# CHAPTER THREE

# IDENTIFICATION OF SEA CUCUMBER SPECIMENS COLLECTED FROM PANGKOR ISLAND AND SABAH, AND MORPHOLOGICAL EVALUATION OF THEIR ASSOCIATED BACTERIAL ISOLATES

## 3.1 Introduction

Malaysia has been acknowledged as the fourth world's top producer of commercial sea cucumber (Choo, 2008). This marine invertebrate is also considered as Malaysia's marine heritage and is not only important in food industry but has high values in traditional and modern-formularised medicine (Lukman *et al.*, 2014). Sea cucumber can be found at shallow and in deep sea places, either under rocks or on the sandy sea floor. Among the locations in Malaysia known to have abundant sea cucumbers are in Sabah of the East Malaysia and Pangkor Island, Perak of the Peninsular Malaysia (Choo, 2004). However, due to their ease of collection from shallow reef flats during low tide, combined with growing coastal populations, as well as the increase in demand and price, sea cucumbers have also faced the threat of widespread overfishing (Choo, 2004; Zulfaqar *et al.,* 2016) and destruction of habitat across the regions.

Kamarudin *et al*. (2015) have documented 52 morphospecies of sea cucumbers found in Malaysia including four orders comprising of twelve genera, with 38 species recorded in Sabah. The dominant taxon of sea cucumbers in Malaysia seawater is from the order Aspidochirotida and genus *Holothuria*, and the most dominant species found is *Holothuria (Mertensiothuria) leucospilota* (Brandt, 1835). Other species from the order Aspidochirotida documented in Malaysia seawaters include *Holothuria (Mertensiothuria) hilla* (Lesson, 1830)*, Holothuria (Metriatyla) scabra* (Jaeger, 1833)*, Holothuria (Halodeima) atra* (Jaeger, 1833), *Holothuria (Halodeima) edulis* (Lesson, 1830) and *Bohadschia vitiensis* (Semper, 1868) (Kamarudin *et al*., 2015). The different types of sea cucumber have been morphologically characterised (Purcell *et al.,* 2012), and some of them were suggested to be a hybrid between different species (Uticke *et al*., 2005; Kerr *et al*., 2006).

Sea cucumbers can be primarily classified through their morphological phenotype particularly the analysis of their anatomy, and nowadays this traditional approach is being complemented by molecular techniques for their taxonomic details (Yang, 2015). In general, the external body parts of sea cucumbers include the tentacles, cuticles, papillae and podia (Kamarudin and Rehan, 2018). The cuticle is the outer surface of the body wall (Yang, 2015), while tentacles are branched from their mouth for acquiring foods (Purcell *et al.,* 2012). The internal body of sea cucumber consists of the digestive system and connected organs, including the intestine that is connected to the anus through rectum and cloaca. Most sea cucumber species have respiratory trees that are also connected to the cloaca, which is their anal cavity. Some species also have Cuvierian tubules, which is the sticky defensive structures function to protect the sea cucumbers from their enemies (Kamarudin, 2018). The body cavity is also filled with coelomic fluid which bathes the internal organs (Shi *et al.,* 2020) and act as a circulatory medium (Lukman *et al*., 2014).

Sea cucumber is one of the marine invertebrates known to provide favourable conditions for bacteria in marine environment, by causing modification in the benthic meiofauna (MacTavish *et al*., 2012). Bacteria have previously been isolated from the surface and internal body parts of some species of sea cucumbers including the skin, surface, coelomic fluid, and intestines (Wibowo *et al.,* 2019; Omran and Allam, 2013; Lukman *et al*., 2014; Farouk *et al*., 2007). However, not much has been documented on the presence of bacteria from other body parts of the sea cucumbers, and for those associated with other species of sea cucumbers from Malaysia seawaters.

Bacteria associated with sea cucumbers play important roles in their development, immunity, metabolism, and physiology (Theis *et al.,* 2016). Sea cucumbers are also considered a promising source of novel bacteria with the potential to produce secondary metabolites and bioactive compounds (Joint *et al*., 2010). In this study, fresh and wild sea cucumber specimens were collected from different locations of Malaysia seawaters, which are Pangkor Island, Perak and Manukan Island, Sabah, and their species were identified. Bacteria were also isolated from the outer and inner body parts of sea cucumbers, sediments and seawaters, then classified using morphological analysis.

## 3.2 Materials and Methods

### 3.2.1 Study Areas

Sampling for sea cucumbers were done at two coastal water areas, one location was selected in Peninsular Malaysia and another in Sabah of the East Malaysia. The locations of the sampling sites were recorded using Global Positioning System (GPS). The sampling sites were chosen referring to previous occurrence of sea cucumber, namely Pangkor Island, Perak located at the Northern region in the West Coast of Peninsular Malaysia (4°14′N, 100°32′E); and Manukan Island, Sabah situated in the East coast of Sabah (5°58′N, 116°00′E) (Figure 3.1).

The mountainous island of Pangkor Island, Perak is best known among locals and tourists with numerous beaches. Specimens were specifically collected from Teluk Nipah Beach (Latitude - 4°14' 03.3"N, Longitude – 100°32' 41.4"E) and Giam Island (Latitude - 4° 14' 09.5"N, Longitude - 100° 32' 22.4"E), the latter is the only island surrounded with corals. Manukan Island, Sabah is the second largest island in the Tunku Abdul Rahman National Park, 6 miles from Kota Kinabalu Harbour. It is well known for its coral reef which attract thousands of visitors every year (Aris *et al*., 2007).

### 3.2.2 Media preparation

All media used in this study were prepared according to manufacturer's instructions. Luria-Bertani (LB) medium Sigma-Aldrich (Missouri, USA) and nutrient agar (NA) medium Oxoid (Basingstoke, UK) were chosen for this study. LB broth is composed of 10 g/l tryptone, 5g/l NaCl and 5 g/l yeast extract. First, 25 g of the powdered medium and 15 g Bacto agar was dissolved in 1000 ml of distilled water. The medium was boiled to dissolve completely then sterilized by autoclaving at 121°C for 15 min. NA medium was prepared by suspending 8 g of premix broth powder (1 g/l Lab lemco powder, 2 g/l yeast extract, 5 g/l peptone and 5g/l sodium chloride) and 15 g bacto agar in 1000 ml distilled water and heated with frequent agitation at 100°C until the powder completely dissolved. Then the medium solution was sterilized in the autoclave at 121°C for 15 min. Both of the media were then cooled inside the laminar flow to below 45°C and poured onto sterile Petri dishes. The plates were left to solidify in the incubator for two hours.

