# Morphological and molecular characterisation of *Carnoya philippinensis* sp. n. (Nematoda: Carnoyidae) from Mindanao Island, Philippines

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**Summary.** The nematode *Carnoya philippinensis* sp. n. is described from spirobolid millipedes (Diplopoda: Spirobolida: Rhinocricidae) from Mindanao Island, Philippines. The presence of a broad cuticular annulation and a long subulate tail in both sexes make *C. philippinensis* sp. n. closest to Australasian and Pacific representatives of the genus. The new species is similar to *Carnoya fimbriata* Hunt & Sutherland, 1984 and *Carnoya perbella* Hunt & Sutherland, 1984 in body/tail length ratio in females and the structure of armature of the female anterior end, but can be distinguished by the shape of the posterior extremity of a lateral ala and the arrangement of the papillae in males. Molecular data (partial LSU and SSU rDNA sequences) were obtained for the representatives of the described species collected from two different collection sites on the Mindanao Island.

**Key words:** description, LSU rDNA, morphology, nematode, SEM, Spirobolida, SSU rDNA, taxonomy.

The type genus of the Carnoyidae family, *Carnoya* Gilson, 1898 (Nematoda: Ransomnematoidea) was first discovered in the intestine of the millipede identified by the author as '*Julus solomonensis*' collected in Viti Levu Island, Fiji (Gilson, 1898). Unfortunately, the authority of the host species was not given and by the date, no species can be found with the specific epithet '*solomonensis*' neither in *Julus* Linnaeus, 1758 nor in *Iulus* Linnaeus, 1758 genera (http://www.biologie.uni-ulm.de/). Moreover, both generic names were used in the body of the manuscript. Currently, *Carnoya* includes about 23 species mostly reported from diplopods of the family Rhinocricidae Brölemann, 1913 (Diplopoda: Spirobolida) from South America (Brazil, Paraguay and Venezuela), Antilles (Martinique, Guadeloupe and Cuba), Australasia and Pacific regions (Fiji, Papua New Guinea, New Britain, Sulawesi and Indonesia) (Artigas, 1926; Dollfus, 1952; Adamson, 1984, 1985; Hunt & Sutherland, 1984; Adamson & Van Waerebeke, 1985; Spiridonov, 1989; Hunt, 1997; Hunt & Moore, 1998; Malysheva, 2014 *etc.*). The new species *Carnoya philippinensis* sp. n. from Mindanao Island is described herein with the molecular data provided.

## MATERIALS AND METHODS

**Isolation and examination of nematodes.** Nematodes were recovered from the hind gut of two millipedes collected by S.E. Spiridonov and A.B. Mohagan in woodland near Tinago Falls, Lanao del Norte Province (Northern Mindanao) and Musuan Peak near Central Mindanao University, Bukidnon Province (Central Mindanao), Philippines. Millipedes were identified by Dr S.I. Golovatch as representatives of Rhinocricidae Brölemann, 1913 (Myriapoda: Diplopoda: Spirobolida), a more precise definition being hampered by the fact that only the female sex was recovered. Nematodes were fixed in hot (60-70ºC) 4% formaldehyde and processed to glycerol according to Seinhorst (1959). Some specimens were frozen for further molecular analysis. Measurements and drawings were made with the aid of microscope drawing attachment for Eclipse E200 (Nikon, Tokyo, Japan). For scanning electron microscopy studies, several nematodes were dehydrated through a graded ethanol series and acetone and then dried to a critical point. Specimens were coated with gold/palladium and examined with a JSM-6380LA (JEOL, Tokyo, Japan) and TM3000 (Hitachi, Tokyo, Japan) electron microscopes.

**DNA extraction, amplification and sequencing.** Nematode specimens were kept at –18ºC prior to DNA extraction. The DNA was extracted according to Holterman *et al.* (2006). The worm-lysis solution was prepared immediately before DNA extraction containing 950 µl of 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 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 homogenate was used as PCR template.

PCR reactions were performed using Encyclo Plus PCR kit (Evrogen®, Moscow, Russia) according to the manufacturer's manual. Primer pairs LSU391 (5'-AGCGGAGGAAAAGAAACT AA-3') and LSU501 (5'-TCGGAAGGAACCAGC TACTA-3') were used to amplify D2-D3 expansion segment of LSU rDNA fragment (Nadler *et al.*, 2006). PCR cycling parameters included primary denaturation at 94°C for 3 min 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'-AGRGGTGAAA TYCGTGGACC-3') and Q39 (5'-TAATGATCCWTCYGCAGGTTCAC CTAC-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.

