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Analysis of genetic variability and differentiation of South Caspian Sea's common kilka (*Clupeonella cultriventris*) using microsatellite markers in two different seasons

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ABSTRACT

In total,120 samples of adult common kilka were collected during the spring and summer seasons from two commercial catch stations in the South Caspian Sea .Fifteen sets of microsatellite primers developed from Clupeidae were tested on genomic DNA. Five primer sets (Cpa6, Cpa8, Cpa104, Cpa125, AcaC051) revealing polymorphic loci were used to analyze the genetic variation found in adults of the common kilka populations. Analyses revealed that the average number of alleles per locus was 13.1(range 5 to 22 alleles per locus in regions, Ne= 9.5). All sampled regions contained private alleles. The average observed and expected heterozygosities were 0.348 and 0.877, respectively. Deviations from Hardy-Weinberg equilibrium were seen in most cases. F_{ST},R_{ST}, *and gene flow estimates in AMOVA indicated significant genetic differentiation among seasons and regions (P≤0.01), indicating that the populations were divergent. The genetic distance between populations indicates that the genetic difference among the studied populations is pronounced. The data generated in this study provides useful information on the genetic variation and differentiation in populations of Caspian common kilka.*

Keywords: Microsatellite, Genetic variability, genetic differentiation, South Caspian Sea, *Clupeonellacultriventris*

INTRODUCTION

The common kilka *Clupeonella cultriventris* is a pelagic schooling species that inhabits the entire Caspian Sea and coastal zones of all Caspian littoral states. They migrate along the eastern and western sea shores, and mature at the age of 2 years old [1]. The common kilka is also regarded as an ecologically key species because it is an important food source for high-value fish such as sturgeon (*Acipenser* spp.; *Husohuso*) and seals (*Phocacaspica*). Kilka has been known to have the greatest commercial fishery potential in the Caspian Sea; its catches amounted up to 80% of the total fish catches in the sea over the last 50years. Prior to the invasion of the comb jelly *Mnemiopsisleidyi* and its mass outburst in 2000-2001, the state of kilka stocks has been satisfactory. Over the past decade, however, the kilka stock in Caspian Sea has drastically declined in number, believed to be mainly due to overfishing, overexploitation, invasion of *M. leidyi*, and environmental changes. Characterizing the population structure of common kilka may lead to a better understanding of the effect of overexploitation and ctenophore invasion, and providenew insights into the maintenance and efficient management of kilka resources [1].

Pelagic marine fishes generally show low levels of genetic differentiation among geographic regions due to the lack of physical barriers to genetic exchange and biological characteristics that include large population sizes and relatively long planktonic early life stages [2].

Here we describe the cross-amplification of Clupeidae for detecting fine-scale population genetic structure, variability and differentiation of *C. cultriventris*. So, the objectives of the present study areto investigate thegenetic structure of the common kilka and test the hypothesis that the common kilka has an identical population in different seasons and regionsofthe South Caspian Sea.

MATERIALS AND METHODS

SAMPLE COLLECTION AND DNA ISOLATION

A total of120 samples of adult fish were collected during the spring and summer seasons from two commercial catches inthe southwest Caspian Sea, Anzali port (37°29′N, 49°17′E) and middle of the South Caspian Sea, Babolsar port (36°42′N, 52°39′ E), Iran (Fig. 1).Briefly, fin clips were collected during from two seasons and two regions: 1- the Anzali water spring-spawner samples (SpAn; n = 30), 2- the Babolsar water spring-spawner samples (SpBa; n = 30), 3- the Anzali water summer-spawner samples (SmAn; n = 30), 4- the Babolsar water summer-spawner samples (SmBa; n = 30), (Figure 1) and tissue samples were preserved in 95% ethanol stored at room temperature.

DNA EXTRACTION MICROSATELLTE DATA SET

Genomic DNA was extracted from fin tissue using a high pure PCR Template preparation kit (Roach, Germany) according to the manufacturer's instructions. The quality and concentration of DNA were assessed by 1% agarose gel electrophoresis and spectrophotometry (CECIL model CE2040) and then stored at -20°C until use.

