Evaluation of Recombinase Polymerase Amplification Assay for Detecting *Meloidogyne javanica*

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Abstract

Meloidogyne javanica is one of the most widespread and economically important nematodes in many countries, including China. In this study, a recombinase polymerase amplification (RPA) assay was evaluated for the detection of *M. javanica* based on the sequences of a sequence-characterized amplified regions marker gene segment. The RPA assay specifically detected *M. javanica* from individual juvenile or adult female, *M. javanica*-induced galls, and nematodes in the soil samples. The detection limit of *M. javanica* RPA assay was 1 pg of purified genomic DNA, 0.01 adult female, or 0.1 second-stage juvenile, which was

10 times more sensitive than conventional PCR assay. Furthermore, combined with lateral flow dipstick (LFD), a visual detection method of LFD-RPA assay was developed, which is suitable for onsite surveys and routine diagnostics. Results indicate that the RPA assay is rapid, sensitive, and reliable for detection and molecular identification of *M. javanica*.

Keywords: detection, lateral flow dipstick, *Meloidogyne javanica*, recombinase polymerase amplification

Root knot nematodes (*Meloidogyne* spp.) are economically the most important plant-parasitic nematodes worldwide; they have a broad host range (Khanal et al. 2016) and cause up to 5% losses in global crop yield (Trudgill and Blok 2001). The most widely distributed *Meloidogyne* species in China are *Meloidogyne incognita*, *Meloidogyne javanica*, *Meloidogyne arenaria*, *Meloidogyne hapla*, and *Meloidogyne enterolobii* (Xu et al. 2004). Among these species, *M. javanica* is responsible for significant damage to vegetables, fruits, and many other crops of economic importance, primarily in south China (Su et al. 2017; Zeng et al. 2018). Therefore, the diagnosis of *M. javanica* becomes essential.

Management options currently available for plant-parasitic nematode are limited to the use of chemicals, biologicals, crop rotation, and host plant resistance (Khanal et al. 2018a). Because plant-parasitic nematodes are highly diverse organisms (Khanal et al. 2016, Khanal et al. 2018b, 2019; Kularathna et al. 2019), an accurate identification of a species is prerequisite for any nematode management programs. Traditional identification of *M. javanica* was based on morphological, biochemical (isozyme phenotyping), and molecular methods (especially PCR-based technology) (Carneiro et al. 2000; Donkers-Venne et al. 2000; Meng et al. 2004). However, an accurate identification of species of the genus *Meloidogyne* using classical morphological methods requires personnel with a high level of expertise (Khanal et al. 2016). Similarly, isozyme phenotyping and conventional molecular identification often require a laboratory, professional background knowledge, and use of advanced instruments and software, making onsite detection infeasible. To overcome the difficulties associated with conventional molecular identification, the loop-mediated isothermal amplification (LAMP) technique has

been developed for onsite detection of several plant-parasitic nematodes, including *M. javanica* (Lin et al. 2016; Niu et al. 2011, 2012; Peng et al. 2012, 2017; Zhang and Gleason 2019; Zhou et al. 2017). Without complex instrumentation, LAMP can yield large amounts of amplified product in a short time (0.5 to 1 h) in isothermal conditions (60 to 65°C) using four or six primers (Notomi et al. 2000). However, the product of LAMP is a series of concatemers of the target DNA region, and it is not useful for cloning and sequencing, hence limiting its application for molecular identification of nematodes.

