DNA BARCODING, PHYLOGENETIC AND SYSTEMATICS OF HOLOTHUROIDEA (SEA CUCUMBERS) IN MALAYSIAN WATERS BASED ON 16S rRNA AND CYTOCHROME OXIDASE I (COI) GENES

by

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bootstrap replication. The tree was rooted with *Mespilia globulus* (AF279178).

LIST OF ABBREVIATIONS

bp	basepair
DNA	Deoxyriboncleic acid
dNTP	Dinucleotide triphosphate
EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium bromide
HCl	Hydrochloric acid
k	Number of groups
kb	kilobase
M	Molar
NaCl	Sodium chloride
SDS	Sodium dodecyl sulphate
TBE	Tris-borate-EDTA
TNES-Urea	Tris-sodium chloride-EDTA-SDS-Urea
UV	Ultra violet

DNA BARKOD, FILOGENETIK DAN SISTEMATIK HOLOTHUROIDEA (TIMUN LAUT) DI PERAIRAN MALAYSIA BERDASARKAN PADA GEN 16S rRNA DAN GEN CYTOCHROME OXIDASE I (COI)

ABSTRAK

Kajian morfologi dan sistematik molekul telah dijalankan ke atas famili Holothuriidae, Stichopodidae, dan juga Caudinidae. Ianya boleh dijumpai di perairan Malaysia. Ini adalah dokumentasi pertama mengenai hubungan filogenetik dan sistematik barkod di antara famili holothuria dan genus yang menghubungkan klasifikasi tradisional dan teknik molekular. Spesies yang dikaji dibahagikan kepada tiga famili iaitu Famili Stichopodidae, spesies yang terlibat adalah adalah *Stichopus hermanni, S. ocellatus, Stichopus* sp.*, S. chloronotus, S. monotuberculatus* dan *S. rubermaculosus* manakala bagi Famili Holothuriidae, famili ini dibahagikan kepada dua genera iaitu genus *Holothuria* dan *Bohadschia*. *Holothuria* yang terlibat adalah *H. atra*, *H. edulis, H. coluber, H. hilla, H. leucospilota*, *H. flavomaculata,* dan *Pearsonathuria graffei* manakala bagi spesies yang terlibat dalam genus *Bohadschia* adalah *B. argus, B. mormorata* dan *B. bivittata.* Famili yang terakhir sekali, adalah Famili Caudinidae dan genusnya adalah *Acaudina.* Sampel tersebut adalah *Acaudina molpadiodes.* Sejumlah 53 sampel mengandungi 17 spesies Holothuroidea telah disampel dan diukur untuk mendapatkan data morfometrik. Ukuran morfometrik diperoleh menggunakan kekunci taksonomi klasik dan perbandingan menggunakan foto sebagai sokongan kepada taksonomi molekular. Keseluruhannya, empat ciri morfologi khusus telah diambil kira dan dipertimbangkan. Ciri-ciri tersebut adalah keratan lintang tubuh, warna tubuh, bentuk tentakel serta kehadiran kaki tiub. Seterusnya, keputusan diperkukuhkan dengan jujukan DNA dari gen 16S rRNA (550bp) dan gen COI (620bp) dari 43 wakil sampel dari spesies Holothuroidea untuk mengkaji hubungan filogenetik. Data jujukan DNA disusun menggunakan perisian Klustal W sebelum analisis filogenetik menggunakan MEGA 4 dijalankan. Filogeni di antara taksa dijalankan melalui kaedah `Neighbour Joining` (NJ), `Maximum Likelihood` (ML) dan `Maximum Parsimony` (MP). Pokok NJ yang diperoleh menunjukkan topologi yang sama seperti yang terbentuk pada analisis ML dan MP kecuali sedikit variasi pada nilai bootstrap dan pembahagian spesies di dalam kumpulan taxa. Dua gugusan utama terbentuk. Gugusan pertama mengandungi Stichopodidae dan Caudinidae dan satu gugusan lagi mengandungi Famili Holothuriidae. Setiap spesies dan genus telah disahkan dengan kaedah morfologi tradisional dari himpunan mereka sendiri. Kajian yang lebih terperinci berdasarkan ciri-ciri morfologi dan kajian populasi telah dicadangkan untuk menangani masalah taksonomi kepada kumpulan ini. Dua teknik iaitu analisis klasikal dan molekular telah menunjukkan kefahaman yang lebih baik serta dapat menyelesaikan beberapa kontroversi yang dikaitkan terutamanya pada keluarga Stichopodidae yang sangat rumit dan kompleks.

