Species Diversity **24**: 137–143
Published online 25 July 2019
DOI: 10.12782/specdiv.24.137

A New Species of *Prosthiostomum* (Platyhelminthes: Polycladida) from Shirahama, Japan

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(Received 31 October 2019; Accepted 17 May 2019)

http://zoobank.org/BA5704AF-2761-4E17-B9EF-65506E3E33B1

We describe a new species of polyclad flatworm, *Prosthiostomum torquatum* sp. nov., from the rocky intertidal zone in Shirahama, on the Pacific coast of middle Honshu, Japan. *Prosthiostomum torquatum* is characterised by a unique dorsal colouration, which is comprised of *i*) numerous orange maculae and blue dots dispersed all over the dorsal surface, *ii*) a transverse dark-brown line in the anterior part of the body running slightly anterior to another transverse white line, both of which are slightly bent backwards at mid-point, and *iii*) an incomplete, mesh-like, median band made by dark-brown pigments, fading away posteriorly. By this dorsal colour pattern, the new species can be distinguished from similar congeners such as *P. trilineatum* Yeri and Kaburaki, 1920 and *P. komaii* Kato, 1944. We performed a molecular phylogenetic analysis based on 462-bp partial mitochondrial cytochrome *c* oxidase subunit I (COI) sequences of four species of Prosthiostomidae currently available in public databases in addition to that of *P. torquatum*. In the resulting tree, *P. torquatum* was sister to *Lurymare clavocapitata* Marquina, Aguado, and Noreña, 2015 originally described from Lizard Island, Australia. While these two share a similar dorsal colouration, *P. torquatum* can be distinguished from *L. clavocapitata* by *i*) the absence of a common muscle bulb/sheath enclosing the whole male copulatory apparatus, *ii*) the median mesh-like band comprised of dark-brown pigments, and *iii*) COI uncorrected *p*-distance being 0.094. As a result, our phylogenetic tree indicates the possibility that *Prosthiostomum* and *Lurymare* as currently diagnosed may not be monophyletic, and that the common muscle bulb enclosing the whole male copulatory apparatus may not be appropriate as a diagnostic character for *Lurymare*.

Key Words: Cotylea, cox1, marine flatworm, phylogeny, taxonomy.

Introduction

The family Prosthiostomidae Lang, 1884 (Platyhelminthes: Polycladida) is characterised by *i*) an elongated body with a ventral sucker, *ii*) a plicate tubular pharynx, and *iii*) paired prostatic ducts, each from a spherical prostatic vesicle, entering the penis or the ejaculatory duct independently, instead of uniting to each other before the entrance. This family consists of six genera, *Prosthiostomum* Quatrefages, 1845, *Enchiridium* Bock, 1913, *Enterogonimus* Hallez, 1913, *Euprosthiostomum* Bock, 1925, *Amakusaplana* Kato, 1938, and *Lurymare* Du Bois-Reymond Marcus and Marcus, 1968. The genus *Prosthiostomum sensu* Faubel (1984) contains 51 species distributed worldwide. Members in this genus are supposed to be distinguished from other prosthiostomids by *i*) lacking a muscle sheath/bulb that encloses two prostatic vesicles, *ii*) possessing a main intestine that is accompanied with a frontal branch, and *iii*) having a penis that is armed with a pointed tubular stylet (Faubel 1984).

In Japan, 21 species of prosthiostomids have been reported, of which 17 belong to *Prosthiostomum* (Kato 1944; Faubel 1984). In the present study, we describe a new *Prosthiostomum* species from Shirahama, Wakayama, Japan, based on morphological and molecular data. In addition, we infer its phylogenetic position in Prosthiostomidae based on the partial mitochondrial cytochrome *c* oxidase subunit I (COI) gene sequences.

