Jurnal Kejuruteraan dan Sains Kesihatan
Journal of Engineering and Health Sciences
Jilid 4 2020: 95 – 110

DNA BARCODING OF *HOLOTHURIA (MERTENSIOTHURIA) LEUCOSPILOTA* FROM PULAU TINGGI, JOHOR

Nursyamfarhani Akashah[a]*, Siti Najihah Solehin[a] , Marsheilla Lia Janing[a], Nur Aina Yusra Manap[a], Nor Shahida Ab Rahman[a], Muhammad Abdul Latiff Abu Bakar[a], 'Aisyah Mohamed Rehan[b], Kamarul Rahim Kamarudin[a]

[a]*Department of Technology and Natural Resources, Faculty of Applied Sciences and Technology (FAST), Universiti Tun Hussein Onn Malaysia (UTHM)*
[b]*Department of Chemical Engineering Technology, Faculty of Engineering Technology (FTK), Universiti Tun Hussein Onn Malaysia (UTHM)*

Abstract

Sea cucumber or locally known as gamat and timun laut is a well-known attraction of Pulau Langkawi, Kedah, Malaysia. Not only limited to Pulau Langkawi, *sea cucumber* can also be found in other coastal water areas of Malaysia. However, the species richness and genetic diversity of *sea cucumber* in Pulau Tinggi, Johor is still unknown to the marine scientific communities, especially for *Holothuria (Mertensiothuria) leucospilota*, the most dominant species in Malaysia. Therefore, this study aimed to generate DNA barcodes of *H. leucospilota* from Pulau Tinggi. Protein-coding cytochrome c oxidase 1 (CO1) mtDNA gene was used as it is commonly and widely used for molecular species identification via DNA barcoding. Polymerase chain reaction (PCR) and DNA sequencing were incorporated to generate the DNA barcodes of CO1 mtDNA gene. The BLAST program confirmed the species status of the *sea cucumber* specimens as *H. leuscospilota* or locally known as bat puntil or white threads fish. The application for GenBank, NCBI, U.S. National Library of Medicine registration was successfully accepted on October 17, 2019 with accession numbers of MN580537 and MN580538). The *phylogenetic* trees i.e neighbor joining tree (NJ, distance-based method), maximum parsimony tree (MP, character-based method) and maximum likehood tree (ML, character-based method) reconstructed using MEGA X software version 10.0.5 (BETA) further supported the species status of the *sea cucumber* specimens from Pulau Tinggi as *H. leuscospilota*. This is the first record of CO1 mtDNA barcodes of *H. leuscospilota* from Pulau Tinggi, Johor, Malaysia.

Keywords: *Holothuria (Mertensiothuria) leuscospilota*, Pulau Tinggi, protein-coding cytochrome c oxidase 1 mtDNA gene, DNA barcodes, phylogenetic trees.

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PENGEKODAN DNA *HOLOTHURIA (MERTENSIOTHURIA) LEUCOSPILOTA* DARI PULAU TINGGI, JOHOR

Abstrak

Timun laut atau lebih dikenali sebagai gamat adalah tarikan terkenal di Pulau Langkawi, Kedah, Malaysia. Tidak hanya terhad di Pulau Langkawi, timun laut juga terdapat di kawasan perairan pesisir lain di Malaysia. Walau bagaimanapun, kekayaan spesies dan kepelbagaian genetik timun laut di Pulau Tinggi, Johor masih belum diketahui oleh komuniti saintifik laut, terutama bagi *Holothuria (Mertensiothuria) leucospilota*, spesies yang paling dominan di Malaysia. Oleh itu, kajian ini bertujuan untuk menghasilkan kod bar DNA *H. leucospilota* dari Pulau Tinggi. Gen *Protein-coding cytochrome c oxidase 1* (CO1) mtDNA digunakan kerana ia biasa dan banyak digunakan untuk identifikasi spesies molekul melalui pengekodan DNA. *Polymerase chain reaction* (PCR) dan penjujukan DNA digabungkan untuk menghasilkan kod bar DNA gen CO1 mtDNA. Program BLAST mengesahkan status spesies-spesies timun laut sebagai *H. leuscospilota* atau dikenali sebagai ikan kelawar atau ikan benang putih. Permohonan pendaftaran GenBank, NCBI, Perpustakaan Negara A.S. berjaya diterima pada 17 Oktober 2019 dengan nombor aksesi MN580537 dan MN580538). Pohon *phylogenetic* iaitu pokok *neighbor joining* (NJ, kaedah berdasarkan jarak), pokok *maximum parsimony* (MP, kaedah berdasarkan watak) dan pokok *maximum likehood* (ML, kaedah berdasarkan watak) dibina semula menggunakan perisian MEGA X versi 10.0.5 (BETA) menyokong lagi status spesies-spesies timun laut dari Pulau Tinggi sebagai *H. leuscospilota*. Ini adalah rekod pertama kod bar CO1 mtDNA *H. leuscospilota* dari Pulau Tinggi, Johor, Malaysia.

