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A new species of slender flatworm in the genus *Eucestoplana* and a record of *E.* cf. *cuneata* (Platyhelminthes, Polycladida) from the Okinawa Islands, Japan, with an inference of their phylogenetic positions within Cestoplanidae

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Abstract

In this study, we describe a new species of elongated marine flatworm, *Eucestoplana ittanmomen* **sp. nov.**, collected from the intertidal zone of the Okinawa Islands, Japan. *Eucestoplana ittanmomen* **sp. nov.** is distinguished from other congeners based on the following characteristics: *i*) its translucent body lacking coloration, *ii*) its dome-shaped penis sheath, *iii*) the absence of cilia on the inner wall of the male atrium except outside the penis sheath, and *iv*) the presence of an adhesive organ at the posterior end of the body. Additionally, we report the occurrence of *E.* cf. *cuneata* (Sopott-Ehlers & Schmidt, 1975) in Japan; *E. cuneata* has previously been documented in the Galapagos and Fiji Islands. We conducted phylogenetic analyses to infer the positions of the two *Eucestoplana* species within Cestoplanidae using a concatenated dataset comprising partial 18S and 28S rDNA sequences from *E.* cf. *cuneata* and *E. ittanmomen* **sp. nov.** from Japan, as well as four known *Cestoplana* species with sequences available in public databases. Our phylogenetic analyses revealed that *Cestoplana* and *Eucestoplana* were reciprocally monophyletic. Furthermore, the genetic distance of the 16S rDNA sequences supported the genetic independence of the two sister species, *E.* cf. *cuneata* and *E. ittanmomen* **sp. nov.**

Key Words

Cotylea, histology, marine flatworms, marine invertebrates, molecular phylogeny, taxonomy

Introduction

Polyclad flatworms in the family Cestoplanidae Lang, 1884 are distinguishable from other flatworms by *i*) their slender bodies without tentacles, *ii*) ruffled pharynx located posterior to the center of the body, *iii*) male copulatory apparatus directed anteriorly, and *iv*) adhesive organ at the posterior end of the body (Faubel 1983; Prudhoe 1985). Currently this family comprises six genera: *Acestoplana* Faubel, 1983; *Cestoplana* Lang, 1884; *Cestoplanella* Faubel, 1983; *Cestoplanides* Faubel, 1983; *Cestoplanoida* Faubel, 1983; and *Eucestoplana* Faubel, 1983 (Faubel 1983).

The genus *Eucestoplana* currently includes two species, *Eucestoplana cuneata* (Sopott-Ehlers & Schmidt, 1975) and *Eucestoplana meridionalis* (Prudhoe, 1982a), which are distinguished from other cestoplanids by *i*) the presence of a tubular penis stylet housed in the male atrium and *ii*) the absence of a Lang's vesicle (Faubel 1983). These species have previously been reported in the Pacific

Ocean, including the Galapagos Islands, the Fiji Islands, and South Australia (Sopott-Ehlers and Schmidt 1975; Prudhoe 1982a; Tajika et al. 1991). During a faunal survey conducted as part of this study, we collected polyclad specimens of a new *Eucestoplana* species, along with *E.* cf. *cuneata*, from the intertidal zone of the Okinawa Islands, Japan. In this paper, we provide morphological descriptions of the new *Eucestoplana* species and *E.* cf. *cuneata*, based on the collected specimens. We calculated the genetic distances among the Japanese *Eucestoplana* specimens using partial 16S rDNA (16S) and cytochrome *c* oxidase subunit I (COI) sequences; the intraspecific genetic distances were also calculated based on partial 28S rDNA sequences among all cestoplanid species available in public databases. Additionally, we infer the phylogenetic positions of these two *Eucestoplana* species among other cestoplanids using molecular phylogenetic analyses of partial 18S and 28S rDNA sequences of all currently available cestoplanids in public databases.

Methods

Specimen collection and fixation

Specimens were collected from the Okinawa Islands, Japan, and processed using methods similar to those described in Tsuyuki et al. (2022, 2023). Gravel samples were collected at depths of about 20 cm from the water surface at low tide (down to about 15 cm from the sediment surface), then agitated in seawater to extract animals. The supernatant was filtered using a dip net with about 1-mm mesh, and the remaining residue was transferred into a bottle filled with fresh seawater. Before fixation, live worms were anesthetized in an $MgCl_2$ solution prepared with tap water to match the seawater salinity using an IS/Mill-E refractometer (AS ONE, Japan). Specimens were photographed using a Nikon D5600 digital camera with external strobe lightning provided by a pair of Morris Hikaru Komachi Di flash units. A portion of the body was preserved in 99.5% ethanol for DNA extraction, whereas the rest of the body was fixed in Bouin's solution for 24 h, then stored in 70% ethanol.

Morphological observation

For histological examination, specimens fixed in Bouin's solution were prestained with acid fuchsin, dehydrated in an ethanol series, cleared in xylene, embedded in paraffin wax, and sectioned serially at a thickness of 4 µm using a microtome. The sections were stained with hematoxylin and eosin, mounted on glass slides, and embedded in Entellan New (Merck, Germany) under coverslips. Specimens were observed and photographed using a Nikon D5600 digital camera under an Olympus BX51 compound microscope.

For comparison, we also examined the type series of *Eucestoplana cuneata* (as *Cestoplana cuneata*), which consists of the holotype ZMUG 25472 (3 slides) and the paratype ZMUG 25473 (5 slides), both of which have been deposited in the Biodiversity Museum Göttingen of the Georg-August-University Göttingen. In addition, we examined serial sagittal sections of *Eucestoplana cuneata* (as *Cestoplana cuneata*) collected from the Fiji Islands in Tajika et al. (1991).

DNA extraction, polymerase chain reaction, and sequencing

Total DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Germany). Prior to extraction, preserved tissues were incubated overnight in 180 µl of ATL buffer (Qiagen, Germany) with 20 µl of proteinase K (>700 U/ml; Kanto Chemical, Japan) at 55 °C. Four gene markers were used for the analysis: a partial sequence (677 bp) of the COI gene and the 16S (444–445 bp) for DNA barcoding, and fragments of the 18S rDNA (18S; 1,735 bp) and 28S rDNA (28S; 1,006 bp) for phylogenetic inference. Amplification of the four markers was performed using polymerase chain reaction (PCR) via a 2720 Thermal Cycler (Applied Biosystems, USA). The PCR reaction volume was 10 µl, including 1 µl of total DNA template, 1 µl of 10× ExTaq buffer (Takara Bio, Japan), 2 mM of each dNTP, 1 µM of each primer, and 0.25 U of Takara Ex Taq DNA polymerase (5 U/µl; Takara Bio, Japan) in deionized water.

