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Research

Loop-Mediated Isothermal Amplification for the Diagnostic Detection of *Meloidogyne chitwoodi* and *M. fallax*

Lei Zhang and **Cynthia Gleason,**† Department of Plant Pathology, Washington State University, Pullman, WA 99164

Abstract

Meloidogyne chitwoodi is a root-knot nematode that parasitizes a broad range of plants. In the Pacific Northwest (PNW) of the United States, *M. chitwoodi* is a major potato pest. The nematodes infect roots and tubers; blemishes caused by the nematodes on the tubers significantly affect potato marketability. *M. chitwoodi* is a quarantine pathogen by many regulatory agencies, limiting potato trade opportunities when it is present. A loop-mediated isothermal amplification (LAMP) assay was developed to amplify the intergenic spacer (IGS2)-18S region of the ribosomal rDNA of *M. chitwoodi.* Using the LAMP assay, we could detect the presence of *M. chitwoodi* from infected Washington State soil samples. The LAMP primers showed specificity for DNA from *M. chitwoodi* and the closely related species *M. fallax*. There was no cross reaction of the LAMP primers with DNA from tropical nematodes *M. incognita, M. arenaria,* and *M. javanica*, or the Northern root-knot nematode *M. hapla.* The LAMP assays can be completed within 45 min, and they were 100 times more sensitive in nematode detection than conventional PCR. The LAMP assay will facilitate detection of potato nematodes *M. chitwoodi* and *M. fallax*. Knowledge of potato nematodes, particularly *M. chitwoodi* in PNW soils, will aid management decisions.

Root-knot nematodes (*Meloidogyne* spp.) are soil-borne, obligate, endoparasitic pests that cause billions of dollars in annual crop losses worldwide (Chitwood 2003; Sasser and Freckman 1987). More than 90 species of root-knot nematodes have been described (OEPP/EPPO-Bulletin 2016). Many species of root-knot nematodes are tropical or subtropical. However, some species are adapted to more temperate climates, such as those found in the Pacific Northwest (PNW). *M. hapla* and *M. chitwoodi* are temperate-climate nematodes that are present in the potato-growing regions of the PNW (Nyczepir et al. 1984). Because *M. chitwoodi* eggs hatch at relatively low temperatures compared with *M. hapla*, *M. chitwoodi* populations can quickly grow within a growing season of potatoes (Charchar and Santo 2001). Therefore, *M. chitwoodi* is the predominant root-knot nematode species in the region (Elling 2013; Nyczepir et al. 1982).

Although *M. chitwoodi* was first identified in potatoes grown in the PNW in 1980 (O'Bannon 1982; Santo et al. 1980), the nematode was subsequently discovered to have patchy worldwide distribution. In addition to its limited distribution in the United States, *M. chitwoodi* is also found in Mexico, South Africa, Turkey, Argentina, and parts of Europe (the Netherlands, Portugal, Belgium, Germany) (CABI 2018; Wesemael et al. 2011). In the PNW, there are least two races of *M. chitwoodi* (race 1 and race 2) (Mojtahedi et al. 1988). These two races are indistinguishable morphologically, but they differ in host range (Mojtahedi et al. 1988; Santo and Pinkerton 1985). In 1992, a new race of *M. chitwoodi* was described in the Netherlands, but subsequent biochemical and morphological analyses determined it was a distinct species called *Meloidogyne fallax* (Karssen 1996; van Meggelen et al. 1994). Although *M. chitwoodi* and *M. fallax* share some common hosts, such as potatoes, they also exhibit some differences in host preference. For example, *M. chitwoodi* can infect corn (*Zea mays*), but *M. fallax* cannot (van Meggelen et al. 1994). Although there is a report of *M. fallax* on turfgrass in the United States, it has not been reported in field crops in the United States (Nischwitz et al. 2013).

†Corresponding author: C. Gleason; E-mail: cynthia.gleason@wsu.edu

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M. chitwoodi is problematic for potato growers because the nematode can infect both roots and tubers (Golden et al. 1980). The tuber infections lead to blemishes and pimple-like galls on the surface of the tubers, and these visual defects can significantly affect the potato market value (Ingham et al. 2007). In fact, if as few as 6% of the potatoes in a field show blemishes, the entire crop can be rejected (Ingham et al. 2000). Without nematode control measures, potato fields infected with *M. chitwoodi* could have total yield loss (Elling 2013). In addition, *M. chitwoodi* is a quarantine pathogen in Europe, and for export markets such as Mexico, there is a near-zero tolerance for the presence of *M. chitwoodi* in potatoes (Ingham et al. 2007). Because *M. chitwoodi* is a pest with important economic impact in regions where it is found, accurate nematode identification is key for implementing nematode eradication strategies.

Because Russet potato cultivars lack resistance to *M. chitwoodi*, potato growers often rely on nematicides to control it. In an effort to reduce the reliance on chemical controls, a nematode resistance gene has been introduced from a wild species of potato into cultivated potato *Solanum tuberosum*, resulting in a breeding line called PA99N82-4 (Brown et al. 1996, 2009; Zhang et al. 2007). Interestingly, a resistance breaking isolate of *M. chitwoodi* race 1 (called pathotype Roza) was discovered in the field of the PA99N82-4 (Brown et al. 2009). Despite efforts to introduce nematode resistance into cultivated potato, the best control strategy currently for *M. chitwoodi* is proper diagnosis followed by nematode control measures, which typically includes the use of nematicides.

