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Morphological and Molecular Characterization of *Gracilacus wuae* n. sp. (Nematoda: Criconematoidea) Associated with Cow Parsnip (*Heracleum maximum*) in Ontario, Canada

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Morphological and Molecular Characterization of *Gracilacus wuae* n. sp. (Nematoda: Criconematoidea) Associated with Cow Parsnip (*Heracleum maximum*) in Ontario, Canada

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Abstract: Gracilacus wuae n. sp. from soil associated with cow parsnip in Ontario, Canada is described and illustrated. Morphologically, females have a long stylet ranging from 80 to 93 μm long, the lip region not offset from the body contour, without lateral lips but with large and flat submedian lobes, the mouth opening slit-like elongated laterally and surrounded by lateral flaps, the excretory pore is anterior to the knobs of the stylet; males without stylet and the pharynx degenerated. The fourth-stage juveniles lack a stylet, the pharynx degenerated, and can be differentiated into preadult females and males based on the position of the genital primordia. The third-stage juveniles are similar to females but smaller. Phylogenetic studies using the rDNA small subunit 18S, large subunit 28S D2/D3, and internal transcribed spacer (ITS) sequences collectively provide evidence of a grouping with other *Gracilacus* and some species of *Paratylenchus* with stylet length of females longer than 41 μm deposited in GenBank.

Key words: Gracilacus, molecular, morphology, morphometric, new species, *Paratylenchus*, taxonomy.

The genus *Gracilacus* was established by Raski (1962) to differentiate species of *Paratylenchus* Micoletzky, 1922, based on primarily stylet length and secondarily on body shape and the position of the excretory pore in females. Eight species with a female stylet longer than 48 μm were separated and placed in the genus *Gracilacus* primarily based on that single character. Shortly after the initial creation of *Gracilacus*, Siddiqi and Goodey (1964) synonymized *Gracilacus* with *Paratylenchus*. Raski (1976) revised *Gracilacus*, lowered the minimum stylet length to 41 um, and added 13 additional species to the genus. Siddiqi (1986) established the subgenera *Gracilacus* and *Paratylenchus* within the genus *Paratylenchus* based on stylet length. Thorne and Malek (1968) accepted *Gracilacus* when they described two new *Paratylenchus* species and evaluated the taxonomic position of *G. audriellae* (Brown, 1959) Raski, 1962. *G. audriellae* was later considered as a junior synonym of *G. straeleni* by Siddiqi (2000). Geraert (1965) divided *Paratylenchus sensu lato* that includes *Gracilacus* into 10 groups based on the stylet length and the V-position and recently Ghaderi et al. (2014) expanded it by dividing the genus into 11 groups based on stylet length, lateral fields, and advulval flaps. With the advent of molecular biology, phylogenetic studies have been conducted to examine the relationships among paratylenchids including very few species of *Gracilacus*. Lopez et al. (2013) used the first ITS1 region to examine paratylenchid relationships including *G. bilineata* Brzeski, 1995 and *G. aculenta* (Brown, 1959) Raski, 1962 in their analyses. Van Den Berg et al. (2014) used 28S D2/D3 and ITS sequence to examine species groupings within *Paratylenchus* including the above two *Gracilacus* species. Wang et al. (2015) described *P. guangzhouensis*, a species with the stylet averaging 47 μm long, and used ITS sequence to show that this new species is clustered with those four previously sequenced *Gracilacus* species. In another study, Wang et al. (2016) also used ITS to demonstrate that their newly described species *P. nanjingensis* with a 66 μm long stylet is grouped with *G. bilineata* and *G. aculenta*. All these studies provided evidence of the possible existence of a genetic grouping of long-stylet-bearing paratylenchids.

This study describes a new species of *Gracilacus* from Canada. There are 43 species listed in the subgenus *Gracilacus* by Siddiqi (2000). Four species have been previously recorded from Canada (Brown, 1959; Wu, 1974). These include *G. acicula* (Brown, 1959) Raski, 1962, *G. aculenta*, *G. audriellus*, and *G. robusta* (Wu, 1974) Raski, 1976. The new species described herein, *G. wuae* n. sp., was found in soil associated with wild parsnip (*Heracleum maximum*) in Ontario, Canada.

Materials and Methods

Nematode samples: Soil samples associated with wild parsnip were collected in Kanata, west of Ottawa, Ontario, Canada, with latitude 45°18′43.33″N and longitude 75°55′54.08″W. The nematodes were extracted with the Baermann funnel method. Freshly extracted nematodes were processed for both morphological and molecular studies.

Morphological study: The method used was as described previously (Yu et al., 2014). The nematodes were all fixed in TAF and mounted in dehydrated glycerin on slides for morphological studies. Specimens were examined using a Leica DM5500 B compound microscope (Wetzlar, Germany) using differential interference contrast and pictures were taken with a Leica

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DFC 420 digital camera. Measurements were made using a Leica micro application system on the images. Scanning electron microscope (SEM) pictures were taken with a FEI Quanta 600 SEM.

Molecular study

DNA extraction: DNA extraction solution consisted of 1× PCR buffer containing Proteinase K at a concentration of 0.25 mg/ml. Five nematodes were crushed in 50 µl of the DNA extraction solution and incubated at 65°C for 2.5 hr, followed by incubation at 95°C for 15 min and on ice for 5 min. The DNA extracts were stored for later use at −20°C.

