Multiplex PCR for the Simultaneous Identification and Detection of *Meloidogyne incognita*, *M. enterolobii*, and *M. javanica* Using DNA Extracted Directly from Individual Galls

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ABSTRACT

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Meloidogyne incognita, *M. enterolobii*, and *M. javanica* are the most widespread species of root-knot nematodes in South China, affecting many economically important crops, ornamental plants, and fruit trees. In this study, one pair of *Meloidogyne* universal primers was designed and three pairs of species-specific primers were employed successfully to rapidly detect and identify *M. incognita*, *M. enterolobii*, and *M. javanica* by multiplex polymerase chain reaction (PCR) using DNA extracted from individual galls. Multiplex PCR from all *M. incognita*, *M. enterolobii,* and *M. javanica* isolates generated two fragments of ≈500 and 1,000, 500 and 200, and 500 and 700 bp, respectively. The 500-bp fragment is the internal positive control fragment of rDNA 28S D2/D3 resulting from the use of the universal primers. Other *Meloidogyne* spp. included in this study generated only one fragment of ≈500 bp in size. Using this approach, *M. incognita*, *M. enterolobii,* and *M. javanica* were identified and detected using DNA extracted directly from individual galls containing the *Meloidogyne* spp. at various stages of their life cycle. Moreover, the percentage of positive PCR amplification increased with nematode development and detection was usually easy after the late stage of the second-stage juvenile. The protocol was applied to galls from naturally infested roots and the results were found to be fast, sensitive, robust, and accurate. This present study is the first to provide a definitive diagnostic tool for *M. incognita*, *M. enterolobii,* and *M. javanica* using DNA extracted directly from individual galls using a one-step multiplex PCR technique.

Root-knot nematodes are widely distributed in South China, a region containing tropical and subtropical areas, and these parasites cause substantial losses to agricultural crops. Previously, the nematodes *Meloidogyne incognita*, *M. javanica*, and *M. arenaria* were considered to be of major economic importance in this region (11,24,30,32). More recently, greater attention has focused on *M. enterolobii,* a species originally described from the Hainan Province in China (31). *M. enterolobii* has been found to damage ≈20 plant species in South China, including members of six plant families: Fabaceae, Cucurbitaceae, Solanaceae, Myrtaceae, Annonaceae, and Marantaceae (34). Furthermore, this species can reproduce on root-knot nematode-resistant tomato, pepper, and tobacco cultivars that carry the *Mi* gene (13,33). It has been suggested that *M. enterolobii* is a senior synonym of *M. mayaguensis* (20,30), which is important due to its wide geographical distribution, host range, and ability to overcome the resistance of tomato and pepper genotypes carrying the *Mi-1*, *N*, and *Tabasco* genes (7,10). During the last 3 years, ≈1,000 samples were collected in South China by our laboratory, and *M. incognita*, *M. enterolobii*, and *M. javanica* were the most common species, occurring in 62, 18, and 11% of the samples, respectively (*unpublished data*).

It is difficult to differentiate these *Meloidogyne* spp. from each other based on the morphology of their perineal patterns alone, because there is considerable morphological similarity between the species and there is also high intraspecies variation. The perineal pattern of *M. enterolobii* is rounded with high dorsal arches, which is very similar to that seen in *M. incognita* (13,33). Identification of *Meloidogyne* spp. using isozyme phenotyping is useful but this technique is limited to females. DNA analyses have been widely used for the identification of major *Meloidogyne* spp., including *M. incognita*, *M. javanica*, and *M. enterolobii* (=*M. mayaguensis*). For examples, *M. incognita*, *M. javanica*, *M. hapla*, *M. arenaria*, and *M. chitwoodi* can be discriminated using *COII* polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) (17); *M. incognita*, *M. javanica* and *M. arenaria* can be identified by PCR using sequence-characterized amplified region (SCAR) primers (15,36); and *M. incognita* or *M. javanica* can be identified by real-time PCR (3,29). More recently, numerous molecular techniques have been developed for identifying *M. enterolobii*, including analyses based on mitochondrial DNA (mtDNA) (5,6,28,30,34) and ribosomal DNA (rDNA) intergenic regions (IGS) (1,4), PCR amplifications using species-specific primers derived from IGS2 (14) and satellite DNA families (20), and random amplification of polymorphic DNA (RAPD)-PCR (27). These studies have demonstrated the effectiveness of identifying and differentiating *Meloidogyne* spp. using molecular techniques. However, a multiplex PCR method for the simultaneous identification of *M. incognita*, *M. enterolobii,* and *M. javanica* has not been reported previously.

For some plant nematodes, such as *Nacobbus* spp. (2) and *Bursaphelenchus xylophilus* (25), infected plant tissue has been used directly for the extraction of DNA for analysis of plant nematode species. More recently, *M. incognita* was detected by PCR *in planta* using template DNA derived from banana roots before gall formation (21). Approaches for extracting DNA directly from

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galls infected with *Meloidogyne* spp. are available but methodologies for detecting *Meloidogyne* spp. from such samples have yet to be reported (16).

In the present study, DNA from individual galls of tomato, cucumber, and water spinach, each known to contain *Meloidogyne* spp. at different stages of the nematode life cycle, were extracted to detect and identify *M. incognita*, *M. enterolobii,* and *M. javanica* via a one-step multiplex PCR. The aims of this present study were to (i) explore methods for extracting DNA from individual *Meloidogyne*-containing galls; (ii) test and develop primers to amplify *Meloidogyne* DNA from individual galls during different stages of the *Meloidogyne* life cycle for the detection and identification of *M. incognita*, *M. enterolobii,* and *M. javanica* by one-step multiplex PCR; and (iii) apply this new methodology to plant samples from the field.

