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Research Note

Characterization of *Meloidogyne enterolobii* intercepted from baobab (*Adansonia digitata* L.) seedlings from Thailand during Japanese import plant quarantine inspection

S. SEKIMOTO[1,*], T. INABA[2]

[1,*]Research Division, Yokohama Plant Protection Station, Ministry of Agriculture, Forestry and Fisheries (MAFF), Yokohama, Kanagawa 231-0801, Japan, E-mail: *pps_yokohama_gaichu@maff.go.jp*; [2]Chubu Airport Sub-station, Nagoya Plant Protection Station, MAFF, Tokoname, Aichi 479-0881, Japan

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Summary

In April 2019, baobab (*Adansonia digitata* L.) seedlings from Thailand, exhibiting galls on the roots, were intercepted during an import plant quarantine inspection at Chubu Centrair International Airport, Japan. Root-knot nematodes (RKNs) were extracted from the galled roots of baobab seedlings and identified by morphological, morphometrical and molecular methods as the guava root-knot nematode, *Meloidogyne enterolobii* Yang & Eisenback. The morphology and morphometrics of the intercepted population were similar to those of the original and subsequent descriptions of *M. enterolobii*. The sequences of D2-D3 of 28S rRNA, mtDNA intergenic COII-16S rRNA and COI genes obtained in this study matched well (99–100% similarity) with each of the gene sequences of *M. enterolobii* deposited in GenBank. Phylogenetic analysis of these genes revealed that the intercepted population clustered with *M. enterolobii* and clearly differed from other RKN species. To the best of our knowledge, this is the first report of *M. enterolobii* from baobab.

Keywords: *Adansonia digitata*; identification; *Meloidogyne enterolobii*; morphology; phylogeny; root-knot nematode

Introduction

The guava root-knot nematode (RKN), *Meloidogyne enterolobii* Yang & Eisenback, 1983 was originally described from a population collected from the roots of the pacara earpod tree (*Enterolobium contortisiliquum* (Vell.) Morong) in Hainan Island, China (Yang & Eisenback, 1983). This species has been recorded from countries in Africa, Asia, North America, Central America and the Caribbean and South America (Subbotin *et al.*, 2021; EPPO, 2022). In Europe, it was first recorded in a greenhouse in France (no longer present) (Blok *et al.*, 2002) and has been reported from two greenhouses in Switzerland (Kiewnick *et al.*, 2008) and some private gardens in Portugal (Santos *et al.*, 2019). This nematode has recently emerged as one of the most important RKN species because of its wide host range, high degree of virulence and its ability to reproduce on crop genotypes resistant to the major RKN species, including resistant tomato (*Mi-1*gene), potato (*Mh* gene), soybean (*Mir1* gene), bell pepper (*N* gene) and sweet pepper (*Tabasco* gene) (Berthou *et al.*, 2003; Brito *et al.*, 2007; Cetintas *et al.*, 2008). Considering the risk of introducing and disseminating this pest, *M. enterolobii* was added to the EPPO A2 list of pests recommended for regulation as quarantine pests in 2010 and is currently subject to quarantine regulations in South Korea, Costa Rica and USA (Arkansas, Florida, Louisiana, Mississippi and North Carolina) (Castagnone-Sereno, 2012; Ye *et al.*, 2021). This species has not occurred in Japan and is also included on the quarantine pest list of Japan (Plant Protection Station, MAFF, 2022).

* – corresponding author

In April 2019, baobab (*Adansonia digitata* L.) seedlings from Thailand exhibiting galls on the roots were intercepted during an import plant quarantine inspection at Chubu Centrair International Airport, Japan. Nematode species identification was performed using morphological, morphometrical and molecular methods at the Research Division, Yokohama Plant Protection Station, MAFF, Yokohama, Kanagawa. The objective of the present study was to provide morphological, morphometrical and molecular characterization of *M. enterolobii* intercepted from baobab seedlings from Thailand during a Japanese import plant quarantine inspection. To our knowledge, this is the first report of *M. enterolobii* from baobab.