Genomic DNA was used as a template to amplify microsatellite loci by touchdown polymerase chain reaction (PCR). Totally 15 primer pairs were designedfor *Alosa*(*AsaC051, 059, 249, 334)* [3], *Clupea*(*Cpa6, 8, 100, 104, 107, 120, 134, 125*)[4]; (*1235, 1014*) [5], and *Sardina (SAR1.12*) [6].

For all primer sets, amplification wasperformed ina reaction volume of 25 µL containing 0.2 mM of dNTPs, 0.2–0.4 µM each primer, 200 ng of template DNA; 0.3–0.4 units of HotStarTaq^{TM} DNA polymerase; 1x HotStarTaq^{TM} PCR buffer and 2.5–4.5 mM $MgCl_2$.

Microsatellites were amplified (see Table 1 for specific annealing temperatures) using a MyCyclerthermocycler (BioRad). An initial denaturing step of 10minutes at 95°C was followed by amplification for 40 cycles withthe following conditions: 30 seconds at 95°C, 40–60 seconds at 51.5–59°C and 45–120 seconds at 70–72°C. A final 5-minute extension at 72°C completed the protocol (See table 1).PCR products were electrophoresed on 10% polyacrylamide gels (29:1 acrylamide: bis-acrylamide; 1X TBE buffer) and followed by silver-staining. Gels were run at 40 mA for 14h. Alleles were sized using Uvitec software, and each gel contained an allelic ladder (100bp) to assist with consistent scoring of alleles.

Data Analysis

Allele frequencies were estimated using *F*-statistics and Nei's genetic distance.The total genetic diversity (heterozygosity) within and among populations can be classified as follows: *Ho*= observed heterozygosity and *He*= expected heterozygosity. Hardy-Weinberg tests of equilibrium were estimated. Wright's *F*-statistics [7] as follows: F_{IS}= inbreeding coefficient within individuals relative to the subpopulation for each locus and common kilka sampling site were assessed; and F_{ST}= inbreeding coefficient within subpopulations relative to the total. $F_{ST}$and R_{ST} were calculated using analysis of molecular variance (AMOVA) to estimate genetic variation among populations and regions. AMOVA calculations and allelic richness (A_R) were performed on Arlequin 3.5 [8] using 10,000 permutations in each case.Nei's genetic identity and distance were determined using a pairwise, individual-by-individual genetic distance, with all codominant data computed in GeanAlex 6 software [9].

RESULTS

Amplification and banding patterns

Out of 15 sets of microsatellite primers, 10sets have not shown any flanking sites in thecommon kilka genome. Five sets of primers (*Cpa6, Cpa8, Cpa104, Cpa125,AcaC051*) were successfully amplified and showed polymorphic

pattern in the 120 individuals assayed (Table 1). All 5 microsatellite primers were able to produce DNA bands displaying a characteristic disomic banding pattern.

Genetic variation within sampling

A total of 133 alleles were identified in 120 individuals, 35 alleles in SpAn, 32 alleles SmAn, 34 alleles in SpBa, and 36 alleles in SmBa, with frequencies >0.05 in all samples. AsaC051showed the maximum variability ranging in frequency from 0.067 to 0.367. Allele sizes ranged from 104 to 402 bp(Table 1).The Na per locus ranged from 5 to 22, with an average of 13.1(±1.03). The number of alleles in *Cpa104* ranged from 15 to 17(A_R= 25.5), in *Cpa6* from 13 to 18(A_R= 27.8), in *Cpa8* from10 to 22(A_R= 20.1), in *Cpa125* from 10 to 17(A_R= 21.5), and in *AcaC051* from 5 to 7 (A_R= 7.1), with a tendency toward being fewer in the Babolsar (Spring and Summer) samples(Table 2).All sampled populations contained a significant number of private alleles.In total, 23 alleles were found,with the number of private alleles being SpAn 3 alleles, SmAn 4 alleles, SpBa7 alleles and SmBa 9 alleles, none of which was found in other seasons.