To determine if *M. chitwoodi* and/or other plant-parasitic nematodes are present in a field requires soil sampling and nematode identification techniques. Identification of root-knot nematode species has been traditionally based on the distinct morphological characteristics of second-stage juveniles, adult males, as well as perineal patterns of adult females (de Oliveira et al. 2011; Eisenback 1982, 1985; Seesao et al. 2017). These observational methods require considerable technical skills and experience. In recent years, many DNA-based methods using polymerase chain reaction (PCR) have been developed to provide faster and more reliable nematode diagnosis, even down to the species level (Carneiro et al. 2017). For example, PCR-based identification methods for root-knot nematode species can distinguish temperate nematodes *M. chitwoodi, M. fallax,* and/or *M. hapla* based on mitochondrial DNA (Powers and Harris 1993), ribosomal DNA (Petersen and Vrain 1996; Petersen et al. 1997; Wishart et al. 2002; Zeng et al. 2015; Zijlstra 1997; Zijlstra et al. 1995), or random-amplified-polymorphic DNA (RAPD) (Adam et al. 2005; Baum et al. 1994; Blok

et al. 1997; Williamson et al. 1997; Zijlstra et al. 2000). The drawback to PCR-based diagnostics is that it requires relatively expensive thermocyclers and imaging systems.

In 2000, Notomi et al. developed loop-mediated isothermal application (LAMP) to amplify DNA (Notomi et al. 2000). This method took advantage of the *Bst* DNA polymerase with strand displacement activity to amplify large amounts of DNA in a short amount of time (<1 h) at single temperature, eliminating the need for the thermocycler. LAMP products can be easily seen by the naked eye using SYBR Green I or other DNA-binding dyes that visibly change color, eliminating the need for expensive imaging systems (Tomita et al. 2008). Thus, the advantages of LAMP assay make it ideal for rapid and accurate identification of various plant pathogens.

In the field of plant nematology, LAMP assays have been established for the detection of *Bursaphelenchus xylophilus* (Kang et al. 2015; Kikuchi et al. 2009; Meng et al. 2018), *Bursaphelenchus cocophilus* (Ide et al. 2017), *Radopholus similis* (Peng et al. 2012), *Tylenchulus semipenetrans* (Lin et al. 2016; Song et al. 2017), *Anguina wevelli* (Yu et al. 2018), and tropical root-knot nematodes *M. incognita* and *M. enterolobii* (Niu et al. 2011, 2012), as well as the temperate root-knot nematode *M. hapla* (Peng et al. 2017) and apple root-knot nematode *M. mali* (Zhou et al. 2017). We have developed a LAMP assay that can specifically identify the potato root-knot nematodes *M. chitwoodi* and *M. fallax*. Because of the importance of *M. chitwoodi* in the PNW, this LAMP assay will be particularly useful for detection of this nematode from infected soil samples from Washington.

Materials and Methods

Nematode isolates and DNA extraction. All three *M. chitwoodi* isolates (race 1, race 2, and Roza) were originally collected in Washington State. The initial egg inoculums were provided by Dr. Charles Brown (USDA-ARS). The *M. hapla* isolate VW9, *M. incognita* isolate VW6, *M. javanica* isolate VW4, and *M. arenaria* isolate HarA were provided by Dr. Valerie Williamson (UC-Davis). All *Meloidogyne* species were maintained on the susceptible tomato *Solanum lycopersicum* cv. Rutgers under greenhouse conditions. The identities of these isolates had been previously confirmed using species-specific PCR assays (Wishart et al. 2002; Zijlstra 1997). Genomic DNA of the nematodes was extracted from eggs and juveniles (J2) using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

LAMP primer design. The *M. chitwoodi* DNA sequence that spans the IGS2 and part of the 18S rDNA was selected as the amplification target region for LAMP primer design. The IGS2-18S rDNA sequences were downloaded from GenBank: *M. chitwoodi* (accession No. AJ421701), *M. fallax* (AJ421703), *M. hapla* (AJ421707), *M. minor* (KC262258), *M. incognita* (GQ395506), and *M. javanica* (GQ395510). Multi sequence comparison was performed using Clustal Omega (Sievers et al. 2011). The primers of LAMP were designed for the IGS2-18S rDNA specific regions of *M. chitwoodi*. Five primers were generated for the LAMP assay, including two outer primers (F3 and B3), one forward inner primer (FIP), one backward inner primer (BIP), and one loop backward primer (LB) (Table 1).

LAMP reactions and product detection. The LAMP reactions were performed according to the previously described protocol (Notomi et al. 2000; Tomita et al. 2008). Briefly, a 25-μl reaction mixture contained 1× isothermal amplification buffer, 1.4 mM of dNTP, 1.6 μM each of inner primers FIP and BIP, 0.2 μM each of outer primers F3 and B3, 0.4 μM of loop primer LB, 6 mM of $MgSO_4$, 1 μl of diluted genomic DNA (~1 ng), and 8 U of Bst 3.0 DNA polymerase (New England Biolabs, USA). To find out the optimal temperature for the LAMP assay, reactions were performed at 60–68°C for 45 min. At the end, the reactions were terminated by heating them to 80°C for 5 min. The products of LAMP reaction were detected using SYBR Green I (ThermoFisher Scientific, MA, USA). One μl of 1:10 diluted SYBR Green I solution was added to the LAMP reaction tube and mixed well. After the addition of the SYBR Green I solution, LAMP reactions with amplified DNA products turned green, while the reactions without successful DNA amplification remained brown. In addition, the amplified LAMP products were monitored by 1.5% agarose gel electrophoresis that was stained with ethidium bromide and visualized under UV light. LAMP reactions were performed at least three times using at least two independent *M. chitwoodi* gDNA samples.

