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Identification of Sea cucumber Holothuria (Lessonothuria) pardalis (Selenka, 1867) and Holothuria (Semperothuria) cinerascens (Brandt, 1835) (Family-Holothuriidae) based on morphological and mitochondrial DNA evidence and phylogenetic analysis from Karachi Coast, Pakistan

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Abstract

The conventional taxonomy on sea cucumbers is challenging due to their morphological complexity. Molecular investigation of the class Holothuroidea was initiated for further clarity regarding the systematics and taxonomy of this class. A molecular phylogeny of the Holothuroidea was constructed by using maximum likelihood methods, and this unveiled variation in the existing taxonomic classification, which is largely based on the morphology of calcareous parts. *H. pardalis* and *H. cinerascens* from Pakistan was identified morphologically but no information was found with regard to molecular identification. This is the first research describing the molecular identification of *H. pardalis* and *H. cinerascens* based on 16S rRNAandCOIgenes from the Karachi Coast of Pakistan. Comparisons of mitochondrial 16S rRNAand COI to accessioned sea cucumber sequences on GenBank confirmed *H.pardalis'and H. cinerascens* identity. This study is the first to report on molecular identification of *H.pardalis* and *H. cinerascens* based on the 16S rRNAand COI gene from the Karachi Coast, Pakistan.

Keywords: Sea cucumber, DNA barcoding, Holothuroidea, marine biodiversity, phylogenetic reconstruction.

Introduction

The family Holothuriidae is the most diverse family in the class Holothuroidea and is predominantly found in the tropical and shallow waters. Usually they are soft-bodied echinoderms constituting a diverse group of worm-like organisms with an elongated, flexible gelatinous body and leathery skin. They play an important ecological role through benthic recycling and bioturbation as they are generally scavengers, feeding on debris in the benthic zone of the ocean. They comprise 90% of the total mass of the macrofauna in certain instances, and hence are a vital component of the marine ecosystem (Miller and Nat. 2007).

The consumption of sea cucumbers is becoming popular around the world (Lovatelli et al., 2004) because of their bioactive compounds (polyunsaturated fatty acids, essential amino acids, minerals, proteins, *etc*.) which have many human health benefits. Concommitant to this growing international trade, it has been observed that often there is intentional species substitution of high value species with low market value species. This can have serious consequences, which include economic fraud, health hazards, and illegal trade of protected species (Rasmussen and Morrissey, 2008).

The taxonomic identification of some groups of sea cucumbers is complex, even for experts, but in the last twenty years, molecular analysis has shed new light on this field. Molecular investigation of the class Holothuroidea has helped to clarify its systematics and taxonomy. Molecular phylogenies of the Holothuroidea conflict with existing taxonomy, which is based largely on the morphology of calcareous parts and inference of acquired and inherited characters (Atifet al. 2008). A systematic aroach combining morphological and molecular aroaches is necessary to make progress in assessing the world's sea-cucumber biodiversity. Implementing molecular species identification as analytical confirmation of sea cucumber taxonomy including commercial and imported food products will help in cases where morphological identification is impossible. The accurate classification of commercial holothurian species can help to overcome practical challenges related to identifying sea cucumbers, largely due to deformation of the marine organisms after collection and drying.

*Holothuriapardalis*occurs in rocky shorelines under stones or in coral rubble and it is widely distributed throughout tropical waters from the intertidal zone to -10m depth. It is distributed from the Red Sea to the Hawaiian Archipelago (Tortonese, 1980) and the Indo-west Pacific Ocean to Australia (Rowe and Gates, 1995).

*Holothuriapardalis*is commercially exploited in China and Indonesia and is exported to be dried and eaten.In dried form this species may be traded after being mixed with other low-value Holothurian species (Purcell et al., 2012).

