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Molecular study of mollusca in Bandar Lengeh using *18S rRNA* gene

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Abstract

Mollusca are one of the most diverse animal phyla whose phylogeny is considered as a controversial subject. Although some groups were traditionally classified as mollusca, they need to be identified again. High diversity of mollusca has created considerable taxonomic problems and despite their importance in marine biota, deep phylogenetic relationships of mollusca have scarcely been investigated. The aim of this study was to determine genetic diversity and differences between mollusca species in waters of Bandar Lengeh (Persian Gulf) of Iran. A clone library of the ribosomal small subunit RNA gene (*18S rDNA*) in the nuclear genome was constructed by PCR, and then, after examining the clones, selected clones were sequenced. The determined clone sequences were analyzed by a similarity search of the NCBI GenBank database using BLAST. Also, the fixation index (F_{ST}) factor was used in order to measure and understand the genetic differences between studied populations. In this study, seventeen sequences were identified belonging to two classes of Bivalvia and Gastropoda and they were used for phylogenetic analysis. According to the allele frequencies at each locus, F_{ST} value was significant in samples of Bandar Lengeh meaning that migration is in the lowest rate in the studied region. The present research project exhibited that clone library of *18S rDNA* might be accounted as a beneficial tool to identify marine specimens and estimate the actual species diversity in marine environments. Moreover, these findings confirmed the effective role of molecular studies for the identification and taxonomy of species from the natural resources to obtain reliable data.

Key words**:** *18S rDNA*, Clone library, Genetic diversity, Mollusca, Persian Gulf.

INTRODUCTION

Mollusca are one of the most diverse animal phyla. Taxonomically, mollusca are classified in several classes including Caudofoveata, Solenogastres, Polyplacophora, Monoplacophora, Gastropoda, Cephalopoda, Bivalvia, Scaphopoda, Rostroconchia and Helcionelloida. However, two Rostroconchia and Helcionelloida classes are extinct (Iijima *et al.,* 2006; Fontoura-da-Silva *et al.,* 2013; Osca *et al.,* 2014; Prié and Puillandre, 2014; Elderkin *et al.,* 2016; Lopes-Lima *et al.,* 2017; Klompmaker *et al.,* 2019). Mollusca phylogeny is a controversial subject and some groups that have been traditionally classified as mollusca need to be reidentified (Goloboff *et al.,* 2009). High diversity of mollusca has created considerable taxonomic

problems and despite their importance in marine biota, deep phylogenetic relationships of mollusca have scarcely been investigated (Kocot, 2013).

Traditionally, the use of morphological characters was a valid approach to identify species and monitor the species composition of environmental water samples. However, this method had many drawbacks to identify species in environmental samples (Lee *et al.,* 2010). Today, molecular biology has been considered as an effective approach to evaluate the species diversity of environmental samples in the fields of taxonomy, ecology, and oceanography that overcome the limitations of pervious morphological studies. Molecular methods such as the use of microRNAs, small non-coding RNAs, molecular markers and DNA sequencing are used to identify and evaluate diversity in environmental water (Sperling *et al.,* 2009; Rota-Stabelli *et al.,* 2010; Alemzadeh *et al.,* 2014; Haddad *et al.,* 2014). The widespread use of 18S ribosomal RNA data in molecular phylogenetic has presented major information about evolutionary relationships among metazoan phyla (Lee *et al.,* 2010; Meyer *et al.,* 2010). The use of environmental clone libraries can estimate species diversity and cover broad taxonomic groups. In addition, preservation of species information is another advantage of environmental clone libraries (Lee *et al.,* 2010).

In this study, we analyzed the biodiversity of mollusca in environmental water samples in Bandar Lengeh (Persian Gulf) by constructing a clone library of *18S rDNA*. Phylogenetic analyses of the *18S rDNA* sequences were performed to analyze seventeen mollusca species. Compared to the classical methods, the employed molecular technique in the present study reduced both time and cost required for constructing community analysis of aquatic systems.

MATERIALS AND METHODS

Study site and sampling

An environmental water sample was collected in Bandar Lengeh (26°33'N, 54°53'E) on 10 July 2012. The water sample collected from the surface using a 50-cm plankton net with 15-micron mesh pore size and stored at -80 °C for subsequent molecular analyses.