LAMP specificity and sensitivity analysis. To determine the analytical sensitivity of the LAMP assay, serial 10-fold dilutions of *M. chitwoodi* genomic DNA, with the initial concentration at 10 ng/μl, were prepared. In addition, DNA extracts from 10-pooled *M. chitwoodi* J2s, single J2, and serial 10-fold dilutions of the single J2 DNA extract were tested by LAMP assay. The PCR was performed using the primer pair of F3/B3 designed in this study. The LAMP products were detected by both SYBR Green I staining and agarose gel electrophoresis. To determine specificity of the LAMP assay, genomic DNA purified from eggs of three isolates of *M. chitwoodi* and several other root-knot nematode species (*M. incognita, M. javanica, M. arenaria,* and *M. hapla*) were used as template DNA. Genomic DNA of the two samples of *M. fallax* was provided by Lieven Waeyenberge, Flanders Research Institute for Agriculture, Fisheries and Food. The LAMP products were monitored by both SYBR Green I staining and agarose gel electrophoresis.

Practical evaluation of LAMP assay. To evaluate the practical application of the LAMP assay, we obtained five unlabeled soil samples from Dr. Charles Brown (USDA-ARS) in Prosser, WA. The soil samples were from pots containing tomato plants with or without nematode infections. The tomato plants were grown in 8-inch terracotta clay pots filled with sandy silt loam soil composed of 84% sand, 10% silt, and 6% clay (Brown et al. 2006). The soil was steam pasteurized before use. The tomato plants were artificially inoculated with 5,000 eggs of either *M. chitwoodi* race 1 or *M. hapla,* or they were left uninoculated as a control. After 6–8 months in the greenhouse, soil was collected from the pots and sent in unlabeled bags to Washington State University, Pullman for blind egg extraction and LAMP assays. Eggs were extracted by mixing ~100 g of each soil sample with 0.5 liters of water and pouring the mixture over a #200 sieve (75 micron diameter pores) and over a #500 sieve (25 micron diameter pores). The eggs were collected off the #500 sieve and were further purified from any remaining plant debris by sucrose flotation (van Bezooijen 2006). Genomic DNA was purified from the eggs using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following manufacturer's instruction. One ng of DNA was subjected for LAMP and PCR assays. For the PCR assays, we used JMV1/

Table 1. Primers used for LAMP and PCR

Primer set	Primer name	Sequence (5′ – 3′)	Usage	Reference
JMV1/JMV2	JMV1	GGATGGCGTGCTTTCAAC	*M. chitwoodi* and *M. fallax*-specific primers	Wishart, et al. (2002)
	JMV2	TTTCCCCTTATGATGTTTACCC		
JMV1/JMV-Hapla	JMV1	GGATGGCGTGCTTTCAAC	*M. hapla*-specific primers	Wishart, et al. (2002)
	JMV-Hapla	AAAAATCCCCTCGAAAAATCCACC		
LAMP	F3	GAAGTCTCTCGTGTGAGAT	LAMP assay for *M. chitwoodi* and *M. fallax*	This study
	B3	CTCGATAAATCGATTAAACTGGT		
	FIP	AGACACGTCGTGTCACCTATATAAAGCATAGCGGTTTGTTC		
	BIP	ATGAAGATATCTGGTTGATCCTGCACATGCATGGCTTAATCTTTG		
	LB	CTGAACTGATGTGTTCATTT		

JMV2 to confirm the presence of *M. chitwoodi* and JMV1/JMV-Hapla to confirm the presence of *M. hapla* (Wishart et al. 2002).

DNA was also extracted directly from nematode-inoculated soil samples and used as the template in LAMP assays. Twelve artificially inoculated soil samples were prepared by mixing 10,000 J2 of *M. chitwoodi* race 1 with 100 g of sandy silt loam soil. Samples of 250 mg were taken from each and ground to a powder using a mortar and pestle. Ground soil was then used for DNA extraction using the DNeasy PowerLyzer PowerSoil Kit (Qiagen), following manufacturer's instructions. Two μl of eluted DNA was used in the LAMP assays. The DNA extraction was also performed on 250 mg of uninoculated soil samples; this and a no-template control were used as LAMP negative controls. Genomic DNA of *M. chitwoodi* race 1 was used as positive control in the experiment.

PCR amplifications. Reactions were performed in a 25-μl reaction volume, including 1× standard Taq buffer, 200 μM dNTP, 0.2 μM primer sets of F3/B3, JMV1/JMV2, or JMV1/JMVHapla, 0.75 U of *Taq* DNA polymerase (New England Biolabs, USA), and 1 μl of template genomic DNA. PCR amplifications were carried out using the procedure: 95°C 2 min, and 35 cycles of 95°C for 20 s, 50°C for 20 s, 68°C for 60 s, and a final incubation at 68°C for 5 min.