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

**Sequence alignment comparison.** Nematode sequences for Tinago Falls (type) population and Musuan population specimens were deposited in GenBank NCBI as KT957945 and KT957944 for D2-D3 LSU rDNA and as KT957946 and KT957947 for SSU rDNA, respectively. Sequence alignments were generated using Clustal X (Thompson *et al.*, 1997) under default values for gap opening and gap extension penalties. PAUP* 4.0b10 (Swofford, 1998) was used to calculate the level of nucleotide differences.

## DESCRIPTION

### *Carnoya philippinensis* sp. n. (Figs 1 & 2)

**Measurements.** See Table 1.

Marked sexual dimorphism in the organisation of the anterior end present.

**Female.** Cephalic end blunt, not swollen, comprising wide cuticular ring followed by three narrower ones. Four cephalic papillae and two rounded amphids present. Oral opening hexagonal in shape with three rounded cuticular plates protruding into its lumen: one dorsal and two subventral. Each plate with smooth upper surface, while lower surface covered with minute spines. Inner edges of plates slightly curved outwards. In cephalic region, 19 transverse rows of flattened cuticular spines present, of which posteriormost interrupted by lateral alae. Spines 25-35 µm in length, with pointed distal tip. Lateral alae 35 µm high appearing immediately behind the 18$^{th}$ row of spines and extending just posterior to anal level. Posterior extremities of alae semi-circular. Cuticle annulation broad, extending onto lateral alae surface. Buccal cavity thick walled, 20-22 µm in length with three small cuticular teeth at base. Pharynx initially long, thin-walled with finely striated cuticle, then widens posteriorly forming well defined cylindrical corpus. Pharyngeal corpus 124 ± 8.8 (110-140) µm in length clearly demarcated from long, flexible isthmus. Basal bulb rounded, 80-90 µm in diameter with cardial lobes protruding into gut lumen. Nerve ring and excretory pore situated 72 ± 10 (55-85) µm and 153 ± 6.5 (145-161) µm from anterior extremity, respectively. Vulva transversal, located 737 ± 128 (630-900) µm from anterior and lined with very thick cuticle forming undulate ridges around its opening. Vagina posteriorly directed, thick-walled, leading into common uterus. Two ovaries anteriorly directed, reaching basal bulb level and entwining isthmus. Eggs 158 ± 5.3 (152-162) × 74 ± 1.2 (73-75) µm in size, with thin and smooth shell. Tail very long, subulate.

