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Morphological and molecular characterisation of *Hemicaloosia guangzhouensis* n. sp. (Nematoda: Caloosiidae) from China

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Abstract A new plant nematode species, *Hemicaloosia guangzhouensis* n. sp., collected from the soil associated with *Schima superba* in a forest park in Guangzhou, China is described. The new species is characterized by having a female with a slender body, 798–961 μm in length, stylet 59–69 μm long, tail 115–145 μm long, a membranous sheath closely adpressed to the entire body, lateral fields marked by one longitudinal line with breaks and anastomoses of transverse striae, total number of annuli 282–313, two lip annuli both projecting slightly anterior, excretory pore 51–58 annuli from anterior end, the number of annulus between vulva and anus 7–11, the number of annulus from vulva to tail terminus 58–69, spermatheca empty; and male unknown. The internal transcribed spacer of ribosomal RNA (ITS rRNA) gene, D2-D3 region of 28S ribosomal RNA (28S rRNA) gene, partial 18S ribosomal RNA (18S rRNA) gene of the new species were amplified and sequenced. The 50 % majority rule consensus trees inferred from ITS rRNA, 28S rRNA, partial 18S rRNA gene sequences of *H. guangzhouensis* n. sp. and some other species in the suborder Criconematina are also presented in this study. PCR-ITS-RFLP diagnostic profile generated by six restriction enzymes for *H. guangzhouensis* n. sp. is given.

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Introduction

Nematodes of the family Caloosiidae are plant parasites widely occurring in India and Sri Lanka, and being detected in Surinam, Ivory Coast, Australia and America (Siddiqi 2000; Zeng et al. 2012), but have not yet been found in China. The family consists of two genera: *Caloosia* Siddiqi & Goodey, 1964 and *Hemicaloosia* Ray & Das, 1978. They are considered to be associated with many plants, and some are able to injure roots and affect growth of their hosts. For example, *C. exilis* was found to be associated with 38 plant species in India (Siddiqi 2000). *C. paxi* feeds on meristematic tissues of rice roots and reduces apical growth (Rao and Mohanadas 1976). *Hemicaloosia nudata* was found to be associated with stunted citrus plants where it produced root galls (Colbran 1963; Duncan 2005). *Caloosia* and *Hemicaloosia* are separated by the membranous body sheath and lateral field in females and juveniles which are absent in *Caloosia*, but present in *Hemicaloosia* (Siddiqi 2000). Raski and Luc (1987) considered the differential characters insufficient to differentiate the two genera and synonymized *Hemicaloosia* with *Caloosia*, but this opinion was not accepted by Siddiqi (2000) and Chitambar and Subbotin (2014). In this study, we adopted the classification proposed by Siddiqi (2000) for this family and considered

Caloosia and *Hemicaloosia* both as valid taxons. In October 2014, a *Hemicaloosia* species was found from the soil associated with *Schima superba* in Guangzhou, China. Morphological and morphometric studies of the nematode revealed that it is a new species and the nematode is here described as *Hemicaloosia guangzhouensis* n. sp. The internal transcribed spacer sequences of ribosomal RNA (ITS rRNA) gene, the D2-D3 region of 28S ribosomal RNA (28S rRNA) gene and the partial 18S small subunit ribosomal RNA (18S rRNA) gene from *H. guangzhouensis* n. sp. were amplified and sequenced.

Material and methods

Nematode population and morphological identification

Soil associated with *Schima superba* was collected in a forest park in Guangzhou, Guangdong Province. Nematodes were extracted from the sample using a modified Baermann funnel method. Nematodes for morphological study were killed by heat, fixed in FG solution (formalin:glycerin:water=10:1:89) (Xie 2005), transferred to anhydrous glycerin according to Seinhorst (1959), and then mounted on permanent slides. Measurements were performed using a Nikon Eclipse 90i microscope with NIS-Elements microscope imaging software (Nikon, Tokyo, Japan). Light photomicrographs of nematodes were taken using an AxioCam MRm camera attached to a Zeiss Axio Scope A1 microscope (Zeiss, Jena, Germany). Drawings were made using an Olympus CX40 microscope equipped with a drawing tube (Olympus, Tokyo, Japan). For scanning electron microscopy (SEM) studies, fixed nematodes were processed according to Wang et al. (2013), and observed with a FEI XL-30-ESEM electron microscope (Philips, Eindhoven, Netherlands).

DNA extraction, PCR and sequencing

DNA was extracted from two hand-picked nematodes using proteinase K according to the method described by Wang et al. (2011). Primers used for amplification of three rRNA gene fragments were as follows: TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) (Tanha Maafi et al. 2003) for amplification of the ITS rRNA gene; D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) (Subbotin et al. 2006) for amplification of the D2-D3 region of the 28S rRNA gene; G18SU (5′-GCTTGTCTCAAAGATTAAGCC-3′) and R18Tyll (5′-GGTCCAAGAATTTCACCTCTC-3′) (Inserra et al. 2013) for amplification of the partial 18S rRNA gene. The PCR products were purified by a Gel Extraction Kit (Omega, Norcross, Georgia, USA), cloned into pJET 1.2/blunt cloning vectors (Thermo Scientific, Waltham, Connecticut, USA), and then sequenced by Sangon Biotech Co. Ltd (Shanghai, PR China).

