RPA assay and the TaqMan genus-specific *atp9-nad9* assay (Table 3). By sequencing the TaqMan amplicon, a variety of species were identified in the field validation, including *Phytophthora* sp. *PgChlamydo, P. cactorum*, *P. cambivora, P. cinnamomi*, *P. citricola* clade E, *P. citrophthora*, *P. inundata, P. multivora*, *P. nicotianae*, *P. palmivora, P. ramorum*, *P. rubi*, *P. syringae*, and *P. tropicalis*. Of those 91 samples positive for a *Phytophthora* sp. using molecular techniques, only 56 yielded a *Phytophthora* culture when plated out with many samples heavily infected with *Pythium* spp. For the *P. ramorum* species-specific RPA assay, 44 plant samples were tested using this species-specific assay and 18 were positive and confirmed to be *P. ramorum* through sequencing. Of those 18 samples, *P. ramorum* was cultured from only 6.

In addition to the plant species listed in Table 3, samples were also assayed from *Acanthus mollis, Anigozanthos manglesii, Asparagus officinalis, Betula* sp., *Bougainvillea* sp., *Cistus* sp., *Coleonema* sp., *Cucurbita* sp., *Cycas revoluta, Cynara cardunculus, Fatsia japonica, Fraxinus* sp., *Leucophyllum langmaniae, Ligustrum* sp., *Loropetalum* sp., *Notholithocarpus densiflorus, Phaseolus vulgaris, Prunus avium, Pseudotsuga menziesii, Trachelospermum jasminoides, Vicia faba*, and *Westringia fruticosa*. *Phytophthora* spp. were not cultured from these plants and all

