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Species identification of processed sea cucumbers from Malaysian market based on concatenated gene sequences of mitochondrial rRNA genes

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Abstract

Species identification of sea cucumbers that have undergone body deformation due to extensive food processing e.g. beche-de-mer is difficult especially with the copresence of cases of unlabelled or mislabelled sea cucumber-based products in the markets. Therefore, a study was done to determine the species identities of processed sea cucumbers from selected Malaysian markets using concatenated gene sequences of non-protein-coding 12S and 16S mitochondrial rRNA genes. 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 47 ingroup sequences representing 37 processed sea cucumber specimens, 6 reference samples, and 4 additional specimens suggested the presence of 3 main clusters i.e. *gamat* family consisting of genus *Stichopus* and genus *Thelenota*; and *timun laut* family comprising family Holothuriidae. A number of 3 *gamat* species i.e. *Stichopus horrens*, *Stichopus vastus*, and *Thelenota anax* were recorded. Meanwhile, the specimens of *Holothuria (Halodeima) atra*, *Holothuria (Halodeima) edulis*, *Holothuria (Metriatyla) lessoni*, *Holothuria (Mertensiothuria) leucospilota*, and *Holothuria (Metriatyla) scabra* were the 5 *timun laut* species that grouped under the family Holothuriidae. The outcomes of this study 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.

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1. Introduction

The seafood industry is facing a critical issue of species substitution of commercial marine products. There are a number of contributing factors to such issue or intentional product mislabelling; among them are increase in global seafood consumption, growing international trade, and fluctuations in the food supply and demand of different marine species (Rasmussen & Morrissey, 2008). According to Rasmussen and Morrissey (2008), economic fraud, health hazards, and illegal trade of protected species could be the serious consequences resulted from species substitution. China have commercialised 26 sea cucumber species placing the country as the second world's top producer (Choo, 2008). However, the mislabelling of 63.6 percent of commercial sea cucumber products from Guangzhou, China was reported by Wen et al. (2011). The same issue can be observed in Malaysian markets by which some sea cucumber-based products have been labelled with incorrect species name and missing manufacturing or packaging details. In fact, Malaysia has been ranked as the fourth world's top producer of commercial sea cucumber with 19 commercial species (Choo, 2008) and the issue could cause problems to the local economy, health and conservation sectors.

For years, the microscopic observation of ossicle shapes; the external anatomy of sea cucumber e.g. the presence and shape of tube feet and feeding tentacles; and the internal anatomy e.g. the types of calcareous rings have been used for morphological species identification of sea cucumber. However, even though the morphological characteristics in sea cucumber species identification are important (Dabbagh et al., 2012; Massin et al., 2002), molecular method using Deoxyribonucleic Acid (DNA) is required as a confirmation tool especially for processed sea cucumbers that have underwent shape deformation due to extensive processing. Processed sea cucumbers including beche-de-mer are available in the forms of frozen, dried, pickled, and canned products, to mention a few.

Species identification, phylogenetic analyses, and phylogeographical analyses of animal have incorporated mitochondrial DNA (mtDNA) as the most preferred model for molecular genetic studies. Small-subunit mitochondrial ribosomal RNA (rRNA) as part of the mtDNA stores informative resources for phylogenies (Freeman & Herron, 2004). 12S rRNA and 16S rRNA are the 2 components of the small subunit. Both mitochondrial rRNA genes are not protein-coding gene, since rRNA only produces polypeptides that are used to make up proteins. In fact, 16S mitochondrial rRNA gene has been frequently used in the molecular genetic analyses of sea cucumber. In terms of species identification of processed sea cucumbers in commercial food products, PCR-RFLP technique and Forensically Informative Nucleotide Sequencing (FINS) technique based on the 16S mitochondrial rRNA gene have been further developed by Wen et al. (2010) to identify six sea cucumber species of the family Stichopodidae. Furthermore, Wen et al. (2011) applied FINS technique to evaluate the incidence of incorrect labelling of sea cucumbers of the family Holothuriidae. The studies reported the presence of product mislabelling issue or species substitution issue in the markets. In contrast to 16S mitochondrial rRNA, there is a lack of study on 12S mitochondrial rRNA gene of sea cucumber to date. Only one study on 12S mitochondrial rRNA gene of live sea cucumber has been found to date (Clouse et al. 2005). Clouse et al. (2005) summarised that *B. marmorata* and *B. bivittata* should be accepted as 2 separate species as both species were not sister species and *B. bivittata* was genetically closer to *B. argus*. In terms of species identification of processed sea cucumbers, Kamarudin et al. (2017) incorporated ossicle shapes and 12S rRNA gene sequences for species identification of *gamat*-based beche-de-mer from Langkawi Island, Kedah, Malaysia. Besides, Kamarudin et al. (2017) used 12S mitochondrial rRNA gene to identify the species of sea cucumber specimens from Kudat, Sabah, Malaysia whereby 3 species were recorded i.e. *Holothuria scabra*, *Stichopus horrens* and *Stichopus ocellatus*.

In Malaysia, issues related to species substitution and product mislabelling of sea cucumber-based products can be observed and investigated at some places. Therefore, the aim of this study was to determine the species identity of processed sea cucumber specimens from selected Malaysian markets by using the concatenated gene sequences of non-protein-coding 12S mitochondrial rRNA gene and 16S mitochondrial rRNA gene. Since both mitochondrial rRNA genes have been known informative, it is believed that their concatenation will give better conclusion on the genetic identity of sea cucumber due to the interconnection of more informative sites in a DNA sequence. The genetic identity and relationship of the sea cucumber specimens in this study were determined through Online Basic Local Alignment Search Tool program for nucleotide (blastn) and 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. A comparison has been made between the findings of the analyses and the manufacturing or packaging details of the sea cucumber specimens. Moreover, this study also highlights the issues of intentional species substitution and product labelling of processed sea cucumbers in selected Malaysian markets. The information may be utilised by the enforcement agencies to tackle issues pertaining to sea cucumber-based products in Malaysia.

2. Materials and methods

2.1 Study site and sampling

Kota Kinabalu, Sabah and Kudat, Sabah (East Malaysia, in Borneo Island); Kuantan, Pahang Darul Makmur (East Coast region of Peninsular Malaysia); Langkawi Archipelago, Kedah Darul Aman (North region of Peninsular Malaysia); Nilai, Negeri Sembilan Darul Khusus (South region of Peninsular Malaysia); and Pangkor Archipelago, Perak Darul Ridzuan (West Coast region in the northern part of Peninsular Malaysia) were included as the sampling sites (**Figure 1**). A number of 112 sea cucumber specimens were sampled including six live and fresh specimens of *gamat* species (SHP1-SHP3, *Stichopus horrens*) and *timun laut* species (HLTNP1-HLTNP3, *Holothuria (Mertensiothuria) leucospilota*)) from 2 sampling sites in Pangkor Archipelago as the reference samples of fresh sea cucumbers; and 7 dried *gamat*-based beche-de-mer specimens from Kuah, Langkawi Archipelago (LKIG1-LKIG6) as the reference samples of processed sea cucumbers.

