

## Phylogenetic Relationships within the Genus *Holothuria* Inferred from 16S Mitochondrial rRNA Gene Sequences

(Hubungan Filogenetik dalam Genus *Holothuria* berdasarkan Jujukan Gen 16S Mitokondria rRNA)

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### ABSTRACT

*This study aimed to resolve the taxonomic status of a morphologically undetermined sea cucumber species of order Apodida from Malaysia (GenBank accession no.: FJ223867) using partial 16S mitochondrial rRNA gene sequences and subsequently to determine the validity of morphological taxonomy of Holothuria species into its current subgenera. The undetermined species clustered with all taxa of Holothuria in previous study. Phylogenetic analyses using maximum parsimony and Bayesian methods suggest that the undetermined species was genetically closer to Holothuria (Lessonothuria) pardalis and Holothuria (Acanthotrapeza) coluber; and its position in both phylogenetic trees further suggests its status as a Holothuria taxon. Subgenera of Holothuria, Merthensiothuria and Metriatyla are monophyletic with strong bootstrap supports and posterior probabilities of clades, thus strengthening their morphological taxonomies. Nonetheless, the non-monophyly of subgenera of Halodeima, Microthele and Platyperona suggests a requirement for their taxonomic revisions using integrative taxonomy. The status of Holothuria (Halodeima) edulis subgroups in the maximum parsimony and Bayesian trees is indistinct and further taxonomic revisions are necessary. In terms of sister relationship, both phylogenetic trees suggest that subgenus Holothuria is a sister taxon of subgenus Roweothuria while the other sister relationships were unclear due to the undetermined species, parafly and polyphyly of a number of subgenera. Further studies with more specimens of genus Holothuria from broader geographical locations and various mtDNA genes along with morphological approaches may facilitate to provide better insights into the molecular phylogeny of subgenera of Holothuria.*

*Keywords: Bayesian inference; Holothuria; partial sequences of 16S mitochondrial ribosomal RNA gene; phylogenetic relationship*

### ABSTRAK

*Kajian ini bertujuan untuk merungkai status taksonomi satu spesies timun laut daripada order Apodida dari Malaysia yang tidak dapat ditentukan status spesiesnya secara morfologi (no akses GenBank: FJ223867) menggunakan jujukan separa gen 16S mitokondria ribosom RNA dan menentukan kelangsungan taksonomi morfologi spesies Holothuria ke subgenus semasanya. Kajian sebelum ini menunjukkan timun laut daripada order Apodida membentuk kluster bersama-sama dengan takson genus Holothuria. Analisis filogenetik menggunakan kaedah hubung kait maksimum dan Bayes mencadangkan spesies timun laut daripada order Apodida yang tidak dapat ditentukan spesiesnya adalah amat rapat secara genetik dengan Holothuria (Lessonothuria) pardalis and Holothuria (Acanthotrapeza) coluber; dan kedudukannya dalam kedua-dua pohon filogeni tersebut selanjutnya mencadangkan statusnya sebagai takson Holothuria. Subgenus Holothuria, Merthensiothuria dan Metriatyla adalah monofiletik dengan sokongan ikatan but yang kuat, seterusnya menyokong kuat pengelasan taksonomi subgenus berdasarkan morfologi. Walau bagaimanapun, ketidakmonofili subgenus Halodeima, Microthele dan Platyperona mencadangkan perlunya semakan taksonomi menggunakan taksonomi berintegratif. Status subkumpulan Holothuria (Halodeima) edulis yang tidak jelas pada pohon-pohon filogenetik hubung kait maksimum dan Bayes memerlukan semakan taksonomi lanjutan. Berdasarkan perhubungan rapat, kedua-dua pohon filogenetik mencadangkan subgenus Holothuria adalah amat rapat secara genetik kepada subgenus Roweothuria sementara perhubungan yang lain adalah tidak jelas disebabkan spesies yang tidak dapat ditentukan status taksonomi, parafili dan polifili beberapa subgenus. Kajian lanjut melibatkan lebih banyak specimen Holothuria dari lokasi geografi lebih luas dan pelbagai gen mitokondria DNA selari dengan pendekatan morfologi mungkin boleh membantu memberikan gambaran lebih jelas tentang filogeni molekul subgenus Holothuria.*

*Kata kunci: Holothuria; pendekatan Bayes; perhubungan filogenetik; separa jujukan gen 16S mitokondria ribosom RNA*

### INTRODUCTION

Sea cucumber is regarded as an essential part of Malaysian marine heritage due to its unique diversity and commercial

value (Kamarudin et al. 2015, 2010a). This marine-living organism is also called sea cuke, holothurian and holothuroid worldwide. This echinoderm from class

