A new species of *Carnoya* Gilson, 1898 (Nematoda: Ransomnematoidea: Carnoyidae) from Sumba Island, Indonesia

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Summary. *Carnoya filipjevi* sp. n. is described from the hind gut of tropical millipede *Salpidobolus* sp. collected in Indonesia and illustrated with the aid of SEM. The new species is characterised by the absence of a knob-like extension in the female cephalic region, the presence of a single cervical spine collar and the position of 12 copulatory papillae in the male and the presence of broad cuticular annulation and very long tail in both sexes. These features show that this species is related to Melanesian *Carnoya* Gilson, 1898 representatives. Partial SSU and LSU rDNA sequences were also obtained for the new species.

Key words: description, millipede, morphology, nematode, new species, SEM, taxonomy.

Among the Ransomnematoidea, the family Carnoyidae Travassos & Kloss, 1960 (Nematoda: Ransomnematoidea) contains the most species, all exclusively reported from the hind gut of diplopod millipedes. The family includes about 17 genera, whose members demonstrate a set of interesting morphological features, such as well-defined sexual dimorphism in cuticular armament and pharynx morphology, and biological adaptations, such as the development of copulatory plug and spermatophore (Adamson, 1987; Hunt, 2001a, b, 2002). Significant diversity of Carnoyidae representatives was discovered in Africa (Adamson & Anderson, 1985; Hunt, 1997a) and in the mainland and insular South-East Asia (Hunt, 1997b; Hunt & Sutherland, 1984; Malysheva & Pham Van Luc, 2012; Malysheva, Pham Van Luc & Spiridonov, 2012). The type genus *Carnoya* Gilson, 1898 was previously reported from South America (Brazil, Paraguay, Venezuela), Antilles (Cuba, Martinique, Guadalupe) and Australasian/Pacific region (Fiji, Papua New Guinea, New Britain, Sulawesi). A new species *Carnoya filipjevi* sp. n. from Sumba Island of Indonesia situated to the East of the Wallace's Line is described below with molecular data provided.

MATERIALS AND METHODS

Nematode material. Nematode specimens were recovered from a single female millipede specimen, collected by Dr D.V. Karelin in February 2011 at Sumba Island, Indonesia (9°41′ S, 119°59′ E). The millipede was identified by Dr S.I. Golovatch from A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences (IPEE RAS) as *Salpidobolus* Silvestri, 1897 (Diplopoda: Spirobolida: Rhinocricidae).

Morphological observation. The hind gut of the diplopod host was cut off and transferred to clean physiological saline. After gut dissection nematodes were picked and fixed in hot 4% formaldehyde and then processed to glycerol according to the method of Seinhorst (1959). Measurements and drawings were made with the aid of a *camera lucida*. Some nematodes were prepared for SEM by dehydration through a graded ethanol series and acetone and dried in a critical point drier. After coating with gold/palladium they were examined in a JEOL JSM-6380LA electron microscope.

Molecular profiles. Some specimens were kept at –18°C prior to DNA extraction. The DNA was extracted from all samples using methods detailed by Holterman *et al*. (2006). The worm-lysis solution was prepared immediately before DNA extraction containing 950 µl of a mixture of 2 ml of 1M NaCl, 2 ml of 1M Tris-HCl (pH 8) plus 5.5 ml of deionized water plus 10 µl of mercaptoethanol and 40 µl of proteinase K (20 mg ml^{-1}). Single nematodes were each transferred to 25 µl of sterile water and after addition of 25 µl of worm-lysis solution the tube was incubated at 65°C for 90 min.

The tubes with homogenate were then incubated at 99°C for 5 min to deactivate proteinase K and 0.8-1.2 µl of the obtained homogenate was used as a template for PCR reactions.

