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Detailed description of scanning electromagnetic microscope (SEM) of the *Holothuria scabra*'s ossicles (Holothuroidea: Echinodermata) collected from Pesawaran Waters, Lampung, Indonesia

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Abstract. *Khatulistiani TS, Dewi AS, Yasman. 2022. Detailed description of scanning electromagnetic microscope (SEM) of the* Holothuria scabra*'s ossicles (Holothuroidea: Echinodermata) collected from Pesawaran Waters, Lampung, Indonesia. Biodiversitas 23: 3697-3704.* Ossicles are microscopic calcified endoskeletons that are unique across sea cucumber species. Furthermore, their integrity is maintained after physical and chemical processes that make them a desired attribute for identification purposes. Understanding ossicle characteristic especially for the sandfish *H. scabra* juveniles is important during mariculture and prevent fraud due to their high economic value. This study aims to identify wild *H. scabra* ossicles from Pesawaran Waters, Lampung Province, Indonesia using scanning electron microscopes (SEM) to get more detailed morphology of their ossicles. Seven specimens of *H. scabra* were collected in Pesawaran, Sari Ringgung and Mutun, around Pesawaran Waters. Morphology identification was carried out by observing the body appearance and tentacles of the specimens as well as measuring their weights and lengths. The observation of the ossicles was performed using a light microscope (LEICA ICC50 HD) at 400x magnification and a SEM (Jeol-JCM6000). The length of the tested specimens was around 140-300 mm and their weight was approximately 99-579 g. These two parameters were found to exhibit positive correlation (R^2 = 0.93; R= 0.96; p = 0.000). All observed specimens displayed peltate tentacles (12-22 tentacles) surrounding their mouths. The correlation between length and tentacle numbers was also positive (R^2= 0.53; R= 0.73; p = 0.101). The shape of the ossicles consists of six to fourteen-hole buttons, tables, spiny perforated plates from the cloaca, tube foot rods, tentacle rods, dorsal rods, dorsal perforated spiny rods, I shaped and branched rods.

Keywords: *Holothuria scabra*, ossicles, scanning electron microscope, SEM

INTRODUCTION

Holothuroids (sea cucumber), together with echinoids (sea urchin), have been grouped as a sister clade based on the absence of branches in the bodies (Mohsen and Yang 2021). Generally, sea cucumber bodies are elongated, curved dorsally and flattened ventrally (Rowe 1969; Massin 1999; Junus et al. 2017). Their mouths are surrounded by 18-30 buccal tentacles and may be either dendritic, peltate, or pinnate in shape (Al Rashdi et al. 2012; Junus et al. 2017). Most sea cucumber species prefer the seagrass bed ecosystem because it provides rich nutrients for their growth (Hamel et al. 2001). Sea cucumbers use ventral sucker tube feet to move along their substrate and also have papillae on the dorsal part as a protector when their body stiffens (Ajayi et al. 2020).

Despite their close cladistic relation, there are several differences between sea cucumbers and sea urchins. Not only do sea cucumbers lack spines surrounding their bodies, but they also have leathery skin. Their ossicles are reduced into a micro size that extremely contrasts with fully jointed and calcified ossicles that function as a compact endoskeleton in the sea urchins (James 1976). The ossicle characteristics among sea cucumber species are various and unique (Rowe 1969; James 1976; Massin et al. 2000; Massin et al. 2009; Kamarudin et al. 2017). Therefore, the identification of sea cucumber species is normally based on species-specific ossicle characteristics (Massin et al. 2000).

Holothuria scabra (order Aspidochirotida, family Holothuriidae) is widely distributed in Indo-Pacific including Indonesian waters (Tuwo and Conand 1992; Setyastuti and Purwati 2015). *H. scabra* has a wide range of body colors from ash grey to black. It also has transversal greenish bands at its grey dorsal. Its ventral is grey-white and speckled with numerous dark tiny dots that refer to its tube feet (Massin 1999). However, using solely the external morphology of *H. scabra* for identification purposes could be misleading. Therefore, a complimentary ossicle description could provide more reliable identification of *H. scabra* than using an external morphological observation alone (Massin 2000). According to Rumlus et al. (2015), H. scabra has potential as antibacterial against *S. aureus* and *E. coli*.

