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Molecular Taxonomy of Sea Cucumbers (Holothuroidea) in the Upper Parts of the Straits of Malacca Inferred from 16S rRNA Gene Sequences

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ABSTRACT: Sea cucumbers (Holothuroidea) are important in Malaysia due to their diversity and commercial value. The sequencing of a 465 base pair (bp) region of the 16S rRNA was utilized to characterize and identify sea cucumbers from the northern region of the Straits of Malacca. The objective of this study was to infer the phylogenetic relationship of the different species collected, based on the DNA sequencing of the mitochondrial 16S rRNA gene. In all 21 samples were extracted, analysed and sequenced for this gene. The sequences were aligned and edited using MEGA 4, followed by the construction of neighbour-joining and maximum parsimony trees. Three genera were identified: namely *Holothuria* and *Stichopus* from the order Aspidochirotida, and the genus *Molpadia* from the order Molpadia. Polymerase Chain Reaction (PCR) gave the desired products of the amplified 16s rRNA mtDNA region with lengths of approximately 525 bp. Multiple aligned partial sequences of 16s rRNA mtDNA ranging from 464 bp to 465 bp were utilized for further analyses. Twenty one ingroups of sea cucumbers and one outgroup represented by *Mespilia globulus* were included in the construction of neighbour-joining (NJ) and maximum parsimony (MP) phylogenetic trees. Further studies are required to clarify the taxonomic status of this group using other molecular markers with additional samples from other sites.

KEY WORDS: holothuroidea, diversity, molecular tools, 16S rRNA gene.

INTRODUCTION

The Holothuroidea (sea cucumbers) are marine animals belonging to one of the five extant classes of echinoderms. According to Brusca and Brusca (2003), they are distributed throughout the world's oceans with about 1,500 species in 6 orders and 25 families. Sea cucumbers have fleshy bodies that are elongated into a sausage-like shape (Brusca and Brusca, 2003). They inhabit soft sediment in intertidal and reef environments (Byrne *et al.*, 2010; Rowe and Doty, 1977; Conand, 1990; Rowe and Gates, 1995; Massin, 1999, 2007; Rowe and Richmond, 2004).

Sea cucumber orders are distinguished on the basis of gross morphological characteristics such as the morphology of the tentacles, the

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presence or absence of tentacular retractor muscles, the presence or absence of an internal respiratory tree, and the distribution of podia on the trunk. At the familial level, taxonomic distinctions are dependent on a finer level of characteristics such as the morphology of dermal ossicles, the form of the esophageal calcareous ring, and the distribution and the morphology of the tube feet (Kerr, 2001; Kerr and Kim, 2001). Identification to species level poses a more challenging task, due to close similarities in the morphology of different species (Massin, 2007; Byrne, Rowe and Uthicke, 2010). Recent studies of the diversity and distribution of sea cucumbers in Malaysia had been done mainly by Forbes *et al.* (1999), Choo (2004), Zulfigar *et al.* (2008) and Kamarul *et al.* (2009), who focused on the Malaysian Peninsula. These studies identified sea cucumbers by using only gross outer morphological characteristics, leaving a number of unresolved species complexes (Massin and Lane, 1991). Among them are the most dominant orders of sea cucumbers in Malaysian waters belonging to the genera *Acaudina, Holothuria* and *Stichopus* which form an important part of a multi-species invertebrate fishery in this region.

The difficulties in identifying sea cucumbers by classical taxonomic methods may be overcome by molecular methods. Molecular tools are very important to clarify taxonomic uncertainty because DNA data can resolve evolutionary history and relationships. DNA marker technologies are widely used, especially in the aquaculture field (Liu and Cordes 2004). In this study, the 16S rRNA mtDNA marker was selected because of its slow evolutionary rate, highly conserved nature, and because it has higher copy numbers than nuclear DNA (Sigang *et al.*, 2011). It is therefore useful for clarifying taxonomic status at species and higher taxonomic levels (Curole and Kocher, 1999; Liu and Cordes, 2004). DNA profiling analysis through the mtDNA gene sequencing technique has lately gained much attention in fisheries science (Lin *et al.*, 2006) and can be applied across a broad range of organisms.

