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Biodiversity and Adaptability of *Holothuria leucospilota* and *Stichopus japonicus* Sea Cucumber Species in Artificial Environment

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Abstract

Sea cucumber species respond to challenging environmental conditions differently thus, to culture them in artificial environment requires a better understanding of their environmental needs. Two species of sea cucumber *Stichopus japonicus* and *Holothuria leucospilota* in Teluk Kamang Port Dickson (PD) (2° 27' 46.61″N 101° 50' 42.98″E), Malaysia, were investigated to determine their biodiversity. *H. leucospilota* was separately investigated to establish its abundance in three (3) separate periods of the year- March, July and November. Global Positioning System (GPS) and Yellow Springs Instruments (YSI) were used to measure the physical parameters. *Stichopus japonicus* and *Holothuria leucospilota* were found in temperature ranges of 31 to 32°C and 30 to 31°C, respectively. Salinity was 30.00 to 31.00 part per thousand (ppt) for *S. japonicus* and 30.00 to 31.00 ppt for *H. leucospilota*. The pH ranged from 7.80 to 8.40 and 8.00 to 8.60 for *S. japonicus* and *H. leucospilota* respectively. *H. leucospilota* species were more abundant in March than in July, and more in July than in November. Amplification of their genomic DNA using the 16S primer, showed *H. leucospilota* to be approximately 550 bp and *S. japonicus* 600 bp in size. The sequenced PCR products analyzed using the Basic Local Alignment Search Tool (BLAST) Version 2.01G identified *Holothuria leucospilota* as another species in Malaysia. These findings provide an insight into Holothurians environment needs and established a nucleotide sequence for *H. leucospilota* in nucleotide database for future use.

Keywords: Biodiversity; *Stichopus japonicas*; Sea cucumber; 16S primer; BLAST; *Holothuria leucospilota*

Introduction

Sea cucumbers are organisms belonging to the phylum Echinodermata from the class Holothuroidea composed of five genera. Even though some are found in shallow waters, they are typically ocean dwellers and are found living in the deep ocean. They live on or near the ocean floor and are often buried beneath it [1]. They are widely distributed in all regions of the oceans worldwide. To date, as many as 1500 species and new species identified each year have been recorded among the six valid orders of Apodida, Aspidochirotida, Elasipodida, Molpadiida, Dendrochirotida and Dactylochirotida [2]. Holothurians have a long history of being consumed by the oriental people, mostly the Chinese and Japanese. Being a source of food and medicine to many has resulted in their being harvested heavily in many communities with tonnes leaving the ocean annually [3]. This situation led some species to the point of extinction. However, some institutions and communities have embarked on aquaculture and breeding projects, in addition to other conservation efforts geared towards protecting the endangered species from complete disappearance. In Malaysia, approximately 50 species from three orders and seven genera have been recorded while 34 species still require further identification [2,4]. Two sea cucumber species, *Stichopus japonicus* and *Holothuria leucospilota*, found around the coastline in Port Dickson are among the types recorded in Malaysia, with *H. leucospilota* being the commonest ones [2,4]. Studies on the identification of local sea cucumbers at the genomic level are limited. Thus, this study was conducted to identify these two species of sea cucumber using DNA sequencing and also to highlight some of the environmental factors that support the survival of one species over the other in an artificial environment.

Materials and Methods

Sampling of sea cucumbers

The sea cucumber samples were collected at low tide at a depth of 30 cm to 100 cm in two sites: a coral reef site at Teluk Kemang beach for *H. leucospilota*, and sandy or muddy areas behind Costa Rica Hotel for *S. japonicus*. The species were collected within three different quadrats, each measuring 5 m x 20 m. YSI was used to take salinity, temperature, and pH measurements, and the GPS was used to determine the location of the species at the sites. The sea cucumber species collected from each quadrat was counted and then stored in buckets containing sea water with battery aerators to provide oxygen [5]. They were then transported to the lab in this condition before being transferred to the aquarium.