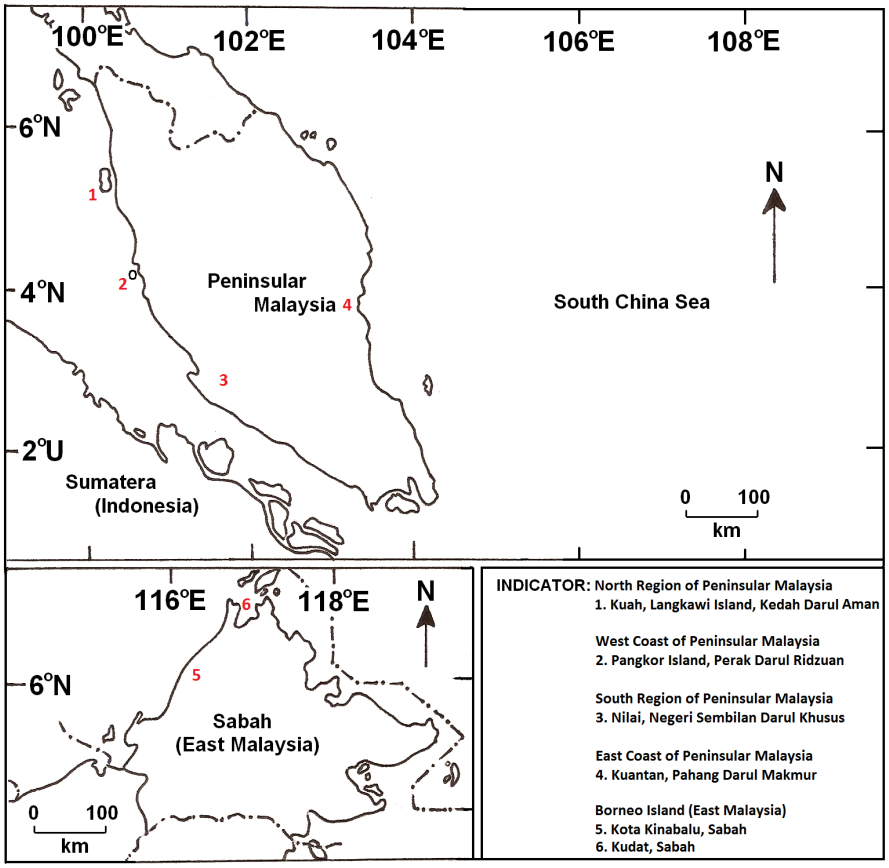

Figure 1 Sampling sites of sea cucumber specimens. Adapted from Kamarudin (2018).

2.2 Total genomic DNA extraction

A number of 3 methods of total genomic DNA extraction were used i.e. modified cetyl trimethyl ammonium bromide (CTAB) method of Grewe et al. (1993) coupled with the Geneaid Genomic DNA Mini Kit (Blood/Cultured Cell), total genomic DNA extraction using the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit, and total genomic DNA extraction using the DNeasy mericon Food Kit by QIAGEN. For the third method, homogenised tissue was prepared by using the QIAGEN TissueRuptor for disrupting and homogenising the tissue. One % 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 –20 °C chest freezer for long-term storage.

2.3 Polymerase chain reaction (PCR)

The gene amplification involved 2 methods:

a) Twenty five µl PCR reaction volume using the 2x TopTaq Master Mix Kit by QIAGEN

b) Fifty 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 sets for mitochondrial rRNA genes are as follows:

a) Primers for 12S mitochondrial rRNA gene (Palumbi et al. (1991), expected length: ~360 bp)):

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)

b) Primers for 16S mitochondrial rRNA gene (Palumbi et al. (1991), expected length: ~650 bp)):

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

16sbr-H (reverse) 5' – CCG GTC TGA ACT CAG ATC ACG T – 3' (22 bases)

The PCR cycles involved 2 parameter batches:

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.

2.4 PCR product purification and DNA sequencing

The PCR fragment purification involved 3 types of kits i.e. 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). Some of the unpurified PCR products were sent directly to the First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor Darul Ehsan, Malaysia as the company also provides PCR products clean up service.

2.5 Phylogenetic analyses

The sequenced PCR products of the mitochondrial DNA genes were displayed using Chromas program version 2.5.1 (Copyright© 1998 - 2016 Technelysium Pty Ltd). The online blastn was used to assign each DNA sequence to a particular sea cucumber species or genus. Prior to the phylogenetic tree reconstruction, ClustalX program version 2.1 (Thompson et al., 1997) was used for multiple sequence alignment of forward reaction sequences. In addition, Molecular Evolutionary Genetics Analysis version 7.0.14 (MEGA7; Kumar et al., 2016) was subsequently used to concatenate the partial sequences of non-protein-coding 12S and 16S mitochondrial rRNA genes, and also to calculate the number of base substitutions per site from between sequences (i.e. pairwise genetic distance matrix) using the Maximum Composite Likelihood model (Tamura et al., 2004) with the elimination of all positions containing gaps and missing data, and then 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). Modeltest (version 3.7) program (Posada and Crandall, 1998) was used to calculate and find the best model for DNA evolution prior to the reconstruction of Maximum Likelihood phylogenetic trees using PAUP* (version 4.0b10) program (Swofford, 1998) with 100 bootstrap replicates. A number of 56 models of DNA substitution were tested in order to choose the model that fitted the data best.

Meanwhile, the reconstructions of consensus Bayesian phylogenetic trees (using Bayesian Analysis method, a character-based method with optimality criterion as the tree building strategy) were done by using MrBayes (version 3.1.2) program (Huelsenbeck and Ronquist, 2001). 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) were used to display and edit the reconstructed phylogenetic trees.

3. Results and discussion

Table 1 indicates the number of base substitutions per site from between concatenated gene sequences of non-protein-coding 12S and 16S mitochondrial rRNA genes. A number of 47 nucleotide sequences and a total of 769 positions were involved in the final dataset. The genetic distance values between specimens that were identified as *S. horrens* ranged from 0 (0 %) to 0.0390 (3.9 %)

with average genetic distance of 0.0131 (1.31 %), thus suggesting their status as single morphospecies i.e. morphospecies *S. horrens*. Besides, the genetic distance values between PKSH1 specimen (from Kudat, Sabah) and other *S. horrens* specimens including the reference samples of SHP ranged from 0.0325 (3.25 %) to 0.0390 (3.9 %). Meanwhile, the genetic distance value between *S. vastus* specimens i.e. PKSO1 (from Kudat, Sabah) and LKIG7 was 0.0185 (1.85 %). The genetic distance values between *T. anax* specimens i.e. KKS specimens (from Kota Kinabalu, Sabah) ranged from 0.0012 (0.12 %) to 0.0375 (3.75 %) with average genetic distance of 0.0181, thus suggesting their status as single morphospecies i.e. morphospecies *T. anax*. Furthermore, the average genetic distance between *Stichopus* specimens was 0.1340 (13.4 %).