MATERIALS AND METHODS

Sample collection and DNA extraction

Specimens of sea cucumbers were collected by SCUBA diving in the sub tidal area of the coast, from fisherman, and by hand in the intertidal zone (Woo, 2010). Locations sampled were from the northern region of the Straits of Malacca covering seven sampling sites off the coasts of Kedah, Penang and Perak (Table 1). The live specimens were relaxed in a bucket of sea water with menthol crystals added to ensure that tentacles, papillae and tube-feet remained extended. After a two to three hour relaxing period, color photographs of the ventral and dorsal sides of the body were taken. The specimens were then preserved by injecting a

small amount of 95% alcohol into the body and then submerging them in 95% alcohol. Identification was done by comparison with *in situ* photographs by Desurmont (2003) and Zulfigar *et al.* (2008). All voucher specimens and corresponding photographs were deposited in the museum collection at the Marine Science Laboratory, Universiti Sains Malaysia and registered accordingly (Table 1).

Approximately 20 mg of tentacle tissue was used for DNA extraction using a modified CTAB (cetyltrimethylammonium bromide) procedure as described by Grewe *et al.* (1993). The concentration of DNA is known to affect banding patterns during amplification. Therefore, quantification of DNA is very important. In many procedures it is necessary to know the amount of DNA that is present. Therefore, the extracted DNA was checked by spectrophotometry for purity and quantity. All samples were adjusted to 40 ng/ml. The purity values obtained ranging from 1.8 to 2.3 based on OD_{260}/OD_{280} were considered to be satisfactory providing DNA free from contamination from protein or RNA.

PCR amplification and sequencing

The amplifications were carried out in a thermo cycle PCR (G-storm) machine. The optimization of PCR was done using 25 µl reaction volumes containing 15.35 µl of sterilized dH_2O, 2 µl of DNA (~15-20 ng), 10X PCR buffer (Promega), 2.0 µl of 10 mM dNTP mix (Promega), 1.5 mM $MgCl_2$ (Promega), 2.5 pmol of each primer (1st Base) and 0.4 µl *Taq* polymerase (Promega). A pair of 16S rRNA primers (Kessing *et al.*, 1989) were used for amplification of the partial region of this gene: 16sar-L (forward) 5′CGCCTGTTTATCAAA AACAT-3′ and 16sbr-H (reverse) 5′CCGGTCGAACTCAGATCACGT -3′. Amplification was set for 40 cycles using the following PCR profile: 60 seconds at 95 °C for initial denaturation, 30 seconds at 95 °C for denaturation, 30 seconds at 50 °C the optimized temperature for annealing, 80 seconds at 72 °C for extension, and 10 minutes at 72 °C for final extension (Atif *et al.*, 2008). Success of DNA amplification was determined by electrophoresis. The PCR product was purified (Promega purification kit) according to the manufacturers' instructions. Sequencing reaction products were purified by Promega Wizard ® SV gel and PCR clean up system. DNA for each sample was sequenced in both directions and the amplicon purification for all sequencing was done by service provider (1st BASE DNA sequencing service; Kuala Lumpur, Malaysia).

Sequence alignment for phylogenetic analysis

The fluorescent based DNA sequences were viewed by Chromas Lite (Version 2.1) before using the Basic Local Alignment Seared Tool (BLAST) in the GenBank in order to find corresponding sequences. This

programme is based on the E-value and Score (S) of the partial DNA sequence. All the sequences were aligned by using CLUSTAL X according to Thompson *et al.* (1997) under default parameters and manually confirmed by eye.

The reconstruction of neighbour-joining (NJ) and maximum parsimony (MP) trees was done by MEGA 4.0 (Tamura *et al.*, 2007, Kumar *et al.*, 2008). The Kimura 2-parameter distance method was incorporated to reconstruct the neighbour-joining (NJ) tree based on equal base frequencies and equal ratio of transition to transversion (ti/tv). Optimality criterion parsimony was performed using the bootstrap method with heuristic search. Neighbour-joining (NJ) analysis is a distance method that associate with evolutionary where its keeps track and distance between each pair of taxa (Felsenstein, 1981; Felsenstein, 1983a,b) while the maximum parsimony (MP) analysis was based on the character method (Hall, 2001). Phylogenetic confidence was estimated by bootstrapping (Felsestein, 1985) with 1000 sequence replications and 100 data sets. The constructed phylogenetic tree was displayed and edited by Tree-View (Win 32) Version 1.6.6. The *Mespilia globulus* (sea urchin) was chosen as the outgroup for this study.

