Species diversity of ring nematodes of the genus *Criconemoides* (Nematoda: Criconematidae) based on three new species from China, using integrative taxonomy

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Abstract Ring nematodes of the genus *Criconemoides* are a group of polyphagous, migratory root-ectoparasites of plants. In a nematological survey of Zhejiang Province, three new *Criconemoides* species were isolated from the rhizosphere of *Citrus* sp., *Euonymus alatus* and *Pterocarya stenoptera* trees. In the present study, the new species characterized using an integrative taxonomic approach and described as *Criconemoides parainformis* n. sp., *C. geraerti* n. sp. and *C. rotundicaudatus* n. sp. Three of the species shares truncated anteriorly flattened lip region comprised of two annuli, body annuli margin smooth with few anastomoses, (except *C. rotundicaudatus* n. sp. it has crenated annuli margins and numerous anastomoses), excretory pore 1-2 annuli posterior to pharyngeal bulb base, (except *C. geraerti* n. sp. it has excretory pore at the same level, or 1-2 annuli anterior to pharyngeal bulb base), vulva closed, anus hardly visible 1-3 annuli posterior to vulva. The new species can be distinguished based on their *en face* view pattern under scanning electron microscope (SEM) and the tail shapes. i.e. *C. parainformis* n. sp. has an ellipsoid oral disc, slightly fused pseudolips and tail ending in a bilobed terminus. *C. geraerti* n. sp. has a hexagonal oral disc, separated pseudolips, and a bluntly conoid tail. *C. rotundicaudatus* n. sp. has a rounded oral disc, well developed pseudolips, and a short broadly rounded tail. Besides detailed morphological studies using SEM, genomic and mitochondrial molecular data were obtained for the three described new species. In inferred phylogenies, these three species clustered with known *Criconemoides* species in 18S, 28S, ITS and *coxI* trees. Currently, only four known *Criconemoides* species occur in China and the discovery of the three new *Criconemoides* species in present study has increased our knowledge on the diversity of *Criconemoides* in China.

Keywords *Criconemoides*, host association, molecular, morphology, new species, phylogeny, SEM, taxonomy

Introduction

The family Cricematidae Taylor, (1914) 1936 comprises a taxonomically complex group of polyphagous, migratory root ectoparasites of many plants including various agricultural crops and trees. These nematodes are commonly referred as ring nematodes, because of the typical wide cuticular annulation (Geraert 2010; Powers et al. 2010; 2014). Cricematids are believed to have a high level of endemism and are some of the most abundant soil-dwelling plant parasites in tropical forests (Wouts 2006). Several cricematids are notorious pest of crops, not only causing damage to roots (Ferris et al. 2012; Westerdahl and Duncan 2015), but also known to predispose plants to the other biotic stresses (Inserra et al. 2014). Moreover, some of them were involved in the decline of cultivated crops (Nath et al. 2008; Liu & Feng 1995).

At present, the family Cricematidae contains 18 genera and 5 subfamilies (Geraert 2010), most of which established using traditional data. However, several studies in recent years have used integrative approaches using molecular tools together with classical methods e.g. Maria et al. (2018a, b; 2019) elucidating the key morphological features of several new and known cricematids using Scanning electron microscopy observations. Powers et al. (2018) provided the 18S phylogenetic classification of Cricematoidea and discussed the taxonomic placement of several species. Jahanshahi Afshar et al. (2019a, b) molecularly characterized (18S, D2-D3 of 28S, ITS and *coxI* fragments) rare *Lobocricema* species and performed species delimitation. Similarly, Van den berg et al., 2014 studied sheathoid nematodes and classify them in different morphological groups using SEM and rRNA sequence based information. These studies demonstrated that morphological studies coupled with SEM and sequence based analyses are helpful to avoid the confusions and are useful to understand the phylogenetic relationships of cricematids.

During a recent survey in Zhejiang and Henan Province, along with other plant parasitic nematodes three different *Cricemoides* Taylor 1936 populations were recovered from soil samples obtained from different sampling sites. The generic name of *Cricemoides* is derived from the Greek word *krikos*=ring, *nema*=nematode, *oides*=shape like (Siddiqi 2000). The important diagnostic characters of the genus are retrorse annuli with smooth or uneven posterior margins; true submedian lobes absent but six pseudolips developed as outgrowth of the first annulus, the lateral ones often reduced to low connections between the submedians which

superficially resemble true submedian lobes; vulva closed, anterior vulval lip neither overhanging nor ornamented; postvulval body part short, conoid; terminus rounded, conoid or acute (Brzeski et al. 2002).

The genus has a nearly global distribution, the species are known to be found from six continents, and however, the majority of the species were reported from the Asian continent (Choi and Geraert, 1975; Choi and Geraert, 1995; Geraert 2010). Several species have been reported as parasitic on the roots of plants but experimental evidence about the damage potential of these nematodes is lacking (Raski 1952; Siddiqi 2000). At present, the genus contains over 40 species, yet only four species *C. informis* Micoletzky 1922) Taylor 1936; *C. parvus* Raski 1952, *C. myungsugae* Choi and Geraert 1975 and *C. zavadskii* (Tulaganov 1941) Raski 1958 were reported from China (Maria et al. 2018a; Maria et al. 2019).

Since criconematid diversity is not well explored in China, therefore the recovered species were investigated thoroughly in order to identify the species status. The morpho-molecular characterization and SEM data of these three species were compared with all the existing species of the genus. Careful examination revealed that the species under investigation present unique characters and are need to be considered new members of the genus *Criconemoides*. Therefore this research describes three new *Criconemoides* species with the following objectives i) to provide an integrative morphological and molecular characterization of these species; ii) to elucidate important morphological details through SEM observations; and iii) to study the phylogenetic relationships of these species with other *Criconemoides* species.

