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Loop-mediated isothermal amplification based on the mitochondrial *COI* region to detect *Pratylenchus zeae*

Xingtong Liu · Honghong Wang · Borong Lin · Ye Tao · Kan Zhuo · Jinling Liao

Abstract *Pratylenchus zeae* parasitizes various crops and damages the host roots, resulting in decreased yield and quality of the host plants. Alignments of mitochondrial DNA (mtDNA) Cytochrome Oxidase I (*COI*) sequences revealed the genetic variation among *Pratylenchus* species. The results indicated 0.2–2.4% intraspecific variations for mtDNA *COI* sequences among eight *P. zeae* populations, and 25.4–35.1% interspecific variations between *P. zeae* and other *Pratylenchus* species. Based on the mtDNA *COI* region, a loop-mediated isothermal amplification (LAMP) assay was developed for the rapid and specific detection of *P. zeae*. The optimal conditions for the LAMP assay were 64 °C for 40 min. The LAMP products were confirmed using conventional polymerase chain reaction (PCR), analysis with the restriction enzyme *Bam* HI and visual inspection by adding SYBR Green I to the products. The LAMP assay could detect *P. zeae* populations from different hosts and different geographical origins specifically. The LAMP assay was also sensitive, detecting 0.1 individual *P. zeae*, which was 10 times more sensitive than conventional PCR. This is the first report of the detection of *Pratylenchus* spp. using LAMP. In addition, the results also suggested that use of the *COI* gene might allow for good resolution at the *Pratylenchus* species level.

Keywords LAMP · *Pratylenchus zeae* · mtDNA *COI* region

X. Liu · H. Wang · B. Lin · Y. Tao · K. Zhuo (✉) · J. Liao
Laboratory of Plant Nematology, South China Agricultural University, Guangzhou 510642, China
e-mail: zhuokan@scau.edu.cn

X. Liu · B. Lin · Y. Tao · K. Zhuo · J. Liao
Guangdong Province Key Laboratory of Microbial Signals and Disease Control, South China Agricultural University, Guangzhou 510642, China

H. Wang · J. Liao
Guangdong Vocational College of Ecological Engineering, Guangzhou 510520, China

Introduction

Root-lesion nematodes of the genus *Pratylenchus* are migratory endoparasites that have a wide host range and are distributed worldwide (Castillo and Vovlas 2007). They feed and migrate within root cortical tissue, causing roots necrosis and a reduction in lateral branching of roots, which are responsible for root lesion disease (Vanstone et al. 1998; Castillo and Vovlas 2007). *Pratylenchus* are considered the third most important economically damaging plant-parasitic nematodes after root-knot nematodes and cyst nematodes (Jones et al. 2013).

To manage root lesion disease, the causative factor must first be identified. Presently, sampling, manual extraction and identification of *Pratylenchus* species is the only method available to correctly diagnose root lesion disease caused by root-lesion nematodes.

Currently, over 70 *Pratylenchus* species have been reported (Castillo and Vovlas 2007; Mizukubo et al. 2007; Bairwa et al. 2008; Troccoli et al. 2008; Múnera et al. 2009; Palomares-Rius et al. 2010; De Luca et al. 2010, 2012; Palomares-Rius et al. 2014; Wang et al. 2015). Among them, *P. zeae* is one of the most important species, which can attack various crops, such as potato, corn, cereal and especially sugarcane, causing serious losses (Castillo and Vovlas 2007). For unskilled observers, it is very difficult to correctly identify *P. zeae*, because there is high intraspecific variability of some morphological characters and some species have very similar morphologies. More recently, *P. parazeae* was found to parasitize sugarcane in China, with *P. parazeae* and *P. zeae* being present in mixed populations (Wang et al. 2015). Polymerase chain reaction (PCR) has been used widely to identify major *Pratylenchus* spp., including *P. zeae*. For example, *P. zeae* from sugarcane can be identified and detected by using SYBR-Green qPCR (Berry et al. 2008); Four *Pratylenchus* species, including *P. zeae*, can be discriminated using rDNA-internal transcribed spacer region (ITS) restriction fragment length polymorphism (RFLP) (Li and Zheng 2013); *P. zeae* can be distinguished from *P. parazeae* using duplex PCR (Wang et al. 2015). These studies showed that *Pratylenchus* spp., including *P. zeae*, can be identified and detected using molecular techniques. However, an expensive thermal cycler is needed for these conventional PCRs, limiting their routine use.

The loop-mediated isothermal amplification (LAMP) assay, a relatively new DNA amplification technique, was developed in 2000 (Notomi et al. 2000). The LAMP reaction is usually performed at an isothermal temperature of 60 to 65 °C, which negates the need for expensive thermal cyclers. All LAMP steps can be done in a single tube within 0.5 to 1h, and the amplification product can be detected by the naked eye using intercalating dyes, such as SYBR Green, propidium iodide or ethidium bromide. LAMP requires a polymerase with a high strand displacement activity and two or three sets of primers that identify six or eight distinct regions on the target gene, which increases the specificity of the reaction (Notomi et al. 2000; Nagamine et al. 2002; Maeda et al. 2005; Duan et al. 2014; Lin et al. 2016). Thus, LAMP has the advantages over conventional PCR in sensitivity, rapidity, specificity and cost.