Table 1. Presumed species of Holothuroidea analysed for the 16S rRNA genes with location and GenBank accession numbers.

Species	Location	Coordinates	Accession Number 16S
Holothuria atra	Pulau Sembilan, Perak	4°09'00.6"N	JN120767
	Pulau Sembilan, Perak	100°19'51.9"E	JN120768
Holothuria leucospilota	Teluk Datai, Langkawi, Kedah	10°30'6.0"N 99°40'54.4"E	JN120769
	Pulau Sembilan, Perak	4°09'00.6"N 100°19'51.9"E	JN120770

Table 1. Presumed species of Holothuroidea analysed for the 16S rRNA genes with location and GenBank accession numbers. (Continued).

Species	Location	Coordinates	Accession Number 16S
Acaudina molpadioides	Balik Pulau, Penang	5°11'45.6"N 100°07'14.5"E	JN120771
	Sungai Udang, Kedah	5°29'34.4"N 100°13'20.2"E	JN120772
	Sungai Udang, Kedah		JN120773
	Balik Pulau, Penang	5°11'45.6"N 100°07'14.5"E	JN120774
	Balik Pulau, Penang		JN120775
	Sungai Udang, Kedah	5°29'34.4"N 100°13'20.2"E	JN120776
	Sungai Udang, Kedah		JN120777
	Sungai Udang, Kedah		JN120778
	Sungai Udang, Kedah		JN120779
	Sungai Udang, Kedah		JN120780
Stichopus chloronotus	Pulau Payar, Kedah	6°03'59.4"N 100°02'17.2"E	JN120781
	Pulau Sembilan, Perak	4°09'00.6"N 100°19'51.9"E	JN120782

Table 1. Presumed species of Holothuroidea analysed for the 16S rRNA genes with location and GenBank accession numbers. (Continued).

Species	**Location**	**Coordinates**	**Accession Number 16S**
Stichopus horrens	Song – Song island, Kedah	5°48'30.0"N	JN120766
	Song – Song island, Kedah	100°17'39.6"E	JN120785
	Song - Song Island, Kedah		JN120786
Stichopus hermanni	Pulau Sembilan, Perak	4°09'00.6"N	JN120783
	Pulau Sembilan, Perak	100°19'51.9"E	JN120784

RESULTS AND DISCUSSION

An approximately 465 bp length of a partial region of the 16S rRNA gene was successfully amplified. The Basic Local Alignment Search Tool (BLAST) program provided by GenBank was used to search for sequences corresponding to the ones obtained in this study. No corresponding sequences were found in the BLAST analysis but the results still indicate that all the 21 DNA sequences of the 16S mtDNA gene are sequences of sea cucumbers as they are genetically close to sequences of other sea cucumbers from three families, namely Stichopodidae, Holothuriidae and Caudinidae.

In the next step, all 21 sequences were aligned to each other prior to analyses. A multiple alignment file using the default setting was generated by CLUSTAL X software (Thompson *et al.,* 1997). This setting was used because increasing the gap penalty made no improvement to the alignments but instead reduced the number of conserved regions along the sequence. Some of the gaps were created as a result of misalignment which was related to the software algorithm. In this case, realignment of sequences using the BioEdit software was applied, since alignment with gaps should be minimized.

In this study, the Caudinidae family showed the most variation from the other sequences recorded from the tree produced. Some moderately large segments with highly or fairly conserved regions were detected in the sequence. In these regions, only a few variations in bases were observed. Large gaps were observed between bases ~168 to ~219 and

bases ~255 to ~304 due to insertion of about 22 bases and 21 bases of nucleotides in the family Stichopodidae. The formation of gaps at other positions along the sequence was less obvious. Next, the multiple sequence alignments were executed and proceeded into neighbour-joining (NJ) and maximum parsimony (MP) analysis for phylogenetic analysis tree construction (Tamura *et al.*, 2007, Kumar *et al.*, 2008).

