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## New Austrognathiidae (Gnathostomulida: Conophoralia) from Hong Kong and Japan: microscopic anatomy, ultrastructure and evolutionary implications

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### Abstract

We describe two new species, *Austrognathia glandifera* and *Austrognatharia orientis* using observations on squeezed, live specimens as well as histological sections and transmission electron microscopy. The protonephridia of *Austrognatharia orientis* are composed of a terminal cell, a canal cell, and a nephroporus cell. The monociliated terminal cell constitutes the so-called filtration area. The canal cell harbors the lacunar system and the protonephridial duct, which is surrounded by six filamentous rods, which originate external to and in between the microvilli of the terminal cell and stretch along the entire length of the canal cell. The female copulatory organs of the investigated species are very different. *Austrognathia glandifera* has a bursa and a vagina whereas *A. orientis* only has a weakly defined bursal tissue and no detectable vagina. The bursa is divided into an anterior and a posterior part; at the anterior end a special area is formed by interdigitations of the cells of the bursal wall. The male copulatory organs in the Conophoralia are uniform, composed of an anterior, glandular portion consisting of a proximal part with medium-grained and a distal part with coarse-grained appearance and a penis that is delineated by a basal lamina and has an ejaculatory duct as well as a gonopore. Parenchymal cells are present and serve to embed the bursa and the male copulatory organ dorsolaterally. Our data on the fine structure of various tissues indicate that the Conophoralia are the “less derived” sister taxon of the Scleroperalia.

**Key words:** ultrastructure, protonephridia, sperm, bursa, male copulatory organ

### Introduction

Gnathostomulida are marine, microscopic, acoelomate, hermaphroditic worms that slowly glide through the interstices of detritus-rich sand and mud by means of their monociliated epidermis. To date, 100 species have been described from shallow sandy bottoms, many with global or circumtropical distribution (Sterrer & Sørensen 2015); another 15 are awaiting description (Sterrer, in prep.).

Most striking (and name-giving) are their complex jaws, which are in stark contrast to the otherwise simple morphology of these animals. Due to their size (they are among the smallest metazoans) and slow movement, they are among the most recently discovered phyla of animals (Riedl 1969), the first description dating back to 1956 (Ax 1956). Because the first species found possessed a stylet and showed similarities in general features as well a 4d-cleavage pattern (Riedl 1969) gnathostomulids have been tied to platyhelminths (Ax 1956; 1960; 1985). However, subsequently described species lacked a stylet and based on features revealed by electron microscopy some authors (Rieger & Mainitz 1977; Sterrer *et al.* 1985; Ruppert 1991) suggested a position intermediate between “turbellarians” and gastrotrichs, implying that these groups are on a common line of evolution, with “turbellarians” being the most primitive. In contrast Ax (1984, 1985) placed the Gnathostomulida as sister group to all other Bilateria based on the monociliated epidermis and features of the protonephridia.

Furthermore, ultrastructural characters suggested a close relationship with other animals having complex jaws, namely the Syndermata (Acanthocephala + “Rotifera”; Ahlrichs 1995; Rieger & Tyler 1995). The name Gnathifera was introduced, and the subsequent discovery of the Micrognathozoa (Kristensen & Funch 2000), showing characters that would have been anticipated for a “missing link” between the two groups, further strengthened this hypothesis of relationship.

Recent phylogenetic studies of molecular data favor the Gnathifera and its placement at the base of the Spiralia (Giribet *et al.* 2004; Struck *et al.* 2014).

The system of the Gnathostomulida was proposed by Sterrer (1972), dividing the phylum into Filospermoidea and Bursovaginoidea. Filospermoidea are long and slender, possess filiform sperm with a single 9+2 flagellar axoneme, and lack any kind of accessory female organ. The Bursavaginoidea have a somewhat bulky shape with conspicuous sensory bristles at the anterior end; they possess female accessory organs (in the simplest case a temporary vagina and an undifferentiated bursal tissue) and have aberrant, aflagellar sperm by which two suborders are defined. Conophoralia have quite large and mushroom-shaped sperm (conuli) and a soft penis. Scleroperalia have quite small sperm with cellular extensions on one side (dwarf sperm), and commonly possess a penis stylet with 8–10 rods.

This paper presents two new species from East Asia, together with new data on fine structure and relationships. This includes data on the male copulatory organ, which has not previously been analysed in Conophoralia (the only studies in gnathostomulids being descriptions in two species of the Filospermoidea (Knauss & Rieger 1979) and various species of the Scleroperalia (Mainitz 1977; Mainitz 1979). We also present data on the ultrastructure of the female accessory organs, protonephridia, and parenchymal cells.

## Material and methods

Geographic coordinates for sites were approximated via Google Earth.

Of 12 sediment samples taken in Hong Kong during July 18–24, 2007, 5 yielded a total of 5 species of Gnathostomulida; and of 6 samples taken in Shirahama during August 6–16, 2007, 4 yielded a total of 4 species.

Field methods, meiofauna extraction and species analysis follow Sterrer (1998). Live animals were viewed with a WILD M11 phase contrast microscope, recorded with a drawing tube and photographed with a Canon PowerShot A620 camera. Specimens processed for histology and electron microscopy were relaxed with magnesium-chloride isotonic to seawater and fixed and stored in 4% glutaraldehyde in 0.2 M cacodylate (pH 7.2) containing 0.1 M NaCl and 0.35 M sucrose. Specimens were washed in cacodylate buffer, postfixed in cacodylate-buffered 1% (v/v) osmium tetroxide, dehydrated in acetone, and embedded in EMBed/Araldite epoxy resin. Single sections with a thickness of 0.5  $\mu\text{m}$  were made using a diamond knife mounted in a Butler trough (Butler 1979) on a Reichert-Jung Ultracut E and stained with Richardson's stain (Richardson *et al.* 1960), mounted with DePeX (SERVA, Heidelberg, Germany), viewed with a Leica DM 5000B compound microscope (Wetzlar, Germany) and photographed with a Leica DFC 490 digital camera (Wetzlar, Germany). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss Libra 120 transmission electron microscope or a Philips EM 201.

Photomicrographs were processed and arranged using Adobe Photoshop CS3; reconstructions were drawn using Adobe Illustrator CS3. Histological sections, including type material, have been deposited at the National Museum of Natural History (NMNH), Smithsonian Institution, Washington DC.

In the descriptions we use a scale of 100 units (U) for body length to indicate the relative location of organs, and various length-width ratios as detailed in Sterrer (1998). In addition to the *sperm index* (= sperm length divided by sperm width) we introduce the *capitulum index* (= sperm length divided by the length of the capitulum, i.e., the "hat" of the conulus).

**TABLE 1.** Collection data for material used in this study.

Region	Sample	Date	Locality	Lat	Long	Depth	Description
Hong Kong	HK2	19-07-07	Pak Lap Tsai	22°21'00"N	114°22'00"E	7m	Coarse shell with detritus among coral heads
Hong Kong	HK3	19-07-07	New Territories, Three Fathoms Cove	22°25'00"N	114°17'00"E	1m	Medium sand with detritus and soil
Shirahama	SH2	08-08-07	Seto Marine Lab, north beach	33°41'38"N	135°20'16"E	1-2m	Medium sand with detritus

## Results

### Phylum Gnathostomulida (Ax, 1956) Riedl, 1969

### Order Bursovaginoidea Sterrer, 1972

### Suborder Conophoralia Sterrer, 1972

### Family Austrognathiidae Sterrer, 1971

### Genus *Austrognathia* Sterrer, 1965

Austrognathiidae with 2 rows of jaw teeth, the dorsal row consisting of 3 or more teeth. Median lobe of basal plate usually prominent, rarely flattened or absent; lateral lobes prominent. Teeth of basal plate rather uniform in size and arrangement.

#### *Austrognathia glandifera* n. sp.

(Figs. 1–3; Table 2)

**Diagnosis.** Small, fairly slender *Austrognathia* (mean length 730  $\mu\text{m}$ , width 97.5  $\mu\text{m}$ ; index 7.47; see Table 2). Basal plate 6.60  $\mu\text{m}$  long, 22.60  $\mu\text{m}$  wide (index 0.29), with conspicuous median and lateral lobes. Jaws 21.2  $\mu\text{m}$  in length; dorsal tooth row with 7 short, even teeth; ventral row with 4–8 longer, uneven-sized teeth. Conuli large and blunt (30.75  $\mu\text{m}$  long by 22.42  $\mu\text{m}$  wide; index 1.37), with capitulum enveloping two thirds of the conulus (capitulum length 20.17  $\mu\text{m}$ ; index 1.62).

