Karyotype analysis in white bream (*Blicca bjoerkna transcaucasica*) from north coast of Iran

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Abstract

Preparation of chromosome spreads and karyotype analysis in *Blicca bjoerkna transcaucasica* were carried out using 0.01% solution of colchicines and Phytohaemagglutinin (PHA) (20 µg g^{-1} body weight). The gill and kidney tissues were collected and let to stand in a hypotonic solution of 0.075 M KCl and then treated with a fixative (Carnoy's solution) in three steps. The chromosomes spreads were then stained with 5% Giemsa solution for 20 min and examined under a light microscope. Appropriate metaphase plates were photographed in order to prepare karyotype. The size of the chromosomes (short and long arms), relative length of chromosomes and centromere index were calculated. Chromosome spreads from gill tissue cultures which were colchicine treated with PHA, had a well defined size, shape and number of chromosomes for karyotype analysis. Based on the 76 metaphase plates studied, chromosome count in 59 metaphase plates was 2n=49.74±0.25. By arranging homologous chromosomes beside each other the chromosome formula was calculated as 6 pairs of Metacentric, 10 pairs of Sub-Metacentric and 9 pairs of Sub-Telocentric (2n=6M+10+Sm+9St) and the chromosome arm number (NF) was 100. The largest chromosome in this species was a pair of metacentric chromosomes. On the basis of the number and type of chromosomes, the karyotype obtained for this species conformed to the findings of other researchers, but the chromosome formula was different, which could be attributed to the existence of different populations for this species.

Keywords: *Blicca bjoerkna transcaucasica*, Phytohaemagglutinin, Colchicines, Karyotype

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Introduction

chromosomes in fish species and other aquatic organisms (Kalbasi et al., 2006). Karyological studies have revealed basic information on the number, size and morphology of chromosomes. These results can also be used to document chromosomal manipulation techniques including induction of polyploidy and to document gynogenesis, androgenesis and production of hybrids within and between two species, in the presence of sex chromosomes (Gold et al., 1990).

Increase in mitotic index to obtain a higher number of metaphase plates plays a vital role in cytogenetic studies (Cucchi and Baruffaldi, 1989). In recent years, various chemicals such as calcium chloride (Subramanyam, 1969), cobalt chloride (Cucchi and Baruffaldi, 1989), phenylhydrazin (Cucchi and Baruffaldi, 1989), phytohaemagglutinin (Fan and Fox, 1990) and bacterial and fungal antigens (Molina, 2001) were used to increase mitotic index, which have produced different results. The objective of the present study were to prepare chromosome spreads and to determine the number and type of chromosomes in *B. bjoerkna transcaucasica* and thus to present the karyotype of this species.

Materials and methods

Thirty specimens of *B. bjoerkna transcaucasica* specimens were collected from the Anzali Lagoon and transferred to an aquarium equipped with aeration in the College of Natural Resources of the Guilan University. The fish were fed 8-10 g of formulated diets (GFTI) and chopped kilka at 9.00 and 15.00 hours every day. Water exchange was carried out at a rate

The family Cyprinidae is one of the most widespread and largest families of fishes in the world with more than 2100 species comprising 8.5% of the total fishes. Most of the species of this family inhabit freshwaters (Coad, 1995; Demirok and Unlu, 2001; Giorgio et al., 2004; Esmaeili and Piravar, 2006). In Iran carps comprise 50% of the freshwater fish fauna (Coad, 1995).

Blicca bjoerkna transcaucasica belongs to the class Osteichthyes, order Cypriniformes, family Cyprinidae and the genus *Blicca* (Coad, 2007). The genus *Blicca*, comprises 2 species and 3 sub-species which are distributed in northern Europe and in the Caspian Sea basin (Coad, 2007). The Caspian Sea basin subspecies is *B. bjoerkna transcaucasica* Berg, 1916, described from the lower reaches of the Kura River, Atraks, Lenkoran District, Transcaucasia and from here this species has found its way into the Aras River (including its middle reaches in Iran) to the Atrak River in the Caspian Sea basin including Anzali Lagoon (Coad, 1995). This species is found in the shallows of warm lakes with heavy vegetation and in the slower reaches of rivers including river estuaries. Maturity is attained at 2-4 years and spawning occurs in intervals beginning in April and runs up to June (Abdoli, 2000; Coad, 2007). This species feeds on planktons, worms, insect larvae and mollusks, and some vegetation (Coad, 1995; Sattari, 2003). This species is listed for its commercial importance and for recreational fishing (Coad, 1995).

