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Full Length Research Paper

Population genetic study on common kilka (*Clupeonella cultriventris* Nordmann, 1840) in the Southwest Caspian Sea (Gilan Province, Iran) using microsatellite markers

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This study represents population genetic analysis of the common kilka *Clupeonella cultriventris* (Nordmann, 1840) in the southwest Caspian Sea (Gilan Province). A total of 60 specimens of adult common kilka were sampled from two seasons (spring and summer), 2010. Fifteen pairs of microsatellites previously developed for American shad (*Alosa sapidissima*), Pacific herring (*Clupea pallasi*), Atlantic herring (*Clupea harengus*) and Sardine (*Sardina pilchardus)* were tested on genomic DNA of common kilka. Alleles frequencies, the fixation index R_{ST}, observed and expected heterozygosity were determined at disomic loci amplified from fin tissue samples. Five pairs of primers (Cpa6, Cpa8, Cpa104, Cpa125 and AcaC051) as polymorphic loci were used to analyze the genetic variation of the common kilka population. Analyses revealed that an average of alleles per locus was 14.4 (range 5 to 21 alleles per locus in regions). All sampled regions contained private alleles. The average observed and expected heterozygosity were 0.153 and 0.888, respectively. All loci significantly deviated from Hardy-Weinberg equilibrium (HWE). Based on AMOVA, R_{ST} values was found to be 0.113 (Nm=1.96, P<0.01). The genetic distance between populations was 0.344, which indicates that the genetic difference among the studied populations is pronounced. These results support the existence of different genetic populations along the Caspian Sea coast (Guilan Province).

Key words: Population genetic, Southwest Caspian Sea, microsatellite, *Clupeonella cultriventris*.

INTRODUCTION

Common kilka, *Clupeonella cultriventris*, belongs to the family Clupeidae, lives in the Caspian Sea, feeds on zooplankton, crustaceans such as copepods, cladocerans and spawn in spring (Abdoli and Naderi, 2009; http://www.fishbase). Common kilka is faced with the challenges resulting from overfishing and invader Ctenophore *Mnemiopsisleidyi* (Karimzadeh, 2011; Velikova et al*.,* 2012). Kilka fishing is an important source of income and protein for Iranians inhabiting in the Caspian Sea's coastal regions. The collapse of kilka fisheries has adverse effects on both the economy and regional protein consumption. Between the years 1989 and 1998, the number of fishing vessels and fishing activities increased progressively and Iran increased its quota of kilka catches to 95000 mt up until 1999.In the next few years, however, the catch sharply decreased to 19500 mt in 2004 due to overexploitation and invasion of jelly fish *Mnemiopsis leidyi*.

During the past 30 years the environmental status of the Caspian Sea changed significantly due to the impacts of various factors, such as fluctuations in sea level and pollution of various toxicants (Ivanov,2000). Recent introductions of invasive species via ballast water from ships have also had a negative impact on fish stocks in the Caspian Sea (Ivanov et al. 2000). In particular, an invasive jellyfish (Ctenophora, *Mnemiopsis leidyi*), which had appeared by November 1999 (Ivanov et al. 2000), affected kilka stocks (Fazli, 2011; Daskalov

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Table 1. Loci, repeat motif, primers sequence, gene bank number, touchdown protocol, PCR product size range (bp) and primer sources used at the present study in common kilka.

Loci	Repeat motif	Primer sequence	GenBank No.	Touchdown protocol / Actual size (bp)	Primer sources
Cpa6	(GATA)14	F: 5'-GTGTGAGTTTGCTCCAAA-3' R: 5'-GTTTGTACCAATGAATGATTACAA-3'	AF309801	52 °C/[40] 104 - 216	Miller et al. (2001)
Cpa8	(GACA)27	F: 5'-GATCCTTCTTTTAAGGAAAA-3' R: 5'-GTTTGACAGAACTTACTATCTCAGA-3'	AF309804	52°C/[40] 104 - 216	
Cpa104	(TG)54	F: 5'-ACGTAGGCGCAGACAT-3' R: 5'-GTTTGCTCAAGTCAATGTGATTTTTA-3'	AF309791	51.5°C/[40] 312 - 390	
Cpa125	(GA)32i (GT)26	F: 5'-GCAAGAAAGAGCAGCAGA-3' R: 5'-GTTTCGACTCAACAGCTGGAA-3'	AF309796	59°C/[40] 216 - 280	
AsaC051	(GAAT)7 (GTAT)13	F: 5'-GTAAGTCGCTTTGGACTACCAG-3' R: 5'- TCTAAATGCCCAGGTAAAGATG-3'	EF014992	54°C/[40] 160 - 180	Julian and Barton (2007)

and Mamedov, 2007). Because of the recent decline in common kilka populations, several management actions have been implemented, which include the closure of select fisheries (Brown et al. 2000). For the sustainable management of this unique species, characterization of genetic variability of wild stocks is essential. Molecular genetic studies on the common kilka in the Caspian Sea were so far limited to a few studies using RFLP (Lalouei et al., 2006). Microsatellites recently have become an extremely popular marker type in a wide variety of genetic investigations (Sekar et al., 2009). Microsatellites are abundantly distributed across the genome, demonstrate high levels of allele polymorphism and can easily be amplified with polymerase chain reaction (PCR).

