

RESEARCH/INVESTIGACIÓN

FIRST REPORT AND MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *MELOIDOGYNE INCOGNITA* FROM *RADERMACHERA SINICA* IN CHINA

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ABSTRACT

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Studies were conducted to characterize morphological and molecular profiles of *Meloidogyne incognita* from *Radermachersa sinica* in China. Affected plants displayed poor growth and significant galling on roots. Serious infections resulted in formation of large numbers of galls, wilted leaves, root rot, and then plant death. The female vulval region was typically surrounded by a gelatinous egg mass with many eggs. Morphometrics and female perineal pattern of the isolate fit within the ranges for *M. incognita*. A single 1200- and 399-bp band produced by sequence-characterized-amplified-region markers with *M. incognita*-specific primers Finc/Rinc and Inc-K14F/Inc-K14R matched those reported for *M. incognita*. The sequences of partial rDNA 18S gene and 28S D2/D3 expansion segment were 99% similar to other *M. incognita* isolates and three major tropical species, namely *M. arenaria*, *M. javanica*, and *M. floridensis*. This is the first report of *M. incognita* in *Radermachersa sinica*.

Key words: 18S small subunit rDNA, *Meloidogyne incognita*, PCR, *Radermachersa sinica*, root-knot nematode, 28S large subunit rDNA, taxonomy.

RESUMEN

Zeng, Y., W. Ye, and J. Kerns. 2014. Primer informe y caracterización morfológica y molecular de *Meloidogyne incognita* en *Radermachersa sinica* en China. *Nematropica* 44:118-129.

Se realizaron estudios para caracterizar los perfiles morfológico y molecular de *Meloidogyne incognita* asociado a *Radermachersa sinica* en China. Las plantas afectadas mostraron crecimiento pobre y un agallamiento significativo en las raíces. Las infecciones más graves mostraron gran número de agallas, hojas marchitas, necrosis en las raíces y muerte de plantas. Típicamente, la región vulvar de la hembra estaba rodeada de una masa gelatinosa con muchos huevos. La morfometría y el patrón perineal de las hembras del aislado se ajustaron a los rangos establecidos para *M. incognita*. Una banda de 1200 pb y otra de 399 pb producidas con marcadores SCAR usando los cebadores específicos para *M. incognita*, Finc/Rinc and Inc-K14F/Inc-K14R, coinciden con aquellos citados para *M. incognita*. La secuencia parcial del gene rDNA 18S y del segmento de expansión 28S D2/D3, fueron 99% similar a otros aislados de *M. incognita* y a tres especies tropicales: *M. arenaria*, *M. javanica* y *M. floridensis*. Este es el primer informe de *M. incognita* asociado con *Radermachersa sinica*.

Palabras clave: *Radermachersa sinica*, *Meloidogyne incognita*, rADN subunidad pequeña 18S, rADN subunidad grande 28S, PCR, nematodos formadores de agallas en raíz, taxonomía.

INTRODUCTION

Root-knot nematodes (RKN) (*Meloidogyne* spp.) are widespread and very destructive pests and cause substantial yield losses mainly in tropical and subtropical areas. Root-knot nematodes have a broad host range and are obligate parasites of the roots of thousands of plant species including monocotyledonous and dicotyledonous herbaceous and woody plants. Root-knot nematodes are considered as one of the three most economically damaging genera of plant-parasitic nematodes on horticultural and field crops. A full account of an early history of genus is given by Hunt and Handoo (2009). Approximately 100 species, including four major species [*M. incognita* (Kofoid and White, 1919) Chitwood, 1949; *M. arenaria* (Neal, 1889) Chitwood, 1949; *M. javanica* (Treub, 1885) Chitwood, 1949; and *M. hapla* Chitwood, 1949], have been described (Skantar *et al.*, 2008; Hunt and Handoo, 2009; Perry *et al.*, 2009; Lunt *et al.*, 2014). Among those, *M. incognita* is one of the most widespread and economically important species. It can be extremely damaging and causes serious economic losses to many agriculturally important plants and ornamentals. Typically, morphological, isozymatic, and molecular characters are collectively used for identification of RKN species. DNA markers, including ribosomal DNA small subunit (SSU) (Powers, 2004), large subunit (LSU) D2/D3 expansion segments (Chen *et al.*, 2003; Palomares-Ruis *et al.*, 2007), internal transcribed spacer (ITS) (Powers and Harris, 1993), mitochondrial DNA (Jeyaprakash *et al.*, 2006), and sequence-characterized-amplified regions (SCAR) (Zijlstra *et al.*, 2000; Randig *et al.*, 2002) have been employed to identify *Meloidogyne* species.

Radermachera sinica (Hance) Hemsley (also called “China Doll”, “Serpent Tree”, or “Emerald Tree”) is an ornamental evergreen tree in the family Bignoniaceae native to mainland China and Taiwan. It is used as a popular indoor house plant in many countries such as China, Japan, Bhutan, India, Myanmar, and Vietnam. Recently, it has been extensively planted in Guangdong, Guangxi, Guizhou, and Yunnan provinces in China. During a survey of nematodes in 2013 in Guangzhou, China, we observed many galls from roots of affected *R. sinica* seedlings growing in an artificial soil mixture. Galls were collected for isolation and identification of potential pathogen(s). A RKN with several stages was isolated from the galls. Morphology of the RKN was performed. Molecular analyses of the RKN including SCAR marker analysis and its relationship to other RKN based upon sequence analysis of the SSU and LSU D2/D3 are described herein. The isolate is identified as *M. incognita*, representing the first report of this nematode from *R. sinica*.

