

[Research]

## A study on genetic differentiation in two species of Iranian bleaks, *Alburnus mossulensis* and *Alburnus caeruleus* (Teleostei, Cyprinidae) using simple sequence repeats

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### ABSTRACT

The genetic structure of the genus *Alburnus* is not well known and the phylogenetic relationships among its species are uncertain. In the present study, simple sequence repeats (SSRs or microsatellites) were used to evaluate genetic diversity and genetic differentiation between *Alburnus mossulensis* Heckel, 1843 from Kashgan River in Lorestan province and *A. caeruleus* Heckel, 1843 from Gamasiab River in Kermanshah province. Thirty specimens from each species were collected and their genomic DNA was extracted. Polymerase chain reaction was performed using four pairs of SSR markers, including CypG24, BL1-2b, BL1-98 and Rser10, from which a total of 480 bands were amplified. The average observed and expected heterozygosities for both species were similar. In both species, except for Rser10 locus, all loci deviated from the Hardy-Weinberg equilibrium ( $P < 0.05$ ). Average genetic distance and *Fst* values between the two species were 0.361 and 0.04, respectively. Analysis of molecular variance (AMOVA) revealed more interspecific (94%) than intraspecific (4%) genetic variation. Although four sets of SSR markers developed for other cyprinids showed high level of polymorphisms in the Iranian bleaks, they showed low genetic differentiation between them. Study on the possibility of genetic differentiation of the examined species by more microsatellite loci or other molecular markers such as amplified fragment length polymorphisms (AFLP) are recommended.

**Keywords:** *Alburnus mossulensis*, *Alburnus caeruleus*, Bleak, Genetic differentiation, Molecular marker

### INTRODUCTION

The cyprinid genus, *Alburnus* Rafinesque, 1820 comprises seven confirmed species from Iranian waters, among which three species including *Alburnus caeruleus* Heckel, 1843, *Alburnus mossulensis* Heckel, 1843 and *Alburnus zagrosensis* Coad, 2009 are present in the Tigris-Euphrates basin. *Alburnus caeruleus* is found in the Tigris-Euphrates and Quwayq River systems (Coad, 2013), while *A. mossulensis* is widely distributed in the Tigris-Euphrates and adjacent basins such as Bushehr, Fars and upper reaches of the Hormuz basins (Keivany *et al.*, 2015). *Alburnus mossulensis* is not considered a trade fish; however, it is used for consumption in some areas of Iraq (Coad, 2013). Morphometric and meristic traits were used to identify these two species. *Alburnus*

*caeruleus* is distinguished from *Alburnus mossulensis* by fewer scales along the lateral line (43-58 vs. 58-89) and a deeper body (2.9-3.5 in standard length) (Coad, 2013; Keivany *et al.*, 2015). In some cases, diagnosis of the species based on morphological traits is very difficult and confusing due to the environmental impact, human judgment and insufficient expertise. Therefore, more robust characters rather than morphological traits are needed to separate these species from each other. Several molecular markers such as Restriction Fragment Length Polymorphisms (RFLP), simple Sequence Repeats (SSRs) and recently, Single Nucleotide Polymorphisms (SNPs) have been developed for different purposes in aquaculture and fisheries sciences (Liu, 2007). Among them SSRs or microsatellite

received much more attention during the last decade because of some advantages including Mendelian fashion inheritance, codominant nature and the highest polymorphic information content (Liu & Cordes, 2004). The marker was frequently applied for the analysis of genetic diversity, population genetic structure, classification and systematic, parentage identification, germplasm conservation and breeding programs (Liu & Cordes, 2004; Balloux & Lugon-Moulin, 2002; Zhang *et al.*, 2006; Liu, 2007). Recently, Shirangi *et al.* (2011) used 5 polymorphic microsatellite loci for genetic differentiation between two migratory forms of the Caspian brown trout, *Salmo trutta caspius*. Because of the some similarities in morphometric and meristic characteristics of two species of bleaks which commonly indwell together in many Iranian water bodies, the present study was conducted to study the genetic relationship between *Alburnus mossulensis* and *A. caeruleus* using highly polymorphic SSRs loci.

