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Axonchoides smokyensis sp. n. (Dorylaimida: Belondiridae) from the Great Smoky Mountains National Park: the second species of a very rare genus

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Summary – *Axonchoides smokyensis* sp. n., collected from a natural forest area in Tennessee, USA, is described and illustrated, including LM and SEM pictures as well as molecular (rRNA SSU-ITS1 and LSU D2/D3) sequences. The new species is characterised by its body length of 2.56-3.18 mm, lip region continuous and 10-12 μm wide, odontostyle 10-13 μm long, neck 662-789 μm long, anterior portion of pharynx enlarging gradually, pharyngeal expansion 407-548 μm long or 60-70% of the total neck length and surrounded by a strong muscular sheath with nearly longitudinal bands, female genital system pseudodidelphic-monodelphic with anterior branch lacking a functional ovary and posterior one bearing a long and tripartite uterus, V = 42-46, female tail slightly clavate (27-34 μm, c = 84-105, c′ = 0.8-1.0), male tail short and rounded (34-38 μm, c = 87-113, c′ = 0.7-0.9), spicules 49-53 μm long, and five or six widely spaced ventromedian supplements outside the range of the spicules. The taxonomy of *Axonchoides* is discussed on the base of morphological and molecular evidence, and an emended diagnosis proposed. It was easily differentiated from other sequenced dorylaimid taxa, but phylogenetic analysis from SSU and LSU failed satisfactorily to resolve its placement in Dorylaimina.

Keywords – Belondirinae, description, diagnosis, LSU D2/D3, molecular, morphology, morphometrics, new species, phylogeny, SSU-ITS1, taxonomy, USA.

Thorne (1967) described *Axonchoides*, with *A. crassus* Thorne, 1967, from El Yunque Mountains, Puerto Rico, as its only and type species. The new taxon was classified under Belondiridae Thorne, 1939, Axonchiinae Thorne, 1964, and distinguished from its relative *Axonchium* Cobb, 1920 "by the semisclerotized labial framework, mammiform cephalic papillae, nonmuscular anterior portion of esophagus, and supplements not rising above body contour". *Axonchoides crassus* was not reported again and no further information is available after its original description.

During a nematological survey conducted in October 1985, our colleague Prof. Ernest Bernard collected *Xiphinema bernardi* Robbins, Bae, Ye & Pedram, 2009. In 2006, collection efforts that acquired *X. bernardi* specimens for expanded morphological and molecular studies also yielded a population of interesting belondirid dorylaims. The detailed morphological and morphometric study of these specimens revealed that they belonged to a non-described form of *Axonchoides*. Molecular analyses on ribosomal DNA were performed to elucidate the nature of its origin and evolutionary relationships. The results obtained are presented below.

Materials and methods

Soil samples were collected from a natural habitat in Sevier County, TN, USA. Nematodes were extracted from the soil by a combination of sieving-decanting

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and sucrose centrifugal-flotation techniques, either killed, fixed, processed to glycerin and permanently mounted on slides as described by Ye & Robbins (2004) or placed in 1 M NaCl for molecular study. Nematodes were observed using a light microscope. Morphometrics included de Man's indices and most of the usual measurements. Some of the best preserved specimens were photographed with a Nikon Eclipse 80i microscope and a Nikon DS digital camera. Raw photographs were edited using Adobe® Photoshop® CS. Drawings were made using a camera lucida.

Scanning Electron Microscopy (SEM)

After their examination and identification, a few specimens preserved in glycerin were recycled for observation under SEM following the protocol by Abolafia & Peña-Santiago (2005). The nematodes were hydrated in distilled water, dehydrated in a graded ethanol and acetone series, critical point-dried, coated with gold, and observed with a ZEISS model Merlin microscope.

DNA extraction, PCR and sequencing and phylogenetic analyses

For molecular study, a single nematode specimen was picked out and transferred to 10 μl AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) on a glass microscope slide, macerated with a pipette tip and collected in final 50 μl AE buffer. DNA samples were stored at −20°C until used as a PCR template. Different sets of primers were used in the PCR reactions. Primers for SSU amplification were forward primer 18S-G18S4 (5′-GCTTGTCTCAAAGATTAAGCC-3′) and reverse primer 18S-18P (5′-TGATCCWKCYGCAGGTTCAC-3′) (De Ley *et al.*, 2002; Dorris *et al.*, 2002), forward primer SSUF07 (5′-AAAGATTAAGCCATGCATG-3′) and reverse primer SSUR26 (5′-CATTCTTGGCAAATGCTTTCG-3′) (Floyd *et al.*, 2002), forward primer 18s965 (5′-GGCGATCAGATACCGCCCTAGTT-3′) and reverse primer 18s1573R (5′-TACAAAGGGCAGGGACGTAAT-3′) (Mullin *et al.*, 2005). Primers for LSU D2/D3 amplification were forward primer D2a (5′-ACAAGTACCGTGAGGGAAAGT-3′) and reverse primer D3b (5′-TGCGAAGGAACCAGCTACTA-3′) (Nunn, 1992). Primers for ITS1 amplification were forward primer rDNA2 (5′-TTGATTACGTTCCCTGCCCTTT-3′) (Vrain *et al.*, 1992) and reverse primer rDNA1.58S (5′-ACGAGCCGAGTGATCCACCG-3′) (Cherry *et al.*, 1997).

The 25 μl PCR was performed using Apex Taq Red Master Mix DNA polymerase (Genesee Scientific) according to the manufacturer's protocol, with 1 μl each of 0.4 μM forward and reverse primers and 1 μl of DNA template. The thermal cycling programme was as follows: denaturation at 95°C for 6 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min. PCR products were cleaned using ExoSap-IT (Affymetrix) according to the manufacturer's protocol. DNA sequencing was performed using PCR primers for direct sequencing by dideoxynucleotide chain termination using an ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) in an Applied Biosystems 3730 XL DNA Analyzer (Applied Biosystems) by the Genomic Sciences Laboratory in North Carolina State University (Raleigh, NC, USA).

The resulting ribosomal DNA SSU-ITS1 and LSU D2/D3 sequences were deposited in GenBank under the accession number JX885740 and JX885739. These sequences were compared with other nematode species stored at GenBank using the BLAST homology search program. The closest sequences were selected in the phylogenetic analysis. The newly obtained sequence was aligned with other available sequences in GenBank using ClustalX 1.83 (Thompson *et al.*, 1997). Sequence alignments were manually edited using GenDoc 2.6.002 (Nicholas *et al.*, 1997). The sequence dataset was analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The best fit model of DNA evolution for BI was obtained using the program MrModeltest 2.2 (Nylander, 2002) with the Akaike Information Criterion in conjunction with PAUP* 4b10 (Swofford, 2003). BI analysis under the GTR + I + G model was initiated with a random starting tree and run with the four Metropolis-coupled Markov chain Monte Carlo (MCMC) for 10^6 generations. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. The Bayesian tree was visualised with the Tree View program (Page, 2001) and drawn with Adobe Acrobat 9 Pro 9.2.0.

Results

Axonchoides smokyensis[*] sp. n.
(Figs 1-4)

MEASUREMENTS

See Table 1.

