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SHORT COMMUNICATION

Morphological and Molecular Identification of *Holothuria* (*Merthensiothuria*) *leucospilota* and *Stichopus horrens* from Pangkor Island, Malaysia

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Abstrak: Kajian awal ini bertujuan untuk mengenalpasti satu spesies komersil gamat iaitu *Stichopus horrens* Selenka, 1867 dan satu spesies timun laut iaitu *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt, 1835) dari Pulau Pangkor, Perak, Malaysia dengan mengaplikasikan teknik-teknik morfologi berdasarkan bentuk-bentuk osikel dan teknik-teknik molekul menggunakan gen sitokrom c osidasi I (COI) mitokondria DNA (mtDNA). Di Malaysia, gamat didefinisikan sebagai spesies timun laut daripada famili Stichopodidae yang memiliki nilai-nilai perubatan manakala timun laut dirujukkan kepada spesies bukan gamat. Spesies *S. horrens* adalah amat popular di Pulau Pangkor sebagai bahan utama dalam penghasilan air gamat dan minyak gamat secara tradisional manakala *H. leucospilota* merupakan spesies paling dominan di Malaysia. Berbeza dengan kajian-kajian sebelum ini, bahagian dalaman tubuh iaitu pohon respirasi dan gastrousus telah disertakan dalam kajian ini untuk mendapatkan kesimpulan yang lebih baik berdasarkan morfologi. Keputusan-keputusan menunjukkan tiada osikel telah hadir di dalam gastrousus *H. leucospilota* dan ciri tersebut dicadangkan sebagai penanda diagnostik yang unik untuk spesies timun laut tersebut. Di samping itu, kehadiran rod berbentuk Y di dalam pohon respirasi *S. horrens* seterusnya menyokong potensi bahagian dalaman tubuh untuk mengenalpasti spesies gamat tersebut. Selanjutnya, analisis-analisis filogenetik gen COI mtDNA spesimen-spesimen timun laut tersebut menggunakan kaedah hubungkait jiran dan kaedah persamaan maksimum seterusnya mengesahkan status spesies *H. leucospilota* dan *S. horrens* dari Pulau Pangkor, Perak, Malaysia. Jujukan-jujukan gen COI mtDNA tersebut telah didaftarkan dengan GenBank, National Center for Biotechnology Information (NCBI), US National Library of Medicine (no. akses GenBank: KC405565-KC405568). Walaupun lebih banyak spesimen dari pelbagai lokasi diperlukan untuk menghasilkan keputusan-keputusan muktamad yang lebih baik, penemuan-penemuan semasa telah memberi gambaran yang lebih baik tentang kepentingan pendekatan-pendekatan yang saling lengkap-melengkapi iaitu teknik-teknik morfologi dan molekul dalam pengenalpastian kedua-dua spesies timun laut Malaysia tersebut.

Kata kunci: *Stichopus horrens*, *Holothuria leucospilota*, Bentuk Osikel, Gen Sitokrom c Osidasi I Mitokondria DNA, Pulau Pangkor

Abstract: This preliminary study aimed to identify a commercial *gamat* species, *Stichopus horrens* Selenka, 1867, and a *timun laut* species, *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt, 1835), from Pangkor Island, Perak, Malaysia, employing morphological techniques based on the shape of the ossicles and molecular techniques based on the cytochrome c oxidase I (COI) mitochondrial DNA (mtDNA) gene. In Malaysia, a *gamat* is defined as a sea cucumber species of the family Stichopodidae with medicinal value, and *timun laut* refers to non-*gamat* species. *S. horrens* is very popular on Pangkor Island as a

