Genetic population structure of Hawksbill turtle (*Eretmochelys imbricata*) using microsatellite analysis

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Abstract

Information on the genetic structure of marine species is essential for stock improvement programs. In order to analyses the genetic diversity of the Hawksbill turtle (*Eretmochelys imbricte*) by the microsatellite genetic method, 64 samples were caught from the beaches located in Kish and Qeshm islands. Polymerase chain reactions (PCR) of genomic DNA extracted from the samples were carried out using 5 pairs of microsatellite primers. The results of this study indicated that all 5 pairs of primers were polymorphic. Average numbers of real allele and effective allele were 4.90 and 2.99, respectively. Average rate of observed heterozygosity was 0.570 and that for expected heterozygosity was 0.616. Study of the Hardy-Weinberg equilibrium was shown the entire locus had not equilibrium except Cm3 and Ei8 locus in Kish area. F_{st} (0.166) and R_{st} (0.634) calculated by the Analysis of Molecular Variance (AMOVA) test illustrated that there are separate populations of Hawksbill turtle in this part of the Persian Gulf (Kish and Qeshm islands). It seems that Kish's turtles live under better conditions in contrast to their Qeshm counterparts. Diminution of genetic variation within examined population decreases its adaptation to environmental alterations. We identified two different E. imbricte populations from north of the Persian Gulf.

Keywords: Microsatellites; Genetic variation; Polymorphism; *Eretmochelys imbricte*, Persian Gulf

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INTRODUCTION

The waters of the Persian Gulf are environmentally unique with regard to their unusual faunal communities (Carpenter *et al*., 1997). Such an environment is associated with Kish, Qeshm, Khark, Kharkoo, Hendourabi, Sheedvar, Lavan, Ommolkaram and Nakhiloo, which are the major and most important islands of the Persian Gulf. There are 7 species of marine turtles in the world that include: *Dermochelys coriacea*; *Caretta caretta*; *Chelonia mydas*; *Chelonia depressa*; *Eretmochelys imbricata*; *Lepidochelys olivacea* and *Lepidochelys kempi* (Storelli and Marcotrigiano, 2003). The main threats to marine turtles worldwide include: destruction and alteration of nesting and foraging habitats; incidental capture in commercial and recreational fisheries; mortification in marine waste; vessel strikes and the use of egg and turtle protein as food (Storelli and Marcotrigiano, 2003; Laurent *et al*., 1996).

There are 2 species of marine turtles in the Persian Gulf and Oman Sea that include the Hawksbill and Green turtles. The Hawksbill sea turtle, *Eretmochelys imbricata* (Linnaeus, 1766), resides mainly in the tropical regions of the Indian, Atlantic and Pacific oceans. An *E. imbricata* adult can weigh up to 45 to 68 kg on average, but can grow as large as 91 kg and reach a length of approximately 60-110 cm. Mating occurs approximately every 2 to 4 years. The mean natural life of a Hawksbill is thought to be approximately 30 to 55 years, however, biologists are not sure exactly how long they can live (Lutz and Musick, 1997;

Marquez, 1990).

Microsatellites are important genetic markers because of their dense distribution in the genome, great differentiation, co-dominant inheritance and simple genotyping. In recent years, they have been widely used in parentage testing, linkage analyses, population genetics and genetic studies (Seyedabadi *et al*., 1994). The low variability at the isozyme loci in the marine species, involving primarily diallel polymorphism, reduces their sensitivity. In contrast, microsatellite DNA markers (one-to-eight-nucleotide tandem repeats, randomly distributed in the genome) have been found useful for detecting high levels of polymorphism and rare alleles. These markers are now widely used for the determination of genetic variation in wild and cultured fish populations (Was and Wenne, 2002; DeWoody and Avise, 2000; Norris *et al*., 1999; Brooker *et al*., 1994).

The study of turtle population genetics has come a long way in the past few decades. In the early 1990s, technologies such as polymerase chain reaction (PCR) and automated DNA sequencing spearheaded a boom in Molecular Ecology. Microsatellite and mitochondrial DNA haplotype markers became the methods of choice for many turtle studies (Lee, 2008). Taxonomy and systematic have undoubtedly benefited from DNA sequencing technology (Hillis *et al*., 1996). The evolutionary history of turtles and how they relate to each other and to other vertebrates has been explored using mitochondrial DNA sequences (Krenz *et al*., 2005; Karl and Bowen, 1999; Kumazawa and Nishida, 1999; Dutton *et al*., 1996; Bowen *et al*., 1993). Ireland *et al*. (2003) determined the level of multiple paternities in a population of green turtles from Ascension island using microsatellite data obtained from 3 females and their offsprings. Aggarwal *et al*. (2004) developed 6 nuclear-encoded microsatellites from a genomic DNA library of olive ridley turtle (*Lepidochelys olivacea*), and used them to screen 83 individual olive ridley turtles.

