Phylogeny of Sea Cucumber (Echinodermata: Holothuroidea) as Inferred from 16S Mitochondrial rRNA Gene Sequences

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INTRODUCTION

Sea cucumber belongs to phylum Echinodermata. This soft-bodied marine-dwelling echinoderm from class Holothuroidea is unique due to the existence of evolved skeleton (i.e. ossicles or spicules) and ancient-looked respiratory system called respiratory tree possessed by few species (Lambert 1997). To date, the systematics of sea cucumbers based on morphology particularly in Malaysia is still unclear (Kamarul et al. 2009) and thus requires molecular methods as alternatives to address the problem (Kamarul & Ridzwan 2005). Among the early studies in Malaysia on the species presence and distribution of sea cucumbers by using morphological characteristics were by George and George...
Dna tree and rna, 2. U.S., and 2.0 μL of 2 u/μL Taq
μL of d (25 mM), 2.5 μL of each universal primer (10 μM), 1.0
volume containing 30.0 μL of sterilized dH
Chain reaction -
Standard thermal cycle amplification (i.e. Polymerase
5'-3'.

Two universal primers were used for isolation of
mitochondrial ribosomal RNA sequences of selected sea cucumbers from
several locations in Malaysia and to apply the partial 16S
mitochondrial rRNA gene were aligned
among and the corresponding sequences from GenBank database in
phylogenetic analyses of sea cucumbers.

Materials and Methods
Total genomic DNA extraction was done using modified
CTAB method of Grew et al. (1993). The total genomic
DNA was extracted from muscle tissue of sea cucumber.
Approximate yields of DNA, the quantity and quality, were
determined by electrophoresis.

Two universal primers were used for isolation of
partial 16S mitochondrial ribosomal RNA (rRNA) region
(approximately 500 bp - 650 bp): 16sra-L (forward)
5'-CGCCGTGTATCAAAAAACAT-3' and 16srb-H (reverse)
5'-CCGCTGCTGACAGATGACGT-3' (Palumbi et al. 1991).
Standard thermal cycle amplification (i.e. Polymerase
Chain Reaction - PCR) was performed in 50 μL reaction
volume containing 30.0 μL of sterilized dH2O, 5.0 μL of
10X PCR reaction buffer, 3.0 μL of magnesium chloride
(25 mM), 2.5 μL of each universal primer (10 μM), 1.0
μL of dNTP mix (10 mM), 4.0 μL of the DNA preparation
and 2.0 μL of 2 u/μL Taq DNA polymerase. Master mix
was used for a large number of samples. Cycle parameters
were 5 min at 96°C for initial denaturation, 45 s at 95°C
for denaturation, 1 min 30 s at optimized temperature for
annealing, 1 min 30 s at 72°C (29 cycles) for extension,
and 7 min at 72°C for final extension. Purification kits
from manufacturer were used for direct purification.
Purified PCR products in suspension form were prepared
prior to sequencing.

A Chromas Lite (Version 2.1) program was used to
display the results of fluorescence-based DNA sequence
analysis. Multiple sequence alignment for forward
reaction sequences was done using ClustalX program
(version 1.81; Thompson et al. 1997), and subsequently
aligned by eyes. PAUP* version 4.0b10 (Swofford 1998)
was used to reconstruct neighbour joining (NJ) tree
(Figure 1) and maximum parsimony (MP) tree (Figure 2)
while PHYLIP version 3.6b (Felsenstein 2004) was used
to reconstruct maximum likelihood (ML) tree (Figure 3).
Kimura 2-parameter distance method was incorporated
to reconstruct the NJ tree based on equal base frequencies
and unequal ratio of transition to transversion (ti/tv).
TreeView (Win32) version 1.6.6 by Page (1996) was
used to display and edit the reconstructed phylogenetic
trees.

Results and Discussion
In total 17 out of 50 species of sea cucumber recorded
recently in Malaysia were included in the phylogenetic
analyses and were registered with GenBank, National
Center for Biotechnology Information (NCBI), U.S.
National Library of Medicine (GenBank accession no.:
fj223854 - fj223872). Thirty seven partial sequences (442
– 487 bp) of 16S mitochondrial rRNA gene were aligned
consisting of 19 sequences of the selected sea cucumbers
from Malaysia, 17 corresponding sequences obtained
from GenBank and one sequence of Ophionereis porrecta
(a brittle star) as outgroup (Table 1). Aligned base (503)
positions including the possible gaps were incorporated
for reconstruction of phylogenetic trees. MP analysis showed
that 205 characters were constant, 76 variable characters
were parsimony-uninformative and 222 characters were
parsimony-informative.