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main ingredient in the traditional production of *air gamat* and *minyak gamat*, while *H. leucospilota* is the most abundant species in Malaysia. In contrast to previous studies, internal body parts (the respiratory tree and gastrointestine) were examined in this study to obtain better inferences based on morphology. The results showed that there were no ossicles present in the gastrointestine of *H. leucospilota*, and this characteristic is suggested as a unique diagnostic marker for the *timun laut* species. In addition, the presence of Y-shaped rods in the respiratory tree of *S. horrens* subsequently supported the potential to use internal body parts to identify the *gamat* species. Phylogenetic analysis of the COI mtDNA gene of the sea cucumber specimens using the neighbour-joining method and maximum likelihood methods further confirmed the species status of *H. leucospilota* and *S. horrens* from Pangkor Island, Perak, Malaysia. The COI mtDNA gene sequences were registered with GenBank, National Center for Biotechnology Information (NCBI), US National Library of Medicine (GenBank accession no.: KC405565-KC405568). Although additional specimens from various localities will be required to produce more conclusive results, the current findings provide better insight into the importance of complementary approaches involving morphological and molecular techniques in the identification of the two Malaysian sea cucumber species.

Keywords: *Stichopus horrens*, *Holothuria leucospilota*, Ossicle Shape, Cytochrome c Oxidase I Mitochondrial DNA Gene, Pangkor Island

Sea cucumbers (Phylum Echinodermata: Class Holothuroidea) are considered to be unique marine animals in Malaysia due to their medicinal properties and commercial value. It is estimated that more than 80 morphospecies of these soft-bodied echinoderms inhabit the seawaters of Malaysia (Kamarudin *et al*. 2010). There are at least five local names for sea cucumbers in Malaysia: *bat*, *balat*, *timun laut*, *gamat* and *brunok*. Among the local names, *gamat* and *timun laut* are the most popular among Malaysians. In Malaysia, *gamat* is a local name for all species of family Stichopodidae. Genus *Stichopus* and genus *Thelenota* are currently the two groups from family Stichopodidae that can be found in the seawaters of Malaysia (e.g., *Stichopus horrens* Selenka, 1867 and *Thelenota anax* H.L. Clark, 1921). Approximately 10 *gamat* species have been identified in the seawaters of Malaysia to date based on Kamarudin *et al.* (2010), Choo (2008) and other documents. Choo (2008) listed *Thelenota ananas* (Jaeger, 1833) as one of the commercial species used in the food industry in Malaysia.

Gamat species have been exploited for their body fluid extracts (*air gamat*) and their lipid extracts (*minyak gamat*). In proportion to the development of science and technology, *gamat*-based products formulated using modern techniques and marketed by Malaysian companies (e.g., Gamat eMas Sdn. Bhd., Nur Af Enterprise, Nutrifes Food & Beverages Industries Sdn. Bhd., and Luxor Network Sdn. Bhd.) can also be found in local and international markets. The spesies *S. horrens* or *gamat emas* has been utilised as the main ingredient and is subject to high demand in Malaysia due to its medicinal properties and commercial values.

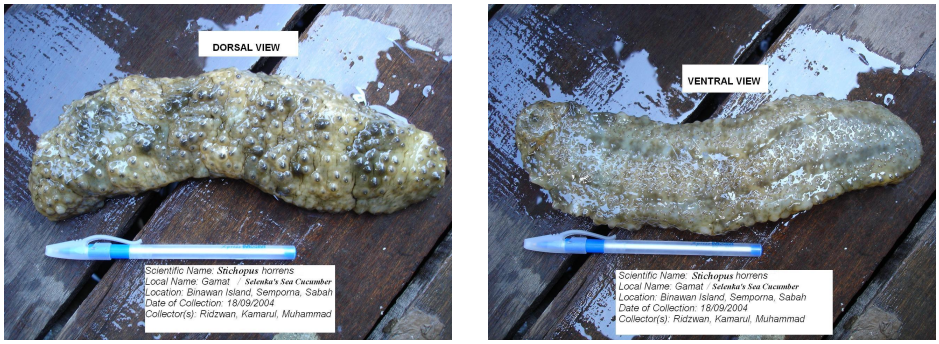
Timun laut is a local name for all species of sea cucumbers in Malaysia, including *gamat* species, and can also be used to refer to all non-*gamat* species. In this study, the latter definition is used. *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt, 1835), a *timun laut* species, is suggested to be the most abundant sea

cucumber species in Malaysia (Kamarudin *et al*. 2010, 2011). This species is also known as *bat puntil*, *bat hitam* or *balat hitam*. The corresponding author prefers to refer to it as *lintah laut*. In terms of commercial value, *H. leucospilota* is one of the commercial species exploited as food in Malaysia, Thailand, Indonesia, the Philippines and Vietnam (Choo 2008).