The PCR reactions were performed using Encyclo Plus PCR kit (Evrogen®, Russia) according to manufacturer's manual. Partial sequences of LSU (28S rDNA) and SSU (18S rDNA) were amplified. Primer pairs #391 (5'-AGCGGAGGAAAAGAAACTAA-3') and #501 (5'-TCGGAAGGAACCAGCTACTA-3') were used to amplify D2-D3 domains of LSU rDNA fragment (Nadler *et al*., 2006). PCR cycling parameters included primary denaturation at 94°C for 3 minutes followed by 34 cycles 94°C for 30 s, 52°C for 30 s and 72°C for 1 min, followed by post-amplification extension at 72°C for 7 min.

Two pairs of primers were used to amplify SSU rDNA. A pair of nematode-specific primers nem18SF (5'-CGCGAATRGCTCATTACAACA GC-3') and nem18SR (5'-GGGCGGTATCTGATC GCC-3') was used to amplify 5' portion of SSU rDNA (Floyd *et al.*, 2005). PCR cycling parameters included primary denaturation at 95°C for 5 min followed by 5 cycles of 94°C for 30 s, 47°C for 30 s and 72°C for 40 s and 35 cycles of 94°C for 25 s, 54°C for 30 s and 72°C for 40 s, followed by post-amplification extension at 72°C for 5 min. Another pair 24F (5'-AGRGGTGAAATYCGTGGACC-3') and Q39 (5'-TAATGATCCWTCYGCAGGTTCA CCTAC-3') was used to obtain the remaining 3' end of SSU rDNA (Blaxter *et al*., 1998). PCR cycling parameters included primary denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 60 s, 53°C for 90 s and 72°C for 90 s, followed by post-amplification extension at 72°C for 6 min.

The PCR reaction products were visualised in agarose gel and bands were excised for DNA extraction with Promega columns (Wizard® SV Gel and PCR Clean-Up System). Samples were directly sequenced using the same primers that were used for the primary PCR reactions.

The nematode sequences were deposited in GenBank NCBI as: JX946703 for D2-D3 LSU rDNA and JX982120 for SSU rDNA.

DESCRIPTION

Carnoya filipjevi sp. n. (Figs 1 & 2)

Measurements: See Table 1.

Adults. Cephalic extremity narrow, body widens gradually up to mid-level, then slowly narrows towards anal level passing into long subulate tail.

Female. Cephalic end comprise of one wide cuticular ring followed by 4-5 narrower ones. There are four cephalic papillae and two rounded amphids. Oral opening hexagonal in shape with three rounded cuticular plates easily discernible in anterior part of buccal cavity: one dorsal and two subventral. Upper surface of plates is smooth and slightly concave, while lower surface and outer edges are covered with cuticular spinelets. In cephalic region 17 rows of big cuticular spines are present, becoming larger and more widely spaced posterior to excretory pore. Spines flattened, 13-30 µm in length, with finally pointed or dissected distal tip. Broad lateral alae *ca* 35 µm high begins behind the spines level and continuous behind anal level becoming abruptly narrow. Body cuticle annulation is well defined with annuli about 17-19 µm wide and extending on lateral alae. Buccal cavity initially short, thick walled, about 19-22 µm in length with three small cuticular teeth located in basal part, then followed by long tubular section lined with striated cuticle. Pharynx initially very narrow with thin walls, then widening posteriorly, forming cylindrical, muscular corpus. Pharyngeal corpus 123 ± 7.6 (115-130) µm in length is followed by narrow isthmus. Basal bulb 80-85 µm in diameter with strong cuticular valves.

Nerve ring 75 ± 5 (70-80) µm from anterior end, located at level of junction of buccal cavity and corpus. Excretory pore 182 ± 5.2 (167-188) µm from anterior extremity leads into longitudinal channel, consisting of some conjoint duct cells, whose nucleus could be easily observed. Longitudinal channel falls into transverse sinus situated ventrally and joining two longitudinal ducts becoming indistinct in hypodermis from left and right sides of the body. Intestine straight, slightly widened anteriorly.

