Indian Journal of Geo-Marine Sciences
Vol. 45(3), March 2016, pp. 405-409

Morphological and Molecular Identification of *Holothuria* (*Selenkothuria*) *parva* from Bostaneh Port, Persian Gulf

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Received 03 February 2015; revised 27 February 2015

This study has been undertaken to investigate morphological and molecular identification of sea cucumber *Holothuria* (*Selenkothuria*) *parva* geographically distributed in the northern part of the Persian Gulf. Specimens were collected from the intertidal zone of Bostaneh Port during 2013. Molecular genetic analysis was done to confirm the morphological identification and taxonomic status of species in phylogenetic trees. Results proved 100% identity of sea cucumber *Holothuria* (*Selenkothuria*) *parva* with previously identified and submitted sequences in GenBank. This species shared a common cluster in phylogenetic tree. This is the first report of *Holothuria* (*Selenkothuria*) *parva* from Bostaneh Port with both morphological and molecular details.

[**Keywords**: Sea cucumber, *Holothuria* (*Selenkothuria*) *parva*, Morphological, MtDNA, Persian Gulf]

Introduction

Holothuria (Selenkothuria) parva occurs in rocky shorelines hiding under stones of intertidal areas, mangroves and coral reefs. This species is widely distributed throughout tropical waters from intertidal zone to up to 20 meters depth in Red sea[1], Africa[2] and Gulf of Aden[1]. Sea cucumbers have high medicinal and nutritional values[3].These echinoderms have a wide range of biological activities including anticancer, antitumor and antibacterial properties[4]. The subgenus *Selenkothuria* composes 12 species of sea cucumbers from tropical shallow waters. In the past, identification of sea cucumbers was based only on morphological characteristics. Molecular analysis has become an important tool in population genetics and molecular identification of marine organisms. Therefore, understanding genetic information will be useful to resolve taxonomic uncertainties.

Molecular barcoding of Holothuridae family has been undertaken by many research groups so far[5]. Present study is to examine morphological characteristics and molecular identification of *Holothuria* (*Selenkothuria*) *parva* from Bostaneh port in Persian Gulf using mtDNA markers.

Materials and Methods

Sea cucumber species were collected from intertidal and sub tidal zones of selected sites located in Bostaneh Port, Persian Gulf during 2013 by scuba diving and transferred to marine biotechnology laboratory. Individuals were washed thoroughly with tap water to remove any sands and residues. Sea cucumber samples were photographed and ossicles were extracted from body wall after anaesthetizing animal with magnesium chloride 5%[6]. Morphological characteristics were investigated using valid identification keys.

Sea cucumber tissues were taken and preserved in 70% ethanol. Total genomic DNA was extracted from 100 mg of body wall tissues using ammonium acetate extraction method with slight modifications[7].Cleaned DNA was re-suspended in TE buffer. The DNA concentration was examined using spectrophotometer (Eppendorf, USA). Quality of extracted DNA was then detected using gel documentation (Kimia Gen, Iran) to ensure the use of suitable *Holothuria* (*Selenkothuria*) *parva* genome for amplification of selected fragments. Genetic characterization was conducted by sequencing segments of the

mitochondrial 16S (large subunit) RNA and cytochrome oxidase I (COI) genes.

PCR amplification and sequencing

The 16SrRNA primers used were designed and optimized by Kerr, et al in 2005.The forward and reverse primers for the fragment of the mitochondrial large ribosomal sub unit (16SrRNA)wereCGCCTGTTTATCAAAAACAT (F),and CTCCGGTTTGAACTCAGATCA(R), respectively. PCR amplification conditions were as follows: in a total volume of 25 µl reaction mixture of approximately 100ng of genome, 2.5 µl 10X PCR buffer, 10 mmol/l dNTP mix (0.5 µl), 50 mmol/l (1µl) $MgCl_2$, 10 pmol/l (1µl) each primer , 5U/µlTaq polymerase (0.5 µl) (SinaGen, Iran) and distilled water up to 25 µl. PCR in Esco thermal cycler involved initial denaturation for 5min at 95 °C, 35 cycles at 95 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min, and a final extension of 10 min at 72 °C.

