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Molecular Phylogeny of *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt 1835) as Inferred from Cytochrome C Oxidase I Mitochondrial DNA Gene Sequences

Filogeni Molekul *Holothuria (Mertensiothuria) leucospilota* (Brandt 1835) Berdasarkan Penjujukan Gen Sitokrom C Oksidase I Mitokondria DNA

KAMARUL RAHIM KAMARUDIN*, 'AISYAH MOHAMED REHAN, RIDZWAN HASHIM, GIRES USUP, HAJAR FAUZAN AHMAD, MOHD HANAFI ANUA & MOHD YAMAN IDRIS

ABSTRACT

Holothuria (Mertensiothuria) leucospilota *(Brandt 1835), white threads fish or locally known as bat puntil is currently considered as the most abundant sea cucumber species in Malaysia. This study aimed to generate the genetic profile of* H. leucospilota *from Malaysia and then to determine the phylogenetic relationship between* H. leucospilota *and other members of genus* Holothuria *using partial sequences of cytochrome c oxidase I (COI) mitochondrial DNA (mtDNA) gene. In this study, specimens of* H. leucospilota *were collected from Intan Besar Island, Langkawi, Kedah Darul Aman in the west coast of Peninsular Malaysia. Three main methods namely neighbour joining, maximum parsimony and maximum likelihood were used for the phylogenetic tree reconstruction. Tree topologies showed that H. leucospilota has its own monophyletic clade clearly distinct from the other species. The pairwise genetic distance calculated further supported these findings. In addition, the results also should that the COI mtDNA gene is capable to unravel the phylogenetic relationship of* H. leucospilota.

Keywords: Holothuria leucospilota*; molecular systematics; partial sequences of cytochrome oxidase I gene; phylogeny tree*

ABSTRAK

Holothuria (Mertensiothuria) leucospilota *(Brandt 1835) atau nama tempatannya* bat puntil *kini dianggap sebagai spesies timun laut yang paling banyak taburannya di Malaysia. Kajian ini bertujuan untuk mendapatkan profil genetik* H. leucospilota *dan seterusnya menentukan perhubungan filogenetik antara* H. leucospilota *dari Malaysia dan ahli-ahli lain daripada genus Holothuria menggunakan jujukan separa gen sitokrom c oksidase I (COI) mitokondria DNA (mtDNA). Dalam kajian ini, spesimen* H. leucospilota *diperolehi dari Intan Besar Island, Langkawi, Kedah Darul Aman di pantai barat Semenanjung Malaysia. Tiga kaedah iaitu kaedah hubungkait jiran, parsimoni maksimum dan kaedah hubungkait maksimum telah digunakan untuk pembinaan pohon-pohon filogenetik. Topologi pohon menunjukkan* H. leucospilota *mempunyai klusternya sendiri yang monofiletik yang jelas berbeza daripada spesies-spesies yang lain. Pengiraan jarak genetik secara berpasangan seterusnya telah menyokong keputusan-keputusan tersebut. Di samping itu, keputusan kini juga menunjukkan bahawa gen COI mtDNA mempunyai kapasiti untuk merungkaikan perhubungan filogenetik* H. leucospilota.

Kata kunci: Sistematik molekul; Holothuria leucospilota*; jujukan separa gen sitokrom c oksidase I mitokondria DNA, pohon filogeni*

INTRODUCTION

Sea cucumber, sea cuke, holothurian or holothuroid is a marine-dwelling organism of phylum Echinodermata. This echinoderm from class Holothuroidea is exclusive due to the existence of the evolved skeleton known as ossicles, and the ancient-looked respiratory system called respiratory tree possessed by a few species (Lambert 1997). In Malaysia, sea cucumbers from other than stichopodidae family such as from genera of *Holothuria* and *Actinopyga* are commonly known as *bat*, *balat*, *brunok* and *timun laut* (Kamarudin et al. 2009). Basically, there are two main economic practices of sea cucumbers in Malaysia: an essential supply for food industry in Sabah, East Malaysia and an important source for traditional medicine (e.g. *gamat* oil and *gamat* water) and modern-formularised health food in Peninsular Malaysia (Hashim 1993).

According to Kamarudin et al. (2009) order Aspidochirotida in general and genus *Holothuria* in particular were found to be the major sea cucumber classes in Malaysia, and *Holothuria (Mertensiothuria) leucospilota* (Brandt 1835) is suggested as the most abundant species. *H. leucospilota* can be found on the sandy sea floor or below the rocks in the sea waters. The English name of this soft-bodied species is *white threads fish* and it is locally known as *bat puntil* in Malaysia. It is a long and black tubular sea cucumber often with reddish body background, that has a

mouth surrounded by tentacles and a terminal anus located at the posterior. In terms of types of ossicle, tegument of *H. leucospilota* contains many tables and buttons as described by Samyn et al. (2006). In general, the table has a perforated base of four holes at the central and four to 12 holes at the peripherals; the disk is oval to quadrangular; and the spire is low and ends by a little thorny crown. The buttons are smooth and perforated with generally 2-4 pairs of uneven holes. Besides, the ventral podia contain buttons, tables as well as major plates while the dorsal podia contain rods, tables and buttons. For defense purposes, the anus will release white sticky tendrils or Cuvierian tubules when the species is agitated. Interestingly, the Cuvierian tubules can regenerate after every expulsion (Hashim 1993).

