Loop-Mediated Isothermal Amplification for the Detection of *Tylenchulus semipenetrans* in Soil

Borong Lin, Honghong Wang, and Kan Zhuo, Laboratory of Plant Nematology, South China Agricultural University, Guangzhou 510642, China; and Guangdong Province Key Laboratory of Microbial Signals and Disease Control, South China Agricultural University, Guangzhou 510642, China; and **Jinling Liao**, Laboratory of Plant Nematology and Guangdong Province Key Laboratory of Microbial Signals and Disease Control, South China Agricultural University; and Guangdong Vocational College of Ecological Engineering, Guangzhou 510520, China

Abstract

Lin, B., Wang, H., Zhuo, K., and Liao, J. 2016. Loop-mediated isothermal amplification for the detection of *Tylenchulus semipenetrans* in soil. Plant Dis. 100:877-883.

Tylenchulus semipenetrans is an economically important plant-parasitic nematode occurring in all citrus-producing regions of the world and causing a disease called “slow decline”. For the rapid detection of this nematode, a loop-mediated isothermal amplification (LAMP) assay was developed, based on the ribosomal DNA internal transcribed spacer sequence. The optimal condition for the LAMP assay was 65°C for 50 min. The LAMP products were confirmed using conventional polymerase chain reaction (PCR) and restriction analysis with the *Bam*HI enzyme, and by adding SYBR Green I to the LAMP products for visual inspection. The LAMP assay was highly specific for the detection of

T. semipenetrans populations from different geographical origins. It was also sensitive, detecting a tenth of the DNA from an individual specimen of *T. semipenetrans*, which was 10 times more sensitive than conventional PCR. The LAMP protocol was applied to natural citrus rhizosphere soil samples from several orchards in China and the results were fast, sensitive, robust, and accurate. This study is the first to provide a diagnostic tool for *T. semipenetrans* using DNA extracted directly from citrus rhizosphere soils. This LAMP assay could be used as a practical molecular tool to identify *T. semipenetrans* and diagnose slow decline disease, even in remote locations.

The plant-parasitic nematode *Tylenchulus semipenetrans* Cobb 1913 is highly pathogenic to citrus and causes “slow decline” disease. Crop loss caused by *T. semipenetrans* can reach 15 to 30% annually (Duncan 2005). This nematode occurs in all citrus-producing regions of the world. In the major citrus-producing regions, infestation rates by this nematode are high. In the United States, 50 to 90% of the citrus orchards in Arizona, California, Florida, and Texas are infested by this nematode (Duncan 2005; Heald and O’Bannon 1987). In Spain and Brazil, the nematode infests 70 to 90% of commercial citrus orchards (Van Gundy and Meagher 1977). In China, *T. semipenetrans* is found in most citrus-producing areas, including Guangdong, Guangxi, Sichuan, Yunnan, and Hunan Provinces (Zhao and Yang 2006). In some citrus orchards within Guangxi and Yunnan Provinces, more than 90% of the citrus trees are infested with *T. semipenetrans* (Huang et al. 2011; Li et al. 2001).

To appropriately manage slow decline, the first task is to diagnose the disease correctly. However, the aboveground symptoms of slow decline are similar to other root diseases and nutrient deficiencies, including stunting, slow growth, yellowing, and reduced foliage, fruit size, and yield. This makes it difficult to correctly diagnosis (Duncan 2005). In China, some citrus orchards infested with *T. semipenetrans* have been misdiagnosed as citrus huanglongbing and destroyed (Huang et al. 2012). To date, the sampling, manual extraction, and identification of *T. semipenetrans* from citrus rhizosphere soil is the only method available to correctly diagnose slow decline (Verdejo-Lucas and Kaplan 2002). Currently, diagnostics rely on the extraction of second-stage juveniles (J2) of *T. semipenetrans*

from soil. For the observer who lacks skills and training, it is very difficult to correctly identify *T. semipenetrans* based on the morphology of only J2 specimens. In particular, *T. semipenetrans* J2 specimens can easily be misidentified as *Meloidogyne* spp. from citrus rhizosphere soils.

In the past decade, methods that utilize the polymerase chain reaction (PCR) have been developed to identify plant-parasitic nematodes based on various target sequences, mainly through the amplification of ribosomal DNA (rDNA) or mitochondrial DNA (mtDNA) sequences (Adam et al. 2007; Blok et al. 2002; Yan et al. 2013). Increasingly, studies have demonstrated the effectiveness of identifying and differentiating nematodes using molecular techniques. At present, several molecular techniques have been developed to identify *T. semipenetrans*, including rDNA-internal transcribed spacer region (ITS) PCR restriction fragment length polymorphism (RFLP) (Maafi et al. 2012; Park et al. 2009; Wang et al. 2004) and PCR amplification using species-specific primers derived from the rDNA-ITS (Liu et al. 2011; Maafi et al. 2012). In contrast to the morphological methods, PCR-based methods are more sensitive and accurate for *T. semipenetrans* identification (Liu et al. 2011). However, for conventional PCR, an expensive thermal cycler is needed; this limits its routine use.

