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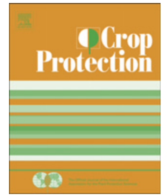

Rapid detection of *Meloidogyne* spp. by LAMP assay in soil and roots

Jun-hai Niu [a,b,1], Quan-xin Guo [a,1], Heng Jian [a,*], Chang-long Chen [a], Dan Yang [a], Qian Liu [a], Yang-dong Guo [b]

[a] Department of Plant Pathology, China Agricultural University, Beijing 100193, China
[b] Department of Olericulture, China Agricultural University, Beijing 100193, China

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ABSTRACT

Loop-mediated isothermal amplification (LAMP), a novel DNA amplification technique, has been used to detect a variety of pathogens including viruses, fungi, bacteria and parasites. However, diagnosis of sedentary plant-parasitic nematode (PPN) species has not yet been attempted. In this study, we developed a universal LAMP set (RKN-LAMP) for the diagnosis of four common *Meloidogyne* species (*Meloidogyne incognita*, *M. arenaria*, *M. javanica* and *M. hapla*), and *M. incognita*-specific LAMP set (Mi-LAMP). In both assays, a typical ladder-like pattern on gel electrophoresis was observed in all positive samples but not in the negative controls. Amplification products were further confirmed using restriction analysis of the *Hpa* II enzyme, detection by visual inspection using SYBR Green I and the lateral flow dipstick (LFD) assay. The two LAMP sets were specifically able to detect four common *Meloidogyne* species and *M. incognita* populations having several different geographical origins and pathotypes. No cross reaction with DNA of other PPNs was observed. Sensitivity of the RKN- and Mi-LAMP was 10 and 100 fg of pure genome DNA respectively. Both LAMP sets could also amplified crude DNA isolated from the galled root tissue and from soil containing juveniles of *M. incognita*. The RKN- and Mi-LAMP sets offer the advantages of simplicity, rapidity and cost effectiveness. Both LAMP sets will be instrumental for the diagnosis of *Meloidogyne* spp. by local extension and regulatory personnel.

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1. Introduction

Root-knot nematodes (RKN) of the genus *Meloidogyne* produce great agricultural damage worldwide. The species primarily responsible for this problem are *Meloidogyne incognita*, *Meloidogyne arenaria*, *Meloidogyne javanica* and *Meloidogyne hapla*. These four common species, especially *M. incognita*, are the most widely distributed species and are important pathogens of more than 3000 plant species, including numerous agricultural crops (Jepson, 1987). Accurate and reliable identification of these nematodes is fundamental for their effective management and for research on the development of novel control measures.

Traditional techniques for species identification of *Meloidogyne* spp. have relied on morphological observation (Eisenback et al., 1981; Eisenback, 1985), host range tests (Hartman and Sasser, 1985; Roberts and Thomason, 1989) and isozyme phenotypes (esterase and malate dehydrogenase isozyme profiles) derived from single females (Esbenshade and Triantaphyllou, 1985, 1990; Carneiro et al., 2000). Within the past two decades, molecular diagnostics targeted different regions of the genome and mitochondrial DNA, has become an alternative strategy for *Meloidogyne* identification. The various PCR-based detection methods that have been developed and shown to be valuable include Random Amplification of Polymorphic DNA (RAPD) (Cenis, 1993; Baum et al., 1994), PCR-restriction fragment length polymorphisms (RFLPs) (Harris et al., 1990; Powers and Harris, 1993; Zijlstra et al., 1995), real-time PCR (Berry et al., 2008), species-specific sequence characterized amplified region (SCAR) primers (Zijlstra et al., 2000; Randig et al., 2002; Meng et al., 2004) and satellite DNA (Castagnone-Sereno and Esparrago, 1995; Piotte et al., 1995; Castagnone-Sereno, 2000). Compared with traditional methods, the PCR-based diagnostics were faster, more accurate and more sensitive tools for *Meloidogyne* identification. However, these technologies often required expensive and sophisticated laboratory instrumentation and trained personnel, the associated diagnostic procedure usually take a few hours to be finished. These drawbacks limited its utility, especially in direct field applications. A preferable detection method would be not only speedy and sensitive but also simple and economical in practical applications.

* Corresponding author. Tel./fax: +86 (0) 10 62731102.
E-mail address: hengjian@cau.edu.cn (H. Jian).
[1] Equal contribution.