The PCR product was then electrophoresed in 1% agarose gel and visualized under UV transiluminator to ensure the quality of PCR products. Amplified PCR products were then sequenced in both directions on an ABI 3770 Automated sequencer. All sequences obtained in this study, have been deposited to GenBank with accession numbers KF135646 – KF135650.

Species specific primers were also designed from COI region of *Holothuria* (*Selenkothuria*) *parva* in this study for molecular identification. To amplify the 350 base pair segment of COI, a pair of primers GACGAGAAGACCCTGTCGAG (F) and CGTTTAGAGCTTCTGCACCC (R) were amplified. Amplification of genome segments were performed in a 25 µl reaction mixture of approximately 100ng of DNA, 3 µl10X PCR buffer, (10nmol/l) dNTP mix(0.3 µl),50 mmol/l (1.5µl)$MgCl_2$,10 pmol/l (0.5µl) each primer, 5U/µlTaq polymerase (0.3 µl)(SinaGen, Iran). PCR program in Esco thermal cycler was conducted as denaturation for30 s at 95 °C, 30 cycles at 95 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min, and a final extension of 72 °C for 10 min. PCR products were verified with agarose-gel electrophoresis before sending out for sequencing. Genome sequences were edited and aligned by Chromas pro and Bio Edit.

Sequences were further analyzed and phylogenetic trees were constructed in two methods of neighbor-joining and fast minimum evolution generated by MEGA6. Neighbor-joining trees and within group genetic distances were also based on Kimura 2- parameter distances.

Results

Morphological observations:

Individuals of this species possess especial morphological characteristics including rods in the body wall and tube feet. The tentacles are modified in this species for suspension feeding and they have cryptic colors.

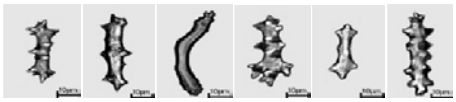
The individuals have soft cylindrical body with blunt end and 22 cm length. Live specimens have brown color with slightly lighter in sides and possess yellow tube feet. Ventral mouth which was surrounded by 20 tentacles was observed. This species has 10 mm polian vesicle and no covarian organs. Dorsal and ventral body wall of organism have 10 µm rod spicules (Figure 1). The tentacles consist of 150 µm length of rods. Spicles of podia were included plates with buttons.

Molecular Observations:

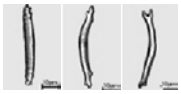
All COI sequences obtained from *Holothuria* (*Selenkothuria*) *parva* species were aligned and sequences already available in GenBank were added to the alignment. DNA barcoding indicated 350 base pair in length sequence from the amplified Mitochondrial DNA COI gene. Comparing the results of the current research with previous sea cucumber sequences in GenBank confirmed *Holothuria* (*Selenkothuria*) *parva* with 100% maximum identity. Phylogenetic analysis of *Holothuria* (*Selenkothuria*) *parva* in this study and other closely related species was carried out. The minimum evolution (Figure 2) and neighbor - joining (Figure 3) methods and bootstrapping with 1000 replicates revealed that all samples in this research formed a cluster together with other specimens of Holothuridae from GenBank. Most

genera also formed monophyletic clades, many of which were not supported by high bootstrap values.