Recombinase polymerase amplification (RPA) is a novel isothermal DNA amplification technology that is based on a combination of polymerases and DNA recombination and repair proteins. In RPA, the recombinase-primer complexes scan double-stranded template DNA for homologous sequences and facilitate strand exchange at cognate sites. Then, the single-stranded DNA binding proteins bind to the displaced strand, and the polymerase starts to extend the primers to produce amplification products (Piepenburg et al. 2006). Unlike LAMP, RPA reacts at lower temperatures (37 to 42°C) and enables the sequence-specific recognition of target sites with only two primers. Furthermore, the amplification product is a “single band,” like that of PCR product on gel, and therefore, it can be used for other molecular biology research. Several studies using RPA have demonstrated high sensitivity and specificity for detecting small amounts of viral, bacterial, and trematode DNA (Boyle et al. 2014; Cabada et al. 2017; Crannell et al. 2016; Feng et al. 2018). RPA has been developed for rapid detection of the root knot nematode *M. enterolobii* (Subbotin 2019). For onsite surveys and routine field monitoring, RPA can be combined with the simple noninstrumentation detection device—lateral flow dipsticks (LFDs; Milenia Biotec)—that is used to detect complex analytes labeled with 5-carboxyfluorescein (FAM) and biotin. RPA uses a specific probe labeled with FAM at the 5′ end and a reverse primer biotinylated at the 5′ end to produce FAM- and biotin-labeled amplification products, which are suitable for LFD detection (Piepenburg et al. 2006). Additionally, the RPA product can also be visualized by adding SYBR Green I, a nucleic acid stain that binds to double-stranded DNA and emits green light. To evaluate the usefulness of RPA assay for identification of *M. javanica*, we established a sensitive and specific RPA method for the rapid detection of *M. javanica*. The applicability of the assay was validated by diagnosis and identification of *M. javanica* from juvenile, adult female, root galls, and soil nematode communities.

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Materials and Methods

Nematode populations and DNA extraction. Seven species of the genus *Meloidogyne* and five species of other plant-parasitic nematodes used in this study are listed in Table 1. *Meloidogyne* spp. collected from various provinces in China were purified from single egg mass and identified by microscopic observation and molecular identification using species-specific primers (Meng et al. 2004). Then, these populations were maintained on a susceptible tomato cultivar (*Solanum lycopersicum* ‘Wanza No. 15’) in a greenhouse. Purified genomic DNA (gDNA) of nematode was extracted using the DNeasy Tissue Kit (Qiagen) according to the manufacturer’s specifications. The purified gDNA of *Meloidogyne graminicola* second-stage juvenile (J2) was provided by Song Zhiqiang (Institute of Bast Fiber Crops, China), and the gDNA of *Meloidogyne mali* and *Meloidogyne camelliae* was provided by Fang Yiwu (Ningbo Academy of Inspection and Quarantine, China).

Primer design and testing. Sequence-characterized amplified region (SCAR) marker genomic sequence of *M. javanica* (GenBank accession no. KP411880.1) was selected as the target for RPA primer design. The primers MJRPAF and MJRPAR were designed using the software Primer Premier 6.0 (Premier Biosoft International), and their overall specificity was examined using BLAST (<https://www.ncbi.nlm.nih.gov/blast> and <https://meloidogyne.inra.fr>). Primers were tested with gDNA extracted from J2 of *M. javanica* using the commercially available TwistAmpBasic Kit (TwistDx). A mixture containing 29.5 µl of rehydration buffer, 1 µl of purified gDNA solution (~10 ng), 2.4 µl (10 µM) of each of MJRPAF and MJRPAR primers, and 12.2 µl of dH₂O was prepared. The supplied dry reagents (enzymes mixture) and 2.5 µl of 280 mM Mg-acetate were added to this mixture to make the final volume of 50 µl. Reactions were incubated at 39°C for 40 min in a water bath. Amplification products were purified with the DiaSpin PCR Product Purification Kit (Sangon Biotech) and visualized in 1.5% agarose gel stained with GoldView I Nuclear Staining Dyes (Solarbio). For visual fluorescence detection, 0.5 µl of SYBR Green I (Sangon Biotech) was added into the product, and the result was detected under ultraviolet light.