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In 2000, Eiken Chemical Company Ltd. developed a novel nucleic acid amplification method termed loop-mediated isothermal amplification (LAMP) and having high sensitivity, specificity and rapidity (Notomi et al., 2000). Unlike traditional PCR, the LAMP reaction requires a DNA polymerase with strand–-displacement activity and a set of 4–6 specially designed primers based on six or eight distinct regions of the target DNA. The reaction occurs under isothermal conditions (60–65 °C) and yields large amounts of product in a short time (0.5–1 h). The positive LAMP reactions can be visualized with the naked eye (Mori et al., 2001; Iwasaki et al., 2003) by adding fluorescence intercalation dye such as ethidium bromide or SYBR green I (Notomi et al., 2000; Iwamoto et al., 2003; Maeda et al., 2005) and measuring the increase in turbidity derived from magnesium pyrophosphate formation to infer increases in amplified DNA concentration (Mori et al., 2001). In addition, all LAMP steps are conducted within one reaction tube, and only a water bath or heating block is needed to provide isothermal conditions. In view of these advantages, LAMP technology has been packaged in commercially available detection kits for a variety of pathogens including viruses, fungi, bacteria and parasitic diseases (Mori and Notomi, 2009).

Although LAMP has been developed successfully for the detection of animal and human parasites including malaria, trypanosomiasis, toxoplasmosis, babesiosis and theileriosis (Poon et al., 2006; Iseki et al., 2007; Thekisoe et al., 2007; Guan et al., 2008; Liu et al., 2008; Njiru et al., 2008; Krasteva et al., 2009; Yamamura et al., 2009; Wang et al., 2010), only limited attempts have been made to detect PPNs by using LAMP assays. To the best of our current knowledge, only one paper has demonstrated the application of this technology to detecting the pinewood nematode *Bursaphelenchus xylophilus* (Kikuchi et al., 2009). The goal of this research was to develop a novel method for diagnosing Meloidogyne species. To this end, a universal LAMP assays capable of detecting four common *Meloidogyne* spp. (*M. incognita*, *M. arenaria*, *M. javanica*, and *M. hapla*; RKN-LAMP) and specifically detecting *M. incognita* (Mi-LAMP) were developed.

2. Methods

2.1. Nematode populations and DNA template preparation

The populations of PPNs used in this work were listed in Table 2. The selected *Meloidogyne* populations, collected in the field from various provinces in China, were maintained on tomato (*Lycopersicum esculentum* cv. Baiguo) in axenic cultures started from a single-egg mass in a greenhouse. These populations had been identified previously by observation of perineal patterns (Eisenback et al., 1981) and by molecular diagnoses using species-specific SCARs markers (Zijlstra et al., 2000; Meng et al., 2004).

Several methods of DNA extraction were used in this study. DNA of single second-stage juveniles and females was extracted by the method described in Zijlstra et al. (1997), and dissolved to a final volume of 15 μl. DNA from a relatively large amount of nematode juveniles was isolated by using the DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Purified DNA was quantified by spectrophotometer, and aliquots were diluted to 100 ng/μl in distilled water as stocks for application.

2.2. LAMP primer design

Various genomic and mitochondrial regions, including ribosomal DNA internal transcribed spacers (rDNA-ITS), 28S D2-D3 expansion regions, β-actin, elongation factor 1α gene (EF1α), β-tubulin gene, heat shock protein 90 (Hsp90) and cytochrome oxidase subunit II (COII), commonly used for phylogenetic analysis, were chosen for candidate LAMP targets of root-knot nematodes. DNA and/or EST sequences of these targets from four common *Meloidogyne* and related species were downloaded from the NCBI, *M. incognita* and *M. hapla* genome databases (http://www.inra.fr/meloidogyne_incognita and http://www.pngg.org/cbnp/index.php) (Abad et al., 2008; Opperman et al., 2008). Target sequence variations within *Meloidogyne* spp. were compared by multiple sequence alignment using Clustal X 2.0 (Larkin et al., 2007). The conserved and variable regions were determined by sliding window analysis using program DnaSP v. 5.10.01 (Librado and Rozas., 2009). Finally, the 18s rDNA-ITS1 regions known to be conserved among four common species and known to exhibit strong dissimilarities with other *Meloidogyne* species were selected for designing universal RKN-LAMP primers (Fig. 1) using Primer-Explorer V4 software (http://primerexplorer.jp).

To distinguish *M. incognita* from other very similar groups, *M. incognita*-specific SCAR primers MI-F/R were used to produce a 999-bp product from *M. incognita* genome DNA (Meng et al., 2004). Amplified products were sequenced and used in a BLAST search performed with *M. incognita* genome databases (Abad et al., 2008; http://meloidogyne.toulouse.inra.fr/). The matched contig elements were downloaded. *M. incognita*-specific SCARs and their flanking sequences were used to designate Mi-LAMP primers.