Specific forward and reverse primer pairs were used for each marker: Acotylea_COI_F and Acotylea_COI_R (Oya and Kajihara 2017) for COI; 16SarL and 16SbrH (Palumbi et al. 1991) for 16S; hrms18S_F and hrms18S_R (Oya and Kajihara 2020) for 18S; and fw1 and rev4 (Sonnenberg et al. 2007) for 28S. The PCR amplification procedures were as follows: 94 °C for 1 min; 35 cycles of 94 °C for 30 s, 50 °C (COI, 16S, and 18S) or 52.5 °C (28S) for 30 s, and 72 °C for 2 min (18S), 1.5 min (28S), or 1 min (COI and 16S); and 72 °C for 7 min. PCR products were purified enzymatically using ExoSAP-IT reagent. Nucleotide sequences were determined by direct sequencing with a BigDye Terminator Kit ver. 3.1 and a 3730 Genetic Analyzer (Life Technologies, California, USA). Four internal primers were used for 18S: hrms18S_Fi1, hrms18S_Fi2, hrms18S_Ri1, and hrms18S_Ri2 (Oya and Kajihara 2020), and two internal primers were used for 28S: hrms_fw2 (Oya and Kajihara 2020) and rev4 (Sonnenberg et al. 2007). In addition to the specimens collected in the present study, a 1,735-bp partial sequence of 18S from the holotype of *Cestoplana nopperabo* Oya & Kajihara, 2019 was obtained using the same methods described above. Sequences were checked and edited using MEGA ver 7.0 (Kumar et al. 2016). The edited sequences were deposited in DDBJ/EMBL/GenBank, with accession numbers of LC740486–LC740495, LC745667, and LC745668.

Molecular phylogenetic analyses

For phylogenetic analyses, a concatenated dataset (2,834 bp) comprising partial 18S (1,735 bp) and 28S (1,099 bp) sequences was prepared (Table 1). Additional 18S and 28S sequences of three cotylean species, *Pericelis flavomarginata* Tsuyuki et al., 2020, *Prosthiostomum siphunculus* (Delle Chiaje, 1828) and *Theama mediterranea* Curini-Galletti et al., 2008, were used as outgroups (Table 1). Sequences were aligned using MAFFT ver. 7.427 (Katoh et al. 2017) with the L-INS-i strategy selected using the "Auto" option. Ambiguous sites were trimmed using Clipkit ver. 1.0 via the "kpic" option (Steenwyk et al. 2020). The optimal substitution models selected using PartitionFinder ver. 2.1.1 (Lanfear et al. 2016) according to the Akaike Information Criterion (Akaike 1974) with the greedy algorithm (Lanfear et al. 2012), were GTR + I + G for both the 18S and 28S partitions. A maximum likelihood (ML) analysis was performed using RAxML ver. 8.2.10 (Stamatakis 2014). A Bayesian phylogenetic inference (BI) was performed using MrBayes ver. 3.2.6 (Ronquist and Huelsenbeck 2003; Altekar et al. 2004) with two independent runs of Metropolis-coupled Markov chain Monte Carlo, each consisting of four chains of 2,000,000 generations. All parameters (*statefreq*, *revmat*, *shape*, and *pinvar*) were unlinked between each position; trees were sampled every 100 generations. The first 25% of trees were discarded as burn-in before a 50% majority-rule consensus tree was constructed. Convergence was confirmed based on an average standard deviation of split frequencies of 0.001138, potential scale reduction factors for all parameters of 1.000–1.025, and effective sample sizes for all parameters ≥ 404. Nodal support within the ML tree was assessed using an analysis of 1,000 bootstrap (BS) pseudoreplicates (Felsenstein 1985). ML BS values ≥70% and posterior probability values ≥90% were considered to indicate clade support. Genetic distances (uncorrected *p*-distances) were calculated using MEGA ver. X (Kumar et al. 2018) with gaps/missing data deleted completely.

Results

Molecular analyses

Molecular phylogeny

The resulting ML and BI trees were identical in terms of topology; all six examined species of Cestoplanidae formed a clade with full support (Fig. 1). Within the clade, *Eucestoplana* and *Cestoplana* were reciprocally monophyletic, each with support of 1.00PP/97% BS and 0.95PP/66% BS, respectively. Within *Cestoplana*, *C. nopperabo* was sister to the remaining three, *C. rubrocincta* (Grube, 1840), *C. salar* Marcus, 1949, and *C. techa* Du Bois-Reymond Marcus, 1957, which received full support. The latter two *C. salar* and *C. techa* were sisters with low support (0.67PP/62% BS).

Genetic distances between cestoplanid species

The interspecific genetic distances between our specimens representing *E.* cf. *cuneata* and *E. ittanmomen* sp. nov. were 3.153–3.378% for 16S and 1.107% for 28S, both of which were greater than the intraspecific ones (0.225% for 16S and 0.000% for 28S) observed within two specimens of *E.* cf. *cuneata*. We failed to amplify the COI sequence of the holotype of *E. ittanmomen* sp. nov. using the primer pair Acotylea_COI_F and Acotylea_COI_R whereas that of the Japanese specimens of *E.* cf. *cuneata* was successfully amplified with the same primers (LC740486–LC740488). The interspecific genetic distance for COI was 0.000–0.148% within three specimens of *E.* cf. *cuneata*.

The interspecific genetic distances for the 28S sequences among five species of Cestoplanidae available in public databases are shown in Table 2. The minimum value was 0.664% between *C. salar* and *C. techa* (both from Brazil), whereas the maximum value within this family was 6.977% between *C. rubrocincta* from Italy and *Eucestoplana ittanmomen* sp. nov. from Japan. Within the same genus, the maximum intraspecific genetic distance was 5.980% between *C. rubrocincta* and *C. nopperabo*.

Table 1. List of species used for the molecular phylogenetic analysis, GenBank accession numbers, and references, respectively.

