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Morphological and Molecular Characterization of *Labrys filiformis* n. sp. (Rhabditida: Tylenchidae) from Iran

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Abstract

Labrys filiformis n. sp., the second species of the rare genus *Labrys*, was recovered from natural forests of Gilan province and is described based upon morphological and molecular characters. The new species is characterized by its smooth cuticle under light microscopy, lateral field with two incisures forming a single plain band, lip region continuous with body contour, dorso-ventrally flattened and forming four poorly prominent lobes, having a dorso-ventrally narrower protuberant labial plate laterally extended to the amphidial margins, oral area (oral plate) dorso-ventrally elongated and embedded in the labial plate with six small labial sensilla surrounding the slightly prominent oral aperture, amphidial apertures as longitudinally lemniscatic slits bordered by the labial plate extensions which are overlapped at the middle length of amphids, stylet delicate, 6 to 7 µm long, elongate weakly developed fusiform median bulb with weak valve, wide excretory pore with long and heavily sclerotized duct, offset spermatheca filled with small spheroid sperm cells, 106 to 127 µm long elongate-conoid tail with filiform distal region and finely rounded tip. Molecular phylogenetic analyses were performed using a near-full length fragment of the 18S rDNA and the D2–D3 expansion segments of the 28S rDNA using Bayesian inference and maximum likelihood methods. In the inferred phylogenetic tree with 18S rDNA, the new species has a close affinity with several isolates of the type species, *Labrys chinensis*. The reconstructed phylogenetic tree using partial 28S rDNA, revealed the new species is nested inside the putative monophyletic group of several populations of *L. chinensis*.

Key words

18S rDNA, 28S rDNA D2–D3, beech forests, *Fagus orientalis*, new species, phylogeny, taxonomy.

The family Tylenchidae (Örley, 1880) is a cosmo politan group of nematodes in the suborder Tylenchina (Chitwood, 1950) and currently contains over 400 species (Siddiqi, 2000; Geraert, 2008). Members of the family have high ecological diversity and abundance, and are associated with algae, mosses, lichens, fungi, and plant roots. Some of the species are considered as weak parasites of plants (Siddiqi, 2000) and typically show low interspecific and high intraspecific morphological variability (Qing and Bert, 2018). Many species are poorly described because of an overreliance on morphological characters using light microscopy (LM) and a limited number of specimens which has led to taxonomic confusion (Qing and Bert, 2018). Recently, molecular data have been used to improve taxonomic studies of the family members (Palomares-Rius et al., 2009; Bert et al., 2010; Atighi et al., 2013; Panahandeh et al., 2014, 2015a, 2015b, 2016, 2018; Soleymanzadeh et al., 2016; Pereira

and Baldwin, 2016; Pereira et al., 2016; Qing et al., 2016, 2017; Pedram et al., 2018; Qing and Bert, 2017, 2018). Unfortunately, molecular data are often not available for all genera and species, and some genera are only rarely recovered after their original descriptions.

According to a comprehensive review of the family Tylenchidae by Geraert (2008), the family includes 42 valid genera. Recently, two other monotypic genera were added, i.e., *Discopersicus* (Yaghoubi et al., 2016) from Iran, and *Labrys* (Qing and Bert, 2018) from China with *L. chinensis* (Qing and Bert, 2018) as its type species (Qing and Bert, 2018). The latter genus is mainly characterized by having a unique offset labial plate, tapering toward both tips and detached from the adjacent cuticle in scanning electron microscopy (SEM) images, protruding lips under LM, laterally elongate amphidial apertures, two incisures in lateral field, delicate stylet, its shaft two times longer than the cone, elongate fusiform weakly developed median bulb having a distinct weakly sclerotized valvular apparatus, wide excretory pore with heavily sclerotized duct, a round spacious postvulval uterine sac (PUS) and spicules with sharp protrusion (Qing and Bert, 2018). Immediately following the description of *L. chinensis,* it was reported from Iran and confirmed to be identical following morphological and morphometric analyses (Konani et al., 2018).

During our extensive study of tylenchids in northern Iran (Panahandeh et al., 2014, 2015a, 2015b, 2016; Soleymanzadeh et al., 2016; Mobasseri et al., 2017; Konani et al., 2018; Pedram et al., 2018), one population of the genus *Labrys* representing the second species of the genus was recovered from a soil sample of the natural forests in Gilan province. It is characterized using molecular and morphological characters and described herein as *L. filiformis* n. sp.

Material and methods

Sampling, extraction and morphological study

The tray method (Whitehead and Hemming, 1965) was used to extract nematodes from several soil samples collected from undisturbed forests of northern Iran. The specimens were collected and concentrated using a 500 mesh sieve (equal to 25 μm openings). Nematodes of interest were hand-picked under a Nikon SMZ1000 stereomicroscope and were heat killed by adding boiling 4% formaldehyde solution, and processed to anhydrous glycerin according to De Grisse (1969) for preparation of permanent slide mounts.

Study and drawings of morphological and morphometric characters were done with a Nikon E600 light microscope equipped with a drawing tube. The hand-drawn sketches were redrawn in CorelDRAW® software version 17. Photographic images of specimens were taken with an Olympus DP72 digital camera attached to an Olympus BX51 microscope equipped with differential interference contrast.

Specimens preserved in glycerine were selected for observation under SEM according to Abolafia (2015). The nematodes were hydrated in distilled water, dehydrated in a graded ethanol-acetone series, critical point dried, coated with gold, and observed with a Zeiss Merlin microscope (5 kV) (Zeiss, Oberkochen, Germany).

