83 PHYLOGENY OF *HOLOTHURIA LEUCOSPILOTA* (ECHINODERMATA: HOLOTHUROIDEA) AS INFERRED FROM CYTOCHROME C OXIDASE I GENE SEQUENCES

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ABSTRACT

This study aims to determine phylogenetic relationship between *Holothuria leucospilota* (Echinodermata: Holothuroidea) from Malaysia and other *Holothuria* species using partial sequences of cytochrome c oxidase I (COI) mitochondrial DNA (mtDNA) gene. *Holothuria (Mertensiothuria) leucospilota* or locally known as *bat puntil* is currently the most abundant sea cucumber species in Malaysia. This coral reef species is not in danger of extinction like commercial *Stichopus* species such as *Stichopus horrens* that are locally known as *gamat*. Until recently, COI mtDNA DNA gene sequences of *H. leucospilota* are unavailable online in GenBank, National Center for Biotechnology Information (NCBI); thus leading to present phylogenetic study. In this study, specimens of *H. leucospilota* were collected from Intan Besar Island, Langkawi, Malaysia. Eight COI mtDNA gene sequences of *H. leucospilota* obtained from this study have been registered with GenBank (Accession no.: FJ223873 - FJ223880). Three main methods namely neighbour joining, maximum parsimony and maximum likelihood were used for phylogenetic tree reconstruction. Present results showed that *H. leucospilota* was genetically closer to *H. coluber* as compared to *H. atra*. Even so, further studies with more samples, broad geographical sampling and various mitochondrial DNA genes are necessary to obtain better information and verification on molecular phylogeny of *H. leucospilota* in particular and *Holothuria* species in general.

Keywords: Phylogenetic relationship; *Holothuria leucospilota*; partial sequences of cytochrome c oxidase I gene; phylogenetic trees.

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. In Malaysia, sea cucumbers from other than genus *Stichopus* such as from genus *Holothuria*, *Actinopyga*, *Pearsonothuria*, *Bohadschia*, *Thelenota* and order Molpadiida are commonly known as *bat*, *balat* and *timun laut* (Kamarul Rahim et al. 2009). *Stichopus* species, frequently used as the main raw material in traditional medicine (i.e. *gamat* oil and *gamat* water) especially in Peninsular Malaysia, are locally known as *gamat*. The same commercial name is used by Sabah and Sarawak residents. In Sabah, sea cucumbers inclusive of *gamat* are commercially marketed as food, and there are minor uses as fishing poison (e.g. holothurins from *Holothuria atra*) and in traditional medication. Approximately 155 tonnes of sea cucumbers were landed in 1995 as estimated by the Sabah Fisheries Department. However, the use of sea cucumbers in Sarawak is not well documented and the information on the species presence and distribution is sparse. It was reported by Ridzwan (1993) that Sarawak fishermen used *brunok* from Order Molpadiida as fishing bait. Basically, there are two main economic practices of sea cucumbers in Malaysia: an important source of traditional medicine as well as modern medicine in Peninsular Malaysia, and an important source in food processing industry in Sabah.

In terms of phylogenetic analyses, Randomly Amplified Polymorphisms of DNA (RAPD) markers were utilized by Norazila et al. (2000) to examine the genetic diversity between and among sea cucumber species from different localities in Malaysia. However, the approach failed to resolve the phylogenetic relationship even at the species level. The inconsistent banding patterns of RAPD might lead to such results. Therefore, DNA sequence analyses were used in this study as currently this technique is capable to give the best resolution as compared to isozyme (i.e. protein analysis) and RAPD. Furthermore, cytochrome c oxidase I (COI) mitochondrial DNA (mtDNA) gene sequences of *Holothuria (Mertensiothuria) leucospilota* (Brandt 1835) are unavailable online in GenBank, National Center for Biotechnology Information (NCBI) to date. Accordingly, partial DNA sequences of COI mtDNA gene were applied for phylogenetic analyses, whereby neighbor joining tree, maximum parsimony tree and maximum likelihood tree were generated to determine phylogenetic relationship between *H. leucospilota* (Echinodermata: Holothuroidea) from Malaysia and other *Holothuria* species represented by corresponding sequences from GenBank.

MATERIALS AND METHODS

Samples collection and identification

Sea cucumber specimens from Intan Besar Island, Langkawi, Kedah Darul Aman, Malaysia were collected during several sampling activities, and one of them was in July 2006. In laboratory, preservation of specimens was done either in 70% ethanol or in -20°C freezer. Identification of sea cucumbers morphologically or based on external characteristics was done by referring to the previous studies, supporting references and also through the information given by local residents.

