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Polymorphism Analysis of Mitochondrial DNA Control Region of Hawksbill Turtles (*Eretmochelys imbricata*) in the Persian Gulf

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ABSTRACT

In Iranian coastal waters of the Persian Gulf as a result of both direct and indirect human impacts such as uncontrolled or illegal hunting, sea turtle populations are drastically declining. Information on the genetic structure of marine species is essential for stock enhancement programs. To estimate the genetic diversity of the hawksbill turtle populations, the mitochondrial DNA control region was used as a matrilineal marker. The captured turtles (69 samples) were obtained from three different islands in the Persian Gulf. From all the samples DNA was isolated. The primers were selected based on a specific sequence in control region of mitochondrial genome. PCR products were restricted by various restriction enzymes and four different haplotypes observed. Measurement of inter-population genetic diversity and evolutionary distance between genotypes showed a low diversity in mitochondrial genome of hawksbill turtle in the studied regions. Therefore, the similarity between these three populations was significant. Present results provide evidences showing that significant genetic variation was not observed between these distinct populations and there is not even enough evidence to show the separation and diversion in the studied populations in haplotype level.

Key words: Polymorphism, *Eretmochelys imbricata*, Persian gulf, mtDNA

INTRODUCTION

A successful conservation planning depends on understanding key aspects of hawksbill biology together with the degree of isolation among nesting colonies and migratory pathways of juveniles and adults and also the source for foraging populations. The hawksbill turtle (*Eretmochelys imbricata*) is a species of endangered sea turtle belonging to the family Cheloniidae (Meylan and Donnelly, 1999). Marine turtles are specific group of vertebrates and creepers who are living in the Atlantic, Pacific and Indian oceans and the warm seas (Venkataraman and Wafar, 2005). There are two species of marine turtles in the Persian Gulf that include the green and hawksbill turtles (Mobaraki, 2004a; Ross and Barwani, 1995). According to the IUCN report (Cheraghi *et al*., 2008), the first species is at risk of extinction and the second one is in critical condition (Baillie and Groombridge, 1996).

Molecular markers such as mitochondrial DNA (mtDNA) show uniparental inheritance which approximately bears the lack of recombination so they can be used as a beneficial tool to study

phylogeographic (Norazila and Patimah, 2002) and speciation (Sanna *et al*., 2008). All organisms due to cellular reaction or interaction with the environment are prone to different mutations which lead to polymorphism and finally genetic diversity (Gharibkhani *et al*., 2009). Two important environmental factors (Rahman *et al*., 2008) include natural selection and genetic drift in one individual or between different individuals or higher level of organization stabilizes the genetic diversity (Atashin *et al*., 2010). Some differences are usually detected in the nucleotide sequence of one gene or a specific part of a gene which create intra or inter species variation (Sultana and Khan, 2007). Although these variations are distinguishable by two methods based on molecular study, in case of first method sequences fragments are directly identified and the other method is based on PCR-RFLP method which elucidate the variations indirectly (Davis and Sterrett, 2011). The provided data from analysis of mtDNA sequencing and RFLP can be used in estimation of each haplotype divergence time and to elucidate the ancient division of different species (Heist *et al*., 1995, 1996).

Main species of marine turtles existing in Iran is *Eretmochelys imbricata* and their main habitat is the Persian Gulf (Mobaraki, 2004b). Although precise census for identifying the number of sea turtles in Qeshm, Kish and Nakhiloo has not been performed yet but according to the IUCN reports in respect to the Persian Gulf pollution, this species is prone to extinction due to mortal environmental effects. Therefore, to study and identify this species, we need more information about gene pool of sea turtle species. The purpose of this research is first, to study the different population of *Eretmochelys imbricata* and compare different habitat based on dominant population in each environment and second, nomination and comparison between various population in Iran and study of their genetics to detect the genetic structure of species based on PCR-RFLP using mtDNA samples (Fayazi *et al*., 2006).

In the present study, we have tested the similarity hypothesis in three distinct islands (Kish, Qeshm and Nakhiloo) in the Persian Gulf (Zolgharnein *et al*., 2010). Kish (26 32 N 53 58 E) is located in the Persian Gulf 19 km from mainland Iran and has an area of around 91 km² with an outer boundary of 40 km and a nearly elliptical shape. The Island is positioned along the 1359 km long Iranian coastline north of the Persian Gulf. Qeshm Island (26 45 N 55 49 E) is located a few kilometers off the southern coast of Iran (Persian Gulf), opposite the port cities of Bandar-Abbas and Bandar Khamir. The island which hosts a 300 km² free zone jurisdiction, is 135 km long and lies strategically in the Strait of Hormuz. Nakhiloo Island (27 49 N 51 28 E) located in the southern coast of the province is devoid of inhabitants. The Nakhiloo Island is the area where giant tortoises inhabit, the presences which have made this area environmentally important (Zolgharnein *et al*., 2011). The object of the study was to findout the heterogeniety between different population in haplotype level.