DNA extraction, PCR and sequencing

For genomic DNA extraction, a single live nematode specimen was picked out and transferred to a small drop of TE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0, QIAGEN Inc., Valencia CA, USA) on a clean slide, studied mainly for its lip region structure, stylet, metacorpus and excretory duct nature, and squashed using a clean slide cover. The suspension was collected by adding 30 μl TE buffer. The DNA sample was stored at –20°C until used as PCR templates. To amplify the near-full length fragment of the 18S rDNA and D2-D3 domains of the 28S rDNA, three sets of primers were used in the PCR reactions. The near-full length fragment of the 18S rDNA was amplified with forward primer 1096 F (5′-GGTAATTCTGGAGCTAATAC-3′) and reverse primer 1912R (5′-TTTACGGTCAGAACTAGGG-3′), forward primer 1813F (5′-CTGCGTGAGAGGTGAAAT-3′) and reverse primer 1573R (5′-TACAAAGGGCAGGGACGTAAT-3′) (Holterman et al., 2006; Mullin et al., 2005). Primers for amplification of the D2-D3 domains of the 28S rDNA were forward primer D2A (5′-ACAAGTACCGTGAGGGAAAGT-3′) and reverse primer D3B (5′ TGCGAAGGAACCAGCTACTA-3′) (Nunn, 1992). The PCR amplification and sequencing were done as described by Panahandeh et al. (2016). The amplicon sizes were verified in 1.2% agarose gel and visualized by staining with DNA Green Viewer™ (0.05 μl/ml). The PCR products were sequenced in both directions using the same primers with an ABI 3730XL sequencer (Bioneer Corporation, South Korea). Newly obtained sequences of the studied species were deposited into the GenBank database under accession numbers: MG686086 for near full-length 18S, and MG686087 for partial 28S rDNA D2–D3.

Phylogenetic analyses

The recently obtained sequences were manually checked, edited, and assembled using CodonCode Aligner v. 6.0.2 (CodonCode Corporation, MA, USA, www.codoncode.com). Using the BLAST homology search program in the GenBank database, these sequences were compared with other relevant available sequences. Several representatives of the family Tylenchidae were selected for both dataset analyses. Multiple alignments of the selected DNA sequences with newly obtained sequences were conducted using MUSCLE (Edgar, 2004) in MEGA6 (Tamura et al., 2013). The ambiguously aligned parts and divergent regions were eliminated using the online version of Gblocks 0.91b (Castresana, 2000) with all three less stringent parameters (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). The best-fitting substitution model for both datasets was selected using PAUP*/MrModeltest.2 (Nylander, 2004). Bayesian analyses were carried out on MrBayes 3.1.2 (Rounquist and Huelsenbeck, 2003) under the Akaike-supported model, a general time reversible model, including among-site rate heterogeneity and estimates of invariant sites (GTR+G+I), for both genes, with five independent runs and 10^7 generations. The Markov chains were sampled every 100 generations for estimating the posterior probabilities of the phylogenetic trees (Larget and Simon, 1999) using the 50% majority rule. Twenty-five percent of the converged runs were regarded as burnin. The Tracer v1.5 software (Rambaut and Drummond, 2009) was used to visualize the results of each run in order to check the effective sample size of each parameter. The maximum likelihood (ML) analysis was performed with 10^3 bootstraps (BS) replicates for both datasets under the same nucleotide substitution model as in the Bayesian inference (BI) using RaxmlGUI 1.1 (Silvestro and Michalak, 2012). The output files of the phylogenetic programs were visualized using Dendroscope v.3.2.8 (Huson and Scornavacca, 2012). The Bayesian posterior probability (BPP) and ML BS values exceeding 0.60 and 50%, respectively, were plotted on Bayesian 50% majority-rule consensus trees after redrawing in CorelDRAW® software version 17.

Results

Labrys filiformis n. sp.*
(Figs. 1–4).
Measurements: See Table 1.

*The specific epithet refers to the filiform tail of the new species.

Female

Body slender and narrow, gradually narrowing toward the posterior end, straight to slightly ventrally curved after fixation. Cuticle slightly annulated, appearing smooth under LM. Lateral field prominent, having two lines forming a simple band with smooth margins in lateral view. Lip region dorso-ventrally flattened forming four poorly prominent lobes, continuous with the adjacent part of body, 4.5 to 5.0 µm wide and 2.5 to 3.0 µm high, with protuberant dorso-ventrally narrower labial plate, frontally flattened and laterally extended to the amphidial margins, thinner distally; oral area (oral plate) embedded in the labial plate, dorso-ventrally elongated, with six small labial sensilla surrounding the oral aperture, this slightly prominent; oral area (oral plate) laterally connected with the amphids through the labial plate by shallow depressions. Amphidial apertures as longitudinally lemniscatic slits on lateral sides of the lip region, bordered by the labial plate extensions which are overlapped at the middle length of amphids. Stylet delicate, slender, with conus ca 39% of the total length, and knobs rounded, small, slightly posteriorly directed. Dorsal pharyngeal gland orifice opening 1 to 2 µm posterior to the stylet knobs. Procorpus slender, median bulb (metacorpus) weakly developed, elongate fusiform with distinct and faint valvular apparatus, isthmus narrow, slender, pharyngeal bulb pyriform. Nerve ring surrounding anterior part of isthmus. Excretory pore wide with long and heavily sclerotized duct, situated at isthmus level, between nerve ring and anterior end of pharyngeal bulb. Reproductive system monodelphic-prodelphic, comprising of an outstretched ovary 13 to 16% of the body length, oocytes mostly arranged in single row, oviduct not well discernible, spermatheca rounded to oval, offset, functional, and filled with spheroid sperm cells, crustaformeria with unclear cell arrangement, vagina with thin wall, perpendicular to body axis and straight or slightly anteriorly sloping. PUS moderately developed, 0.4 to 0.7 times corresponding body diam. long. Vulva a small transverse slit, with very small lateral flap-like differentiation of the cuticle in both sides. Tail elongate-conoid, filiform, very gradually narrowing toward end, 1.7 to 2.5 times longer than vulva–anus distance with faintly rounded tip.