In recent years, considerable attention has been focused on the development of genetic studies on

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chromosomes of the same size (Levan et al., 1964).

The karyotyping data for each chromosome was calculated using the length and the centromeric index (CI), ratio of chromosome arms (the length of the longer arm divided by the length of the short arm) and the relative length (total length of chromosome divided by total length of all chromosomes in a set of haploid chromosomes). The number of chromosomes was calculated based on the following formula (Levan et al., 1964):

$$2n = \bar{X} \pm [\ Z_{\frac{a}{2}} \frac{\sigma}{\sqrt{n}}\ ]$$

Where $\bar{X}$ = average, Z= confidence level, σ= confidence coefficient, n= number of counted metaphase plate, $Z_{\frac{a}{2}}$ in 95% confidence level was equal to 1.96.

Results

A total of 76 metaphase plates were prepared in which the chromosomes were clearly distinguishable from its adjacent segments and counted (Fig. 1). The number of chromosomes in all the metaphase plates was not the same. The difference in chromosome number in the different metaphase plates was due to method of mounting slide, overlapping of chromosomes and so on. Therefore all metaphase plates were counted and the number of chromosomes was calculated on the basis of their frequency. Of the 76 metaphase plates counted in the present study, 77.6% showed 2n=50 with the lowest number found being 46 (in one metaphase plate) and the highest number found being 51 (in 4 metaphase plates) (Table 1, Fig. 2).

of 20% per day. PHA was injected following Gold et al. (1990) and colchicine was injected following Baruffaldi et al. (1992). Specimens under study received an intramuscular injection of PHA at a dose of 20 µg per kg body weight in two stages at an interval of 15 h and an injection of 0.5 ml/100 mg body weight of colchicine 2 h after the second dose of PHA. After injection the fish were kept at 15-18 °C for a period of 3-3.5 h after which the gills and the head kidney of 12 specimens were removed. Sample tissues were squashed and placed in a hypotonic solution of KCl (0.075 M) for 35 min. To induce cytoplasmic swelling, the samples were incubated at 35 °C. Hypotonised tissue were centrifuged at the rate of 1300 rpm for 5 min, the supernatant was discarded and cold Carnoy's solution (3 parts methanol:1 part glacial acetic acid) was added to them. The suspension was centrifuged once again and after discarding the supernatant, cold Carnoy's solution was added. This was repeated two times. Then a few drops of the suspension were mounted on hot (heated on a hot plate after rinsing in alcohol) and cold (placed in a freezer after rinsing in alcohol) slides. The slides were air dried and stained with 5% Giemsa solution (pH=6.8). Slides were rinsed in distilled water and dried at room temperature for 4-5 h.

The metaphase plates were observed under an optical microscope (Olympus, Made in Japan) (X1000) and photographed. The length of the chromosomes and its arms were measured using the Biocom visiol software and the results were analyzed using Microsoft Excel 2003. The karyotype was prepared by size and position of centromere for

