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16S rRNA Barcoding Technique for Species Identification of Processed Sea Cucumbers from selected Malaysian Markets

Pengenalpastian Spesis Gamat (Timun Laut) yang telah diproses di beberapa pasar di Malaysia dengan teknik bar kod 16S rRNA

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Abstract

In food industry, wide-ranging processing of sea cucumbers (Phylum Echinodermata: Class Holothuroidea) including gutting, boiling, roasting, and subsequent preservation procedures are frequently needed prior to marketing. These processes cause body deformation of the sea cucumbers, thus leading to difficulties in species identification and confirmation of the processed sea cucumbers or beche-de-mer. Furthermore, beche-de-mer products in Malaysian markets are often unlabelled or mislabelled. Economic fraud, health hazards, and illegal trade of protected species are the potential major consequences of the issues. Therefore, a reliable, reproducible, and rapid technique for species identification is required. For that reason, this study was conducted to determine species identity of 25 beche-de-mer specimens that were not tagged with species details from four selected Malaysian markets. Five reference samples were also included in the analyses consisting of fresh samples that were morphologically identified as *Stichopus horrens* and *Holothuria* (*Mertensiothuria*) *leucospilota* from Pangkor Island, Perak, Malaysia. Phylogenetic analyses of 30 partial sequences of non-protein-coding 16S mitochondrial ribosomal RNA (rRNA) gene using five main methods i.e. Neighbour-Joining (NJ), Maximum Likelihood (ML), Minimum Evolution (ME), Unweighted Pair Group Method with Arithmetic Mean (UPGMA), and Maximum Parsimony (MP) showed the presence of two main clusters of the beche-de-mer specimens: Stichopodidae (*gamat* family) and Holothuriidae (*timun laut* family). The beche-de-mer specimens of *S. horrens*, *Stichopus herrmanni*, and *Thelenota anax* were the three *gamat* species that clustered under the family Stichopodidae. Meanwhile, the beche-de-mer specimens of *H. leucospilota*, *Holothuria* (*Halodeima*) *edulis*, *Holothuria (Metriatyla) scabra*, and *H. scabra* var. versicolor were the four *timun laut* species that clustered under the family Holothuriidae. In fact, the outcomes of this study suggested the potential of 16S mitochondrial rRNA gene sequencing technique to be used by the enforcement agencies in monitoring and overcoming the issues of species substitution and product mislabeling of beche-de-mer products in Malaysian markets.

Keywords gamat, sea cucumbers, beche-de-mer, mitochondrial DNA sequencing, 16S rRNA gene, phylogenetic trees.

Abstract

Dalam industri makanan, gamat (Phylum Echinodermata: Class Holothuroidea) diproses dengan menyiang, merebus, membakar dan diawet sebelum dipasarkan. Proses-proses ini menyebabkan rupa bentuk asli gamat bertukar dan menyukarkan spesis gamat ditentukan keaslian produk timun laut atau *beche-de-mer*. Tambahan lagi produk gamat di pasaran Malaysia sering kali tidak berlabel dan salah label. Penipuan, ancaman kesihatan dan pemasaran gelap spesis yang dikawal adalah antara isu-isu besar yang dihadapi oleh industri ini. Oleh itu, satu teknik mengenalpasti spesis yang andal, dipercayai dan segera diperlukan. Oleh kerana itu, satu kajian telah dijalankan bagi mengenal pasti 25 spesis spesimen beche-de-mer yang dijual di empat pasar terpilih di Malaysia. Lima sampel rujukan telah dianalisis yang terdiri dari spesimen segar telah dikenalpasti dari segi morfologinya sebagai *Stichopus horrens* dan *Holothuria* (*Mertensiothuria*) *leucospilota* dari Pulau Pangkor, Perak, Malaysia. Analisis pilogenetik 30 jujukan kod gen 16S *mitochondrial ribosomal* RNA (rRNA) dengan lima kaedah utama berikut iaitu *Neighbour-Joining* (NJ), *Maximum Likelihood* (ML), *Minimum Evolution* (ME), *Unweighted Pair Group Method with Arithmetic Mean* (UPGMA), dan *Maximum Parsimony* (MP) menunjukan terdapatnya dua kluster utama spesimen *beche-de-mer*: *Stichopodidae* (keluarga gamat) dan *Holothuriidae* (keluarga timun laut). Spesimen *beche-de-mer* daripada *S. horrens*, *Stichopus herrmanni*, dan *Thelenota anax* adalah tiga spesis gamat yang berada dalam klaster keluarga *Stichopodidae.* Manakala *H. leucospilota*, *Holothuria* (*Halodeima*) *edulis*, *Holothuria (Metriatyla) scabra*, dan *H. scabra* var. versi warna merupakan empat timun laut dibawa kluster keluarga Holothuriidae. Dengan itu, hasil kajian ini mencadangkan potensi jujukan kod gen 16S mitochondrial rRNA digunakan oleh agensi penguatkuasa bagi memantau dan menyelesaikan isu-isu tentang perlabelan produk *beche-de-mer* dipasaran Malaysia.