Male

Unknown.

Table 1. Morphometrics of *Labrys filiformis* n. sp. All measurements are in µm and in the form: mean ± standard deviation (range).

Character	Holotype	Paratype
n	female	8 females
L	427	440 ±17 (425–463)
a	38.8	41.6 ± 2.3 (38.7–46.2)
b	4.9	5.1 ± 0.2 (4.9–5.4)
c	3.9	3.8 ± 0.2 (3.5–4.0)
c′	13.8	14.6 ± 1.1 (13.2–15.9)
V	60.4	59.9 ± 1.21 (57.7–61.5)
V′	81.4	81.5 ± 1.3 (79.9–84.1)
Stylet length	6	6.2 ± 0.4 (6–7)
Median bulb valve – anterior end	44	43.7 ± 1.8 (40–46)
Excretory pore – anterior end	69	65.5 ± 2.6 (62–69)
Neck length (stoma + pharynx)	87	86.1 ± 2.3 (82–90)
Lip region to vulva	258	263 ± 8 (256–277)
Body width	11	10.6 ± 8.3 (10–11)
Anal body width	8	8.0 ± 0.5 (7–9)
Anterior genital branch length	63	62.5 ± 5.7 (56–72)
Postvulval uterine sac	6	6.4 ± 1.1 (5–8)
Vulva – anterior end	258	263 ± 8 (256–277)
Vulva to anus distance	59	60.1 ± 5.2 (49–66)
Tail length	110	116.6 ± 8.6 (106–127)

Type habitat and locality

The new species was recovered from a soil sample collected from the rhizosphere of a beech tree (*Fagus orientalis* L.) in a forest from the Salkisar region (south of Rasht), Gilan province, northern Iran, in December 2015. GPS coordinates: 37°07.307′N, 49°39.257′E.

Type material

Holotype female and three paratype females were deposited at Nematode Collection of the Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran. Two paratype females deposited at UGent Nematode Collection of the Nematology Research Unit, Department of Biology, Ghent University, Ghent, Belgium. Two paratype females deposited at the Nematode Collection of the Department of Animal Biology, Plant Biology and Ecology of the University of Jaén, Jaén, Spain.

Diagnosis and relationships

Labrys filiformis n. sp. is characterized by its body length (425 to 463 µm in females), cuticle slightly annulated, lateral field with two incisures, lip region with dorso-ventrally narrower labial plate laterally extended to the amphids margins, oral area (oral plate) dorso-ventrally elongated and embedded in the labial plate with six small labial sensilla surrounding the slightly prominent oral aperture, amphidial apertures as longitudinally lemniscatic slits bordered by the labial plate extensions which are overlapped at the middle length of amphids, stylet 6 to 7 µm long, elongate fusiform weakly developed median bulb with a weak valve, wide excretory pore at isthmus level with long and heavily sclerotized duct, 106 to 127 µm long elongate-conoid filiform tail with faintly rounded tip.

The genus *Labrys* currently has only one nominal species, *L. chinensis* the type species, which was discussed earlier. The new species can be separated