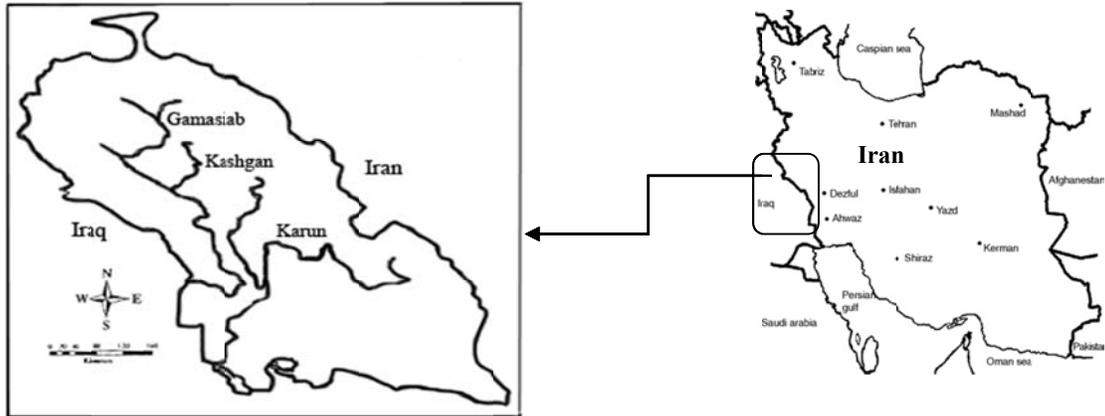
#### MATERIALS AND METHODS

A total of 60 specimens (30 from each) were collected in summer 2009 from two major rivers of the Tigris-Euphrates basin in Iran. Gamasiab River in Kermanshah Province (47° 26' 51" E, 34° 24' 20" N) and Kashgan River in Lorestan Province (47° 57' 49" E, 33° 22' 31" N) were the sampling sites for *A. caeruleus* and *A. mossulensis*, respectively (Fig. 1). The fishes were identified using the available literatures (Coad, 2013; Keivany *et al.*, 2015). For DNA extraction, about 2-3 g of the fish caudal fins was preserved in 96% ethanol at 4°C.

Genomic DNA was extracted using FastPure™ DNA kit based on the manufacturer manual (Takara Bio Inc. Japan). The quality and quantity of the extracted DNA were determined by running on 1% agarose gel and spectrophotometry, respectively (Sambrook *et al.*, 1989). Four microsatellite loci CypG24, BL1-2b, BL1-98 and Rser10

(Dubut *et al.*, 2010) were used. For Polymerase chain reaction (PCR) was performed in a 25 µl reaction mixture containing 4-40 ng/µL of DNA, 0.4 mM primers, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each nucleotides (dNTPs) and 1-1.5 unit *Taq* DNA polymerase (Cinnagen, Iran). Thermocycling parameters were 5 min at 94 °C for an initial denaturation, 35 cycles of denaturation at 95°C for 1 min, primer annealing at 56°C for 30 seconds and extension at 72 °C for 1 min followed by a final extension step at 72 °C for 10 min (Table 1). The PCR was followed by electrophoresis of the products in 12% polyacrylamide gel. The DNA fragments were visualized by silver staining method (Sambrook *et al.*, 1989).

Allelic variations at the four microsatellite loci in the two species were determined as number of alleles per locus (A) and heterozygosity (H) using POPGENE 3.1 (Raymond & Rousset, 1995). To test for departures from Hardy-Weinberg equilibrium (HWE), comparisons were made between the observed heterozygosity (Ho), and expected heterozygosity (He) using exact tests implemented in POPGENE 3.1. This software employs the Markov chain method to estimate the probability of significant deviation from HWE (Rice, 1989) and to determine heterozygote deficiencies per locus for each population by comparing the observed and expected heterozygosities for deviations from the HWE. The expected frequencies of null alleles were estimated using equation of Brookfield (1996),  $r = (He - Ho) / 1 + He$ . Genetic differences between the two species were evaluated by calculating pairwise  $F_{st}$  values and testing their significance by bootstrapping analysis (1000 replicates) using ARLEQUIN 2.000 (Schneider *et al.*, 2000). Genetic distances between the species were estimated by the DA distance of Nei *et al.* (1983), using PowerMarker 3.0 software (Liu & Muse, 2004).