Understanding the ossicle characteristics of *H. scabra* was important for conservation, mariculture, and disease management in aquaculture and quality control in trading. Instead of studying its growth factor genes and its sex steroids and steroidogenesis-related genes (Kornthong et al. 2021; Thongbuakaew et al. 2021), ossicle tagging can be used to study *H. scabra* growth and stock in the wild. It indeed also helps mariculture to collect the correct *H. scabra* juveniles in the wild (Purcell and Blockmans 2009; Simoes and Knauer 2012; Turner 2015). The skin ulceration disease of cultured *H. scabra* also can be detected by observing the integrity of the ossicles (Becker et al. 2004; Delroisse et al. 2020). Understanding ossicle characteristics can also be applicable to the post-harvest industry of economically important sea cucumbers. The appearances (color and shape) of sea cucumbers are usually altered during processing, thus make the identification difficult. In the case where the integrity of ossicles of sea cucumbers is maintained throughout physical and chemical processes, an ossicle-based identification method could be beneficial for fraud detection (Setyastuti and Purwati 2015). In the previous study, the application of a molecular approach to identify sea cucumbers was attempted by Kamarudin et al. (2018) and Patantis et al. (2019). This particular method is convenient when the observed samples were lacking common ossicles or whose ossicles have been severely damaged during processing.

The study of ossicle characteristics of *H. scabra* (120 and 175 mm long) using a light microscope was done by Massin (1999). Ossicles characteristics during early juvenile stages (0.9-15 mm long) and adult specimens with body lengths up to 110 mm were also reported a year later by the same group using a scanning electron microscope (SEM) (Massin et al. 2000). It was found that the specific features of ossicles (i.e., size, shape/appearance and prevalence) of the early juveniles *H. scabra* are unique and different from those of the bigger size of juvenile specimens. Juveniles with 30 mm in length and above display similar ossicle characteristics to those of the adult specimens (Massin et al. 2000).

The ossicles of adult *H. scabra* have been illustrated using light microscopes by Panning (1935; 1941), Cherbonnier (1980; 1988), and Mary Bai (1980). However, the detailed study of adult wild *H. scabra* from the western part of Indonesia with size >110 mm using SEM has not been reported yet. This study aims to describe the morphology of *H. scabra* that was not found by Setyastuti et al. (2018) from Pesawaran Waters, Lampung, Indonesia, compared to those collected from the eastern part of Indonesia as reported by Massin (1999). The morphology identification was performed by observing the external morphology with special focus on the spicules characteristic using SEM. We also analyzed the correlation of length vs weight as well as length vs number of tentacles of *H. scabra* using Simple Linear Regression analysis.

MATERIALS AND METHODS

Sampling area

Samples were collected in Pesawaran, Sari Ringgung and Mutun of Pesawaran Waters, Lampung Province, Indonesia (Figure 1), in October-November 2020.

Procedures

Specimens sampling

Holothuria scabra that were recognized from their out morphology as described by Massin (1999) were collected by hand in Pesawaran, Sari Ringgung and Mutun of Pesawaran Waters, Lampung, Indonesia. Seven specimens of *H. scabra* with various body sizes from 140 to 300 mm in length were obtained. The specimens were frozen immediately after collection. During transportation to the Research Center for Marine and Fisheries Product Processing and Biotechnology in Jakarta, the specimens were packed with ice in a styrofoam box.

