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Short Communication

Identification of *Perna viridis* based on mitochondrial COI sequence

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Abstract
The Asian green mussel *(Perna viridis*), a bivalve species of high economic importance, is widely distributed in the Indo-Pacific region. Here, we aimed to investigate the *P. viridis* species from Pakistan, a biogeographic area where nearly no *P. viridis* species were genetically characterized using mitochondrial cytochrome C oxidase subunit I (COI) gene to correlate it with morphologically identified species of other countries. Our results of Basic Local Alignment search Tool (BLAST) analyses show 98.7% similarity with the partial sequences of *P. viridis* reported from India. This study confirmed the molecular identification of *P. viridis* for the first time from Karachi, Pakistan and this finding is important for further taxonomic identification.

Keywords: bivalve; green mussel; mtDNA COI; Karachi; Pakistan

1 | INTRODUCTION

Mussels (Class Bivalvia, Phylum Mollusca) are primitive in origin as well as worldwide in distribution (Graf and Cummings 2007; Shafiullah *et al.* 2021) The muscles have high nutritive and medicinal importance (Rajagopal 2006; Chakraborty *et al.* 2016; Dehghan *et al.* 2021) These significantly important animals occupy a profound position in our ecosystem as bio indicators and biofilters and perform a key role in the food web. The members of the Mytilidae family are distributed globally (Distel 2000) and it has three recognized species, *Perna viridis* or the green mussel (Linnaeus 1758*) P. canaliculus* (Gmelin 1791) or the green-lipped mussel and *P. perna* or (Linnaeus 1758) brown mussel (Siddall 1980; Vakily 1989; Noor *et al.* 2019). *Perna viridis* is distributed along the coasts and estuaries of the Asia-Pacific region and is regarded as a warm water bivalve species. This mollusc species are highly prized as a food source and an important aquaculture component in Southeast Asia (Tan and Ransangan 2017; Wang *et al.* 2018; Guo *et al.* 2019).

These mussels are morphologically identified by the shape of the shell and the colour difference. The pattern of the scars is the most important distinctive feature by which *Perna* and *Mytilus* can be distinguished which is left at the area of muscle attachment on the shell (Siddall 1980). The shell this mollusc features concentrically lines and narrows as it extends towards the anterior side.

The north Arabian Sea seemed to have the highest diversity of molluscs (Melvill 1928). During the 18th century studies on molluscs were started in the northern Arabian Sea. Many researchers (e.g. Khan and Dastagir 1971; Ahmed 1977; Ahmed *et al.* 1982; Barkati and Asif 1984; Timizi and Zehra 1984; Tirmizi and Kazmi 1995; Siddiqui and Ahmed 2002; Kazmi and Naushaba 2004; Barkati and Rahman 2005; Naseem and Mozazzam 2007; Afsar 2009; Ali *et al*. 2011; Gondal *et al*. 2012; Jahangir *et al*. 2012; Rahman and Barkati 2012; Jahangir *et al*. 2014) have revealed high varieties of molluscs in Pakistan. Almost 92 taxa of molluscs comprising 37 gastropods and 54 bivalves from Manora Island, mangroves and coastal areas along the Karachi coast (Woodward 1856), Ormara, Gawadar and Astola Island, have been documented (Mel-

vill and Abercrombie 1893). New species of molluscs were also reported in the 20th century from Indo-Pakistan (Satyamurti 1956).

Ten molluscan species were reported by the Zoological Survey Department of Pakistan (Ashraf 1969). Despite that, noteworthy contributions to the mollusc diversity have been made during the last few decades, covering different aspects together of reproduction, growth, and population dynamics of bivalves, including oysters (Siddiqui and Mustaquim 1988; Siddiqui 2005); mussels (*Perna viridis*) (Barkati and Ahmed 1974); rock borer (Barkati and Asif 1984); and hard clam (Barkati and Khatoon 1994). Formerly Iqbal (2014) reported 273 gastropods and 160 bivalves from the Pakistan coast and concluded that the Balochistan coast is extremely diverse concerning the molluscan community (Aslam *et al*. 2020).

