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Identification of Microorganisms Associated with Sea Cucumbers in Johor Coastal Seawater

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ABSTRACT

Sea cucumbers have been reported to host diverse microorganisms, including pigment-producing microorganisms. Investigating these microorganisms is essential for understanding ecological functions, potential biotechnology applications, and impacts on human health. However, despite their importance, the microbial diversity of sea cucumbers remains largely understudied. Thus, this study aims to identify the microorganisms associated with three species of sea cucumbers: *Holothuria pardalis*, *Holothuria leucospilota*, and *Holothuria scabra* collected from Johor coastal seawater. Identification of these isolates revealed that there were twenty-two strains of bacteria and three strains of fungi in total, representing 11 taxa, including 9 taxa from bacteria, namely *Staphylococcus*, *Bacillus*, *Brevibacillus*, *Psychrobacter*, *Stenotrophomonas*, *Chryseobacterium*, *Sphingomonas*, and *Pseudoxanthomonas*, and two taxa from fungi: *Aspergillus* and *Rhodotorula*. The isolates were identified using 16S rRNA for bacteria and internal transcribed spacer for fungi. Among these species, *Chryseobacterium* sp., *Sphingomonas* sp., and *Pseudoxanthomonas* sp. were first reported as part of the pigment-producing microorganisms found in sea cucumbers in Malaysia. Thus, these findings offer a novel insight into pigment-producing microorganisms in sea cucumbers and their potential as natural alternatives for colourants.

Keywords: 16S rRNA, bacteria, fungi, identification, ITS, phylogenetic analysis, pigment-producing microorganisms, sea cucumbers

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INTRODUCTION

The sea cucumber, belonging to Holothuroidea and the phylum Echinodermata, is globally distributed in deep seas and benthic areas (Gianasi et al., 2021; Liu et al., 2023). Its consumption is widespread in China, Korea, Japan, Malaysia, Indonesia, and Russia due to its numerous biological activities (Hossain et al., 2020). It has leathery skin and a soft, cylindrical body with a single-branched gonad, which has been commercially utilised for food and health purposes over the past few decades (Halder & Pahari, 2020). These marine invertebrates, known for their application in food, cosmetics, and traditional medicine, encompass around 100 species harvested for commercial use, particularly in Asian countries like China, Indonesia, Japan, Korea, and Malaysia. Malaysia and Singapore were acknowledged as the Asia’s biggest importers of sea cucumbers (Louw & Bűrgener, 2020). More than 80 species of sea cucumbers have been documented in Malaysia (Solehin et al., 2021). The Aspidochirotida order exhibits Malaysia’s highest distribution and diversity of sea cucumbers.

The distribution of sea cucumbers in Malaysia has been documented across several Peninsular Malaysia, Sabah and Sarawak states. *Stichopus horrens* are the most popular species found in Langkawi and Pangkor Island, which are commercially exploited for medicine and food supplements (Kamarudin et al., 2015). In Sabah, four genera were recorded: *Holothuria*, *Stichopus*, *Actinopyga*, and *Molpadia*. Sea cucumbers are commercially marketed in Sabah and serve as food and traditional medicine. In addition, species of sea cucumbers from the Molpadiida order have been documented in Sarawak (Kamarudin et al., 2016). In Johor, *Holothuria* (*Halodeima*) *edulis* and *Stichopus chloronotus* were the first documentation of sea cucumbers in Pulau Aur, Johor, by Zulfigar et al. (2007).

For centuries, sea cucumbers have been popular throughout Asia as a medicine, delicacy and nutritious food (Song et al., 2020). Furthermore, today’s market offers a diverse array of products sourced from various parts of sea cucumbers, such as extracts from the body wall, liquid extracts, skin, and all body parts of sea cucumbers (Marchese et al., 2020; Tolon et al., 2021). Recent studies indicate that the isolation of culturable microorganisms associated with sea cucumbers has been identified with five genera: *Holothuria*, *Cucumaria*, *Stichopus*, *Apostichopus*, and *Eupentacta* (Wingfield et al., 2024).

Sea cucumbers obtain their food through the ingestion of marine sediments or filtering seawater (Mohsen et al., 2020). Besides, they also consume microfauna, bacteria, and decomposed organic and inorganic matter present on the surface of ocean sediments (Ennas et al., 2023). It is believed that seafloor bacterial colonies serve as both a direct food source and an indirect provider of essential nutrients for sea cucumbers (Chakraborty, 2022). Moreover, the natural products derived from bacteria associated with marine organisms offer a promising novelty for new research findings (Chu et al., 2020; Khalifa et al., 2019).

Therefore, this study aimed to identify microorganisms associated with three sea cucumbers: (1) *Holothuria* (*Lessonothuria*) *pardalis* Selenka (1867) from Pulau Tinggi, (2) *Holothuria* (*Metriatyla*) *scabra* (Jaeger, 1833) from Tanjong Surat, and (3) *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt, 1835) collected from various locations in Johor's coastal seawater: Pulau Tinggi, Sedili Kechil, and Tanjung Surat using 16S rRNA and internal transcribed spacer (ITS). The discovery of these microorganisms, particularly those capable of producing pigments, could lead to new findings in the field of natural colourants.