MATERIALS AND METHODS

Nematode isolates and DNA extraction. All the nematodes listed in Table 1 were collected and morphologically identified in our laboratory. *Meloidogyne* spp. were confirmed by isozyme and molecular data, while other plant nematodes were confirmed using only molecular data. *Meloidogyne* populations were purified from single egg masses and reared on the appropriate plant hosts, except that *M. hapla*. *M. incognita*, *M. javanica*, *M. enterolobii,* and *M. arenaria* isolates were reared on tomato while *M. graminicola* and *M. graminis* were reared on rice and bermudagrass, respectively. DNA was extracted from individual nematodes (12) and this was used immediately for PCR or stored at –80°C until later use.

Primer design and selection. Using alignments obtained from GenBank, the *Meloidogyne* spp.-specific forward primer was designed based on a region conserved across *Meloidogyne* nematodes in the 28S rRNA D2D3 expansion domain (MF 5′-GGGGATGTTTGAGGCAGATTTG-3′; inside the D2 region), while the universal reverse primer was designed based on a region conserved across plant nematodes in the 28S rRNA D2D3 expansion domain (MR 5′-AACCGCTTCGGACTTCCACCAG-3′; inside the D3 region). *M. incognita*-specific primers (Mi-F 5′-GTGAGGATTCAGCTCCCCAG-3′ and Mi-R 5′-ACGAGGAA CATACTTCTCCGTCC-3′) and *M. javanica*-specific primers (Fjav 5′-GGTGCGCGATTGAACTGAGC-3′ and Rjav 5′-CAG GCCCTTCAGTGGAACTATAC-3′) that amplify SCAR markers were described by Meng et al. (15) and Zijlstra et al. (36), respectively. rDNA-IGS2 internal primers (Me-F 5′-AACTTTTG TGAAAGTGCCGCTG-3′ and Me-R 5′-TCAGTTCAGGCAGG ATCAACC-3′) were described by Long et al. (14) to amplify a specific sequence from *M. enterolobii*.

The Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast) was first used to examine the overall specificity of the primer pairs. Subsequently, these primer sets were tested separately in a PCR prior to multiplex analysis using DNA template from nematodes listed in Table 1. PCR amplification using the Fjav/Rjav primers was performed as described by Zijlstra et al. (36). PCRs with the Me-F/Me-R and the SCAR Mi-F/Mi-R primers were conducted according to the descriptions of Long et al. (14) and Meng et al. (15), respectively, with the following minor modifications: 94°C for 4 min; 35 cycles of 94°C for 30 s, 64°C for 30 s, and 68°C for 1 min; and a final 10-min incubation at 72°C. PCR using the new MF/MR primers were performed in a 25-µl reaction volume comprising 1 µl of template DNA from an individual nematode, 0.2 µM each primer, 2× buffer, 0.4 mM each dNTP, and 0.5 U of KOD FX polymerase (Toyobo, Shanghai, China). PCR amplifications were performed in a thermocycler (TaKaRa TP600; Shiga, Japan) using the following parameters: 94°C for 2 min; 35 cycles of 94°C for

TABLE 1. *Meloidogyne* spp. and other nematodes used to evaluate the species-specific primers used for multiplex polymerase chain reaction

Species	Isolate	Host	Geographical location
Meloidogyne incognita	GNq	Eggplant	Guangxi, China
M. incognita	ZCd	White gourd	Guangdong, China
M. incognita	HZ1	Tomato	Henan, China
M. incognita	GLs	Towel gourd	Guangdong, China
M. incognita	JS2	Unknown	Jiangsu, China
M. incognita	SSs	Towel gourd	Shandong, China
M. javanica	YZy1	Tobacco	Yunnan, China
M. javanica	GNs	Towel gourd	Guangxi, China
M. javanica	ZCf	Tomato	Guangdong, China
M. javanica	GNk	Towel gourd	Guangxi, China
M. javanica	SMj	Unknown	Guangdong, China
M. javanica	ZH8	Peanut	Henan, China
M. enterolobii	PYl	Pepper	Guangdong, China
M. enterolobii	HYz	Arrowroot	Hainan, China
M. enterolobii	GNj	Chrysanthemum	Guangxi, China
M. enterolobii	GNc	Crimson bottle brush	Guangdong, China
M. enterolobii	PYk	Bitter gourd	Guangdong, China
M. enterolobii	HNh	Black pepper	Hainan, China
M. arenaria	YZy2	Tobacco	Yunnan, China
M. arenaria	HK1	Palm	Hongkong, China
M. hapla	MH1	Tomato	Liaoning, China
M. hapla	HB2	Peanut	Hebei, China
M. graminicola	ZCs	Rice	Hainan, China
M. graminis	YJg	Bermudagrass	Guangdong, China
Ditylenchus destructor	D	Sweet potato	Shandong, China
Radopholus similis	Rs	Anthurium	Guangdong, China
Pratylenchus zeae	Pz	Sugarcane	Guangdong, China
Pratylenchus coffeae	Pc	Banana	Guangdong, China
Heterodera glycines	SCN	Soybean	Shenyang, China
Tylenchulus semipenetrans	T	Litchi	Guangdong, China
Hirschmanniella oryzae	Hir	Rice	Guangdong, China
Rotylenchulus reniformis	Rr	Cucumber	Anhui, China
Aphelenchoides sp.	Apo	Cucumber	Guangdong, China
Aphelenchus sp.	Aps	Cucumber	Guangdong, China
Cephalobus sp.	Cep	…	Guangdong, China

30 s, 64°C for 30 s, and 68°C for 1 min; and a final 5 min incubation at 72°C.