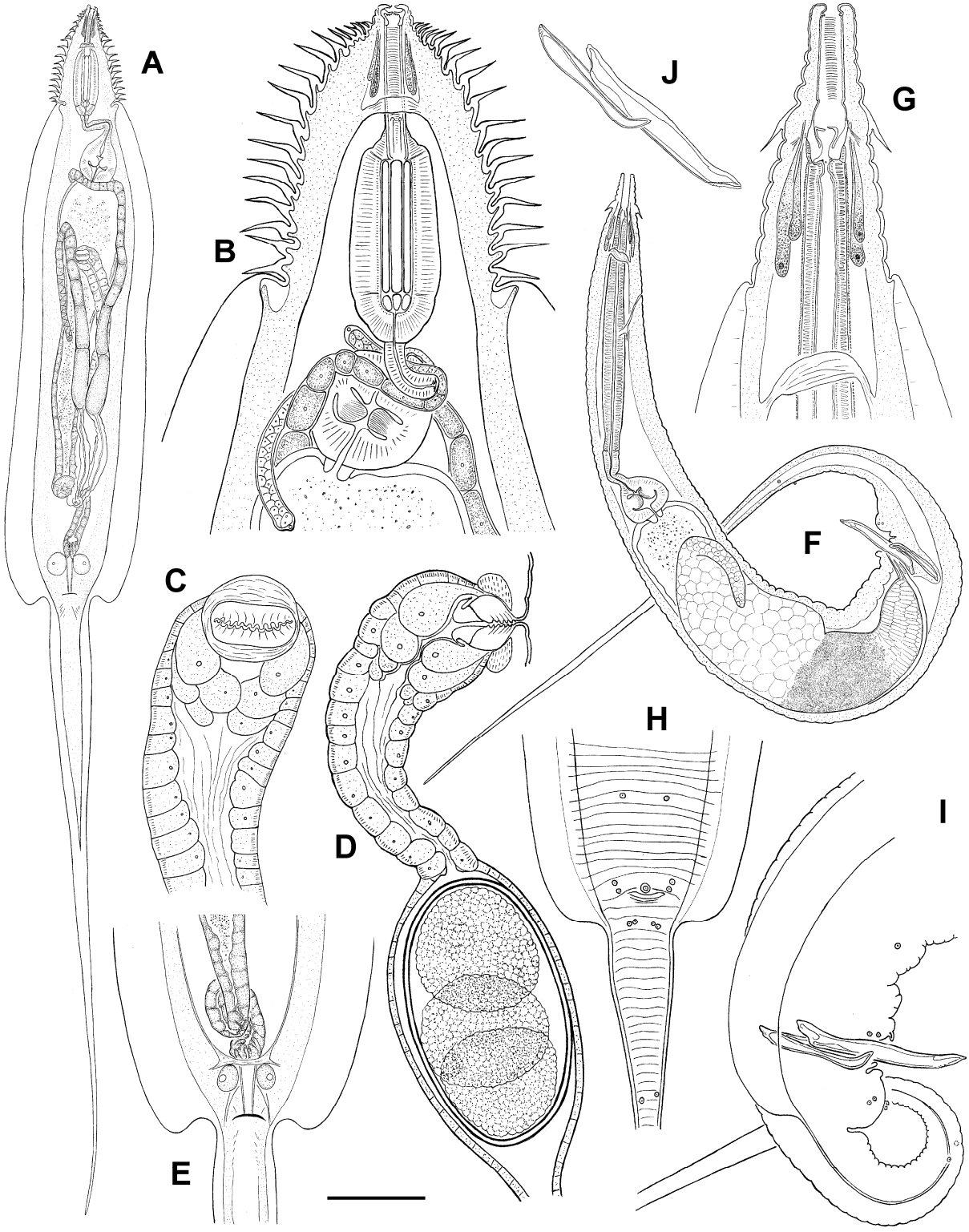


**Fig. 1.** *Carnoya philippinensis* sp. n. Female (A-E): A: total view; B: optical section through anterior end, ventral view; C, D: vulva and anterior end of genital tract, ventral and lateral view, respectively; E: anal region, ventral view. Male (F-J): F: total view; G: optical section through anterior end, ventral view; H, I: cloacal region, ventral and lateral view, respectively; J: spicule and gubernaculum. (Scale bar for A = 360 µm, B = 75 µm, C, D = 50 µm, E, H, I = 160 µm, F = 150 µm, G = 40 µm, J = 60 µm).

**Male.** Cephalic end narrow, comprising three narrow and four wide cuticular rings followed by cervical collar. Cervical collar bearing 22-24 cuticular spines protruding from common base as wide as spine length. Oral opening triangular with one dorsal and two subventral lips. Amphidial openings rounded, four cephalic papillae present. Lateral alae 25-30 µm high, appearing in 6$^{th}$ cuticular ring behind the cervical collar and extending posterior to cloacal level similarly to female. Cuticle annulation broad, extending onto lateral alae surface. Buccal cavity 57-62 µm long, divided into three parts: very short anterior part delimited by small cuticular ridges, long medium part with finely striated cuticle and expanded posterior one, embedded in pharyngeal tissues with one dorsal and two subventral cuticular teeth. Pharyngeal corpus fusiform, gradually narrowing before junction with flexible isthmus. Basal bulb pear-like, 65-75 µm in diameter. Nerve ring and excretory pore situated 115 ± 12.5 (105-145) µm and 239 ± 9.1 (228-250) µm from anterior extremity, respectively. Spicules paired, equal in size and morphology. Spicule distal tips bearing four shallow creases. Gubernaculum long, shovel-like, with ventrally curved distal tip. Twelve genital papillae present: anteriormost pair in ventro-sublateral position, two precloacal pairs situated ventrally in tandem just anterior to cloacal aperture, two subventral pairs posterior to cloaca and one small pair on subulate tail appendage. Tail long, subulate, slowly attenuating into a final point.

**Type host and locality.** Female of spirobolid millipede (Spirobolida: Rhinocricidae) collected in November 2013 by S.E. Spiridonov near Tinago Falls, Lanao del Norte Province, Northern Mindanao, Philippines. Coordinates: 8°14' N and 125°02' E, about 200 m above sea level (a.s.l.). Also, representatives of the described species were found in the same millipede host collected by A.B. Mohagan in the woodland near Musuan Peak of Bukidnon Province, Central Mindanao. Coordinates: 7°53' N and 125°04' E, about 400 m a.s.l.

