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Microbial Population in the Coelomic Fluid of *Stichopus chloronotus* and *Holothuria (Mertensiothuria) leucospilota* Collected from Malaysian Waters

(Populasi Mikrob dalam Cecair Selom *Stichopus chloronotus* dan *Holothuria (Mertensiothuria) leucospilota* dari Perairan Malaysia)

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ABSTRACT

This preliminary study aimed to isolate and identify microbes that inhabit the coelomic fluid of two local species of sea cucumbers collected from Malaysian waters - a 'gamat' species i.e. Stichopus chloronotus *Brandt, 1835 and the most abundant 'timun laut' species in Malaysia i.e.* Holothuria (Mertensiothuria) leucospilota *(Brandt 1835). Phylogenetic analyses of partial 16S rRNA mtDNA gene sequences suggested the presence of at least eight microbial genera i.e. five bacterial genera -* Bacillus, Exiguobacterium, Pseudomonas, Stenotrophomonas *and* Vibrio *- isolated from the coelomic fluid of H. leucospilota and three genera of gram-positive bacteria from the* Micrococcaceae *family -* Kytococcus, Micrococcus *and either* Kocuria *or* Rothia *- isolated from the coelomic fluid of* S. chloronotus. *We speculate that less diverse microbial population in* S. chloronotus *as compared to* H. leucospilota *could be due to a number of environmental factors e.g. penetration of light surrounding the habitats of both species, the feeding behaviour of* H. leucospilota *and the higher level of antimicrobial properties of coelomic fluid in* S. chloronotus. *In terms of antimicrobial-resistance capability test, an isolate from genus* Pseudomonas *that is suspected to be* P. alcaligenes *exhibited high resistance towards streptomycin. Another isolate from genus* Stenotrophomonas *that was suspected to be* S. maltophilia *showed moderate resistance towards streptomycin and lower resistance towards kanamycin. Both isolates were from the coelomic fluid of* H. leucospilota. *Tetracycline inhibited the growth of all bacterial isolates tested. Further studies with more specimens of* S. choronotus *and* H. leucospilota *from broader geographical locations and the use of complete mtDNA genes along with morphological approaches for species identification may facilitate to provide better insights into the microbial population in the coelomic fluid of both local sea cucumber species.*

Keywords: Antimicrobial-resistance; coelomic fluid; microbial diversity; partial sequences of 16S ribosomal RNA mitochondrial gene; phylogenetic analyses; sea cucumbers

ABSTRAK

Kajian awal ini bertujuan untuk memencil dan mengenal pasti mikrob yang mendiami cecair selom dua spesies timun laut dari perairan Malaysia – satu spesies gamat iaitu Stichopus chloronotus *Brandt, 1835 dan spesies timun laut yang mempunyai taburan paling tinggi di Malaysia iaitu* Holothuria (Mertensiothuria) leucospilota *(Brandt, 1835). Analisis filogenetik menggunakan separa jujukan gen mitokondria 16S ribosom RNA mencadangkan kehadiran sekurang-kurangnya lapan genus mikrob iaitu lima genus bakteria -* Bacillus, Exiguobacterium, Pseudomonas, Stenotrophomonas *dan* Vibrio *- yang dipencilkan daripada cecair selom* H. leucospilota *dan tiga genus bakteria gram-positif daripada famili* Micrococcaceae *-* Kytococcus, Micrococcus *dan samada* Kocuria *atau* Rothia *- yang dipencilkan daripada cecair selom* S. chloronotus. *Spekulasi kami adalah kepelbagaian populasi mikrob yang lebih rendah pada* S. chloronotus *berbanding dengan* H. leucospilota *mungkin disebabkan oleh faktor-faktor alam sekitar seperti pencahayaan yang melingkungi habitat kedua-dua spesies tersebut, tabiat pemakanan* H. leucospilota *dan tahap kandungan antimikrob yang lebih tinggi dalam cecair selom* S. chloronotus. *Untuk ujian kebolehrintangan antimikrob, satu spesies daripada genus* Pseudomonas *yang berkemungkinan* P. alcaligenes *menunjukkan rintangan tinggi terhadap streptomisin. Spesies lain daripada genus* Stenotrophomonas *yang berkemungkinan* S. maltophilia *menunjukkan rintangan sederhana terhadap streptomisin dan rintangan rendah terhadap kanamisin. Kedua-duanya dipencilkan daripada cecair selom* H. leucospilota. *Tetrasiklin merencat pertumbuhan semua bakteria yang diuji. Kajian lanjut melibatkan lebih banyak spesimen* S. choronotus *dan* H. leucospilota *daripada lokasi geografi lebih luas dan penggunaan gen mitokondria DNA yang lengkap selari dengan pendekatan-pendekatan morfologi untuk pengenalpastian spesies mungkin boleh membantu memberikan gambaran lebih jelas tentang populasi mikrob dalam cecair selom kedua-dua spesies timun laut tempatan.*

Kata kunci: Analisis filogenetik; cecair selom; kepelbagaian mikrob; kerintangan antimikrob; separa jujukan gen mitokondria 16S ribosom RNA; timun laut

Introduction

Phylum Echinoderm is a large group of marine animals worldwide, consisting of starfish, brittle star, sea urchin, feather star and sea cucumber. Among the most popular echinoderms in Malaysia is the sea cucumber; especially the 'gamat' species, known for its health beneficial values e.g. *Stichopus horrens* Selenka, 1867 and *Stichopus herrmanni* Semper, 1868 (Kamarudin et al. 2010; Ridzwan 2007, 1993). Approximately more than 80 species of sea cucumber can be found in marine environment surrounding Malaysia (Kamarudin et al. 2010; Sim et al. 2008; Zulfigar et al. 2008, 2007). They are locally known by many names including 'timun laut', 'bat', 'balat', 'trepang', 'brunok', 'gamat', and 'hoi sum' or 'hai shen'. 'Beche-de-mer' is the other name given by the Portuguese for the 'sea ginseng'. In fact, there are different definitions of 'gamat' from previous studies in Malaysia and 'gamat' is exclusively referred to sea cucumbers of family Stichopodidae. Some Malaysian sea cucumber species are consumed as food while others are being used in traditional and modern-formularised medicines.