A: Dorsal Spicles of *Holothuria parva*



B: Ventral Spicles of *Holothuria parva*



C: Tentacular Spicles of *Holothuria parva*

Figure 1. Dorsal (A), Ventral (B) and Tentacular spicles of *Holothuria parva*

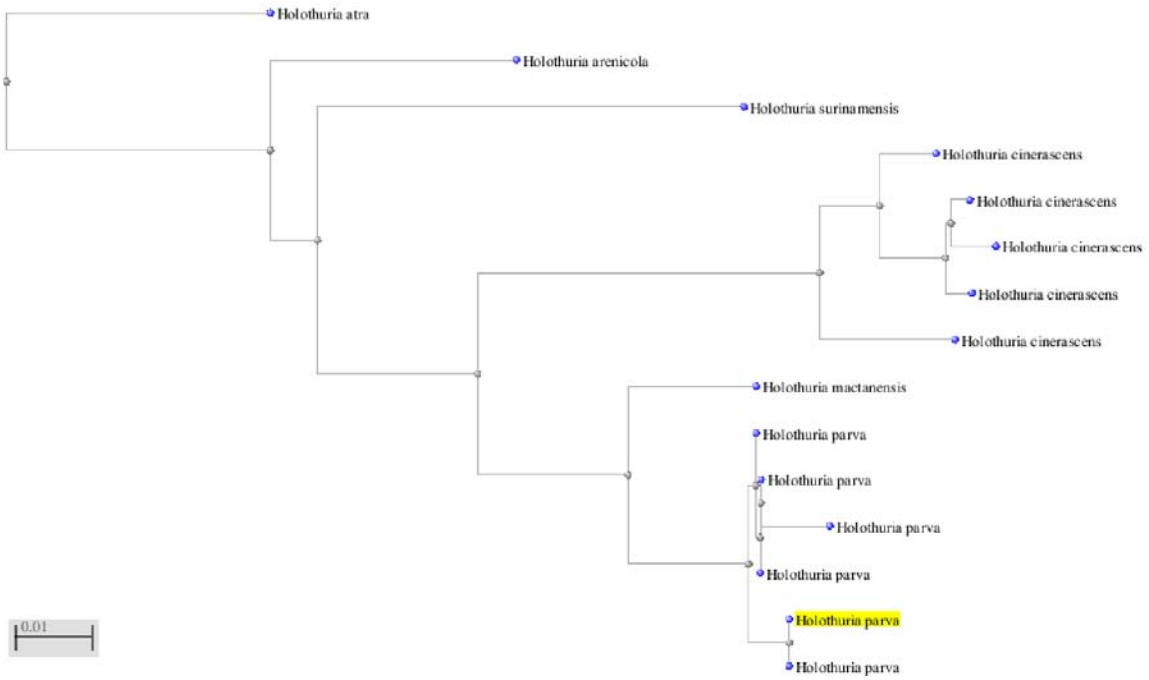


Figure 2. Minimum evolution tree obtained for the phylogeny of *Holothuria parva*