Therefore, in consideration of the afore mentioned information, the principal objectives of this study were to assess the intra- and inter-population genetic variations and genetic differentiation in 2 populations of Hawksbill turtle inhabiting the northern coasts of the Persian Gulf (Kish and Qeshm islands) using the microsatellite DNA markers developed by FitzSimmons *et al*. (1995).

MATERIALS AND METHODS

In this study, 64 specimens of immature *Eretmochelys imbricata* were hand-captured from their beach nests at 2 locations (Kish island, # 34; Qeshm island, number # 30) during 3 weeks, between February and April of 2007 from the areas indicated in Figure 1. The zones are situated 346 km of each other in the northern coasts of the Persian Gulf. Tissue samples from the immature Hawksbill turtles were collected and fixed immediately in 95% ethanol onsite, and later frozen (-18ºC), until further use.

Approximately, 40 mg of internal muscular tissue was cut into small pieces with scissors. Total genomic DNA was extracted by proteinase-K digestion, phenol: chloroform: isoamyl alcohol extraction followed by ethanol precipitation as described by Alam *et al*. (1996). Finally, DNA was dissolved in 100 µl of double distilled water and stored at -20ºC.

Five primer pairs for Hawksbill sea turtle microsatellite markers, CM3, Ei8, Cc117, CM84 and CM72 developed by Fitzsimmons *et al*. (1995), were used in this study (Table 1). The polymerase chain reaction (PCR) condition, especially the annealing temperature, was optimized for the 5 microsatellite loci as necessary to produce scoreable amplification products. The annealing temperature during the reaction varied from 55°C for CM72 and CM3 and 57ºC for Ei8 and CM84 to 58°C for Cc117. PCR was performed in a 25 µl reaction volume containing 50 ng of genomic DNA, 2 µM of each primer, 0.4 mM each of the dNTPs, 1 U of *Taq* DNA polymerase (Fermentas,

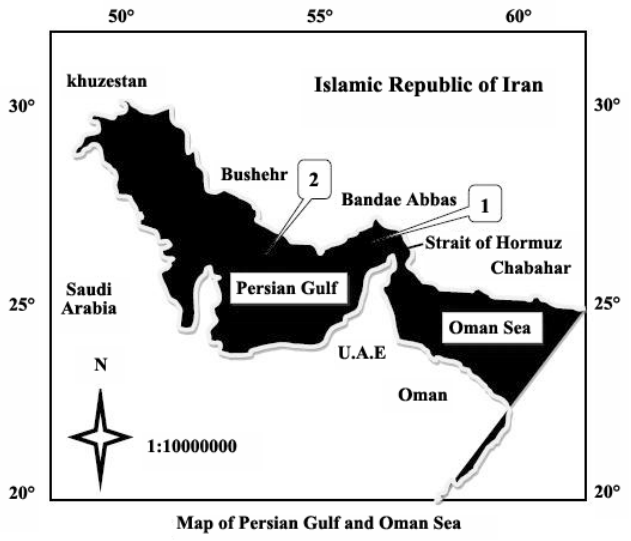

Figure 1. Map of localities sampled for the Hawksbill sea turtle (*E. imbricata*) in the northern coasts of the Persian Gulf, Iran (1, Qeshm Island; 2, Kish Island).

Table 1. Sequences of 5 microsatellite markers and their specific annealing temperature for PCR amplification in the Hawksbill sea turtle, *E. imbricata*.

Locus	Anneal (ºC)	Repeat motif	Primer sequences
Cm3	53	(CA)13	F: 5' -AATACTACCATGAGATGGGATGTG-3'
			R: 5' -ATTCTTTTCTCCATAAACAAGGCC-3'
Cm72	55	(CA)33	F: 5' -CTATAAGGAGAAGCGTTAAGACA-3'
			R: 5' -CCAAATTAGGATTACACAGCCAAC-3'
Cm84	52	(CA)15	F: 5' -TGTTTTGACATTAGTCCAGGATTG-3'
			R: 5' -ATTGTTATAGCCTATTGTTCAGGA-3'
Cc117	53	(CA)17	F: 5' -TCTTTAACGTATCTCCTGTAGCTC-3'
			R: 5' -CAGTAGTGTCAGTTCATTGTTTCA-3'
Ei8	51	(CA)19	F: 5' -ATATGATTAGGCAAGGCTCTCAAC-3'
			R: 5' -AATCTTGAGATTGGCTTAGAAATC-3'

