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# Species Identification and Molecular Phylogenetics of Processed Sea Cucumbers from Malaysian Market based on 12S Mitochondrial rRNA Gene

**Kamarul Rahim Kamarudin[1*], Maryam Mohamed Rehan[1] and 'Aisyah Mohamed Rehan[2]**

*[1]Department of Technology and Natural Resources, Faculty of Applied Sciences and Technology (FAST), Universiti Tun Hussein Onn Malaysia (UTHM), Pagoh Campus, Pagoh Education Hub, Km 1, Jalan Panchor, 84600 Muar, Johor Darul Takzim, Malaysia*

*[2]Department of Biotechnology, Kulliyyah of Science, International Islamic University Malaysia (IIUM), Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang Darul Makmur, Malaysia*

## ABSTRACT

Extensive processing of sea cucumber causes body deformation of the marine organism, hence causing difficulties in species identification of processed sea cucumbers. Due to the copresence of cases of unlabelled or mislabelled sea cucumber products in Malaysian markets and worldwide, a study was conducted to determine the species identities of processed sea cucumbers from selected Malaysian markets using non-protein-coding 12S mitochondrial rRNA gene. Phylogenetic analyses based on the distance-based Neighbour Joining method, and the character-based methods i.e. the Maximum Parsimony method, Maximum Likelihood method, and the Bayesian Analysis method of 81 ingroup sequences representing 63 processed sea cucumber specimens, 13 fresh and processed reference samples for species identification, and five fresh additional specimens from Teluk Nipah Beach, Pangkor Archipelago and Manukan Island, Sabah suggested the presence of three main clusters i.e. a *gamat* family cluster consisting of family Stichopodidae (*Stichopus horrens*, *Stichopus vastus*, and *Thelenota anax*) and two clusters of *timun laut* family comprising family Holothuriidae (*Holothuria (Mertensiothuria) leucospilota*, *Holothuria (Metriatyla) scabra*, *Holothuria (Metriatyla) lessoni*, *Holothuria (Halodeima) atra*, and *Holothuria (Halodeima) edulis*) and family Caudinidae (*Acaudina molpadioides*). The outcomes of this study also highlighted the availability of 40 new 12S mitochondrial rRNA gene sequences deposited in the

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*E-mail addresses:*
physique481@yahoo.co.uk (Kamarul Rahim Kamarudin)
maryam@usim.edu.my (Maryam Mohamed Rehan)
mraisyah@iium.edu.my ('Aisyah Mohamed Rehan)
* Corresponding author

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GenBank that can be utilised by the enforcement agencies to monitor and overcome the issues of species substitution and product mislabelling of processed sea cucumber products in Malaysian markets.

*Keywords*: 12S rRNA gene, *gamat*, Malaysian markets, phylogenetic analysis**,** processed sea cucumber, *timun laut*

## INTRODUCTION

Species substitution of commercial marine products is one of the critical issues in the seafood industry. Growing international trade, increase in global seafood consumption, fluctuations in the food supply and demand of different marine-dwelling species are among the contributing factors of species substitution or intentional product mislabelling. Species substitution can have serious consequences, which include economic fraud, health hazards, and illegal trade of protected species (Rasmussen & Morrissey, 2008). Wen, Hu, Zhang and Fan (2011) reported the mislabelling of seven samples of commercial sea cucumber products from Guangzhou, China. In fact, China was ranked as the second world's top producer of commercial sea cucumber with 26 commercial species (Choo, 2008). Processed sea cucumbers in the forms of dried and frozen products sold in local markets in Malaysia (Kota Kinabalu, Sabah and at supermarkets in Peninsular Malaysia) were observed as mislabelled with incorrect species name, as well as missing manufacturing or packaging details for some sea cucumber-based products. Malaysia was the fourth world's top producer of commercial sea cucumber with 19 commercial species (Choo, 2008) and the occurrence of product mislabelling and species substitution could cause major economic and health problems as well as conservation issues in Malaysia.

Species identification of sea cucumber was typically done based on the external anatomy e.g. the presence and shape of feeding tentacles and tube feet; the internal anatomy e.g. the types of calcareous rings; and the microscopic observation of ossicle shapes. Among the common shapes of ossicles are perforated plate, anchor, roxette, button, rod, and table. Ossicles are the small pieces of calcified materials in the body tissues of a sea cucumber species considered informative and useful to identify the species of sea cucumbers in any forms e.g. fresh, salted, and dried forms (Toral-Granda, 2005). Nonetheless, different body parts of a particular sea cucumber species may contain different types and sizes of ossicles. Massin, Zulfigar, Hwai and Boss (2002) reported that the table-shaped ossicles in the ventral body part of *Stichopus chloronotus* was larger than the table-shaped ossicles in its dorsal body part. Dabbagh, Keshavarz, Mohammadikia, Afkhami and Nateghi (2012) reported the presence of rod-shaped ossicles in the tentacles, and table- and button-shaped ossicles in the dorsal body wall of *Holothuria (Metriatyla) scabra* from the Persian Gulf, Iran and the presence of uncommon ossicles of *H. scabra* in their specimens i.e. the branched rods. Moreover, Massin et al. (2002) also mentioned that the abundance of rosette-shaped ossicles, and

the shapes of the rod-shaped ossicles from the tube feet and dorsal papillae of *Stichopus herrmanni* were unsuitable to be used as a characteristic to differentiate the curryfish from the other species; thus indicating disadvantages of using ossicles in sea cucumber species identification. Therefore, in order to address the issues of mislabelled products and species substitution; a prompt, reliable, and reproducible molecular method is needed as an identification tool specifically for processed sea cucumbers that underwent shape deformation such as dried, frozen, canned, and pickled products as ossicle shape identification was commonly used for sea cucumber specimens with intact body forms. Approximetely, every cell in the body of a living organism has the same Deoxyribonucleic Acid (DNA). Processed sea cucumbers including the beche-de-mer are available in the forms of dried, frozen, canned, and pickled products worldwide.