Table 1 Pairwise genetic distance matrix of sequences of concatenated gene sequences of non-protein-coding 12S and 16S mitochondrial rRNA genes of sea cucumber specimens from selected Malaysian markets and other sampling sites including the reference samples and processed specimens.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. HL1																									
2. HLTNP1	0.0278																								
3. HLTNP2	0.0197	0.0105																							
4. HLTNP3	0.0092	0.0211	0.0131																						
5. KKS1	0.3075	0.3135	0.3051	0.3075																					
6. KKS2	0.2967	0.3067	0.2983	0.3007	0.0132																				
7. KKS3	0.2991	0.3091	0.3007	0.3031	0.0145	0.0013																			
8. KKS4	0.2967	0.3067	0.2983	0.3007	0.0145	0.0026	0.0039																		
9. KKS5	0.3468	0.3489	0.3441	0.3468	0.0323	0.0364	0.0379	0.0365																	
10. KKS7	0.3003	0.3063	0.3019	0.3043	0.0185	0.0052	0.0065	0.0078	0.0365																
11. KKS9	0.2967	0.3067	0.2983	0.3007	0.0132	0.0000	0.0013	0.0026	0.0364	0.0052															
12. KPTS1	0.1661	0.1661	0.1627	0.1661	0.3091	0.3003	0.3027	0.3003	0.3466	0.3007	0.3003														
13. LKIG1	0.3304	0.3284	0.3300	0.3284	0.2184	0.2086	0.2106	0.2086	0.2469	0.2106	0.2086	0.2596													
14. LKIG2	0.3325	0.3346	0.3362	0.3346	0.2254	0.2121	0.2141	0.2121	0.2543	0.2141	0.2121	0.2672	0.0157												
15. LKIG3	0.3304	0.3325	0.3341	0.3325	0.2201	0.2069	0.2089	0.2069	0.2488	0.2089	0.2069	0.2615	0.0118	0.0039											
16. LKIG4	0.3346	0.3367	0.3383	0.3367	0.2254	0.2138	0.2158	0.2104	0.2525	0.2158	0.2138	0.2653	0.0171	0.0092	0.0052										
17. LKIG5	0.3304	0.3284	0.3300	0.3284	0.2166	0.2069	0.2089	0.2069	0.2451	0.2089	0.2069	0.2596	0.0039	0.0118	0.0078	0.0131									
18. LKIG6	0.3284	0.3304	0.3321	0.3304	0.2184	0.2051	0.2071	0.2051	0.2469	0.2071	0.2051	0.2615	0.0052	0.0105	0.0065	0.0118	0.0013								
19. LKIG7	0.3518	0.3518	0.3492	0.3455	0.2093	0.1997	0.2017	0.1997	0.2356	0.2017	0.1997	0.2811	0.0497	0.0470	0.0429	0.0429	0.0456	0.0470							
20. PFKK1	0.1480	0.1576	0.1542	0.1544	0.3152	0.3043	0.3067	0.3043	0.3551	0.3039	0.3043	0.1273	0.3075	0.3115	0.3055	0.3095	0.3075	0.3055	0.3279						
21. PFKK11	0.1463	0.1558	0.1525	0.1526	0.3177	0.3067	0.3091	0.3067	0.3578	0.3063	0.3067	0.1256	0.3099	0.3140	0.3079	0.3119	0.3099	0.3079	0.3254	0.0013					
22. PFKK12	0.1479	0.1542	0.1541	0.1542	0.3197	0.3087	0.3112	0.3087	0.3556	0.3043	0.3087	0.1271	0.3119	0.3160	0.3099	0.3140	0.3119	0.3099	0.3274	0.0026	0.0013				
23. PFKK13	0.1774	0.1823	0.1821	0.1823	0.3442	0.3442	0.3468	0.3442	0.3864	0.3395	0.3442	0.1543	0.3432	0.3496	0.3432	0.3475	0.3432	0.3432	0.3596	0.0294	0.0281	0.0267			
24. PFKK14	0.1514	0.1609	0.1576	0.1577	0.3218	0.3107	0.3132	0.3107	0.3621	0.3103	0.3107	0.1305	0.3140	0.3180	0.3119	0.3160	0.3140	0.3119	0.3325	0.0039	0.0039	0.0052	0.0322		
25. PFKK15	0.1479	0.1542	0.1541	0.1542	0.3197	0.3087	0.3112	0.3087	0.3556	0.3043	0.3087	0.1271	0.3119	0.3160	0.3099	0.3140	0.3119	0.3099	0.3274	0.0026	0.0013	0.0000	0.0267	0.0052	