DESCRIPTION

Adults

Very slender nematodes (a-ratio nearly 60) of medium to large size, 2.56-3.18 mm long. Habitus upon fixation curved ventrad, more so in posterior body region, J-shaped in female and G-shaped in male. Cuticle two-layered, smooth under LM, but bearing very fine transverse striation throughout body when observed under SEM. Cuticle 2.5-3.0 μm thick at anterior region and mid-body, and 8-10 μm on tail, outer layer thin and with constant thickness throughout body, inner layer thicker than outer and becoming distinctly widened in posterior body region. Cervical lacunae always present, more or less (often well) developed in specimens examined, *ca* 50 μm long. Lateral chord 6-10 μm wide or occupying 12-22% of mid-body diam., lacking any special differentiation. Lip region rounded, nearly continuous with adjacent body, 1.8-2.3 times as wide as high and one-fifth to one-fourth (19-26%) of body diam. at neck base, lips amalgamated in part since their perioral parts are separated by short radial incisures, while their basal regions are also separated by deep groves, which are especially marked between each lateral lip and its corresponding subdorsal and subventral lips, lip region therefore displaying a bi-radial symmetry, papillae button-like, cephalic visibly larger than labial ones. Amphid fovea cup-like, its aperture 8-10 μm wide and occupying majority (73-90%) of lip region diam. Cheilostom cylindrical, without any special differentiation. Odontostyle cylindrical to slightly fusiform, 5.0-6.5 times as long as wide, equal or barely longer (1.0-1.2 times) than lip region diam. and 0.34-0.47% of total body length; aperture short, 2.5-3.0 μm or *ca* one-fourth its length. Guiding ring simple but easily seen, located 10-11 μm from anterior end. Odontophore rod-like, with a small but perceptible thickening at its middle, hence apparently consisting of two parts. Anterior region of pharynx slender, weakly muscular, enlarging gradually, lacking any constriction or isthmus-like area separating both pharyngeal sections, basal expansion very muscular, 13-19 times as long as wide, 8-10 times as long as corresponding body diam., occupying *ca* two-thirds (60-70%) of total neck length, enveloped by a strong muscular sheath whose bands are weakly spiral, nearly longitudinal. Cardia cylindrical to tongue-like, (24-35) $\times$ (10-15) μm, surrounded by intestinal tissue.

Female

Genital system pseudomonodelphic-opisthodelphic. Anterior genital branch 170-380 μm long, 1.9-6.9 times body diam. or 6-14% of body length, consisting of a very long, tube-like uterine sac, often containing sperm cells, followed by a visible narrowing and a terminal cell mass 16-64 μm long, probably representing rudiment of oviduct and maybe also ovary. Posterior genital branch very well developed, 208-370 μm long or occupying 7-14% of body length, but certainly larger than these measurements as uterus is always convoluted, reducing its real size, ovary of variable size, 41-104 μm long, often not reaching sphincter level, oviduct 59-152 μm long or 1.3-2.9 times body diam., consisting of slender portion of prismatic cells and a poorly developed *pars dilatata* lacking a distinct lumen, marked sphincter separating oviduct and uterus, the latter a long tube consisting of three sections: a distal, small and spherical region close to sphincter, a long and convoluted intermediate and slender part with narrow lumen, and a proximal region with wide lumen and often containing sperm cells. Vagina 26-31 μm long, extending inwards to *ca* three-fifths (55-66%) of body diam., *pars proximalis* (16-20) $\times$ (13-23) μm with convergent walls distally and surrounded by a weak circular musculature, *pars refringens* absent although two small and weak sclerotisations visible in some specimens at junction of *pars proximalis* and *pars distalis*, latter being 4-7 μm long. Vulva a pre-equatorial, transverse slit. Prerectum very long, 11-20 times anal body diam. Rectum separated from prerectum by a marked sphincter, 32-38 μm long and nearly equal to anal body diam. Anus a curved and somewhat asymmetrical transverse slit. Caudal region short and rounded, slightly clavate, two pairs of caudal pores, one lateral and other subdorsal, at anterior end of tail.

Male

Genital system diorchic, with opposed testes. Prerectum very long, 14.7-16.5 times body diam. at level of cloacal aperture. In addition to adcloacal pair, located at 8-11 μm from cloacal aperture, a series of five or six,

[*] The specific epithet refers to geographical origin of the new species in the Great Smoky Mountains.

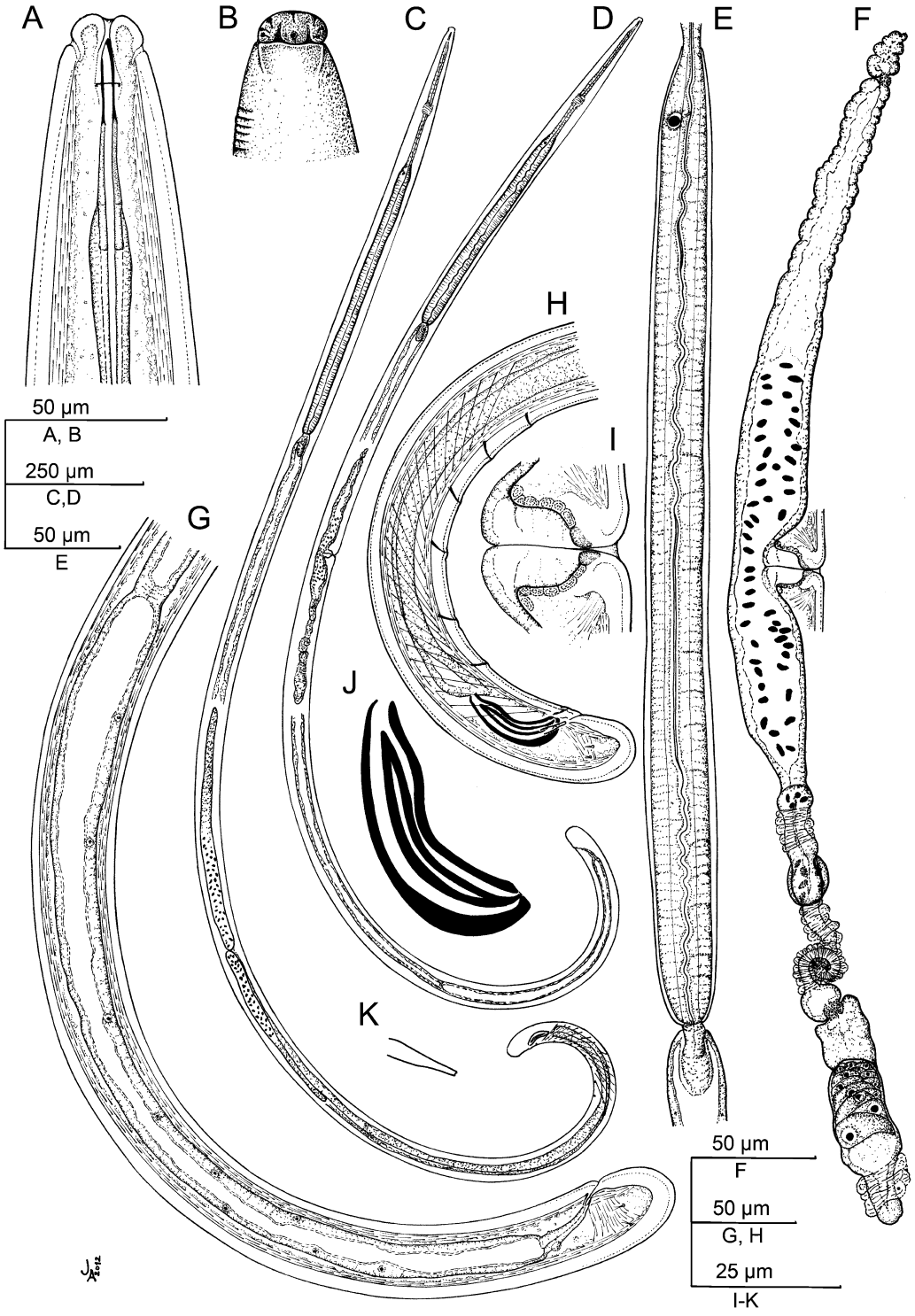

Fig. 1. *Axonchoides smokyensis* sp. n. A: Anterior region in median view; B: Lip region in surface, lateral view; C: Male, entire; D: Female, entire; E: Pharyngeal expansion; F: Female genital system; G: Female posterior body region; H: Male posterior body region; I: Vagina; J: Spicule; K: Lateral guiding piece.

