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Checklist of Marine Fungi and Yeasts Associated with *Holothuria* (*Mertensiothuria*) *leucospilota* from Pangkor Island

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Abstract: *Marine microorganisms such as fungi and yeasts can adapt to extreme marine environment conditions and play different roles especially in the nutrient cycling and as bioindicator of ocean change. This study was carried out to isolate and identify fungi and yeasts associated with Holothuria (Mertensiothuria) leucospilota from Pangkor Island, Perak, Malaysia in order to determine their species richness. Two specimens of H. leucospilota were collected from Giam Island and Teluk Nipah Beach of Pangkor Island. Nine samples of fungi and ten samples of yeasts were isolated from the internal and external parts of the H. leucospilota specimens such as cuticle, tentacle, coelomic fluid, cloaca, cuvierian tubules, and surrounding sediment and seawater. Polymerase Chain Reaction (PCR) and DNA sequencing of the Internal Transcribed Spacer (ITS) region were applied for species identification of the microorganisms. Sequence analyses of the ITS region resulted in the identification of five genera of fungi i.e. Cladosporium, Curvularia, Polyporaceae, Acremonium, and Penicillium; and four genera of yeasts i.e. Sterigmatomyces, Pichia, Debaryomyces, and Candida with some of them could be identified up to the species level. The findings have significantly contributed to the recent information on the checklist of fungi and yeasts isolated from the H. leucospilota specimens from Pangkor Island.*

Keywords: Fungi, *Holothuria leucospilota*, Internal Transcribed Spacer region, Pangkor Island, yeasts

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1. Introduction

Marine environment provides a high level of microbial biodiversity for biotechnological exploitation. It represents a vast resource that can provide a source of food, medicines, and raw materials. [1] noted that marine microorganisms have potential applications in metal nutrient cycling, greenhouse gas reduction, detoxification, and the basis of the food web. The ability to synthesize functional biomolecules is a unique potential of marine microorganisms, including marine fungi and yeasts [2]. Such ability helps the marine microorganisms to adapt to extreme marine environmental conditions such as high or low temperature, alkaline or acidic water, high pressure, and limited substrates in sea water.

There have been quite a number of studies done focussing on marine yeasts. In fact, irregular account and description of marine fungi existed as early as 1850. The first known record of marine yeast was in 1894 when Fischer isolated yeasts from the Atlantic Ocean waters, by which the majority was identified as white and red "Torula" species [5]. There were some researchers who had isolated over 30 different yeast species have already been isolated from various marine environments by since the mid 1960's [6]. In early studies, yeasts from both the Ascomycota and the Basidiomycota were isolated, although it was originally thought that the sea was almost devoid of Basidiomycota [7]. For example, various species of the genus *Candida* (Ascomycota) and *Rhodotorula* (Basidiomycota) were isolated from the Biscayne Bay, Florida [4]. Furthermore, recent investigations of yeast isolated from various substrates and marine habitat had been done. However, these information on the yeasts are is still incomplete [4] and many locations have yet to be surveyed [8].

Due to evolution in a different environment, the characteristics of marine fungi and yeasts are different from their terrestrial counterparts in terms of their structures and properties. Therefore, marine fungi and yeasts can offer a wide range of development and applications in biotechnology with their own unique characteristics. In Malaysia, only few studies regarding association of microorganisms with local sea cucumbers were conducted to date. Accordingly, the aims of this study were to isolate fungi and yeasts from the interior and exterior parts of *Holothuria (Mertensiothuria) leucospilota* specimens and their surrounding sediments and seawater from Giam Island and Teluk Nipah Beach, Pangkor Island, Perak; and to identify the yeast and fungal isolates using Polymerase Chain Reaction (PCR) of the Internal Transcribed Spacer (ITS) region as well as DNA sequencing analysis of the target region. The sea cucumber species chosen in this study, known as white threads fish in English and *bat puntil* among the Malaysians, was considered as the most dominant sea cucumber species in Malaysia [9], and the findings have significantly contributed to the recent information on the checklist of fungi and yeasts isolated from the local species. It may contain microorganisms that could help it to adapt in the marine environment [10].

