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***Benthodytes palauta*, a new species of deep-sea holothuroid (Elasipodida: Psychropotidae) from the western Pacific Ocean**

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Abstract

A new species of Psychropotidae holothuroid, *Benthodytes palauta* sp. nov., was collected from the Kyushu-Palau Ridge at a depth of 2 666 m. This new species is characterized by a leathery body wall, red-violet skin, five pairs of dorsal papillae, nineteen pairs of tube feet, and a narrow brim. The internal organs include one Polian vesicle, two tufts of gonads, and no respiratory trees. Ventral ossicles are large and spinous, with crosses of four arms with central bipartite apophyses. Papillae ossicles are crosses with four arms with bipartite apophyses. The dorsal ossicles were few and large, and cross-shaped with four arms and central bipartite apophyses. Tentacle ossicles were large and rod-shaped or slender rods. Gonad ossicles were primary crosses of four arms and brim ossicles were cross-shaped with spines. The phylogenetic analyses of this species support that *B. palauta* sp. nov. belongs to *Benthodytes*. Furthermore, the paraphyletic relationships were confirmed; however, a revision of the genus *Benthodytes* is needed to resolve its phylogenetic relationship.

Key words: 16S, COI, Kyushu-Palau Ridge, phylogenetic analyses, Psychropotes, Psycheotrephes

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1 Introduction

Holothurians of the family Psychropotidae that can swim in the water column were first identified by Théel (1882), who described the deep-sea sea cucumbers discovered on the *H.M.S. Challenger* Expedition and defined the genus of *Benthodytes*. *Benthodytes* is characterized by a brim that enables the sea cucumber to swim through undulating or flapping. The ossicles in *Benthodytes* are cross-shaped, or rod-shaped. The swimming activity of *Benthodytes gosarsi* was first reported in the northern mid-Atlantic ridge (Rogacheva et al., 2012).

The genus *Benthodytes* is widely distributed, and the least studied deep-sea holothurians. *Benthodytes* was established by Théel (1882) with the characteristics of dorsal retractile or non-retractile inconsiderable processes. Hansen (1975) revised this genus and described eight species, two of which were identified as new species. In the last 12 years, five new species of *Benthodytes* have been described (Gebruk, 2008; Rogacheva et al., 2009; Martinez et al., 2014; Xiao et al., 2018; Li et al., 2018). According to WoRMS (WoRMS, 2021), the genus includes 14 valid species*:* *B. abyssicola* Théel, 1882; *B. gosarsi* Gebruk, 2008; *B. incerta* Ludwig, 1894; *B. lingua* Perrier, 1896; *B. manusensis* Xiao et al., 2018; and *B. marianensis* Li et al., 2018; *B. plana* Hansen, 1975; *B. sanguinolenta* Théel, 1882; *B. sibogae* Sluiter, 1901a; *B.superba* Koehler & Vaney, 1905; *B. typica* Théel, 1882; *B. valdiviae* Hansen, 1975; *B. violeta* Martinez, SolísMarín & Penchaszadeh, 2014; and *B. wolffi* Rogacheva & Cross in Rogacheva et al., 2009.

During cruise DY59 of the China Ocean Mineral Resources R & D Association (COMRA), one specimen of the genus *Benthodytes* was collected from a seamount on the Kyushu-Palau Ridge. Based on the analysis of the external morphological characters and ossicles, it was identified as a new species of the genus *Benthodytes*. In addition, we analyzed the phylogenetic relationships of family Psychropotidae, which were the least studied.

2 Materials and methods

2.1 *Sampling and morphological observations*

The specimen (catalog number: RSIO591006) used in this study was collected by a remotely operated vehicle, *Hailong-IV*, from the Kyushu-Palau Ridge (13°23.98′N, 134°34.74′E) at a depth of 2 666 m. The specimen was fixed in 10% seawater formalin, and a piece of tissue was cut and frozen under –20°C for DNA extraction. Upon arrival at the laboratory, the intact specimen, fixed in seawater formalin, was dissected, and pictures of the worm body were taken using a Canon EOS 5DII camera (Canon Inc., Tokyo, Japan). Small pieces of the body tissues of tentacles, dorsum, ventrum, brim and gonad were dissolved in 60% sodium hypochlorite and the ossicles were examined using a scanning electron microscope (TM 1000; Hitachi, Ltd., Tokyo, Japan).