Kata kunci: *Holothuria (Mertensiothuria) leuscospilota*, Pulau Tinggi, gen *protein-coding cytochrome C Oxidase* 1 mtDNA, pengekodan DNA, pokok *phylogenetic*.

1.0 INTRODUCTION

Pulau Tinggi is located about 20 nautical miles or 37 km southeast of Mersing Town, on the east coast of Johor Mainland (Majlis Daerah Mersing, 2020). This island is one of the 42 islands that have been gazetted as Marine Park of Malaysia (Jabatan Taman Laut Malaysia, 2019). Specifically, Pulau Tinggi was gazetted under Sultan Iskandar Marine Park, clustering together with other four islands which are Pulau Aur, Pulau Pemanggil, Pulau Sibu and Pulau Besar (Johor National Parks, 2019). Besides, Pulau Tinggi is also one of the main populated islands from 62 islands of Seribuat Archipelago, with a broad range of ecological zones from the mountains to the sea containing significant values of distinct biodiversity richness (Malaysian Nature Society, 2013). Being gazetted as Marine Park, precautionary actions and sustainable management plans towards controllable recreational activities for tourism development should be implemented to diminish any risk in the future.

Pulau Tinggi is the host of diverse marine-living organisms including sea cucumbers. Sea cucumber is a marine invertebrate of the phylum Echinodermata and class Holothuroidea. This marine invertebrate has a physical often soft and cylindrical body with longitude elongated body (Steven *et al.*, 2012). Its size range is usually from 1.9 centimetres and it can elongate until 1.8 meters. The unique sea creatures live across the oceans of the world from seashore to the deepest trenches of the sea, often partially buried in the sand. Globally, sea cucumbers are also known as sea cuke, holothuroid and holothuria. In Malaysia, sea cucumber are locally and commonly named as *timun laut, balat, bat, brunok,* and *gamat*; while the Malaysian Chinese community usually calls it *hoi sum* or *hai shen*.

It was estimated that more than 80 sea cucumber species inhabited Malaysia seawaters (Kamarudin *et al*., 2010). Order Aspidochirotida was reported as the most diverse order of sea cucumber in Malaysia (Kamarudin *et al*. 2006, Kamarudin *et al*. 2009). *Holothuria, Stichopus, Bohadschia, Thelenota* and *Actinopyga* are the genera that make up the order Aspidochirotida in Malaysian coastal areas (Kamarudin & Hashim, 2005). The presence of nine species of order Dendrochirotida was also documented in Malaysia (Lane *et al.*, 2000). However, there is still a lack of official information on the diversity and distribution of sea cucumber species in Pulau Tinggi, Johor, Malaysia.

Furthermore, there was also no record on the genetic information of sea cucumber species from Pulau Tinggi. In fact, genetic or molecular approaches have been identified as an excellent tool to support the outcomes of morphological approaches for sea cucumber species identification and genetic

relationship. Mitochondrial DNA (mtDNA) of organisms has been justified as an efficient way to specify the population genetic structure, taxonomic status, conservation, zoogeography and geographic variation (Harrison, 1989, Amos & Hoelzel, 1992, Daniels *et al.,* 2002). Besides, mtDNA contains genetic inheritance that is informative for understanding how species and populations respond, adapt and evolve in the natural environment (Wang *et al.*, 2000, Wilkinson *et al.,* 2002). Maternal inheritance, apparent haploid genome, non-recombination, and continuous replication are among the characteristics of mtDNA gene that make it the most chosen approach in many studies including molecular ecology (Amos & Hoelzel, 1992).

Due to the absence of information on the genetic information of sea cucumber species from Pulau Tinggi, this study was done to generate DNA barcodes of *Holothuria (Mertensiothuria) leucospilota* from Pulau Tinggi. *H. leucospilota* was selected since it was regarded as the most dominant species in Malaysia (Kamarudin *et al*., 2010). Protein-coding cytochrome c oxidase 1 (CO1) mtDNA gene was used as mtDNA representative in order to generate DNA barcodes of *H. leucospilota* from Pulau Tinggi since it is commonly and widely used for molecular species identification. The outcome of this study is very significant as it is the first record of CO1 mtDNA barcodes of *H. leuscospilota* from Pulau Tinggi, Johor, Malaysia.

