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Morphological and Molecular Identification of Sea Cucumber species *Holothuria scabra, Stichopus horrens* and *Stichopus ocellatus* from Kudat, Sabah, Malaysia

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ABSTRACT

The abundance of sea cucumbers (Phylum Echinodermata: Class Holothuroidea) in Malaysian waters has been gradually declining in past decades. Due to the lack of recent studies on the status of sea cucumber populations in Kudat, Sabah, Malaysia, this study was conducted. This study aimed to identify the species of a *timun laut* morphospecies i.e. *Holothuria (Metriatyla) scabra* and two *gamat* morphospecies i.e. *Stichopus horrens* and *Stichopus ocellatus* that were collected from Limau Limawan based on ossicle shapes and non-protein-coding 12S mitochondrial rRNA gene sequences. A number of five main ossicle shapes were microscopically identified without microscopic size measurement i.e. rod, plate, rosette, button and table. However, a number of ossicle shapes for *S. horrens* and *S. ocellatus* recorded in the previous studies were not observed in this study and this could be due to the body deformation of the specimens. Interestingly, five specimens of *H. scabra* exhibited additional ossicle shapes other than the smooth button and the table. Despite the absence of common ossicles and the presence of additional ossicle shapes, 12S mitochondrial rRNA gene sequences analysed using the Basic Local Alignment Search Tool programme for Nucleotide (blastn) resulted in the species identification of the specimens of morphospecies *H. scabra* and *S. horrens* as *H. scabra* and *S. horrens*; however, the specimen of morphospecies *S. ocellatus* was identified only up to the genus level i.e. genus *Stichopus*, showing the lack of 12S mitochondrial rRNA gene sequences of *S. ocellatus* in the GenBank until 15 September, 2016. In total, 31 partial sequences of 12S mitochondrial rRNA gene were registered with the GenBank (Accession No.: KX913672-KX913702). The findings also suggested that species identification based on 12S mitochondrial rRNA gene sequence

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showed better inference than the ossicle shape identification. In summary, the three morphospecies were morphologically and genetically verified as *H. scabra, S. horrens* and *S. ocellatus*. Despite the fact that more specimens and more molecular techniques are required to generate better conclusive outcomes, the current findings give better insight into the importance of morphological and molecular approaches and the present status of the *timun laut* species and *gamat* species in Kudat.

Keywords: 12S mitochondrial rRNA gene, *gamat, Holothuria scabra*, Kudat, ossicle shape, *Stichopus horrens, Stichopus ocellatus, timun laut*

INTRODUCTION

In Malaysia, sea cucumber (Phylum Echinodermata: Class Holothuroidea) species can be categorised into two groups i.e. *gamat* species and *timun laut* species (Kamarudin et al., 2015). *Gamat* species refers to all species of sea cucumber from the family Stichopodidae including the genus *Stichopus* and the genus *Thelenota*. The term *gamat* also refers to sea cucumber species that are believed traditionally or proven scientifically to contain medicinal properties e.g. *Stichopus horrens* (Selenka, 1867) or the dragonfish. The *timun laut* or non-*gamat* species refers to all species of sea cucumber from other than the family Stichopodidae. *Timun laut* species are usually exploited as food in the *beche-de-mer* or *trepang* industry in Sabah, Malaysia. Choo (2008) reported that 19 sea cucumber species are commercialised by Malaysia, making the country the fourth top producer of sea cucumber products in the world after Indonesia (35 commercial species), China (27 commercial species) and the Philippines (26 commercial species).

Holothuria (Metriatyla) scabra (Jaeger, 1833) is a *timun laut* species. However, in Malaysia, some also regard it as a *gamat* species. It is locally known in Malaysia as *bat putih* (Kamarudin et al., 2015) or *balat harimau putih* (Abdullah, 2016). Its English name is sandfish. It has a brackish grey dorsal part, dark wrinkle lines (upper side) and a light grey ventral part (underside). The sandfish is one of Malaysia's commercial species of sea cucumber that are exploited as food (Choo, 2008). However, the species is regarded as "endangered, or at a high risk of extinction" based on the International Union for Conservation of Nature (IUCN) Red List for aspidochirotid holothuroids (Conand et al., 2014). In order to restore the sea cucumber species in Langkawi, sea ranching of *H. scabra* is being carried out by the Langkawi Development Authority (LADA) in Teluk Yu, Temoyong, Langkawi and Tuba Island, Langkawi (Sharif & Osman, 2016). Langkawi and Pangkor Islands are well known in the sea cucumber-based traditional medicine industry in Peninsular Malaysia for the production of body fluid extracts (*air gamat*) and lipid extracts (*minyak gamat*).