A relatively new DNA amplification technique, loop-mediated isothermal amplification (LAMP), was developed in 2000 (Notomi et al. 2000). During LAMP, the reaction is performed at an isothermal temperature of 60 to 65°C, eliminating the need for expensive thermal cyclers. LAMP requires a polymerase with high-strand displacement activity, and two or three sets of primers that identify six or eight distinct regions on the target gene; these requirements strongly increase the specificity of the reaction. All LAMP steps can be done in a single tube over a short time period of 0.5 to 1 h, and detection of the amplification product can be determined by the naked eye using intercalating dyes, such as SYBR green, propidium iodide, or ethidium bromide, or by measuring the turbidity caused by the formation of magnesium pyrophosphate (Duan et al.

Corresponding author: K. Zhuo; E-mail: zhuokan@scau.edu.cn

Accepted for publication 4 December 2015.

2014; Maeda et al. 2005; Mori et al. 2001; Nagamine et al. 2002; Notomi et al. 2000). Hence, compared with conventional PCR technologies, LAMP offers some major advantages, including simplicity, sensitivity, rapidity, specificity, and low cost. These factors make it well suited for field diagnosis applications.

Currently, LAMP has been successfully applied in plant pathology to detect plant pathogens, including fungi (Dai et al. 2012; Duan et al. 2014; Niessen and Vogel 2010) and bacteria (Harper et al. 2010). Only a few studies have developed LAMP detection methods for the identification of plant-parasitic nematodes (Kikuchi et al. 2009; Niu et al. 2011, 2012; Peng et al. 2012). At present, LAMP assays have been developed only for *Bursaphelenchus xylophilus* (Kikuchi et al. 2009), *Meloidogyne* spp. (Niu et al. 2011, 2012), and *Radopholus similis* (Peng et al. 2012). In this study, a LAMP assay for the rapid detection of *T. semipenetrans* based on its rDNA-ITS sequence was developed. The LAMP assay was evaluated by comparing it with conventional PCR methods, and by conducting tests using field citrus rhizosphere soil samples.

Materials and Methods

Nematode populations and DNA extraction. Five populations of *T. semipenetrans* collected from citrus orchards in China were used. Other nematode species were also used to verify the specificity of the primers (Table 1). These included specimens isolated from

Table 1. *Tylenchulus semipenetrans* and other nematodes from China used in this study to test the specificity of the loop-mediated isothermal amplification assay

Species	Isolate	Host	Geographical location in China
<i>T. semipenetrans</i>	Ts1	Citrus	Dali, Yunnan
<i>T. semipenetrans</i>	Ts2	Citrus	Yangjiang, Guangdong
<i>T. semipenetrans</i>	Ts3	Citrus	Fogang, Guangdong
<i>T. semipenetrans</i>	Ts4	Citrus	Yuantan, Guangdong
<i>T. semipenetrans</i>	Ts5	Citrus	Guangzhou, Guangdong
<i>Aphelenchoides</i> sp.	Apco	Citrus	Fujian, Fuzhou
<i>Aphelenchus</i> sp.	Apcu	Citrus	Yangjiang, Guangdong
<i>Ditylenchus</i> sp.	Di	Citrus	Nanchang, Jiangxi
<i>Helicotylenchus</i> sp.	He	Citrus	Yuantan, Guangdong
<i>Meloidogyne javanica</i>	MJ1	Towel gourd	Shenzhen, Guangdong
<i>M. incognita</i>	ZCd	Cucumber	Zengcheng, Guangdong
<i>Pratylenchus brachyurus</i>	Fj908	Sugarcane	Zhangzhou, Fujian
<i>P. coffeae</i>	PC	Citrus	Yangjiang, Guangdong
<i>P. vulnus</i>	JXP	Peach	Jiujiang, Jiangxi
<i>Paratylenchus</i> sp.	Pa	Citrus	Yangjiang, Guangdong
<i>Radopholus similis</i>	RS	Anthurium	Guangzhou, Guangdong
<i>Rotylenchulus reniformis</i>	RR	Citrus	Yangjiang, Guangdong
<i>Trichodorus</i> sp.	Tr	Citrus	Yuantan, Guangdong
<i>Caenorhabditis elegans</i>	CE	...	Yangjiang, Guangdong
Dorylaimidae	Do	...	Yongsheng, Hunan
<i>Iotonchus</i> sp.	Io	...	Yuantan, Guangdong
Monhysterida	Mo	...	Yuantan, Guangdong
<i>Prismatolaimus</i> sp.	Pr	...	Yongsheng, Hunan
Qudsianematidae	Qu	...	Yongsheng, Hunan
<i>Tobrilus</i> sp.	To	...	Yongsheng, Hunan