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
26. PFKK16	0.1514	0.1609	0.1576	0.1577	0.3177	0.3067	0.3091	0.3067	0.3578	0.3063	0.3067	0.1305	0.3099	0.3140	0.3079	0.3119	0.3099	0.3079	0.3304	0.0026	0.0039	0.0052	0.0322	0.0065	0.0052
27. PFKK2	0.1479	0.1574	0.1541	0.1542	0.3156	0.3047	0.3071	0.3047	0.3556	0.3043	0.3047	0.1256	0.3099	0.3140	0.3079	0.3119	0.3099	0.3079	0.3254	0.0026	0.0013	0.0026	0.0294	0.0052	0.0026
28. PFKK3	0.1465	0.1560	0.1526	0.1528	0.3132	0.3023	0.3047	0.3023	0.3530	0.3019	0.3023	0.1273	0.3095	0.3136	0.3075	0.3115	0.3095	0.3075	0.3300	0.0013	0.0026	0.0039	0.0308	0.0052	0.0039
29. PFKK4	0.1465	0.1560	0.1526	0.1528	0.3132	0.3023	0.3047	0.3023	0.3530	0.3019	0.3023	0.1273	0.3095	0.3136	0.3075	0.3115	0.3095	0.3075	0.3300	0.0013	0.0026	0.0039	0.0308	0.0052	0.0039
30. PFKK5	0.1465	0.1560	0.1526	0.1528	0.3132	0.3023	0.3047	0.3023	0.3530	0.3019	0.3023	0.1273	0.3095	0.3136	0.3075	0.3115	0.3095	0.3075	0.3300	0.0013	0.0026	0.0039	0.0308	0.0052	0.0039
31. PFKK6	0.1683	0.1715	0.1681	0.1666	0.3313	0.3172	0.3197	0.3193	0.3692	0.3176	0.3172	0.1486	0.2959	0.2939	0.2919	0.2939	0.2959	0.2939	0.3070	0.1100	0.1084	0.1099	0.1372	0.1131	0.1099
32. PFKK7	0.1463	0.1558	0.1525	0.1526	0.3177	0.3067	0.3091	0.3067	0.3578	0.3063	0.3067	0.1256	0.3099	0.3140	0.3079	0.3119	0.3099	0.3079	0.3254	0.0013	0.0000	0.0013	0.0281	0.0039	0.0013
33. PFKK8	0.1905	0.1989	0.1970	0.1972	0.3638	0.3520	0.3547	0.3520	0.4076	0.3515	0.3520	0.1646	0.3491	0.3512	0.3448	0.3491	0.3491	0.3470	0.3709	0.0378	0.0393	0.0406	0.0696	0.0420	0.0406
34. PFKK9	0.1809	0.1859	0.1856	0.1858	0.3606	0.3563	0.3589	0.3563	0.3949	0.3515	0.3563	0.1646	0.3489	0.3532	0.3489	0.3531	0.3489	0.3489	0.3654	0.0364	0.0350	0.0336	0.0199	0.0392	0.0336
35. PKS1	0.1729	0.1761	0.1727	0.1761	0.3156	0.2999	0.3023	0.3019	0.3525	0.2983	0.2999	0.0158	0.2556	0.2594	0.2537	0.2593	0.2556	0.2537	0.2730	0.1305	0.1288	0.1304	0.1594	0.1338	0.1304
36. PKS2	0.1712	0.1778	0.1743	0.1745	0.3156	0.2999	0.3023	0.3019	0.3525	0.2983	0.2999	0.0171	0.2574	0.2612	0.2556	0.2612	0.2574	0.2556	0.2711	0.1321	0.1304	0.1320	0.1610	0.1354	0.1320
37. PKS21	0.1834	0.1867	0.1832	0.1867	0.3284	0.3124	0.3148	0.3144	0.3660	0.3108	0.3124	0.0265	0.2673	0.2711	0.2654	0.2711	0.2673	0.2654	0.2850	0.1436	0.1418	0.1434	0.1731	0.1469	0.1434
38. PKS3	0.1729	0.1761	0.1727	0.1761	0.3156	0.2999	0.3023	0.3019	0.3503	0.2983	0.2999	0.0184	0.2574	0.2612	0.2556	0.2612	0.2574	0.2556	0.2749	0.1274	0.1257	0.1273	0.1561	0.1307	0.1273
39. PKS4	0.1745	0.1778	0.1743	0.1778	0.3177	0.3019	0.3043	0.3039	0.3525	0.3003	0.3019	0.0197	0.2593	0.2632	0.2575	0.2631	0.2593	0.2575	0.2769	0.1258	0.1242	0.1257	0.1545	0.1291	0.1257
40. PKSH1	0.3263	0.3242	0.3258	0.3242	0.2219	0.2086	0.2106	0.2086	0.2506	0.2106	0.2086	0.2673	0.0331	0.0304	0.0264	0.0318	0.0291	0.0278	0.0484	0.3075	0.3099	0.3120	0.3454	0.3140	0.3120
41. PKSO1	0.3383	0.3404	0.3378	0.3362	0.2111	0.1980	0.1999	0.1980	0.2374	0.1999	0.1980	0.2733	0.0414	0.0387	0.0346	0.0400	0.0373	0.0360	0.0158	0.3148	0.3123	0.3144	0.3479	0.3193	0.3144
42. PM1	0.1479	0.1574	0.1541	0.1542	0.3156	0.3047	0.3071	0.3047	0.3556	0.3043	0.3047	0.1256	0.3099	0.3140	0.3079	0.3119	0.3099	0.3079	0.3254	0.0026	0.0013	0.0026	0.0294	0.0052	0.0026
43. PM3	0.1683	0.1715	0.1681	0.1666	0.3313	0.3172	0.3197	0.3193	0.3692	0.3176	0.3172	0.1486	0.2959	0.2939	0.2919	0.2939	0.2959	0.2939	0.3070	0.1100	0.1084	0.1099	0.1372	0.1131	0.1099
44. PM4	0.2921	0.2920	0.2859	0.2901	0.4813	0.4828	0.4862	0.4828	0.5069	0.4820	0.4828	0.2754	0.4357	0.4309	0.4309	0.4309	0.4357	0.4333	0.4523	0.2244	0.2224	0.2242	0.2420	0.2283	0.2242
45. SHP1	0.3304	0.3283	0.3300	0.3283	0.2201	0.2104	0.2123	0.2103	0.2488	0.2123	0.2104	0.2615	0.0118	0.0039	0.0026	0.0078	0.0078	0.0092	0.0429	0.3095	0.3119	0.3140	0.3453	0.3160	0.3140
46. SHP2	0.3304	0.3283	0.3300	0.3283	0.2201	0.2104	0.2123	0.2103	0.2488	0.2123	0.2104	0.2615	0.0118	0.0039	0.0026	0.0078	0.0078	0.0092	0.0429	0.3095	0.3119	0.3140	0.3453	0.3160	0.3140
47. SHP3	0.3283	0.3304	0.3320	0.3304	0.2219	0.2086	0.2106	0.2086	0.2506	0.2106	0.2086	0.2634	0.0131	0.0026	0.0013	0.0065	0.0092	0.0078	0.0442	0.3075	0.3099	0.3119	0.3453	0.3140	0.3119

	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47
26. PFKK16																						
27. PFKK2	0.0052																					
28. PFKK3	0.0039	0.0039																				
29. PFKK4	0.0039	0.0039	0.0000																			
30. PFKK5	0.0039	0.0039	0.0000	0.0000																		
31. PFKK6	0.1131	0.1084	0.1085	0.1085	0.1085																	
32. PFKK7	0.0039	0.0013	0.0026	0.0026	0.0026	0.1084																
33. PFKK8	0.0406	0.0393	0.0365	0.0365	0.0365	0.1477	0.0393															
34. PFKK9	0.0392	0.0363	0.0378	0.0378	0.0378	0.1458	0.0350	0.0771														
35. PKS1	0.1338	0.1288	0.1305	0.1305	0.1305	0.1422	0.1288	0.1681	0.1698													
36. PKS2	0.1354	0.1304	0.1321	0.1321	0.1321	0.1438	0.1304	0.1698	0.1714	0.0013												
37. PKS21	0.1469	0.1418	0.1436	0.1436	0.1436	0.1522	0.1418	0.1804	0.1816	0.0105	0.0118											
38. PKS3	0.1307	0.1257	0.1274	0.1274	0.1274	0.1390	0.1257	0.1648	0.1664	0.0026	0.0039	0.0131										
39. PKS4	0.1291	0.1242	0.1258	0.1258	0.1258	0.1375	0.1242	0.1632	0.1648	0.0039	0.0052	0.0145	0.0013									
40. PKSH1	0.3099	0.3099	0.3095	0.3095	0.3095	0.2880	0.3099	0.3491	0.3510	0.2556	0.2575	0.2673	0.2575	0.2594								
41. PKSO1	0.3172	0.3123	0.3168	0.3168	0.3168	0.2962	0.3123	0.3548	0.3536	0.2616	0.2634	0.2734	0.2635	0.2654	0.0373							
42. PM1	0.0052	0.0000	0.0039	0.0039	0.0039	0.1084	0.0013	0.0393	0.0363	0.1288	0.1304	0.1418	0.1257	0.1242	0.3099	0.3123						
43. PM3	0.1131	0.1084	0.1085	0.1085	0.1085	0.0000	0.1084	0.1477	0.1458	0.1422	0.1438	0.1522	0.1390	0.1375	0.2880	0.2962	0.1084					
44. PM4	0.2283	0.2224	0.2226	0.2226	0.2226	0.1245	0.2224	0.2716	0.2527	0.2758	0.2777	0.2865	0.2739	0.2720	0.4285	0.4388	0.2224	0.1245				
45. SHP1	0.3119	0.3119	0.3115	0.3115	0.3115	0.2959	0.3119	0.3491	0.3510	0.2575	0.2593	0.2692	0.2594	0.2612	0.2612	0.0373	0.3119	0.2959	0.4357			
46. SHP2	0.3119	0.3119	0.3115	0.3115	0.3115	0.2959	0.3119	0.3491	0.3510	0.2575	0.2593	0.2692	0.2594	0.2612	0.0291	0.0373	0.3119	0.2959	0.4357	0.0000		
47. SHP3	0.3099	0.3099	0.3095	0.3095	0.3095	0.2939	0.3099	0.3470	0.3510	0.2556	0.2575	0.2673	0.2575	0.2594	0.0278	0.0360	0.3099	0.2939	0.4333	0.0013	0.0013	