Recently, LAMP assays have been developed to identify and detect plant-parasitic nematodes, such as several economically important plant-parasitic nematodes, including *Bursaphelenchus xylophilus*, *Meloidogyne* spp., *Radopholus similis* and *Tylenchulus semipenetrans* (Kikuchi et al. 2009; Niu et al. 2011, 2012; Peng et al. 2012; Lin et al. 2016). However, LAMP has not yet been developed to detect *Pratylenchus* species. The aim of the current study was to develop a LAMP assay to detect *P. zeae* based on the mitochondrial DNA (mtDNA) Cytochrome Oxidase I (*COI*) sequence, and to evaluate its efficiency.

Materials and methods

Nematode populations and DNA extraction

Eight populations of *P. zeae* from distant geographical areas and different hosts were used. Seventeen other plant-parasitic nematode species collected from various hosts, including eight *Pratylenchus* species (*P. parazeae*, *P. brachyurus*, *P. coffeae*, *P. loosi*, *P. vulus*, *P. neglectus*, *P. scribneri* and *P. thornei*), *Pratylenchoides ritteri*, *Radopholus similis*, *Hirschmanniella* sp., *Meloidogyne incognita*, *M. javanica*, *Tylenchorhynchus annulatus*, *Helicotylenchus dihystera*, *Filenchus* sp. and *Criconemoides* sp., were also used to verify the specificity of the primers (Table 1). All the nematodes were identified using morphological characteristics, together with molecular data. DNA was extracted from individual nematode specimens as described by Subbotin et al. (2008). For *Meloidogyne* spp., the mtDNA *COII/rrnL* region was amplified. For the other nematodes, two rDNA fragments the D2D3 expansion domains of large subunit (LSU D2D3) or the internal transcribed spacer (ITS) were amplified. Primers for mtDNA *COII/rrnL* amplification were C2F3 (5′-GGTCAATGTTCAGAAATTTGTGG-3′) and 1108 (5′-ACCTTTGACCAATCACGCT-3′) (Powers and Harris 1993). Primers for ITS amplification were TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) (Subbotin et al. 2000). Primers for LSU D2D3 amplification were D2A (5′-ACAAGTACCGTGGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) Subbotin et al. (2006).

Table 1 *Pratylenchus* species and other nematodes used in this study

Species	Collection code	Host	Geographical location	GenBank accession number for *COI*
Pratylenchus zeae	A24	Sugarcane	Beihai, Guangxi, China	KX349412
P. zeae	A39	Sugarcane	Liuzhou, Guangxi, China	KX349413
P. zeae	GX1004	Rice	Liuzhou, Guangxi, China	KX349414
P. zeae	HN297	Corn	Wenchang, Hainan, China	KX349415
P. zeae	SG209	Sugarcane	Shaoguan, Guangdong, China	KX349416
P. zeae	U19	Corn-Bean	North Carolina, USA	KX349417
P. zeae	YNZT	Tobacco	Honghe, Yunnan, China	KX349418
P. zeae	ZC13	Corn	Guangzhou, Guangdong, China	KX349419
P. brachyurus	GX655	Corn	Beihai, Guangxi, China	KX349420
P. coffeae	JS26	Peanut	Yangzhou, Jiangsu, China	KX349421
P. loosi	FJGYT	Tea	Ningde, Fujian, China	KX349422
P. neglectus	GSY24S	Sesame	Lanzhou, Gansu, China	KX349423
P. parazeae	A65	Sugarcane	Chongzuo, Guangxi, China	KX349424
P. scribneri	SD1275	Corn	Yantai, Shandong, China	KX349425
P. thornei	GSY24L	Sesame	Lanzhou, Gansu, China	KX349426
P. vulnus	JXP	Peach	Jiujiang, Jiangxi, China	KX349427
Hirschmanniella sp.	Miti	Taro	Qionghai, Hainan, China	KX349428
Pratylenchoides ritteri	NMG	Cherry tomato	Chifeng, Neimenggu, China	KX349429
Radopholus similis	RS	Anthurium	Guangzhou, Guangdong, China	KX349430
Tylenchorhynchus annulatus	TA	Sugarcane	Guangzhou, Guangdong, China	-
Filenchus sp.	SW	Sugarcane	Guangzhou, Guangdong, China	-
Criconemoides sp.	CB	Sugarcane	Guangzhou, Guangdong, China	-
Helicotylenchus dihystera	LX	Sugarcane	Guangzhou, Guangdong, China	-
Meloidogyne incognita	MI	Tomato	Yuxi, Yunnan, China	-
M. javanica	MJ	Water spinach	Shenzhen, Guangdong, China	-

Detailed protocols of the PCR amplification were described in Tanha Maafi et al. (2003). DNA sequencing was performed as described by Zhuo et al. (2010).

PCR amplification of the partial mtDNA *COI* gene

The partial mtDNA *COI* sequences of all *Pratylenchus* species, *Pratylenchoides ritteri*, *Radopholus similis* and *Hirschmanniella* sp. were amplified. The primers used were COI F (5′-GATTTTTTGGKCATCCWGARG-3′) and COI R (5′-CWACATAATAAGTATCATG-3′) (He et al. 2005). Detailed protocols of the PCR amplification were described in He et al. (2005). The newly obtained sequences were deposited in the GenBank database under the accession numbers listed in Table 1. The sequences were compared in GenBank using the BLASTn program to analyze intraspecific and interspecific differences among the specimens.