PCR and sequencing: Internal transcribed spacer was amplified with the primers ITS-F (5′-TTGATTACGTC CCTGCCCTTT-3′) and ITS-R (5′-TTTCACTCGCCGT TACTAAGG-3′) (Vrain et al., 1992) using the following thermocycling conditions: an initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation (30 sec at 95°C), annealing (30 sec at 56°C), and extension (1 min at 68°C), and a final extension step (3 min at 68°C). 18S rRNA gene (18S) was amplified with the primers 18S-F (5′-CGATCAGATACCGCCCTAG-3′) and 18S-R (5′-TACAAAGGGCAGGGACGTAAT-3′) (Powers et al., 2010) using the following thermocycling conditions: an initial denaturation at 95°C for 3 min, followed

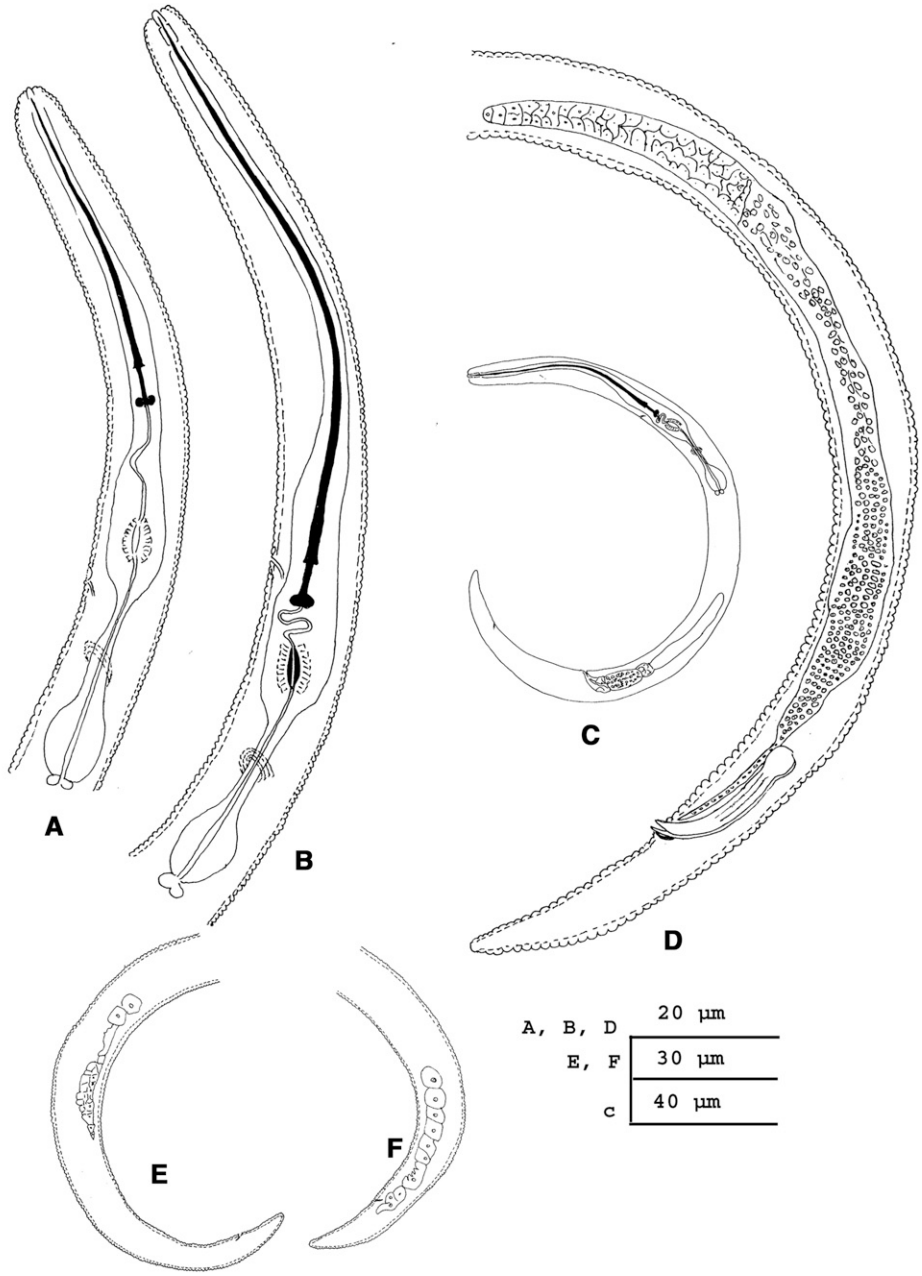

Fig. 1. Line drawing of *Gracilacus wuae* n. sp. associated with cow parsnip in Ontario, Canada, A. Head and pharynx of the third-stage juvenile. B. Head and pharynx of female. C. Female body. D. Male genital system. E. Posterior half of preadult female. F. Posterior half of preadult female.

by 30 cycles of denaturation (30 sec at 95°C), annealing (30 sec at 62°C), and extension (1.5 min at 68°C), and a final extension step (3 min at 68°C). D2/D3 region of the 28S rRNA gene (28S D2/D3) was amplified with the primers D2A (5′-GACCCGTCTTGAAACACGGA-3′) (Nunn, 1992) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) (De Ley et al., 1999), using the following thermocycling conditions: an initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation (30 sec at 95°C), annealing (30 sec at 50°C), and extension (1 min at 68°C), and a final extension step (3 min at 68°C).

All PCR reactions were prepared using the Titanium *Taq* PCR kit (Clontech Laboratories Inc., Mountain View, CA) and contained 5 or 10 ul of DNA, 2.5 µl of 10× PCR buffer, 1.0 µl of 2.0 mM dNTPs, 0.4 µl 10 µM primers, and 0.2 µl of Titanium *Taq* DNA polymerase. One microliter of PCR product was used per sequencing reaction. Sequencing was performed as described previously (Marchand et al., 2014) and samples were run on an ABI 3130xl Avant sequencer (Applied Biosystems). Consensus sequences of forward and reverse sequence reactions were obtained and used for a homology search of the National Centre for Biotechnology Information's GenBank database using Basic Local Alignment Search Tool (BLAST).

