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Microsatellite Polymorphism in Iranian Populations of Cobia (*Rachycentron canadum* G.)

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Abstract: Genetic divergence within and between wild populations of cobia, *Rachycentron canadum* (L.) was assessed by means of microsatellite analysis in the Persian Gulf and Oman Sea. Ten microsatellite markers were used to estimate the level of genetic diversity within six wild populations of cobia and the degree of genetic differentiation between them was compared. Mean observed and effective allele number was 12.357 and 8.319, respectively. Mean observed and expected heterozygosity was 0.655 and 0.874, respectively. Based on Analysis of Molecular Variance highest F-statistics (0.063) was observed when comparing specimens from Dayer Port zone and Pozm of Chabahar zone. Highest genetic distance (0.258) and lowest genetic resemblance (0.223) were observed between specimens from Dayer Port zone and Beris of Chabahar zone. The present study showed that at least three different populations of *Rachycentron canadum* were found in the northern coasts of Persian Gulf and Oman Sea.

Key words: Microsatellites, Iran, polymorphism, genetic variation, PCR, *Rachycentron canadum*

INTRODUCTION

Rachycentron canadum Goode, 1884 or cobia, the sole representative of the family Rachycentridae, has an elongated, strongly rounded body with a broad flat head. The mouth is terminal in position with a projecting lower jaw. The first dorsal fin is comprised of seven to nine short, stout isolated spines and the second dorsal fin is long with anterior rays forming a raised lobe in adults. The anal fin is similar to second dorsal and both are covered in thick skin. The caudal fin is lunette and crescent shaped in adults with the upper lobe longer than the lower lobe. The caudal fin in the very young is paddle shaped. Scales are small and embedded in the skin. Cobia is a large, pelagic fish that is primarily distributed throughout warm temperate, subtropical and tropical waters worldwide except for the eastern pacific, with substantial concentrations located in the Gulf of Mexico (Turner *et al*., 2005). Cobia can reach a total length of 2 m and the maximum weight has been reported as 68 kg (Ditty and Shaw, 1992). It is a highly prized food and recreational trophy fish and is considered a prime candidate for aquaculture (Benetti *et al*., 2003). Adult cobia are absent along the continental shelf between March and October and are believed to move into tropical waters to over winter (Ditty and Shaw, 1992).

Molecular genetic assays can contribute to conservation of taxa by assessing their evolutionary and taxonomic distinctiveness, the levels of genetic variation within and between populations and the degree of introgression from introduced taxa (Frankham *et al*., 2002). Highly polymorphic genetic markers, such as microsatellites, can solve most problems related with family relationships in fish reared communally (García de León *et al*., 1998; Jackson *et al*., 2003; Castro *et al*., 2004; Hayes *et al*., 2005). Recently, microsatellite markers which are characterized as codominant and highly polymorphic systems have become the most widely applicable DNA technology and, in many fish species, have been used for monitoring changes in genetic variation of farmed stocks, parentage assignment and fine-scale studies of population structure (Desvignes *et al*., 2001; Nielsen *et al*., 1997; Holland, 2001; Norris *et al*., 2000). Microsatellites also have many applications in breeding programs (Garcia de Leon *et al*., 1998) and for assessing population structure of wild populations as a means to improve assessment and allocation of resources.

Cobia is an important species for commercial fishing, its meat is white, having different composition for each part of the body especially in large sized fish and is considered an excellent table fish. Cobia is normally a

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bycatch of commercial fisheries. Due to the solitary behavior they are difficult targets for a species specific fishery. Cobia is caught in trawls, drift nets and by hook and line. In Iran, the volume of product in the market is low and many consumers have probably never tasted cobia. Pruett *et al.* (2005) developed twenty nuclear-encoded microsatellites from a genomic DNA library of cobia and screened among a sample of 24 fish from the Gulf of Mexico. Renshaw (2005) also developed 35 pairs of microsatellite markers for *R. canadum* and reported the numbers of alleles and heterozygosity observed in a single sample comprising 32 fish from the Gulf of Mexico. The aim of this study was to compare levels of genetic polymorphism between six wild populations of cobia, *R. canadum* in the northern coasts of Persian Gulf and Oman Sea using microsatellite loci.