H. cinerascens is a red-brown to purple sea cucumber and belongs to family Holothuriidae they have thick fleshy bodies and consist of several rows of tube feet which are used for moving around and for adhering to the surface. They occurcircumglobablly at low to middle latitudes, they live incoral reefs and nearby sandy habitat types and some occur in deeper waters. The conventional morphological taxonomy of *H. pardalis*from Pakistan (Clark and Rowe 1971;Tahera, 1992; Ahmed and Ali, 2014) and *H. cinerascens*(Ahmed *et al.,*2016) were reported but no information describing molecular identification of this species is available from Pakistan.

Kerr *et al.,* (2005) described eight species (Holothuriidae: Aspidochirotida) from the 5 currently recognized genera based on aroximately 540 nucleotides from the conserved 3' section of 16S mitochondrial ribosomal DNA. Kamarudin*et al.* (2017) describe the morphological and molecular identification of sea cucumber species *Holothuriascabra, Stichopushorrens*and *Stichopusocellatus*from Kudat, Sabah, Malaysia. Kamarudin and Rehan (2015), studied the morphological and molecular identification of *Holothuria (Merthensiothuria) leucospilota*based on the cytochrome c oxidase I (COI) mitochondrial DNA (mtDNA) gene. Clouse *et al.* (2005), resurrected *Bohadschiabivittata*from *B. marmorata*(Holothuroidea: Holothuriidae) based on behavioral, morphological, and mitochondrial DNA evidence. Borrero-Pérez *et al.* (2010) describe the molecular systematics of the genus *Holothuria* in the Mediterranean and Northeastern Atlantic and a molecular clock for the diversification of the Holothuriidae (Echinodermata: Holothuroidea). Yang etal.(2019) studied the complete mitochondrial genome of *Holothurialeucospilata* (Holothuroidea, Holothuriidae) with phylogenetic analysis. Xia *et al.* 2016 studied the complete mitochondrial genome of the sandfish *Holothuriascabra* (Holothuroidea, Holothuriidae). The aim of the present study was to identify sea cucumber to species level by using mitochondrial DNA.

The aim of this study was to confirm, for the first time, the molecular identification of *H. pardalis*and *H. cinerascens*from the Pakistan coast, using mitochondrial (mtDNA) 16S rRNA and COI gene sequences since these have been demonstrated to be informative. This will serve as a basis for future studies in this field and region, and as a demonstration of the strengths of a systematic aroach that considers both morphological and molecular characters in species delineation. Accurate species delineation is the first step to quantifying biodiversity and associated natural resources, and without reliable data with regard to identification, downstream research and decision making becomes fundamentally flawed. Using a combination of taxonomic and molecular methods we demonstrate the value of this aroach in the Holothuroidea.

Materials and Methods

Sample collection and morphological identification

In total 156 sea cucumber specimens were collected from Buleji (24°50'20.41" N, 66°49'24.15" E) and Sunehri (24°52'33.49" N, 66°40'40.20" E) during low tide from the intertidal zone in September to December 2018. For molecular studies the specimens were collected and placed in an ice box which relaxed the specimens by cooling them to temperatures close to freezing (0°C). Samples were immediately transported to the laboratory and cleaned with sea water to remove sand and residue particles. Gut, gonads and tentacles of fresh specimens were removed and preserved at -30°C until DNA extraction. For morphological studies specimens were stored in 70% ethanol. 8 to 10 specimens of *H. Pardalis* (ECH-64) and 5 to 6 specimens of *H.cinerascens* (Cat. No. Holo. 15.) were collected from Buleji and Sunehri. The specimens are deposited in the repository of the Marine Reference Collection and Resource Centre, University of Karachi.
For taxonomic purposes small pieces of tissue (tentacles, dorsal and ventral body wall) were cut with a sterile blade and at once placed on a glass microscope slide with a few drops of household bleach added. Samples were crushed for three to four minutes with a sterile needle to dissolve the soft tissues after which the sample slide was covered with a cover slip. The prepared slide was placed under a microscope for examination at 10 × magnification (Nikon LABOPHOT-2). Microphotography was also performed using a digital camera (Fujifilm 16 MP).