MATERIALS AND METHODS

Morphological characterization

Galls caused by the RKN were collected from infected roots of *R. sinica* and were opened with a scalpel and placed in distilled water. All stages observed, including adult females, egg masses, second-stage (J2) and other juveniles, and males were kept in petri dishes at room temperature in a small amount of water. Juveniles, females, and males were fixed in 3% formaldehyde and processed to glycerin by the formalin-glycerin method (Hooper, 1970; Golden, 1990). Specimen preparation and measurements were as described in Golden and Birchfield (1972). Perineal patterns of eight females were cut and mounted in a clear lactophenol solution. Measurements of nematodes were performed with the aid of a camera lucida and a stage micrometer. The morphometric data were processed using Excel software (Ye, 1996). Photomicrographs were taken with a Leica video camera (DFC490) attached via a C-mount Adapter fitted on a Leica microscope (DM4000B), and edited using Adobe Photoshop CS5.

Molecular characterization

Three RKN females were handpicked separately into distilled water for DNA extraction, amplification, and sequencing. They were placed into 50 µl of worm lysis buffer (WLB) containing Proteinase K for DNA extraction (Williams *et al.*, 1992). DNA samples were stored at -20°C until used as a PCR template.

The primers used for polymerase chain reaction (PCR) and DNA sequencing are presented in Table 1. Two pairs of primers (Finc/Rinc and Inc-K14F/Inc-K14R) are species-specific for *M. incognita*, Fjav/Rjav for *M. javanica*, and Far/Rar for *M. arenaria*. Primers SSUF07, SSUR26, 18S965, and 18S1573R were used for PCR amplification and DNA sequencing for small subunit 18S, rDNA2 and rDNA1.58S for internal transcribed spacer 1 (ITS1), and D2a/D3b for large subunit 28S rDNA.

The 25-µl PCR was performed using TaqMix DNA polymerase (Guangzhou Dongsheng Biotech Ltd., Guangzhou, China) according to the manufacturer's protocol. The thermal cycler program for PCR was as follows: denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C with 30 s; annealing at 55°C for 45 s, and extension at 72°C for 2 min. A final extension was performed at 72°C for 10 min (Ye *et al.*, 2007).

Sequence-characterized-amplified region (SCAR) PCR products were checked using 1% agarose gel under electrophoretic running conditions (120V, 45min.). PCR products were cleaned using an EZ Spin Column DNA Gel Extraction Kit (Bio Basic

Inc., Markham, Ontario, Canada) according to the manufacturer's protocol before being sequenced by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China) using an ABI PRISM 3730 sequencing system.

The nematode sequences from this project were deposited in GenBank. We used DNA sequences with the highest matches with our isolate from the GenBank database for phylogenetic analysis. DNA sequences were aligned using ClustalW (<http://workbench.sdsc.edu>, Bioinformatics and Computational Biology Group, Department of Bioengineering, UC San Diego, San Diego, CA, USA). The model of base substitution in the SSU and LSU sets was evaluated using MODELTEST version 3.06 (Posada and Crandall, 1998). The Akaike-supported model, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates were used in phylogenetic analyses. Bayesian analysis was performed to confirm the tree topology for each gene separately using MrBayes 3.1.0 (Huelsenbeck and Ronquist, 2001) running the chain for 106 generations and setting the 'burn in' at 1000. We used MCMC (Markov Chain Monte Carlo) methods within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget and Simon, 1999) using the 50% majority rule.

RESULTS

Symptoms and reproductive characterization

In fields where many seedlings of *R. sinica* were planted in an artificial soil mixture, we observed that

numerous plants were infected by the RKN. The RKN feeding caused swellings or galls in the infected roots of *R. sinica*. Galls were irregularly shaped, variable in size (approximately 0.5 cm in diameter), and located in the zone of maturation of roots. Affected plants typically displayed chlorotic leaves indicative of nutrient deficiencies, and with severe infections, leaves wilted, became necrotic, and resulted in plant death (Fig. 1 A, B). Infected roots had numerous galls, and roots appeared necrotic with necrosis expanding upward from the roots into the lower portions of the stem (Fig. 1 C–E). The gelatinous egg mass contained many eggs, surrounding in the female vulval region under light microscopy. Newly produced egg masses were hyaline and turned brown with age. The average number of J2 that hatched from each egg mass was 190.

Morphological description

Morphometrics of males, females, and J2 of this isolate are presented in Table 2. Measurements of this isolate lie within the ranges previously reported for *M. incognita* (Chitwood, 1949; Whitehead, 1968).

Females

The body is soft, pear-shaped with relatively long projecting neck (Fig. 2 L), 650 to 785 μm in length, usually embedded in root tissue, which is often swollen or galled. The stylet is 14 to 15 μm long and with rounded knobs. The distance from the dorsal pharyngeal gland orifice to the base of the stylet (DGO) is 2 to 4 μm . The excretory pore is located

Table 1. Primers used for polymerase chain reaction and DNA sequencing.

Primer	Marker	Sequence (5' to 3')	Reference
Finc	SCAR	CTCTGCCCAATGAGCTGTCC	Zijlstra <i>et al.</i> 2000
Rinc	SCAR	CTCTGCCCTCACATTAAG	Zijlstra <i>et al.</i> 2000
Inc-K14F	SCAR	CCCGCTACACCCTCAACTTC	Randig <i>et al.</i> 2002
Inc-K14R	SCAR	GGGATGTGTAATGCTCCTG	Randig <i>et al.</i> 2002
Fjav	SCAR	GGTGC GCGATTGAACTGAGC	Zijlstra <i>et al.</i> 2000
Rjav	SCAR	CAGGCCCTTCAGTGGA ACTATAC	Zijlstra <i>et al.</i> 2000
Far	SCAR	TCGGCGATAGAGGTAAATGAC	Zijlstra <i>et al.</i> 2000
Rar	SCAR	TCGGCGATAGACACTACAAACT	Zijlstra <i>et al.</i> 2000
SSUF07	18S	AAAGATTAAGCCATGCATG	Floyd <i>et al.</i> 2002
SSUR26	18S	CATTCTTG GCAAATGCTTTCG	Floyd <i>et al.</i> 2002
18S965	18S	GCGATCAGATACCGCCCTAGTT	Mullin <i>et al.</i> 2005
18S1573R	18S	TACAAAGGGCAGGGACGTAAT	Mullin <i>et al.</i> 2005
rDNA2	ITS1	TTGATTACGTTCCCTGCCCTTT	Vrain <i>et al.</i> 1992
rDNA1.58S	ITS1	ACGAGCCGAGTGATCCACCG	Cherry <i>et al.</i> 1997
D2a	28S	ACAAGTACCGTGAGGGAAAGTTG	Nunn 1992
D3b	28S	TGCGAAGGAACCGACTACTA	Nunn 1992

Table 2. Morphometrics of male, female, and J2 of *Meloidogyne incognita* mounted in formalin–glycerin. All measurements in micrometer and in the format: mean \pm s.d. (Range).