Results

LAMP primer design and reaction optimization. The LAMP primers were designed based on DNA sequence differences in the intergenic spacer 2 (IGS2) region between 5S rDNA and 18S rDNA of *M. chitwoodi* (Fig. 1). The sequences of the primers F3, F2, and F1c are located in the IGS2 region, and sequences of B3, B2, and B1c are located in 18S region. Previous sequencing of these regions in several root-knot nematode species, including *M. chitwoodi*, *M. hapla*, *M. incognita,* and *M. javanica*, has shown that the IGS2 contains species-polymorphisms, while the 18S region is conserved among the root-knot nematode species (Adam et al. 2007; Wishart et al. 2002). For the optimization of the LAMP assay using the *M. chitwoodi* LAMP primers, we determined the optimal reaction temperature. The reactions were performed at temperatures between 60°C and 68°C for 45 min using 1 ng genomic DNA of *M. chitwoodi* race 1 as the template. One hallmark of successful LAMP amplification is that the LAMP products produce ladder-like banding patterns when they are separated on an agarose gel. When visualized on an agarose gel, the LAMP products from all incubation temperatures showed ladder-like banding patterns. However, the most efficient amplification and brightest banding pattern on the agarose gel occurred at an incubation temperature of 68°C (Fig. 2A).

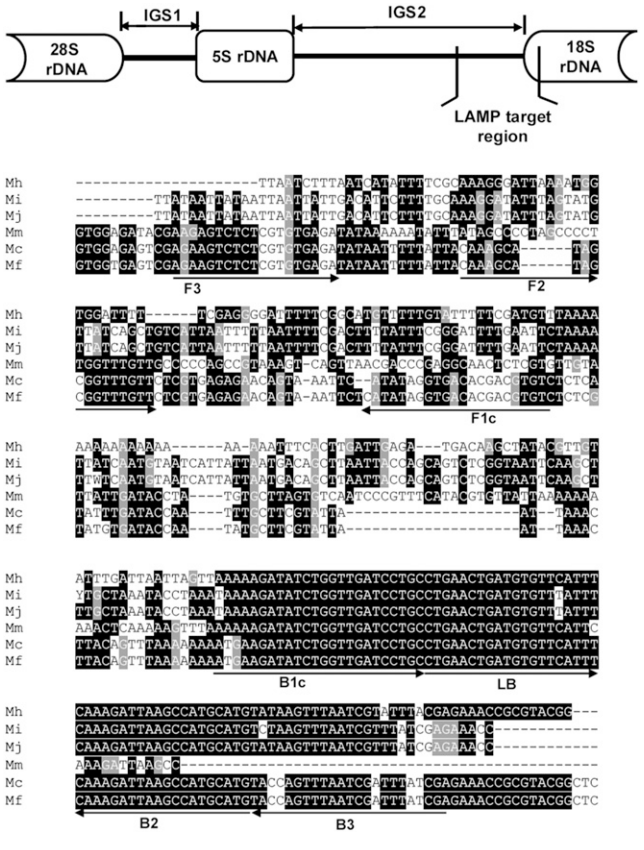

Fig. 1. Primer design for *Meloidogyne chitwoodi* LAMP assay based on IGS2-18S rDNA sequence. Scheme of the rDNA-IGS regions of *M. chitwoodi* is shown. Multiple alignment of the LAMP target region (partial IGS2 and partial 18S rDNA) among *M. chitwoodi* and other related root-knot nematode species is shown. The position and direction of primers are shown with arrows. Mc, *Meloidogyne chitwoodi* (GenBank: AJ421701); Mf, *Meloidogyne fallax* (GenBank: AJ421703); Mh, *Meloidogyne hapla* (GenBank: AJ421707); Mm, *Meloidogyne minor* (GenBank: KC262258); Mi, *Meloidogyne incognita* (GenBank: GQ395506); Mj, *Meloidogyne javanica* (GenBank: GQ395510).

An easy method for detection of LAMP products, which does not require gel electrophoresis equipment, is to add a DNA stain to the reaction mixture. After the addition of SYBR Green I stain, the *M. chitwoodi* LAMP products could be detected by a visual color change of the reaction from brown to green. Meanwhile, the nontemplate control (NTC) produced no ladder-like bands on the agarose gel, and the LAMP reaction was brown in color after the addition of SYBR Green I stain (Fig. 2B and C).

Analytical specificity test of LAMP assay. We wanted to determine that the LAMP primers we designed could amplify products from *M. chitwoodi*. We obtained DNA from three isolates of *M. chitwoodi* found in the PNW: race 1, race 2, and Roza. We then performed LAMP assays using genomic DNA from the three *M. chitwoodi* isolates and from DNA obtained from the tropical root-knot nematodes (*M. incognita, M. javanica,* and *M. arenaria*) and the Northern root-knot nematode *M. hapla*. Both gel electrophoresis (Fig. 3A) and SYBR Green I staining of the LAMP products (Fig. 3B) showed that the LAMP primers could specifically amplify products from all three *M. chitwoodi* isolates. No products were observed from DNA templates of the 4 other *Meloidogyne* species: *M. hapla*, *M. incognita, M. javanica,* and *M. arenaria* (Fig. 3A and B).

