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Short Communication

Identification of Nine Gastropod Species with Three New Records using Cytochrome Oxidase Sub-Unit 1 Marker

Qurat-ul-Ain Ijaz[1], Amna Sulaman[1], Dost Muhammad Baloch[2], Muhammad Shafi[2], Faiz Muhammad[1]* and Shahnaz Rashid[1]

[1]*Center of Excellence in Marine Biology, University of Karachi, Karachi-75270, Pakistan*

[2]*Lasbella University of Agriculture, Water and Marine Sciences, Lasbella, Uthal*

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ABSTRACT

Gastropods are ecologically significant and can be found in a wide range of habitats. Their taxonomy is complicated by their perplexing morphological characteristics. Therefore, a molecular based identification approach was used to confirm the variety of gastropod species commonly found on the coast during low tide at rocky shores. In the present investigation, nine species of gastropods were identified using the foresaid modern technique such as (*Thalessa savignyi, Purpura persica, Turbo sparverius, Lunella coronata, Cellana karachiensis, Nerita albicilla, Nerita tristis, Ischnochiton australis, Astralium tentorriformis*). T our knowledge among them three are news records (*A. tentoriiformes, Ischnochiton australis* and, *Nerita tristis*). The highest evolutionary divergence within the family of Nacellidae (0.917) followed by Turbinidae (0.169) and Neritidae (0.100) was noted. The results of this study will be helpful to taxonomists because meager attention was given to molecular- based identification from this region.

The gastropods, a dominant class of Mollusca are ecologically important animals, dwell in variety of environments and have perplexing morphological characteristics (Loker, 2010; Ran *et al*., 2020). Marine gastropods are abundant in intertidal zone and are exposed during low tide. These can be grazers, scavengers, and predators, these have variety of colour patterns, and Euryhaline in marine environment (Mienis, 1973; Vermeij, 1984; Krijnen *et al*., 1996; Haynes, 2005; Tan and Clements, 2008).

The complex morphological characteristics confuse the identification of species, therefore, it becomes intuitive to identify the species accurately to understand the diversity, determine the reference basis of fishery resources and assess potential natural resources of the region (Fontanilla *et al*., 2014; Ran *et al*., 2020) using modern techniques like the DNA barcode-based identifications are appreciated worldwide especially for the animals of similar morphological characteristics, juveniles, or the animals that undergo different life stages (Hausmann *et al*., 2009).

The mitochondrial DNA (mtDNA) are potential markers because of maternal inheritance, fast evolutionary rate. Among mtDNA markers the Cytochrome oxidase subunit 1 has been extensively used to identify the species, construct a phylogenetic relationship, and monitor the population for conservation (Liu and Cordes, 2004).

In Pakistan, with exception of a few studies, the gastropod species are traditionally identified, the molecular-based taxonomic investigations on the subject species are limited to the work of (Zafar *et al*., 2016; Humayun *et al*., 2019; George *et al*., 2021); whereas the gastropods have significant variations in morphological features at different growth stages which creates confrontations to morphologically based identifications (Wilke and Falinowski, 2001). Therefore, present study was designed to identify the molluscan species using molecular based approaches and with support of morphological characteristics.

Materials and methods

Bulleji rocky shore is popular in Karachi division of Sindh. It supports variety of animals including gastropod species because of boulders, flat rocks and tide pools (Afsar *et al*., 2012, 2013; Rahman and Barkati, 2010; Karim *et al*., 2021). Target samples were handpicked and brought to the laboratory. The morphological based

* Corresponding author: balouch_23@yahoo.com
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identifications were completed using the authentic local and international keys (Tirmizi and Zehra, 1982; Bosch *et al*., 1995).

Genomic DNA (gDNA) was isolated from muscle tissue using the phenol-chloroform method (Sambrook *et al*., 1989). The cytochrome oxidase subunit 1 (*COX1*) gene was amplified by polymerase chain reaction (PCR) using universal primers LCO1490:5'~ and HCOR2:5',~ their sequences were GGTCAACAATCATAAAGATATTGG~ 3' and TAAACTTCAGGGTGACCAAAAAATCA~ 3' (Folmer *et al*., 1994). The PCR was performed using 100mg DNA template, 2.5 µL dNTP (2.5mM each), 2.5 µL10 X buffer, 2 µL MgCl2, (20mM), 1 µM primers (10 µM each) and 0.25 µLOf Taq polymerase (5 µU Ml*1). PCR conditions were as: denaturation at 94 °C for 5 min; 35 cycles, each of 94 °C for 30 s, annealing at 50°C for 30 s, and a final extension for 7 min at 72 °C. Successful amplification was verified by visualizing PCR product on 1 % agarose gel with ethidium bromide.