All the phylogenetic trees (Figure 1-3) supported
the presence of five main genera namely Holothuria,
Actinopyga, Bohadschia, Stichopus and Molpadia.
Actinopyga, Bohadschia, Stichopus and Molpadia were
monophyletic with 100% bootstrap support, while
Holothuria was paraphyletic. Stichopus was considered
sister taxon to Molpadia with strong bootstrap support
(NJ-93%; MP-87%; ML-100%), hence it is suggested that
Stichopus was genetically very close to Molpadia from
order Molpadiida. As a result, the status of Stichopus
as one of Aspidochirotida members is questionable and
this requires further verification. The other members of
order Aspidochirotida such as Bohadschia, Actinopyga
and paraphyletic Holothuria formed a cluster of family

(1987) and later on by Ridzwan (1993) and the focus region
was Sabah, East Malaysia. Stichopus horrens was formerly
identified as S. hermanni and the validation was done based
on the findings by Baine and Forbes (1998), Baine and Sze
(1999), Sze and Williams (2004) and Zulfigar et al. (2000).
The Randomly Amplified Polymorphisms of DNA (RAPD)
analysis carried out by Norazila et al. (2000) was the first
and pioneering effort in Malaysia in the use of molecular
phylogeny method. However, phylogenetic analysis using
DNA sequences is considered as more powerful tool as
compared to RAPD in molecular approach. It has the ability
to study the synonymous substitution occurred between
and among the nucleotide sequences. Mitochondrial DNA
(mtDNA) and nuclear DNA are two main sources of genetic
materials used as the target sites for phylogenetic studies
using DNA sequencing.

There are a few characteristics of mtDNA that make this
component the most preferred model in molecular
ecology such as effective maternal inheritance,
apparent haploid genome, non-recombination,
continuous replication. The rate of substitution in mtDNA is within
the range of 5 to 10 times greater than in ‘single-copy’
Phylogenetic inference from 16S mitochondrial rRNA gene
shown by previous studies suggests the ability of such
gene to correlate the relationship between morphology
and genetics (Clouse et al. 2005; Kerr et al. 2005).

The previously unclear and problematic identification
of sea cucumber species as well as the not up-to-date and
incomplete documentation on the species presence and
distribution in Malaysia have led the way to this study. This
study aimed to obtain partial 16S mitochondrial ribosomal
RNA gene sequences of selected sea cucumbers from
several locations in Malaysia and to apply the partial 16S
mitochondrial ribosomal RNA gene sequences along with
their corresponding sequences from GenBank database in
phylogenetic analyses of sea cucumbers.

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RNA gene sequences of selected sea cucumbers from
several locations in Malaysia and to apply the partial 16S
mitochondrial ribosomal RNA gene sequences along with
their corresponding sequences from GenBank database in
phylogenetic analyses of sea cucumbers.
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<th>GenBank Accession No.</th>
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</table>

Note: G - GenBank, ECPM - East Coast of Peninsular Malaysia, WCPM - West Coast of Peninsular Malaysia and SBEM - Sabah, East Malaysia.
Holothuriidae. Apart from that, *B. marmorata* (G1) was successfully verified as *B. bivittata* thus supporting the findings from NJ, MP and ML analyses. On the whole, the phylogenetic trees likely resolved the genetic relationship of sea cucumbers at the genus and family level but not thoroughly at higher taxonomic level i.e. order level.

At the genus level, basically, the species classification was supported by high bootstrap percentage. However, the clustering showed that *H. excellens* was not grouped within *Holothuria* group. Even though the position of *H. excellens* shown by NJ tree (Figure 1) was different from the MP tree (Figure 2) and furthermore by ML tree (Figure 3), all trees still did not support *H. excellens* as the member of genus *Holothuria*. The genetic distance between *H. excellens* and the other *Holothuria* species was relatively high, ranging from 0.1992 to 0.2710 (Table 2), thus supporting the paraphyly of *Holothuria*. The inclusion of *H. excellens* into the phylogenetic analyses with the incorporation of more individuals from different species was done to verify the paraphyly of *Holothuria* as summarized by Kerr et al. (2005). This study strengthened and strongly supports the status of *Holothuria* as paraphyletic. However, in terms of taxonomic validity, the status of *H. excellens* as one of *Holothuria* species needs to be further verified, and the possibility of wrong morphological identification of *H. excellens* as one of *Holothuria* species must not be ruled out.

Six out of 32 unknown species of sea cucumber from Malaysia identified morphologically were incorporated in the phylogenetic analyses. Interestingly, all the phylogenetic trees based on 16S mitochondrial rRNA gene (Figure 1-3) principally showed the clustering of

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**Figure 1.** Topology of neighbour joining tree (consensus tree) of sea cucumber species inferred from 16S mitochondrial ribosomal rRNA gene using PAUP*®* version 4.0b10 (Swofford 1998). Abbreviation of G refers to corresponding sequences obtained from GenBank. Each partial sequence detail is described in Table 1. The tree was rooted with a sequence of *Ophionereis porrecta*, a brittle star (GenBank accession number: AY365184). Kimura 2-parameter distance method with 1000 replications was used.

