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Description of *Discocriconemella sinensis* n. sp. (Nematoda: Criconematidae) from the rhizosphere of *Camellia sinensis* in China

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Summary – *Discocriconemella sinensis* n. sp. isolated in Hangzhou, Zhejiang, China, from the rhizosphere of *Camellia sinenis* is described. The new species was characterised morphologically and molecularly. Important morphological details were elucidated by SEM photographs. The new species is characterised by an uninterrupted rounded cephalic disc, *en face* showing a rectangular labial plate with slit-like oral apertures, labial plate surrounded by slightly elevated projections resembling rudimentary lobes, R = 66 (64-69), Rex = 21 (17-24), stylet 74 (67-81) μm long, excretory pore located at the base of the pharyngeal bulb, vulva open, tail short and conoid with a lobed terminus. The species belongs to the group 1 lip pattern. Morphologically, it is most similar to *D. discolabia*, *D. mauritiensis*, *D. mineira* and *D. perseae*. This is the first new *Discocriconemella* species described from China. Phylogenetic analyses based on analysis of the D2-D3 expansion segments of the 28S rRNA, ITS rRNA, partial 18S rRNA, and *COI* gene revealed that the new species formed a separate clade from other criconematid species, thereby supporting its status as a new species of the genus. The new species showed close relationships with *Criconemoides informis*.

Keywords – first record, molecular, morphology, morphometrics, new species, phylogeny, plant-parasitic nematode, SEM, taxonomy, tea.

The genus *Discocriconemella* De Grisse & Loof, 1965 was erected by De Grisse & Loof (1965), the species in the new genus being characterised by the presence of a distinctly offset cephalic disc. Some species were later transferred to *Madinema* Khan, Chawla & Saha, 1976 by Khan *et al*. (1976). Orton Williams (1981) revised the genus and distinguished cephalic disc type as: simple, circular disc with uninterrupted margin; disc with one dorsal and one ventral deep indentation; and disc ventrally bearing a pair of lobes and large and open amphids (*macramphidia*-group). Nematodes having the latter disc type were placed in a new genus, *Acrozostron* Orton Williams, 1981. Raski & Luc (1987) discussed the problems of this grouping and agreed with Ebsary (1982) who had synonymised *Acrozostron* with *Discocriconemella*. Vovlas (1992) did not recognise the validity of *Madinema* or *Acrozostron*, but divided all *Discocriconemella* species into four groups based on the configuration of the cephalic disc: cephalic disc round with uninterrupted margins (group 1); disc with ventral and dorsal deep indentations (group 2); disc indentations giving a four-lobed appearance (group 3); and rounded disc with paired dorsal and ventral projections (group 4). Currently, this genus contains 28 species (Geraert, 2010; Maria *et al*., 2018a). The species of *Discocriconemella* are mainly reported from warmer areas of the world. To our knowledge, none of the new *Discocriconemella* species was described from China; however, *D. hengsungica* Choi & Geraert, 1975 and *D. limitanea* (Luc, 1959) De Grisse & Loof, 1965 were reported to be associated with native Chinese woody plantations

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(Maria *et al*., 2018a), while *D. mineira* Vovlas, Ferraz & Dos Santos, 1989 was reported by Ye *et al*. (1997) from the rhizosphere of *Ulmus pumila* L. imported from Hong Kong.

During a routine nematological survey, a population of *Discocriconemella* species was recovered from the rhizosphere of tea, *Camellia sinensis* (L.) Kuntze. The rare reports of this genus from China, and detection of this species from the rhizosphere of an economic plant such as tea, led us to perform detailed morphological, molecular and scanning electron microscopy analyses, which revealed the species to be new to science and it is described herein as *D. sinensis* n. sp. The objectives of this study were: *i*) to provide morphological and molecular characterisation of *D. sinensis* n. sp.; and *ii*) to study the phylogenetic relationships of this species with other criconematid species using rRNA and *COI* gene sequences.

Materials and methods

Nematode population sampling, extraction and morphological identification

Nematodes were extracted from soil samples using the Cobb sieving and flotation-centrifugation method (Jenkins, 1964). For morphometric studies, the nematodes were killed and fixed with hot formalin and processed to glycerin (Seinhorst, 1959) as modified by De Grisse (1969). The drawings, measurements and light micrographs of nematodes were completed with the help of a Zeiss compound microscope (Stemi 2000-C).

For the SEM examination, the nematodes were fixed in a mixture of 2.5% paraformaldehyde and 2.5% glutaraldehyde, washed three times in 0.1 M cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated in a series of ethanol solutions and critical point-dried with CO_2. After mounting on stubs, the samples were coated with gold (Maria *et al*., 2018b).

For molecular analysis several other nematodes were included: *Criconemoides parvus* Raski, 1952; *D. hengsungica*, and *D. limitanea*, isolated in Hangzhou, China, from the rhizosphere soil of woody perennials (Maria *et al*., 2018a); two unidentified *Mesocriconema* Andrássy, 1965 species from Washington state, USA; and an unidentified *Mesocriconema* species from Kyrgyzstan.

Molecular analyses

DNA samples were prepared according to Zheng *et al*. (2003). Five sets of primers (synthesised by Invitrogen, Shanghai, China) were used in the PCR analyses to amplify the nearly full-length 18S, D2-D3 expansion segments of 28S, ITS rRNA and partial *COI* genes. Nearly full length 18S region was amplified with two sets of primers, the first set being 18s39F (5′-AAA GAT TAA GCC ATG CAT G-3′) and 18s977R (5′-TTT ACG GTT AGA ACT AGG GCG G-3′), and the second, 18s900F (5′-AAG ACG GAC TAC AGC GAA AG-3′) and 18s1713R (5′-TCA CCT ACA GCT ACC TTG TTA CG-3′) (Olson *et al*., 2017). Primers for amplification were: TW81 (5′-GTT TCC GTA GGT GGT GAA CCT GC-3′) and AB28 (5′-ATA TGC TTA AGT TCA GC GGG T-3′) for the ITS-rRNA gene (Joyce *et al*., 1994), the forward D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and the reverse D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) for the D2-D3 28S rRNA gene (De Ley *et al*., 1999). The primers used for *COI* amplification were COI-F5 (5′-AAT WTW GGT GTT GGA ACT TCT TGA AC-3′) and COI-R9 (5′-CTT AAA ACA TAA TGR AAA TGW GCW ACW ACA TAA TAA GTA TC-3′) (Powers *et al*., 2014). PCR conditions were as described by Ye *et al*. (2007), Powers *et al*. (2010, 2014). PCR products were evaluated on 1% agarose gels stained with ethidium bromide. PCR products of sufficiently high quality were sent for sequencing by Invitrogen, Shanghai, China. The newly obtained sequences were submitted to the GenBank database under accession numbers MK249990, MK253536-MK253546 and as indicated on the phylogenetic trees.