In terms of phylogenetic studies, Kamarudin et al. (2010) obtained a partial 16S mitochondrial ribosomal RNA (rRNA) gene sequence of *H. leucospilota* from Tioman Island, Pahang Darul Makmur, Malaysia (GenBank accession no.: FJ223871) that best aligned with an available corresponding sequence (GenBank accession no.: AY338419, species locality – Guam, Mariana Islands) in the GenBank, National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine. Besides, they also suggested that *Holothuria (Mertensiothuria) hilla* Lesson, 1830 and *H. leucospilota* are sister species based on the consensus phylogenetic relationship. In fact, mitochondrial DNA (mtDNA) is the most preferred model in molecular genetic ecology due to its effective maternal inheritance, apparent haploid genome, non-recombination, continuous replication and the rate of substitution in mtDNA is within the range of 5 to 10 times greater than in 'single-copy' nuclear DNA (Amos & Hoelzel 1992; Hartl & Clark 1989). More information from various mtDNA genes are essential to support the current genetic information of *H. leucospilota* from 16S mitochondrial rRNA. Nevertheless, cytochrome c oxidase I (COI) mtDNA gene sequences of *H. leucospilota* were previously unavailable online in the GenBank until 19 September 2008; thus leading to the present study. Such unavailability indicated the lack of studies and information on the genetic profile and phylogenetic relationship of *H. leucospilota* as the most abundant species in Malaysia (Kamarudin et al. 2009).

In this study, specimens of *H. leucospilota* were collected from Intan Besar Island, Langkawi, Kedah Darul Aman in the west coast of Peninsular Malaysia, Malaysia (Figure 1). Partial DNA sequencing of COI mtDNA gene was applied to generate the genetic profile of *H. leucospilota*. Besides, the phylogenetic relationship between *H. leucospilota* from Malaysia and other members

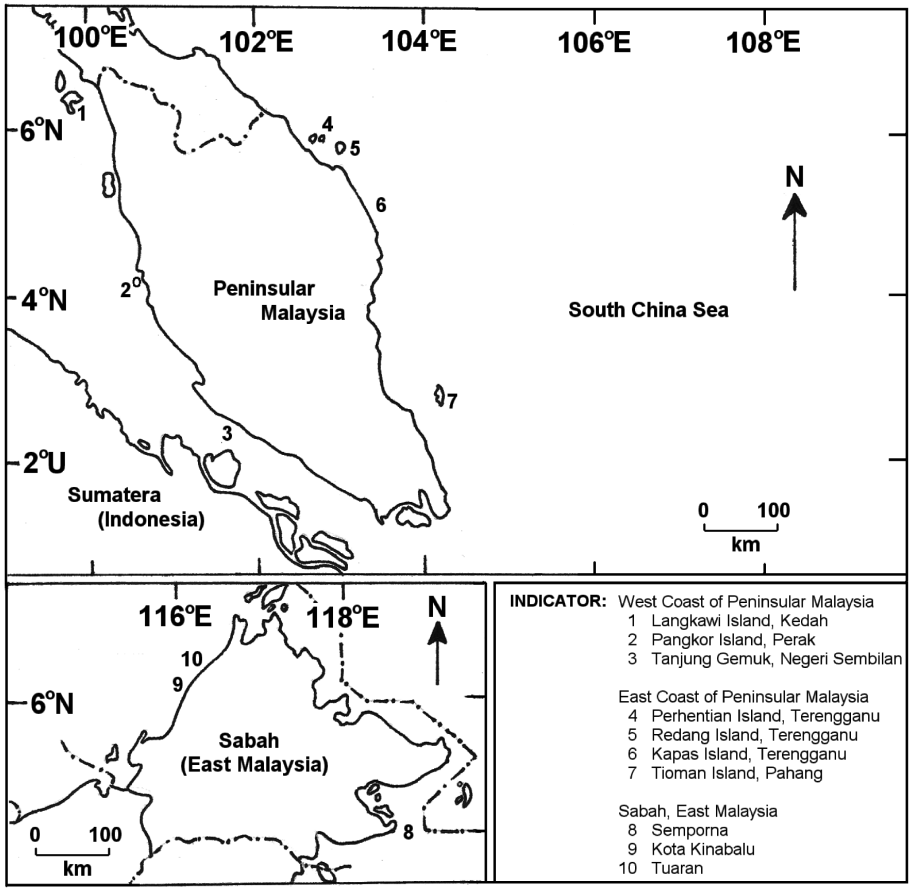

FIGURE 1. Specimens of *Holothuria leucospilota* used in this study were collected from Intan Besar Island, Langkawi, Kedah Darul Aman, Malaysia (sampling site 1). Modified from Kamarudin et al. (2009)