The *H*o and *H*e per locus ranged from zero to 0.967 and from 0.752 to 0.944, with an average of 0.348 (±0.06) and 0.877 (±0.01), respectively (Table 2). The Cpa6 locus had the highest level of heterozygosity, and lower heterozygosities were consistently observed in most samples screened, which may be due to the presence of null allelesor small sample sizes.

Estimates of inbreeding coefficient or F_{IS} values of five microsatellites were positive and between 0.299 at Cpa6 and 0.845 at Cpa104 (mean F_{IS} = 0.607 ±0.10; Table 2), and positive F_{IS} values a relative dearth of heterozygotes. However, Cpa6 had lower F_{IS} and higher heterozygosity than all loci in the populations assayed.In all cases, significant deviations from Hardy-Weinberg equilibrium (P≤ 0.01) were only found at one locus, which was in Hardy-Weinberg equilibrium in SmBasamples (Table 2). All departures from this equilibrium resulted from fewer heterozygotes than expected under equilibrium conditions (Table 2).

Pairwise population F_{ST} values and estimates of Nm

The *Nm*and F_{ST}via frequency ranged from 3.843 to 7.99 and from 0.030 to 0.061, with an average of 5.53 and 0.046, respectively (Tables 3, 4). In practice, $F_{ST}$is rarely larger than 0.5 and often very much less.F_{ST}, R_{ST} and gene flow estimates in *AMOVA* indicated significant genetic differentiation among seasons and regions (P≤0.01), indicating that the populations were divergent from each other. Values of pairwise R_{ST} among samples were consistently much higher (as much as an order of magnitude) than equivalent F_{ST} values (Table 4) but differences were not significant. Nei's genetic identity ranged from 0.430 to 0.701. Consequently, Nei's genetic distance ranged from 0.355 to 0.843 [10](Table 5).

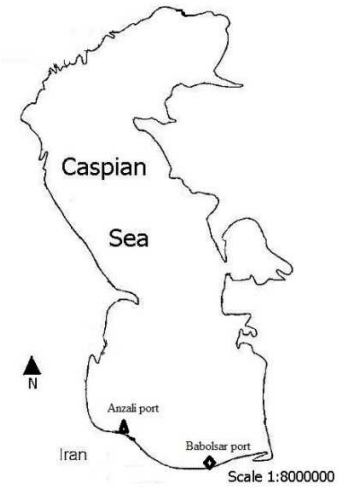

Figure 1 Map showing sampling locations of populations of *Clupeonellacultriventris*: Anzali port (▲) and Babolsar port (♦)

Table 1.Loci, repeat motif, Actual size (bp), gene bank number and primer sources used at present study in commomkilka

Loci	Repeat motif	Gene bank no	Touchdown protocol Actual size (bp)	Primer sources
Cpa6	(GATA)14	AF309801	52°C/40 104-216	Miller et al. 2001
Cpa8	(GACA)27	AF309804	52°C/40 104-216	
Cpa104	(TG)54	AF309791	51.5°C/40 312-390	
Cpa125	(GA)32i (GT)26	AF309796	59°C/40 216-280	
AsaC051	(GAAT)7 (GTAT)13	EF014992	54°C/40 160-180	Julian &Barton, 2007