Phylogenetic analyses

The newly obtained sequences for each gene were aligned using ClustalX 1.83 with default parameters with corresponding published gene sequences of criconematids and related genera (Subbotin *et al*., 2005, 2006; Powers *et al*., 2010, 2014, 2016a, b; Van den Berg *et al*., 2012; Maria *et al*., 2018a). Outgroup taxa for each dataset were chosen according to the results of previously published data (Subbotin *et al*., 2005, 2006). Sequence datasets were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). BI analysis under the GTR + I + G model for each gene was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations.

Two runs were performed for each analysis. The log likelihood values of the sample points stabilised after approximately 1000 generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Nematode species name on phylogenetic trees are given as they indicated in the GenBank database.

Results and discussion

Discocriconemella sinensis[*] n. sp.
(Figs 1-3)

Measurements

See Table 1.

Description

Female

Body curved ventrad to open C-shape after heat relaxation. Cuticle finely annulated, Annuli with posterior margins smooth, 1-2 anastomoses present at mid-body on ventral side of body and one anastomosis immediately anterior to annuli where vulva is situated. Second lip annulus forming an uninterrupted disc, face pattern matching that of group 1 proposed for *Discocriconemella* species by Vovlas (1992). SEM observation showing rectangular labial plate with slit-like oral apertures. Labial plate surrounded by first lip annulus and slightly elevated projections resembling rudimentary lobes. Stylet long and robust, mostly straight. Stylet knobs indented anteriorly and rounded posteriorly. Dorsal pharyngeal gland opening *ca* 3.0-4.0 μm posterior to stylet base. Pharyngeal lumen looped in median pharyngeal bulb, which has medium sized refractive valvular apparatus. Isthmus narrow, short, encircled by nerve ring. Basal pharyngeal bulb elongated. Excretory pore inconspicuous, situated at base of pharyngeal bulb. Vulva wide open (SEM observation), without vulval flaps but in a few specimens rudimentary flaps were observed under SEM. Vagina straight, extending for less than half of body diam. Spermatheca rounded to oblong, filled with sperm cells. Oviduct short, ovary single, prodelphic. Oocytes arranged in single file except for a short region of multiplication near anterior end. Anus small, indistinct located 3-4 annuli posterior to vulva. Postvulval body elongated conoid. Tail short, conoid, with a lobed terminus.

Male and juvenile

Not observed.

Type host and locality

The type specimens were extracted from the rhizosphere of *Camellia sinenis*, Longwu tea village, Hangzhou, Zhejiang Province, P.R. China, on 24 May 2018. The geographical position of the sampling site is 30°10′28′′N; 120°1′38′′E.

Type material

Holotype female, 11 female paratypes (slide numbers ZJU-20-01-ZJU-20-05) deposited in the nematode collection of Zhejiang University, Hangzhou, P.R. China; four paratype females deposited in the Nematological collection of the University of California, Davis, CA, USA.

Diagnosis and relationships

Discocriconemella sinensis n. sp. is characterised by uninterrupted rounded cephalic disc, *en face* view showing rectangular labial plate with slit-like oral apertures surrounded by slightly elevated projections resembling rudimentary lobes. Total number of body annuli (R = 66 (64-69)), number of annuli from anterior end to excretory pore (Rex = 21 (17-24)), stylet length of 74 (67-81) μm, excretory pore located at the base of the pharyngeal bulb, vulva open, post-vulval body elongated conoid, and tail short and conoid with a lobed terminus.

Based on the lip pattern scheme proposed by Vovlas (1992), this new species belongs to group 1 in having the cephalic disc round with an uninterrupted margin. It shares the same lip type with *D. discolabia* (Diab & Jenkins, 1966) De Grisse, 1967, *D. mauritiensis* (Williams, 1960) De Grisse & Loof, 1965, *D. mineira*, and *D. perseae* Cid del Prado Vera & Loof, 1985. It differs from *D. discolabia* by longer body length (378 (344-418) *vs* 190-300 μm), longer stylet length (74 (67-81) *vs* 38 (35-47) μm, total number of body annuli (R = 66 (64-69) *vs* 167 (155-174), number of annuli from anterior end to excretory pore (Rex = 21 (17-24) *vs* 47-56), number of annuli between vulva to tail terminus (RV = 7 (6-8) *vs*

[*] This is the first new species of *Discocriconemella* described from China, hence the specific epithet that is formed from the latinised name of the country of origin.