With regard to the *timun laut* specimens, the average genetic distance value between *Holothuria* specimens including the reference samples of HLTNP was 0.1112 (11.12 %). Moreover, the genetic distance values between *H. leucospilota* specimens (HLTNP specimens and HL1 specimen from Pangkor Archipelago, Perak) ranged from 0.0086 (0.86 %) to 0.0277 (2.77 %) with an average genetic distance of 0.0169 (1.69 %). The genetic distance values between HL1 specimen (from Pangkor Archipelago, Perak) and other *H. leucospilota* specimens ranged from 0.0086 (0.86 %) to 0.0277 (2.77 %). Furthermore, the genetic distance values between *H. scabra* specimens (PKS specimens from Kudat, Sabah) ranged from 0.0013 (0.13 %) to 0.0140 (1.4 %) with an average genetic distance of 0.0066 (0.66 %), while the genetic distance values between *H. lessoni* specimen i.e. KPTS1 (from Kuantan, Pahang) and *H. scabra* specimens ranged from 0.0179 (1.79 %) to 0.0284 (2.84 %). As for the *H. atra* specimens (PFKK6 specimen from Kota Kinabalu, Sabah and specimens of PM3 and PM4 from Manukan Island, Sabah), the average genetic distance was 0.0835 (8.35 %) ranging from 0 (0 %) to 0.1253 (12.53 %), with the genetic distance value between PFKK6 specimen and PM3 specimen was 0 (0 %). The average genetic distance between *H. edulis* specimens (PFKK specimens from Kota Kinabalu, Sabah and PM1 specimen from Manukan Island, Sabah) excluding PFKK6 specimen was 0.0293 (2.93 %) ranging from 0 (0 %) to 0.1539 (15.39 %), 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 0.1182 (11.82 %).

With regard to the Neighbour Joining analyses using concatenated gene sequences of non-protein-coding 12S and 16S mitochondrial rRNA genes, the optimal tree with the sum of branch length = 7.50559484 is shown in **Figure 2**. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (i.e. 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. All positions containing gaps and missing data were eliminated. A number of 48 taxa consisting of 47 ingroup taxa and one outgroup taxon, and 749 characters representing aligned base positions (after multiple alignment) were involved in the phylogenetic analyses of the sequences of concatenated gene sequences of non-protein-coding 12S and 16S mitochondrial rRNA genes using Neighbour Joining method. The outgroup taxon was an individual of *Peniagone* sp., a deep-sea swimming sea cucumber species with GenBank Accession No. KF915304.

Figure 2 illustrates the presence of 2 main groups of the specimens: the *timun laut* family with 47 % bootstrap support and a few clusters representing family Stichopodidae (the *gamat* family). A number of 3 *gamat* species i.e. *S. horrens*, *S. vastus*, and *T. anax* were representing the family Stichopodidae. *T. anax* was divided into 2 clusters and *S. horrens* cluster was supported by 96 % bootstrap value. Nonetheless, the specimens of *S. vastus* were also grouped into the *S. horrens* due to their close genetic relationship. As mentioned earlier, the genetic distance value between *S. vastus* specimens i.e. PKSO1 (from Kudat, Sabah) and LKIG7 was low i.e. 0.0185 (1.85 %). *S. vastus* cluster was supported with 97 % bootstrap value. In addition, the specimens of PFKK excluding PFKK6 specimen were clustered as *H. edulis* with 91 % bootstrap support. Purcell et al. (2012) recorded a processed *H. lessoni* that is similar to the dried *tip-sum* (KPTS1), therefore the specimen was regarded as *H. lessoni*. In terms of the species status of PM specimens, the analyses identified PM1 specimen as *H. edulis*, and the specimens of PM3 and PM4 as *H. atra*. Therefore, the specimens of *H. leucospilota* that formed a cluster with 92 % bootstrap value, the specimens of *H. edulis* that formed a

cluster with 91 % bootstrap value, the specimens of *H. scabra* that formed a cluster with 66 % bootstrap value, the dried specimen of *H. lessoni* (KPTS1), and the specimens of *H. atra* that formed a cluster with 79 % bootstrap value were the 5 *timun laut* species that grouped under the family Holothuriidae with 47 % bootstrap support. *H. atra* was genetically closer to *H. edulis* with 68 % bootstrap support. Furthermore, 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 46 % bootstrap value, thus supporting their taxonomic classification.

