ANALYSES OF MICROSCOPIC OSSICLES AND MITOCHONDRIAL GENOME OF *Holothuria (Mertensiothuria) leucospilota* FROM SEDILI KECHIL, JOHOR, MALAYSIA

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ABSTRACT

The white threads fish *Holothuria (Mertensiothuria) leucospilota* (Brandt, 1835) or locally known as *bat puntil* is a widely distributed sea cucumber species in Malaysia. The species is categorised as low commercial value species, but it will be sought after if high-value species at fishing zones are depleted. Despite issues of sea cucumber taxonomic identification, the species has no representative of genetic documentation of mitogenome from Malaysia. In this study, the mitogenome of *H. leucospilota* collected from Malaysia, specifically from Sedili Kechil, Kota Tinggi, Johor was sequenced and annotated using Illumina Novaseq6000 next-generation sequencing (NGS) technology. A sufficient quantity and acceptable quality of DNA extract from *H. leucospilota* was necessary to ease the process of NGS. The calcareous ossicle shape and external morphological characteristics of *H. leucospilota* from Sedili Kechil were also recorded to assist early identification and provide an update of morphological characters of *H. leucospilota* from Sedili Kechil. The current findings showed that there were five ossicle shapes: button-shaped, rod-shaped, perforated plate, table, and anchor-shaped ossicles found in different body parts of *H. leucospilota*. Then, a sufficient amount of DNA extract was obtained due to an additional step and modification of centrifugation speed process during DNA filtration of column based FavorPrep DNA extraction kit. The PCGs phylogenetic analyses show that *H. leucospilota* is in order Holothuriida in a monophyletic group, forming sister group with *H. hilla*. This study recorded the first mitogenome of *H. leucospilota* from Malaysia. Along with the species-specific ossicle shape documentation, these findings will provide useful taxonomic and genetic reference of *H. leucospilota* in Malaysia.

ABSTRAK

Bat puntil *Holothuria (Mertensiothuria) leucospilota* (Brandt, 1835) atau dikenali sebagai *white threads fish* ialah spesies timun laut yang tersebar secara meluas di Indo Pasifik, termasuk Malaysia. Meskipun ia dikategorikan sebagai spesies bernilai komersial rendah, tetapi ia akan dicari sekiranya spesies bernilai tinggi di zon perikanan berkurangan. Walaupun terdapat isu pengenalpastian taksonomi gamat, spesies ini tidak mempunyai rekod genom mitokondria dari Malaysia, yang merupakan penanda molekul bermaklumat untuk mengkaji pengenalpastian spesies, taksonomi molekul dan genetik populasi timun laut. Dalam kajian ini, genom mitokondria *H. leucospilota* yang diperoleh dari Malaysia, khususnya dari Sedili Kechil, Kota Tinggi, Johor telah dijujuk dan dianotasi dengan menggunakan teknologi *Illumina Novaseq6000* Penjujukan Generasi Seterusnya (*NGS*). Kuantiti dan kualiti ekstrak DNA *H. leucospilota* yang mencukupi adalah diperlukan bagi memudahkan proses NGS. Bentuk osikel berkapur dan pemerhatian morfologi luaran *H. leucospilota* juga dilakukan untuk membantu dalam pengecaman awal dan memberikan maklumat terkini berkenaan ciri-ciri morfologi spesimen *H. leucospilota* dari Sedili Kechil. Penemuan semasa menunjukkan lima bentuk osikel: bentuk butang, bentuk batang, plat berlubang, meja, dan bentuk sauh dijumpai pada beberapa tempat bahagian badan *H. leucospilota*. Kemudian, jumlah ekstrak DNA yang mencukupi terhasil daripada penambahan langkah dan pengubahsuaian proses kelajuan pengemparan semasa penurasan DNA kit pengekstrakan DNA FavorPrep berasaskan turus. Analisis filogenetik PCG menunjukkan bahawa *H. leucospilota* adalah mengikut urutan Holothuriida dalam kumpulan monofiletik, membentuk kumpulan saudara dengan *H. hilla*. Kajian ini merekodkan mitogenom pertama *H. leucospilota* dari Malaysia., Dengan dokumentasi bentuk osikel khusus spesies, penemuan ini akan memberikan rujukan taksonomi dan genetik yang berguna untuk *H. leucospilota* di Malaysia.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATION

