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Eubostrichus fertilis sp. n., a new marine nematode (Desmodoridae: Stilbonematinae) with an extraordinary reproductive potential from Belize, Central America

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Summary – *Eubostrichus fertilis* sp. n. is described from fine subtidal sands in the Belize Barrier Reef system using LM and SEM illustrations and the sequence of the 18S rRNA gene. The new species is one of the smallest (mature specimens ranging from 1.88 to 3.03 mm) and the stoutest (a = 36-80) of all previously described *Eubostrichus* species. The closest relatives are *E. parasitiferus* and *E. hopperi*. It differs from the former in the more posterior position of the vulva and the postanal porids, and from the latter in the smaller size of the amphids, the shorter cephalic setae and the shape of the tail. Furthermore, it is remarkable for the prominent extent of the female genital system. Females have up to 18 eggs of similar size in their uteri. The body of the worm is covered by large (up to 45 μm long) crescent-shaped bacteria attached with both poles to the cuticle of the worm in a spiral pattern. The genus *Eubostrichus* is phylogenetically well supported on the basis of the 18S rRNA gene sequence. *Eubostrichus gerlachi* nom. nov. (= *E. parasitiferus apud* Gerlach, 1963 *nec* Chitwood, 1936) is proposed.

Keywords – Caribbean Sea, description, *Eubostrichus gerlachi* nom. nov., meiofauna, molecular, morphology, morphometrics, new name, new species, sulphide system, symbiosis, taxonomy, thiotrophic bacteria.

The subtidal porous back-reef sediments of the Belize Barrier Reef system harbour a rich meiofauna in which the subfamily Stilbonematinae (Desmodoridae) plays a prominent role, occasionally dominating the nematode fauna (Ott & Novak, 1989). Stilbonematinae are remarkable for their symbiosis with sulphur-oxidising Gammaproteobacteria, which cover the body cuticle in an often regular pattern (Ott *et al.*, 1991; Polz *et al.*, 1992; Hentschel *et al.*, 1999). In all cases where the identity of the symbiotic bacteria has been established, the coat proved to be a monospecific biofilm specific to each host species (Polz *et al.*, 1994; Bayer *et al.*, 2009; Bulgheresi *et al.*, 2011). The mechanism of symbiont/host recognition has been attributed to lectins secreted by the worms from unique hypodermal glands (glandular sensory organs, gso) (Bauer-Nebelsick *et al.*, 1995) interacting with sugars in the bacterial cell wall in a specific fashion (Nussbaumer *et al.*, 2004; Bulgheresi *et al.*, 2006, 2011).

In *Eubostrichus* Greeff, 1869, the symbiotic bacteria grow to exceptionally large sizes, forming non-septate filaments. In *E. dianeae* Hopper & Cefalu, 1973 these filaments may exceed 100 μm in length (Polz *et al.*, 1999; Ott *et al.*, 2004) and are attached by only one pole to the host cuticle. In all other so far described species of *Eubostrichus*, the bacteria are crescent-shaped, up to 30 μm long and usually attached by both poles. The symbionts are arranged in a spiral pattern along the anterior/posterior axis of the host giving the worm a rope-like appearance (Ott *et al.*, 2004).

To date, nine *Eubostrichus* species have been described, two of which are regarded as *species inquirendae*. Except for *E. hortulanus* Leduc, 2013, which has been retrieved from a depth of 350 m, all other species have been found in intertidal or shallow subtidal sediments. Here we describe *Eubostrichus fertilis* sp. n., which shows a remarkable expansion of the female genital system

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and occurs in fine subtidal sands in the Belize Barrier Reef, occasionally in very high numbers. The new taxonomic name is registered in ZooBank (publication ID Zoobank: urn:lsid:zoobank.org:pub:69B84D1E-1560-4233-88F0-AEAC8911B5FE).

Materials and methods

Nematode collection

Specimens of *E. fertilis* sp. n. were collected in December 2011 and January 2012 at the type locality. The nematodes were extracted by stirring the sand in seawater and pouring the supernatant through a 63 μm pore sieve. The contents of the net were transferred into a Petri dish and single individuals were then picked by hand under a dissecting microscope.

Microphotographs of live animals were taken with a Canon EOS 500D camera mounted on a Zeiss microscope. For permanent slides, animals fixed in methanol were slowly transferred to and mounted in pure glycerin. Drawings from permanent mounts were made using a Reichert Diavar equipped with a *camera lucida*.

For genomic DNA extraction, nematodes were fixed in methanol and stored deep-frozen for transportation and storage. For scanning electron microscopy (SEM), nematodes were fixed with 2.5% glutaraldehyde and stored and transported in buffer at 4°C.

Scanning electron microscopy (SEM)

Worms were post-fixed with 1% osmium tetroxide for 2 h at room temperature and then dehydrated in a graded ethanol series, transferred into pure acetone and critical point dried with a CPD 300 unit (Leica). After mounting on stubs they were gold-sputter coated with an AGAR B7340 sputter-coater unit. Images were taken with a XL20 (Philips) using the Microscope control program (v. 7.00, FEI).

