

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

(Filogeni Timun Laut (Echinodermata: Holothuroidea) Berdasarkan
Jujukan Gen 16S Mitokondria rRNA)

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ABSTRACT

This study aimed to determine phylogenetic relationship between and among selected species of sea cucumbers (Echinodermata: Holothuroidea) using 16S mitochondrial ribosomal RNA (rRNA) gene. Phylogenetic analyses of 37 partial sequences of 16S mitochondrial rRNA gene using three main methods namely neighbour joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) showed the presence of five main genera of sea cucumbers: Molpadia from order Molpadiida and four genera of order Aspidochirotida namely Holothuria, Stichopus, Bohadschia and Actinopyga. All of the 17 species obtained from Malaysia distributed among the main genera except within Actinopyga. Interestingly, Holothuria excellens was out of Holothuria group causing Holothuria to be paraphyletic. High bootstrap value and consistent clustering made Molpadia, Stichopus, Bohadschia and Actinopyga monophyletic. The relationship of Actinopyga with the other genera was unclarified and Stichopus was sister to Molpadia. The latter finding caused the resolution at order level unclear. The pairwise genetic distance calculated using Kimura 2-parameter model further supported and verified findings from the phylogenetic trees. Further studies with more samples and different mitochondrial DNA genes need to be done to get a better view and verification on the molecular phylogeny of sea cucumbers.

Keywords: Phylogenetic relationship; partial sequences of 16S mitochondrial ribosomal RNA gene; sea cucumbers

ABSTRAK

Kajian ini bertujuan untuk menentukan perhubungan filogenetik antara spesies-spesies terpilih timun laut (Echinodermata: Holothuroidea) dengan menggunakan gen subunit 16S mitokondria ribosom RNA (rRNA). Analisis filogenetik melibatkan 37 separa jujukan gen 16S mitokondria ribosom RNA yang menggunakan tiga kaedah utama iaitu kaedah hubung kait jiran (NJ), parsimoni maksimum (MP) dan kaedah hubung kait maksimum (ML) telah menunjukkan kehadiran lima kumpulan utama timun laut iaitu Molpadia daripada order Molpadiida dan empat genus daripada order Aspidochirotida iaitu Holothuria, Stichopus, Bohadschia dan Actinopyga. Semua 17 spesies yang diperolehi dari Malaysia tergolong dalam kumpulan-kumpulan utama tersebut kecuali dalam kumpulan Actinopyga. Penemuan paling menarik adalah Holothuria excellens tidak tergolong dalam kumpulan Holothuria menyebabkan Holothuria bersifat parafiletik. Nilai bootstrap yang tinggi dan pengelasan yang konsisten menyebabkan Molpadia, Stichopus, Bohadschia dan Actinopyga bersifat monofiletik. Perhubungan genetik antara Actinopyga dengan genus yang lain tidak dapat diklarifikasikan dan Stichopus pula mempunyai hubungan genetik yang rapat dengan Molpadia dari order Molpadiida. Perhubungan kedua tersebut menyebabkan resolusi pada peringkat order adalah tidak jelas. Pengiraan jarak genetik secara berpasangan menggunakan model Kimura 2-parameter seterusnya telah menyokong dan mengesahkan keputusan-keputusan yang diperolehi daripada pokok-pokok filogenetik yang diperolehi. Kajian lanjut melibatkan bilangan sampel yang lebih banyak serta gen mitokondria DNA yang berbeza perlu dilakukan untuk mendapatkan gambaran serta pengesahan yang lebih baik terhadap filogeni molekul timun laut.

Kata kunci: Perhubungan filogenetik; separa jujukan gen 16S mitokondria ribosom RNA; timun laut

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-looking 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

(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 genetic component the most preferred model in molecular genetic 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' nuclear DNA (Amos & Hoelzel 1992; Hartl & Clark 1989). 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.