˚C - Degree celcius

% - Percentage

> - Greater than

≈ - Almost equal to/ approximately

µg - Unit for micro gram

µl - Unit for micro per litre

A - Adenine

BLAST - Basic Local Alignment Search Tool

bp - base pair

C - Cytosine

cm - Unit for centimetre

COI - Cytochrome c oxidase (I) gene

CYTB - Cytochrome b gene

DNA - Deoxyribonucleic acid

G - Guanine

g - Gram

Gb - Gigabyte

gDNA - Genomic DNA

hr - Hour

hrs - Hours

Ind	-	Individual
IUCN	-	International Union for Conservation of Nature
Kbp	-	Kilo-base pair
Kg	-	Kilogram
Min	-	Minute
ml	-	millilitre
mtDNA	-	Mitochondrial DNA
NCBI	-	National Centre for Biotechnology Information
nm	-	nano meter
NGS	-	Next Generation Sequencing
PCR	-	Polymerase Chain Reaction
PCGs	-	Protein Coding Genes
pH	-	Potential of Hydrogen
SRA	-	Sequence Read Archive
RNA	-	Ribonucleic acid
rpm	-	Rotary per minute
T	-	Thymine
UV	-	Ultraviolet light
y	-	Year

LIST OF APPENDICES

LIST OF PUBLICATION AND AWARD

- **GenBank mitogenome**

  Badrulhisham, Nur Sabrina; Solehin, Siti Najihah; Ming Gan, Han; Jahari, Puteri Nur Syahzanani; Mohd Salleh, Faezah; Rehan, Aisyah Mohamed; Kamarudin, Kamarul Rahim (2022), "*Holothuria leucospilota* voucher HL2 mitochondrion", GenBank, NCBI. Accession number: ON584426.1.
- **SRA**

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- **BioProject**

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- **BioSample**

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- **Mendeley Data**

  Badrulhisham, Nur Sabrina; Solehin, Siti Najihah; Ming Gan, Han; Jahari, Puteri Nur Syahzanani; Mohd Salleh, Faezah; Rehan, Aisyah Mohamed; Kamarudin, Kamarul Rahim (2022), "The Mitogenome Data of *Holothuria (Mertensiothuria) leucospilota* (Brandt, 1835) from Malaysia.", Mendeley Data, V1, doi: 10.17632/k6nsv8vycc.1.

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CHAPTER 1

INTRODUCTION

1.1 Background of research

Sea cucumbers (Phylum Echinodermata: Class Holothuroidea) are a group of the most populated and abundant species in the marine benthic ecosystem (Purcell *et al.* 2016) that are found well distributed worldwide. They are ecologically significant as a marker for healthy marine sediment ecosystems (Azari *et al.* 2021) and coral reef ecosystems (Cheng *et al.* 2019) as they significantly contribute towards physicochemical processes in the ecosystem functions (Purcell *et al.* 2016). According to expert estimates, there are currently approximately 1,400 species of sea cucumbers, and new species continue to be identified and classified among six valid orders: Dendrochirotida, Elasipodida, Holothuriida, Molpadiida, Persiculida, and Synallactida (Miller et al., 2017). For centuries, different species of sea cucumbers have been fished primarily for food (Conand & Byrne, 1995; Lawrence *et al.* 2010). According to a review in 2008, a total of 52 species are commercially exploited as food in Asia with majority of them comprising tropical and sub-tropical species from the families Holothuriidae and Stichopodidae, including the genera *Holothuria*, *Actinopyga*, *Bohadschia*, and *Stichopus* (Choo, 2008). Nowadays, sea cucumbers are processed as dry product (*bêche-de-mer*) or frozen product and sold on the markets, whereas biomedical products derived from them have also been used locally and internationally in traditional medications (Choo, 2008).

