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Taxonomy and Phylogeny of Acotylea (Platyhelminthes: Polycladida) from Japan

（日本産無吸盤亜目ヒラムシ類（扁形動物門：多岐腸目）の系統分類学的研究）

Yuki Oya

A Ph.D. dissertation presented

to

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1. Oya, Y. and Kajihara, H. 2017. Description of a new *Notocomplana* species (Platyhelminthes: Acotylea), new combination and new records of Polycladida from the northeastern Sea of Japan, with a comparison of two different barcoding markers. Zootaxa 4282: 526–542.
2. Oya, Y. and Kajihara, H. 2019. A new species of *Phaenoplana* (Platyhelminthes: Polycladida) from the Ogasawara Islands. Species Diversity 24: 1–6.
3. Oya, Y., Kimura, T. and Kajihara, H. 2019. Description of a new species of *Paraplehnia* (Polycladida, Stylochoidea) from Japan, with inference on the phylogenetic position of Plehniidae. ZooKeys 864: 1–13.
4. Oya, Y. and Kajihara, H. 2020. Molecular phylogenetic analysis of Acotylea (Platyhelminthes: Polycladida). Zoological Science 37: 271–279.
5. Oya, Y., Tsuyuki, A. and Kajihara, H. 2020. A new species of *Zygantroides* (Platyhelminthes: Polycladida) from Amakusa, Japan. Species Diversity 25: 189–196.
6. Oya, Y. and Kajihara, H. Description and phylogenetic relationships of a new genus of Planoceridae (Polycladida, Acotylea) from Shimoda, Japan. Journal of the Marine Biological Association of the United Kingdom. [In review]
7. Oya, Y., Tsuyuki, A. and Kajihara, H. Two new stylochoplanid polyclads (Platyhelminthes: Polycladida) from Misaki, Japan. [In preparation]

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CONTENTS

I. GENERAL SECTION

INTRODUCTION

Polyclad systematics, morphology, and biology

Polycladida (Platyhelminthes) is a group of marine flatworms, traditionally placed at the taxonomic rank of order, and characterized by having entolecithal eggs and a numerously branched intestine (Cannon 1986). Phylogenetically, Polycladida is the sister to Prorhynchida in Platyhelminthes (Egger *et al*. 2015; Laumer *et al.* 2015). Polycladida currently contains more than 800 species (Tyler *et al.* 2006–2020). Polyclad flatworms have been divided into two suborders, Cotylea and Acotylea, based on the presence or absence, respectively, of the cotyl, a ventral sucker-like structure (Lang 1884).

The polyclad body is commonly flattened dorso-ventrally. The body shape in dorsal view varies from oval to elongated or ribbon-like (Prudhoe 1985). In length, polyclads vary from 1 mm to 150 mm but are mostly 10–60 mm (Prudhoe 1985). Their color and pattern also have a diversity among species. Polyclads in cold or temperate waters often have dull colors; on the other hand, many of tropical species have brilliant colors or flamboyant patterns (Newman and Cannon 2003). Their colorful appearance is indicated to be aposematic colorations (Ang and Newman 1998) or mimicry of particular sea slugs (Newman and Cannon 1994). In the head, polyclad flatworms frequently have tentacles or pseudotentacles; the latter are an upfolding of the anterior margin that looks like tentacles. In addition, polyclads usually possess numerous eyespots forming clusters such as marginal eyespots, located in the periphery of the body; cerebral eyespots, situated at the brain region; frontal eyespots, scattered between the brain and the anterior margin of body; and tentacular eyespots, sited at around or in the tentacles (Faubel 1983). On the ventral side, polyclads have a plicated pharynx contained in a pharyngeal cavity. The pharynx is separated into two types, ruffled or tubular, based on its structure (Prudhoe 1985). The pharyngeal cavity is located more or less centrally but may be located anteriorly or posteriorly and opens as the mouth on the ventral surface. The male and female gonopores open posterior to the mouth; some polyclads have the common gonopore where the male and female reproductive systems open together. The cotyl, when present, is situated posteriorly to the female or common gonopore.

The copulatory apparatuses in polyclads show a morphological diversity. All polyclads are simultaneous hermaphrodites, generally having the male and female copulatory organs posterior to the pharynx. The male copulatory apparatus consists of several sac-like organs (prostatic vesicle, seminal vesicle, and spermiducal bulbs) and an intromittent organ (Faubel 1983). The sperms transferred by a pair of sperm ducts are given off though these sac-like and intromittent organs. The intromittent organs are classified into two types, the penis and cirrus. The penis is a papillose or rod-like structure and sometimes possesses a cuticularized part called stylet. The cirrus is an eversible organ and usually lined with hard structures (*e.g.,* spines, thorns, or teeth). The female copulatory apparatus consists of the vagina and various accessory structures (Faubel 1983). At the proximal end of the vagina, one of the following three structures may be found: *i*) a sac-shaped organ called the Lang's vesicle, *ii*) the genito-intestinal duct, which connects the proximal end of the vagina to the intestine, and *iii*) the vaginal duct, which leads to another opening apart from the female gonopore. Some species lack the Lang's vesicle, the genito-intestinal duct, and the vaginal duct altogether. In the tract of the vagina, a blind sac called the bursa copulatorix or a region called the vagina bulbosa is sometimes developed. These are observable by histological techniques and treated as important taxonomic characters in the polyclad systematics.

Polyclads are found in various marine environments. Most of polyclads live in shallow coastal areas (Prudhoe 1985). However, their vertical distribution ranges from the supralittoral zone (Newman and Cannon 1997) to the bathyal zone down to 3,232 m (Quiroga *et al.* 2006). The majority of polyclads are benthic, living on various substrates such as rocks, muddy bottom, seaweeds and seagrasses. Some polyclads are associated with other invertebrates such as crustaceans (crabs, hermit crabs), echinoderms (brittle stars, sea urchins), cnidarians (gorgonians, black corals), mollusks (bivalves, chitons, gastropods), and tunicates (*cf.* Graff 1903; Bock 1925; Kato 1933; Prudhoe 1985; Bo *et al.* 2019). A few species of interstitial (*e.g.,* Curini-Galletti *et al.* 2008) and pelagic (*e.g.,* Kato 1938a) polyclads are also described.

Generally, polyclads are predators feeding on other invertebrates such as ascidians (Newman and Cannon 2003), crustaceans (Merory and Newman 2005), and mollusks (Philips and Chiarappa 1980); some polyclads are significant pests in commercial oyster cultures (Newman and Cannon 2003). Polyclads tend to be specialists; they eat only particular preys (Galleni *et al.* 1980).

Polyclads employ diverse strategies for insemination. They undergo internal fertilization (Prudhoe 1985), where three methods of insemination are known: direct copulation, dermal impregnation, and hypodermic insemination. The direct copulation is reciprocal, with each partner contributing and receiving sperm, while in the latter two methods, sperm transfer may be unilateral from one individual to the other (Rawlinson *et al.* 2008). The dermal impregnation is performed by depositing spermatophores onto the dorsal surface of the partner; sperm are absorbed through the epidermis and then migrate through the parenchyma to the eggs. The hypodermic insemination is executed by injecting sperm anywhere through the epidermis of another individual using the penis stylet.

The ontogeny of polyclads is unique among turbellarians. Polyclads are the only turbellarians along with lecithoepitheliates that exhibit a clear spiral cleavage and are therefore important models for studying embryonic development in turbellarians (Gammoudi *et al.* 2012a). In addition, not only direct but also indirect development modes are known in polyclads. Free-living flatworms commonly hatch as juvenile worms (direct development) (Martín-Durán and Egger 2012). In indirect-developing species, planktonic larvae such as Götte's, Kato's, or Müller's larva are produced before metamorphosing into juveniles (Martín-Durán and Egger 2012).

Suborder Acotylea

Acotylea is an assemblage of polyclads lacking the cotyl and contains more than 350 species in 29 families (Faubel 1983; Dittmann *et al.* 2019; Litvaitis *et al.* 2019). In external characters, nuchal tentacles located near the cerebral organ are almost exclusively observed in Acotylea among Polycladida. In addition, most acotylean species have a ruffled pharynx but only a few species have a tubular pharynx. In the internal morphology, a posteriorly directed male copulatory apparatus and a Lang's vesicle are representative characters in Acotylea (*cf.* Faubel 1983); some acotyleans lack nuchal tentacles or a Lang's vesicle.

Taxonomic studies of Acotylea in Japan

In Japan, taxonomic studies of Acotylea had been actively conducted in shallow waters until 1940s along with those of Cotylea. Polyclads were reported from Japan for the first time by Stimpson (1855) as to species collected in Okinawa Islands and Kikaishima Island. Later, Stimpson (1857) described several species from Amami Island, Shizuoka, and Hokkaido. The descriptions by Stimpson (1855, 1857) contain both acotyleans and cotyleans but are only brief statements based on observations of external morphology without accompanied by any illustration; large part of Stimpson's species are now treated as "*incertae sedis*" by subsequent researchers (*e.g.,* Faubel 1984). In the 20th century, Yeri and Kaburaki (1918a, 1918b, 1920) investigated polyclads in Kanagawa, Shizuoka, and Wakayama; Kaburaki (1923) reported two acotylean species from Hokkaido. In almost the same period, Bock (1923, 1924) described three acotyleans in Kanagawa and Hyogo. From the 1930s to 1940s, Kojiro Kato (1906–1981) actively surveyed Japanese polyclad fauna in Hokkaido (Kato 1937a, 1937b), Aomori (Kato 1939a), Miyagi (Kato 1939b), Kanagawa (*e.g.,* Kato 1937c), Shizuoka (*e.g.,* Kato 1934, 1937d), Wakayama (Kato 1938b) and Kumamoto (Kato 1938c). Finally, Kato (1944) summarized his and previous studies in a monograph entitled *Polycladida of Japan*. Unfortunately, the specimens collected by Kato were destroyed by fire during World War II (Sudzuki 1981).

After Kato (1944), taxonomic studies of Acotylea have been sporadically carried out (Hyman 1955; Cannon and Grygier 1991; Hagiya 1992, 1993, 2013; Hagiya and Gamo 1992; Tajika and Ishida 1999). In addition to these studies, a single species of acotylean was illustrated in pictorial books (Iwase *et al.* 1990; Nunomura 1992). Thus, 79 species of Acotylea had been in Japan reported before I initiated my research (Kato 1944; Hyman 1955; Iwase *et al.* 1990; Cannon and Grygier 1991; Hagiya 1992;

Nunomura 1992).

It is expected that there are numerous undescribed acotylean species in Japan. Compared to cotylean polyclads, the majority of acotyleans lack conspicuous characters in the external morphology; even representatives from different families may have a similar appearance to each other. Thus, species identification of acotylean polyclads requires histological examination. However, polyclads tend to be ignored even if they are captured because of the difficulty of handling (*cf.* Prudhoe 1985). Considering *i*) the absence of specialists for many years, *ii*) the elusive nature of visual recognition of species based on the external appearance, and *iii*) the time-consuming species identification method, the alpha taxonomy of Japanese acotyleans was anticipated to be incomplete even as to species occurring in the intertidal zone with relatively easy accessibility, much less to deep-water species. In this thesis, taxonomic accounts on shallow- and deep-water species are presented in Chapters II and III, respectively.

Acotylean systematics and phylogenetics

Currently, the classification of Acotylea is in a transition stage from a morphology-based system to a phylogeny-based one. Families in Acotylea have been classified into three superfamilies on the basis of morphological characters. First, Bock (1913) distinguished acotyleans into three “Sectio” (sections) based on eyespot distributions: Craspedommata (with marginal eyes), Emprosthommata (with frontal eyes and without marginal eyes), and Schematommata (without frontal and marginal eyes). Later, Bock’s (1913) sections were regarded as superfamilies by Poche (1926) and renamed Stylochides, Cestoplanides, and Planocerides, respectively; these superfamily names were emended to Stylochoidea Stimpson, 1857, Cestoplanoidea

Lang, 1884, and Planoceroidea Stimpson, 1857 by Nicoll (1935, 1936) and Prudhoe (1982). Marcus and Marcus (1966) also raised three sections of Bock (1913) to the superfamilies: Craspedommatidea Marcus and Marcus, 1966, Emprosthommatidea Marcus and Marcus, 1966, and Schematommatidea Marcus and Marcus, 1966. This system, based on the eyespot distributions, was also followed by Prudhoe (1985). On the other hand, Faubel (1983, 1984) established a new classification of Acotylea based on the morphology of copulatory apparatuses, consisting of Ilyplanoidea Faubel, 1983 (without prostatic vesicle), Leptoplanoidea Ehrenberg, 1831 (with interpolated prostatic vesicle), and Stylochoidea *sensu* Faubel (1983) (with free prostatic vesicle). At lower taxonomic levels, Faubel (1983) split many existing families and genera, whereas Prudhoe (1985) tended to retain the earlier taxonomy. The first attempt to reconstruct the polyclad classification using molecular sequence data was performed by Bahia *et al.* (2017). Bahia *et al.* (2017) inferred a phylogenetic relationship in Polycladida using partial sequences of 28S ribosomal DNA, and indicated that their phylogenetic tree conflicted the superfamily concepts of Faubel (1983, 1984) and Prudhoe (1985). In addition, two families formerly placed in the Acotylea, Cestoplanidae Lang, 1884 and Theamatidae Marcus, 1949, were encompassed in the cotylean clade (Bahia *et al.* 2017). In the clade of Acotylea, Bahia *et al.* (2017) detected three major clades which were defined by morphological characters as Cryptoceloidea Laidlaw, 1903, Leptoplanoidea, and Stylochoidea. Later, Cryptoceloidea was renamed Discoceloidea Laidlaw, 1903 based on an extended majority-rule consensus of 12 trees based on 28S sequences from polyclad flatworms (Dittmann *et al.* 2019) and Stylochoidea was revealed to lack any morphological synapomorphies by a 28S phylogeny with improved taxon sampling (Litvaitis *et al.* 2019). It eventually turned out that none of the three superfamilies could be effectively circumscribed by morphology (Dittmann *et al.* 2019). Acotylea currently comprises the superfamilies Discoceloidea, Leptoplanoidea, and Stylochoidea, which are recognizable only through molecular data (Dittmann *et al*. 2019; Litvaitis *et al.* 2019).

Acotylean molecular phylogenetics should be improved by denser and broader taxon sampling. Previous studies provided frameworks for a new classification of Acotylea (Aguado *et al*. 2017; Bahia *et al.* 2017; Tsunashima *et al.* 2017; Dittmann *et al.* 2019; Litvaitis *et al.* 2019). However, more than half of the families within Acotylea have not been employed in the molecular phylogenetic analyses. In addition, quite a lot of family- and genus-level taxa defined morphologically appear to be not monophyletic when different tree shapes of previous works are integrated (*cf.* Dittmann *et al.* 2019; Kenny *et al.* 2019; Litvaitis *et al.* 2019). To establish a more systematic classification, as many families and genera as possible must be incorporated in analyses. This topic is addressed in Chapter IV, where I present the results of molecular phylogenetic analyses of Acotylea based on a broader taxon sampling and expanded molecular markers. In line with the obtained tree topology, I will also discuss the superfamily-, family-, and genus-level taxonomy of Acotylea.

MATERIAL AND METHODS

Specimen collections. All specimens were collected from the coasts around Japan from 2015 to 2019 (Fig. I-1, Table I-1). In the Ogasawara Islands, I carried out sampling under a permission from the Tokyo Metropolitan Government to collect wild animals (Permission No. 28, Special Permission No. 29). In the intertidal zone, acotyleans were collected from undersurface of stones or dead corals, seaweeds, and mud sediments. In the subtidal zone, flatworms were captured from undersurface of stones or dead corals by hand with SCUBA diving. Bathyal specimens were collected by dredging during the research cruise No. 1722 by Training/Research Vessel (TRV) *Seisui-maru*, Mie University and the 18th Japanese Association for Marine Biology (JAMBIO) Coastal Organism Joint Survey by TRV *Tsukuba II*, University of Tsukuba.

Observations in life and fixation. Live specimens were anesthetized in a $MgCl_2$ solution prepared with tap water so that it had the same refractive index (or 'salinity') as seawater (as determined using an IS/Mill-E refractometer (AS ONE, Japan)), and then photographed with a Nikon D5300 digital camera with external strobe lighting provided by a pair of Morris Hikaru Komachi Di flash units. A piece of the body margin was cut off from each specimen and fixed in 100% ethanol for DNA extraction. The rest of the body was fixed in Bouin's solution for 24 h and preserved in 70% ethanol.

Histological observation. Material fixed in Bouin's solution was later dehydrated in an ethanol series, cleared in xylene, embedded in paraffin, sectioned at 7 µm thickness, stained with Delafield's hematoxylin and eosin, and mounted on glass slides in Entellan New (Merck, Germany). Sections were observed under an Olympus BX51 compound microscope and photographed with a Nikon D5300 digital camera. Schematic diagrams were prepared with Adobe Illustrator CS6 based on the photographs of serious sections stacked by ImageJ (Rasband *et al.* 1997−2018).

Specimens were identified morphologically to the genus or species level. Type and voucher specimens have been deposited in the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo, Japan. The specimens I used in this study are listed in Table I-2.

Primer design. Primers newly designed in this study were configured by using PerlPrimer ver. 1.1.21 (Marshall 2003–2011). The primers for cytochrome *c* oxidase subunit I (COI) (Acotylea_COI_F and Acotylea_COI_R) were designed based on the complete mitochondrial genome sequences (Golombek *et al.* 2015; Aguado *et al.* 2016) from four polyclad species: *Stylochoplana maculata* (Quatrefages, 1845) (Accession number: KP965863); *Hoploplana elisabelloi* Noreña, Rodríquez, Pérez and Almon, 2015 (KT363735); *Enchiridium* sp. (KT363734); and *Prosthiostomum siphunculus* (Delle Chiaje, 1822) (KT363736). The primers for nested PCR of COI (nestCOI_F1 and nestCOI_R1) were based on the COI sequences of personal specimens. The internal primer for 28S rDNA (28S) (hrms_fw2) and the primer set of 18S rDNA (18S) (hrms18S_F, hrms18S_Fi1, hrms18S_Fi2, hrms18S_R, hrms18S_Ri1, hrms18S_Ri2) were prepared by sequences derived from personal specimens preliminary determined with a pair of PCR primers, 1F and 9R, developed by Giribet *et al.* (1996). The primers I used in this study are listed in Table I-3.

DNA extraction, PCR amplification, and sequencing. Total DNA was extracted by using a DNeasy Blood and Tissue Kit (Qiagen, Germany) or the protocol by Boom *et al.* (1990). Four gene markers, 16S rDNA (16S), 18S, 28S, and COI, were amplified by polymerase chain reaction (PCR) in 10 µl reaction volumes each containing 1 µl of total DNA template, 1 µl of 10 × Ex Taq buffer (Takara Bio, Japan), 2 mM each dNTP, 1 µM each primer (Table I-3), and 0.25 U of Takara Ex Taq DNA polymerase (5 U/µl; Takara Bio) in deionized water. PCR amplification conditions were 94°C for 1 min; 35 cycles of 94°C for 30 s, 50°C (16S, 18S, and COI) or 52.5°C (28S) for 30 s, and 72°C for 45 sec (16S), 2 min (18S), 1.5 min (28S), or 1 min (COI); and

72°C for 7 min. PCR products were purified enzymatically by using ExoSAP-IT^{TM} reagent. All nucleotide sequences were determined by direct sequencing with a BigDye Terminator Kit ver. 3.1 and 3730 Genetic Analyzer (Life Technologies, USA) using the primers listed in Table I-3. Sequences were checked and edited with MEGA ver. 7 (Kumar *et al.* 2016). The sequences determined in this study have been deposited in DDBJ/EMBL/GenBank databases.

Phylogenetic analyses. Additional sequences from Acotylea were downloaded from GenBank. Alignment of 16S, 18S, and 28S sequences was done with MAFFT ver. 7 (Katoh and Standley 2013). Ambiguous sites in the aligned 16S, 18S, and 28S alignments were removed with Gblocks ver. 0.91b (Castresana 2002). Alignment of COI was done manually with MEGA ver. 7 (Kumar *et al.* 2016). Optimal substitution models for the analyses were selected with Partitionfinder ver. 2.1.1 (Lanfear *et al.* 2016) under the Akaike information criterion (AIC) (Akaike 1974) using the greedy algorithm (Lanfear *et al.* 2012).

Phylogenetic analyses were performed with the maximum-likelihood (ML) method executed in IQtree ver. 1.6 (Nguyen *et al.* 2015) under a partition model (Chernomor *et al*. 2016) and with Bayesian inference (BI) executed in MrBayes ver. 3.2.2 (Ronquist and Huelsenbeck 2003) using a concatenated dataset from the four genes. Nodal support within the ML tree was assessed by analyses of 1000 bootstrap pseudoreplicates (Felsenstein 1985). For BI, the Markov chain Monte Carlo (MCMC) process used random starting trees and involved four chains run for 1,000,000–10,000,000 generations, with the first 25% of trees discarded as burn-in. I considered posterior probability (PP) values ≥ 0.90 and ML bootstrap (BS) values ≥ 70% as indicating clade support; in the text, combined nodal support is indicated as PP/BS.

II. DESCRIPTIONS OF ACOTYLEA IN THE INTERTIDAL ZONE IN JAPAN

INTRODUCTION

Update of Japanese acotylean taxonomy has been seldom performed since Kato's (1944) monograph, with only three new species, *Notoplana comes* Cannon and Grygier, 1991, *Notoplana rugulosa* Hagiya, 1992, and *Pseudostylochus ostreophagus* Hyman, 1955, being described from Okinawa, Iwate, and Miyagi, respectively (Hyman 1955; Cannon and Grygier 1991; Hagiya 1992). These species show an inconspicuous appearance in term of body coloration and closely resemble other known congeners. Therefore, it is anticipated that numerous undescribed acotylean species will be discovered if detailed observations with histological technique are carried out.

In addition, before I started my research, little molecular information for DNA barcoding such as partial sequences of COI was available for species from not only Japan but also other regions of the world. To make DNA barcoding possible, accumulation of nucleotide sequence data tagged with taxon names based on reliably identified material is prerequisite.

In this chapter, I describe eight species (five genera, three families, two superfamilies) of Acotylea from the intertidal zone along the Japanese coasts. I provide a partial sequence of COI gene for each species for the purpose of future DNA barcoding. Four of five genera (*Allioioplana* Plehn, 1896, *Armatoplana* Faubel, 1983, *Phaenoplana* Faubel, 1983, *Zygantroides* Faubel, 1983) dealt with in this chapter have not been reported from Japanese waters.

MATERIAL AND METHODS

Polyclads were collected from the sites numbered 2A–C, 5, 6B, and 11 in Fig. I-1 (Table I-1). Methods of observations in life, fixations, and histological observations are described in Chapter I; for *Zygantroides*, living specimens were photographed without anesthetization. The COI sequences were determined following the methods of DNA extraction, PCR amplification, and sequencing described in Chapter I; for *Phaenoplana*, the COI sequence was amplified by nested PCR. The first PCR amplification conditions followed the standard protocol showed in Chapter I. The second PCR conditions were 94°C for 5 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min; and 72°C for 7 min, with the nested internal primer pair, nestCOI_F1 and nestCOI_R1 (Table I-3). The template for the second PCR was used 1 μl of the first PCR product diluted by 50 times with deionized water; other components of the PCR mixture was follwed the protocol described in Chapter I. Genetic distances of COI were calculated using MEGA ver. 7.

TAXONOMY

Superfamily **Discoceloidea** Laidlaw, 1903

Family **Ilyplanidae** Faubel, 1983

Genus ***Zygantroides*** Faubel, 1983

Redefinition. Ilyplanidae without tentacles and marginal eyespots. Pharynx somewhat oriented posteriorly. Genital tracts entering separately into common genital atrium; male complex with seminal vesicle and papillate penis. Distal part of ejaculatory duct lined with glandular ciliated epithelium. Lang's vesicle present (see Remarks).

Zygantroides serpulidicola Oya, Tsuyuki and Kajihara, 2020

(Figs II-1 to II-4)

Zygantroides serpulidicola Oya, Tsuyuki and Kajihara, 2020: pp. 189–196, figs 1–4 [Tsujishima Island, Kumamoto, Japan].

Etymology. The new specific name serpulidicola is a noun in apposition and refers to the habit that the flatworms dwell on tubes of serpulid annelids (Fig. II-1).

Material examined. Holotype: ICHUM 6023, sagittal sections, 6 slides, intertidal (32°33′09″N, 130°06′32″E), Tsujishima Island, Kumamoto, Japan, 6 June 2019, collected by Y. Oya and A. Tsuyuki.

Paratypes (3 specimens, sagittal sections, all from the type locality, collected by Y. Oya and A. Tsuyuki): ICHUM 6024, 4 slides, 6 June 2019; ICHUM 6025, 4 slides, 13 June 2018; ICHUM 6026, 5 slides, 13 June 2018.

Diagnosis. *Zygantroides* with oval body, elongated Lang's-vesicle duct, Lang's vesicle positioned posterior to common gonopore, and without common sperm duct (Figs II-2, II-3).

Description. Live specimens 6.7–8.1 mm (7.9 mm in holotype) in length, 2.7–4.8 mm (3.8 mm in holotype) in maximum width. Body oval, narrow toward posterior end when elongated (Fig. II-2A, B). Ground body color translucent to whitish opaque (Fig. II-2A–C). Pale brown maculae sparsely scattered on dorsal surface (Fig. II-2A, B).

General appearance of body whitish to orangish (Fig. II-2A, B). Tentacles lacking (Fig. II-2D). Pair of tentacular eye clusters, each containing 8–19 eyespots (11 in right cluster, 11 in left cluster in holotype, Fig. II-2D). Pair of cerebral eye clusters, each consisting of 8–22 eyespots (10 in right cluster, 8 in left cluster in holotype, Fig. II-2D), arranged near median line and congregated anterior to tentacular eye cluster. Pharynx whitish, ruffled in shape, occupying about three-eighths of body length (2.6–2.9 mm in length, 2.7 mm in holotype), located at almost center of body (Fig. II-2C). Intestine not anastomosed, spreading throughout body except margin and brain region. Pair of whitish sperm ducts visible through ventral body wall (Fig. II-2C).

Mouth situated posterior to pharynx and near common gonopore (Figs II-3, II-4A–C). Male copulatory apparatus located posterior to pharynx, consisting of seminal vesicle and penis papilla; prostatic vesicle lacking (Fig. II-4A–C). Pair of sperm ducts running anteriorly, then turning medially at point about one-fourth length of pharynx from posterior end, subsequently running posteriorly along both sides of pharynx and extending further posteriorly for short distance beyond level of posterior end of pharynx, before turning medially (Fig. II-2C) to enter separately proximal end of bean-shaped seminal vesicle with strong muscular wall (Fig. II-4A, C). Distal end of seminal vesicle connecting to ejaculatory duct. Ejaculatory duct having thin muscular wall lined with prostatic-like glandular epithelium; shape of ejaculatory duct varying from vesicular to cylindrical depending on fixation (Fig. II-4B, C). Distal end of ejaculatory duct connecting to penis papilla. Penis papilla small, without stylet, projecting into cylindrical male atrium. Male atrium lined with smooth epithelium.

Pair of oviducts forming common oviduct, latter running posteriorly to enter vagina. From this point, elongated Lang’s-vesicle duct, lined with smooth ciliated epithelium, running ventrally, then curving posteriorly to connect to Lang's vesicle (Fig. II-4D). Lang's vesicle horseshoe-shaped, with tips orienting anteriorly and reaching to level where Lang's-vesicle duct connects to vagina (Fig. II-3). Inner epithelium of Lang's vesicle similar to that in vagina and Lang's-vesicle duct (Fig. II-4D). Vagina lacking ampulla, lined with smooth, ciliated epithelium, curving postero-dorsally, running anteriorly, then recurving postero-ventrally to exit at common genital atrium (Figs II-3, II-4D). Cement glands surrounding about two-thirds of distal part of vagina. Lang's-vesicle duct and vagina surrounded by circular muscle fibers. Common genital atrium opening posterior to mouth as common gonopore.

Habitat. Found on tubes of serpulid annelids on the undersurface of stones in the intertidal zone (Fig. II-1).

Sequence of COI. The partial COI sequences (712 bp) from the four specimens (LC528160–LC528163) almost coincided with each other. The uncorrected p-distance among specimens showed 0.000–0.007.

Remarks. I extend the generic diagnosis of *Zygantroides*. Faubel (1983, p. 40) included "Female apparatus directed backwards and oriented dorsal to the male complex" in the generic diagnosis. The female copulatory apparatus in the present species directs somewhat posteriorly but it is not arranged dorsally to the male copulatory apparatus (Fig. II-3). However, *Z. serpulidicola* satisfies other diagnostic characters of *Zygantroides*: absence of tentacles, eyespot distribution, and structure of male and female copulatory organs (Figs II-2, II-4). Therefore, I judge it is more appropriate to redefine *Zygantroides* by eliminating the character of orientation of female apparatus than to establish a new genus-level taxon. The horseshoe-shaped Lang's vesicle such as that in *Z. serpulidicola* has been often employed in genus-level definition (*cf.* Faubel 1983); however, I do not reflect this trait in the redefinition because the shape of Lang's vesicle in other *Zygantroides* is not described (Corrêa 1949).

Zygantroides serpulidicola can be distinguished from the two congeners *Z. henriettae* (Corrêa, 1949) and *Z. plesia* (Corrêa, 1949) by the following characters: *i*) the position of the mouth (posterior to pharynx in *Z. serpulidicola*; at one-third from posterior end of the pharynx in *Z. henriettae*; at two-fifths from posterior end of the pharynx in *Z. plesia*), *ii*) the presence/absence of the common sperm duct (absent in *Z. serpulidicola*; present in *Z. henriettae* and *Z. plesia*), *iii*) the shape of the seminal vesicle (bean-shaped in *Z. serpulidicola*; spindle-shaped in *Z. henriettae*; spherical in *Z. plesia*), *iv*) the length of the Lang's-vesicle duct compaired with that of the vagina (about one-third in *Z. serpulidicola*; about one-tenth in *Z. henriettae*; about one-twentieth in *Z. plesia*), *v*) the position of the Lang's vesicle (posterior to the common gonopore in *Z. serpulidicola*; anterior to the common gonopore in *Z. henriettae* and *Z. plesia*), and *vi*) the presence/absence of an ampulla in the vagina (absent in *Z. serpulidicola* and *Z. henriettae*; present in *Z. plesia*) (Table II-1).

This is the first report of *Zygantroides* from the Pacific Ocean. Other two congeners are only reported from the Atlantic coast of southeastern Brazil (Corrêa 1949; Bahia and Schrödl 2018). The two Brazilian species have been captured from between algae, whereas this species was found on tubes of annelids.

Superfamily **Leptoplanoidea** Ehrenberg, 1831

Family **Notocomplanidae** Litvaitis, Bolaños and Quiroga, 2019

Remarks. After publication of Oya and Kajihara (2017), Litvaitis *et al.* (2019) established a new family Notocomplanidae based on their molecular phylogeny. However, the clade of Notocomplanidae in the sense of Litvaitis *et al.* (2019) did not contain *Notocomplana humilis* (Stimpson, 1857), the type species of the type genus of Notocomplanidae (see Chapter IV).

Genus ***Notocomplana*** Faubel, 1983

Remarks. In Oya and Kajihara (2017), *Melloplana japonica* (Kato, 1937) then assigned in Pleioplanidae Faubel, 1983 was transferred to *Notocomplana* based on the morphological observation and comparison with other *Notocomplana* species. Faubel (1983) defined *Melloplana* Faubel 1983 as pleioplanids without a penis stylet, while *Notocomplana* as notoplanids without a penis stylet. Litvaitis *et al.* (2019) synonymized *Melloplana* with *Notocomplana* and Pleioplanidae with Notoplanidae, separating Notocomplanidae from Notoplanidae. However, this taxonomic change made by Litvaitis *et al.* (2019) is questionable (see Chapter IV).

Notocomplana hagiyai Oya and Kajihara, 2017

(Figs II-5 to II-7)

?*Notoplana* sp. Hagiya (1993), p. 36, pl. 4, figs 1–3, pl. II, fig. A [Otsuchi, Iwate, Japan]; Hagiya (2013), pp. 73–74, pl. I, figs 4, 13, 14, pl. II, fig. 5 [Rebun

Island, Hokkaido, Japan].

?*Notoplana japonica*: Tokinova (2008), pp. 55–56, pl. IV, figs 1–3 [Kamenka Bight, Peter the Great Bay, Russia].

Notocomplana hagiyai Oya and Kajihara, 2017: pp. 529–532, figs 1, 2 [Oshoro, Hokkaido, Japan].

Etymology. The specific name is a noun in the genitive case honoring Morio Hagiya (1948–2015), a former high-school teacher in Japan (Kajihara 2016) who studied Japanese polyclads for a long time with great fortitude and indomitable spirit. Hagiya (1993, p. 36; 2013, p. 75) indicated that his "*Notoplana* sp." likely represented an undescribed species, but unfortunately passed away before describing it.