Materials and Methods

Nematode extraction and morphological characterization

Females were extracted from the galled roots under a stereomicroscope and perineal patterns were prepared as described by Hartman & Sasser (1985). Second-stage juveniles (J2s) extracted from the galled roots by the Baermann funnel method were heat-killed, mounted in water on temporary slides and measured immediately. Microphotographs were taken using a digital camera Olympus FX380 attached to a compound Olympus BX51 microscope equipped with differential interference contrast (DIC).

DNA extraction, PCR and sequencing

DNA was extracted from single females using a DNA extraction kit, ISOHAIR® (Nippon Gene, Tokyo, Japan). A single nematode was placed into a drop of sterile distilled water on a clean glass slide. After the water dried, the nematode was crushed with a small sterile filter paper chip under a stereo microscope using forceps (Iwahori *et al.*, 2000). The paper chip was then dropped into a 1.5 mL plastic tube containing 10 µL of "nematode-dissolving solution", whereupon the tube was incubated at 60°C for 20 min (Tanaka *et al.*, 2012). After the incubation, 90 µL of sterile distilled water was added to yield 100 µL of lysate for each specimen, which was then stored at –20°C. The D2-D3 expansion segments of the 28S rRNA gene were amplified using primers D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG-3') and D3B (5'-TCG GAA GGA ACC AGC TAC TA-3') (Nunn, 1992). The mtDNA intergenic COII-16S rRNA gene was amplified using primers C2F3 (5'-GGT CAA TGT TCA GAA ATT TGT GG-3') and 1108 (5'-TAC CTT TGA CCA ATC ACG CT-3') (Powers & Harris, 1993). The partial mtDNA COI gene was amplified using primers JB3 (5'-TTT TTT GGG CAT CCT GAG GTT TAT-3') and JB5 (5'-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3') (Derycke *et al.*, 2005). PCR amplification was performed in a final volume of 20 µL reaction mixture containing 2 µL 10 × Ex Taq buffer (20 mM Mg^{2+} plus) (Takara Bio, Shiga, Japan), 1.6 µL dNTP mixture (2.5 mM each), 0.4 µL (10 µM) of each primer, 0.1 µL TaKaRa Ex Taq® Hot Start Version (5 U/µL) (Takara Bio), 1 µL DNA template and 14.5 µL distilled water. The amplification conditions for D2-D3, COII-16S and COI were as follows: a single step of pre-denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s (D2-D3 and COI) or 1 min (COII-16S) and extension at 72°C for 30 s