DNA extraction

Total DNA was extracted according to the method outlined by Doyle and Doyle (1987). The sample was suspended in the CTAB extraction buffer (3%CTAB, 0.1 mol L^{-1}Tris-HCl, pH 8.0, 0.01 mol L^{-1} EDTA,1.4 mol L^{-1}NaCl, 0.5% β-mercaptoethanol, 1% PVP). The mixture was incubated at 60 °C for one h, with shaking at every fifteen minutes, and was cooled down to room temperature. Subsequently, one ml of chloroform: isoamyl alcohol (1:24 v/v) was added, mixed for 15 min at room temperature, and centrifuged (Allegra 64R High-Speed Centrifuge, Beckman Coulter) at 12,000 rpm for 10 min. The supernatant was transferred into new tubes and the chloroform:isoamyl alcohol extraction was repeated once more. The supernatant was transferred into a new tube containing an equal volume of ice-cold isopropanol and incubated at –20 °C for 30-60 min. After centrifugation at 12,000 rpm and 4 °C for 10 min, the resulting pellet was washed by 70% ethanol. Finally, the pellet was dissolved in TE buffer. The quality of DNA was checked by electrophoresis on the 1% agarose gel stained with ethidium bromide for the following PCR amplification.

Eukaryotic rRNA gene libraries

Eukaryotic *18S rRNA* gene was amplified with forward primer A (5′-AACCTGGTTGATCCTGCCAG-3′) and reverse primer SSU-inR1 (5′-CACCAGACTTGCCCTCCA-3′) based on the conserved domain region of *18S rDNA* (Lee *et al.,* 2010). A 50 µl PCR reaction volume contained 4 µl of template DNA mix (20 ng), 1 µl of primer mix (1 pmol), PCR master mix (Fermentas, USA) and RNAse-Free Water. Reactions were carried out in an automated thermal cycler (Techne TC-212, UK) with the following conditions: initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. PCR product (50 µl) was pooled and run on 0.8% agarose gel. Then, the amplified fragment with the expected size was excised from the gel and purified using Gel Purification Kit (Bioneer).

Cloning, unique clone selection, and sequencing

PCR products were used to construct one clone library according to the method outlined by Sambrook and Russell (2001). PCR product was ligated into pTG19-T plasmid vector (Vivantis) and subcloned into the competent *Escherichia coli* strain DH5α based on the calcium chloride transformation process. Then several hundred white colonies were selected after growing at 37 °C for 15 h on a Luria–Bertani (LB) plate. White colonies were used as template DNA for colony PCR. The presence of the *18S rRNA* gene inserts in positive colonies were checked by PCR amplification using the same primer set (A and SSU-inR1) employed to evaluate the environmental samples. The colony PCR products were then electrophoresed on a 1.8% agarose gel to evaluate the presence of the inserts. To confirm the presence of the *18S rRNA* gene insert, plasmids

were extracted from bacteria and applied to enzymatic digestion *Bam*HI and *Eco*RI for 12 h at 37 °C and separated by electrophoresis on a 2.5% low-melting-point agarose gel (NuSieve). After screening, selected clones (fifty clones) were cultured again and their recombinant plasmids were isolated and purified using a High Pure Plasmid Isolation Kit (Bioneer). Finally, the fifty plasmid products were sent for commercial sequencing (SinaClon).

Phylogenetic analysis

To determine the first phylogenetic affiliation, sequences were compared with sequences available in the NCBI database and the Ribosomal Database Project using BLAST. The nucleotide sequences with the highest max scores were identified by the BLAST search and selected for data analysis. The *18S rDNA* sequences were aligned using Clustal X ver. 1.8 (Larkin *et al.,* 2007) and then manually adjusted. Phylogenetic trees were constructed using MEGA 4.0 [16]. Also, in this study, 17 alleles from samples collected from Bandar Lengeh (Persian Gulf) and 37 alleles of samples obtained from NCBI were compared. The fixation index (F_{ST}) factor was also used in order to measure and understand the genetic differences between the studied populations.

