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Description of *Gracilacus paralatescens* n. sp. (Nematoda: Paratylenchinae) found from the rhizosphere of Bamboo in Zhejiang, China

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Abstract

Gracilacus paralatescens n. sp., isolated in Anji County, Zhejiang Province, China from the rhizospheric soil of bamboo. The new species can be characterized by the female lateral field with three incisures, stylet 71.5 to 78.8 µm long, lip region truncated, excretory pore located anterior to basal knobs. Vulval lips non-protruding and without vulval flap, spermatheca large, elongated squarish shaped filled with sperms. Tail slender, relatively straight having wedge shape terminus. The spicule slender, slightly curved and 17.5 to 18.9 µm long. In the phylogenetic analysis based on 18S, D2-D3 of 28S and ITS regions of rDNA, the new species is clustered with Paratylenchid species having longer stylet length. Morphologically, the new species belongs to Group 9 of *Paratylenchus sensu lato* and is most similar to *G. latescens.*

Key words

DNA sequencing, Morphology, Morphometrics, Nematode, New species, Phylogeny, Scanning electron microscopy.

The genus *Gracilacus* was erected by Raski (1962) for those *Paratylenchus* species having stylet length more than 48 µm. The validity of the genus *Gracilacus* has been questioned several times. The species were either synonymised to *Paratylenchus* (Brzeski, 1963; Siddiqi and Goodey, 1964; Geraert, 1965; Brzeski, 1998; Nguyen et al., 2004; Decraemer and Hunt, 2006; Ghaderi et al., 2014), treated as subgenus (Siddiqi, 2000) or maintained (Raski and Luc, 1987; Maggenti et al., 1988; Raski, 1991; Esser, 1992; Andrássy, 2007) reflecting the validity of the genus *Gracilacus* in the subfamily Paratylenchinae Thorne, 1949.

The species of pin nematodes are widely distributed in China. So far, 30 species have been reported from Beijing, Fujian, Gansu, Guandong, Guangxi, Hebei, Heilongjiang, Henan, Hunan, Jiangsu, Jilin, Liaoning, Ningxia, Inner Mangolia, Shandong, Shanxi, Sichuan, Taiwan, Xinjiang, and Zhejiang Provinces. These includes *G. aculenta* Brown, 1959; *P. audriellus* Brown, 1959; *P. alleni* Raski, 1975; *G. bilineata* Brzeski, 1995; *P. bukowinensis* Micoletzky, 1922; *P. ciccaronei* Raski, 1975; *P. curvitatus* Raski, 1975b; *P. dianthus* Jenkins and Taylor, 1956; *P. elachistus* Steiner, 1949; *G. epacris* Allen and Jensen, 1950; *G. goodeyi* Oostenbrink, 1953; *G. latescens* Raski, 1976; *P. lepidus* Raski, 1975; *P. mexicanus* Raski, 1975; *P. microdorus* Andrássy, 1959; *P. minutus* Linford et al., 1949; *P. nanus* Cobb, 1923; *P. neoamblycephalus* Geraert, 1965; *P. neoprojectus* Wu and Hawn, 1975; *P. perlatus* Raski, 1975; *P. projectus* Jenkins, 1956; *P. prunii* Sharma, Sharma and Khan, 1986; *G. raskii* Phukan and Sanwal, 1979; *G. steineri* Golden, 1961; *G. straeleni* Oostenbrink, 1960; *G. verus* Brzeski, 1995; *P. veruculatus* Wu, 1962; and *P. vexans* Thorne

and Malek, 1968. Among those, *P. guangzhouensis* Wang et al., 2015; *P. nanjingensis* Wang et al., 2014; *P. shenzhenensis* Wang et al., 2013 are three new species described from China (Chen et al., 2002, 2008; Zhou et al., 2005; Fang et al., 2012; Wang et al., 2014, 2015). However, *P. curvitatus* has *species inqurenda* status in Ghaderi et al. (2014).

A nematode survey was conducted in bamboo plantation of Anji County, Zhejiang Province, China. Bamboo has deep cultural and economic roots in China, a country with the largest bamboo resources in the world (Ruiz Pérez et al., 2014). It is an important forest resource and has a major use in tool making, housing, musical instruments, food, paper, irrigation systems, and developing a variety of transporting devices and infrastructures (Zou and Liang, 2008).

In the present study, a *Gracilacus* population was isolated from the rhizospheric soil of bamboo. Preliminary identification revealed that the species belongs to group 9 of Ghaderi et al. (2014). Detailed morphological and molecular analysis indicated the status of this species as a new species and it is herein described as *Gracilacus paralatescens* n. sp.

Materials and methods

Nematode samplings, extraction and morphological study

Nematodes were extracted from soil and root samples using modified Cobb sieving and flotation-centrifugation method (Jenkins, 1964). For morphometric studies, nematodes were killed and fixed in hot Formalin (4% with 1% glycerol) and processed to glycerin (Seinhorst, 1959). The drawings, measurements, and light micrographs of nematodes were made with a Nikon Eclipse Ni-U 931845 compound microscope. 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., 2018).

