Plant Disease • 2024 • 108:987-995 • https://doi.org/10.1094/PDIS-04-23-0722-RE

Specific Detection of *Phytophthora parasitica* by Recombinase Polymerase Amplification Assays Based on a Unique Multicopy Genomic Sequence

Rongsheng Wang,[1,2] Ran Zhou,[2] Yuling Meng,[2] Jie Zheng,[2] Wenqin Lu,[2] Yang Yang,[2] Jiapeng Yang,[2] Yuanhua Wu,[1,†] and Weixing Shan[2,†]

[1] Liaoning Key Laboratory of Plant Pathology, College of Plant Protection, Shenyang Agricultural University, Shenyang 110866, Liaoning, China
[2] State Key Laboratory of Crop Stress Biology for Arid Areas and College of Agronomy, Northwest A&F University, Yangling 712100, Shaanxi, China

Abstract

Phytophthora parasitica is a highly destructive oomycete plant pathogen that is capable of infecting a wide range of hosts including many agricultural cash crops, fruit trees, and ornamental garden plants. One of the most important diseases caused by *P. parasitica* worldwide is black shank of tobacco. Rapid, sensitive, and specific pathogen detection is crucial for early rapid diagnosis, which can facilitate effective disease management. In this study, we used a genomics approach to identify repeated sequences in the genome of *P. parasitica* by genome sequence alignment and identified a 203-bp *P. parasitica*-specific sequence, PpM34, that is present in 31 to 60 copies in the genome. The *P. parasitica* genome specificity of PpM34 was supported by PCR amplification of 24 genetically diverse strains of *P. parasitica*, 32 strains representing 12 other *Phytophthora* species, one *Pythium* species, six fungal species, and three bacterial species, all of which are plant pathogens. Our PCR and real-time PCR assays showed that the PpM34 sequence was highly sensitive in specifically detecting *P. parasitica*. Finally, we developed a PpM34-based high-efficiency recombinase polymerase amplification assay, which allowed us to specifically detect as little as 1 pg of *P. parasitica* total DNA from both pure cultures and infected *Nicotiana benthamiana* at 39°C using a fluorometric thermal cycler. The sensitivity, specificity, convenience, and rapidity of this assay represent a major improvement for early diagnosis of *P. parasitica* infection.

Keywords: molecular detection, multicopy genomic sequences, *Phytophthora parasitica*, recombinase polymerase amplification

Phytophthora parasitica Dastur, also known as *P. nicotianae* van Breda de Haan, is one of the top 10 oomycete pathogens (Kamoun et al. 2015) and a typical soilborne pathogen with a broad range of hosts containing more than 250 genera of plants in 90 botanical families, many economically important plants included (Cline et al. 2008). *P. parasitica* is widespread in garden nurseries and fruit trees (Olson et al. 2013), which can potentially increase the speed that *P. parasitica* can spread in the global agricultural trade (Brasier 2008). Moreover, diseases caused by *P. parasitica* are difficult to control because their oospores or chlamydospores can survive in the soil (Li et al. 2011). Additionally, it is too late and would be extremely difficult to reduce the economic losses once the crops begin to show symptoms since the disease has an asymptomatic period. Therefore, rapid and accurate detection or diagnosis of pathogens in the early stages of infection is essential to making decisions on fungicide application or other effective strategies to control disease (Huang et al. 2010).

The most accurate technique to detect *P. parasitica* is DNA-based molecular diagnostics, which relies on nucleic acid sequences. Detection based on molecular methods has been widely used to detect *P. parasitica* because of its high sensitivity, speed, specificity, accuracy, and reproducibility; examples are conventional PCR (Kong et al. 2003; Meng and Wang 2010), nested PCR (Grote et al. 2002; Ippolito et al. 2002; Li et al. 2015), quantitative PCR (Blaya et al. 2016; Huang et al. 2010; Li et al. 2013), and loop-mediated isothermal amplification (LAMP) (Hieno et al. 2019, 2020; Li et al. 2015).

The candidate target sequences are crucial to ensure the specificity, accuracy, and sensitivity of molecular detection. Several target sequences have been designed for the specific detection of *P. parasitica*, including the nuclear ribosomal internal transcribed spacer (nrITS) region (Grote et al. 2002; Ippolito et al. 2002; Kong et al. 2003), the elicitin gene *parA1* (Kamoun et al. 1993; Lacourt and Duncan 1997), and the *Ypt1* gene (Meng and Wang 2010). The nrITS region and *Ypt1* are the two most commonly used targets for *P. parasitica* detection. rDNA genes generally exist in multiple copies, and detection methods for rDNA genes showed high sensitivity (Grote et al. 2002; Ippolito et al. 2002; Kong et al. 2003). However, the high level of similarity among rDNA sequences in closely related *Phytophthora* species, such as *P. cactorum* and *P. infestans*, makes it difficult to distinguish them from one another (Martin and Tooley 2004; Schena et al. 2008). Highly repeated and specific sequences in the genome are potentially ideal target candidates because the sensitivity of molecular detection methods depends on the copy number of the target genes, and the specificity depends on genome specificity of the target genes. With the development of the high-throughput next-generation DNA sequencing technology, whole-genome sequences are increasingly available (Afandi et al. 2019; Grenville-Briggs et al. 2017; Haas et al. 2009; Tyler et al. 2006), allowing comparative analyses for ideal target sequences in *P. parasitica* detection with higher sensitivity and specificity.

Unlike PCR-based methods which require a thermal cycler to achieve DNA amplification, isothermal nucleic acid amplification methods do not require expensive, temperature-controlled equipment with a reduced number of steps and reduced time. The most commonly used isothermal nucleic acid amplification methods for detection of plant pathogens include LAMP (Huang et al. 2017; Li et al. 2015; Notomi et al. 2000), helicase-dependent amplification (Vincent et al. 2004), and rolling circle amplification (Lizardi et al. 1998). In recent years, the recombinase polymerase amplification

[†]Corresponding authors: Y. H. Wu; wuyh09@syau.edu.cn, and W. X. Shan; wxshan@nwafu.edu.cn

Funding: This work was supported by the Chongqing Tobacco Major Program of Integrated Pest Management (NY20140401070002).

The author(s) declare no conflict of interest.

Accepted for publication 22 October 2023.

(RPA) assay has been developed as a novel method to efficiently amplify DNA by using recombinase, DNA polymerase, and DNA-binding proteins, which could work at low temperatures between 37 and 42°C (Piepenburg et al. 2006). Compared with other nucleic acid amplification methods, RPA assays have several significant advantages in terms of reaction time, reaction temperature, and result-reading mode. Using the RPA assay, the amplified products can be detected in 6 to 10 min at lower temperatures (Euler et al. 2013). Several types of RPA kits (TwistDx, U.K.) have been developed for different assays through the design of specific primer pairs (30 to 35 nucleotides) (Kim and Lee 2016; Piepenburg et al. 2006; Priti et al. 2021; Jiang et al. 2020).

Recently, RPA-based diagnostic techniques have been developed for genus-specific (Miles et al. 2015; Rojas et al. 2017) and species-specific detection in several *Phytophthora* species, including *P. infestans* (Lu et al. 2020; Si Ammour et al. 2017), *P. parasitica* (Chen et al. 2021), *P. sojae* (Dai et al. 2019; Rojas et al. 2017), *P. capsici* (Yu et al. 2019), *P. cactorum* (Lu et al. 2021), and *P. fragariae* (Munawar et al. 2020). It is well documented that nearly all 180 species in the genus *Phytophthora* are plant pathogens, and they are diverse in terms of host ranges (Kronmiller et al. 2023), making it very useful in the development and application of both species- and genus-specific detection methods.

The purposes of this study were (i) to identify species-specific multicopy sequences in the genome of *P. parasitica* for their specific detection, (ii) to test the specificity of the multicopy sequences for molecular detection, (iii) to develop a real-time multicopy sequence-based RPA assay for *P. parasitica* detection, and (iv) to validate the application of assays with artificially inoculated plant samples.