S. horrens and *Stichopus ocellatus* (Massin, et al., 2002) are among the *gamat* species in Malaysia. The total number of Malaysia's *gamat* species to date is 10, including eight *Stichopus* species and two *Thelenota* species (Kamarudin et al., 2015).

S. horrens or dragonfish is locally known as *gamat emas* or golden sea cucumber in Malaysia due to its grey-brown colour and its use as the main ingredient in the production of *air gamat* and *minyak gamat*. Its old scientific name was *Stichopus variegatus* because its body is often variegated with dark patches; however; the scientific name is no longer accepted. Meanwhile, a living specimen of *S. ocellatus* is yellow-orange mottled with a green-grey colour and has eye-like, large papillae in its dorsal part (Massin et al., 2002). The dragonfish is also one of Malaysia's commercial species of sea cucumber but *S. ocellatus* was not listed by Choo (2008). Moreover, *S. horrens* and *S. ocellatus* are not included in the IUCN Red List for aspidochirotid holothuroids as endangered or at risk of extinction and by Conand et al. (2014) as vulnerable or at risk of extinction; however *Thelenota ananas* and *Stichopus herrmanni* from the gamat species group are in their lists.

There are a lot of sea cucumber studies focussing on Sabah, Malaysia as the main study site (Kamarudin et al., 2015). The report by Ridzwan and Che Bashah (1985) on the distribution of sea cucumbers in Sabah and their use as a food resource is believed to be the first documentation of the sea cucumber. According to Kamarudin et al. (2009), at least three factors have contributed to the unique level of richness of the sea cucumber species in Sabah i.e. the extensive distribution of coral reefs, low level marine pollution and the possibility of biogeographical factors within and out of the Sunda Platform area. Furthermore, Sabah is the most significant sea cucumber fishery in Malaysia and the Sabah Fisheries Department reported that about 139 tonnes of sea cucumber was landed in Sabah between 2000 and 2005 (DOF, 2000-2005). Kudat, a town in the state of Sabah, is geographically located near the northernmost point of Borneo Island in East Malaysia. Due to the lack of recent studies on sea cucumber in Kudat, this town was chosen as the study site for this study.

In general, the aims of this study were to identify the species of a *timun laut* morphospecies i.e. *Holothuria (Metriatyla) scabra* and two *gamat* morphospecies i.e. *Stichopus horrens* and *Stichopus ocellatus* that were collected from Limau Limawan based on ossicle shapes and non-protein-coding 12S mitochondrial rRNA gene sequences. Ossicles are small parts of calcified materials from sea cucumber. Their shapes, in fact, have continued to be an important characteristic for morphological identification of sea cucumber (Kamarudin & Mohamed Rehan, 2015). Furthermore, mitochondrial DNA containing the 12S mitochondrial rRNA gene has been the main subject of interest in zoological genetic studies due to its considerably effective maternal inheritance, continuous replication, non-recombination, haploid genome and greater rate of substitution as compared to 'single-copy' nuclear cells (Nabholz et al., 2008). Therefore, ossicle and non-protein-coding 12S mitochondrial rRNA genes were used in this study for

morphological and molecular sea cucumber species identification and verification.

MATERIALS AND METHODS

Study Site and Sampling

A number of 30 specimens of *H. scabra* (PKS 1-PKS 30), one specimen of *S. horrens* (PKSH1) and one specimen of *S. ocellatus* (PKSO1) were collected from Limau Limawan, Kudat, Sabah, Malaysia (Figure 1) in February 2015. The specimens of *H. scabra* were fresh and in good form while the bodies of specimen of *S. horrens* and *S. ocellatus* became deformed before the transportation by flight from Kudat, Sabah to Science Research Lab 3.2 (SRL 3.2), Faculty of Science and Technology, Universiti Sains Islam Malaysia (USIM), Nilai, Negeri Sembilan (Figure 2). The morphospecies identification was done based on the outward body appearance or external morphology and the information given by the collectors. Prior to the transportation, each specimen was packed and sealed in a plastic bag, left in the freezer for a few days and wrapped in old newspaper before being transferred to an ice box. In the SRL 3.2, the specimens were stored in a -20oC chest freezer for long-term storage with proper cataloguing.