Materials and Methods

Six polyclad specimens were collected from under rocks at low tide in Tanabe Bay, Wakayama. One was collected by N. Jimi in 2015 and the other five were by Y. Oya in 2017. Worms were anaesthetised in seawater containing menthol before fixation. Except for the 2015 material, the relaxed worms were photographed by Y. Oya with a Nikon D5200 digital camera with external strobe lightning provided by a pair of Morris Hikaru Komachi Di flash units. For DNA extraction, a posterior piece of the body was removed and stored in 99.5% ethanol. The rest of the body was fixed in Bouin's solution for 24 h and preserved in 70% ethanol for long-term storage.

For histological examination, tissues were dehydrated in an ethanol series, cleared in xylene, embedded in paraffin wax, and sectioned sagittally at a thickness of 7 μm using a microtome. Sections were stained with either haematoxylin

and eosin (HE) or Mallory's trichrome method (MT), mounted on glass slides in Entellan New (Merck, Germany), and then observed and photographed under an Olympus BX51 compound microscope. All voucher and type slides have been deposited in the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo, Japan.

Total DNA was extracted using a silica-based method (Boom *et al.* 1990) after specimens were homogenised. A fragment of the COI (462 bp) was amplified with the primer pair Hoso_COI_F (5′-ATG GAC GTC CTT TGC GTG AT-3′) and Hoso_COI_R (5′-CAG GAT GTG TTC TAG GAG AGC C-3′). Primer3 online software (http://bioinfo.ut.ee/primer3/) was used to design these primers, based on the COI sequence of *Lurymare clavocapitata* Marquina, Aguado, and Noreña, 2015 (GenBank MF371153) (Aguado *et al.* 2017). Polymerase chain reaction (PCR) amplification conditions were 94°C for 5 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and 72°C for 7 min. All nucleotide sequences were determined by direct sequencing with a BigDye Terminator Kit ver. 3.1 and a 3730 Genetic Analyzer (Life Technologies, California, USA). Sequences were checked and edited by using MEGA ver. 7.0 (Kumar *et al.* 2016). The edited sequences have been deposited in DDBJ/EMBL/GenBank databases.

Additional COI sequences were downloaded from GenBank; *Pseudobiceros uniarborensis* Newman and Cannon, 1994 (Pseudocerotidae) and *Prostheceraeus vittatus* (Montagu, 1815) (Euryleptidae) were chosen as outgroups (Table 1). Sequences were aligned manually using AliView (Larsson 2014). That these sequences have not experienced substitution saturation was confirmed by DAMBE ver. 7.0.51 (Xia 2018). The optimal substitution model selected with MEGA ver. 7.0 (Kumar *et al.* 2016) under the Akaike Information Criterion (AIC) (Akaike 1974) was GTR+G. A phylogenetic analysis based on partial COI sequences (462 bp) was performed with maximum likelihood (ML) method by RAxML ver. 8.2.10 (Stamatakis 2014). Nodal support values were estimated based on 500 bootstrap pseudoreplicates. Genetic distances were calculated using MEGA ver. 7.0 (Kumar *et al.* 2016).

All graphical treatments were done with Adobe Photoshop CC. Illustrations were prepared with Adobe Illustrator CC.

Taxonomy

Prosthiostomum torquatum sp. nov.

(Figs 1–3)

Material examined. Holotype: ICHUM 5563, 13 slides (HE), collected by Y. Oya in Shirahama (33.6926°N, 135.3332°E), Wakayama, Japan, on 22 August 2017. Paratypes (4 specimens, all from the type locality): ICHUM 5562, sections through reproductive structures (7 slides) and anterior part (4 slides) (HE), collected by N. Jimi on 16 May 2015; ICHUM 5565, 9 slides (HE), collection data same as holotype; ICHUM 5566, 12 slides (HE), collection data same as holotype; ICHUM 5567, 13 slides (MT), collected by Y. Oya in Shirahama (33.6951°N, 135.3440°E), Wakayama, Japan, on 23 August 2017. Non-type specimen: ICHUM 5564, unsectioned, preserved in 70% ethanol, collection data same as holotype.

