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Description of a new bathyal species of *Notocomplana* (Platyhelminthes, Polycladida) collected from *Bathymodiolus* aggregations in a deep-sea hydrocarbon seep

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Abstract

Polyclad flatworms are free-living platyhelminths that inhabit various marine environments; however, their biodiversity in the deep sea remains poorly understood. Herein, we describe a new species of *Notocomplana* Faubel, 1983, *Notocomplana profunda* **sp. nov.**, based on specimens collected from *Bathymodiolus* mussel aggregations in a deep-sea hydrocarbon seep off Hatsushima, Japan. This is the first report of a bathyal species of *Notocomplana*, which is dominated by shallow-water species. The new species is characterized by the absence of tentacles, eyespots, and any color pattern on the dorsal surface of the body and the presence of a seminal vesicle larger than a prostatic vesicle and a long, tubular Lang's vesicle. A molecular phylogenetic analysis based on the partial sequences of two mitochondrial and two nuclear genes suggested that the strategy of *N. profunda* **sp. nov.** to use mussel aggregations was acquired by its shallow coastal ancestors.

Key words: Acotylea, chemosynthesis-based ecosystem, Leptoplanoidea, systematics, turbellarian

Introduction

Polycladida is an order of marine free-living flatworms that includes approximately 1000 species worldwide (Tyler *et al*. 2006–2025). The vertical distribution of polyclads ranges from the supralittoral zone (Newman & Cannon 1997) to the bathyal zone down to 3232 m (Quiroga *et al*. 2006). Most polyclads are epibenthic, inhabiting various substrates such as rocks, muddy bottoms, algae, and seagrasses in shallow coastal areas (Prudhoe 1985), although interstitial (e.g., Tsuyuki *et al*. 2023) and pelagic (e.g., Kato 1938) species have also been described. Some species are known to be commensal or parasitic (e.g., Oya *et al*. 2022a)

Several polyclads are known to have colonized habitats unique to the deep-water realm. Four polyclad species were described from wood falls at depths of 330 m (Oya *et al.* 2024a), 610 m (Quiroga *et al*. 2008), and 2642–3232 m (Quiroga *et al*. 2006). Two species of polyclads were reported from mussel aggregations formed at cold seeps, at depths of 650 m (Quiroga *et al.* 2008) and 1350–1525 m (Li *et al.* 2023). In addition, unidentified polyclad flatworms have been detected at a whale fall (760 m depth) (Silva *et al.* 2021), and at hydrothermal vents on Mokuyo Seamount (around 1256 m depth) (Chen *et al.* 2024) and in the *Kairei* vent field (2415–2460 m depth) (Van Dover *et al*. 2001).

In this study, we describe a new species of *Notocomplana* Faubel, 1983 (Leptoplanoidea Ehrenberg, 1831: Notocomplanidae Litvaitis, Bolaños, & Quiroga, 2019) based on specimens found in *Bathymodiolus* aggregations

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around a hydrocarbon seep at a depth of 911 m off Hatsushima, Japan. We also infer the phylogenetic position of *N. profunda* **sp. nov.** based on two mitochondrial (16S rRNA and cytochrome *c* oxidase subunit I (*COI*) and two nuclear (18S rRNA and 28S rRNA) genes.

Material and methods

Seven intact polyclad individuals—and an undetermined number of fragments and specimens that went unnoticed—were found at a depth of 911 m at the seep site off Hatsushima Island (35°00.94′N, 139°13.38′E) during the research cruise KM21-08 [R/V *Kaimei* belonging to the Japan Agency for Marine-Earth Science and Technology (JAMSTEC)]. *Bathymodiolus* mussels were collected using a suction sampler attached to the ROV, KM-ROV (cf. Hookabe *et al.* 2024). Samples from the mussels, including bottom sediments, were gently stirred in cold (4°C) seawater, then the supernatant was filtered through a 500 µm mesh plankton net. Living specimens were anesthetized using $MgCl_2$ solution and photographed using a Nikon D5600 Digital SLR Camera (Nikon, Japan). One individual was fixed and preserved in 99.5% ethanol for DNA extraction, and the other six were fixed in Bouin's solution for 24 h and preserved in 70% ethanol for morphological studies.

Out of six samples prepared for morphological observation, four specimens with favorable fixation conditions (less curling or twisting) were used in this study. Two specimens were dehydrated in an ethanol series and cleared in xylene, then embedded in paraffin wax and sectioned sagittally at a thickness of 7 µm. The sections were stained with hematoxylin and eosin (HE) following the protocol of Oya *et al.* (2024b) or Mallory's triple (MT) stain according to the method of Gibson (1994) and mounted in Entellan New (Merck, Germany). The third specimen was divided into two portions; anterior 1/3 and posterior 2/3, then, these two subsamples were dehydrated and cleared following the same protocol. The anterior portion was prepared as a whole-mounted specimen in Canada balsam (Kanto Chemical, Japan), whereas the posterior portion was cross-sectioned at a thickness of 7 µm, then stained with HE and mounted in Entellan New. The fourth specimen was dehydrated, cleared using the same procedure, and then mounted in Canada balsam. The histological and whole-mounted specimens were observed using a BX53 Upright Microscope (Evident, Japan) and photographed with an ARTCAM-150P5-WOM (Artray, Japan) attached to the microscope.

According to the method of Tsuyuki *et al.* (2025), a 3D model of the seminal vesicle with a part of the prostatic vesicle was reconstructed to obtain a reliable image of its shape. A series of photomicrographs of 90 cross-sections of the male copulatory apparatus of the paratype (NSMT-Pl-9513) was prepared using the same camera on the microscope. The photographs were stacked and aligned using the StackReg and TurboReg plugins based on Thévenaz *et al.* (1998) in the program Fiji (Schindelin *et al.* 2012), employing a "Rigid Body" transformation. The outlines of the seminal vesicle and a part of the prostatic vesicle were manually traced on each aligned image using Adobe Photoshop CC. After calibrating the voxel depth to reflect the section thickness, 3D structures were created for each organ using a 'surface' display and were then combined using the 3D Viewer in Fiji.