**Type material.** Holotype: USNM 1270621, one adult from Hong Kong (sample #2) in squeeze preparation.

**Type locality.** Sample HK2 from Hong Kong (see Table 1).

**Other material examined.** Four adults and two juveniles from sample #2, one adult from sample #9. Semithin and ultrathin sections of two mature specimens.

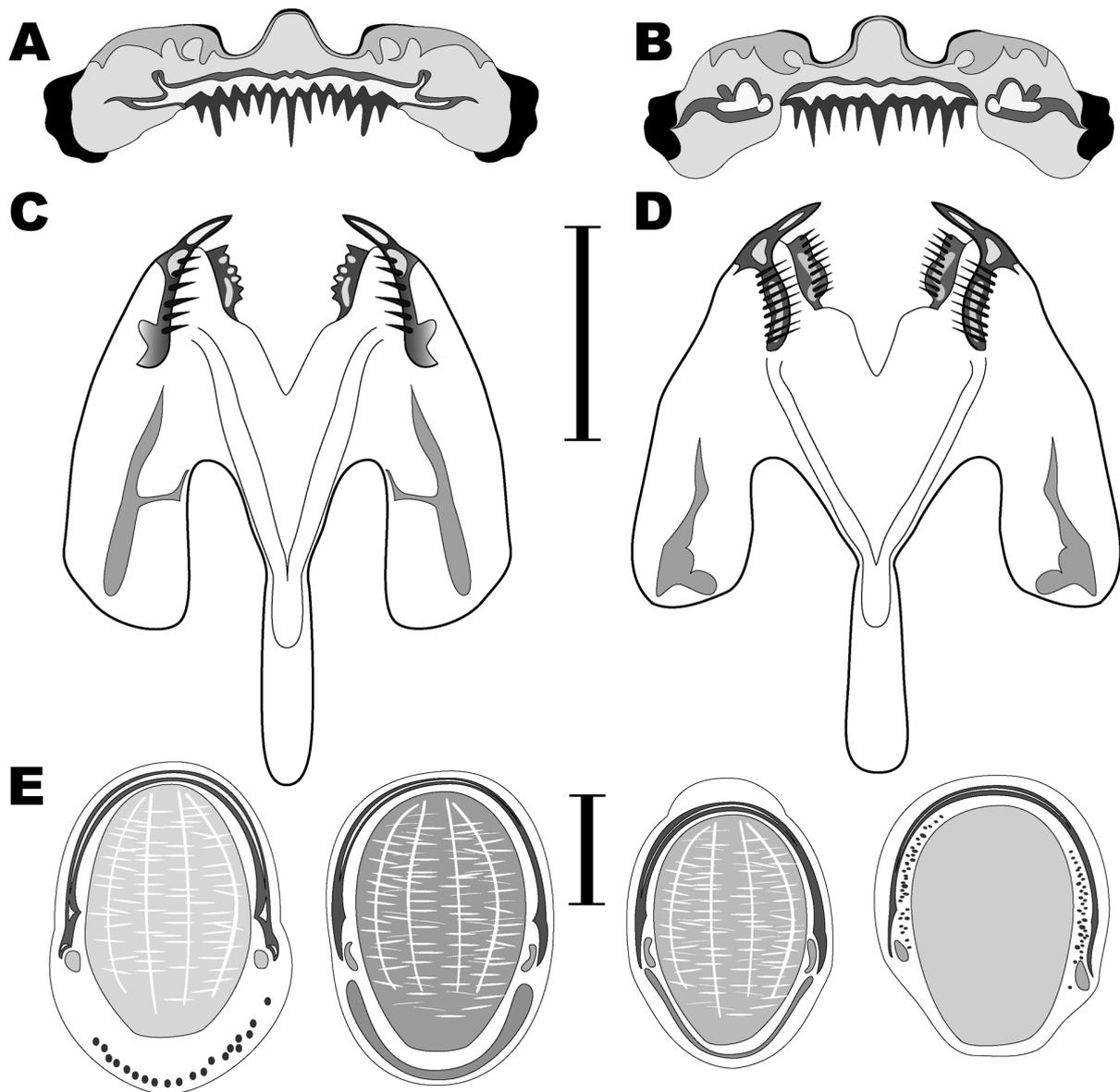
**Etymology.** Referring to the acorn-shape of mature conuli (from Latin *glans* = acorn).

**Description.** *Organization* (Fig. 2A). Adults measure 730  $\mu\text{m}$  in length (600–800  $\mu\text{m}$ ), and 97.50  $\mu\text{m}$  in width at U55 (body index 7.47), with a short rostrum (index 0.94). The epidermis of the tail region is studded with adhesive glands.

*Sclerotized pharyngeal parts* (Figs. 1A–D; 2C–F). The basal plate is 6–8  $\mu\text{m}$  long and 20–24  $\mu\text{m}$  wide (index 0.29). It has well-defined lobes, with the lateral pair pointing medio-rostrally. Caudally the basal plate is set with up to 15 teeth of which the largest 3 are each flanked by a pair of smaller teeth; even smaller ones separate the three groups. Jaws are 20–22  $\mu\text{m}$  long, with a strong terminal tooth and two rows of teeth: a dorsal row of up to 7 even teeth, and a ventral row in which a larger tooth is followed by up to 8 uneven teeth.

*Male system* (Figs. 2A, B, G, H, I; 3B, C, F, G). The dorsal testis contains 5–8 mature conuli; one specimen had but a single conulus located in the penis. Mature conuli range from 27  $\mu\text{m}$  to 35  $\mu\text{m}$  in length (mean 30.75  $\mu\text{m}$ ), and 18  $\mu\text{m}$  to 28  $\mu\text{m}$  in width (mean conulus index 1.34). They are plump and blunt, with the semi-circular capitulum reaching 20.17  $\mu\text{m}$  in length, which is two-thirds of the conulus length (capitulum index 1.62). The pointed end of the conulus is usually enveloped in a matrix (i.e., the sperm cytoplasm), and is often bilobed, an indication of the peculiar conulus composition along two longitudinal axes. The cingulum is inconspicuous but long, barely protruding caudally past the capitulum. A granular layer below the capitulum is conspicuous in the conuli (Fig. 2G, H, I). The male copulatory organ consists of a glandular part and an epidermal penis that measures 30  $\mu\text{m}$  in length and 15  $\mu\text{m}$  in width. The glandular part (pg) is ~70  $\mu\text{m}$  long; the spermatic duct runs through it from the dorsal to the ventral side, where it proceeds into the ejaculatory duct of the penis (p). The cells constituting the glandular part are filled with vesicles, which are refractive in live observation (Fig. 2B) and electron-dense by transmission electron microscopy (Fig. 3F, G). A group of cells, which lie adjacent to the penis, contain spherical vesicles that measure up to 1  $\mu\text{m}$  (Fig. 3B, F, G). The vesicles in the more anterior part (ag) are potato-shaped and are more electron-dense when smaller, and larger in size the further posteriorly and ventrally they are positioned (pg), starting with ~200 nm in diameter and reaching up to 1  $\mu\text{m}$  (Fig. 3B, F, G). The cells of the penis contain numerous electron-dense vesicles (~150 nm) and rods, which measure up to 5  $\mu\text{m}$  in length and 200 nm in width. The rods are