Kata kunci gamat, timun laut, *beche-de-mer*, jujukan mitochondrial DNA, gen16S rRNA, pokok pilogenetik

INTRODUCTION

Sea cucumber is an echinoderm of the class Holothuroidea (Phylum Echinodermata: Class Holothuroidea) and known in Malaysia as sea ginseng, with an estimate of more than 52 species populating the sea waters of Malaysia (Kamarudin *et al.* 2015b; Hashim, 2011; Kamarudin *et al*. 2010; Hashim, 1993). *Gamat*, *timun laut*, *brunok/beronok, bat,* and *balat* are among the most common names of the sea cucumbers used by the Malaysians. The names *bat* and *balat* are commonly used by the local residents in Sarawak and Sabah. *Gamat* is the most renowned name and it refers to all species of family Stichopodidae e.g. *Stichopus horrens* or dragonfish (Figure 1)*, Stichopus ocellatus*, *Stichopus chloronotus* or greenfish, *Stichopus herrmanni* or curryfish (Lukman *et al.* 2014), and species of genus *Thelenota* e.g. *Thelenota anax* or amberfish. Meanwhile, non-*gamat* species are known as *timun laut*. *Holothuria* (*Mertensiothuria*) *leucospilota* or white threads fish, one of *timun laut* species, is considered as the most dominant sea cucumber species in Malaysia (Figure 2, Kamarudin *et al.* 2015b).

In fact, sea cucumber trading has been contributing to the economic growth of Malaysia. There are two major commercial values of sea cucumbers in Malaysia, i.e., (1) as an important resource for the food industry; and (2) as an important resource for traditional

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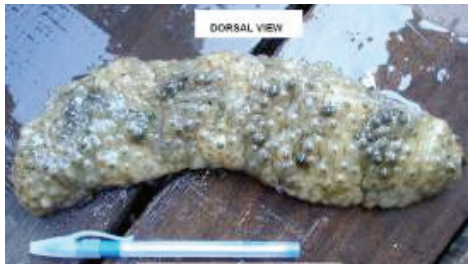

Figure 1 *Stichopus horrens* or dragonfish, a *gamat* species. Adapted from Kamarudin *et al.* (2015a)

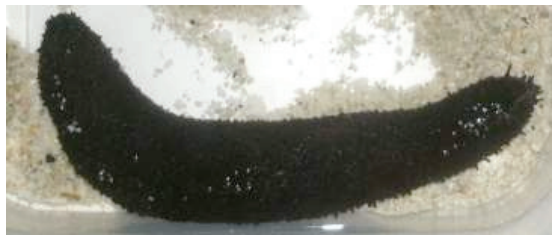

Figure 2 *Holothuria* (*Mertensiothuria*) *leucospilota* or white threads fish, a *timun laut* species. Adapted from Kamarudin *et al.* (2015a).

and modern medicine (Kamarudin *et al.* 2015b; Hashim, 2011; Kamarudin *et al*. 2010; Hashim, 1993). Pangkor Island, Perak Darul Ridzuan and Langkawi Archipelago, Kedah Darul Aman in the west coast are well-known in the *gamat*-based traditional medicine industry in Peninsular Malaysia in the forms of commercial lipid extracts (i.e. *minyak gamat*) and body fluid extracts (i.e. *air gamat*). Besides, Gamat eMas Sdn Bhd (GESB) and Luxor Network Sdn. Bhd. are among the multi-level marketing companies that market *gamat*-based health products produced through the modern technologies for the national and international demands. In contrast, food processing industry of sea cucumbers i.e. beche-de-mer or *trepang* (dry tunics) is popular in Sabah in Borneo Island. However, in Sarawak as part of the Borneo Island, up-to-date reports on the sea cucumber species richness and commercial exploitation are still unavailable, although *Acaudina molpadioides* (English name: sea potato) or *brunok/beronok* from the order Molpadiida was reported by Hashim (1993) as being used as fishing bait.

In terms of commercial sea cucumber species in Asia, Choo (2008) listed 52 species from both families of Stichopodidae and Holothuriidae. According to Table 1, Indonesia is the top producer with 35 commercial sea cucumber species, followed by China (27 species), the Philippines (26 species), Malaysia (19 species), Japan and Vietnam (11 species each) and Thailand (8 species). *Holothuria (Metriatyla) scabra* or sandfish, *Holothuria (Halodeima) atra* or lollyfish, *Holothuria (Halodeima) edulis* or pinkfish, *H. leucospilota*, *S. herrmanni*, *S. chloronotus*, and *Thelenota ananas* known as prickly redfish are the sea cucumber species commercialised by Malaysia, Thailand, Indonesia, the Philippines, and Vietnam.