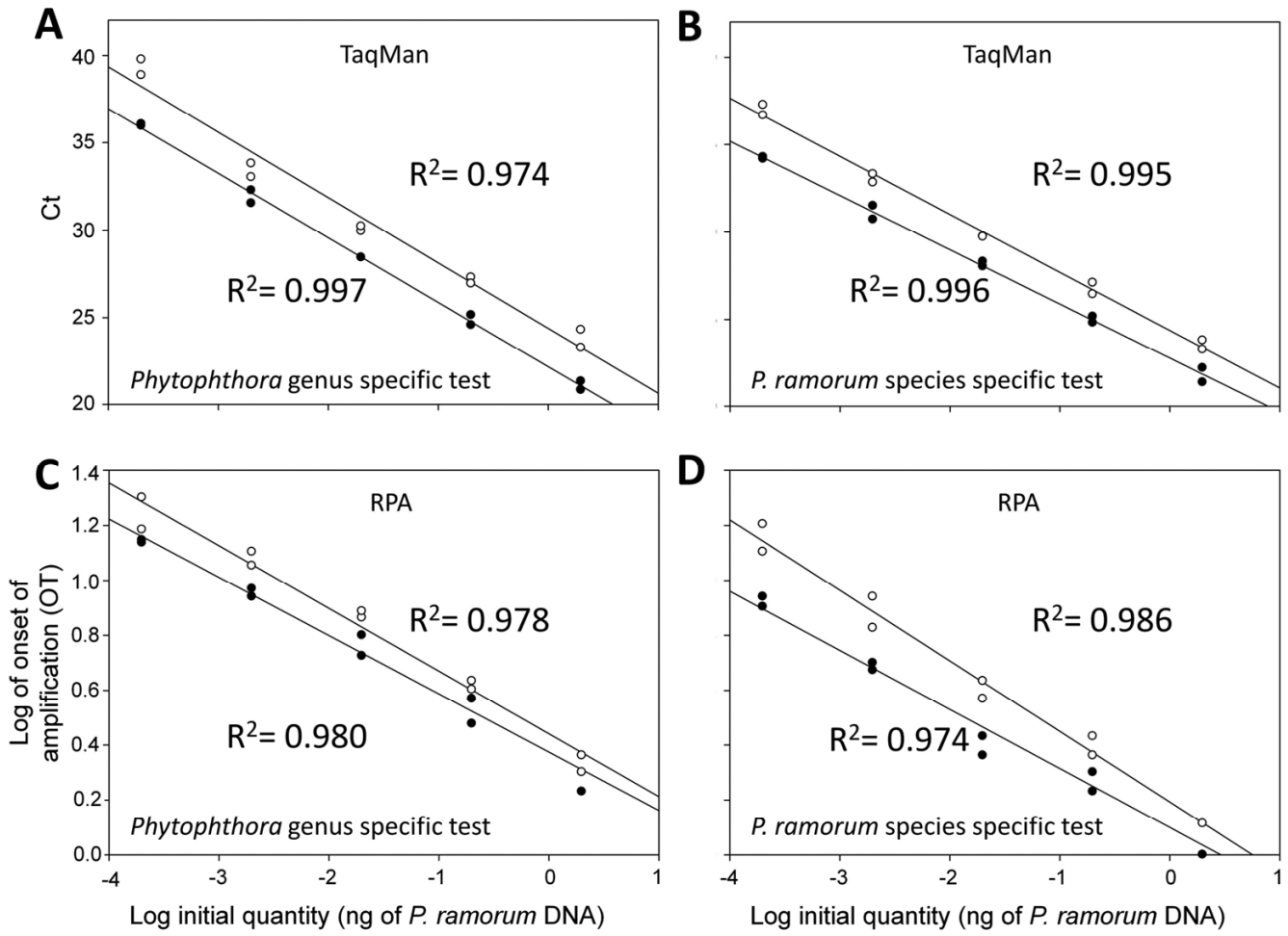

Fig. 4. Comparison of genus-specific and *Phytophthora ramorum* species-specific assays using TaqMan and recombinase polymerase amplification (RPA) with a serial dilution of purified DNA of *P. ramorum* in the presence or absence of plant DNA from *Umbellularia californica*. **A and B,** TaqMan *Phytophthora* genus-specific and *P. ramorum* species-specific assays with the log of the initial DNA quantity of *P. ramorum* against cycle threshold values (Ct), where the absence or presence of *U. californica* DNA is denoted by closed and open circles, respectively. **C and D,** *Phytophthora* genus-specific and *P. ramorum* species-specific RPA assays with the log of the initial DNA quantity of *P. ramorum* against the log of the onset of amplification minus the agitation step (OT), where the absence or presence of a crude leaf extract of *U. californica* DNA is denoted by closed and open circles, respectively.

were negative for this pathogen with both the TaqMan and RPA diagnostic assays. The *Phytophthora* RPA assays did not generate any false positives from plant or *Pythium* DNA from these samples.

Nested PCR and sequencing is an effective way to confirm a positive RPA product and identify the species present. A technique to confirm a positive result in an RPA assay and identify which species was present was developed using nested PCR and sequencing. This was tested against 10 samples which were found to be positive for *Phytophthora* spp. in our earlier field validation experiments (Table 3). Initial experiments showed that a nested PCR amplification using the *trnM-trnP-trnM* TwistAmp exo amplicon as a target was able to generate a template capable of being sequenced. Due to the low level of interspecific polymorphisms observed for this locus, it was difficult in many cases to confirm which species was present; however, the sequence information could easily discern between a *Phytophthora* or *Pythium* sp. in the event that there was a mispriming because of significant variation in the first spacer region of *trnM-trnP* (Supplemental Figure 1). Interestingly, a ladder-type banding pattern was observed with this technique when amplified products were separated by gel electrophoresis (data not shown).

In order to identify the species that gave a positive detection in the genus-specific RPA assay, an alternative approach using the *atp9-nad9* locus was developed. Although RPA amplification with primers and probe was observed, it was not possible to generate a PCR-amplifiable template using the TwistDx exo kit (data not shown). Another RPA amplification technique (TwistDx nfo kit) was tested that does not have exonuclease activity. Template amplification was fluorometrically detected and a 1:10 dilution of the amplified template was successfully amplified by nested PCR. DNA sequence data from this template confirmed the species identification of the isolate that was detected (Table 3).

RPA assay detection can be read on different assay platforms. All three machines tested (Twista, SMART-Dart, and CFX96) can be used with the RPA technology using the TwistAmp exo kits. The Twista unit was simpler to use with the technology and gave the most accurate results in terms of the time of the onset of amplification and the overall reduced background noise level and uniformity of the data (Fig. 6A). The assay also worked well in the SMART-Dart unit with a similar time frame of amplification, although the amplification curve was not as uniform as the Twista unit (Fig. 6B). The Bio-Rad CFX96 also worked well for data collection and generated a uniform amplification curve; however, because the data are collected by a cassette of sensors moving across the top of the tubes, the 20-s cycle for data collection means the machine is in an almost continuous data collection mode (Fig. 6C).