### 3.2.3 Field Methodology

Eight fresh sea cucumbers that were available within the vicinity of the sampling sites were randomly handpicked during low tides. Upon collection, each sea cucumber specimen was placed in individual plastic bags and immediately transported to the working area for identification and processing. Two environmental sources i.e., seawater and sediment around each of the collected sea cucumbers were also sampled and placed into a 50ml falcon tube. The water temperature and pH were also recorded.

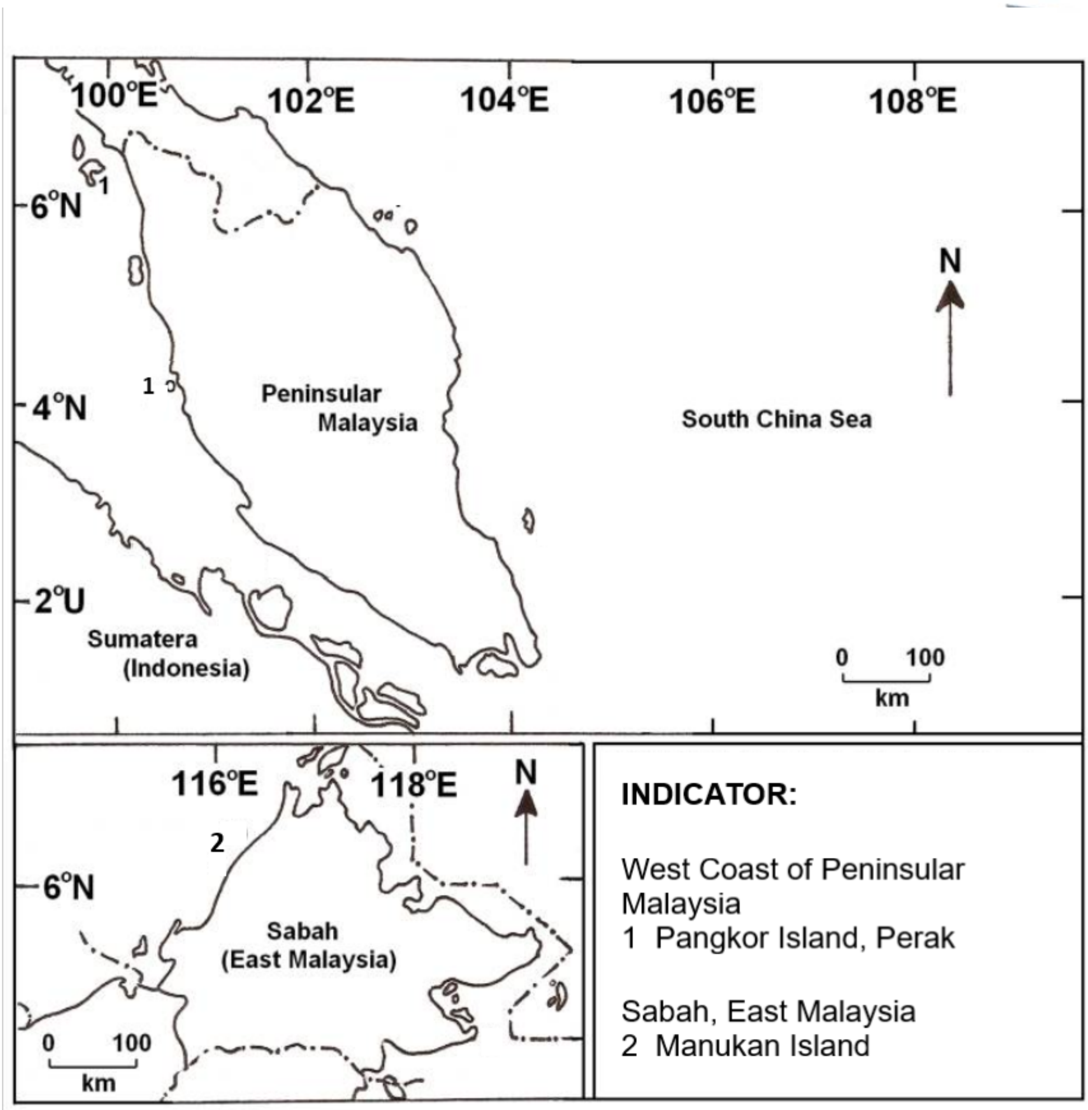


**Figure 3.1.** Sampling areas for sea cucumber. 1- Pangkor Island, Perak; 2- Manukan Island, Sabah (Kamarudin *et al.,* 2009)

### 3.2.4 Sea cucumber Observation and Identification

The sea cucumber specimens were identified using visual examination of their morphology according to Purcell *et al.* (2012), which include observation of their body colour, appearance, tentacles and the presence of Cuvierian tubules. The sea cucumber species were then further validated using genetic identification by Kamarudin (2018), through Polymerase Chain Reaction and DNA sequencing of the protein-coding cytochrome c oxidase I (COI) mitochondrial DNA (mtDNA) gene, and non-protein-coding 12S and 16S mitochondrial ribosomal RNA (rRNA) genes. The sequence obtained were analysed using the online Basic Local Alignment Search Tool program for nucleotide (blastn) for their closest relatives if available, from the GenBank, National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine.

### 3.2.5 Bacterial Isolation

Sea cucumbers were processed directly after collection. All apparatus were sterilised before being used. Bacteria were isolated from eight body parts of the sea cucumber specimen i.e., cuticle, tentacle, coelomic fluid, cloaca, gastrointestinal tissue, respiratory tree, Cuvierian tubules, polian vesicle, and their surrounding sediments and seawater by direct streaking method (Figure 3.2). The sources were swabbed using sterile cotton swabs and streaked on growth media as in previous studies (Wibowo *et. al.,* 2019; Tangestani & Kunzmann, 2019; Wijaya *et. al.,* 2020). Two rich growth media for bacteria, Luria-bertani (LB) and Nutrient agar (NA) were used for isolation, as these media favours bacterial growth and have been widely applied for growing bacterial cultures.