Genomic DNA extraction and PCR amplification of the nematode 18S rRNA genes

DNA was extracted from two single *E. fertilis* sp. n. individuals as previously described (Schizas *et al.*, 1997). Two μl of DNA were used as template in each 50 μl PCR reaction. An almost complete (*ca* 1800 bp) fragment of the 18S rRNA-gene was amplified by PCR with the general eukaryotic primers, 1f (5′-CTGGTTGATYCTGCC AGT-3′) (Winnepenninckx *et al.*, 1998) and 2023r (5′-GGTTCACCTACGGAAACC-3′) (Pradillon *et al.*, 2007). Cycling conditions for the 18S rRNA-gene amplification were as follows: 95°C for 3 min followed by 35 cycles of 95°C for 45 s, 48°C for 45 s and 72°C for 120 s, and a final elongation of 72°C for 10 min. The PCR products were cleaned using the MinElute PCR purification kit (Qiagen) and directly sequenced using the PCR primers.

18S rRNA phylogenetic analysis

The two *E. fertilis* sp. n. 18S rRNA sequences and all sequences from the Stilbonematinae available in GenBank, as well as two Draconematidae sequences as outgroup representatives, were aligned using MAFFT Q-INS-I, which considers the predicted secondary structure of the RNA for the alignment (Katoh *et al.*, 2005). Alignments were manually inspected and 5′- and 3′-end-trimmed using Geneious software version 6 (Drummond *et al.*, 2011). The optimal substitution model was assessed using the Akaike information criterion as implemented in MEGA 5.3 (Tamura *et al.*, 2011) and the GTR + G + I model was chosen. Phylogenetic trees were reconstructed using maximum likelihood- (PHYML) (Guindon *et al.*, 2010) and Bayesian inference-based (MrBayes) (Ronquist & Huelsenbeck, 2003) methods. MrBayes was run for 2×10^6 generations using four chains. Convergence was evaluated by plotting the generations *vs* log L and the burn-in was set to 5×10^5 generations. Node stability was evaluated using posterior probabilities (pp, Bayesian inference) and aLRT (maximum likelihood) (Anisimova & Gascuel, 2006) with values above 0.80 (pp), 80 (aLRT) considered significant.

Results

Eubostrichus fertilis[*] sp. n.
(Figs 1-5)

Measurements

See Table 1.

Description

Adults

Body stout, cylindrical, tapering only slightly towards anterior end, head diam. at level of cephalic setae =

[*] Specific epithet formed from the Latin *fertilis* = fertile, and referring to the high number of eggs in the female gonads.

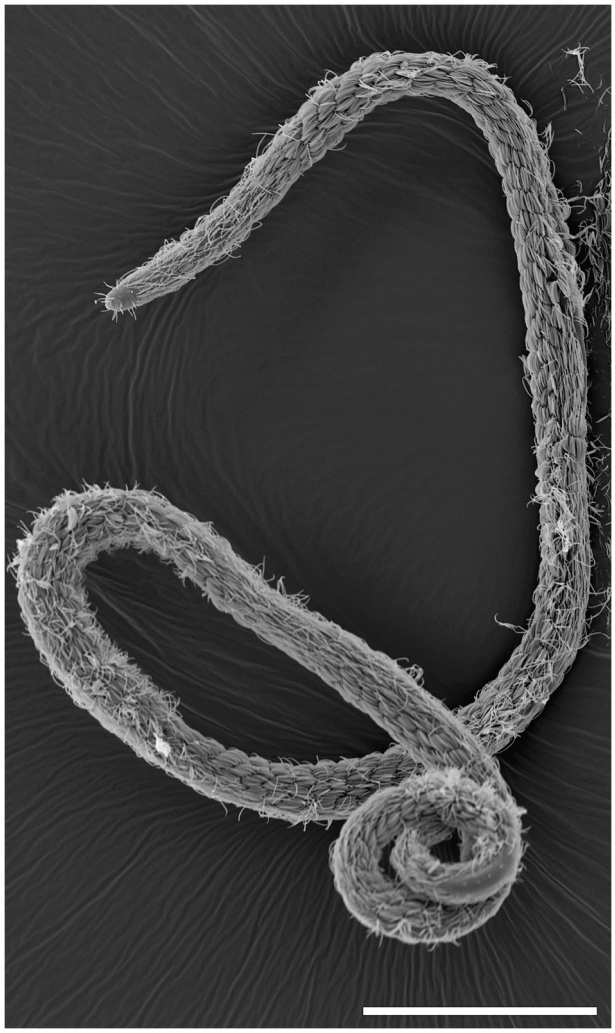

Fig. 1. *Eubostrichus fertilis* sp. n. SEM of entire female. (Scale bar = 200 μm.)