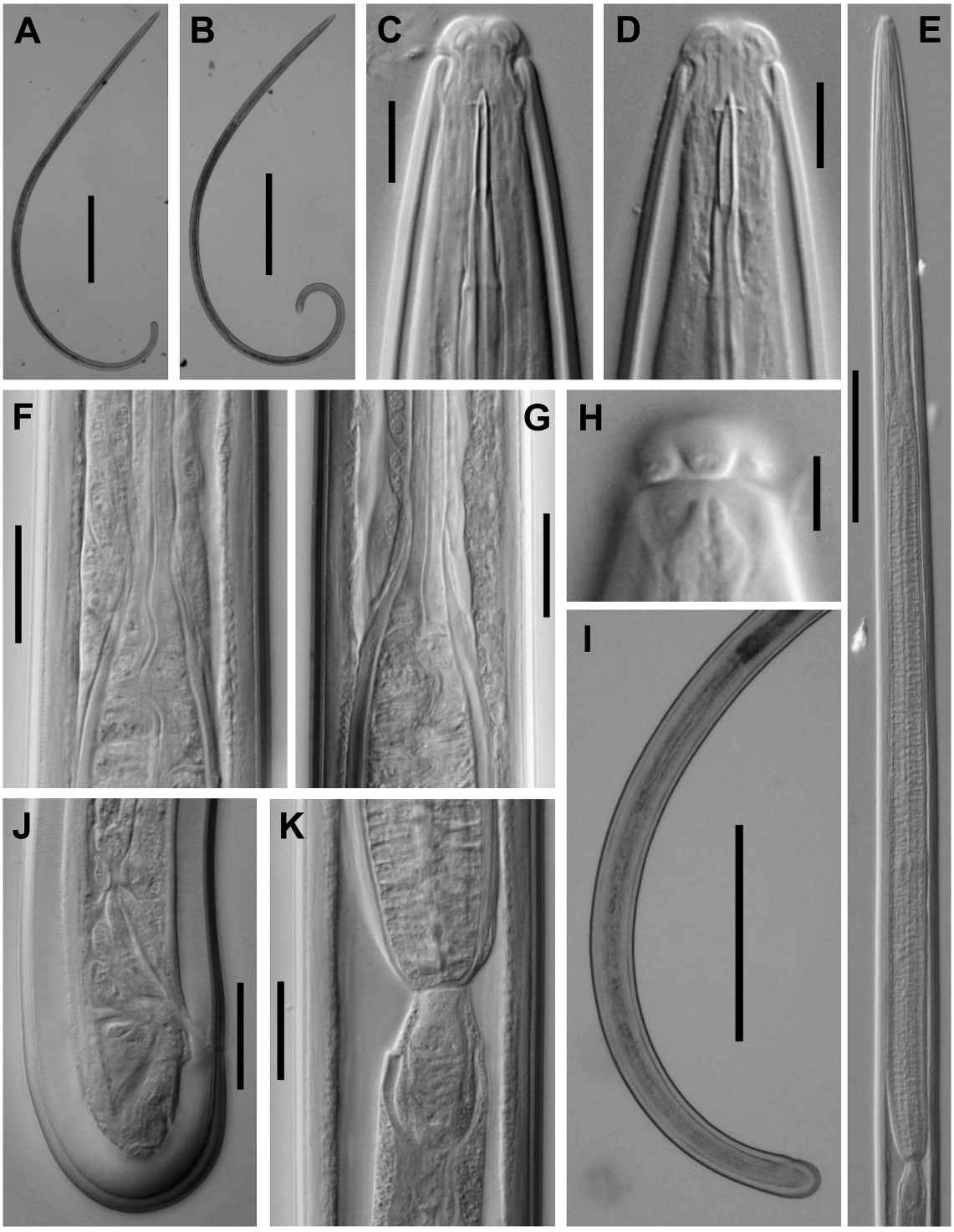

Fig. 2. *Axonchoides smokyensis* sp. n. (LM). A: Female, entire; B: Male, entire; C, D: Anterior region in median view; E: Neck region; F, G: Pharyngeal enlargement; H: Lip region in sublateral view; I: Female, posterior body region; J: Female, caudal region; K: Pharyngo-intestinal junction. (Scale bars: A, B = 500 μm; C, D = 10 μm; E = 50 μm; F, G, J, K = 20 μm; H = 5 μm; I = 200 μm.)