Phylogenetic analyses

The sequences of *H. guangzhouensis* n. sp. were compared with other *Hemicaloosia* sequences in the GenBank database by the BLAST program implemented in NCBI. The newly obtained sequences for each gene and other corresponding gene sequences published before (Van den Berg et al. 2011; Inserra et al. 2013) were used for phylogenetic analyses. Outgroup taxa for each dataset were chosen according to Van den Berg et al. (2011) and Inserra et al. (2013). Sequences were aligned by ClustalW in MEGA 5.05 (Tamura et al. 2011) and edited using Gblocks in Phylogeny. fr (Dereeper et al. 2008). Phylogenetic analyses were performed by Bayesian inference (BI) using MrBayes3.1.1 (Huelsenbeck and Ronquist 2001). The best-fit model was determined by the Akaike Information Criterion (AIC) implemented in MrModeltest 2.3 (Nylander 2004). BI analysis for each dataset was initiated with a random starting tree and was run with four Markov chains for 1,000,000 generations. The Markov chains were sampled every 100 generations. Two runs were performed for each analysis. 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.

PCR-ITS-RFLP

The purified PCR product of the ITS rRNA gene was digested by each of the following six restriction enzymes: *Ava*I, *Bsh1236*I, *Dra*I, *Hin*fI, *Hin*6I and *Msp*I following manufacturer's protocols. The digested fragments were electrophoresed on a 2 % TAE buffered agarose gel, stained, and

photographed under UV light. The length of each restriction fragment from the PCR products was predicted using Primer Premier 5 (Lalitha 2000).

Results

Hemicaloosia guangzhouensis n. sp. (Figs. 1, 2 and 3)

Measurements of holotype female and 20 paratype females are listed in Table 1.

Description

Female

Body slender, slightly ventrally curved when relaxed. A membranous sheath thinner than body cuticle and closely adpressed to the entire body. Body annuli 2.9±0.1 (2.7–3.2) μm wide at mid-body. Lateral field on sheath begin from second to fifth annulus, marked by breaks and anastomoses of transverse striae with one

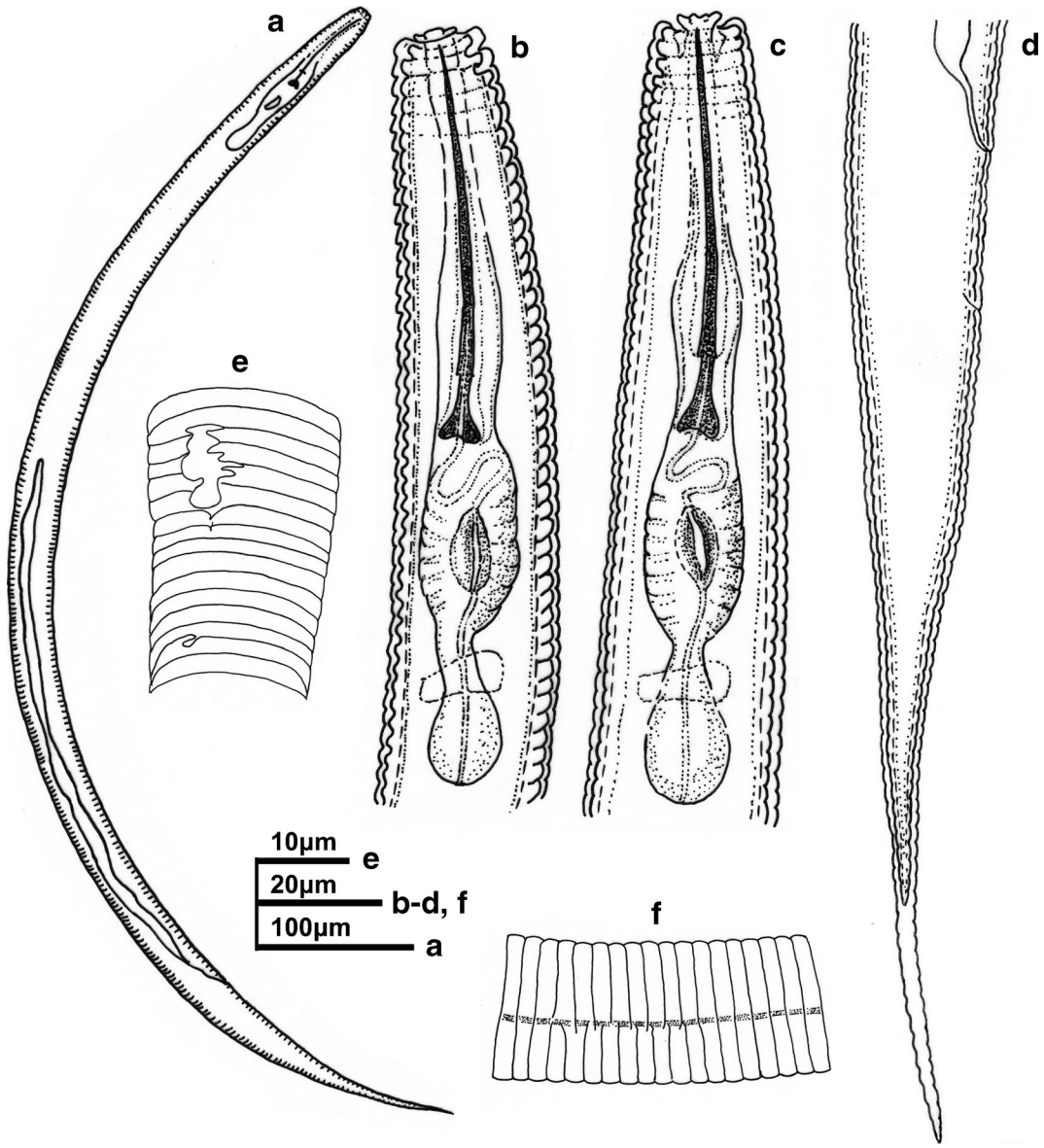
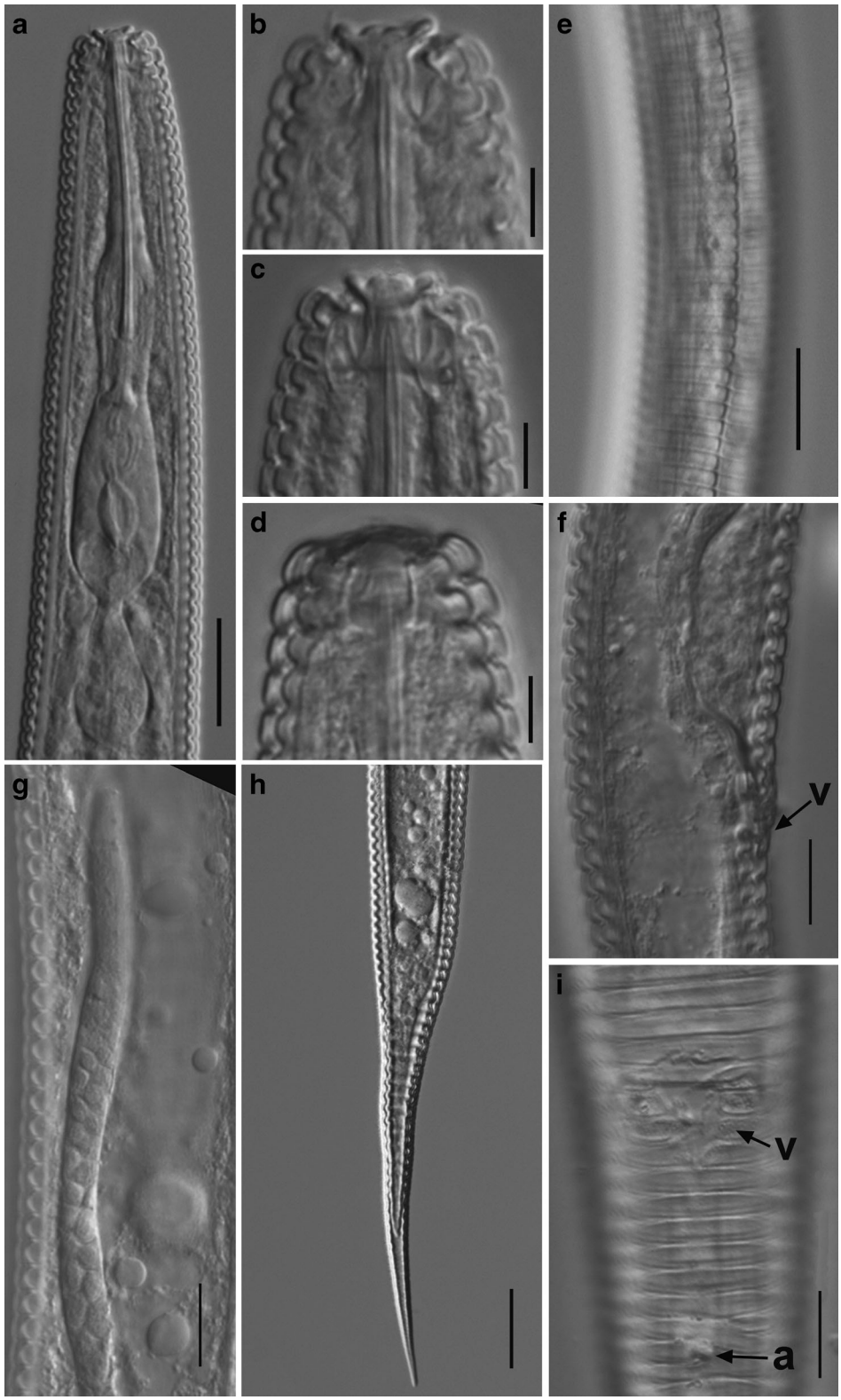