For comparison, we also examined digital photomicrographs of the male copulatory apparatus in the holotype (AM W. 44692, MI QLD 2395) and paratype (AM W.44065, MI QLD 2351) of *Lurymare clavocapitata* deposited at the Australian Museum, Sydney.

Type locality. Shirahama, Wakayama, Japan.

Description. Body elongated, anterior margin rounded, slightly tapered posteriorly, mid-point of posterior margin acute, 9–18 mm long (14 mm in holotype) and 2.5–5 mm maximum width (4 mm in holotype) in living state (Fig. 1A, B). Tentacles absent. Dorsal surface smooth, translucent, covered with numerous orange maculae and blue dots; orange maculae denser medially, blue dots uniformly scattered; each orange macula larger than single blue dot. Single transverse narrow dark-brown line running on dorsal surface of body in front of cerebral eyespots; its mid-point slightly arched backwards. Another transverse white line on dorsal surface, situated in short distance behind dark-brown line, likewise bending posteriorly at mid-point. Dark-brown pigments aggregating mid-dorsally to form incomplete, mesh-like, posteriorly-fading band, which runs from behind transverse white line (Fig. 1A). Body margin transparent. Ventral surface transluscent, without colour pattern. Intestine highly branched, spreading all over body. Pair of cerebral-eyespot clusters situated between transverse dark-

Table 1. List of species used for the molecular phylogenetic analysis and respective database accession numbers.

Species	Accession number	Reference
Prosthiostomidae		
Enchiridium sp.	MF371137	Aguado *et al.* (2017)
Lurymare sp.	MF371156	Aguado *et al.* (2017)
Lurymare clavocapitata Marquina, Aguado, and Noreña, 2015	MF371153	Aguado *et al.* (2017)
Prosthiostomum siphunculus (Delle Chiaje, 1822)	NC_028201	Aguado *et al.* (2016)
Prosthiostomum torquatum sp. nov.	LC429589–LC429592	this study
Outgroup		
Prostheceroeus vitattus (Montagu, 1815)	MF371140	Aguado *et al.* (2017)
Pseudobiceros uniarborensis Newman and Cannon, 1994	MF371146	Aguado *et al.* (2017)

