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Karyology study on Bleak (*Alburnus alburnus*) from the South Caspian Sea region

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ABSTRACT
The chromosomal spread and karyotype of Bleak (*Alburnus alburnus*) from Anzali lagoon were identified using tissue squashing techniques with injection of 0.5ml/100g body weight of 0.01% Colchicines solution in fish fingerlings. Kidney and gill tissues were then extracted and chopped in KCl 0.045M for 20 min and fixed in Carnoy solution in 3 stages. The chromosomal spreads were stained in 20% Gimsa for 30 min. From 347 chromosomal spread counts, the results showed diploid chromosome number of this species 2n=50. Karyotype composed of 7 metacentric, 13 submetacentric and 5 acrocentric or subtelocentric choromosome pairs, and the number of chromosome arms (NF) was determined as NF=90.

Keywords: *Alburnus alburnus, Anzali Lagoon, Bleak, Chromosome, Iran, Karyotype.*

INTRODUCTION
Bleak, *Alburnus alburnus* (Hohenackeri Kessler, 1877) belongs to teleostei, class Cypriniformes, order Actionopterygii, family Cyprinidae and genus Alburnus (Abdouly, 1999). Its main habitat is located in Southern part of Caspian Sea in Iran, but it was artificially transferred to Sistan Hamoon Lake and Zarivar (Marivan) (Abdouly, 1999; Vosoughi & Mostajeer, 2000). Among 23000 identified species, standard karyotype has been reported only for 2400 species (10.4%). While chromosomal study is very applicable for taxonomic, genetic, cytotoxicological, race improvement and biotechnological investigations, it also has application in chromosome set manipulation and triploidy production as a tool to enhance success of chromosome alteration (Hosseini & Kalbassi, 2003; Gold *et al*., 1990; Al-Sabti, 1991).

Chromosomal studies have been conducted on several Cyprinidae species such as *Rutilus frisii kutum* (Nowruzfashkhami & Khosroshahi, 1995), *Abramis brama* (Nahavandi *et al*., 2001), *Hypophthalmichthys molitrix* (Varasteh *et al*., 2002), *Ctenopharyngodon idella* (Nowruzfashkhami *et al*., 2002), *Schizothorax zarudnyi* (Hosseini & Kalbassi, 2002), *Barbus capito* and *Copoeta copoeta gracilis* (Pourali Darestani *et al*., 2006), *Petroleuciscus pradis* (Esmaeili & Piravar, 2006, *Garra persica* (Esmaeili *et al*., 2009), *Vimba vimba persa* (Pourkazemi *et al*., 2010), *Chondrostoma regium* (Esmaeili *et al*., 2010), *Blicca bjoerkna transcaucasica* (Pourkazemi *et al*., 2010), *Alburnoides bipunctatus* (Khosravanizadeh, 2010) and *Alburnus filippii* (Nazari *et al*., 2011). However to date there are no reports on the number of chromosomes in *Alburnus alburnus* in Iran. Hence the objective of the present study was to determine chromosome number and also karyotype of this species in Anzali Lagoon, in Iran.

MATERIALS AND METHODS
Totally, 30 fingerlings were caught by electroshocker in the mouth of the rivers entering the Anzali Lagoon. All fish samples were transferred to the genetic department of International Sturgeon Research Institute and maintained in well aerated aquaria. Aquarium temperature was maintained at 27°C during the experiment period. The fish were fed with formulated diets. Tissue squashing