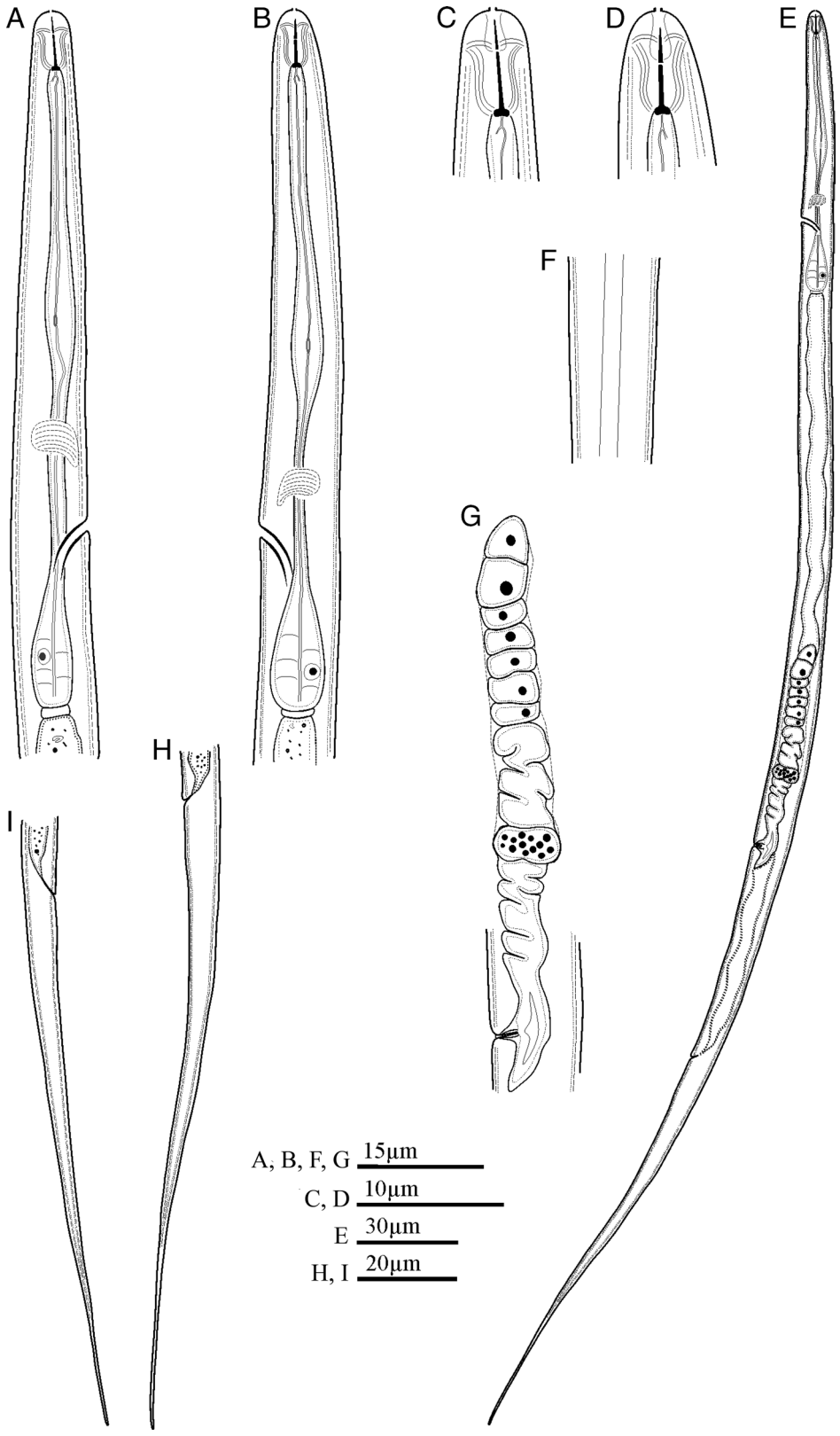

Figure 1: *Labrys filiformis* n. sp. (female). A, B: Neck (stoma + pharynx); C, D: Anterior end, E: Entire body; F: Lateral field at mid-body; G: Reproductive system; H, I: Tail.

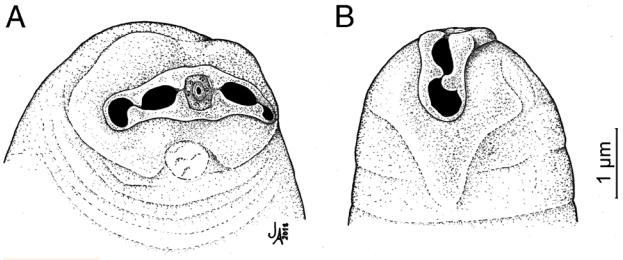

Figure 2: Interpretative drawing of lip region of *Labrys filiformis* n. sp. based on scanning electron microscopy (SEM) observations in frontal (A) and lateral (B) view.

from *L. chinensis*, by its smaller lateral vulval flaps, shorter body (425-463 vs 580-637 µm), lower *b* ratio (4.9-5.4 vs 5.4-7.3), greater *V'* ratio (80-84 vs 75-78), shorter stylet (6-7 vs 9-10 µm), greater tail length/vulva to anus distance ratio (1.7-2.5 vs 1.3-1.7), shorter PUS (5-8 vs 13-17 µm), shorter tail (106-127 vs 132-171 µm) and difference in tail terminus morphology (faintly rounded vs broadly rounded).

Molecular characterization

To determinate the phylogenetic affinity of the new species with the type species of the genus and other genera/species of the family Tylenchidae, the near-full length sequence of the 18S rDNA and the D2–D3 expansion segments of the 28S rDNA were used in BI and ML analyses.

For reconstructing the 18S rDNA phylogenetic trees (Bayesian and ML trees), a total of 64 species/

