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Taxonomy of Stilbonematinae (Nematoda: Desmodoridae): description of two new and three known species and phylogenetic relationships within the family

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A taxonomic study of the subfamily Stilbonematinae (Nematoda: Desmodoridae) based on collected specimens from a coral reef in the Caribbean Sea revealed two new species, ***Laxus parvum* sp. nov**. and ***Leptonemella brevipharynx* sp. nov.** ***L. parvum*** is characterized by the small body size (2738 μm), large cephalated spicules (63 μm), wide gubernaculum, and coccoid-shaped ectosymbiotic bacteria. The diagnosis of the genus *Laxus* is emended and a dichotomous identification key is given for the seven valid species. ***L. brevipharynx*** is characterized by the shape of the amphidial fovea, 'open' spiral with 1.25 turns, a short pharynx, hook-shaped gubernaculum, and male tail relatively short. Three known sympatric species, *Eubostrichus hopperi*, *Robbea porosum*, and *Stilbonema brevicolle*, were re-described and illustrated based on morphometric features and morphology from light microscope and scanning electronic microscope observations. For each species, relationships are discussed as well as the diagnostic value of morphological features. Phylogenetic relationships amongst desmodorid species were explored based on *small subunit* rDNA and *cytochrome oxidase c subunit 1* partial loci. The three subfamilies within Desmodoridae (Desmodorinae, Spiriniinae, and Stilbonematinae) are polyphyletic; four of the genera of Stilbonematinae proved to be paraphyletic. Convergent evolution would reconcile the presence of glandular sensory organs and ectosymbiosis with the paraphyly of stilbonematins. The cryptic diversity of *R. porosum* could be explained by morphological stasis owing to obligate ectosymbiosis with bacteria. The current classification of nine genera is still the most tractable system for Stilbonematinae in spite of the evidence of its paraphyletic nature based on molecular data.

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ADDITIONAL KEYWORDS: Desmodorida – marine – morphology – nematodes – new species – revision – systematics.

INTRODUCTION

The family Desmodoridae (Filipjev, 1922) Steiner, 1927, consists of a diverse and heterogeneous group of free-living nematodes, mostly marine (Decraemer & Smol, 2006). The family belongs to the superfamily Desmodoroidea Filipjev, 1922, that also includes the families Draconematidae Filipjev, 1918, and Epsilonematidae Steiner, 1927. Currently, the Desmodoridae groups six subfamilies (amongst them the Stilbonematinae), 35 genera, and around 318 species (Hodda, 2011). At present the phylogenetic relationships within the family are still poorly understood based on morphology as well as on molecular data, which cover only a small percentage of the family's taxa. The family is recognized as a clade and within it also the subfamily Stilbonematinae (Meldal *et al*., 2007; van Megen *et al*., 2009).

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The Stilbonematinae Chitwood, 1936, have received much attention because of their unique ectosymbiotic relationships with chemolithoautotrophic bacteria. The bacteria serve as a food source to their host (Schiemer, Novak & Ott, 1990; Ott *et al*., 1991; Ott, 1996; Ott, Bright & Bulgheresi, 2004a, b) and probably act as a shield against sulphide poisoning (Hentschel *et al*., 1999). The monophyly of stilbonematins has been stated based on the ultrastructure of the body cuticle (Urbancik, Bauer-Nebelsick & Ott, 1996a; Urbancik, Novotny & Ott, 1996b), pharynx (Hoschitz, Bright & Ott, 2001), and glandular sensory organs (Bauer-Nebelsick *et al*., 1995). Data from DNA sequences also support Stilbonematinae as a clade (van Megen *et al*., 2009). Within the subfamily, Kampfer, Sturmbauer & Ott (1998) explored the relationships based on 18S rDNA and morphological data; they reported that species of *Eubostrichus* formed a clade, *Leptonemella* and *Stilbonema* were sister groups, and that the relationships of *Catanema* and *Laxus* could not be resolved.

The taxonomy of the group has been rather confused mainly because of the following reasons: (1) the typical coiling of the body and presence of ectosymbiotic bacteria often mask the internal morphology; (2) evolutionary convergence of some features (e.g. head capsule, associated bacteria); and (3) poor descriptions and species diagnoses. Revisions of single genera have been carried out for *Catanema* (Platt & Zhang, 1982), *Eubostrichus* (Hopper & Cefalu, 1973), *Laxus* (Ott, Bauer-Nebelsick & Novotny, 1995), and *Leptonemella* (Riemann, Thiermann & Bock, 2003). Recently, Tchesunov (2013) gave a comprehensive taxonomic revision based on light microscopic observations, with generic diagnoses, lists of valid species, and an outline of the main differences amongst the genera. He proposed nine valid genera: *Adelphos* Ott, 1997; *Catanema* Cobb, 1920; *Eubostrichus* Greeff, 1869; *Laxus* Cobb, 1984; *Leptonemella* Cobb, 1920; *Parabostrichus* Tchesunov, Ingels & Popova, 2012; *Robbea* Gerlach, 1956; *Squanema* Gerlach, 1963, and *Stilbonema* Cobb, 1920. The main changes that Tchesunov (2013) proposed were: the validity of genera *Robbea* and *Squanema*, the transfer of several species from *Catanema* to *Robbea* and from *Leptonemella* to *Laxus* based on emended diagnoses, and the proposal of three species inquirendae.

In the current study, we analysed several species of stilbonematin nematodes collected during two surveys of a Cuban coral reef with two aims. The first was to provide novel information about morphology from light and scanning electronic microscopic observations and the description of two new species and three poorly known species of the subfamily. The second aim was to test the phylogenetic hypotheses of monophyly of Stilbonematinae and the genera within the subfamily based on molecular data. Phylogenetic analyses based on morphological features have been performed previously by Kampfer *et al*. (1998). We partially sequenced the *small subunit* (*SSU* rDNA) and cytochrome oxidase *c* subunit 1 (*COI*) loci; *SSU 18S partim* to clarify relationships amongst the genera of Desmodoridae (i.e. sequences of Stilbonematinae and also of Desmodorinae and Spiriniinae) with sequences from GenBank included, and *COI partim* to clarify relationships of species within the genera. They are the first *COI* sequences published for this subfamily and they contribute to the development of the DNA barcoding of nematodes.

MATERIAL AND METHODS

Collection and processing of samples

Nematodes were collected in the south-west region of the Cuban Archipelago, Punta Francés Reef (21°36′29.68″N, 83°10′34.40″W), during two sampling campaigns, in July 2009 and July 2010. The survey was part of an ecological study of the meiobenthos in this reef system; results on diversity and distribution have already been published (Armenteros *et al*., 2012). Samples were taken from the top 6 cm of sediment using plastic cores (internal diameter 2.5 cm) in two different habitats within the reef lagoon: sand flats with scarce vegetation and seagrass meadows of *Thalassia testudinum*.

In the field, samples were sieved through a 45 μm mesh size sieve and preserved in 70% ethanol until processing in the laboratory. Nematodes were extracted from the samples using a high power stereomicroscope, left for 36 h in a mixture of ethanol and glycerine within an incubator at 35 °C and mounted in wax-ring fix preparations as described in Vincx (1996).

Morphological description and identification

Sixty-eight nematodes belonging to five sympatric species of Stilbonematinae were recovered, identified, and described. For each species, all available developmental stages were measured (i.e. juvenile, female, male). Measurements and drawings of specimens were carried out using a light microscope Leica DM2500 with interference contrast and drawing tube. Light microscope (LM) images were taken with a Color View digital camera on a microscope Olympus BX41 with interference contrast and using the software Olympus cell^D. High magnification images were taken with a scanning electronic microscope (SEM) FEI Quanta 200.

Males, females, and juveniles were scrutinized but the morphological description was mostly based on the adult stages because these provide most of the diagnostic features. Fourteen morphometric continu-

ous variables were considered and another 18 discontinuous morphological characters were also recorded. For the known species, the descriptions include only the new information gathered from our observations (i.e. using SEM and interference LM); for the new species more complete descriptions are supplied.

The Bremerhaven checklist of nematodes (Gerlach & Riemann, 1973) was used as an overview of the literature prior to 1973. We used the primary literature (i.e. taxonomic papers) for the identification of the species and the morphological comparative analyses; most of the original descriptions were obtained from the NeMys database (http://nemys.ugent.be).

DNA extraction and sequencing

Several specimens of nematodes from the July 2010 survey were picked out using a stereomicroscope and mounted, one by one, on temporary microscopic preparations. Specimens were identified to species and photo-vouchered with a camera coupled to a Olympus BX41 microscope. Only species belonging to the family Desmodoridae were included in this analysis. Identified and photographed nematodes were removed from the temporary slide, put independently in an Eppendorf vial with worm lysis buffer [50 mM KCL, 10 Mm Tris, pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP 40 (Tergitol Sigma) and 0.45% Tween 20] and posteriorly frozen for 24 h at –20 °C to break the cell walls. Following this, we added 1 μL of K-proteinase (10 mg mL^{-1}) and incubated samples for 1 h at 65 °C to digest the proteins and finally for 10 min at 95 °C to inactivate the enzyme. Samples were centrifuged for 1 min at 21 000 *g* and the DNA extracts stored at –80 °C.

For the amplification of the SSU rDNA locus we used the primer set G18S4 (f): 5′-GCT TGT CTC AAA GAT TAA GCC and 4R (r): 5′-GTA TCT GAT CGC CKT CGA WC, which amplified a fragment of *c.* 1000 bp (Sofie Derycke pers. comm.). For the amplification of the *COI* gene we used the primer set JB3 (f): 5′-TTT TTT GGG CAT CCT GAG GTT TAT and JB5 (r): 5′-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG, which amplified a fragment of 426 bp (Derycke *et al*., 2010b).

DNA amplification was carried out by PCR in 25 μL reaction volume with the following mix: 1 μL DNA template (i.e. sample), 2.5 μL of 10× PCR buffer, 2.5 μL Coral Load 10×, 2.0 μL $MgCl_2$ (25 mM), 0.5 μL deoxynucleotides (10 mM each), 0.125 μL primers (25 μM), and 0.125 μL TopTaq DNA polymerase (Qiagen, 5 U μL^{-1}). PCR cycling conditions were: initial denaturation of 5 min at 95 °C, 35 cycles of (94 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s), followed by a final extension of 10 min at 72 °C.

