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Molecular phylogeny, diagnostics, and diversity of plant-parasitic nematodes of the genus *Hemicycliophora* (Nematoda: Hemicycliophoridae)

SERGEI A. SUBBOTIN[1,2*], JOHN J. CHITAMBAR[1], VLAMIDIR N. CHIZHOV[2], JASON D. STANLEY[3], RENATO N. INSERRA[3], MARCELO E. DOUCET[4], MICHAEL McCLURE[5], WEIMIN YE[6], GEORGE W. YEATES[7†], DIMITRE S. MOLLOV[8], CAROLINA CANTALAPIEDRA-NAVARRETE[9], NICOLA VOVLAS[10], ESTHER VAN DEN BERG[11] and PABLO CASTILLO[9]

[1]*Plant Pest Diagnostic Center, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento, CA 95832-1448, USA*
[2]*Center of Parasitology of A.N. Severtsov, Institute of Ecology and Evolution of the Russian Academy of Sciences, Leninskii Prospect 33, Moscow, 117071, Russia*
[3]*Florida Department of Agriculture and Consumer Services, DPI, Nematology Section, P.O. Box 147100 Gainesville, FL 32614-7100, USA*
[4]*Centro de Zoología Aplicada, Facultad de Ciencias Exactas, Físicas y Naturales, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina*
[5]*School of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA*
[6]*Nematode Assay Section, Agronomic Division, North Carolina Department of Agriculture & Consumer Services, 1040 Mail Service Center, Raleigh, NC 27699-1040, USA*
[7]*P.O. Box 1758, Palmerston North 4440, New Zealand*
[8]*Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108, USA*
[9]*Instituto de Agricultura Sostenible (IAS), Consejo Superior de Investigaciones Científicas (CSIC), Campus de Excelencia Internacional Agroalimentario, ceiA3, Apdo. 4084, 14080 Córdoba, Spain*
[10]*Istituto per la Protezione delle Piante (IPP), Sezione di Bari, Consiglio Nazionale delle Ricerche, (C.N.R.), Via G. Amendola 165/A, 70126 Bari, Italy*
[11]*National Collection of Nematodes, Biosystematics Programme, ARC-Plant Protection Research Institute, Private Bag X134, Queenswood 0121, South Africa*

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The genus *Hemicycliophora* (Nematoda: Hemicycliophoridae) contains 132 valid species of plant-parasitic nematodes, collectively known as 'sheath nematodes'. *Hemicycliophora* spp. are characterized morphologically by a long stylet with rounded basal knobs and a cuticular sheath, present in juvenile and adult stages. Populations of 20 valid and 14 putative species of *Hemicycliophora* and *Loofia* from several countries were characterized morphologically using light (LM) and scanning electron microscopy (SEM) and molecularly using the D2-D3 segments of 28S rRNA and internal transcribed spacer (ITS) rRNA gene sequences. LM and SEM observations provided new details on the morphology of these species. PCR-restriction fragment length polymorphisms (PCR-RFLPs) of the D2-D3 of 28S rDNA were proposed for identification of the species. Phylogenetic relationships within populations of 36 species of the genus *Hemicycliophora* using 102 D2-D3 of 28S rDNA and 97 ITS rRNA gene sequences as inferred from Bayesian analysis are reconstructed and discussed. Ancestral state reconstructions of diagnostic characters (body and stylet length, number of body annuli, shape of vulval lip and tail), using maximum parsimony

*Corresponding author. E-mail: subbotin@ucr.edu
†Passed away in August 2012.

and Bayesian inference, revealed that none of the traits are individually reliable characters for classifying the studied sheath nematode. The Shimodaira–Hasegawa test rejected the validity of the genus *Loofia*. This is the most complete phylogenetic analysis of *Hemicycliophora* species conducted so far.

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ADDITIONAL KEYWORDS: ancestral state reconstructions – Bayesian inference – cryptic species – D2-D3 – PCR-ITS-RFLP – rDNA – sheath nematodes.

INTRODUCTION

The phylum Nematoda includes the genus *Hemicycliophora* de Man, 1921, which represents a large group of plant-parasitic nematodes that are polyphagous, migratory root-ectoparasites of many plants including various agricultural crops and trees (Siddiqi, 2000). These nematodes are generally found inhabiting moist soils and aquatic environments. *Hemicycliophora* spp. are characterized morphologically by the presence of a long stylet (60 to 150 μm) with rounded basal knobs and an outer, separated and loosened cortical layer, which forms a cuticular sheath in both adult and juvenile stages. As the body cuticle and cuticular sheath are produced simultaneously at each moult, both are an integral part of the cuticle. Because of the presence of a cuticular sheath, these nematodes received the common name of ‘sheath nematodes’. Presently the genus *Hemicycliophora* contains 132 valid species (Chitambar & Subbotin, 2014).

Although all *Hemicycliophora* spp. are obligate plant-parasites, only some species have been reported as damaging parasites of crops, *viz*. *Hemicycliophora arenaria* Raski, 1958, causing root-tip galls in citrus as well as other crops in the families Cucurbitaceae, Leguminosae, Rutaceae, Solanaceae, and Umbelliferae (Van Gundy, 1958; Van Gundy & Rackham, 1961); *Hemicycliophora parvana* Tarjan, 1952, is pathogenic to celery in Florida, USA (Tarjan, 1952); *Hemicycliophora similis* Thorne, 1955, has been associated with maple decline in Wisconsin, USA (Riffle, 1962); *Hemicycliophora typica* caused stubby-root symptoms in carrots in sandy soil in the Netherlands (Thorne, 1961); *Hemicycliophora conida* Thorne, 1955, caused stunted growth and aberrant root development in forage crops (Spaull & Newton, 1982); *Hemicycliophora ripa* Van den Berg, 1981, caused stunted and terminally thickened roots in Swiss chard (Malan & Meyer, 1993); and *Hemicycliophora poranga* Monteiro & Lordello, 1978, caused similar symptoms in tomato (Chitambar, 1993). Consequently, accurate and timely identification of *Hemicycliophora* spp. infesting crops is a prerequisite for designing effective management strategies and the separation of species having agricultural and regulatory relevance, such as *H. arenaria*. The morphological identification and delimitation of several of these species remains very problematic because of their high morphological plasticity at the species level and the large number of described species within the genus (Loof, 1968, 1976; Brzeski, 1974; Brzeski & Ivanova, 1978; Raski & Luc, 1987; Siddiqi, 2000). The application of molecular methods to study these nematodes may reveal that some long-assumed single species are in fact cryptic taxa consisting of species almost indistinguishable morphologically, but phylogenetically and genetically different, as has been shown for many other plant-parasitic species (Gutiérrez-Gutiérrez *et al*., 2010; Cantalapiedra-Navarrete *et al*., 2013).

Two classifications of sheath nematodes were proposed and are presently in use. Raski & Luc (1987) considered only two genera *Hemicycliophora* and *Caloosia* Siddiqi & Goodey, 1964, within the subfamily Hemicycliophorinae Skarbilovich, 1959, whereas Siddiqi (2000) recognized the superfamily Hemicycliophoroidea Skarbilovich, 1959, with the families Hemicycliophoridae Skarbilovich, 1959, and Caloosiidae Siddiqi, 1980. According to Siddiqi (2000), the family Hemicycliophoridae is represented by the subfamily Hemicycliophorinae, which contains the genera *Aulosphora* Siddiqi, 1980, *Colbranium* Andrássy, 1979, *Hemicycliophora*, and *Loofia* Siddiqi, 1980. The genus *Colbranium* was erected by Andrássy (1979) with a single species *Colbranium truncatum* (Colbran, 1960), which is characterized by a lip region separated from the rest of the body by a deep groove and a very short postvulval body portion deeply recessed at vulval level. Siddiqi (1980) used vulval lip and spicule structure for establishing the genera *Aulosphora* and *Loofia*. The latter genus was erected as a new taxon differing from *Hemicycliophora* by females having a conoid lip region, and rounded and nonmodified vulval lips. Loof (1985) did not recognize *Loofia* in his scanning electron microscopy (SEM) studies and consequently synonymized this genus with *Hemicycliophora*. Likewise, Siddiqi’s proposal was not supported by Raski & Luc (1987) who, in their reappraisal of Tylenchina, considered *Loofia* together with *Aulosphora* and *Colbranium* as junior synonyms of *Hemicycliophora*. The validity of these genera has not been tested with molecular analyses in other studies of sheath nematodes.

In the last decade, nuclear ribosomal RNA gene sequences have been used for molecular diagnostics, reconstruction of phylogenetic relationships, and testing the reliability of the classification thus far proposed for Hemicycliophoroidea (Subbotin *et al.*, 2005; Holterman *et al.*, 2009; Van den Berg, Subbotin & Tiedt, 2010; Van den Berg, Tiedt & Subbotin, 2011; Cordero López, Robbins & Szalanski, 2013; Inserra *et al.*, 2013). These studies showed that several *Hemicycliophora* species can be identified using the D2-D3 of 28S rRNA and internal transcribed spacer (ITS) rRNA gene sequences. Analyses also showed that the genus *Hemicycliophora* was monophyletic and phylogenetically related to the genera *Caloosia* and *Hemicaloosia* but clearly separated from sheathoid nematodes of the genus *Hemicriconemoides* Chitwood & Birchfield, 1957. However, only a few *Hemicycliophora* species were molecularly characterized in these studies, and consequently, the relationships amongst all species in the genus and validities of some genera listed above remain unknown and untested.

The aims of the present study were to: (1) carry out a detailed morphological and morphometric characterization of a wide range of *Hemicycliophora* species and populations from several countries; (2) provide molecular characterization of the species and populations of *Hemicycliophora* using sequences of the D2-D3 of the 28S rRNA and the ITS of the rRNA gene; (3) analyse phylogenetic relationships within *Hemicycliophora* species using rRNA gene sequences; (4) to evaluate the validity of the genus *Loofia*, another genus included in this study and represented by populations of two species; (5) analyse the evolutionary histories of several diagnostic morphological characters by mapping them onto the phylogenetic trees; and (6) develop diagnostic PCR-ITS-RFLP profiles for rapid identification of *Hemicycliophora* species.

MATERIAL AND METHODS

Taxonomic sampling and morphological studies

Nematode populations used in this study were obtained from several sources and geographical areas (Table 1). The topotypes of five *Hemicycliophora* species (*Hemicycliophora floridensis*, *Hemicycliophora halophila*, *Hemicycliophora hellenica*, *Hemicycliophora iberica*, and *Hemicycliophora italiae*) were also collected and added to the populations studied. Two species, namely *Hemicycliophora thienemanni* and *Hemicycliophora vaccinii*, considered to belong to the genus *Loofia sensu* Siddiqi (1960) were also included. All populations were morphologically identified using the polytomous and dichotomous keys and species descriptions according to Chitambar & Subbotin (2014). Populations from nontype localities were used for molecular analyses and are proposed to be used as standard and reference populations for each given species until topotype material becomes available and molecularly characterized. Specimens were extracted from soil samples with a centrifugal flotation method (Coolen, 1979). Specimens for light microscopy were killed by gentle heat, fixed in a solution of 4% formaldehyde + 1% propionic acid and processed to pure glycerine using Seinhorst's (1966) method. Nematode specimens were examined and measured in four laboratories (IAS-CSIC, Spain; CDFA, USA; CPSIEE, Russia; and FDACS, USA) using compound microscopes equipped with differential interference contrast. Additional light microscopic photographs of nematodes were taken with an automatic Infinity 2 camera attached to an Olympus BX51 microscope equipped with a Nomarski differential interference contrast. Heat-killed specimens fixed in formalin-acetic acid-alcohol were processed for scanning electron microscopy according to Chitambar (1992). Species delimitation of *Hemicycliophora* in this study was performed using an integrated approach that considered morphological and morphometric evaluation combined with molecular-based phylogenetic inference (tree-based methods) and sequence analyses (genetic distance methods) (Sites & Marshall, 2004).

DNA extraction, PCR, and DNA sequencing

For molecular analyses, nematode DNA from *Hemicycliophora* samples was extracted from single or several individuals using proteinase K as described by Castillo *et al.* (2003). PCR and sequencing were completed in two laboratories: IAS-CSIC, Spain, and CDFA, USA. All detailed protocols were as described by Castillo *et al.* (2003) and Tanha Maafi, Subbotin & Moens (2003), respectively. The forward D2A (5′-ACAAGTACCGT GAGGGAAAGTTG-3′) and the reverse D3B (5′-TCGGAAGGAACCAGCTACTA-3′) primers (Subbotin *et al.*, 2006) amplifying the D2-D3 expansion segments of 28S rRNA gene and the forward primer TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and the reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) amplifying the ITS1-5.8S-ITS2 of rRNA (Curran *et al.*, 1994) were used in the present study. Two μl of the PCR product were run on a 1% Tris-acetate-EDTA (TAE) buffered agarose gel.

PCR products were purified after amplification with Geneclean turbo (Q-BIOgene SA, Illkirch Cedex, France) or QIAquick (Qiagen, USA) gel extraction kits and used for direct sequencing in both directions with the primers referred above or for cloning. The PCR products were cloned into the pGEM-T vector and transformed into JM109 High Efficiency Competent Cells (Promega, USA). Several clones of each sample were isolated using blue/white selection and submitted to PCR with

Table 1. *Hemicycliophora* species and populations used in the present study

Species	Locality	Host	Sample code	Morphological (Mo) and morphometric (Mr) studies	Molecular study: PCR-ITS-RFLP	Molecular study: D2-D3 of 28S rRNA sequence	Molecular study: ITS-rRNA sequence	Reference, collector or identifier
H. californica	Yolo County, CA, USA	*Salix* sp.	CD826B	Mo, Mr	+	KF430518, KF430519	KF430576	S. A. Subbotin, J. Chitambar
H. chilensis	Venezuela	*Theobroma cacao*	–	–	–	AY780977	–	Subbotin *et al*. (2005)
H. conida	Belgium	Unknown plant	–	–	–	FN433875	–	I. Tandingan De Ley *et al*. (unpubl. data)
H. conida	Football pitch, Madrid, Spain	Turf grasses	Bernabeu	Mo, Mr	–	KF430447	KF430580	P. Castillo
H. conida	Clallam County, WA, USA	Unknown plant	CD939	Mo, Mr	+	KF430448	KF430579	S. A. Subbotin, J. Chitambar
H. epicharoides	Serranova, Brindisi, Italy	*Ammophila arenaria*	Serranova	Mo, Mr	–	KF430512	KF430607	N. Vovlas
H. epicharoides	S. Barrameda, Cádiz, Spain	*Ammophila arenaria*	J229	Mo, Mr	+	KF430513	KF430608	P. Castillo
H. epicharoides	Epiros, Greece	*Pragmites* sp.	Greece	–	–	–	KF430606	N. Vovlas
H. floridensis	*Highway 441, Lake City, FL, USA	*Pinus elliotti*	CD772	Mo, Mr	+	KF430506	KF430536	R. N. Inserra, J. D. Stanley
H. gracilis	Hamilton City, Glenn County, CA, USA	*Prunus domestica*	CD45	Mo, Mr	–	KF430478, KF430480	KF430561, KF430562	S. A. Subbotin, J. Chitambar
H. gracilis	Butte City, Glenn County, CA, USA	*Prunus domestica*	CD63	Mo	–	KF430477, KF430481	KF430560, KF430564	S. A. Subbotin, J. Chitambar
H. gracilis	Oliverhurst, Yuba County, CA, USA	*Prunus domestica*	CD441	Mo	+	KF430479	KF430563, KF430565	S. A. Subbotin, J. Chitambar
H. gracilis†	Mendocino County, CA, USA	Unknown plant	–	–	–	–	FN435301	De Ley *et al*. (unpublished)
H. gracilis	Brooklyn Park, MN, USA	Unknown plant	CD1169	Mo	–	KF430482		D. S. Mollov, S. A. Subbotin
H. halophila	*Taylors Mistake, New Zealand	*Desmoschoenus spiralis*	CD705	Mo	+	KF430444, KF430445	KF430582, KF430583	G. Yeates
H. hellenica	*Filippias, Epirus, Greece	*Arundo donax*	Hel	Mo	–	KF430453	KF430584	N. Vovlas
H. iberica	*Arroyo Frío, Jaén, Spain	*Populus nigra*	CD337	Mo	+	KF430461	KF430539, KF430540	P. Castillo
H. iberica	Hinojos, Huelva, Spain	*Quercus suber*	978, H37	Mo, Mr	–	KF430462	–	P. Castillo
H. iberica	Santa Elena, Jaén, Spain	*Quercus suber*	Despeñaperros	Mo, Mr	–	KF430463	KF430541	P. Castillo
H. italiae	*Zapponeta, Foggia, Italy	*Ammophila arenaria*	Zapponeta	Mo	–	KF430458	–	N. Vovlas
H. lutosa	South Africa	Unknown plant	–	–	–	GQ406241, GQ406240	GQ406237	Van den Berg *et al*. (2010)
H. lutosoides	S. Pablo de Buceite, Cádiz, Spain	*Juncus* sp.	CD701	Mo	+	KF430456, KF430457	KF430537, KF430538	P. Castillo
H. lutosoides	Los Palacios y Vill., Sevilla, Spain	*Solanum tuberosum*	BT-631-10	Mo, Mr	–	KF430455	–	P. Castillo
H. lutosoides	Football pitch, Madrid, Spain	Turf grasses	Larga-Bernabeu	Mo, Mr	–	KF430454	–	P. Castillo
H. obtusa	Moguer, Huelva, Spain	*Pinus pinea*	H133	Mo, Mr	+	KF430521	KF430578	P. Castillo
H. poranga	Bajo Seco, Venezuela	Unknown plant	–	–	–	AY780975	–	Subbotin *et al*. (2005)
H. poranga	Spanish Bay, CA, USA	*Poa annua*	CD714	Mo, Mr	–	KF430432, KF430434	KF430598, KF430599	M. McClure
H. poranga	Argentina	Apium graveolens	CD513, 1873	Mo	–	KF430435, KF430442	KF430596	M. Doucet
H. poranga	Argentina	Unknown plant	CD763, 2308-1	Mo	+	KF430433	KF430594, KF430595	M. Doucet
H. poranga	San Francisco, CA, USA	*Lepidorrhachis mooreana*	CD219	Mo, Mr	–	KF430441, KF430443	KF430597, KF430600	S. A. Subbotin
H. poranga	Benamahoma, Cádiz, Spain	*Ficus carica*	CD700	Mo, Mr	–	KF430431, KF430440	–	P. Castillo

H. poranga	Bari, Italy	*Pistacia vera*	H02	Mo, Mr	–	KF430439	–	N. Vovlas
H. poranga	Santa Rosa, CA, USA	*Salix* sp.	CD800, CD801, CD802	Mo, Mr	+	–	KF430590-KF430593, KF430601	S. A. Subbotin
H. poranga	San Jose, CA, USA	*Urtica* sp.	CD902	–	–	KF430438	–	S. A. Subbotin
H. poranga	Austin Creek Road, Guerneville, CA, USA	Grasses	CD808	Mo, Mr	–	KF430437	–	S. A. Subbotin
H. poranga	Nursery, Los Angeles County, USA	*Neoregelia* sp.	CD1186	–		KF430436	–	S. A. Subbotin
H. raskii	River Bend Park, Sacramento County, CA, USA	Grasses	CD843	Mo, Mr	+	KF430520	KF430577	S.A. Subbotin, J. Chitambar
H. ripa	Moguer, Huelva, Spain	*Pinus pinea*	H167, H33	Mo, Mr	+	KF430449, KF430450	KF430587, KF430588	P. Castillo
H. similis	Cartaya, Huelva, Spain	*Fragaria x ananassa*	IAS-6–13	Mo, Mr	–	KF430464, KF430465	–	P. Castillo
H. thienemanni	Vall di Non, Trento, Italy	*Malus domestica*	–	Mo, Mr	–	AY780976	–	Subbotin *et al*. (2005)
H. thienemanni	Moscow, Russia	*Salix* sp.	CD696, CD695	Mo, Mr	+	KF430468-KF430471	KF430569-KF430572	V. N. Chizhov
H. thienemanni	Castillo de Locubin, Jaén, Spain	*Populus nigra*	Locubin	Mo	–	KF430467	KF430568	P. Castillo
H. thienemanni	Tera river, Garray, Soria, Spain	*Phragmites* sp.	Tera	Mo, Mr	–	KF430466	KF430567	P. Castillo
H. thornei	La Rambla, Córdoba, Spain	*Vitis vinifera*	M157	Mo, Mr	+	KF430452	KF430581	P. Castillo
H. typica	South Africa	Sugarcane	–	–	–	–	GQ406238, GQ406239	Van den Berg *et al*. (2010)
H. typica	South Africa	Unknown plant	CD831, Tyl2055	Mo	–	KF430515	KF430603	E. Van den Berg
H. vaccinii	Carnota, Coruña, Spain	*Pinus pinaster*	Carnota	Mo, Mr	–	KF430522	KF430542	P. Castillo
H. wyei	NC, USA	Grasses	–	–	–	–	JQ708145	Cordero López *et al*. (2013)
H. wyei	New Hanover County, NC, USA	Turf grasses	CD676	Mo, Mr	–	KF430495, KF430500	KF430528, KF430529	W. Ye
H. wyei	New Hanover County, NC, USA	Turf grasses	CD684	Mo, Mr	+	KF430497, KF430499	KF430527, KF430532	W. Ye
H. wyei	New Hanover County, NC, USA	Turf grasses	CD679	Mo, Mr	–	KF430498, KF430501	KF430534, KF430535	W. Ye
H. wyei	New Hanover County, NC, USA	Turf grasses	CD683	Mo, Mr	–	KF430504, KF430505	KF430531	W. Ye
H. wyei	Wayne County, NC, USA	Turf grasses	CD682	Mo, Mr	–	KF430503	KF430533	W. Ye
H. wyei	Carteret County, NC, USA	Turf grasses	CD685A	–	–	KF430502	KF430530	W. Ye
H. wyei	Paines Praire, FL, USA	*Andropogon virginicus*	CD759, CD760, CD791	Mo, Mr	+	KF430496	KF430523, KF430524, KF430525, KF430526	R. N. Inserra, J.D. Stanley
H. wyei‡	TX, USA	Bentgrass	–	–	–	KC329574	KC329575	X. Ma, P. Agudelo (unpubl. data)
Hemicycliophora sp. 1	Terovo, Epirus, Greece	Grasses	852	–	+	AY780974	KF430602	Subbotin *et al*. (2005), N. Vovlas
Hemicycliophora sp. 2	Birdlings Flat, New Zealand	Unknown plant	CD713	–	+	KF430516, KF430517	KF430609, KF430610	G. Yeates
Hemicycliophora sp. 3	Tingle Farms, Willcox, AZ, USA	*Zea mays*	CD715	Mo, Mr	+	KF430472, KF430473	KF430573, KF430574	M. McClure
Hemicycliophora sp. 4	Brunswick, NC, USA	Turf grasses	CD675	Mo, Mr	–	KF430487, KF430488, KF430492	KF430545, KF430546	W. Ye
Hemicycliophora sp. 4	Fort Lauderdale, FL, USA	*Phoenix roebelenii*	CD656	Mo	–	–	KF430552, KF430554	S. A. Subbotin
Hemicycliophora sp. 4	Cedar Island, FL, USA	*Borrichia* sp.	CD753	–	–	KF430490	KF430555	R.N. Inserra, J.D. Stanley

Table 1. *Continued*

Species	Locality	Host	Sample code	Morphological (Mo) and morphometric (Mr) studies	Molecular study			Reference, collector or identifier
					PCR-ITS-RFLP	D2-D3 of 28S rRNA sequence	ITS-rRNA sequence	
Hemicycliophora sp. 4	Fort Myers, FL, USA	Cynodon sp.	CD766	Mo, Mr	–	KF430489	KF430553	M. McClure
Hemicycliophora sp. 4	Indian Hills, CA, USA	Turf grasses	CD764	–	–	KF430491	KF430549, KF430556	M. McClure
Hemicycliophora sp. 4	La Cantera, San Antonio, TX, USA	Turf grasses	CD793	Mo	–	–	KF430543, KF430544	M. McClure
Hemicycliophora sp. 4	St. Augustine, FL, USA	*Borrichia* sp.	CD748	Mo, Mr	–	–	KF430550, KF430551	R. N Inserra, J. D. Stanley
Hemicycliophora sp. 4	Osteen, FL, USA	Grasses	CD789	Mo, Mr	+	–	KF430547, KF430548	R. N. Inserra, J. D. Stanley
Hemicycliophora sp. 4§	Wayne County, NC, USA	Turf grasses	–	–	–	–	JQ708144	Cordero López *et al.* (2013)
Hemicycliophora sp. 5	Carteret County, NC, USA	Turf grasses	CD685B	–	–	–	KF430575	W. Ye
Hemicycliophora sp. 6	Kaitoke Waterworks, New Zealand	*Nothofagus* forest	CD560	–	+	KF430446	KF430585, KF430586	G. Yeates
Hemicycliophora sp. 7	Almonte, Huelva, Spain	*Pinus pinea*	H144	–	–	KF430451	KF430589	P. Castillo
Hemicycliophora sp. 8	Monterey, CA, USA	Turf grasses	CD765	Mo, Mr	+	KF430494	KF430559	M. McClure
Hemicycliophora sp. 8	Henrieville, UT, USA	Unknown plant	347	–	–	KF444173	–	L. Poiras *et al.* (unpubl. data)
Hemicycliophora sp. 9¶	Brake, Germany	Unknown plant	–	–		AY780973	–	Subbotin *et al.* (2005)
Hemicycliophora sp. 9	Jaroslavl region, Russia	*Agrostis* sp.	CD755	Mo, Mr	+	KF430510	KF430604	V. N. Chizhov
Hemicycliophora sp. 9	Preveza, Preveza, Greece	*Trifolium repens*	CD702	–	–	KF430509, KF430511, KF430514	KF430605	N. Vovlas
Hemicycliophora sp. 10	Yolo County, CA, USA	*Salix* sp.	CD826A	Mo, Mr	+	KF430483-KF430486	KF430566	S. A. Subbotin, J. Chitambar
Hemicycliophora sp. 11	Paines Praire, FL, USA	*Andropogon virginicus*	CD790	Mo, Mr	+	KF430493	KF430557, KF430558	R. N. Inserra, J. D. Stanley
Hemicycliophora sp. 12	Brooklyn Park, MN, USA	Unknown plant	CD1160	–	–	KF430475	–	D. S. Mollov, S. A. Subbotin
Hemicycliophora sp. 12	Saint Paul, MN, USA	Grasses	CD1087	–	–	KF430474	–	D. S. Mollov, S. A. Subbotin
Hemicycliophora sp. 12	Sedona, AZ, USA	Unknown	CD1316	–	–	KF430476	–	S. A. Subbotin
Hemicycliophora sp. 13	Nursery, Los Angeles County, CA, USA	*Neoregelia* sp	CD1187	–	–	KF430507, KF430508	–	S. A. Subbotin
Hemicycliophora sp. 14	Monteagudo Isl., Pontevedra, Spain	*Pinus pinaster*	J74, J75	–	–	KF430459, KF430460	–	P. Castillo

*Type locality.
†Originally identified as *H. conida*.
‡Originally identified as *H. uniformis*.
§Originally identified as *H. pruni*.
¶Originally identified as *H. typica*.
ITS, internal transcribed spacer; RFLP, restriction fragment length polymorphism.

same primers. PCR products from each clone were sequenced in both directions. The newly obtained sequences were submitted to the GenBank database under accession numbers KF430431–KF430610 as indicated in Table 1 and on the phylogenetic trees.

Sequence and phylogenetic analyses

The newly obtained sequences of the D2-D3 of 28S rRNA and the ITS rRNA were aligned using ClustalX 1.83 (Thompson *et al*., 1997) with default parameters with their corresponding published gene sequences (Subbotin *et al*., 2005; Van den Berg *et al*., 2010; Cordero López *et al*., 2013). Outgroup taxa for each dataset were chosen according to the results of a previous study (Subbotin *et al*., 2006). Three alignments were developed: (1) D2-D3 of 28S rRNA gene sequence alignment; (2) ITS rRNA gene sequence alignment; (3) combined D2-D3 and ITS rRNA gene sequence alignment with reduced sequence number. The last alignment contained one sequence from each morphologically characterized species.

The alignments were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The best fit model of DNA evolution was obtained using the program jModeltest 0.1.1 (Posada, 2008) under the Akaike information criterion. The general time reversible substitution model with estimation of invariant sites and assuming a gamma distribution with four categories (GTR + I + G) was selected as the optimal nucleotide substitution model for the analyses. BI analysis for each gene was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. Two runs were performed for each analysis. The Markov chains were sampled at intervals of 100 generations. After discarding burn-in samples other trees were used to generate a 50% majority rule consensus tree.

Maximum likelihood (ML) and maximum parsimony (MP) trees were reconstructed from the combined reduced data set alignment using PAUP* 4.0b 10 (Swofford, 2003) with 1000 bootstrap replicates. For testing of alternative topologies in ML, we used the Shimodaira–Hasegawa (SH) test as implemented in PAUP. Trees were visualized using TreeView (Page, 1996). Sequence analyses of alignments were performed with PAUP. Pairwise divergences between taxa were computed as absolute distance values and as percentage mean distance values based on whole alignment, with adjustment for missing data.

The evolution of six morphological/morphometric characters: body length, stylet length, R (total number of body annuli), RV (number of annuli between posterior end of body and vulva), vulval lips, and tail shape, and one biological character (presence or absence of males), frequently used in taxonomy for their diagnostic value, were traced over the molecular phylogeny on the tree obtained from the combined data set using MP and BI approaches. Information on the characters was obtained from the present study and retrieved from referenced literature (Van den Berg *et al*., 2010; Cordero López *et al*., 2013; Chitambar & Subbotin, 2014). Characters were recorded as binary and multistate characters. Character changes were traced on the MP tree using the parsimony ancestral state reconstruction (ASR) methods with MESQUITE 2.75 (Maddison & Maddison, 2010) under a Markov one-rate model. To account for topological uncertainty, we used the 'trace character over trees' option. Because the statistical properties of ASR models are not well stated, ancestral character states were also estimated according to their posterior probability distributions in a Bayesian approach using the program SIMMAP (Bollback, 2006). The equal-rates model for multistate data was selected. The character states were set as unordered. The gamma distribution for the overall evolutionary rate was chosen and default parameters were maintained ($\alpha = 1.25$, $\beta = 0.25$, $k = 60$).

Molecular diagnostics of Hemicycliophora with PCR-ITS-RFLP

Five to seven µl of purified PCR product of ITS rDNA was digested by one of the following restriction enzymes: *Ava*I, *Bsh*1236I, *Dra*I, *Hin*fI, or *Hin*6I in the buffer stipulated by the manufacturer. The digested DNA was separated on a 1.4% TAE buffered agarose gel, stained with ethidium bromide, visualized on a UV transilluminator, and photographed. The length of each restriction fragment from the PCR products was obtained by a virtual digestion of the sequences using WebCutter 2.0 (http://www.firstmarket.com/cutter/cut2.html) or estimated from a gel.

RESULTS

Species identification and delimiting

Integrating traditional morphological taxonomic characters and molecular criteria, we distinguished 20 valid species within the studied samples: *Hemicycliophora californica* Brzeski, 1974, *Hemicycliophora conida* Thorne, 1955, *Hemicycliophora epicharoides* Loof, 1968, *Hemicycliophora floridensis* (Chitwood & Birchfield, 1957) Goodey, 1963, *Hemicycliophora gracilis* Thorne, 1955, *Hemicycliophora halophila* Yeates, 1967, *Hemicycliophora hellenica* Vovlas, 2000, *Hemicycliophora iberica* Castillo, Gómez-Barcina & Loof, 1989, *Hemicycliophora italiae* Brzeski & Ivanova, 1978, *Hemicycliophora lutosoides* Loof, 1984, *Hemicycliophora obtusa* Thorne, 1955, *Hemicycliophora poranga* Monteiro & Lordello, 1978, *Hemicycliophora raskii* Brzeski, 1974, *Hemicycliophora ripa* Van den Berg, 1981, *Hemicycliophora similis* Thorne 1955, *Hemicycliophora thienemanni* (Schneider, 1925) Loos, 1948, (= *Loofia thienemanni* (Schneider, 1925) Siddiqi, 1980) *Hemicycliophora thornei* Goodey, 1953, *Hemicycliophora typica* de Man, 1921, *Hemicycliophora vaccinii* Reed & Jenkins, 1963 [= *Loofia vaccini* (Reed & Jenkins, 1963) Siddiqi, 1980], and *Hemicycliophora wyei* Cordero López, Robbins & Szalanski, 2013 (Table 1). Most of the soil samples examined in this study were monospecific and only two samples contained a mixture of two species.