Following isolation, the plate media were incubated at 30°C for 24 to 48 hours to allow bacterial growth. Bacterial colonies with different morphologies (i.e., shape and colour) were then randomly picked and regularly sub-cultured onto fresh media by making streak plates to obtain pure cultures. For the preservation of bacterial strains, a loopful of the overnight culture were added into Eppendorf tube containing 500 µl of 20% glycerol and stored at -20°C and -80°C freezer.

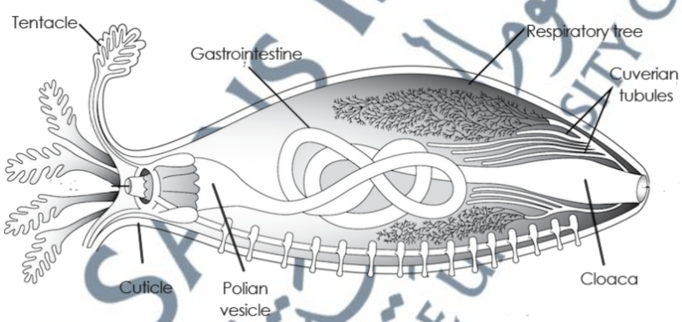


**Figure 3.2**. General body parts of sea cucumber as the source of bacterial isolation. (Inouye, n.d)

### 3.2.6 Morphological Observation

Bacterial isolates were morphologically analysed with regard to their Gram-stain, which may facilitate in confirmation of their bacterial groups or classes in the microscopic identification (Lukman *et al.*, 2014). One loopful of bacterial cells was applied on a clean glass slide using an inoculation loop. The smear was then heat fixed by passing it over mild flame of the Bunsen burner (Lima-Bittencourt *et al*., 2007). The slide was then flooded with crystal violet solution for 40 seconds. The stain was washed off before adding Gram's iodine solution for 20 seconds. The smear was washed off and decolorised by adding 95% (v/v) ethyl alcohol. The smear was counter stained with safranin for 40 seconds before it was washed off. The smear was then blot dried before it was microscopically analysed using compound microscope (Olympus CX21) with magnifications of 1000× with oil immersion.

## 3.3 Results and Discussion

### 3.3.1 Sampling and Identification of Sea Cucumber Specimens

Eight individuals of sea cucumbers were successfully collected randomly at two main locations in Malaysia; specifically at the Giam Island and Teluk Nipah Beach of Pangkor Island, Perak of Peninsular Malaysia in March 2016; and at Manukan Island, Tunku Abdul Rahman Park, Sabah of East Malaysia in September 2016. These particular sites of Malaysia seawaters were selected to represent the two major locations based on the record of availability of sea cucumbers (Reef Check Malaysia, 2018). The physical parameters during sampling in both locations were recorded as pH 6 and temperature 26°C at Pangkor Island, Perak, while pH 6 and temperature 30°C at Manukan Island, Sabah.

The eight collected sea cucumber samples include two individuals from Pangkor Island, Perak (labelled as PPA and PPB) and six individuals from Manukan Island, Sabah (labelled as PMA to PMF). The species were further verified through genetic identification by Kamarudin (2018), except for PPA and PMF. Three separate markers were amplified and their sequence analysed, which are the protein-coding COI mtDNA as well as the non-protein-coding 12S and 16S mitochondrial rRNA genes. The sea cucumber species were identified based on the BLASTN results of the highest identity percentage, in combination with their morphological observation.

#### 3.3.1.1 Identification of Sea Cucumbers from Pangkor Island, Perak

In Pangkor Island, one sea cucumber specimen was recovered from the rocky shores of Giam Island and labelled as PPA, while another one was found under a large rock closest to the coast of Teluk Nipah beach and labelled as PPB (Figure 3.3). These rocky shore areas in Pangkor Island where the two specimens were discovered are preferable habitat for sea cucumbers that can protect these species from wave action (Bakus, 1973). The two specimens were observed to have long and black body colour, with mouth surrounded by brown tentacles, indicating that both are from the species of *Holothuria (Mertensiothuria) leucospilota* (Brandt, 1835). Both PPA and PPB also have similar outer appearance with each other, and with eight *H. leucospilota* samples previously found in Langkawi Island, Kedah (Kamarudin *et al*., 2011).

PPA PPB

**Figure 3.3**. Two sea cucumber specimens collected from Pangkor Island, designated PPA and PPB.

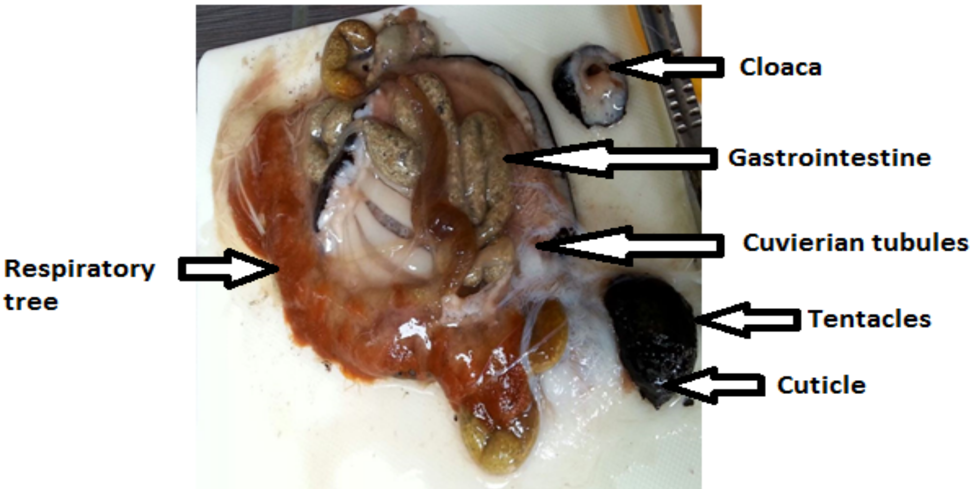


**Figure 3.4**. Body parts of *Holothuria leucospilota* (PPB) from Pangkor Island, Perak after dissection.

Sample PPB from Teluk Nipah Beach, Pangkor Island, Perak was further identified by Kamarudin (2018) using genetic approach, based on the BLASTN results of the highest identity percentage of three markers, which are the protein-coding COI mtDNA, the non-protein-coding 12S and 16S mitochondrial rRNA genes (Table 3.1). Sample PPB is confirmed to be *Holothuria leucospilota* based on all three markers with 99% to 100% identity. Meanwhile, sample PPA was identified as *H. leucospilota* solely based on morphological identification.