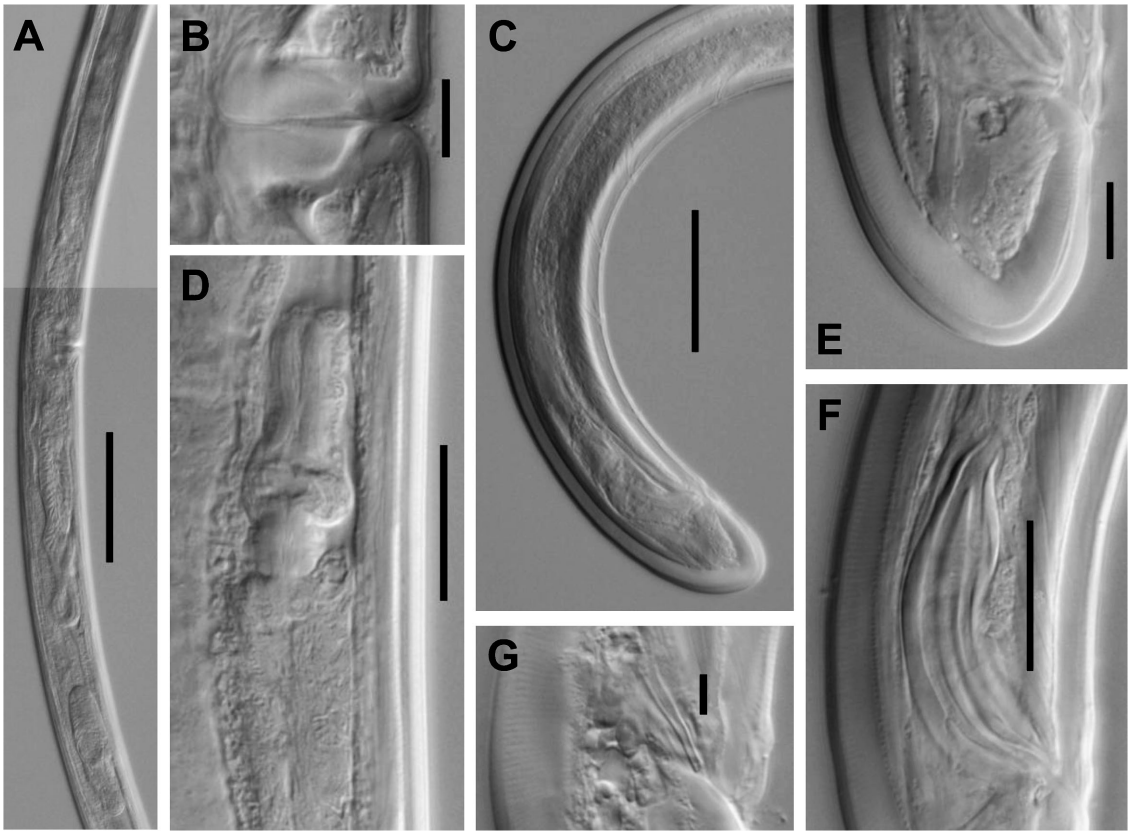

Fig. 3. *Axonchoides smokyensis* sp. n. (LM). A: Female, genital system; B: Vagina; C: Male, posterior body region; D: Oviduct-uterus junction; E: Male, caudal region; F: Spicules; G: Lateral guiding pieces. (Scale bars: A = 100 μm; B, E = 10 μm; C = 50 μm; D, F = 20 μm; G = 5 μm.)

widely spaced (26-35 μm apart), ventromedian supplements, posteriormost of which situated outside range of spicules at 34-44 μm from adcloacal pair. Spicules dorylaimoid, curved ventrad, relatively robust, 3.6-4.5 times as long as wide and 1.3-1.5 times body diam. Lateral guiding pieces rather slender, 13-15 μm long, 6.5-7.5 times as long as wide and slightly tapering at tip. Caudal region short and rounded but not visibly swollen or clavate, two pairs of caudal pores, one lateral and other subdorsal, at anterior end of tail.

Type locality and habitat

Great Smoky Mountains National Park, Sevier County, TN, USA, Laurel Falls Trail, at an elevation of 1008 m a.s.l., in a mixed maple (*Acer* sp.), hemlock (*Tsuga* sp.), and silverbell (*Halesia carolina* L.) forest. Collected by K. Felderhoff, M. MacCarroll and M. Moore on 6 July 2006. GPS coordinates: 35°40.874N, 83°36.149W.

Type material

Female holotype, four female and two male paratypes deposited at USDANC (Beltsville, MD, USA); other paratypes at Nematode Collection of the University of Jaén (Jaén, Spain).

Diagnosis and relationships

The new species is characterised by its body length of 2.56-3.18 mm, lip region continuous and 10-12 μm wide, odontostyle 10-13 μm long with aperture occupying *ca* one-fourth its length, neck 662-789 μm long, anterior portion of pharynx weakly muscular and enlarging gradually, pharyngeal expansion 407-548 μm long or 60-70% of the total neck length and surrounded by a strong muscular sheath with nearly longitudinal bands, female genital system pseudodidelphic-opisthodelphic with long anterior branch lacking a functional ovary and posterior one bear-

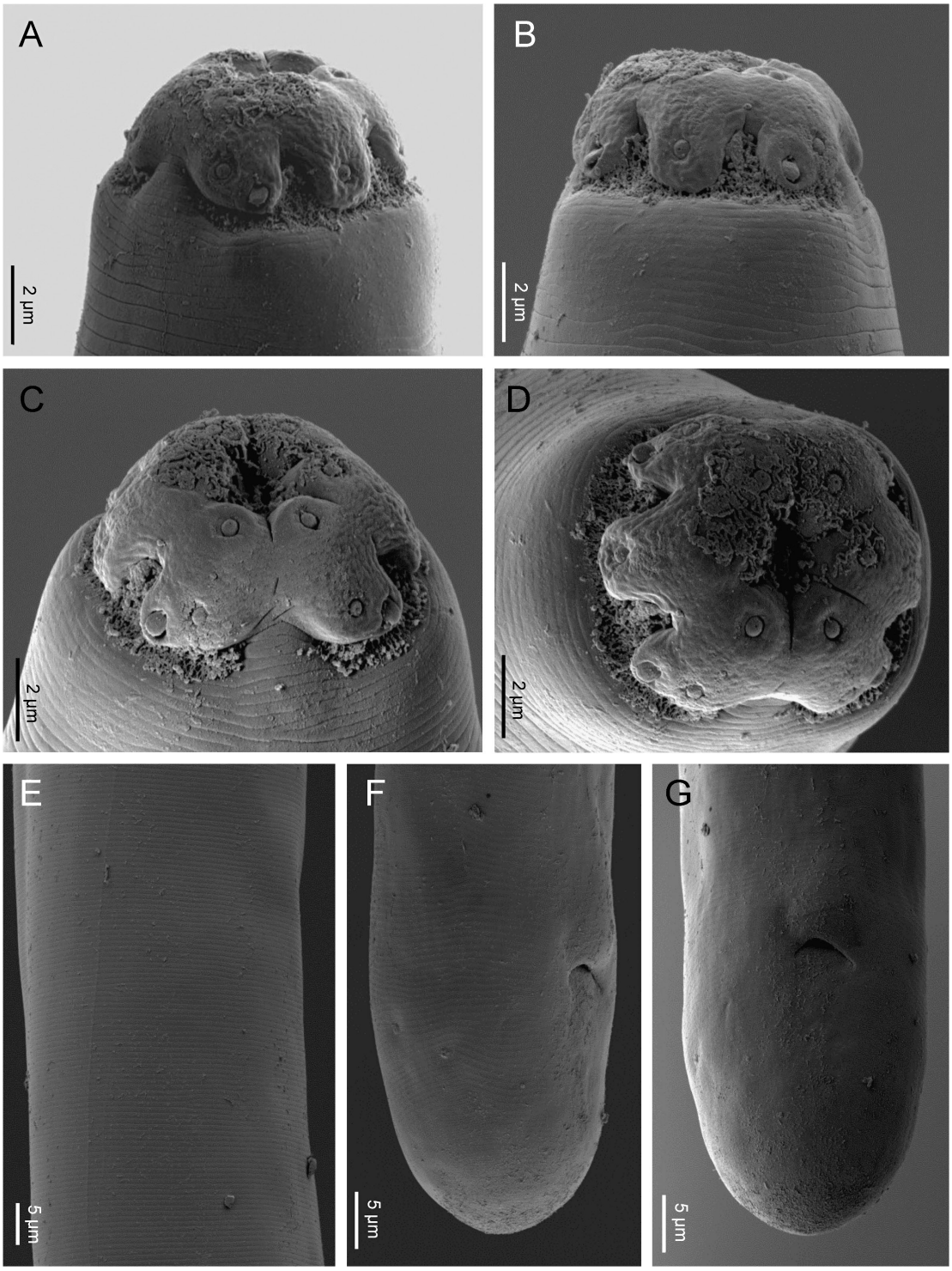

Fig. 4. *Axonchoides smokyensis* sp. n. (SEM). A: Lip region in sublateral view; B: Same in lateral view; C: Same in ventral, subfrontal view; D: Same in frontal view; E: Mid-body region, showing fine transverse striation; F: Female caudal region in sublateral view; G: Same in ventral view.

Table 1. Morphometric data of *Axonchoides smokyensis* sp. n. from the USA. All measurements are in μm (apart from L in mm) and are in the form: mean ± s.d. (range).

