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Molecular and protein characterization of two species of the latrunculin-producing sponge *Negombata* from the Red Sea

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ABSTRACT

Red Sea sponges offer a potential for production of novel drugs and prototypes. The genus *Negombata* is a type of sponges abundant in the Red Sea. This sponge produces latrunculins that have well documented antitumor activity in addition to antimicrobial and antiviral effects. The identification of *Negombata* species is based on morphology and microscopical examination of megascleres of spicules. However, these criteria have proven to be unreliable for identification. Therefore, this study was established to test the accuracy of the spicules based taxonomy against molecular and protein profiles for the two species of *Negombata*: *N. magnifica* and *N. corticata*. About 700 bp of cytochrome c oxidase I gene was sequenced from the tissues of the two *Negombata* species. Additionally total proteins were extracted from *Negombata* samples collected from different locations during different seasons and separated by denaturing polyacrylamide gel electrophoresis. Characteristic different protein profiles were obtained for both species. The data obtained from cytochrome c oxidase I gene sequencing and protein profiles can reliably differentiate between different species of *Negombata* in the Red Sea.

1. Introduction

The marine environment is a wealthy source of plants, animals and microorganisms, which due to their adaptations to this unique habitat, produce a wide variety of secondary metabolites, unlike those found in terrestrial species [1].

The majority of marine natural products currently in clinical trials or under pre-clinical evaluation are produced by invertebrates such as sponges, tunicates, molluscs, bryozoans and cnidarians. These natural products have two main ecological roles towards marine invertebrates especially sponges. First, these bioactive metabolites protect sessile or soft bodied marine invertebrates against their predators [2]. Second, they help to fight off neighbors competing for space [3]. Moreover, studies have proved that the bioactive metabolites produced by sponges have potent cardiovascular, gastrointestinal and respiratory effects in addition to anti-tumor, anti-inflammatory, anti-viral, anti-fungal and antibacterial properties [4].The Red Sea contains representatives of all major tropical marine communities. Such a high diversity of habitat is occupied by a large and diverse number of marine animals producing biologically active natural products. Among the diverse number of marine invertebrates, sponges produce the highest and most diverse quantity of natural products. Therefore, they have become the focus of natural product studies for many years [5]. The most prominent sponges in the Red Sea that grow exposed are *Negombata* sponges [6]. Genus *Negombata* is represented by four species, *N. magnifica*, *N. corticata*, *N. kenyensis* and a new undescribed species from Indonesia. The three described species have a brilliant orange-red coloration and a digitate or leafy gross morphology. The two species present in the Red Sea are *N. magnifica* and *N. corticata* [7].

Negombata sponge was shown to produce potent cytotoxic macrolides called latrunculins (e.g. latrunculins A and B) in addition to other cytotoxic compounds [8]. Latrunculin B differs from latrunculin A in containing 14 versus 16 membered macrocycles [9]. Experiments performed in vitro revealed that latrunculins could inhibit force development in muscles, the microfilament-mediated processes of meiosis [10], fertilization, and early development [11] and even affect protein kinase C signaling [12]. Latrunculin A has been found to disrupt the actin cytoskeleton, leading to deterioration of microfilament bundles, loosening of cell–cell attachment, and cell retraction [13]. These results have raised interest in the potential use of latrunculins as growth inhibitors of some tumor cell lines, and therefore, the possibility for them to serve as prototypes in the discovery and development of novel antitumor agents [14]. Moreover, latrunculins are patented as possible antiglaucoma leads. They were reported to decrease intraocular pressure and increase outflow facility without corneal effects in monkeys [15].

Quantitative determination of latrunculins A and B from *N. magnifica* collected from different locations in the Red Sea

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during different seasons was performed by high performance liquid chromatography by Khalifa et al. [16]. The results revealed that *N. magnifica* samples from different locations produced both latrunculins A and B, but in different concentrations. *N. magnifica* collected from Ras Mohamed produced the highest concentration of latrunculin A. Whereas *N. magnifica* collected from Safaga produced the highest concentration of latrunculin B. Moreover, a comparison of latrunculin concentrations in the summer and winter indicated that latrunculin concentrations were generally higher in the winter than in summer [16].