Mitochondrial DNA (mtDNA) of animal has been the most preferred model for molecular genetic studies, specifically in the species identification, phylogenetic analyses, and phylogeographical analyses. According to Freeman and Herron (2004), small-subunit rRNA in the mitochondria remained an informative resource for whole-life phylogenies. One of the component of the small subunit of the mitochondrial ribosome is 12S rRNA. In fact, 12S mitochondrial rRNA gene is a non-protein-coding gene, as rRNA only produces polypeptides that are used to make up proteins. In terms of studies related to the non-protein-coding 12S mitochondrial rRNA gene of sea cucumber, only one study by Clouse, Janies and Kerr (2005) has been found to date. In the study, two morphs from the *Bohadschia marmorata* species complex in Micronesia were examined based on the spicules, colour, body size, behavior, and mitochondrial DNA including the 12S mitochondrial rRNA region in order to investigate the taxonomy of the two morphs. Clouse et al. (2005) concluded that *B. marmorata* and *B. bivittata* were not sister species, and *B. bivittata* was genetically closer to *B. argus*, the leopardfish or tigerfish; therefore *B. marmorata* and *B. bivittata* should be regarded as two separate species. The study also suggested the capability of 12S mitochondrial rRNA gene in addressing issues related to species complexity and classification.

Furthermore, there were studies on the use of non-protein-coding 12S mitochondrial rRNA gene to conduct forensics of food products. Wang, Duan and Zhang (2017) used 12S mitochondrial rRNA gene sequences to identify the animal species that were used to produce three types of aquatic and commercial collagens. Liao, Liu, Ku, Liu and Huang (2017) successfully addressed the issue of milk powder adulteration using PCR-based methods of 12S mitochondrial rRNA gene through the detection of cow milk compositions in goat milk powder. Meanwhile, Di Domenico, Di Giuseppe, Wicochea Rodríguez and Cammà (2017) developed and validated Fast real-time PCR TaqMan assays based on 12S mitochondrial rRNA gene and cytochrome b (cytB) gene for species

identification in dairy products. The studies highlighted the need of implementing molecular species identification as analytical checks on commercial and imported food products in order to address issues of species substitution and product mislabelling.

Issues related to species substitution and product mislabelling of sea cucumber-based products can be observed and investigated at some Malaysian markets. As a result, this study is aimed at determining the species identity of processed sea cucumber specimens from selected Malaysian markets by using forensically informative nucleotide sequencing (FINS) technique by Bartlett and Davidson (1992). Online Basic Local Alignment Search Tool program for nucleotide (blastn) was included to resolve the species status of the specimens based on the partial sequences of non-protein-coding 12S mitochondrial rRNA gene. There are scarce studies related to the 12S mitochondrial rRNA gene of sea cucumber that cause lack of 12S mitochondrial rRNA gene sequences of sea cucumber species in the GenBank. Phylogenetic analyses based on the distance-based method with clustering algorithm as the tree building strategy i.e. the Neighbour Joining method, and the character-based methods with optimality criterion as the tree building strategy i.e. the Maximum Parsimony method, Maximum Likelihood method, and the Bayesian Analysis method were incorporated to determine the genetic relationships of the sea cucumber species. The findings of the analyses were compared with the manufacturing or packaging details of the specimens. This study highlights the issues of intentional species substitution and product labelling of processed sea cucumbers in selected Malaysian markets. The information will be useful to tackle issues pertaining sea cucumber-based products in Malaysia.

## MATERIALS AND METHODS

### Study Site and Sampling

A total of 112 sea cucumber specimens from Pangkor Archipelago, Perak Darul Ridzuan (West Coast region in the northern part of Peninsular Malaysia, n=7); Kuah, Langkawi Archipelago, Kedah Darul Aman (North region of Peninsular Malaysia, n=7); Nilai, Negeri Sembilan Darul Khusus (South region of Peninsular Malaysia, n=8); Kuantan, Pahang Darul Makmur (East Coast region of Peninsular Malaysia, n=27); Kota Kinabalu, Sabah and Kudat, Sabah (East Malaysia, in Borneo Island, n=63, Kamarudin, Mohamed Rehan, & Bahaman, 2017a) were included (Figure 1). Three live and fresh specimens of *Holothuria (Mertensiothuria) leucospilota* from Teluk Nipah Beach, Pangkor Archipelago (HLTNP1-HLTNP3, Kamarudin & Mohamed Rehan, 2015) were collected as reference samples of fresh *timun laut* species, and three live and fresh specimens of *Stichopus horrens* from Pangkor Laut, Pangkor Archipelago (SHP1-SHP3; Kamarudin & Mohamed Rehan, 2015; Kamarudin, Mohamed Rehan, Mohd Noor, Ramly, & Mohamed Rehan, 2017b) were used as reference samples of fresh *gamat* specimens. Besides, seven dried *gamat*-based beche-de-mer specimens

from Kuah, Langkawi Archipelago (North region of Peninsular Malaysia; Figure 1; LKIG1-LKIG7; Kamarudin et al., 2017b; Kamarudin & Mohamed Rehan, 2015) were used as reference samples of processed sea cucumbers. For unlabelled specimens, more information was gained from the salespersons.

