

CHAPTER FOUR

SPECIES IDENTIFICATION OF MARINE BACTERIA ASSOCIATED WITH SEA CUCUMBERS FROM PANGKOR ISLAND AND SABAH SEAWATER

4.1 Introduction

Sea cucumber is one of the marine invertebrates that is known to host diverse bacterial communities. Identification of bacteria associated with marine host have been the focus of many recent research since they can provide information about the microbial-host symbiotic interaction (Blockley *et al.*, 2017), diseases related to the host (Tangestani and Kunzmann, 2019) and may also results in the discovery of marine bacterial strains with the ability to produce secondary metabolites and bioactive compounds (Leal *et al.*, 2014). The microbial associates may also provide defence against pathogens and serve as bioindicators of changing environmental conditions (Morrow *et al.*, 2012).

Sea cucumbers are filter feeders that obtain their foods from sediments and seawater, consuming all microorganisms within their vicinity. This opens the prospect of biotechnological applications of bacterial inhabitants in the internal body parts of the sea cucumber, particularly their intestinal symbionts (Pringgenies *et al.*, 2020). These intestinal bacteria also have important role in modulation of the immune system, proliferation of intestinal epithelium and regulation of food energy intake of the host (Sulardiono *et al.*, 2020). Therefore, isolation and identification of bacterial communities in the internal parts of the sea cucumber could be important to be applied in aquaculture industry. For example, bacteria isolated from the gut of sea cucumber *A.*

japonicus have been developed as probiotics for juvenile sea cucumbers. These bacterial strains of *Bacillus cereus* and *Paracoccus marcusii* showed positive effect on the growth performance and immune response in the sea cucumber's coelomocytes and the intestine, and provide protection against infection caused by pathogenic bacterial species (Yan *et al.*, 2014; Yang *et al.*, 2015).

Apart from the internal body parts, the marine host surfaces may also provide optimum growing conditions and nutrient sources for surface-attached bacteria. The relationship between invertebrates and surface-associated bacteria varies from sessile to motile invertebrates in terms of maintaining overall health and settlement issues (Alipiah *et al.*, 2016). Many surface-attached bacteria secrete antagonistic metabolites, as a reaction to inhibit the attachment and growth of competitors and predators, for them to survive in the highly competitive environments (Long and Azam 2001). Therefore, the symbiotic bacteria hosted by sea cucumbers could be an important source of chemically diverse and biologically active secondary metabolites.

Bacteria can be identified using morphological and molecular-based methods, and the most widely applied molecular approach involve analysis of the 16S rRNA gene sequence. This gene sequence has long been used as a taxonomic gold standard in determining the phylogenies of bacterial species (Weose, 1987). The 16S rRNA gene possesses a region that is highly conserved as well as a region that contains distinctive species sequences (Ahmadian *et al.*, 2006). Besides, there are extensive availability of the 16S rRNA gene sequence in public database such as GenBank (Benson *et al.*, 2004), which allow a reliable characterisation of a bacterial community associated with sea cucumbers. This study identified bacteria isolated from sea cucumber specimens

collected from Pangkor Island, Perak and Manukan Island, Sabah including *Holothuria leucospilota*, *Holothuria edulis*, *Holothuria atra*, *Holothuria hilla* and *Bohadschia vitiensis* based on analyses of their 16S rRNA gene sequence, alignment and phylogenetic relationship.

4.2 Materials and Methods

4.2.1 Bacterial Genomic DNA Extraction

Genomic DNA extraction was carried out using two methods, which are using the Wizard Genomic DNA Purification Kit (Promega, Southampton, UK) to obtain pure DNA, and another method of the colony PCR (alkaline lysis) technique (Rehan, 2012).

4.2.1.1 DNA Extraction using Wizard Genomic DNA Purification Kit

Genomic DNA extraction using the Wizard Genomic DNA Purification Kit was carried out following the manufacturer's instructions. First, 1 ml of the bacterial overnight culture was centrifuged at 13,000 rpm for 2 min, and the supernatant was removed. Then, 600 µl of Nuclei Lysis Solution was added and the mixture was resuspended. The cells were incubated at 80°C for 5 minutes, before adding 3 µl of RNase Solution and mixed by inverting the tube. This is followed by incubation at 37°C for 45 minutes and then cooled to room temperature. Then, 200 µl of Protein Precipitation Solution was added to the RNase-treated cell lysate and vortex vigorously at high speed for 20

seconds. The sample was then incubated on ice for 5 minutes and centrifuged at 13,000 rpm for 3 minutes.

The supernatant was transferred to a fresh 1.5 ml microcentrifuge tube that contains 600 µl of room temperature isopropanol and centrifuged at 13,000 rpm for 2 minutes. Then, the supernatant was discarded before adding 600 µl of 70% (v/v) ethanol to the pellet and mixed by gentle inversion of the tube several times. This is followed by centrifugation at 13,000 rpm for 2 minutes and the supernatant was removed. The pellet was left to air-dry for 15 minutes. Then, 100 µl of DNA Rehydration Solution was added to the tube, gently mixed, and incubated at 65°C for 1 hour. The DNA was stored at -20°C for further used.

4.2.1.2 DNA Extraction using Colony Polymerase Chain Reaction (PCR)

DNA from was also extracted using colony PCR (alkaline lysis technique) according to Rehan (2012). A pure colony from freshly streaked agar plate was scraped using sterile pipette tip and placed into the PCR tube containing 20 µL of 0.02M NaOH. Next, the sample was incubated at 95°C for 10 min. Sample were then placed on ice for 5 min, followed by centrifugation (Eppendorf Minispin) at maximum speed for 2 min. After that, the 2 µL supernatant was saved for later use.

4.2.3 DNA Quantification and Purify Determination

Genomic DNA that was extracted using the Wizard Genomic DNA Purification Kit was quantified using the NanoPhotometer® P-Class (Implen, Germany). 1 µl of DNA Rehydration Solution was used as control or blank sample to obtain zero value, and 1 µl of extracted DNA was measured by reading at 260 nm to 280 nm (A_{260}/A_{280}). Pure DNA was considered at a range of 1.6 to 2.0.