To further test the specificity of the LAMP primers, we used *M. fallax* DNA as a template in the LAMP assay. *M. fallax* is a closely related nematode species to *M. chitwoodi* (Holterman et al. 2009; van Megen et al. 2009). In LAMP assays using two different DNA samples of *M. fallax*, we found amplification products visible by gel electrophoresis and by SYBR Green I staining (Fig. 3C and D). Therefore, the LAMP primers can specifically amplify from DNA of *M. chitwoodi* (race1, race 2, and race 1 Roza) and the closely related *M. fallax.*

Analytical sensitivity of LAMP assay. To compare the analytical sensitivity of LAMP assay with conventional PCR, a series of 10-fold dilutions of *M. chitwoodi* race 1 genomic DNA was used as the reaction templates for both a LAMP assay and PCR. We used the primer set F3/B3 for conventional PCR. Both SYBR Green I staining and gel electrophoresis of LAMP products produced positive results up to a 10^{-5} dilution (0.1 pg DNA) (Fig. 4A and B). Meanwhile, the conventional PCR was only able to detect DNA at the dilution level of 10^{-3} (10 pg DNA) (Fig. 4C). Therefore, the LAMP assay is about 100 times more sensitive than the conventional PCR.

To further evaluate the analytical sensitivity of the LAMP assay, we isolated genomic DNA from 10 *M. chitwoodi* juveniles and a single *M. chitwoodi* juvenile. The LAMP assay was able to detect nematodes in both cases (Fig. 4D). To test the limits of detection of the LAMP assay, we made a serial 10-fold dilution of the DNA from a single juvenile to use as the template for the LAMP assays. We could positively detect *M. chitwoodi* down to a 10^{-2} dilution of DNA from a single nematode (Fig. 4D).

Evaluation of LAMP assay using soil samples. Because *M. fallax* has been reported once on turfgrass and is not established on crop plants in the United States (Nischwitz et al. 2013), we focused on optimizing our LAMP primers for identification of *M. chitwoodi* that is isolated from soil samples to create a useful tool for potato growers in the PNW. As a proof of concept, we wanted to determine if the LAMP assay could detect *M. chitwoodi* from artificially inoculated

soil samples. We obtained five soil samples from the USDA Agricultural Research Service in Prosser, WA. In a blind test, we were given four samples inoculated with either M. *chitwoodi* or *M. hapla* and one uninoculated soil sample. Nematode eggs were extracted from 4 out of the 5 soil samples. The fifth sample contained no nematode eggs. It was the uninoculated sample and used as a no-egg control. DNA was extracted from the eggs from the 4 samples, and 1 ng of DNA from each sample was used as the template for the LAMP assays. SYBR Green I staining of LAMP products showed that *M. chitwoodi* was present in samples 1, 2, and 3, but not in samples 4 and 5 (no-egg control)