DNA Extraction

Total DNA was extracted from three soft tissues (tentacles, gonads and gut) of *H. pardalis* and H.*cinerascens* using a modified hexadecyltrimethyl-ammonium bromide (CTAB) method by Greweet *al.* (1993). 8 to 10 specimens of *H. Pardalis*and 5 to 6 specimens of H.*cinerascens* were selected for molecular studies. 100 mg of tissue were transferred in a mortar with liquid N_2 and ground with a pestle. The ground tissues were transferred to a marked *eendorf*tube (1.5 mL) containing 700 µL CTAB and 20-30 µL proteinase K and incubated in a water bath (Elmasonic S 30H) at 55 ^{o}C for three hours at which point samples were completely digested. During this time the sample was vortexed (HeidolphReax 2000) for about 20 minutes in order to reduce the lysis time. Four hundred µL of phenol:chloroform (24:1) was added to each tube and gently inverted 5-10 times before centrifuging at 13 000 rpm for 10 minutes. Only the uer layer of the supernatant was transferred to a newly labeled tube. Next 300 µL of supernatant and 200 µL of isopropanol was mixed well and centrifuged at 13,000 rpm for 10 min. The supernatant was then discarded and the DNA pellet was washed with 1 mL 70 % cold ethanol (4°C) without disturbing the pellet, and subsequently centrifuged for 5 min at 10,000 rpm. The supernatant was removed and the pellet dried for 3 hours and finally dissolved in 200 µl TE buffer.

Sequences editing and analysis

Gel preparation: 50 ml of TAE buffer (Tris-Acetate-EDTA) and 0.5 (g) agarose were mixed on hot plate for 2 minutes, until complete dissolution. The hot agarose solution was cooled down, after which 1.5 µl of Ethidium Bromide solution was added and gently mixed to avoid the formation of bubbles. The agarose solution was transferred immediately to a gel tray, aropriate combs were placed in the solution and bubbles were pushed away from the slots using pipette tips. After about 30 minutes, polymerization was complete and the agarose gel was used for electrophoresis. The gel tray was submerged in the electrophoresis tray, wells on the top and the plastic comb removed. TAE buffer was added into the gel bath, and the DNA samples were loaded into the wells. The power unit was set to 100 volts for 30 minutes. After 30 minutes agarose gels were placed on a UV-transilluminator for DNA band visualisation.

PCR Amplification and Purification

Polymerase chain reaction (PCR) was employed to amplify the 16s rRNA gene region from the mtDNA. Primers for the 16s rRNA marker were 5'-CGCCTGTTATCAAAAACAT (forward) and 5'-CTCCGGTTTGAACTCAGATCAGC (reverse). The PCR amplification cycle was as follows; initial denaturation at 94°C for 4 min, followed by 34 cycles of 96ºC/ 5 min, 95ºC/ 30 s, 55ºC/ 30 s, 72°C/ 30 s, and a final elongation step of 72ºC/ 5min. After amplification, products were separated by electrophoresis on 1% agarose gels and visualized under a UV trans-illuminator to ensure the quality of PCR product. The PCR products were purified by Bio Basic; EZ-10 Spin Column Purification Kit (Canada Inc.) according to the manufacturers' instructions. Both strands were sequenced on a 3130 genetic analyzer (ABI) using dye-terminator sequencing.

Data Analysis

MEGA 5 and Ugene were used to conduct sequence editing and alignment (Tamura et al. 2011, Okonechnikov et al. 2012). The Basic Local Alignment Search Tool (BLAST) was used to identify sequence origin (Altschul et al. 1990). The accessioned sequences with the highest maximum identity to the amplicon sequence were downloaded from the GenBank sequence database for phylogenetic analysis (Table 1). Out groups were chosen from the Asteroidea and used to root the phylogenetic tree (Table 1). Maximum likelihood phylogenetic trees were reconstructed with 1000 bootstrap replicates under the General time reversible parameter model (Tavare, 1986) using R (R Core Team, 2020) and the packages APE (Paradis and Schliep, 2018), PHANGORN (Schliep, 2011) and PHYTOOLS (Revell, 2012). The sequence recovered was compared to sequences from GenBank using BarcodingR (Zhang et al. 2016), and adegenet (Jombart 2008), species identification was inferred using both protein coding barcodes and fuzzy-set k-mer algorithms.

