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First observation of the "double-faced X-framed cup ossicle" extracted from a deep sea holothurian in Japan

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Abstract

A New type ossicle form, “double-faced x-framed cup ossicle” is discovered from the undescribed deep sea holothuroid. To give a definite view on the substantial qualities of this ossicle, a SEM observation on the ossicles and a DNA barcoding analysis are conducted. Although the most internal and external morphologies of the present species agree well with the characteristics of the family Cucumariidae Ludwig, 1894, the ossicles morphologies mostly does not agree with the congeners of Cucumariidae. On the other hand, our molecular study indicates a possibility that the specimens are not cucumariids, but belong to a sister group of Cucumariidae. In our present observation, some of the peri-oral ossicles show a very similar property with the small x-framed cup-shaped structures (which sometimes occurs in cucumariids). Therefore, the double-faced x-framed cup ossicle probably could be considered as the results of derivation from the x-body: in which four extra-arms developed on the bottom face of a cup ossicle, and finally these arms equipped with an extra-rim.

Key words: Cucumariidae, Dendrochirotida, DNA barcoding, molecular phylogeny

Introduction

In the studies on holothurians, highly variegated forms of ossicles have been reported. Although many species show specific ossicle structures, the various shapes of ossicles are considered as the results of derivation from the fundamental forms of the following basic types: plate; table; button; scale; rosette; cup; wheel; sigmoid hook; anchor; granule; needle; and rod. However recently, one taxonomically undescribed species was discovered from the deep water of Japan, which possesses a peculiar ossicle shape different from all the fundamental forms previously reported. Here, we report the first observation of a double-faced x-framed cup ossicle from an undescribed deep sea holothuroid, collected from the Satsuma Peninsula in Kagoshima, Japan. SEM observation on the ossicles was carried out to give a definite view on the substantial qualities of this ossicle. We also conducted a DNA barcoding analysis using the mitochondrial gene COI and the nuclear Histone H3 gene to examine the phylogenetic position of this species, in order to provide a systematics background for the explanation of this ossicle.

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Materials and Methods

Eleven specimens were collected from off the Satsuma Peninsula (Kagoshima) at 200 m depth by R/V Kaiyo-maru using ROV (Kowa Co.Ltd). The specimens were then fixed and preserved in 80% ethanol. All specimens and the permanent slides of the ossicles are deposited in the Invertebrate Collection (INV) of the Wakayama Prefectural Museum of Natural History (WMNH), in Kainan, Wakayama, Japan.

For SEM study, the ossicles were extracted from several parts of a single specimen. First, tissue samples were dissolved in sodium hypochlorite (NaClO, 5%). Afterward, the samples were rinsed in deionized water, and then dehydrated in 99% ethanol. Dehydrated samples were then mounted on aluminium stubs using conductive tapes (Nisshin NEM Tape), dried at room temperature, and finally observed using a scanning electron microscope (Nippondenshi JEOL JSM-6480LV). SEM materials were also deposited in WMNH.

We also observed the outer body-wall ossicles of *Hemiocnus tegulatus* (Augustin, 1908, also see Yamana & Kohtsuka, 2018) (specimen registration: WMNH-INV-2015-307), as an example of the normal single-faced x-framed cups (x-bodies) of the congeners of Cucumariidae.

For DNA analysis, a piece of the muscle tissue (ca. 25 mg) were extracted from three of the vouchered, EtOH-fixed samples (WMNH-INV-2018-7, 8, and 9). Fragments of the mitochondrial COI gene and the nuclear Histone H3 gene were amplified and then sequenced. PCR reactions follow standard protocols, and sequencing was outsourced. PCR primers used for COI were COIceF and COIceR (Hoareau and Boissin 2010); and for Histone H3 were H3aF and H3aR (Colgan *et al*. 1998) we were successful in obtaining COI and Histone H3 sequences of all three individuals. Obtained sequences were visualized in MESQUITE ver. 3.5 (Maddison and Maddison 2018) for editing prior and post-alignment. Sequence alignment were conducted using the online version of MAFFT ver. 7 (Katoh *et al*. 2017). After alignment and editing, the two gene sequences were concatenated and used in subsequent phylogenetic analyses. Sequences for selected taxa from Miller *et al*. (2017) were also data-mined and included in the analyses (Table 1). Maximum Likelihood phylogenetic analyses were conducted using the program RAxML-GUI ver 1.5 beta (Silvestro and Michalak 2012) under the GTR+GAMMA model, and Neighbor Joining analyses were conducted using MEGA 7 (Kumar *et al*. 2016) under the Maximum Composite Likelihood substitution model, GTR nucleotide substitution rate, and 30% cutoff for each site. Both analyses were done with 1000 bootstrap replications to assess the robustness of each node. To minimize the effect of homoplasy caused by oversaturation, we conducted phylogenetic analyses with the third codon of the mitochondrial COI gene excluded.