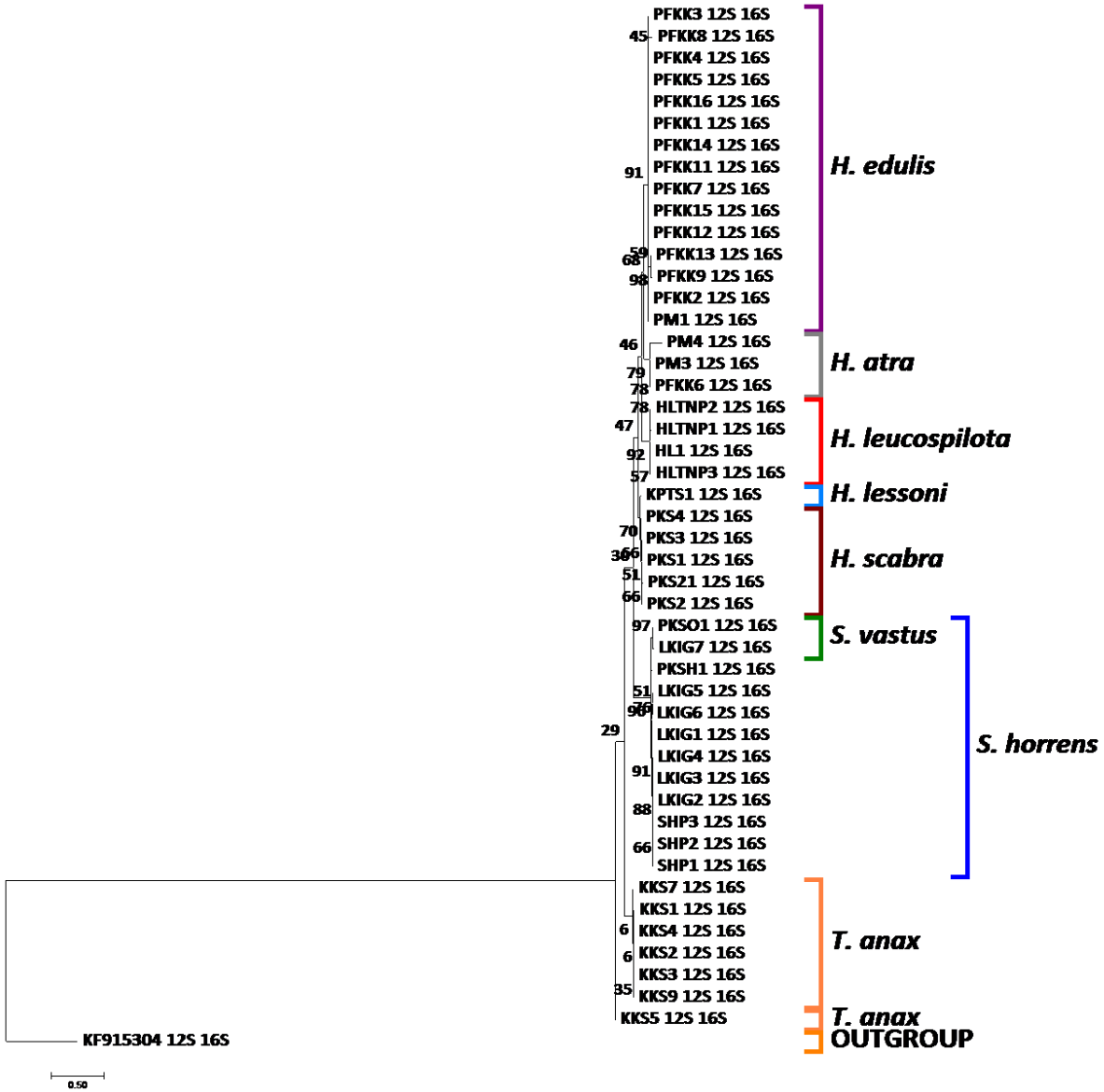

Figure 2 Topology of 50 % majority-rule consensus tree of Neighbour Joining of sea cucumber specimens from selected Malaysian markets and other sampling sites including the reference samples and processed specimens inferred from concatenated gene sequences of non-protein-coding 12S and 16S mitochondrial rRNA genes using MEGA7 program (Kumar et al., 2016) with 1000 bootstrap replicates. Numbers at nodes indicate the bootstrap values in percentage (%).

As for the Maximum Parsimony analyses using concatenated gene sequences of non-protein-coding 12S and 16S mitochondrial rRNA genes, the bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates were collapsed. 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 Maximum Parsimony tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei & Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated. Besides, a number of 48 taxa consisting of 47 ingroup taxa and one outgroup taxon, and 749 characters representing aligned base positions (after multiple alignment) were involved in the phylogenetic analyses.

Figure 3 illustrates the presence of 2 main clusters of the specimens: family Stichopodidae (the *gamat* family) and the *timun laut* family, both with 90 % bootstrap support and 99 % bootstrap support, respectively. A number of 3 *gamat* species i.e. *S. horrens*, *S. vastus*, and *T. anax* were clustered under the family Stichopodidae. *T. anax* cluster was supported by 99 % bootstrap value, *S. horrens* cluster was supported by 76 % bootstrap value, and *S. vastus* cluster was supported by 93 % bootstrap value. *S. vastus* was genetically closer to *S. horrens* with 99 % bootstrap support. Furthermore, the specimens of *H. leucospilota* that formed a cluster with 99 % bootstrap value, the specimens of *H. edulis* that formed a cluster with 99 % bootstrap value, the specimens of *H. scabra* that formed a cluster with 88 % bootstrap value, the dried specimen of *H. lessoni* (KPTS1), and the specimens of *H. atra* that formed a cluster with 99 % bootstrap value were the 5 *timun laut* species that grouped under the family Holothuriidae with 98 % bootstrap support. *H. atra* was genetically closer to *H. edulis* with 51 % bootstrap support. Likewise the Neighbour Joining analyses, 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.

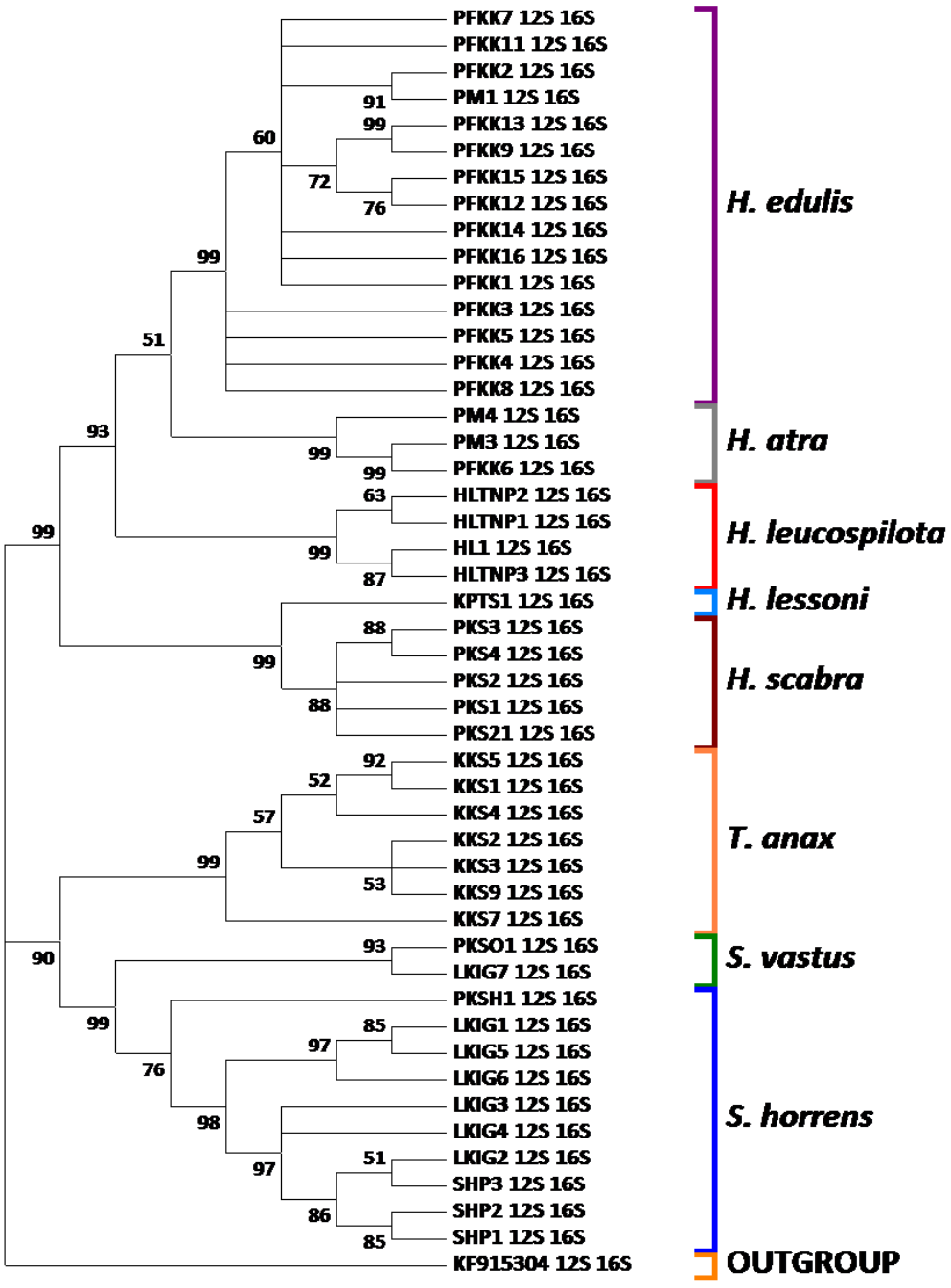

Figure 3 Topology of 50 % majority-rule consensus tree of Maximum Parsimony of sea cucumber specimens from selected Malaysian markets and other sampling sites including the reference samples and processed specimens inferred from concatenated gene sequences of non-protein-coding 12S and 16S mitochondrial rRNA genes using MEGA7 program (Kumar et al., 2016) with 1000 bootstrap replicates. Numbers at nodes indicate the bootstrap values in percentage (%).

