

***Filenchus spirocaudatus* sp. n. (Tylenchomorpha: Tylenchidae) from the rhizosphere of wheat in Isfahan province, Iran: morphological and molecular characterisation**

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Summary. A new species of the genus *Filenchus*, *F. spirocaudatus* sp. n., recovered from a wheat (*Triticum aestivum*) field in Isfahan province, Iran, is described and illustrated based on light microphotographs and scanning electron microscopy, as well as molecular data (partial small subunit ribosomal DNA, SSU rDNA). The new species is mainly characterised by its terminal tail annulus, which is irregularly twisted in all examined specimens and appears star-like under light microscopy. Females (387–453 µm long) possess an annulated lip region, amphidial openings short longitudinal lateral slits, small lateral vulval flaps, and an elongate-conoid tail. Morphological and morphometric differences from three closely similar species, viz. *F. australis*, *F. microdorus* and *F. misellus*, are discussed. *Filenchus misellus* is considered a cryptic form relative to the new species, but *F. spirocaudatus* sp. n. is distinguished from it and the other congeners primarily by tail tip morphology and molecular evidence. Phylogenetic relationships with other relevant genera and species were inferred from a partial SSU rDNA sequence. In the resulting tree, the SSU sequence of *F. spirocaudatus* sp. n. formed a clade with three sequences assigned to *F. misellus*.

Key words: cryptic species, morphometrics, phylogeny, sequence, SSU rDNA, *Triticum aestivum*.

The family Tylenchidae Örley, 1880 represents an abundant and diverse group of early diverging nematodes (Qing & Bert, 2019). Ecologically, they are one of the main parts of soil fauna, which may constitute up to 30% of the nematodes in a given soil sample (Yeates & Bird, 1994; Ferris & Bongers, 2006). The subfamily Tylenchinae Örley, 1880 currently includes 12 genera (Siddiqi, 2000; Qing & Bert, 2018). The genus *Filenchus* Andrassy, 1954 (Tylenchinae) contains over 90 species and constitutes one of the species-rich tylenchomorph genera. Among these, some are fungal feeders and, in general, they do not affect plants (Okada *et al.*, 2002, 2005; Geraert, 2008; Bert *et al.*, 2010; Mortazavi *et al.*, 2021; Monemi *et al.*, 2023). Most species within the genus have been described using traditional

approaches. So far, approximately 30 species of the genus *Filenchus* have been reported from Iran, of which only a small number have been studied at the molecular level (Karegar, 2018; Hosseinvand, 2020). Recently, two species, *Filenchus multistriatus* Monemi, Atighi, Abolafia, Castillo & Pedram, 2023 and *F. pseudodiscus* Mortazavi, Heydari, Abolafia, Castillo & Pedram 2021 have been described from Iran (Monemi *et al.*, 2023; Mortazavi *et al.*, 2021).

Bickford *et al.* (2007) defined ‘cryptic species’ as two or more distinct species that are erroneously classified (and hidden) under a single species name. Cryptic species represent an important component of biodiversity, such speciation being common among plant-parasitic nematodes and occurs in diverse groups with different life styles, including the spiral,

virus vector, root-lesion, false root-knot nematodes, *etc.* (Palomares-Rius *et al.*, 2014). There are examples of cryptic forms in Tylenchidae. For example, *Lelenchus brevislitis* Soleymanzadeh, Pedram, Pourjam & Álvarez-Ortega, 2016 and *L. leptosoma* (de Man, 1880) Andrassy, 1954 look morphologically and morphometrically indistinguishable under a light microscope, but scanning electron microscopy revealed remarkable differences in their major morphological characters of the anterior body end. These differences were confirmed by molecular analysis (Soleymanzadeh *et al.*, 2016). In addition, the use of molecular markers for species identification of pin nematodes has indicated over the last few years that many widespread species actually comprise multiple genetically divergent and morphologically similar cryptic species (Van den Berg *et al.*, 2014; Munawar *et al.*, 2020, 2021; Clavero-Camacho *et al.*, 2021; Singh *et al.*, 2021). *Paratylenchus variabilis* Raski, 1975 is morphologically and morphometrically almost indistinguishable from *P. zurgenerus* Clavero-Camacho, Cantalapiedra-Navarrete, Archidona-Yuste, Castillo & Palomares-Rius, 2021 (Clavero-Camacho *et al.*, 2021), from which it can be separated using molecular markers, and both are cryptic species. The other examples relate to different groups of soil-dwelling and plant and insect-related taxa (Cantalapiedra-Navarrete *et al.*, 2013; Cai *et al.*, 2020; Kanzaki *et al.*, 2020).