METHODOLOGY

Research Sampling

Sea cucumbers were collected around coastal seawater during low tide from three different locations in Johor state, as shown in Figure 1, which were Pulau Tinggi (Figure 1a), Sedili Kechil (Figure 1b), and Tanjong Surat (Figure 1c). Three individuals, *Holothuria* (*Lessonothuria*) *pardalis* Selenka (1867) from Pulau Tinggi, *Holothuria* (*Metriatyla*) *scabra* (Jaeger, 1833) from Tanjong Surat, and *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt, 1835) from Sedili Kechil, were sampled. The position and sampling sites were marked using the Global Positioning System (GPS) at 2.3047° N, 104.1176° E for Pulau Tinggi, 1.5876° N, 104.1466° E for Tanjong Surat, and 1.8258° N, 104.1587° E for Sedili Kechil. Fresh specimens of sea cucumbers were stored in ice boxes containing seawater during sampling for short-term storage. For long-term storage, the specimens were stored in a -20°C chest freezer with proper cataloguing (Kamarudin & Rehan, 2018).

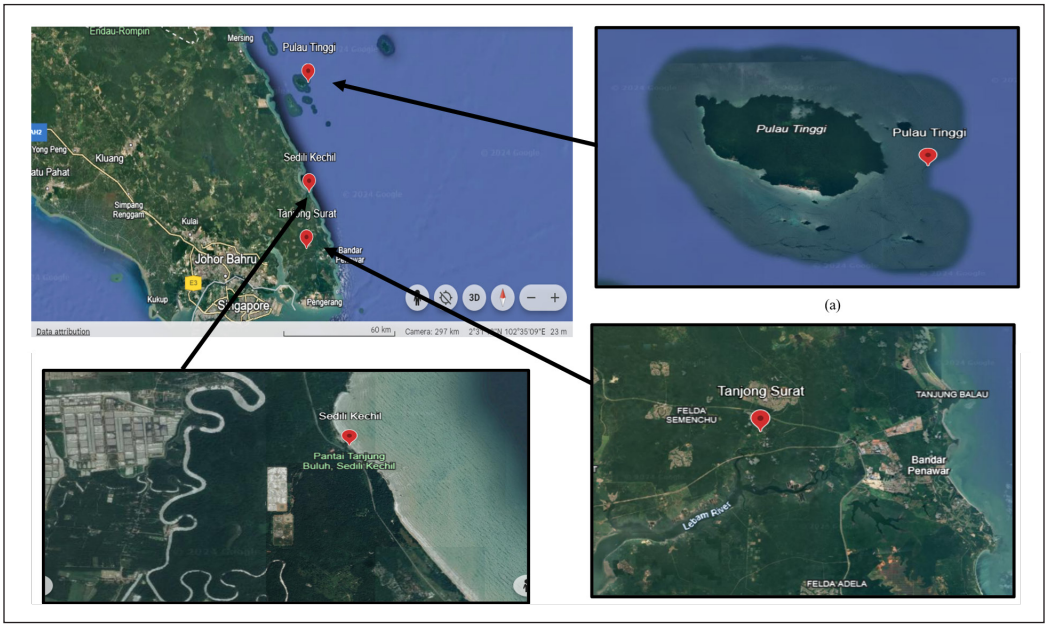

Figure 1. Sampling location for (a): Pulau Tinggi, (b): Sedili Kechil, and (c): Tanjong Surat (adapted from Google map images)

Culture Media and Cultivation

All specimens were dissected with a sterile blade in the Biological Safety Cabinet in the Food Microbiology Laboratory at the Faculty of Applied Science and Technology, Universiti Tun Hussein Onn Malaysia (UTHM), Pagoh, to mitigate possible contamination from the surroundings. All bacteria and fungi strains from the three sea cucumbers were isolated from their external body parts: the ventral podium, anus, and tentacles, as well as their internal body parts: coelomic fluid, the stomach, the cloaca, the respiratory tree, and the intestine. Each specimen body was swabbed using a sterile cotton swab and spread over Tryptone Glucose Yeast Extract (TGYE) agar (HiMedia Laboratories Private Limited, India) at pH 6.8 (Bajwa et al., 2018). The morphologies of the bacterial colonies were evaluated after overnight incubation at 37°C (since the average seawater temperature recorded in the sample sites was 37°C), and various colonies were repeatedly sub-cultured in new TGYE agar to purify each target individual of bacterium and fungus.

DNA Extraction, Amplification, and Sequencing

The FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Taiwan) was used to extract total DNA from each bacterium and fungus associated with sea cucumbers. The bacterial strains' 16S rRNA genes were amplified using primers with V3-V4 target regions, which are the S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and the S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') primers, and the expected length of the amplified polymerase chain reaction (PCR) products is approximately 464 bp (Klindworth et al., 2013). As for fungal identification, two universal primers, ITS1 (forward) and ITS4 (reverse), were used for the isolation of the ITS region, which are ITS1 (5' – TCCGTAGGTGAACCTGCGG – 3'; 19 bases) and ITS4 (5'– TCCTCCGCTTATTCATATGC–3'; 20 bases) (Mirhendi et al., 2007), with an expected length of the amplified PCR product of approximately 800 bp. The PCR reaction mixture was prepared to a total volume of 25 µl comprising 12.5 µl PCR master mix (Axil Scientific Pte Ltd, Singapore), 1.5 µl of each primer (forward and reverse primer) (Axil Scientific Pte Ltd. Singapore), 6.5 µl of DNA sample and 3.0 µl of sterile ultra-pure water. Amplification was conducted using a PCR Max Alpha Cycler (Cole-Parmer, USA) with the following temperature profiles: for bacteria, initial denaturation for 3 min at 95°C was followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 5 min; for fungi, initial denaturation for 5 min at 95°C was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 1 min, and a final extension step at 72°C for 8 min.