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2.2 *PCR amplification and phylogenetic analyses*

Total genomic DNA was extracted from 100 mg muscle tissue using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The partial sequences of 16S rRNA and COI genes were amplified with primers that were used in Miller et al. (2017). The PCR amplification reactions were carried out in a 50 μL volume system, containing 25 μL 2× Phanta Max Master Mix (Vazyme, Biotech CO., Ltd., NanJing, China), 20 μL DNase free ddH_2O, 2 μL of each primer, and 1-μL template DNA, as suggested by the manufacturer. The PCR amplification procedure of 16S rRNA was as follows: an initial denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 40 s, 50°C for 40 s and 68°C for 50 s, and a final extension at 68°C for 5 min. The PCR amplification procedure of COI was as follows: an initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 40 s, 45°C for 40 s, and 72°C for 50 s, and a final extension at 72°C for 5 min. The seizes of the PCR products were checked through 1.5% agarose gel electrophoresis, and purified using OMEGA PCR kit (Omega, Biotek, Norcross). The purifed PCR products were sequenced on an ABI 3730XL sequencer (Sangon, Biotec CO., Ltd., Shanghai). Sequence data were edited with Geneious R6.1.6 (Kearse et al., 2012) and deposited in GenBank (Table 1).

For phylogenetic analyses, thirteen COI gene sequences and twelve 16S rRNA gene sequences were downloaded from GenBank, and two species of Elpidiidae were chosen as the outgroup (Table 1). COI and 16S sequences were aligned using MAFFT 7 (Katoh and Standley, 2013) with the G-INS-I strategy for COI and the E-INS-I strategy for 16S rRNA. Later, COI and 16S were concatenated into the dataset (COI/16S=678/571 bp). Maximum likelihood (ML) and Bayesian inference (BI) analyses were performed for the dataset. To obtain the best-fit tree, JModelTest 2.1.10 (Darriba et al., 2012) was performed to find the best-fit model from 88 competing models using Akaike Information Criterion (AIC) calculations. The best-fit model was GTR+I+G. BI analysis was conducted with Mrbayes 3.2 (Huelsenbeck and Ronquist, 2001). Markov Chains Monte Carlo (MCMC) iterations were run for 1 000 000 generations, with topologies being sampled every 100 generations. The first 25% of trees were discarded as burn-in and the remaining trees were summarized in 75% majority rule consensus tree. ML analysis was performed with RAxML GUI 1.5 (Silvestro and Michalak, 2012; Stamatakis, 2014) using the GTR+GAMMA+I substitution model for 1 000 bootstraps, as recommend by Miller et al. (2017).

3 Results and discussion

3.1 *Morphological observations*

Order Elasipodida Théel, 1882

Suborder Psychropotina Hansen, 1975

Family Psychropotidae Théel, 1882

Genus *Benthodytes* Théel, 1882

Diagnosis (according to Hansen, 1975)

Anus dorsal. Unpaired dorsal appendages absent. Circum-oral (or post-oral) papillae present. Tentacles soft, pliable, and retractile.

Benthodytes palauta sp. nov.

(Figs 1– 4)

Diagnosis

Body elongated and subcylindrical when fixed. Skin red with light violet, thick, soft, and leathery. Tentacles ten, retracted after fixing. Mouth ventral, anus dorsal. Five pairs of dorsal papillae, conical, with filiform tips, arranged in two single rows. Brim narrow, thin, flattened, with visible pigment canals. Gonads branched, with two tufts. Ossicles, with large, spinous crosses of four arms with central bipartite apophyses scattered on the ventrum and papillae. Brim ossicles cross-shaped with spines and rods present in tentacles. Dorsal ossicles very few, cross-shaped with four arms and central bipartite apophyses, and rod-shaped.

Description of holotype. The central body of the specimen was inflated after collection and was approximately 28 cm in length before being preserved in 10% seawater formalin (Figs 2a and b). When the specimen arrived at the laboratory, the dorsum was damaged (Fig. 2c) and approximately 22 cm in length (Fig. 2d). The skin was red with light violet on the deck and decolorized into white violet after fixing.