Material examined. Holotype: ICHUM 5267, sagittal sections, 7 slides, intertidal, (43°12′36″N, 140°51′28″E), Oshoro, Hokkaido, Japan, 22 March 2016, collected by Y. Oya.

Paratypes (11 specimens, all from the type locality, collected by Y. Oya): ICHUM 5262, sagittal sections, 7 slides, 26 February 2015; ICHUM 5263, sagittal sections, 10 slides, 26 February 2015; ICHUM 5264, sagittal sections, 4 slides, 11 May 2015; ICHUM 5265, sagittal sections, 8 slides, 11 May 2015; ICHUM 5266, sagittal sections, 5 slides, 25 May 2015; ICHUM 5268, sagittal sections, 4 slides, 9 May 2016; ICHUM 5269, sagittal sections, 6 slides, 9 May 2016; ICHUM 5270, cross sections, 7 slides, 9 May 2016; ICHUM 5271, sagittal sections, 5 slides, 16 May 2016; ICHUM 5272, cross sections, 5 slides, 16 May 2016; ICHUM 5273, sagittal sections, 4 slides, 30 May 2016.

Diagnosis. *Notocomplana* with distally projecting intra-prostatic ejaculatory duct, prostatic vesicle larger than seminal vesicle, anteriorly curved vagina, and Lang's-vesicle duct shorter than long axis of Lang's vesicle and without common sperm duct (Figs II-5, II-6).

Description. Live specimens 8.9–15 mm (12 mm in holotype) in length, 4.0–6.5 mm (4.5 mm in holotype) in maximum width. Body elongate oval, narrow toward posterior end. Ground body color translucent to whitish opaque (opaque in holotype). General appearance of body varying from light yellowish brown to orange or brown (light yellow brown in holotype; Fig. II-5A, B). Dorsal body tinged with brown due to minute granules scattered over entire surface except around margin. Dorsal surface of body around pharynx brown. Body margin translucent. Pair of small tentacular knobs present at about one-fifth body length (1.6–2.7 mm, 2.4 mm in holotype) from anterior end but hardly visible in anesthetized state. Tentacular eye clusters consisting of 11–21 eyespots (14 in right cluster, 12 in left cluster in holotype, Fig. II-5C). Cerebral eye clusters consisting of 12–31 eyespots (16 in right cluster, 18 in left cluster in holotype, Fig. II-5C), arranged along median line and congregated anterior to tentacular eye clusters. Pharynx cream colored, ruffled in shape, occupying one-third of body length (2.6–4.9 mm, 3.6 mm in holotype) (Fig. II-5B). Mouth opening at almost center of pharyngeal cavity. Intestine not anastomosed, spreading throughout body except margin. Pair of whitish sperm ducts and oviducts visible through ventral body wall (oviducts not seen in holotype). Male and female gonopores separate; male gonopore opening at about three-eights of body length (3.1–4.7 mm, 4.6 mm in holotype) from posterior end; female gonopore situated 0.7–1.1 mm (0.8 mm in holotype) posterior to male gonopore (Fig. II-5B).

Male copulatory apparatus located immediately posterior to pharynx, consisting of seminal vesicle, interpolated prostatic vesicle, and penis papilla (Figs II-6, 7A). Pair of sperm ducts running anteriorly, then turning medially at point about one-fourth length of pharynx from posterior end, and subsequently curving posteriorly along both sides of pharynx and running for short distance beyond level of posterior end of pharynx, before turning medially (Fig. II-5B) to enter separately proximal end of bean-shaped seminal vesicle with strong muscular wall. Intra-prostatic ejaculatory duct projecting deeply and distally into prostatic vesicle. Canals of extra-vesicular glands penetrating wall of prostatic vesicle. Prostatic vesicle pear-shaped, possessing 9–10 longitudinal tubular chambers surrounding intra-prostatic ejaculatory duct (n = 2; Fig. II-7B), with strong muscular wall, larger than seminal vesicle, coated with thick muscular wall. Distal end of prostatic vesicle connecting to post-prostatic ejaculatory duct. Post-prostatic ejaculatory duct with thin muscular wall leading to penis papilla. Penis papilla muscular, conical, without stylet, projecting into cylindrical male atrium. Male atrium lined with ciliated, smooth epithelium.

Pair of oviducts forming common oviduct, latter running dorso-anteriorly to enter vagina (Fig. II-7C). From this point, Lang's-vesicle duct, lined with folded ciliated epithelium, leading posteriorly to Lang's vesicle. Lang's vesicle short (half of Lang's-vesicle duct), sac-shaped, lined with columnar cells (Fig. II-7C). Vagina lined with smooth and ciliated epithelium, curving antero-ventrally, running posteriorly, then recurving ventrally to exit at female gonopore (Fig. II-7C). Lang's-vesicle duct and vagina surrounded by circular muscle fibers; vagina surrounded by numerous cement glands (not much developed in holotype).

Habitat. Found intertidally on the undersurface of stones along sheltered rocky shore.

Sequence of COI. The partial COI sequences (712 bp) from the eight specimens (LC176036–LC176043) almost coincided with each other. The uncorrected p-distance among specimens showed 0.001–0.010.

Remarks. Among 31 species currently classified in *Notocomplana* (Tyler *et al.* 2006–2020), *N. hagiyai* most closely resembles *N. acticola* (Boone, 1929) and *N. sanjuania* (Freeman, 1933) in that they share the following four characters: *i*) the sperm ducts enter the seminal vesicle separately, *ii*) the intra-prostatic ejaculatory duct projects distally into the prostatic vesicle, *iii*) the vagina is directed dorso-anteriorly from the female gonopore, then curves backward, and *iv*) the Lang's vesicle assumes a short sac in shape. *Notocomplana hagiyai* differs from *N. acticola* in the length of the Lang's vesicle duct (shorter than the long axis of the Lang's vesicle in *N. hagiyai*; longer than the long axis of the Lang's vesicle in *N. acticola*); it differs from *N. sanjuania* in the size of the prostatic vesicle (larger than the seminal vesicle in *N. hagiyai*; smaller than the seminal vesicle in *N. sanjuania*) (Table II-2).

The morphological characters of *N. hagiyai* agree with those of "*Notoplana* sp." reported from Iwate and Rebun Island, Japan (Hagiya 1993, 2013). Hagiya (1993, 2013) indicated "*Notoplana* sp." resembles "*Notoplana japonica*" of Kato (1937d), but can be distinguished from the latter by *i*) the width between the male and female gonopores (wide in "*Notoplana* sp."; narrow in "*Notoplana japonica*"), and *ii*) the course of the vagina (curved anteriorly in "*Notoplana* sp."; almost vertical in "*Notoplana japonica*"). The width between the two gonopores in "*Notoplana* sp." is almost twice as wider as that in "*Notoplana japonica*" (Hagiya 1993, 2013). These characters are also observed in *N. hagiyai*.

Notocomplana hagiyai also seems to be identical to "*Notoplana japonica*" reported from Peter the Great Bay by Tokinova (2008). Tokinova's (2008, pl. 4, fig. 3) specimens also had the above-mentioned two morphological characters indicated by Hagiya (1993, 2013). Future molecular data from these localities should confirm the actual distribution of *N. hagiyai*.

Notocomplana japonica (Kato, 1937)

(Figs II-8, 9)

Notoplana japonica Kato, 1937d: pp. 215–216, text-figs 5, 6, pl. XIV, figs 6, 7 [Shimoda, Shizuoka, Japan]; Kato (1938b), p. 582 [Seto, Wakayama, Japan]; Kato (1944), p. 276 [Misaki, Kanagawa, Japan]; Hagiya (1993), pp. 35–36, pl. 3, figs. 7–9, pl. I, fig. C [Otsuchi, Iwate, Japan]; Hagiya (2013), p. 73, pl. I, figs 2, 9, 10, pl. II, fig. 2 [Rebun Island, Hokkaido, Japan].

Melloplana japonica: Faubel (1983), p. 117 [secondary literature].

Notocomplana japonica: Oya and Kajihara (2017), pp. 532–534, figs 3, 4A [Toyoi Beach, Hokkaido, Japan].

Material examined. Five specimens (three sagittal and two cross sections), all collected in Toyoi Beach (43°13′33″N, 141°00′57″E), Hokkaido, Japan, 10 June 2016, by Y. Oya: ICHUM 5282, sagittal sections, 8 slides; ICHUM 5283, sagittal sections, 6 slides; ICHUM 5284, cross sections, 6 slides; ICHUM 5285, sagittal sections, 11 slides; ICHUM5286, cross sections, 6 slides.

Description. Live specimens 14–17 mm in length, 6.0–6.8 mm in maximum width. Body elongate oval, or narrow toward posterior end. Ground body color pale pinkish white under natural light, pinkish around pharynx. General appearance pinkish milky white (Fig. II-8A, B). Dark brown spots scattered on dorsal surface around pharynx (Fig. II-8A), also on anterior part of body in some specimens, but lacking entirely in other specimens. Body margin milky white and translucent (Fig. II-8A). Pair of small tentacular knobs present at one-fifth body length (2.9–3.2 mm) from anterior end but hardly visible in anesthetized state. Cerebral eye clusters arranged anterior to tentacular ones, each consisting of 8–20 and 10–18 eyespots, respectively (Fig. II-8C). Pharynx pinkish white, ruffled in shape, occupying three-sevenths to three-eighths of body length (5.8–5.9 mm). Mouth opening at almost center of pharyngeal cavity. Intestine not anastomosed, spreading throughout body except margin. Pair of whitish sperm ducts and oviducts visible through ventral body wall. Male and female gonopores separate; male gonopore opening at about one-third body length (4.2–5.8 mm) from posterior end; female gonopore situated 0.3–0.4 mm posterior to male gonopore (Fig. II-8B).

Male copulatory apparatus located immediately posterior to pharynx, consisting of seminal vesicle, interpolated prostatic vesicle, and penis papilla (Fig. II-9A). Pair of sperm ducts running anteriorly, turning medially at about one-fourth length of pharynx from posterior end, subsequently curving posteriorly along both sides of pharynx and running beyond level of posterior end of pharynx for short distance, before turning medially (Fig. II-8B) to enter separately proximal end of bean-shaped seminal vesicle with strong muscular wall (Fig. II-9A). Intra-prostatic ejaculatory duct projecting deeply and distally into prostatic vesicle. Prostatic vesicle pear-shaped, larger than seminal vesicle, possessing eight longitudinal tubular chambers (n = 2; confirmed in transversely sectioned specimens, Fig. II-9B) surrounding intra-prostatic ejaculatory duct, coated with strong muscular wall. Distal end of prostatic vesicle connecting post-prostatic ejaculatory duct. Post-prostatic ejaculatory duct with thin muscular wall leading to penis papilla. Penis papilla muscular, conical, without stylet, projecting into cylindrical male atrium. Male atrium lined with ciliated, smooth epithelium.

Pair of oviducts forming common oviduct, latter running dorsally to enter vagina. From this point, Lang's-vesicle duct, lined with folded ciliated epithelium, running almost ventrally, then curving posteriorly to connect to Lang's vesicle. Lang's vesicle subspherical, lined with columnar cells (Fig. II-9C). Vagina lined with smooth and ciliated epithelium, curving almost ventrally to exit at female gonopore. Lang's-vesicle duct and vagina surrounded by circular muscle fibers.

Habitat. Found intertidally on undersurface of stones in more-or-less sheltered rocky shore.

Sequence of COI. The partial COI sequences (712 bp) from the four specimens (LC176051–LC176054) almost coincided with each other. The uncorrected p-distance among specimens showed 0.003–0.011.

Remarks. The specimens collected from Hokkaido are generally consistent with Kato's (1937d) original description of *Notoplana japonica* from Shimoda, Shizuoka, Japan. Although specimens (14–17 mm) were smaller than Kato's (1937d) material (30 mm), the former agree with the latter in that *i*) the body is generally pinkish under natural light, *ii*) the cerebral eye clusters are arranged anterior to the tentacular eye clusters, *iii*) the sperm ducts separately enter the seminal vesicle, *iv*) the prostatic vesicle is pear-shaped, *v*) the intra-prostatic ejaculatory duct deeply protrudes into the prostatic vesicle, *vi*) the vagina runs almost vertically to open to the female pore, and, *vii*) the Lang's-vesicle duct runs abruptly ventrally from its junction between the vagina, then curves posteriorly before connecting to the Lang's vesicle.

This species should be placed in *Notocomplana* rather than *Melloplana* in the sense of Faubel (1983) because its morphology is much more similar to that of *N. humilis* (type species of *Notocomplana*) than to that of *M. ferruginea* (type species of *Melloplana*). In *M. ferruginea*, the epithelium of the prostatic vesicle is chambered but each chamber is perpendicular to the axis of the intra-prostatic ejaculatory duct; the chambers are not elongated as commonly found in *Notoplana* (Bock 1913). On the other hand, Kato (1937d, p. 215) explicitly stated that the “prostate gland ... consists of a few tubular glands...”. This character state, clearly depicted in Kato (1937d, text-fig. 6), resembles that of *N. humilis*. The lumen of the prostatic vesicle in this material is also consistent with Kato’s (1937d) description.

Notocomplana koreana (Kato, 1937)

(Figs II-10, 11)

Notoplana koreana Kato, 1937e: pp. 234–235, text-figs 2, 3, pl. XVI, figs 3, 4 [Gunsan, Korea]; Kato (1939b), p. 72, text-fig. 10 [Onagawa, Miyagi, Japan]; Hagiya and Gamo (1992), p. 16, pl. II, fig. 6, pl. III, fig. 5 [Manazuru, Kanagawa, Japan]; Hagiya (1993), p. 35, pl. 3, figs 4–6, pl. I, fig. B [Otsuchi, Iwate, Japan]; Hagiya (2013), p. 73, pl. I, figs 3, 11, 12, pl. II, figs 3, 4 [Rishiri Island, Hokkaido, Japan].

Notocomplana koreana: Faubel (1983), p. 114 [secondary literature]; Oya and Kajihara (2017), pp. 534–536, figs 4B, 5 [Oshoro, Hokkaido, Japan].

Material examined. Eight specimens (seven sagittal and one cross sections), all collected in Oshoro (43°12′36″N, 140°51′28″E), Hokkaido, Japan, by Y. Oya:

ICHUM 5274, sagittal sections, 20 slides, 26 February 2015; ICHUM 5275, sagittal sections, 6 slides, 11 May 2015; ICHUM 5276, sagittal sections, 8 slides, 25 May 2015; ICHUM 5277, sagittal sections, 7 slides, 25 May 2015; ICHUM 5278, sagittal sections, 6 slides, 22 March 2016; ICHUM 5279, cross sections, 5 slides, 9 May 2016; ICHUM 5280, sagittal sections, 5 slides, 6 June 2016; ICHUM 5281, sagittal sections, 6 slides, 6 June 2016.

Description. Live specimens 10–20 mm in length, 4.5–8.7 mm in maximum width. Body elongate oval, narrow toward posterior end. Ground body color whitish. General appearance varying from yellowish khaki (Fig. II-10A, B) to goldenrod. Brown granules scattered over entire dorsal body surface except at body margin. Dorsal surface above pharynx dark brown (Fig. II-10A). Body margin translucent. Pair of small tentacular knobs present at one-fifth or one-sixth body length (1.9–3.8 mm) from anterior end but hardly visible in anesthetized state. Tentacular eye clusters consisting of 17–41 eyespots (Fig. II-10C). Cerebral eye clusters consisting 16–27 eyespots (Fig. II-10C), arranged along median line and congregated anterior to tentacular eye clusters,. Pharynx white, ruffled in shape, occupying two-sevenths to two-fifths body length (3.1–7.0 mm) (Fig. II-10B). Mouth opening at almost center of pharyngeal cavity. Intestine not anastomosed, spreading throughout body except margin. Pair of whitish sperm ducts and oviducts visible through ventral body wall (Fig. II-10B). Male and female gonopores separate; male gonopore opening at about two-sevenths to two-fifths body length from posterior end (3.6–9.3 mm), female gonopore situated 0.3–1.1 mm posterior to male gonopore.

Male copulatory apparatus located immediately posterior to pharynx, consisting of seminal vesicle, interpolated prostatic vesicle, and penis papilla (Fig.

II-11A). Pair of sperm ducts running anteriorly, then turning medio-posteriorly to extend along pharynx, running posteriorly beyond level of posterior end of pharynx, subsequently curving medio-anteriorly and fusing to form common sperm duct. Common sperm duct anteriorly to enter proximal end of elongated, bean-shaped seminal vesicle with strong muscular wall. Intra-prostatic ejaculatory duct projecting deeply and distally into prostatic vesicle. Prostatic vesicle pear-shaped, smaller than seminal vesicle, possessing six longitudinal tubular chambers (n = 1; confirmed in transversely sectioned specimen, Fig. II-11B) surrounding intra-prostatic ejaculatory duct, coated with strong muscular wall. Distal end of prostatic vesicle connecting post-prostatic ejaculatory duct. Post-prostatic ejaculatory duct with thin muscular wall leading to penis papilla. Penis papilla muscular, conical, without stylet, projecting into large or anteriorly elongate male atrium. Male atrium lined with ciliated, smoooth epithelium.

Pair of oviducts forming common oviduct, latter running dorsally to enter vagina. From this point, Lang's-vesicle duct, lined with folded ciliated epithelium, posteriorly leading to Lang's vesicle. Lang's vesicle small, spherical, lined with columnar cells (Fig. II-11C). Vagina, lined with smooth and ciliated epithelium, curving ventrally to exit at female gonopore. Vagina and Lang's-vesicle duct surrounded by circular muscle fibers; vagina lined with smooth, ciliated epithelium.

Habitat. Found intertidally on undersurface of stones along sheltered rocky shore and occasionally in *Mytilus* spp. mussel beds (*M. galloprovincialis* Lamarck, 1819 and/or *M. trossulus* Gould, 1850) on exposed rocky areas.

Sequence of COI. The partial COI sequences (712 bp) from the seven specimens (LC176044–LC176050) almost coincided with each other. The uncorrected p-distance among specimens showed 0.000–0.020.

Remarks. Kato (1937e) originally described *Notocomplana koreana* from Korea based on a single, fixed specimen. The material from Hokkaido is consistent with the original description in that *i*) the intra-prostatic ejaculatory duct projects deeply into the prostatic vesicle, *ii*) the common sperm duct is present, *iii*) the male atrium is large, and *iv*) the Lang's vesicle is small and spherical. The specimens have a pair of small tentacular knobs, which became indistinct after anesthetization and fixation; this can explain Kato's (1937e) statement about the absence of the tentacles in his preserved material. The original description (Kato 1937e) mentions that the post-prostatic ejaculatory duct curves in an S-shape before entering the penis papilla. However, I observed that the shape of the post-prostatic ejaculatory duct varies from straight to sigmoid among individuals, suggesting that its shape is affected by fixation.

Notocomplana septentrionalis (Kato, 1937)

(Figs II-12, 13)

Notoplana septentrionalis Kato, 1937b: pp. 127–129, text-figs 5, 6, pl. 8, figs 4–6 [Muroran, Hokkaido, Japan].

Notocomplana septentrionalis: Faubel (1983), p. 115 [secondary literature]; Oya and Kajihara (2017), pp. 536–539, figs 4C, 6, 7 [Oshoro and Zenibako. Hokkaido, Japan].

Material examined. Fourteen specimens (eleven sagittal and two cross sections, and one whole specimens preserved in 70% ethanol), collected by Y. Oya: eleven from Oshoro (43°12′36″N, 140°51′28″E), Hokkaido, Japan, of which four

(ICHUM 5287, sagittal sections, 4 slides; ICHUM 5288, cross sections, 11 slides; ICHUM 5289, sagittal sections, 9 slides; ICHUM 5290, sagittal sections, 6 slides) were collected on 25 May 2015, one (ICHUM 5294, sagittal sections, 9 slides) on 30 May 2016, and six (ICHUM 5295, cross sections, 7 slides; ICHUM 5296, sagittal sections, 8 slides; ICHUM 5297, sagittal sections, 7 slides; ICHUM 5298, sagittal sections, 7 slides; ICHUM 5299, sagittal sections, 9 slides; ICHUM 5300, sagittal sections, 9 slides) on 6 June 2016; and three specimens (ICHUM 5291, sagittal sections, 7 slides; ICHUM 5292, sagittal sections, 6 slides; ICHUM 5293, whole specimen preserved in 70% ethanol) from Zenibako (43°09′07″N, 141°11′16″E), Hokkaido, Japan, on 28 May 2016.

Description. Live specimens 12–23 mm in length, 4.8–9.6 mm in maximum width. Body elongate oval, narrow toward posterior end. Ground body color whitish. General appearance of body varying from yellowish khaki mottled with dark (Fig. II-12A, D), to milky white with black dots above pharynx (Fig. II-12B), to uniform chocolate color (Fig. II-12C); in each form, dorsal surface above pharynx appearing darker, with scattered small, brown granules. Body margin translucent. Pair of small tentacular knobs present at about one-fifth of body length (2.2–4.0 mm) from anterior end but hardly visible in anesthetized state. Tentacular eye clusters consisting of 26–78 eyespots (Fig. II-12E). Cerebral eye clusters consisting of 24–81 eyespots (Fig. II-12E), arranged along median line and congregated anterior to tentacular eye clusters. Pharynx white, ruffled in shape, occupying three-sevenths to one-half of body length (4.8–10 mm) (Fig. II-12D). Mouth opening at almost center of pharyngeal cavity. Intestine not anastomosed, spreading throughout body except margin. Pair of whitish sperm ducts and oviducts visible through ventral body wall. Male and female gonopores separate;

male gonopore opening at about one-fifth to one-fourth of body length (1.8–5.4 mm) from posterior end; female gonopore situated 0.3–0.8 mm posterior to male gonopore (Fig. II-12D).

Male copulatory apparatus located posterior to pharynx, consisting of seminal vesicle, interpolated prostatic vesicle, and penis papilla (Fig. II-13A). Pair of sperm ducts running anteriorly, turning medially at point about one-fourth of pharynx length from posterior end of pharynx, curving posteriorly along both sides of pharynx, and continuing beyond level of posterior end of pharynx for short distance, before turning medially to enter separately proximal end of bean-shaped seminal vesicle with strong muscular wall. Intra-prostatic ejaculatory duct projecting deeply and distally into prostatic vesicle. Canals of numerous extra-vesicular glands penetrating wall of prostatic vesicle. Prostatic vesicle pear-shaped, larger than seminal vesicle, possessing 11 longitudinal tubular chambers (n = 2; confirmed in transversely sectioned specimens, Fig. II-13B) surrounding intra-ejaculatory duct, coated with strong muscular wall (Fig. II-13A). Distal end of prostatic vesicle connecting post-prostatic ejaculatory duct. Post-prostatic ejaculatory duct with thin muscular wall leading to penis papilla. Penis papilla muscular, conical, without stylet, projecting into cylindrical male atrium. Male atrium lined with ciliated, folded epithelium (Fig. II-13C, D).

Pair of oviducts forming common oviduct, latter running dorsally to enter vagina (Fig. II-13E). From this point, Lang's-vesicle duct, lined with folded ciliated epithelium, leading posteriorly to Lang's vesicle. Lang's vesicle elongate, lined with columnar cells. Vagina lined with folded and ciliated epithelium, running anteriorly, turning postero-ventrally, then recurving ventrally to exit at female gonopore. Vagina and Lang's-vesicle duct surrounded by circular muscle fibers; vagina surrounded numerous cement glands.

Habitat. Kato (1937b) did not mention details about the habitat. The present specimens were collected from beds of *Mytilus* spp. mussels (*M. galloprovincialis* Lamarck, 1819 and/or *M. trossulus* Gould, 1850) in the intertidal zone and from concrete blocks forming a breakwater.

Sequence of COI. The partial COI sequences (712 bp) from the seven specimens (LC176023–LC176035) almost coincided with each other. The uncorrected p-distance among specimens showed 0.000–0.015.

Remarks. Kato (1937b) originally described this species from Muroran, Hokkaido, Japan. The specimens are consistent with the original description in that *i*) the small tentacular knobs are present, *ii*) the sperm ducts separately enter the seminal vesicle, *iii*) the intra-prostatic ejaculatory duct projects deeply into the prostatic vesicle, *iv*) the vagina is directed dorso-anteriorly from the female gonopore, then curves backward, *v*) the vaginal epithelium is folded, and *vi*) the Lang's vesicle is posteriorly elongate.

The male atrial epithelium varied among individuals from weakly (Fig. II-13C) to extensively (Fig. II-13D) folded. The folds in the type material illustrated by Kato (1937b, text-fig. 6) fall within the range of variation I observed. The variation of this character does not correlate to the body size and maturity in the specimens.

Family **Stylochoplanidae** Meixner, 1907

Genus ***Alloioplana*** Plehn, 1896

Alloioplana sp.

(Figs II-14 to II-16)

Material examined. Six specimens from Arai Beach (35°09′34″N, 139°36′42″E), Misaki, Kanagawa, Japan, collected by H. Kohtsuka, K. Oguchi, and T. Miura, consisting of sections and unsectioned anterior body preserved in 70% ethanol: ICHUM 6084, sagittal sections, 9 slides, 27 March 2019; ICHUM 6085, sagittal sections, 5 slides, 27 March 2019; ICHUM 6086, sagittal sections, 10 slides, 27 March 2019; ICHUM 6087, sagittal sections, 6 slides, 21 February 2019; ICHUM 6088, sagittal sections, 10 slides, 21 February 2019; ICHUM 6089, cross sections, 8 slides, 21 February 2019.

Diagnosis. *Alloioplana* without nuchal tentacles, with ejaculatory duct projecting into prostatic vesicle. Gonopore separated (Figs II-14, II-15).

Description. Live specimens 16–17 mm in length, 5.1–5.9 mm in maximum width. Body elongate oval, narrow toward posterior end (Fig. II-14A). Dorsal body tinged with light brown due to minute granules scattered over entire surface except around margin. Dorsal surface of body around pharynx brown. Body margin translucent. General appearance of body brownish (Fig. II-14A, B). Tentacles lacking. Pair of cerebro-tentacular eye clusters, each containing 24–46 eyespots (Fig. II-14C), arranged near median line. Pharynx whitish, ruffled in shape, occupying about one-fourth of body length (3.8–4.6 mm in length), located at center of body (Fig. II-14B). Mouth opening at almost center of pharyngeal cavity. Intestine not anastomosed, spreading throughout body except margin. Pair of whitish sperm ducts and oviducts visible through ventral body wall (Fig. II-14B). Male and female gonopores separate; male gonopore opening at about one-fourth of body length (3.3–4.6 mm) from posterior end; female gonopore situated 0.3–0.5 mm posterior to male gonopore.

Male copulatory apparatus located posterior to pharynx, consisting of seminal vesicle, interpolated prostatic vesicle, and penis stylet (Fig. II-15). Pair of sperm ducts running anteriorly, turning medially at point about one-fourth length of pharynx from posterior end, subsequently running posteriorly along both sides of pharynx and extending further posteriorly for short distance beyond level of posterior end of pharynx, then turning anteriorly and fusing to common sperm duct (Fig. II-14B). Common sperm duct entering curved-oval seminal vesicle with strong muscular wall (Figs II-15, II-16A). Distal end of seminal vesicle slender, long, meandering, entering proximal end of prostatic vesicle and projecting as intra-prostatic ejaculatory duct (Fig. II-16B). Intra-prostatic ejaculatory duct lacking muscular wall, reaching to half length of prostatic vesicle (Fig. II-16B, C). Prostatic vesicle oval, lacking tubular chambers, smaller than seminal vesicle, coated with thick muscular wall and lined with glandular epithelium (Fig. II-16B). Post-prostatic ejaculatory duct with thin muscular wall running and connecting to penis stylet. Penis stylet straight, projecting into penis pocket (Fig. II-16D). Penis pocket lined with non-ciliated epithelium, opening to male atrium. Male atrium cylindrical, lined with ciliated epithelium; latter smooth to folded depending on fixation (Fig. II-16A, E).

Pair of oviducts running posteriorly and entering separately to vagina; Lang’s vesicle lacking (Fig. II-16F). Vagina lacking ampulla, lined with smooth, ciliated epithelium and surrounded by cement glands, curving antero-dorsally, running anteriorly, then turning postero-ventrally to exit at female gonopore (Fig. II-16F).

Habitat. Intertidally, the surface on coralline algae.

Sequences of COI. The partial COI sequences (712 bp) from the four specimens (LC582941–LC582944) almost coincide with each other. The uncorrected p-distance among specimens showed 0.004–0.010.

Remarks. Faubel (1983) assigned four species in *Alloioplana* (Table II-3). *Alloioplana* sp. is distinguished from *A. aulica* (Marcus, 1947) by the state of gonopores (separated in *Alloioplana* sp.; common in *A. aulica*), from *A. delicata* Plehn, 1896 by the presence or absence of the nuchal tentacles (absent in *Alloioplana* sp.; present in *A, delicata*), from *A. stylifera* (Hyman, 1953) by a shape of the prostatic vesicle (oval in *Alloioplana* sp.; curved oval in *A. stylifera*), from *A. wyona* (Du Bois-Reymond Marcus and Marcus, 1968) by the size of the prostatic vesicle compared with the seminal vesicle (small in *Alloioplana* sp.; almost the same size in *A. wyona*) as well as the presence or absence of an ampulla at the terminal of the vagina (absent in *Alloioplana* sp.; present in *A. wyona*). In addition, the intra-prostatic ejaculatory duct is only observed in *Alloioplana* sp. among the congeners. This species will be formally described in Oya *et al*. (in prep.).

This is the first report of *Alloioplana* from the West Pacific. Two Atlantic species, *A. aulica* and *A. wyona*, were described from Palmas Island, Brazil (Marcus 1947) and Saint Martin Island, the Caribbean Sea (Du Bois-Reymond Marcus and Marcus 1968), respectively. Other congeners are known from the East Pacific: *A. delicata* from Payta, Peru (Plehn 1896) and *A. stylifera* from the Gulf of California (Hyman 1953).

Genus ***Armatoplana*** Faubel, 1983

Remarks. Molecular phylogeny based on partial sequences of the 28S rDNA by Litvaitis *et al.* (2019, fig. 5) indicated *Armatoplana* was not monophyletic in that *A. divae* (Marcus, 1947) and *A. leptalea* (Marcus, 1947) were positioned in separated clades. However, the phylogenetic position of *A. panamensis* (Plehn, 1896), the type species of *Armatoplana*, has not been inferred yet.

Armatoplana sp.

(Figs II-17 to II-19)

Diagnosis. *Armatoplana* without nuchal tentacles and common sperm duct, and with seminal vesicle, prostatic vesicle larger than seminal vesicle and located above seminal vesicle, straight penis stylet, elongated Lang's vesicle without accessory vesicles (Figs II-17, II-18).

Material examined. Two specimens from Arai Beach (35°09′34″N, 139°36′42″E), Misaki, Kanagawa, Japan, 27 March 2019, collected by H. Kohtsuka, K. Oguchi, and T. Miura, consisting of sagittal sections and unsectioned anterior body preserved in 70% ethanol: ICHUM 6090, 5 slides; ICHUM 6091, 9 slides.

Description. Live specimens 13–14 mm in length, 3.8–4.2 mm in maximum width. Body elongate oval, narrow toward posterior end (Fig. II-17A). Dorsal body tinged with light brown due to minute granules scattered over entire surface except around margin. Dorsal surface of body around pharynx pale yellow. Body margin translucent. General appearance of body yellowish translucent (Fig. II-17A, B). Tentacles lacking. Pair of cerebro-tentacular eye clusters, each containing 26–31 eyespots (Fig. II-17C), arranged near median line. Pharynx whitish, ruffled in shape, occupying about one-fourth of body length (3.4–3.7 mm in length), located at center of body (Fig. II-17B). Mouth opening at almost center of pharyngeal cavity. Intestine not anastomosed, spreading throughout body except margin. Pair of whitish sperm ducts and oviducts visible through ventral body wall (Fig. II-17B). Male and female gonopores separate; male gonopore opening at about one-fourth to two-sevenths of body length (3.6–3.7 mm) from posterior end; female gonopore situated 0.07–0.13 mm posterior to male gonopore.

Male copulatory apparatus located posterior to pharynx, consisting of seminal vesicle, interpolated prostatic vesicle, and penis stylet (Fig. II-18). Pair of sperm ducts running anteriorly, turning medially at point about one-fifth length of pharynx from posterior end, subsequently running posteriorly along both sides of pharynx and extending further posteriorly for short distance beyond level of posterior end of pharynx, then turning anteriorly (Fig. II-17B) and entering separately proximal end of elongated ovate seminal vesicle. Seminal vesicle directing anteriorly and having strong muscular wall (Fig. II-19A). Distal end of seminal vesicle slender, running dorsally before connecting to prostatic vesicle. Prostatic vesicle oval-shaped, located dorsally above seminal vesicle, having thick muscular wall lined with smooth, thick epithelium (Fig. II-19A). Distal end of prostatic vesicle forming penis papilla with stylet (Fig. II-19B). Penis stylet straight, projecting into penis pocket. Penis pocket lined with ciliated epithelium opening to male atrium (Fig. II-19C). Male atrium small, cylindrical, and lined with ciliated epithelium.