**Type specimens.** Holotype female № 84YT1 and paratype male № 84YT2 of *C. philippinensis* sp. n. from the type locality and two voucher specimens from Musuan population № 85YT1 and № 85YT2 are deposited in the collection of the Musée National d'Histoire Naturelle, Paris, France.

**Etymology.** The species name is proposed to designate this species as the first representative of the genus described from the Philippines.

**Differential diagnosis.** *Carnoya philippinensis* sp. n. is characterised by the cuticular armament of the male and female anterior end; by the arrangement of copulatory papilla in males; by the presence of broad lateral alae and a long subulate tail in both sexes.

The presence of such morphological features as a broad cuticular annulation and a long tail in both sexes immediately distinguishes the described species from all South American and Caribbean species. The absence of a knob-like expansion of the female head makes *C. philippinensis* sp. n. close to the representatives of the genus from Australasia and Pacific: *C. fimbriata* Hunt & Sutherland, 1984, *C. perbella* Hunt & Sutherland, 1984, *C. wallacei* Hunt, 1997, *C. janiceae* Hunt & Moore, 1998 and *C. filipjevi* Malysheva, 2014.

*Carnoya philippinensis* sp. n. can be differentiated from *C. filipjevi* by almost twice longer female tail (1392 (1220-1630) µm *vs* 805 (760-870) µm) and a longer spicule and gubernaculum in males (153 (145-158) µm and 108 (102-113) µm *vs* 82 (80-87) µm and 47 (45-50) µm, respectively). Females of *C. philippinensis* sp. n. also differ from females of *C. filipjevi* in the presence of 19 *vs* 17 rows of cuticular spines of an anterior end. Additionally, the last row of spines in *C. philippinensis* sp. n. is interrupted laterally by the lateral alae, which was not observed for *C. filipjevi*. *Carnoya philippinensis* sp. n. can be distinguished from *C. wallacei* and *C. janiceae* by the greater number of spine rows in females (19 *vs* 13 and 12-13, respectively) and the presence *vs* absence of a spine collar in males. By the female body/tail length ratio *C. philippinensis* sp. n. is closest to *C. fimbriata* and *C. perbella*, but can be distinguished by the shape of the posterior extremity of a lateral ala in males (more rounded in *C. philippinensis* sp. n.). *Carnoya philippinensis* sp. n. also differs from *C. fimbriata* by significantly longer spicules and a gubernaculum (153 (145-158) µm and 108 (102-113) µm *vs* 95, 100 µm and 58, 64 µm, respectively).

**Molecular characterisation.** Partial sequences for LSU and SSU rDNA were obtained for two specimens of *C. philippinensis* sp. n. collected in two different collection sites at Mindanao Island. By the date of submission, the only available sequences for *Carnoya* genus deposited in the GenBank NCBI belong to *C. filipjevi* described from Indonesia, which was used herein for comparative purposes to determine the level of nucleotide differences within the genus. The analysis of the alignment for LSU sequences revealed the difference in single nucleotide between two studied representatives of *C. philippinensis* sp. n. and 10 bp difference with *C. filipjevi* (for the 1007 bp long alignment). Nucleotide differences for SSU rDNA between these