Ossicle shapes remain an important characteristic employed for the morphological identification of sea cucumbers. Ossicles are small pieces of calcified material that form part of the skeleton of a sea cucumber. A morphological approach is simpler and easier to apply compared with a genetic approach. The common shapes of ossicles in the sea cucumber body include table, button, rod, anchor, perforated plate and roxette. Conducing species identification using ossicle shapes as part of a morphological approach is as important as applying a genetic approach (e.g., using mitochondrial DNA [mtDNA] gene sequencing techniques), and each approach complements the other in producing more accurate results. In fact, mtDNA has become the most preferred model due to its effective maternal inheritance, apparent haploid genome, non-recombination, continuous replication, and the fact that its rate of substitution is within the range of 5 to 10 times greater than that of 'single-copy' nuclear DNA (Hartl & Clark 1989; Amos & Hoelzel 1992).

Many studies suggest the usefulness of ossicle shapes for the species identification of sea cucumbers based on morphology. Ossicles from external body parts, such as the tentacles, cuticles, papillae and podia, are commonly examined. However, the use of ossicles from internal body parts, such as the gastrointestine and respiratory tree, is uncommon, or has yet to be incorporated in such analyses, as no studies employing these characters could be found by the corresponding author. Accordingly, the objective of the present study was to identify a commercial *gamat* species, *S. horrens* and a *timun laut* species, *H. leucospilota*, from Pangkor Island, Perak, Malaysia, employing morphological techniques based on the shape of ossicles, including ossicles from the gastrointestine and respiratory tree, and molecular techniques based on the cytochrome c oxidase I (COI) mtDNA gene.

Specimens of *S. horrens* (Fig. 1) and *H. leucospilota* (Fig. 2) were collected from Teluk Nipah and Pangkor Laut, Pangkor Island. A total of three individuals of each species were sampled. A global positioning system (GPS) was used to mark and record the position of sampling sites on Pangkor Island (not shown specifically, refer to Fig. 3). The samplings took place for approximately two days, from the 8th to the 9th of November 2011. Documentation and collection were performed during low tide. There were no fixed or standard sampling hours for all sites. For short-term storage, fresh specimens of sea cucumbers were stored in ice boxes containing sea water or ice cubes during sampling. In the Science Research Laboratory (Faculty of Science and Technology, Universiti Sains Islam Malaysia), the specimens were transferred to a freezer for long-term storage with proper cataloguing.



(a) (b)

Figure 1: Image of *S. horrens* Selenka, 1867: (a) dorsal view; (b) ventral view.
Source: Ridzwan Hashim

Figure 2: An individual of *H.* (*Mertensiothuria*) *leucospilota* (Brandt, 1835) from Pangkor Island, Perak, Malaysia.
Source: Kamarul Rahim Kamarudin

A small piece of tissue from each of the external body parts (the tentacles, dorsal cuticle, and ventral cuticle) and internal body parts (the respiratory tree and gastrointestine) was cut with a sterile blade. Each piece of tissue was placed on a glass microscope slide and then covered with several drops of liquid household bleach to dissolve the soft tissue. The ossicles, usually in the form of white pellets, remained in the liquid, and a cover slip was placed gently on each microscope slide covering the ossicles. The prepared slide was then observed under a Nikon (Tokyo) ECLIPSE 80i digital compound microscope, and all of the captured images were saved for the identification of ossicle shapes. As the main focus in this study was to identify the shapes of ossicles and then to compare their varieties between *H. leucospilota* and *S. horrens*, the size of each ossicle type was not precisely measured under the microscope.