DNA BARCODING, PHYLOGENETIC AND SYSTEMATICS OF HOLOTHUROIDEA (SEA CUCUMBERS) IN MALAYSIAN WATERS BASED ON 16S rRNA AND CYTOCHROME OXIDASE I (COI) GENES

ABSTRACT

A morphological and molecular systematic study was conducted on family Holothuriidae, Stichopodidae, and also Caudinidae. They can be found in Malaysian waters. This is the first documentation of the Phylogenetic relationship and Barcoding systemic among Holothurians family and genera that integrates traditional classification and molecular techniques. The species investigated were divided into three families which is Family Stichopodidae, Holothuriidae and Family Caudinidae. In Family Stichopodidae, the species involved were *Stichopus hermanni, S. ocellatus, Stichopus* sp., *S. chloronotus, S. monotuberculatus* and *S. rubermaculosus* while for Family Holothuriidae, the family was divided into two genera which is genus *Holothuria* and *Bohadschia.* The Holothurian involved was *Holothuria atra, H. edulis, H. coluber, H. hilla, H. leucospilota, H. flavomaculata* and *Pearsonathuria graffei* while for the second genus was the genus *Bohadschia* which is *B. argus, B. mormorata* and *B. bivittata.* The last but no least family was the Family Caudinidae and the genus was Acaudina. The sample was *Acaudina molpadiodes.* A total of 53 samples consisting of 17 Holothuroidea species were sampled and measured to obtain the morphometric data. Brief morphometric measurement was done by classical taxonomy keys and comparison by photos as a support for molecular taxonomy. Overall, four characteristics were taken into consideration. They are the body cross section, body colouration, shape of the tentacles and also present or absent of tube feet. The results were further supported by sequencing of partial 16S rRNA (550bp) and COI gene (620bp) from 43 representative samples of Holothurian species to investigate the phylogenetic relationships. DNA sequence data were aligned by Clustal W software before phylogenetic analysis using MEGA 4 was conducted. Phylogeny among taxa was conducted through Neighbour Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP) methods. The NJ trees showed similar topology as in the ML and MP analysis except for slight variations in bootstrap values and species forming the sister taxon to the ingroup taxa. Two main clusters were formed. The first cluster comprising of Stichopodidae and Caudinidae and the other cluster comprising of the family Holothuriidae. Each species and genus was defined by traditional morphology from their own body characteristics. More detailed studies of the morphological characteristics and population studies is suggested to address the problematic taxonomy of this sea cucumber group by merging two techniques i.e classical and molecular analysis were shown to provide better understanding as well as resolving several of the arguments associated expecially on Stichopodidae family which was very complicated and complex.

CHAPTER 1

INTRODUCTION

1.1 General introduction

The Phylum Echinodermata (derived from the Greek language meaning *spiny skin*) is a group of marine animals which are widely distributed throughout the world's oceans (Brusca and Brusca, 2003). The class Holothuriidea in which sea cucumber is classified into, is one of the five existent classes in Echinoderms which include asteroids (starfish), ophiuroids (brittle stars), echinoids (sea urchins and sand dollars), holothuroids (sea cucumbers) and as well as crinoids. Within this holothuroidea (sea cucumbers), there are namely Aspidochirotida, Apodida, Daktilokirotida, Dendrokirotida, Elasipodida and Molpadida orders. Currently, there are about 1500 species of sea cucumbers recorded globally (Massin and Lane, 1991) with approximately 30 species recorded in Malaysia (Kamarul et al., 2009).

Research on the taxonomy, biology and ecology of commercially important sea cucumber species is crucial in strategizing sea cucumber stock management (Choo, 2004). Sea cucumbers are distinguished into the various taxonomic orders on the basis of gross morphological characteristics such as the morphology of the tentacles, the presence or absence of tentacular retractor muscles, the presence and absence of an internal respiratory tree and the distribution of podia on the trunk (Rowe and Doty, 1977; Kerr, 2001; Kerr and Kim, 2001).

At the familial level, the taxonomic distinctions are dependent on a finer level of characteristics such as the morphology of dermal ossicles, the form of the esophageal calcareous ring, distribution and the morphology of the tube feet (Kerr, 2001; Kerr and Kim, 2001). However, identification to species level poses a challenging task when based on the classical approach due to close similarities in the morphology of different species (Massin, 2007; Byrne et al., 2010).

Sea cucumbers or collectively known as gamat to the locals are one of the highly demanded sea product in Malaysia. This is because they are consumed as food and also used in many traditional pharmaceutical products by local people (Choo, 2004). For example, ‘gamat’ oil production is very popular in Langkawi, Kedah, Malaysia. This has resulted in uncontrolled harvesting of many gamat populations (Baine, 2005). Unfortunately, the precise taxonomy of the captured species is generally unknown with no record of its distribution. Such knowledge on the biodiversity and taxonomy of sea cucumber is crucial for stock management and regulation of a conservation programme of this important group (Baine and Sze, 1999; Frieddman et al., 2011) in Malaysia.

