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Cotylenchus cleo gen. n., sp. n., a new plant-parasitic nematode (Tylenchida: Anguinidae) parasitising on leaves of western sword fern plants from rainforests in Washington State, USA

Sergio ÁLVAREZ-ORTEGA [1,*] and Sergei A. SUBBOTIN [2,3]

[1] *Departamento de Biología y Geología, Física y Química Inorgánica, Universidad Rey Juan Carlos, Campus de Móstoles, 28933 Madrid, Spain*

[2] *Plant Pest Diagnostic Center, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento, CA 95832, USA*

[3] *Center of Parasitology of A.N. Severtsov Institute of Ecology and Evolution of the Russian, Academy of Sciences, Leninskii Prospect 33, 117071 Moscow, Russia*

ORCID iDs: Álvarez-Ortega: 0000-0003-2077-5041; Subbotin: 0000-0001-6648-5889

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Summary – A new genus and species of anguinid nematode, *Cotylenchus cleo* gen. n., sp. n., was recovered from leaves of the western sword fern, *Polystichum munitum*, growing in rainforests in several locations of Olympic National Park, Washington State, USA. This new anguinid nematode induced distinct necrotic and chlorotic symptoms on fern leaves characterised by yellowing, light to brown areas between veins. The new species is characterised by a long and slender body, six incisures in the lateral field, robust stylet (12.0-13.5 μm) with large and rounded knobs, pyriform to elongate and abutting basal pharyngeal bulb, and long conical tail with pointed terminus; females having posteriorly located vulva (V = 74-80%) and well-developed post-vulval uterine sac (26-59 μm) and males having spicules 20-23 μm long. Phylogenetic analysis of the partial 18S rRNA and the D2-D3 expansion segments of the 28S rRNA genes showed that this anguinid nematode formed a separate evolutionary lineage different from all other Anguinidae taxa. The new species was also characterised by sequencing the ITS rRNA and *COI* genes.

Keywords – fern, leaf necrosis, new genus, new species, phylogeny, taxonomy.

In 2019-2022, surveys for plant-parasitic nematodes were conducted in natural areas of Washington State, USA, and leaves with necrotic symptoms were observed and collected from western sword fern *Polystichum munitum* (Kaulf.) C.Presl plants, growing in rainforests in several locations of Olympic National Park. Numerous nematode specimens were extracted from these fern leaves when dissected in water in a Petri dish. The nematodes were examined under a stereomicroscope and initially identified as *Aphelenchoides fragariae* (Ritzema Bos, 1891) Christie, 1932. However, further detailed morphological and molecular analyses revealed that this nematode actually belonged to an unknown species of the family Anguinidae Nicoll, 1935. Phylogenetic analysis showed that this nematode represented a separate lineage within this family and should be affiliated to a new genus. Therefore, morphological and molecular characterisations of the new anguinid nematode, *Cotylenchus cleo* gen. n., sp. n. are herein provided.

The western sword fern is an evergreen perennial fern native to western North America. It occurs along the Pacific coast, from southeastern Alaska to southern California, and can be found from the east to southeastern regions of British Columbia, northern Idaho and western Montana. It is one of the most abundant ferns inhabiting forested areas. Up to now, only *A. fragariae* has been reported as a foliar plant-parasitic nematode of *P. munitum* in western states (Sandeno & Jensen, 1962).

The goals of the present work were to provide: *i*) morphological description of a new tylenchid species,

* Corresponding author, e-mail: sergio.aortega@urjc.es

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Cotylenchus cleo gen. n., sp. n. infecting fern leaves; *ii*) morphological diagnosis of the new genus, *Cotylenchus* gen. n.; *iii*) molecular characterisation of *C. cleo* gen. n., sp. n. using rRNA and *COI* gene sequences; and *iv*) reconstruction of the phylogenetic relationships of *Cotylenchus* gen. n. with other Anguinidae members using rRNA gene sequences.

Materials and methods

Nematode samples

Necrotic and chlorotic leaves were collected from western sword fern plants growing along Barnes Creek and Falls Creek, Storm King Ranger station and other locations of Olympic National Park, Washington, USA.

Nematode extraction and morphological study

Nematodes were extracted from chopped fern leaf samples using the standard Baermann funnel method (Ayoub, 1980). Nematodes were relaxed and killed by heat, fixed in 4% formaldehyde, and processed to anhydrous glycerin following Siddiqi's (1964) method. Specimens were mounted on permanent glass slides for light microscopic (LM) observation. Specimens were examined and measured using a Nikon Eclipse NiU light microscope (Nikon) equipped with differential interference contrast (DIC). Morphometrics included de Man's indices and standard measurements. Some of the best-preserved specimens were photographed with an Olympus DP23 digital camera (Olympus). Digital images were edited using Adobe® Photoshop® CS (Adobe Systems). Drawings were made using a *camera lucida* attached to the microscope. After examination and identification, one male and one female specimen preserved in glycerin were selected for observation under scanning electron microscopy (SEM) following the protocol of Álvarez-Ortega & Peña-Santiago (2016). The nematode was hydrated in distilled water, dehydrated in a graded ethanol and acetone series, critical point-dried, coated with gold, and observed with a Nova NanoSEM 230 microscope.

Experiment with nematode cultivation on fungal cultures

Twenty to 30 adult and juvenile specimens of *Cotylenchus cleo* gen. n., sp. n. in 30 μl double distilled water were inoculated to fungal cultures of *Fusarium* sp. and *Botrytis* sp. growing on agar in Petri dishes. Forty days after inoculation, Petri dishes were observed for the presence of nematodes.