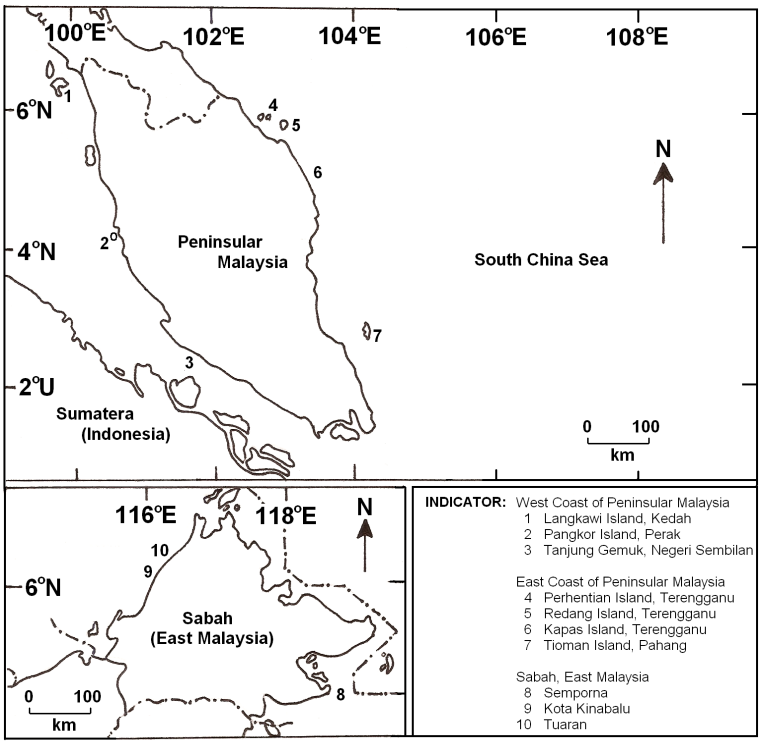


Figure 3: Specimens of *H.* (*Mertensiothuria*) *leucospilota* and *S. horrens* used in this study were collected from Pangkor Island, Perak, Malaysia (sampling site 2).
Source: Adapted from Kamarudin *et al.* (2009).

Total genomic DNA (tgDNA) extraction was performed using the modified cetyl trimethyl ammonium bromide (CTAB) method of Grewe *et al*. (1993) coupled with the Geneaid Genomic DNA Mini Kit (New Taipei City, Taiwan) (blood/cultured cell). The approximate yield of tgDNA, including its quantity and quality, was determined via electrophoresis (on a 1% agarose gel, using ethidium bromide as a gel stain). For polymerase chain reaction (PCR) analysis, approximately 650 base pair (bp) sequences of the COI mtDNA gene of *H. leucospilota* and *S. horrens* were amplified using standard PCR procedures. Two universal primers were used for the PCR: COI (forward) 5'- ATA ATG ATA GGA GGR TTT GG -3' (20 bases) and COI (reverse) 5'- GCT CGT GTR TCT ACR TCC AT -3' (20 bases) (Arndt *et al*. 1996).

Standard thermal cycle amplification (i.e., PCR) was performed in a 50 µL reaction volume containing 33.75 µL of sterilised dH_2O, 5.0 µL of 10× PCR buffer, 3.0 µL of magnesium chloride (25 mM), 2.5 µL of each universal primer (5 µM), 1.0 µL of dNTP mix (10 mM), 2.0 µL of the DNA extract and 0.25 µL of 5 u/µL *Taq* DNA polymerase. A master mix was used for a large number of samples. The cycling parameters were 5 min at 95°C for initial denaturation; 45 s at 95°C for denaturation, 90 s at the optimised temperature for annealing (i.e.,

55°C), and 1 min 30 s at 72°C (60 s/kb; 29 cycles) for extension; 7 min at 72°C for final extension; and then holding at 4°C. The approximate yield of amplified DNA, including its quantity and quality, were determined via electrophoresis (on a 1% agarose gel, using ethidium bromide as a gel stain).

The Geneaid Gel/PCR DNA Fragments Extraction Kit (New Taipei City, Taiwan) was used for direct purification of the PCR products. Purified PCR products in suspension form were prepared prior to sending the samples for sequencing. Sequencing was performed using the BigDye® Terminator v3.0 Cycle Sequencing Kit (Thermo Fisher Scientific Inc., Massachusetts, USA) (ACGT). The cycle sequencing reaction was carried out in a programmable cycler (Tpersonal Combi Thermocycler, Biometra GmbH, Goettingen, Germany) and was run for 35 cycles of 96°C: 10 s, 55°C: 5 s, 60°C: 4 min hold, followed by ethanol/sodium acetate precipitation. A rapid thermal ramp of 1°C/s was applied. Sequencing was carried out on an ABI 377 automated sequencer (Thermo Fisher Scientific Inc., Massachusetts, USA).