Pair of oviducts forming common oviduct, latter running postero-dorsally to enter vagina. From this point, elongated Lang's-vesicle duct, lined with folded ciliated epithelium, running postero-ventrally to connect to Lang's vesicle (Fig. II-19D). Lang's vesicle elongate, sac-shaped, and lined with columnar cells, lacking accessory vesicles (Fig. II-19D). Vagina curving antero-ventrally, then running postero-ventrally to exit at female gonopore. Lang's-vesicle duct and vagina surrounded by circular muscle fibers and lined with ciliated and smooth epithelium.

Habitat. Intertidally, the surface on coralline algae.

Sequences of COI. The partial COI sequences (712 bp) from the two specimens (LC582945 and LC582946) almost coincide with each other. The uncorrected p-distance among specimens showed 0.004.

Remarks. Among 15 species currently classified in *Armatoplana* (Noreña *et al.* 2015), *Armatoplana* sp. resembles *A. lactoalba* (Verrill, 1900), *A. leptalea* (Marcus, 1947), *A. reishi* (Hyman, 1959), and *A. snadda* (Du Bois-Reymond Marcus and Marcus, 1968) in that they share the following four characters: *i*) nuchal tentacles absent, *ii*) a common sperm duct absent, *iii*) a seminal vesicle present, *iv*) a penis stylet present, and *v*) a pair of accessory vesicles lacking in the Lang's vesicle. I list these species in Table II-4. *Armatoplana* sp. is distinguished from *A. lactoalba* and *A. leptalea* by the shape of the stylet (straight in *Armatoplana* sp.; curved in *A. lactoalba* and *A. leptalea*) as well as arrangement of the prostatic vesicle (dosally above the seminal vesicle in *Armatoplana* sp.; in alignment with the seminal vesicle in *A. lactoalba* and *A. leptalea*). The present *Armatoplana* sp. differs from *A. reishi* in the shape of the Lang's vesicle (elongated sac in *Armatoplana* sp.; laterally broadened in *A. reishi*) and from *A. snadda* in the size of the prostatic vesicle compared with that of the seminal vesicle (large in *Armatoplana* sp.; small in *A. snadda*). This species will be formally described in Oya *et al*. (in prep.).

This is the first report of *Armatoplana* from the West Pacific. Seven of 15 known *Armatoplana* species have been recorded from the Atlantic coast of the Central and South America (Noreña *et al.* 2015). Two species, *A. panamensis* and *A. reishi*, were recorded from the Pacific coast of North and Central America (Plehn 1896; Hyman 1953, 1959). Five species were described from the coast of Africa: *A. affinis* (Palombi, 1940) and *A. vesiculata* (Palombi, 1940) were reported form the Atlantic (Palombi 1940); *A. lactea* (Laidlaw, 1903), *A. tenuis* (Palombi, 1936), and *A. robusta* (Palombi, 1928) were recorded from the Indian (Laidlaw 1903; Palombi 1928, 1936). Other congeners are known from the West Eurasia: *A. celta* Noreña, Rodríquez, Pérez and Almon, 2015 was reported from the northern Atlantic coast of Spain (Galicia) (Noreña *et al.* 2015) and *A. taurica* (Jacubowa, 1909) was found in the Black Sea (Jacubowa 1909).

Genus ***Phaenoplana*** Faubel, 1983

Phaenoplana kopepe Oya and Kajihara, 2019

(Figs II-20 to II-22)

Phaenoplana kopepe Oya and Kajihara, 2019: pp. 2–5, figs 2, 3 [Chichijima Island, Tokyo, Japan].

Etymology. The specific name is an indeclinable noun, taken from the type locality, Kopepe Beach. According to a local anecdote, *Kopepe* was the name of a man who came from the Gilbert Islands all the way across the Pacific to Chichijima Island and lived near the beach.

Material examined. Holotype: ICHUM 5343, 10 slides (1 slide for whole mount of the anterior body and 9 slides for serial sagittal sections), intertidal, Kopepe

Beach (27°03′52″N, 142°11′32″E), Chichijima Island, the Ogasawara Islands, Japan, 3 September 2016, collected by Y. Oya. Paratype (collection data same as holotype): ICHUM 5344, 5 slides (1 slide for whole mount of the anterior body and 4 slides for serial sagittal sections).

Diagnosis. *Phaenoplana* with nuchal tentacles, anteriorly curved vagina, Lang's-vesicle duct shorter than vagina, and horseshoe-shaped Lang's vesicle (Figs II-20, II-21).

Description. Live specimens 17–21 mm (21 mm in holotype) in length, 5.5–7.0 mm (7.0 mm in holotype) in maximum width. Body elongate oval, narrow toward posterior end. Ground body color translucent to whitish opaque. General appearance of body light brown (Fig. II-20A, B). Dorsal body tinged with light brown due to minute granules scattered over entire surface except around margin. Dorsal surface of body around pharynx brown. Body margin translucent. Pair of nuchal tentacles present at between one-fifth to one-sixth body length (3.4–3.5 mm, 3.5 mm in holotype) from anterior end; each nuchal tentacle containing at least 30 eyespots (Fig. II-20C). Cerebral eye clusters arranged along median line and congregated anterior to nuchal tentacles, consisting of 29–44 eyespots (29 in right cluster, 30 in left cluster in holotype, Fig. II-20D). Pharynx whitish, ruffled in shape, occupying two-ninths to one-third of body length (3.7–7.0 mm, 7.0 mm in holotype), located slightly anterior to center of body (Fig. II-20B). Mouth opening at near posterior end of pharyngeal cavity (Fig. II-22A). Intestine not anastomosed, spreading throughout body except margin. Pair of whitish sperm ducts and oviducts visible through ventral body wall (Fig. II-20B). Male and female gonopores separate; male gonopore opening at about one-third of body length (5.6–7.0 mm, 7.0 mm in holotype) from posterior end; female gonopore situated 0.5–0.7

mm (0.7 mm in holotype) posterior to male gonopore.

Male copulatory apparatus located posterior to pharynx (Fig. II-22A), consisting of seminal vesicle, interpolated prostatic vesicle, and penis rod (Fig. II-22A, B). Pair of sperm ducts running anteriorly, then turning medially at point about one-fifth length of pharynx from posterior end, subsequently running posteriorly along both sides of pharynx and extending further posteriorly for short distance beyond level of posterior end of pharynx, before turning medially to enter separately proximal end of elongated ovate seminal vesicle. Seminal vesicle directing anteriorly and having strong muscular wall (Fig. II-22B). Distal end of seminal vesicle slender, running dorsally before connecting to prostatic vesicle. Prostatic vesicle oval-shaped, having thick muscular wall lined with smooth, thick epithelium (Fig. II-22B). Distal end of prostatic vesicle forming elongated penis rod (Fig. II-22C). Penis rod muscular, without stylet, projecting into cylindrical male atrium; ejaculatory duct passing through penis rod (Fig. II-22C). Male atrium lined with smooth epithelium. Prostatic vesicle and male atrium enclosed by muscular bulb (Fig. II-22B–D).

Pair of oviducts forming common oviduct, latter running postero-dorsally to enter vagina. From this point, Lang's-vesicle duct, lined with folded ciliated epithelium, shorter than vagina, running posteriorly, then curving ventrally, and leading posteriorly to Lang's vesicle (Fig. II-22D). Lang's vesicle horseshoe-shaped (Fig. II-21) and lined with columnar cells (Fig. II-22D). Vagina lined with ciliated and smooth epithelium, curving antero-ventrally for short distance, then recurving postero-dorsally, then twisting again, eventually turning ventrally to exit at female gonopore (Figs II-21, II-22D). Lang's-vesicle duct and vagina surrounded by circular muscle fibers; vagina surrounded by cement glands.

Habitat. Found intertidally on the undersurface of stones and dead corals along sheltered beach.

Sequence of COI. The partial COI sequences (610 bp) from the two specimens (LC369778 and LC369779) almost coincide with each other. The uncorrected p-distance among specimens showed 0.002.

Remarks. Among the five species of *Phaenoplana*, *P. kopepe* resembles *P. challengeri* (Graff, 1892) and *P. conoceraea* (Schmarda, 1859) (Table II-5). They are from the Indo-Pacific, and share the following characters: *i*) a pair of nuchal tentacles are present, *ii*) the mouth opens near posterior end of the pharyngeal cavity, and *iii*) the Lang's vesicle is horseshoe shaped (Table II-5). *Phaenoplana kopepe* differs from *P. challengeri* in the shape of vagina (anteriorly curved in *P. kopepe*; almost vertical in *P. challengeri*) and the distance between male and female gonopores (well separated in *P. kopepe*; close in *P. challengeri*), and from *P. conoceraea* in the length of the Lang's-vesicle duct compared with that of the vagina (short in *P. kopepe*; long in *P. conoceraea*) in addition to the two characters mentioned above.

This is the first report of *Phaenoplana* from Japan. The congeners have been reported from tropical and subtropical areas (Schmarda 1859; Graff 1892; Hyman 1953; Pérez-García *et al.* 2019). In West Pacific Ocean, *P. challengeri* and *P. taiwanica* (Kato, 1943) have been reported (Schmarda 1859; Graff 1892; Kato 1943).

III. TAXONOMIC STUDIES OF ACOTYLEAN POLYCLADS IN THE BATHYAL ZONE AROUND JAPAN

INTRODUCTION

The biodiversity of deep-sea polyclads remains poorly understood. Although the majority of polyclads have been reported from shallow coastal areas (Prudhoe 1985), the deepest record of polyclad flatworm is represented by specimens collected from 3,232 m depth in the East Pacific (Quiroga *et al.* 2006). Deep-sea polyclads have been reported from muddy bottoms or sunken woods (*cf.* Bock 1913; Quiroga *et al.* 2006, 2008); some unidentified flatworms, seemingly polyclads, were found in hydrothermal vents in the Indian Ocean (Van Dover *et al*. 2001, fig. 2E). Before I started my research, 14 species (ten acotyleans and four cotyleans) have been described from sea bottoms deeper than 200 m (Fig. III-1, Table III-1). As a reason of shortage of information, Quiroga *et al.* (2006) has pointed out that flatworms can be easily disintegrated or wafted away during dredging. In addition to the difficulty of collections, the problems in the treating of living polyclad specimens indicated by Prudhoe (1985) are deemed to affect the delay of the researches.

In Japan, the polyclad fauna in deep waters (deeper than 200 m) had been unknown. Previous studies were mainly carried out in shallow waters, especially in the intertidal zone. Before my graduate research, the deepest record of acotylean from Japanese waters identified to species was *Paraplehnia pacifica* (Kato, 1939) collected at a depth of 78 m in Otsuchi Bay, Iwate (Hagiya 1993). However, the Marine Biological Samples Database provided by Japan Agency for Marine-Earth Science and Technology

(JAMSTEC) (2016 onward) records several unidentified polyclad specimens captured from the bathyal zone (200–2000 m depths) around Japan. Therefore, there was no doubt that various polyclads inhabit in Japanese deep waters.

In this chapter, I describe two stylochoid acotyleans collected from the bathyal zone by dredging offshore of Japan. One species Planoceridae gen. et sp. indet. (Planoceridae Stimpson, 1857) was collected from 245 m depth, Suruga Bay, Shizuoka (site number 7 in Fig. I-1). The member of Planoceridae are mainly reported from intertidal to sublittoral zones and the deepest record is 77.4 m for *Planocera hawaiensis* Heath, 1907 (Heath 1907). *Paraplehnia seisuiae* Oya, Kimura and Kajihara, 2019 (Plehniidae Bock, 1913) was captured from 298–310 m depths in the Kumano Sea, Mie (site number 8 in Fig. I-1). The majority of plehniids have been reported from sublittoral zones by dredging (*e.g.,* Bock 1913, 1923; Kato 1939b; Hyman 1953; Hagiya 1993); some species were described from more than 200 m depths (Bock 1913; Hyman 1953).

MATERIAL AND METHODS

Polyclads were collected from two sampling sites by dredging (site numbers 7, 8 in Fig. I-1, Table I-1). Methods of observations in life, fixations, and histological observations were described in Chapter I. In the description of Planoceridae gen. et sp. indet., I observed the sagittal sections of *Paraplaocera oligoglena* (Schmarda, 1859) and *Planocera reticulata* (Stimpson, 1855) for morphological comparison. In the description of *Paraplehnia*, sections containing part of copulatory apparatus, mounted on one of the slides, were re-stained by Mallory's trichrome method to yield clear contrast between the muscular and connective tissues. The cover glass was removed by steeping the preparation in xylene for 24 h. The sections on the slide were hydrated in an ethanol series. HE staining was then removed by washing in 50% ethanol containing 0.5% HCl for 2 h. After Mallory's staining, the sections were likewise embedded in Entellan New.

The sequences of COI in *Paraplehnia* and 16S, 18S, 28S, and COI in Planoceridae gen. et sp. indet. were determined followed by methods of DNA extraction, PCR amplification, and sequencing described in Chapter I. In the section of Planoceridae gen. et sp. indet., I calculated genetic distances of COI using by MEGA ver. 7.

In addition, I inferred the phylogenetic position of Planoceridae gen. et sp. indet. in Planoceridae following the protocol described in Chapter I to evaluate the propriety of the establishment of new genus. For the phylogenetic analyses, I downloaded additional sequences of Planoceridae and outgroup species from GenBank; three acotylean species, *Discocelis* sp., *Imogine* cf. *aomori* (Kato, 1937) and *Notocomplana humilis*. The 16S, 18S, and 28S sequences were aligned using MAFFT ver. 7, with the L-INS-i strategy selected by the "Auto" option. Ambiguous sites were removed with Gblocks ver. 0.91b using a more stringent selection. The COI sequences were aligned manually using MEGA ver. 7 and I obtained the concatenated dataset comprised four genes (3708 bp long) of 12 terminal taxa (Table III-2). The optimal substitution models for the ML analysis were GTR + G (16S), GTR + I (18S), GTR + I + G (28S, first codon position in COI), TIM + G (third codon position in COI), and TVM + I + G (second codon position in COI); those for BI were GTR + G (16S, third codon position in COI), GTR + I (18S, second codon position in COI), GTR + I + G (28S, first codon position in COI). Nodal support within the ML tree was assessed by analyses of 1000 bootstrap pseudoreplicates. For BI, the MCMC process used random starting trees and involved four chains run for 1,000,000 generations.

TAXONOMY

Superfamily **Stylochoidea** Stimpson, 1857

Family **Planoceridae** Stimpson, 1857

Planoceridae gen. et sp. indet.

(Figs III-2 to III-5)

Material examined. Two specimens, off the coast of the Izu Peninsula (34°39′11″N 138°43′12″E to 34°39′15″N 138°43′21″E; 245 m depth), Shizuoka, Japan, 12 December 2018, collected by Y. Oya, consisting of sagittal sections and unsectioned anterior body preserved in 70% ethanol: ICHUM 6081, 22 slides; ICHUM6082, 20 slides.

For comparison, I also observed two planocerids: *Paraplanocera oligoglena* (Schmarda, 1859), non-type, ICHUM 6083, sagittal sections, 20 slides, Bonomisaki (31°15′15″N, 130°12′54″E; subtidal), Kagoshima, Japan, 26 July 2018, collected by Y. Oya; and *Planocera reticulata* (Stimpson, 1855), non-type, ICHUM 6018, sagittal sections, 16 slides, collection data same as ICHUM 6083.

Description. Live specimens 18–21 mm in length, 12–14 mm in maximum width. Body oval (Fig. III-2A, B). Ground body color translucent to whitish opaque (Fig. III-2A). Pair of nuchal tentacles located about three-tenths of body length (5.4–5.9 mm) from anterior end (Fig. III-2A). Tentacular eye clusters congregated in bases of each tentacle and containing 10–14 eyespots (Fig. III-2C). Pair of cerebral eye clusters, each consisting of 4–6 eyespots (Fig. III-2C), arranged near median line and congregated anterior to tentacular eye clusters. Pharynx whitish, ruffled in shape, occupying about two-ninths of body length (4.1–4.9 mm in length), located at center of body (Fig. III-2B). Intestine not anastomosed, spreading throughout body except margin. Mouth opening at near center of pharyngeal cavity. Ovary distributed around pharynx. Common gonopore opening at about three-tenths (5.1–6.6 mm) from posterior end of body (Fig. III-2D).

Male copulatory apparatus located immediately posterior to pharynx, consisting of pair of spermiducal bulbs, free prostatic vesicle, cirrus, and pair of accessary organs; seminal vesicle lacking (Figs III-3, III-4A–F). Sperm ducts running anteriorly, then turning medially at point posterior to pharynx, and forming spermiducal bulbs (Figs III-2D, III-4C). Distal end of spermiducal bulbs becoming slender, forming distal part of sperm duct (Fig. III-4D), latter running anteriorly (Fig. III-3E), then forming common sperm duct (Fig. III-4F). Common sperm duct entering middle part of ejaculatory duct from ventral side. Ejaculatory duct lined with prostatic-like glandular epithelium (Fig. III-4A, F). Prostatic vesicle elongated oval shaped, having tubular folded epithelium, and connecting proximal end of ejaculatory duct (Figs III-3, III-4A, E). Prostatic vesicle and ejaculatory duct coated with thick muscular wall (Fig. III-4A, F). Distal end of ejaculatory duct opening to cirrus cavity. Inner epithelium of cavity having three types of teeth. Teeth in proximal part of cirrus cavity hook shaped, large, becoming smaller and denser in distal part (Fig. III-5A); base of tooth grooved (Fig. III-5B), becoming smooth towards tip. Teeth in middle part of cirrus same shape and size as small ones in proximal part, and distributed densely (Fig. III-5C); base of tooth not grooved. Teeth in distal part of cirrus curved-hook shaped, small, and distributed sparsely (Fig. III-5D). Cirrus cavity, prostatic vesicle, and ejaculatory duct enclosed by muscular bulb. Distal part of cirrus cavity connecting to cylindrical male atrium, latter opening at common gonopore (Fig. III-4A). Ventral surface of male atrium smooth, lined with ciliated epithelium. Proximal dorsal surface of male atrium folded, lined with ciliated epithelium (Fig. III-4E). Distal dorsal surface of male atrium smooth, lined with cuticularized epithelium, and connecting to that of vagina bulbosa (Fig. III-4A, B). Pair of accessory pouches, lined with ciliated epithelium, opening to ventral side of male atrium (Figs III-4A, III-5E). Accessory organs located at end of each accessory pouch, and consisting of eosinophilic glands, developed muscle, and several hook-shaped teeth (Fig. III-5E).

Pair of oviducts forming common oviduct, latter running postero-dorsally to enter vagina. From this point, short Lang's-vesicle duct running postero-ventrally to connect to Lang's vesicle (Fig. III-5F). Lang's vesicle rudimentary, spherical, lined with epithelium similar to that in Lang's-vesicle duct (Fig. III-5F). Vagina running anteriorly, then curving posteriorly, and connecting to vagina bulbosa (Fig. III-4A); vagina lined with smooth, ciliated epithelium, and coated with thick muscular wall. Proximal part of vagina surrounded by cement glands. Vagina bulbosa with cuticularized epithelium possessing several spines, surrounded by developed muscular wall, and exiting common gonopore (Figs III-4A, III-5G). Bursa copulatrix absent. Mass of sperm-like structure observed in atrium of vagina bulbosa and male atrium (Fig. III-4A).

Habitat. Judging from the nature of the dredged material, the sediment type of the species' habitat is likely to be mud.

Sequence of COI. The partial COI sequences (712 bp) from the two specimens (LC545561 and LC545562) almost coincided with each other. The uncorrected p-distance between specimens showed 0.004.

Molecular phylogeny. The resulting BI and ML trees were identical to each other in topology; I show only the BI tree (Fig. III-6). Within Planoceridae clade, Planoceridae gen. et sp. indet. was sister to the clade of *Planocera* with high support (0.99/91).

Remarks. Among the seven existing genera in Planoceridae *sensu* Faubel (1983), Planoceridae gen. et sp. indet. is morphologically similar to *Paraplanocera* Laidlaw, 1903 and *Planocera* Blainville, 1828. It shares the following traits with the two genera: *i*) a pair of nuchal tentacles, *ii*) muscular bulb surrounding cirrus cavity, *iii*) free prostatic vesicle, and *iv*) Lang's vesicle (Fig. III-7A, C; Table III-3). In addition, Planoceridae gen. et sp. indet. resembles *Paraplanocera* in possessing *i*) a pair of spermiducal bulbs instead of a seminal vesicle and *ii*) a pair of accessory organs on the ventral side of male atrium (Fig. III-7B). On the other hand, Planoceridae gen. et sp. indet. also shows resemblance to *Planocera* in that it has a rudimentary Lang's vesicle and a developed vagina bulbosa with a cuticularized epithelium (Fig. III-7D).

The present Planoceridae gen. et sp. indet. differs from *Paraplanocera* in that the accessory organ in the former has several teeth, whereas that in the latter is glandular and unarmed (Fig. III-7B). In addition, the present Planoceridae gen. et sp. indet. lacks the bursa copulatrix and a developed Lang's vesicle unlike *Paraplanocera*. The present Planoceridae gen. et sp. indet. is distinguished from *Planocera* by lacking the seminal vesicle and having the accessory organs in the male genital complex (Fig. III-7C, D). Moreover, a common gonopore is only observed in the present Planoceridae gen. et sp. indet. among Planoceridae (Fig. III-4A). Furthermore, the present Planoceridae gen. et sp. indet. is phylogenetically separate from *Paraplanocera* and *Planocera* (Fig. III-6). A new genus for the present species will be established in Oya and Kajihara (in review).

The homology of the accessory organ in Planoceridae is unclear. The components of the organ differ between the present Planoceridae gen. et sp. indet. and *Paraplanocera* (Figs III-5E, III-7B). Planocerids in the genus *Neoplanocera* Yeri and Kaburaki, 1918 have a structure similar to the accessory organ. The structure in *Neoplanocera* has a developed muscle like that in the accessory organ of Planoceridae gen. et sp. indet. but it is papilla-like in shape and does not exist in pair (Yeri and Kaburaki 1918b, text-fig. 18; Kato 1937d, text-fig. 14, pl. 14, fig. 8). Besides, the phylogenetic position of *Neoplanocera* has not been tested by molecular analyses. It is possible that the genus is not even encompassed in Planoceridae because the external morphology of *Neoplanocera* (*e.g.,* the body shape and formation of eye-clusters) is more similar to that of leptoplanoids rather than planocerids (Yeri and Kaburaki 1918b, text-fig. 17, pl. 2, fig. 4; Kato 1937d, text-figs 11, 12). To evaluate the homology of the accessory organs among planocerids, not only detailed morphological and phylogenetic examination but also developmental observation should be done in future studies.

The accessory organs and their surrounding structure in the present Planoceridae gen. et sp. indet. suggest that the organs are everted along with the cirrus during the course of mating. The cirrus in planocerids is turned itself inside out (*e.g.*, Kato 1944, text-fig. 27) and performs as an intromittent organ (Prudhoe 1985). The accessory organs in this species possess hook-shaped teeth and a developed muscular part as well as the cirrus (Fig. III-5E). The muscle will support eversion of the organs and the teeth will help holding the mating partner during copulation.

The cuticularized epithelium on the vagina bulbosa in the present Planoceridae gen. et sp. indet. is likely a result of co-evolution between the male and female copulatory apparatuses. In some insects that have armed male copulatory apparatuses, the inner surface of the female tract tends be thickened or sclerotized (*e.g.,* Rönn *et al*. 2007; Kamimura 2012). This 'hardening' of copulatory apparatuses in both sexes is one of the trends observed in male–female co-evolution relating to traumatic mating (Lange *et al*. 2013). Polyclad flatworms employ three methods for sperm transfer: direct copulation, dermal impregnation, and hypodermic insemination (Rawlinson *et al*. 2008). Lang (1884, p. 307) presumed that polyclads possessing hard structures (penis stylet or teeth) in the intromittent organ and a vagina bulbosa ("bursa copulatrix" in Lang (1884)) in the female copulatory apparatus employed the direct copulation. Although the copulatory behavior of the present Planoceridae gen. et sp. indet. has not been observed, I speculate that these species perform a direct copulation, with the cuticle on the vagina bulbosa acting as a protection against the numerous teeth on the male genitalia of their mating partner.

One of the significant findings in the phylogenetic analysis is that the spines on the vagina bulbosa evolved at least two times within the present Planoceridae gen. et sp. indet. + *Planocera* clade. Other planocerids lack the vagina bulbosa with the cuticularized epithelium and spines. Among the species employed in the analyses, only *P. multitentaculata* Kato, 1944 has distinct spines on the vagina bulbosa, other than the present Planoceridae gen. et sp. indet.; whether it is also present in *Planocera* sp. of Litvaitis *et al*. (2019) is unknown. I suppose that the vagina bulbosa was lined with cuticularized tissue in the last common ancestor of the present Planoceridae gen. et sp. indet. + *Planocera* clade, and then the spines evolved independently in each lineage of the present Planoceridae gen. et sp. indet. and *P. multitentaculata* (Fig. III-8). Among *Planocera* species not included in the present analysis, the spines are also known in *P.*

gilchristi Jacubowa, 1906 and *P. uncinata* Palombi, 1939 (Jacubowa 1907; Palombi 1939). Adding these species in future studies should reveal the entire picture of the evolution of these 'female spines' among planocerids.

Family **Plehniidae** Bock, 1913 *sensu* Prudhoe (1985)

Genus ***Paraplehnia*** Hyman, 1953

Remarks. I adopted Prudhoe's (1985)—instead of Faubel's (1983)—classification system as to the infra-familial classification of Plehniidae, because this system was followed by some of the subsequent researchers (*e.g.,* Hagiya 1993; Newman and Cannon 1997). Hyman (1953) established *Paraplehnia* and assigned *Plehnia japonica* Bock, 1923 and *Plehnia pacifica* Kato, 1939 to this genus; Prudhoe (1985) followed this opinion. Faubel (1983) did not accept *Paraplehnia* because he considered that "the presence (*P. japonica* [= *Paraplehnia japonica*]) or the absence (*P. pacifica* [= *Paraplehnia pacifica*]) of Lang's vesicle demands a separation of both these species" (Faubel 1983, pp. 54, 55) and classified *Paraplehnia pacifica* into *Diplehnia* which was characterized by lacking a Lang's vesicle (Faubel 1983); *Paraplehnia japonica* was transferred to *Plehnia*. However, Kato (1939b) clearly stated that the "Lang's vesicle is small and irregularly elongated, disposed immediately behind the vagina bulbosa in the ventral part of the body" in the original description of *Paraplehnia pacifica* (Kato 1939b, p. 68), and at the same time also included a line drawing of the Lang's vesicle as a schematic figure of the copulatory apparatus of *Paraplehnia pacifica* (Kato 1939b, text-fig. 3). The validity of *Diplehnia* should be tested by future molecular studies along with *Diplehnia caeca* (Hyman, 1953), the type species of the genus.

Paraplehnia seisuiae Oya, Kimura and Kajihara, 2019

(Figs III-9 to III-11)

Paraplehnia seisuiae Oya, Kimura and Kajihara, 2019: pp. 4–9, figs 1, 2 [Sea of Kumano, Mie, Japan].

Etymology. The specific name is a noun in the genitive case and taken from the TRV *Seisui-maru*.

Material examined. Holotype: ICHUM 5345, sagittal sections, 44 slides (14 slides for the anterior part and 30 slides for the posterior part of the body), Sea of Kumano (34°08′00″N 136°37′48″E to 34°07′48″N 136°37′54″E; 298–310 m depths), Japan; 9 November 2017, collected by Y. Oya.

Diagnosis. *Paraplehnia* without common sperm duct and with genital pit (Figs III-9, III-10).

Description. Live specimen 26 mm in length, 11 mm in width. Body thick, elongate, oval, narrow toward posterior end (Fig. III-9A, B). Anterior and posterior ends pointed. Ground body color translucent to whitish opaque. General appearance of body light brown. Dorsal body without any pattern. Body margin translucent. Tentacles lacking. Pharynx, ruffled in shape, 7.4 mm in length, located at center of body. Mouth opening at center of pharyngeal cavity (Fig. III-9B). Intestine not anastomosed, spreading throughout body except margin. Pair of sperm ducts and oviducts whitish, visible through ventral surface (Fig. III-9B). Male and female gonopores separate; male gonopore opening at 9 mm from posterior end; female gonopore situated 2.5 mm posterior to male gonopore.

Marginal and cerebral eyespots small and embedded in parenchyma (Fig. III-9C, D). At least 47 and 28 eyespots arranged in anterior body margin and from just behind brain to anterior to brain, respectively, but detailed distribution of eyespots could not be observed.

Male copulatory apparatus located immediately posterior to pharynx, consisting of pair of spermiducal bulbs, prostatic vesicle, and penis papilla (Figs III-10, III-11A–E). Distal end of each sperm duct forming oval spermiducal bulb, latter having thick muscular wall (Fig. III-11A). Distal end of each spermiducal bulb slender, forming distal part of sperm duct; latter separately connecting to neck of prostatic vesicle (Fig. III-11B–D). Prostatic vesicle pear-shaped, having strong muscular wall occupying its proximal one-third, distally coated with connective tissue and enclosed by muscular bulb (Fig. III-11F). Canals of extra-vesicular gland penetrating prostatic-vesicle wall. Glandular epithelium with numerous tear-drop-shaped cells folded in prostatic vesicle (Fig. III-11E). Ejaculatory duct lacking; distal end of prostatic vesicle directly forming a part of penis papilla. Penis papilla large, conical, lacking stylet and projecting posteriorly into male atrium. Male atrium lined with thin, non-ciliated epithelium.

Pair of oviducts forming common oviduct, latter running postero-dorsally to enter vagina (Fig. III-11E). From this point, Lang's-vesicle duct, lined with ciliated epithelium, running posteriorly. Length of Lang's-vesicle duct about one-third of that of vagina. Lang's vesicle sac-shaped, lined with squamous cells, positioned posterior to female gonopore. Vagina lined with smooth ciliated epithelium, running antero-dorsally, curving postero-ventrally as it becomes slenderer, turning postero-dorsally as it becomes wider, eventually leading ventrally to exit at female atrium (or vagina externa). Medial part of vagina surrounded by numerous cement glands (Fig. III-11E). Female atrium large, folded, with thick basement membrane, opening at female gonopore.

Genital pit with smooth epithelium and basement membrane similar to those in vagina (Fig. III-11G), located between male and female gonopores (Fig. III-11F).

Habitat. Judging from the nature of the dredged material, the sediment type of the species' habitat is likely to be sandy mud.

Remarks. *Paraplehnia* has contained two species, *P. japonica* (Bock, 1923) and *P. pacifica*, both were originally described from the sublittoral zone in Japan (Bock 1923; Kato 1939b; Hagiya 1993). *Paraplehnia seisuiae* can be distinguished from the two congeners by the occupancy of the developed muscular wall relative to the entire prostatic vesicle (about one-third in *P. seisuiae*; about one-half in *P. japonica* and *P. pacifica*), the presence/absence of a common sperm duct between spermiducal bulbs and prostatic vesicle (absent in *P. seisuiae*; present in *P. japonica* and *P. pacifica*), and the presence/absence of a genital pit between the male and the female gonopores (present in *P. seisuiae*; absent in *P. japonica* and *P. pacifica*) (Table III-4). In addition, *P. seisuiae* differs from *P. japonica* by the length of the Lang's-vesicle duct compared with that of the vagina (about one-third in *P. seisuiae*; about one-sixth in *P. japonica*) and from *P. pacifica* by the range of developed connective tissues in the female copulatory apparatus (from the female atrium to around the female gonopore and the genital pit in *P. seisuiae*; only around the female atrium in *P. pacifica*).

It is for the first time that a genital pit (or genital sucker) was found in plehniid species. Among Acotylea, genital pits have been known in *Itannia ornata* Marcus, 1947 (Hoploplanidae Stummer-Traunfels, 1933), three species of *Leptoplana* (Leptoplanidae

Ehrenberg, 1831) (Gammoudi *et al.* 2012b), and *Notoplana qeshmensis* (Maghsoudlou, Bulnes and Rahimian, 2015) (Notoplanidae Marcus, 1947) (Maghsoudlou *et al.* 2015). Genital pits in *I. ornata* are present in a pair, situated on both sides of the female gonopore (Marcus 1952). On the other hand, a single genital pit is present between the male and female gonopores in three *Leptoplana* species and *N. qeshmensis*, as well as in *P. seisuiae* (Fig. III-11F).