Sensitivity and specificity of RPA assay. To test the sensitivity of RPA, a serial 10-fold dilution of purified *M. javanica* gDNA (at an initial concentration of 1 ng/µl) was subject to RPA assay. A total volume of 50 µl of RPA mixture containing 29.5 µl of rehydration buffer, 1 µl of gDNA solution, 2.4 µl (10 µM) of each of MJRPAF and MJRPAR primers, and 12.2 µl of dH₂O was mixed with the supplied dry reagents, and then, it was added with 2.5 µl of 280 mM Mg-acetate. The mixture was incubated at 39°C for 40 min in a water bath. The same amount of *M. javanica* gDNA was also amplified using conventional PCR assay. The PCR reactions were prepared for a

25-µl volume of 25 µl having 12.5 µl of Taq PCR Master Mix (Sangon Biotech), 1 µl of each of 10 µM *M. javanica*-specific primers MJF and MJR (Table 2), 9.5 µl of DNase-free water, and 1 µl of gDNA template. PCR cycling parameters were 94°C for 4 min followed by 35 PCR cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s and a final incubation at a 72°C for 10 min.

To determine the specificity of RPA assay for *M. javanica* within and across genus, *M. javanica* of four different geographical origins, six other *Meloidogyne* species, and plant-parasitic nematodes of five other genera were subject to RPA assay (Table 1). Purified gDNA was extracted with the DNeasy Tissue Kit (Qiagen) as described above. For each RPA reaction, 1 µl of gDNA (~10 ng) solution was used. Both sensitivity and specificity tests were performed in triplicate. The amplification products of RPA and PCR were visualized in 1.5% agarose gel.

RPA assays of individual juvenile and adult female. To test whether RPA assay can detect *M. javanica* by using crude gDNA of individual J2 or adult female, an individual nematode was placed in 10 µl of worm lysis buffer (WLB; 50 mM KCl, 10 mM Tris, pH 8.2, 2.5 mM MgCl₂, 60 µg/ml proteinase K, 0.45% Tween 20, and 0.01% gelatin) on a glass slide and cut into several pieces using a sterilized scalpel under stereoscopic microscope (Castagnone-Sereno et al. 1995). After incubating at 65°C for 1 h and 95°C for 10 min in a water bath, the crude gDNA was serially 10-fold diluted to 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴, and then, it was subject to RPA and PCR assays as described above.

Detection of *M. javanica* in artificially inoculated plant root galls and soil. Four-leaf-old tomato seedlings (Wanza No. 15) were each inoculated with 400 *M. javanica* J2s and kept at 25°C for 2 weeks. The root galls developed were then collected and assessed using the RPA assay. The crude *Meloidogyne* gDNA from galls was extracted as described by Niu et al. (2012). Briefly, 5 to 10 mg of root gall was crushed in 40 µl of WLB followed by incubation of the lysate at 65°C for 1 h and 95°C for 5 min. After centrifugation at 12,000 rpm for 2 min, 5 µl of the supernatant was taken for RPA and PCR assays. *M. incognita*-induced root galls and healthy roots were used as negative controls.

Ten artificially inoculated soil samples were prepared by mixing 500 g of root knot nematode (RKN)-free soil with 500 J2 of *M. javanica*. Three RKN-free soil samples and three soil samples mixed with *M. incognita* J2 were used as negative controls. The gDNA of *M. javanica* from soil samples was extracted using two different methods. In method 1, total gDNA of soil was extracted directly from 0.5 g soil using the FastDNA SPIN Kit for soil (MP Biomedicals) following the manufacturer’s instructions. In method 2, nematodes were collected from 100 g of soil by the modified Baermann funnel method (Viglierchio and Schmitt 1983), and then, crude gDNA of

Table 1. Plant-parasitic nematode species used in assessing specificity of recombinase polymerase amplification assay