2. Materials and Methods

2.1 Sample Collection and Handling

Two specimens of *H. leucospilota* were collected from Giam Island (4º 14' 09.5" N 100º 32' 22.4" E) and Teluk Nipah Beach (4º 14' 03.3"N 100º 32' 41.4"E) in Pangkor Island, Perak as sources for fungi and yeasts samples. The specimens were labeled as HL (Fig. 1) and HL1 (Fig. 2). Non-protein-coding 12S and 16S mitochondrial ribosomal RNA (rRNA) gene sequences of the HL1 specimen (Fig. 2) were registered with the GenBank, National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine (Accession No.: KX768273 - KX768274). Both specimens were then subjected to the microbial isolation procedures immediately after the collection. The isolation media were placed in iceboxes during transportation to keep them in cold condition.

Fig. 1 - Specimen of *Holothuria (Mertensiothuria) leucospilota* collected from Giam Island, Pangkor Island, Perak (labelled as HL)

Fig. 2 - Specimen of *Holothuria (Mertensiothuria) leucospilota* collected from Teluk Nipah Beach, Pangkor Island, Perak (labeled as HL1)

2.2 Isolation of Yeasts and Fungi

The internal and external body parts of both *H. leucospilota* specimens such as cuticle, tentacle, coelomic fluid, cloaca, cuvierian tubules, polian vesicles, gastrointestine, respiratory tree, surrounding sediment and seawater were swabbed using sterilied cotton swabs. The microbial samples on the swabs were then directly streaked on PDA (Potato Dextrose Agar) plates and NA (Nutrient Agar) plates simultaneously, and the agar plates were incubated at 26˚C for three to five days. The microbial colonies grown on the agar plates were then restreaked into new PDA and NA plates, and then incubated at 26˚C for 3 to 5 days to get single colonies.