Taxonomic identification of sponges by morphology alone is difficult due to the lack of diagnostic characteristics in their simplified asymmetrical bodies. Body coloration is of little use for classification in *Negombata* species because it varies with habitat, and can even change immediately when the animals are moved. Species are the basic units of taxonomy, and the delineation of species boundaries is clearly a fundamental requirement for improving the accuracy and validity of biodiversity assessments.

In recent years, the development of molecular biological techniques has enabled the rapid compilation of nucleotide sequence databases for a variety of animal phyla. This has led to the establishment of suitable genetic markers to detect genetic variation [17].

Mitochondrial genomes have become widely used to infer metazoan phylogeny. Most metazoan mtDNA molecules contain the same set of 37 genes. These include 13 genes for proteins of the electron transport chain (cytochrome b, cytochrome c oxidase subunits I–III, subunits 6 and 8 of the F_1F_o ATP synthase complex, and NADH dehydrogenase subunits 1–6 and 4L (*nad1–nad6* and *nad4L*). They include also two genes for the small and large subunits of ribosomal RNA (rRNA) and 22 genes for transfer RNA (tRNA). Mitochondrial cytochrome c oxidase subunit I (COI) is the most frequently used genetic marker to infer phylogeographic relationships in most marine organisms. It can be easily amplified using universal primers and has been widely applied in phylogenetic studies [18].

Traditionally, systematics of the genus *Negombata* has been based mostly on skeletal morphology and spicule geometry and diversity [19]. In particular, the shape and size of the large structural spicules (megascleres) and/or of the small reinforcing or packing spicules (microscleres) have been used as taxonomic characters for *Negombata* classification. However, these skeletal tools are insufficient for classification and distinguishing of different species present in the same genus. The advent of molecular studies brings new ideas in the field of sponge systematics. Since different species of the *Negombata* sponges produce the bioactive metabolites latrunculins A and B with different concentrations, it is important to explore different tools that can reliably differentiate between the different species.

The goal of this study was to classify the latrunculin-producing sponge *Negombata* in the Red Sea and examine genetic diversity within this genus. Sequence diversity in COI gene has been shown to be an effective tool for species identification in the same genus [18]. Therefore, COI gene sequencing was applied in this work for more accurate classification. This study focused also on testing the reliability of the morphological and spicules-based taxonomy against the protein profile for both *Negombata* species and hence offers new insights for a more precise classification of the genus.

2. Experimental

2.1. Sponge collection and processing

Specimens of the Red Sea sponge *Negombata* (class *Demospongiae*, order *Poecilosclerida*, family *Podospongiidae*) were collected by SCUBA at two different locations in the Red Sea; Ras Mohamed and Safaga, Sinai, Egypt at a water depth of 10 m in June, 2004 and January, 2005 (Table 1). Sponge samples were cut with a dive knife while wearing latex gloves and individual pieces were put into separate plastic sample collection bags. Samples were brought to the surface, maintained at ambient seawater temperature, and transported to a land-based laboratory for processing within 2 h of collection. A section of the sponge specimens was immediately frozen on dry ice and stored at −80 °C. This sponge tissue was then freeze-dried and used for molecular applications. Fragments of collected specimens were stored in 70% ethanol for morphological characterization and taxonomic classification.

Table 1
Detailed sample designation of collected *Negombata* samples.

Sponge	Location	Time of collection	Sample designation
Negombata magnifica	Ras Mohammed	January 2005	SAA-RM1
		June 2004	SAA-RM6
Negombata corticata	Safaga	January 2005	SAA-SA1
		June 2004	SAA-SA6

2.2. Morphological and taxonomical characterization of *Negombata* samples

Morphological identification of the collected samples of the sponge *Negombata* was done by comparison of their morphological features such as color, shape, texture and length according to the principles of Nèeman et al. [20].

Taxonomical classification of *Negombata* was done by microscopical examination of megascleres by Rob. W. M. van. Soest at the Institute for Systematic and Ecology, University of Amsterdam, Amsterdam, Netherlands. The voucher specimens were deposited at the Zoological Museum of the University of Amsterdam under registration numbers ZMAPOR 18568 for *N. magnifica* and ZMAPOR 18569 for *N. corticata*.