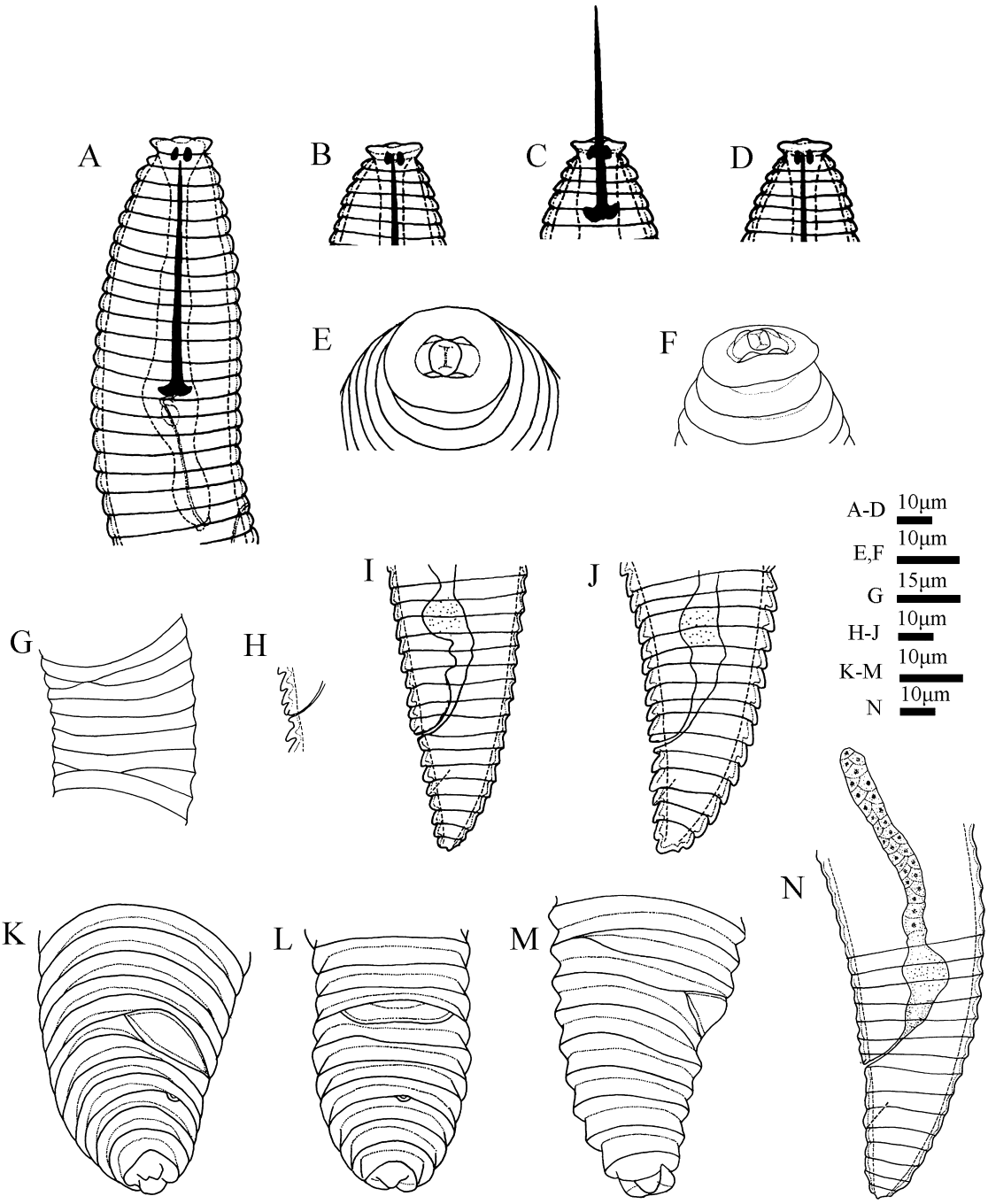

Fig. 1. Line drawings of *Discocriconemella sinensis* n. sp. Female. A: Pharyngeal region; B-D: Various lip regions; E, F: *En face* view of lip region; G: Mid-body showing anastomoses; H: Vulval region; I, J: Posterior region; K-M: Posterior region (drawn from SEM); N: Gonad. (Scale bars: A-F, H-N = 10 μm, G = 15 μm.)