PCR products (5 μl) were separated on standard 2% agarose gels stained with Goldview (EB substation; Toyobo). A DS2000 DNA ladder (Dongsheng Biotech, Shanghai, China) was used to determine the molecular sizes of the bands. Band patterns were photographed under UV light using the Alphalmager (Alpha Innotech). All primers were synthesized by Invitrogen Biotech (Shanghai, China).

Multiplex PCR. The multiplex PCR was optimized by varying the reaction components and cycling conditions. The annealing temperature was increased from 58 to 66°C by single incremental temperature units. At the selected annealing temperature, the amplifications were carried out for different extension times (30, 60, 90, and 120 s) and at different concentrations of primers (0.02 to 0.4 μM) and dNTPs (0.02, 0.06, 0.1, 0.2, 0.3, 0.4, and 0.5 mM). Finally, optimal PCR amplifications were performed in 25-μl reaction volumes comprising 1 μl of template DNA from an individual nematode or DNA mixture (DNA from different *Meloidogyne* spp. were mixed in a ratios of 1:1); 0.1 μM MF and MR primers; 0.12 μM Me-F and Me-R primers; 0.16 μM Mi-F, Mi-R, Fjav, and Rjav primers; 2× buffer; 0.4 mM each dNTP; and 0.5 U of KOD FX polymerase. Multiplex-PCR amplification parameters were as follows: 94°C for 2 min; 35 cycles of 94°C for 30 s, 64°C for 30 s, and 68°C for 1 min; and a final 5-min incubation at 72°C.

DNA extraction from individual galls infected with *Meloidogyne* spp. and assessments of efficiency. Tomato plants infected with *Meloidogyne* spp. were collected from fields. The roots were flushed with water to remove soil and then washed with a 0.52% NaClO soap for 10 min. Individual root knots were obtained following three to five washes. DNA extraction was performed using the following four methods.

Method 1: DNA was extracted using a method modified from Stanton et al. (23). Individual galls were placed into 1.5-ml centrifuge tubes with 45 μl of 50 mM NaOH and ground with a self-made glass muller. Tubes were incubated at 95°C for 10 min. Following this incubation, 5 μl of 1 M Tris-HCl (pH 8.0) was added to each tube and then mixed thoroughly. The tubes were subsequently centrifuged at 10,000 × *g* for 1 min, and then the supernatant was transferred to PCR tubes for PCR amplification or stored at –20°C until later use.

Method 2: DNA was extracted following the protocol of Thomson and Henry, with minor modifications (26). Individual galls were placed in 1.5-ml centrifuge tubes with 50 μl of buffer A (100 mM Tris-HCl [pH 9.5], 1 M KCl, and 10 mM EDTA) and ground with a self-made glass muller. Tubes were incubated at 95°C for 10 min. Subsequently, the samples were centrifuged at 10,000 × *g* for 1 min, and then the supernatant was transferred to PCR tubes for PCR amplification or stored at –20°C until later use.

Method 3: DNA was extracted using a method modified from Iwahori et al. (9). Individual root knots were placed in PCR tubes and ground under liquid nitrogen. Then, to each tube was added 700 μl of lysis buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate, and proteinase K at 20 mg/ml) (TaKaRa). The tubes were incubated for 4 h at 50°C with occasional mixings. An equal volume (700 μl) of phenol was added and the tubes were centrifuged at 10,000 × *g* for 10 min. Then, the supernatants were transferred to new tubes and an equal volume of chloroform:isoamyl alcohol (24:1) was added. The tubes were subsequently centrifuged at 10,000 × *g* for 10 min, and then the supernatants were transferred to new tubes. The DNA solutions received 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 99.5% ethanol (–20°C) and were then kept at –20°C for 2 h, before centrifugation at 10,000 × *g* for 10 min. The precipitated DNA pellet was washed twice with 70% ethanol (–20°C), then dried and resuspended in 50 μl of Tris-EDTA (TE) buffer (10 mM Tris-HCl and 1 mM EDTA [pH 8.0]).

Method 4: DNA was extracted using the 3S Spin Genomic DNA Miniprep Kit v3.0 (Shenergy Biocolor, Shanghai, China) according to the manufacturer's protocol after grinding individual root knots in a 1.5-ml sterile tube under liquid nitrogen. Finally, 50 μl of TE buffer was used to elute the DNA.

The efficiency of DNA isolation from individual galls using the four methods described above was assessed by performing the same multiplex-PCR as above, except that 4 μl of template DNA from individual galls was used and 40 cycles of PCR amplification were performed.

Discrimination of *M. incognita*, *M. enterolobii,* and *M. javanica* in artificially inoculated roots. Twenty-five-day-old tomato (*Lycopersicon esculentum*), cucumber (*Cucumis sativus*), and water spinach (*Ipomoea aquatica*) seedlings were transplanted into pots 15 cm in diameter containing autoclaved soil and maintained under greenhouse conditions at 25°C. Two hundred *Meloidogyne* second-stage juveniles (J2), including six isolates each of *M. incognita*, *M. enterolobii,* and *M. javanica*, were inoculated onto the plant roots. Each host plant species was inoculated with water as a negative control. Nematodes were observed at 5, 10, 15, 20, 25, and 30 days after inoculation using the method of Feng (8) and photographed with a digital camera (Olympus CX21). DNA was extracted from individual galls at 5, 10, 15, 20, 25, and 30 days postinoculation and one section of healthy root (to serve as the negative control) using method 1 described above. Ten replicates of individual galls from each sample were selected for DNA extraction. The PCRs were the same as those employed for the multiplex PCR above, except that 40 cycles and 4 μl of template DNA were used. Finally, the percentage of positive PCR amplifications was calculated according to the following ratio: (numbers of galls showing positive PCR/numbers of total galls tested) × 100%.