Materials and methods

Soil sampling, nematode population extraction and morphological identification

Several soil samples were collected from Zhejiang and Henan Provinces of China. Nematodes were extracted from soil samples using a modified Cobb sieving and flotation-centrifugation method (Jenkins 1964). For morphometric studies, nematodes were killed and fixed with hot formalin and processed to glycerine (Seinhorst 1959) as modified by De Grisse (1969). The drawings, measurements and light micrographs of nematodes were made with a Nikon Eclipse Ni-U 931845 compound microscope. For the SEM examination, 40-50 handpicked nematodes of

each new species were fixed in a mixture of 2.5% paraformaldehyde and 2.5% glutaraldehyde, washed three times in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated in a series of ethanol solutions and critical-point dried with CO₂. After mounting on stubs, the samples were coated with gold at 6 to 10-nanometer thickness and the micrographs were made at 3 to 5 kV operating system of Hitachi SU8010 (Maria et al. 2018b). Based on the condition of the specimens 5-10 nematodes of each species were examined for every morphological character.

DNA extraction, PCR and sequencing

DNA samples were prepared according to Zheng et al. (2003). Four sets of primers (synthesized by Invitrogen, Shanghai, China) were used in the PCR analyses to amplify the near full-length 18S, D2-D3 expansion segments of 28S, and ITS region of rRNA. Partial 18S region was amplified with two sets of primers 18s39F (5'- AAAGATTAAGCCATGCATG -3') and 18s977R (5'- TTTACGGTTAGAACTAGGGCGG -3'), the second set was 18s900F (5'- AAGACGGACTACAGCGAAAG-3') and 18s1713R (5'- TCACCTACAGCTACCTTGTTACG-3') (Olson et al. 2017). Primers for amplification of ITS were TW81 (5'- GTTTCCGTAGGTGGTGAACCTGC -3') and AB28 (5'- ATATGCTTAAGTTCAGCGGGT -3') (Joyce et al. 1994), the forward D2A (5'- ACAAGTACCGTGAGGGAAAGTTG -3') and the reverse D3B (5'- TCGGAAGGAACCAGCTACTA -3') primers for amplification of D2-D3 28S (De Ley et al. 1999), and finally the primers used for *coxI* amplification were COI-F5 (5'- AATWTWGGTGTGGAACCTTCTTGAAC -3') and COI-R9 (5'- CTAAAACATAATGRAAATGWGCWACWACATAATAAGTATC -3') (Powers et al. 2014). PCR conditions were as described by Ye et al. (2007) and Powers et al. (2010). PCR products were evaluated on 1% agarose gels and 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 indicated on the phylogenetic trees.

Phylogenetic analyses

1
2 Newly obtained sequences of D2-D3 expansion segments of 28S, ITS, partial 18S rRNA
3 gene sequences, partial *coxI*, and available sequences of other nematodes obtained from GenBank
4 were used for phylogenetic reconstructions of *Criconemoides* species. Outgroup taxa for the
5 dataset were chosen according to previous published data (Maria et al. 2018a; Jahanshahi Afshar
6 et al., 2019 a,b). Multiple sequence alignments of the different genes were made using the FFT-
7 NS-2 algorithm of MAFFT V.7.450 (Kato et al. 2017). Sequence alignments were manually
8 visualised using BioEdit (Hall 1999) and edited by Gblocks ver. 0.91b (Castresana 2000) in
9 Castresana Laboratory server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html)
10 using options for a less stringent selection (minimum number of sequences for a conserved or a
11 flanking position: 50% of the number of sequences +1; maximum number of contiguous non-
12 conserved positions: 8; minimum length of a block: 5; allowed gap positions: with half).
13 Percentage similarity between sequences was calculated using the sequence identity matrix using
14 BioEdit. For that, the score for each pair of sequences was compared directly and all gap or place-
15 holding characters were treated as a gap. When the same position for both sequences had a gap it
16 was not treated as a difference. Phylogenetic analyses of the sequence datasets were based on
17 Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). The best-fit model
18 of DNA evolution was obtained using JModelTest V.2.1.7 (Darriba et al. 2012) with the Akaike
19 information criterion (AIC). The best-fit model, the base frequency, the proportion of invariable
20 sites, and the gamma distribution shape parameters and substitution rates in the AIC were applied
21 in the MrBayes for the phylogenetic analyses. The unlinked general time-reversible model with
22 invariable sites and a gamma-shaped distribution (GTR + I + G) was used for all analyses. These
23 BI analyses were run separately per dataset using a random starting tree and four chains for $2 \times$
24 10^6 generations for all of molecular markers. A combined analysis of the four genes was not
25 performed due to unavailability of sequences for all species. The Markov chains were sampled at
26 intervals of 100 generations. Two runs were conducted for each analysis. After discarding burn-in
27 samples and evaluating convergence, the remaining samples were retained for further analyses.
28 The topologies were used to generate a 50% majority-rule consensus tree. Posterior probabilities
29 (PP) are given on appropriate clades. Trees from all analyses were visualised using FigTree
30 software V.1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

31

Results and descriptions

*Criconemoides parainformis*¹ n. sp. (Figs 1, 2, and 3, Table 1)

Female: Body slightly curved ventrally after heat-relaxation. Body annuli smooth rough or crenated margins were not observed. Anastomoses few, 1-2 at the posterior half of the body. Lip region anteriorly flattened in profile, comprised of two annuli, the second labial annulus larger than the first one. Cephalic annuli seem appressed at low magnification (under LM). *En face* view using SEM data showing an ellipsoidal oral disc, "I" shaped oral aperture, flanked by eye-shaped amphidial opening. True submedian lobes absent, pseudolips six, each subdorsal and subventral lips slightly fused. Stylet robust with anchor-shaped basal knobs, dorsal gland orifice (DGO) hardly visible. Pharyngeal lumen looped in median pharyngeal bulb, having medium-sized refractive valvular apparatus. Isthmus narrow, short, basal pharyngeal bulb distinct. Nerve ring encircling isthmus. Excretory pore 1-2 annuli posterior to pharyngeal bulb base. Gonad monodelphic, outstretched, spermatheca spherical, filled with sperm. Vagina straight, vulva closed, vulval lips not projecting above body contour. Anterior and posterior annuli surrounding vulva larger than the preceding body annuli. Anus hardly visible, 2-3 annuli posterior to vulva. Tail short, bluntly conoid ending in a bilobed terminus.