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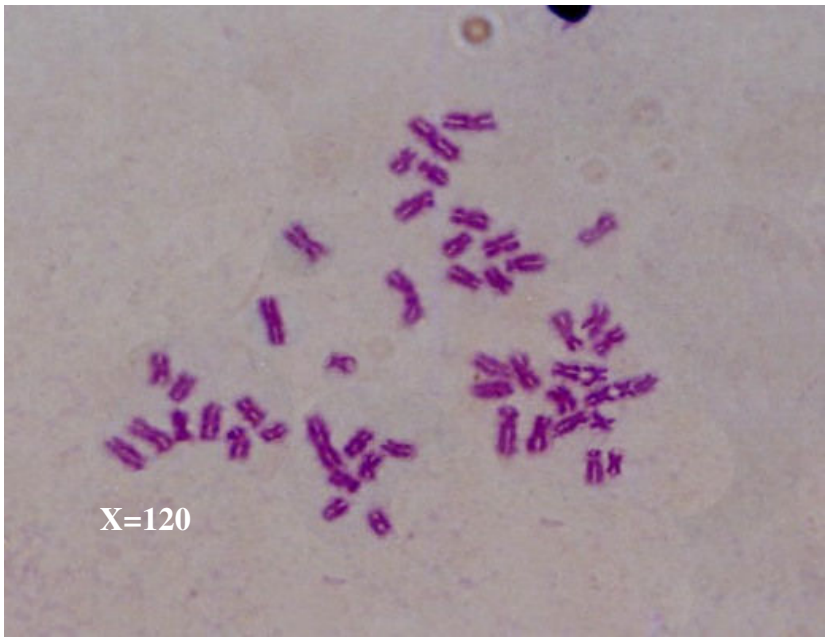

Figure1: Chromosome spreads prepared from *B. bjoerkna transcaucasia* (2n=50)

Table 1: Frequency of chromosome number in *B. bjoerkna transcaucasia*

Plate number	1	2	3	4	5	6
Number of chromosomes in each plate	51	50	49	48	47	46
Number of metaphase plates	4	59	6	4	2	1

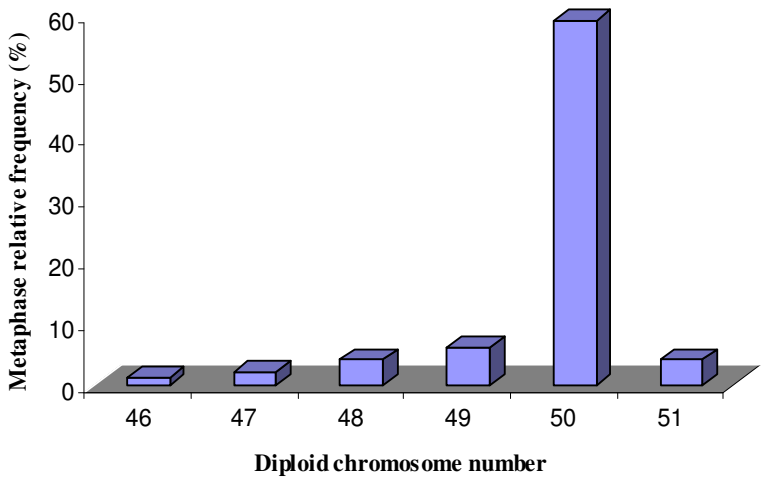

Figure 2: Distribution of chromosomes in the 76 metaphase plates counted in the present study