MATERIALS AND METHODS

Total genomic DNA extraction was done using modified CTAB method of Grewe 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): 16sar-L (forward) 5'-CGCCTGTTTATCAAAAACAT-3' and 16sbr-H (reverse) 5'-CCGGTCTGAACTCAGATCACGT-3' (Palumbi et al. 1991). Standard thermal cycle amplification (i.e. Polymerase Chain Reaction - PCR) was performed in 50 μ L reaction volume containing 30.0 μ L of sterilized dH₂O, 5.0 μ L of 10X PCR reaction buffer, 3.0 μ L of magnesium chloride (25 mM), 2.5 μ L of each universal primer (10 μ M), 1.0 μ L of dNTP mix (10 mM), 4.0 μ L of the DNA preparation and 2.0 μ L of 2 u/ μ L 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

TABLE 1. Taxa used for phylogenetic analyses of sea cucumbers inferred from 16S mitochondrial ribosomal RNA gene

Taxa	Sample Size	Individual No.	Locality	GenBank Accession No.
Order Aspidochirotida				
Family Stichopodidae				
<i>Stichopus chloronotus</i>	2	1	Tioman, ECPM	FJ223860
		G	Unknown	AY338422
<i>Stichopus horrens</i>	1	2	Pangkor, WCPM	FJ223859
<i>Stichopus ocellatus</i>	1	1	Tioman, ECPM	FJ223855
<i>Stichopus</i> sp. 7	1	1	Tioman, ECPM	FJ223863
Family Holothuriidae				
<i>Holothuria edulis</i>	1	1	Tioman, ECPM	FJ223854
<i>Holothuria atra</i>	1	1	Tioman, ECPM	FJ223858
<i>Holothuria coluber</i>	1	1	Tioman, ECPM	FJ223866
<i>Holothuria hilla</i>	2	1	Tioman, ECPM	FJ223864
		2	Tioman, ECPM	FJ223856
<i>Holothuria leucospilota</i>	2	1	Tioman, ECPM	FJ223871
		G	Unknown	AY338419
<i>Holothuria scabra</i>	5	2	Semporna, SBEM	FJ223862
		G1	Unknown	AY509140
		G2	Unknown	AY509138
		G3	Unknown	AY509136
<i>Holothuria excellens</i>	1	G4	Unknown	AY509135
		G	Unknown	AY338418
<i>Holothuria</i> sp. 7	1	1	Terengganu, ECPM	FJ223869
<i>Holothuria</i> sp. 8	1	1	Terengganu, ECPM	FJ223872
<i>Holothuria notabilis</i>	1	2	Tioman, ECPM	FJ223861
<i>Holothuria</i> sp. 11	1	1	Tioman, ECPM	FJ223857
<i>Holothuria</i> sp. 12	1	1	Tioman, ECPM	FJ223867
<i>Bohadschia bivittata</i>	2	G1	Micronesia	AY574872
		G2	Micronesia	AY574873
<i>Bohadschia marmorata</i>	4	G1	Unknown	AY338417
		G2	Micronesia	AY574875
		G3	Micronesia	AY574874
		G4	Micronesia	AY574877
<i>Bohadschia vitiensis</i>	1	1	Tioman, ECPM	FJ223868
<i>Bohadschia argus</i>	2	G1	Micronesia	AY574870
		G2	Micronesia	AY574869
<i>Actinopyga mauritania</i>	1	G	Unknown	AY338414
<i>Actinopyga</i> sp.	1	G	Unknown	AY338413
Order Molpadida				
<i>Molpadia</i> sp. 1	2	1	Port Dickson, WCPM	FJ223870
		2	Port Dickson, WCPM	FJ223865
Outgroup				
<i>Ophionereis</i>				
<i>Porrecta</i>	1	G	Unknown	AY365184

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. Eventhough 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