Molecular study

DNA was extracted from several specimens using a standard protocol with proteinase K as described by Subbotin (2021a). Nematodes placed in drops of distilled water were cut with a stainless-steel dental needle under a stereomicroscope. Fragments of nematodes in 25 μl water were transferred into a 0.2 ml Eppendorf tube containing 3 μl proteinase K (600 ug ml^{-1}) (Promega) and 2 μl 10× PCR buffer (Taq PCR Core Kit, Qiagen). The tubes were incubated at 65°C (1 h) and 95°C (15 min) consecutively. After incubation, the tubes were centrifuged and kept at −20°C until use. PCR and sequencing were performed as described by Subbotin (2021a). Several primer sets were used in the present study: *i*) D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) primers for amplifying the D2-D3 expansion segments of 28S rRNA gene; *ii*) TW81 (5′-GTT TCC GTA GGT GAA CCT GC-3′) and AB28 (5′-ATA TGC TTA AGT TCA GCG GGT-3′) primers for amplification of the ITS1-5.8S-ITS2 rRNA gene; *iii*) G18SU (5′-GCT TGT CTC AAA GAT TAA GCC-3′) and R18Tyl1 (5′-GGT CCA AGA ATT TCA CCT CTC-3′) primers for amplifying the partial 18 rRNA gene; and *iv*) JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) and JB5 (5′-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3′) primers for amplifying the partial *COI* gene. Amplicons were purified and directly sequenced with forward and reverse primers by Azenta (CA, USA). New sequences are submitted to GenBank under accession numbers: OR880372 (ITS rRNA gene), OR877940 (18S rRNA gene), OR877941 (28S rRNA gene), OR878546 (*COI* gene).

New sequences of the 18S rRNA and the D2-D3 of 28S rRNA genes were aligned with corresponding published sequences of representatives of the family Anguinidae (Subbotin *et al*., 2006; Oliveira *et al*., 2013; Medina-Gómez *et al*., 2016; Barrantes-Infante *et al*., 2018; Davies *et al*., 2020; Velandia *et al*., 2021; Azimi & Abdolkhani, 2022; Munawar *et al*., 2022, 2023; Gu *et al*., 2023; and others) using ClustalX 1.83 (Chenna *et al*., 2003) with default parameters for 18S rRNA gene dataset and with modified parameters (gap opening = 5, gap extension = 3) for 28S rRNA gene dataset. Alignments were analysed with Bayesian inference (BI)

using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) as described by Subbotin (2021b).

Results and discussion

During surveys in 2019-2022, leaves with specific necrotic and chlorotic symptoms (Fig. 1) were collected from western sword fern plants growing in rainforests in several locations of Olympic National Park, Washington. Numerous nematodes were extracted from symptomatic leaves, whereas no nematodes were observed in extracts from healthy leaves. Similar symptoms were not found in other plants growing near infected ferns, except for finding one unidentified small plant with necrotic symptoms from which nematodes were also extracted and then molecularly identified as *A. fragariae*.

Molecular and morphological analysis and feeding habits indicated that the tylenchid nematode extracted from fern plants, *Polystichum munitum*, differed from

Fig. 1. Symptoms of *Polystichum munitum* fern plants infected with a new anguinid nematode, *Cotylenchus cleo* gen. n., sp. n., in rainforest of Olympic National Park, Washington, USA.

all currently described representatives of the family Anguinidae. Rather than place this nematode species in the genus *Ditylenchus sensu lato*, we proposed that this new species should be classified under a new genus. Descriptions of this new genus and new species are given below.

Cotylenchus gen. n.*

Diagnosis

Anguinidae, Anguininae Nicoll, 1935. Body small (1.0-1.2 mm), slender cylindrical, straight to slightly arcuate upon relaxation. Cuticle striated. Lateral field with six incisures. Lip region low, continuous with body contour. Cephalic framework weakly sclerotised. Stylet robust (12.0-13.5 μm), knobs distinctly rounded with anterior surface slightly sloped posteriorly. Corpus nearly cylindrical; median bulb oval, distinct, with well-developed valve; basal pharyngeal bulb pyriform to elongate, abutting intestine. Vulva a transverse slit. Vagina at right angles to body axis. Post-vulval uterine sac present. Spermatheca oblong, with or without rounded sperms. Ovary outstretched, oocytes in single row. Tail elongate-conical, terminus pointed. Bursa well developed, extending less than half length of tail.

Type species

Cotylenchus cleo gen. n., sp. n.

Other species

Sequences of the D1-D2 expansion segments of 28S rRNA gene obtained from environmental soil samples collected in the Duke Forest, NC, USA, and deposited in the GenBank by Mueller *et al*. (2014) were identified here as belonging to an unidentified *Cotylenchus* gen. n. species. Presently, 985 plant species were identified in the Duke Forest (https://dukeforest.duke.edu/forest-environment/plants). Although *Polystichum munitum*, host plant for *C. cleo* gen. n. sp. n., was not found there, several other fern species, including *Polystichum acrostichoides* and *Dryopteris marginalis* from family Dryopteridaceae, and representatives of genera *Thelypteris*, *Woodsia*, *Athyrium* and *Onoclea*, were found in this forested land and could be potential hosts for this nematode species.

Etymology

The genus name is derived from the two first letters of Cocker (Spaniel, dog breed) adjoined to *Tylenchus*.

Relationships

Representative of the genus *Cotylenchus* gen. n. induces necrotic and chlorotic spots on leaves, similar to those induced by species of the anguinid genera *Litylenchus* Zhao, Davies, Alexander & Riley, 2011 and *Zeatylenchus* Zhao, Davies, Alexander & Riley, 2013. However, unlike certain other genera such as *Afrina* Brzeski, 1981, *Anguina* Scopoli, 1777, *Heteroanguina* Chizhov, 1980, *Mesoanguina* Chizhov & Subbotin, 1985, *Nothanguina* Whitehead, 1959, *Orrina* Brzeski, 1981, *Pterotylenchus* Siddiqi & Lenne, 1984, and *Subanguina* Paramonov, 1967, *Cotylenchus* gen. n. does not induce plant galls. Furthermore, it does not cause stem swellings like those induced by plant-parasitic species of *Ditylenchus* Filipjev, 1936.

The new genus is morphologically similar to the plant-parasitic nematode genera *Ditylenchus*, *Litylenchus*, *Pterotylenchus* and *Zeatylenchus*.

Females of the genus *Cotylenchus* gen. n. are characterised by a slender and cylindrical body and easily distinguished from the obese females with a spiral form of *Anguina* and other gall-inducing anguinid genera or from the semi-obese female forms found in *Litylenchus* species. Besides, the stylet of adult *Cotylenchus* gen. n. is more robust and with large and rounded knobs compared to the delicate stylet and small knobs of *Ditylenchus*.