The morphological identification of sea cucumber, particularly a dry or frozen product that has undergone multiple working procedures is usually difficult and doubtful due to physical changes (Uthicke *et al.* 2010; Kamarudin *et al.* 2019). On the market, sea cucumbers are mostly labelled based on their colour and place of production, such as *gamat emas*, *ibu gamat*, U.S.'s red ginseng, or African sea cucumber, which lack the scientific name, making sea cucumber market labelling frequently confusing (South China Sea Institute of Oceanology of CAS, 2013). In Malaysia, sea cucumber fishery is collected in nature and they are commonly collected by hand, with some collected through snorkelling and diving (Choo, 2008). In some cases, sea cucumber fishing shifted to target many low value species after stocks of the more valuable species have been depleted (Choo, 2008). According to a recent report, from June 2020 to July 2020 in Malaysia and Singapore, sea cucumber online trade and international trafficking have been concerning, with trades in the form of dried, frozen, and cooked products. The online trades remained active until January 2022 and continue to sell CITES-listed species (Ong & Chin, 2022). Malaysia has also implemented the National Policy on Biological Diversity (NPBD) 2016–2025, which focuses on improving Malaysia’s current biodiversity management and meeting Malaysia’s obligations under the United Nations Convention on Biological Diversity (CBD). Thus, many conservation approaches from various multidisciplinary fields have thus been used in Malaysia to evaluate and monitor biodiversity (Li *et al.* 2019).

Morphological or phenotypic identification is the basis for species identification approaches. Wagner (1989) uses three concepts to define biologically similar organisms, also known as a homologue, as having three concepts: 1) The conservation of the features that define a homologue, 2) the individualisation of the homologue relative to the rest of the body, and 3) the uniqueness of homologues, i.e., their specificity for monophyletic groups. In sea cucumber morphological identification, external features and ossicle shapes are the main components of morphological approach. External features such as body colour and shape, number of tentacles, and habitat description are crucial along with ossicle shapes, which are species-specific microscopic calcified skeletons in the dermis of sea cucumber that are informative for preliminary observation and verification of sea cucumber species during collection (Kamarudin and Mohamed Rehan, 2015; Utzeri *et al.* 2020). Aside from morphological identification of species, molecular biology is a field that is required for species identification and is important in monitoring biodiversity conservation (Coker, 2017). Knowledge on genetics or genotyping in species identification would aid conservation efforts such as monitoring species from small populations, tackling taxonomic difficulties, identifying management units within species, using molecular genetics analyses in forensics, and understanding species biology (Coker, 2017).

In DNA-based identification, the Sanger sequencing method is the first-generation sequencing that is most commonly applied for DNA barcoding by sequencing small fragments of target genes, usually mitochondrial genes such as cytochrome c oxidase 1 (*COX1*) for animals (Batovska *et al.* 2017) or also known as partial mitochondrial DNA (mtDNA) genes. Mitochondrial genes are preferred as the best markers for DNA barcoding because they are highly evolved, are present in multiple copies, and have conserved DNA region (Xin *et al* 2017; Li *et al.* 2020). Molecular identification such as DNA barcoding is advantageous in species identification, complementing traditional morphological identification approaches (Batovska *et al.* 2017) such as distinguishing species of similar looking (cryptic species), species with colour variants, undescribed species, and species that have undergone physical changes (processed products such as *bêche-der-mer*). However, DNA barcoding has limited application in systematic relationship; some gene regions have limited use for identifying certain species . Furthermore, some DNA gene markers such as *COX1* are not effectively applied for every species in polymerase chain reaction (PCR) in all animal taxa (Chen *et al.* 2012). Large DNA regions such as the mitochondrial genome (or known as mitogenome) are more suitable to find good species relationship and provide better resolution compared to using DNA barcoding.

NGS technology can perform massive parallel sequencing of DNA strands thus resulting in high-throughput data and speed at low cost, compared to Sanger sequencing. NGS enables the sequencing of millions of DNA fragments, effectively determining the nucleotide order of an entire genome. As a result, this technology can overcome the limitations of the Sanger sequencing method for DNA barcoding, allowing for new perspective on genome study and research (Kchouk *et al.* 2017) such as mitogenome. The mitochondrial genome is all of the DNA found in mitochondria (mitogenome). The use of NGS to sequence the whole genome allows for the sequencing of the mitogenome in a short period of time with a low cost. The mitogenome in bilaterians is double helix, circular, and smaller in size (~14 to 18 kbp) which consists of 37 genes, including protein-coding genes (PCGs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and a putative control region (D-loop) (Xin *et al.* 2017; Li et al., 2020). This DNA region especially PCGs is crucial in the construction of phylogenetic trees, as PCGs impart more statistical confidence of inferred phylogenetic trees with higher resolution compared to analyses based on partial mtDNA genes. The mitogenome also helps in wildlife forensics (metabarcoding) for biodiversity monitoring (Miga *et al.* 2022). The sequence of mitogenome obtained through NGS is valuable and can be deposited in GenBank or Mendeley Data. GenBank is a public database under the National Center for Biotechnology Information (NCBI) that stores all publicly available DNA sequences, which are essential for genetic documentation for all uses (Dennis *et al.* 2012), while Mendeley Data is a general cloud-based repository to store research data in any versions of file (Swab, 2016; Mendeley Data, n.d.).