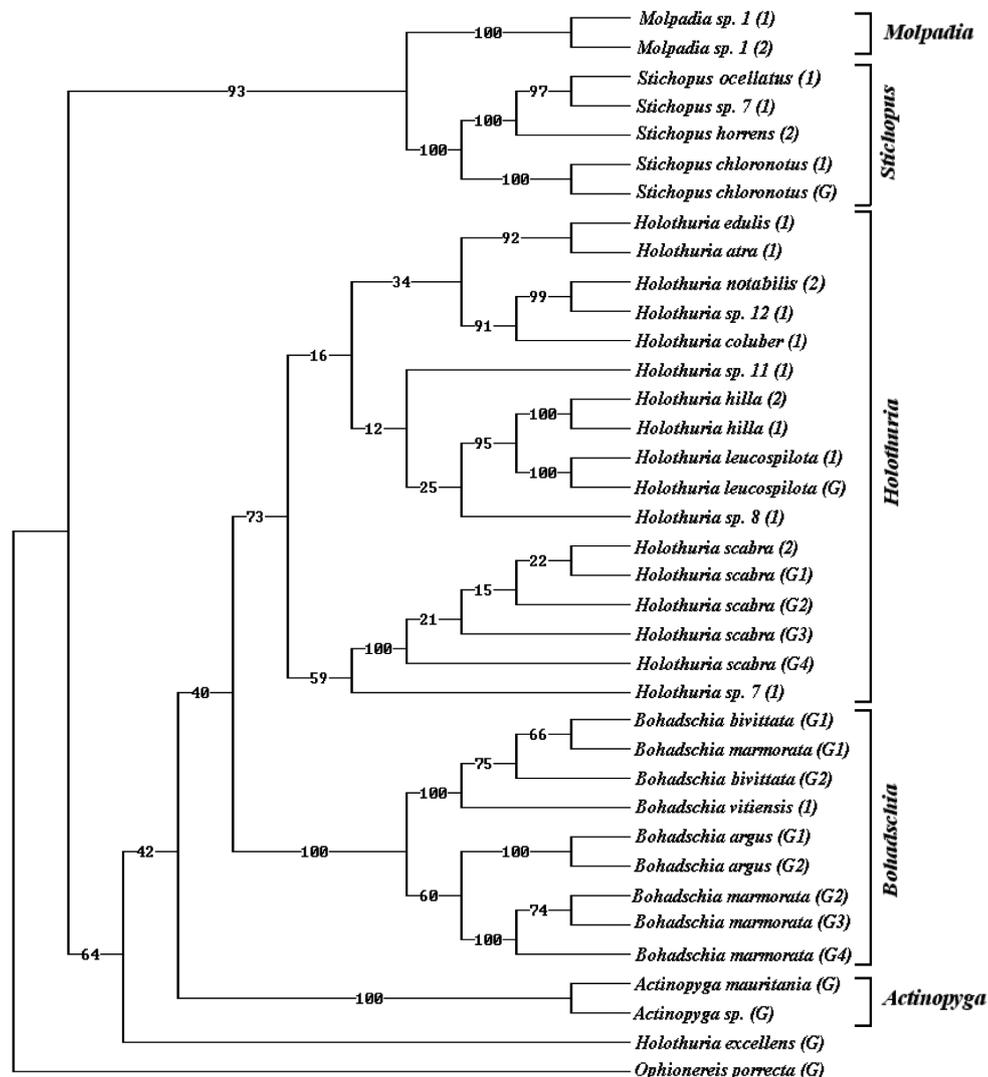


FIGURE 1. Topology of neighbour joining tree (consensus tree) of sea cucumber species inferred from 16S mitochondrial ribosomal RNA gene using PAUP* version 4.0b10 (Swofford 1998). Abbreviation of G refers to corresponding sequences obtained from GenBank. Each partial sequence detail is described in TABLE 1. The tree was rooted with a sequence of *Ophioneis 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 (%)