Phylogenetic study: Phylogenetic analysis was performed as described previously (Zeng et al., 2013). The sequences were deposited into the GenBank database. DNA sequences were aligned by Clustal W using default settings. The DNA sequences of *G. wuae* n. sp. were compared with those of the other nematode species available at the sequence database using the BLAST homology search program. The model of base substitution was evaluated using MODELTEST, and the Akaike information criterion (AIC) 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 using GTR + I + G was performed to confirm the tree topology for each gene separately using MrBayes 3.1.0 (Ronquist and Huelsenbeck, 2003) running the chain for 1×10^6 generations and setting the "burnin" at 1,000. We used the Markov Chain Monte Carlo method within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees using 50% majority rule.

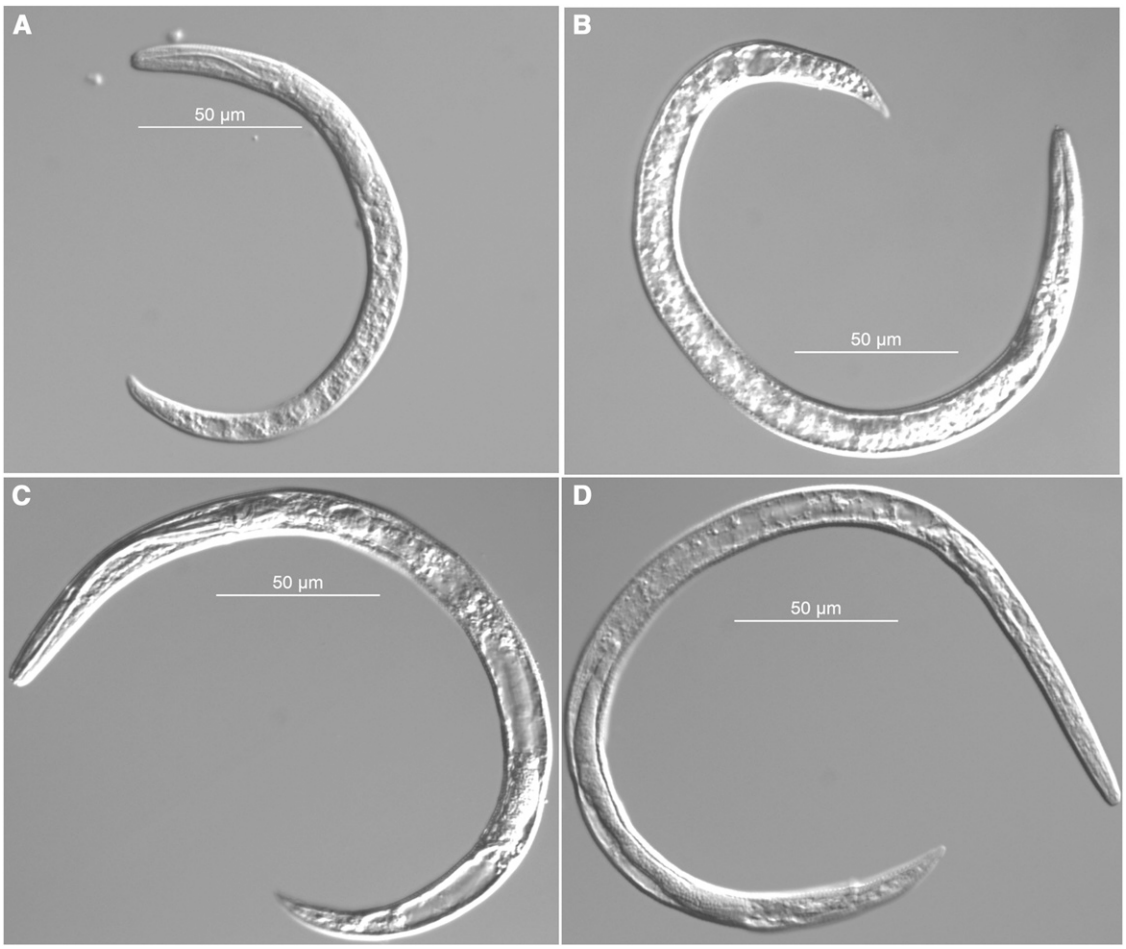

Fig. 2. Light micrographs of *Gracilacus wuae* n. sp. associated with cow parsnip in Ontario, Canada. A. Body of the third-stage juvenile. B. Body of the fourth-stage juvenile. C. Body of female. D. Body of male.

Results and Description

Systematics

Gracilacus wuae n. sp.*
(Figs. 1–4)

Measurements: Morphometrics of the holotype, paratype of females, males, and the third- and fourth-stage juveniles of *G. wuae* n. sp. are given in Table 1.

Description

Female: Body not obese, C-shaped when relaxed; annuli delicate but distinct, lateral fields, each occupy one quarter of the body width, with four straight lines with equal space extending to the full length of the body. Lip region with pronounced large and flat submedian lobes, but without lateral lips (Fig. 4A,B), the mouth aperture is slit like, elongated laterally and surrounded by lateral flaps, submedian lobes rounded, lip region not offset from the body contour. Cephalic frame well developed. Stylet is elongated, 80 to 93 μm long, slightly curved ventrally, cone is about 20 times length of shaft and knobs combined, and stylet knobs large, and robust. Metacorpus is large, well developed, and occupying about three-fourth of body width. Valvular apparatus large and muscular. Isthmus is narrow and surrounded by nerve ring. Basal bulb pyriform. Cardia consists of two rounded cells. Hemizonid is about 3 μm anterior to the excretory pore, which is positioned anterior to stylet knobs, and in the range of the stylet shaft. Gonad is prodelphic, short, and outstretched; spermatheca round, offset, filled with globular sperms. Advulval flaps absent. Post uterine sac absent. Anus rather prominent. Tail terminus finely rounded with distinct annuli.