The collected sea cucumber specimens were dissected to isolate the internal parts' bacteria. Some sea cucumber species are known to have the presence of certain organs that can be absent in other sea cucumbers, hence dividing them into six orders. In this study, both *H. leucospilota* PPA and PPB showed the presence of Cuvierian tubules, as would be expected from *H. leucospilota* species (Figure 3.4) (Purcell *et al*., 2012). A mass of sticky Cuvierian tubules is emitted by *H. leucospilota* as its defence mechanism (Eissa *et al*., 2017). These tubules are also present in several other holothuroids of the genera *Holothuria*, *Bohadschia* and *Pearsonothuria* (Hamel and Mercier, 2000).

Overall, only two samples of sea cucumbers were found in Pangkor Island, Perak during the sampling and both are from the species of *H. leucospilota*. The *H. leucospilota* species is similar with those previously found by Kamarudin *et al*. (2009) at the same area, in which the researchers also observed low levels of sea cucumber diversity in this area. One reason for this observation could be due to the limited coral distribution along the West Coast of Peninsular Malaysia that include the Pangkor Island (Ho, 1992). Pankor (2004) also documented that sea cucumbers from other species including *Stichopus* spp. were plentiful in Pangkor Island in the year 1994 as claimed by local residents and became increasingly reduced. The commercially important species were almost disappeared potentially due to the overexploitation by fishermen for gamat oil and gamat water (Baine and Sze, 1998).

Another possibility for the lower diversity of sea cucumber species observed in Pangkor Island could be due to the increasing numbers of tourists and development in recent years, since this small island is one of the leading tourism destinations in Malaysia. Ismail *et al.* (2004) have reported that sea cucumbers in Pangkor Island have high content of certain heavy metals in their body walls, while Husin *et al.* (2021) recently demonstrated microplastic contamination in the intestine of sea cucumber *Stichopus horrens* from Pangkor Island. These indicate potential pollution and uncontrolled dumping of industrial affluent in the area, which may also affect the diversity of sea cucumbers.

#### 3.3.1.2 Identification of Sea Cucumbers from Manukan Island, Sabah

Six sea cucumber specimens were recovered from the second sampling location, in Manukan Island, Sabah, and labelled as PMA to PMF. Their morphological characteristics were also observed and recorded (Figure 3.5; Table 3.1). PMA was collected on sandy areas of the shore and observed to have light pink on the ventral body wall and dark reddish black colour on its dorsal side, hence was morphologically identified as *Holothuria (Halodeima) edulis.* The next sea cucumber sample, PMB was found half-buried in the sediment and has the largest size among all the sea cucumber samples collected. It has loaf-like bodies, and light brown colour with dark brown spots, which is the characteristic of *Bohadschia vitiensis*.

**Table 3.1**. List of Sea Cucumbers Collected from Pangkor Island, Perak and Manukan Island, Sabah

| Sample ID | Location | Morphology | Morphological identification | Genetic Identification | % Similarity & Corresponding Match from GenBank | Registered Gene Accession No |
|---|---|---|---|---|---|---|
| PPA | Giam Island, Pangkor Island, Perak | Black, elongated | *Holothuria (Mertensiothuria) leucospilota* | Not identified genetically | - | - |
| PPB | Teluk Nipah Beach, Pangkor Island, Perak | Black, elongated | *Holothuria (Mertensiothuria) leucospilota* | *Holothuria (Mertensiothuria) leucospilota* | 99% (COI) - FJ223877<br>100% (16S) - KX768274<br>99% (12S) - DQ777101 | COI - Not registered<br>16S - MF139518<br>12S - KX768273 |
| PMA | Manukan Island, Sabah | Reddish black dorsally and light pink ventrally. Sausage-shaped. | *Holothuria (Halodeima) edulis* | *Holothuria (Halodeima) edulis* | 87% (COI) -EU220829<br>99% (16S) - EU822446 | COI - MF787780<br>16S - MF616466 |
| PMB | Manukan Island, Sabah | Light brown body with dark brown spots and stout body. | *Bohadschia vitiensis* | *Bohadschia vitiensis* | 88% (COI) - EU848217 | COI - MF787781 |
| PMC | Manukan Island, Sabah | Black, elongated | Unidentified but suspected to be morphospecies *Holothuria (Halodeima) atra* | *Holothuria (Halodeima) atra* | 95% (COI) -EU848225<br>85% (16S) -FJ223858 | COI - MF787782<br>16S - MF616469 |

| | | | | | | |
|---|---|---|---|---|---|---|
| PMD | Manukan Island, Sabah | Black, elongated | Unidentified but suspected to be morphospecies *Holothuria (Halodeima) atra* | *Holothuria (Halodeima) atra* | 95% (COI) - JN543448<br>99% (16S) -HM056229 | COI - MF787792<br>16S - MF616468 |
| PME | Manukan Island, Sabah | Black, elongated | Unidentified but suspected to be morphospecies *Holothuria (Halodeima) atra* or *Holothuria hilla* | *Holothuria hilla* | 87% (12S) - KX856663 | 12S - MF188880 |
| PMF | Manukan Island, Sabah | Black, elongated | *Holothuria (Halodeima) atra* | Genetically unidentified due to poorly obtained sequence | - | - |

Note: COI: cytochrome c oxidase I (COI) mitochondrial DNA (mtDNA) gene; 12S: 12S mitochondrial ribosomal RNA (rRNA) gene. 16S: 16S mitochondrial ribosomal RNA (rRNA) gene. Genetically identified by Kamarudin (2018).