Validation of *Meloidogyne* spp. identification in naturally infested roots. Thirty naturally infested root samples from eight different host plant species (tomato, eggplant, pepper, cucumber, bitter gourd, white gourd, pumpkin, and lettuce) were collected from Guangdong, Guangxi, Hainan, and Yunnan, China. Five appropriate galls of each sample were selected for the extraction of DNA and multiplex-PCR was performed as described above. To validate the protocol, the *Meloidogyne* spp. in each sample were identified based on their morphology and isozyme phenotype.

Sequencing. To verify whether the PCR products obtained from the primers were the expected sequences, the differently sized bands resulting from the PCRs were separately excised and purified using a universal DNA gel purification kit (Tiangen Biotech, Beijing). The fragments were cloned individually into the pMD 18-T vector and sequenced. Sequencing reactions were run on an ABI3730 Stretch DNA sequencing system (Applied Biosystems, Foster City, CA). Mega 4 or BLAST was used to conduct homology comparisons on the sequences generated.

RESULTS

PCR primer specificity. BLAST analysis of the primer sequences indicated that the *Meloidogyne* forward-specific primer MF exhibited 100% matches with *M. hispanica* (GQ375158 and EU443606 to EU443608), *M. thailandica* (EU364890), *M. arenaria* (AF435803, EU364889, U42339, and U42342), *M. paranaensis* (AF435798 to AF435800), *M. konaensis* (AF435797), *M. incognita* (AF43579), *M. silvestris* (EU570214), *M. graminicola* (HQ420904, HQ420905, and AF435793), *M. fallax* (FN429017), *M. dunensis* (EF612712), *M. exigua* (AF435795, AF435796, and AF435804), *M. chitwoodi* (AF435802), and *M. trifoliophila* (AF435801) sequences. The universal reverse primer MR exhibited 100% matches with different groups of nematodes including plant nematodes, freeliving nematodes, and entomopathogenic

nematodes (accession numbers were omitted). *M. enterolobii*-specific primer pairs Me-F/Me-R only matched *M. enterolobii* and *M. mayaguensis* (=*M. enterolobii*) (GQ395524 to GQ39556 and DQ641506). *M. incognita*-specific primer pairs Mi-F/Mi-R and *M. javanica*-specific primer pairs Fjav/Rjav showed that there were no sequences exhibiting high matching.

Primer specificity was further examined by PCR using genomic DNA extracted separately from the nematodes listed in Table 1. All *Meloidogyne* isolates generated one fragment of ≈500 bp using the *Meloidogyne*-universal MF/MR primers, *M. incognita* generated a fragment of ≈1,000 bp using the Mi-F/Mi-R primers, *M. javanica* generated a fragment of ≈700 bp using the Fjav/Rjav primers, and *M. enterolobii* gave a fragment of ≈200 bp using the Me-F/Me-R primers. No band was observed in the other PCRs. The specificity of all PCR products was confirmed by DNA sequencing. Sequencing results indicated that the ≈500-bp fragment was, in fact, 478 bp for *M. enterolobii*, *M. incognita*, *M. javanica*, and *M. arenaria*; 481 bp for *M. hapla*; and 485 bp for *M. graminicola* and *M. graminis*. The ≈1,000-bp fragment was 955 bp for *M. incognita*, while the 200-bp fragment was 236 bp for *M. enterolobii* and the 700-bp fragment was 657 or 660 bp for *M. javanica*. These sequences have been deposited in GenBank with sequence accession numbers JN005834 to JN005875.

Multiplex PCR. The multiplex-PCR conditions were optimized as described above. Optimal multiplex PCR revealed that each *M. incognita* isolate generated two fragments of ≈500 and 1,000 bp, each *M. javanica* isolate gave fragments of ≈500 and 700 bp, and each *M. enterolobii* isolate generated fragments of ≈500 and 200 bp. All other *Meloidogyne* spp. gave only one fragment of ≈500 bp in length. The mixture of *M. incognita* and *M. javanica* produced three fragments of ≈500, 700, and 1,000 bp. The mixture of *M. incognita* and *M. enterolobii* produced three fragments of ≈200, 500, and 1,000 bp. The mixture of *M. enterolobii* and *M. javanica* produced three fragments of ≈200, 500, and 700 bp. Finally, the mixture of *M. incognita*, *M. enterolobii,* and *M. javanica* produced four fragments of ≈200, 500, 700, and 1,000 bp. No bands were observed from the non-*Meloidogyne* spp. tested (Fig. 1).

DNA extraction efficiency applying different methods. No bands were observed with template DNA extracted from individual galls when method 2 was used, whereas the other three methods yielded DNA template sufficient for PCR amplifications with the four pairs of primer. Indeed, bands were all clearly visible using template DNA extracted with these three methods (Fig. 2).

Discrimination of *M. incognita*, *M. enterolobii,* and *M. javanica* in artificially inoculated roots. Five days after inoculation, small galls were visible with the naked eye, and the nematodes were early parasitic J2s. Root galls enlarged quickly and turned from spindle shaped to round in appearance, while the nematode developed from vermiform to sausage-shaped and eventually to pear-shaped, undergoing late J2 (10 days), third- or fourth-stage juvenile (15 days), and female stages (20 days). At 25 days, eggs were produced, and these were apparent after 30 days.