2.3. Optimization of the LAMP conditions

The LAMP reaction was performed according to the method previously described (Notomi et al., 2000). The procedure used 25 μl of reaction mixture containing 2.5 μl of 10x *Bst*-DNA polymerase buffer, 0.35 mM each dNTP, 1.6 μM (each) of FIP and BIP primers, 0.2 μM (each) of each F3 and B3 outer primers, 0.8 μM of LB primer (for RKN-LAMP only), 0.8 M betaine (Sigma–Aldrich Co., St. Louis, MO, USA), 8 U *Bst*-DNA polymerase (New England Biolabs Ltd. UK) and 1 μl purified *M. incognita* genome DNA (~100 ng). For improving amplification efficiency, template DNA was denatured at 95 °C for 5 min and then cooled on ice before adding *Bst*-DNA polymerase. To find the optimum temperature and time for the visual LAMP amplification,

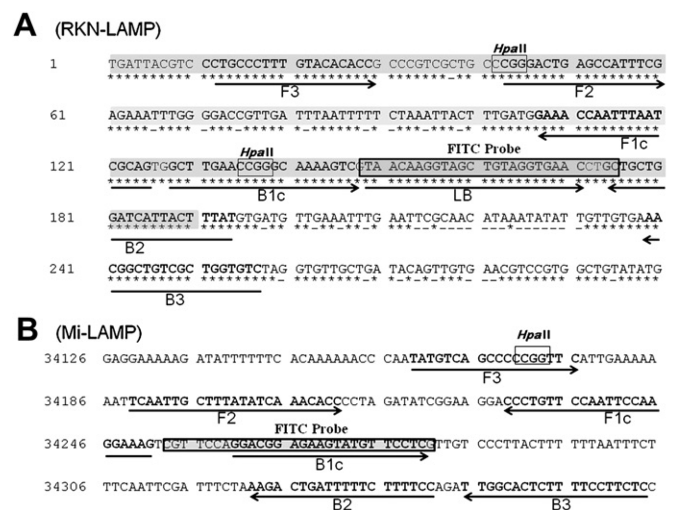

Fig. 1. Localization of target sequences used for primer construction. (A) RKN-LAMP targeted partial 18s rDNA-ITS1. 18s rDNA sequence is highlighted in gray. The rest part is ITS1. "*"and "-" represent the consensus and mutation sites. (B) Mi-LAMP target to a portion of Minc08401. Arrows indicate the direction and location of the primers. The restriction sites for *Hpa* II are indicated by small boxes. The sequences used for the FITC-labeled probe are shown by gray boxes. Numbers at the left end correspond to the positions in 18s rDNA-ITS1 (GeneBank accession no. FJ534516) and in the Minc08401 gene (from the complementary sequence *M. incognita* genome scaffold Miv1ctg272), respectively.

the reactions were carried out in a 60–65 °C water bath for 30, 45, 60 and 75 min, respectively. Finally, the mixture was heated at 80 °C for 5 min to terminate the reaction. The LAMP amplification results were visually inspected by naked eye and under UV by adding fluorescent dye SYBR Green I, and were monitored using 1.8% agarose gel electrophoresis stained with ethidium bromide.

2.4. Traditional PCR

PPNs rDNA-ITS universal primer pairs VRF1/VRF2 were used for evaluating plate DNA qualification. *M. incognita*-specific SCAR primer Mi-F/Mi-R was adapted for acquiring polymorphic fragments (Meng et al., 2004). Two pairs of LAMP outer primers, RKN-F3/RKN-B3 and Mi-F3/Mi-R3, were used to confirm that the LAMP amplified the correct target. Each PCR mix had a total volume of 25 μl and contained 2.5 μl of 10x *Ex* Taq buffer, 2 μl of 2.5 mmol/L dNTP, 0.5 μl of 10 μM forward and reverse primers, 2 U *Ex* Taq-polymerase (Takara Bio Inc. Japan) and 1 μl plate DNA. Initial denaturation was conducted at 94 °C for 4 min, followed by 30 cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C for VRF1/F2, 62 °C for MI-F/R, 57 °C for RKN-F3/RKN-B3, 56 °C for Mi-F3/Mi-R3) and extension (60 s at 72 °C). Subsequently, 5 μl of PCR products were subjected to 1.8% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.