The diversity and distribution of sea cucumbers in Malaysia have been documented by Forbes et al. (1999) and more recently by Choo (2004), Zulfigar et al. (2008) and Kamarul et al. (2009), mainly focusing on the Peninsular Malaysia. These studies have been based on traditional external morphological characters which are often variable and plastic and greatly influenced by the environment. Thus, taxonomy of many species complexes remains unresolved

(Massin and Lane, 1991). Among them are the most dominant members of sea cucumbers in Malaysian waters belonging to the genera *Acaudina, Holothuria* and *Stichopus.* These groups form an important part of a multi-species invertebrate fishery in this region (Bruckner et al., 2003; Conand, 2004).

The difficulties in precise taxonomy of sea cucumbers by classical taxonomical methods, is well recognized (Conan, 2004; Binder et al., 2012). In this regard molecular methods provide a complementary tool to support morphological data. Molecular tools are important because DNA is stable throughout life stages and can reveal the evolutionary history and relationships among the studied subjects as well as to clarify taxonomic uncertainties of ambiguous species. DNA marker technologies are widely used especially in aquaculture field and conservation (Liu and Cordes, 2004; Yan et al., 2005; Ferguson et al., 2006, Sigang et al., 2011).

The mitochondrial DNA (mtDNA) has been used widely in molecular taxonomic study as reviewed by Ferris and Berg (1987); Lansman et al. (1981); Curole and Kocher (1999) and Hurst and Jiggins (2005). These mitochondrial genes can be amplified *in vitro* by polymerase chain reaction (PCR) and provide an unusually sensitive method to explore the differences between populations and higher taxonomic levels. This development of DNA sequencing technology has provided a powerful tool for elucidating evolutionary relationships among species as stated by Billington and Herbert (1991) and Hillis et al. (1996). Thus, advances in molecular biology provide potentially efficient tools in understanding the biology and the relationships among this fascinating group of sea cucumbers in Malaysian waters.

In this study, the mitochondrial 16S rRNA marker was selected because of its several advantageous characteristics in resolving higher level taxonomic and systematic relationships: slow evolving, conserved, and with large copy numbers compared to the nuclear DNA (Sigang et al., 2011). It is therefore useful in identification of species that are difficult to separate based on their morphology especially of cryptic species (Curole and Kocher, 1999; Liu and Cordes, 2004).

However, the application of various genetic markers in tandem has become more popular since 1980`s (Wang and Szmidt, 2001). Thus, in the last few years, the DNA barcoding approach based on the cytochrome oxidase subunit I (COI) gene is widely used to complement other molecular markers in identifying and resolving phylogenetic, phylogeography and population genetics of various organisms (Uthicke et al., 2010; Suzana et al., 2011; Tun et al., 2012). This technique was introduced due to the ability of the mitochondrial COI gene to resolve complex identification of species (Hebert et al., 2003a).

Based on the present knowledge of this group of organisms, the objectives of the present study were to:

1. assess the species diversity and distribution of sea cucumber in Malaysian water based on morphological characteristics.
2. investigate the taxonomic and systematic status of the Holothuroidea (sea cucumbers) in Malaysian waters utilizing mitochondrial 16S rRNA gene.

3. elucidate species-level identification using the barcoding gene, mitochondrial cytochrome oxidase I (COI) and provide biodiversity assessment.

CHAPTER II

LITERATURE REVIEW

2.1 Echinoderms: Holothuroidea (sea cucumbers)

The sea cucumber, Class Holothuroidea, is one of the five extant classes in echinoderms. They are distributed in six (6) orders and 25 families (Lawrence, 1897). The sea cucumbers (Class Holothuroidea) are an abundant group of worm-like and usually soft-bodied echinoderm. Holothuroidea are globally distributed in almost all depths, latitudes and environments in the ocean. They are found in nearly every marine environment, but are most diverse in tropical shallow-water coral reefs. They range from the intertidal, where they may be exposed briefly at low tide, to the floor of the deepest oceanic trenches (Gilliland, 1993).

Sea cucumber orders are distinguished on the basis of gross morphological characteristics such as the morphology of the tentacles, the presence or absence of tentacular retractor muscles, the presence and absence of an internal respiratory tree and the distribution of podia on the trunk. At familial level the taxonomic distinctions are dependent on a finer level of characteristics such as the morphology of dermal ossicles, the form of the esophageal calcareous ring, distribution and the morphology of the tube feet (Clark and Rowe, 1971). Identification to species poses a challenging task due to close similarities in the morphology of different species (Massin, 2007; Kamarul et al., 2009; Byrne et al., 2010). Several studies (Bruckner et al., 2003; Carpenter and Springer, 2005; Kamarul et al., 2009), have also documented that Malaysia, which lies in the Coral Triangle of a rich apogee of marine biodiversity also harbours a rich diversity of sea cucumber species. Among them are the most dominant members of sea cucumbers in Malaysian waters belonging to the genera *Bohadschia, Holothuria* and *Stichopus* (order Aspidochirotida) and *Acaudina* (order Molpadida) which form an important part of a multi-species invertebrate fishery in this region. Kamarul and Ridzwan (2005) revealed problematic taxonomic status of sea cucumbers in Malaysia, based on the documentation of a large number of undetermined species. Other studies of diversity and distribution of sea cucumbers in Malaysia have included those by Massin and Lane (1991), Forbes et al. (1999), Choo (2004), Zulfigar et al. (2008) and Kamarul et al. (2009), which focused on the Peninsular Malaysia. These studies identified sea cucumbers based on general outer morphology in compliance with another study based on spicule, thus, leaving a number of unresolved species complexes.