An upsurge in global seafood consumption, rising international trade, and variations in the food demand and supply of different commercial marine species are among the

Table 1 Commercial sea cucumber species in Asia. Species of commercial importance in each country are marked in red. Source: Choo (2008)

	China	Taiwan PC	China HK SAR	Japan	Malaysia	Thailand	Indonesia	Philippines [1]	Viet Nam	Singapore	Spratly
O: Aspidochirotida F: Holothuriidae											
Actinopyga lecanora	x	x			x	x	x	x		x	x
Actinopyga mauritiana	x	x		x	x	x	x	x			x
Actinopyga echinites	x	x			x	x	x	x			
Actinopyga miliaris	x				x	x	x	x			
Bohadschia argus	x	x		x	x	x	x	x	x		x
Bohadschia graeffei					x	x	x	x			x
Bohadschia marmorata	x	x			x	x	x	x	x		
Bohadschia similis							x				
Bohadschia vitiensis						x	x		x		
Bohadschia tenuissima							x				
Bohadschia bivittata				x					x		
Holothuria atra	x	x		x	x	x	x	x	x		
Holothuria coluber					x	x	x	x			
Holothuria rigida						x	x	x			
Holothuria pulla								x			
Holothuria edulis	x			x	x	x	x	x	x		
Holothuria pardalis	x	x	x		x	x	x				
Holothuria cinerascens	x	x				x					
Holothuria moebii	x	x	x		x	x					
Holothuria whitmaei	x	x			x	x	x	x	x		
Holothuria fuscogilva	x				x		x	x	x		
Holothuria difficilis		x				x		x			
Holothuria arenicola	x	x				x	x				
Holothuria hilla		x			x	x	x	x			
Holothuria impatiens	x	x			x	x	x				
Holothuria leucospilota	x	x	x		x	x	x	x	x		x
Holothuria pervicax	x	x			x		x				
Holothuria conusalba						x	x				
Holothuria scabra	x		x	x	x	x	x	x	x	x	
Holothuria scabra versicolor					x		x	x			
Holothuria similis							x				
Holothuria fuscopunctata					x	x	x	x	x		
Holothuria ocellata					x	x	x				
Holothuria fuscocinerea	x	x	x		x	x		x			
Holothuria vagabunda							x				
Holothuria vatiensis							x				
Pearsonothuria graeffei		x			x		x				
O: Aspidochirotida F: Tichopodidae											
Stichopus chloronotus	x			x	x	x	x	x	x		x
Stichopus sp.					x						
Stichopus horrens	x	x			x		x	x	x		
Stichopus herrmanni	x	x			x	x	x	x	x		
Stichopus vastus					x		x				
Stichopus quadrifaciatus							x				
Parastichopus nigripunctatus				x							
Thelenota ananas	x	x		x	x	x	x	x	x		x
Thelenota anax	x				x		x	x			x
Apostichopus japonicus	x			x							
O: Dendrochirotida F: Cucumariidae											
Mensamaria sp.	x										
Cucumaria frandosa japonica				x		x					
Pentacta quadragulis						x			x		
O: Moldavia F: Caudinidae											
Acaudina leucoprocta	x										
O: Molpadida F: Molpadiidae											
Paracaudina sp.					x						
No. of commercial species	**27**	**0**	**0**	**11**	**19**	**8**	**35**	**26**	**11**	**0**	**0**

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contribution factors of species substitution or intentional product mislabelling (Rasmussen and Morrissey, 2008). Additionally, species substitution can have severe consequences, e.g. illegal trade of protected species, health hazards, and economic fraud. In terms of food processing, sea cucumbers are gutted, boiled, and roasted then preserved through drying, smoking, or freezing. Prickling and canning are also done prior to marketing. Due to these processes, the species identity of the processed sea cucumbers or beche-de-mer is difficult to be identified and confirmed morphologically at the markets due to their body deformation. Some beche-de-mer products in Malaysian markets have not been properly labelled or being mislabeled, consequently leading to greater difficulties in species identification.

Hence, a reliable, reproducible, and rapid technique is required for species identification of beche-de-mer products from the Malaysian markets. Therefore, this study aimed to determine the species identity of beche-de-mer specimens in the forms of dried and frozen products from selected Malaysian markets by using modified forensically informative nucleotide sequencing (FINS) technique that was first described by Bartlett and Davidson (1992). Partial sequences of non-protein-coding 16S mitochondrial ribosomal RNA (rRNA) gene were acquired and subsequent analysis using online Basic Local Alignment Search Tool program (BLAST) was incorporated to resolve the species status. Mitochondrial DNA (mtDNA) was chosen due to its obvious haploid genome, non-recombination, effective maternal inheritance, continuous replication, and its rate of substitution is within the range of five to 10 times greater than in 'single-copy' nuclear DNA (Nabholz *et al.* 2008; Amos and Hoelzel, 1992). 16S mitochondrial rRNA gene has also been suggested to be able to correlate the relationship between the morphology and genetics of sea cucumber (Clouse *et al.* 2005; Kerr *et al.* 2005). Beside that, the genetic relationships of the sea cucumber species were also determined through phylogenetic analyses. All information generated from the analyses were compared with the species and source details of the products. The outcomes of this study highlight some information on the issues of product labelling and intentional species substitution of processed sea cucumbers in selected Malaysian markets, and the information are useful and helpful to enforcement agencies in order to tackle the issues through 16S mitochondrial rRNA gene sequencing technique.