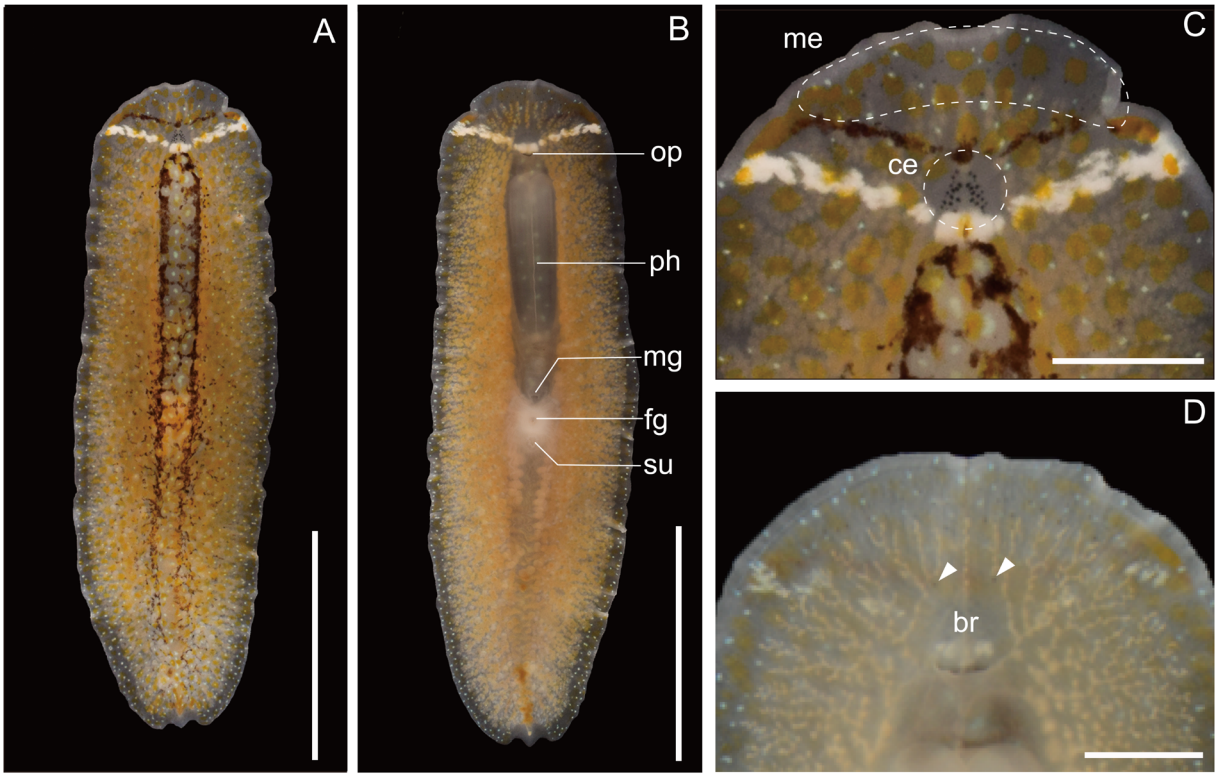

Fig. 1. *Prosthiostomum torquatum* sp. nov., photographs taken in anaesthetised living state. A–C, ICHUM 5563 (holotype); D, ICHUM 5562 (paratype). A, Entire animal, dorsal view; B, entire animal, ventral view; C, magnification of anterior body, dorsal view, showing position and arrangement of cerebral and marginal eyespots; D, magnification of anterior body, ventral view, showing position of ventral eyespots (indicated by arrowheads). Abbreviations: br, brain; ce, cerebral eyespots; fg, female gonopore; me, marginal eyespots; mg, male gonopore; op, oral pore; ph, pharynx; su, sucker. Scale bars: 5 mm (A, B), 1 mm (C, D).

brown and white lines near midline, each consisting of 7–13 eyespots (13 eyespots each in holotype); cerebral-eyespot clusters medially approaching each other at two or three points; two size categories among eyespots, larger one being more than twice the size of smaller one (Fig. 1C). Marginal eyespots sparsely anterior to transverse dark-brown line; marginal eyespots smaller than cerebral ones (Fig. 1C). Pair of ventral eyespots anterior to brain (Fig. 1D). Plicated pharynx tubular in shape, one third of body length, located in anterior half of body (Fig. 1B). Oral pore situated at anterior end of pharynx, behind brain. Male gonopore, female gonopore, and sucker closely set on body center (Fig. 1B). Male copulatory apparatus consisting of large seminal vesicle, pair of prostatic vesicles, and armed penis papilla, located immediately posterior to pharynx (Fig. 2A–D). Spermiducal vesicles forming single row on each side of midline, separately entering into seminal vescle. Ejaculatory duct wide, with thick muscular layer, entering penis papilla. Prostatic ducts narrow, with muscular layer, attached to ejaculatory duct at proximal end of penis papilla. Pair of prostatic vesicles and seminal vesicle closely set to each other (Fig. 2B). Muscular sheath/bulb enclosing three vesicles not found (Fig. 2B). Pair of spherical prostatic vesicles coated with thin nonnucleated muscular wall, located on both sides of ejaculatory duct. Seminal vesicle oval, coated with thick muscular wall. Penis papilla armed with pointed tubular stylet, enclosed in penis pouch, protruding into male atrium (Fig. 2C, D). Penis sheath present between penis pouch and male antrum (Fig. 2C, D). Male atrium elongated anteriorly, lined with cilliated and muscularised epithelium (Fig. 2A). Female reproductive system immediately posterior to male reproductive system. Vagina short, leading from uterus to cement pouch (Fig. 2A, D). Cement glands numerous, concentrated around vagina and releasing their contents in cement pouch (Fig. 2A). Oviduct running on each side of main intestine, extending anteriorly and posteriorly to female copulatory apparatus; anterior and posterior branches of oviduct converging before joining proximal end of vagina (Fig. 2A, D). Lang's vesicle absent.