Table 1. Sequence of sample 8-F. DNA barcoding indicated 480 bp sequences were amplified.

5'GGCCGCGGTATCTTGACCGTGCAAAGGTASCWTAATCATTTGTCTTTTAAATGGAGACTTGTATGAA
CGGCGAAACCTTTTTTAACTGTCTCCCCTCCTACCCTTATAAATTTCCTTCTGCGTGAAGAGGCGCAGA
TTAAACAGAAAGACGAGAAGACCCTGTCGAGCTTCAGTCTACAAGTGAACACATCACAATCACCCAA
AGACTTTGGTTGGGGCAACCCTGGAGAAAAAAAATCCTCCAGAAGCAATAGAAGAAACCCACTCCCT
ATTTTCATTATCAAGAACCAGAACCTGGTATACGGAAAAAGTTACCGCAGGGATAACAGCGTCATCCC
CTCTAAGAGTCCTTATTGACGAGGGGGTTTGCGACCTCGATGTTGGATTAGGGCCCCCTTAGGGTGCA
GAAGCTCTAAAAGGTTAGACTGTTCGTCTATTAAAGCCCCACATGATCTGAGTTCAAACCGGAGA-3'

Table 2 Ten (10) search results based on Holothuria pardalis 16s DNA using the BLAST algorithm on the GenBank database (NCBI) (Sequences producing significant alignments).

FJ223861.1	*Holothuriapardalis* clone HNTKE2 16S ribosomal RNA gene,partial sequence; mitochondrial	804	804	88%	0.0	**98.46%**
MK564647.1	*Holothuriapardalis* isolate CN13 large subunit ribosomal RNA gene, partial sequence; mitochondrial	756	756	95%	0.0	94.32%
FJ223867.1	Apodida sp. Undetermined 2 KKR-2008 clone H12RE1 16S ribosomal RNA gene, partial sequence; mitochondrial	678	678	88%	0.0	93.44%
LR694133.1	*Holothuriapolii*mitochondrion, complete genome	621	621	98%	9e-174	88.67%
FJ223866.1	*Holothuriacoluber*clone HCTKE1 16S ribosomal RNA gene, partial sequence; mitochondrial	621	621	88%	9e-174	91.25%
JN207561.1	*Holothuriaimitans* isolate A52 voucher UNAM-ICML 5.28.79 16S ribosomal RNA gene, partial sequence; mitochondrial	612	612	93%	6e-171	89.59%
MK564644.1	*Holothuriaarenicola* isolate CN10 large subunit ribosomal RNA gene, partial sequence; mitochondrial	601	601	99%	1e-167	87.72%
JN207530.1	*Holothuriaimitans* isolate N237 voucher FRM174 16S ribosomal RNA gene, partial sequence; mitochondrial	601	601	93%	1e-167	89.18%
LC176660.1	*Holothuriapoli*mitochondrial gene for 16S ribosomal RNA, partial sequence, isolate: Hpo-16S4	599	599	95%	4e-167	88.40%

Table 3. Sequences results of sample QA-F. DNA barcoding indicated (522 bp) in length sequences was amplified.

CCCCTATCCAGAAACATCGCACACGCCGGGGGATCAGTGGACCTAGCCATTTTCTCCCTACACTTAGCC
GGGGCTTCCTCAATCTTAGCCTCAATAAATTTCATAACAACAATCATAAAAATGCGAACTCCCGGAGT
AACTTTTGATCGTCTCCCACTATTCGTATGGTCCGTATTCATAACAGCATTCCTTCTTTTACTTAGACTA
CCCGTCCTAGCAGGAGCAATAACAATGCTCTTAACCGACCGGAACATAAATACCACATTCTTCGACCC
TGCTGGAGGAGGAGATCCTATACTATTCCAACATCTCTTCTGGTTTTTTGGACATCCAGAAGTATATAT
TCTTATCCTACCAGGGTTTGGAATGATATCCCACGTAATTGCCCACTACAGAGGAAAGCAAGAACCAT
TTGGATACCTAGGAATGGTATACGCAATGGTAGCAATAGGAATTCTAGGATTCTTGGTCTGAGCCCAC
CACATGTTCACCG

Table 4. Seven (07) Homology results of Holothuriacinerascens COI- DNA using the BLAST algorithm on the GenBank database (NCBI) (Sequences producing significant alignments).