PCR reaction products were loaded on 1.2% agarose gels, run in an electrophoresis chamber, and the results visualized with a BioRed UV system. Amplifications were considered successful when a band of the expected size was observed in the agarose gel and then PCR products were further purified using the ExoSAP-IT kit following the manufacturer's instructions (Affymetrix, Inc.) and sequenced. Sequencing was performed in two directions with an ABI Biosystem automatic sequencer using a BigDye v. 1.1 kit; precipitation and purification were carried out following the manufacturer's instructions using formamide, ethanol, and ethylenediaminetetraacetic acid.

Data mining and phylogenetic reconstruction

Complementary chromatograms were used for sequence quality control and sequences edited by hand when necessary (i.e. gaps, double peaks). Complementary sequences were assembled with the software CHROMAS v. 2.0 (www.technelysium.com.au) and a previous database of all the sequences was built in BioEdit v. 7.0.5 (Hall, 1999). All the sequences were aligned using the algorithm ClustalW in the software MEGA 5.10 (Tamura *et al*., 2011) with the default parameters. The ends of the obtained sequence matrix were trimmed and we manually deleted the intermediate gaps, ambiguously aligned regions, and identical haplotypes; as *COI* codes for a protein we ensured that there were no stop codons in the sequences.

We obtained 27 sequences of the *SSU* rDNA *partim* locus; after the deletion of identical sequences and the addition of 23 desmodorid sequences from GenBank and one outgroup species (*Viscosia viscosia*), we had a final matrix of 37 sequences × 586 sites (130 of them were parsimony informative). The best fitted model of nucleotide substitution was the Kimura two-parameter with non-uniform evolutionary rates amongst sites (K2 + G). We obtained 29 sequences of the *COI partim* locus; after deletion of identical sequences and addition of one outgroup species (*Viscosia viscosia*) we had a final matrix of 20 sequences × 386 sites (187 of them were parsimony informative). There were no desmodorid *COI* sequences available from GenBank; therefore, these are the first *COI* sequences available for this group. The best fitted model of nucleotide substitution was the Hasegawa–Kishino–Yano with non-uniform evolutionary rates amongat sites (HKY + G). Both evolutive models were assessed by the Bayesian information criterion in MEGA 5.10.

The phylogenetic trees, based on the two loci independently, were built using the maximum likelihood method in the software MEGA 5.10 with the abovementioned models of nucleotide substitution. The statistical support of the branches was calculated by the bootstrap method with 1000 permutations.

TAXONOMY

SUBFAMILY STILBONEMATINAE CHITWOOD, 1936

EUBOSTRICHUS HOPPERI (HOPPER & CEFALU, 1973) MUTHUMBI, VERSCHELDE & VINCX, 1995

Measurements: Table 1; Figures 1, 2.

Material examined: Two ♂, five ♀, four juveniles (J).

Description: Body habitus slender. Cuticle finely striated (annuli ~0.2 μm width), often covered by a coat of mucus; dense groups of crescent-shaped bacteria grown in a 'rope-like' pattern; in most of the specimens the pattern was disrupted or bacteria were detached during processing. Head shape rounded, no head capsule, striation surrounding the amphidial fovea. Six inner and outer labial sensilla both papilliform (< 1 μm long). Four cephalic sensilla setiform (4–8 μm long) at anterior border of amphidial fovea and very close to the outer labial

Table 1. Mean (range) of morphological measurements for three known species of nematodes belonging to the subfamily Stilbonematinae

Species	*Eubostrichus hopperi*	*Robbea porosum*	*Stilbonema brevicolle*
N	2♂, 5♀, 4J	5♂, 1♀, 2J	1♂, 2♀, 2J
Body length	2327 (1475–2745)	4323 (3781–4701)	1876
	2080 (1905–2256)	3789	2460 (2095–2825)
	1454 (1168–1606)	2668 (2657–2679)	1143 (1000–1285)
de Man's ratio a	81 (64–98)	188 (164–214)	54
	74 (30–95)	146	56 (52–60)
	59 (45–73)	133 (133–134)	39 (29–48)
de Man's ratio b	26 (23–29)	44 (43–46)	*
	33 (28–36)	49	23
	20 (15–22)	29 (28–30)	14 (11–16)
de Man's ratio c	30 (27–33)	54 (50–61)	31
	34 (23–39)	*	30 (27–33)
	23 (19–25)	43 (42–45)	19 (16–21)
Head diameter	15 (14–16)	15 (11–18)	21
	13 (12–15)	12	19 (18–20)
	13 (12–15)	12 (11–12)	19
Amphidial fovea c.b.d. (%)	*	47 (42–50)	*
	59 (50–67)	59	*
	46 (36–56)	60 (52–67)	*
Pharynx length	81 (79–83)	92 (83–102)	*
	78 (73–83)	77	124*
	73 (66–76)	93 (88–97)	85 (81–89)
Anal diameter	21 (21–21)	22 (22–23)	28
	16 (15–19)	*	32 (30–33)
	19 (16–23)	18 (17–19)	25 (20–29)
Maximum body diameter	27 (23–30)	23 (22–24)	35
	34 (27–50)	26	44 (40–47)
	25 (20–32)	20 (20–20)	31 (27–34)
Tail length	70 (69–70)	77 (69–84)	60
	69 (64–78)	*	82 (78–85)
	64 (62–71)	62 (59–64)	62 (61–63)
c′	3.3 (3.3–3.3)	3.5 (3.0–3.8)	2.1
	4.4 (4.1–4.7)	*	2.6
	3.6 (3.1–3.9)	3.4 (3.0–3.8)	2.6 (2.2–3.0)
V %	49 (43–52)	55	47*
Spicule length	44 (39–48)	34 (32–36)	*
Gubernaculum length	16 (13–18)	15 (8–15)	*

c′, tail length relative to the anal body diameter; c.b.d., corresponding body diameter; *N*, number of specimens; V %, distance (relative to body length) of vulva to anterior apex.

All measurements are in μm.

*indicates that measurements could not be taken (or with higher associated error) because of the position of the specimens. For each measure, the first row is males, second row females, and third row juveniles (J).

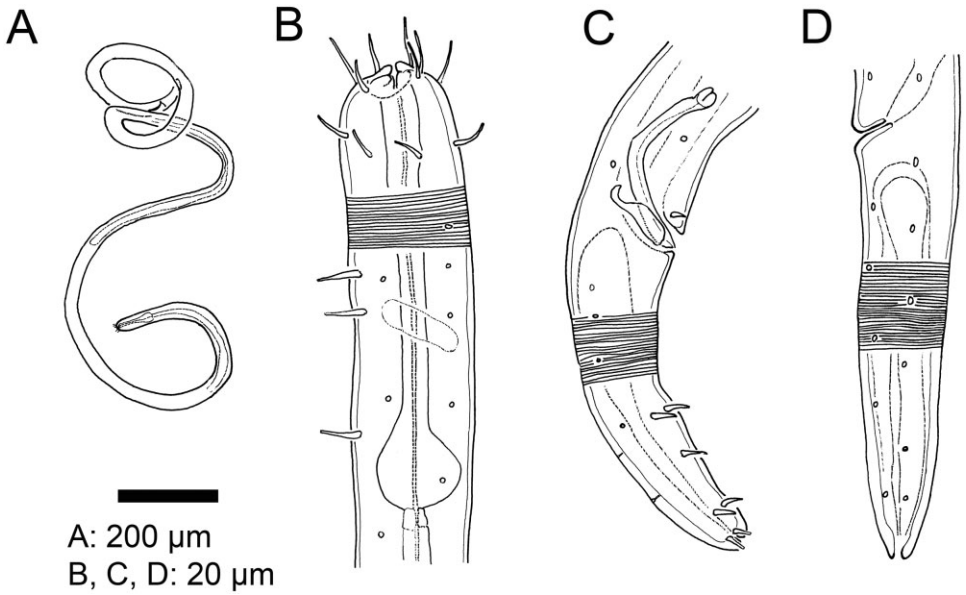

Figure 1. *Eubostrichus hopperi*. A, habitus, specimen 1♂; B, anterior region, specimen 2♂; C, tail, specimen 1♂; D, tail, specimen 3♀.

sensilla. Four subcephalic setae (5–10 µm long) at level of the fovea: two subdorsal and two subventral. Four additional subcephalic setae (5–10 µm long) laterally at the posterior border of the fovea. Other two pairs of setae (5–10 µm) (two subdorsal and two subventral) not always present. Eight longitudinal rows of somatic setae associated with epidermal glands running along body length; the first circles longer (5–10 µm), two to three subventral pairs of enlarged setae at the level of posterior pharynx; on the rest of the body, somatic setae considerably shorter or replaced by porids. Amphidial fovea spiral, difficult to observe in most of the specimens because of film on the cuticle, diameter approximately 0.5 corresponding body diameter (c.b.d.), located lateral in the head. Pharynx muscular without anterior widening, terminal bulb almost round, cardia small, rounded. Tail conical-tapered, tip rounded and possessing two separate outlets of caudal glands; four subventral pairs of enlarged setae in male; two to three caudal pairs in females and juveniles.

Male monorchic, anterior testis to the left of the intestine. Spicules paired and curved, capitulum present; gubernaculum a narrow rod with distal end curved hook-like, no supplements.

Female didelphic, ovaries antidromously reflexed, both genital branches to the left of intestine.