Molecular analyses

DNA was extracted by transferring individual nematodes into an Eppendorf tube containing 16 µL ddH_2O. Two µL PCR buffer solution was added to each tube. Nematodes were crushed using a sterilized pipette tip, the tubes were centrifuged at 12,000 rpm for 1 min and frozen at −68°C for at least 30 min. Tubes were heated to 85°C for 2 min, then 2 µL proteinase K was added. The tubes were incubated at 56°C for 1 to 2 hr, followed by 10 min at 95°C. After incubation, these tubes were cooled to 4°C and used for conducting PCR (Zheng et al., 2003). Several sets of primers (synthesized by Invitrogen, Shanghai, China) were used in the PCR analyses to amplify the near-full-length 18S region, D2-D3 of 28S, and ITS region of rDNA. Primers for amplification of 18S were 18s39F-18s977R and 18s900-18s1713 (Olson et al., 2017). Primers for amplification of ITS were TW81-AB28 (Joyce et al., 1994). The primers for amplification of D2-D3 of 28S were D2A and D3B (De Ley et al., 1999). PCR conditions were as described by Ye et al. (2007) and Powers et al. (2010). 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).

Phylogenetic analysis

The sequences were deposited into the GenBank database. DNA sequences were aligned by MEGA7 (Kumar et al., 2016.) using default settings. The DNA sequences were compared with those of the other nematode species available at the GenBank sequence database using the BLAST homology search program. The model of base substitution was evaluated using MODELTEST (Posada and Crandall, 1998; Huelsenbeck and Ronquist, 2001). The Akaike-supported model, the base frequencies, the proportion of invariable sites and the gamma distribution shape parameters and substitution rates were used in phylogenetic analyses. Bayesian analysis was performed to confirm the tree topology for each gene separately using MrBayes 3.1.0 (Huelsenbeck and Ronquist, 2001) running the chain for 1×10^6 generations and setting the "burnin" at 2,500. We used the Markov Chain Monte Carlo (MCMC) method within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget and Simon, 1999) using 50% majority rule.

Results

Systematics
*Gracilacus paralatescens** n. sp.

(Figs. 1–3)

Measurements

Measurements of the holotype, females and male paratype of *G. paralatescens* n. sp. are given in Table 1.

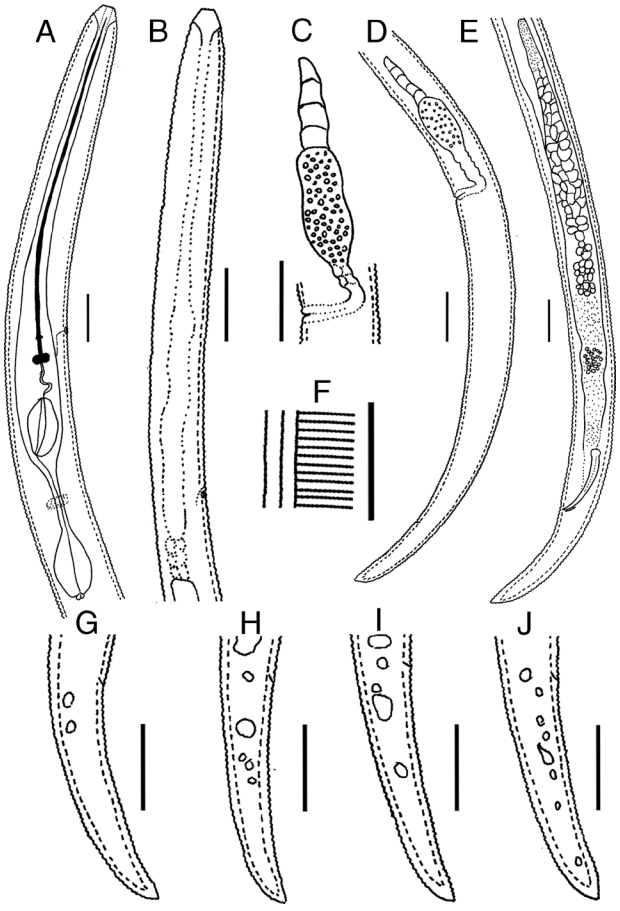

Figure 1: Line drawings of *Gracilacus paralatescens* n. sp. A: Female pharynx; B: Male pharynx; C: Vulval region and reproductive branch; D: Female posterior body; E: Male posterior body; F: Lateral lines; G-J: Female tails (Scale bars =10 µm).

Description

Female

The female body can be described as follows: Body slender, not obese, ventrally arcuate when heat relaxed; cuticle finely annulated; lateral field with three incisures; *en face* view showed smooth lip region without submedian lobes, oral aperture slit-like; lip region narrow, truncated without submedian lobes, not offset from body; cephalic sclerotization weak; stylet flexible, elongated, ventrally curved, cone *ca* 90% of the total stylet length; stylet knobs rounded; pharynx constitutes almost two-fifths of the total body length; dorsal oesophageal gland opening inconspicuous; median pharyngeal bulb elongate, bearing distinct large valve; isthmus short slender, surrounded by nerve ring; basal bulb pyriform, cardia inconspicuous, lobe-shaped; excretory pore anterior to the level of stylet knobs; hemizonid immediately posterior to excretory pore; gonad short, prodelphic, spermatheca elongated squarish (the double size of body width) filled with sperm; vulva a transverse slit occupying half of the body width, vulval lips not protruding without advulval flaps, post uterine sac absent; anus indistinct; tail slender, finely annulated, relatively straight, gradually tapers to form a wedge-shaped (V shape) terminus.