Holothuroidea is exclusive due to the existence of the evolved skeleton known as ossicles and the ancient-looking respiratory system called respiratory tree possessed by a few species (Lambert 1997). Most interestingly, the sticky Cuvierian tubules - used for defense purposes when certain species feel disturbed - can regenerate after every expulsion or evisceration (Hashim 1993). In Malaysia, at least six local names of sea cucumber are well known namely *timun laut*, *bat*, *balat*, *brunok*, *gamat* and *hoi sum* or *hai shen* called by the Chinese community, which means *sea ginseng* referring to its healing properties. There were different definitions of *gamat* from previous studies in Malaysia (i.e. from 1985 until April 2015) and the most appropriate definition is that *gamat* is exclusively referred to sea cucumbers of family Stichopodidae such as genera of *Stichopus* and *Thelenota*. Beside being popular as a main resource for the world's beche-de-mer or *trepan* industry, sea cucumbers are also important for traditional medicine for instances *gamat* oil (i.e. *gamat* lipid extracts) and *gamat* water/fluid (i.e. *gamat* water extracts) and modern-formularised health food in Peninsular Malaysia.

Several studies related to sea cucumbers (Echinodermata: Holothuroidea) in Malaysia have been published since 1985 (Kamarudin et al. 2015, 2010a). It has been estimated that more than 80 species of sea cucumber are present in Malaysia (Kamarudin et al. 2015, 2010a, 2009). According to Kamarudin et al. (2009), order Aspidochirotida is found to be the major sea cucumber class in Malaysia. Among members of order Aspidochirotida are genera of *Actinopyga*, *Pearsonothuria*, *Stichopus*, *Thelenota* and *Holothuria*. *Holothuria* from family Holothuriidae is considered as the most dominant genus in Malaysia (Kamarudin et al. 2015, 2010a). This genus has been morphologically divided into a number of subgenera for better taxonomic classification e.g. *Acanthotrapeza*, *Halodeima*, *Holothuria*, *Lessonothuria*, *Mertensiothuria*, *Metriatyla*, *Microthele*, *Panningothuria*, *Platyperona*, *Roweothuria*, *Theelothuria* and *Thymiosycia*. A number of 16 species of *Holothuria* were recorded in Malaysia by Kamarudin et al. (2015) and *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt, 1835) was suggested as the most dominant species.

In terms of molecular ecological studies of sea cucumber, mitochondrial DNA (mtDNA) has been the most preferred model. Effective maternal inheritance, apparent haploid genome, non-recombination and continuous replication are among its main characteristics. Apart from that, the rate of substitution in mtDNA was within the range of 5 to 10 times greater than in 'single-copy' nuclear DNA (Amos & Hoelzel 1992; Hartl & Clark 1989). Overall, mtDNA consists of 13 protein-coding regions, 22 tRNAs and two ribosomal RNA regions including 16S mitochondrial ribosomal RNA (rRNA) gene. Clouse et al. (2005) and Kerr et al. (2005) suggested the ability of 16S mitochondrial rRNA gene to correlate the relationship between the morphology and genetics of sea cucumber. Needless to say, genetic information from mtDNA genes including 16S mitochondrial rRNA gene are essential to

support the current morphological information of sea cucumber particularly from Malaysian seawater.

A number of molecular ecological studies of sea cucumber have been recorded in Malaysia since 1999 (Kamarudin et al. 2015, 2010a). For instance, Kamarudin et al. (2010b) obtained 11 partial 16S mitochondrial rRNA gene sequence of *Holothuria* species from Malaysia representing 10 different morphospecies. All the sequences have been registered with GenBank, National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine (Kamarudin et al. 2010b). However, the phylogenetic trees of neighbour joining, maximum parsimony and maximum likelihood (Kamarudin et al. 2010a) showed that a morphologically undetermined species of order Apodida from Malaysia (GenBank accession no.: FJ223867) has clustered with all taxa of *Holothuria* and *Holothuria* (*Platyperona*) *excellens* (Ludwig 1875) formed a basal group in every tree causing genus *Holothuria* to not be monophyletic. Other genera were found monophyletic. Kerr et al. (2005) have also reported the paraphyly of genus *Holothuria* thus supporting the findings of Kamarudin et al. (2010a, 2010b). Besides, the trees also suggest that *Holothuria* (*Halodeima*) *edulis* Lesson, 1830 is a sister to *Holothuria* (*Halodeima*) *atra* Jaeger, 1833, *Holothuria* (*Mertensiothuria*) *hilla* Lesson, 1830 is a sister to *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt 1835), *Holothuria* (*Metriatyla*) *scabra* Jaeger, 1833 is a sister to *Holothuria* (*Metriatyla*) *ocellata* Jaeger, 1833 and *Holothuria* (*Lessonothuria*) *pardalis* Selenka, 1867 is a sister to the undetermined species of order Apodida. In addition, *Holothuria* (*Acanthotrapeza*) *coluber* Semper, 1868 was suggested to be basal to *H. pardalis* and the undetermined species of order Apodida, *Holothuria* (*Theelothuria*) *notabilis* Ludwig, 1875 is basal to *H. hilla* and *H. leucospilota* and *Holothuria* (*Thymiosycia*) aff. *impatiens* is basal to the latter. Regardless the unclear status of *H. excellens*, it was also apparent that the clusterings support the subgenus classification of *Holothuria*.