**Fig. 1.** The two sampling locations, Gamasiab River in Kermanshah province and Kashgan River in Lorestan province. Both rivers are parts of the Tigris-Euphrates basin in Iran

**Table 1.** Flanking primers, observed size range and Genbank accession number of four microsatellite loci (Dubut et al., 2010)

Locus	GenBank Accession ID	Size (bp)	Primer 5' → 3'	Motif
BL1-2b	FJ468347	143-179	F:TTTGCACTAGTAACGAGCATCA R:CAGCACAGTTTCTCCATCCA	(TG) <sub>12</sub>
BL1-98	FJ468349	271-296	F:ATTGTTTTTCATTTTGTTCAG R:CCGAGTGTCTCAGAGTTATT	(CA) <sub>9</sub> CTAA(CA) <sub>3</sub> N <sub>50</sub> (CA) <sub>4</sub>
Rser10	AJ312850	193-222	F:TGCCTAATCGTGAAGCGGTG R:GCCACTAAAGCGCAGAAGCC	(GT) <sub>12</sub>
CypG24	AY439142	140- 272	F:CTGCCGCATCAGAGATAAACACTT R:TGGCGGTAAGGGTAGACCAC	(CAGA) <sub>19</sub>

## RESULTS AND DISCUSSION

All the four SSR loci for both species showed polymorphism with numbers of alleles ranging from 8 (BL1-98, BL1-2b) to 12 (CypG24) and averaging 9.75 alleles per locus for *A. caeruleus* and 6 (BL1-98) to 11 (CypG24, Rser10) with an average of 9 alleles per locus for *A. mossulensis* (Tables 2-3). There were substantial overlaps between the two species in the alleles present at each locus. However, some private alleles were observed in different loci for each species (Table 2). For both species, number of the effective alleles was lower than that of the

observed alleles, in all the loci. Observed heterozygosities ranged between 0.024-1 and 0.03-1 for *A. caeruleus* and *A. mossulensis*, respectively, while the mean expected heterozygosities was measured as 0.83 and 0.84 for the two species, respectively (Table 3).

Except for Rser10 locus, all loci in both species showed deviation from Hardy-Weinberg equilibrium ( $P < 0.05$ ). For BL1-98 locus of both species, the expected heterozygosity was higher than the observed heterozygosity, while this was reversed in the two other loci, BL1-2b and CypG24.

**Table 2.** The investigated loci and the alleles found in *A. mossulensis* and *A. caeruleus*\*

locus	Rser10	BL1-98	BL1-2b	CypG24
	<i>176</i>	<u>268</u>	<u>141</u>	<u>153</u>
	<i>184</i>	<u>272</u>	<u>145</u>	<u>158</u>
	<i>188</i>	<u>277</u>	<u>150</u>	<u>162</u>
	<i>192</i>	<u>279</u>	<u>156</u>	<u>168</u>
	<i>196</i>	<u>284</u>	<u>160</u>	<u>171</u>
	<i>200</i>	<u>286</u>	<u>167</u>	<u>176</u>
	<i>206</i>	<u>290</u>	<u>171</u>	<u>183</u>
	<i>212</i>	<u>295</u>	<u>175</u>	<u>187</u>
	<i>225</i>		<u>180</u>	<u>193</u>
	<i>231</i>			<u>200</u>
	<i>236</i>			<u>210</u>
	<i>240</i>			<u>215</u>

\*Alleles are shown by length of the repeat in base pairs. Underlined are alleles present in *A. caeruleus* and italics are the alleles in *A. mossulensis*.