Male: Body slender than female body. Lip region differs to that of females without distinct lips or mouth opening (Fig. 4C). Cephalic framework less developed. Pharynx rudimentary, only vague contour of procorpus and metacorpus can be discerned. Stylet lacking. Positions of hemizonid and excretory pore equivalent to those of females. Body annuli more visible than those of females, especially in tail region. Lateral fields with four lines. Gonad about 110 μm long. Cloacal opening elevated, forming a short penial sheath. Spicules well

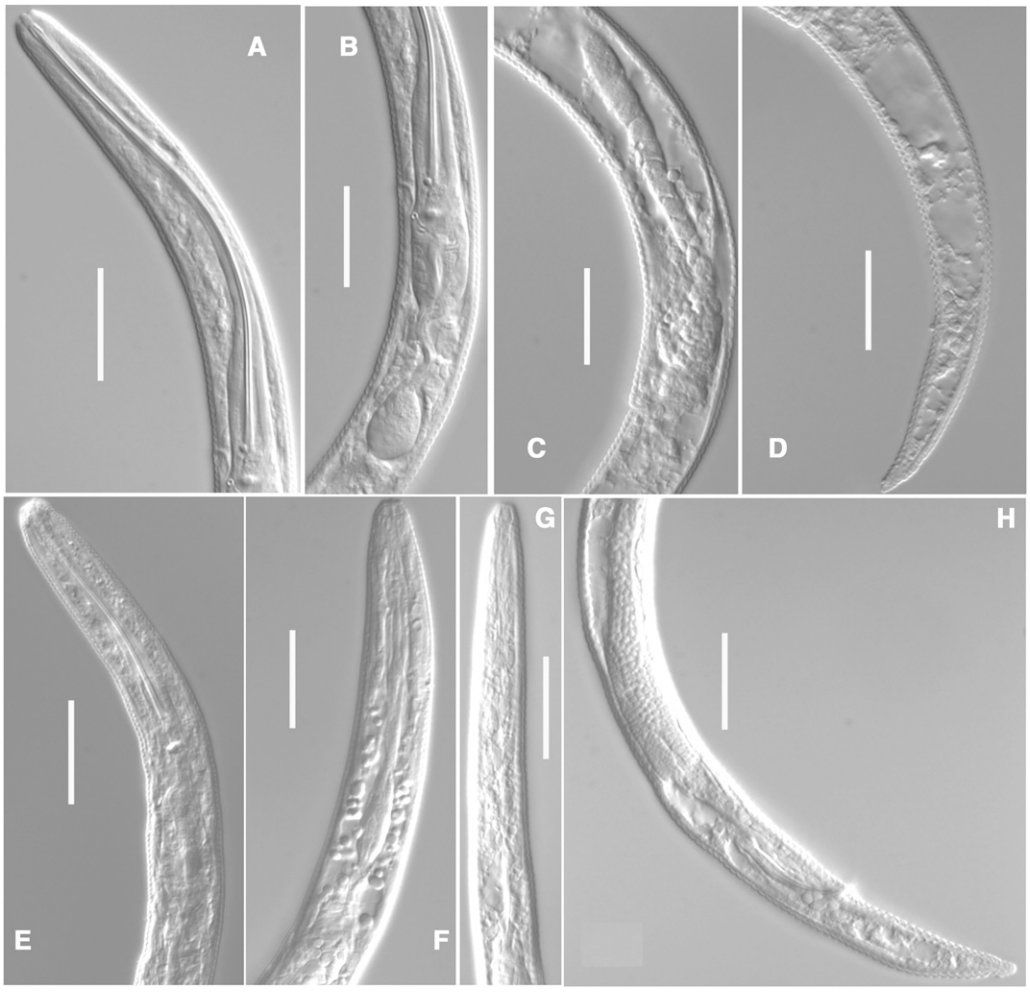

Fig. 3. Light micrographs of *Gracilacus wuae* n. sp. associated with cow parsnip in Ontario, Canada. A. Head and pharynx of female. B. Metacorpus and basal bulb of pharynx of female. C. Female genital. D. Female tail. E. Head of the third-stage juvenile. F. Head of the fourth-stage juvenile. G. Head of male. H. Male genital and tail region.

developed, slightly curved ventrally, protruding from body. Gubernaculum about 4 μm long. Bursa absent. Tail terminus similar to that of female.

The second-stage juvenile: Not found.

The third-stage juvenile: Similar to females, but smaller. The body C shaped when relaxed. The stylet is 46 to 52 μm long, almost half of the stylet length of the female, pharynx well developed like in female. Genital primordia visible, located at about 50 μm from the posterior end of the nematode or about 30 μm anterior to the anus.

The fourth-stage juvenile: The fourth-stage juveniles have no stylet, pharynx reduced but clearly defined, no valvar apparatus, anterior portion of lumen somewhat dark, sclerotized. They can be clearly classified into preadult female (Fig. 1E) and preadult male (Fig. 1F) based on the positions of the genital primordia. Preadult male: the posterior end of the genital primordia is near the anus. Preadult female: the posterior end of the genital primordia is 38 to 51 μm anterior to the anus. The ratio of preadult female/preadult male is 2 (total number of preadult female recovered is 24, preadult male 12); the ration is nearly the same of adults (1.9): female (40)/male (21).