Male

The male body can be described as follows: Body more slenderer than female, curved to open 'C' shaped when heat relaxed; cuticle smooth with fine annulations; cephalic region narrower than female, continuous from body, no obvious sclerotization in cephalic region; pharynx rudimentary, procorpus, metacorpus, and basal bulb inconspicuous; stylet lacking; excretory pore located at the level of metacorpus; hemizonid immediately posterior to excretory pore; testis outstretched, with small spermatozoa; spicule slender, slightly curved; gubernaculum curved; penile sheath short, only observed when spicule protrudes from cloaca; bursa absent; tail cylindrical, slightly ventral curved, tapering gradually to a bluntly pointed tip.

Juveniles

Juveniles were not observed in this study.

Type host and locality

The type material was collected from rhizospheric soil samples of bamboo trees from Anji County, Zhejiang Province, China on November 22, 2017.

Type specimens

Holotype female, 22 paratype females and 4 paratype males were deposited in the nematode collection of Zhejiang University (ZJU), P.R. China (slides no. N01-1 to N01-12). Three female paratypes were deposited in the Nematode collection in Institute for Sustainable Agriculture, CSIC, Córdoba, Spain.

Differential diagnosis

Gracilacus paralatescens n. sp. is characterized by the lateral field with three incisures, stylet 71.5 to 78.8 µm

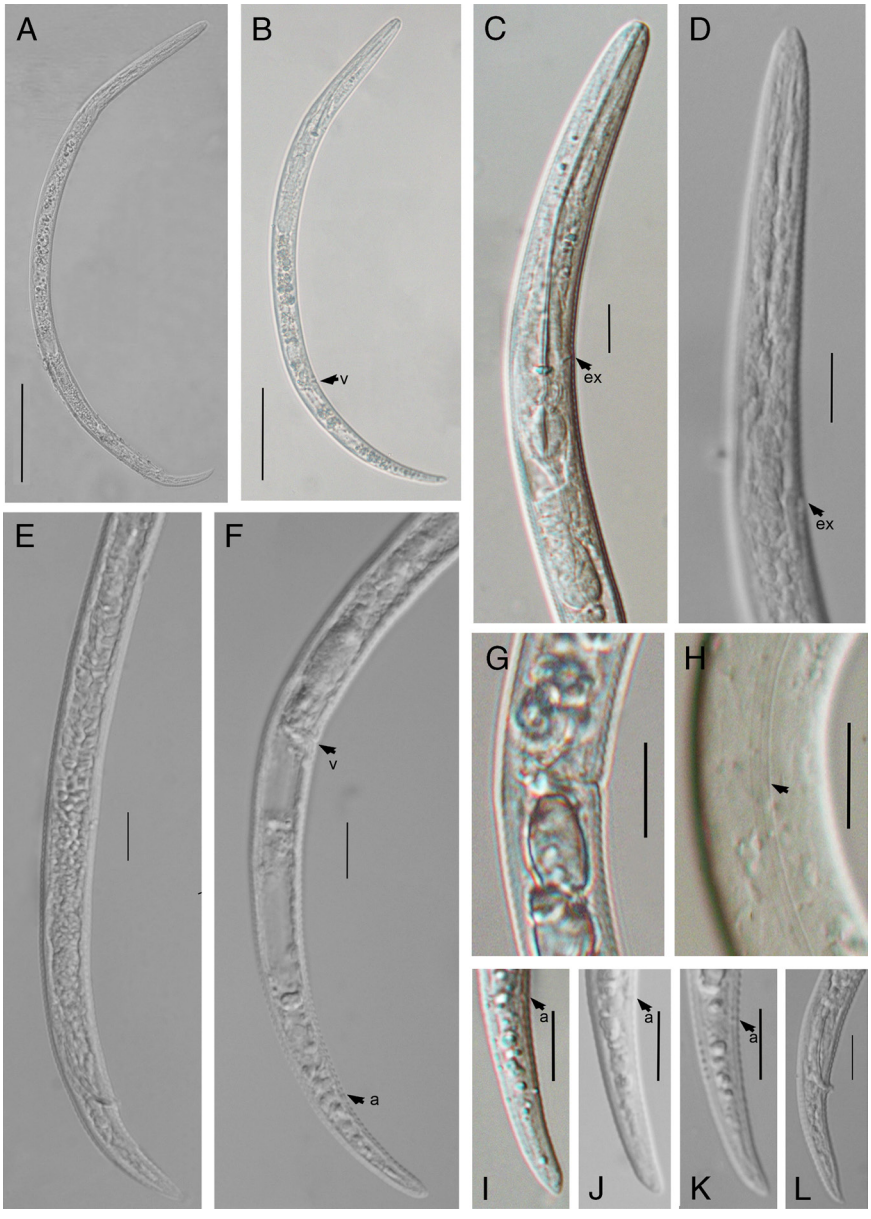

Figure 2: Light photomicrographs of *Gracilacus paralatescens* n. sp. A: Entire male; B: Entire female; C, D: Female and male anterior regions; E, F: Male and female posterior regions; G: Vulval region; H: Lateral lines; I-K: Female tails; L: Male tail (Scale bars = A, B = 50 µm, C-L 10 µm) C-L = 10 µm; Abbreviations: a = anus; ex = excretory pore; v = vulva).

long, vulval lips non-protruding, without vulval flap; lip region truncated, continuous, and narrow; excretory pore located at the level of stylet shaft; gonad prodelphic, with large spermatheca; tail slender, relatively straight with wedge-shaped terminus; the spicule slender, slightly curved and 17.5 to 18.9 µm long.