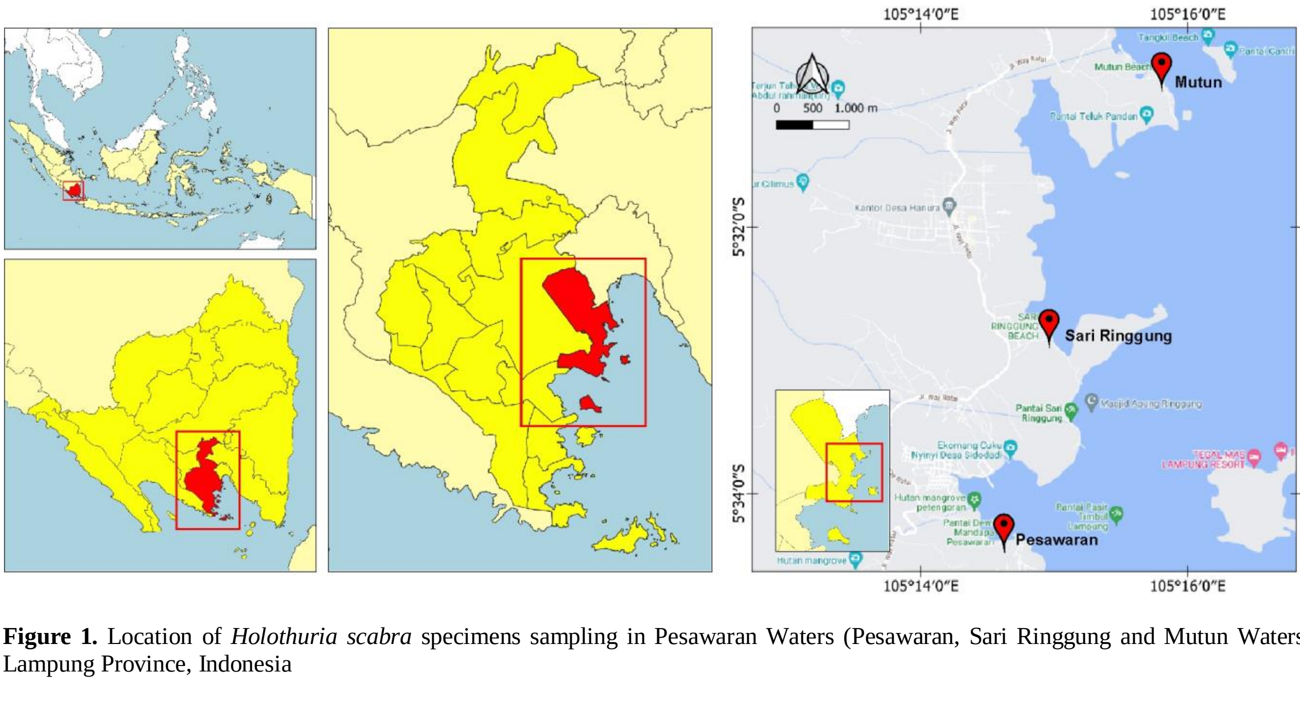

Figure 1. Location of *Holothuria scabra* specimens sampling in Pesawaran Waters (Pesawaran, Sari Ringgung and Mutun Waters), Lampung Province, Indonesia

External morphology observation

The external morphology observation of *H. scabra* specimens was directly performed upon arrival to the laboratory. The specimens were thawed prior to analysis. External morphology inspections of the specimens were carried out by observing the appearances of dorsal and ventral parts, the shapes and numbers of tentacles, and measuring their weights and lengths (Rowe 1969; James 1976). Subsequently, the specimens were gutted and cleaned. Both viscera and the body wall of the specimens were then stored at -20 ºC.

Ossicles observation

Ossicle observation was performed following methods by Massin et al. (2000) with modification. The body wall and tentacles (±1.00 cm^2) of small (~ 140-165 mm; D and F specimens), medium (~ 235-260 mm; A, B, C, and E specimens) and large (~ 300 mm; G specimens) were decayed separately by using household bleach (Bayclin® containing 5.25% NaOCl). After the tissues decayed, the ossicles were rinsed with distilled water three times to remove the bleach residues. For ossicles observation using a light microscope at 400x magnification (Leica ICC50 HD), the ossicles were dropped onto the object glass by the splash method and the shape and size of yielded scattered ossicles on the object glass were observed.