In recent times, *P. viridis* was supposed to be geographically isolated from other *Perna* species and subsequently, morphological differentiation was of least concern. The most accurate way to distinguish between the different species of *Perna* is through molecular studies. Mitochondrial cytochrome c oxidase subunit I gene (COI) evolved slowly enough for DNA studies but fast enough to perfectly determine changes among closely-related species (Hebert *et al*. 2003).

mtDNA markers are currently used broadly to spot differences amongst species, populations or individuals. Because of the fast sequence evolution and maternal non-recombining nature of animal inheritance, mitochondrial genes are being extensively used as an influential tool in species identification and phylogeny. Furthermore, the COI sequence is fairly well conserved within species and is therefore now being broadly used in invertebrate taxonomy (Baldwin 1996; Yu *et al.* 2003; An *et al.* 2005). The COI seems to hold a larger series of phylogenetic indications than other mitochondrial genes and the evolution of this gene is speedy enough to let discrimination of closely allied species (Hebert *et al*. 2003; Divya *et al*. 2009).

For confirmation of the species' identity, DNA barcoding was done and we concluded that this species is *P. viridis*. To our knowledge, no previous bar-code-based identification is available of Pakistani mussel species.

2 | METHODOLOGY

2.1 Samples Collection

Fifteen individuals of *P. viridis* were collected from Clifton (24°48′12.59″N 67°01′13.80″E) by random handpick method and then they were brought to the laboratory in a cool box. The shells were cleaned to remove any external material. Species morphological identification was done using (Peter Dance 1974; Bosch *et al.* 1995). The morphology of the *Perna viridis* is shown in Figure 1. Shells were opened and their soft tissues from the valves were removed, labelled and preserved in an Eppendorf containing 95% ethanol at –20°C for further analysis.

FIGURE 1 The morphological and anatomical structure of *Perna viridis*.

2.2 DNA isolation

The phenol-chloroform method (Sambrook *et al*. 1989) was used to isolate total genomic DNA from muscle tissue.

2.3 PCR amplification and gel electrophoresis

The sequences of a fragment of DNA were obtained by using a set of universal primers (Folmer *et al.* 1994) COX1 (LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′) and COX1 (HCOR2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). A fragment of the 490 bp sequence of cytochrome oxidase subunit 1 was used to identify the *P. viridis*. Polymerase chain reaction (PCR) was performed in a 25 µl volumes reaction, containing 100 mg of template DNA, 1 µM primers (10 µM each of each), 0.25 µLof Taq polymerase (5 µU Ml*1) 2.5 µL dNTP (2.5 mM each), 2.5 µL10 X buffer, 2 µL $MgCl_2$, (20 mM). Reactions conditions were as follows: after an initial denaturation at 94°C for 5 min, then 35 cycles were followed at 94°C for 30 s, 50°C for 30 s and final elongation at 72°C for 7 min. The PCR successful amplification was then examined on the electrophoresis machine (1 % agarose gel with ethidium bromide (stain))

2.4 Sequencing and construction of phylogenetic tree

PCR products were subsequently sequenced with the Sanger sequencing method. The sequences are then aligned, edited and deleted whether there are stop codons or not by using software BIOEDIT and MEGA 6 (Tamura *et al*. 2013). A phylogenetic analysis of the mitochondrial cytochrome c oxidase subunit 1 gene (mtDNA COI) was performed by bootstraps 1000 replicates using the Kimura-2-parameter model to create a Neighbour-Joining tree (Kimura 1980). The genetic result of the *P. viridis* species is compared with various species of *Perna* with reference GenBank samples.

