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Morphological and molecular characterization of *Trophurus wuhuensis* n. sp. (Nematoda: Telotylenchinae) from soil associated with *Cinnamomum camphora* in China

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Research Paper

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Abstract

A new plant nematode species, *Trophurus wuhuensis* n. sp., was collected from the soil associated with *Cinnamomum camphora* in Wuhu, Anhui Province, China. The new species is characterized by having a female with a slender body 660.5–801.5 µm in length, stylet 12–14 µm long, knobs directed laterad, lateral field marked by short and scattered grooves, post-vulval uterine sac shorter than vulval body diameter, post-rectal intestinal sac absent, tail cylindroid, terminus with deep wrinkles; and male with a pointed tail terminus and spicules 16–18 µm long. The internal transcribed spacer sequences of ribosomal DNA (ITS rDNA) and partial 18S ribosomal DNA (18S rDNA) from *T. wuhuensis* n. sp. were amplified and sequenced. A phylogenetic analysis based on sequences of 18S rDNA fragments is given in this study.

Introduction

The subfamily Telotylenchinae, which contains *Trophurus* Loof, 1956, and related genera, represents a large group of plant-parasitic nematodes (Siddiqi, 2000; Geraert, 2011). According to Geraert (2011), the subfamily Telotylenchinae contains nine genera: *Histotylenchus* Siddiqi, 1971, *Neodolichorhynchus* Jairajpuri & Hunt, 1984, *Paratrophurus* Arias, 1970, *Quinisulcius* Siddiqi, 1971, *Sauertylenchus* Sher, 1974, *Telotylenchus* Siddiqi, 1960, *Trichotylenchus* Whitehead, 1960, *Trophurus* and *Tylenchorhynchus* Cobb, 1913. These nematodes are known as stunt nematodes, and are obligate root ectoparasites of a large variety of plants (Siddiqi, 2000; Handoo *et al.*, 2014). In the subfamily Telotylenchinae, the female reproductive system of most genera is didelphic; however, *Trophurus* is the only genus where the posterior branch of the female reproductive system is reduced to a sac (Siddiqi, 2000; Geraert, 2011; Handoo *et al.*, 2014). *Trophurus* is a small genus, and only 15 species have been detected and described worldwide (Siddiqi, 2000; Geraert, 2011; Sen *et al.*, 2012). According to the literature, the species of *Trophurus* are found mostly in cultivated soils in Africa, Asia, Europe, North and South America (Kleynhans & Cadet, 1994; Sen *et al.*, 2012). For example, *Trophurus impar* Ganguly & Khan, 1983 was described from the rhizosphere soil of the betel vine (*Piper betel*) in India (Ganguly & Khan, 1983). *Trophurus pakendorfi* De Waele & Bolton, 1988 was collected from soil associated with sunflowers (*Helianthus annuus*) in Transvaal (De Waele & Bolton, 1988). In China, *Trophurus minnesotensis* (Caveness, 1958) Caveness, 1959 has been identified and reported from soil associated with tobacco plants (*Nicotiana tabacum*) in Guizhou province (Li & Zhao, 2012). In addition, several unidentified *Trophurus* species have been reported from Taiwan, Tianjin and the provinces of Guangdong and Hainan, China (Li & Zhao, 2012). In 2015, a *Trophurus* species was collected from the soil associated with *Cinnamomum camphora* in Wuhu, Anhui Province, China, and is described here as a new species, *Trophurus wuhuensis* n. sp. The internal transcribed spacer sequences of ribosomal DNA (ITS rDNA) and partial 18S ribosomal DNA (18S rDNA) from *T. wuhuensis* n. sp. were amplified and sequenced. Phylogenetic relationships of the new species with other species in related genera were studied based on the 18S rDNA sequences.

Materials and methods

Collection and examination of nematodes

Soil associated with *C. camphora* was collected in Wuhu, Anhui Province, China. Nematodes were extracted from the soil sample using a modified Baermann funnel method (Hooper *et al.*, 2005), and the *Trophurus* population was picked out by hand for examination.

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Nematodes were killed by gentle heat, fixed in FG solution (formalin : glycerin : water = 10 : 1 : 89), transferred to anhydrous glycerin, using the method described by Seinhorst (1959), and then mounted on permanent slides. The nematodes were measured, photographed and illustrated using a Nikon Eclipse Ti-S microscope equipped with a Nikon DS-Ri2 camera (Nikon, Tokyo, Japan). For scanning electron microscopy (SEM) studies, nematodes were fixed and processed according to the method described previously (Wang *et al.*, 2013), and photographed with a Hitachi S-3400N electron microscope (Hitachi, Tokyo, Japan).