Pertaining to the Maximum Likelihood analyses using concatenated gene sequences of non-protein-coding 12S and 16S mitochondrial rRNA genes, Modeltest (version 3.7) program suggested general time-reversible (GTR) model (Tavaré, 1986) with the rate variation among sites (+G) (i.e. GTR+G) as the best model of DNA substitution based on the Akaike Information Criterion (AIC); and Tamura and Nei (TrN) model (Tamura & Nei,

1993) with the rate variation among sites (+G) (i.e. TrN+G) as the best model of DNA substitution based on the Hierarchical Likelihod Ratio Tests (hLRTs). The GTR model and the TrN model were based on unequal base frequencies. According to Posada and Buckley (2004), the AIC and Bayesian Analysis are good at the evaluation of model selection uncertainty, capable to compare multiple nested or non-nested models at once, and allow for the use of all available models for the estimation of phylogenies and model parameters. Hence, between the AIC and the hLRTs used in Modeltest program, the model of DNA substitution suggested by the AIC is better. The GTR+G model suggested by the AIC was chosen for the Maximum Likelihood tree reconstruction (Lset Base=(0.3343 0.2070 0.1869) Nst=6 Rmat=(2.4444 4.2221 1.8990 1.0703 7.6112) Rates=gamma Shape=0.7426 Pinvar=0)).

A number of 48 taxa consisting of 47 ingroup taxa and one outgroup taxon, and 859 characters representing aligned base positions (after multiple alignment) were involved in the phylogenetic analyses. Overall, the base frequencies were unequal (i.e. Adenine (A) = 33.43 %, Cytosine (C) = 20.70 %, Guanine (G) = 18.69 %, and Thymine (T) = 27.18 %)), thus supporting the selection of GTR+G model by the AIC as the best model. In addition, **Figure 4** illustrates the presence of 2 clusters of family Stichopodidae (the *gamat* family) i.e. genus *Stichopus* cluster with 86 % bootstrap support and genus *Thelenota* cluster with 66 % bootstrap support; and the *timun laut* family with 59 % bootstrap support. A number of 3 *gamat* species i.e. *S. horrens*, *S. vastus*, and *T. anax* under the family Stichopodidae were identified. *T. anax* cluster was supported by 66 % bootstrap value, *S. horrens* cluster was supported by 62 % bootstrap value, and *S. vastus* cluster was supported by 94 % bootstrap value. *S. vastus* was genetically closer to *S. horrens* with 86 % bootstrap support. Besides, the specimens of *H. leucospilota* that formed a cluster with 91 % bootstrap value, the specimens of *H. edulis* that formed a cluster with 90 % bootstrap value, the specimens of *H. scabra* (with the inclusion of the dried specimen of *H. lessoni* (KPTS1)) with 90 % bootstrap value, and the specimens of *H. atra* that formed a cluster with 98 % bootstrap value were the 5 *timun laut* species that grouped under the family Holothuriidae with 59 % bootstrap support.

Furthermore, the phylogenetic analysis of Bayesian Analysis method was ended when the standard deviation of split frequencies was below 0.01. Accordingly, for the Bayesian Analysis analyses of concatenated gene sequences of non-protein-coding 12S and 16S mitochondrial rRNA genes, the standard deviation of split frequencies was 0.007697 at 1,780,000 generations. **Figure 5** illustrates the presence of 3 main groups of the specimens: family Stichopodidae (the *gamat* family) with 66 % posterior probability, and 2 subclusters of the *timun laut* family. A number of 3 *gamat* species i.e. *S. horrens*, *S. vastus*, and *T. anax* were clustered under the family Stichopodidae. *T. anax* cluster was supported by 87 % posterior probability, *S. horrens* cluster was supported by 50 % posterior probability and *S. vastus* cluster was supported by 99 % posterior probability.

The specimens of *H. leucospilota* that formed a cluster with 98 % posterior probability, the specimens of *H. edulis* that formed a cluster with 95 % posterior probability, and the specimens of *H. atra* that formed a cluster with 89 % posterior probability were the 3 *timun laut* species that formed one of the subclusters of the *timun laut* family with 65 % posterior probability. The specimens of *H. scabra* that formed a cluster with 94 % posterior probability and the dried specimen of *H. lessoni* (KPTS1) were the 2 *timun laut* species that formed the other subcluster of the *timun laut* family with 90 % posterior probability. Moreover, *H. edulis* was closer to *H. atra* with 75 % posterior probability, and *H. scabra* was closer to *H. lessoni* with 90 % posterior probability. Likewise the Neighbour Joining analyses and the Maximum Parsimony analyses, 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 65 % posterior probability.

In addition, the Maximum Likelihood tree (**Figure 4**) and the Bayesian Analysis tree (**Figure 5**) supported that *H. scabra* was genetically closer to *H. lessoni* with 90 % bootstrap value/posterior probability, thus supporting their taxonomic classification as from the subgenus *Metriatyla*. Except the Bayesian Analysis tree, the other phylogenetic trees show the clustering of *timun laut* specimens into clusters with 47 - 99 % bootstrap values, thus suggesting the formation of *timun laut* group. In summary, 8 sea cucumber species were recorded in this study including 5 *timun laut* species i.e. *H. leucospilota*, *H. atra*, *H. edulis*, *H. scabra,* and *H. lessoni*; and 3 *gamat* species i.e. *S. horrens*, *S. vastus,* and *T. anax*. *H. leucospilota*, *H. atra*, *H. edulis*, *H. scabra, S. horrens*, and *T. anax* were the commercial Malaysian sea cucumber species (Choo, 2008). Nevertheless, *H. lessoni* and *S. vastus* were not listed as commercial Malaysian sea cucumber species. Among the species recorded in this study, 2 *timun laut* species were included in the International Union for Conservation of Nature (IUCN) Red List for aspidochirotid holothuroids, whereby *H. lessoni* and *H. scabra* were regarded as "endangered, or at a high risk of extinction" (Conand et al., 2014). Apart from that, the outcomes of this study provide better information on the level of species substitution and product mislabelling issues of processed sea cucumbers in Malaysian markets which may subsequently assists the enforcement agencies to monitor and overcome the issues through the introduction of mtDNA sequencing technique.