Male and Juvenile: Not found.

Type host and locality

The type specimens were extracted from the rhizosphere of *Citrus* sp. from Zijingang Campus, Zhejiang University, Hangzhou Zhejiang Province, P. R. China, 23 January, 2019. The geographical position of the sampling site is N: 30°18'28", E: 120°4'45".

Type material

¹ Specific epithet formed from the Latin word *para* = beside or near, and the species epithet *informis*, thereby reflecting its close similarity to *C. informis*.

Holotype female and 13 female paratypes (slide numbers ZJU-25-01-ZJU-25-03) deposited in the nematode collection of Zhejiang University, Hangzhou, China. Five female paratypes were deposited in Nematode collection in Institute for Sustainable Agriculture, CSIC, Córdoba, Spain (slide number IAS-19-012). Additional slides of voucher materials having 12 females (slide number T-7380p, T-7381p) were deposited at USDA nematode collection, Beltsville, Maryland, USA.

Diagnosis and relationships

The new species can be characterized by having a truncate-conoid lip region comprised of two annuli, ellipsoid oral disc, eye-shaped amphidial openings, true submedian lobes absent, pseudolips six, each subdorsal and subventral lips slightly fused (*en face* view under SEM). Body annuli smooth and with few anastomoses, stylet robust 63.7 (59.4-67.9) μm long, excretory pore 1-2 annuli posterior to pharyngeal bulb base, vulva closed, vulval lips not projecting above body contour, anus hardly visible, 2-3 annuli posterior to vulva and tail short, bluntly conoid, ending in a bilobed terminus.

Based on the number of body annuli, stylet length, and shape of postvulval body region, *C. parainformis* n. sp. is compared with the followings: *C. amorphus* De Grisse 1967; *C. ihlathum* Van den Berg and Tiedt 2001; *C. informis*; *C. insignis* Siddiqi 1961; and *C. justus* (Eroshenko, 1981) Brzeski, Loof and Choi 2002. *Criconemoides parainformis* n. sp. can be differentiated from *C. amorphus* by lower number of annuli between vulva and tail terminus (RV= 5.7 (5-6) vs 6-10), more posteriorly located vulva (V= 93.4 (92.3-95.4) vs 88-92), elevated labial disc absent (vs present), smooth annuli margins (vs rough), position of excretory pore (1-2 annuli posterior to pharyngeal bulb base vs 4-5 annuli) and tail terminus bilobed (vs multilobed). It differs from *C. ihlathum* by higher number of body annuli (R= 63 (58-66) vs 50-57), shorter stylet (63.7(59.4-67.9) vs 68-85 μm), smooth annuli margins (vs irregular), number of cephalic annuli (2 vs 1), shape of labial annuli (rounded vs projecting outward) stylet knob shape (anchor shaped vs cupped), vulval lips with no projections (vs present) and lobed tail terminus (vs rounded). From *C. informis* by rounded lip annuli margins (vs directed sideways), labial disc absent (vs present), subdorsal and

1 subventral lips slightly fused (*vs* separate), bulging of vulval lips absent (*vs* present), and tail
 2 terminus lobed (*vs* rounded). From *C. insignis* by shorter body (382.1 (339.6-438.1) *vs* 470 (400-
 3 530) μ m), cephalic annuli margins simple (*vs* projected forward and outwards), labial disc absent
 4 (*vs* present), eye shaped amphidial apertures (*vs* slit like) and lobed tail terminus (*vs* rounded).
 5 From *C. justus* by higher number of body annuli (R= 63 (58-66) *vs* 70-72), shorter stylet
 6 (63.7(59.4-67.9) μ m *vs* 78-56 μ m), lower number of annuli from excretory pore to anterior end
 7 (Rex= 48 (44-50) *vs* 20.4 (19.0-22.0)), continuous lip region (*vs* offset), anterior vulval lip simple
 8 (*vs* hanging) and tail terminus lobed (*vs* rounded or bifurcate).

9

10 *Criconemoides geraerti*² n. sp. (Figs 4, 5, and 6, Table 2)

11

12 *Female*: Body plump, ventrally curved after heat-relaxation. Body annuli smooth, rough or
 13 crenated margins were not observed. Anastomoses few, two anterior to vulva and one near vulval
 14 region. Lip region truncate conoid, anteriorly flattened in profile, comprised of two annuli, the
 15 second labial annulus larger than the first one. *En face* in SEM showing a hexagonal oral disc, "I"
 16 shaped oral aperture, flanked by amphidial opening. True submedian lobes absent, pseudolips six,
 17 separated, lateral ones reduced, the subdorsal and subventral ones enlarged, appearing as
 18 submedian lobes (forming not true submedian lobes). Stylet robust with anchor-shaped basal knobs,
 19 dorsal gland orifice (DGO) hardly visible. Pharyngeal lumen looped in median pharyngeal bulb,
 20 having medium-sized refractive valvular apparatus, isthmus narrow, short, basal pharyngeal bulb
 21 small. Nerve ring encircling isthmus. Excretory pore at the same level, or 1-2 annuli anterior to
 22 pharyngeal bulb base. Gonad monodelphic, outstretched, spermatheca spherical. Vagina straight,
 23 vulva closed, vulval lips not projecting above the body contour. Anterior and posterior annuli
 24 surrounding vulva larger than the preceding body annuli. Anus hardly visible, 1-2 annuli posterior
 25 to vulva. Tail short, bluntly conoid, ending in a single or trilobed terminus.

26

27 *Male and Juvenile*: Not found.