IV. MOLECULAR PHYLOGENETIC ANALYSIS OF ACOTYLEA (PLATYHELMINTHES: POLYCLADIDA)

INTRODUCTION

Molecular phylogenetic analyses have been used to elucidate the superfamily-level systematics in Acotylea (Bahia *et al.* 2017; Dittmann *et al.* 2019; Litvaitis *et al.* 2019). Acotylea is currently divided into three superfamilies, Discoceloidea Laidlaw, 1903, Leptoplanoidea Ehrenberg, 1831, and Stylochoidea Stimpson, 1857 (Dittmann *et al.* 2019). These superfamilies are recognizable only through molecular data (Dittmann *et al.* 2019; Litvaitis *et al.* 2019).

Although molecular studies have revised acotylean family- and genus-level classifications (Bahia *et al.* 2017; Dittmann *et al.* 2019; Litvaitis *et al.* 2019), it remains necessary to integrate the taxonomic insights provided by these studies because the taxonomic views by these authors conflict partially with each other. Furthermore, differences in taxon sampling may have affected the conclusions in some cases; for instance, Notocomplanidae, established by Litvaitis *et al.* (2019), is questionable in view of Tsunashima *et al.* (2017) and Dittmann *et al.* (2019).

Another issue pertaining to previous studies is data comparability. The partial region of the 28S ribosomal RNA gene (28S) determined by Tsunashima *et al.* (2017) does not completely overlap with those in Bahia *et al.* (2017), Litvaitis *et al.* (2019), and Dittmann *et al.* (2019). Dittmann *et al.* (2019) also utilized 18S ribosomal RNA gene (18S), but their taxonomic conclusions were mainly grounded on their 28S trees because of limited availability of 18S sequences.

In this study, I reconstructed the phylogeny of Acotylea (including taxa representing 27 genera and 16 families) using a concatenated dataset of partial sequences from four genetic markers: the 16S ribosomal RNA gene (16S), 18S, the D1–D2 region of 28S, and the cytochrome *c* oxidase subunit I gene (COI). I determined sequences from 24 acotylean species collected from Japan, including nine of the 14 species of Japanese acotyleans for which only the data was available in Tsunashima *et al.* (2017). The goals of this study were to investigate family- and genus-level monophyly in Acotylea and to integrate the results of other, recent phylogenetic studies.

MATERIAL AND METHODS

Polyclads were collected from 12 sampling sites in Japan (Fig. I-1, Table I-1). Methods of observations in life, fixations, and histological observations were described in Chapter I. Specimens were identified morphologically to the genus or species level. Two polyclads, *Amemiyaia pacifica* Kato, 1944 and *Planocera multitentaculata*, that died before fixation in Bouin's solution were identified only from external morphology.

DNA extraction, PCR amplification, and sequencing were followed the protocol described in Chapter I. Additional sequences from Acotylea were downloaded from GenBank; two cotylean species, *Cestoplana rubrocincta* (Grube, 1840) and *Pericelis tectivorum* Dittmann, Dibiasi, Noreña and Egger, 2019, were chosen as outgroup taxa. Alignment of 16S, 18S, and 28S sequences was done with MAFFT ver. 7, using the "unalignlevel: 0.8" and "Leave gappy regions" options under the G-INS-i strategy. Ambiguous sites in the aligned 16S, 18S, and 28S alignments were removed with Gblocks ver. 0.91b using the "Allow smaller final blocks" option. Alignment of

COI was done manually with MEGA ver. 7. The concatenated dataset from the four genes was 3527 bp long and contained 60 terminal taxa (Table IV-1). The optimal substitution models for the ML analysis were GTR + I + G (18S, 28S, third codon position in COI), TRN + I + G (first codon position in COI), and TVM + I + G (16S, second codon position in COI); that for BI were GTR + I + G (16S, 18S, 28S, all codon positions in COI). Nodal support within the ML tree was assessed by analyses of 1000 bootstrap pseudoreplicates. For BI, the MCMC process used random starting trees and involved four chains run for 10,000,000 generations

RESULTS

The resulting BI and ML trees (Figs IV-1, IV-2) are almost identical in topology and show three clades, corresponding to superfamilies, supported by high PP (1.00) and moderately high to high BS (80–98%) values. The clade Discoceloidea + Leptoplanoidea is strongly supported (1.00/99). Callioplanidae Hyman, 1953 is the sister clade to Stylochoidea in both trees, but the nodal support indicated by PP is lower than 0.90 (Fig. IV-1).

Nodal support for Discoceloidea is 1.00/89. In this clade, Cryptocelidae Laidlaw, 1903 (represented by *Amemiyaia pacifica* and *Phaenocelis* Stummer-Traunfels, 1933) is the sister clade to Ilyplanidae Faubel, 1983 + Discocelidae Laidlaw, 1903. Ilyplanidae, represented by *Discoplana gigas* (Schmarda, 1859), is the sister clade to Discocelidae, with nodal support of 1.00/99.

Leptoplanoidea branches into two subclades, with high nodal support (1.00/98). One (1.00/99) contains *Gnesioceros sargassicola* (Martens, 1933) and *Styloplanocera* *fasciata* (Schmarda, 1859) (Gnesiocerotidae Marcus and Marcus, 1966) + *Comoplana agilis* (Lang, 1884) and *Phaenoplana* Faubel, 1983 (Stylochoplanidae Meixner, 1907). The other (1.00/93) contains *Leptoplana tremellaris* (Müller, 1773) and all other leptoplanoids, with the internal topology differing somewhat between the ML and BI trees. The latter clade includes a clade formed by *Pseudostylochus* Yeri and Kaburaki, 1918 (Pseudostylochidae Faubel, 1983) and *Comoplana pusilla* (Bock, 1924) (Stylochoplanidae), with nodal support of 1.00/98. The specimens representing the four species of *Pseudostylochus* included in the phylogenetic analyses all possessed prostatic vesicles of the 'free' type (Fig. IV-3A–D). Species of *Notocomplana* Faubel, 1983 (Notocomplanidae) are distributed in three well supported clades that each contains representatives of other genera.

Stylochoidea, with nodal support of 1.00/80, contains four robust subclades corresponding to Stylochidae Stimpson, 1857 (represented by *Stylochus ellipticus* (Girard, 1850)), Hoploplanidae Stummer-Traunfels, 1933, Planoceridae Stimpson, 1857, and Plehniidae Bock, 1913 (represented by *Paraplehnia* Hyman, 1953). *Latocestus* sp. (Latocestidae Laidlaw, 1903) and *Mirostylochus akkeshiensis* Kato, 1937 (Stylochidae) form a clade supported by 1.00/74. However, the relationships among clades remain unclear.

DISCUSSION

Superfamily-level systematics in Acotylea

The results generally support the superfamily-level phylogeny proposed by Dittmann *et al.* (2019), which was based on molecular analyses covering 14 families and 20 genera as an ingroup within Acotylea. The multi-locus analyses of four gene markers (16S, 18S, 28S, and COI) from 16 families and 27 genera of acotyleans showed robust clades corresponding to the three superfamilies Discoceloidea, Leptoplanoidea, and Stylochoidea. In the trees, the families Latocestidae and Plehniidae—not included in Dittmann *et al.* (2019)—are nested within Stylochoidea, corroborating the results of Litvaitis *et al.* (2019) for Latocestidae and Oya *et al.* (2019) for Plehniidae. In addition, this study confirms that *Pseudostylochus* (Pseudostylochidae) belongs in Leptoplanoidea, a phylogenetic position that has been debatable in previous studies.

On the basis of morphology, *Pseudostylochus* had been placed in Stylochoidea (Faubel 1983). The leptoplanoid affiliation of *Pseudostylochus* was suggested not only by 28S phylogenies (Tsunashima *et al.* 2017; Dittmann *et al.* 2019), but also by one based on concatenated 16S and COI sequences (Aguado *et al*. 2017), with the latter depending on Sato *et al.* (2001) for 16S and COI sequences from *Pseudostylochus intermedius* Kato, 1939. Membership of *Pseudostylochus* in Leptoplanoidea is counterintuitive, because the prostatic vesicles in Leptoplanoidea are the interpolated type, whereas those in *Pseudostylochus* are the free type (*e.g.,* Kato 1939a, text-fig. 5). Because of this morphological discordance, Aguado *et al.* (2017) doubted the species identification of *Pseudostylochus intermedius* by Sato *et al.* (2001). Moreover, neither Tsunashima *et al.* (2017) nor Dittmann *et al.* (2019) provided any morphological information on their *Pseudostylochus* material. The *Pseudostylochus* specimens included in this study, belonging to the leptoplanoid clade (Figs IV-1, IV-2), clearly possessed free prostatic vesicles (Fig. IV-3A–D), which are typically found in stylochoid polyclads. This means that Leptoplanoidea cannot be defined as having an interpolated prostatic vesicle, as formerly believed (Faubel 1983; Bahia *et al.* 2017;

Litvaitis *et al.* 2019). A morphological transformation in the opposite direction seems to have occurred in the lineage leading to *Hoploplana* Laidlaw, 1902 (Hoploplanidae) within Stylochoidea, in which the majority of species have a free prostatic vesicle, while *Hoploplana* has an interpolated one (*e.g.,* Yeri and Kaburaki 1918b, text-figs 15, 16). Previous molecular studies (Aguado *et al.* 2017; Bahia *et al.* 2017; Tsunashima *et al.* 2017; Dittmann *et al*. 2019; Litvaitis *et al.* 2019) indicated a stylochoid affiliation for *Hoploplana*, and the present results confirm this. I also detected a robust Discoceloidea clade formed by *i*) polyclads lacking a prostatic vesicle (*Adenoplana* Stummer-Traunfels, 1933; *Discocelis* Ehrenberg, 1836, and *Discoplana* Bock, 1913), as well as *ii*) acotyleans having an interpolated prostatic vesicle (*Amemiyaia* Kato, 1944 and *Phaenocelis*) (Figs IV-1, IV-2), a taxonomic composition suggested by Dittmann *et al.* (2019) and Litvaitis *et al.* (2019). These character-state changes mean that none of the three superfamilies can be defined by a synapomorphy involving prostatic-vesicle morphology (Dittmann *et al.* 2019).

It is possible to find morphological diagnostic characters to classify these "exceptions" into each superfamily. *Amemiyaia* and *Phaenocelis* (discoceloids with an interpolated prostatic vesicle) have marginal eyespots, while other leptoplanoid flatworms lack them. *Pseudostylochus* (leptoplanoids with a free prostatic vesicle) can be distinguished from other stylochoid species in that they lack marginal and frontal eyespots and possess a seminal vesicle, a penis papilla, and a Lang's vesicle. *Hoploplana* (stylochoids with an interpolated prostatic vesicle) has a pair of spermiducal bulbs instead of a seminal vesicle. I add morphological diagnoses to the definitions of each superfamily by Dittmann *et al.* (2019) (see below).

The phylogenetic position of *Callioplana* Stimpson, 1857 remains unclear, leaving the superfamily affiliation of Callioplanidae indeterminate. All previous 28S-based studies failed to place *Callioplana* with certainty in the acotylean phylogeny (Tsunashima *et al.* 2017; Dittmann *et al.* 2019; Litvaitis *et al.* 2019). The multi-locus analyses indicated a sister-taxon relationship of *Callioplana* to all other stylochoids, with relatively high BS support but a PP lower than 0.90 (Figs IV-1, IV-2). Morphologically, *Callioplana* is similar to Stylochoidea in having a free prostatic vesicle. However, the placement of Callioplanidae within Stylochoidea should be regarded as provisional, especially in light of the homoplasic changes in prostatic-vesicle morphology that appear to have occurred multiple times in acotylean evolution (see above). For a fully resolved superfamily-level classification, the phylogenetic position of *Callioplana* needs to be ascertained with the use of additional molecular markers.

Family- and genus-level classifications

Family- and genus-level relationships, especially in Leptoplanoidea and Stylochoidea, were poorly resolved in the resulting trees (Figs IV-1, IV-2). Even so, some taxa were clearly not monophyletic. In the following paragraphs, I discuss problematic families and genera detected in the analyses.

Gnesiocerotidae. The family Gnesiocerotidae Marcus and Marcus, 1966 has been diagnosed so that it contains *Echinoplana* Haswell, 1907 (Faubel 1983; Prudhoe 1985). In the trees (Figs. IV-1, IV-2), *Echinoplana celerrima* Haswell, 1907 was more closely related to some other genera bearing family-group names—such as *Leptoplana* Ehrenberg, 1831, *Notoplana* Laidlaw, 1903, and *Notocomplana* Faubel, 1983—than to *Gnesioceros sargassicola* (Mertens, 1833); this means that *Echinoplana* cannot be placed in Gnesiocerotidae, if Leptoplanidae, Notoplanidae, and Notocomplanidae (and possibly many other families) were to be separate, monophyletic taxa. Although other studies (Bahia *et al.* 2017; Tsunashima *et al.* 2017; Dittmann *et al.* 2019) have implied the position of *Echinoplana*, this study demonstrates that Gnesiocerotidae in the sense of Faubel (1983) is non-monophyletic.

How can a monophyletic Gnesiocerotidae be circumscribed by morphological characters? In the trees (Figs IV-1, IV-2), *Gnesioceros sargassicola* forms a clade along with *Comoplana agilis*, *Phaenoplana kopepe* Oya and Kajihara, 2019, and *Styloplanocera fasciata* (Schmarda, 1859). These four species more or less share the following four characters: *i*) the nuchal tentacles are present, *ii*) the mouth opens at near the posterior end of the pharyngeal cavity, *iii*) the prostatic vesicle is interpolated, and *iv*) the Lang's vesicle is either horseshoe-shaped or crescent-shaped (Lang 1884; Graff 1892; Bock 1913; Stummer-Traunfels 1933; Oya and Kajihara 2019); exceptions would include *Gnesioceros sargassicola* identified by Faubel (1983, figs 38, 39), in which the mouth opens centrally and Lang's vesicle is rounded, and *C. agilis*, in which the shape of Lang's vesicle is not described by Lang (1884). Should the name Gnesiocerotidae be given to this clade, these characters might be diagnostic for it.

Here, however, I refrain from redefining Gnesiocerotidae as a valid taxon because I cannot rule out the possibility that Gnesiocerotidae Marcus and Marcus, 1966 is a junior synonym of Stylochoplanidae Meixner, 1907, until the nominal species *Stylochus maculatus* Quatrefages, 1845 (= *Stylochoplana maculata*, the type species of *Stylochoplana* Stimpson, 1857, which, in turn, is the type genus for Stylochoplanidae) can be placed in the context of a molecular phylogeny. Lang (1884, p. 457) mentioned that *C. agilis* closely resembles *Stylochoplana maculata*. Now that *C. agilis* has turned out to be closely related to *Gnesioceros sargassicola* (Figs IV-1, IV-2), the family-group-name-bearing genera *Gnesioceros* and *Stylochoplana* may also be closely related to each other, given Lang's (1884) observation and remarks. This is why Gnesiocerotidae and Stylochoplanidae possibly refer to the same taxon, in which case the two names compete for nomenclatural precedence.

Comoplana. Faubel (1983) defined *Comoplana* as stylochoplanids with a pair of nuchal tentacles, a common genital pore, and Lang's vesicle, and without a penis stylet. However, the two species of *Comoplana* included in these analyses, *C. agilis* and *C. pusilla*, were not sister taxa (Figs IV-1, IV-2). This means that the set of morphological characters used by Faubel (1983) to define *Comoplana* does not pin down a monophyletic group. Given the possible close relationship between *C. agilis* (type species of *Comoplana*) and *Stylochoplana maculata* (type species of *Stylochoplana*) (see above), *Comoplana* might be a junior synonym of *Stylochoplana*. *Comoplana pusilla*, on the other hand, is the type species of the genus-group name *Stylochoplanoides* Bock, 1924. The latter was originally established as a subgenus within *Stylochoplana* (Bock 1924), but Prudhoe (1985) regarded it as synonymous with *Stylochoplana*. The name *Stylochoplanoides* may become valid for *Comoplana pusilla* (*i.e.,* '*Stylochoplanoides pusilla*') in future studies.

Notocomplanidae. Litvaitis *et al.* (2019) established Notocomplanidae for a clade comprising *Notocomplana ferruginea* (Schmarda, 1859)—which, however, is different from *Notocomplana ferruginea* sequenced by Rawlinson *et al.* (2011) and used in the analyses of Bahia *et al.* (2017), Tsunashima *et al.* (2017), and Dittmann *et al.* (2019)—and *Notocomplana lapunda* Du Bois-Reymond Marcus and Marcus, 1968, defining Notocomplanidae as "notoplanids with an unarmed penis papilla", a character shared by the two species. The species included in the analyses that fit this definition are *Notocomplana ferruginea* (Schmarda, 1859); *Notocomplana hagiyai* Oya and Kajihara, 2017; *Notocomplana humilis* (Stimpson, 1857); *Notocomplana japonica* (Kato, 1937); *Notocomplana koreana* (Kato, 1937); *Notocomplana lapunda*; and *Notocomplana septentrionalis* (Kato, 1937). These species, however, did not form a single clade in the trees (Figs IV-1, IV-2). Needless to say, the name 'Notocomplanidae' should be applied to a family that includes *Notocomplana humilis* (type species of *Notocomplana*). In the trees (Figs IV-1, IV-2), the least inclusive clade containing *Notocomplana humilis* and *Notoplana atomata* was highly supported with 1.00/100. If a family name were to be applied to this clade, either Notoplanidae Marcus, 1947 or Pleioplanidae Faubel, 1983—rather than Notocomplanidae—would be the valid name, because the nominal species *Planaria atomata* Müller, 1776 (= *Notoplana atomata*) is the type species of *Pleioplana* Faubel, 1983, which is the type genus of Pleioplanidae; Litvaitis *et al.* (2019) synonymized the latter with Notoplanidae. Moreover, once the nominal species *Centrostomum dubium* Schmarda, 1859 (= *Notoplana dubia*, the type species of *Notoplana* Laidlaw, 1903) is included in future studies, the name Notoplanidae Marcus, 1947 may also come to compete for nomenclatural precedence with Pleioplanidae and Notocomplanidae. With these competing names, there seems little chance that Notocomplanidae is valid.

Pseudostylochus. In the trees (Figs IV-1, IV-2), *Pseudostylochus elongatus* is the sister taxon to *P. takeshitai*, and *P. intermedius* to *P. obscurus*; these four species form a sister clade to *Comoplana pusilla*. Previous studies (Tsunashima *et al.* 2017; Dittmann *et al.* 2019) showed only that *P. elongatus* and *P. obscurus* comprise a highly supported clade distantly related to *Callioplana*. The analyses, which include more species, better resolve the relationships within *Pseudostylochus*.

The results affirm that *Pseudostylochus* belongs in its own family, Pseudostylochidae Faubel, 1983 (Tsunashima *et al.* 2017; Dittmann *et al.* 2019), rather than Callioplanidae Hyman, 1953 (Prudhoe 1985). In the trees, *Pseudostylochus* and *Callioplana* appear in different superfamilies, Leptoplanoidea and Stylochoidea, respectively (though with some reservations; see above).

The alleged morphological separation of *Koinostylochus* Faubel, 1983 from *Pseudostylochus* purported by Faubel (1983) is baseless. Faubel (1983) assigned *P. takeshitai* and *P. elongatus* to *Koinostylochus* under Callioplanidae, and *P. intermedius* and *P. obscurus* to *Pseudostylochus* under Pseudostylochidae. Focusing on the inner lining of the prostatic vesicle, Faubel (1983) categorized this structure into several types that he regarded as defining different families. According to Faubel (1983), the inner epithelium of the prostatic vesicle in *Koinostylochus* is “smooth” and that in *Pseudostylochus* is “tubularly chambered”. Faubel (1983) judged the inner lining of the prostatic vesicle of *P. obscurus* to be “tubularly chambered”, possibly based on an illustration by Yeri and Kaburaki (1918b, text-fig. 34). In fact, however, the character state in *P. obscurus* is “smooth” in the sense of Faubel (1983) (Fig. IV-3B; Morita *et al.* 2018, fig. 3C). In addition, *Pseudostylochus intermedius*—assigned to *Pseudostylochus* in the sense of Faubel (1983), which should have had a “tubularly chambered” lining—actually possesses a “wavy” lining (Fig. IV-3D; Tajika and Ishida 1999, figs 3, 4); the latter is found in callioplanid species in the sense of Faubel (1983, p. 56). Therefore, ‘*Koinostylochus*’ *takeshitai* and ‘*Koinostylochus*’ *elongatus* cannot be differentiated from *Pseudostylochus elongatus* and *P. intermedius* in the way Faubel (1983) envisioned, and thus I consider *Koinostylochus* to be synonymous with

Pseudostylochus.

***Mirostylochus*.** The familial affiliation of *Mirostylochus* Kato, 1937 should be tested in future studies with additional latocestid species. This study shows that *Mirostylochus* may belong to Latocestidae or its own new family, rather than to Pseudostylochidae (Faubel 1983) or Stylochidae (Prudhoe 1985). In the trees (Figs IV-1, IV-2), *M. akkeshiensis*, the type species of *Mirostylochus*, is the sister taxon to *Latocestus* sp. (Latocestidae). *Mirostylochus akkeshiensis* shares the following morphological characters with latocestids: *i*) the frontal eyespots are spread on the anterior part of the body, *ii*) the nuchal tentacles are absent, *iii*) the paired spermiducal bulbs are united into a common sperm duct that leads to the ejaculatory duct, and *iv*) the mouth opens at the posterior end of the pharyngeal cavity (in *Mirostylochus*, the mouth opens at the common genital atrium). At present, however, it remains unclear whether *Mirostylochus* is the sister taxon to Latocestidae, or is completely nested in a latocestid clade.

Reclassification of Acotylea

Bahia *et al.* (2017), Dittmann *et al.* (2019), and Litvaitis *et al.* (2019) modified the superfamily-level acotylean classification on the basis of molecular phylogenetic analyses. However, I perceive that additional minor revisions are necessary. Below, I slightly amend the extensional definitions of Dittmann *et al.* (2019) and add morphological diagnoses.

Superfamily **Discoceloidea** Laidlaw, 1903

Definition. Acotylea containing at least Discocelidae, Ilyplanidae, *Amemiyaia*, and *Phaenocelis*.

Diagnosis. Not having prostatic vesicle; or, having *i*) interpolated prostatic vesicle and *ii*) marginal eyespots.

Remarks. Dittmann *et al*. (2019) defined Discoceloidea as comprising "Cryptocelididae", Discocelidae, and Ilyplanidae. I consider "Cryptocelididae Poche, 1926" to be a junior homonym of Cryptocelididae Bergendal, 1893 and simultaneously a junior synonym of Cryptocelidae Laidlaw, 1903. Poche (1926), for unknown reasons, proposed the unnecessary new replacement name Cryptocelididae for Cryptocelidae Laidlaw, 1903. Cryptocelididae Poche, 1926, which had been previously occupied by Cryptocelididae Bergendal, 1893, includes *Cryptocelis* Lang, 1884 but not *Cryptocelides* Bergendal, 1890.

In addition, *Cryptocelis* (and thus Cryptocelidae) is probably not a member of Discoceloidea, judging from the molecular phylogeny of Kenny *et al*. (2019, fig. 4) based on nucleotide sequences from 14 mitochondrial genes. Kenny *et al*. (2019) found *Cryptocelis alba* (Schmidtlein, 1880), the type species of *Cryptocelis*, to be the sister group to three superfamilies and separately positioned from *Discocelis tigrina*. Although Kenny *et al.* (2019, fig. 6B) showed a clade of *Cryptocelis* + *Discocelis* in an analysis based on amino-acid alignments of five mitochondrial protein-coding genes, the nodal supports were extremely low.

Amemiyaia and *Phaenocelis* were formerly assigned to 'Phaenocelidae Stummer-Traunfels, 1933', but Marcus (1952) and Hyman (1953) treated Phaenocelidae as a junior synonym of Cryptocelidae; Faubel (1983) and Prudhoe (1985) followed this taxonomic view. To judge the validity of 'Phaenocelidae', phylogenetic studies including *Amemiyaia*, *Cryptocelis*, and *Phaenocelis* will be required.

Superfamily **Leptoplanoidea** Ehrenberg, 1831

Definition. Acotylea containing at least Leptoplanidae, Notoplanidae, Pseudostylochidae, and Stylochoplanidae.

Diagnosis. Not having marginal eyespots. Having interpolated prostatic vesicle; or, having *i*) free prostatic vesicle with seminal vesicle, *ii*) penis papilla, and *iii*) Lang's vesicle.

Remarks. The above definition is essentially that proposed by Dittmann *et al.* (2019), except for the phrase "at least", which I added here. The BI tree (Fig. IV-1) shows two specimens of *Leptoplana tremellaris*, type species of the type genus of the type family of Leptoplanoidea, positioned separately; in the ML tree (Fig. IV-2), the two form a clade, but with a support value lower than 70%. This does not affect the superfamily-level taxonomy, but the true identity of *L. tremellaris* should be ascertained by future studies.

Superfamily **Stylochoidea** Stimpson, 1857

Definition. Acotylea containing at least Hoploplanidae, Idioplanidae, Planoceridae, and Stylochidae.

Diagnosis. Having free prostatic vesicle; or, having interpolated prostatic vesicle with—instead of seminal vesicle—a pair of spermiducal bulbs.

Remarks. The above definition is essentially that proposed by Dittmann *et al.*

(2019), except for the phrase “at least”, which I added here. It remains uncertain whether Callioplanidae is encompassed in Stylochoidea (see above). For the time being, I leave Callioplanidae in Stylochoidea to avoid confusion.

V. SUMMARY

Acotylea is an assemblage of polyclads lacking the cotyl and contains more than 350 species. During my Ph.D course, I conducted taxonomic and phylogenetic studies of Acotylea in Japan. This thesis is compiled from Oya and Kajihara (2017, 2019, 2020, in review) and Oya *et al.* (2019, 2020, in prep.).

Chapter I is a general section of this thesis. I introduced overviews of Polycladida and reviewed systematics and phylogenetics of Acotylea. In addition to the introduction, I summarized sampling and observation methods common to each chapter.

In Chapter II, I described eight acotyleans (five genera, three families) from the intertidal zone in Japan. Four of five genera (*Alloioplana*, *Armatoplana*, *Phaenoplana*, *Zygantroides*) have not been reported from Japanese waters until I started this research. In the descriptions of each species, I provided COI sequences tagged to morphological identification for the first time in Polycladida. The descriptions of four species of *Notocomplana*, one species of *Phaenoplana*, and one species of *Zygantroides* have been published as Oya and Kajihara (2017, 2019) and Oya *et al.* (2020), respectively. Two stylochoplanids, *Alloioplana* sp. and *Armatoplana* sp. will be formally described as new species in Oya *et al.* (in prep.).

In Chapter III, I described bathyal acotyleans; these are the first descriptions of the bathyal acotyleans around Japan. In the description of Planoceridae gen. et sp. indet., I judged the present species should be assigned to an undescribed genus based on its morphological characters and the phylogenetic position in Planoceridae; a new genus will be established in Oya and Kajihara (in review). In the description of *Paraplehnia seisuiae*, I reported a genital pit for the first time in plehniids (Oya *et al.* 2019).

In Chapter IV, I discussed phylogeny of Acotylea. I newly determined partial sequences of four genes (16S, 18S, 28S, and COI) from 24 acotylean species (12 families and 14 genera). Based on these sequences in addition to those available in public databases, I inferred the phylogeny of 16 families and 27 genera of Acotylea from molecular phylogenetic analyses based on concatenated gene sequences. The phylogeny supported three clades corresponding to Discoceloidea, Leptoplanoidea, and Stylochoidea; I amended definitions of each superfamily and add morphological diagnoses. The phylogenetic position of Callioplanidae remains unclear. Among family- or genus-level taxa, I indicated Gnesiocerotidae, Stylochoplanidae, and *Comoplana* were not monophyletic. In addition, I discussed the validities of Notocomplanidae and *Koinostylochus*, and the family-level assignment of *Mirostylochus*. These contents have been published in Oya and Kajihara (2020).

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FIGURES AND TABLES

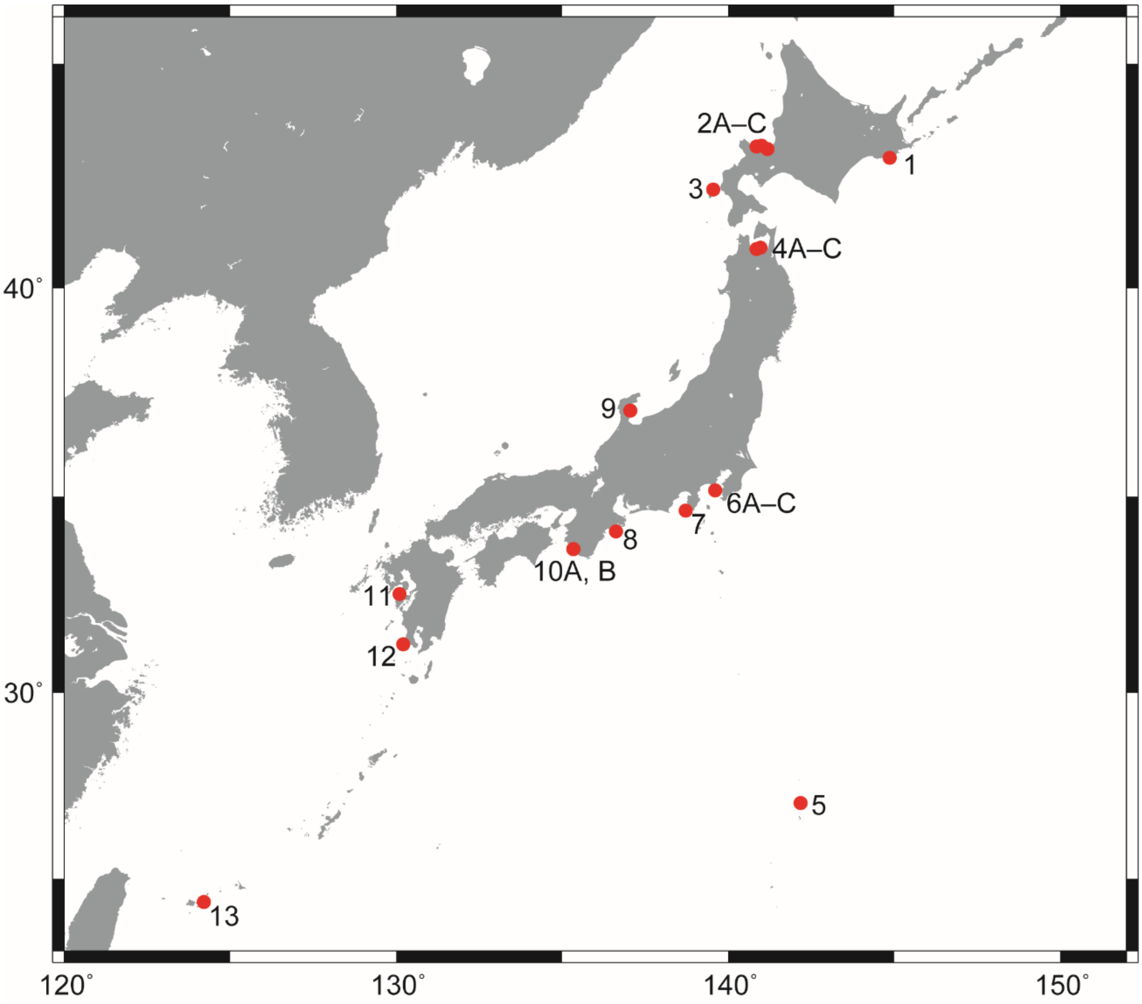

Figure I-1. Sampling sites in this study. The site numbers correspond to those in Table I-1.

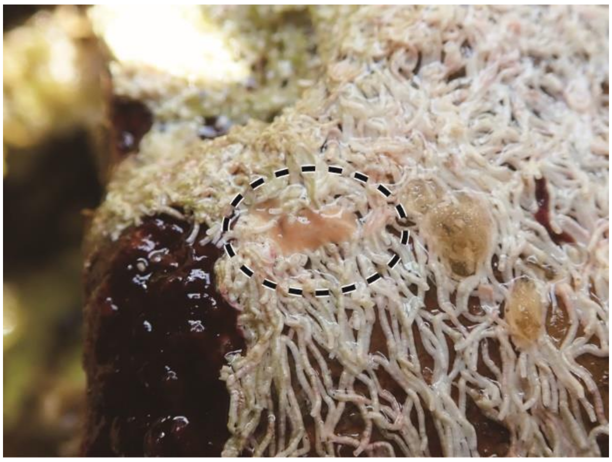

Figure II-1. Photograph of a living individual of *Zygantroides serpulidicola* Oya, Tsuyuki and Kajihara, 2020 on tubes of serpulid annelids.