Subsequently, sequences were visualised and edited using Chromas Version

2.6.6 (http://www.technelysium.co-m.au/chromas.html). The results were obtained as nucleotides in FASTA format. Species identification was performed using the resultant nucleotide base pairs through the Basic Local Alignment Search Tool (BLAST) algorithm analysis by direct blasting on http://blast.ncbi.nlm.nih.gov (Altschul et al., 1990). A read was performed for each set of isolates, and the top hit with a minimum E-score for every BLAST result showing the species name was utilised to name the specific organism. Similarity values of 99 to 100% indicated the same species. In comparison, 95 to 99% similarity values were regarded as the same genus, and values below 95% were categorised within the same family.

Phylogenetic Analyses

The neighbour-joining (NJ) tree and the maximum likelihood (ML) tree were reconstructed using MEGA 11. A distance matrix was generated through pairwise alignment of the sequences, and the neighbour-joining method (Saitou & Nei, 1987) was employed to construct a phylogenetic tree based on this matrix. The branch lengths of the resulting tree were proportional to the estimated divergence along each branch. Confidence levels of the phylogenies were determined using the bootstrap method of Felsenstein (1985). The Kimura 2-parameter (K2P) nucleotide substitution model was applied for bacterial sequences, while the K2P with gamma distribution was employed for fungal sequences.

GenBank Submission

The BankIt sequence submission tool takes precedence in organising the sequence data for GenBank submission. Subsequently, all sequences were submitted to GenBank: each acquires a distinct accession number serving as a unique identifier in the GenBank database. BankIt thoroughly examines submissions, detects common errors, and utilises Vecscreen, a variant of BLASTn, to identify any potential vector contamination (Benson et al., 2018).

RESULTS

Twenty-five pure cultures were isolated from four body parts of the sampled sea cucumbers collected from three locations in Johor's coastal seawater: Pulau Tinggi, Tg. Surat, and Sedili Kechil. Genetic identification of all bacterial strains was conducted using 16S rRNA analysis, while fungal strains were identified using ITS. The bacterial strains exhibited an approximate length of 500 bp, as shown in Figure 2, whereas the fungal strains displayed an approximate length of 800 bp, as illustrated in Figure 3.

To ascertain the genus of the isolated bacteria and fungi, the 16S rRNA gene and ITS rRNA were sequenced. The resulting sequences were submitted to GenBank, NCBI. BLAST analysis of these DNA sequences identified strains belonging to ten different genera. Pairwise sequence comparisons were performed using BLAST analysis to determine sequence similarity (Kapli et al., 2020). The accession numbers for the isolated strains from GenBank were provided in Table 1.

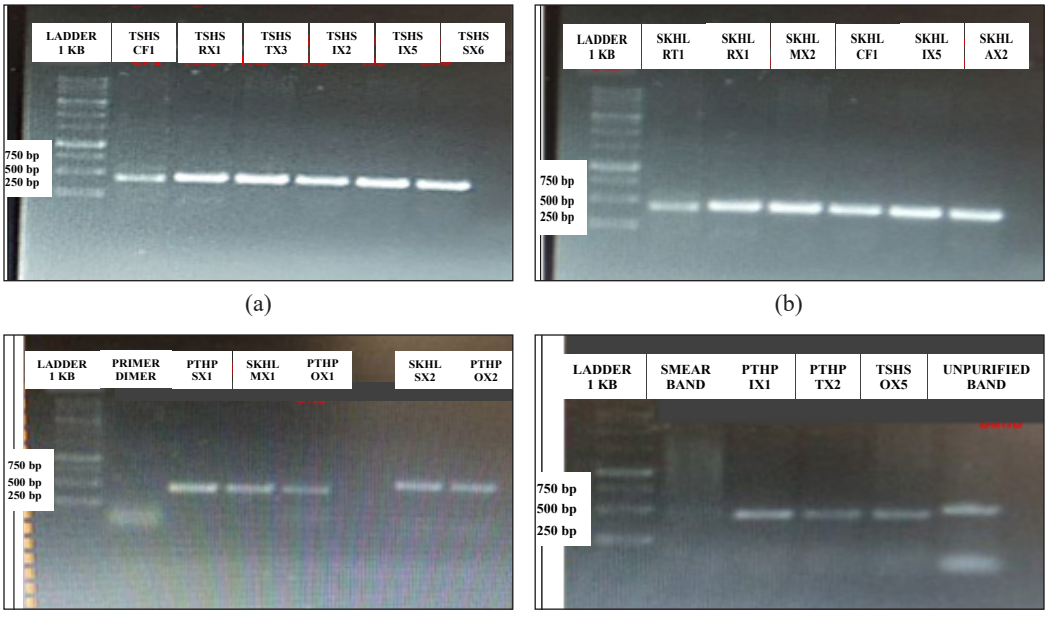

Figure 2. The DNA amplification for the isolated bacteria after polymerase chain reaction analysis on the agarose gel: (a) TSHS_CF1, TSHS_RX1, TSHS_TX3, TSHS_IX2, TSHS_IX5, and TSHS_SX6; (b) SKHL_RT1, SKHL_RX1, SKHL_MX2, SKHL_CF1, SKHL_IX5, and SKHL_AX2; (c) PTHP_SX1, SKHL_MX1, PTHP_OX1, SKHL_SX2, and PTHP_OX2; (d) PTHP_IX1, PTHP_TX2, and TSHS_OX5