Antioxidant properties, analgesic effects, therapeutic effects, anti-anaphylaxis effects, and many more medicinal properties from Malaysian sea cucumbers have been discovered by previous studies (Fredalina et al. 1999; Khartini et al. 2003; Osama et al. 2009; Ridzwan et al. 1995). Antimicrobial activity was also found associated with Malaysian sea cucumbers (Farouk et al. 2007; Kaswandi et al. 2007; Ridzwan et al. 2003). Coelomic fluid of sea cucumber - the fluid within the coelom functioning as a hydrostatic skeleton and as a circulatory medium - has been shown to contain medicinal properties. Hawa et al. (1999) indicated that some form of antioxidant activities were present in the coelomic fluid of Malaysian sea cucumbers and Tan et al. (2005) showed that the coelomic fluid of *S. herrmanni* caused vasorelaxation effect on rat coronary arteries. Coelomocytes as immune cells in the coelomic fluid of sea cucumbers are able to kill infecting agents such as bacteria, and we also believe that the medicinal properties possessed by the sea cucumbers may also be due to the existence and contribution of some microorganisms associated with them.

In this present study, two species of Malaysian sea cucumber - *Stichopus chloronotus* Brandt, 1835 and *Holothuria (Mertensiothuria) leucospilota* (Brandt, 1835) – were used to isolate and identify microbes inhabiting the coelomic fluid of these species. Both species can be found on reef flat zone. *S. chloronotus* is one of the known 'gamat' species in Malaysia. It is a deep black-green sea cucumber. Known as greenfish for its English name, it is locally known as 'talifan varieti hitam' in Malaysia. Osama et al. (2009) studied the *in vitro* antioxidant and antiproliferative activities of *S. chloronotus*, *H. leucospilota* and *H. scabra* Jaeger, 1833 and they suggested that all three species of Malaysian sea cucumber were potential sources of natural antioxidant and anticancer. Among other studies were on the activity of crude extracts mixture from *S. badionotus* (suspected as *Stichopus vastus* Sluiter, 1887) and *S. chloronotus* on fungal growth (Kaswandi et al. 2007) and the antinociceptive effects of extracts from *S. chloronotus* (Ridzwan et al. 2001). Besides, aqueous and ethanol extracts of *S. chloronotus* showed high antimycotic activity against experimentally induced dermatophytosis in guinea pigs (Dayang et al. 2004).

H. leucospilota, a well-known 'timun laut', was suggested as the most abundant species in Malaysia (Choo 2008; Kamarudin et al. 2011). The English name of this soft-bodied species is white threads fish and it is locally known as 'bat puntil' or 'lintah laut'. It is a long and black tubular sea cucumber often with reddish body background, with its mouth surrounded by tentacles and a terminal anus located at the posterior (Brandt 1835). Ridzwan et al. (2003) suggested the potential of water extract from *H. leucospilota* and *Bohadschia marmorata vitiensis* and the coelomic fluid from *S. herrmanni* as alternative analgesic drug sources. *H. leucospilota* was also suggested as a potential source of natural antioxidant and anticancer (Osama et al. 2009).

Considering the high level of abundance of *H. leucospilota* in the marine environment of Malaysia as compared to *S. chloronotus*, the local species may contain indigenous microorganisms or microbes that help it to adapt and exist in various conditions. In fact, not many studies were done to investigate the existence and association of microbes in Malaysian sea cucumbers. Farouk et al. (2007) isolated 30 bacterial strains from *H. (Halodeima) atra* Jaeger, 1833 from Malaysia waters and seven strains recorded moderate antibacterial activity against *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Therefore, this study primarily aimed to isolate and identify microbes inhabiting the coelomic fluid of the local sea cucumber species i.e. *H. leucospilota* and *S. chloronotus*. Subsequently, antimicrobial-resistance capability of the microbes was then studied using Kirby-Bauer diffusion disk test with a little modification.

Materials and Methods

Sampling of *H. leucospilota* was held in July 2007 at Dayang Bunting Island, Yan, Kedah Darul Aman, Malaysia (West Coast) and the sampling of *S. chloronotus* was done in August 2007 at Tioman Island, Pahang Darul Makmur, Malaysia (East Coast). The study sites were on sandy and rocky shores and most documentation were done during the low tide. No fixed or standard sampling hours were allocated for all sites. Global positioning system (GPS) was used to mark and to record the position of each sampling site (not shown specifically). Three specimens from each species - designation of *H. leucospilota* specimens as HL1 until HL3, and *Stichopus chloronotus* specimens as SC1 until SC3 - were dissected immediately at the sampling areas in a sterile condition. Coelomic fluid was collected using 5 mL Eppendorf micropipette and stored in separate

falcon tubes that were later kept in an ice box. Coelomic fluid samples were taken back to the laboratory for isolation of microbes.

A portion from each coelomic fluid sample was diluted with sterilised distilled water (dH_2O) with the ratio of 1:9. A volume of 500 µL of each coelomic fluid sample i.e. the diluted (designated as 10×) and the undiluted samples, making a total of 12 samples, were pipetted and then spread onto separate nutrient agar (NA) plates. After overnight incubation at 37°C, the morphologies of colonies were observed and colonies with different morphologies were subcultured onto new NA plates until pure cultures were obtained. Each single colony was tagged with alphabet only/and number. The cultures were maintained on NA plates and also in nutrient broth (NB).

NA plates containing single colonies were observed under dissecting microscope for its morphological characteristics. The characteristics observed were optical density, shape, colour, edge, elevation and texture. Gram staining was done to further assist in confirmation of preliminary grouping of microbes by previous morphological observation. The slides of Gram-stained samples were observed under light microscope with magnifications of 4, 10, 20, 40 and 100× with oil immersion.