15-18 μm, at level of centre of amphid = 17-20 μm. Tail conical, cuticle finely annulated except for anterior part of head and tail tip, annulation beginning at level of centre of amphid, annuli in pharyngeal region 0.4-0.5 μm wide (20-25 annuli 10 μm^{-1}), in mid-body region 0.30-0.35 μm (28-32 annuli 10 μm^{-1}), non-annulated tail tip 2-3 μm long. Somatic setae short (1.2 μm), arranged in eight longitudinal rows in submedian and sublateral position. Distance between setae within a row 10-11 μm in pharyngeal region, 18-22 μm in mid-body region. One or two pairs of small (<2 μm) terminal setae on non-annulated tail tip. Mouth surrounded by a circle of six papilliform outer labial sensilla, in lateral, laterodorsal and lateroventral position, whether there is an inner circle of labial sensilla could not be ascertained, four cephalic setae close to anterior margin of amphid, in some specimens setae on ventral side are double, two circles of eight subcephalic setae each, first at level of amphid with sublateral setae bordering amphid, submedian setae in a slightly more anterior position, length variable within same specimen. Second circle 15-25 μm from anterior end. Amphids situated close to anterior end, spiral with 1.5 turns, indistinct in live animals and glycerin whole mounts, in SEM preparations conspicuous because of *corpus gelatum* filling fovea. Mouth opening in some specimens slightly protruding, forming a 'snout', buccal cavity minute, conical, pharynx with a cylindrical elongated corpus slightly thicker than isthmus, isthmus surrounded by nerve ring. Terminal bulb slightly wider than long, weakly muscular. No ventral gland. Gso in eight rows in cervical region, subcentral rows merging in posterior body region. Gso opening through the somatic setae.

Male

Monorchic, testis on right side of intestine; spicule cephalate, curved, gubernaculum simple, directed anteriodorsal, with small lateral flanges, proximal end with small hook. One pair of precloacal porids (thorn-like setae), 10 μm anterior to cloacal aperture, and three pairs of postcloacal porids, first of which situated at 33-46% of tail length, last at level where annulation ends. Porids 5-8 μm long. One symbiont-free male showing two pairs of long subventral setae in pharyngeal region, at 45 and 80% of pharynx length. Unfortunately these are obscured by bacterial coat in specimens used for drawings.

Female

Didelphic, amphidelphic, ovaries reflexed, position in relation to gut not determined unambiguously, at point of reflexion a *receptaculum seminis* filled with sperm. Ripe eggs *ca* 30 × 30 μm in size, one most distal from ovary up to 40 μm long. Always several (up to ten) eggs of similar size in each branch of uterus, apparently produced almost at same time and accumulating proximally to point of flexure of female gonad, which they seem to pass simultaneously *en route* to vulva. Long uteri leading to vagina, distal 50-60 μm of uterus branches consisting of large vesicular cells and never containing eggs. Vulva and vagina strongly cuticularised. Female genital system occupying up to two-thirds of body length.