2.3. Molecular analysis of Negombata species

2.3.1. Total genomic DNA extraction from sponge

Freeze-dried 1 cm^3-sponge tissues (SAA-RM6 and SAA-SA6) were ground using a sterile mortar and pestle. Total genomic DNA was extracted using the bead beater method modified for sponge tissue as described by Enticknap et al. [21]. Briefly, the sponge powder was placed in a bead beater (Biospec Products, Bartlesville, OK, USA) and an equal volume of sterile 0.1 and 1 mm Zirconia/silica beads were added to fill one third of the chamber. The sponge material was mechanically homogenized in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) for three times, each for 30 s with a 1-min pause between each beating. The chamber was cooled by crushed ice in the surrounding plastic container. An equal volume of guanidium thiocyanate buffer was added and mixed gently. The buffer was prepared by dissolving 60 g of guanidium thiocyanate in 20 ml of 100 mM EDTA while heating to 65 °C, and then the solution was cooled, completed to a volume of 100 ml and filtered. The samples were transferred to ice. Ammonium acetate was added to 2.5 M final concentration. The samples were extracted with an equal volume of equilibrated phenol followed by two extractions with a half volume of chloroform/isoamylalchol (24:1). DNA in the resulting supernatant was precipitated with a half volume of isopropanol on ice followed by centrifugation at 13,000 × *g* for 20 min. The pellets were washed with 70% ethanol, air-dried, and suspended in 100 μl of PCR water.

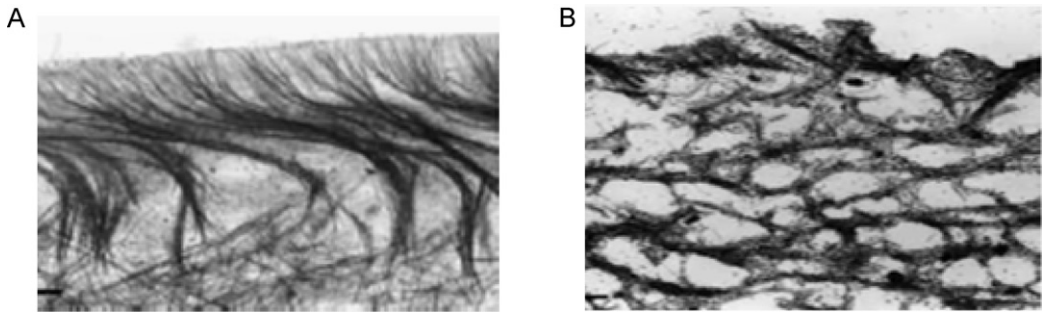

Fig. 1. Microscopical examination of the Red Sea sponge *Negombata*: (A) *Negombata magnifica*; (B) *Negombata corticata*.

2.3.2. Cytochrome c oxidase I gene sequence analysis

Approximately 700 bp fragment of the cytochrome c oxidase I (COI) gene was amplified from the genomic DNA extracted from sponges using the primer set LCO1490 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HCO2198 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ [22]. The PCR reaction mixture (50 μl) contained 30–50 ng/μl DNA, 10 pmol/μl (each primer), 0.2 mM (each dNTP) and 2 mM $MgCl_2$. Thermal cycling was performed with an Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany) using the Qiagen Taq DNA Polymerase kit (Qiagen, Crawley, West Sussex, UK). Amplifications were initiated with denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 45 °C for 1 min, extension at72 °C for 1 min, and a final extension step at 72 °C for 7 min. Size of the PCR product was confirmed by visualization on a 1.2% agarose gel amended with ethidium bromide. The amplified products were purified with the EZ-10 Spin Column Purification kit (Bio Basic Inc., Markham, Ontario, Canada). Purified products were sequenced on an ABI 377 automated sequencer using the PRISM Ready Reaction Kit (Applied BioSystems, Foster City, CA, USA) using LCO1490 and HCO2198 primers.

2.3.3. Phylogenetic analysis

The COI gene sequences from sponge samples were analyzed using the BLASTn tool at the National Centre of Biotechnology Information (NCBI) GenBank database using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were presumptively identified according to the identity of the closest relative in the top BLAST hits. Closely related COI gene sequences were retrieved from GenBank database and included in the analysis. COI gene sequences of the sponge samples were aligned with those of other sponges of the phylum Porifera. Multiple sequence alignments were created with the ClustalX program (http://ftp-igbmc.u-strasbg.fr/pub/ClustalX). Trees were constructed using the PHYLIP software package (http://evolution.genetics.washington.edu/phylip.html). Distances were calculated using the Jukes–Cantor algorithm of DNADIST, and branching order was determined via the neighbor-joining algorithm of NEIGHBOR. Each tree was consensus of 100 replicate trees.