The remaining ossicles of specimens F, E, and G to represent small, medium and large specimens, respectively, were dried using a vacuum concentrator (Scanvac, scan speed 40 cool safe 110-4). The obtained dried ossicles were then patched onto the carbon tape (NEM TAPE) on the specimen block, followed by spraying with Gas Duster 720 Chromax to eliminate excess dried ossicles. The patched ossicles were coated with gold using Smart Coater for a minute, then inserted and examined using SEM (Jeol-JCM6000). The magnification was adjusted depending on the size of the ossicles.

Data analysis

This study analyzed the correlation of *H. scabra* length vs weight as well as length vs tentacle numbers usingSimple Linear Regression analysis. The statistical analysis was performed by SPSS 25. Other data such as color and shape were discussed descriptively.

RESULTS AND DISCUSSION

External morphology observation

Seven specimens of *H. scabra* (coded as specimens A-G) from Pesawaran Waters had various body lengths and weights, ranging from 140-300 mm and 99-579 g, respectively. Specimen G was the largest (300 mm, 579 g), while specimen F was the smallest (140 mm, 99 g) (Table 1). Based on the statistical analysis, the length of *H. scabra* has a strong positive correlation with its weight (R^2 = 0.93; R= 0.96; p = 0.000); the longer the body, the heavier the weight of the specimen. This is due to the increasing thickness of the body wall along with the increasing body size (Pitt and Nguyen 2004). Our finding is consistent with that reported by Yanti et al. (2019). Their study also reported a positive correlation between length and weight as well the exponential correlation between length and the gutted body weight of *H. scabra* during three years of observation in South Sulawesi waters.

All observed specimens have short peltate tentacles (Figure 2.A) surrounding their mouths at the anterior ends. While other reports did not specify the type of tentacles of *H. scabra* specimen that they assessed (Massin 1999; Dabbagh et al. 2012; Kizakhudan and Asha 2017; Yaghmour and Whittington-Jones 2018). James (1976) reported that the tentacle shape of *H. scabra* juvenile was also peltate. Different from previous studies, we found that the tentacles varied in numbers (12-22 tentacles) and had positive correlations with the size of the specimens (R^2= 0.53; R= 0.73; p = 0.101). Other literature reported the presence of 20 tentacles both during juvenile (James 1976) and the adult stage of *H. scabra* (Massin 1999; Dabbagh et al. 2012; Kizhakudan and Asha 2017; Yaghmour and Whittington-Jones 2018).

Commonly, *H. scabra* was gritty to touch because of the papillae, the wrinkled skin on the dorsal part, and the dotted tube feet on the ventral part. Specimens of *H. scabra* from Pesawaran Waters have light to dark grey color with black lines in their dorsal part. Among others, specimens B and F were the darkest and lightest in color, respectively. Additionally, specimen B had a distinct yellow spot that was superimposed with the black line on the dorsal part. This type of appearance has been observed on the specimens collected from Bengkulu Indonesia, Persian Gulf Iran and Indian Seas (Dewi et al. 2011; Dabbagh et al. 2012; Junus et al. 2017), but was not observed from specimens collected in Sulawesi (Massin 2000). Hamel et al. (2001) noted that *H. scabra* with yellow spots are distinctive for specimens originated from Indian Ocean, but not from the Pacific. All tested specimens showed lighter color (white) on their ventral body parts that were speckled with many dark tiny dots corresponding to their tube feet (Figure 2.B).

The gradient coloration from light to dark grey of *H. scabra* in the present study was consistent with the specimens of *H. scabra* from Queensland and Tanzania that were grey to black in color (Uthicke and Benzie 1999; Kithekani et al. 2002). Molecular analysis done by Uthicke and Benzie (1999) revealed that they all belong to the same species suggesting that the color variation of *H. scabra* could be affected by environmental factors. In our study, we found that the color of sediment inside the viscera of light grey *H. scabra* (specimen F) was brittle white sand, meanwhile, the color of sediment inside the viscera of dark grey *H. scabra* (specimen B) was soft black mud. This finding suggested that the color of the substrates might affect the skin coloration of *H. scabra*. As shown by other holothuroids species, this technique is presumably employed by *H. scabra* to camouflage itself as a defense mechanism to deter predators (Kamyab et al. 2020).