Species	GenBank accession		Reference
	18S rDNA	28S rDNA	
Cestoplanidae			
Eucestoplana cf. *cuneata* (Sopott-Ehlers & Schmidt, 1975)	LC740491	LC740493	This study
Eucestoplana ittanmomen sp. nov.	LC740492	LC740495	This study
Cestoplana nopperabo Oya & Kajihara, 2019	LC745668	LC322284	Oya and Kajihara (2019); this study
Cestoplana rubrocincta (Grube, 1840)	MW376751	MW377504	Rodríguez et al. (2021)
Cestoplana salar Marcus, 1949	–	KY263653.2	Bahia et al. (2017)
Cestoplana techa Du Bois-Reymond Marcus, 1957	–	KY263654.2	Bahia et al. (2017)
Outgroup			
Pericelis flavomarginata Tsuyuki et al., 2020	LC672041	LC568535	Tsuyuki et al. (2020); Tsuyuki et al. (2021)
Prosthiostomum siphunculus (Delle Chiaje, 1828)	MZ292836	MZ292816	Rodríguez et al. (unpub.)
Theama mediterranea Curini-Galletti et al., 2008	MN384707	MN384705	Dittmann et al. (2019)

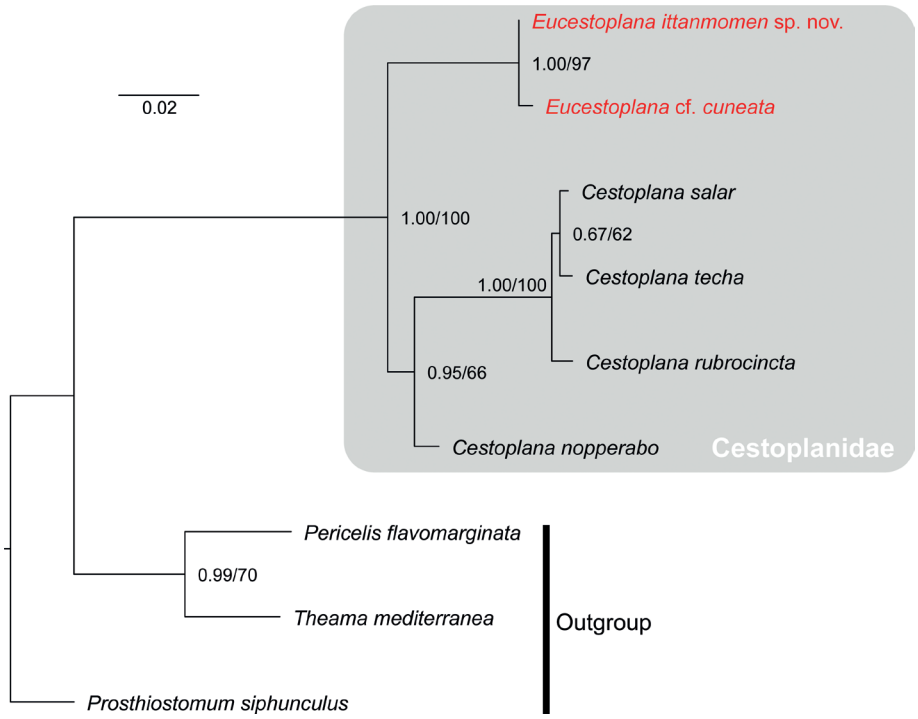

Figure 1. Maximum likelihood phylogenetic tree based on a concatenated dataset of partial 18S and 28S rDNA sequences. Numbers near nodes are posterior probabilities and bootstrap values. The species names of sequences which are newly determined in this study are indicated in the red.

Table 2. Interspecific uncorrected *p*-distances (%) for the 28S gene fragments between cestoplanid species of which sequences are available in public databases.

	C. nopperabo	***C. rubrocincta***	***C. salar***	***C. techa***	***E. ittanmomen* sp. nov.**
C. nopperabo LC322284.1	–	–	–	–	–
C. rubrocincta MW377504.1	5.980	–	–	–	–
C. salar KY263653.2	5.094	1.772	–	–	–
C. techa KY263654.2	4.873	1.883	0.664	–	–
E. ittanmomen sp. nov.	4.430	6.755	6.091	5.759	–
E. cf. *cuneata*	4.651	6.977	6.312	5.980	1.107

Taxonomy

Family Cestoplanidae Lang, 1884

Genus *Eucestoplana* Lang, 1884

Type species. *Cestoplana cuneata* Sopott-Ehlers & Schmidt, 1975.

Eucestoplana cf. *cuneata* (Sopott-Ehlers & Schmidt, 1975)

Figs 2, 3

?*Cestoplana cuneata* Sopott-Ehlers & Schmidt, 1975: 210–212, figs 9, 10; Tajika et al. 1991: 335.

?*Eucestoplana cuneata* (Sopott-Ehlers & Schmidt, 1975): Faubel 1983: 95.

Material examined. Japan •1; Okinawa Prefecture, the Okinawa Islands, Kouri Island, Tokei Beach; 26°42.86'N, 128°1.108'E; intertidal gravelly sediments; 7 Aug. 2021; A. Tsuyuki and Y. Oya leg.; sagittal sections (3 slides); GenBank: LC740488 (COI) and LC740489 (16S); ICHUM 8440. Japan •1; same data as above, except for the date (11 Aug. 2021); sagittal sections (4 slides); GenBank: LC740486 (COI), LC740491 (18S), LC740493 (28S); ICHUM 8441. Japan •1; Okinawa Prefecture, the Okinawa Islands, Okinawa Island, Nagahama Beach; 26°37.45'N, 128°11.06'E; under rocks; 9 Aug. 2021; A. Tsuyuki leg.; sagittal sections (4 slides); GenBank: LC740487 (COI), LC745667 (16S), LC740494 (28S); ICHUM 8442.

For comparison, we also examined eight serial sections of *Eucestoplana cuneata* (as *Cestoplana cuneata*) (ZMUG 25472 (holotype, three slides) and ZMUG 25473 (paratype, five slides)) and four serial sagittal sections of *Eucestoplana cuneata* (as *Cestoplana cuneata*) collected from the Fiji Island.

Description. Body slender and elongated, 24–30 mm long and 0.71–0.82 mm wide in living state (Fig. 2A). Pair of eyespot-clusters, each composed of 11–19 eyespots, distributed along midline in front of brain (Fig. 2B).