Character	Male	Female	J2
n	10	10	10
L	1572.0 \pm 192.5 (1400–1780)	728.3 \pm 70.1 (650.0–785.0)	446.7 \pm 30.1 (412.0–466.0)
a	50.1 \pm 2.1 (48.3–52.4)		28.0 \pm 2.6 (25.8–30.8)
b1	19.5 \pm 1.1 (18.7–20.7)		4.7 \pm 0.6 (4.0–5.1)
c	225.1 \pm 7.3 (219.4–233.3)		6.9 \pm 0.5 (6.4–7.4)
c'	0.4 \pm 0.1 (0.4–0.5)		5.0 \pm 0.3 (4.7–5.1)
Body diam. (greatest body diam.)	31.3 \pm 2.5 (29.0–34.0)		16.0 \pm 1.0 (15.0–17.0)
Stylet length	25.3 \pm 2.5 (23.0–28.0)	14.3 \pm 0.6 (14.0–15.0)	11.0 \pm 1.0 (10.0–12.0)
DGO	2.8 \pm 0.3 (2.5–3.0)	3.0 \pm 0.8 (2.0–4.0)	3.0 \pm 1.0 (2.0–4.0)
Pharynx length (head to metacarpus center)	80.3 \pm 5.5 (75.0–86.0)		96.3 \pm 5.5 (91.0–102.0)
Spicule length ^a	35.7 \pm 2.5 (33.0–38.0)	–	–
Anal body diam. (males = cloacal body diam.)	15.7 \pm 2.1 (14.0–18.0)		13.0 \pm 1.0 (12.0–14.0)
Tail length	7.0 \pm 1.0 (6.0–8.0)		64.7 \pm 8.1 (56.0–72.0)
Excretory pore from anterior end			96.0 \pm 5.3 (90.0–100.0)

^athe distance between the condylus and the posterior–most point of the lamina measured in a straight line.

posterior to stylet knobs. The metacarpus is nearly spherical and well developed (Fig. 2 L). The perineal pattern is round, and has a relatively high, squarish dorsal arch, with very wavy striae. The dorsal arch is relatively flattened at the top (Fig. 3 A–D).

Males

Males are 1400 to 1780 μ m long. The head is not offset, annulated, and has a characteristic large, rounded labial disc, which appears concave in lateral view. The head–cap has a stepped outline in lateral view. The stylet is 23 to 28 μ m long and robust, and the conus is longer than the shaft. Stylet knobs are prominent with flat, concave or ‘toothed’ anterior margins and are greater in width than length. The excretory pore is situated at the posterior end of the isthmus. Tails are bluntly rounded, terminus, and unstriated. Spicules slightly curved, gubernaculum

crescentic. The DGO is 2.5 to 3.0 μ m (Fig. 2 I–K).

Second–stage juveniles (J2)

J2s are 412 to 466 μ m long. Heads are not offset, and sub–hemispherical in dorso–ventral view. The stylet is 10 to 12 μ m long, with prominent, rounded stylet knobs. Tails taper to a subacute terminus and are striae coarsening posteriorly. The rectum is inflated. DGO is 2 to 4 μ m. The poorly defined hyaline tail terminus is 22 to 31 μ m long (Fig. 2 C–E).

SCAR marker analysis

Amplification of DNA extracted from an adult female with *M. incognita*–specific SCAR primers Finc/Rinc and Inc–K14F/Inc–K14R yielded a 1200–bp (Fig. 4, lane B) and a 399–bp product respectively (Fig. 4, lane E), while no amplification occurred