Total DNA was extracted from a small piece of tissue of the specimen fixed in 99.5% ethanol using a DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions; the rest of the body was preserved in 99.5% ethanol. Partial sequences of two mitochondrial (16S rRNA and *COI*) and two nuclear (18S rRNA and 28S rRNA) genes were determined according to the protocol of Oya & Kajihara (2020). Fragments of each gene were amplified by PCR using the primers 16Sar-L and 16Sbr-H (Palumbi *et al.* 1991) for 16S; hrms18S_F and hrms18S_R (Oya & Kajihara 2020) for 18S; fw1 and rev2 (Sonnenberg *et al.* 2007) for 28S; and Acotylea_COI_F and Acotylea_COI_R (Oya & Kajihara 2017) for *COI*. Each reaction volume was 10 µl, containing 1 µl of total DNA template, 1 µl of 10 × Ex Taq buffer (Takara Bio, Japan), 2 mM each dNTP, 1 µM of each primer, and 0.25 U of Takara Ex Taq DNA polymerase (5 U/µl; Takara Bio) in deionized water. The PCR amplification conditions were as follows: initial denaturation at 94°C for 1 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C (16S, 18S, and *COI*) or 52.5°C (28S) for 30 s, and extension at 72°C for 45 s (16S), 1 min (*COI*), 2 min (18S), or 1.5 min (28S); and with a final extension at 72°C for 7 min. The PCR products were purified enzymatically by using ExoSAP-IT^{TM} reagent (Thermo Fisher Scientific, USA). All nucleotide sequences were determined by direct sequencing with a BigDye Terminator Kit ver. 3.1 and 3730 Genetic Analyzer (Life Technologies, USA). Sequences were checked and edited using MEGA ver. 7.0 (Kumar *et al.* 2016).

Additional 41 sequences of Leptoplanoidea, of which at least 28S sequences are available, and outgroup species were retrieved from GenBank; two acotylean species, *Discocelis* sp. (Discocelidae Laidlaw, 1903) and *Stylochus* cf. *aomori* Kato, 1937 (Stylochidae Stimpson, 1857), were selected as outgroup taxa following Oya *et al.* (2021, 2022b) (Table 1). The sequences of 16S, 18S, and 28S were aligned using MAFFT ver. 7 (Katoh & Standley 2013), with the L-INS-i strategy selected by the "Auto" option. Sites with ambiguous alignments were removed using Gblocks ver. 0.91b (Castresana 2000) with the "With Half" option. The sequences of *COI* were automatically translated into amino acid sequences and visually aligned using MEGA ver. 7.0 (Kumar *et al.* 2016). The final blocks were 417 bp of 16S, 939 bp of 18S, 917 bp of 28S and 516 bp of *COI* in length and a concatenated dataset (2789 bp) was obtained.

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

Family	Species	16S	18S	28S	*COI*
Gnesiocerotidae	*Echinoplana celerrima* 1	-	MN421936	MN421930	-
Gnesiocerotidae	*Echinoplana celerrima* 2	MW376599	MW376754	MW377507	MW375911
Gnesiocerotidae	*Gnesioceros sargassicola*	-	-	MH700309	-
Gnesiocerotidae	*Parabolia megae*	MW376589	MW376744	MW377497	MW375901
Gnesiocerotidae	*Styloplanocera fasciata*	-	-	MH700408	-
Leptoplanidae	*Leptoplana tremellaris* 1	-	MN421937	MN421931	-
Leptoplanidae	*Leptoplana tremellaris* 2	-	-	KY263696	-
Notocomplanidae	*Notocomplana ferruginea* 1	-	-	HQ659014	-
Notocomplanidae	*Notocomplana ferruginea* 2	-	-	MH700322	-
Notocomplanidae	*Notocomplana hagiyai*	LC176041	LC508152	LC508129	LC176003
Notocomplanidae	*Notocomplana humilis*	LC508187	LC508168	LC508144	LC508204
Notocomplanidae	*Notocomplana japonica*	LC176051	LC508154	LC508131	LC176018
Notocomplanidae	*Notocomplana koreana*	LC176048	LC508151	LC508128	LC176014
Notocomplanidae	*Notocomplana lapunda*	-	-	MH700325	-
Notocomplanidae	*Notocomplana profunda* **sp. nov.**	LC899822	LC899823	LC899824	LC899825
Notocomplanidae	*Notocomplana septentrionalis*	LC176059	LC508153	LC508130	LC176028
Notoplanidae	*Amyris hummelincki*	-	-	MH700269	-
Notoplanidae	*Notoplana atomata*	-	-	MH700329	-
Notoplanidae	*Notoplana australis* 1	-	AJ228786	AY157153	-
Notoplanidae	*Notoplana australis* 2	MW376595	MW376750	MW377503	MW375907
Notoplanidae	*Notoplana delicata*	LC508188	LC508169	LC508145	LC508205
Notoplanidae	*Notoplana felis*	MW376598	MW376753	MW377506	MW375910
Notoplanidae	*Notoplana qeshmensis*	-	-	MH700331	-
Notoplanidae	*Notoplana queruca*	-	-	MH700333	-
Notoplanidae	*Notoplana* sp. 1	-	-	KY263651	-
Notoplanidae	*Notoplana* sp. 2	-	-	MH700334	-
Pseudostylochidae	*Pseudostylochus elongatus*	-	LC508171	LC508147	LC508207
Pseudostylochidae	*Pseudostylochus intermedius*	LC508183	LC508164	LC508141	LC508201
Pseudostylochidae	*Pseudostylochus obscurus*	LC508180	LC508160	LC508137	LC508197
Pseudostylochidae	*Pseudostylochus takeshitai*	LC508184	LC508165	LC508142	LC508202
Pseudostylochidae	*Tripylocelis typica*	MW376597	MW376752	MW377505	MW375909
Stylochoplanidae	*Allioplana yerii*	-	LC651420	LC651421	LC582944
Stylochoplanidae	*Armatoplana albomaculata*	LC672052	LC672049	LC672048	LC672054
Stylochoplanidae	*Armatoplana divae*	-	-	MH700273	-
Stylochoplanidae	*Armatoplana kaburakii*	LC672053	LC672051	LC672050	LC582946

......*continued on the next page*

TABLE 1. (Continued)

Family	Species	16S	18S	28S	*COI*
Stylochoplanidae	*Armatoplana leptalea* 1	-	-	KY263649	-
Stylochoplanidae	*Armatoplana leptalea* 2	-	-	MH700275	-
Stylochoplanidae	*Ceratoplana falconerae*	MW376585	MW376740	MW377493	MW375897
Stylochoplanidae	*Comoplana agilis*	-	MN334199	MN384685	-
Stylochoplanidae	*Comoplana pusilla*	LC508177	LC508157	LC508134	LC508194
Stylochoplanidae	*Phaenoplana kopepe*	LC508176	LC508156	LC508133	LC369778
Stylochoplanidae	*Stylochoplana clara*	MW376586	MW376741	MW377494	MW375898
Outgroup					
Discocelidae	*Discocelis* sp.	LC508189	LC508170	LC508146	LC508206
Stylochidae	*Stylochus* cf. *aomori*	LC508182	LC508163	LC508140	LC508200