DNA from individual cucumber galls at different stages of the *Meloidogyne* life cycle was amplified successfully using the four pairs of primers MF/MR, Mi-F/Mi-R, Fjav/Rjav, and Me-F/Me-R. The DNA amplification banding patterns achieved from extracting the DNA directly from individual galls (Fig. 3A) were consistent with those obtained from genomic DNA purified from *Meloidogyne* isolates. Thus, two fragment bands of ≈500 and 1,000 bp were detected for the *M. incognita* isolates, fragments of 500 and 700 bp were observed for the *M. javanica* isolates, and fragments of 500 and 200 bp were obtained for *M. enterolobii*. The multi-

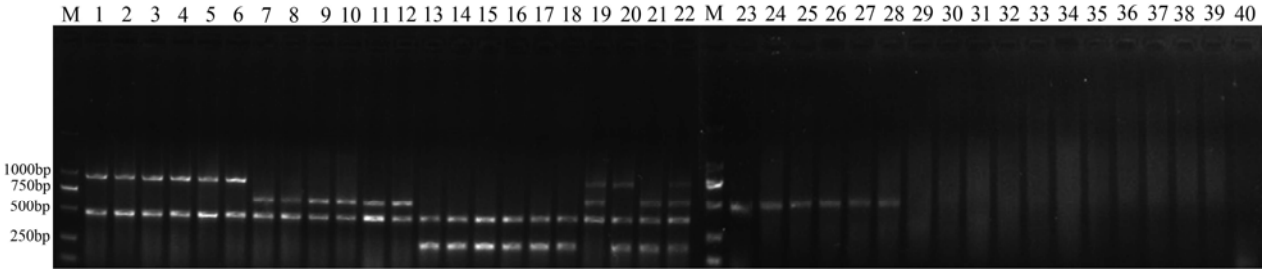

Fig. 1. Agarose gel of multiplex polymerase chain reaction products. M: DS2000 DNA marker; lanes 1 to 6: *Meloidogyne incognita* isolates GNq, ZCd, HZ1, GLs, JS2, and SSs; lanes 7 to 12: *M. javanica* isolates GNs, YZy1, ZCf, GNk, SMj, and ZH8; lanes 13 to 18: *M. enterolobii* isolates PY1, Hyz, GNj, GNc, PYk, and HNh; lane 19: mixture of *M. incognita* and *M. javanica*; lane 20: mixture of *M. incognita* and *M. enterolobii*; lane 21: mixture of *M. javanica* and *M. enterolobii*; lane 22: mixture of *M. incognita*, *M. javanica*, and *M. enterolobii*; lanes 23 and 24: *M. arenaria* isolates YZ2 and HK1; lanes 25 and 26: *M. hapla* isolates MH1 and HB2; lane 27: *M. graminicola*; lane 28: *M. graminis*; lane 29: *Cephalobus* sp.; lane 30: *Aphelenchoides* sp.; lane 31: *Aphelenchus* sp.; lane 32: *Heterodera glycine*; lane 33: *Ditylenchus destructor*; lane 34: *Tylenchulus semipenetrans*; lane 35: *Radopholus similis*; lane 36: *Hirschmanniella oryzea*; lane 37: *Pratylenchus coffea*; lane 38: *Pratylenchus zeae*; lane 39: *Rotylenchulus reniformis*; lane 40: water control.

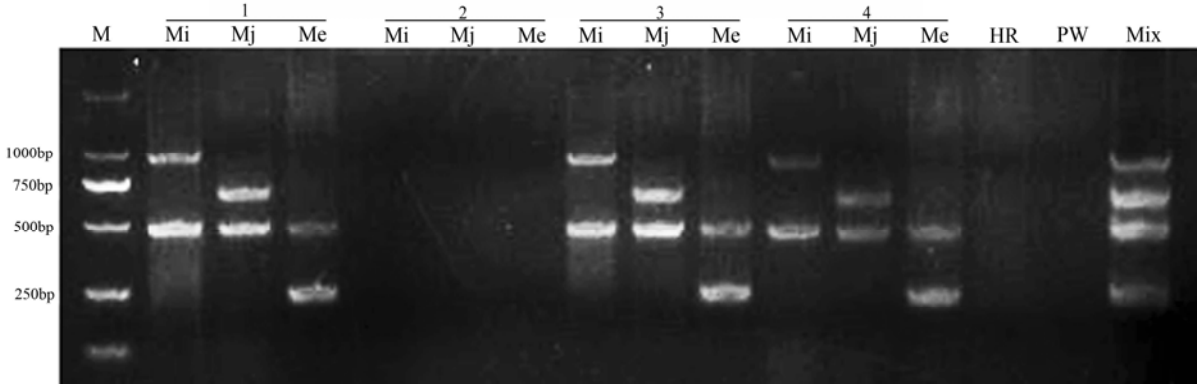

Fig. 2. Multiplex polymerase chain reaction results from individual galls using different DNA preparation methods (1 to 4). M: DS2000 DNA marker; Mi: *Meloidogyne incognita*; Mj: *M. javanica*; Me: *M. enterolobii*; HR: negative control of root DNA without nematodes; PW: negative control without DNA template; Mix: positive control with a mixture of bulk DNA from *M. incognita*, *M. javanica*, and *M. enterolobii*.

plex PCR test results were the same for individual galls taken from tomato and water spinach plants, except that, at 5 days after inoculation with *M. incognita*, no fragments were observed from water spinach galls (Fig. 3B and C). The percentage of positive PCR amplifications using DNA extracted directly from individual galls found on the three plant species during any stage of the *Meloidogyne* sp. life cycle are shown in Table 2. Of the galls containing *M. enterolobii* isolates, 100% generated two fragments at all stages of the nematode life cycle in all three of the crop plants. For the *M. javanica* isolates, at 10 to 30 days postinoculation of cucumber and tomato, 100% of the galls showed positive PCR amplification while, in water spinach, 100% of the galls gave successful amplifications at 15 to 30 days after inoculation. For the *M. incognita* isolates, 100% of the galls gave positive PCR amplification at 20 to 30 days postinoculation.