Morphology and morphometrics of *Hemicycliophora* species

Brief morphological descriptions with illustrations (Figs 1–5, S1–S17) and morphometric values (Tables S1–S8) are given for the populations of the earlier mentioned 20 *Hemicycliophora* species. All topotype populations of *H. floridensis*, *H. halophila*, *H. hellenica*, *H. iberica*, and *H. italiae* were compared with paratypes and were coincident with the original descriptions (*viz*. Chitwood & Birchfield, 1957; Yeates, 1967; Vovlas, 2000; Castillo *et al*., 1989; Brzeski & Ivanova, 1978; respectively) in their morphology and morphometrics (Figs 2A, F, K, 4, S4, S5; Table S2).

Hemicycliophora californica Brzeski, 1974 (Fig. 1F, L, R, Tables 1, S1)

Hemicycliophora californica was first found in association with apricot roots in southern California. Morphological analysis of a population from Yolo County, California, showed that females were characterized by a straight or ventrally arcuate body, lateral fields marked by breaks and anastomoses of transverse striae, forming an occasional discontinuous, single longitudinal line over short distances in mid- and prevulval body regions, annuli outside lateral fields smooth or inconsistently marked with fine lines or ridges, lip region round to hemispherical with three annuli, labial disc slightly protruding and rounded in lateral view, sometimes low and flat, vulval lips modified (slightly elongated), postvulval body constricted immediately behind vulva then cylindrical to anus and tail cylindrical then tapering abruptly to a uniformly conical posterior third region, occasionally with a greater dorsal curvature, with a short conical to slightly attenuated conical spike and narrowly rounded terminus. Tail terminus distinctly annulated.

Except for the infrequent presence of fine lines or ridges and occasional irregular lines on annuli outside the lateral fields, and three lip annuli (vs. two annuli), the morphology and morphometrics of the Californian population of *H. californica* studied here agreed well with the original and subsequent descriptions by Brzeski (1974) and Costa-Manso (1996), respectively. This species has been found only in California and Idaho, USA.

Distinguishing characters include a conoid tail with a narrowly rounded to acute terminus, and presence of males. *Hemicycliophora californica* is differentiated from the morphologically similar species *H. raskii* and *Hemicycliophora montana* Eroshenko, 1980 (Chitambar & Subbotin, 2014).

Hemicycliophora conida Thorne, 1955 (Figs 1A, G, M, S1; Tables 1, S1)

Hemicycliophora conida was described by Thorne from a population collected from sugarbeet near Ballyculane, Ireland. Two populations of this species found in Spain and Washington, USA, are described in this study. Females were characterized by a straight or ventrally arcuate body, lateral fields marked by breaks and anastomoses with three longitudinal lines starting as a distinct central line from just posterior to pharyngeal base to near the midbody region where two faint lines appear adjacent on either side forming two rows of blocks with the transverse striae, lip region with rounded anterior margins, two distinct annuli, three annuli suggested with first annulus less distinct and narrower than remaining two distinct lip annuli, vulval lips modified, vulval sheath about one to two annuli long and annuli at tail tip smaller than at anterior tail. No males were found.

Morphology and morphometrics of the Spanish and Washington populations were coincident with those provided for this species (Thorne, 1955; Loof, 1968), except for minor intraspecific morphometric differences (e.g. body length 683–912 vs. 660–990 μm; stylet 70–89 vs. 73–96 μm). *Hemicycliophora conida* has been widely reported in European countries (Loof, 1968; Brzeski, 1974, 1998; Peña-Santiago *et al*., 2004) and Iran (Loof, 1984). This is the first report of *H. conida* from the Americas. The specimens recently identified as *H. conida* by Zeng *et al*. (2012) from grasses in North and South Carolina do not fully fit the original and other *H. conida* descriptions [e.g. tail length 100.5–128.2 vs. 60–93 μm; Rex (number of annuli between anterior end of body and excretory pore) 49–67 vs. 32–52 and others] and probably belong to *H. wyei* or other species.

Distinguishing characters of this species include lateral fields marked by anastomoses bordered on both sides by rows of ornamentations, R 175–278, stylet 69–101 μm, a short conoid tail ending in a narrowly rounded to acute terminus, and presence of males. *Hemicycliophora conida* is differentiated from the morphologically similar species *Hemicycliophora iwia* Brzeski, 1974 and *Hemicycliophora ovata* Colbran, 1962 (Chitambar & Subbotin, 2014).

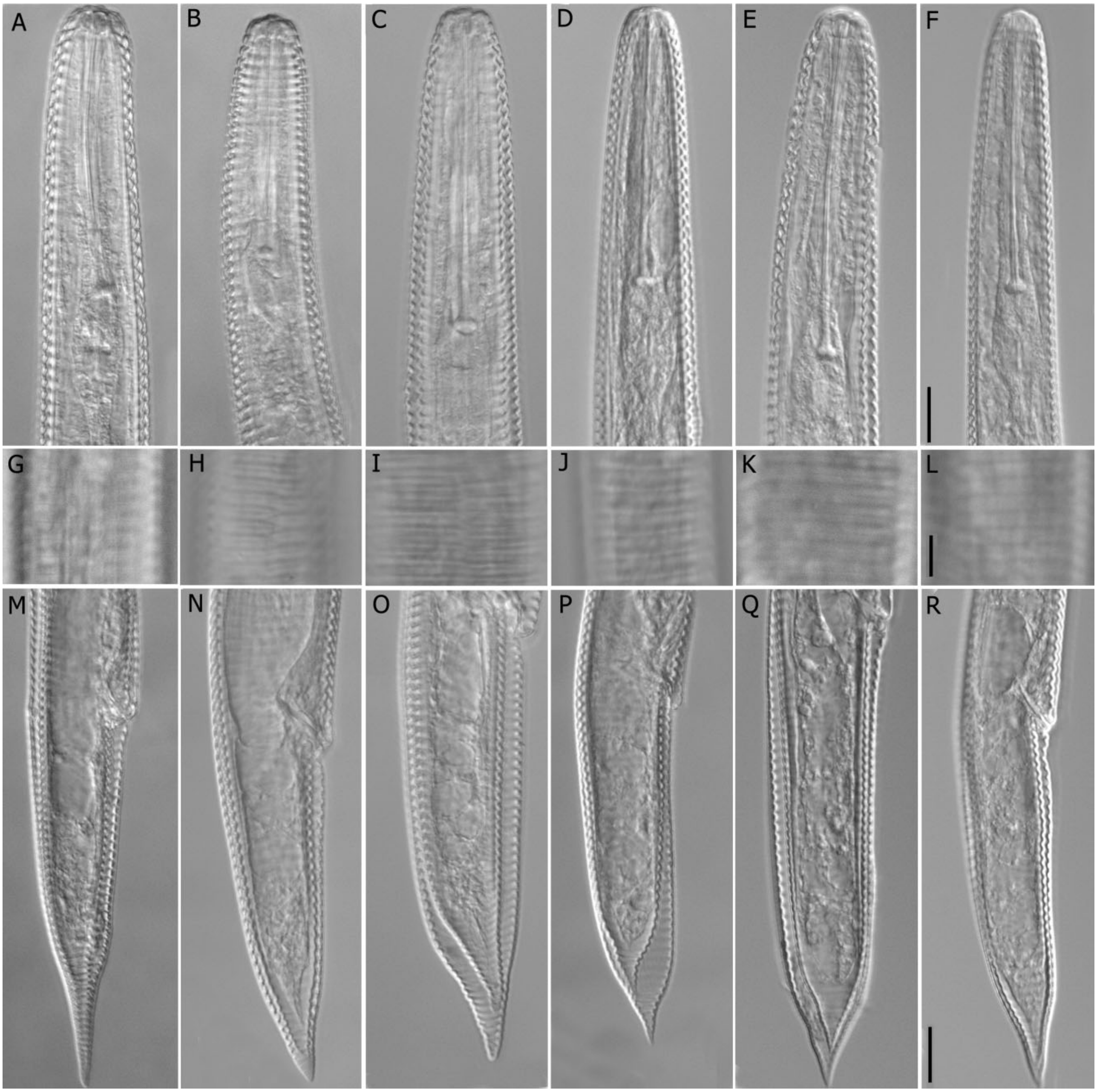

Figure 1. Photomicrographs of specimens of populations of selected *Hemicycliophora* species. A–F, anterior region; G–L, lateral field; M–R, posterior region. A, G, M, *Hemicycliophora conida* (Washington State, USA); B, H, N, *Hemicycliophora* sp. 3 (Arizona, USA); C, I, O, *Hemicycliophora* sp. 8 (California, USA); D, J, P, *Hemicycliophora raskii* (California, USA); E, K, Q, *Hemicycliophora* sp. 10 (California, USA); F, L, R, *Hemicycliophora californica* (California, USA). Scale bars: A–F, M–R = 10 µm; G–L = 5 µm.

HEMICYCLIOPHORA EPICHAROIDES LOOF, 1968 (FIG. S2, TABLES 1, S1)

Hemicycliophora epicharoides was described by Loof from sandy soil in the Netherlands. The populations of this species described in this study were collected from sandy soils in Italy and Spain. Females of these populations were characterized by coarse body annuli (5–7 µm wide), the number of body annuli fewer than 190, a rounded lip region (18.5–22 µm wide), lateral fields marked by breaks in the annuli, vulval lips modified, slightly elongate, and tail cylindroid, then becoming bluntly triangular distally, without sharp demarcation between the two parts. Morphologically and

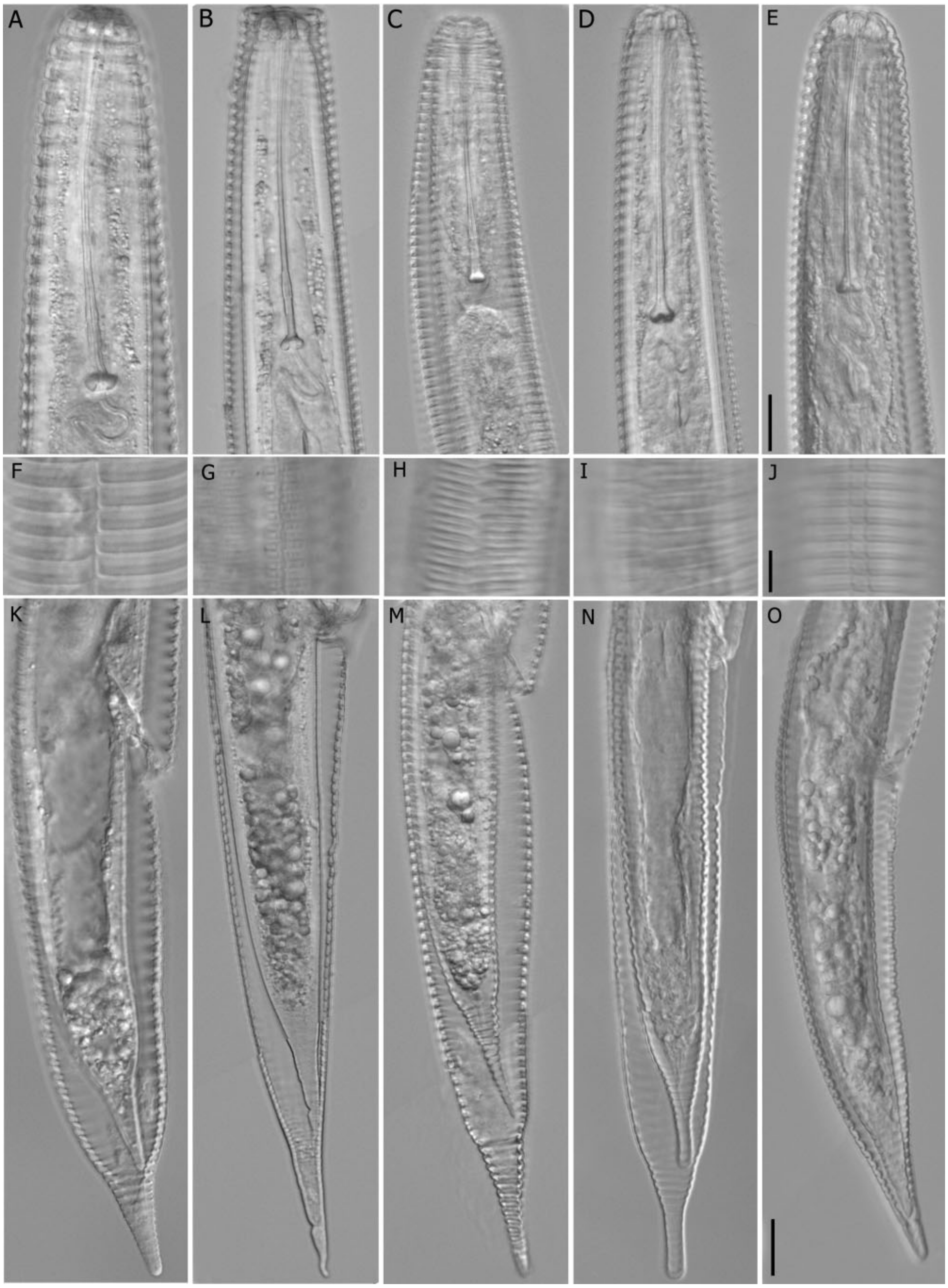

Figure 2. Photomicrographs of specimens of populations of selected *Hemicycliophora* species. A–E, anterior region; F–J, lateral field: K–O, posterior region. A, F, K, *Hemicycliophora floridensis* (topotype, Florida, USA); B, G, L, *Hemicycliophora poranga* (California, USA); C, H, M, *Hemicycliophora* sp. 11 (Florida, USA), D, I, N, *Hemicycliophora* sp. 4 (North Carolina, USA); E, J, O, *Hemicycliophora wyei* (North Carolina, USA). Scale bars: A–E, K–O = 10 μm; F–J = 5 μm.

morphometrically the Italian and Spanish populations are similar to the original description (Loof, 1968), except for minor differences including a looser cuticular sheath, smaller body length/maximum body width (17.1–22.2 vs. 21–27) value, and a slightly smaller tail length/body width at anus value (1.5–2.1 vs. 2.1–2.7).

This species was found in several European countries including France, Italy, the Netherlands, Poland, and Spain (Loof, 1968; Germani & Luc, 1973; Brzeski 1974; Vovlas & Inserra, 1980; Peña-Santiago *et al*., 2004), Korea (Choi & Geraert, 1995) and South Africa (Van den Berg & Tiedt, 2001).

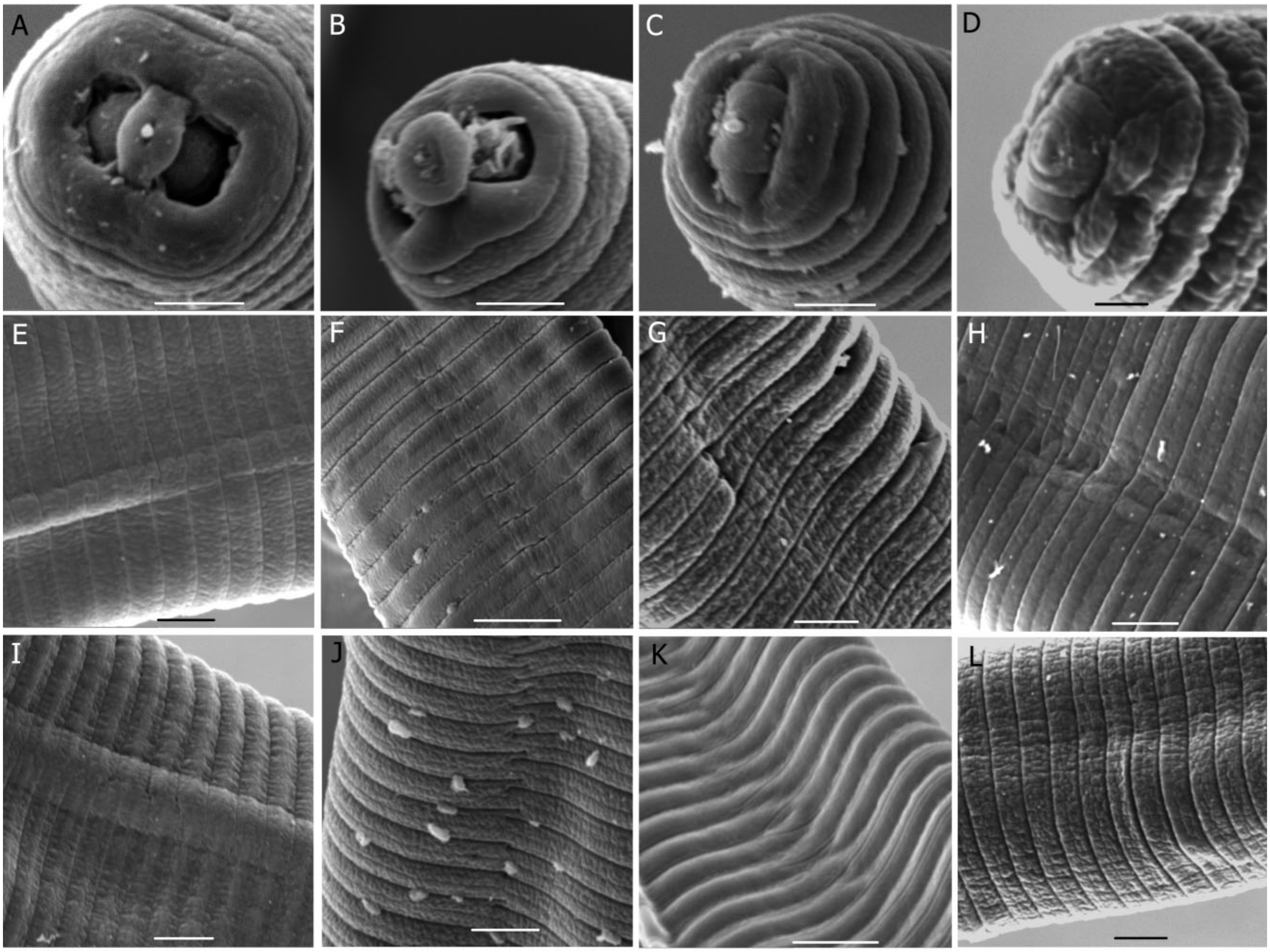

Figure 3. Scanning electron microscope (SEM) micrographs of specimens of populations of selected *Hemicycliophora* species. A–D, lip region; E–L, lateral field. A, *Hemicycliophora wyei* (North Carolina, USA) (CD683); B, *Hemicycliophora poranga* (California, USA) (CD714); C, *Hemicycliophora* sp. 3 (Arizona, USA) (CD715); D, *Hemicycliophora californica* (California, USA) (CD826B); E, *Hemicycliophora gracilis* (California, USA) (CD45); F, *H. wyei* (North Carolina, USA) (CD679); G, *H. californica* (CD826B); H, *H. wyei* (CD683); I, *H. poranga* (CD714); J, *Hemicycliophora* sp. 3 (CD715); K, *Hemicycliophora* sp. 4 (North Carolina, USA) (CD675); L, *H. californica* CD826B). Scale bars: A–C, E, G–J, L = 5 μm; D = 2 μm; F, K = 10 μm.

Distinguishing characters of this species include lip annuli not separated from the rest of the body, stylet knobs with large cavity, lateral fields with two lines, a conoid tail with a rounded to acute terminus, and presence of males. *Hemicycliophora epicharoides* is differentiated from the morphologically similar species *Hemicycliophora demani* Edward & Rai, 1971, and *Hemicycliophora koreana* Choi & Geraert, 1971 (Chitambar & Subbotin, 2014).

HEMICYCLIOPHORA GRACILIS THORNE, 1955 (FIGS 3E, S3; TABLES 1, S2)

Hemicycliophora gracilis was described by Thorne from specimens collected in five states in the USA. The populations described in this study are from California and one from Minnesota. Females of these populations were characterized by a ventrally arcuate body, cuticular sheath loosely fitting, often extending over anterior end, lateral fields marked by breaks and anastomoses in between two longitudinal lines, annuli smooth outside lateral field, sometimes marked with few short, irregular longitudinal lines that mark midbody region near lateral fields, lip region rectangular to slightly hemispherical, three lip annuli with first annulus narrower than second and third lip annuli and often difficult to discern, labial disc slightly elevated, vulval lips modified, about two annuli long, tail anteriorly cylindrical then tapering uniformly to an attenuated conical posterior third with almost cylindrical attenuation with a rounded terminus. Annulation more or less distinct on terminus. One male was found (Fig. S3). This is the first report of males for this species.

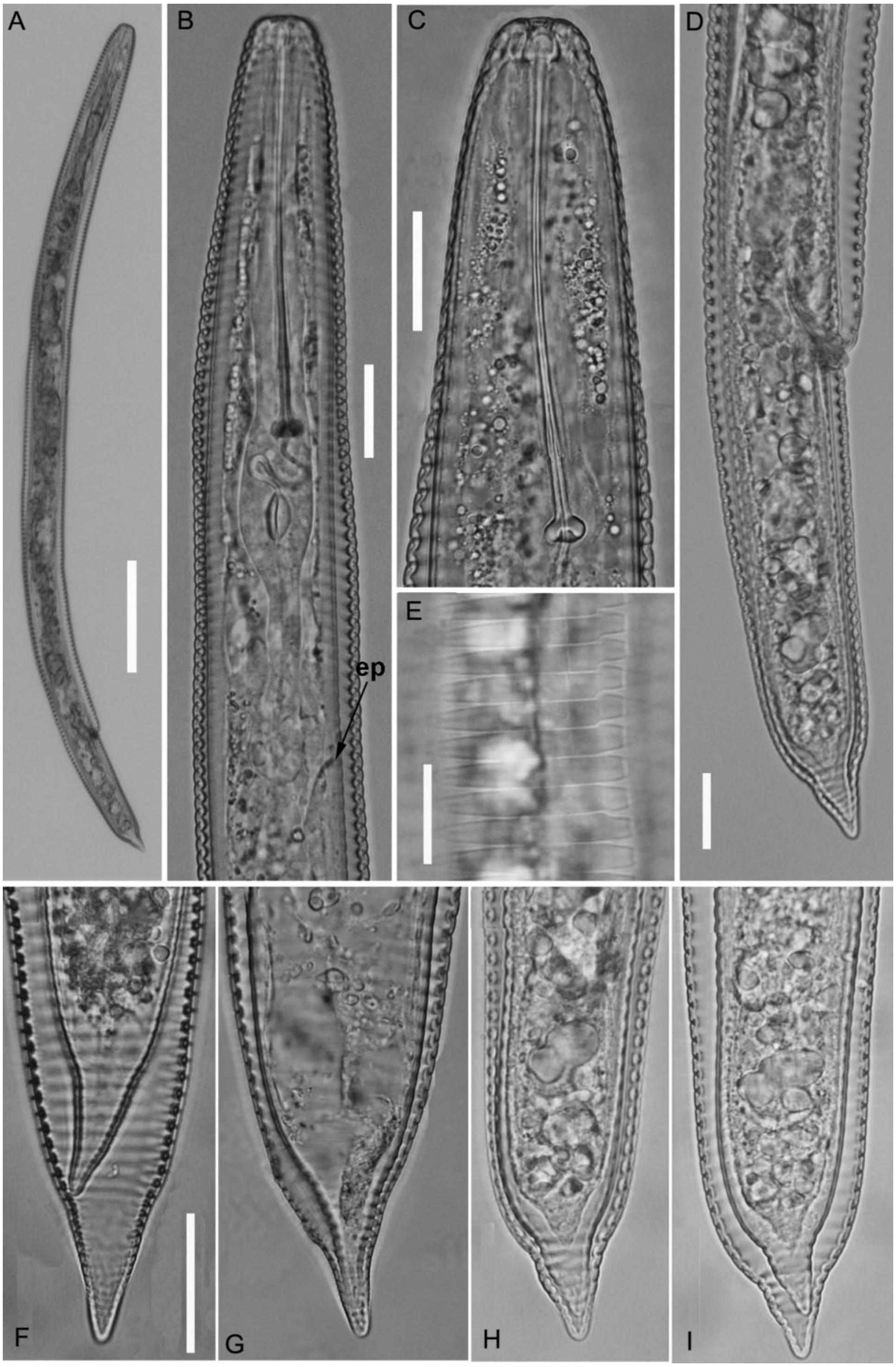

Figure 4. Photomicrographs of specimens of a new Spanish population of *Hemicycliophora iberica* Castillo *et al.*, 1989. A, entire female body; B, female pharyngeal region; C, female anterior region; D, posterior region; E, detail of lateral field; F–I, female tail tips. Scale bars: A = 100 μm; B–D, F–I = 20 μm; E = 10 μm. ep, excretory pore.

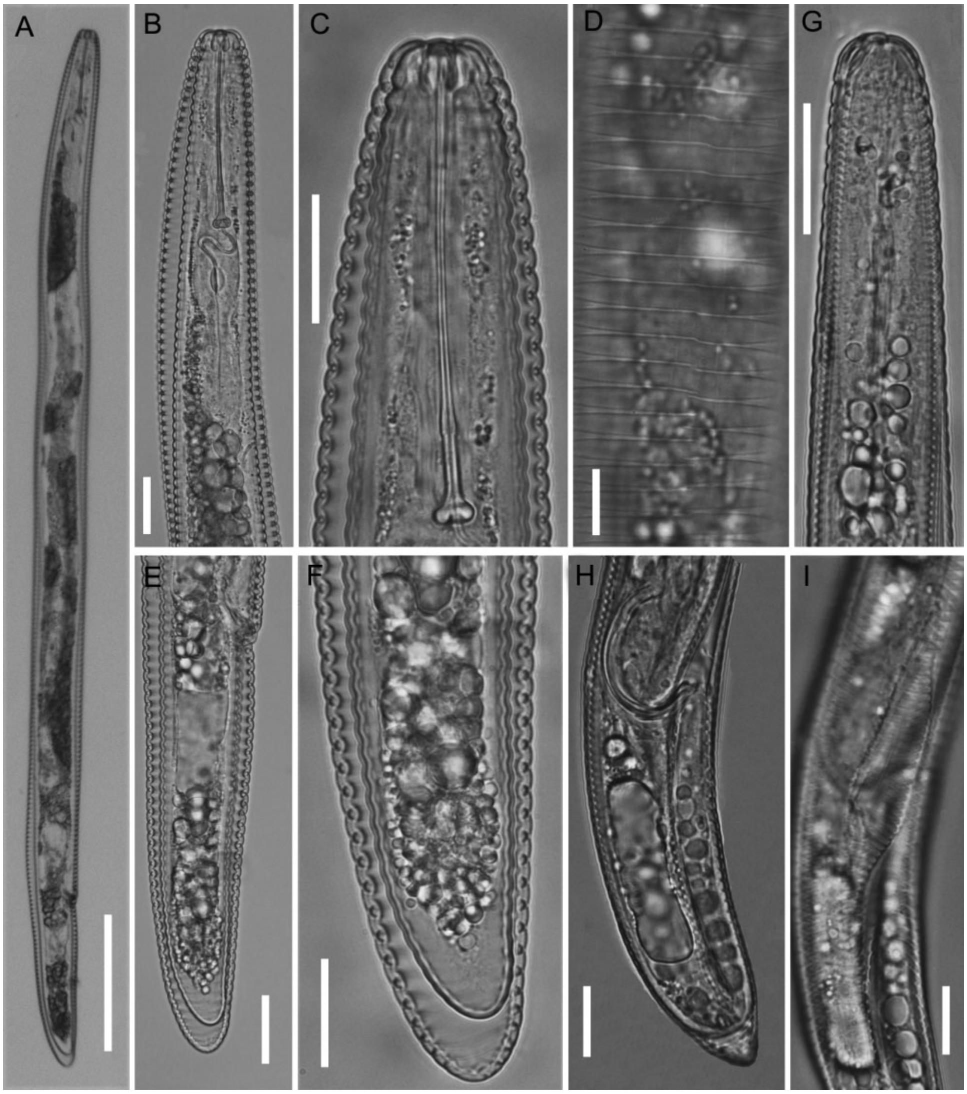

Figure 5. Photomicrographs of specimens of a Spanish population of *Hemicycliophora obtusa* Thorne, 1955. A, entire female body; B, female pharyngeal region; C, female anterior region; D, detail of lateral field; E, F, vulval and tail regions; G, pharyngeal region of pre-adult male showing absence of stylet; H, I, detail of spicules and bursa of pre-adult male. Scale bars: A = 100 µm; B, C, E–I = 20 µm; D = 10 µm.

The Californian population of *H. gracilis* closely resembles other populations reported elsewhere in the USA (Thorne, 1955; Brzeski, 1974), but differs from them by a slightly shorter female body (1044–1212 vs. 1230–1700 µm), shorter stylet (91–116 vs. 111–132 µm), smaller R (322–380 vs. 341–395), and three vs. two lip annuli.

Distinguishing characters of this species include a long body (1.2–1.5 mm) and stylet (111–132 µm), posterior part of tail distinctly offset with a spicate terminus, and presence of males. *Hemicycliophora gracilis* is differentiated from the morphologically similar species *Hemicycliophora litorea* Van den Berg, 1988 (Chitambar & Subbotin, 2014).

HEMICYCLIOPHORA IBERICA CASTILLO, GÓMEZ-BARCINA & LOOF, 1989 (FIG. 4; TABLES 1, S2)

Two new Spanish populations of *H. iberica* from Hinojos, Huelva Province, and Santa Elena, Jaén Province, were detected and found to be similar in morphology and morphometrics to the original description (Castillo *et al.*, 1989), except for minor differences such as a shorter stylet (80–91 vs. 79.94 µm) and a smaller Rst (number of annuli between anterior end of body and stylet base) value (15–27 vs. 18–31). As the species was only known from the type locality, the new records of the species extend its distribution within southern Spain.

Distinguishing characters of this species include two incisures, truncated lip region with three annuli, a long stylet, absence of males, tail elongate-triangular with distal part offset (Castillo *et al.*, 1989).

HEMICYCLIOPHORA LUTOSOIDES LOOF, 1984 (FIG. S6; TABLES 1, S3)

Hemicycliophora lutosoides was described by Loof (1984) from populations collected in Iran and Iraq. Two female populations of *H. lutosoides* from San Pablo de Buceite, Cádiz, and Los Palacios y Villafranca, Seville Province in Spain are described here. Females of these populations were characterized by a truncate-rounded lip region with a barely protruding labial disc, body annuli more than 300, lateral fields marked by breaks, vulval lips modified, and an elongate tail tapering uniformly to an acute terminus. Morphology and morphometrics of these populations were similar to the original description (Loof, 1984), except for minor intraspecific differences including a smaller number of body annuli (303–364 vs. 297–376) and a smaller Rex (54–65 vs. 57–70). The present record of *H. lutosoides* is the second one from Spain, after Bello (1979), and the third after its original description. An additional population with similar morphometrics was collected in Hinojos, Huelva Province, Spain, associated with wild olive.

Costa-Manso (1998) synonymized this species with *Hemicycliophora lutosa* Loof & Heyns, 1969, from South Africa considering described differences in body and tail annuli as intraspecific variations. However, our molecular data do not support this synonymy and indicate that *H. lutosoides* is a valid species.

Distinguishing characters of this species include a truncated and not elevated labial disc, lateral fields marked by breaks and anastomoses, slightly elongated vulval lips, a conical postvulval region, a uniformly tapering elongate conoid tail with an acute terminus, and absence of males. *Hemicycliophora lutosoides* is differentiated from the morphologically similar species *H. lutosa* (Chitambar & Subbotin, 2014).

HEMICYCLIOPHORA OBTUSA THORNE, 1955 (FIG. 5; TABLES 1, S3)

Hemicycliophora obtusa was described by Thorne from a population found in Utah, USA. The population described in this study was found in the rhizosphere of Scotch pine growing in sandy soil in Spain. Females of this population were characterized by a cuticular sheath closely adpressed to the cuticle, lip region continuous with body and bearing two annuli, labial disc undistinguishable, lateral fields with anastomoses or breaks in striae, vulval lips modified, slightly modified and tail typically hemispheroid with rounded terminus and usually smooth tip (although annulation was observed in some specimens). Spermatheca almost hemispherical (20–22 μm wide), filled with rounded sperm cells, 1.5–2.0 μm in diameter. Males very rare, only a pre-adult stage was detected without a stylet, a rounded tail tip, a short bursa, and semicircular spicules. The morphology and morphometrics of the Spanish population agree with the original description and redescription (Thorne, 1955; Brzeski, 1974), except for minor intraspecific differences: R (190–219 vs. 219–268), VL/VB (distance between vulva and posterior end of body divided by body width at vulva) (2.9–4.2 vs. 3.0–4.4), V [(distance from head end to vulva/ body length)*100] (82–86 vs. 86–90%), and a slightly shorter stylet (62–88 vs. 78–98 μm). *Hemicycliophora obtusa* is known only from the type population from Utah, USA. The present record of *H. obtusa* is the first from Europe and the second world record after the original description. An additional population with similar morphometrics was collected in Sanlúcar de Barrameda, Cádiz Province, Spain, associated with wild olive.