citrus rhizosphere soils in this study (*Paratylenchus* sp., *Rotylenchulus reniformis*, *Helicotylenchus* sp., *Pratylenchus coffeae*, *Aphelenchoides* sp., *Ditylenchus* sp., *Aphelenchus* sp., *Trichodorus* sp., *Prismatolaimus* sp., *Tobrilus* sp., *Caenorhabditis elegans*, *Iotonchus* sp., Dorylaimidae, Qudsianematidae, and Monhysterida) and other nematodes that have previously been reported in citrus rhizosphere soils (*P. brachyurus*, *P. vulnus*, *Meloidogyne incognita*, *M. javanica*, and *Radopholus similis*) (Duncan 2005). All the nematodes were identified using morphological characteristics (Abebe et al. 2006; Nickle 1991) in combination with molecular data. DNA was extracted from individual nematode specimens following the protocol described by Subbotin et al. (2008). The mtDNA *COII/rnL* region of *Meloidogyne* spp. was amplified. Two rDNA fragments (i.e., partial small subunit [SSU] or ITS) of other nematodes were amplified. Primers for mtDNA *COII/rnL* amplification were C2F3 (5'-GGTC AATGTTTCAGAAATTTGTGG-3') and 1108 (5'-ACCTTTGACCA ATCACGCT-3') (Powers and Harris 1993). Primers for partial SSU amplification were G18SU (5'-GCTTGCTCAAAGATTAAGCC-3') and R18Ty1 (5'-GGTCCAAGAATTTACACTCTC-3') (Chizhov et al. 2006). Primers for ITS amplification were TW81 (5'-GTTT CCGTAGGTGAACCTGC-3') and AB28 (5'-ATATGCTTAAGT TCAGCGGGT-3') (Subbotin et al. 2000). Detailed protocols of PCR amplification were as described by Maafi et al. (2003).

Primer design. Sequences of the rDNA-ITS region were chosen as the candidate targets for primer design. The sequences of the target region in *Tylenchulus* spp., including *Tylenchulus semipenetrans* (JN112276), *T. graminis* (JN112286), *T. palustris* (JN112268), *T. musicola* (JN112281), *T. furcus* (JN112285), and other nematode species, including *Paratylenchus* sp. (KF150664), *P. coffeae* (EU130845), *P. brachyurus* (HQ641385), *P. vulnus* (JQ003994), *M. incognita* (LC030364), *Heterodera avenae* (HQ450302), *Aphelenchoides besseyi* (EF371500), *Aphelenchus avenae* (KJ155784), *C. elegans* (JN636101), *R. similis* (FJ455832), *Rotylenchulus reniformis* (KP018580), *Helicotylenchus digonicus* (GQ906352), and *Trichodorus viruliferus* (KC160453), were obtained from the National Center for Biotechnology Information. A multiple sequence alignment was performed to compare the sequences, using the MegAlign program in Lasergene software (DNASTAR Inc., Madison, WI). Then, the primers TSF3 (5'-GCATCTGGCGAGT CTGTG-3'), TSB3 (5'-GCACCGAATCTGGAATCAT-3'), TSFIP (F1c+BamHI+F2: 5'-CRGGTAAGAGCCGAGAAGGACAggatccG TCATACTTCCTCYGCCGCT-3'), and TSBIP (B1c+ttttt+B2: 5'-TGTAACGCTGAGCGACTGTTGAtttttGCGACATGTGGAGAA GGC-3') were designed, using PrimerExplorer V4 software (Eiken Chemicals, Tokyo, Japan) (Fig. 1). All of the primers were synthesized by Invitrogen Biotech (Shanghai, China).

LAMP protocol and optimization. According to the user manual (New England Biolabs Ltd., London) and previous reports (Niu et al. 2011, 2012), the LAMP reaction was performed in a 25- μ l volume comprising 2.5 μ l of the 10 $\times$ isothermal amplification buffer, 3.5 μ l of 10 mM dNTP, 1.5 μ l of 100 mM MgSO₄, 2.5 μ l of 10 $\times$ primers (1.6 μ M each for the TSFIP and TSBIP primers and 0.2 μ M each for the TSF3 and TSB3 outer primers), 1 μ l of Bst 2.0-DNA polymerase (New England Biolabs Ltd.), 4 μ l of 5 M betaine (Sigma-Aldrich, St. Louis), and 1 μ l of the template DNA.

To optimize the reaction temperature for the LAMP assay, the reactions were performed in a water bath (YiHeng Technical Co. Ltd.,

Fig. 1. Primers used in loop-mediated isothermal amplification for the detection of *Tylenchulus semipenetrans*. Arrows and boxes indicate the direction and location of the primers.

Shanghai, China) at 58, 60, 62, 63, 65, 67, and 68°C for 90 min. Then, the products (10 µl) were separated on standard 2% agarose gels stained with Goldview (EB substitution) (Toyobo Co., Shanghai, China). A DS2000 DNA marker (Dongsheng Biotech, Shanghai, China) was used to determine the molecular size of the bands, which were photographed under UV light using an Alphamager system (Alpha Innotech Co., San Leandro, CA). The optimal temperature was determined according to the band intensity obtained at different temperatures. The experiment was performed three times. One sample was included at each temperature at each experiment.

The reaction time was optimized by real-time PCR analysis using the TP860 system (Takara, Dalian, China): the reactions were performed at the determined optimal temperature in a 25-µl reaction volume (as described above) and 1.5 µl of 20× EVA green (Maygene, Guangzhou,

China) was added as an intercalator. Then, 35 reaction cycles were performed for 2 min each and the signal after each cycle was determined.