of genus *Holothuria* was also determined. For the later purpose, three main methods namely neighbour joining, maximum parsimony and maximum likelihood were used for the phylogenetic tree reconstruction. This study has significantly contributed to the recent availability of partial COI mtDNA gene sequences of *H. leucospilota* in the GenBank for future research.

Materials and Methods

Specimens of *H. leucospilota* from Intan Besar Island, Langkawi, Kedah Darul Aman, in the west coast of Peninsular Malaysia (Figure 1) were collected during several sampling activities, and one of them was in July 2006. The sampling sites in Intan Besar Island were on intertidal zone, and most documentation were done during the low tide. No fixed or standard sampling hours were allocated for all sites. Global Positioning System (GPS) was used to mark and to record the position of each sampling site (not shown). Preservations of specimens were done either in 70% ethanol or in cold temperature containers at the sampling sites, and the specimens are kept for long storage period in the -20°C and -80°C freezers in the laboratories.

Total genomic DNA extraction was done using DNeasy® QIAGEN blood and tissues kit. The total genomic DNA was extracted from muscle tissue. Quantification of extracted DNA was done using Perkin Elmer Lambda 25 UV/Vis spectrophotometer and 1% (w/v) agarose gel electrophoresis. For the polymerase chain reaction (PCR), approximately 550 base pairs (bp) section of the COI mtDNA gene was amplified using standard PCR procedures. Two universal primers were used for the PCR: COI (forward) 5'- CCT GCA GGA GGA GGA GGA GAY CC -3' and COI (reverse) 5'- CCA GAG ATT AGA GGG AAT CAG TG -3' (Palumbi et al. 1991). Each PCR reaction volume contained 30.0 µL of sterilised dH_2O, 5.0 µL of 10X PCR reaction buffer, 3.0 µL of magnesium chloride ($MgCl_2$, 25 mM), 2.5 µL of each primer (10 mM), 1.0 µL of nucleotide/dNTP mix (10 mM), 1.0 µL of acetylated bovine serum albumin (BSA, 10 µg/µL), 0.5 µL of glycerol, 4 µL of DNA template and 0.5 µL of 5 u/µL *Taq* DNA polymerase.

The PCR was programmed for 30 cycles. Cycle parameters for the PCR were 7 min at 96°C for initial denaturation, 1 min at 95°C for denaturation, 45 s at optimised temperature for annealing, 1 min 30 s at 72°C for extension, repetition of step 2-4 for another 29 cycles and 10 min at 72°C for final extension. Quantification of the PCR products was analysed by 1% (w/v) agarose gel electrophoresis. Purification kits from manufacturers were used for direct purification of the positive PCR products. The purified products were directly sent for sequencing using BigDye® Terminator v3.1 Sequencing Kit and analysed on ABI PRISM® 377 Genetic Analyzer. Cycle sequencing reaction was done in a programmable cycler. Cycle sequencing reaction was done for 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min.

Chromas Lite (Version 2.1) program was used to display the results of fluorescence-based DNA sequence analysis. Basic Local Alignment Search Tool (BLAST) in the GenBank was used to find the corresponding sequences. Multiple sequence alignment for forward reaction sequences was done using ClustalX program (version 1.81; Thompson et al. 1997), and subsequently aligned by eyes. The reconstructions of neighbour joining tree, maximum parsimony tree and maximum likelihood tree were done by using several programs of PHYLIP version 3.6b (Felsenstein 2004) with 1000 sequence replications and 100 multiple data sets. Felsenstein (1984) distance method (i.e. F84 evolutionary model) was incorporated to reconstruct the neighbour joining tree using dnadist program and then Neigbor program based on the unequal empirical base frequencies calculated in this study (i.e. Adenine = 29.20%, Thymine – 26.86%, Cytosine = 25.70% and Guanine = 18.24%). The ratio of transition to transversion (*ti/tv*) was set to 2.0 and the sequence sites were unweighted. As for maximum parsimony tree, dnapars program performed a modified Templeton test using ordinary parsimony, and the phylogeny tree generated was a majority rule consensus tree. The other settings for reconstruction of the maximum parsimony tree were as follows: sequence sites were unweighted, random number seed was 999, number of times to jumble was 10, search option was more thorough search, interleaved sequences were put in and 10 000 trees were saved prior to majority rule consensus. Maximum likelihood method with molecular clock program or dnamlk program performed a Kishino-Hasegawa test as the model to generate a majority rule consensus tree, with the *ti/tv* was set to 2.0. Empirical base frequencies, one category of sites, constant variation rate among sites, unweighted sequence sites, interleaved sequences input and speedier but rougher analysis were set for the maximum likelihood analysis. TreeView (Win32) version 1.6.6 by Page (1996) was used to display and then to edit the reconstructed phylogenetic trees.