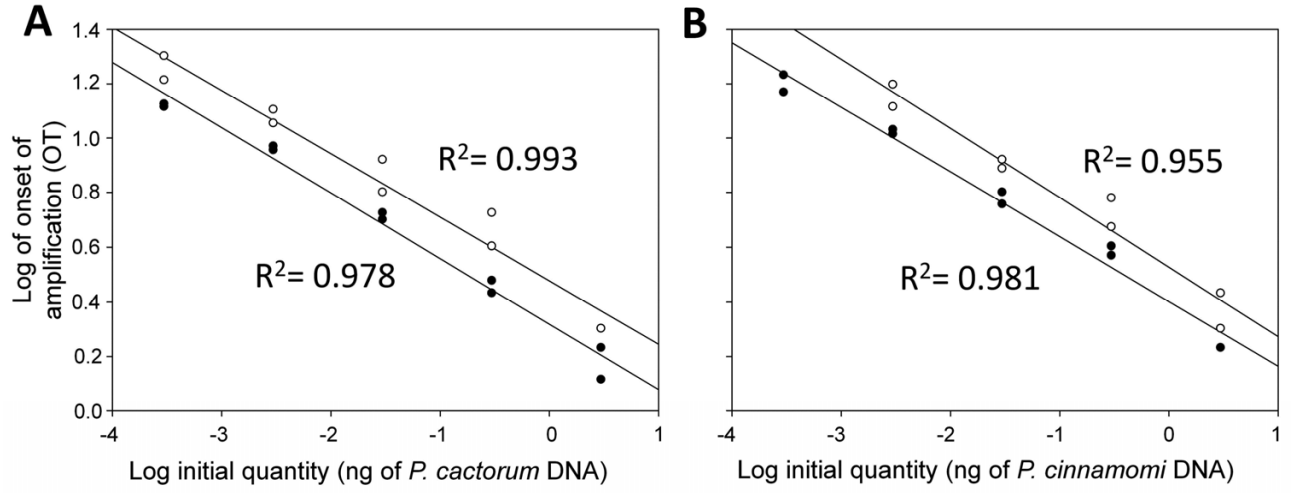

Fig. 5. Comparison of the *Phytophthora* genus-specific recombinase polymerase amplification (RPA) assay using a serial dilution of purified *Phytophthora* DNA in the presence or absence of a crude tissue extract of plant DNA. **A,** Log of the initial DNA quantity of *Phytophthora cactorum* against the log of the onset of amplification minus the agitation step (OT), where the absence or presence of a crude crown extract of *Fragaria* × *ananassa* DNA is denoted by closed and open circles, respectively. **B,** Log of the initial DNA quantity of *P. cinnamomi* against the OT, where the absence or presence of a crude root extract of *Persea americana* DNA is denoted by closed and open circles, respectively.

