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A New Species of the Rare Genus *Anguillonema* Fuchs, 1938 (Nematoda: Hexatylina, Sphaerularioidea) with Its Molecular Phylogenetic Study

Mahyar Mobasseri, Majid Pedram, and Ebrahim Pourjam

Abstract: Anguillonema amolensis n. sp. is described and illustrated based on its morphological, morphometric, and molecular characters. The new species is characterized by its 575 to 820 μm long and wide body (body width at vulva = 30 to 59 μm), irregularly ventrally curved after fixation, five to six lines in lateral fields, 6.0 to 7.5 μm long stylet with small rounded knobs, pharynx lacking a median bulb, pharyngo-intestinal junction anterior to nerve ring and excretory pore, females with monodelphic-prodelphic reproductive system, 15 to 19 μm long conical tail with broad rounded tip, and males absent. The new species is compared with two known species of the genus, *Anguillonema poligraphi* and *A. crenati*. Molecular phylogenetic studies of the new species using partial sequences of small subunit (SSU) rDNA revealed that it forms a clade with an unidentified nematode species and two species of the genus *Howardula*. In phylogenetic analyses using partial sequences of the 28S rDNA (D2-D3 segment), the new species formed a monophyletic group with species belonging to two genera *Howardula* and *Parasitylenchus*.

Key words: Anguillonema poligraphi, *A. crenati*, bayesian inference, maximum likelihood, Mazandaran province, new species, taxonomy.

The genus *Anguillonema* Fuchs, 1938 belongs to suborder Hexatylina Siddiqi, 1980, and there was not a consensus on its taxonomic position until 2000 (a *genus dubium* in Siddiqi, 2000). Andrássy (2007), in his second volume of book series on free-living nematodes of Hungary, proposed a resolved taxonomic position for the genus under the same suborder, Hexatylina, the family Neotylenchidae Thorne, 1941 and subfamily Gymnotylenchinae Siddiqi, 1980. This is one of the rarest nematode genera, with poor data on its morphology; and some details of its body structure such as the nature of pharynx and pharyngo-intestinal junction are not well known due to poor illustrations and/or lacking of other reports or redescriptions (Sumenkova, 1989). As expected, there is no molecular data for the genus in GenBank database.

Recently, two genera are added to the suborder Hexatylina (Yaghoubi et al., 2014; Miraeiz et al., 2015), and in a recent study (Pedram, 2017), a history of some conducted taxonomic studies of insect-related nematodes is given. In our samplings from several ports of northern forests of Iran, a population belonging to the genus *Anguillonema* was recovered from rotten wood of a dead trunk of a forest tree. The objectives of this work were a morphological study of this rare and poorly known genus and a first molecular phylogenetic study using two genomic fragments.

Materials and Methods

Sampling, extracting, and taxonomy: Several soil, wood, bark, and rotten organic material samples were collected from different natural locations and forests of Mazandaran province, northern Iran, during 2015 and 2016. All samples were kept in a cool and dark place. Nematodes were extracted from the collected samples using the tray method (Whitehead and Hemming, 1965) and examined under a Nikon SMZ1000 stereomicroscope. Nematodes were hand-picked and heat-killed by adding boiling 4% formalin solution, transferred to anhydrous glycerin according to De Grisse (1969), mounted on permanent slides, and examined using a Nikon Eclipse E600 light microscope. Photographs were taken using an Olympus DP72 digital camera attached to an Olympus BX51 microscope powered with differential interference contrast. Drawings were made using a drawing tube attached to the microscope and were redrawn using CorelDRAW® software version 16.

PCR: DNA was extracted from one single female nematode. The specimen was picked out, studied onto a temporary slide, transferred to a small drop of TE buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0; 100 QIAGEN Inc., Valencia, CA) on a clean slide and squashed using a clean slide cover glass. The suspension was collected by adding 15 μl of the aforementioned buffer (Alvani et al., 2016). The DNA sample was stored at −20°C until using as PCR templates. Primers for amplification of 18S rDNA were forward primer SSU F22 (5′-TCCAAGGAAGGCAGCAGGC-3′) and reverse primer SSU R13 (5′- GGGCATCACAGACCTGTTA-3′) as used by Dorris et al. (2002). Primers for 28S rDNA D2/D3 amplification were forward primer D2A (5′-ACAAGTACCGTGAGGGAAAGT-3′) and reverse primer D3B (5′-TGCGAAGGAACCAGCTACTA-3′) (Nunn, 1992). PCR reaction was carried out in a total volume of 30 μl (19.2 μl distilled water, 3 μl 10× PCR buffer, 0.6 μl 10 mM dNTP mixture, 1.2 μl 50 mM MgCl2, 1.2 μl of each primer (10 pmol/μl), 0.6 μl of *Taq* DNA polymerase (5 unit/μl, CinnaGen, Tehran, Iran), and 3 μl of DNA template. The thermal cycling program for amplifying two genomic fragments (18S rDNA, 28S rDNA D2/D3) was as follows: denaturation at 95°C for 4 min, followed by 32 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 40 sec, and extension at 72°C for 80 sec. A final extension was performed at 72°C for 10 min.

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Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran.

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E-mail: majid.pedram@modares.ac.ir.

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The PCR products were sequenced in both directions using the same primers with an ABI 3730XL sequencer (Applied Biosystems) at Macrogen (Seoul, South Korea). Newly obtained sequences of the studied species were deposited in GenBank (accession numbers: MF134423 for partial 18S and MF134424 for partial 28S rDNA).