In this study, 145 16S mitochondrial rRNA gene sequences of *Holothuria* species available in the GenBank until June 13th, 2010 were added to 11 sequences of *Holothuria* species and a sequence of a morphologically undetermined species of order Apodida from Malaysia (GenBank accession no.: FJ223867) used by Kamarudin et al (2010a, 2010b) in order to get a better insight into the molecular phylogeny of the undetermined species of order Apodida that clustered with all taxa of *Holothuria* (Kamarudin et al. 2010a). On top of that, this study has also aimed at determining the validity of morphological taxonomy of *Holothuria* species into its current subgenera using 16S mitochondrial rRNA gene sequences. For the phylogenetic analyses, two main methods namely maximum parsimony and Bayesian were used for the phylogenetic tree reconstruction. Maximum parsimony, as one of the popular character-based methods, infers a phylogenetic tree by minimising the total number of evolutionary steps required to explain a given set of data;

while Bayesian inference of phylogeny is based upon a quantity called the posterior probability distribution of trees accomplished using Bayes's theorem, which is the probability of a tree conditioned on the observations (Huelsenbeck & Ronquist 2001). The Bayesian analysis is not included in the previous molecular ecological study by Kamarudin et al. (2010b).

#### MATERIALS AND METHODS

For the phylogenetic analyses, 11 partial 16S mitochondrial rRNA gene sequences of *Holothuria* species and a sequence of a morphologically undetermined species of order Apodida (Figure 1) from Malaysia (GenBank accession no.: FJ223867) analysed by Kamarudin et al. (2010a, 2010b) were incorporated. PhyLoTA Browser (rel. 1.5) database (Sanderson et al. 2008) was used to find all other 16S mitochondrial rRNA gene sequences of *Holothuria* species available in the GenBank until June 13th, 2010. The database gave 145 sequences registered as *Holothuria* species (i.e. based on the NCBI taxon name) in the GenBank, with GenBank accession no. However, in this study, only 72 sequences were selected (excluding the 12 sequences taken from Kamarudin et al. (2010a, 2010b)). As for rooting the 84 ingroup sequences, four partial 16S mitochondrial rRNA gene sequences of *Stichopus ocellatus* Massin, Zulfigar, Hwai and Boss, 2002 were included as the outgroup sequences.



FIGURE 1. A morphologically undetermined species of order Apodida from Malaysia (GenBank accession no.: FJ223867).  
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Chromas Lite (version 2.1) program (Copyright © 2005 Technelysium Pty Ltd) was used to display the results of fluorescence-based DNA sequence analysis. Multiple sequence alignment for forward reaction sequences was done using ClustalX (version 2.0.12) program (Thompson et al. 1997), and subsequently aligned by eyes. The multiple sequence alignment resulted in 4510 aligned nucleotides including the gaps. MrModeltest (version 2.3) program (Nylander 2004) was then used to calculate and find the best model for DNA evolution in order to construct the maximum parsimony tree. The best model suggested by the program was general time-reversible (GTR) (i.e. GTR+I+G

model as selected by the Akaike Information Criterion (AIC) in MrModeltest (version 2.3) program; Lanave et al. 1984; Rodriguez et al. 1990) with rates across sites were assumed to follow a continuous gamma distribution. The GTR model is based on unequal base frequencies with estimations of all six substitutions rate classes.

The reconstruction of the consensus maximum parsimony tree was done by using PAUP\* (version 4.0b10) program (Swofford 1998) with 1000 bootstrap replicates with estimations of six substitutions rate classes. Furthermore, MrBayes (version 3.1.2) program (Huelsenbeck & Ronquist 2001) was used for the reconstruction of consensus Bayesian phylogenetic tree with 4000000 generations and the addition of all compatible groups to the tree with the posterior probabilities of clades and the branch lengths. The potential scale reduction factor (PSRF) is reasonably close to 1.000 for all parameters. FigTree (version 1.4.2) program (Rambaut 2006-2009) 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 then to edit the reconstructed phylogenetic trees. All partial 16S mitochondrial rRNA gene sequences of sea cucumber from Malaysia analysed by Kamarudin et al. (2010b) were underlined in both consensus trees (Figures 2 & 3). The asterisk symbol (\*) indicates a non-monophyletic group.