Distinguishing characters of this species include the absence of longitudinal lines outside lateral fields, lateral fields without lines, a hemispherical tail terminus, and absence of males. *Hemicycliophora obtusa* is differentiated from the morphologically similar species *H. arenaria* (Chitambar & Subbotin, 2014).

HEMICYCLIOPHORA PORANGA MONTEIRO & LORDELLO, 1978 (FIGS 2B, G, L, 3B, I, S7; TABLES 1, S4)

Hemicycliophora poranga was described by Monteiro and Lordello from a population in a cabbage field in Brazil. The populations examined in this study were collected in Italy, Spain, and California, USA. Females of these populations were characterized by a rounded to hemispherical lip region in lateral view with an elevated labial disc. Lateral fields marked by breaks and anastomoses, which, in some populations, are between two more or less distinct longitudinal lines starting near base of pharynx and extending to the posterior part of the body. Vulval lips modified, about one to two annuli long, posteriorly directed with short vulval sleeve. Tail tapering uniformly to a narrow, attenuated conical posterior part with a finely rounded terminus. Annulation on tail almost distinct up to terminus. Morphology and morphometrics of Italian and Spanish populations are generally similar to the original description (Monteiro & Lordello, 1978). The present records of *H. poranga* are the first from Italy and Spain, and constitute the first records in Europe. The species has been reported from Argentina (Doucet, 1982; Chaves, 1983), Iran (Jamali *et al.*, 2004), Venezuela (Crozzoli & Lamberti, 2006), and California, USA (Chitambar, 1994).

Distinguishing characters of this species include an elevated labial disc and elongate conoid tail uniformly tapering to a narrowly rounded to acute terminus, and presence of males. *Hemicycliophora poranga* is differentiated from the morphologically similar species *Hemicycliophora ornamenta* Bajaj, 1998, *H. ripa* Van den Berg, 1981, *Hemicycliophora subaolica* Jairajpuri & Baqri, 1973, and *Hemicycliophora micoletzkyi* Goffart, 1951 (Chitambar & Subbotin, 2014).

Hemicycliophora raskii Brzeski, 1974 (Fig. 1D, J, P; Tables 1, S3)

Hemicycliophora raskii was described by Brzeski from a population collected from the rhizosphere of almond trees in Oakley, Contra Costa County, California, USA. This species is known only from California. Another population was found in Sacramento County, California, and described in this study. Females of this population were characterized by a slightly ventrally arcuate body, cuticular sheath closely or loosely fitting, lateral fields marked by anastomoses and continuous transverse striae – a short, irregular longitudinal line was observed in one specimen, lip region rounded to hemispherical with three annuli usually with first lip annuli faintly visible labial disc not protruding, vulval lips modified, about one annulus long posteriorly directed, tail cylindrical then abruptly conoid to wedge-shaped in posterior third tapering uniformly to a narrow, slightly attenuated spike with a finely rounded terminus; variable forms having a more conoid posterior region with a wider conical spike or with a rounded terminus.

The population closely resembles the original description of *H. raskii* (Brzeski, 1974) in lip region shape and number of annuli, markings of the lateral fields and annuli, shape of vulval lips and tail. The presence of distinct irregular annular lines outside and near the lateral fields have not been reported earlier for this species, and the studied population differed from the original description by a slightly shorter stylet (77–85 vs. 86–94 μm), slightly larger R (194–234 vs. 166–201), and RV-anterior end (number of body annuli from anterior end to vulva) (161–194 vs. 134–163).

Distinguishing characters of this species include the presence of longitudinal lines or scratches outside lateral fields, very small stylet knobs with very small or no cavity, a conical to wedge-shaped tail with a finely rounded terminus, and absence of males. *Hemicycliophora raskii* is differentiated from the morphologically similar species *H. californica* (Chitambar & Subbotin, 2014).

Hemicycliophora ripa Van den Berg, 1981 (Fig. S9; Tables 1, S3)

Hemicycliophora ripa was described by Van den Berg from a population found in South Africa. A population of this species was also found in Spain and described in this study. Females of the Spanish population were characterized by a loosely fitting cuticular sheath and typical cuticular ornamentation, appearing as blocks inside annuli, a rounded lip region with two annuli and an elevated labial disc, 7.7 ± 0.6 (7.0–8.0) μm wide, body annuli more than 240, lateral fields marked by anastomoses or breaks, vulval lips modified, and an elongate tail tapering uniformly to an acute terminus. Morphology and morphometrics of the Spanish population are coincident with the original description (Van den Berg, 1981) and those of Argentinean populations (Doucet, 1982; Chaves, 1983) except for minor intraspecific differences including a slightly wider lip region (20–23 vs. 16.2–21.1 μm), a slightly smaller number of body annuli (229–248 vs. 246–288, 241–316), a slightly longer stylet (94–110 vs. 81.5–98.6, 65–97 μm), a slightly larger VA%T value (54.2–86.3 vs. 25.1–70.7, 35–65), and a slightly shorter tail (78–103 vs. 84–172, 60–113 μm). The present record of *H. ripa* is the first from Spain, and constitutes the first record in Europe.

Distinguishing characters of this species include a loosely fitting cuticular sheath, lateral fields marked only by anastomoses or breaks in the striae occasionally forming a faint line, and the absence of males. *Hemicycliophora ripa* is distinguished from the morphologically similar species *H. subaolica*, *Hemicycliophora popaensis* Van den Berg & Tiedt, 2005, and *Hemicycliophora catarinensis* Costa-Manso, 1996 (Chitambar & Subbotin, 2014).

Hemicycliophora similis Thorne 1955 (Fig. S10; Tables 1, S3)

Hemicycliophora similis was described by Thorne from agricultural and noncultivated fields in the Pacific North West, USA. A population of this species was found in strawberry rhizosphere soil in Cartaya, Huelva Province, Spain and described in this study. This Spanish population was characterized by a rounded lip region with two or three annuli and a barely protruding labial disc (6.0–6.5 μm wide), body annuli around 300, lateral fields marked by breaks or anastomoses of body annuli without longitudinal lines, stylet knobs rounded, without distinct cavity, hemizonid usually undistinguishable, occupying two annuli; vulval lips not modified (not elongated, rounded) and tail with a conoid-rounded end, clearly offset with a terminal somewhat elongate spike (39–52 μm long). Morphology and morphometrics of this population were similar to those of the original description and redescription (Thorne, 1955; Brzeski, 1974), except for minor intraspecific differences including a slightly longer body (1022–1578 vs. 1110–1200 μm) and stylet (91–108 vs. 88–96 μm), and larger VL/VB ratio (4.8–7.6 vs. 4.1–5.2). The present record of *H. similis* is the first for Europe and Spain, and the second after the original description from Utah, USA (Thorne, 1955). Arias, López-Pedregal & Jiménez-Millán (1963) and Jiménez-Millán *et al*. (1965) recorded this species from southern Spain in Seville Province, but according to Bello (1979) this material belongs to *H. thornei*.

Distinguishing characters of this species include a rounded labial disc, vulval lips modified and with an elongated posterior lip, an elongate conoid tail tapering to a distal, offset and narrower elongated portion with a narrowly rounded terminus, and the absence of males. *Hemicycliophora similis* is differentiated from morphologically similar species, *Hemicycliophora filicauda* Doucet, 1982, *H. thornei*, *H. vaccinii*, and *Hemicycliophora zuckermani* Brzeski, 1963 (Chitambar & Subbotin, 2014).

Hemicycliophora thienemanni (Schneider, 1925) Loos, 1948 (= *Loofia thienemanni* (Schneider, 1925) Siddiqi, 1980) (Fig. S11; Tables 1, S5)

Hemicycliophora thienemanni was described by Schneider from a population found in Germany. Populations of this species examined in our study were found in Italy (southern), Spain (northern and southern), and Russia. These populations were characterized by a slender body, a conoid-rounded lip region with indistinct lips, lateral fields marked by breaks or anastomoses, vulval lips unmodified and tail long (88–128 µm) with posterior end distinctly offset, annulations on posterior part of tail usually distinct but annuli smaller than other tail annuli in females. Morphology and morphometrics of the three studied populations were quite similar to each other, showing few intraspecific differences in body annuli and width of lip region, and fitted with data for several populations from the Netherlands and Poland, e.g. in stylet length (86–105 vs. 85–99, 77–106 µm, respectively) and V (80–85 vs. 81–84, 78–85%, respectively) (Loof, 1968; Brzeski, 1974). This is probably the most widespread species of the genus, and has been reported in several European countries including Belgium, England, France, Germany, Greece, Italy, Spain, and Switzerland (Loof, 1968; Brzeski, 1974; Boag & Orton Williams, 1976; Koliopanos & Vovlas, 1977; Peña-Santiago *et al*., 2004; Subbotin *et al*., 2005), as well as in Oregon, USA (Hafez *et al*., 1992), Argentina (Chaves, 1983), Brazil (Rashid, Geraert & Sharma, 1987), Martinique (Van den Berg & Cadet, 1992), Egypt (Ibrahim, Mokbel & Handoo, 2010), and South Africa (Van den Berg & Tiedt, 2001).

Distinguishing characters of this species include lateral fields with breaks or anastomoses, an offset tail end, and the absence of males. *Hemicycliophora thienemanni* is differentiated from the morphologically similar species *H. thornei*, *H. poranga*, *Hemicycliophora indica* Siddiqi, 1961, and *Hemicycliophora postamphidia* Rahaman, Ahmad & Jairajpuri, 1966 (Chitambar & Subbotin, 2014).

Hemicycliophora thornei Goodey, 1953 (Fig. S12; Tables 1, S5)

Hemicycliophora thornei was described by Goodey from a population found in the Netherlands. The population examined in our study was found in La Rambla, Córdoba Province, Spain. This Spanish population was characterized by a conoid-rounded, slightly elevated lip region, body annuli without longitudinal lines or scratches outside lateral fields, lateral fields marked only with breaks and irregularities in the transverse striae, vulval lips modified, tail conical, end tapering and distinctly offset (Fig. S12). This species was previously reported from southern Spain by Arias *et al*. (1963), but no information of taxonomic interest was given about the Iberian populations. Morphology and morphometrics of the Spanish population were similar to the original description and redescriptions of specimens from the Netherlands (Loof, 1968; Brzeski, 1974), except for minor intraspecific differences including a slightly longer stylet (94–114 vs. 89–103 µm) and slightly smaller VL/VB ratio (4.0–4.3 vs. 4.4–4.7). The species has also been reported in Germany, Poland, Spain, and Hungary (Loof, 1968; Brzeski, 1974; Peña-Santiago *et al*., 2004; Andrássy, 2007).

Distinguishing characters of this species include a conoid lip region, lateral lips higher than submedian ones, a subcylindroid elongate conoid tail distally spicate and distinctly offset with a finely rounded terminus, and the presence of males. *Hemicycliophora thornei* is differentiated from the morphologically similar species *H. thienemanni* and *Hemicycliophora monticola* Mehta, Raski &Valenzuela, 1983 (Chitambar & Subbotin, 2014).

Hemicycliophora vaccinii Reed & Jenkins, 1963 (= *Loofia vaccinii* (Reed & Jenkins, 1963) Siddiqi, 1980) (Fig. S13; Tables 1, S5)

Hemicycliophora vaccinii was described by Reed and Jenkins from a population associated with cranberry in Massachusetts, USA. The population examined in our study was collected from maritime pine in Carnota, Coruña Province, Spain. Females of the Spanish population were characterized by a truncate lip region with two annuli and a slightly protruding labial disc, body annuli more than 300, lateral fields marked by breaks or anastomoses of body annuli, without longitudinal lines, stylet knobs thick, rounded with a moderate cavity (1–1.5 µm), vulval lips not modified, and an elongate conoid tail with offset terminal region. Morphology and morphometrics of this population were similar to those of the original description (Reed & Jenkins, 1963), except for minor intraspecific differences including a slightly shorter stylet (84–96 vs. 95–112 μm), smaller b ratio (6.3–7.4 vs. 5.9–8.7) and larger c ratio (9.4–11.1 vs. 14.9–16.7). The present record of *H. vaccinii* is the first for Europe and Spain, and the fourth after the report from Iran (Chenari Bouket, Niknam & Eskandari, 2010). This species was also found on highbush blueberries from Ottawa County, Michigan, USA (Knobloch & Bird, 1981).

Distinguishing characters of this species include a truncate lip region with two or three annuli, lateral fields marked mostly by breaks of striae, an elongate conoid tail tapering to a distal offset and narrower elongated portion with a narrowly rounded terminus, and the absence of males. *Hemicycliophora vaccinii* is differentiated from the morphologically similar species *Hemicycliophora nucleata* Loof, 1968, *H. zuckermani*, *Hemicycliophora mettleri* Jenkin & Reed, 1964, and *H. similis* (Chitambar & Subbotin, 2014).

HEMICYCLIOPHORA WYEI CORDERO LÓPEZ, ROBBINS & SZALANSKI, 2013 (FIGS 2E, J, O, 3A, F, H, S14; TABLES 1, S6)

Hemicycliophora wyei was recently described by Cordero López *et al*. from a population associated with turf grasses in North Carolina, USA. Five additional populations were found in North Carolina and described in our study. Females of these populations were characterized by a straight or slightly ventrally arcuate body; cuticular sheath loosely fitting body. Lateral fields marked by breaks and anastomoses, and relatively often by a broken, irregular longitudinal line, sometimes with one or two additional outer, fairly distinct, irregular, offset, short, broken lines demarcating the lateral field, extending from the intestinal to vulval region. Sometimes instead of outer lines, two short, shadowy, often indistinct, narrow longitudinal ridges mark the pre- and postvulval regions, which through SEM observation appear as two narrow rows of blocks bordering a wider central row of blocks. Breaks and anastomoses continue in the postvulval region; outside lateral fields, annuli smooth often marked with a transverse median line, lip region hemispherical with flattened anterior end, two lip annuli, labial disc not protruded, *en face*: oblong, connected to 'pouch-like' dorsal and ventral extensions of first lip annulus, vulval lips modified, anterior and posterior lips about one to three annuli in length and equally long, vulval sheath short, rounded, two to three annuli long, postvulval body contracted immediately posterior to vulva, tail elongate conoid, tapering uniformly, sometimes slightly offset dorsally, to an attenuated, almost cylindrical terminal portion with narrowly rounded to narrowly blunted tip. Morphology and morphometrics of these North Carolina populations are similar to the original description (Cordero López *et al*., 2013).

Distinguishing characters of this species include an elevated rounded oral disc, two lip annuli, lateral fields demarcated by two faint lines with dot-like structures, an elongated uniformly conoid and elongate conoid tail uniformly tapering to a narrowly rounded or narrowly blunted terminus, and the absence of males. *Hemicycliophora wyei* is differentiated from the morphologically similar species *Hemicycliophora vietnamensis* Nguyen &Nguyen, 2001, *Hemicycliophora acuta* (Reay, 1985) Raski & Luc, 1987, *Hemicycliophora ritteri* Brizuela, 1963, and *Hemicycliophora belemnis* Germani & Luc, 1973 (Chitambar & Subbotin, 2014).

NON-IDENTIFIED *HEMICYCLIOPHORA* SPECIES

Twenty-five populations of sheath nematodes were classified as representatives of 14 unidentified species (Table 1). Samples of *Hemicycliophora* sp. 1 from Greece, *Hemicycliophora* sp. 7 and sp. 14 from Spain, *Hemicycliophora* sp. 2 and sp. 6 from New Zealand, and *Hemicycliophora* sp. 8, sp. 12, and sp. 13 from USA were too poorly fixed or had insufficient numbers of adults for their morphological identification and determination of their taxonomic status. Thus, these populations were only molecularly characterized, without attempting any morphological and morphometric study. Other *Hemicycliophora* samples (sp. 3, sp. 4, sp. 5, sp. 9, sp. 10, and sp. 11) were morphometrically characterized but their morphological variation prevented their reliable identification because their morphology fitted that of several valid species, which presently are poorly or incompletely morphologically described. At this point, these species have been left unnamed but differentiated from other *Hemicycliophora* species that they most closely resemble. These unsuccessful identification attempts point out the need for complete morphological and molecular data of *Hemicycliophora* species collected from the type localities in order to make possible the identification of our samples in future studies.

HEMICYCLIOPHORA SP. 3 (FIGS 1B, H, N, 3J; TABLES 1, S7)

This species is characterized by a ventrally arcuate body; sheath closely adpressed to body, but detached over postvulval body area, lateral fields marked by breaks and anastomoses occasionally with a faint, short, and irregular longitudinal line, annuli outside lateral fields marked with numerous more or less distinct fine longitudinal scratches or ridges, sometimes a few distinct short, irregular lines mark cuticle near lateral fields in the midbody to vulval region, lip region rounded anteriorly with three annuli, labial disc dome-shaped, elevated, vulval lips modified, about one annulus long, tail more or less conical, more so in posterior quarter, usually gradually tapering to a finely rounded or acute terminus; sometimes this portion is slightly offset by a greater dorsal curvature. No males were found. This species was found only in Arizona, USA.

This species resembles *H. raskii* but differs from it mainly by a protruded labial disc (vs. not protruded) and shorter stylet length (63–71 vs. 80–94 μm) and smaller RVan (number of annuli between vulva and anus) (5–8 vs. 9–14). This species is clearly different from *H. raskii* in the ITS and D2-D3 of 28S rRNA gene sequences.

HEMICYCLIOPHORA SP. 4
(FIGS 2D, I, N, S15; TABLES 1, S7)

This species is characterized by a straight or slightly ventrally arcuate body, cuticular sheath loosely fitting body, lateral fields marked by breaks and anastomoses throughout body, sometimes with a central longitudinal line, annuli outside lateral field coarse or smooth, several anastomoses observed in anterior body region, lip region rectangular to truncate, or with slightly rounded anterior edges, and with three annuli, labial disc not protruded or slightly elevated, vulval lips modified, about one annulus long, vulval sleeve about one annulus long, tail cylindrical then abruptly curved dorsally in posterior third with less to no curvature ventrally, continuing to an attenuated narrow conical, almost cylindrical posterior portion with rounded terminus. No males were found. This species has a wide distribution in the USA where it was detected at one site in California, North Carolina, and Texas and in three localities in Florida.

The species is similar to *Hemicycliophora epicharoides* but differs from it mainly by a more narrowly conical to cylindrical terminal portion of the tail, and larger values than those reported for R (241–254 vs. 144–209), Rst (24–28 vs. 15–21), Roes (number of annuli between anterior end of body and pharynx base) (41–46 vs. 33–42), Rex (46–49 vs. 32–41), RV (50–64 vs. 31–46), RVan (16–25 vs. 9–17), and Ran (number of annuli between posterior end of body and anus) (29–39 vs. 20–33). This species is clearly different from *H. epicharoides* in the ITS and D2-D3 of 28S rRNA gene sequences.

HEMICYCLIOPHORA SP. 8
(FIG. 1C, I, O; TABLES 1, S8)

One female *Hemicycliophora* specimen was detected in California, USA. It is characterized by a body ventrally arcuate in death; cuticular sheath closely fitting most of body, but loosely fitting over vulva and postvulval areas, lateral fields marked by anastomoses and breaks in transverse striae, short, one broken longitudinal line observed in posterior, prevulval region and suggested in anterior body region (but not observed), annuli outside lateral fields, smooth, lip region rectangular with slightly rounded edges and marked by three lip annuli, labial disc slightly elevated, vulval lips modified, about one to 1.5 annulus long, with vulval sheath about two annuli long, tail abruptly conical in posterior third, with greater dorsal curvature than ventral, tapering to a rounded tip. Annulation coarse and distinct over entire body.

This non-identified species resembles *H. californica*, *H. raskii*, *H. epicharoides*, and *H. iwia*. It differs from *H. californica* by larger R (275 vs. 210–241), RV-anterior end (229 vs. 172–195), Rst (26 vs. 22), Rex (49 vs. 38–46), number of lip annuli (three vs. two). It differs from *H. raskii* by longer body length, larger R, Rex, RV-anterior end, RVan and Ran, smooth or few ridges on annuli vs. numerous scratches, basal bulb not offset vs. offset from isthmus. It differs from *H. epicharoides* in having more annuli, i.e. larger values for R (275 vs. 144–209), Rst (26 vs. 15–21), Roes (48 vs. 29–42), Rex (49 vs. 32–41), RV-anterior end (229 vs. 142–167), and RVan (19 vs. nine to 17), annuli smooth vs. with fine scratches. From *H. iwia* it differs by the absence of irregular longitudinal striae, presence of a moderate-sized isthmus and basal bulb vs. very short isthmus and large, round basal bulb, tail end annuli shorter than other tail annuli vs. about equal size (according to Brzeski & Ivanova, 1978), larger R (275 vs. 188–245), RV-anterior end (229 vs. 158–181), RVan (19 vs. ten to 16), and Ran (27 vs. 16–24). This species is clearly different from the abovementioned species in the ITS and D2-D3 of 28S rRNA gene sequences.

HEMICYCLIOPHORA SP. 9
(FIG. S16; TABLES 1, S8)

Female body straight or ventrally arcuate, cuticular sheath closely adpressed to inner cuticle over entire body, occasionally loosely fitting on one side over pre- or postvulval body. Lateral fields marked by irregular continuous, breaks and anastomoses of transverse striae throughout body configuring a short, broken, faint central line, additionally one to two short, irregular often faint lines mark each annulus at midbody region, often joining to form one or two irregular, broken longitudinal lines on either side of central portion, forming an irregular row of blocks. Sometimes, a few additional scattered longitudinal lines mark annuli in central body region. Outside lateral fields, annuli marked with more or less distinct fine longitudinal lines, lip region with two annuli, rounded anteriorly, labial disc rounded-rectangular in lateral view, elevated, stylet knobs elongate oblong, posteriorly sloped with moderately large cavity, vulval lips not modified, very slightly bulged if at all, almost no discontinuity in body contour, vulval sheath absent. Postvulval body not contracted behind vulva, cylindrical to anus. Tail tapering uniformly and gradually in anterior half, then more abruptly conoid, sometimes dorsally offset in posterior portion extending to a narrower elongate conoid, almost spike-like terminal portion with a narrowly rounded to subacute terminus. Tail terminus annulation irregular, often distinct. Males were not found. This species was detected in Germany, Greece, and Russia.

Hemicycliophora sp. 9 is morphometrically, and for the most part, morphologically similar to *Hemicycliophora labiata* Colbran, 1960. It differs from this species mainly by the shape of the vulval lips (not modified, only slightly bulged vs. modified, elongate), absence of vulval sheath vs. present and two to three annuli long, and contour of the postvulval body immediately behind vulva (not contracted vs. contracted). These characters alone are insufficient to morphologically distinguish this species from *H. labiata*, and indeed, they may be considered intraspecific variations. However, pending further detailed study, *Hemicycliophora* sp. 9 is currently left as non-identified.

HEMICYCLIOPHORA SP. 10
(FIGS 1E, K, Q, S17; TABLES 1, S8)

A mixed population of *H. californica* and an unidentified *Hemicycliophora* sp. 10 were found in one sample collected in California, USA. The latter species was characterized by a straight or ventrally arcuate body; cuticular sheath loosely fitting body, lateral fields marked anteriorly by anastomoses as slanted lines connecting transverse striae, followed by a single, sometimes continuous longitudinal line extending from base of pharyngeal bulb to vulva, then continuing as breaks and anastomoses on postvulval area. Outside lateral fields annuli usually smooth or sometimes marked with irregularly scattered, short lines or scratches. Lip region hemispherical and marked by two lip annuli, first lip annulus larger than second one, labial disc not protruding. Vulval lips modified, about one to 1.5 annulus in length. Postvulval body area not contracted or slightly contracted immediately posterior to vulva. Tail cylindrical, then abruptly conical in posterior quarter, tapering uniformly or slightly offset dorsally to a short spike with a finely rounded terminus, or pyramidal, acute terminus. Annulations distinct on tail terminus.

Hemicycliophora sp. 10 is similar to *H. californica* but differs from it by longer body length (1017–1026 vs. 780–980 μm) and larger R (253–277 vs. 210–266). This species is clearly different from *H. californica* in the ITS and D2-D3 of 28S rRNA gene sequences.

HEMICYCLIOPHORA SP. 11
(FIG. 2C, H, M; TABLES 1, S8)

This species from Florida is characterized by a ventrally arcuate body; cuticular sheath loosely fitting and attached to body only at anterior end and vulva; annulation coarse, lateral fields marked by irregularities, breaks, and some anastomoses. Outside lateral field annuli smooth, lip region hemispherical to slightly rounded with three annuli: first annulus narrower than succeeding ones, separate, somewhat anteriorly directed, labial disc slightly elevated or not. Stylet straight or curved; basal knobs rectangular to elongate rounded, posteriorly sloped, with distinct, large cavity. Vulval lips modified, about 1.5 annuli long. Spermatheca oval, without sperm. Postvulval body contracted immediately posterior to vulva. Tail tapers gradually, then more abruptly posteriorly to an elongate conical narrower portion with a finely rounded, more or less distinctly annulated terminus. No males were found for this species.

The non-identified *Hemicycliophora* sp. 11 is similar to *H. thienemanni*, *H. similis*, and *H. vaccinii*. It differs from *H. thienemanni* and *H. similis* by the number of labial annuli (three vs. two), shape of first labial annulus (separate from other labial annuli and anteriorly directed vs. not separate and laterally directed), and fitting of cuticular sheath (loosely fitting vs. very closely adpressed to body). It further differs from *H. thienemanni* by a slightly shorter stylet (65–80 vs. 76–105 μm) and contour of the postvulval body immediately behind the vulva (contracted vs. not contracted). It differs from *H. similis* by a shorter stylet (65–80 vs. 88–108 μm), shorter body (700–978 vs. 920–1578 μm), and the presence of a large stylet knobs cavity vs. very small or absent. From *H. vaccinii* it differs by a shorter stylet (65–80 vs. 84–112 μm), smaller RV (50–67 vs. 70–76), VL/VB (2.7–4.6 vs. 4.7–9.0), shape of labial region (rounded vs. truncate), and vulval lips (modified, elongate vs. not modified, not elongate). This species is clearly different from the above-mentioned species in the ITS and D2-D3 of 28S rRNA gene sequences.

MOLECULAR CHARACTERIZATION OF *HEMICYCLIOPHORA* SPECIES

The D2-D3 of the 28S rRNA gene alignment included 102 sequences of *Hemicycliophora* and two *Paratylenchus* sequences selected as outgroup taxa and was 685 bp in length. Ninety-two new D2-D3 of 28S rRNA gene sequences were obtained in the present study. Intraspecific sequence diversity (uncorrected p-distance) for the populations of some species were: *H. thienemanni* – 0–0.8% (0–5 bp), *H. gracilis* – 0–0.5% (0–3 bp), *H. iberica* – 0.2–1.0% (2–7 bp), *Hemicycliophora* sp. 4 – 0–0.3% (0–2 bp), *Hemicycliophora* sp.9 – 0–0.4% (0–2 bp), *H. poranga* – 0–0.7% (0–7 bp), *H. wyei* – 0–0.7% (0–5 bp), *H. conida* – 0–0.6% (0–4 bp). The minimal interspecific differences observed were for *Hemicycliophora* sp. 9 vs. *Hemicycliophora* sp. 13 – 0.3–0.4% (2–3 bp), vs. *H. epicharoides* – 0.3% (2 bp), and vs. *H. typica* – 0.4–0.6% (3–5 bp).

The ITS of the rRNA gene alignment included 97 sequences of *Hemicycliophora* and two sequences selected as outgroups from the genera *Paratylenchus* and *Gracilacus* and was 874 bp in length. Eighty-eight new ITS of rRNA gene sequences were obtained in the present study. Intraspecific sequence diversity for populations of *H. thienemanni* – 0.1–1.2% (1–9 bp), *H. gracilis* – 0.1–0.8% (1–6 bp), *H. iberica* – 0.4–1.7% (3–13 bp), *Hemicycliophora* sp. 4 – 0–1.5% (0–10 bp), *H. epicharoides* – 0–1.5% (0–11 bp), *H. poranga* – 0–1.3% (0–9 bp), *H. wyei* – 0–0.9% (0–7 bp). Minimal interspecific differences were for *Hemicycliophora* sp. 13 vs. *H. epicharoides* – 2.0–2.6% (15–19 bp) and vs. *H. typica* – 2.7–3.6% (20–26 bp).

PCR-RFLP study

The PCR-ITS-RFLP profiles generated by five restriction enzymes for populations of 15 valid and nine unidentified species of *Hemicycliophora* are given in Figure 6. The results of PCR-RFLP analysis based on all enzymes studied were identical to those expected from *in silico* analysis for all sequences that correspond to species. Lengths of restriction fragments from RFLP for the ITS rDNA of 26 species, including those published by Van den Berg *et al*. (2010) and populations of two types of *H. epicharoides*, are presented in Table 2. Restriction of the five restriction enzymes *Ava*I, *Bsh*1236I, *Dra*I, *Hin*fI, and *Hin*6I separated populations of all valid and putative species.

New rRNA gene sequence data set allows correction of species identification

The sequence and phylogenetic analyses conducted in our study allowed the correction of the identity of several *Hemicycliophora* species that were wrongly identified in GenBank. Comparison of the ITS (KC329575) and D2-D3 of 28S rRNA (KC329574) gene sequences from GenBank with our data set revealed that these sequences belong to *H. wyei*, rather than to *Hemicycliophora uniformis* as originally considered by X. Ma & P. Agudelo (unpubl. data). A *Hemicycliophora* population from Germany previously identified as *H. typica* (AY780973) by Subbotin *et al*. (2005) was found to be an unidentified *Hemicycliophora* species, *Hemicycliophora* sp. 9, based on the present analysis of D2-D3 of the 28S rRNA gene sequence. The gene sequence (JQ708144) labelled and deposited as *Hemicycliophora pruni* by Cordero López *et al*. (2013) should be considered as our *Hemicycliophora* sp. 4. *Hemicycliophora* sp. 4 populations are clearly different from *H. pruni* in the lateral fields (marked by breaks and anastomoses vs. four longitudinal lines in *H. pruni*). The gene sequence (FN435301) deposited as *H. conida* by I. Tandingan De Ley *et al*. (unpubl. data) was found to belong instead to *H. gracilis*.

Phylogenetic relationships of the genus *Hemicycliophora*

A majority consensus phylogenetic tree generated by the BI analysis of the D2-D3 of the 28S rRNA gene sequence alignment under the GTR + G + I model is presented in Figure 7. The tree contains nine moderate or highly supported major clades. Clade I is moderately supported [posterior probability (PP) = 86] containing the following species: *H. poranga*, *H. halophila*, *H. conida*, *H. ripa*, *H. thornei*, *H. hellenica*, and *Hemicycliophora* sp. 1, sp. 6, and sp. 7. Clade II (PP = 79) includes *H. wyei*, *H. floridensis*, *H. lutosoides*, *H. lutosa*, and *H. italiae*. Clade III (PP = 99) contains a group of molecularly similar species: *H. epicharoides*, *Hemicycliophora* sp. 9, sp. 13, *H. typica*, and an unidentified species (sp. 2) from New Zealand. The highly supported (PP = 100) clade IV includes *H.* (= *Loofia*) *thienemanni*, *H. similis*, *H. gracilis*, and *Hemicycliophora* sp. 3, sp. 4, sp. 8, sp. 10, sp. 11, and sp. 12. The three clades named here as Va, VIb, and VII each include

Figure 6. PCR-restriction fragment length polymorphisms of the internal transcribed spacer of the rRNA gene for populations of selected *Hemicycliophora* species. A, *Hemicycliophora californica* (California, USA); B, *Hemicycliophora conida* (Washington, USA); C, *Hemicycliophora epicharoides* (Spain) (type A); D, *Hemicycliophora floridensis* (topotype, Florida, USA); E, *Hemicycliophora gracilis* (California, USA); F, *Hemicycliophora halophila* (topotype, New Zealand); G, *Hemicycliophora iberica* (topotype, Spain); H, *Hemicycliophora lutosoides* (Spain); I, *Hemicycliophora obtusa* (Spain); J, *Hemicycliophora wyei* (North Carolina, USA); K, *Hemicycliophora poranga* (California, USA); L, *Hemicycliophora raskii* (California, USA); M, *Hemicycliophora ripa* (California, USA); N, *Hemicycliophora thienemanni* (Spain); O, *Hemicycliophora thornei* (Spain); P, *Hemicycliophora* sp. 1 (Greece); Q, *Hemicycliophora* sp. 2 (New Zealand); R, *Hemicycliophora* sp. 3 (Arizona, USA); S, *Hemicycliophora* sp. 4 (Florida, USA); T, *Hemicycliophora* sp. 6 (New Zealand); U, *Hemicycliophora* sp. 8 (California, USA); V, *Hemicycliophora* sp. 9 (Russia); W, *Hemicycliophora* sp. 10 (California, USA); X, *Hemicycliophora* sp. 11 (Florida, USA). Lines: M, 100 bp DNA marker (Promega); 1, *Ava*I; 2, *Bsh*1236I; 3, *Dra*I; 4, *Hin*fI; 5, *Hin*6I.