Character	Female		Male
	Holotype	Paratypes	Paratypes
n	–	10	4
L	2.56	2.84 ± 0.20 (2.56-3.18)	2.88 ± 0.04 (2.85-2.94)
a	55	60 ± 2.2 (56.6-63.7)	63 ± 1.3 (60.8-64.0)
b	3.9	4.0 ± 0.6 (3.4-5.5)	4.0 ± 0.3 (3.7-4.4)
c	85	93 ± 7.4 (83.6-104.9)	101 ± 9.9 (86.6-113.2)
c′	0.8	0.9 ± 0.1 (0.8-1.0)	0.8 ± 0.1 (0.7-0.9)
V	43	44 ± 1.2 (42-46)	–
Lip region diam.	11	10 ± 0.5 (10-11)	12 ± 0.5 (11-12)
Odontostyle length	12	12 ± 0.9 (10-13)	13 ± 0.4 (12-13)
Odontophore length	16	16 ± 0.4 (16-17)	17 ± 0.4 (16-17)
Guiding ring from ant. end	10	10 ± 0.3 (10-11)	11 ± 0.5 (10-11)
Neck length	662	723 ± 46.7 (662-789)	711 ± 40.6 (676-775)
Pharyngeal expansion length	418	473 ± 61.9 (383-562)	465 ± 48.5 (408-538)
Diam. at neck base	46	47 ± 3.2 (42-52)	48 ± 2.1 (46-51)
at mid-body	47	48 ± 3.1 (42-52)	46 ± 0.7 (45-47)
at anus/cloaca	36	34 ± 2.0 (32-38)	36 ± 1.5 (34-38)
Prerectum length	485	517 ± 89.6 (415-693)	562 ± 36.4 (515-617)
Rectum length	37	35 ± 2.8 (32-38)	11 ± 1.3 (9-12)
Tail length	30	31 ± 2.2 (27-34)	29 ± 2.7 (26-33)
Spicule length	–	–	51 ± 1.5 (49-53)
Ventromedian supplements	–	–	6.0 ± 0.5 (5-6)

ing a large tripartite uterus, vulva pre-equatorial (V = 42-46), female tail slightly clavate (27-34 μm, c = 84-105, c′ = 0.8-1.0), male tail short and rounded (34-38 μm, c = 87-113, c′ = 0.7-0.9), spicules 49-53 μm long, and five or six widely spaced ventromedian supplements out the range of spicules.

The new species fits well with the pattern of *Axonchoides*: lip region continuous, odontophore consisting of two parts and anterior region of pharynx weakly muscular and enlarging gradually, among other features. It differs from the type species, *A. crassus*, by its much more slender body (a = 55-64 *vs* 31-33), narrower lip region (10-12 *vs ca* 14.5 μm wide, calculated from Thorne's Fig. 12A) and lacking (*vs* having) distinctly protruding papillae, shorter neck (b = 3.4-4.8 *vs* 2.7-3.2), absence (*vs* presence) of a spindle-shaped swelling of the slender portion of the pharynx anterior to the nerve ring, more anterior vulva (V = 42-46 *vs* 48), female tail visibly swollen or clavate (*vs* short and rounded but not clavate), and smaller spicules (1.3-1.5 *vs ca* twice the body diam. at neck base, as calculated from Thorne's Fig. 12B).

Molecular characterisation

Two sequences (Fig. 5) were obtained; one consists of 2296-bp near-full-length SSU-ITS1 (Fig. 5A), and the other 812-bp LSU D2/D3 (Fig. 5B).

Taxonomy of *Axonchoides*

The discovery and characterisation of the new species herein described provides new relevant data and allows a better definition of the genus, as follows.

Diagnosis (emended)

Belondiridae, Belondirinae. Medium- to large-sized nematodes, 2.5-3.5 mm long. Cuticle dorylaimoid. Lip region narrow, rounded, continuous. Odontostyle cylindrical to slightly fusiform, about as long as lip region diam., with aperture occupying one-fourth to one-third its length. Guiding ring simple. Odontophore bearing a weak thickening at its middle, hence consisting of two parts. Anterior region of pharynx weakly muscular and

A

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   1 ACTGCGGCAA TTCTAGAGCT AATACATGCA ACAAAGCTCT GCCCGCAAGG AACGAGCGCA
  61 TTTATTAGAA TAAAAACCAA TCGGGCTTCG GCCCGTAATT TGGTGAATCT GAATAACTTT
 121 GCCAATCGCA CAGTCCTAGT ACTGGCGATG TATCTTTCAA GTGTCTGCCT TATCAACTTT
 181 CGATGGTAGG CTATACGCCT ACCATGGTAG TAACGGGTAA CGGAGAATAA GGGTTCGACT
 241 CCGGAGAGGG AGCCTGAGAA ACGGCTACCA CATCCAAGGA AGGCAGCAGG CGCGCAAATT
 301 ACCCACTTCC AGAACGGAGA GGTAGTGACG AAAAATAACG AGACAGTCCT CTTCGAGGTC
 361 TGTCATCGGA ATGGGTACAA TTTAAATCCT TTAACGAGGA TCTATTGGAG GGCAAGTCTG
 421 GTGCCAGCAG CCGCGGTAAT TCCAGCTCCA ATAGCGTATA TTAAAGTTGT TGCGGTTAAA
 481 ACGCTCGTAG TTGGATCTGC GGCCGCGGAG AACGGTCCCC CGAAAGGGCG GTCACTGTCT
 541 CTCCTAGCCT AAATTCGAGT CGTCCCTAGG TGCTCTTTAT TGAGTGCTTA GGGGTGACTA
 601 GAACGTTTAC TTTGAAAAAA TTAGAGTGCT TAAAGCAGGC GAAATAGCCT GAATAAGGGC
 661 ATGGAATAAT GGAATAGGAC CTCGGTTCTA TTTTGTTGGT TTTCGGAGCC CGAGGTAATG
 721 ATTAAGAGGA ACAAACGGGG GCATTCGTAT TCCGGCGCTA GAGGGAAAAT TCTTGGGACG
 781 CCAGAAGACG GACAACTGCA AAGCATTTGC CAAGAATGTT TTCATTAATC AAGAACGAAA
 841 GTTAGAGGTT CGAAGGCGAT CAGATACCGC CCTAGTTCTA ACCGTAAACG ATGACAACCA
 901 GCGATTAATC GGCGTTAATA TATGACCCGA TTAGCAGCTT CCGGGAAACC AAAGTTTTTC
 961 GTTTCCGGGG GAAGTATGGT TGCAAAGCTG AAACTTAAAG GAATTGACGG AAGGGCACCA
1021 CCAGGAGTGG AGCCTGCGGC TTAATTTGAC TCAACACGGG AAAACTCACC CGGCCCGGAC
1081 ACCGTAAGGA TTGACAGACT GAGAGCTCTT TCTTAATTCG GTGGGTGGTG GTGCATGGCC
1141 GTTCTTAGTT GGTGGAGCGA TTTGTCTGGT TAATTCCGAT AACGAACGAG ACTCTGGCCT
1201 ATTAAATAGA CGGTATATTA AAAAGTATAT CGCACTTCTT AGAGGGACAA GCGGCGTCTA
1261 GCCGCATGAA ATAGAGCAAT AACAGGTCTG TGATGCCCTT AGATGTCCGG GGCTGCACGC
1321 GCGCTACACT GAAAGAATCA GTGTGCATTG TGCCTAGTTC GGAAGAACCG GGTAACCCAA
1381 GTAAATTCTT TCGTGCTTGG GATAGGGAAT TGCAATTATT TCCCTTAAAC GAGGAATTCC
1441 CAGTAAATGC GGGTCATAAG CTCGCGTTGA TTACGTCCCT GCCCTTTGTA CACACCGCCC
1501 GTCGCTACTA CCGATTGGAT GACTTAGTGA GGTCTTAGGA CCGAGGTCAA GGGTGCTTTA
1561 ACGAGTGCTT TTTGCTTTGG AAATTTGATC GAACTACGTT ATCTAGAGGA AGTAAAAGTC
1621 GTAACAAGGT TTCCGTAGGT GAACCTGCGG AAGGATCATT ACCGAGCTAA ACAAACGAGA
1681 AAACGCGCGT GCTTATGAAA TACTGACACG GGTCGGAAAG TACTTATGTA CGTATGCCCG
1741 TTGAAGTTAA AGTACCCGTG TAAGTCTGAT TACGTACCGG TGCGAATGCG GTAAGGCGCC
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1981 CTCGAAGATC TAAATACCCA CTAGTGGCTG CTCTTGAAAA TTAATCACAA GTCGAAAAAT
2041 AGCGAGTCAC TAGTCGGGAT CGGAAGGTTT AAAGAGTCAC TTGGCGATTC TAGCGGCTGG
2101 TCGAAAAGTA TAAAGCACCA CGTACCACAC CGGAAAAGAG TAGTACGGTC TCCTTATTAT
2161 TTACCGGGCT CGGAGGTGTG AAACGCTCGG GTGACCACAA GAAAAAAACA TGAAATTACA
2221 CTATTAGTTT CTCTTCGCGC ACCCCTTCAT ATTTTGGAGC GATAAAAAAG CGGATTCTGA
2281 GTCGCGGTTC GATTCAA
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B