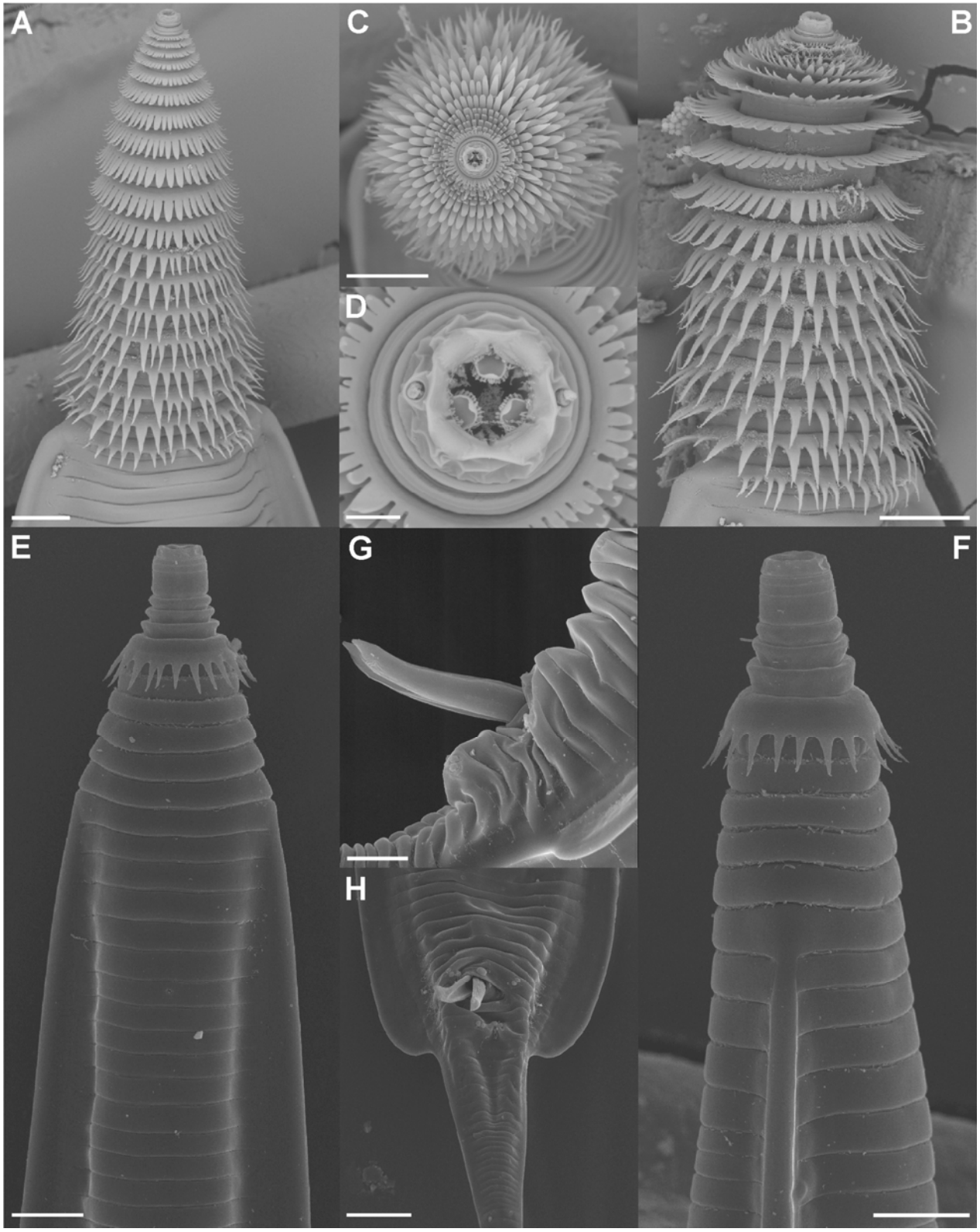


**Fig. 2.** SEM micrographs of *Carnoya philippinensis* sp. n. A, B, C: female anterior end, ventral, sublateral and apical view, respectively; D: female head end, apical view; E, F: male anterior end, ventral and lateral view, respectively; G: male cloaca region with spicules protruding, lateral view; H: male posterior end, ventral view. (Scale bars for A-C = 40 µm, D = 5 µm, E, F = 20 µm, G = 30 µm, H = 50 µm).

**Table 1.** *Carnoya philippinensis* sp. n. Morphometrics for adults from two collection sites. All measurements are in μm and in form: mean ± s.d. (range)