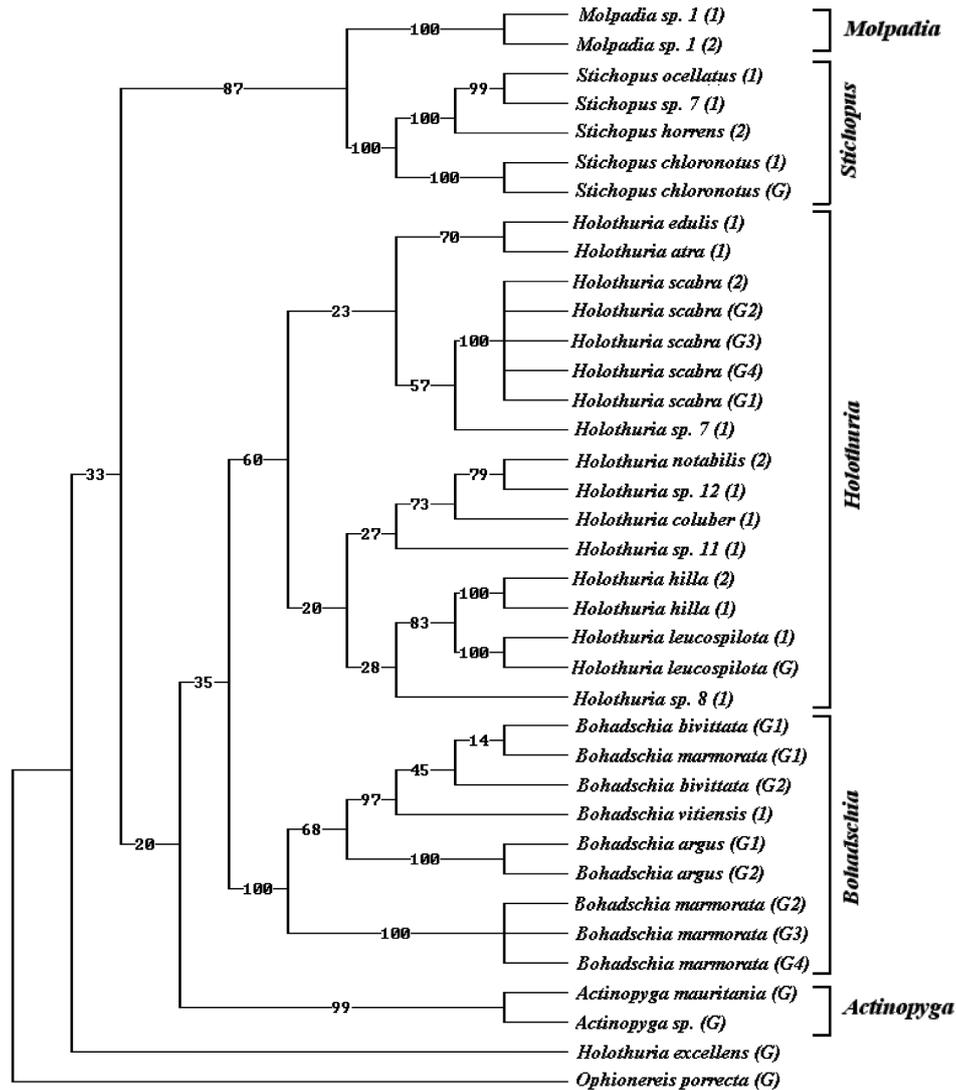


FIGURE 2. Topology of maximum parsimony tree (consensus tree) of sea cucumber species inferred from 16S mitochondrial ribosomal RNA gene using PAUP* version 4.0b10 (Swofford 1998). Abbreviation of G refers to the corresponding sequences obtained from GenBank. Each partial sequence detail is described in TABLE 1. The tree was rooted with a sequence of *Ophioneis 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).