2.5. Verification of LAMP detection specificity

To confirm the specificity of LAMP amplification for the target sequences, the RKN- and Mi-LAMP products were digested separately with *Hpa* II restriction enzyme. The restriction enzyme was selected on the basis of appropriate sequence information (shown in Figs. 2 and 3). In addition, the products generated from conventional PCR using primer pairs of RKN-F3/RKN-B3 and Mi-F3/Mi-B3 were cloned using the pMD18-T vector system (TaKaRa Bio Inc. Japan) and were sequenced.

To determine the LAMP specificity for the target *Meloidogyne* species, forty-three plant nematode populations (Table 2), including several different *Meloidogyne* and other plant nematode species, were subjected to both LAMP assays and conventional PCR amplifications. Specificity tests were repeated three times.

2.6. Lateral flow dipstick (LFD) assay

The primers cited in Section 2.3 were used for LAMP-LFD reactions. Additionally, 5′ biotin-labeled inner primer FIP was used for this assay. DNA probes were designed and labeled with FITC at the 5′ end (Fig. 1A and B). Hybridization was carried out using the method recommended in previous reports (Kiatpathomchai et al., 2008). Then, 20 pmol of the probe was added to the LAMP products and incubated at 63 °C for 5 min. After hybridization, 8 μl of the hybridized product were added to 100 μl of the assay buffer in a new tube. Finally, the commercially prepared LFD strips (Milenia Biotec GmbH, Germany) were dipped into the mixer for 5 min to detect the amplicon-probe hybrid.

2.7. Verification of LAMP detection sensitivity

To determine LAMP sensitivity, serial 10-fold dilutions of *M. incognita* genome DNA (at initial concentration ~10 ng/μl) were prepared in ddH_2O and were subjected to both LAMP and

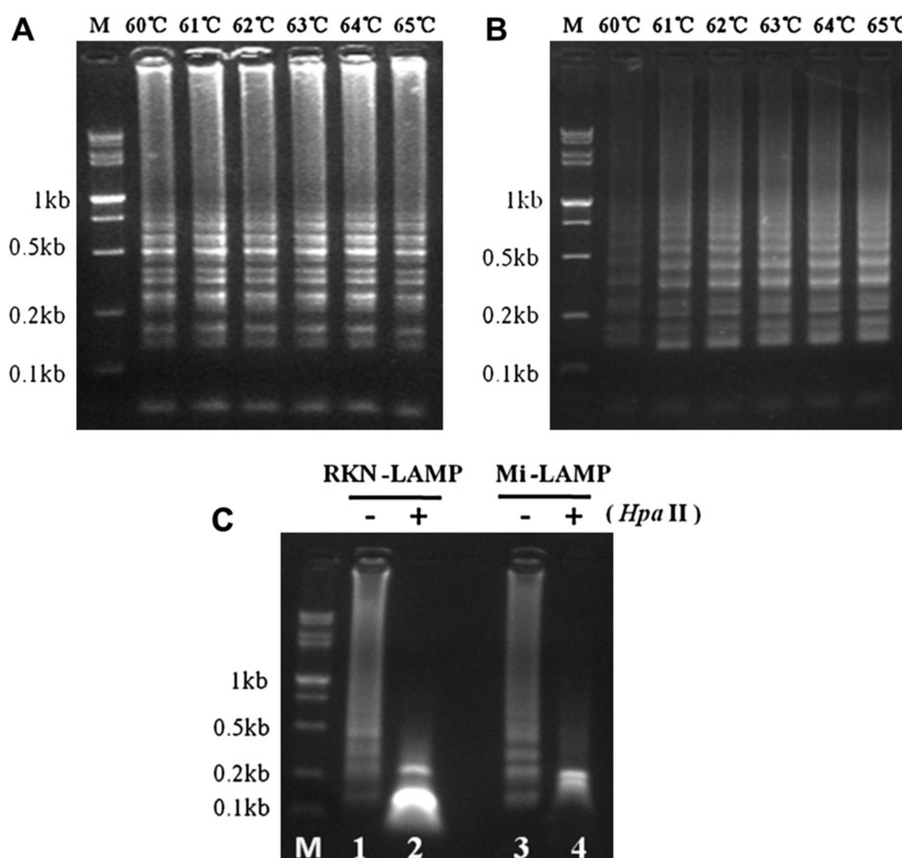

Fig. 2. LAMP reaction optimization and product confirmation. (A) Temperature effect of the RKN-LAMP assay. (B) Temperature effect of the Mi-LAMP assay. (C) Restriction enzyme analysis of the LAMP products. Lanes 1 and 3: RKN- and Mi-LAMP reactions performed on the *M. incognita* genome DNA. Lanes 2 and 4: LAMP products of lanes 1 and 3, respectively, after digestion with *Hpa* II in which the two expected fragments were obtained. Lane M: molecular marker.