Phylogenetic analyses: The newly obtained 18S and 28S rDNA sequences were compared with those of other nematode species available in GenBank using the BLAST homology search program. The selected DNA sequences were aligned using MUSCLE (Edgar, 2004) as implemented in MEGA6 (Tamura et al., 2013). The most appropriate model of nucleotide substitution was selected using the Akaike information criterion in MrModeltest 2 (Nylander, 2004). The general time reversible model, including a gamma distribution for rates across sites and a proportion of invariant sites (GTR + G + I), was selected. Bayesian inference (BI) was performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) running the chains for five million generations (nruns = 4). After discarding burn-in samples, the remaining samples were retained for further analyses. The Markov chain Monte Carlo method within a Bayesian framework was used to estimate the posterior probabilities of the phylogenetic trees (Larget and Simon, 1999) using the 50% majority rule. Suitability of the posterior sample size was evaluated using autocorrelation statistics as implemented in TRACER v.1.5 (Drummond and Rambaut, 2007). A maximum likelihood tree was reconstructed by using RaxmlGUI 1.1 (Silvestro and Michalak, 2012) software using the same nucleotide substitution model as in the BI including 1,000 bootstrap pseudoreplicates. For the 28S rDNA phylogenetic analyses (BI and maximum likelihood), *Poikilolaimus oxycerca* de Man, 1895 and *Poikilolaimus piniperdae* Fuchs, 1930 (accession numbers DQ059059 and DQ059060, respectively), and for the 18S rDNA phylogeny, the species *Pseudacrobeles* sp., *Acrobeloides maximus* Thorne, 1925, and *Acrobeles ciliatus* von Linstow, 1877 (accession numbers KU180672, EU196016, and AF202148, respectively) were used as outgroup taxa. The resultant files of phylogenetic software were visualized using Dendroscope V.3.2.8 (Huson and Scornavacca, 2012) and redrawn in CorelDRAW software version 16.

Results

Systematics

Anguillonema amolensis n. sp. (Table 1; Figs. 1–4).

Description

Female: Vermiform, wide, irregularly ventrally curved after fixation. Cuticle thin, finely annulated with five to six lines in the lateral field. Lip region low, continuous with the body. Amphidial openings not visible. Stylet short, conus as long as the shaft or slightly shorter, small knobs distinct. Dorsal gland orifice close to the stylet knobs. Corpus muscular, wide, slender, isthmus lacking, pharyngeal glands forming long dorsal overlapping. Phyaryngo-intestinal junction anterior to the nerve ring and excretory pore. Hemizonid indistinct. Intestine simple, rectum and anus functional. Reproductive system monodelphic-prodelphic, ovary long, usually reaching nerve ring, reflexed once or twice in some individuals, oocytes in multiple rows close to the germinal zone, at two rows distally, oviduct and spermatheca indistint, crustaformeria formed from multiple cells, joining to the uterus, the latter sometimes containing mature egg, vagina with sclerotized walls, vulva a transverse slit with protruding lips and the postvulval uterine sac absent. Tail conical, short, with wide rounded tip dorsally bent in fresh females in water.

Male: Not found.

Type habitat and locality: Recovered from rotten barks of a beech tree in a forest in Mazandaran province, northern Iran, during February 2015. GPS coordinates: N 36°23′48″, E 52°19′46″.

Type material: Holotype female and five paratype females were deposited in the Nematode Collection at the Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran. One paratype female deposited in each of the following collections: UGent Nematode Collection of the Nematology Research Unit, Department of Biology, Ghent University, Ghent, Belgium and WANECO collection, Wageningen, The Netherlands (http://www.waneco.eu/).

Table 1. Morphometrics of *Anguillonema amolensis* n. sp. All measurements are in μm and in the form: mean ± SD (range).

Characters	Holotype Female	Paratype Female
n		7
L	778	716.0 ± 89.3 (575–820)
L′	763	700.1 ± 89.9 (556–805)
a	25.9	16.3 ± 4.3 (13.6–25.9)
b	14.8	13.5 ± 2.1 (11.5–15.6)
c	51.9	45.1 ± 0.7 (35.9–54.7)
c′	1	1.2 ± 0.2 (0.9–1.6)
V	95.2	95.7 ± 0.5 (94.9–96.2)
V′	97.1	97.9 ± 0.7 (97.1–98.9)
Head height	1	1.0 ± 0.0 (1.0–1.0)
Head width	6	5.1 ± 0.7 (4.0–6.0)
DGO	0.7	0.9 ± 0.3 (0.7–1.1)
Stylet	7.5	6.8 ± 0.5 (6.0–7.5)
Conus	3.6	3.7 ± 0.6 (3.0–4.5)
Excretory pore	61	59.1 ± 8.2 (43.0–64.6)
Pharyngo-intestinal junction to anterior end	52.5	53.3 ± 4.1 (50.0–59.3)
Pharyngo intestinal junction to end of glands	116	138 ± 21.5 (116–178)
Head-vulva	741	685.3 ± 87.1 (550–789)
Mid-body width	30	43.7 ± 9.4 (30–59)
Body width at vulva	30	41.9 ± 8.8 (30–57)
Anal body width	15	13.7 ± 2.4 (11–18)
Tail	15	16.0 ± 1.5 (15–19)