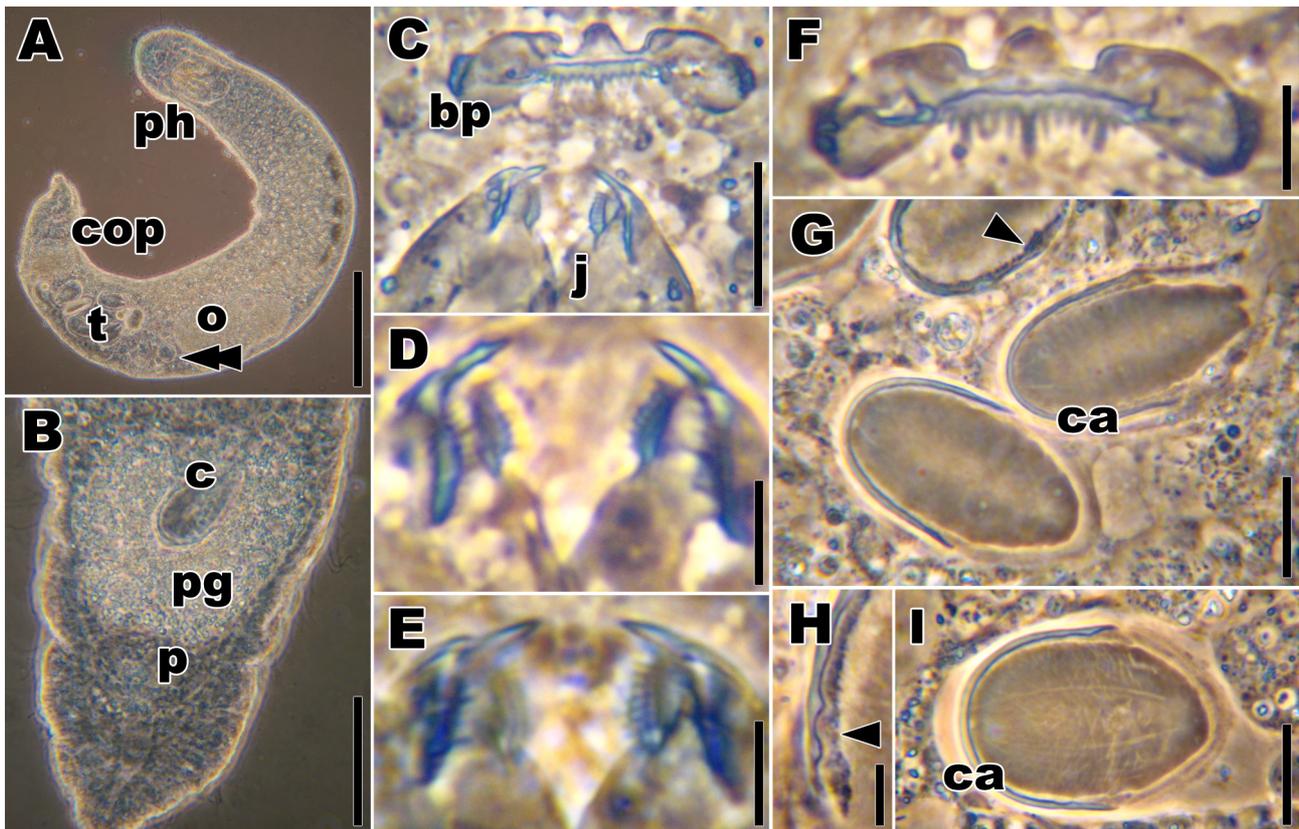
especially numerous around the gonopore (Fig. 3C). Some obliquely striated longitudinal muscles run along the glandular and epidermal part of the penis (Fig. 3F).



**FIGURE 1.** *Austrognathia glandifera* sp. nov.; reconstructions to show structures important for species determination. Sclerotized pharyngeal structures are shown as dorsal views and are shaded according to the phase effects of the phase-contrast microscope. These effects, such as the dark of the teeth and of the edges of the basal plate, are artefacts and don't represent real structural differences as implied. Some viewing them with bright-field, oblique illumination, or DIC would not see them. A, B. Basal plates. C, D. Jaws. E. Different developmental stages of conuli. Scale bars: 10  $\mu$ m.

*Female system* (Fig. 3A, D). The ovary including the mature egg extends from about U25 to U66; the single mature egg can be 340  $\mu$ m long and 65  $\mu$ m wide, and often wraps around the bursa, which lies immediately behind it, at U57 to U65.

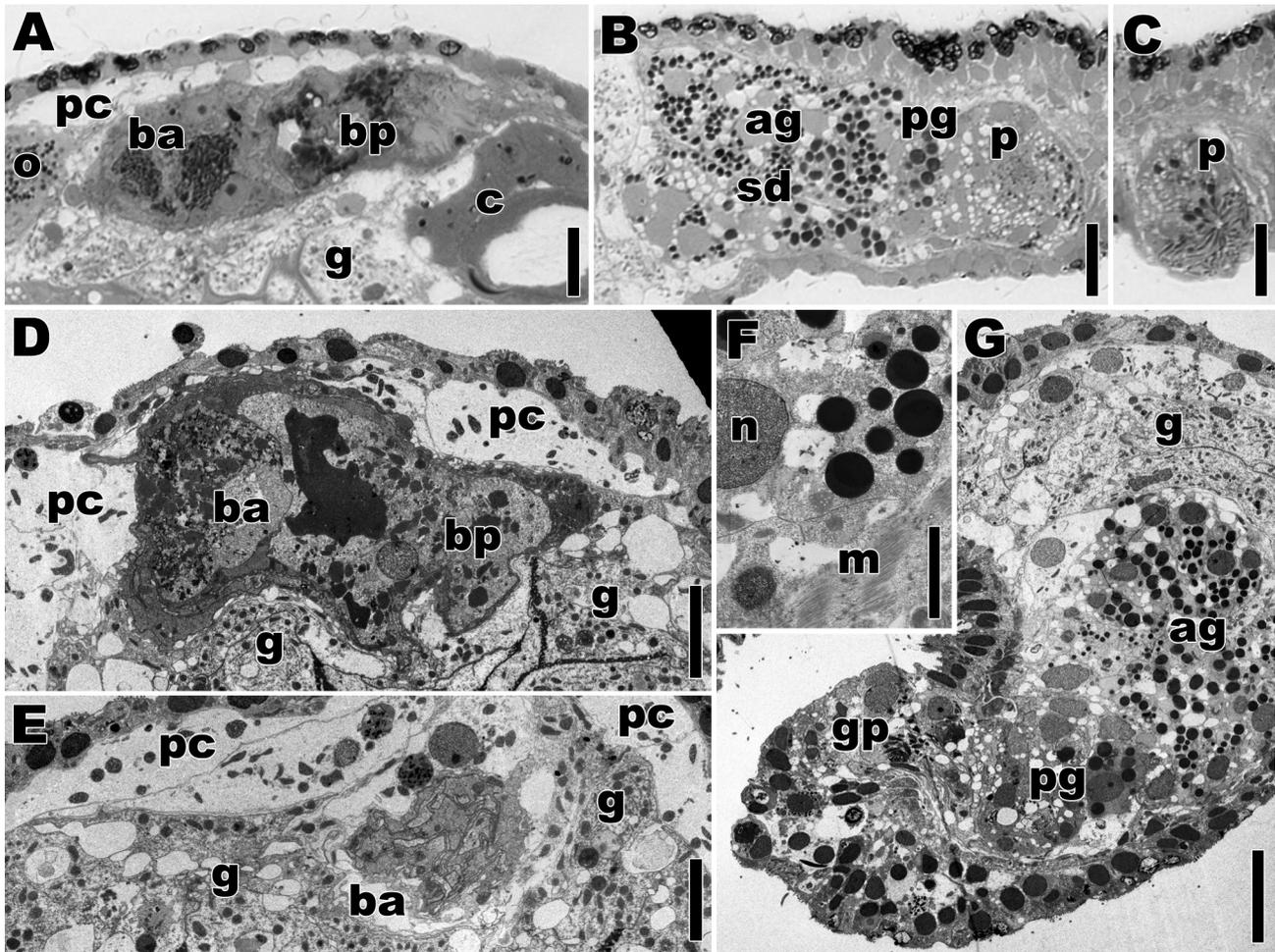
The bursa is ~50  $\mu$ m long and consists of a cellular wall (4–5 layers) and a syncytial, multinucleated interior. Even though a distinction into an anterior and posterior part is not conspicuous in its form it is by its content. The posterior part (bp) often contains large electron-dense material of undefined shape, whereas the anterior part (ba) contains filamentous material (Fig. 3A, D). Overall the content of the bursa is nearly devoid of cell organelles, e.g. mitochondria. The origin and function of the electron-dense material is not known. On the anterior end the cells of the bursal wall are highly intermingled, but in an unorganized fashion (Fig. 3E). On the dorsolateral side the bursa is enclosed by parenchymal cells and connects to the body wall at the posterior end through a vagina that lacks a lumen (Fig. 3A, D, E).