#### RESULTS AND DISCUSSION

In total, 88 partial sequences of 16S mitochondrial rRNA gene of sea cucumber consisting of the ingroup and outgroup sequences were aligned. The sequences consist of 84 ingroup sequences and four outgroup sequences. Of the 451 characters inclusive of the gaps, maximum parsimony analysis showed that all characters were of type 'unord' and had equal weight, 108 characters were constant, 16 variable characters were parsimony-uninformative and 327 characters (approximately 73%) were parsimony-informative. The high number of parsimony-informative character suggests that 16S mitochondrial rRNA gene is capable to be an informative locus candidate for phylogenetic studies. Besides, in comparison to other mtDNA genes, the rate of mutation in mitochondrial rRNA gene is deemed the lowest and this criterion may lead to more reliable data collection. In general, the frequencies for all bases i.e. Adenine (A), Cytosine (C), Guanine (G) and Thymine (T) were unequal (i.e. A = 308.48%, T = 23.35%, C = 16.84% and G = 21.33%).

For the maximum parsimony analysis, the consensus phylogenetic tree (Figure 2) suggests that the morphologically undetermined species of order Apodida (i.e. H12RE1, Figure 1) is a sister taxon of *H. pardalis* from Malaysia (i.e. HNTKE2, GenBank accession no.: FJ223861) with 72% bootstrap support. For that reason, along with its clustering position among other *Holothuria* members in the maximum parsimony tree, the current results obviously suggested that the undetermined species of order Apodida