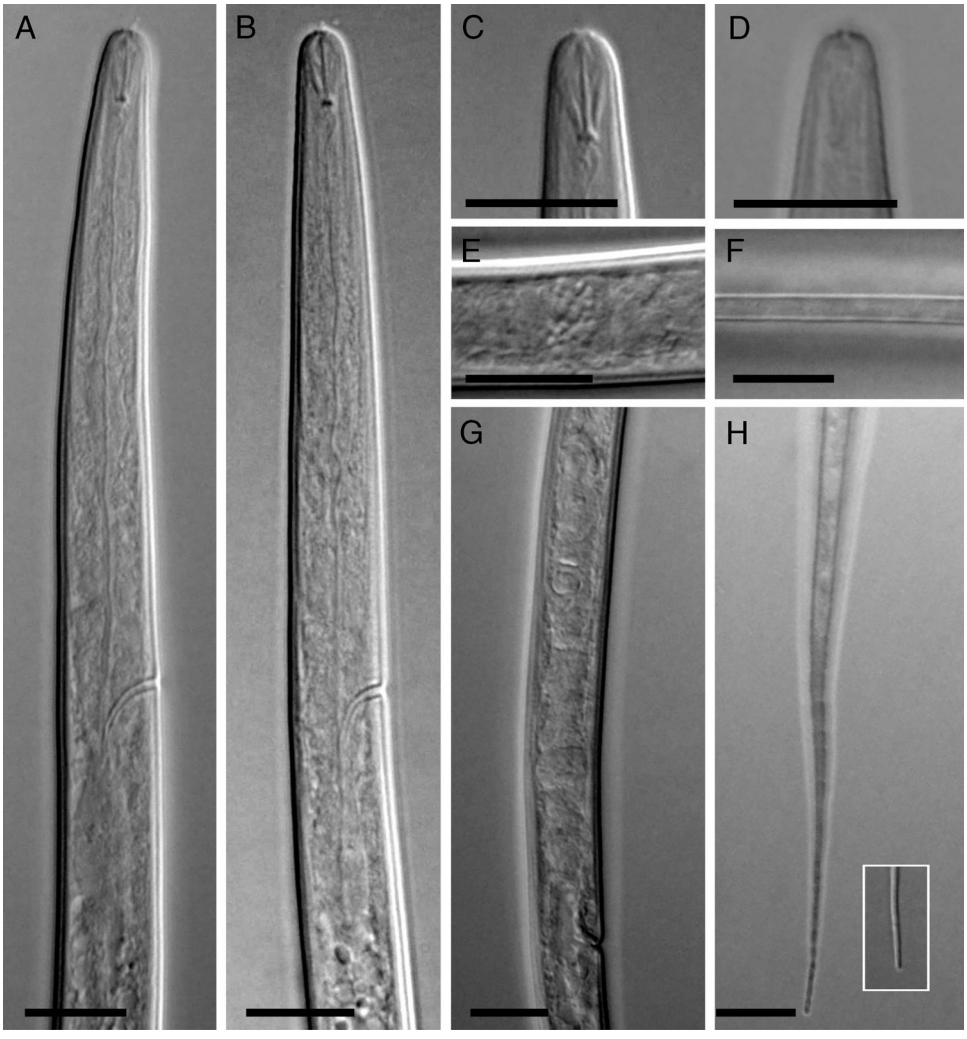

Figure 3: *Labrys filiformis* n. sp. (light microscopy, female). A, B: Pharyngeal region; C: Anterior end; D: Amphidial aperture E: Spermatheca filled with sperm; F: Lateral field at mid-body; G: Reproductive system; H: Tail tip. All scale bars =10 µm.