| Character | ***Carnoya philippinensis* sp. n.** | | | | |
|---|---|---|---|---|---|
| | **Tinago Falls population** | | | **Musuan Peak population** | |
| | **Female** | | **Male** | **Female** | **Male** |
| | **Holotype** | **Paratypes** | **Paratypes** | **–** | **–** |
| n | – | 10 | 9 | 6 | 5 |
| L | 2873 | 2763 ± 191 (2500-3079) | 1750 ± 285 (1380-2330) | 2653 ± 245 (2420-2960) | 2011 ± 66 (1935-2090) |
| L’ | 1323 | 1371 ± 128 (1250-1619) | 732 ± 162 (518-952) | 1252 ± 163 (1100-1460) | 1132 ± 79 (1010-1200) |
| a | 14.4 | 13.2 ± 1.5 (12.5-16.4) | 10.7 ± 1.6 (8.6-13.3) | 8.9 ± 0.6 (8-9.6) | 14 ± 0.4 (13.6-14.4) |
| b | 7.7 | 7.4 ± 1.1 (6.5-10.2) | 3.0 ± 0.5 (2.5-4.0) | 6.8 ± 0.9 (6-7.8) | 3.5 ± 0.2 (3.3-3.8) |
| c | 1.8 | 1.9 ± 0.1 (1.8-2.1) | 1.8 ± 0.2 (1.6-2.2) | 1.9 ± 0.1 (1.8-2) | 2.3 ± 0.2 (2.0-2.5) |
| c’ | 10.2 | 11 ± 0.7 (9.2-14.7) | 9 ± 1.1 (7.3-10.5) | 11 ± 1.7 (9-13.2) | 6.8 ± 0.8 (6.1-8.3) |
| V | 19.6 | 18.1 ± 1.6 (16.6-20.3) | – | 19.4 ± 0.8 (18.5-20.3) | – |
| V’ | 42.7 | 36.4 ± 4.0 (29.2-40.4) | – | 41.5 ± 2.6 (38.4-44) | – |
| Pharynx length | 370 | 374.5 ± 46.2 (290-430) | 587 ± 27 (535-620) | 392 ± 14.6 (375-412) | 561 ± 18.4 (537-575) |
| Head to vulva | 565 | 495.5 ± 41.3 (450-570) | – | 516 ± 42.3 (450-560) | – |
| Max. body diam. | 200 | 211 ± 25 (180-240) | 162 ± 18.4 (120-180) | 296 ± 27 (250-320) | 143 ± 6.7 (135-150) |
| Anal/cloacal body diam. | 152 | 123 ± 20 (90-160) | 109 ± 16.8 (88-140) | 125 ± 17 (100-150) | 128 ± 9.1 (120-140) |
| Spicule length (arc) | – | – | 153 ± 4.6 (145-158) | – | 149 ± 2.6 (145-152) |
| Gubernaculum length | – | – | 108.7 ± 4.4 (102-113) | – | 93 ± 10 (80-107) |
| Tail | 1550 | 1392 ± 114 (1220-1630) | 928 ± 56 (815-1020) | 1400 ± 80 (1320-1500) | 879 ± 77 (800-990) |

**Table legends:** n: number of specimens examined; L: total body length; L’: body length from head to anal/cloacal aperture; a: body length/greatest body diameter; b: body length/distance from anterior to esophago-intestinal valve; c: body length/tail length; c’: tail length/tail diameter at anus or cloaca; V: % distance of vulva from anterior; V’: position of vulva from anterior end expressed as percentage of distance from head to anal aperture.

species of *Carnoya* are found in five positions, while sequences of *C. philippinensis* sp. n. from different localities are identical (for 1627 bp long alignment).

## DISCUSSION

*Carnoya philippinensis* sp. n. is the first species of *Carnoya* described from Philippines, which demonstrates a clear morphological similarity with other representatives of the genus from Australasian region, especially with two Papua New Guinean species. Unfortunately, the absence of molecular data for the aforementioned species does not enable comparison of nucleotide sequences, and only the comparison with the Indonesian species provides some data on the intrageneric variability of sequences in *Carnoya*. Similar level of nucleotide differences in LSU rDNA was reported earlier for the closely related species of *Heth* Cobb, 1898 (8-16 bp or 1.7-3.4% of compared sequences), while for two representatives of *Cattiena* Hunt & Spiridonov,

2001, the level of nucleotide differences for the same segment was reported as a significantly higher (39 bp = 5.5%) (Malysheva *et al.*, 2015). The pronounced gap between intraspecific and intrageneric (1 bp *vs* 10 bp) nucleotide differences between two *Carnoya* indicates the taxonomic independence of these species.

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**S.V. Malysheva, A.B. Mohagan and S.E. Spiridonov.** Морфологическая и молекулярная характеристика *Carnoya philippinensis* sp. n. (Nematoda: Carnoyidae) с острова Минданао, Филиппины.

**Резюме.** Дано морфологическое описание нового вида *Carnoya philippinensis* sp. n. из тропических многоножек семейства Rhinocricidae Brölemann, 1913 (Diplopoda: Spirobolida). Новый вид характеризуется кутикулярным вооружением переднего конца тела самцов и самок, а также расположением копулятивных папилл у самцов. Наличие широкого латерального крыла и длинного хвостового отростка у взрослых особей обоих полов морфологически сближает данный вид с другими представителями рода из Тихоокеанского региона.