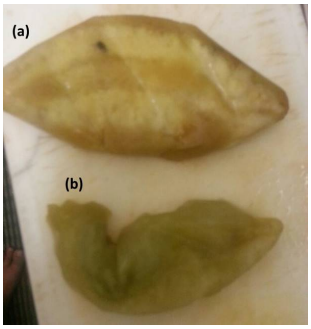

Figure 2. Deformed specimens of *Stichopus horrens* (a) and *Stichopus ocellatus* (b) from Limau Limawan, Kudat, Sabah, Malaysia

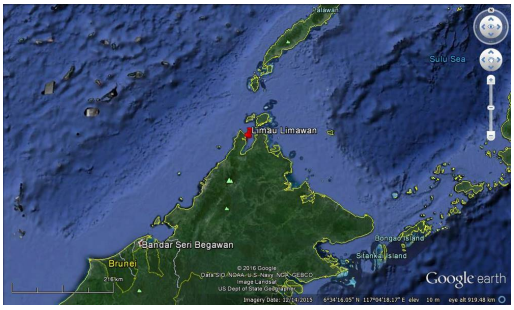

Figure 1. The collection site of *Holothuria (Metriatyla) scabra, Stichopus horrens* and *Stichopus ocellatus* in Kudat, Sabah, Malaysia highlighted in red. [Adapted from Google earth Version 7.1.5.1557 (December 14, 2015)].

Ossicle Extraction and Shape Observation

A little modification was done to the related methods by Kamarudin and Mohamed Rehan (2015). A small piece of tissue from the ventral cuticle of each of the specimens was cut with a sterile blade. The tissue portion was placed on a glass microscope slide. Three tissue portions of different specimens were allocated for each glass microscope slide. Several drops of liquid household bleach were used to dissolve the soft tissue while the ossicles that were usually in the form of white pellets remained in the liquid solution. The prepared slides were observed under the Olympus culture microscope model CKX41 with 400x magnification. The captured images of ossicle shapes were saved for morphological identification. The definite

microscopic size of each ossicle type was not entirely counted due to the specification of the Olympus culture microscope model CKX41. The main focus of this study was to record and identify the shapes of ossicles and then to compare their varieties between *H. scabra,* the *timun laut* morphospecies and *S. horrens* and *S. ocellatus*, the two *gamat* morphospecies.

Amplification of 12S Mitochondrial rRNA Gene

Total genomic DNA extraction was done using the DNeasy mericon Food Kit by QIAGEN with a little modification to the protocol. Standard PCR procedures were then used to amplify the non-protein-coding 12S mitochondrial rRNA gene using the 2x TopTaq Master Mix Kit by QIAGEN [~360 bp of fragment length based on Palumbi et al. (1991)].

AB12SA-Lf (forward) 5'- AAA CTG GGA TTA GAT ACC CCA CTA T -3' (25 bases)

AB12SB-Hr (reverse) 5'- GAG GGT GAC GGG CGG TGT GT -3' (20 bases)

Cycle parameters for the PCR run were 2 min at 95°C for initial denaturation, 30 s at 95°C for denaturation, 30 s at 50.3oC for annealing and 45 s at 72°C for extension. Repetition of step 2-4 was done for another 34 cycles and final extension was for 5 min at 72°C. The purified PCR products were sent for DNA sequencing at the First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor, Malaysia. QIAquick PCR Purification Kit by QIAGEN (for direct purification of single PCR fragment) and QIAquick Gel Extraction Kit by QIAGEN (for purification of desired PCR fragment from agarose gel) were used for the PCR product purification.

Basic Local Alignment and GenBank Submission

The online Basic Local Alignment Search Tool programme for Nucleotide (blastn) was used to align and match each gene sequence (i.e. the query sequence) from this study with a particular sea cucumber species or genus. The Sequin Version 15.10 programme was then used to prepare sequence data for the GenBank submission in order to obtain the accession numbers from the GenBank, National Centre for Biotechnology Information (NCBI), U. S. National Library of Medicine.