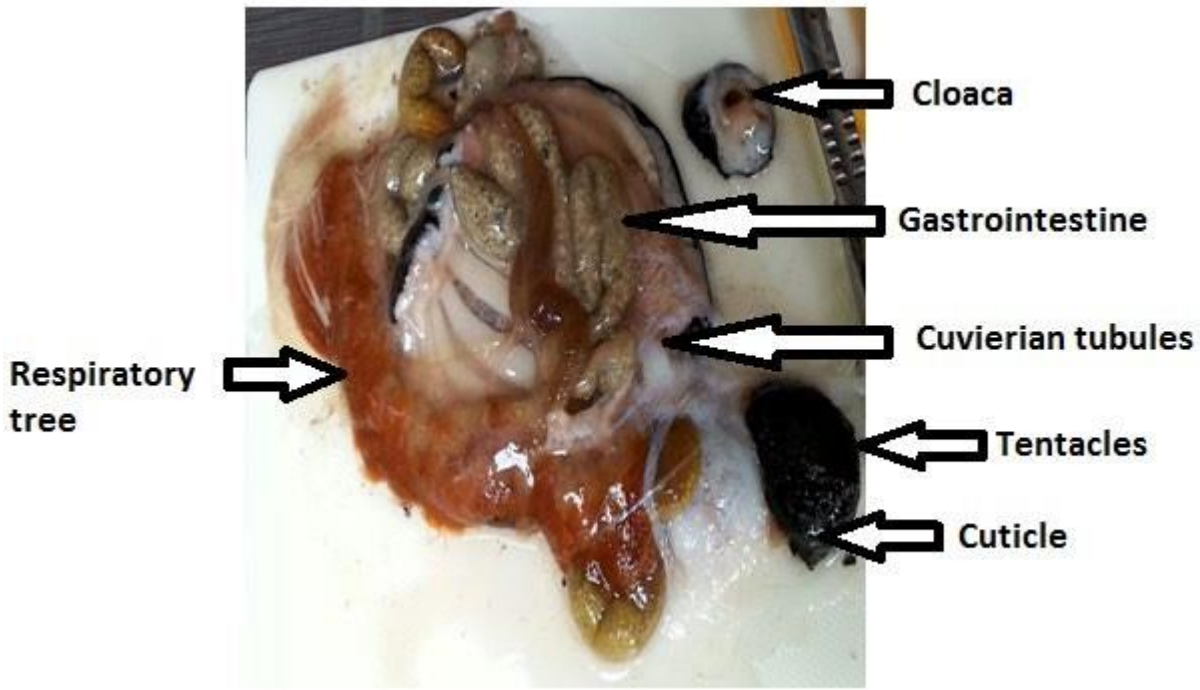

Fig. 3 - Body parts of *Holothuria (Mertensiothuria) leucospilota* from Pangkor Island, Perak that was used for microbial isolation. Coelomic fluid, polian vesicles, surrounding seawater and sediment were also involved (not depicted)

2.3 Microscopic Observation

A single colony of each isolate was mixed with a droplet of sterile distilled water on a glass slide and then smeared. The smear was passed over the flame for heat-fixation until the smear dried off. The smear was stained using simple staining method by adding methylene blue for 2 min. The glass slide was then washed with tap water and air-dried. The glass slide was covered with a glass cover and observed under the light microscope (Olympus model CX21) at 100X magnification using oil immersion.

2.4 Total Genomic DNA Extraction

The total genomic DNA extraction was done following the methods of [11] with little modification to the protocol.

2.5 Polymerase Chain Reaction (PCR)

The amplification processes were carried out in 25 µL total reaction mixtures using Promega PCR Master Mix (Promega Corporation, Madison, USA). The 5.8S-ITS fragments of the yeast isolates were amplified using PCR with the forward primer ITS1 (5´TCCGTAGGTGAACCTGCGG´3) and reverse primer ITS4 (5´TCCTCCGCTTATTGATATGC´3). These primers were used for amplification of the 5.8S ITS region which was in the range of 350 to 880 base pairs (bp) in length. Meanwhile, primers from [12] i.e. ITS5 (5´GGAAGTAAAAGTCGTAACAAGG´3) and ITS4 (5´TCCTCCGCTTATTGATATGC´3) were used to amplify the ITS region that highly specific for fungi targetting the gene encoding for 18S rRNA.

The amplification of the ITS region was done using T100^{TM} Thermal Cycler (Bio-Rad, California, USA). The PCR condition for the DNA extracts of yeast isolates: 94ºC for 5 min (initial denaturation), 94ºC for 30s (denaturation), 56.5ºC for 30s (annealing), 72°C for 1 min (extension), followed by another 34 cycles, and 72°C for 5 min (final extension). Meanwhile, the PCR condition for the DNA extracts of fungal isolates: 94ºC for 5 min (initial denaturation), 94ºC for 45s (denaturation), 55ºC for 1 min (annealing), 72°C for 1 min (extension), followed by another 34 cycles, and 72°C for 7 min (final extension).

2.6 DNA Sequencing and Molecular Species Identification

The amplified DNA fragments were sent for PCR clean up (sample purification) and DNA sequencing at the Apical Scientific Sdn. Bhd., Seri Kembangan, Selangor, Malaysia. The sequencing results were viewed using the Chromas Lite software, version 2.1.1 (Technelysium Pty Ltd). The sequences were aligned and compared with corresponding sequences in the GenBank database using the Basic Local Alignment Search Tool program (BLAST) to identify the species or genus of each isolate.

3. Results and Discussions

Fungi and yeasts were successfully isolated from various parts of *H. leucospilota* specimens collected from Giam Island and Teluk Nipah Beach of Pangkor Island, Perak. There were nine isolates of fungi and ten isolates of yeasts isolated from seven different parts of the specimens, such as cuticle, tentacle, coelomic fluid, cloaca, cuvierian tubules, surrounding sediments and seawater (Table 1). No microbial isolates were observed for Polian vesicles, gastrointestinal tract, and respiratory trees of both *H. leucospilota* specimens.

