mtDNA marker reveals the first record of *Sepiella japonica* (Sasaki, 1929) in the marine waters of Pakistan

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Abstract

Cephalopods are the most intelligent, mobile and also the largest of all mollusks, with all members marine. Cuttlefish is a valuable fishery source and is used as human food because of its high nutritive value. Previously, only one Sepiidae species, *Sepiella inermis*, had been reported in Pakistan. Morphological species identification is now being replaced by molecular-based approaches due to their fast and reliable results. Here, we used mitochondrial fragments, cytochrome c oxidase I (COI) to investigate *whether Sepiella japonica* could be identified by the DNA barcoding technique. In the present investigation, a 588 bp fragment of Cytochrome oxidase subunit 1 sequence revealed 98.40% identity with *S. japonica*. The evolutionary distance between the *S. japonica* from Pakistan and India is 0.012. The neighbor-joining tree showed a close relationship between sequences of this species from India and Pakistan. The *S. japonica* sequence from this region was submitted to NCBI under accession number ON430600. To the best of our knowledge, this study is the first that report *S. japonica* in Pakistani coastal waters. Presence of this commercially important species will be a valuable addition to the Pakistani coastal waters. Molecular information provides important assistance for taxonomic decisions.

Keywords: *Sepiella japonica*, CO1, DNA barcoding, Pakistan; Cuttlefish, PCR

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Introduction

Cephalopods, which include octopuses, squids, cuttlefishes, and nautiluses inhabit diverse marine environments, and distributed worldwide especially in Indo-Pacific, Australian, Mediterranean, and African coastal waters. Recently, three genera are documented in the family Sepiidae including Sepiella, Sepia and Metasepia, (Sanchez *et al*., 2018; Zhao *et al*., 2020). It comprises 28 families and over 845 known species and is ranked the third largest class among phylum Mollusca (Hoving *et al*., 2014). They are commercially important (Kim *et al*., 2019; Liu *et al*., 2002; Xu *et al*., 2022) and regarded as a model animal (Wang *et al*., 2021). The species of the family Sepiidae has significant commercial value to artisanal and industrial fisheries. The cuttlefishes are characterized by several features such as having a buoyancy device made up of calcium carbonate, the body is compressed and oval and the presence of chromophores and a gland.

Sepiella japonica (Japanese spineless cuttlefish), is one of the four major kinds of seafood having ecological, commercial (Lü *et al*., 2022), medicinal and high edible values in the East China Sea (Wang *et al*., 2021; Ye *et al*., 2022; Lü *et al*., 2022). It plays a key role in the marine ecosystem (Li *et al*., 2018). Due to ocean environmental damage and overfishing since the 1980s *S. japonica*'s production has sharply decreased (Zhao *et al*., 2020). Their short life cycle and high growth rates make them ideal for artificial breeding (Zhao *et al*., 2020). Among other cuttlefishes, the *Sepiella japonica* has profound commercial importance and is used as a human food because of its high nutritive value including the source of protein (Li *et al*., 2014). Besides, its nutritive importance cuttlebone is also used for ornamental and medicinal purposes, (Lei *et al*., 2012; Zheng, 2004; Farhadi and Anderson, 2021). The migration of this species is restricted and influenced by anthropogenic activities and over-exploitation, therefore its population decreases respectively in the countries where its commercial demand is high enough (Guo *et al*., 2016).

In Pakistan, only a single species (*S. inermis*) of the family Sepiidae was reported and no previous record of *S. japonica* is available, nevertheless, it has been reported from India, and Oman (Farhadi and Anderson, 2021). Mitochondrial DNA molecular markers are extensively being used for the identification of various species (Xia, 2017), nevertheless, nuclear DNA markers are also being used. Because of the high accuracy, traditional morphological-based identifications are frequently being replaced with molecular-based identifications (Taberlet *et al.*, 2012). Traditional morphological features are thought to be less trustworthy than the output of molecular markers (Packer *et al.*, 2009). As a result, this method of identification is commonly utilized to identify and comprehend phylogenetic relationships.

Polymerase chain reaction (PCR) is a widely used technique that may amplify a single or a small number of copies of certain DNA sequences with remarkable accuracy and speed found in a heterogeneous population to millions of copies, making it possible to detect even very low DNA concentrations in samples. It is a method for extracting significant quantities of a particular DNA sequence from a DNA sample. The replication of a double-stranded DNA template serves as the foundation for this amplification (Kadri, 2019). The PCR amplification has three steps: denaturation, annealing, and extension.

The goal of this study was to better understand the molecular taxonomy of *S. japonica*, which was discovered in Pakistani coastal waters. To our knowledge, this is the first record of this species from Pakistani coastal waters that provides taxonomists and ecological managers with valuable information in science and fishery management.

Materials and methods

Sampling

Sepiella japonica individuals were collected from Ibrahim Hyderi, Karachi and kept in the icebox, transported to laboratory, morphologically identified with available literature. The muscle tissues were taken and preserved in 95% ethanol and kept at -20°C until DNA extraction.