During a faunistic study on nematodes associated with agricultural fields in Iran, a Tylenchidae population having characteristics of *Filenchus* was found in a wheat field in Isfahan province. The morphological studies, combined with molecular data, showed that it belongs to an unknown species. Hence, the present study aims to describe the new species using an integrative approach.

MATERIALS AND METHODS

Soil sampling and nematode extraction. Total of 180 soil samples were collected from different gardens, fields and natural regions of the Isfahan province, Iran, in May 2021. The nematodes were extracted from soil using the tray method (Whitehead & Hemming, 1965) and specimens of interest were handpicked under a Nikon SMZ1000 dissecting microscope.

Morphological characterisation. The specimens were heat-killed by adding hot 4% formaldehyde solution, transferred to anhydrous glycerin according to De Grisse (1969) and mounted on permanent slides. Measurements and drawings were performed using a drawing tube attached to a Nikon E600 light

microscope (LM) and were digitally drawn using CorelDraw software version 2020. Light microphotographs were taken with Olympus BX51 microscope (Olympus, Tokyo, Japan) powered with differential interference contrast (DIC) equipped with a digital DP72 camera (Olympus) and DIC optics. Morphometric indices and ratios were measured and calculated according to Siddiqi (2000).

Scanning electron microscopy (SEM). Three females of the new species preserved in glycerin were selected for SEM observations. 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 scanning electron microscope (5 kV) (Zeiss, Oberkochen, Germany) (Abolafia, 2015).

Molecular methods and phylogenetic study. DNA was extracted from three individual female specimens of the new species separately. Each specimen was squashed in 15 µl Tris EDTA buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) after examination on a temporary slide. The DNA samples were stored at -20°C until used as PCR templates. The SSU rDNA was amplified using forward primer 1096F (5' - GGT AAT TCT GGA GCT AAT AC - 3') and reverse primer 1912R (5' - TTT ACG GTC AGA ACT AGG G - 3'), and forward primer 1813F (5' - CTG CGT GAG AGG TGA AAT - 3') and reverse primer 2646R (5' - GCT ACC TTG TTA CGA CTT TT - 3') (Holterman *et al.*, 2006). Several commonly used primer pairs (Nunn, 1992; Nadler & Hudspeth, 1998; Kiontke *et al.*, 2004) were tested for amplification of the D2-D3 expansion segments of the large subunit (LSU) rDNA, but all attempts failed to produce amplicons. The 30 µl PCR mixture contained: 15 µl *Taq* DNA Polymerase 2X Master Mix RED, 2 mM MgCl₂ (Ampliqon-Denmark), 8 µl distilled water, 1 µl of each primer (10 pmol µl⁻¹), and 5 µl of DNA template. The thermocycling program for PCR was as follows: denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 40 s, and extension at 72°C for 80 s. A final extension was performed at 72°C for 10 min. PCR amplicons were sequenced on an ABI 3730XL sequencer (Bioneer Corporation, South Korea) using the same primers employed for the PCR reactions. The newly generated SSU sequence was deposited into the GenBank database under the accession number PQ135939 for the SSU rDNA.

Phylogenetic analyses. The quality of the newly obtained sequences was manually checked using Chromas software (<http://technelysium.com.au/wp/chromas>) and edited. The newly obtained SSU sequence was compared with other available

sequences already deposited into the GenBank database using the basic local alignment search tool (BLAST) program. Several relevant sequences of representatives of the family Tylenchidae were selected for SSU phylogeny. Sequences of representatives of Aphelenchoidea Fuchs, 1937, including *Aphelenchoides fragariae* (Ritzema Bos, 1890) Christie, 1932 (AY284645), and *Bursaphelenchus mucronatus* Mamiya & Enda, 1979 (AY284648), were used as outgroups. The SSU dataset was aligned using Clustal X2.1 (<https://clustalx.software.informer.com/2.1/>) and the resulting alignment was manually edited using MEGA6 (Tamura *et al.*, 2013). The best-fitting substitution model was selected using PAUP*/MrModeltest2 (Nylander, 2004). The Akaike-supported model, a general time reversible model, including among site rate heterogeneity and estimates of invariant sites (GTR + G + I) was selected and applied in the SSU phylogenetic analysis. Bayesian analysis was performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) with a random starting tree and running the chains for 5×10^6 generations. After discarding burn-in samples, the remaining samples were retained for further analyses. The Markov chain Monte Carlo (MCMC) method within a Bayesian framework was used to estimate the posterior probabilities of the phylogenetic tree (Larget & Simon, 1999) using the 50% majority rule. The convergence of model parameters and topology were assessed based on average standard deviation of split frequencies and potential scale reduction factor values. Adequacy of the posterior sample size was evaluated using autocorrelation statistics as implemented in Tracer v.1.6 (Rambaut & Drummond, 2009). The output file of the tree was visualised using Dendroscope v3.2.8 (Huson & Scornavacca, 2012) and the tree was digitally drawn in CorelDRAW software version 2020.