The DNA extraction procedure and the buffer preparation were adopted from Kuske et al. (1998) and other studies. A pure colony that grew on each NA plate was scooped out using inoculation loop and mixed with 200 µL sterilised dH_2O in a 1.5 mL microcentrifuge tube. A volume of 500 µL of extraction buffer (2× cetyl trimethyl ammonium bromide (CTAB)) and 40 µL of 10 mg/mL Proteinase K were added into the microcentrifuge tube and vortexed. The mixture was incubated at 37°C for 30 min. Thirty µL of RNase was added later on and the mixture was incubated again at 37°C for 15 min. After that, 200 µL of phenol/chloroform/isoamyl alcohol (25:24:1) solution was added into the mixture, followed by shaking and vortexing for 15 min. The mixture in the microcentrifuge tube was then centrifuged at 13000 rpm for 1 min. The supernatant was then transferred into a new microcentrifuge tube and equal volume of isopropanol was added and the mixture was placed at -20°C for 30 min. The microcentrifuge tube was then centrifuged at 13000 rpm for 10 min. The supernatant was discarded and the tube was left to air-dry for approximately 2 h before the DNA was eluted with 50 µL of Tris-EDTA (TE) storage buffer. The total genomic DNA was stored at 4°C. Approximate yields of DNA, the quantity and quality, were determined by electrophoresis.

Two universal primers were used for isolation of partial 16S rRNA mtDNA region (approximately 1.5 kilobase pairs (kb)): PA (forward) 5′ - AGA GTT TGA TCC TGG CTC AG - 3′ and PH (reverse) 5′ - AAG GAG GTG ATC CAG CCG CA - 3′. Standard thermal cycle amplification (i.e. polymerase chain reaction (PCR)) was performed in 100 µL reaction volume containing 71.8 µL of sterilised dH_2O, 10.0 µL of 10× PCR reaction buffer, 3.0 µL of magnesium chloride (25 mM), 2.0 µL of each universal primer (10 µM), 2.0 µL of dNTP mix (10 mM), 8.0 µL of the DNA preparation, 0.2 µL of acetylated bovine serum albumin (BSA, 100 mg/mL) and 1.0 µL of 2 u/µL *Taq* DNA polymerase. Master mix was used for large number of samples. Cycle parameters were 1 min at 95°C for pre-heating (before putting samples), 5 min at 95°C for initial denaturation, 1 min at 95°C for denaturation, 1 min at optimised temperature (i.e. 55°C) for annealing, 1 min at 72°C (29 cycles) for extension, 10 min at 72°C for final extension and the temperature was held at 4°C until the samples were taken out. Purification kit from manufacturer were used for direct purification. Purified PCR products in suspension form were prepared prior to sending samples for DNA sequencing.

Three types of restriction enzymes were used for restriction fragment length polymorphism (RFLP) analysis i.e. *Kpn* I (5′…G GTAC$^{\vee}$C…3′, 3′…C$^{\wedge}$CATG G…5′, optimal assay temperature = 37°C), *Sac* I (5′… GAGCT$^{\vee}$C…3′, 3′…C$^{\wedge}$TCGA G…5′, optimal assay temperature = 37°C), and *Sma* I (5′ …CCC$^{\vee}$GGG …3′, 3′…GGG$^{\wedge}$CCC …5′, optimal assay temperature = 25°C). The digestion of purified DNA product was done in a total volume of 20 µL consisting of 13.8 µL of sterilised dH_2O, 3.0 µL of purified DNA product, 2.0 µL of Buffer J, 1.0 µL of restriction enzyme and 0.2 µL of BSA. The mixture was then incubated according to individual optimal assay temperature for 4 h. The pattern of fragmentation or band formed during the endonucleases activity was determined by gel electrophoresis.

The DNA sequences obtained were analysed using multiple bioinformatics softwares for microbial species identification and phylogenetic analyses. The sequence similarity search was done by using the Basic Local Alignment Search Tool program (BLAST), which is available at National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine sequence database (retrieved at www.ncbi.nlm.nih.gov). Three most similar sequences were selected for every sample. All DNA sequences were aligned using ClustalX software (Thompson et al. 1997).

Neighbour-joining (NJ) method and maximum parsimony (MP) method were used to reconstruct phylogenetic trees using phylogeny inference package (Phylip) software version 3.65 (Felsenstein 2004). Bootstrapping was performed to estimate the reliabilities of the nodes in the phylogenetic trees. The bootstrap values were obtained from 100 trees generated randomly with Seqboot and Consense programs in the Phylip software package. Treeview 3.2 software by Page (1996) was used to display and then to edit the reconstructed phylogenetic trees.

As for antimicrobial-resistance capability test, the resistance of the isolated microbes towards broad-spectrum antimicrobial agents were tested. Three common antibiotics were used i.e. tetracycline, streptomycin and kanamycin. One hundred ppm and 50 ppm of these antimicrobial agents were mixed together with the NA prior to pouring them into the plates. Sterilised filter paper disks soaked

with microbial samples (in NB) were placed on the agar surface. Microbial growth was observed for five days.

Results and Discussion

All NA plates spread with the coelomic fluid of *H. leucospilota* (designated as HL1-HL3) exhibited massive growth of microbes covering the whole plates, while NA plates spread with the coelomic fluid of *S. chloronotus* (designated as HL1-HL3) showed fair growth of microbes, each microbe covered at most half of the plate, with some already produced single colonies. After a series of subculturing in order to obtain single and pure colonies of microbes, there were a total of 101 NA plates of microbial colonies. 92 NA plates were microbes isolated from the coelomic fluid of *H. leucospilota* and only nine plates were microbes isolated from the coelomic fluid of *S. chloronotus*.