**FIGURE 2.** *Austrognathia glandifera* sp. nov.; photomicrographs of adult specimens. A. Dorsal view of whole specimen. Double arrowhead points to conuli embedded in bursal tissue. B. Posterior part with male copulatory organ. C. Pharynx with basal plate and jaws. D, E. Jaws. F. Basal plate. G. Conuli. Arrowhead points to granular layer. H. Lateral part of cingulum. Arrowhead points to granular layer. I. Conulus. Abbreviations: bp basal plate, c conulus, cop male copulatory organ, j jaw, o oocyte, pg glandular part of male copulatory organ, ph pharynx, p penis, t testis. Scale bars: A 100  $\mu$ m; B 50  $\mu$ m; C, G, I 10  $\mu$ m; D, E, F 5  $\mu$ m; H 2  $\mu$ m.

**TABLE 2.** Morphometric data (in  $\mu$ m) for *Austrognathia glandifera* n. sp.

Hong Kong	Mean	StDev	Max	Min	n
Body length of adults	730.00	89.07	800	600	4
Body width of adults	97.50	5.00	100	90	4
Body index of adults	7.47	0.57	8.00	6.67	4
Rostrum index of adults	0.94	0.22	1.10	0.79	2
Jaw length	21.20	0.84	22	20	5
Basal plate length	6.60	0.89	8	6	5
Basal plate width	22.60	1.52	24	20	5
Basal plate index	0.29	0.03	0.33	0.26	5
Sperm length	30.75	2.34	35	27	12
Sperm width	22.42	2.71	28	18	12
Sperm index (sp l/w)	1.37	0.19	1.78	1.20	12
Capitulum length	20.17	2.48	27	18	12
Capit. index (sp l/cap l)	1.62	0.18	1.84	1.43	5



**FIGURE 3.** *Austrognathia glandifera* sp. nov.; sagittal sections through copulatory organs. A–C. Photomicrographs of histological sections. D–G. Electronmicrographs of ultrathin sections. A. Bursa. B. Male copulatory organ. C. Penis. D. Sagittal section through bursa. E. Cross section through bursa. F. Detail of posterior portion of glandular part of male copulatory organ. G. Male copulatory organ. Abbreviations: ag anterior portion of glandular part of male copulatory organ, ba anterior part of bursa, bp posterior part of bursa, c conulus, g gut, gp gonopore, m muscle, n nucleus, o oocyte, pc parenchymal cell, p penis, pg posterior portion of glandular part of male copulatory organ, sd spermatid duct. Scale bars: A, B, C, D, G 10  $\mu$ m; E 5  $\mu$ m; F 2  $\mu$ m.

**Remarks.** Members of the genus *Austrognathia* are mainly distinguished by the dimensions and proportions of their sperm (conuli). The mean sperm index of the 10 described species ranges from 2.79 (in *A. australiensis*) to 1.71 (in *A. hymanae*); none has a sperm index as low as 1.37. Mature sperm in 5 species are considerably smaller (from 8.0  $\mu$ m in *A. nannulifera* to 22.8  $\mu$ m in *A. hymanae*) than in *A. glandifera*, and considerably larger in another four (from 36.73  $\mu$ m in *A. singatokae* to 52.50  $\mu$ m in *A. macroconifera*); only the conuli of *A. christiana*e (31.00  $\mu$ m) and *A. riedli* (34.29  $\mu$ m), two closely related species, are of comparable length but both are significantly more slender (conulus index 1.72 viz. 1.73). The consistency in shape and dimensions of 12 conuli measured in five specimens also suggests this species to be new.

#### Genus *Austrognatharia* Sterrer, 1971 (emend. Sterrer, 1991)

Austrognathiidae with 2 rows of jaw teeth, the dorsal row usually consisting of 1, sometimes 2 teeth. Median lobe of basal plate absent, lateral lobes mostly flattened or absent. Teeth of basal plate regionated, of non-uniform size and arrangement.

***Austrognatharia orientis* n. sp.**

(Figs. 4–7; Table 3)

**Diagnosis.** Small, fairly slender *Austrognatharia* (mean length 738  $\mu\text{m}$ , width 96  $\mu\text{m}$ ; index 8.40; see Table 3). Basal plate 6.11  $\mu\text{m}$  long, 20.44  $\mu\text{m}$  wide (index 0.30), with inconspicuous median but pronounced lateral lobes; with at least 8 uneven-sized teeth of which the median and the lateral pair are largest. Jaws 20.78  $\mu\text{m}$  in length, with a strong terminal tooth; dorsal tooth row with two teeth sharing a single root, ventral row with 3–6 uneven-sized teeth. Conuli small (12.80  $\mu\text{m}$  long by 7.60  $\mu\text{m}$  wide; index 1.76), with a shallow capitulum (length 2.55  $\mu\text{m}$ ; index 5.24). Conuli often appear as twins, and may have a flaring cingulum.

**Type material.** Holotype one adult from Hong Kong (sample #2) in squeeze preparation, USNM 1270622.

**Type locality.** Sample HK3 from Hong Kong (see Table 1).

**Other material examined.** Seven more specimens (4 adults) from Hong Kong, and five specimens (3 adults) from Shirahama. Semithin and ultrathin sections of one mature specimen from Hong Kong and one mature specimen from Shirahama.

**Etymology.** From Latin *orientis* = from the East.

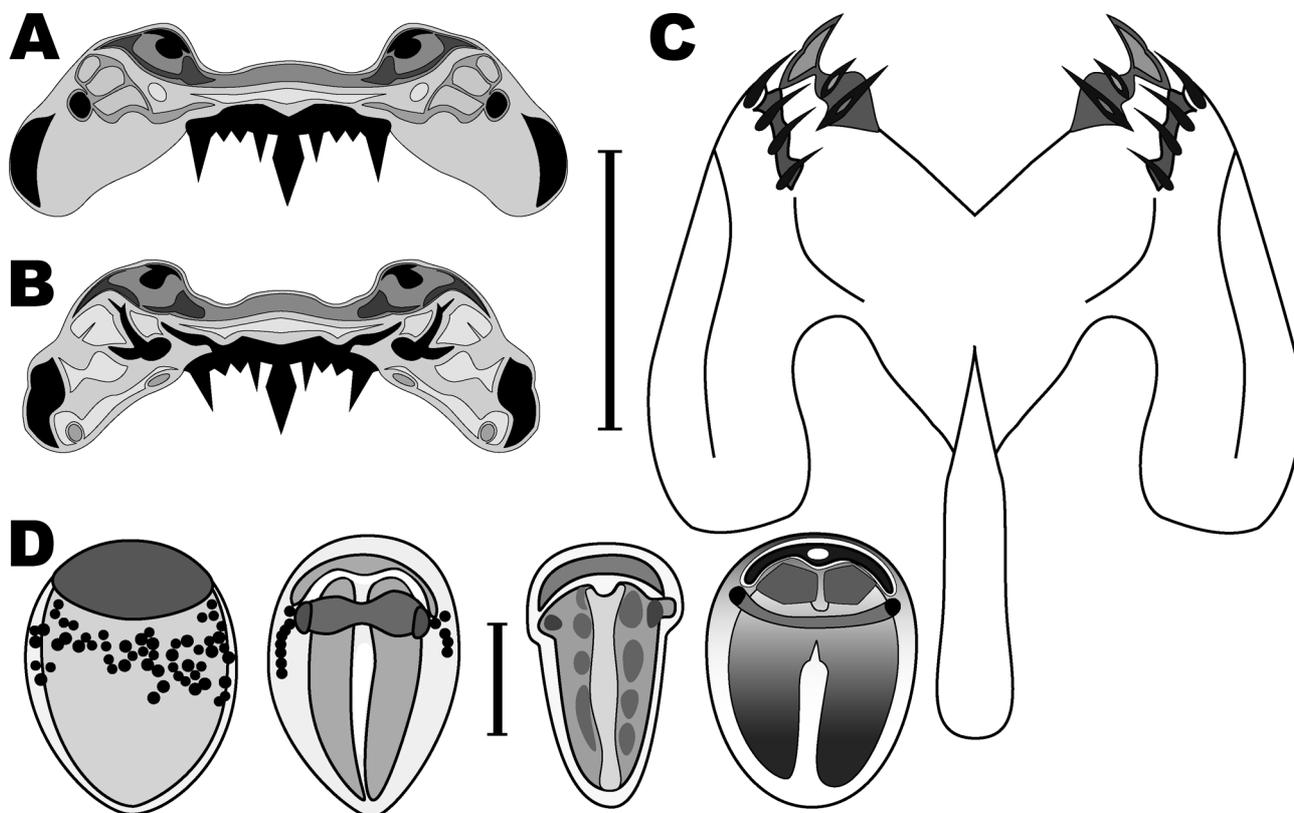
**Description.** *Organization* (Fig. 5A). Adults measure 600–850  $\mu\text{m}$  in length and 80–120  $\mu\text{m}$  in width (body index 8.40), with a short rostrum (index 0.97).