Remarks: The specimens of *Eubostrichus* collected mostly agree with the original description of *E. hopperi* in Hopper & Cefalu (1973) based on the most important diagnostic features: body size, relative tail length, and cephalic setae pattern. The closest congeneric species is *Eubostrichus parasitiferus* but it differs by being larger (♂: 2800–2920 µm, ♀: 2800 µm) than the specimens of *E. hopperi* described by Hopper & Cefalu (1973) (♂: 2140–2180 µm, ♀: 2640–2680 µm). The specimens measured by us have a broader body size range (♂: 1905–2256 µm, ♀: 1475–2745 µm) but includes the size range of the type population of *E. hopperi*. For our specimens, the ratio of the tail length to anal diameter (c′) (♂: 3.3, ♀: 4.1–4.7) was also closer to *E. hopperi* (♂: 3.4–3.9, ♀: 4.5–4.9 in type population) than to *E. parasitiferus* described by Chitwood (1936) (♂: 2.5–2.6; ♀: 3.5). Hopper & Cefalu (1973) stated the existence of 16 subcephalic setae in a pattern of 8 + 8 for *E. hopperi* but this is not in agreement with our observations of a more complicated pattern of subcephalic setae (i.e. 4 + 4 + 4). Unfortunately, we could not check the type material of *E. hopperi* to resolve this apparent mismatch.

The number of enlarged subventral setae (i.e. porids *sensu* Hopper & Cefalu, 1973) is not always easy to determine and appears to be variable as we recorded some females and juveniles with fewer pairs of setae.

The disposition of cephalic sensilla of *E. hopperi* is quite similar to *Robbea porosum*; the main difference is that first two circles of subcephalic setae in the former are longer compared to the remarkably short setae in *R. porosum*. Two juvenile specimens of *Eubostrichus* had bacteria attached in a 'fuzzy' pattern, i.e. attached to a single point on the cuticle. Therefore, we suspect they belong to *Eubostrichus*

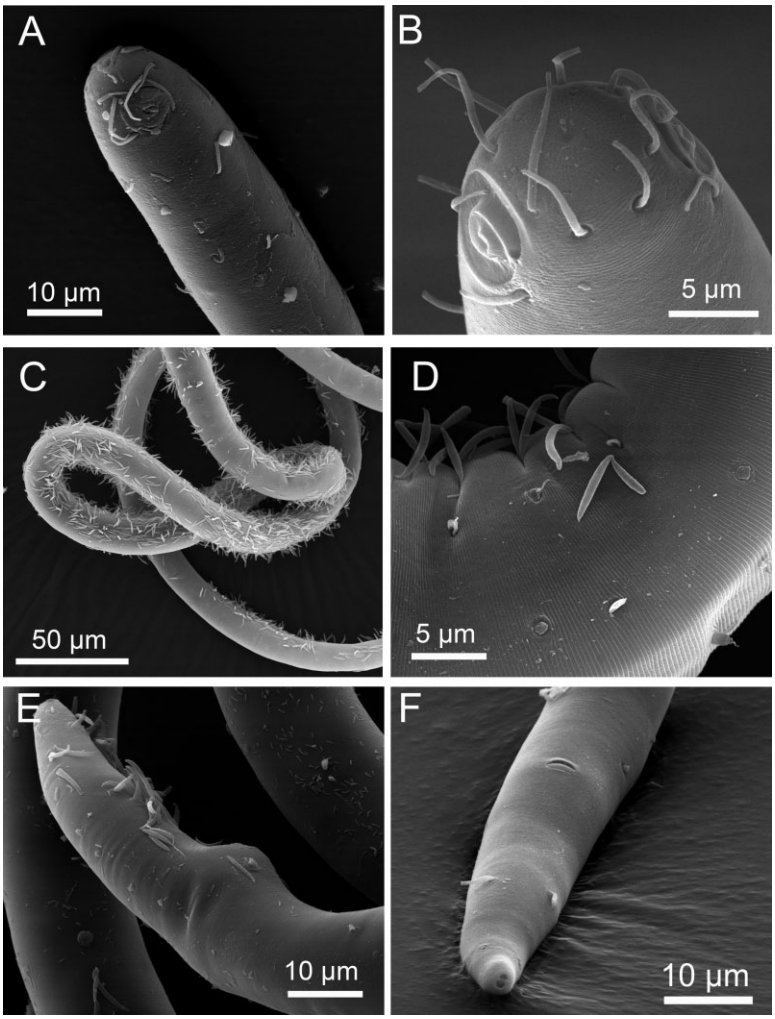

Figure 2. *Eubostrichus hopperi*. A, anterior region (lateral view), specimen 1♂; B, head (lateral–frontal oblique dorsoventral view), specimen 2♀; C, habitus (*partim*), specimen 3♂; D, mid-body with crescent-shaped bacteria, specimen 4 (indeterminate sex); E, tail (lateral view), specimen 1♂; F, tail, specimen 2♀.

dianeae but we could not recover any adult of this putatively sympatric species. The cuticle often has a coating that is mucous in nature and therefore is probably secreted by the epidermal glands to keep the bacteria adhered.

Eubostrichus hopperi has a few pairs of subventral setae enlarged at the level of pharynx that differ from the features given in Table 1 by Tchesunov (2013) (i.e. no male anterior structures). Two to three pairs of these enlarged setae were depicted by Hopper & Cefalu (1973) and we noted a similar number of these structures.

Laxus Cobb, 1894

Diagnosis (emended from Ott et al., 1995 and Tchesunov, 2013): Stilbonematinae. Cuticle finely striated. Cephalic cuticle reinforced by a 'block-layer' inserted in the median layer of the cuticle. Amphidial fovea small, spirally coiled with about 1.5 turns, located close to the apex. Subcephalic lateral setae (four) at level of amphidial fovea and very close to cephalic setae. Anterior region of the pharynx slightly swollen and not sharply marked from the narrow median region. Spicule strongly cephalated. Gubernaculum directed dorsally. Tail short, conical, 1.4–2 anal diameters long. Symbiotic bacteria coccoid or rod shape.

Tchesunov (2013) included as a diagnostic feature the presence of 'fingerprint' pattern in the surface of the head cuticle but this feature is also present in other noncongeneric species examined by us (e.g. *E. hopperi* and *R. porosum*). In *Laxus parvum* sp. nov. we could not observe the relief of the cuticle surface clearly because it was covered by a film of secretion. We included the presence of the 'block-layer' in the

head capsule as a diagnostic feature as stated by Ott *et al*. (1995); the reinforcement of the head capsule is also present in other genera of Stilbonematinae (e.g. *Robbea*, *Stilbonema*) but in *Laxus* it is clearly visible under the LM as small internal vertical rods in the layer. The diagnosis of the genus *Laxus* provided by Ott *et al*. (1995) indicated that ectosymbiotic bacteria were rod-shaped, with the longitudinal axis perpendicular to the cuticle surface; Tchesunov (2013) mentioned only coccoid bacteria. Therefore we included in the diagnosis both bacteria shapes: rod-shape (e.g. *Laxus cosmopolitus* and *Laxus oneistus*) and coccoid shape (e.g. *La. parvum* and *Laxus sigma*).

Type species: Laxus longus Cobb, 1894.

Valid species (6): Laxus cobbi (Inglis, 1968) Ott, Bauer-Nebelsick & Novotny,1995; *La. cosmopolitus* Ott, Bauer-Nebelsick & Novotny, 1995; *La. gerlachi* (Hopper & Cefalu, 1973) Tchesunov, 2013; *La. oneistus* Ott, Bauer-Nebelsick & Novotny, 1995; *La. parvum* sp. nov.; *La. sigma* (Gerlach, 1963) Tchesunov, 2013.

LAXUS PARVUM SP. NOV.

Measurements: Table 2; Figures 3, 4.

Type material: Holotype male, deposited at the Center for Marine Collections, National Aquarium (Habana, Cuba) with collection number ANC_04.063. Paratypes: Two male paratypes, collection numbers CN_1533.10 and CN_1541.7, one female paratype CN_1552.9 and two paratype juveniles CN_1541.3 and CN_1541.9 deposited in the Nematode Collection at Centro de Investigaciones Marinas, Universidad de La Habana (Habana, Cuba). Four deposited at the Royal Belgian Institute of Natural Science (Brussels, Belgium): collection numbers RIT 816 (two females) and RIT 817 (one juvenile and one male).

Habitat type: Coralline sand in the reef lagoon, ~2 m depth, Punta Francés coral reef (21°36′29.68″N, 83°10′34.40″W).

Etymology: The specific epithet (i.e. *parvum*) is the neuter Latin word for 'small' and refers to the small body size of the species compared with most of the other stilbonematins.

Description: Body habitus filiform and coiled, largest body diameter at neck region. Cuticle largely covered by a thin film of mucus, with faint fine striation (annuli ~0.2 μm width), surrounding the anterior part of the amphidial fovea; under SEM, lip region appears smooth. Head capsule with thickened cuticle with a marked median layer. Six inner labial sensilla and six outer labial sensilla setiform papillae. Four cephalic sensilla setiform (8–12 μm long) at level of anterior border of fovea and very close to outer labial papillae. Four short subcephalic setae (< 3 μm long) located very close to the cephalic setae. Another four short sublateral setae (< 3 μm long), each pair located just

Table 2. Mean (range) of morphological measurements for ***Laxus parvum* sp. nov.** (Nematoda: Stilbonematinae)

	Holotype	Paratypes		
N		8♂	7♀	8J
Body length	2738	2421 (1672–2847)	3013 (2905–3322)	1289 (883–2132)
de Man's ratio a	57	66 (46–89)	69 (59–79)	46 (35–63)
de Man's ratio b	32	31 (28–35)	36 (32–39)	19 (15–28)
de Man's ratio c	52	45 (42–52)	51 (44–61)	25 (19–32)
Head diameter	20	17 (13–20)	18 (15–22)	14 (12–16)
Amphidial fovea c.b.d. (%)	38	39 (25–48)	44 (33–48)	40 (36–45)
Pharynx length	85	81 (76–85)	84 (76–97)	68 (58–78)
Max. body diameter	48	37 (32–48)	44 (41–49)	28 (25–34)
Anal body diameter	32	31 (27–36)	28 (22–33)	19 (14–21)
Tail length	51	56 (49–63)	60 (49–74)	52 (41–67)
c′	1.6	1.8 (1.6–2.0)	2.2 (1.5–2.7)	2.8 (2.2–3.4)
V %			51 (49–53)	
Spicule length	63	55 (36–63)		
Gubernaculum length	22	19 (11–24)		

Holotype measurements are included in the male averages and ranges.
c′, tail length relative to the anal body diameter; c.b.d., corresponding body diameter; J, juveniles; *N*, number of specimens; V %, distance (relative to body length) of vulva to anterior apex.
All measurements are in μm.