LAMP specificity analysis. To determine whether the LAMP amplified the correct target sequence, the LAMP products were digested with the *Bam*HI restriction enzyme (Thermo Fisher Scientific, Shanghai, China) and analyzed by electrophoresis. The restriction enzyme site was selected based on the appropriate sequence information (shown in the primer TSFIP). The restriction digestion system consisted of 2 µl of the 10× FastDigest green buffer, 2 µl (0.5 µg) of LAMP products, 1 µl of *Bam*HI, and 15 µl of double-distilled (dd)H₂O. The reaction was carried out at 37°C for 30 min. Additionally, the PCR product generated by using the primers TSF3/TSB3 was then sequenced and the Basic Local Alignment Search Tool (BLAST) was used to examine the specificity of the primers. The PCR was performed in a 25-µl volume, comprising 12.5 µl of the 2× EasyTaq PCR SuperMix (Transgen, Beijing), 0.5 µl of primer TSF3, 0.5 µl of primer TSB3, and 1 µl of the template DNA. The amplification was carried out with an initial denaturation step at 95°C for 3 min; 30 reaction cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s; followed by an extension at 72°C for 5 min.

To determine whether the primers were specific to *Tylenchulus semipenetrans*, DNA was extracted from 25 nematode populations composed of 21 different nematode species (Table 1). Each sample was loaded in triplicate and a negative control sample was also prepared, using ddH₂O instead of a DNA template. The LAMP results were visually inspected with the naked eye in daylight by adding 1 µl of 1,000× SYBR Green I solution (Dingguo Biotech, Beijing) to the amplification products.

LAMP sensitivity assay for *T. semipenetrans*. DNA was first extracted from 1 or 100 individuals of *T. semipenetrans*. Subsequently, a 10-fold dilution of the DNA from 1 *T. semipenetrans*, the DNA from 1 *T. semipenetrans*, 10-fold dilution of the DNA from 100 *T. semipenetrans*, and the DNA from 100 *T. semipenetrans* were separately subjected to LAMP and conventional PCR. The LAMP assay was performed as described above, and the conventional

Fig. 2. Optimization of temperature requirement for the loop-mediated isothermal amplification reaction. The reaction was performed for 90 min at temperatures ranging from 58 to 68°C; lane M represents the Ds2000 DNA molecular weight marker ladder and lane CK represents the control (the DNA template was replaced with H₂O).

Fig. 3. Optimization of the duration of time for the loop-mediated isothermal amplification reaction. Real-time polymerase chain reaction was used to optimize the reaction time. Numbers on the x-axis represent the reaction time. Lines labeled 0.1, 1, 10, 50, 80, and 100 represent 1,000-fold, 100-fold, 10-fold, 2-fold, 1.25-fold, and 1-fold dilution of the DNA template from 100 *T. semipenetrans*, respectively. CK represents the control (the DNA template was replaced with double-distilled H₂O).

PCR was conducted using the primers Ts-SF (5'-TACCAGGTT GAGCAGAGTCTT-3') and Ts-SR (5'-TCCTACCCTTCCA CAGCG-3'), as described previously (Liu et al. 2011), at 95°C for 5 min; then, 35 reaction cycles of 95°C for 45 s, 59°C for 30 s, and 72°C for 30 s; followed by an extension period at 72°C for 5 min. A negative control sample was also prepared, using ddH₂O instead of a DNA template. Each sample was run in triplicate and the experiment was performed three times.

Field application of LAMP. In order to evaluate the application of LAMP as a diagnostic tool for *T. semipenetrans* in the field, 24 rhizosphere soil samples from citrus orchards in different regions across China were collected. DNA was extracted from 1 g of soil using a PowerSoil DNA Isolation Kit and further purified with an UltraClean PCR Clean-up Kit (Mo-Bio Laboratories, Carlsbad, CA). Then, 1 µl of DNA was added into the 25 µl of LAMP reaction mixture; the LAMP reactions were performed as described above. To validate the protocol, the nematodes in each sample were also identified based on morphology. The purified DNA from *T. semipenetrans* was used as a positive control and ddH₂O instead of the DNA of *T. semipenetrans* was used as a negative control. Three independent experiments were performed.

Results

Optimization of LAMP reaction conditions. When LAMP was conducted using *T. semipenetrans* DNA at 58 to 68°C for 90 min, the typical ladder-like bands produced with gel electrophoresis were observed. The bands obtained were most intense at 65°C and no ladder-like bands were observed in the negative control (Fig. 2). The fluorescence intensity of DNA extracted from 0.1 to 100 nematodes started to increase after 30 min of reaction time, and reached a plateau at 50 min in the sample containing 0.1 nematode (Fig. 3). Therefore, the optimal condition for the LAMP assay was determined to be 65°C for 50 min.

Specificity of the LAMP assay. Electrophoresis showed that the products of the LAMP assay were digested completely using the restriction enzyme *Bam*HI (Fig. 4). In addition, the product generated using the primers TSF3/TSB3 was sequenced and the resulting 233-bp sequence was compared with known ITS sequences of *T. semipenetrans*; the sequence was 100% identical to the ITS sequences of original *T. semipenetrans* population (data not shown). The LAMP

assay was highly specific for *T. semipenetrans*, based on the visual evaluation with SYBR Green I using DNA isolated from *T. semipenetrans* and other nematodes found in citrus rhizosphere soil (Table 1); the *T. semipenetrans* samples resulted in positive reactions (SYBR Green I turned yellow), while the other nematode samples resulted in negative reactions (SYBR Green I remained orange to brown) (Fig. 5).