Phylogenetic analyses

At the time of analysis, there were nineteen mtDNA *COI* sequences of root-lesion nematodes available in GenBank. Additionally, some mtDNA genome-, SSU- and LSU-based phylogenetic studies have indicated that *Meloidogyne* is closely related to the Pratylenchidae (Subbotin et al. 2006; Holterman et al. 2009; Rybarczyk-Mydłowska et al. 2013; Humphreys-Pereira and Elling 2014; Sun et al. 2014). Thus, to confirm the identity of the newly acquired *COI* sequences, the new sequences, the known *COI* sequences of *Pratylenchus* and several *COI* sequences of *Meloidogyne* from GenBank were used in phylogenetic analyses. Outgroup taxa were chosen according to previous molecular phylogenetic analyses of plant-parasitic nematodes (Subbotin et al. 2006; Holterman et al. 2009; Humphreys-Pereira and Elling 2014; Sun et al. 2014).

DNA sequences were aligned using MEGA4.0 (Tamura et al. 2003), with default parameters and the alignment quality was examined by eye. The model of base substitution was evaluated using MODELTEST (Posada and Crandall 1998; Huelsenbeck and Ronquist 2001). The Akaike information criterion indicated that we should use the TVM + I + G substitution model to construct the Bayesian consensus tree (lnL = 5479.3257; freqA =0.2849; freqC =0.0975; freqG =0.2188; freqT =0.3988; R(a) = 0.0339; R(b) = 6.1549; R(c) = 3.6032; R(d) = 6.9599; R(e) = 6.1549; R(f) = 1; Pinva =0.2618; Shape =0.9910). The Bayesian analysis was performed using MrBayes 3.1.0 (Huelsenbeck and Ronquist 2001), running the chain for 1×10^6 generations and setting the 'burn-in' at 2500. The Markov Chain Monte Carlo method was used within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget and Simon 1999) using the 50% majority rule.

Primer design

Sequences of the partial mtDNA *COI* region in all *Pratylenchus* species, *Pratylenchoides ritteri*, *Radopholus similis* and *Hirschmanniella* sp. obtained from this study (Table 1) and in *Meloidogyne*, including *M. incognita* (KM887154) and *M. javanica* (KM887156) obtained from GenBank, were chosen as the candidate targets for primer design. A multiple sequence alignment was performed to compare these sequences, using MegAlign program in the Lasergene software (DNASTAR Inc., Madison, WI, USA). The primers PZF3 (5′-GGTTTAGATCTTGATTCTCGG-3′), PZB3 (5′-GTAAAAATAAATCCAAAGTGGCA-3′), PZFIP (F1c + *Bam*HI + F2: 5′-ACCAGGTAAAAACC TTAATTCCGGggatccGCTTATTTTAGGGGGGCT-3′) and PZBIP (B1c + aaaaaa + B2: 5′-TATT CGTTTTGGTCCGGTTTTTTTaaaaaaCAAAATAA CCCCAGAGCAAC-3′) were then designed using PrimerExplorer V4 software (Eiken Chemicals, Tokyo, Japan) (Fig. 1). All primers were synthesized by Invitrogen Biotech (Shanghai, China).

LAMP protocol optimization

According to the user manual (New England Biolabs Ltd., London, UK), the LAMP reaction was performed in a 25 μl volume comprising 2.5 μl of the 10 × isothermal amplification buffer, 3.5 μl of 10 mM dNTPS, 1.5 μl of 100 mM MgSO$_4$, 2.5 μl of the 10 × primers (1.6 μM each for the PZFIP and PZBIP primers, and 0.2 μM each for the PZF3 and PZB3 primers), 1 μl of Bst 2.0-DNA polymerase (New England Biolabs Ltd., London, UK), 4 μl of 5 M betaine (Sigma Aldrich Co., St. Louis, MO, USA), and 1 μl of the template DNA.

To optimize the reaction temperature of the LAMP assay, the reactions were performed in a water bath (YiHeng Technical Co. Ltd., Shanghai, China) at 62, 64, 66 and 68 °C for 90 min. Then, 10 μl of the products were separated on standard 2% agarose gels stained with Goldview (Toyobo Co., Shanghai, China). Trans2K DNA markers (Transgene, Beijing, China) were used to determine the molecular size of the bands, which were photographed under UV light using an Alphalmager system (Alpha Innotech Co., San Leandro, CA, USA). The optimal temperature was determined according to the band intensity obtained at different temperatures. The experiment was performed three times.

To optimize the reaction time, the LAMP assay was carried out using the TP860 system (Takara, Dalian, China). Firstly, DNA was isolated from 1 or 100 individuals of *P. zeae*. Subsequently, a 10-fold dilution of the DNA from one *P. zeae*, and 1.25-fold, 2.5-fold and 10-fold dilutions of the DNA from the 100 *P. zeae* were obtained. All these DNA samples were subjected to real-time PCR. The real-time LAMP reaction was performed in a 25-μL volume comprising 2.5 μL of 10 × isothermal amplification buffer, 3.5 μL of 10 mM dNTPs, 1.5 μL of 100 mM MgSO$_4$, 2.5 μL of the 10 × primers, 1 μL of Bst 2.0-DNA polymerase, 1 μL of template DNA, 4 μL of 5 M betaine and 2 μL EVA green (Mayene, Guangzhou, China). Thirty-five reaction cycles were performed at 64 °C, and each cycle lasted 2 min: the signal after each cycle was determined.