28

² Specific epithet is named in honour of Professor Etienne Geraert for his extraordinary contribution to our knowledge of nematode taxonomy.

Type host and locality

The type specimens were extracted from the rhizosphere of burning bush (*Euonymus alatus* (Thunb.) Sieb.) at the Yellow River National Geopark of Zhengzhou City, Henan Province on July, 2019. The geographical position of the sampling site is N: 34°57'25, E: 113°32'20".

Type material

Holotype female and 11 female paratypes (slide numbers ZJU-26-01-ZJU-26-03) deposited in the nematode collection of Zhejiang University, Hangzhou, China. Five female paratypes were deposited in Nematode collection in Institute for Sustainable Agriculture, CSIC, Córdoba, Spain (slide number IAS-19-013). Additional slide of voucher material having 6 females (slide number T-7382p) were deposited at USDA nematode collection, Beltsville, Maryland, USA.

Diagnosis and relationships

Criconemoides geraerti n. sp. is characterised by its truncate lip region comprised of two annuli, a hexagonal oral disc and probable "I" shaped oral, true submedian lobes absent, pseudolips six, separated, lateral ones reduced, the subdorsal and subventral ones enlarged, submedian lobe-like (*en face* view under SEM). Body annuli smooth and with few anastomoses, stylet robust 60.2 (57.0-62.7) μ m long, excretory pore at the same level or 1-2 annuli anterior to pharyngeal bulb base, vulva closed, vulval lips not projecting above body contour, anus hardly visible, 1-2 annuli posterior to vulva and tail short, bluntly conoid ending in a single or trilobed terminus.

Based on the number of body annuli, stylet length, and shape of postvulval body region, *C. geraerti* n. sp. is compared with the followings: *C. amorphus*; *C. ananasi* Misra and Edward 1972; *C. informis*; *C. insignis*; *C. inusitatus* Hofmann 1974 and *C. wologicus* (Choi and Geraert 1975) Brzeski, Loof and Choi 2002. *Criconemoides geraerti* n. sp. can be differentiated from *C. amorphus* by higher number of body annuli (R= 80 (71-84) vs 55-76), higher number of annuli from excretory pore to anterior end (Rex= 25(24-27) vs 17-22), stylet length (60.2 (57-62.7) vs 62-84 μ m), lower number of annuli between vulva and tail terminus (RV= 6 (5-7) vs 6-10), posteriorly located vulva (V= 93.6 (92.6-94.2) vs 88-92), elevated labial disc absent (vs present) and position

1 of excretory pore (anterior to pharyngeal bulb base *vs* posterior). It differs from *C. ananasi* by
 2 higher number of body annuli ($R = 80$ (71-84) *vs* 58-75), higher number of annuli from excretory
 3 pore to anterior end ($R_{ex} = 25$ (24-27) *vs* 20), tail terminus lobed (*vs* rounded or forked). From *C.*
 4 *informis* by higher number of body annuli ($R = 80$ (71-84) *vs* 48-77), higher number of annuli from
 5 excretory pore to anterior end ($R_{ex} = 25$ (24-27) *vs* 15-24), elevated labial disc absent (*vs* present),
 6 cephalic annuli margins rounded (*vs* directed sideways), bulging of vulval lips absent (*vs* present)
 7 and tail terminus lobed (*vs* rounded). From *C. insignis* by shorter (366 (321.7-455.5) *vs* 470 (400-
 8 530) μm) and slimmer ($a = 9.6$ (8.1-10.6) *vs* 12 (11-13)) body, higher number of body annuli ($R =$
 9 80 (71-84) *vs* 68 (65-69)), higher number of annuli from excretory pore to anterior end ($R_{ex} = 25$
 10 (24-27) *vs* 22-23) and cephalic annuli margins simple (*vs* projected forward and outwards).

11 From *C. inusitatus* by shorter 366 (321.7-455.5) *vs* 459 (337-514) μm) and slimmer ($a = 9.6$
 12 (8.1-10.6) *vs* 14.7 (11.6-16.7)) body, longer stylet (60.2 (57-62.7) *vs* 48 (42-50) μm), and cephalic
 13 annuli continuous (*vs* offset). From *C. wologicus* by greater c value (24.4 (20.3-30.5) *vs* 16-19),
 14 posteriorly located vulva ($V = 93.6$ (92.6-94.2) *vs* 88-92), lower number of annuli between vulva
 15 and tail terminus ($R_V = 6$ (5-7) *vs* 8-9), longer stylet (60.2 (57.0-62.7) *vs* 53-56 μm), and
 16 indentations on the terminal annulus absent (*vs* present). Morphologically *C. geraerti* n. sp. is also
 17 close to *C. parainformis* n. sp. but can be differentiated by having higher number of body annuli
 18 ($R = 80$ (71-84) *vs* 63 (58-66)), higher number of annuli from excretory pore to anterior end ($R_{ex} =$
 19 25 (24-27) *vs* 20.4 (19-22)), hexagonal oral disc and pseudolips separated (*vs* ellipsoidal oral disc
 20 and pseudolips fused) and position of excretory pore (at the same level or slightly anterior to
 21 pharyngeal bulb base *vs* posterior).

22

23 *Criconemoides rotundicaudatus*³ n. sp. (Figs. 7, 8, and 9, Table 3)

24

25 *Female*: Body plump, curved ventrally after heat-relaxation. Annuli margins smooth to
 26 crenated. Anastomoses numerous. Lip region anteriorly flattened in profile, comprised of two
 27 annuli, the second lip annulus larger than the first one. *En face* view in SEM showing a rounded
 28 oral disc, "I" shaped oral aperture (the shape is not clearly seen due to dirt particles on the corners
 29 of oral aperture), flanked by amphidial opening. True submedian lobes absent, pseudolips six, all

³ Specific epithet is based on the specific tail morphology, *rotundum* = round and *cauda* = tail.