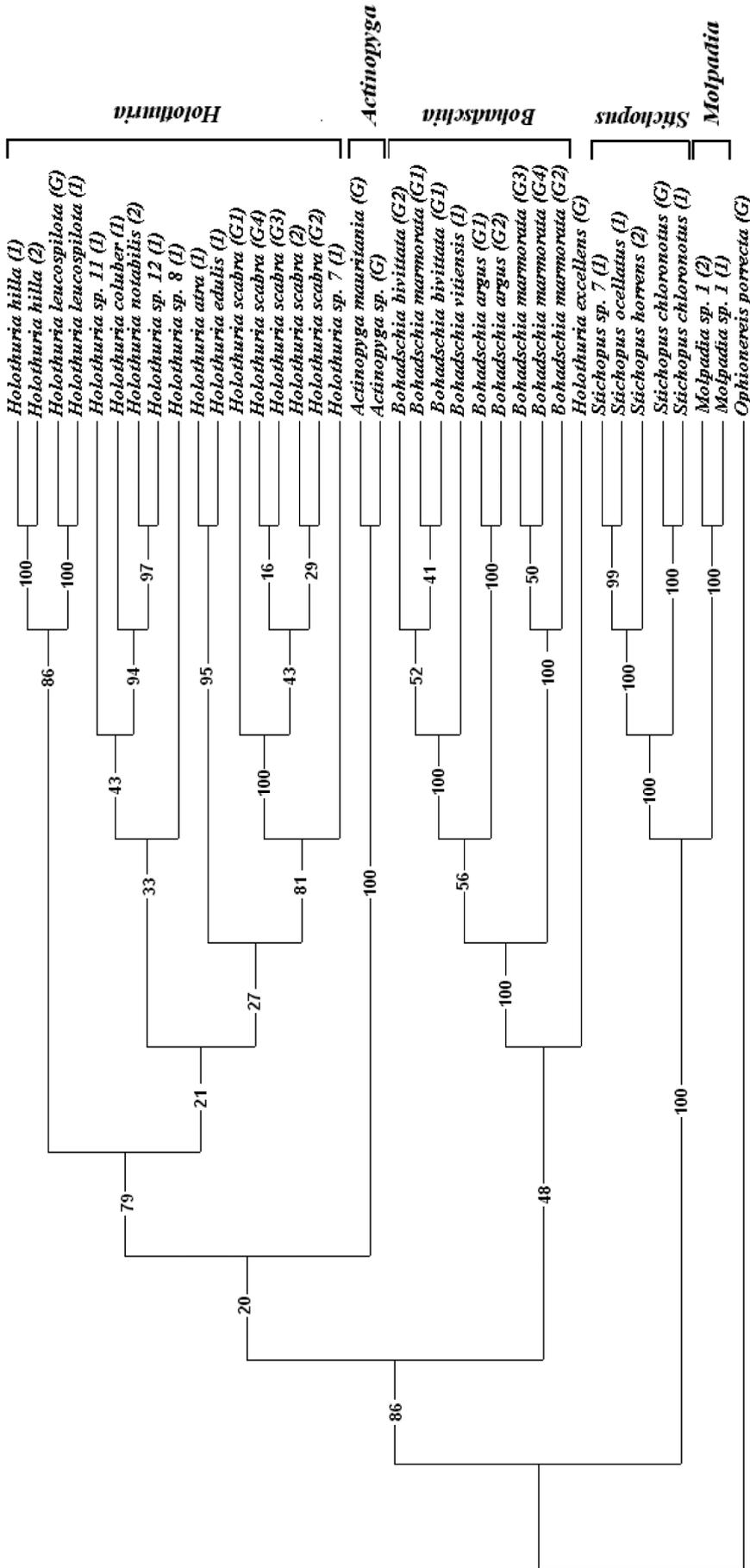


FIGURE 3. Topology of maximum likelihood tree (consensus tree) with molecular clock of sea cucumber species inferred from 16S mitochondrial ribosomal RNA gene using PHYLIP version 3.6b (Felsenstein 2004). Abbreviation of G refers to the corresponding sequences obtained from GenBank. Each partial sequence detail is described in TABLE 1. The tree was rooted with a sequence of *Ophioneis 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 (%)