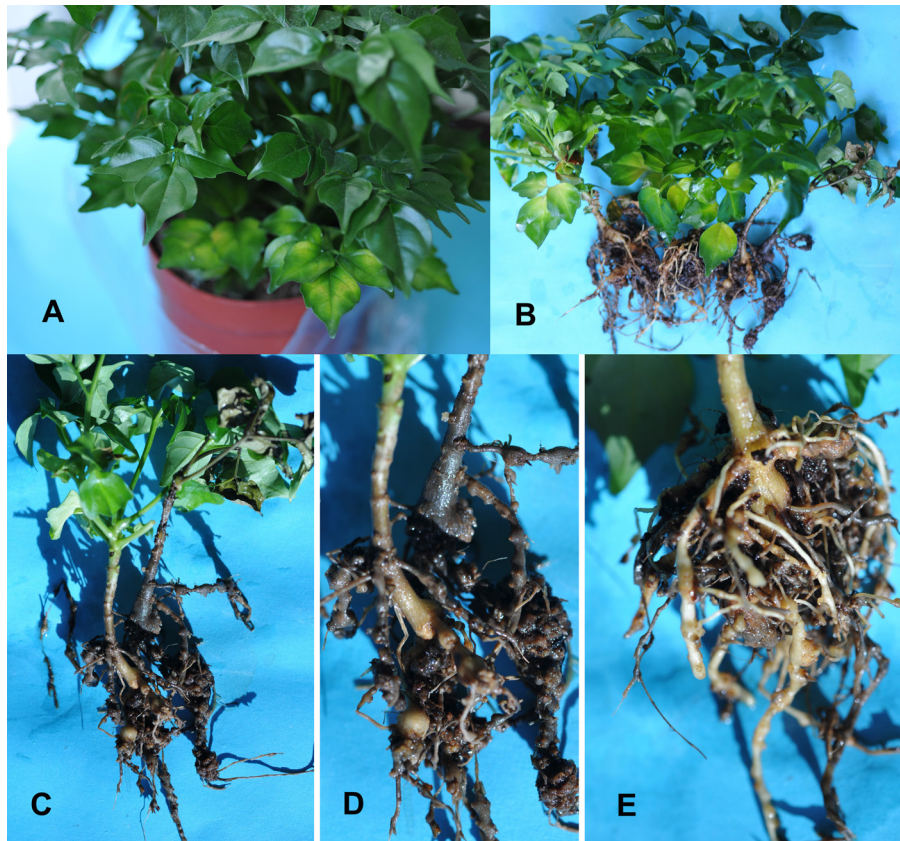


Fig. 1. Symptoms caused by root-knot nematode damage to *Radermachera sinica*. A and B: Plants with yellowing leaves; C–E: Diseased roots with galls.

with water negative control (Fig. 4, lanes C and F). Amplification was not observed with the *M. javanica*-specific primers Fjav/Rjav and the *M. arenaria*-specific primers Far/Rar (gel photo not shown). Primers Finc/Rinc and Inc-K14F/Inc-K14R successfully amplified positive control DNA from *M. incognita* (Voucher No. 11-6325) collected from tomato in North Carolina (Fig. 4, lanes A and D) and this reaction yielded the same sized PCR product as the adult female collected from *R. sinica*.

Molecular phylogenetic relationships

A 1948-bp 18S rDNA gene plus ITS1 and part of the 5.8S rDNA gene and a 764-bp 28S D2/D3 expansion segment were amplified and sequenced. Sequences of the rDNA were compared using blastN search from a diverse collection of *Meloidogyne* species present in GenBank. The identity of the 18S sequence of this isolate (GenBank accession No. KJ641552) with *M. chitwoodi* (AY593885), *M. exigua* (AY942627), *M. fallax* (EU669936), *M. graminicola* (AF442196), *M. naasi* (AY593901) and *M. oryzae* (AY942631) are 96%, 97%, 96%, 97%, 96%, and 96%, respectively. However, with *M. incognita* (AY284621, AY942624, AY268120, and EU669939),

M. arenaria (AY942623), *M. floridensis* (AY942621), and *M. javanica* (EU669938), the identity is 99%. Similar results were observed for the D2/D3 sequence of this isolate (KJ641553), i.e., the identity with *M. chitwoodi* (AF435802), *M. exigua* (AF435795), *M. fallax* (KC241962), *M. graminicola* (KF201162), *M. minor* (KC241977), and *M. naasi* (JN019291) is 89%, 88%, 87%, 88%, 87%, and 86%, respectively, while with *M. incognita* (KC464470, AF435794, JX100425 and JX100424), *M. arenaria* (KC287192), *M. arenaria* (KF112873), and *M. javanica* (JX100426), the identity is all 99%.

Phylogenetic analysis of the near-full-length SSU and LSU D2/D3 was performed to examine the relationships of *M. incognita* from *R. sinica* among other *Meloidogyne* species sequenced using the same loci. The dendrogram inferred from SSU (Fig. 5) using *Pratylenchus crenatus* Loof, 1960 and *P. neglectus* (Rensch, 1924) Filipjev and Schuurmans-Stekhoven, 1941 as outgroups suggested that: i) all the selected *Meloidogyne* are in a monophyletic clade in relation to *P. crenatus* and *P. neglectus* with 100% posterior probability; ii) this isolate of *M. incognita* is in a monophyletic clade with four other isolates of *M. incognita* (AY284621, AY942624, AY268120, and EU669939), three isolates of *M. javanica* (AY268121,