RESULTS AND DISCUSSION

All specimens of *H. scabra* from Kudat, Sabah, Malaysia shared two main shapes of ossicles i.e. smooth button and table (Figure 3). In terms of quantity, more smooth tables were observed compared to the tables.

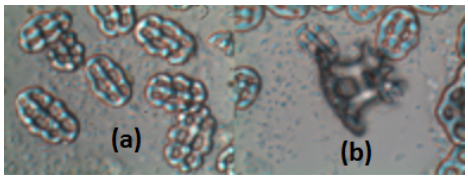

Figure 3. Two common ossicle shapes in the ventral cuticles of *H. scabra* specimens from Kudat, Sabah, Malaysia. (a) smooth button, and (b) table

Regardless of the microscopic size of each shape, the results showed good congruence with the study of Massin et al. (2000), who found that from the specimen length of 30 mm and above i.e. for the adult specimens, the ossicles of *H. scabra* contained more buttons and fewer tables than did the juveniles. In this study, the average length of the specimens of *H. scabra* adults was 70 mm. Therefore, the results supported the species identification based on the outward body appearance or external morphology of *H. scabra* from Kudat, Sabah.

Interestingly, five out of 30 specimens of *H. scabra* from Kudat, Sabah (approximately 17%) also showed additional ossicle shapes in their ventral cuticles. Figure 4 indicates that at least six different types of ossicle shape were present in specimens PKS 14, PKS 19, PKS 20, PKS 21 and PKS 27 (Figure 5). In terms of outward body appearance, all 30 specimens of *H. scabra* had the same body colour, shape and length; thus, the presence of the additional ossicles may represent some uniqueness of the specimens. Dabbagh et al. (2012) reported the presence of large perforated I-shaped rod in the tube feet of *H. scabra* from the Persian Gulf (see Figures 4b-4c) and branched rod from the ventral body wall and tube feet (see Figure 4e). The presence of a large perforated I-shaped rod in specimens PKS 14 and PKS 19 from Kudat, Sabah (Figures 4b-4c) and a branched rod in specimen PKS 21 (Figure 4e) could have originated from the tube feet of *H. scabra* from Kudat, Sabah. The presence of a Y-shaped rosette in the ventral cuticle of specimen PKS 14 (Figure 4a), a J-shaped rod in specimen PKS 20 (Figure 4d), an H-shaped rod in specimen PKS 21 (Figure 4f) and an I-shaped rod in specimen PKS 27 (Figure 4g) were in very small quantity. It is speculated that the Y-shaped rosette in specimen PKS 14 (Figure 4a) and the H-shaped rod in specimen PKS 21 (Figure 4f) could be the broken parts of a button-shaped ossicle.

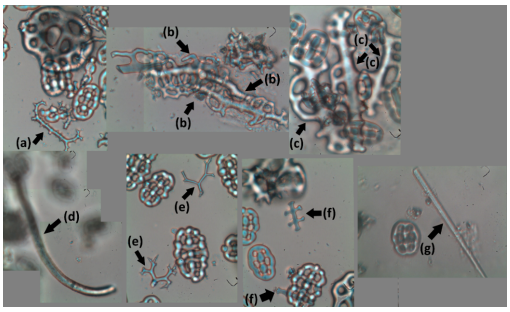

Figure 4. Six additional ossicle shapes in the ventral cuticles of five *H. scabra* specimens from Kudat, Sabah apart from the three common shapes (Figure 3). (a) Y-shaped rosette from specimen PKS 14, (b) large perforated I-shaped rod from specimen PKS 14, (c) large perforated I-shaped rod from specimen PKS 19, (d) J-shaped rod from specimen PKS 20, (e) branched rod from specimen PKS 21, (f) H-shaped rod from specimen PKS 21, and (g) I-shaped rod from specimen PKS 27

Figure 5. Five specimens of *H. scabra* from Kudat, Sabah, Malaysia with additional ossicle shapes extracted from their ventral cuticles (Figure 4).

Nevertheless, all the specimens remained to be verified as *H. scabra* despite the presence of the additional ossicles.