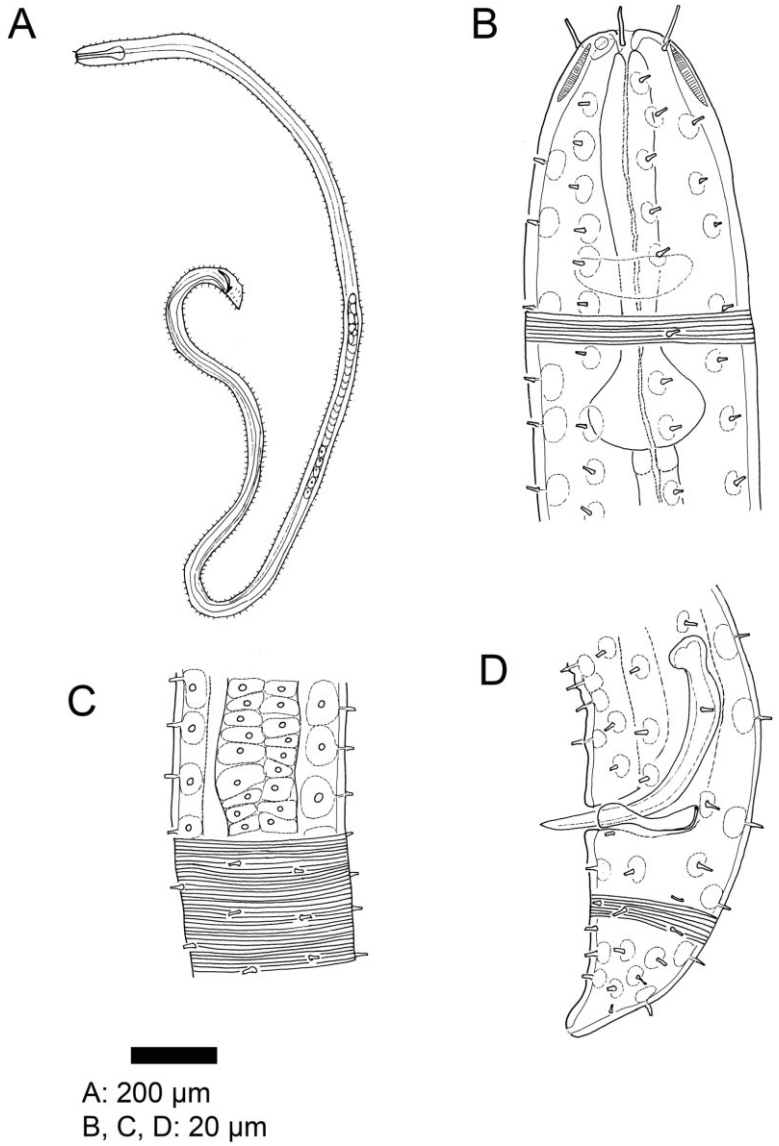

Figure 3. ***Laxus parvum*** **sp. nov.** holotype ♂. A, habitus; B, anterior region; C, testis and epidermal glands; D, tail region and copulatory apparatus.

at the posterior border of the amphidial fovea. Eight longitudinal rows of short somatic setae (1–2 µm long), connected to conspicuous epidermal glands, and extending from the head to tail; setae slightly longer in tail region. Amphidial fovea spirally coiled in 1.5 turns, located laterally, subterminally on the head, approximately 0.4 c.b.d. of relative size. Buccal cavity minute, no sclerotized structures. Pharynx muscular, relatively short, corpus hardly swollen, posterior bulb almost round, lumen not sclerotized, cardia shape rounded and inconspicuous; nerve ring approximately around mid-pharynx. Ventral gland not observed. Tail conical and relatively short, tip smooth, with swollen spinneret having two apertures for the caudal glands. Coccoid bacteria attached to the cuticle and also in the intestine lumen; the arrangement patterns on the cuticle could not be ascertained.

Male monorchic, anterior testis to the left of the intestine. Spicules paired and curved, with marked capitulum; gubernaculum broad and curved with apophysis directly dorsally, no precloacal supplements.

Female didelphic, ovaries antidromously reflexed, both genital branches to the left of intestine. Vulva approximately in the mid-body.

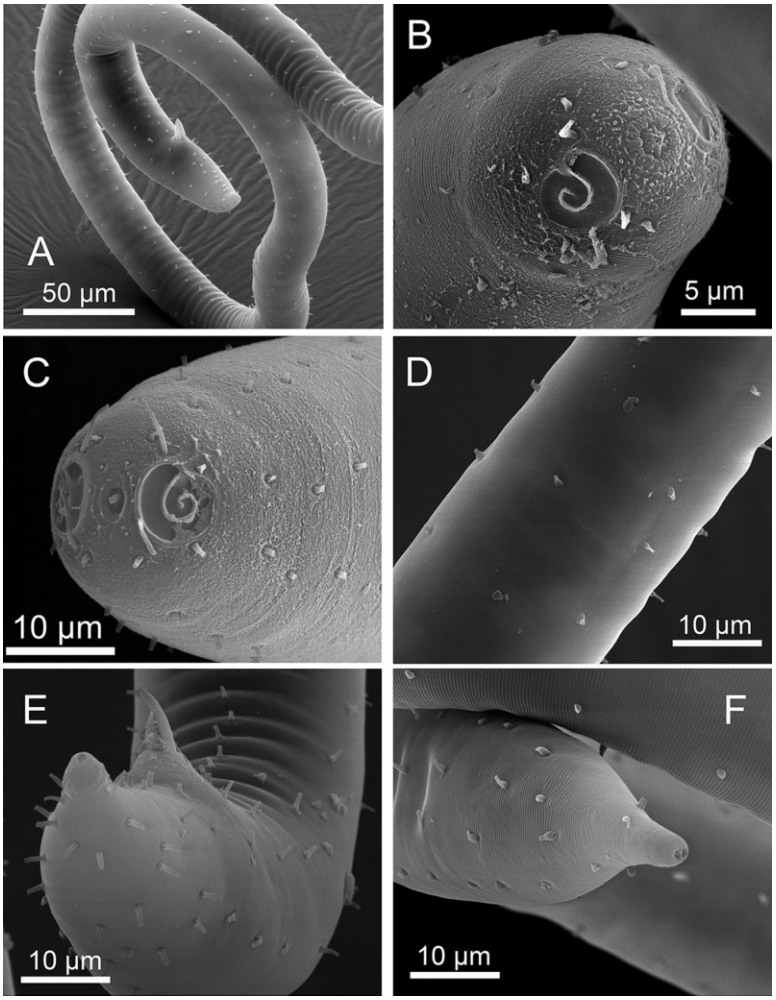

Figure 4. ***Laxus parvum*** **sp. nov.** A, habitus (posterior region), specimen 1♂; B, head (lateral-frontal view), specimen 2 (indeterminate sex); C, head (lateral-frontal view), specimen 3♂; D, mid-body, specimen 3♂; E, tail region, specimen 3♂; F, tail region, specimen 4 (indeterminate sex).

Diagnosis: The new species is characterized by a body length less than 4 mm, pharynx corpus rather slender, spicule relatively long [> 1.8 anal body diameter (a.b.d.)] and strongly cephalated, gubernaculum broad and curved, tail length of female equal or larger than 2 a.b.d., ectosymbiotic bacteria coccoid-shape.

Differential diagnosis and relationships: Laxus parvum sp. nov. belongs to the genus *Laxus* based on the particular structure of the head capsule constituted by a block-layer located on the basal layer of the cuticle, the amphidial fovea spirally coiled in 1.5 turns and located laterally close to the apex, the relatively short conical tail, the spicule capitulum cephalated, and the characteristic somatic setae protruding from the body surface giving a thorny aspect.

Within *Laxus*, currently two species group can be differentiated based on body length: (1) a group with a body length of less than 4 mm consisting of two species (*Laxus gerlachi* and *La. sigma*) and the new species, and (2), a group of four species with a body that is at least twice as long as those in group (1) (*La. cobbi*, *La. cosmopolitus*, *La. longus*, and *La. oneistus*). As far as we know, the shorter species possess coccoid bacteria, whereas for two of the longer species rod-shaped bacteria have been described. Within the short-bodied species, *La. parvum* can be differentiated from the other two species by having long spicules strongly cephalated and the presence of a thick gubernaculum with dorsally slightly curved apophyses.

Leptonemella Cobb, 1920

Diagnosis (adapted from Tchesunov, 2013): Stilbonematinae. Cuticle with fine but distinct striation. Head capsule convex, surface smooth or punctuated, well

KEY TO THE SPECIES OF THE GENUS *LAXUS*

1. Body length < 4 mm 2
   Body length > 4 mm 4
2. Tail > 2.0 a.b.d., spicule capitulum not marked ..... *Laxus sigma*
   Tail < 2.0 a.b.d., spicule strongly cephalated 3
3. Spicule longer (> 1.5 a.b.d.), gubernaculum thick and curved ..... *Laxus parvum*
   Spicule shorter (< 1.5 a.b.d.), gubernaculum thin ..... *Laxus gerlachi*
4. Pharynx corpus enlarged (> 0.5 diameter of isthmus and > 0.3 pharynx length), de Man ratio a > 175 ..... *Laxus oneistus*
   Pharynx corpus different, de Man ratio a < 175 5
5. Subcephalic setae about as long as the cephalic setae, gubernaculum thick ..... *Laxus cobbi*
   Subcephalic setae shorter than cephalic setae, gubernaculum relatively thin 6
6. Pharynx relatively longer (de Man ratio b < 70), spicule relatively long (> 1.3 a.b.d.) ..... *Laxus cosmopolitus*
   Pharynx shorter (b > 70), spicule shorter (< 1.3 a.b.d.) ..... *Laxus longus*

demarcated from annulated body cuticle. Amphidial fovea latero-subterminal, small, spirally coiled in 1.5 turns, loop-shaped or formed as a shepherd's crook, sausage-like corpus gelatum may be protruded. Pharynx very slightly swollen anteriorly. Gubernaculum without dorsocaudal apophysis. Males of some species equipped with stout postcervical, preanal, and postanal subventral setae. Tail elongate-conical. Symbiotic bacteria coccoid to short stick-shaped.