Germany), 2 mM $MgCl_2$ and 2.5 µl of 10X reaction buffer. The temperature profile consisted of initial denaturation for 3 min at 94°C followed by 30 cycles of: 30 s at 94°C, 45 s at the respective annealing temperature, and 1 min at 72°C, and a final extension for 10 min at 72°C. The PCR products were separated on an 8% denaturing polyacrylamide gel containing 19:1 acrylamide: bis-acrylamide and 5 M urea. Electrophoresis was conducted using a SequiGen sequencing gel electrophoresis system (BIO-RAD Laboratories, Hercules, CA). DNA fragments were visualized by silver nitrate staining (Bassam *et al*., 1991). Allele sizes (base pairs) were obtained by comparison to a pBR322 DNA/AluI Marker, 20 (Fermentas, Germany) sequencing ladder. A typical example of stained gel is shown in Figure 2.

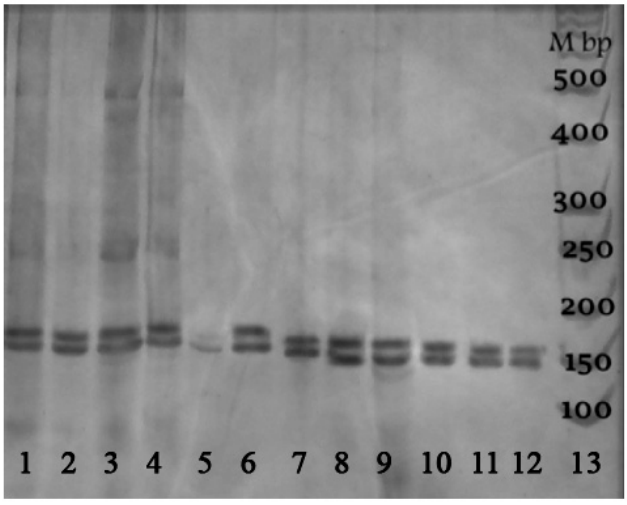

Figure 2. Microsatellite profiles for 12 samples at the Cm3 locus. Amplification products were separated on a denaturing polyacrylamide gel and visualized by silver staining. From left; lanes 1-12 represent Kish island genotypes and lane 13 is Ladder (pBR322 DNA/AluI Marker, 20; Fermentas, Germany).

The size of each allele was estimated using the DNAfrag, version 3.03 (Nash, 1991) software. A genotypic data matrix was constructed for all loci. The program MICROCHECKER version 2.2.3 (Van Oosterhout *et al*., 2004) was used to identify possible null alleles, large allele dropout, scoring error due to stutter peaks and possible typographic errors, before proceeding with further analyses. Allele and genotype frequencies that were identified as affected by the presence of null alleles were adjusted according to the Brookfield I method (Van Oosterhout *et al*., 2004). Fit of genotype data to Hardy-Weinberg proportions was estimated using the software POPGENE version 1.31 (Yeh *et al*., 1999) with 1000 simulated samples. POPGENE is a user-friendly Microsoft Window-based computer package for the analysis of genetic variation among and within natural populations using co-dominant and dominant markers and quantitative traits. The GenAlEx version 6 software package (Peakall and Smouse, 2006) was used for estimating allele frequencies and for applying the homogeneity test between populations. GenAlEx 6 is written in Visual Basic for applications (VBA) within Excel. GenAlEx requires all data to be coded as numbers and formatted within Excel as numeric data. The dendrogram was constructed and drawn using MEGA version 4 (Tamura *et al*., 2007). The MEGA 4 software includes distance matrix and phylogeny explorers as well as advanced graphical modules for the visual symbol of input data and output results.

RESULTS

All 5 microsatellite loci were polymorphic in the stud-

Table 2. Allelic variation at 5 microsatellite loci in 2 populations of *E. imbricata*. (N = Number of alleles per locus; N_e= Number of effective alleles; H_o=observed heterozygosity; H_e=expected heterozygosity).