Vulva transversally orientated, oval, located 683 ± 100 (570-760) from anterior and surrounded by undulate cuticular ridges. Vagina muscular, posteriorly directed, leading into common uterus. Two ovaries anteriorly directed extend until basal bulb level and beyond, surrounding its junction with isthmus. Eggs 167 ± 5.2 (160-172) × 80 ± 3 (77-83) µm in size, with thin flexible shell. Tail initially conical, turning into long subulate tip.

Male. Cephalic extremity narrow, cylindrical, comprised of three narrow cuticular rings followed by four wide ones prior to cervical collar. Cervical collar represent a wide cuticular ring fringed with 22-24 flattened cuticular spines. Oral opening triangular in shape with one dorsal and two subventral lips. Amphidial openings rounded, four cephalic papillae present. Broad lateral alae start three cuticular rings behind the collar level becoming

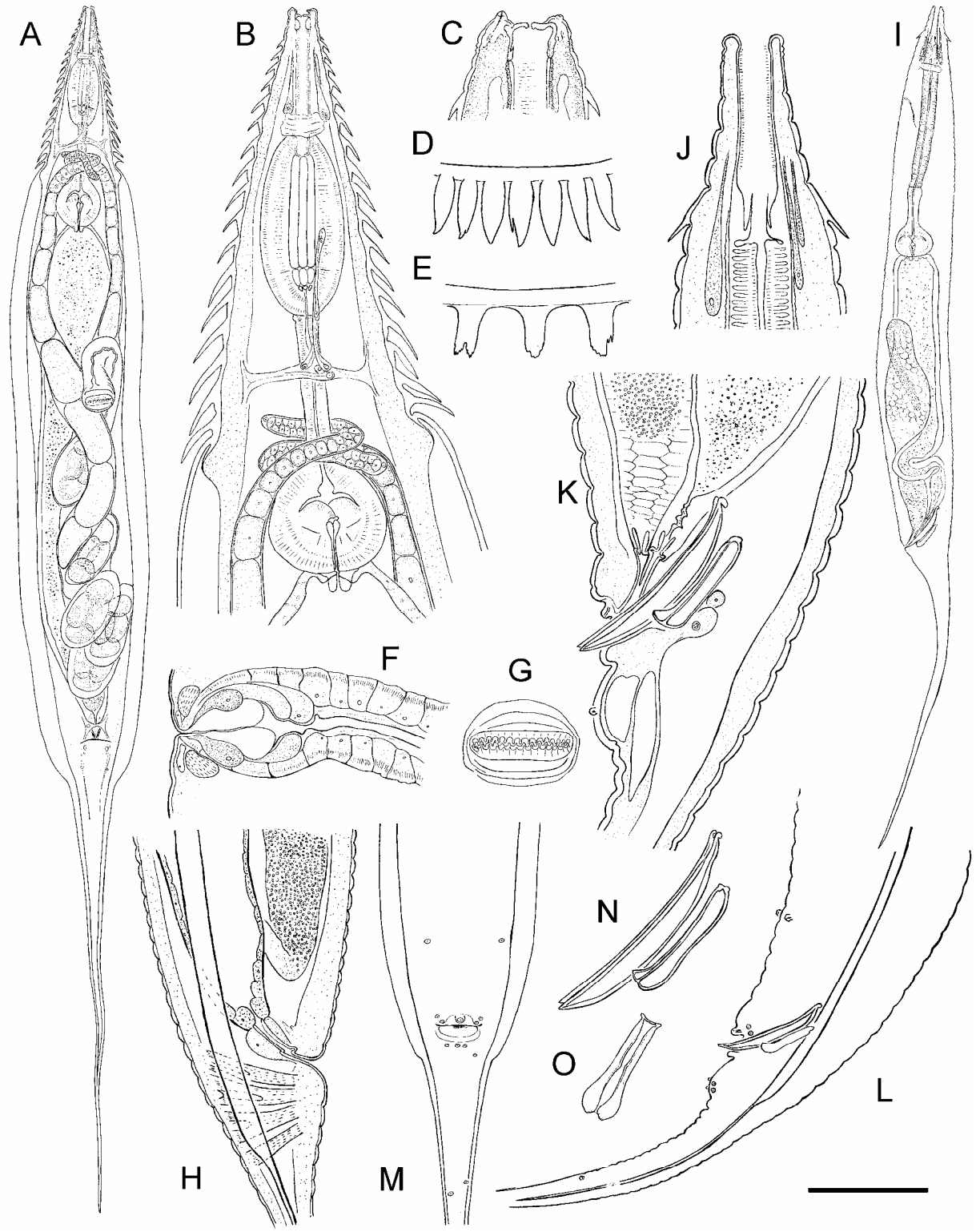

Fig. 1. *Carnoya filipjevi* sp. n., female (A-H). A: total view; B: optical section through anterior end, ventral view; C: optical section through buccal cavity, lateral view; D & E: spines of cervical region; F: superficial structures of vulva region, lateral view; G: vulva, ventral view; H: anal region, lateral view. Male (I-O). I: total view; J: optical section through buccal cavity, lateral view; K: posterior end, ventral view; L & M: cloacal region, lateral and ventral view, respectively; N & O: spicules and gubernaculum. (Scale bars for A = 200 µm, B = 80 µm, C, F, G, J, N, O = 40 µm, D & E = 30 µm, H = 100 µm, I = 250 µm, K = 65 µm, L = 85 µm, M = 150 µm).