JN207581.1	Holothuriacinerascens isolate G103 voucher UF4683a cytochrome oxidase subunit I gene, partial cds; mitochondrial	898	898	76%	0.0	99.00%
JN207584.1	Holothuriacinerascens isolate A165 voucher UF9489 cytochrome oxidase subunit I gene, partial cds; mitochondrial	893	893	76%	0.0	98.80%
JN207583.1	Holothuriacinerascens isolate A162 voucher UF4093 cytochrome oxidase subunit I gene, partial cds; mitochondrial	887	887	76%	0.0	98.60%
JN207582.1	Holothuriacinerascens isolate A151 voucher MRAC1867 cytochrome oxidase subunit I gene, partial cds; mitochondrial	865	865	76%	0.0	97.80%
JN207579.1	Holothuriacinerascens isolate A61 voucher UF6875a cytochrome oxidase subunit I gene, partial cds; mitochondrial	865	865	76%	0.0	97.80%
JN207580.1	Holothuriacinerascens isolate A62 voucher UF6875b cytochrome oxidase subunit I gene, partial cds; mitochondrial	837	837	76%	0.0	96.61%
MK562378.1	Holothuriacinerascens cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	826	826	71%	0.0	98.50%

Results

Taxonomic Identification of *Holothuria pardalis*

Live specimens of *H. pardalis* have dark brown dorsally, with two rows of large dark brown spots; ventrally light yellowish with dark brown bands. Body wall tables with rounded disc with four distinct central and a several small marginal holes; spire of moderate height with about eight teeth; pseudo-buttons mostly complete, only few incomplete, always smooth. 20 tentacles. Ossicles of body wall tables with rounded disc with four distinct central and a several small marginal holes; spire of moderate height with about eight teeth; pseudo-buttons mostly complete, only few incomplete, always smooth. Ventral podia with curved rods with 1–5 perforationsat each end, elongated plates with a double series of holes and a smooth undulating margin, never serrate. Tentacle rods branched at ends, holes absent. Taxonomic details of *H.pardalis* already published by (Ahmed et al., 2020) see detail in references.

Taxonomic Identification of *Holothuriacinerascens*

The specimen measured 280 mm and weighed 248 g**.** Colouration of the fresh specimen was rusty brown and papillae were orange. The body was cylindrical with relatively long podia on the ventral side. The dorso-ventral skin was thin. The terminal mouth was surrounded by 20 dendro-peltate retractile tentacles. Large, clearly tube feet were present on the ventral side. The anus was terminal with three small papillae. Dorso-ventral spicules were similar to tables and rods. Tables were more numerous on the dorsal side than on the ventral body wall. Tentacle spicules were rod shaped. Taxonomic details of *H.cinerascens*already published by (Ahmed et al., 2016) see detail in references.

DNA sequence alignment and species identification

The fluorescent based DNA sequences were viewed by sequence scanner (1.0 v) software. The DNA run sequencer configuration for *H. pardalis* were: base caller version (1.4 to 1.8), average raw signal intensity = A(133), C(158), G, (92), T(147) and average noise=A(5), C(4), G(3), T (5), average raw signal to noise ratio=A(25), C(39), G(30),T(30). QV20 was 480 (Base w QV >=20). DNA barcoding indicated 480 bp length sequences were amplified (Table 1). The DNA run sequencer configuration for *H.cinerascens*were: base caller version (1.4 to 1.8), average raw signal intensity = A(245), C(256), G, (132), T(235) and average noise=A(4), C(4), G(3), T (4), average raw signal to noise ratio=A(63), C(67), G(42),T(55). QV20 was 522 (Base w QV >=20). DNA barcoding indicated 522 bp length sequences were amplified (Table 3).The numbers of base pairs were estimated using a Gene Ruler marker set (1kb DNA ladder). For sequence identification BLAST was run on the GenBank nucleotide sequence database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?).