Fig. 1 *Hemicaloosia guangzhouensis* n. sp. **a** total view; **b** anterior body region, lateral view; **c** anterior body region, ventral view; **d** posterior region; **e** vulva and anus at the membranous sheath; **f** lateral field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◀ **Fig. 2** Photomicrographs of *Hemicaloosia guangzhouensis* n. sp. **a** anterior body region, ventral view; **b**, **c** lip region, ventral view; **d** lip region, lateral view; **e** lateral field; **f** vulva, lateral view; **g** oocytes at anterior part in two rows; **h** posterior region; **i** vulva and anus at the membranous sheath. Abbreviations: *v* vulva; *a* anus. (Scale bars: **a**, **e**, **h**=20 μm; **b-d**=5 μm; **f-g**, **i**=10 μm)

longitudinal line forming a depression. Lip region with two annuli, first lip annulus slightly narrower than second, and both projecting slightly anterior. SEM *en face* view showing a large, oval, forward-projecting labial disc with a prominent, oval oral disc. Oral opening I-shaped. Cephalic framework weak. Stylet slender, slightly curved, 65.5±2.2 (59–69) μm long; stylet knobs rounded, sloping backwards; cone occupying 80.9 ±2.2 (77.0–83.7) % of stylet length. Median pharyngeal bulb well developed, valve 10.8±0.6 (9.5–12) μm long; basal pharyngeal bulb pyriform in shape, amalgamated with short broad isthmus. Nerve ring at level of isthmus and basal bulb fusion. Hemizonid not observed. Excretory pore located from nine to fourteen annuli posterior to basal pharyngeal bulb. Vulva flush with body contour, 63±2.9 (58–69) annuli from tail terminus; vulva opening irregular in shape at the membranous sheath. Reproductive system monoprodelphic, outstretched, oocytes at anterior part in two rows. Vagina sigmoid. Post-vulval uterine sac absent. Spermatheca indistinct, empty. Tail slender, tapering gradually, becoming filiform with an acute terminus.