Table 1. *Carnoya filipjevi* sp. n. Morphometric data in the form: mean ± s.d. (range). All linear measurements are in µm.

Character	Females		Males
	Holotype	Paratypes	Paratypes
n	–	3	6
L	2410	2185 ± 134 (1990-2290)	1708 ± 57 (1620-1770)
L′	1480	1377 ± 98 (1230-1430)	1086 ± 72.6 (1000-1171)
a	12	10 ± 0.6 (10-11.3)	13 ± 1.3 (11.3-14.4)
b	5.8	5.5 ± 0.3 (5.2-5.9)	3.55 ± 0.2 (3.4-3.8)
c	2.6	2.7 ± 0.1 (2.6-2.8)	3 ± 0.2 (2.4-3)
c′	8.5	8 ± 0.7 (7-8.7)	7.5 ± 0.4 (7-8)
V	30	31 ± 2.4 (29-34)	–
V′	48.6	50 ± 3 (46.3-53)	–
Total pharynx length	415	395 ± 24 (370-420)	480 ± 23 (450-510)
Max. body diam.	200	205 ± 6 (200-210)	133 ± 12 (120-150)
Anal/cloacal body diam.	110	100 ± 10 (90-110)	83 ± 4.2 (80-90)
Spicules length (arc)	–	–	82 ± 2.8 (80-87)
Gubernaculum length	–	–	47 ± 1.9 (45-50)
Tail length	930	805 ± 48 (760-870)	621 ± 60 (560-730)

Table legend. n: number of specimens examined; L: total body length (head to tail tip); L′: body length from head to anal/ cloacal aperture; a: total body length divided by maximum body diameter; b: total body length divided by pharyngeal length (the pharynx is defined as head end to the pharyngo-intestinal junction); c: total body length divided by tail length; c′: tail length divided by body diameter at the anal/ cloacal aperture; V: position of vulva from anterior end expressed as percentage of body length; V′: position of vulva from anterior end expressed as percentage of distance from head to anal aperture.