2.3.4. Nucleotide sequence accession numbers

The COI gene sequences of the sponges were deposited in GenBank under accession numbers HQ728489 for *N. magnifica* and HQ728490 for *N. corticata*.

2.4. Total protein analysis of Negombata species

Total proteins were extracted from freeze-dried sponge tissues of both *Negombata* species collected from Ras Mohamed and Safaga in different seasons. Equal amounts of the sponge tissue and the protein extraction buffer were manually homogenized over ice. The protein extraction buffer was composed of Tris–HCl (pH 8; 50 mM), dithiothreitol (DDT) (1 mM), EDTA (50 mM), NaCl (0.5 M), Triton-X 100 (1%, v/v), benzamidine (1 mM) and phenyl methyl sulfonyl fluoride (1 mM). Proteins were separated by centrifugation at 13,900 × *g* for 15 min at 4 °C. The supernatant was boiled for 3 min in sample buffer (0.125 M Tris–HCl; pH 6.8, 4% sodium dodecyl sulphate (SDS), 20% glycerol, 0.2 M DTT and 0.02% bromophenol blue). One dimensional SDS-PAGE was carried out following the method of Laemmli [23]. Proteins were electrophoresed on a 10% separating gel (0.75 mm thickness) overlaid with 4% stacking gel. Protein bands were visualized by Coomassie blue R 250 staining. Molecular weight estimation was done by comparison to a full range molecular weight marker (from 205 to 14 kDa) (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The gel plate was photographed and gel image data were stored as TIFF files and analyzed using the GelCompar II software, version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium). Similarity between protein profiles was calculated by using the band-matching Dice coefficient with optimization at 0.5% and tolerance level at 0.5%. Cluster analysis was performed by using the unweighted pair-group method with arithmetic averages to produce a similarity dendrogram.

3. Results and discussion

Morphological comparisons among the collected samples of the Red Sea sponge *Negombata* showed that all samples had nearly the same morphological characters. Each sponge had a branching conspicuous brightly red-colored, thickly encrusting, massive tubular and digitate or leafy gross morphology. Texture was flexible, rubbery and compressible due to richness of sponges in collagenous mesophyl matrix. The simplicity in morphology and structure led to a poor taxonomy, most prominently at the species level.

Taxonomic identification of sponge samples was done by microscopical examination of megascleres. Sponge samples collected from Ras Mohamed (SAA-RM1 and SAA-RM6) were identified as *N. magnifica*, while those collected from Safaga (SAA-SA1 and SAA-SA6) were identified as *N. corticata*.

Megascleres of *N. magnifica* were arranged uniformly within fibers and clearly differentiated into primary and secondary tracts (Fig. 1A). In *N. corticata*, megascleres are not arranged uniformly within fibers, but are scattered singly or in groups and oblique or occasionally perpendicular to the fiber axis (Fig. 1B). Although sponge systematics has been based almost entirely on skeletal traits and, in particular, on the spicules, information and complexity is limited. Additionally, heavy reliance on skeletal characters in sponge systematics may reflect what characters are readily available rather than confidence in their reliability. The simple comparisons of spicules cannot be used for definitive species identification.

Molecular analysis was used to provide a better perspective of molecular taxonomy of the different *Negombata* species. The