Detection of *Meloidogyne* spp. in naturally infested roots. The multiplex PCR was evaluated for its ability to detect and identify *Meloidogyne* spp. in 30 root samples from the field. In 20 cases, all five individual galls from each sample exhibited positive PCR amplifications; in six of the samples, four individual galls

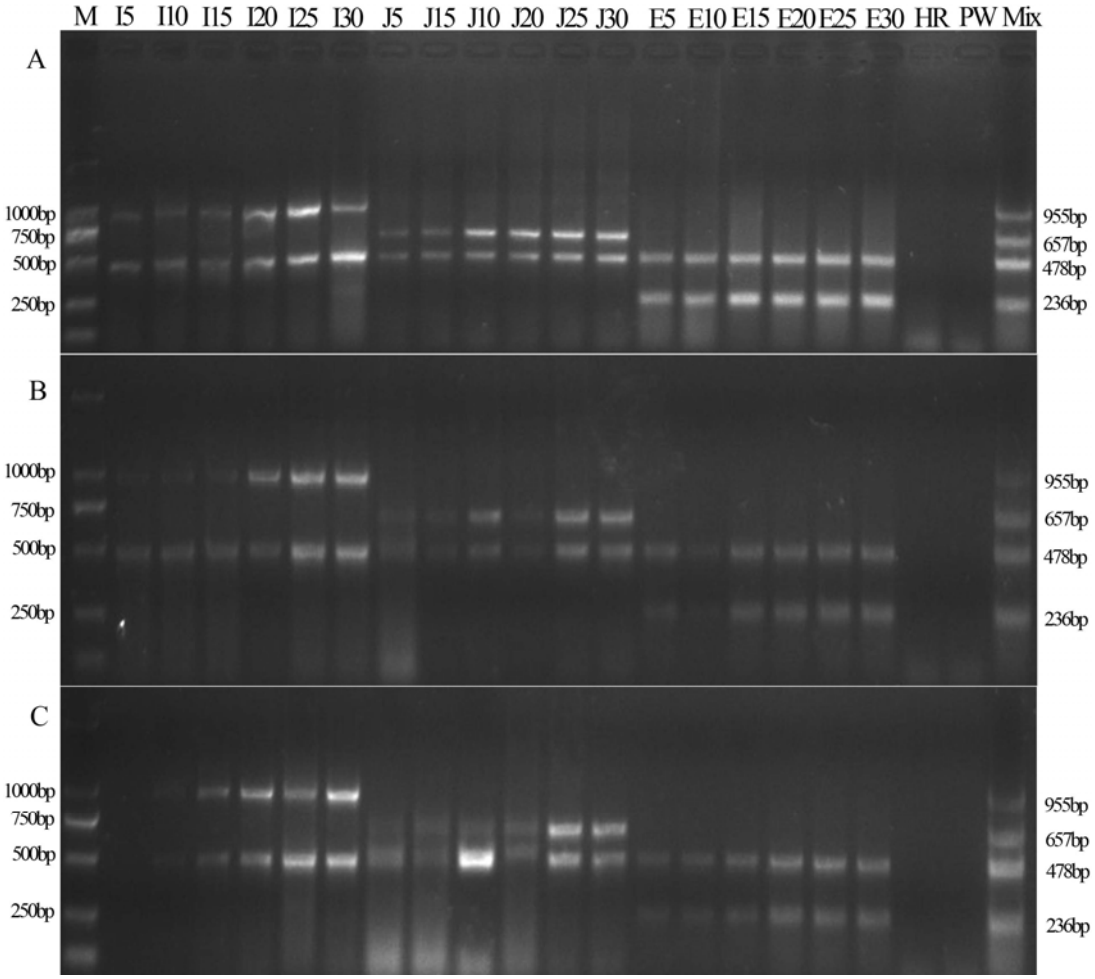

Fig. 3. Agarose gel of multiplex polymerase chain reaction products using DNA extracted from individual galls from **A,** cucumber; **B,** tomato; and **C,** water spinach inoculated with different *Meloidogyne* spp. M: DS2000 DNA marker; I5, I10, I15, I20, I25, and I30 indicate galls from plants at 5, 10, 15, 20, 25, and 30 days after inoculation with *Meloidogyne incognita*, respectively; J5, J10, J15, J20, J25, and J30 indicate galls from plants at 5, 10, 15, 20, 25, and 30 days after inoculation with *M. javanica*, respectively; E5, E10, E15, E20, E25, and E30 indicate galls from plants at 5, 10, 15, 20, 25, and 30 days after inoculation with *M. enterolobii*, respectively; HR: negative control of healthy roots without nematodes; PW: negative control with no DNA added; Mix: positive control with a mixture of bulk DNA from *M. incognita*, *M. javanica*, and *M. enterolobii*.

TABLE 2. Percentage of positive polymerase chain reaction (PCR) amplifications using DNA extracted directly from individual galls at different days after inoculation (DAI) of the *Meloidogyne* spp.