RESULTS

The objective of this work was to study the molecular phylogeny of Mollusca found in Bandar Lengeh in the Persian Gulf coast. We analyzed one clone library. PCR amplification using the SSU-inR1/A primer pairs produced a DNA fragment of approximately 560 bp (Figure 1A). The presence of the *18S rRNA* gene insert in positive colonies was confirmed by colony PCR (Figure 1B). PCR amplification products containing the correct insert size were digested with 1 U of restriction enzymes *Eco*RI and *Pst*I µl-1 for 6-12 h at 37 °C (Figure 1C) and the selected clones (fifty clones) were sequenced. BLAST searches of the GenBank database was conducted using *18S rDNA* clone library which revealed 17 unique ribotypes among the fifty clones (Table 1). Then, sequences were aligned with *18S rDNA* sequences available in the GenBank. Phylogenetic analyses were conducted to compare affinities and were classified in group of mollusca. All of the species of animal planktons belonged to two classes of *Bivalvia* and *Gastropoda*. All of the determined sequences showed high sequence similarity (Table 1; >97% and 99% coverage) at the level of species and genus. However, ten *18S rDNA* partial sequences exhibited significant identities (more than 99%) and 7 sequences exhibited less similarity with known eukaryotic *18S rDNA*, ranging from 97% to 98%. In the meantime, six sequences (HA/BL16, HA/BL6, HA/BL3, HA/BL14, HA/BL7, and HA/BL8) had different conditions and their sequence similarity and distinctive taxonomic position in the phylogenetic tree

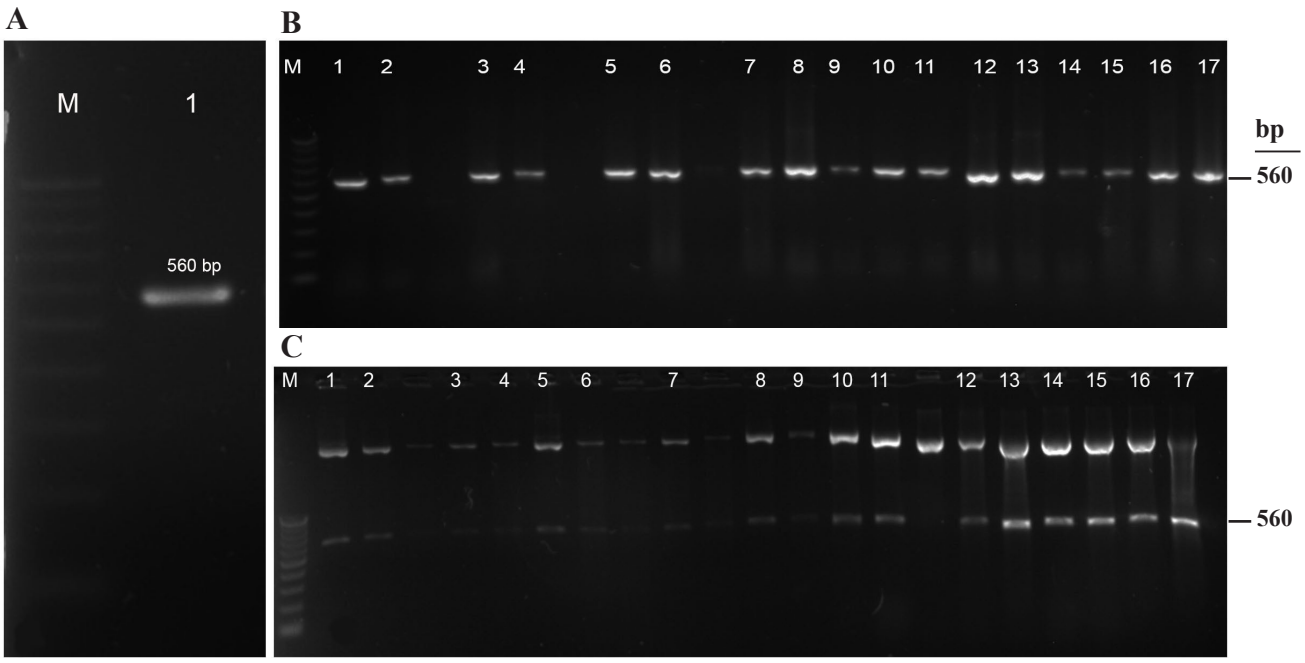

Figure 1. A: Polymerase chain reaction (PCR) products of environmental water samples by agarose gel electrophoresis, **B:** Colony PCR products were directly amplified from positive transformants in the 18S rDNA clone library and **C:** Restriction analysis of recombinant plasmids with *Eco*RI and *Pst*I enzymes. M) molecular weight marker, 1-17) the mentioned clones in Table 1.

Table 1. Clones in the 18S rDNA clone library of the environmental water sample identified based on the results of BLAST search of the GenBank database.