Abundance of *H. leucospilota* at Port Dickson

To determine the abundance of *H. leucospilota,* a survey was first conducted from March to July 2010, and then in November of the same year. The number of species per survey was determined randomly due to the vastness of the area and it was conducted in three quadrats measuring 100 m^{-2} (5 m x 20 m) along a 100 m transect line. The sea cucumber species were collected and counted before transferring them in improvised aquariums. Upon completion of the work the species were returned to their respective locations [5]. The physical parameters were also determined during each sampling period.

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Estimation of population size

To determine the estimated population size of *H. leucospilota* sea cucumber we collected data such as quadrat size (100 m^{-2}), the size of the survey area (8000 m^{-2}) and the number of species per quadrat. The mean ($\Re$) value of all the quadrat counts generates the population density expressed in numbers of individuals per quadrat area. To estimate the total population size of *H. leucospilota* at the survey site, we calculated N for the quadrat data using the formula: $N = (A/a) \times n$, where: N = the estimated total population size, A = the total study area, a = the area of the quadrat and n = the mean number of organisms per quadrat. To also determine the distribution of the species at the site we calculated the variance (s^2) and divided the variance by the mean ($\Re$) to compute the variance-to-mean ratio ($s^2/\Re$) which tells us whether the species were aggregated, random or uniformly dispersed. To calculate the variance and variance-to-mean ratio, we used the following set of formulas: (i) = quadrat number, $\Sigma (x_i)$ = total number of organisms, $\Sigma (x_i) / n$ = mean, $\Sigma (d^2)_i$ = sum of squared deviations, $s^2 = \Sigma (d^2)_i / (n-1)$ = sample variance and $s^2 / \Re$ = variance-to-mean ratio.

The values are given below:

A = 8000 m^{-2}, a = 100 m^{-2}, n = 19, 61, 38, 13, 10, 14, 4, 3, 3

N=?

Aquarium cultures

The two sea cucumber species, *H. leucospilota* and *S. japonicus* each 6 individuals were cultured in two separate aquariums of the same size (145cm x 85cm x 63cm). Fresh water mixed with sodium chloride was used to culture each species. Some sand, stones, and a few corals were placed in the aquariums to mimic their natural habitat. The species were left in the aquarium for a week prior to the experiment under normal conditions. *S. japonicus* was cultured in artificial sea water at 27.00 ppt below its optimum salinity level of approximately 30.00 ppt and left in that condition for two days. *H. leucospilota* was also cultured in artificial sea water at salinity level of 27.00 ppt, below its optimum salinity level of 30.00 ppt, and left in such a condition for two days. The salinity was adjusted to 28.00 ppt on the third day when organisms showed signs of stress. Temperature on the other hand was control at 31 and 32°C for *S. japonicus* and *H. leucospilota* respectively using heaters for the first week but when the salinity was normalized, the temperature was left ambient and was normally between 24 and 30°C. Their conditions were monitored both in the morning and in the afternoon. Observations made on each species were recorded on a daily basis.

Total genomic DNA Extraction

DNA extraction was carried out using Epicenter's DNA and RNA extraction kit (Epicenter Biotechnologies) according to the manufacturer's instructions. Five to ten milligram (5-10 mg) of *H. leucospilota* and *S. japonicus* body tissue and intestine samples collected from one individual sacrificed from each species were mixed with 300 µL of the tissue and cell lysis buffer containing 1 µL proteinase K in a microcentifuge tube and incubated at 65°C for 15 mins, vortexing every 5 mins. Samples were then placed on ice for 3-5 minutes before addition of the MPC protein, and centrifuged for 10 mins at 4°C at 8,500 x g. The supernatant was collected and the pellet discarded. Ice cold 99% isopropanol was added and the tube was inverted gently 35 times. The samples were centrifuged (as above) to pellet total nucleic acid. The isopropanol was then carefully poured off without dislodging the nucleic acid pellet. The pellet was rinsed twice with 70% ethanol, and all traces of ethanol were removed before resuspending the total nucleic acid in 35 µL TE buffer. The qualitative and quantitative yield of DNA was determined by biophotometric measurements.