Sea cucumbers PMC and PMD were encountered in sandy grounds close to the coast, while PME and PMF were found among the coral rubble on sandy grounds surrounded by sea urchins. The presence of sea urchins could be due to the existing corals, and the area is less disturbed from snorkeling activities. The remaining four samples PMC, PMD, PME and PMF were observed to be tan to black in colour. However, the three samples of PMC, PMD, PME were unable to be completely identified by morphological observation, but PMC and PMD were suspected to be the morphospecies of *Holothuria (Halodeima) atra*, while PME was suspected to be either *Holothuria atra* or *Holothuria hilla.* Meanwhile, the sample PMF was morphologically identified as *Holothuria atra.*

The sea cucumber specimens were further identified using genetic approach by Kamarudin (2018), except for PMF due to poorly obtained sequence. PMA was verified as *Holothuria edulis* based on COI mtDNA and 16S mitochondrial rRNA gene with 87% and 99% identity respectively; while PMB was confirmed as *Bohadschia vitiensis* from COI sequence analysis with 88% identity. Sea cucumber samples PMC and PMD were genetically identified as *Holothuria atra* based on their COI mtDNA and 16S mitochondrial rRNA gene with 85% to 99% similarity, verifying their morphological identification. Sample PME showed 87% identity as *Holothuria hilla* based in its 12S mitochondrial rRNA gene, therefore it is resolved as *Holothuria hilla* after considering both its physical appearance and genetic sequence analysis. Meanwhile, PMF was genetically unidentified due to poorly obtained sequence, hence it is regarded as *Holothuria atra* according to its physical appearance.

PMA

PMB

PMC

PMD

PME

PMF

**Figure 3.5**. Six sea cucumber specimens collected from Sabah, designated PMA to PMF

Upon dissection, only *B. vitiensis* (PMB) showed the presence of Cuvierian tubules, while no tubules were found from other specimens, which are three *Holothuria atra* samples (PMC, PMD and PMF), a *Holothuria edulis* (PMA) and a *Holothuria hilla* (PME). Purcell *et al.* (2012) have stated that one of the diagnostic features of *Holothuria atra* and *Holothuria edulis* is that Cuvierian tubules are absent in these species. However, *Holothuria hilla* has been previously reported to produce Cuvierian tubules, however, they were not observed in this study for sample PMF. *Holothuria hilla* has been known to never ejected their Cuvierian tubules (Purcell *et al.,* 2012), which could explain why the tubules were not observed in PMF specimen.

A higher number of sea cucumber specimens consisting of four species were discovered in Manukan Island, Sabah as compared to the two samples found in Pangkor Island. This may suggest higher diversity of sea cucumbers in Manukan Island compared to Pangkor Island, which could be related to the proximity of the area to the Wallace's line. Besides, Manukan Island has been gazetted as Tunku Abdul Rahman National Park and could have proper management in terms of their sustainability, which contribute to the species diversity.

The habitat preference of each sea cucumber could be related to their necessities such as food availability and shelter. It has been suggested that physical factors that include salinity degrees, water temperature, turbidity of the water, depth at which species occur and nutrient composition may have a direct impact on holothurians distribution and occurrence (Lampe, 2013). The highest number of sea cucumber found in this study is *H. atra*, comprising of 37.5% of the total sea cucumbers, and all of them were collected from Manukan Island, Sabah. *H. atra* is known to live in a wide range of habitat (Lampe, 2013), and has a fairly strong susceptibility from the influence of the quality of the surrounding environment, especially the effect of infection on the body of sea cucumbers (Sulardiono *et al.,* 2020).

The presence of certain species such as *H. atra* and *H. edulis* has been previously recorded in Sabah by Kamarudin *et al*. (2009), as the species that are commonly discovered, whereas *B. vitiensis* collected in this study is not considered to be a common species found in Sabah. Furthermore, *H. hilla* is also a new record of sea cucumber species found in Manukan Island Sabah, since this species has not been previously reported in the area as to our knowledge. However, there are five common genera of sea cucumber previously documented in Manukan Island, Sabah that are not found in this study, which include *Actinopya* spp., *Pearsonothuria* spp., *Stichopus* spp., *Molpadia* spp. and *Synapta* spp. (Kamarudin *et al*., 2009).

Choo (2004) stated that data of sea cucumber populations recorded in Sabah has suggested a severe decline, potentially due to overfishing and degradation of natural habitats. Moreover, direct exploitation, habitat loss and degradation and coral reef destruction may also contribute to the decline of species diversity, since coral reef is their food source and main habitat (Kamarudin *et al.,* 2015; Gaston and Spicer, 2004). Nevertheless, all eight of the collected sea cucumber species in Pangkor Island, Perak and Manukan Island, Sabah in this study is not in the list of aspidochirotid holothuroids that are vulnerable, endangered or at risk of extinction in the International Union for Conservation of Nature (IUCN) Red List (Conand *et al.,* 2014).

### 3.3.2 Isolation and Morphological Evaluation of Bacterial Isolates

Bacteria were successfully isolated from the external (tentacle, cuticle and cloaca) and most of the internal parts (polian vesicle, gastrointestinal tissue, respiratory tree, Cuvierian tubules, coelomic fluid) of each sea cucumber specimen immediately after dissection, and from the sea cucumbers' surrounding seawater and sediments. However, no bacterial growth was observed on agar media following isolation from the respiratory tree of *H. leucospilota* PPA and the Polian vesicle of *H. hilla* PME, which are both the internal parts of the sea cucumbers. Similarly, no bacterial colonies were present on the LB and NA agar media when isolated from all internal parts of *B. vitiensis* PPB.

Some factors that could contribute to the absence of bacteria from the sea cucumbers' internal regions may include their feeding behaviour, the penetration of light surrounding their habitats and higher levels of antimicrobial activities from their internal fluids as a defence mechanism against microorganisms (Lukman *et al.,* 2014). Furthermore, the coelomic fluid of *B. vitiensis* collected from Terengganu, Malaysia has been reported to contain considerably high levels of crude proteins and total antioxidant activity (Hawa *et al.,* 1999), hence may also affect the species' internal microbial composition. However, the cultivation media used for bacterial isolation in this study may also be unsuitable for the growth of some unique marine bacterial strains that inhabit the sea cucumbers' internal regions, which could explain the total absence of bacteria. For the plates that have bacterial growth, colonies were observed to grow on agar media after approximately one to two days of incubation at 30°C. Selected representatives of morphologically distinct bacterial colonies were randomly picked and purified by successive streaking onto fresh agar plates.