techniques with injection of colchicines solution were used in this study (Reddy & John, 1986). The process is as follows: First, feeding was interrupted. After 6 hours, the colchicine solution of 0.01% was administrated at a dose of 0.5 ml/l00g body weight into the intraperitoneal muscle and dorsal fin. After injection fish were kept in a well aerated aquarium. After 200 minutes, kidney and gill tissues were removed and placed in 1 ml of potassium chloride (0.075M) and chopped by scalpel. After 10 minutes, the tissues were squashed well by homogenizing glass Muller. Then by adding 3 ml of hypotonic solution, the volume of the solution was made up to 4 ml. After 10 minutes, the solution was centrifuged at 1300 rpm/min. Then the supernatant was collected. At this stage, the remaining sediments were slowly mixed with 4 ml of cooled Carnoy's fluid (3 parts methanol and 1 part acetic acid). After 40 minutes and extraction of the solution, the old fixative was replaced by the new fixative. Then after 30 minutes, the sample was centrifuged. After 15 minutes and settling of very large particles, the suspension was ready for slide preparation. At first, the slides were washed and heated up to 50°C, then 3-4 drops of the upper and clear parts of the fixing solution obtained after settling of large particles, fell on to the slides at 50 cm height by Pasteur pipette. Thereafter, the fixative was dried in laboratory temperature and stained by Giemsa (20%) at pH=6.8 for 30 minutes. After washing and drying, the slides were examined under a light microscope (Nikon, Labophot2T).

The number of chromosomes was determined by counting various spreads and calculating the mean and standard deviation. To determine chromosome formula, each arm of the chromosomes and centromeric index were measured. So, the best metaphase spread picture was selected among all metaphase plates. The morphometric measurements of chromosome pictures were conducted with photographic software Photoshop (Middle Eastern Version) by determining the coordinate of beginning and end of chromosome arms and centromer. Then the length of each arm was identified using line formula by Microsoft office Excel 2003. First, centromeric Index (length of the chromosome, short arm divided by its total length) was calculated. Finally, to determine homologous pairs and chromosome formula, the chromosomes were arranged based on centromeric index in the descending order. The chromosome type was identified by method of Levan *et al.,* (1964). According to this method, the chromosome pairs were classified into Metacentric (M), Submetacentric (Sm), Subtelocentric (St) and Telocentric (T), with CI ranges of 0.375-0.5, 0.250-0.375, 0.125-0.250 and 0-0.125, respectively. Then the karyotype was constructed using Adobe Photoshop CS (Middle Eastern Version) and the ideogram was drawn using Microsoft Office Excel 2003.

RESULTS

After standardization of the method, in order to determine the number of chromosomes in bleck (*Alburnus alburnus*), the examination was carried out on four different fish in four replicates. The chromosome number was counted based on 347 chromosome spreads in the four examined fish. The number of chromosomes was 2n=47, 2n=48, 2n=49, 2n=50, 2n=51 and 2n=52 in 7%, 9%, 16%, 63%,3% and 2% of the spreads, respectively. The most frequent value was 2n=50 (Fig 1)

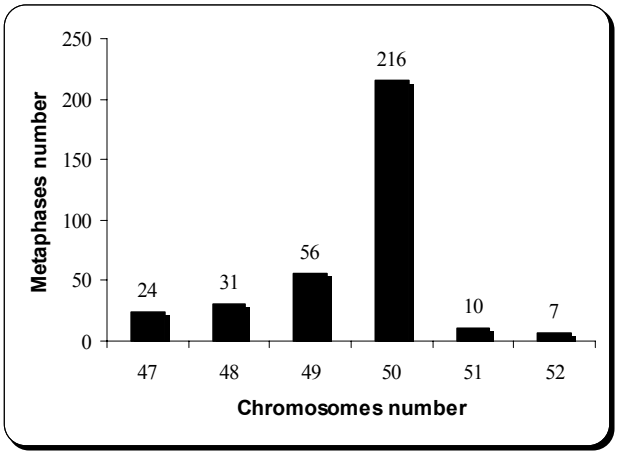

Fig 1. Distribution of chromosome number observed at 347 diploid metaphases in *Alburnus alburnus*

Then metaphase spreads were counted. The mean and standard deviation was considered at a probability level of 95%. According to the results, the number of chromosomes in this species was n=49.56±0.1 (Fig 2).