Materials and Methods

Pathogen strains and culture conditions

A total of 56 pathogen strains were used in this study: 24 *P. parasitica* strains and 32 other control strains. These 24 *P. parasitica* strains were obtained from different host plants and different locations. Thirty-two control strains include the following: 19 strains representing other *Phytophthora* species (*P. cactorum* [2 different strains], *P. sojae* [2 different strains], *P. infestans* [4 different strains], *P. capsici* [3 different strains], *P. hedraiandra* [1 strain], *P. pseudotsugae* [1 strain], *P. iranica* [1 strain], *P. tentaculate* [1 strain], *P. mirabilis* [1 strain], *P. heveae* [1 strain], *P. insolita* [1 strain], and *P. palmivora* [1 strain]); 2 *Pythium* strains (*P. aphanidermatum* [2 different strains]); 7 strains of fungal species (*Fusarium oxysporum* [1 strain], *Alternaria alternata* [1 strain], *Drechslera sofokiniana* [1 strain), *Verticillium dahliae* [1 strain], *Rhizoctonia solani* [1 strain], and *Thielaviopsis basicola* [2 different strains]); and 4 strains of the bacterial pathogens (*Ralstonia solanacearum* [2 different strains], *Pseudomonas syringae* pv. *tabaci* [1 strain], and '*Candidatus* Liberibacter asiaticus' [1 strain]).

Phytophthora species were cultured on 5% CA (carrot agar) plates or RSA (rye sucrose agar) plates at 18 or 23°C, depending on the species (Erwin and Ribeiro 1996). Bacterial strains were cultured on Luria–Bertani plates at 28°C, and the fungi were cultured on PDA plates at 28°C.

DNA preparation

All microbial isolates of total DNA were extracted using the Plant Genomic DNA Kit (Tiangen, China) for oomycetes and fungi and the TIANamp Bacteria DNA Kit (Tiangen, China) for bacteria. The concentration and quality of the extracted DNA were determined by measuring the absorbance at 260, 230, and the ratio of OD260/280 and OD260/230 using the ND-1000 Spectrophotometer (NanoDrop, U.S.A.). The DNA samples were all stored at −20°C for further use.

Genomics analysis for the identification of genome-specific multicopy sequences in *P. parasitica*

To identify novel multicopy sequences that were suitable for *P. parasitica* detection, the *P. parasitica* reference genome INRA-310 version 2.0 (GenBank assembly accession no. GCA_000247585.2) was used for genome sequence alignment. We used BLASTN (version 2.10.1) to identify potential multicopy sequences using a self-blast strategy with the default parameters. We screened the multicopy sequence through two rounds of BLAST searches. First, the sequences longer than 150 bp and with more than two high-confidence hits (E value < 0.01) were kept by "self-to-self" comparisons of the genome. Then, bedtools (version 2.30.0) was implemented to combine the overlapping multicopy sequences, and based on these collapsed regions, the potential multicopy sequences were collected with samtools (1.9). Finally, the multicopy sequences were used as query sequences in BLAST searches of the genome, and the number of hits and the mean identity value for each multicopy sequence were counted from the results. The multicopy sequence in the INRA-310 genome was aligned with the genomes of 13 sequenced *P. parasitica* strains. The genome information of the 13 sequenced *P. parasitica* strains was downloaded from the NCBI database, and then conserved and high copy number sequences were selected for BLAST in the RefSeq Genome Database in the NCBI database. This database included almost all known genomes, including those of 203 *Phytophthora* species and 123 *Pythium* species. Finally, a novel multicopy sequence was identified from *P. parasitica.*

PCR and real-time PCR amplification

Each 25-μl conventional PCR reaction mixture contained 2.5 μl of 10 × Ex buffer (Mg^{2+} plus), 2 μl of dNTP mix (2.5 mM), 0.5 μl of each forward and reverse primers (10 μM), 10 μl of template DNA solution, 0.125 μl of Ex Taq DNA polymerase (5 U/μl) (TaKaRa, Japan), and 9.375 μl of sterile water. The amplifications were performed in an S1000 Thermal Cycler (Bio-Rad, U.S.A.) as follows: an initial denaturation step of 94°C for 2 min, followed by 33 cycles of amplification at 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s, with a final extension for 10 min at 72°C. The PCR products (5 μl) were analyzed by electrophoresis in 2% agarose gels. Each experiment was independently repeated at least three times.

The real-time PCR reactions were performed in a total volume of 25 μl containing 12.5 μl of 2 × SYBR MIX (CWBIO, China), 0.625 μl of forward and reverse primers (10 μM each), 10 μl of template DNA solution, and 25 μl of sterile deionized water. The amount of template DNA is different in each experiment, as shown in the results. A three-step method was used for amplification: an initial denaturing step at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Fluorescence was monitored at 60°C using a fluorometric thermal cycler (ABI, U.S.A.) for real-time data collection in the annealing-extension steps. This experiment was independently repeated at least three times.

Specificity and sensitivity assays of PpM34 by PCR and real-time PCR

The 56 representative pathogen strains (Table 1) were used to confirm the specificity of PCR and real-time PCR based on the new multicopy sequence from *P. parasitica*. To compare the detection sensitivity between the unique multicopy sequence and the *Ypt1* gene, total DNA extracted from the *P. parasitica* strain Pp042 was serially diluted to 10, 1 ng, 100, 10, 1 pg, 100, and 10 fg and added to the PCR and real-time PCR reaction mixture. Sterile distilled water was used as a negative control. All reactions were performed as described earlier and independently repeated three times.

RPA assays for *P. parasitica* detection

The RPA primers and probes were designed using Primer Premier 3 software (Premier Biosoft International, U.S.A.) based on the sequence of the unique multicopy *P. parasitica* sequence. Primer sizes are typically 30 to 35 bp, and ideally, the product size is between 100 and 200 bp. Primer GC content was set between 30 and 70%. The size of the exo probe should be 46 to 52 bp, at least 30 of which are placed 5′ to the THF site, and at least a further 15 bases are located on the 3′ side.

RPA reactions were performed with the TwistAmp Liquid exo Kit (TwistDx). The reactions contained 2.1 μl of the forward and reverse

primers (10 μm each), 0.6 μl of exo probe (10 μm), 3.2 μl of nuclease-free water, and 10 μl of template DNA solution in 29.5 μl of rehydration buffer (at least containing dNTP, recombinase, single-stranded DNA-binding protein, and strand-displacing DNA polymerase) from the TwistAmp Liquid exo Kit. Then, 2.5 μl of MgAc (280 mM) was added. The reactions were immediately mixed, and the 50 μl of real-time RPA reaction mixtures were placed in a fluorometric thermal cycler (ABI. The system was maintained at 39°C, and the fluorescence intensity was detected 40 times at 30-s intervals using a fluorometric thermal cycler (ABI).

Fifty-six representative plant pathogen strains (Table 1), which were relative species, soilborne pathogens, or tobacco pathogens, were used to determine the specificity of the real-time RPA assay. In order to detect the sensitivity of the RPA, serial 10-fold dilutions of the *P. parasitica* strain Pp042 total DNA (at an initial concentration of 10 ng/μl) were prepared in sterile deionized water. This experiment was independently repeated at least three times.