2.0 MATERIAL AND METHODS

2.1 Total Genomic DNA Extraction

A 25 mg of muscle tissue was cut from the sea cucumber specimen. The total genomic DNA extraction was done by following the protocols of FavorPrep Tissue Genomic DNA Extraction Mini Kit by Favorgen. The quantity and quality of each total genomic DNA yield were determined by using NanoDrop Spectrophotometer and agarose gel electrophoresis.

2.2 Polymerase Chain Reaction (PCR)

A set of primers for COI mtDNA gene with expected length of PCR fragment lenth approximately 550 base pairs (bp) was used. The primers are HS_SCCOI_F (forward) 5’- CCT GCA GGA GGA GGA GGA GAY CC -3’ (23 bases) and HS_SCCOI_R (reverse) 5’- CCA GAG ATT AGA GGG AAT CAG TG -3’ (23 bases) (Palumbi *et al*., 1991).

The PCR recipe contained 25µL reaction volume. The volume for each PCR reaction comprised of 2.5µL DNA template, 0.5µL 10µM forward primer,

0.5µL 10µM reverse primer, 12.5µL PCR master mix and 9µL ultrapure H_2O. The PCR cycle settings were 95°C for 4 min for initial denaturation, 95°C for 30 sec for denaturation, 50°C for 30 sec for annealing, and 72°C for 45 sec for extension. Repetition of steps 2-4 was done for another 35 cycles and followed by a final extension for 10 min at 72°C. Agarose gel electrophoresis was used to determine the quantity and quality of each PCR fragment or PCR product.

2.3 PCR Product Purification and DNA Sequencing

Unpurified positive PCR products in suspension form were directly sent to Apical Scientific Sdn. Bhd. for DNA purification and DNA sequencing services.

2.4 BLAST Analysis and Phylogenetic Analyses

Sequence editing, multiple alignment and phylogenetic analyses were conducted using MEGA X Software version 10.0.5 (BETA). Sample identification was performed using the Basic Local Assignment Search Tool or BLAST (Altschul *et al*., 1990). One or more GenBank corresponding sequences with the highest maximum identity scores were included in the phylogenetic analyses. Neighbor joining (NJ) method, maximum likelihood (ML) method and maximum parsimony (MP) method were use for the phylogenetic tree reconstruction, with 1000 replicates of bootstrap value.

2.5 GenBank Submission

The DNA sequences were prepared following the GenBank requirements prior to the GenBank submission. Sequence submission was done via a web-based submission tool called BankIt. ExPASy Translate tool was used to translate the DNA sequences to protein sequences during the preparation via BankIt.

3.0 RESULTS AND DISCUSSION

A number of two partial COI mtDNA gene sequences of *H. leuscospilota* from Pulau Tinggi were successfully obtained in this study. The species status of the sea cucumber specimens was confirmed as *H. leuscospilota* or locally known as bat puntil or white threads fish by the BLAST program. Both partial sequences have been successfully registered with the GenBank database maintained by the National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine on October 17, 2019 with accession numbers of MN580537 and MN580538, thus significantly contributing to the current availability of the gene sequences of the species for future research. This new registration and deposition via GenBank has contributed to the availability of COI mtDNA gene sequences

of *H. leucospilota* that had been deposited previously, including those partial sequences by Kamarudin *et al*. (2011).

For the phylogenetic analyses, a total number of 27 COI mtDNA gene sequences were included in the analyses as shown in Table 1, including 25 sequiences from the GenBank. The results of the phylogenetic analyses indicated that the neighbour-joining (NJ) method (Figure 1), maximum likelihood (ML) method (Figure 2), and maximum parsimony (ML) method (Figure 3) grouped the COI mtDNA gene sequences of *H. leucospilota* from Malaysia in one cluster of *H. leucospilota* with high bootstrap values ranging from 88% to 97%, thus showing strong genetic relationship and single species status between *H.leucospilota* from Pulau Tinggi, Johor and Intan Besar Island, Langkawi, Kedah. The other GenBank sequences formed clusters of *Holothuria atra*, *Holothuria coluber*, and *Holothuria austrinabassa* from Papua New Guinea, Australia, Indonesia and New Caledonia with high bootstrap values ranging from 95% to 99%.