DNA extraction

Total genomic DNA extraction was done using using DNeasy® QIAGEN blood and tissues kit. The total genomic DNA was extracted from muscle tissue. Quantification of extracted DNA was done using spectrophotometer and 1% (w/v) agarose gel electrophoresis.

DNA amplification

Approximately 550 base pairs (bp) section of the COI mtDNA region was amplified using standard polymerase chain reaction (PCR) procedures.

CO1 (forward) 5'- CCT GCA GGA GGA GGA GGA GAY CC -3'
CO1 (reverse) 5'- CCA GAG ATT AGA GGG AAT CAG TG -3'
(Palumbi et al. 1991)

The PCR reaction mixture was prepared in 1.5 ml microcentrifuge tube and then dispensed into 0.2 mL PCR tubes with the addition of 4 µL DNA template. Standard thermal cycle amplification was performed in 50 µL reaction volume containing 30.0 µL of sterilized 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 µM), 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.

DNA amplification was then performed in the Mastercycler (Eppendorf), which at first had been programmed according to the desired temperature cycling profile. The PCR cycle was then repeated for 29 cycles. Cycle parameters for PCR were 7 min at 96°C for initial denaturation, 1 min at 95°C for denaturation, 45 s at optimized temperature for annealing, 1 min 30 s at 72°C for extension, repetition of step 2-4 for another 29 cycles (total: 30 cycles) and 10 min at 72°C for final extension. Quantification of the PCR products was analyzed by 1% (w/v) agarose gel electrophoresis. Purification kits from manufacturer were used for direct purification.

DNA sequencing

The purified products were directly sent for sequencing using BigDye® Terminator v3.1 Sequencing Kit and analyzed 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 seconds, 50°C for 5 seconds and 60°C for 4 min at rapid.

Phylogenetic analysis

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. The reconstruction of neighbor joining tree, maximum parsimony tree and maximum likelihood tree was done by using PHYLIP version 3.6b (Felsenstein 2004) with 1000 sequence replications and 100 data sets. TreeView (Win32) version 1.6.6 by Page (1996) was used to display and edit the reconstructed phylogenetic trees.

RESULTS AND DISCUSSION

Eight COI mtDNA gene sequences of *H. leucospilota* obtained from present study have been registered with GenBank, National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine (GenBank accession no.: FJ223873 - FJ223880). 26 partial sequences of COI mtDNA gene were aligned consisting of 8 sequences of *H. leucospilota* from Malaysia, 17 sequences obtained from GenBank and one sequence of *Cucumaria piperata*, a sea cucumber, as outgroup (GenBank accession number: U32211; Figure 1-3).

For neighbour joining tree (Figure 1), the results showed the presence of two major groups. All specimens of *H. atra* formed a separate cluster with 82% bootstrap value while specimens of *H. coluber*, *H. leucospilota* and an unknown sea cucumber species (GenBank accession no.: EU220818) formed another cluster with low bootstrap value of 37%. The latter cluster showed that the unknown species was basal to subgroups of *H. coluber* and *H. leucospilota*. Besides, *H. coluber* and *H. leucospilota* clustered together exhibiting their close genetic relationship. All specimens of *H. leucospilota* clustered together with 100% bootstrap value.