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   1 TGAAAAGAAC TTTGAAGAGA GAGTTCAAAA GGGCGTGAAA CCGCTTAGAA TAAAACGGAC
  61 GGAGCCATCG GGAGCCATCG GGAATCAGTC GTATGGTATG TGAACGTACG TCGGTACCGT
 121 AACGAAAGTA CGGCCGGAGT ATACACTAGT AGCATCCGCT CGACGCATTT CTCGACGGTA
 181 AGCGCCGCGA CCGATACGAA AGGCCAGCGA AGTGAATGCG ATTGAGGTCG AGGCCCGCAA
 241 GGGTTTCGCT ACCAGTCCGT TTCGCGATAG TCGCTGGACC GCGCAGTATG TATCGAGCGC
 301 CTAAGATATA TATATACGTA TACGTCGGCG TAAAAGCTCG CCGAGCCGTG GATAATTGGT
 361 CGATTAGTTG GTACCTGCGC GCAGGTGCCG GCGACTCTGG CCGATTAAAA GCGGCCGGCG
 421 ACGTAAGACG TATTGGCGTC GGCGGCGTCA GTGTTGGCCA CCCGTCCGAC CCGTCTTGAA
 481 ACACGGACCA AGGAGTCTAA CATATGCGCG AGCTAATGGG CGGAAAACCC AAAGGCGCAA
 541 TAAAAGTAAA AGCTGTTTCC GACAGCTAAG GTACGATCCC GGAGTCGAGG CTCCGGGCGC
 601 AGTACCGTCC CGTCTCGACC GCACGTCGGT AGGGCGGCGA TAGAGTGCAT ACGTTGGGAC
 661 CCGAAAGATG GTGAACTATG CCTGAGCAGA GCGAAGCCAG AGGAAACTCT GGTGGAGGTT
 721 CGTAGCGGTT CTGACGTGCA AATCGATCGT CAGACTTGGG TATAGGGGCG AAAGACTAAT
 781 CGAACCATCT AGTAGCTGGT CCCTTCCCGA GA
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Fig. 5. *Axonchoides smokyensis* sp. n. (sequences). A: Nearly-full-length SSU-ITS1 rDNA of 2296 bp; B: LSU D2/D3 rDNA gene sequence of 812 bp.

enlarging gradually, lacking a constriction or an isthmus-like section, basal expansion distinctly muscular, surrounded by a strong muscular sheath of nearly longitudinal bands and occupying *ca* two-thirds of total neck length. Cardia long, tongue-shaped. Female genital system pseudomonodelphic-pseudodidelphic, with long anterior branch lacking a functional ovary, posterior branch well developed, including a tripartite uterus, *pars refringens vaginae* absent or poorly developed, vulva a transverse slit. Spicules typical dorylaimid. Ventromedian supplements five or six, well and regularly spaced, outside range of spicules. Caudal region short and rounded in both sexes, may be slightly clavate in female.

Remarks

In defining the (new) genus *Axonchoides*, Thorne (1964) emphasised (p. 44) that its lip region displayed a "conspicuous semisclerotized framework". Nevertheless, Thorne's Figure 12A, which illustrates the anterior region of *A. crassus*, does not show such a remarkable feature. Our observations of *A. smokyensis* sp. n. (Fig. 2C, D) do not confirm the presence of a conspicuous framework in the lip region, although the LM surface view (Fig. 2H) and SEM pictures (Fig. 4A-D) show a rather atypical 'image' of the lip region with partially amalgamated lips which, especially under LM, might be interpreted by Thorne as a "semisclerotized framework".

Taxonomical affinities of *Axonchoides* as derived from morphological data

The two representatives of *Axonchoides* much resemble the type and only species of the rare genus *Immanigula* Andrássy, 1991, *I. laeta* Andrássy, 1991, which is only known to occur in Hungary. In his original description of *Immanigula*, Andrássy separated it from *Axonchoides* in having (p. 181) "small labial papillae, much thinner cuticle, sclerotized vulva, other-shaped spicula and differently arranged supplements". Nevertheless, the taxonomical significance of such differences to support the separation of two generic taxa is questionable: *i*) In describing *A. crassus*, Thorne (1967) mentioned (pp. 44-45) that labial papillae are mammiform and illustrated them (original Fig. 2A) as distinctly protruding above the labial contour, while Andrássy, regarding this feature, stated (p. 181) "...and non-elevated papillae". Although the degree of elevation or protrusion of labial papillae in both taxa is distinctly different, it should be interpreted as intrageneric variation, as usually observed in many dorylaimid genera. Actually, the new species herein described shows low labial papillae; *ii*) the original Figure 12A of Thorne shows that the body cuticle is rather thick in the anterior region, but not unusually thick at mid-body (Thorne's Fig. 12E). Thorne probably misinterpreted the morphology of cuticle in the cervical region, including the thickness of the cervical lacunae as part of the cuticle, since his Figure 12A is totally comparable to our Figure 2D. *Immanigula laeta* has the cuticle 2.5-3.0 μm thick throughout the body but 9-10 μm in the caudal region, an entirely comparable pattern to that observed in the new species herein described, hence no significant difference is observed between *Axonchoides* and *Immanigula*; *iii*) the morphology of the vagina, in particular the presence/absence of *pars refringens*, seems to be a relevant difference between *Axonchoides* (*pars refringens* absent or very small, see above) and *Immanigula* ("...Vulva transverse with sclerotized lips", see p. 184). Nevertheless, Andrássy's original Figure 20(4) apparently shows a well developed *pars distalis vaginae*, continuous with the body cuticle and with indication of a weak inner differentiation, which is a rather similar scheme to that observed in *A. smokyensis* sp. n.; *iv*) Andrássy (1991) certainly overestimated the shape of the spicules in his comparison of both genera since the spicules of their respective type species (as illustrated in Thorne's Fig. 12b and Andrássy's Fig. 21(3)) do not differ significantly at all. Moreover, differences in slenderness and shape of spicules are regarded as part of intrageneric variation, except if such differences are exceptional, which is not the case here; and finally *v*), the number and arrangement of the ventromedian supplements are the most relevant differences between the two genera since *Axonchoides* species bear few (four or five) ventromedian supplements outside the range of the spicules whereas *I. laeta* has 11 supplements with the posteriormost being located within the range of the spicules. However, such differences have been only exceptionally used (see, for instance the case of *Allodorylaimus* Andrássy, 1986 and *Eudorylaimus* Andrássy, 1959) to separate genera. In conclusion, both *Axonchoides* and *Immanigula* are certainly closer than previously accepted and might be identical. Nevertheless, the two species of *Axonchoides* share a very special apomorphic condition (apparently absent in *I. laeta*) concerning the morphology of the odontophore which bears a thickening at its middle and which therefore consists of two parts. This is a significant difference that, in addition to the number and the arrangement of ventromedian supple-