Phylogenetic analyses

a) Neighbour-joining (NJ) tree analysis

This Neighbour-joining analysis showed that there are three main clusters formed comprising the genera *Acaudina*, *Holothuria* and *Stichopus*. This condition is due to the grouping of a few individuals of *S. horrens, S. hermanni* and *S. chloronatus* into the cluster that contain *Stichopus* species, grouping the individuals of *H. atra* and *H. leucospilota* into the cluster that contain *Holothuria* species and to grouping all *Acaudina molpadioides* into the *Acaudina* group. The genus *Acaudina* from the order Caudinidae is genetically close to *Stichopus*. Even though all individuals in the primary cluster of the genus *Acaudina* were morphologically identified as *Acaudina molpadioides*, a few individuals of the same species were not clustered together and perform a minor cluster support by a 60 % bootstrap value.

b) Maximum parsimony (MP) analysis

Maximum parsimony analysis of the partial 16S rRNA sequence data set comprising of 21 taxa recorded a single most-parsimonious tree rooted when all characters were equally weighted, gap coded as missing data and tree rooted with the genera *Acaudina*, *Holothuria* and *Stichopus*. The phylogenetic tree is best described with a tree length (L) of 460 steps, a consistency index (CI) of 0.774, retention index (RI) of 0.899 and rescaled consistency index (RCI) of 0.695 as presented in Table 2.

Among the 525 characters, 460 were constant. A total of 0.748 sites was variable characters were parsimony-informative for informative consistency index (iCI) while 0.899 sites were considered as parsimony-informative for informative retention index and lastly informative rescaled consistency index (iCRI) recorded as 0.672 sites. The number of bootstrap replicates was 1000. The strict consensus of the tree is presented in Figure 2 with bootstrap values at the appropriate internodes. The outgroup species *Mespilia globulus* (sea urchin) was chosen to form a sister group to the ingroup investigated. Three main clusters forming minor clusters or subclades A to C were formed with *Mespilia globulus* as a sister taxon to all other sea cucumber species.

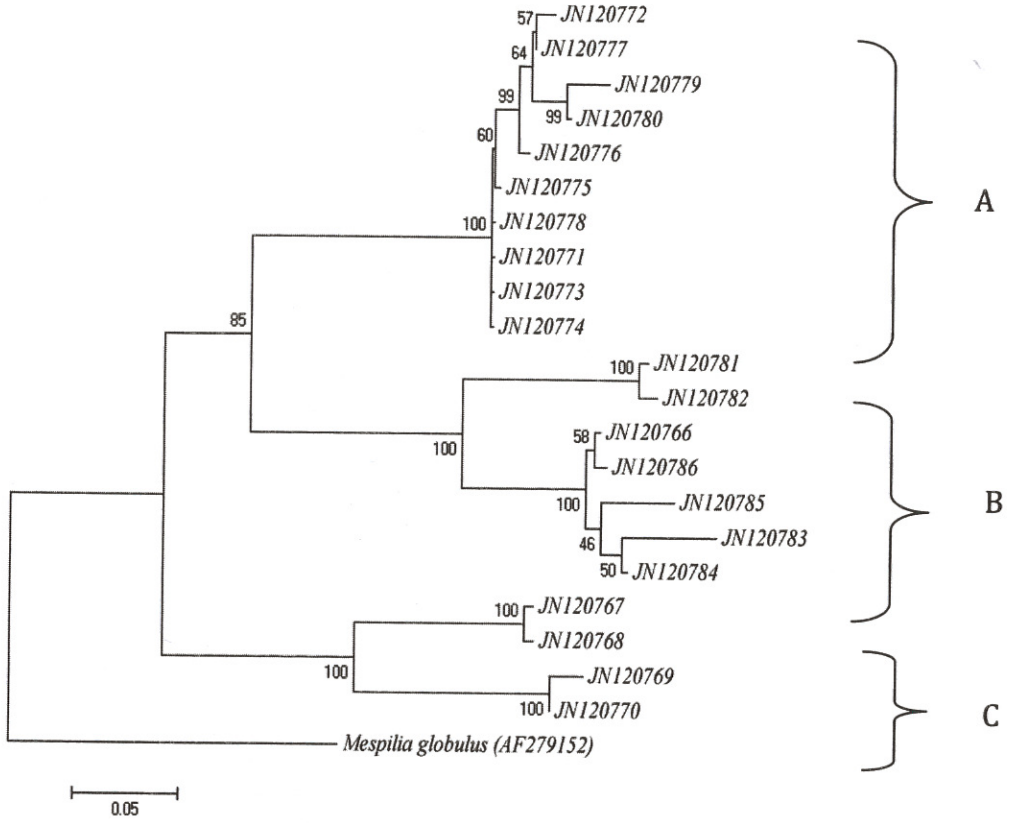

Figure 1. Phylogenetic relationships of the Holothuroidea based on the partial 16S rRNA gene (465 bp) generated by the neighbour-joining (NJ) method. Values at nodes represent bootstrap confidence levels (1000 replicates).