Preliminary grouping based on the morphological observation and Gram staining reduced the number of microbial isolates from 101 to 27 (Table 1). About 73% of the microbial isolates had been discarded because they were considered as morphologically similar to the 27 microbial isolates. There were microbes that shared almost

TABLE 1. Summary of results of restriction fragment length polymorphism (RFLP) analyses and DNA sequencing of 16S rRNA mtDNA gene of 27 microbial isolates from the coelomic fluid of *Holothuria leucospilota* and *Stichopus horrens*

Microbial isolate	Remarks on RFLP band patterns	DNA Sequencing
Holothuria leucospilota		
HL1 A2	Band patterns could not be seen at all	√
HL1 B3	Not similar to any other isolates	√
HL1 10× A3	Not similar to any other isolates	√
HL1 10× A4	Similar to HL1 10× C1	
HL1 10× B1	Not similar to any other isolates	√
HL1 10× B2	Not similar to any other isolates	√
HL1 10× C1	Similar to HL1 10× A4	√
HL2 A1	Similar to HL2 C2, HL2 C3, HL2 10× C2, and HL2 10× D2	
HL2 A2	Similar to HL2 D4	
HL2 C2	Similar to HL2 A1, HL2 C3, HL2 10× C2, and HL2 10× D2	√
HL2 C3	Similar to HL2 A1, HL2 C2, HL2 10× C2, and HL2 10× D2	
HL2 D4	Similar to HL2 A2	√
HL2 10× C2	Similar to HL2 A1, HL2 C2, HL2 C3, and HL2 10× D2	
HL2 10× D2	Similar to HL2 A1, HL2 C2, HL2 C3 and HL2 10× C2	
HL3 B1	Similar to HL3 10× B1	
HL3 D4	Not similar to any other isolates	√
HL3 10× B1	Similar to HL3 B1	√
HL3 10× C1	Not similar to any other isolates	√
Stichopus chloronotus		
SC1 A	Similar to SC1 D and SC3	
SC1 B	Similar to SC1 C and SC2 B	
SC1 C	Similar to SC1 B and SC2 B	√
SC1 D	Similar to SC1 A and SC3	√
SC1 E	Not similar to any other isolates	√
SC1 F	No bands seen on *Sac*I and *Sma*I	√
SC2 A	Not similar to any other isolates	√
SC2 B	Similar to SC1 B and SC1 C	
SC3	Similar to SC1 A and SC1 D	

The similar ones were considered as from the same species and only one of them was sent for DNA sequencing 16S rRNA mtDNA gene i.e. 16 isolates in total (labelled √). HL1-HL3 – from the coelomic fluid of *Holothuria leucospilota*, SC1-SC3 – from the coelomic fluid of *Stichopus chloronotus*, 10× – 10× dilution of coelomic fluid and alphabet only/and number – microbial colony tags

similar characteristics but it was difficult to determine whether they were from the same group or not. Therefore, these microbial strains were chosen for molecular test. Since there were only nine plates from the coelomic fluid of *S. chloronotus*, all were selected for molecular test. Based on the RFLP analysis, 16 isolates were shortlisted and sent for DNA sequencing (Table 1). Nonetheless, the shortlisting would be more accurate if more restriction enzymes were used in order to produce more distinctive fragmentation or band patterns.

Isolates of HL1 A2 (Gram-negative bacterium) and HL1 B3 (Gram-negative bacterium), both isolated from the coelomic fluid of *H. leucospilota*, produced too much noisy data after DNA sequencing. Noisy data mean there are a lot of undetermined nucleotides (whether they are adenine (A), thymine (T), guanine (G), or cytosine (C)) that cause difficulties in multiple sequence alignment and finally in molecular species identification. Though the noise could be reduced manually, the results could be less reliable. Therefore, these DNA sequences were not included in phylogenetic analyses and hence were unable to be identified due to time constraint. The remaining 14 microbial isolates produced good DNA sequencing results. Forty two corresponding sequences from the GenBank, NCBI, U.S. National Library of Medicine were incorporated for the phylogenetic analyses. Blast results gave expect values (E-values) of zero (i.e. 0.0), which means the matches were significant. The phylogenetic trees were rooted with an airborne bacterium, *Enterococcus faecalis*. Based on NJ tree (Figure 1) and MP tree (Figure 2), all microbial isolates could be identified up to genus level and some of them could be further identified up to species level (Table 2).