TABLE 1. List of selected OTU from Miller *et al*. (2017) acquired from Genbank.

Order	Species	COI	H3
	Abyssocucumis abyssorum	KX874335	KX874442
	Afrocucumis africana	KX874348	KX874451
	Aslia pygmaea	KX874339	KX874450
	Crucella scotiae 1	KX874366	KX874433
	Crucella scotiae 2	KX874367	KX874434
	Echinocucumis cf. hispida	KX874395	KX874437
	Echinocucumis hispida	KX874396	KX874438
Dendrochirotida	*Euthyonidiella huwi*	KX874371	KX874448
	Heterothyone alba	KX874390	KX874444
	Lissothuria nutriens	KX874341	KX874443
	Massinium magnum	KX874351	KX874467
	Pachythyone rubra	KX874387	KX874446
	Paracucumis turricata	KX874368	KX874439
	Pentactella leonina	KX874369	KX874440
	Pentactella sp.	KX874372	KX874441

...Continued on next page

TABLE 1. (Continued)

Order	Species	COI	H3
Dendrochirotida	*Phyrella mookiei*	KX874346	KX874477
	Placothuria squamata	KX874391	KX874445
	Psolidium dorsipes	KX874350	KX874435
	Psolidium whittakeri	KX874349	KX874432
	Sclerodactyla briareus	KX874342	KX874447
	Thyonella gemmata	KX874340	KX874449
	Ypsilothuria cf. bitentaculata	KX874370	KX874436
Synallactida	*Deima validum*	KX874364	KX874426
	Orphnurgus glaber	KX874361	KX874428
	Oneirophanta setigera	KX874363	KX874427
	Paelopatides sp. 2	KX874355	KX874419
	Stichopus chloronotus	KX874352	KX874424
	Synallactes sp.	KX874365	KX874420
	Thelenota anax	KX874375	KX87442
Molpadida	*Acaudina molpadioides*	KX874336	KX874455
	Heteromolpadia tridens	KX874362	KX874431
	Paracaudina chilensis	KX874343	KX874414
Persiculida	*Gephyrothuria alcocki*	KX874377	KX874406
	Paroriza prouhoi	KX874378	KX874405
	Pseudostichopus sp. 2	KX874389	KX874454
Holothuriida	*Actinopyga varians*	KX874345	KX874409
	Holothuria hilla	KX874337	KX874407
	Mesothuria oktaknemus	KX874394	KX874429
Elasipodida	*Amperima robusta*	KX874381	KX874457
	Enypniastes eximia	KX874383	KX874465
	Pannychia cf. moseleyi	KX874379	KX874464
	Protelpidia murrayi	KX874382	KX874456
Apodida	*Chiridota laevis*	KX874399	KX874473
	Chiridota rigida	KX874401	KX874469
	Euapta tahitiensis	KX874402	KX874475
	Paradota sp.	KX874400	KX874470
Outgroups	*Patiria miniata*	KX874393	KX874430
	Hemicentrotus pulcherrimus	JQ742947.1	LC275143.1

Results

Morphological observation

For familial assignments, body internal and external morphology were checked based on eleven specimens. The morphological characterization result is as follows.

Body medium, approximately 20–80 mm in length and 6–25 mm in width. Body color yellow in living (Fig. 1a), white in preservation (Fig. 1b). The body was cylindrical, the skin soft, with five oral valves present.