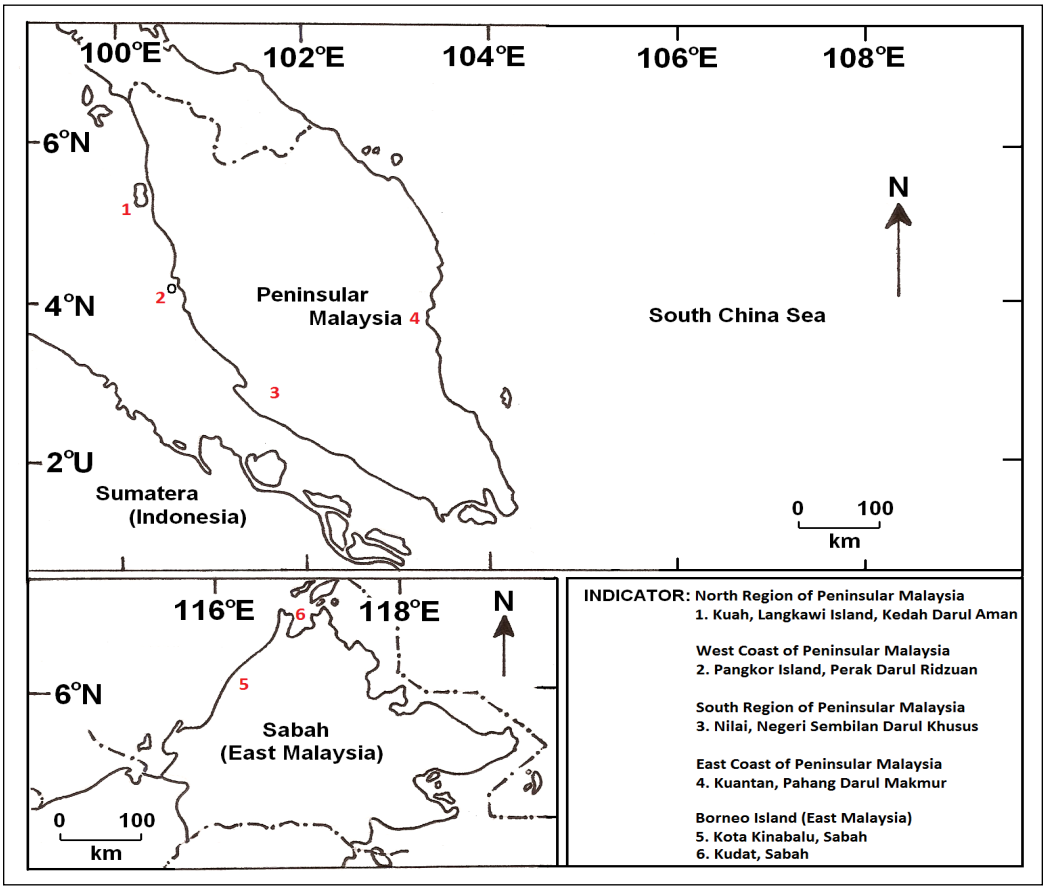


*Figure 1.* Sampling sites of sea cucumber specimens

**Morphological Species Identification**

The sea cucumber specimens were morphologically identified by referring to information provided by local residents, experts [Assoc. Prof. Alexander M. Kerr (Marine Laboratory, University of Guam, USA) and the participants of National Science Foundation (NSF) Partnerships for Enhancing Expertise in Taxonomy (PEET) Holothuroid Systematics Workshop held on 7-16 June 2010 at the Marine Laboratory, University of Guam, USA)]; Hashim (2011), Purcell, Samyn and Conand (2012), and the World Register of Marine Species database at http://www.marinespecies.org/index.php.

**Total Genomic DNA Extraction**

Three methods of total genomic DNA extraction were employed to obtain a better yield of total genomic DNA, i.e. modified cetyl trimethyl ammonium bromide (CTAB) method of Grewe et al. (1993) together with the Geneaid Genomic DNA Mini Kit (Blood/Cultured Cell), total genomic DNA extraction using the DNeasy mericon Food Kit by QIAGEN, and total genomic DNA extraction using the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit. For the second method, homogenised tissue was prepared by using the QIAGEN TissueRuptor whereby ~0.2 g tissue from each specimen was disrupted and homogenised. Furthermore, 1% agarose gel with FloroSafe DNA Stain was used to determine the approximate yield of the total genomic DNA through horizontal gel electrophoresis. The extracts were kept in 4°C chiller for short-term storage prior to the next analysis or -20°C chest freezer for long-term storage.

**Polymerase Chain Reaction (PCR)**

The non-protein-coding 12S mitochondrial rRNA gene was amplified using two methods:

(a) 25 µl PCR reaction volume using the 2x TopTaq Master Mix Kit by QIAGEN with 1.0 µl of the DNA extract

(b) 50 µl PCR reaction volume containing 33.75 µl of sterilised $dH_2O$, 5.0 µl of 10X PCR reaction buffer, 3.0 µl of 25 mM magnesium chloride, 2.5 µl of each 5 µM universal primer, 1.0 µl of 10 mM dNTP mix, 2.0 µl of the DNA extract and 0.25 µl of 5 u/µl *Taq* DNA polymerase.

The primer set for non-protein-coding 12S mitochondrial rRNA gene (Palumbi et al. (1991), expected length: ~360 bp)) is as follows:

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)

Furthermore, two batches of PCR cycle parameters were used:

(a) 2 min at 95°C for initial denaturation, 30 s at 95°C for denaturation, 30 s at optimised temperature for annealing, 45 s at 72°C for extension, repetition of step 2-4 for another 34-39 cycles, 5 min at 72°C for final extension, and forever hold at 4°C.

(b) 5 min at 95°C for initial denaturation, 45 s at 95°C for denaturation, 90 s at optimised temperature for annealing, 1 min 30 s at 72°C (60 s/kb; 29 cycles) for extension, 7 min at 72°C for final extension, and forever hold at 4°C.

**PCR Product Purification and DNA Sequencing**

The QIAquick PCR Purification Kit by QIAGEN (for direct purification of single

PCR fragment), Geneaid Gel/PCR DNA Fragments Extraction Kit (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. The unpurified PCR products were sent directly to the First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor Darul Ehsan, Malaysia since the company also provides PCR products clean up service prior to the DNA sequencing.

**Phylogenetic Analyses based on DNA Sequences**

Chromas program version 2.5.1 (Copyright© 1998-2016 Technelysium Pty Ltd) was used to display all the sequenced PCR products of the non-protein-coding 12S mitochondrial rRNA gene. Each DNA sequence was assigned to a particular sea cucumber species or genus by using the online Basic Local Alignment Search Tool program for nucleotide (blastn). ClustalX program version 2.1 (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) was used for multiple sequence alignment of forward reaction sequences before the phylogenetic tree reconstruction. Molecular Evolutionary Genetics Analysis version 7.0.14 (MEGA7; Kumar, Stecher, & Tamura, 2016) was subsequently used to reconstruct phylogenetic trees using Neighbour Joining method (a distance-based method with clustering algorithm as the tree building strategy) and Maximum Parsimony method (a character-based method with optimality criterion as the tree building strategy). Prior to the reconstruction of Maximum Likelihood phylogenetic trees, Modeltest (version 3.7) program (Posada & Crandall, 1998) was used to calculate and find the best model for DNA evolution. A number of 56 models of DNA substitution were tested in order to choose the model that fitted the data best. PAUP* (version 4.0b10) program (Swofford, 1998) with 100 bootstrap replicates was then used to reconstruct the Maximum Likelihood phylogenetic tree. Furthermore, the reconstructions of consensus Bayesian Analysis phylogenetic trees were done by using MrBayes (version 3.1.2) program (Huelsenbeck & Ronquist, 2001). The run was stopped when the standard deviation of split frequencies reached below 0.01 (Reference: Tutorial – A Simple Analysis by Huelsenbeck & Ronquist, 2001). The reconstructed phylogenetic trees were displayed and edited by using TreeView (version 1.6.6) program (Page, 1996) and paint.net 4.0.6 (Final 4.6.5693.28) program (Copyright © 2015 dotPDN LLC, Rick Brewster, and contributors).

**GenBank Registration and Accession**

In terms of GenBank submission, sequence data were prepared by using the submission entry of Sequin version 15.10 program prior to obtaining the accession numbers from the GenBank, National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine.