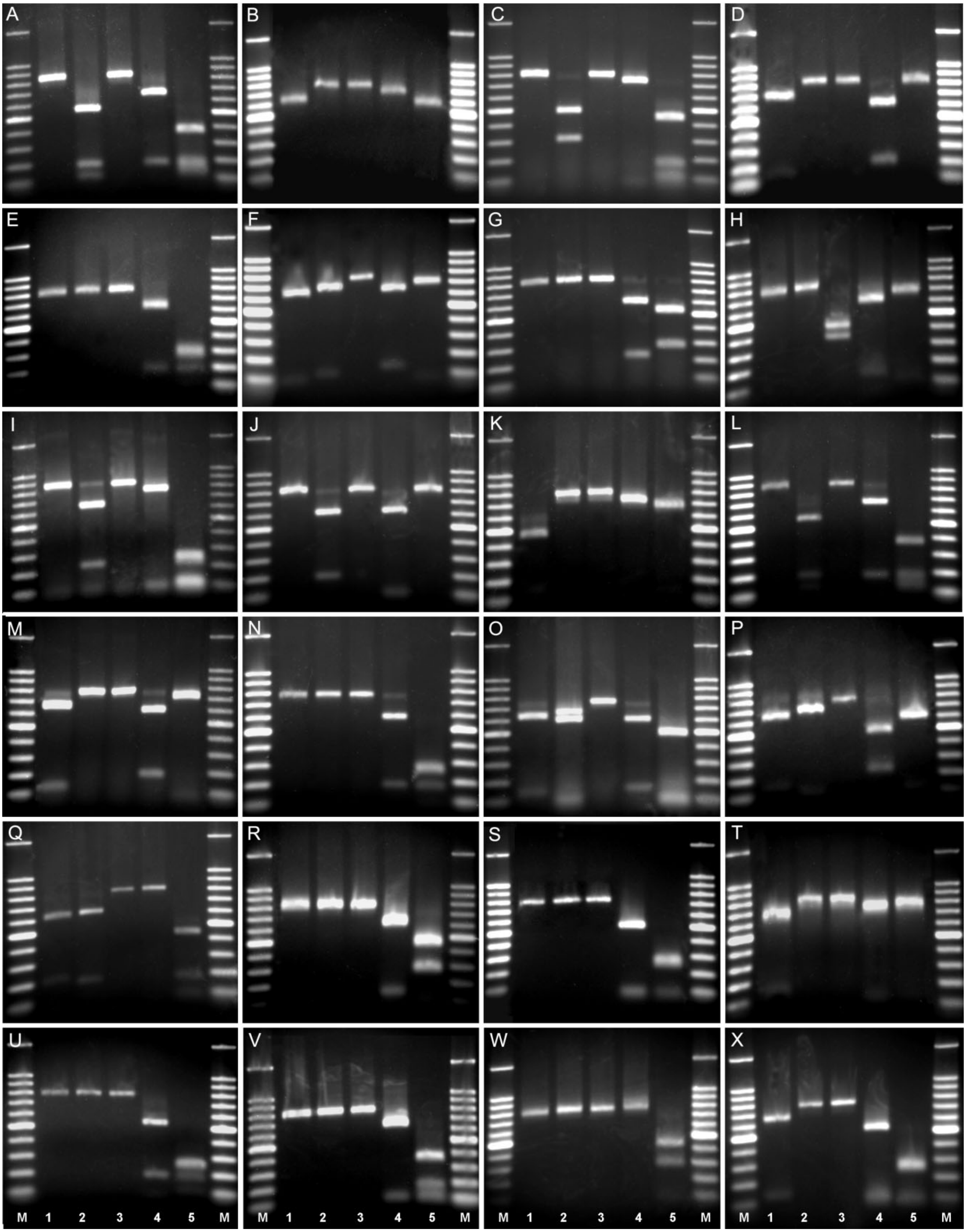
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Table 2. Approximate sizes (in bp) of restriction fragments generated by five restriction enzymes after digestion of PCR-internal transcribed spacer products amplified by TW81 and AB28 primers for *Hemicycliophora*

Species	Unrestricted PCR	Restriction enzymes					Reference
		*Ava*I	*Bsh*1236I	*Dra*I	*Hin*fI	*Hin*6I	
H. californica	**826**	**826**	**530, 181, 115**	**826**	**647, 179**	**363, 145, 181, 112, 25,**	This study
H. conida	**753**	**620, 72, 61**	**753**	**753**	**679, 74**	**577, 70, 69, 35**	This study
H. epicharoides (type A)	**791**	**791**	**484, 307**	**791**	**723, 68**	**437, 176, 111, 67,**	This study
H. epicharoides (type B)	792	792	792	792	724, 68	356, 176, 111, 82, 67	This study
H. floridensis	**808**	**672, 136**	**808**	**808**	**596, 198, 14**	**808**	This study
H. gracilis	**779**	**779**	**779**	**779**	**603, 176**	**283, 252, 178, 66**	This study
H. halophila	**766**	**632, 74, 60**	**671, 95**	**766**	**638, 128**	**699, 67**	This study
H. iberica	**806**	**806**	**806**	**806**	**587, 219**	**524, 282**	This study
H. lutosa	**788**	**655, 133**	**788**	**418, 370**	**593, 82, 68, 33, 12**	**670, 118**	Van den Berg *et al.* (2010)
H. lutosoides	**766**	**766**	**766**	**417, 349**	**591, 130, 33, 12**	**667, 99**	This study
H. obtusa	**799**	**799**	**625, 174**	**799**	**717, 82**	**243, 174, 116, 105, 91, 70**	This study
H. poranga	**773**	**449, 130, 70, 67, 57**	**773**	**773**	**701, 72**	**663, 110**	This study
H. raskii	**836**	**836**	**534, 185, 117**	**836**	**656, 180**	**365, 185, 145, 115, 26**	This study
H. ripa	**781**	**655, 126**	**781**	**781**	**593, 188**	**714, 67**	This study
H. thienemanni	**767**	**767**	**767**	**767**	**584, 183**	**272, 250, 180, 65**	This study
H. thornei	**749**	**614, 135**	**592, 94, 63**	**749**	**592, 157**	**481, 96, 91, 66, 15**	This study
H. typica	**791**	**791**	**419, 260, 112**	**791**	**756, 35**	**354, 175, 87, 67, 45, 39, 24**	Van den Berg *et al.* (2010)
H. wyei	**798**	**798**	**604, 194**	**798**	**608, 108, 82**	**798**	This study
Hemicycliophora sp. 1	**761**	**622, 139**	**663, 98**	**761**	**475, 222, 64**	**584, 112, 65**	This study
Hemicycliophora sp. 2	**802**	**604, 198**	**619, 183**	**802**	**802**	**437, 185, 94, 68, 18**	This study
Hemicycliophora sp. 3	**783**	**783**	**783**	**783**	**597, 134, 52**	**439, 275, 69**	This study
Hemicycliophora sp. 4	**762**	**762**	**762**	**762**	**511, 107, 80, 64**	**277, 240, 104, 75, 66**	This study
Hemicycliophora sp. 6	**802**	**662, 140**	**802**	**802**	**718, 84**	**733, 69**	This study
Hemicycliophora sp. 8	**774**	**774**	**774**	**774**	**525, 188, 61**	**249, 246, 179, 100**	This study
Hemicycliophora sp. 9	**791**	**791**	**791**	**791**	**633, 90, 68**	**355, 176, 111, 82, 67**	This study
Hemicycliophora sp. 10	**772**	**772**	**772**	**772**	**772**	**424, 283, 65**	This study
Hemicycliophora sp. 11	**767**	**640, 127**	**767**	**767**	**513, 111, 80, 63**	**276, 242, 108, 75, 66**	This study

Bold font indicates fragments verified by PCR-restriction fragment length polymorphism.

Figure 7. Phylogenetic relationships within populations and species of the genus *Hemicycliophora* as inferred from Bayesian analysis using the D2-D3 of the 28S rRNA gene sequence data set with the general time reversible substitution model with estimation of invariant sites and assuming a gamma distribution with four categories. Posterior probabilities of over 70% are given for appropriate clades. Newly obtained sequences are indicated in bold.

one species only: *H. obtusa*, *H.* (= *Loofia*) *vaccinii*, and *Hemicycliophora chilensis*, respectively. The highly supported clades (PP = 100) named as VIa and Vb both include two species: *H. iberica* and *Hemicycliophora* sp. 14, and *H. californica* and *H. raskii*, respectively.

The BI tree inferred from the analysis of the ITS rRNA gene sequence alignment is given in Figure 8. The tree includes six major highly supported clades (PP = 99–100). Clade I contains the following populations of species: *H. poranga*, *H. halophila*, *H. conida*, *H. ripa*, *H. thornei*, *H. hellenica*, and *Hemicycliophora* sp. 1, sp. 6, and sp. 7. Clade II includes *H. wyei*, *H. floridensis*, *H. lutosoides*, and *H. lutosa*. Clade III contains *H. epicharoides*, *H. typica*, and *Hemicycliophora* sp. 2 and sp. 9. Clade IV includes *H.* (= *Loofia*) *thienemanni*, *H. gracilis*, and *Hemicycliophora* sp. 3, sp. 4, sp. 5, sp. 8, sp. 10, and sp. 11. Clade V contains *H. californica*, *H. raskii*, and *H. obtusa*. Clade VI consists of *H. iberica* and *H.* (= *Loofia*) *vaccinii*.

MP, ML, and BI analysis of the combined D2-D3 and ITS of the rRNA gene sequence alignment with reduced sequence number resulted in trees (Figs 9, 10) with similar species grouping generally corresponding to the BI trees obtained from the full alignments. Differences in topologies were observed in weakly or moderately supported clades.

Shimodaira–Hasegawa tests for alternative hypotheses

The SH testing of an alternative topology with the D2-D3 of the 28S and ITS-rRNA gene fragment data sets strongly rejected the hypothesis, where the genus *Loofia* was a monophyletic lineage outside *Hemicycliophora* (Table 3). The phylogenetic analysis also revealed that *H.* (= *Loofia*) *thienemanni* and *H.* (= *Loofia*) *vaccinii* do not form a monophyletic lineage and that the monophyly of the clade within the genus *Hemicycliophora* was rejected by the SH test.

Ancestral state reconstruction

The characters that were selected for the ASR are listed in Table S9. These characters were morphological and morphometric (binary). The absence or presence of males was also included as a biological feature of diagnostic value, which is commonly used in combinations of binary characters (multistate). The results of the ASR of these characters are shown in Figures 9 and 10. The reconstruction yielded no conflicts between parsimony and Bayesian analyses. The mapping results suggested repeated origins of sheath nematodes with different body sizes, stylet lengths, and total numbers of body cuticle annuli and/or number of annuli from tail terminus to vulva. Our results also support the scenario that the ancestral nonmodified lip structure and amphimictic mode of reproduction appeared several times independently during sheath nematode evolution. The evolutionary history of tail shapes clearly also suggests multiple evolutions of this diagnostic character.

DISCUSSION

The primary objective of this study was to identify and characterize morphometrically and molecularly a wide range of populations of *Hemicycliophora* spp. from cultivated and natural environments in distant geographical areas and different continents including Africa, Europe, North and South America, and Oceania. Molecular markers were designed based on nuclear rDNA and proved useful for the identification of populations of *Hemicycliophora* species. The diagnostics and identification of *Hemicycliophora* species based solely on morphological and morphometric characters is a complicated and not always reliable procedure because of the overlap of morphological features of sheath nematode species. Although a few species stand out from the other representatives of the genus for their distinct morphological characteristics, many populations in our study were not properly identifiable using only their morphology. Our study shows that currently accurate identification of samples can be achieved by integrative taxonomy including morphological and DNA sequence analysis.

For the molecular identification, the rRNA genes of a nematode are amplified and sequenced or PCR-rDNA products are digested by several enzymes and then the results are compared to the reference sequences or RFLP profile data sets. RFLP-rDNA-PCR still remains an effective, cheap, and rapid method of identification of nematodes from soil samples. Although RFLP-PCR diagnostic profiles have been developed for many nematode species, the comparison of DNA sequences is the most comprehensive approach for nematode identification. It is imperative that reference sequences from nematode type materials should be obtained and deposited in public databases for reliable and correct diagnostics. This need is particularly important for nematode genera with great phenotypic plasticity, intraspecific variability in morphometrics, and minor interspecific differences, such as the genus *Hemicycliophora*. We should note that in our data set only five *Hemicycliophora* species were characterized from the type localities. The characterization and identification of the populations of other species from nontype localities still require additional verification and molecular comparisons with the type materials. We propose to consider these characterized populations from nontype localities as reference and standard material to be used for comparisons in future diagnostic and taxonomic studies of the

Figure 8. Phylogenetic relationships within populations and species of the genus *Hemicycliophora* as inferred from Bayesian analysis using the internal transcribed spacer rRNA gene sequence data set with the general time reversible substitution model with estimation of invariant sites and assuming a gamma distribution with four categories. Posterior probabilities of over 70% are given for appropriate clades. Newly obtained sequences are indicated in bold.

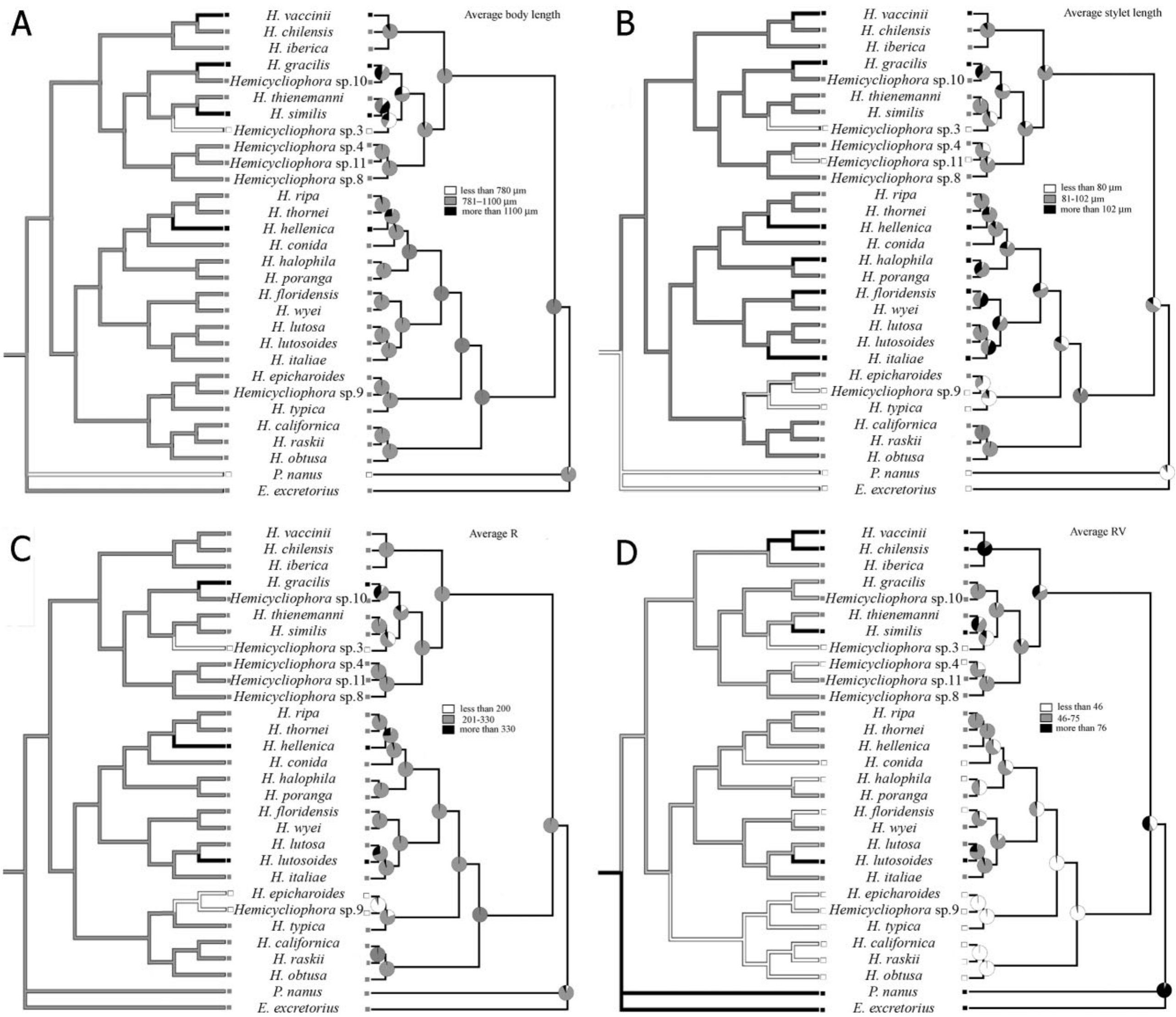

Figure 9. Ancestral state reconstructions for the genus *Hemicycliophora* based on parsimony (left maximum parsimony tree) and Bayesian inference (BI; right: BI tree) of A, average body length; B, average stylet length; C, average R (total number of body annuli); D, average RV (number of annuli between posterior end of body and vulva). Posterior probabilities for each character state are indicated as pie charts in the majority consensus BI tree.

representatives of the genus *Hemicycliophora* until topotype populations of each species become available and molecularly characterized. We are aware that future molecular analyses of type material of the studied species may contradict the results obtained in this work.

Our study revealed the presence of cryptic species pairs or complexes including our populations of *H. ripa* with *Hemicycliophora* sp. 7. Small sequence divergences within the complex may be interpreted as various stages in the speciation process, from recently diverged populations to distinct biological species. The results of the present study also suggest that the observed genetic diversity of *Hemicycliophora* is significantly higher than that shown by morphological observations. Thus, species diversity in *Hemicycliophora* based on morphological characters needs a thorough re-examination. In fact, our results suggest that the biodiversity of sheath nematodes is still not fully clarified and requires further study. We believe that the successful approach will be to carry out regional surveys of sheath nematodes using molecular tools to identify species and supplement these identifications with morphological observations in order to verify the species diversity at regional level.

In addition to the six characters used in this study, several morphological characters of sheath nematodes have been evaluated by several researchers for possible species grouping within the genus

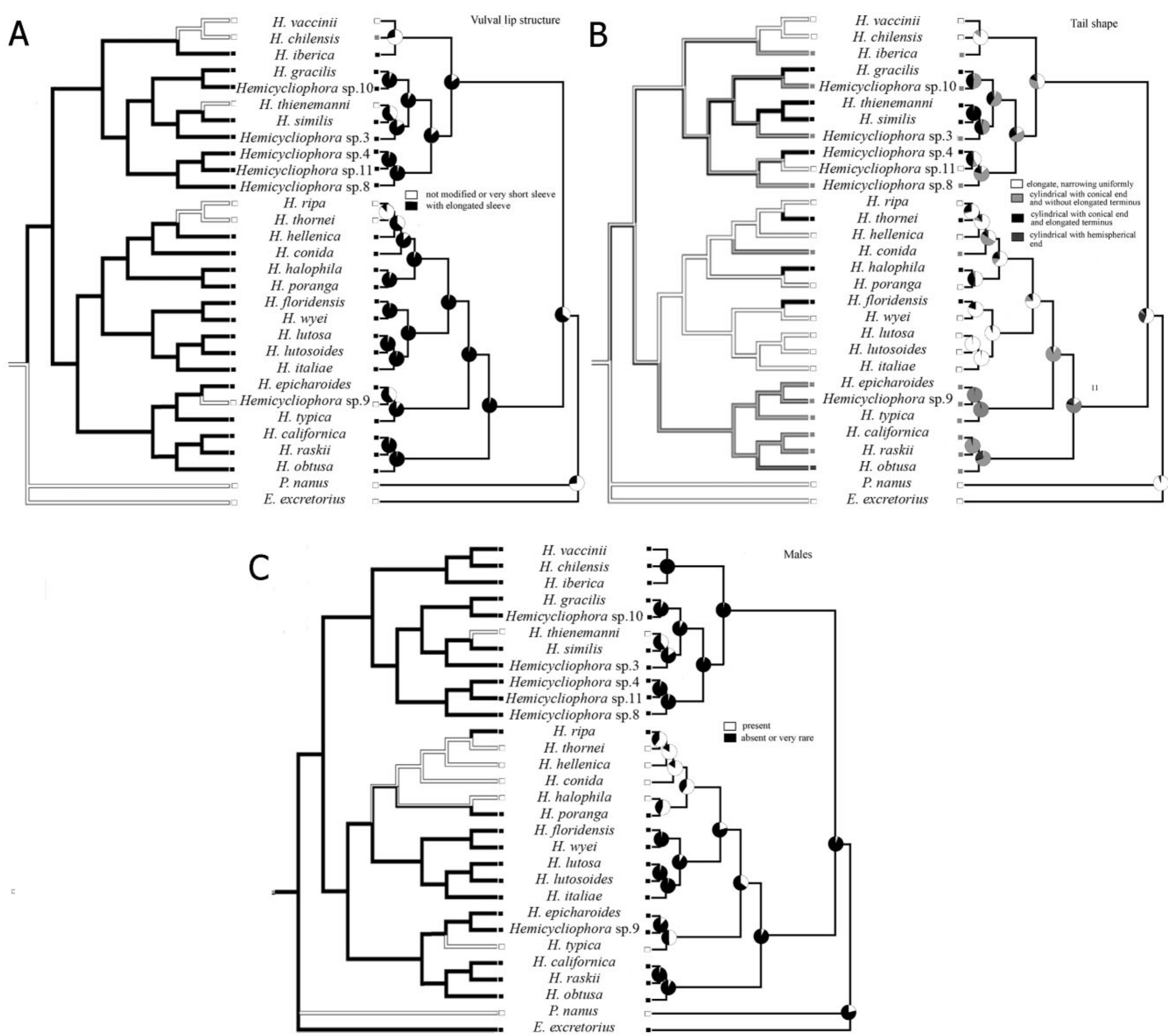

Figure 10. Ancestral state reconstructions for the genus *Hemicycliophora* based on parsimony (left maximum parsimony tree) and Bayesian inference (BI; right: BI tree) of A, vulval lip structure; B, tail shape; C, presence of males. Posterior probabilities for each character state are indicated as pie charts in the majority consensus BI tree.

Hemicycliophora and for revision of this genus and the subfamily Hemicycliophorinae. Considering the lip region, which is set off by a deep groove, subterminal vulva and bursa positions, as generic characters, Andrássy (1979) erected the genus *Colbranium* with a single species, *Colbranium truncatum*. Siddiqi (1980) also used vulval lips with or without sleeves and spicule structure for establishing the genera *Aulosphora* and *Loofia*. In agreement with these taxonomic reorganizations, Siddiqi (2000) included in the classification of Hemicycliophorinae, in addition to *Hemicycliophora*, the genera *Aulospora*, *Colbranium*, and *Loofia*. We were not able to obtain representative populations of these genera, except for two species of the genus *Loofia*, which belonged to *L. thienemanni* (type species of the genus *Loofia*) and *L. vaccinii*, both respectively named in our study as *H. thienemanni* and *H. vaccinii*. Our phylogenetic analyses using the D2-D3 of 28S rRNA and ITS of rRNA gene sequences showed that the studied populations of these two species are not related and formed two clearly separate clades with representatives of the genus *Hemicycliophora*. Moreover, ancestral state reconstructions showed that nonmodified vulval lips or with very short sleeves appeared several times during evolution. Thus, our molecular analysis did not support the erection of the genus *Loofia* and justified its synonymization with *Hemicycliophora*.

Table 3. Results of the Shimodaira–Hasegawa tests for alternative hypotheses using maximum likelihood (ML) trees

Gene	D2-D3 of 28S rRNA			Internal transcribed spacer rRNA		
Hypothesis tested	-*Ln*L	Difference of -*Ln*L	*P**	-*Ln*L	Difference of -*Ln*L	*P**
ML tree	13661.80	Best	–	13734.08	Best	–
Hemicycliophora (= *Loofia*) *thienemanni* and *Hemicycliophora* (= *Loofia*) *vaccinii* constrained into a monophyletic group within the genus *Hemicycliophora*	13715.18	53.38	0.005*	13827.24	93.16	0.000*
Validity of the genus *Loofia* or *Hemicycliophora* (= *Loofia*) *thienemanni* and *Hemicycliophora* (= *Loofia*) *vaccinii* are placed outside the genus *Hemicycliophora*	3729.32	67.52	0.001*	13831.82	97.74	0.000*

*$P < 0.05$ indicates significant differences between the two inferred tree topologies. *Ln*L, the log-likelihood.

The value of the tail shape as a taxonomic character became quite controversial after it was reported to be variable in *H. zuckermani* (Brzeski & Zuckerman, 1965; Minton & Golden, 1966). Indeed, during the 1960s, several taxonomists avoided the use of tail shape in their identification keys. However, tail shape is a fairly consistent character for the differentiation of most *Hemicycliophora* species (Brzeski, 1974). Comparatively, only a small number of species have variable shapes that can mislead identification, especially when a single specimen is involved. Most species have one of four broad categories of tail shape. Ancestral state reconstructions revealed multiple origins of these types, although there is some uncertainty in this picture. Likewise, on the basis of the results of the ancestral state reconstructions, the four morphometric characters studied, namely, body and stylet lengths, total number of body annuli, and number of annuli to vulva from body posterior end, which are important differentiating features used in the identification of *Hemicycliophora* spp., *Caloosia* spp., and *Hemicaloosia* spp. (Brzeski, 1974; Ganguly & Khan, 1983; Inserra *et al*., 2013), also have multiple origins. Our phylogenetic hypothesis testing and ancestral state reconstructions indicate that none of the six morphological/morphometric traits is by itself a good tool to classify the sheath nematode species that we studied and support the decision of grouping sheath nematodes within a single genus.

CONCLUSIONS

In summary, the present study establishes the importance of using integrative taxonomic identification by highlighting the time-consuming aspect and difficulty of correct identification at species level within the genus *Hemicycliophora*. This study also provides molecular markers for precise and unequivocal diagnosis of some species of sheath nematodes in order to differentiate species of agricultural and quarantine relevance because their morphology is quite similar and several sheath nematode species may be present in the same soil sample. The present study suggests that the genus *Hemicycliophora* harbours one or probably more complexes of species that have simply diverged in morphology and rRNA gene sequences. Consequently, these data strengthen the argument that nematode species delimitation should be the result of integrated studies based on morphology, ecology, and genetics with molecular taxonomic identification and phylogeny. Future phylogenetic studies should consider additional genetic markers including mitochondrial DNA genes and nuclear protein-coding genes such as *cytochrome c oxidase subunit 1* or *heat shock protein* (*hsp90*) genes in order to resolve the relationships within *Hemicycliophora* and other genera of the family Criconematidae. Additionally, a more comprehensive molecular analysis on such genetic markers based on worldwide sampling of *Hemicycliophora* isolates might further clarify the relationships amongst sheath nematodes characterized in this study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Photomicrographs of specimens of a population of *Hemicycliophora conida* Thorne, 1955 from Spain. A, entire female body; B, female pharyngeal region showing excretory pore (ep); C, D, female anterior regions; E, detail of lateral fields; F, G, posterior regions showing anus (a); H, female tail tip. Scale bars: A = 100 μm; B–D, F–H = 20 μm; E = 10 μm.

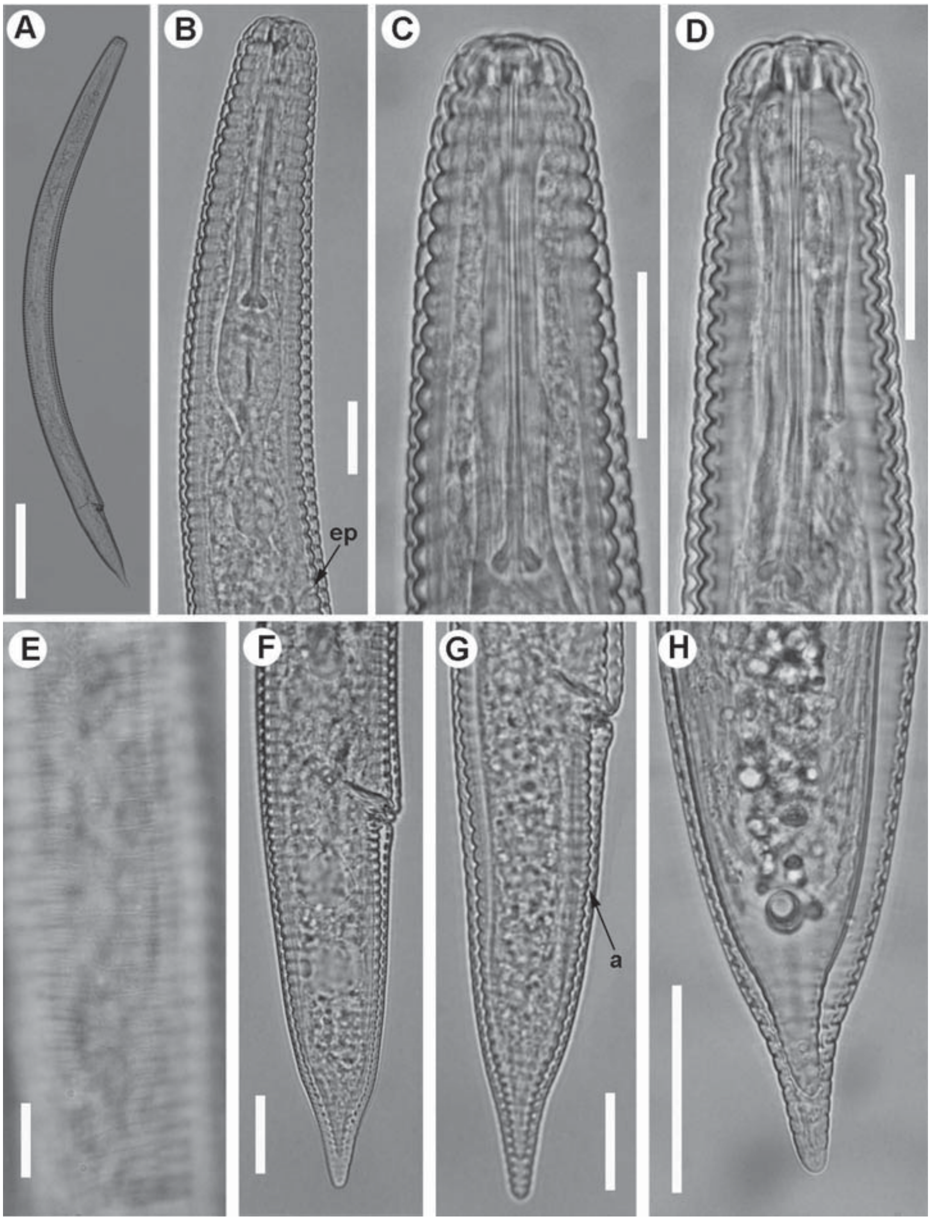
Figure S2. Photomicrographs and scanning electron microscopy (SEM) plate of specimens of populations of *Hemicycliophora epicharoides* Loof, 1968, from Italy and Spain. A, entire female body; B, female pharyngeal region; C, female anterior region; D, SEM plate of lateral fields; E, posterior region; F–H, female tail tips. Scale bars: A = 100 μm; B–C, E–H = 20 μm; D = 10 μm.

Figure S3. Photomicrographs of specimens of a population of *Hemicycliophora gracilis* Thorne, 1955, from California, USA. A, entire female and male bodies; B–D, female pharyngeal region; E, F, posterior regions; G–I, female tails; J, male pharyngeal region; K, male spicules; L, male tail tip. Scale bars: A = 35 μm; B–L = 20 μm.

Figure S4. Photomicrographs of topotype specimens of *Hemicycliophora hellenica* Vovlas, 2000, from Greece. A, entire female and male body; B, D, E, G, female pharyngeal regions; C, F, posterior regions; H, male tail; I, J, male anterior region; K, detail of male lateral fields; L, male spicules. Scale bars = 25 μm.

Figure S5. Photomicrographs and scanning electron microscopy (SEM) plates of topotype specimens of *Hemicycliophora italiae* Brzeski & Ivanova, 1978, from Italy. A, entire female body; B, female pharyngeal region; C, posterior region; D, detail of stylet knobs; E, detail of lateral fields; F, detail of vulval lips; G, detail of female mid-body and tail tip; H, I, SEM plate *en face* view; J, SEM plate of female posterior body portion; K, SEM plate of lateral fields. Scale bars: A = 100 μm; B, C, F, G, J = 20 μm; D, E = 10 μm; H, I, K = 5 μm.