4.2.4 Polymerase Chain Reaction (PCR) Amplification

All bacterial isolates were identified through amplification, sequencing, and sequence analysis of their 16S rRNA gene. Amplification was carried out using the standard polymerase chain reaction (PCR) using the universal primer pair 27F and 1492R (Table 4.1) targeting the 16S rRNA gene with the expected size of approximately 1,500 bp (Weisburg *et al.*, 1991). All PCR reactions were conducted in a total volume of 25 µL. The PCR components for each tube and standard protocol used are detailed in Table 4.2 and Table 4.3. PCR reaction was performed with the following conditions: Initial denaturation 95°C for 2 min and then 35 amplification cycles at 95°C for 40 sec, 55°C for 40 sec, and 72°C for 90 min. A final extension at 72°C for 10 min. This PCR amplification process was carried out using T100 Thermal Cycler (Bio Rad, Singapore).

4.2.5 Agarose Gel Electrophoresis and DNA Visualisation

The PCR products were analysed by 1% (w/v) agarose gel electrophoresis. The agarose gel was made by dissolving 0.4 g agarose powder in 40 ml of 1× TAE buffer and then heated on hotplate until the gel solution turns clear. In order to make the DNA visible in the gel, 1.6 µL of FloroSafe DNA Stain (1st BASE) were added to the gel solution after it was left to cool down. A 1 kb DNA ladder (GeneRuler) were loaded into the well of agarose gel as reference marker for the aliquots of DNA amplified products. The electrophoresis was conducted with a constant voltage of 100V for 35 min. After electrophoresis, the fractionate DNA were visualised under UV light using computer-based gel documentation system, FireReader V4 (Uvitec, Cambridge).

4.2.6 Data Analysis

The PCR products were outsourced for purification and sequencing to 1st Base Laboratories Sdn. Bhd. (Seri Kembangan, Selangor). Following sequencing, Basic Local Alignment Search Tool (BLAST) programme were used to detect the results of fluorescence-based DNA sequence similarity in a non-redundant sequence database from gene bank (Altschul *et al.*, 1997). The DNA sequence results were displayed using Chromas Version 2.5.1. Then, multiple alignment of DNA sequences was done with ClustalX Version 1.81 (Thompson *et al.*, 1997). The phylogenetic analysis was performed using Molecular Evolutionary Genetic Analysis (MEGA) software Version 6.0 (Tamura *et al.*, 2011) and clustering was performed using the neighbour-joining method (Saitou and Nei; 1987).

Table 4.1. Primers used in this study

Gene	Primer	Sequences	Direction	Reference
16S	27F	5'- AGAGTTTGATCCTGGCTCAG-3'	Forward	Weisburg <i>et al.</i> (1991)
	1492R	5'-GGTACCTTGTTACGACTT-3'	Reverse	

Table 4.2. PCR components

Component	1x reaction (25 µl)
ddH ₂ O	7.9
PCR master mix	12.5
Forward 16s rRNA (10mM)	1.3
Reverse 16s rRNA (10mM)	1.3
Template DNA	2.0

Table 4.3. Parameter of PCR process

Step	Temperature (°C)	Time	Cycle
Initial denaturation	95	2 min	1
Denaturation	95	40 sec	35
Annealing	55	40 sec	
Extension	72	90 min	
Final extension	72	10 min	1

4.3 Results and Discussion

4.3.1 Analysis of the Amplified 16S rRNA Gene Product on Agarose Gel

All 115 bacterial samples isolated from the eight sea cucumber specimens and their surrounding water and sediments were subjected to genetic identification procedure, which include DNA extraction, PCR amplification and DNA sequencing of the 16S rRNA gene. However, only 60 samples (comprising of 50% of total isolates) were successfully amplified using PCR and showed bands of the expected size, which is 1,500 bp (Figure 4.1). These include 22 bacterial samples from Pangkor Island, Perak and 38 isolates from Manukan Island, Sabah.



Figure 4.1: PCR products of amplified 16S rRNA gene for selected bacterial isolates with approximately 1,500 bp in size

The absence of bands in certain samples could potentially be due to the unsuccessful extraction of DNA from these bacterial strains. Another possibility could also be caused by the single pair of universal primers used in this study, in which its sequence may not covering some species of bacteria (Ben-Dov *et al.*, 2006; Barghouthi, 2011). This may explain the failure of amplification and obtaining bands from the samples and could be overcome by multiplexing using various primer pairs targeting

the 16S rRNA gene sequence (Barghouthi, 2011). Due to the time limitation and financial constraints of this project, samples that did not show visible bands after at least two attempts of DNA extraction and amplification were not proceeded to be sequenced and genetically identified.

4.3.2 Bacterial Identification using BLAST

Bacteria isolated from sea cucumbers and their surrounding seawater and sediments were further identified using 16S rRNA gene sequencing and sequence analysis. Out of the 60 amplified samples, only 58 sequences were obtained and further analysed. The sequences of other two amplified samples were excluded due to poor sequence data, which could be due to the low amplification efficiency since the PCR products also showed faint bands on gel electrophoresis even after repeated DNA extraction and amplification procedure.

The sequences for the 16S rRNA gene obtained were compared to the sequences available in the NCBI database using BLAST (Table 4.4 and 4.5). Overall, 58 bacterial isolates were identified and displayed 97% to 99% similarities with sequences in the database, except for PMEC2 sample isolated from the cuticle of *H. atra* PME that showed 81.82% similarity with *Exiguobacterium profundum*. Low percentage of identity (<95%) obtained is potentially due to unavailability of the sequence in the database for alignment or can also be explained by detection of a new bacterial species (Barghouthi, 2011). However, more studies are needed to confirm if the isolated strain is from a unique or new species, such as using whole genome sequencing of the strain.

4.3.2.1 Identification of Bacterial Samples from Pangkor Island, Perak

A total of 22 sequences of the partial 16S rRNA gene were successfully obtained and identified for bacterial strains isolated from the two *Holothuria leucospilota* specimens (PPA and PPB) as well as the sediment and seawater samples collected in Pangkor Island, Perak. From the BLAST analysis, *Vibrio* is the predominant bacterial genus identified from the Pangkor Island samples with 19 isolates, comprising of 86% of the identified bacteria from Pangkor Island, followed by two samples of *Acinetobacter* and one isolate from the *Bacillus* genus. These include fourteen isolates of *Vibrio parahaemolyticus*, four isolates of *Vibrio harveyi*, two samples of *Acinetobacter schindleri*, and one each of *Vibrio maritimus* and *Bacillus thuringiensis* (Table 4.4). The identified genera were consistent with their morphological observations under the microscope, in which short-curved rods observed for *Vibrio* spp., round forms for *Acinetobacter* spp., and long curved rod for *Bacillus* spp. (Chapter 3).