Species	Code	Original host	Geographical origin	Sample source
<i>Meloidogyne javanica</i>	Mj1	Tomato	Kunming, Yunnan Province	Institute of Bast Fiber Crops, China
<i>Meloidogyne javanica</i>	Mj2	Pseudoginseng	Wenshan, Yunnan Province	
<i>Meloidogyne javanica</i>	Mj3	Tomato	Shouguang, Shandong Province	
<i>Meloidogyne javanica</i>	Mj4	Industrial hemp	Kunming, Yunnan Province	
<i>Meloidogyne incognita</i>	Mi1	Tomato	Yantai, Shandong Province	
<i>Meloidogyne arenaria</i>	Mi2	Eggplant	Bozhou, Anhui Province	Institute of Bast Fiber Crops, China
	Ma1	Grape	Jinan, Shandong Province	
	Ma2	Peanut	Huaiyuan, Anhui Province	
<i>Meloidogyne hapla</i>	Mh1	Pseudoginseng	Wenshan, Yunnan Province	
<i>Meloidogyne graminicola</i> ^a	Mg1	Rice	Changsha, Hunan Province	
<i>Meloidogyne mali</i> ^a	Mm1	Japanese maple	Japan	Ningbo Academy of Inspection and Quarantine, China
<i>Meloidogyne camelliae</i> ^a	Mc1	Crape myrtle	Japan	Ningbo Academy of Inspection and Quarantine, China
<i>Heterodera avenae</i>	Ha1	Wheat	Suzhou, Anhui Province	
<i>Heterodera filipjevi</i>	Hf1	Wheat	Taihe, Anhui Province	
<i>Aphelenchoides besseyi</i>	Ab1	Rice	Wangjiang, Anhui Province	
<i>Ditylenchus destructor</i>	Dd1	Sweet potato	Xuzhou, Jiangsu Province	
<i>Pratylenchus neglectus</i>	Pn1	Wheat	Suzhou, Anhui Province	

^a Only genomic DNA of second-stage juvenile was provided.

nematode was extracted using WLB as described by Qiu et al. (2006). Briefly, nematode isolated from soil was transferred into a 1.5-ml microfuge tube and concentrated by spinning at 12,000 × g for 10 min at 4°C. The supernatant was discarded, and 50 µl of WLB was added to the tube. The tubes were stored at –80°C overnight (or in liquid nitrogen for 5 min). The tubes were incubated at 65°C for 1 h followed by 95°C for 5 min and centrifugation at 12,000 × g for 5 min; 5 µl of supernatant was used as the template DNA.

LFD-RPA assay. LFD-RPA assay was evaluated using purified gDNA of *M. javanica* J2, crude gDNA of individual *M. javanica* J2, an individual adult female, root galls, and crude gDNA of total nematode isolated from soil. An FAM-labeled hybridization probe MJPro and a biotin-labeled reverse primer MJBioR were designed for RPA reaction (Table 2). For RPA reaction, a mixture containing 29.5 µl of rehydration buffer, 2.1 µl of forward primer MJRPAF (5 µM), 2.1 µl of biotinylated reverse primer MJBioR (5 µM), 0.6 µl of probe MJPro (2.5 µM), 1 µl of DNA solution (~10 ng), and 12.2 µl of dH₂O was thoroughly mixed and added to the supplied dry reagents from the TwistAmpnf0 Kit (TwistDx). Finally, 2.5 µl (280 mM) of Mg-acetate was added to the mixture to make the final volume of 50 µl. After incubating at 39°C for 40 min, 5 µl of the reaction mix was added directly to the sample application area of LFD (Milenia Biotec), and then, the application area was incubated in the assay buffer (1× phosphate buffered saline with 0.1% Tween 20) for

5 min at room temperature. The positive result was visualized by the formation of an intensely colored test line and control line, whereas a negative result was visualized by the formation of control line only.

To determine the practical application of LFD-RPA onsite field diagnostics, a total of 18 natural field soil samples suspected of having *M. javanica* infestation were collected from Shandong (6 samples), Yunnan (6 samples), and Anhui (6 samples) in China. Nematode isolation was performed using the Baermann funnel technique (Christie and Perry 1951), and crude gDNA was extracted using the WLB method as described by Qiu et al. (2006). The results were confirmed by microscopic observation and single-nematode PCR assay. RKN-free and *M. incognita* J2-inoculated soil samples were used as negative controls, and *M. javanica* J2-inoculated soil sample was used as positive control.