Polian vesicle and stone canal single, with ten dendritic tentacles arranged in a single circle. Calcareous ring was short, stout, with no posterior prolongations. The medioventral radial element and two inter-radial elements were not fused. Ten anal papillae and five anal teeth in radii were present. The tube feets were distributed in three ventral radii, retractile, and lacking in the dorsal side. The gonad was situated in the anterior side of body in two clumps, one on each side of the dorsal mesentery, with most tubules branched.

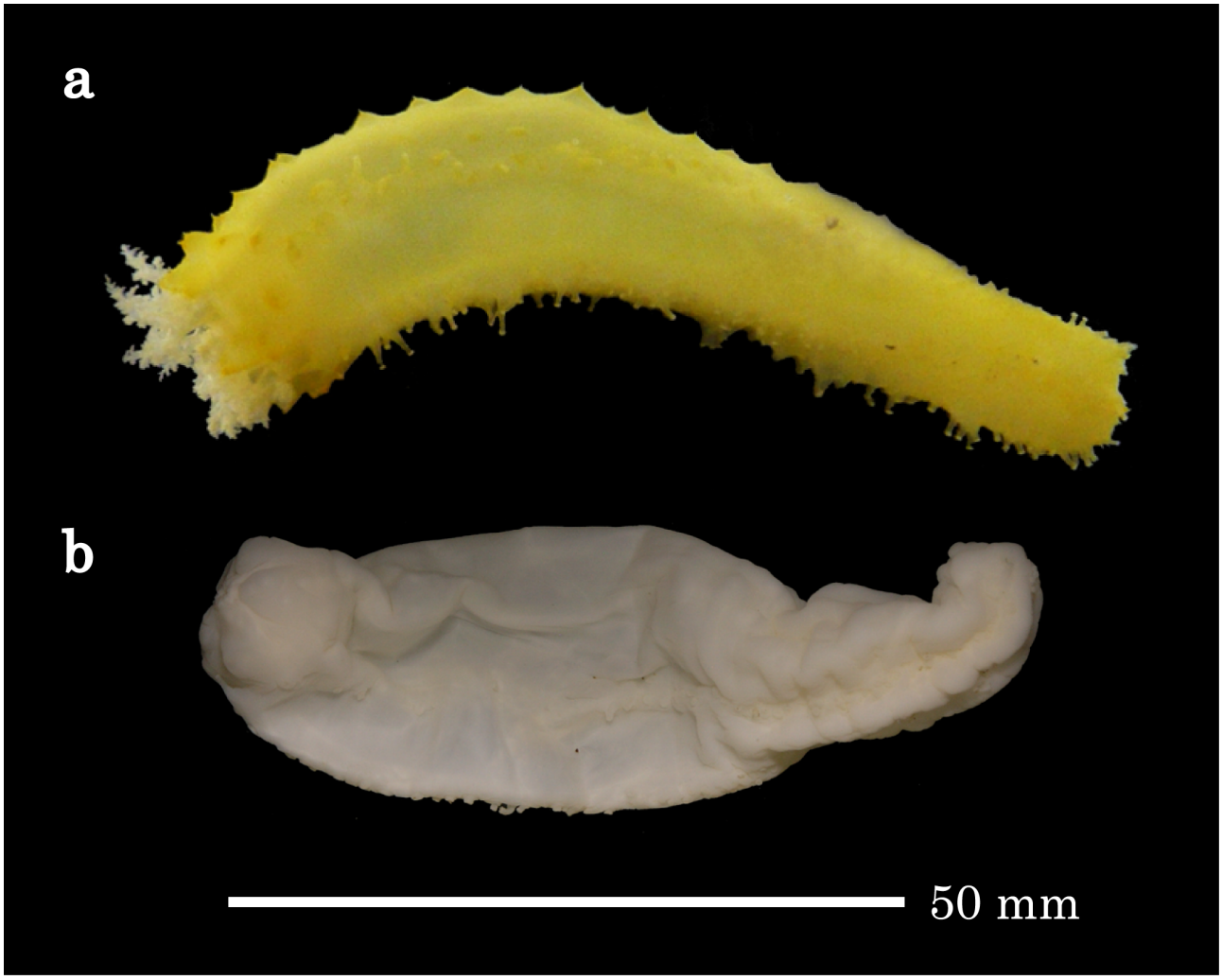

FIGURE 1. Lateral views of taxonomically unsolved species collected from deep sea Japan. (a) Live specimen, (b) preserved specimen (the same individual of Fig. 1a).