Table 4.4. Closest Relatives of Bacteria Isolated from Sea Cucumbers and Surrounding Water and Sediments in Pangkor Island, Kedah

Location	Sea Cucumber	Sources	Isolates	Corresponding Match in Genbank (NCBI)	Query Cover	Identity (%)	Genbank Accession number
Giam Island, P.Pangkor	<i>Holothuria (Mertensiothuria) leucospilota</i> (PPA)	Water	PPAW	<i>Vibrio parahaemolyticus</i> strain Ramsar	99%	99.67%	KJ704113.2
		Sediment	PPAS	<i>Vibrio parahaemolyticus</i> strain HH101313	99%	99.59%	NR_113604
		Cuticle	PPAC1	<i>Vibrio harveyi</i> strain NCIMB1280	99%	99.65%	NR_043165.1
		Cuticle	PPAC2	<i>Vibrio parahaemolyticus</i> strain K5	100%	99.92%	MN810388.1
		Cloaca	PPAL	<i>Vibrio parahaemolyticus</i> strain VP-ABTNL	99%	99.86%	MG589511.1
		Tentacle	PPAT1	<i>Vibrio harveyi</i> strain B10-2	100%	99.28%	MK102609.1
		Coelomic fluid	PPACOE	<i>Vibrio parahaemolyticus</i> strain Ramsar	99%	97.84%	KJ704113.2
		Gastrointestine	PPAG1	<i>Vibrio parahaemolyticus</i> strain ATCC 17802	100%	99.13%	NR_119058.1
		Gastrointestine	PPAG2	<i>Vibrio harveyi</i> strain S-39	99%	100.00%	JF412246.1
		Gastrointestine	PPAG3	<i>Vibrio maritimus</i> strain Disc2	100%	98.01%	KP843717.1
Teluk Nipah, Pangkor	<i>Holothuria (Mertensiothuria) leucospilota</i> (PPB)	Water	PPBW	<i>Acinetobacter schindleri</i> strain H3 chromosome	100%	99.67%	CP030754.1
		Sediment	PPBS	<i>Vibrio parahaemolyticus</i> strain SC2	100%	99.70%	MK308579.1
		Cuticle	PPBC	<i>Vibrio parahaemolyticus</i> strain BC36	100%	99.86%	MT325878.1
		Cloaca	PPBL2	<i>Vibrio parahaemolyticus</i> strain Vp-4	100%	100.00%	MK377081.1
		Tentacle	PPBT1	<i>Vibrio parahaemolyticus</i> strain Ramsar	99%	99.72%	KJ704113.2
		Tentacle	PPBT2	<i>Vibrio parahaemolyticus</i> strain Vp-4	100%	99.92%	MK377081.1

		Cuvierian tubules	PPBV	<i>Bacillus thuringiensis</i> strain ODPY	99%	99.86%	HM770098.1
		Coelomic fluid	PPBCOE	<i>Acinetobacter schindleri</i> strain SGAir0122	100%	99.93%	CP025618.2
		Gastrointestine	PPBG	<i>Vibrio parahaemolyticus</i> strain Ramsar	99%	99.79%	KJ704113.2
		Polian vesicle	PPBP1	<i>Vibrio harveyi</i> strain NBRC 15634	100%	99.64%	NR_113784.1
		Polian vesicle	PPBP2	<i>Vibrio parahaemolyticus</i> strain SC2	100%	99.85%	MK308579.1
		Respiratory tree	PPBR	<i>Vibrio parahaemolyticus</i> strain Vp-F7	99%	99.57%	MH298562.1

Vibrio is the main bacterial genus associated with the *H. leucospilota* specimens from Pangkor Island. *Vibrio* spp. are commonly found in the marine, estuarine, and freshwater environment worldwide (Romalde *et al.*, 2014). The presence of bacteria from *Vibrio* genus were also previously reported in sea cucumbers of *Apostichopus japonicas* (Enomoto *et al.*, 2012; Gao *et al.*, 2014; Kim *et al.*, 2017), from coelomic fluid of *Holothuria forskali* (Offret *et al.*, 2019) intestine of *H. atra* (Ward-Rainey *et al.*, 1999) and skin and intestine of *S. vastus* and *H. leucospilota* (Wibowo *et al.* 2019). A previous research has also isolated bacteria of *Vibrio* sp. from the coelomic fluid of *H. leucospilota* from Dayang Bunting Island, Kedah, with the probable species of *Vibrio vulnificus* or *Vibrio furnissii* (Lukman *et al.*, 2014).

Vibrio parahaemolyticus is the most abundant bacterial species isolated from the external and internal body parts of both *H. leucospilota* PPA and PPB, comprising of 63.6% of total identified bacterial strain from Pangkor Island samples. The presence of *V. parahaemolyticus* in the internal body parts of the two sea cucumbers are most likely due to the prevalence of the species in the location where the sea cucumbers are collected. This is because sea cucumbers obtain food by ingesting marine sediment, or by filtration of seawater (Sulardiono *et al.*, 2020). Consistently, the species of *V. parahaemolyticus* was also found from the samples collected at the surrounding sediments of the two sea cucumbers (designated as PPAS and PPBS) and from seawater samples at Giam Island where *H. leucospilota* PPA was collected (PPAW).

V. parahaemolyticus was also previously found from the surface of a sea cucumber *Stichopus badionotus* collected from the west coast of Peninsular Malaysia (Alipiah *et al.*, 2016). Furthermore, *V. parahaemolyticus* was reported to be frequently

found in sediments, plankton, fish, crustaceans, and bivalve molluscs (Iida *et al.*, 2006). Iida *et al.* (2006) further suggested that water temperature and salinity played important roles in the growth of this species. During the summer season in Europe and the United States, as the temperature rose around 25°C and above, this species was frequently found; and interestingly, in Southeast Asia the *Vibrio* species could be found all year round (Zulkifli *et al.*, 2009). In Malaysia, a high marine temperature between 25 and 35°C favours the outbreaks of *V. parahaemolyticus* (Zulkifli *et al.*, 2009). This bacterial species can be pathogenic to human, and it has been shown that the species isolated from cultured sea cucumber *Apostichopus japonicas* in China possess antimicrobial resistance properties which may pose risk to public health and the environments (Jiang *et al.*, 2014). Since *V. parahaemolyticus* is the prevalent bacteria associated with *H. leucospilota* in the present study, further characterization on the bacterial pathogenicity could be useful for public consumption on the sea cucumbers.