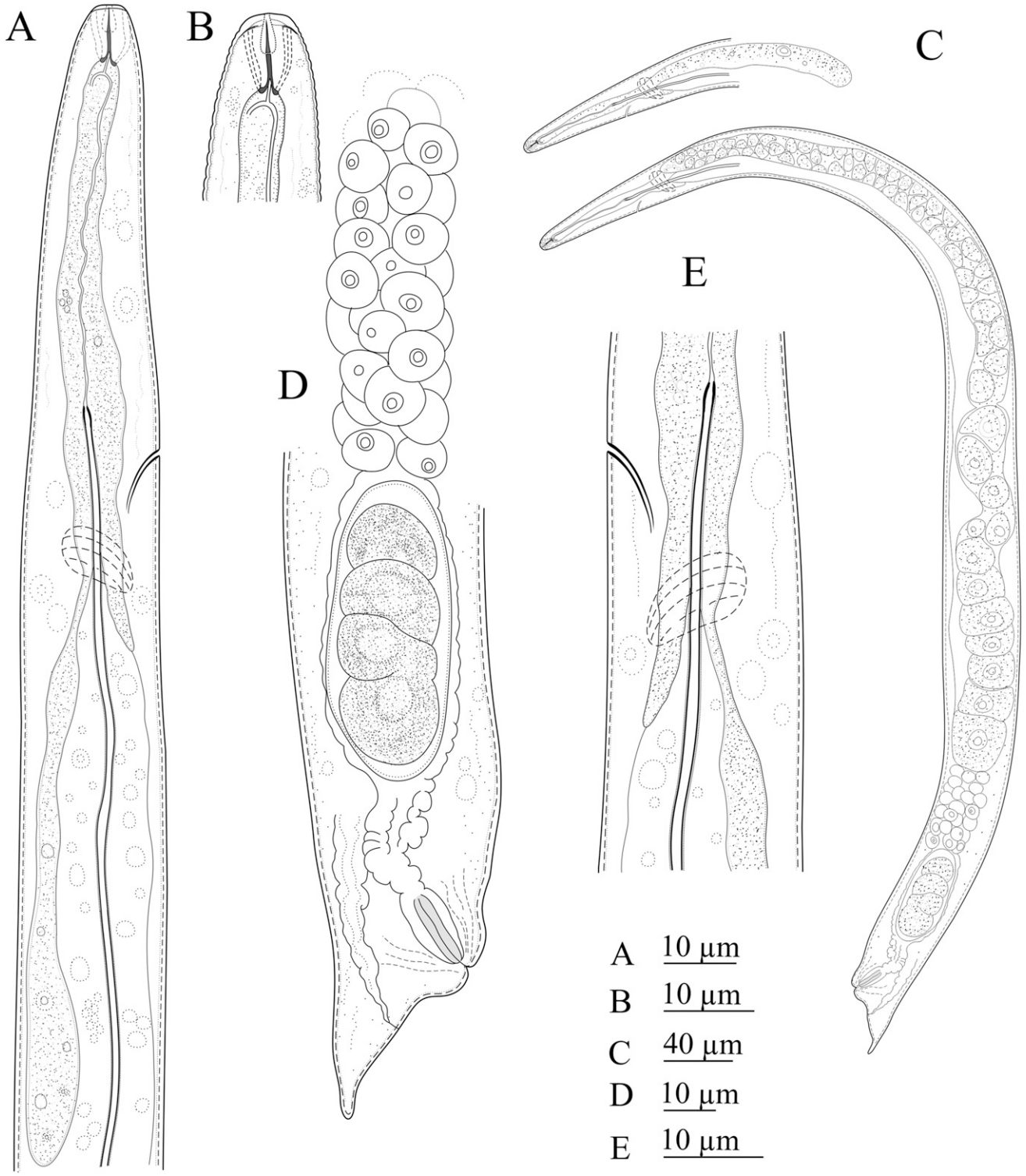

Fig. 1. Line drawings of *Anguillonema amolensis* n. sp. A. Pharyngeal region. B. Details of anterior end. C. Entire female. D. Details of posterior body end. E. Pharyngo-intestinal junction details.

Etymology: The specific epithet "*amolensis*" refers to the city of Amol, the original geographic point where the new species was recovered.

Diagnosis and relationships: Anguillonema amolensis n. sp. is characterized by its long (575 to 820 µm) and wide (30 to 59 µm) females, low lip region continuous with body contour, five to six lines in lateral fields, small stylet with small rounded knobs, monodelphic-prodelphic reproductive system, and conical 15- to 19-µm long tail. It is morphologically close to the type species of the genus, *A. poligraphi* Fuchs, 1938, and compared with it, has a continuous head (vs. offset, according to original drawings),