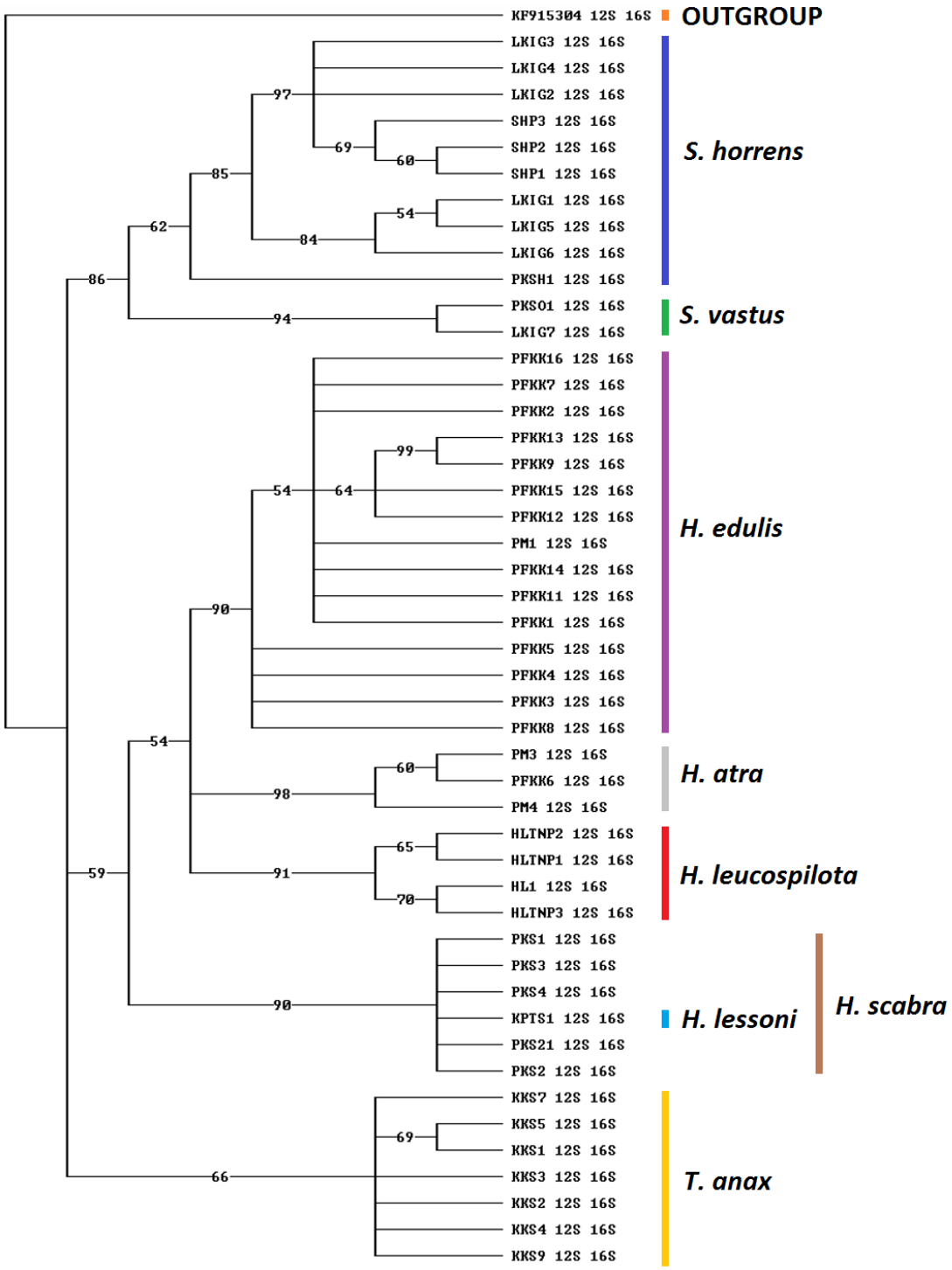

Figure 4 Topology of 50 % majority-rule consensus tree of Maximum Likelihood of sea cucumber specimens from selected Malaysian markets and other sampling sites including the reference samples and processed specimens inferred from concatenated gene sequences of non-protein-coding 12S and 16S mitochondrial rRNA genes using PAUP* (version 4.0b10) program (Swofford, 1998) with 100 bootstrap replicates. Numbers at nodes indicate the bootstrap values in percentage (%).

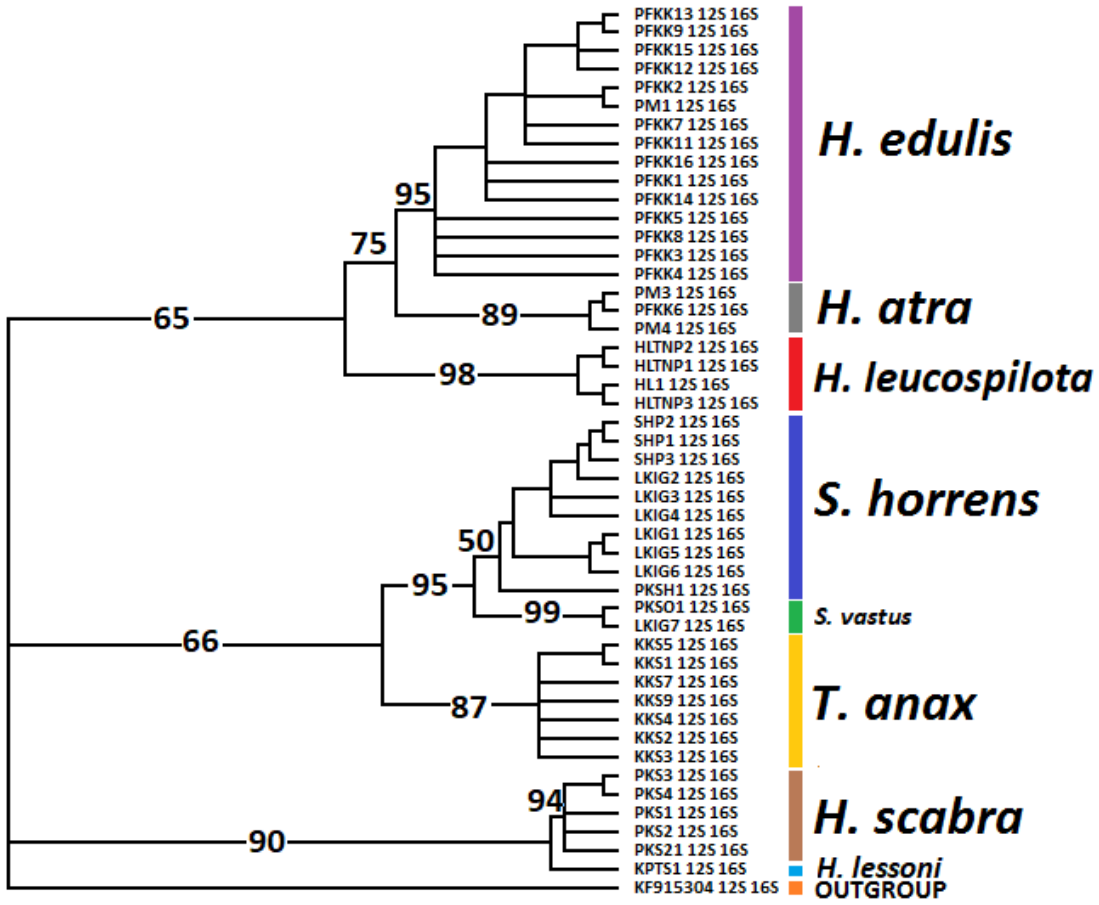

Figure 5 Topology of consensus Bayesian Analysis tree of sea cucumber specimens from selected Malaysian markets and other sampling sites including the reference samples and processed specimens inferred from from concatenated gene sequences of non-protein-coding 12S and 16S mitochondrial rRNA genes using MrBayes (version 3.1.2) program (Huelsenbeck & Ronquist, 2001), with the addition of all compatible groups to the tree. Numbers at nodes indicate the posterior probabilities of clades in percentage (%).

4. Conclusions

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 3 main clusters of the specimens i.e. *gamat* family consisting of genus *Stichopus* and genus *Thelenota*; and *timun laut* family comprising family Holothuriidae. Three *gamat* species i.e. *S. horrens*, *S. vastus*, and *T. anax*; and 5 *timun laut* species i.e. *H. atra, H. edulis, H. lessoni, H. leucospilota, and H. scabra* were recorded. This study also highlights the presence of issues of intentional species substitution or product mislabeling due to the observation of unlabelled products in the selected Malaysian markets. The outcomes of this study may assist the enforcement agencies to monitor and address the said issues.

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