The mouth was ventral, close to the anterior edge of the body, with circum-oral papillae, and anus was at the dorsal terminal (Fig. 1a). Ten tentacles were retracted into the stalks. Five pairs of papillae were arranged in two rows: the first pair was located anterior to the dorsum; the second pair was distributed one-fourth of the distance along the dorsum but on the outer edge; the third and fourth pairs increased in size toward inner dorsum; the last pair was close to the terminal of the dorsum; and some minute papillae were scattered on the dorsal surface. Nineteen pairs of tube feet were arranged in two rows along the mid-ventrum and retracted to the body when fixed (Fig. 1b).

The Polian vesicle was drop-shaped, and 30 mm in length. No

Table 1. The Gene sequences of the specimens used in this study

Family	Species	GenBank accession number	
		16S	COI
Psychropotidae	*Benthodytes manusensis*	MH627223.1	MH627222.1
	Benthodytes sanguinolenta		HM196507.1
	Benthodytes marianensis	MH049433.1	MH049435.1
	Benthodytes sp. SB-1-11	MH627223.1	KU519513.1
	Benthodytes cf. *sanguinolenta*	MN313657.1	KU519546.1
	***Benthodytes palauta* sp. nov.**	MW465752	MW458948
	Psycheotrephes exigua		KX874392.1
	Psychropotes longicauda	KU987549.1	KU987478.1
	Psychropotes moskalevi	MN310400	MN313650
	Psychropotes raripes	MN310403.1	MN313656.1
	Psychropotes verrucicaudatus	MH077589.1	MH077588.1
	Psychropotes cf. *semperiana*	KU519526.1	
Elpidiidae	*Peniagone diaphana*	KX856725.1	KX874384.1
	Peniagone sp. AKM-2016	KX856726.1	KX874385.1

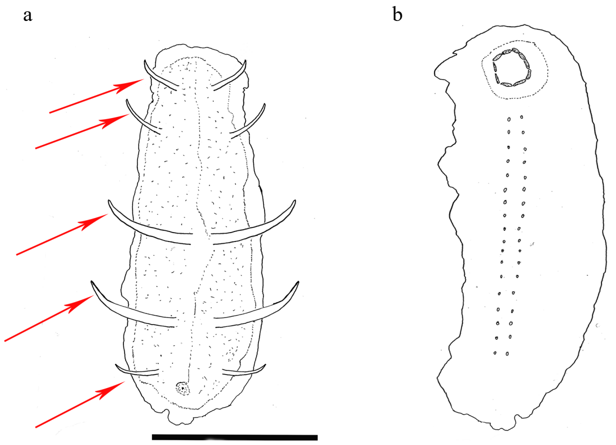

Fig. 1. *Benthodytes palauta* sp. nov. a. Dorsal view, red arrows pointed to dorsal papillae; b. ventral view. Scale bar: 10 cm.

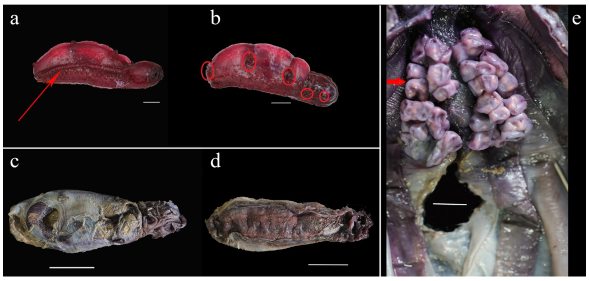

Fig. 2. Photograph of *Benthodytes palauta* sp. nov. a–b. The speciemen photographed on the deck before fixation, showing retracted brims (red arrow) and five pairs of dorsal papillae (red circles); c–d. the specimen photographed in the lab after preservation in 10% seawater formalin, dorsal view (c) and ventral view (d); e. Photograph of internal organs of the specimen, red arrow indicating to the oocytes. Scale bars were 5 cm (a–d) and 1 cm (e).

respiratory trees were observed. Gonads were branched with two tufts (Fig. 2e), and each tuft included 12 oocytes. The maximum size of oocytes was nearly 1 cm and each contained 6–8 yellow globules.