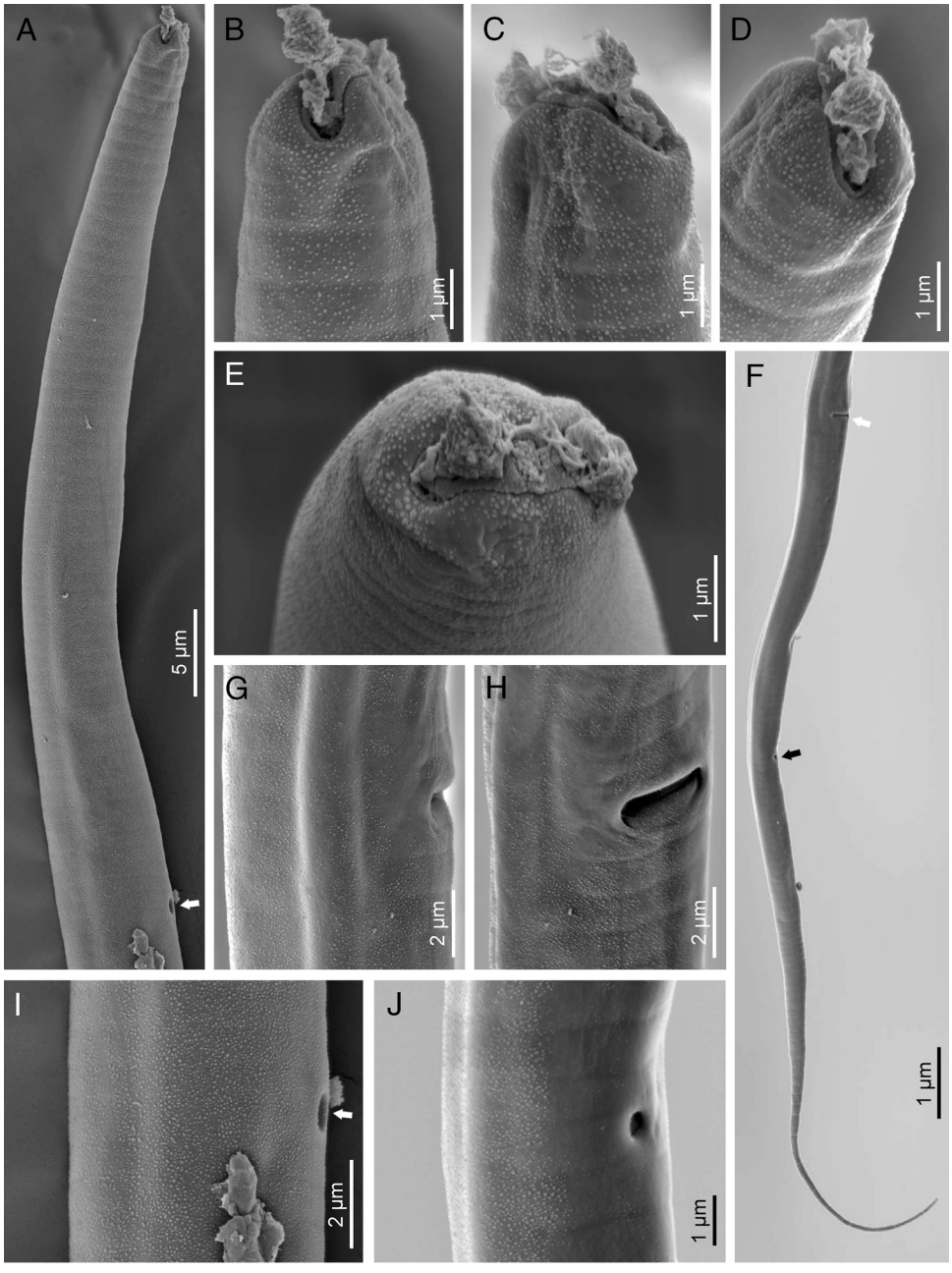

Figure 4: *Labrys filiformis* n. sp. (scanning electron microscopy [SEM], female). A: Anterior region (arrow at the excretory pore); B–E: Lip region in right lateral, subdorsal, sublateral and frontal views, respectively; F: Posterior region (white arrow at the vulva, black arrow at the anus); G, H: Vulva in lateral and subventral views, respectively; I: Excretory pore (arrow); J: Anus.

isolates, including the newly generated sequence of the new species, an aphelenchid and two aphelenchoidid outgroups (species and accession numbers in Fig. 5) were selected. The 18S dataset was composed of 1,574 characters with 763 variable characters. The average nucleotide composition was 25.1% A, 22.2% C, 28.2% G, and 24.5% T. Figure 5 represents the Bayesian phylogenetic tree inferred using the above-mentioned dataset. In this tree, the new species and four isolates of *L. chinensis* (KY776630, KY776631, KY776632, and KY776633) formed a strongly supported clade in BI (0.98 BPP). The putative clade of *Labrys* spp. is in poor putative sister relationship with *Filenchus misellus* (Andrássy, 1958) (Raski and