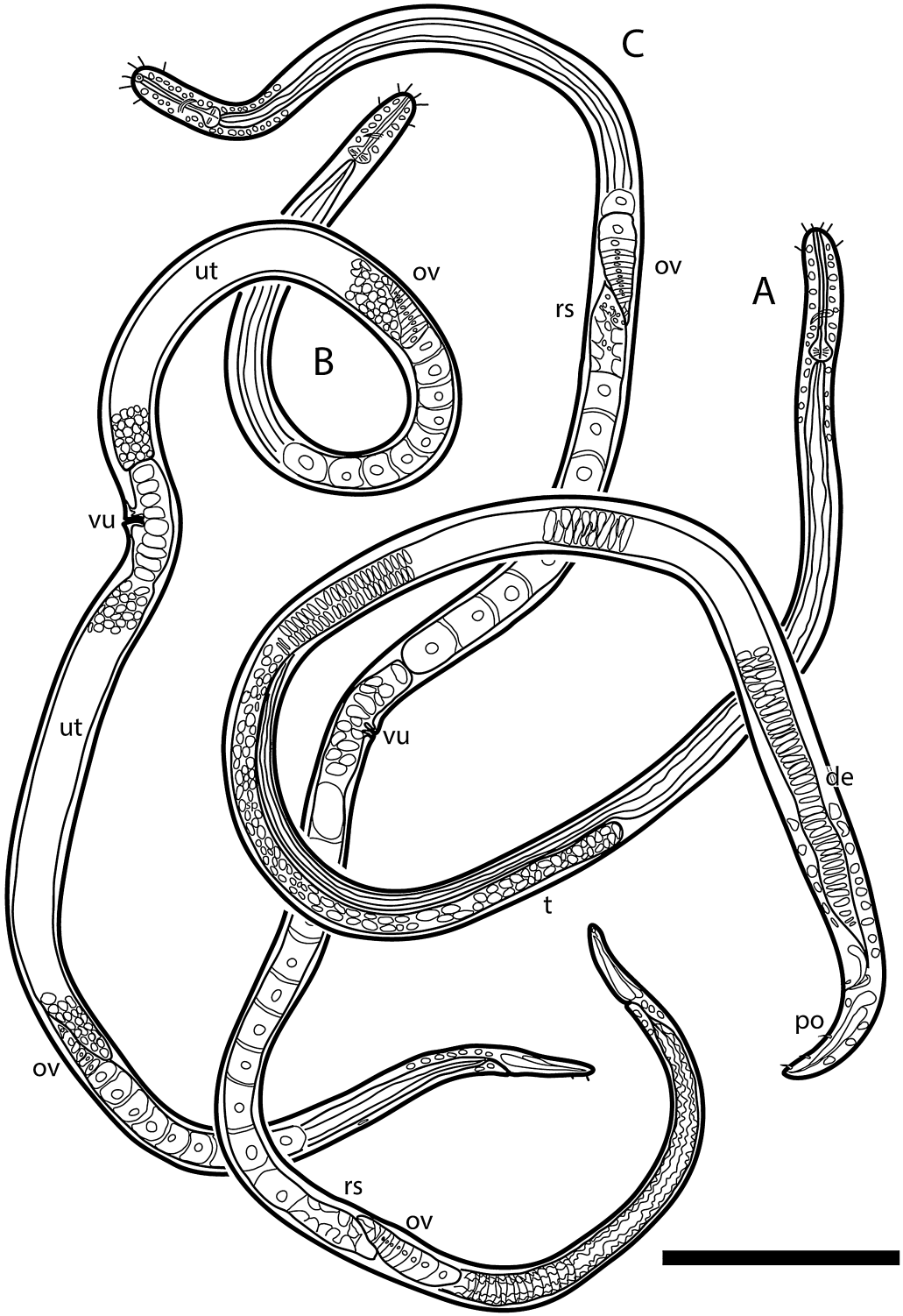

Fig. 2. *Eubostrichus fertilis* sp. n., entire view. A: Male, holotype. B: Female, paratype. C: Female, paratype. (Abbreviations: de = *ductus ejaculatorius*; ov = ovary; po = porid; rs = *receptaculum seminis*; t = testis; ut = uterus; vu = vulva.) (Scale bar = 200 μm.)

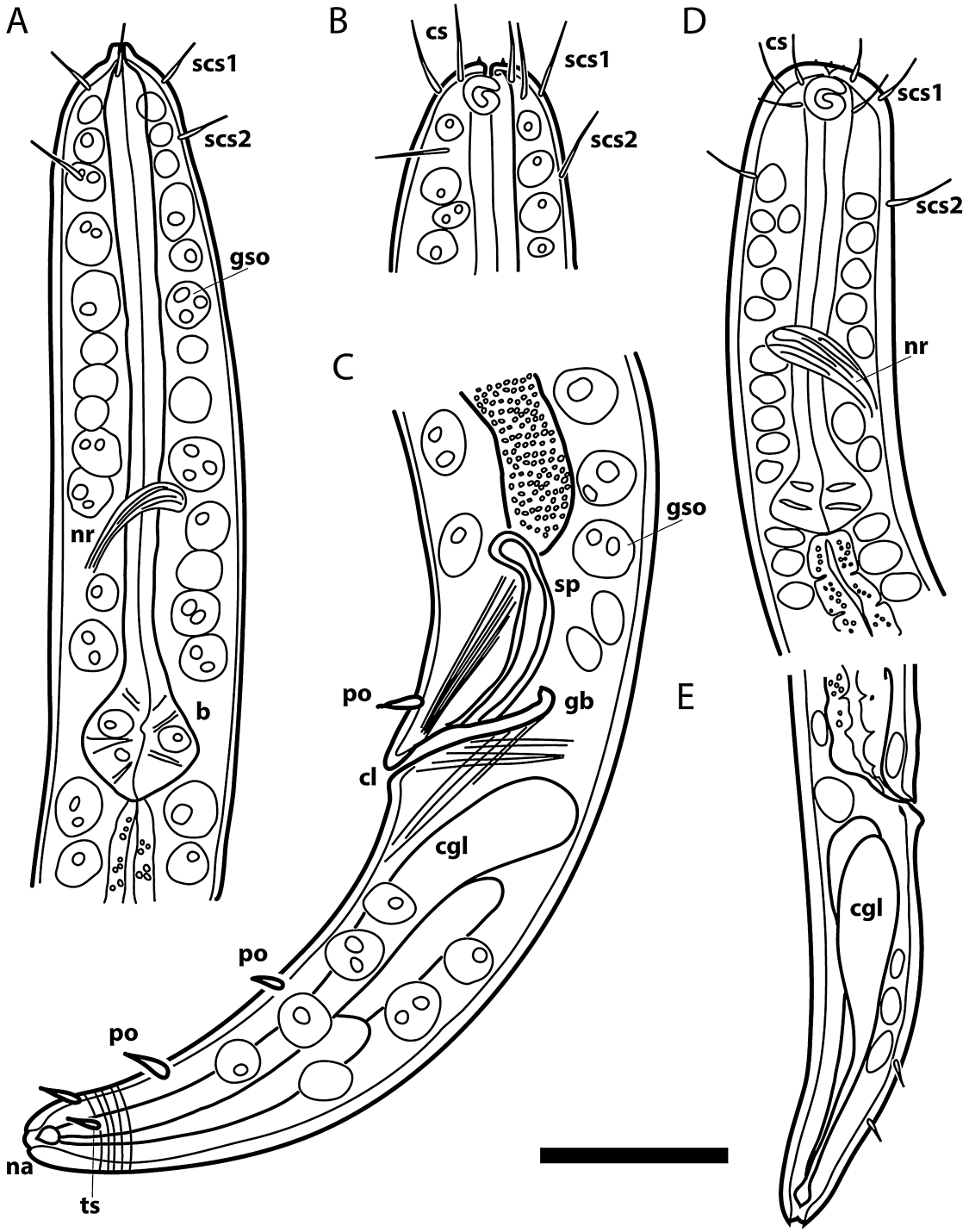

Fig. 3. *Eubostrichus fertilis* sp. n. A: Male holotype, anterior region; B: Male paratype, head; C: Male holotype, tail and spicular apparatus; D: Female paratype, anterior region; E: Female paratype, tail. (Abbreviations: b = *bulbus*; cgl = caudal gland; cl = cloacal aperture; cs = cephalic seta; gb = gubernaculum; gso = glandular sensory organ; na = non-annulated tail tip; nr = nerve ring; po = porid; scs1 = first circle of subcephalic setae; scs2 = second circle of subcephalic setae; sp = spiculum; ts = terminal seta.) (Scale bar = 30 μm.)