**Table 3.** Summary of microsatellite data: number of the observed and effective alleles per locus, tests for deviation from Hardy-Weinberg equilibrium, expected (He) and observed (Ho) heterozygosities in *A. caeruleus* and *A. mossulensis*

Species	Test	Rser10	BL1-98	BL1-2b	CypG24	Mean
<i>A. caeruleus</i>	No. of observed alleles	11	8	8	12	9.75
	No. of effective alleles	6.64	5.11	6.69	5.38	5.97
	H <sub>O</sub>	1.00	0.024	1.00	1.00	0.81
	He	0.85	0.80	0.85	0.82	0.83
	HWE	ns	(D) *	(E) *	(E) *	
<i>A. mossulensis</i>	No. of observed alleles	11	6	8	11	9
	No. of effective alleles	8.07	4.40	5.28	9.61	6.84
	H <sub>O</sub>	1.00	0.30	1.00	0.93	0.81
	He	0.88	0.77	0.81	0.89	0.84
	HWE	ns	(D) *	(E) *	(E) *	

\*Significant at  $P < 0.001$ ; HWE. Hardy-Weinberg equilibrium

Based on the number of different alleles ( $F_{st}$ ), the distance between these two species was very low ( $F_{st}=0.04$ ,  $P < 0.05$ ). Genetic distance between the two species was estimated at 0.361 (Table 4). The AMOVA test showed a low differentiation between the two species based on the SSR analysis, in which, about 94% was intra-species genetic variation and only 6% was inter-species variation. Many of the molecular markers such as SSRs can be used as a valuable tool for assessing the persistence of a fish species by calculating allelic diversity, gene diversity; effective population size and population

structure (Liu, 2007; Pourkazemi *et al.*, 2012) and sex differentiation in fish (Pourkazemi & Razikazemi, 2011). Despite the importance of *A. mossulensis* and *A. caeruleus* as two widely distributed native fish in Iranian inland waters, there is very little information on them. For better understanding of an ecosystem function, it is necessary to separate different fish species. Unfortunately, morphological characteristics, as defining tools, are weak in cases when the species are very close to each other or when they are small/juvenile fish and it is necessary to distinguish them by molecular

markers. The study indicated high levels of polymorphism in *A. caeruleus* and *A. mossulensis* detectable by microsatellite primers developed from the other cyprinid fishes. Results of the present study demonstrated that the four sets of microsatellite primers produce replicable amplicons for the species. It is highly recommended to use species-specific SSR markers to evaluate genetic structure of an aquatic in question (Okumus & Ciftci, 2003), but it is possible to use primers which have been developed for the related fishes (Dubut et al., 2009). Results suggest that there is evolutionary conservation of the flanking regions for these loci among related taxa. The cross amplification between cyprinid species is consistent with earlier findings that primers developed in one species often work in other related one (Dubut et al., 2010). Genetic differentiation within and between the species are affected by several factors such as common history, current and past gene flow, species-specific processes including genetic drift and natural selection (Beaumont & Hoare, 2003). The role of geographic distance and implicit gene flow, in patterning the spatial genetic variation is well-known both theoretically and empirically (Epperson, 2003; Rousset, 2004). Low level of genetic distance as well as low number of private alleles at the loci may reflect the existence of a common ancestor for the two species. Species which have been derived from a common ancestor, usually showed very similar pattern of allele frequency (Freeland, 2007). Some rare private alleles in the loci may reflect the existence of differences in habitat characteristics (natural and anthropogenic effect) which can cause selection pressure or different mutation rates.

The discrepancies in allele's frequencies even in different populations of the same species have been previously reported (Shirangi et al., 2011; Fallahbagheri et al., 2013). Pollution of aquatic ecosystems is recognized as one of the main anthropogenic effect on fish population (Belfiore & Anderson, 2001). Probably, interference of the pollutants with nucleotide synthesis causes abnormalities in DNA and affects allele frequency over generations (Matter et al., 1992). Although, the effectiveness of pollution on genetic

diversity is affected by several factors such as exposure time and duration, aquatic life cycle and pollution concentration (Freeland, 2007; Wang et al., 2006). Nevertheless, the observed private alleles were not sufficient to genetically discriminate between the two species as supported by low level of genetic distance and high level of genetic variability which observed intra- instead of inter-species (AMOVA test). The low level of genetic differentiation was also supported by low  $F_{st}$  value as 0.04. Generally,  $F_{st}$  ranges between 0-0.05 denote a low level of genetic differentiation (Wright, 1951).