Molecular analysis

DNA of one individual nematode was extracted using proteinase K, as described by Wang *et al.* (2011). Primers used for amplification of the ITS rDNA were as follows: rDNA1 (5′-TTGATTAC GTCCCTGCCCTTT-3′) and rDNA2 (5′-TTTCACTCGCC GTTACTAAGG-3′) (Vrain *et al.*, 1992). The 18S rDNA was amplified as two partially overlapping fragments using two pairs of primers, according to the method described previously, and the primers were as follows: 988 F (5′-CTCAAAGATTAAGCC ATGC-3′) and 1912R (5′- TTTACGGTCAGAACTAGGG-3′) for amplification of the first fragment; 1813 F (5′-CTGC GTGAGAGGTGAAAT-3′) and 2646R (5′-GCTACCTTGTTAC GACTTTT-3′) for amplification of the second fragment (Holterman *et al.*, 2006). Polymerase chain reaction (PCR) amplifications were performed using KOD FX DNA polymerase (Toyobo, Osaka, Japan) in 25 µl of reaction mixture, as described by Wang *et al.* (2016). The following PCR cycling steps were used to amplify the ITS rDNA: pre-denaturation at 94°C for 2 min, followed by 35 cycles (denaturation at 98°C for 10 s, annealing at 58.2°C for 30 s, extension at 68°C for 90 s) and final extension at 72°C for 10 min. The PCR conditions for 18S rDNA amplification were as described above, just changing the annealing temperature to 54°C. The PCR products were purified with a gel extraction kit (Omega, Norcross, Georgia, USA), ligated with pJET1.2/blunt cloning vectors (Thermo Scientific, Waltham, Connecticut, USA) and then sequenced by Sangon Biotech Co. Ltd (Shanghai, PR China). The newly obtained sequences in this study were submitted to the GenBank database.

The 18S rDNA sequences of *T. wuhuensis* n. sp. were compared with sequences of other nematode species in related genera in the GenBank database, using the nucleotide BLAST program from the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence (AY993976) of *Boleodorus thylactus* was chosen for the outgroup taxon, according to Handoo *et al.* (2014). Multiple alignments of the 18S rDNA sequences were performed using ClustalW in MEGA 5.05 (Tamura *et al.*, 2011). Phylogenetic analysis of the sequence dataset was performed by Bayesian inference (BI) using MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001). The best-fit model (GTR+I+G) was obtained by Akaike Information Criterion (AIC) using MrModeltest 2.3 (Nylander, 2004). BI analysis was initiated with a random starting tree. Four Markov chains were run for 1×10^6 generations and sampled at intervals of 100 generations. After discarding burn-in samples, the remaining samples were used to generate a 50% majority rule consensus tree. Posterior probabilities (pp) were given on appropriate clades.

Results

Description of Trophurus wuhuensis *n. sp.*

Taxonomic summary

Type material. Holotype female, 16 paratype females and 4 paratype males are deposited in the Laboratory of Plant Pathology, Henan Agricultural University, Zhenzhou, Henan Province, PR China. Two paratype females are deposited at the University of California Riverside Nematode Collection (Riverside, California, USA).

Type habitat and locality. The new species was collected from the soil associated with *C. camphora* in Wuhu, Anhui Province, China.

Morphological description

Measurements. See table 1.

Female (figs 1a, c–i, 2a–l, 3a–i). Body vermiform, slender, straight to ventrally curved after heat relaxation. Body annuli very fine, 0.8 ± 0.1 (0.7–0.9) µm wide at mid-body. Lateral field 6 ± 0.4 (5–7) µm wide, with four distinct lines forming three bands. SEM photographs show that the three lateral bands are not smooth, but are marked by short and scattered grooves. Head sub-truncate, without transverse striae, and continuous with body contour. Cephalic framework weak. SEM view of the head shows a kind of rim on the top of the head of about 1.5 µm in diameter, surrounding a sunken area with apparently six papillae around an indistinct mouth opening; amphidial apertures not distinct, outside the rim, on the lateral side of the head. Stylet slender and straight, 13 ± 0.5 (12–14) µm long; stylet knobs rounded, directed laterad; cone occupying 47.3 ± 2.9 (41.3–53.7)% of stylet length. Isthmus slender, 29 ± 1.8 (23.5–31.5) µm long. Nerve ring at middle or slightly anterior region of isthmus. Basal bulb pyriform in shape, abuts intestine or the dorsal gland slightly overlaps intestine; cardia prominent. Excretory pore situated opposite middle of isthmus. Hemizonid situated 3–8 annuli posterior to excretory pore, 3–4 annuli wide. Vulva 425.5 ± 19.5 (394–456) µm from anterior end. Ovary single, outstretched, oocytes arranged in two rows. Spermatheca rounded or oval, 11 ± 1.2 (9–13) µm long and 10 ± 1.4 (7.5–13) µm wide, filled with round spermatozoa. Post-vulval uterine sac 14 ± 1.9 (11.5–17.5) µm long, about seven-tenths of vulval body diameter. Post-rectal intestinal sac absent. Tail cylindroid, hyaline tail portion 9.5 ± 0.9 (8–11.5) µm long. Tail terminus not smooth, slightly rough when being observed by the light microscope. SEM photographs show a broadly rounded terminus with deep wrinkles.