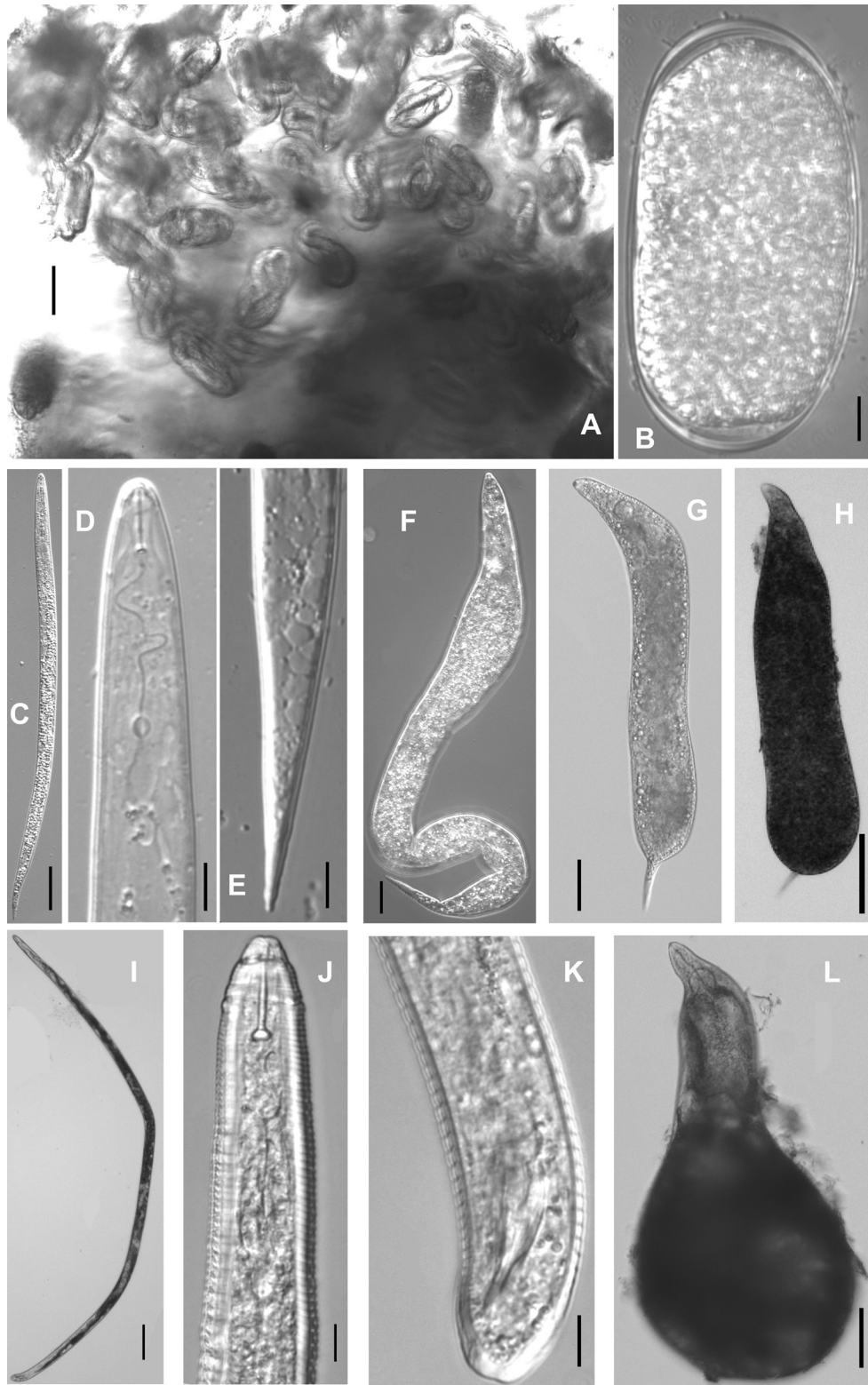


Fig. 2. Light micrographs of *Meloidogyne incognita* from *Radermachera sinica*. A: Egg mass with J1 and J2; B: Egg; C: Entire body of J2; D: Anterior body of J2; E: Tail of J2; F and G: Entire body of J3; H: Entire body of J4; I: Entire body of male; J: Anterior body of male; K: Tail of male; L: Entire body of adult female. Scale bars: A, C, G = 50 μ m; B, D, E, J, K = 10 μ m; F = 20 μ m; H, I, L = 100 μ m.

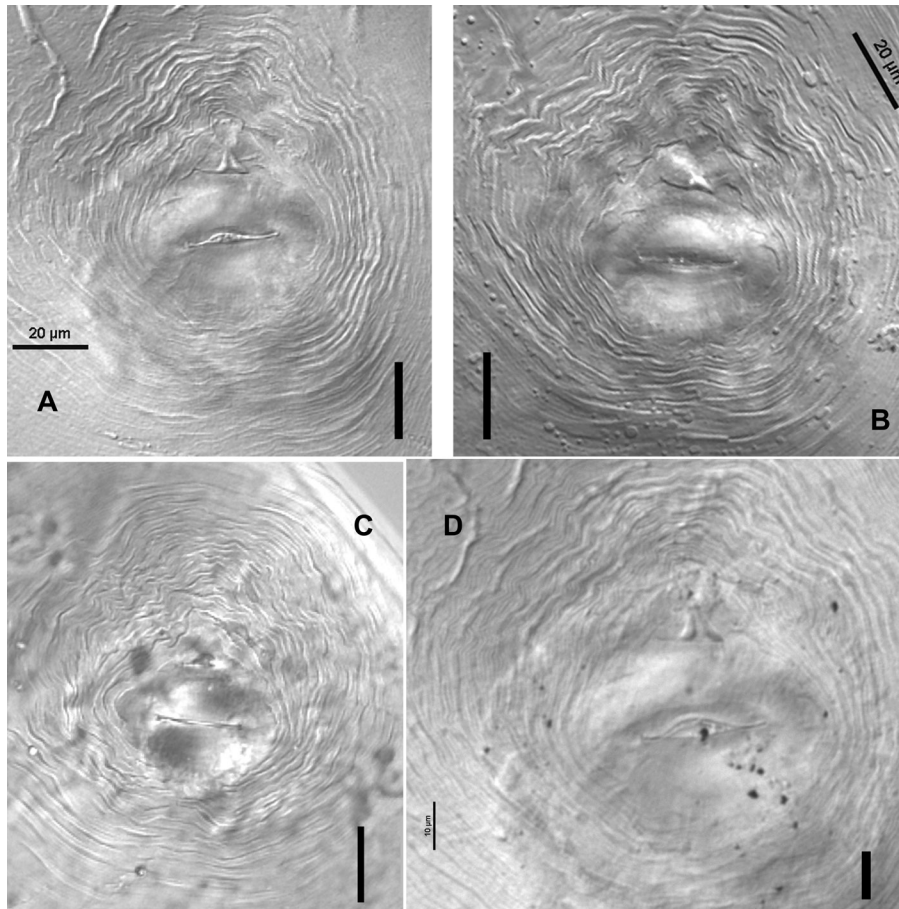


Fig. 3. Light micrographs of perineal patterns of females from *Radermachera sinica*. Scale bars: A, B, C = 20 µm; D = 10 µm.