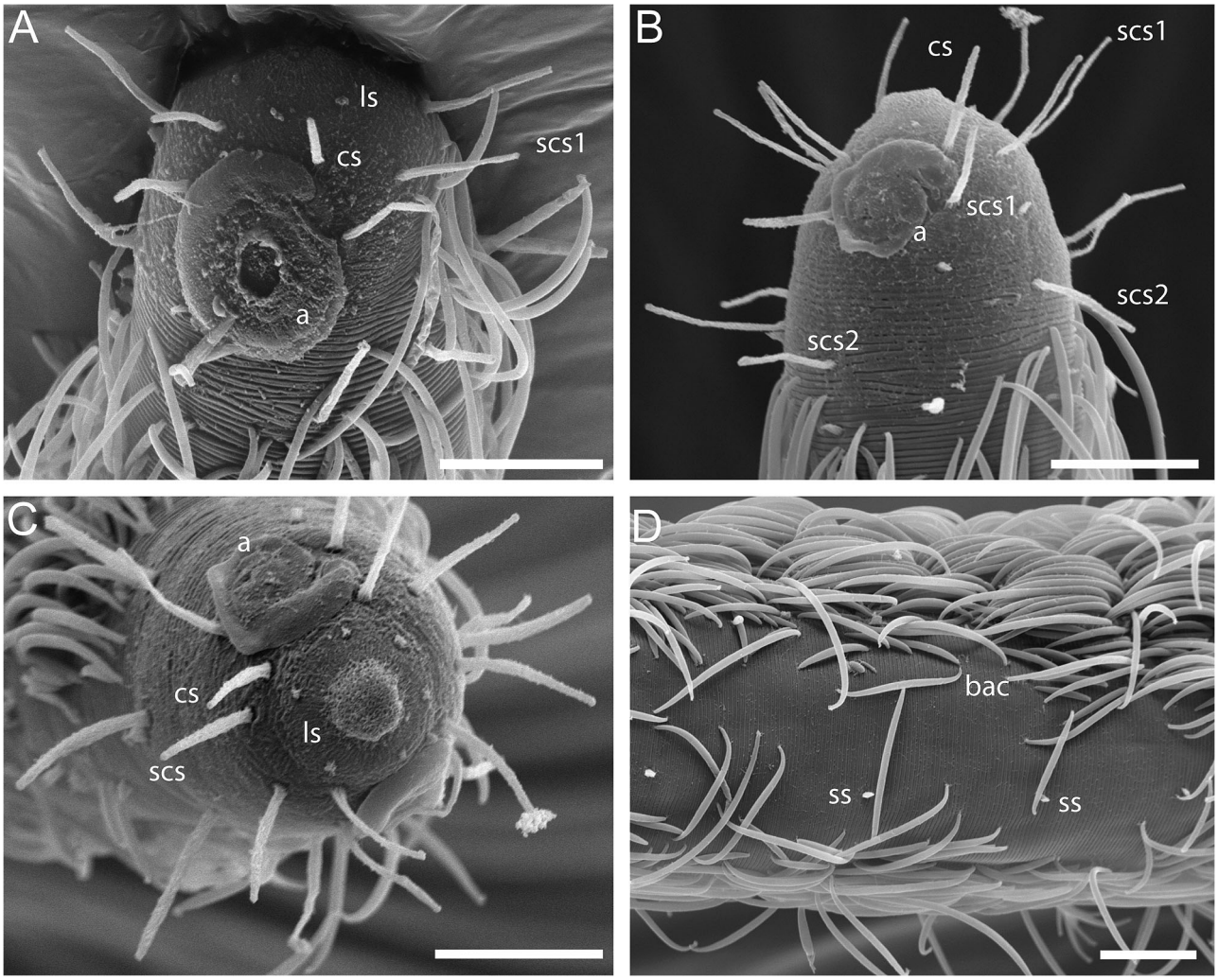

Fig. 4. *Eubostrichus fertilis* sp. n., SEM. A: Male, lateral view of head region; B: Female, lateral view of head region; C: Male, *en face* view of anterior end; D: Female, mid-body region, symbiotic bacteria partly removed. (Abbreviations: a = amphid; bac = bacteria; cs = cephalic seta; ls = labial sensilla; scs = subcephalic seta; scs1 = first circle of subcephalic setae; scs2 = second circle of subcephalic setae; ss = somatic seta.) (Scale bars = 10 μm.)

Type habitat and locality

Subtidal fine calcareous sand at depth of 1 m adjacent to the Fisheries Department Warden Station on Twin Cayes, Belize ('Fisheries Beach'; 16°49′25″N, 88°06′21″W).