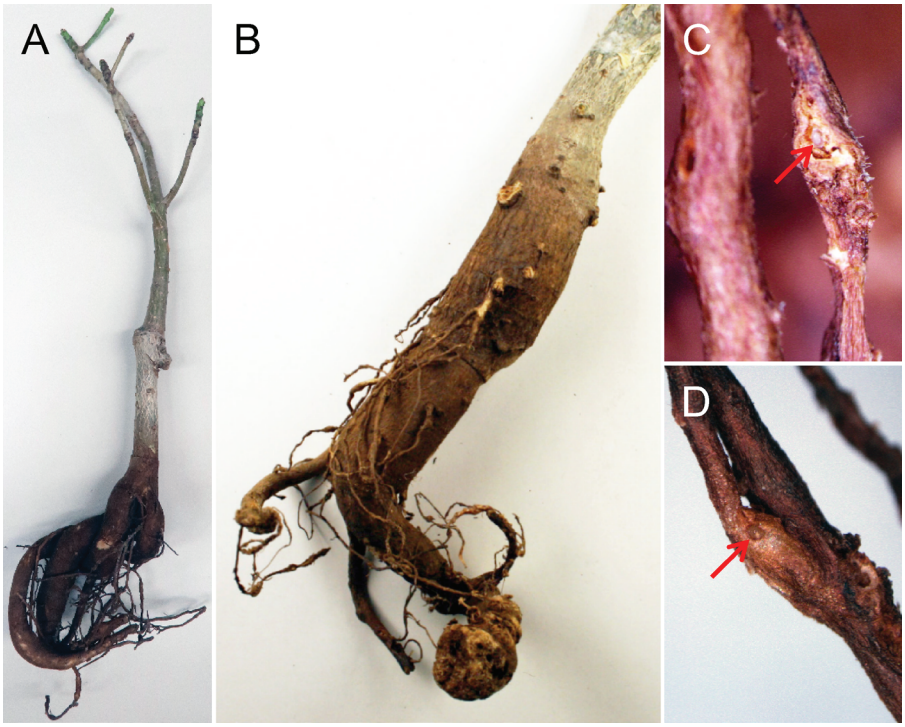

Fig. 1. Baobab seedling infested by *Meloidogyne enterolobii*. (A) Infested baobab seedling; (B) Baobab roots showing galls caused by *M. enterolobii*; (C, D) Females of *M. enterolobii* parasitizing roots of baobab (red arrow indicating female).

Table 1. Morphometrics of second-stage juveniles of *Meloidoyne enterolobii*. All measurements are in µm and in the form: mean ± s.d. (range).

Character	Thailand	China	Puerto Rico
	This study	Yang & Eisenback (1983)	Rammah & Hirschmann (1988)
n	10	30	35
L	466.7 ± 33.8 (423.8 – 527.3)	436.6 ± 16.6 (405.0 – 472.9)	453.6 ± 28.4 (390.4 – 528.0)
a	31.2 ± 1.4 (29.0 – 32.9)	28.6 ± 1.9 (24.0 – 32.5)	30.9 ± 1.9 (26.4 – 34.7)
c	8.8 ± 0.8 (8.0 – 10.0)	7.8 ± 0.7 (6.8 – 10.1)	8.3 ± 0.4 (7.0 – 9.2)
Body diam. at mid-body	14.8 ± 0.7 (13.8 – 16.3)	15.3 ± 0.9 (13.9 – 17.8)	14.7 ± 0.5 (13.8 – 15.8)
Stylet length	11.0 ± 0.5 (10.5 – 11.8)	11.7 ± 0.5 (10.8 – 13.0)	11.6 ± 0.3 (11.1 – 12.2)
DGO	2.7 ± 0.5 (2.0 – 3.4)	3.4 ± 0.3 (2.8 – 4.3)	3.9 ± 0.2 (3.3 – 4.3)
Anterior end to median bulb valve	58.0 ± 2.5 (54.5 – 61.3)	–	58.2 ± 1.8 (55.2 – 62.9)
Anterior end to excretory pore	92.0 ± 2.1 (89.0 – 94.4)	91.7 ± 3.3 (84.0 – 98.6)	87.6 ± 3.3 (79.9 – 97.9)
Tail length	53.1 ± 4.7 (44.0 – 60.9)	56.4 ± 4.5 (41.5 – 63.4)	54.4 ± 3.6 (49.2 – 62.9)
Length of hyaline part of tail	9.8 ± 3.9 (5.1 – 15.3)	–	–

(D2-D3 and COI) or 1 min (COII-16S). PCR products were purified with ExoSAP-IT® (USB Products, Affymetrix, Cleveland, OH, USA) and used for direct sequencing. PCR products were directly sequenced bidirectionally using the primers described above. The resulting products were purified with BigDye® XTerminator™ Purification Kit (Life technologies, Bedford, MA, USA) and analyzed in an ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The new sequences were submitted to the GenBank database under accession numbers: LC738960 (D2-D3), LC738961 (COII-16S) and LC738962 (COI).