## RESULTS AND DISCUSSION

In the context of product labelling, no specimens of processed sea cucumbers were labelled with species details and manufacturing details were absent for most of the specimens. Intentional species substitution and mislabelling of sea cucumber products have been reported worldwide and have influenced the trading of processed sea cucumbers (Rasmussen & Morrissey, 2008). The identification of *H. atra* species i.e. PFKK6 specimen among the 16 PFKK specimens as *H. edulis* was also identified. Shape deformation of sea cucumber upon extensive processing steps could lead to such situation as the morphologies or physical appearances of all the dried PFKK specimens are similar and difficult to be differentiated from each other. Besides, such difficulty could lead to human handling errors during the packaging process. Similar issue was also observed for the *gamat*-based beche-de-mer LKIG specimens, the reference samples from Kuah, Langkawi Archipelago, Kedah whereby two species i.e. *S. horrens* and *S. vastus* were present instead of one species in a packet (Kamarudin et al., 2017b).

In terms of the number of base substitutions per site from 81 nucleotide sequences of 299 aligned positions of non-protein-coding 12S mitochondrial rRNA gene sequences, the genetic distance values between specimens identified as *S. horrens* ranged from 0 (0%) to 0.0393 (3.93%) with average genetic distance of 0.0114 (1.14%), thus suggesting their status as single morphospecies i.e. morphospecies *S. horrens*. In addition, the genetic distance values between PKSH1 specimen and other *S. horrens* specimens including the reference samples of SHP ranged from 0.0291 (2.91%) to 0.0393 (3.93%). Furthermore, the genetic distance values between KPUI7 specimen and other *S. horrens* specimens including the reference samples of SHP ranged from 0 (0%) to 0.0291 (2.91%). Meanwhile, the genetic distance value between *S. vastus* specimens i.e. PKSO1 and LKIG7 was 0.0256 (2.56%). The genetic distance values between *T. anax* specimens i.e. KKS specimens ranged from 0 (0%) to 0.0094 (0.94%) with average genetic distance of 0.0029, thus suggesting their status as single morphospecies i.e. morphospecies *T. anax*. As for the *timun laut* specimens, the average genetic distance value between the *Holothuria* specimens including the reference samples of HLTNP was 0.0380 (3.8%). The genetic distance values between *H. leucospilota* specimens ranged from 0.0062 (0.62%) to 0.0288 (2.88%) with an average genetic distance of 0.0164 (1.64%). In addition, the genetic distance values between *H. scabra* specimens ranged from 0 (0%) to 0.0128 (1.28%) with an average genetic distance of 0.0038 (0.38%), while the genetic distance values between *H. lessoni* specimen i.e. KPTS1 and *H. scabra* specimens ranged from 0.0063 (0.63%) to 0.0128 (1.28%). As for the *H. atra* specimens, the average genetic distance was 0.0068 (0.68%) ranging from 0 (0%) to 0.0094 (0.94%), with the genetic distance value between PFKK6 specimen and PM3 specimen was 0 (0%). The average genetic

distance between *H. edulis* specimens excluding PFKK6 specimen was 0.0135 (1.35%) ranging from 0 (0%) to 0.1003 (10.03%), thus suggesting their status as single morphospecies i.e. morphospecies *H. edulis*. The genetic distance values between PM1 specimen and PFKK specimens excluding PFKK6 specimen ranged from 0 (0%) to 0.0962 (9.62%). Furthermore, the genetic distance values between *A. molpadioides* specimens i.e. specimens of GK and GN ranged from 0.0062 (0.62%) to 0.0391 (3.91%). Overall, the base frequencies were unequal (i.e. Adenine (A) = 34.14%, Cytosine (C) = 21.12%, Guanine (G) = 18.00%, and Thymine (T) = 26.74%)).

With regard to the availability of the non-protein-coding 12S mitochondrial rRNA gene of sea cucumber species in the GenBank, NCBI, U.S. National Library of Medicine; this study showed the absence of DNA sequences of at least three *timun laut* species i.e. *Holothuria (Halodeima) atra*, *Holothuria (Halodeima) edulis*, and *Holothuria (Metriatyla) lessoni*; and a gamat species i.e. *Stichopus vastus*; thus showing the lack of 12S mitochondrial rRNA gene sequences of some sea cucumber species in the GenBank until May 2017. For example, the *H. edulis* specimens from Kota Kinabalu, Sabah (PFKK) were identified as *Holothuria (Metriatyla) scabra* with 89% identity value (GenBank Accession No. of corresponding matches: KP257577, except for PFKK6 specimen) based on the blastn analyses (Table 1). Meanwhile, the morphospecies *H. atra* specimens from Manukan Island, Kota Kinabalu, Sabah (PM) were identified as *Holothuria (Mertensiothuria) hilla* with 87% identity value (GenBank Accession No. of corresponding match: KX856663, except for PM1 specimen). Moreover, the *H. lessoni* specimen from Kuantan, Pahang (KPTS1) was identified as *H. scabra* with 98% identity value (GenBank Accession No. of corresponding match: KP257577) and the *S. vastus* specimen from Kudat, Sabah (PKSO1) was identified as from genus *Stichopus* with 96% identity value (GenBank Accession No. of corresponding match: HM853683). The partial non-protein-coding 12S mitochondrial rRNA gene sequences obtained from this study were registered with the GenBank, and the deposition of the sequences have contributed to the availability of more DNA sequences of different sea cucumber species in the GenBank database. Therefore, researchers in future who work when using the blastn program especially in the species identification of sea cucumber could obtain higher percentage of identity value.

Even though the Neighbour Joining tree (Figure 2) shows the formation of family Stichopodidae cluster (the *gamat* family) with 83% bootstrap support, the cluster formation of the *timun laut* family was negligible due to the very low bootstrap support i.e. 41%. Thus, the clustering of the specimens of *Acaudina molpadioides* (GN) or the *U-in* specimen from family Caudinidae with the family Holothuriidae cluster is very weak. In contrast, the Maximum Parsimony tree (Figure 3) based on the non-protein-coding 12S mitochondrial rRNA gene sequences show the formation of