LAMP specificity analysis

To determine the specificity of the LAMP reaction, the LAMP products were digested with *Bam* HI restriction enzyme (Thermo Fisher Scientific, Shanghai, China) and analyzed by electrophoresis. The restriction enzyme site was chosen based on the appropriate sequence information (shown in the inner primer PZFIP). The restriction digestion system comprised 2 μl of the 10 × FastDigest green buffer, 2 μl of LAMP products, 1 μl of *Bam* HI and 15 μl of double-distilled water. The reaction was conducted at 37 °C for 30 min. In addition, the PCR product generated using the outer primers PZF3/PZB3 was sequenced to determine the specificity
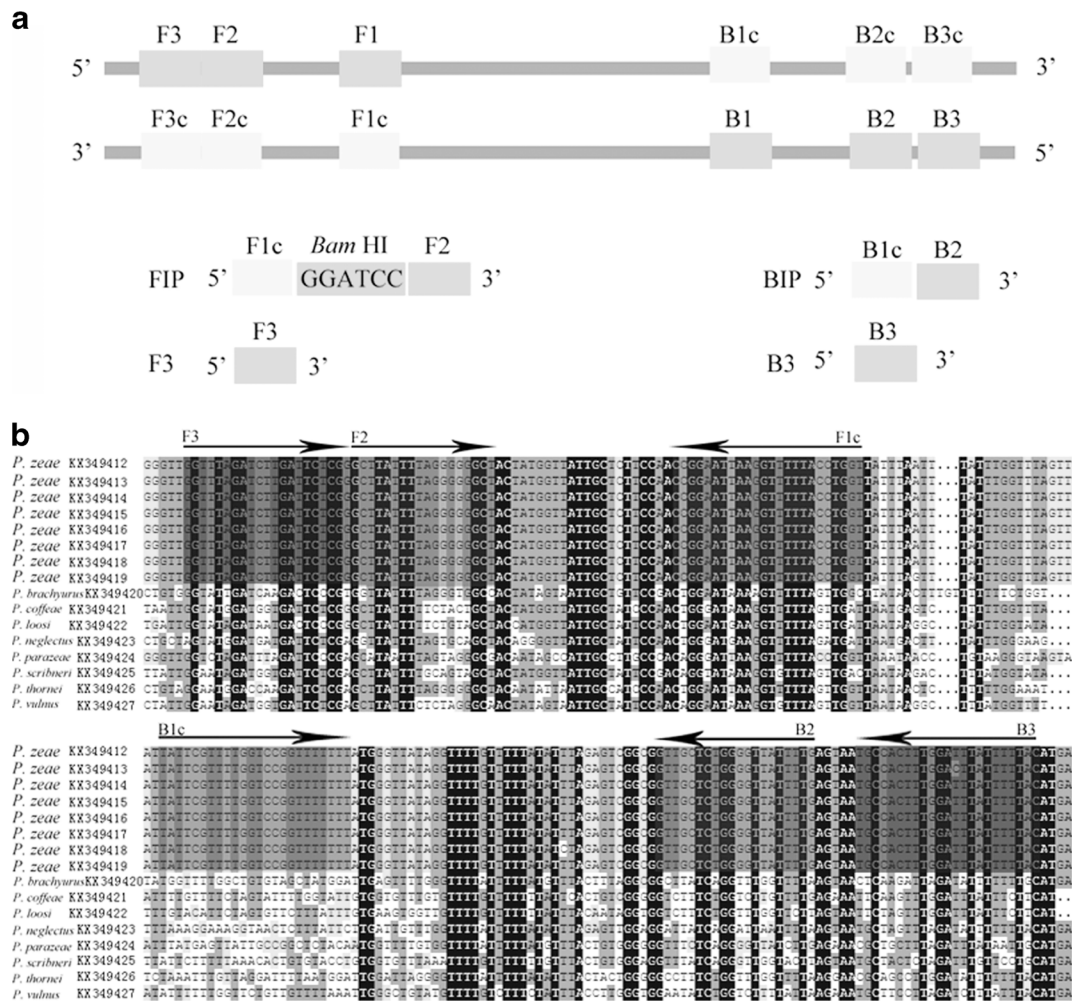
Fig. 1 Primers used for loop-mediated isothermal amplification to detect *Pratylenchus zeae*. **a** schematic representation of the double-stranded target DNA and loop-mediated isothermal amplification inner (FIP and BIP) and outer (F3 and B3) primers. The FIP primer consists of F1c, *Bam* HI and F2; the BIP primer consists of B1c and B2. **b** Multiple sequence alignment of partial *COI* sequences of eight *P. zeae* isolates and other *Pratylenchus* species. Arrows and shadows indicate direction and location of the primers

of the outer primers. The PCR reaction was performed in a 25 μl volume comprising 12.5 μl of the 2 × Taq Green Mix (Dingguo, Guangzhou, China), 0.5 μl each of the outer primers and 1 μl of the template DNA. The amplification program was as follows: 95 °C for 3 min; followed by 35 cycles at 95 °C for 30 s, 40 °C for 30 s and 72 °C for 40 s; and an extension at 72 °C for 7 min.