Comparing the Pakistan *H. pardalis* sequence with previous results of sea cucumber sequences found on GenBank confirmed that these specimens were likely *Holothuriapardalis* (98.46%) (Accession no. MT808203) (Table 2) and *H.cinerascens* (99.00%) (Accession no. MT982203) (Table 4).

The results of the BLAST search demonstrate that no direct correspondence between sequences was found, but the results indicate that all 21 sequences of the 16S mtDNA gene were sequences of sea cucumber as they are genetically similar to sequences of other sea

cucumbers deposited on GenBank (NCBI) from three families of the class Holothuroidea (Holothuroidae, Stichopodidae, and Caudinidae).

Phylogenetic reconstruction

Phylogenetic analysis employed the likelihood method, and illustrates that the specimens collected in Pakistan were most likely *H. pardalis*. Samples and 16S gene sequences from Genbank (Table 1) indicated that nearly all species form distinct monophyletic clades (Figure 2). The phylogenetic tree constructed here indicates that the specimen collected from Pakistan's shoreline is most closely related to specimens identified as *H. pardalis* on Genbank. It should be noted, however, as observed in Figure 2, that there are multiple instances of specimens that aear to be misidentified morphologically and deposited on Genbank as such. The clades of genera identified on the tree do indicate general consensus in terms of identification, but caution is urged in terms of relying on similarity results from Genbank for identification of specimens. This is exemplified by the aarent misidentification of multiple species such as the *Apodida* sp. in the *pardalis* clade, and the *H. nigralutea* in the *edulis* clade. The utility of the NCBI Genbank database is however, evident from the consensus in lineage sorting amongst the Holothuroidea in Figure 2.

Species identification in BarcodingR

Species identification using protein-coding barcodes and fuzzy-set logic analyses revealed that the 16S query sequence from Pakistan was most likely *Holothuriapardalis* (reference accession number: FJ223861) with base-pair probability of 0.71 and anfuzzy membership function value (FMF) of 0.1. The COI sequence was most likely *Holothuriacinerascens*, with a base-pair probability of 0.74 and an FMF value of 1, an indication of suort for the correct identification of the sequence.

Discussionand Conclusion

The results from the molecular analysis and comparison of the holothurian 16S DNA revealed in both the phylogenetic tree and the barcode species identification demonstrated that the query specimen was *Holothuriapardalis*. This suorts the taxonomic identification of this specimen and serves as a useful case study demonstrating the utility of molecular methods (Ahmed et al., 2016 and Ahmed et al., 2020).There is no information on molecular identification of sea cucumbers from Pakistan, but Kamarul et al. (2010) sequenced *Holothuriapardalis* from Malaysia which aears closely related to sequences from the present study.As compared to morphological observation in isolation, DNA barcoding has advantages in precision and objectivity for the identification of species as it is based purely on systematic analysis of hundreds of observed characters.

This method has been successful in different marine organisms including sea cucumbers (Kamarul and Ridzwan, 2005; Kamarul, *et al.*, 2006; Kamarudin, et al. 2010; Ismail et al. 2012; Jefri et al. 2015; Prehadi et al. 2015; Sembiring et al., 2015; Kamarudin and Rehan, 2015; Maddua et al. 2016; Maulid et al. 2016; Saleky et al. 2016; Zahra et al. 2016; Kamarul et al. 2018). Ismail et al. (2012) studied the molecular taxonomy of commercially important species of the class