DNA isolation and PCR

Genomic DNA (gDNA) was extracted using phenol-chloroform method from muscle tissue (Sambrook *et al*., 2001).

A set of universal primer of Cytochrome oxidase subunit 1 (COX1) gene were used for amplification (Table 1). 100 mg DNA template, 2.5 L dNtp (2.5 mM each), 2.5 L10 X buffer, 2L Mgcl2, (20mM), 1M primers (10M eac h), and 0.25Lof Taq polymerase (5U Ml*1) were used in the PCR. Denaturation at 94 oC for 5 min; 35 cycles of 94 oC for 30 s, annealing at 50 oC for 30 s, and extension at 72 oC for 30 s; and a final extension at 72 oC for 7 min. Gel electrophoresis (1 percent agarose gel with ethidium bromide (stain)) was used to confirm successful amplification.

Table 1: The set of primers was used in this study.

Gene	Primer name and Sequence	Tm (°C)	Size (bp)	Reference
Cytochrome oxidase subunit1 (COX1)	LCO1490:5'GGTCAACAAATCATAAAGATATTGG-3'	50	588	Folmer *et al*., 1994
	HCOR2198:5'-TAAACTTCAGGGTGACCAAAAAATCA-3'			

Sequencing and construction of phylogenetic tree

The Sanger sequencing method was used to sequence the PCR products. Software (BIOEDIT and MEGA 6) was used to do the necessary insertion and deletion. Using the Kimura 2 parameter (K2P) model and MEGA 6, a neighbour

joining tree was created to establish the genetic links among the populations (Tamura *et al*., 2013).

Results and discussion

In this study, we successfully used single-gene DNA barcoding for cephalopods along Pakistani waters. A fragment of the 588 bp sequence of Cytochrome oxidase subunit 1 was used to identify the *S. japonica*. The NCBI-based nucleotide blast result showed a similarity of 98.40%. The morphological structure of *S. japonica* is shown collected from the marine water of Pakistan (Fig. 1). The similarity score of the one hundred best matches from the BOLD System is shown in Figure 2 (https://www.boldsystems.org/index.php/IDS_OpenIdEngine). The species sequence was submitted to NCBI under accession number ON430600. The evolutionary distance of *S. japonica* from Pakistan and India was 0.012. The histogram (Fig. 3) was obtained by using the ABGD website (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html). The neighbor-joining tree shows *S. japonica* clustered with the sequence from India under accession number KC409394, while the sequences from the Gulf of Oman differed with 66% bootstrap support (Fig. 4).

Cephalopods are important to commercial fisheries, however, local fishermen do not use specialized gear for cephalopod catch in Pakistan. From, 1987 onward their importance increased and 460 tons worth 0.31 million U.S. dollars were exported annually (Rashad *et al*., 2010). The exported products consist mainly of frozen squids, filleted cuttlefish and tentacles (Majid and Khaliuddin, 1992).

Figure 1: The morphological structure of ***S. japonica*** is shown collected from the marine water of Pakistan.

Of the twenty-seven species of cephalopods in Pakistani coastal waters, only a few have high commercial value, such as *Sepiella inermis, Sepia pharoanis, Sthenoteuthis oualaniensis, Sepioteuthis lesoniana and Loligo duvauceli* (Moazam and Ahmed, 1994).

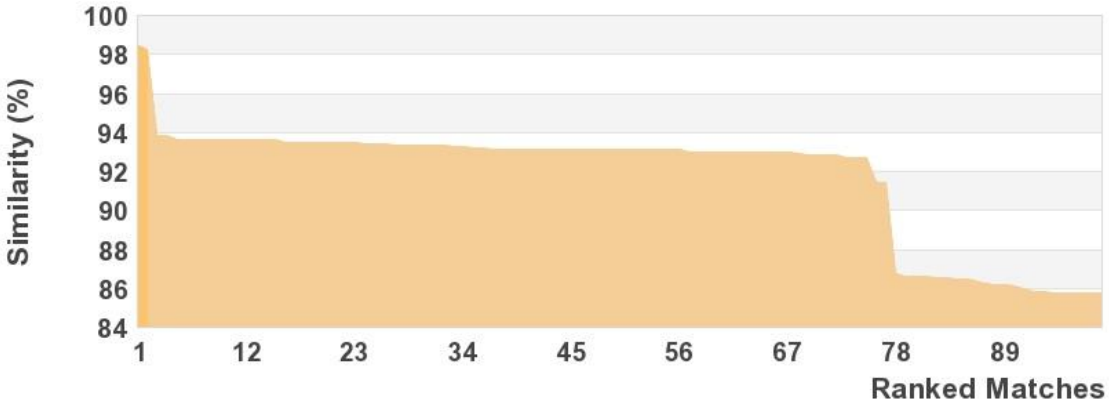

Figure 2. The similarity score of the top 100 matches is taken from BOLD System V3. (https://www.boldsystems.org/index.php/IDS_OpenIdEngine).