Table 1. Fifty-six plant pathogen strains used and their derived host plants[a]

No.	Strains	Host	Mating type
1	*Phytophthora parasitica*	Eggplant	A2
2	*P. parasitica*	Tobacco	A2
3	*P. parasitica*	Tobacco	A1
4	*P. parasitica*	Tobacco	A2
5	*P. parasitica*	Paw-paw	A2
6	*P. parasitica*	Paw-paw	A2
7	*P. parasitica*	Paw-paw	A2
8	*P. parasitica*	*Banksia* species	A2
9	*P. parasitica*	*Chamelaucium* species	A2
10	*P. parasitica*	*Banksia* species	A2
11	*P. parasitica*	Citrus	A1
12	*P. parasitica*	Citrus	A1
13	*P. parasitica*	Citrus	A1
14	*P. parasitica*	Citrus	A1
15	*P. parasitica*	*Dendrobium candidum*	A2
16	*P. parasitica*	*D. candidum*	A2
17	*P. parasitica*	*D. candidum*	A2
18	*P. parasitica*	Tobacco	A2
19	*P. parasitica*	Tobacco	A2
20	*P. parasitica*	Tobacco	A2
21	*P. parasitica*	Tobacco	A2
22	*P. parasitica*	Tobacco	A2
23	*P. parasitica*	Tobacco	A2
24	*P. parasitica*	Tobacco	A2
25	*P. infestans*	Potato	
26	*P. infestans*	Potato	
27	*P. infestans*	Potato	
28	*P. infestans*	Potato	
29	*P. capsici*	Peppers	
30	*P. capsici*	Peppers	
31	*P. capsici*	Peppers	
32	*P. sojae*	Soybean	
33	*P. sojae*	Soybean	
34	*P. palmivora*	NA	
35	*P. cactorum*	Ginseng	
36	*P. cactorum*	Ginseng	
37	*P. hedraiandra*	*Viburnum* sp.	
38	*P. pseudotsugae*	*Pseudotsuga menziesii*	
39	*P. iranica*	*Solanum melongena*	
40	*P. tentaculate*	*Calendula arvensis*	
41	*P. mirabilis*	*Mirabilis jalapa*	
42	*P. heveae*	Avocado	
43	*P. insolita*	NA	
44	*Ralstonia solanacearum*	Tobacco	
45	*R. solanacearum*	Tomato	
46	*Fusarium oxysporum*	Cotton	
47	*Alternaria alternata*	Tobacco	
48	*Drechslera sofokiniana*	Wheat	
49	*Verticillium dahliae*	Cotton	
50	*Pseudomonas syringae* pv. *tabaci*	Tobacco	
51	*Rhizoctonia solani*	Tobacco	
52	'*Candidatus* Liberibacter asiaticus'	Citrus	
53	*Pythium aphanidermatum*	Tobacco	
54	*P. aphanidermatum*	Tobacco	
55	*Thielaviopsis basicola*	Tobacco	
56	*T. basicola*	Tobacco	

[a] NA = not available.

Detection of *P. parasitica* in artificially inoculated plants

To evaluate the use of conventional PCR, real-time PCR, and RPA assays, artificially inoculated plant samples were used in *P. parasitica* detection assays. The leaves of 5-week-old greenhouse-grown *Nicotiana benthamiana* plants were inoculated with the *P. parasitica* strain Pp042 mycelia that had been cultivated for 3 days and were then kept at 23°C as previously reported (Huang et al. 2019). The infected leaves showed obvious water-soaked lesions 48 h postinoculation and were divided into four different regions for genomic DNA extraction (Chen et al. 2021; Lu et al. 2020). At 48 h postinoculation, DNA was extracted from 0.1 to 0.2 g of infected leaf tissue using the cetyltrimethylammonium bromide method and dissolved in 50 μl of water. Three independent experiments were performed. Aliquots of the extracted DNA samples (10 μl) were used in the PCR, real-time PCR, and RPA assays. Healthy *N. benthamiana* leaf samples were included as the negative control. This experiment was independently repeated at least three times.

Results

Genomic DNA sequence alignment of *P. parasitica*

Scanning the whole *P. parasitica* reference genome INRA-310 version 2.0 (GenBank assembly accession no. GCA_000247585.2) yielded 202,279 repetitive sequences ranging in size from 25 bp to 1.6 Mb. In order to acquire the species-specific repeated DNA sequences, we further kept and collapsed the high-confidence repeated regions (E value < 0.01) with lengths >150 bp, and 914 collapsed sequences were mapped to the genomes of other plant pathogens that contained closely related *Phytophthora* and *Pythium* species. Next, we screened five specific multicopy sequences with copy numbers >20 in the genome of *P. parasitica*. After multiple sequence alignments of these five sequences with their homologous sequences in 13 strains of *P. parasitica*, we identified a conserved sequence with a higher copy number and named it PpM34. The specific multicopy sequence PpM34 is 203 bp in length and is repeated 31 to 60 times in the genomes of the 13 sequenced *P. parasitica* strains (Table 2). The results of sequence alignment suggest that the novel multicopy sequence PpM34 can be used as a target for the specific detection of *P. parasitica*.

Table 2. Copy numbers of the PpM34 sequence in 13 different *Phytophthora parasitica* genomes

Strains	GenBank accession number	Copy number
INRA-310	GCA_000247585.2	31
CJ02B3	GCA_000509465.1	33
IAC_01/95	GCA_000509525.1	32
CHvinca01	GCA_000509505.1	35
CJ05E6	GCA_000509485.1	31
CJ01A1	GCA_000365545.1	43
P10297	GCA_000367145.1	49
P1569	GCA_000365505.1	40
P1976	GCA_000365525.1	44
JM01	GCA_003328465.1	40
BL162	GCA_012658955.1	60
Race 1	GCA_001482985.1	35
Race 0	GCA_001483015.1	57

The genome specificity of PpM34 in *P. parasitica*

Conventional PCR using the primer pair PpM34F/PpM34R (Table 3) was able to amplify a unique DNA fragment with the expected size of 149 bp from all 24 *P. parasitica* strains but gave no PCR amplification products for all of the other 32 isolates of oomycetes, bacteria, and fungi (Table 4). The real-time PCR assays yielded consistent results (Table 4). At least three replicates were tested to confirm the specificity of the primers for both PCR and real-time PCR. These results indicate that the multicopy sequence PpM34 is highly specific for *P. parasitica* detection.

Sensitivity tests using the multicopy sequence PpM34

In conventional PCR, the assay could detect the PpM34 sequence using as little as 10 pg of purified total DNA in a 25-µl reaction volume (Fig. 1A), while PCR amplification of the *Ypt1* gene required at least 100 pg of purified DNA in a 25-µl reaction volume (Fig. 1B). The minimum detection limits for the real-time PCR assay that targeted the PpM34 sequence and the *Ypt1* gene were 100 fg and 1 pg of total DNA, respectively (Fig. 1C and D). This indicates that the sensitivity of the PpM34 sequence was 10-fold higher than the *Ypt1* gene in detecting *P. parasitica* using both conventional PCR and real-time PCR. Our results showed that PCR amplification of the PpM34 sequence had high sensitivity in the detection of *P. parasitica*.

Detection of *P. parasitica* using the PpM34-based RPA assay

Four forward and four reverse RPA primers and two exo probes were designed based on the PpM34 sequence (Table 5) to screen for the optimal primer–probe combinations for detection of *P. parasitica*. All reverse primers were tested against a single forward primer, while the best reverse primer was chosen and used to screen all the forward primers. All reverse primers (designated PpRPAR1 to PpRPAR4) were screened with the forward primer PpRPAF1 in the first screening experiment and scored for amplification performance. The best combination (PpRPAF1/PpRPAR4 [PpRPA-Probe1] and PpRPAF1/PpRPAR3 [PpRPA-Probe2]) was obtained. Then, these two reverse primers were paired with all forward candidate primers, respectively, and finally, the primers PpRPAF2 and PpRPAR3 and the probe PpRPA-Probe2 were chosen as the best combination because of the high yield of amplification and the corresponding fluorescence. The primer combination PpRPAF2/PpRPAR3 targets the PpM34 sequence, directing the amplification of a 183-bp internal DNA fragment (Fig. 2).

The specificity of the RPA assay was evaluated on 24 *P. parasitica* strains and 32 control strains (Table 1) with the primer pair PpRPAF2/PpRPAR3 and the probe PpRPA-Probe2. We used 1 ng of total DNA per reaction in the RPA assay, and all amplification products could be detected from the assay of 24 *P. parasitica* strains, while no amplification fluorescent signals were detected from the 32 control strains (Table 4). These results show that the RPA assay targets the multicopy sequence PpM34 and is highly specific for the detection of *P. parasitica*.

Analytical sensitivity was further assessed under optimal conditions using 10-fold serial dilutions of *P. parasitica* total DNA ranging from 10 ng to 100 fg. The lowest concentration for detection in the RPA assays was 1 pg (Fig. 3). This result showed that the RPA assay can specifically detect 1 pg of *P. parasitica* total DNA at 39°C within 20 min.