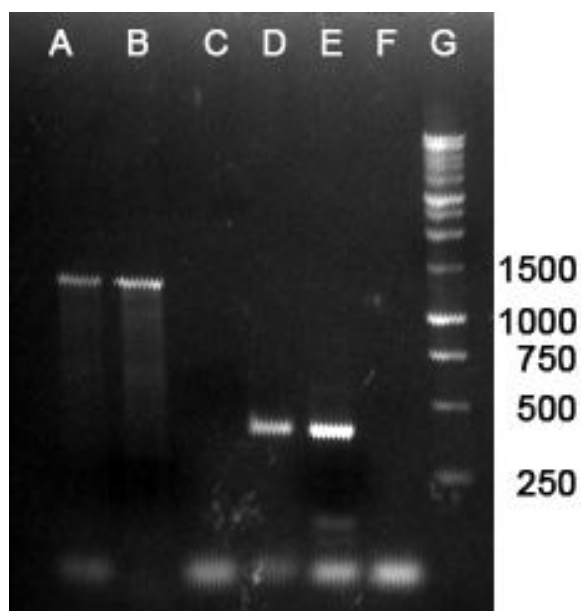


Fig. 4. Sequence-characterized-amplified-region PCR with *Meloidogyne incognita*-specific primers. Lane A: *M. incognita* positive control (Voucher No. 11-6325) from tomato in North Carolina with primers Finc/Rinc; Lane B: *M. incognita* from *Radermachera sinica* in China with primers Finc/Rinc; Lane C: negative control (water) with primers Finc/Rinc; Lane D: *M. incognita* positive control (11-6325) with primers Inc-K14F/Inc-K14R; Lane E: *M. incognita* from *R. sinica* in China with primers Inc-K14F/Inc-K14R; Lane F: negative control (water) with primers Inc-K14F/Inc-K14R; Lane G: 1-kb DNA ladder.

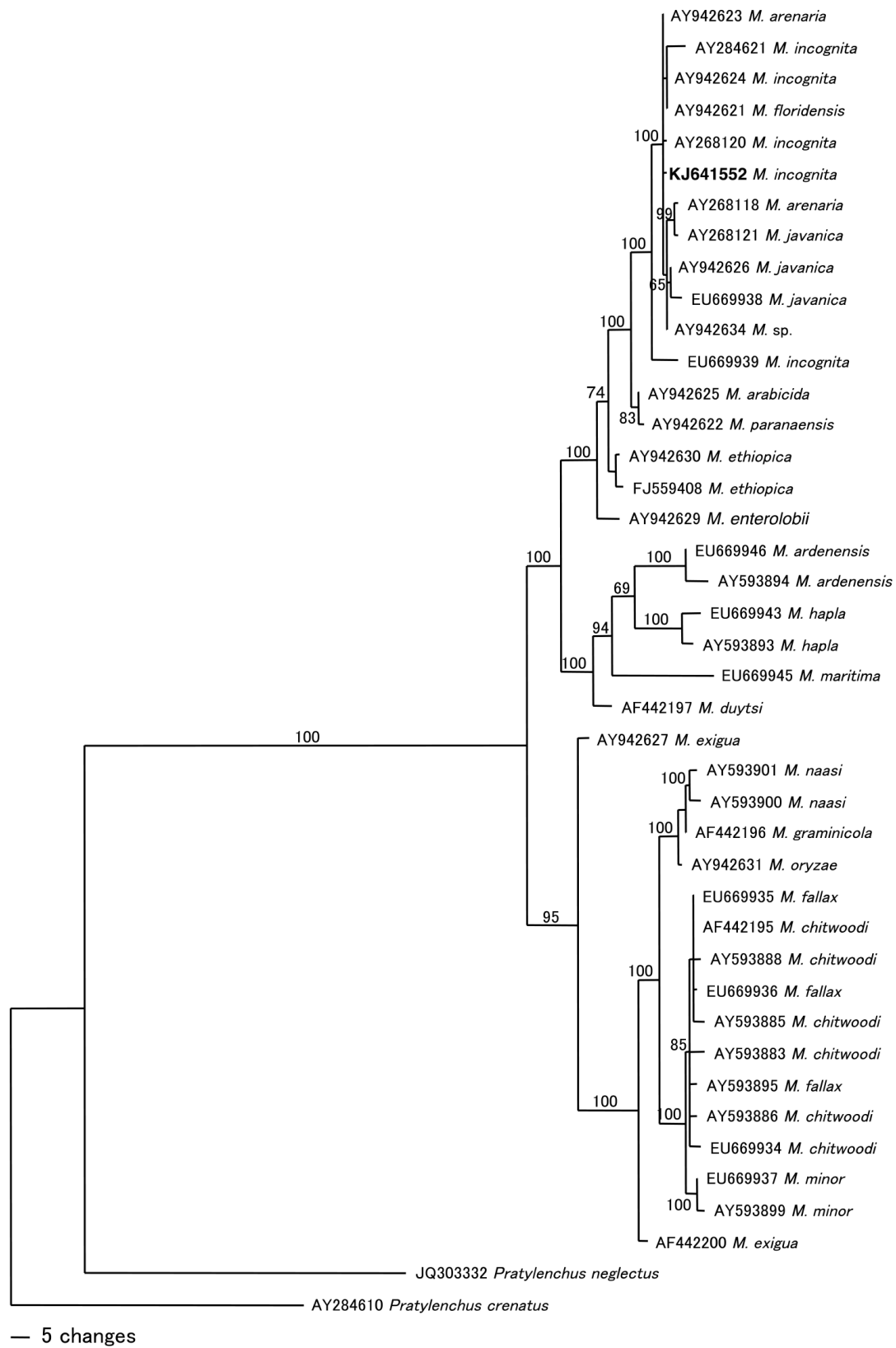


Fig. 5. The 10001st Bayesian tree inferred from 18S under GTR+I+G model ($-\ln L = 5395.4136$; $\text{freqA} = 0.2628$; $\text{freqC} = 0.2168$; $\text{freqG} = 0.2661$; $\text{freqT} = 0.2543$; $R(a) = 1.3861$; $R(b) = 3.6103$; $R(c) = 3.2178$; $R(d) = 0.8607$; $R(e) = 8.037$; $R(f) = 1$; $\text{Pinva} = 0.5043$; $\text{Shape} = 0.8658$). Posterior probability values exceeding 50% are given on appropriate clades.