In this study, the classification of the Holothuroidea studied was listed as below (Table 2.1) to differenciate from the phylum, class, order, family and genus of the sea cucumber. The classification was referring to the hierarchy in Indo-West Pacific by Clark and Rowe (1971) and Arnold and Birtles (1989).

Table 2.1: Classification of the studied Holothuroidea hierarchy in the Indo-West Pacific, based on Clark and Rowe (1971) and Arnold and Birtles (1989).

Phylum	Echinodermata
Class	Holothuroidea
Order	Aspidochirotida Molpadida
Family	Stichopodidae Holothuriidae Caudinidae
Genus	*Stichopus* *Holothuria* *Bohadschia* *Acaudina*

To date, there are about 1500 presumed species of sea cucumber that have been recorded in the world’s oceans (Massin and Lane, 1991) with approximately 50 documented species registered with GeneBank, National Center for Biotechnology Information (NCBI), U.S (Kamarul et al., 2010). Currently in Malaysia, there are three orders in total that can be found. They are Aspidochirotida, Molpadida and Apodida (results not amplified) with seven genera and about 50 species distributed among the orders (Kamarul et al., 2009). Several have been known to have high nutritional and pharmacological values especially species that are found in Malaysia (Hawa et al., 2004).

Understanding the taxonomy and systematics of this group and a firmly established phylogeny is required for the conservation and management of this important group. This is especially important because of the rapidly decreasing population and population sizes, a consequence of over-harvesting and extensive destruction of its natural habitat to make way for tourism or for industrial purposes. Currently, supply of sea cucumber is mainly from the wild. Unfortunately, many studies have revealed that populations in Malaysia are depleting at an alarming rate (Sim et al., 2008; Woo et al., 2010). Thus, without ensuring sufficient management and conservation programme of the wild and replenishment through restocking from wild or cultured stocks, it could eventually lead to their extinction (Choo, 2004). Such efforts require data on precise identification of the group as a whole and genetic variability assessment of target species.

2.2 Geographical distribution and population dynamics of sea cucumber in Malaysia

Malaysia has some of the richest sea cucumber microhabitats in the world (Ridzwan, 1993). The tropical coral reefs in Malaysia have a broad and wide range of flora and faunal microhabitats in Western Pacific suitable to support the high diversity of sea cucumber species and populations (Kinch et al., 2008). However, the distribution of sea cucumber species has decreased in recent times

(Sim et al., 2008; Woo et al., 2010) in the surrounding seas of Malaysia as compared to previous records (Zulfigar and Tan, 1990; Lane et al., 2000). This is due to several factors including overfishing and destructive harvesting such as fish bombing activities around the coral reef and previously undisturbed area of sea cucumber habitats. The distribution of sea cucumber species is also affected by coastal processes such as sediment granulometry, the area`s climatic, geological past, ecological constrain and ocean acidification (Hoeksema, 2007). According to Massin and Doumen (1986), the hydrodynamics influence of sediment is one of the key to defining the niches and larval dispersion of sea cucumbers, while study by Hoeksema (2007), stated that oceanic currents also contribute to the dispersal of sea cucumber larvae and diversity.

2.3 Sea cucumber fisheries

Sea cucumbers stocks are now under intense fishing pressure throughout the world. This has been reviewed by FAO reports (Choo, 2004) of overfishing practices of sea cucumber occurring in the majority of the countries lying between the African and the Indian Oceans. Likewise in the Asian Pacific regions, the most sought-after species such as *Stichopus* family sea cucumber, are largely depleted. Sea cucumber fisheries in Peninsular Malaysia are on a very small scale and limited to only a few locations compared to East Malaysia. In Peninsular Malaysia, a few locations that are involved in sea cucumber harvesting include Perak, Kedah and Terengganu while in East Malaysia, Sabah is the main location for harvesting. The scale of harvesting sea cucumbers in Sabah (139 tonnes), is higher compared to other areas in Malaysia but in comparison to other countries such as China (5500 tonnes), it is relatively low due to its mostly artisanal fishing methods (Choo, 2008). Nevertheless, populations are seen to be decreasing.