Results

Primer test. A 262-bp band was obtained from RPA reaction using gDNA of four *M. javanica* isolates, whereas negative control did not produce any amplification. None of the RPA reactions had non-specific amplification. Under visual fluorescence detection with SYBR Green I, positive amplification glowed bright fluorescent green under ultraviolet light (Fig. 1). The results indicated that the primers MJRPAF and MJRPAR can be used for RPA reaction, whereas the product can be detected by both agarose gel electrophoresis and visual fluorescence detection with SYBR Green I.

Table 2. Primers and DNA probe used in *Meloidogyne javanica* recombinase polymerase amplification (RPA) assay and PCR assay

Code	Primer and probe sequence (5'–3')	Size of amplicon, base pair	Application	Source
MJRPAF	GAAGTGAGCCAGACTGAAACGAGGTGTACCC	262	RPA	This study
MJRPAR	GGCGTTTATTTTCAATTGGCCTCCTTGAGTG			This study
MJF	ACGCTAGAATTCGACCCCTGG	517	PCR	Meng et al. (2004)
MJR	GGTACCAGAAGCAGCCATGC			Meng et al. (2004)
MJBioR ^a	Bio-GGCGTTTATTTTCAATTGGCCTCCTTGAGTG		LFD-RPA ^b	This study
MJPro ^c	FAM-GCTCTAATTAAAGCCCGGTTCTATTAGATGC (dSpacer) CATGTCTAATAGATGC-C ₃ spacer		LFD-RPA ^b	This study

^a MJBioR was labeled at its 5' end with a biotin.

^b LFD, lateral flow dipstick.

^c MJPro consisted of an oligonucleotide backbone with a 5-carboxyfluorescein (FAM) group at the 5' end, a dSpacer that replaced a cytosine, and a C₃ spacer at the 3' end.

Fig. 1. Primer test of recombinase polymerase amplification (RPA) assay for *Meloidogyne javanica*. Genomic DNA extracted from *M. javanica* population 1 (Mj1), *M. javanica* population 2 (Mj2), *M. javanica* population 3 (Mj3), and *M. javanica* population 4 (Mj4) second-stage juveniles was used as amplification template for RPA reaction. The amplification products were detected by both 1.5% agarose gel electrophoresis and visual observation with SYBR Green I fluorescence dye. Amplification products using *M. javanica* A, showed single bands on the gel and B, glowed bright fluorescent green under ultraviolet light. M, marker; NC, negative control (water).

Sensitivity and specificity of RPA assay. To test the sensitivity of RPA assay, a dilution series of *M. javanica* gDNA (1 ng to 10 fg) was detected by both RPA assay and traditional PCR assay. The RPA assay showed high sensitivity, with the lowest detection limit of 1 pg of *M. javanica* gDNA, which was 10-fold more sensitive than the conventional PCR assay (Fig. 2).

For specificity, the gDNA of *M. javanica* and 11 other plant-parasitic nematodes was subjected to RPA assay. RPA reactions using the gDNA of *M. javanica* as template showed a single band in agarose gel electrophoresis and bright fluorescent green under ultraviolet light after staining with SYBR Green I. No amplification was obtained with DNA of other plant-parasitic nematodes, including six *Meloidogyne* species (*M. incognita*, *M. graminicola*, *M. arenaria*, *M. hapla*, *M. mali*, and *M. camelliae*), *Heterodera avenae*, *Heterodera glycines*, *Aphelenchoides besseyi*, *Ditylenchus destructor*, and *Pratylenchus neglectus* (Fig. 3). Sensitivity and specificity tests revealed that the primers MJRPAF/MJRPAR could be used specifically for the detection of *M. javanica* DNA.