Table 2. Summary of genetic variability of the studied populations

	Anzali		**Babolsar**		
	Spring	**Summer**	**Spring**	**Summer**	**Average**
Locus /n	*30*	*30*	30	30	
Cpa6					
Na(Ne)	18(12.4)	16(10.1)	14(9.7)	13(9.7)	15.2
Ho(He)	0.267(0.919)	0.400(0.901)	0.9(0.897)	0.967(0.897)	0.633(0.904)
A_R	18	16	14	13	27.8
Fis ($Signif_{HW}$)	0.710***	0.556***	-0.003*	-0.077*	0.299
Cpa8					
Na(Ne)	16(11.653)	22(17.8)	10(7.2)	13(9.89)	15.2
Ho(He)	0.200(0.913)	0.200(0.944)	0.7(0.826)	0.9(0.899)	0.5(0.904)
A_R	16	22	10	13	20.1
Fis ($Signif_{HW}$)	0.781***	0.788***	0.188*	-0.001ns	0.447
Cpa104					
Na(Ne)	17(11.6)	16(10.2)	15(12.7)	17(12.3)	16.2
Ho(He)	0.133(0.914)	0.067(0.902)	0.200(0.922)	0.167(0.919)	0.142(0.914)
A_R	17	16	15	17	25.5
Fis ($Signif_{HW}$)	0.854***	0.926***	0.783***	0.819***	0.845
Cpa125					
Na(Ne)	10(7.7)	17(13.5)	11(6.5)	14(9.045)	13
Ho(He)	0.067(0.871)	0.200(0.926)	0.467(0.848)	0.367(0.889)	0.275(0.884)
A_R	10	17	11	14	21.5
Fis ($Signif_{HW}$)	0.923***	0.784***	0.450***	0.588***	0.689
AsaC051					
Na(Ne)	5(4.2)	6(5)	6(4.7)	7(4)	6
Ho(He)	0(0.762)	0(0.800)	0.300(0.79)	0.467(0.752)	0.192(0.776)
A_R	5	6	6	7	7.1
Fis ($Signif_{HW}$)	1***	1***	0.621 ***	0.380*	0.753
Allele frequency >0.05	35	32	34	36	
Total of alleles	66	77	56	64	
Average					
Na(Ne)	13.2(9.5)	15.4(11.3)	11.2(8.2)	12.8(9)	13.1(9.5)
Ho(He)	0.133(0.876)	0.173(0.895)	0.513(0.864)	0.573(0.871)	0.348(0.877)
Fis	0.854	0.811	0.408	0.341	0.607(±0.076)

*n: number of samples, Na : number of alleles, Ne : effective number of alleles, A_R: allelic richness , Ho : observed heterozygosity, He : expected heterozygosity, Fis : fixation index, loci in accordance with H-W unequilibrum *P< 0.05 ; **P<0.01; P<0.001; n.s., non-significant using 5 sets of microsatellite primers.*

Table 3.F-Statistics and estimates of Nm over all populations for each locus

	Cap6	**Cap8**	**Cap104**	**Cap 125**	**Asac051**	**Average**
F_{ST}	0.061	0.038	0.044	0.057	0.03	0.046
Nm	3.843	6.322	5.404	4.11	7.99	5.53

Table 4. Pair wise estimates of genetic differentiation detected at 5 loci in common kilka samples, using F_{ST} values (above diagonal) and R_{ST} (below diagonal)

		Fst			
	Samples	**SpAn**	**SmAn**	**SpBa**	**SmBa**
Rst	**SpAn**		0.019	0.033	0.035
	SmAn	0.126		0.035	0.036
	SpBa	0.643	0.663		0.030
	SmBa	0.566	0.602	0.052	

Table 5. Genetic Distance (Nei, 1972) detected at 5 loci in common kilka samples

		Genetic Identity			
	Samples	**SpAn**	**SmAn**	**SpBa**	**SmBa**
Genetic Distance	**SpAn**		0.701	0.540	0.501
	SmAn	0.355		0.467	0.430
	SpBa	0.616	0.762		0.586
	SmBa	0.69	0.843	0.534	

DISCUSSION

Five out offifteen primer sets designed originally from American shad (*Alosasapidissima*) and Pacific herring (*Clupeapallasi*) DNA sequences (see table 1) amplified in *Clupeonellacultriventris* indicate a high degree of conservation of primer sites between two species of *Clupea* and *Clupeonella*. These results suggest that there is evolutionary conservation of the flanking regions for these loci among related taxa. The cross-amplification between American shad, Pacific herring and The Caspian Sea's common kilka is consistent with earlier findingsclosely related [3,4]. Totally ten sets of primers were not amplified in the PCR reaction. There is a significant and negative relationship between microsatellite performance and evolutionary distance between the species. The proportion of polymorphic loci among those markers that amplified decreased with relatively high genetic distance [11].