V. harveyi is the second major species found from the external and internal body parts of *H. leucospilota* PPA and PPB, making up to 18.1% of total bacterial strain identified from Pangkor Island samples. This bacterial species has also been isolated from the surface of *Stichopus badionotus* collected from Port Dickson beach, Negeri Sembilan in a previous study by Alipiah *et al.* (2016). *V. harveyi* is known as light-emitting producer or luminous bacterium (Hui & Sherkat, 2005). Many marine animals generate their light by developing symbiotic relationships with luminous bacteria from the genera *Vibrio* or *Photobacterium* as protection, and in return the host provides a nutrient rich environment for the growth of the bacterium (Lin *et al.*, 2001).

The rest of the bacterial species isolated from the *H. leucospilota* PPA and PPB were *Vibrio maritimus* isolated from gastrointestinal of PPA, as well as *Bacillus thuringiensis* and *Acinetobacter schindleri* from the Cuvierian tubules and coelomic fluid of PPB respectively. The bacteria *A. schindleri* found in the coelomic fluid of *H. leucospilota* PPB is similar to the same bacterial species identified in the seawater sample surrounding PPB at Teluk Nipah Beach, Pangkor Island, hence is likely obtained by the sea cucumber from its surrounding. The genera *Acinetobacter* and *Bacillus* were common among sea cucumbers in previous studies (Yasoda *et al.*, 2006, Gao *et al.*, 2014; Wibowo *et al.*, 2019). *B. thuringiensis* in particular was identified in farmed sea cucumber *Apostichopus japonicus* from China which increased in density during possible different stages of skin ulceration (Yang *et al.*, 2018). However, no case reports of disease in sea cucumbers due to *A. schindleri* and *B. thuringiensis* have been reported to date. There is also little information about the association of *V. maritimus* with sea cucumbers, and a novel strain of this species has been isolated by Chimetto *et al.* (2011) from a coral species in Brazil, *Palythoa caribaeorum*.

4.3.2.2 Identification of Bacterial Samples from Manukan Island, Sabah

Altogether there are 36 bacterial strains identified using BLAST analysis for the samples isolated from sea cucumbers in Manukan Island, Sabah including their surrounding seawater and sediments, which include 15 isolates of *Bacillus cereus*, three isolates of *Staphylococcus saprophyticus*, two samples each of *Bacillus megaterium*, *Acinetobacter pittii*, *Staphylococcus warneri* and *Paracoccus zeaxanthinifaciens*, and one sample each of *Bacillus wiedmanii*, *Bacillus vietnamensis*, *Staphylococcus*

pasteuri, *Staphylococcus warneri*, *Staphylococcus equorum*, *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Microbacterium oxydans*, *Brevundimonas vesicularis*, *Exiguobacterium profundum* and *Stenotrophomonas maltophilia* (Table 4.5).

The dominant genus identified from samples collected in Manukan Island, Sabah is *Bacillus*, which include 52.8% of the total bacterial isolates from Sabah samples. *Bacillus* spp. were found from body parts of the six sea cucumber specimens, which is also consistent with the bacteria identified from their surrounding sediments and seawater samples, particularly the seawater samples surrounding *B. vitiensis* PMB and *H. atra* PMD as well as sediments nearby *H. edulis* PMA and *B. vitiensis* PMB. In terms of bacterial species, *Bacillus cereus* is the most abundant, comprising of 42% of the total bacterial species identified from Sabah samples. Furthermore, two samples of *Bacillus megaterium* were identified, which were isolated from coelomic fluid of *H. edulis* PMA and cuticle of *H. atra* PMD. Other *Bacillus* spp. include *Bacillus vietnamensis* isolated from cuticle of *H. atra* PMD and *Bacillus wiedmanii* from seawater samples surrounding *B. vitiensis* PMB.

Bacteria from the genus *Bacillus* were previously indicated to form a major group in the marine environment (Jensen *et al.*, 1996). Even though studies have demonstrated the epidemiological and pathogenesis effect *B. cereus* to humans (Rasko *et al.*, 2005), some strains of this species have also been characterized for various applications including for productions of numerous enzymes and metabolites, removal of various heavy metals and persistent organic pollutants, besides promoting growth of animals and plants as probiotics (Liu *et al.*, 2017). Marine bacteria from the genus

Bacillus isolated from sea cucumber *Aspotechopus japonicus* have also been studied for their effectiveness to be used as probiotics for sea cucumbers.

For example, Lu *et al.* (2021) demonstrated that dietary composition that include *B. licheniformis* and *B. amyloliquefaciens* at an optimal concentration can enhance growth performance, up-regulate innate immunity and alter microbial colonization in the gut of sea cucumbers, hence can be used as their probiotics in artificial farms. Similarly, Li *et al.* (2015) demonstrated that *A. japonicus* feed containing *B. subtilis* and *B. cereus* can stimulate non-specific immune responses and enhance the growth performance of sea cucumbers and was effective to control infections caused by *V. alginolyticus*.

Furthermore, *B. cereus* added with dietary rhubarb and yeast polysaccharide were also shown to improve the growth, intestinal morphology, and immune responses of juvenile *A. japonicus* (Yang *et al.*, 2015). Since most of the bacterial samples identified from the internal body parts of sea cucumbers from Manukan Island, Sabah in this study are from the *Bacillus* genus, therefore, these *Bacillus* spp. could be further characterized for their potential to be developed as probiotics for improving the health of sea cucumbers in Malaysia.