Other habitat and locality

Also been found in intertidal and shallow subtidal sand north of the main channel entrance ('Candy's Trail'; 16°49′47.96″N, 88°6′29.67″W).

Type material

Holotype male, three paratype males and four paratype females (accession numbers: 1231525-1231532) deposited at the National Museum of Natural History, Washington, USA. Two female paratypes from the type locality and one female from the additional locality are in the collection of the first author (J.A.O.).

Diagnosis and relationships

Eubostrichus fertilis sp. n. is among the smallest and the stoutest *Eubostrichus* species described so far. Mature

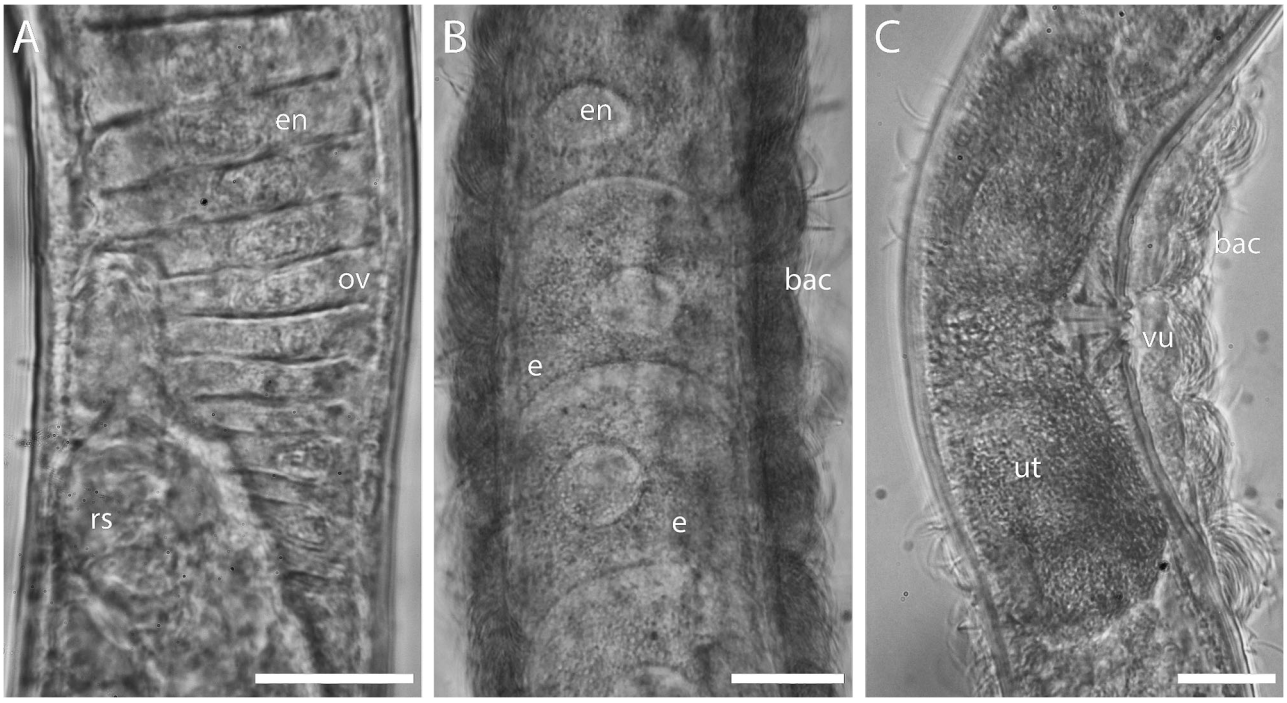

Fig. 5. *Eubostrichus fertilis* sp. n., LM. A: Anterior ovary of female paratype 2; B: Eggs in uterus of female paratype 2; C: Vulva and uterus of female paratype 1. (Abbreviations: bac = bacteria; e = egg; en = egg nucleus; ov = ovary; rs = *receptaculum seminis*; ut = uterus; vu = vulva.) (Scale bars = 20 μm.)

specimens vary considerably in body length, which ranges in males from 2.00 to 2.41 mm and in females from 1.87 to 3.03 mm. Long specimens tend to be more slender, with a values above 70, whereas short specimens are stouter (a = 36-70). Cephalic setae 6-11 μm long, amphids in male 8-10 μm (50-65% of corresponding diam. wide), in female 6-8 μm (38-50%), respectively. Tail short, 2.6-3.1 cloacal diam. long in male, 3.2-3.9 anal body diam. in female. Males with three pairs of thorn-like setae (porids) on ventral side of tail, the first pair at 33-48% of tail length. Female V = 51-57, both ovaries containing up to nine oocytes maturing at the same time and being simultaneously transferred to the uterus. Body covered by large (up to 45 μm long) crescent-shaped symbiotic bacteria arranged in a spiral pattern around the nematode's body.