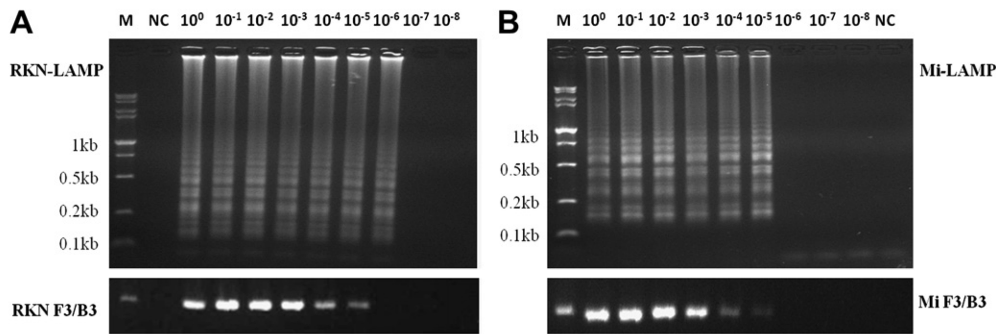

Fig. 3. Comparison of conventional PCR and LAMP sensitivity using various amounts of template. (A) RKN-LAMP and RKN F3/B3 products. (B) Mi-LAMP and Mi F3/B3 products. The initial template concentration was 100 ng of *M. incognita* DNA. The line 10^0–10^{-8} indicates serial dilutions of DNA solutions as templates. Lane NC: no-template control. Lane M: 100 bp DNA molecular marker.

conventional PCR amplifications (using primer pairs of RKN-F3/RKN-B3 and Mi-F3/Mi-B3, respectively). In addition, the LAMP assays were also performed with 15 μl 10-fold serial dilutions of DNA template isolated from single juveniles and females of *M. incognita* (see Section 2.1). Amplification was monitored as described in Section 2.3. Sensitivity tests were repeated three times.

2.8. LAMP testing in host roots and soil samples

To demonstrate the field application of LAMP as a diagnostic tool for *Meloidogyne* surveying and management, 20 tomato root galls and artificially-inoculated soil samples collected from a greenhouse were surveyed using the RKN- and Mi-LAMP methods. The presence of *M. incognita* in galls from the same sampled root system was confirmed by using acid fuchsin stain and microscopic observation. To obtain crude *Meloidogyne* DNA from galls of nematode-infested tomato roots, approximately 5 mg sections of galls were crushed using a tapered glass rod in 40 μl worm lysis buffer (WLB: 50 mM KCl, 10 mM Tris pH 8.2, 2.5 mM MgCl, 60 μg/mL Proteinase K, 0.45% Tween 20 and 0.01% Gelatin) (Castagnone-Sereno et al., 1995). The lysis tissue was centrifuged slightly, and 3 μl supernatant was then used for a 25 μl LAMP reaction mixture. Experimental soil samples were prepared by adding 10,000 fresh-hatched *M. incognita* juveniles to 100 g RKN-free and moist soil and mixing well. 50 μl DNA eluate of Meloidogyne from 100 mg soil samples was prepared by using a PowerSoil™ DNA Isolation Kit (Mo-Bio Laboratories, Inc., Carlsbad, CA, USA) following the instruction manual, and 1 μl of the undiluted DNA eluate was used for a 25 μl LAMP reaction mixture. The LAMP reactions were performed at 63 °C for 60 min. Amplification was monitored as described in Section 2.3.

3. Results

3.1. Primer design

The RKN- and Mi-LAMP primers were designed to target the *M. incognita* 18s rDNA-ITS1 and Minc08407 (a retrieved gene from *M. incognita* genome annotation) separately. The positions of the LAMP primers were shown in Fig. 1, and the sequences were listed in Table 1. The outer primers were F3 and B3. The inner primer FIP consisted of F2 and F1c (the complementary sequence of F1). Another inner primer BIP consisted of B2 and B1c (the complementary sequence of B1). To avoid misdiagnoses, primers were selected for the RKN-LAMP assay so that the mutations were located outside of the 3′ end in F2 (B2), the 5′ end of F1c (B1c) or the 3′ end of F3 (B3). The primers were then less susceptible to the effect of the mutation, and all four common species would be detectable by using a universal set of primers.