Table 1. Notes of gross morphology of *Holothuria scabra* specimens

Specimens	Length (mm)	Weight (g)	Color	Number of tentacles	Picture
A	250	510	Light grey with black line	20	
B	235	417	Dark grey with black line and yellow spot	18	
C	260	503	Grey with black line	20	
D	165	292	Dark Grey with black line	NA*	
E	240	407	Grey with black line	22	
F	140	99	Light Grey with black line	12	
G	300	579	Grey with black line	18	

Notes: *NA= data not available

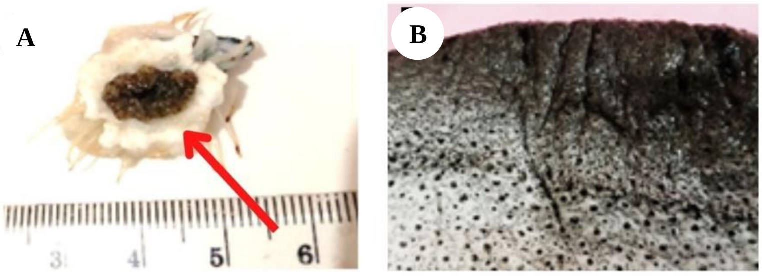

Figure 2. External morphology observation of *Holothuria scabra* from Lampung waters: A. Short peltate tentacles; B. Dorsal part (wrinkled and darker color) and ventral part (lighter color with tiny black dotted sucker tube)

Ossicles identification

In this study, the ossicle types of the tested specimens were dominated by six hole-buttons which were slightly oval in shape (Figure 3), while some others contain eight-, ten-, twelve-, and fourteen-hole buttons. The size of the buttons in specimens D and F (small-size specimens), specimens A, B, C, and E (medium-size specimens), and specimens G (large-size specimen) were 37-118 µm, 35-102 µm and 37-88 µm, respectively. This finding is in agreement with that reported by Massin et al. (2000) in which the size of the buttons decreases as the length of *H. scabra* increases. Massin et al. (2000) assessed the ossicle changes in *H. scabra* from early juvenile (0.9 mm) to adult stage (up to 110 mm). Meanwhile, our present study found that that button size pattern was also observed in the adult stage ranging from 140-300 mm in length.

The SEM data showed that the buttons were smooth and had medium nodules (Figure 3.A). This type of button is unique to subgenus *Metriatyla* which *H. scabra* belongs to. Other subgenus members of *Metriatyla* such as *H. lessoni* (formerly *H. scabra* var. *versicolor*) also have a similar characteristics of button (Hamel et al. 2013; Kamarudin et al. 2017). In comparison, subgenus *Microthele* has a medium nodule with ellipsoidal button shape, whereas *Thymiosycia* has smooth button ossicles without nodules (Rowe 1969). Buttons with more holes are more often found in small specimens. This result is consistent with previous study reporting that a decreasing number of pairs of buttonholes coincide with the increasing body size of sea cucumbers (Massin et al. 2000). The hole diameter in the present study was around 6 to 12 µm. There were also found spiny perforated plates from cloaca (Figure 3.B) that were found only in specimens C and D around 96-118 µm respectively. In this study, the number of holes of spiny perforated plates from the cloaca was 4-8. The spiny perforated plates from the cloaca were also mentioned in Massin et al. (2009).

The table ossicles were different in size between the small and larger specimens (medium and large). The diameter of the spiny crown table of the small specimens was around 16-46 µm and that of the disc table was around 70-101 µm. Meanwhile, the table height was around 58-87 µm (Table 2), thus creating a pyramid-like shape (Figure 4.A). Our findings were consistent with Massin et al. (2000) these pyramid tables are only found in the small specimens, and will shift to cube-like tables as the crown grow wider and the length of *H. scabra* increases. The diameters of the spiny crown table in medium and large specimens were around 39-52 µm and 59-64 µm, respectively; while those of the disc table was around 62-89 µm and 72-95 µm, respectively. The table heights were around 55-71 µm in medium specimens and 62 µm in large ones. The size of the spiny crown tables in large *H. scabra* can increase up to 70 % of the disc table diameter so that it becomes more like a cube in shape (Massin et al. 2000).