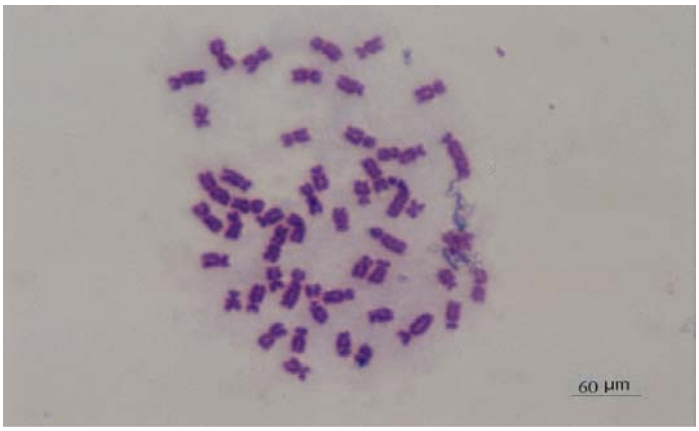

Fig 2. Metaphas spread of Bleak *(Alburnus alburnus)* ×10000 .

With regard to the results, it was determined that *Alburnus alburnus* has 7 pairs of metacentric (M), 13 pairs of submetacentric (Sm) and 5 pairs of subtelocentric (St) or acrocentric (A), so the chromosome formula can be expressed as 2n=7M+13Sm+5(St-A) and the number of chromosome arms is NF=90 (Table 1, Fig 3&4).

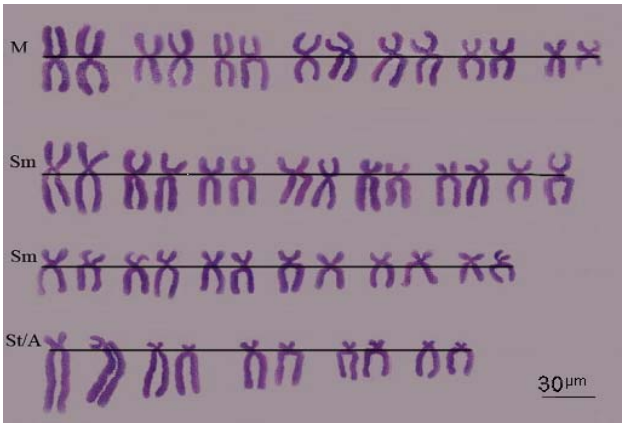

Fig 3. Karyotype of Bleak (*Alburnus alburnus)* from Anzali Lagoon, 2n=50.

Table 1. Numeral characteristics of the karyotype of *A.alburnu* showing the mean values of measurements from ten best mitotic metaphases