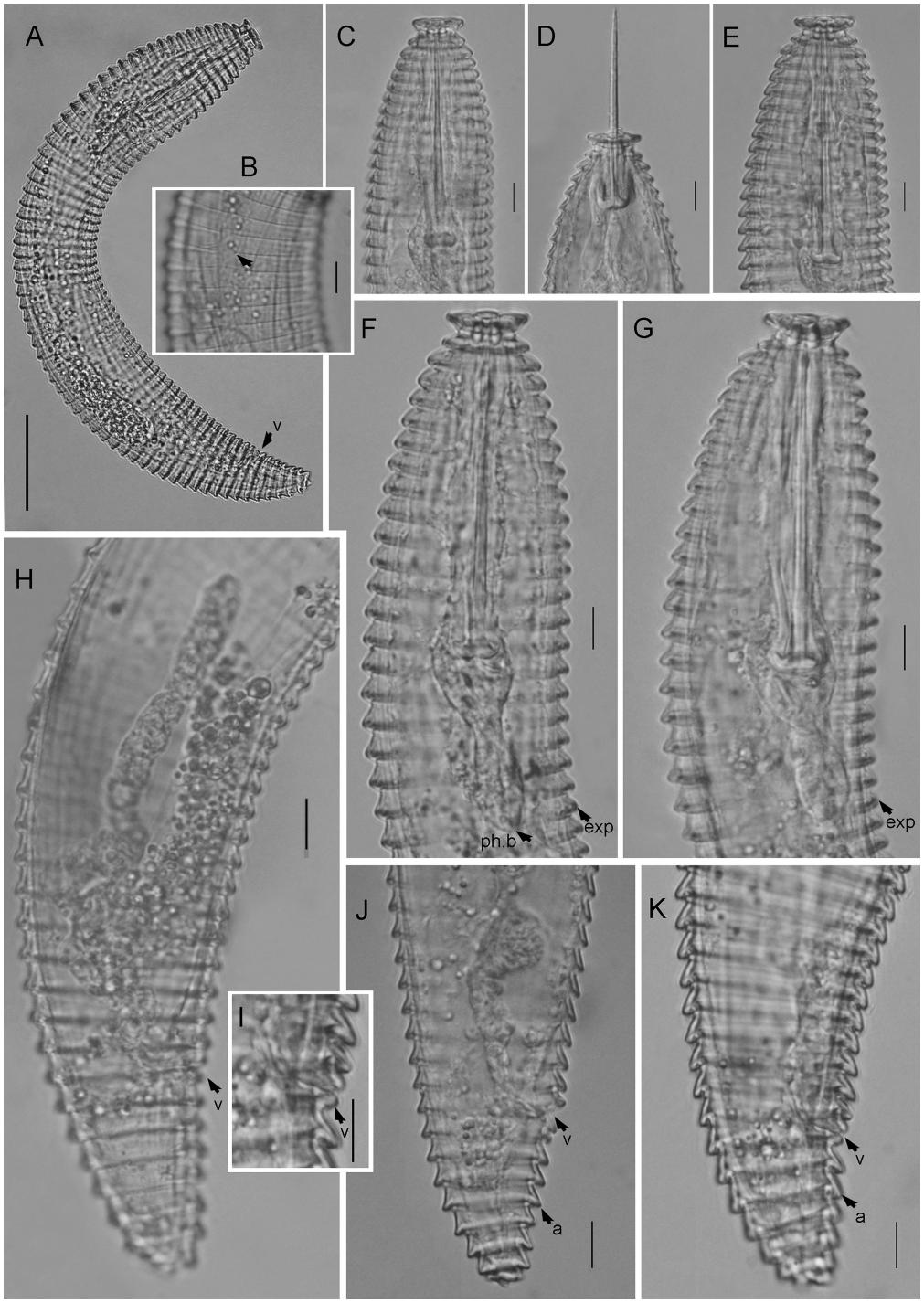

Fig. 2. Light photomicrographs of *Discocriconemella sinensis* n. sp. female. A: Entire body (v = vulva); B: Mid-body (arrow showing anastomoses); C-E: Lip regions; F, G: Pharyngeal region, arrows showing position of base of pharyngeal bulb (ph.b) and excretory pore (exp); H: Posterior region showing gonad and vulva (v); I: Vulval region (v = vulva); J, K: Tail region, arrows showing position of vulva (v) and anus (a). (Scale bars: A = 40 μm, B-K = 10 μm.)

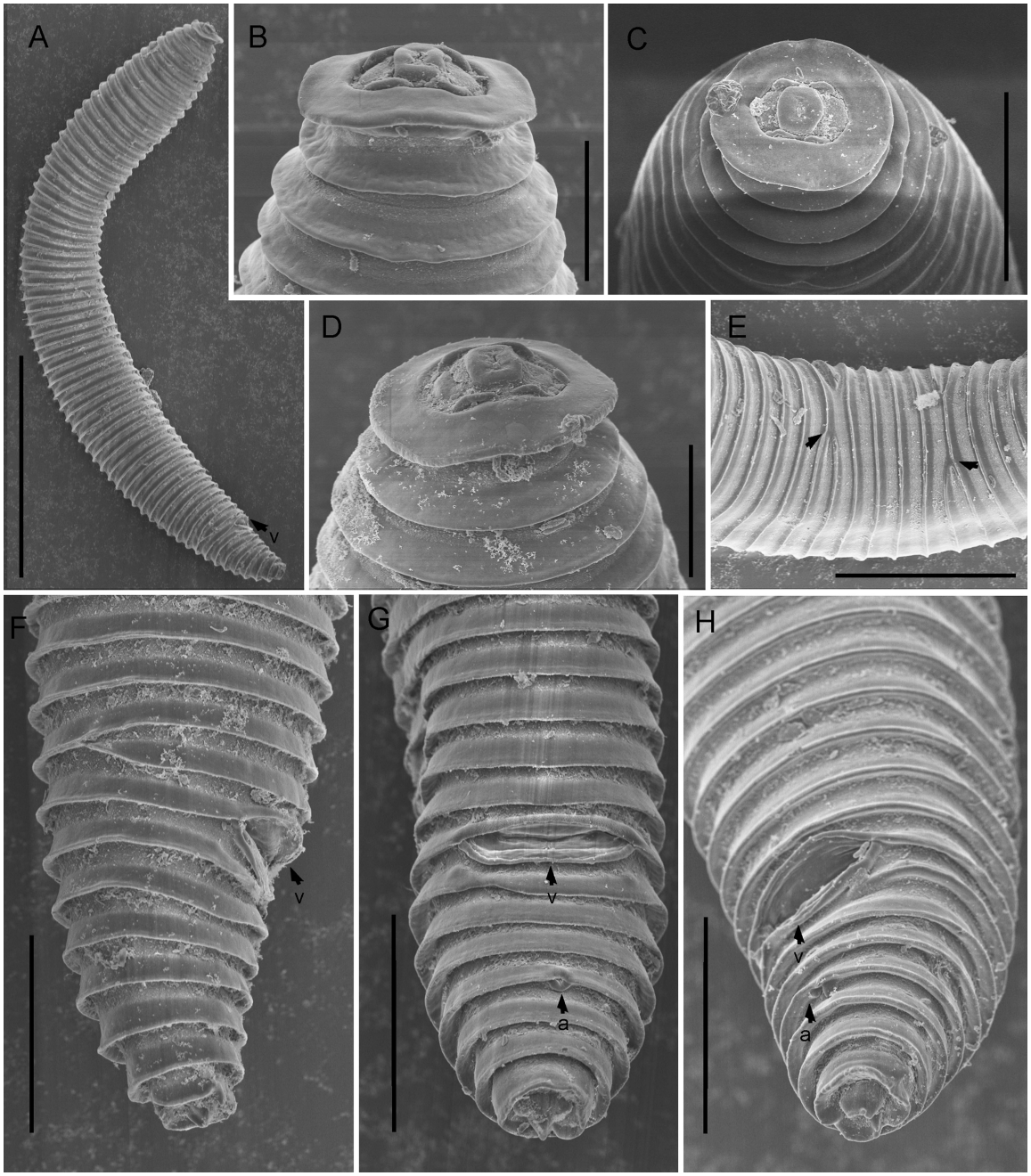

Fig. 3. Scanning electron micrographs of *Discocriconemella sinensis* n. sp. female. A: Entire body (v = vulva); B-D: *En face* view of lip region; E: Cuticular annuli at mid-body (arrows showing anastomoses); F-H: Posterior region showing vulva (v) and anus (a). (Scale bars: A = 100 μm; B-D = 10 μm; E = 30 μm; F-H = 20 μm.)

Table 1. Morphometrics of female *Discocriconemella sinensis* n. sp. All measurements are in μm and are in the form: mean ± s.d. (range).