MATERIALS AND METHODS

To test 10 paired microsatellite primers, 184 specimens of *R. canadum* were collected from six zones (Booshehr, Dayer Port, Lengeh Port, Bandarabass, Pozm and Beris of Chabahar) between June and April of 2007 (Fig. 1). The zones are located within 140-310 km of each other in the northern coasts of Persian Gulf and Oman Sea. DNA was obtained from a small sample of the pectoral fin in the individuals. Samples were preserved in 96% ethanol at 4°C. Genomic DNA was extracted following a standard SDS-proteinase K/phenol-chloroform (Hillis *et al.*, 1996). Briefly, pieces of tissue of approximately 100 μL in volume were digested for 3 h in 500 μL of extraction buffer containing 0.1 M Tris HCl, 10 mM EDTA, 0.1 M NaCl, 2% SDS and 0.1 mg proteinase K mL^{-1}. The cellular debris was removed using phenol/chloroform extraction and the DNA precipitated with absolute ethanol. Finally, DNA was dissolved in 100 μL of double distilled water and stored at -20°C.

PCR conditions were optimized for ten microsatellite loci. Annealing temperatures varied from 55°C for *Rca* 1B-F07 and *Rca* 1B-E08B, 58°C for *Rca* 1B-F06, 60°C for *Rca* 1B-D09, *Rca* 1B-H09 and *Rca* 1-A04, 61°C for *Rca* 1B-A10 and 62.8 °C for *Rca* 1B-G10 to 63°C for *Rca* 1B-E02 and *Rca* 1B-E08A. $MgCl_2$ concentration was 2 mM and primer concentrations were 0.2 μM for all microsatellites except for *Rca* 1B-G10, for which it was 0.3 μM. Concentration of Taq polymerase (1 U/25 μL) and nucleotides (0.4 mM) was the same for all loci. Thermal profile for PCR: 95 °C 3 min, (95°C 30 sec, X°C 45 sec, 72°C 1 min)×30, 95°C 30 sec, X°C 45 sec, 72°C 10 min (X°C-annealing temperature). PCR was followed by electrophoresis of products in 8% polyacrylamide. DNA fragments were visualized by silver staining (Bassam *et al.*, 1991). Allele sizes were obtained

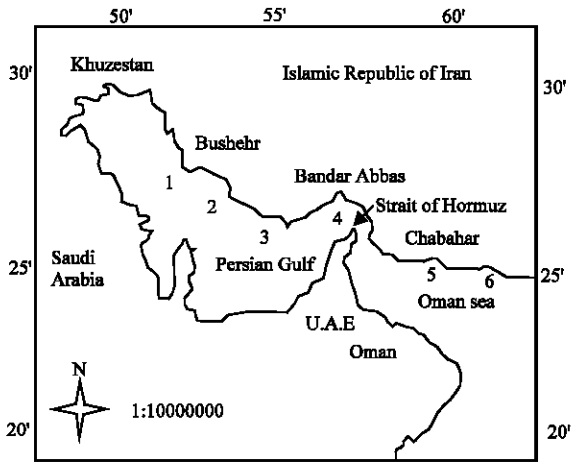

Fig. 1: Map of localities sampled for cobia (*R. canadum*) in the northern coasts of Persian Gulf and Oman Sea, Iran. populations 1-6 (1, Booshehr; 2, Dayer Port; 3, Lengeh Port; 4, Bandarabass; 5, Pozm of Chabahar; 6, Beris of Chabahar)

by comparison to pBR322 DNA/AluI Marker, 20 (Fermentas company) sequencing ladders by their size in base pairs.

The recorded microsatellite genotypes were used as input data for the GENALEX software package (Peakall and Smouse, 2006) in order to calculate allele and genotype frequencies, observed and expected heterozygosities and to test for deviations from Hardy-Weinberg equilibrium. Genetic distance between two populations was estimated from Nei standard genetic distance and genetic similarity index (Nei, 1972). Genetic differentiation between populations was also evaluated by the calculation of pairwise estimates of F_{st} values. All calculations were conducted using the GENALEX version 6 (Peakall and Smouse, 2006). The dendrogram was constructed and drawn using MEGA version 4 (Tamura *et al.*, 2007).