No.	Clones	GenBank accession number	Size (bp)	Taxonomic lineage	Closest known species based on a BLAST search	Accession and similarity index
1	HA/BL1	KC904728	572	Mollusca: Bivalvia	*Brachidontes Variabilis*	AJ389643, Identities=558/565(99%) Gaps=1/565(0%)
2	HA/BL4	KC904731	572		*Brachidontes variabilis*	AJ389643, Identities=560/565(99%) Gaps=1/565(0%)
3	HA/BL5	KC904732	572		*Brachidontes variabilis*	AJ389643, Identities=557/565(99%) Gaps=1/565(0%)
4	HA/BL11	KC904738	564		*Brachidontes variabilis*	AJ389643, Identities=527/533(99%) Gaps=1/533(0%)
5	HA/BL12	KC904739	572		*Brachidontes variabilis*	AJ389643, Identities=552/565(98%) Gaps=1/565(0%)
6	HA/BL13	KC904740	572		*Brachidontes variabilis*	AJ389643, Identities=559/565(99%) Gaps=1/565(0%)
7	HA/BL15	KC904742	572		*Brachidontes variabilis*	AJ389643, Identities=560/565(99%) Gaps=1/565(0%)
8	HA/BL16	KC904743	572		*Brachidontes variabilis*	AJ389643, Identities=544/567(97%) Gaps=5/567(0%)
9	HA/BL17	KC904744	572		*Brachidontes variabilis*	AJ389643, Identities=559/565(99% Gaps=1/565(0%)
10	HA/BL2	KC904729	575		*Pinctada albina*	AB214453, Identities=570/574(99%) Gaps= 574(0%)
11	HA/BL6	KC904733	577		*Mya truncata*	AY570556, Identities=548/564(97%) Gaps=0/564(0%)
12	HA/BL3	KC904730	571	Mollusca: Gastropoda	*Planaxis sulcatus*	HQ833993, Identities=560/572(98%) Gaps=2/572(0%)
13	HA/BL10	KC904737	571		*Planaxis sulcatus*	HQ833993, Identities=565/5672(99%) Gaps=2/572(0%)
14	HA/BL14	KC904741	572		*Planaxis sulcatus*	HQ833993, Identities=557/572(97%) Gaps=1/572(0%)
15	HA/BL7	KC904734	572		*Batillaria zonalis*	HQ833995, Identities=557/571(98%) Gaps=1/571(0%)
16	HA/BL8	KC904735	574		*Serpulorbis imbricatus*	HQ833992, Identities=563/574(98%) Gaps= 5/574(0%)
17	HA/BI2	KC904766	571		*Cerithidea cingulata*	AM932845, Identities=550/550(99%) Gaps=0/550(0%)