Sensitivity of the LAMP assay. The LAMP and conventional PCR assays, performed on different serial dilutions of the DNA extracted from *T. semipenetrans*, demonstrated the increased sensitivity of the LAMP assay. The conventional PCR could only detect one individual nematode but the LAMP assay could detect approximately a tenth of the DNA extracted from a single nematode (10-fold dilution of template DNA from 1 nematode; Fig. 6). This demonstrates that the LAMP assay exhibited a 10-fold greater sensitivity than conventional PCR.

Evaluation of the LAMP assay using citrus rhizosphere soil.

The LAMP assay was able to detect *T. semipenetrans*, when present, in the 24 soil samples collected from the citrus orchards. Amplification occurred in 14 samples and the remaining 10 samples produced negative results. These results were confirmed by morphological observations: all of the positive samples did contain *T. semipenetrans*, ranging from 202 to 2,300 individuals per 100 g of soil. In the negative samples, although there were other nematodes, no *T. semipenetrans* specimens were isolated (Table 2).

Discussion

PCR is one of the most valuable tools for the identification of plant pathogens and plant-parasitic nematodes and diagnosis of plant

Fig. 4. Restriction enzyme analysis of the loop-mediated isothermal amplification products **A**, without and **B**, with *Bam*HI digestion, respectively. Lane M represents the Ds2000 DNA molecular weight marker ladder; lanes 1 to 5 represent the DNA templates from five different isolates of *Tylenchulus semipenetrans*; and lanes 6 to 8 represent the DNA templates of control from *Aphelenchoides* sp., *Aphelenchus* sp., and *Ditylenchus* sp., respectively.

Fig. 5. Specificity of the loop-mediated isothermal amplification (LAMP) assay for *Tylenchulus semipenetrans* and other nematodes. SYBR Green I was added to the LAMP products from tubes 1 to 5 = five different populations of *T. semipenetrans*; and tubes 6 to 25 = populations of *Pratylenchus brachyurus*, *P. coffeae*, *P. vulnus*, *Meloidogyne incognita*, *M. javanica*, *Ditylenchus* sp., *Aphelenchoides* sp., *Aphelenchus* sp., *Caenorhabditis elegans*, *Radopholus similis*, *Paratylenchus* sp., *Rotylenchulus reniformis*, *Helicotylenchus* sp., *Iotonchus* sp., *Trichodorus* sp., *Prismatolaimus* sp., *Tobrilus* sp., Qudsianematidae, Dorylaimidae, and Monhysterida. Tube 26 = control (the DNA template was replaced with double-distilled H₂O).

diseases. Conventional PCR requires different temperatures to achieve amplification; therefore, an expensive thermal cyclers are needed. This limits its routine use, especially in the field and in some developing countries. Other techniques of sequence-specific DNA amplification, which do not require changes in temperature during the reaction, have been developed. One of these alternative approaches is LAMP, a fast and inexpensive technique. LAMP does not require thermal cyclers and has been successfully applied to detect pathogenic microorganisms for more than 10 years (Duan et al. 2014; Fu et al. 2011). However, only in recent years has this technique been used to identify plant-parasitic nematodes. Several

important plant-parasitic nematode species, including *B. xylophilus*, *M. incognita*, *M. enterolobii*, and *R. similis*, have been successfully identified using LAMP (Kikuchi et al. 2009; Niu et al. 2011, 2012; Peng et al. 2012). In this study, a new LAMP method was developed to detect *T. semipenetrans*. The LAMP assay was successfully applied to identify *T. semipenetrans* in crude DNA extracts from citrus rhizosphere soil.

Many studies indicate that the rDNA-ITS sequence located between the repeating array of nuclear 18S and 28S rDNA genes is an important marker for differentiating nematode species (Gasser and Newton 2000; Powers et al. 1997; Subbotin et al. 2001). More

Fig. 6. Sensitivity of **A**, the loop-mediated isothermal amplification (LAMP) and **B**, conventional polymerase chain reaction methods. **C**, LAMP products visualized with SYBR green I using natural daylight. The initial DNA template was extracted from 1 or 100 *Tylenchulus semipenetrans* individuals. Lane M represents the Ds2000 DNA molecular weight marker ladder, lane 0.1 represents 10-fold dilution of the DNA template from one *T. semipenetrans*, lane 1 represents the DNA template from one *T. semipenetrans*, lane 10 represents 10-fold dilution of the DNA template from 100 *T. semipenetrans*, lane 100 represents the DNA template from 100 *T. semipenetrans*, respectively, and CK represents the control (the DNA template was replaced with double-distilled H₂O).