*Body wall.* The epidermis is cellular, monociliated, has glandular components and rod-shaped secretory products. The “potatoe-shaped” nuclei of the epidermis lie at the basal side of the cells and possess a dark, homogeneous chromatin. The nuclei are up to 2.5  $\mu\text{m}$  long. The cilia originate from a ciliary pit, which is surrounded by eight microvilli. The ciliary pit has a diameter of 500 nm and is ~500 nm deep. The apical cell surface is covered by microvilli, which are 200 nm long, 50 nm wide and occur at a density of ~64/ $\mu\text{m}^2$ . Many epidermal cells contain vacuoles with glandular content that can nearly fill the entire cell and mitochondria that are ~500 nm long and ~300 nm in diameter. Specialized cells contain up to 7 rods, which are bluntly pointed at both ends, and measure up to 3.5  $\mu\text{m}$  long and 800–850  $\mu\text{m}$  wide. They are composed of numerous hollow tubes of unknown material, which are arranged parallel to each other in a longitudinal orientation. Occasionally electron-lucent vesicles, which have a diameter of ~500 nm, are found underneath the apical cell membrane. The epidermal cells are connected with zonula adherentes and apical septate junctions to one another. On the basal side, the epidermis has a distinct basal lamina. Underneath the epidermis lies the body-wall musculature with outer circular muscles and inner longitudinal muscles. However, in the region of the male copulatory organ this pattern is inverted (see below).

*Sclerotized parts of the pharynx.* The basal plate (Figs. 4A, B; 5B, C) is 5–7  $\mu\text{m}$  long and 18–23  $\mu\text{m}$  wide (index 0.30). Its median lobe is shallow whereas its lateral lobes are slightly more defined. Caudally the basal plate is set with up to 9 uneven-sized teeth, of which only three stand out in size. Jaws (Figs. 4C; 5B, C) are 19–24  $\mu\text{m}$  long, with a strong terminal tooth and two rows of teeth: a dorsal row of two (more rarely one) teeth of which only the posterior is rooted, and a ventral row in which a larger tooth is followed by up to 5 long but uneven teeth.

*Excretory system.* The protonephridia (Fig. 6A–E) are positioned below the epidermis and are accompanied by two lateral muscles (one muscle shown in Fig. 6B and C). We found three pairs of protonephridia in the posterior part. They are arranged serially, separated ~50  $\mu\text{m}$  from another, the most posterior pair lying 10  $\mu\text{m}$  in front of the male gonopore. Each protonephridium is composed of three cells: a terminal cell, a canal cell, and a nephroporus cell (Fig. 6A–E). The terminal cell encloses the canal cell along its entire apical half or more, its nucleus is situated eccentrically, reaching from the terminal end distally, overlapping with the nucleus of the canal cell for an undetermined distance (Fig. 6B, C). At the apical part, the terminal cell lacks cytoplasmic organelles and has lobes with 30-nm small clefts in between, constituting the so-called filtration area. In the central part, the terminal cell, the canal cell and the two adjacent muscles are enclosed by a basal lamina (Fig. 6C). The terminal cell has a cilium with the typical 9+2 arrangement and eight microvilli. The cilium of the terminal cell projects into the protonephridial duct of the canal cell, the microvilli of the terminal cell originate from pits and terminate at about half the length of the protonephridial duct (Fig. 6A–E). The nucleus of the canal cell is also situated eccentrically and stretches along the distal half of the cell. The cell has many mitochondria and a lacunar system, the tubules of which often appear collapsed in our preparations. The protonephridial duct is surrounded by six clusters of unidentified filamentous material (Fig. 6A–E). These clusters form rods, which originate in a position that lies approximately in between the microvilli of the terminal cell (Fig. 6A, B) and stretch along the entire length of the

canal cell. The nephridial duct opens into the nephroporus, its opening is surrounded either by a stiffened wall or collar or by very long microvilli (at least 250 nm long; Fig. 6E). The pyriform nephroporus cell encloses the protrusion of the nephridial duct, bears numerous microvilli on its surface, it lacks a cilium but a centriole is present (Fig. 6E).



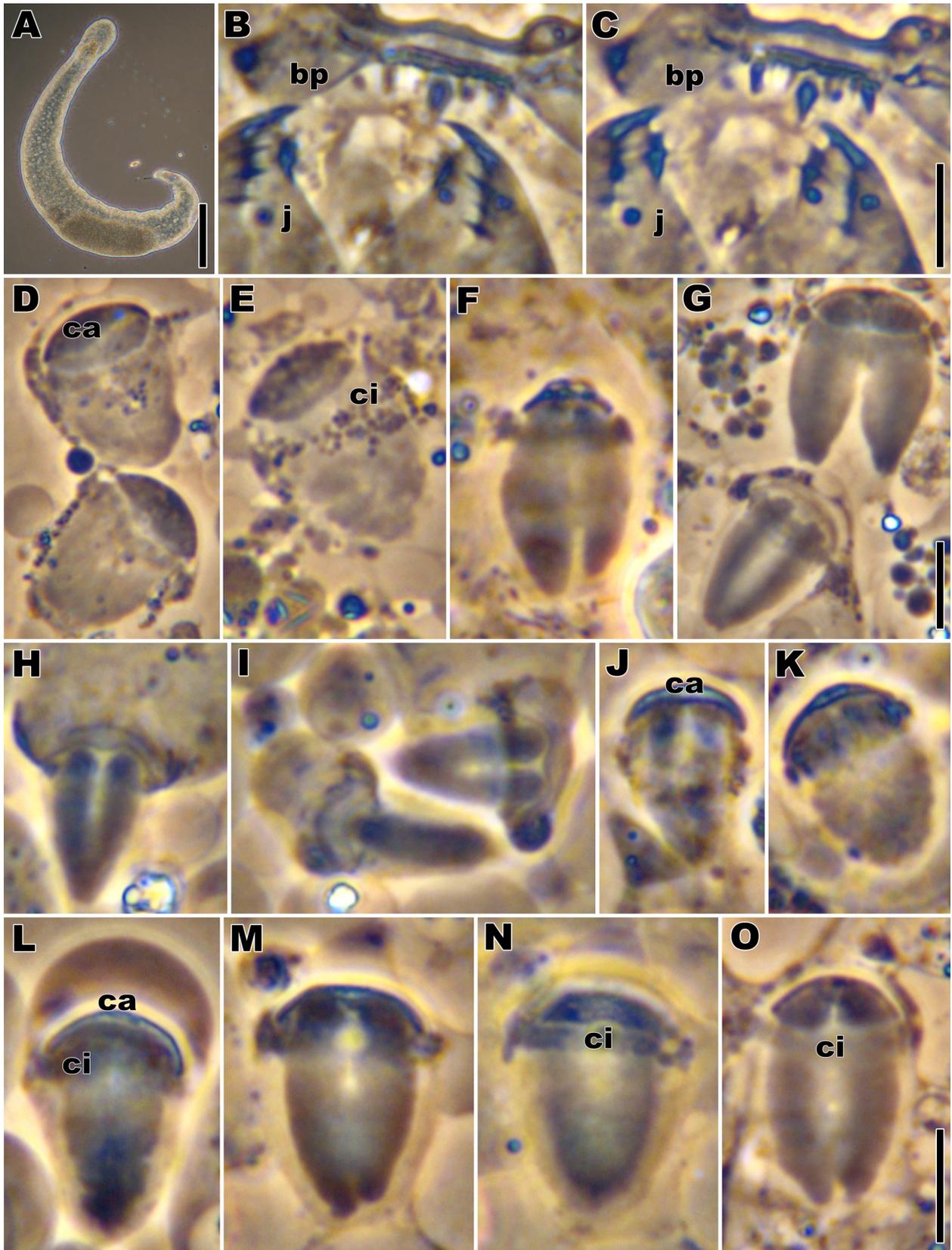
**FIGURE 4.** *Austrognatharia orientis* sp. nov.; reconstructions to show structures important for species determination. Sclerotized pharyngeal structures are shown as dorsal views and are shaded according to the phase effects of the phase-contrast microscope. These effects, such as the dark of the teeth and of the edges of the basal plate, are artefacts and don't represent real structural differences as implied. Some viewing them with bright-field, oblique illumination, or DIC would not see them. A, B. Basal plates. C. Jaws. D. Different developmental stages of conuli. Scale bars: A, B, C 10  $\mu$ m; D 5  $\mu$ m.