To determine whether the primers were specific to *P. zeae*, DNA was isolated from 25 nematode populations comprising 18 different nematode species (Table 1). Each sample was loaded in triplicate and a negative control sample was also prepared, using double-distilled water instead of the DNA template. LAMP was then performed as described above. The

LAMP results could be inspected visually with the naked eye in daylight by the addition of 1 μl of 1000 × SYBR Green I solution (Dingguo, Guangzhou, China) to the amplification products.

LAMP sensitivity analysis

DNA was firstly extracted from one and 100 individuals of *P. zeae*. Then, a 10-fold dilution of the DNA from one *P. zeae* or 100 *P. zeae* was obtained. Subsequently, the four concentrations of DNA were subjected to LAMP and conventional PCR separately. The LAMP reaction was performed as described above. The conventional PCR was conducted using the primers COI F and COI

Fig. 2 Bayesian consensus tree inferred from partial *COI* sequences among different species of *Pratylenchus* under the TVM + I + G model. Posterior probability values exceeding 50% are provided for the appropriate clades
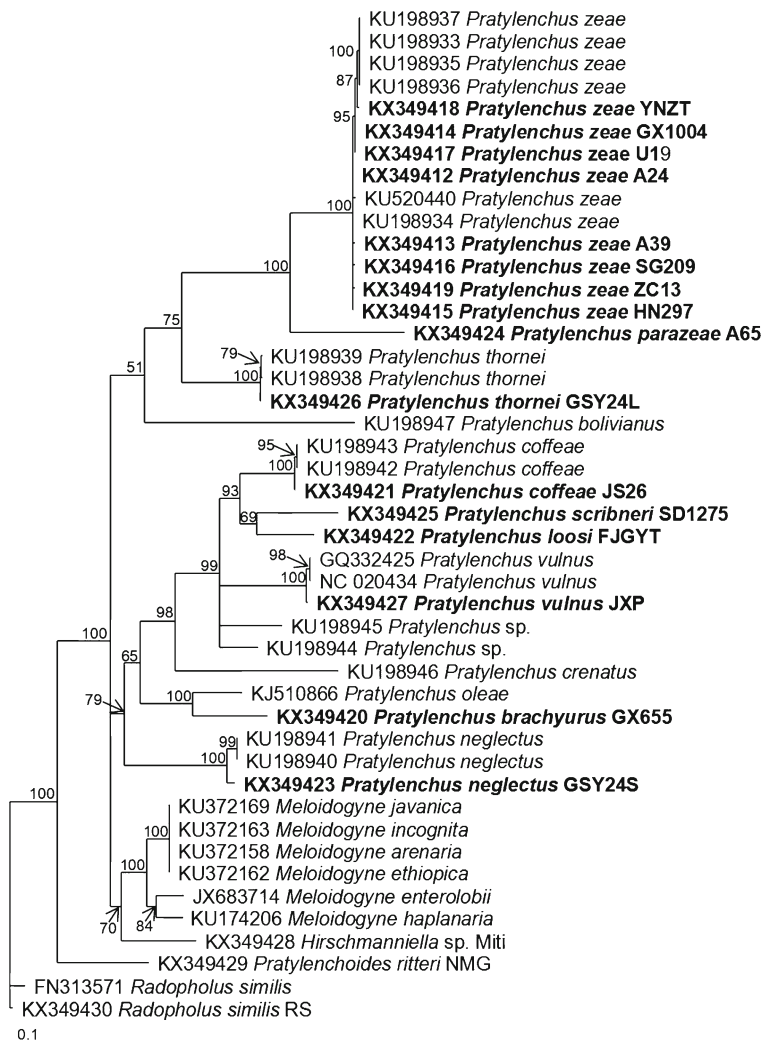
R, as described above. A negative control sample was also prepared, using double-distilled water instead of DNA template. Each sample was run in triplicate and the experiment was performed three times.

Results

Analysis of the partial sequences of mtDNA *COI*

PCR amplifications of the partial mtDNA *COI* regions indicated that all *Pratylenchus* species, *Pratylenchoides ritteri*, *Radopholus similis* and *Hirschmanniella* sp. could generate a single fragment of approximately 400 bp. The sequencing results showed that these *COI* amplified fragments varied from 414 to 417 bp. All

these sequences were deposited in GenBank and the accession numbers are listed in Table 1. When using *R. similis* as the outgroup taxon, the Bayesian consensus tree (Fig. 2) demonstrated that all the newly acquired *COI* sequences of *Pratylenchus* and some known *COI* sequences of *Pratylenchus* were clustered within the same clade. All the newly acquired *COI* sequence of the eight *P. zeae* populations and the six known *COI* sequences of *P. zeae* formed a strongly supported clade with 100% support and short branch differences, which was sister to the *P. parazeae* clade, with 100% support. Thus, the Bayesian consensus tree confirmed the identities of these newly acquired *COI* from *Pratylenchus*.