abruptly narrower posterior to cloaca level. Body cuticle with fine annulation extending on lateral alae. Buccal cavity 67-70 µm long, consisting of three parts: short anterior followed by tubular medium part lined with striated cuticle and the most wide posterior one, surrounded with pharyngeal tissues with one dorsal and two subventral cuticular processes at its base. Pharyngeal corpus long, fusiform, gradually narrowing before junction with well defined isthmus. Basal bulb 80-85 µm in diameter. Nerve ring 115 ± 3.1 (112-120) µm from anterior extremity, surrounding pharyngeal corpus just posterior to its junction with buccal cavity. Excretory pore 159 ± 14 (150-180) µm from anterior end. Excretory duct is organised similarly as in females. Flexure of testis situated 640 ± 55 (550-700) µm posterior to basal bulb. Spicules paired, closely adpressed, equal in size and shape. Spicules heads dorsally curved. Gubernaculum shovel-like, with strongly curved lateral edges and lacking a dorsal aperture. Twelve genital papillae present: anteriormost pair ventro-sublateral, two precloacal pairs situated ventrally in tandem on both sides of the cuticularised median duct, two subventral pairs posterior to cloaca and one small pair present on the subulate tail appendage. Two anteriormost postcloacal pairs can be arranged as: two groups of two papillae, of which one (usually left) group is situated more posterior; transverse row of four papillae present in one line or three papillae situated in one line with one strongly displaced posteriorly. Tail initially conical, gradually narrowing in long subulate distal tip.

Type host and locality. Diplopod *Salpidobolus* Silvestri, 1897 (Spirobolida: Rhinocricidae), collected at Sumba Island, Indonesia (9°41′ S, 119°59′ E).