Population	Parameters	Locus				
		Cm3	Cm72	Cm84	Cc117	Ei8
Kish Island	N	3	8	7	5	4
	Ne	1.97	6.61	3.30	2.84	1.77
	Ho	0.65	0.96	0.44	1.00	0.30
	He	1.00	1.00	0.00	0.35	0.00
Qeshm Island	N	2	4	5	6	5
	Ne	2.00	3.38	2.77	1.98	3.25
	Ho	0.50	0.85	0.69	0.65	0.44
	He	0.50	0.70	0.64	0.50	0.69

ied population of Hawksbill sea turtles, *Eretmochelys imbricate*, and the level of polymorphism variation depended on the locus (Table 2). Figure 2 illustrates the results obtained with the Cm3 locus in Kish area for 12 samples on an 8% denaturing polyacrylamide gel. A total of 49 different alleles were found over all loci for all populations. Allele frequencies at 5 loci in all populations are shown in Figure 3. The number of allele per locus ranged from 3 to 10. The average of observed and expected heterozygosities ranged from 0.30 to 1.00 and from 0.44 to 0.85, respectively. The maximum and minimum numbers of the unique alleles were found at loci Ei8 (9) and loci Cm84 (1), respectively. Considerable differences among 2 populations in the number of alleles were found at some of these loci (Table 2). The number of alleles in Cm72 ranged from 4 to 8 and Cm84 from 5 to 7 with a tendency to a reduction in the Qeshm island population. Allele sizes ranged from 151 to 349 bp across the microsatellite loci. The effective number of alleles varied from 1.77 for Ei8 to 6.61 for Cm72. In all populations, the effective number of alleles was lower than the observed number of alleles.

Significant deviations from Hardy-Weinberg equilibrium (HWE) at the locus level are shown in Table 3. All five loci used in this study were tested for departure the from HWE. 8 out of 10 (5 loci × 2 populations) possible tests for HWE were statistically significant ($P < 0.05$).

The population differentiation (F_{ST}) was modest

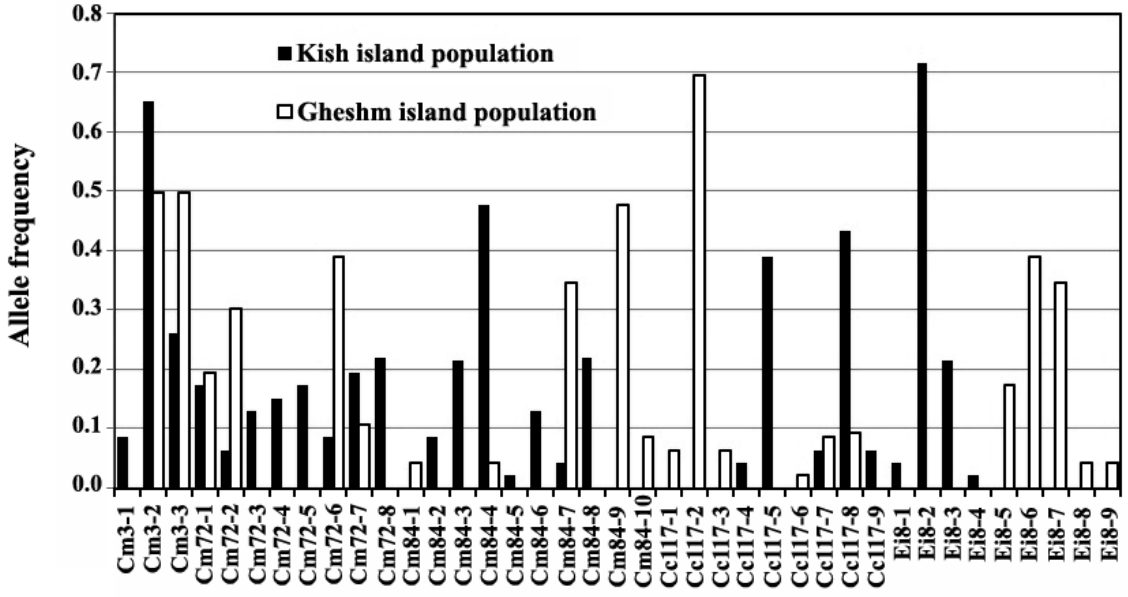

Figure 3. Allele frequencies at 5 loci in all populations of the Hawksbill sea turtle (*E. imbricata*).

Table 3. Deviation from the Hardy-Weinberg genotype frequency expectations in 2 different populations of *E. imbricate*.