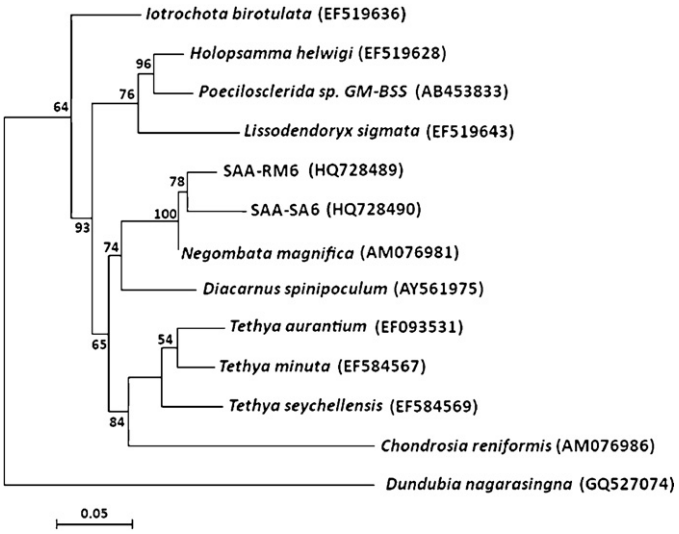

Fig. 2. Neighbor-joining tree based on mitochondrial COI gene sequences showing relation between Red Sea Negombata sponges and other sponges of the phylum Porifera. Genbank accession numbers for the sequences are shown in parentheses. Numbers at nodes denote bootstrap values based on 100 replicates, only values greater than 50 are shown. Bar, 5 nucleotide substitutions per 100 nt.

results of amplification of COI gene fragment showed that both *Negombata* species had similar band size (700 bp). Phylogenetic trees were constructed with DNA sequences of COI gene of both *Negombata* species, *N. magnifica* (SAA-RM6) and *N. corticata* (SAA-SA6). Neighbor-joining analysis approved diagnostic differences between *N. magnifica* and *N. corticata* from geographically different locations. Trees constructed by other methods e.g. maximum likelihood, Fitch-Margoliash, also confirmed the differences between the two *Negombata* species. The difference between both *Negombata* species was implied by strong branch support value. The bootstrap value at the node *N. magnifica* from Ras Mohamed (SAA-RM6) and *N. corticata* from Safaga (SAA-SA6) was 78. The phylogenetic tree shows closest relationship of *N. magnifica* (SAA-RM6) and *N. corticata* (SAA-SA6) to *N. magnifica* [24], *Diacarnus spinipoculum,* and *Tethya* spp. as shown in Fig. 2. The data indicate that sequencing of the COI gene is an effective tool for differentiating and identifying different *Negombata* sponges from the Red Sea.

In order to corroborate the molecular genetic differences, another tool was studied to find out the difference between two *Negombata* species. SDS-PAGE analysis of total proteins extracted from samples of *Negombata* species collected from different locations at different seasons was performed. The use of the GelCompar II software allowed the analysis of all protein fingerprints simultaneously. The dendogram constructed from profiles of *Negombata* species proteins showed that the two sponge species have different banding patterns from each other (<40% similarity) and demonstrated a clear clustering of protein profiles from the same sponge species (Fig. 3). Although each sponge had different protein profiles, all samples shared characteristic common protein bands of molecular weights between 55 and 44 kDa and a band with a molecular weight of 66 kDa, indicating the commonality of some proteins in the two sponge species.

N. magnifica samples (SAA-RM1 and SAA-RM6) were characterized by a distinctive protein band of a molecular weight between 30 kDa and 21 kDa. On the other hand, *N. corticata* samples (SAA-SA1 and SAA-SA6) had also characteristic protein bands of a molecular weight between 66 kDa and 55 kDa. These bands were not present in *N. magnifica* collected in the same seasons. Therefore, these protein bands may be used as a fingerprint for each species.

The differences in protein bands between both *Negombata* species might be due to different locations for collecting *Negombata* samples where *N. magnifica* samples were collected from Ras Mohamed and *N. corticata* samples were collected from Safaga. HPLC analysis of latrunculins A and B of several *N. magnifica* samples from different locations in the Red Sea by Khalifa et al. [16] revealed that sponge from Ras Mohamed had the highest concentration of latrunculin A, while that from Safaga had the highest concentration of latrunculin B. Latrunculin A is more potent than latrunculin B [25], therefore, the locations in which there is more stress may produce the substance that is more toxic. Ras Mohamed, being rich in its biodiversity, the competition level is high. There is also heavy human impact due to boating and diving activities. The reef in the location of collection is also very much exposed. On the other hand, Safaga area has much less human impact, biodiversity and competition and the reef is much more sheltered. Accordingly, there is much more stress in Ras Mohamed than in Safaga [16].