	Positive PCR (%)[a]								
	CRG with			TRG with			WRG with		
DAI	*Mi*	*Mj*	*Me*	*Mi*	*Mj*	*Me*	*Mi*	*Mj*	*Me*
5	20	63.3	100	33.3	60	100	0	23.3	100
10	71.7	100	100	78.3	100	100	55	73.3	100
15	88.3	100	100	80	100	100	68.3	100	100
20	100	100	100	100	100	100	100	100	100
25	100	100	100	100	100	100	100	100	100
30	100	100	100	100	100	100	100	100	100

[a] Percentage of positive PCR = (numbers of galls showing positive PCR/numbers of total galls tested) × 100%. CRG = root gall of cucumber, TRG = root gall of tomato, WRG = root gall of water spinach, *Mi* = *M. incognita*, *Mj* = *M. javanica*, and *Me* = *M. enterolobii.*

gave positive PCR amplifications; while in the remaining four samples, three individual galls gave positive PCR amplifications (Table 3). Overall, 90.7% of the galls showed positive PCR amplifications, and all 30 samples contained a root-knot nematode species or mixture of species. Based on the multiplex-PCR amplification patterns, 18 samples were identified as containing *M. incognita*, five samples contained *M. javanica*, four samples contained *M. enterolobii*, one sample had mixed populations of *M. incognita* and *M. javanica*, and another sample had mixed populations of *M. incognita* and *M. enterolobii*. All these results were confirmed using standard morphological and isozyme methodologies, except for one tomato sample (from Shenzhen, Guangdong). In this sample, the PCRs from all five galls selected gave fragments of ≈500 and 700 bp (indicating the presence of *M. javanica*) but morphological observations and isozyme assessments indicated that there were, in fact, mixed populations of *M. incognita* and *M. javanica*. Finally, the remaining sample (a lettuce sample from Zengcheng, Guangdong) gave one fragment of ≈500 bp from all five galls, and the infestation was identified as *M. arenaria* based on morphology and isozyme assay.

DISCUSSION

For multiplex PCR using more than one primer set, it is important to determine the specificity and sensitivity of the primers and optimize the PCR reaction conditions (22). In this study, serial PCRs were tried using different annealing temperatures, primer concentration ratios, and primer pairs, and this eventually led to the determination of the best program and primers for the multiplex PCR. In the multiplex PCR, a fragment of ≈500 bp was amplified from all galls containing *Meloidogyne* spp. using the MF/MR primers as was expected. This also confirmed the presence of template nematode DNA that had been extracted from individual galls. PCRs using the specific primer pairs Mi-F/Mi-R, Fjav/Rjav, and Me-F/Me-R produced DNA fragments of 955, 657 or 660, and 236 bp, respectively, which corresponded to the galls containing *M. incognita*, *M. javanica*, and *M. enterolobii*, respectively. Using these specific primer pairs, no fragments were generated from galls containing any other *Meloidogyne* spp. These results indicated the absence of mutual interference in the PCR amplification, that the primer pairs were species-specific and sensitive, and that the PCR conditions were ideal for the rapid detection and discrimination of *M. incognita*, *M. javanica*, and *M. enterolobii* from individual plant galls. This approach requires only one PCR reaction using DNA extracted directly from an individual gall, meaning that it is simple, rapid, and reliable.

M. incognita, M. enterolobii, and *M. javanica* have become the most important root-knot nematode species in South China (*unpublished data*). In this study, multiplex PCR using species-specific primers performed on DNA extracted from individual root-knot galls was employed to rapidly detect and identify these three species of nematodes. Though there are many molecular techniques for detecting these *Meloidogyne* spp. (1,3–6,15,17, 20,27–30,36), none of these other methods identify and discriminate these three important species simultaneously by multiplex PCR, a method that has been used to differentiate other *Meloidogyne* spp. (19,35). Moreover, these techniques were developed using DNA extracted from nematode specimens isolated from plant tissues or soils but not using DNA extracted directly from individual galls. Direct molecular detection of plant-parasitic

TABLE 3. Identification results of *Meloidogyne* spp. by multiplex polymerase chain reaction (PCR) using DNA extracted from individual galls from naturally infested root samples[a]

Geographical origin	Host	Single gall amplification pattern[b]					Confirmation[c]
		Gall 1	Gall 2	Gall 3	Gall 4	Gall 5	
Panyu, Guangdong, China	Eggplant	3	3	3	3	3	*Me*
Panyu, Guangdong, China	Pepper	3	3	3	3	3	*Me*
Panyu, Guangdong, China	Bitter gourd	3	3	3	3	3	*Me*
Nama, Guangxi, China	Cucumber	1	1	1	1	1	*Mi*
Nama, Guangxi, China	Bitter gourd	1	1	2	2	1	*Mi/Mj*
Nama, Guangxi, China	Eggplant	1	1	0	1	1	*Mi*
Nama, Guangxi, China	Pepper	1	1	1	1	0	*Mi*
Zengcheng, Guangdong, China	Tomato	2	2	2	2	2	*Mj*
Zengcheng, Guangdong, China	Lettuce	4	4	4	4	4	*Ma*
Zengcheng, Guangdong, China	White gourd	1	1	1	1	1	*Mi*
Guangzhou, Guangdong, China	Tomato	1	1	1	1	1	*Mi*
Guangzhou, Guangdong, China	Bitter gourd	1	1	1	1	1	*Mi*
Guangzhou, Guangdong, China	Cucumber	1	1	1	1	1	*Mi*
Guangzhou, Guangdong, China	Pepper	0	1	1	1	1	*Mi*
Shenzhen, Guangdong, China	Cucumber	1	1	1	1	1	*Mi*
Shenzhen, Guangdong, China	Eggplant	1	1	0	0	1	*Mi*
Shenzhen, Guangdong, China	Cucumber	1	1	1	1	1	*Mi*
Shenzhen, Guangdong, China	Tomato	2	2	2	2	2	*Mi/Mj*
Hainan, China	Tomato	3	1	3	3	3	*Mi/Me*
Panyu, Guangdong, China	Bitter gourd	2	2	2	2	2	*Mj*
Panyu, Guangdong, China	Eggplant	1	0	1	1	0	*Mi*
Panyu, Guangdong, China	Pumpkin	1	1	1	1	1	*Mi*
Panyu, Guangdong, China	White gourd	1	1	0	1	1	*Mi*
Shaoguan, Guangdong, China	Eggplant	1	1	1	0	1	*Mi*
Shaoguan, Guangdong, China	Eggplant	2	0	2	2	2	*Mj*
Shaoguan, Guangdong, China	Pepper	0	2	2	0	2	*Mj*
Shaoguan, Guangdong, China	Bitter gourd	1	1	1	1	1	*Mi*
Shaoguan, Guangdong, China	Pepper	3	3	3	3	3	*Me*
Yuanmou, Yunnan, China	Pepper	1	1	0	1	0	*Mi*
Yuanmou, Yunnan, China	Tomato	1	1	1	1	1	*Mi*

[a] Results were confirmed by morphology and isozyme phenotypes.