Table 1
*GenBank accession number, and blastn results based on non-protein-coding 12S mitochondrial rRNA gene of sea cucumber specimens*

| Location | Type of Sample | Registration - GenBank Accession Number | Blastn Results | | Corresponding Match(s) from GenBank |
|---|---|---|---|---|---|
| | | | Query Cover | Identity Value | |
| Teluk Nipah Beach, Perak | Reference samples of *Holothuria (Mertensiothuria) leucospilota* (live and fresh) | HLTNP1-HLTNP3-KY986419-KY986421 | 98-99%; 98.67% | 98-99%; 98.33% | KX768273 |
| Teluk Nipah Beach, Pangkor Archipelago, Perak | Additional samples (live and fresh) | HL1- KX768273 | 87% | 99% | DQ777101 |
| Manukan Island, Kota Kinabalu, Sabah | Additional samples (live and fresh) | PM1- MF188877<br>PM3- MF188878<br>PM4- MF188879<br>PM5- MF188880 | PM1-95%<br>PM3-95%<br>PM4-95%<br>PM5-96% | PM1-88%<br>PM3-87%<br>PM4-87%<br>PM5-87% | PM1- KP257577<br>PM3- KX856663<br>PM4- KX856663<br>PM5- KX856663 |
| Nilai, Negeri Sembilan | Processed samples (frozen) | GN7- MF667552<br>GN8- MF188876 | 90% | 93.5% | KX856677 |
| Kuantan, Pahang | Processed samples (dried) | KPTS1- MG682240 | 95% | 98% | KP257577 |
| Kuantan, Pahang | Processed samples (frozen) | KPUI7- MG922854<br>KPUI11- MG682241 | 95%<br>88% | 99%<br>86% | HM853683<br>AY574864 |
| Kuantan, Pahang | Processed samples (frozen) | GK10- MF667553<br>GK13- MF667554 | 89% | 92% | KX856677 |
| Kota Kinabalu, Sabah | Processed samples (dried) | KKS1-KKS9 - MF188867-MF188875 | 96.56% | 96.11% | KX856681 |
| Kota Kinabalu, Sabah | Processed samples (dried) | PFKK1-PFKK16 - MG682224- MG682239 | PFKK1-PFKK5, PFKK7-PFKK16 – 91.13%<br>PFKK6-94% | PFKK1-PFKK5, PFKK7-PFKK16 – 87.8%<br>PFKK6-87% | PFKK1-PFKK5, PFKK7-PFKK16 - KP257577<br>PFKK6- KX856663 |

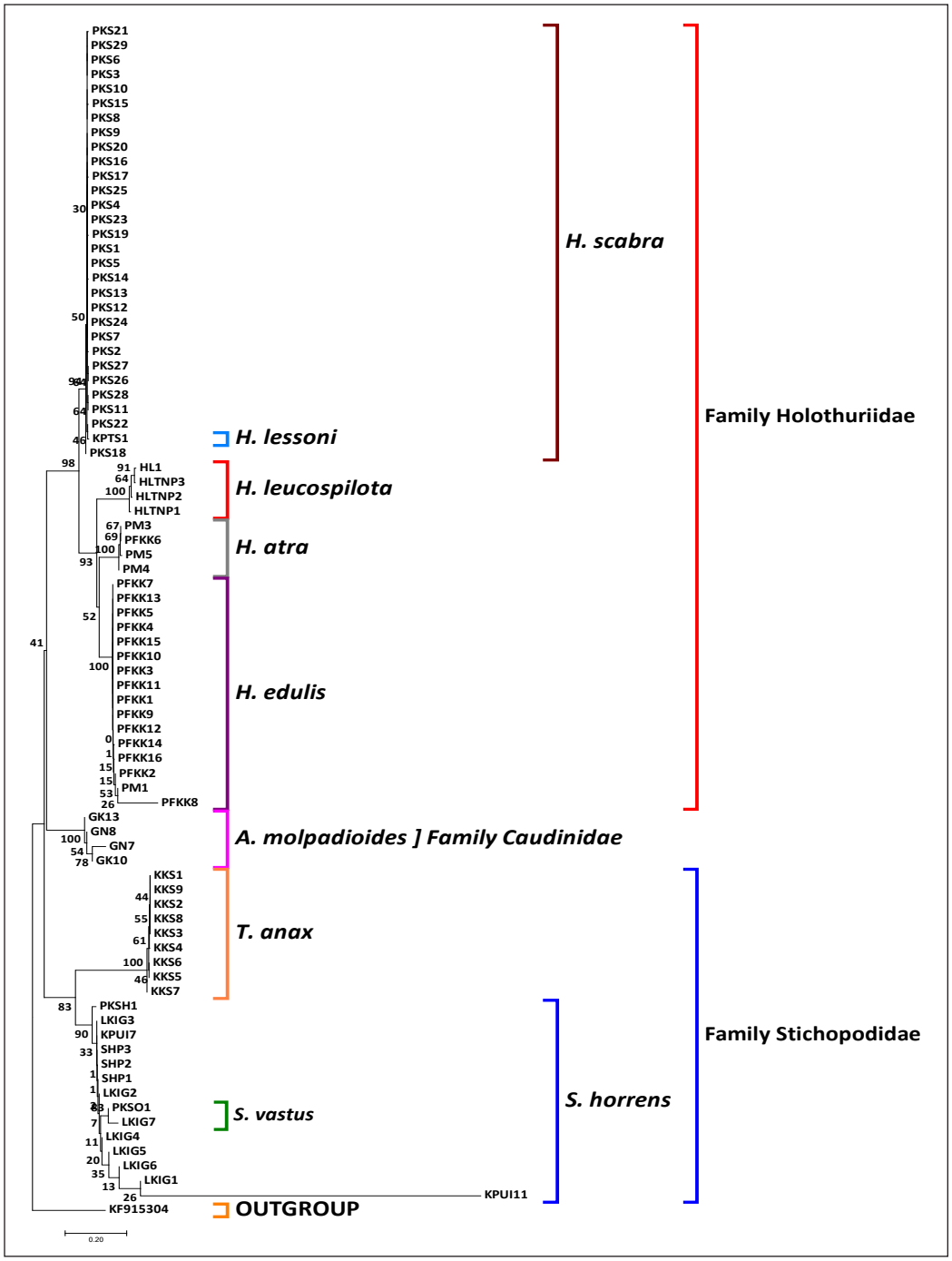


*Figure 2.* Topology of 50% majority-rule consensus tree of Neighbour Joining of sea cucumber specimens from selected Malaysian markets and other sampling sites including the reference samples and processed specimens inferred from non-protein-coding 12S mitochondrial rRNA gene sequences using MEGA7 program (Kumar et al., 2016) with 1000 bootstrap replicates. Numbers at nodes indicate the bootstrap values in percentage (%)