Character	Holotype	Paratypes
n	–	15
L	374	378 ± 19.1 (344-418)
a	7.2	7.4 ± 0.6 (6.2-8.1)
b	3.1	3.2 ± 0.2 (2.9-3.5)
c	26.2	25.0 ± 2.3 (20.9-28.2)
c′	0.6	0.7 ± 0.1 (0.6-0.8)
V	92.6	92.0 ± 0.6 (90.7-92.6)
VL/VB	1.0	1.1 ± 0.1 (1.0-1.3)
R	66	66.0 ± 1.5 (64-69)
Rex	21	21.0 ± 1.8 (17-24)
RV	6	7.0 ± 0.5 (6-8)
RVan	3	3.4 ± 0.5 (3-4)
Ran	3	3.5 ± 0.5 (3-4)
Lip height	7.8	8.1 ± 0.7 (7.3-9.9)
Lip diam.	20.3	20.9 ± 1.5 (18.4-23.9)
Stylet length	77	74 ± 3.8 (67-81)
Stylet (% L)	20.6	19.6 ± 1.2 (17.1-22.0)
Pharynx	121	118 ± 4.9 (109-128)
Max. body diam.	52.1	51.5 ± 6.6 (44.1-64.4)
Vulval body diam.	29.1	29.4 ± 2.8 (24.9-33.8)
Vulva to tail terminus	27.8	31.5 ± 2.6 (27.8-35.4)
Anal body diam.	22.1	22.2 ± 1.9 (18.5-25.9)
Tail length	14.3	15.4 ± 1.5 (13.4-17.7)

14-17), crenations on the margins of body annuli absent *vs* present, anastomoses few *vs* common, and presence of rudimentary lobes *vs* absent; from *D. mauritiensis* by longer stylet length 74 (67-81) *vs* 33-38 μm, total number of body annuli (R = 66 (64-69) *vs* 140-152), number of annuli from anterior end to excretory pore (Rex = 21 (17-24) *vs* 42-45), number of annuli between anus to tail terminus (Ran = 3.5 (3-4) *vs* 8-10), position of vulva (V = 92 (90.7-92.6) *vs* 93-96), crenations on the margins of body annuli absent *vs* present, and vulval lip morphology (not enlarged *vs* anterior lip enlarged); from *D. mineira* by longer body length (378 (344-418) *vs* 304 (253-342) μm), stylet length (74 (67-81) *vs* 65 (61-71) μm), total number of body annuli (R = 66 (64-69) *vs* 82 (78-88)), number of annuli from anterior end to excretory pore (Rex = 21 (17-24) *vs* 25 (23-27)), position of vulva (V = 92.0 (90.7-92.6) *vs* 93.5 (93-94)), and vulval lips simple *vs* bilobed; from *D. perseae* by longer body length (378 (344-418) *vs* 220-370 μm), total number of body annuli (R = 66 (64-69) *vs* 118 (108-126)), number of annuli from anterior end to excretory pore (Rex = 21 (17-24) *vs* 35 (32-38)), number of annuli between vulva to tail terminus (RV = 7 (6-8) *vs* 18 (14-20)), number of annuli between anus to tail terminus (Ran = 3.5 (3-4) *vs* 10 (9-12)), position of vulva (V = 92.0 (90.7-92.6) *vs* 85-89), shape of post-vulval body (elongated conoid with lobed terminus *vs* convex conoid, terminus subacute, directed dorsad), and rudimentary lobes present *vs* absent.

Molecular characterisation and phylogenetic relationships of *Discocriconemella sinensis* n. sp. with other criconematid species

The new species was molecularly characterised using D2-D3 expansion segments of 28S, ITS, 18S and *COI* fragments, sequences being deposited in GenBank. D2-D3 of 28S and ITS sequences of *D. hengsungica* and *D. limitanea* obtained in a previous study (Maria *et al*., 2018a) were also deposited in GenBank and used for phylogenetic analysis.

The D2-D3 of 28S rRNA tree was 667 bp in length and was used in a multiple edited alignment of 28 criconematid sequences (Fig. 4). Three *Paratylenchus* species were selected as outgroup taxa. Seven new sequences of

