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Description of a new marine flatworm of *Prosthiostomum* (Platyhelminthes, Polycladida, Prosthiostomidae) from the South China Sea

Hai-Long Liu[1,2], Da-Hao Lin[1], An-Tai Wang[1], Zhang-Li Hu[1], Yu Zhang[1]

1 *Shenzhen Key Laboratory of Marine Bioresource and Eco-environmental Science, Guangdong Engineering Research Center for Marine Algal Biotechnology, College of Life Sciences and Oceanography, Shenzhen University, Shenzhen, China*

2 *Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province, College of Physics and Optoelectronic Engineering, Shenzhen University, Shenzhen, China*

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Corresponding author: Yu Zhang (biozy@szu.edu.cn)

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Abstract

A new species of the polyclad genus *Prosthiostomum* is described from the intertidal zone of the South China Sea, Huidong, China, based on morphological and molecular analyses. *Prosthiostomum huidongense* **sp. nov.** is characterized by i) few marginal eyes scattered between the marginal band and the cerebral eyes; ii) sucker located at two-thirds of the body length, being removed from the female gonopore by twice the distance between the male and female gonopores; iii) shallow male atrium with slightly ruffled inner wall, positioned approximately perpendicular to the body wall. Molecular phylogenetic analyses based on 28S rDNA sequence showed that the new species was nested in a clade composed of *Prosthiostomum* species. The uncorrected p-distance of COI between *P. huidongense* **sp. nov.** and other *Prosthiostomum* species ranged from 20.3 to 24.3%, and the high genetic divergence further supports *P. huidongense* as a new species.

Key Words

Cotylea, genetic distance, molecular phylogeny, morphology, taxonomy

Introduction

Polyclads are free-living, almost exclusively marine flatworms with an extremely ramified intestine. They inhabit a variety of environments ranging from the intertidal zone to the deep-sea, such as rocky shores, sand/mud flats, coral reefs, and deep-sea hydrothermal vents (Newman and Cannon 2003; Wolff 2005; Quiroga et al. 2006). Polyclads are important predators in hard bottom environments (Rawlinson et al. 2011), and prey on crustaceans, ascidians, cnidarians, gastropods, and bivalves (Jennings 1957; Newman and Cannon 2003; Lee 2006; Barton et al. 2020; Teng et al. 2022). Some species feed on scallops and oysters (Newman et al. 1993; Heasman et al. 1998; Gutiérrez et al. 2023), damaging commercial shellfish farming (Sluys et al. 2005).

About 1000 species of Polycladida have been described worldwide; they are classified into two suborders, namely Cotylea and Acotylea, on the basis of the presence or absence of a ventral sucker (Faubel 1983, 1984; Prudhoe 1985). The cotylean polyclad genus *Prosthiostomum* Quatrefages, 1845 includes the largest number of species in the family Prosthiostomidae Lang, 1884. It currently contains 54 species distributed worldwide (Tsuyuki et al. 2019, 2021), which are characterized by i) a pair of free prostatic vesicles without a muscular envelope, ii) a main intestine accompanied by a frontal branch, and iii) a penis armed with a pointed tubular stylet (Faubel 1984). To date, four species of *Prosthiostomum* have been reported from Hong Kong and Taiwan, China: *P. obscurum* Stimpson, 1855, *P. grande* Stimpson, 1857, *P. tenebrosum*

Stimpson, 1857 and *P. formosum* Kato, 1943. Stimpson's original descriptions were simple and incomplete, lacking illustrations. With the exception of *P. grande*, the above-mentioned three species have never been recorded again, and Stimpson's materials were destroyed during the Great Chicago Fire in 1871 (see Tsuyuki et al. 2021). Therefore, these species remain questionable and cannot be reidentified (cf. Lang 1884). In the last 80 years, since Kato's (1943) research, species of *Prosthiostomum* have not been reported from Chinese waters. In this paper, we describe a new species of *Prosthiostomum* from the coast of the South China Sea based on morphological and molecular data. We selected 28S rDNA for a phylogenetic analysis, considering the fact that this sequence is available for most species of prosthiostomids.

Materials and methods

Sample collection and morphological studies

Three specimens were collected under rocks at the intertidal zone in Huidong, Guangdong Province, China (Fig. 1). The worms were measured and then photographed alive with a digital camera. Two specimens were fixed for histological examination following the method modified from Newman and Cannon's (1995), in which i) worms are placed onto filter paper, which is then placed onto frozen 10% formalin seawater, ii) an additional fixative is added to just cover the worm, then worms are smoothened with a soft brush to ensure they are fixed flat. For histological examination, specimens were dehydrated in an ethanol series and cleared in xylene, thereafter, embedded in paraffin wax. Serial sections were cut at intervals of 7 μm and were stained with modified Cason's Mallory-Heidenhain stain solution (see Yang et al. 2020). One specimen was fixed in 95% ethanol for DNA extraction. Histological preparations are deposited at the Marine Biological Museum, Chinese Academy of Sciences (**MBMCAS**), Qingdao, China.