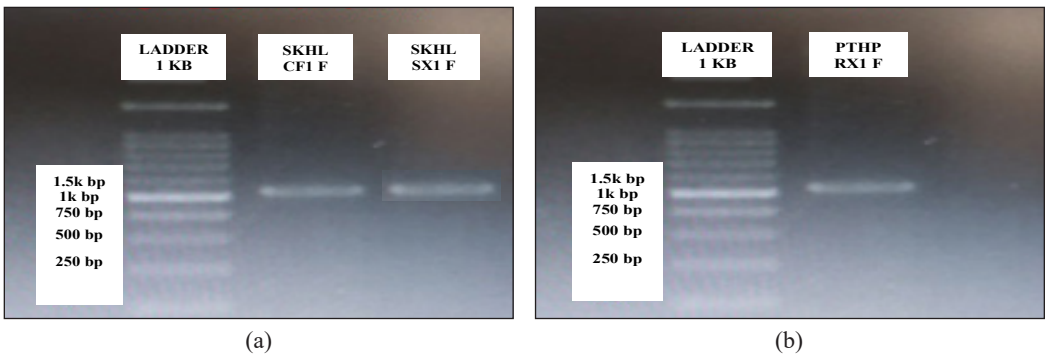

Figure 3. The DNA amplification for the isolated fungi after polymerase chain reaction analysis on the agarose gel: (a) SKHL_CF F and SKHL_SX1 F; (b) PTHP_RX1 F

Table 1
Accession numbers of isolated bacterial and fungal strains deposited in GenBank

Isolates	GenBank accession number	Species identification	% similarities
PTHP_B12	OP693446	*Chryseobacterium* sp.	100
PTHP_AX1	OP698237	*Sphingomonas* sp.	100
PTHP_SX1	OP684251	*Staphylococcus* sp.	100
PTHP_TX2	OP684252	*Psychrobacter* sp.	100

Table 1 *(continue)*

Isolates	GenBank accession number	Species identification	% similarities
PTHP_IX1	OP684253	*Stenotrophomonas* sp.	100
PTHP_OX1	OP684254	*Brevibacillus* sp.	99
PTHP_OX2	OP684255	*Brevibacillus* sp.	99
TSHS_T1	OP684296	*Bacillus* sp.	100
TSHS_CF1	OP684297	*Brevibacillus* sp.	99
TSHS_IX2	OP684298	*Stenotrophomonas* sp.	100
TSHS_IX5	OP684299	*Pseudoxanthomonas* sp.	99
TSHS_SX6	OP684300	*Pseudoxanthomonas* sp.	99
TSHS_RX1	OP698234	*Pseudoxanthomonas* sp.	99
TSHS_TX1	OP684301	*Stenotrophomonas* sp.	100
TSHS_OX5	OP698235	*Pseudoxanthomonas* sp.	99
SKHL_CF1	OP684290	*Psychrobacter* sp.	100
SKHL_IX5	OP684291	*Stenotrophomonas* sp.	100
SKHL_AX2	OP684246	*Psychrobacter* sp.	100
SKHL_MX2	OP684292	*Psychrobacter* sp.	100
SKHL_RT1	OP684293	*Psychrobacter* sp.	100
SKHL_MX1	OP684294	*Psychrobacter* sp.	100
SKHL_SX2	OP684295	*Psychrobacter* sp.	100
SKHL_CF1 F	OP703319	*Aspergillus* sp.	100
SKHL_SX1 F	OP703320	*Aspergillus* sp.	100
PTHP_RX1 F	OP703321	*Rhodotorula* sp.	100

NJ Tree

The evolutionary history was elucidated using the NJ tree method (Saitou & Nei, 1987), with the optimal tree depicted in Figure 4. The percentage of replicate trees in which the linked taxa clustered together in the bootstrap test with 1,000 replicates was shown next to the branches (Felsenstein, 1985). Furthermore, evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004), quantified as the number of base substitutions per site. The study included 77 nucleotide sequences, with positions having less than 95% site coverage, and those with less than 5% alignment gaps, missing data, or ambiguous bases were excluded (utilising the partial deletion option). The resulting datasets comprised a total of 38 positions. Evolutionary patterns were analysed using MEGA11 (Tamura et al., 2021).

The bacterial phylogenetic tree depicted 77 taxa, comprising 74 ingroup taxa and three outgroup taxa. The outgroup taxa consisted of three individuals from the *Pseudomonas aeruginosa* species with GenBank accession no. NR026078, FJ9722533, and FJ972538, respectively. Within the tree, PTHP SX1 was found within the same clade as the *Staphylococcus* taxa, albeit with less than 50% bootstrap support. Similarly, TSHS_T1 clustered

alongside the *Bacillus* taxa, with less than 50% bootstrap support. Conversely, TSHS CF1, PTHP_OX2, and PTHP_OX2 formed a different cluster within the *Brevibacillus* taxa, supported by a 94% bootstrapping. Notably, the TSHS_CF1 isolates showed close proximity to *Brevibacillus parabrevis* NBRC 12334 (NR113589) with 94% bootstrap support. Moving on, SKHL_SX2, SKHL_RT1, SKHL_MX2, SKHL_MX1, SKHL_CF1, SKHL_AX2, and PTHP_TX2 were grouped under the *Psychrobacter* taxa. These isolates clustered alongside *Psychrobacter lutiphocae* IMMIIB L-1110 (NR044602) and *Psychrobacter sanguinis* 13983 (NR117833), supported by a 63% bootstrap. TSHS_IX2, SKHL_IX5, PTHP_IX1, and TSHS_TX1 were clustered under the *Stenotrophomonas* taxa. Specifically, TSHS_IX2 and SKHL_IX5 were grouped under the clade *Stenotrophomonas geniculate* ATCC 19374 (NR024708), while PTHP_IX1 clustered with the clade *Stenotrophomonas maltophilia* LMG958 (NR119220). Similarly, TSHS_TX1 was associated with *Stenotrophomonas malthophilia* IAM 12423 (NR041577). Additionally, PTHP_B12 isolates formed a cluster within the *Chryseobacterium* taxa, supported by a 55% bootstrap and grouped with the clade *Chryseobacterium timonianum* G972 (NR164881). Meanwhile, PTHP_AX1 was grouped under the *Sphingomonas* taxa with 95% bootstrap support and clustered alongside the clade *Sphingomonas olei* K-1-16 (NR157757). Lastly, TSHS_IX5, TSHS_OX5, TSHS_RX1, and TSHS_SX6 formed a cluster within the *Pseudoxanthomonas* taxa, supported by a 95% bootstrap and grouped with the clade *Pseudoxanthomonaskalamensis* JA40 (NR043110) as illustrated in Figure 4.