Cotylenchus gen. n. can be distinguished from the genus *Litylenchus* by its lip region (low and continuous with body contour *vs* offset from adjacent body) and bursa (leptoderan, extending less than half of the tail *vs* peloderan, reaching to near the tail tip); from the genus *Pterotylenchus* by its median bulb and valve (distinct, well developed *vs* non-differentiated), basal pharyngeal bulb (abutting intestine *vs* dorsal pharyngeal gland overlapping intestine) and vulva (without cuticular flaps *vs* with large cuticular flaps partially covering vulva); and from the genus *Zeatylenchus* by its lip region (low and continuous with body contour *vs* offset from adjacent body), excretory pore position (anterior to or at basal pharyngeal bulb *vs* at level of stylet knobs), and basal pharyngeal

* https://zoobank.org/urn:lsid:zoobank.org:act:12A2A6AC-7C42-4094-8628-F3E31A004C1C

bulb (abutting intestine *vs* abutting or slightly overlapping intestine dorsally).

Cotylenchus cleo **gen. n., sp. n.**[*]
(Figs 2-5)

MEASUREMENTS

See Table 1.

DESCRIPTION

Female

Body cylindrical, long, slender, slightly ventrally arcuate or straight when relaxed. Cuticular annulation distinct. Lateral field with six incisures, 4-5 μm wide, occupying 21-26% of body width. Under light microscopy in lateral view, lip region low, anteriorly flattened, with rounded margins, continuous with body contour. In SEM view, labial area with slightly rectangular outline, first lip annulus discontinuous due to position of amphidial apertures, giving the appearance of lip region with 4-5 annuli. Stoma opening pore-like, in the middle of a slightly raised and small, circular oral disc. Amphidial apertures situated laterally on first lip annulus. Cephalic framework weak. Stylet robust, conus 40-46% of stylet length, knobs large and rounded, 3-4 μm wide. Dorsal pharyngeal gland orifice (DGO) located close to the stylet knobs. Median bulb oval, 7.5-9.5 μm wide, muscular, with distinct valve. Isthmus cylindrical and narrower than procorpus, 42-58 μm long. Nerve ring surrounding isthmus. Excretory pore located anterior to or in the range of basal pharyngeal bulb. Hemizonid *ca* three cuticular annuli long, located 1-2 annuli anterior to the excretory pore. Basal pharyngeal bulb pyriform to elongated, and abutting intestine. Crustaformeria consisting of four rows of four cells, forming a quadricolumella. Ovary mono-prodelphic, outstretched, oocytes in a single row. Spermatheca oblong, usually filled with rounded sperms. Vagina straight 9.5-11.0 μm long reaching 49-60% of vulval body width. Vulva a simple transverse slit. In SEM view, transverse striae discontinuous and broken longitudinally for 3-4 annuli length around vulva. Vulva lips slightly to non-protruding. Post-vulval uterine sac empty, tube-like, 1.5-3.2 times vulval body width long. Anus a transverse, anteriad curved, slit. Tail long conical, tapering to a pointed terminus.

[*] https://zoobank.org/urn:lsid:zoobank.org:act:50C5090A-8656-46CB-9E7C-AA4F0744DEF9

Male

Similar to females in general body characteristics and tail shape. Lateral field with six incisures. Testis single, anteriorly outstretched. Spermatocytes arranged in one row. Spicules slightly ventrally arcuate, gubernaculum simple, crescent-shaped. Bursa with crenate margin, well developed, starting anterior to cloacal opening and extending less than half of the tail, 46-56 μm long.

Juveniles

Juveniles of different stages were extracted from infected leaves. Body shapes of juveniles were similar to those of the adults.

TYPE HOST AND LOCALITY

Western sword fern, *Polystichum munitum* (Kaulf.) C.Presl. (family Dryopteridaceae), Storm King Ranger station, Port Angeles, Clallam County, Olympic National Park, Washington, USA (48.051730°N, 123.787441°W). Fern plants growing along Barnes Creek and Falls Creek.

OTHER LOCALITY

Fern plants growing along trails (47.861414°N, 123.934211°W) from Hoh Rail Forest Visitor Center, Jefferson County, Washington, USA. Our surveys for plant-parasitic nematodes in ferns growing in forests along Pacific coast of California did not reveal this species.

SYMPTOMS OF INFECTION

Necrotic and chlorotic symptoms characterised by yellowing, light to brown interveinal areas were observed in diseased leaves of the fern. Leaf symptoms in fern caused by the new species were similar to those induced by other foliar plant-parasitic nematodes, *Aphelenchoides fragariae* and *A. ritzemabosi* (Schwartz, 1911) Steiner & Buhrer, 1932. No swellings, abnormal growths of leaves or growth suppression were observed in nematode-infected ferns.

TYPE MATERIALS

Holotype female and 11 female and 12 male paratypes, mounted on glass slides, were deposited in the nematode collection of the National Museum of Natural Sciences, Madrid, Spain. An additional three female and three male paratypes were sent to the United States Department of Agriculture Nematode Collection, Beltsville, MD, USA.