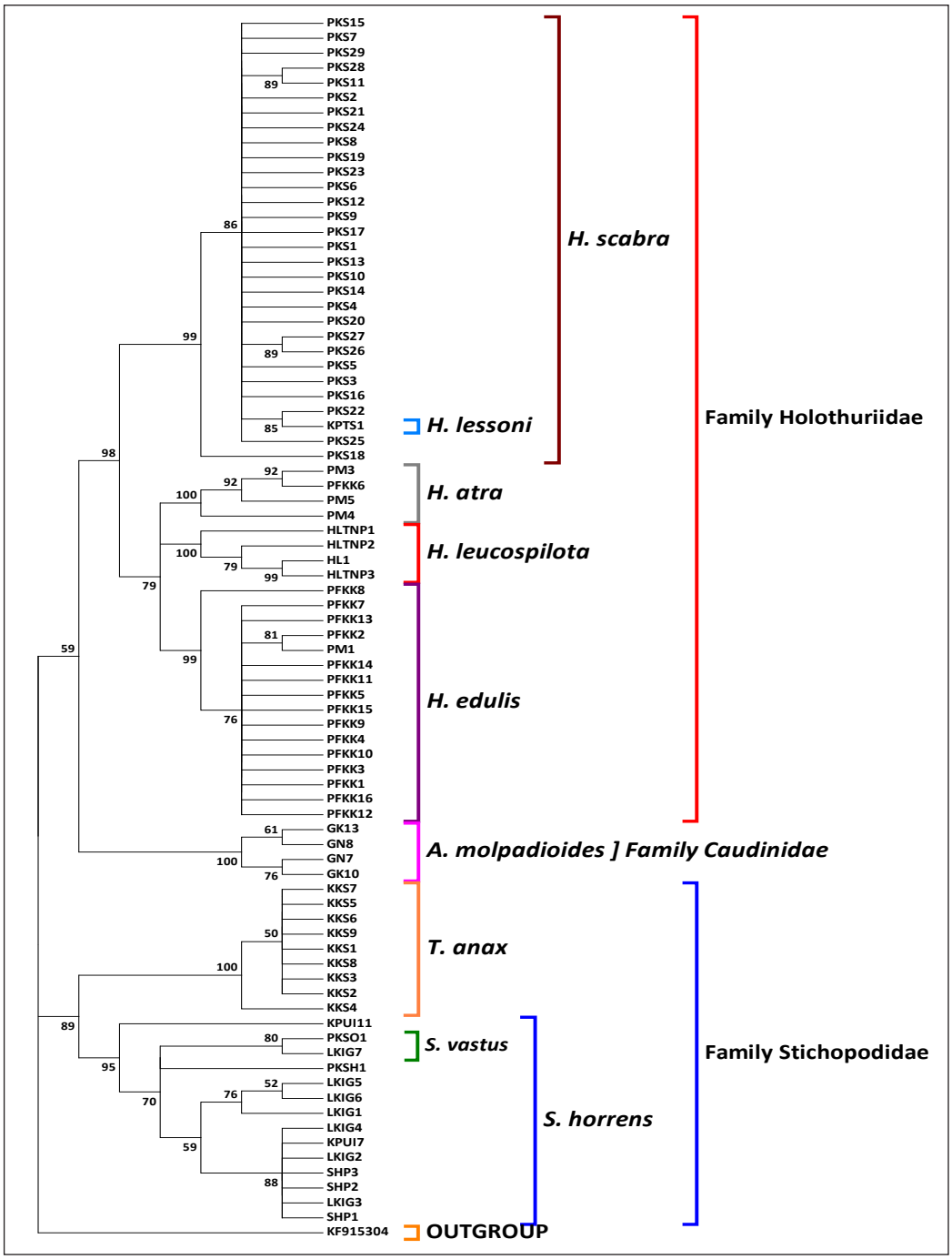


*Figure 3.* Topology of 50% majority-rule consensus tree of Maximum Parsimony of sea cucumber specimens from selected Malaysian markets and other sampling sites including the reference samples and processed specimens inferred from non-protein-coding 12S mitochondrial rRNA gene sequences using MEGA7 program (Kumar et al., 2016) with 1000 bootstrap replicates. Numbers at nodes indicate the bootstrap values in percentage (%)

two main clusters of the specimens i.e. family Stichopodidae (the *gamat* family) with 89% bootstrap support and the *timun laut* family with 59% bootstrap support. Species from the family Stichopodidae e.g. *S. vastus* and *Thelenota ananas* (the prickly redfish) were known as *gamat* while the term *timun laut* refers to all non-*gamat* species including the species of family Holothuriidae (Kamarudin, Rehan, Hashim, & Usup, 2010; Kamarudin, Usup, Hashim, & Mohamed Rehan, 2015). As for choosing the best model of DNA substitution for the Maximum Likelihood tree reconstruction using the Modeltest program, the model suggested by the Akaike Information Criterion (AIC) was chosen instead of the Hierarchical Likelihod Ratio Tests (hLRTs). According to Posada and Buckley (2004), the AIC and Bayesian Analysis were perceived as a good evaluation of model selection uncertainty, which were capable to compare multiple nested or non-nested models at once, and allowed for the use of all available models for the estimation of phylogenies and model parameters. Regarding the Bayesian Analysis tree reconstruction using the MrBayes (version 3.1.2) program, the standard deviation of split frequencies was 0.009766 at 3 020 000 generations. In contrast to the results of the Neighbour Joining tree (Figure 2) and the Maximum Parsimony tree (Figure 3), the Maximum Likelihood tree (Figure 4) and the Bayesian Analysis tree (Figure 5) show the formation of three main clusters of the specimens i.e. family Stichopodidae (the *gamat* family) and two subclusters of the *timun laut* family i.e. the family Holothuriidae cluster and the family Caudinidae clade/cluster. In other words, both the character-based phylogenetic analyses with optimality criterion suggested the same inference. In conclusion, the phylogenetic analyses suggested the presence of three main clusters of the specimens i.e. family Stichopodidae (the *gamat* family), family Holothuriidae (the *timun laut* family), and family Caudinidae (the *timun laut* family). With regard to the taxonomy, family Holothuriidae and family Stichopodidae are the members of Order Aspidochirotida, and family Caudinidae is from Order Molpadiida.