Morphologically, yeasts isolates differed in color. Some colonies were of cream, white, orange, and yellowcolors. [13] stated that several yeasts were visualised on surface-grown colonies following colours: cream (*S. cerevisiae*), white (*Geotrichum candidum*), black (*Aureobasidium pullulans*), pink (*Phaffia rhodozyma*), red (*Rhodotorula rubra*), orange (*rhodosporidium* spp.), and yellow (*Cryptococcus laurentii*). [14] reported that the colonies formed by cells of different yeast genera could be smooth, fluffy, rough, and slimy; depending on the ability of the particular yeast to form capsules or other extracellular matrix material. The morphologies also depended on the capability of the cells to enter different stages of the yeast life cycle, for examples mating, sporulation or pseudohyphal growth [14]. Meanwhile, the fungal isolates showed whitish, blackish, and brownish colonies with cottony texture. The selected single colonies were observed under the microscope for morphological observation. Most fungal isolates grew like a flower shape and diagonally which consist of white, black, and brown fungi, and could not be identified morphologically.

Table 1 - The isolated fungi and yeasts from different parts of *Holothuria (Mertensiothuria) leucospilota* specimens from Pangkor Island, Perak and from their surrounding sediment and seawater

Sample number	Name of sample	Source of isolate
Fungi		
1	HLS1	Sediment
2	HLS2	Sediment
3	HLW	Seawater
4	HLL	Cloaca
5	HL1S1	Sediment
6	HL1C1	Cuticle
7	HL1C2	Cuticle
8	HL1CT2	Cuvierian Tubules
9	HL1T2	Tentacle
Yeasts		
1	HLW	Seawater
2	HLC	Cuticle
3	HL1S1	Sediment
4	HL1S2	Sediment
5	HL1S3	Sediment
6	HL1C1	Cuticle
7	HL1C2	Cuticle
8	HL1T2	Tentacle
9	HL1Coe2	Coelomic Fluid
10	HL1CT2	Cuvierian Tubules

Total genomic DNA of the isolates was successfully extracted and analysed using agarose gel electrophoresis. The species identities of the isolates were determined based on the ITS sequences from the DNA sequencing results. Searches in Basic Local Alignment Search Tool (BLAST) were performed to determine the closest known relative of DNA fragment. From the BLASTn result, nine isolates of fungi and eight isolates of yeasts were identified with a very good accuracy up to the genus level, however, the other two isolates of yeasts had poor identification since their

identity scores were below 95%. (Table 2). In total, five genera of fungi i.e. *Cladosporium, Curvularia, Polyporaceae, Acremonium, and Penicillium* and four genera of yeasts i.e. *Sterigmatomyces, Pichia, Debaryomyces, and Candida* were identified with some of them could be identified up to the species level (Table 2). A higher number of isolates and species was obtained for HL1 specimen and its surrounding sediment and seawater, as compared to HL specimen and its environment sources i.e. the surrounding sediment and seawater.

Regarding the molecular species identification of fungi, two isolates of *Curvularia lunata* from the cuticle of HL1 specimen were recorded (Table 2). Previously, [18] reported that *C. lunata* was isolated from the marine sponge *Niphates olemda*. Besides, *Cladosporium halotolerans* was isolated from both the surrounding sediments of HL and HL1 specimens. The halophilic and halotolerant mycobiota from hypersaline aqueous habitats worldwide frequently contain *Cladosporium* Link isolates [19][20] thus supporting the presence of *C. halotolerans* in the sediment in Pangkor Island, Perak. *Penicillium* was regarded as the second most common genus of marine fungi [21]. Likewise, the presence of an unknown *Penicillium* species in the surrounding sediment of HL specimen and the presence of *Penicillium funiculosum* in the cloaca of HL specimen and the cuverian tubule of HL1 specimen were recorded in this study. Isolates of genus *Acremonium* and genus *Polyporaceae* from the surrounding seawater of HL specimen and the tentacle of HL1 specimen, respectively, were also recorded (Table 2). [22] stated that marine fungus *Acremonium strictum* collected from a *Choristida* sponge off the coast of Korea produced the novel natural product, *Acremostrictin*. However, there was no recent study on *Polyporaceae* species from the marine environment could be found to date.