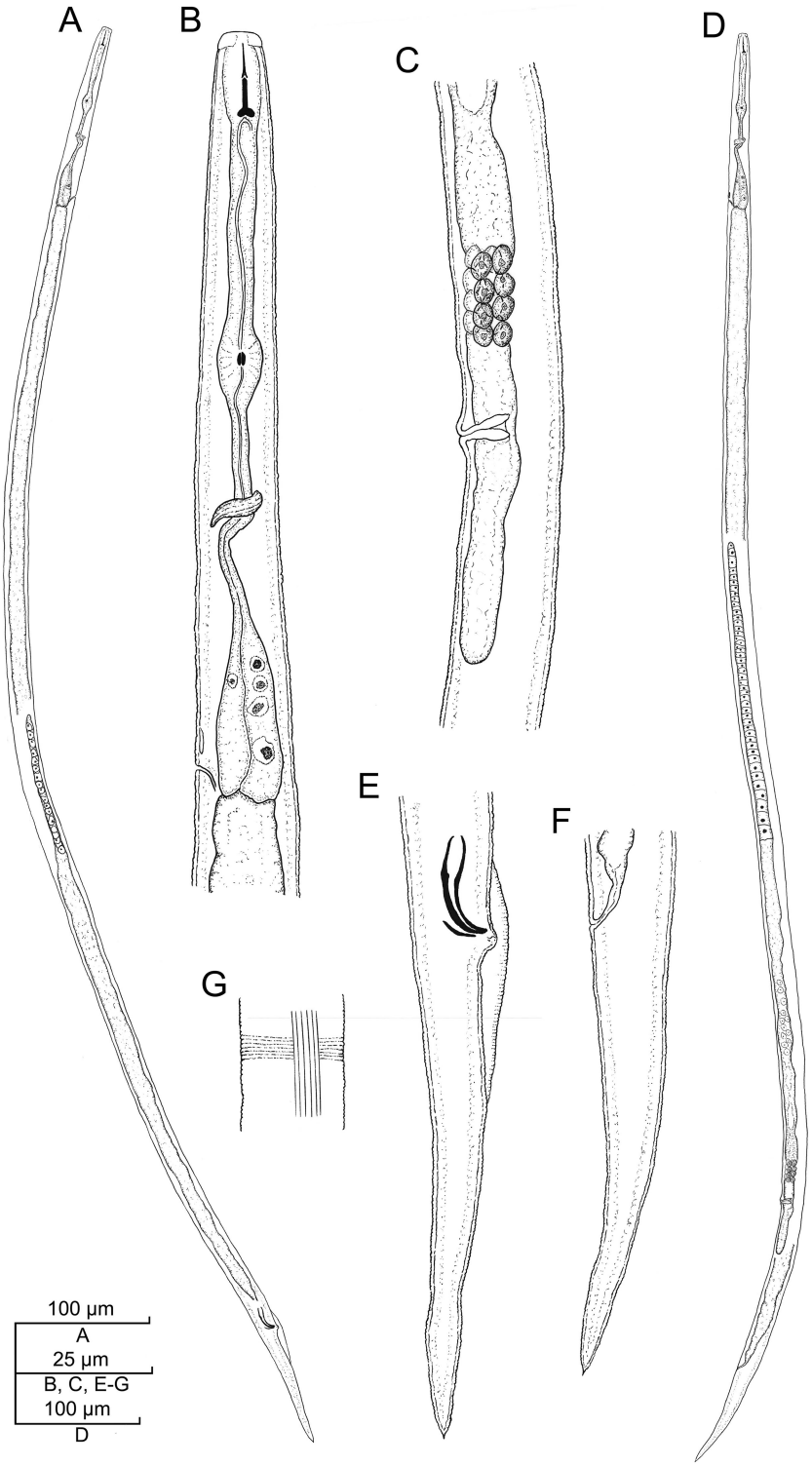

Fig. 2. *Camera lucida* drawings of *Cotylenchus cleo* gen. n., sp. n. A: Male entire body; B: Female anterior region; C: Vulva region and post-vulval uterine sac (PUS); D: Female entire body; E: Male posterior body region; F: Female posterior body region; Lateral field.

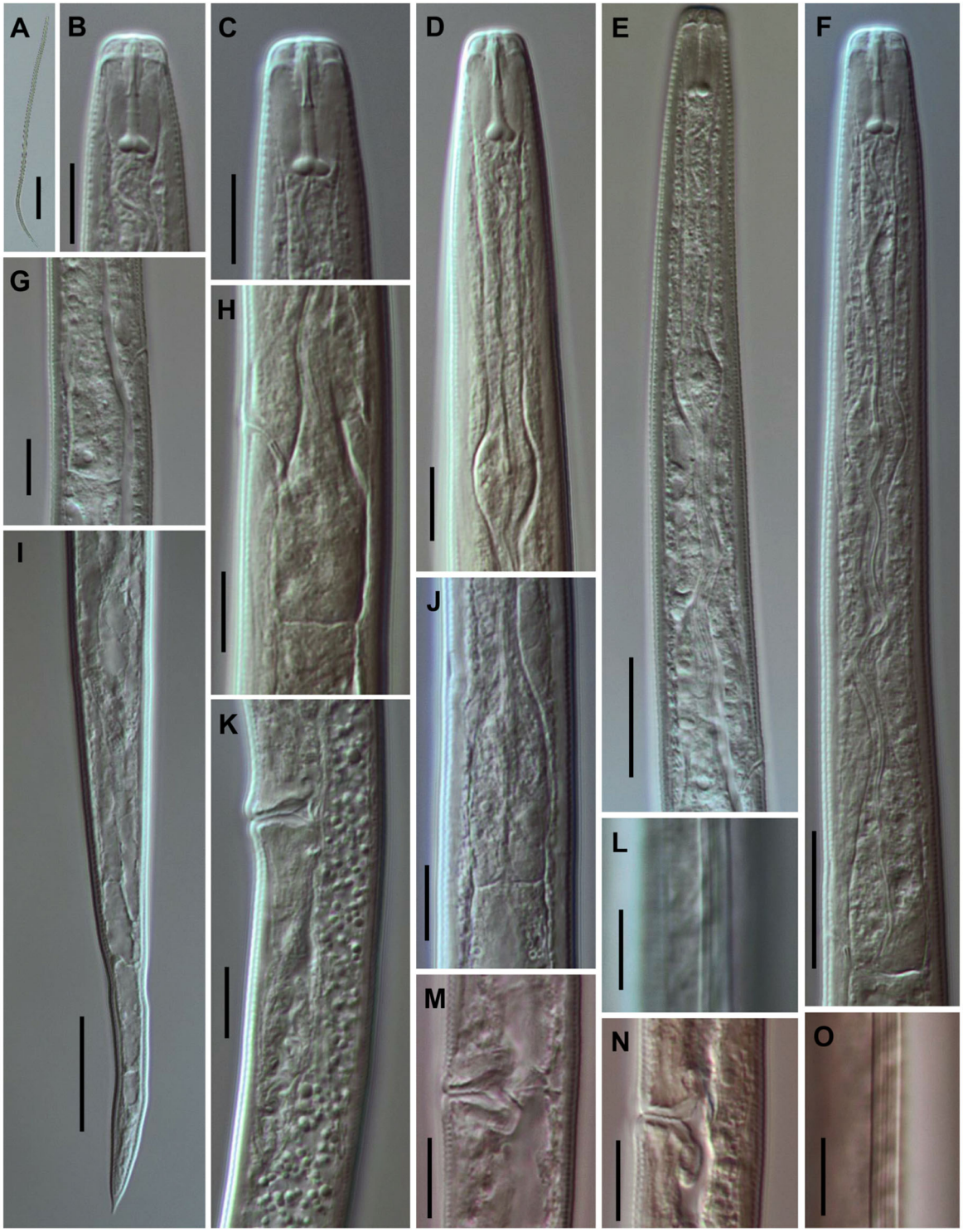

Fig. 3. Light microphotographs of *Cotylenchus cleo* gen. n., sp. n. Female. A: Entire body; B, C: Lip region; D-F: Anterior body region; G, H, J: Basal bulb; I: Posterior body region; K: Vulva region and post-vulval uterine sac; M, N: Vulva region; L, O: Lateral field. (Scale bars: A = 200 μm; B-D, G, H, J-O = 10 μm; E, F, I = 20 μm.)