Phylogenetic analysis

The new sequences for each gene were aligned with default parameters with their corresponding published gene sequences of *M. enterolobii* and other RKN species using MUSCLE (Edgar, 2004) as implemented in MEGA 5.2.2 (Tamura *et al.*, 2011). Sequence datasets were analyzed with Bayesian inference (BI) using MrBayes 3.2.2 (Ronquist *et al.*, 2012) under the GTR + G model using the Akaike Information Criterion (AIC) as implemented in MrModeltest 2.3 (Nylander, 2004) in conjunction with PAUP* 4.0b10 (Swofford, 2003). BI analysis was initiated with a random starting tree and was run with four chains for 1.0 × 10^6 generations.

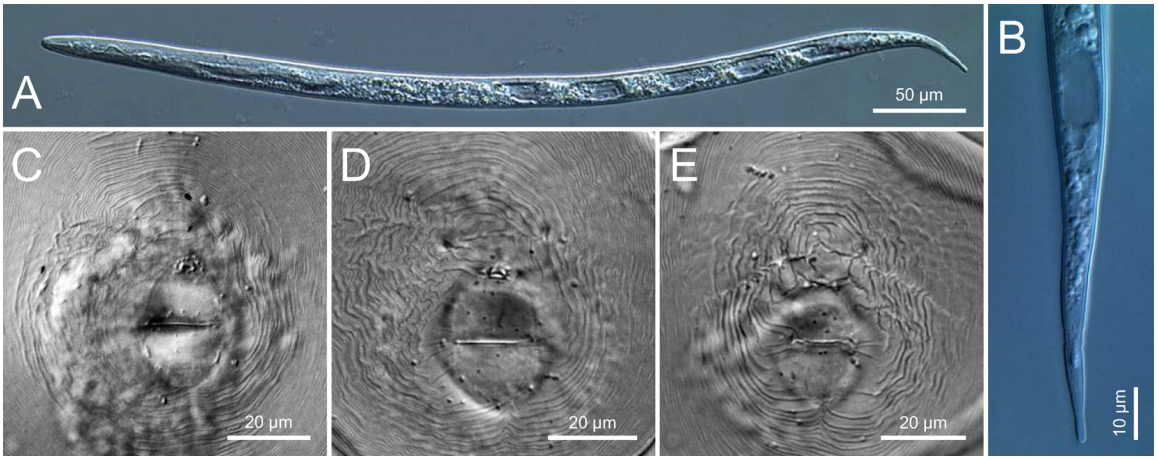

Fig. 2. Light micrographs of *Meloidogyne enterolobii* intercepted from baobab seedlings. (A) Whole body of second-stage juvenile (J2); (B) Tail of J2; (C–E) Perineal patterns of females.