MATERIALS AND METHODS

Specimen Collection

A total number of 25 processed sea cucumber or beche-de-mer specimens were collected. The specimens were sampled in Kuantan, Pahang (East Coast region of Peninsular Malaysia); Langkawi Island, Kedah (the Northern region); Kudat, Sabah (near the northernmost point of Borneo, East Malaysia), and Kota Kinabalu, Sabah (in the northwestern Borneo). Three frozen specimens and one dried specimen were from Kuantan, Pahang; six dried specimens were from Kuah, Langkawi Island; six frozen specimens from Kudat, Sabah; and nine dried specimens from Kota Kinabalu, Sabah. For specimens with absent species details, the information were obtained from the salespersons. Two fresh specimens of *Stichopus horrens* (i.e. *gamat* species) and three fresh specimens of *Holothuria (Mertensiothuria) leucospilota* (i.e. *timun laut* species) from Pangkor Island, Perak (North region of Peninsular Malaysia) were used as the commercial species standard or reference samples.

Morphological species identification of the beche-de-mer specimens was done by referring to Purcell *et al.* (2012), Hashim (2011), experts [Prof. Alexander M. Kerr (Marine Laboratory, University of Guam, USA) and the participants of National Science Foundation's Partnerships for Enhancing Expertise in Taxonomy (NSF PEET) Holothuroid Systematics Workshop held on 7-16 June 2010 at the Marine Laboratory, University of Guam, USA)], the World Register of Marine Species database at http://www.marinespecies.org/index.php, and also through the information given by the local residents.

Total Genomic DNA Extraction

Total genomic DNA (tgDNA) extraction was done using the DNeasy mericon Food Kit by QIAGEN. Approximate yields of tgDNA, the quantity and quality, were determined by electrophoresis (on 1% agarose gel, with ethidium bromide as gel stain).

Polymerase Chain Reaction (PCR)

For the PCR using 2× TopTaq Master Mix Kit by QIAGEN, non-protein-coding 16S mitochondrial rRNA gene was amplified using standard PCR procedures (Kamarudin *et al*. 2011, approximately 570 base pairs (bp) of fragment length).

16Sar (forward) 5' - CGC CTG TTT ATC AAA AAC AT – 3' (20 bases)

16Sbr (reverse) 5' - CTC CGG TTT GAA CTC AGA TCA – 3' (21 bases)

(Kerr *et al*. 2005)

Approximate yields of amplified DNA fragments, the quantity and quality, were determined by electrophoresis (on 1% agarose gel, with ethidium bromide as gel stain).

PCR Product Purification and DNA Sequencing

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 purification of the PCR products. Purified PCR products in suspension form were prepared prior to sending samples for sequencing at First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor, Malaysia.

BLAST Analysis for Species Identification of Processed Sea Cucumbers

The BLAST search available at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was assessed to assign each sea cucumber DNA sequence acquired from this study to a particular genus or species.

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Phylogenetic Analyses

Chromas Lite (version 2.1) program (Copyright © 2005 Technelysium Pty Ltd) was used to display the results of fluorescence-based DNA sequence analysis received from the First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor, Malaysia. Multiple sequence alignment of forward reaction sequences was done using ClustalX (version 2.0.12) program (Thompson *et al*. 1997), and subsequently aligned by eyes. The reconstruction of Neighbour-Joining (NJ) tree, Maximum Likelihood (ML) tree, Minimum Evolution (ME) tree, Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree, and Maximum Parsimony (MP) tree were done by using Molecular Evolutionary Genetics Analysis 6 (MEGA6) (Tamura *et al*. 2013).

RESULTS AND DISCUSSION

A number of 30 partial DNA sequences of non-protein-coding 16S mitochondrial rRNA gene were obtained to date. Based on the BLAST results, the specimens from Kuantan, Pahang were genetically identified as *Holothuria* (*Metriatyla*) *scabra* var. versicolor (a dried specimen) and *Holothuria* (*Mertensiothuria*) *leucospilota* or white threads fish (three frozen specimens); the dried specimens from Kuah, Langkawi Island were genetically identified as *Stichopus horrens* or dragonfish (five specimens) and *Stichopus herrmanni* or curryfish (a specimen); the frozen specimens from Kudat, Sabah were genetically identified as *Holothuria* (*Metriatyla*) *scabra* or sandfish (four specimens), *S. horrens* (one specimen) and *S. herrmanni* (one specimen); the dried specimens from Kota Kinabalu, Sabah were genetically identified as *Thelenota anax* or amberfish (six specimens) and *Holothuria* (*Halodeima*) *edulis* or pinkfish (three specimens); and finally the fresh specimens from Pangkor Island, Perak were morphologically and genetically identified as *H. leucospilota* and *S. horrens*. Nevertheless, the beche-de-mer products were not tagged with species details upon sampling (Figure 3). Products with absent species details could be resulted from or lead to intentional species substitution or negative trading where a supply does not

Figure 3 Processed sea cucumber or beche-de-mer products unlabelled with species details. Left: from Kuantan, Pahang; right: from Kota Kinabalu, Sabah.

meet the demand for a specific species. Intentional species substitution and mislabelling of marine products have been reported worldwide (Rasmussen and Morrissey, 2008), and these issues are also affecting the trading of processed sea cucumbers or beche-de-mer.