1 well developed with crenate margins, the lateral ones large and well-developed, not fused with
 2 subdorsal and subventral pseudolips, each subdorsal and subventral pseudolips separated or fused.
 3 Stylet robust with anchor-shaped basal knobs, dorsal gland orifice (DGO) hardly visible.
 4 Pharyngeal lumen looped in median pharyngeal bulb, having medium-sized refractive valvular
 5 apparatus, isthmus narrow, short, basal pharyngeal bulb small. Nerve ring encircling isthmus.
 6 Excretory pore 1-2 annuli posterior to pharyngeal bulb base. Gonad monodelphic, outstretched,
 7 spermatheca spherical. Vagina straight, vulva closed. Anterior and posterior annuli surrounding
 8 vulva smaller than the preceding body annuli. Anus hardly visible, 1-3 annuli posterior to vulva.
 9 Tail short, broadly rounded .

10

11 *Male and Juvenile:* Not found.

12

13 Type host and locality

14

15 The type specimens were extracted from the rhizosphere of Chinese wingnut (*Pterocarya*
 16 *stenoptera* C. DC.) at the Yellow River National Geopark of Zhengzhou City, Henan Province on
 17 July, 2019. The geographical position of the sampling site is N: 34°57'14, E: 113°30'41".

18

19 Type material

20

21 Holotype female and 13 female paratypes (slide numbers ZJU-27-01-ZJU-27-05) deposited in
 22 the nematode collection of Zhejiang University, Hangzhou, China. Five female paratypes were
 23 deposited in Nematode collection in Institute for Sustainable Agriculture, CSIC, Córdoba, Spain
 24 (slide number IAS-19-014). Additional slide of voucher material having 7 females (slide number
 25 T-7383p) were deposited at USDA nematode collection, Beltsville, Maryland, USA.

26

27 Diagnosis and relationships

28

29 *Criconemoides rotundicaudatus* n. sp. is characterised by its anteriorly flattened lip region
 30 comprised of two annuli a rounded oral disc and "I" shaped oral aperture, true submedian lobes
 31 absent, pseudolips well-developed with crenate margins, the lateral pseudolips large and well-

1 developed, not fused with subdorsal and subventral pseudolips, each subdorsal and subventral
 2 pseudolips separated or fused, annuli margins smooth to crenated with numerous anastomoses,
 3 stylet robust 36.0 (32.5-38.6) μ m long, excretory pore 1-2 annuli posterior to pharyngeal bulb base,
 4 vulva closed, anterior and posterior annuli surrounding vulva smaller than the preceding body
 5 annuli, anus hardly visible, 1-3 annuli posterior to vulva and tail short and broadly rounded.

6

7 Based on the number of body annuli, stylet length, and shape of postvulval body region, *C.*
 8 *rotundicaudatus* n. sp. is compared with the followings: *C. humilis* Raski and Riffle 1967; *C.*
 9 *parvus*; *C. parvulus* Raski 1952; and *C. zavadskii* (Tulaganov 1941) De Grisse and Loof 1965.
 10 *Criconemoides rotundicaudatus* n. sp. can be differentiated from *C. humilis* by its longer body
 11 (290.6 (246.6-332.1) vs 450 (360-510) μ m), shorter stylet (36 (32.5-38.6) vs 38-46 μ m), higher
 12 number of body annuli (R= 155 (150-164) vs 113 (105-118) μ m), higher number of annuli from
 13 excretory pore to anterior end (Rex= 48 (44-50) vs 30 (28-34)), higher number of annuli from anus
 14 to tail terminus (Ran= 8 (7-9) vs 5 (4-6)), anastomosis numerous (vs few, only 6-7).

15 It differs from *C. parvus* by pseudolips morphology under SEM (pseudolips with crenated
 16 margins, lateral pseudolips large, subdorsal and subventral pseudolips separated or partially fused
 17 vs pseudolips with smooth margins and fused subdorsal and subventral pseudolips, after Maria et
 18 al., 2018), vulval lip morphology (simple vs bulging out and anterior lip faintly bilobed),
 19 postvulval body region morphology (conoid vs trapezoid), tail morphology (broadly rounded vs
 20 bluntly conoid). From *C. parvulus* by lower number of body annuli (R= 155 (150-164) vs 168-
 21 194), higher number of annuli from excretory pore to anterior end, lower number of annuli between
 22 vulva and tail terminus (RV= 9.0 (8-11) vs 13 (12-15)), position of anus (1-3 annuli posterior to
 23 vulva vs 5-8 annuli), number of annuli from anus to tail terminus (Ran= 8.2 (7-9) vs 6), position
 24 of excretory pore related to pharyngeal bulb base (1-2 annuli posterior to pharyngeal bulb base vs
 25 6-7 annuli, after original drawing), postvulval body morphology (tapers gradually to broadly
 26 rounded terminus vs abruptly arrows down to the terminus). From *C. zavadskii* by lower number
 27 of body annuli (R= 155 (150-164) vs more than 200), higher number of annuli between vulva and
 28 tail terminus (RV= 9.0 (8-11) vs 7-8), higher number of annuli from anus to tail terminus (Ran=
 29 8.2 (7-9) vs 5-6), posteriorly located vulva (V= 96 (95-97) vs 94-95), lip region morphology
 30 (truncate vs saucer-shaped) and postvulval body region shape (conoid vs straight).

31

Molecular characterization and phylogenetic relationships of the three new *Criconemoides* species

The new *Criconemoides* species were molecularly characterized using D2-D3 expansion segments of 28S, ITS, 18S and *coxI* fragments and sequences were deposited in the GenBank. Phylogenetic relationships among *Criconemoides* and *Mesocriconema* species were inferred using the aforementioned gene sequences using BI and are given in Figures 10-13 respectively.