Figure S6. Photomicrographs of specimens of a population of *Hemicycliophora lutosoides* Loof, 1984, from Spain. A, entire female bodies; B, female pharyngeal region; C, D, female anterior regions; E, detail of lateral fields; F–H, vulval and tail regions showing anus (a). Scale bars: A = 100 μm; B–D, F–H = 20 μm; E = 10 μm.
Figure S7. Photomicrographs of specimens of populations of *Hemicycliophora poranga* Monteiro & Lordello, 1978, from Italy and Spain. A, female pharyngeal region; B–D, female anterior regions showing labial disc (ld); E, detail of lateral fields; F–I, vulval and tail regions showing anus (a). Scale bars: A = 100 μm; B–D, F–I = 20 μm; E = 5 μm.
Figure S8. Photomicrographs of specimens of populations of *Hemicycliophora poranga* Monteiro & Lordello, 1978, from California, USA. A, entire female body; B–D, female pharyngeal region; E, F, lateral fields; G, female posterior region; H, I, rare variation of female tails. Scale bars: A = 50 μm; B–D, H, I = 20 μm; E–G = 30 μm.
Figure S9. Photomicrographs of specimens of a population of *Hemicycliophora ripa* Van den Berg, 1981, from Spain. A, entire female; B, C, female pharyngeal region; D, female anterior region showing labial disc (ld); E, detail of annuli showing typical cuticular ornamentation (blocks inside annuli); F, detail of lateral fields; G–I, vulval and tail regions showing anus (a); J, detail of terminal tip. Scale bars: A = 100 μm; B, C, G, H = 20 μm; D–F, I = 10 μm.
Figure S10. Photomicrographs of specimens of a population of *Hemicycliophora similis* Thorne, 1955, from Spain. A, whole females; B, female pharyngeal region; C, detail of excretory pore (ep); D, detail of lateral fields; E, F, vulval regions showing vulva (V); G–K, tail regions showing anus (a). Scale bars: A = 100 μm; B, C, E–K = 20 μm; D = 10 μm.
Figure S11. Photomicrographs of specimens of populations of *Hemicycliophora thienemanni* (Schneider, 1925) Loos, 1948, from: A–I, Castillo de Locubín (southern Spain); J–N, Garray (northern Spain); O–U, Trentino (northern Italy). A, entire female; B, O, female pharyngeal regions; C, D, female anterior regions; E–G, J–L, P–S, posterior regions; H, I, M, N, T, U, female tail tips. Scale bars: A = 100 μm; B, C, E–U = 20 μm; D = 10 μm.
Figure S12. Photomicrographs of specimens of a population of *Hemicycliophora thornei* Goodey, 1953, from Spain. A, female pharyngeal region; B, C, female anterior region; D, pharyngeal region showing excretory pore (ep); E, detail of lateral fields; F, vulval region showing vulva (v) and anus (a); G, H, female tail tip. Scale bars: A–D, F–H = 20 μm; E = 5 μm.
Figure S13. Photomicrographs of specimens of a population of *Hemicycliophora vaccinii* Reed & Jenkins, 1963, from Spain. A, female pharyngeal region; B, C, female anterior regions; D, detail of lateral fields; E, vulval and tail regions showing anus (a). Scale bars: A–C, E = 20 μm; D = 10 μm.
Figure S14. Photomicrographs of specimens of populations of *Hemicycliophora wyei* Cordero López, Robbins & Szalanski, 2013, from North Carolina, USA. A, entire female bodies; B–D, female pharyngeal region; E, F, detail of lateral fields; G, posterior region; H–J, female tails. Scale bars: A = 40 μm; B–D, H–J = 10 μm; E–G = 15 μm.
Figure S15. Photomicrographs of specimens of populations of *Hemicycliophora* sp. 4 from California. A, entire female body; B–D, female pharyngeal region; E–G, lateral fields; H–J, female posterior region. Scale bars: A = 65 μm; B–D, H–J = 13 μm; E–G = 19 μm.
Figure S16. Photomicrographs of specimens of a population of *Hemicycliophora* sp. 9 from Russia. A, B, female pharyngeal region; C, D, female posterior region. Scale bars = 20 μm.
Figure S17. Photomicrographs of specimens of a population of *Hemicycliophora* sp. 10 from California, USA. A, entire female body; B, C, female pharyngeal region; D, E, lateral field; F–H, female posterior region. Scale bars: A = 50 μm; B, C, F, G = 10 μm; D, E = 15 μm.
Table S1. Morphometrics of specimens of populations of *Hemicycliophora californica*, *Hemicycliophora conida*, and *Hemicycliophora epicharoides* analysed in the present study.
Table S2. Morphometrics of topotype specimens of *Hemicycliophora floridensis* and specimens of populations of *Hemicycliophora gracilis* and *Hemicycliophora iberica* analysed in the present study.
Table S3. Morphometrics of specimens of populations of *Hemicycliophora lutosoides*, *Hemicycliophora obtusa*, *Hemicycliophora ripa*, *Hemicycliophora raskii*, and *Hemicycliophora similis* analysed in the present study.
Table S4. Morphometrics of specimens of populations of *Hemicycliophora poranga* analysed in the present study.
Table S5. Morphometrics of specimens of populations of *Hemicycliophora thienemanni*, *Hemicycliophora thornei*, and *Hemicycliophora vaccinii* analysed in the present study.
Table S6. Morphometrics of specimens of populations of *Hemicycliophora wyei* analysed in the present study.
Table S7. Morphometrics of specimens of populations of *Hemicycliophora* sp. 3 and sp. 4 analysed in the present study.
Table S8. Morphometrics of *Hemicycliophora* sp. 8, sp. 9, sp. 10, and sp. 11 analysed in the present study.
Table S9. Morphometric, morphological, and biological characters of *Hemicycliophora* used for ancestral state reconstruction.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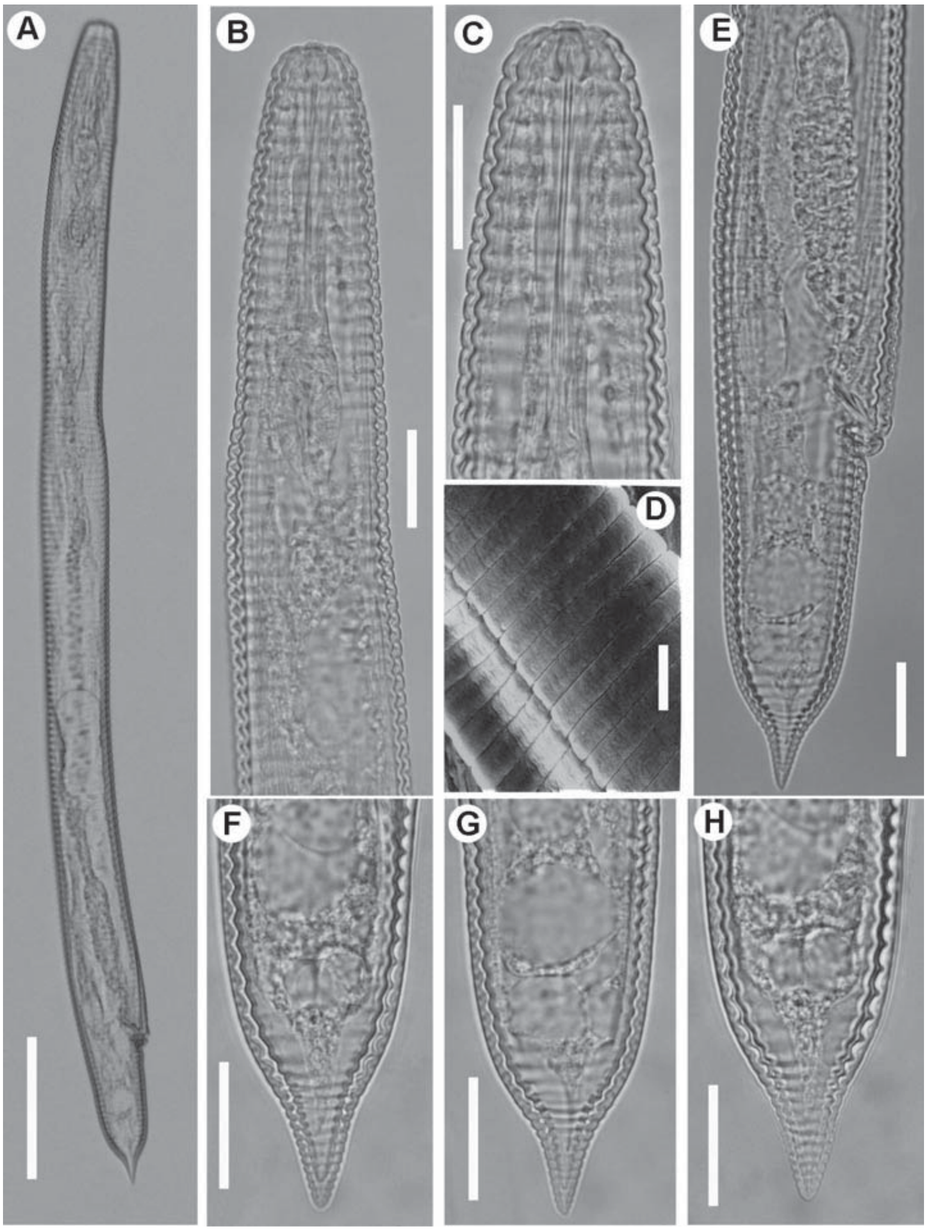
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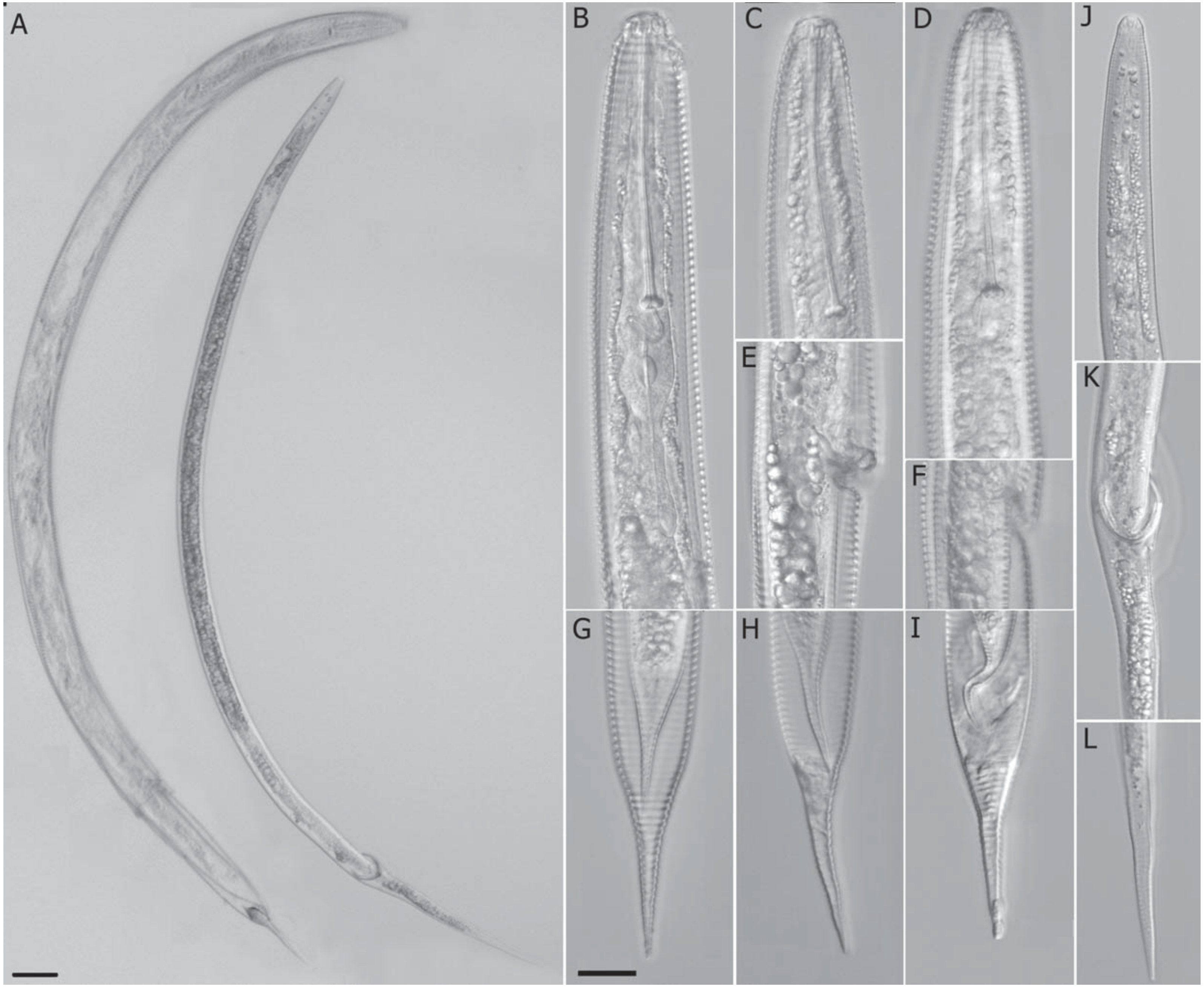
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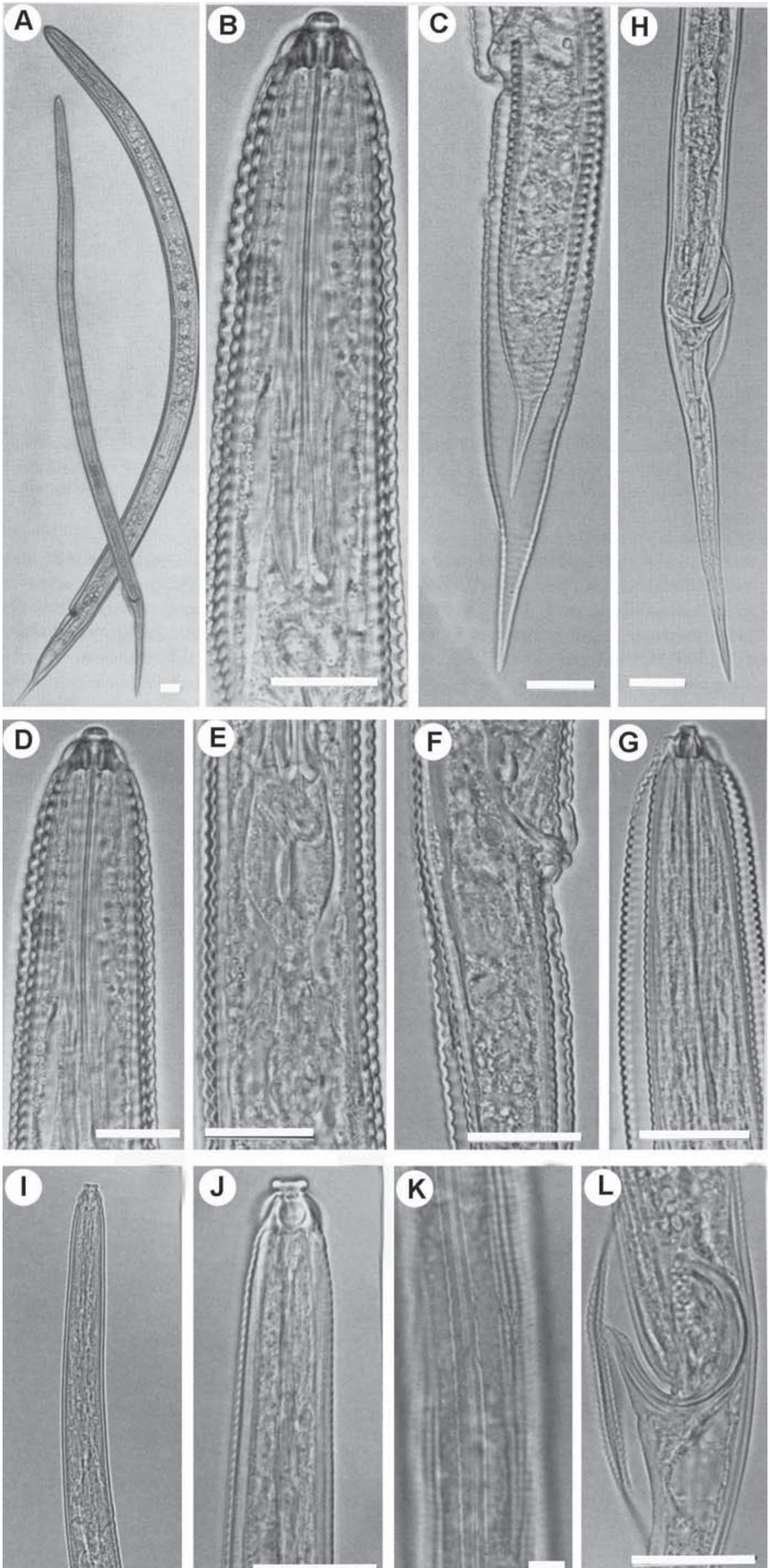
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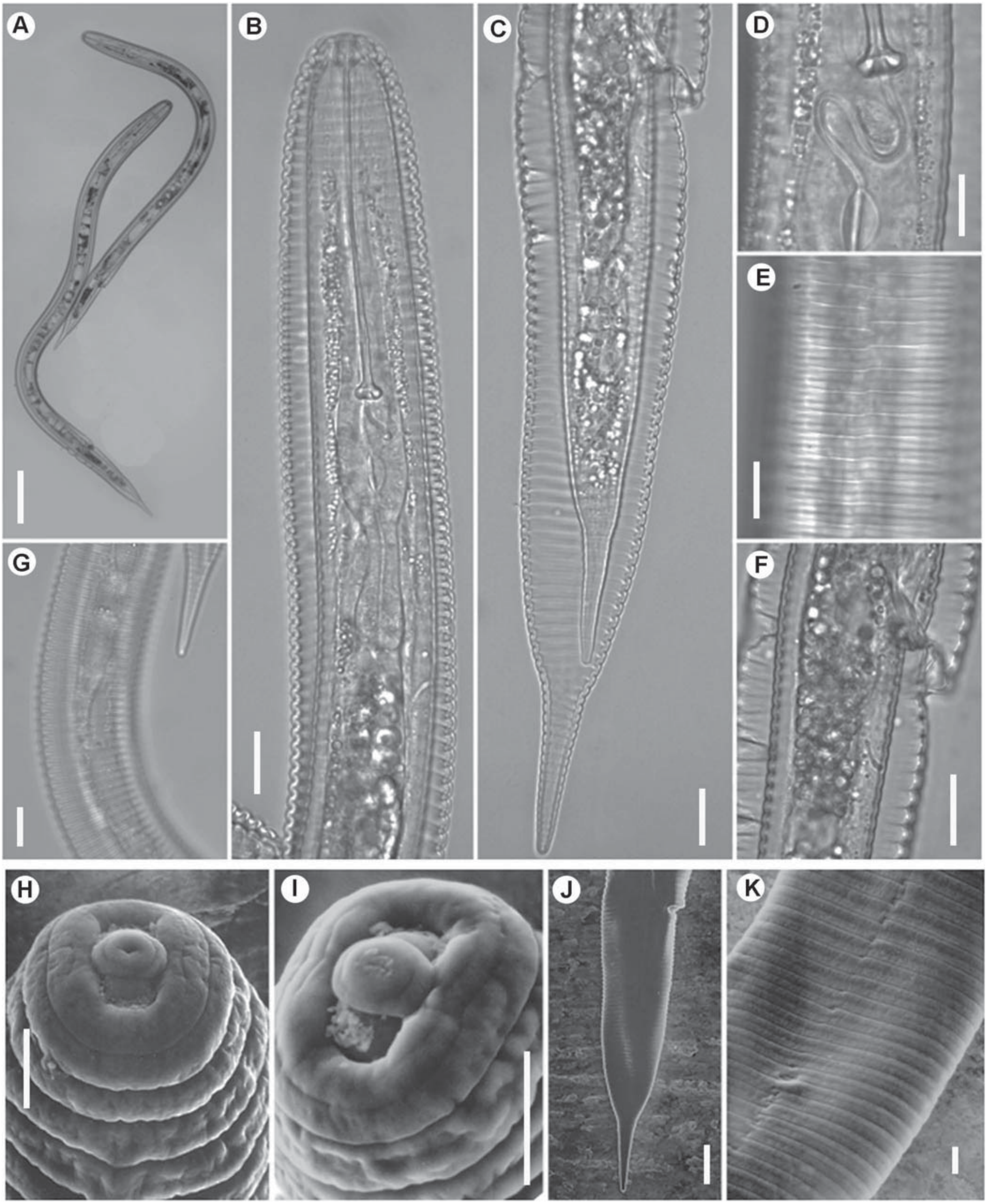