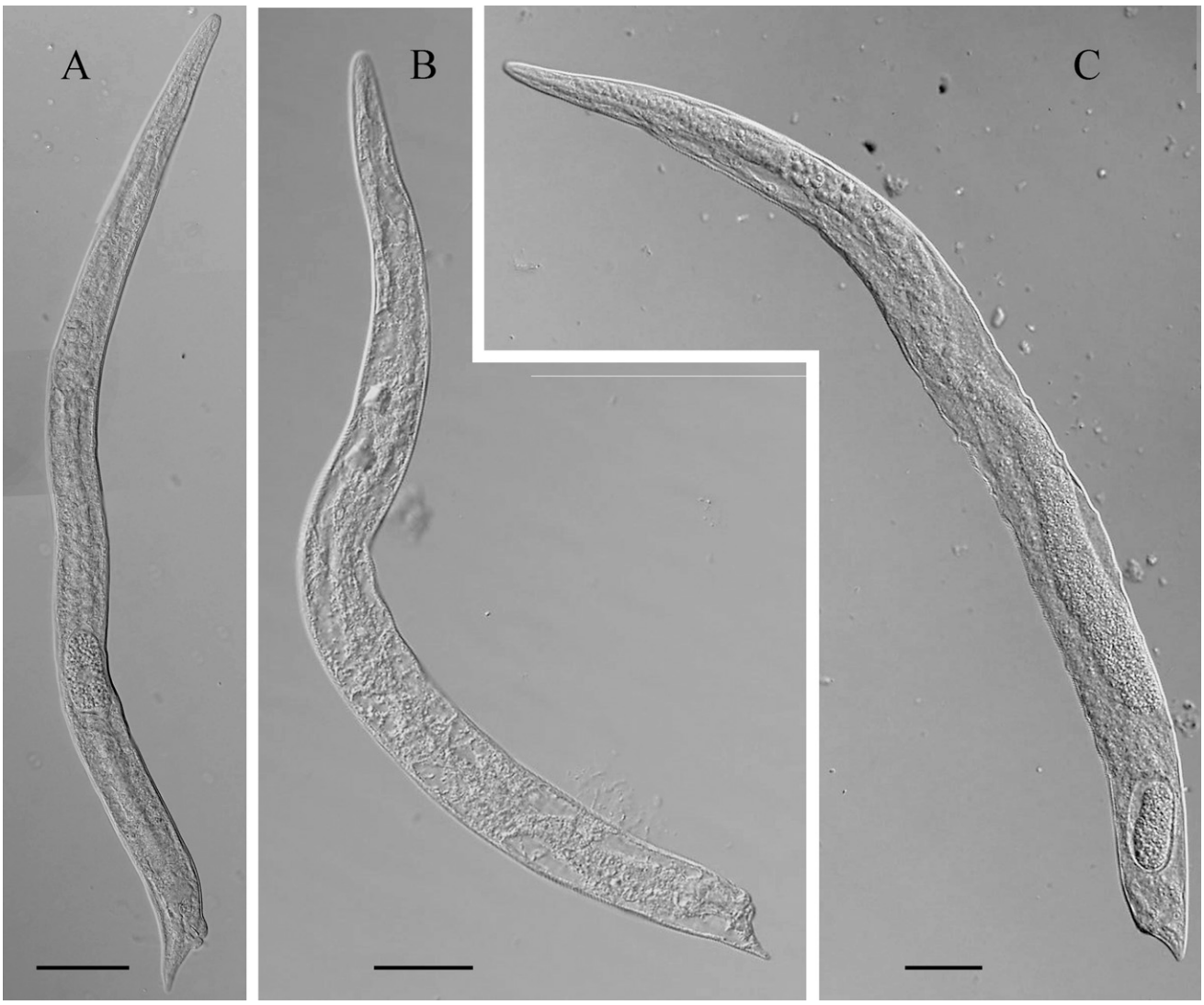

FIG. 2. Light microphotographs of *Anguillonema amolensis* n. sp. A to C. Entire female (all scale bars = 50 μm).

shorter tail (average length of 16.0 ± 1.5 vs. 28 μm, as calculated), smaller c′ (1.2 ± 0.2 vs. 2.1, as calculated from drawing), greater c (45.1 ± 0.7 vs. 23), and broadly rounded tail tip not dorsally bent in mounted specimens (vs. tail tip dorsally curved, apparently sharp). Compared with *A. crenati* Fuchs, 1938, the new species has shorter body (average length of 700.0 ± 89.3 vs. 1,082 μm), smaller a value (an average of 16.3 ± 4.3 vs. 42), greater V value (an average of 95.7 ± 0.5 vs. 92.6), and shorter tail (c′ = 1.2 ± 0.2 vs. 4, as calculated according to original drawing) not dorsally bent in mounted specimens (vs. dorsally bent).

Molecular phylogenetic analysis: Sequencings of 18S and 28S rDNA D2/D3 fragments of the new species yielded single sequences of 882 and 870 nt (accession numbers MF134423 and MF134424, respectively). The blast search of the GenBank nucleotide database using these sequences revealed they are both unique. A 96% to 98% identity was achieved for BLAST search of partial 18S rDNA sequence for some species belonging to the genera *Howardula* Cobb, 1921, *Rubzovinema* Slobodyanyuk, 1991, *Deladenus* Thorne, 1941, and *Parasitylenchus* Micoletzky, 1922. The BLAST search using partial 28S rDNA, revealed it has the highest identity (88%) with *Howardula phyllotretae* Oldham, 1933 (accession number DQ328728). A total number of 49 hexatylenchid species/isolates, and 55 species/isolates of hexatylenchids and anguinids were used for reconstructing of 18S and 28S phylogenetic trees. The multiple alignment of 18S dataset was composed of 1964 total characters with 574 variable characters. The 28S dataset was composed of 431 total characters of which 269 characters were variable.