Results and Discussion

In total, 26 partial sequences of COI mtDNA gene were aligned. The sequence consisted of eight sequences of *H. leucospilota* from Malaysia, 17 sequences of selected *Holothuria* species from the GenBank based on the BLAST results (i.e. *Holothuria (Halodeima) atra* Jaeger, 1833 – 13, *Holothuria (Acanthotrapeza) coluber* Semper, 1868 – 3 and *Holothuria* (*Panningothuria*) *austrinabassa* O'loughlin, Paulay, Vandenspiegel & Samyn, 2007 - 1) and one sequence of *Cucumaria piperata*, a sea cucumber (GenBank accession number: U32211; Figure 2-4) as the outgroup (Table 1). The COI mtDNA gene sequences of *H. leucospilota* obtained from this study have been registered with the GenBank (accession no.: FJ223873 - FJ223880, submission date: 19 September 2008) thus significantly contributing to the recent availability of the gene sequences of the species for future research. A number of 494 aligned base positions including the possible

TABLE 1. Taxa incorporated for phylogenetic analyses of *Holothuria leucospilota* using partial cytochrome c oxidase I mtDNA gene

Taxa	Sample Size	Individual No.	Locality	GenBank Accession No.
Order Aspidochirotida Family Holothuriidae				
Holothuria (Halodeima) atra Jaeger 1833	2	AtraPNG1	Papua New Guinea: Milne Bay	EU848225
		AtraPNG5	Papua New Guinea: Milne Bay	EU848224
	2	AtraAUS6	Australia: Lizard Island	EU848223
		AtraAUS14	Australia: Lizard Island	EU848283
	3	AtraAUS8	Australia: Great Barrier Reef	EU848217
		AtraAUS13	Australia: Great Barrier Reef	EU848233
		AtraAUS17	Australia: Great Barrier Reef	EU848286
	1	AtraAUS15	Australia	EU220820
	1	AtraINDO9	Indonesia: Pulao Doi	EU848244
	4	AtraNC7	New Caledonia	EU848222
		AtraNC10	New Caledonia	EU848265
		AtraNC11	New Caledonia	EU848266
		AtraNC12	New Caledonia	EU848264
Holothuria (Acanthotrapeza) coluber Semper, 1868	2	ColubrAUS2	Australia: Lizard Island	EU848297
		ColubrAUS4	Australia: Lizard Island	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, Malaysia	FJ223873
		FJ223874	Malaysia: Intan Besar Island, Langkawi, Malaysia	FJ223874
		FJ223875	Malaysia: Intan Besar Island, Langkawi, Malaysia	FJ223875
		FJ223876	Malaysia: Intan Besar Island, Langkawi, Malaysia	FJ223876
		FJ223877	Malaysia: Intan Besar Island, Langkawi, Malaysia	FJ223877
		FJ223878	Malaysia: Intan Besar Island, Langkawi, Malaysia	FJ223878
		FJ223879	Malaysia: Intan Besar Island, Langkawi, Malaysia	FJ223879
		FJ223880	Malaysia: Intan Besar Island, Langkawi, Malaysia	FJ223880
Outgroup *Cucumaria piperata*	1	OUTGROUP	Unknown	U32211

gaps were incorporated for the reconstruction of the phylogenetic trees. Maximum parsimony analysis showed that 149 characters were constant, 24 variable characters were parsimony-uninformative and 321 characters were parsimony-informative. The high number of parsimony-informative character suggests that COI mtDNA is capable to be an informative locus candidate for phylogenetic studies.

Overall, all phylogenetic trees showed weak clustering with 50% bootstrap values (Figure 2-4). For the neighbour joining tree (Figure 2), the results showed the presence of two major groups. All specimens of *H. atra* formed the first group with 82% bootstrap value while the specimens of *H. coluber*, *H. leucospilota* and *H. austrinabassa* formed the second group with a low bootstrap value of 37%. The latter group showed that *H. austrinabassa* was basal. All specimens of *H. leucospilota* clustered together with 100% bootstrap value showing its monophyly. Besides, *H. coluber* and monophyletic *H. leucospilota* clustered together with 73% bootstrap value exhibiting their close