Male

Not found.

Type locality and habitat

The new species was recovered from the soil associated with *Schima superba* in a forest park in Guangzhou, Guangdong Province, China.

Etymology

The species name "*guangzhouensis*" refers to the city where the species was found.

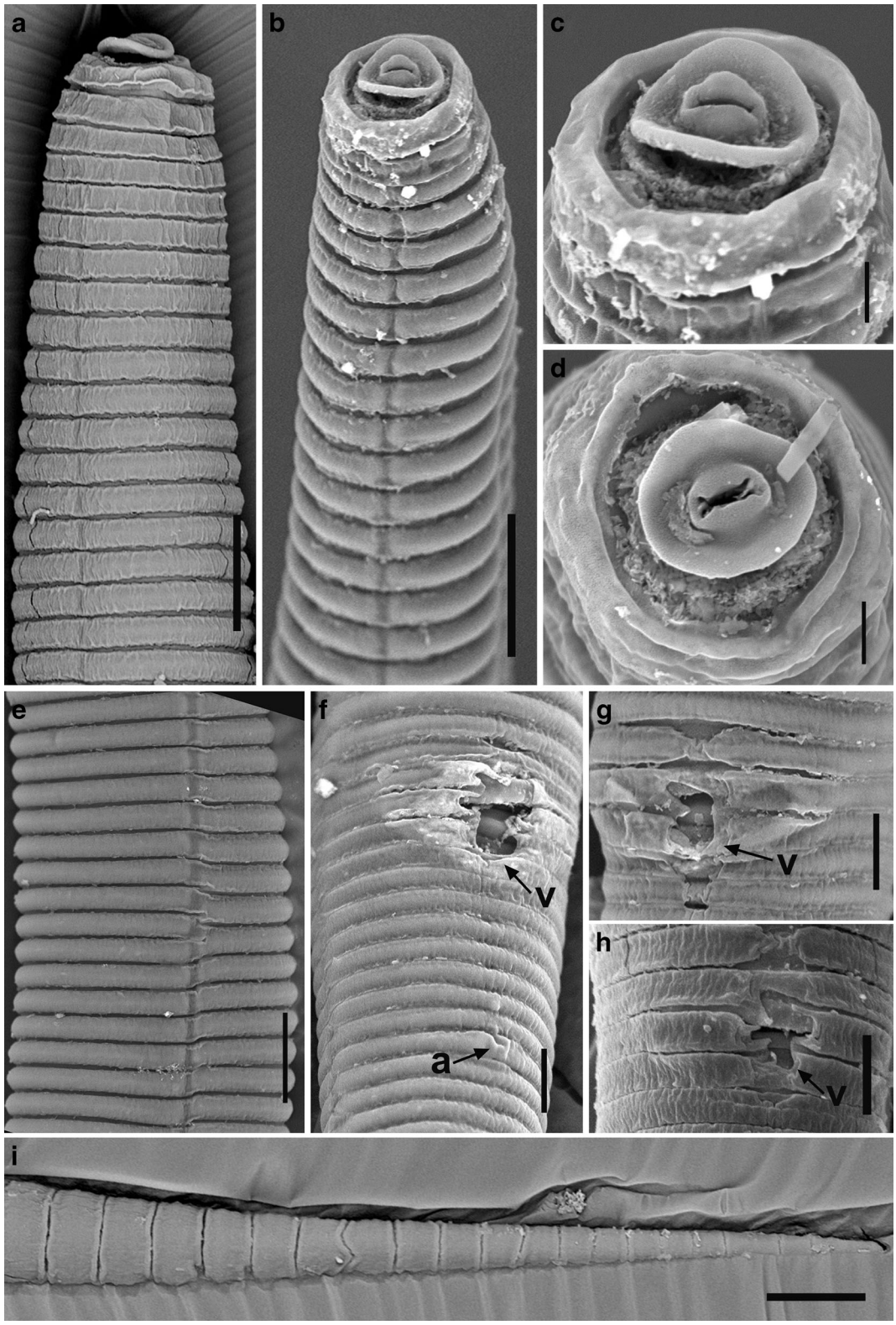
Type material

Holotype female and 18 paratype females are deposited in the Laboratory of Plant Nematology, South China Agricultural University, Guangzhou, Guangdong Province, PR China. Two paratype females are deposited at the University of California Riverside Nematode Collection (Riverside, CA, USA).

Diagnose and relationships

Hemicaloosia guangzhouensis n. sp. is characterized by having a female with a slightly ventrally curved body, 798–961 μm long, total number of annuli (R) 282–313, stylet 59–69 μm long, a membranous sheath closely adpressed to the entire body, lateral fields marked by one longitudinal line with breaks and anastomoses of transverse striae, two lip annuli both projecting slightly anterior, spermatheca empty, tail 115–145 μm long, number of annulus between vulva and anus (RVan) 7–11, and male unknown.