MATERIALS AND METHODS

In this study, sampling was performed in the end of spring season (2010) by digging the marked nests where turtles put their eggs and some random samples were taken from each hole. Tissue specimens were obtained from their beach nests at three locations (Qeshm Island, #40; Kish Island, #25 and Nakhiloo Island, #4) in the Persian Gulf. Embryos of each nest were collected separately and fixed immediately in absolute ethanol onsite and later frozen (-18°C), until further use. Total genomic DNA from 69 specimens was extracted using phenol-chloroform method (Alam *et al*., 1996). Finally, DNA was dissolved in 100 μL of double distilled water and stored at -20°C. PCR primers (TAG Copenhagen, Denmark) were designed based on a sequence of control region in D-loop of mitochondrial genome, each primer had 20 bp nucleotide length and their

sequences were 5'-GTCTCGGATTTAGGGGTTTG-3' and 5'-GCTTAACCCTAAAGCATTGG-3' for LCM15382 (Diaz-Fernandez *et al*., 1999) and H950 (Laurent *et al*., 1998), respectively. Amplified fragments were restricted by seven (EcoRI, HindIII, HinfI, HindII, AfaI, BamHI and NdeI) different restriction enzymes (Fermentas GmbH, Germany) then the samples along with 50 bp ladders loaded on Polyacrylamide gel electrophoresis (Bio-Rad, Germany).

Statistical analysis: To test the significance of hypothesis and to estimate the diversity between three studied populations, chi-square test was used and likelihood ratio for these populations calculated (Eyduran *et al*., 2006). According to the test result ($p<0.05$), enough evidence was not found to eliminate the hypothesis. The estimated G2 elucidate the similarity between these populations. In addition to the number of genotypes (na) and gene diversity (h) for each population, we also tried to calculate total na and h in all samples simultaneously. Another factor which we calculated was genetic distance to identify the level of similarity between three different populations (Bjorndal *et al*., 2005).

RESULTS

We analyzed sixty nine samples of unknown haplotypes in order to determine if RFLP analysis could be used to discriminate haplotypes in a blind test or not. Four different haplotypes were detected by seven restriction enzymes cut sites (Table 1). Digestion of the 890 bp segment PCR product with restriction endonucleases showed diverse genotypes. Three different genotypes were observed based on number and size of fragments created by restriction enzymes (Table 2). First enzyme was NdeI which produced two genotypes, one of them showed two electrophoretic bands and was called genotype A which was the dominant genotype in this population. Another genotype showed only one band since there was no cut in DNA and was named genotype B. The restriction of DNA samples by AfaI enzyme produced three different genotypes which were called A, B and C. The restricted DNA samples by HinfI produced only genotype A. The results of cutting by

Table 1: Different types of genotypes which are produced due to restriction of PCR products by seven various Restriction enzymes

	Haplotypes			
Enzymes	AAAAAAA	BAAAAAA	CAAAAAA	ABAAAAA
AfaI	A	B	C	A
NdeI	A	A	A	B
HinfI	A	A	A	A
HindII	A	A	A	A
HindIII	A	A	A	A
EcoRI	A	A	A	A
BamHI	A	A	A	A
Total No. of genotypes from each haplotype	70	3	1	3

Table 2: The size of DNA fragments in each genotype after cutting by enzymes

	Enzymes name		
Genotype	RsaI (AfaI) NdeI	HinfI	
A	420+265+210+30 bp	650+240 bp	840+50 bp
B	680+420+265+210+30 bp	Without cut	-
C	Without cut -	-	

Table 3: The calculation of χ^2 test values based on Restriction enzyme

	AfaI	NdeI	HinfI
Degree of freedom (df)	4	2	0
χ^2	3.221769	0.391757	0
Probability	0.521422	0.822112	1

Table 4: The estimation of Similarity ratio (G2) between three populations

	AfaI	NdeI	HinfI
Degree of freedom (df)	4	2	0
Similarity ratio (G2)	4.704600	0.566686	0
Probability	0.318972	0.753261	1