A total of 115 bacterial isolates were recovered and labelled according to their source of isolation, then the general morphologies were recorded. These include the colony colour, cell arrangement of bacterial colonies when observed under microscope and Gram reaction (Table 3.2). The bacterial isolates were predominantly Gram-negative, comprising of approximately 64% of the total isolated strains. The colonies were observed to exhibit a range of colours, mainly white to cream, and seven colonies (6.1%) showed vivid coloration that include light pink (1 sample), yellow (4 samples), orange (1 sample) and green (1 sample). These pigmented bacterial colonies were selected for further analysis (discussed in Chapter 4). Under microscopic examination, all bacterial isolates were observed to vary in shape, ranging from long rods, short rods and coccoid. Most of the bacteria have rod-shape which makes up to 81% of the total isolates, followed by coccoid shape with 17% of the bacterial samples.

**Table 3.2**. Bacteria isolated from each sea cucumber specimen and their general morphology

| Sampling station | Sea cucumber species | Samples ID | Sources of bacterial isolates | Bacteria ID | Colony colour | Cell characterization | |
|---|---|---|---|---|---|---|---|
| | | | | | | Cell arrangement | Gram reaction |
| Giam Island, Pangkor Island, Perak | *Holothuria leucospilota* | PPA | Water | PPAW | Cream-yellowish | Rod-shaped | - |
| | | | Sediment | PPAS | Creamy white | Rod-shaped | - |
| | | | Cuticle | PPAC1 | White translucent | Rod-shaped | - |
| | | | | PPAC2 | White transparent | Rod-shaped | - |
| | | | Cloaca | PPAL | Creamy white | Rod-shaped | - |
| | | | Tentacle | PPAT1 | Creamy white | Rod-shaped | - |
| | | | | PPAT2 | Creamy white | Rod-shaped | - |
| | | | | PPAT3 | Cream-yellowish | Rod-shaped | - |
| | | | Cuvierian tubules | PPAV | Creamy white | Rod-shaped | - |
| | | | Coelomic fluid | PPACOE | Creamy white | Rod-shaped | - |
| | | | Gastrointestine | PPAG1 | Creamy white | Rod-shaped | - |
| | | | | PPAG2 | White translucent | Rod-shaped | - |
| | | | | PPAG3 | Creamy white | Rod-shaped | - |
| | | | | PPAG4 | Creamy white | Rod-shaped | - |
| | | | Respiratory tree | No growth observed | | | |
| Teluk Nipah Beach, Pangkor Island, Perak | *Holothuria leucospilota* | PPB | Water | PPBW | Creamy white | Coccoid-shaped | + |
| | | | Sediment | PPBS | White translucent | Rod-shaped | - |
| | | | Cuticle | PPBC | White transparent | Rod-shaped | - |
| | | | Cloaca | PPBL1 | White translucent | Rod-shaped | - |
| | | | | PPBL2 | Cream-yellowish | Rod-shaped | - |
| | | | Tentacle | PPBT1 | Creamy white | Rod-shaped | - |
| | | | | PPBT2 | Milky white | Rod-shaped | - |
| | | | Cuvierian tubules | PPBV | Creamy white | Rod-shaped | - |
| | | | Coelomic fluid | PPBCOE | Creamy white | Coccoid-shaped | + |
| | | | Gastrointestine | PPBG | White translucent | Rod-shaped | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | Polian vesicle | PPBP1 | Creamy white | Rod-shaped | - |
| | | | | PPBP2 | White transparent | Rod-shaped | - |
| | | | Respiratory tree | PPBR | Creamy white | Rod-shaped | - |
| Manukan Island, Sabah | *Holothuria edulis* | PMA | Water | PMAW1 | White translucent | Rod-shaped | + |
| | | | | PMAW2 | White translucent | Rod-shaped | + |
| | | | Sediment | PMAS1 | Creamy white | Rod-shaped | + |
| | | | | PMAS2 | Creamy white | Coccoid-shaped | + |
| | | | Cuticle | PMAC1 | Creamy white | Rod-shaped | + |
| | | | | PMAC2 | Creamy white | Rod-shaped | + |
| | | | Cloaca | PMAL1 | Creamy white | Rod-shaped | + |
| | | | | PMAL2 | Creamy white | Rod-shaped | + |
| | | | Tentacle | PMAT | Creamy white | Rod-shaped | - |
| | | | Coelomic fluid | PMACOE1 | Rough creamy white | Rod-shaped | + |
| | | | | PMACOE2 | | Rod-shaped | + |
| | | | | PMACOE3 | Creamy white | Rod-shaped | + |
| | | | | PMACOE4 | Creamy white | Rod-shaped | + |
| | | | Gastrointestine | PMAG1 | White translucent | Rod-shaped | + |
| | | | | PMAG2 | Creamy white | Rod-shaped | + |
| | | | Polian vesicle | PMAP1 | Creamy white | Rod-shaped | - |
| | | | | PMAP2 | Creamy white | Rod-shaped | + |
| | | | Respiratory tree | PMAR | White translucent | Rod-shaped | + |
| | *Bohadschia vitiensis* | PMB | Water | PMBW1 | Creamy white | Rod-shaped | + |
| | | | | PMBW2 | White translucent | Rod-shaped | + |
| | | | Sediment | PMBS1 | Milky white | Rod-shaped | - |
| | | | | PMBS2 | White translucent | Rod-shaped | + |
| | | | | PMBS3 | White translucent | Coccoid-shaped and form in grape-like clusters | + |
| | | | Cuticle | PMBC1 | White translucent | Rod-shaped | + |
| | | | | PMBC2 | Creamy white | Rod-shaped | + |
| | | | Cloaca | PMBL1 | Creamy yellow | Rod-shaped | + |
| | | | | PMBL2 | Milky white | Rod-shaped | + |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | Tentacle | PMBT | Creamy yellow | Rod-shaped | + |
| | | | Cuvierian tubules | No growth observed | | | |
| | | | Coelomic fluid | No growth observed | | | |
| | | | Gastrointestine | No growth observed | | | |
| | | | Polian vesicle | No growth observed | | | |
| | | | Respiratory tree | No growth observed | | | |