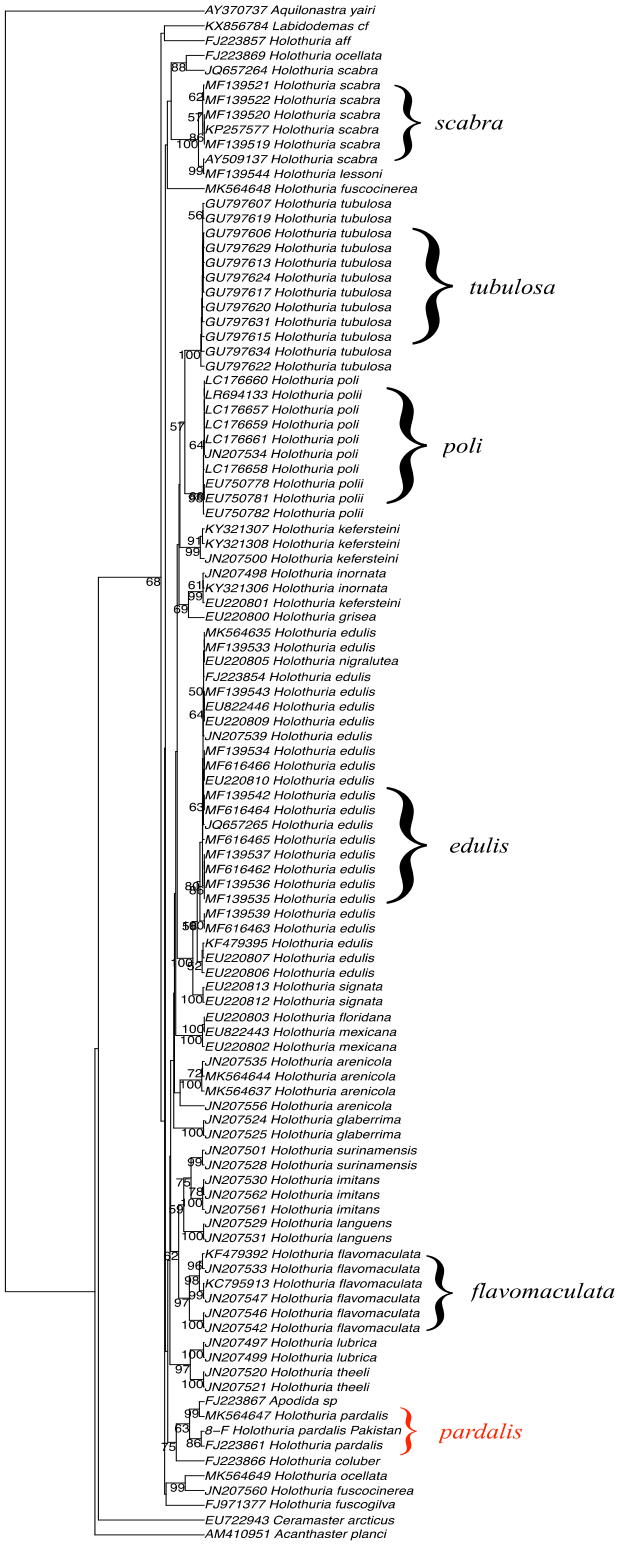

Figure 2. Phylogenetic tree reconstruction of the studied sample (8-F) compared with sea cucumber species 16Ssequences from the NCBI Genbank based on maximum likelihood with 1000 bootstrap re sampling.