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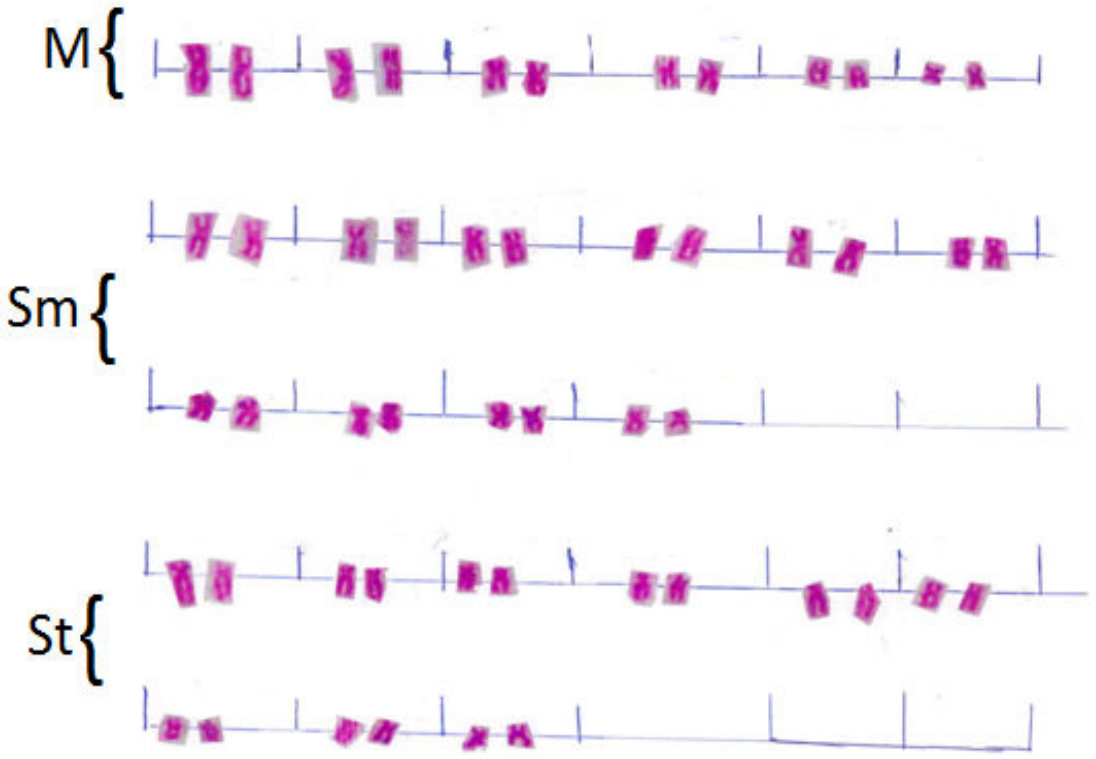

Figure 3: Karyotype in *B. bjoerkna transcaucasia* (2n=6M + 10 SM + 9St)

The centromere index, ratio of arms and the relative and total length of chromosomes in the different types of chromosomes studied in the present study were different. Chromosome length varied from a minimum of 0.47 µm to a maximum of 0.50 µm in metacentric chromosomes, from 0.38 to 0.45 µm in sub metacentric chromosomes and from 0.15 to 0.29 µm in sub telocentric chromosomes (Table 2). The ratio of arms varied from 0.97 to 1.08 µm in metacentric chromosomes, from 1.2 to 1.58 µm in sub metacentric chromosomes and from 2.44 to 5.24 µm in sub telocentric chromosomes (Table 2). The relative length for metacentric chromosomes varied from a minimum of 2.81 µm to a maximum of 6.11 µm. Relative length in sub metacentric chromosomes varied from 3.12 to 5.85 µm and that in sub telocentric chromosomes varied from 3.18 to 5.72 µm (Table 2). Total chromosome length varied from 1.42 to 3.09 µm in metacentric chromosomes, from 1.54 to 2.96 µm in sub metacentric chromosomes and from 1.61 to 2.89 µm in sub telocentric chromosomes (Table 2).

Based on the present study the chromosome number for *B. bjoerkna transcaucasia* was calculated as 2n=49.74 ± 0.25 or 2n=50 and the chromosome formula was determined as 2n=6M + 10 SM + 9St (NF=100) (Fig. 3).

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Table 2: Centromere index, arm ratio, relative length of chromosomes in *B. bjoerkna transcaucasia*