The NJ tree (Figure 1) shows the presence of two major groups. All sequences of *H. atra* formed the first group with 95% bootstrap value showing its monophyly while the sequences of *H. coluber* and *H. leucospilota* formed the second group with 74% bootstrap value exhibiting their close genetic relationship. The sequence of *H. austrinabassa* became the basal. Likewise the NJ tree, all sequences of *H. atra* in the ML tree (Figure 2) clustered together with a strong boostrap support of 99%. The two groups of *H. leucospilota* and *H. coluber* were clustered with 89% bootstrap values indicating their close genetic relationship. The ML tree supported NJ tree in the positioning of *H. austrinabassa* as the basal. Even though the MP tree (Figure 3) supported the close genetic relationship between *H. leucospilota* and *H. coluber* with 83% bootstrap value, the phylogenetic tree indicated that *H. austrinabassa* clustered together with *H. atra* showing their close genetic relationship.

Generally, the present results suggested that *H. coluber* was genetically closer to monophyletic *H. leucospilota*, while *H. atra* has the strongest cluster on its own species. The phylogenetic status of *H. austrinabassa* was unresolved due to its inconsistent clustering position in the trees with unresolved branching, thus needs additional specimens in order to confirm its molecular phylogeny particularly within the genus *Holothuria*. The molecular data on 16S mitochondrial rRNA gene and COI mtDNA gene suggested that *H. (Panningothuria) austrinabassa* and *H. (Panningothuria) forskali* Delle Chiaje, 1823 are sister (O'Loughlin *et al*.,2007). Despite the differences, this study strongly supported the species status of *H. leucospilota* specimens from Pulau Tinggi, Johor, Malaysia as it is genetically.

Table 1: Taxa incorporated for phylogenetic analyses of *Holothuria leucospilota* from Pulau Tinggi, Johor, Malaysia using partial cytochrome c oxidase I mitochondrial DNAgene

Taxa	**Sample Size**	**Individual No.**	**Locality**	**GenBank Acession No.**
Order Aspidochirotida Family Holothuriidae				
Holothuria (Halodeima) atra Jaeger 1833	2	AtraPNG1 AtraPNG5	Papua New Guinea: Milne Bay Papua New Guinea: Milne Bay	EU848225 EU848224
	2	AtraAUS6 AtraAUS14	Australia: Lizard Island Australia: Lizard Island	EU848223 EU848283
	3	AtraAUS8 AtraAUS13 AtraAUS17	Australia: Great Barrier Reef Australia: Great Barrier Reef Australia: Great Barrier Reef	EU848217 EU848233 EU848286
	1	AtraAUS15	Australia	EU220820
	1	AtraINDO9	Indonesia: Pulao Doi	EU848244
	4	AtraNC7 AtraNC10 AtraNC11 AtraNC12	New Caledonia New Caledonia New Caledonia New Caledonia	EU848222 EU848265 EU848266 EU848264
Holothuria (Acanthotrapeza) coluber Semper, 1868	2	ColubrAUS2 ColubrAUS4	Australia: Lizard Island Australia: Lizard Island	EU848297 EU848295
	1	ColubrAUS3	New Caledonia	EU848284

Holothuria (Panningothuria) austrinabassa O'loughlin, Paulay, Vandenspiegel & Samyn 2007	1	SpAUS16	Australia	EU220818
Holothuria (Mertensiothuria) leucospilota (Brandt 1835)	8	FJ223873	Malaysia: Intan Besar Island, Langkawi	FJ223873
		FJ223874	Malaysia: Intan Besar Island, Langkawi	FJ223874
		FJ223875	Malaysia: Intan Besar Island, Langkawi	FJ223875
		FJ223876	Malaysia: Intan Besar Island, Langkawi	FJ223876
		FJ223877	Malaysia: Intan Besar Island, Langkawi	FJ223877
		FJ223878	Malaysia: Intan Besar Island, Langkawi	FJ223878
		FJ223879	Malaysia: Intan Besar Island, Langkawi	FJ223879
		FJ223880	Malaysia: Intan Besar Island, Langkawi	FJ223880
	2	*MN580537	Malaysia: Pulau Tinggi, Johor	
		*MN580538	Malaysia: Pulau Tinggi, Johor	

Notes: * specimens from Pulau Tinggi, Johor, Malaysia used in this study. The ones without the asterisk symbol were corresponding sequences obtained from the GenBank, NCBI, US National Library of Medicine.