Ossicles in the ventral body wall were approximately 300–350 μm in length, with four arms crosses-shaped of central bipartite apophyses. The large arms were approximately 150 μm in length, and the small one was approximately 80 μm (Fig. 3a). Papillae ossicles were similar to the ventral type, but larger. The papillae ossicles were approximately 350–400 μm in length, and the arms were approximately 200 μm. Furthermore, the central bipartite apophyses of the papillae ossicles were fully developed, with clear spines (Fig. 3b). The ossicles in the dorsal body wall were very few and large. They were approximately 1 100 μm in length with four arms in a cross with central bipartite apophyses, or rod-shaped and approximately 650 μm in length. The large arms were approximately 600 μm, and the arms of the apophyses were approximately 200 μm. The opening angles of the arms of central bipartite apophyses of the dorsal ossicles were smaller than those of the other ossicles (Fig. 3c).

Ossicles in the tentacles were rod-shaped, but with variations: (1) large rods, approximately 300 μm in length; (2) smaller, approximately 200 μm in length, with an apophysis in the middle area; (3) slender, but the longest with a length of 600 μm (Fig. 4a). Gonad ossicles had three or four axes. The arms and the apophyses were approximately 400 μm (Fig. 4b). Brim ossicles were primarily cross-shaped with spines, with a width of approximately 350 μm (Fig. 4c).

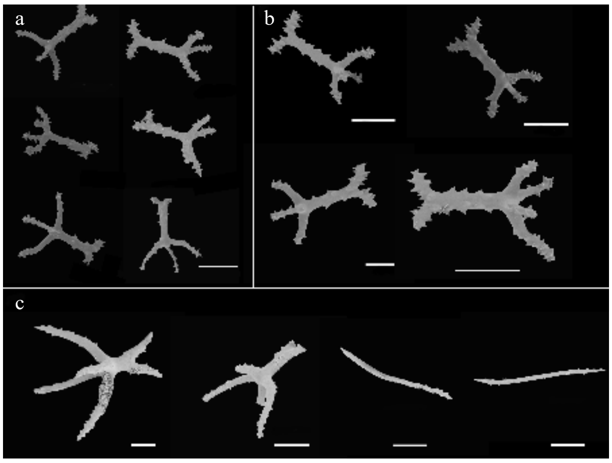

Fig. 3. Scanning electron microscope images of ossicles from ventral body wall (a), papillae (b), and dorsal body wall (c). Scale bars: 200 μm.

Material examined. Holotype: 1 adult specimen, catalog number RSIO591006.

Type locality. Kyushu-Palau Ridge, tropical western Pacific (13°23.98′N, 134°34.74′E). Depth 2 666 m, muddy sediments, August 5, 2020, Ruiyan Zhang, Bo Lu.

Distribution. Known only from the type locality.

Etymology. The specific epithet palauta refers to the type locality Kyushu-Palau Ridge.

Remarks. According to its external morphology, *B. palauta* sp. nov. is similar to *B. sibogae* Sluiter, 1901a. Both species share five pairs of dorsal papillae, an inflated dorsum, and a narrow brim. In addition, ossicles from the ventrum and papillae are similar. *B. sibogae* was described by Sluiter in 1901 based on specimens from the Siboga-Expedition. Hansen (1975) re-examined *B. sibogae*, and proposed *B. hystrix* as a junior synonym of *B. sibogae*. Hansen (1975) believed that the original description of the ossicles was not typical. In his report, dorsal ossicles were spinous crosses with bipartite apophyses; ventral ossicles were