RESULTS

Allele frequencies and estimates of gene diversity were assembled in Table 1 for each locus and population. All loci showed polymorphism, except for locus *Rca* 1B-D09, *Rca* 1B-E08B and *Rca* 1B-G10 which were monomorphic in the all populations. A total of 90 alleles were found in the pooled *R. canadum* populations, ranging from 13 for locus *Rca* 1B-E08A to 18 for locus *Rca* 1B-H09 (Table 1). Fourteen alleles were private, i.e. found in only one population. The highest total number of private alleles at the population level was

Table 1: Population genetic statistics summarizing variation at 10 microsatellite loci in six *R. canadum* populations sampled from the northern coasts of Persian Gulf and Oman Sea, Iran

		Locus				
Population	Parameters	*Rca* 1B-A10	*Rca* 1B-D09	*Rca* 1B-E02	*Rca* 1B-E08A	*Rca* 1B-E08B
Booshehr	N	39	39	39	39	39
	NA	13	1	13	13	1
	NE	8.3	1	11.1	7.9	1
	HO	0.538	0	0.615	0.385	0
	HE	0.88	0	0.91	0.873	0
	PHWE	0	1	0	0	1
	Significant	***	ns	***	***	ns
Dayer Port	N	22	22	22	22	22
	NA	8	1	11	9	1
	NE	4.7	1	7.4	5.9	1
	HO	0.273	0	0.727	0.364	0
	HE	0.788	0	0.865	0.83	0
	PHWE	0	1	0	0	1
	Significant	***	ns	***	***	ns
Lengeh Port	N	32	32	32	32	32
	NA	14	1	12	13	1
	NE	7.7	1	10.5	10.2	1
	HO	0.625	0	0.625	0.313	0
	HE	0.87	0	0.905	0.902	0
	PHWE	0	1	0	0	1
	Significant	***	ns	***	***	ns
Bandarabas	N	41	41	41	41	41
	NA	14	1	12	13	1
	NE	7.6	1	10.4	9	1
	HO	0.659	0	0.634	0.366	0
	HE	0.869	0	0.904	0.889	0
	PHWE	0	1	0	0	1
	Significant	***	ns	***	***	ns
Pozm	N	30	30	30	30	30
	NA	11	1	13	11	1
	NE	6.3	1	9.4	8.8	1
	HO	0.533	0	0.7	0.267	0
	HE	0.841	0	0.893	0.887	0
	PHWE	0	1	0.012	0	1
	Significant	***	ns	*	***	ns
Beris	N	20	20	20	20	20
	NA	11	1	11	10	1
	NE	7.4	1	8	7.3	1
	HO	0.5	0	0.45	0.15	0
	HE	0.865	0	0.875	0.864	0
	PHWE	0	1	0.001	0	1
	Significant	***	ns	***	***	ns
		Rca 1B-F06	*Rca* 1B-F07	*Rca* 1B-G10	*Rca* 1B-H09	*Rca* 1-A04
Booshehr	N	39	39	39	39	39
	NA	9	13	1	18	14
	NE	6.1	8.3	1	11.6	8.7
	HO	0.641	1	0	0.641	0.821
	HE	0.837	0.879	0	0.914	0.886
	PHWE	0	0	1	0	0
	Significant	***	***	ns	***	***
Dayer Port	N	22	22	22	22	22
	NA	14	12	1	13	12
	NE	8.6	8	1	8	6.9
	HO	0.773	1	0	0.5	0.727
	HE	0.883	0.875	0	0.875	0.854
	PHWE	0	0	1	0	0
	Significant	***	***	ns	***	***
Lengeh Port	N	32	32	32	32	32
	NA	8	16	1	16	15
	NE	4.3	12.1	1	12.6	8.1
	HO	0.781	1	0	0.75	0.813
	HE	0.767	0.917	0	0.92	0.877
	PHWE	0	0	1	0. 014	0
	Significant	***	***	ns	*	***
Bandarabas	N	41	41	41	41	41
	NA	15	16	1	15	15