Table 4.5. Closest Relatives of Bacteria Isolated from Sea Cucumbers and Surrounding Water and Sediments in Manukan Island, Sabah

Sea Cucumber	Sources	Isolates	Corresponding Match in Genbank (NCBI)	Query Cover	Identity (%)	Genbank Accession number
<i>Holothuria</i> (<i>Halodeima</i>) <i>edulis</i> (PMA)	Sediment	PMAS1	<i>Bacillus cereus</i> strain HZLJC 2-7	100%	99.86%	MT605417.1
	Coelomic fluid	PMACOE2	<i>Bacillus cereus</i> strain S8	100%	100.00%	MT611946.1
	Coelomic fluid	PMACOE3	<i>Bacillus megaterium</i> strain PF18X	99%	99.82%	MK574950.1
	Polian vesicle	PMAP2	<i>Bacillus cereus</i> strain S8 16S	100%	99.92%	MT611946.1
<i>Bohadschia</i> <i>vitiensis</i> (PMB)	Water	PMBW1	<i>Bacillus cereus</i> strain NRC215	100%	99.91%	MT229271.1
	Water	PMBW2	<i>Bacillus wiedmannii</i> strain SN2-2	100%	100.00%	MT071682.1
	Sediment	PMBS2	<i>Bacillus cereus</i> strain HYS02	99%	99.82%	MF101471.1
	Sediment	PMBS3	<i>Staphylococcus pasteurii</i> strain F71053	99%	99.56%	HQ908689.1
	Cuticle	PMBC1	<i>Bacillus cereus</i> strain JS10	100%	99.71%	MT102922.1
	Cuticle	PMBC2	<i>Bacillus cereus</i> strain HZLJC 2-7	100%	100.00%	MT605417.1
	Tentacle	PMBT	<i>Bacillus cereus</i> strain FSI-4	100%	100.00%	MG737476.1
<i>Holothuria</i> (<i>Halodeima</i>) <i>atra</i> (PMC)	Cuticle	PMCC1	<i>Pseudomonas aeruginosa</i> strain MLTBM2	100%	100.00%	MT646431.1
	Cuticle	PMCC2	<i>Paracoccus zeaxanthinifaciens</i> strain ATCC 21588	100%	99.32%	NR_025218.1
	Cuticle	PMCC3	<i>Bacillus cereus</i> strain FSI-4	99%	99.81%	MG737476.1
	Tentacle	PMCT1	<i>Microbacterium oxydans</i> strain E3	100%	99.56%	KC967221.1
	Tentacle	PMCT2	<i>Brevundimonas vesicularis</i> strain BAC1070	100%	99.90%	HM355660.1
	Coelomic fluid	PMCCOE2	<i>Bacillus cereus</i> strain JS10	100%	100.00%	MT102922.1

<i>Holothuria</i> (<i>Halodeima</i>) <i>atra</i> (PMD)	Water	PMDW	<i>Bacillus cereus</i> strain HZLJC 2-7	100%	100.00%	MT605417.1
	Sediment	PMDS1	<i>Acinetobacter pittii</i> strain BGRI EBC SK18-S8	100%	99.90%	MK332521.1
	Sediment	PMDS2	<i>Pseudomonas stutzeri</i> strain U2-n-1	100%	97.91%	MT126494.1
	Cuticle	PMDC2	<i>Acinetobacter pittii</i> strain NQ-003	100%	98.96%	CP035109.1
	Cuticle	PMDC3	<i>Paracoccus zeaxanthinifaciens</i> strain ATCC 21588	100%	99.81%	NR_025218.1
	Cuticle	PMDC4	<i>Bacillus megaterium</i> strain AK4	100%	100.00%	MK966390.1
	Cloaca	PMDL	<i>Staphylococcus saprophyticus</i> SPB40-5 gene	100%	99.75%	LC511705.1
	Tentacle	PMDT1	<i>Staphylococcus saprophyticus</i> strain IF1SW-B3	100%	99.90%	KY218803.1
	Coelomic fluid	PMDCOE	<i>Staphylococcus warneri</i> strain DK131	100%	99.90%	MT642942.1
	Gastrointestine	PMDG	<i>Bacillus cereus</i> strain HYM88	100%	100.00%	KT982245.1
	Polian vesicle	PMDP	<i>Staphylococcus saprophyticus</i> strain IIHR-NFCBW33	100%	98.91%	MT734014.1
<i>Holothuria hilla</i> (PME)	Cuticle	PMEC1	<i>Bacillus cereus</i> strain HYM88	100%	100.00%	KT982245.1
	Cuticle	PMEC2	<i>Exiguobacterium profundum</i> strain APBSDSB170	90%	81.82%	MG705716.1
	Tentacle	PMEL1	<i>Stenotrophomonas maltophilia</i> strain IAM 12423	100%	98.69%	NR_041577.1
	Tentacle	PMET1	<i>Bacillus cereus</i> ATCC 14579	99%	99.73%	NR_074540.1
	Respiratory tree	PMER2	<i>Staphylococcus warneri</i> strain AW 25	100%	100.00%	NR_025922.1
<i>Holothuria</i> (<i>Halodeima</i>) <i>atra</i> (PMF)	Coelomic fluid	PMFCOE	<i>Staphylococcus equorum</i> strain PA 231	99%	100.00%	NR_027520.1
	Gastrointestine	PMFG	<i>Bacillus cereus</i> strain CUMB NS 07	100%	100.00%	MN197732
	Respiratory tree	PMFR2	<i>Bacillus vietnamensis</i> strain 15-1	100%	98.77%	NR_024808.1

Staphylococcus spp. is the second major genus identified from Sabah samples, comprising of 19.4% of the total bacteria from Sabah. This includes three *Staphylococcus saprophyticus* isolated from tentacle, cloaca and polian vesicle of *H. atra* PMD, while two samples of *Staphylococcus warneri* were isolated from coelomic fluid of *H. atra* PMD and respiratory tree of *H. hilla* PME. In addition, *Staphylococcus equorum* and *Staphylococcus pasteurii* were isolated from coelomic fluid of *H. atra* PMF and sediment surrounding *B. vitiensis* PMB, respectively. The association of *Staphylococcus* spp. with sea cucumber is in line with a previous study that detected *Staphylococcus* spp. from samples of the sea cucumber *A. japonicus* from South Korea, in particular *S. warneri* and *S. sciuri* (Kim *et al.*, 2017). In addition, an orange pigmented *S. kloosii* was also isolated from the respiratory tree of *H. leucospilota* from Teluk Nipah Island, Pangkor Island, Perak (Kamarudin *et al.*, 2013).

Other bacterial species identified include two samples of *Acinetobacter pittii* isolated from *H. atra* PMD cuticle and its surrounding sediment, while two samples of *Paracoccus zeaxanthinifaciens* were isolated from cuticle of *H. atra* PMC and PMD. The rest of identified species occur as singletons, which are *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Microbacterium oxydans*, *Brevundimonas vesicularis*, *Exiguobacterium profundum* and *Stenotrophomonas maltophilia* isolated from external body parts of *H. atra* PMC, *H. atra* PMD and *H. hilla* PME. *Pseudomonas* spp. have also been previously isolated from the coelomic fluid of *H. leucospilota* and in the intestine of *Stichopus japonicus* (Lukman *et al.*, 2014; Gao *et al.*, 2017), which are both the internal parts of the sea cucumbers unlike the two *Pseudomonas* strains that were isolated from the sea cucumber external parts in this study.