Evaluation of the RPA assays using individual J2 and adult female. The crude gDNA extracts from an individual *M. javanica* J2 and adult female were used as amplification templates for conventional PCR and RPA assays. All of 20 J2 and 20 female DNA samples were detected by RPA assay, whereas 17 of 20 single

J2 samples and 19 of 20 female adult samples were successfully amplified by PCR assay. None of the PCR and RPA assays exhibited false-positive result using *M. incognita* or DNA-free samples (Table 3). RPA allowed the detection of as low as 0.01 fraction of an adult female or 0.1 fraction of a J2, making it 10-fold more sensitive than conventional PCR assay (Fig. 4).

Evaluation of the RPA assay using artificially inoculated plant root galls and soil. RPA assay was also evaluated for the detection of *M. javanica* using artificially inoculated plant root and soil nematodes. All 20 crude gDNA samples of *M. javanica*-induced galls were successfully amplified and detected by RPA assay, whereas the healthy roots and *M. incognita*-induced galls were not detected. The two different methods used for DNA extraction from 16 soil samples (10 *M. javanica* artificially inoculated samples, three healthy samples, and three *M. incognita* artificially inoculated samples) differed in sensitivity. Using total DNA extracted from soil directly, only three *M. javanica* of 10 artificially inoculated samples were detected successfully by RPA assay, whereas using crude gDNA extracted from nematodes isolated from 100-g soil samples by the modified Baermann funnel method resulted in successful detection

Table 3. Detection of *Meloidogyne javanica* from different samples using recombinase polymerase amplification assay

Samples	DNA extraction method	Positive/trials
Single nematode		
Single second-stage juvenile	Crude extraction	20/20
Single female	Crude extraction	20/20
Root galls		
<i>M. javanica</i> induced	Crude extraction	10/10
<i>Meloidogyne incognita</i> induced	Crude extraction	0/3
Healthy root	Crude extraction	0/3
Soil		
<i>M. javanica</i> infected	Method 1 ^a	3/10
<i>M. javanica</i> infected	Method 2 ^b	10/10
<i>Meloidogyne incognita</i> infected	Method 1	0/3
<i>Meloidogyne incognita</i> infected	Method 2	0/3
RKN ^c free	Method 1	0/3
RKN free	Method 2	0/3

^a Crude genomic DNA was extracted directly from soil samples.
^b Total crude genomic DNA was extracted from nematodes isolated from soil samples by the modified Baermann funnel method.
^c RKN, root knot nematode.

Fig. 2. Detection of a serially diluted *Meloidogyne javanica* genomic DNA by **A**, recombinase polymerase amplification and **B**, conventional PCR assays. The products were separated in 1.5% agarose gel and visualized under ultraviolet light. M, marker; NC, negative control (water).

Fig. 3. Specificity of the recombinase polymerase amplification assay to *Meloidogyne javanica*. The amplification products were detected by both **A**, 1.5% agarose gel electrophoresis and **B**, visual observation with SYBR Green I fluorescence dye. Ab1, *Aphelenchoides besseyi*; Dd1, *Ditylenchus destructor*; Ha1, *Heterodera avenae*; Hf1, *Heterodera filipjevi*; M, marker; Ma1, *Meloidogyne arenaria* population 1; Ma2, *M. arenaria* population 2; Mc1, *Meloidogyne camelliae*; Mg1, *Meloidogyne graminicola*; Mh1, *Meloidogyne hapla*; Mi1, *Meloidogyne incognita* population 1; Mi2, *M. incognita* population 2; Mj1, *M. javanica* population 1; Mj2, *M. javanica* population 2; Mm1, *Meloidogyne mali*; NC, negative control (water); Pn1, *Pratylenchus neglectus*.

of all *M. javanica* artificially inoculated samples. All of the RKN-free soil and artificially inoculated *M. incognita* soil samples were not detected (Table 3).

LFD-RPA assay. For the easy-to-use LFD-RPA assay, the labeled reverse primer MJBioR and a labeled probe MJPro were added to the reaction mixture, and an LFD was used for terminal detection. The result showed an intense band that appeared at the test line on the strips when hybridized with the RPA products of *M. javanica* DNA extracted from nematode, root galls, or soil nematodes. No false-positive result was obtained from DNA of other samples (Fig. 5).