Apart from that, the molecular species identification of yeasts showed that there were four different species present in the specimens of HL and HL1 and in their environment sources, i.e. the surrounding sediments and the seawater (Table 2). In this study, *Debaryomyces hansenii* was the most common species isolated from the surrounding sediments and seawater, cuticles, tentacle, and cuverian tubule of the *H. leucospilota* specimens. *D. hansenii* can be found initially in many habitats with low water activity, such as seawater, from which it was initially isolated; cheese, meat, wine, beer, fruit, and soil [23]. Moreover, *Debaryomyces* species are osmotolerant and extremophilic yeasts, which proved to be genetically and biochemically interesting yeasts with the considerable biotechnological promise [24]. Furthermore, *Sterigmatomyces halophilus* was isolated from the surrounding sediment of HL1 specimen. [25] stated that *Sterigmatomyces halophilus* was usually found in association with marine environments, and species of *Sterigmatomyces* are osmotolerant. The ecological niche of many species, however, remains unknown. *Candida* sp. was isolated from the coelomic fluid of HL1 specimen, while *Pichia* sp. was isolated from the surrounding sediment of HL1 specimen. Previously, [26] stated that *Candida* and *Pichia* were common in shallow water.

For future research, more *H. leucospilota* specimens from different localities in Malaysia are required in order to obtain better results on the yeast and fungal communities in a specific population. Moreover, different molecular techniques such as PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) and Total Genomic DNA-RFLP (TgDNA-RFLP) need to be incorporated for better species identification and validation of yeast and fungi associated with *H. leucospilota*. The fungi and yeasts isolated from the *H. leucospilota* specimens from Pangkor Island could have potential biotechnological applications which can be further characterised.

Table 2 - Molecular species identification of fungi and yeasts associated with *Holothuria (Mertensiothuria) leucospilota* specimens from Pangkor Island, Perak based on ITS sequence analysis using BLAST. Note: ID – based on Identityscore (Ident)

Isolate name	Identification	% ID	Accession number of corresponding sequences
Fungi			
HLS1	*Cladosporium halotolerans*	99%	LN834369.1
HLS2	*Penicillium* sp.	99%	KM066554.1
HLW	*Acremonium* sp.	99%	EF042103.1
HLL	*Penicillium funiculosum*	99%	JQ717348.1
HL1S1	*Cladosporium halotolerans*	99%	LN834369.1

HL1C1	*Curvularia lunata*	99%	KR815445.1
HL1C2	*Curvularia lunata*	99%	KR815445.1
HL1CT2	*Penicillium funiculosum*	99%	JQ717348.1
HL1T2	*Polyporaceae* sp.	99%	LC133841.1
Yeasts			
HLW	*Debaryomyces hansenii*	99%	JN851059.1
HLC	*Debaryomyces hansenii*	100%	JN851059.1
HL1S1	*Debaryomyces hansenii*	99%	KR264906.1
HL1S2	*Pichia kudriavzevii*	84%	FJ231421.1
HL1S3	*Sterigmatomyces halophilus*	97%	NR073302.1
HL1C1	*Debaryomyces hansenii*	100%	JN837098.1
HL1C2	*Debaryomyces hansenii*	99%	JN851059.1
HL1T2	*Debaryomyces hansenii*	99%	JN851059.1
HL1Coe2	*Candida* sp.	85%	GU126458.1
HL1CT2	*Debaryomyces hansenii*	99%	JN851059.1

4. Conclusion

As a conclusion, ten yeast strains and nine fungal strains from different parts of *Holothuria (Mertensiothuria) leucospilota* specimens from Pangkor Island, Perak and their environment sources, i.e. the surrounding sediment and seawater were isolated. The BLAST analysis of the ITS region sequences resulted in the identification of five genera of fungi, i.e. *Cladosporium, Curvularia, Polyporaceae, Acremonium,* and *Penicillium;* and four genera of yeasts, i.e. *Sterigmatomyces, Pichia, Debaryomyces,* and *Candida*. A high number of yeast and fungal isolateswere obtained for HL1 specimen and its surrounding sediment and seawater, as compared to HL specimen and its environment sources. In summary, the findings have contributed to the recent information on the checklist of fungi and yeasts isolated from the local sea cucumber species.

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