Chromosome type	Number of paired chromosomes	Relative length (µm)	Ratio of arms	Centromere index (CI)	Length of short arm (µm)	Length of long arm (µm)	Total length (µm)
metacentric	**1**	6.11	1.03	0.49	1.52	1.57	3.09
metacentric	**2**	5.85	1.02	0.49	1.46	1.5	2.96
metacentric	**3**	3.68	0.97	0.5	0.94	0.92	1.86
metacentric	**4**	3.34	1.08	0.47	0.81	0.88	1.69
metacentric	**5**	2.9	1.04	0.48	0.72	0.75	1.47
metacentric	**6**	2.81	1.05	0.48	0.69	0.73	1.42
X ± sd		4.12±1.48	1.03±0.04	0.49±0.01	1.02±0.37	1.06±0.38	2.08±0.75
Sub metacentric	7	5.85	1.57	0.38	1.15	1.81	2.96
Sub metacentric	8	5.68	1.58	0.38	1.11	1.76	2.87
Sub metacentric	9	4.31	1.27	0.44	0.96	1.22	2.18
Sub metacentric	10	3.97	1.31	0.43	0.87	1.14	2.01
Sub metacentric	11	3.74	1.36	0.42	0.8	1.09	1.89
Sub metacentric	12	3.48	1.2	0.45	0.8	0.96	1.76
Sub metacentric	13	3.28	1.27	0.43	0.73	0.93	1.66
Sub metacentric	14	3.24	1.21	0.45	0.74	0.9	1.64
Sub metacentric	15	3.12	1.28	0.43	0.69	0.89	1.54
Sub metacentric	16	3.62	1.23	0.44	0.82	1.01	1.83
X ± sd		4.03±0.98	1.33±0.14	0.43±0.03	0.87±0.16	1.17±0.34	2.03±0.50
Sub telocentric	17	5.72	4.25	0.19	0.55	2.34	2.89
Sub telocentric	18	4.96	5.12	0.16	0.41	2.1	2.51
Sub telocentric	19	4.88	4.36	0.18	0.46	2.01	2.47
Sub telocentric	201	4.77	5.24	0.15	0.38	2.03	2.41
Sub telocentric	21	4.8	4.42	0.18	0.45	1.99	2.44
Sub telocentric	22	3.69	2.44	0.29	0.54	1.32	1.86
Sub telocentric	23	3.3	3.17	0.23	0.4	1.27	1.67
Sub telocentric	24	3.2	2.95	0.25	0.41	1.21	1.62
Sub telocentric	25	3.18	3.12	0.24	0.39	1.22	1.61
X ± sd		4.28±0.94	3.90±1.00	0.21±0.05	0.44±0.06	1.72±0.45	2.16±0.48
Over all Chromosome X ± sd		4.14±1.06	2.18±1.44	0.36±0.12	0.75±0.32	1.34±0.48	2.09±0.54

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Discussion

Based on the results obtained the chromosome number for this species was 2n=50 which conforms to the chromosome number reported for other species belonging to the genus *Blicca* (Klinkhardt, et al., 1995).

In recent years, different chemicals have been used to increase mitotic coefficient while preparing chromosome spreads *in vivo*. Nowadays with regard to the fact that it is time consuming, labor intensive and expensive and it is at the risk of bacterial contamination, cell culture is not very much used in cytogenetic analysis. (Cucchi and Baruffaldi,1990; Gold et al., 1990; Fujiwara et al., 2003). The improved conditions provided by the use of colchicine play an important role in producing appropriate metaphase plates. The application of higher concentrations and doses of colchicines and/or the longer periods of application decreased chromosome size and made it difficult to determine their exact size and to determine the exact position of the centromere. On the other hand when used in lower concentrations and doses and/or shorter periods of application colchicines may induce chromosomal aberrations and thus result in a decrease in the number of suitable metaphase plates (Ojima and Hitotsumachi, 1967; Hartley and Horne 1985; Cucchi and Baruffaldi, 1990; Gold et al., 1990; Klinkhardt, 1991; Foresti et al., 1993). Based on the chromosome spreads obtained it is evident that best metaphase plates were obtained with colchicines injected at a rate of 0.0125% and PHA injected at a rate of 20 µg g^{-1} body weight.

Allowing the tissues under study to stand for 55 min in a hypotonic solution of 0.075 M KCl resulted in the cells to swell and the cytoplasmic membrane to stretch producing good quality metaphases. Incubation of cultures at 37 °C for 20 min was considered suitable for cells to swell. Results obtained indicate that higher concentration (0.080 M) of KCl was not suitable for did not produce good metaphases. Similarly lower concentration (0.070 M) of KCl also caused excess swelling of cells and thus did not produce good metaphases. These findings conform with that of other researchers (Ojima and Hitotsumachi 1967; Cataudell et al., 1977; Vujosevic et al., 1983; Gold et al., 1990; Klinkhardt, 1991; Cucchi and Baruffaldi, 1990; Giorgio et al., 2004; Colak and Sezgin, 2004).