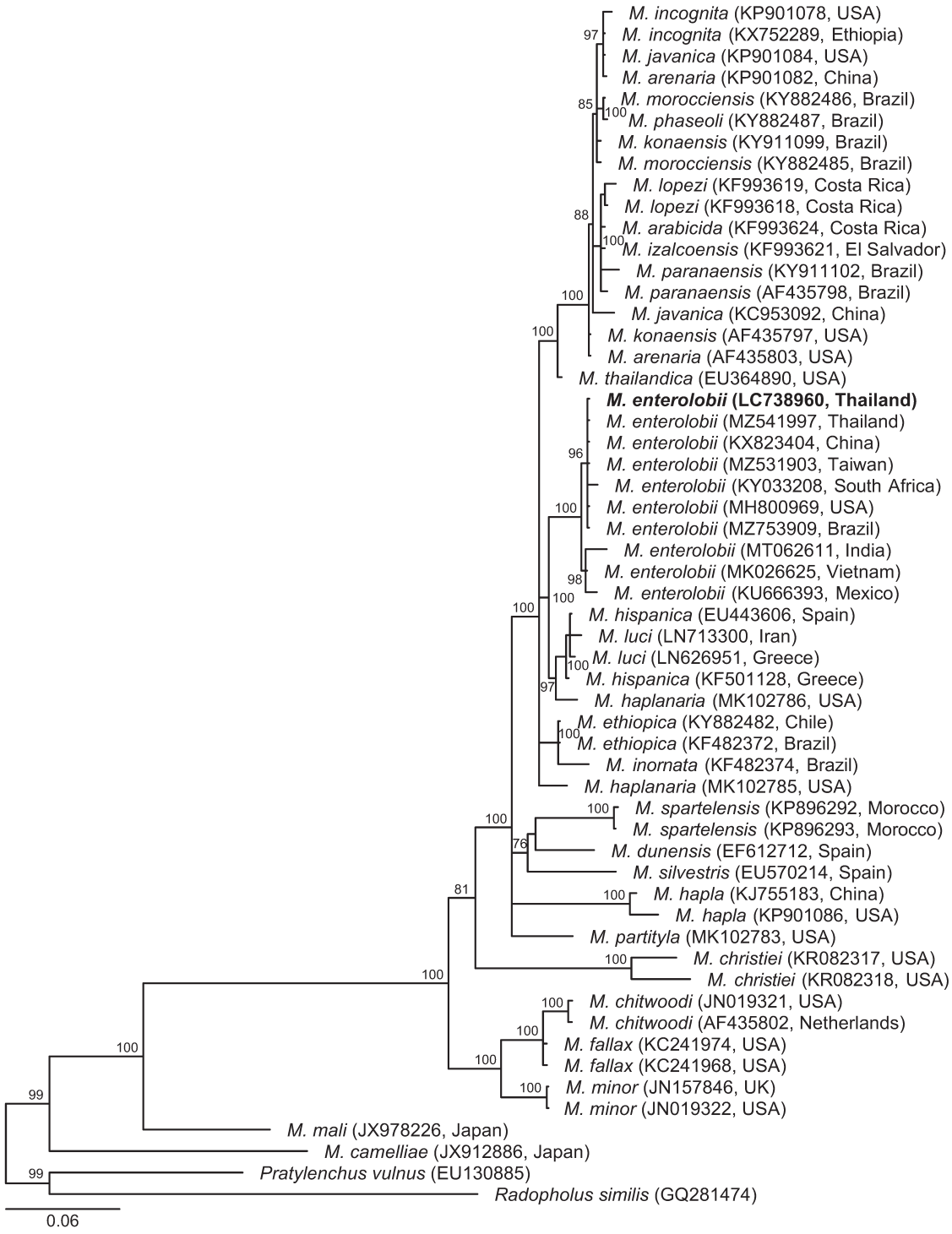

Fig. 3. Phylogenetic relationships within *Meloidogyne* species: Bayesian 50% majority rule consensus trees from two runs as inferred from analysis of the D2-D3 of 28S rRNA gene sequence alignment under the GTR + G model. Posterior probabilities equivalent to or exceeding 70% are given for appropriate clades. New sequence is indicated in bold.

The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The topologies were used to generate a 50 % majority rule consensus tree. Posterior probabilities (PP) were given on appropriate clades.

Ethical Approval and/or Informed Consent

For this study, formal consent is not required.

Results

Morphological characterization

The measurements of J2s of the RKN population intercepted from baobab seedlings from Thailand (Fig. 1) are shown in Table 1. The J2s were vermiform in shape, slender and tapering at both ends (Fig. 2A). The body length was 423.8 – 527.3 µm. The labial region was slightly offset from the body. The stylet was delicate with sharply pointed cone and rounded knobs and 10.5 – 11.8 µm in length. The tail was conoid with a bluntly rounded tip (Fig. 2B). The hyaline terminus was distinct. The perineal patterns of the females were round to oval with distinct phasmids (Fig. 2C–E). The dorsal arch was moderately high to high and rounded or square. The lateral lines were mostly lacking, but indistinct lateral lines were sometimes present. The striae were fine and smooth, sometimes coarse. The fine striae were sometimes present on the lateral sides of the vulva.

Molecular characterization

The amplification of the D2-D3 of 28S rRNA, COII-16S rRNA and the partial COI genes yielded a single fragment of *ca* 750, 700 and 400 bp, respectively.