Numbers at nodes indicate the bootstrap values in percentage (%).
FIGURE 2. Topology of maximum parsimony tree (consensus tree) of sea cucumber species inferred from 16S mitochondrial ribosomal RNA gene using PAUP* version 4.0b10 (Swofford 1998). Abbreviation of G refers to the corresponding sequences obtained from GenBank. Each partial sequence detail is described in Table 1. The tree was rooted with a sequence of Ophionereis porrecta, a brittle star (GenBank accession number: AY365184). 1000 replications were used.

Numbers at nodes indicate the bootstrap values in percentage (%)

Each the unknown species into the expected genus. Even though the species status was unknown, the phylogenetic trees suggested the possible genetic relationship of each unknown species to the other identified species within the same genus. For instance, close genetic relationship was observed between H. sp. 7 and H. scabra, H. sp. 12 and H. notabilis and between S. sp. 7 and S. ocellatus.

One of the interesting parts from the phylogenetic analyses of 16S mitochondrial rRNA gene in this study was the species validation of B. marmorata (G1) incorporated in the tree reconstruction (Table 1; Figure 1-3) to B. bivittata. The similar genetic distance between B. marmorata (G1) and individuals of B. bivittata (G1 and G2) apparently suggested the wrong identification of B. marmorata (G1) by morphology. Furthermore, high average of genetic distance between B. marmorata (G1) and the other individuals of B. marmorata (G2, G3 and G4), with high bootstrap support for B. marmorata subclade and B. bivittata subclade strongly proved that B. marmorata (G1) is actually B. bivittata. As a result, B. vitiensis from Malaysia was supported as sister taxon to B. bivittata, revealing their close genetic relationship. Data from the calculation of genetic distance (Table 2) supported such close genetic relationship as the average of genetic distance between B. vitiensis (1) and B. bivittata (0.0077) was much lower than the average of genetic distance between B. vitiensis (1) and B. marmorata (0.0897). The apparent different morphology between B. vitiensis and B. bivittata is the absence of two broad, dark-brown bands across the entire breadth of B. vitiensis as mentioned by Clouse et al. (2005).
FIGURE 3. Topology of maximum likelihood tree (consensus tree) with molecular clock of sea cucumber species inferred from 16S mitochondrial ribosomal RNA gene using PHYLIP version 3.6b (Felsenstein 2004). Abbreviation of G refers to the corresponding sequences obtained from GenBank. Each partial sequence detail is described in TABLE 1. The tree was rooted with a sequence of Ophionereis porrecta, a brittle star (GenBank accession number: AY365184).

1000 sequence replications and 100 data sets were used. Numbers at nodes indicate the bootstrap values in percentage (%).
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TABLE 2. The distance matrix of the pairwise distance calculation. The calculation incorporated Kimura 2-parameter distance method. Each partial distance detail is described in Table 1.
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Continued (Table 2)
By using 18S rRNA gene, Lacey et al. (2005) suggested the paraphyly of genus Bohadschia, as Actinopyga miliaris and Bohadschia vitiensis/marmorata were grouped together. Such finding also supported the status of both Bohadschia vitiensis and Bohadschia vitiensis/marmorata as separate species, however, the paraphyly status put forward question about the effectiveness of 18S rRNA gene to resolve the taxonomic status at the genus level. In contrast, the phylogenetic relationship of sea cucumbers in this study inferred from 16S mitochondrial rRNA gene strongly supported the monophyly of genus Bohadschia with average of 100% bootstrap value. Likewise the status of separate species as shown by Lacey et al. (2005), B. marmorata from Micronesia and the only single individual of B. vitiensis from Malaysia were proven as separate species. It seems that 16S mitochondrial rRNA gene better resolves the taxonomic status of genus Bohadschia at the genus level as compared to 18S rRNA gene.

CONCLUSION

The current phylogenetic relationship of sea cucumbers using 37 partial sequences of 16S mitochondrial ribosomal RNA (rRNA) gene indicated the presence of five main genera namely Molpadia from order Molpadiida and four genera of order Aspidochirotida namely Holothuria, Stichopus, Bohadschia and Actinopyga. Interestingly, H. excellens was out of genus Holothuria causing Holothuria to be a paraphyletic. High bootstrap value and consistent clustering made Molpadia, Stichopus, Bohadschia and Actinopyga monophyletic. Moreover, Stichopus was a sister taxon to Molpadia and this finding made the resolution at order level of sea cucumber unclear and problematic. Furthermore, in terms of taxonomic validity, the phylogenetic inference strongly suggested the wrong identification of B. marmorata (G1) whereby its partial sequence was obtained from GenBank database. The outcome suggested that the actual status of the said taxon is B. bivittata. Even if the phylogenetic analyses failed to resolve and verify the actual taxonomic status of the six unknown species from Malaysia at species level, such analyses suggested the possible relationship of the unknown species with the known species utilized in this present study of molecular phylogeny. Further studies with more samples and different mtDNA genes need to be done in near future as attempts to get better view and verification on the molecular phylogeny of sea cucumbers.

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REFERENCES


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