RESULTS

Filenchus spirocaudatus sp. n. (Figs 1-3, Table 1)

Female. Body slightly ventrally curved to C-shape after fixation. Cuticle with faint annuli. Lateral fields with three bands, formed by four longitudinal incisures, ending at anterior part of the tail. Lip region scarcely narrower than the adjacent body, almost continuous with the body, composed of six poorly differentiated lips, the lateral ones smaller and divided into four quadrangular annuli with indentation on each side. The amphidial apertures short longitudinal lateral slits, arranged diagonally.

Oral opening very small, rounded. Stoma with stylet (stomatostylet) weak and fine, the conus about one-third of the total stylet length, shaft thin, and knobs small, rounded. Dorsal gland orifice (DGO) slightly posterior to the stylet base. Pharynx tylenchoid, with slender procorpus, joining an oval metacorpus with valvular apparatus, isthmus similar in morphology to the procorpus, and basal bulb pyriform. Cardia small, conoid. Nerve ring surrounds the isthmus at its posterior half. Excretory system with distinct duct and excretory pore located at the level of the anterior part of the pharyngeal bulb. Hemizonid barely distinct, located at the level of the nerve ring. Deirids located slightly behind the excretory pore. Intestine simple, rectum very thin, shorter than the body width, and anus functional, pore-like. Reproductive system monodelphic-prodelphic, composed of an outstretched ovary with the oocytes in a single row, tubular oviduct, rounded to slightly oval offset spermatheca filled with ellipsoid sperm in most individuals, crustaformia apparently quadricolumellate, uterus with visible lumen and thin walls, vagina with thin walls, vulval slit transverse with small lateral flaps, and post-vulval uterine sac (PUS) short, 0.6-0.8 times vulval body width diameter. Tail elongate-conical with tip showing the last annulus irregularly twisted. Phasmids not observed.

Male. Not found.

Type habitat and locality. The new species was recovered from a soil sample collected from a wheat field (*Triticum aestivum*) in Daran, Isfahan province, Iran, collected on May 2021. GPS coordinates: 33°0.1910'N and 50°26.5780'E.

Type material. Holotype female and ten paratype females of the new species were deposited in the WaNeCo collection, Wageningen, the Netherlands (<http://www.waneco.eu/>). Eight paratype females were deposited in the Nematology Collection at the Faculty of Agriculture, Tarbiat Modares University. The LSID code of this publication is: urn:lsid:zoobank.org:act:87BB717D-5C5E-44B2-A05F-43AF402A135B

Etymology. The specific epithet refers to the twisted shape of the last annulus of tail tip.

Diagnosis and relationships. The new species is mainly characterised by the irregularly twisted last annulus of the tail, visible in SEM images, appearing as a star-like differentiation in LM. It is further characterised by having continuous lip region with four annuli in SEM, short lateral amphidial slits, stylet delicate with finely rounded knobs and small lateral vulval flaps. Based on similarities in cuticle annulation, stylet morphology, tail shape and vulval position, the new species was compared to three closely related species: *F. australis* Xie & Feng, 1996

