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Research Article

Molecular phylogeny of bivalve families (Arcidae, Chamidae, Margaritidae, Ostreidae, Veneridae) in the Persian Gulf

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Abstract

Bivalvia is one of the least studied classes of the Persian Gulf. A survey and molecular analysis was conducted to determine bivalve species diversity in the Persian Gulf. To the best of our knowledge, this is the first report of barcoding data on bivalves of the Persian Gulf. We examined 40 individuals representing 8 species, 6 genera and 5 families. We collected samples from Hengam, Larak and Qeshm Islands and Bandar Lengeh in Persian Gulf, Iran. After DNA extraction, mitochondrial 16S ribosomal DNA (16SrDNA) and Cytochrome Oxidase subunit I were amplified by polymerase chain reaction (PCR). Based on obtained 16SrDNA and COI gene sequences and maximum parsimony, neighbor joining and maximum likelihood trees of these genes there was no overlap between maximum Kimura 2- parameter distance among conspecifics. Most species formed agglutinate sequence units with a small amount of changes. Eventually, comparison of the 8 selected studied species with metadata from India, Brazil, Japan, China, and America exposed that these species in Persian Gulf are classified in sister clades with high bootstraps except *Pinctada*. Since there is not much work on bivalves identification in the Persian Gulf, larger sampling and more research is needed to investigate mollusc diversity in this area.

Keywords: Bivalvia, Persian Gulf, 16SrDNA, DNA sequencing, COI

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Introduction

One powerful use of molecular characters in systematics is to examine basal divergences for the groups which morphology could not identify in species level. Many scientists have used sequence data to investigate the relationships between animal phyla and the classes within them (Philippe *et al*., 1994; Boore *et al*., 1995; Halanych *et al*., 1995; Winnepenninckx *et al*., 1994). Of the protostome phyla, mollusca are among the most ancient and diverse classes. Living representatives of this phylum are usually divided into seven classes. Bivalves and gastropods are the most common classes in which most species are placed in the modern fauna (Halanych *et al*., 1995). To date, molecular investigations of the molluscs have focused primarily on relationships of other phyla to Mollusca, demonstrated by the classes Gastropoda, Bivalvia, and the Polyplacophora, the latter being considered as basic (Canapa *et al*., 2000). Bivalvia is a class of marine and freshwater molluscs that have laterally compressed bodies enclosed by a shell consisting of two hinged parts. The first bivalve fossils are found in Lower Cambrian sediments. But changes were not found in the fossil records both taxonomically and ecologically until the Lower Ordovician. These changes continued non-stop throughout the Phanerozoic, with relatively small reductions during the end-Permian and end-Cretaceous extinction incidents. Gradually diversified and expanded molluscs species became dominant in most marine ecosystems (Waller, 1998).

Approximately, 20,000 species of marine bivalves exist worldwide, so this class offers a rich diversity of life (Pearse *et al*., 1987). Following that, studies in the Persian Gulf continued by Melvill (1904), Smythe (1972) and Hosseinzadeh Sahafi *et al*. (2001). Most of these studies have focused on identification of bivalves.

According to the available literature, in the Arabian side of the Persian Gulf some of the molluscs including bivalves are studied by a small number of conchologists (Basson *et al*., 1977).

Mollusca with almost 100,000 species organize the second largest animal phylum (Barnes *et al*., 2009). Many mollusc species are used as bioindicators (Astani *et al*., 2012; Saeedi, 2012). In terms of biodiversity and ecology, regardless of great variety in Persian Gulf molluscs, little is known about subtidal species. Some studies on molluscs of the Iranian coasts of Persian Gulf are conducted but are mostly centralized on specimens greater than about 5 mm in size, therefore small species are often neglected (Tadjallipour, 1974;

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Rezai Marnani *et al*., 1994; Hosseinzadeh Sahafi *et al*., 2001; Nassaj *et al*., 2010; Asgari *et al*., 2012). Morphological studies are sometimes unable to perform reliable species identification; therefore molecular studies are necessary to be done (Ardura *et al*., 2010).