Molecular analyses

Total DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Germany). Three markers (partial nuclear 28S rDNA, mitochondrial 16S rRNA, and cytochrome c oxidase subunit I (COI) sequences) were amplified by polymerase chain reaction (PCR). The PCR was carried out using the primers: Acotylea_COI_F and Acotylea_COI_R (Oya and Kajihara 2017) for COI; 16sar-L and 16sbr-H (Palumbi et al. 2002) for 16S; LSU5 and LSU3 (Littlewood 1994) for 28S. Thermal cycling was initiated with 3 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C (COI) or 49 °C (16S) or 52 °C (28S) for 45 s, and extension at 72 °C for 1 min. The cycling ended with a 7-min sequence extension at 72 °C. All amplified

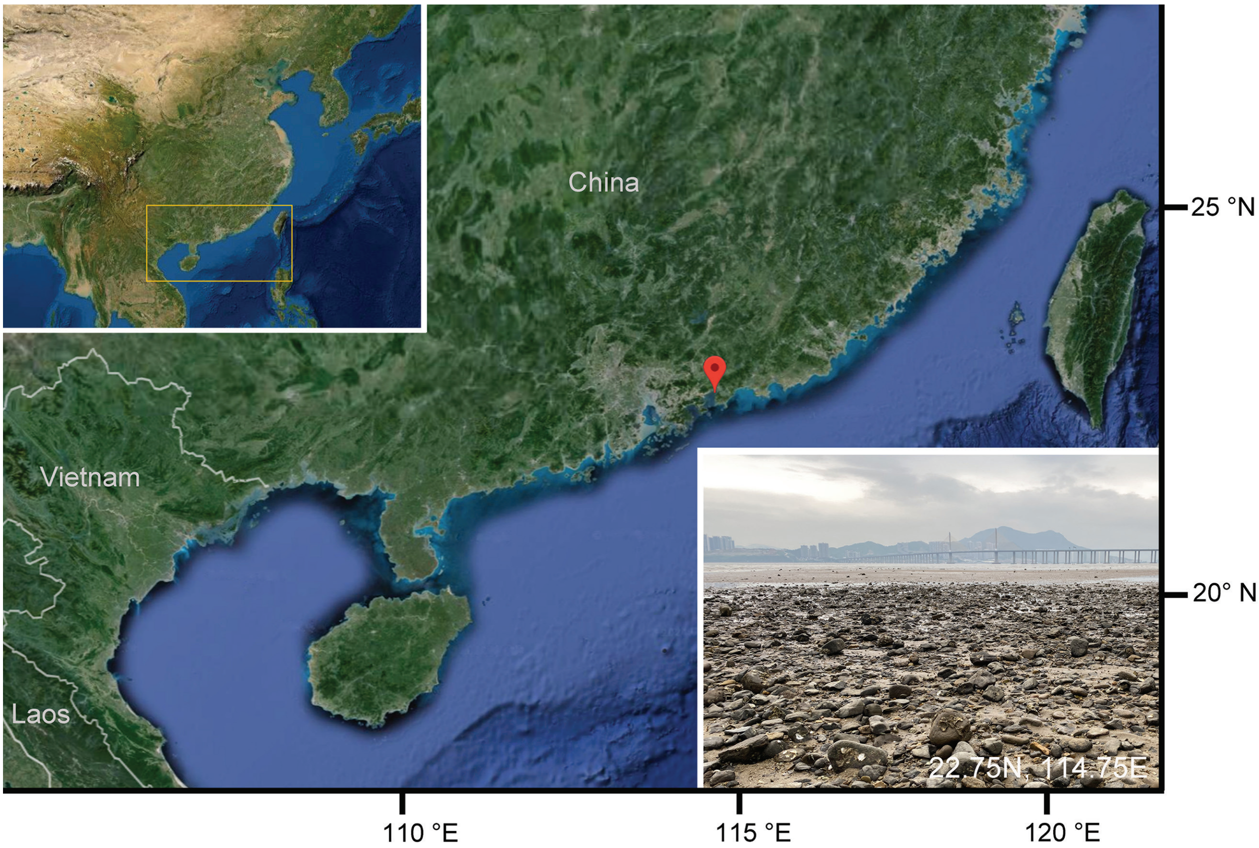

Figure 1. Map showing the sampling site of *Prosthiostomum huidongense* sp. nov. and photograph of the habitat.

products were purified using QIA-quick gel purification kit (Qiagen) and sequenced at BGI (Shenzhen, China), by means of double-stranded Sanger sequencing to verify accuracy. Sequences were checked and edited using SeqMan software (DNAStar Inc.). All the new sequences were submitted to GenBank.