Hardy-Weinberg equilibrium theory is based on the distribution of genes and different genotypes in a species/population, and the accuracy of the theory depends on fulfilling some conditions such as no migration, large number of the population, no genetic drift, random reproduction, no selection and no mutation (Beaumont & Hoar, 2003). In addition to these biological parameters, some other factors such as heterozygosity deficiency and Wahlund effect can cause disequilibrium (Freeland, 2007). Heterozygosity deficiencies usually exist because of technical errors or null alleles (Castric et al., 2002). Although, null alleles are not specific to SSRs, the results obtained from this co-dominant marker usually affected by this phenomenon (Liu, 2007). Some other factors such as genetic drift and small size of population were found to be responsible for the deviation from Hardy-Weinberg equilibrium in Chinese sturgeon, *Acipenser sinensis* (Zhao et al., 2005) and pike, *Esox lucius* (Birgitte et al., 2005).

## CONCLUSION

This study indicates high levels of polymorphism inside *Alburnus mossulensis* and *A. caeruleus* detectable with microsatellite primers developed for other cyprinids, although they show low genetic differentiation between the two species. This is a preliminary study and it is necessary to be continued. Therefore, there is a need to assess genetic structure of the two species using more microsatellite loci and/or other molecular markers such as AFLP which may provide useful detectable molecular markers for each species.

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## مطالعه تمایز ژنتیکی دو گونه ماهی کولی ایرانی (*Alburnus mossulensis*) و (*Alburnus caeruleus*) (ماهیان استخوانی، کپورماهیان)، با استفاده از توالی‌های تکراری ساده

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### چکیده

تنوع ژنتیکی گونه‌های مختلف جنس *Alburnus* به خوبی ارزیابی نشده و رابطه فیلوژنتیک گونه‌های مختلف این جنس به طور کامل مشخص نیست. در این مطالعه، توالی‌های تکراری ساده (ریزماهوره) برای ارزیابی تنوع و تمایز ژنتیکی بین دو گونه ماهی کولی *Alburnus mossulensis* Heckel, 1843 و *Alburnus caeruleus* Heckel, 1843 به ترتیب از رودخانه کشکان در استان لرستان و رودخانه گاماسیاب از استان کرمانشاه مورد استفاده قرار گرفت. تعداد 30 قطعه ماهی از هر گونه صید و پس از استخراج DNA ژنومی، واکنش زنجیره‌ای پلی‌مرز با استفاده از چهار جفت نشانگر Rser10 و Bl1-98، CypG24، BL1-2b انجام شد. در مجموع 480 باند تکثیر شدند. متوسط هتروزایگوسیتی مشاهده شده و مورد انتظار برای هر دو گونه تا حدود زیادی مشابه بود. در هر دو گونه، تمامی جایگاه‌ها به جز جایگاه Rser10، انحراف از تعادل هاردی-واینبرگ را نشان دادند. متوسط فاصله ژنتیکی و شاخص تمایز *Fst* برای دو گونه به ترتیب معادل 0/361 و 0/04 بود. آنالیز واریانس مولکولی نشان داد که بخش اعظم تنوع ژنتیکی (94٪) مربوط به تنوع درون گونه‌ای و بخش اندکی از آن (4٪) مربوط به تنوع بین گونه‌ای بود. چهار جفت نشانگر توالی-های تکراری ساده طراحی شده برای سایر کپورماهیان، چندشکلی بالایی را در دو گونه ماهی کولی ایرانی نشان دادند، با این وجود، تمایز ژنتیکی قابل توجهی بین آنها مشاهده نشد. ارزیابی تمایز ژنتیکی این دو گونه با استفاده از تعداد بیشتری نشانگر ریزماهوره یا سایر نشانگرهای مولکولی نظیر چندشکلی طول قطعات تکثیر شده (AFLP) پیشنهاد می‌شود.

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