Most Holothuriidae have table ossicles, but the shapes vary between subgenus and species (Rowe 1969). *H. scabra* and *H. lessoni* have similar types of buttons, but both have very different types of table ossicles (Massin et al. 2009). The disc table of *H. scabra* is smooth, flat, and has a central hole (Figure 4.C); the size of crown table is medium with a blunt spine (Figure 4.D with arrow); and the height of the spire is also medium (Massin et al. 2009). On the other hand, *H. lessoni* disc table is spiky, the table crown size is large with a sharp spine (Rowe 1969). Even though *H. scabra* is closely related to *H. lessoni* with occasionally similar gross morphology, the characteristics of their table ossicles could distinguish the two species (Massin et al. 2009).

Another type of ossicles that characterize specimens of *H. scabra* in the present study is rod ossicles. Dorsal rods of *H. scabra* from Pesawaran Waters had various numbers of holes (Figure 5.C) in all specimens. At least they had one or two holes in the central dorsal rod. The tube foot rod had a curved shape that was texturized with blunt spines (Figure 5.A with arrow), while the tentacle rod shapes were not uniform which they have rough tips on both ends (Figure 5.B). Most of the observed rod ossicles were more than 100 µm in length; only a few of them were 68-91 µm. (Table 2; Figure 5.A,B,C,D,E). The longest dorsal and tube foot rods (107-213 µm; 71-244 µm) were found in medium specimens, while the longest tentacle rods (159-259 µm) were found in the large specimens. Moreover, this study also found the dorsal perforated spiny rods (155 µm) in specimen E (Figure 5.D), branched rod (91 µm) in specimen E (Figure 5.F) and I-shaped rods (289 µm) in specimens F (Figure 5.E).

Table 2. Type and size of ossicles of *Holothuria scabra* from Pesawaran Waters, Lampung Province, Indonesia

Specimen	Button holes	Buttons (µm)	Disc table (µm)	Spiny crown table (µm)	Full table (µm)	Tentacle rods (µm)	Tube foot rods (µm)	Dorsal rods (µm)
A	6-14	42-95	67-89	44-53	56-71	68-140	NA	107-161
B	6-14	38-115	62-78	39-48	57-65	128	242	131-160
C	6-12 *	35-97	64-70	52-60	55-67	91-203	107-244	125-213
D	6-10 *	40-118	73-85	21-46	62-68	NA	NA	136-176
E	6-12	35-102	62-72	44-56	57-65	80	71-117	122-212
F	6-14	37-95	70-101	16-45	58-87	140	168-171	158-204
G	6-14	37-88	72-95	59-64	62	159-259	99-167	165-170

Notes: * Found the spiny buttons in the specimens. NA: Data not available

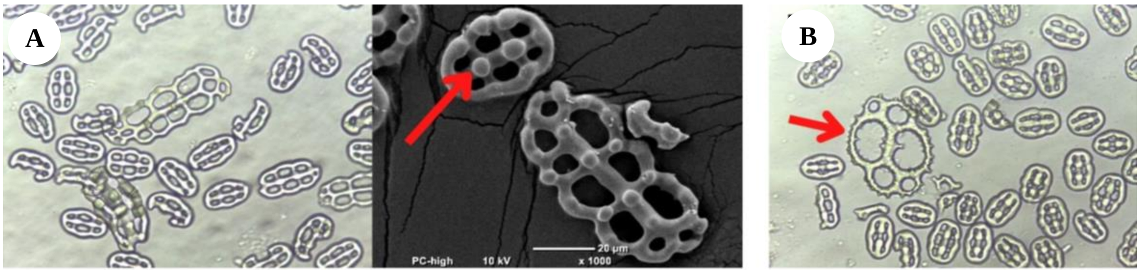

Figure 3. Ossicle identification of *Holothuria scabra* from Pesawaran Waters: A. Button ossicles using light microscope 400x (left) and nodule button ossicles using SEM (right); B. Perforated plates from the cloaca (light microscope 400x)