Detection of *P. parasitica* from inoculated plant samples

The reliability of the *P. parasitica* detection assays based on the multicopy sequence PpM34 and the *Ypt1* gene was tested on artificially inoculated plant samples. The PCR assay based on the PpM34 sequence could accurately detect the presence of *P. parasitica* in sections I, II, and III, whereas real-time PCR and RPA assays based on the PpM34 sequence accurately detected the presence of the pathogen in all four sections (Fig. 4). Meanwhile, as a comparison, PCR and real-time PCR assays based on the *Ypt1* gene could accurately detect the presence of *P. parasitica* in sections I, II and I, II, III, respectively (Fig. 4). Conventional PCR, real-time PCR, and RPA assays based on the PpM34 sequence can detect *P. parasitica* in two asymptomatic sections (sections II and III). Real-time PCR and RPA assays accurately detected the pathogen in the leaf section that was the farthest away from the inoculation site (section IV), indicating that molecular detection based on the PpM34 sequence has a high potential for detecting *P. parasitica* in asymptomatic infected plant samples.

Table 4. Specificity tests of the multicopy sequence PpM34 by PCR, real-time PCR, and recombinase polymerase amplification (RPA) assay developed in this study[a]

Pathogen species	Number of isolates	PCR	Real-time PCR	RPA
Phytophthora parasitica	24	+	+	+
P. capsici	3	–	–	–
P. sojae	2	–	–	–
P. infestans	4	–	–	–
P. palmivora	1	–	–	–
P. cactorum	2	–	–	–
P. hedraiandra	1	–	–	–
P. pseudotsugae	1	–	–	–
P. iranica	1	–	–	–
P. tentaculate	1	–	–	–
P. mirabilis	1	–	–	–
P. heveae	1	–	–	–
P. insolita	1	–	–	–
Ralstonia solanacearum	2	–	–	–
Pythium aphanidermatum	2	–	–	–
Thielaviopsis basicola	2	–	–	–
Fusarium oxysporum	1	–	–	–
Alternaria alternata	1	–	–	–
Drechslera sofokiniana	1	–	–	–
Verticillium dahliae	1	–	–	–
Pseudomonas syringae pv. *tabaci*	1	–	–	–
Rhizoctonia solani	1	–	–	–
'*Candidatus* Liberibacter asiaticus'	1	–	–	–

[a] + = positive amplification; – = negative amplification.

Table 3. Names and nucleotide sequences of the primers designed and used for PCR and real-time PCR amplification of PpM34 and the *Ypt1* gene from *Phytophthora parasitica* mycelial DNA

Primer	Sequence (5′–3′)	Target	GC%	TM (°C)	Amplicon size (bp)
QYpt1F	GACATGATATCAACTGTTCTGC	Ypt*1*	41	58	150
QYpt1R	CAGACACACACGTGATTTGGT		48		
QM34F	GGCGGATTCTCCCTTTCTAC	PpM34	55	55	149
QM34R	GGGTACTTTTGACCTGGCTG		55		
Pn1[a]	GACTTTGTAAGTGCCACCATAC	Ypt*1*	45	60	389
Pn2[a]	CTCAGCTCTTTTCCTTGGATCT		45		

[a] These primers were cited by Meng and Wang (2010).

Discussion

As a soilborne pathogen, how to effectively control the diseases caused by *P. parasitica* is an immense challenge (Huang et al. 2010). Rapid detection is a crucial step toward effective management of soilborne pathogens (Dai et al. 2019). Sensitivity and specificity are the two most important evaluation indices of a good diagnostic method. Compared with traditional detection methods, such as