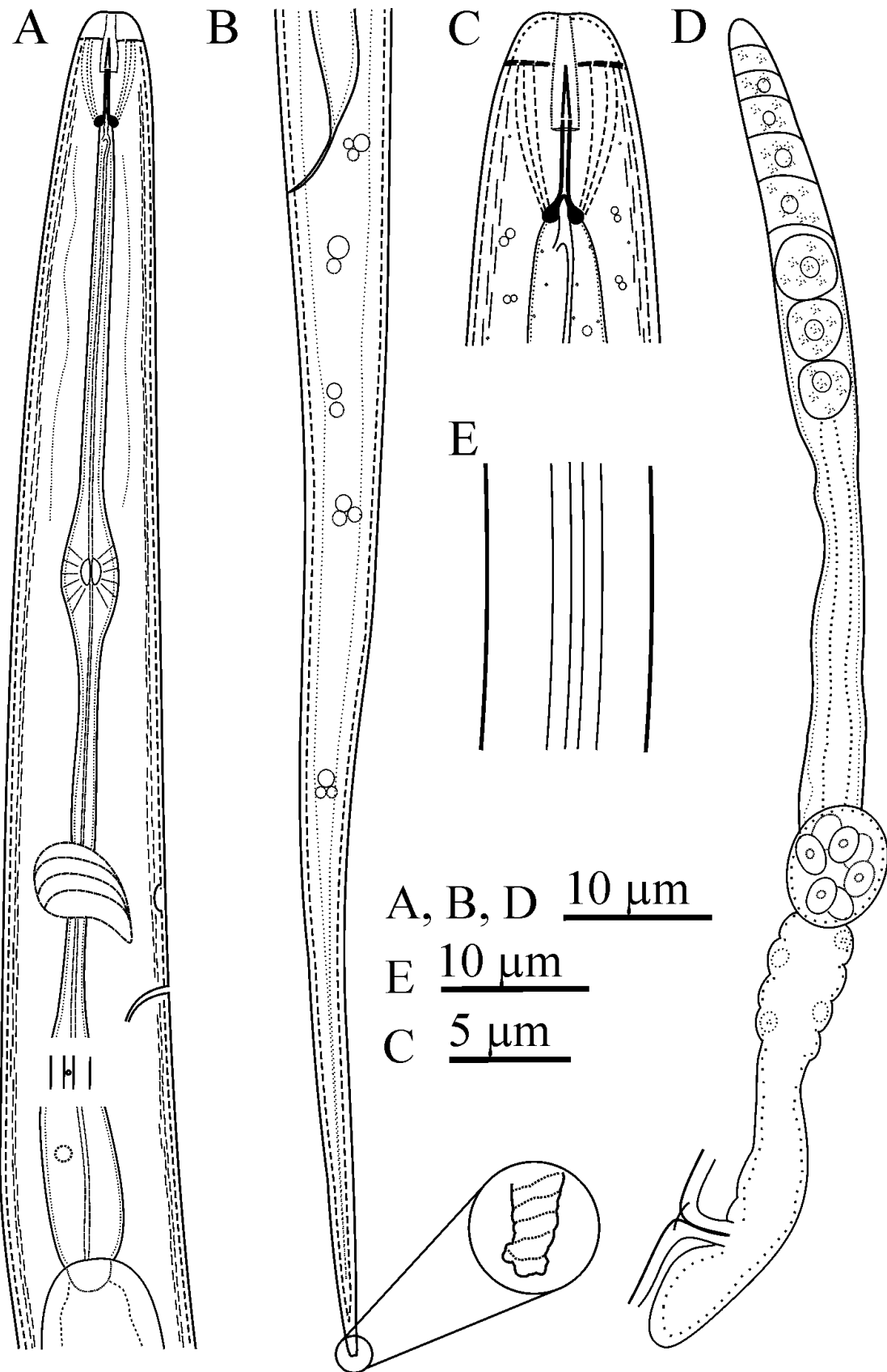


Fig. 1. Line drawings of *Filenchus spirocaudatus* sp. n. (female). A: Neck region (stoma and pharynx); B: Tail; C: Anterior body end; D: Reproductive system; E: Lateral field.