In terms of local names, the dried specimen from Kuantan, Pahang is called *tip-sum* by the Malaysian Chinese community. All specimens from Kuah, Langkawi Island are locally known as *gamat*. The BLAST results have suggested that *tip-sum* is referred to *H. scabra* var. versicolor, a *timun laut* species; and the specimens from Kuah, Langkawi Island consisted of two *gamat* species namely *S. horrens* and *S. herrmanni*. Six dried specimens from Kota Kinabalu, Sabah were also suggested by the BLAST results as *gamat* species or *T. anax*. Moreover, both genetic and morphological approaches supported each other in identifying all fresh specimens from Pangkor Island, Perak (i.e. the commercial species standard) as *S. horrens* and *H. leucospilota*. Overall, the BLAST results suggested the presence of three *gamat* species from family Stichopodidae (43%) and four *timun laut* species from family Holothuriidae (57%).

There were a total of 515 positions/nucleotide bases after a multiple alignment using ClustalX (version 2.0.12) program, including one sequence of *Cucumaria frondosa* (GenBank accession no: KF479389) as the outgroup. In terms of phylogenetic analyses, 30 nucleotide sequences of 16S mitochondrial rRNA gene were involved. All positions containing gaps and missing data were eliminated. As a result, there were a total of 474 positions (approximately 92 percent out of the 515 positions) in the final dataset. For the NJ method, the optimal tree with the sum of branch length equals to 0.68661945 is shown in Figure 4. The percentage of replicate NJ trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*. 2004) and are in the units of the number of base substitutions per site. The NJ tree shows the presence of two main groups i.e. family Stichopodidae clade with 100% bootstrap support and family Holothuriidae cluster with 95% bootstrap support. *S. horrens*, *S. herrmanni,* and *T. anax* are the members of family Stichopodidae or *gamat*, while *H. scabra*, *H. scabra* var. versicolor, *H. edulis*, and *H. leucospilota* are the members of family Holothuriidae or *timun laut*. *H. edulis* became the basal to the clusters of *H. scabra* and *H. scabra* var. versicolor. Moreover, *H. leucospilota* became the basal of genus *Holothuria* cluster or family Holothuriidae cluster with 95% bootstrap support. However, a specimen from Kudat, Sabah that was marked with a red circle (Figure 4) was not grouped with *S. horrens* specimens and became basal to the genus *Stichopus* clade (with 100% bootstrap support), even though the BLAST results suggested it as *S. horrens*. The species were all listed by Choo (2008) as commercial sea cucumber species in Asia.

The ME tree with the sum of branch length equals to 0.68661945 is shown in Figure 5. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood (MCL) method (Tamura *et al*. 2004) and are in the units of the number of base substitutions per site. The ME tree was searched using the

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Close-Neighbour-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 1. The Neighbour-joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree. The ME tree supported all the outcomes of NJ tree.

Figure 6 shows the ML tree with the highest log likelihood (i.e. 0.68661945). The percentage of ML trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using the MCL approach. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The MP tree also supported the findings of NJ tree and ME tree except for the clusterings of genus *Stichopus* specimens. The specimen from Kudat, Sabah that was marked with a red circle (Figure 6) was not the basal taxon of genus *Stichopus* clade, but it became the basal to *S. herrmanni* cluster; and all the other *S. horrens* specimens did not form a distinct cluster of *S. horrens*.

For the UPGMA method, the optimal tree with the sum of branch length equals to 0.68661945 is shown in Figure 7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The UPGMA tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the MCL method (Tamura *et al*. 2004) and are in the units of the number of base substitutions per site. The UPGMA tree also supported the outcomes of NJ, ML and ME trees, but interestingly, the specimen from Kudat, Sabah that was marked with a red circle (Figure 7) became basal to *S. horrens* cluster with 60% bootstrap value.