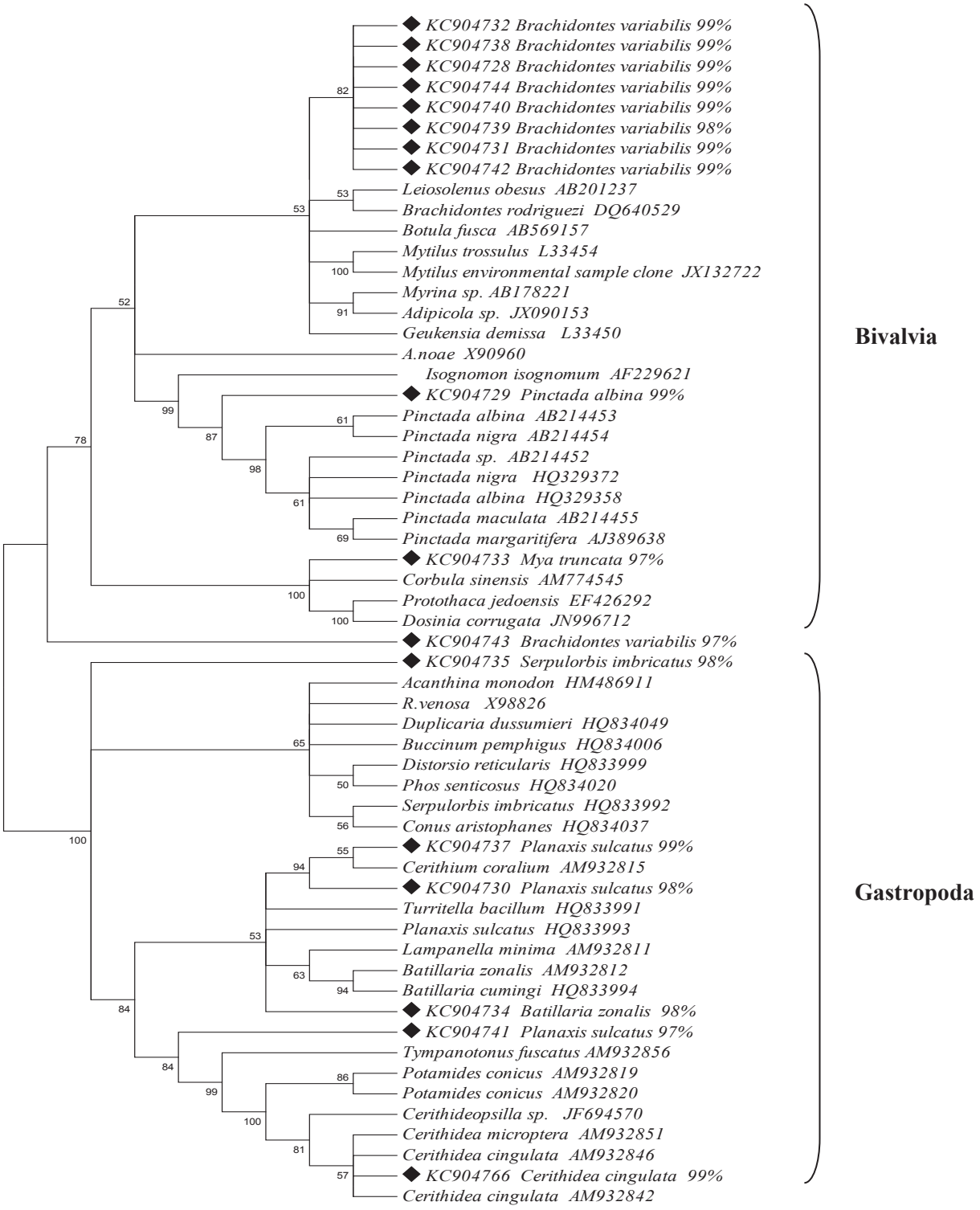

Figure 2. Neighbor-joining tree obtained from the *18S rDNA* of the determined clone sequences. The *18S rDNA* sequences of the related taxonomic groups deposited in the GenBank database were included. Numbers above the branches indicate bootstraps of NJ analysis.

suggested the possibility of hidden biodiversity (Table 1 and Figure 2). The sequences were aligned using Clustal X ver. 1.8 and a phylogenetic tree was constructed to reveal the taxonomic position of the new sequences. The phylogenetic tree was constructed based on the environmental clone sequences compared with those available in the GenBank database using NCBI BLAST (Figure 2).

Table 2. Population pairwise F_{STs}.

Distance method: Pairwise difference		
	samples of Persian Gulf	samples of NCBI
Samples of Persian Gulf	0.00000	
Samples of NCBI	60947	0.00000

According to the allelic frequencies at each locus, F_{ST} value was significant in samples of Bandar Lengeh and this means that migration rates are the lowest in Bandar Lengeh.

Table 3. Molecular diversity indexes. The number and the position of the transition (Displacement bases A and G to each other and C and T to each other) in the Persian Gulf samples are 89 and 78. The number and position of trasversion, in the Persian Gulf samples are 71 and 78.

Statistics	Persian Gulf	NCBI	Mean	SD	
No. of transitions	89	104	96.500	10.607	
No. of transversions	71	78	74.500	4.950	
No. of substitutions	160	182	171.000	15.556	
No. of ts.sites	78	89	83.500	7.778	
No. of tv. sites	78	89	74.500	4.950	
No. of subst. sites	131	131	131.000	0.000	Total: 1864
No. private subst. sites	21	32	26.500	7.778	

According to the allele frequencies at each locus, F_{ST} value was significant in Bandar Lengeh samples meaning that migration rates were the lowest in Bandar Lengeh (Table 2).

Table 3 showed the number and the position of the transition (displacement of bases A and G to each other and C and T to each other) in the Bandar Lengeh samples were 89 and 78, respectively. Also, the number and position of trasversion, in the Bandar Lengeh samples were found to be 71 and 78, respectively.

DISCUSSION

The study of the genetic diversity of small eukaryotes provides useful information about the marine ecology (Yuan *et al.,* 2004; Semmouri *et al.,* 2020). Sequencing of small subunit rRNA genes (*16S rDNA* for Prokaryota and *18S rDNA* for Eukarya) from the clone libraries is a suitable method for the investigation of diversity of microbial communities (Madigan *et al.,* 2009). It is clear that complete sequence analysis of the entire gene is the best option but the use of partial sequence analysis is also able to provide accurate and useful information about genetic diversity (Harris *et al.,* 2004; Valentin *et al.,* 2005). In this study, partial sequences were employed for the structural analysis of the genes.