TABLE 2. The distance matrix of the pairwise distance calculation. The calculation incorporated Kimura 2-parameter distance method. Each partial sequence detail is described in Table 1

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
1 <i>Molpadiia</i> sp. 1 (2)	-																				
2 <i>Molpadiia</i> sp. 1 (1)	0.00206	-																			
3 <i>Stichopus ocellatus</i> (1)	0.24136	0.23776	-																		
4 <i>Stichopus</i> sp. 7 (1)	0.24425	0.24064	0.02346	-																	
5 <i>Stichopus horrens</i> (2)	0.24582	0.24222	0.04335	0.04342	-																
6 <i>Stichopus chloronotus</i> (1)	0.28699	0.28624	0.21166	0.20310	0.19945	-															
7 <i>Stichopus chloronotus</i> (3)	0.24764	0.24395	0.15977	0.15726	0.14862	0.04071	-														
8 <i>Holothuria edulis</i> (1)	0.26198	0.25814	0.33903	0.32807	0.33333	0.37461	0.33313	-													
9 <i>Holothuria atra</i> (1)	0.27647	0.27249	0.33609	0.32524	0.33355	0.32831	0.29254	0.13157	-												
10 <i>Holothuria scabra</i> (2)	0.23567	0.23195	0.27952	0.26651	0.28450	0.26593	0.23222	0.14082	0.14952	-											
11 <i>Holothuria scabra</i> (G2)	0.23567	0.23195	0.27952	0.26651	0.28450	0.26593	0.23222	0.14082	0.14952	0.00000	-										
12 <i>Holothuria scabra</i> (G3)	0.23567	0.23195	0.27952	0.26651	0.28450	0.26593	0.23222	0.14082	0.14952	0.00000	0.00000	-									
13 <i>Holothuria scabra</i> (G4)	0.23567	0.23195	0.27952	0.26651	0.28450	0.26593	0.23222	0.14082	0.14952	0.00000	0.00000	0.00000	-								
14 <i>Holothuria scabra</i> (G1)	0.23881	0.23506	0.28285	0.26977	0.28784	0.26919	0.23534	0.14358	0.15234	0.00220	0.00220	0.00220	0.00220	-							
15 <i>Holothuria</i> sp. 7 (1)	0.25157	0.24764	0.30040	0.29714	0.30905	0.31295	0.27690	0.16422	0.17908	0.11272	0.11272	0.11272	0.11272	0.11538	-						
16 <i>Holothuria natibitis</i> (2)	0.29153	0.28727	0.31832	0.31137	0.30602	0.34498	0.30402	0.14102	0.15819	0.14891	0.14891	0.14891	0.14891	0.15176	0.14344	-					
17 <i>Holothuria</i> sp. 12 (1)	0.31334	0.30892	0.34640	0.33924	0.33360	0.36295	0.31765	0.14294	0.16009	0.15143	0.15143	0.15143	0.15143	0.15427	0.13788	0.06247	-				
18 <i>Holothuria coluber</i> (1)	0.29574	0.29149	0.32469	0.32136	0.30890	0.34028	0.29629	0.16625	0.16078	0.15992	0.15992	0.15992	0.15992	0.16280	0.15771	0.09944	0.12250	-			
19 <i>Holothuria</i> sp. 11 (1)	0.28288	0.27878	0.31397	0.30026	0.31903	0.34072	0.30402	0.18044	0.18421	0.15771	0.15771	0.15771	0.15771	0.16062	0.14735	0.13596	0.14694	0.14868	-		
20 <i>Holothuria hilla</i> (2)	0.31932	0.31511	0.35535	0.33782	0.34587	0.38421	0.34611	0.18646	0.19890	0.16374	0.16374	0.16374	0.16374	0.16089	0.17848	0.17656	0.19005	0.18708	0.18612	-	
21 <i>Holothuria hilla</i> (1)	0.32597	0.32171	0.35876	0.34117	0.34925	0.38772	0.34943	0.19466	0.20731	0.17193	0.17193	0.17193	0.17193	0.16906	0.17570	0.18487	0.19845	0.19551	0.19437	0.00644	-
22 <i>Holothuria leucospilata</i> (1)	0.30647	0.30235	0.36283	0.35217	0.35340	0.34428	0.30802	0.17978	0.17445	0.18205	0.18205	0.18205	0.18205	0.18505	0.18045	0.17169	0.19192	0.20411	0.16748	0.14478	-