The Chromas Lite (version 2.01) program (Technelysium Pty. Ltd., Queensland, Australia) was used to display the results of fluorescence-based DNA sequence analyses. Multiple sequence alignment for the forward reaction sequences was carried out using the ClustalX (version 2.1) program (Thompson *et al*. 1997), with subsequent alignment by eye. Molecular Evolutionary Genetics Analysis 5 (MEGA5) (Tamura *et al*. 2011) was then used to reconstruct phylogenetic trees using the neighbour-joining method (Saitou & Nei 1987) and maximum likelihood method. Both analyses involved eight nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 594 positions in the final dataset. The codon positions included were 1st+2nd+3rd+Noncoding. Phylogenetic confidence was estimated via bootstrapping (Felsenstein 1985) with 1000 replicate data sets. For the neighbour-joining tree, the optimal tree with the sum of branch lengths = 0.29416820 was shown. Evolutionary distances were computed using the Tamura-Nei method (Tamura & Nei 1993) and are presented in units of the number of base substitutions per site. For the maximum likelihood tree, branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree was drawn to scale, with branch lengths measured in terms of the number of substitutions per site.

The list of ossicle shapes observed in the five examined body parts of *H. leucospilota* (*timun laut*) and *S. horrens* (*gamat*) is summarised in Table 1. Most interestingly, the gastrointestine of *H. leucospilota* showed no ossicles (Fig. 4), suggesting that this characteristic of this internal body part of *H. leucospilota* can be used as a unique diagnostic marker to identify the non-*gamat* species. In addition, both the dorsal and ventral cuticles of *H. leucospilota* and *S. horrens* shared table-shaped ossicles (Figs. 5 and 6). Button-shaped ossicles were observed in both cuticle areas in *H. leucospilota*. The dorsal and ventral regions of *H. leucospilota* were determined based on the position of its body when it moved on the sea floor. Moreover, roxette-shaped ossicles were found in both cuticle areas in *S. horrens*.

Table 1: Ossicle shapes in five body parts of *H. (Mertensiothuria) leucospilota* (*timun laut*) and *S. horrens* (*gamat emas*) from Pangkor Island, Perak, Malaysia. Microscopic observations were done using Nikon ECLIPSE 80i digital compound microscope.

No.	Body part		Ossicle shape	
			H. leucospilota	*S. horrens*
1	Dorsal cuticle	1	Table	Table
		2	Button	x
		3	Perforated plate	x
		4	x	Roxette
2	Ventral cuticle	1	Table	Table
		2	Anchor-shaped button	x
		3	Button	x
		4	x	Roxette
		5	x	Terminal plate
		6	x	Major I-shaped rod (boomerang-shaped rod)
3	Tentacle	1	C-shaped rod	C-shaped rod
		2	I-shaped rod	I-shaped rod
		3	F-shaped rod	x
		4	L-shaped rod	x
		5	x	X-shaped rod
		6	x	Table
		7	x	Roxette
4	Respiratory tree	1	C-shaped rod	C-shaped rod
		2	I-shaped rod	I-shaped rod
		3	x	Y-shaped rod
5	Gastrointestine	1	x	X-shaped rod
		2	x	Y-shaped rod

Note: x = absent
Source: Kamarudin (2011)

The tentacles and respiratory trees of *H. leucospilota* and *S. horrens* contained C-shaped and I-shaped rods (Figs. 7 and 8). In contrast to the respiratory tree of *H. leucospilota*, the respiratory tree of *S. horrens* consisted of Y-shaped rods (Fig. 8). In terms of the variety of ossicle shapes, the tentacles of *H. leucospilota* and *S. horrens* displayed the greatest variety, with seven different shapes of ossicles (Table 1), followed by the ventral cuticles (6), dorsal cuticles (4), respiratory trees (3), and gastrointestines (2). Thus, the external body parts of both sea cucumber species (i.e., the tentacles, dorsal cuticles and ventral cuticles) presented a greater variety of ossicle shapes compared with the internal body parts (i.e., the respiratory tree and gastrointestine).