The D2-D3 of the 28S rRNA gene alignment contained 54 sequences of RKN species and two sequences of the outgroup taxa and was 850 bp in length. The phylogenetic relationships within RKNs are given in Figure 3. The sequence of the RKN population intercepted from baobab from Thailand (LC738960) clustered with those of *M. enterolobii* with a high support value (PP = 100) and completely matched those of *M. enterolobii* from Thailand (MZ541997), China (KX823404), Taiwan (MZ531903), USA (MH800969) and Brazil (MZ753909).

The COII-16S rRNA gene alignment contained 50 sequences of RKN species and two sequences of the outgroup taxa and was 1936 bp in length. The phylogenetic relationships within RKNs are given in Figure 4. The sequence of the RKN population intercepted from baobab from Thailand (LC738961) clustered with those of *M. enterolobii* with a high support value (PP = 100) and showed 99 % similarity with those of *M. enterolobii* from Brazil (KX767844), China (KX823370), Costa Rica (KF993632), Kenya (KX214350), Mexico (KF360358), Niger (MF927970), Portugal (MK387171), South Africa (JX522542), Taiwan (KP411229),Thailand (MW167103) and USA (MN809527).

The partial COI gene alignment contained 39 sequences of RKN species and two sequences of the outgroup taxa and was 450 bp in length. The phylogenetic relationships within RKNs are given in Figure 5. The sequence of the RKN population intercepted from baobab from Thailand (LC738962) clustered with those of *M. enterolobii* with a high support value (PP = 100) and completely matched those of *M. enterolobii* from India (MT075847), China (JX683714), Portugal (MK387170), Kenya (KT936633), South Africa (KY203704), USA (MH128530), Costa Rica (KP202351) and Puerto Rico (KU372161).

Discussion

Accurate identification of RKN species is crucial to implement appropriate quarantine of imported and exported plant materials to prevent the introduction and spread of exotic and quarantine RKNs. Species identification of RKN has been traditionally based on microscopic examination of female perineal patterns and J2s (Hunt & Handoo, 2007). These methods, however, require considerable technical skill, expertise and time and the morphological and morphometrical characteristics are often unreliable due to significant inter- and intraspecific variation. For example, *M. enterolobii* might have been misidentified as *M. incognita* in a number of past surveys because of its morphological resemblance to *M. incognita* considering only the perineal patterns (Carneiro *et al.*, 2001; Brito *et al.*, 2004). Various DNA-based approaches have been recently successfully applied to identify RKN species and characterize the phylogenetic relationships within RKNs (Blok & Powers, 2009; Rashidifard *et al.*, 2018; Subbotin *et al.*, 2021). A combination of morphological/morphometrical characters and molecular methods are therefore essential to identify RKN species accurately.

The morphology and morphometrics of the RKN population intercepted from baobab from Thailand were similar to those of the original description of *M. enterolobii* from China (Yang & Eisenback, 1983) and were also within the ranges reported for *M. enterolobii* populations from India (Ghule *et al.*, 2020), Puerto Rico (Rammah & Hirschmann, 1988), South Africa (Rashidifard *et al.*, 2019), USA (Brito *et al.*, 2004) and Vietnam (Trinh *et al.*, 2022). The sequences of D2-D3 of 28S rRNA, COII-16S rRNA and COI genes obtained in this study matched well (99 – 100 % similarity) with each of the *M. enterolobii* gene sequences deposited in GenBank. Phylogenetic analysis of these genes revealed that the RKN population intercepted from baobab from Thailand clustered with *M. enterolobii* with a high support value (PP = 100) and clearly differed from other RKN species. Our results were in congruence with those of previous studies by Archidona-Yuste *et al.* (2018) and Trinh *et al.* (2022). Accordingly, the RKN population intercepted from baobab from Thailand was identified as *M. enterolobii* by morphological/morphometrical and molecular methods.