Phylogenetic analyses were conducted using the maximum likelihood (ML) method executed in RAxML-NG (Kozlov *et al.* 2019) and using Bayesian inference (BI) executed in MrBayes ver. 3.2.2 (Ronquist & Huelsenbeck 2003). The optimal substitution models for the ML analysis selected with PartitionFinder ver. 2.1.1 (Lanfear *et al.* 2016) under the Akaike information criterion (Akaike 1974) using a greedy algorithm (Lanfear *et al.* 2012) were GTR+I (18S), GTR+I+G (16S, 28S, third codon position in *COI*), K81UF+I (second codon position in *COI*), and TRN+I+G (first codon position in *COI*); and those for BI were GTR+I (18S, second codon position in *COI*) and GTR+I+G (16S, 28S, first and third codon positions in *COI*). Nodal support within the ML tree was evaluated by analyzing 1000 bootstrap pseudoreplicates. For BI, the Markov chain Monte Carlo process used random starting trees and involved four chains run for 20,000,000 generations, with a sampling frequency of 1000. A total of 40002 trees were obtained and the initial 25% of trees discarded as burn-in. Convergence was confirmed using an average standard deviation of split frequencies of 0.003795, potential scale reduction factors for all parameters of 1.000–1.003, and effective sample sizes for all parameters of >100.

The specimens used in this study have been deposited in the National Museum of Nature and Science, Tsukuba (NSMT), Japan. All sequences determined in this study have been deposited in DDBJ/EMBL/GenBank databases with the accession numbers LC899822–LC899825 (Table 1). Terminology in this study mostly followed that of Hyman (1953). To distinguish the parts of the ejaculatory duct, the terms “intra-prostatic ejaculatory duct” and “post-prostatic ejaculatory duct,” which were introduced by Oya & Kajihara (2017), were also adopted.

Abbreviations used in the figures. cg, cement 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; o, ovary; pc, pharyngeal cavity; ped, post-prostatic ejaculatory duct; ph, pharynx; pp, penis papilla; pv, prostatic vesicle; sd, sperm duct; sv, seminal vesicle; v, vagina.

Results

Family Notocomplanidae Litvaitis, Bolaños, & Quiroga, 2019

Genus *Notocomplana* Faubel, 1983

Notocomplana profunda sp. nov.

[New Japanese name: *shinkai-usu-hiramushi*]
(Figs 1–4)

Etymology. The new specific name *profunda* (*-us*, *-a*, *-um*) is a Latin adjective meaning ‘deep’ and refers to the habitat of the flatworms. The new Japanese name for the new species is derived from *shinkai* (deep sea) and *usu-hiramushi* (*Notocomplana* polyclad) in the Japanese language.

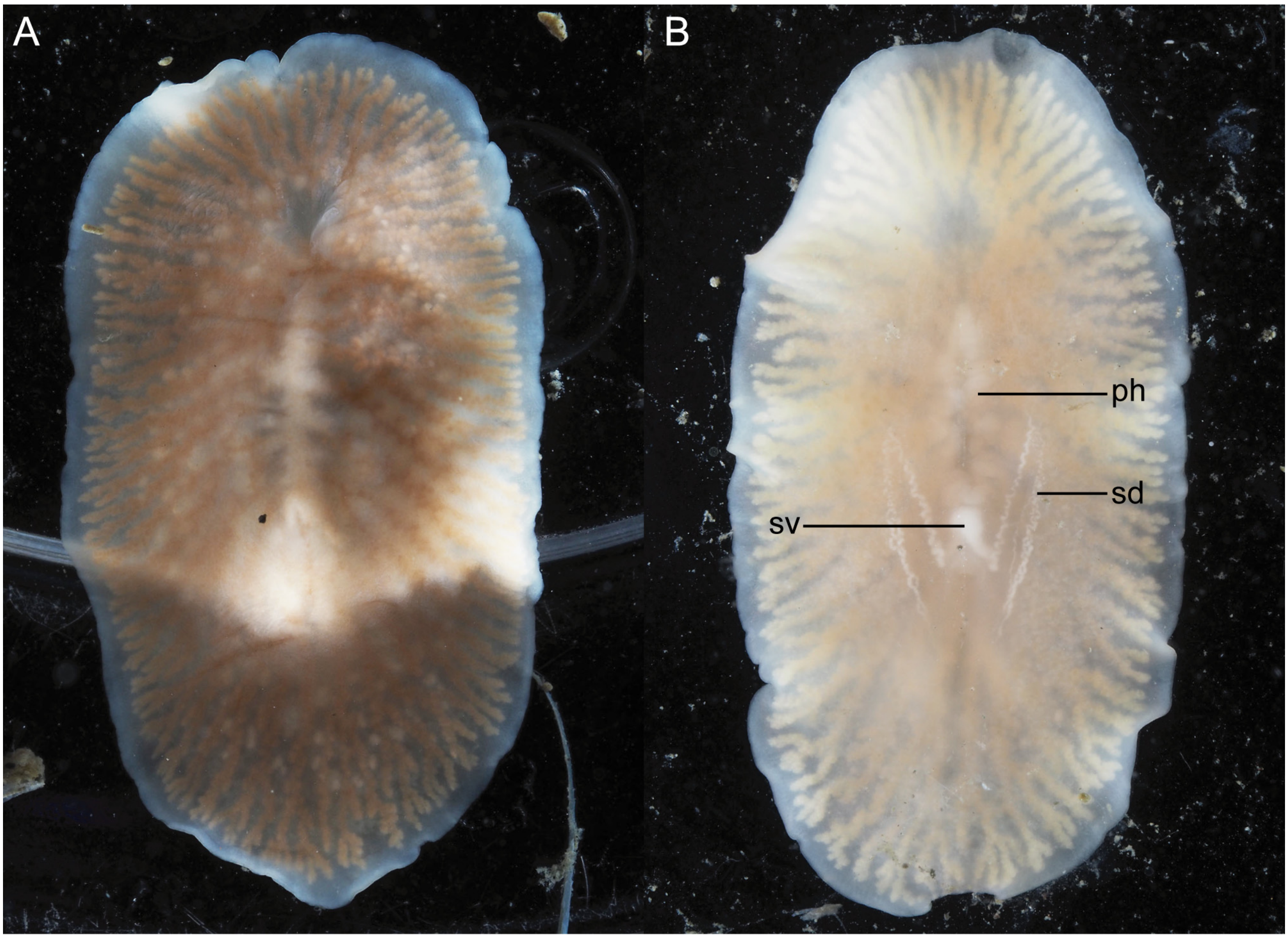

FIGURE 1. *Notocomplana profunda* **sp. nov.**, photographs taken in life, anterior to the top. A, dorsal view; B, ventral view. Scales unknown.