Ossicle morphology

Body wall ossicles were mostly peculiar shape: small, x-framed, and dice-shaped, namely "double-faced x-framed cup ossicle" (Fig. 2a), and rarely show dendriformed rods in the inner body-wall (Fig. 2b). However, interestingly, the body ossicles completely lack buttons, plates, x-bodies, cups and/or baskets. The tube feet ossicles also mostly double-faced x-framed cup ossicle, with small numbers of terminal supporting plates (Fig. 2c) and usual endplate. The peri-oral ossicles were x-framed cup-shaped structures (Fig. 2d), with large rods (Fig. 2e) and double-faced x-framed cup ossicle.

Front views of the rims of body wall double-faced x-framed cup ossicles are square, centrally with x-frame, marginally with spinous processes (Fig. 2, a1–a6). There are more numbers of processes on the upside rim than on the downside rim. Side views are also square, because of four pillars that united the both rims together (Fig. 2, a7–a11). However, some ossicles are partially or completely lacking of rims of upside and/or downside (Fig. 2, a6 and a12). On the other hand, most of the peri-oral ossicles are completely lacking of downside rim (Fig. 2d), resulted in the normal x-framed cup ossicles possessing central x-frame and upside rim that partially reduced frequently.

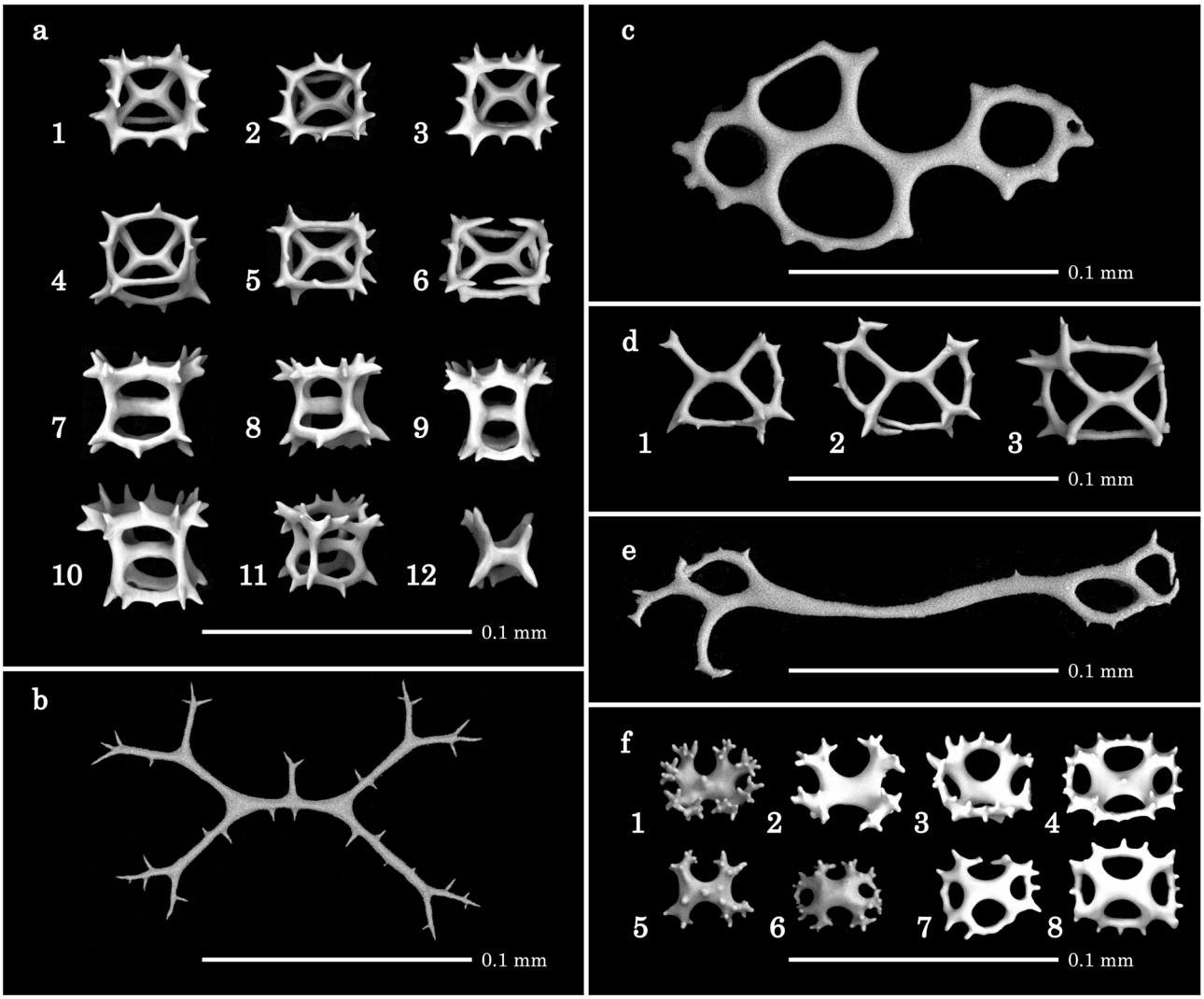

FIGURE 2. (a–e) Ossicles of taxonomically unsolved species collected from deep sea Japan, and (f) the outer body-wall ossicles of *Hemiocnus tegulatus* (Augustin, 1908). (a) double-faced x-framed cup ossicle from outer body-wall, a1–a3: top views, a4–a6: bottom views, a7–a12: side views, (b) dendriformed rod from inner