A total of 27 sequences of 28S rDNA of prosthiostomid species were used for molecular analyses; *Pseudobiceros stellae* Newman & Cannon, 1994 was selected as outgroup (Suppl. material 1: table S1). Alignments were performed with MAFFT ver. 7 (Katoh and Standley 2013) using the Auto strategy. Ambiguous regions were removed with the web version of Gblocks ver 0.91b using default parameters (Castresana 2000). The Maximum likelihood (ML) analysis was performed at the CIPRES Science Gateway web with RAxML (Stamatakis 2014) on XSEDE, in which 1000 rapid bootstrap replicates and the GTRGAMMA model were used to evaluate and optimize the likelihood of the final tree. Bayesian inference (BI) was performed using MrBayes ver 3.2.2 (Ronquist et al. 2012), and the substitution model (GTR + G + I) was selected by MrModeltest ver 2.3 (Nylander 2004) according to the Akaike information criterion. MrBayes was run for 10 million generations with a tree being sampled every 1000 generations, with two parallel runs and four independent Markov chains per run and with the first 25% of trees discarded as burn-in. The standard deviation of the split frequencies (<0.01) was used as the criterion to validate the convergence of the analysis. In addition, we determined cytochrome c oxidase subunit I (COI) sequences for DNA barcoding. COI sequences of the *Prosthiostomum* species currently available from the GenBank were used for the genetic distances analysis (Suppl. material 1: table S1). Uncorrected p-distances of COI were calculated in MEGA ver 10 (Kumar et al. 2018). Voucher specimens of the material that was analyzed molecularly have been deposited at the Shenzhen Key Laboratory of Marine Bioresource and Eco-environmental Science.

Results

Family Prosthiostomidae Lang, 1884
Genus *Prosthiostomum* Quatrefages, 1845

Prosthiostomum huidongense Liu, sp. nov.

https://zoobank.org/9A6C8F9B-5664-4D50-806A-A50A4E4D119E

Material examined. ***Holotype***: MBM287880, Huidong, 22°44.95'N, 114°45.05'E, Guangdong Province, China; under rocks at the intertidal zone, 21 April 2023, coll. Hai-Long Liu; sagittal sections on 10 slides, deposited at MBMCAS. ***Paratypes***: MBM287881, sagittal sections on 11 slides, same data as for holotype. Molecular voucher specimens: 20230421A1, material of the following GenBank accession numbers have been deposited at the Shenzhen Key Laboratory of Marine Bioresource and Eco-environmental Science, GenBank: OR680085 (COI), OR680128 (16S), and OR680129 (28S). Collection data are the same as for the holotype and paratype.

Diagnosis. Body oval-elongated; dorsal surface cream-colored with numerous yellowish-brown maculae; a pair of cerebral eyes clusters forming an approximately inverted “V” shape; band of marginal eyes extending backwards to behind brain, few eyes scattered between the marginal band and the cerebral eyes; a pair of prostatic vesicles distinctly separated; male atrium shallow, inner wall slightly ruffled; sucker located at two-thirds of body length, removed from the female gonopore by twice the distance between the male and female gonopores.

Description. Body oval-elongated with rounded ends, 10.8–12.1 mm long and 5.6–7.0 mm wide at its widest in living state (n = 3) (Fig. 2A). Tentacles absent. Dorsal surface smooth, cream-colored, uniformly covered with numerous yellowish-brown maculae, pharynx slightly bulging (Fig. 2A). Ventral surface translucent, without color pattern. Cerebral region without pigmentation and provided with a pair of clusters of cerebral eyes, located at approximately 1 mm behind the anterior margin, each cluster comprising 15–20 eyes (Fig. 2B, C). About 140 marginal eyes irregularly scattered along the anterior body margin, extending backwards to a level just behind the brain; few eyes scattered between the marginal band and the cerebral eyes (Fig. 2B, C). Ventral eyes not observed. Frontal branch of the main intestine extending anteriorly to the brain (Fig. 3B). Tubular pharynx in a pharyngeal pocket, positioned in the anterior half of the body. Mouth opening ventrally, located shortly behind the brain (Fig. 3B). Male and female gonopores closely set at the body center, distance between male and female gonopores being 0.6–0.8 mm; sucker situated 1.4–1.8 mm posterior to the female gonopore, twice distance between the male and female gonopores (Fig. 3A, E). Male copulatory apparatus comprising a large seminal vesicle, a pair of prostatic vesicles, and an armed penis papilla, located immediately posterior to the pharyngeal pocket (Fig. 3A, C, E). A pair of spherical prostatic vesicles (0.05–0.07 mm in diameter) with a thin muscular wall (0.009–0.012 mm thick), located at both sides of the ejaculatory duct (Fig. 3A, D). Seminal vesicle oval, about 0.2 mm in diameter, with a muscular wall about 0.04 mm thick (Fig. 3A, E). Spermiducal vesicle separately open into the anterior portion of the seminal vesicle (Fig. 3A, D). Penis papilla armed with pointed tubular stylet (0.14–0.17 mm in length), enclosed in penis pouch, protruding into male atrium (Fig. 3C). Shallow male atrium with slightly ruffled inner wall, positioned approximately perpendicular to the body wall (Fig. 3A, E). Female reproductive system consisting of vagina, cement pouches, and uteri. Vagina short about 0.1 mm long, lined with a ciliated epithelium; the dorsal portion of the vagina enlarged and anteriorly curved, connecting to the uteri (Fig. 3A, E). Cement glands numerous, concentrated around the vagina (Fig. 3A, E). Pair of oviducts converging before joining proximal end of vagina. Ovaries not discerned.