The genetic variation among *Pratylenchus* species was examined by aligning the mtDNA *COI* sequences. The results indicated that intraspecific variations among
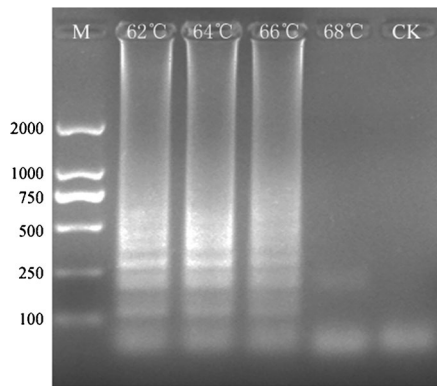
Fig. 3 Temperature optimization for the loop-mediated isothermal amplification reaction. The reaction was performed for 90 min at temperatures ranging from 62 °C to 68 °C. Lane M: Trans 2 K DNA Markers; CK: negative control (distilled water)

the mtDNA *COI* sequences of the eight *P. zeae* populations were 0.2–2.4% (1–7 bp) without gaps, and the interspecific variations between *P. zeae* and other *Pratylenchus* species were 25.4–35.1% (103–130 bp) with 2–19 gaps.

Optimization of LAMP reaction conditions

When LAMP was performed based on *P. zeae* DNA at 62–68 °C for 90 min, electrophoresis showed the typical ladder-like bands produced at 62–66 °C. The bands were most intense at 64 °C, and no ladder-like bands appeared in the negative control (Fig. 3). The fluorescence intensity of DNA extracted from 0.1 to

100 nematodes started to increase after 25 min of reaction time, and reached a plateau at 40 min in the DNA sample of 0.1 nematode (Fig. 4). Therefore, the most suitable conditions for the LAMP assay were determined as 64 °C for 40 min.

Specificity of the LAMP assay

Electrophoresis indicated that the products of the LAMP assay were digested completely using the restriction enzyme *Bam* HI (Fig. 5). In addition, the sequence of the 220-bp product amplified using the outer primers PZF3/PZB3 was determined. The sequence revealed a 100% match with the mtDNA *COI* sequence of original *P. zeae* population (data not shown). Based on the visual evaluation with SYBR Green I using DNA isolated from *P. zeae* and other nematodes, the *P. zeae* samples produced positive reactions, i.e. the SYBR Green I turned yellow-green, while the other nematode samples and the control double-distilled water sample produced negative reactions, i.e. the SYBR Green I remained brown or orange (Fig. 6). These results demonstrated that the LAMP assay was highly specific for *P. zeae*.

Sensitivity of the LAMP assay

The LAMP and conventional PCR assays, performed on different serial dilutions of the DNA extracted from *P. zeae*, demonstrated the increased sensitivity of the LAMP assay. The conventional PCR could
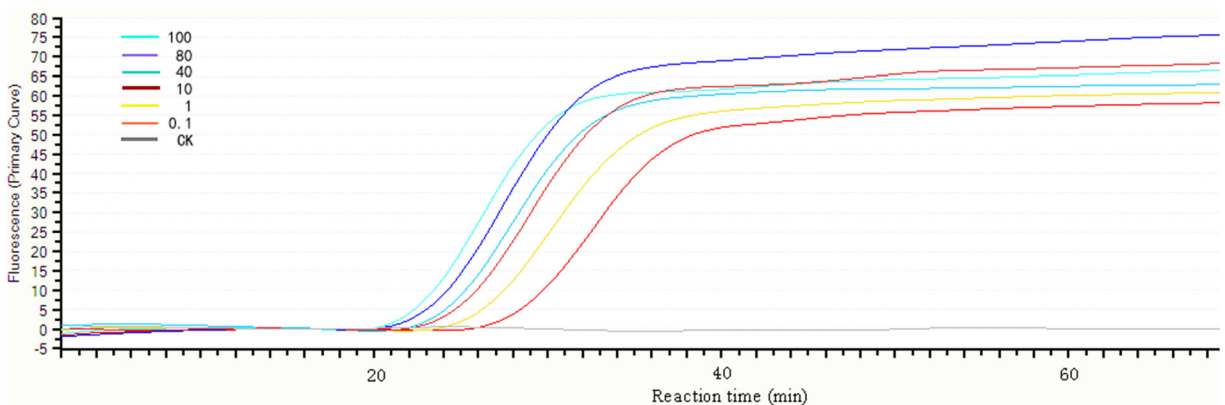