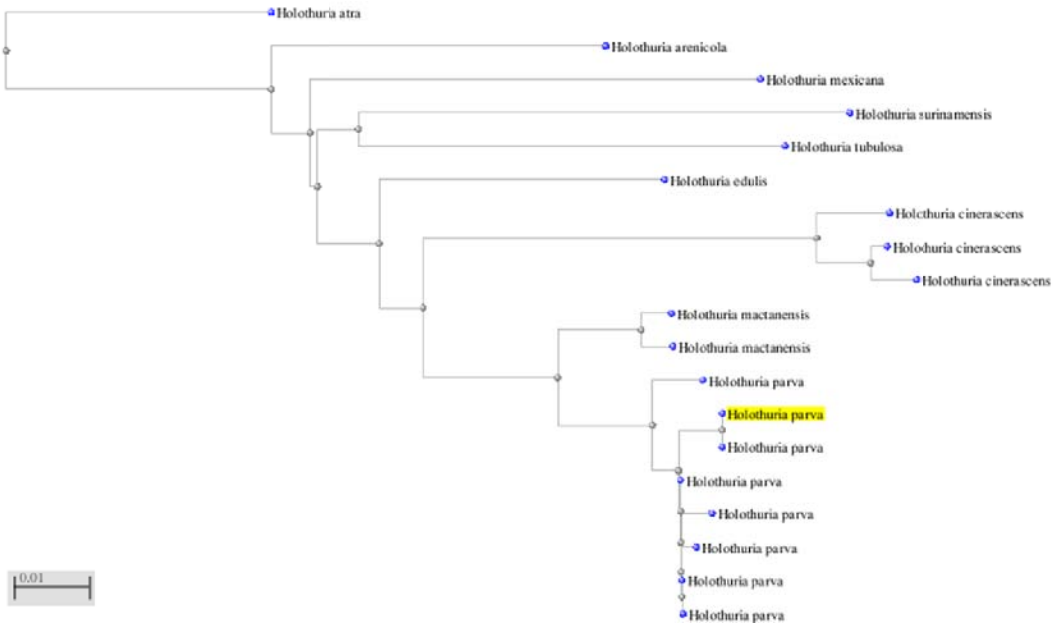


Figure 3. Neighbor- joining tree obtained for the phylogeny of *Holothuria parva*

Discussion

Morphological evaluation:

Individuals have terminal tentacles which are modified for suspension feeding. The main characteristics of this species are spinous rods of the dorsal body wall and yellow tube feet.

There is limited information about the identification of holothurians in the Persian Gulf which almost are confined to the same considerations as Heding [9], Price[10], Dabbagh[11] (2011), Dabbagh and Kamrani [12]. Moreover, Price[10]focused only on fauna (Holothuroidea) of the western Persian Gulf. Therefore, *Holothuria* (*Selenkothuria*) *parva* has only been investigated morphologically in few coastal areas of Persian Gulf region. This report is the first to both morphological and molecular study of this species of sea cucumber in the Persian Gulf.

Molecular evaluation:

Results showed that sea cucumbers of this species formed a strong monophyletic group. Therefore, the mtDNA COI gene was a suitable marker for species identification of Holothuridae family of sea cucumbers.

On the basis of DNA barcoding, species specific primer for *Holothuria* (*Selenkothuria*) *parva* was designed in this research. Amplification of *Holothuria* (*Selenkothuria*) *parva* fragments showed high specificity. Hence, specific primers can be applied and facilitate molecular identification of morphologically similar sea cucumbers both in Persian Gulf coastal waters and other parts of the world. Fast and easy molecular identification of sea cucumber species is also applicable for commercially important species of this family especially for beˆche-de-mer specimens.

COI has been an ideal marker for a wide range of molecular studies including within-species holothurians population genetics[12]and for phylogenetic and taxonomic work at finer scales below genus levels [14]. It is to note that for phylogenetic analysis between genus and family level, the slowly evolving region of 16S is a more suitable marker for Holothuridae family[7]. Apart from identification of adult sea cucumbers, COI sequences are useful to identify

juveniles which are normally different in morphological characteristics. Results of this study confirmed that COI is a good marker for genetic barcoding of most aspidochirotid and dendrochirotid beˆche-de-mer species as it is revealed for other species of sea cucumbers.

Comparing the results of phylogenetic analysis with previous investigation by Magali [15]et al 2012 confirmed that geographical split divides the species of this subgenus into three different groups: one Indo-West-Pacific (IWP) group and two American groups. The IWP group is more closely related to *Holothuria* (*Semperothuria*) *cinerascens* and two other subgenera such as *Roweothuria*, *Holothuria*, and *Vaneyothuria*. These results of phylogenetic tree suggest multiple parallel originations and diversification within the subgenus *Selenkothuria*[15].

In conclusion, COI barcode proved useful in species clarification of sea cucumbers from Holothuridae family in northern Persian Gulf.

The main objective of marine management strategies is to conserve and protect biodiversity. It should be continued to value sea cucumber species as the main contributors to marine communities' biodiversity. Therefore, future management strategies should be rely on how to define and design conservation efforts and take Persian Gulf sea cucumbers community into account.

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