3.2. Detection and confirmation of LAMP products

Optimization of LAMP reaction conditions (temperature and time) revealed that the ideal settings for both primer sets were 63 °C and 60 min (data not shown). A typical ladder-like pattern on gel electrophoresis was observed in all positive samples, but not in the negative controls. The samples giving positive reactions exhibited a green color upon addition of SYBR Green I, whereas the negative control remained orange (Day light) or weak fluorescence (UV light) (Fig. 4). Specificity of the amplification products was confirmed by restriction analysis of the *Hpa* II enzyme (Fig. 2c).

Table 1
DNA primers used for LAMP and traditional PCR detection.

Primer set	Oligonucleotide	Sequence (5′–3′)	Length	Usage	Reference
VRF1/F2	VRF1	cgtaacaaggtagctgtag	19 nt	rRNA-ITS universal primers	Ferris et al., 1993
	VRF2	tcctccgctaaatgatatg	19 nt		
Mi-F/R	Mi-F	gtgaggattcagctccccag	20 nt	*M. incognita*-specific SCAR	Meng et al., 2004
	Mi-R	acgaggaacatacttctccgtcc	23 nt		
RKN-LAMP	RKN–F3	ctgccctttgtacacacc	18 nt	Universal LAMP assay for four common Meloidogyne species	This study
	RKN–B3	gacaccagcgacagccgtt	19 nt		
	RKN–FIP	ctgcgattaaattggtttccatcaacgggactgagccatttcg	43 nt		
	RKN–BIP	gcttgaaccgggcaaaagtccataaagtaatgatccagcagc	42 nt		
	RKN–LB	gtaacaaggtagctgtaggtgaac	24 nt		
Mi-LAMP	Mi–F3	tatgtcagcccccggttc	18 nt	*M. incognita* - specific LAMP	This study
	Mi–B3	gagaaggaaaagagtgccaa	20 nt		
	Mi–FIP	ctttccttggaattggaacagggtcaattgctttatatcaaacacc	46’ nt		
	Mi–BIP	ggacggagaagtatgttcctctctggaaaagaaaaatcagtctt	44 nt		

3.3. Determination of specificity for LAMP assay

Specificity of the LAMP primers was tested using the plant nematodes *Meloidogyne*, *Heterodera glycines*, *H. avenae*, *H. filipjevi*, *Ditylenchus destructor* (race A and B), *B. xylophilus*, and *B. mucronatus* (Table 2). In the RKN-LAMP assay, all four common *Meloidogyne* species were successfully detected using reaction conditions with 100 pg of template DNA after incubation for 60 min. A total of 22 populations of *M. incognita* could be detected in the Mi-LAMP assay. On the contrary, all other PPN species were negative in both assays under the same reaction conditions.

3.4. Determination of sensitivity for LAMP assay

For analytical sensitivity tests, both LAMP reactions were performed using 10-fold serial dilutions of pure DNA of *M. incognita*. As shown in Fig. 3A, the minimum detection concentration of DNA required for the RKN-LAMP assay was 100 fg. This assay was 10 times more sensitive than conventional PCR (primer pairs of RKN F3/B3 with detection limit of 1 pg) in a 60-min reaction. However, the Mi-LAMP assay exhibited sensitivity equivalent to that of the conventional PCR (primer pairs of RKN F3/B3), about 1 pg DNA of detection limitation (Fig. 3B). In addition, the LAMP assays were also performed successfully with 1% genome DNA isolated from single juveniles or females of *M. incognita*.

3.5. Specificity of LFD

In this study, tests showed that RKN- and Mi-LAMP-LFD detection methods were specific for common *Meloidogyne* species and *M. incognita*, respectively. No cross reactions occurred with other related species (Fig. 5). These findings were consistent with the results of gel electrophoresis and SYBR Green I stain used in visual detection. The LFD test confirmed the presence of positive amplicons by hybridization in a sequence-dependent manner. In addition, the LFD detection method was time saving and was not equipment dependent.

3.6. Evaluation of the LAMP assay using root and soil samples

We also evaluated the practicability of LAMP methods using root material infested with *M. incognita* and artificially-inoculated soil. For the 20 replicate LAMP reactions using root gall samples, amplification from both primer sets was observed. Several amplifications generated different smears in agarose gels, whereas no amplification was observed from the healthy roots. Additionally, as shown in Table 3, 20 (100%) and 19 (95%) of the 20 soil samples were positively detected by the RKN- and Mi-LAMP assays, respectively. These results indicate the high detection capability of both LAMP assays.

Table 2
Species or populations of PPN used to evaluate the analytical specificity of the LAMP assay.