These features provide the underlying basis for their successful application in a wide range of fundamental and applied fields of fisheries and aquaculture (Sekar et al., 2009). Microsatellite genotypes are particularly helpful to detect structure in closely related populations, regardless of whether they are in an evolutionary equilibrium. Additionally, primers designed for one species can often be used with other related species (Chistiakov et al., 2005).

Recently, many microsatellite loci were used to investigate the genetic structure of various Clupeidae species including Atlantic herring (Shaw et al., 1999; McPherson et al., 2001), Sardine (Gonzalez and Zardoya, 2007a), Pacific sardine (Pereyra et al., 2004), Pacific herring (Miller et al., 2001; Semenova et al., 2012) and American shad (Julian and Barton, 2007).

The objectives of the present study were to investigate on genetic structure of common kilka and also to test the hypothesis that common kilka has identical population in different seasons in the Southwest Caspian Sea.

MATERIALS AND METHODS

Sample collection and DNA isolation

A total of 60 specimens of adult common kilka were sampled from a single sampling location, Anzali port (37° 29′ N, 49° 17′ E; Iran), but the fish were caught during different seasons (spring and summer) and preserved in 95% ethanol.

DNA extraction

Genomic DNA was extracted from the fin tissue using High Pure PCR Template Preparation Kit (Roche Applied Science, Germany) according to manufacturer's instructions. The quality and concentration of DNA were assessed by 1% agarose gel electrophoresis and spectophotometry (CECIL model CE2040) and stored at −20°C until use.

Microsatellite data set

Genomic DNA was used as a template to amplify microsatellite loci by touchdown PCR. Totally, 15 primer pairs were designed for *Alosa* (AsaC051, 059, 249, 334, Julian and Barton, 2007), *Clupea* (Cpa6, 8, 100, 104, 107, 120, 134, 125, Miller et al., 2001; 1235, 1014, McPherson et al., 2001) and *Sardina* (SAR1.12, Gonzalez and Zardoya, 2007a).

For all primer sets, amplification was performed in a reaction volume of 25 µl containing 0.2 mM of deoxynucleotide triphosphates (dNTPs), 0.2 to 0.4 µM of each primer, 200 ng of template DNA; 0.3 to 0.4 unit of HotStarTaq^{TM} DNA polymerase; 1x HotStarTaq^{TM} PCR buffer and 2.5 to 4.5 mM of $MgCl_2$. Microsatellites were amplified (Table 1 for specific annealing temperatures) using a thermocycler (MyCycler, BioRad). An initial denaturing step of 10 min at 95°C was followed by amplification for 40 cycles at the following conditions: 30 s at 95°C, 40 to 120 s at 51.5 to 59°C and 45 to 120 s at 70 to 72°C. A final 5-min extension at 72°C completed the protocol (Table 1).

PCR products were separated on 10% polyacrylamide gels (29:1 acrylamide: bis-acrylamide; 1×TBE buffer) followed by silver staining. Gels were run at 40 mA for 14h. Alleles were sized using