The height at which the cell suspension is released on to the slide is an important factor when preparing chromosome spreads. In this study a height of 150 cm was considered very appropriate. The addition of 1 ml of freshly prepared cold fixative (1:3 methanol:glacial acetic acid) to each culture was also an important factor in the quality of chromosomes. Fixative should be made fresh before each use, because it can absorb water upon standing and the pH changes with time (Nowruzfashkhami et al., 2002). The fixative should also be cooled at 4 °C in a refrigerator before being used for better fixation of the cells and better metaphases (Gold et al., 1990).

Staining with 5% Giemsa stain diluted for use with buffered water to pH 6.8 for 15 min produced very good staining

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results and is recommended in the present study. The same concentration of Giemsa stain has been reported by other investigators (Ojima and Hitotsumachi 1967; Gold et al., 1990; Vujosevic et al., 1983; Porto et al., 1990; Giorgio et al., 2004).The mean chromosome number determined in *B. bjoerkna transcaucasia* in the present study was 2n=49.74 ± 0.25 which is almost similar to that (2n=50) reported in most of the species belonging to the family Cyprinidae (Gul et al., 2004). The chromosome number (2n=50) for *B. bjoerkna transcaucasia* was the same as that reported for other species belonging to the same genus (Klinkhardt et al., 1995). Chromosome number for the species *B. argyroleuca* in the Volga and Sura Rivers has also been reported as 2n=50, however the chromosome formula (CF=14M + 36 SmSt) for this species (Vasilev, 1985; Klinkhardt et al., 1995) differs from that obtained for the species *B. bjoerkna transcaucasia* in the present study. The karyotype for *B. bjoerkna* was reported as 2n=50, NF=76 and CF=12M + 12 Sm + 14 St + 12t by Simovic et al. (1994). Chromosome analysis of the species *B. bjoerkna bjoerkna* (Linneaus, 1758) was reported as 2n=50 and NF=76 (Klinkhardt et al., 1995) and for the species *B. bjoerkna derjavini* (Dadikyan, 1970) from the Bevdzhure River was reported as 2n=50 and NF=76 (Klinkhardt et al., 1995)

Table 3: Chromosome formula, chromosome number (2n) and chromosome arms in different species belong to the genus *Blicca*

Species	2n	Chromosome formula	NF	Reference
B. argyroleuca	50	14M + 36SMSt	_	Vasilev, 1985
B. bjoerkna	50	12M + 12Sm + 14St + 12t	76	Simovic, 1994
B. bjoerkna bjoerkna	50	12M + 12Sm + 14St + 12t	76	Klinkhardt et al., 1995
B. bjoerkna transcaucasica	50	12M + 12Sm + 14St + 12t	76	Klinkhardt et al., 1995
B. bjoerkna transcaucasica	50	6M+10Sm+9St	100	Present study
B. bjoerkna derjavini	50	12M + 12Sm + 14St + 12t	76	Klinkhardt et al., 1995

(Table 3).

The presence of different populations, races and/or sub species arising from mutation, race improvement and hybridization with other indigenous species could be the possible explanation for differences in number and type of chromosome reported for in a species found distributed in different aquatic ecosystems. These differences may also be caused by differences in the research methodology used in counting and measuring chromosomes. Sex, age as well as the hygienic state of the species and also the concentration of colchicines and the time of mitotic arrest may also influence chromosome characteristics particularly the chromosome arm number (NF) and chromosome length (Kalbasi et al., 2006). However based on the results obtained from the present study, chromosome analysis of *B. bjoerkna transcaucasica* is reported as 2n=50, CF=6M + 10 Sm + 9St and NF=100. With regard to the results obtained from the karyotype analysis of *B. bjoerkna transcaucasica* the use of 20 µg g^{-1} body weight PHA to stimulate cultures prepared from gill tissues treated with 0.0125% colchicines is recommended to conduct

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cytogenetic studies on other species belonging to the family cyprinidae.

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