In 18S tree, the new species, representing the only currently sequenced species of the genus has formed a clade with an unidentified isolate (accession number EU880149), both of which forming a clade with two species of *Howardula* (accession numbers JX291137, AF519234) (Fig. 5). In the 28S phylogenetic tree, the new species has formed a clade with an isolate of *Howardula* (accession number DQ328728) and two isolates of *Parasitylenchus* (accession numbers DQ328729

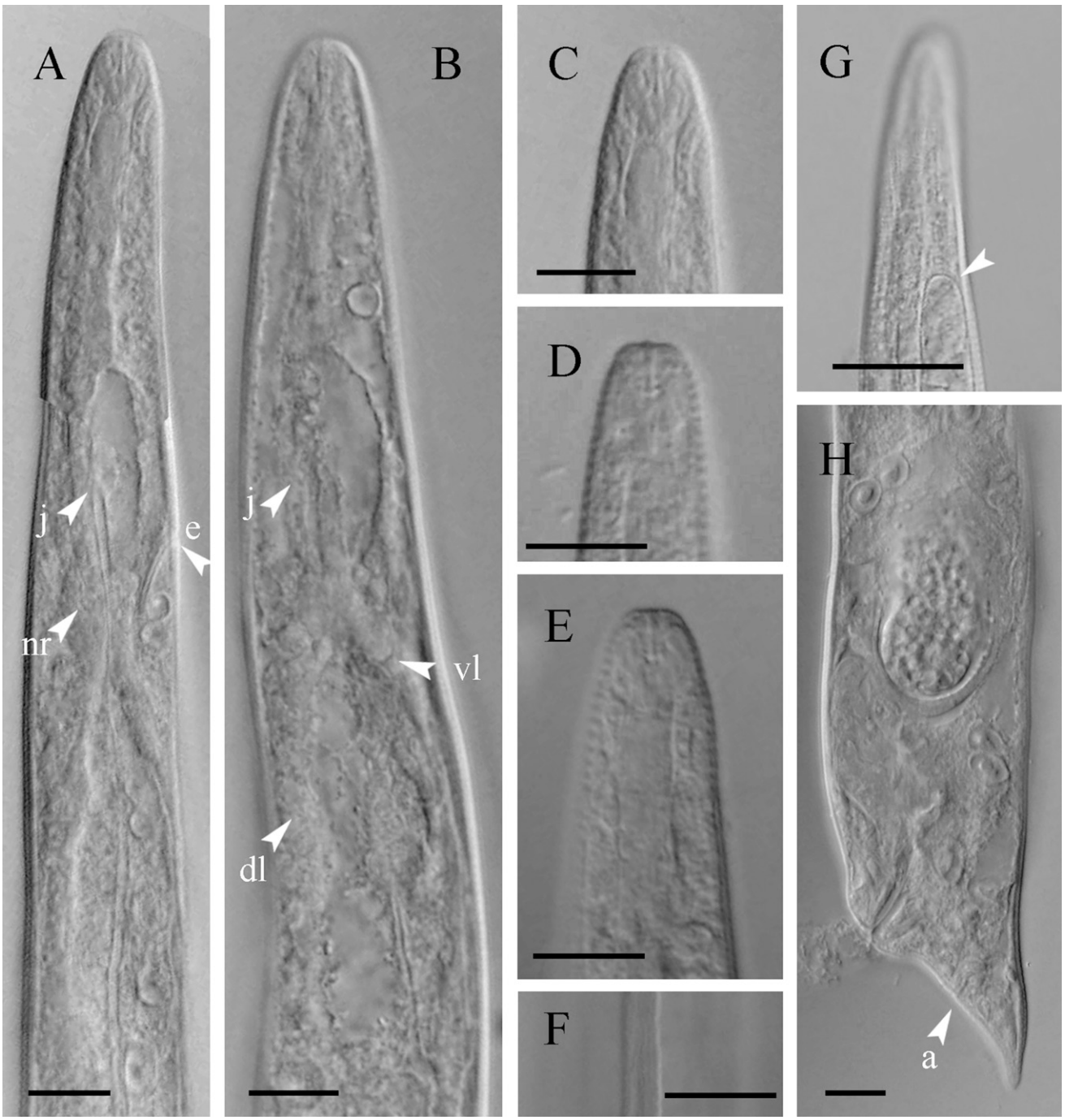

Fig. 3. Light microphotographs of *Anguillonema amolensis* n. sp. A, B. Details of pharyngeal region. C to E. Details of anterior end. F. Lateral field. G. Ovary tip (arrow). H. Details of posterior body end (all scale bars = 10 μm). j = pharyngo-intestinal junction, e = excretory pore, nr = nerve ring, vl = ventral lobe of pharyngeal glands, dl = dorsal lobe of pharyngeal glands, a = anus.

and KM245038) (Fig. 6). The monophyletic nature is not seen for most families and subfamilies of Hexatylina in either the 18S or 28S trees, however, genomic sequences are not available for most representatives of the suborder.

Discussion

The genus *Anguillonema* belongs to one of the rarest Tylenchomorpha De Ley and Blaxter, 2002 genera and currently includes two species, *A. poligraphi* and *A. crenati,* both of which are reported in the shape of original descriptions (no other reports or redescriptions are available for these two species). The "*dubium*" status of the genus (Siddiqi, 2000) and its uncertain taxonomic position was revised by Andrássy (2007), and the genus was placed inside the family Neotylenchidae, subfamily Gymnotylenchinae. It seems, however, that the type materials of these two known species are not accessible.