Type host and locality: Roadside in Kanata, west of Ottawa, Ontario, Canada, with latitude 45°18′43.33″N and longitude 75°55′54.08″W. It was found in soil associated with cow parsnip (*H. maximum*), a fairly common weed in the region. Subsequent sampling in other places with cow parsnip did not recover the nematode. The roadside location of the positive collection site had recently been excavated, so it is not known if the disturbed roadside habitat fully represented the original soil and vegetation prior to road construction.

Type specimens: Holotype female, 10 paratype females, 10 males, 10 third-stage juveniles, and 10 preadult

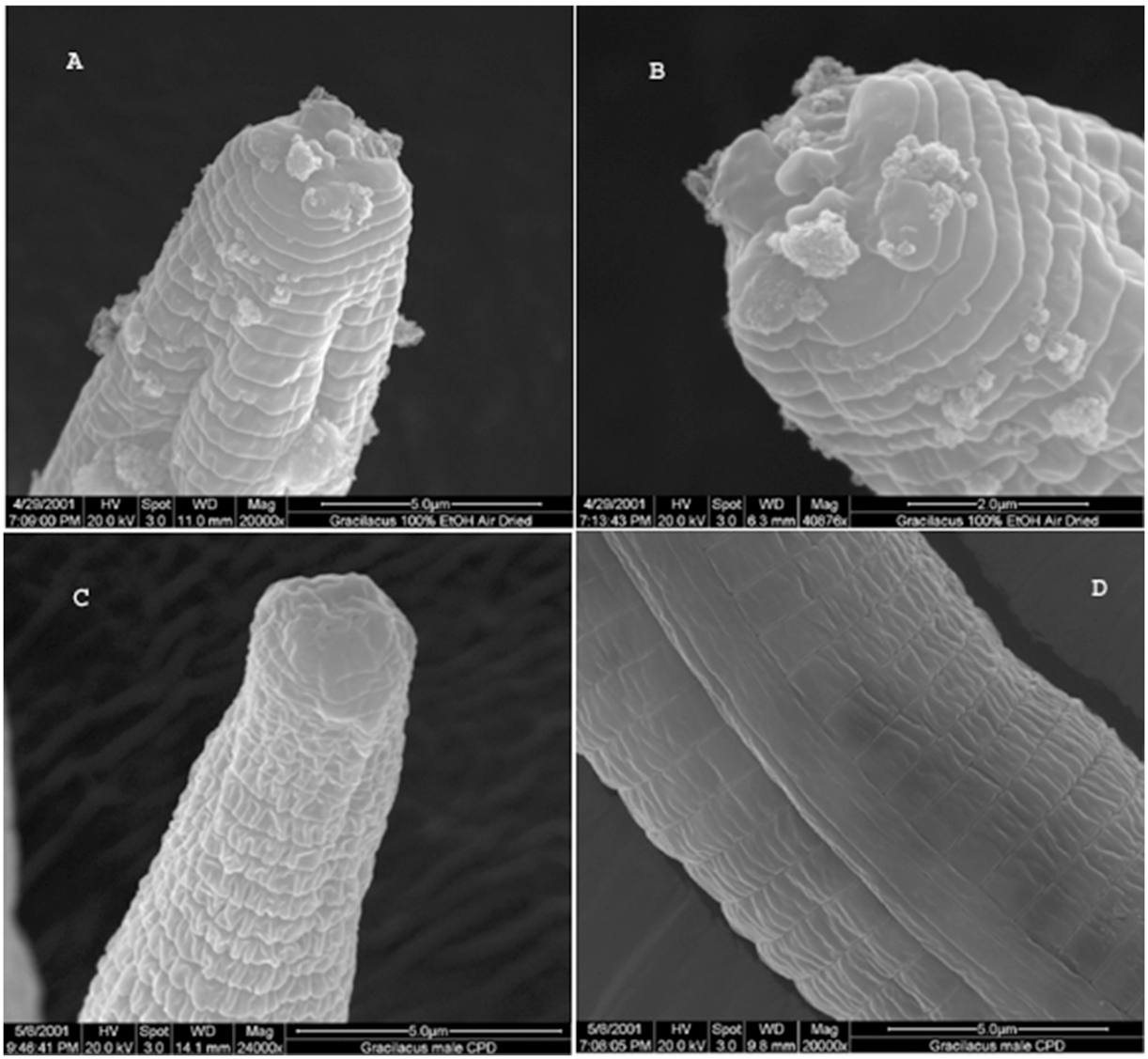

Fig. 4. Scanning electron microscope pictures of *Gracilacus wuae* n. sp. associated with cow parsnip in Ontario, Canada. A. Head of female. B. Lip region of female. C. Head of male. D. Lateral field of male.

TABLE 1. Morphometrics of *Gracilacus wuae* n. sp. All measurements are in μm and in the form: mean ± standard deviation (range).