There were at least four different shapes of ossicle extracted from the ventral cuticle of *S. horrens* specimen from Kudat, Sabah (Figure 6). The morphospecies name of the specimen was determined based on the information given by the collectors. Even though its body became deformed prior to the ossicle extraction (Figure 2a), the ossicles were still observable. Figure 6 shows the presence of a large boomerang-shaped rod (Figure 6a), a large perforated plate (Figure 6b), a table (Figure 6c) and an X-shaped rosette (Figure 6d). These findings were supported by Kamarudin and Mohamed Rehan (2015), who also recorded the same observation for three *S. horrens* specimens from Pangkor Island, Perak, Malaysia. However, Massin et al. (2002) listed a C-shaped rod as one of the ossicle shapes in the ventral cuticle of *S. horrens* from Pulau Aur, Johor; this was not observed in this study. The body deformation of *S. horrens* in this study could have led to the absence. Notwithstanding the absence of the C-shaped rod, Kamarudin and Mohamed Rehan (2015) successfully confirmed the species status of the specimens from Pangkor Island, Perak as *S. horrens* using the cytochrome c oxidase I (COI) mitochondrial DNA (mtDNA) gene-sequencing technique.

Regarding the specimen of *S. ocellatus* from Kudat, Sabah, at least five ossicle shapes were recorded i.e. C-shaped rod, X-shaped rod, rosette, X-shaped rosette and table (Figure 7). Nonetheless, the large rod and large plate shapes were not observed in this study although the two shapes were recorded by Massin et al. (2002). In the specimen of *S. horrens* from Kudat, Sabah, the body deformation of *S. ocellatus* (Figure 2b) in this study could have led to the absence. According to Toral-Granda (2005), the ossicles of *Isostichopus fuscus* samples in the forms of fresh, salted and dried specimens showed no difference in

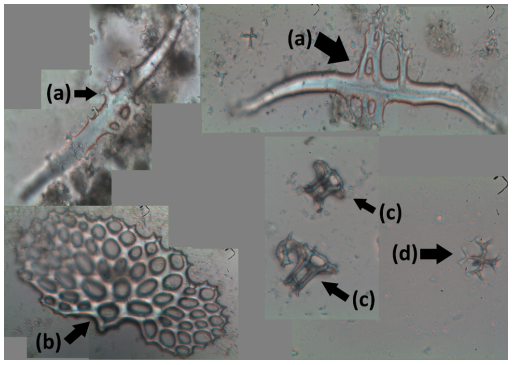

Figure 6. Ossicle shapes of *Stichopus horrens* specimen from Kudat, Sabah, Malaysia. (a) large boomerang-shaped rod, (b) large perforated plate, (c) table, and (d) X-shaped rosette

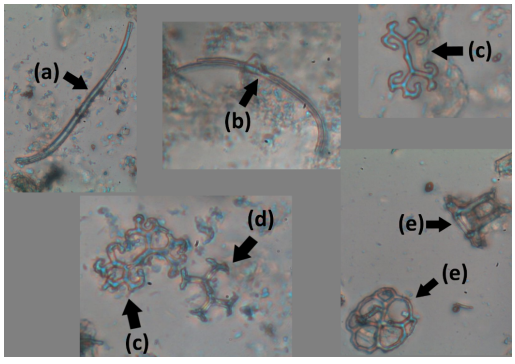

Figure 7. Ossicle shapes of *Stichopus ocellatus* specimen from Kudat, Sabah, Malaysia. (a) C-shaped rod, (b) X-shaped rod, (c) rosette, (d) X-shaped rosette, and (e) table

terms of the proportion, size and shape. The results suggested that ossicle shape as a feature is still useful and informative for morphological identification of sea cucumber in any form. The observable ossicles from *S. horren*s and *S. ocellatus* specimens from Kudat, Sabah in this study further support this suggestion.