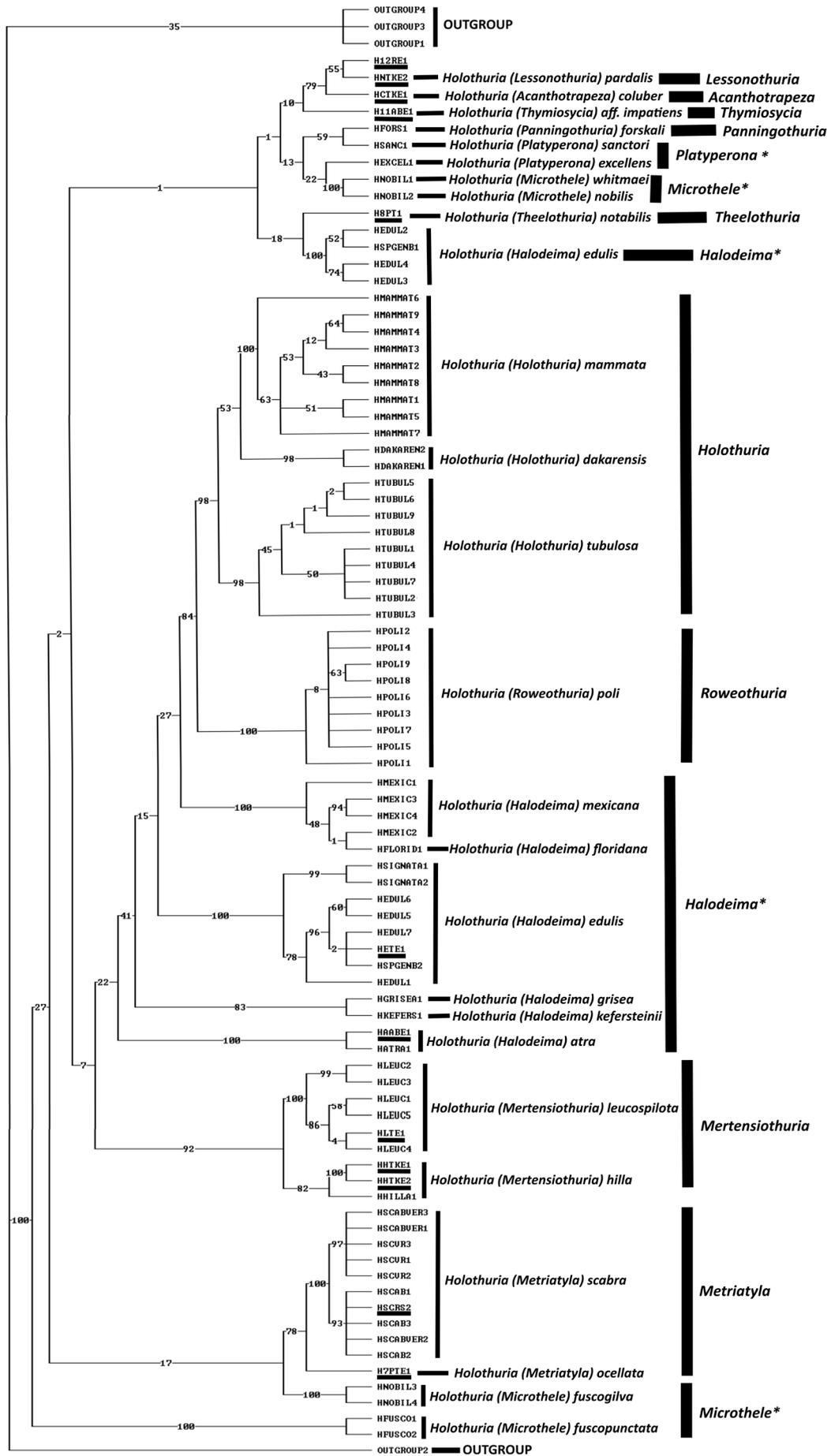


FIGURE 2. Topology of extended majority rule consensus tree of maximum parsimony of *Holothuria* species inferred from 16S mitochondrial rRNA gene sequences using PAUP\* (version 4.0b10) program (Swofford 1998) with 1000 bootstrap replicates. Each partial sequence detail is described in Table 1. Numbers at nodes indicate the bootstrap values in percentage (%).

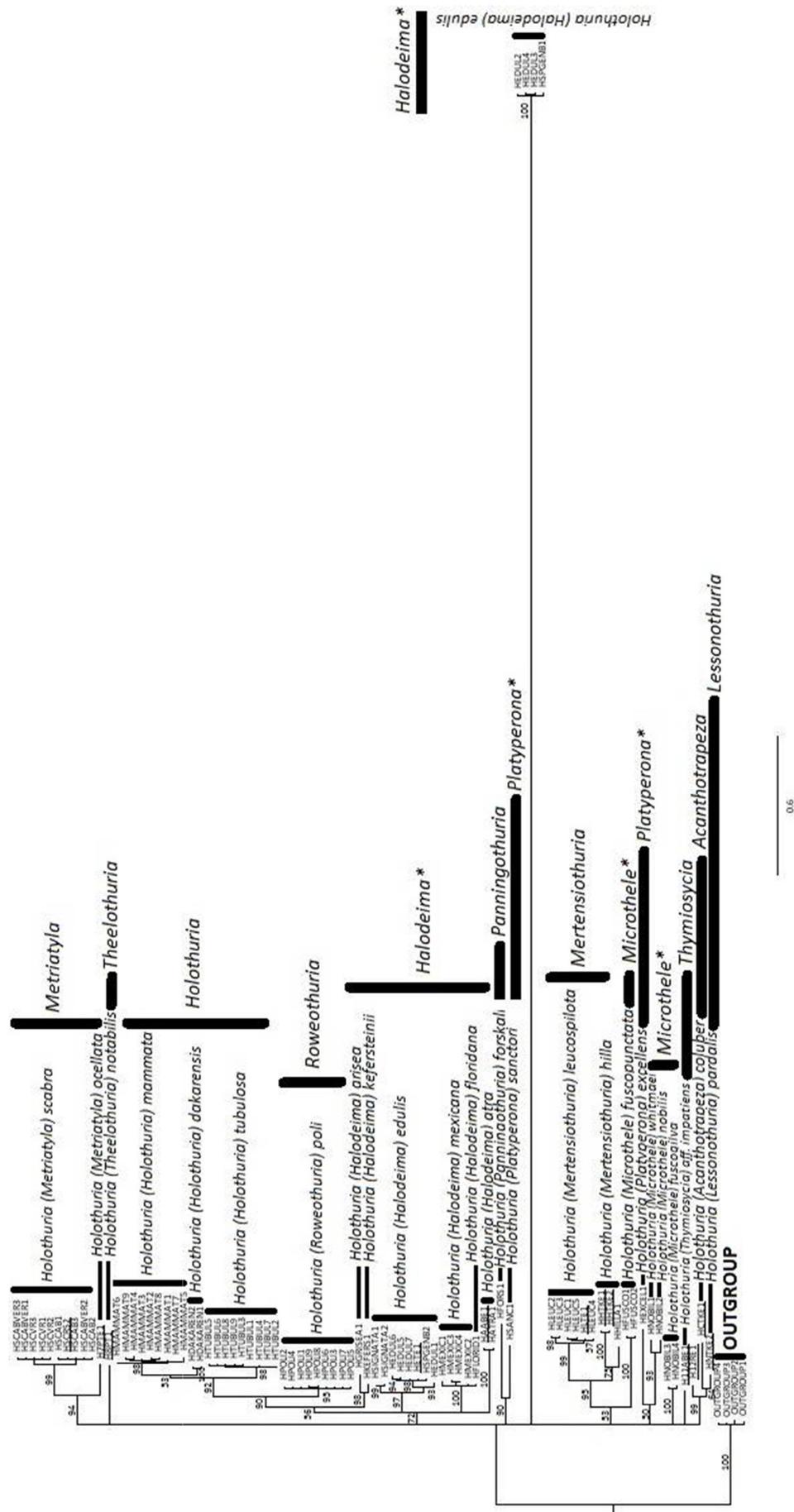


FIGURE 3. Topology of consensus Bayesian tree of Holothuria species inferred from 16S mitochondrial rRNA gene sequences using MrBayes (version 3.1.2) program (Huelsenbeck & Ronquist 2001), with the addition of all compatible groups to the tree. Each partial sequence detail is described in Table 1. Numbers at nodes indicate the posterior probabilities of clades in percentage (%)

belongs to genus *Holothuria*. Species identification by morphology e.g. based on the characteristics of calcareous ring, tentacle and ossicle along with the genetic information by Kamarudin et al. (2010b) is important to revise the taxonomic status of the undetermined species of order Apodida.