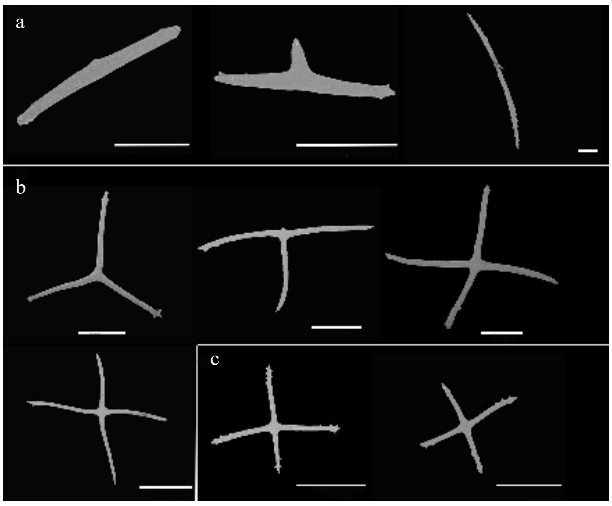

Fig. 4. Scanning electron microscope images of ossicles from tentacles (a), gonads (b), and brims (c). Scale bars: 200 μm.

rod-shaped, tripartite, or cross-shaped, without apophyses, or rudimentary and undivided; and gonad ossicles were cross-shaped with four arms. Xiao et al. (2020) reported a new record of *B. sibogae* from the South China Sea, and described papillae ossicles which was not mentioned before. The papillae ossicles were cross-shaped with spinous arms and, central bipartite apophyses.

Three morphological distinguishing characteristics were detected between *B. palauta* sp. nov. and *B. sibogae*: (1) the ventral ossicles of *B. palauta* sp. nov. only presented as four arms cross with central bipartite apophyses (Fig. 3a), while *B. sibogae* have rod-shape, tripartite; (2) the arm and apophyses of the papillae cross ossicles were similar to *B. sibogae* (Fig. 3b), but larger; (3) both cross and rod ossicles are found in the dorsum of the new species, but rod ossicle was absent and the cross ossicles were smaller in *B. sibogae* (Fig. 3c).

In addition, the body wall of *B. palauta* sp. nov. differed from that of *B. sibogae*, and was red-violet before preservation. These evidences in differences in external morphology and ossicles support *B. palauta* sp. nov. as a valid new species.

3.2 *Phylogenetic analyses*

Both ML and BI trees were consistent, and showed that species of the genus *Benthodytes* were divided into two clades. The new species was closely related with *B. manusensis*, *Benthodytes* sp. SB-1-11 and *B. marianensis*, while *B. sanguinolenta* and *B.* cf. *sanguinolenta* were clustered together and placed at the root of Psychropotidae. Our results suggested that *Benthodytes* was a paraphyletic group (Li et al., 2018) (Fig. 5).

In addition, *Psychropotes* showed paraphyletic relationships with *Psycheotrephes*. The clade of *Psychropotes moskalevi* and *Psychropotes raripes* were clustered together, and *Psychropotes longicauda* was their sister group. *Psychropotes verrucicaudatus* and *Psychropotes* cf. *semperiana* were clustered into a group, but the probability of the clade was very low. *Psycheotrephes exigua* was located in the lateral clade of the sister group.

In general, the relationships of Psychropotid can be presented as follows: (((*Psychropotes* + *Psycheotrephes*) + *Benthodytes*) + (*Benthodytes sanguinolenta* + *Benthodytes* cf. *sanguinolenta*)). However, since only 10 sequences were available among the 37 existing species, more molecular data combined with morphological data are required to explore the phylogenetic relationships of Psychropotid.

4 Discussion

Based on the morphology and phylogenetic analyses, *B. palauta* sp. nov. clearly belongs to *Benthodytes*. However, a revision of the genus *Benthodytes* is required based on the divergences of the morphology and phylogenetic analyses.

According to the review by Hansen (1975), the genus *Benthodytes* was well defined, particularly on its circum-oral papillae, but the species of *Benthodytes* were difficult to evaluate taxonomically. Hansen (1975) proposed that *Benthodytes*, except *Benthodytes superba*, can be divided into two distinct groups. The first group included five species (*B. incerta*, *B. lingua*, *B. valdiviae*, *B. sibogae* and *B. plana*) according to the regular crosses ossicles with large bipartite central apophysis and 15 tentacles. The remaining species *B. typica* and *B. sanguinolenta* consisted of another group by the strongly reduced rod ossicles, a high tentacle number and the minute dorsal papillae. Recently, more five spe-