Character	Holotype	Paratypes					
					J4 (Preadult)		
	Female	Females	Males	J3	Female	Male
n	-	13	9	9	9	9
L	329	326 ± 17.9 (300–360)	380 ± 12.2 (369–405)	233 ± 4.9 (225–239)	323 ± 8.2 (320–343)	329 ± 8.4 (320–353)
a	20.5	20.7 ± 0.8 (20.1–22.2)	29.1 ± 1.1 (26.4–30.8)	17.6 ± 1.3 (15.3–19.8)	22.7 ± 1.7 (20–26)	21.7 ± 1.8 (19–25)
b	2.5	2.4 ± 0.1 (2.3–2.6)	3.9 ± 0.2 (3.9–4.1)	2.5 ± 0.1 (2.4–2.6)	3.5 ± 0.1 (3.4–3.7)	3.4 ± 0.2 (3.1–3.5)
c	11.0	10.9 ± 0.3 (10.5–11.3)	12.3 ± 0.7 (11.2–13.2)	10.9 ± 0.4 (10.4–11.4)	13.1 ± 1.3 (11.8–15.6)	12.9 ± 1.3 (11.2–15.1)
c′	3.6	3.2 ± 0.2 (3.4–3.8)	3.7 ± 0.1 (3.6–3.9)	2.0 ± 0.1 (1.8–2.2)	2.5 ± 0.1 (2.2–2.6)	2.3 ± 0.1 (2.2–2.6)
V	75.9	74.7 ± 3.8 (69.9–80.1)	-	-	-	-
G or T	20.1	20.1 ± 0.5 (18.8–20.3)	37.4 ± 1.2 (35.7–38.9)	-	-	-
Stylet	91.6	88 ± 4.7 (80–93)	-	48 ± 2.4 (46–52)	-	-
Body diameter	16.1	15.7 ± 0.5 (18.8–20.4)	13.0 ± 0.6 (12–14)	13.3 ± 0.9 (12–15)	14.8 ± 0.9 (13–16)	12.8 ± 0.9 (11–15)
Tail	30	29.8 ± 1.6 (27.1–32.1)	31.1 ± 1.1 (30–33)	21.3 ± 0.7 (20–22)	25.6 ± 2.6 (22–28)	26.6 ± 2.7 (23–30)
Spicules	-	-	21.8 ± 0.4 (21–22)	-	-	-
Distance of the posterior end of the genital primordia to the tail tip	-	-	-	-	60 ± 6.5 (51–67)	25 ± 2.5 (23-28)
Length of the genital primordia	-	-	-	-	42 ± 3.8 (39–47)	48 ± 4.0 (44–52)

females and males each were deposited in the Canadian National Collection of Nematodes under accession number T530. Five paratypes for each stage were in USDA Nematode Collection, Beltsville, MD, with accession numbers T-6778p to T-6797p.

Diagnosis: This new species is characterized by the combination of the stylet 80 to 93 μm long, the lip region continuous without lateral lips, with large and flat submedian lobes, lateral fields with four lines, the anterior positioned excretory pore relative to the knobs of the stylet, and lateral vulvar membranes absent in females.

Relationships: According to the grouping scheme of *Paratylenchus sensu lato* by Ghaderi et al. (2014), this new species belongs to Group 11 (stylet >40 μm; four lateral lines; and advulval flaps absent). Within this group, the species with stylet longer than 67 μm includes *P. laocaiensis* Nguyen, Baldwin & Choi, 2004, *G. teres* Raski, 1976, *G. enata* Raski, 1976, *G. oostenbrinki* (Misra and Edward, 1971) Raski, 1976, *G. steineri* (Golden, 1961) Raski, 1962, and *G. macrodorus*. Scanning electron microscope *en face* views of *G. teres* (Pena and Geraert, 1990) were also used for comparison. This new species differs from *P. laocaiensis* by a longer stylet (88 [80–93] vs. 73 [67–82] μm), more posterior positioned vulva (V 70–80 vs. 65–67), submedian lobes, and rounded tail terminus vs. pointed terminus. It differs from *G. teres* by having a longer stylet (88 [80–93] vs. 73 [69–83] μm), the absence of lateral lips, submedian lobes larger and flatter, spermatheca filled with sperms, and conspicuous vulva. It differs from *G. enata* by having a shorter stylet (88 [80–93] vs. 93 [83–104] μm), a wider female body (a 20–22 vs. 23–28), longer male (309–405 vs. 340–350). It differs from *G. oostenbrinki* by longer female body (300–360 vs. 250–280 μm), longer stylet (80–93 vs. 72–88 μm), more anterior positioned excretory pore, and rounded tail terminus vs. acute. It differs from *G. teineri* by having longer stylet (88 [80–93] vs. 73 [68–75] μm), and rounded tail terminus vs. subacute pointed.

Etymology: *The species was named after Dr. Liangyu Wu, a former nematode taxonomist at the Agriculture and Agri-Food Canada, who made significant contribution to the taxonomy of *Paratylenchus* and *Gracilacus*.

Phylogenetic analysis

Three regions of the rDNA repeating region were sequenced and compared with sequences deposited in GenBank. The stylet length greater than 41 μm was mapped to the species in phylogenetic trees (Figs. 5–7).

18S: A 979-bp fragment of the 3′ region of 18S rDNA gene was sequenced for *G. wuae* n. sp. Rooted with *Hemicriconemoides wessoni*, the 18S tree (Fig. 5) placed *G. wuae* n. sp. in a clade with *G. latescens* (AY912039) from Konza Prairie, KS, *Paratylenchus colinus* (KP966494) from apple in Iran and *Gracilacus* sp. (KR232931) from pine tree in China with 82% support. These species have