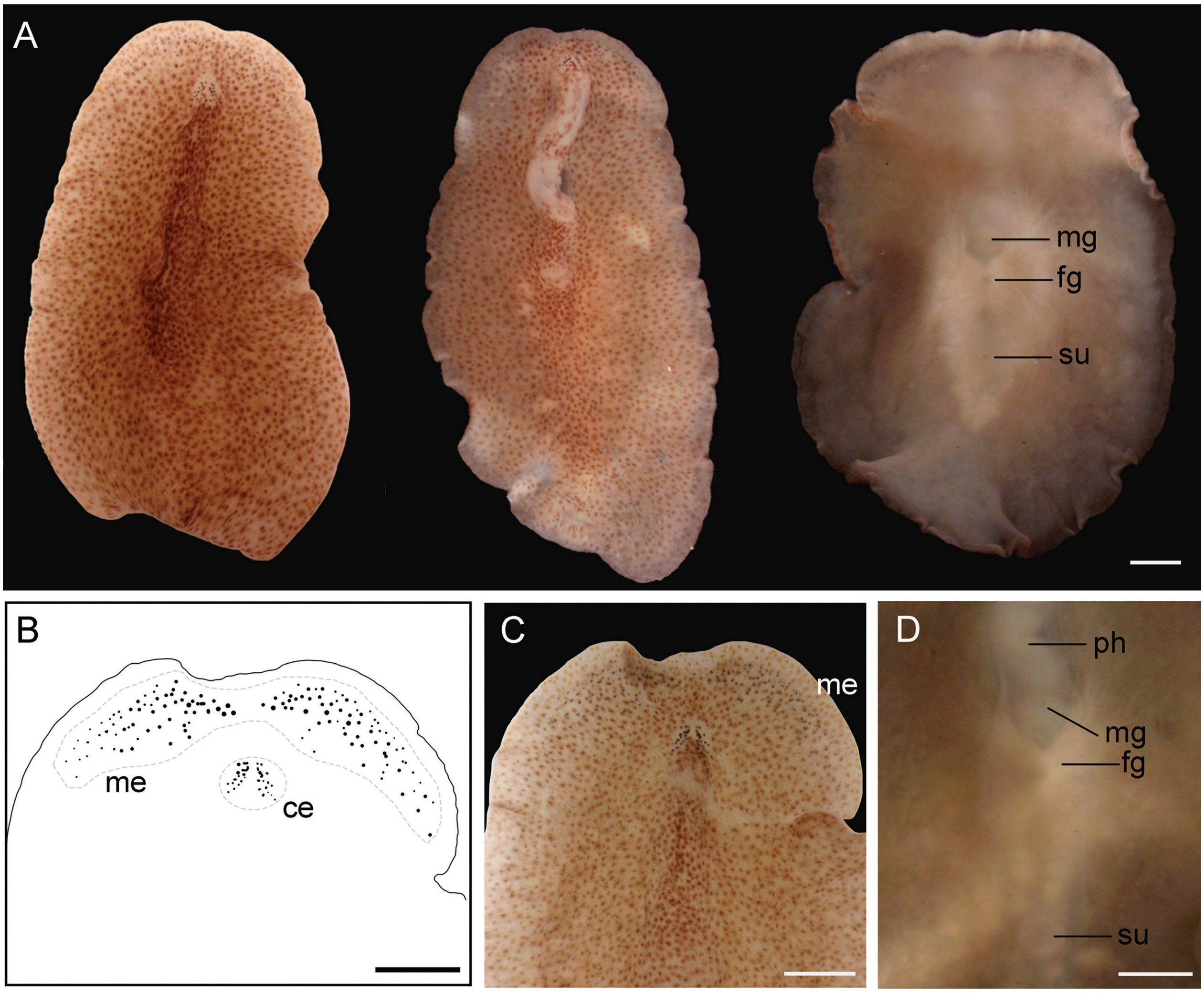

Figure 2. *Prosthiostomum huidongense* sp. nov. **A.** Photograph of entire body, 20230421A1 (left, living specimen in dorsal view), MBM287881 (middle, preserved specimen in dorsal view, paratype), MBM287880 (right, preserved specimen in ventral view, holotype); **B.** Schematic diagram of head, showing arrangement of the eyes; **C.** Magnification of head, dorsal view (holotype); **D.** Magnification of genital pore and sucker (paratype). Abbreviations: **fg** – female gonopore; **mg** – male gonopore; **ph** – pharynx; **su** – sucker. Scale bars: 1 mm.

Etymology. The name of the new species is originated from the name of Huidong City, Guangdong Province, China.

Distribution. So far only known from Huidong, Guangdong, China.

Habitat. Intertidal, under rocks.

Molecular phylogeny and genetic distances. The BI and ML trees based on the partial 28S sequences (904 bp) are almost identical in their general topology, and we show only the ML tree (Fig. 4). In the phylogenetic tree, all *Prosthiostomum* species form a clade with high support values (BP = 81%, PP = 1). *Prosthiostomum huidongense* sp. nov. is sister to the clade formed by all other species of the genus, excluding *P. lobatum*, with high support values (BP = 78%, PP = 1). The uncorrected p-distances of the partial COI sequences (658 bp) of the 11 *Prosthiostomum* species/molecular entities is 5.7–24.3% (Suppl. material 1: table S2).

Discussion

Our specimens are characterized by i) a median frontal branch of the main intestine, ii) a pair of prostatic vesicles that are distinctly separated, and iii) a penis armed with a pointed tubular stylet, features consistent with the generic diagnosis of the genus *Prosthiostomum* as proposed by Faubel (1984). The new species is easily distinguished from the four species reported from China (*P. obscurum*, *P. tenebrosum*, *P. grande*, and *P. formosum*) by the dorsal coloration and arrangement of the eyes. Among these species, *P. obscurum* is pale reddish-brown with red spots, a light-colored middle band and a pair of linear clusters of cerebral eyes, each composed of 8–10 eyes; *P. tenebrosum* is dark gray or sub-black; *P. grande* has reddish-brown maculae or spots, with a darker longitudinal band in the middle; *P. formosum* is uniformly chestnut brown without any color pattern.

Table 1. Comparison of characters between nine *Prosthiostomum* species that have similar arrangement of marginal eyes or color pattern.

Species	***P. awaensa***	***P. griseum***	***P. latocelis***	***P. milcum***	***P. huidongense* sp. nov.**	***P. dohrni***	***P. grande***	***P. nozakensis***	***P. sonorum***
Body size	22 mm long; 5 mm wide	4 mm long	17 mm long; 4.5 mm wide	about 7 mm long	10.8–12.1 mm long; 5.6–7.0 mm wide	25 mm long; 6 mm in wide	22 mm long; 5 mm wide	15 mm long	20 mm long; 2 mm wide