Chromosome pair no.	Short arm (µm)	Long arm (µm)	Short arm (µm)	Centromeric index	Arm ratio	Classification
1	6.964	9.303	16.267	0.428	0.027	Metacentric
	7.970	10.404	18.374	0.433	0.026	Metacentric
2	8.876	10.963	19.840	0.447	0.023	Metacentric
	9.688	14.252	23.940	0.404	0.022	Metacentric
3	10.018	10.826	20.845	0.480	0.021	Metacentric
	10.538	14.080	24.618	0.428	0.021	Metacentric
4	10.932	14.534	25.467	0.429	0.021	Metacentric
	11.261	14.972	26.233	0.429	0.020	Metacentric
5	11.560	12.767	24.327	0.475	0.020	Metacentric
	12.175	12.912	25.087	0.485	0.020	Metacentric
6	12.433	13.274	25.707	0.483	0.017	Metacentric
	12.767	14.746	27.513	0.464	0.016	Metacentric
7	16.475	14.867	31.343	0.525	0.015	Metacentric
	16.646	16.507	33.153	0.502	0.013	Metacentric
8	13.511	21.100	34.612	0.390	0.029	Submetacentric
	12.806	19.957	32.763	0.390	0.027	Submetacentric
9	11.583	16.359	27.942	0.414	0.023	Submetacentric
	9.992	17.341	27.334	0.365	0.022	Submetacentric
10	9.704	14.120	23.825	0.407	0.021	Submetacentric
	9.100	14.603	23.703	0.383	0.020	Submetacentric
11	9.433	15.507	24.941	0.378	0.020	Submetacentric
	9.708	15.153	24.862	0.390	0.020	Submetacentric
12	7.570	17.147	24.718	0.306	0.020	Submetacentric
	7.961	13.601	21.562	0.369	0.020	Submetacentric
13	7.747	13.997	21.745	0.356	0.019	Submetacentric
	8.324	14.832	23.156	0.359	0.019	Submetacentric
14	8.819	13.049	21.868	0.403	0.019	Submetacentric
	11.289	14.043	25.332	0.445	0.019	Submetacentric
15	9.640	14.771	24.411	0.394	0.018	Submetacentric
	8.072	11.827	19.899	0.405	0.018	Submetacentric
16	9.126	13.792	22.919	0.398	0.018	Submetacentric
	7.247	15.508	22.755	0.318	0.018	Submetacentric
17	8.348	12.406	20.755	0.402	0.017	Submetacentric
	7.971	12.975	20.946	0.380	0.017	Submetacentric
18	7.685	13.897	21.582	0.356	0.017	Submetacentric
	8.626	11.811	20.438	0.422	0.016	Submetacentric
19	6.177	11.360	17.537	0.352	0.016	Submetacentric
	7.7466	12.382	20.128	0.384	0.014	Submetacentric
20	6.603	11.200	17.803	0.370	0.032	St - A$^{\circ}$
	5.841	9.5520	15.393	0.379	0.031	St - A
21	7.655	30.974	38.629	0.198	0.022	St - A
	7.392	29.931	37.323	0.198	0.020	St - A
22	3.940	23.161	27.102	0.145	0.020	St - A
	4.652	19.813	24.466	0.190	0.018	St - A
23	5.663	18.248	23.912	0.236	0.015	St - A
	4.939	16.572	21.511	0.229	0.015	St - A
24	5.197	13.257	18.454	0.281	0.015	St - A
	5.060	13.685	18.746	0.269	0.014	St - A
25	3.780	14.120	17.901	0.211	0.013	St - A
	3.383	12.808	16.191	0.208	0.012	St - A

¤ St - A= Subtelocentric or Acrocenteric

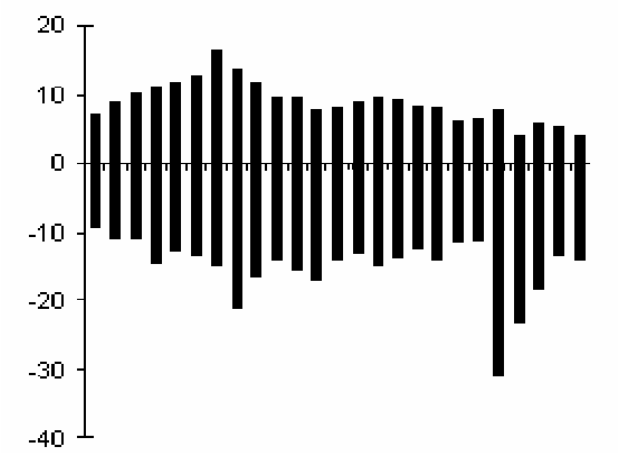

Fig 4. Idiogram of Bleak *(Alburnus alburnus*) from Anzali Lagoon, n =25.

DISCUSSION

Method of tissue squashing has also been used for different species including *Abramis brama* (Nahavandi *et al*., 2001), *Schizothorax zarudnyi* (Hosseini & Kalbassi, 2002), *Hypophthalmichthys molitrix* (Varasteh *et al*., 2002), *petroleuciscus persidis* (Esmaeli & Piravar, 2006) and *Garra rufa* (Esmaaeli & Piravar, 2007) in Iran.

In chromosome studies, improvement of colchicine treatment has a basic role in obtaining suitable metaphase spreads. In this study, the optimum colchicine concentration was determined to be 0.015 and 0.005 ml/mg Bw for 200 minutes exposure time which is similar to that of Nahavandi *et al*., (2001) and Nowruzfashkhami *et al*., (2002) (Table 2).

In this examination, the hypotonic solution potassium chloride (KCl) was applied and the best results were obtained with 0.045M KCl for 20 min. Table 2 shows the type and concentration of solution and necessary exposure time used for Cyprinids in recent studies.