In the past, species were identified primarily on the basis of morphology, a main problem here was that the border line between intra-specific variation and inter-specific morphological similarities were sometimes unclear (Kyle and Wilson, 2007). Accurate and relatively simple identification of species is based on the nucleotide sequence of widely used species-level, usually a short DNA fragment. DNA barcoding is introduced in a bold decision to overcome some of these shortcomings (Hebert *et al*., 2003). Sometimes, short sequences of different markers such as a mitochondrial or nuclear target gene confirm the COI sequence data (Monaghan *et al*., 2006; Sonnenberg *et al*., 2007). These findings allow barcoding tools to be used not only for species identification, but also for biodiversity related issues. After two hundred years of morphology based taxonomic studies, species are now identified in a more effective molecular method (Waugh, 2007). In addition to systematics, such protocols can potentially be applied to important subjects in ecology, conservation and issues related to economy (Armstrong and Ball, 2005; Markmann and Tautz, 2005; Savolainen *et al*., 2005; Smith and Fisher, 2009). The phylogenetic reconstruction of a bivalve family (Pectinidae) based on mitochondrial 12S and 16SrRNA and nuclear histone H3 sequence study (Puslednik and Serb, 2008), was quite in contrast with the then morphological hypothesis of Pectinid evolution (Waller, 2006). An important difficulty in Pectinidae systematics remains the evolutionary relationships of the subfamilies and major tribes (Puslednik and Serb, 2008). Various tools, allowing phylogenetic investigation of RNA and protein sequences at the sequence structure level, are developed (Jow *et al*., 2002; Smith *et al*., 2004; Seibel *et al*., 2006). DNA barcoding is introduced as a faster and more accessible method for species identification (Hebert *et al*., 2004; Blaxter *et al*., 2005; Kress *et al*., 2005; Saunders, 2005). The feasibility of identifying species by DNA barcodes is related to the sequence variation among living groups. Bivalvia is a great and diverse class among animals, but little is known about molecular level variations within this class of molluscs (Smith *et al*., 2004). It is

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estimated that only half of the existing species of molluscs are studied (Coleman, 2007). Additionally, dominance of mollusc species is grossly underestimated in ecological surveys (Bouchet *et al*., 2008). The incidence of secret species also makes identification more difficult. A quicker and easier way for species recognition would therefore be useful for molecular level identification of bivalves (Schander and Willassen, 2005).

Materials and methods

Marine molluscs were sampled from several sites in Bandar Abbas, Bandar Lengeh, Qeshm, Larak and Hengam Islands in Persian Gulf, during May and July 2015 by wading and scuba diving (Fig. 1). The stations and number of replicates are shown in Table 1.

All specimens were kept in refrigerator of agriculture research center. Species were identified based on conchology. DNA was extracted from living bivalves or frozen somatic tissue (Barber *et al*., 2006). Three DNA extraction methods were used for DNA extracting. DNA was extracted with a DNeasy tissue extraction kit from CINAGEN and MBST kit, using the tissue protocol as recommended by the manufacture. A piece of tissue (150-200mg) was removed and placed in 850µl extraction buffer (Tris–HCl, EDTA, SDS, NaCl) and 5 µl proteinase K was added into a micro centrifuge tube, then incubated in water bath at 55°C for 12 hr (overnight). Then Phenol chloroform isoamyl alcohol (25:24:1) was added into the tube and inverted 5-6 times (Selig *et al*., 2008). After centrifuge, the same steps were repeated two times. NaCl and cold absolute ethanol was added and was kept in -20°C overnight. After spin and pouring out the ethanol, the DNA pellets stayed at the bottom of the tube under the following thermal profile: 94°C for 10 min; five cycles of 94°C for 45 s, 45°C for 45 s, 72°C for 45 s; 40 cycles of 94°C for 45 s, 51°C for 45 s, 72°C for 45 s; 72°C for 10 min (Gil, 2007). Each reaction mixture contained 25 µL of 10% Trehalose, 5 µL of 10·PCR buffer, 2 µL of 50 mM MgCl2, 1 µL of 10mM dNTP mix, 0.5 µL of each primer (10pmol), 1–2 µL of DNA and 1–2 U *SmarTaq* DNA polymerase (Cinnagen Tehran, Iran). Those were also identified in our study. Deionized water was added to obtain a reaction volume of 50 µL (Gil, 2007). PCR products were visualized on 1% agarose gels. Sequencing was performed on samples that produced a single band.

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Figure 1: Map of sampling locations in the Persian Gulf.