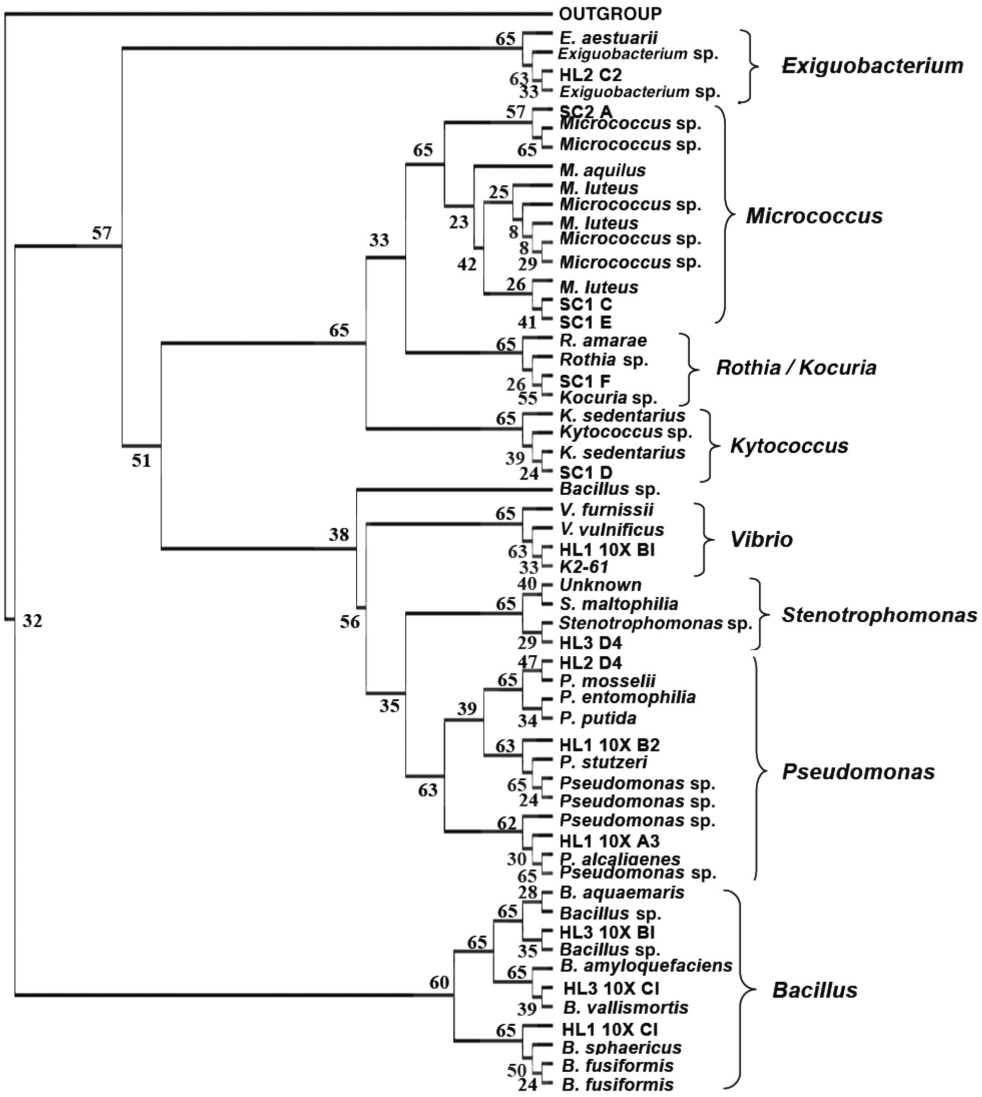

FIGURE 1. Neighbour-joining (NJ) tree (50% majority rule consensus tree) of 14 bacteria isolated from the coelomic fluid of *Holothuria leucospilota* (HL1-HL3) and *Stichopus chloronotus* (SC1-SC3) inferred from 16S rRNA mtDNA gene sequences using Neighbor program of PHYLIP version 3.6b (Felsenstein 2004). A number of 42 corresponding sequences from the GenBank, NCBI, U.S. National Library of Medicine were also incorporated. The tree was rooted with an airborne bacterium, *Enterococcus faecalis*. Kimura 2-parameter distance (1980) with 1000 sequence replications and 100 data sets were used. Numbers at nodes indicate the bootstrap values in percentage (%)

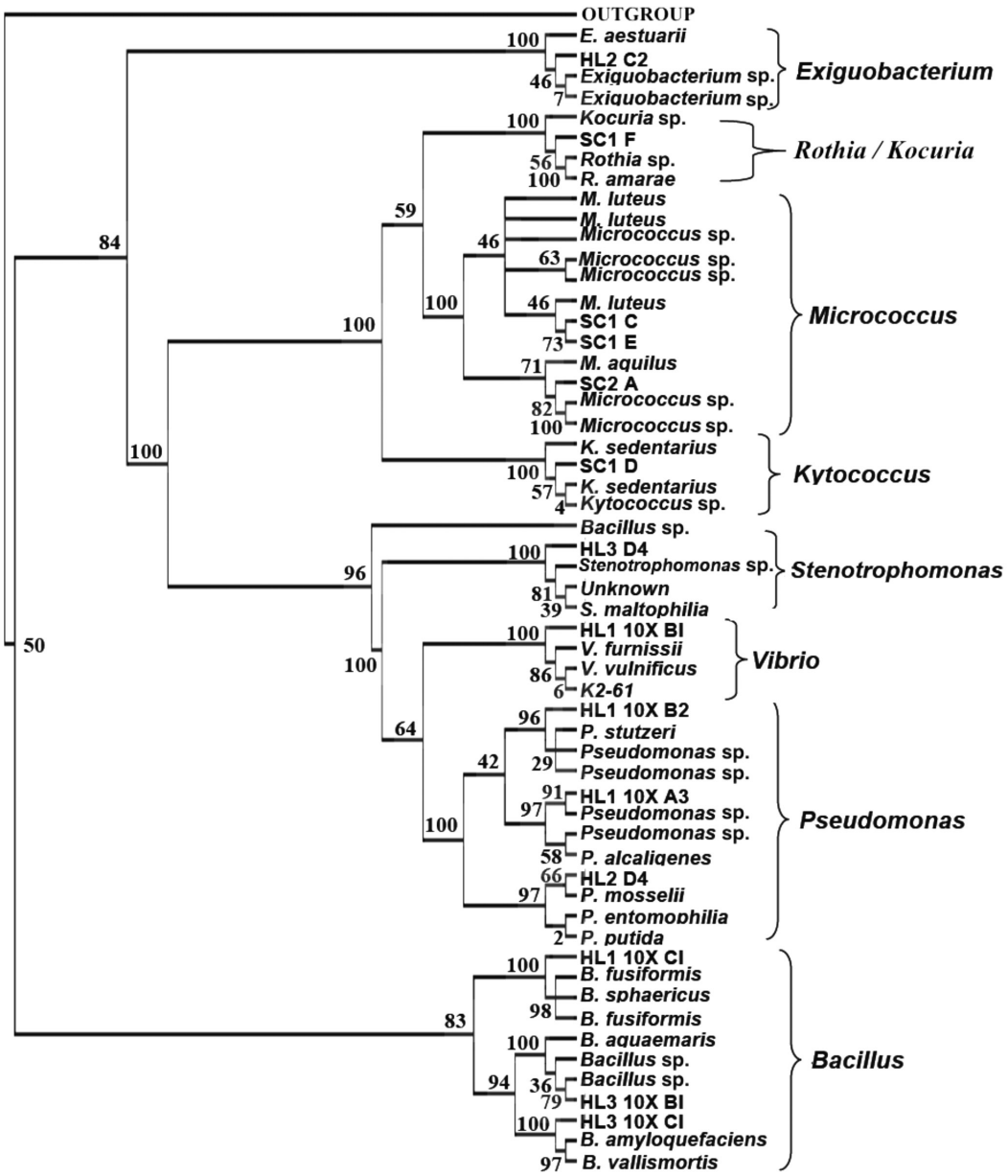

FIGURE 2. Maximum parsimony (MP) tree (50% majority rule consensus tree) of 14 bacteria isolated from the coelomic fluid of *Holothuria leucospilota* (HL1-HL3) and *Stichopus chloronotus* (SC1-SC3) inferred from 16S rRNA mtDNA gene sequences using dnapars program of PHYLIP version 3.6b (Felsenstein 2004). Modified Templeton test using ordinary parsimony was incorporated. A number of 42 corresponding sequences from the GenBank, NCBI, U.S. National Library of Medicine were also incorporated. The tree was rooted with an airborne bacterium, *Enterococcus faecalis*. Numbers at nodes indicate the bootstrap values in percentage (%)