Table 2. Phylogenetic tree description.

Description	Value
Tree length (L)	460
Consistency Index (CI)	0.774
Retention Index (RI)	0.899
Rescaled Consistency Index (RC)	0.695

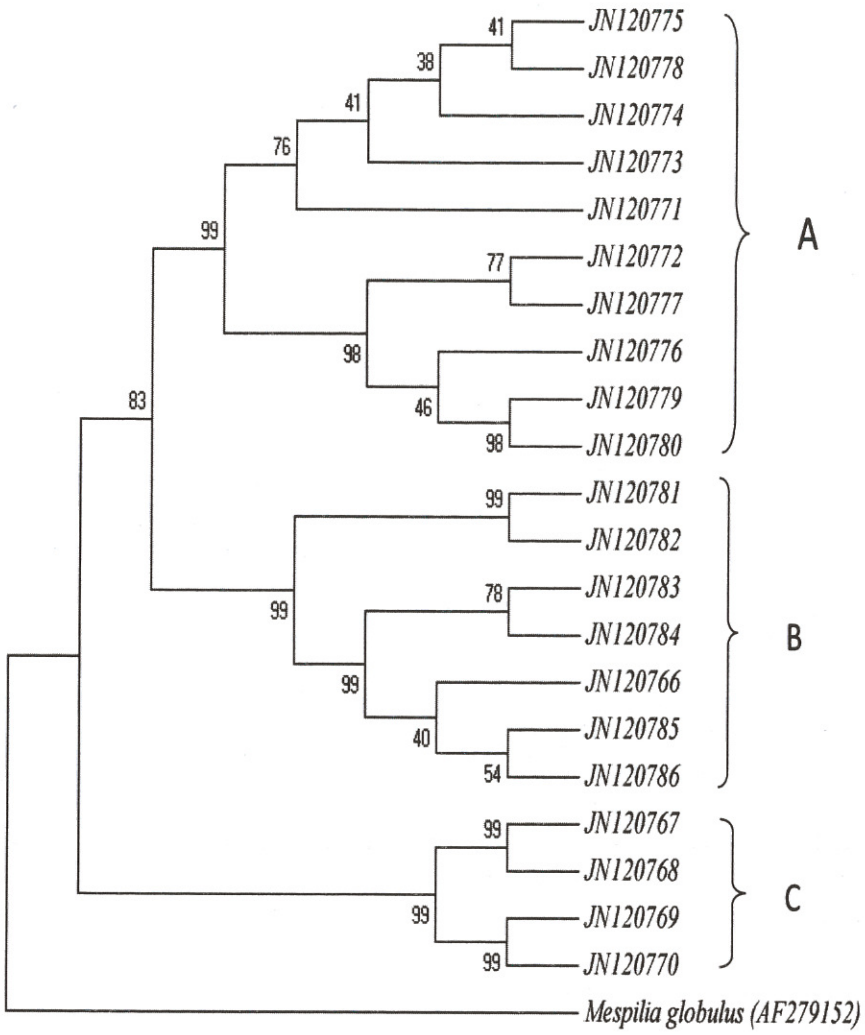

Figure 2. Phylogenetic relationships of the Holothuroidea based on the partial 16S rRNA gene (465 bp) generated by Maximum Parsimony (MP) method. Values at nodes represent bootstrap confidence level (1000 replicates). A: genus *Acaudina* ; B: genus *Stichopus* ; C: genus *Holothuria*.

Just as in the neighbour-joining (NJ) analysis, all three genera were well resolved with high bootstrap value (99%). In fact each species formed its own monophyletic assemblage in general agreement with neighbour-joining (NJ) analysis. However, while there was a slight ambiguity with the *S. horrens* (JN120785) which grouped with the *S. hermanni* subcluster in the neighbour-joining (NJ) analysis, the maximum parsimony (MP) analysis was well resolved for these two species. Again, as shown in the neighbour-joining (NJ) analysis, *Stichopus* and

Acaudina were closely related while *Holothuria* formed the basal and presumably ancestral group.