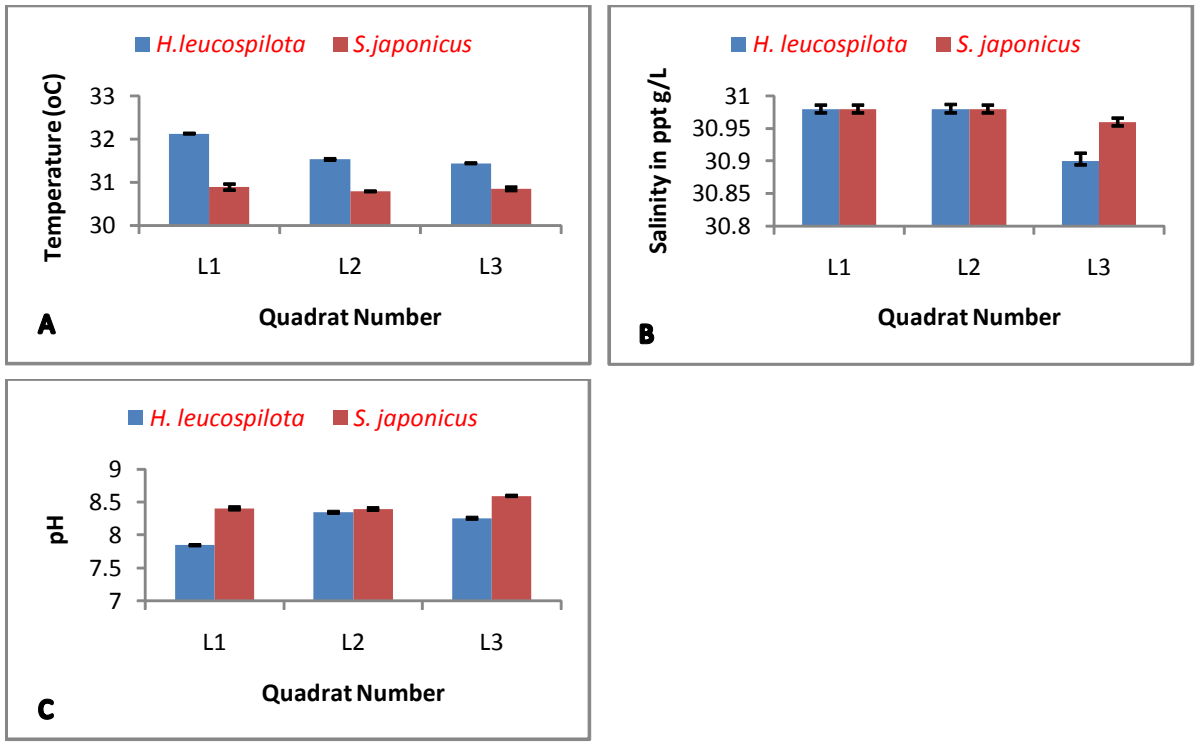

Figure 1: The graphs represent the environmental parameters of sampling sites at Port Dicksons for *S. japonicus* and *H. leucospilota* and also show a comparison on the two species based on these parameters.

Polymerase chain reaction (PCR)

PCR was done in a thermocycler and the sample preparations were carried out in a lamina flow in an aseptic condition. The components of the PCR reaction included ddH_2O, 2 mM of 50 mM magnesium chloride ($MgCl_2$), 1.5 mM of 10 mM deoxyribonucleotide triphosphate (dNTPS), 2.5 µL (1X) of 10X PCR reaction Buffer A, 0.3 mM of 50 mM 16S primers (forward and reverse), 1 µL (7.9 mg/mL) DNA template and 0.2 µL (1 U) of 5 U/µL Taq Polymerase. The total volume of the reaction mixture in each tube was 25 ÛL. A portion of approximately 540 nucleotides from the conserved 3' end of the mitochondrial gene coding for the 16S like, large ribosomal RNA subunit using the echinoderm specific universal primers 16Sar (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr (5'CTCCGGTTTGAACTCAGATCA-3') were amplified then a standard PCR was executed under standard conditions. PCR cycling parameters comprised of initial denaturation at 95°C for 5 mins, followed by 29 cycles of denaturation at 95°C for 45 secs, annealing at 55°C for 90 sec, and elongation at 72°C for 90 secs, and a final elongation step at 72°C for 7 mins [6]. Purified PCR products in suspension forms were prepared prior to sending the samples for commercial sequencing to Repfon Glamor SDN BHD, Malaysia.