When using male unknown and spermatheca without sperm as a guide, the new species comes close to *H. delpradi* (Maas, 1970) Siddiqi 1980, *H. langola* (Pramodini, Mohilal & Gambhir, 2007) Van den Berg et al. 2011, *H. luci* Dhanachand & Jairajpuri, 1980, *H. psidii* Gambhir & Dhanachand, 1997 and *H. uarki* Cordero López., Robbins & Szalanski, 2013. When following the key of Chitambar and Subbotin (2014), the new species is closest to *H. luci*. The new species differs from *H. luci* by the female having a lateral field marked by one longitudinal line with breaks and anastomoses of transverse striae vs. two incisures, a less number of annulus from anterior end to base of stylet knob (RSt) and RVan (21–26 vs. 27–35 and 7–11 vs. 11–18, respectively) and a greater number of annulus from tail terminus to anus (Ran=49–59 vs. 23–48). It differs from *H. delpradi* by the female having a shorter stylet (59–69 μm vs. 75–78 μm), a greater R value (282–313 vs. 261–283), a greater number of annulus from tail terminus to vulva (RV=58–69 vs. 50–58), a lower RVan (7–11 vs.13–18) and a greater Ran value (49–59 vs.35–42). It differs from *H. langola* by the female having a greater number of annulus from anterior end to excretory pore (Rex =51–58 vs. 45–51), a greater a, R, RV and Ran values (24.9–34.4 vs. 20–24, 282–313 vs. 243–259, 58–69 vs. 38–45 and 49–59 vs. 29–36, respectively). It differs from *H. psidii* by the female having a longer body length, stylet length and tail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◀ **Fig. 3** Scanning electron micrographs of *Hemicaloosia guangzhouensis* n. sp. **a**, **b** anterior body region, lateral view; **c**, **d** en face view; **e** lateral field showing one longitudinal line with breaks and anastomoses of transverse striae; **f** vulva and anus, ventral view; **g**, **h** vulva, ventral view; **i** posterior part of tail. Abbreviations: *v* vulva; *a* anus. (Scale bars: **a**-**b**, **e**=10 μm; **c**-**d**= 2 μm; **f**-**i**=5 μm)

length (798–961 vs. 560–740 μm, 59–69 vs. 46–58 μm and 115–145 vs. 57–87 μm, respectively), and a greater R, Rex, RV and Ran (282–313 vs. 212–266, 51–58 vs. 42–45, 58–69 vs. 51–52 and 49–59 vs. 19–23, respectively). It differs from *H. uarki* by the female having a shorter stylet length (59–69 vs. 106–128 μm), a lower R, RV and RVan (282–313 vs. 338–377, 58–69 vs. 72–81, 7–11 vs. 22–29, respectively).

Table 1 Morphometrics of *Hemicaloosia guangzhouensis* n. sp. from the soil associated with *Schima superba* from Guangzhou, Guangdong Province, China. All measurements in μm and in the form: mean±s.d. (range)

Character	Female	
	Holotype	Paratypes
n	1	20
L	871	906±38.3 (798–961)
a	29.5	28.8±2.6 (24.9–34.4)
b	7.2	7.4±0.2 (7.1–8.0)
c	6.7	7.0±0.3 (6.5–7.6)
c’	6.1	5.8±0.4 (5.1–6.5)
V	82.1	82.4±0.8 (81.2–84.2)
Stylet length (St)	65.5	65.5±2.2 (59–69)
Metenchium length	54.5	53±1.8 (47–56)
Telenchium length	11	12.5±1.7 (10.5–16)
m	83.2	80.9±2.2 (77.0–83.7)
Stylet knob height	4	4±0.5 (2.5–4.5)
Stylet knob width	6	6±0.5 (5–6.5)
Excretory pore(Ep)	147	157±7.6 (146–175)
Pharynx (Ph)	121	122±5 (109–130.5)
Head to vulva	714.5	747±34 (659–802)
Max body diam.	29.5	32±3.2 (27–37)
Tail length	131	130±6.8 (115–145)
St%L	7.5	7.3±0.3 (6.8–7.8)
St%Ph	54.1	53.7±2 (50.6–57.8)
Ep%L	16.9	17.4±0.6 (16.4–18.4)
R	307	298±7.8 (282–313)
RSt	26	24±1.4 (21–26)
Rex	54	54±2 (51–58)
RV	66	63±2.9 (58–69)
RVan	10	9±1 (7–11)
Ran	55	53±2.7 (49–59)

Phylogenetic relationships of *H. guangzhouensis* n. sp.

The two newly obtained ITS-rRNA gene sequences of *H. guangzhouensis* n. sp. were 805 bp in length, and were submitted to the GenBank database under accession numbers KT381014-KT381015. Variation of the two ITS sequences was 0 bp. However, no sequences of the morphological close species were available to compare genetic relationship with *H. guangzhouensis* n. sp. The BLAST search showed the ITS-rRNA gene sequences from the new species were closest to the sequences from *H. vagisclera* Inserra et al. 2013 (JQ246427-JQ246429), and the sequence identities were 76–77 %. The ITS-rRNA gene alignment contained 21 sequences with 589 positions in length. The 50 % majority rule consensus tree inferred from the ITS data set by Bayesian analysis is shown in Fig. 4. In this tree, the two ITS-rRNA gene sequences of *H. guangzhouensis* n. sp. clustered together, and formed a 100 % supported clade with that of *H. vagisclera*.

The two 769-bp D2-D3 regions of the 28S rRNA gene sequences of *H. guangzhouensis* n. sp. were submitted to the GenBank database under accession numbers KT381016-KT381017. Variation of the two D2-D3 sequences was 1 bp. At present, only three D2-D3 sequences from other *Hemicaloosia* species are available in the Genbank database. The three sequences were from *H. vagisclera* with accession numbers JQ246422-JQ246424. The identities of the D2-D3 sequences from *H. guangzhouensis* n. sp. and *H. vagisclera* were 89 %. Alignment of the D2-D3 region of 28S rRNA gene contained 39 sequences with 525 positions in length. The 50 % majority rule consensus tree reconstructed from the D2-D3 data set by the Bayesian analysis is shown in Fig. 5. Two D2-D3 sequences of *H. guangzhouensis* n. sp. clustered together, and formed a 100 % supported clade with that of *H. vagisclera*.