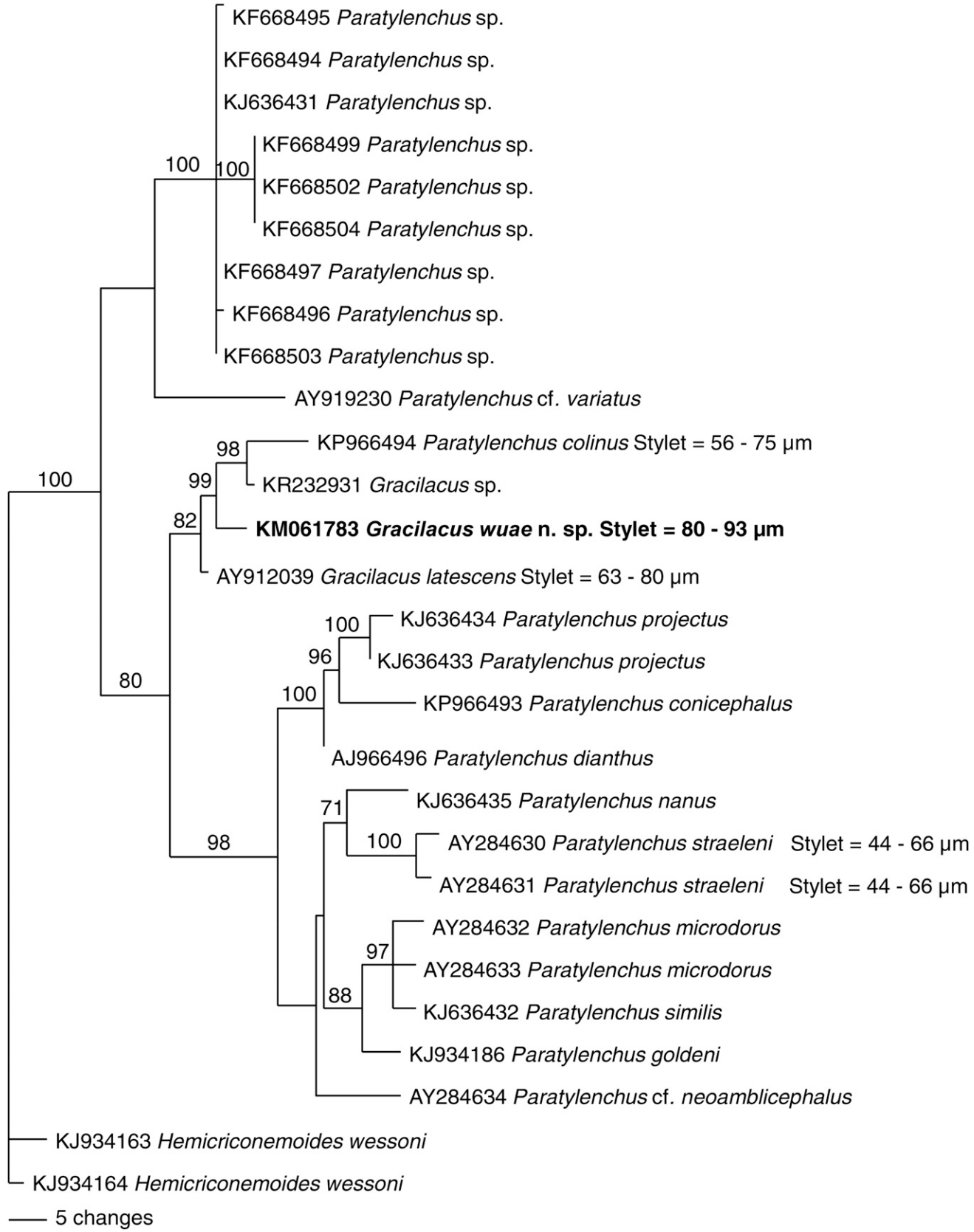

Fig. 5. The 10,001st Bayesian tree inferred from 18S under GTR + I + G model (-lnL = 2,216.9043; AIC = 4,453.8086; freqA = 0.2305; freqC = 0.2442; freqG = 0.2862; freqT = 0.2391; R(a) = 0.2285; R(b) = 1.7446; R(c) = 1.0079; R(d) = 0.875; R(e) = 4.7408; R(f) = 1; Pinva = 0.7656; shape = 1.1055). Posterior probability values exceeding 50% are given on appropriate clades.