Male (figs 1b, j, 2m–o). Similar to female except for reproductive system and tail shape. Testis outstretched, with small spermatozoa. Spicules distinct, 17 ± 1 (16–18) µm long. Tail tapering gradually to a pointed terminus. Bursa large, surrounding tail tip.

Diagnosis and relationships

Trophurus wuhuensis n. sp. is characterized by having females with a slender body 660.5–801.5 µm in length, stylet 12–14 µm long, knobs directed laterad, lateral field marked by short and scattered grooves, post-vulval uterine sac shorter than vulval body diameter, post-rectal intestinal sac absent and tail terminus with deep wrinkles; and by males with spicules 16–18 µm long.

When using the key to the species of *Trophurus* proposed by Kleynhans & Cadet (1994) and Geraert (2011), and using the

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Table 1. Morphometics (μm) of *Trophurus wuhuensis* n. sp. from China. All measurements are in the form: mean ± SD (range); *n*, number of specimens observed; *L*, body length; *a, L*/max. width; *b, L*/pharyngeal length; *c, L*/tail length; *c′* = tail length/anal body diameter; *V*, distance of vulva from anterior end × 100/*L*; *V′*, distance from anterior end to vulva × 100/distance from anterior end to anus; *T*, distance between cloaca and anterior-most part of testis × 100/*L*; *m*, metenchium length × 100/stylet length.

	Female		Male
Character	Holotype	Paratypes	Paratypes
n	–	18	4
L	709	729.5 ± 37.9 (660.5–801.5)	670 ± 42.6 (611.5–709.5)
a	33.8	34.1 ± 2.3 (27.6–38.6)	39.3 ± 1.8 (37.7–41.8)
b	5.5	5.6 ± 0.3 (5.2–6.1)	–
c	32.2	30.9 ± 2.6 (25.9–34.8)	22.1 ± 1.2 (20.9–23.3)
c′	1.4	1.5 ± 0.1 (1.3–1.7)	2.3 ± 0.2 (2.2–2.5)
V	59.0	58.4 ± 1.4 (56.2–61.3)	–
V′	60.8	60.4 ± 1.4 (58.3–63.1)	–
T	–	–	30.7 ± 3.1 (27.6–34.9)
Stylet length (St)	13.5	13 ± 0.5 (12–14)	12.5 ± 1.3 (11–13.5)
Metenchium length	6.5	6 ± 0.6 (5–7)	–
Telenchium length	7	7 ± 0.3 (6–7)	–
m	48.1	47.3 ± 2.9 (41.3–53.7)	–
Stylet knob height	1.5	1.3 ± 0.2 (1.1–1.8)	1.2 ± 0.2 (1–1.4)
Stylet knob width	2.1	2 ± 0.2 (1.8–2.5)	2.1 ± 0.1 (1.9–2.2)
Excretory pore (Ep)	84.5	88.5 ± 3.8 (83.5–95)	85.5 ± 3.9 (81.5–89)
Pharynx (Ph)	128	130 ± 5.5 (115.5–138)	133.5 ± 4.2 (128–138.5)
Head to vulva	418	425.5 ± 19.5 (394–456)	–
Max. body diameter	21	21.5 ± 2 (18–26.5)	17 ± 1.2 (15.5–18.5)
Vulval body diameter	18.5	19.5 ± 2.2 (16–24.5)	–
Anal body diameter	15.5	16 ± 1.7 (11.5–19)	–
Post-vulval uterine-sac length	11	14 ± 1.9 (11.5–17.5)	–
Tail length	22	23.5 ± 2.4 (19–27.5)	30.5 ± 1.4 (29–32)
Hyaline portion	8	9.5 ± 0.9 (8–11.5)	–
St%*L*	1.9	1.8 ± 0.1 (1.7–2.0)	1.9 ± 0.2 (1.6–2.1)
St%Ph	10.5	10.1 ± 0.7 (9.2–11.3)	9.5 ± 1 (8.1–10.5)
Ep%*L*	11.9	12.2 ± 0.6 (11.2–13.5)	12.8 ± 0.6 (12.2–13.6)
Spicule length	–	–	17 ± 1 (16–18)
Gubernaculum length	–	–	4.5 ± 0.1 (4.3–4.6)