Regarding the bootstrap support, within the *gamat* family group, the Neighbour Joining method (Figure 2), the Maximum Parsimony method (Figure 3), the Maximum Likelihood tree (Figure 4), and the Bayesian Analysis tree (Figure 5) show the formation of *Thelenota anax* clade with 100% bootstrap values/posterior probability. The strong bootstrap values supported the grouping of the specimens of *T. anax* under the Stichopodidae family i.e. the *gamat* family. In Malaysia, sea cucumbers of the genus *Stichopus* and the genus *Thelenota* were regarded as the members of *gamat* family or the family Stichopodidae (Kamarudin et al., 2009, 2010, 2015). All the phylogenetic trees grouped the KKS specimens from Kota Kinabalu, Sabah with 100% bootstrap value, thus confirming their species status as *T. anax*. Within the genus *Stichopus* cluster, all the phylogenetic trees grouped the specimens of *S. vastus* i.e. LKIG7 specimen

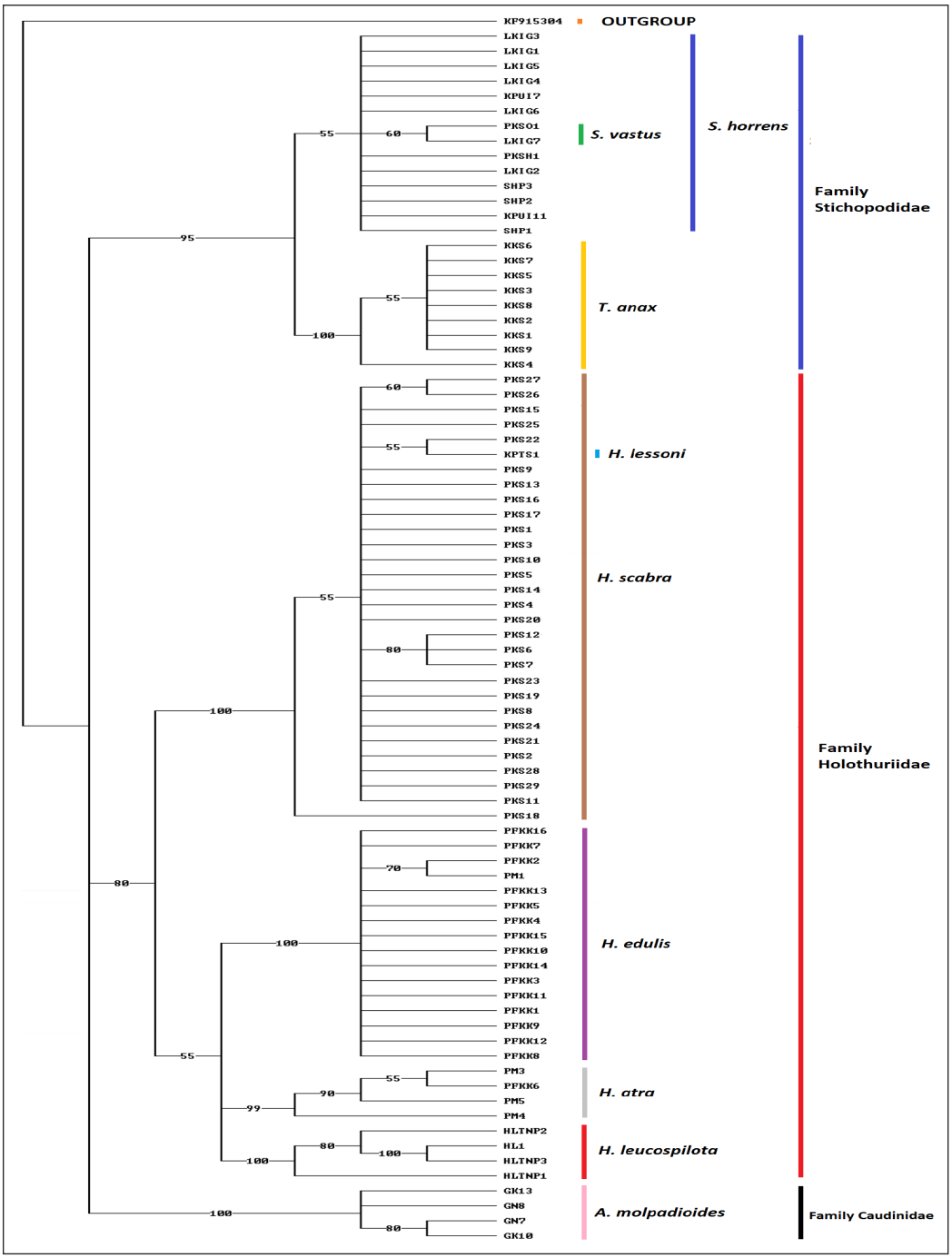


*Figure 4*. Topology of 50% majority-rule consensus tree of maximum likelihood of sea cucumber specimens from selected Malaysian markets and other sampling sites including the reference samples and processed specimens inferred from non-protein-coding 12S mitochondrial rRNA gene sequences using PAUP* (version 4.0b10) program (Swofford, 1998) with 100 bootstrap replicates. Numbers at nodes indicate the bootstrap values in percentage (%)