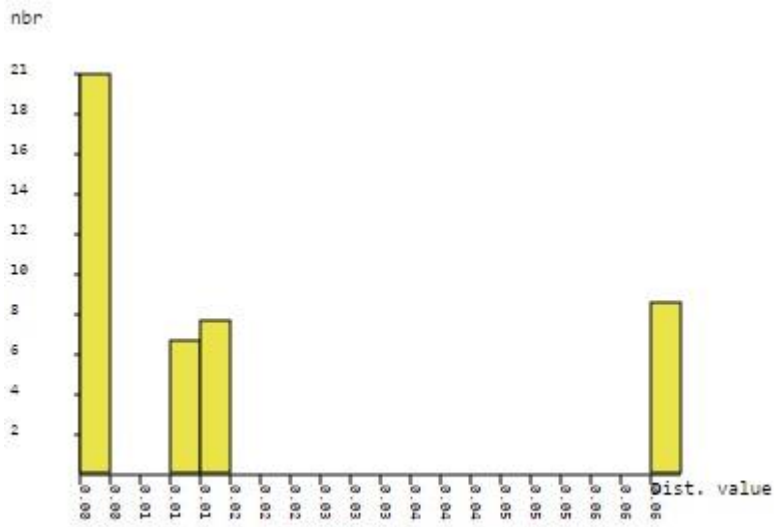

Figure 3: Showing histogram of distance among *Sepiella japonica* individuals sequenced from different parts of the world.

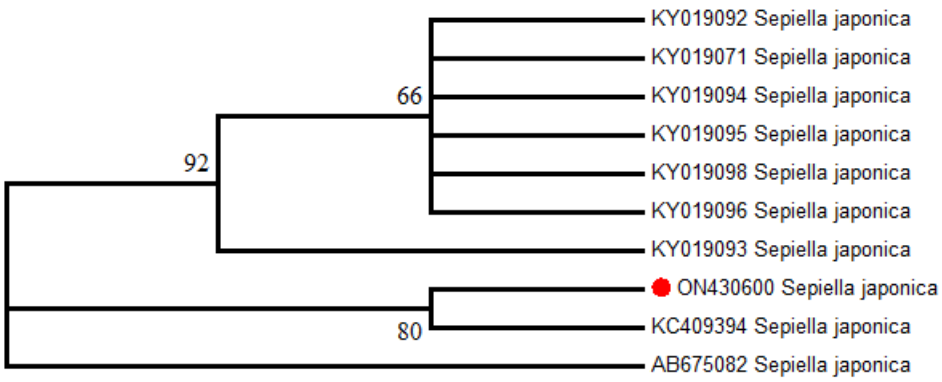

Figure 4: Neighbor-joining phylogenetic tree of *Sepiella japonica*. The sequence from Pakistani waters was circled with red and it clustered with the sequence from India.

Stock assessment of cephalopods from Pakistan was also assessed by (Soomro *et al.*, 2015 and Kalhoro *et al.*, 2018). However, neither specifically nor collectively *Sepiella japonica* was previously reported by any of the researchers from the coast of Pakistan. Withinthe genus *Sepiella*, a single cuttlefish species (*Sepiella inermis*) has been reported from Pakistani (Ashraf, 1969; Voss *et al.*, 1998; Kazmi and Sultana 2003; Kazmi *et al.*, 2018) coastal waters.

To the best of our knowledge, this studyis the first to report the presence of *S. japonica* in Pakistani coastal waters. Presence of this species will be a valuable addition to the Pakistani coastal waters. The exploitation of this species in neighboring countries was well established. In 1957, the wild stock production in Zhejiang Province reached 60,000 tonnes, which accounted for more than 9.3% of the province's fishery catch (Liu, 2002; Li *et al.*, 2016). Nevertheless, overfishing and pollution have reduced the stocks of *S. japonica* in Chinese marine waters since the 1980s (Jiang *et al.*, 2014). Therefore, artificial farming techniques have been emphasized in China to increase productivity and successful aquaculture techniques have been developed in recent years (Yin *et al.*, 2013). Because *S. japonica* is a potentially valuable species in Pakistani marine waters, its stock assessment, and management research is recommended.

Conclusion

Cephalopods from Pakistan gained less attention and to our knowledge this is the first time to report the presence of this species, which identified using molecular based approach. At present we are unable to compare / comment between the earlier identified species *S. intermis* and species identified in present study, because there is no preserved specimen of *S. inermis*. It is recommended for future studies to compare the *S. inermis* and *S. japonica* using molecular techniques in order to know that either there are two different species in the Pakistani marine waters or a single species of S. japonica which might possibly misidentified earlier.

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Ethics statement

All the methods were carried out in line with international norms for an invertebrate.

Declaration

We declare no conflict of interest.

Author contribution

Amna Sulaman collected samples**,** conducted experiments and analyzed the data Haqdil Hakeem Shad**,** Muhammad Saleem Chang participated in writing the manuscript**,** Muhammad Shafi and Faiz Muhammad designed experiments, generated funds and wrote the article.

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