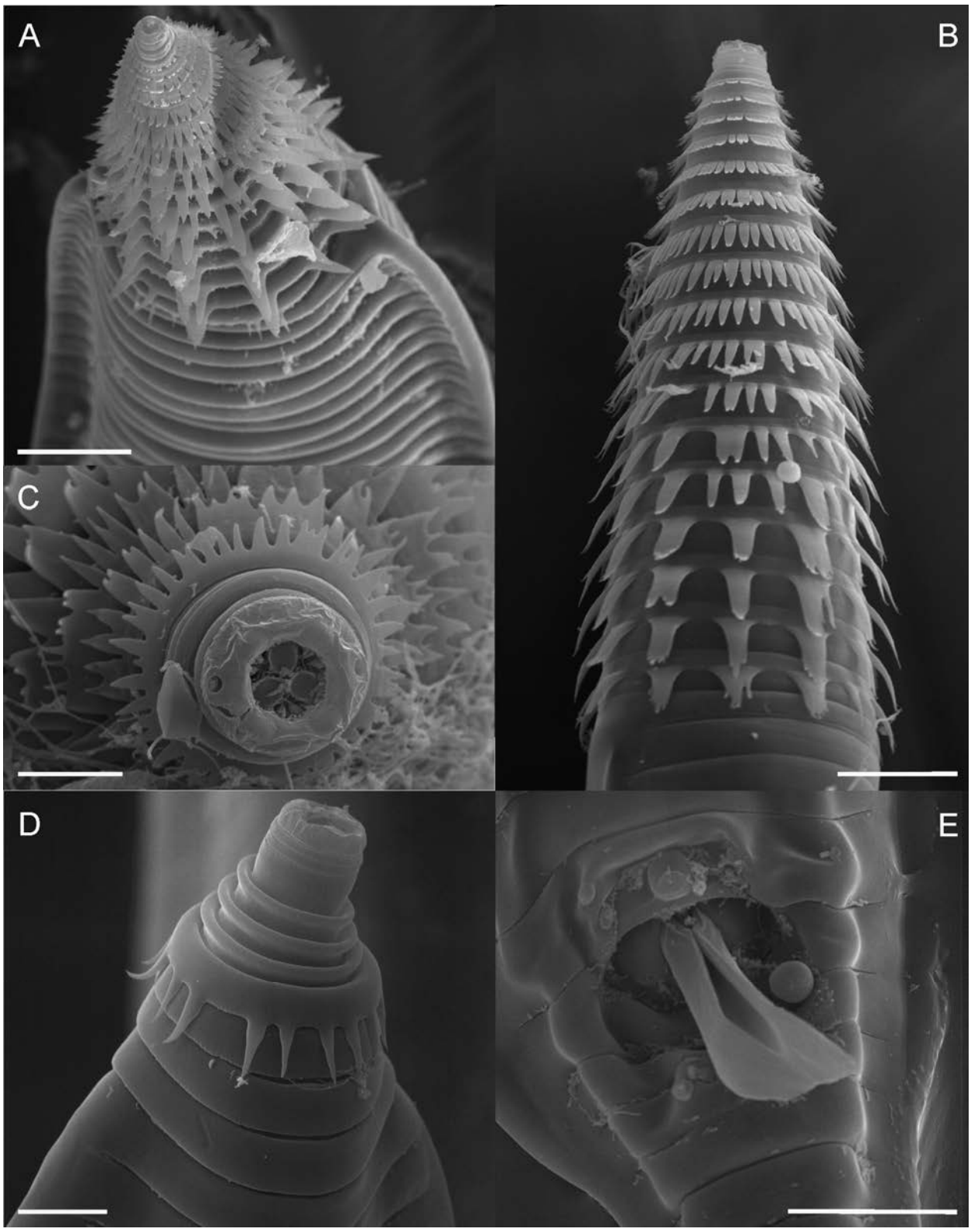

Fig. 2. SEM micrographs of *Carnoya filipjevi* sp. n. A & B: female anterior end, lateral view; C: female head end, apical view; D: male anterior end, ventrally; E: male cloaca region with gubernaculum protruding, ventral view. (Scale bars are 50, 30, 10, 15 and 30 μm, respectively.

Type material. Holotype female № 1253 and paratype male № 1254 of *C. filipjevi* sp. n. are deposited in the collection of the Centre of Parasitology, Institute of Ecology and Evolution, Russian Academy of Sciences.

Etymology. The species is named after Ivan Nikolaevich Filipjev (1889-1940) the outstanding Russian researcher, zoologist, who made a significant contribution to the study and systematics of Nematoda including its free-living and parasitic representatives.

Differential diagnosis. *Carnoya filipjevi* sp. n. is characterised by the organisation of female cephalic end; the armament of the male cephalic region and the position of copulatory papillae; the presence of broad cuticular alae and very long, subulate tail in both sexes. The presence of broad cuticular annulation and very long tail in both sexes make *C. filipjevi* sp. n. mainly close to the following Melanesian species: *C. fimbriata* Hunt & Sutherland, 1984, *C. perbella* Hunt & Sutherland, 1984, *C. strobilina* Hunt & Sutherland, 1984, *C. wallacei* Hunt, 1997, *C. caputbulla* Hunt & Moore, 1998, *C. posterovulva* Hunt & Moore, 1998, *C. janiceae* Hunt & Moore, 1998 and *C. vitiensis* Gilson, 1898.