Table 1: Continued

Population	Parameters	Locus *Rca* 1B-F06	*Rca* 1B-F07	*Rca* 1B-G10	*Rca* 1B-H09	*Rca* 1-A04
	NE	9	12.9	1	9.8	9.4
	HO	0.659	1	0	0.732	0.854
	HE	0.889	0.923	0	0.898	0.894
	PHWE	0	0	1	0.024	0
	Significant	***	***	ns	*	***
Pozm	N	30	30	30	30	30
	NA	8	11	1	13	12
	NE	5.1	8.9	1	9.9	4.6
	HO	0.6	1	0	0.767	0.733
	HE	0.805	0.888	0	0.899	0.781
	PHWE	0.137	0	1	0.348	0.022
	Significant	ns	***	ns	ns	*
Beris	N	20	20	20	20	20
	NA	9	8	1	13	12
	NE	7.1	6.6	1	8.4	6.5
	HO	0.45	1	0	0.65	0.9
	HE	0.86	0.848	0	0.881	0.845
	PHWE	0.001	0.006	1	0.004	0.175
	Significant	**	**	ns	**	ns

N = Sample size, N_A = No. of alleles per locus; N_E = No. of effective alleles; H_O = Observed heterozygosity; H_E Expected heterozygosity; P_{HWE} = Probability of rejecting hypothesis of Hardy Weinberg equilibrium; ns = Not significant, **p<0.01, ***p<0.001

Table 2: Analysis of genetic differentiation between pairs of populations across all loci based on estimates of F_{st} values (below diagonal) and N_m (above diagonal)

Populations	Zones Booshehr	Dayer port	Lengeh port	Bandarabas	Pozm	Beris
Booshehr	****	108	14.20	11.90	5.5	5.0
Dayer Port	0.002	****	7.50	08.50	3.7	3.8
Lengeh Port	0.017*	0.032*	****	346.00	17.0	9.3
Bandarabas	0.021*	0.029*	0.000	****	16.1	16.4
Pozm	0.044*	0.063*	0.014*	0.015*	****	204
Beris	0.047*	0.062*	0.026*	0.015*	0.001	****

*Statistically significant (p<0.01)

Table 3: AMOVA analyses of microsatellite loci, considering variation among and within all 6 populations

Source of variation	df	Variation (%)	Variance components	p*
Among region	2	10	0.100	0.01
Among populations/region	3	2	0.019	0.01
Within populations	362	88	0.117	0.01

*: Based on 1000 permutations, df: Degree freedom, p: Probability

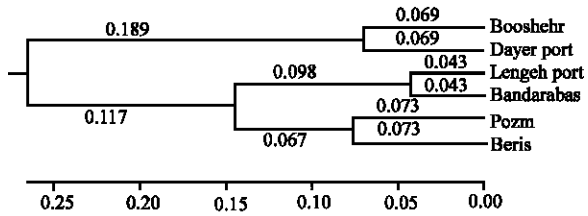

Fig. 2: UPGMA dendrogram based on the genetic distance computed by Nei (1972) between *R. canadum* populations, according to microsatellite DNA analysis

observed in Booshehr. Unique alleles, that is, alleles unique to just one individual, were detected for two loci. The highest values of mean number of alleles and of expected heterozygosity were detected in the Bandarabas population and the lowest in the Lengeh Port population. Significant deviations from Hardy-Weinberg equilibrium at the locus level are shown in Table 1. All ten loci used in this study were tested for departure from Hardy-Weinberg equilibrium. 39 out of 60 (ten loci×six populations) possible tests for HWE were statistically significant (p<0.05).

