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Short Communication

DNA Bar-Coding Based Identification of *Neverita didyma* (Roding 1798) and its Phylogeny

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AS collected samples, conducted experiments, data analysis. QI participated in the collection, typing, and reviewing. MS revised the manuscript. FM designed an experiment, generated funds, and wrote the article.

Key words
DNA barcode, Gastropods, Littorinimorpha, Naticidae, Pakistan

ABSTRACT

The Phylum Mollusca's class Gastropoda contains a diverse range of organisms that live in several environments. *Neverita didyma* is a commercially important snail that is also known as the moon shell or bladder moon snail. Its molecular identification as *N. didyma* was confirmed in this investigation. The current study's sequence has been submitted to NCBI under the accession number ON358159. It showed 91.36% similarity with the sequence under accession number NC046594. Neighbour- joining tree revealed that *N.didyma* from Pakistani coastal waters is distinctly clustered with other species of *N.* didyma. The present molecular-based confirmation of this species will be helpful for taxonomists, and biodiversity monitors.

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Gastropods live in several environments, including terrestrial, freshwater, and marine environments (Loker, 2010). During low tide, a variety of gastropods can be found along the coast. The gastropods in the marine environment are Euryhaline and have a diverse colour pattern (Haynes, 2005; Tan and Clements, 2008).

Neverita didyma belongs to the family of Naticidae commonly called moon shell or bladder moon snail. It is a carnivore animal and has sexual reproduction (Lee, 1999; Liu and Sun, 2009). It inhabits in the marine benthic environment. The species is widely distributed along the China coast, Indian Ocean, Madagascar, Mozambique, and South Africa. In China, this species has a significant economic and nutritional value (Liu *et al*., 2013; Zhao *et al*., 2018), yet no commercial use of this species has been reported in Pakistan and its research is also restricted to the work of (Khan and Dastgir, 1971; Tirmizi and Zehra, 1983; Barkati and Rehman, 2005; Kazmi *et al*., 2018; Ghani *et al*., 2018, 2019; Aslam *et al*., 2020). Khan and Dastgir (1971) described it as *N. didyma*, and Barkati and Rehman reported it as *N. lamarkii* in 2005. Until now, no molecular-based identification of this species was reported from this region.

It is significantly important to determine the potential natural resources of the region with an accurate taxonomic approach to understand the diversity of species in the area (Ran *et al*., 2020). Many species are very complex morphologically, therefore, the DNA bar code-based identifications are being appreciated worldwide. The molecular-based taxonomic investigations on Mollusca species from Pakistani coastal waters are limited to the work of (Zafar *et al*., 2016; Humayun *et al*., 2019; George *et al*., 2021).

The cytochrome oxidase subunit 1 (COX1) is considered suitable for investigations of genetic differentiation, taxonomy, and evolution of species. In the present investigation, we focused to confirm its identification and understanding of phylogenetic relationships. The molecular-based confirmation of this species will be helpful for bio-diversity monitors.

Materials and methods

N. didyma individuals were hand-picked at random and transported to the laboratory. The morphological identification was done using the available literature (Bosch *et al*., 1995; Khan and Dastagir, 1971). The tissues

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were taken and maintained at -20 0C for further study.

For PCR amplification of *COX1* gene the phenol-chloroform procedure was used to isolate total genomic DNA from muscle tissue (Sambrook, 1989). A 100mg DNA template, 2.5ul dNtp (2.5mM each), 2.5ul10 X buffer, 2ul Mgcl2, (20mM), 1µl primers for cytochrome oxidase 1 (*COX1*) gene (10M each), and 0.25µlof Taq polymerase (5U Ml*1) were used for amplification. The thermocycler conditions were denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 sec., annealing at 50°C for 30 sec., extension at 72°C for 30 sec.; and a final extension at 72°C for 7 min (Table I). Gel electrophoresis (1 percent agarose gel with ethidium bromide) was done to confirm successful amplification.

The successful PCR product was sequenced. Softwares (BIOEDIT and MEGA 6) were used to do the necessary insertion and deletion (Tamura *et al*., 2013). A neighbour joining tree (NJ) was constructed using the Kimura 2 parameter (K2P) model in MEGA 6 (Tamura *et al*., 2013).

Results

Initially, the individuals of *N. didyma* were identified using morphological characteristics (Fig. 1).

After species confirmation as *N. didyma* using NCBI's Nucleotide BLAST TOOL, a 671 bp fragment of the *COX1* gene was submitted to the National Center for Biotechnology Information under accession number (ON358159). The sequence of the present study showed significant alignment with the mitochondrial complete genomes of *N. didyma* accession numbers as NC046594 and MK548644, the query coverage was 100 percent and the percent identity was 91.36 %. The difference within the Naticidae family was calculated to be 0.109, the pairwise differentiation among the specie of *N. didyma* depicted that the sequence under accession number (NC046594) is nearest to the Pakistani specimen (Table II).

Nine COX1 sequences of related gastropods were downloaded from NCBI and used to build the phylogenetic tree. The accession number of each is presented in Figure 2. The phylogenetic tree based on neighbour joining indicated significant distinction among the Naticidae species. The *N. didyma* from Pakistan showed within-group distinction with 97% bootstrap support (Fig. 2).