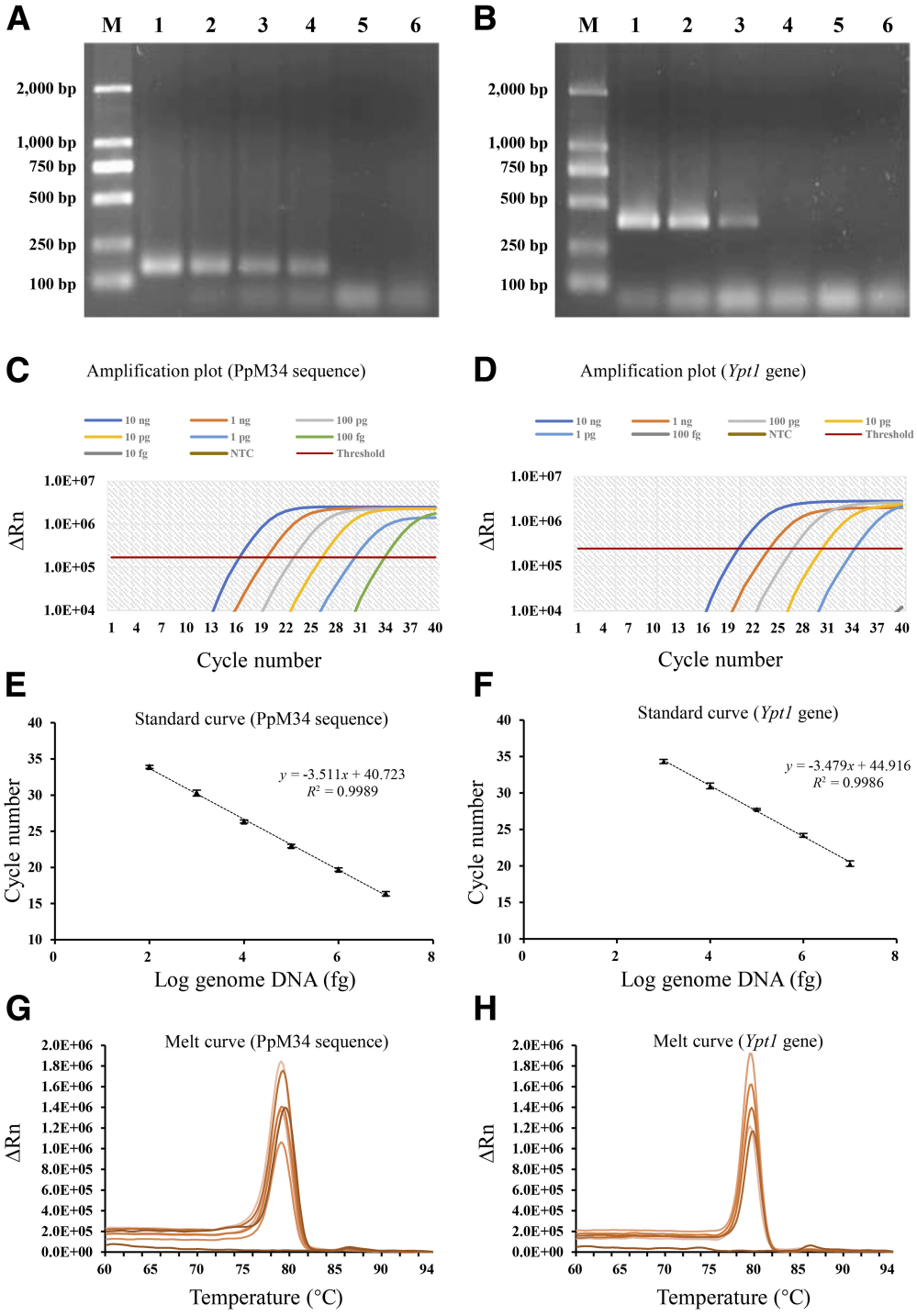

Fig. 1. Comparison of the sensitivity of conventional PCR and real-time PCR assays based on the PpM34 sequence and the single-copy *Ypt1* gene. **A,** PCR based on the PpM34 sequence using different concentrations of mycelial DNA for the detection of *Phytophthora parasitica*. Each DNA sample was tested three times, with consistent results. **B,** PCR based on the *Ypt1* gene for the detection of *P. parasitica*. Each DNA sample was tested three times, with consistent results. Lane M, GL2000 DNA marker; Lanes 1 to 5, amplified products using mycelial DNA at concentrations of 10, 1 ng, 100, 10, and 1 pg in 25-µl PCR reactions. Lane 6, nuclease-free water was used as the negative control. Similar, consistent results were obtained from three biological replicates. **C,** Real-time PCR amplification curves based on the PpM34 sequence using 10-fold dilutions of *P. parasitica* mycelial DNA (10 ng to 10 fg per reaction). NTC, the negative control (sterile nuclease-free water). **D,** Real-time PCR amplification curves based on the *Ypt1* gene using 10-fold dilutions of *P. parasitica* mycelial DNA (10 ng to 100 fg per reaction). NTC, the negative control (sterile nuclease-free water). **E,** Standard curve of the real-time PCR assay based on the PpM34 sequence. The linear regression equation of Ct (x) versus log of the starting quantity of DNA (y) was $y = -3.511x + 40.723$. The error bars represent the mean and standard error of three real-time PCR repeats. **F,** Standard curve of real-time PCR based on the *Ypt1* gene. The linear regression equation of Ct (x) versus the log of the starting quantity of DNA (y) was $y = -3.479x + 44.916$. The error bars represent the mean and standard error of three real-time PCR repeats. **G,** Melt curve of real-time PCR based on the PpM34 sequence. **H,** Melt curve of real-time PCR based on the *Ypt1* gene.

culture or serology, molecular detection, based on direct pathogen DNA, offers a more sensitive and specific diagnosis. The choice of the target DNA sequence plays a crucial role in the specificity of DNA-based detection.

The target sequence is the core problem for molecular detection methods, and molecular detection based on the high copy number sequence will suggest a higher sensitivity (González-Salgado et al. 2009; Homan et al. 2000; Jones et al. 2000). In fact, high copy number repetitive sequences have been identified and used as ideal targets in many molecular detection assays (Chng et al. 2021; Papaiakovou et al. 2017; Pilotte et al. 2016). Fortunately, the genome of a broad range of species, from prokaryotes to eukaryotes, possesses a large number of repetitive sequences (Gebre et al. 2016; Treangen and Salzberg 2011). Recently, the multicopy mitochondrial genome *atp9-nad9* region has been widely used in the detection of *Phytophthora* species (Munawar et al. 2020; Rojas et al. 2017). However, considering the various numbers of mitochondria in each cell, nuclear genomic repetitive sequences could become a better option in terms of quantitative detection. In this study, in order to develop a highly sensitive and specific DNA-based method for detecting *P. parasitica*, we identified a novel nuclear genomic multicopy sequence, PpM34, using whole-genome sequence alignment. We characterized PpM34 and found that this unique multicopy sequence is conserved among the 13 sequenced *P. parasitica* strains and is repeated 31 to 60 times in the genome of *P. parasitica* (Table 2).

Considering *P. parasitica* has shown a high degree of genetic diversity (Afandi et al. 2019), it is necessary to assay as many as possible different *P. parasitica* strains to evaluate the conservation of the multicopy PpM34 sequence in the genome of *P. parasitica*. Twenty-four *P. parasitica* strains isolated from different host plants and regions were used to determine whether the sequence is conserved, and all 24 *P. parasitica* strains were detected by amplification using both conventional PCR and real-time PCR. In addition, the sequence alignment of PpM34 in the NCBI database also showed high specificity in the *P. parasitica* genome. In our experiments (Table 4), 32 other control pathogen strains, including seven closely related taxa (*P. cactorum*, *P. infestans*, *P. hedraiandra*, *P. pseudotsugae*, *P. iranica*, *P. tentaculate*, and *P. mirabilis*), showed negative results in molecular detection assays with the same primers. The sensitivity tests comparing PpM34 with the *Ypt1* gene, which was used in previous detection of *P. parasitica*, by conventional PCR and real-time PCR indicated that the PCR amplification targeting the PpM34 sequence is 10 times more sensitive than the assay based on the single-copy *Ypt1* gene (Fig. 1). Therefore, the PpM34 sequence could be used as an ideal candidate target sequence for sensitive and specific detection of *P. parasitica*. Our results also showed that the genome sequence alignment is an effective and fast way to identify a repeated sequence target for the molecular detection of pathogens.

Isothermal amplification technology has developed rapidly because it does not require expensive, programmable, temperature-controlled instruments and trained operators. The RPA assay is considered an effective isothermal amplification method for nucleic acid detection (Munawar et al. 2020) because of its many advantages such as straightforward primer design, low instrumentation requirements, a relatively low reaction temperature, and short assay times. These advantages make isothermal amplification technology widely applicable for the detection of pathogens (Bentahir et al. 2018; Miles et al. 2015; Rojas et al. 2017). RPA combined with the lateral flow strip (RPA-LF) assay has been used for rapid and equipment-free detection of *P. parasitica*. In both this and our study, the RPA showed sensitivity (1-pg DNA of *P. parasitica* detection limit) and was rapid (20 min amplification time) in *P. parasitica* detection (Chen et al. 2021). Compared with the RPA-LF, real-time RPA based on an exo probe is more suitable for large-scale indoor quantitative detection.

Considering the success of the RPA reaction depending on the quality of the primer–probe combination, we designed four forward primers, four reverse primers, and two exo probes that targeted the PpM34 sequence. Based on the screening strategy, which was described in materials and methods, we developed an optimized RPA assay with a primer–probe combination for *P. parasitica* detection. Our results showed that the RPA assays could specifically detect 1 pg of *P. parasitica* total DNA at an amplification temperature of 39°C within 20 min.

In terms of applicability, high sensitivity is so essential that the detection assay can successfully detect very low levels of pathogen DNA in soil samples and asymptomatic plant tissues; more sensitive assays can reduce the risk of false negatives, and this is especially important for the disease control. The RPA method showed high sensitivity and could detect 1 pg of *P. parasitica* total DNA, which is 100 times higher than conventional PCR assays that target the *Ypt1* gene. Tests conducted with artificially inoculated plants showed that RPA assays could accurately detect pathogens in asymptomatic plant tissues. Therefore, the RPA assay developed in this study has great potential to be used for detecting *P. parasitica* in soil and plant samples.

For soilborne pathogens, the detection of pathogens in the soil and rhizosphere samples is necessary for effective management (Dai et al. 2019). Diagnosis of *P. parasitica* in soil and rhizosphere samples, based on our high-sensitivity sequence of PpM34, may have two

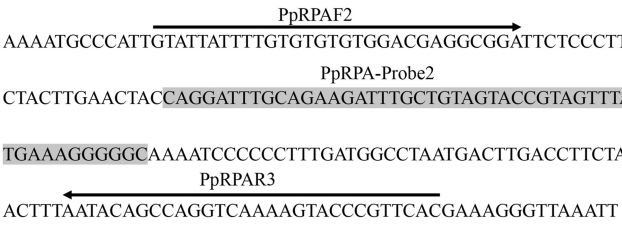

Fig. 2. Location and sequence of the primer pairs and probe used in the RPA assay that targets the *Phytophthora parasitica* multicopy sequence PpM34. RPA, recombinase polymerase amplification.

Table 5. Names and nucleotide sequences of primers and probes used for the recombinase polymerase amplification (RPA) assay

Primers/probes	Sequence (5′–3′)	Length (bp)
PpRPAF1	TGCCCATTGTATTATTTTGTGTGTGTGGAC	30
PpRPAF2	GTATTATTTTGTGTGTGTGGACGAGGCGGA	30
PpRPAF3	TGTGTGGACGAGGCGGATTCTCCCTTTCTAC	31
PpRPAF4	ACGAGGCGGATTCTCCCTTTCTACTTGAAC	30
PpRPAR1	TAGAAGGTCAAGTCATTAGGCCATCAAAGG	30
PpRPAR2	TGTATTAAAGTATAGAAGGTCAAGTCATTAG	31
PpRPAR3	GTGAACGGGTACTTTTGACCTGGCTGTATT	30
PpRPAR4	CCTTTCGTGAACGGGTACTTTTGACCTGGCTG	32
PpRPA-Probe1[a]	AGTTTATGAAAGGGGGCAAAATCCCCCCTT[FAM-dT][THF]A[BHQ1-dT]GGCCTAATGACTTGA[C3 spacer]	49
PpRPA-Probe2[a]	CAGGATTTGCAGAAGATTTGCTGTAGTACCG[FAM-dT][THF]G[BHQ1-dT]TTATGAAAGGGGGC[C3 spacer]	49

[a] FAM = thymidine nucleotide carrying fluorescein; THF = tetrahydrofuran; BHQ1 = thymidine nucleotide carrying Black Hole Quencher 1.

major applications in the future. The RPA assay combining rapid soil DNA extraction methods may also be an important research direction in future studies.