ments and their provisional distant geographical origin, justifies the separation of both genera.

The new taxon also resembles a few, atypical, species of *Axonchium* Cobb, 1920, namely *A. geminum* Coomans & Nair, 1975 and *A. variabile* Coomans & Nair, 1975, which are characterised by having the odontostyle cylindrical rather than spindle-shaped or fusiform, and the anterior region of the pharynx slender and weakly muscular and the pharyngeal enlargement gradual. These species, however, also have a lip region with separate lips and vagina with very peculiar morphology since its *pars distalis* bears a dilated portion, including an internal cavity anterior to its junction with the *pars proximalis*.

In having a continuous lip region, the new species also resembles representatives of *Syncheilaxonchium* Coomans & Nair, 1975. However, this taxon is distinguished (see recent compendium by Naz & Ahmad, 2012) by its short and fusiform odontostyle as well as by the presence of a strong constriction or a short, but distinct, isthmus-like section separating both pharyngeal regions.

Evolutionary relationships as derived from molecular analyses

Sequencing of ribosomal DNA was employed to investigate the origin and phylogenetic relationships of the new species. The sequences obtained are unique and are significantly different from other sequenced dorylaimid taxa. A Blastn search of *Axonchoides smokyensis* sp. n. on the SSU region revealed the highest match as *Aporcelaimellus* sp. (GenBank accession number AJ875154 and AJ875155). Alignment of these two sequences yielded 1639 total characters with 1607 identical nucleotides (98.04%) and five insertions/deletions (0%). Alignment of *Axonchoides smokyensis* sp. n. and *Axonchium propinquum* (AY593020), the only available near-full-length SSU sequence in *Axonchium*, yielded 1554 total characters with 1616 identical nucleotides (96.16%) and six insertions/deletions (0%). Other species had 96-98% identities and were selected in further phylogenetic analysis. The LSU D2/D3 sequence of *A. smokyensis* sp. n. had less than 84% identity with other dorylaims, the highest being *Ecumenicus monohystera* (AY593013) with 670 identical nucleotides over 795 total characters (84.28%) and 18 insertions/deletions (2.3%). Alignment of *A. smokyensis* sp. n. and *Axonchium propinquum* (AY593022), the only available LSU D2/D3 sequence in *Axonchium*, yielded 662 identical nucleotides over 799 total characters (82.85%) and 25 insertions/deletions (3.1%). ITS is a highly variable DNA fragment and the corresponding Blastn search failed to result in good matches for any nematode species, thus further phylogenetic analysis using this sequence was not pursued.

The evolutionary relationships of the new species, as derived from the nearly-full-length SSU-ITS1 rDNA and the LSU D2/D3 and using several species of mononchs (Mononchida) and nygolaims (Nygolaimina) as outgroups, are presented in Figures 6 and 7, respectively. In both trees, *A. smokyensis* sp. n. is included in the Dorylaimina clade, but it is not very close to any species in the trees, thus the origin of this new species is not clear. The most remarkable (and intriguing) result is that the new species is very distant from *Axonchium propinquum* in both molecular trees, despite the latter being morphologically similar to *A. smokyensis* sp. n., and indeed actually the most similar one among those represented in the trees. In other words, these results suggest that morphological and molecular data do not match at all.

The molecular tree presented in Figure 6 provides very poor resolution to elucidate evolutionary relationships in Dorylaimina, which produced similar results to Holterman *et al.* (2008), van Megen *et al*. (2009) and Álvarez-Ortega & Peña-Santiago (2012) in that none of the families Qudsianematidae, Nordiidae or Dorylaimidae is monophyletic. *Axonchoides smokyensis* sp. n. forms part of a well supported clade dominated by representatives of the families Qudsianematidae (genera *Allodorylaimus*, *Crassolabium*, *Epidorylaimus*, *Eudorylaimus* and *Microdorylaimus*) and Nordiidae (*Californidorus*, *Enchodelus*, *Longidorella*, *Rhyssocolpus* and *Pungentus*), but also with a member of Dorylaimidae (*Prodorylaimus*). This clade has a problematic interpretation from a morphological perspective, especially due to the presence of *Prodorylaimus mas.* On the other hand, Figure 7 shows that Dorylaimina consists of two large subgroups, both with representatives of several families and/or superfamilies. The new species is now included in a well supported clade, together with a series of heterogeneous taxa (discolaims, tylencholaims and *Aporcella*). These dorylaimid taxa, however, have in common the absence of *pars refringens vaginae*, a not very frequent morphological feature in dorylaims — usually it is present — which traditionally was not given much taxonomic weight, at least to distinguish taxa of higher rank such as superfamilies or families.

Finally, both trees confirm, yet again, that the order Dorylaimida and its two suborders Dorylaimina and Nygolaimina are well supported, natural (monophyletic) taxa (*cf*., Mullin *et al.*, 2005; Holterman *et al.*, 2008), but also