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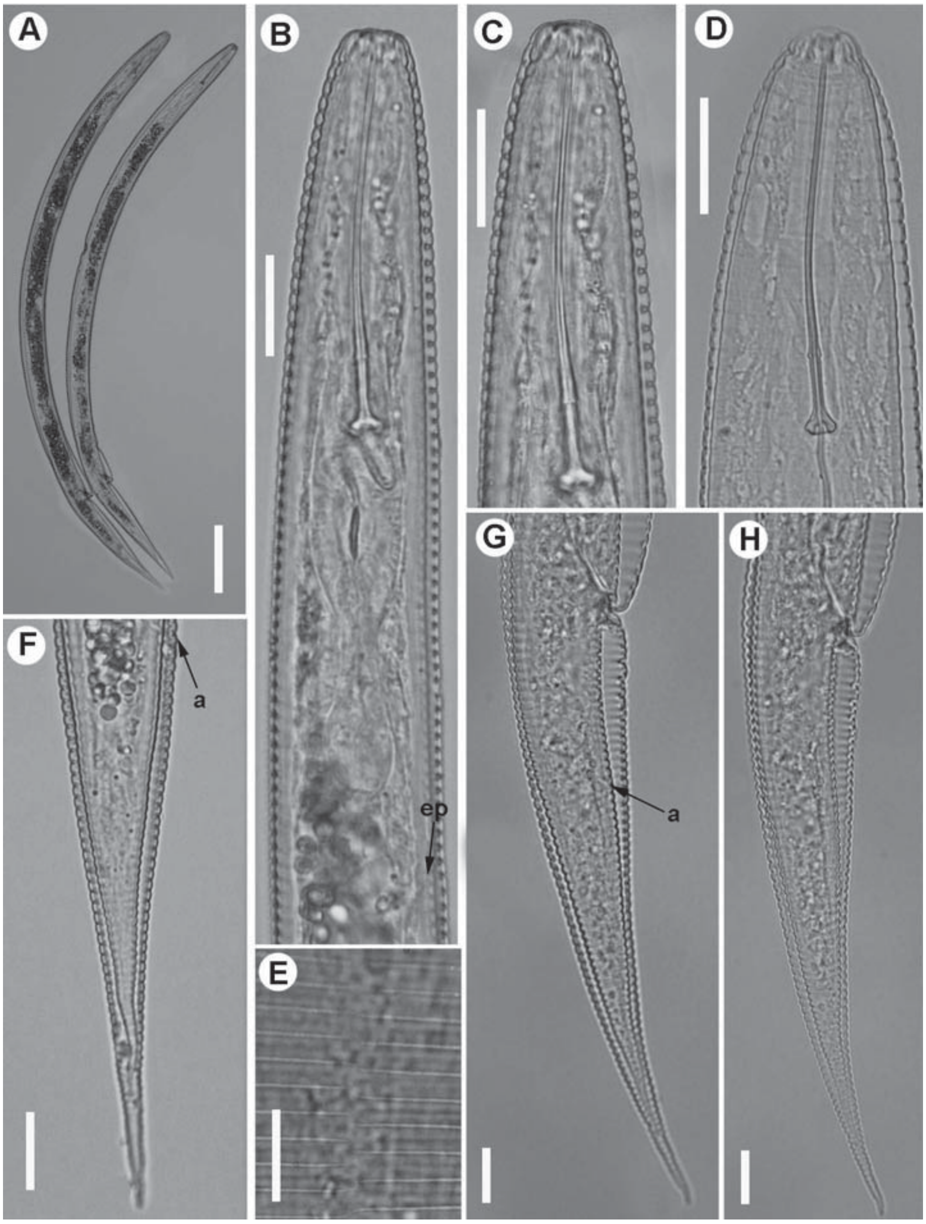
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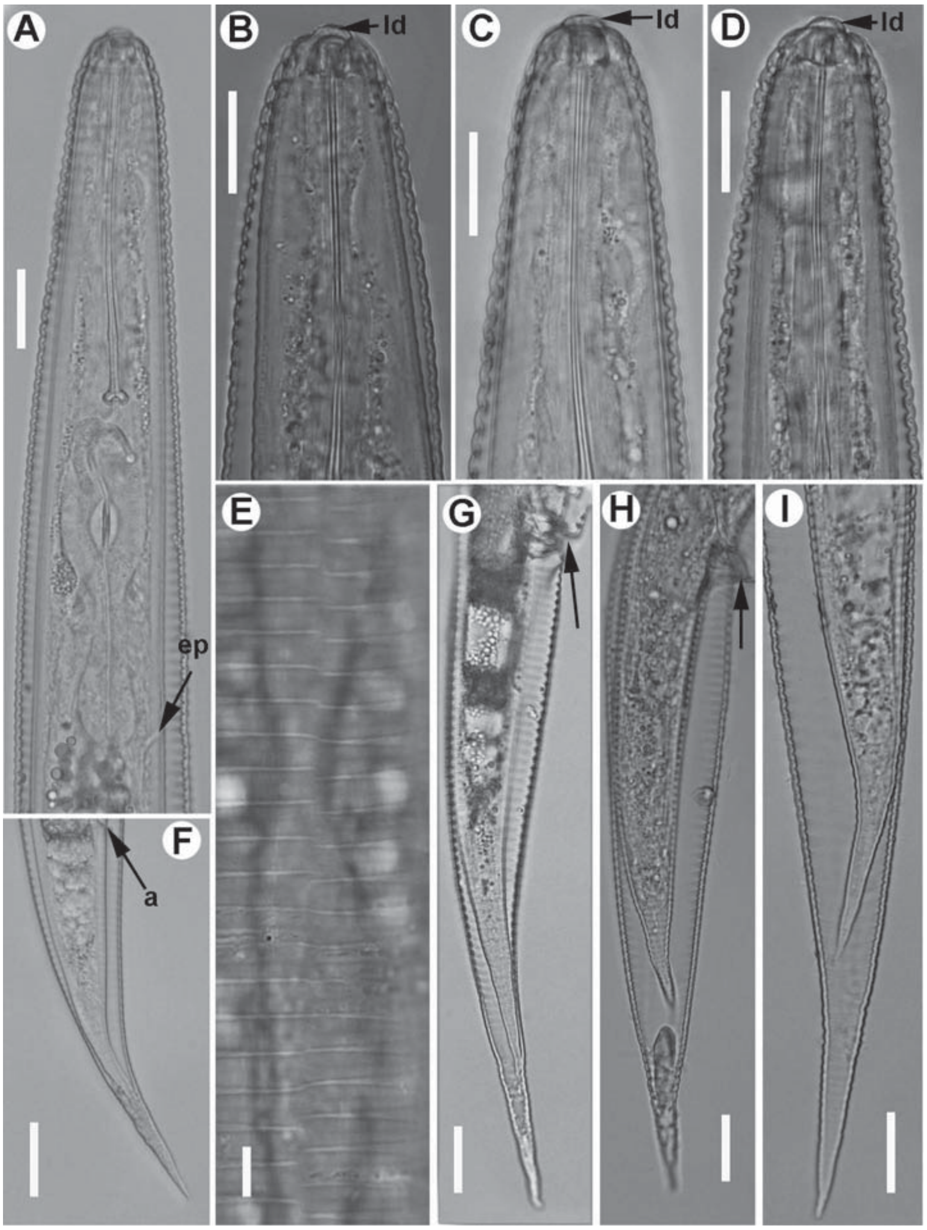
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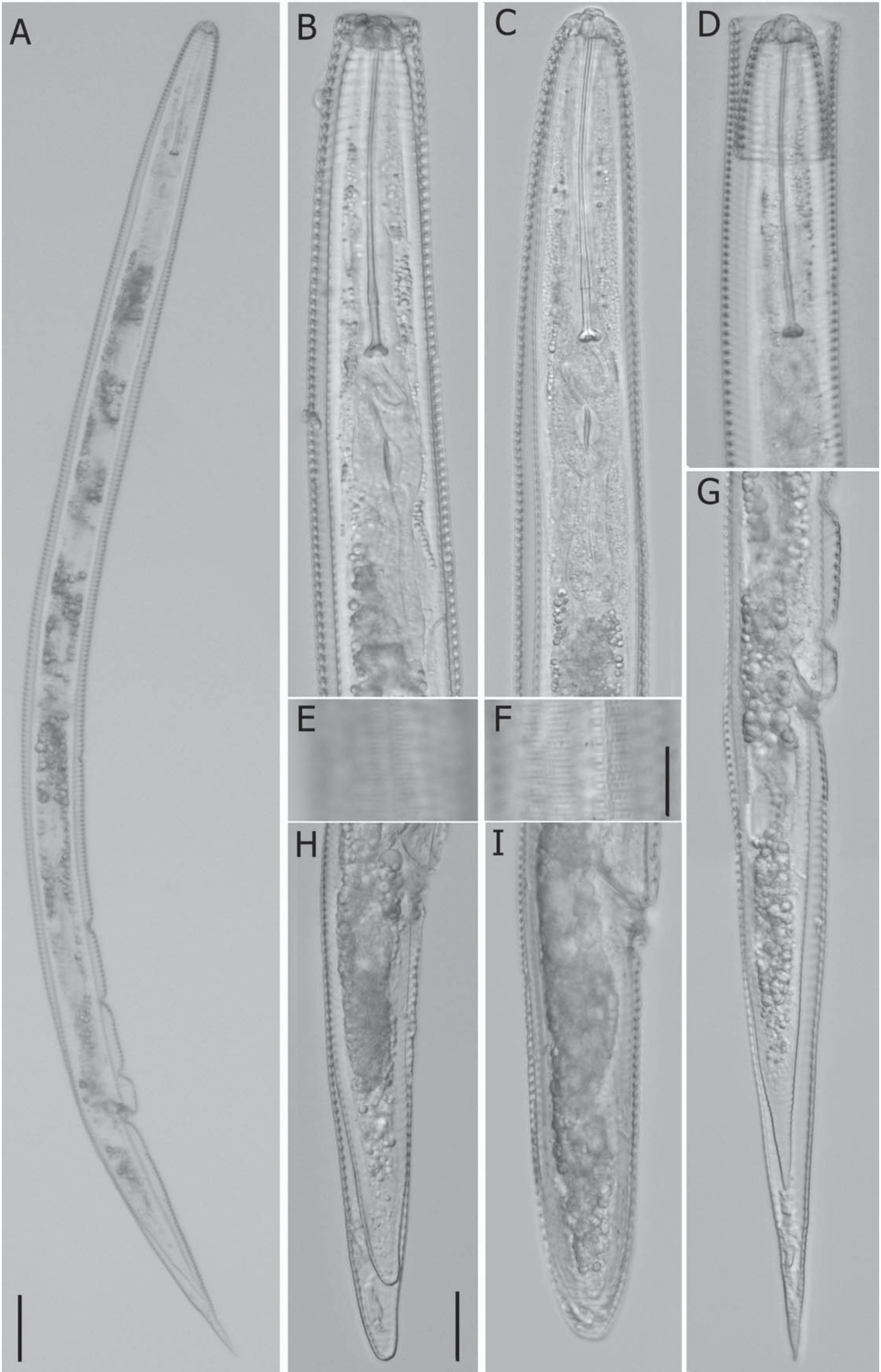
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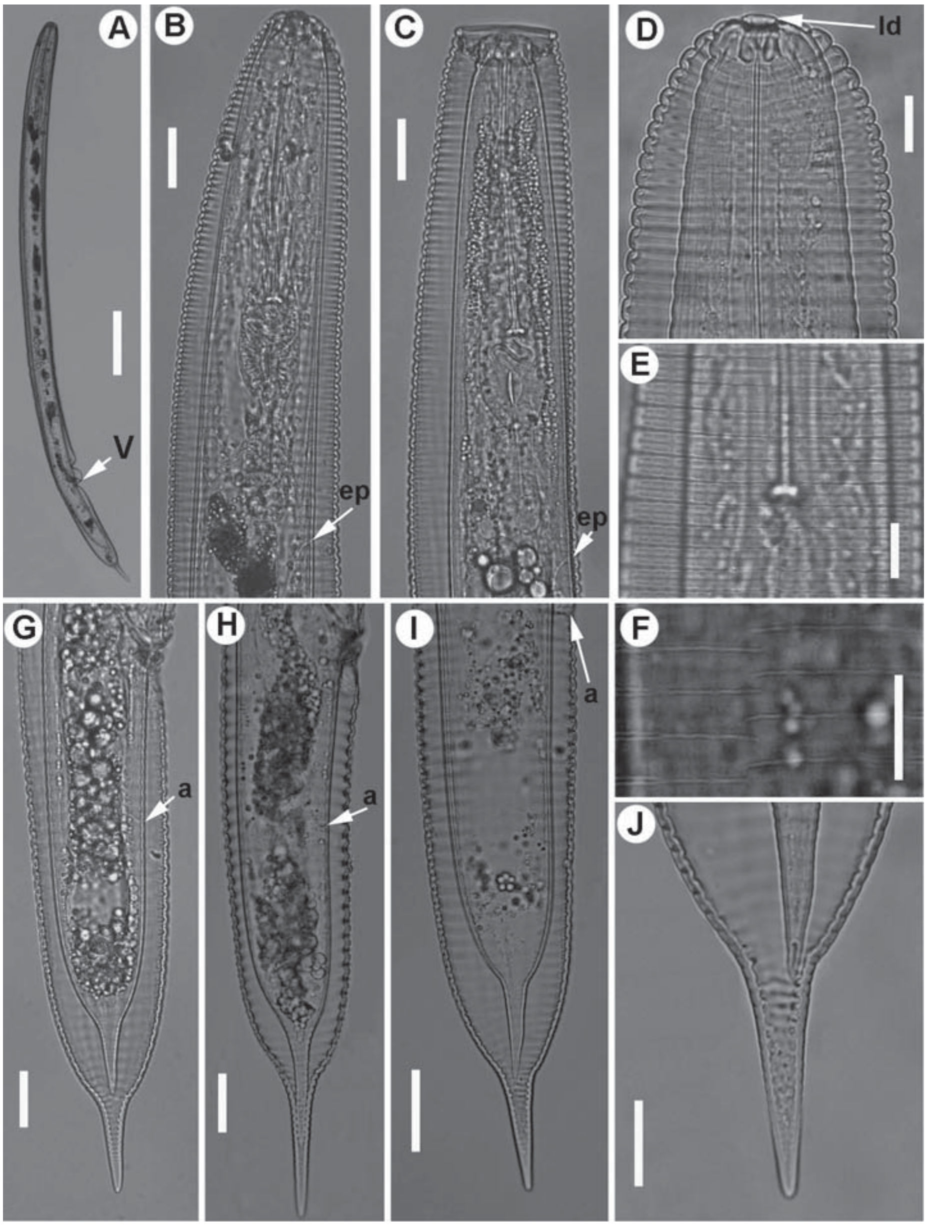
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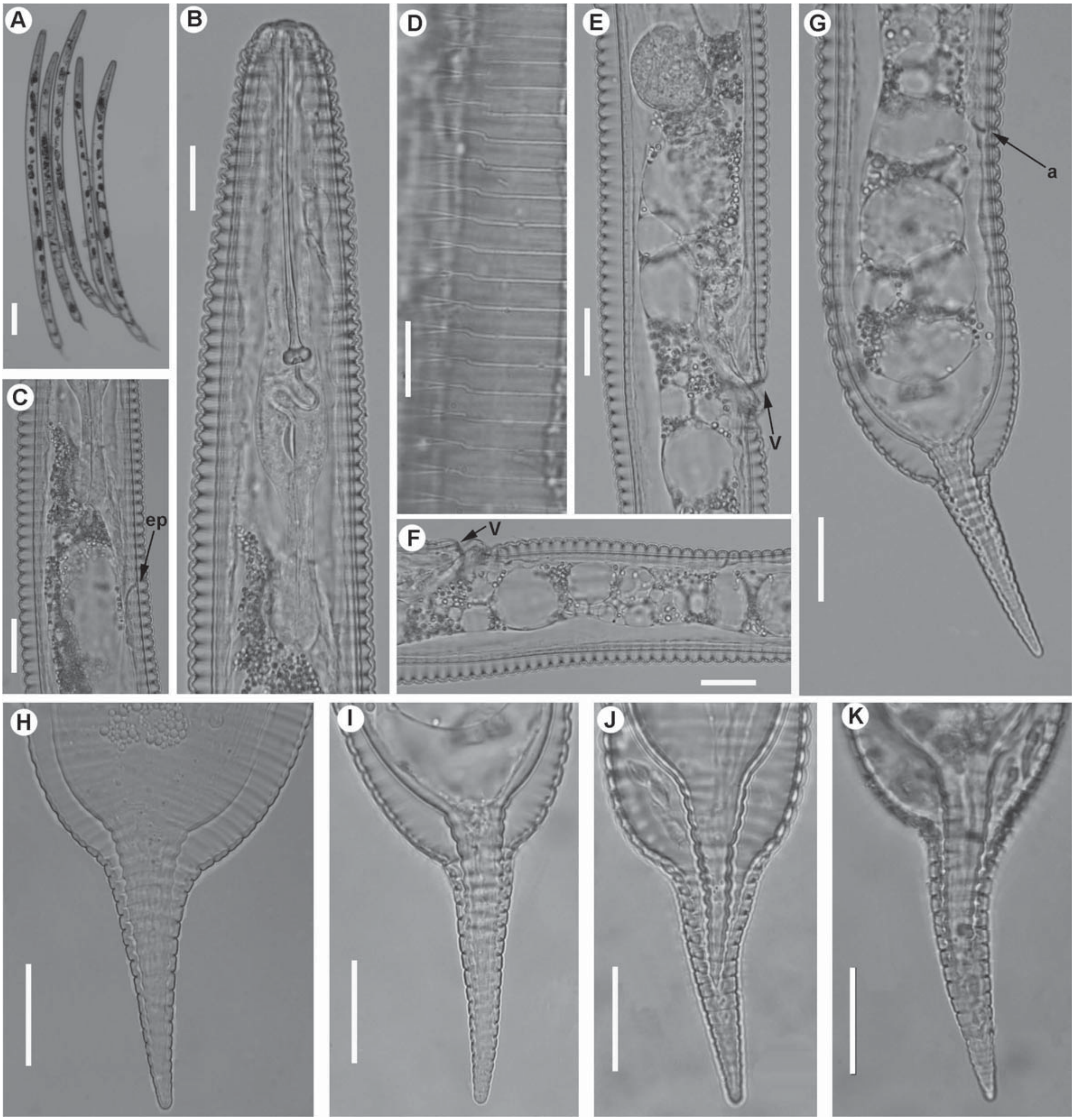
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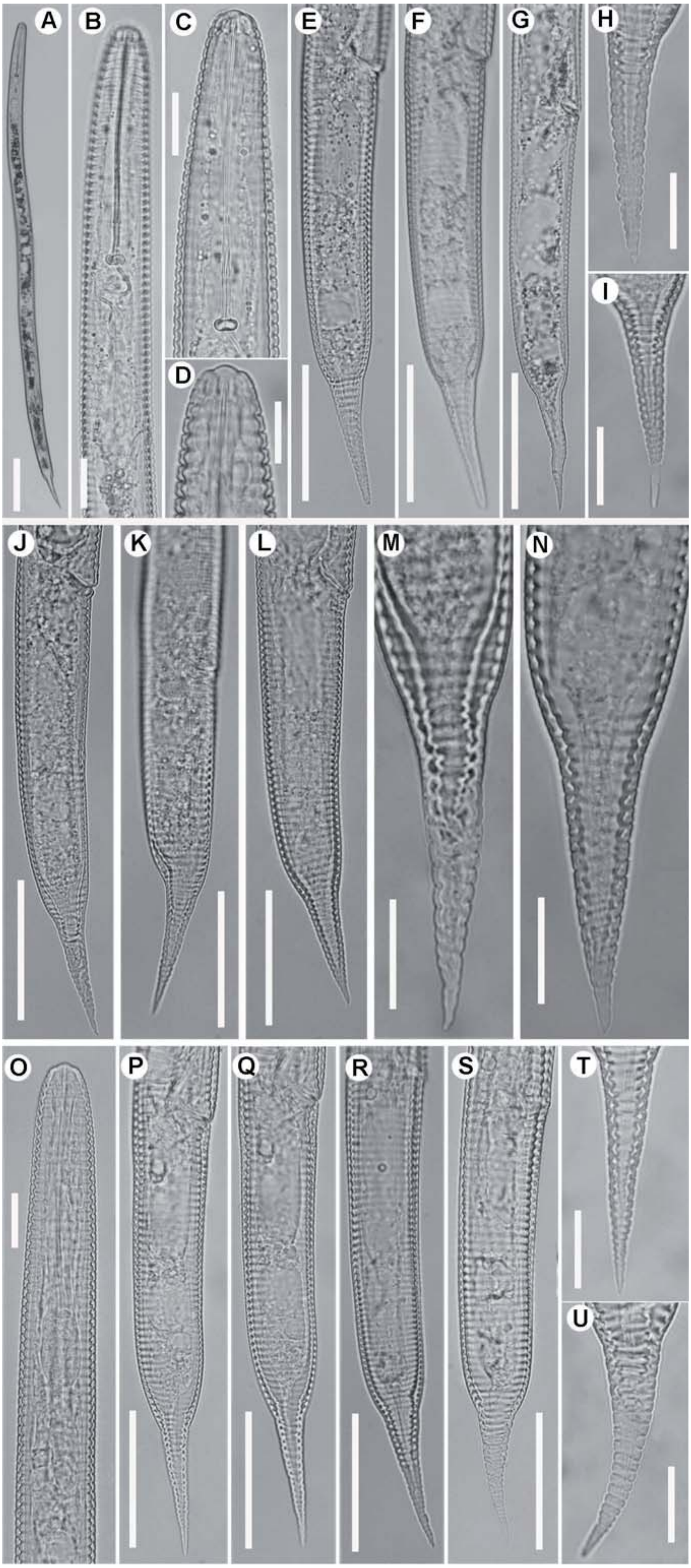
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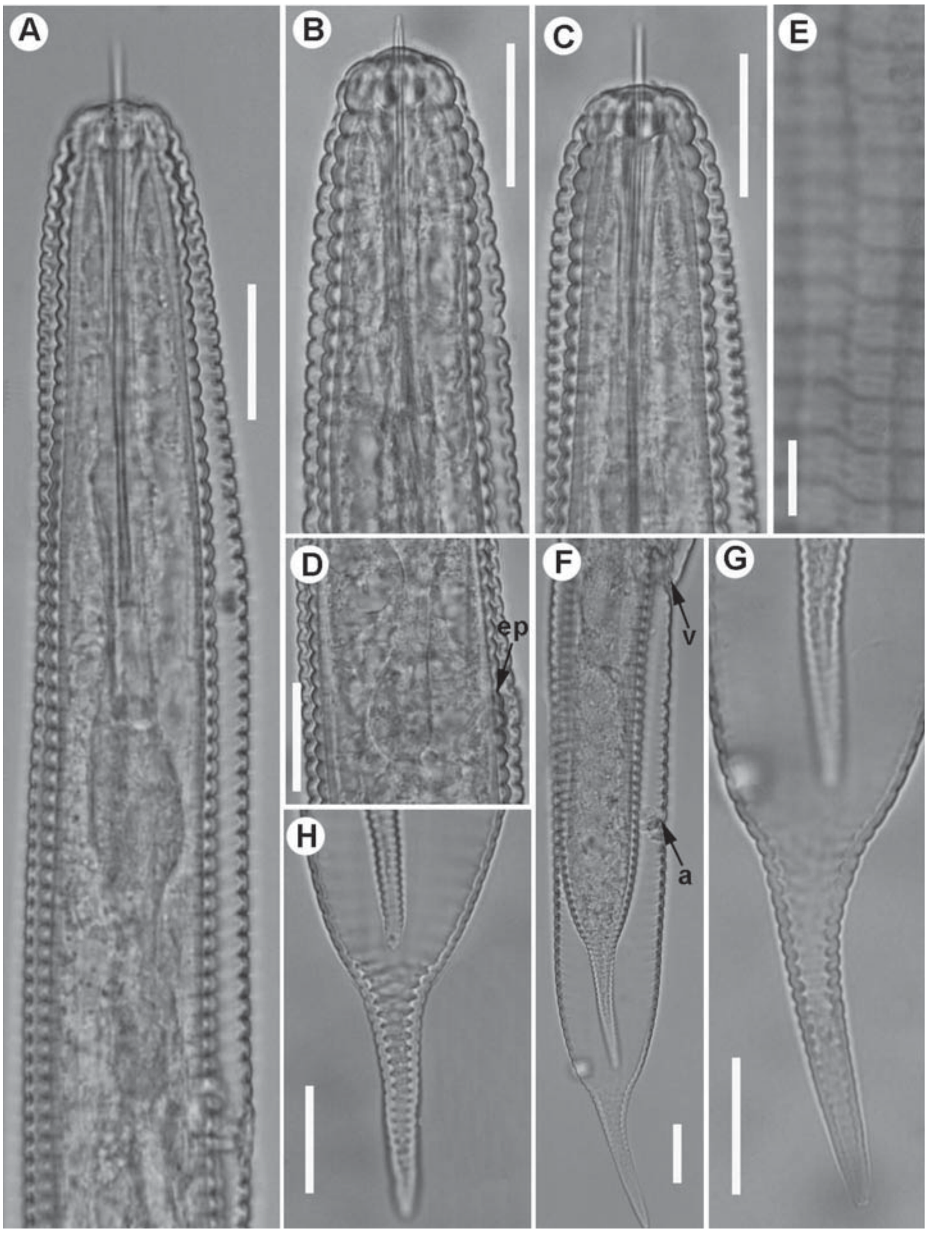
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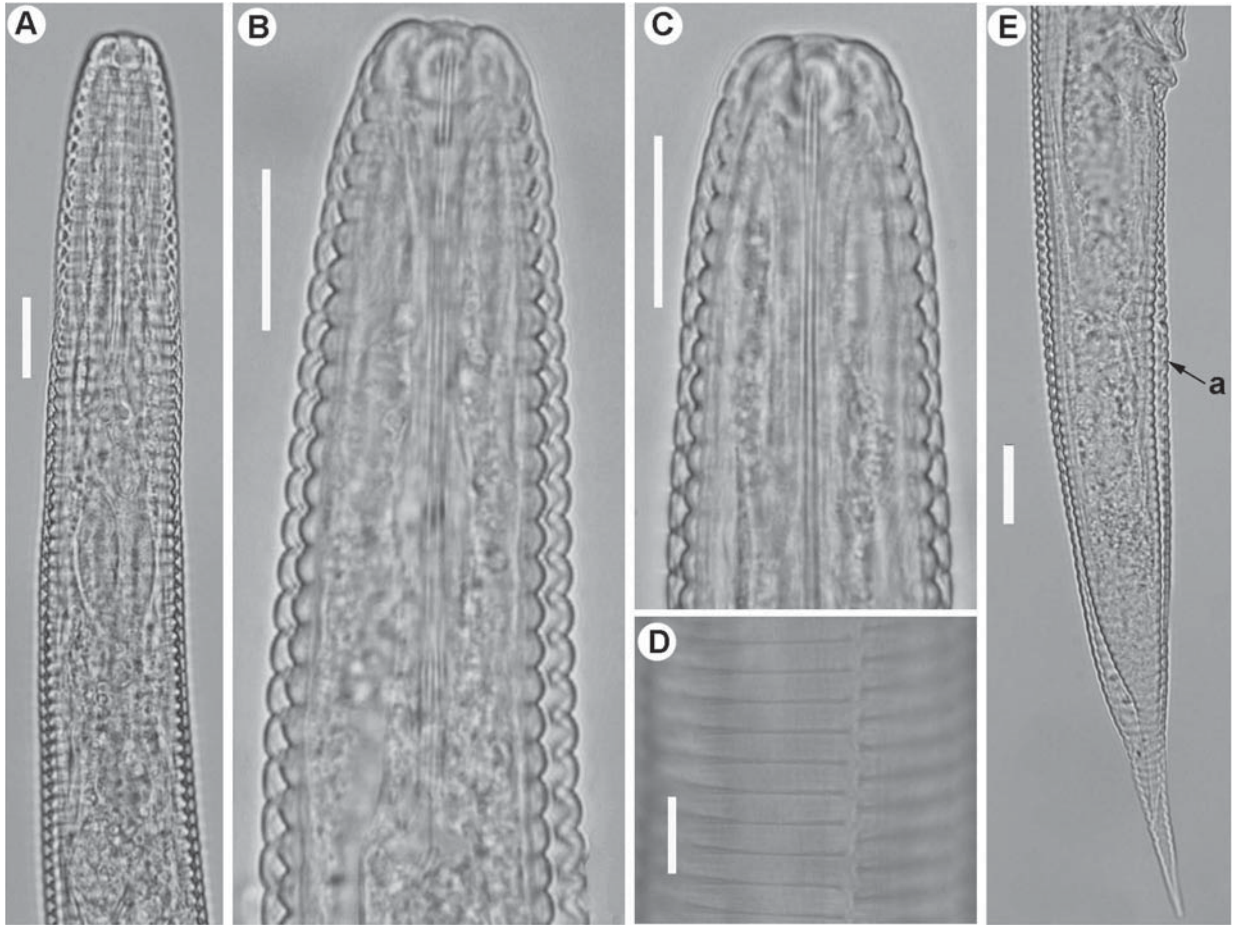

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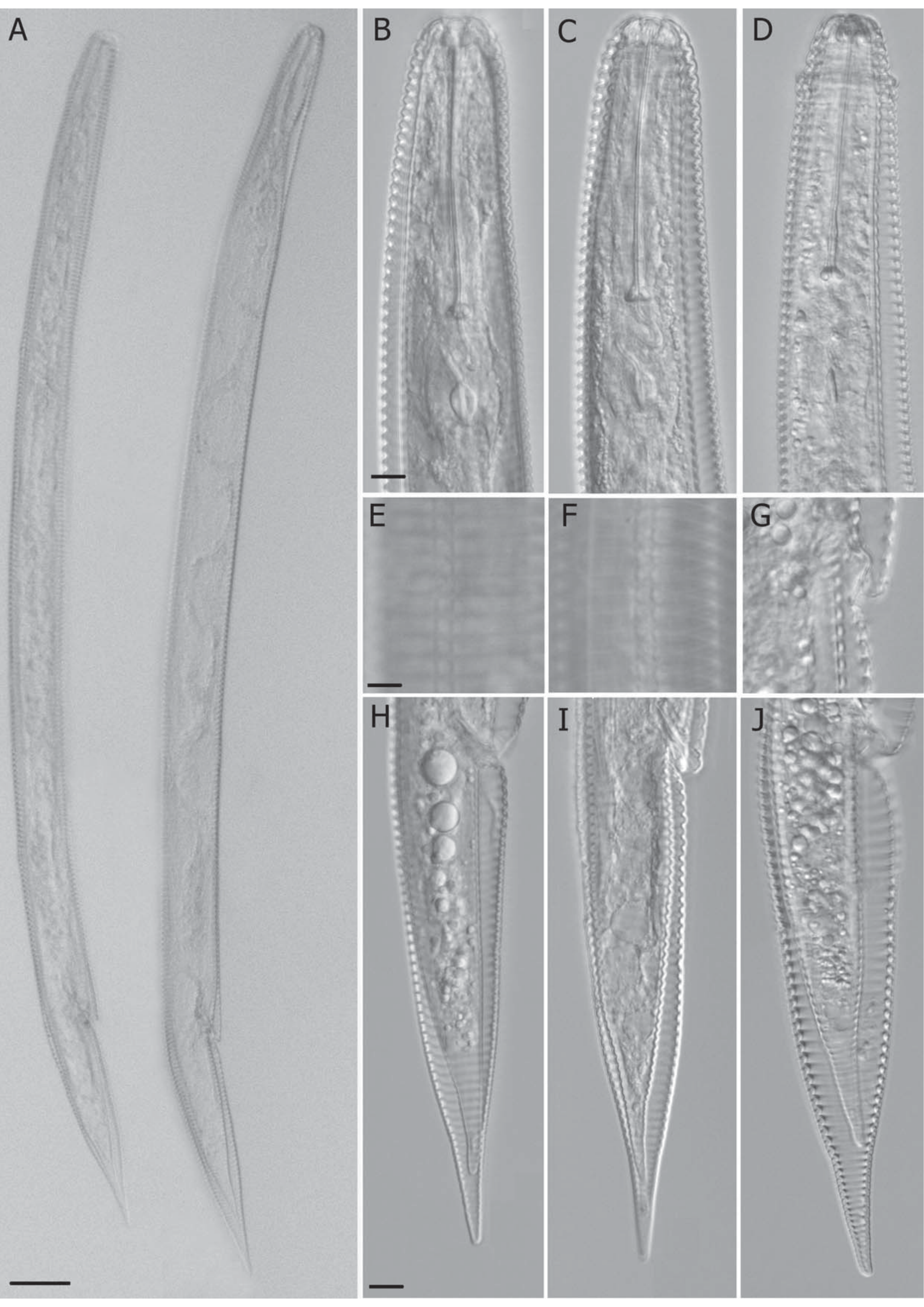
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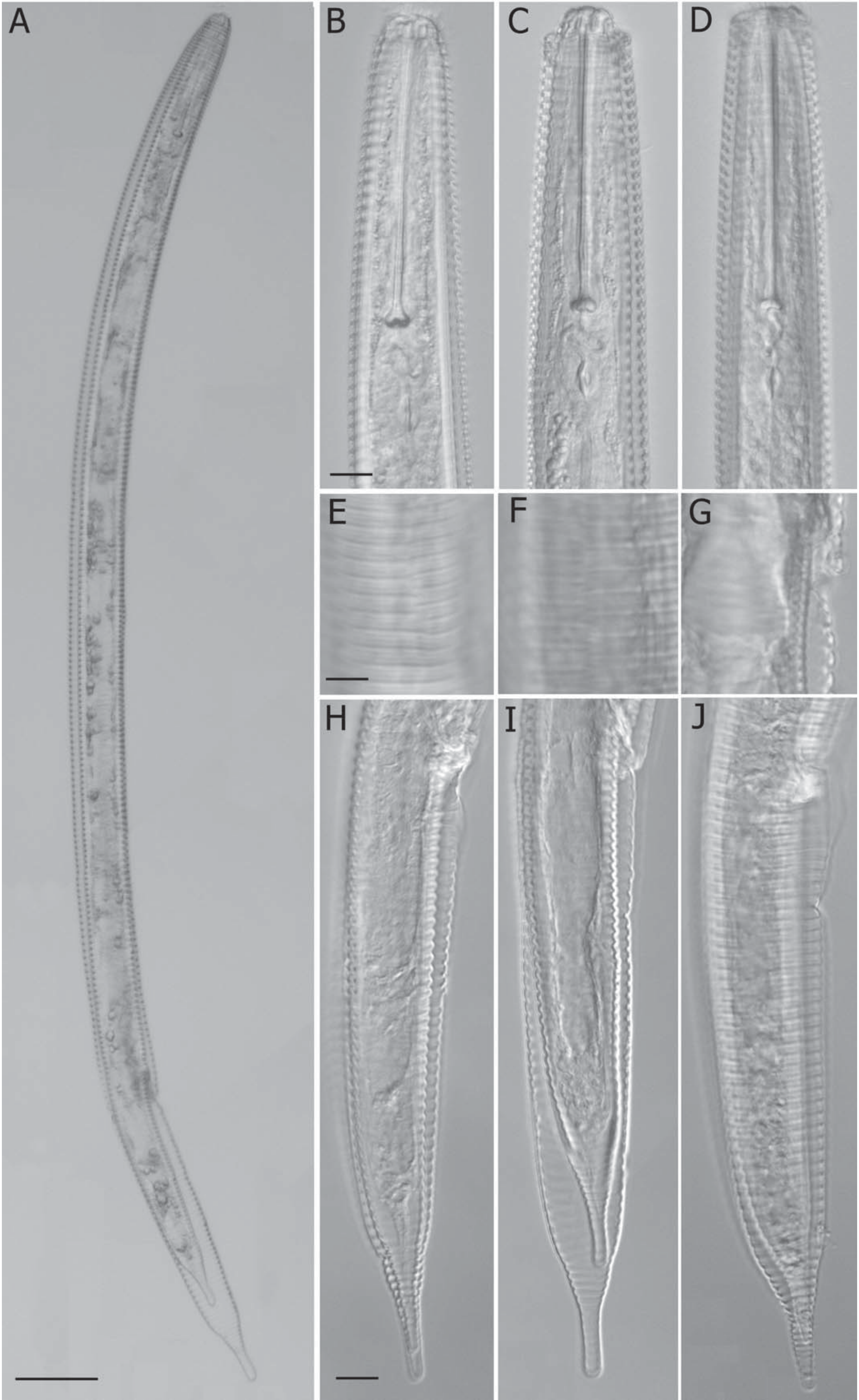
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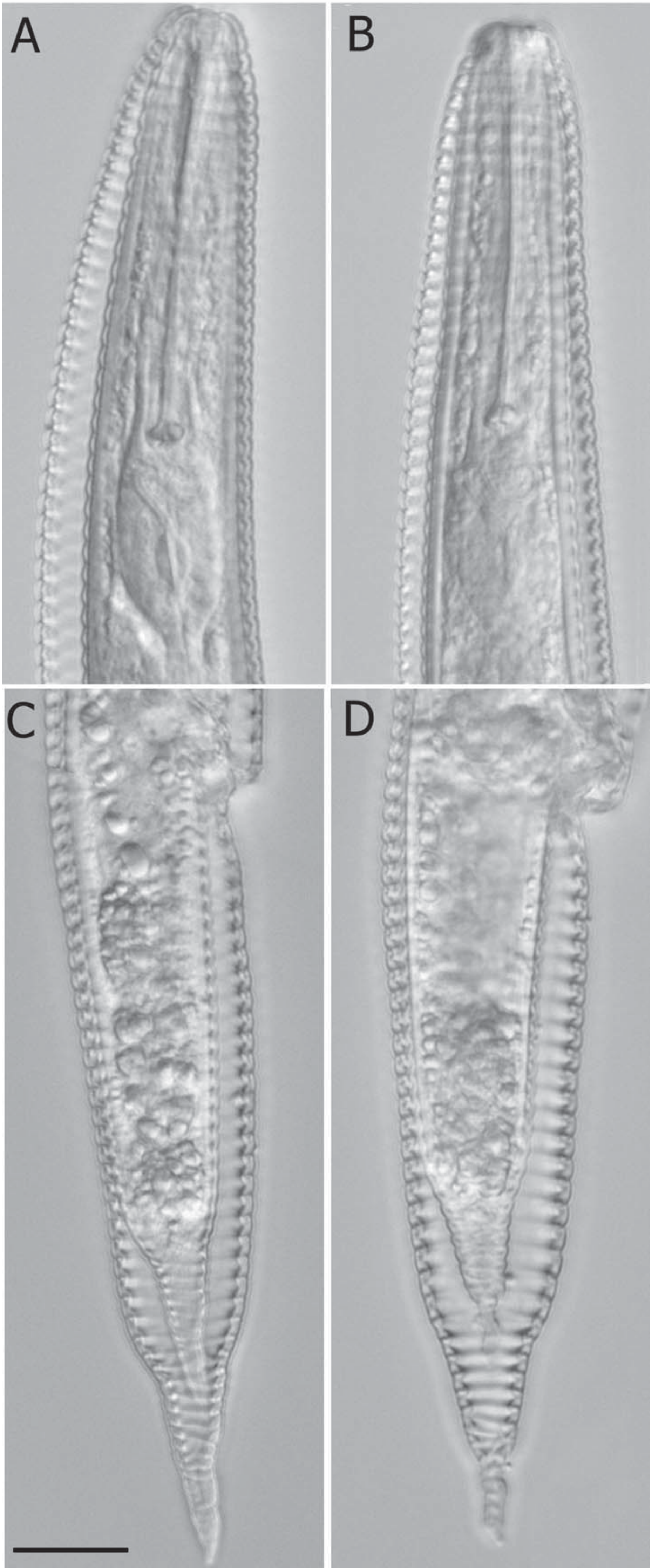
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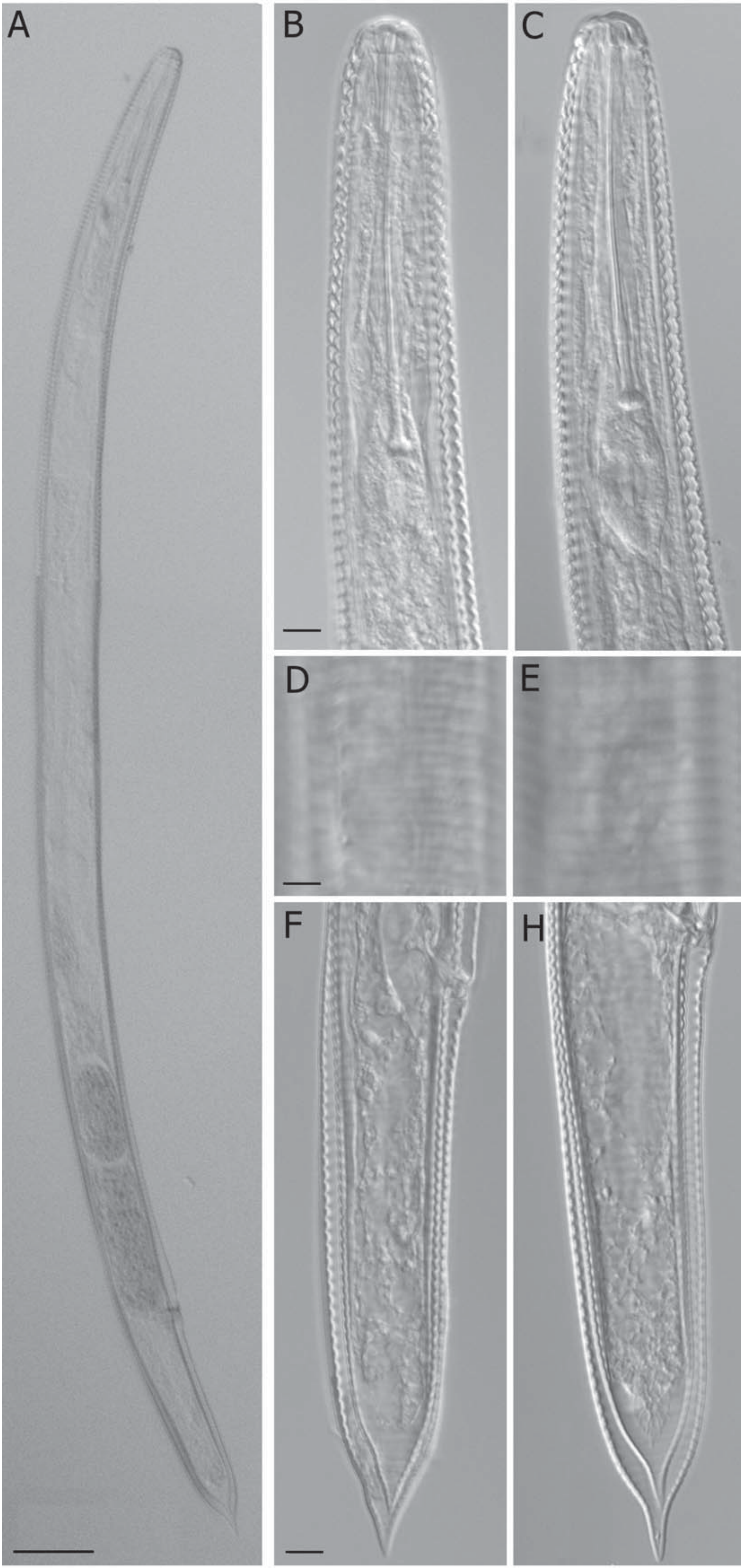
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Table S1. Morphometrics of *Hemicycliophora californica, H. conida* and *H. epicharoides* analysed in the present study. (All measurements in µm.)