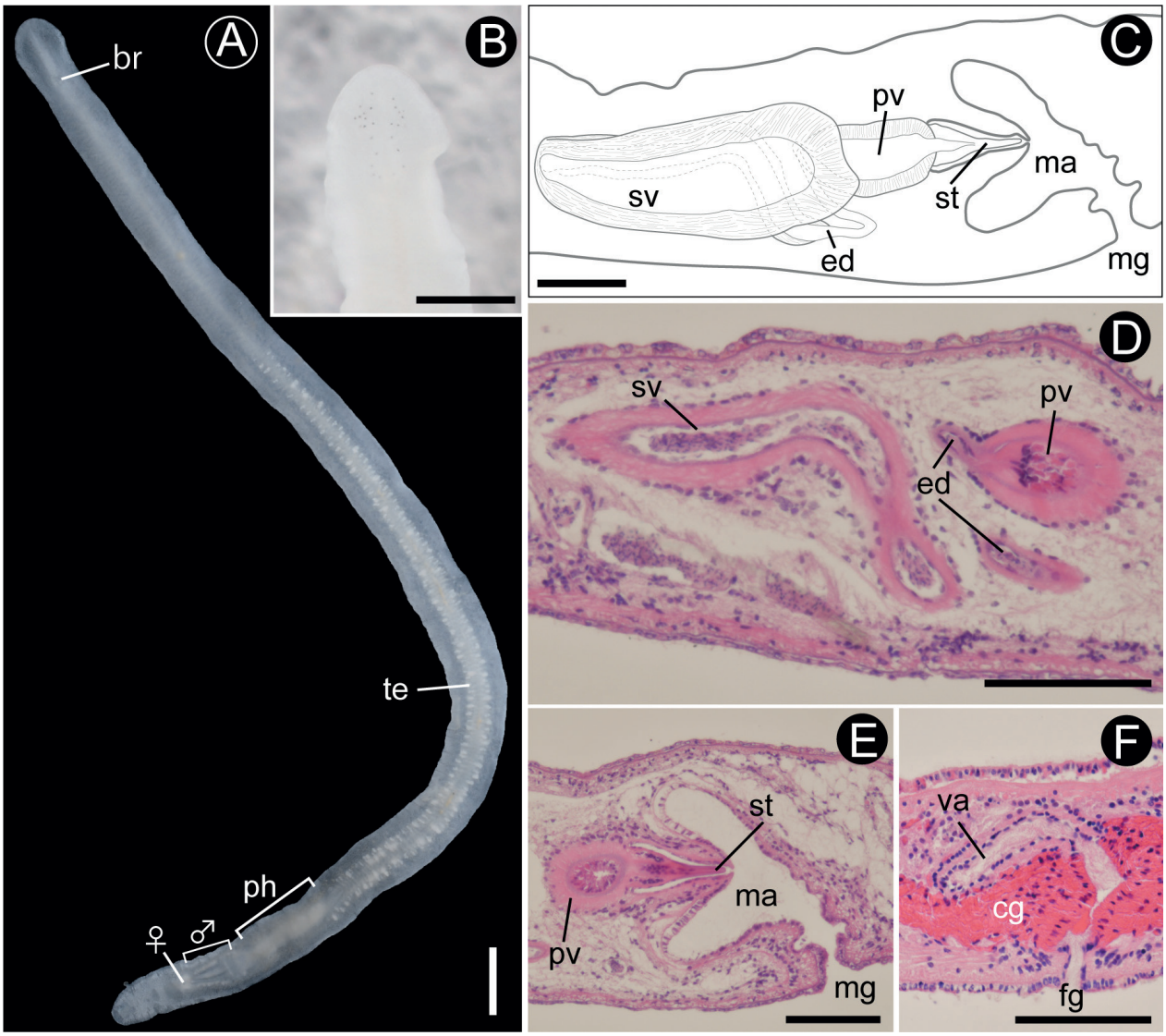

Figure 2. *Eucestoplana* cf. *cuneata* (Sopott-Ehlers & Schmidt, 1975). **A.** ICHUM 8442, whole animal in living state, dorsal view; **B.** ICHUM 8442, magnification of anterior body in living state, dorsal view, showing eyespot distribution; **C.** ICHUM 8440, schematic diagram of male copulatory apparatus in sagittal view, anterior to the right; **D, E.** ICHUM 8440, photomicrographs of sagittal sections, anterior to the right, showing male copulatory apparatus; **F.** ICHUM 8441, photomicrograph of sagittal section, showing female copulatory apparatus, anterior to the right. Abbreviations: **br** — brain; **cg** — cement glands; **ed** — ejaculatory duct; **fg** — female gonopore; **ma** — male atrium; **mg** — male gonopore; **ph** — pharynx; **pv** — prostatic vesicle; **st** — stylet; **sv** — seminal vesicle; **te** — testicular follicle; **va** — vagina; ♀ — female copulatory apparatus; ♂ — male copulatory apparatus. Scale bars: 1 mm (**A**, **B**); 100 µm (**C**–**F**).

Male copulatory apparatus composed of true seminal vesicle, interpolated prostatic vesicle, and penis papilla with stylet (Fig. 2C–E). Testicular follicles arranged in two lateral, longitudinal rows, about half length of body, running anteriorly from area in front of pharynx (Fig. 2A). Seminal vesicle antero-posteriorly elongated, posteriorly turning 180° right in front of female copulatory apparatus before running forward for short distance and then descending ventrally; thick muscular wall coating seminal vesicle, being thinner toward distal portion with forming ejaculatory duct seamlessly (Fig. 2C). Ejaculatory duct 942 µm long, extending from proximal end of prostatic vesicle to proximal end of seminal vesicle. Prostatic vesicle oval, with 19-µm thick muscular wall, lined with thick glandular epithelium (Fig. 2C–E). Penis papilla with wedged, strongly sclerotized stylet (about 60 µm long) (Fig. 2C, E). Penis sheath cone-shaped (Fig. 2C, E). Male atrium lined with cilia (Fig. 2E), opening to exterior via male gonopore with depth of about 67 µm (Fig. 2C, E). Pair of oviducts running posteriorly, then connecting to the proximal end of vagina independently. After receiving pair of oviducts, vagina curving dorsally and leading to female gonopore without evident cement pouch (Fig. 2F). Adhesive organ present at posterior end of body.