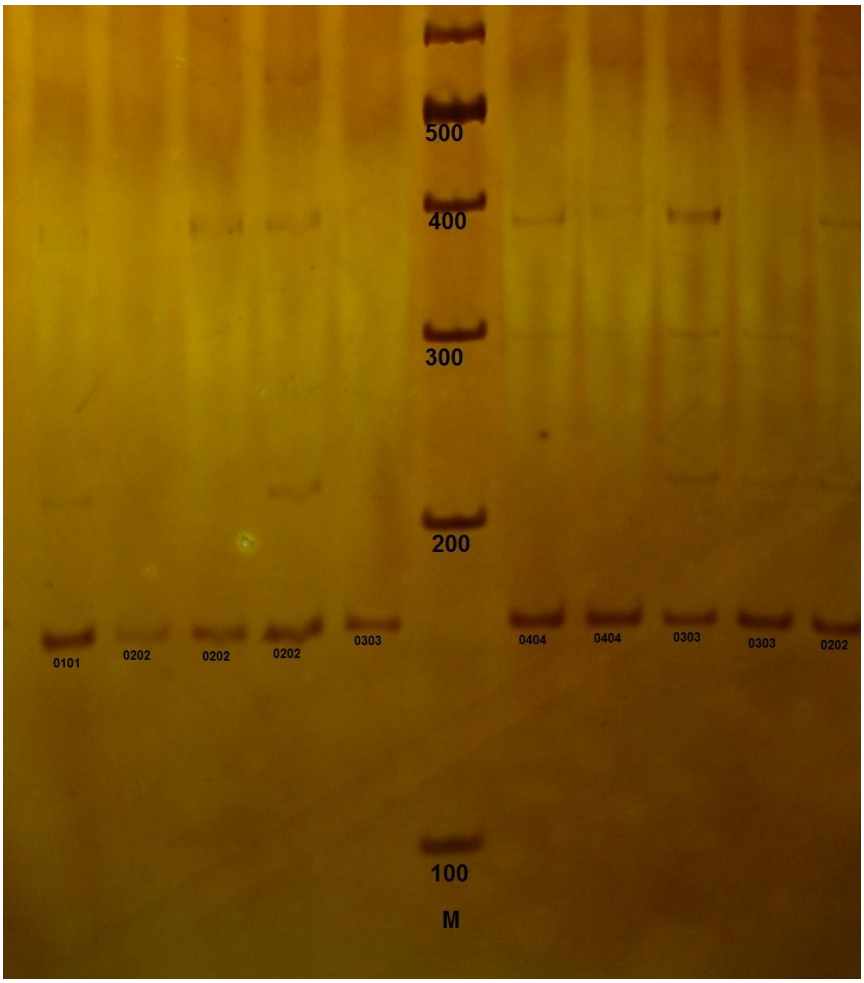

Figure 1. Microsatellite banding profile of *C. cultriventris* from Anzali spring using primer pair AcaC051.

Uvitec software, and each gel contained an allelic ladder (100 bp) to assist in consistent scoring of alleles.

Data analysis

Allelic frequencies, observed and expected heterozygosities, genetic distance (Nei, 1972), genetic identity (Nei, 1972), F_{IS}, F_{ST} and R_{ST} value, Nm, Hardy-Weinberg (HW) tests of equilibrium, analysis of molecular variance (AMOVA) via codominant data were computed in the GeanAlex 6 software (Peakall and Smouse, 2006).

RESULTS

Amplification and banding patterns

Of the 15 pairs of microsatellite primers, 10pairs did not show any flanking sites on the common kilka genome. Five pairs of primers (Cpa6, Cpa8, Cpa104, Cpa125 and AcaC051) were amplified successfully and they showed polymorphic pattern in the 60 individuals assayed. All microsatellite primers that were able to produce DNA bands displayed a characteristic disomic banding pattern (Figure 1).

Genetic variation within sampling

The total number of alleles found in each population ranged from 68 in spring to 73 in summer (Table 2). Out of 93 observed alleles, in spring and summer, 35 and 33 alleles, respectively occurred at frequencies of >0.05 in all samples. AsaC051 showed the maximum variability ranging in frequency from 0.067 to 0.333. All sampled populations contained private alleles at the significant level (Table 2). For example, marker Cpa125 identified 4 private alleles; 3 private alleles for spring samples (one at a frequency 0.133 and the others at 0.117 and 0.067), and 1 private allele for summer samples (at a frequency 0.083). Cpa104 identified 3 private alleles; 2 private alleles for spring samples and 1 private allele for summer samples (all at a frequency 0.067). Cpa8 identified 2 private alleles; 1 private allele for spring samples (at a

Table 2. PCR product size range (bp), using five pairs of microsatellites.

Locus/n	Spring	Summer	Average
	30	30	
Cpa6			
Na(Ne)	19 (14)	16 (9.7)	17 (11.8)
Ho(He)	0.267 (0.929)	0.400 (0.897)	0.333 (0.913)
Fis	0.713	0.554	
Cpa8			
Na(Ne)	17 (12)	21 (17.8)	19 (15)
Ho(He)	0.200 (0.917)	0.200 (0.944)	0.200 (0.930)
Fis	0.782	0.788	
Cpa104			
Na(Ne)	17 (11.6)	17 (11.6)	17 (11.6)
Ho(He)	0.133 (0.914)	0.100 (0.914)	0.117 (0.914)
Fis	0.854	0.891	
Cpa125			
Na(Ne)	10 (7.7)	16 (13.2)	13 (10.49)
Ho(He)	0.067 (0.871)	0.167 (0.924)	0.117 (0.898)
Fis	0.923	0.820	
AsaC051			
Na(Ne)	5 (4)	6 (5.2)	5.5 (4.66)
Ho(He)	0.756	0 (0.809)	0 (0.783)
Fis	1	1	
Allele frequency >0.05	35	33	
Total of alleles	68	73	
Average			
Na(Ne)	13.6 (10)	15.2 (11.5)	14.4 (10.72)
Ho(He)	0.133 (0.877)	0.173 (0.898)	0.153 (0.888)