Species Locality	*H. californica* CA, USA	*H. conida* Madrid, Spain	*H. conida* WA, USA	*H. epicharoides* Serranova, Italy	*H. epicharoides* Cádiz, Spain
n	8	10	3	11	20
L	856.4 ± 30.7 (801-903)	769.7 ± 76.5 (683-912)	836.1 ± 32.9 (810-873)	834.7 ± 49.3 (766-905)	822.4 ± 42.3 (766-886)
Maximum body width	29.7 ± 2.3 (28-34)	39.3 ± 0.9 (38-41)	33.2 ± 2.9 (31-37)	44.0 ± 4.1 (40-53)	44.1 ± 2.7 (39-47)
Pharynx length	156.1 ± 6.1 (152-170)	139.5 ± 7.1 (132-156)	164.9 ± 6.0 (160-172)	151.4 ± 6.5 (141-159)	145.0 ± 5.5 (137-152)
Tail length	75.6 ± 5.6 (68-84)	72.9 ± 4.2 (66-79)	82.8 ± 7.0 (78-91)	65.4 ± 4.2 (60-72)	63.4 ± 3.2 (59-70)
Anal body diameter	23.8 ± 1.7 (22-26)	28.4 ± 0.8 (27-30)	23.3 ± 2.4 (22-26)	38.7 ± 1.8 (37-42)	35.0 ± 0.8 (34-36)
Stylet length	89.7 ± 6.6 (83-102)	74.1 ± 3.0 (70-80)	83.6 ± 5.7 (77-89)	80.5 ± 5.8 (74-91)	86.7 ± 3.2 (83-94)
Stylet knob width	6.8 ± 0.7 (5.6-8)	6.8 ± 0.4 (6.5-7.0)	7.2 ± 0.4 (6.8-7.6)	8.2 ± 0.5 (7.5-9.0)	7.8 ± 0.3 (7.0-8.0)
Stylet knob height	3.4 ± 0.2 (2.8-3.6)	4.8 ± 0.5 (4.5-5.0)	4.2 ± 0.2 (4-4.4)	4.6 ± 0.5 (4.0-5.0)	4.5 ± 0.6 (4.0-5.0)
DGO	11.2 ± 2.4 (9.6-14)	9.8 ±1.25 (9.0-12.0)	9.3 ± 2.3 (7.6-12)	6.6 ± 0.5 (6.0-7.0)	6.7 ± 0.8 (6.0-8.0)
Nerve ring-anterior end	-	112.5 ± 3.9 (108-117)	-	127.0 ± 5.2 (119-133)	116.7 ± 3.8 (111-122)
Excr. pore-anterior end	166.3 ± 11.7 (153-186)	153.0 ± 5.6 (146-158)	173.5 ± 2.9 (170-175)	170.0 ± 8.2 (164-182)	161.5 ± 4.5 (158-168)
Lip width	15.0 ± 0.7 (13.6-15.6)	18.3 ± 0.4 (18.0-18.5)	16.6 ± 1.1 (16-18)	21.1 ± 0.7 (20-22)	19.3 ± 0.7 (18.5-20.0)
Lip height	8 ± 0.4 (7.2-8.4)	6.3 ± 0.4 (6.0-6.5)	8.7 ± 0.3 (8.4-9)	9.8 ± 0.5 (9.0-10.5)	9.8 ± 0.8 (9.0-11.0)
Annuli width	3.7 ± 0.4 (3.3-4.3	3.8 ± 0.4 (3.5-4.0)	3.5 ± 0.1 (3.4-3.7)	6.0 ± 0.7 (5-7)	6.1 ± 0.7 (5-7)
R	245 ± 19.7 (219-266)	238.3 ± 22.3 (209-278)	250 ± 4.0 (246-254)	175.1 ± 7.4 (164-185)	178.9 ± 5.2 (170-185)
Rst	26 ± 1.6 (23-28)	18.5 ± 0.7 (18-19)	25 ± 2.0 (23-27)	19.0 ± 1.3 (17-21)	18.3 ± 0.9 (17-20)
Roes	43 ± 2.7 (40-48)	33.5 ± 0.7 (33-34)	47 ± 2.6 (45-50)	32.4 ± 1.6 (30-34)	32.5 ± 1.8 (30-35)
Rex	48 ± 4.2 (42-55)	38.5 ± 0.7 (38-39)	51 ± 1.5. (49-52)	36.4 ± 1.6 (33-38)	35.9 ± 2.0 (36-43)
RV from terminus	44 ± 8.0 (37-61)	43.7 ± 2.1 (42-49)	45 ± 4.0 (41-49)	31.6 ± 3.6 (22-35)	35.7 ± 2.5 (32-35)
RV anterior end	202 ± 15.8 (182-220)	194.6 ± 21.4 (167-229)	206 ± 4.0 (202-210	143.5 ± 6.3 (134-154)	139.2 ± 5.6 (129-147)
RVan	15 ± 3.3 (11-19)	11.3 ± 0.9 (10-13)	13 ± 2.3 (10-14)	11.0 ± 1.2 (9-13)	11.9 ± 2.2 (8-15)
Ran	27 ± 2.2 (23-29)	32.6 ± 1.6 (31-36)	32 ± 2.6 (30-35)	20.6 ± 3.8 (10-23)	26.6 ± 1.3 (25-28)
VL/VB	.4 ± 0.4 (3.9-5.3)	2.8 ± 0.1 (2.7-2.8)	4.3 ± 0.2 (4.2-4.6)	2.7 ± 0.2 (2.5-3.0)	2.7 ± 0.2 (2.5-3.2)
PV/ABW	5.0 ± 0.3 (4.6-5.5)	4.7 ± 0.3 (4.0-5.0)	5.2 ± 0.3 (5-5.6)	2.9 ± 0.2 (2.6-3.2)	3.2 ± 0.2 (3.0-3.6)
a	28.6 ± 1.5 (25.7-30.9)	19.6 ± 1.7 (17.2-22.2)	25.2 ± 1.2 (23.9-26.4)	19.0 ± 1.0 (17.1-20.4)	18.7 ± 1.4 (17.2-22.2)
b	5.4 ± 0.1 (5.2-5.7)	5.5 ± 0.3 (5.1-6.0)	5.0 ± 0.2 (4.8-5.3)	5.5 ± 0.2 (5.1-5.8)	5.7 ± 0.2 (5.3-6.0)
c	11.4 ± 0.9 (10.2-12.6)	10.5 ± 0.6 (9.2-11.5)	10.1 ± 0.4 (9.6-10.4)	12.8 ± 0.7 (12.1-14.1)	13.0 ± 0.5 (12.4-14.1)
c'	2.9 ± 0.7 (1.3-3.6)	2.6 ± 0.1 (2.4-2.8)	3.5 ± 0.05 (3.5-3.6)	1.7 ± 0.1 (1.5-1.9)	1.8 ± 0.1 (1.7-2.1)
V (%)	85.6 ± 0.9 (84-87)	84.6 ± 1.2 (83-86)	87.3 ± 1.5 (86-88)	84.7 ± 1.5 (82-87)	84.6 ± 1.7 (81-87)
G1 (%)	-	56.2 ± 2.5 (54.5-58.0)	-	53.3 ± 7.2 (45.6-62.7)	47.0 ± 3.1 (42.9-51.6)
St%L	10.4 ± 0.5 (9.7-11.3)	9.7 ± 0.7 (8.7-10.5)	10.0 ± 0.6 (9.6-10.8)	9.7 ± 0.6 (8.9-10.8)	10.6 ± 0.5 (9.7-11.6)
St%Oes	-	53.2 ±1.51 (51.3-56.2)	-	53.2 ± 4.1 (46.5-58.9)	59.9 ± 3.2 (55.9-68.1)
VA%T	61.9 ± 16.8 (44.8-89.8)	81.2 ± 5.7 (69.7-87.8)	46.1 ± 9.5 (36.5-55.6)	69.1 ± 12.8 (43.1-83.3)	75.9 ± 6.6 (70.3-87.1)
O	-	13.6 ± 1.9 (12.2-17.1)	-	8.2 ± 0.9 (7.2-9.5)	11.6 ± 1.2 (10.3-12.9)

Table S2. Morphometrics of *Hemicycliophora floridensis, H. gracilis* and *H. iberica* analysed in the present study. (All measurements in μm.)

Species Locality	*H. floridensis* FL, USA	*H. gracilis* CA, USA	*H. iberica* Huelva, Spain	*H. iberica* Jaén, Spain
n	10	5	6	10
L	1050 ± 57 (954-1116)	1124 ± 80.7 (1044-1212)	847.2 ± 71.5 (752-932)	881.8 ± 69.6 (774-978)
Maximum body width	54 ± 3.3 (50-62)	35.6 ± 2.0 (34-38)	33.2 ± 0.8 (32.0-34.0)	33.5 ± 1.6 (31.0-36.0)
Pharynx length	204 ± 24 (180-268)	200.1 ± 10.8 (188-216)	139.8 ± 9.2 (127-150)	140.8 ± 9.6 (129-154)
Tail length	84 ± 11 (68-97)	116.2 ± 7.5 (103-122)	78.2 ± 3.3 (74-82)	79.8 ± 3.6 (74-84)
Anal body diameter	40 ± 2.5 (35-43)	26.6 ± 2.3 (25-30)	25.8 ± 1.0 (25.0-27.0)	27.2 ± 1.2 (26.0-29.0)
Stylet length	121 ± 4.0 (115-125)	103.3 ± 8.9 (91-116)	83.5 ± 2.6 (80-87)	85.8 ± 3.2 (82-91)
Stylet knob width	9.6 ± 0.5 (9.1-11)	8.4 ± 0.5 (8-9.2)	7.3 ± 0.5 (7.0-8.0)	7.0 ± 0.7 (6.0-8.0)
Stylet knob height	7.0 ± 0.7 (6.0-7.6)	4.4 ± 0.5 (3.6-5.2)	3.8 ± 0.3 (3.5-4.0)	4.3 ± 0.3 (4.0-4.5)
DGO	6.6 ± 0.6 (5.3-7.6)	11.4 ± 1.1 (10.4-12.8)	7.7 ± 0.6 (7.0-8.5)	7.3 ± 0.5 (7.0-8.0)
Nerve ring-anterior end	166 ± 6.9 (156-176)	-	113.2 ± 7.8 (106-124)	114.2 ± 7.0 (108-124)
Excr. pore-anterior end	219 ± 12.2 (200-237)	182.5 ± 13.5 (162-197)	145.7 ± 9.0 (133-156)	147.2 ± 7.3 (136-156)
Lip width	25 ± 1.2 (24-27)	16.3 ± 1.7 (14.8-19.20)	8.6 ± 0.5 (8.0-9.0)	8.7 ± 0.4 (8.0-9.0)
Lip height	9.8 ± 0.3 (9.1-10)	9.3 ± 1.0 (8.4-10.8)	4.5 ± 0.4 (4.0-5.0)	4.6 ± 0.4 (4.0-5.0)
Annuli width	5.2 ± 0.7 (4.2-6.3)	3.5 ± 0.3 (3.2-4)	2.9 ± 0.3 (2.5-3.0)	2.6 ± 0.3 (2.5-3.0)
R	229 ± 17 (209-265)	355 ± 29.6 (322-380)	268.3 ± 10.6 (256-281)	264.5 ± 12.8 (243-279)
Rst	26 ± 2.6 (22-30)	40 ± 1.4 (39-41)	21.8 ± 8.3 (5-27)	24.8 ± 1.5 (23-27)
Roes	48 ± 6.7 (37-55)	67 ± 3.0 (64-70)	42.7 ± 1.4 (41-44)	44.8 ± 3.1 (41-49)
Rex	51 ± 3.2 (46-55)	66 ± 4 (62-70)	44.8 ± 1.6 (43-47)	45.8 ± 3.0 (43-51)
RV from terminus	41 ± 7.1 (31-50)	71 ± 4.6 (64-77)	51.7 ± 4.9 (46-58)	50.3 ± 3.8 (45-56)
RV-anterior end	190 ± 18 (170-220)	299 ± 8.6 (291-308)	216.7 ± 8.1 (206-224)	214.2 ± 12.3 (194-227)
RVan	12 ± 1.7 (10-15)	23 ± 1.1 (22-25)	15.2 ± 2.1 (13-18)	13.8 ± 1.5 (12-16)
Ran	29 ± 7.4 (21-39)	47 ± 4.2 (41-52)	36.5 ± 3.0 (33-41)	36.0 ± 2.3 (33-39)
VL/VB	2.7 ± 0.4 (1.9-3.6)	5.7 ± 0.4 (5.4-6.5)	3.7 ± 0.2 (3.4-3.9)	3.8 ± 0.2 (3.5-3.9)
PV/ABW	3.4 ± 0.7 (2.4-4.6)	6.7 ± 0.3 (6.4-7.3)	4.7 ± 0.1 (4.5-4.8)	4.6 ± 0.2 (4.3-4.8)
a	20 ± 1.2 (18-21)	31.5 ± 2.2 (28.6-34.7)	25.5 ± 1.8 (22.8-27.4)	26.3 ± 0.9 (25.0-27.2)
b	5.2 ± 0.6 (4.0-6.0)	5.6 ± 0.4 (5.2-6.3)	6.1 ± 0.2 (5.7-6.3)	6.3 ± 0.6 (5.9-7.6)
c	13 ± 2.2 (10-17)	9.6 ± 0.6 (8.9-10.4)	10.8 ± 0.7 (9.8-11.6)	11.0 ± 0.8 (9.8-12.0)
c'	2.1 ± 0.3 (1.5-2.4)	4.3 ± 0.2 (4.1-4.8)	3.0 ± 0.1 (2.9-3.1)	2.9 ± 0.1 (2.8-3.1)
V (%)	87 ± 2.4 (82-90)	83.9 ± 1.5 (82-86)	84.5 ± 1.4 (82-86)	84.3 ± 1.2 (82-85)
G1 (%)	42 ± 5 (36-53)	-	41.9 ± 5.7 (36-47)	38.4 ± 4.2 (35.2-43.2)
St%L	12 ± 0.7 (11-13)	9.1 ± 0.4 (8.7-9.5)	9.9 ± 0.6 (9.3-10.8)	9.8 ± 0.7 (9.3-11.1)
St%Oes	59 ± 6.7 (44-66)	-	59.9 ± 3.0 (57.3-65.4)	61.7 ± 5.8 (57.3-71.6)
VA%T	73 ± 14 (59-95)	53.4 ± 7.3 (43.3-63.5)	53.7 ± 1.8 (50.6-55.4)	55.1 ± 1.6 (53.6-58.2)
O	5.5 ± 0.5 (4.6-6.6)	-	9.2 ± 0.5 (8.5-9.8)	8.9 ± 0.7 (8.1-9.8)

Table S3. Morphometrics of *Hemicycliophora lutosoides, H. obtusa, H. ripa, H. raskii* and *H. similis* analysed in the present study. (All measurements in µm.)

Species Locality	*H. lutosoides* Sevilla, Spain	*H. lutosoides* Madrid, Spain	*H. obtusa* Huelva, Spain	*H. ripa* Huelva, Spain	*H. raskii* CA, USA	*H. similis* Cartaya, Spain
n	10	10	5	10	7	12
L	982.0 ± 43.2 (911-1058)	940.8 ± 57.0 (875-1032)	763.5 ± 70.3 (683-928)	970.7 ± 42.5 (900-1017)	773.7 ± 27.6 (720-798.1)	1182.4 ± 146.5 (1022-1578)
Maximum body width	44.7 ± 1.7 (42.0-48.0)	37.5 ± 2.8 (30.0-40.0)	34.9 ± 3.2 (31.0-40.0)	41.7 ± 2.0 (40.0-45.0)	29.6 ± 2.8 (25-34)	41.8 ± 3.0 (38.0-49.0)
Pharynx length	157.5 ± 5.8 (149-167)	152.7 ± 8.1 (143-166)	134.4 ± 10.6 (119-153)	167.0 ± 5.1 (158-174)	143.4 ± 8.3 (130-167)	184.8 ± 12.0 (164-209)
Tail length	132.2 ± 10.1 (121-149)	116.8 ± 8.5 (103-129)	63.5 ± 6.0 (53-73)	83.8 ± 7.9 (78-103)	73 ± 7.5 (61-84)	112.6 ± 14.8 (80-125)
Anal body diameter	30.2 ± 2.5 (26.0-34.0)	24.4 ± 1.0 (23.0-26.0)	30.2 ± 4.5 (26.0-39.0)	30.2 ± 2.5 (27.0-34.0)	24.7 ± 3.4 (21-30)	32.4 ± 2.5 (28.0-36.0)
Stylet length	80.8 ± 1.5 (79-84)	75.5 ± 2.0 (73-80)	68.7 ± 7.3 (62-88)	101.8 ± 4.4 (94-110)	80.4 ± 2.7 (77-85)	100.3 ± 4.9 (91-108)
Knobs width	6.2 ± 0.3 (6.0-6.5)	6.9 ± 0.3 (6.5-7.5)	6.6 ± 0.5 (6.0-7.0)	6.3 ± 0.3 (6.0-6.5)	6.9 ± 0.5 (6.4-8)	8.9 ± 0.6 (8.0-10.0)
Knobs height	2.8 ± 0.3 (2.5-3.0)	4.2 ± 0.2 (4.0-4.5)	3.4 ± 0.2 (3.0-3.5)	3.3 ± 0.3 (3.0-3.5)	3.3 ± 0.4 (2.8-4)	4.1 ± 0.4 (3.5-5.0)
DGO	15.3 ± 1.8 (13.0-19.0)	9.8 ± 1.2 (8.0-12.0)	5.8 ± 0.3 (5.5-6.0)	8.3 ± 0.6 (8.0-9.0)	14.2 ± 3.0 (10.8-16)	11.6 ± 1.5 (9.0-14.0)
Nerve ring-anterior end	131.7 ± 3.1 (129-135)	118.8 ± 7.3 (112-128)	107.4 ± 11.5 (97-123)	133.6 ± 6.6 (125-143)	-	147.5 ± 10.8 (132-168)
Excr. pore-anterior end	175.3 ± 10.1 (166-186)	162.0 ± 6.4 (157-171)	139.3 ± 19.5 (121-173)	173.7 ± 6.8 (164-184)	151.3 ± 11.0 (130-167)	197.3 ± 13.3 (177-217)
Lip width	11.0 ± 2.0 (9.0-13.0)	14.5 ± 0.5 (14.0-15.0)	15.3 ± 0.6 (14.5-16.0)	21.8 ± 1.1 (20.0-23.0)	14.7 ± 0.5 (14-15.6)	18.1 ± 0.8 (17.0-20.0)
Lip height	4.2 ± 0.3 (4.0-4.5)	4.82 ± 0.3 (4.5-5.0)	5.7 ± 0.6 (5.0-6.5)	8.5 ± 0.3 (8.0-9.0)	7.5 ± 0.7 (6.4-8.4)	4.1 ± 0.3 (3.5-4.5)
Annuli width	3.0 ± 0.5 (2.5-3.5)	3.2 ± 0.3 (3.0-3.5)	3.9 ± 0.4 (3.5-4.5)	4.1 ± 0.2 (4.0-4.5)	4.2 ± 0.2 (3.8-4.6)	4.1 ± 0.2 (4.0-4.5)
R	335.5 ± 20.0 (303-364)	328.5 ± 19.0 (302-359)	207.2 ± 9.9 (190-219)	238.8 ± 6.0 (229-248)	208 ± 13.9 (194-234)	290.4 ± 15.3 (262-309)
Rst	24.7 ± 2.4 (21-28)	26.2 ± 1.4 (24-29)	20.7 ± 2.4 (17-25)	25.8 ± 1.5 (24-29)	24 ± 1.6 (21-25)	25.2 ± 1.9 (23-29)
Roes	54.7 ± 2.3 (51-58)	56.3 ± 2.5 (52-60)	40.0 ± 3.7 (37-45)	43.1 ± 2.8 (41-50)	40 ± 3.1 (37-44)	45.1 ± 3.2 (42-52)
Rex	58.8 ± 2.4 (54-62)	62.0 ± 2.3 (58-65)	42.8 ± 3.7 (39-49)	46.1 ± 3.6 (43-55)	42 ± 3.6 (38-49)	50.3± 6.3 (46-69)
RV from terminus	81.2 ± 6.1 (73-95)	77.5 ± 3.6 (72-84)	39.9 ± 2.1 (37-43)	53.8 ± 1.8 (51-56)	37 ± 2.7 (33-41)	64.4 ± 4.1 (57-70)
RV-anterior end	254.3 ± 22.2 (224-282)	251.0 ± 16.6 (230-275)	167.3 ± 8.5 (153-178)	185.0 ± 5.4 (178-194)	172 ± 11.6 (161-194)	237.4 ± 34.4 (192-314)
RVan	21.0 ± 1.5 (19-23)	21.6 ± 1.4 (20-24)	18.0 ± 2.5 (14-22)	17.1 ± 0.8 (15-18)	11.8 ± 1.5 (10-14)	21.6 ± 1.3 (19-24)
Ran	60.2 ± 5.3 (52-72)	54.7 ± 4.3 (49-62)	21.9 ± 1.6 (20-25)	36.7 ± 1.7 (34-39)	25 ± 2.4 (22-29)	42.8 ± 4.3 (36-51)
VL/VB	5.1 ± 0.3 (4.6-5.4)	6.0 ± 0.2 (5.6-6.3)	3.5 ± 0.4 (2.9-4.2)	4.3 ± 0.3 (3.7-4.7)	3.9 ± 0.6 (3.1-4.9)	5.9 ± 0.8 (4.8-7.6)
PV/ABW	6.6 ± 0.5 (6.0-7.4)	7.0 ± 0.3 (6.7-7.7)	4.0 ± 0.7 (2.9-4.6)	5.0 ± 0.5 (4.3-5.6)	4.4 ± 0.7(3.5-5.5)	7.0 ± 0.6 (6.3-8.1)
a	22.0 ± 1.2 (19.0-23.0)	25.3 ± 2.9 (23.3-33.2)	22.0 ± 1.9 (18.2-24.1)	23.3 ± 1.5 (20.0-24.7)	24.3 ± 3.6 (20.4-29.7)	28.3 ± 2.5 (24.9-34.3)
b	6.2 ± 0.4 (5.6-7.0)	6.2 ± 0.3 (5.6-6.7)	5.7 ± 0.4 (5.1-6.3)	5.8 ± 0.2 (5.5-6.1)	5.3 ± 0.4 (4.7-6.1)	6.4 ± 0.4 (5.9-7.6)
c	7.5 ± 0.6 (6.3-8.2)	8.1 ± 0.6 (7.4-9.1)	12.1 ± 1.1 (10.6-14.0)	11.6 ± 1.0 (9.4-13.0)	10.6 ± 0.9 (9.3-11.8)	10.6 ± 1.2 (9.4-12.9)
c'	4.4 ± 0.3 (4.1-4.8)	4.8 ± 0.2 (4.5-5.2)	2.1 ± 0.3 (1.6-2.5)	2.8 ± 0.3 (2.4-3.4)	2.9 ± 0.5 (2.3-3.9)	3.5 ± 0.4 (2.4-3.8)
V (%)	80.8 ± 1.0 (80-83)	80.3 ± 1.5 (77-82)	84.1 ± 1.4 (82-86)	81.2 ± 1.2 (80-83)	86 ± 0.8 (85-87)	79.8 ± 0.9 (78-81)
G1 (%)	30.2 ± 10.1 (24-45)	50.8 ± 16.4 (38-69)	34.0 ± 5.3 (27-42)	38.8 ± 2.0 (37-41)	-	33.0 ± 5.4 (26-43)
St%L	8.2 ± 0.3 (7.9-9.0)	8.0 ± 0.4 (7.6-8.3)	8.7 ± 0.8 (7.2-9.4)	10.5 ± 0.5 (9.8-11.3)	10.4 ± 0.4 (9.8-10.9)	8.6 ± 0.7 (6.8-9.7)
St%Oes	51.4 ± 2.0 (47.9-55.6)	49.5 ± 2.0 (46.0-52.4)	50.2 ± 5.0 (43.8-56.3)	60.9 ± 1.9 (58.4-63.6)	-	54.4 ± 2.2 (51.3-57.6)
VA%T	50.4 ± 6.1 (39.2-59.7)	47.4 ± 3.6 (41.1-51.2)	97.3 ± 20.9 (74.0-119.0)	77.8 ± 6.8 (54.2-86.3)	50.1 ± 10.9 (39.6-71.7)	85.5 ± 6.2 (71.8-93.8)
O	18.9 ± 2.0 (16.5-23.2)	13.0 ± 1.8 (10.4-16.4)	8.9 ± 0.6 (8.2-9.7)	8.0 ± 0.5 (7.4-8.8)	-	11.6 ± 1.6 (8.6-14.1)

Table S4. Morphometrics of *Hemicycliophora poranga* analysed in the present study. (All measurements in µm.)

Species	*H. poranga*					
Locality	Cádiz, Spain	Bari, Italy	Santa Rosa, CA, USA	Guerneville, CA, USA	San Francisco, CA, USA	Spanish Bay, CA, USA
n	10	10	4	14	4	12
L	993.3 ± 113 (876-1210)	1015.3 ± 37 (972-1084)	987.9 ± 97 (867-1104)	961.2 ± 70 (819-1068)	1076 ± 60 (1005-1140)	801.6 ± 56 (693-912)
Maximum body width	35.3 ± 0.9 (34.0-36.5)	39.6 ± 2.5 (37.0-45.0)	34.8 ± 5.1 (28-39)	35.5 ± 3.7 (29-42)	33.5 ± 3.3 (31-37)	35.9 ± 4.1 (25-42)
Pharynx length	173.3 ± 9.5 (158-185)	171.5 ± 7.7 (158-181)	191 ± 20.9 (172-221)	174 ± 14.6 (137-189)	179 ± 6.4 (172-187)	160 ± 7.0 (148-169)
Tail length	96.4 ± 3.6 (89-101)	98.2 ± 8.0 (86-109)	89.2 ± 24.5 (68-120)	97.9 ± 11.5 (78-113)	114 ± 8.6 (104-123)	91.2 ± 8.7 (70-101)
Anal body diameter	25.1 ± 0.7 (24.0-26.0)	27.1 ± 0.9 (26.0-28.0)	23 ± 2 (20-30)	23 ± 2 (20-28)	24.5 ± 0.5 (24-25)	24.1 ± 2.2 (20-28)
Stylet length	92.9 ± 3.0 (88-97)	91.2 ± 3.7 (86-96)	95.6 ± 4.1 (90-99)	93.4 ± 5.9 (77-98)	97.7 ± 1.7 (97-100)	92.2 ± 3.4 (86-99)
Stylet knob width	8.0 ± 0.8 (7.0-9.0)	7.2 ± 0.3 (7.0-7.5)	7.3 ± 0.5 (6.8-8)	6.8 ± 0.7 (5.6-8)	8.8 ± 2.1 (7.4-12)	7.1 ± 0.7 (5.6-8.4)
Stylet knob height	6.6 ± 0.5 (6.0-7.0)	5.2 ± 0.3 (5.0-5.5)	3.6 ± 0.4 (3.2-4)	3.6 ± 0.3 (3.2-4.4)	3.7 ± 0.2 (3.6-4)	3.1 ± 0.3 (2.8-4)
DGO	14.2 ± 1.6 (12.0-17.0)	7.9 ± 0.7 (7.0-9.0)	16.3 ± 1.5 (15.2-18)	16.1 ± 1.6 (12.8-18)	15.3 ± 1.7 (13-16.8)	16.8 (n = 1)
Nerve ring-anterior end	140.3 ± 5.4 (134-147)	126.7 ± 13.6 (114-145)	-	-	-	-
Excr. pore-anterior end	180.5 ± 7.0 (172-189)	176.3 ± 6.5 (170-183)	182 ± 30 (157-225)	173 ± 14.7 (141-193)	189 ± 15.5 (167-200)	165 ± 11.6 (135-178)
Lip width	16.6 ± 0.5 (16.0-17.0)	15.5 ± 0.7 (15.0-16.0)	16.4 ± (16-17.2)	16.6 ± 1.2 (14.4-18.4)	16.8 ± 1.3 (15.2-18.4)	15.8 ± 0.7 (14.8-17.6)
Lip height	7.2 ± 0.8 (6.0-8.0)	6.5 ± 0.7 (6.0-7.0)	8.9 ± 0.9 (8-10)	9.4 ± 0.7 (8-11.2)	9.7 ± 0.2 (9.6-10)	8.8 ± 0.6 (7.6-10))
Annuli width	3.5 ± 0.4 (3.0-4.0)	3.5 ± 0.4 (3.0-4.0)	3.3 ± 0.4 (2.8-3.9)	3.7 ± 0.3 (3.1-4.1)	4.2 ± 0.2 (3.9-4.5)	2.7 ± 0.3 (2.2-3.5)
R	294.5 ± 17.7 (275-321)	328.8 ± 9.9 (317-345)	317 ± 15.1 (300-335)	306 ± 9.9 (292-326)	314 ± 10.2 (303-327)	311 ± 12.8 (285-330)
Rst	31.4 ± 1.7 (29-34)	31.3 ± 1.5 (29-34)	34 ± 3.1 (29-36)	30 ± 2.9 (21-35)	30 ± 1.7 (28-32)	31 ± 1.6 (28-34)
Roes	59.7 ± 2.3 (56-64)	63.2 ± 2.9 (59-67)	65 ± 2.9 (62-69)	60 ± 4.8 (53-69)	56 ± 4.0 (52-61)	56 ± 2.2 (52-60)
Rex	60.8 ± 2.4 (57-65)	64.5 ± 2.8 (60-68)	61 ± 2.5 (58-64)	60 ± 1.7 (57-63)	60 ± 0.8 (59-61)	60 ± 2.3 (50-58)
RV from terminus	68.5 ± 2.6 (64-72)	75.3 ± 4.7 (68-83)	57 ± 10.2 (43-67)	60 ± 3.4 (54-66)	65 ± 6.1 (61-74)	59 ± 2.9 (54-64)
RV-anterior end	226.0 ± 15.7 (211-250)	253.5 ± 8.8 (236-267)	260 ± 11.7 (250-277)	247 ± 8.3 (233-260)	249 ± 5.5 (242-254)	253 ± 11.1 (230-270)
RVan	20.4 ± 1.7 (17-23)	21.2 ± 1.8 (18-24)	20 ± 0.9 (19-21)	21 ± 1.6 (18-24)	22 ± 2.8 (20-26)	21 ± 2.9 (18-27)
Ran	48.1 ± 1.9 (46-51)	54.1 ± 4.3 (46-59)	38 ± 9.6 (24-47)	40 ± 3.6 (34-45)	43 ± 3.7 (39-48)	38 ± 3.2 (33-45)
VL/VB	5.3 ± 0.5 (4.7-6.1)	5.6 ± 0.4 (4.8-6.1)	4.9 ± 0.9 (3.7-6)	5.3 ± 0.5 (4.6-6.4)	5.8 ± 0.9 (4.9-6.8)	4.5 ± 0.5 (3.5-5.8)
PV/ABW	5.4 ± 1.4 (3.7-6.9)	4.6 ± 1.0 (3.4-5.6)	5.7 ± 1.1 (4.2-6.8)	6.5 ± 0.3 (5.8-7.3)	7.0 ± 0.8 (6-8)	5.4 ± 0.5 (4.6-6.4)
a	28.1 ± 2.7 (25.5-33.6)	25.7 ± 1.0 (24.0-27.0)	28.6 ± 2.2 (25.7-31.2)	27.2 ± 2.1 (23.7-30.8)	31.9 ± 2.6 (29.2-35.6)	21.8 ± 1.8 (18.9-25.3)
b	5.7 ± 0.4 (5.2-6.6)	5.9 ± 0.3 (5.5-6.4)	5.1 ± 0.6 (4.4-5.9)	5.5 ± 0.5 (4.8-7.3)	5.9 ± 0.1 (5.8-6.1)	5.0 ± 0.2 (4.7-5.5)
c	10.3 ± 0.8 (9.5-12.0)	10.4 ± 1.0 (9.2-12.5)	11.5 ± 2.5 (9.2-14.8)	9.9 ± 1.2 (8.1-12.3)	9.4 ± 0.2 (9.2-9.6)	8.8 ± 0.7 (7.6-9.9)
c'	3.8 ± 0.1 (3.7-4.1)	3.6 ± 0.2 (3.3-4.0)	3.5 ± 0.9 (2.3-4.5)	4.0 ± 0.7 (1.8-4.9)	4.6 ± 0.3 (4.3-5)	3.6 ± 0.5 (2.2-4.3)
V (%)	81.1 ± 1.2 (79-83)	81.4 ± 2.0 (78-84)	83.6 ± 3.4 (79-87)	84.8 ± 1.0 (82-86)	84.4 ± 1.8 (82-86)	84.2 ± 1.5 (82-88)
G1 (%)	41.9 ± 4.2 (38-47)	37.2 ± 6.6 (30-46)	-	-	-	-
St%L	9.4 ± 0.8 (8.0-10.3)	9.0 ± 0.4 (8.4-9.5)	9.7 ± 0.9 (8.9-10.9)	9.7 ± 0.4 (8.9-10.8)	9.1 ± 0.3 (8.8-9.6)	11.5 ± 0.6 (10.2-12.7)
St%Oes	53.7 ± 1.5 (51.7-55.7)	53.2 ± 1.8 (50.6-55.5)	-	-	-	-
VA%T	67.5 ± 6.1 (59.6-75.5)	53.3 ± 9.8 (43.1-68.6)	64 ± 19.6 (44.3-85.3)	54.7 ± 10.4 (42.3-81.5)	46.7 ± 12 (35.3-60)	43.8 ± 3.9 (38.4-50)
O	15.3 ± 1.8 (12.6-18.5)	8.8 ± 1.0 (7.8-10.3)	-	-	-	-

Table S5. Morphometrics of *Hemicycliophora thienemanni, H. thornei* and *H. vaccinii* analysed in the present study. (All measurements in μm.)