Redescription of holotype of *E. cuneata*. Male copulatory apparatus composed of true seminal vesicle, interpolated prostatic vesicle, and penis papilla with stylet (Fig. 3A). Seminal vesicle elongated, posteriorly turning 180° right, and then leading to ejaculatory duct; thick muscular wall coating seminal vesicle, being thinner

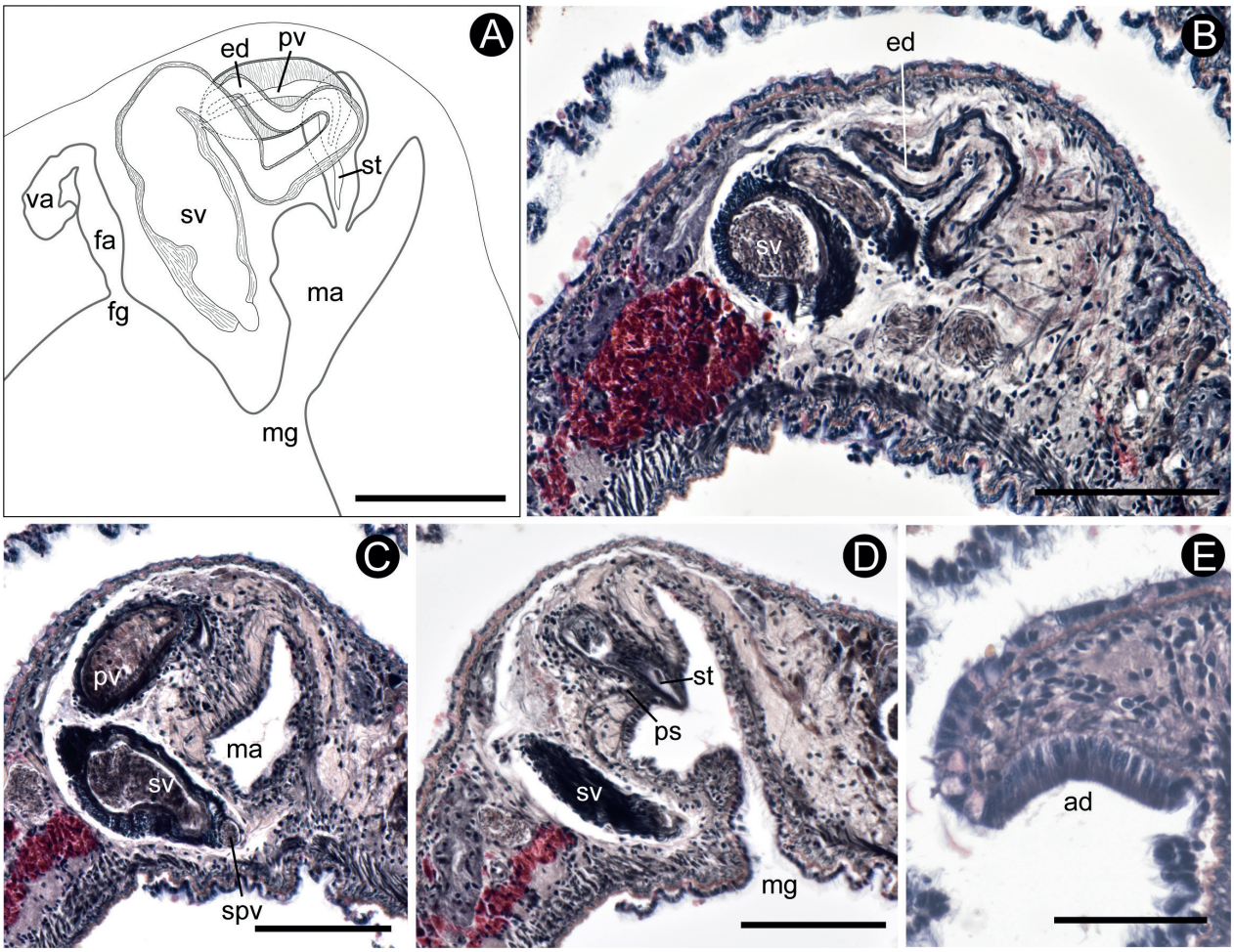

Figure 3. *Eucestoplana cuneata* (Sopott-Ehlers & Schmidt, 1975), holotype (ZMUG 25472), schematic diagram (**A**) and photomicrographs of sagittal sections (**B**–**E**) (anterior to the right). **A.** Male and female copulatory apparatuses; **B**–**D.** Male copulatory apparatus; **E.** Adhesive organ. Abbreviations: **ad** — adhesive organ; **ed** — ejaculatory duct; **fa** — female atrium; **fg** — female gonopore; **ma** — male atrium; **mg** — male gonopore; **ps** — penis sheath; **pv** — prostatic vesicle; **spv** — spermiducal vesicle; **st** —stylet; **sv** — seminal vesicle; **va** — vagina. Scale bars: 100 µm (**A**–**E**).

toward distal portion (Fig. 3A–D). Ejaculatory duct 455 µm long, extending from proximal end of prostatic vesicle to proximal end of seminal vesicle. Prostatic vesicle oval, with 13-µm thick muscular wall, lined with thick glandular epithelium (Fig. 3A, C). Penis papilla with sclerotized stylet (Fig. 3A, D). Penis sheath cone-shaped (Fig. 3A, D). Male atrium lined with cilia, opening to exterior via male gonopore with depth of about 95 µm (Fig. 3D). Pair of oviducts running posteriorly, then connecting to proximal end of vagina independently. Vagina curving dorsally after receiving oviducts. Adhesive organ present at posterior end of body (Fig. 3E).

Supplementary description of the specimen of *E. cuneata* from the Fiji Islands. Male copulatory apparatus composed of true seminal vesicle, interpolated prostatic vesicle, and penis papilla. Stylet not well observed possibly due to fixation state. Seminal vesicle elongated, posteriorly turning 180° right, and then leading to ejaculatory duct. Ejaculatory duct running to anterior, curving posteriorly behind male atrium, then connecting to proximal end of prostatic vesicle; part of ejaculatory duct from proximal end of seminal vesicle to proximal end of prostatic vesicle ca. 1 mm long. Prostatic vesicle oval; internal glandular epithelium not well observed possibly due to fixation state. Penis sheath cone-shaped. Male atrium lined with cilia, opening to exterior via male gonopore. Female reproductive organs and adhesive organ not available to be observed possibly due to fixation state.