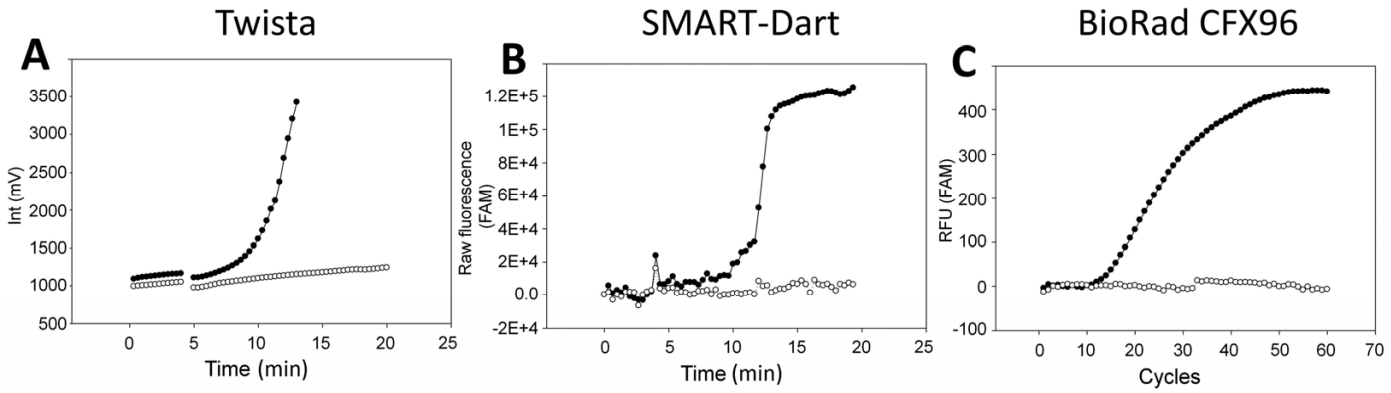

Fig. 6. *Phytophthora* genus-specific RPA assay using different detection devices with crude DNA leaf extracts, where *Phytophthora ramorum*-free *Umbellularia californica* and *P. ramorum*-infected *U. californica* extracts are denoted by closed and open circles, respectively. Devices included **A,** a Twista unit; **B,** a Bluetooth-enabled-SMART-Dart machine; and **C,** a Bio-RadCFX96 PCR machine.

DISCUSSION

The development of a rapid isothermal molecular assay that does not require DNA extraction and is capable of detecting *Phytophthora* at both a genus- and species-specific level would enhance the diagnostic capabilities for this pathogen and would perhaps bring us one step closer to having a specific and sensitive detection technology that could be used directly in the field. This, in turn, would improve disease management decision-making and monitoring of pathogen spread (especially important for quarantine species). The ability to detect the pathogen at the genus level and identify the species present by sequence analysis would be an important component of this diagnostic capability because it would allow the assay to focus on a broader level than just an individual species, a feature that would be very useful when conducting pathogen surveys. The RPA isothermal assays described herein provide a technique that should help address these goals.

The assays are based on two loci spanning mitochondrial gene order differences between *Phytophthora* spp. and other related organisms (*Pythium, Phytopythium*, and plant species), thereby enhancing specificity. The genus-specific marker at the *trnM-trnP-trnM* locus was able to detect all *Phytophthora* spp. tested but did not cross react with any *Pythium, Phytopythium*, or plant species evaluated. These results paralleled what was observed with the TaqMan assay for this locus (5). In *Pythium* and *Phytopythium* spp., the *trnM-trnP* gene order is conserved but the final *trnM* is at least 10 kb away and in the opposite orientation (F. Martin, *unpublished data*); therefore, even if mispriming occurred, template amplification under the reaction conditions used would not take place and sequence analysis afterward would not be possible. The species-specific RPA markers that were developed based on the *atp9-nad9* spacer locus were also highly specific and did not amplify plants or *Pythium, Phytopythium*, or other *Phytophthora* spp. This same locus was also used for development of a TaqMan real-time PCR assay for genus- and species-specific detection of *Phytophthora* (5) with the amplification primers in the *atp9* and *nad9* genes and the species-specific TaqMan probe designed from the intervening spacer region. In *Pythium* and *Phytopythium* spp., these genes are 18 to 30 kb apart (F. Martin, *unpublished data*) and cannot be amplified in the event of mispriming. With the RPA assay, the forward primer and probe are located in a conserved portion of the *atp9* gene and the reverse primer, which confers species specificity, is located in the spacer region. Given the level of interspecific polymorphisms observed in the spacer sequences and success in developing species-specific TaqMan probes from this region (5) (T. Miles and F. Martin, *unpublished data*), using this approach allows for a more systematic development of other species-specific RPA assays. Based on sequence alignments of this region, it should be possible to develop species-specific primers for this RPA assay for a wide range of *Phytophthora* spp. (Supplemental Figure 2).