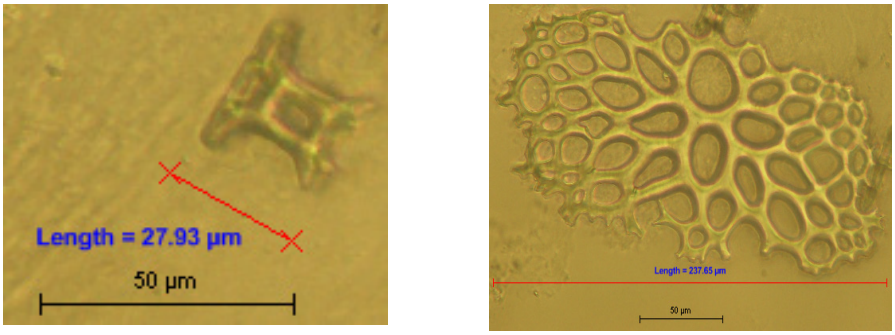
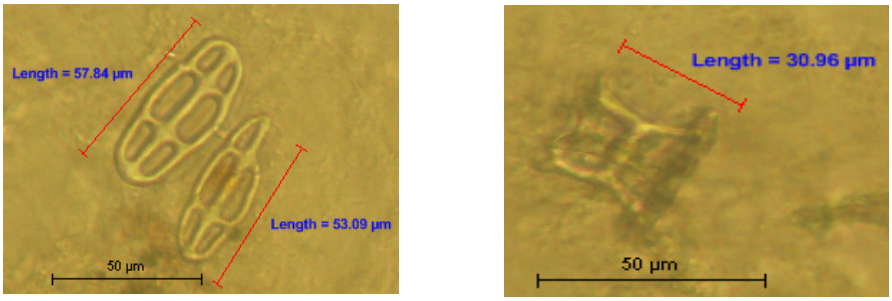
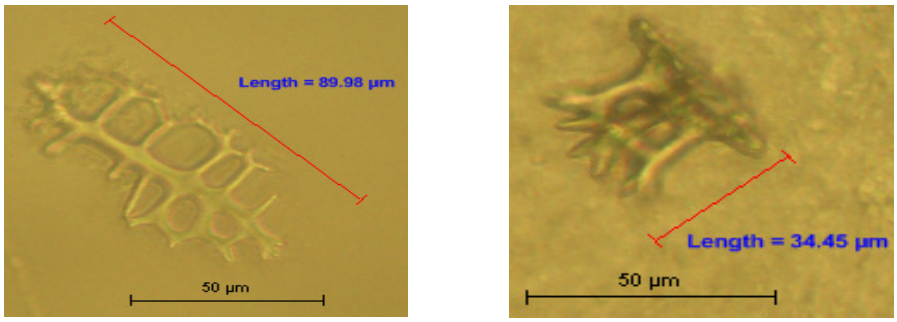


Figure 4: Ossicle shapes in the gastrointestine of *S. horrens*.
Source: Kamarudin (2011)



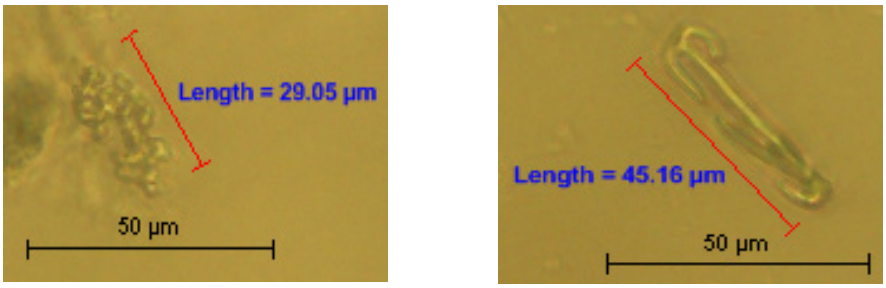
(a) (b)