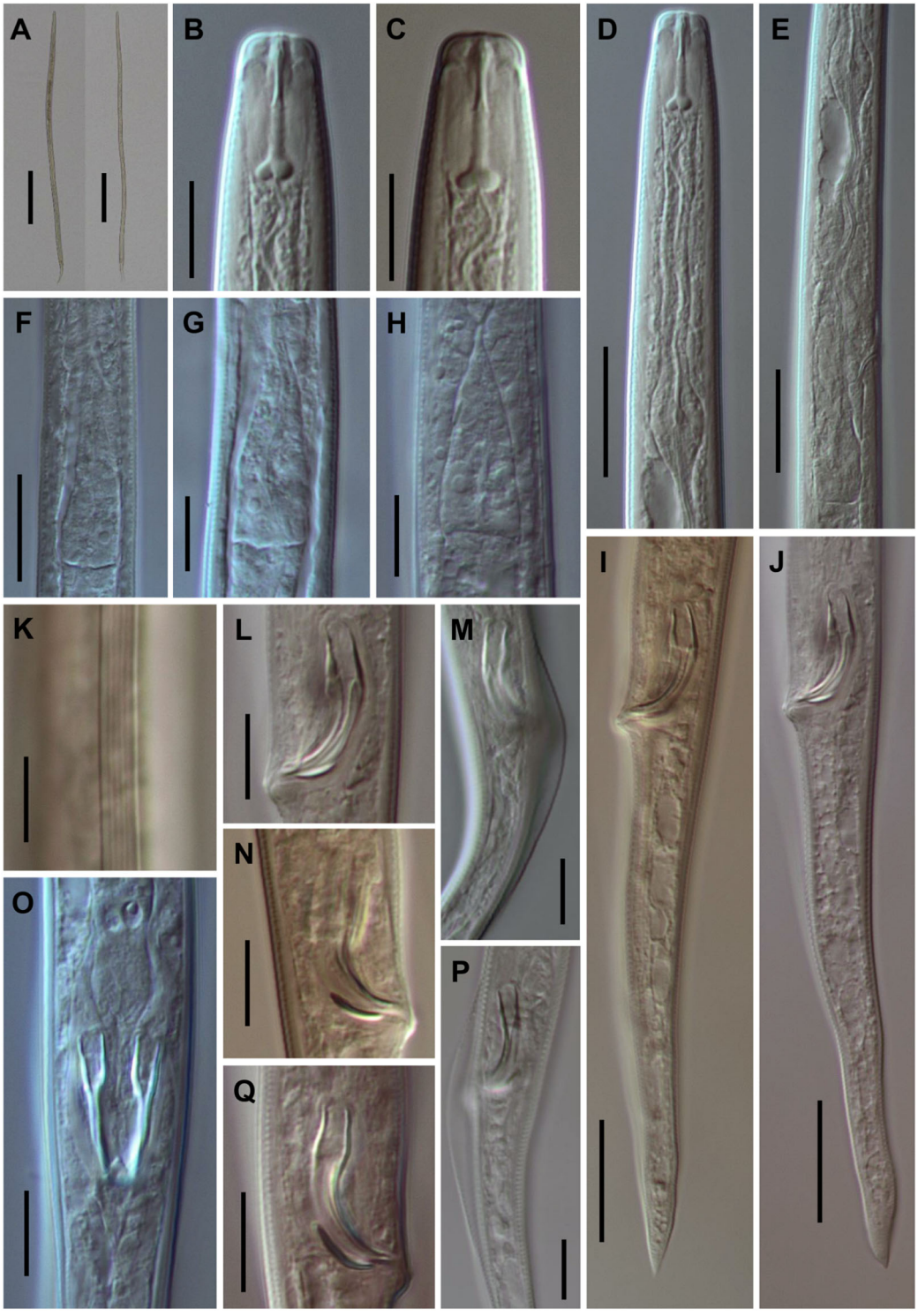

Fig. 4. Light microphotographs of *Cotylenchus cleo* gen. n., sp. n. Male. A: Entire body; B, C: Lip region; D, E: Anterior body region; F-H: Basal bulb; I, J: Posterior body region; K: Lateral field; L, N, O, Q: Spicules; M, P: Bursa region. (Scale bars: A = 200 μm; B, C, F-H, K-Q = 10 μm; D, E, I, J = 20 μm.)