According to the fungal phylogenetic tree depicted in Figure 5, there are 19 taxa, comprising 18 ingroup taxa and one outgroup taxa, represented by *Candida albicans* CBS 562 (NG070791). SKHL_SX1 F and SKHL_CFI F were found to cluster together within the *Aspergillus* taxa, with a bootstrap support of 66%, alongside *Aspergillus arenarioides* CBS 138200 (NR135460). Conversely, PTHP_RX1 F isolates formed a cluster within the *Rhodotorula* taxa, with a strong bootstrap support of 99%, along with *Rhodotorula alborubescens* JCM5352 (NR153197).

Maximum Likelihood Tree

The phylogenetic tree, generated through maximum likelihood analysis, was constructed using the K2P model (Kimura, 1980) for bacteria and fungi. In the bacterial category, the tree with the highest log likelihood of -5235.88 was presented, with the percentage of trees wherein associated taxa clustered together indicated next to the branches. This analysis encompassed 77 nucleotide sequences, with a total of 1,455 positions in the final dataset.

According to Figure 6, PTHP_SX1 isolates formed a cluster within the *Staphylococcus* taxa, exhibiting strong bootstrap support at 99% — similarly, TSHS_T1 isolates clustered under the *Bacillus* taxa, with a strong 99% bootstrap support. PTHP_OX1, TSHS_CF1, and

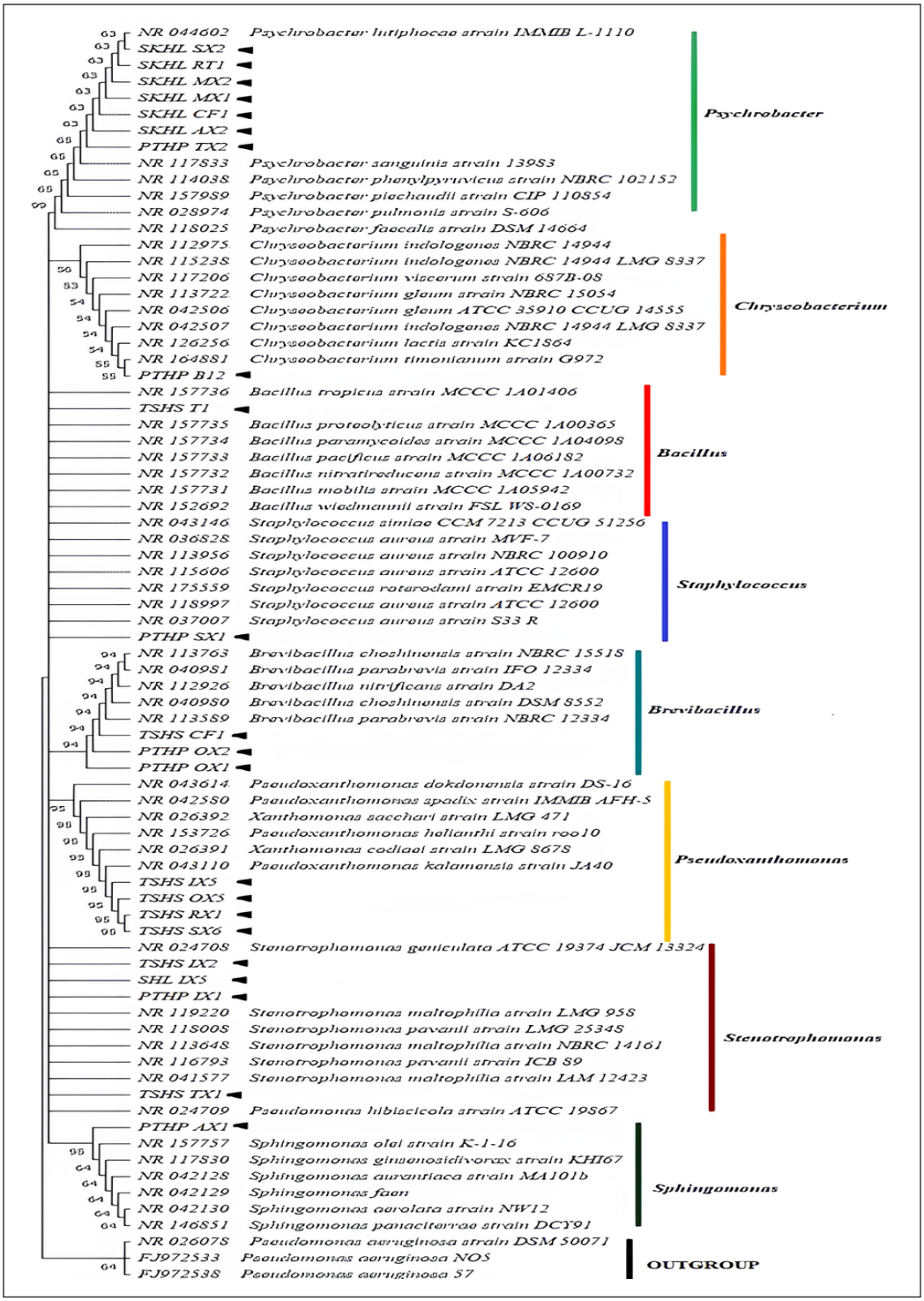

Figure 4. Neighbour-joining tree of 8 taxa of bacteria with *Peudomonas aeruginosa* as an outgroup. Alignment was done using MUSCLE W, and the tree was calculated using MEGA11 based on 1,000 bootstraps