Remarks. *Eucestoplana cuneata* was originally described from the Galapagos Islands. Our re-examination of the holotype revealed that the ejaculatory duct from proximal end of prostatic vesicle to proximal end of seminal vesicle was over twice as long as that in the original description (Sopott-Ehlers and Schmidt 1975, fig. 9).

We tentatively identified the present specimens from Kouri Island as *Eucestoplana* cf. *cuneata*. The specimens were consistent with the type specimens of *E. cuneata* in having: *i*) the eyespots distributed only anterior to the brain, *ii*) the wedged sclerotized stylet, *iii*) an adhesive organ at posterior end of body, *iv*) the conical penis sheath, and *v*) the fully ciliated inner wall of male atrium (Sopott-Ehlers and Schmidt 1975). The following morphological differences between the specimens from Japan and the Galapagos Islands should be tested by

genetic analyses if these are interspecific or intraspecific: *i*) body length (24–30 mm in our specimens; 10 mm in the original description), *ii*) eyespot number (about 30 in our specimens; 35–40 in the original description), and *iii*) length of ejaculatory duct from the proximal end of prostatic vesicle to proximal end of seminal vesicle (over 900 µm in our specimens; 455 µm in the holotype).

The wide range of distribution of *E. cuneata* needs to be verified in future studies. So far, this species has been collected from the Galapagos Islands (Sopott-Ehlers and Schmidt 1975) and the Fiji Islands (Tajika et al. 1991). Our re-examination of a specimen collected from the Fiji Islands suggested that it corresponded to the holotype of *E. cuneata* in having the *i*) conical shape of penis sheath and *ii*) the fully ciliated inner wall of male atrium. However, the Fiji specimen might be identical to *E.* cf. *cuneata* from the Okinawa Islands because it was more similar to Japanese specimens by having a long ejaculatory duct (about 1 mm). Future studies will resolve the doubt of the actual distribution of *E. cuneata* by comparing their morphology such as the body length, the eyespot number, and the male reproductive organs in more detail between different populations from the Galapagos Islands and the Fiji Islands.

Eucestoplana ittanmomen sp. nov.

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Figs 4, 5

Material examined. ***Holotype*: Japan** •1; Okinawa Prefecture, the Okinawa Islands, Kouri Island, Tokei Beach; 26°42.86'N, 128°1.108'E; intertidal gravelly sediments; 11 Aug. 2021; A. Tsuyuki and Y. Oya leg.; sagittal sections (6 slides); GenBank: LC740490 (16S), LC740492 (18S), and LC740495 (28S); ICHUM 8443. ***Paratype*: Japan** •1; same data as for holotype; sagittal sections (4 slides); ICHUM 8444.

Type locality. Japan, Okinawa Prefecture, Kunigami, Nakijin, Kouri Island, Tokei Beach (26°42.86'N, 128°1.108'E).

Diagnosis. Body slender and elongated; anterior margin rounded; dorsal surface translucent white without any color pattern; pair of eyespot-clusters distributed along midline in front of brain; penis papilla with heavily sclerotized stylet; penis sheath dome-shaped with external epithelium covered with cilia; cilia absent in inner wall of male atrium; adhesive organ present at posterior end of body.

Description of holotype. Body slender and elongated, 26 mm long and 0.75 mm wide in living state (Fig. 4A); anteriorly rounded, spreading like fan; posteriorly tapered. Dorsal surface smooth, translucent, without any color pattern. Ventral surface translucent. Tentacles absent. Pair of eyespot-clusters, each composed of 12–14 eyespots (12 on left; 14 on right), distributed along midline in front of brain (Fig. 4B), spreading out in fan shape anteriorly. Intestine highly branched without anastomosing, spreading throughout body, not reaching body margin. Pharynx ruffled, 1.94 mm long, situated on last fourth of body (Fig. 4A, C). Mouth opening at last third of pharyngeal pouch (Fig. 4C). Male gonopore opening at last ninth of body (Fig. 4A). Female gonopore situated posterior to male gonopore. Male copulatory apparatus consisting of true seminal vesicle, interpolated prostatic vesicle, and penis papilla with stylet (Fig. 4A). Testicular follicles arranged in single, lateral, longitudinal row on each side, about half length of body, running anteriorly from area in front of pharynx (Fig. 4A). Pair of sperm ducts separately entering proximal end of seminal vesicle; each duct forming spermiducal vesicle before entering seminal vesicle (Fig. 5A, B). Seminal vesicle extending posteriorly, about 700 µm long and 90 µm wide at its widest point, posteriorly turning 180° right in front of female copulatory apparatus before running anteriorly to lead to ejaculatory duct at position of proximal end of

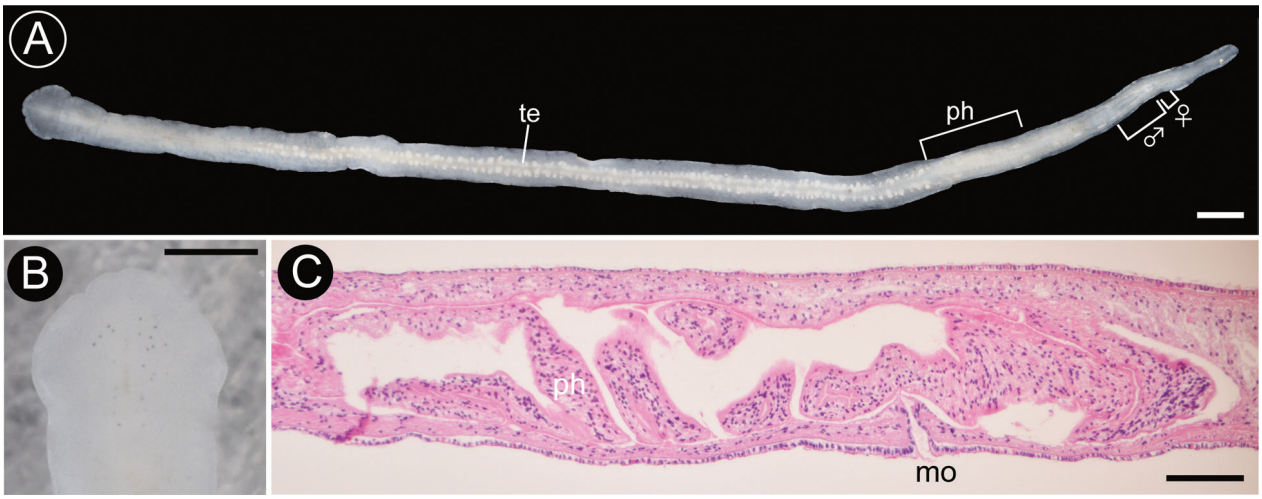

Figure 4. *Eucestoplana ittanmomen* sp. nov., holotype (ICHUM 8443). **A.** Whole animal in living state, dorsal view; **B.** Magnification of anterior body, dorsal view, showing eyespot distribution; **C.** Photomicrograph of sagittal section (anterior to the right), showing pharynx and mouth. Abbreviations: **mo** — mouth; **ph** — pharynx; **te** — testicular follicle; ♀ — female copulatory apparatus; ♂ — male copulatory apparatus. Scale bars: 1 mm (**A**, **B**); 100 µm (**C**).