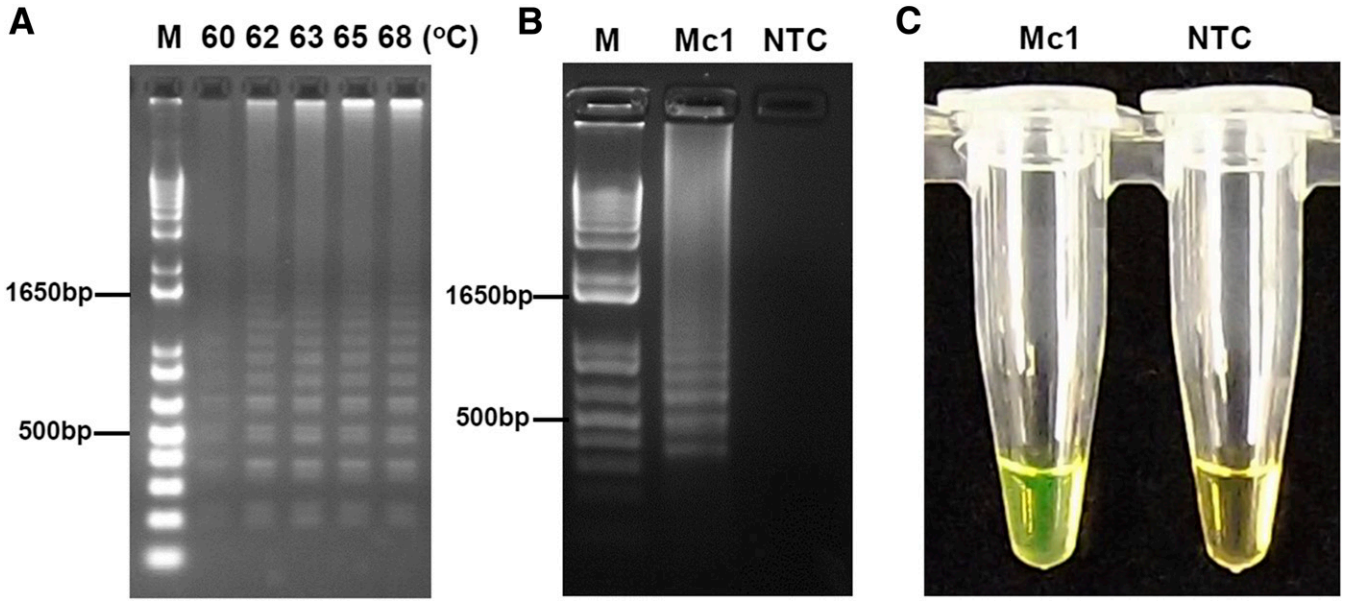

Fig. 2. LAMP reaction optimization and product detection. **A,** To optimize the LAMP assay temperatures, reactions were carried out at temperatures ranging from 60–68°C for 45 min. 1 μl of the LAMP reaction at each temperature was separated on a 1.5% agarose gel. The LAMP assay conducted at 68°C produced the brightest banding pattern on the gel. **B,** Agarose gel electrophoresis of 1 μl of the LAMP reaction using 1 ng of *Meloidogyne chitwoodi* DNA or water (no template control, NTC). Reaction was carried out at 68°C for 45 min. The LAMP reactions were positive for *M. chitwoodi* and negative for the NTC, as seen in the agarose gel and **C,** the reactions were stained with SYBR Green I fluorescence dye.

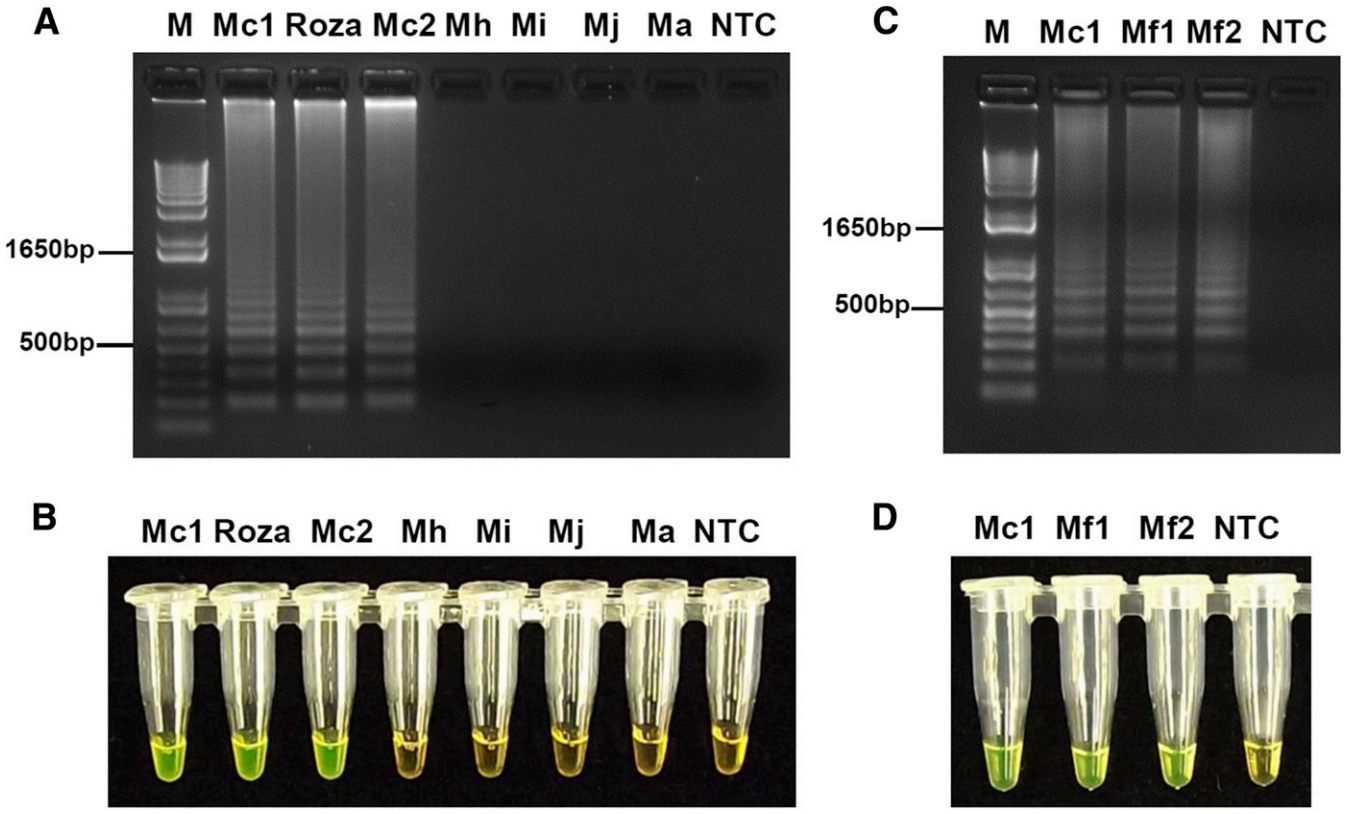

Fig. 3. Specificity test of LAMP assay. LAMP assays were performed using genomic DNA from Mc1 (*Meloidogyne chitwoodi* race 1), Roza (*M. chitwoodi* pathotype Roza), Mc2 (*M. chitwoodi* race 2), Mh (*M. hapla* VW9), Mi (*M. incognita* VW6), Mj (*M. javanica* VW4), and NTC (nontemplate control). Positive LAMP results were visualized by **A,** banding patterns on an agarose gel electrophoresis, and **B,** SYBR Green I staining of the LAMP reactions. There were positive reactions for all three *M. chitwoodi* isolates tested. There were no positive reactions for *M. hapla* (Mh), *M. incognita* (Mi), *M. javanica* (Mj), or *M. arenaria* (Ma) samples. When two isolates of *M. fallax* DNA was used as template in the LAMP assays, positive results were detected by **C,** agarose gel electrophoresis and **D,** SYBR Green I staining of the reactions.