Figure 5: Ossicle shapes in the dorsal cuticles of (a) *H. (Mertensiothuria) leucospilota* and (b) *S. horrens*.
Source: Kamarudin (2011)



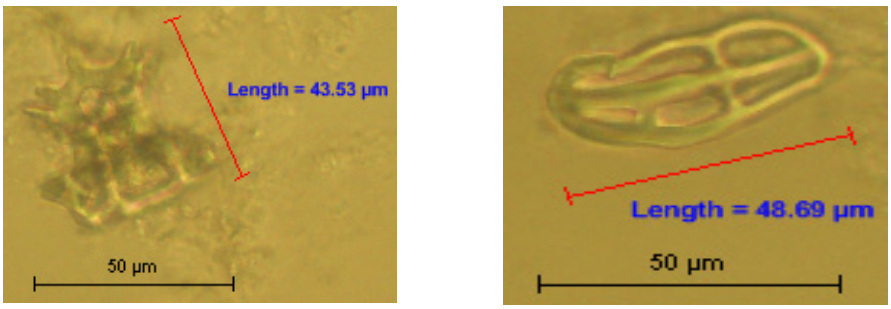
(a) (b)

Figure 6: Ossicle shapes in the ventral cuticles of (a) *H. (Mertensiothuria) leucospilota* and (b) *S. horrens*.
Source: Kamarudin (2011)



(a) (b)

Figure 7: Ossicle shapes in the tentacles of (a) *H. (Mertensiothuria) leucospilota* and (b) *S. horrens*.
Source: Kamarudin (2011)



(a) (b)

Figure 8: Ossicle shapes in the respiratory trees of (a) *H. (Mertensiothuria) leucospilota* and (b) *S. horrens*.
Source: Kamarudin (2011)

A total of three specimens of *H. leucospilota* and three specimens of *S. horrens* were used for tgDNA extraction and PCR analysis of the COI mtDNA gene. However, only two specimens of each species showed successful results enabling them to be employed for COI mtDNA gene sequencing. All four COI mtDNA gene sequences obtained from the Malaysian sea cucumber species were registered with GenBank, National Center for Biotechnology Information (NCBI), US National Library of Medicine (Table 2, GenBank accession no.: KC405565-KC405568).

A total of four COI mtDNA gene sequences from known species (Table 2) were obtained from GenBank via the Basic Local Alignment Search Tool (BLAST, National Library of Medicine, Maryland, USA) program as corresponding sequences for the phylogenetic analyses. Thus, a total of eight COI mtDNA gene sequences were included in the analyses. The results of the phylogenetic analyses indicated that the neighbour-joining method (Fig. 9) and maximum likelihood method (Fig. 10) grouped the COI mtDNA gene sequences of *H. leucospilota* from Malaysia with all of the corresponding sequences from

GenBank, confirming the species status as *H. leucospilota*. The same clustering result was obtained for the COI mtDNA gene sequences of *S. horrens* from Malaysia.

Table 2: Taxa incorporated for the phylogenetic analyses of COI mtDNA gene of *H.* (*Mertensiothuria*) *leucospilota* and *S. horrens* from Pangkor Island, Perak, Malaysia. A number of four sequences of known species were obtained from the GenBank, NCBI, US National Library of Medicine as corresponding sequences.

Taxa	Sample size	Individual no.	GenBank accession no.
H. leucospilota	4	*HLTNP1	KC405565
		*HLTNP3	KC405566
		HLJN207617	JN207617
		HLFJ971394	FJ971394
S. horrens	4	*SHP1	KC405567
		*SHP2	KC405568
		SHEU848282	EU848282
		SHHQ000092	HQ000092

Notes: * specimen from Malaysia used in this study. The ones without the asterisk symbol were corresponding sequences obtained from the GenBank, NCBI, US National Library of Medicine.