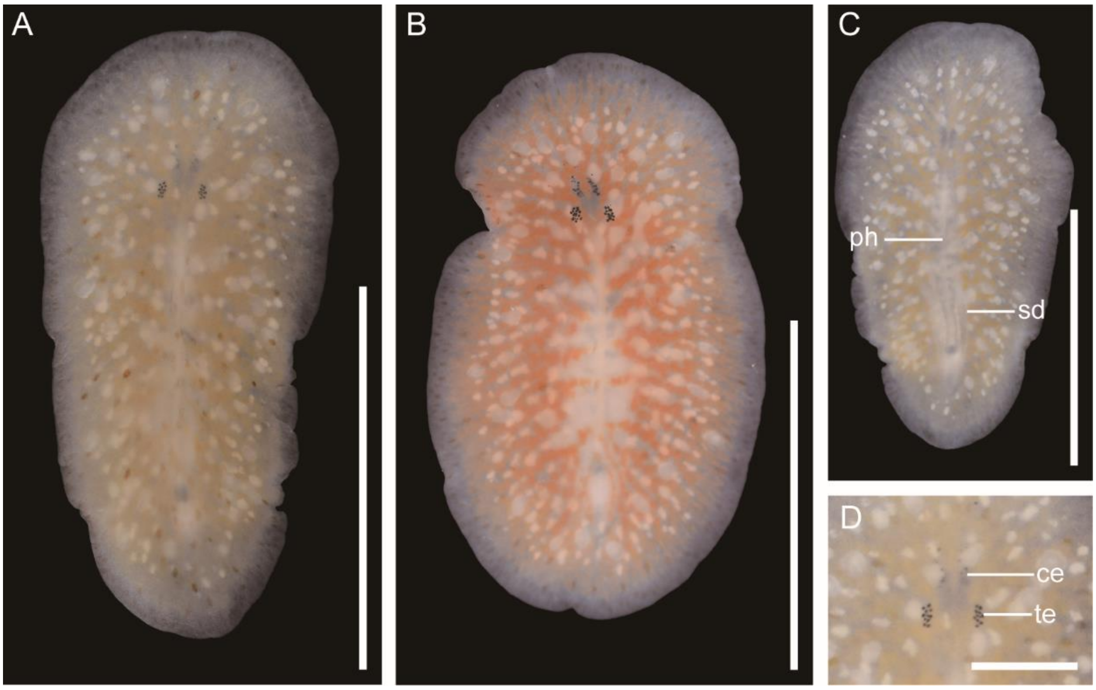

Figure II-2. *Zygantroides serpulidicola* Oya, Tsuyuki and Kajihara, 2020, photographs taken in life. A, B, Dorsal view, ICHUM 6023 (holotype, A), ICHUM 6025 (paratype, B); C, ventral view, ICHUM 6023 (holotype); D, eyespots, ICHUM 6023. Abbreviations: ce, cerebral eyespot; ph, pharynx; sd, sperm duct; te, tentacular eyespot. Scale bars: 5 mm (A–C); 1 mm (D).

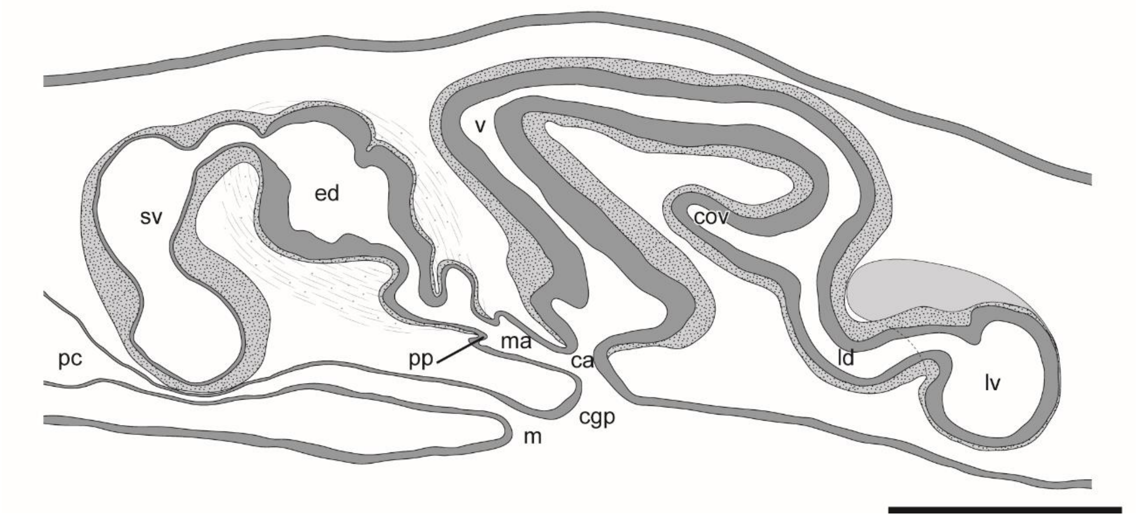

Figure II-3. *Zygantroides serpulidicola* Oya, Tsuyuki and Kajihara, 2020, schematic diagram of male and female copulatory apparatuses, anterior to the left. Abbreviations: ca, common genital atrium; cgp, common gonopore; cov, common oviduct; ed, ejaculatory duct; ld, Lang's-vesicle duct; lv, Lang's vesicle; m, mouth; ma, male atrium; pc, pharyngeal cavity; pp, penis papilla; sv, seminal vesicle; v, vagina. Scale bar: 200 μm.

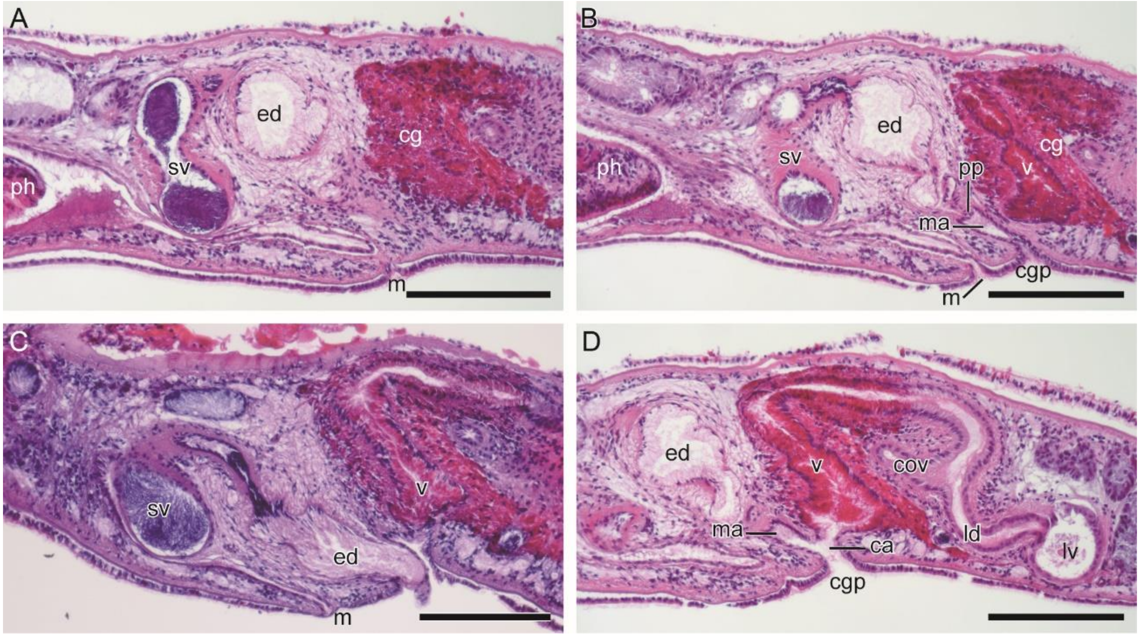

Figure II-4. *Zygantroides serpulidicola* Oya, Tsuyuki and Kajihara, 2020, photomicrographs of sagittal sections, anterior to the left. A–C, Male copulatory apparatus, ICHUM 6023 (holotype, A and B), ICHUM 6025 (paratype, C); D, female copulatory apparatus, ICHUM 6023 (holotype). Abbreviations: ca, common genital atrium; cg, cement glands; cov, common oviduct; cgp, common gonopore; ed, ejaculatory duct; ld, Lang's-vesicle duct; lv, Lang's vesicle; m, mouth; ma, male atrium; ph, pharynx; pp, penis papilla; sv, seminal vesicle; v, vagina. Scale bars: 200 µm (A–D).

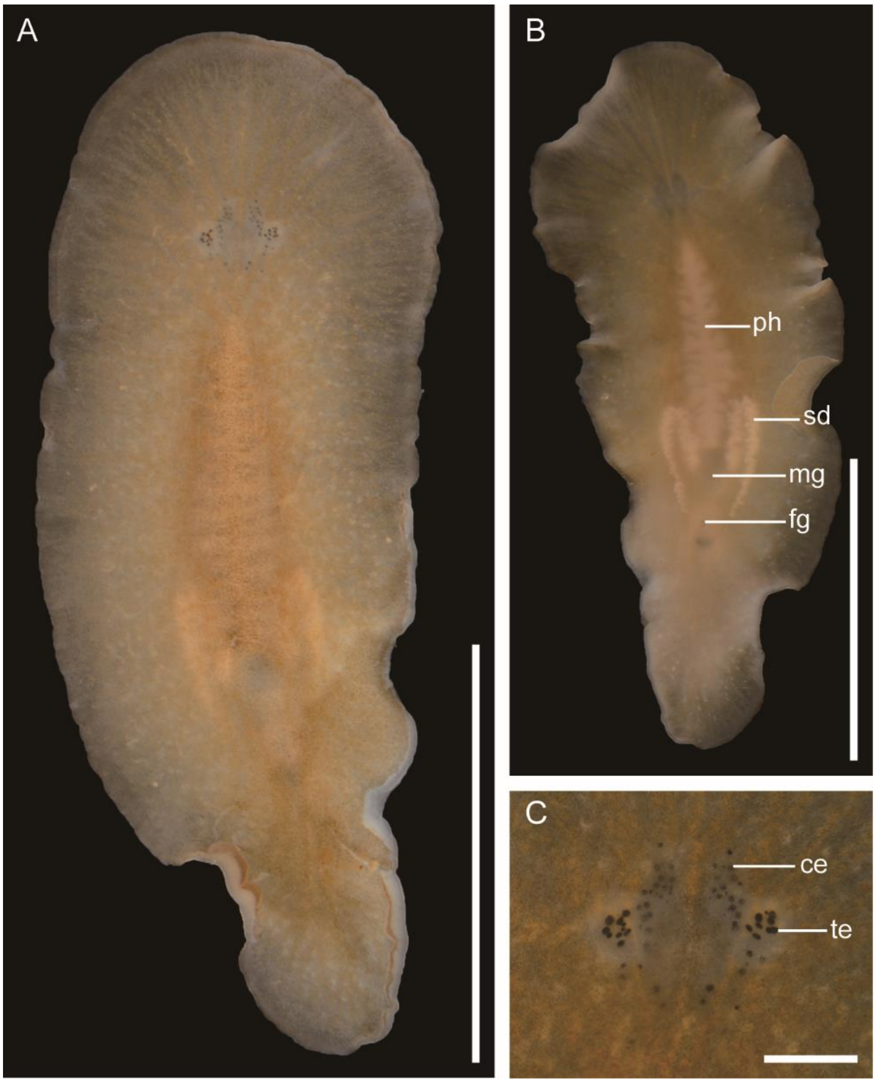

Figure II-5. *Notocomplana hagiyai* Oya and Kajihara, 2017, photographs taken in life, ICHUM 5267 (holotype). A, Dorsal view; B, ventral view; C, eyespots. Abbreviations: ce, cerebral eyespot; fg, female gonopore; mg, male gonopore; ph, pharynx; sd, sperm duct; te, tentacular eyespot. Scale bars: 5 mm (A, B); 500 µm (C).

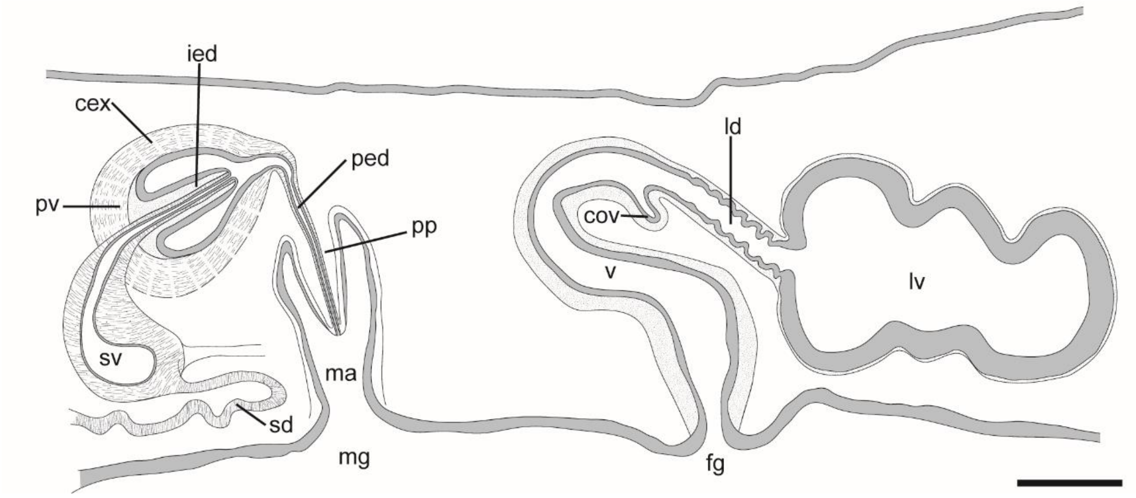

Figure II-6. *Notocomplana hagiyai* Oya and Kajihara, 2017, schematic diagram of male and female copulatory apparatuses, anterior to the left. Abbreviations: cex, canal of extra-vesicular gland; cov, common oviduct; fg, female gonopore; ied, intra-prostatic ejaculatory duct; ld, Lang's-vesicle duct; lv, Lang's vesicle; ma, male atrium; mg, male gonopore; ped, post-prostatic ejaculatory duct; pp, penis papilla; pv, prostatic vesicle; sd, sperm duct; sv, seminal vesicle; v, vagina. Scale bar: 200 µm.

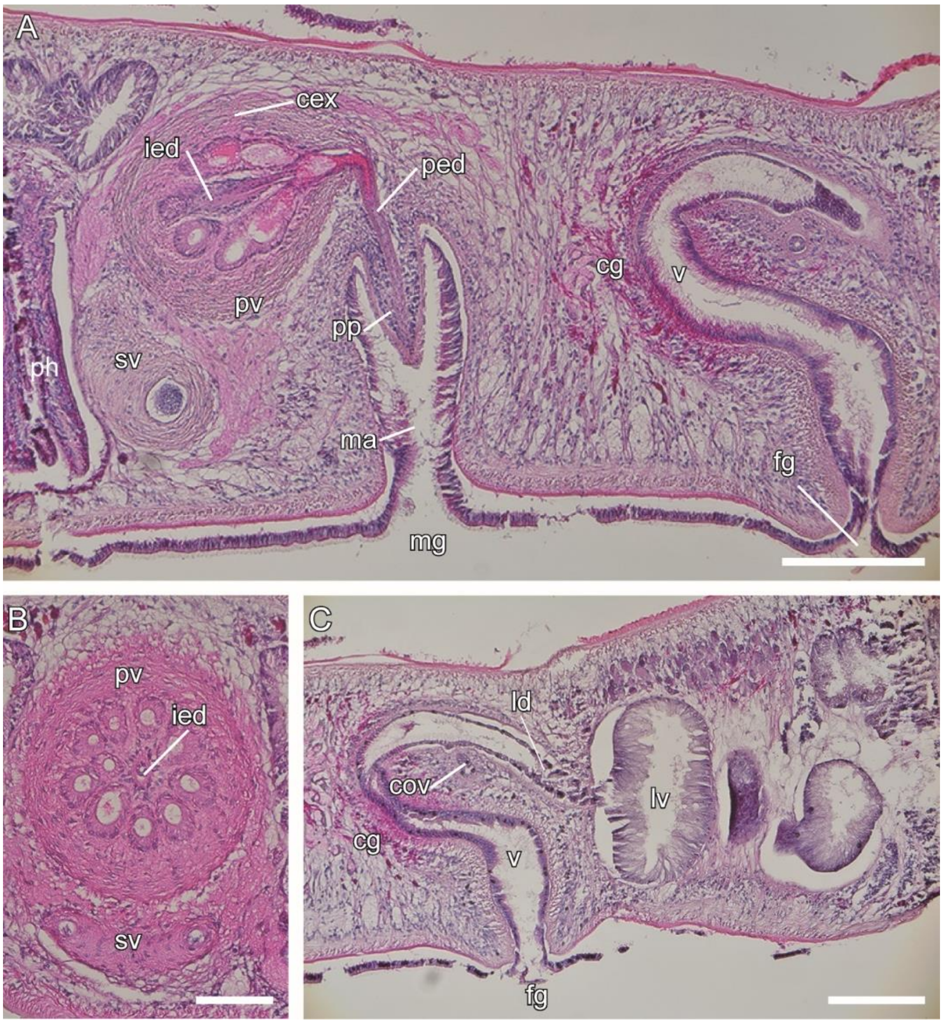

Figure II-7. *Notocomplana hagiyai* Oya and Kajihara, 2017, photomicrographs of sagittal (A, C, anterior to the left) and cross (B) sections. A, Male copulatory apparatus, ICHUM 5267 (holotype); B, prostatic vesicle, ICHUM 5272 (paratype); C, female copulatory apparatus, ICHUM 5267 (holotype). Abbreviations: cex, canal of extra-vesicular gland; cg, cement glands; cov, common oviduct; fg, female gonopore; ied, intra-prostatic ejaculatory duct; ld, Lang’s-vesicle duct; lv, Lang’s vesicle; ma, male atrium; mg, male gonopore; ped, post-prostatic ejaculatory duct; ph, pharynx; pp, penis papilla; pv, prostatic vesicle; sv, seminal vesicle; v, vagina. Scale bars: 200 µm (A, C); 100 µm (B).

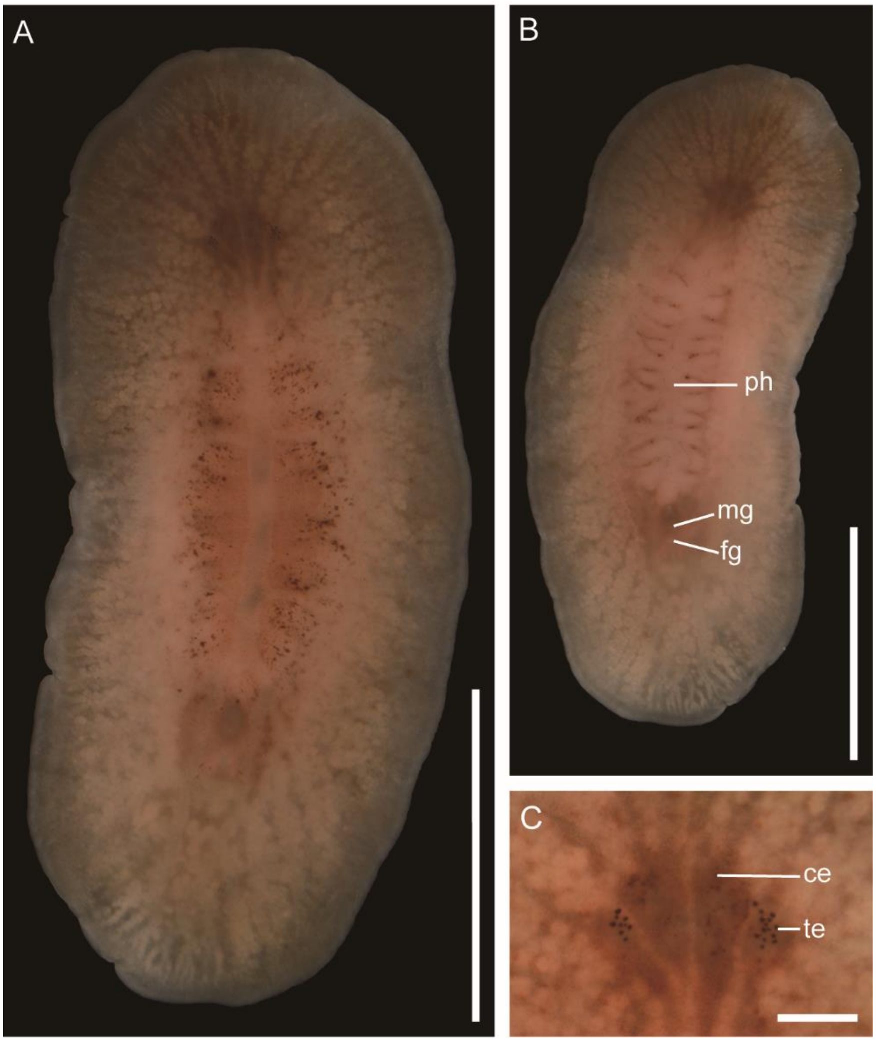

Figure II-8. *Notocomplana japonica* (Kato, 1937), photographs taken in life, ICHUM 5282 (non-type). A, Dorsal view; B, ventral view; C, eyespots. Abbreviations: ce, cerebral eyespot; fg, female gonopore, mg, male gonopore; ph, pharynx; te, tentacular eyespot. Scale bars: 5 mm (A, B); 500 µm (C).

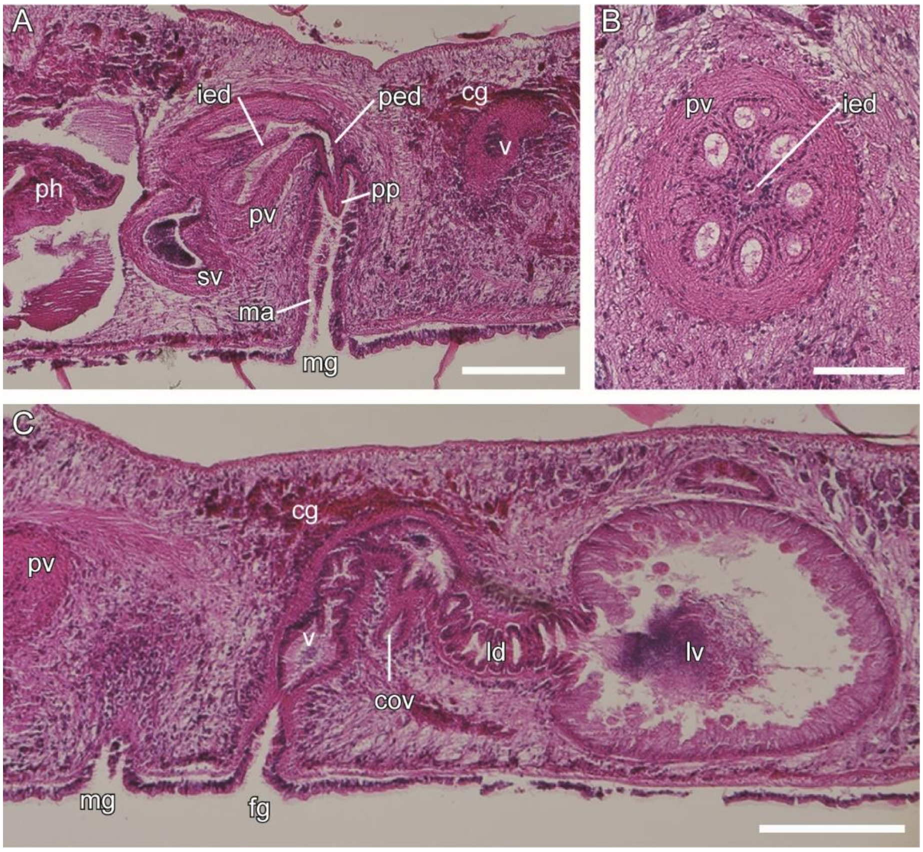

Figure II-9. *Notocomplana japonica* (Kato, 1937), photomicrographs of sagittal (A, C, anterior to the left) and cross (B) sections. A, Male copulatory apparatus, ICHUM 5282 (non-type); B, prostatic vesicle, ICHUM 5286 (non-type); C, female copulatory apparatus, ICHUM 5282 (non-type). Abbreviations: cg, cement glands; cov, common oviduct; fg, female gonopore; ied, intra-prostatic ejaculatory duct; ld, Lang's-vesicle duct; lv, Lang's vesicle; ma, male atrium; mg, male gonopore; ped, post-prostatic ejaculatory duct; ph, pharynx; pp, penis papilla; pv, prostatic vesicle; sv, seminal vesicle; v, vagina. Scale bars: 200 µm (A, C); 100 µm (B).

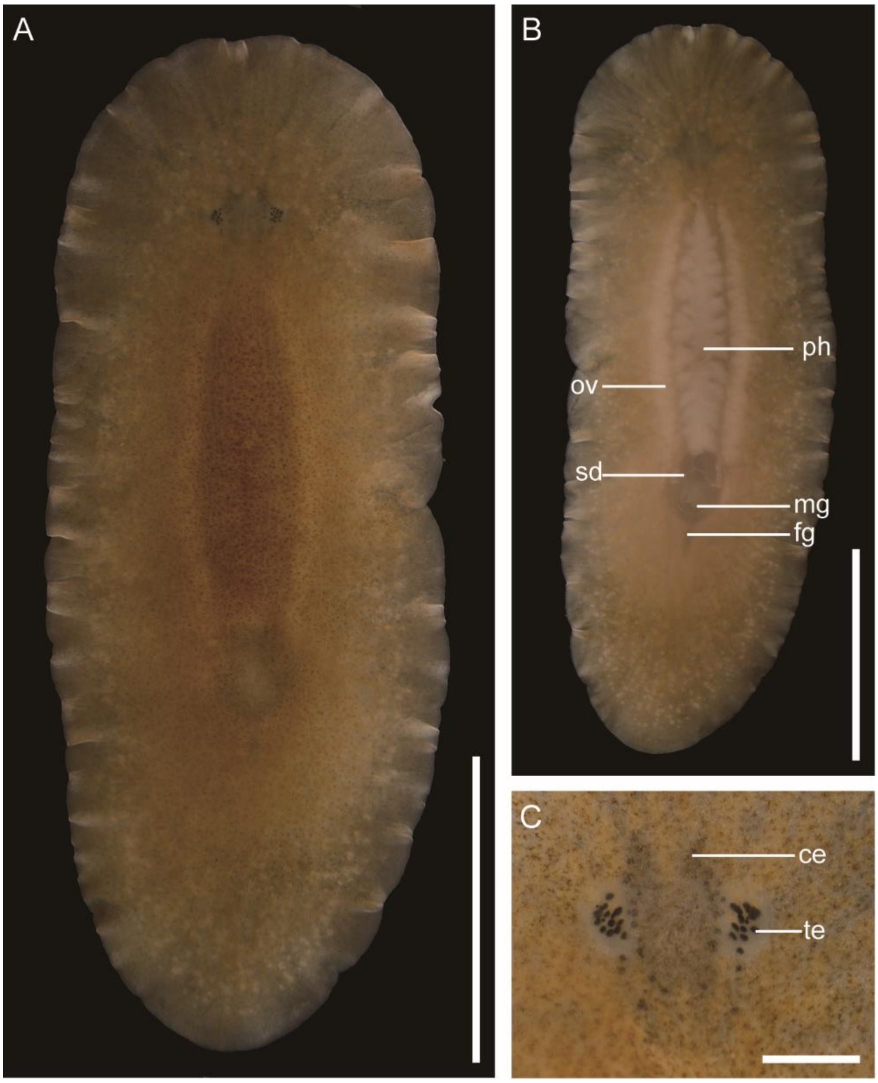

Figure II-10. *Notocomplana koreana* (Kato, 1937), photographs taken in life, ICHUM 5281 (non-type). A, Dorsal view; B, ventral view; C, eyespots. Abbreviations: ce, cerebral eyespot; fg, female gonopore, mg, male gonopore; ov, oviduct; ph, pharynx; sd, sperm duct; te, tentacular eyespot. Scale bars: 5 mm (A, B); 500 µm (C).

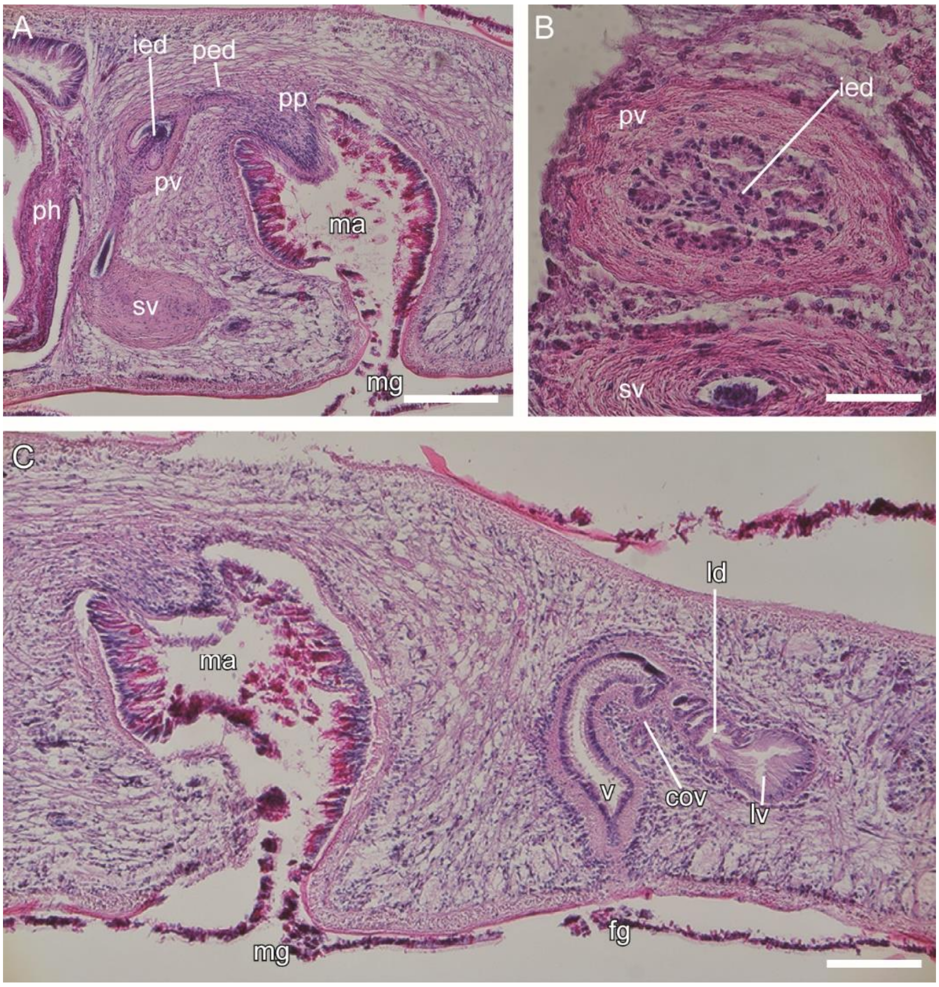

Figure II-11. *Notocomplana koreana* (Kato, 1937), photomicrographs of sagittal (A, C, anterior to the left) and cross (B) sections. A, Male copulatory apparatus, ICHUM 5278 (non-type); B, prostatic vesicle, ICHUM 5279 (non-type); C, female copulatory apparatus, ICHUM 5278 (non-type). Abbreviations: cov, common oviduct; fg, female gonopore; ied, intra-prostatic ejaculatory duct; ld, Lang's-vesicle duct; lv, Lang's vesicle; ma, male atrium; mg, male gonopore; ped, post-prostatic ejaculatory duct; ph, pharynx; pp, penis papilla; pv, prostatic vesicle; sv, seminal vesicle; v, vagina. Scale bars: 200 µm (A, C); 50 µm (B).

Figure II-12. *Notocomplana septentrionalis* (Kato, 1937), photographs taken in life. A–C, Dorsal view, ICHUM 5292 (non-type) (A), ICHUM 5289 (non-type) (B), ICHUM 5298 (non-type) (C); D, ventral view, ICHUM 5292 (non-type); E, eyespots, ICHUM 5292 (non-type). Abbreviations: ce, cerebral eyespot; fg, female gonopore, mg, male gonopore; ov, oviduct; ph, pharynx; te, tentacular eyespot. Scale bars: 5 mm (A–D); 500 μm (E).