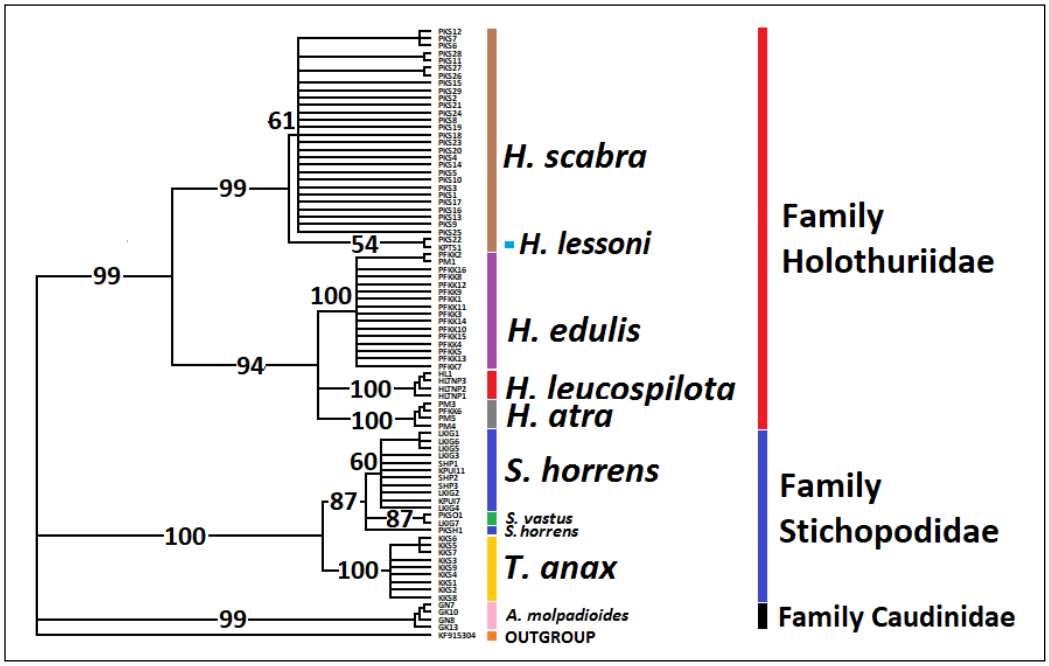


*Figure 5.* Topology of consensus Bayesian Analysis tree of sea cucumber specimens from selected Malaysian markets and other sampling sites including the reference samples and processed specimens inferred from non-protein-coding 12S mitochondrial rRNA gene sequences using MrBayes (version 3.1.2) program (Huelsenbeck and Ronquist, 2001), with the addition of all compatible groups to the tree. Numbers at nodes indicate the posterior probabilities of clades in percentage (%)

from Langkawi Archipelago, Kedah and PKSO1 specimen from Kudat, Sabah into the *S. horrens* cluster, with bootstrap values ranging from 55% to 90%. This could be due to the very close genetic characteristics between the two *gamat* species, even though their physical appearances are significantly different. Besides, *S. vastus* clusters were supported with bootstrap values ranging from 60% to 87%, as shown in all the phylogenetic trees.

Regarding the *timun laut* family, all the phylogenetic trees supported the formation of Holothuriidae family group with strong bootstrap values ranging from 80% to 99%. The phylogenetic trees also supported the formation of *H. leucospilota* clade and *H. edulis* clade each with 100% bootstrap support, thus confirming their species status. Besides, the Neighbour Joining method (Figure 2), the Maximum Parsimony method (Figure 3), and the Bayesian Analysis tree (Figure 5) supported the formation of *H. atra* clade with 100% bootstrap values/posterior probability, while the Maximum Likelihood tree (Figure 4) supported the formation of *H. atra* cluster with 99% bootstrap values. In addition, all the phylogenetic trees also show the presence of two genera of *timun laut* i.e. genus *Holothuria* and genus *Acaudina*. The specimens of *A. molpadioides* became basal within the *timun laut* family group in the Neighbour Joining tree and the Maximum Parsimony

tree. Such clustering suggested the presence of *timun laut* cluster (family Holothuriidae and family Caudinidae) and *gamat* cluster (family Stichopodidae). Nonetheless, the Maximum Likelihood tree and the Bayesian Analysis tree did not support the basal formation. Interestingly, all the phylogenetic trees show no distinct separation between the specimen of *H. lessoni* (KPTS1) and the specimens of *H. scabra* (PKS) as the specimens were mixed with each other with strong bootstrap values/posterior probability ranging from 94% to 100%. Purcell et al. (2012) recorded a processed *H. lessoni* that is similar to the dried *tip-sum* specimen from Kuantan, Pahang (KPTS1), therefore the specimen was regarded as *H. lessoni*. In addition, *H. scabra* and *H. lessoni* are taxonomically from the subgenus *Metriatyla*. From another point of view, *H. lessoni* was previously known as *Holothuria scabra* var. versicolor (Purcell et al., 2012), thus the clustering of KPTS1 specimen with the *H. scabra* specimens could be due to the close genetic contents between *H. scabra* and *H. lessoni*. Furthermore, only the Neighbour Joining tree (Figure 2) shows that *H. edulis* was genetically closer to *H. atra* with 52% bootstrap value, and the subgenus *Mertensiothuria* represented by the specimens of *H. leucospilota* was genetically closer to the subgenus *Halodeima* represented by the specimens of *H. atra* and *H. edulis* with 93% bootstrap value. *H. edulis* and *H. atra* are from the subgenus *Halodeima*, thus the grouping supported their taxonomic classification.

Overall, nine sea cucumber species were recorded in this study including six *timun laut* species i.e. *H. leucospilota*, *H. atra*, *H. edulis*, *H. scabra*, *H. lessoni*, and *A. molpadioides*; and four *gamat* species i.e. *S. horrens*, *S. vastus*, and *T. anax*. According to Choo (2008), *H. leucospilota*, *H. atra*, *H. edulis*, *H. scabra*, *S. horrens*, *S. vastus*, and *T. anax* were the commercial Malaysian sea cucumber species. Nonetheless, *A. molpadioides* and *H. lessoni* were not listed as commercial Malaysian sea cucumber species. Among the species recorded in this study, two *timun laut* species are included in the International Union for Conservation of Nature (IUCN) Red List for aspidochirotid holothuroids. *H. scabra* and *H. lessoni* were regarded as "endangered, or at a high risk of extinction" based on the IUCN Red List for aspidochirotid holothuroids (Conand et al., 2014). Apart from that, the outcomes of this study provide further information on the level of species substitution and product mislabelling issues of processed sea cucumbers in Malaysian markets. The enforcement agencies can use the information for monitoring and overcoming the issues through the introduction of mtDNA sequencing technique.

## CONCLUSION

In conclusion, the phylogenetic trees based on the distance-based method with clustering algorithm as the tree building strategy i.e. the Neighbour Joining method, and the character-based methods with optimality criterion as the tree building

strategy i.e. the Maximum Parsimony method, Maximum Likelihood method, and the Bayesian Analysis method suggested the presence of three main clusters of the specimens i.e. family Stichopodidae (the *gamat* family), family Holothuriidae (the *timun laut* family), and family Caudinidae (the *timun laut* family). Three *gamat* species i.e. *S. horrens*, *S. vastus*, and *T. anax* were clustered under the family Stichopodidae and the specimens of *H. leucospilota*, *H. atra*, *H. edulis*, *H. scabra*, *H. lessoni*, and *A.* molpadioides are the five *timun laut* species grouped under the family Holothuriidae. Moreover, *A. molpadioides* from the family Caudinidae and Order Molpadiida was also present. Issues of intentional species substitution or product mislabeling were recorded due to the observation of unlabelled products in the selected Malaysian markets. The information may assist the enforcement agencies to monitor and address the issues. Pertaining to the export and import transactions, in order to ensure that the right species are utilised in processing and trading matters, the traders and the manufacturers may practise the molecular approach used in this study. Furthermore, the enforcement agencies may apply the mtDNA sequencing technique as an additional identification tool and the non-protein-coding 12S mitochondrial rRNA gene sequences deposited in the GenBank in order to establish facts in legal investigation prior to deciding the charges to the offenders.

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