| | *Holothuria atra* | PMC | Water | PMCW | Creamy white | Rod-shaped | + |
| | | | Sediment | PMCS | White translucent | Rod-shaped | + |
| | | | Cuticle | PMCC1 | Green | Rod-shaped | - |
| | | | | PMCC2 | Yellow | Rod-shaped | - |
| | | | | PMCC3 | White translucent | Rod-shaped | + |
| | | | Cloaca | PMCL1 | White translucent | Coccoid-shaped | + |
| | | | | PMCL2 | White translucent | Coccoid-shaped | + |
| | | | Tentacle | PMCT1 | Yellow | Coccoid-shaped | + |
| | | | | PMCT2 | Creamy white | Rod-shaped | - |
| | | | Coelomic fluid | PMCCOE1 | White translucent | Rod-shaped | + |
| | | | | PMCCOE2 | White transparent | Rod-shaped | + |
| | | | Gastrointestine | PMCG | Creamy white | Coccoid-shaped | + |
| | | | Polian vesicle | PMCP | Creamy white | Rod-shaped | + |
| | | | Respiratory tree | PMCR | White translucent | Rod-shaped | + |
| | *Holothuria atra* | PMD | Water | PMDW | Creamy white | Rod-shaped | + |
| | | | Sediment | PMDS1 | Milky white | Coccoid-shaped | - |
| | | | | PMDS2 | White translucent | Rod-shaped | - |
| | | | Cuticle | PMDC1 | White translucent | Coccoid-shaped | - |
| | | | | PMDC2 | Light pink | Coccoid-shaped | - |
| | | | | PMDC3 | Milky white | Rod-shaped | - |
| | | | | PMDC4 | White translucent | Rod-shaped | + |
| | | | Cloaca | PMDL | Creamy white | Coccoid-shaped | + |
| | | | Tentacle | PMDT1 | Yellow | Coccoid-shaped | + |
| | | | | PMDT2 | Creamy white | Rod-shaped | + |
| | | | Coelomic fluid | PMDCOE | White translucent | Coccoid-shaped | + |
| | | | Gastrointestine | PMDG | Creamy white | Rod-shaped | + |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | Polian vesicle | PMDP | Yellow | Coccoid-shaped | + |
| | | | Respiratory tree | PMDR1 | White translucent | Rod-shaped | + |
| | | | | PMDR2 | Creamy white | Rod-shaped | + |
| | *Holothuria hilla* | PME | Water | PMEW | Creamy white | Rod-shaped | - |
| | | | Sediment | PMES1 | White translucent | Rod-shaped | + |
| | | | | PMES2 | Creamy white | Coccoid-shaped | + |
| | | | | PMES3 | Creamy white | Rod-shaped | + |
| | | | Cuticle | PMEC1 | Creamy white | Rod-shaped | + |
| | | | | PMEC2 | Light orange | Rod-shaped | + |
| | | | | PMEC3 | White translucent | Rod-shaped | - |
| | | | Cloaca | PMEL1 | Creamy white | Coccobacillus | - |
| | | | | PMEL2 | Milky white | Coccoid-shaped | + |
| | | | Tentacle | PMET1 | Cream-yellowish | Rod-shaped | + |
| | | | | PMET2 | White translucent | Rod-shaped | + |
| | | | Coelomic fluid | PMECOE1 | Creamy white | Rod-shaped | + |
| | | | | PMECOE2 | White translucent | Rod-shaped | + |
| | | | | PMECOE3 | White transparent | Rod-shaped | - |
| | | | Gastrointestine | PMEG | Creamy white | Rod-shaped | - |
| | | | Polian vesicle | No growth observed | | | |
| | | | Respiratory tree | PMER1 | Creamy white | Rod-shaped | + |
| | | | | PMER2 | White translucent | Coccoid-shaped | + |
| | | | | PMER3 | White transparent | Rod-shaped | + |
| | | | | PMER4 | White transparent | Coccoid-shaped | + |
| | | | | PMER5 | Milky white | Rod-shaped | + |
| | | | | PMER6 | Creamy white | Rod-shaped | + |
| | *Holothuria atra* | PMF | Water | PMFW | Creamy white | Rod-shaped | + |
| | | | Sediment | PMFS | Creamy white | Rod-shaped | + |
| | | | Cuticle | PMFC | Creamy white | Rod-shaped | + |
| | | | Cloaca | PMFL | Creamy white | Rod-shaped | + |
| | | | Tentacle | PMFT | Creamy white | Rod-shaped | + |
| | | | Coelomic fluid | PMFCOE | White translucent | Coccoid-shaped | + |
| | | | Gastrointestine | PMFG | Creamy white | Rod-shaped | + |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | Polian vesicle | PMFP | White translucent | Rod-shaped | + |
| | | | Respiratory tree | PMFR1 | Creamy white | Rod-shaped | + |
| | | | | PMFR2 | Milky white | Rod-shaped | + |

Gram-negative rod-shaped bacteria have previously been reported to be the dominant microorganisms in marine environments, which could be due to their outer membrane structure that is evolutionarily adapted to the low nutrition, high pressure and high salinity conditions (Soliev, 2012). These Gram-negative marine bacteria can be a source of economically important natural substances, however some of them is associated with substantial diseases to the marine organisms due to the unique cell wall component termed lipopolysaccharide (LPS) (Anwar & Choi, 2014). This may indicate potential threat to the sea cucumber samples that are associated with high amount of the Gram-negative bacteria, particularly the two *H. leucospilota* from Pangkor Island, Perak. Nevertheless, these wild sea cucumbers collected from the marine environment did not present any detectable diseases, and further studies are needed to determine whether the bacterial isolates are pathogenic.

The relative abundance and diversity of marine Gram-positive bacteria may also be greater than previously thought, even though their ecological function in the sea has not been completely understood. Marine Gram-positive bacteria could also be an important source of biotechnologically valuable products, since they are well known for their capacity to produce structurally diverse classes of secondary metabolites and antimicrobial compounds, particularly *Actinobacteria* (Leiva *et. al*., 2015).