Analysis of Amplified PCR product by agarose gel electrophoresis

Eight hundred and forty milligrams (0.84 grams (1.4%)) of agar and 1 uL of gelRed was prepared in 60 mL of 1X (Tris Borate EDTA) TBE buffer. A 1 kb DNA standard ladder (300 – 10,000 bp) and Hypper Ladder-IV (100 – 1,000 bp) were used for size separation references. The gel was run at 70V for 90 mins. Upon completion, the gel was removed and viewed under UV light.

Results

Sea cucumber Sampling

The environmental parameters collected at the sampling sites for the two sea cucumber species showed that *S. japonicus* was found in a salinity range of 30.00 – 31.00 part per thousand (ppt) while *Holothuria luecospilota* was found in 30.00 – 31.00 ppt salinity (Figure 1B). Temperature values for *S. japonicus* ranged from approximately 31 – 32°C, while the range for *Holothuria leucospilota* was 30 – 31°C (Figure 1A). The pH readings ranged from 7.80 – 8.40 and 8.00 – 8.60 for *S. japonicus* and *H. leucospilota*, respectively (Figure 1C). The salinity mean value for both species was 31.00 ppt. Mean values in temperature for the two species were 32°C and 31°C, respectively. The mean pH value for *S. japonicus* was 8.00 and for *H. leucospilota* it was 8.13.

Population Study on *H. leucospilota*

Estimation of population size and distribution pattern: The data obtained from the analysis showed that an estimated population size of 1464 (Table 3) species may have colonized the survey site. According to the variance-to-mean ratio ($s^2/\Re$), if the $s^2/\Re \approx 1$, the species are randomly dispersed because statisticians have demonstrated that the variance-to-mean ratio has generated a value close to 1 in a randomly dispersed population, because in samples from a random distribution the variance is equal to the mean whereby if ratio is significantly greater than 1 indicates aggregation and a ratio less than one indicates a trend toward uniformity. In this study the value obtained in variance-to-mean ratio was 9.7 (Table 4) suggesting that the species were aggregately distributed during periods of the survey.

Laboratory cultures: The two species, *S. japonicus* and *H. leucospilota*, cultured outside their natural habitats showed some marked differences based on their ability to adapt to changes outside their natural environments. Observations showed that after 2 days of culture in artificial sea water, *S. japonicus* started to shrink, gradually becoming smaller, and on the third day of culture all samples of this species eviscerated their internal organs and died. *H. leucospilota* on the other hand showed a little bit of stress on the first day when salinity was reduced to 27.00 ppt against their normal salinity tolerance of 30.00 ppt. However, when the salinity value was normalized (28-30 ppt) and the temperature ambient, the sea cucumber continued to thrive in the aquarium with the same number of organisms for more than 6 weeks.

Polymerase Chain Reaction (PCR)

The PCR results showed the amplified fragments from body tissue and intestines of *S. japonicus* to be approximately 600 bp (Figure 2B), and from the body tissue of *H. leucospilota* to be 550 bp (Figure 2A).

Sequencing and alignment: The PCR product was commercially sequenced by Repfon Glamor SDN BHD. The sequences were received in the form of DNA Chromatograms, indicating the total nucleotide bases contained in the PCR product. Both the forward and reverse sequences returned 530 nucleotide bases (Figure 3A &B) which were used as query sequences to search for, and sequences to search against (also called the target sequence) a sequence database containing multiple such sequences. When the sequences were inserted for BLAST similarity search, the software found subsequences in the database