We propose only two minor changes to the diagnosis by Tchesunov (2013): the deletion of (1) the presence of buccal cavity not developed as this is a diagnostic feature to subfamily level, and (2) the range of c′ values being 3–5 because the new species *Leptonemella brevipharynx* has c′ < 3.

Type species: Leptonemella cincta Cobb, 1920.

Valid species (seven): Leptonemella aphanothecae Gerlach, 1950; *Le. brevipharynx* sp. nov.; *Leptonemella granulosa* Boucher, 1975; *Leptonemella gorgo* Gerlach, 1950; *Leptonemella juliae* Hoschitz, Buchholz & Ott, 1999; *Leptonemella vestari* Hoschitz, Buchholz & Ott, 1999; *Leptonemella vicina* Riemann, Thiermann & Bock, 2003.

LEPTONEMELLA BREVIPHARYNX SP. NOV.

Measurements: Table 3; Figures 5, 6.

Type material: Holotype male, deposited at the Center for Marine Collections, National Aquarium (Habana, Cuba) with collection number ANC_04.064. Paratypes: one male paratype, collection number CN_1525.2, one female paratype CN_1525.9, and two paratype juveniles CN_1516.3 and CN_1554.3 deposited in the Nematode Collection at Centro de Investigaciones Marinas, Universidad de La Habana (Habana, Cuba). Three deposited at the Royal Belgian Institute of Natural Sciences (Brussels, Belgium): collection numbers RIT 818 (male), RIT 819 (two juveniles).

Habitat type: Coralline sand in the reef lagoon, ~2 m depth, Punta Francés coral reef (21°36′29.68″N, 83°10′34.40″W).

Etymology: The specific epithet (i.e. *brevipharynx*) is a combination of the Latin words '*brevis*' (meaning short) and '*pharynx*'; it refers to the characteristic short length of the pharynx of the species compared with the body length.

Description: Body habitus filiform and usually tightly coiled; often body diameter decreasing posterior to the pharyngeal region. Cuticle finely striated (annuli ~0.3 μm width). Anterior profile of the head rectangular, head capsule clearly demarcated, inner cuticular wall with vertical rods that give a punctuated surface pattern under LM. Inner labial sensilla papilliform (~1 μm long), outer labial sensilla papilliform (1–2 μm long), four cephalic sensilla setiform (13–18 μm long), four circles of eight subcephalic setae (10–12 μm long), very close and posterior to the cephalic setae. Eight longitudinal rows of somatic setae (8–10 μm long) running from cervical region to tail, each somatic seta connected to an epidermal gland. Amphidial fovea an open spiral of 1.25 turns, located at head border, appearing almost frontal in position, diameter approximately 0.4 c.b.d. Buccal cavity minute, no sclerotized structures. Pharynx muscular, very short, with almost rounded posterior bulb, cardia rounded and inconspicuous; nerve ring at mid-pharynx. Ventral gland not observed. Tail conical, final portion without striae but with internal vertical rods (similar to those

Table 3. Mean (range) of morphological measurements for ***Leptonemella brevipharynx* sp. nov.** (Nematoda: Stilbonematinae)

	Holotype	Paratypes		
N		5♂	2♀	5J
Body length	3358	3297 (2738–3723)	3438 (3387–3489)	1952 (1270–3161)
de Man's ratio a	71	70 (60–74)	80 (79–81)	45 (37–61)
de Man's ratio b	37	39 (35–45)	46 (45–48)	25 (20–34)
de Man's ratio c	30	30 (28–34)	35 (34–35)	24 (18–33)
Head diameter	33	32 (29–33)	35	29 (25–41)
Amphidial fovea c.b.d. (%)	*	32 (14–42)	38*	36 (24–45)
Pharynx length	90	84 (78–90)	75 (71–78)	74 (62–94)
Max. body diameter	47	47 (45–53)	43 (43–43)	42 (30–55)
Anal body diameter	44	43 (35–49)	34 (29–38)	27 (21–36)
Tail length	111	110 (97–118)	100 (99–100)	81 (69–97)
c′	2.5	2.6 (2.2–2.9)	3.0 (2.6–3.4)	3.0 (2.7–3.3)
V %			46 (45–46)	
Spicule length	57	52 (31–61)		
Gubernaculum length	29	27 (22–29)		

Holotype measurements are included in the male averages and ranges.
c′, tail length relative to the anal body diameter; c.b.d., corresponding body diameter; J, juveniles; *N*, number of specimens; V %, distance (relative to body length) of vulva to anterior apex.
All measurements are in μm.
*indicates that measurements could not be taken (or with higher associated error) because of the position of the specimens.

in the head capsule); tail shorter in males (c′ = 2.6) compared to females (c′ = 3.0); caudal glands with spinneret present and three apertures and a central pore; we could not ascertain how glands connect to the central pore. Coccoid-shape bacteria covering most of the body.

Male monorchic, anterior testis to the left of the intestine. Spicules curved, paired, with marked capitulum; gubernaculum with short corpus and clear dorsal apophysis, hooked at tip; no precloacal supplements.

Female didelphic, ovaries antidromously reflexed; vulva not sclerotized, located at mid-body.

Diagnosis: The new species is characterized by the shape of the amphidial fovea as an 'open' spiral, very short pharynx b > 39; gubernaculum with short corpus 22–29 μm long and dorsal apophysis, hooked at tip; tail short in males, less than 120 μm (< 3 a.b.d.).

Differential diagnosis and relationships: Le. brevipharynx sp. nov. belongs to the genus *Leptonemella* Cobb, 1920 because of the clearly demarcated cephalic capsule not surrounded by fine but distinctive cuticle striation, circles of relatively long subcephalic setae on the cephalic capsule, amphidial fovea as an 'open' spiral (i.e. not tightly coiled) located in laterofrontal position and the coccoid-shape ectosymbiotic bacteria and gubernaculum without dorsocaudal apophysis.

Hoschitz *et al*. (1999) recognized four valid species of *Leptonemella*, transferred *Le. sigma* Gerlach, 1963 to the genus *Laxus* Cobb, 1920, and proposed two new species (*Le. juliae* and *Le. vestari*). These authors considered the following diagnostic features as the most important for the genus (Hoschitz *et al*., 1999: table 1): the amphidial fovea shape (in females); length of cephalic, subcephalic, and somatic setae; and shape of the gubernaculum. Riemann *et al*. (2003) made an outline of the genus recognizing seven species, including a new one (*Le. vicina*), and discussed valuable information about ecology, distribution, physiology, and taxonomy. The authors stressed the difficulty in discriminating amongst the known species because of the minor differences amongst them. The most recent revision of the Stilbonematinae (Tchesunov, 2013) recognized seven species (see above) and also supports the taxonomy proposed by Riemann *et al*. (2003).

Leptonemella brevipharynx sp. nov. can be discriminated from the other seven species based on three unique features: the very short length of the pharynx, the very short length of the tail in males, and the shape of gubernaculum with curved, hook-like proximal end.

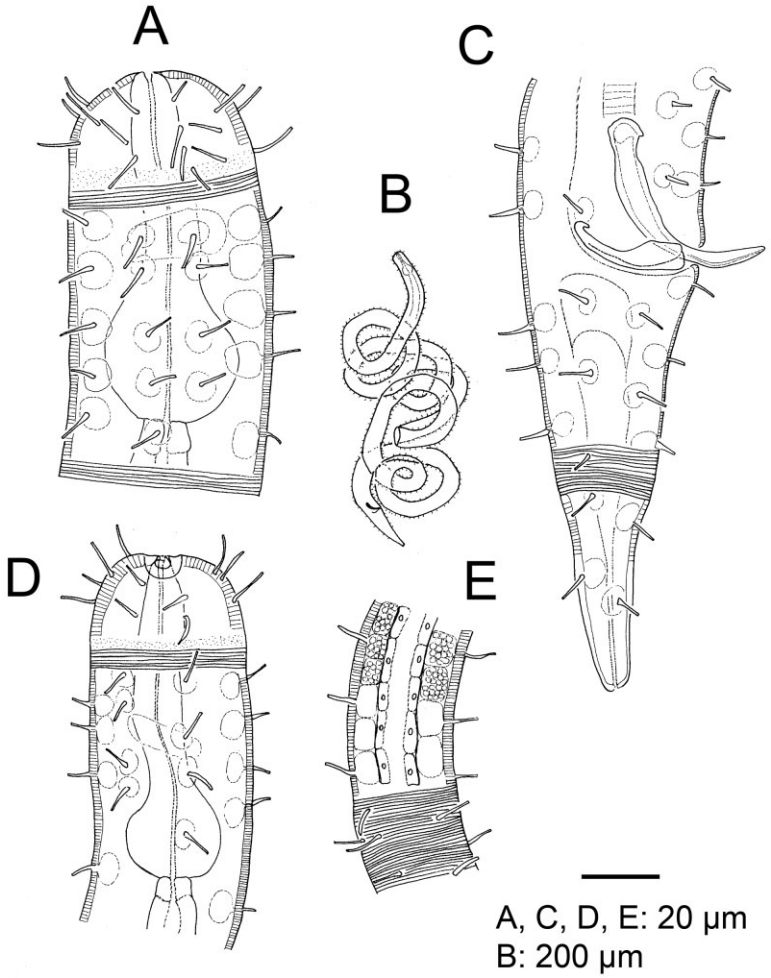

Figure 5. ***Leptonemella brevipharynx*** **sp. nov.** A, anterior region, paratype 1♂; B, habitus, holotype ♂; C, tail region, holotype ♂; D, anterior region, paratype 2♀; E, intestine and epidermal glands connected to setae, paratype 2♀.

ROBBEA POROSUM (HOPPER & CEFALU, 1973) TCHESUNOV, 2013

Measurements: Table 1; Figures 7, 8.