At the subgenus level, subgenera of *Holothuria* (with 98% bootstrap support), *Merthensiothuria* (with 92% bootstrap support) and *Metriatyla* (with 100% bootstrap support) are monophyletic with all their respective species clustered together forming clades, thus strengthening their current morphological taxonomy or classification by morphology. Subgenera of *Acanthotrapeza*, *Lessonothuria*, *Panningothuria*, *Roweothuria* (with 92% bootstrap support), *Theelothuria* and *Thymiosycia* are represented by one sequence or individuals of the same species. Meanwhile, subgenera of *Halodeima*, *Microthele* and *Platyperona* are polyphyletic due to their scattered species.

Surprisingly, within the subgenus *Halodeima*, there are five subgroups with *Holothuria* (*Halodeima*) *edulis* Lesson, 1830 splitted into two subgroups both supported by 100% bootstrap values. The first subgroup of *H. edulis* (with the 100% bootstrap support) consists of *H. edulis* from Malaysia (i.e. HETE1, GenBank accession no.: FJ223854), thus confirming the status of the subgroup members as *Holothuria* (*Halodeima*) *edulis* Lesson, 1830. With regard to the second subgroup of *H. edulis* (also with 100% bootstrap support), it is speculated that they might be of different *Holothuria* species but under subgenus *Halodeima*. In general, the current results from maximum parsimony analysis suggested that the genetic information do not fully support the current morphological taxonomy of *Holothuria* species at subgenus level, therefore taxonomic revisions for subgenera of *Halodeima*, *Microthele* and *Platyperona* are required by integrating the genetic information and morphological data.

In terms of sister relationship, subgenus *Holothuria* is a sister to subgenus *Roweothuria* with 84% bootstrap support while the other sister relationships are not clear due to the presence of undetermined species and polyphyly of some subgenera. In the meantime, subgenera of *Holothuria* and *Roweothuria* comprising of *Holothuria* (*Roweothuria*) *poli* Delle Chiaje, 1823, *Holothuria* (*Holothuria*) *mammata* Grube, 1840, *Holothuria* (*Holothuria*) *dakarensis* Panning, 1939 and *Holothuria* (*Holothuria*) *tubulosa* Gmelin, 1790 were suggested as the most recently evolved groups of genus *Holothuria*.

The Bayesian tree (Figures 3) shows that the undetermined species of order Apodida (i.e. H12RE1, GenBank accession no.: FJ223867) grouped with *H. pardalis* and *H. coluber* with 99% posterior probability and its position in the tree further suggested its status as a *Holothuria* taxon. Subgenera of *Holothuria* (with 92% posterior probability), *Merthensiothuria* (with 95% posterior probability) and *Metriatyla* (with 94% posterior probability) are monophyletic, thus complementing their taxonomic classification based on morphology. Polyphyly of subgenera of *Microthele* and *Platyperona* are also

shown by the Bayesian tree, suggesting a requirement for taxonomic revisions using integrative taxonomy i.e. morphological and genetic approaches.

Likewise the maximum parsimony tree, the consensus Bayesian tree suggests the polyphyly of subgenus *Halodeima* with five subgroups. However, the maximum parsimony tree suggested that one of the subgroups of *H. edulis* (i.e. with the 100% bootstrap support) is genetically closer to *H. notabilis* from Malaysia (GenBank accession no.: FJ223872). Despite the latter, the consensus Bayesian tree still suggested that the subgroup individuals might be of different *Holothuria* species but under subgenus *Halodeima*. Thus, the status of *H. edulis* was questionable and further taxonomic revisions were needed.