According to the grouping scheme of *Paratylenchus sensu lato* by Ghaderi et al. (2014), this new species belongs to Group 9 (stylet> 40 µm; three lateral lines; and advulval flaps absent). There are seven species in this group, namely, *Gracilacus acicula* (Brown, 1959) Raski, 1962, *G. aculenta* (Brown, 1959) Raski, 1962, *G. anchora* Mohilal and Dhanachand, 2004, *G. costata* Raski, 1976, *G. latescens*, *Paratylenchus musae* (Shahina and Maqbool, 1993) Brzeski, 1998, *G. solivaga* Raski, 1976.

The new species differs from *G. aciculus* by stylet length 74.8 (71.5–78.8) vs 67 (61–69) µm, a value = (22–29 vs 15–24), spicule length (17.5–18.9 vs 14.5–16.5 µm), female tail terminus (wedge-shaped vs

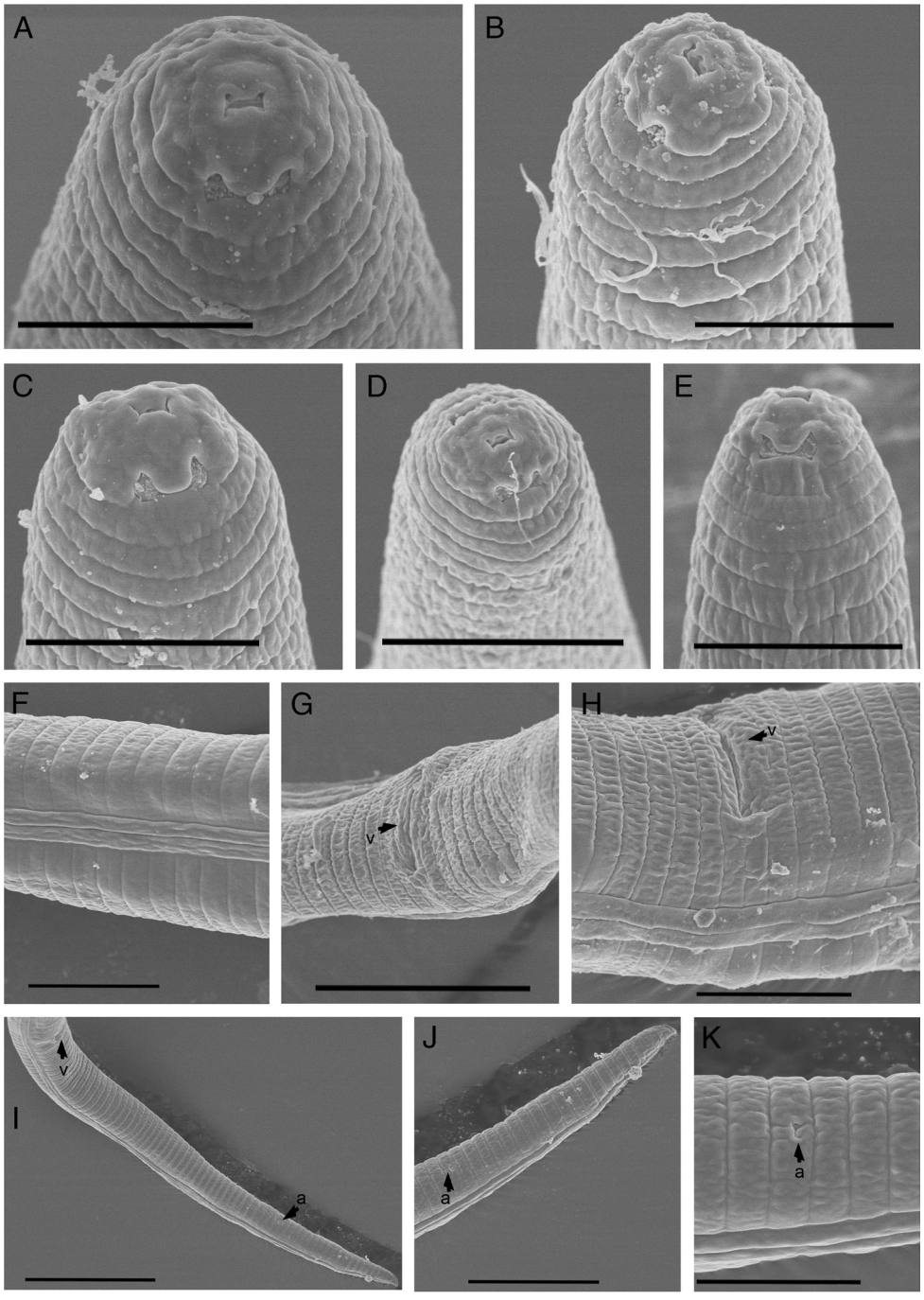

Figure 3: Scanning electron microscopy of *Gracilacus paralatescens* n. sp. A-E: Lip region; F: Lateral lines; G, H: Vulval regions; I: Posterior region arrows showing position of vulva (v) and anus (a); J: Tail region arrow showing position of anus; K: Anal region (Scale bars, A-C = 3 µm; E, K = 4 µm; D, H = 5 µm; J, G = 10 µm; I = 20 µm).

subacute to finely rounded); from *G. aculenta* by stylet length 74.8 (71.5-78.8) vs 58 (52-66) µm, spicule length (17.5-18.9 vs 15 µm), female tail terminus (wedge-shaped vs subacute to bluntly rounded); from *G. anchora* by lip region (truncated narrow vs rounded), position of the excretory pore (in the stylet shaft area vs anterior to stylet shaft), tail shape (relatively straight with wedge shape terminus vs conoid with subacute terminus), male (present vs absent); from *G. costata* by stylet length 74.8 (71.5-78.8) vs 77 (70-87) µm, vulval

Table 1. Morphometrics data for *Gracilacus paralatescens* n. sp.