In summary, our study identified a unique multicopy sequence, designated as PpM34, specifically conserved in the *P. parasitica* genome. We used the PpM34 sequence to develop very sensitive and specific PCR and qPCR assays for the diagnosis of *P. parasitica*. At last, we developed a rapid, real-time RPA-based method to detect *P. parasitica* strains isolated from a variety of plant hosts. Our method could be used to detect the presence of less than 1 pg of total DNA in 20 min at 39°C using a fluorometric thermal cycler.

Acknowledgments

The authors thank Prof. Mingzhu Li of Shaanxi Normal University for providing *Phytophthora hedraiandra*, *P. pseudotsugae*, *P. iranica*, *P. tentaculate*, *P. mirabilis*, *P. heveae*, and *P. insolita* isolates; Prof. Jie Gao of Jilin Agricultural University for providing *P. cactorum* isolates; Dr. Guangjin Fan of Southwest University for the *P. palmivora* isolate; Dr. Guiyan Huang of Gannan Normal University for providing '*Candidatus* Liberibacter asiaticus' genomic

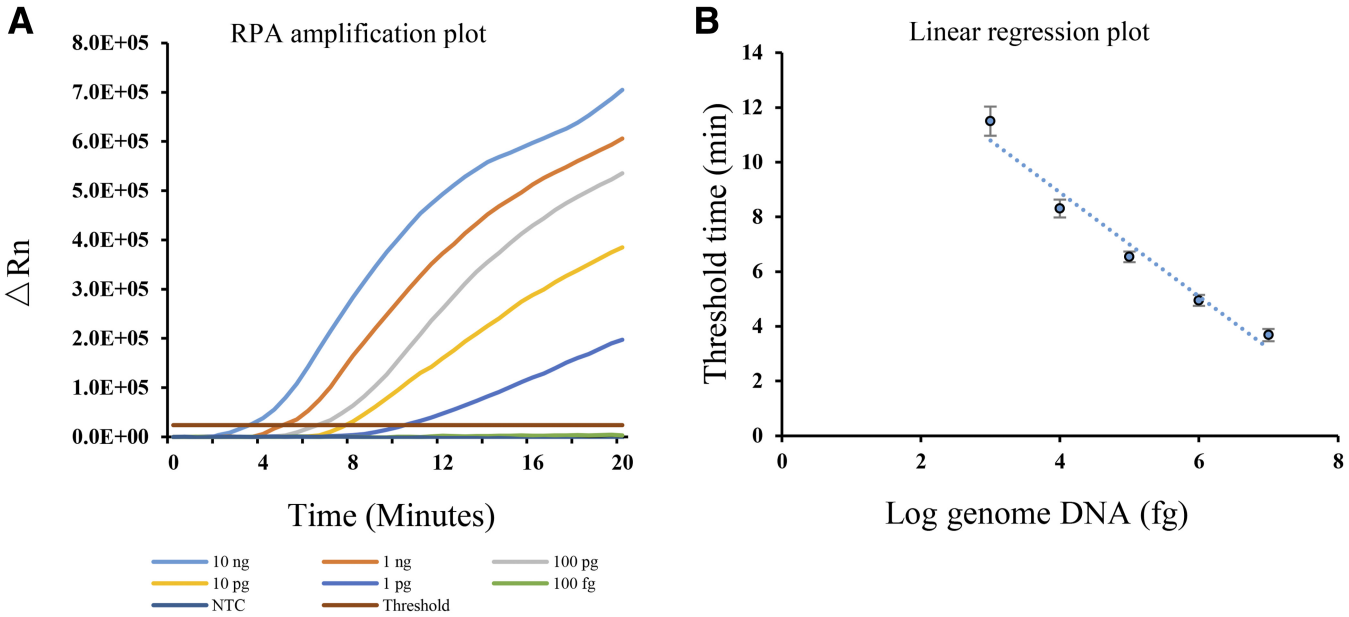

Fig. 3. Real-time recombinase polymerase amplification (RPA) assay performance. **A,** Fluorescence development over time using 10-fold serial dilutions of *Phytophthora parasitica* mycelial DNA. Real-time RPA amplification curves of *P. parasitica* mycelial DNA at 39°C for 20 min are shown, with starting mycelial DNA amounts between 10 ng and 1 pg. NTC, the negative control (sterile nuclease-free water). **B,** Semilogarithmic regression analysis of the real-time RPA assay. The error bars represent the mean and standard error of three real-time RPA repeats.

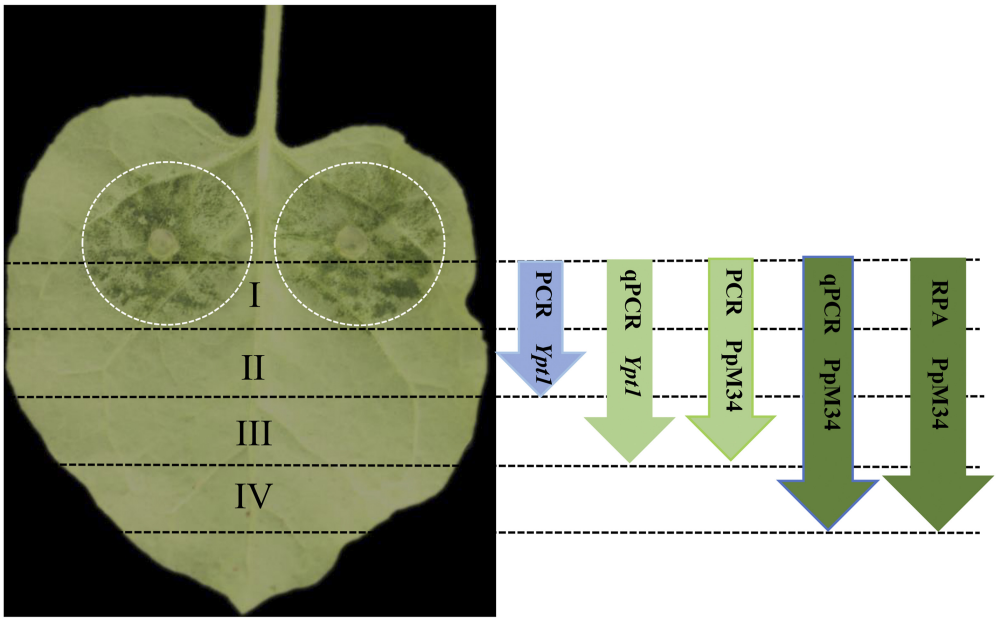

Fig. 4. Detection of *Phytophthora parasitica* in inoculated *Nicotiana benthamiana* by PCR, real-time PCR, and recombinase polymerase amplification (RPA) assays based on the PpM34 sequence and *Ypt1* gene. Total DNA was isolated from detached *N. benthamiana* leaves 48 h after inoculation with the *P. parasitica* strain Pp042. Three different pictures represent three independent experiments.

DNA; and Dr. Chong Zhang of Shenyang Agricultural University for providing *Pseudomonas syringae* pv. *tabaci* and *Rhizoctonia solani* genomic DNA.

Literature Cited

Afandi, A., Hieno, A., Wibowo, A., Subandiyah, S., Afandi, Suga, H., Tsuchida, K., and Kageyama, K. 2019. Genetic diversity of *Phytophthora nicotianae* reveals pathogen transmission mode in Japan. J. Gen. Plant Pathol. 85:189-200.