*Parenchyma.* Wide and flat parenchyma cells occur between the copulatory organs and the body wall. They have more or less rounded nuclei with a diameter of up to 2.5  $\mu$ m with a small nucleolus and the most electron-lucent chromatin of all cell types. The cytoplasm contains only a few organelles. Mitochondria, which can be slightly larger than the ones in the epidermis, are the most numerous ones.

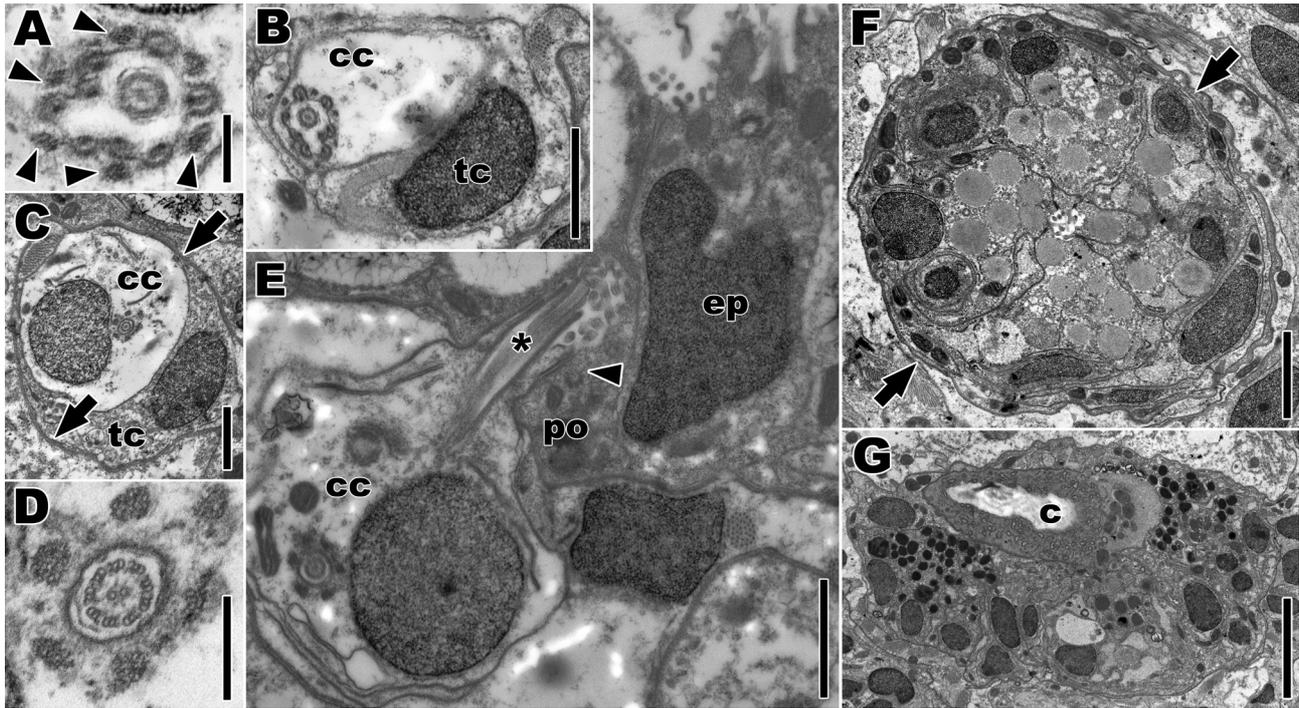
*Male system.* The single testis is saccular, unpaired and lies dorsally. It contains 6–9 conuli that are usually uniform in size but of various shapes. Conuli of the Hong Kong specimens measured 12–15  $\mu$ m in length and 7–10  $\mu$ m in width (index 1.60), with a very shallow capitulum (index 5.40) sitting atop a narrow, rarely flaring cingulum. The larger conuli usually appear as more or less distinct 'twin conuli'. Conuli of the three mature Shirahama specimens were quite similar in size and proportions but showed the 'twin' aspect only once, in a conulus wedged in the penis. The overall structure of the testis as well as the development of the gametes is in concordance with the description in *Austrognathia* sp. by Lanfranchi and Falleni (1998). Spermatogenesis starts from primary spermatogonia, with a homogeneously condensed nucleus with pronounced nucleolus (Fig. 7A) and proceeds to spermatogonia, primary spermatocytes and secondary spermatocytes, which no longer show a nuclear envelop, to spermatids. Late spermatids are completely enclosed by a nurse cell and the chromatin starts to change from an ovoid to a mushroom-like shape with the chromatin showing three grades of condensation (Fig. 7B). The cytoplasm of spermatids and sperm is rich in mitochondria, and the more mature the sperm are the more restricted the mitochondria are to the area lateral and below the cingulum (Fig. 7B, C, D). In mature sperm, a layer of flocculent electron-dense material appears 150 nm apart from the top of the cingulum (Fig. 7E, F, G). The usually single conulus within the bursal tissue goes through a disintegration whereby the restricted distribution of the

**TABLE 3.** Morphometric data (in  $\mu\text{m}$ ) for *Austrognatharia orientis* n. sp.

Hong Kong	Mean	StDev	Max	Min	n
Body length of adults	760.00	95.39	850	660	3
Body width of adults	92.50	10.61	100	85	2
Body index of adults	8.78	0.40	9.06	8.50	2
Rostrum index of adults	0.97				1
Jaw length	19.75	0.96	21	19	4
Basal plate length	6.25	0.50	7	6	4
Basal plate width	20.25	2.22	23	18	4
Basal plate index	0.31	0.02	0.33	0.29	4
Sperm length	13.50	1.38	15	12	6
Sperm width	8.50	1.05	10	7	6
Sperm index (sp l/w)	1.60	0.15	1.80	1.40	6
Capitulum length	2.60	0.55	3	2	5
Capit. index (sp l/cap l)	5.40	0.55	6	5	5
Japan	Mean	StDev	Max	Min	n
Body length of adults	716.67	104.08	800	600	3
Body width of adults	100.00	28.28	120	80	2
Body index of adults	8.02	1.92	9.38	6.67	2
Rostrum index of adults					
Jaw length	21.60	1.52	24	20	5
Basal plate length	6.00	0.71	7	5	5
Basal plate width	20.60	1.34	23	20	5
Basal plate index	0.29	0.04	0.35	0.25	5
Sperm length	12.33	0.71	13	11	9
Sperm width	7.00	1.80	10	4	9
Sperm index (sp l/w)	1.87	0.52	3	1.30	9
Capitulum length	2.50	0.55	3	2	6
Capit. index (sp l/cap l)	5.11	0.98	6	4	6
ALL DATA	Mean	StDev	Max	Min	n
Body length of adults	738.33	92.39	850	600	6
Body width of adults	96.25	17.97	120	80	4
Body index of adults	8.40	1.21	9.38	6.67	4
Rostrum index of adults	0.97				1
Jaw length	20.78	1.56	24	19	9
Basal plate length	6.11	0.60	7	5	9
Basal plate width	20.44	1.67	23	18	9
Basal plate index	0.30	0.03	0.35	0.25	9
Sperm length	12.80	1.15	15	11	15
Sperm width	7.60	1.68	10	4	15
Sperm index (sp l/w)	1.76	0.42	3	1.30	15
Capitulum length	2.55	0.52	3	2	11
Capit. index (sp l/cap l)	5.24	0.79	6	4	11



**FIGURE 5.** *Austrognatharia orientis* sp. nov.; photomicrographs of adult specimens. A. Dorsal view of whole specimen. B, C. Pharynx with basal plate and jaws at different focal planes. D–G. Different developmental stages of conuli of specimens from Hong Kong. H–O. Different developmental stages of conuli of specimens from Japan. Scale bars: A 100  $\mu$ m; B, C 5  $\mu$ m; D–G 5  $\mu$ m; H–O 5  $\mu$ m.