In order to decrease the changes in cell structure, Carnoy's solution was used 3 times for fixation and dying induction. High fixation power of Carnoy's makes it widely applicable for different treatments (Table 2). It should be mentioned that the lasting time for this solution had no effect on the quality of chromosome spreads. In this study the suspension was dropped on to the slides from a height of 50 cm. In the previous studies, different heights ranging from 20-30 cm (Al-Sabti, 1985) and 200 cm (Gold, 1974) had been used.

In the recent study the slides were first warmed and then the suspension fell on to them which is similar to the studies carried out by Baksi and Means, (1988), Nahavandi *et al*., (2001), Varasteh *et al*., (2002), Nowruzfashkhami *et al*., (2002) and Hosseini and Kalbasii *et al*., (2002).

In cytogenetic studies, optimum staining of chromosomes is very important. Because a good staining can show the morphology of chromosomes in a better manner. In this examination 20% Giemsa (pH=6.8) at 30 minutes was suitable. It differs from other studies in terms of concentration used and lasting time (Table 2).

In 70% of the examined Cyprinids, the chromosome number was 2n=50 and it is considered as a model and base number for Cyprinids (Khuda-Bukhsh *et al*., 1986). Nevertheless, cytogenetic studies on Cyprinids showed four different models of chromosomes including octaploidy 2n=200, Hexaploidy 2n=150, tetraploidy 2n=100 and diploidy 2n=50 (Nahavandi *et al*., 2001).

According to the previous studies, *Alburnus alburnus* is classified as diploid Cyprinid (Schmid *et al*., 2006; Ziegler *et al*., 2003 ; Arkhipchuk, 1999; Klinkhardt *et al*., 1995). Moreover the results of this study indicate that bleck is a diplioid (2n=50) species.

Table 2. Comparison used methods in some karyotype researches on Cyprinidea

References	species	Colchicine		Used tissue	Hypotonic			Carnoy repeat	Giemsa	
		Concentration for 100g BW	Time (min)		type	Concentration	Time (min)		Concentration %	Time (min)
Nowruzfashkhami & Khosroshahi, 1995	*Rutilus frisii kutum*	1ml colchicin 0.00001 mol	240	Leukocyte	KCl	0.075M	45	2	5	20
Nahavandi *et al.*, 2001	*Abramis brama*	0.5ml colchicin 0.01%	200	kidney & gill	KCl	0.075M	20-40	2	20-40	20-30
Kilic-Demirok & Unlu, 2001	*Capoeta trutta* & *C.capoeta umbla*	1ml colchicin 0.06%	210-240	kidney & gill	-----	----------	-----	----	0.5	----
Nowruzfashkham *et al.*, 2002	*Ctenopharyngodon idella*	1ml colchicin 0.00001 mol	240	Leukocyte	KCl	0.075M	45	2	30	45
Varasteh *et al.*, 2002	*Hypophthamichthys molitrix*	0.5ml colchicin 0.01%	200	Kidney	KCl	0.075M	20	2	15	30
Ziegler *et al.*, 2003	*Alburnus alburnus*	30ml colchicin 0.03%	60	kidney & gill	KCl	46 m/l	60	1	------	----
Gul *et al.*, 2004	*Alburnus heckeli*	1ml colchicin 0.6%	190	Gill	KCl	0.046M	45	1	20	7
Kilic-Demirok & Unlu, 2004	*Alburnoides bipunctatus*	100ml colchicin 0.01%	120	Kidney	------	---------	----	---	0.5	----
Zhao *et al.*, 2004	*Carassius auratus*	----------	---------	Kidney	------	---------	----	---	----	----
Pourali Darestani *et al.*, 2006	*Barbus capito* & *B.mursa* & *Capoeta capoeta*	0.2ml colchicin 0.01%	180	kidney & gill	KCl	0.075M	45	3	5-10	5-15
Ueda *et al.*, 2006	*Tanakia spp*	----------	--------	gastrula & kidney	------	----------	----	---	---	---
Naran *et al.*, 2006	*Pseudobarbus spp.*	10ml colchicin 0.01%	--------	kidney & gill	NaCl	0.4%	20	---	6-4	5
Nirchio *et al.*, 2006	*Hoplosternum littorale*	----------	--------	---------	------	----------	----	---	10	20
Esmaeli & Piravar, 2006	*Petroleuciscus persidis*	2ml colchicin 0.025%	200-240	kidney & gill	KCl	0.36%	45	1	10	10
Schmid *et al.*, 2006	*Alburnus alburnus*	0.3ml colchicin 0.003%	120	kidney & gill	KCl	0.046M	60	1	0.5	6
Esmaeili & Piravar, 2007	*Garra rufa*	2ml colchicin 0.025%	180-200	kidney & gill	KCl	0.36%	40	1	10	15
Sahoo *et al.*, 2007	*Garra spp.*	1ml colchicin 0.05%	120-180	kidney & gill	-----	--------	----	---	4	---
Kalbassi *et al.*, 2008	*Schizothorax zarudnyi*	2500-5000 µg colchicin	300-600	Kidney	KCl Sodium citrate	0.075M 1%	45-60 45-60	3	5-15	7-10