PCR products were subsequently sequenced with the Sanger sequencing method. Necessary insertion and deletion were done using software like Molecular evolutionary genetic analysis (MEGA) and BIOEDIT (Tamura *et al*., 2013; Hall, 1999). The maximum likelihood option was used to construct the phylogenetic relationship among species using MEGA 6 (Tamura *et al*., 2013). The average evolutionary distance analysis was also detected using MEGA 6.

Results and discussion

The samples were carefully identified and found to be nine species belonging to six families. The brief morphological characters are described in (Supplementary Table I), whereas, the photo morphs of these species are shown in Supplementary Figure 1. Each sequence of 640 base pair (bp) fragments of *COX1* was analyzed using NCBI nucleotide blast parameters which confirmed the identification of nine species of gastropods such as *Thallesa savignyi, Purpura persica, Turbo sparverius, Lunella coronata, Cellana karachiensis, Nerita albicilla, Nerita tristis, Ischnochiton australis, Astralium tentorriformis*, among them three are new records (*A. tentoriiformes, Ischnochiton australis* and, *Nerita tristis*) for this region. The sequences of the present study were submitted to National Center for Biotechnology Information (NCBI) under accession numbers (OL373543, OL373542, OL373868, OL372634, OL373916, OL362012, OL362211, OL373913, OL355305), respectively.

1. *Tylothais savignyi* (Deshayes 1844): The blast result revealed 96.73% resemblance with *Thallesa savignyi* (HE584390) (Supplementary Fig. 1A). This species is commonly found on the rocky shores of Pakistani coastal waters and was reported by Tirmizi and Zehra (1982), Nasreen *et al*. (2000), Afsar *et al*. (2012), Ghani *et al*. (2018), Aslam *et al*. (2020) (Supplementary Fig. 1A). Nasreen *et al*. (2000) reported this species as *Thais hippocastanum*.

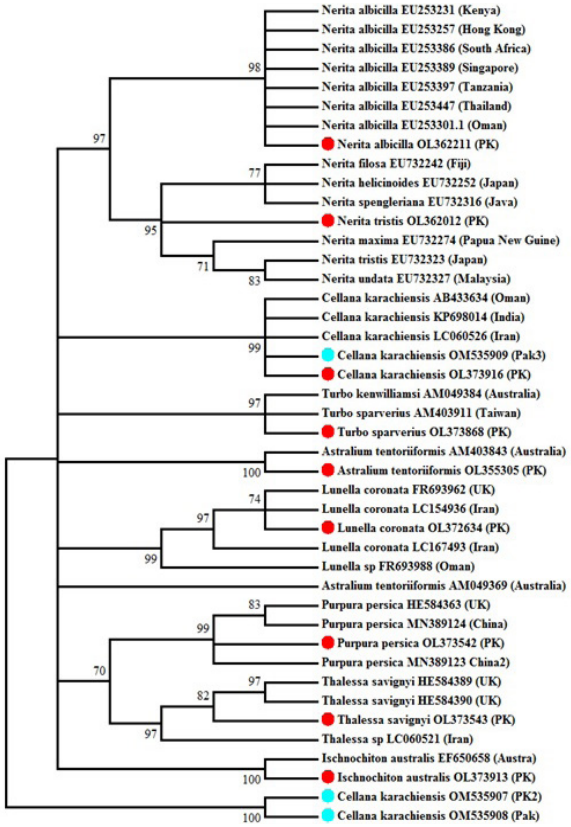

Fig. 1. Molecular phylogenetic analysis by maximum likelihood method. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The red circles are showing the individuals of the present investigation, the blue circles are showing individuals other than this study from Pakistan.

2. *Purpura persica* (Linnaeus, 1758): Nucleotide blast result showed 94.3% similarity with the sequence of *P. persica* submitted under accession number HE584363 (Supplementary Fig. 1B). In Pakistani coastal waters, it was reported as *Thais rudolphi* by Khan and Dastgir (1971) and Afsar *et al* (2012). The *T. rudolphi* is an unacceptable name for this species (https://www.marinespecies.org/aphia.php?p=taxdetailsandid=208648)
3. *Turbo sparverius* (Gmelin 1791): It showed 89.47% identity with the sequence (HE584363) of similar species. Tirmizi and Siddiqui (1984) reported this species as *Turbo* (*Marmarostoma*) *chrysostomus*

(Supplementary Fig. 1C).