The two 18S rRNA gene sequences of *H. guangzhouensis* n. sp. were obtained and submitted to the GenBank database under accession numbers KT381018-KT381019. The length of the two sequences was 882 bp. Variation of the two 18S sequences was 1 bp. The BLAST search showed the 18S rRNA gene sequences from *H. guangzhouensis* n.sp. were closest to

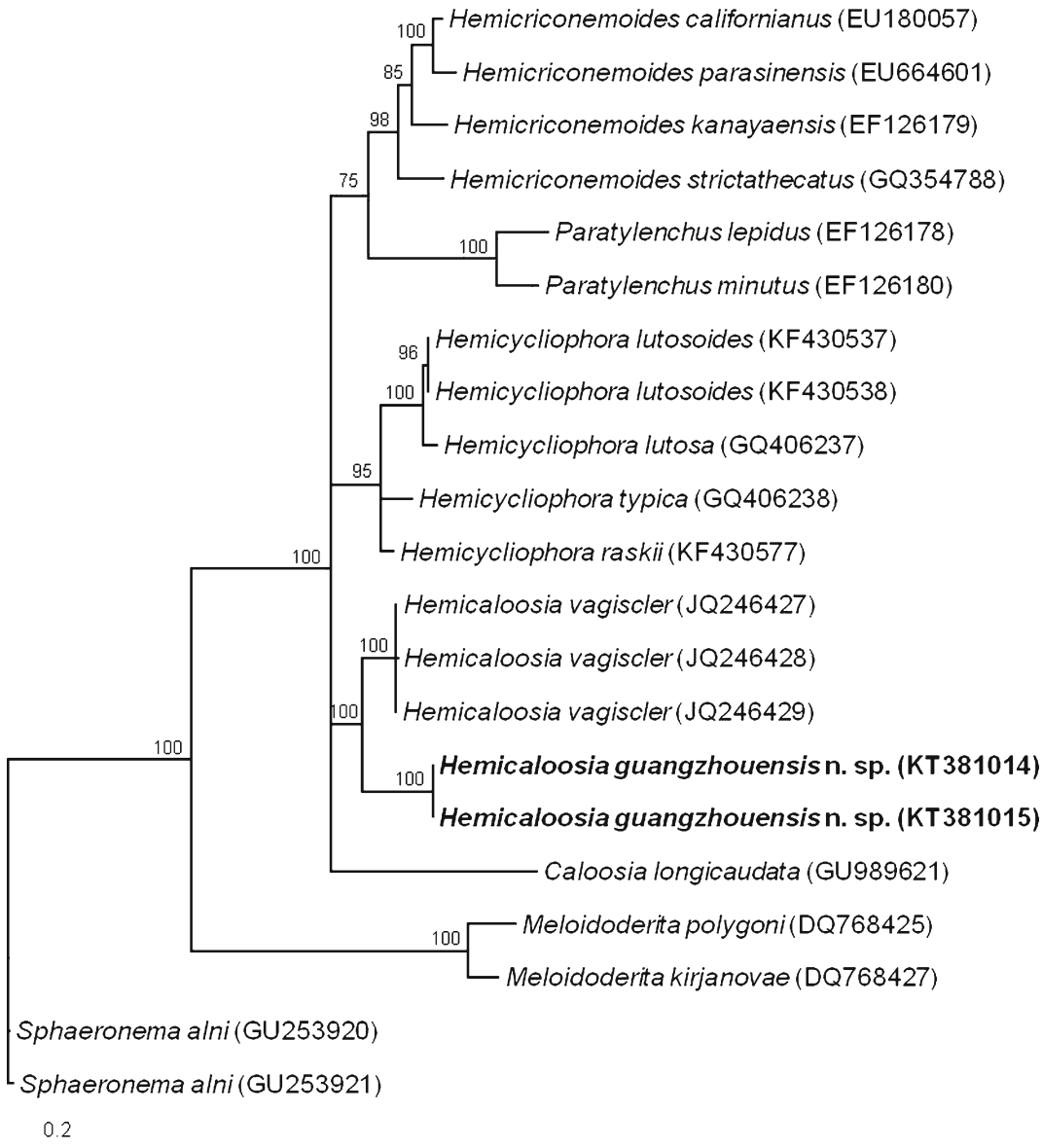

Fig. 4 The 50 % majority rule consensus tree inferred from the ITS-rRNA gene sequences of *Hemicaloosia guangzhouensis* n. sp. and some other species in the suborder Criconematina under GTR+I+G model. Posterior probabilities more than 50 % are given for appropriate clades. Newly obtained sequences are indicated in bold font

the sequences from *H. vagisclera* (JQ246425, JQ246426 and JQ446376), and the sequence identities were 98 %. The 18S rRNA gene alignment contained 23 sequences with 1492 positions in length. The 50 % majority rule consensus tree reconstructed from the 18S data set by the Bayesian analysis is shown in Fig. 6. The two 18S rRNA gene sequences of *H. guangzhouensis* n. sp. clustered together, and were positioned within a 100 %-supported clade with that of *H. vagisclera*.