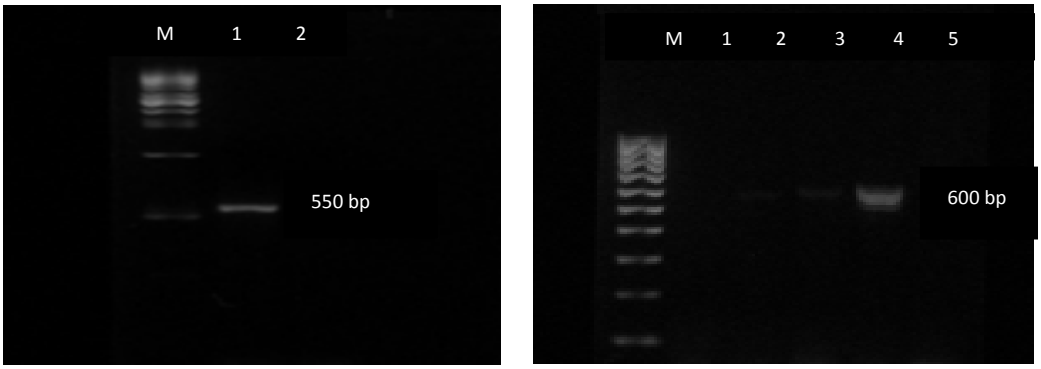

Figure 2: Amplified PCR products of (A) *H. leucospilota* and (B) *S. japonicus* using 1 kb and 100 bp DNA Ladders as size reference, respectively. In both (A) and (B), lanes M bear the DNA markers and lanes 2 are the negative controls. The amplified PCR product in (A) lane 1 is approximately 550 bp, and in (B) lane 4 is approximately 600 bp.

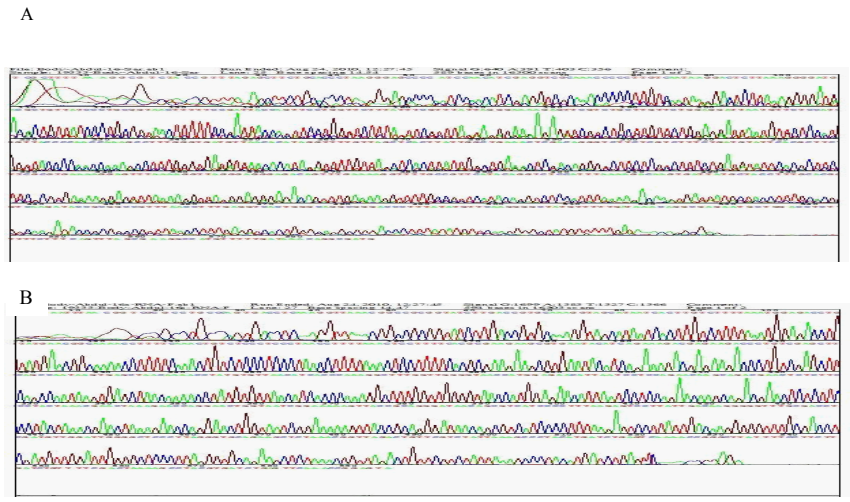

Figure 3: The reverse (16Sbr) and forward (16Sar) sequences of *H. leucospilota* in A and B respectively

Month	Physical Parameter									
	Temperature (o C)	SEM	Salinity (g/L)	SEM	pH	SEM	Dissolved Oxygen (mg/L)	SEM	Total Dissolved Solids (g/L)	SEM
March	33.3011_a	±0.1411	31.1300_a	±0.0201	8.47_a	±0.3018	11.2988_a	±0.4108	31.2733_a	±0.0029
July	30.8433_b	±0.0145	31.0444_b	±0.1108	8.04_b	±0.0366	11.2567_b	±0.1183	31.2567_b	±0.0044
November	30.2167_c	±0.0203	29.4933_c	±0.1513	7.93_c	±0.0107	10.5900_c	±0.3341	31.0867_c	±0.0096

Data represent means of 3 replicates. In each column values with the same letters are not significantly different with Duncan (p= 0.05%). SEM represents standard mean error

Table 1: shows the environmental parameter values recorded in the survey for H. leucospilota in PD from March to November, 2010.

which were similar to subsequences in the query thus returning 100% similarity for the forward sequence and 98% similarity for the reverse sequence [7].