Diagnosis. *Notocomplana* without tentacles, eyespots, and color pattern on dorsal surface of body. Copulatory apparatus consisting of tripartite-like seminal vesicle, prostatic vesicle smaller than seminal vesicle, and long, tubular Lang's vesicle (Figs 1–4).

Material examined. Holotype: NSMT-Pl-9511, 911 m depth, off Hatsushima, Shizuoka Prefecture, Japan, 24 October 2021, coll. N. Hookabe and N. Jimi, sagittal sections of whole body on 37 slides, MT stain. Paratypes: NSMT-Pl-9512, ibid., sagittal sections of anterior 1/3 of body on 19 slides and posterior 2/3 of body containing pharynx and copulatory apparatus on 33 slides, HE stain; NSMT-Pl-9513, ibid., whole mount of anterior 1/3 of body and cross sections of posterior 2/3 of body containing pharynx and copulatory apparatus on 27 slides, HE stain; NSMT-Pl-9514, whole mount of body; NSMT-Pl-9515, whole body fixed and preserved in 99.5% ethanol.

Sequenced material. Paratype: NSMT-Pl-9515.

Description. Living worms elongated oval (Fig. 1A, B). Body whitish translucent, no color pattern (Fig. 1A). Intestine not anastomosed, spreading throughout body except into the margins. Pharynx, sperm ducts and seminal vesicle visible whitish through ventral body wall (Fig. 1B).

Fixed specimens 13–19 mm in length (19 mm in holotype), 6.6–9.7 mm in maximum width (9.7 mm in holotype) (Fig. 2A, B). Pharynx ruffled, about one-fourth of body length (3.1–4.5 mm in length, 4.5 mm in holotype), located at slightly anterior to center of body (Fig. 2B). Mouth opening at center of pharyngeal cavity. Male and female gonopores separate; male gonopore opening at about two-fifths to one-half of body length (5.4–8.9 mm, 8.9 mm in holotype) from posterior end; female gonopore situated 0.6–1.2 mm (1.1 mm in holotype) posterior to male gonopore (Fig. 2B). Tentacles and eyespots absent (Fig. 2A, C).

Male copulatory apparatus located immediately posterior to pharynx (Fig. 2D), consisting of seminal vesicle, interpolated prostatic vesicle, and penis papilla (Figs 3, 4A). Pair of sperm ducts running anteriorly from posterior to female gonopore, turning medially at about one-half length of pharynx, subsequently running posteriorly along both sides of pharynx to short distance beyond posterior end of pharynx, then turning anteriorly (Fig. 1B) to open separately to seminal vesicle; common sperm duct lacking (Fig. 3B–D). Seminal vesicle nearly tripartite-type, with 35–176 μm (25–176 μm in holotype) thick muscular wall formed by intermingle fibers (Figs 2D, 3, 4A). Distal end of seminal vesicle forming intra-prostatic ejaculatory duct of 187–213 μm in length (213 μm in holotype) that projecting deeply into prostatic vesicle. Prostatic vesicle bulbiform in shape, 311–360 μm on long axis (360 μm in holotype), 295–345 μm on short axis (345 μm in holotype), with 41–113 μm (46–113 μm in holotype) thick muscular wall (Figs 3A, 4A). Inner lining of prostatic vesicle glandular, forming six tubular chambers surrounding intra-prostatic ejaculatory duct (Fig. 4B–E). Post-prostatic ejaculatory duct lined with glandular epithelium similar to that of prostatic vesicle and surrounded by circular muscles (Fig. 4A, E). Penis papilla conical in shape, not sclerotized. Male atrium cone-shaped, lined with ciliated epithelium, lacking penis sheath.

Pair of oviducts forming common oviduct, latter running antero-dorsally to enter vagina (Figs 3A, 4E). Lang's vesicle duct 693–737 μm in length (737 μm in holotype), lined with folded ciliated epithelium (Fig. 4F). Lang's vesicle tubular in shape, 1540–1650 μm in length (1650 μm in holotype), lined with columnar cells (Fig. 4F). Lang's vesicle duct and Lang's vesicle surrounded by thin muscular wall. Vagina 1450–1697 μm in length (1697 μm in holotype), surrounded by circular muscle fibers and lined with ciliated epithelium, running anteriorly, then turning posteriorly to exit at female gonopore. About two-thirds to one-fourth of vagina surrounded by cement glands (Fig. 4F). Vagina bulbosa not developed.

Type locality. 911 m depth, off Hatsushima (35°00.94′N, 139°13.38′E), Sagami Bay, Shizuoka Prefecture, Japan.

Habitat. Mussel beds of *Bathymodiolus* species. Other invertebrates such as polychaetes, nemerteans, sea spiders, and acoels were also found in the habitat (Hookabe *et al*. 2024).

Molecular phylogeny. *Notocomplana profunda* **sp. nov.** is nested in a robust clade (97 in bootstrap value (BS)/ 1.00 in posterior probability (PP)) formed by *Notoplana atomata* (Müller, 1776) and five *Notocomplana* species: *N. hagiyai* Oya & Kajihara, 2017, *N. humilis* (Stimpson, 1857), *N. japonica* (Kato, 1937), *N. koreana* (Kato, 1937), and *N. septentrionalis* (Kato, 1937) (Fig. 5). Here, we call this clade "*humilis* clade" named after the clade containing *N. humilis*, the type species of *Notocomplana*. In this clade, the present species is the sister to *Notoplana atomata* with a 99% BS in the ML tree and 1.00 PP in the BI tree.