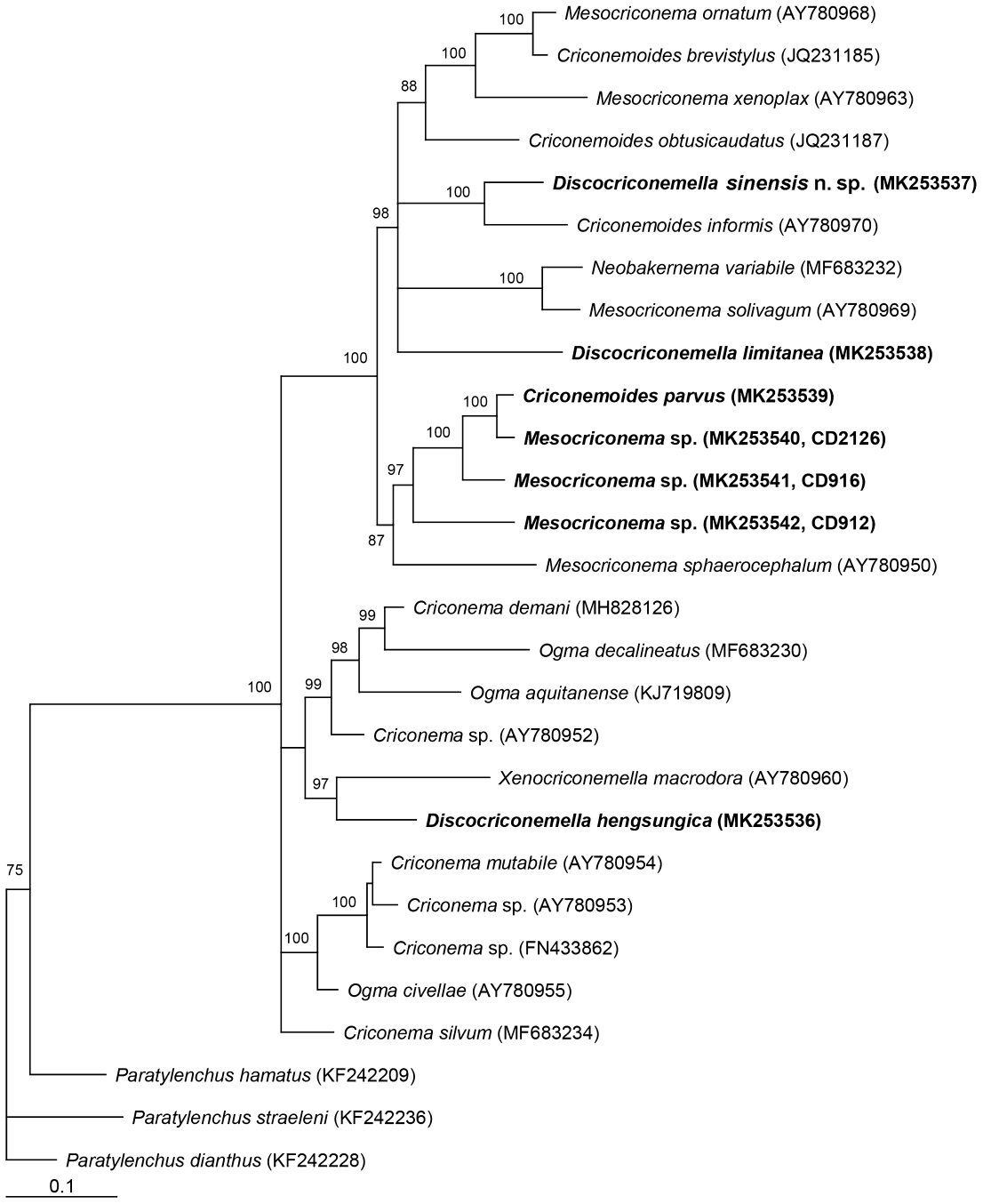

Fig. 4. Phylogenetic relationships of *Discocriconemella sinensis* n. sp. with Criconematidae as inferred from Bayesian analysis using the D2-D3 of 28S rRNA gene sequence dataset with the GTR + I + G model. Posterior probability more than 70% is given for appropriate clades. Newly obtained sequences are indicated in bold.

D2-D3 of the 28S gene were obtained in the present study, including sequence of *D. sinensis* n. sp. and other species of *Discocriconemella*, *Criconemoides* Taylor, 1936, and *Mesocriconema* which appear in bold on the trees. The tree revealed four highly supported major clades, where *D. sinensis* n. sp. had a sister relation with *Criconemoides informis* (Micoletzky, 1922) Taylor, 1936, differing by 9.8% (53 bp) from this species. *Discocriconemella limitanea* appeared basal to this clade, while *D. hengsungica* grouped with *Xenocriconemella macrodora* (Taylor, 1936) De Grisse & Loof, 1965 in the following clade.

The ITS1 rRNA sequence was 556 bp in length, the tree (Fig. 5) being based on a multiple edited alignment of 22 criconematid sequences. Two *Paratylenchus* species were selected as outgroup taxa. Three new sequences were obtained in the present study, including *D. sinensis* n. sp. *D. hengsungica* and *D. limitanea*. Like the D2-D3 tree, the ITS tree also presented four major clades where the new species and *D. limitanea* grouped in one clade while *D. hengsungica* and *X. macrodora* grouped in the next. The partial 18S rRNA sequence was 1662 bp in length, the tree (Fig. 6) being based on a multiple edited alignment of 32 sequences of criconematids with three sequences of *Paratylenchus* selected as outgroup taxa. In this tree *D. sinensis* n. sp. had a sister relation with *C. informis* and differed by 1.0% (18 bp) from this species. The other two *Discocriconemella* species grouped in different clades. The *COI* gene sequence was 721 bp in length, the tree (Fig. 7) being based on a multiple edited alignment of 31 sequences of criconematids and two sequences of *Paratylenchus* used as outgroup taxa. As with the 18S tree, in the *COI* tree the new species shares a branch with *C. informis*, although differing by 8.2% (57 bp).

In the present study, we provided sequences for several representatives of Criconematina. Our analysis showed that the new species, *D. sinensis* n. sp., is nested within the criconematids and has phylogenetic affinities with *Criconemoides* and *Mesocriconema* species. Three *Discocriconemella* species clustered separately representing a unique evolutionary lineage. The *Discocriconemella* species studied belong to three different groups of Vovlas (1992), *i.e.*, *D. hengsungica* – group 4, *D. limitanea* – group 2 and *D. sinensis* n. sp. – group 1. Several authors (Orton Williams, 1981; Vovlas, 1992; Siddiqi, 2000) noticed that species of this genus showed considerable variation in distinguishing characters. Maria *et al*. (2018a) had already shown that *Discocriconemella* is not monophyletic and it is likely that a large cephalic disc is a homoplastic character, independently appearing in several criconematid lineages.

In the phylogenetic analysis, it appears that *D. limitanea* grouped with the new species or in the adjacent clade. However, in all the trees *D. hengsungica* and *X. macrodora* grouped in the same clade, *i.e.*, away from the new species clade. Morphologically, *Xenocriconemella* and *Discocriconemella* are related genera as suggested by Loof & De Grisse (1989) who explained that *X. macrodora* has an enlarged second cephalic annule that surrounds the first one, a feature very similar to members of *Discocriconemella*. However, *Discocriconemella* has a distinct disc-like head whereas *Xenocriconemella* does not. Here, we assume that the stylet length may have some role in the phylogenetic grouping of the studied species. For example, *D. limitanea* and the new species both have a stylet length shorter than 100 μm while *D. hengsungica* and *Xenocriconemella* have a stylet length greater than 100 μm. There are only four species of *Discocriconemella* with a stylet length greater than 100 μm, *i.e.*, *D. hengsungia*, *D. pannosa* Sauer & Winoto, 1975, *D. spermata* Mohilal & Dhanachand, 1998, and *D. baforti* De Grisse, 1967. The first two species belong to group 4, *D. spermata* has an unknown lip pattern and *D. baforti* belongs to group 2 of Vovlas (1992). At this point we only have the morphological details of these four species, and molecular sequencing of *Discocriconemella* species with longer stylets may provide better insights into this grouping. Phylogenetically, *D. sinensis* n. sp. is related to *Criconemoides* and *Mesocriconema* species. This close relationship is problematic from a taxonomic perspective because the new species has a characteristic disc-shaped cephalic configuration and an overall morphological similarity with *Discocriconemella*. Therefore, the placement of this new species into *Discocriconemella* is primarily based on the presence of a distinct cephalic disc. Powers *et al*. (2014) transferred *D. inarata* Hoffman, 1974 to *Mesocriconema* based on the results of phylogenetic analysis. Accordingly, we emphasise the need for detailed molecular characterisation of *Discocriconemella* species in order to facilitate their phylogenetic grouping.