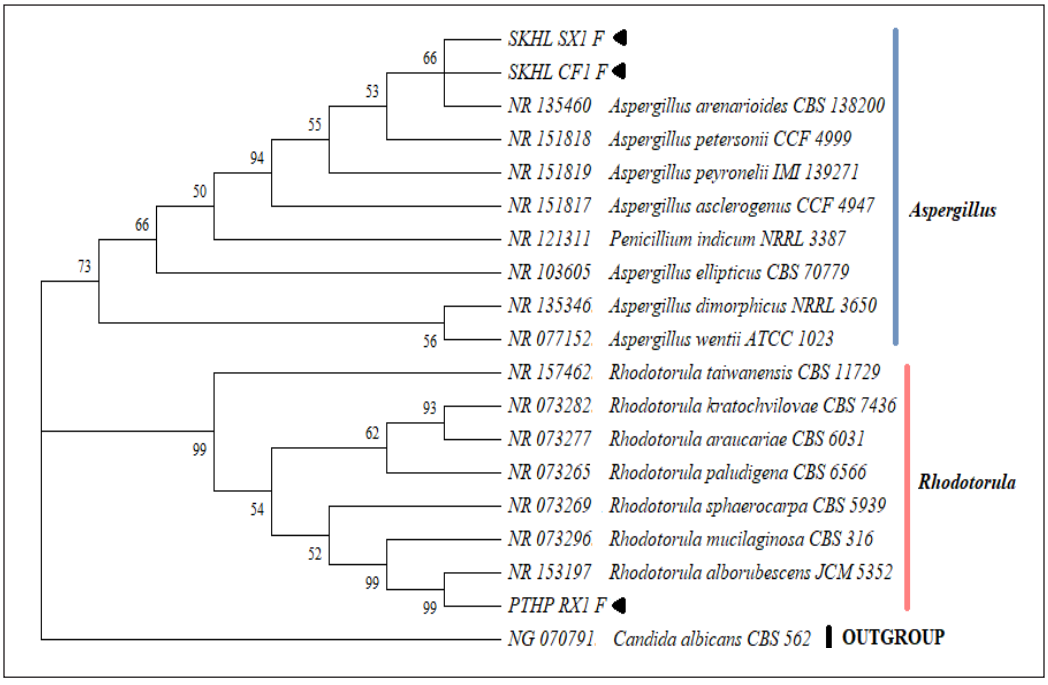

Figure 5. Neighbour-joining tree of 3 taxa of bacteria with *Candida albicans* as an outgroup. Alignment was done using MUSCLE W, and the tree was calculated using MEGA11 based on 1,000 bootstraps

PTHP_OX2 were grouped within the *Brevibacillus* taxa, supported by a 100% bootstrap. Additionally, SKHL_AX2, SKHL_CF1, SKHL_MX1, SKHL_RT1, SKHL_MX2, PTHP_TX2, and SKHL_SX2 formed a cluster under the *Psychrobacter* taxa, sharing a 99% bootstrap support and clustering with *Psychrobacter piechaudii* strain CIP 110854 (NR157989). SKHL_IX5, PTHP_IX1, and TSHS_IX2 clustered together with sequences from BLASTn, including *Pseudomonas hibiscicola* ATCC 19867 (NR024709), *Stenotrophomonas pavanii* LMG 25348 (NR041577), and *Stenotrophomonas malthophilia* IAM 12423 (NR041577), supported by a 59% bootstrap. PTHP_B12 exhibited 100% bootstrap support within the *Chryseobacterium* taxa, while PTHP_AX1 was classified under the *Sphingomonas* taxa, also with 100% bootstrap support. However, TSHS_TX1 and PTHP_AX1 formed a clade with a 45% bootstrap. Furthermore, TSHS_RX1, TSHS_SX6, TSHS_IX5, and TSHS_OX5 clustered within the *Pseudoxanthomonas* taxa, supported by a 70% bootstrap. Among them, TSHS_RX1, TSHS_SX6, and *Pseudoxanthomonas spadix* IMMIB AFH-5 (NR042580) formed a cluster, while TSHS_IX5 and TSHS_OX5 clustered with *Pseudoxanthomonas kalamensis* JA40 (NR043110), supported by a 54% bootstrap.

Similarly, the evolutionary history of the fungal tree was inferred using the K2P model with a discrete gamma distribution, accounting for evolutionary rate variations