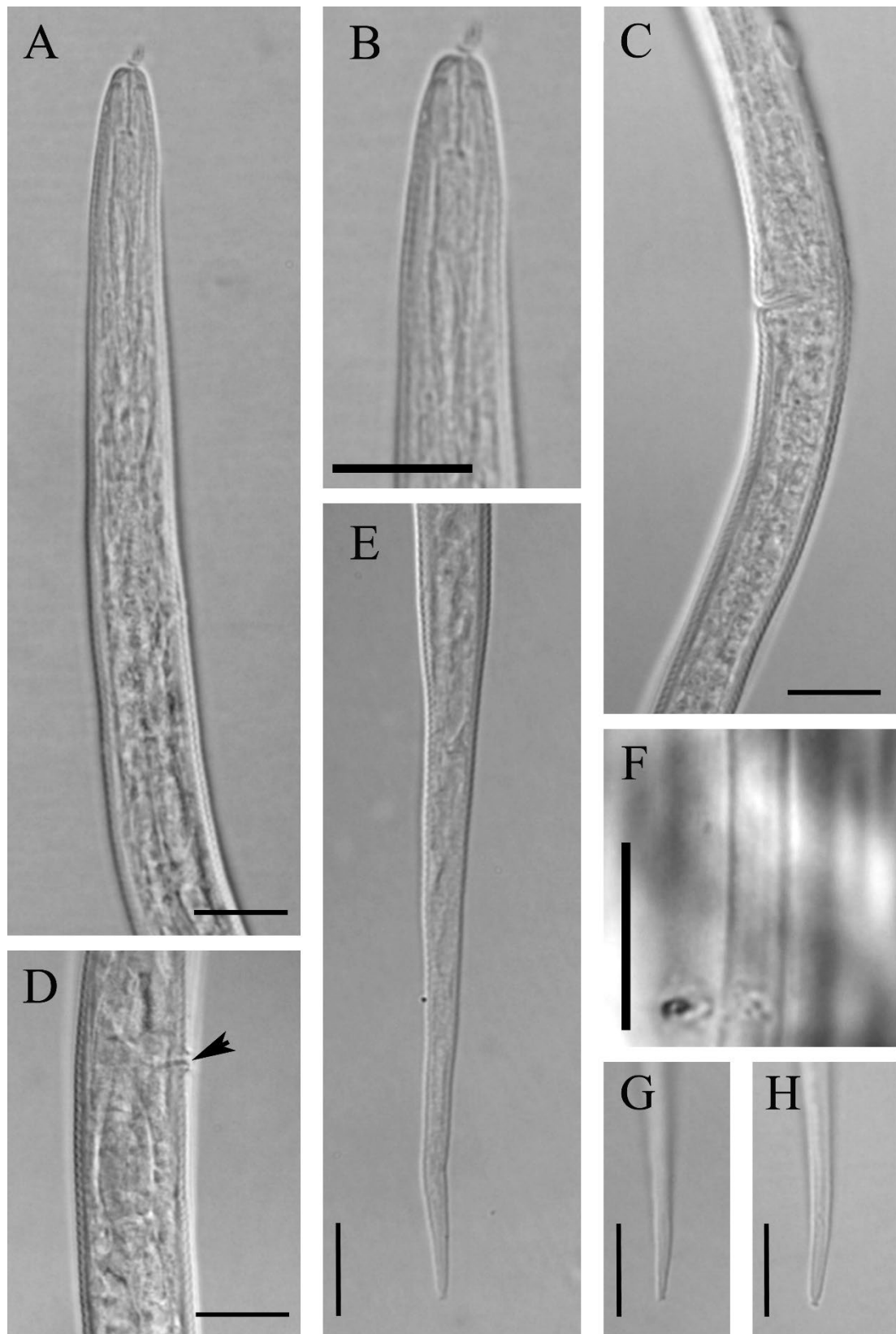


Fig. 2. Light microphotographs of *Filenchus spirocaudatus* sp. n. (female). A: Neck region (stoma and pharynx); B: Anterior body region; C: Vulva in lateral view; D: Basal bulb and excretory pore (arrow); E: Tail; F: Lateral field; G, H: Tail tip. All scale bars = 10 μ m.