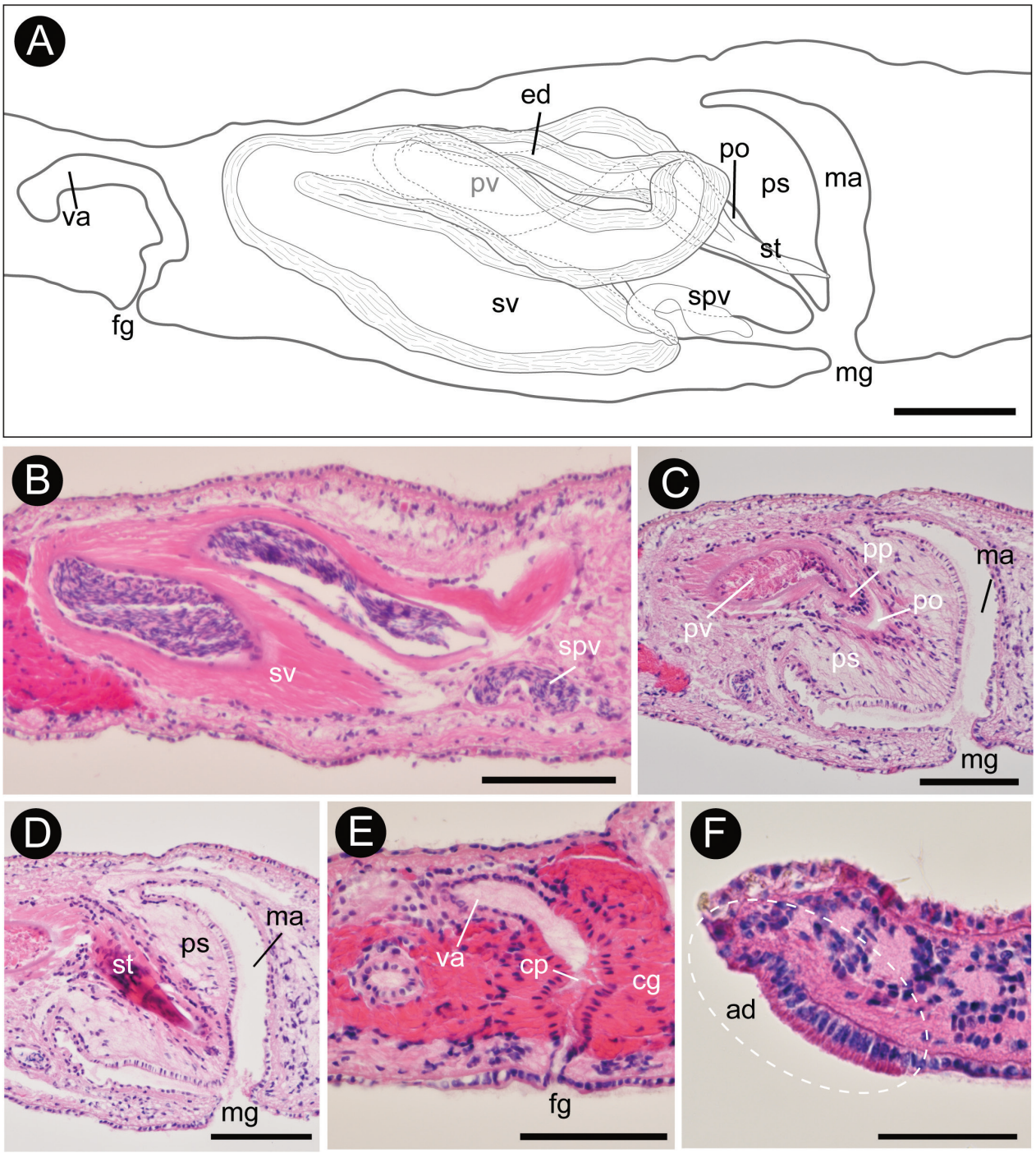

Figure 5. *Eucestoplana ittanmomen* sp. nov., schematic diagram (**A**) and photomicrographs of sagittal sections (**B**–**F**) (anterior to the right). **A.** ICHUM 8443 (holotype), male and female copulatory apparatuses; **B–D.** ICHUM 8443 (holotype), male copulatory apparatus; **E.** ICHUM 8443 (holotype), female copulatory apparatus; **F.** ICHUM 8444 (paratype), adhesive organ. Abbreviations: **ad** — adhesive organ; **cg** — cement glands; **cp** — cement pouch; **ed** — ejaculatory duct; **fg** — female gonopore; **ma** — male atrium; **mg** — male gonopore; **po** — penis pouch; **pp** — penis papilla; **ps** — penis sheath; **pv** — prostatic vesicle; **spv** — spermiducal vesicle; **st** —stylet; **sv** — seminal vesicle; **va** — vagina. Scale bars: 100 µm (**A**–**F**).

penis stylet; thick muscular wall, about 19 µm thickness, coating seminal vesicle and ejaculatory duct (Fig. 5A, B). Prostatic vesicle oval, elongated, with about 18-µm thick muscular wall, lined with thick glandular epithelium; distal end of prostatic vesicle forming penis papilla (Fig. 5A, C). Penis papilla with wedged, strongly sclerotized stylet (131 µm long), projecting into male gonopore (Fig. 5A, D). Penis sheath dome-shaped, about 184 µm wide at its widest point, housing penis stylet (Fig. 5A, C, D); external epithelium being exposed to male atrium, former being lined with cilia (Fig. 5C, D); penis pocket lined with non-ciliated epithelium. Male atrium lined with thin epithelium without cilia (Fig. 5C, D). Male gonopore about 27 µm deep. Female copulatory organ lacking Lang's

vesicle. Pair of oviducts running posteriorly, then connecting to proximal end of vagina independently. Vagina narrow, curved dorsoventrally, lined with ciliated epithelium, leading to female gonopore via narrow cement pouch (Fig. 5A, E). Numerous cement glands releasing their contents into cement pouch (Fig. 5E). Adhesive organ located at posterior end of body.