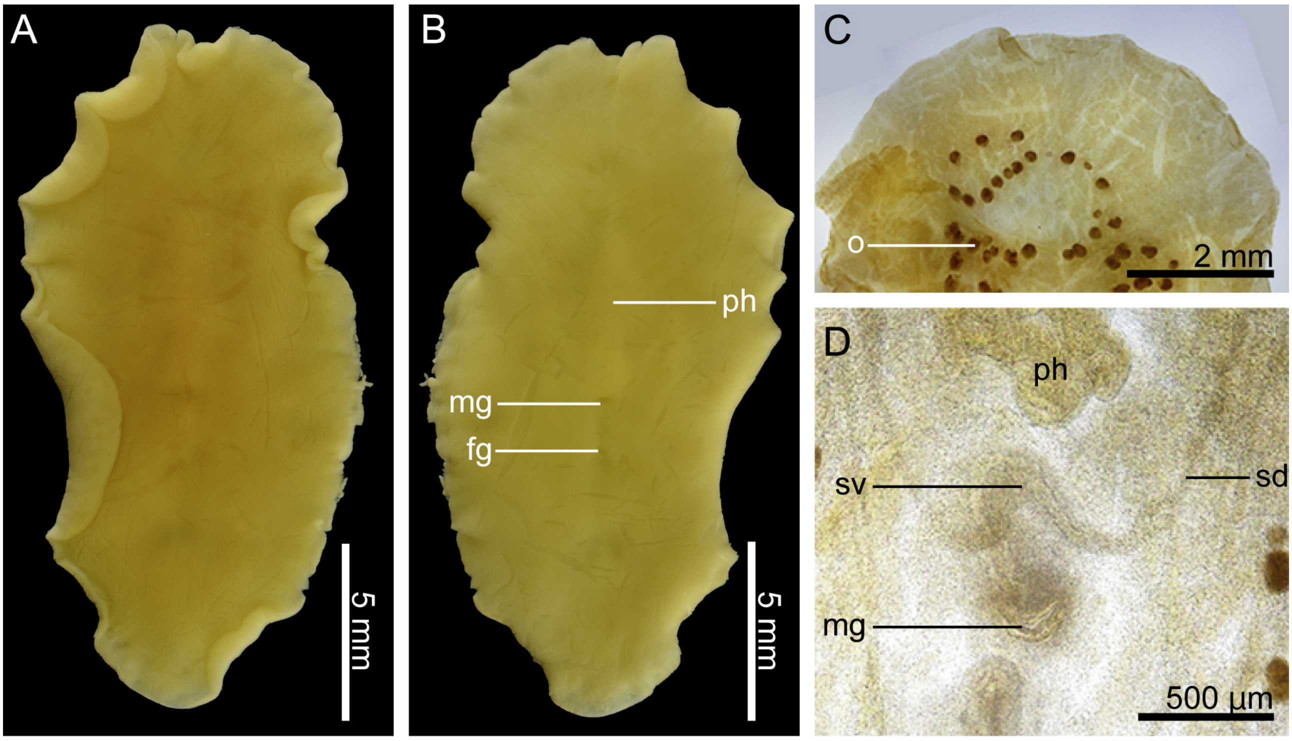

FIGURE 2. *Notocomplana profunda* **sp. nov.** photographed in preserved condition before sectioning (A, B) and whole-mounted state in Canada balsam (C, D), anterior to the top. A, holotype NSMT-Pl-9511, dorsal view; B, holotype NSMT-Pl-9511, ventral view; C, paratype NSMT-Pl-9513, dorsal view of anterior body; D, paratype NSMT-Pl-9514, ventral view of male copulatory apparatus.

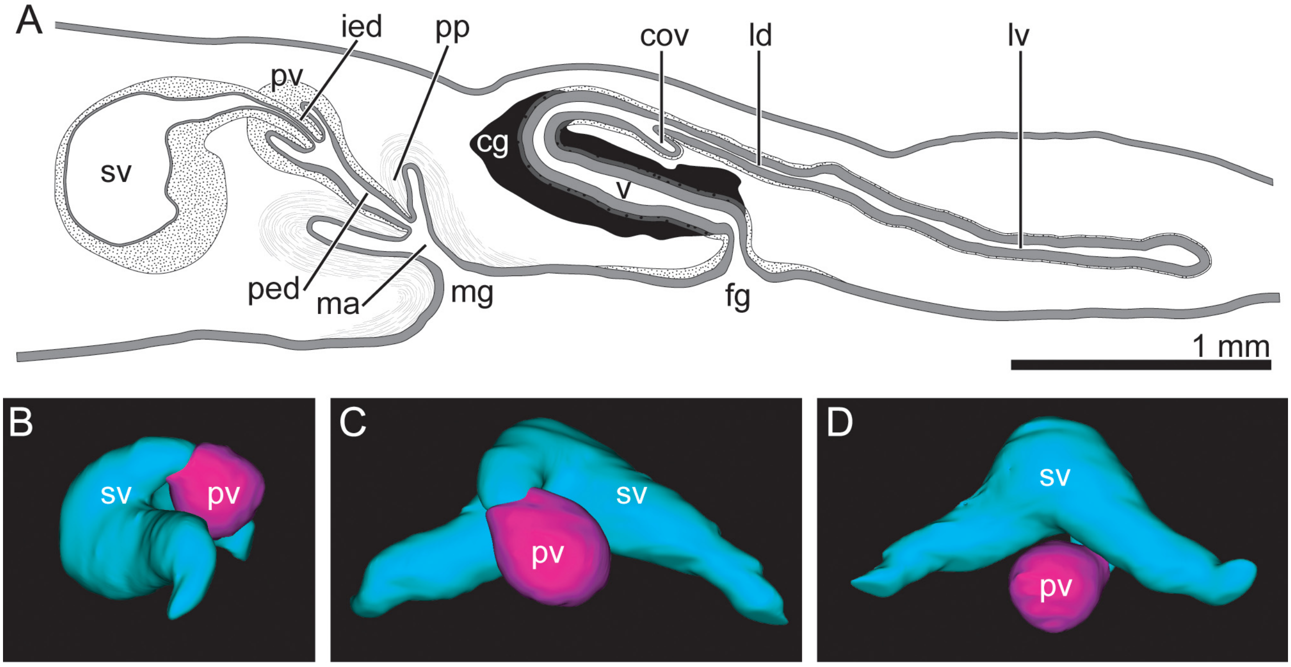

FIGURE 3. Copulatory apparatuses of *Notocomplana profunda* **sp. nov.**, schematic figure of section (A) and 3D model of seminal vesicle and part of prostatic vesicle displayed by Fiji (B–D). A, sagittal view, anterior to the left; B, lateral view, anterior to the left; C, dorsal view, anterior to the top: D, ventral view, anterior to the top.