In the 28S tree (Fig. 10), *C. parainformis* n. sp. (MN738723-MN738724) and *C. geraerti* n. sp. (MN738725-MN738727) clustered with *C. informis* (KU722386, AY780970), while *C. rotundicaudatus* n. sp. (MN738728-MN738729) clustered with *C. parvus* (MK253539) and two unidentified *Mesocriconema* sp. (MK253540-MK253541). The molecular difference between new species and the phylogenetically related species is as follows. The sequence divergence between *C. parainformis* n. sp. and *C. geraerti* n. sp. was 4.67% (35 bp difference), since *C. informis* is the most closely related species it showed 9.74-9.96% (53-54 bp difference) and 9.36-9.56% (51-52 bp difference) sequence divergence from *C. parainformis* n. sp. and *C. geraerti* n. sp. respectively. The sequence divergence of *C. parvus* and unidentified *Mesocriconema* from *C. rotundicaudatus* n. sp. is 5.98% (37 bp difference) and 5.99-6.06% (33-37 bp difference) respectively.

In the ITS tree (Fig. 11), *C. parainformis* n. sp. (MN738717) and *C. geraerti* n. sp. (MN738718-MN738719) grouped with *C. informis* (KF908246), while *C. rotundicaudatus* n. sp. (MN738720-MN738722) grouped with *C. myungsugae* (MH444640). The molecular difference between new species and the related species is as follows. The sequence divergence between *C. parainformis* n. sp. and *C. geraerti* n. sp. is 12.56% (84 bp difference), as *C. informis* is the most closely related species it showed 1.1% (2 bp difference) and 12.82% (65 bp difference) sequence divergence from *C. parainformis* n. sp. and *C. geraerti* n. sp. This sequence of *C. informis* (KF908246) was submitted from Haidan, China in 2013. No morphological details are available for this sequence. Since *C. informis* presents a large morphometric range, we believe the population from Haidan may be misidentified. Lastly, the sequence divergence between *C. rotundicaudatus* n. sp. and *C. myungsugae* was 14.77 % (82 bp difference).

In the 18S tree (Fig.12), The *C. parainformis* n. sp. (MN738711) and *C. geraerti* n. sp. (MN738712-MN738714) grouped together whereas *C. rotundicaudatus* n. sp. (MN738715-

MN738716) grouped with *C. parvus* (MF795587). The molecular difference between new species and the related species is as follows. The sequence divergence between *C. parainformis* n. sp. and *C. geraerti* n. sp. is 1 % (7 bp difference), whereas sequence divergence between *C. rotundicaudatus* n. sp. and *C. parvus* is 1.91 % (32 bp difference). This close similarity is not surprising, several studies pointed out that closely related nematode species can have identical 18S nucleotide sequences (Tang et al. 2012; Armenteros et al. 2014; Olson et al. 2017).

In the *coxI* gene tree (Fig. 13), *C. parainformis* n. sp. (MN791124-MN791126) grouped with *C. informis* (MF770962), while *C. rotundicaudatus* n. sp. (MN791127-MN791128) grouped with *C. annulatus* Taylor 1936 (MF770956), *C. parvus* (MF770968) and *Mesocriconema sphaerocephala* (Taylor 1936) Loof 1989 (KY574837). The molecular difference between new species and the related species is as follows. The sequence divergence between *C. parainformis* n. sp. and *C. informis* is 15.9% (113 bp difference). The sequence divergence of *C. annulatus* and *M. sphaerocephala* from *C. parainformis* n. sp. is 9.97% (72 bp difference) and 14.98% (106 bp difference) respectively.

15

16 Discussion

17

The *Criconemoides* species have recorded as associated with a broad range of hosts from cultivated, forests and grassland plants (Eskandari et al. 2010; Van den Berg et al. 2012), indicating that species within this genus are quite generalized and can be found in a multitude of environments and plant hosts. Unlike other agricultural pest species of plant-parasitic nematodes, there is little evidence of long-distance dispersal of *Criconemoides* species as only *C. informis* and *C. parvus* were reported to have a cosmopolitan distribution (Geraert 2010). Biogeographically the majority of species were described from Asia (27 spp.) the rest of the species were found in Americas (7 spp.), Africa (5 spp.) Australia (1) and Europe (5 spp.) (Geraert 2010).

Morphological studies concerning *Criconemoides* are numerous in literature, but data on the molecular characterization of these nematodes is still insufficient (Cordero et al. 2012). On the other hands, molecular approaches using different loci have exploited in taxonomy of criconematids (Maria et al., 2018 a, b; 2019; Jahanshahi Afshar et al., 2019 a, b; Van den berg 2017). In the presently inferred phylogenetic trees (Figs 10-13), the new *Criconemoides* species grouped with known *Criconemoides* species, it is also observed that *Mesocriconema* species forms

1 a separate clade except *M. sphaerocephala* and *M. solivaga*. The key differentiating character
2 between the genera *Criconemoides* and *Mesocriconema* is the absence/presence of true submedian
3 lobes respectively. It is imperative to state that a strict description of submedian lobes is often
4 difficult to establish, as these traits cannot be adequately examined on lateral view of the lip region
5 under LM. The origin of sublateral lobes is uncertain. It is also possible that lobes result from the
6 modification of an annulus (Raski 1952) or the lip (De Grisse 1964). The division of lips, in either
7 case, may have occurred primarily because of the sensory organs on the lips (Raski and Golden
8 1965), whatever their origin these are valuable taxonomic guides.

9 Therefore, in a discussion of the phylogenetic position of *Criconemoides* and *Mesocriconema*
10 species, we recommend it is important to find morphological characters that might be markers for
11 different inferred branches of lineages on the phylogeny.

12 In addition, we agreed with Ryss et al. (2013) that phylogeny is an independent way of verifying
13 if the clustered species shares the similar morphological features, as it is not strict framework of
14 assigning species to their morphogroups.

15

16 In *Criconemoides* species female morphological characters are important than males. Male
17 are rare and out of 45 valid species (Geraert, 2010), males were unknown for 34 species. It is likely
18 that *Criconemoides* male play little or no role in reproduction. During our survey, no
19 *Criconemoides* males were encountered in the examined collections.