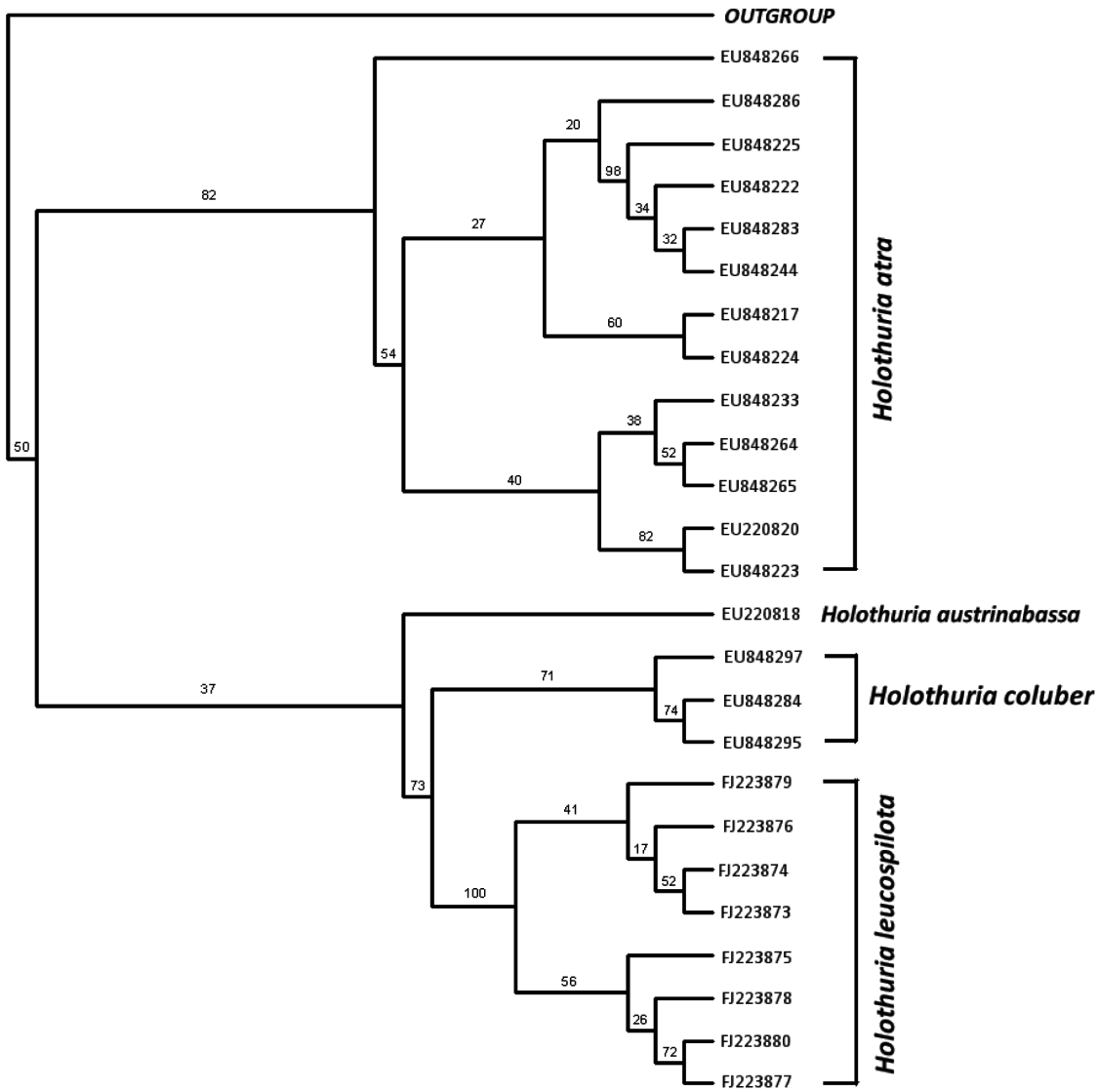

FIGURE 2. Topology of neighbour joining tree (50% majority rule consensus tree) of *Holothuria* species inferred from cytochrome c oxidase I (COI) mtDNA gene using Neighbor program of PHYLIP version 3.6b (Felsenstein 2004). Felsenstein (1984, F84) evolutionary model was incorporated. Accession number of EU refers to sequences obtained from GenBank. The tree is rooted with a sequence of *Cucumaria piperata*, a sea cucumber (GenBank accession number: U32211). 1000 sequence replications and 100 data sets were used. Numbers at nodes indicate the bootstrap values in percentage (%)

genetic relationship. Likewise the neighbour joining tree, all specimens of *H. leucospilota* in the maximum parsimony tree (Figure 3) clustered together with a strong boostrap support of 100%. Very low average genetic distance between specimens of *H. leucospilota* (i.e. 0.013) has further supported the findings. The clusters of *H. atra* and *H. coluber* were also supported by 100% bootstrap values with very low average genetic distances between the species individuals (i.e. 0.019 and 0.011, respectively) showing their respective origins from single species. *H. austrinabassa*, *H. leucospilota* and *H. coluber* formed a cluster with 35% bootstrap value but unlike the neighbour joining tree, the maximum parsimony tree indicated that *H. coluber* was basal. In addition, the maximum parsimony tree suggests that *H. austrinabassa* is the closest to monophyletic *H. leucospilota* with 53% bootstrap value followed by *H. coluber* and *H. atra*.