In an effort to protect fisheries and diversity, the Malaysian government has gazette several islands as marine parks or Marine Protected Areas (MPA) for *in situ* conservation and management for underwater creatures. By definition, a marine park is a sea-zoned area of two nautical miles from the shore at the lowest sea level of low tide. These parks are managed by strict and specific rules and regulations for protection and conservation of various habitats and aquatic marine life. In Peninsular Malaysia, there are four main islands that are managed by the federal government and gazetted as marine parks. These are located in the Straits of Malacca and South China Sea (George, 2012). The Pulau Payar MPA is located in the Straits of Malacca, while three clusters of Marine Protected Area (MPA) namely Pulau Redang, Pulau Tioman and Johor group of islands are located in the South China Sea. In Sabah and Sarawak, the marine parks are under the jurisdiction of the state marine park departments or relevant authorities. In Sabah all protected islands are gazetted under Sabah Parks while in Sawarak all the islands are gazetted under Sarawak National Park and Reserve Avoidance (George, 2012).

Sea cucumber management plans are specific to local circumstances and need to be developed comprehensively (Choo, 2004). This should include a measurement and establishment of catch quotas and minimum size limits, closures during breeding seasons and monitoring of the status of stock available. According to Harborne et al. (2000), because of lower fishing pressure on the East Coast of Peninsular Malaysia, edible species of sea cucumbers can still be found in abundance for example at the Redang Island, Tioman Island and Tinggi Island.

In view of the strict regulations on gazetted MPAs, sea cucumber fisheries are concentrated on ungazzetted islands. According to Mark and Choo (1999), in the Straits of Malacca, fishing activities are mainly focused in the Langkawi group of islands in Kedah, Penang group of islands in Penang, Sembilan and Pangkor groups of islands in Perak. In Pulau Pangkor, there is a small commercial fishery set up, mainly for production of traditional medicinal products from the species *Stichopus horrens* and *Stichopus hermanni* (Mark and Choo, 1999; Choo, 2004). This particular practice has been sustainable because *Stichopus* family owned and specimens are only collected at low tide for approximately five days a month, with each catch reaching up to a total of two gallon-drums per day (Baine and Choo, 1999).

Another important site for sea cucumber fisheries can be found is Pulau Langkawi. The target species in Pulau Langkawi are also from the genus *Stichopus*. The fishery on Pulau Langkawi dates back to the mid 1900`s but markedly disappeared in the early 1940`s when the target species *Stichopus hermanni* and *S. horrens* were fished almost to depletion (Forbes and Ilias, 1999; Choo, 2004). At present, most of the raw supplies to be processed to become consumable product come from Pulau Adang, Thailand (Kamarul et al., 2009).

As compared to Peninsular Malaysia, sea cucumber fisheries in Sabah are larger and more important, not due to the fact that it contributes to foreign exchange but rather because it provides a livelihood, supplement incomes in poor coastal communities and also food for the indigenous people in the coastal area. Sea cucumbers are collected from the shallow reefs by hand picking, snorkelling as well as free diving. Though the fishing methods used seem harmless to the population of sea cucumbers, the intensity of collection as well as number of fishers was staggering. The Annual Fisheries Statistics of Sabah in the year 2000 registered only sea cucumber collectors but the actual number was believed to be at least five to six times higher (Choo, 2004).

The landing tonnage of sea cucumbers captured in the Annual Fisheries Statistics, Sabah, indicated that more than 90 % of sea cucumber catches were landed from trawlers in year 2005 (Choo, 2008). This number did not really reflect the actual amount of sea cucumber landings because those harvested by hand-picking are difficult to capture in the official fisheries statistics as artisanal fishing of these products are not required to report to the authority. A survey conducted by Forbes and Ilias (1999) in Sabah, from July 1996 to December 1999 revealed that high value species such as *Holothuria scabra* were already rare. This result in increased pressure on middle to low value species including several species from the family Stichopodiidae namely *Thelenota ananas* and other *Stichopus* spp. Fresh sea cucumber is processed to become beche-de-mer or ‘curry fish’ in the form of chilled, fresh or frozen before exported to target countries which include China, Hong Kong and Singapore. China remains to be the main major market and consuming country for sea cucumber in the world amounting to about 5500 tonnes in year 2001 (Ferdouse, 2004).

2.4 DNA markers in taxonomic and genetic variability assessments

DNA markers have become widely used in order to acquire details about gene flow, allele frequency as well as other parameters that are crucial in population biology (Neigel, 1997). Methods for DNA cloning, sequencing as well as hybridization developed in the 1970`s and DNA amplification and programmed sequencing throughout the 1980`s led to the growth and development of a variety of classes of DNA markers. Okumus and Çiftci (2003) defined molecular markers as a DNA sequence utilized to “mark” or possibly track a selected site (locus) on a specific chromosome (marker gene). It is in fact a gene with an acknowledged specific location or clear phenotypic expression which is usually determined by analytical methods as well as a recognizable DNA sequence. This marker nevertheless has to be conveniently identifiable in the phenotype, such as by controlling an easily observable feature or by being quickly detectable by molecular means (Zaid et al., 1999; Williamson, 2001).