n, Number of samples; Na, number of alleles; Ne, effective number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; Fis : fixation index using five primer pairs microsatellite.

frequency 0.083) and 1 private allele for summer samples (at a frequency 0.067). Cpa6 identified 3 private alleles; 1 private allele for spring samples (at a frequency 0.083) and 2 private alleles for summer samples (both at a frequency 0.167). AsaC051 identified 1 private allele for summer samples (at a frequency 0.067), neither of which was found in other seasons (Table 3).

Average observed and expected heterozygosity were 0.153 and 0.888, respectively, and ranged of observed heterozygosity from 0 in two seasons (at AsaC051) to 0.4 in summer (Cpa6) (Table 2), and lower observed heterozygosities (H_E) than expected consistently in all samples screened, which may be due to the presence of null alleles. In all cases, significant deviations from Hardy-Weinberg equilibrium (HWE) were significant at ($P \leq 0.001$), (Table 2). All of the departures from HWE resulted from fewer heterozygotes than expected under equilibrium conditions. Estimate of inbreeding coefficient or F_{IS} values of five microsatellite loci were positive (0.635 Cpa6, 0.785 Cpa8, 0.872 Cpa104, 0.870 Cpa125 and 1.00 AcaC051; Table 4), positive F_{IS} values a relative dearth of heterozygotes. Pairwise population F_{ST} value was 0.018. Based on AMOVA, R_{ST} values was found to be 0.113 ($Nm=1.96$, $P<0.01$). The genetic distance (Nei, 1972) between sampling seasons was 0.344.

DISCUSSION

The study of population genetic variation of marine pelagic fish species has proven to be particularly challenging because of the biological peculiarities of these fishes including large effective population sizes and high dispersal capacities, as well as the apparent lack of

Table 3. Number of private allele, actual size (bp) and allele frequency in spring and summer seasons.

Seasons	Parameter	Cap6	Cap8	Cap104	Cap 125	Asac051	Total
Spring	Number of private allele (actual size)	1 (164)	1 (144)	2 (334, 364)	3 (216, 230, 248)	-	7
	Allele frequency	0.083	0.083	0.067,0.067	0.067, 0.133, 0.117		
Summer	Number of private allele (actual size)	2 (120, 124)	1 (188)	1 (388)	1 (260)	1 (180)	6
	Allele frequency	0.0167	0.067	0.067	0.083	0.067	

Table 4. F-statistics and estimates of Nm over all populations for each locus using five pairs of microsatellites.

Parameter	Cap6	Cap8	Cap104	Cap 125	Asac051	Average
F_{ST}	0.025	0.016	0.013	0.026	0.010	0.018
Nm	9.66	15.08	19	9.4	25.14	05.64

physical barriers to gene flow in the marine realm (Gonzalez and Zardoya, 2007b). Microsatellites are nuclear markers with higher mutation rates that have been proved to be more efficient and informative for detecting fine-scale population structure in marine pelagic fishes (Gonzalez and Zardoya, 2007b).

Although DNA depended methodology such as microsatellite loci is an important tool in fisheries management and aquaculture, the application of population genetic data to management in Caspian Sea common kilka is in its early stage and little information is available about the genetic population structure subdivision. This is the first report of a microsatellite analysis of population structure study in common kilka. Five out of 15 primer sets designed originally from American shad (*Alosa sapidissima*) and Pacific herring (*Clupea pallasi*) DNA sequences (Table 1) amplified in *C. cultriventris* indicates 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 findings closely related species of fish (Julian and Barton, 2007; Miller et al., 2001). Totally, 10 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 were amplified decreased with increasing genetic distance (Cui et al., 2005).