Table1: Sample sites, location and molecular identification of each specimen.

Code	Sampling Location	Map Coordinates	Sampling Date	Species Molecular Identification
A	Hengam Island	$26^0 40' 19.67^0$N-$55^0 51' 42.16^0$E	24.06.2015	*Saccostrea cucullata*
B	Larak Island	$26^0 53' 16.36^0$N-$56^0 22' 11.25^0$E	15.07.2015	*Crassostrea virginica*
C	Qeshm Island	$26^0 54' 38.85^0$N-$56^0 13' 44.08^0$E	25.07.2015	*Paratapes undulatus*
D	Bandar Lengeh	$26^0 33' 26.95^0$N-$54^0 54' 11.83^0$E	14.08.2015	*Pinctada imbricata radiata*
E	Bandar Lengeh	$26^0 33' 26.95^0$N-$54^0 54' 11.83^0$E	14.08.2015	*Pinctada imbricata fucata*
F	Qeshm Island	$26^0 54' 38.85^0$N-$56^0 13' 44.08^0$E	25.07.2015	*Barbatia obliquata*
G	Larak Island	$26^0 53' 16.36^0$N-$56^0 22' 11.25^0$E	15.07.2015	*Chama* sp.
H	Hengam Island	$26^0 40' 19.67^0$N-$55^0 51' 42.16^0$E	24.06.2015	*Saccostrea scyphophilla*

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A band of almost 500 bp in addition to the band of expected size was observed in a few cases. In this condition, DNA in the 1000 bp band was removed using a gel extraction kit (Fermentas International Inc., ON, Canada) and sequenced. Sequencing was carried out using the ABI Big Dye terminator chemistry and an ABI prism 3700 instrument, Applied Biosystems, Korea (Wolf *et al*., 2008). To further query existence of cryptic species flagged by COI sequences, 16srDNA fragments were used. For amplification of the 500 bp fragment encoding 16srRNA, the 16Sar-5 and 16Sbr-3 primers were used (Ivanova *et al*., 2007).

Barcode sequence analysis

Chromatograms were first examined visually to avoid reading mistakes, sequencer software (Technelysium, Australia) was used. Sequences were then aligned in Mega 6.0 (Tamura *et al*., 2007). Fasta alignment was checked onto the NCBI website. Computing of Kimura 2-parameter (K2P) distances (Kimura, 1980) and output of neighbor-joining (NJ) tree (Blaxter *et al*., 2005) of K2P distances were done using the BOLD Management and Analysis System. Computing K2P intervals and output of NJ tree of 16srDNA sequences were done using MEGA 6.0 (Nassaj *et al*., 2010).

Meta data analysis were done using BOLD management and analysis system to contrast 16s sequences of selected species of the present study with conspecifics from Australia, India, China and South Africa. Two types of dividing values were calculated. A common global divergence value was calculated for each species which was the average of all pairwise intervals of sequences depending to the identical species regardless of location of origin. Territorial divergences were computed as mean interval of all con special sequences from the same position (Selig *et al*., 2008). A number of experimental molecular operational taxonomic units (MOTUs) were derived based on patterns of sequence clustering and grade of variegate for each nominal species. NJ tree covering for all sequences was built. Finally degrees of observed divergence at different taxonomic levels of samples of the present study were compared with those of BOLD project encompassing broader geographical ranges (Blaxter *et al*., 2005).

Results

In this research 5 bivalve families including Arcidae, Chamidae, Margaritidae, Ostreidae and Veneridae were studied. Also intra specific variation and inter specific divergence in six genera of marine bivalves was investigated. 16srRNA from species *Pinctada imbricata radiata, Pinctada imbricata fucata* (family Margaritidae), *Paratapes undulatus* (family Veneridae), *Barbatia obliquata* (family Arcidae), *Chama sp.* (family Chamidae), *Saccostrea cucullata, Saccostrea scyphophilla, Crassostrea virginica* (family Ostreidae) was

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sequenced to compare divergence within and between the species. The resulting phylogenetic tree for the aligned mitochondrial 16s ribosomal DNA sequences is shown in "Figure 2".