Dorsal coloration	rather deep buffy, with a brownish longitudinal band in the median line	uniform dark gray	Yellow	whitish body with brown mottles	cream-colored surface uniformly covered with numerous yellowish-brown maculae	soft bright orange yellow, with darker orange yellow spots scattered over the body especially denser along midline	buffy ground color, with numerous small spots of ochraceous color distributed all over body	light brown, with numerous dark brown spots over the whole dorsal surface	translucent white covered with brown mottles, a number of which aggregate along the median line to form a brown stripe
Cerebral eyes	two oblong clusters, each composed of 28 eyes	each cluster composed of 6–8 eyes	two wedge-shaped clusters, each composed of about 50 eyes	oblong clusters, each composed of 22–23 eyes	each cluster consisting of 15–20 eyes	two oval clusters, composed of numerous eyes	two wedge clusters, each composed of about 25 eyes	two oblong clusters, each composed of about 10 eyes	two cluster, each composed of about 30 eyes
Marginal eyes	about 60, distributed anterior to brain; few eyes scattered between the marginal band and the cerebral eyes	about 30, distributed anterior to brain; few eyes scattered between the marginal band and the cerebral eyes	about 80, distributed anterior to brain; some eyes scattered between the marginal band and the cerebral eyes	95, elongated to the half position of the brain; 25 small scattered eyes between the marginal band and the cerebral eyes	about 140, elongated to the level behind brain; few eyes scattered between the marginal band and the cerebral eyes	numerous; elongated to the level behind brain	two irregular rows along the anterior margin	about 60, elongated to the half position of the brain	about 50, distributed anterior to brain
Male atrium	NA	NA	deep; anterior wall strongly folded	deep; inner wall slightly ruffled	shallow; inner wall slightly ruffled	NA	NA	NA	NA
Seminal vesicle	NA	NA	oval; lumen oval	elongated oval; lumen narrow	spherical or oval; lumen spherical or oval	NA	spherical; lumen shape unknown	oval; lumen oval	oval; lumen oval
Sucker	nearly central	located slightly behind body center	nearly central; distance between the female gonopore and the sucker similar to the distance between the female gonopore and the male gonopore	nearly central; distance between the female gonopore and the sucker similar to the distance between the female gonopore and the male gonopore	located at the two-thirds of body; distance between the female gonopore and the sucker twice the distance between the female gonopore and the male gonopore	present; details not described	located slightly behind body center	nearly central	nearly central; distance between the female gonopore and the sucker similar to the distance between the female gonopore and the male gonopore
Distribution	Shirahama, Amakusa, Japan	Parry Island, USA	California, USA	Bonaire, Netherlands; Florida, USA	Huidong, China	Naples, Italy	Noto, Misaki, Shimoda, Shirahama, Amakusa, Amami Oushima, Japan	Nozaki, Noto, Japan	Tomoe-zaki, Amakusa, Japan
Reference	Yeri and Kaburaki 1918	Hyman 1959	Hyman 1953	Marcus and Marcus 1968	this study	Lang (1884)	Stimpson (1857); Yeri and Kaburaki (1918); Tsuyuki et al. 2021	Kato 1944	Kato 1938