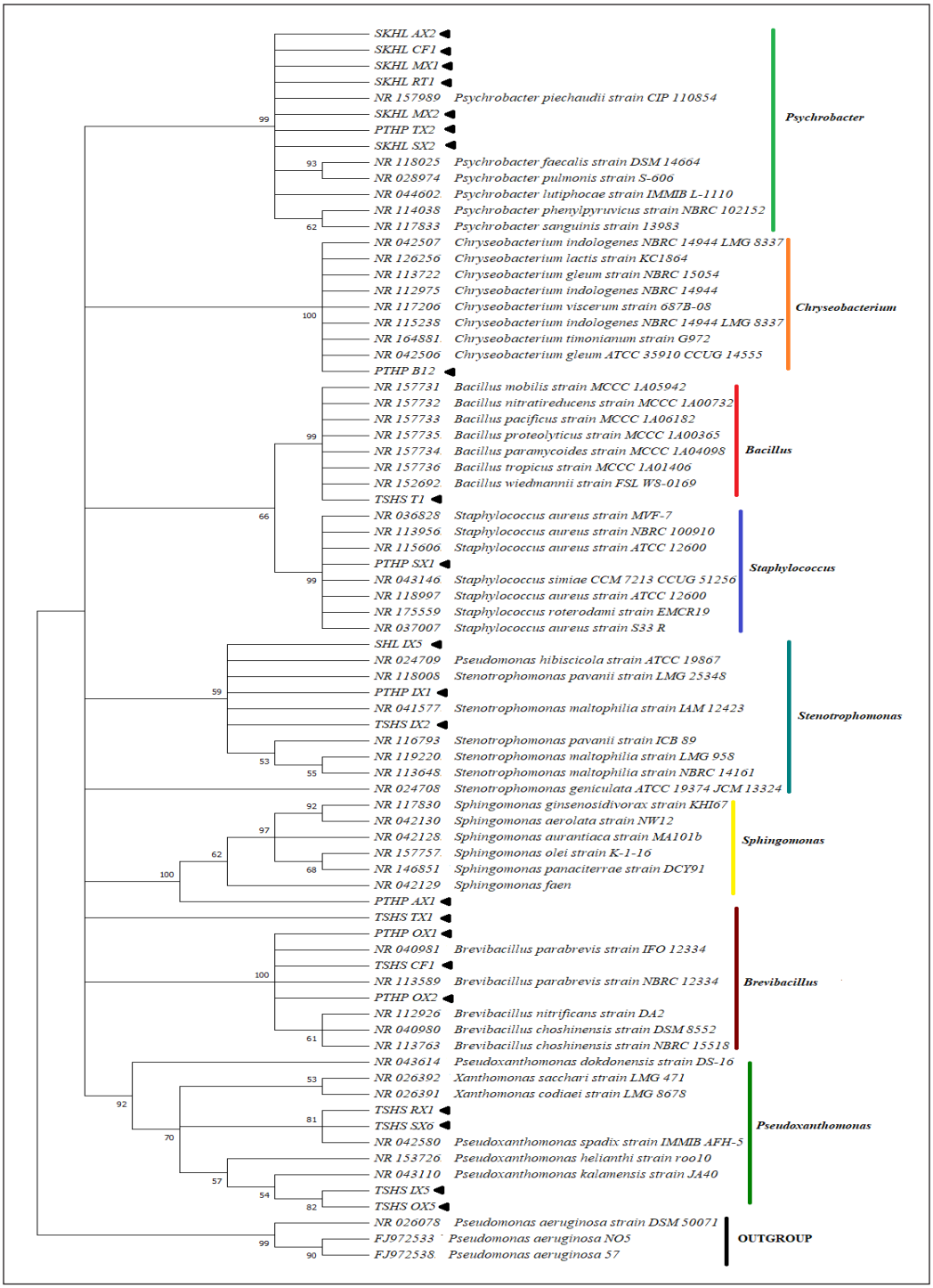

Figure 6. Maximum likelihood tree of 8 taxa of bacteria with *Pseudomonas aeruginosa* as an outgroup. Alignment was done using MUSCLE W, and the tree was calculated using MEGA11 based on 1,000 bootstraps

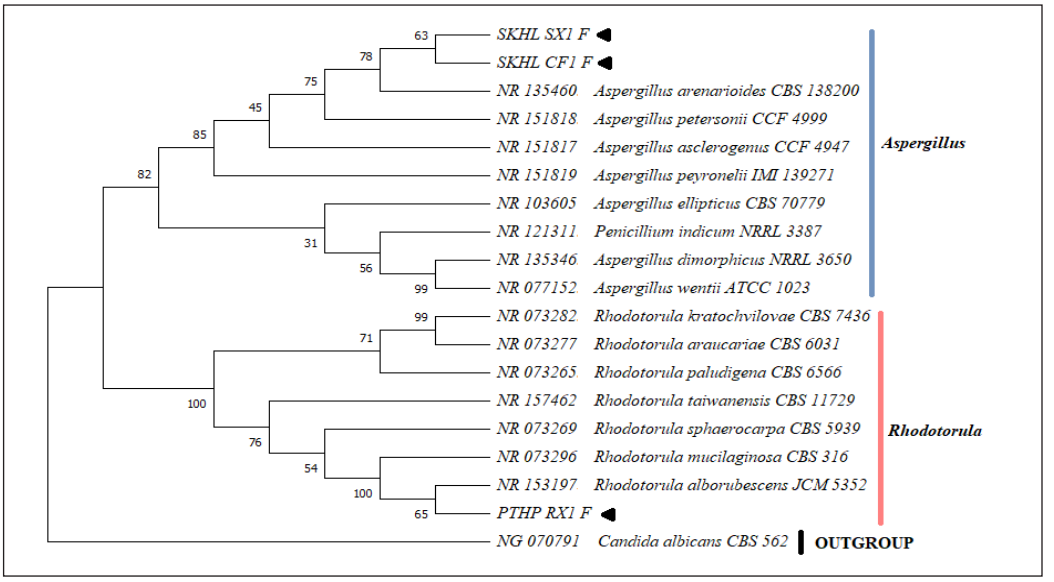

Figure 7. Maximum likelihood tree of 3 fungus taxa with *Candida albicans* as an outgroup. Alignment was done using MUSCLE W, and the tree was calculated using MEGA11 based on 1,000 bootstraps

among sites. This analysis involved 19 nucleotide sequences and incorporated 18 ingroup taxa and one outgroup taxa, represented by *Candida albicans* CBS 562 (NG070791), as illustrated in Figure 7. SKHL_SX1 F and SKHL_CF1 F formed a cluster within the *Aspergillus* taxa, supported by an 82% bootstrap. These isolates clustered alongside *Aspergillus arenarioides* CBS 138200 (NR135460) with a 78% bootstrap support. Conversely, PTHP_RX1 F was categorised under the *Rhodotorula* taxa with 100% bootstrap support and exhibited 65% bootstrap support with the *Rhodotorula alborubescens* JCM 5352 (NR152197) clade.