Characters/Ratios	Holotype	Paratypes	
		Female	Male
n	1	26	4
L	285.4	290.4 ± 11.8 (271.1–308.1)	312.8 ± 12.4 (300.3–324.2)
a	25.0	25.3 ± 1.7 (22.4–29.0)	30.2 ± 2.0 (27.6–32.1)
b	2.2	2.3 ± 0.1 (2.2–2.5)	3.5 ± 0.2 (3.3–3.7)
c	11.0	11.2 ± 1.1 (9.3–13.8)	11.1 ± 0.8 (10.3–12.1)
c’	4.5	4.5 ± 0.4 (3.2–5.4)	3.6 ± 0.3 (3.3–4.0)
V/T	70.6	70.3 ± 1.2 (68.2–72.9)	31.5 ± 5.3 (23.8–35.7)
Lip region height	1.7	1.9 ± 0.3 (1.2–2.3)	2.0 ± 0.1 (1.9–2.1)
Lip region diam.	4.8	4.5 ± 0.2 (4.0–4.9)	3.5 ± 0.2 (3.2–3.8)
Stylet	72.3	74.8 ± 2.4 (71.5–78.8)	–
Median bulb length	14.6	15.6 ± 1.3 (13.4–18.7)	–
Median bulb diam.	7.4	7.5 ± 0.6 (6.2–8.6)	
Median bulb clip length	8.2	8.5 ± 1.3 (5.8–9.7)	–
Median bulb clip diam.	4.0	4.2 ± 0.2 (4.0–4.5)	–
Ant. end to excretory pore	69.5	68.8 ± 2.8 (62.8–73.4)	66.3 ± 1.9 (63.6–67.8)
Pharynx length	128.9	125.6 ± 4.6 (118.7–133.5)	90.4 ± 2.4 (87.8–93.4)
Max. body diam.	11.4	11.5 ± 0.7 (10.3–12.7)	10.4 ± 0.4 (10.1–10.9)
Vulval body diam.	11	10.9 ± 0.6 (9.7–12.5)	–
Vulva to tail terminus	83.9	86.3 ± 5.9 (73.6–93.7)	–
Anal/cloacal body diam.	5.8	5.9 ± 0.5 (5.0–6.8)	8.0 ± 0.7 (7.4–8.8)
Tail length	25.9	26.2 ± 2.5 (21.3–29.9)	28.2 ± 1.5 (26.7–29.5)
Spicule	–	–	18.1 ± 0.7 (17.5–18.9)
Gubernaculum	–	–	4.5 ± 0.6 (4.0–5.3)

All measurements are in μm and in the form of mean ± SD (range).

lips (not protruding vs protruding), position of excretory pore (at the range of stylet shaft vs anterior to shaft), spermatheca (conspicuous vs inconspicuous), tail shape (relatively straight with wedge shape terminus vs curved ventrally with subacute to finely rounded terminus), spicule length 18.1 (17.5–18.9) vs 20 (18–22) μm; from *G. latescens* by the obese females (absent vs present), lip region morphology (truncated narrow vs rounded), submedian lobes (absent vs present), position of excretory pore (at the range of stylet shaft vs at the base of stylet), female vulva lips (non-protruding vs protruding), a value = 25.3 (22.4–29) vs 19 (14–23), tail shape (relatively straight with wedge shape terminus vs conoid with to bluntly rounded terminus), spicule length 18.1 (17.5–18.9) vs 20 (18–23) μm, T value = 31.5 (23.8–35.7) vs 37 (27–47); from *G. musae* by the stylet length (71.5–78.8 vs 42–44) μm, c value = (9.3–13.8 vs 20–22), V value = (68.2–72.9 vs 82–84); from *G. solivaga* by female body length 290 (271–308) vs 240 (220–250) μm, position of excretory pore (at the range of stylet shaft vs anterior to shaft), spermatheca (distinct filled with sperm vs indefinite without sperm), tail shape (relatively straight with wedge shape terminus vs conoid with lobed irregular terminus), male (present vs absent).