The advent of the Polymerase Chain Reaction (PCR) technique had given further impetus to the utilization of genetic markers in taxonomic and population studies. This process permits manifold amplification of small amounts of DNA taken from various tissues; scales or fin clips of fish, respiratory tree of an Echinodermata, a sample of blood, hair from humans etc, most of which are non-invasive to the organisms to obtain. With each cycle of copying, the quantity of the target allele is doubled resulting in an exponential amplification of the gene in the PCR and therefore ease of analysis.

2.5 Importance of molecular approaches in sea cucumber taxonomy

In general the morphological differences characterising species have been efficient in discriminating among species as well as in addressing the evolutionary morphological changes in the sea cucumber group especially between their families (Conan, 2008). However, there are several limitations. As an example, the classical methods using spicules (Arndt et al., 1986) has been shown to be insufficient for explicit taxonomy. Thus, despite its extensive taxonomic history (Pawson and Fell, 1965; Clark and Rowe, 1971; Rowe and Doty, 1977; Miller and Pawson, 1984; Arnold and Britles, 1989), the systematics of the sea cucumbers based on morphology is still incomplete. Many researchers have now turn to molecular method as a complementary tool to resolve these ambiguities (Kumar and Filipski, 2001; Lawrence et al., 2004; Kamarul and Ridzwan, 2005; He et al., 2008). It is clear from previous studies and concordant with this study that there is a large gap between the morphological and molecular phylogenies.

Molecular studies have now become very important because DNA sequences have permitted the evolutionary, histories, changes and relationships of organisms at various taxonomic levels to be elucidated. Molecular phylogenetic studies could reveal presence of cryptic or even sibling species as well as occurrence of speciation over time (Katrina et al., 2005; Atif et al., 2008; Borrero-Perez et al., 2010; Wen et al., 2010; Uthicke et al., 2010). However, resolving phylogenetics relationships based on only a single gene could be very limiting. Thus, the utilization of increased number of markers should provide more significant resolving power as was conducted in this study.

2.6 Mitochondrial DNA

Precise recognition of a species is crucial to prevent incorrect information and database error. In certain scenarios, accurate identification may be problematical to establish because of overlapping morphological characteristics among closely related species or plasticity as a result of different environments. Traditionally, authenticity has been reliant on certain sturdy phenotypic traits but due to afore mentioned factors DNA profiling analysis for example through gene sequencing technique is required (Morozova, 2009). This approach can be utilized across a broad range of organisms.

The mitochondrion is a large power generating cellular organelle which includes its own small, circular DNA molecule. This genetic material is termed mitochondrial DNA or mtDNA. This genetic material is presumed to have evolved independently from nuclear DNA. Two to ten copies of mtDNA are present in every mitochondrial cell and this mtDNA is assumed and believe to occur from the circular genomes of bacteria which have been consumed by the early predecessor of eukaryotic cells (Wiesner et al., 1992).

Mitochondrial DNA is amenable to various taxonomic and population genetic investigations. It is a lot more sensitive towards genetic modifications as compared to nuclear DNA, and also has a high mutation rate (Brown et al., 1979), high copy number, has limited or does not undergo recombination. According to Elson et al. (1983) mtDNA evolves more rapidly compared to nuclear DNA (Brown et al., 1982), at approximately 5-10 times higher than nuclear DNA, presumablydue to its inefficient replication repair mechanism (Clayton, 1984). As mitochondrial DNA is maternally inherited in the majority of species (Gyllesten et al., 1991; Zouros et al., 1992), each and every molecule will possess a single genealogical background via maternal lineages and thus evolutionary history can be easily investigated. Such advantages make it ideal for studying evolution, migration patterns, diseases, species identification, forensics as well as maternal lineage correlation. In addition, phylogenetic relationships among closely related taxa can also be addressed by the utilization of mtDNA (Moritz et al., 1987). Although there is some uncertainty of mitochondrial evolutionary neutrality, sufficient diagnosed synapomorphies still permit the verification of monophyletic clades. Knowledge of mtDNA neutrality is crucial for analyses with regards to genetic range approximations and also a molecular clock (Avise et al., 1987).

Whether or not mtDNA is strictly neutral, it is nevertheless a sensitive indicator of population level process. Investigation of mtDNA variation is useful to explain geographic clusters of affiliated molecules (individuals) and maternal relationships within populations. This can also be applied to track down historical events such as bottlenecks, as well as to explore hybrid regions. MtDNA is furthermore very useful in addressing phylogenetic relationships between closely related taxa (Moritz et al., 1987).