Material examined: Five ♂, one ♀, two J.

Description: Body habitus filiform. Cuticle finely striated (annuli ~0.3 µm width), covered by coccoid bacteria and mucus (also adhered to the setae). Head capsule weak marked by thickening of the cuticle, revealing in SEM photographs a pattern-like fingerprint anteriorly and fine transverse striations posteriorly, surrounding the amphidial fovea; six lips partially fused, each provided with an inner labial papilla, and surrounded by a differentiated collar; six outer labial papillae (~1 µm long) at border of labial region. Four cephalic sensilla setiform (11–14 µm long) at level of outer labial sensilla. Four shorter subcephalic setae at anterior level of amphidial fovea (7–8 µm long): two subdorsal and two subventral. Additional four subcephalic setae (5–8 µm long) flanking posteriorly the fovea. Eight longitudinal rows of short setae connected to epidermal glands (1–2 µm long) running along the body length. Amphidial fovea spiral, lateral, and far anteriorly on the head, diameter approximately 0.5 c.b.d. Pharynx divided into three portions, an anterior muscular corpus with sclerotized lumen, a narrow isthmus, and a posterior bulb with glandular aspect and lumen nonsclerotized; cardia rounded and inconspicuous. Tail conical, striations extending subterminally, tail tip rounded. Males with spicules paired, blade ventrally curved and marked capitulum; gubernaculum with relatively large dorsally orientated caudal apophysis; neither precloacal nor postcloacal supplements.

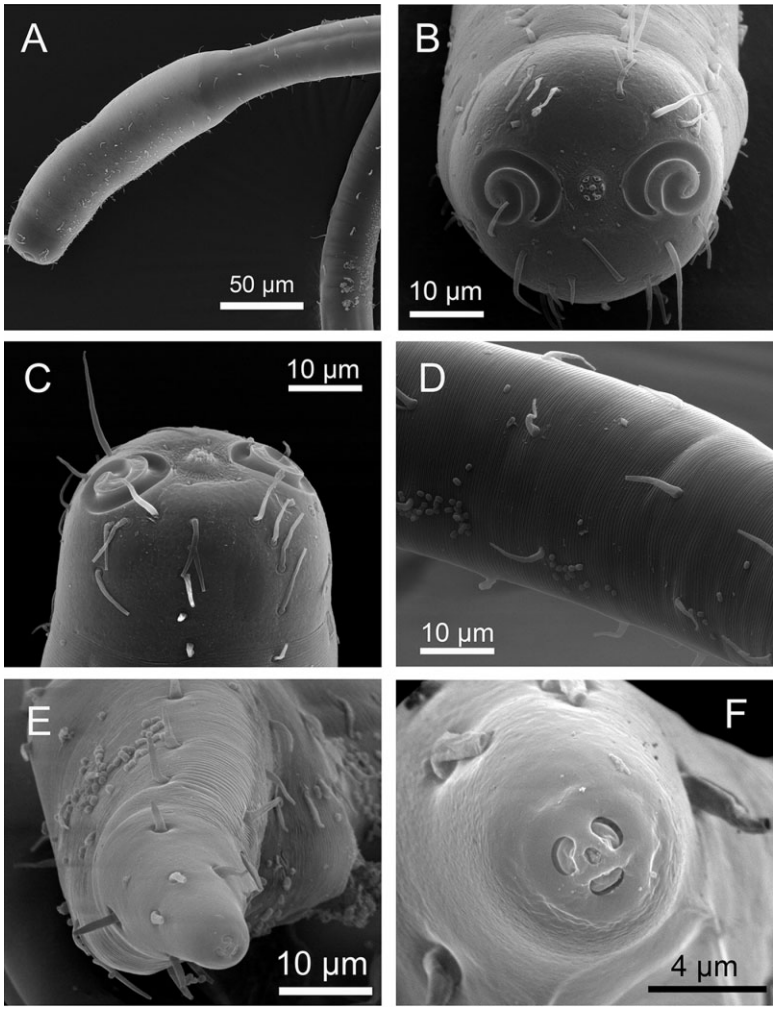

Figure 6. ***Leptonemella brevipharynx*** **sp. nov.** A, anterior region, specimen 1 (indeterminate sex); B, head (frontal view), specimen 2 (indeterminate sex); C, head (lateral view), specimen 2 (indeterminate sex); D, mid-body with coccoid-shaped bacteria, specimen 1 (indeterminate sex); E, tail region with coccoid-shape bacteria, specimen 2 (indeterminate sex); F, spinneret aperture, specimen 2 (indeterminate sex).

Female didelphic, ovaries antidromously reflexed, vulva at mid-body position, no other features could be ascertained because of the strong coiling of the single female examined.

Remarks: Robbea porosum has recently been transferred from the genus *Catanema* to *Robbea* based on its large, spirally coiled amphidial fovea in lateral position (Tchesunov, 2013). The fingerprint pattern in the head capsule appears to be a diagnostic feature in this species as it has not been recorded for the other five known species of the genus. The arrangement of the bacterial coat could not be ascertained because in most of the specimens the bacteria were detached from the host upon processing. Several specimens of *R. porosum* had the intestine full of bacteria, supporting the hypothesis that the latter serve as a food source for the nematode.

Stilbonema brevicolle Cobb, 1920

Measurements: Table 1; Figures 9, 10.

Material examined: One ♂, two ♀, two J.

Description: Body habitus slender. Cuticle coarsely striated (annuli 1.4–2.0 µm width). A coat of rod-shaped bacteria covering the cuticle; the axis of bacteria cells perpendicular to the cuticle. Head profile anteriorly flattered (but may look globular in some specimens), head capsule well developed, surface with slight depressions. Inner labial sensilla papilliform, outer labial sensilla setiform (~6 µm long).

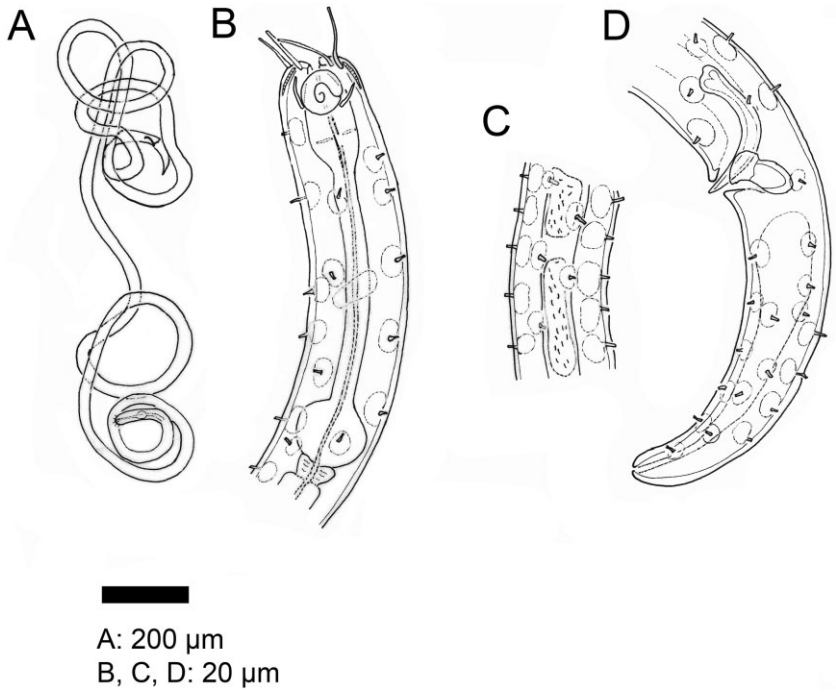

Figure 7. *Robbea porosum*. Specimen 1♂. A, habitus, B, neck region, C, testis and epidermal glands connected to somatic setae, D, tail region.

Four cephalic sensilla setiform (10–15 µm long), two circles of eight subcephalic setae (10–12 µm long), first one anteriorly on the head, second one close to the border of the cephalic capsule; one or two pairs of additional subcephalic setae can occur between both circles. Somatic setae short (5–6 µm long) probably arranged in eight longitudinal rows but few are visible because of the bacterial coat or broken off; somatic setae connected to epidermal glands but not always clear. Amphidial fovea pore-like with corpus gelatum protruding, located subterminally on the head. Pharynx muscular, corpus largely cylindrical but slightly wider near head region, posteriorly widened to a small pyriform bulb; cardia triangular/elongated and conspicuous. Tail conical, tip smooth and spinneret present.

Male monorchic, anterior testis to the left of intestine; spicules curved and paired. Six to eight conspicuous cup-like precloacal supplements present at level of pharynx.

Female didelphic, vulva not conspicuous, ovaries antidromously reflexed, both genital branches to the left of intestine.

Remarks: There are four species of *Stilbonema* formally described: *Stilbonema annulatum* Gerlach, 1963; *S. brevicolle* Cobb, 1920; *Stilbonema majum* (Cobb, 1920), and *Stilbonema smurovi* Tchesunov, 2013. The descriptions of the first three species are rather poor in morphological details and with only a single male specimen per species. The name *S. majum* appeared in Urbancik *et al*. (1996a) but without comments on the transfer of *Laxonema majum* Cobb, 1920, to *Stilbonema majum*. Tchesunov (2013) formally transferred *L. majum* to the genus *Stilbonema* but without explanation. We agree with this classification because *L. majum* in Cobb (1920) resembles some features characteristic of the genus *Stilbonema* such as cephalic capsule clearly set-off and coarse annulation. Additionally, specimens identified as *S. majum* in Urbancik *et al*. (1996a) were 8.5 mm in average length, which agrees roughly with the single specimen described as *L. majum* by Cobb (10 mm). Our specimens fit well with the original description by Cobb (1920) based on specimens from the Caribbean Basin (Kingston Harbor, Jamaica). The male specimen of *S. annulatum* described by Gerlach (1963) is larger than our male specimen (5977 vs. 1870 µm) and was collected far away (i.e. Indian Ocean).