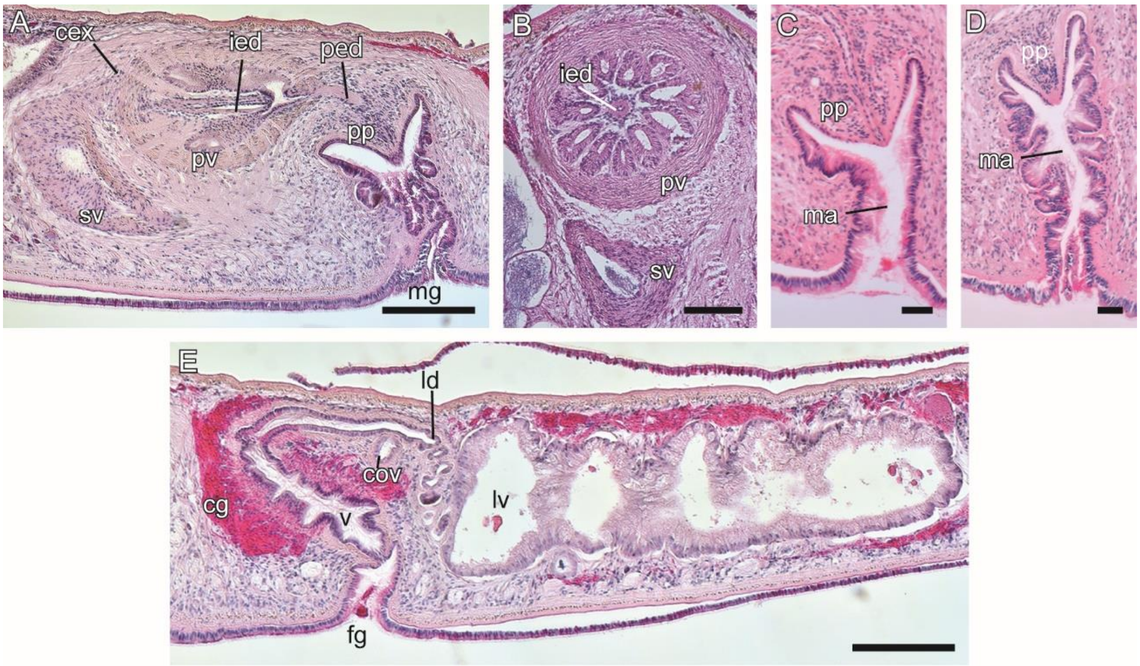

Figure II-13. *Notocomplana septentrionalis* (Kato, 1937), photomicrographs of sagittal (A, C–E, anterior to the left) and cross (B) sections. A, Male copulatory apparatus, ICHUM 5292 (non-type); B, prostatic vesicle, ICHUM 5295 (non-type); C, weakly folded epithelium of the male atrium, ICHUM 5291 (non-type); D, extensively folded epithelium of the male atrium, ICHUM 5294 (non-type); E, female copulatory apparatus, ICHUM 5292 (non-type). Abbreviations: cex, canal of extra-vesicular gland; cg, cement glands; cov, common oviduct; fg, female gonopore; ied, intra-prostatic ejaculatory duct; ld, Lang's-vesicle duct; lv, Lang's vesicle; ma, male atrium; mg, male gonopore; ped, post-prostatic ejaculatory duct; pp, penis papilla; pv, prostatic vesicle; sv, seminal vesicle; v, vagina. Scale bars: 200 µm (A, E); 100 µm (B); 50 µm (C, D).

Figure II-14. *Alloioplana* sp., photographs taken in life, ICHUM 6084. A, Dorsal view; B, ventral view; C, eyespots. Abbreviations: cte, cerebro-tentacular eyespot; fg, female gonopore, mg, male gonopore; ov, oviduct; ph, pharynx; sd, sperm duct. Scale bars: 5 mm (A, B); 1 mm (C).

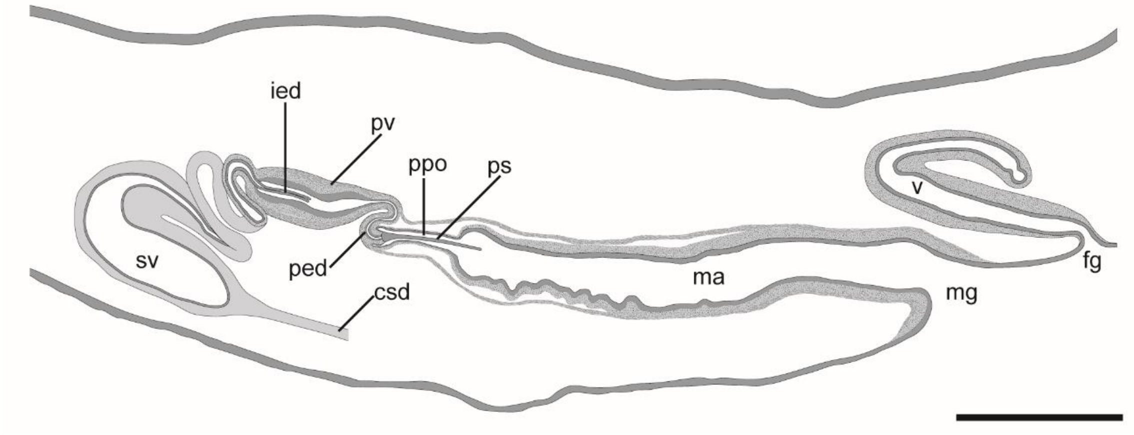

Figure II-15. *Alloioplana* sp., schematic diagram of male and female copulatory apparatuses, anterior to the left. Abbreviations: csd, common sperm duct; fg, female gonopore; ied, intra-prostatic ejaculatory duct; ma, male atrium; mg, male gonopore; ped, post-prostatic ejaculatory duct; ppo, penis pocket; ps, penis stylet; pv, prostatic vesicle; sv, seminal vesicle; v, vagina. Scale bar: 300 µm.

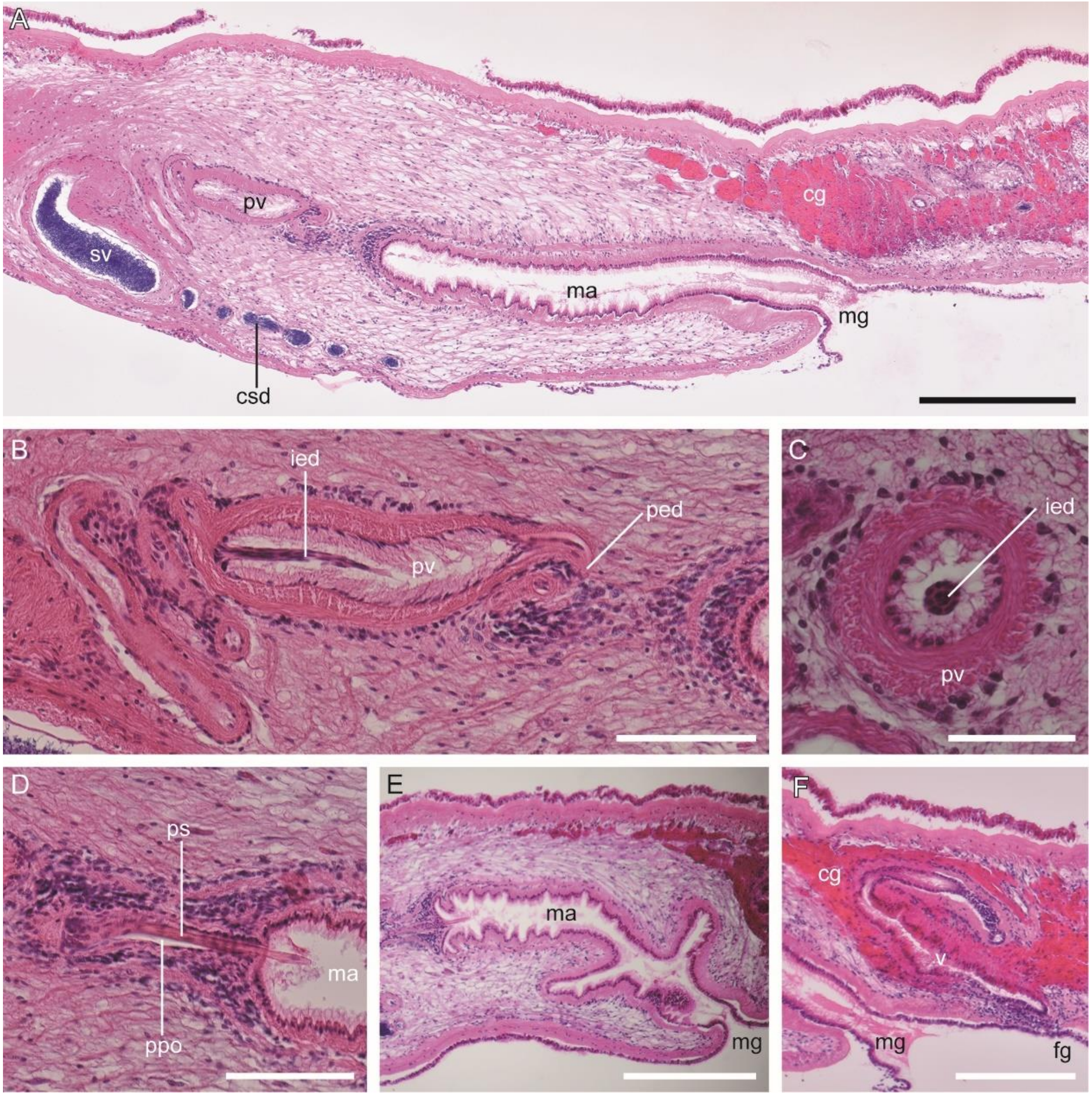

Figure II-16. *Alloioplana* sp., photomicrographs of sagittal (A, B, D–F, anterior to the left) and cross (C) sections. A, Male copulatory apparatus, ICHUM 6084; B, prostatic vesicle and intra-ejaculatory duct, ICHUM 6084; C, prostatic vesicle, ICHUM 6089; D, penis stylet, ICHUM 6084; E, male atrium, ICHUM 6082; F, female copulatory apparatus, ICHUM 6084. Abbreviations: cg, cement glands; csd, common sperm duct; fg; female gonopore; ied, intra-prostatic ejaculatory duct; ma, male atrium; mg, male gonopore; ped, post-prostatic ejaculatory duct; ppo, penis pocket; ps, penis stylet; pv, prostatic vesicle; sv, seminal vesicle; v, vagina. Scale bars: 300 μm (A, E); 100 μm (B, D); 50 μm (C); 200 μm (F).

Figure II-17. *Armatoplana* sp., photographs taken in life, ICHUM 6090. A, Dorsal view; B, ventral view; C, eyespots. Abbreviations: cte, cerebro-tentacular eyespot; fg, female gonopore, mg, male gonopore; ov, oviduct; ph, pharynx; sd, sperm duct. Scale bars: 5 mm (A, B); 1 mm (C).

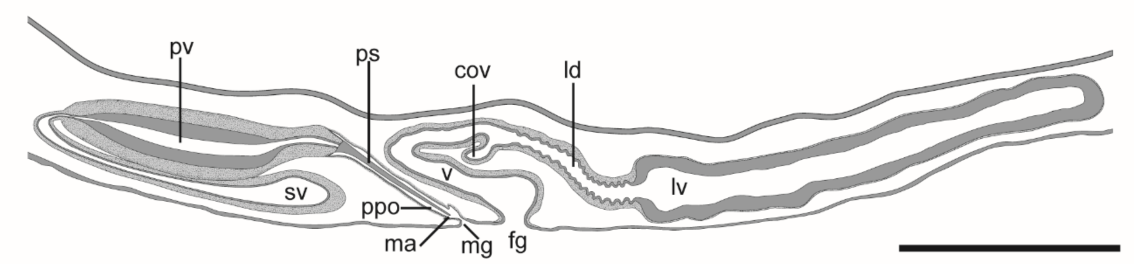

Figure II-18. *Armatoplana* sp., schematic diagram of male and female copulatory apparatuses, anterior to the left. Abbreviations: cov, common oviduct; fg, female gonopore; ld, Lang's-vesicle duct; lv, Lang's vesicle; ma, male atrium; mg, male gonopore; ppo, penis pocket; ps, penis stylet; pv, prostatic vesicle; sv, seminal vesicle; v, vagina. Scale bar: 500 µm.

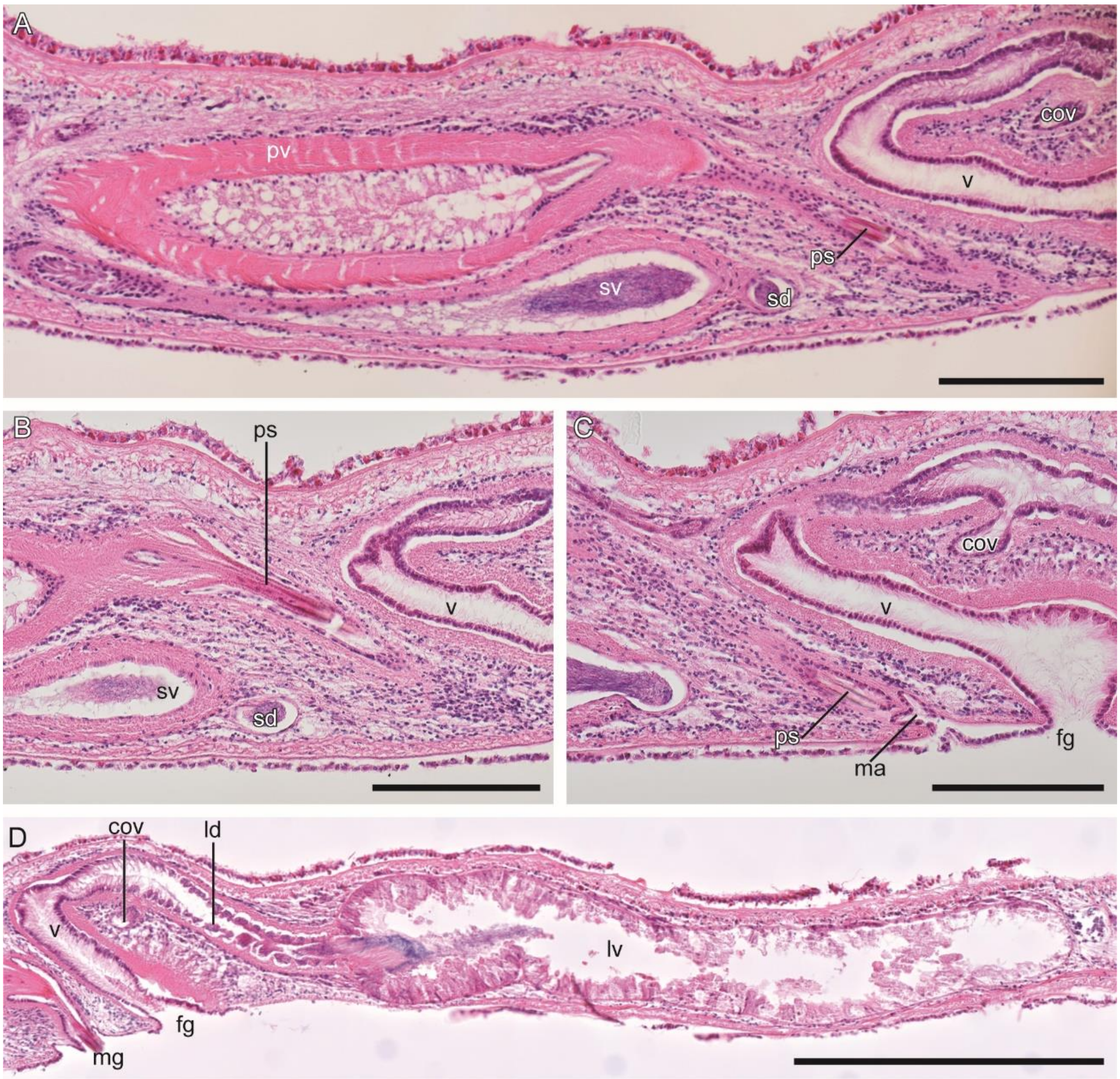

Figure II-19. *Armatoplana* sp., photomicrographs of sagittal sections, anterior to the left. A–C, Male copulatory apparatus, ICHUM 6090; D, female copulatory apparatus, ICHUM 6091. Abbreviations: cov, common oviduct; female gonopore; ld, Lang's-vesicle duct; lv, Lang's vesicle; ma, male atrium; mg, male gonopore; ps, penis stylet; pv, prostatic vesicle; sd, sperm duct; sv, seminal vesicle; v, vagina. Scale bars: 300 μm (A–C); 500 μm (D).

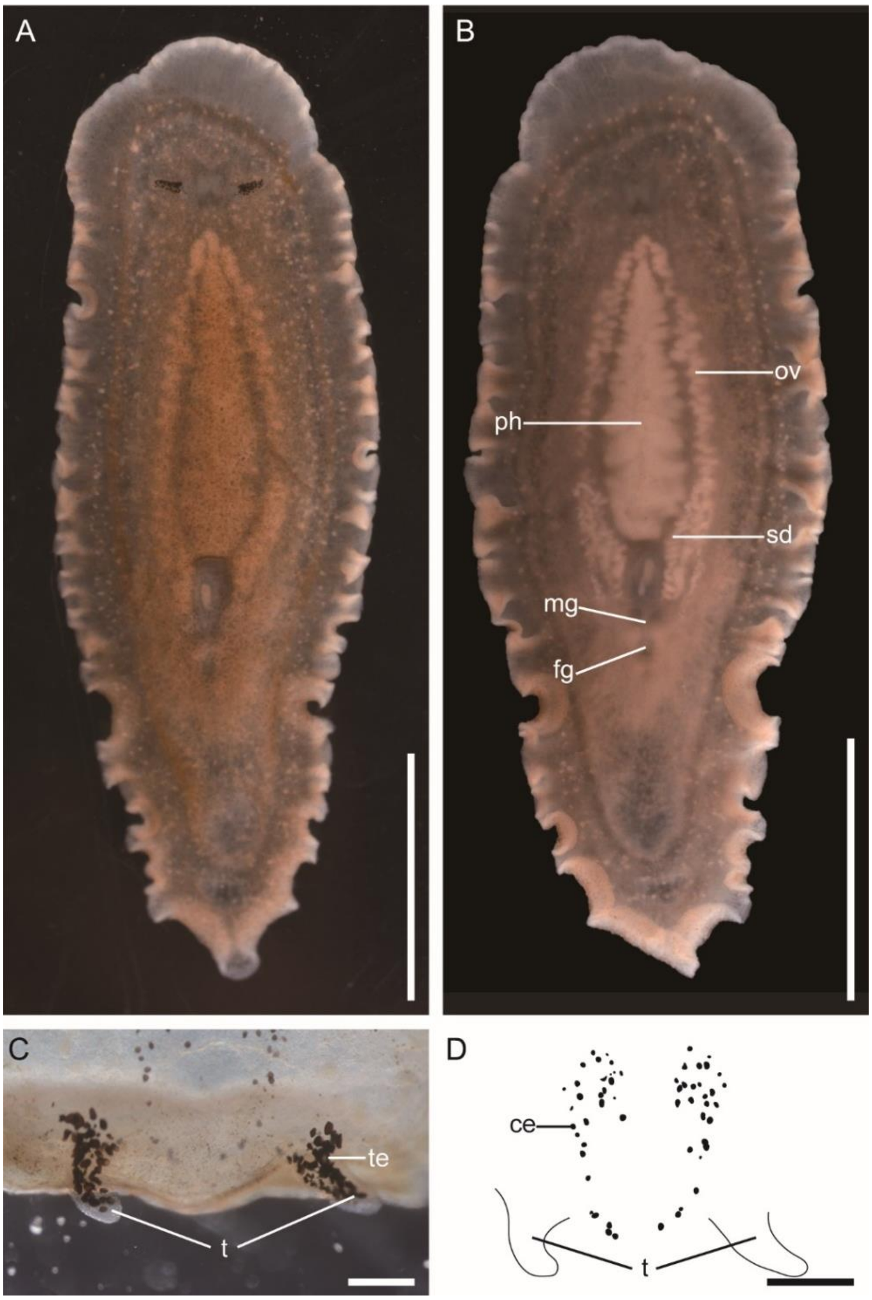

Figure II-20. *Phaenoplana kopepe* Oya and Kajihara, 2019, photographs taken in life (A, B), photograph (C) and sketch (D) of whole mount of anterior part, ICHUM 5343 (holotype). A, Dorsal view; B, ventral view; C, tentacles and eye clusters; D, tentacles and cerebral eye clusters. Abbreviations: ce, cerebral eyespot; fg, female gonopore, mg, male gonopore; ov, oviduct; ph, pharynx; sd, sperm duct; t; tentacle; te, tentacular eye. Scale bars: 5 mm (A, B); 300 μm (C, D).

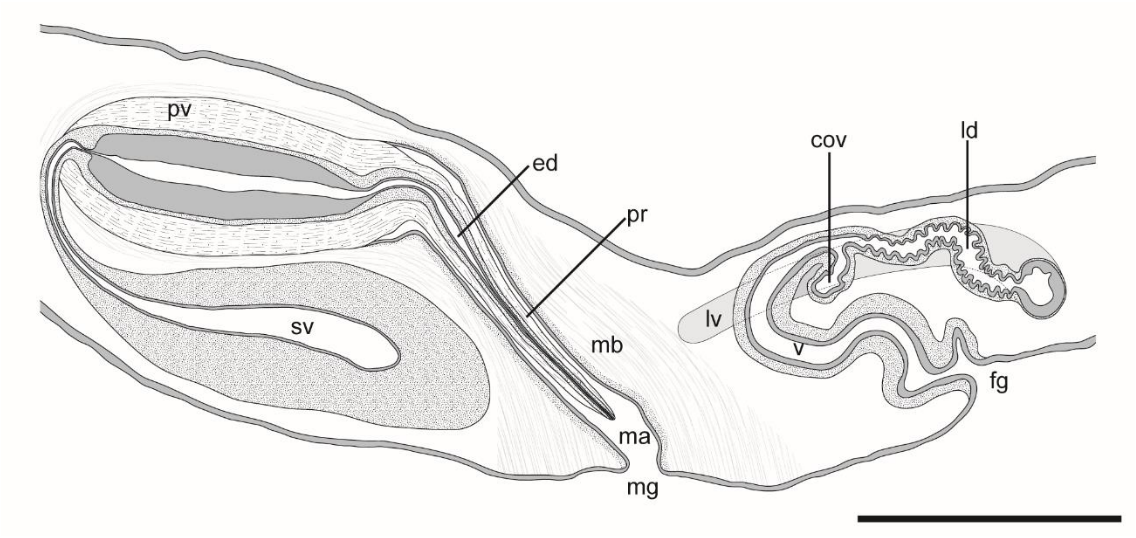

Figure II-21. *Phaenoplana kopepe* Oya and Kajihara, 2019, schematic diagram of male and female copulatory apparatuses, anterior to the left. Abbreviations: cov, common oviduct; ed, ejaculatory duct; fg, female gonopore; ld, Lang's-vesicle duct; lv, Lang's vesicle; ma, male atrium; mb, muscular bulb; mg, male gonopore; pr, penis rod; pv, prostatic vesicle; sv, seminal vesicle; v, vagina. Scale bar: 500 µm.

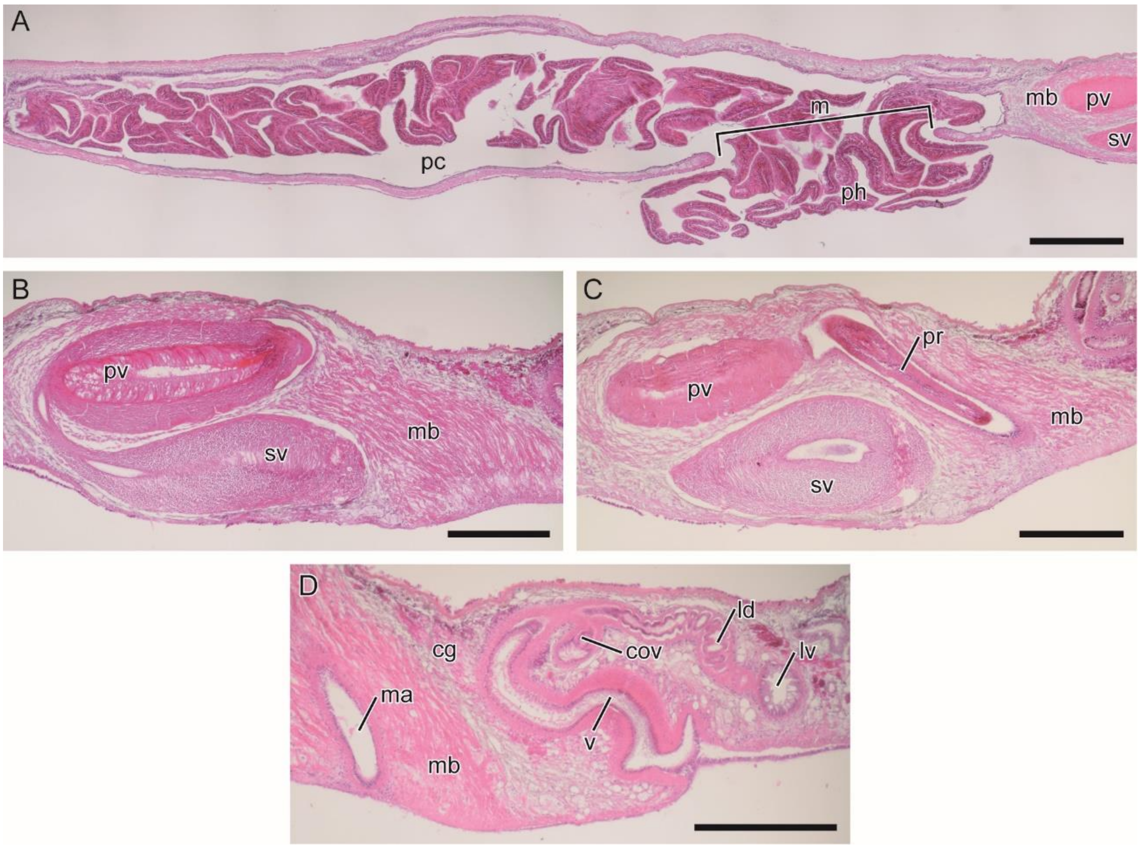

Figure II-22. *Phaenoplana kopepe* Oya and Kajihara, 2019, photomicrographs of sagittal sections, anterior to the left, ICHUM 5343 (holotype). A, Pharynx and mouth; B, C, male copulatory apparatus; D, female copulatory apparatus. Abbreviations: cg, cement glands; cov, common oviduct; ld, Lang's-vesicle duct; lv, Lang's vesicle; m, mouth; ma, male atrium; mb, muscular bulb; pc, pharyngeal cavity; ph, pharynx; pr, penis rod; pv, prostatic vesicle; sv, seminal vesicle; v, vagina. Scale bars: 500 µm (A); 300 µm (B–D).

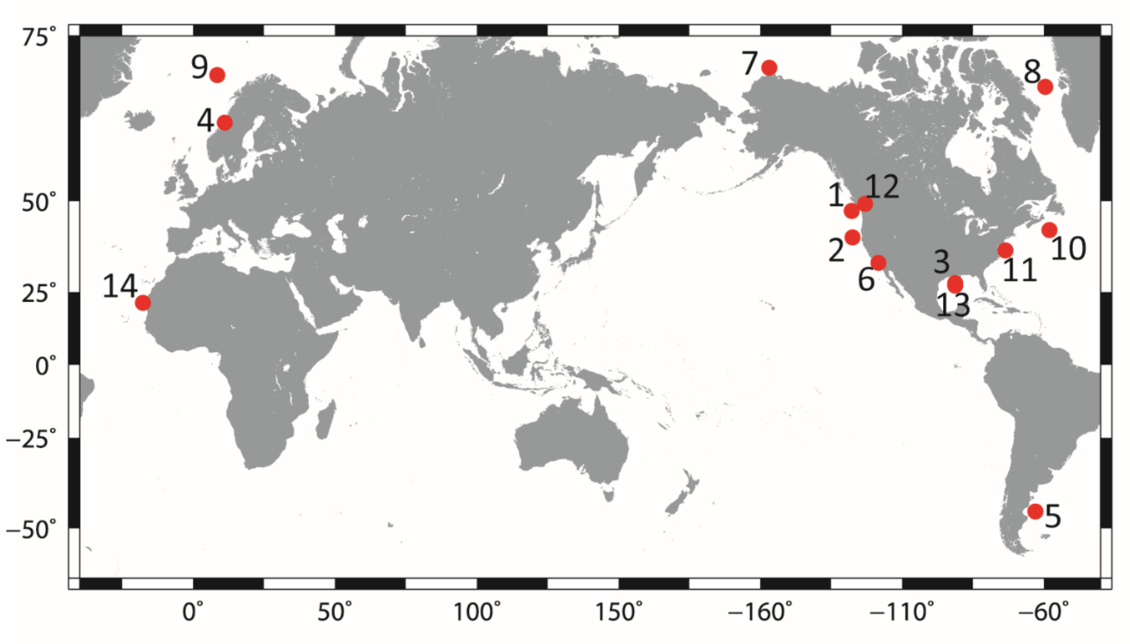

Figure III-1. Map showing locations where deep-sea polyclads have been reported:1, Cascadia Basin (Quiroga *et al.* 2006); 2, Escanaba Trough (Quiroga *et al.* 2006); 3 and 13, continental slope of the Gulf of Mexico (Quiroga *et al.* 2008); 4, Trondheim Fjord (Bock 1913); 5, coast of Argentina (Faubel 1983); 6, coast of California (Hyman 1953); 7, Point Barrow, Alaska (Hyman 1953); 8, Paffin Bay (Bock 1913); 9, coast of Jan Mayen Island (Bock 1913); 10, off Nova Scotia, Canada (Hyman 1940); 11, coast of Delmarva Peninsula (Verrill 1893); 12, Spieden Island (Freeman 1933); 14, coast of Mauritania (Faubel 1984).

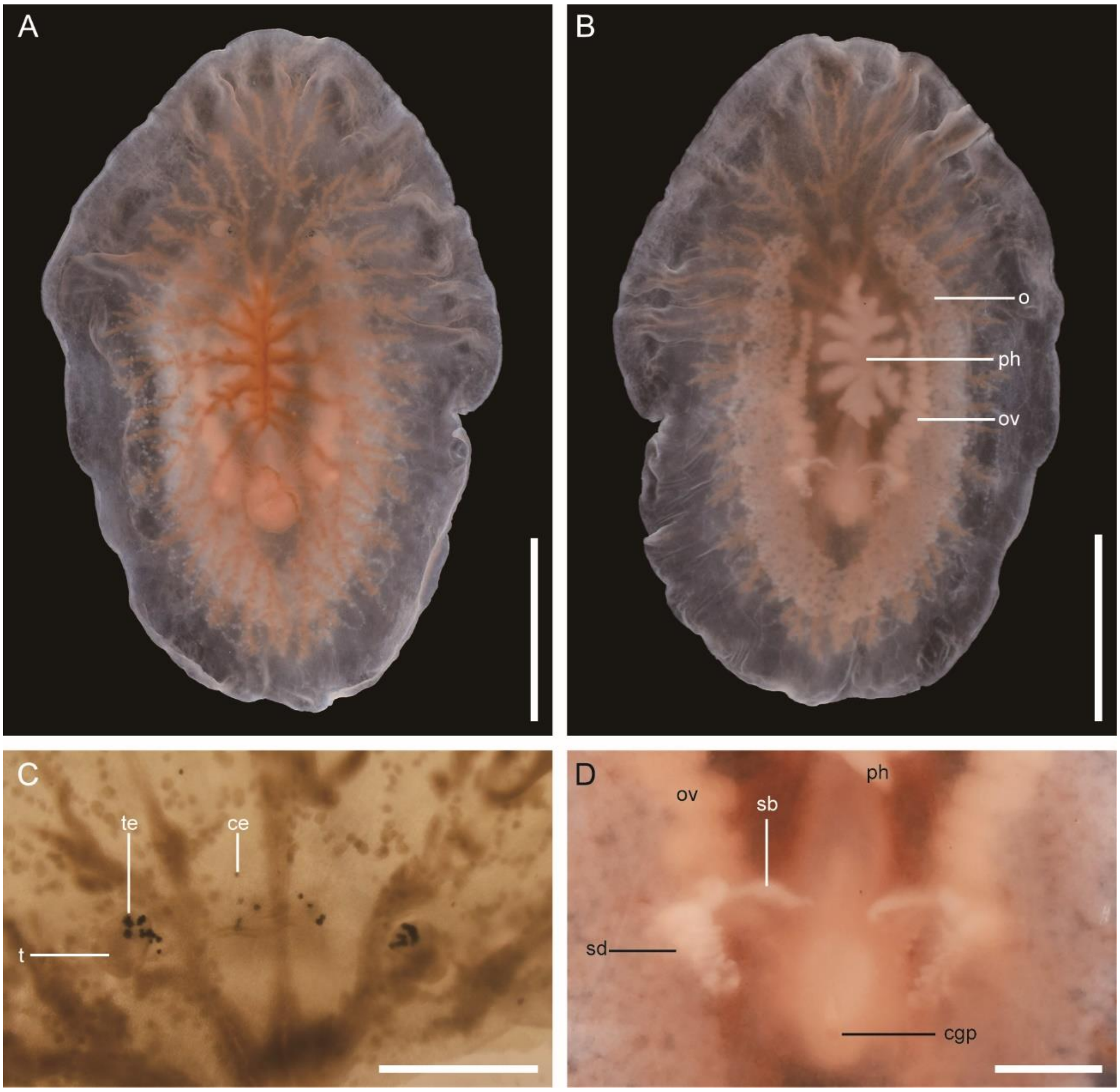

Figure III-2. Planoceridae gen. et sp. indet. (ICHUM 6081), photographs taken in life (A, B, D) and in preserved state cleared with xylene (C). A, Dorsal view; B. ventral view; C, eyespots; D, sperm ducts and oviducts observed from ventral view. Abbreviations: ce, cerebral eyespot; cgp, common gonopore; o, ovary; ov, oviduct; ph, pharynx; sb, spermiducal bulb; sd, sperm duct; t, tentacle; te, tentacular eyespot. Scale bars: 5 mm (A, B); 1 mm (C, D).

