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

Amna Sulaman¹, Qurat-ul-Ain Ijaz¹, Muhammad Shafi² and Faiz Muhammad¹*  
¹Center of Excellence in Marine Biology, University of Karachi, Karachi-75270, Pakistan  
²Lasbella University of Agriculture, Water, and Marine Sciences, Lasbella, Uthal.

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.

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...
were taken and maintained at -20°C 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 0.25μl of 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).

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 |
|-----------------|---|---|---|---|---|---|---|---|---|----|
| Littorina saxatilis | - |   |   |   |   |   |   |   |   |    |
| Marcia marmorata | 0.367 | - |   |   |   |   |   |   |   |    |
| Meretrix casta | 0.377 | 0.300 | - |   |   |   |   |   |   |    |
| Naticarius hebraeus | 0.185 | 0.348 | 0.357 | - |   |   |   |   |   |    |
| Nerita albicilla | 0.267 | 0.417 | 0.397 | 0.249 | - |   |   |   |   |    |
| Nerita tristis | 0.273 | 0.407 | 0.382 | 0.219 | 0.183 | - |   |   |   |    |
| Neverita didyma (Pak) | 0.214 | 0.371 | 0.382 | 0.137 | 0.250 | 0.247 | - |   |   |    |
| Neverita didyma | 0.204 | 0.357 | 0.361 | 0.115 | 0.260 | 0.245 | 0.115 | - |   |    |
| Polinices didyma | 0.198 | 0.359 | 0.356 | 0.119 | 0.272 | 0.242 | 0.114 | 0.081 | 0.081 | - |
| Neverita didyma | 0.194 | 0.354 | 0.366 | 0.100 | 0.259 | 0.234 | 0.097 | 0.066 | 0.066 | 0.059 |
Online First Article

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, 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.

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 COI 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

We deeply thank Petroleum Marine Development Committee Malir District, Karachi for support of this study.

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.

References

Aslam, S., Dekker, H., Siddiqui, G., Mustaquim, J., and Kazmi, S.J.H., 2020. Reg. Stud. Mar. Sci., 39: 101415. https://doi.org/10.1016/j.rsma.2020.101415

Barkati, S., and Rahman, S., 2005. Pakistan J. Zool., 37: 17.

Bosch, D.T., Dance, S.P., Moolenbeek, R.G., and Oliver, P.G., 1995. Seashells of eastern Arabia. Dubai Motivate Publishing.

Deza, M.M., and Deza, E., 2013. Distances in biology. In: Encyclopedia of distances (eds. M.M. Deza and E. Deza). Heidelberg, Berlin: Springer Verlag. pp. 387-434. https://doi.org/10.1007/978-3-642-30958-8_23

Doherty, P.J., Planes, S., and Mather, P., 1995. Ecology, 76: 2373-2391. https://doi.org/10.2307/2265814

Fontanilla, I.K.C., Torres, A.F., Canasa, J.A.D., Yap, S.L., and Ong, P.S., 2014. Philipp. Sci. Lett., 7: 104-137.

George, N., Siddiqui, G., Muhammad, F., George, Z., and Lucena, R., 2021. J. Anim. Pl. Sci., 31: 1530-1536. https://doi.org/10.36899/JAPS.2021.5.0355

Ghani, A., Afsar, N., Ahmed, R., Qadir, S., Saleh, S., Majeed, S., and Imam, N., 2019. Pak. J. Mar. Sci.,
Ghani, A., Afsar, N., and Qadir, S., 2018. Int. J. Boil. BiotechH., 15: 163-166.

Haynes, A., 2005. Molluscan Res., 25: 75-84. https://doi.org/10.1159/000086287

Huelsken, T., Marek, C., Schreiber, S., Schmidt, I., and Hollmann, M., 2008. Zootaxa, 1770: 1-40. https://doi.org/10.11646/zootaxa.1770.1.1

Humayun, F., Ayub, Z., Haider, M.S., and Ali, S.A., 2019. Biologia (Pak.), 65: 1-10. https://www.tehqeeqat.org/downloadpdf/8310

Kabat, A.R., 1990. Zool. J. Linn. Soc., 100: 1-25. https://doi.org/10.1111/j.1096-3642.1990.tb01859.x

Kazmi, Q.B., Moazzam, M., and Sultana, R., 2018. Marine molluscan fauna of the Pakistani coastal waters. BCC and T Press, University of Karachi, Pakistan.