body-wall, (c) supporting plate from tube feet tip, (d) x-framed cup-shaped structures from peri-oral skin, d1–d3: bottom views, and (e) large rod from peri-oral skin. (f) Outer body-wall ossicles of *Hemiocnus tegulatus* (Augustin, 1908) (specimen registration: WMNH-INV-2015-307), as the examples of the single-faced x-framed cups (x-bodies), f1–f4: top views, f5–f8: bottom views.

Molecular phylogeny

In this study, we sequenced portions of the mitochondrial gene COI and the nuclear gene Histone H3, and conducted phylogenetic analyses together with selected holothuroid sequences included in Miller *et al*. (2017). From eleven samples morphologically examined, we selected three individuals to be sequenced (WMNH-INV-2018-7, 8, and 9, Genbank accession numbers are LC425500, LC425501, and LC425502 for COI, and LC425503, LC425504, and LC425505 for H3, respectively). We obtained 659 bp (and 439 bp after the exclusion of the 3rd codon) of COI and 334 bp of Histone H3 post-alignment, which were then used in the subsequent analyses. Although bootstrap supports were low (especially for the nodes of higher phylogeny and/or for datasets with the third codon of COI included), the topology of our phylogenetic trees are congruent with that of Miller *et al*. (2017) (Fig. 3). Our phylogenetic analyses have repeatedly placed the undescribed species inside Dendrochirotida. BLAST results using sequences of the specimens as queries, also support this result (Table 2). Phylogenetic trees with the third codon of the COI gene included are also provided as supplementary results (Fig. 4).

TABLE 2. BLAST results of the genes amplified from specimens used in this study.