In Japanese import plant quarantine inspection, *M. enterolobii* was first intercepted from baobab from Thailand in January 2017 (MAFF, 2022). However, this record has never been published

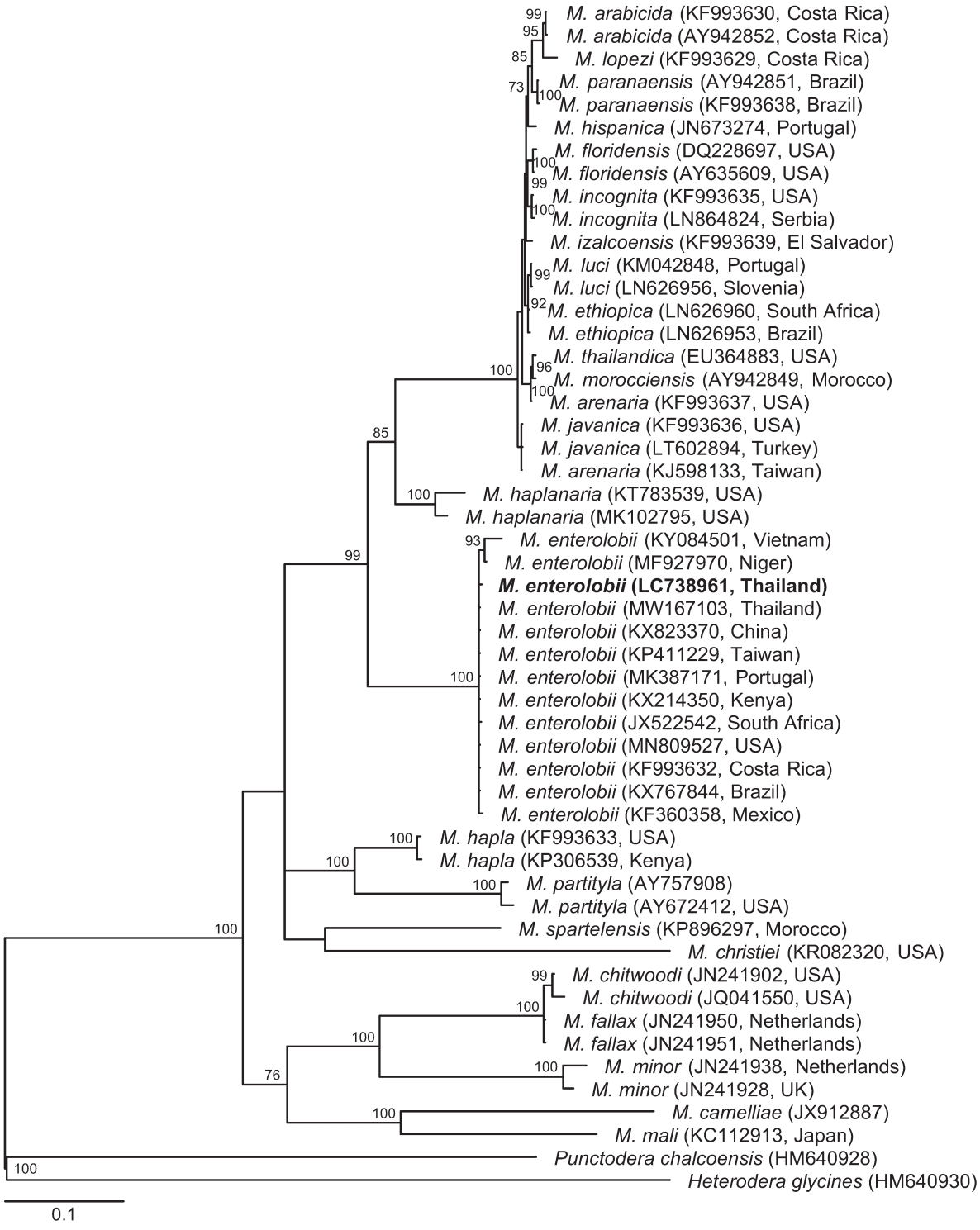

Fig. 4. Phylogenetic relationships within *Meloidogyne* species: Bayesian 50% majority rule consensus trees from two runs as inferred from analysis of the intergenic COII-16S rRNA gene sequence alignment under the GTR + G model. Posterior probabilities equivalent to or exceeding 70% are given for appropriate clades. New sequence is indicated in bold.