Code	PPN Species	No. of populations	Positive numbers by		
			VRF1/F2	RKN-LAMP	Mi-LAMP
1	*Meloidogyne incognita*	22	22	22	22
2	*M. javanica*	4	4	4	0
3	*M. arenaria*	3	3	3	0
4	*M. hapla*	2	2	2	0
5	*M. enterolobii*	2	2	0	0
6	*Heteroder aglycines*	1	1	0	0
7	*H. avenae*	3	3	0	0
8	*H. filipjevi*	1	1	0	0
9	*Ditylenchus destructor*	3	3	0	0
10	*Bursaphelenchus xylophilus*	1	1	0	0
11	*B. mucronatus*	1	1	0	0

For each population, PCR and LAMP reactions were performed in triplicate. The products were assessed using gel electrophoresis and SYBR Green I stain.

4. Discussion

The two novel LAMP systems presented here offer a rapid, simple, accurate and cheap method for *Meloidogyne* diagnosis. The RKN-LAMP system described above uses a universal primer set for four common *Meloidogyne* species, including *M. incognita*, *M. arenaria*, *M. javanica* and *M. hapla*, but not other species of *Meloidogyne*. Furthermore, the Mi-LAMP primer set described above can specifically detect *M. incognita* populations (Table 2). Among samples from serial dilutions of the template, LAMP results showed equivalent or greater sensitivity relative to conventional PCR. These results correspond to those found in previous reports (Notomi et al., 2000; Kikuchi et al., 2009; McKenna et al., 2011; Njiru et al., 2010). However, the LAMP assay is timesaving and less equipment dependent. Furthermore, our preliminary results indicate that the LAMP assay described here could be applied to the diagnosis of root and soil samples using rough DNA isolated from those samples.

The nematode rRNA array consists of three ribosomal genes (18S, 5.8S and 28S rRNA) and two internal transcribed spacer regions (ITS1 and ITS2), aligned in the chromosome and present as multiple copies. The rRNA array is generally an attractive target and has been applied routinely for nematode characterization (ZijIstra et al., 1995; Harris et al., 1990; McKeand, 1998; Clapp et al., 2000; Madani et al., 2004). However, *Meloidogyne* rRNA genes are so highly conserved among different species that they have no diagnostic value at the species level (Powers and Harris, 1993; Stanton et al., 1997; Powers et al., 1997). Conversely, variation is found in the

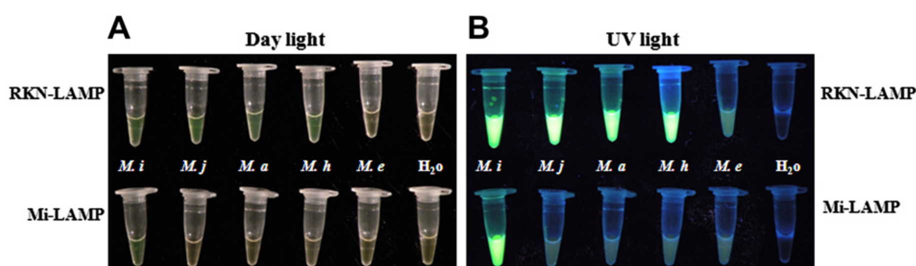

Fig. 4. The specificity of the LAMP assay products visualized by adding SYBR Green I. (A) Direct visualization by the naked eye. (B) Observation under UV transillumination. M. i, M. j, M. a, M. h, and M. e represent *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla* and *M. enterolobii*, respectively. The upper tubes contained RKN-LAMP reaction products, and the lower contained Mi-LAMP reaction products. The H_2O tube was used as a negative control without DNA template. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

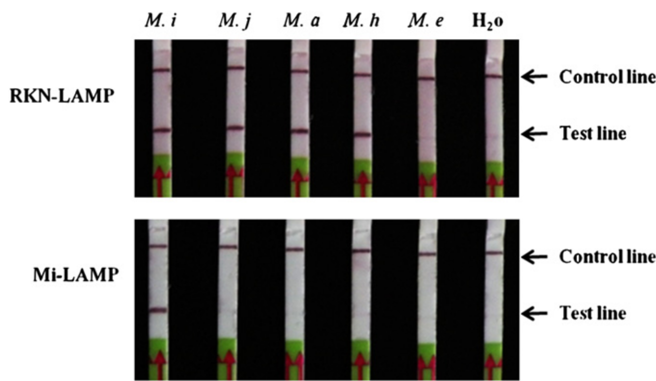

Fig. 5. The specificity of LAMP-LFD for the detection of root-knot nematodes. Samples *M. i*, *M. j*, *M. a*, *M. h*, and *M. e* represent *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla* and *M. enterolobii*, respectively. H_2O represents a no-template negative control.

length and sequence of the ITS1 and ITS2 regions. In this study, the universal RKN-LAMP primer set was designed based on the 3′ end of 18S rRNA combined with the 5′ sequence of ITS1. The B2 primer located at the junction of 18S rRNA and ITS1, and the F3 annealed with regions of ITS1, both proved to be highly conserved among four common *Meloidogyne* species and greatly divergent from other species.