Even though the maximum likelihood tree (Figure 4) supported that the genetic relationship between monophyletic *H. leucospilota* and *H. coluber* was close with 69% bootstrap value, the phylogenetic tree indicated that *H. austrinabassa* clustered together with *H. atra* with 41% bootstrap value. Statistically, such low bootstrap value suggests that the clustering position for *H. austrinabassa* is likely changeable, as previously indicated by the neighbour joining tree and the maximum parsimony tree. Furthermore, the bootstrap support for *H. leucospilota* cluster was still robust with 100% value summarising that *H. leucospilota* has its own monophyletic clade clearly distinct from the other species. The pairwise

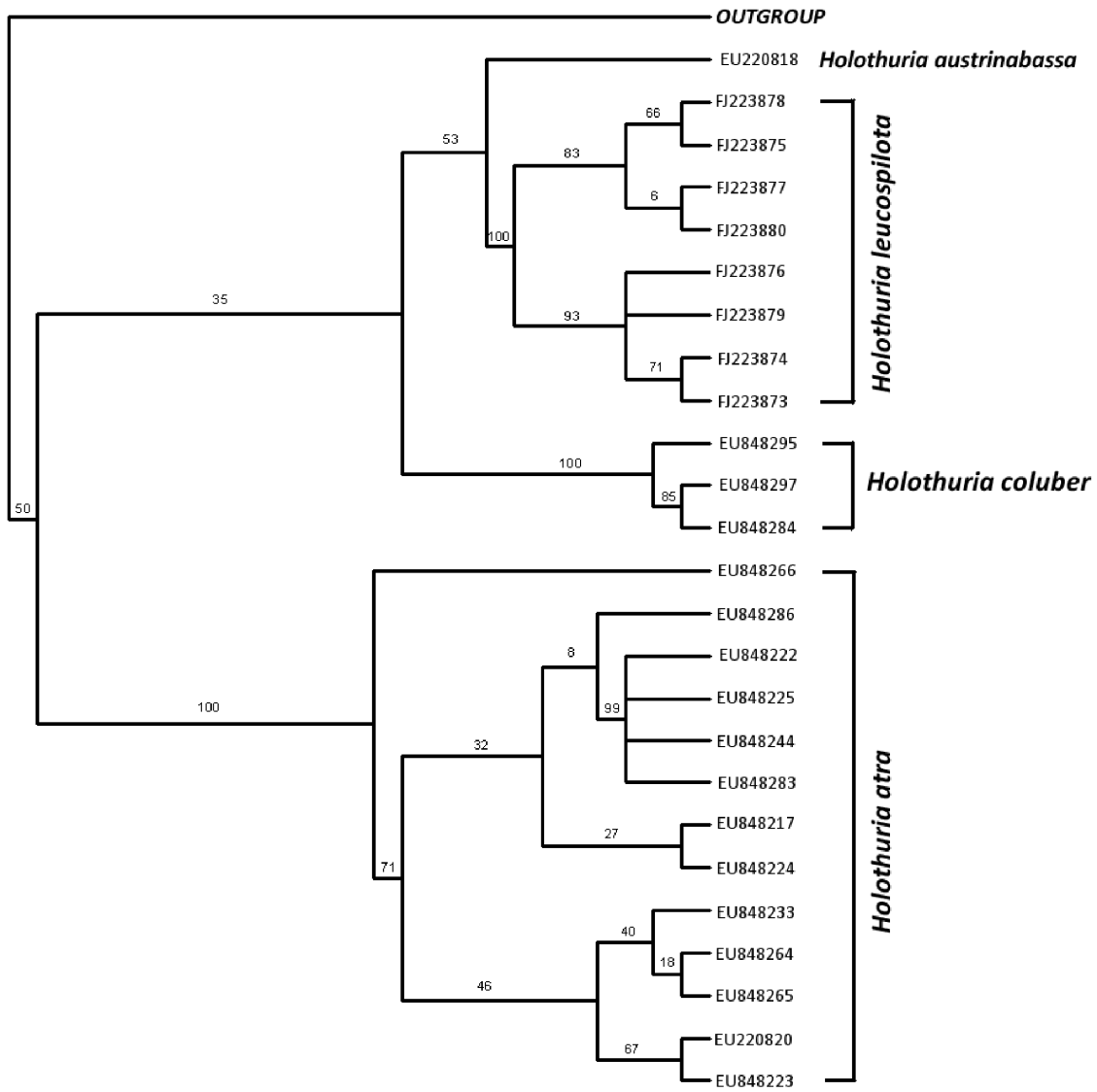

FIGURE 3. Topology of maximum parsimony tree (50% majority rule consensus tree) of *Holothuria* species inferred from cytochrome c oxidase I (COI) mtDNA gene using dnapars program of PHYLIP version 3.6b (Felsenstein 2004). Modified Templeton test using ordinary parsimony was incorporated. Accession number of EU refers to sequences obtained fromGenBank. The tree is rooted with a sequence of *Cucumaria piperata*, a sea cucumber (GenBank accession number: U32211). 1000 sequence replications and 100 data sets were used. Numbers at nodes indicate the bootstrap values in percentage (%)