3 | RESULTS AND DISCUSSION

Blast results revealed that our sample is 98.7% similar to several Genebank samples (OL362212) under accession numbers MH664002, KU743182, KP892924 and MW722974 respectively. These sequence submissions originated in Kerala (India), Kadiapatinam (India), Zhejiang (China) and Kerala (India). The findings revealed that it is a *Perna viridis* species that clustered together with members of the Mytilidae family (Figure 2). The graphic blast visualization from the Kabalmoo is shown in Figure 3. The evolutionary divergence was minimal (3.8%) among the sequences of India (Accession numbers: MH664002, KU743182, MW722974, JF520794 and MN119648) and Pakistan whereas the considerable (4.4%) divergence was denoted (Figure 4) between the sequence from Pakistan and Australia (Accession number DQ343589).

The phylogenetic tree was constructed with the 12 similar sequences obtained from the GenBank database, the accession numbers are shown in Figure 2. The NJ tree revealed that the species from Pakistan is distinct, however, the rest of the sequences clustered together with two distinctions of two sub-clades (Figure 2) therefore, it is necessary to have extensive future studies on population genetics.

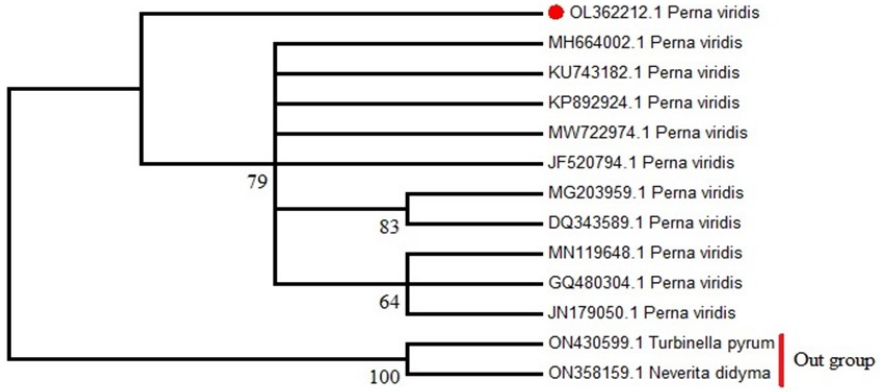

FIGURE 2 Neighbour-joining tree showing distinguished clades of *Perna viridis* species. The red symbol is showing a sequence from Pakistan.

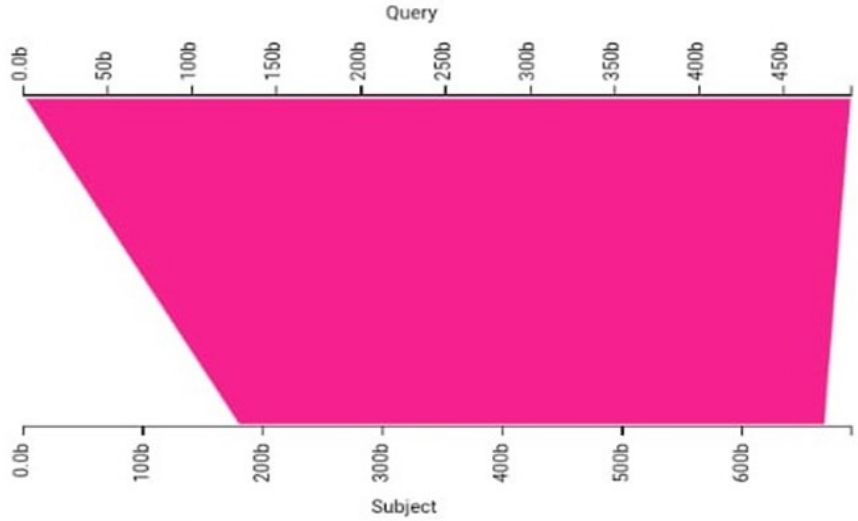

FIGURE 3 Graphic blast visualization of *Perna viridis* showing the results from Kabalommo (source: https://kablammo.wasmuthlab.org).