NA: not available from reference.

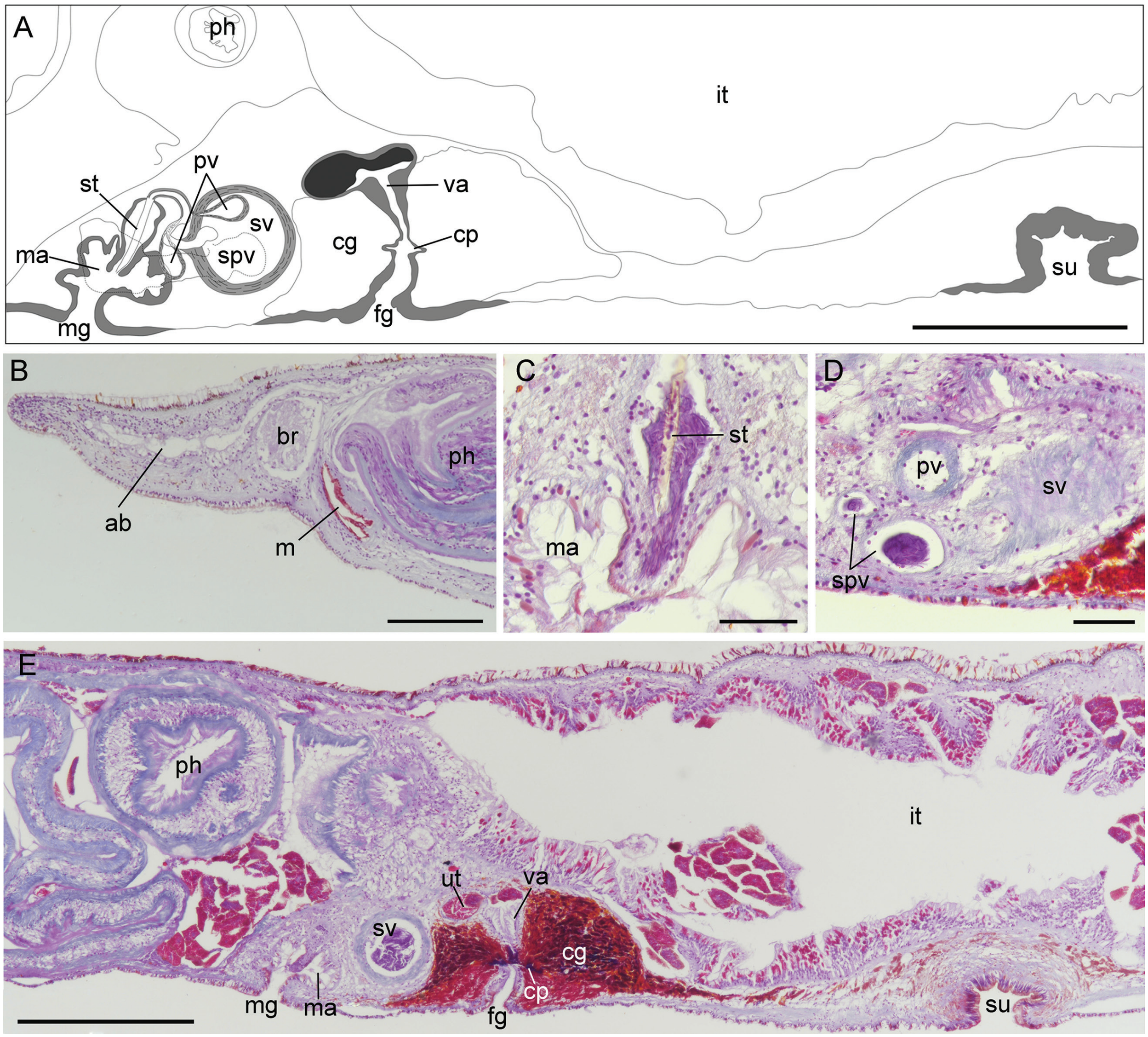

Figure 3. *Prosthiostomum huidongense* sp. nov., MBM287880 (holotype), schematic diagram (**A**) and photomicrographs of sagittal sections (**B–E**), anterior to the left. **A.** Copulatory complex and sucker; **B.** Anterior end of the body; **C.** Penis stylet; **D.** Male copulatory apparatus; **E.** Copulatory complex and sucker. Abbreviations: **ab** – anterior branch of main intestine; **br** – brain; **cg** – cement glands; **cp** – cement pouch; **fg** – female gonopore; **it** – intestine; **m** – mouths; **ma** – male atrium; **mg** – male gonopore; **ph** – pharynx; **pv** – prostatic vesicle; **spv** – spermiducal vesicle; **st** – stylet; **su** – sucker; **sv** – seminal vesicle; **va** – vagina; **ut** – uterus. Scale bars: 0.5 mm (**A, E**); 200 µm (**B**); 50 µm (**C, D**).

The new species has few eyes scattered between the marginal band and the cerebral eyes and thus can be easily distinguished from most congeners. Only five known species have been described with the above characteristics: *P. awaensa* Yeri & Kaburaki, 1918, *P. griseum* Hyman, 1959, *P. latocelis* Hyman, 1953, *P. milcum* Marcus & Marcus, 1968, and *P. notoensis* Kato, 1944. However, these species can be easily distinguished from the new species by the dorsal coloration or pigmentation pattern (except *P. milcum*) and the number of the marginal eyes (see Table 1). In addition, *P. awaensa*, *P. griseum*, and *P. latocelis* are also different from *P. huidongense* sp. nov. in the position of the band of marginal eyes (distributed anterior to the brain for these species; extending to the level of the posterior margin of the brain in *P. huidongense* sp. nov.). Although the dorsal coloration or pigmentation pattern of *P. milcum* is similar to that of the new species, it differs from *P. huidongense* sp. nov. by the deep male atrium and the distance between the female gonopore and sucker (Table 1). *Prosthiostomum sonorum* Kato, 1938, *P. dohrnii* Lang, 1884, *P. nozakensis* Kato, 1944, and *P. grande* also have yellow to brown mottles or spots on the dorsal surface, but they are different from *P. huidongense* sp. nov. in the arrangement of the eyes (Table 1). In general, identification of *Prosthiostomum* species is mainly achieved by features such as body size, dorsal color and pattern, eye arrangement and characteristics of the male and female reproductive system. Apart from that, the distance between the female gonopore and the sucker may be a noteworthy taxonomic feature within the genus, as in species *P. latocelis*, *P. milcum* and