The first cluster branched into 3 major clusters. Minor cluster A grouped all Caudinids. The A cluster is divided into 2 minor clades and these clades formed from Balik Pulau Penang and Sungai Udang, Kedah. The first mini clade with 76 % bootstrap support consists of specimens JN120774, JN120778, JN120773, JN120771 and JN120775. The specimens were collected from Balik Pulau, Penang and there are two specimens, JN120778 and JN120773, collected from Sungai Udang, Kedah. The second minor clade was from the other individuals (JN120772, JN120777, JN120776, JN120779 and JN120788) which were collected from Sungai Udang, Kedah, Malaysia formed the second subclade with 98% bootstrap support. However, the bootstrap value at the internodes connected both subclades as sister group of minor cluster 99 %. Each species (as morphologically identified) in this minor B cluster was grouped into its own sub clusters with high confidence levels; *Stichopus chloronotus* (99 %), *Stichopus hermanni* (78 %), and *Stichopus horrens* (40 %). Two subclades were formed by *Stichopus horrens*. This subclade was weakly supported by a bootstrap value of 54 %. However, *S. horrens* species and phylogenetic relationship could not be confirmed as the more basal internodes showed <50 % bootstrap values. *Stichopus chloronotus* (accession number: JN120781 and JN120782) was found in Pulau Payar, Malaysia and formed a taxon with 99% bootstrap value. *S. chloronotus* and *S. hermanni* were grouped with high confidence level. *S. hermanni* (accession number JN120784 and JN120783) collected from Pulau Sembilan, Perak, Malaysia recorded moderate support at 78% bootstrap value.

Some population structuring was observed in the Caudinids where the Balik Pulau, Penang populations (Accession nos JN120775, JN120774, and JN120771) formed one subcluster with the inclusion of two Sungai Udang, Kedah individuals (JN120778 and JN120773). The other subcluster was formed of only Sungai Udang, Kedah individuals. Two samples in the first subcluster were collected from fisherman nets in Sungai Udang, Kedah, and according to the fisherman that provided the specimens, they were from the Penang area. Due to the limited sample sizes, population structuring could not be investigated in *Stichopus* and *Holothuria*.

In the cluster C all Holothuriidae grouped as a monophyletic lineage. As observed in the dendrogram, *Holothuria atra* (JN120767 and JN120769) and *H. leucospilota* (JN120769 and JN120770) formed the ancestral taxon with 99 % bootstrap each. The first minor cluster compromised of *H. atra*. The next minor subclade was made up of *H. leucospilota* individuals with a 99 % bootstrap value.

Interspecies Genetic Variation

Genetic distance among individuals of Holothuroidea species lies in the range of 0.002 – 0.460. The highest divergence occurred between *S. hermanni* and *H. leucospilota* taxa. Referring to the distance matrix generated, *H. leucospilota* has a very distant relationship with all other species, having genetic distance mean values of around 0.36. Even pair wise comparisons of *Acaudina molpadioides*, the outgroup from the family Caudinidea, with the other taxa had much lower genetic distances of 0.01. All other pair wise comparisons within the Stichopodid group were <1.

A good example of the value of molecular taxonomy is the differentiation of *Stichopus hermanni* and *Stichopus horrens* and between *Holothuria atra* and *Holothuria leucospilota*. Both pairs of sea cucumbers have a lot of similarity in morphology, such as body coloration, types of tentacles and also the cross section of their bodies. A molecular marker is essential to address the uncertainties between different species which look similar morphologically. Molecular data is the most efficient tool in resolving the taxonomy of sea cucumbers. The 16S rRNA gene has proven in the past to be a good marker in resolving species identification and differentiation (Vences *et al.*, 2005a,b).

CONCLUSION

In conclusion, this molecular study shows that the 16S rRNA gene was successful in addressing some of the taxonomic and systematic issues of sea cucumber classification. However, more samples are required from various genera and families in future to generate more data both morphologically and genetically to resolve the remaining taxonomic ambiguities and provide better insight into the phylogeny of sea cucumbers in Malaysian waters.

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