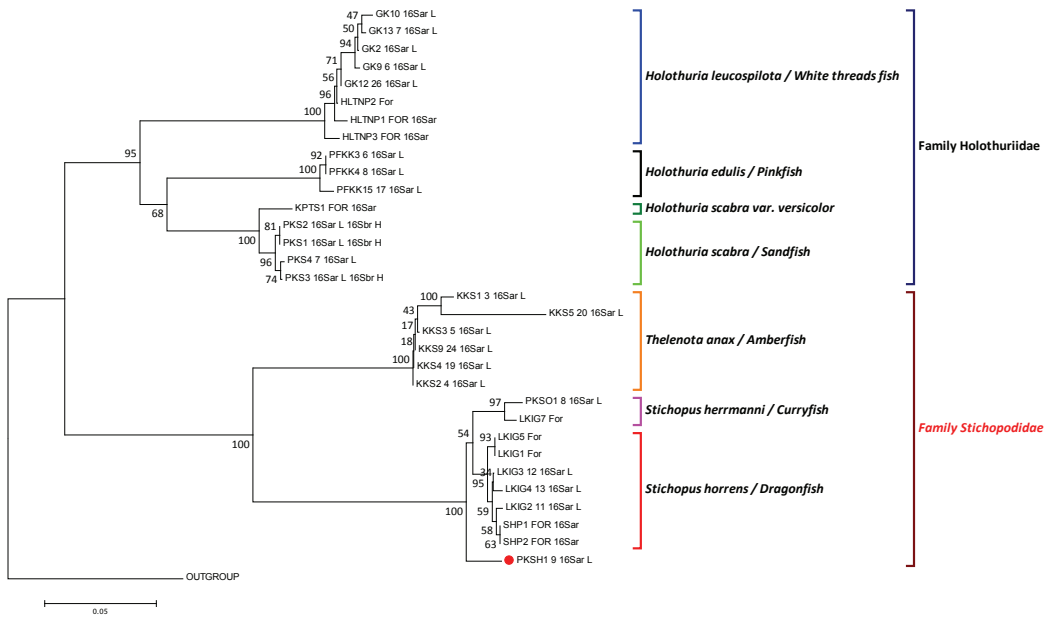

Figure 4 The evolutionary history of processed sea cucumbers from selected Malaysian markets inferred using the Neighbour-Joining (NJ) method (Saitou and Nei, 1987)

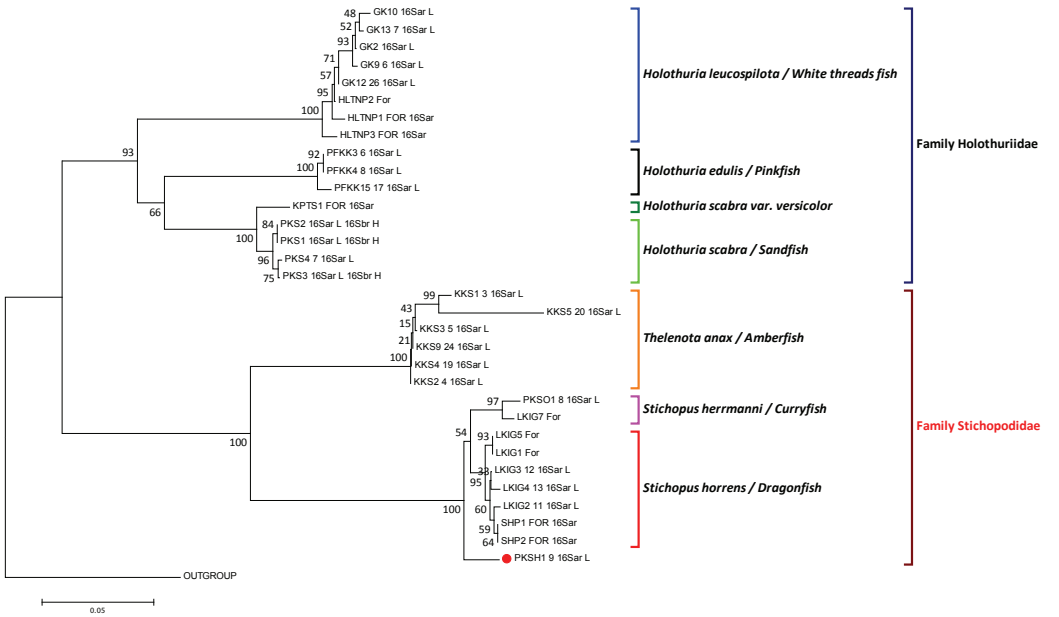

Figure 5 The evolutionary history of processed sea cucumbers from selected Malaysian markets inferred using the Minimum Evolution (ME) method (Rzhetsky and Nei, 1992)