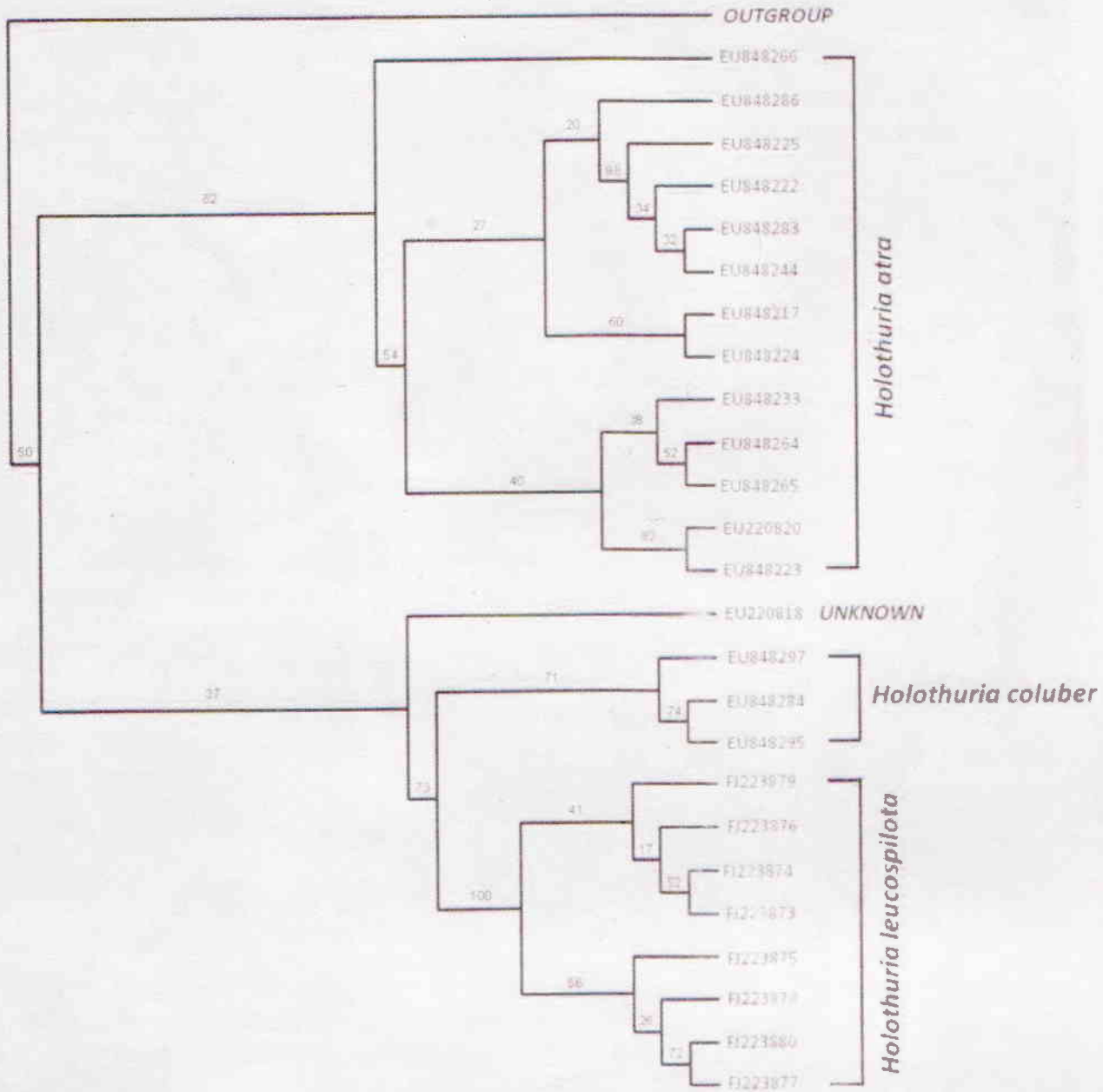

Figure 1 Topology of neighbor joining tree (consensus tree) of *Holothuria* species inferred from cytochrome c oxidase I (COI) mtDNA gene using PHYLIP version 3.6b (Felsenstein 2004). Accession number of EU refers to sequences obtained from GenBank. The tree is rooted with a sequence of *Cucumaria piperata*, 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 (%).

Likewise neighbour joining tree, all specimens of *H. leucospilota* in maximum parsimony tree (Figure 2) clustered together with strong boostrap support of 100%. Clustering of all specimens of *H. atra* also presented 100% bootstrap value showing their origins from single species. The unknown species, *H. leucospilota* and *H. coluber* formed a cluster but unlike neighbour joining tree, maximum parsimony tree indicated that *H. coluber* was basal to *H. leucospilota* and the unknown species. The maximum parsimony tree also suggested that *H. leucospilota* was closer to *H. coluber* as compared to *H. atra*.