20 Taxonomic records presently list only three known *Criconemoides* species from China
21 indicating the gaps in knowledge and the complexity of this group to be studied in more detail
22 (Maria et al. 2018a). The discovery of new *Criconemoides* species from Chinese native plants
23 illustrates that the considerable criconematid diversity is hidden within the Chinese soils. However,
24 increased surveys and research opportunities are needed in order to explore the full diversity of
25 criconematids occurring in the country.

26

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals No specific permits were required for the described fieldwork studies. The samples from wild plants were obtained in public areas, forests, and other natural areas studied and do not involve any species endangered or protected in China. The sites are not protected in any way.

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- 19

Figure legends

Fig. 1 Line drawing of *Criconemoides parainformis* n. sp. Female. A: Pharyngeal region; B,C: Lip region (under LM); D, E: *En face* view of lip region; F: Gonad; G: Posterior region showing anastomosis; H: Tail region (under LM); I: Tail region (under SEM). (Scale bars = A-H=10 μ m, I =30 μ m).

Fig. 2 Light micrographs of *Criconemoides parainformis* n. sp. Female. A: Entire body; B-C: Lip region; D-F: Pharyngeal region (arrows showing position of base of pharyngeal bulb(ph.b) and excretory pore (exp)); G: Gonad; H: Posterior region showing anastomosis; I-K: Tail regions arrows showing position of vulva (v) and anus (a). (Scale bars: A=40 μ m, B-K =10 μ m).

Fig. 3 Scanning electron micrographs of *Criconemoides parainformis* n. sp. Female. A: Entire body; B-D: *En face* view; E: Cuticular annuli; F-H: Posterior region showing vulva (v) and anus (a) (Scale bars: A=100 μ m; B-D=10; F, H= 30 μ m; G=20 μ m).

Fig. 4 Line drawing of *Criconemoides geraerti* n. sp.. Female. A: Pharyngeal region; B,C: *En face* view of lip region; D: Anastomosis at middle of the body; E: Gonad; F, G: Tail region (under LM); H: Tail region (under SEM). (Scale bars: A; D-G=10 μ m, B, C; H =20 μ m).

Fig. 5 Light micrographs of *Criconemoides geraerti* n. sp. Female. A: Entire body; B-C: Pharyngeal region arrows showing position of base of pharyngeal bulb (ph.b) and excretory pore (exp); D: Gonad; E-F: Tail regions arrows showing position of vulva (v) and anus (a). (Scale bars = A=40 μ m; B-F =10 μ m).

Fig. 6 Scanning electron micrographs of *Criconemoides geraerti* n. sp. Female. A-C: *En face* view of lip region; D-F: Posterior body region showing vulva (v) and anus (a). (Scale bars: A-C=10 μ m; D-F=20 μ m).

Fig. 7 Line drawing of *Criconemoides rotundicaudatus* n. sp. female. A: Pharyngeal region;

B,C: *En face* view of lip region; D: Gonad; E: Tail region (under SEM); F: Anastomosis at middle of the body; G: Tail region (under LM). (Scale bars = A;-D-G=10 µm, B, C =5 µm).

Fig. 8 Light photomicrographs of *Criconemoides rotundicaudatus* n. sp. Female. A: Entire body; B-C: Pharyngeal region (arrows showing position of base of pharyngeal bulb (ph.b) and excretory pore (exp)); D: Gonad; E-F: Tail regions arrows showing position of vulva (v) and anus (a). (Scale bars = A=40 µm; B-F =10 µm).

Fig. 9 Scanning electron micrographs of *Criconemoides rotundicaudatus* n. sp. Female. A: Entire body; B-D: *En face* view; E, F: Posterior region showing vulva (v) and anus (a). (Scale bars = A=10 µm; B-D=5µm; E, F= 10 µm).

Fig.10 Phylogenetic relationships of *Criconemoides* Taylor, (1914) 1936 and *Mesocriconema* Andrassy, 1965 species as inferred from Bayesian analysis using the D2-D3 expansion segments of 28S rRNA gene with the GTR + I + G model. Posterior probability values more than 70% are given for appropriate clades. Newly obtained sequences are indicated in bold.

Fig. 11 Phylogenetic relationships of *Criconemoides* Taylor, (1914) 1936 and *Mesocriconema* Andrassy, 1965 species as inferred from Bayesian analysis using the ITS rRNA gene sequence dataset with the GTR + I + G model. Posterior probability values more than 70% are given for appropriate clades. Newly obtained sequences are indicated in bold.

Fig. 12 Phylogenetic relationships of *Criconemoides* Taylor, (1914) 1936 and *Mesocriconema* Andrassy, 1965 species as inferred from Bayesian analysis using the partial 18S rRNA gene sequence dataset with the GTR + G model. Posterior probability values more than 70% are given for appropriate clades. Newly obtained sequences are indicated in bold.

Fig. 13 Phylogenetic relationships of *Criconemoides* Taylor, (1914) 1936 and *Mesocriconema* Andrassy, 1965 species as inferred from Bayesian analysis using *coxI* gene sequence dataset with the GTR + G model. Posterior probability values more than 70% are given for appropriate clades. Newly obtained sequences are indicated in bold.

Table 1 Morphometrics of *Criconemoides parainformis* n. sp. female. All measurements are in μm and in the form: mean $\pm$ s.d. (range).