Khan, M.D., and Dastagir, S.G., 1971. Rec. Zool. Surv. Pak., 1: 17-130.

Lee, J.H., 1999. Korean J. Biol. Sci., 3: 375-383. https://doi.org/10.1080/12265071.1999.9647511

Liu, H.H., Xu, M.Y., and Wu, C.W., 2013. Fd. Sci., 34: 228-231.

Liu, Q., and Sun, Z.X., 2009. Trans Ocean Limnol., 2: 67-72.

Loker, E.S., 2010. Gastropod immunobiology. IN: Invertebrate immunity, pp. 17-43. https://doi.org/10.1007/978-1-4419-8059-5_2

Packer, L., Gibbs, J., Sheffield, C., and Hanner, R., 2009. Mol. Ecol. Resour., 9: 42-50. https://doi.org/10.1111/j.1755-0998.2009.02631.x

Palumbi, S.R., Grabowski, G., Duda, T., Geyer, L., and Tachino, N., 1997. Evolution, 51: 1506-1517. https://doi.org/10.1111/j.1558-5646.1997.tb01474.x

Poutiers, J.M., 1998. Gastropods. In: FAO identification guides for fishery purposes. The living marine resources of the Western Central Pacific, Vol. 1. Seaweeds, corals, bivalves and gastropods (K.E. Carpenter and V.H. Niemeds), FAO, Rome. pp. 363–648. https://www.fao.org/3/x2401e/x2401e.pdf

Ran, K., Li, Q., Qi, L., Li, W., and Kong, L., 2020. Fish. Res., 225: 105504. https://doi.org/10.1016/j.fishres.2020.105504

Riginos, C., and Nachman, M.W., 2001. Mol. Ecol., 10: 1439-1453. https://doi.org/10.1046/j.1365-294X.2001.01294.x

Sambrook, J., 1989. In vitro amplification of DNA by the polymerase chain reaction. In: Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, N.Y.

Schmidt, P.S., and Rand, D.M., 1999. Evolution, 53: 135-146. https://doi.org/10.1111/j.1558-5646.1999.tb05339.x

Stepien, C.A., 1999. Mol. Ecol., 8: 923-939. https://doi.org/10.1046/j.1365-294X.1999.00643.x

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S., 2013. Mol. Biol. Evol., 30: 2725-2729. https://doi.org/10.1093/molbev/mst197

Tan, S.K., and Clements, R., 2008. Zool. Stud., 47: 481-494. https://doi.org/10.7506/spkx1002-6630-201305047

Tirmizi, N.M. and Zehra, I., 1983. Illustrated key to families of Pakistani marine molluses. Published by Pakistan Science Foundation. pp. 1-46.

Wang, Z., Liu, C., Liu, H., Wang, B., Pang, M., and Zheng, F., 2019. Mitochondrial DNA B, 4: 1545-1546. https://doi.org/10.1080/23802359.2019.1601535

Werner, F.E., Cowen, R.K., and Paris, C.B., 2007. Oceanography, 20: 54-69. https://doi.org/10.5670/oceanog.2007.29

Zafar, F.H.S., Ayub, Z., Begum, S., Siddiqui, G., and Roberts, D., 2016. Mitochondrial DNA A, 27: 2868-2872. https://doi.org/10.3109/19401736.2015.1053133

Zhang, S., 2016. Fauna sinica. Invertebrata, Vol. 56. Mollusca, Gastropoda, Strombacea and Naticacea. Science Press, Beijing.

Zhao, D., Kong, L., Yu, H., and Li, Q., 2018. Conserv. Genet., 19: 275-282. https://doi.org/10.1007/s10592-017-0998-7