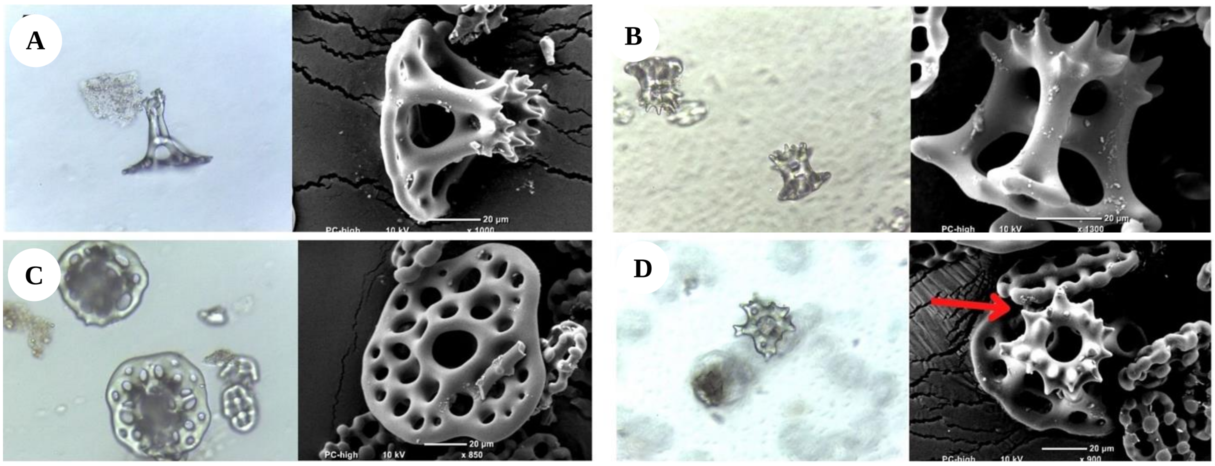

Figure 4. Ossicle shapes of *Holothuria scabra* from Lampung waters: A. Pyramid tables in small specimens (14-16.5 cm); B. Square tables in medium and large specimens; C. Table plate with holes; D. Spiny table crown