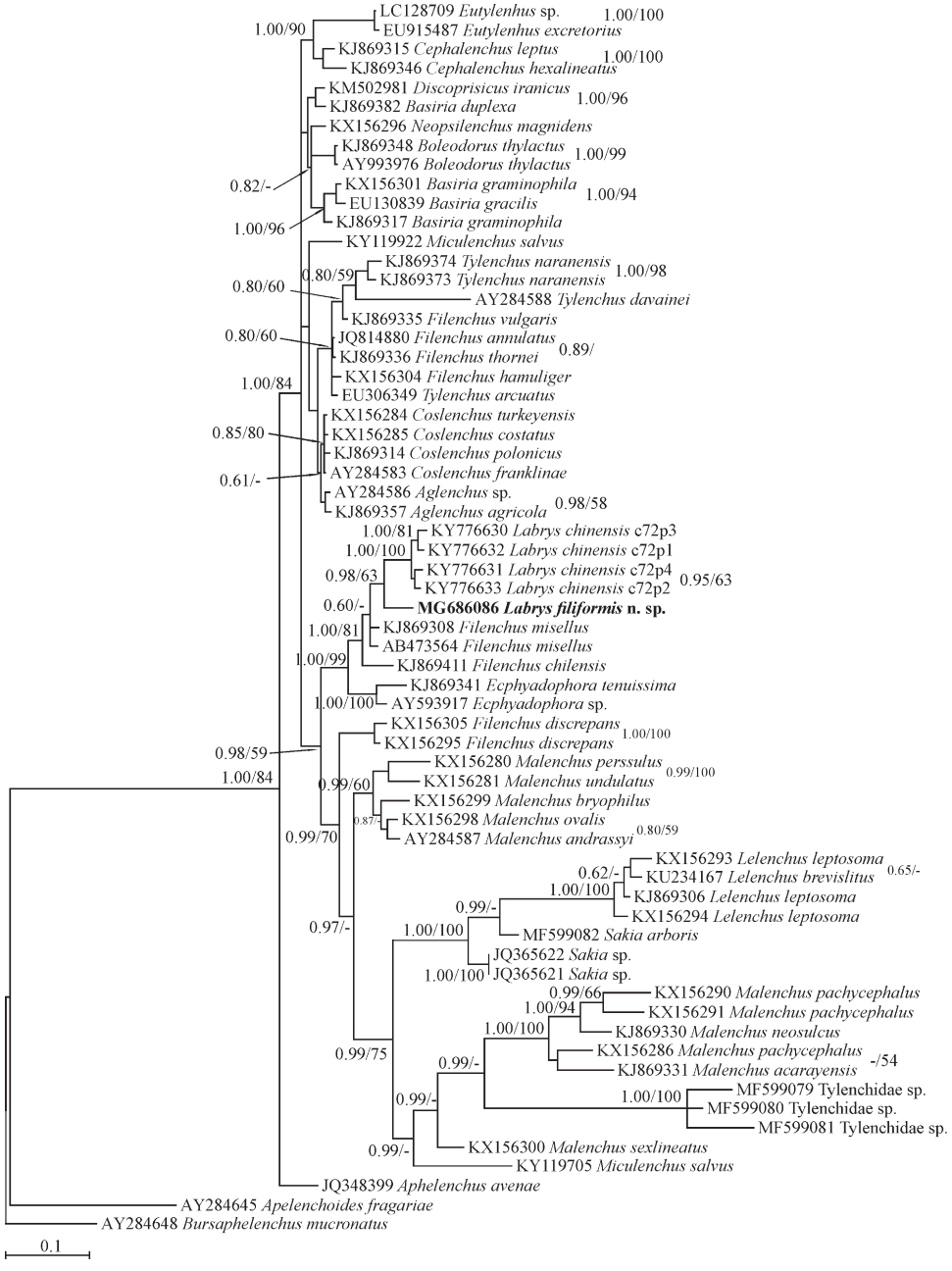

Figure 5: Bayesian 50% majority rule consensus tree inferred from 64 sequences of 18S rDNA (GenBank accession number and species name) under the GTR+G+I model. Bayesian posterior probabilities and maximum likelihood bootstrap values are given for appropriate clades in the form of BPP/ML BS. The new species is in bold.

Geraert, 1987) (KJ869308, AB473564). The major clade, including *Labrys* spp., *Filenchus misellus*, *F. chilensis* (Raski and Geraert, 1987) (KJ869411) and two species of the genus *Ecphyadophora* (de Man, 1921): *E. tenuissima* (de Man, 1921) (KJ869341) and *Ecphadophora* sp. (AY593917) received maximal BPP and high ML BS values (1.00/99).

A total of 66 species/isolates (including newly generated sequence of the new species and two aphelenchid and aphelenchoidid outgroups) were selected for reconstructing the 28S rDNA D2–D3 phylogenetic Bayesian and ML trees (species and accession numbers in Fig. 6). They yielded an alignment with 553 total characters having 404 varia-

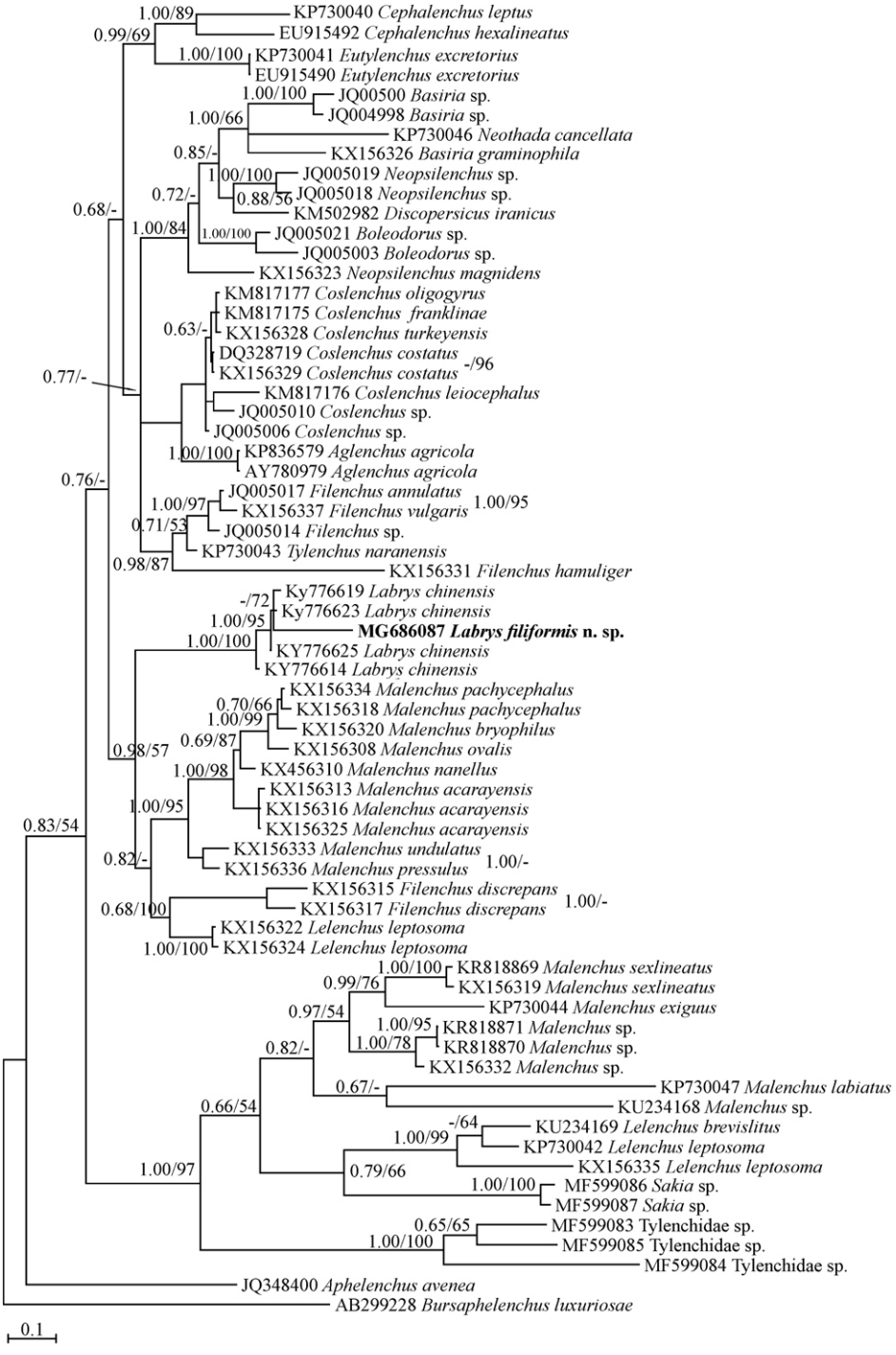

Figure 6: Bayesian 50% majority rule consensus tree inferred from 66 sequences of 28S rDNA D2-D3 (GenBank accession number and species name) under the GTR+G+I model. Bayesian posterior probabilities and maximum likelihood bootstrap values are given for appropriate clades in the form of BPP/ML BS. The new species is in bold.