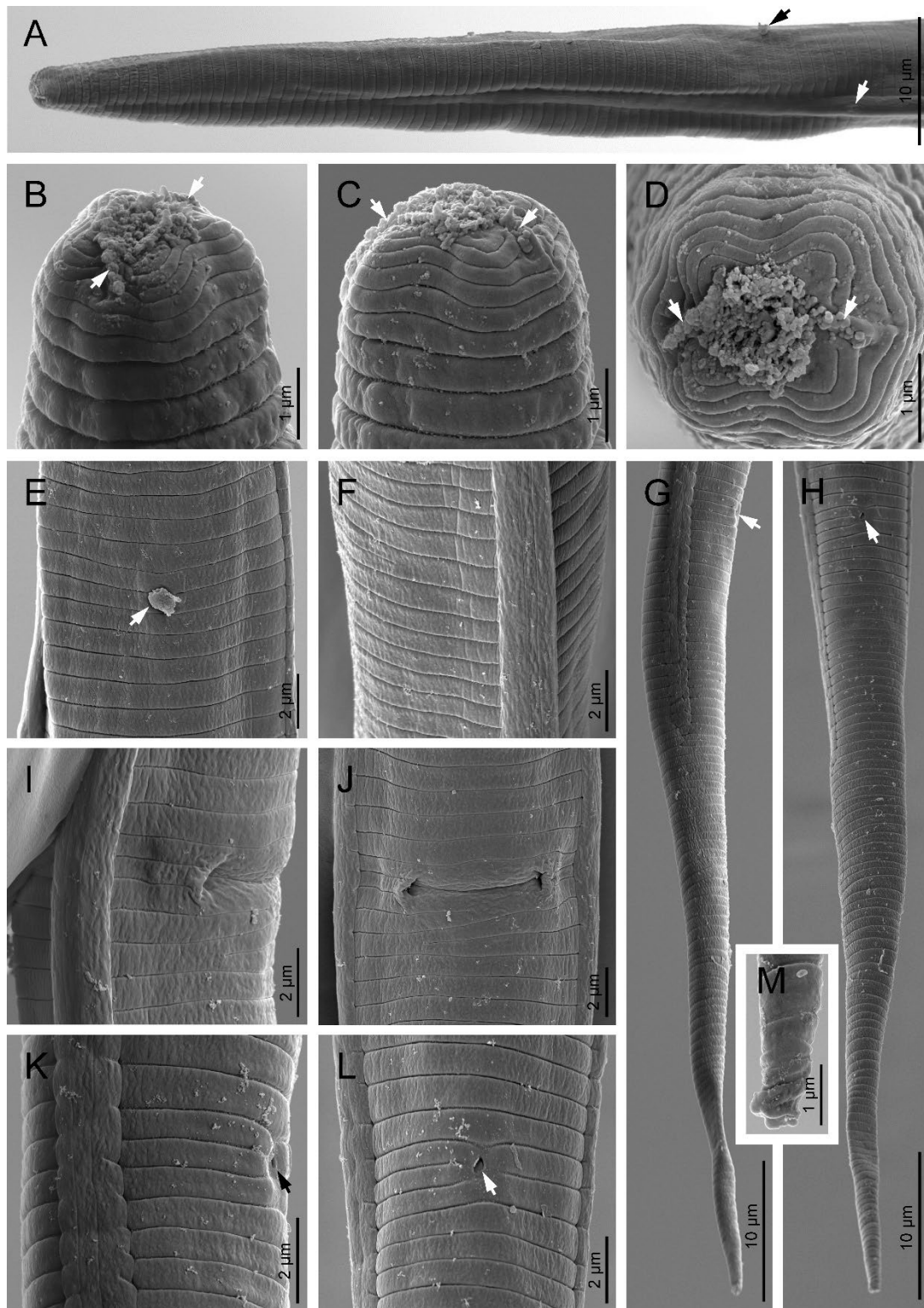


Fig. 3. Scanning electron microphotographs of *Filenchus spirocaudatus* sp. n. (female). A: Anterior body region (black arrow pointing to the excretory pore, white arrow pointing to the deirid); B-D: Lip region in lateral, ventral and frontal views, respectively (arrows pointing to the amphidial apertures); E: Excretory pore (arrow); F: Lateral field; G, H: Posterior body region in lateral and ventral views, respectively (arrows pointing to the anus); I, J: Vulva in lateral and ventral views, respectively; K, L: Anus (arrow) in lateral and ventral views, respectively; M: Tail tip showing the twisted last annulus.

Table 1. Morphometrics of *Filenchus spirocaudatus* sp. n. All measurements are in μm and in the form mean $\pm$ SD (range).

Characters	Holotype Female	Paratypes Females
n	1	18
L	450	418 $\pm$ 22 (387-453)
L'	369	350 $\pm$ 17 (317-376)
a	37.2	38.0 $\pm$ 2.3 (34-43)
b	5.1	5.0 $\pm$ 0.2 (4.5-5.3)
c	5.5	5.9 $\pm$ 0.3 (5.6-6.6)
c'	10.1	9.7 $\pm$ 0.6 (8.4-10.6)
V	66.8	67.3 $\pm$ 0.8 (65.9-68.9)
V'	81.5	81.0 $\pm$ 0.8 (79.8-82.2)
Lip region height	1.7	1.9 $\pm$ 0.1 (1.7-2.1)
Lip region width	4.3	4.4 $\pm$ 0.2 (4-5)
Stylet	6	6.4 $\pm$ 0.5 (6.0-6.5)
Conus	2	2.0 $\pm$ 0.0 (2-2)
Excretory pore	68.7	64.5 $\pm$ 3.0 (60.0-68.7)
MB	43.5	44.2 $\pm$ 0.8 (42.7-45.3)
Pharynx	87.5	84.8 $\pm$ 3.0 (80.0-90.5)
Anterior end-vulva	301	281 $\pm$ 15 (258-303)
Body width (BW)	12	11.0 $\pm$ 0.8 (9.5-12.2)
Anal body width	8	7.4 $\pm$ 0.3 (7.0-8.0)
Vulva-anus	68	66.7 $\pm$ 4.2 (57.6-73.1)
Tail length	81	71.8 $\pm$ 5.8 (60-81)
Tail/V-A	1.2	1.1 $\pm$ 0.1 (0.9-1.2)
PUS	8.9	8.0 $\pm$ 1.3 (6-11)

(original description), *F. microdorus* (Chawla, Prasad, Khan & Nand, 1969) Siddiqi, 1986 (data from Geraert, 2008) and *F. misellus* (Andrássy, 1958) Raski & Geraert, 1986 (original description).

The new species can be distinguished from *F. australis* by having a longer body (387-453 vs 334-387 μm), number of lateral field (four vs three), annulation of the lip region (four vs not distinct), greater a value (34-43 vs 28.5-33.0), greater c value (5.6-6.6 vs 4.7-5.6), shorter stylet (6.0-6.5 vs 7.8 μm), longer pharynx (80.0-90.5 vs 70.2-78 μm), slightly longer PUS (6-11 vs 5.2-7.8), presence of the lateral vulval flaps (small vs not seen), and tail tip with irregularly twisted last annulus vs pointed or filiform.