Characters	Holotype	Paratypes
n	-	19
Body length	382.5	382.1 $\pm$ 30.4 (339.6-438.1)
R	60.0	63.0 $\pm$ 2.2 (58.0-66.0)
Rst	11.0	12.0 $\pm$ 0.7 (11.0-13.0)
Rex	20.0	20.4 $\pm$ 0.8 (19.0-22.0)
RV	6.0	5.7 $\pm$ 0.4 (5.0-6.0)
Rvan	2.0	2.2 $\pm$ 0.5 (2.0-3.0)
Ran	4.0	3.5 $\pm$ 0.5 (3.0-4.0)
a	9.7	10.2 $\pm$ 1.0 (8.8-11.9)
b	3.2	3.7 $\pm$ 0.3 (3.2-4.2)
c	25.3	29.6 $\pm$ 4.7 (21.8-42.1)
c'	0.7	0.7 $\pm$ 0.1 (0.5-0.8)
V	93.1	93.4 $\pm$ 0.8 (92.3-95.4)
VL/VB	0.9	0.9 $\pm$ 0.1 (0.8-1.1)
Lip height	5.7	6.5 $\pm$ 0.7 (5.7-7.6)
Lip diam.	15.9	16.7 $\pm$ 1.1 (15.1-19.2)
Stylet length	67.2	63.7 $\pm$ 2.6 (59.4-67.9)
*Stylet percentage	17.6	16.8 $\pm$ 1.3 (14.2-18.9)
Pharynx length	119.8	102.6 $\pm$ 4.9 (95.7-119.8)
Maximum body width	39.5	37.4 $\pm$ 1.2 (34.4-39.5)
Vulval body diam.	28.7	27.3 $\pm$ 2.3 (23.7-31.8)
Anal body diam.	22.8	20.1 $\pm$ 2.6 (15.4-24.6)
Distance from vulva to tail terminus	26.5	24.9 $\pm$ 2.2 (20.3-29.5)
Tail length	15.1	13.2 $\pm$ 1.8 (9.5-16.6)

*Stylet percentage calculated as (stylet length/total body length)*100

Table 2 Morphometrics of *Criconemoides geraerti* n. sp. female. All measurements are in μm and in the form: mean $\pm$ s.d. (range).

Characters	Holotype	Paratype
n	-	19
Body length	369.6	366 $\pm$ 33.9 (321.7-455.5)
R	76	80 $\pm$ 3.9 (71.0-84.0)
Rst	13	14.0 $\pm$ 0.8 (13.0-16.0)
Rex	25	25.0 $\pm$ 0.8 (24-27)
RV	6	6.0 $\pm$ 0.6 (5.0-7.0)
RVan	2	1.7 $\pm$ 0.5 (1.0-2.0)
Ran	4	4.5 $\pm$ 0.6 (3.0-5.0)
a	9.9	9.6 $\pm$ 0.8 (8.1-10.6)
b	3.6	3.6 $\pm$ 0.3 (3.2-4.3)
c	25.3	24.4 $\pm$ 3.1 (20.3-30.5)
c'	0.7	0.7 $\pm$ 0.1 (0.5-0.8)
V	93.7	93.6 $\pm$ 0.5 (92.6-94.2)
VL/VB	0.9	0.9 $\pm$ 0.1 (0.8-1.0)
Lip height	5.7	6.1 $\pm$ 0.4 (5.5-6.8)
Lip width	15.7	15.9 $\pm$ 0.7 (15.0-17.4)
Stylet length	56.7	60.2 $\pm$ 2.0 (57.0-62.7)
*Stylet percentage	15.3	16.5 $\pm$ 1.4 (13.6-19.4)
Pharynx length	101.7	103.0 $\pm$ 2.9 (100.0-109.8)
Maximum body width	37.4	38.6 $\pm$ 3.5 (34.3-43.6)
Vulval body diam.	25.1	25.8 $\pm$ 2.4 (22.3-31.2)
Anal body diam.	22.0	23.0 $\pm$ 1.8 (20.1-26.3)
Distance from vulva to tail terminus	23.2	23.5 $\pm$ 2.2 (19.4-27.4)
Tail length	14.6	15.2 $\pm$ 2.1(11.5-18.8)

*Stylet percentage calculated as (stylet length/total body length)*100

Table 3 Morphometrics of *Criconemoides rotundicaudatus* n. sp. female. All measurements are in μm and in the form: mean $\pm$ s.d. (range).

Characters	Holotype	Paratype
n		19
Body length	292.6	290.6 $\pm$ 19.9 (246.6-332.1)
R	154.0	155.0 $\pm$ 4.5 (150.0-164.0)
Rst	24.0	23.0 $\pm$ 1.2 (21.0-25.0)
Rex	50.0	48.0 $\pm$ 1.7 (44-50)
RV	10.0	9.0 $\pm$ 0.7 (8.0-11.0)
RVan	1.0	1.4 $\pm$ 0.5 (1.0-3.0)
Ran	9.0	8.2 $\pm$ 0.7 (7.0-9.0)
a	13.5	11.8 $\pm$ 0.9 (9.7-13.5)
b	3.6	3.5 $\pm$ 0.3 (3.1-4.3)
c	47.2	43.6 $\pm$ 6.1 (32.4-56.8)
c'	0.4	0.4 $\pm$ 0.1 (0.3-0.5)
V	96.6	96.3 $\pm$ 0.5 (95.4-97.0)
VL/VB	0.5	0.6 $\pm$ 0.1 (0.4-0.7)
Lip height	3.0	3.5 $\pm$ 0.5 (3.0-4.4)
Lip width	9.1	9.3 $\pm$ 0.6 (8.5-10.3)
Stylet length	35.8	36.0 $\pm$ 1.6 (32.5-38.6)
*Stylet percentage	12.2	12.4 $\pm$ 0.7 (10.8-13.5)
Pharynx length	81.3	82.0 $\pm$ 2.0 (77.8-86.4)
Maximum body width	21.7	24.7 $\pm$ 2.0 (21.7-28.7)
Vulval body diam.	18.7	19.4 $\pm$ 1.3 (17.6-22.1)
Anal body diam.	16.5	16.7 $\pm$ 1.3 (14.8-19.3)
Distance from vulva to tail terminus	10.0	10.7 $\pm$ 1.3 (8.4-13.1)
Tail length	6.2	6.8 $\pm$ 1.1 (5.4-9.3)

*Stylet percentage calculated as (stylet length/total body length)*100