Table 2. Detection of *Tylenchulus semipenetrans* in citrus soil samples using the loop-mediated isothermal amplification assay

Sample	Geographical location in China	Amplification result ^a	Number of <i>T. semipenetrans</i> ^b	Other species identified ^c
1	Fogang, Guangdong	–	0	14,16,17
2	Fuzhou, Fujian	+	860	1,13
3	Kunming, Yunnan	–	0	13,14
4	Kunming, Yunnan	+	304	13
5	Kunming, Yunnan	+	202	13
6	Loudi, Hunan	–	0	13
7	Nanchang, Jiangxi	+	1020	2,4,13
8	Nanning, Guangxi	–	0	1
9	Nanning, Guangxi	–	0	2
10	Nanning, Guangxi	+	2300	2,13
11	Nanning, Guangxi	+	1160	2,13
12	Nanning, Guangxi	–	0	13
13	Yangjiang, Guangdong	–	0	3,8,10
14	Yangjiang, Guangdong	–	0	2,8,9,16
15	Yangjiang, Guangdong	–	0	9,14,16
16	Yangjiang, Guangdong	+	569	3,7
17	Yangjiang, Guangdong	+	482	2,3
18	Yangshan, Guangdong	–	0	2,6,13
19	Yongsheng, Hunan	+	1000	1,12
20	Yongsheng, Hunan	+	550	2
21	Yongsheng, Hunan	+	1500	2,5,7,11,15
22	Yongsheng, Hunan	+	550	2,12
23	Yongsheng, Hunan	+	500	11
24	Yuantan, Guangdong	+	1100	7,8,16

^a Symbols + and – indicate a positive and negative result, respectively.

^b Morphologically identified and extracted from 100 g soil.

^c Species 1 = Aphelenchoides sp., 2 = Aphelenchus sp., 3 = Caenorhabditis elegans, 4 = Ditylenchus sp., 5 = Dorylaimoididae, 6 = Helicotylenchus sp., 7 = Iotonchus sp., 8 = Monhysterida, 9 = Paratylenchus sp., 10 = Pratylenchus coffeae, 11 = Pristionotus sp., 12 = Qudsiatidae, 13 = Rhabditidae, 14 = Rotylenchulus reniformis, 15 = Tobrilus sp., 16 = Trichodorus sp., and 17 = Xiphenema sp.

recently, five *Tylenchulus* spp., including *T. semipenetrans*, were distinguished and identified from their PCR-ITS-RFLP profiles, and through PCR with species-specific primers based on ITS sequences (Maafi et al. 2012). In this study, LAMP primers that targeted the ITS sequence of *T. semipenetrans* were designed. The results showed that the LAMP assay using these primers was highly specific and able to differentiate *T. semipenetrans* from other nematodes in citrus rhizosphere soils. Unfortunately, aside from *T. semipenetrans*, we were unable to obtain other *Tylenchulus* spp. to examine whether primers would cross-react with other species in the genus. Thus far, the genus *Tylenchulus* contains five valid species (*T. semipenetrans*, *T. furcus*, *T. graminis*, *T. palustris*, and *T. musicola*) (Maafi et al. 2012), of which only *T. semipenetrans* is able to infest citrus. *T. graminis* and *T. palustris* only occur in Florida and parasitize monocotyledons and non-rutaceous dicots (including *Baccharis halimifolia*, *Fraxinus caroliniana*, peach, and sea oxeye), respectively. *T. furcus* occurs in South Africa only, infecting monocotyledons, while *T. musicola* only occurs in Iran, infecting banana (Dow et al. 1990; Eisenback et al. 2007; Inserra et al. 1988, 1990; Maafi et al. 2012). Because the other species have very different host ranges and narrow distributions, the LAMP system from this study is still suitable for the detection of *T. semipenetrans* in citrus rhizosphere soils. In addition, four primers that identify six distinct regions within the ITS region of *T. semipenetrans* were used in this study, which increases the specificity of the reaction. More *Tylenchulus* samples could be included to validate the specificity in the future.

The validity of the LAMP assay was confirmed in 24 soil samples collected from different citrus-producing regions in China. All soil samples infested with *T. semipenetrans* were successfully detected with LAMP, whereas those without *T. semipenetrans* but with other nematodes produced negative results. There were no false-positive reactions. These results indicate that the LAMP technology is sensitive and stable, and can overcome the impact of soil substances such as humic acids, as previously reported (Niu et al. 2011, 2012).

Compared with conventional PCR (Liu et al. 2011), the LAMP assay was 10-fold more sensitive. The results are also easier to evaluate than those from conventional PCR because they can be visualized with the naked eye using SYBR Green I staining. Moreover, the LAMP assay is rapid (50 min was found to be optimal for the detection of *T. semipenetrans*) and only requires a single constant temperature to be maintained (in this case, 65°C). In other words, the developed LAMP assay provides a good alternative to conventional PCR for the rapid and simple detection of *T. semipenetrans*. LAMP can be used to detect target nematode DNA from citrus rhizosphere soils with high sensitivity and specificity. The LAMP assay developed in this study has the potential to be a useful tool for the quick detection of *T. semipenetrans* in citrus orchards and, thus, the diagnosis of slow decline disease.

Acknowledgments

This work was supported by grants from the National Key Basic Research Program of China (973 Program, numbers 2013CB127501 and 2013CB127502), the National Natural Science Foundation of China (numbers 31171824, 31471750, and 31071666), and the Special Fund for Agro-scientific Research in Public Interest of China (number 201103018). We thank M. Hu for helping to collect *Meloidogyne incognita* and *M. javanica* and L. Hu for helping to collect *Tylenchulus semipenetrans* in Yunnan Province.