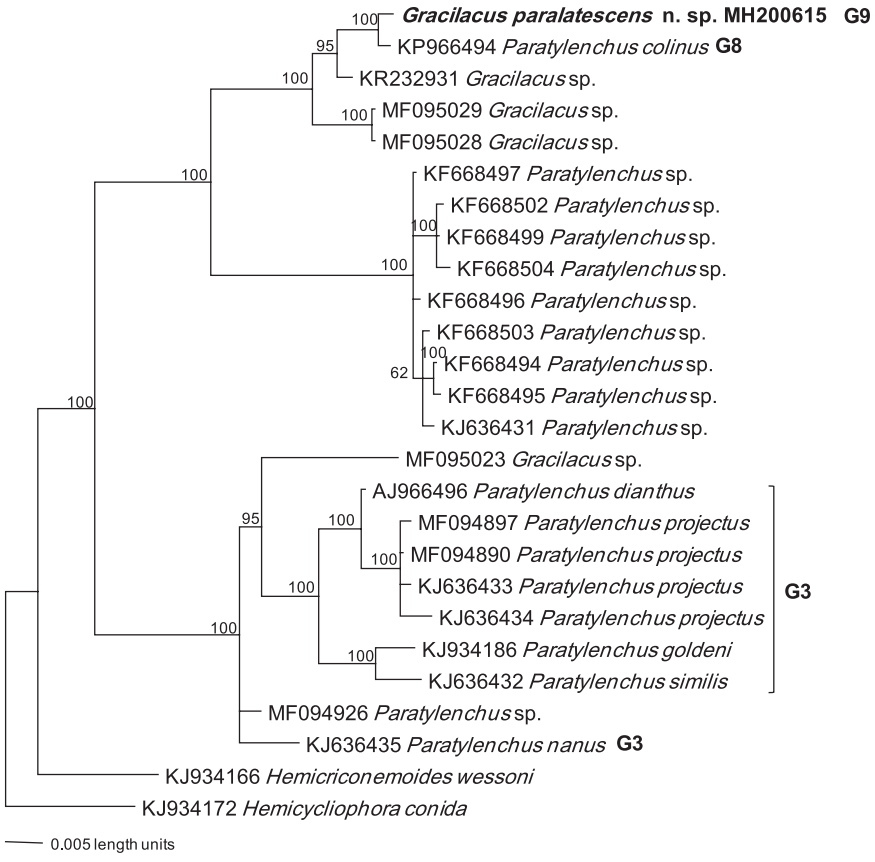

Figure 4: Bayesian consensus tree of *Gracilacus paralatescens* n. sp. inferred from combined 18S and ITS under TIM+I+G model (-lnL = 3318.9573; AIC = 6653.9146; freqA = 0.2748; freqC = 0.2016; freqG = 0.2586; freqT = 0.265; R(a) = 1; R(b) = 2.5867; R(c) = 0.479; R(d) = 0.479; R(e) = 6.0139; R(f) = 1; Pinva = 0.4072; Shape = 0.814). Posterior probability values exceeding 50% are given on appropriate clades.

Etymology

*The species name is derived from the most similar species *Gracilacus latescens* Raski, 1976.

Molecular profiles and phylogenetic status

The size of sequenced fragments of near-full-length 18S, D2-D3 of 28S and ITS was of *ca* 1718, 1051, and 861bp, respectively. Sequences from *G. paralatescence* n. sp. matched well with the paratylenchid nematode sequences deposited in GenBank but with distinct differences. The partial 18S sequence of *G. paralatescence* n. sp. (MH200615) showed 98.6% (9bp difference) sequence similarity with *G. latescence* (AY912039), 98 to 99% similarity (differing from 5 to 29 nucleotides) with *P. colinus* (KP966494), *Gracilacus* sp. (KR232931) from China and two *Gracilacus* spp. (MF095028-29) from the USA. The D2-D3 of 28S rRNA sequence of *G. paralatescence* n. sp. (MH200616) showed 91 to 98% similarity values (differed in a range from 12 to 71 nucleotides) with several paratylenchid nematode spp. such as *P. aculentus* (KR270597), *G. audrielus* (KU291238), *P. colinus* (KP966492), *G. wuae* (KM061782), and *Gracilacus* sp. (KR232932) from China. ITS sequence for *G. paralatescence* n. sp. (MH200615) showed 81 to 97% similarity (differing from 30 to 168 nucleotides) with *G. aculenta* (EU247526), *P. aculentus* (KR270603), *G. bilineata* (EU247525), *P. guangzhouensis* (KT725625-26), *P. nanjingensis* (KM366101,103), *G. wuae* (KM061783), and *Gracilacus* sp. (KR232933) from China.

Phylogenetic relationships among paratylenchid nematode species inferred from analyses of 18S, D2-D3 of 28S, and ITS rRNA gene sequences using BI are given in Figures 4, 5, and 6, respectively. A combined 18S and ITS tree (Fig. 4) is based on a multiple edited alignment of 28 sequences using *Hemicricon-*