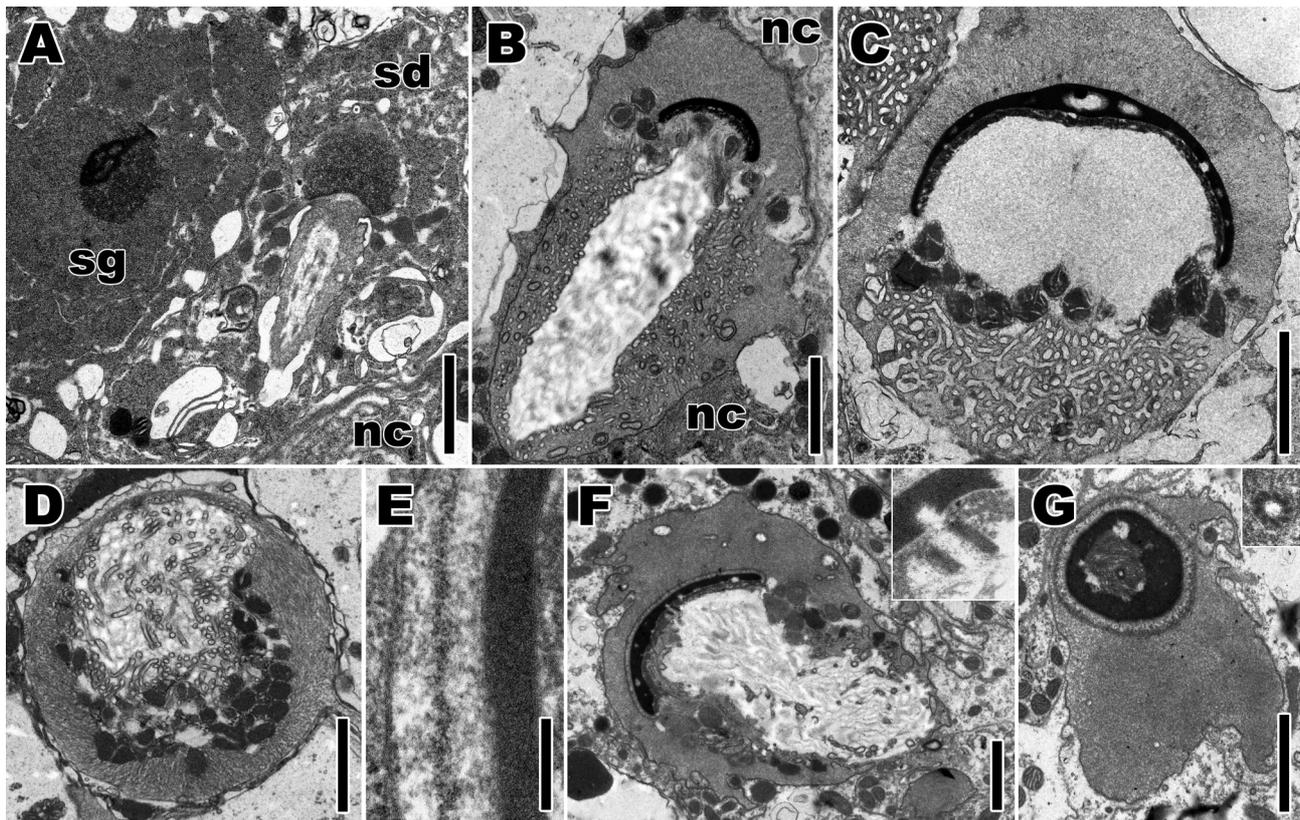
**FIGURE 6.** *Austrognatharia orientis* sp. nov.; electronmicrographs of protonephridia (A–E) and the male copulatory organ (F, G). A. Cilium and microvilli with indentation of terminal cell. Arrowheads point to origin of filamentous rods. B. Cross section through terminal cell and canal cell at a plane proximal to the termination of the microvilli of the terminal cell. C. Cross section through terminal cell and canal cell distal to the termination of the microvilli of the terminal cell. Arrows point to ECM. D. Detail of C showing the protonephridial canal and the surrounding six rods of filamentous material. Note that the rods are clearly an intracellular part of the canal cell. E. Section through the nephroporus showing the nephroporus cell and the canal cell. Note the cilium reaching from the protonephridial duct into the nephroporus and the many microvilli of the nephroporus cell. Asterisk marks the cilium of the terminal cell, arrowhead points to centriole of the nephroporus cell. F. Distal part of penis with electron-lucent vesicles. Arrows point to the basal matrix at the base of the penis cells. Note microvilli in the ejaculatory duct. G. Distal, glandular part of male copulatory organ enclosing a mature conulus. Note the numerous electron-dense vesicles in the gland cells. Abbreviations: c conulus, cc canal cell, ep epidermal cell, po nephroporus cell, tc terminal cell. Scale bars: A, D 250 nm; B, C, E 1  $\mu$ m; F 2  $\mu$ m; G 5  $\mu$ m.

mitochondria is lost and the tubular system disappears (Fig. 7F, G). Like Lanfranchi and Falleni (1998), we found single basal bodies below the cingulum in disintegrating conuli (Fig. 7F, G, inserts). The anterior, glandular part of the male copulatory organ (Fig. 6G) comprises 24 gland cells, which have nuclei with dark chromatin and a distinct nucleolus, lack microvilli on the apical surface, are rich in cytoplasmic organelles, and produce electron-dense, potato-shaped vesicles with a diameter of  $\sim$ 500 nm. More distally a ring of 6 cells, which produce unshaped and chiseled secretion and spherical, electron-dense vesicles with a diameter of  $\sim$ 1  $\mu$ m joins the penis, which is  $\sim$ 10  $\mu$ m long, has a diameter of  $\sim$ 9  $\mu$ m, and consists of  $\sim$ 24 cells. The male genital duct lies in the center of the tube. Distally the penis tube is composed of 16 cells with peripherally positioned nuclei with dark, homogeneous heterochromatin. These cells contain a large number of cytoplasmic organelles, mitochondria measuring  $\sim$ 500 nm in length and  $\sim$ 300 nm in diameter, and electron-lucent vesicles with a diameter of  $\sim$ 800 nm (Fig. 6F). These cells lack cilia but bear microvilli with a length of up to 700 nm and are connected with zonulae adherentes and septate junctions to adjacent cells; the basal part is delineated from other tissues by a basal lamina (Fig. 6F). The zonulae adherentes stretch 300 nm from apical to basal, the septate junctions up to 1  $\mu$ m further. The male gonopore lies  $\sim$ 20  $\mu$ m in front of the posterior end. All ventrally situated longitudinal body-wall muscles bend around the gonopore caudally. In the region from 6  $\mu$ m in front of the gonopore to 6  $\mu$ m posterior to the gonopore the circular muscles are positioned inside the longitudinal muscles. About six muscle fibers run along the male copulatory organ and connect it to the testis. At the distal part a thin sphincter encloses the penis tube.

*Female system.* In a Hong Kong specimen, the ovary (including the single mature, 220  $\mu$ m long egg) extends from U51.8 to U82.4. No distinct bursa is present but various conuli were found in a state of disintegration close to the most posteriorly lying oocyte (Fig. 7FL, G). The conuli were partially enclosed either by gut cells or cells that

closely resemble parenchymal cells, except that their nucleus is darker, their cytoplasm richer in organelles, and their cell surface highly interdigitated with gut cells. In one case a laterally lying parenchymal cell extended towards the center of the body and partially covered a conulus.

**Remarks.** The species is similar to *A. homunculus* Sterrer, 1991, and *A. moorensis* Sterrer, 1991, in having one or two teeth in the dorsal row of its jaws, two teeth being more common than one. Both of these species, however, differ in having much larger jaws and basal plates and vastly larger conuli (mean conulus length in *A. homunculus* is 43.47  $\mu\text{m}$  and in *A. moorensis* 39.78  $\mu\text{m}$ ). *A. orientis* has the second smallest conuli in the genus, and *A. barbadensis* Sterrer, 2011, has the smallest (length 6.00  $\mu\text{m}$ ). The heterogeneity of conuli in *A. orientis* (within specimens and populations as much as between populations) is puzzling and leaves room for gaining better knowledge of this species.