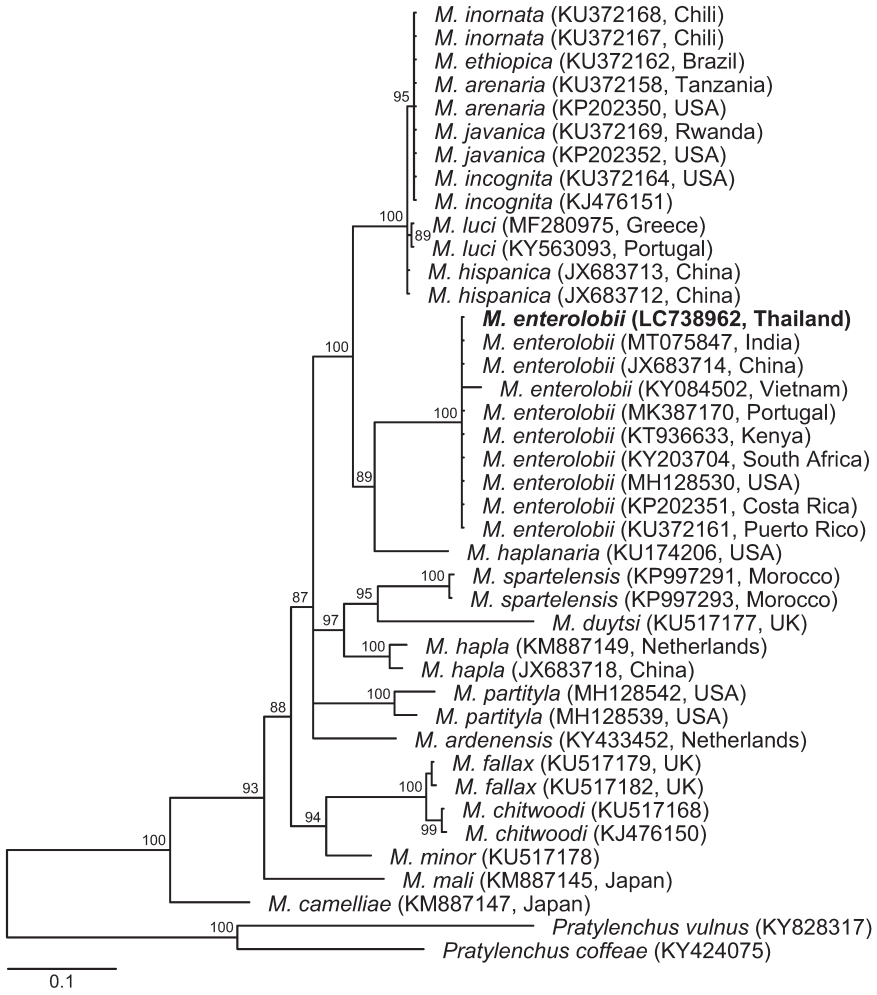

Fig. 5. Phylogenetic relationships within *Meloidogyne* species: Bayesian 50% majority rule consensus trees from two runs as inferred from analysis of the COI gene sequence alignment under the GTR + G model. Posterior probabilities equivalent to or exceeding 70% are given for appropriate clades. New sequence is indicated in bold.

with morphological and molecular data. In the present study, we identified the RKN population intercepted again from baobab from Thailand as *M. enterolobii* morphologically and molecularly. To the best of our knowledge, therefore, this is the first report of *M. enterolobii* from baobab.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgment

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