Only two microbial isolates from the coelomic fluid of *S. chloronotus* – SC2 A and SC1 F – were grouped differently by NJ tree and MP tree. MP tree suggested that SC2 A was genetically closer to *Micrococcus aquilus* with 71% bootstrap value and SC1 F was closer to *Rothia amarae* with 56% bootstrap value. With regard to NJ tree, the species for SC2 A was hard to determine and it was grouped in different cluster from *M. aquilus* and the phylogenetic tree suggested that SC1 F was closer to genus *Kocuria* with 55% bootstrap value rather than *Rothia*. Both genera of *Rothia* and *Kocuria,* members of the family *Micrococcaceae*, were previously classified into the genus of *Micrococcus*, but were dissected later from *Micrococcus* based on the phylogenetic and chemotaxonomic analyses by Stackerbrandt et al. (1997, 1995). Apart from that, SC1 D was clustered in genus *Kytococcus*, also one of family members of *Micrococcaceae* with 65% bootstrap value in NJ tree and 100% bootstrap value in MP tree.

Isolates of SC1 C, SC1 E and SC2 A from the coelomic fluid of *S. chloronotus* were clustered together in genus *Micrococcus* with bootstrap values of 65% for NJ tree and 100% for MP tree. SC1 C and SC1 E were closely clustered together in both phylogenetic trees, with bootstrap values of 41 and 73% for NJ tree and MP tree, respectively. This

explained why both isolates had only one different band in RFLP analyses. There is also a possibility that they were actually of the same species, but technical error(s) had occurred during the RFLP analyses. Both were closely related to *M. luteus*.

Based on the phylogenetic trees (Figures 1 & 2), isolates of HL1 10× A3, HL1 10× B2 and HL2 D4 from the coelomic fluid of *H. leucospilota* (i.e. undiluted and 10× diluted coelomic fluid) were identified as from genus *Pseudomonas* with bootstrap values of 63% for NJ tree and 100% for MP tree, while HL1 10× C1, HL3 10× B1 and HL3 10× C1 also from the coelomic fluid of *H. leucospilota* (i.e. 10× diluted coelomic fluid) were clustered under genus *Bacillus* with bootstrap value of 60% for NJ tree and 83% for MP tree. HL2 C2 was classified under genus *Exiguobacterium* and the closest species related to it was *E. aestuarii*. HL1 10× B1 was identified as from genus *Vibrio*, either *V. furnissii* or *V. vulnificus*. Finally, HL3 D4 was classified under genus *Stenotrophomonas* with 65% bootstrap value based on NJ tree and 100% bootstrap value based on MP tree. The closest species to it was *S. maltophilia*.

In general, it was suggested that nine microbial species were present in the coelomic fluid of *H. leucopilota* and five microbial species in the coelomic fluid of *S. chloronotus* (Table 2). All of them were bacteria. More interestingly, all bacterial isolates from the coelomic fluid of *S. chloronotus* were from the *Micrococcaceae* family. This explained why the isolates had very slight different characteristics during the morphological observation and vaguely different band patterns for RFLP analysis. Based on the findings, the microbial population in the coelomic fluid of *H. leucospilota* was more diverse as compared to *S. chloronotus*, possibly because the lack of antimicrobial activity inside the coelomic fluid of *H. leucospilota*. Ridzwan et al. (2003) suggested the potential of the coelomic fluid from *S. herrmanni* as alternative analgesic drug sources.

In view of environmental factors, the specimens of *H. leucospilota* collected in this study were found in shallow water that allowed a better penetration of light and heat as compared to *S. chloronotus* that inhabited deeper water level and the specimens of *H. leucospilota* were more affected with the changing water level during the low and high tide due to their habitat condition. Besides that, the specimens of *H. leucospilota* used their elongated and adhesive tentacles to get organic matters from the sand. The proximity of habitat of *H. leucospilota* to the land with influx of water from rivers and other sources was also observed. The environmental factors, the feeding behaviour of *H. leucospilota* and the higher level of antimicrobial properties of coelomic fluid in *S. chloronotus* were speculated to be the contributing factors to the most diverse of microbial population in the coelomic fluid of *H. leucospilota* as compared to *S. chloronotus*.

Genera of *Pseudomonas* and *Bacillus* formed the major microbial classes in the coelomic fluid of *H. leucospilota*; while genus *Micrococcus* in general and *Micrococcus luteus* in particular were the major microbial classes in the coelomic fluid of *S. chloronotus*. More interestingly, all bacteria isolated from the coelomic fluid of *S. chloronotus* were gram-positive bacteria. Approximately 82% of the coelomic fluid of *H. leucospilota* consisted of

TABLE 2. Identification of 14 microbial isolates from the coelomic fluid of *Holothuria leucospilota* and *Stichopus chloronotus* based on phylogenetic analyses of 16S rRNA mtDNA gene sequences (Figures 1 & 2)

Microbial isolate	Probable genus	Probable species	Gram
Holothuria leucospilota			
HL1 10× A3	*Pseudomonas*	*Pseudomonas alcaligenes*	*-ve*
HL1 10× B2	*Pseudomonas*	*Pseudomonas stutzeri*	*-ve*
HL2 D4	*Pseudomonas*	*Pseudomonas mosselii*	*-ve*
HL1 10× C1	*Bacillus*	*Bacillus sphaericus* or *Bacillus fusiformis*	*-ve*
HL3 10× B1	*Bacillus*	*Bacillus aquaemaris*	*+ve*
HL3 10× C1	*Bacillus*	*Bacillus vallismortis* or *Bacillus amyloquefaciens*	*+ve*
HL3 D4	*Stenotrophomonas*	*Stenotrophomonas maltophilia*	*-ve*
HL2 C2	*Exiguobacterium*	*Exiguobacterium aestuarii*	*-ve*
HL1 10× B1	*Vibrio*	*Vibrio vulnificus* or *Vibrio furnissii*	*-ve*
Stichopus chloronotus			
SC1 C	*Micrococcus*	*Micrococcus luteus*	*+ve*
SC1 E	*Micrococcus*	*Micrococcus luteus*	*+ve*
SC2 A	*Micrococcus*	*Micrococcus aquilus*	*+ve*
SC1 D	*Kytococcus*	*Kytococcus sedentarius*	*+ve*
SC1 F	*Kocuria* / *Rothia*	*Rothia amarae*	*+ve*