Bacteria from genera *Staphylococcus* and *Pseudomonas* have long been associated with human diseases, however, there are also strains from these bacterial group that are known to be non-pathogenic and may act as good probiotics to humans or animals (Khusro *et al.*, 2018; Divya *et al.*, 2018; Sung *et al.*, 2010). For example, *Staphylococcus* sp. bacteria have been isolated from the intestine of *H. leucospilota* and *H. atra* from Bandengan Beach, Jepara, Indonesia, with the potential as probiotics for human health, besides other bacteria such as *Micrococcus* sp., *Rothia* sp., and *Bacillus* sp. (Pringgenies *et al.*, 2020). Gao *et al.* (2014) also demonstrated that potential probiotic candidates for aquaculture application were detected in the gut of the sea cucumber *A. japonicas* from Shandong Province, China, which include the genera *Pseudomonas*, *Bacillus* and some lactic acid bacteria.

Likewise, members of the genus *Acinetobacter* have been known as important opportunistic human pathogens, and the species *A. pittii* has been isolated from a diseased fish *Megalobrama amblycephala* in China, suggesting that it is pathogenic to the fish (Li *et al.*, 2017). However, a recent study by Bunnoy *et al.* (2019) isolated a new bacterial strain closely related to *A. pittii* from the skin mucus of healthy catfish in Thailand, which showed the potential as probiotic in catfish farming and have antagonistic activity against various fish pathogens. The strain is also believed to be safe for host and human health (Bunnoy *et al.* 2019). Bacteria from the genus *Acinetobacter* were also previously detected in asymptomatic sea cucumbers collected from the suburbs of Dalian in north-eastern China and were not detected in symptomatic or diseased sea cucumbers (Li *et al.*, 2010). This suggests that *Acinetobacter* spp. are not associated with diseases of the sea cucumbers.

Apart from that, the presence of *E. profundum* on cuticle of *H. hilla* PME concurs well with a previous finding of the same species on the surface of sea cucumber *Stichopus badionotus* collected from the west coast of peninsular Malaysia (Alipiah *et al.*, 2016). Similarly, the existence of *S. maltophilia* on tentacle of *H. hilla* PME confirms previous results by Lukman *et al.* (2014) in which the species was shown to be present in the coelomic fluid of *H. leucospilota* from Dayang Bunting Island, Yan, Kedah among other genera including *Bacillus*, *Exiguobacterium*, *Pseudomonas*, *Stenotrophomonas* and *Vibrio*. The authors also reported that the *S. maltophilia* showed moderate resistance towards the antibiotic streptomycin and lower resistance towards kanamycin.

Three other bacterial species identified from the Manukan Island samples, which are *Paracoccus zeaxanthinifaciens*, *Microbacterium oxydans* and *Brevundimonas vesicularis*, all of which were isolated from external parts (tentacle and cuticle) of *Holothuria atra* (PMC), have not yet been previously documented to be associated with sea cucumbers as to our knowledge. However, bacteria from the genus *Paracoccus* have previously been found in both *H. leucospilota* and *S. vastus* from Lampung, Indonesia, which exhibited antimicrobial activity (Wibowo *et al.*, 2019). Besides, Yang *et al.* (2015) showed that a strain of *Paracoccus marcusii* could act as a probiotic along with *B. cereus* and showed a positive effect on the growth performance and immune response in coelomocytes and the intestine of *A. japonicus*. Bacteria from the genus *Microbacterium* spp. have been indicated to be present in the digestive tracts of juvenile *A. japonicus* in Qingdao, China (Sha *et al.*, 2016). Meanwhile, the genus *Brevundimonas* has not been previously found to be associated with sea cucumbers,

although *B. vesicularis* has been detected from the surface water of pond in Germany (Beilstein *et al.*, 2006).

4.3.3 Phylogenetic Analysis and Overall Evaluation of Bacterial Isolates from Pangkor Island, Perak and Manukan Island, Sabah

Phylogenetic analysis combining both bacterial isolates from Pangkor Island and Manukan Island was carried out to analyse the bacterial isolates relatedness between these two different locations and to confirm the relationship between bacterial isolates and known reference strains from NCBI database. The phylogenetic analysis was performed with the neighbour-joining method using the Kimura 2-parameter model (Kimura, 1980). A number of 75 taxa and 952 characters representing aligned base positions were involved in the final dataset. Of the 75 taxa, 19 taxa were corresponding sequences from the Genbank. Sequences from the present study are in code, whereas the corresponding sequences from GenBank are shown with their full name.

The optimal tree with the sum of branch length = 2.32369329 is shown in Figure 4.2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (i.e., 1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated. Based on the sequence similarity and confirmed by the phylogenetic clustering, the most closely related type strains of all isolates were

determined. The taxonomic relatedness of bacterial isolates between two different locations which are Pangkor Island and Manukan Island were also shown.

The tree demonstrated that the bacteria isolated from sea cucumber specimens as well as the sediment and seawater samples belonged to four classes of three bacterial phyla, the Firmicutes (Bacilli), Proteobacteria (Alphaproteobacteria, Gammaproteobacteria) and Actinobacteria (Actinobacteria). The phylum Firmicutes was represented by 37 strains from three genera *Bacillus*, *Staphylococcus* and *Exiguobacterium* in class Bacilli with 93% bootstrap support, with *Bacillus* being the dominant genus. Meanwhile, class Alphaproteobacteria and Gammaproteobacteria were clustered together under phylum Proteobacteria with 93% bootstrap support. Five strains consisting of representatives of genera *Paracoccus* and *Brevundimonas* belonged to class Alphaproteobacteria with 99% bootstrap support and 29 strains from four genera *Stenotrophomonas*, *Acinetobacter*, *Pseudomonas* and *Vibrio* belonged to Gammaproteobacteria with 99% bootstrap support. *Vibrio* was the dominant genus from the phylum Proteobacteria. The other two strains belonged to phylum Actinobacteria. They were affiliated with the genus *Microbacterium* from class Actinobacteria with 99% bootstrap support. The Neighbour Joining method grouped the identified bacterial isolates with their corresponding reference strains from the GenBank in similar clusters. Only two bacterial isolates, PPAS and PPBG from sediment and gastrointestinal of *H. leucospilota* were grouped differently by NJ tree.