Gene	Specimen	BLAST result	E-value	Identity	Genbank ID
H3	INV-2018-7	*Psolus phantapus* isolate D histone H3 (H3) gene, partial cds	3.00E-151	336/356 (94)	KP 113611.1
		Echinocucumis cf. hispida AM-2017 histone H3 (H3) gene, partial cds	4.00E-150	321/335 (96)	KX 874437.1
H3	INV-2018-8	*Psolus phantapus* isolate D histone H3 (H3) gene, partial cds	7.00E-153	337/356(95)	KP 113611.1
		Echinocucumis cf. hispida AM-2017 histone H3 (H3) gene, partial cds	4.00E-150	321/335 (96)	KX 874437.1
H3	INV-2018-9	*Psolus phantapus* isolate D histone H3 (H3) gene, partial cds	3.00E-151	336/356(94)	KP 113611.1
		Echinocucumis cf. hispida AM-2017 histone H3 (H3) gene, partial cds	4.00E-150	321/335 (96)	KX 874437.1
COI	INV-2018-7	*Afrocucumis africana* voucher SIO: BIC: E6844 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1.00E-126	396/468(85)	KX 874348.1
		Cucumaria miniata voucher BIOUG: BAM00037 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	3.00E-123	381/449 (85)	HM 542157.1
COI	INV-2018-8	*Cucumaria miniata* voucher BIOUG: BAM00037 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	0	570/676 (84)	HM 542157.1
		Psolus chitonoides cytochrome oxidase 1 (CO1) gene, partial cds, and large subunit rRNA gene, partial sequence, mitochondrial genes encoding mitochondrial products	0	562/664 (85)	U 32220.1
COI	INV-2018-9	*Psolus chitonoides* voucher BIOUG: BAM00144 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	3.00E-177	539/637 (85)	HM 542342.1
		Psolus chitonoides voucher BIOUG: BAM00038 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	1.00E-175	538/637 (84)	HM 542341.1