(Fig. 5A). To confirm that the eggs in samples 1–3 were *M. chitwoodi*, the DNA was used as a template for PCR using the primer set JMV1/JMV2 (Wishart et al. 2002). To determine the identity of the eggs in sample 4, a PCR was performed using the primer set JMV1/JMV-Hapla, which had been previously shown to specifically amplify a 440-bp band from *M. hapla* DNA (Wishart et al. 2002). The PCR results show that samples 1–3 were *M. chitwoodi* and sample 4 contained *M. hapla* eggs (Fig. 5B and C). Hence, the LAMP assay is able to specifically detect *M. chitwoodi* present in soil samples.

To further demonstrate the utility of the LAMP assay, we inoculated 12 soil samples with *M. chitwoodi* juveniles and then directly extracted DNA from each soil sample. The LAMP assay was able to detect the presence of *M. chitwoodi* in all 12 DNA samples extracted directly from the nematode-inoculated soils (Fig. 5D). Thus, the LAMP assay can detect nematode DNA from soil DNA extracts. This eliminates the need to first isolate the nematodes from the soil before the DNA extraction step.

Discussion

Because of its capability of DNA amplification at isothermal conditions with high sensitivity and efficiency, the LAMP assay serves as a useful tool for plant-parasitic nematode identification. In this study, we developed a LAMP assay to detect potato root-knot nematodes *M. chitwoodi* and *M. fallax*. The LAMP primers were designed for the IGS2-18S region of *M. chitwoodi* rDNA. Our LAMP primers amplified products from three *M. chitwoodi* isolates found in the western United States: race1, Roza, and race 2. Although these isolates are nearly morphologically indistinguishable, race 1 and race 2 differ in their ability to infect carrot and alfalfa, and Roza overcomes the natural resistance found in the wild potato species *Solanum bulbocastanum* (Brown et al. 2009; Humphreys-Pereira and Elling \2013). Our results showed that our LAMP primers are stable across the different *M. chitwoodi* isolates; and thus, the LAMP assay is a reliable identification tool regardless of *M. chitwoodi* race.

Interestingly, our LAMP assay showed specificity for both *M. chitwoodi* and its close relative *M. fallax*. *M. fallax* is referred to as

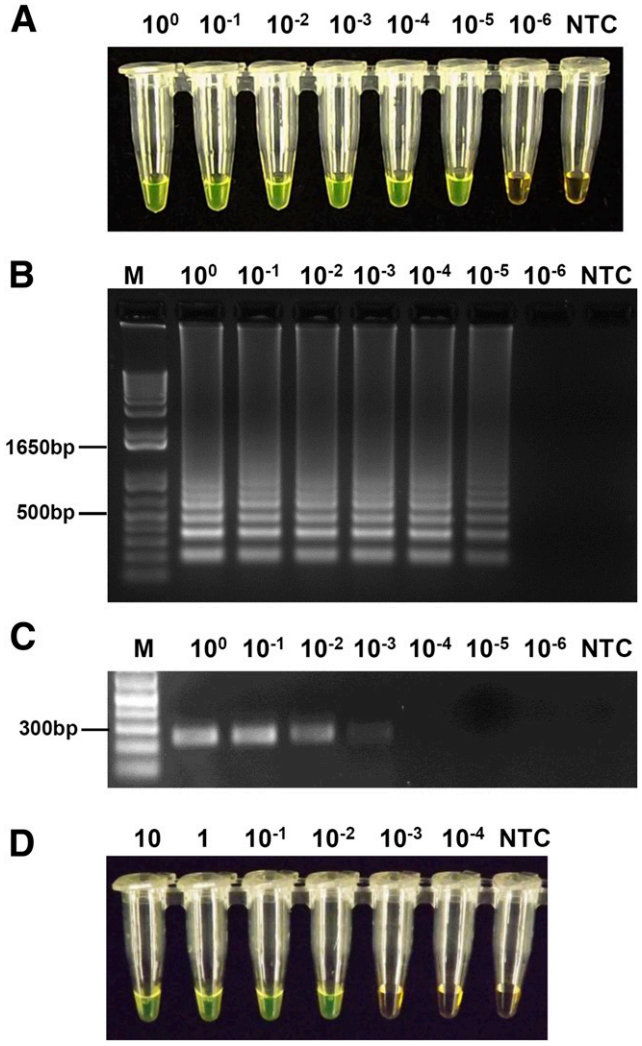

Fig. 4. Sensitivity test of LAMP assay compared with conventional PCR. **A,** SYBR Green I staining and **B,** agarose gel electrophoresis of LAMP products indicate that the LAMP assay can detect *Meloidogyne chitwoodi* DNA that has been diluted from 100 (10 ng DNA) to 10^{-5} (0.1 pg DNA). **C,** PCR can detect *M. chitwoodi* DNA that has been diluted from 100 (10 ng DNA) to 10^{-3} (10 pg DNA). **D,** SYBR Green I staining of LAMP reactions showed that the assay detects *M. chitwoodi* DNA extracted from 10 J2, one J2, and up to a 100-fold dilution of DNA extracted from single J2. NTC, no template control.