Variation. Our specimens exhibited variation in the colour pattern on the dorsal surface. The body colour in general appearance ranged from orange to white depending on the gut contents. In addition, the density and distribution of the dark-brown pigments that form the mid-dorsal, mesh-like band were different among the specimens examined; the transverse narrow dark-brown line in front of cerebral eyespots was interrupted in some specimens; another transverse white line in short distance behind dark-brown line was not clear in the largest specimen (Fig. 3).

Diagnosis. Body elongated, usually rounded anteriorly; dorsal surface speckled with numerous orange maculae, blue dots, and dark-brown pigments, with dark-brown mesh-like band along median line; transverse dark-brown line running a short distance in front of similar, transverse

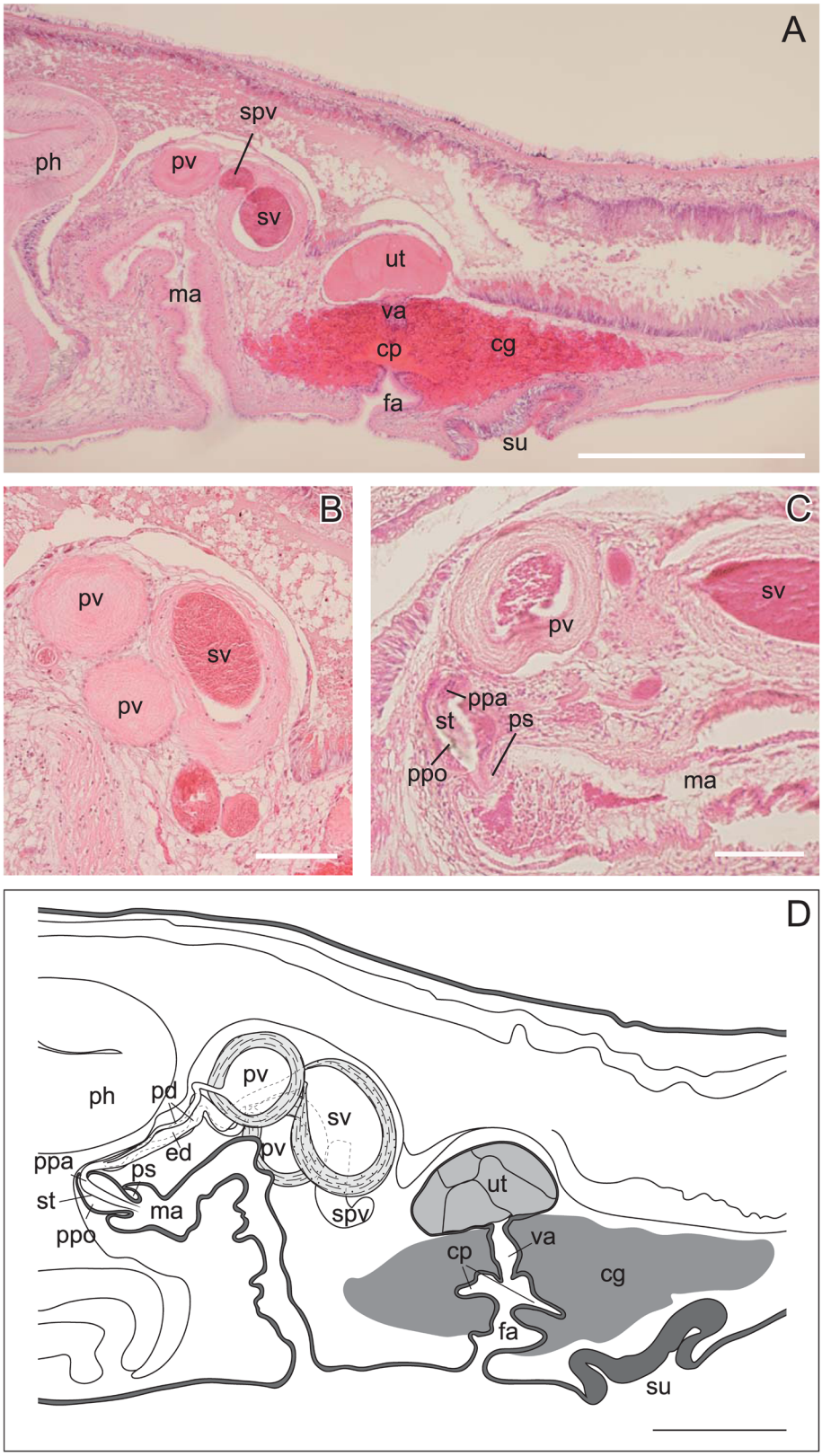

Fig. 2. *Prosthiostomum torquatum* sp. nov., sagittal sections, head to the left. A, B, ICHUM 5563 (holotype); C, ICHUM 5566 (paratype). A, Copulatory complex; B, seminal vesicle and paired prostatic vesicles; C, penis pouch; D, diagrammatic reconstruction of copulatory complex. Abbreviations: cg, cement glands; cp, cement pouch; ed, ejaculatory duct; fa, female atrium; ma, male atrium; pd, prostatic duct; ph, pharynx; ppa, penis papilla; ppo, penis pouch; ps, penis sheath; pv, prostatic vesicle; spv, spermiducal vesicle; st, stylet; su, sucker; sv, seminal vesicle; va, vagina; ut, uterus. Scale bars: 500 µm (A), 100 µm (B, C), 300 µm (D).

Fig. 3. Dorsal colour pattern variation in *Prosthiostomum torquatum* sp. nov. A, ICHUM 5563 (holotype); B, ICHUM 5565 (paratype); C, ICHUM 5566 (paratype); D, ICHUM 5564 (non-type); E, ICHUM 5562 (paratype). Scale bar: 5 mm.

white line on anterior part of body; a pair of free prostatic vesicles and a seminal vesicle located close together.