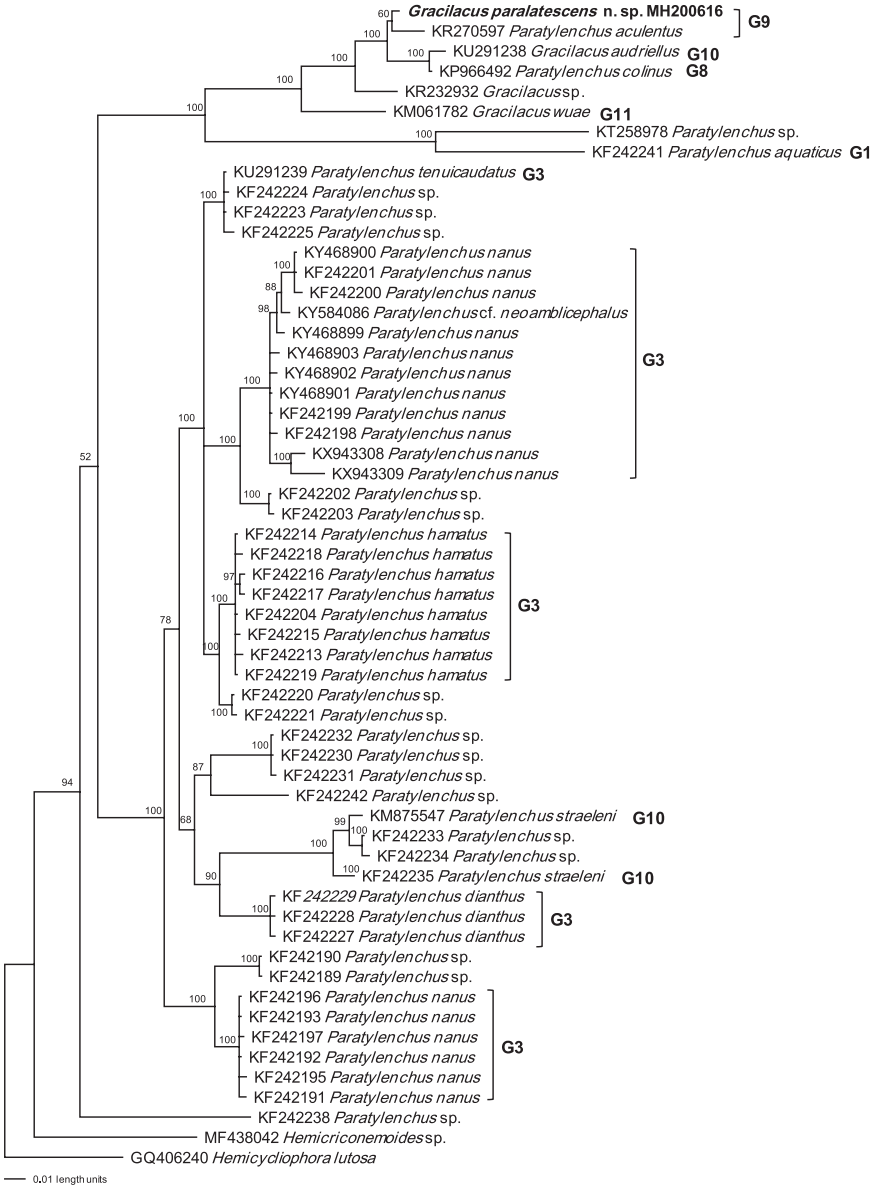

Figure 5: Bayesian consensus tree of *Gracilacus paralatescens* n. sp. inferred from 28S D2-D3 under GTR+I+G model (-lnL = 6253.7139; AIC = 12527.4277; freqA = 0.2129; freqC = 0.1946; freqG = 0.3023; freqT = 0.2902; R(a) = 0.7174; R(b) = 7.1524; R(c) = 2.4855; R(d) = 0.6881; R(e) = 12.1391; R(f) = 1; Pinva = 0.2537; Shape = 1.3274). Posterior probability values exceeding 50% are given on appropriate clades.

emoides wessoni (KJ934166) and *Hemicycliophora wessoni* (KJ934172) as out groups. In this tree, the *G. paralatescence* n. sp. is clustered with *P. colinus* and three unidentified *Gracilacus* species with higher probability support (PP = 100). *G. latescence* was not included in the phylogenetic analysis as only 637bp of this gene at 3′ end was sequenced from Konza, Kansas, USA. The D2-D3 of 28S rRNA gene tree (Fig. 5) based on a multiple edited alignment of 58 sequences using *Hemicycliophora lutosa* (GQ406240) and *Hemicriconemoides* sp. (MF438042) as out groups. In this tree, the *G. paralatescence* n. sp. is clustered with *P. aculentus*, *G. audrielus*, *P. colinus*, *G. wuae*, and *Gracilacus* sp. with higher support (PP = 100). The ITS tree (Fig. 6) based on a multiple edited alignment of 71 sequences using *Hemicycliophora conida* (FN435301)