Table 3. Karyotype comparison in different cytogenetic studies in Bleak (*Alburnus alburnus*)

References	Chromosome number	Chromosome type			Arms number
		Metacentric	Submetacentric	Acrocenteric, Subtelocentric or Telocentric	
Klinkhardt *et al.*, 1995	2n=50	16	20	14	86
Arkhipchuk, 1999	2n=50	16	20	14	86
Ziegler *et al.*, 2003	2n=50	14	14	22	78
Bianco *et al.*, 2004	2n=50	16	26	8	92
Schmid *et al.*, 2006	2n=50	14	14	22	78
Rab *et al.*, 2008	2n=50	16	26-30	8-4	92-96
This research	2n=50	14	26	10	90

Despite, the similarities in chromosome numbers between this study and the previous studies, differences in chromosome formula and number of arms (NF) were observed (Table 3).

This may be due to various factors including differences in population and also sub species in sampling region, or may be related to interspecific polymorphism. It may also depend on technical and procedural experimental condition, loss of chromosomes during the preparation of spreads, incorrect moving of fixed cells during spread preparation, addition of chromosomes from adjacent cells, un-recognizable micro arms in chromosomes, inadequate number of samples, variety of population and subspecies in each region, errors in measuring chromosome arms and determining chromosome type, etc (Unlu *et al.*, 1997; Khuda-Bukhsh *et al.*, 1986). Therefore, more studies should be conducted on banding staining, FISH and other techniques.

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کاریولوژی مروارید ماهی (*Alburnus alburnus*) منطقه جنوب دریای خزر

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چکیده:

گسترش کروموزومی و کاریوتایپ مروارید ماهی *Alburnus alburnus* تالاب انزلی با استفاده از تکنیک لـه کـردن بافت و با تزریق ml ۰/۵ کلشی سین ۰/۰۱ درصد به ازای هر ۱۰۰ گرم وزن به بچه ماهیـان انگـشت قـد، اسـتخراج و خرد کردن بافت های کلیه و آبشش در محلول KCl ۰/۰۴۵ مولار به مدت ۲۰ دقیقه و تثبیت سلول هـای حاصـل در سه مرحله توسط کارنوی تهیه شد. رنگ آمیزی گسترش های حاصل با گیمسا ۲۰ درصد طی ۳۰ دقیقه صورت گرفت. شمارش کروموزومی در ۳۴۷ گسترش کروموزومی نشان داد تعداد کرومـوزوم هـای ایـن گونـه ۲n=۵۰ اسـت، فرمـول کروموزومی آن ۷ جفت کروموزوم متاسانتریک، ۱۳ جفت ساب متاسانتریک، ۵ جفت ساب تلوسانتریک یا آکروسانتریک (۷M +۱۳Sm + ۵St-A) می باشد و تعداد بازوهای کروموزومی این گونه نیز NF=۹۰ تعیین شد.