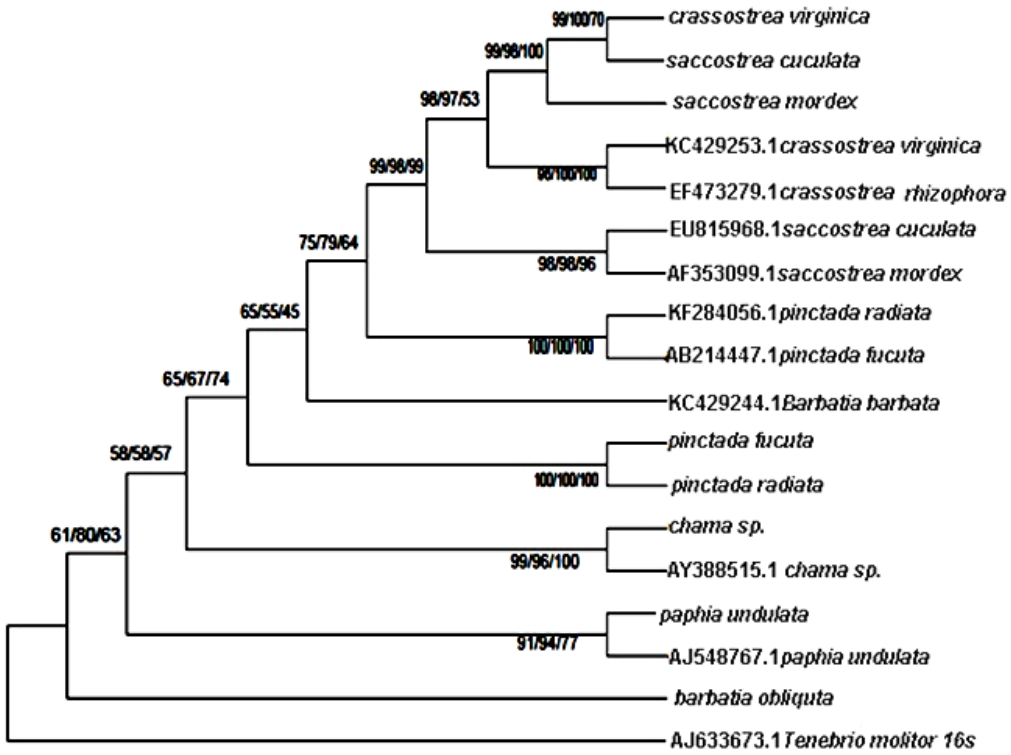


Figure 2: Neighbor joining, maximum parsimony and maximum likelihood tree for specimens in the Persian Gulf from mitochondrial 16S ribosomal DNA sequences. First numbers on left show maximum likelihood, second numbers in middle show neighbor joining, third numbers on right show maximum parsimony.

In the current study maximum parsimony (MP), neighbor joining (NJ) and maximum likelihood (ML) trees based on 16srRNA data were used for the families of bivalvia. In this study *Paratapes undulatus* from Persian Gulf with *Paratapes undulatus* from Northern Atlantic Ocean were classified in sister groups (MP:77 ,NJ:94,ML:91). The Iranian species of *Chama* sp. with (MP: 100 bootstrap, NJ: 96, ML: 99) was similar to the same species from the Western North Atlantic and both were classified in the same sister group and classified in separate clade with high bootstrap from *Pinctada* clade and classified in almost similar sister groups. Iranian *Pinctada imbricata fucata* from Bandar Lengeh were totally different from *Pinctada imbricata fucata* from Australia and Japan, so they were classified in two different clades. In this research *Crassostrea virginica* and *Saccostrea cucullata* of Persian Gulf (MP: 70, NJ: 100, ML: 99) were classified in one sister clade. They were similar to *Saccostrea scyphophilla* from Persian Gulf with MP: 100, NJ: 98, ML: 99. These species were similar to the same species from Brazil and

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were relatively close to each other; so they were located in the same sister clade. In current study, *Saccostrea cucullata* from South China Sea with *Saccostrea scyphophilla* from the Indo-West Pacific were classified in a sister groups (MP: 96, NJ: 98, ML: 98). High quality photographs of bivalves are shown in Figure 3.