PCR-ITS-RFLP study

The PCR-ITS-RFLP diagnostic profile generated by six restriction enzymes for *H. guangzhouensis* n. sp. is given in Fig. 7. Lengths of restriction fragments from

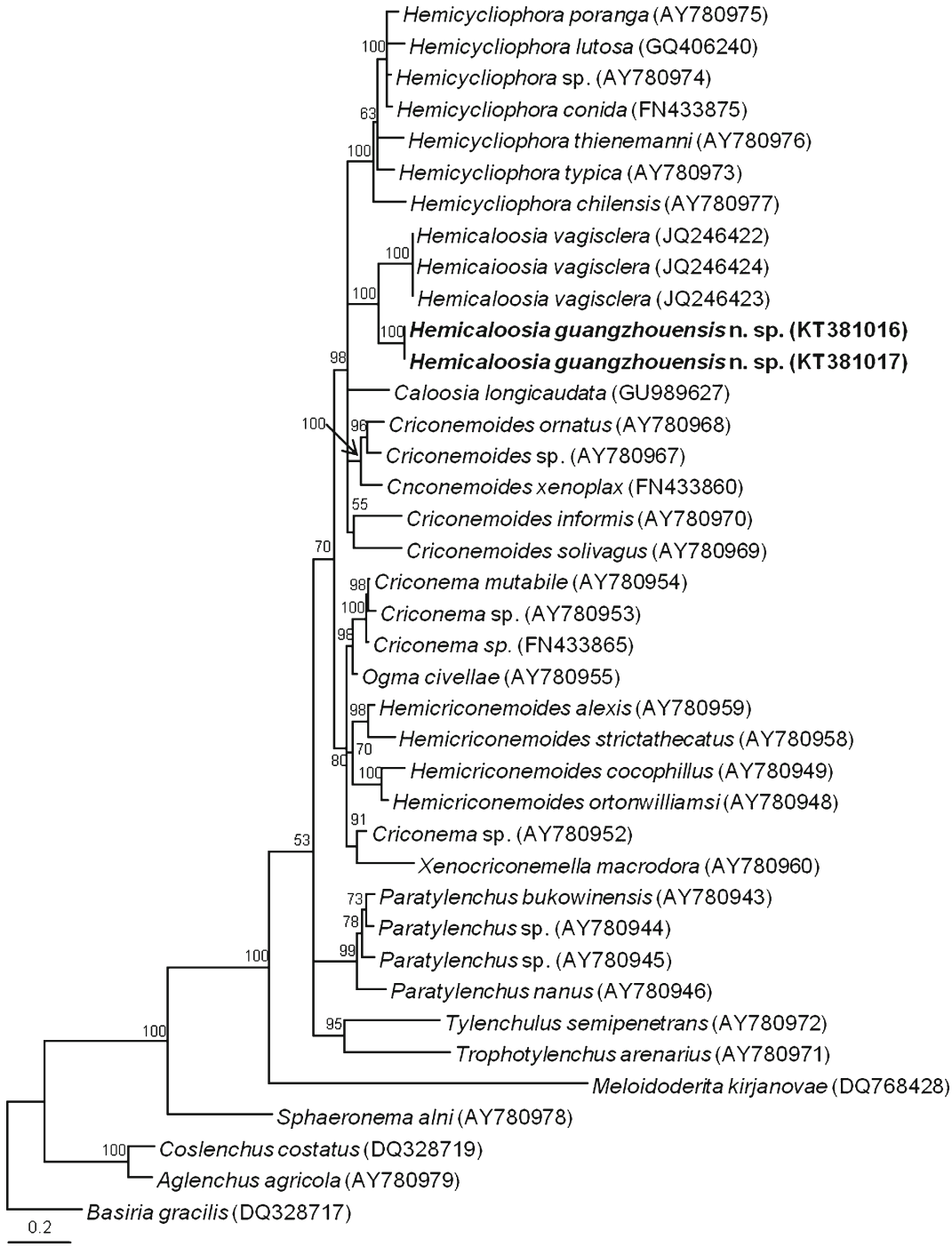

Fig. 5 The 50 % majority rule consensus tree inferred from the D2-D3 region of 28S rRNA gene sequences of *Hemicaloosia guangzhouensis* n. sp. and some other species in the suborder Criconematina under GTR+I+G model. Posterior probabilities more than 50 % are given for appropriate clades. Newly obtained sequences are indicated in bold font

RFLP for the ITS rRNA gene were: *Ava*I - 805 bp (not restricted); *Bsh*1236I - 805 bp (not restricted); *Dra*I- 805 bp (not restricted); *Hin*fI - 625, 132, 48 bp; *Hin6*I – 586, 130, 78, 11 bp; *Msp*I - 424, 303, 78 bp.