+ve – Gram-positive, *-ve* – Gram-negative

gram-negative bacteria, including isolates of HL1 A2 (gram-negative bacterium) and HL1 B3 (gram-negative bacterium) that had no good DNA sequences. The species identification based on 16S rRNA mtDNA gene sequences supported the results of gram staining and morphological observation. Gram stain showed that *B. sphaericus* consisted of a gram-negative sheath enclosing long bacilliform rods, which were both gram-positive and gram-negative (Isaacson et al. 1976, Table 2).

Furthermore, all 14 identified bacterial isolates were tested on streptomycin, kanamycin and tetracycline in two different concentrations i.e. 50 and 100 ppm. Only three tests involving two out of the 14 bacterial isolates produced positive results in either concentration. Other bacterial isolates showed no sign of growth. An isolate from genus *Pseudomonas* that is suspected to be *P. alcaligenes* i.e. HL1 10× A3 exhibited high resistance towards streptomycin. Another isolate from genus *Stenotrophomonas* that is suspected to be *S. maltophilia* i.e. HL2 D4 showed moderate resistance towards streptomycin and lower resistance towards kanamycin. Both isolates were from the coelomic fluid of *H. leucospilota*. Streptomycin has been widely used and many bacteria had developed resistance towards it. Beside that, tetracycline inhibited the growth of all microbes tested. According to Black (2002), tetracyline has a very broad spectrum of activity and it affects both gram-positive and gram-negative bacteria by inhibiting protein synthesis.

P. alcaligenes is classified under the same group as *P. aeruginosa*. It is increasingly recognised as an emerging opportunistic pathogen of clinical relevance. Several different epidemiological studies indicated that antibiotic resistance was increasing in clinical isolates. In immunocompetent individuals, *S. maltophila* is a relatively unusual cause of pneumonia, urinary tract infection, or blood stream infection; in immunocompromised patients, however, *S. maltophilia* is a growing source of latent pulmonary infections (McGowan 2006). *S. maltophilia* colonization rates in individuals with cystic fibrosis have been increasing. *S. maltophilia* is naturally resistant to many broad-spectrum antibiotics (including all carbapenems) and is thus often difficult to eradicate (Al-Jasser 2006).

Conclusion

Less diverse microbial population in a 'gamat' species i.e. *S. chloronotus* as compared to the most abundant 'timun laut' species in Malaysia i.e. *H. leucospilota* is speculated to be due to the environmental factors e.g. penetration of light, the feeding behaviour of *H. leucospilota*, and the higher level of antimicrobial properties of coelomic fluid in *S. chloronotus*. Phylogenetic analyses of partial 16S rRNA mtDNA gene sequences suggested the presence of at least eight microbial genera i.e. five bacterial genera - *Bacillus*, *Exiguobacterium*, *Pseudomonas*, *Stenotrophomonas*, and *Vibrio* - isolated from the coelomic fluid of *H. leucospilota* and three bacterial genera - *Kytococcus*, *Micrococcus* and either *Kocuria* or *Rothia* - isolated from the coelomic fluid of *S. chloronotus*. More interestingly, all bacterial isolates from the coelomic fluid of *S. chloronotus* were Gram-positive bacteria from the family *Micrococcaceae*. In terms of antimicrobial-resistance capability test, an isolate from genus *Pseudomonas* that was suspected to be *P. alcaligenes* exhibited high resistance towards streptomycin. Another isolate from genus *Stenotrophomonas* that was suspected to be *S. maltophilia* showed moderate resistance towards streptomycin and lower resistance towards kanamycin. Both isolates were from the coelomic fluid of *H. leucospilota*. Tetracycline inhibited the growth of all 14 bacterial isolates tested. For future research, we suggest incorporating more specimens of *S. choronotus* and *H. leucospilota* from broader geographical locations including Sabah and Sarawak, and the use of complete mtDNA genes along with morphological approaches for better species identification as these could provide better insights into the microbial population in the coelomic fluid of both local sea cucumber species.

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References

Al-Jasser, A. 2006. *Stenotrophomonas maltophilia* resistant to trimethoprim-sulfamethoxazole: An increasing problem. *Annals of Clinical Microbiology and Antimicrobials* 5: 23.

Black, J.G. 2002. *Microbiology: Principles and Explorations*. 5th ed. USA: John Wiley and Sons.

Brandt, J.F. 1835. Echinodermata ordo Holothurina. Pp. 42-62. In *Prodromus Descriptionis Animalium Ab. H. Mertensio In Orbis Terranum Circumnavigatione Observatorum*. Fasc. I: 75 pps. Petropoli.

Choo, P.S. 2008. Population status, fisheries and trade of sea cucumbers in Asia. In *Sea Cucumbers. A Global Review of Fisheries and Trade*, edited by Toral-Granda, V., Lovatelli, A. & Vasconcellos, M. FAO Fisheries and Aquaculture Technical Paper. No. 516.

Dayang, F.B., Jacinta, S., Chong, S.L. & Rohasmizah, I. 2004. Antimycotic activity of the extracts of *Stichopus chloronotus* brandt in the treatment of experimental dermatophytosis. *Jurnal Sains Kesihatan Malaysia* 2(2): 97-102.

Farouk, A.E., Ghouse, F.A.H. & Ridzwan, B.H. 2007. New bacterial species isolated from Malaysian sea cucumbers with optimized secreted antibacterial activity. *American Journal of Biochemistry and Biotechnology* 3(2): 60-65.

Felsenstein, J. 2004. *'Phylip', Phylogeny Inference Package*. version 3.6b (beta release). Seattle, WA: Department of Genome Sciences, University of Washington.