Discussion

The process of establishing data on sea cucumbers in Malaysia, especially biodiversity data, is very challenging because very little is recorded for the very many species of sea cucumbers [2,4]. However, the data collected on *S. japonicus* and *H. leucospilota* based on salinity, temperature, and pH optimum ranges are partly in line with the data recorded by the FAO Corporate Document Repository for *S. japonicus* in China. According to the data, 27 to 35 ppt is the optimum salinity tolerant limit for *S. japonicus* [8]. Comparing this with the salinity value obtained for *S. japonicus* and *H. leucospilota* in the study, no differences were shown even though the geographical locations are different. However, the temperature tolerant values obtained for these species from the current study were compared with FAO Corporate Repository Document, showing a totally different optimal temperature tolerance. The optimal temperature tolerance range according to the FAO recorded data was 5 to 15°C. These values are far below the 31 to 32°C and 30 to 31°C recorded for *S. japonicus* and *H. leucospilota*, respectively. These differences in optimal temperature tolerance could be due to the difference in geographical locations of these sea cucumber species.

In this study all species of *S. japonicus* perished within a week of culture but unfortunately a detailed explanation could not be ascertain simply because both species were cultured in conditions similar to their environmental conditions whereas *H. leucospilota* made it through *S. japonicus* failed. The simplest explanation that could be associated with this scenario could partly be that species of *S. japonicus* reacted more to stress than species of *H. leucospilota* and thus, all *S. japonicus* species eviscerated leading to their death. Evisceration by sea cucumbers according to some authors is one of the defensive mechanisms of sea cucumbers responding to stress during collection and shipping. According to Ruppert and Barnes [9], evisceration is a common response among the sea cucumbers to severe stress and can be induced in a variety of ways including such factors as chemical stress, physical manipulation and crowding among others. Species of sea cucumber may eviscerate, slowly shrinks and eventually die in the aquarium even just by moving from one location to another.

Sea cucumbers are organisms that cannot be cultured successfully without a prior knowledge of their feeding habits. Sea cucumbers are either suspension or deposit feeders, thus, keeping them in the aquarium requires providing an immediate environment where there is easy access to food. Most Holothuria species that are in high demand are deposit-feeding that consume organic detritus and ingest fine grained sands to digest off the bacteria, microalgae and diatoms among others that cover the surface of each sand particle. Since all of them feed on the same kind of food, over stocking in an aquarium will cause rapid consumption of the available food and organisms starved to death. One important thing about sea cucumbers is that they can be without food for months as observed, however, when starved it consumes itself gradually and eventually die.

The environmental parameters recorded in the survey showed some significant differences (p=0.05%) as indicated in Table 1. Making a close analysis of the parameters in Table 1, one realizes that the highest values were recorded in March which translates in the number of animals recorded in this month (Table 2). November showed the lowest

Month	Quadrat Number	Number of sea cucumber species	% number of species per quadrat
March	L1	19	11.5
	L2	61	37
	L3	38	23.0
July	L1	13	7.9
	L2	10	6.1
	L3	14	8.5
November	L1	4	2.4
	L2	3	1.8
	L3	3	1.8
Total	9	165	100.0

Table 2: shows the number of sea cucumber species recorded for each survey month, (March to November).

Number of species	Number of quadrat	Area of survey site (m-2) (A)	Quadrat size (m-2) (a)
165	9	8000	100
Mean n =	18.3	A/a =	80
	Population estimate N =		1464

Table 3: shows the population size estimation of *H. leucospilota* sea cucumber at survey area.

Quadrat number ($_i$)	Number of organism (x_i)	- $\Re$	Deviations (d i)	Squared deviations $(d^2)_i$
1	19	-18.3	0.7	0.49
2	61	-18.3	42.7	1823.29
3	38	-18.3	19.7	388.09
4	13	-18.3	-5.3	-28.09
5	10	-18.3	-8.3	-68.89
6	14	-18.3	-4.3	-18.49
7	4	-18.3	-14.3	-204.49
8	3	-18.3	-15.3	-234.09
9	3	-18.3	-15.3	-234.09
Total number of organisms $\Sigma (x_i)$ =	165	Sum of Squared Deviations $\Sigma (d^2)$ i =		1423.73
Mean $\Sigma (x_i) / n$ =	18.3	Sample Variance $s^2 = \Sigma (d^2)_i / (n-1)$ =		177.97
	Variance/Mean ($s^2 / \Re$) =	9.7		

Table 4: shows the statistical analysis of distribution pattern of *H. leucospilota*.

environmental parameter values which also translated in the number of animals recorded for that month (Table 2). Nevertheless, these physical parameter values were all recorded at low tide, thus there is need to collect similar data at high tide using scuba diving.