Literature Cited

Abebe, E., Trautspurger, W., and Andrassy, I. 2006. Freshwater Nematodes: Ecology and Taxonomy. CABI Publishing, Cambridge, MA.

Adam, M. A. M., Phillips, M. S., and Blok, V. C. 2007. Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.). Plant Pathol. 56:190-197.

Blok, V. C., Wishart, J., Fargette, M., Berthier, K., and Phillips, M. S. 2002. Mitochondrial DNA differences distinguishing *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. Nematology 4: 773-781.

Chizhov, V. N., Chumakova, O. A., Subbotin, S. A., and Baldwin, J. G. 2006. Morphological and molecular characterization of foliar nematodes of the genus *Aphelenchoides*: *A. fragariae* and *A. ritcemabosi* (Nematoda: Aphelenchoididae)

from the Main Botanical Garden of the Russian Academy of Sciences. Russ. J. Nematol. 14:179-184.

Dai, T. T., Lu, C. C., Lu, J., Dong, S. M., Ye, W. W., Wang, Y. C., and Zheng, X. B. 2012. Development of a loop-mediated isothermal amplification assay for detection of *Phytophthora sojae*. FEMS Microbiol. Lett. 334:27-34.

Dow, R. L., Inserra, R. N., Esser, R. P., and Langdon, K. R. 1990. Distribution, hosts, and morphological characteristics of *Tylenchulus palustris* in Florida and Bermuda. J. Nematol. 22:724-728.

Duan, Y. B., Ge, C. Y., Zhang, X. K., Wang, J. X., and Zhou, M. G. 2014. Development and evaluation of a novel and rapid detection assay for *Botrytis cinerea* based on loop-mediated isothermal amplification. PLoS One 9: e111094.

Duncan, L. W. 2005. Nematode parasites of citrus. Pages 437-466 in: Plant Parasitic Nematodes in Subtropical and Tropical Agriculture. M. Luc, R. A. Sikora, and J. Bridge, eds. CABI Publishing, Cambridge, MA.

Eisenback, J. D., Reaver, D., and Ashley, J. E. 2007. First report of the nematode, *Tylenchulus palustris*, parasitizing peach in Virginia. Plant Dis. 91:1683.

Fu, S. J., Qu, G. G., Guo, S. J., Ma, L., Zhang, N., Zhang, S. L., Gao, S. Y., and Shen, Z. Q. 2011. Applications of loop-mediated isothermal DNA amplification. Appl. Biochem. Biotechnol. 163:845-850.

Gasser, R. B., and Newton, S. E. 2000. Genomic and genetic research on bursate nematodes: Significance, implications and prospects. Int. J. Parasitol. 30: 509-534.

Harper, S. J., Ward, L. I., and Clover, G. R. G. 2010. Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. Phytopathology 100:1282-1288.

Heald, C. M., and O'Bannon, J. H. 1987. Citrus declines caused by nematodes. V. Slow decline. Nematol. Circ. No. 143. Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Tallahassee, FL.

Huang, J. L., Liu, Z. M., Lu, X. H., and Lu, G. Y. 2011. Preliminary investigation on *Tylenchulus semipenetrans* of citrus in Guangxi. S. China Fruits 40:60-61.

Huang, J. L., Liu, Z. M., Lu, X. H., Qin, B. X., Ai, H. J., and Qiao, L. Y. 2012. Trial on controlling citrus *Tylenchulus semipenetrans*. J. South. Agric. 43: 1139-1141.

Inserra, R. N., Vovlas, N., Nyczepir, A. P., Wehunt, E. J., and Golden, A. M. 1990. *Tylenchulus palustris* parasitizing peach trees in the United States. J. Nematol. 22:45-55.

Inserra, R. N., Vovlas, N., O'Bannon, J. H., and Esser, R. P. 1988. *Tylenchulus graminis* n. sp. and *T. palustris* n. sp. (Tylenchulidae), from native flora of Florida, with notes on *T. semipenetrans* and *T. furcus*. J. Nematol. 20: 266-287.

Kikuchi, T., Aikawa, T., Oeda, Y., Karim, N., and Kanzaki, N. 2009. A rapid and precise diagnostic method for detecting the pinewood nematode *Bursaphelenchus xylophilus* by loop-mediated isothermal amplification. Phytopathology 99:1365-1369.

Li, Y. Q., Wang, Y., and Yu, S. P. 2001. Damage of *Tylenchulus semipenetrans* disease on citrus in Yunnan Province. J. Shenyang Agric. Univ. 32:236-237.

Liu, G. K., Chen, J., Xiao, S., Zhang, S. S., and Pan, D. M. 2011. Development of species-specific PCR primers and sensitive detection of the *Tylenchulus semipenetrans* in China. Agric. Sci. China 10:252-258.