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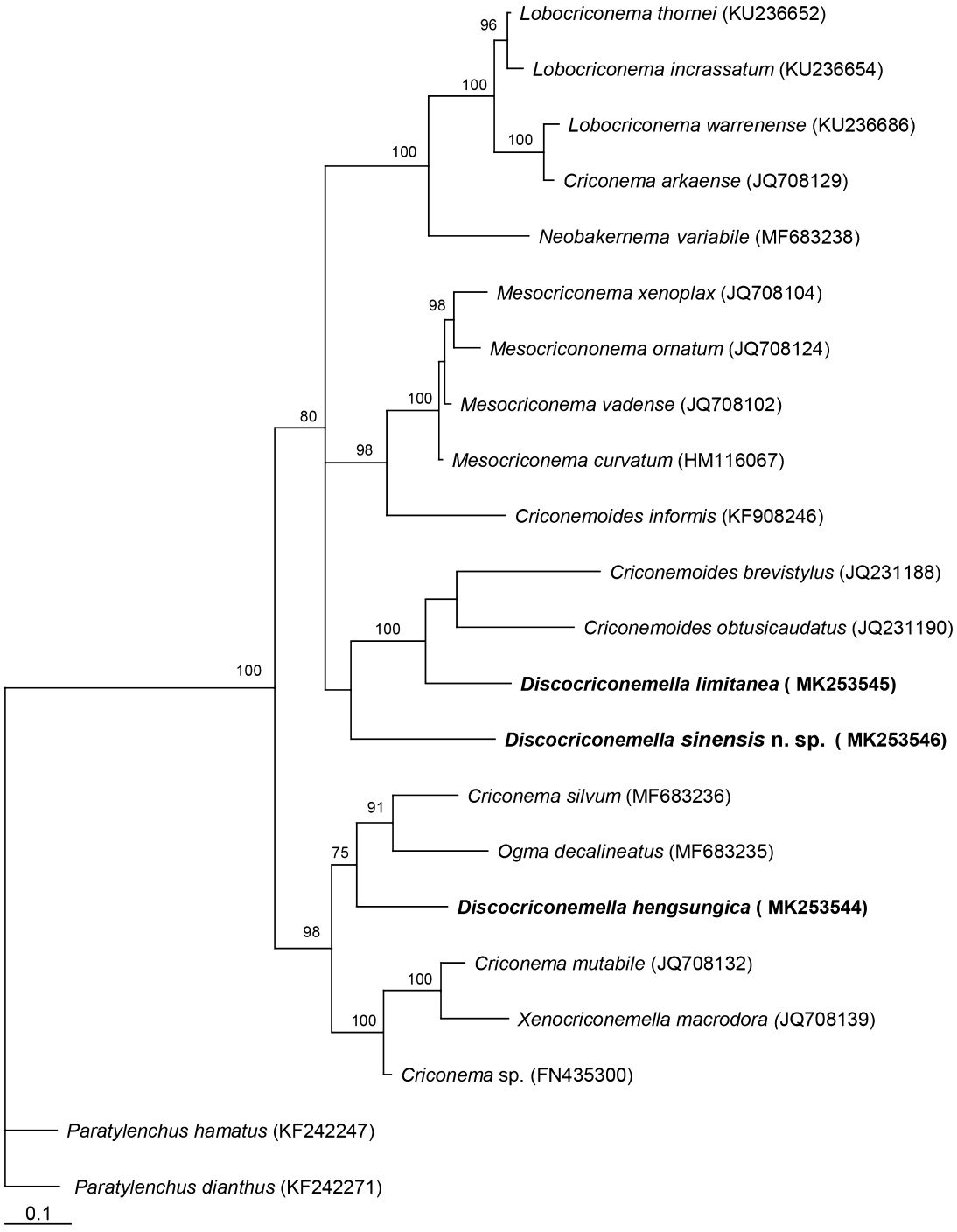

Fig. 5. Phylogenetic relationships of *Discocriconemella sinensis* n. sp. with Criconematidae as inferred from Bayesian analysis using the ITS1 rRNA gene sequence dataset with the GTR + I + G model. Posterior probability more than 70% is given for appropriate clades. Newly obtained sequences are indicated in bold.