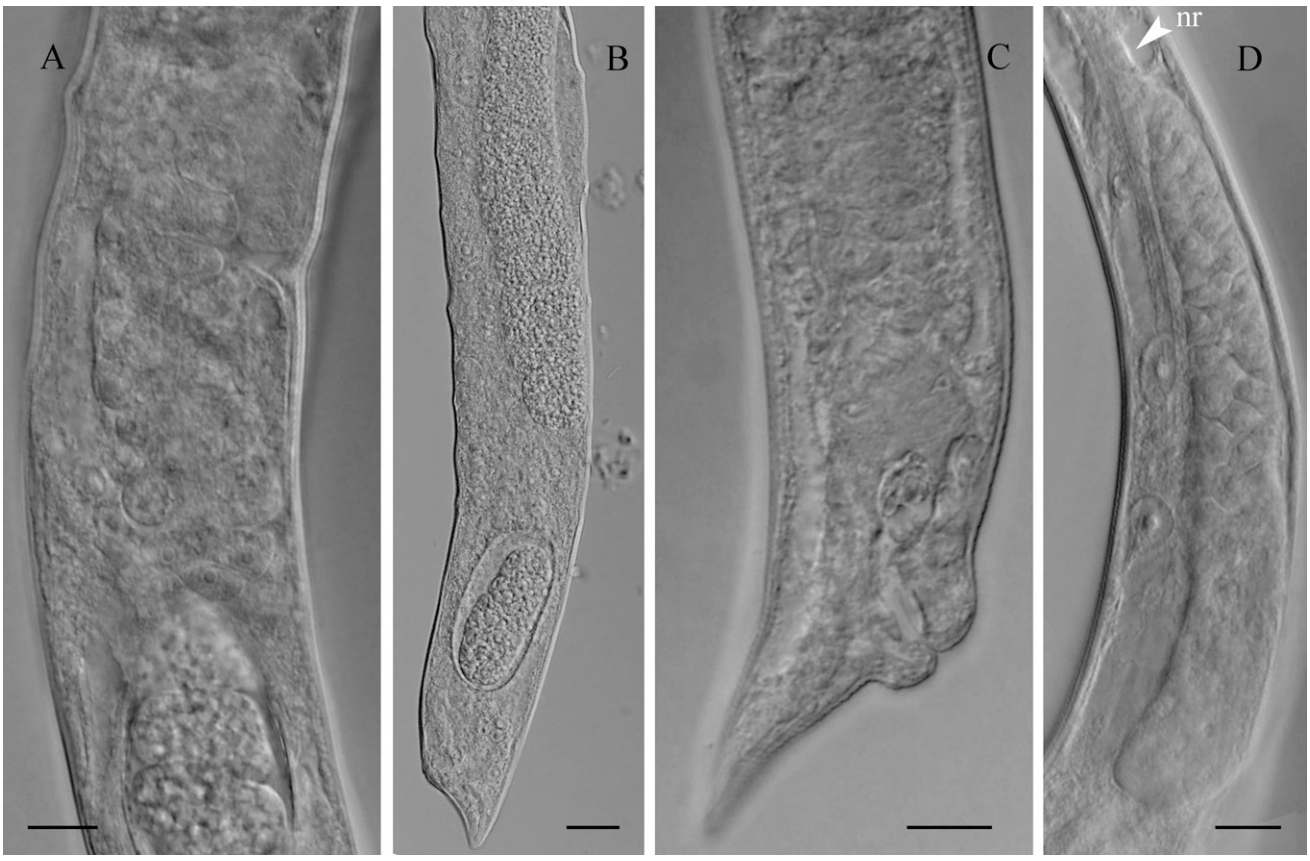

Fig. 4. Light microphotographs of *Anguillonema amolensis* n. sp. A. Part of female reproductive system. B. Female posterior body region. C. Vulval region and part of reproductive system. D. Nerve ring, ovary tip and end of pharyngeal glands seen at the same focus (all scale bars = 10 μm, except B = 20 μm). nr = nerve ring.

According to Siddiqi (2000), the suborder Hexatylina contains two superfamilies: Sphaerularioidea Lubbock, 1861 and Iotonchioidea Goodey, 1953. The first superfamily, contains three families Sphaerulariidae Lubbock, 1861, Allantonematidae Pereira, 1931, and Neotylenchidae. The latter family, Neotylenchidae, as Siddiqi (2000) stated, has "two types of generation, one free-living, fungus- or plant-feeding, another involving a heterosexual female parasitic in the insect haemocoel". Thus, with regard to the free-living mycetophagous or probably, the plant feeding habit of the recovered new species, the placement of the genus by Andrássy (2007) inside the family Neotylenchidae is confirmed, but the lack of knowledge about other type of generation in the tentative insect host, the assigning of the genus *Anguillonema* to either of four subfamilies of Neotylenchidae (*Sensu* Siddiqi, 2000) or even to a new subfamily was not conducted in this study. However, the assigning of *Anguillonema* to Gymnotylenchinae by Andrássy (2007) could be logical, as, morphology could support such placement.

The suborder Hexatylina (*sensu* Siddiqi, 2000) comprises a diverse group of taxa which are separated from each other based on their morphological and/or biological characters. This is an artificial grouping and molecular phylogenetic studies do not infer such classification. Besides, using the currently available sequences of representatives of several genera of Hexatylina, members of the families and even genera, do not form monophyletic groups in phylogenetic trees using the SSU and large subunit (LSU) rDNA sequences.

In our present SSU tree, members of Sphaerularioidea and Iotonchioidea have occupied separate clades within the phylogenetic tree. For example, the subfamilies of Neotylenchidae are in separate clades, distantly related to each other. The nonmonophyletic nature is also seen for several genera such as *Deladenus*, *Howardula,* and *Rubzovinema*. In this tree, the new species from the family Neotylenchidae has formed a clade with an unidentified nematode species, EU880149, both of which forming a well-supported clade with two species of *Howardula* (JX291137, AF519234) from the family Allantonematidae. Similar to the former phylogenetic analysis by Koshel et al. (2014) using SSU-ITS1-5.8S-LSU rDNA sequences, the nonmonophyletic nature of families such as Neotylenchidae, Allantonematidae, and Parasitylenchidae Siddiqi, 1986 is documented.