Fredalina, B.D., Ridzwan, B.H., Zainal, A.A., Kaswandi, M.A., Zaiton, H., Zali, I., Kittakoop, P. & Mat Jais, A.A. 1999. Fatty acid compositions in local sea cucumber, *Stichopus chloronotus*, for wound healing. *General Pharmacology* 33(4): 337-340.

Hawa, I., Zulaikah, M., Jamaludin, M., Zainal Abidin, A.A., Kaswandi, M.A. & Ridzwan, B.H. 1999. The potential of the coelomic fluid in sea cucumber as an antioxidant. *Malaysian Journal of Nutrition* 5(1&2): 55-59.

Isaacson, P., Jacobs, P.H., Mackenzie, A.M.R. & Mathews, A.W. 1976. Pseudotumour of the lung caused by infection with *Bacillus sphaericus*. *Journal of Clinical Pathology* 29: 806-811.

Kamarudin, K.R., Rehan, A.M., Hashim, R., Usup, G., Ahmad, H.F., Anua, M.H. & Idris, M.Y. 2011. Molecular phylogeny of *Holothuria (Mertensiothuria) leucospilota* (Brandt 1835) as inferred from cytochrome C oxidase I mitochondrial DNA gene sequences. *Sains Malaysiana* 40(2): 125-133.

Kamarudin, K.R., Rehan, A.M., Hussin, R. & Usup, G. 2010. An update on diversity of sea cucumber (Echinodermata: Holothuroidea) in Malaysia. *Malayan Nature Journal* 62(3): 315-334.

Kaswandi, M.A., Azraul-Mumtazah, R., Sahalan, A.Z., Normalawati, S., Aziz, A.H.A. & Abu Bakar, M.A. 2007. Activity of crude extracts mixture from sea cucumbers *Stichopus badionotus* and *Stichopus chloronotus* on fungal growth. *Journal of Microscopy Society of Thailand* 24: 151-154.

Khartini, A.W., Dayang Fredalina, B., Kaswandi, A., Hing, H.L., Oon, K.W. & Sit, N.W. 2003. Antimicrobial and cytotoxic activities of *Holothuria sp*. and *B. marmorata vitiensis*. *Malaysian Journal of Biochemistry and Molecular Biology* 8: 49-53.

Kuske, C.R., Banton, K.L., Adorada, D.L., Stark, P.C., Hill, K.K. & Jackson, P.J. 1998. Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. *Applied and Environmental Microbiology* 64(7): 2463-2472.

McGowan, J. 2006. Resistance in nonfermenting gram-negative bacteria: Multidrug resistance to the maximum. *American Journal of Medicine* 119(6 Suppl 1): S29-S36, discussion S62-S70.

Osama, Y.A., Ridzwan, H., Muhammad, T., Jamaludin, M.D., Masa-Aki, I. & Zali, B.I. 2009. *In vitro* antioxidant and antiproliferative activities of three Malaysian sea cucumber species. *European Journal of Scientific Research* 37(3): 376-387.

Page, R.D.M. 1996. Treeview: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12: 357-358.

Ridzwan, B.H. 1993. *Sumber Makanan Persisiran Laut Sabah*. Kuala Lumpur: Dewan Bahasa dan Pustaka.

Ridzwan, B.H. 2007. *Sea Cucumber: The Malaysian Heritage*. Malaysia: Research Management Centre, International Islamic University Malaysia (IIUM).

Ridzwan, B.H., Kaswandi, M.A., Azman, Y. & Fuad, M. 1995. Screening for anti-bacterial agents in three species of sea cucumber from coastal areas of Sabah. *General Pharmacology* 26(7): 1539-1543.

Ridzwan, B.H., Leong, T.C. & Idid, S.Z. 2003. The antinociceptive effects of water extracts from sea cucumber *Holothuria leucospilota* brandt, *Bohadschia marmorata vitiensis* jaeger and coelomic fluid from *Stichopus hermanii*. *Pakistan Journal of Biological Sciences* 6(24): 2068-2072.

Ridzwan, B.H., Zarina, M.Z., Kaswandi, M.A., Nadirah, M. & Shamsuddin, A.F. 2001. The antinociceptive effects of extracts from *Stichopus chloronotus* brandt. *Pakistan Journal of Biological Sciences* 4(3): 244-246.

Sim, Y.K., Tan, S.H., Zulfigar, Y., Wong, W.S., Chai, J., Ng, B.W. & Luar, L. 2008. The diversity and distribution of Holothuroidea in Pulau Aur, Johor. In *Research and Information Series of Malaysian Coast 2*. Pusat Penyelidikan Ekosistem Marin (EKOMAR), UKM. pp. 13-22.

Stackebrandt, E., Rainey, F.A. & Ward-Rainey, N.L. 1997. Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *International Journal of Systematic Bacteriology* 47: 479-491.

Stackebrandt, E., Koch, C., Gvozdiak, O. & Schumann, P. 1995. Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. *International Journal of Systematic Bacteriology* 45: 682-692.

Tan, W.T., Fong, Y.S. & Ridzwan, B.H. 2005. The effect of coelomic fluid *Stichopus hermanii* on isolated perfused rat hearts and the involvement of prostaglandin in its mechanism of action. *Pakistan Journal of Biological Sciences* 8(1): 78-84.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. 1997. The clustalX windows interface: Flexible strategies for multiple sequence alignment aided by the quality analysis tools. *Nucleic Acids Research* 24: 4876-4882.

Zulfigar, Y., Sim, Y.K. & Tan, S.H. 2007. The distribution of sea cucumbers in Pulau Aur, Johore, Malaysia. In *Publications of the Seto Marine Biological Laboratory, Special Publication Series* Volume 8 (from Selected Paper of NaGISA World Congress 2006). Japan: Seto Marine Biological Laboratory, Field Science Education and Research Center, Kyoto University. pp 73-86.

Zulfigar, Y., Sim, Y.K., Tan, S.H. & Shirayama, Y. 2008. *Field Guide to the Echinoderms (Sea Cucumber and Sea Stars) of Malaysia*. The Nippon Foundation, Japan: Kyoto University Press. p. 103.

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