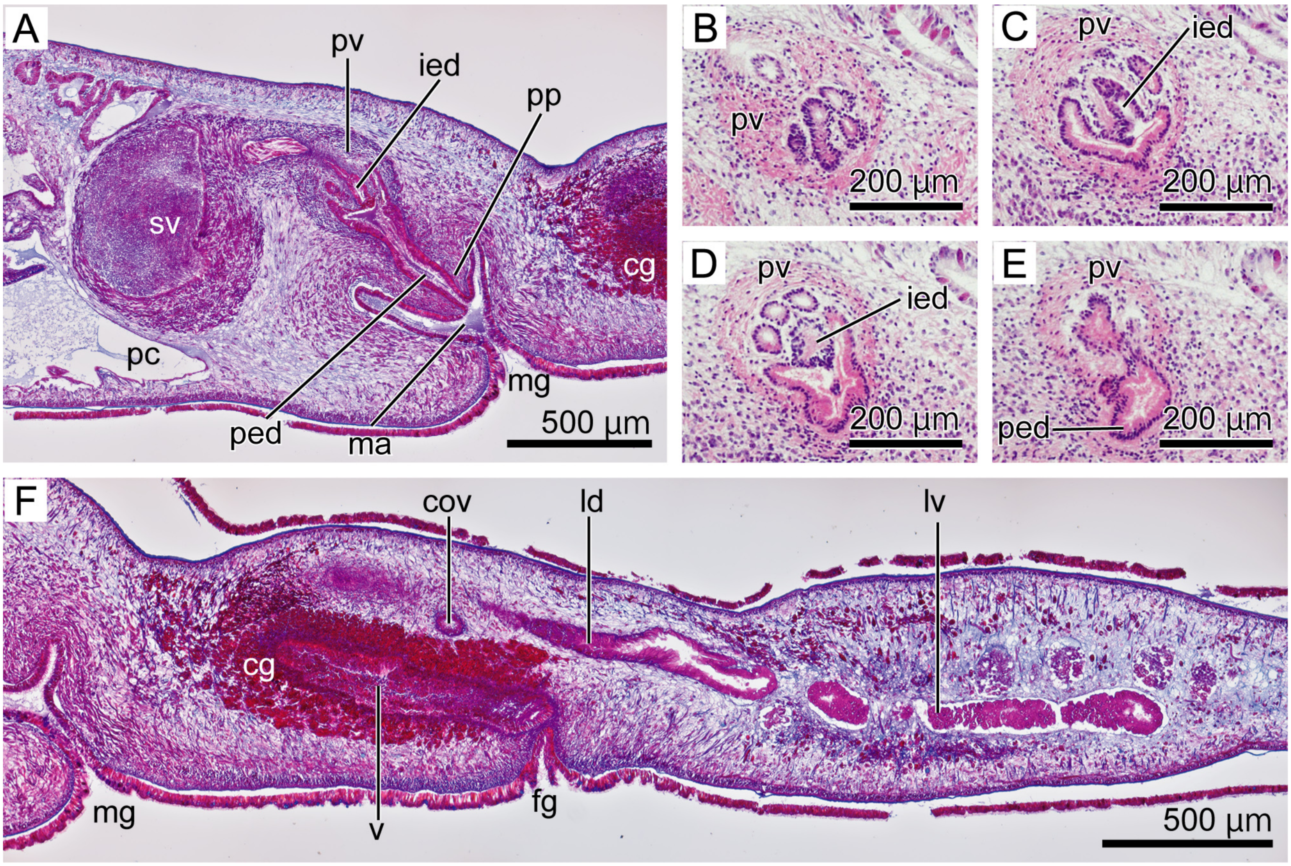

FIGURE 4. Copulatory apparatuses of *Notocomplana profunda* **sp. nov.** A, male copulatory apparatus of holotype NSMT-Pl-9511 (sagittal section), anterior to left; B–E, prostatic vesicle (cross-section) of paratype NSMT-Pl-9513, anterior to the front; F, female copulatory apparatus of holotype NSMT-Pl-9511 (sagittal section), anterior to the left.