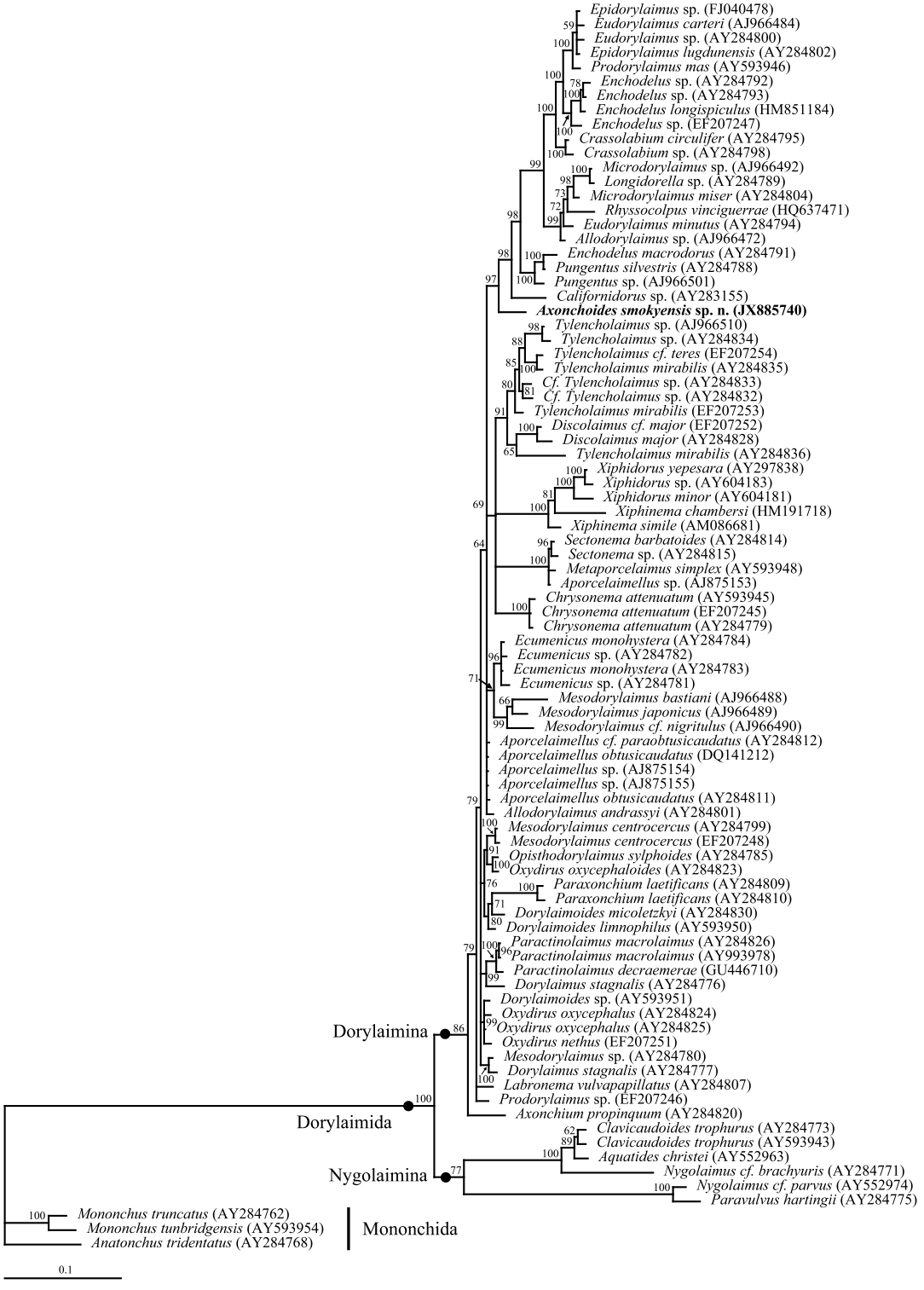

Fig. 6. Bayesian 50% majority rule consensus trees as inferred from SSU-ITS1 rDNA gene sequence alignments under the GTR + I + G model. Posterior probabilities are given for appropriate clades. Newly obtained sequence indicated by bold letters.

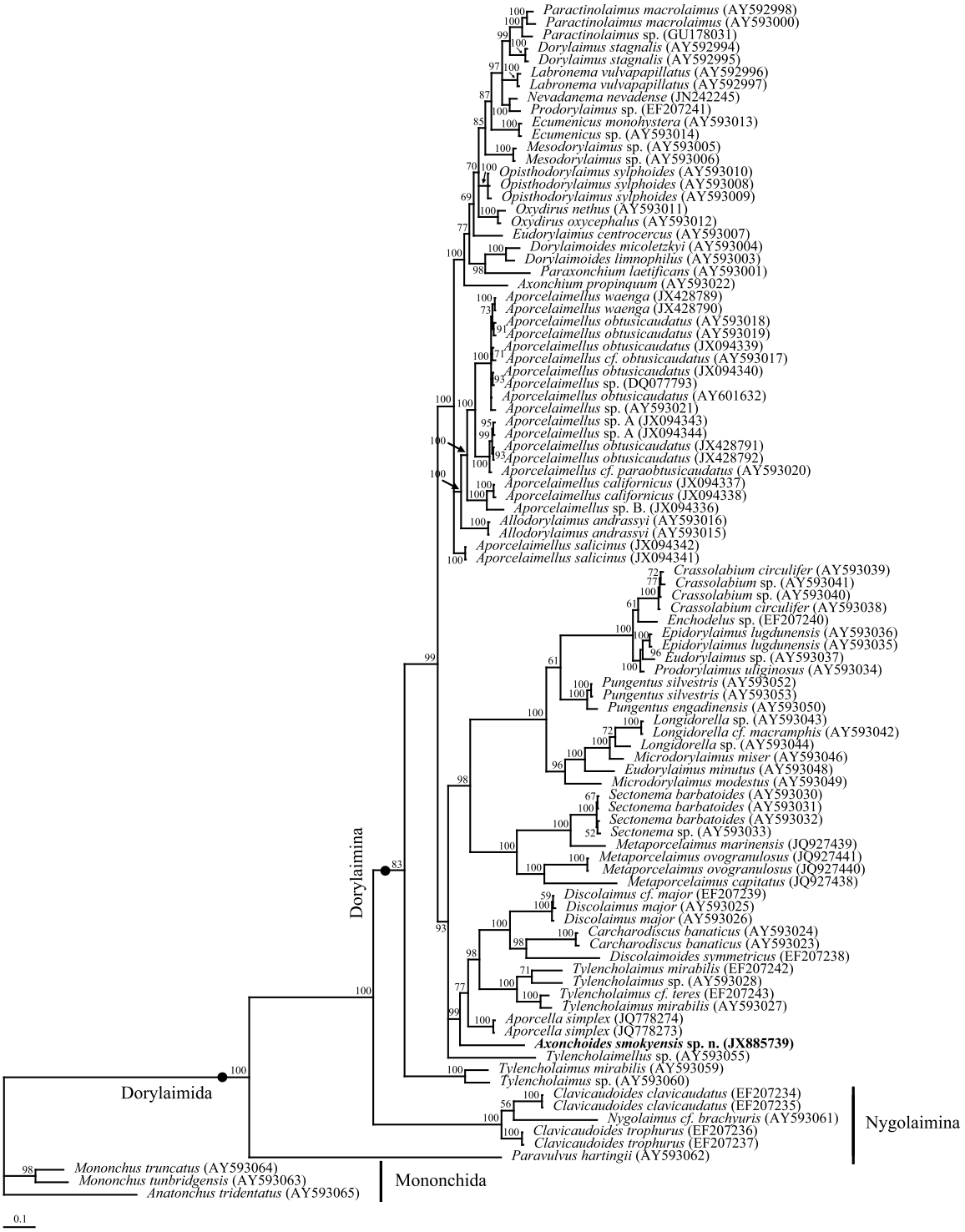

Fig. 7. Bayesian 50% majority rule consensus trees as inferred from D2/D3 expansion segments of LSU rDNA gene sequence alignments under the GTR + I + G model. Posterior probabilities are given for appropriate clades. Newly obtained sequence indicated by bold letters.

that evolutionary relationships within the Dorylaimina are difficult to establish and that the monophyly of their superfamilies and families, as traditionally (morphologically) conceived, should be questioned (*cf.*, Holterman *et al.*, 2008; Álvarez-Ortega & Peña-Santiago, 2012). The large sequence divergence in SSU (less than 98%) and LSU D2/D3 (less than 86%) between *A. smokyensis* sp. n. and other sequenced dorylaims revealed that LSU gene has evolved more rapidly than SSU. The poor resolution in our phylogenetic trees using these markers indicated that these DNA fragments (size and mutation rate) are not suitable for studying high level taxonomy in Dorylaimina. Sequencing the full ribosomal gene or other genes and more species is needed to examine the phylogenetic relationships in Dorylaimina in the future.

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