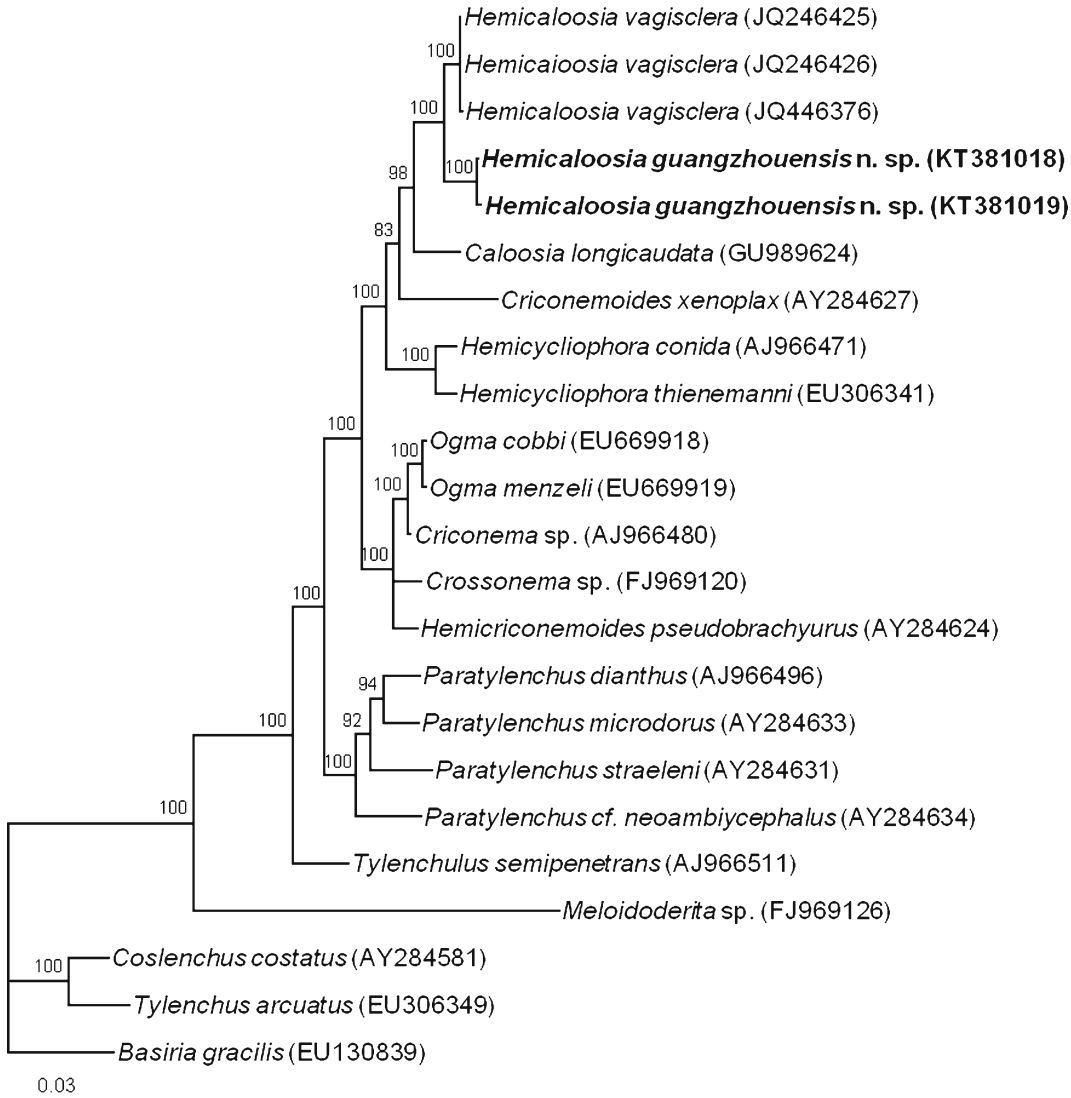

Fig. 6 The 50 % majority rule consensus tree inferred from the 18S rRNA gene sequences of *Hemicaloosia guangzhouensis* n. sp. and some other species in the suborder Criconematina under GTR+I+G model. Posterior probabilities more than 50 % are given for appropriate clades. Newly obtained sequences are indicated in bold font

Hemicaloosia guangzhouensis n. sp. is the first member of *Hemicaloosia* genus recovered from China, and thus the current number of *Hemicaloosia* species raises to ten. Presently, several characters can be used for differentiating *Hemicaloosia* species, such as body length, stylet length, R, RV, Ran, RVan, spermatheca features etc. However, the variability of these characters needs to be further investigated. In recent years, some rRNA gene sequences have been used to reconstruct the phylogenetic relationships within the suborder Criconematina, and provided some support for the validity of the family Caloosiidae (Zeng et al. 2012; Inserra et al. 2013). In the 18S rRNA gene tree, *H. guangzhouensis* n. sp., *H. vagisclera* and *C. longicaudata* are in a monophyletic clade with 98 % support, and the genera *Hemicaloosia* and *Caloosia* have a sister relationship. These results confirmed the close relationship between *Hemicaloosia* and *Caloosia*, and provided further evidence supporting the classification for the family Caloosiidae which contains the genera *Hemicaloosia* and *Caloosia* (Siddiqi 1980,

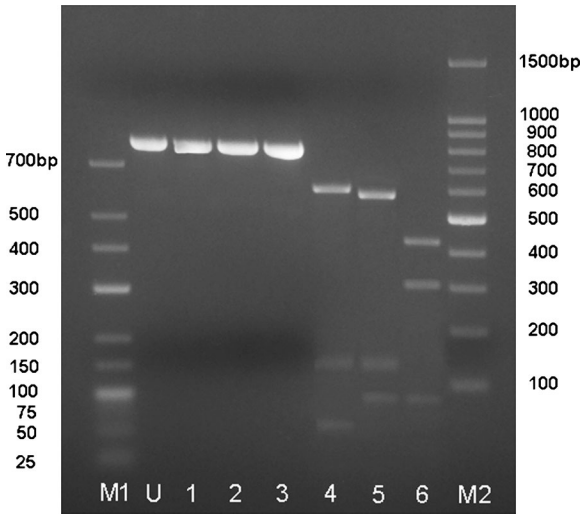

Fig. 7 Diagnostic PCR-ITS rRNA-RFLP profile for *Hemicaloosia guangzhouensis* n. sp. M1 - low ladder (Dongsheng Biotech, China), U=unrestricted PCR product, 1 – *Ava*I, 2 - *Bsh*1236I, 3 - *Dra*I, 4 - *Hin*fI, 5 - *Hin6*I, 6 - *Msp*I, M2 – 100 bp ladder (Dongsheng Biotech, China)

2000). However, most *Hemicaloosia* species have no molecular information available. We believe that, the use of molecular data will help to diagnose species in *Hemicaloosia* genus.

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