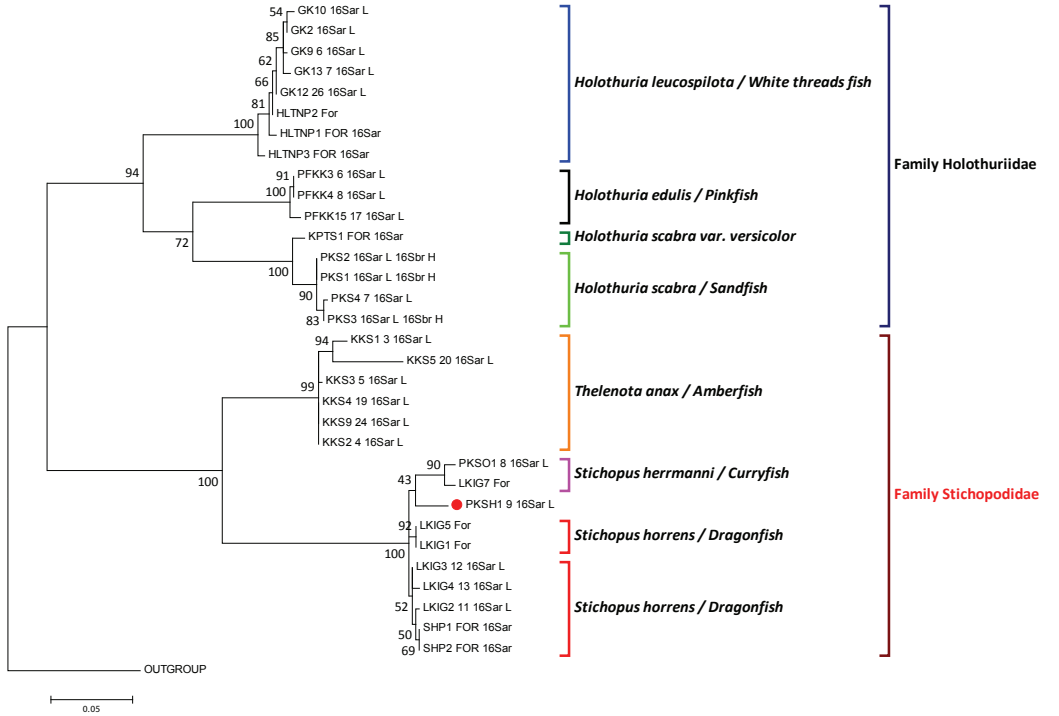

Figure 6 The evolutionary history of processed sea cucumbers from selected Malaysian markets inferred by using the Maximum Likelihood (ML) method based on the Tamura-Nei model (Tamura and Nei, 1993).

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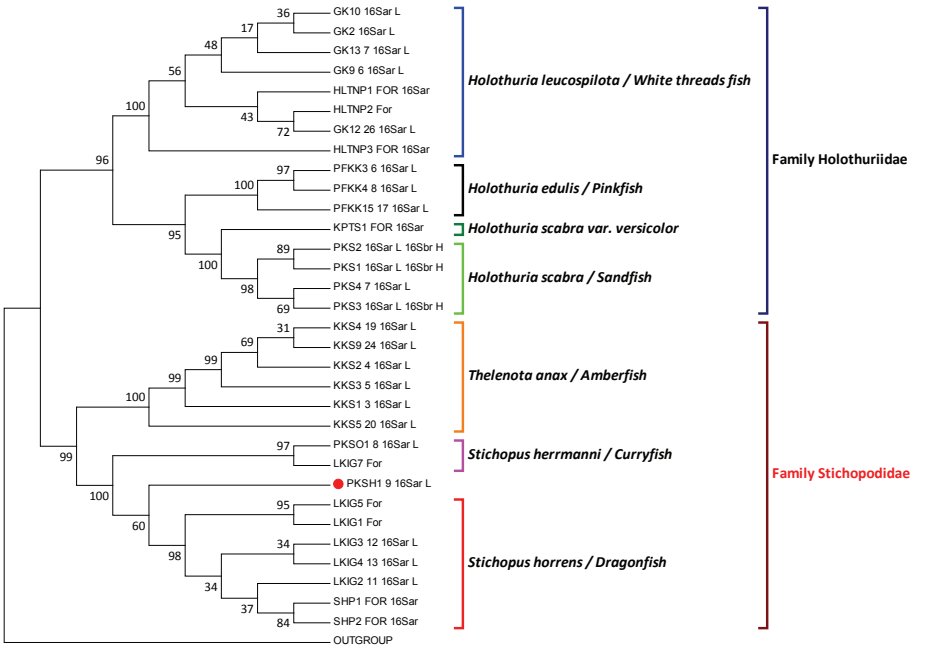

Figure 7 The evolutionary history of processed sea cucumbers from selected Malaysian markets inferred using the UPGMA method (Sneath and Sokal, 1973)