PHYLOGENETIC RECONSTRUCTION

The phylogenetic trees based on *SSU* rDNA and *COI* shows that the three subfamilies of Desmodoridae included in our analysis are polyphyletic

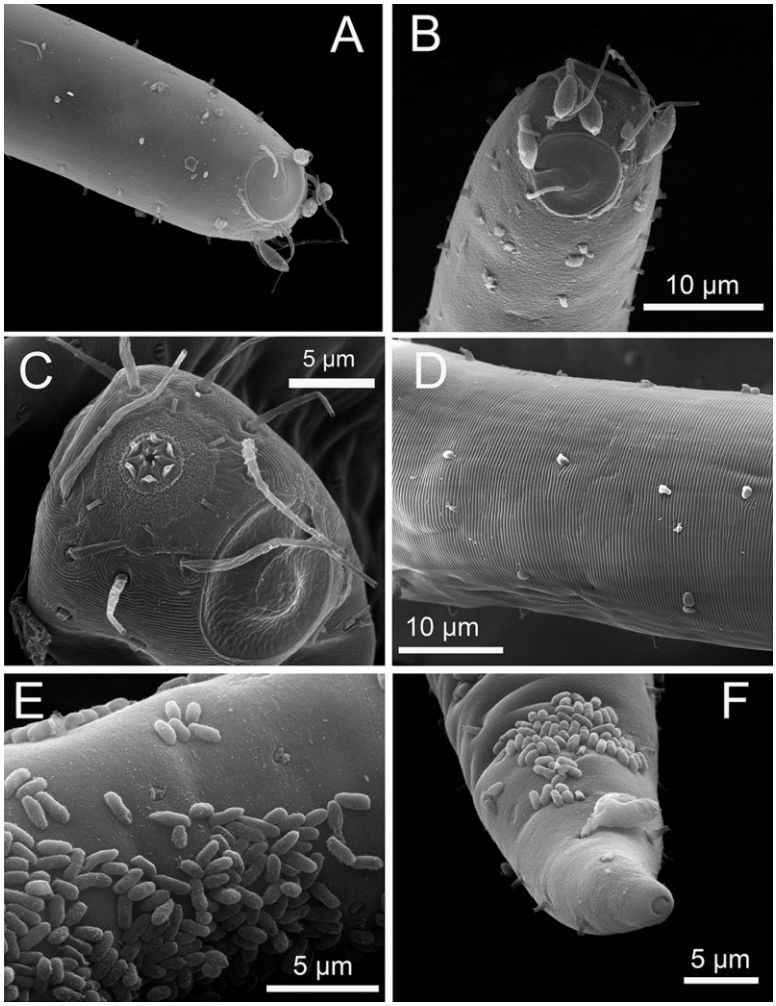

Figure 8. *Robbea porosum*. A, anterior region (lateral view), specimen 1♂; B, head (lateral-frontal view), specimen 1♂; C, head (frontal view), specimen 2 (indeterminate sex); D, mid-body, specimen 3 (indeterminate sex); E, symbiotic bacteria, specimen 1♂; F, tail region, specimen 1♂.

(Figs 11, 12). In other words, our evidence, based on two independent loci, supports the rejection of the hypothesis of monophyly of the subfamily Stilbonematinae; but also the monophyly of the other two sampled subfamilies: Desmodorinae and Spiriniinae. It implies that the relationships within the family are not completely solved based on the current scheme of the subfamilies even when Desmodoridae appears as a clade in phylum-wide studies (e.g. Van Megen *et al*., 2009). Two previous studies (Kampfer *et al*., 1998; Bayer *et al*., 2009) found very low support for the monophyly of Stilbonematinae based on *SSU* rDNA but despite this both still accepted the taxa as clades.

Our study includes the highest number of stilbonematin species sequenced so far and reinforces the absence of a natural grouping of the genera within the subfamily. At least four genera are paraphyletic (i.e. *Eubostrichus*, *Laxus, Robbea*, and *Stilbonema*) based on *SSU* rDNA (Fig. 11). These genera differ in important morphological features such as head capsule, cuticle, amphidial fovea, pharynx, supplements in males, and outlet of caudal glands. Two morphological traits were proposed as synapomorphies of Stilbonematinae by Bauer-Nebelsick *et al*. (1995): the complex glandular sensory organ and symbiosis with chemoautotrophic sulphur-oxidizing bacteria. An alternative explanation for the polyphyly of Stilbonematinae is that these features evolved independently several times in a process of convergent evolution; this idea has been also previously suggested by Bayer *et al*. (2009) to explain the polyphyletic nature of *Robbea* specimens. More extensive studies are necessary to solve this incongruence between

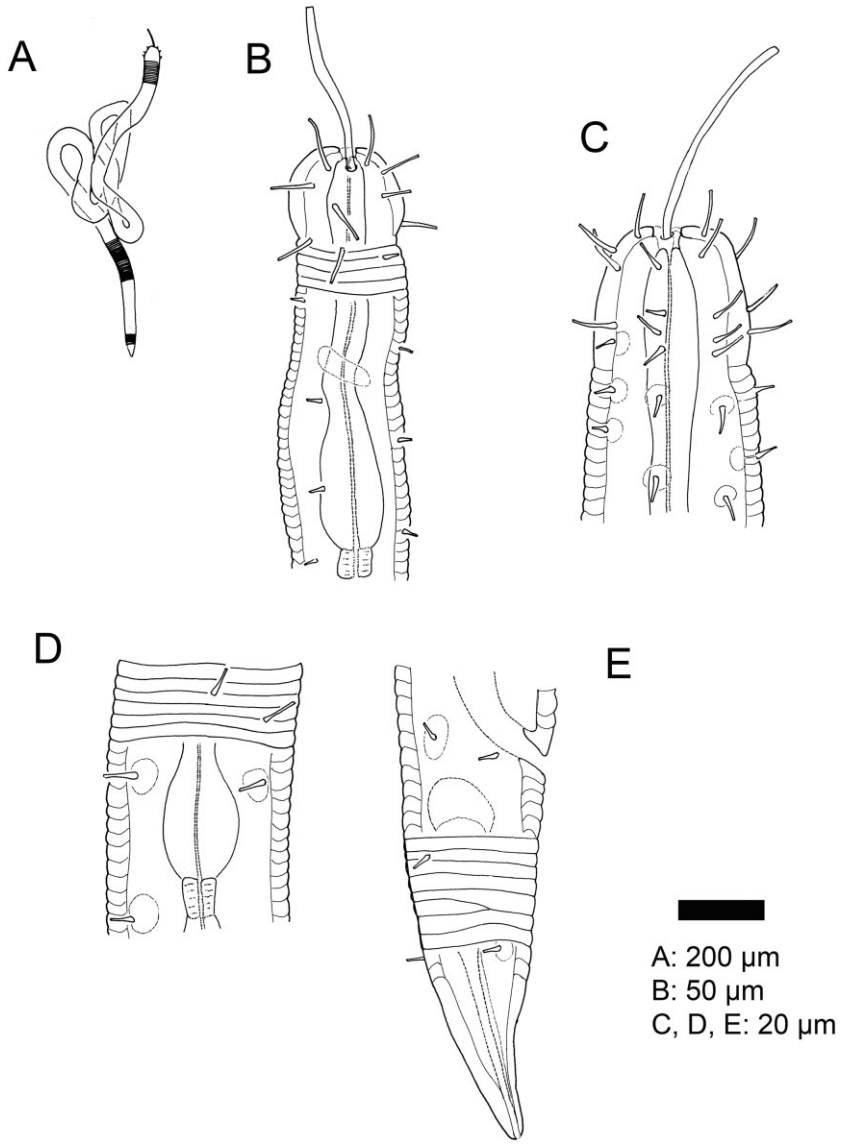

Figure 9. *Stilbonema brevicolle*. A, habitus, specimen 1 juvenile; B, anterior region, specimen 1 juvenile; C, anterior region, specimen 2♀; D, pharyngeal bulb and epidermal glands, specimen 2♀; E, tail region, specimen 2♀.

molecular and morphological data; for instance describing the ultrastructure of the setae in more species of Desmodoridae and sequencing more genes [e.g. Large Subunit rDNA (*LSU*) and Internal Transcribed Spacer (*ITS*)].

The *COI* phylogenetic tree shows two well-supported lineages within *Zalonema* sp. and *R. porosum*, and within *Paradesmodora immersa* (Fig. 12); the two lineages for *R. porosum* are also supported by the *SSU* rDNA marker (Fig. 11). Divergent lineages within these nominal species are evidence of cryptic species because the photo-vouchers of specimens did not indicate any clear morphological discontinuity between them. Cryptic species occur homogeneously across metazoan and also across biogeographical regions (Pfenninger & Schwenk, 2007); therefore, it is also expected in tropical nematode species. Reports of cryptic diversity for free-living marine nematodes refer to several orders, such as Enoplida (*Thoracostoma trachygaster*; Silva de Oliveira *et al*., 2012); Monhysterida (*Halomonhystera disjuncta* Fonseca, Derycke & Moens, 2008; *Terschellingia* spp., Bhadury *et al*., 2008); Rhabditida (*Rhabditis marina* Fonseca *et al*., 2008); and Desmodorida (present study).