body length, stylet length and post-rectal intestinal sac absence as a guide, *T. wuhuensis* n. sp. is close to *Trophurus clavicaudatus* Sen, Chatterjee & Manna, 2012, *Trophurus deboeri* Kleynhans & Cadet, 1994, *Trophurus lomus* Saha, Chawla & Khan, 1974, *Trophurus longimarginatus* Roman, 1962, *Trophurus marathwadensis* Suryawanshi, 1971, *Trophurus scognamiglii* Talamé, 1974, *Trophurus sculptus* Loof, 1956 and *Trophurus similis* Khan & Nanjappa, 1971. *Trophurus wuhuensis* n. sp. can be separated easily from these eight species by having a rough tail terminus with deep wrinkles in the female. Some other characters separating *T. wuhuensis* n. sp. from these species are described below. The new species differs from *T. clavicaudatus* by having a shorter stylet length (12–14 μm vs. 17–17.5 μm), a shorter distance from excretory pore to anterior end (83.5–95 μm vs. 103–110 μm), a shorter tail length (19–27.5 μm vs. 51.5–59 μm), a higher *c* value (25.9–34.8 vs. 12.5–15.5) and a lower *c′* value (1.3–1.7 vs. 3.0–3.9) in the female; and a shorter spicule length (16–18 μm vs. 22.5–24.5 μm) in the male (Sen *et al.*, 2012). It differs from *T. deboeri* by having a lower *a* value (27.6–38.6 vs. 42–56), a lower *c′* value (1.3–1.7 vs. 1.7–2.9), stylet knobs directed laterad vs. back-sloped, spermatheca rounded or oval vs. lobed, and post-vulval uterine sac shorter than vulval body diameter vs. about equal to vulval body diameter in the female (Kleynhans & Cadet, 1994). It differs from *T. lomus* by having a shorter stylet length (12–14 μm vs. 16–18 μm), a shorter pharynx length (115.5–138 μm vs. 160 μm), a shorter tail length (19–27.5 μm

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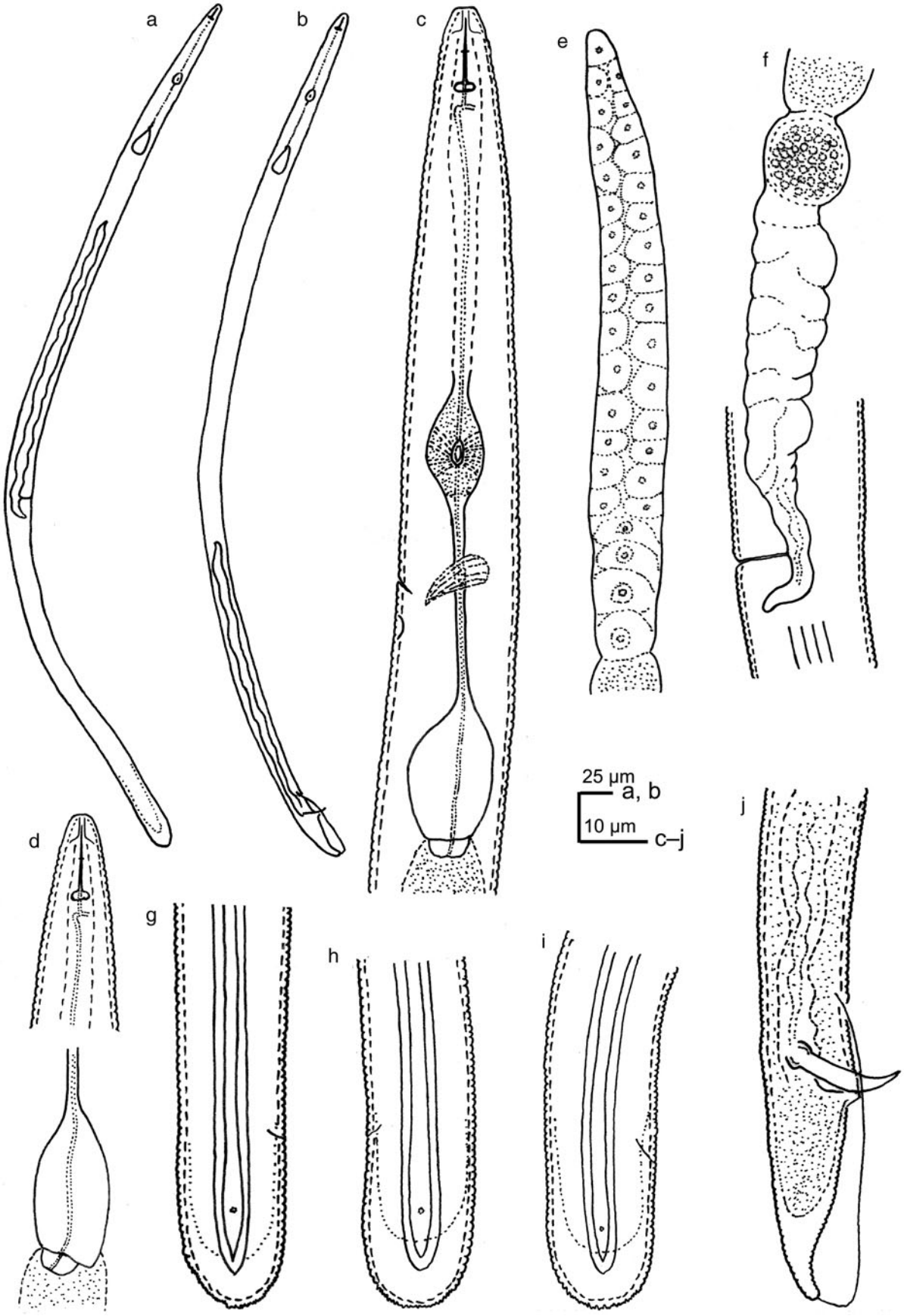