The new species is similar to the two species previously described from the West Atlantic, *E. parasitiferus* Chitwood, 1936 and *E. hopperi* (Hopper & Cefalu, 1973) Muthumbi, Verschelde & Vincx, 1995. It differs from the former in the position of the vulva (V = 53-57 *vs* 43). Chitwood (1936) does not depict porids; however, the type specimen deposited in the USNM and material collected by one of the authors (J.A.O.) close to the type locality, including males, show that the first pair of postcloacal porids is at >50% of tail length. *Eubostrichus hopperi* has larger amphids, longer cephalic setae and a longer, more slender, tail (Table 1). The small species that Gerlach (1963) identified as *E. parasitiferus* has only two pairs of porids on the tail, the first of which is at 44% of the tail length. It should therefore be regarded as a separate species, for which we propose herein *E. gerlachi* nom. nov. (= *E. parasitiferus apud* Gerlach, 1963) *nec* Chitwood, 1936). The female individual that Gerlach (1964) reported from the Red Sea most probably also belongs to that species.

Molecular sequences

The sequences of the 18S rRNA genes of two individuals are available from GenBank and under accession numbers KF453617 and KF453618.

Bionomics

A remarkable feature in the biology of the new species is the high number of eggs that are presumably produced

Table 1. Morphometric data for *Eubostrichus fertilis* sp. n. and the two most similar species, *E. parasitiferus* Chitwood, 1936 and *E. hopperi* (Hopper & Cefalu, 1973) Muthumbi, Verschelde & Vincx, 1995. For *E. fertilis*, ranges are given for the male and female types and for all measured individuals, including three additional females from the collection of the first author (see *Other material*). All measurements are in μm.

Character	*E. fertilis* sp. n.				*E. parasitiferus*	*E. hopperi*
	Male		Female	All measured individuals		
	Holotype	Paratypes	Paratypes			
n	–	3	4	11	n.a.	n.a.
L	2010	2077-2412	2000-3032	1876-3032	2800-2920	2140-2680
a	36.5	65.5-69.0	40.0-79.7	36.5-79.7	75.0-100.0	78.0-90.0
b	17.0	24.0-27.0	20.0-34.4	17.0-34.4	30.0-33.0	20.0-25.4
c	21.1	27.7-28.4	24.7-33.6	21.1-33.6	26.0-30.0	25.0-29.0
V	n.a.	n.a.	51-57	51-57	43*	50-53
Max. diam.	55	30-37	38-50	30-55	n.d.	22-34
Pharynx length	118	77-100	76-100	70-118	82	85-108
Tail length	98	75-85	70-85	65-98	85	75-107
Nerve ring (% pharynx length)	59	58-59	56-59	56-59	61	65
Corresponding body diam. (cbd)	30	25-30	28-30	25-30	32	n.d.
Bulbus length/diam.	18/18	14-18/16-18	12-16/18-20	12-18/16-20	13/20**	13-16/18-21
Bulbus cbd	30	24-30	27-30	24-30	31	n.d.
Vulval cbd	n.a.	n.a.	40-45	40-45	n.d.	n.d.
Anal (cloacal) body diam.	32	25-33	18-28	18-33	35	21-24
Tail length: anal diam. male/female	3.1	2.6-3.1	3.2-3.9	2.6-3.1/3.2-3.9	2.4/n.d.	3.4-3.9/4.5-4.9*
Spicule length arc/chord	50/40	45-50/38-42	n.a.	45-50/38-42	50/40**	37-53/28-41
Gubernaculum length	30	23-29	n.a.	23-30	25**	19-22
Amphid males width/% cbd	10/50	8-10/60-65	n.a.	8-10/50-65	n.d.	13-15/73*
Amphid females width/% cbd	n.a.	n.a.	6-8/38-50	6-8/38-50	n.d.	6-8/n.d.*
Cephalic setae number/length	4/7-8	4/10-11	4/9-11	4/6-11	4/11	4/13-15*
Subcephalic setae 1 number/length	8/9	8/10-15	8/9-15	8/9-15	8/8**	8/12-16
Subcephalic setae 2 number/length	8/8-11	8/10-14	8/10-13	8/8-14	8/10**	8/10-16
Subventral elongated setae number of pairs/length	n.d.	2/12	n.a.	2/12	2/10**	2/8-10 and 6-8
Postanal thorn-like setae (porids) number of pairs	3	3	n.a.	3	3**	3
Position of first postanal porid pair (% tail length)	46	33-48	n.a.	33-48	61*,**	50

Abbreviations: n.d. = not determined; n.a. = not applicable.

* Diagnostically significant morphometric features.

** Data obtained from the holotype and a co-type of *E. parasitiferus* deposited in the US National Museum.

simultaneously in both ovaries. In all other *Eubostrichus* species the eggs are – when described – fewer in number (maximum of seven eggs per animal in *E. topiarius*) and much larger (up to 320 μm long in *E. hortulanus*). The high egg number in *E. fertilis* sp. n. can be interpreted as an indication of a high reproduction rate and presumably an r-strategist life history. This agrees well with the observation that *E. fertilis* sp. n. was only a rare member of the nematode community at the Candy's trail locality, which has been intensively sampled for many years, during which this habitat has remained nearly unchanged. The subtidal fine sand deposit at Fisheries beach, however, has been recently formed due to changes in coastal morphology associated with the development of the ranger station. Here the new species is abundant and conspicuous. It is also here where the smallest mature specimens

are found. A combination of early sexual maturity and high reproduction rate would make *E. fertilis* sp. n. an ideal colonising species.