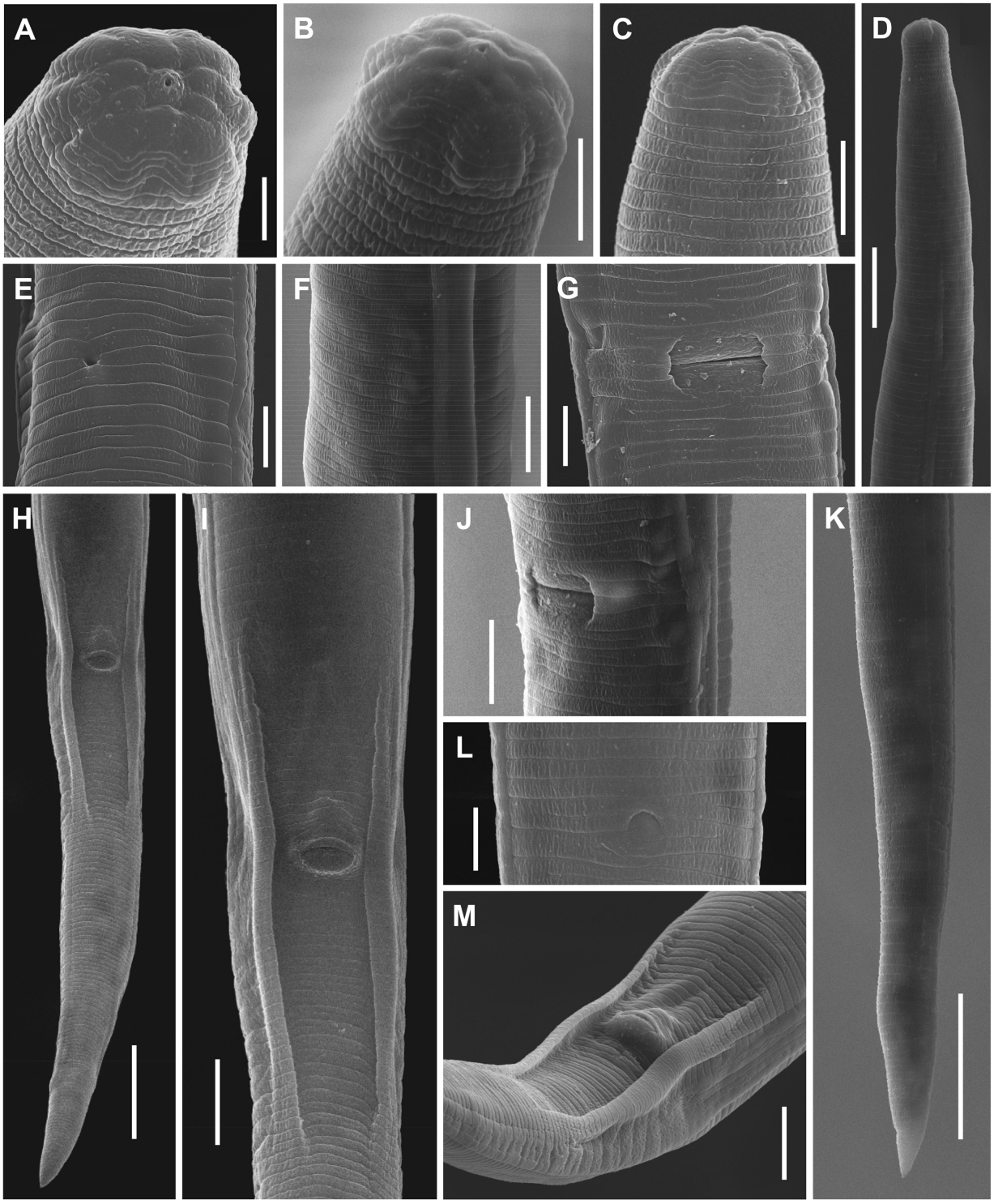

Fig. 5. Scanning electron microphotographs of *Cotylenchus cleo* gen. n., sp. n. Female. A: Lip region; E: Excretory pore; F: Lateral field; G, J: Vulva region; K: Posterior body region. Male. B, C: Lip region; D: Anterior region; H, I, M: Posterior body region. (Scale bars: A-C = 2.5 μm; D, H, K = 10 μm; E-G, I, J, L, M = 5 μm.)

Table 1. Morphometrics of *Cotylenchus cleo* gen. n., sp. n. All measurements are in μm and in the form: mean ± s.d. (range).

Character	Female		Male
	Holotype	Paratypes	Paratypes
n	1	9	10
L	1141.6	1104.3 ± 46.2 (1033.3-1159.3)	1120.8 ± 43.6 (1068.2-1212.2)
L′	1060.1	1018.2 ± 44.0 (949.3-1064.5)	1033.4 ± 40.6 (984.7-1118.0)
a	56.0	57.8 ± 2.7 (51.9-61.4)	60.3 ± 3.3 (55.3-64.3)
b	8.2	7.7 ± 0.4 (7.3-8.5)	8.2 ± 0.6 (7.2-9.3)
c	14.0	12.8 ± 0.6 (12.2-14.2)	12.9 ± 0.6 (12.1-14.3)
c′	5.6	6.6 ± 0.4 (6.1-7.3)	5.8 ± 0.4 (5.0-6.4)
V	80.3	76.9 ± 1.6 (73.9-78.6)	–
V′	86.5	83.4 ± 1.8 (80.0-85.1)	–
Lip region width	7.9	8.0 ± 0.2 (7.9-8.3)	7.7 ± 0.2 (7.3-8.0)
Stylet length	13.3	13.1 ± 0.4 (12.2-13.6)	12.6 ± 0.3 (12.3-13.1)
Median bulb length	12.9	13.2 ± 1.0 (12.0-15.1)	12.1 ± 0.7 (11.4-13.1)
Median bulb width	8.7	8.2 ± 0.6 (7.3-9.3)	8.0 ± 0.6 (7.2-9.3)
Anterior end to excretory pore distance	131.0	118.0 ± 7.4 (110.0-127.3)	119.6 ± 6.9 (108.0-128.9)
Pharynx length	139.6	143.1 ± 8.8 (127.0-151.0)	137.4 ± 6.8 (130.8-154.1)
Max body width	20.4	19.1 ± 1.1 (17.1-20.3)	18.6 ± 1.3 (17.4-20.8)
Lateral field width	4.7	4.6 ± 0.4 (4.2-5.1)	3.8 ± 0.6 (3.4-5.1)
Body width at vulva (BWV)	19.2	17.8 ± 1.2 (15.8-19.8)	–
Genital tract length	554.5	420.1 ± 59.4 (314.8-507.7)	422.4 ± 51.1 (338.8-500.9)
Post-vulval uterine sac (PUS) length	48.5	47.2 ± 9.8 (26.0-58.6)	–
Lip region-vulva distance	917.2	849.5 ± 43.3 (769.0-897.1)	–
Vulva-tail terminus distance	224.4	254.8 ± 18.6 (237.3-296.1)	–
Vulva-anus distance (V-A)	140.7	170.3 ± 18.7 (152.0-208.0)	–
PUS/BWV	2.5	2.6 ± 0.5 (1.5-3.2)	–
PUS/V-A%	34.4	28.1 ± 6.4 (12.5-35.0)	–
Body width at anus	14.6	13.1 ± 0.8 (11.5-13.8)	15.1 ± 1.0 (13.0-16.3)
Tail length	81.5	86.1 ± 4.5 (79.7-94.8)	87.4 ± 5.0 (78.2-94.3)
Spicules (arc)	–	–	21.7 ± 1.2 (20.2-23.2)
Spicules (chord)	–	–	19.3 ± 1.0 (17.7-20.5)
Gubernaculum	–	–	7.1 ± 0.4 (6.7-7.8)

Abbreviations: n = number of measured specimens; L = overall body length; G = genital tract length/body length %; L′ = lip region to anus body distance; a = body length/greatest body diam.; b = body length/pharynx length; c = body length/tail length; c′ = tail length/body diam. at anus or cloaca; V = distance of anterior body end to the vulva/body length %. V′ = position of the vulva as a percentage (%) of the lip region to anus body distance.