Fig. 4 Optimization of the duration of the loop-mediated isothermal amplification reaction. Real-time PCR was used to optimize the reaction time. Numbers on the x-axis represent the reaction time. Lines labeled 0.1 and 1 represent 10-fold and 1-fold dilutions of the DNA template from one nematode, respectively. Lines labeled 10, 40, 80 and 100 represent 10-fold, 2.5-fold, 1.25-fold, and 1-fold dilutions of the DNA template from 100 nematodes, respectively. CK: negative control (distilled water)
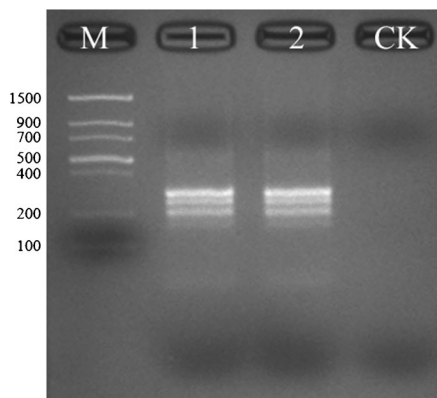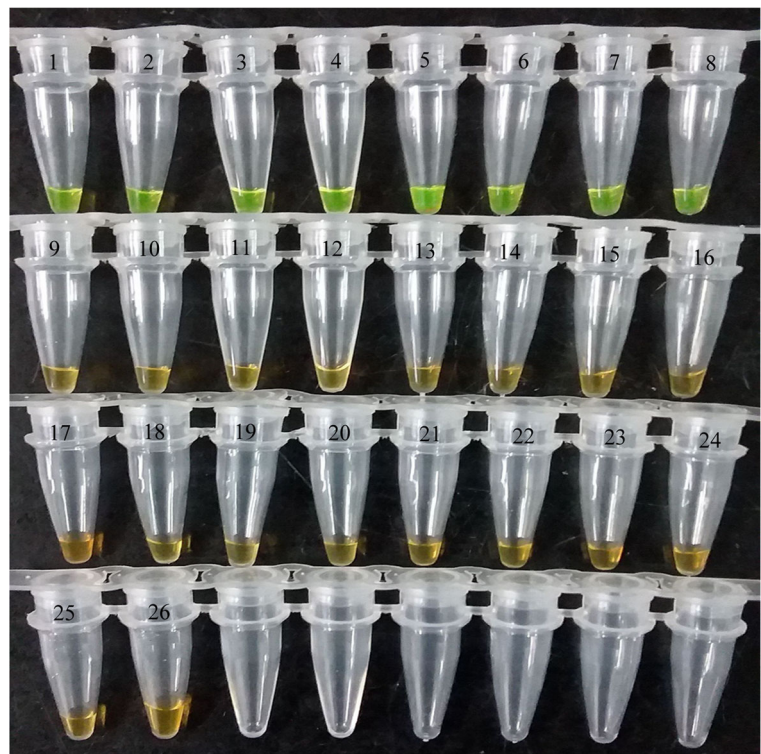
Fig. 5 Restriction enzyme analysis of the loop-mediated isothermal amplification products. Lanes 1 and 2 represent DNA templates from two different populations of *Pratylenchus zeae*. M: Trans DNA Marker II; CK: negative control (distilled water)

only detect the DNA equivalent to one individual nematode; however, the LAMP assay could detect approximately a tenth of the DNA extracted from a single nematode, i.e. 10-fold dilution of the template DNA from one nematode (Fig. 7). These demonstrated that the LAMP assay is 10 times more sensitive than conventional PCR.

Discussion

It is challenging to identify *Pratylenchus* species because of the high intraspecific variability and the inter-specific overlap of some morphological characteristics. Therefore, different PCR techniques, such as PCR amplification using species-specific primers, PCR-RFLP and qPCR based assay, have been used to identify and detect *Pratylenchus* spp. (Castillo and Vovlas 2007; Berry et al. 2008; Li and Zheng 2013; Wang et al. 2015). These conventional PCR techniques require an expensive thermal cycler to achieve amplification at different temperatures, which limits their routine use, particularly in certain developing countries and in the field (Lin et al. 2016). About ten years ago, a fast and inexpensive technology for sequence-specific DNA amplification, namely LAMP, was invented. LAMP does not require changes in temperature during the reaction, meaning that LAMP does not require expensive thermal cyclers. LAMP has been applied successfully to detect pathogenic microorganisms, including plant-parasitic nematodes (Fu et al. 2011; Duan et al. 2014; Lin et al. 2016). Several important plant-parasitic nematodes species, including *B. xylophilus*, *M. incognita*,

Fig. 6 Specificity of the loop-mediated isothermal amplification (LAMP) assay for *Pratylenchus zeae* and other nematodes. SYBR Green I was added to the LAMP products from: (tubes 1–8) eight different populations of *Pratylenchus zeae*; (tubes 9–25) populations of *P. parazeae*, *P. brachyurus*, *P. coffeae*, *P. loosi*, *P. neglectus*, *P. scribneri*, *P. thornei*, *P. vulnus*, *Hirschmanniella* sp., *Pratylenchoides ritteri*, *Tylenchorhynchus annulatus*, *Filenchus* sp., *Criconemoides* sp., *Helicotylenchus dihystera*, *Meloidogyne incongnita*, *M. javanica* and *Radopholus similis*; (tube 26) negative control (distilled water)
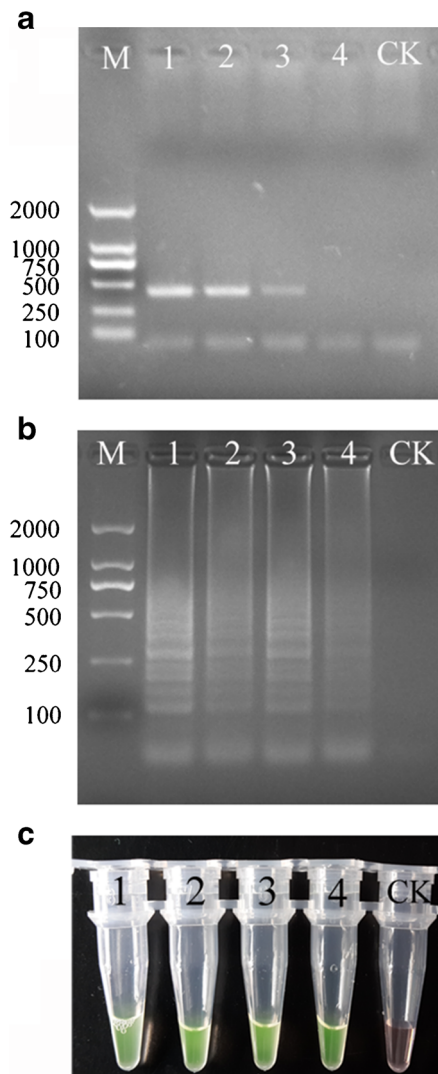
Fig. 7 Sensitivity of the loop-mediated isothermal amplification (LAMP) and convention PCR methods. **a** conventional PCR and **b** LAMP products. **c** LAMP products visualized with SYBR green I using natural daylight. Lane 1 or tube 1 represent the DNA template from 100 *Pratylenchus zeae*; lane 2 or tube 2 represent 10-fold dilution of the DNA template from 100 *P. zeae*; lane 3 or tube 3 represent the DNA template from one *P. zeae*; and lane 4 or tube 4 represent 10-fold dilution of the DNA template from one *P. zeae*; M: Trans 2 K DNA Markers; CK: negative control (distilled water)