4. *Lunella coronata* (Gmelin 1791): It showed 94% affinity with the similar (LC154936) sequence (Supplementary Fig. 1D). Khan and Dastagir (1971) reported it as *Turbo coronatus* while Tirmizi and Zehra (1982) also elaborated this species. Furthermore, Afsar *et al*. (2012) also collected and described this species as *L. coronatus*. Its present accepted name is *L. coronata*.
5. *Cellana karachiensis* (Winckworth 1930): Its blast results revealed 97.39% homology with the sequence submitted under accession number LC060526 (Supplementary Fig. 1E). Ahmed and Hameed (1999) reported this species from Pakistani marine waters as *Patella granularis*. Earlier Zafar *et al*. (2016) described three morphs of this species from Pakistan and the probability to be the same species. The three sequences were submitted to NCBI under accession numbers (OM535909, OM535908 and OM535907) however, it is unclear which sequence belongs to which morph. Poriya *et al*. (2016) also described three morphs named X, Y and Z, different color pattern; but using *COX1* analysis has shown that all three forms belong to one species *C. karachinesis*. The changes in the color pattern were supposed to be due to environmental factors (Samantaray, 1979; Underwood and Creese, 1976).
6. *Nerita albicilla* (Linnaeus 1758): It has a 92.63% resemblance with the sequence submitted under accession of EU253386 (Supplementary Fig. 1F). It was earlier identified using 16S rDNA from Pakistan (Humayun *et al*, 2019), however, the reference sample is not available for comparison. Khan and Dastaghir (1971) also reported it.
7. *Nerita tristis* (d'orbigny 1842): It showed 89.77% affinity with the sequence available under accession number EU732323 (Supplementary Fig. 1G). At present its accepted scientific name in world register of marine species is *Puperita pupa* (Linnaeus, 1767). This is a new record from Pakistani coastal waters.
8. *Ischnochiton australis* (G.B. Soweby II, 1840): Its similarity with another sequence under accession number EF650658 is 99.55%, whereas to our knowledge it has no previous record from the Pakistan coast; therefore it is being considered the first record from the area (Supplementary Fig. 1H).
9. *Astralium tentorriforme* (Jonas, 1845): It showed 99.38% similarity with other sequences of *Astralium tentorriforme* accession number AM403843. This is a new record for this region (Supplementary Fig. 1I).

The average evolutionary distance within the family members of Nacellidae is 0.917 followed by Tubinidae (0.169), Neritidae (0.100), Muricidae (0.034), Melongenidae (0.025) and Ischnochitonidae (0.003). A very high genetic divergence was shown by two sequences of *Cellana karachiensis* (OM535907 and OM535908), the genetic divergence between the sequences of *Cellana karachiensis* (OM535909 and OL373916) the later sequence is part of this investigation.

The phylogenetic tree was constructed using 43 sequences (COX1), sequences other than this study were downloaded from NCBI, and the accession number of each is shown in Figure 1. The species of the family Neritidae were showing two separate clades and the *N. albicilla* from Pakistani coastal waters clustered separately with similar species from other countries and closely related to the Omanian species (Fig. 1). The *N. tristis* showed distinct clustering within the clade of the Neritidae family. *C.karachienesis* showed a close relationship with one of the morphs early reported from the area (Zafar *et al*., 2016), however, two of the morph in the same study separately clustered, and we are unable to describe that which sequence among (OM535909, OM535908, OM535907) these three belongs to morpho1, 2 or 3. Porya *et al*. (2016) also elaborated 3 morphs as a single species C. *karachiensis*. *T. sparverius* clustered with its similar species and showed close relation with the species from Taiwan. *A. tentoriiformis* showed more closeness with Australian found species, whereas *L. coronata* showed an affinity with Iranian and United Kingdom species. *P. persica* showed closeness with a species from China accession number MN389123. *T. savignyi* from Pakistan depicted differentiation among the same species. *Australis* sp. from Pakistan showed an affinity with the species from Australia.

The molecular-based studies from Pakistani waters on the subject are very few and the available data is only restricted to the work of George *et al*. (2021), Ali *et al*. (2018) and Zafar *et al*. (2016). The present study re-confirms the identification of nine species, among them, three species *A. tentorriformes, I. australis* and, *N. tristis* are new records from the region.

Conclusions

This preliminary study would be helpful for Taxonomists to design a future plan for investigation of Mollusca found in Pakistani coastal waters.

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Ethics statement

All the methods were carried in line with international

norms for invertebrate.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20220701070727

Statement of conflict of interest

The authors have declared no conflict of interest.

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