To evaluate the performance of the LFD-RPA assay in field application, a total of 18 soil samples were collected from natural field and

subject to LFD-RPA assay. All of the samples were identified as having natural *Meloidogyne* spp. infestation by traditional microscopic observation, whereas the result of single nematode PCR assay showed that only six samples were *M. javanica* positive. Using crude gDNA of nematodes as amplification template, LFD-RPA assay exhibit the same detection result with PCR assay (Fig. 6).

Discussion

M. javanica is one of the most important species of root knot nematode in southern provinces of China. To accurately identify *M. javanica*, herein we presented the RPA assay, a novel and effective isothermal amplification method. The RPA reactions can be completed in 20 to 40 min, and the amplification product can be detected

Fig. 4. Detection limits of the **A** and **B**, recombinase polymerase amplification (RPA) and **C** and **D**, PCR assays using serial dilution of crude genomic DNA extract of individual second-stage juvenile and female *Meloidogyne javanica*. M, marker; NC, negative control (water).

Fig. 5. Lateral flow dipstick-recombinase polymerase amplification assay of *Meloidogyne javanica*. Genomic DNA (gDNA) of second-stage juvenile (J2), crude gDNA extract of individual J2, female, crude gDNA of galls, and crude gDNA extract of nematodes isolated from soil by modified Baermann funnel method were used as amplification templates. gDNA were extracted from J2 of *M. javanica* population 1 (Mj1), *Meloidogyne incognita* population 1 (Mi1), *Meloidogyne arenaria* population 1 (Ma1), and *Meloidogyne hapla* (Mh1). Crude gDNA of J2 and female was extracted using individual J2 or female of Mj1 and Mi1. Crude gDNA of galls was extracted from plant roots artificially inoculated with Mj1 and Mi1, and healthy root was used as negative control. The nematodes were isolated from soil samples artificially inoculated with Mj1 and Mi1, and the root knot nematode (RKN)-free soil sample was used as negative control (NC). Test line indicated detection of analytes labeled with both 5-carboxyfluorescein and biotin, and a control line validates lateral flow dipstick tests.

by different techniques. RPA assay was more sensitive, more convenient, and faster than the conventional PCR method. Furthermore, using nucleic acid dye or LFDs for terminal detection, RPA can provide rapid and visual detection of *M. javanica* from different types of samples without the need for complex equipment or procedures, suggesting increased applicability of this assay for use in onsite surveys and routine diagnostics.

SCAR markers enable specific and reliable discrimination of *M. javanica* from other plant-parasitic nematode species by using PCR-based methods (Donkers-Venne et al. 2000; Meng et al. 2004). In this study, the RPA primers and probe were designed based on the SCAR marker gene sequence showing specificity for *M. javanica*; however, more plant-parasitic nematode species need to be tested in the future to validate the assay. Previous studies show that the RPA assay is more sensitive than conventional PCR assay for detecting pathogens (Cabada et al. 2017; Liu et al. 2016). We found the detection limit of RPA to be 1 pg of gDNA, which was 10 times more sensitive than conventional PCR assay; it was a little lower than that of LAMP assay, which was recorded to detect as low as 10 fg of gDNA (Niu et al. 2011; Zhou et al. 2017).

WLB has been widely used for DNA extraction for DNA-based diagnostics of plant-parasitic nematode. For both conventional PCR and LAMP assays, WLB can be used for preparing DNA template from individual nematode, root galls, and nematodes collected by the Baermann funnel method from soil (Adam et al. 2007; Niu et al. 2012; Qiu et al. 2006; Zhou et al. 2017; Zijlstra 2000). In fact, the crude gDNA extracted with WLB was a mixture of nucleic acid, proteins, salts, and nematode tissues, which may contain inhibitors of amplification reaction. In this study, RPA assay successfully detected *M. javanica* using crude gDNA of different samples, indicating that RPA assay is a stable and efficient DNA amplification method suitable for plant-parasitic nematodes.