In the last two centuries, there has been no consensus on the phylogeny of Bivalvia from the many developed classifications. In earlier classification systems, choosing among shell morphology, type of hinges, and gills were used to classify them (Weersing and Toonen, 2009; Ruzzante *et al.,* 2013; Lemer *et al.,* 2016). In May 2010, a new taxonomy of the Bivalvia was published in the journal Malacologia, which is used for a variety of phylogenetic information including molecular analysis, anatomical analysis, shell morphology, and shell microstructure as well as bio-geographic, paleo biogeographic, and stratigraphic information. In such a classification, 324 families were recognized as valid, 214 of which were known exclusively from fossils, and 110 of which occur in the recent past, with or without a fossil record (Meyer *et al.,* 2010; Philippe *et al.,* 2010). In our study, eleven species of Bivalvia were characterized into three different species of *Brachidontes variabilis, Pinctada albino,* and *Mya truncata*.

At all taxonomic levels, Gastropods are second only to the insects in terms of their diversity (McArthur and Harasewych, 2003). Gastropods have the greatest numbers of named mollusca species. However, estimates of the total number of Gastropod species vary widely, depending on cited sources (Chapman, 2009). From the results, we found six species (KC904730, KC904737, KC904741, KC904734, KC904766, and KC904735) related to Gastropods that were found in Bandar Lengeh.

The F_{ST} is a standard factor for populations differentiation based on polymorphism. This factor is

used to measure the total genetic variation of organism in the subpopulation and its comparison with the total genetic variation. This value is always between zero and one. Number one shows complete separation of the two populations and number zero shows that there is no difference between the populations. In fact, the value of zero is indicator of a panmictic population that two populations have free sexual relationship. Also, the value of one indicates the allelic variation among populations so that two populations are completely separated and there is no gene flow between populations (Czarnecki *et al.,* 2008; Holsinger and Weir, 2009; Leaché *et al.,* 2014). Gene flow (also known as gene migration) is the transfer of alleles or genes from one population to another (Avise, 1994).

In relation to F_{ST} values, it has been shown that the values between 0 to 0.05 indicates low genetic differentiation and the values between 0.05 to 0.15 indicate medium differentiations, values between 0.15 to 0.25 indicate high differentiations between populations and the values higher than 0.25 show very high differentiation (Wright, 1978). F_{ST} typically arises below 0.05 which might be thought that the structure of the population is poor but the obtained number does not always reflect the actual population and in most cases, the F_{ST} values do not reach one because the effect of the polymorphism (caused by mutation) dramatically reduces the value of F_{ST} (Wright, 1978; Bhatia *et al.,* 2013). Wright (1978) believes that F_{ST} values less than 0.05 can also show an important genetic differentiation (Bhatia *et al.,* 2013).

Considering the F_{ST} values recorded for the samples collected from Bandar Lengeh, it seems that they have moderate genetic differentiation with more gene flow than other regions from the GenBank samples. One reason for this level of genetic differentiation in Bandar Lengeh might be the hydrodynamic structure of the region (marine currents) between the Strait of Hormuzand and Goiter. It has been documented that genetic flow between regions could be affected by this factor (Ruzzante *et al.,* 1998; Hauquier *et al.,* 2018; Rodrigues *et al.,* 2019). Also, Weersing and Toonen (Weersing and Toonen, 2009) believe that many factors including marine currents and eddy currents can cause prevention of the combination and release of pelagic larvae between regions.

In this case, crossing of marine flow that begin from Strait of Hormuz (Entrance to Strait of Hormuz from East to West and exit of West to East) can combine and increase gene flow between different regions of Bandar Lengeh that can be effective in increasing gene flow observed in samples from Bandar Lengeh (Reynolds, 1993; Azizpour *et al.,* 2014).

Commonly indicators such as heterozygosity are used in assessing genetic diversity and this index can be useful in the study of genetic structure of populations of different species. This index represents a wide range of genotypes in assessing the compatibility of environmental conditions and is affected by many important features such as growth, resistance to diseases and fertility (Beardmore *et al.,* 1997).

Taken together, our findings showed that clone library of *18S rDNA* is a beneficial tool to identify marine specimens and estimate the actual species diversity in marine environments. Moreover, such a method is recommended as an efficient method for phylogenic studies in marine environments. These findings can also be used to study migration patterns among the populations studied and play an important role in studies of relationships between populations.

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