Table 5: The estimation of na and h for all samples based on Restriction enzymes

Enzyme	No. of genotypes (na)	Genotype diversity (h)	Total No. of samples
HinfI	1	0	66
NdeI	2	0.0868	66
RsaI	3	0.1034	74

Table 6: Matrix of Similarity and Genetic distance between three populations based on restriction of PCR products by HinfI, NdeI, AfaI enzymes

Population	Qeshm			Kish			Nakhiloo		
Enzyme	HinfI	NdeI	AfaI	HinfI	NdeI	AfaI	HinfI	NdeI	AfaI
Qeshm	****	****	****	1.0000	0.9997	0.9966	1.0000	0.9981	0.9966
Kish	0.0000	0.0003	0.0035	****	****	****	1.0000	0.9993	1.0000
Nakhiloo	0.0000	0.0019	0.0035	0.0000	0.0007	0.0000	****	****	****

HindII, EcoRI, BamHI and HindIII were similar and only genotype A was observed in all cases. RFLP analysis from two polymorphic enzymes, three and four restriction and non restriction enzymes , respectively, shows four different genotypes in studied populations. The frequency of one haplotype from 4 haplotypes was only 1, so it is called "rare haplotype". Although the less amount of variation was observed in these populations but most of the variation belonged to Qeshm Island. Comparison between the obtained results due to cutting by three enzymes of NdeI, AfaI (RsaI), HinfI shows that HinfI enzyme is the best proof of geographical similarity between these three populations. The highest level of differences in genotype frequency was observed in Kish and Qeshm populations. According to the χ^2 test, we did not have significant evidence to eliminate the similarity hypothesis (Table 3). Estimated similarity ratio (G2) elucidated the similarity between these populations (Table 4). Gene diversity was calculated and indicated the highest level of Gene diversity (h) present in Qeshm populations while DNA was restricted using AfaI enzyme. The calculated total "na" (number of genotypes) and "h" reveals that the highest and lowest amount of similarity ratio belonged to AfaI and HindI enzymes which are equal to 0.1034 and 0.0, respectively (Table 5). Moreover, we calculated standard genetic distance (Ds) based on the level of genetic divergence between different populations, the highest and lowest level of similarity between three populations observed when DNA samples were restricted using HinfI and AfaI

(RsaI) enzymes, respectively (Table 6). Actually, the highest genetic distance was very less (equal to 0.0035) so it can prove that the similarity between these populations is significantly high.

DISCUSSION

The sensitivity of the RFLP technique was demonstrated by coordination between banding patterns and nucleotide sequence haplotypes assigned for sixty nine samples of three populations tested (Rozihan and Ismail, 2011). We were able to identify a dominant haplotype pattern (haplotye A) which can be registered as a new haplotype of the hawksbill turtles in the Persian Gulf. According to the findings, dominant haplotype dispersions in these three different populations were approximately equal and the less number of haplotypes show that the variation in mitochondrial genome of *Eretmochelys imbricata* is low in studied populations. In studied haplotypes, one rare haplotype was observed with frequency equal to 1, it could be possible that this rare haplotype was a dominant haplotype in the past and its frequency has decreased or it might be a new haplotype which is produced in the population and may increase due to life condition over times or perhaps they are produced by new mutation or crossing over phenomenon. Finally, this study proved that the level of genetic variation in these populations was very less and it seems that there is a significant genetic relationship between these islands population. These results show that the PCR-RFLP method applied to the analysis of sea turtle mtDNA control region is efficient in distinguishing the species in the Cheloniidae family and its haplotypes. This simple and fast technique could also be employed to reduce the sequencing efforts (Kaska, 2000; Kavan *et al*., 2009).

Among these seven enzymes only three of them restricted PCR products which included AfaI, NdeI and HinfI. Among these three restriction enzymes, AfaI had the highest amount of restriction sites. The highest level of genetic distances were detected between Qeshm and Kish populations (GD = 0.0035) and also between Qeshm and Nakhiloo (GD = 0.0035) using AfaI enzymes although the highest level of genetic distance was observed between Kish and Nakhiloo population (GD = 0.0007) using NdeI enzyme. Four different haplotypes with increasing the frequencies include CAA, ABA, BAA, AAA , respectively, were produced by these three enzymes. Among these haplotypes, a rare haplotype with frequency 1.00 has been seen. In addition, the results showed no considerable heterogeneity between different populations and there are no enough evidences at least in haplotypic level for separation and distinction of studied samples.

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