Research on the biology and ecology of commercially important sea cucumber species have traditionally been considered as the main factors in strategizing management of sea cucumber stocks (Choo, 2004). But in recent years, the importance of genetics information for precise taxonomy and genetic variability assessment have also been recognized as necessary data for this same purpose. Sea cucumber orders are distinguished on the basis of gross morphological characteristics such as the morphology of the tentacles, the presence or absence of tentacular retractor muscles, the presence and absence of an internal respiratory tree and the distribution of podia on the trunk. At familial level, the taxonomic distinctions are dependent on a finer level of characteristics such as the morphology of dermal ossicles, the form of the esophageal calcareous ring, distribution and the morphology of the tube feet (Clark and Rowe, 1971; Arnold and Birtles, 1989).

However, identification to species level poses a more challenging task due to close similarities in the morphology of different species. Although the studies of diversity and distribution of sea cucumber in Malaysia have been previously documented (Forbes et al., 1999; Zulfigar et al., 2008; Kamarul et al., 2009), most were focused on Peninsular Malaysia. Identification have been based on outer morphology and several taxonomic status of species have remained unresolved. These include members of the most dominant groups of sea cucumber in our Malaysian waters from the genus *Holothuria* and *Stichopus* which are important resources of a multi-species invertebrate fishery in this region.

Phylogenetic studies of mitochondrial 16S rRNA gene have demonstrated its ability to elucidate the evolutionary relationships among species and higher order due to its slow and gradual evolution (Katrina et al., 2005). Presently, there is uncertain and also problematic identification of sea cucumber species along with the out of date and incomplete documentation on the species existence and distribution in Malaysia (Atif et al., 2008). Although numerous studies on the diversity and distribution of sea cucumber have been conducted in Malaysian waters, there is nevertheless a large amount of ambiguities on the taxonomic status and phylogenetic relationships of sea cucumber. Hence, this research was conducted to address this problem.

2.7 Molecular taxonomy and the DNA barcoding approach

Dealing with species limitations between closely connected species is notoriously challenging when they cannot be exclusively diagnosed according to specific characteristics. This occurs when the morphological variations between the species is very subtle, complicated to explain and also appropriate only to certain life history stages or gender. Therefore, DNA sequences offer an obvious and thus extremely rich source of additional data. For the last few decades, DNA sequence has played an important role in systematics studies to deal with difficult taxa (Frost et al., 1998). Thus, DNA sequences have been commonly used to provide the additional information which is required to decide on species boundaries or even the status of the organism as a species or sub species (Gray, 1992; Matthews et al., 2002; Mayden, 2002; McCafferty et al., 2002).

The taxonomic identification of species utilizing an approximately 640 base pair of the mitochondrial cytochrome oxidase subunit I (COI) gene or commonly referred to as the DNA barcode has gained broad popularity since it was first introduced in 2003 (Hebert et al., 2003a; 2003b). Vaneechoutte et al. (1995) was the first to recommend the utilization of DNA taxonomy for species identification. Its capability to effectively discriminate among species across the animal (Hebert et al., 2003a), plant (Chase et al., 2005), and fungi (Seena et al., 2010) kingdoms, stem from its higher variation among congeneric species as compared to conspecific individuals. This strategy has seen successful taxonomic verification of several groups of taxa including fishes (Ward et al., 2008; Hubert et al., 2008; Steinke et al., 2009), birds (Kerr et al., 2009), insects (Hebert et al., 2004; Virgilio et al., 2010), and also terrestrial mammals, such as opossum, rodents and bats (Borisents et al., 2008). In marine biodiversity its effectiveness has also been discovered in mollusks (Mikkrlser et al., 2007; Suzana et al., 2011; Chee et al., 2011; Tun et al., 2012), echinodermata (Uthicke et al., 2010) and cnidarian (Moura et al., 2007).

On many occasions, DNA barcoding helps to resolve these kinds of issues by allowing even non-experts to precisely identify specimens at various life stages and gender. However, it is of course not a substitute for morphological taxonomy, but is a complementary tool for taxonomist to recognize and additionally verify present species status as well as to discover new and cryptic species (Tun et al., 2012). With the phenomenal database collection, the Barcode of Life Data System (BOLD) was established to function as the biodiversity catalogue that consolidates information of worldwide barcodes, impart an identification engine and also monitor sequence record around the globe (Ratnasingham and Hebert, 2007). Thus, the DNA barcoding approach acts as the major platform to resolve present taxonomic difficulties, discover new species, identify biological tissues, associate various life story phases and also modernize the concept of taxonomy.

In the last decade there have been an explosion of studies based on mitochondrial and nuclear genes in many marine fisheries species to assess taxonomic integrity and differentiation. For instance, reports by Asensio et al. (2005), Garcia and Martin (2009), Iacumin et al. (2005), Lin (2008), Tsai et al. (2009) and Zhang et al. (2006) focused on fish, whereas Pascoal et al. (2008) on shrimp and Fernandea-Tajes and Mandez (2007), Fernandez-Tajes et al. (2010) on shrimp. However, studies on the identification of sea cucumbers are very limited (Borrero-Perez et al., 2010; Wen et al., 2010; Uthicke et al., 2010) due to the problems in sea cucumber species identification (Wen et al., 2011).The relationships among subgenera of *Holothuria* hypothesiszed by Rowe (1969) have been reviewed by several authors using morphological and molecular approach (Kerr et al., 2005; Kamarul et al., 2006).