In molecular diagnostics assays, an individual J2 or adult female of *Meloidogyne* spp. has often been used for target sequence amplification and sequencing. Both PCR and LAMP assays have been developed to discriminate *Meloidogyne* species by using individual J2 or female (Adam et al. 2007; Zijlstra 2000). LAMP has been reported to detect as low as 0.01 RKN J2 or 0.001 RKN adult female (Zhou et al. 2017), which is 10 times more sensitive than RPA assay. However, the result of LAMP assay cannot be distinguished by the size of band in the agarose gel owing to the fact that the amplification product is a series of concatemers of the target DNA region, showing a “ladder” or banding pattern on gel.

Moreover, the product is also not suitable for cloning, sequencing, and other molecular biology applications (Iseki et al. 2007). RPA assay, however, can detect *M. javanica* by using crude gDNA extracts from an individual J2 or an adult female, resulting in a single-band amplification product that can be sequenced directly, which suggests its practicability in molecular identification of nematodes.

Soil samples are frequently submitted to diagnostic laboratories to determine whether a species of RKN is present. Molecular identification methods play a key role in RKN detection from soil samples, because limited morphological characters of J2s make it difficult to accurately identify the species (Qiu et al. 2006). Additionally, several species of nematode are present in the soil, making it more difficult to identify a species. Direct isolation of community DNA from soil is available for molecular analysis on bacteria and fungus from environmental soil (Soliman et al. 2017). However, the current commercial soil DNA extraction kits only use 0.1 to 0.5 g of soil for each DNA extraction. Such small samples are inappropriate for nematode assays, because nematodes are unevenly distributed in the field, and their population density may be much lower than one nematode per gram of soil. Here, we used a Baermann funnel method to collect nematode first with increased sample size (100 g), giving a 100% (10 of 10) positive detection, although the RPA reactions were performed on crude nematode gDNA extracted through a simple procedure. This indicated that, combined with Baermann funnel method, RPA assay is sensitive and reliable in diagnosis of *M. javanica* from soil samples.

The sandwich assay device—LFDs (Milenia Biotec)—is based on immune colloidal gold technique and can rapidly detect analytes, including DNA fragments labeled with FAM and biotin, without the need for specialized and costly equipment. The LFD-LAMP method was developed to increase the applicability of LAMP in onsite surveys and routine diagnostics of *Meloidogyne* spp. and other pathogens (Ding et al. 2010; Kiatpathomchai et al. 2008; Niu et al. 2011, 2012; Zhou et al. 2017). However, the combination of RPA and LFDs can also constitute a rapid amplification and visual detection system (Piepenburg et al. 2006). To avoid false-positive result, LFD-RPA used a dSpacer and a C₃ spacer to prevent the production of biotin/FAM-containing primer artifacts (Piepenburg et al. 2006). In this study, the specific and sensitive detection of *M. javanica* from various samples and field soil samples by LFD-RPA revealed a new RKN detection technique, which is potentially accessible for routine field monitoring.

Fig. 6. Evaluation of the lateral flow dipstick-recombinase polymerase amplification (LFD-RPA) assay using field soil samples. **A**, A total of 18 field soil samples from Shandong (1 to 6), Yunnan (7 to 12), and Anhui (13 to 18) in China were subject to LFD-RPA assay, and **B**, the result was confirmed using PCR assay. Soil samples artificially inoculated with *Meloidogyne incognita* (Mi), *Meloidogyne javanica* (Mj), and root knot nematode (RKN)-free samples (negative control [NC]) were used as controls.

This study describes a rapid and highly specific RPA assay for the detection of *M. javanica*. The newly described RPA assay could potentially be utilized in molecular identification and routine diagnostics of *M. javanica* from a mixture of nematode species, plant tissues, and soil samples.

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