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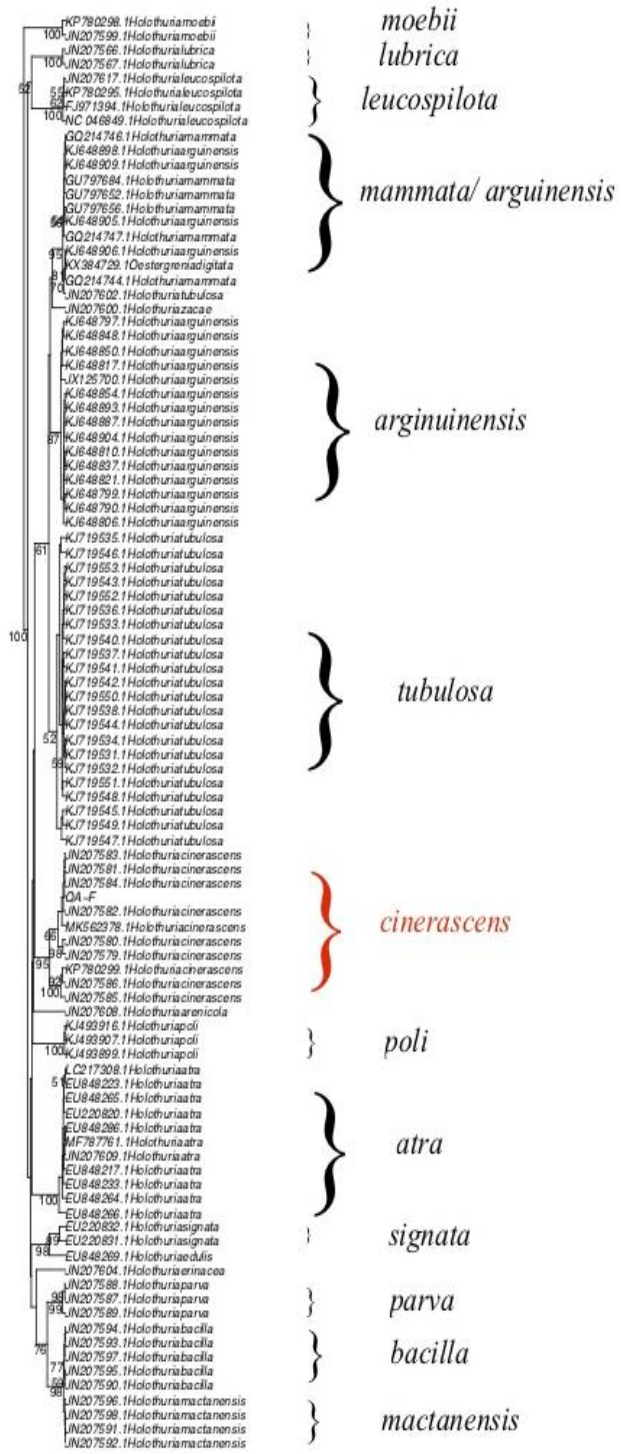

Figure 3. Phylogenetic tree reconstruction of the studied sample (QA-F) compared with sea cucumber species COI sequences from the NCBI Genbank based on maximum likelihood with 100 bootstrap re sampling.

Holothuroidea by sequencing a 465 bp region of the 16S rRNA to characterize and identify sea cucumbers.

Zahra et al. (2016) investigated molecular identification in *Holothuriaparva*, and DNA barcoding indicated 350 bp sequences from the amplified mitochondrial DNA COI gene was useful. They also noted that for phylogenetic analysis at genus and family level, the slowly evolving 16S region is a more suitable marker for consistent delineation. Kamarudin and Rehan (2015) studied the morphological and molecular identification of *Holothurialeucospilota*and *Stichopushorrens* from Pangkor Island, Malaysia. They undertook species identification using ossicle shapes as part of a morphological aroach combined with a genetic aroach (the COI mtDNA gene sequencing technique). Evidence presented here suorts the utility of COI and 16S mtDNA as a molecular barcoding locus for the *holothuria* and presents a useful tool for the delineation of products of uncertain origin.

Genetic drift and natural selection are among several factors which drive genetic differences through accumulation of substitutions in separated populations (Freeland 2005). Uthicke*et al.* (2010) have analyzed the relationships among many commercial species and come to similar conclusions regarding the use of molecular methods as a useful means of identification.

We suggest that molecular methods be combined with traditional morphological methods (based on established taxonomy) as a practical strategy of specimen identification especially when specialist taxonomists are not available for consultation. The combination of molecular characters and morphological characters is an effective method of aroaching the identification of specimens that aear duplicitous in morphological character. This methodology has been adopted in other fields of molecular ecology and has proven its value (Schmidt-Roach *et al.* 2014). In surveys of biodiversity, which form the foundation for understanding habitat ecology, correct identification of specimens is a vitally important first step.

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