From *F. microdorus* the described new species can be distinguished by a shorter body (387-453 vs 400-500 μm), annulation in the lip region (four vs not seen), greater a value (34-43 vs 26-34), greater c value (5.6-6.6 vs 4.2-5.8), longer pharynx (80.0-90.5 vs 74 μm), presence of the lateral vulval flaps (small vs not seen) and tail tip with irregularly twisted last annulus vs acute.

From *F. misellus*, the tentative cryptic form of the new species, the described new species differs by having a longer body (387-453 vs 328 μm), greater a value (34-43 vs 32.5), and tail tip with irregularly twisted last annulus vs sharply to minutely pointed.

Molecular characterisation. Sequencing of the SSU rDNA of the new species using the primer pairs 1813F/2646R failed. Amplification with primer pair 1096F/1912R yielded a single 791 nt sequence (PQ135939). All attempts to amplify the D2-D3 fragment expansion segments failed despite repeated trials with several primer combinations.

A BLAST search using the newly generated SSU sequence showed identity values of 98.67%, 98.06%, 98.48 and 97.68% with three sequences PQ896995 (8 mismatches, 3 gaps), KJ869308 (13 mismatches, 3 gaps), PP478510 (9 mismatches, 3 gaps), and AB473564 (10 mismatches, 8 gaps), respectively. All these sequences are assigned to *Filenchus misellus*. Identity values for all remaining sequences were below 94%. In the SSU phylogenetic tree (Fig. 4), the SSU sequence from *F. spirocaudatus* sp. n. formed a clade with the three aforementioned sequences.