The registration and deposition of the four COI mtDNA gene sequences from Malaysian sea cucumber species with GenBank contributes to the availability of COI mtDNA gene sequences from *S. horrens* from Malaysia and provides additional COI mtDNA gene sequences from *H. leucospilota* from Malaysia in the database, in which eight sequences (GenBank accession no.: FJ223873-FJ223880) had been deposited previously by Kamarudin *et al*. (2011). Furthermore, performing species identification using ossicle shapes as part of a morphological approach together with a genetic approach (the COI mtDNA gene sequencing technique) was shown to be complementary in producing more concrete and reliable results.

In conclusion, the present study suggests that the absence of ossicles in the gastrointestine of *H. leucospilota* and the presence of Y-shape rods in the respiratory tree of *S. horrens* are unique characteristics of *S. horrens* and *H. leucospilota*. Such characteristics showed the potential for ossicles in the internal body parts of the two sea cucumber species to be utilised for identifying the *gamat* species and *timun laut* species, especially in the early stage of species identification. However, further studies involving more specimens and various localities are suggested to achieve more concrete conclusions. Additionally, the tree topologies obtained through neighbour-joining and maximum likelihood showed that the COI mtDNA gene sequences of *H. leucospilota* and *S. horrens* from Malaysia clustered according to the species classification, thus further confirming the species status of *H. leucospilota* and *S. horrens* from Pangkor Island, Perak, Malaysia. Moreover, the current findings provide better insight into the importance of complementary approaches involving morphological and molecular techniques in the identification of the two Malaysian sea cucumber species.

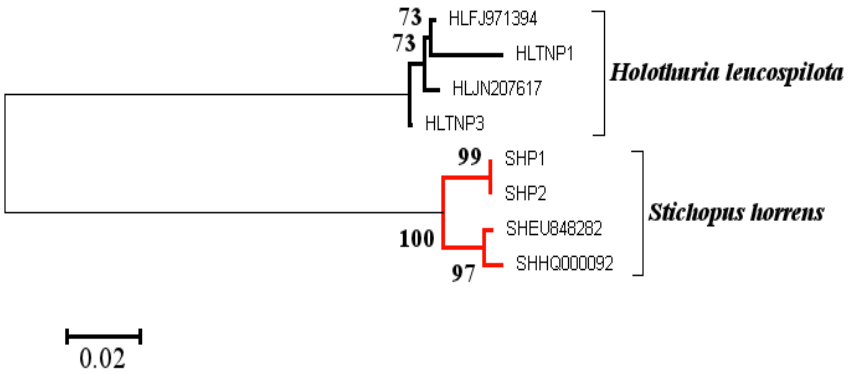


Figure 9: The evolutionary history of COI mtDNA gene of *H.* (*Mertensiothuria*) *leucospilota* and *S. horrens* from Pangkor Island, Perak, Malaysia inferred using the neighbor-joining method (Saitou & Nei 1987).

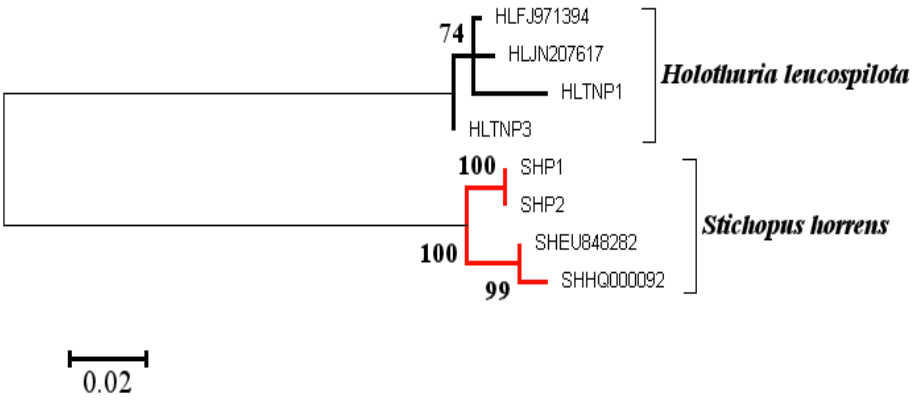


Figure 10: The evolutionary history of COI mtDNA gene of *H.* (*Mertensiothuria*) *leucospilota* and *S. horrens* from Pangkor Island, Perak, Malaysia inferred by using the maximum likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa *et al*. 1985).

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