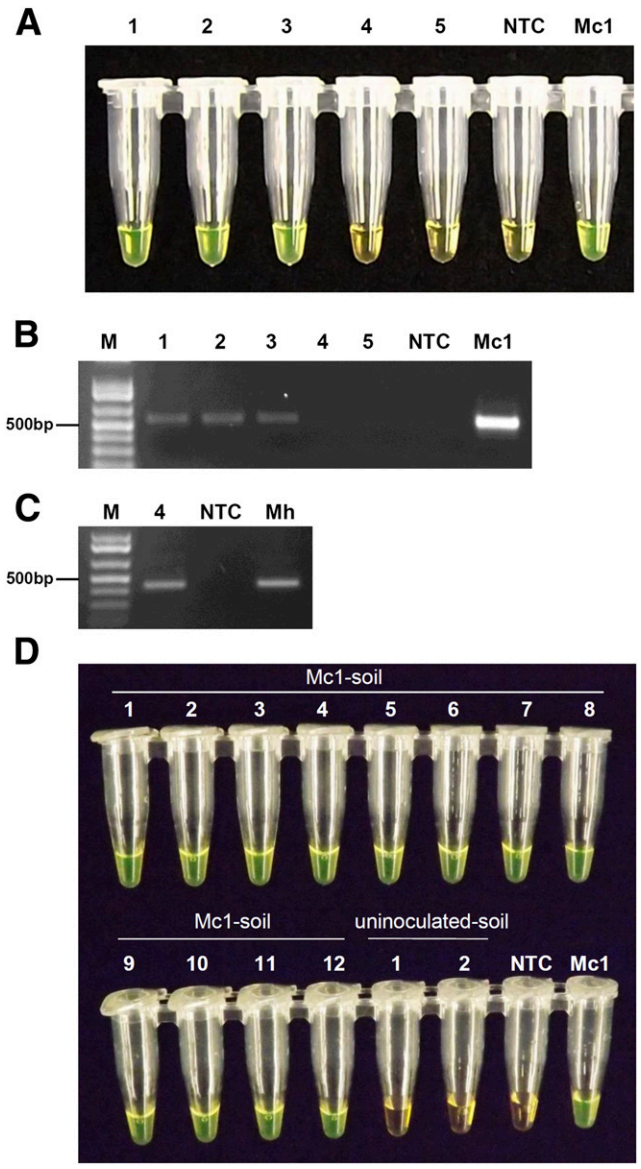

Fig. 5. Evaluation of LAMP assay using soil samples. **A,** LAMP assays on DNA isolated from nematode eggs extracted from four nematode infested soil samples (1–4) and soil from an uninoculated pot (5). As a positive control, 1 ng genomic DNA of *Meloidogyne chitwoodi* race 1 was used as a template (Mc1) in the LAMP assay and PCR. **B,** PCR using JMV1/JMV2 primers confirmed that the eggs in soil samples 1–3 were *M. chitwoodi.* **C,** PCR using *M. hapla* specific primers JMV1/JMV-Hapla confirmed that the eggs present in soil sample 4 were from *M. hapla.* Lane Mh indicates a PCR using 1 ng genomic DNA from *M. hapla* isolate VW9. NTC, no template control. **D,** DNA was extracted from *M. chitwoodi* J2 inoculated soil samples (Mc1-soil), or uninoculated-soil samples. SYBR Green I staining of LAMP products showed *M. chitwoodi* in all 12 Mc1-soil samples. The LAMP reactions from two uninoculated-soil samples, NTC and Mc1 genomic DNA, were used as controls.

the "false Columbia root-knot nematode," and it was previously known as *M. chitwoodi* (Baexem) B-type (van Meggelen et al. 1994). Additional morphological and biochemical studies determined that *M. fallax* and *M. chitwoodi* are different species (Karssen 1995; Zijlstra 1997). Currently, *M. fallax* is primarily found in parts of Europe, Australia, and South America (CABI/EPPO 2017). Although in a recent report, *M. fallax* was reported in turf grass on a California golf course (McClure et al. 2012), there are no reports of *M. fallax* on crops in the United States, and it is not known to be present in Washington State. Since *M. fallax* has not been reported in crop fields in the PNW, a positive LAMP assay using our primers would most likely indicate the presence of *M. chitwoodi*. If there were a positive result in the LAMP assay, concerned parties could distinguish the two species by PCR amplification of the rDNA intergenic spacer (IGS) region (Petersen and Vrain 1996; Petersen et al. 1997; Wishart et al. 2002) or by looking at the restriction fragment length polymorphisms (RFLPs) of the ITS of rDNA (Zijlstra 1997). The drawback to these techniques is that they require specialized equipment, such as a thermocycler and electrophoresis unit. Therefore, we propose that our LAMP assay can be a quick, easy assessment of potato root-knot nematodes in samples, and if necessary, a positive sample can be further examined using techniques that are more sophisticated.

In summary, our study has developed a sensitive and fast LAMP assay that positively detects *M. chitwoodi* and *M. fallax,* but it could not amplify products from three tropical root-knot nematode species (*M. incognita*, *M. arenaria*, and *M. javanica*) or temperate root-knot nematode *M. hapla*. The LAMP assay is more sensitive than conventional PCR, and it does not require complicated equipment. Therefore, it can be used as a simple tool for nematode identification with future potential for in-field surveys.

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