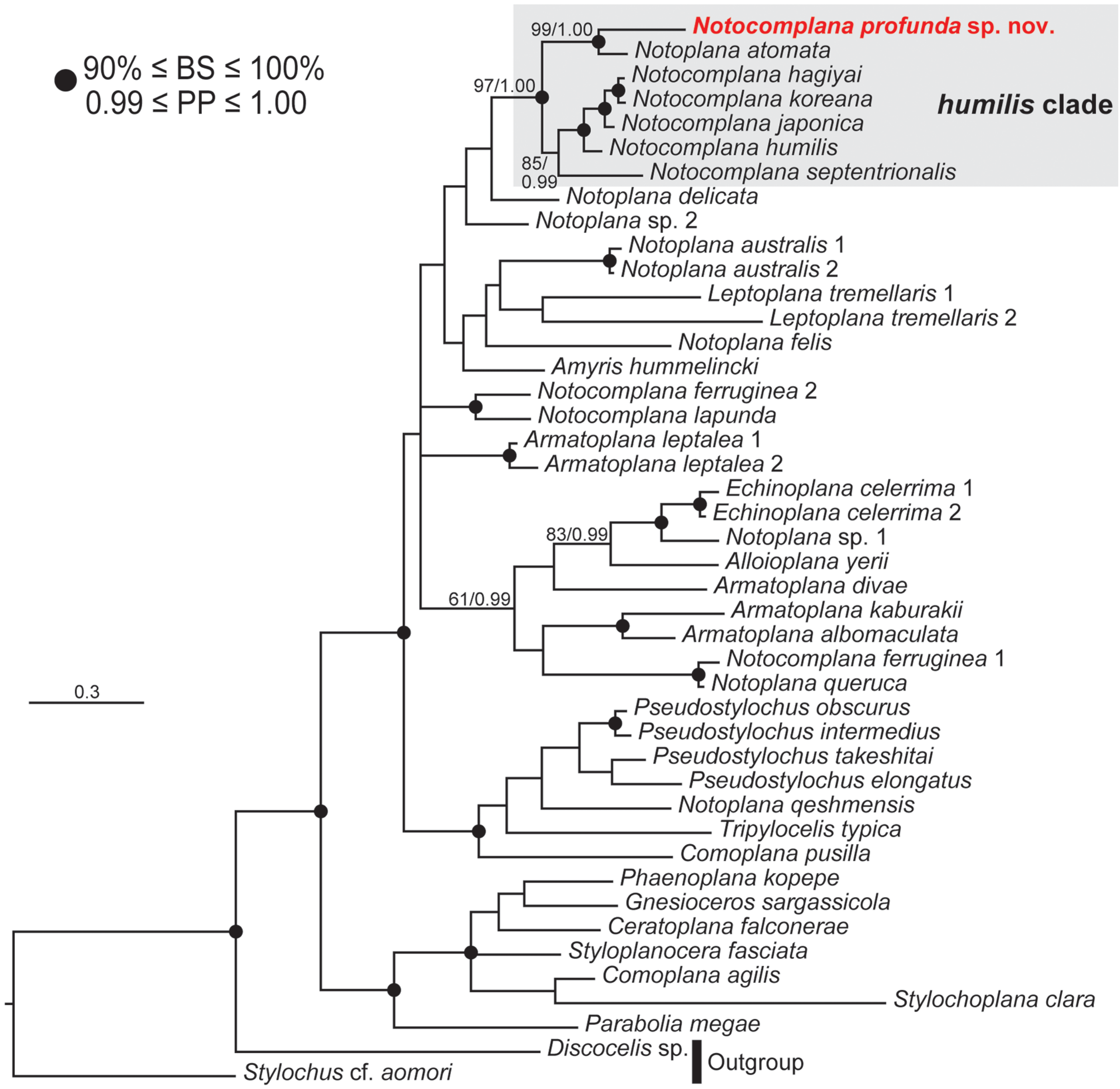

FIGURE 5. Bayesian tree based on concatenated data from four genes (16S, 18S, 28S, and *COI*; concatenated length: 2789 bp). Black circles indicate that nodal supports are ML bootstrap values (BS) ≥90% and posterior probabilities (PP) ≥0.99. Nodes whose support rate satisfies either BS ≥ 90% or PP ≥ 0.99 display numerical values near them. Nodes without black circles or numbers have support values below these thresholds (BS <90% and PP <0.99). Scale indicates substitutions per site.

Remarks. The present polyclads are characterized by possessing *i*) a seminal vesicle, *ii*) an interpolated prostatic vesicle with an intra-prostatic ejaculatory duct, *iii*) several tubular chambers in the prostatic vesicle, *iv*) a conical penis papilla lacking a penis stylet, *v*) a Lang's vesicle, and *vi*) separated gonopores. The morphology of the species meets the diagnosis of *Notocomplana* (cf. Faubel 1983; Litvaitis *et al.* 2019). Our molecular phylogenetic analyses demonstrated that the present species is the sister to a notoplanid species, *Notoplana atomata*, with high nodal supports; however, the morphology of the species is clearly distinguished from that of *Notoplana atomata* as mentioned later. In addition, both *Notocomplana* and *Notoplana* are not monophyletic (Fig. 5). Here, we assigned the present species to *Notocomplana* based on the morphological criteria.

The copulatory apparatus of the newly described species *Notocomplana profunda* **sp. nov.** is characterized by the presence of a prostatic vesicle smaller than the seminal vesicle and a tubular elongated Lang's vesicle as well as the absence of a common sperm duct (Figs 2D, 3, 4). Among the 33 valid species of *Notocomplana* (López-Márquez *et al.* 2025), the same combination of characters is only observed in *N. sanguinea* (Freeman, 1933). However, *N. profunda* **sp. nov.** is distinguished from *N. sanguinea* by *i*) the absence of eyespots (present in *N. sanguinea*), *ii*) the absence of color patterns on the body surface (a red saddle across the central part of the dorsal surface in *N. sanguinea*), *iii*) the length of the pharynx relative to the body length (one-fourth in *N. profunda* **sp. nov.**; two-fifths in *N. sanguinea*), *iv*) the shape of the seminal vesicle (nearly tripartite shaped in *N. profunda* **sp. nov.**; bean-shaped in *N. sanguinea*), and *v*) the presence/absence of a tooth-like elevation in the vagina (absent in *N. profunda* **sp. nov.**; present in *N. sanguinea*) (Freeman 1933, pl. XV, figs 11–13; Hyman 1953, figs 81–83).

Notocomplana profunda **sp. nov.** differs significantly from *Notoplana atomata* in terms of morphology, despite forming a robust clade with the species in the molecular phylogeny. *Notoplana atomata* has a bean-shaped seminal vesicle and a large sac-shaped or reduced Lang's vesicle as well as eyespots and a penis stylet (Bock 1913, text-fig. 39; Hyman 1939, pl. 2, fig. 5; Marquina *et al.* 2015, fig. 3E). In addition, the inner chambers of the prostatic vesicle in *N. atomata* are tightly packed with each other and fill the interior of the prostatic vesicle (Marquina *et al*. 2015, fig. 3F) whereas the chambers in *N. profunda* **sp. nov.** are separated from each other and similar to those in other *Notocomplana* in the *humilis* clade (Fig. 4B–E; Oya & Kajihara 2017, figs 1E, 3E, 5E, 6E). In this study, we were unable to identify any morphological synapomorphies within the clade.

Discussion

Ecology of *Notocomplana profunda* sp. nov. *Notocomplana profunda* **sp. nov.** is the second polyclad flatworm formally described from a chemosynthesis-based ecosystem. The first polyclad species, *Oligocladus bathymodiensis* Quiroga, Bolaños & Litvaitis, 2008, a cotylean polyclad flatworm, was described from an aggregation of the mussels *Gigantidas childressi* (Gustafson, Turner, Lutz & Vrijenhoek, 1998) [= *Bathymodiolus childressi* in Quiroga *et al*. (2008)] surrounding a cold seep in the continental slope of the Gulf of Mexico (Quiroga *et al*. 2008). In addition to *O. bathymodiensis*, an unidentified discocelid polyclad species was reported from mussel aggregations in seeps in the South China Sea (Li *et al*. 2023). Our findings support that seep-based mussel assemblages provide an important habitat for polyclads inhabiting deep waters.

We speculate that *Notocomplana profunda* **sp. nov.** is a polyphagous predator in the community. As an example of *Notocomplana* species associating with mussel beds, Page & Hubbard (1987) stated that *N. acticola* (Boone, 1929) consumes 4–15 mm of *Mytilus edulis* Linnaeus, 1758. However, *N. acticola* is also abundant on the undersurface of stones and feeds on small nudibranchs or brine shrimps in laboratories (Boone 1929; Koopowitz *et al*. 1976). Considering the feeding habitat in *N. acticola*, we expect that *N. profunda* **sp. nov.** will feed on other organisms such as small polychaetes or arthropods, as well as *Bathymodiolus* mussels.