However, in the absence of common ossicles, the DNA sequencing technique using mtDNA genes, for instance, is required to confirm the species status of a sea cucumber specimen. For this reason, non-protein-coding 12S mitochondrial rRNA gene sequencing was incorporated in this study. Approximately 360 bp non-protein-coding 12S mitochondrial rRNA gene fragments were successfully amplified. In terms of DNA sequencing results, a range of 327-372 nucleotide bases of the 12S mitochondrial rRNA gene was successfully obtained except for specimen PKS 30. The DNA sequencing failure for specimen PKS 30 was due to the presence of bad sequence parts. Moreover, the blastn results showed that the specimens of morphospecies *H. scabra* were specifically identified as *H. scabra* with identity scores (Ident) ranging from 98% to 100% when aligned against the corresponding sequence (GenBank accession number: KP257577), while the specimen of morphospecies S. horrens was specifically identified as *S. horrens* with an Ident of 99% when aligned against the corresponding sequence (GenBank accession number: HQ000092). Interestingly, the morphospecies specimen, *S. ocellatus*, was identified only up to the genus level i.e. genus *Stichopus*, with an Ident of 96% when aligned against the corresponding sequence (GenBank accession number: HM853683.2, an unknown *Stichopus* species). However, the morphospecies identification based on outward body appearance and the information given by the collectors suggested that the specimen was *S. ocellatus*. The score of Query cover for the blastn of the morphospecies *S. ocellatus* was 99% and the Expect value (E value) was $2e^{-160}$, showing the most significant score and alignment with the corresponding sequence. The other scores were maximum score and total score; each was 575. Therefore, the findings suggested that 12S mitochondrial rRNA gene sequence for *S. ocellatus* was lacking in the GenBank until 15 September, 2016. The genetic information using the non-protein-coding 12S mitochondrial rRNA gene supported the species status of the specimens as *H. scabra* (the *timun laut* species), *S. horrens* and *S. ocellatus* (the *gamat* species) as suggested earlier through the morphological information. All the 31 partial sequences of the 12S mitochondrial rRNA gene were registered with the GenBank, NCBI, U. S. National Library of Medicine (Accession No.: KX913672-KX913702). The results from ossicle shape observation as well as the non-protein-coding 12S mitochondrial rRNA gene sequencing complemented each other for a more concrete conclusion.

Despite the fact that more specimens, especially for *S. horrens* and *S. ocellatus* (the *gamat* species), and more molecular techniques are required to generate better

conclusive outcomes, the current findings gave a better insight into the importance of morphological and molecular approaches and the present status of H. scabra (the *timun laut* species) and the two *gamat* species in Kudat, Sabah, Malaysia. The non-protein-coding 16S mitochondrial rRNA gene is among the most common genes used in genetic studies. It was suggested as being able to correlate the relationship between the morphology and genetics of sea cucumber (Clouse et al., 2005; Kerr et al., 2005). Moreover, protein-coding genes such as COI and *cytochrome b* mtDNA genes are also common in genetic studies (Kamarudin & Mohamed Rehan, 2015; Kamarudin & Esa, 2009) and useful for confirming results from non-protein-coding mitochondrial rRNA gene sequencing.

CONCLUSION

Ossicles were successfully extracted from the specimens of *H. scabra, S. horrens* and *S. ocellatus*, even though the specimens of *S. horrens* and *S. ocellatus* became deformed prior to the analyses. Rod, plate, rosette, button and table were the five main ossicle shapes extracted from the specimens. However, a number of ossicle shapes for *S. horrens* and *S. ocellatus* recorded in the previous studies were not observed in this study; this could have been due to the body deformation of the specimens. In terms of ossicle varieties, five specimens of *H. scabra* showed additional ossicle shapes other than the common shapes i.e. smooth button and table. Despite the presence of additional ossicle shapes and the absence of common ossicle shapes, all the specimens from Kudat, Sabah, Malaysia remained to be morphologically identified and verified as *H. scabra* (the *timun laut* species), *S. horrens* and *S. ocellatus* (the *gamat* species). Generally, species identification and species status verification using ossicles have continued to be useful. In addition, 12S mitochondrial rRNA gene sequences analysed using the blastn resulted in species identification of the specimens of morphospecies *H. scabra* and *S. horrens* as *H. scabra* and *S. horrens*; however, the specimen of morphospecies *S. ocellatus* was only identified as being from genus *Stichopus*, showing that 12S mitochondrial rRNA gene sequences of *S. ocellatus* was lacking in the GenBank until 15 September, 2016. A number of 31 partial sequences of 12S mitochondrial rRNA gene were registered with the GenBank (Accession No.: KX913672-KX913702). Despite the absence of common ossicles and the presence of additional ossicle shapes, the three morphospecies were morphologically and genetically verified as *H. scabra, S. horrens* and *S. ocellatus*. Moreover, the current findings gave a better insight into the importance of morphological and molecular approaches and the present status of the *timun laut* species and *gamat* species in Kudat. More specimens of different species and molecular techniques are required in order to generate better conclusive outcomes in the future. Future studies may focus on this.

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