Although ITS may perform well as a universal diagnostic region for four common *Meloidogyne* species, the use of the ITS regions for identification of the *M. incognita* populations could easily lead to misdiagnosis. Because the mitotically parthenogenetic species *M. incognita*, *M. arenaria* and *M. javanica* usually occur in similar geographical regions and in similar hosts, they have closely related evolutionary lineages and genome compositions (Hugall et al., 1999; Powers, 2004; Perry et al., 2007). There was not enough nucleotide variation present in the ITS regions or in other candidate targets used for designing *M. incognita*-specific LAMP primers. Other candidate targets exhibited the same limitation. Previously published studies report several pairs of species-specific primers that were developed and widely confirmed for use in identification *of M. incognita* using sequence characterized amplified regions (SCAR) (Zijlstra et al., 2000; Dong et al., 2001; Randig et al., 2001; Meng et al., 2004). These SCAR portions may represent the species-specific genes or divergence from ancient alleles and could facilitate *M. incognita*-specific LAMP designation. Based on this observation, we developed six LAMP sets based on the *M. incognita* SCARs. Their diagnostic potential was evaluated by amplification of DNA isolated from *M. incognita* populations and from a wide variety of other related *Meloidogyne* species. The best-characterized sets that targeted the retrieved Minc08401 gene were then identified (Fig. 1B). To take into account the fact that the Mi B1c sequence

Table 3
Detection of *M. incognita* in root and soil samples using Loop-mediated isothermal amplification.

Samples	Nematode density	RKN-LAMP		Mi-LAMP	
		Positive/ trial	Positive rate	Positive/ trial	Positive rate
Root galls[a]					
M. i-induced galls	1.22 females/gall	20/20	100%	20/20	100%
M. a-induced galls	1.25 females/gall	20/20	100%	0/20	0
Healthy root	No female or gall	0/10	0	0/10	0
Soil					
M. incognita	100 juveniles/g	20/20	100%	19/20	95%

M. i: *M. incognita*, *M. a*: *M. arenaria*.
[a] Root galls were sampled from *Meloidogyne*-infested tomato roots.

(5′ end of Mi BIF primer) annealing region was consistent with the widely used *M. incognita* SCAR marker (the forward primer of Mi-F/Mi-R), and in the light of our primary evaluation, the Mi-LAMP set should be considered reliable for differentiating *M. incognita* efficiently from other similar and closely related species.

Meloidogyne is a very diverse genus containing more than 90 species (Eisenback et al., 1991; Karssen and Moens, 2006; Palomares Rius et al., 2007). Possibly, the primer set originally designed to be species-specific could cross-react with other species or with natural intraspecific variants, leading to mismatches at the primer-binding site. To avoid the risk of misdiagnosis, a larger-scale investigation of the evaluation capabilities of the method will be necessary in the future.

The sensitivity of the RKN-LAMP assay was high. The method could detect as little as 100 fg of genomic DNA and was therefore 10-fold more sensitive than the conventional PCR. In contrast, the sensitivity of Mi-LAMP was equivalent to that of conventional PCR, with detection limits below 1 pg of DNA. This result is probably associated with the use of a low copy gene and four primers.

Moreover, the LAMP assay is easy to perform. It requires only a heating block or water bath in order to maintain the temperature at 60–65 °C. In addition, the LAMP reaction requires less than 1 h for detection, based on the fact that amplification results can be performed by naked eye observation of the turbidity or color change after adding SYBR Green I or LFD assay. These advantages make it more economical and practical than conventional PCR.

In summary, we developed two LAMP sets, one for identifying four common *Meloidogyne* species universally and the other for identifying *M. incognita* separately and species specifically. Our results suggest that LAMP methods represent a promising tool for molecular diagnosis of *Meloidogyne* infections in field surveys. Furthermore, our findings suggest that these methods may be applicable to plant breeding projects as a simple methodology for preliminary screening of *Meloidogyne* spp.-resistant progeny.

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