Etymology

The new species is named in memory of the second author's American Cocker Spaniel dog, Cleo, in recognition of her service.

Diagnosis

The new species is characterised by a long (L = 1.03-1.21) and slender (a = 52-64) body, six incisures in lateral field, robust stylet (12.2-13.6 μm) with large and rounded knobs, pyriform to elongated and offset basal pharyngeal bulb, and long conical tail with pointed terminus. Females having posteriorly located vulva (V = 74-80%) and well-developed post-vulval uterine sac 26-59 μm) and males having spicules of 20-23 μm long. The status of the new species was confirmed by molecular analysis.

Experiment with nematode cultivation on fungal cultures

There was no success in culturing the new nematode species on fungal cultures. No nematodes were observed on fungal cultures of *Fusarium* sp. and *Botrytis* sp. in 40 days after nematode inoculation.

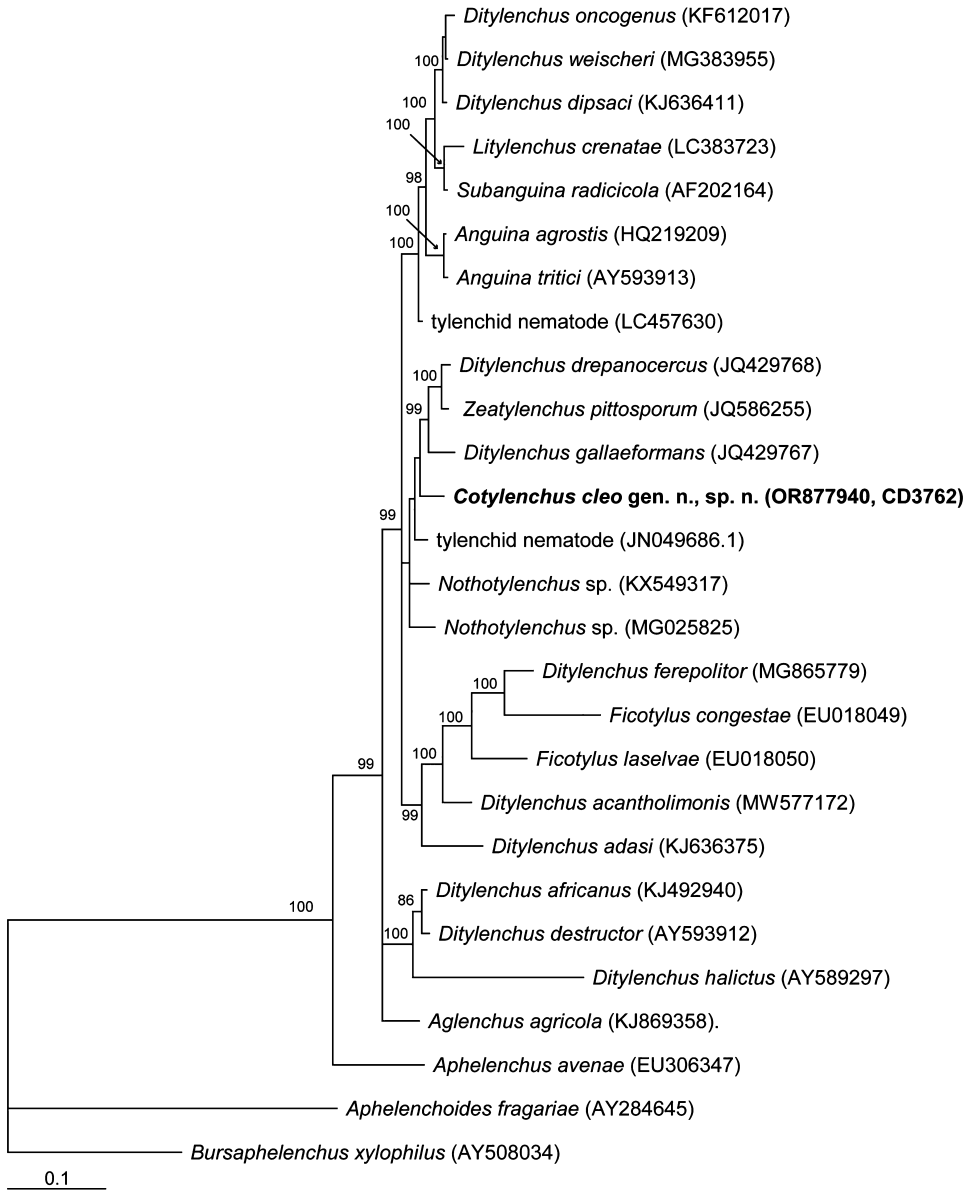

Fig. 6. Phylogenetic positions of a new anguinid nematode, *Cotylenchus cleo* gen. n., sp. n. within some representatives of the family Anguinidae as inferred from Bayesian analysis of sequences of the 18S rRNA gene using GTR + I + G model of DNA evolution. Posterior probability values more than 70% are given on appropriate clades. New sequence is indicated in boldface.

Molecular characterisation and phylogenetic relationships

Partial 18S rRNA gene

The alignment contained 23 sequences of the representatives from Anguinidae and four sequences of the outgroup taxa and was 841 bp long. One new sequence was obtained in this study. Phylogenetic relationships of *Cotylenchus cleo* gen. n., sp. n. with other Anguinidae taxa are given in Figure 6. Phylogenetic position of the new species was not well resolved.

D2-D3 expansion segments of 28S rRNA gene

The alignment contained 43 sequences of the representatives from Anguinidae and two sequences of the out-