Population differentiation was modest, especially among the same region populations. The population differentiation (F_{st}) value between Dayer Port zone and Pozm of Chabahar zone was the highest (0.063) and significant among the population pair, while the F_{st} value between the Bandarabass zone and Lengeh port zone populations (0.000) was the lowest and not significant (Table 2). The estimated gene flow (N_m) value between the Dayer Port zone and Pozm of Chabahar zone (N_m = 3.7) populations across all the studied loci was the

lowest, while the N_m value between the Bandarabass zone and Lengeh port zone (N_m = 346) populations was the highest (Table 2).

The genetic distance was the smallest (0.043) between the Bandarabass zone and Lengeh port zone populations, whereas the largest distance (0.258) was between Dayer Port zone and Beris of Chabahar zone (Fig. 2).

Variance analysis (AMOVA) using microsatellite showed 88% of variation inside populations. The percentage of variation among populations/region (2%) and among regions (10%) was low but significant (Table 3). Furthermore, the contingency table analyses of allele frequencies showed a significant differentiation among pairwise population comparisons.

DISCUSSION

Conservation of flanking regions is a general property of microsatellite loci and has been specifically reported in fishes (Rico *et al*., 1996). The conservation of fish species has traditionally been carried out through restocking. Genetic variability is pivotal to maintaining the capability of restocked fish to adapt to a changing environment (Avise, 1994). One approach that has been successfully used in uncovering cryptic population structure is microsatellite markers. Microsatellites have been isolated and characterized in a large number of fish species and have been used in a wide range of applications, as in evolutionary biology, population genetics and ecology (Dunham, 2004). By characterizing the geographical distribution of allele frequencies, population sub-structuring can be detected and local populations can be identified.

In the present study, microsatellites were used to assess the genetic variability and the population structure of cobia, *R. canadum* from Persian Gulf and Oman Sea.

It was also interesting to compare levels of diversity in these Iranian populations with those in the northern Gulf of Mexico (Pruett *et al*., 2005). The 7 from 10 microsatellite loci chosen for analysis were polymorphic in all six populations. Because the number of alleles observed in microsatellite loci is usually large and the frequency of each allele may be low, a large sample size is necessary for satisfying subsequent statistic analyses. The microsatellite loci used for cobia populations had enough genetic variation, with around 90 alleles across 7 polymorphic loci. The loci were polymorphic and the genotypic distribution frequencies for all pairs of populations across all loci were significantly different, suggesting genetic structuring among populations. Microsatellites allowed the identification of some unique alleles. In fact, as they were in low frequencies, it is probably that these unique alleles are rare alleles, once hyper-variable loci need a bigger sample to identify real unique alleles. Specific markers were also identified for of the Booshehr zone population identified. The Booshehr population zone can be identified using primers *Rca* 1B-E08A, *Rca* 1B-F07, *Rca* 1B-H09 and *Rca* 1-A04.

Heterozygote deficiencies observed were different for different loci. Heterozygote deficiency can be interpreted as 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 sample sites. Mean Heterozygosity per population was highly for each of these seven loci in the Iranian populations than in the northern Gulf of Mexico (Pruett *et al*., 2005). The mean value of Iranian populations was 0.655 and the northern Gulf of Mexico value was 0.589.

Significant deviations from Hardy-Weinberg (HW_E) expectations were observed in all six populations. Null alleles and homoplasy, frequently found in microsatellite loci, are likely causes for the H-W disequilibrium (Callen *et al*., 1993; Estoup *et al*., 1995).

The partitioning of variability of populations seen after F-statistics comparisons with total types of markers shows that most of genetic variation is within populations. There was a high level of genetic differentiation among the six populations, with a highly significant overall F_{ST} value of 0.063 ($p<0.01$). Based on Analysis of Molecular Variance (AMOVA) highest F_{st} (0.063) was observed when comparing specimens from Dayer Port zone and Pozm of Chabahar zone (N_m = 3.7).

CONCLUSION

The present study showed that at least three different populations of *R. canadum* are found in the northern coasts of Persian Gulf and Oman Sea. These include the Booshehr region population, Bandarabass region population and the Chabahar region population. The present study also point out to the potentiality of the marker collections employed for a variety of purposes such as parentage testing, population monitoring and traceability in wild fish populations.

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