[b] PCR scale: 0 indicates a single gall giving no fragments, 1 indicates a single gall giving two fragments of ≈500 and 1,000 bp (showing the presence of *M. incognita*), 2 indicates a single gall giving two fragments of ≈500 and 700 bp (showing the presence of *M. javanica*), 3 indicates a single gall giving two fragments of ≈500 and 200 bp (showing the presence of *M. enterolobii*), and 4 indicates a single gall giving one fragment of 500 bp (showing the presence of other *Meloidogyne* spp. except *M. incognita*, *M. javanica*, and *M. enterolobii*).

[c] Morphology and isozyme confirmation; *Mi* = *M. incognita*, *Mj* = *M. javanica*, *Me* = *M. enterolobii*, and *Ma* = *M. arenaria*.

nematodes using template DNA from plant tissues containing nematodes may be useful for early detection but only a few cases of such an approach have been reported to date. Takeuchi et al. (25) developed a nested PCR-based method to identify the pine wood nematode, *B. xylophilus*, while Atkins et al. (2) refined a molecular diagnostic approach to detect *Nacobbus* spp. in soil and potato tubers. Methods for the early detection of *Meloidogyne* spp. directly from banana roots before gall formation have also been reported (21). However, due to the difficulty in collecting such non-gall roots for diagnosing *Meloidogyne* spp. infection, in practice, we aimed to diagnose *Meloidogyne* spp. from individual galls using a molecular approach. A one-step multiplex PCR method was designed and employed successfully to identify and discriminate the three tropical and subtropical nematode species *M. incognita, M. enterolobii,* and *M. javanica* from individual nematodes as well as individual galls. This is the first time that such a method has been reported and this present study may help in the design of new tests for the practical detection of other root-knot nematodes. In the present study, we tested the four methods to obtain effective template DNA from individual galls. Expected band sizes were produced using three of these methods; however, fewer reagents and time were required for method 1 compared with methods 3 and 4 and, therefore, method 1 was used throughout to provide DNA easily and quickly from individual galls.

In order to determine which life stages of *Meloidogyne* spp. could be detected effectively in the gall by PCR, PCRs were performed using template DNA from individual galls at 5, 10, 15, 20, 25, and 30 days after inoculation, which spans the entire life cycle of the nematode. The results indicated that *Meloidogyne* spp. could be detected at any stage of the life cycle in galls of cucumber and tomato. In water spinach, only at 5 days after inoculation was *M. incognita* not detected. Nevertheless, the percentage of positive PCR amplifications increased with *M. incognita* development and it could be detected more easily after the late stage of the second-stage juvenile. In the present study, *M. incognita*-specific primers were designed based on RAPD fragments from Meng et al. (15). The fragment was sequenced by us and subjected to BLASTN search against the National Center for Biotechnology Information database but no homologous sequences were found. Thus, the sequence may be a low-copy fragment in *M. incognita* and water spinach roots may contain high levels of PCR inhibitors relative to the target DNA at the early stages of J2, and these reasons could have led to the failure in detecting *M. incognita* in the galls 5 days after inoculation. The *Meloidogyne* universal primers and *M. enterolobii*-specific primers were designed from multicopy sequences of nuclear ribosomal genes, and 100% of galls selected gave successful PCR amplifications. Using real-time PCR or changing the primers for those designed against multicopy DNA sequences, such as rDNA or mtDNA, may increase the sensitivity of the assay. Unfortunately, the rDNA of *M. incognita* and *M. javanica* is so homologous that rDNA-based differentiation is impossible (4,18, 37). In addition, it is also difficult to design primers to discriminate *M. incognita* and *M. javanica* based on mtDNA using a one-step PCR approach because the mtDNA nucleotide sequences of some *M. incognita* populations are highly similar (>99%) to those of *M. javanica* (*unpublished data*). In this study, two primer pairs from RAPD fragments were chosen by us for differentiating *M. incognita* and *M. javanica* simultaneously.

M. incognita, *M. enterolobii,* and *M. javanica* were detected successfully in individual galls using the same PCR conditions at 5, 10, 15, 20, 25, and 30 days after inoculation in artificially inoculated roots of plant species representing divergent plant families, including cucumber (Cucurbitaceae), tomato (Solanaceae), and water spinach (Convulvulaceae). Moreover, the validity of the PCR detection system was confirmed in naturally infested roots of eight plant species. In 29 of the 30 natural samples, PCR detection gave satisfactory results. In only one case did the PCR method fail to identify that galls were infected by mixed populations of *M. incognita* and *M. javanica*; rather, the PCR detected infection by *M. javanica* alone. If more galls in the sample had been collected for PCR detection, perhaps this problem would have been overcome, though more tests are needed to confirm this. In conclusion, this one-step multiplex PCR method can be applied to different crops infected with *Meloidogyne* spp., which will reduce the time needed to diagnose these nematodes.

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