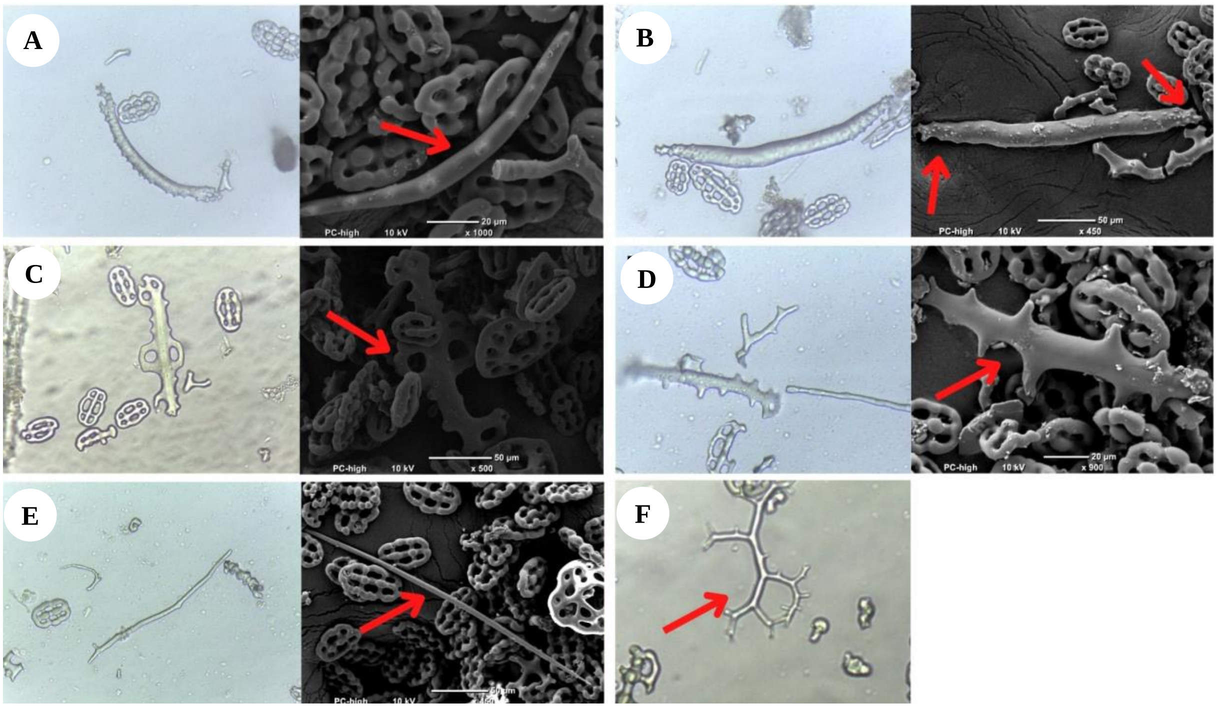

Figure 5. Shapes of *Holothuria scabra* rods: A. Tube foot rod; B. Tentacle rod; C. Dorsal rod; D. Dorsal perforated spiny rod; E. I shaped rod; F. Branched rod (No SEM figure)

According to Massin et al. (2000), the dorsal rods and dorsal perforated spiny rods of *H. scabra* can be detected as early as the specimen is 6 mm long. Meanwhile, their tube foot and tentacle rods can be observed when the specimen reaches 1.5 mm in length (Massin et al. 2000). We found that the dorsal rods were easier to observe compared to other rods were quite difficult to find. The dorsal perforated spiny rods found in 240 mm *H. scabra* (specimen E) were also illustrated by Massin et al. (2000) in 6 mm *H. scabra* specimen, its dorsal perforated spiny rods have > 100 µm length. We also observed branched rod from specimen E that was also mentioned in Dabbagh et al. (2012) and Kamarudin et al. (2017). The length of branched rod was about 80 to 110 µm (Dabbagh et al. 2012). I shaped rod was found in the F specimen. This finding is in agreement with those reported by Kamarudin et al. (2017) but he did not mention the length of its rods. Based on SEM figure in this study, the I shaped rod has a smooth surface.

Based on the morphology identification and ossicle observation of *H. scabra* specimens from Pesawaran Waters, we conclude that their body morphology and ossicles shape were similar to those from East Pacific (Rowe 1969), Indian Seas (James 1976), Solomon Island (Massin et al. 2000), New Caledonia (Massin et al. 2009), and Malaysia (Kamarudin et al. 2017). The ossicle shape of *H. scabra* from Pesawaran Waters were consist of 6-14 holes buttons, tables, spiny perforated plates from the cloaca, tube foot rods, tentacle rods, dorsal rods, dorsal perforated spiny rods, I shaped rods and branched rod. The SEM data showed that the button types of the tested specimens were smooth and nodulous. The size of the buttons decreased along with the increasing length of *H. scabra*. The table spicules of *H. scabra* from Pesawaran Waters consisted of holes with smooth disc, spire, and spiny crown shapes. The small specimens had a large disc and small table crown, creating a pyramid-like shape, meanwhile, the medium and large specimens had a cube-shaped table crown. Most of rod ossicles had a length of more than 100 µm.

Based on the SEM figures, the observed specimens had bluntly spine tube foot rods, rough tentacle rods in both tips, smooth dorsal rods, dorsal perforated spiny rods and smooth I-shaped rods. These results support the importance of complimentary ossicles description in addition to an external morphological observation to provide more reliable identification of *Holothuria* species, *H. scabra*. The fact that the external morphology and set of ossicle types of *H. scabra* from Lampung waters (western part of Indonesia) in the present study are slightly different from specimens from Sulawesi (eastern part of Indonesia) as described by Massin (1999), may be used as a tradeoff tracing of this heavily exploited Indo-Pacific holothurid species. Further study of more *H. scabra* specimens both from western and eastern parts of Indonesia is needed to confirm this suggestion.

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