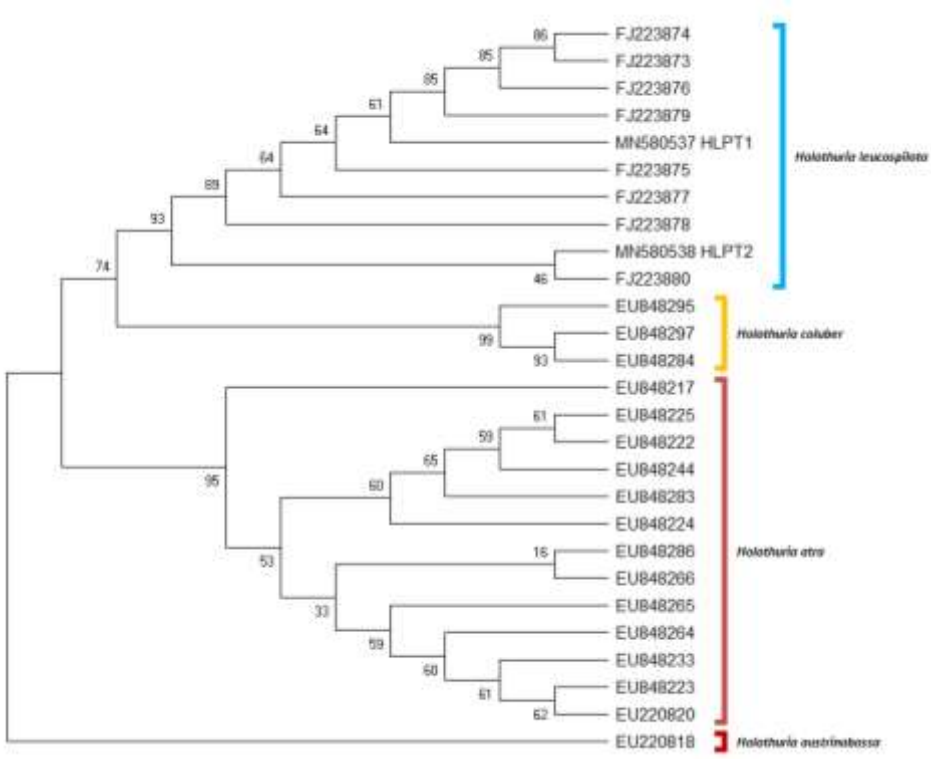

Figure 1. The evolutionary history was inferred using the Neighbor Joining method. The optimal tree with the sum of branch length = 0.38319863 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is 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 and are in the units of the number of base substitutions per site. This analysis involved 27 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 156 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

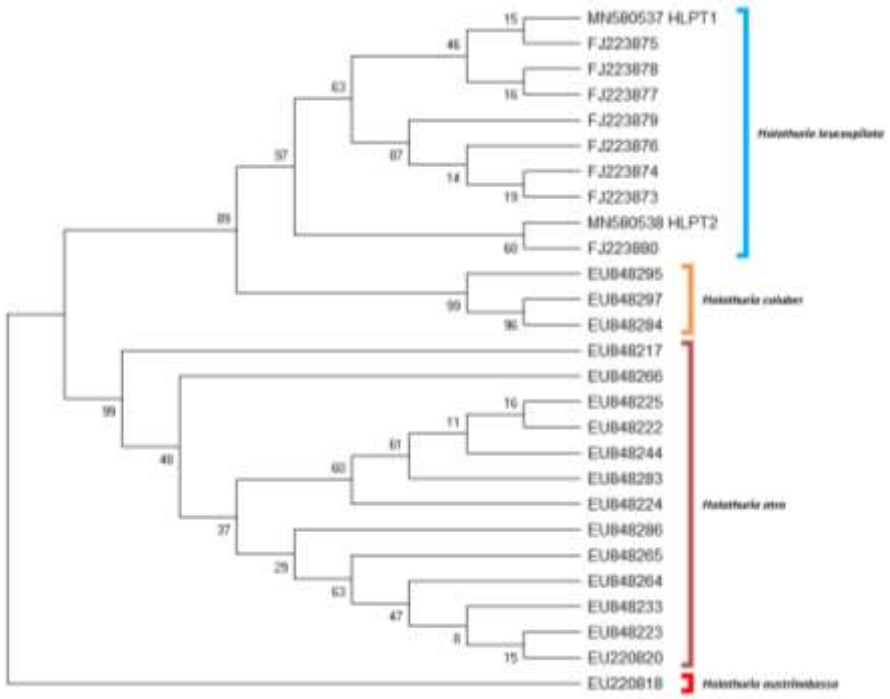

Figure 2. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-503.38) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 27 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 156 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