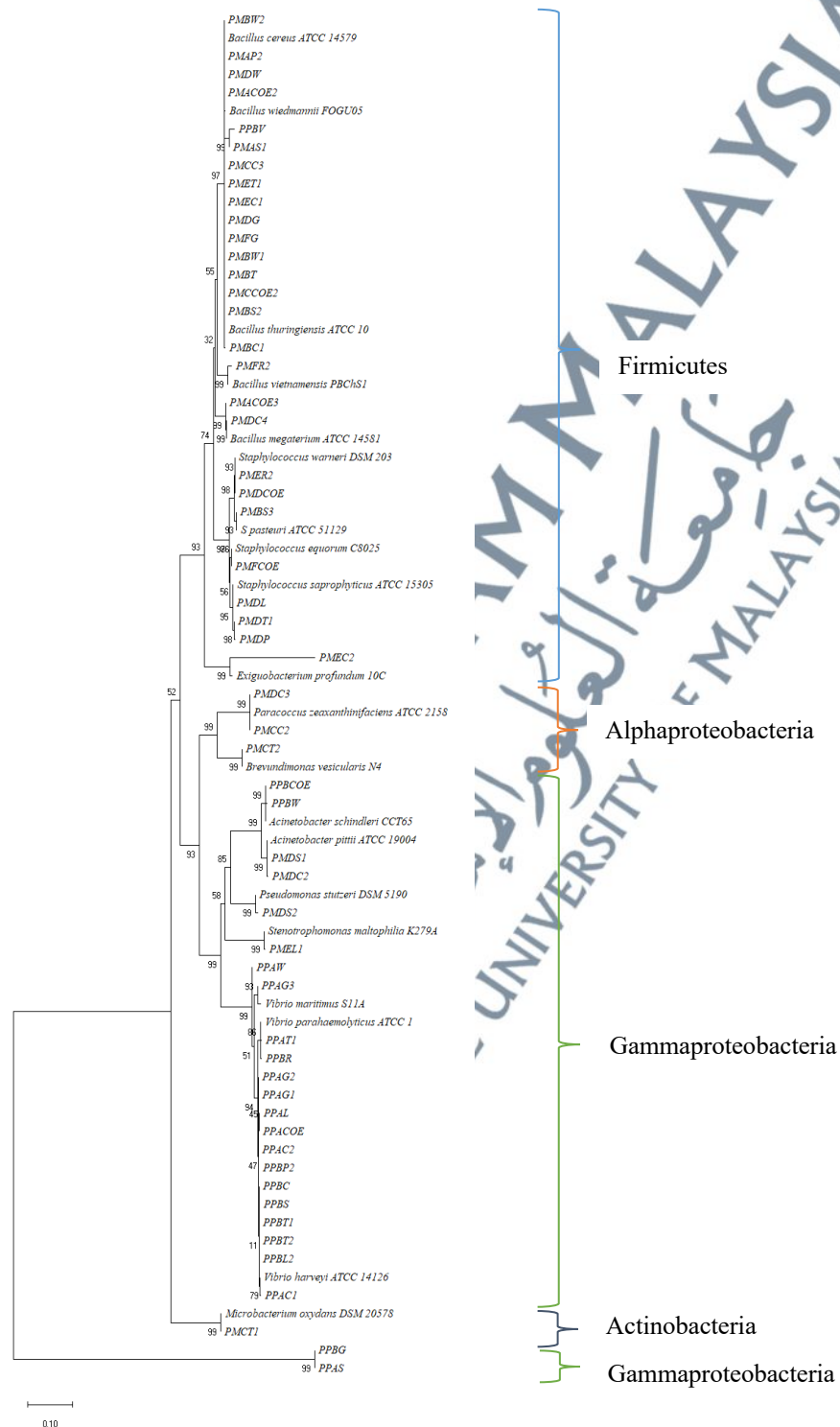


Figure 4.2. Phylogenetic tree built with the neighbour-joining method with the aligned 16S rRNA gene sequences of bacterial strains associated with the sea cucumbers from Pangkor Island and Manukan Island.

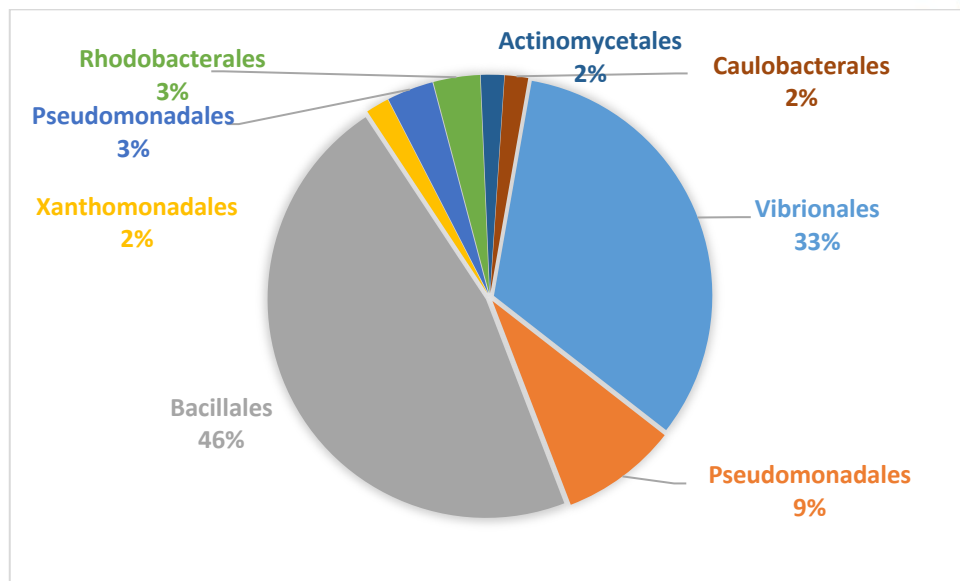


Figure 4.3. Overall order of identified bacteria isolated from sea cucumbers and surrounding sediments and seawater from Pangkor Island, Perak and Manukan Island, Sabah