genetic distance calculated using Felsenstein (1984) model supported the closer genetic relationship between monophyletic *H. leucospilota* and *H. coluber* shown by the neighbour joining tree and the maximum likelihood tree (Table 2). Table 2 shows that the average pairwise genetic distance between monophyletic *H. leucospilota* and *H. coluber* is the lowest (i.e. 1.216) strengthening their closer relationship. In terms of 16S mitochondrial rRNA gene, Kamarudin et al. (2010) found that the maximum parsimony tree supported the closer genetic relationship of *H. leucospilota* to *H. coluber* than to *H. atra*, however the phylogenetic trees of neighbour joining and maximum likelihood suggest that *H. coluber* is genetically closer to *H. atra*. The latter inference summarised that *H. coluber* was genetically closer to *H. atra* as compared to *H. leucospilota* based on the 16S mitochondrial rRNA gene which is in contrary to the results of the present study. Despite such difference, this study suggests that the COI mtDNA gene has shown the capability to unravel the phylogenetic relationship of *H. leucospilota.*

In general, the present results suggest that *H. coluber* was genetically the closest to monophyletic *H. leucospilota* followed by *H. austrinabassa* and *H. atra*. The phylogenetic status of *H. austrinabassa* was unresolved due to its inconsistent clustering position in all trees with unresolved branching (i.e. <55% bootstrap values) thus requiring more specimens of it to confirm its