Furthermore, the consensus maximum parsimony tree validates HSPGENB2 (Figures 2) as *H. edulis* due to its clustering with *H. edulis* from Malaysia (i.e. HETE1, GenBank accession no.: FJ223854) and other other sequences of *H. edulis* with 100% bootstrap support. This finding was supported by the Bayesian tree with 97% posterior probability of clade. HSIGNATA1 and HSIGNATA2 (Figures 2-3) have been registered with the GenBank as *Holothuria signata* (GenBank accession no.: EU220812-EU220813), however the scientific name was not accepted by the World Register of Marine Species (WoRMS - <http://www.marinespecies.org/index.php>). The accepted scientific name was *Holothuria* (*Halodeima*) *edulis* Lesson, 1830 instead of *Holothuria signata*, accordingly the accepted name has been applied to the sequences of *Holothuria signata*. The maximum parsimony tree and the Bayesian tree then support such taxonomic status due to their robust clusterings with other sequences of *H. edulis* with 100% bootstrap support and 97% posterior probability of clade, respectively. Apart from that, HSPGENB1 (Figures 2 & 3, GenBank accession no.: EU220804) is speculated to be under the subgenus *Halodeima* due to its position in the 'Halodeima subgroup of *H. edulis*' (Figures 2 & 3). As mentioned earlier, with regard to the second subgroup of *H. edulis* (i.e. based on the NCBI taxon name) comprising HEDUL2 (GenBank accession no.: EU220807), HEDUL3 (GenBank accession no.: EU220808), HEDUL4 (GenBank accession no.: EU220809) and HSPGENB1; it is speculated that they might be of different *Holothuria* species but under subgenus *Halodeima*.

In terms of sister relationship, the Bayesian tree also suggests that subgenus *Holothuria* is a sister taxon of subgenus *Roweothuria* with 90% posterior probability of clade while the other sister relationships were undecided due to the undetermined species, paraphyly and polyphyly of a few subgenera. In relation to the maximum parsimony tree, subgenera of *Holothuria* and *Roweothuria* comprising *Holothuria* (*Roweothuria*) *poli* Delle Chiaje, 1823, *Holothuria* (*Holothuria*) *mammata* Grube, 1840, *Holothuria* (*Holothuria*) *dakarensis* Panning, 1939 and *Holothuria* (*Holothuria*) *tubulosa* Gmelin, 1790 are suggested as the most recently evolved groups of sea cucumber. Overall, this study has significantly contributed to the better insights into the molecular phylogeny of the

TABLE 1. Taxa incorporated for the phylogenetic analyses of genus *Holothuria* using partial 16S mitochondrial rRNA gene sequences. Refer to Kamarudin et al. (2010a, 2010b) for the details of all 12 sequences of sea cucumber from Malaysia

| Taxa  | Sample size | Individual No.        | GenBank Accession No.                   |  |
|---|-------------|-----------------------|---|--|
| Order Aspidochirotida   |             |                       |   |  |
| Family Holothuriidae  |             |                       |   |  |
| Genus <i>Holothuria</i>   | 72          |                       |   |  |
| Subgenus <i>Roweothuria</i>                                     |             |                       |   |  |
| <i>Holothuria (Roweothuria) poli</i> Delle Chiaje, 1823         | 9           | HPOLI1 – HPOLI9       | EU191981, EU750754 – EU750761           | Atlanto-Mediterranean area, Mar Menor coastal lagoon (Spain) |
| Subgenus <i>Holothuria</i>                                      |             |                       |   |  |
| <i>Holothuria (Holothuria) mammata</i> Grube, 1840              | 9           | HMAMMAT1 – HMAMMAT9   | EU191948 – EU191956,                    | Atlanto-Mediterranean area                                   |
| <i>Holothuria (Holothuria) dakarensis</i> Panning, 1939         | 2           | HDAKAREN1 – HDAKAREN2 | EU191979 – EU191980                     | Cape Verde Islands (Africa)                                  |
| <i>Holothuria (Holothuria) tubulosa</i> Gmelin, 1790            | 9           | HTUBUL1 – HTUBUL9     | EU191971 – EU191978, FJ231192           | Mediterranean Sea  |
| Subgenus <i>Halodeima</i>                                       |             |                       |   |  |
| <i>Holothuria (Halodeima) floridana</i> Pourtalés, 1851         | 1           | HFLORID1              | EU220803                                | Australia  |
| <i>Holothuria (Halodeima) mexicana</i> Ludwig, 1875             | 4           | HMEXIC1 – HMEXIC4     | EU220802, EU822443, FJ971378, GQ240831  | Australia, Australia, China, China                           |
| <i>Holothuria (Halodeima) edulis</i> Lesson, 1830               | 7           | HEDUL1 – HEDUL7       | EU220806 – EU220811,                    | Australia,   |
|   | 2           | HSIGNATA1 – HSIGNATA2 | EU822446, EU220812 – EU220813           | Australia, Australia   |
|   | 1           | HSPGENB1              | EU220804                                | Australia  |
|   | 1           | HSPGENB2              | EU220805                                | Australia  |
| <i>Holothuria (Halodeima) kefersteini</i> (Selenka, 1867)       | 1           | HKEFERS1              | EU220801                                | Australia  |
| <i>Holothuria (Halodeima) grisea</i> Selenka, 1867              | 1           | HGRISEA1              | EU220800                                | Australia  |
| <i>Holothuria (Halodeima) atra</i> Jaeger, 1833                 | 1           | HATRA1                | EU220799                                | Australia  |
| Subgenus <i>Metriatyla</i>                                      |             |                       |   |  |
| <i>Holothuria (Metriatyla) scabra</i> Jaeger, 1833              | 3           | HSCAB1 – HSCAB3       | AY509130 – AY509132,                    | Australia  |
|   | 3           | HSCVR1 – HSCVR3       | AY509142 – AY509144                     | Australia  |
|   | 3           | HSCABVER1 – HSCABVER3 | AY509137 – AY509139                     | Australia  |
| Subgenus <i>Mertensiothuria</i>                                 |             |                       |   |  |
| <i>Holothuria (Mertensiothuria) leucospilota</i> (Brandt, 1835) | 5           | HLEUC1 – HLEUC5       | AY338419, EU822444 – EU822445,          | Indo-West Pacific coral reefs,                               |
| <i>Holothuria (Mertensiothuria) hilla</i> Lesson, 1830          | 1           | HHILLA1               | FJ971389, GQ240842, EU822442            | Australia, China, China, Australia                           |
| Subgenus <i>Microthele</i>                                      |             |                       |   |  |
| <i>Holothuria (Microthele) fuscopunctata</i> Jaeger, 1833       | 2           | HFUSCO1 – HFUSCO2     | FJ971379, GQ240832                      | China, China, Australia                                      |
| <i>Holothuria (Microthele) whitmaei</i> Bell, 1887              | 1           | HNOBIL1               | AY509147                                | Australia  |
| <i>Holothuria (Microthele) nobilis</i> (Selenka, 1867)          | 1           | HNOBIL2               | EU822441                                | China, China   |
| <i>Holothuria (Microthele) fuscogilva</i> Cherbonnier, 1980     | 2           | HNOBIL3 – HNOBIL4     | EU822441                                | China, China   |
| <i>Holothuria (Platyperona) excellens</i> (Ludwig, 1875)        | 1           | HEXCEL1               | FJ971377, GQ240830                      | Indo-West Pacific coral reefs                                |
| <i>Holothuria (Platyperona) sanctori</i> Delle Chiaje, 1823     | 1           | HSANC1                | AY338418, EU191982                      | Atlanto-Mediterranean area                                   |
| Subgenus <i>Panningothuria</i>                                  |             |                       |   |  |
| <i>Holothuria (Panningothuria) forskali</i> Delle Chiaje, 1823  | 1           | HFORS1                | EU191983                                | Atlanto-Mediterranean area                                   |
| OUTGROUP  |             |                       |   |  |
| <i>Stichopus ocellatus</i> Massin, Zulfigar, Hwai & Boss, 2002  | 4           | OUTGROUP1 – OUTGROUP4 | EU220793, FJ223855, EU856675 – EU856676 | Australia, Malaysia, Australia, Australia                    |