Sensitivity of the RPA assay was similar to traditional TaqMan assays using the same locus as a target. Using culture-purified DNA as a template, genus-specific detection using the *trnM-trnP-trnM* locus was linear in both assays down to 200 fg. Although the addition of *U. californica* DNA (purified by standard techniques for the TaqMan assay and a crude tissue maceration for the RPA assay) reduced amplification efficiency and caused a limited delay in amplification, this did not affect the final level of sensitivity of either assay. Similar results were observed for TaqMan and RPA species-specific detection using the *atp9-nad9* locus; sensitivity of detection to 200 fg was observed for both. However, for the RPA assay, this may be a function of the host tissue examined. Although there was a reduction in sensitivity of detection of *Phytophthora cinnamomi* in the presence of macerated avocado root tissue (3 pg) this was not observed for detection of *P. cactorum* when macerated strawberry crown tissue was added to the amplification (300-fg sensitivity). It should be noted that there appeared to be a high level of polysaccharides present in avocado root tissue, leading to a more viscous solution that was added to the amplification mixture; dilution of this sample may reduce this inhibition of amplification but will, in turn, dilute pathogen DNA and may reduce the sensitivity of detection. The level of sensitivity we observed with RPA was similar but not as sensitive as the TaqMan marker system reported by Bilodeau et al. (5), where the linear limit of detection was observed to be 100 fg in most cases. However, the RPA's level of detection is more sensitive than some LAMP detection assays that have been reported for *P. kernoviae* and *P. sojae,* where the authors found the limit of detection using purified DNA to be 17 and 10 pg, respectively (12,30). However, a recent study by Chen et al. (9) has developed a LAMP detection assay for *P. melonis* based on the *ytp1* gene that is sensitive down to 10 fg. These differences could be due to the copy number of the locus being targeted and the complexity in designing primers for LAMP assays.

The ability to confirm a positive in the RPA assay by template sequencing is a valuable tool, particularly when working with a genus-specific assay because this will allow for a broader analysis of the taxa present rather than detection of just a particular species. The described RPA assays provide two means for accomplishing this. If confirmation is needed only at a genus level so that the appropriate control measures for *Phytophthora* spp. can be initiated, then sequencing of the *trnM-trnP-trnM* TwistAmp exo amplicon will confirm whether a *Phytophthora* sp. is present versus other potential oomycete genera. The drawback of this locus is that its relatively small polymorphic *trnM-trnP* spacer limits the number of *Phytophthora* spp. that can be identified. From a research and regulatory perspective, it can be important to identify an RPA positive to a species level; for this purpose, a new set of primers and probe was designed to amplify the entire spacer region and flanking coding regions of the *atp9-nad9* locus. Unlike the *trnM-trnP-trnM* locus, nested PCR amplification of the TwistAmp exo product from the *atp9-nad9* locus was not successful in generating a sequencing template (likely due to the exonuclease activity in the amplification digesting the primer annealing sites needed for amplification of the entire amplicon); therefore, a second template amplification of the *atp9-nad9* locus was done using a TwistAmp nfo kit (similar to the exo kit but does not have exonuclease activity degrading the amplified template). Although this technology is not as robust or sensitive as the TwistAmp exo kit, it did provide a template that could be PCR-amplified and sequenced without the need for DNA extraction. BLAST analysis of the sequence to a sequence database for this locus provided the species identification (Supplemental Files 1 and 2). However, because these markers are genus-specific if more than one species of *Phytophthora* is present, cloning would be required to get clean sequence reads.

Primer and probe design for RPA assays is very similar to conventional PCR but requires greater length. Amplification primer design is straightforward; however, to optimize specificity, it may be necessary to evaluate several primers. For example, it took only one primer validation to find the reverse primer conferring specificity for *P. ramorum*; however, for *P. kernoviae*, 15 reverse primers were evaluated before a sensitive and specific assay was developed. Among those 15 primers were 2 with locked nucleic acid (LNA) primers, 1 with a single modification at the 3′ end, and 1 with a modification 1 bp in from the 3′ end; neither of these primers showed significant increases in specificity. Although it is not entirely clear why the final primers that were chosen were more specific, we did find that, in general, a mixture of bases at the 3′ end was the most effective and LNAs at the locations chosen had no effect on specificity. More research is required to better characterize the requirements for optimal primer design. Although this technology does require optimization, the

ability to transfer existing PCR technology to an isothermal platform like RPA is a huge advantage over other techniques such as LAMP, where primer design can be complex, requires specific software to design assays, and use of AT-rich targets such as mitochondrial DNA can complicate LAMP assay design.

Another approach that was used to optimize specificity was altering the amplification primer concentrations. To achieve the greatest sensitivity, the manufacturer recommended that a total of 0.84 micromoles of primers be used, with an equal amount for the forward and reverse primers. However, early in the research, it was noticed that reducing the concentration of the conserved forward primer with a corresponding increase in the concentration of the reverse primer improved specificity. This technique was particularly important in our assays because the reverse primer provided the species specificity of the assay. The ability to focus on design of species-specific reverse primers while using a conserved forward primer and probe reduces the cost of development of additional species-specific assays because the labeled probe does not need to be redesigned.