The average number of alleles per locus and observed H_E were comparable in Caspian Sea's populations as reported earlier using RFLP analysis of the same populations (Lalouei et al., 2006). All five loci tested were highly polymorphic in *C. cultiventris*, with 6 to 23 alleles per locus over all samples, and observed heterozygosities (Ho) within samples ranging from 0 to 0.4 (mean = 0.153), expected heterozygosities (H_E) within samples ranging from 0.756 to 0.944 (mean = 0.888). Allele size ranges and levels of polymorphism at all five loci within the spring common kilka samples are very similar to those observed in the summer common kilka sample, and also to those reported previously for common kilka in South Caspian Sea (Lalouei et al., 2006). The other studies of marine fish to date have shown similar levels of microsatellite polymorphism for H_E but not in Ho in Atlantic herring (Ho ranging from 0.65 to 0.98, H_E ranging from 0.90 to 0.93; Shaw et al., 1999), sardine (Ho = 0.772, H_E = 0.94; Gonzalez and Zardoya, 2007a), Pacific sardine (Ho ranging from 0.667 to 0.967, H_E ranging from 0.606 to 0.959, Pereyra et al., 2004), Atlantic herring (Ho ranging from 0.522 to 0.903, H_E ranging from 0.743 to 0.948, McPherson et al., 2001), Pacific herring (Ho ranging from 0.46 to 1, H_E ranging from 0.743 to 0.948, Miller et al., 2001) and American shad (Ho ranging from 0.522 to 0.903, H_E ranging from 0.743 to 0.948, Julian and Barton, 2007).

In fact, although the populations do not differ in the amount of genetic variation expressed as expected heterozygosity or alleles per locus, they are very different in the nature of the genetic variation, which depends on the private alleles and genotypes. Unfortunately, the Caspian stocks of kilka, is faced with the challenges resulting from overfishing and invader Ctenophore *M. leidyi* (Karimzadeh, 2011). During recent years, in Guilan region, the catch amounts of kilka fishes in the Caspian Sea have decreased the increasing fishing effort, overfishing, intrapogenic and natural factors are the main reasons for decreasing kilka fishes in the Southeast Caspian Sea in Guilan region. The losses of alleles and heterozygosity may increase with bottlenecking and inbreeding. On the other hand, reduced genetic diversity may increase the susceptibility to disease and other selective factors, resulting in further decline in population size (Shen and Gong, 2004). A heterozygote deficiency

can be attributable to other phenomena including inbreeding, population admixture (the Wahlund effect) or the presence of a non-expressed (non-amplifying or null) allele. At the present study, deviation from the HWE observed in all loci was significant ($p<0.001$). The significant deviations from HWE could be explained either by sample bias or not using species specific primers, the presence of null alleles in these populations. In the presence of null alleles, heterozygotes possessing a null allele could be erroneously recorded as homozygotes for the variant allele leading to a deficiency of heterozygotes in the respective population. Similar results have been reported in Pacific sardine (Pereyra et al., 2004), American shad (Julian and Barton, 2007), Sardine (Gonzalez and Zardoya, 2007a), Pacific herring (Miller et al. 2001), Atlantic herring (Shaw et al., 1999) and it also may be related to sampling from mixtures of migrating population.

F_{ST} and R_{ST} are very commonly used to describe population differentiation at various levels of genetic structuring. In our study, F_{ST} was 0.018, it has been suggested that a value lying in the range 0 to 0.05 indicates little genetic differentiation (Balloux et al., 2002) and R_{ST} in all sampling site was significant ($P \leq 0.01$), suggesting that at least two populations are genetically differentiated and do not represent a single panmictic population.

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 (Balloux et al., 2002). In fish, negative correlation has been demonstrated between F_{ST} values and dispersal capability (Waples, 1987). Marine species often have low levels of genetic differentiation, because few migrants per generation are sufficient to eliminate genetic evidence of stock structure (Waples, 1998) and marine species generally have high fecundities and dispersal abilities. On these bases, 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 error which may also contribute to the loss of regional genetic differentiation.

The most important finding of the present study was the degree of genetic structuring indicated within the spring and summer population of the Caspian Sea area. All tests showed that these samples are genetically identical to a degree which suggests high gene flow.

The genetic distance between populations was 0.344. Shaklee et al. (1982) and Thorpe and Sol-Cave (1994) showed that genetic distance values (Nei, 1972) for conspecific populations averaged 0.05 (range: 0.002 to 0.07) and for congeneric species averaged 0.30 (range: 0.03 to 0.61). The distance value obtained in the present study falls within the average value of congenerics, which indicates that the genetic difference among the studied populations is pronounced. In summary, this study provides preliminary evidence for the existence of at least two differentiated populations in the Southwest Caspian Sea existence private alleles and significant R_{ST} confirm spring and summer populations. Probably, extra populations should be present in the Caspian Sea; therefore, investigation using more samples may prove such finding. Characterizing the genetic structure of common kilka currently being used in the fishery industry help and improve the management and conservation of the unique species.

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