Fig. 1. Morphology of *Trophurus wuhuensis* n. sp. Female: (a) entire body, (c) anterior part, (d) anterior end and basal bulb, (e–f) reproductive system and lateral lines, (g–i) tails. Male: (b) entire body, (j) posterior part.

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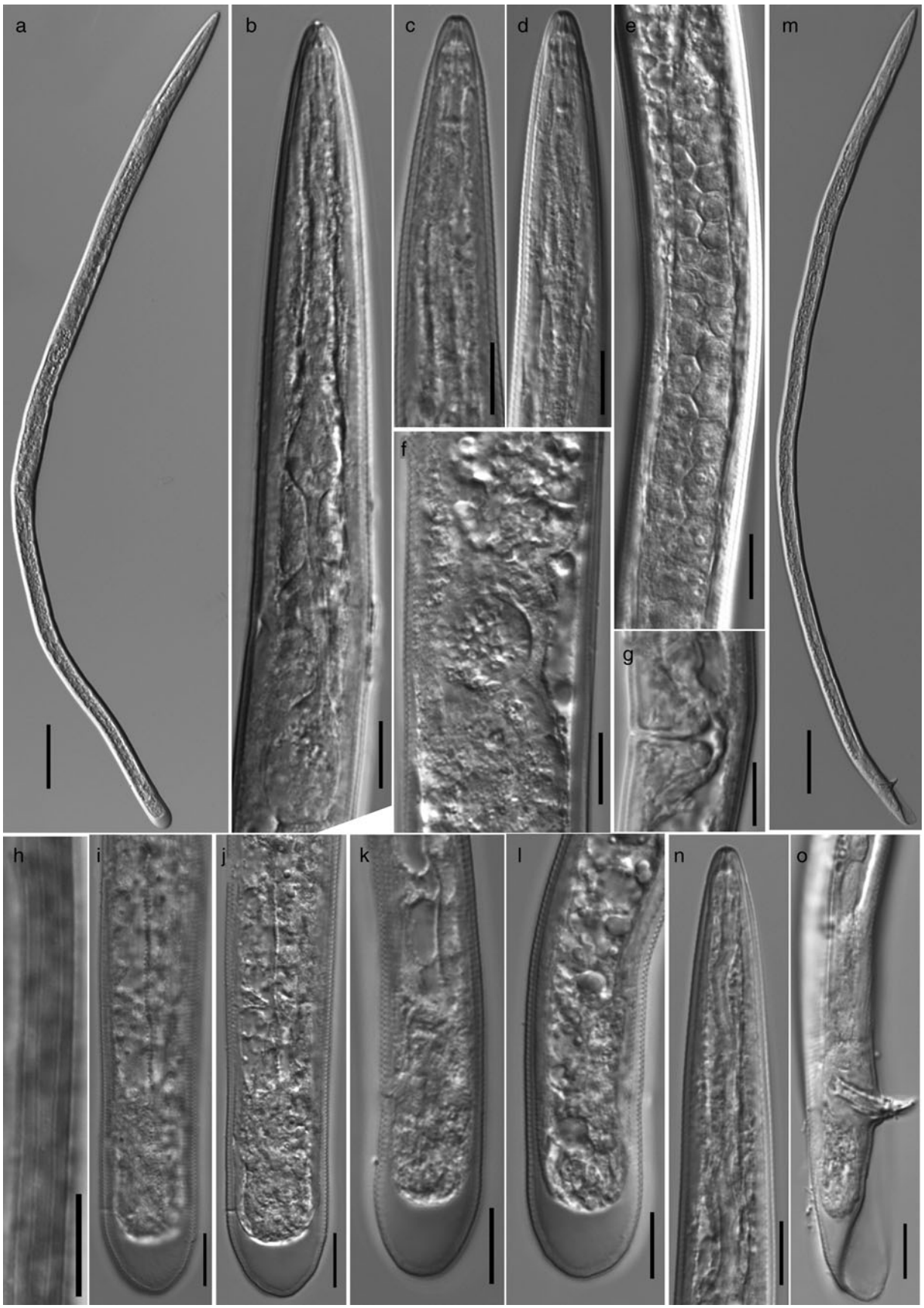