One significant advantage of the RPA isothermal assay over PCR-based diagnostic assays is that there is no need for DNA extraction; a simple and rapid tissue maceration step was all that was needed to provide template DNA for amplification. This represents a meaningful savings in time and expense and, rather than having to run the assay in a laboratory setting, facilitates the field deployment of the technology. Because commercially available buffers commonly used for ELISA work well in the RPA assay, it is possible to follow up a positive ELISA assay with the RPA assay for confirming presence of a *Phytophthora* sp. using the same macerated tissue. One potential utility of RPA technology is to test soil samples. A sensitive and specific LAMP assay was recently developed for plant and soil samples of the soilborne banana pathogen *Fusarium oxysporum* f. sp. *cubense* (35). However, for soil samples, a full DNA extraction was done prior to running the LAMP assay. This would not be possible in a field situation; however, using the types of RPA assays which are tolerant of inhibitors may improve the detection capability from soil over standard TaqMan assays. Future research on rapid DNA extraction from soil in the field would aid in the utility of these isothermal technologies (either LAMP or RPA).

With a level of sensitivity similar to that of a TaqMan assay using the same loci as targets and the ability to amplify template from crude tissue macerations, the described RPA assays for *Phytophthora* spp. are ideally suited for use directly in the field. The Twista unit used to monitor amplification can be used with an optional battery pack for this purpose but needs to be connected to a computer in order to see the actual amplification curve (without the computer the unit will give a ± result). Although it was originally developed for use with LAMP assays, the software in the SMART-Dart unit can adjusted to run the RPA assays and, in laboratory tests, the unit generated data that were comparable with the Twista unit. Advantages of this system are its portability (about 25% the size of the Twista unit) and the ability to run the system and monitor amplification with an app on a smart phone connected to the unit via a Bluetooth connection. The SMART Dart system is still in development but the beta version we were working with has been used successfully directly in the field. Efforts are underway with the company to add features to improve compatibility of the unit with RPA assays.

Although using mitochondrial sequences for diagnostic assays has the advantage that the target is present in a high copy number, thereby allowing for increased sensitivity, it is important to remember that there is only one type of mitochondrial DNA present in an isolate. If a hybrid species is the target, the use of a mitochondrial marker alone may not detect the hybrid nature of the isolate (although a species-specific TaqMan real-time PCR assay has been developed for *P. alni*) (5). Although not common, hybrid species of *Phytophthora* do exist (23). Furthermore, if several species are expected in a particular sample, as has been reported in natural ecosystems of Australia and South Africa (16,20,26), more conventional approaches (e.g., isolation) to detect *Phytophthora* spp. may be needed in conjunction with these RPA techniques.

The use of the RPA assay directly in the field requires some adjustments in the amplification procedure. Rather than using a portable microfuge to pellet the amplification mixture to the bottom of the tube, this can be accomplished by tapping the tubes on a hard surface or by a sharp rotating hand motion. To ensure optimum sensitivity, the manufacturer of the TwistAmp exo kit recommends remixing the tubes 4 min after initiating amplification to evenly distribute RPA amplicons in the amplification mixture. Amplification can still occur if this mixing step is omitted; however, the sensitivity of detection will be reduced (data not shown). Recently, the company that developed the TwistAmp kits (TwistDx) has released a new fluorimeter for data collection (model T-16) that eliminates this need for mixing; a single ball bearing is added to the microfuge tube in which the assays are run and a magnetic mixer in the unit ensures an even mixing during amplification.

The described RPA techniques should have a significant impact on our ability to rapidly detect *Phytophthora* spp. directly in the field and make management decisions immediately. Also, due to the fact that PCR technologies can be easily transferred to the RPA platform, it is possible that new assays for other species or taxa of plant pathogens could be quickly developed. We have already demonstrated an ability to make species-specific RPA assays by taking advantage of a TaqMan marker system that was developed targeting a polymorphic spacer region present in *Phytophthora* spp. Furthermore, making these assays genus specific will facilitate development of an array type of detection capability so that new pathogens that have been previously undetected could be identified.

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