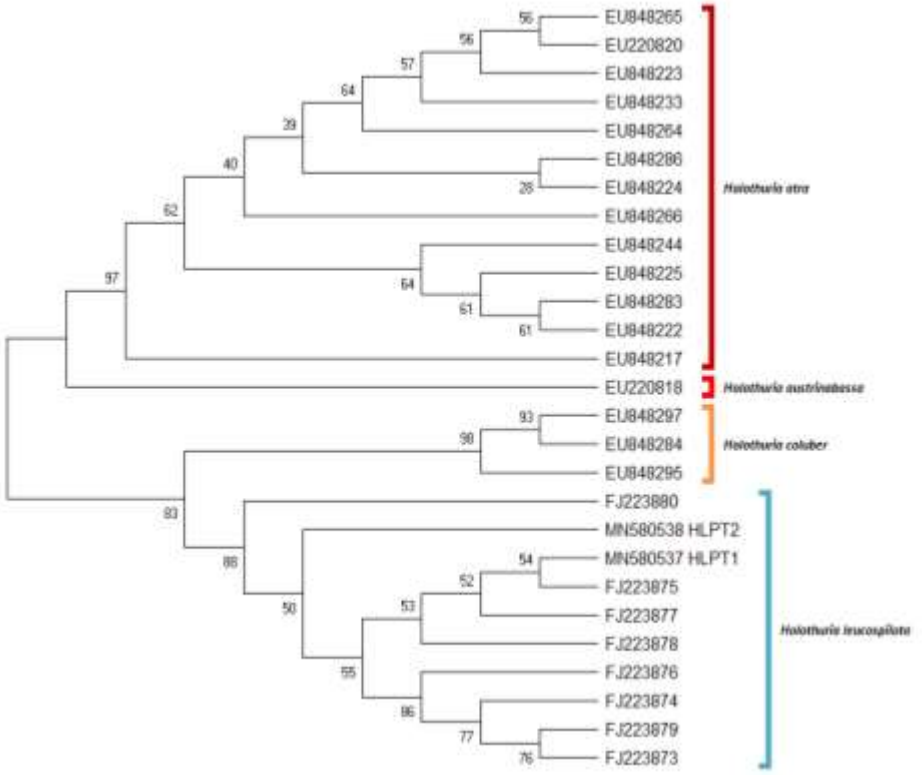

Figure 3. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 9 most parsimonious trees (length = 58) is shown. The consistency index is (0.840909), the retention index is (0.965517), and the composite index is 0.848989 (0.811912) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). This analysis involved 27 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 156 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

4.0 CONCLUSION

A number of two partial CO1 mtDNA sequences of *H. leucospilota* from Pulau Tinggi, Johor, Malaysia were successfully obtained and then registered with the GenBank with accession numbers of MN580537 and MN580538. The phylogenetic analyses suggested the single species status of *H. leucospilota* specimens from Malaysia, thus confirming the species identity of *H. leucospilota* specimens from Pulau Tinggi. Therefore, the COI mtDNA barcodes supported the morphological status of the *H. leucospilota* specimens. Needless to say, this study has significantly contributed to the recent availability of partial COI mtDNA gene sequences of *H. leucospilota* in the GenBank, NCBI, U.S. National Library of Medicine for future research as well as DNA barcodes of *H. leucospilota* from Pulau Tinggi. Further studies need to be done to identify more species of sea cucumber including *gamat* species and other *timun laut* species from Pulau Tinggi. In conclusion, this study have contributed the first data on species presence and genetics of *H. leucospilota* from Pulau Tinggi, Mersing, Johor, Malaysia.

ACKNOWLEDGMENTS

This study was funded by the Industrial Grant of Universiti Tun Hussein Onn Malaysia (UTHM) with code no. M004 and partially supported by the Fundamental Research Grant Scheme (FRGS) from the Malaysian Ministry of Education (FRGS/1/2019/WAB09/UTHM/03/2). It is part of a thesis and a dissertation which was and to be submitted as partial fulfilment to meet requirements for the Degree of Bachelor in Science (Biodiversity and Conservation) with Honours and the Degree of Master in Science (Biodiversity Conservation) at UTHM.

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