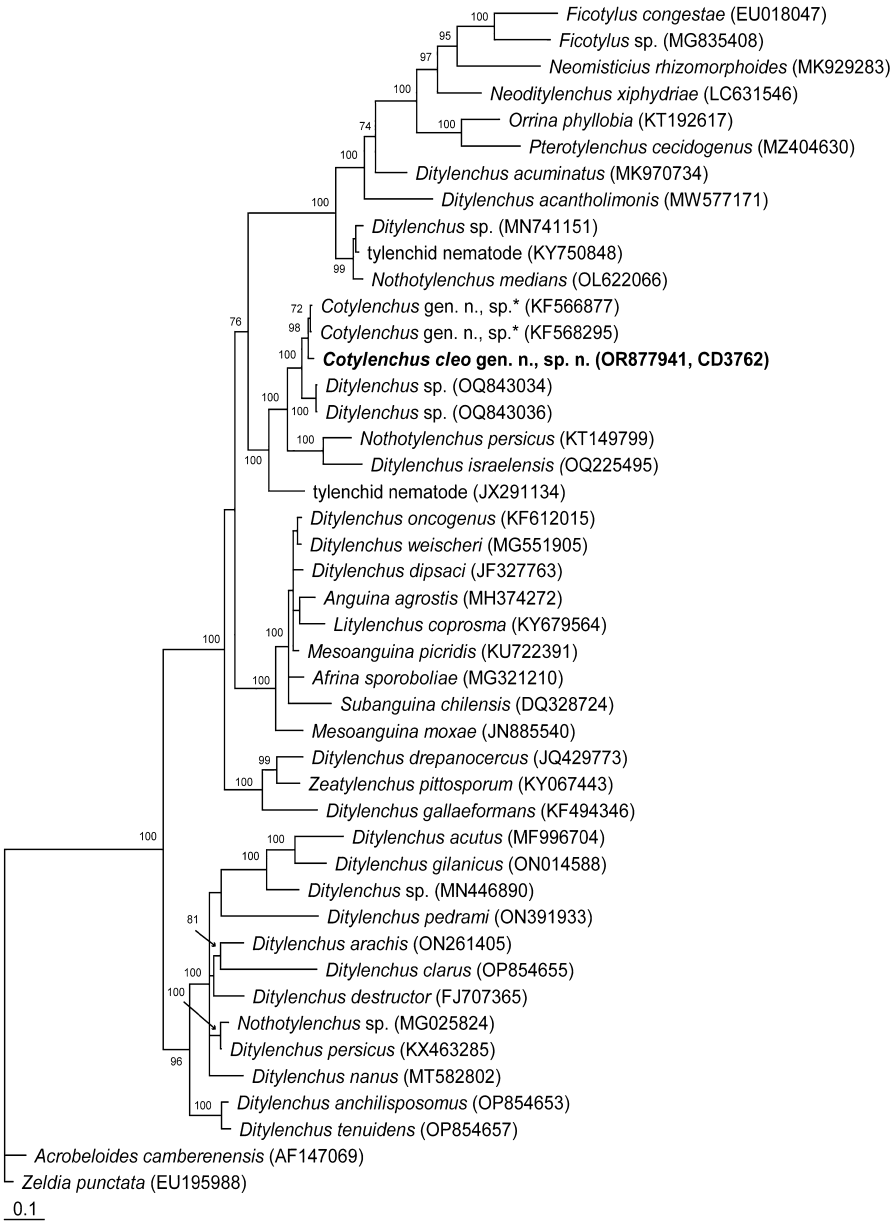

Fig. 7. Phylogenetic positions of a new anguinid nematode, *Cotylenchus cleo* gen. n., sp. n. within some representatives of the family Anguinidae as inferred from Bayesian analysis of sequences of the D2-D3 of 28S rRNA gene using GTR + I + G model of DNA evolution. Posterior probability values more than 70% are given on appropriate clades. New sequence is indicated in boldface. * Originally identified as fungus sequence.

group taxa and was 804 bp long. One new sequence was obtained in this study. Phylogenetic relationships of *C. cleo* gen. n., sp. n. with other Anguinidae taxa are given in Figure 7. The D2-D3 of 28S rRNA gene sequence of *C. cleo* gen. n., sp. n. formed a clade (PP = 100%) with the sequences obtained from environmental soil samples collected in the Duke Forest, North Carolina, USA (Mueller *et al*., 2014) and originally identified as fungal sequences. Sequences obtained from these environmental samples was considered here as those belonging to

undescribed species of *Cotylenchus* gen. n. Sequences of the partial 28S rRNA gene of *C. cleo* gen. n., sp. n. and *Cotylenchus* gen. n., sp. n. differed from each other in 1.7-1.9% (7-8 bp). Sequences of *Cotylenchus* gen. n. and two sequences from China identified as *Ditylenchus* sp. formed a separated lineage within the family Anguinidae.

ITS rRNA gene

The new sequence of *C. cleo* gen. n., sp. n. was 698 bp long. BLASTn search of the ITS rRNA gene sequence showed highest similarity with *Ditylenchus israeliensis* (OQ225512) – coverage = 52%, identity = 89.73%.

Partial COI gene

The new sequence of *C. cleo* gen. n., sp. n. was 364 bp long. BLASTp search of the *COI* amino acid sequence showed highest similarity with that of *Filenchus vulgaris* (QLC27093) – coverage = 100%, identity = 81.67%.

The family Anguinidae includes mycophagous and plant-parasitic nematodes. The latter are obligate specialised parasites of higher plants, mosses, and seaweeds on which they often induce swellings and galls. Several species are considered of economic importance as agricultural and quarantine pests in various countries (Subbotin & Riley, 2012). In this study, the new anguinid plant-parasitic nematode that was discovered and described can be also considered as a new pest of fern plants. Ferns are not only important members of the natural ecosystems but are also considered ornamental garden plants. The distribution, biology, host range and pathogenicity of the new nematode pest are still unknown and require intensive study.

Phylogenetic analysis of rRNA gene sequences revealed that the new nematode parasitising fern leaves could not be placed in any existing anguinid genus, and therefore, was placed in a new genus. Phylogenetic analysis of rRNA gene sequences also revealed that the ability to parasitise plant leaves and induce necrotic spots appeared at least three times during the evolution of nematodes from the family Anguinidae, *e.g.*, in representatives of the genera *Litylenchus*, *Zeatylenchus* and *Cotylenchus* gen. n. Parasitism of nematodes in mesophyll tissue without inducing abnormal cell growth could be considered a more primitive step of nematode relationships with their plant-hosts compared to a more advanced ability to induce gall induction leading to cell hyperplasia and hypertrophy and forming a new food source for parasites. It is remarkable that the genera *Litylenchus*, *Zeatylenchus* and *Cotylenchus* gen. n. are not distinctly related taxa, but they are clearly related to other nematode taxa inducing plant galls or swellings.

Practically all nematologists recognise the family Anguinidae, while discrepancies exist only over its composition of subfamilies and genera. The validities and positions of several genera and subfamilies have been the subject of intensive discussions and speculation and remain unresolved. Comprehensive morphological and molecular analyses are still needed to understand relationships within these nematode groups and replacement of the present artificial classification by a natural one that accurately reflects the evolutionary trends within this group (Subbotin & Riley, 2012).

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