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	
1		98.7	98.7	98.7	98.7	98.7	98.6	98.6	98.5	84.0	37.6	71.8	71.5	1
2	3.8		100.0	100.0	100.0	100.0	99.9	99.9	99.9	85.4	39.0	72.3	71.8	2
3	3.8	0.0		100.0	100.0	100.0	99.9	99.9	99.9	85.4	39.0	72.3	71.8	3
4	3.8	0.0	0.0		100.0	100.0	99.9	99.9	99.9	85.4	39.0	72.3	71.8	4
5	3.8	0.0	0.0	0.0		100.0	99.9	99.9	99.9	85.4	39.0	72.3	71.8	5
6	3.8	0.0	0.0	0.0	0.0		99.9	99.9	99.9	85.4	39.0	72.3	71.8	6
7	4.0	0.2	0.2	0.2	0.2	0.2		100.0	99.9	85.3	38.9	72.3	71.8	7
8	4.0	0.2	0.2	0.2	0.2	0.2	0.0		99.9	85.3	38.9	72.3	71.8	8
9	4.2	0.4	0.4	0.4	0.4	0.4	0.2	0.2		85.2	38.8	72.2	71.8	9
10	4.2	0.4	0.4	0.4	0.4	0.4	0.6	0.6	0.8		49.1	78.3	78.7	10
11	4.4	0.6	0.6	0.6	0.6	0.6	0.8	0.8	1.0	0.6		31.2	32.4	11
12	65.1	62.3	62.3	62.3	62.3	62.3	62.3	62.3	62.9	58.2	59.4		89.1	12
13	59.2	57.1	57.1	57.1	57.1	57.1	57.1	57.1	57.6	54.3	53.4	23.2		13
	1	2	3	4	5	6	7	8	9	10	11	12	13	

Divergence

OL362212.1
MH664002.1
KU743182.1
KP892924.1
MW722974.1
JF520794.1
MN119648.1
GQ480304.1
JN179050.1
MG203959.1
DQ343589.1
ON430599.1
ON358159.1

FIGURE 4 Showing the similarity (above diagonal) and distance (%) below diagonal. *Perna viridis* (OL362212) is from Pakistan. The details of the remaining accession numbers can be seen in Figure 2.

Perna viridis is one of the potential mussels for commercial cultivation in tropical countries such as Indonesia, Malaysia, Thailand and other Asian countries. It is cultivated because of its nutritive value as a cheap source of animal protein for human consumption (Monirith *et al.* 2003; Noor *et al.* 2019).

Globally bivalve mussels constitute a very important component of hard bottom communities in coastal waters. Among them, mussels are a very successful group that often produces large amounts of biomass in rocks, jetty piers, navigation buoys etc. In the warm coastal waters off the coast of the Indo-Pacific regions, *P. viridis* and related species (*P. perna* and *P. canaliculus*) have been reported as important species and therefore, a good amount of work has already been done on them (Lee 1988; Cheung 1993; Rajagopal *et al.* 2006).

In Pakistan several researchers have worked on the species (e.g. Fatima *et al.* 1985; Barkati and Choudhry 1988). *Perna viridis* is a fast-growing mussel species found on beaches and creeks of the coastal belt of Sindh province Pakistan. Many researchers have discussed the effects of various environmental factors on mussels such as temperature (Lutz 1980; Mason 1991), salinity (Bardach *et al.* 1972; Ahmed *et al.* 1982) and food supply (Hickman 1992; Awan *et al.* 2012).

5 | CONCLUSIONS

The bar-code-based identification re-confirmed *Perna viridis* and it is suggested to have its population genetics for a better understanding of its diversity and stability.

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CONFLICT OF INTEREST

The author of this article declares that there is no conflict of interest.

AUTHORS' CONTRIBUTION

As conducted the experiments and analysed data, FI collected samples and participated in conducting experiments, SK generated funds, SR and MS edited the manuscript, FM designed experiments and wrote the article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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