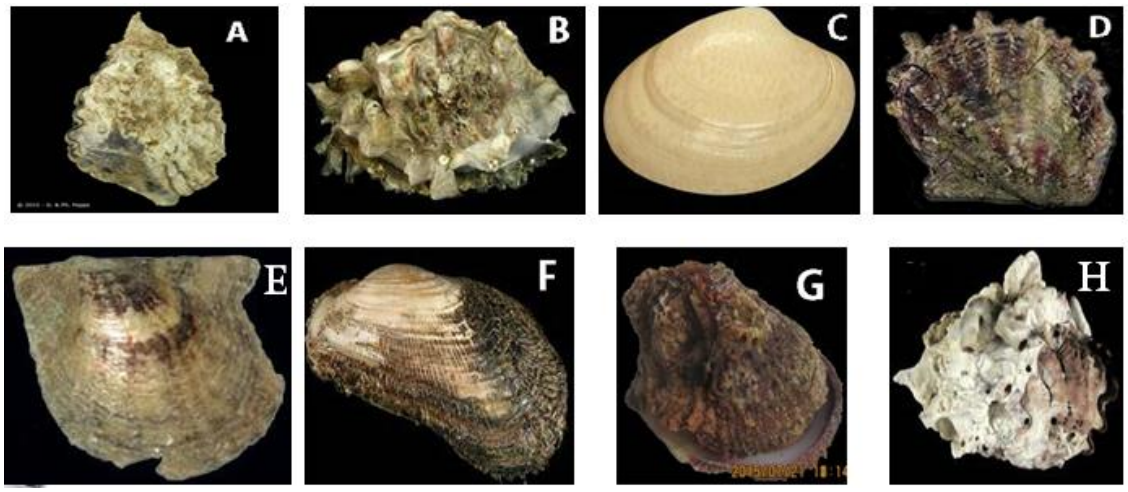


Figure 3: ***In situ*** **images of bivalve specimens collected in this study; A:** ***S. cucullata*****, B:** ***C. virginica*****, C:** ***P. undulatus*****, D:** ***P. imbricata radiata*****, E:** ***P. imbricata fucata*****, F:** ***Barbatia obliquata*****, G:** ***Chama sp*****., H:** ***S. scyphophilla.***

Discussion

Reduction in aquatic resources in many parts of the world stresses the importance of fisheries and aquaculture resources management. Before any kind of action, study and determining the genetic structure of valuable species via molecular methods is necessary. This is very important in sustainable utilization programs of marine reserves, aquatic industry and breeding programs (Lin *et al*., 2002). The data related to DNA sequence is used to determine ancestral relations in development of most animals. These data are less influenced by selection index and phylogenetic relations; as a result the real genetic structure is demonstrated (Saavedra and Peňa, 2006).

The reason for this difference can be special condition of the geographical area, i.e. existence of estuaries and mangrove forests. These factors can affect genetic diversity of the area. For example mangrove forests are important nursery and feeding areas for larva of crustaceans and molluscs (Barber *et al*., 2006). One of the main causes of isolation of species is geographical distance which can affect the genetic distance due to physical and natural barriers which can reduce genetic transfer (Bisby *et al*., 2010). It is reported that animal immigration behavior is an important factor on the transfer of gene and change in population structure. Mollusca often select nests in different areas in their life cycle and for the completion of

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their life cycle have to move to different areas (Lin *et al*., 2002). Geographic movement of bivalves into each area is related to the environmental conditions, especially due to variability of salinity, or other important factors such as the seabed and hydrological conditions, existence of estuaries and also dispersion of the bivalves which can have an effect on gene transfer (Weersing and Toonen, 2009).

In conclusion, results obtained from the present study, as the first study on diversity and genetic structure of bivalvia in coastal waters of Hormozgan using mt DNA sequencing 16srRNA, demonstrated that molecular markers, especially mt marker, can be a suitable parameter for separation of aquatic populations such as bivalvia. These kinds of markers can be used in the study of species and related common ancestor (Puslednik and Serb, 2008). In this study haplotype diversity, in a limited extent, and high nucleotide diversity is found in species of different studied areas (Selig *et al*., 2008).

In order to complete this research further studies like application of mitochondrial gene with other methods such as microsatellite, RFLP, AFLP are needed. Because of intensive reduction of populations and due to reduced genetic diversity, evaluation of this study and the application of results of further studies can help in rebuilding the population of these valuable species (Asgari *et al*., 2012).

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