DISCUSSION

The NJ and maximum likelihood analyses consistently revealed that isolates were grouped within the same taxa and confidently identified under their respective taxa: PTHP_SX1 as *Staphylococcus* sp.; TSHS_T1 as *Bacillus* sp.; TSHS_CF1, PTHP_OX2, and PTHP_OX1 as *Brevibacillus* sp.; SKHL_SX2, SKHL_RT1, SKHL_MX2, SKHL_MX1, SKHL_CF1, SKHL_AX2, and PTHP_TX2 as *Psychrobacter* sp.; TSHS_IX2, PTHP_IX1, and SKHL_IX5 as *Stenotrophomonas* sp.; PTHP_B12 as *Chryseobacterium* sp.; PTHP_AX1 as *Sphingomonas* sp.; TSHS_SX6, TSHS_RX1, TSHS_IX5, and TSHS_OX5 as *Pseudoxanthomonas* sp. However, while TSHS_TX1 was initially grouped under the *Stenotrophomonas* taxa in the NJ analysis, it exhibited weak bootstrap support and clustered with *Sphingomonas* sp. in the maximum likelihood analyses. The NJ method is the reconstruction for the distance-based method, while maximum likelihood is based on character-based methods (Kapli et

al., 2020). Most of microbial associated with selected sea cucumbers were predominantly normal microbiota.

Psychrobacter sp. was initially identified in the intestine of sea cucumber *Stichopus japonicus* (Gao et al., 2014) and later found in the external part of *H. leucospilota* collected from Lampung, Indonesia by Wibowo et al. (2019). However, there is no documentation of *Psychrobacter* sp. isolated from *H. leucospilota* and *H. pardalis* in Malaysia up to recent data. *Bacillus* sp., *Stenotrophomonas* sp., and *Brevibacillus* sp. were first reported by Lukman et al. (2014), who isolated them from the gastrointestine of *S. horrens* collected from Pangkor Island, Perak. Li et al. (2016) also reported *Bacillus* and *Brevibacillus* as the most abundant bacterial species in the gastrointestine. Kamarudin and Rehan (2018) also found *Bacillus* and *Brevibacillus* in *H. leucospilota* and *S. horrens* collected from Pangkor Island in 2018. *Staphylococcus* sp., isolated from the coelomic fluid of *H. leucospilota*, was first documented by Kamarudin et al. (2013) in Pangkor Island, Perak. However, no records of *Chryseobacterium* sp., *Pseudoxanthomonas* sp., and *Sphingomonas* sp. isolated from sea cucumber species have been documented in Malaysia or other Asian countries. Although *Aspergillus* sp. is commonly found in *H. polii* (Marchese et al., 2020), there is no data on *Aspergillus* sp. in *H. pardalis*, especially in Malaysia. While *Rhodotorula* sp. was found in *Apostichopus japonicas* by Wang et al. (2015) and Yang et al. (2019), there is no recorded data for *H. pardalis* and other sea cucumbers collected in Malaysia. Intriguingly, no data on microorganisms isolated from sea cucumbers collected from Johor Island, Malaysia, has been documented.

Of the eleven different bacterial strains taxa identified, *Chryseobacterium* sp., *Sphingomonas* sp., and *Pseudoxanthomonas* sp. emerged as the most promising candidates for further evaluation as potential food-grade microbial pigments. *Chryseobacterium* species, belonging to the Flavobacteriaceae family, typically produce colonies ranging from pale yellow to orange due to carotenoid content or flexirubin-type pigments (Hugo et al., 2019), while *Sphingomonas* sp. is known to produce various pigments ranging from yellow, red, or orange to white or non-pigmented (Busse et al., 2003). In this analysis, *Sphingomonas* sp. exhibited a bright yellow pigment on TGYE agar. According to Lipski and Stackebrandt (2015), *Pseudoxanthomona*s sp. produces yellow pigment on agar. Hence, these three pigmented bacteria provided new insights and discoveries on natural colourants derived from microorganisms.

CONCLUSION

In this study, a total of 22 bacterial strains and three fungal strains were successfully identified. The bacteria were categorised into seven clades: *Psychrobacter*, *Chryseobacterium*, *Bacillus*, *Brevibacillus*, *Staphylococcus*, *Pseudoxanthomonas*, *Stenotrophomonas*, and *Sphingomonas*. Meanwhile, the three fungal strains were classified under the *Aspergillus* and *Rhodotorula* clades. Notably, this study

marks the first documentation of these species in Pulau Tinggi, Tanjong Surat, and Sedili Kechil in Johor. Among these species, *Chryseobacterium* sp., *Sphingomonas* sp., and *Pseudoxanthomonas* were first documented as part of pigment-producing microorganisms found in sea cucumbers in Malaysia.

The microorganisms isolated in this study are interesting discoveries for future research applications. The pigment-producing microorganisms can be further evaluated in terms of their potential and performance, such as growth factor, which can be used in colourant industries as one of the natural colourant alternatives.

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