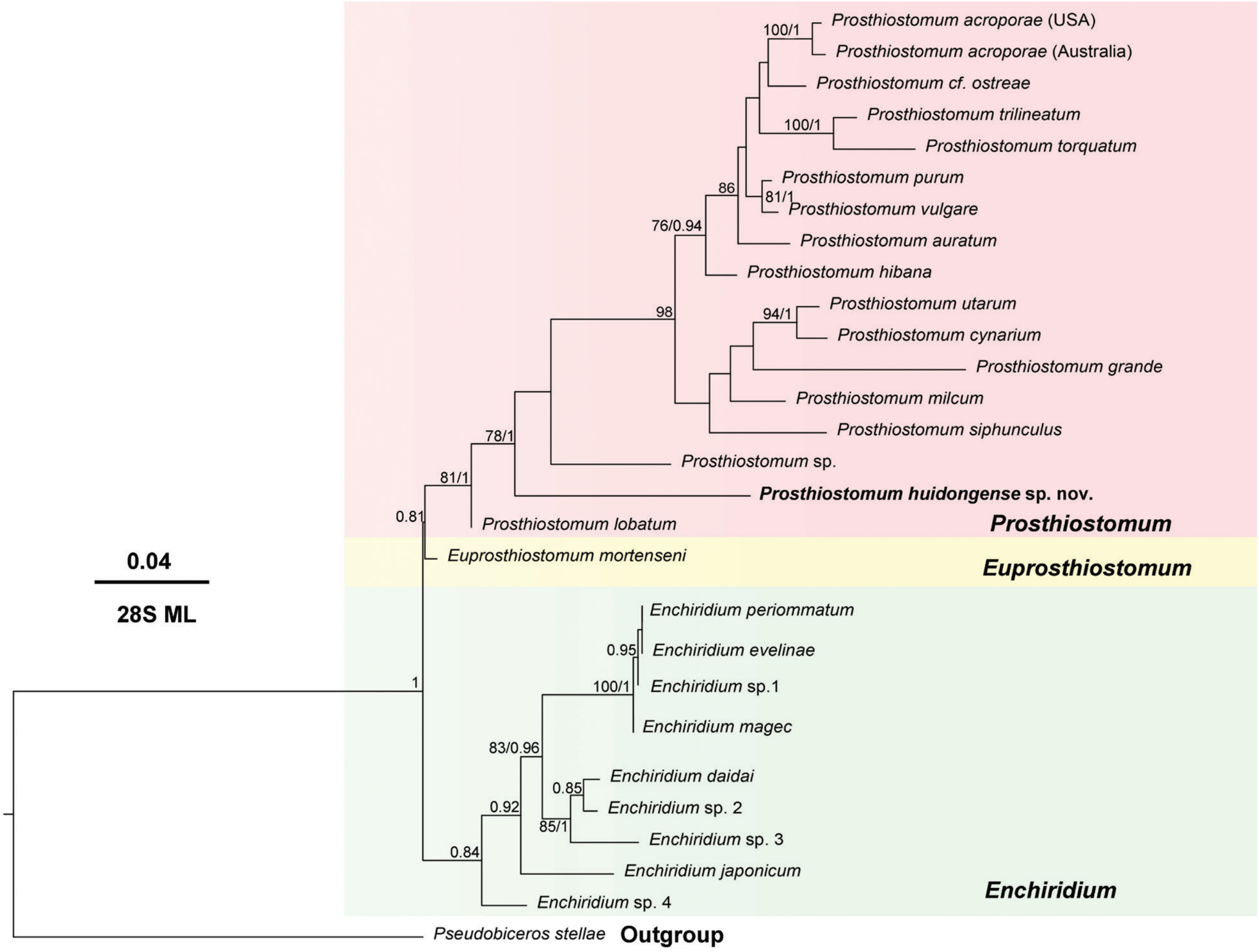

Figure 4. Maximum likelihood phylogenetic tree of species of the family Prosthiostomidae based on 28S rDNA. For clades that received either ≥70% ML bootstrap or ≥0.80 BI posterior probability, the support values are presented at the nodes.

P. sonorum this distance differs from that in the new species (see Table 1).

Our molecular phylogenetic analysis of Prosthiostomidae based on partial 28S rDNA is largely consistent with that of Tsuyuki et al. (2021), in which the genera *Prosthiostomum* and *Enchiridium* form well-supported taxa. Although some *Enchiridium* species (e.g., *E. evelinae*, *E. japonicum* and *E. punctatum*) have similar dorsal patterns to that of *P. huidongense* sp. nov., they can be easily distinguished from the new species by lacking a muscular sheath that completely encloses the prostatic vesicles. This feature distinguishing these two genera is further corroborated by the molecular phylogenetic tree. *Euprosthiostomum mortenseni* Marcus, 1948 is sister to the *Prosthiostomum* clade, with poor support (Fig. 4). According to Faubel (1984), absence of the frontal branch of the main intestine in *Euprosthiostomum* distinguishes this genus morphologically from *Prosthiostomum*, but whether that character can be reliably used to discriminate the genus has not been clarified in the present study due to the limited number of available sequences (only one sequence available for *Euprosthiostomum*).

Prosthiostomum huidongense sp. nov. is nested in the clade *Prosthiostomum*, thus corroborates our generic assignment based on the morphology. Uncorrected p-distance of COI among 11 species/molecular entities of *Prosthiostomum* are 5.7–24.3% (Suppl. material 1: table S2), while the average interspecific distance is 19% among these Prosthiostomum species that are morphologically distinguishable from each other. The uncorrected p-distance between *P. huidongense* sp. nov. and other tested species ranged from 20.3 to 24.3%, which is greater than the average interspecific distance among *Prosthiostomum* species, thus, provided further support for the notion that *P. huidongense* is a new species.

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Supplementary material 1

Supplementary information

Authors: Hai-Long Liu, Da-Hao Lin, An-Tai Wang, Zhang-Li Hu, Yu Zhang

Data type: docx

Explanation note: **table S1.** GenBank accession numbers of sequences for species taxa used in the phylogenetic analyses or genetic distance calculation; **table S2.** Interspecific uncorrected p-distances for the COI gene fragments; **figure S1.** The tree is reconstructed by Bayesian inference analyses based on the 28S rDNA.

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