The only currently available sequence of a Gymnotylenchinae member (cf. *Gymnotylenchus* sp., AY912040) in our SSU tree has placed in a distantly related clade with the new species, and likewise, concerning the uncertainty of its generic identity (the "*cf.*" status) and the

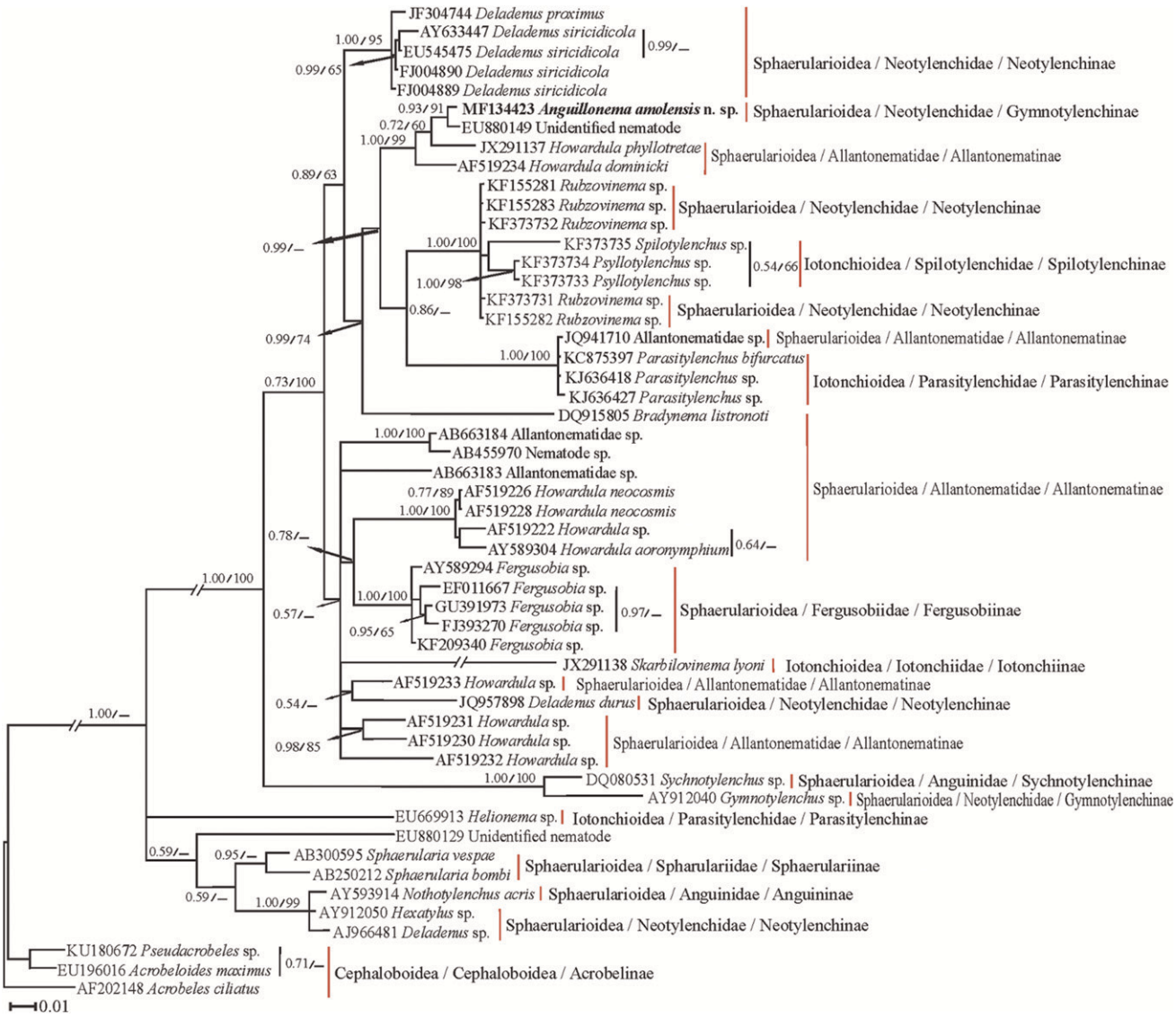

FIG. 5. Bayesian tree inferred under the GTR + G + I model (−lnL = 9561.6309; AIC = 19143.2617; freqA = 0.2705; freqC = 0.1883; freqG = 0.2443; freqT = 0.2969; rAC = 0.9005; rAG = 2.8077; rAT = 1.6605; rCG = 0.6144; rCT = 5.2285; Pinvar = 0.4158; Shape = 0.5844) using 18S rDNA sequence of *Anguillonema amolensis* n. sp. Posterior probability and bootstrap values exceeding 50% are given on appropriate clades in the form Bayesian posterior probability/maximum likelihood bootstrap value (BPP/BS). The new species is in bold font.

nonmonophyletic nature of most hexatylenchid taxa, this placement could neither confirm nor reject the placement of the genus *Anguillonema* under the subfamily Gymnotylenchinae.