Etymology. The new specific name *torquatum* is a Latin adjective (*-us*, *-a*, *-um*), meaning "adorned with a neck chain or collar". It was named after the anterior transverse white line, bending backward at the mid-point.

Distribution. Only known from the type locality, Shirahama, Wakayama, Japan.

Habitat. Intertidal, under rocks.

Sequences. Partial COI sequences (462 bp) from four individuals: LC429590 from the holotype (ICHUM 5563), LC429589 and LC429591 from two paratypes (ICHUM 5562, ICHUM 5565), and LC429592 from a non-type specimen (ICHUM 5567). These four sequences were completely identical to each other.

Phylogeny and genetic distance. In the phylogenetic tree, *Prosthiostomum torquatum* sp. nov. appeared as sister to *Lurymare clavocapitata*; these two species differed by 0.094 uncorrected *p*-distance in terms of the 462-bp COI sequence. *Prosthiostomum torquatum* and *P. siphunculus* (Delle Chiaje, 1822), the type species of *Prosthiostomum*, did not form a monophyletic group. *Enchiridium* sp. was sister to the rest of prosthiostomids (Fig. 4).

Remarks. Our specimens belong to *Prosthiostomum* because they conform to Faubel's (1984) generic diagnosis by having *i*) a pair of prostatic vesicles that are separated from each other, *ii*) a median frontal branch from the main intestine, and *iii*) a penis armed with a pointed tubular stylet. This definition applies to the type species, *Planaria siphunculus* Delle Chiaje, 1822, and thus there is no serious taxonomic issue here. However, probably due to either misconception or oversight, Faubel (1984, pp. 232, 233) also included the three nominal species *Prosthiostomum purum* Kato, 1937, *Prosthiostomum cynarium* Marcus, 1950, and *Prosthiostomum utarum* Marcus, 1952, which actu-