Carnoya filipjevi sp. n. distinguishes from *C. vitiensis* by markedly longer tail length in both sexes and by the presence of one instead of two spine collars in males. Females of *C. filipjevi* sp. n. can be easily differentiated from females of *C. strobilina*, *C. caputbulla* and *C. posterovulva* by the absence *vs* presence of a knob-like expansion of the cephalic end, number of cuticular spine rows (17 *vs* 11-13, 12 and 11, respectively) and shape of the lateral alae when merging along the body (sharp in *C. filipjevi* sp. n. and slowly tapering in the rest species). In addition, males of *C. filipjevi* sp. n. possess well developed spine collar and 12 copulatory papillae while males of *C. strobilina* and *C. caputbulla* lack the spine collar and have only 10 copulatory papillae. By the organisation of cephalic region, *C. filipjevi* sp. n. is closer to *C. wallacei* and *C. janiceae*, but differ from both species by greater number of spine rows in females (17 *vs* 13 and 12-13, respectively), presence *vs* absence of cervical collar and number of copulatory papillae (12 *vs* 10) in males. Furthermore, in *C. filipjevi* sp. n. all spine rows are complete, whereas in *C. wallacei* and *C. janiceae* the most posterior spine row is incomplete and interrupted by the alae. *Carnoya filipjevi* sp. n. is close to *C. fimbriata* and *C. perbella* by number of spine rows in female cervical region, shape of alae posterior end in males (for both species) and females (only for *C. perbella*), and number of copulatory papillae, but can be differentiated by shorter female tail (805 (760-870) µm *vs* 1477 (1310-1575) µm and 1377 (1274-1469) µm, respectively) and shorter spicule (82 (80-87) µm *vs* 95, 100 µm and 141 (129-149) µm, respectively) and gubernaculum length (47 (45-50) µm *vs* 58, 64 µm and 98 (89-108) µm, respectively).

Molecular characterisation. The sequences obtained for *C. filipjevi* sp. n. are the first ones for the representative of the genus. The only available sequences for Carnoyidae that present in the GenBank NCBI belong to two species of *Cattiena* Hunt & Spiridonov, 2001, two specimens of *Brumptaemilius justini* Adamson & Anderson, 1984 and *Insulanema longispiculum* Malysheva, Luc & Spiridonov, 2012. The intergeneric level of differences found for SSU rDNA sequences is 32-43 bp (for 1630 bp long alignment). Nucleotide differences between *C. filipjevi* sp. n. and the aforementioned Carnoyidae representatives for D2-D3 LSU rDNA sequences is at the level of 124-133 bp (for 440 bp long alignment), when the intrageneric difference between two species of the *Cattiena* genus is equal to 5 bp. Unfortunately, the absence of molecular data for other *Carnoya* species does not enable the use of the obtained data for the differentiation of the new species, although the pronounced nucleotide differences with other Carnoyidae are obvious.

DISCUSSION

Common morphological characters, such as the presence of a very long tail, broad cuticular annulation and development of lateral alae in both sexes demonstrate the evident relationship of *C. filipjevi* sp. n. with other representatives of the genus from the Australasian/Pacific region. At the same time the lack of smooth, broad, knob-like annule at the cephalic end show affinities with American species (Hunt & Moore, 1998). The fine morphology of female buccal cavity armature has not been thoroughly studied yet in a range of *Carnoya* species, but the rounded concave cuticular plates found in *C. filipjevi* sp. n. are almost identical to those of *Carnoya perbella* García & Morfee, 2014. The variability of anteriormost postcloacal papillae arrangement in males found in *C. filipjevi* sp. n. was also mentioned earlier for *C. janiceae* (Hunt & Moore, 1998), although lack of one of the papillae was not observed in *C. filipjevi* sp. n. It is worth noting that the arrangement of postcloacal papillae as two trios was mentioned as being usual for two Cuban species – *C. perbella* and *Carnoya guantanamera* Spiridonov, 1989 (García & Morfee, 2014; Spiridonov, 1989).

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S.V. Malysheva. Новый вид *Carnoya* Gilson, 1898 (Nematoda: Ransomnematoidea: Carnoyidae) с острова Сумба, Индонезия.

Резюме. Приводится морфологическое описание нового вида *Carnoya filipjevi* sp. n. из многоножек *Salpidobolus* Silvestri, 1897 (Spirobolida: Rhinocricidae). Вид характеризуется отсутствием утолщенного кутикулярного кольца на головном конце самок, присутствием одного воротника кутикулярных шипиков в шейной области, а также характером расположения копулятивных папилл у самцов и наличием широкой кутикулярной кольчатости и длинного хвостового отростка у взрослых особей обоих полов, что сближает данный вид с меланезийскими видами рода. Особенностью вида является непостоянство расположения двух пар постклоакальных папилл относительно друг друга.