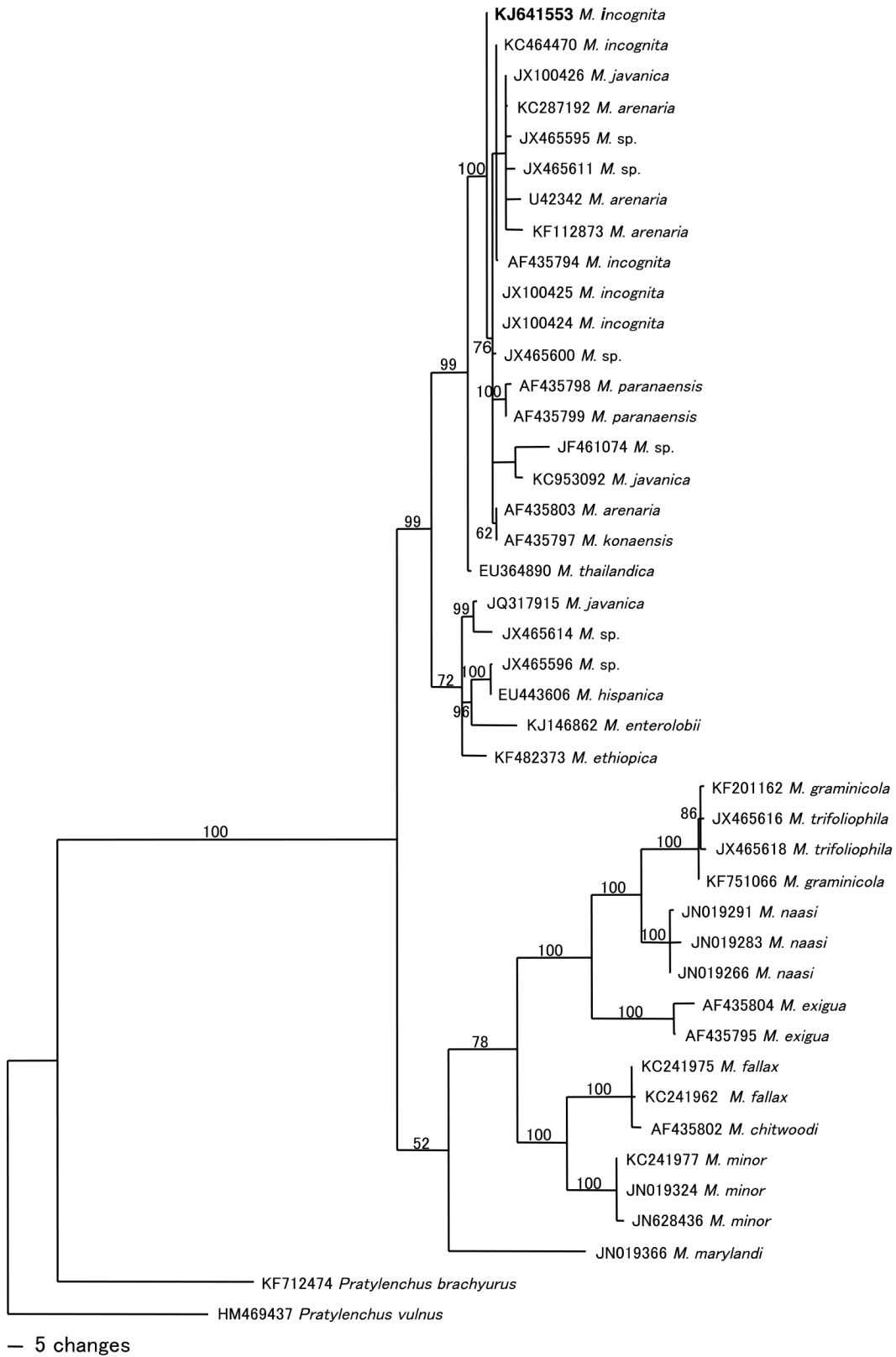


Fig. 6. The 10001st Bayesian tree inferred from 28S D2/D3 under GTR+I+G model ($-\ln L = 4226.5239$; $\text{freqA} = 0.2483$; $\text{freqC} = 0.1885$; $\text{freqG} = 0.2792$; $\text{freqT} = 0.284$; $R(a) = 0.7156$; $R(b) = 2.3615$; $R(c) = 1.4936$; $R(d) = 0.2897$; $R(e) = 3.1454$; $R(f) = 1$; $\text{Pinva} = 0.3144$; $\text{Shape} = 1.0913$). Posterior probability values exceeding 50% are given on appropriate clades.

AY942626, and EU669938), two isolates of *M. arenaria* (AY942623 and AY268118), and one isolate of *M. floridensis* (AY942621) with 100% posterior probability. This clade is more closely grouped with *M. arabicida*, *M. arenaria*, *M. enterolobii*, *M. ethiopica*, *M. floridensis*, *M. javanica*, and *M. paranaensis* in the same highly supported monophyletic clade than with the other sequenced *Meloidogyne* species; iii) the isolate of *M. incognita* from *R. sinica* is clearly different from *M. ardenensis*, *M. maritima*, *M. chitwoodi*, *M. exigua*, *M. fallax*, *M. graminicola*, *M. hapla*, *M. minor*, *M. naasi*, and *M. oryzae*.

The tree inferred from D2/D3 of LSU (Fig. 6) using *P. vulnus* Allen and Jensen, 1951 and *P. brachyurus* (Godfrey, 1929) Filipjev and Schuurmans–Stekhoven, 1941 as outgroups suggested that: i) all the sequenced *Meloidogyne* species are divided into two monophyletic clades with 100% support; ii) the isolate of *M. incognita* from *R. sinica* is in a well-supported monophyletic clade with four other isolates of *M. incognita* (KC464470, AF435794, JX100425, and JX100424), four isolates of *M. arenaria* (KC287192, KF112873, U42342, and AF435803), two isolates of *M. javanica* (JX100426 and KC953092), two isolates of *M. paranaensis* (AF435798 and AF435799), and one isolate of *M. konaensis* (AF435797) with 100% posterior probability; iii) this isolate of *M. incognita* appears to be more distant from *M. marylandi*, *M. chitwoodi*, *M. exigua*, *M. fallax*, *M. graminicola*, *M. minor*, *M. naasi*, and *M. trifoliophila*.