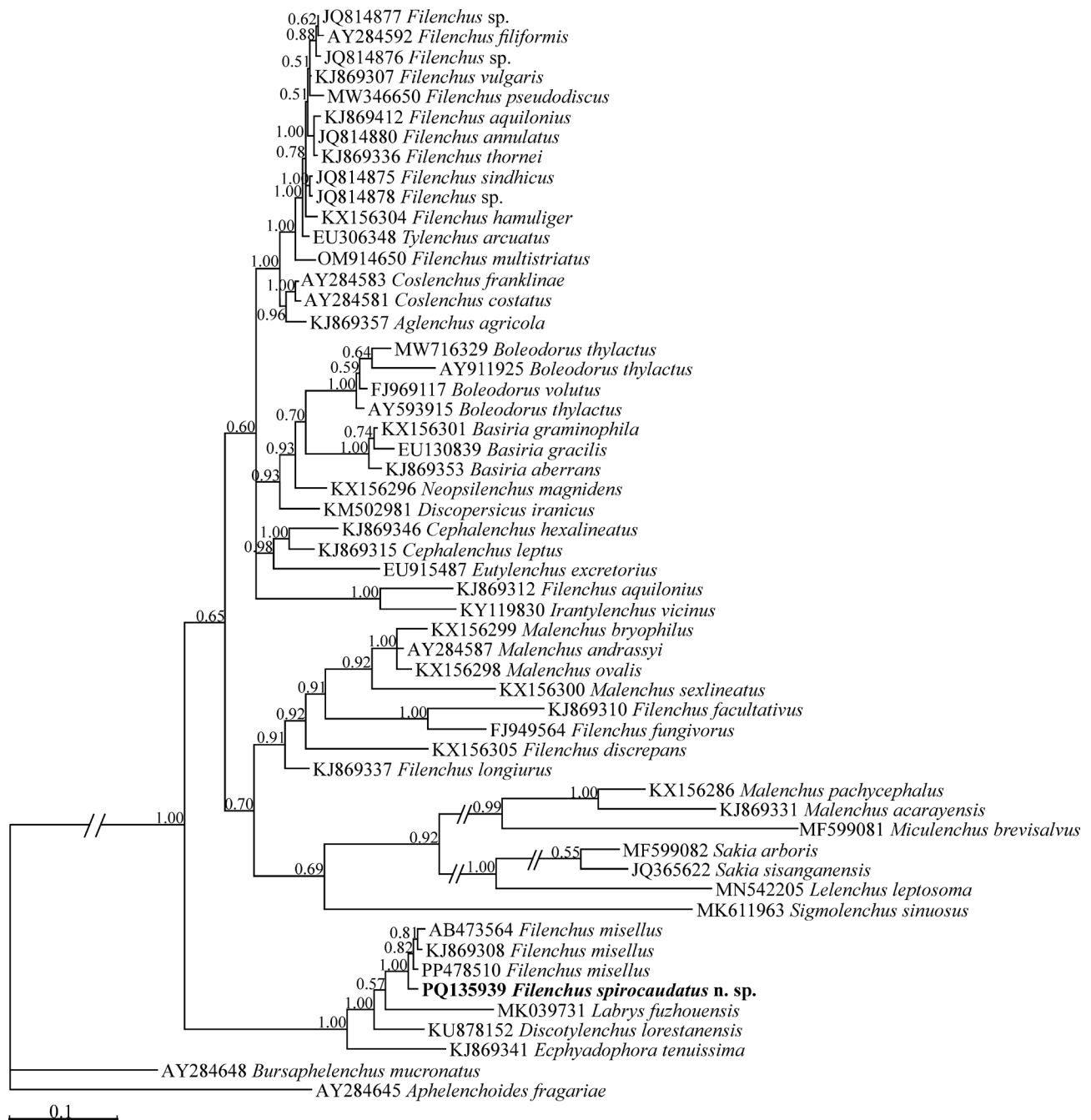


Fig. 4. Bayesian inference tree of sequences of species of the infraorder Tylenchomorpha, including *Filenchus spirocaudatus* sp. n., based on sequences of the SSU rDNA region. Bayesian posterior probabilities (%) are given for each node. The scale bar shows the number of substitutions per site.

DISCUSSION

Taxonomic studies within the family Tylenchidae, particularly the genus *Filenchus*, face considerable challenges. Most Tylenchidae species have been described solely based upon line drawings, light microphotographs, SEM data and molecular sequences are not available for their type or topotype

populations. Several successive reports have extended the range of morphometric data for several species (Geraert, 2008) and several divergent sequences have been deposited into the GenBank for a given species (Munawar *et al.*, 2022; Monemi *et al.*, 2023). By extended morphometric data ranges and no available sequences for type and topotype populations of several Tylenchidae spp., Mahboubi *et*

al. (2023) urged that the original data of type populations of Tylenchidae spp. are the most reliable sources for comparative purposes. During the present study, the same rule was followed and the original data of type population of two compared species were used.

Recent integrative taxonomic studies have increasingly revealed the presence of cryptic speciation in soil nematodes, including members of Tylenchidae (Palomares-Rius *et al.*, 2014; Soleymanzadeh *et al.*, 2016; Munawar *et al.*, 2022). The newly described species, *F. spirocaudatus* sp. n., is morphologically very similar to *F. misellus*. Without careful examination of the tail tip, these two species could be considered conspecific. However, consistent differences in tail tip shape justify their separation as distinct species. This is supported by molecular data. The results of the present study, in line with recent findings (Munawar *et al.*, 2022), highlight the importance of integrative taxonomy (exploiting both morphological and molecular data) for accurate species delimitation in *Filenchus*.

Molecular phylogenies of the Tylenchidae have primarily relied on ribosomal markers (Atighi *et al.*, 2013; Qing *et al.*, 2017; Qing & Bert, 2019, Mofrad & Azimi, 2026), although some recent studies have incorporated mitochondrial *COI* data (Bai *et al.*, 2020). Consistent with previous work (Mahboubi *et al.*, 2023), several cladogenetic events were observed in the SSU phylogeny resolved herein. Given the slow evolutionary rate of this locus, the observed numbers of mismatches and gaps were considered sufficient to distinguish between two cryptic species, *Filenchus misellus* and *F. spirocaudatus* sp. n., based on morphological differences at the tail tip.

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Z. Mahboubi, M. Keramat, E. Pourjam, M. R. Atighi, J. Abolafia, P. Castillo and M. Pedram. *Filenchus spirocaudatus* sp. n. (Tylenchomorpha: Tylenchidae) из ризосферы пшеницы в провинции Исфахан, Иран: морфологическая и молекулярная характеристика.

Резюме. На основе фотографий, полученных с помощью световой микроскопии и сканирующей электронной микроскопии, а также молекулярных данных (частичная последовательность малой субъединицы рибосомальной ДНК), описан и проиллюстрирован новый вид рода *Filenchus*, *F. spirocaudatus* sp. n., обнаруженный на пшеничном поле (*Triticum aestivum*) в провинции Исфахан, Иран. Новый вид отличается в первую очередь тем, что у всех исследованных особей терминальное хвостовое кольцо скручено неравномерно и при световой микроскопии имеет звездообразную форму. У самок (длина тела 387–453 мкм) имеется кольчатая область губы, короткие продольные боковые щели амфидиальных отверстий, небольшие боковые складки вульвы и удлинено-конусовидный хвост. Обсуждаются морфологические и морфометрические отличия от трех близкородственных видов: *F. australis*, *F. microdorus* и *F. misellus*. Вид *Filenchus misellus* считается криптической формой по отношению к новому виду, но *F. spirocaudatus* sp. n. отличается от него и других близких видов в первую очередь морфологией кончика хвоста и молекулярными характеристиками. Филогенетические связи с другими соответствующими родами и видами были установлены на основе частичной последовательности рДНК. На полученном дереве последовательность малой субъединицы рибосомной ДНК *F. spirocaudatus* sp. n. сгруппировалась с тремя последовательностями, отнесенными к *F. misellus*.