Comparisons of protein bands were also performed among the samples of only one species and same location but in different seasons. For *N. magnifica* samples collected in January (SAA-RM1), they were characterized by two protein bands; one of a molecular weight of 97 kDa and the other between 116 and 97 kDa. These protein bands were not characteristic for the other *N. magnifica* sample collected in June (SAA-RM6). On the other hand, *N. magnifica* sample (SAA-RM6) had two characteristic protein bands; one of molecular weight between 97 kDa and 88 kDa and the other of a molecular weight of 21 kDa. Comparisons were performed also for *N. corticata* between June, 2004 and January, 2005. *N. corticata* samples collected from Safaga in January (SAA-SA1) were characterized by the presence of two consecutive protein bands of molecular weights between 30 kDa and 21 kDa. In addition, it had two characteristic protein bands; one of a molecular weight 14 kDa and the other of molecular weights between 116 kDa and 97 kDa. These bands were

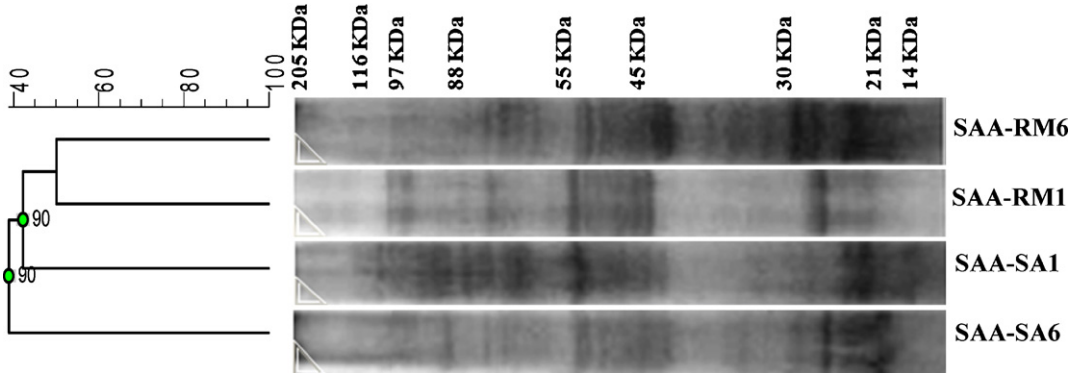

Fig. 3. Dendrogram constructed from 10% SDS-PAGE of *Negombata* species proteins in different seasons. Levels of similarity between protein profiles were calculated by using the band-matching Dice coefficient. Cluster analysis was performed by using the unweighted pair-group method with arithmetic averages (UPGMA).

not characteristic for samples collected from Safaga in June (SAA-SA6). On the other hand, *N. corticata* samples (SAA-SA6) had two distinctive protein bands; one of a molecular weight 30 kDa and the other of molecular weights between 30 kDa and 21 kDa.

The differences in protein bands between both *Negombata* species and also among the samples of each species collected from the same location in different seasons might be due to environmental conditions that induce the production of heat shock proteins (Hsps) which are found in diverse organisms from bacteria to mammals. These sponges, being sessile organisms, experience constant direct exposure to the surrounding environmental conditions and are exposed to local and global environmental fluctuations that may lead to cellular protein damage and subsequent death [26]. They undergo irreversible protein damage on exposure to temperature fluctuations on a seasonal basis [27]. One of the most intriguing habitats occupied by these sponges is that of the rocky intertidal zone, which is characterized by steep gradients in environmental factors such as temperature, ultraviolet (UV) radiation, and salinity [28]. Sponges may inhabit also several niches in the intertidal zone and lack any developed physiological regulatory systems. Thus they are expected to possess well-developed cellular adaptation abilities. Induction of Hsps constitutes an important defence mechanism that protects these organisms from deleterious stress conditions. Hsps are known to be induced by various stresses (e.g., extreme temperature, UV, the presence of heavy metals, and salinity changes) [29]. In addition, the variation in proteins might be also due to the altered gene expression as well as posttranslational modification of polypeptides. These changes can be caused by environmental stress and/or aging [30].

4. Conclusions

The advent of molecular studies brought new ideas for accurate classification at the species levels. The study has shown that COI gene sequencing and comparison of protein profile can be used to effectively differentiate between the two different species of the Red Sea *Negombata*. In conclusion, genetic and proteomic tools can reliably differentiate among different species of the genus *Negombata* in the Red Sea.

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