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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.