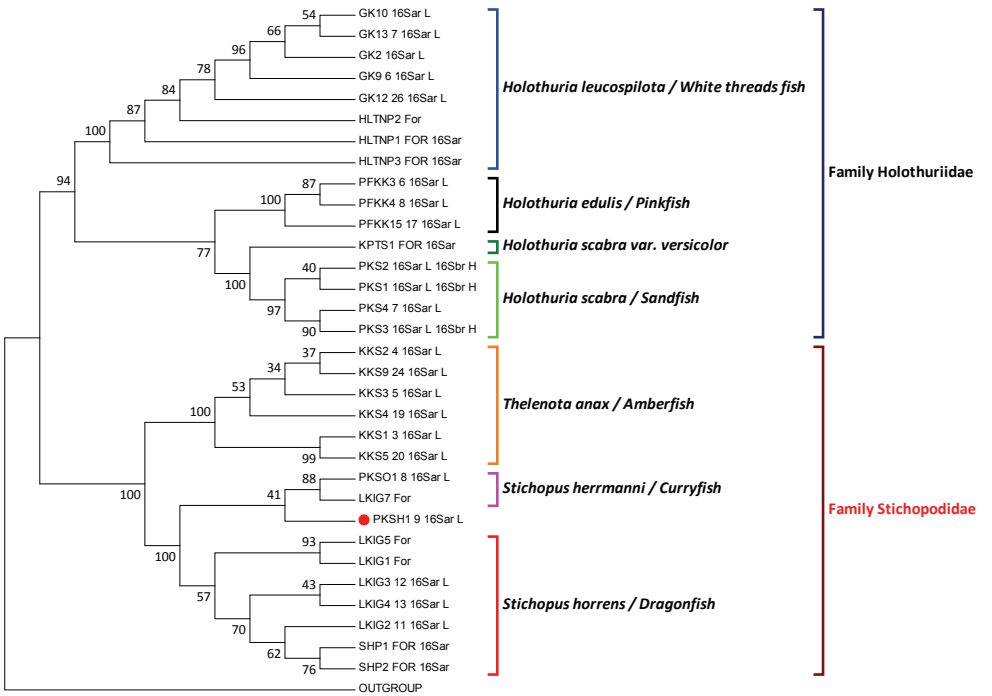

Figure 8 The evolutionary history of processed sea cucumbers from selected Malaysian markets inferred using the Maximum Parsimony (MP) method.

Furthermore, the bootstrap consensus MP tree with the most parsimonious tree with length equals to 291 is shown in Figure 8. The consistency index is (0.811808), the retention index is (0.811808), and the composite index is 0.780790 (0.768546) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate MP trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (pg. 126 in ref. Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The MP tree also supported the outcomes of the other phylogenetic trees. Likewise the ML tree, the specimen from Kudat, Sabah that was marked with a red circle (Figure 8) became the basal to *S. herrmanni* clade.

In summary, most of the phylogenetic trees show the presence of two main clusters of sea cucumbers i.e. Stichopodidae (*gamat* family) and Holothuriidae (*timun laut* family). Overall, there were seven sea cucumber species recorded in this study. Specimens of *S. horrens*, *S. herrmanni,* and *T. anax* were the three *gamat* species that clustered under the family Stichopodidae and specimens of *H. leucospilota*, *H. edulis*, *H. scabra* and *H. scabra* var. versicolor were the four *timun laut* species that clustered under the family Holothuriidae. All the species are commercial sea cucumber species in Asia (Choo, 2008). Further morphological and molecular studies with more samples, especially for the specimen from Kudat, Sabah that was marked with a red circle (Figures 4-8), more different types of mtDNA genes, and additional techniques e.g. microsatellite, randomly amplified polymorphic DNA (RAPD), and restriction fragment length polymorphism (RFLP) need to be done to get a better view and verification.

The issue of intentional species substitution cannot be disregarded due to the presence of unlabelled products in the selected Malaysian markets. The information on product labelling status and possibility of intentional species substitution can be used by the enforcement agencies to search for evidence and then establish facts in their legal investigation prior to bringing any charges to offenders. Apart from that, 16S mitochondrial rRNA sequencing or 16S rDNA barcoding has the potential to be used as a tool to help manufacturers and traders to ensure that the right species are used in their trading transactions. It can also be used by the local enforcement agencies in monitoring and overcoming the issues of species substitution and product mislabeling of beche-de-mer products in Malaysian markets.

CONCLUSION

In conclusion, the phylogenetic analyses of 30 partial sequences of non-protein-coding 16S mitochondrial ribosomal RNA (rRNA) gene using five main methods i.e. Neighbour-Joining (NJ), Maximum Likelihood (ML), Minimum Evolution (ME), Unweighted Pair Group Method with Arithmetic Mean (UPGMA), and Maximum Parsimony (MP) supported the presence of two main clusters of the beche-de-mer specimens i.e. Stichopodidae (*gamat* family) and Holothuriidae (*timun laut* family). The beche-de-mer specimens of *S. horrens*, *Stichopus herrmanni*, and *Thelenota anax* were the three *gamat* species that clustered under the family Stichopodidae and the beche-de-mer specimens of *H. leucospilota*, *Holothuria* (*Halodeima*) *edulis*, *Holothuria (Metriatyla) scabra*, and *H. scabra* var. versicolor were the four *timun laut* species that clustered under the family Holothuriidae. The species

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status of the specimen from Kudat, Sabah that was marked with a red circle (Figures 4-8) needs to be resolved through further morphological and molecular studies in order to get a better view and verification. In general, the current findings suggested the potential of 16S mitochondrial rRNA gene sequencing technique to be used by the enforcement agencies in monitoring and overcoming the issues of species substitution and product mislabeling of beche-de-mer products in Malaysian market

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