Fig. 2. Light micrographs of *Trophurus wuhuensis* n. sp. Female: (a) entire body, (b) anterior part, (c–d) anterior end, (e) oocytes in two rows, (f) spermatheca, (g) post-uterine sac, (h) lateral lines, (i–l) tails. Male: (m) entire body, (n) anterior end, (o) tail and spicule. Scale bars: (a, m) 50 μm; (b–l, n–o) 10 μm.

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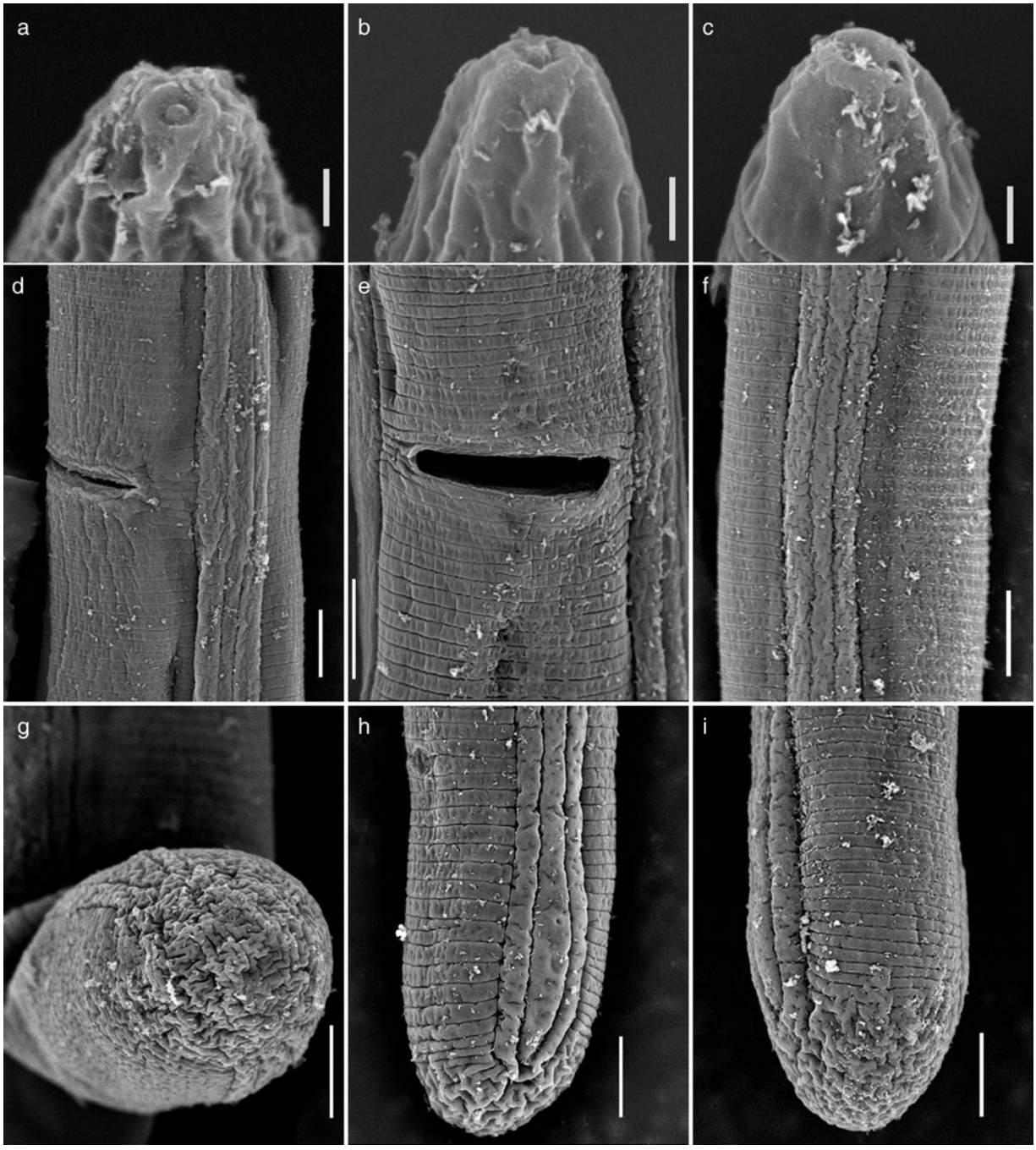