23 <i>Holothuria leucospilata</i> (G)	0.30647	0.30235	0.36283	0.35217	0.35340	0.34428	0.30802	0.17978	0.17445	0.18205	0.18205	0.18205	0.18205	0.18505	0.18045	0.17169	0.19192	0.20411	0.16748	0.14478	-
24 <i>Holothuria</i> sp. 8 (1)	0.28658	0.28261	0.33930	0.33601	0.33420	0.34499	0.30222	0.17498	0.19029	0.14650	0.14650	0.14650	0.14650	0.14927	0.17034	0.15272	0.16009	0.16780	0.15551	0.18714	-
25 <i>Bohadschia hirtellata</i> (G1)	0.30500	0.30093	0.34604	0.33581	0.31723	0.41708	0.36308	0.20683	0.22185	0.20184	0.20184	0.20184	0.20184	0.20492	0.18171	0.18608	0.20530	0.20530	0.19727	0.19843	-
26 <i>Bohadschia marmorata</i> (G1)	0.30523	0.30114	0.34587	0.33566	0.31714	0.41678	0.36284	0.20962	0.22473	0.20468	0.20468	0.20468	0.20468	0.20777	0.18454	0.18887	0.20815	0.20815	0.20007	0.20120	-
27 <i>Bohadschia marmorata</i> (G2)	0.30809	0.30400	0.34937	0.33910	0.32044	0.42070	0.36646	0.20405	0.21898	0.20468	0.20468	0.20468	0.20468	0.20777	0.18454	0.18887	0.20815	0.20815	0.20007	0.20120	-
28 <i>Bohadschia marmorata</i> (G3)	0.31223	0.30809	0.35386	0.34349	0.32462	0.41797	0.36384	0.21059	0.22564	0.20570	0.20570	0.20570	0.20570	0.20882	0.18501	0.18972	0.20603	0.20603	0.20093	0.20196	-
29 <i>Bohadschia argus</i> (G1)	0.30831	0.30421	0.34921	0.34937	0.33036	0.41649	0.36261	0.24285	0.24432	0.23455	0.23455	0.23455	0.23455	0.23780	0.21338	0.21837	0.22880	0.20726	0.22957	0.22550	-
30 <i>Bohadschia argus</i> (G2)	0.30171	0.29767	0.34572	0.34587	0.32700	0.41262	0.35902	0.24285	0.24432	0.22812	0.22812	0.22812	0.22812	0.23132	0.21338	0.22151	0.23196	0.20426	0.22957	0.21942	-
31 <i>Bohadschia marmorata</i> (G2)	0.27713	0.27330	0.35567	0.34529	0.33297	0.40431	0.35849	0.24156	0.21877	0.21714	0.21714	0.21714	0.21714	0.22031	0.19302	0.20184	0.22784	0.20280	0.21306	0.23141	-
32 <i>Bohadschia marmorata</i> (G3)	0.27713	0.27330	0.35567	0.34529	0.33297	0.40431	0.35849	0.24156	0.21877	0.21714	0.21714	0.21714	0.21714	0.22031	0.19302	0.20184	0.22784	0.20280	0.21306	0.23141	-
33 <i>Bohadschia marmorata</i> (G4)	0.28028	0.27642	0.35567	0.34529	0.33297	0.40431	0.35849	0.24479	0.22181	0.22031	0.22031	0.22031	0.22031	0.22351	0.19302	0.20184	0.22784	0.20280	0.21306	0.23451	-
34 <i>Holothuria exellens</i> (3)	0.28349	0.27964	0.31491	0.30842	0.31660	0.37146	0.32081	0.23233	0.26304	0.23396	0.23396	0.23396	0.23396	0.23719	0.19918	0.21426	0.20978	0.21791	0.21306	0.26797	-
35 <i>Actinopyga mauritiana</i> (G)	0.28523	0.28765	0.32954	0.32626	0.32438	0.35917	0.31581	0.24070	0.27632	0.22195	0.22195	0.22195	0.22195	0.22510	0.23619	0.24799	0.24435	0.25064	0.23531	0.24666	-
36 <i>Actinopyga</i> sp. (G)	0.30546	0.30794	0.33433	0.32085	0.33895	0.38181	0.32675	0.25772	0.30525	0.22886	0.22886	0.22886	0.22886	0.23206	0.24534	0.25908	0.26039	0.26959	0.25102	0.24820	-
37 <i>Ophionereis porrecta</i> (G)	0.45011	0.44417	0.50849	0.51393	0.50564	0.55327	0.51529	0.46709	0.44534	0.42993	0.42993	0.42993	0.42993	0.43452	0.43495	0.46787	0.45811	0.47201	0.47884	0.47884	-