Compared with the heterozygosity between regions, the heterozygosity in samples from Babolsar Port was higher than Anzali Port's samples (Table 2). The genetic diversitywas relatively low, and the population structure and resource have significantly declined,especially in Anzali Port's samples. In fact, although the populations do not differ in the amount of genetic variation expressed as heterozygosity or alleles per locus, they are very different in the nature of the genetic variation, which depends on the private alleles and genotypes.The heterozygosity of the common kilka in this study was lower (Table 2) compared to other Clupeidae species such as Pacific sardine (0.667 to 0.967)[12]; (0.522 to 0.903)[5]; Pacific herring (0.46 to 1)[4]; Sardine (0.772) [6]; American shad (0.522 to 0.903)[3].Unfortunately, introduction of *Mnemiopsisleidyi*and overfishing have been the main factors for such a huge resource loss. Undoubtedly, the kilka stock in the Caspian Sea is in a critical condition and is suffering more damage every year. Recently, unfavorable hydrological conditions related to climate change have had a negative impact on the sustainability of exploitable yield of kilka; the thermal structure of the upper layers of the sea has been detrimental to the species, and there has also been poor vertical mixing of surface and deeper water [1].

The losses of alleles and heterozygosity may increase with bottlenecking and inbreeding. Positive inbreeding coefficient values are implied by a relative dearth of heterozygotes.Whether the explanation for decreased levels of heterozygosity is biological. On the other hand, reduced genetic diversity may increase the susceptibility to disease and other selective factors, resulting in further declines in population size [13]. A heterozygote deficiency can also be attributable to other phenomena including inbreeding, or population admixture (Wahlund effect).

In the present study, deviation from the H-W equilibrium was observed for most loci ($P<0.001$).The significant deviations from H-W equilibrium could be explained either by sample bias, the Wahlund effect, not using species specific primers.

F_{ST}represents the degree of population genetic differentiation, that is, the proportion of the total genetic diversity (~heterozygosity) that separates the populations. The range of F_{ST} via frequency for codominant data was 0.021–0.055. In fact, in the great majority of cases, F_{ST} is low, because the effect of polymorphism (due to mutations) drastically deflates F_{ST} expectations [14]. In fish, negative correlations have been demonstrated between F_{ST} values and dispersal capability. On this basis, the Caspian Sea common kilka might present high dispersal capability, presumably due to the absence of physical or ecological barriers to individuals. However, the loss of genetic

variability also might be caused by sampling errors contributing to the loss of regional genetic differentiation.F_{ST}andR_{ST}via AMOVA for Codominant Data in all sampling site were significant (P≤0.01), suggesting that at least four populations are genetically differentiated and do not represent a single panmictic population.The most important finding of the present study was the degree of genetic structuring found in the spring and summer populations of the South Caspian Sea area. All tests showed these samples are genetically identical to a degree that suggests low gene flow. The range of genetic distance between populations was 0.355–0.843. [15,16] showed that genetic distance values [10] for conspecific populations averaged 0.05 (range: 0.002–0.07) and for congeneric species averaged 0.30 (range: 0.03–0.61). The distance value obtained in the present studyare above the average value of congenerics, indicating that the genetic difference among the studied populations is pronounced.

CONCLUSION

In summary, this study provides preliminary evidence for the existence of at least four differentiated populations in the South Caspian Sea. The existence private alleles and significant F_{ST} and R_{ST} confirm that spring and summer populations in Anzali and Babolsar Ports. Probably, extra populations are present in the Caspian Sea; therefore, a comprehensive investigation using more samples from the entire Caspian Sea may confirm this hypothesis. Characterizing the genetic structure of common kilka in the fishery industry will help and improve the future management and conservation of the unique species. The losses of genetic diversity may increase with bottlenecking. So we should protect the genetic diversity by reducing pollution, controlling Catch Per Unit Effort (CPUE) and control the *Mnemiopsis* proliferation. Apart from *Mnemiopsis*, possible drivers of the kilka's stock decline could be of natural origin and/or various anthropogenic activities related to overfishing, pollution, and habitat loss.

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