Bentahir, M., Ambroise, J., Delcorps, C., Pilo, P., and Gala, J.-L. 2018. Sensitive and specific recombinase polymerase amplification assays for fast screening, detection, and identification of *Bacillus anthracis* in a field setting. Appl. Environ. Microbiol. 84:e00506-18.

Blaya, J., Lloret, E., Santísima-Trinidad, A. B., Ros, M., and Pascual, J. A. 2016. Molecular methods (digital PCR and real-time PCR) for the quantification of low copy DNA of *Phytophthora nicotianae* in environmental samples. Pest Manag. Sci. 72:747-753.

Brasier, C. M. 2008. The biosecurity threat to the UK and global environment from international trade in plants. Plant Pathol. 57:792-808.

Chen, W.-y., Yu, J., Xu, H., Lu, X.-y., Dai, T.-t., Tian, Y.-e., Shen, D.-y., and Dou, D.-l. 2021. Combining simplified DNA extraction technology and recombinase polymerase amplification assay for rapid and equipment-free detection of citrus pathogen *Phytophthora parasitica*. J. Integr. Agric. 20:2696-2705.

Chng, L., Holt, D. C., Field, M., Francis, J. R., Tilakaratne, D., Dekkers, M. H., Robinson, G., Mounsey, K., Pavlos, R., Bowen, A. C., Fischer, K., Papenfuss, A. T., Gasser, R. B., Korhonen, P. K., Currie, B. J., McCarthy, J. S., and Pasay, C. 2021. Molecular diagnosis of scabies using a novel probe-based polymerase chain reaction assay targeting high-copy number repetitive sequences in the *Sarcoptes scabiei* genome. PLoS Negl. Trop. Dis. 15:e0009149.

Cline, E. T., Farr, D. F., and Rossman, A. Y. 2008. A synopsis of *Phytophthora* with accurate scientific names, host range, and geographic distribution. Plant Health Prog. 9. https://doi.org/10.1094/PHP-2008-0318-01-RV

Dai, T., Yang, X., Hu, T., Jiao, B., Xu, Y., Zheng, X., and Shen, D. 2019. Comparative evaluation of a novel recombinase polymerase amplification-lateral flow dipstick (RPA-LFD) assay, LAMP, conventional PCR, and leaf-disc baiting methods for detection of *Phytophthora sojae*. Front. Microbiol. 10:1884.

Erwin, D. C., and Ribeiro, O. K. 1996. *Phytophthora* Diseases Worldwide. American Phytopathological Society, St. Paul, MN.

Euler, M., Wang, Y., Heidenreich, D., Patel, P., Strohmeier, O., Hakenberg, S., Niedrig, M., Hufert, F. T., and Weidmann, M. 2013. Development of a panel of recombinase polymerase amplification assays for detection of biothreat agents. J. Clin. Microbiol. 51:1110-1117.

Gebre, Y. G., Bertolini, E., Pè, M. E., and Zuccolo, A. 2016. Identification and characterization of abundant repetitive sequences in *Eragrostis tef* cv. Enatite genome. BMC Plant Biol. 16:39.

González-Salgado, A., Patiño, B., Gil-Serna, J., Vázquez, C., and González-Jaén, M. T. 2009. Specific detection of *Aspergillus carbonarius* by SYBR Green and TaqMan quantitative PCR assays based on the multicopy ITS2 region of the rRNA gene. FEMS Microbiol. Lett. 295:57-66.

Grenville-Briggs, L. J., Kushwaha, S. K., Cleary, M. R., Witzell, J., Savenkov, E. I., Whisson, S. C., Chawade, A., and Vetukuri, R. R. 2017. Draft genome of the oomycete pathogen *Phytophthora cactorum* strain LV007 isolated from European beech (*Fagus sylvatica*). Genom. Data 12:155-156.

Grote, D., Olmos, A., Kofoet, A., Tuset, J., Bertolini, E., and Cambra, M. 2002. Specific and sensitive detection of *Phytophthora nicotianae* by simple and nested-PCR. Eur. J. Plant Pathol. 108:197-207.

Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H. Y., Handsaker, R. E., Cano, L. M., Grabherr, M., Kodira, C. D., Raffaele, S., Torto-Alalibo, T., Bozkurt, T. O., Ah-Fong, A. M. V., Alvarado, L., Anderson, V. L., Armstrong, M. R., Avrova, A., Baxter, L., Beynon, J., Boevink, P. C., Bollmann, S. R., Bos, J. I. B., Bulone, V., Cai, G., Cakir, C., Carrington, J. C., Chawner, M., Conti, L., Costanzo, S., Ewan, R., Fahlgren, N., Fischbach, M. A., Fugelstad, J., Gilroy, E. M., Gnerre, S., Green, P. J., Grenville-Briggs, L. J., Griffith, J., Grünwald, N. J., Horn, K., Horner, N. R., et al. 2009. Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. Nature 461:393-398.

Hieno, A., Li, M., Afandi, A., Otsubo, K., Suga, H., and Kageyama, K. 2019. Rapid detection of *Phytophthora nicotianae* by simple DNA extraction and real-time loop-mediated isothermal amplification assay. J. Phytopathol. 167:174-184.

Hieno, A., Li, M., Afandi, A., Otsubo, K., Suga, H., and Kageyama, K. 2020. Detection of the genus *Phytophthora* and the species *Phytophthora nicotianae* by LAMP with a QProbe. Plant Dis. 104:2469-2480.

Homan, W. L., Vercammen, M., De Braekeleer, J., and Verschueren, H. 2000. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. Int. J. Parasitol. 30:69-75.

Huang, G., Liu, Z., Gu, B., Zhao, H., Jia, J., Fan, G., Meng, Y., Du, Y., and Shan, W. 2019. An RXLR effector secreted by *Phytophthora parasitica* is a virulence factor and triggers cell death in various plants. Mol. Plant Pathol. 20:356-371.

Huang, J., Wu, J., Li, C., Xiao, C., and Wang, G. 2010. Detection of *Phytophthora nicotianae* in soil with real-time quantitative PCR. J. Phytopathol. 158:15-21.

Huang, W., Zhang, H., Xu, J., Wang, S., Kong, X., Ding, W., Xu, J., and Feng, J. 2017. Loop-mediated isothermal amplification method for the rapid detection of *Ralstonia solanacearum* phylotype I mulberry strains in China. Front. Plant Sci. 8:76.

Ippolito, A., Schena, L., and Nigro, F. 2002. Detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soils by nested PCR. Eur. J. Plant Pathol. 108:855-868.

Jiang, W., Ren, Y., Han, X., Xue, J., Shan, T., Chen, Z., Liu, Y., and Wang, Q. 2020. Recombinase polymerase amplification-lateral flow (RPA-LF) assay combined with immunomagnetic separation for rapid visual detection of *Vibrio parahaemolyticus* in raw oysters. Anal. Bioanal. Chem. 412:2903-2914.

Jones, C. D., Okhravi, N., Adamson, P., Tasker, S., and Lightman, S. 2000. Comparison of PCR detection methods for B1, P30, and 18S rDNA genes of *T. gondii* in aqueous humor. Invest. Ophthalmol. Vis. Sci. 41:634-644.

Kamoun, S., Furzer, O., Jones, J. D. G., Judelson, H. S., Ali, G. S., Dalio, R. J. D., Roy, S. G., Schena, L., Zambounis, A., Panabières, F., Cahill, D., Ruocco, M., Figueiredo, A., Chen, X.-R., Hulvey, J., Stam, R., Lamour, K., Gijzen, M., Tyler, B. M., Grünwald, N. J., Mukhtar, M. S., Tomé, D. F. A., Tör, M., Van Den Ackerveken, G., McDowell, J., Daayf, F., Fry, W. E., Lindqvist-Kreuze, H., Meijer, H. J. G., Petre, B., Ristaino, J., Yoshida, K., Birch, P. R. J., and Govers, F. 2015. The top 10 oomycete pathogens in molecular plant pathology. Mol. Plant Pathol. 16:413-434.