Isolation of bacteria from the two *H. leucospilota* specimens from Pangkor Island, Perak including the surrounding sediments and seawater resulted in 27 bacterial strains, and 92.6% of them are Gram-negative rod-shaped bacteria. Previous studies have also reported on bacteria isolated from *H. leucospilota* including their skin (Wibowo et al., 2019) and internal regions such as the coelomic fluid (Lukman *et al.,* 2014), respiratory tree (Kamarudin *et al*., 2013) and intestine (Kamarudin, & Rehan, 2018). In particular, Lukman *et al.* (2014) isolated at least five genera of bacteria comprising of seven Gram-negative and two Gram-positive bacteria from the coelomic fluids of *H. leucospilota* from Dayang Bunting Island, Kedah. Furthermore, six Gram-positive bacterial genera were isolated from the gastrointestines of *H. leucospilota* from Pangkor Island, Perak (Kamarudin and Rehan, 2018), and an orange pigmented Gram-positive bacteria was isolated from respiratory tree of *H. leucospilota* from the same location (Kamarudin *et al*., 2013).

From the three *H. atra* specimen collected from Manukan Island, Sabah, 32 bacterial samples were isolated from their body parts, with 81.3% of them are Gram-positive bacteria. Bacteria associated with *H. atra* have also been documented in previous studies, mostly isolated from the intestine (Sulardiono *et al*., 2020; Farouk *et al*., 2007; Ward-Rainey *et al.,* 1999). Farouk *et al.* (2007) isolated 30 bacterial strains from the coelomic fluid, intestines, and outer skin layer of two *H. atra* juvenile specimens from Pangkor Island, Perak, and 7 of them recorded moderate antibacterial activity against selected pathogenic bacteria, suggesting that these associated bacteria can be a source of bioactive compounds.

The highest number of bacteria isolated from the sea cucumbers' body parts is from *H. hilla* with 17 bacteria (18.9%) followed by *H. edulis* with 12 bacterial strains (13.3%), while the lowest number of bacteria are those isolated from the body parts of *B. vitiensis* (5.6%) (Figure 3.6). Even though previous studies have documented on bacteria associated with various species of sea cucumber, however, limited studies have reported on those associated with *H. edulis*, while no previous research could be found on bacteria from *H. hilla* and *B. vitiensis* to date. Further characterization on these sea cucumbers-associated bacterial isolates in various aspect such as their secondary metabolites, antibiotics, antitumor agents, antitoxins, and enzymes production in future studies could be beneficial for a broad range of applications.

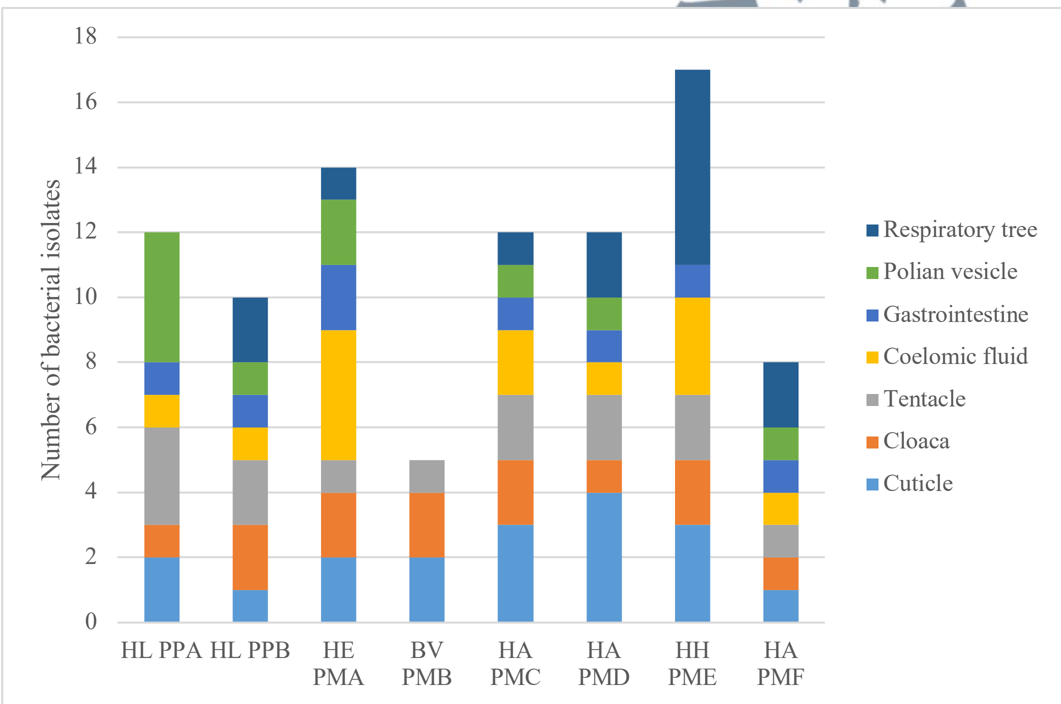


**Figure 3.6**. Comparison between total number of bacteria isolated from various parts of sea cucumbers. HL: *Holothuria leucospilota,* HE: *Holothuria edulis,* BV: *Bohadschia vitiensis,* HA: *Holothuria atra,* HH: *Holothuria hilla*

### 3.4 Conclusion

Eight sea cucumber specimens were collected and identified in this study, which are two *H. leucospilota* specimens from Pangkor Island, Perak, while the six specimens collected from Manukan Island, Sabah were identified as three samples of *H. atra* and one each of *H. edulis, H. hilla* and *B. vitiensis.* In general, low species variation of sea cucumbers was observed and collected in this study, which may suggest the decline in diversity of sea cucumbers in both areas. However, more studies are needed to confirm this observation. A total of 115 bacterial samples were isolated from internal and external body parts of the sea cucumbers, and their surrounding sediments and seawaters. The bacterial isolates showed variation in their morphology. A higher proportion of the bacterial isolates are Gram-negative rod-shaped bacteria, especially the bacterial samples isolated from two *H. leucospilota* specimens from Pangkor Island, Perak.