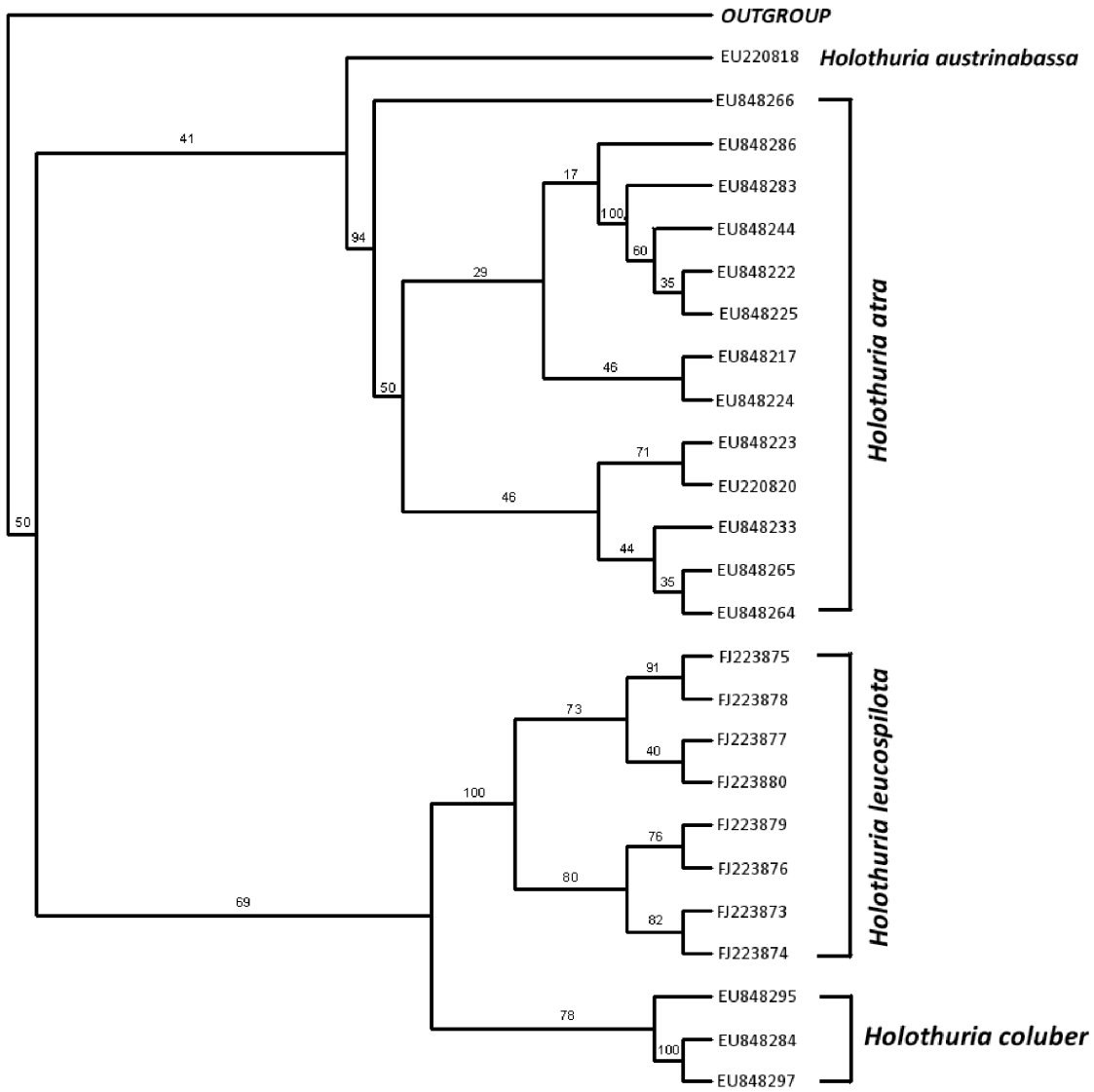

FIGURE 4. Topology of maximum likelihood tree with molecular clock (50% majority rule consensus tree) of *Holothuria* species inferred from cytochrome c oxidase I (COI) mtDNA gene using dnamlk program of PHYLIP version 3.6b (Felsenstein 2004). Kishino-Hasegawa model was incorporated. Accession number of EU refers to sequences obtained from GenBank. The tree is rooted with a sequence of *Cucumaria piperata*, a sea cucumber (GenBank accession number: U32211). 1000 sequence replications and 100 data sets were used. Numbers at nodes indicate the bootstrap values in percentage (%)

TABLE 2. The average distance matrix between *Holothuria leucospilota* and other selected members of genus *Holothuria*. The pairwise distance calculation was generated by dnadist program of PHYLIP version 3.6b (Felsenstein 2004). The calculation incorporated Felsenstein (1984) distance method

Species	*H. leucospilota*	*H. atra*	*H. coluber*	*H. austrinabassa*	*Outgroup*
H. leucospilota	-				
H. atra	1.268	-			
H. coluber	1.216	0.209	-		
H. austrinabassa	1.271	0.226	0.241	-	
Outgroup	1.384	0.193	0.202	0.246	-

molecular phylogeny particularly within genus *Holothuria*. According to O'Loughlin et al. (2007), their molecular data on 16S mitochondrial rRNA gene and COI mtDNA gene suggest that *H. (Panningothuria) austrinabassa* and *H. (Panningothuria) forskali* Delle Chiaje, 1823 are sister species.

CONCLUSION

The present phylogenetic study of *H. leucospilota* from Intan Besar Island, Langkawi, Kedah Darul Aman, Malaysia using 26 partial sequences of COI mtDNA gene showed that *H. leucospilota* has its own monophyletic clade clearly distinct from the other species. *H. coluber* was genetically the closest to monophyletic *H. leucospilota* followed by *H. austrinabassa* and *H. atra* based on the phylogenetic trees of neighbour joining, maximum parsimony and maximum likelihood. The pairwise genetic distance calculated using Felsenstein (1984) model further supported and verified the closer genetic relationship between *H. leucospilota* and *H. coluber*. Beside suggesting that the COI mtDNA gene has the capability to unravel the phylogenetic relationship of *H. leucospilota*, this study has also 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. Further studies with more *Holothuria* species, broader geographical locations of *H. leucospilota* including Sabah and Sarawak, East Malaysia and other mtDNA genes along with the morphological approaches such as the characterisation of calcareous ring, tentacle and ossicle may help to provide a better view on the molecular phylogeny of *H. leucospilota* in particular and *Holothuria* species in general.

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Hajar Fauzan Ahmad, Mohd Hanafi Anua & Mohd Yaman Idris, Aisyah Mohamed Rehan, Ridzwan Hashim, Hajar Fauzan Ahmad, Mohd Hanafi Anua & Mohd Yaman Idris
Sea Cucumber (Echinodermata: Holothuroidea)
Research Group
Institute of Oceanography & Maritime Studies (INOCEM)

Kulliyyah of Science
International Islamic University Malaysia (IIUM)
Jalan Istana, Bandar Indera Mahkota
25200 Kuantan, Pahang Darul Makmur
Malaysia

Gires Usup
Marine Science Programme
School of Environmental & Natural Resource Sciences
Faculty of Science & Technology
Universiti Kebangsaan Malaysia (UKM)
43600 UKM Bangi, Selangor D.E.
Malaysia

Kamarul Rahim Kamarudin*
School of Biological Sciences (SBS)
The University of Auckland
Private Bag 92019
Auckland 1142, New Zealand

*Corresponding author; email: physique481@yahoo.co.uk

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