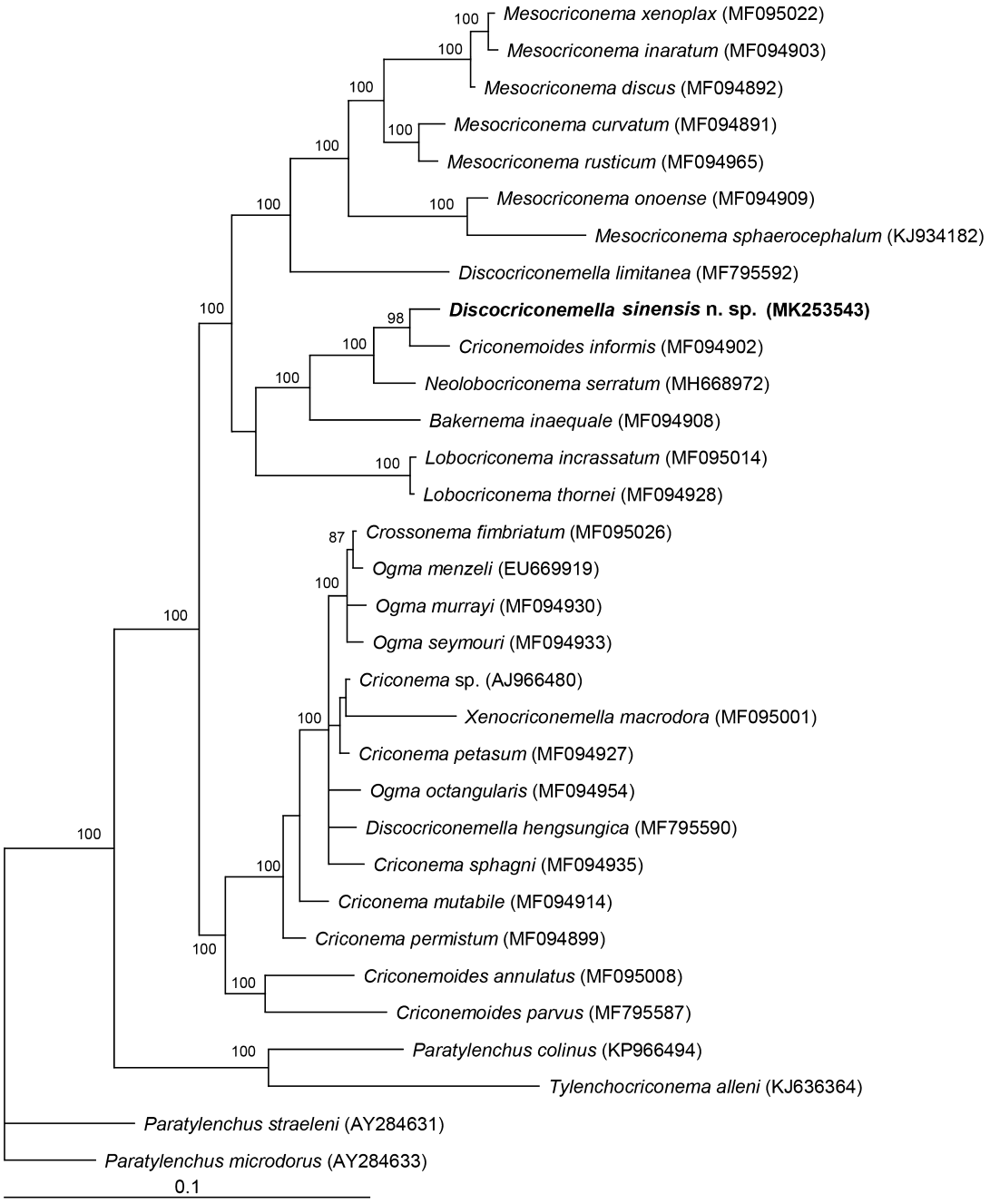

Fig. 6. Phylogenetic relationships of *Discocriconemella sinensis* n. sp. with Criconematidae as inferred from Bayesian analysis using the partial 18S rRNA gene sequence dataset with the GTR + I + G model. Posterior probability more than 70% is given for appropriate clades. Newly obtained sequences are indicated in bold.

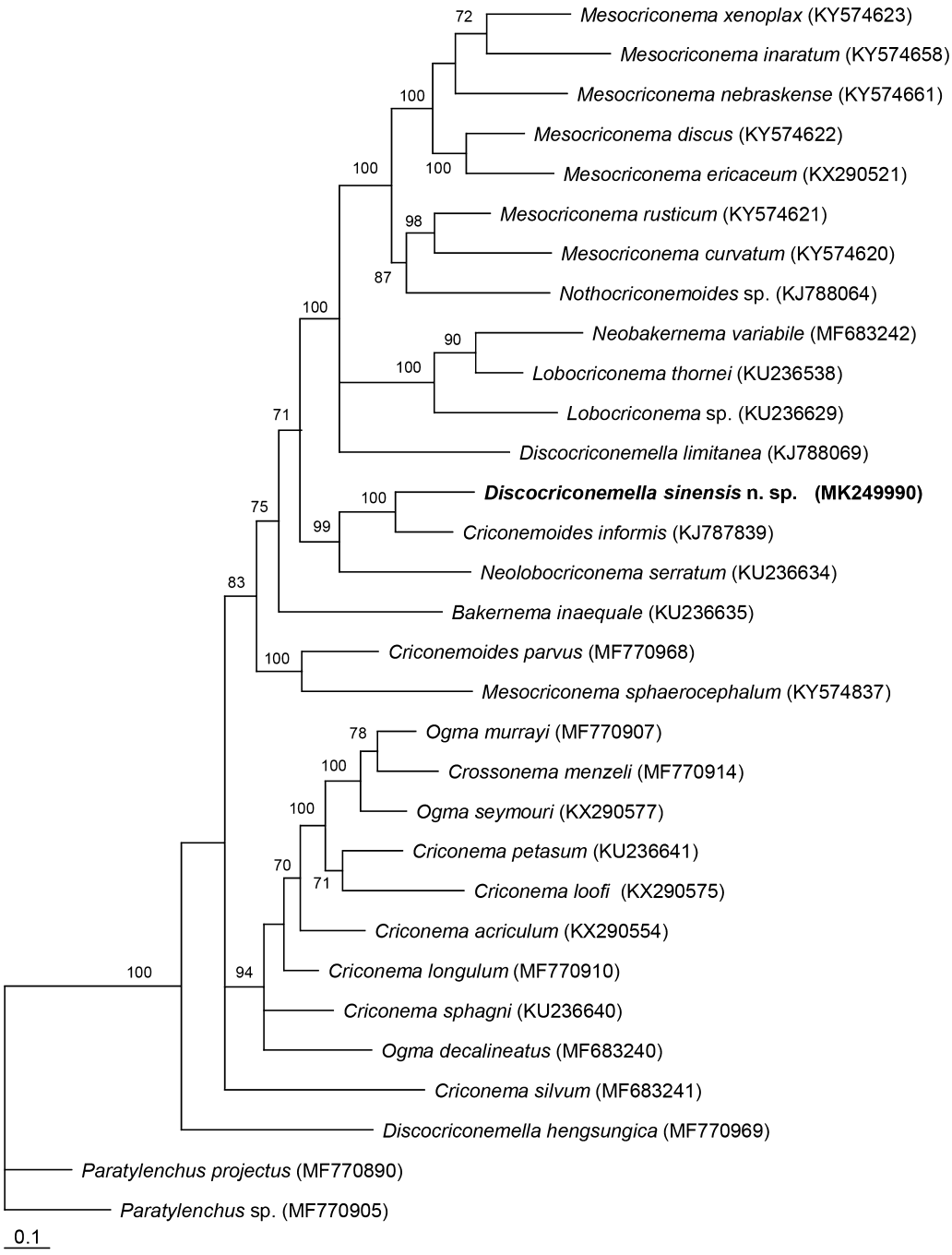

Fig. 7. Phylogenetic relationships of *Discocriconemella sinensis* n. sp. with Criconematidae as inferred from Bayesian analysis using the partial *COI* gene sequence dataset with the GTR + I + G model. Posterior probability more than 70% is given for appropriate clades. Newly obtained sequences are indicated in bold.

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