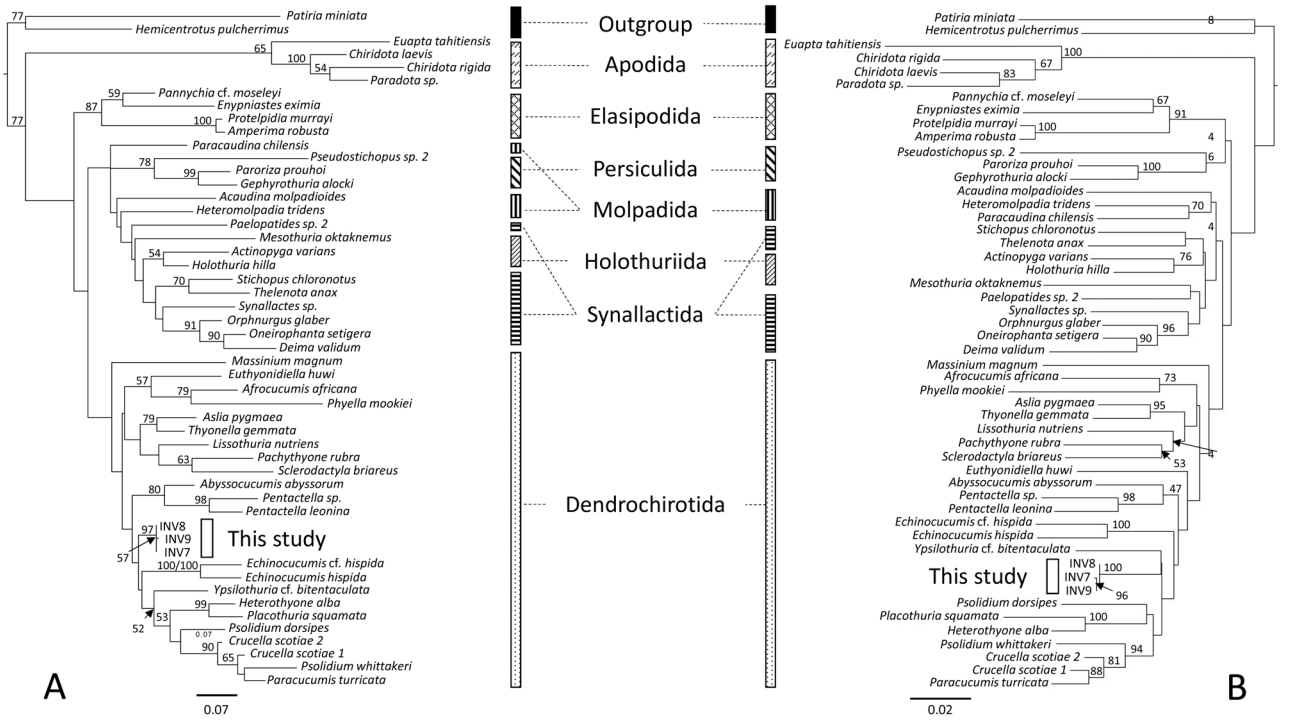

FIGURE 3. Phylogenetic tree (third codon of the COI gene excluded) depicting the position of the specimens in Dendrochirotida. Both of the (A) Maximum Likelihood tree and (B) Neighbor Joining tree show the inclusion of the specimens in Dendrochirotida, and their affinities to the genera *Echinocucumis*, *Ypsilothuria*, *Heterothyone*, *Placothuria*, *Psolidium*, *Crucella*, and *Paracucumis*, with *Afrocucumis* and *Pentactella* as sister clades.

Discussion

Taxonomic placement of the specimens

Although the most internal and external morphologies of the present species agree well with the characteristics of the family Cucumariidae Ludwig, 1894, the present species have none of the plate, single-faced x-framed cup or x-body (Fig. 2f), or button ossicles, which are usually observed in the body wall of the congeners of Cucumariidae. On the other hand, the body ossicles of the present species have the similar structures to those of some species of the aspidochirotid holothurians, such as the small tables of the genus *Labidodemas* Selenka, 1867, the dendriformed rods of the genera *Thelenota* Clark, 1921 and *Stichopus* Brandt, 1835.

Meanwhile, our molecular phylogenetic tree suggested that the specimens studied here belong to Dendrochirotida, in a clade together with the genera *Echinocucumis*, *Ypsilothuria*, *Heterothyone*, *Placothuria*, *Psolidium*, *Crucella*, and *Paracucumis*, with *Afrocucumis* and *Pentactella* (previous nominal genus of the taxonomic meaning of "Cucumariidae") reported by Miller *et al*. (2017), as the sister clade to the whole group (Fig. 3). Thus, our molecular study indicates a possibility that the specimens are not cucumariids, but belong to a sister group of Cucumariidae. However, we were unable to place our specimens with certainty in any lower taxonomic group because of the low supports on most nodes. Miller *et al*. (2017) has suggested that a more exhaustive molecular systematics work is still needed to resolve the interrelationships among the families of dendrochirotids, including to test the monophyly of each family. Therefore future taxonomic and systematics studies will still be needed, not only on our specimens, but also on Dendrochirotida itself.

Although we were unable to pinpoint the specimens' lower taxonomic affinity with certainty, we are confident with the placement of the specimens in Dendrochirotida, because this taxonomic placement has been repetitively consistent, regardless of the phylogenetic method (ML or NJ) used for inference, and the inclusion/exclusion of the third codon position. Moreover, this result is in congruence with the morphological observation.