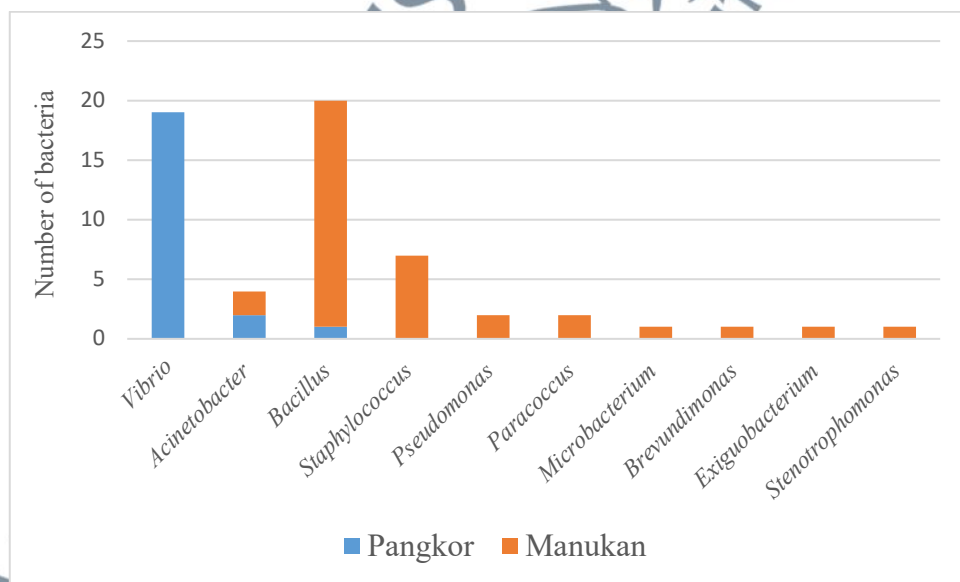


Figure 4.4. Bacterial genera identified from samples collected at Pangkor Island, Perak and Manukan Island, Sabah

Overall, the bacterial isolates can be assigned to three different phyla, consisting of nine different orders (Figure 4.3). The dominant phylum is Proteobacteria, with the highest abundance of samples from Pangkor Island, Perak. This concurs with previous studies that showed the dominance of Proteobacteria associated with sea cucumbers (Gao *et al.*, 2016; Alipiah *et al.*, 2016; Kim *et al.*, 2017; Xu *et al.*, 2019; Zhang *et al.*, 2019). *Microbacterium oxydans* is the only representative of phylum Actinobacteria in this study. This observation is consistent with previous findings by Wibowo *et al.* (2019), in which Actinobacteria were represented by a small number of associated bacteria as compared to the overall sea cucumber microbiome of *S. vastus* and *H. leucospilota*, with the majority of bacteria belong to the phylum Proteobacteria.

In terms of composition of the bacterial community associated with the eight sea cucumber specimens, diverse bacterial species was observed between the two locations i.e., Pangkor Island, Perak and Manukan Island, Sabah, rather than between sea cucumber specimens at the same region (Figure 4.4). For example, sea cucumber samples in both locations harboured different bacterial communities, with *Bacillus* and *Acinetobacter* are the only bacterial genera that were detected in both sites. Furthermore, Pangkor Island samples were predominated with *Vibrio* spp., while the main bacterial genus found in Manukan Island, Sabah is *Bacillus* spp. This indicates geographical variation in terms of the sea cucumber-associated bacterial species, as compared to variation of bacterial communities between different sea cucumber species.

This geographical difference could be due to environmental factors such as water quality, location, depth, temperature, and the presence of other sessile organisms in close proximity (Morrow *et al.*, 2012). Furthermore, Taylor *et al.* (2005) suggested

that allopatric speciation or changes in environmental gradients may also drive site-specificity of associated bacteria, although its underlying mechanism is not yet known. Besides, this difference of sea cucumber-associated bacterial species between Pangkor Island, Perak and Manukan Island, Sabah could also be due to the difference of anthropogenic activities at both locations. This is because Pangkor Island is known to be one of the leading tourist destinations in Malaysia, while Manukan Island has been gazetted as a National Park which could be more properly managed, hence this may have significant effects on the microbial diversity.

The prevalence of pathogenic members of *Vibrio* genus have also been reported in *Stichopus horrens* collected from Pangkor Island, Perak (Chanderan *et al.*, 2019). This pathogenic bacterial outbreak may also be caused by the warm temperature of the marine environment along with poor hygienic practices at the location (Elhadi *et al.*, 2004). Therefore, information on bacterial species associated with sea cucumbers in this study could be helpful for developing proper intervention procedures to protect sea cucumbers and consumers, specifically in Pangkor Island, Perak.

The diversity of bacterial communities associated with the sea cucumbers as well as seawater and sediment is also predicted to be much higher at both Pangkor Island, Perak and Manukan Island, Sabah than was observed in this study. This is because not all of the isolated bacteria were successfully identified. Besides, the culture-dependent technique used in this study may allow only a small proportion of the total community in the marine environment to be identified (Nugraheni *et al.*, 2010), and the actual diversity of bacterial community in sea cucumber can be further characterized using culture-independent techniques of the next generation sequencing along with

increasing the sample size. Nevertheless, the isolated cultivable bacteria found in this study can be further characterized for their potential benefit such as bioactive and probiotic activities, besides investigating their potential pathogenicity to sea cucumbers and consumers.

4.4 Conclusion

A total of 58 bacterial samples isolated from internal and external sea cucumbers body parts, surrounding seawaters and sediments from Pangkor Island, Perak and Manukan Island, Sabah were successfully identified using 16S rRNA gene sequencing and phylogenetic analysis. The identified bacteria are from three phyla, which are Proteobacteria (52%), Firmicutes (46%) and Actinobacteria (2%), and comprising of nine genera. Genus *Bacillus* is the major bacterial strain found associated with the six sea cucumber specimens in Sabah, while *Vibrio* is the main genus found from the two *H. leucospilota* collected in Pangkor Island, Perak. Other genera include *Acinetobacter*, *Staphylococcus*, *Pseudomonas*, *Microbacterium*, *Brevundinomas*, *Exiguobacterium* and *Stenotrophomonas*. Results suggested that there is geographical variation between the sea cucumber-associated bacterial community at the two locations. Further studies are needed to determine the potential pathogenicity of associated bacterial species and to further characterize the non-pathogenic bacteria for various applications.