Description of paratype. Due to lack of anterior part of body, body length, width and eyespot arrangements unknown. Body coloration same as holotype. Pharynx ruffled, 1.27 mm in length; mouth opening at posterior region of pharyngeal pouch. Male copulatory apparatus composed of elongate seminal vesicle, interpolated prostatic vesicle, and penis papilla with wedged stylet (106 µm long); penis stylet slenderer than that of holotype. Penis sheath dome-shaped, with external epithelium ciliated; numerous eosinophilic glands piercing distal part of penis sheath. Male atrium covered with non-ciliated epithelium. Female copulatory apparatus same as holotype except for shape of cement pouch being more expanded than that of holotype. Adhesive organs present at posterior end of body (Fig. 5F).

Etymology. The specific name *ittanmomen* (Ittan-momen) is a Japanese noun, representing the name of one of the "yokai" (a class of supernatural entities and spirits in Japanese folklore). It is named after the long and narrow cloth-like white body of the flatworm, which evokes the similar-looking yokai, Ittan-momen.

Distribution. To date, only from the Okinawa Islands, Japan.

Remarks. Our specimens belong to *Eucestoplana* based on the following characteristics: *i*) the evident sclerotized penis stylet and *ii*) a female copulatory apparatus without any accessory ducts or Lang's vesicle. *Eucestoplana ittanmomen* sp. nov. can be easily distinguished from *E. meridionalis* by the following characteristics: *i*) translucent body, *ii*) fewer eyespots distributed only anterior to the brain, and *iii*) the presence of the adhesive organ (Table 3). Our new species is most similar to *E. cuneata* in having the following characteristics: *i*) around 30 eyespots distributed only anterior to the brain, *ii*) a wedge-shaped stylet, and *iii*) the adhesive organ located on the posterior end of the body. However, *E. ittanmomen* sp. nov. is differentiated from *E. cuneata* by the following characteristics: *i*) the shape of the penis sheath (dome-shaped in *E. ittanmomen* sp. nov.; cone-shaped in *E. cuneata*), *ii*) the arrangement of the cilia in the inner wall of the male atrium (only present along the outside of the penis sheath in *E. ittanmomen* sp. nov.; surrounding the whole male atrium in *E. cuneata*), and *iii*) the stylet length (106–131 µm in *E. ittanmomen* sp. nov.; 70 µm in *E. cuneata*). *Eucestoplana ittanmomen* sp. nov. can be also distinguished from *E.* cf. *cuneata* collected from the same locality by the same morphological differences as mentioned above. In addition, the genetic distance for 16S and 28S sequences between them could support that the two entities are likely to be genetically independent. The values for 16S (3.153%–3.378%) were much larger than the three interspecific values 0.5–1.8%, which were observed among three species of *Notocomplana* (*N. hagiyai*, *N. japonica*, and *N. koreana*) (Oya and Kajihara 2017). The *p*-distance between the 28S sequences of *E. ittanmomen* sp. nov. and *E.* cf. *cuneata* was also much larger than that between *C. salar* and *C. techa*, which are clearly different species because of their morphological difference (Table 2).

Discussion

The molecular phylogeny presented here reveals that the two *Eucestoplana* species, *E.* cf. *cuneata* and *E. ittanmomen* sp. nov., were most closely related to each other (Fig. 1). This phylogenetic closeness suggests synapomorphic traits within the cestoplanid lineage. Indeed, the *i*) heavily sclerotized penis stylet, *ii*) reduced number of eyespots, and *iii*) preference for gravelly interstitial habitats may be unique features of representatives

Table 3. Comparison of the selected characteristics among the known *Eucestoplana* species and our new species.

	E. cuneata	***E. ittanmomen* sp. nov.**	***E. meridionalis***
Body length (mm)	10[a]	26	20
Body width (mm)	?(slender, ribbon-shaped)	0.7	3
Anterior body shape	Rounded	Rounded	Slightly pointed
Eyespots	35–40[a], only anterior to the brain	About 20–30, only anterior to the brain	Numerous, distributed around brain
Dorsal coloration	? [a]; translucent white[b]	Translucent white	Chocolate-brown
Dorsal color pattern	? [a]	Absent	Absent
Mouth position	Near posterior end of pharynx	Near posterior end of pharynx	In posterior region of pharyngeal cavity
Seminal vesicle	Elongate, bending 180°	Elongated, bending 180° at position posterior to female reproductive organ	Elongate-oval
Stylet	70-µm long; wedge-shaped	106–131-µm long; wedge-shaped	Present
Penis sheath	Cone-shaped	Dome-shaped	Cone-shaped
Cilia along inner wall of male atrium	Surrounding the whole male atrium	Only present along the outside of the penis sheath	?
Adhesive organ	Present	Present	Absent
Distribution	The Galapagos Islands[a]; Fiji[b]	The Okinawa Islands, Japan	South Australia
Reference	[a]Sopott-Ehlers and Schmidt (1975); [b]Tajika et al. (1991)	This study	Prudhoe (1982a); Prudhoe (1982b)

of *Eucestoplana*. Although we were unable to include *Cestoplana nexa* Sopott-Ehlers & Schmidt, 1975 in our phylogenetic analyses, future studies may show that this species should affiliate with *Eucestoplana* rather than *Cestoplana* because the latter two characteristics of eyespot number and habitats are also found in this species. Further investigations involving more cestoplanid species are necessary to confirm the monophyly of *Cestoplana* and *Eucestoplana*. Additional species such as *E. meridionalis*, the other four species of *Cestoplana*, and representatives of the other four genera, viz. *Acestoplana*, *Cestoplanella*, *Cestoplanides*, and *Cestoplanoida* should be included in future studies to gain a more comprehensive understanding of the relationships within this family.

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