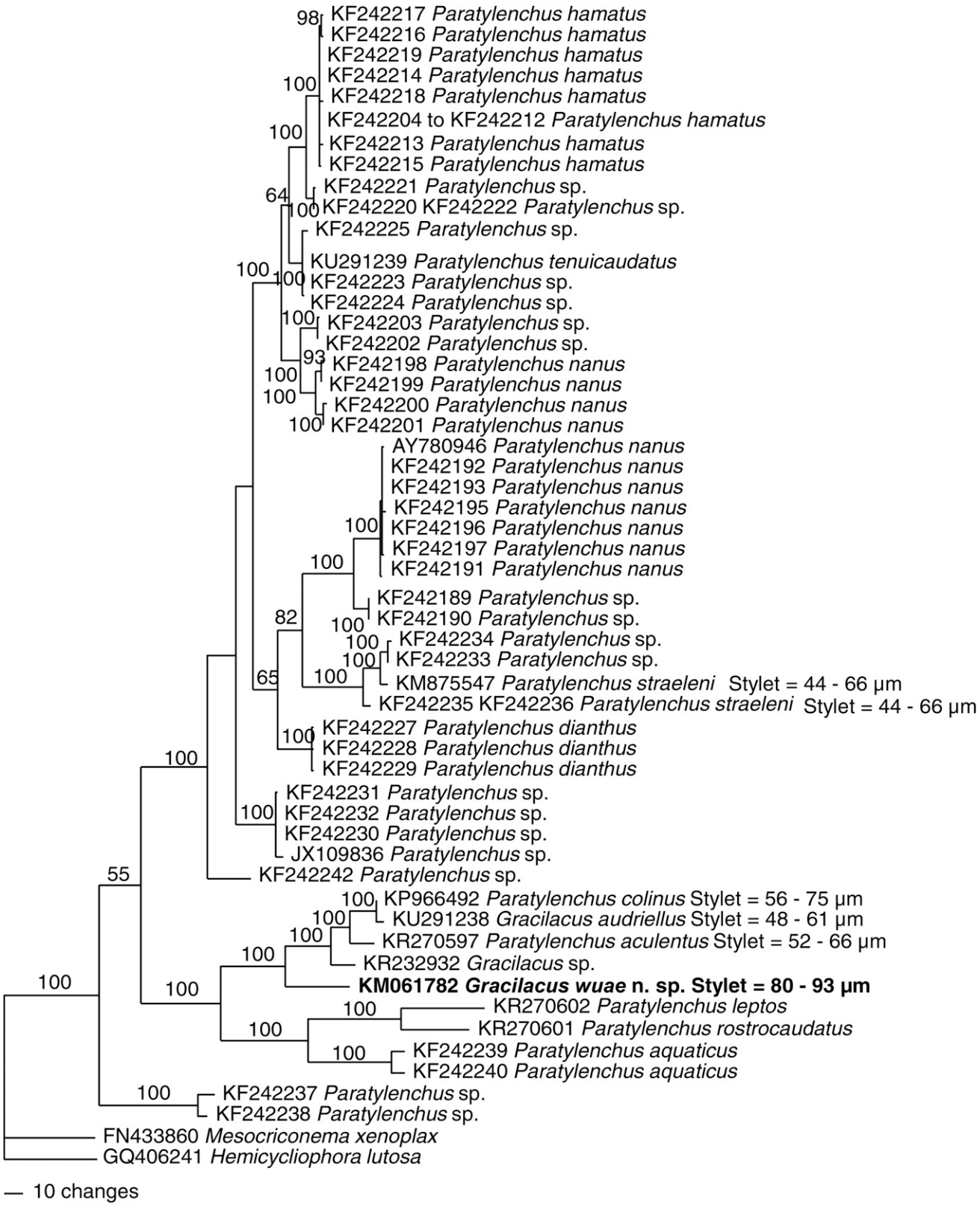

Fig. 6. The 10,001st Bayesian tree inferred from 28S D2/D3 under GTR + I + G model (-lnL = 5,683.3032; AIC = 11,386.6064; freqA = 0.2094; freqC = 0.2291; freqG = 0.3176; freqT = 0.2438; R(a) = 0.513; R(b) = 2.0052; R(c) = 0.7642; R(d) = 0.4773; R(e) = 4.4825; R(f) = 1; Pinva = 0.2154; shape = 0.5762). Posterior probability values exceeding 50% are given on appropriate clades.

Fig. 7. The 10,001st Bayesian tree inferred from ITS under GTR + I + G model (-lnL = 12,135.0107; AIC = 24,290.0215; freqA = 0.2228; freqC = 0.2549; freqG = 0.2804; freqT = 0.2419; R(a) = 1.3527; R(b) = 3.0954; R(c) = 2.0549; R(d) = 0.8779; R(e) = 3.8983; R(f) = 1; Pinva = 0.2508; shape = 1.1435). Posterior probability values exceeding 50% are given on appropriate clades.

stylet more than 41 μm long. *Paratylenchus straeleni* (AY284630, AY284631) has a stylet 44 to 66 μm long, but grouped with other *Paratylenchus* species. *P. straeleni* has been considered as members of the subgenus *Gracilacus* (Wouts, 2006). This tree revealed long-stylet paratylenchids are not monophyletic.

28S D2/D3: The 873-bp DNA fragment of the 28S D2/D3 was sequenced for *G. wuae* n. sp. Rooted with *Hemicycliophora lutosa*, the tree (Fig. 6) placed *G. wuae* n. sp. in a 100%-supported monophyletic clade with other long-stylet species, namely *G. audriellus* (KU291238), *G.* sp. (KR232932), *P. aculentus* (KR270597), and *P. colinus* (KP966492). Two populations of *P. straeleni* (KF242235, KF242236) from Mendocino and Napa Counties, CA (Van Den Berg et al., 2014) were grouped with other species of *Paratylenchus*. As in the 18S tree, the long-stylet paratylenchids are not monophyletic if *P. straeleni* is considered as a member of *Gracilacus*.

ITS: A 929-bp fragment of ITS1 was sequenced for *G. wuae* n. sp. Rooted with *Hemicriconemoides chitwoodi*, the ITS tree (Fig. 7) placed *Gracilacus* in a 100%-supported monophyletic clade with other long-stylet species, namely *P. guangzhouensis* (KT725625, KT725626), *P. nanjingensis* (KM366102-KM366103), *G. bilineata* (EU247525), *G.* sp. (KR232933), and *G. aculenta* (EU247526). This clade is equivalent to Clade III in the ITS tree by Van Den Berg et al. (2014). Another long-stylet species *P. goldeni* (KJ934186) is together with short stylet paratylenchids. As in 18S and 28S D2/D3, the long-stylet paratylenchids are not monophyletic. Unfortunately, no ITS sequence data are available for *P. straeleni* to compare the result.

Species of paratylenchids are small nematodes with stylet lengths that vary from 10 to 120 μm. This large variation is rare in tylenchids. In this study, the new species *G. wuae* n. sp. is grouped with other long-stylet species when nucleotide sequences were compared, but with exceptions in all three DNA fragments sequenced. Based on this study, the character "stylet length greater than 41 μm" is not homologous and has evolved more than once within the *Paratylenchus* lineage. Given this combination of molecular and morphological evidence, we believe it would be taxonomically appropriate to recognize the new species as a member of the genus *Gracilacus*, although few species are available for comparison. It is clear, however, that considerable taxonomic effort and DNA sequencing on the vast majority paratylenchids will be necessary to clarify relationships and classification within Paratylenchidae.

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