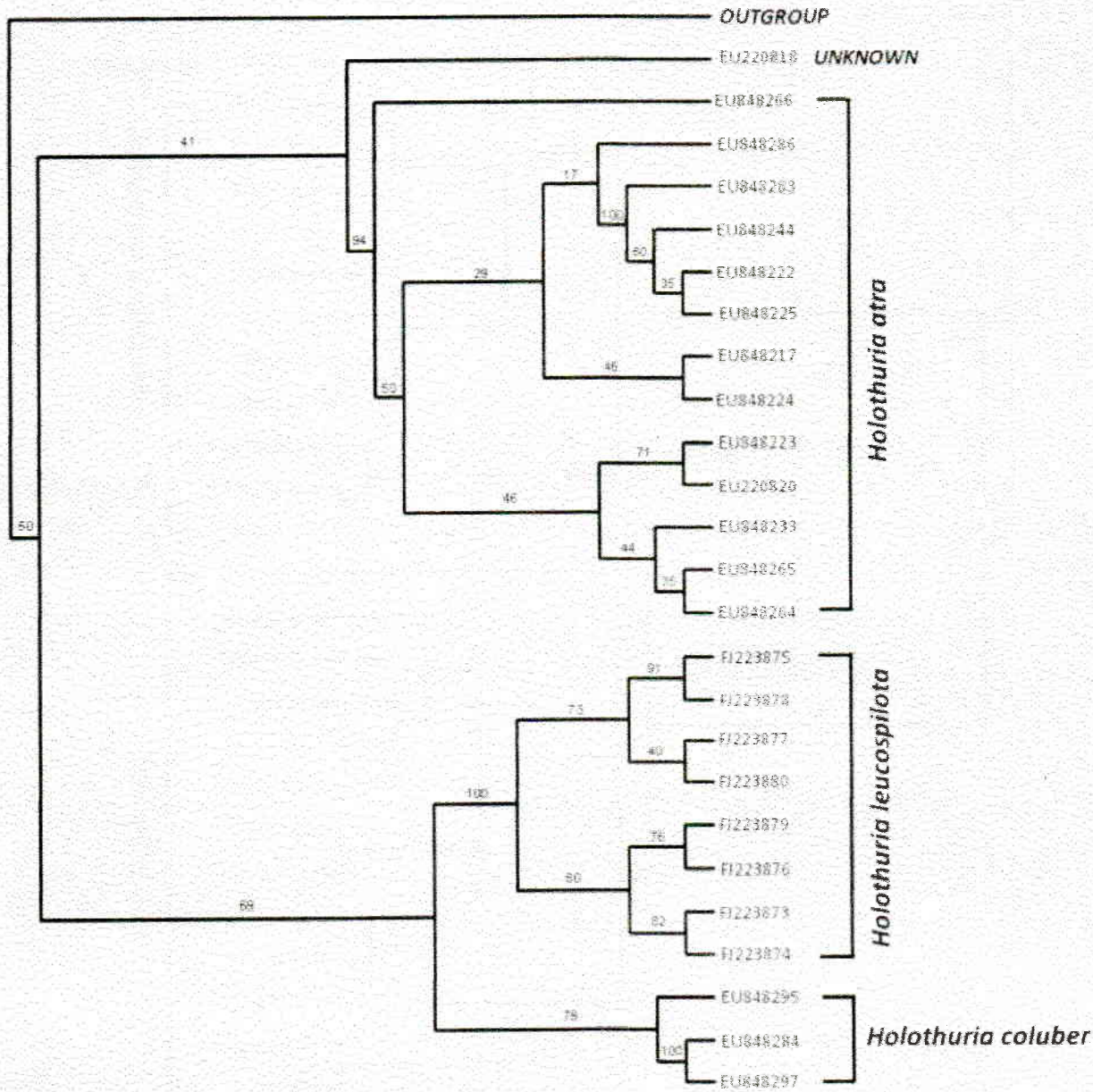

FIGURE 3. Topology of maximum likelihood tree (consensus tree) of *Holothuria* species inferred from cytochrome c oxidase I (COI) mtDNA gene using PHYLIP version 3.6b (Felsenstein 2004). Accession number of EU refers to sequences obtained from GenBank. The tree is rooted with a sequence of *Cucumaria 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 (%).

In general, present results suggested that *H. leucospilota* was genetically closer to *H. coluber* as compared to *H. atra*. The taxonomic status of the unknown sea cucumber species was unclear 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 molecular phylogeny particularly in genus *Holothuria*.