Discussion

A diagnosis of the genus *Eubostrichus* was recently given by Tchesunov (2013) and has subsequently been modified by Leduc (2013) to accommodate *E. hortulanus* Leduc, 2013. All species so far described belong to the FA (faintly annulated) group *sensu* Urbancik *et al.* (1996a), in which the annulation involves <50% of the cuticle thickness. In *E. topiarius*, for example, the basal layer of the cuticle contributes 70% of the cuticle and is composed exclusively of longitudinal fibres. The annuli extend far anterior and enclose the posterior half of the amphid. The cuticle of the anterior end (cephalic cuticle) shows no reinforcement (Urbancik *et al.*, 1996b). The arrangement of the cephalic sensilla follows a consistent pattern. The first circle of six inner labial sensilla is hard to see and requires SEM. In *E. topiarius* they are represented by finger-like papillae within the mouth opening (Berger *et al.*, 1996) in mediolateral and subdorsal and subventral positions. Nipple-like papillae or small setae in the lateral and laterodorsal and lateroventral position constitute the second circle of six outer labial sensilla. A circle of four cephalic setae is generally situated close to the anterior margin of the amphid. A first circle of eight subcephalic setae occurs at the general level of the amphids, a second one a short distance posterior to the amphid. The somatic setae are the outlets of complex hypodermal glands (gso) that are characteristic for the Stilbonematinae and are 'porids' in the sense of Cobb (1925). Hopper & Cefalu (1973) used this term in the description of *E. dianeae*, pointing out that in males there are special thorn-like pairs developed close to the ventral line in the cloacal and tail region. In subsequent descriptions of new *Eubostrichus* species the term 'porid' has been used just for these. There is one pair situated a short distance anterior to the cloacal aperture and 2-3 pairs on the tail. In addition there are one or two pairs of terminal setae on the non-annulated tip of the tail. Pairs of subventral elongated setae in the pharyngeal region have been reported for *E. dianeae*, *E. hopperi* and *E. topiarius*, for the specimen of *E. africanus* described by Tchesunov (2013), and for the current new species. They are also visible in a co-type for *E. parasitiferus* deposited by Chitwood. It is unclear whether they occur in all *Eubostrichus* species since the symbionts make observation of setae very difficult.

According to the 18S rRNA gene sequences so far available, the genus *Eubostrichus* is phylogenetically well-supported (Fig. 6). It appears to be the sister group to all other genera of Stilbonematinae for which genomic data exist. The genus probably split off early in the evolution of the Stilbonematinae.

In addition to the morphological similarities and the genomic relationship, the species of *Eubostrichus* share the association with large bacteria, which are non-septate filaments with several to many nucleoids. The filaments are attached by one (*E. dianeae*) or both poles (all other

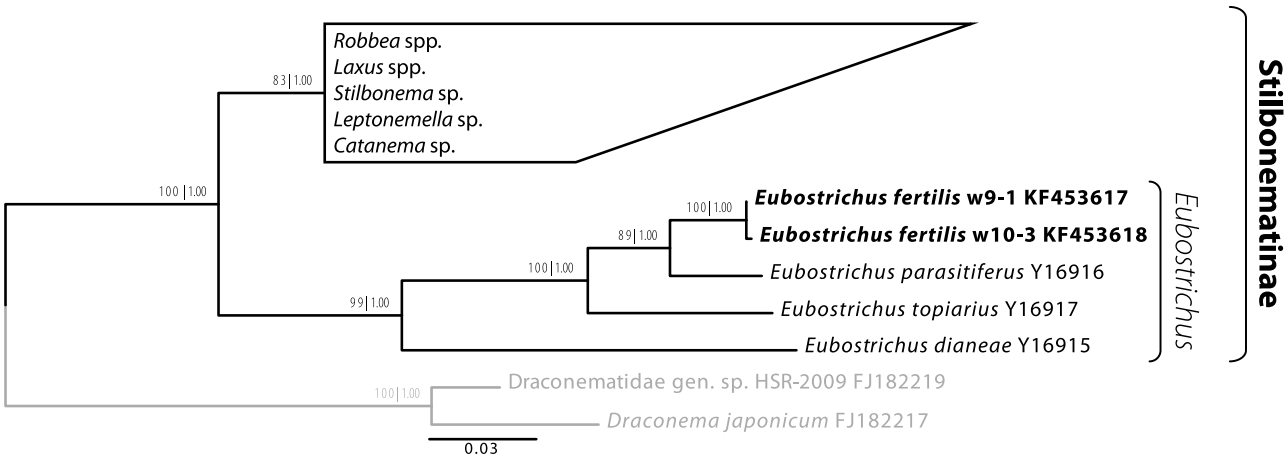

Fig. 6. Phylogenetic relationship of the *Eubostrichus* clade within the Stilbonematinae based on the 18S rRNA gene. The tree shown was calculated using maximum likelihood (PHYML) and node support is given as aLRT as well as Bayesian posterior probabilities. (Scale bar = 0.03 nucleotide substitutions per site.)

species) in a highly ordered fashion. The symbionts of *E. fertilis* sp. n. are the longest so far observed of the latter type. There are two other genera of Stilbonematinae, *Adelphos* Ott, 1997 and *Squanema* Gerlach, 1963 that bear similar symbionts. Whether they fall into the same clade as the *Eubostrichus* species could be explored with 18S rRNA data from members of the two genera.

Acknowledgements

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