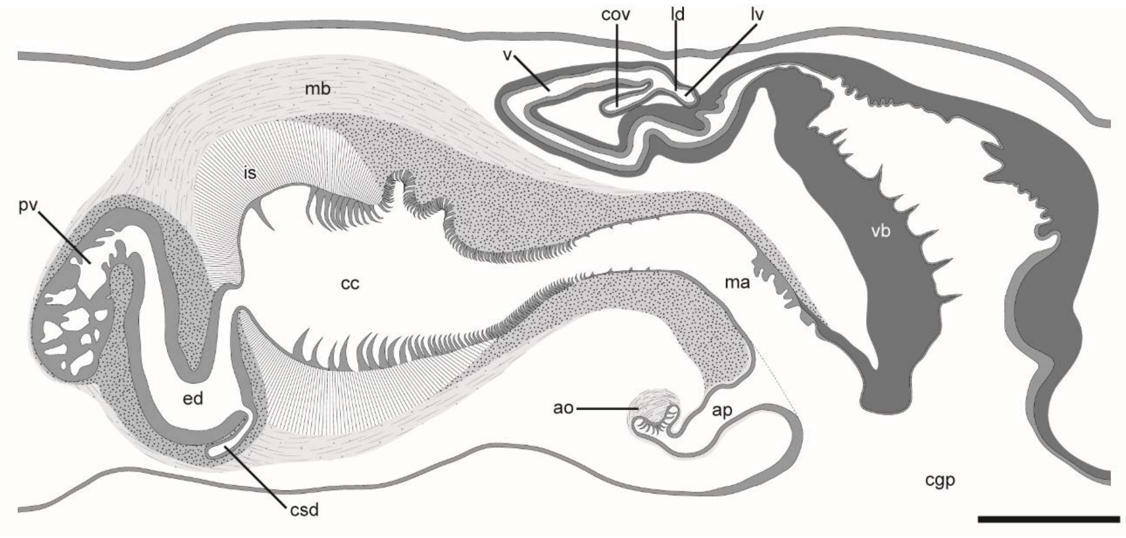

Figure III-3. Planoceridae gen. et sp. indet., schematic diagram of male and female copulatory apparatuses, anterior to the left. Abbreviations: ao, accessory organ; ap, accessory pouch; cc, cirrus cavity; cgp, common gonopore; cov, common oviduct; csd, common sperm duct; ed, ejaculatory duct; is, intermuscular space; ld, Lang's-vesicle duct; lv, Lang's vesicle; ma, male atrium; mb, muscular bulb; pv, prostatic vesicle; v, vagina; vb, vagina bulbosa. Scale bar: 300 µm.

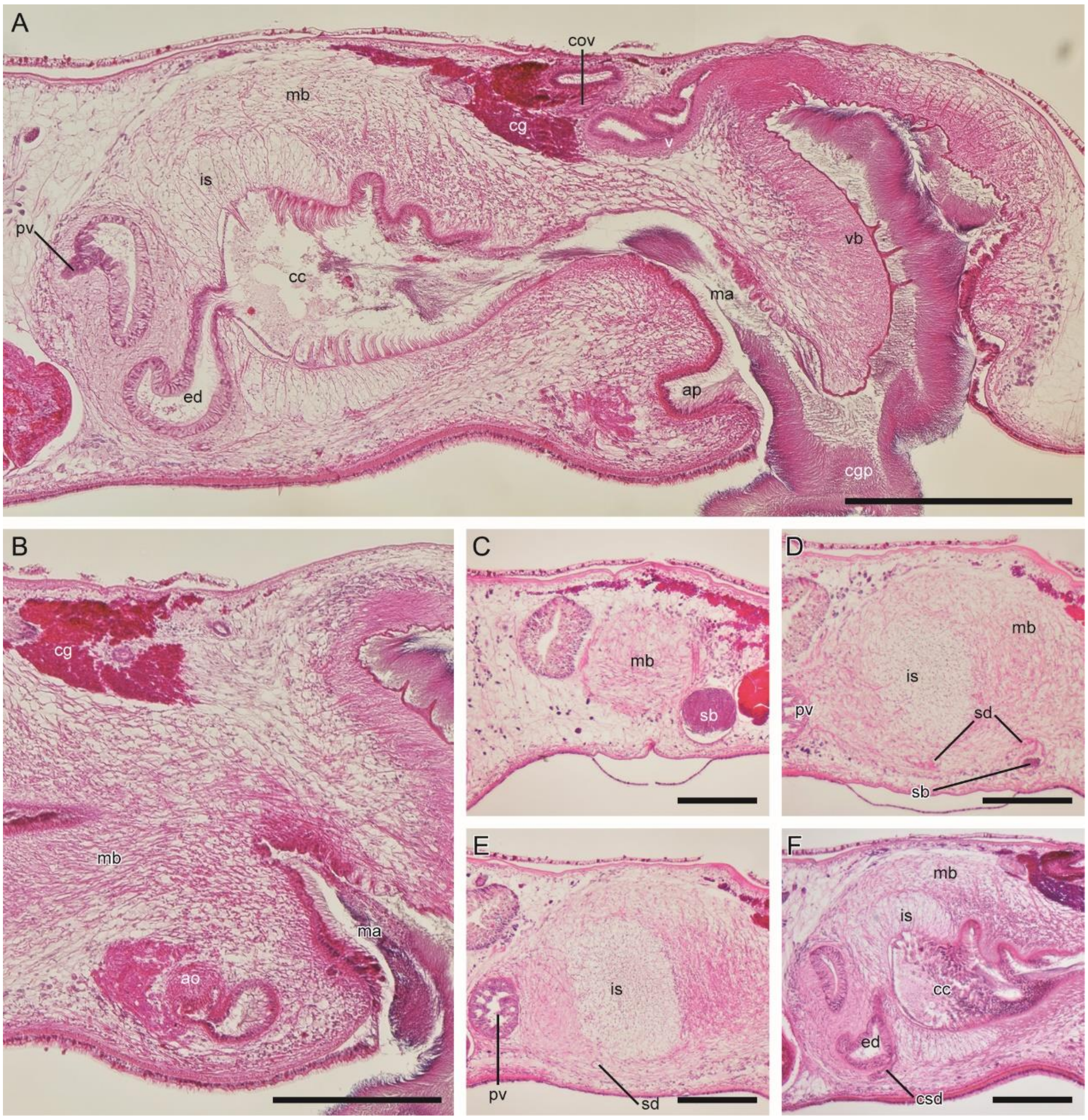

Figure III-4. Planoceridae gen. et sp. indet., photomicrographs of sagittal sections, anterior to the left, ICHUM 6081. A, B, Male and female copulatory apparatuses; C–F, running of sperm duct. Abbreviations: ao, accessory organ; ap, accessory pouch; cc, cirrus cavity; cg, cement glands; cgp, common gonopore; cov, common oviduct; csd, common sperm duct; ed, ejaculatory duct; is, intermuscular space; ma, male atrium; mb, muscular bulb; pv, prostatic vesicle; sb, spermiducal bulb; sd, sperm duct; v, vagina; vb, vagina bulbosa. Scale bars: 500 µm (A); 300 µm (B–F).

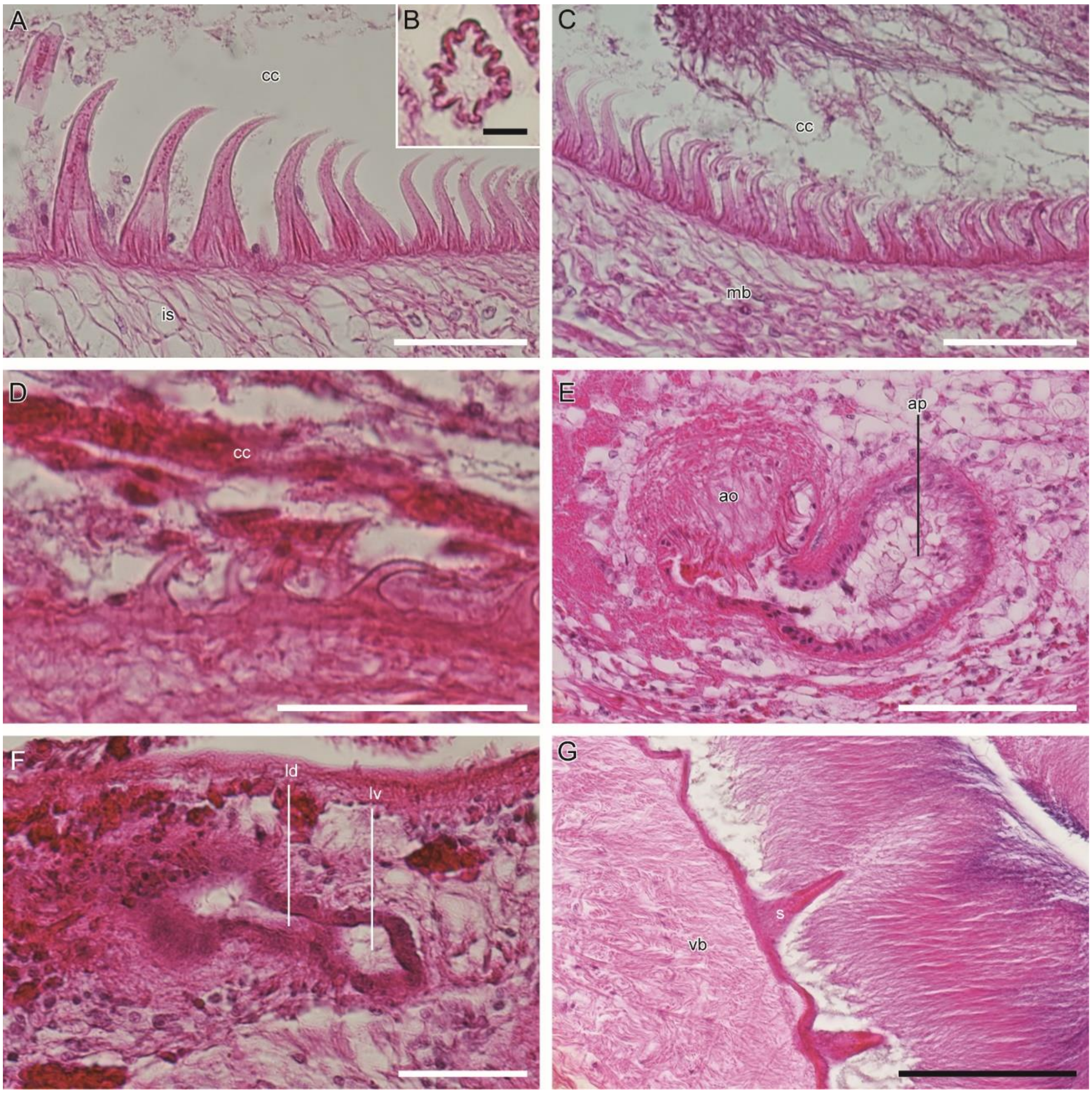

Figure III-5. Planoceridae gen. et sp. indet., photomicrographs of sagittal sections, anterior to the left, ICHUM 6081. A, C, D, Teeth in cirrus; B, cross view of large tooth; E, accessory organ; F, Lang's vesicle; G, spines of vagina bulbosa. Abbreviations: ao, accessory organ; ap, accessory pouch; cc, cirrus cavity; is, intermuscular space; ld, Lang's-vesicle duct; lv, Lang's vesicle; mb, muscular bulb; s, spine; vb, vagina bulbosa. Scale bars: 50 μm (A, C, D, F); 10 μm (B); 100 μm (E, G).

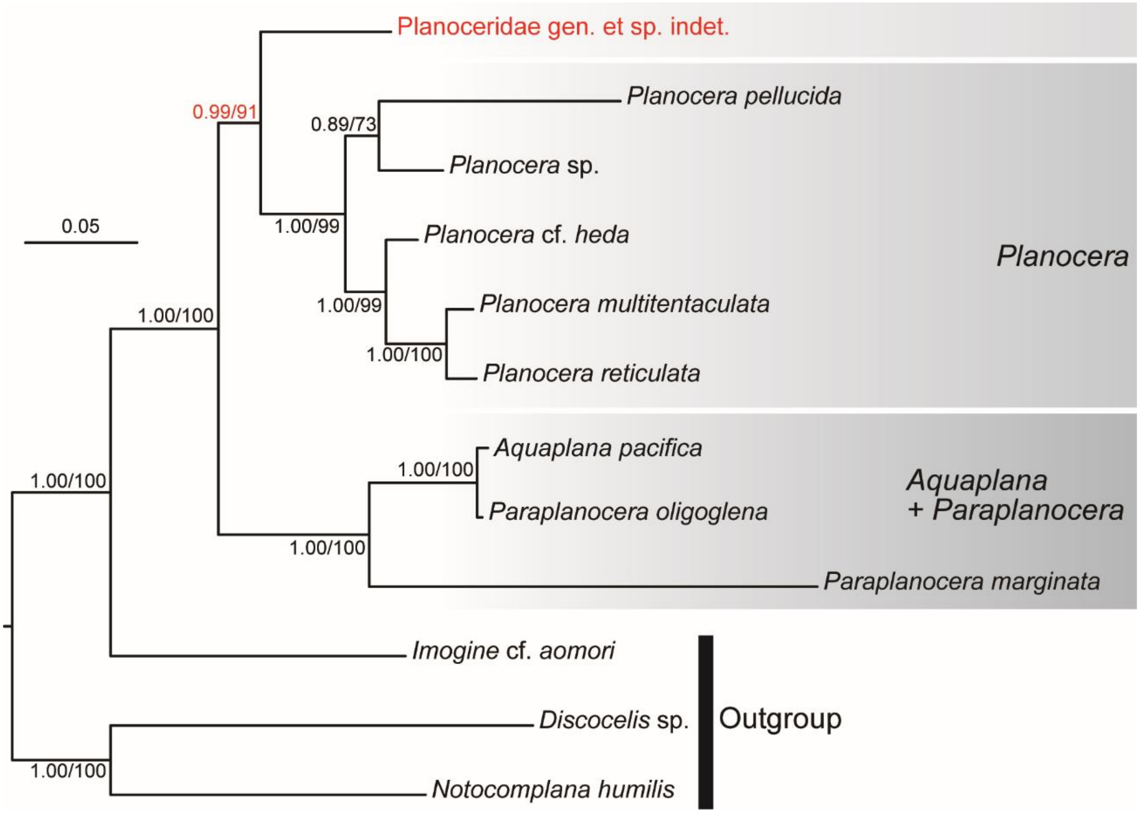

Figure III-6. Bayesian phylogenetic tree based on sequences from four genes (16S, 18S, and 28S ribosomal RNA; cytochrome *c* oxidase subunit I; concatenated length 3708 bp): Numbers near nodes represent posterior probability and bootstrap values, respectively.

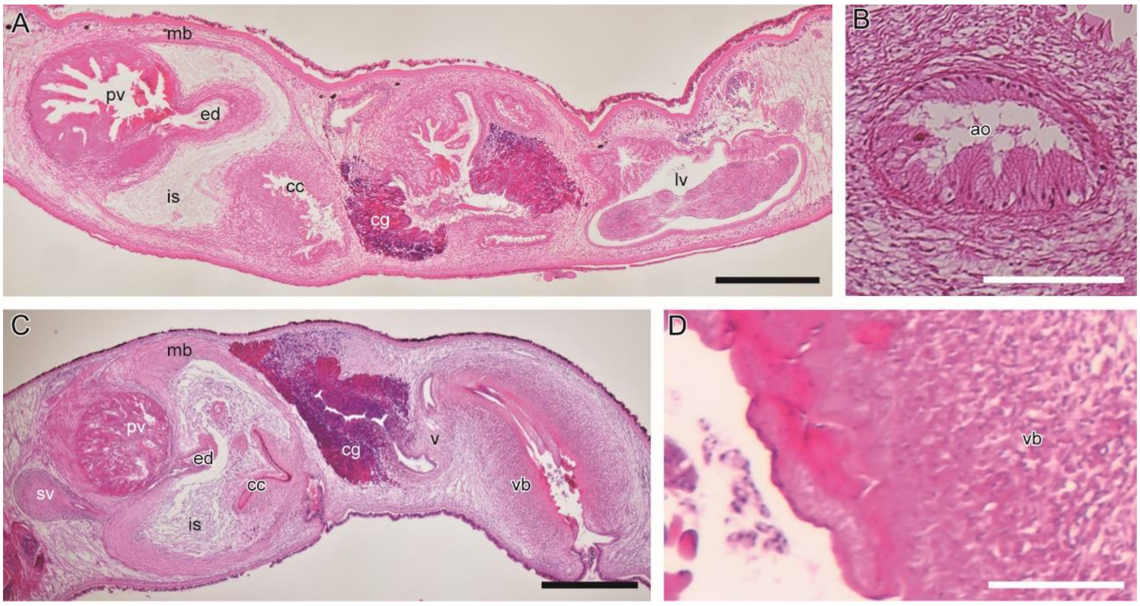

Figure III-7. Photomicrographs of sagittal sections of *Paraplanocera oligoglena* (Schmarda, 1859) (A, B; ICHUM 6083) and *Planocera reticulata* (Stimpson, 1855) (C, D; ICHUM 6018), anterior to the left: A, C, Copulatory apparatuses; B, accessory organ; D, cuticularized epithelium of vagina bulbosa. Abbreviations: ao, accessory organ; cc, cirrus cavity; cg, cement glands; ed, ejaculatory duct; is, intermuscular space; lv, Lang's vesicle; mb, muscular bulb; pv, prostatic vesicle; sv, seminal vesicle; v, vagina; vb, vagina bulbosa. Scale bars: 500 µm (A, C); 100 µm (B, D).

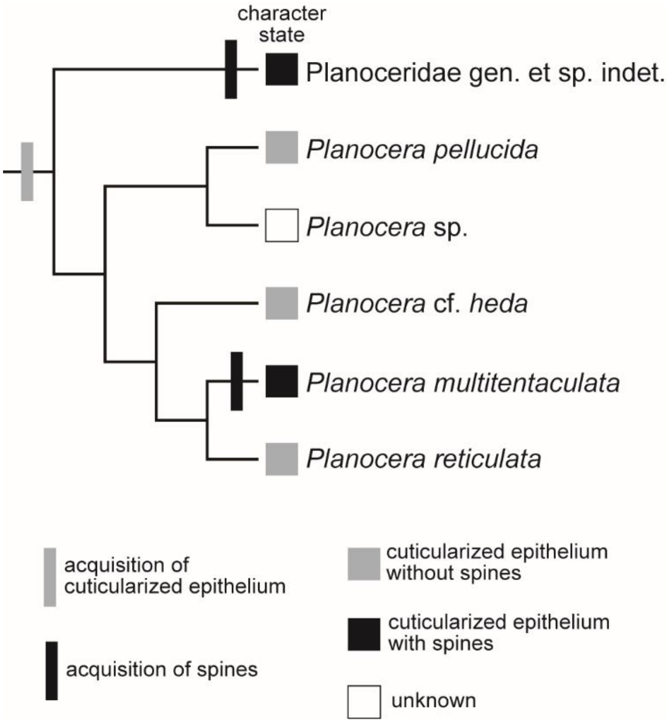

Figure III-8. Hypothesis for the evolution of spines in the vagina bulbosa based on a tree topology obtained from the molecular phylogenetic analysis in the present study.

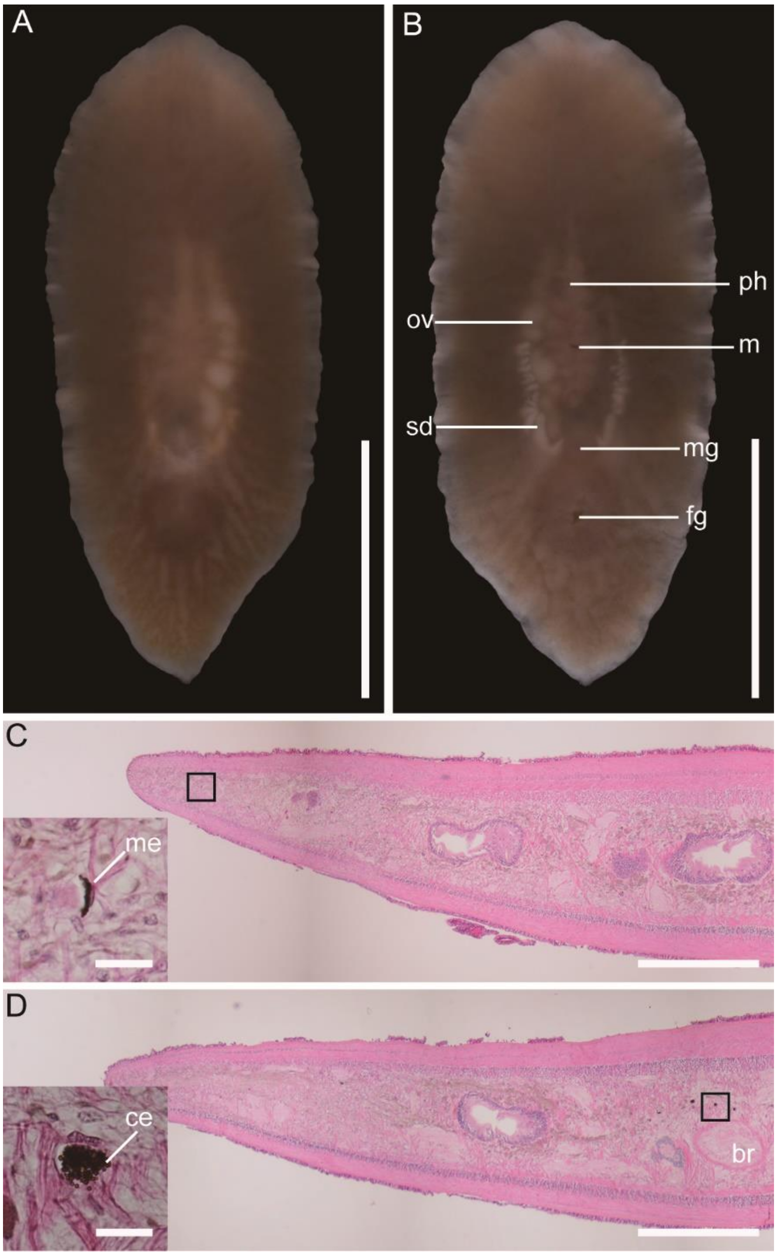

Figure III-9. *Paraplehnia seisuiae* Oya, Kimura and Kajihara, 2019, photographs taken in life (A, B) and eyespots observed in sections, anterior to the left (C, D), ICHUM 5345 (holotype). A, Dorsal view; B, ventral view; C, marginal eyespots (inset showing magnification of black box); D, cerebral eyespots (inset showing magnification of black box). Abbreviations: br, brain; ce, cerebral eyespot; fg, female gonopore; m, mouth; me, marginal eyespot; ov, oviduct; ph, pharynx; sd, sperm duct. Scale bars: 10 mm (A, B); 600 µm (C, D); 20 µm (insets in C, D).

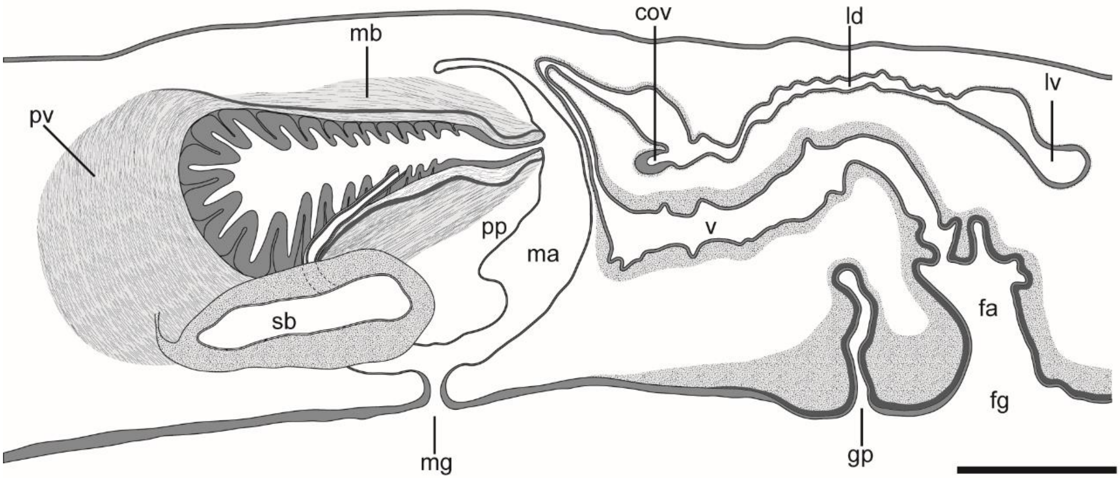

Figure III-10. *Paraplehnia seisuiae* Oya, Kimura and Kajihara, 2019, schematic diagram of male and female copulatory apparatuses, anterior to the left. Abbreviations: cov, common oviduct; fa, female atrium; fg, female gonopore; gp, genital pit; ld, Lang's-vesicle duct; lv, Lang's vesicle; ma, male atrium; mb, muscular bulb; mg, male gonopore; pp, penis papilla; pv, prostatic vesicle; sb; spermiducal bulb; v, vagina. Scale bar: 600 μm.

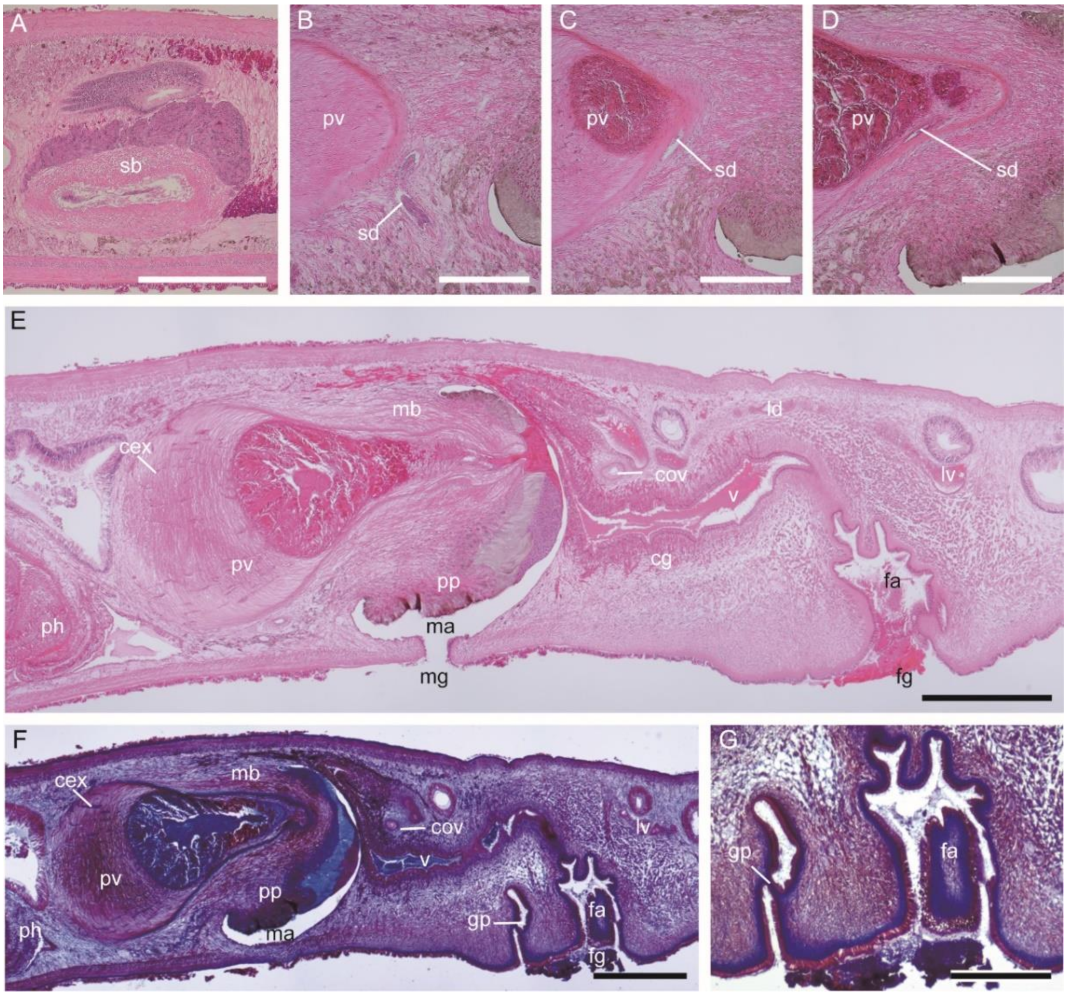

Figure III-11. *Paraplehnia seisuiae* Oya, Kimura and Kajihara, 2019, photomicrographs of sagittal sections, anterior to the left, ICHUM 5345 (holotype). A, Spemiducal bulb; B–D, connection between spermiducal bulb and prostatic vesicle; E, F, male and female copulatory apparatuses; G, genital pit. Staining: hematoxylin and eosin stain (A–E); Mallory's triple stain (F, G). Abbreviations: cex, canal of extra-vesicular gland; cg, cement glands; cov, common oviduct; fa, female atrium; fg, female gonopore; gp, genital pit; ld, Lang's-vesicle duct; lv, Lang's vesicle; ma, male atrium; mb, muscular bulb; mg, male gonopore; ph, pharynx; pp, penis papilla; pv, prostatic vesicle; sb; spermiducal bulb; sd, sperm duct; v, vagina. Scale bars: 600 µm (A, E, F); 300 µm (B–D, G).

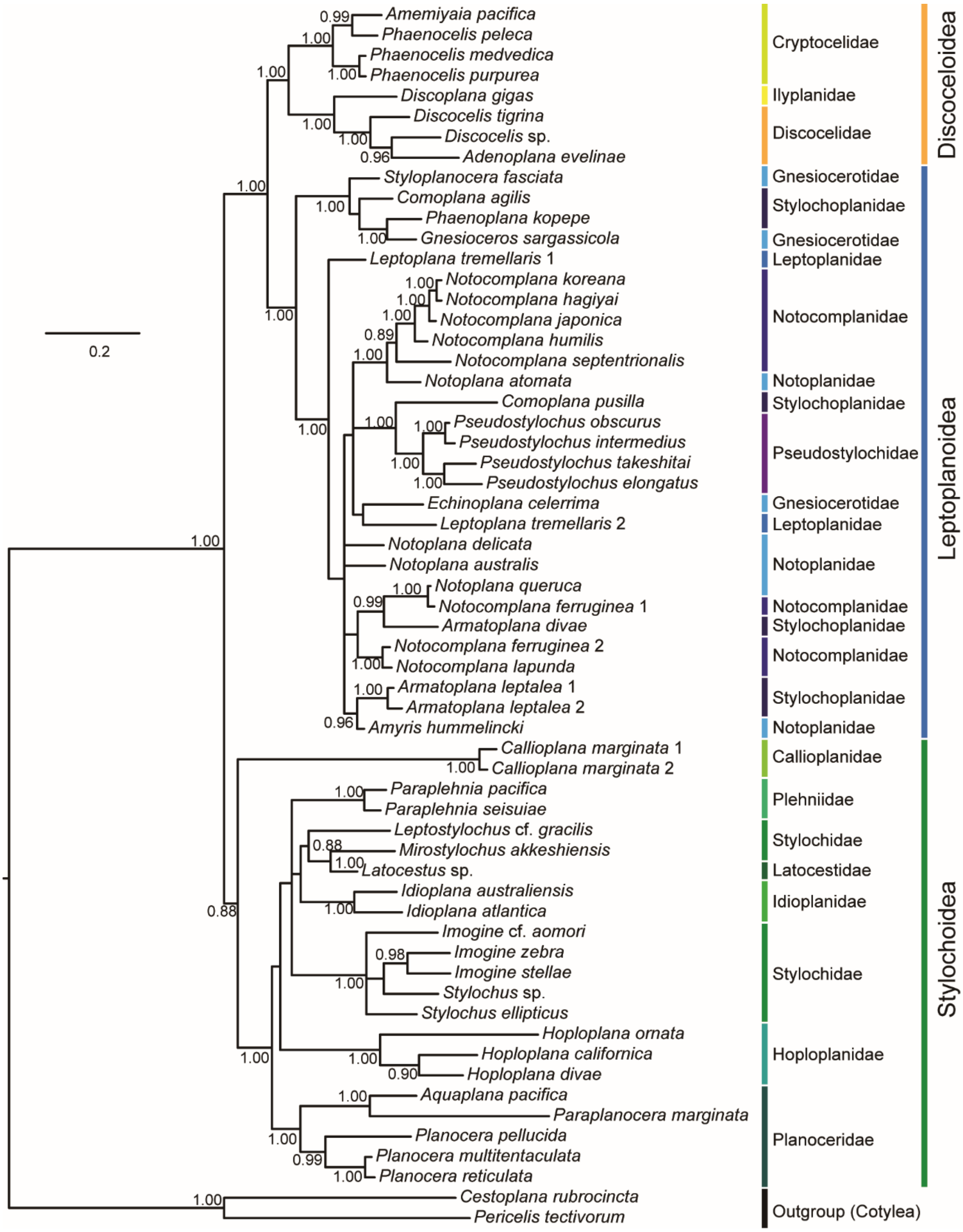

Figure IV-1. Bayesian phylogenetic tree based on sequences from four genes (16S, 18S, 28S, COI) (concatenated length 3527 bp). Numbers near nodes are posterior probability (PP) values greater than 0.80.