The interstitial lifestyle and type of sensorial structures of nematodes suggest that chemical signals are

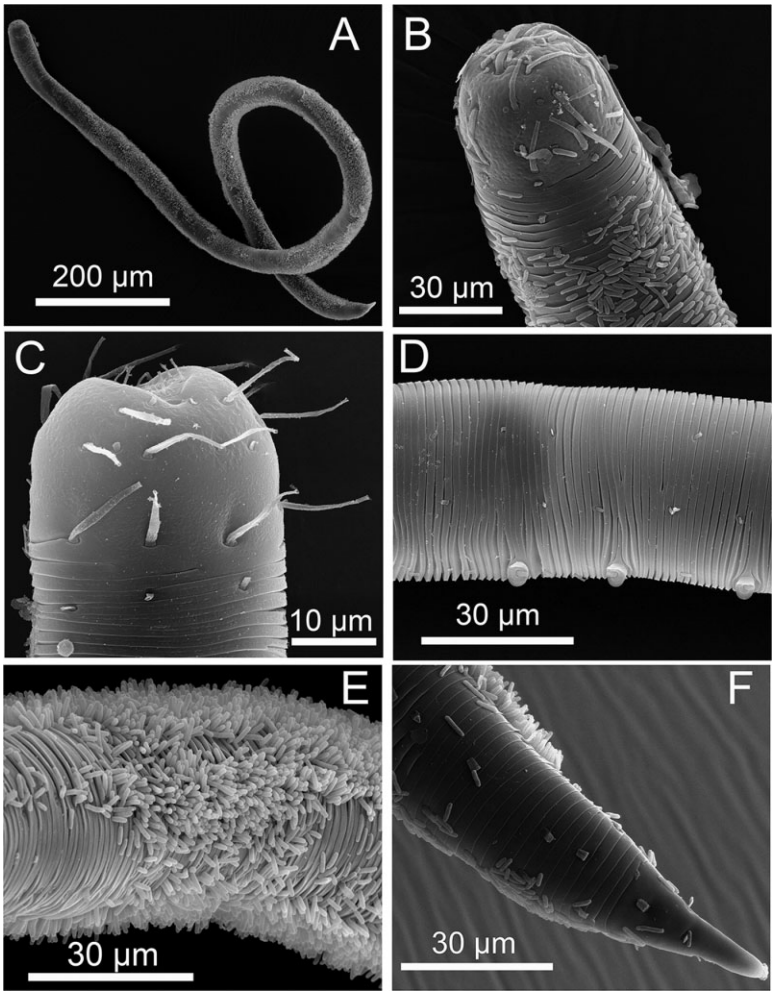

Figure 10. *Stilbonema brevicolle*. A, habitus, specimen 1 (indeterminate sex); B, anterior region, specimen 1; C, head (lateral view), specimen 2♂; D, anterior region with ventral supplements (pharynx level), specimen 2♂; E, mid-body with rod-shaped bacteria, specimen 1; F, tail region with rod-shaped bacteria, specimen 1.

more important than visual ones; this is one of the most important causes of cryptic diversity (Bickford *et al*., 2006). According to Bickford *et al*. (2006), the second cause of cryptic diversity is stabilizing selection, which is conducive to morphological stasis; *Robbea* (and other stilbonematins) would be subject to this stabilizing selection as a result of their obligate symbiosis with bacteria. This interspecific association demands particular morphological (e.g. exudation of mucus, coiling of the body) as well behavioural features (e.g. repeated migrations across the chemocline, Ott *et al*., 1991). Evolutionary convergence of morphological features also supports the above-mentioned paraphyletic nature of *Robbea*.

The current classification of Stilbonematinae into nine genera (Tchesunov, 2013) remains the most tractable system to deal with this heterogeneous group in spite of the evidence of its paraphyletic nature based on molecular data.

ACKNOWLEDGEMENTS

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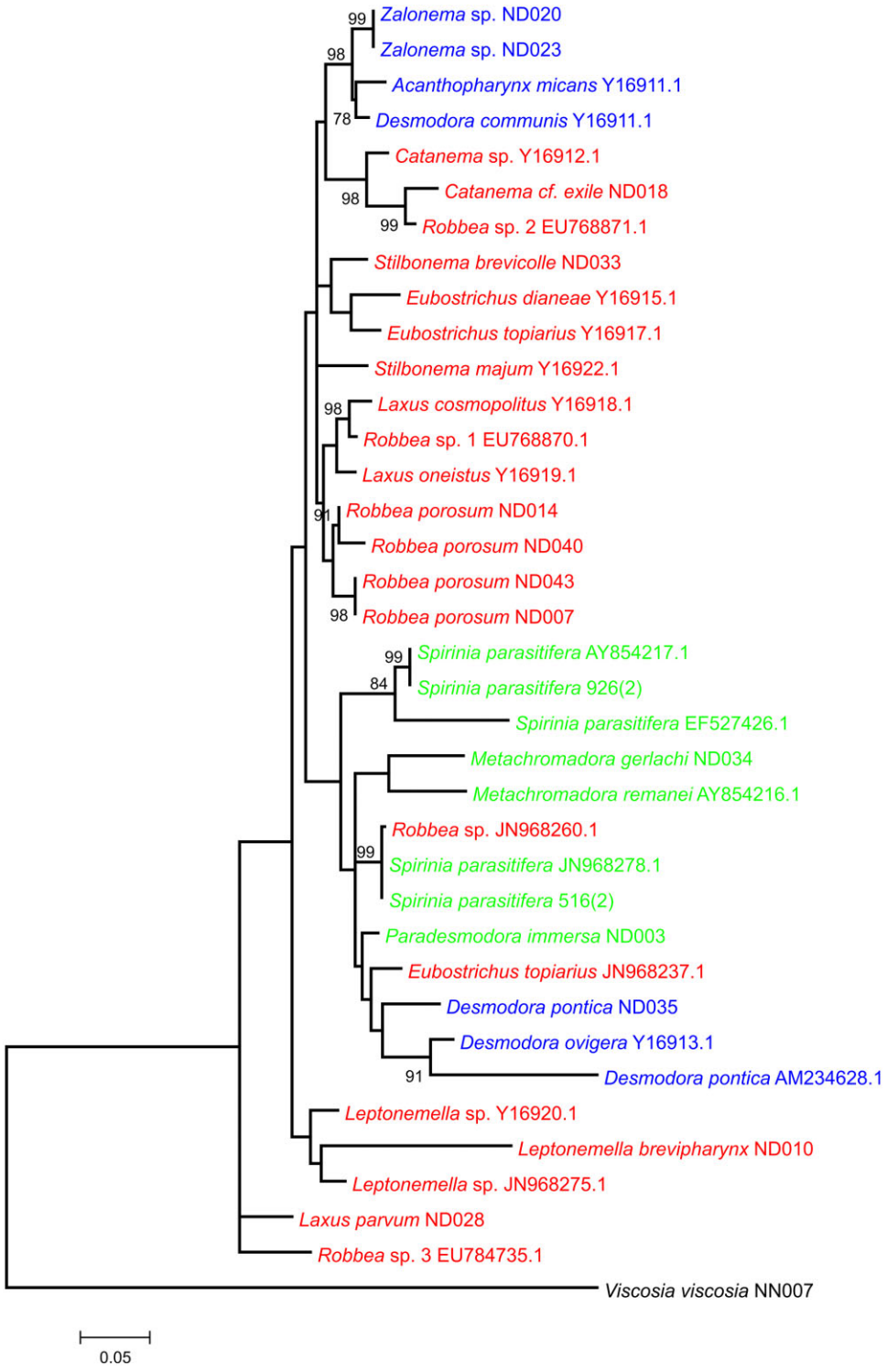

Figure 11. Maximum likelihood phylogenetic tree of 37 specimens of desmodorids (including one outgroup) based on 586 nucleotide sites of the *small subunit* rDNA locus. Statistical support values (1000 bootstrap permutations) > 75% are shown above or below the branches. Species names are coloured after the three subfamilies as defined in Decraemer & Smol (2006): Desmodorinae (blue), Spiriniinae (green), and Stilbonematinae (red).

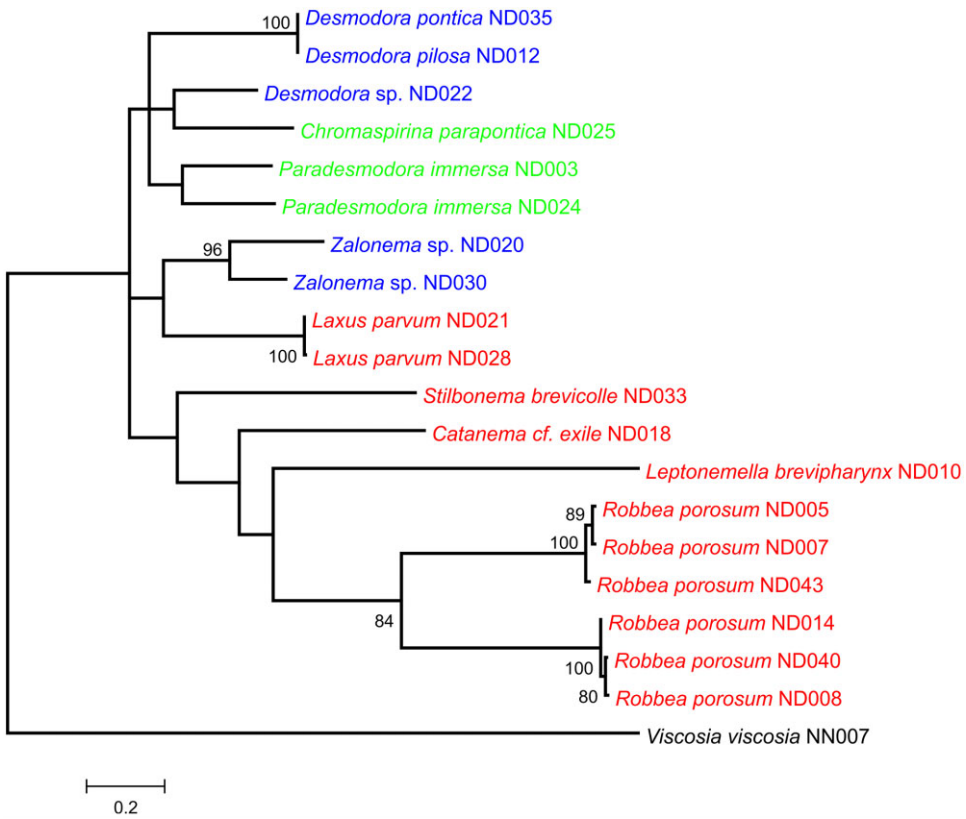

Figure 12. Maximum likelihood phylogenetic tree of 20 specimens of desmodorids (including one outgroup) based on 386 nucleotide sites of the *cytochrome oxidase c subunit 1* locus. Statistical support values (1000 bootstrap permutations) > 75% are shown above or below the branches. Species names are coloured after the three subfamilies as defined in Decraemer & Smol (2006): Desmodorinae (blue), Spiriniinae (green), and Stilbonematinae (red). Specimens labelled as ND are new sequences, others are from GenBank.

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