Fig. 3. Scanning electron micrographs of *Trophurus wuhuensis* n. sp. Female: (a) *en face* view; (b–c) head, oblique view; (d) vulva, lateral view; (e) vulva, ventral view; (f) lateral field marked by short and scattered grooves; (g) tail terminus with deep wrinkles; (h–i) tails. Scale bars: (a–c) 1 µm; (d–i) 5 µm.

vs. 33 µm) and a shorter distance from excretory pore to anterior end (83.5–95 µm vs. 105 µm) in the female; and a shorter spicule length (16–18 µm vs. 20–22 µm) and a shorter gubernaculum length (4.3–4.6 µm vs. 7–8 µm) in the male (Geraert, 2011). It differs from *T. longimarginatus* by having a shorter body length (660.5–801.5 µm vs. 840–1050 µm), a shorter stylet length (12–14 µm vs. 14–16 µm), a shorter tail length (19–27.5 µm vs. 32 µm) and a lower *a* value (27.6–38.6 vs. 41–50) in the female; and a longer spicule length (16–18 µm vs. 11–15 µm) in the male (Geraert, 2011). It differs from *T. marathwadensis* by having a shorter body length (660.5–801.5 µm vs. 1030–1210 µm), a shorter stylet length (12–14 µm vs. 15–16 µm), a shorter tail length (19–27.5 µm vs. 37 µm), oocytes arranged in two rows vs. in a single row, a lower *a* value (27.6–38.6 vs. 43–50) and a lower *b* value (5.2–6.1 vs. 6.2–7.4) in the female; and a shorter spicule length (16–18 µm vs. 19–22 µm) in the male (Kleynhans & Cadet, 1994; Geraert, 2011). It differs from *T. scognamiglii* by having a shorter body length (660.5–801.5 µm vs. 850–1010 µm), a shorter stylet length (12–14 µm vs. 14.5–17 µm), a shorter pharynx length (115.5–138 µm vs. 160 µm) and ovary outstretched vs. plicate in the female; and a shorter spicule length (16–18 µm vs. 22 µm) in the male (Geraert, 2011). It differs

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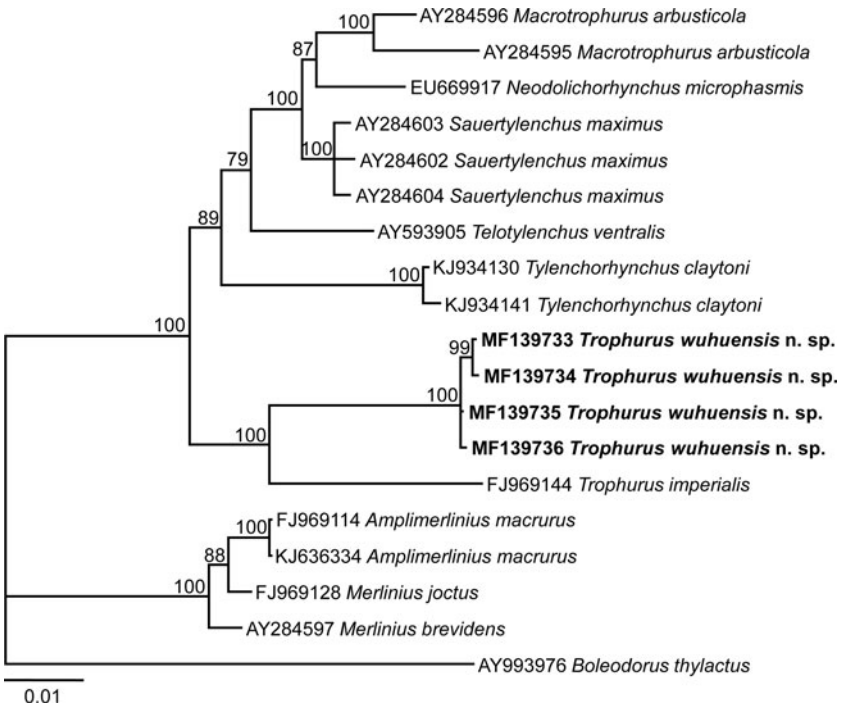