**FIGURE 7.** *Austrognatharia orientis* sp. nov.; electronmicrographs of conuli of specimens from Japan (A–E) and Hong Kong (F, G). A. Spermatogonium and late spermatid lying next to each other. B. Late stage of spermatid with slightly chaotic distribution of mitochondria. Note that the nurse cell entirely encloses the spermatid, especially the small rim of cytoplasm on the left side. C. Later stage of conulus with restricted distribution of mitochondria below the cingulum but no maturation layer. D. Cross section through the same developmental stage as in C. E. Sagittal section through apical part of mature conulus showing the cellular membrane, the maturation layer and the electron-dense cingulum. F. Sagittal section through conulus in stage of disintegration embedded within the bursal tissue. Note maturation layer above the cingulum. Inset: Magnification of basal body. G. Cross section through conulus in stage of disintegration embedded within the bursal tissue. Note maturation layer surrounding the cingulum. Inset: Magnification of basal body. Abbreviations: nc nurse cell, sd spermatid, sg spermatogonium. Scale bars: A, B, C, D, F, G 2  $\mu\text{m}$ ; E 200 nm.

## Discussion

*Protonephridia.* In concordance with former work performed on gnathostomulids, the protonephridia in *A. orientis* consist of three cells, a monociliated terminal cell, a canal cell with a protonephridial duct and an intracellular lacunar system, and a nephroporus cell. Similar to what Lammert (1985) described for *Gnathostomula paradoxa* and *Haplognathia rosea* we found the distal part of the terminal cell to form lobes with interjacent small clefts, constituting the filtration area of the excretory organ (contrary to Graebner 1968). Also similar to *G. paradoxa* is

the eccentric position of nucleus, the numerous cisternae in the canal cell, and the extension of the canal cell into the nephroporus (Fig. 6E). With regards to the filamentous rods, it is striking that Lammert (1985) shows structures resembling ciliary rootlets in the canal cells of juvenile *G. paradoxa* and we think that the rods we describe are the equivalent to these structures. Having only cross sections through the protonephridial duct we cannot verify or reject the striated morphology of the rods we found in *A. orientis* and more complete and comprehensive data is needed to settle this point. However, it must be emphasized that they are clearly intracellular components of the canal cell and lie outside of the protonephridial duct (Fig. 6B, C, D) and from sections that lie in the planes where the terminal cell and the canal cell are intersecting it is obvious that these rods are not the microvilli, rootlets of microvilli, or ridges of microvilli.

In contrast to *G. paradoxa*, and more similar to *H. rosea*, the microvilli terminate at about half of the length of the protonephridial duct and the nucleus of the terminal cell is not positioned at the distal end but more centrally and overlaps for an undetermined distance with the nucleus of the canal cell (Fig 6C). By comparison within gnathostomulids and other bilaterians, the central position of the nucleus of the canal cell, surrounding the protonephridial canal, and the absence of a nephridial duct in *H. rosea* must be considered as apomorphies of the species (Ax 1985).

The continuation of the protonephridial duct into the nephroporus as found in *A. orientis* would be expected of any protonephridium, actually, at least of other animals, and shows that some characters have retained the plesiomorphic condition in the Austrognathiidae, opposite to *G. paradoxa* and *H. rosea*, in which the protonephridial duct is blind ended.

*Parenchyma.* Gnathostomulids are considered acoelomate and lacking any kind of parenchyme or mesenchyme (Mainitz 1979; Rieger 1985; Sterrer 1996). Potential parenchymal cells have previously only been reported as large translucent cells in *Valvognathia pogonostoma* (Kristensen & Nørrevang 1978) and in the caudal intestinal epithelium in Filospermoidea (Lammert 1986). However, Mainitz (1979; Figure 2) shows the stylet apparatus of *Gnathostomula jenneri* to be embedded in parenchymal tissue that is at least partly derived from gut cells; and with regard to the tissue surrounding the testes and spermatid ducts in filospemoideans Knauss & Rieger (1979) pointed out that: "...the presence of mesenchyme and its spatial arrangement need further EM investigation...by the use of complete serial sections". We found large cells with only few cell organelles in both species investigated in the area of the copulatory organs. Possibly this evident occurrence of such parenchymal cells is restricted to the Austrognathiidae but future comparative investigations have to shed light on this issue.

*Female system.* The investigated species show a high variability in that *A. glandifera* has a bursa and a vagina whereas *A. orientis* only has a weakly defined bursal tissue; a vagina could not be identified. These observations are representative for the diversity of the female organs found in the Conophoralia even though Sterrer *et al.* (1985) note that a vagina is a permanent feature in the Conophoralia. According to the same authors the bursa is a simple, hyaline, soft pouch and may only be present temporarily (Sterrer *et al.* 1985). However, it could well be that the bursa is only recognized in specimens that have recently copulated and have their bursa filled with allosperm. With regards to the characteristic appearance of conuli found in the bursa (Sterrer 1970; Sterrer *et al.* 1985) and our observations on the content of the bursa it must be noted that the transformation of the conuli on the way to fertilization is rather unique, and puzzling. The interior division of the bursa into an anterior and a posterior part and the special area at the anterior end of the bursa with interdigitations formed by cells of the bursal wall are reminiscent of the bipartition of the bursa and the unification of the layered cells of the anterior bursa as a nozzle-like "mouthpiece" in the Scleroperalia (Mainitz 1983; Sterrer *et al.* 1985). If we take into consideration that the bursa is simpler and more rounded and with fewer or no cristae in lower Scleroperalia (Sterrer *et al.* 1985; Sørensen *et al.* 2006), the organization found in *A. glandifera* could well represent the ground pattern for the Bursovaginoidea. However, a more detailed study of this organ and a phylogenetic scheme for the Austrognathiidae, preferably based on molecular markers, seem to be necessary to solve this issue. Nevertheless, the presence of bursal tissue, either being organized loosely or as a regionalized pouch, supports the unification of the Conophoralia and the Scleroperalia as the Bursovaginoidea.

*Sperm.* The development of the male gametes is in concordance with the description in *Austrognathia* sp. by Lanfranchi and Falleni (1998) and in line with the spermatogenesis described in Filospermoidea (Sterrer 1969; Knauss & Rieger 1979); the development of sperm in tetrads can be taken as the ground pattern in gnathostomulids. We further support the hypothesis of Sterrer *et al.* (1985; see Knauss & Rieger 1979) that the many "multiple conuli" cases found in live observations are due to a failed complete separation during

spermiogenesis. Like Lanfranchi and Falleni (1998), we found single basal bodies below the cingulum in some conuli (Fig. 7F, G), and together with the facts mentioned above we are convinced that each conulus is a single sperm and not a spermatophore (Sterrer 1965).

Species-specific differences in sperm morphology in the Austrognathiidae arise largely from differences in the maturation process from spermatids to sperm, namely spermiogenesis and not early spermatogenesis. The early-stage spermatids are still very similar in the investigated species mentioned above, but the change of shape and variations in the condensation and differentiation of the chromatin as well as the amount and positional restriction of intracellular components (e.g. mitochondria) finally determine the shape of the mature conuli.

*Male copulatory organ.* Our observations confirm light microscopy findings that the male copulatory organ is tripartite, composed of an anterior, glandular portion with medium-sized granules proximally and coarse-grained secretions distally and a posterior portion of a concentrically structured, sometimes twisted funnel (Sterrer 1997; 2001). This funnel represents a penis in both described species, being delineated by a basal lamina and having an ejaculatory duct as well as a gonopore. Differences occur first in the dimensions of the structures, the male copulatory organ of *A. glandifera* being massive compared to that of *A. orientis*, and second in the content of the cells constituting the penis, namely electron-lucent vesicles with a diameter of ~800 nm (Fig. 6F) in the former species versus electron-dense vesicles (~150 nm) and rods in the latter species (Fig. 3B, C, G). Summarizing, the male copulatory organs in the Conophoralia are uniform and we speculate that the minor differences found might be related to the presence or absence of a bursa.

On one hand the male copulatory organs are similar to those of the Scleroperalia, having an ejaculatory duct with a gonopore and part of the copulatory organ being an invagination of the body wall, as evidenced by the basal lamina of the penis. On the other hand they are similar to those of the Filospermoidea in that they are glandular and lack any kind of hard structure. With respect to the gland cells and their vesicles, it is tempting to speculate that these are homologous to the dorsal and ventral gland cells of the penis of *H. gubbarnorum* (as *H. cf. lyra*) as described by Knauss & Rieger (1979). In the same species, similar to *A. orientis*, the layers of the body-wall muscles are inverted in the area of the gonopore. Consequently, concerning the male copulatory organ, the Conophoralia show traits of the Filospermoidea (soft, glandular penis) and the Scleroperalia (penis is an invagination of the body wall), supporting the notion to be the “less derived” sister group of the Scleroperalia.

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