Maafi, Z. T., Amani, M., Stanley, J. D., Inserra, R. N., Van den Berg, E., and Subbotin, S. A. 2012. Description of *Tylenchulus musicola* sp. n. (Nematoda: Tylenchulidae) from banana in Iran with molecular phylogeny and characterisation of species of *Tylenchulus* Cobb, 1913. Nematology 14:353-369.

Maafi, Z. T., Subbotin, S. A., and Moens, M. 2003. Molecular identification of cyst-forming nematodes (Heteroderidae) from Iran and a phylogeny based on the ITS sequences of rDNA. Nematology 5:99-111.

Maeda, H., Koikeguchi, S., Fujimoto, C., Tanimoto, I., Yoshizumi, W., Nishimura, F., and Takashiba, S. 2005. Detection of periodontal pathogen *Porphyromonas gingivalis* by loop-mediated isothermal amplification method. FEMS Immunol. Med. Microbiol. 43:233-239.

Mori, Y., Nagamine, K., Tomita, N., and Notomi, T. 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289:150-154.

Nagamine, K., Hase, T., and Notomi, T. 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Probes 16: 223-229.

Nickle, W. R. 1991. Manual of Agricultural Nematology. Marcel Dekker, Inc., New York.

Niessen, L., and Vogel, R. F. 2010. Detection of *Fusarium graminearum* DNA using a loop-mediated isothermal amplification (LAMP) assay. Int. J. Food Microbiol. 140:183-191.

Niu, J. H., Guo, Q. X., Jian, H., Chen, C. L., Yang, D., Liu, Q., and Guo, Y. D. 2011. Rapid detection of *Meloidogyne* spp. by LAMP assay in soil and roots. Crop Prot. 30:1063-1069.

Niu, J. H., Jian, H., Guo, Q. X., Chen, C. L., Wang, X. Y., Liu, Q., and Guo, Y. D. 2012. Evaluation of loop-mediated isothermal amplification (LAMP) assays based on 5S rDNA-IGS2 regions for detecting *Meloidogyne enterolobii*. Plant Pathol. 61:809-819.

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

- Park, B. Y., Park, S. N., Lee, J. K., and Bae, C. H. 2009. Morphometric and genetic variability among *Tylenchulus semipenetrans* populations from citrus growing area in Korea. *Plant Pathol. J.* 25:236-240.
- Peng, H., Peng, D. L., Hu, X. Q., He, X. F., Wang, Q., Huang, W. K., and He, W. T. 2012. Loop-mediated isothermal amplification for rapid and precise detection of the burrowing nematode, *Radopholus similis*, directly from diseased plant tissues. *Nematology* 14:977-986.
- Powers, T. O., and Harris, T. S. 1993. A polymerase chain reaction method for identification of five major *Meloidogyne* species. *J. Nematol.* 25:1-6.
- Powers, T. O., Todd, T. C., Burnell, A. M., Murray, P. C. B., Fleming, C. C., Szalanski, A. L., Adams, B. A., and Harris, T. S. 1997. The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. *J. Nematol.* 29:441-450.
- Subbotin, S. A., Ragsdale, E. J., Mullens, T., Roberts, P. A., Mundo-Ocampo, M., and Baldwin, J. G. 2008. A phylogenetic framework for root lesion nematodes of the genus *Pratylenchus* (Nematoda): Evidence from 18s and D2-D3 expansion segments of 28s ribosomal RNA genes and morphological characters. *Mol. Phylogenet. Evol.* 48:491-505.
- Subbotin, S. A., Vierstraete, A., De Ley, P., Rowe, J., Waeyenberge, L., Moens, M., and Vanfleteren, J. R. 2001. Phylogenetic relationships within the cyst-forming nematodes (Nematoda, Heteroderidae) based on analysis of sequences from the ITS regions of ribosomal DNA. *Mol. Phylogenet. Evol.* 21:1-16.
- Subbotin, S. A., Waeyenberge, L., and Moens, M. 2000. Identification of cyst forming nematodes of the genus *Heterodera* (Nematoda: Heteroderidae) based on the ribosomal DNA-RFLPs. *Nematology* 2:153-164.
- Van Gundy, S. D., and Meagher, J. W. 1977. Citrus nematode (*Tylenchulus semipenetrans*) problems worldwide. *Proc. Int. Soc. Citricult.* 3:823-826.
- Verdejo-Lucas, S., and Kaplan, D. T. 2002. The citrus nematode: *Tylenchulus semipenetrans*. Pages 207-219 in: *Plant Resistance to Parasitic Nematodes*. J. L. Starr, R. Cook, and J. Bridge, eds. CABI Publishing, Cambridge, MA.
- Wang, Y., Yu, S. F., Li, Y. Q., Hu, X. Q., and Pu, W. Q. 2004. Studies on rDNA of *Tylenchulus semipenetrans* and *Pratylenchus* spp. by ITS-RFLP. *J. Laiyang Agric. Coll.* 21:151-153.
- Yan, G., Smiley, R. W., Okubara, P. A., Skantar, A. M., and Reardon, C. L. 2013. Developing a real-time PCR assay for detection and quantification of *Pratylenchus neglectus* in soil. *Plant Dis.* 97:757-764.
- Zhao, W. X., and Yang, B. J. 2006. *A List of Plant Nematodes in China*. China Forestry Publishing House, Beijing.