Notocomplana profunda **sp. nov.** has a similar external morphology to the polyclad flatworm collected by Chen *et al.* (2024) from white bacterial mats on Mokuyo Seamount. Both flatworms share *i*) a similar body shape, *ii*) the absence of eyespots, and *iii*) the length of the pharynx (Chen *et al*. 2024, fig. 4l). It may imply that the *N. profunda* **sp. nov.** is closely related to (or the same as) Chen *et al*.'s (2024) polyclad species. However, polyclads sharing the characters of *i*) and *iii*) are also found in phylogenetically distant families, such as Discocelidae (e.g., Maghsoudlou & Rahimian 2013, figs 2, 7). In addition, the character *ii*) does not necessarily indicate that *N. profunda* **sp. nov.** is closely related to the species since it appears to be a general tendency observed in bathyal polyclads (Oya & Kajihara 2019; Oya *et al*. 2024a). Further studies with histological observations and molecular evaluations of specimens collected by Chen *et al*. (2024) are required to draw a conclusion .

Notocomplana profunda **sp. nov.** is the first *Notocomplana* species described from the bathyal zone. *Notocomplana* species are mostly found in intertidal to low tidal zones (e.g., Heath & McGregor 1912; Freeman 1933; Hyman 1953; Oya & Kajihara 2017); only a few species have been collected from sublittoral zones (e.g., Marcus 1954; Brusa & Damborenea 2011) to the present. The deepest record of the genus is a depth of 104 fathoms [= ca. 190 m] in *N. rupicola* Heath & McGregor, 1912 by Hyman (1953). Nevertheless, the understanding that *Notocomplana* is dominated by shallow-water species may be biased because taxonomic studies of Polycladida have been conducted primarily in such regions. In addition, intact specimens are difficult to collect from deep waters due to the fragility of their bodies (Quiroga *et al*. 2006). These facts suggest that the diversity of *Notocomplana* in the deep sea may be underestimated, as is the case with other polyclads. It is possible that *Notocomplana* flatworms are actually abundant in sublittoral to bathyal zones as well as in the intertidal zone.

The strategy of using mytilid aggregations by *N. profunda* **sp. nov.** is assumed to have been acquired before its ancestor inhabited the deep sea. The polyclads in the *humilis* clade are primarily found on the undersurface of stones; however, *N. hagiyai* [mentioned as '*Notoplana japonica*' in Tokinova (2008) (cf. Oya & Kajihara 2017; López-Márquez *et al.* 2025)], *N. humilis*, *N. koreana*, *N. septentrionalis*, and *Notoplana atomata* have also been recorded from the aggregations of *Mytilus* spp. in coastal regions (Tsuchiya & Nishihira 1985; Tokinova 2008; Oya & Kajihara 2017; Khalaman *et al.* 2021). The sister relationship between *N. profunda* **sp. nov.** and *Notoplana atomata* suggests that the utilization of mytilid aggregations as a habitat or resource was already established in their common ancestor dwelling in shallow waters rather than evolving after divergence from the ancestor and the advance into deep waters. Given that the widespread opportunistic use of mussel beds is among the *humilis* clade, it would not be surprising that the common ancestor of the seven species has already acquired this strategy.

Systematics of *Notocomplana* and *Notoplana*. Our phylogenetic analysis suggests the need to revise *Notocomplana* and *Notoplana*, as previous studies (e.g., Oya & Kajihara 2020; Rodríguez *et al*. 2021; Oya *et al*. 2021, 2022b) have demonstrated. The tree topology in this study is consistent with the previous studies in that *i*) *Notoplana atomata* and Japanese *Notocomplana* species form a robust clade (= the *humilis* clade), *ii*) *Notoplana delicata* Yeri & Kaburaki, 1918 is the sister to the *humilis* clade with low supports, *iii*) two OTUs of *Notocomplana ferruginea* (Schmarda, 1859) do not form a monophyletic clade and show sister relationships with *Notocomplana lapunda* (Du-Bois Reymond Marcus & Marcus, 1968) and *Notoplana queruca* (Du-Bois Reymond Marcus & Marcus, 1968), respectively, *iv*) *Notoplana qeshmensis* (Maghsoudlou, Bulnes & Rahimian, 2015) is nested within a least inclusive clade of Pseudostylochidae Faubel, 1983 and *Comoplana pusilla* (Bock, 1924), and *v*) the sister taxa of *Notoplana australis* (Schmarda, 1859) and *Notoplana felis* Rodríguez, Hutchings & Williamson, 2021 are unclear (Rodríguez *et al.* 2021; Oya *et al.* 2022b).

To revise these two genera, the phylogenetic positions of the following four nominal taxa must be considered: *Notoplana dubia* (Schmarda, 1859), *Notocomplana humilis*, *Pleioplana atomata* [= *Notoplana atomata*], and *Melloplana ferruginea* [= *Notocomplana ferruginea*] (cf. Oya & Kajihara 2020). They are the type species of *Notoplana* Laidlaw, 1903, *Notocomplana*, *Pleioplana* Faubel, 1983, and *Melloplana* Faubel, 1983, respectively. Faubel (1983) established three new genera from the previous *Notoplana* based on the presence/absence of the penis stylet and the difference in the internal structure of the prostatic vesicle. In particular, Faubel (1983) defined the character state of the latter in *Melloplana* and *Pleioplana* as "*Atomata*-type"; however, the morphology expressed by the term was inconsistent throughout the text (cf. Oya & Kajihara 2017). Later, Litvaitis *et al.* (2019) indicated uncertainty regarding the interpretation of the internal character of the prostatic vesicle and synonymized *Melloplana* and *Pleioplana* with *Notocomplana* and *Notoplana*, respectively. Currently, phylogenetic information on *Notoplana dubia*, which is crucial for revising these genera, is unavailable. It is hard to estimate the phylogenetic position of the species within the leptoplanoid phylogeny based on its morphological traits.

The *humilis* clade includes two type species: *Notocomplana humilis* and *Pleioplana atomata* (= *Notoplana atomata*). We may be able to validate *Pleioplana* focused on the clade comprising *Notocomplana profunda* **sp. nov.** and *Notoplana atomata*; however, morphological diagnoses that can distinguish these two species from other *Notocomplana* and *Notoplana* polyclads in our phylogeny have yet to be found (See Remarks). This situation is the same even if we regard the *humilis* clade as a single genus. Although the seven species in the *humilis* clade share the presence of an intra-prostatic ejaculatory duct that deeply projects into the prostatic vesicle (Fig. 4A; Bock 1913; Yeri & Kaburaki 1918; Oya & Kajihara 2017), this characteristic is also observed in *Notoplana australis* and *Notoplana felis* (Rodríguez *et al.* 2021). Furthermore, it cannot be excluded the possibility that *Notoplana dubia* is nested within the *humilis* clade. Accordingly, we provisionally adopt the opinion of Litvaitis *et al.* (2019) and propose accepting the non-monophyly of *Notoplana* and *Notocomplana* until comprehensive studies, including *Notoplana dubia*, are conducted.

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