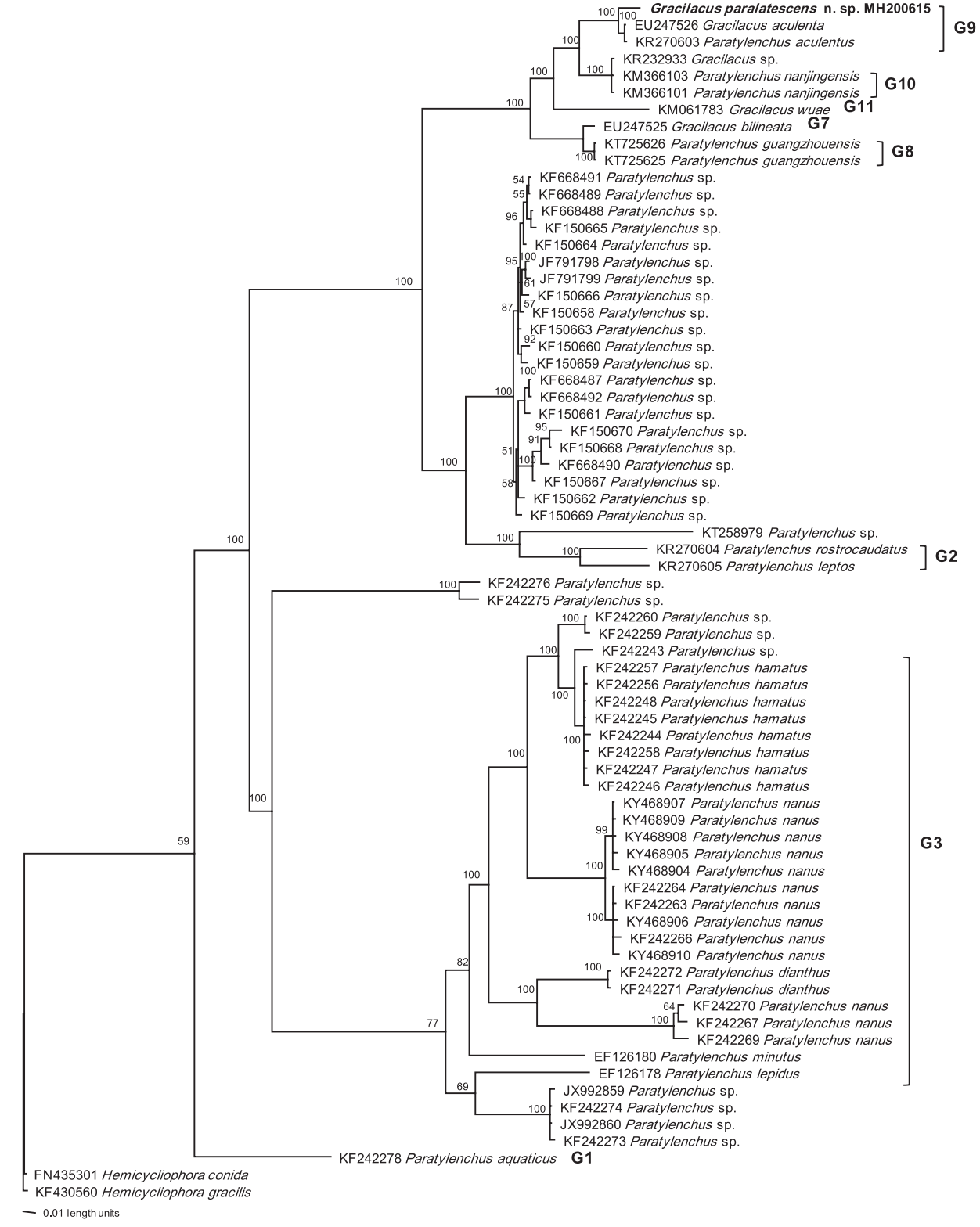

Figure 6: Bayesian consensus tree of *Gracilacus paralatescens* n. sp. inferred from ITS under SYM+I+G model (-lnL = 9907.1797; AIC = 19828.3594; freqA = 0.278; freqC = 0.2355; freqG = 0.2265; freqT = 0.2601; R(a) = 1.2457; R(b) = 6.0854; R(c) = 2.081; R(d) = 0.4505; R(e) = 7.1498; R(f) = 1; Pinva = 0.0908; Shape = 1.0971). Posterior probability values exceeding 50% are given on appropriate clades.

and *H. gracilis* (KF430560) as out groups. In this tree, the *G. paralatescence* n. sp. is clustered with *G. aculenta*, *P. aculentus*, *G. bilineata*, *P. guangzhouensis*, *P. nanjingensis*, *G. wuae*, and *Gracilacus* sp. from China with higher probability support (PP = 100).

Discussion

Ghaderi et al. (2014) proposed an 11-group scheme based on stylet length, the presence/absence of ad-vulval flap and number of lateral lines to identify *Paratylenchus* species, i.e., Group 1. Stylet < 40 µm; lateral lines = 2; advulval flaps present, Group 2. Stylet < 40 µm; lateral lines = 3; advulval flaps present, Group 3. Stylet < 40 µm; lateral lines = 4; advulval flaps present, Group 4. Stylet < 40 µm; lateral lines = 4; advulval flaps absent, Group 5. Stylet < 40 µm; lateral lines indistinct; advulval flaps present, Group 6. Stylet > 40 µm; lateral lines = 2; advulval flaps present, Group 7. Stylet > 40 µm; lateral lines = 2; advulval flaps absent, Group 8. Stylet > 40 µm; lateral lines = 3; advulval flaps present, Group 9. Stylet > 40 µm; lateral lines = 3; advulval flaps absent, Group 10. Stylet > 40 µm; lateral lines = 4; advulval flaps present, Group 11. Stylet > 40 µm; lateral lines = 4; advulval flaps absent. According to this scheme, the maximum number of species reported from China belongs to Group 3 (18 species) followed by Group 10 (6 spp.), Groups 9 and 11 represent only two species and single species was reported from Groups 7 and 8. The new species is described from bamboo species. Several other Chinese populations of *G. aculentus*, *G. bilineata*, and *P. guanzhouensis* were also reported from bamboo.

Paratylenchids have a diverse range of stylet length ranging from 10 to 120 µm. In our phylogenetic analysis, the new species is grouped with long stylet paratylenchids. Yu et al. (2016) suggested that the stylet length greater than 41 µm is not homologous and has evolved more than once within the *Paratylenchus* lineage. The phylogenetical studies of Paratylenchid nematodes conducted by Lopez et al. (2013), Van den Berg et al. (2014), Wang et al. (2014, 2015) demonstrated that the Paratylenchid nematode with longer stylets grouped together. This provides the strong support of genetic grouping of Paratylenchid nematodes into two genera based on the stylet length. This supports that the possibility of *Gracilacus* as a valid genus cannot excluded (Van den Berg et al., 2014; Yu et al., 2016). However, only a few species with longer stylets are available for molecular comparisons. Hence, future taxonomic studies coupled with DNA sequencing on the paratylenchids will help to clarify relationships within family Paratylenchidae.

Acknowledgments

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