Molecular taxonomy studies of the sea cucumbers in Malaysia are very limited. Most of the early studies on the species presence and distribution of sea cucumbers was conducted based on morphological characteristics (George and George, 1987; Ridzwan, 1993), focusing mainly in the Sabah waters. Through various studies taxonomic revision for example the status of *S. hermanni* which was identified based on the findings by Baine and Forbes (1998), Baine and Sze (1999), Sze and Williams (2004) and Zulfigar et al. (2000) were revised to *S. horrens*.

Recognising the importance of molecular methods, Norazila (2000) conducted the first pioneering molecular investigation of Malaysian species using Randomly Amplified Polymorphisms of DNA (RAPD) technique. Later, Kamarul et al. (2010) conducted a more comprehensive study utilising the mitochondrial 16S rRNA of 17 Malaysian sea cucumbers species with additional sequences from the GenBank to investigate their phylogenetic relationships. Their study indicated the presence of five monophyletic genera namely *Molpadia* from order Molpadiida and four genera of order Aspidochirotida namely *Holothuria*, *Stichopus*, *Bohadschia* and *Actinopyga.* They also recorded the genus *Holothuria* to be paraphyletic due to the outgroup position of a GenBank *H. excellens* sequence.

At a higher level, they also found genetic discrepancies with morphological classification; *Stichopus* of the order Aspidochirotida was found to be a sister taxon to *Molpadia* of the order Molpadiida requiring verification of the status of *Stichopus* within the present order. Their study also revealed possible errors in morphological identification of GenBank sequences. Specifically the GenBank *B. marmorata* phylogenetically clustered with *B. bivittata* clade suggesting misidentification of the former.

Taxonomic misidentifications of species has been frequently reported in the GenBank database (Nilsson et al., 2006; Uwe Fritz et al., 2012). In addition, their study recorded six unknown species from Malaysia which required further verification. Thus, this project aimed to elucidate the taxonomic and phylogenetic status of the Malaysian sea cucumbers, family Holothuriidae and Stichopodidae of the order Aspidochirotida as well as the relationship of the former to the order Molpadida. Two mitochondrial genes; the barcoding cyctochrome oxidase subunit I (CO1) and ribosomal RNA subunit (16S RNA) would be utilized to investigate the Malaysian sea cucumber.

CHAPTER III

MATERIALS AND METHODS

3.1 Samples collection and sampling technique

Three families of class Holothuroidea (sea cucumber) namely family Stichopodidae, family Holothuroiidea, and family Caudinidae were collected. These samples were collected from Malaysian waters using various techniques (Gillialand, 1993); i.e SCUBA diving in the sub tidal area of the coast for example coral reefs area, by hand during reef walk and in the intertidal zone and also from the fisherman trawl nets.

When sample collection was conducted underwater, each whole individual collected was placed in different plastic bags to avoid contact to each other and to minimize the expulsion of the Cuverian tubules and auto evisceration (Samyn et al., 2004). Identification methods were mainly qualitative, since the quantitative assessment such as the type of the tentacle observation was not possible underwater within the available time. The survey was conducted both during the night and day since most of the sea cucumber species are nocturnal although some may be found in the day.

3.2 Sampling sites and ecological description

This study was conducted throughout the Malaysian waters covering the Straits of Malacca, South China Sea, Sulu Sea and Sulawesi Sea. The sampling sites were surveyed to cover as many representative habitats as possible ranging from the outer reef to the associated coral reef lagoon in the sandy area and adjacent sea grass bed.

Ecological notes were recorded *in-situ* for each individual collected at the sampling site. The parameters recorded were substrate type, depth and also the general habitat where the samples were found. For samples collected from the trawler nets, information was obtained from the fisherman on the fishing and dredging area to determine the substrate, area and habitat of sampled individuals. The flow chart of the procedure of sampling and preservation have been simplified as stated in Figure 3.1.

Specimens were obtained from Kedah (Pulau Langkawi, Pulau Payar, Pulau Songsong and Kuala Sungai Udang), Pulau Pinang (Balik Pulau), Perak (Pulau Sembilan and Pulau Lalang), Johore Archipelago (Pulau Besar, Pulau Mentinggi, Telok Kampar, Telok Jawa and Pulau Pinang) and Sabah (Terumbu Peninjau, Terumbu Mantanani, Pulau Banggi, Pulau Lankayan, Lobster Wall, Mummiang and Mabul) and illustrated in Figure 3.2.