Population	Parameters	Locus				
		Cm3	Cm72	Cm84	Cc117	Ei8
Kish island	χ^2	3.412	84.128	55.040	69.000	5.965
	Signif	ns	***	***	***	ns
	df	3	28	21	10	6
Qeshm island	χ^2	23.000	31.214	92.000	48.915	92.000
	Signif	***	**	***	***	***
	df	1	6	10	15	10

χ^2 = Chi-square values; df = degrees of freedom; Signif = Statistically significant values are marked with asterisks (** P<0.01, *** P<0.001 and ns = not significant).

and F_{ST} value between the Qeshm and Kish islands' populations was 0.17 and significant. R_{ST} value between the 2 populations was high (0.64) and significant. The estimated gene flow (N_m) value between the Qeshm and Kish islands' populations across all the studied loci was 1.26. Genetic distances among the respective populations were small. The genetic distances and genetic similarity, as computed by Saitou and Nei (1987) between the Qeshm and Kish islands' populations were 0.33 and 0.78, respectively.

DISCUSSION

In this study, 64 specimens of *E. imbricata* were collected from 2 locations. This sample number was regarded as suitable for this purpose because the number of alleles observed in the microsatellite loci is usually large and the frequency of each allele may be low, hence a large sample size is necessary for satisfying subsequent statistic analyses (Salari Aliabadi *et al*., 2009).

The microsatellite loci used for Hawksbill sea turtle populations had genetic variations, with approximately 49 alleles across 5 polymorphic loci. All of the loci were polymorphic and the genotypic distribution frequencies across all the loci were significantly different, suggesting genetic structuring among these 2 populations. Frequencies of alleles in the Kish island samples are higher from the Qeshm island samples, except at one locus (Ei8). It is likely that the Qeshm island population had originated from Kish, and that it had lost some alleles during the course of fisheries and environmental management in the northern coasts of the Persian Gulf. The losses of alleles and heterozygosities in the sea turtle stocks may increase with bottlenecking and inbreeding through time. Heterozygosity is an important measurement of population diversity at the genetic level and has drawn much attention from ecologists and aquaculturists (Xu *et al*., 2001). In the current study, the observed heterozygosity for 5 microsatellite loci was greater than the expected heterozygosity in two populations. In a study with Hawksbill sea turtle populations by FitzSimmons et al. (1995) was showed similar results that found in our study. The Kish island population even showed 100% heterozygosity at Cc117. However, the Qeshm island population showed the lowest genetic diversity among the 2 populations in terms of the average number of alleles and genotypes per locus, the number of unique alleles, and low-frequency alleles. The results of this study indicated that considerable heterozygosity excess was observed in intra-population based on allelic and genotypic frequencies. Heterozygote deficiencies were observed at varied in different loci. Heterozygote deficiency can be interpreted as the increase in homozygotes, which might be a result of increased inbreeding. The significant heterozygote deficiency might reflect the fact that there is restricted gene flow between these populations. Although heterozygosities were low, there was enough variation present to examine any potential genetic differences among the sample sites. Results from this study showed that the mean heterozygosity per population for all loci was in agreement with the work of FitzSimmons *et al*. (1995).

Significant deviations from Hardy-Weinberg expectations (HWE) were observed in the 2 populations. Genetic drift, inbreeding and divergent evolution are likely to be the causes for deviation from the H–W disequilibrium. These results are compatible with a previous study that had been conducted for Hawksbill sea turtle populations (FitzSimmons *et al*., 1995), and with studies on other types of fish (Salari Aliabadi *et*

al., 2009; Bradshaw *et al*., 2007; Alam and Islam, 2005; Hansen and Mensberg, 1998).

The partitioning of variability of populations observed after F-statistics comparisons with total types of markers showed that most of the genetic variation is within populations. There was a high level of genetic differentiation among the 2 populations, with a highly significant overall F_{ST} value of 0.167 ($P < 0.01$). Based on analysis of molecular variance (AMOVA), F_{ST} (0.167) was observed between the Qeshm and Kish islands' populations (N_m=1.260). Therefore, geological structures separate the Qeshm island from the Kish island stocks and may limit gene flow between these 2 populations. The genetic distance between these 2 populations is estimated as 0.331, which indicates that the genetic difference among the studied populations is pronounced. However, the loss of genetic variability in the Qeshm island population also might be caused by sampling error, as the sampling sizes were relatively little for characterizing allele frequencies at marker loci as changeable as microsatellites.

The results obtained from the present study show that at least 2 different populations of Hawksbill sea turtle is found in the northern coasts of the Persian Gulf, which include the Qeshm and Kish islands' populations.

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