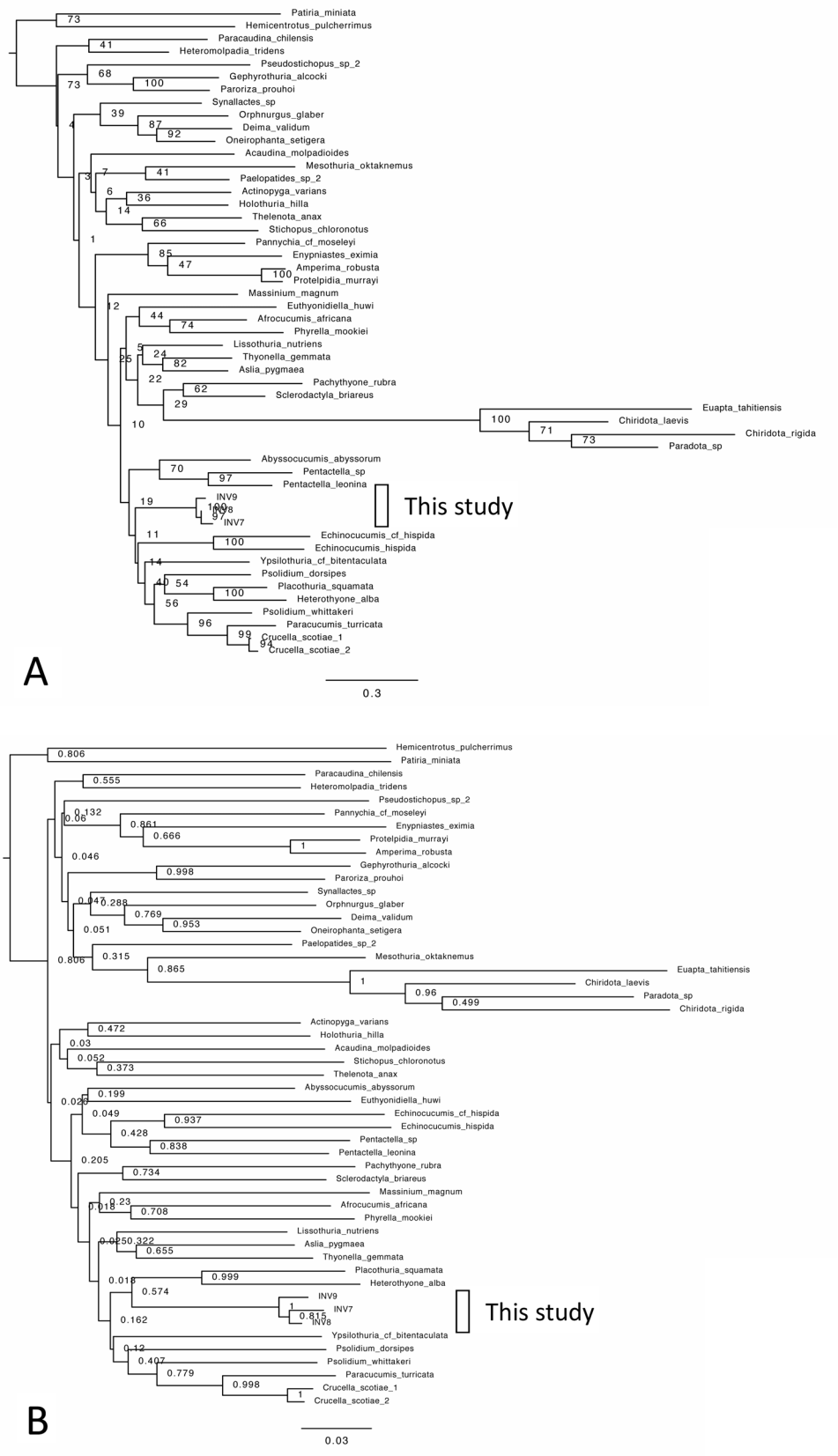

FIGURE 4. Phylogenetic tree (third codon of the COI gene included) depicting the position of the specimens in Dendrochirotida. Both of the (A) Maximum Likelihood tree and (B) Neighbor Joining tree show the inclusion of the specimens in Dendrochirotida, and their affinities to the genera *Echinocucumis*, *Ypsilothuria*, *Heterothyone*, *Placothuria*, *Psolidium*, *Crucella*, and *Paracucumis*, with *Afrocucumis* and *Pentactella* as sister clades.

Possible origin of double-faced x-framed cup ossicle

In dendrochirotid holothurians, some studies show that the fundamental form of ossicle tends to be exist in the peri-oral skin (*e.g.*, O'Loughlin *et al*. 2012; Yamana *et al*. 2015). In our present observation, some of the peri-oral ossicles show a very similar property with the small x-framed cup-shaped structures. Furthermore, we found that the structures of the double-faced x-framed cup ossicles (Fig. 2a) and normal x-framed cups (Fig. 2f) resemble each other in several points: the presence of the central x-frame, the marginal rim connectted to the x-frame, and the spinous processes developing on the marginal rim. Judging from these similarity, if a normal x-framed cup ossicle developed four extra-arms on the bottom face, and finally these arms equipped with an extra-rim, it could be considered as a "double-faced x-framed cup ossicle. As we have seen, the new ossicle form "double-faced x-framed cup ossicle," could be a derivation pattern of the x-body which sometimes occurs in cucumariids, and could not be rerated to the small table ossicle of some species of the aspidochirotid and dendrochirotid holothurians.

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