Kamoun, S., Klucher, K. M., Coffey, M. D., and Tyler, B. M. 1993. A gene encoding a host-specific elicitor protein of *Phytophthora parasitica*. Mol. Plant-Microbe Interact. 6:573-581.

Kim, J. Y., and Lee, J.-L. 2016. Rapid detection of *Salmonella Enterica* serovar enteritidis from eggs and chicken meat by real-time recombinase polymerase amplification in comparison with the two-step real-time PCR. J. Food Saf. 36:402-411.

Kong, P., Hong, C., Jeffers, S. N., and Richardson, P. A. 2003. A species-specific polymerase chain reaction assay for rapid detection of *Phytophthora nicotianae* in irrigation water. Phytopathology 93:822-831.

Kronmiller, B. A., Feau, N., Shen, D., Tabima, J. F., Ali, S. S., Armitage, A. D., Arredondo, F., Bailey, B. A., Bollmann, S. R., Dale, A., Harrison, R. J., Hrywkiw, K., Kasuga, T., McDougal, R., Nellist, C. F., Panda, P., Tripathy, S., Williams, N. M., Ye, W., Wang, Y., Hamelin, R. C., and Grünwald, N. J. 2023. Comparative genomic analysis of 31 *Phytophthora* genomes reveals genome plasticity and horizontal gene transfer. Mol. Plant-Microbe Interact. 36:26-46.

Lacourt, I., and Duncan, J. M. 1997. Specific detection of *Phytophthora nicotianae* using the polymerase chain reaction and primers based on the DNA sequence of its elictin gene *ParA1*. Eur. J. Plant Pathol. 103:73-83.

Li, B., Liu, P., Xie, S., Yin, R., Weng, Q., and Chen, Q. 2015. Specific and sensitive detection of *Phytophthora nicotianae* by nested PCR and loop-mediated isothermal amplification assays. J. Phytopathol. 163:185-193.

Li, M., Asano, T., Suga, H., and Kageyama, K. 2011. A multiplex PCR for the detection of *Phytophthora nicotianae* and *P. cactorum*, and a survey of their occurrence in strawberry production areas of Japan. Plant Dis. 95:1270-1278.

Li, M., Inada, M., Watanabe, H., Suga, H., and Kageyama, K. 2013. Simultaneous detection and quantification of *Phytophthora nicotianae* and *P. cactorum*, and distribution analyses in strawberry greenhouses by duplex real-time PCR. Microbes Environ. 28:195-203.

Lizardi, P. M., Huang, X., Zhu, Z., Bray-Ward, P., Thomas, D. C., and Ward, D. C. 1998. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat. Genet. 19:225-232.

Lu, X., Xu, H., Song, W., Yang, Z., Yu, J., Tian, Y., Jiang, M., Shen, D., and Dou, D. 2021. Rapid and simple detection of *Phytophthora cactorum* in strawberry using a coupled recombinase polymerase amplification–lateral flow strip assay. Phytopathol. Res. 3:12.

Lu, X., Zheng, Y., Zhang, F., Yu, J., Dai, T., Wang, R., Tian, Y., Xu, H., Shen, D., and Dou, D. 2020. A rapid, equipment-free method for detecting *Phytophthora infestans* in the field using a lateral flow strip-based recombinase polymerase amplification assay. Plant Dis. 104:2774-2778.

Martin, F. N., and Tooley, P. W. 2004. Identification of *Phytophthora* isolates to species level using restriction fragment length polymorphism analysis of a polymerase chain reaction-amplified region of mitochondrial DNA. Phytopathology 94:983-991.

Meng, J., and Wang, Y. 2010. Rapid detection of *Phytophthora nicotianae* in infected tobacco tissues and soil samples based on its *Ypt1* gene. J. Phytopathol. 158:1-7.

Miles, T. D., Martin, F. N., and Coffey, M. D. 2015. Development of rapid isothermal amplification assays for detection of *Phytophthora* spp. in plant tissue. Phytopathology 105:265-278.

Munawar, M. A., Toljamo, A., Martin, F., Oksanen, E., and Kokko, H. 2020. Development and evaluation of a recombinase polymerase amplification assay for rapid detection of strawberry red stele pathogen. Phytopathol. Res. 2:26.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28:e63.

Olson, H. A., Jeffers, S. N., Ivors, K. L., Steddom, K. C., Williams-Woodward, J. L., Mmbaga, M. T., Benson, D. M., and Hong, C. X. 2013. Diversity and mefenoxam sensitivity of *Phytophthora* spp. associated with the ornamental horticulture industry in the Southeastern United States. Plant Dis. 97:86-92.

Papaiakovou, M., Pilotte, N., Grant, J. R., Traub, R. J., Llewellyn, S., McCarthy, J. S., Krolewiecki, A. J., Cimino, R., Mejia, R., and Williams, S. A. 2017. A

novel, species-specific, real-time PCR assay for the detection of the emerging zoonotic parasite *Ancylostoma ceylanicum* in human stool. PLoS Negl. Trop. Dis. 11:e0005734.

Piepenburg, O., Williams, C. H., Stemple, D. L., and Armes, N. A. 2006. DNA detection using recombination proteins. PLoS Biol. 4:e204.

Pilotte, N., Papaiakovou, M., Grant, J. R., Bierwert, L. A., Llewellyn, S., McCarthy, J. S., and Williams, S. A. 2016. Improved PCR-based detection of soil transmitted helminth infections using a next-generation sequencing approach to assay design. PLoS Negl. Trop. Dis. 10:e0004578.

Priti, Jangra, S., Baranwal, V. K., Dietzgen, R. G., and Ghosh, A. 2021. A rapid field-based assay using recombinase polymerase amplification for identification of *Thrips palmi*, a vector of tospoviruses. J. Pest Sci. 94:219-229.

Rojas, J. A., Miles, T. D., Coffey, M. D., Martin, F. N., and Chilvers, M. I. 2017. Development and application of qPCR and RPA genus- and species-specific detection of *Phytophthora sojae* and *P. sansomeana* root rot pathogens of soybean. Plant Dis. 101:1171-1181.

Schena, L., Cardle, L., and Cooke, D. E. 2008. Use of genome sequence data in the design and testing of SSR markers for *Phytophthora* species. BMC Genom. 9:620.

Si Ammour, M., Bilodeau, G. J., Tremblay, D. M., Van der Heyden, H., Yaseen, T., Varvaro, L., and Carisse, O. 2017. Development of real-time isothermal amplification assays for on-site detection of *Phytophthora infestans* in potato leaves. Plant Dis. 101:1269-1277.

Treangen, T. J., and Salzberg, S. L. 2011. Repetitive DNA and next-generation sequencing: Computational challenges and solutions. Nat. Rev. Genet. 13: 36-46.

Tyler, B. M., Tripathy, S., Zhang, X., Dehal, P., Jiang, R. H. Y., Aerts, A., Arredondo, F. D., Baxter, L., Bensasson, D., Beynon, J. L., Chapman, J., Damasceno, C. M. B., Dorrance, A. E., Dou, D., Dickerman, A. W., Dubchak, I. L., Garbelotto, M., Gijzen, M., Gordon, S. G., Govers, F., Grunwald, N. J., Huang, W., Ivors, K. L., Jones, R. W., Kamoun, S., Krampis, K., Lamour, K. H., Lee, M.-K., McDonald, W. H., Medina, M., Meijer, H. J. G., Nordberg, E. K., Maclean, D. J., Ospina-Giraldo, M. D., Morris, P. F., Phuntumart, V., Putnam, N. H., Rash, S., Rose, J. K. C., Sakihama, Y., et al. 2006. *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. Science 313:1261-1266.

Vincent, M., Xu, Y., and Kong, H. 2004. Helicase-dependent isothermal DNA amplification. EMBO Rep. 5:795-800.

Yu, J., Shen, D., Dai, T., Lu, X., Xu, H., and Dou, D. 2019. Rapid and equipment-free detection of *Phytophthora capsici* using lateral flow strip-based recombinase polymerase amplification assay. Lett. Appl. Microbiol. 69:64-70.