Fig. 4. The 50% majority rule consensus tree inferred from the 18S rDNA sequences of *Trophurus wuhuensis* n. sp. and some other species in related genera under the GTR+I+G model; posterior probabilities more than 50% are given for appropriate clades; newly obtained sequences are indicated in bold font.

from *T. sculptus* by having a shorter tail length (19–27.5 µm vs. 29–44 µm), a higher *c* value (25.9–34.8 vs. 16–24), spermatheca rounded or oval vs. bilobed, and vagina without epiptygma vs. with small double epiptygma in the female (Geraert, 2011); and from *T. similis* by having a lower *a* value (27.6–38.6 vs. 40–58), a longer stylet length (12–14 µm vs. 9–11 µm), a shorter tail length (19–27.5 µm vs. 36 µm) and a higher *c* value (25.9–34.8 vs. 18–25) in the female (Geraert, 2011).

Molecular characterization and phylogenetic relationships

Two ITS rDNA sequences of *T. wuhuensis* n. sp. were obtained and submitted to the GenBank database under accession numbers MF139731–MF139732. The length of the two ITS sequences was 1045–1046 bp with 6 bp variation. At present, no other ITS sequences of *Trophurus* species are available in the GenBank database.

Four 18S rDNA sequences of *T. wuhuensis* n. sp. were obtained and submitted to the GenBank database under accession numbers MF139733–MF139736. The length of the four 18S sequences was 1748 bp with 1–3 bp variation. The BLAST search showed that the 18S sequences of the new species were closest to the sequence from *T. imperialis* Loof, 1956 (FJ969144) within the sequenced *Trophurus* species. Identity between the 18S sequences from *T. wuhuensis* n. sp. and *T. imperialis* was 95%. Alignment of the 18S rDNA contained 19 sequences with 1685 positions in length. The 50% majority rule consensus tree reconstructed from the 18S dataset by the Bayesian analysis is shown in fig. 4. In this tree, four 18S rDNA sequences of *T. wuhuensis* n. sp. clustered together, and formed a 100% supported clade with that of *T. imperialis*. Other selected nematode species in the subfamily Telotylenchinae, including *Neodolichorhynchus microphasmis*, *Sauertylenchus maximus*, *Telotylenchus ventralis* and *Tylenchorhynchus claytoni* were in a 89% supported monophyletic clade and sister to the two *Trophurus* species (*T. wuhuensis* n. sp. and *T. imperialis*) with high support (pp = 100%).

Discussion

In this study, a new species, *T. wuhuensis* n. sp. was obtained from *C. camphora* in Anhui Province, China, and thus the current number of *Trophurus* species is increased to 16. Kleynhans & Cadet (1994) gave a dichotomous key to species of *Trophurus* in which body length, stylet length, tail shape, shape of stylet knobs, *a* value, *c* value, etc. were used to differentiate *Trophurus* species. These species usually show considerable variation in many characters. For example, the basal bulb is usually offset from the intestine, but the dorsal gland may extend slightly over the intestine; the posterior branch of the female reproductive system is completely regressed and represented by a uterine sac, but often carries rudiments of posterior ovary; spermatheca oval, rounded, lobed or bilobed; post-rectal intestinal sac absent or present; female tail terminus may be distinctly annulated, appearing smooth or rough with deep wrinkles (Kleynhans & Cadet, 1994; Siddiqi, 2000; Geraert, 2011). Sher & Bell (1975) reported that *Trophurus* sp. did not show any structures on the lip region, not even the amphid apertures or oral opening, when examined by SEM. In the observation of *T. wuhuensis* n. sp., the SEM view of the head showed a kind of rim on the top of the head, surrounding a sunken area with apparently six papillae around an indistinct mouth opening; amphidial apertures were outside the rim, on the lateral side of the head. In addition, the tail terminus of the new species was slightly rough when observed by light microscopy; however, SEM photographs showed a broadly rounded terminus with deep wrinkles. Thus, we believe that the SEM micrographs will provide many useful characters with which to identify *Trophurus* species.

Molecular data become more and more valuable in the identification of closely similar species. However, there is no molecular information available on most *Trophurus* species. Therefore, we believe that, in the future, the use of molecular data will also make the identification of *Trophurus* more accurate.

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Conflict of interest. None.

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