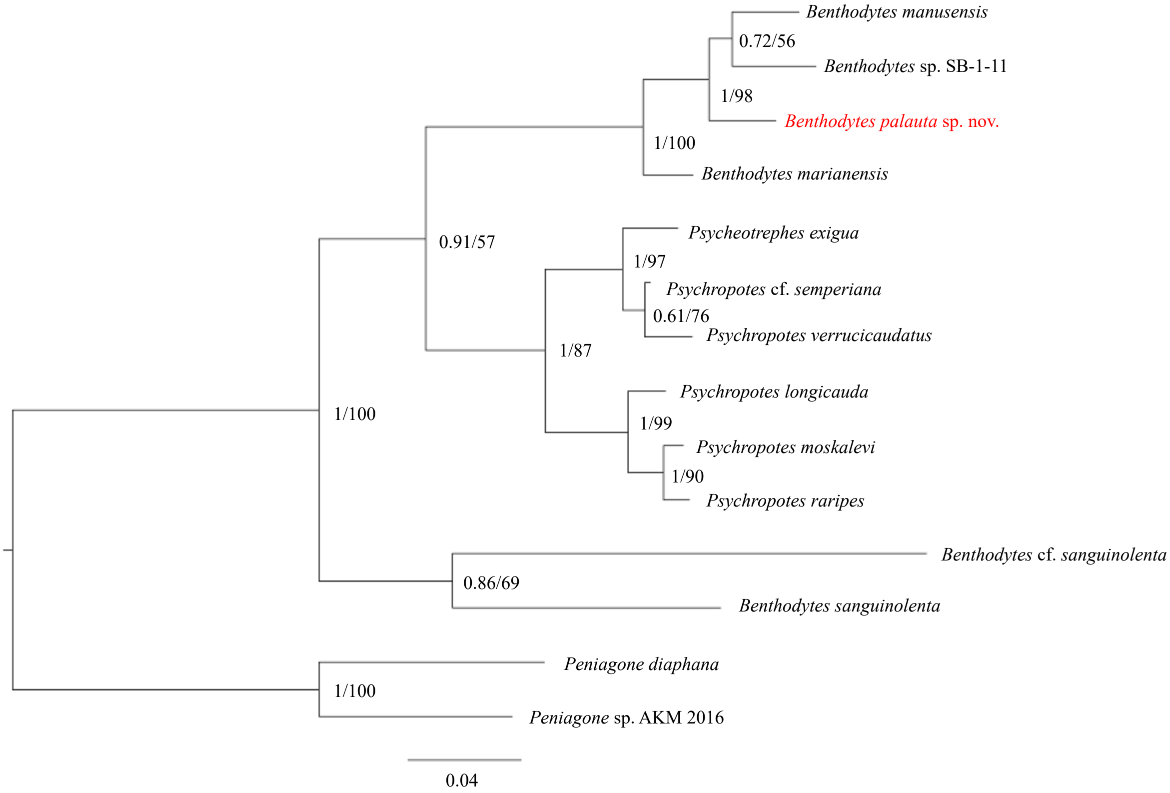

Fig. 5. Bayesian inference (BI) and maximum likelihood (ML) trees based on concatenated sequences. The Bayesian posterior probabilities (BI) and Maximum likelihood bootstrap (BS) values are shown as BI/ML at each node. Scale bar presented the evolutionary branch length.

cies were identified: *B. gosarsi*, *B. violeta*, *B. wolffi*, *B. manusensis*, and *B. marianensis*. Together with *Benthodytes palauta* sp. nov., these species can be assigned into the first group by the well-developed papillae and crosses ossicles.

There were few works on the molecular phylogeny of the Psychropotid (Miller et al., 2017; Li et al., 2018). Phylogenetic analyses in this study supported that *Benthodytes* is a paraphyletic group. The clade of *B. sanguinolenta* and *Benthodytes* cf. *sanguinolenta* was placed at the lateral clade of other Psychropotid species. This result was consistent with the views of Hansen (1975) and Li et al. (2018).

Nevertheless, the gene sequences are limited in the genus *Benthodytes* or the family Psychropotidae, and only few studies on molecular phylogeny have been conducted. Thus, the relationships within *Benthodytes* remain unresolved and await more works combine morphology and molecular data in depth work.

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