Species Locality	*H. thienemanni* Soria, Spain	*H. thienemanni* Trento, Italy	*H. thienemanni* Jaen, Spain	*H. thienemanni* Moscow, Russia	*H. thornei* Córdoba, Spain	*H. vaccinii* Carnota, Spain
n	10	10	10	10	10	8
L	971.6 ± 40.7 (898-1024)	962.3 ± 106.0 (798-1124)	962.3 ± 106.0 (798-1124)	1004 ± 89.3 (806-1146)	880.2 ± 60.8 (806-1011)	1188.6 ± 101.5 (1067-1328)
Maximum body width	33.3 ± 0.7 (32.0-34.0)	34.3 ± 1.4 (31.0-36.0)	34.3 ± 1.4 (31.0-36.0)	36 ± 3.1 (31-43)	39.7 ± 1.0 (38.0-41.0)	38.6 ± 1.6 (35.0-40.0)
Pharynx length	164.6 ± 6.2 (154-172)	167.1 ± 7.5 (154-176)	167.1 ± 7.5 (154-176)	173 ± 7.9 (185-160)	166.8 ± 9.8 (152-179)	175.3 ± 7.4 (166-187)
Tail length	103.3 ± 4.1 (97-109)	102.6 ± 4.7 (94-110)	102.6 ± 4.7 (94-110)	103 ± 13.0 (88-128)	72.7 ± 4.7 (67-79)	116.5 ± 2.9 (113-121)
Anal body diameter	27.6 ± 1.2 (26.0-30.0)	26.5 ± 1.2 (25.0-28.0)	26.5 ± 1.2 (25.0-28.0)	27 ± 2.4 (23-30)	26.0 ± 2.9 (22.0-29.0)	25.3 ± 1.0 (24.0-26.5)
Stylet length	96.4 ± 4.3 (90-103)	98.3 ± 4.8 (91-105)	98.3 ± 4.8 (91-105)	94 ± 3.8 (86-100)	101.7 ± 5.6 (94-114)	89.6 ± 4.4 (84-96)
Stylet knob width	7.8 ± 0.4 (7.0-8.0)	7.8 ± 0.4 (7.0-8.0)	7.8 ± 0.4 (7.0-8.0)	6.8 ± 0.5 (5.6-7.8)	7.4 ± 0.5 (7.0-8.0)	9.6 ± 0.5 (9.0-10.5)
Stylet knob height	2.8 ± 0.3 (2.5-3.0)	2.8 ± 0.4 (2.5-3.0)	2.8 ± 0.4 (2.5-3.0)	4.4 ± 0.4 (3.4-5.7)	4.3 ± 0.5 (4.0-4.5)	3.9 ± 0.2 (3.5-4.0)
DGO	10.7 ± 1.1 (9.0-12.0)	10.7 ± 1.1 (9.0-12.0)	10.7 ± 1.1 (9.0-12.0)	-	7.9 ± 0.3 (7.5-8.5)	9.6 ± 0.5 (9.0-10.5)
Nerve ring-anterior end	126.0 ± 4.7 (120-131)	138.0 ± 8.5 (130-147)	138.0 ± 8.5 (130-147)	137 ± 7.4 (123-151)	136.3 ± 8.2 (127-148)	136.0 ± 5.0 (130-145)
Excr. pore-anterior end	165.3 ± 4.6 (162-172)	167.3 ± 11.9 (159-181)	167.3 ± 11.9 (159-181)	181 ± 12.0 (160-200)	158.0 ± 14.8 (144-192)	196.9 ± 10.2 (182-210)
Lip width	13.8 ± 1.0 (13.0-15.0)	9.5 ± 0.5 (9.0-10.0)	9.5 ± 0.5 (9.0-10.0)	15.3 ± 0.7 (14.0-17.0)	17.6 ± 0.5 (17.0-18.0)	18.1 ± 0.3 (17.5-18.5)
Lip height	4.5 ± 0.5 (4.0-5.0)	5.0 ± 0.5 (4.5-5.5)	5.0 ± 0.5 (4.5-5.5)	5.9 ± 0.6 (4.5-8.0)	7.4 ± 0.5 (7.0-8.0)	5.1 ± 0.2 (5.0-5.5)
Annuli width	3.2 ± 0.3 (3.0-3.5)	2.8 ± 0.3 (2.5-3.0)	2.8 ± 0.3 (2.5-3.0)	-	3.5 ± 0.0 (3.5-3.5)	4.0 ± 0.0 (4.0-4.0)
R	303.6 ± 11.9 (287-327)	308.5 ± 14.8 (287-326)	308.5 ± 14.8 (287-326)	314 ± 40.1 (275-387)	249.3 ± 12.5 (234-268)	304.5 ± 23.8 (273-339)
Rst	27.3 ± 1.2 (26-29)	31.1 ± 0.7 (30-32)	31.1 ± 0.7 (30-32)	27 ± 1.6 (25-30)	29.7 ± 2.9 (27-34)	27.1 ± 1.1 (26-29)
Roes	46.5 ± 1.4 (45-49)	53.0 ± 1.1 (51-54)	53.0 ± 1.1 (51-54)	48 ± 3.1 (44-52)	53.7 ± 4.3 (48-62)	52.8 ± 2.8 (50-57)
Rex	47.8 ± 1.8 (46-51)	52.9 ± 1.2 (51-55)	52.9 ± 1.2 (51-55)	49 ± 3.1 (44-54)	50.7 ± 6.0 (46-63)	59.9± 4.8 (50-66)
RV from terminus	66.0 ± 1.8 (64-69)	71.3 ± 2.3 (68-74)	71.3 ± 2.3 (68-74)	61 ± 3.5 (57-65)	57.9 ± 2.7 (54-62)	73.3 ± 2.0 (70-76)
RV-anterior end	237.6 ± 11.9 (220-258)	237.2 ± 13.4 (219-255)	237.2 ± 13.4 (219-255)	253 ± 40.0 (211-322)	191.4 ± 10.5 (177-208)	231.3 ± 24.8 (200-266)
RVan	22.1 ± 1.1 (20-24)	25.0 ± 0.8 (24-26)	25.0 ± 0.8 (24-26)	38 ± 3.0 (18-28)	14.6 ± 1.1 (13-17)	23.6 ± 0.5 (23-24)
Ran	43.9 ± 1.7 (42-47)	46.3 ± 1.7 (44-49)	46.3 ± 1.7 (44-49)	22 ± 3.0 (34-42)	43.3 ± 2.9 (39-47)	49.6 ± 1.9 (46-52)
VL/VB	5.3 ± 0.2 (5.1-5.6)	5.3 ± 0.2 (5.1-5.5)	5.3 ± 0.2 (5.1-5.5)	5.5 ± 0.5 (4.6-6.7)	4.1 ± 0.1 (4.0-4.3)	5.0 ± 0.4 (4.7-5.9)
PV/ABW	6.0 ± 0.1 (5.7-6.2)	6.3 ± 0.1 (6.1-6.5)	6.3 ± 0.1 (6.1-6.5)	6.1 ± 0.7 (4.9-7.1)	4.6 ± 0.2 (4.4-5.0)	7.4 ± 0.6 (5.9-7.8)
a	29.2 ± 1.2 (26.7-31.3)	28.0 ± 2.4 (24.2-32.1)	28.0 ± 2.4 (24.2-32.1)	28.9 ± 1.5 (24.1-32.3)	22.2 ± 1.1 (21.1-24.7)	30.9 ± 3.4 (27.4-37.3)
b	5.9 ± 0.2 (5.5-6.2)	5.7 ± 0.5 (5.2-6.7)	5.7 ± 0.5 (5.2-6.7)	5.8 ± 0.4 (5.0-6.3)	5.3 ± 0.3 (4.6-5.6)	6.8 ± 0.4 (6.3-7.4)
c	9.4 ± 0.3 (9.0-10.0)	9.4 ± 0.7 (8.5-10.4)	9.4 ± 0.7 (8.5-10.4)	9.0 ± 1.0 (8.4-11.4)	12.1 ± 0.6 (11.3-12.8)	10.2 ± 0.6 (9.4-11.1)
c'	3.7 ± 0.2 (3.6-4.0)	3.9 ± 0.1 (3.8-4.0)	3.9 ± 0.1 (3.8-4.0)	3.6 ± 0.4 (2.9-4.4)	2.8 ± 0.2 (2.6-3.1)	4.6 ± 0.1 (4.5-4.7)
V (%)	81.5 ± 1.1 (80-83)	81.0 ± 0.7 (80-82)	81.0 ± 0.7 (80-82)	83 ± 1.2 (81-85)	80.7 ± 1.4 (79-84)	81.0 ± 1.2 (79-83)
G1 (%)	41.2 ± 7.9 (35-50)	28.8 ± 3.6 (26-33)	28.8 ± 3.6 (26-33)	-	36.6 ± 4.2 (32-46)	33.2 ± 3.0 (31-38)
St%L	9.9 ± 0.5 (9.2-10.8)	10.3 ± 0.7 (9.2-11.7)	10.3 ± 0.7 (9.2-11.7)	9 ± 0.8 (8-11)	11.6 ± 0.7 (10.8-13.0)	7.6 ± 0.4 (7.2-8.2)
St%Oes	58.6 ± 2.5 (54.7-63.1)	58.8 ± 1.7 (56.3-61.3)	58.8 ± 1.7 (56.3-61.3)	-	61.0 ± 2.9 (58.6-67.8)	51.1 ± 1.5 (49.4-53.1)
VA%T	60.6 ± 4.9 (50.5-66.4)	63.4 ± 2.1 (60.6-67.7)	63.4 ± 2.1 (60.6-67.7)	60 ± 6.2 (46-69)	65.2 ± 5.3 (58.2-70.9)	63.8 ± 5.2 (54.3-71.1)
O	11.1 ± 0.9 (9.7-12.2)	10.8 ± 0.8 (9.7-12.4)	10.8 ± 0.8 (9.7-12.4)	-	7.9 ± 0.4 (7.0-8.4)	10.6 ± 0.7 (9.7-11.2)

Table S6. Morphometrics of *Hemicycliophora wyei* analysed in the present study. (All measurements in µm.)

Species	*Hemicycliophora wyei*					
Locality, code	FL, USA, CD791	NC, USA, CD684	NC, USA, CD676	NC, USA, CD679	NC, USA, CD683	NC, USA, CD682
n	10	5	3	3	6	1
L	942 ± 39 (866-1001)	964.6 ± 40.5 (900.1-1007)	949.1 ± 30.7 (915.1-975.1)	998.1 ± 42.6 (969-1047)	938.1 ± 104.6 (786-1038)	945.1
Maximum body width	44 ± 1.7 (42-47)	43.6 ± 6.3 (39-55)	40.6 ± 2.1 (38-43)	39.1 ± 0.8 (38-40)	40.2 ± 4.5 (32-45)	37.2
Pharynx length	151 ± 4.6 (142-156)	169.7 ± 26.8 (157-218)	160 ± 11.4 (149-172)	163 ± 6.0 (157-169)	146 ± 11.7 (128-157)	145.8
Tail length	108 ± 8.5 (88-115)	128.3 ± 24.9 (102-166)	109.4 ± 2.7 (107-112)	113.8 ± 17.7 (95-131)	109.4 ± 21.3 (71-133)	74.4
Anal body diameter	34 ± 2.1 (30-37)	30.9 ± 4.3 (28-38)	29.5 ± 1.9 (28-32)	30 ± 3.1 (28-34)	27.8 ± 3.4 (22-31)	28.2
Stylet length	81 ± 2.3 (78-85)	84.7 ± 16.8 (76-115)	90.8 ± 19.5 (79-113)	82.7 ± 2.1 (80-84)	77.7 ± 4.1 (73-83)	76.8
Stylet knob width	7.8 ± 0.3 (7.6-8.4)	7.5 ± 0.6 (6.8-8.4)	8.4 ± 0.0 (8.4-8.4)	8.2 ± 0.4 (8.0-8.8)	7.8 ± 0.5 (7.2-8.8)	7.2
Stylet knob height	4.9 ± 0.3 (4.5-6.1)	4.3 ± 0.1 (4-4.4)	4.4 ± 0.2 (4.2-4.6)	4.5 ± 1.0 (3.6-5.6)	3.9 ± 0.5 (3.2-4.8)	3.4
DGO	5.5 ± 0.8 (4.6-6.8)	13.8 ± 4.3 (10.8-18.8)	9.7 ± 1.9 (8.4-12)	12.6 ± 0.8 (12-13.2)	12.6 ± 2.4 (9.2-15.2)	10.8
Nerve ring-anterior end	126 ± 8.5 (108-138)	-	-	-	-	-
Excr. pore-anterior end	172 ± 12 (154-185)	198.7 ± 35.0 (180-261)	174.8 ± 9.7 (166-185)	180.6 ± 6.0 (175-187)	174.7 ± 14.6 (152-192)	168.6
Lip width	21 ± 1.3 (19-23)	19.9 ± 0.8 (18.8-21.2)	19.2 ± 1.6 (17.6-20.8)	20.4 ± 1.8 (18.8-22.4)	18.8 ± 0.9 (17.6-20)	18.4
Lip height	7.7 ± 0.5 (7.0-8.4)	9.2 ± 0.5 (8.4-9.6)	8.8 ± 0.0 (8.8-8.9)	8.9 ± 0.2 (8.8-9.2)	8.4 ± 0.4 (8-9.2)	8.4
Annuli width	4.4 ± 0.3 (3.8-4.6)	4.3 ± 0.0 (4.2-4.4)	3.5 ± 0.8 (2.6-4.1)	4.3 ± 0.3 (3.9-4.6)	4.1 ± 0.6 (3-5)	4.2
R	238 ± 16 (218-264)	260 ± 5.9 (255-270)	259 ± 8.5 (250-267)	254 ± 4.0 (250-258)	256 ± 9.5 (243-267)	256
Rst	26 ± 4.2 (20-32)	21 ± 1.9 (19-24)	25 ± 5.1 (22-31)	22 ± 0.5 (21-22)	21 ± 1.6 (19-23)	22
Roes	53 ± 8.2 (42-66)	42 ± 2.7 (38-45)	44.6 ± 3.0 (42-48)	43 ± 1.5 (42-45)	40 ± 1.7 (37-42)	43
Rex	61 ± 8.5 (52-72)	50 ± 5.5 (42-54)	51 ± 0.0 (50-51)	49 ± 1.1 (48-50)	50 ± 2.2 (46-53)	51
RV from terminus	54 ± 6.3 (48-70)	53 ± 4.3 (49-60)	57 ± 8.5 (51-67)	50 ± 1.5 (48-51)	53 ± 3.2 (49-57)	51
RV-anterior end	180 ± 15 (160-206)	206 ± 2.7 (203-210)	201 ± 3.2 (199-205)	204 ± 3.4 (202-208)	203 ± 9.9 (187-214)	205
RVan	22 ± 2.6 (19-26)	16 ± 3.0 (14-20)	14 ± 2.3 (13-17)	14 ± 1.7 (13-16)	16 ± 3.3 (13-22)	16
Ran	34 ± 7.0 (28-51)	37 ± 4.9 (29-41)	43 ± 10.1 (34-54)	36 ± 2.0 (34-38)	37 ± 5.2 (28-43)	35
VL/VB	4.0 ± 0.3 (3.7-4.5)	5.4 ± 1.4 (4.5-8)	4.7 ± 0.2 (4.6-5)	4.7 ± 0.6 (4.3-5.5)	4.9 ± 0.2 (4.5-5.2)	3.9
PV/ABW	4.9 ± 0.4 (4.1-5.5)	6.1 ± 0.6 (5.4-6.8)	5.3 ± 0.6 (4.7-5.9)	5.3 ± 0.2 (5.1-5.6)	5.7 ± 0.1 (5.6-5.9)	4.7
a	22 ± 1.5 (19-23)	22.4 ± 3.0 (17.8-25.4)	23.3 ± 0.4 (22.9-23.8)	24.8 ± 0.6 (24.2-25.5)	23.4 ± 2.4 (19.7-26.8)	25.4
b	6.3 ± 0.3 (5.7-6.7)	5.7 ± 0.7 (4.4-6.3)	5.9 ± 0.2 (5.7-6.1)	6.1 ± 0.3 (5.8-6.4)	6.4 ± 0.2 (6.2-6.6)	6.5
c	8.8 ± 0.9 (7.6-11)	7.7 ± 1.6 (5.8-9.9)	8.7 ± 0.3 (8.4-9)	8.8 ± 1.1 (8-10.2)	8.7 ± 1.5 (6.7-11.1)	12.7
c'	3.2 ± 0.2 (2.9-3.5)	4.1 ± 0.5 (3.6-5)	3.7 ± 0.2 (3.4-3.9)	3.8 ± 0.3 (3.4-4.1)	3.8 ± 0.3 (3.2-4.3)	2.6
V (%)	84 ± 2.5 (81-89)	84 ± 1.3 (83-86)	84 ± 0.7 (83-85)	84.5 ± 0.8 (84-85)	83.3 ± 1.1 (81-85)	84
G1 (%)	41 ± 3.8 (33-45)	-	-	-	-	-
St%L	8.6 ± 0.5 (8.1-9.8)	8.7 ± 1.8 (7.5-11.9)	9.5 ± 1.7 (8.4-11.6)	8.3 ± 0.3 (8-8.6)	8.3 ± 0.5 (7.6-9.2)	8.1
St%Oes	54 ± 1.5 (52-56)	-	-	-	-	-
VA%T	55 ± 13 (40-83)	49.1 ± 11.6 (37.7-66.7)	43.8 ± 6.1 (39.6-50.8)	42.3 ± 9.1 (36.2-52.8)	48.7 ± 14.6 (33.5-75.4)	77.4
O	6.8 ± 0.9 (5.8-8.7)	-	-	-	-	-

Table S7. Morphometrics of *Hemicycliophora* sp. 3 and sp. 4 analysed in the present study. (All measurements in µm.)

Species	*Hemicycliophora* sp. 3	*Hemicycliophora* sp. 4			
Locality	AZ, USA	St. Augustine, FL, USA	Fort Myers, FL, USA	Osteen, FL, USA	Brunswick, NC, USA
N	8	20	10	10	6
L	641.3 ± 41 (609-726)	936 ± 56 (828-1026)	989 ± 69 (892-1105)	995 ± 65 (931-1123)	976.1 ± 60 (897-1074)
Maximum body width	33.7 ± 3.0 (30-40)	37 ± 1.8 (34-41)	37 ± 2.5 (33-40)	39 ± 1.4 (36-41)	36.9 ± 2.8 (34-40)
Pharynx length	126.3 ± 6.2 (121-138)	165 ± 6.2 (154-178)	184 ± 9.0 (170-200)	170 ± 3.2 (167-175)	158.9 ± 10.2 (144-175)
Tail length	71.8 ± 9.5 (59-85)	118 ± 11 (102-144)	121 ± 16 (93-141)	126 ± 16 (102-148)	100.8 ± 13.1 (88-121)
Anal body diameter	24.4 ± 2.5 (21-27)	33 ± 1.7 (30-35)	31 ± 2.5 (26-34)	34 ± 1.8 (32-38)	27 ± 0.7 (26-28)
Stylet length	67.8 ± 2.6 (63-71)	82 ± 3.8 (72-87)	94 ± 2.7 (91-97)	88 ± 2.9 (83-92)	90.2 ± 3.1 (87-96)
Stylet knob width	6.3 ± 0.5 (5.6-7.2)	7.2 ± 0.7 (6.1-8.3)	7.3 ± 0.3 (6.1-8.3)	7.5 ± 0.4 (6.8-8.0)	7.8 ± 0.8 (6.8-9.2)
Stylet knob height	2.7 ± 0.1 (2.4-2.8)	4.1 ± 0.5 (3.1-4.6)	3.7 ± 0.3 (3.0-3.8)	4.4 ± 0.4 (3.8-4.9)	3.9 ± 0.3 (3.6-4.4)
DGO	13.0 ± 2.4 (10.4-15.2)	11 ± 2.8 (6.8-15)	7.1 ± 0.3 (6.8-7.6)	7.2 ± 0.6 (6.2-8.0)	10.8 ± 2.2 (8.8-13.2)
Nerve ring-anterior end	-	133 ± 10 (110-144)	146 ± 6.9 (140-163)	140 ± 4.6 (132-148)	-
Excr. pore-anterior end	139.7 ± 9.3 (128-156)	169 ± 11 (148-186)	184 ± 11 (174-205)	184 ± 8.9 (168-201)	170.4 ± 8.4 (163-185)
Lip width	15.1 ± 0.8 (14-16.4)	17 ± 1.0 (15-18)	15 ± 0.9 (13-16)	16 ± 1.6 (14-19)	17.2 ± 1.1 (15.6-19.2)
Lip height	7.7 ± 0.4 (7.2-8.4)	7.5 ± 0.4 (6.8-7.6)	8.0 ± 0.5 (7.6-9.1)	7.4 ± 0.5 (6.8-8.3)	8.5 ± 0.3 (8.2-9.2)
Annuli width	3.6 ± 0.1 (3.3-3.9)	3.8 ± 0.4 (3.4-4.5)	3.8 ± 0.3 (3.0-3.8)	3.7 ± 0.4 (3.1-4.5)	4.3 ± 0.3 (3.8-4.9)
R	192 ± 6.6 (183-202)	252 ± 17 (231-288)	250 ± 26 (215-283)	241 ± 19 (220-289)	245 ± 5.0 (241-254)
Rst	21 ± 2.0 (18-24)	25 ± 2.0 (21-30)	31 ± 3.7 (27-38)	25 ± 3.8 (19-32)	25 ± 1.5 (24-28)
Roes	37 ± 2.4 (33-40)	62 ± 10 (51-81)	58 ± 5.0 (50-67)	46 ± 4.5 (37-55)	44 ± 2.3 (41-46)
Rex	41 ± 2.4 (38-44)	50 ± 5.1 (43-61)	59 ± 7.6 (51-76)	50 ± 4.7 (42-56)	48 ± 1.0 (46-49)
RV from terminus	30 ± 2.2 (27-33)	63 ± 5.3 (52-75)	61 ± 7.4 (52-73)	56 ± 4.4 (48-63)	55 ± 5.5 (50-64)
RV-anterior end	162 ± 5.0 (156-171)	200 ± 19 (172-257)	194 ± 21 (170-228)	187± 19 (165-234)	188 ± 6.6 (179-197)
RVan	7 ± 1.4 (5-8)	24 ± 4.9 (17-36)	20 ± 1.4 (18-22)	19 ± 2.0 (16-23)	21 ± 3.2 (16-25)
Ran	23 ± 1.1 (21-24)	39 ± 3.8 (34-46)	39 ± 8 (28-50)	40 ± 4.1 (33-47)	35 ± 4.5 (29-39)
VL/VB	3.4 ± 0.6 (2.8-4.7)	5.1 ± 0.4 (4.4-5.9)	5.5 ± 0.9 (4.6-7.6)	5.2 ± 0.4 (4.7-6.0)	5.5 ± 0.5 (4.9-6)
PV/ABW	3.7 ± 0.5 (3.1-4.7)	5.6 ± 0.5 (4.8-6.7)	6.5 ± 0.9 (5.4-8.6)	6.1 ± 0.3 (5.5-6.7)	6.2 ± 0.4 (5.7-6.7)
a	19.0 ± 1.1 (17.2-20.6)	25 ± 1.4 (23-29)	27 ± 1.9 (24-30)	26 ± 1.6 (24-30)	26.5 ± 3.1 (22.3-31.7)
b	5.0 ± 0.3 (4.5-5.7)	5.6 ± 0.4 (4.8-6.4)	5.4 ± 0.4 (4.7-6.2)	5.8 ± 0.3 (5.4-6.4)	6.1 ± 0.5 (5.7-6.9)
c	8.9 ± 1.0 (7.3-10.7)	8.8 ± 0.7 (6.8-9.3)	8.3 ± 0.8 (7.2-9.8)	8.0 ± 0.8 (6.8-9.4)	9.7 ± 1.1 (8.1-11.2)
c'	3.0 ± 0.3 (2.6-3.7)	3.6 ± 0.4 (3.1-4.4)	3.9 ± 0.4 (3.3-4.7)	3.8 ± 0.4 (3.2-4.5)	3.7 ± 0.4 (3.2-4.4)
V (%)	86.8 ± 0.8 (86-88)	79 ± 0.3 (70-83)	80 ± 2.8 (75-85)	80 ± 0.8 (79-81)	82.8 ± 1.2 (81-84)
G1 (%)	-	40 ± 5.8 (35-51)	43 ± 9.4 (32-62)	42 ± 3.8 (35-47)	-
St%L	10.6 ± 0.8 (8.7-11.3)	8.7 ± 0.5 (8.0-9.6)	9.5 ± 0.5 (8.7-10)	8.9 ± 0.6 (7.5-9.4)	9.2 ± 0.6 (8.4-10.2)
St%Oes	-	50 ± 2.2 (45-55)	51 ± 2.3 (48-54)	52 ± 1.8 (49-55)	-
VA%T	35.4 ± 22.4 (21-87.6)	63 ± 9.7 (53-91)	58 ± 7.5 (48-71)	61 ± 12 (42-80)	68.5 ± 13.1 (50-81.8)
O	-	14 ± 3.3 (8.1-18)	7.5 ± 0.4 (7.0-8.3)	8.2 ± 0.7 (6.8-9.1)	-

Table S8. Morphometrics of *Hemicycliophora* sp. 8, sp. 9, sp. 10 and sp. 11 analysed in the present study. (All measurements in µm.)

Species Locality	*Hemicycliophora* sp. 8 CA, USA	*Hemicycliophora* sp. 9 Jaroslav region, Russia	*Hemicycliophora* sp. 10 CA, USA	*Hemicycliophora* sp. 11 FL, USA
n	1	29	4	10
L	927.1	816.6 ± 46.1 (738- 895)	1022 ± 3.8 (1017-1026)	839± 86 (700-978)
Maximum body width	43.2	36.0 ± 2.2 (31-40)	37.9 ± 5.6 (30-44)	39 ± 3.7 (32-44)
Pharynx length	180.6	144 ± 4.9 (134-151)	168.9 ± 7.2 (159-176)	144 ± 12 (126-161)
Tail length	70.8	82 ± 5.4 (74-94)	75.8 ± 5.8 (72-84)	102 ± 17 (75-116)
Anal body diameter	26.4	24 ± 1.8 (20-26)	26.7 ± 2.0 (25-29)	32 ± 3.2 (27-38)
Stylet length	95.4	68.0 ± 2.9 (63-77)	97.7 ± 5.8 (92-106)	76 ± 5.1 (65-80)
Stylet knob width	8.8	6.2 ± 0.4 (5.5-7.1)	7.7 ± 0.8 (6.8-8.8)	6.9± 0.5 (6.1-7.6)
Stylet knob height	3.2	4.2 ± 0.3 (2.5-5.1)	4 ± 0.3 (3.6-4.4)	3.8 ± 0.1 (3.5-4.0)
DGO	9.6	-	11.2 ± 2.4 (9.6-14)	5.1± 0.6 (4.6-6.1)
Nerve ring-anterior end		114 ± 3.3 (111-123)	-	119 ± 11 (102-133)
Excr. pore-anterior end	180.6	159 ± 9.4 (142-180)	184.9 ± 13.3 (167-199)	154 ± 12 (133-169)
Lip width	17.6	16.9 ± 0.5 (14.0-19.0)	17.3 ± 1.0 (16.4-18.8)	18.3 ± 2.5 (15-22)
Lip height	9.4	6.6 ± 0.3 (5.5-8.6)	9.0 ± 0.4 (8.4-9.4)	8.1 ± 0.4 (7.6-8.4)
Annuli width	3.8	4.2 ± 0.4 (3.2-5.5)	4.3 ± 0.2 (4-4.5)	3.5 ± 0.4 (3.0-3.8)
R	275	194 ± 13.1 (167-217)	268 ± 11.1 (253-277)	260 ± 26 (228-289)
Rst	26	19 ± 1.6 (17-23)	28 ± 0.9 (27-29)	26 ± 2.4 (22-29)
Roes	48	40 ± 2.6 (36-46)	46 ± 2.5 (43-49)	51 ± 4.2 (44-59)
Rex	-	45 ± 2.8 (41-45)	51 ± 2.5 (48-54)	59 ± 5.2 (52-70)
RV from terminus	46	40 ± 2.6 (37-45)	46 ± 4.5 (41-52)	61 ± 6.0 (50-67)
RV-anterior end	229	146 ± 12.0 (135-172)	215 ± 15.9 (193-231)	211± 24 (180-260)
RVan	19	9 ± 1.2 (7-12)	20 ± 4.1 (15-25)	21 ± 3.0 (16-26)
Ran	27	31 ± 2.2 (28-36)	27 ± 3.3 (22-30)	43 ± 3.2 (39-50)
VL/VB	4.3	3.8 ± 0.3 (3.2-4.6)	5.1 ± 0.4 (4.6-5.5)	4.0 ± 0.7 (2.7-4.6)
PV/ABW	5	4.8 ± 0.6 (3.4-6.0)	5.1 ± 0.4 (4.6-5.5)	4.8 ± 0.9 (3.0-5.6)
a	21.5	22.1 ± 1.3 (20.8-27.2)	27.3 ± 4.4 (23.5-33.6)	22 ± 1.4 (19-24)
b	5.1	5.7 ± 0.2(5.3-6.2)	6.0 ± 0.2 (5.8-6.4)	5.8 ± 0.3 (5.1-6.4)
c	13.1	9.6 ± 0.6 (8.9-11.1)	13.5 ± 0.9 (12.1-14.3)	8.4 ± 1.4 (7.1-12)
c'	2.7	3.5 ± 0.3 (2.8-4.1)	2.8 ± 0.1 (2.6-2.9)	3.2 ± 0.5 (2.0-3.7)
V (%)	86.7	85 ± 1.1 (82-87)	86.1 ± 0.9 (85-87)	83 ± 3.8 (79-90)
G1 (%)	-	-	-	41 ± 5.0 (34-47)
St%L	10.3	8.3 ± 0.4 (7.9- 9.6)	9.5 ± 0.6 (9-10.4)	9.1 ± 0.5 (8.2-10)
St%Oes	-	49 ± 1.6 (46-52)	-	53 ± 2.1 (49-56)
VA%T	85.6	45 ± 8.9 (32-65)	83.7 ± 20.0 (59.7-108)	59 ± 13 (34-78)
O	-	-	-	6.9 ± 1.1 (5.8-9.4)

Table S9. Morphometrical, morphological and biological characters of *Hemicycliophora* used ancestral state reconstruction.

Character / Species	Body length (1)	Stylet length (2)	R (3)	Vulval lips (4)	Tail shape (5)	Male 6	Lateral fields (7)	RV (8)
H. californica	1	1	1	1	1	1	1	0
H. chilensis	1	1	1	0	0	1	0	2
H. conida	1	1	1	1	1	0	0	0
H. epicharoides	1	1	0	1	1	1	1	0
H. floridensis	1	2	1	1	2	1	0	0
H. gracilis	2	2	2	1	2	1	1	1
H. halophila	1	2	1	1	2	0	1	0
H. hellenica	2	2	2	1	0	0	1	1
H. iberica	1	1	1	1	1	1	0	1
H. italiae	1	2	1	1	0	1	1	1
H. lutosa	1	1	1	1	0	1	1	1
H. lutosoides	1	1	2	1	0	1	1	2
H. obtusa	1	1	1	1	3	1	1	0
H. poranga	1	1	1	1	0	1	1	1
H. raskii	1	1	1	1	1	1	1	0
H. ripa	1	1	1	0	0	1	1	1
H. similis	2	1	1	1	2	1	1	2
H. thienemanni	1	1	1	0	2	0	1	1
H. thornei	1	1	1	0	2	0	1	1
H. typica	1	0	1	1	1	0	1	0
H. vaccinii	2	2	1	0	0	1	1	2
H. wyei	1	1	1	1	0	1	1	1
Hemicycliophora sp. 3	0	0	0	1	1	1	1	0
Hemicycliophora sp. 4	2	2	2	2	3	2	2	1
Hemicycliophora sp. 8	1	1	1	1	1	1	1	1
Hemicycliophora sp. 9	1	0	0	0	1	1	1	0
Hemicycliophora sp. 10	1	1	1	1	1	1	1	1
Hemicycliophora sp. 11	1	0	1	1	0	1	1	1
P. nanus	0	0	?	0	0	0	0	2
E. excretorius	1	0	?	0	0	1	0	2

1. Average body length: 0- < 781 µm; 1- 781-1100 µm; 2- >1100 µm
2. Average stylet length: 0- < 80 µm; 1- 81-102 µm; 2- >102 µm
3. Average R: 0-< 200; 1- 200-330; 2- > 330
4. Vulval lips: 0- not modified or very short sleeve; 1- with elongated sleeve
5. Tail shape: 0- elongate conical narrowing uniformly; 1- cylindrical with conical end and without elongated terminus; 2- cylindrical with conical end and elongated terminus; 3- cylindrical with hemispherical end
6. Male: 0- present; 1- absent or very rare
7. Lateral fields: 0- without anastomose; 1- with anastomose
8. Average RV from terminus: 0- < 46; 1- 46-75; 2->75.