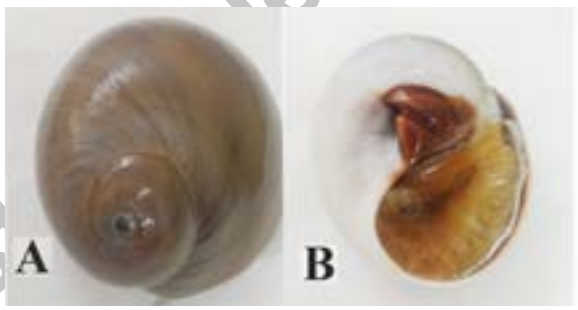

Fig. 1. Photomorph of *N. didyma* collected at Keti Bandar during low tide on the Pakistan coast. The dorsal side of the *N. didyma* is shown in A, and the pod view is shown in B.

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Table I. The set of primer used in this study.

Gene	Primer name and sequence	Tm (°C)	Size (bp)	Reference
Cytochrome oxidase subunit1 (COX1)	LCO1490:5'GGTCAACAAATCATAAAGATATTGG-3'	50	710	Folmer *et al*., 1994
	HCOR2198:5'-TAAACTTCAGGGTGACCAAAAAATCA-3'			

Table II. Estimates of evolutionary divergence between sequences.

Name of species	1	2	3	4	5	6	7	8	9	10
1- *Littorina saxatilis*	-									
2- *Marcia marmorata*	0.367	-								
3- *Meretrix casta*	0.377	0.300	-							
4- *Naticarius hebraeus*	0.185	0.348	0.357	-						
5- *Nerita albicilla*	0.267	0.417	0.397	0.249	-					
6- *Nerita tristis*	0.273	0.407	0.382	0.219	0.183	-				
7- *Neverita didyma (Pak)*	0.214	0.371	0.382	0.137	0.250	0.247	-			
8- *Neverita didyma*	0.204	0.357	0.361	0.115	0.260	0.245	0.115	-		
9- *Polinices didyma*	0.198	0.359	0.356	0.119	0.272	0.242	0.114	0.081	0.081	-
10- *Neverita didyma*	0.194	0.354	0.366	0.100	0.259	0.234	0.097	0.066	0.066	0.059

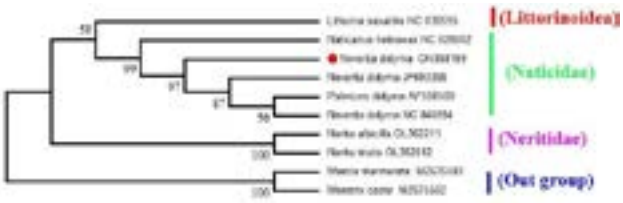

Fig. 2. A neighbor joining tree depicting gastropod species evolutionary relationships. The species obtained from the Pakistani marine environment are shown in red circles.

Discussion

The family Naticidae of order Littorinimorpha has 260 to 270 species worldwide (Huelsken *et al*., 2008) and over 200 species are inhabiting in Indo-Pacific region (Kabat, 1990). The members of the family Naticidae has immense commercial importance as food along the coast of the western Pacific and Southeast Asia (Zhang, 2016; Poutiers, 1998). However, in Pakistan the consumption of seafood is much lesser than in Southeast Asian countries, therefore, less attention was given to certain species of commercial importance. In scientific investigations, the exact identification is difficult based on morphological features (Fontanilla *et al*., 2014). It is said that traditional taxonomy sometimes fails to identify species (Packer *et al*., 2009). The *N. didyma* was first time reported from Pakistan by Khan and Dastgir (1971) later on reported by Barkati and Rehman (2005) as *N. lamarkii*. However, Ghani *et al*. (2018, 2019) and Kazmi (2018) reported this species *as N. didyma*. To our knowledge no early bar-code-based identification was carried out in this species from South Asia. Nevertheless, Wang *et al*. (2019) published the full mitochondrial genome of *N. didyma,* while Zhao *et al*. (2018) analyzed the species' cryptic genetic diversity. The NJ tree of the present study revealed its distinction from the similar species found other than Pakistan.

Geographic location is important in evolutionary divergence, and it may grow as geographic distance increases (Deza and Deza, 2013). Geographic barriers physical, climatic, limiting adaptation, partial dispersal ability and ocean currents have all been caused for limiting gene flow across populations (Riginos and Nachman, 2001; Schmidt and Rand, 1999; Werner *et al*., 2007; Doherty *et al*., 1995; Schmidt and Rand, 1999; Stepien, 1999; Palumbi *et al*., 1997).

Conclusion

The COXI gene has been a valuable method of identifying organisms. *N. didyma*, a member of the Naticidae family is evaluated morphologically and genetically in present research. The CO1 result of this investigation confirmed the *N. didyma* species. To our knowledge, this is the first molecular-based identification of *N. didyma* from the Pakistani marine environment. The outcome of the present investigation will be useful for taxonomists and biodiversity monitors.

Acknowledgment

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Ethics statement

No mandatory ethical approval was needed for the animals studied; however, all the methods were carried out in line with international norms for invertebrates.

Declaration

We declare no conflict of interest.

Data availability statement

The sequences which support this study are openly available in GenBank of NCBI at (https://www.ncbi.nlm.nih.gov/nucleotide).

Statement of conflict of interest

The authors have declared no conflict of interest.

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