In our partial LSU tree (Fig. 6), and similar to SSU tree, members of superfamilies Sphaerularioidea and Iotonchioidea have occupied separate clades. Some genera from different subfamilies (e.g. *Psyllotylenchus* Poinar and Nelson, 1973, *Spilotylenchus* Launay, Deunff and Bain, 1983, *Paurodontella* Husain and Khan, 1968 and *Rubzovinema*) formed the clade A. The new species has also formed a clade with a specimen of *Howardula* (DQ328728) from Allantonematidae and two species of *Parasitylenchus* (DQ328729, KM245038) from the family Parasitylenchidae.

In conclusion, the fragments studied in this work, 18S rDNA and LSU D2-D3, along with the partial 28S rDNA analysed by Koshel et al. (2014), do not infer congruent topologies with the currently available classic taxonomic frameworks for Hexatylina. Although some genomic or nongenomic fragments remain to be tested for their usefulness in resolving of phylogenetic relations among this group of nematodes, the uncommon nature of these nematodes and paucity of their sequences in databases such as GenBank further complicate phylogenetic studies of these nematodes.

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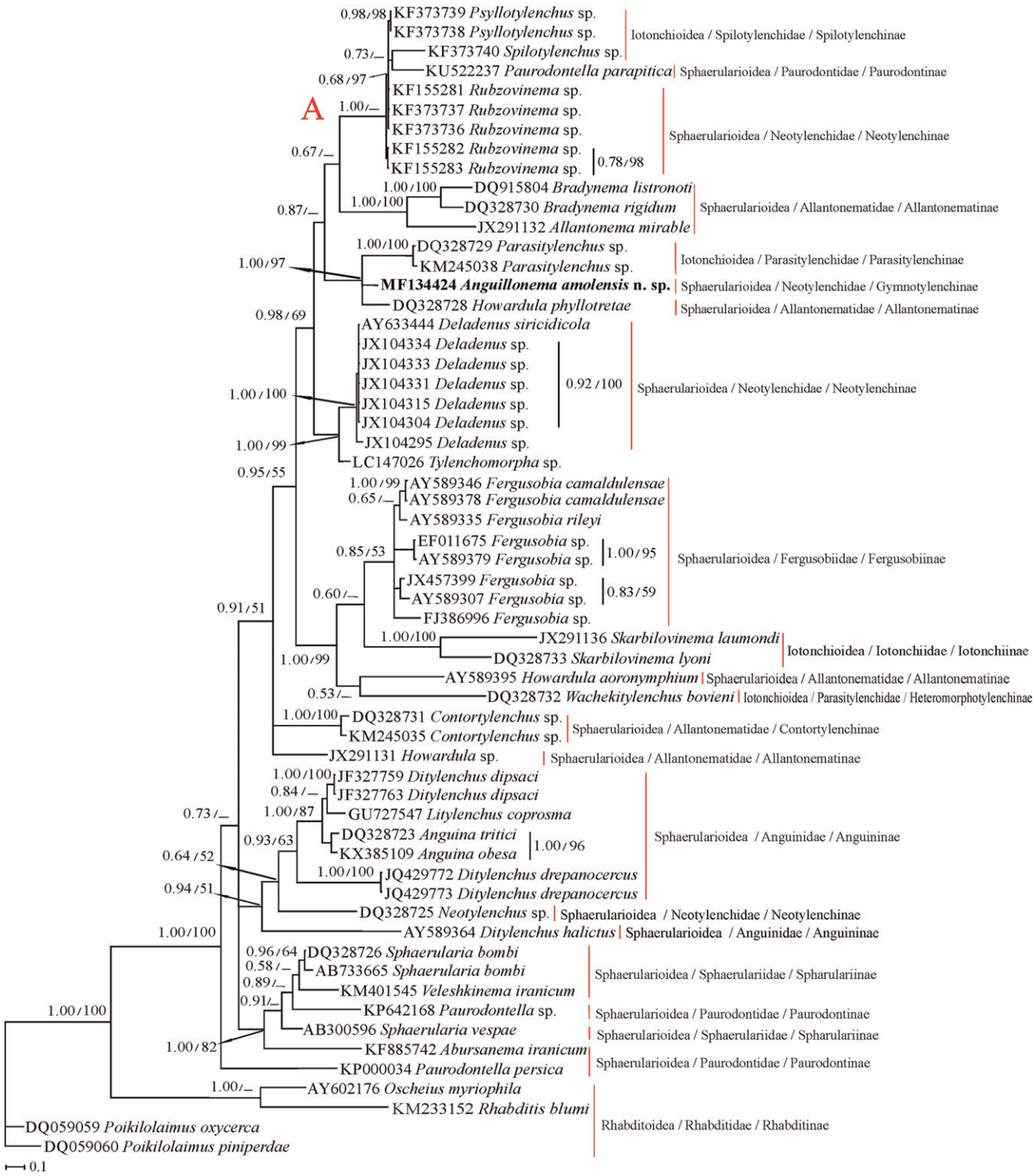

FIG. 6. Bayesian tree inferred under the GTR + G + I model (−lnL = 11238.0518; AIC = 22496.1035; freqA = 0.1766; freqC = 0.1757; freqG = 0.3425; freqT = 0.3052; rAC = 1.7104; rAG = 5.2788; rAT = 2.0787; rCG = 1.2116; rCT = 8.5988; Pinvar = 0.2490; Shape = 0.6558) using LSU D2-D3 sequence of *Anguillonema amolensis* n. sp. Posterior probability and bootstrap values exceeding 50% are given on appropriate clades in the form Bayesian posterior probability/maximum likelihood bootstrap value (BPP/BS). The new species is in bold font.

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