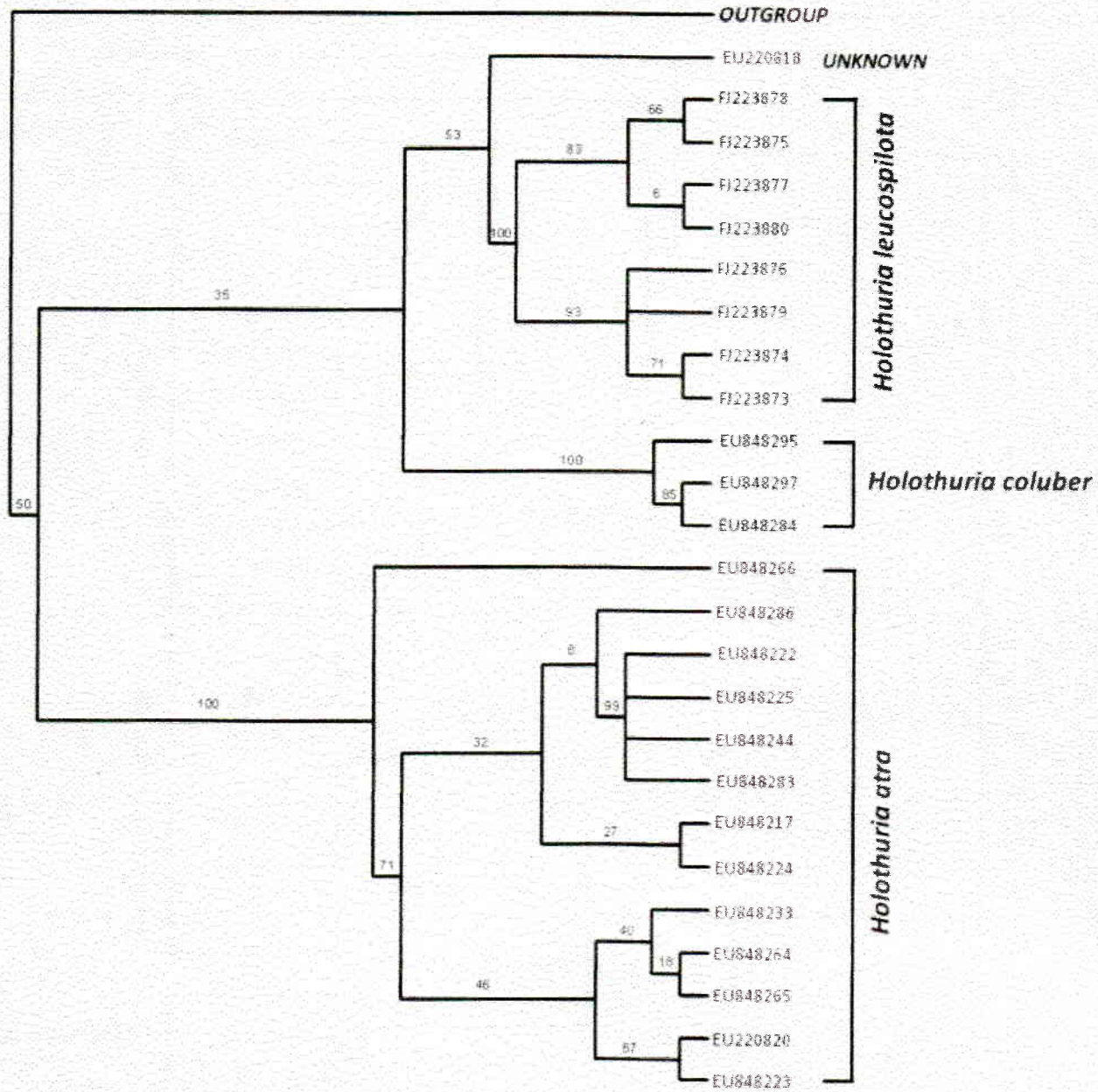

Figure 2 Topology of maximum parsimony tree (consensus tree) of *Holothuria* species inferred from cytochrome c oxidase I (COI) mtDNA gene using PHYLIP version 3.6b (Felsenstein 2004). Accession number of EU refers to sequences obtained from GenBank. The tree is rooted with a sequence of *Cucumaria piperata*, 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 (%).

Even though maximum likelihood tree (Figure 3) supported that the genetic relationship between *H. leucospilota* and *H. coluber* was closer, the phylogenetic tree indicated that the unknown species clustered together with *H. atra* with 41% bootstrap value. Statistically, such low bootstrap value suggested that the clustering position for the unknown species was likely changeable, as previously indicated by neighbour joining tree and maximum parsimony tree. Furthermore, the bootstrap support for *H. leucospilota* cluster was still robust with 100% value.

CONCLUSION AND RECOMMENDATIONS

More studies on *H. leucospilota* in Malaysia are needed to fully resolve the taxonomic status as well as the phylogeography. This will require the use of other molecular characters, especially nuclear gene that may provide resolution at higher level. Among other mtDNA genes available that can be used for further studies are 12S, 16S, and cytochrome *b* (cyt-*b*). Future studies with broad geographical sampling for *H. leucospilota* (e.g. inclusion of Sabah and Sarawak regions) may help to provide better view on molecular phylogeny of *H. leucospilota* in particular and *Holothuria* species in general.

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