(continue)

Continued (TABLE 2)

Species	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
<i>Holohuria hilla</i> (I)	-																
<i>Holohuria leucospicata</i> (I)	0.14980	-															
<i>Holohuria leucospicata</i> (G)	0.14710	0.00214	-														
<i>Holohuria</i> sp. 8 (I)	0.19535	0.16844	0.16844	-													
<i>Bohadschia bivitata</i> (G1)	0.20409	0.21852	0.21852	0.23617	-												
<i>Bohadschia marmorata</i> (G1)	0.20688	0.22135	0.22135	0.23903	0.00209	-											
<i>Bohadschia bivitata</i> (G2)	0.20132	0.21868	0.21868	0.23903	0.00209	0.00418	-										
<i>Bohadschia vitensis</i> (I)	0.20739	0.22223	0.22223	0.23702	0.00629	0.00840	0.00840	-									
<i>Bohadschia argus</i> (G1)	0.23141	0.23363	0.23363	0.24621	0.06153	0.06376	0.05931	0.06391	-								
<i>Bohadschia argus</i> (G2)	0.22528	0.23363	0.23363	0.24311	0.06153	0.06376	0.05931	0.06391	0.00417	-							
<i>Bohadschia marmorata</i> (G2)	0.23738	0.21922	0.21922	0.23024	0.08640	0.08870	0.08410	0.08890	0.06430	0.06430	-						
<i>Bohadschia marmorata</i> (G3)	0.23738	0.21922	0.21922	0.23024	0.08640	0.08870	0.08410	0.08890	0.06430	0.06430	0.00000	-					
<i>Bohadschia marmorata</i> (G4)	0.24050	0.21922	0.21922	0.23024	0.08888	0.09119	0.08658	0.09140	0.06667	0.06667	0.00209	0.00209	-				
<i>Holohuria excellens</i> (G)	0.27096	0.26445	0.26769	0.24503	0.24344	0.24628	0.24628	0.24690	0.21934	0.22229	0.22846	0.22846	0.22846	-			
<i>Actinopyga mauritania</i> (G)	0.25236	0.22834	0.22834	0.22001	0.22300	0.22582	0.22019	0.22676	0.25070	0.24441	0.24744	0.24744	0.24744	0.27370	-		
<i>Actinopyga</i> sp. (G)	0.25385	0.27363	0.27029	0.25148	0.25646	0.25941	0.25941	0.26055	0.29939	0.29595	0.30204	0.30204	0.30551	0.27740	0.14596	-	
<i>Opionereis porrecta</i> (G)	0.48657	0.46709	0.46709	0.46637	0.45028	0.45433	0.45433	0.45302	0.44451	0.44451	0.43513	0.43513	0.43513	0.44422	0.47027	0.48665	-

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 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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