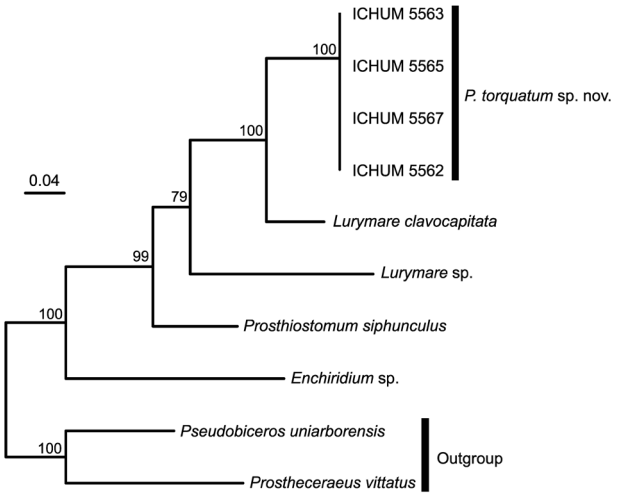

Fig. 4. Maximum likelihood phylogenetic tree based on partial mitochondrial COI gene. Bootstrap support values are indicated above nodes.

ally have a pair of prostatic vesicles enclosed together by a muscle bulb, and thus do not strictly fit the generic diagnosis. While the latter two have been transferred to *Lurymare* (Bahia *et al.* 2014; Bahia and Schrödl 2018), *P. purum* is still left in the genus, awaiting further taxonomic scrutiny. As a result, there are 49 species currently included in *Prosthiostomum*. Of these, *P. torquatum* sp. nov. is similar to *P. trilineatum* Yeri and Kaburaki, 1920 and *P. komaii* Kato, 1944 in the dorsal colour pattern, which includes several transverse lines in the anterior part of the body and longitudinal stripes along the median line (Yeri and Kaburaki 1920; Kato 1944). However, *P. torquatum* can be easily distinguished from both species by the orange maculae and blue dots scattered all over the dorsal surface. In actuality, we could have placed our new species in *Lurymare* due to its phylogenetic closeness to *Lurymare clavocapitata*, but we did not do so because our material lacked a common muscle bulb, a diagnostic character for *Lurymare* (Faubel 1984) (see below).

Prosthiostomum torquatum and *L. clavocapitata* share a unique dorsal colour pattern among prosthiostomids: *i*) a translucent white body covered with numerous orange maculae and blue dots, *ii*) a transverse dark-brown line in front of the cerebral eyespots, directing backwards at the mid-point, and *iii*) another transverse white line running slightly behind the dark-brown one, likewise pointing posteriorly at the mid-point. *Lurymare clavocapitata* was established based on two specimens, the holotype and a paratype; while the white line was present in the holotype, it was absent in the paratype. The similarity between the two species is very likely due to their genetic/phylogenetic closeness (Fig. 4).

Prosthiostomum torquatum differs from *L. clavocapitata* by the absence of the common muscle bulb, which encloses the whole male copulatory apparatus including the seminal vesicle, the two prostatic vesicles, the armed penis papilla, and the male atrium. We confirmed the difference by comparative examination on digital images of histological photomicrographs of the type specimens of *L. clavocapitata*. *Prosthiostomum torquatum* is also distinguishable from *L. clavocapitata* by their dorsal colour pattern; our new species has a mesh-like band along the mid-dorsal line formed by dark-brown pigments, although the density of the dark-brown pigments varies intraspecifically (Fig. 3); *L. clavocapitata* has two discontinuous longitudinal lines composed of dark-brown pigments, instead of a mesh-like band. The COI *p*-distance between the two species, 0.094, is greater than the value of 0.045, which was observed between sympatric *Notocomplana* species morphologically distinguishable from each other (Oya and Kajihara 2017), a value that is currently only available among the Polycladida, although these values may not be directly comparable as they belong to different suborders. While barcoding gaps in Prosthiostomidae are yet to be examined, we consider that *P. torquatum* and *L. clavacapitata* are likely to be two genetically independent entities.