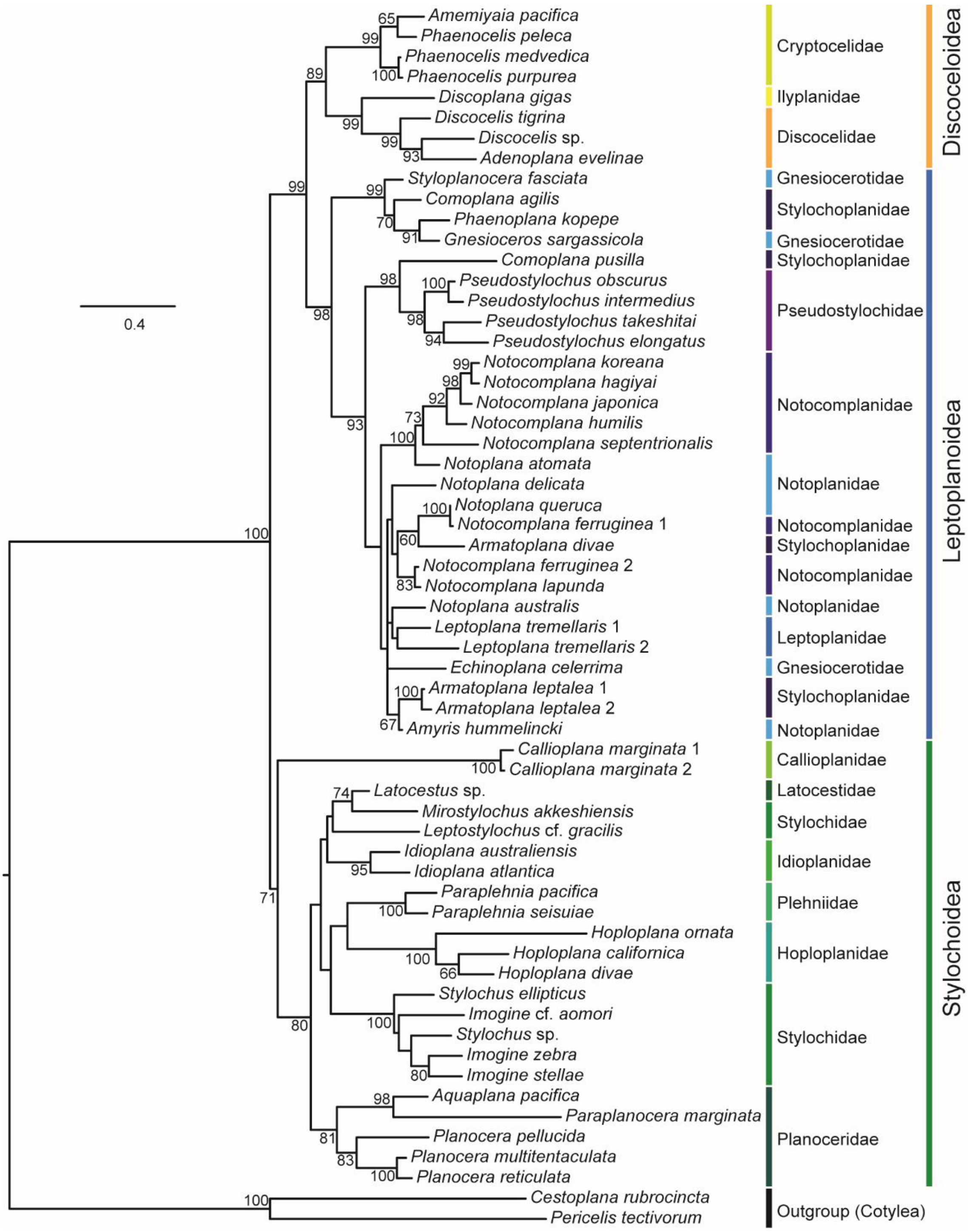

Figure IV-2. Maximum-likelihood tree based on sequences from four genes (16S, 18S, 28S, COI) sequences (concatenated length 3527 bp). Numbers near nodes are bootstrap values (BS) greater than 50%.

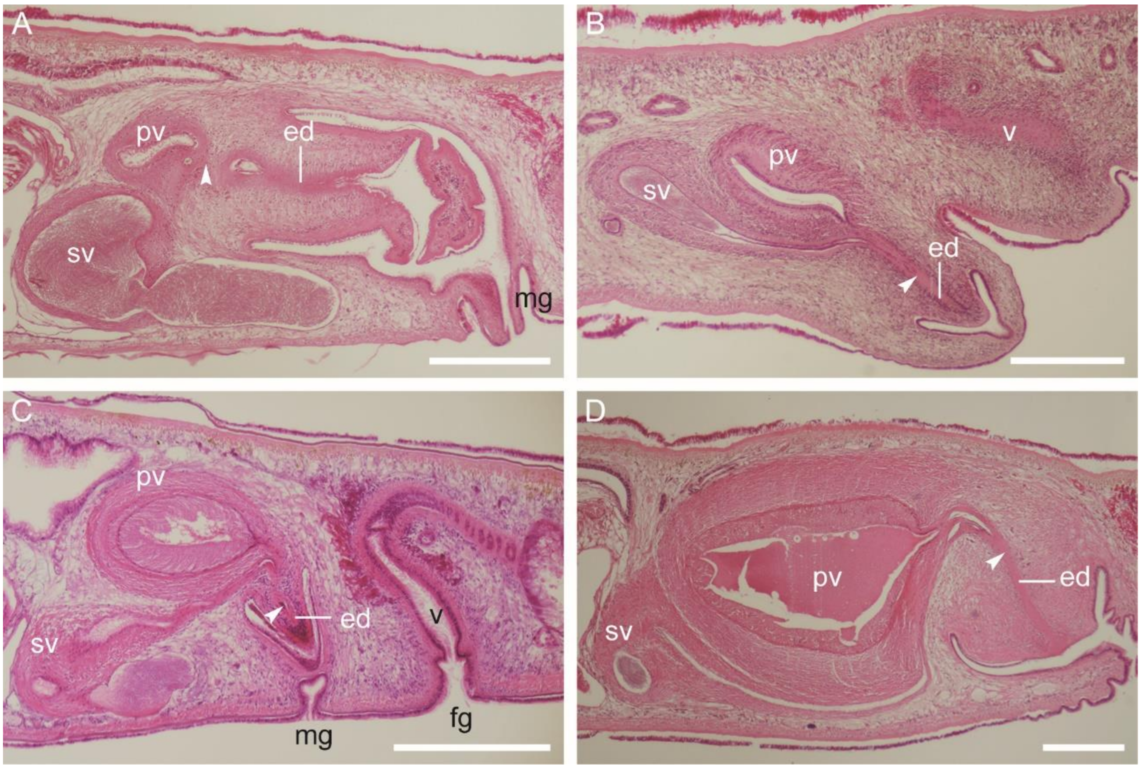

Figure IV-3. Male copulatory apparatus in *Pseudostylochus* species, anterior to the left. A, *Pseudostylochus takeshitai* Yeri and Kaburaki, 1918; B, *Pseudostylochus obscurus* (Stimpson, 1857); C, *Pseudostylochus elongatus* Kato, 1937; D, *Pseudostylochus intermedius* Kato, 1939. Arrowheads, connection of seminal vesicle and ejaculatory duct. Abbreviations: ed, ejaculatory duct; fg, female gonopore; mg, male gonopore; pv, prostatic vesicle; sv, seminal vesicle; v, vagina. Scale bars: 300 µm.

Table I-1. List of sampling sites in this study.

Site number[1]	Prefecture	Locality	Latitude (N)	Longitude (E)	Depth (m)	Collector or vessel[2]
1	Hokkaido	Akkeshi: Daikoku Island	42°57′45″	144°52′24″	intertidal	YO
2A	Hokkaido	Otaru: Oshoro	43°12′36″	140°51′28″	intertidal	YO
2B	Hokkaido	Otaru: Toyoi Beach	43°13′33″	141°00′57″	intertidal	YO
2C	Hokkaido	Otaru: Zenibako	43°09′07″	141°11′16″	intertidal	YO
3	Hokkaido	Okushiri Island: Anama	42°15′06″	139°33′26″	intertidal	YO
4A	Aomori	Asadokoro Beach	40°56′16″	140°58′22″	intertidal	YO
4B	Aomori	Asamushi: near Asamushi Marine Biological Station	40°54′10″	140°51′19″	intertidal to 6 m	YO
4C	Aomori	Asamushi: near Asamushi Marine Biological Station	no details	no details	20 m	NJ, SF
5	Tokyo	Chichijima Island: Kopepe Beach	27°03′52″	142°11′32″	intertidal	YO
6A	Kanagawa	Misaki: near Misaki Marine Biological Station	35°09′29″	139°36′44″	intertidal	YO
6B	Kanagawa	Misaki: Arai Beach	35°09′34″	139°36′42″	intertidal	HK, KO, TM
6C	Kanagawa	Misaki	no details	no details	(aquarium)	HK
7	Shizuoka	Suruga Bay	34°39′11″	138°43′12″	245 m	TRV *Tsukuba II*
8	Mie	Sea of Kumano	34°08′00″	136°37′48″	298–310 m	TRV *Seisui-maru*
9	Ishikawa	Noto: Kannonzaki	37°06′31″	137°03′27″	intertidal	YO
10A	Wakayama	Shirahama: Banshozaki	33°41′32″	135°20′04″	intertidal	YO
10B	Wakayama	Shirahama: Edura Beach	33°41′40″	135°20′50″	intertidal	YO
11	Kumamoto	Amakusa: Tsujishima Island	32°33′09″	130°06′32″	intertidal	YO, AT
12	Kagoshima	Bonomisaki	31°15′15″	130°12′54″	10 m	YO, AT, NJ, DU, MM
13	Okinawa	Ishigaki Island	no details	no details	intertidal	YT

[1] Numbers indicate the points on the map in Fig. I-1.

[2] Abbreviations: AT, Aoi Tsuyuki; DU, Daisuke Uyeno; HK, Hisanori Kohtsuka; KO, Kohei Oguchi; MM, Midori Matsuoka; NJ, Naoto Jimi; SF, Shinta Fujimoto; TM, Toru Miura; YO; Yuki Oya; YT, Yuki Tanabe.

Table I-2. List of specimens used in this study.

Superfamily	Family	Genus	Species	ICHUM	Site number	Chapter
Discoceloidea	Cryptocelidae	*Amemiyaia*	*Amemiyaia pacifica* Kato, 1944	*	10B	IV
	Discocelidae	*Discocelis*	*Discocelis* sp.	6009	11	IV
	Ilyplanidae	*Discoplana*	*Discoplana gigas* (Schmarda, 1859)	6008	13	IV
		Zygantroides	*Zygantroides serpulidicola* Oya, Tsuyuki and Kajihara, 2020	6023–6026	11	II
Leptoplanoidea	Notocomplanidae	*Notocomplana*	*Notocomplana hagiyai* Oya and Kajihara, 2017	5262–5273	2A	II, IV
			Notocomplana humilis (Stimpson, 1857)	6010	10A	III, IV
			Notocomplana japonica (Kato, 1937)	5282–5286	2B	II, IV
			Notocomplana koreana (Kato, 1937)	5274–5281	2A	II, IV
			Notocomplana septentrionalis (Kato, 1937)	5287–5300	2A, 2C	II, IV
	Notoplanidae	*Notoplana*	*Notoplana delicata* Yeri and Kaburaki, 1918	6011	10A	IV
	Pseudostylochidae	*Pseudostylochus*	*Pseudostylochus elongatus* Kato, 1937	6014	9	IV
			Pseudostylochus intermedius Kato, 1939	6012	4B	IV
			Pseudostylochus obscurus (Stimpson, 1857)	6013	6A	IV
			Pseudostylochus takeshitai Kato, 1937	6015	4B	IV
	Stylochoplanidae	*Alloioplana*	*Alloioplana* sp.	6084–6089	6B	II
		Armatoplana	*Armatoplana* sp.	6090–6091	6B	II
		Comoplana	*Comoplana pusilla* (Bock, 1924)	5482	3	IV
		Phaenoplana	*Phaenoplana kopepe* Oya and Kajihara, 2019	5343–5344	5	II, IV

*The voucher specimen is not available because it died before fixation in Bouin’s solution.

Table I-2. Continued.

Superfamily	**Family**	**Genus**	**Species**	**ICHUM**	**Site number**	**Chapter**
Stylochoidea	Callioplanidae	*Callioplana*	*Callioplana marginata* Stimpson, 1857	6016	6C	IV
	Hoploplanidae	*Hoploplana*	*Hoploplana ornata* Yeri and Kaburaki, 1918	6017	6A	IV
	Planoceridae	Planoceridae gen. et sp. indet.		6081–6082	7	III
		Paraplanocera	*Paraplanocera oligoglena* (Schmarda, 1859)	6083	12	III
		Planocera	*Planocera multitentaculata* Kato, 1944	*	6C	III, IV
			Planocera reticulata (Stimpson, 1855)	6018	12	III, IV
	Plehniidae	*Paraplehnia*	*Paraplehnia pacifica* (Kato, 1939)	6019	4C	IV
			Paraplehnia seisuiae Oya, Kimura and Kajihara, 2019	5345	8	III, IV
	Stylochidae	*Imogine*	*Imogine* cf. *aomori* (Kato, 1937)	6020	4A	III, IV
		Leptostylochus	*Leptostylochus* cf. *gracilis* Kato, 1934	6021	6A	IV
		Mirostylochus	*Mirostylochus akkeshiensis* Kato, 1937	6022	1	IV

* The voucher specimen is not available because it died before fixation in Bouin’s solution.

Table I-3. List of primers used in this study.

Gene	Primer name	Sequence	Application	Reference
16S	16SarL	CGCCGTTTATCAAAAACAT	for amplification and sequencing	Palumbi *et al.* (1991)
	16SbrH	CCGGTCTGAACTCAGATCACGT	for amplification and sequencing	Palumbi *et al.* (1991)
18S	hrms18S_F	ATCCTGCCAGTAGTCATATGC	for amplification and sequencing	this study
	hrms18S_Fi1	GCCGCGGTAATTCCAGCTCC	for sequencing	this study
	hrms18S_Fi2	GGGTTCCGGGGGAAGTATG	for sequencing	this study
	hrms18S_R	CTACGGAAACCTTGTTACGAC	for amplification and sequencing	this study
	hrms18S_Ri1	CTTTAATATACGCTATTGGAGCTGG	for sequencing	this study
	hrms18S_Ri2	CTATTTAGTGGCTAGAGTCTCGTTCG	for sequencing	this study
28S	fw1	AGCGGAGGAAAAGAAACTA	for amplification and sequencing	Sonnenberg *et al.* (2007)
	hrms_fw2	AGAAGTACCGCGAGGGAARGTTG	for sequencing	this study
	rev4	GTTAGACTYCTTGGTCCGTG	for sequencing	Sonnenberg *et al.* (2007)
	rev2	ACGATCGATTTGCACGTCAG	for amplification and sequencing	Sonnenberg *et al.* (2007)
COI	Acotylea_COI_F	ACTTTATTCTACTAATCATAAGGATATAGG	for amplification and sequencing	this study
	Acotylea_COI_R	CTTTCCTCTATAAAATGTTACTATTTGAGA	for amplification and sequencing	this study
	nestCOI_F1	GGTGTATGGTCAGGTCTAATRGGWAC	for nested PCR	this study
	nestCOI_R1	ATACTTCCGGATGACCAAAGAAYCA	for nested PCR	this study

Table II-1. Comparison of morphological characters between species of *Zygantroides*.

	Z. henriettae **(Corrêa, 1949)**	***Z. plesia*** **(Corrêa, 1949)**	***Z. serpulidicola*** **Oya, Tsuyuki and Kajihara, 2020**
Mouth	one-third from posterior end of pharynx	two-fifths from posterior end of the pharynx	posterior to pharynx and near common gonopore
Common sperm duct	present	present	absent
Seminal vesicle	spindle-shaped	spherical	bean-shaped
Ejaculatory duct ("granule vesicle" in Corrêa 1949)	ovoid	approximately cordiform	varied
Length of Lang's-vesicle duct	about one-tenth of that of vagina	about one-twentieth of that of vagina	about one-third of that of vagina
Lang's vesicle	positioned anterior to common gonopore	positioned anterior to common gonopore	positioned posterior to common gonopore
Ampulla in vagina	absent	present	absent

Table II-2. Comparison of morphological characters between *N. acticola*, *N. hagiyai*, and *N. sanjuania*.

	***N. acticola* (Boone, 1929)**	***N. hagiyai* Oya and Kajihara, 2017**	***N. sanjuania* (Freeman, 1933)**
Prostatic vesicle	larger than the seminal vesicle	larger than the seminal vesicle	smaller than the seminal vesicle
Lang's-vesicle duct	longer than the long axis of Lang's vesicle	shorter than the long axis of Lang's vesicle	shorter than the long axis of Lang's vesicle

Table II-3. Comparison of morphological characters between species of *Alloioplana*.

	A. aulica **(Marcus, 1947)**	***A. delicata*** **Plehn, 1896**	***A. stylifera*** **(Hyman, 1953)**	***A. wyona*** **(Du Bois-Reymond Marcus and Marcus, 1968)**	***Alloioplana*** **sp.**
Tentacles	present	present	absent	absent	absent
Common sperm duct	present	?	?	absent	present
Prostatic vesicle	oval, slightly larger than seminal vesicle	elongated oval, larger than seminal vesicle	posteriorly curved, size unknown	oval, same size as seminal vesicle	oval, smaller than seminal vesicle
Intra ejaculatory duct	absent	absent	absent	absent	present
Ampulla of terminal of vagina	absent	absent	absent	present	absent
Gonopores	common	separated	separated	separated	separated

Table II-4. Comparison of morphological characters between species of *Armatoplana* with a seminal vesicle and without nuchal tentacles, a common sperm duct, and a pair of accessory vesicles in the Lang's vesicle.

	A. lactoalba **(Verrill, 1900)**	***A. leptalea*** **(Marcus, 1947)**	***A. reishi*** **(Hyman, 1959)**	***A. snadda*** **(Du Bois-Reymond Marcus and Marcus, 1968)**	***Armatoplana*** **sp.**
Common sperm duct	absent	absent	absent	absent	absent
Position of prostatic vesicle	in alignment with seminal vesicle	in alignment with seminal vesicle	dorsally above seminal vesicle	dorsally above seminal vesicle	dorsally above seminal vesicle
Size of prostatic vesicle	smaller than seminal vesicle	larger than seminal vesicle	same as seminal vesicle	smaller than seminal vesicle	larger than seminal vesicle
Stylet	long, slightly curved	long, curved	short	long, straight	short, straight
Lang's vesicle	large, elongated	large, elongated	laterally broadened	present	large, elongated

Table II-5. Comparison of morphological characters between species of *Phaenoplana.*

	P. caetaria **Pérez-García, Noreña and Cervera, 2018**	***P. challengeri*** **(Graff, 1892)**	***P. conoceraea*** **(Schmarda, 1859)**	***P. kopepe*** **Oya and Kajihara, 2019**	***P. longipenis*** **(Hyman, 1953)**	***P. taiwanica*** **(Kato, 1943)**
Nuchal tentacles	absent	present	present	present	absent	absent
Mouth	?	near posterior end of pharyngeal cavity	near posterior end of pharyngeal cavity	near posterior end of pharyngeal cavity	center of pharyngeal cavity	?
Prostatic vesicle	spherical	oval	oval	oval	V-shaped	oval
Gonopores	well separated	close	close	well separated	close	close
Vagina	anteriorly curved	almost vertical	almost vertical	anteriorly curved	anteriorly curved	anteriorly curved
Lang's-vesicle duct	shorter than vagina	shorter than vagina	longer than vagina	shorter than vagina	shorter than vagina	shorter than vagina
Lang's vesicle	sac-shaped	horseshoe-shaped	horseshoe-shaped	horseshoe-shaped	sac-shaped	horseshoe-shaped

Table III-1. List of polyclad species described from the deep sea (below 200 m).

Species	**Depth**	**Point on the map**[1]	**Reference**
Acotylea			
Anocelidus profundus Quiroga, Bolaños and Litvaitis, 2006	2642–3232 m	1, 2	Quiroga *et al*. (2006)
Didangia carneyi Quiroga, Bolaños and Litvaitis, 2008	610 m	3	Quiroga *et al*. (2008)
Discocelides langi Bergendal, 1893	5–300 m	4	Bock (1913)
Discoprosthides patagoniensis Faubel, 1983	bathyal zone	5	Faubel (1983)
Diplehnia caeca (Hyman, 1953)	23.7–393.19 m	6	Hyman (1953)
Notoplana atomata (Müller, 1776)	222.3 m	7	Hyman (1953)
N. kuekenthali (Plehn, 1896)	304.2 m	8	Bock (1913)
Plehnia arctica (Plehn, 1896)	12–1275 m	9	Bock (1913)
P. ellipsoides (Girard, 1853)	intertidal to 347 m	10	Hyman (1940)
Stylochus crassus Verrill, 1893	About 2000 m	11	Verrill (1893)
Cotylea			
Eulylepta leoparda Freeman, 1933	243 m	12	Freeman (1933)
Oligocladus bathymodiensis Quiroga, Bolaños and Litvaitis, 2008	610–650 m	3, 13	Quiroga *et al*. (2008)
O. voightae Quiroga, Bolaños and Litvaitis, 2006	3232 m	2	Quiroga *et al*. (2006)
Stygolepta hjalmari Faubel, 1984	603 m	14	Faubel (1984)

[1] Numbers indicate the points on the map in Fig. III-1.

Table III-2. List of species included in the molecular phylogenetic analysis and the respective GenBank accession numbers.

Family	**Species**	**16S**	**18S**	**28S**	**COI**	**Reference**
Planoceridae	Planoceridae gen. et sp. indet.	LC545564	LC545566	LC545568	LC545562	this study
	Aquaplana pacifica	—	—	MH700272	—	Litvaitis *et al*. (2019)
	Paraplanocera marginata	—	—	MH700335	—	Litvaitis *et al*. (2019)
	Paraplanocera oligoglena	LC545565	LC545567	LC545569	LC545563	this study
	Planocera cf. *heda*	—	—	MH700363	—	Litvaitis *et al*. (2019)
	Planocera multitentaculata	LC508174	LC508150	LC508127	LC508192	this study
	Planocera pellucida	—	MN334203	MN384696	—	Dittmann *et al*. (2019)
	Planocera reticulata	LC508190	LC508172	LC508148	LC508208	this study
	Planocera sp.	—	—	MH700364	—	Litvaitis *et al*. (2019)
Discocelidae	*Discocelis* sp.	LC508189	LC508170	LC508146	LC508206	this study
Notoplanidae	*Notocomplana humilis*	LC508187	LC508168	LC508144	LC508204	this study
Stylochidae	*Imogine* cf. *aomori*	LC508182	LC508163	LC508140	LC508200	this study

Table III-3. Comparison of morphological characteristics between genera in Planoceridae *sensu* Faubel (1983).

	Planoceridae gen. et sp. indet.	[1]***Aquaplana*** **Hyman, 1953** ***sensu*** **Faubel (1983)**	***Disparoplana*** **Laidlaw, 1903**	[2]***Neoplanocera*** **Yeri and Kaburaki, 1918**	***Paraplanocera*** **Laidlaw, 1903**	***Planocera*** **Blainville, 1828**	***Pseudoplanocera*** **Bulnes, 2010**	[2]***Spinicirrus*** **Hyman, 1953**
Nuchal tentacles	present	present	absent	absent	present	present	absent	absent
Muscular bulb enclosing cirrus	present	present	absent	absent	present	present	present	present
Prostatic vesicle	present	present	present	present	present	present	present	two
Seminal vesicle/spermiducal bulbs	spermiducal bulbs	spermiducal bulbs	seminal vesicle	seminal vesicle	spermiducal bulbs	seminal vesicle	seminal vesicle	absent
Accessory organ	present	absent	absent	present	present	absent	absent	absent
Bursa copulatrix	absent	absent	absent	absent	present	absent	absent	present
Lang's vesicle	present	present	present	absent	present	present	present	absent

[1] *Aquaplana* should be treated as an invalid taxon because Faubel (1983) has transferred *Aquaplana oceanica* Hyman, 1953, the type species of *Aquaplana*, to *Paraplanocera*. It is therefore included in this table for comparison purposes only.

[2] Prudhoe (1985) classified these genera into Gnesiocerotidae Marcus and Marcus, 1966.

Table III-4. Comparison of morphological characters between species of *Paraplehnia.*

	P. japonica **(Bock, 1923)**	***P. pacifica*** **(Kato, 1939)**	***P. seisuiae*** **Oya, Kimura and Kajihara, 2019**
Muscular wall in posterior end of prostatic vesicle	about one-half	about one-half	about one-third
Common sperm duct	present	present	absent
Genital pit	absent	absent	present
Length of Lang's-vesicle duct	about one-sixth of that of vagina	almost same length as that of vagina	about one-third of that of vagina
Developed connective tissues in female copulatory apparatus	?	only around female atrium	from female atrium to genital pit and female gonopore

Table IV-1. List of species included in the molecular phylogenetic analysis and respective GenBank accession numbers.

Superfamily/ Family	Species	16S	18S	28S	COI	Specimen	Reference
Discoceloidea							
Cryptocelidae	*Amemiyaia pacifica*	LC508185	LC508166	LC508143	LC508203		this study
	Phaenocelis medvedica	—	—	KY263706	—		Bahia *et al.* (2017)
	Phaenocelis peleca	—	—	MH700342	—		Litvaitis *et al.* (2019)
	Phaenocelis purpurea	—	—	MH700346	—		Litvaitis *et al.* (2019)
Ilyplanidae	*Discoplana gigas*	—	LC508162	LC508139	LC508199	ICHUM 6008	this study
Discocelidae	*Adenoplana evelinae*	—	—	MH700268	—		Litvaitis *et al.* (2019)
	Discocelis tigrina	—	MN334200	MN384690	—		Dittmann *et al.* (2019)
	Discocelis sp.	LC508189	LC508170	LC508146	LC508206	ICHUM 6009	this study
Leptoplanoidea							
Gnesiocerotidae	*Echinoplana celerrima*	—	MN421936	MN421930	—		Dittmann *et al.* (2019)
	Gnesioceros sargassicola	—	—	MH700309	—		Litvaitis *et al.* (2019)
	Styloplanocera fasciata	—	—	MH700408	—		Litvaitis *et al.* (2019)
Leptoplanidae	*Leptoplana tremellaris* 1	—	—	KY263696	—		Bahia *et al.* (2017)
	Leptoplana tremellaris 2	—	MN421937	MN421931	—		Dittmann *et al.* (2019)

Table IV-1. Continued

Family	**Species**	**16S**	**18S**	**28S**	**COI**	**Specimen**	**Reference**
Notocomplanidae	*Notocomplana ferruginea* 1	—	—	HQ659014	—		Rawlinson *et al.* (2011)
	Notocomplana ferruginea 2	—	—	MH700322	—		Litvaitis *et al.* (2019)
	Notocomplana hagiyai	LC176041	LC508152	LC508129	LC176003	ICHUM 5267	this study
	Notocomplana humilis	LC508187	LC508168	LC508144	LC508204	ICHUM 6010	this study
	Notocomplana japonica	LC176051	LC508154	LC508131	LC176018	ICHUM 5282	this study
	Notocomplana koreana	LC176048	LC508151	LC508128	LC176014	ICHUM 5278	this study
	Notocomplana septentrionalis	LC176059	LC508153	LC508130	LC176028	ICHUM 5292	this study
	Notocomplana lapunda	—	—	MH700325	—		Litvaitis *et al.* (2019)
Notoplanidae	*Amyris hummelincki*	—	—	MH700269	—		Litvaitis *et al.* (2019)
	Notoplana australis	—	AJ228786	AY157153	—		Littlewood *et al.* (1999); Lockyer *et al.* (2003)
	Notoplana atomata	—	—	MH700329	—		Litvaitis *et al.* (2019)
	Notoplana delicata	LC508188	LC508169	LC508145	LC508205	ICHUM 6011	this study
	Notoplana queruca	—	—	MH700333	—		Litvaitis *et al.* (2019)

Table IV-1. Continued

Family	**Species**	**16S**	**18S**	**28S**	**COI**	**Specimen**	**Reference**
Pseudostylochidae	*Pseudostylochus intermedius*	LC508183	LC508164	LC508141	LC508201	ICHUM 6012	this study
	Pseudostylochus obscurus	LC508180	LC508160	LC508137	LC508197	ICHUM 6013	this study
	Pseudostylochus elongatus	—	LC508171	LC508147	LC508207	ICHUM 6014	this study
	Pseudostylochus takeshitai	LC508184	LC508165	LC508142	LC508202	ICHUM 6015	this study
Stylochoplanidae	*Armatoplana divae*	—	—	MH700273	—		Litvaitis *et al.* (2019)
	Armatoplana leptalea 1	—	—	KY263649	—		Bahia *et al.* (2017)
	Armatoplana leptalea 2	—	—	MH700275	—		Litvaitis *et al.* (2019)
	Comoplana agilis	—	MN334199	MN384685	—		Dittmann *et al.* (2019)
	Comoplana pusilla	LC508177	LC508157	LC508134	LC508194	ICHUM 5482	this study
	Phaenoplana kopepe	LC508176	LC508156	LC508133	LC369778	ICHUM 5343	this study
Stylochoidea							
Callioplanidae	*Callioplana marginata* 1	LC508179	LC508159	LC508136	LC508196	ICHUM 6016	this study
	Callioplana marginata 2	—	—	MH700284	—		Litvaitis *et al.* (2019)

Table IV-1. Continued

Family	Species	16S	18S	28S	COI	Specimen	Reference
Hoploplanidae	*Hoploplana californica*	KC869753	KC869797	KC869850	—		Laumer and Giribet (2014)
	Hoploplana divae	—	—	KY263693	—		Bahia *et al.* (2017)
	Hoploplana ornata	LC508178	LC508158	LC508135	LC508195	ICHUM 6017	this study
Latocestidae	*Latocestus* sp.	—	—	MH700317	—		Litvaitis *et al*. (2019)
Planoceridae	*Aquaplana pacifica*	—	—	MH700272	—		Litvaitis *et al*. (2019)
	Paraplanocera marginata	—	—	MH700335	—		Litvaitis *et al.* (2019)
	Planocera multitentaculata	LC508174	LC508150	LC508127	LC508192		this study
	Planocera pellucida	—	MN334203	MN384696	—		Dittmann *et al*. (2019)
	Planocera reticulata	LC508190	LC508172	LC508148	LC508208	ICHUM 6018	this study
Plehniidae	*Paraplehnia pacifica*	LC508175	LC508155	LC508132	LC508193	ICHUM 6019	this study
	Paraplehnia seisuiae	LC508186	LC508167	LC467000	LC466999	ICHUM 5345	this study
Idioplanidae	*Idioplana atlantica*	—	—	MH700310	—		Litvaitis *et al*. (2019)
	Idioplana australiensis	—	—	HQ659008	—		Rawlinson *et al*. (2011)

Table IV-1. Continued

Family	**Species**	**16S**	**18S**	**28S**	**COI**	**Specimen**	**Reference**
Stylochidae	*Imogine stellae*	—	MN334201	MN384692	—		Dittmann *et al*. (2019)
	Imogine zebra	—	AF342801	AF342800	—		Mallatt and Winchell (2002)
	Imogine cf. *aomori*	LC508182	LC508163	LC508140	LC508200	ICHUM 6020	this study
	Leptostylochus cf. *gracilis*	LC508181	LC508161	LC508138	LC508198	ICHUM 6021	this study
	Mirostylochus akkeshiensis	LC508191	LC508173	LC508149	LC508209	ICHUM 6022	this study
	Stylochus ellipticus	—	Suppl. File 1 in Dittmann *et al.* (2019)	Suppl. File 1 in Dittmann *et al.* (2019)	—		Dittmann *et al*. (2019)
	Stylochus sp.	—	—	KY263743	—		Bahia *et al.* (2017)
Outgroup							
Cestoplanidae	*Cestoplana rubrocincta*	—	MN334198	MN384689	—		Dittmann *et al*. (2019)
Pericelidae	*Pericelis tectivorum*	—	MN334202	MK181524	—		Dittmann *et al*. (2019)