M. enterolobii, *R. similis* and *T. semipenetrans*, have been detected using LAMP (Kikuchi et al. 2009; Niu et al. 2011, 2012; Peng et al. 2012; Lin et al. 2016). In this study, a new LAMP method, based on the partial mtDNA *COI* region, was developed successfully to detect *P. zeae*. This is the first application of LAMP to identify a root-lesion nematode. To develop the LAMP

method, eight *P. zeae* populations, one from the USA and seven from China, were included. The seven populations from China were collected from remote locations and isolated from several different hosts. It has been reported that *P. zeae* exhibits variation in the shape of its stylet knobs, from more or less rounded to markedly cup-shaped anteriorly; and in its tail shape, including almost pointed, narrowly rounded to subacute (Roman and Hirschmann 1969; Castillo and Vovlas 2007). All of these variations were observed in our populations. More recently, the intraspecific variation for mtDNA *COI* sequences of *P. zeae* from distant localities, including the USA, South Africa, Suriname and Kenya (Troccoli et al. 2016), was reported as 0–1.1%, which was lower than the 0.2–2.4% in our populations. Thus we believe that the *P. zeae* populations used in this study have enough diversity.

Some studies have shown that ITS-rDNA is an important marker to identify and differentiate nematode species (Gasser and Newton 2000; Powers et al. 1997; Subbotin et al. 2001). However, ITS sequences within a species or an individual species of some *Pratylenchus* might have extensive polymorphisms (Waeyenberge et al. 2009; De Luca et al. 2011), making it difficult to design species-specific primers. Wang et al. (2015) reported that intraspecific variations in the ITS sequences of *P. zeae* could reach 9.5%. In this study, the intraspecific variations in the ITS sequences of eight *P. zeae* populations were 3.0–9.1% (data not shown), while intraspecific variations for mtDNA *COI* sequences were only 0.2–2.4%. In addition, interspecific variations of the *COI* region between *P. zeae* and other *Pratylenchus* species ranged from 25.4–35.1%. Therefore, we decided to use the *COI* sequences to design LAMP primers to detect *P. zeae*. The results showed that the LAMP assay could detect *P. zeae* specifically and there was no cross-reaction with other *Pratylenchus* species. The mtDNA *COI* gene is one of the most popular markers for identifying animals (Derycke et al. 2010); however, only a few *COI* sequences have been obtained from *Pratylenchus* species (Sultana et al. 2013; Palomares-Rius et al. 2014; Troccoli et al. 2016). In the present study, sixteen *COI* sequences from eight *P. zeae* populations and eight other *Pratylenchus* species were obtained. Among these sequences, the *COI* sequences of *P. scribneri*, *P. loosi* and *P. brachyurus* were amplified for the first time. Intraspecific comparisons within *P. zeae* showed that the *COI* sequences varied by <0.03, while the interspecific comparisons between the

nine *Pratylenchus* species gave values >0.2. Moreover, the LAMP assay using the primers that targeted the *COI* sequence of *P. zeae* was highly specific and could distinguish *P. zeae* from other *Pratylenchus* species. These results suggested that the *COI* gene might provide good resolution at the *Pratylenchus* species level. More *Pratylenchus* samples could be included to confirm the utility of *COI* to identify *Pratylenchus* species.

In the present study, the LAMP assay was 10 times more sensitive than conventional PCR, which was similar to the results for the detection of *Tylenchulus semipenetrans* in soil (Lin et al. 2016). In addition, the results of the LAMP reaction could be observed visually by the addition of SYBR Green I staining to the products, which is not possible with conventional PCR. The LAMP is also rapid, detecting *P. zeae* in 40 min. In addition, LAMP only requires a constant-temperature bath of 64 °C. In conclusion, the developed LAMP method provides a good alternative to conventional PCR for the rapid, specific and simple detection of *P. zeae*. Hence, it will be useful for the rapid diagnosis of root lesion disease caused by *P. zeae*.

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