Survey should be planned according to the available resources. It is impractical if not impossible for one to be able to count every individual organism in a large area, thus, different approaches have been developed to ease the process. Ecologists randomly choose small portions of the whole area which they classify and count the organisms in each small portion to estimate the size of population in larger community is called quadrat sampling. Quadrat sampling is a high quality tool for the study of ecology, most especially biodiversity. Passive quadrat sampling (done without removing the organisms found within the quadrat) is done either by hand with researchers sorting through each individual quadrat or even more efficiently by taking a photograph of the quadrat for future analysis. They are time- tested sampling methods that are best suitable for coastal areas where access to a habitat is relatively easy. The method allows one to collect standardized data at locations separated by vast area of land where one can compare the sites and determine how the abundance or diversity of organisms varies at different locations.

The abundance of *H. leucospilota* sea cucumber at the site was determined by randomly developing quadrats within the survey area. However, the results obtained from the survey clearly showed that the species have habitat preferences. More sea cucumber species were found within corals and seaweeds infested areas and less in areas where these are scarce or absent and it is further manifested by the variance-to-mean ratio (Table 4). In this survey the number of species encountered reduced with time (Table 2). This is because the species preferred to be closed to corals, seaweeds and boulders, thus, the quadrat with very few species where found to be away from corals, seaweeds and boulders. This could also explain why the number of sea cucumbers counted was far less compared to the first two months (Table 2) but also not forgetting the other contributing environmental condition like rain and storm. Quadrat size is another factor that determines the number of organisms involved in a survey and their distribution pattern. If quadrat size is too small more species will likely be left out and there distribution will not be clearly established. On the other hand if it is also too large there will be some difficulty to search through the entire quadrat and count all species. Quadrat sampling to estimate the population size of a species may not be the best of options because it could be bias and data obtained may not reflect the true population size of species [10]. One frequent source of biasness is when the selection of sample plots are nonrandom with respect to the abundance of the target organism and this may lead to underestimation or overestimation of the true population sizes of species. In this study the selection of quadrats was random but just that few values were obtained from the survey (Table 2) as a result of the few number of quadrats setup which may underestimated the population size of the species but also too many quadrats may not have been feasible for us because of the fact that tides rise quickly and most often does not give us time and even to meet the 1 hour set target. We recommend for more advanced survey methods which would employ more advanced survey techniques and the use of scuba diving instead of the passive method which interferes too much with the species and also one that will minimize the effects of environmental factors.

The BLAST analysis for *H.leucospilota* from the library returned seven sequences that resembled the 16Sar reverse primer in various degree showing 98% and 99% maximum identity and a 95% query coverage. For 16Sar forward primer, the library again returned seven sequence alignments that express resemblances to the 16Sar primer showing 97% to 100% maximum identity and 94% to 99% query coverage. These library or database sequences include isolates, clones, clone of 16 ribosomal RNA gene, large subunit ribosomal RNA genes, and 16s ribosomal RNA gene [11]. These findings will therefore contribute significantly to the existing mechanisms put in place to protect the sea cucumber species which are disappearing due to overfishing and destruction of their natural habitats and the recent availability of the gene sequences of the species for future research.

Sea cucumbers are organisms famous for their skin. In Malaysia and in many parts of the world where sea cucumber trade has flourished, many valuable species of sea cucumbers also called gamat in Malaysia have disappeared over the years due mainly to over fishing. In Malaysia less valuable species like *H.leucospilota* are most common, valuable species like *S. japonicus* and many others are rarely encountered and with time the less valuable species will become the inevitable targets. Thus, aquaculture practices will without doubt reduced the over exploitation of wild types.

However, the findings highlighted here may not be entirely conclusive, and further studies should be carried out using more advanced techniques which could reduce mortality in more vulnerable species to stress.

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