DISCUSSION

Accurate identification of *Meloidogyne* species is crucial to implement effective RKN management practices such as crop rotation and using resistant varieties (Hussey, 1990; Cenis, 1993; Zijlstra *et al.*, 2000; Zijlstra and van Hoof, 2006). Given that high intraspecific and interspecific variations of molecular and morphological characters occur (Karssen and van Aelst, 2001; Brito *et al.*, 2004; Skantar *et al.*, 2008; García and Sánchez–Puerta, 2012;), enzymatic character analysis is challenging in some cases and relatively conserved nature of SSU rRNA and LSU D2/D3 expansion segments from major species such as *M. arenaria*, *M. incognita*, and *M. javanica* (Blok and Powers, 2009; Huang *et al.*, 2013), make species identification in the genus *Meloidogyne* challenging. Therefore, a multidimensional approach that uses morphological and molecular characters is needed for discrimination of species. In this study, SCAR marker analysis was performed, indicating that two pairs of specific–species primers positively amplified DNA extracted from this isolate, and a single *M. incognita*–specific band of 1200–bp and a single *M. incognita*–specific band of 399–bp was produced through PCR by *M. incognita*–specific primers Finc/Rinc and Inc–K14F/Inc–K14R, respectively. These results revealed

this isolate is *M. incognita* as reported by Zijlstra *et al.* (2000) and Randig *et al.* (2002). Although ribosomal DNA is very conserved among root–knot nematodes, the DNA sequences of 18S, ITS, and 28S D2/D3 of this study isolate are in the tropical species group including *M. incognita*, *M. javanica*, and *M. arenaria*. In addition, measurements of this isolate lie within the ranges previously reported for *M. incognita* (Chitwood, 1949; Whitehead, 1968), and features of female perineal patterns of this population belong to *M. incognita*. Thus, based upon morphological and molecular characterization, this RKN isolate from the galls on roots of *R. sinica* was identified as *M. incognita* (Kofoid and White, 1919) Chitwood 1949. To our knowledge, this is its first report on *R. sinica*.

Meloidogyne incognita is the most frequently encountered RKN species (Khan and Ahmad, 2000; Guzman–Plazola *et al.*, 2006; Anwar and McKenry, 2010; Kayani *et al.*, 2013). It is extremely polyphagous, with a host range of up to 3,000 plant species (Castagnone–Sereni, 2002). It can infect many species of vegetables, cereals, ornamentals, pasture grasses, trees and shrubs, sugarcane, tobacco, cotton, and potatoes (<http://plpnemweb.ucdavis.edu/nemaplex/Taxadata/G076S3.htm#EconomicImportance>), and it can be detrimental in warmer regions and greenhouses. In the present study, we observed that the isolate of *M. incognita* from *R. sinica* lays eggs in gelatinous masses that form on the surface of the galls that hatched numerous J2. Serious infections resulted in prolific gall formation on roots, wilted and chlorotic leaves, root rot and then ultimately plant mortality, indicating *M. incognita* is of economic importance to *R. sinica* production and related trade.

For DNA analysis, ribosomal DNA SSU and LSU D2/D3 expansion segments have been useful to identify species of nematodes (Zijlstra *et al.*, 2000; Chen *et al.*, 2003; Powers, 2004; Palomares–Ruis *et al.*, 2007; Zeng *et al.*, 2011, 2013). In the present study, we observed that the 18S region is more conserved than 28S D2/D3. The divergence of the isolate of *M. incognita* from *R. sinica* from other *Meloidogyne* species obtained from GenBank is 4%, 3%, 4%, 3%, 4%, and 4% compared with *M. chitwoodi* (AY593885), *M. exigua* (AY942627), *M. fallax* (EU669936), *M. graminicola* (AF442196), *M. naasi* (AY593901), and *M. oryzae* (AY942631), respectively. But the divergence of D2/D3 is 14%, 13%, 11%, 12%, 12%, and 13% with *M. naasi* (JN019291), *M. fallax* (KC241962), *M. chitwoodi* (AF435802), *M. graminicola* (KF201162), *M. exigua* (AF435795), and *M. minor* (KC241977), respectively. However, minor differences in 18S sequences were observed with several tropical species such as *M. incognita* (AY284621, AY942624, AY268120, and EU669939), *M. arenaria* (AY942623), *M. javanica* (EU669938), and *M. floridensis* (AY942621) and in 28S D2/D3 with *M. incognita* (KC464470, AF435794,

JX100425, and JX100424), *M. arenaria* (AF435803 and KC287192), and *M. javanica* (JX100426).

Phylogenetic trees inferred from 18S and 28S D2/D3 (Fig. 5, 6) showed that the isolate of *M. incognita* from *R. sinica* is clearly different from *M. ardenensis*, *M. chitwoodi*, *M. exigua*, *M. fallax*, *M. graminicola*, *M. hapla*, *M. maritima*, *M. minor*, *M. naasi*, and *M. oryzae*. This isolate is in a monophyletic clade with other isolates of *M. incognita*, and other tropical species of *Meloidogyne* including *M. incognita*, *M. arenaria*, *M. javanica*, and *M. floridensis* with 100% posterior probability. However, these four major tropical species could not be separated based on these molecular markers due to their limited sequence polymorphisms. Previous studies also revealed that the parthenogenic *M. arenaria*, *M. incognita*, and *M. javanica* are difficult to distinguish using nucleotide sequences, including ITS, 18S rDNA, and mitochondrial DNA (mtDNA) (Blok and Powers, 2009; Huang *et al.*, 2013). In this study, 18S rDNA and D2/D3 expansion segment sequencing aided understanding molecular phylogenetic relationships of this isolate of *M. incognita* and other *Meloidogyne* species, but could not discriminate several major tropical species including *M. incognita*, *M. arenaria*, and *M. javanica*. The final species identification could be achieved through PCR by species-specific primers.

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