Holothuria (Mertensiothuria) leucospilota (Brandt, 1835), a sea cucumber species from family Holothuriidae or also known as white threads fish is a widely distributed species in the Indo-Pacific reefs region and is the most abundant species in Malaysia, including Sabah and Sarawak (Kamarudin *et al.* 2009; Harith *et al.* 2018). This species is found in shallow water of many coastal areas, usually under sandy bottoms and under rocks, specifically inhabiting depths up to 10 m (Choo, 2008). This species has been studied and found to contain abundant of collagen in the body wall as well as bioactive compounds such as phenolics, terpenoids, tannins, and various trace elements in all body organs, making it a potential source for nutritive food value as well as nutraceutical and pharmaceutical uses (Ceesay *et al.* 2019). *Holothuria leucospilota* is one of the many species exploited for food in Asia, particularly in Thailand and Vietnam (Choo, 2008) despite being classified as low-value species in markets and species of least concern by IUCN Red List of Threatened Species (IUCN Red List, 2022). This species would also be sought after if the high-value species are depleted (Choo, 2008). There have also been numerous reports of species mislabelling and the difficulty in identifying the taxonomy after processing (example: Bêche-de-mer (dried form), and frozen), which could lead to species substitution (Choo, 2008). Regardless of the issues, there is still no genetic documentation of *H. leucospilota* mitogenome particularly those from Malaysian region, based on BLAST nucleotide search. There are only three available mitogenomes of *H. leucospilota* which are sourced from China (accession number: MK940237, MN594790, and MN276190).

Thus, in this study, fresh specimens of *H. leucospilota* were collected at Sedili Kechil, Kota Tinggi, Johor Darul Ta'zim, Malaysia. Morphological identification including identification of variety of ossicle shapes was done prior to obtaining good quantity and quality of total genomic DNA (total gDNA) extraction for DNA library.

Obtaining high quantity and quality of total gDNA of *H. leucospilota* is important for achieving good performance and complete run during the NGS sequencing process (Marg-Haufe & Zymo Research, n.d.). The complete sequence data of *H. leucospilota* mitogenome were submitted to the public bioinformatics library GenBank, NCBI and general cloud-based data repository Mendeley Data, and phylogenetic trees were compared with those of other sea cucumbers (Xia *et al.* 2015) based on 13 concatenated PCGs. The complete mtDNA of *H. leucospilota* will provide useful genetic markers and genomic sequences for species management, trades, and genetic assessment due to their conserved structures and active structure evolutions (Xin *et al.* 2017; Li *et al.* 2020). Moreover, public repositories such as GenBank, NCBI and Mendeley Data provide an important role in conservation of genetic resources especially in many research exploration and comprehensive genetic information management. *H. leucospilota* mitogenome would be an excellent molecular marker for future references for species identification, molecular taxonomy, phylogenetic relationship of sea cucumbers, genome evolution, and population genetics (Mu et al., 2018). Hence, the *H. leucospilota* mitogenome would assist researchers in providing useful genetic data of *H. leucospilota* in Malaysia for future use.

1.2 Problem statement

Issues of taxonomic identification has commonly occurred among sea cucumbers. For example, tiger tail sea cucumber is named after two different species: *H. hilla* and *H. thomasi* (SeaLifeBase, n.d.). Meanwhile, Sven Uthicke reported that all *H. nobilis* are actually *H. whitmaei*, due to the problems of taxonomic names of teatfish groups (Choo, 2008). Moreover, *H. leucospilota* are often confused with *H. atra*, which have similar body shapes, colour, and habitat (Bonham & Held, 1963; Setyastuti, 2015). Incidentally, the issues of taxonomic identification would lead to species substitution, overexploitation that takes place in markets which cause effects on health, economic and food security (Bruckner, 2005; Choo, 2008; Anderson *et al.* 2011; Hashem *et al.* 2022). Moreover, despite being widely distributed, there are currently no mitogenome records of *H. leucospilota* collected in Malaysia and deposited in GenBank, NCBI. Then, the use of conventional Sanger sequencing imposes many limitations to obtain *H. leucospilota* mitogenome in terms of cost and speed compared to using NGS

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