The taxonomy of *Lurymare* in relation to *Prosthiostomum* requires revision because our phylogenetic tree based on COI indicates that these two genera as currently diagnosed are not monophyletic. The genus *Lurymare* was established by Du Bois-Reymond Marcus and Marcus (1968) for seven species, *P. drygalskii* Bock, 1931, *P. purum* Kato, 1937, *P. delicatum* Palombi, 1939, *P. russoi* Palombi, 1939, *P. gabriellae* Marcus, 1949, *P. matarazzoi* Marcus, 1950, and *P. utarum* Marcus, 1952, on the basis of the presence of prostatic vesicles bound in a common muscle sheath which may include seminal vesicle. Later, Faubel (1984) gave a more strict definition to *Lurymare* so that it would contain members having a common muscle bulb that encloses the entire male copulatory apparatus including both of a pair of prostatic vesicles and a seminal vesicle. As a result, all species in *Lurymare* except for the type species, *L. drygalskii* (Bock, 1931), were transferred to other genera, such as *Prosthiostomum, Enchiridium*, and *Euprosthiostomum*. At the same time, Faubel (1984) transferred another three species of *Prosthiostomum*, *viz*., *P. monosorum* (Schmarda, 1859), *P. singulare* Laidlaw, 1904, and *P. katoi* Poulter, 1975, into *Lurymare*. In contrast, Prudhoe (1985) supported Du Bois-Reymond Marcus and Marcus' (1968) taxon concept of *Lurymare*, retaining the original seven species in the genus, whereas Poulter (1975) considered that *Lurymare* should be a subgenus of *Prosthiostomum*. Bahia *et al.* (2014) redescribed *P. utarum* based on material collected in Praia das Conchas, about 250 km away from the type locality, Ilha de São Sebastião (Brazil), and placed the species back in *Lurymare*, because the Praia-das-Conchas specimen possessed a muscle sheath, which was actually illustrated—but not mentioned—in Marcus' (1952) original description, and was thus probably overlooked by Faubel (1984). Likewise, Bahia (2016) redescribed *Euprosthiostomum matarazzoi* as *Lurymare matarazzoi*. In addition, Bahia and Schrödl (2018) transferred *P. cynarium* Marcus, 1950 to *Lurymare* based on examination of the type material. A new species, *L. clavocapitata*, has been described by Marquina *et al.* (2015) from Lizard Island (Queensland, Australia) and placed in the genus based on Faubel's (1984) strict definition, which currently amounts to eight species. According to Prudhoe (1989), the validity of *Lurymare* appears to be uncertain because a muscle sheath enclosing prostatic vesicles could develop as maturity increases in several *Prosthiostomum* species. Therefore, there is a possibility that the muscular developmental state observed in *Lurymare* represents a later ontogenetic stage of *Prosthiostomum*, and thus these two genera are actually synonymous. The results of our molecular phylogenetic analysis support this view. However, molecular information of the type species *L. drygalskii* is currently not available; synonymization of *Lurymare* with *Prosthiostomum* is thus premature. A taxonomic revision of *Lurymare* should involve a phylogenetic analysis of prosthiostomids with more gene markers and a dense taxon sampling including the type species of the constituent six genera, as well as careful examination of intra- and interspecific variation of various morphological characters that have been used to diagnose *Lurymare*.

Acknowledgments

We are grateful to Dr. Naoto Jimi (National Institute of Polar Research, Japan) for offering a precious specimen. YO is thankful to the staff at the Seto Marine Biological Laboratory.

We thank Dr. Carolina Noreña (National Museum of Natural History, Spain), Mr. Jorge Rodriguez Monter (Australian Museum), and Dr. Stephen Keable (Australian Museum) for arrangement of the histological photomicrographs of *Lurymare clavocapitata*. We are also very grateful to Dr. Carolina Noreña, Mr. Daniel Marquina (Swedish Museum of Natural History), and Mr. Jorge Rodriguez Monter for their helpful comments about the generic placement of our new species. This study was partially supported by the Research Institute of Marine Invertebrates (No. 2017 IKU-3) for YO.

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