ble characters. The average nucleotide composition was as follows: A: 21.1%, T: 21.8%, C: 22.6% and G: 34.5%. Figure 6 represents the Bayesian phylogenetic tree inferred using this dataset. Based upon the resolved topology, the new species was embedded between four isolates of *L. chinensis* representing its

four populations, in a putative clade which was maximally supported (1.00 BPP, 100 BS). The *Labrys* clade is in putative sister relationship with the clade including several species/isolates of *Malenchus* (Andrássy, 1968) (KX156318, KX156320, KX156308, KX456310, KX156313, KX156316, KX156325, KX156333, KX156334, KX156336), two isolates of *Filenchus discrepans* (Andrássy, 1954) (Andrássy, 1972) (KX156315, KX156317) and two isolates of *Lelenchus leptosoma* (de Man, 1880) (Andrássy, 1954) (KX156322, KX156324).

Discussion

The lip pattern of the members of *Labrys* is the main generic diagnostic character separating it from other genera of the Tylenchidae. According to the comprehensive study of morphology of the family Tylenchidae by Geraert and Raski (1987), seven lip patterns were distinguished. Recently, the eighth unique pattern was recognized in *Labrys* (Qing and Bert, 2018). Based upon SEM images from the lip region of the genus provided for the type species (Qing and Bert, 2018; Konani et al., 2018), it is dorso-ventrally flattened forming four poorly prominent lobes, the labial plate is offset, narrowing toward both corners, and protuberant from the adjacent cuticle and appearing as a disc-like structure in lateral view with LM (Fig. 4a–c in Qing and Bert (2018), Figure 5C in Konani et al. (2018), and Fig. 3C in present study). The amphidial apertures are also typically longitudinally lemniscatic slits bordered by lateral extensions of the labial plate which protrude into and overlap at the middle length of the amphids (Fig. 6a,b in Qing and Bert (2018), Fig. 6A,B,C,E in Konani et al. (2018) and Fig. 4B–E in the present study). This was not represented accurately in the 3-D illustrations provided by Qing and Bert (2018, Figs. 7a,b,i,j).

Large amphidial pouches, such as those recently discussed by Soleymanzadeh et al. (2016) and Panahandeh et al. (2018) as a character separating *Sakia* (Khan, 1964) and *Lelenchus* (Andrássy, 1954), were not observed in the genus *Labrys* under LM. The weakly valved and elongate fusiform metacorpus which is not clearly demarcated from the procorpus and isthmus is another generic character (Qing and Bert, 2018) observed for the second population of *L. chinensis* recently recovered from Iran too (Konani et al., 2018). This character is well conserved for the new species as well. The wide and sclerotized duct of the excretory pore is also an important conserved trait among the three aforementioned populations, although its position shows variation between the type species and the Iranian population of the type species (Konani et al., 2018). Qing and Bert (2018) designated a spacious PUS as a generic character in the genus *Labrys*. Thus, the smaller PUS observed in *L. filiformis* n. sp. updates the characters delimiting the genus. In contrast, the PUS could have extensive intraspecific (e.g., in the case of the genus *Filenchus* (Andrássy, 1954)) and interspecific variation in the family. From the molecular phylogenetic vantage point, the genus *Labrys*, with its two known species, is monophyletic using both 18S and 28S rDNA D2-D3 markers, and other future tentative species should help corroborate its status.

In the present study, we found two *Ottolenchus* (Husain and Khan, 1967) species that closely resemble *Labrys*. Siddiqi (2000) and Siddiqi and Lal (1992) considered all species of *Filenchus* that possessed two incisures in the lateral field and sinuate-shaped amphidial apertures as members of the genus *Ottolenchus*, whereas Raski and Geraert (1986) and Geraert (2008) synonymized *Ottolenchus* with *Filenchus*. In a recent study by Qing and Bert (2017), *Ottolenchus* was considered a valid genus. We agree with this opinion. Nevertheless, two species of *Ottolenchus*, namely *O. crassistylus* (Siddiqi and Lal, 1992), and *O. porosus* (Siddiqi and Lal, 1992) have similar morphology to *Labrys* as below: both of the aforementioned species have a disk-like structure on the cephalic region. This character was described in the case of the first species as “cephalic region smooth, broadly rounded with 4, small papilla-like elevations around oral opening”. This is also drawn in Figure 3B in Siddiqi and Lal (1992). In the case of the second species, the disc-like structure is drawn in Figure 3I (Siddiqi and Lal, 1992). More importantly, both species have an elongate fusiform metacorpus, but without a distinct valvular apparatus. Surprisingly, the excretory duct in both species is well sclerotized. However, the two species of *Labrys* (*L. chinensis* and *L. filiformis* n. sp.) differ from those two abovementioned species of *Ottolenchus* by having an elongate broader amphidial aperture and smooth cuticular appearance under LM (*vs* coarsely annulated). Thus, SEM studies of the lip regions as well as molecular phylogenetic analyses of these two species of *Ottolenchus* should yield critical results about their taxonomic placement, and the potential validity of *Labrys* as a genus.

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