morphologically undetermined species of order Apodida from Malaysia (i.e. H12RE1, GenBank accession no.: FJ223867) and then the subgenera of *Holothuria* and has also given a better understanding on the needs for further taxonomic revisions by integrating the genetic information and morphological data.

#### CONCLUSION

The current phylogenetic trees of maximum parsimony and Bayesian suggests that the morphologically undetermined species of order Apodida from Malaysia (i.e. H12RE1, Figure 1, GenBank accession no.: FJ223867) is genetically closer to *Holothuria (Lessonothuria) pardalis* Selenka, 1867 and *Holothuria (Acanthotrabeza) coluber* Semper, 1868; and its position in both trees further suggested its status as a *Holothuria* taxon. Subgenera of *Holothuria*, *Merthensiothuria* and *Metriatyla* are monophyletic with strong bootstrap supports and posterior probabilities of clades, thus complementing their morphological taxonomy. Moreover, the non-monophyly of subgenera of *Halodeima*, *Microthele* and *Platyperona* suggests a requirement for their taxonomic revisions using integrative taxonomy i.e. morphological and genetic approaches. In terms of sister relationship, both phylogenetic trees suggested that subgenus *Holothuria* is a sister taxon of subgenus *Roweothuria* while the other sister relationships were indistinguishable due to the undetermined species, paraphyly and polyphyly of a number of subgenera. In relation to the maximum parsimony tree, subgenera of *Holothuria* and *Roweothuria* comprising *Holothuria (Roweothuria) poli* Delle Chiaje, 1823, *Holothuria (Holothuria) mammata* Grube, 1840, *Holothuria (Holothuria) dakarensis* Panning, 1939 and *Holothuria (Holothuria) tubulosa* Gmelin, 1790 were suggested as the most recently evolved groups of genus *Holothuria*. As a result, further studies with more specimens of genus *Holothuria* from broader geographical locations and various mtDNA genes along with morphological approaches such as the characterisation of calcareous ring, tentacle and ossicle may help to provide a better view on the molecular phylogeny of subgenera of *Holothuria*.

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