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KURA-ARAS HAVZASINA ENDEMİK *Acanthalburnus microlepis* (DE FILIPPI, 1863) KROMOZOMLARININ C, G VE RESTRİKSİYON ENDONÜKLEAZLAR (*Alu I, Nhe I, Hae III, Mbo I, Hinf I*) İLE BANTLANMASI

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Özet

Acanthalburnus microlepis'in kromozom sayı ve standart karyotipik bilgileri araştırılmıştır. Bu araştırmada kullanılan balıklar, Kura-Aras havzası'ndan elektroşokerle yakalanarak laboratuvara getirilmiştir. Balıkların karın boşluğuna her bir g. vücut ağırlığı için %0.6'lık kolşisin solüsyonundan 0.01 ml enjekte edilmiş ve balık kesilmeden önce 190 dakika beklenilmiştir. Metafaz incelemeleri ile *A.microlepis*'in 2n=50 kromozoma sahip olduğu belirlenmiştir. Bunların karyotiplerinin ise 8 metasentrik, 7 submetasentrik ve 10 akrosentrik kromozom çiftinden (NF: 80) oluştuğu saptanmıştır. Bu türde cinsiyete bağlı herhangi bir kromozom tespit edilememiştir.

A.microlepis kromozomları beş restriksiyon endonükleazla muamele edilmiş, Giemsa ile boyanmış ve bant örnekleri incelenmiştir. *Alu I* enzimi baryum hidroksit etkileşimi ile ortaya çıkan C-banda benzer bant örnekleri üretmiştir. *Hae III, Hinf I, Nhe I* ve *Mbo I* enzimleri ise G-banda benzer bant örnekleri üretmiştir. Restriksiyon endonükleazlar, Giemsa ile boyanma oranını belirgin bir şekilde düşürmüştür. İlk defa bu çalışma ile Kura-Aras Havzasında Cyprinidlere ait endemik bir balığın detaylı karyotipi belirlenmiştir.

Anahtar kelimeler: *Acanthalburnus microlepis, cyprinidae,* karyotip, restriksiyon endonükleazlar, Kura-Aras Havzası.

C, G and Restriction Endonuclease (*ALU I, NHE I, HAE III, MBO I, HINF I*) Banging of The Chromosomes in *Acanthalburnus microlepis* (DE FILIPPI, 1863) Endemic to Kura-Aras River Basin

Abstract

Chromosome numbers and the standard karyotypic details for the Blackbrow bleak, *Acanthalburnus microlepis,* (De Filippi, 1863) (Fam: *Cyprinidae*) were ascertained. The fish used in this study were caught by electrofishing from the Kura-Aras river basin (Çıldır Lake) and taken to the laboratory. Fishes were injected intraperitoneally (i.p.) with doses of 0.01 ml/g body weight of 0.6 % solution of colchicine and left for 190 minutes before sacrification. It was determined that *A. microlepis* had 2n=50 chromosomes by metaphase investigation. Their karyotypes were determined as being composed of 8 metacentric, 7 submetacentric and 10 acrocentric chromosome pairs with NF: 80. We were unable to identify any sex-related chromosomes in this species.

A. microlepis chromosomes were treated with 5 restriction endonucleases stained with Giemsa and examined for banding patterns. The enzymes *Alu I* revealed banding patterns similar to the C-bands produced by treatment with barium hydroxide. The enzymes *Hae III, Hinf I, Nhe I* and *Mbo I* revealed banding patterns similar to the those G-bands. The restriction endonucleases markedly reduced the extent of Giemsa staining. For the first time, it is this study that determined in detail the karyotype of the endemic cyprinid fish in the Kura-Aras river basin.

Keywords: *Acanthalburnus microlepis, cyprinidae,* karyotype, restriction endonucleases, Kura-Aras river basin.

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INTRODUCTION

The carp or minnow family (Cyprinidae), is one of the most widespread and speciose families of fish in the world; certainly the most speciose in freshwater and possibly the largest family of vertebrates [1]. This family is found in North America, Eurasia and Africa. There are over 2100 species, almost 10% of the world's fish [1]. A vast majority of boned fish belongs to this family in Turkey, and they are distributed widely in freshwater sources. Cyprinid fish are a taxonomically complex group, due to the high number of endemic species with restricted distribution areas. *Acanthalburnus microlepis* (De Filippi, 1863), an endemic fish restricted to the Kura-Aras river basin, is a member of this complex group [2].

Cytogenetic studies on fish have received considerable attention in recent years [3, 4]. Chromosomal analysis is important for fish breeding from the viewpoint of genetic control, the rapid production of inbred lines, taxonomy and evolutionary studies. Genetic divergence of populations and their local adaptation are a potential resource for breeding programs in aquaculture and for fishery management [5].

Since the introduction of banding techniques for human chromosomes in the early 1970s and the more recent advances in banding using elongated chromosomes, knowledge of human and primate evolutionary relationships, medical genetics, and gene mapping has vastly expanded. In contrast, only limited success has been obtained with chromosome banding in plants, amphibia, and fishes [6-8].

A new banding method employing restriction enzymes has recently been applied to the chromosomes of a variety of animal species [9-12].

As the considerable chromosomal diversity in fish becomes better known, it has become clear that various methods, both basic and more advanced, are necessary for an adequate cytogenetic characterization of Neotropical fish. Diploid number, chromosome Formula and chromosomal banding, from the simplest techniques to those that provide high resolution and specificity, have become very important as cytogenetic markers for understanding chromosome diversity in Neotropical fish. These markers facilitate the pairing of homologs, highlight differences between apparently similar karyotypes, and can even reveal mechanisms of chromosome rearrangements [4, 13].

Standart karyotypes (chromosome and chromosome arm number) have been reported less than 10 % of the more than 20,000 species of fishes. The application of chromosome banding methodologies to fish chromosomes has been minimal [14, 15]. The main difficulty in working with fish chromosomes was to obtain high quality metaphase spreads. A few studies have used fish standart karyotypes to examine taxonomic or systematic problems [16, 17].

We examined metaphase chromosomes of *Acanthalburnus microlepis* digested with five restriction enzymes. Reproducible and distinct bands were produced by some restriction enzymes suggesting that this method may prove useful in fish chromosomes.

MATERIAL AND METHODS

Specimens of *A. microlepis* were collocted from the Çıldır Lake in Kars province, eastern Turkey (lat 38° 35'E, long 48° 49'N) by electrofishing. The fishes were transported live to the laboratory, and kept in a well-aerated aquarium at 20-25^{0}C before analysis. Fishes were injected intraperitoeally with doses of 0.01 ml/g body weight of 0.6 % solution of colchicine and left for 190 minutes before sacrification. The gill filament tissues were removed and placed in hypotonic fetal calf serum (fetal calf serum diluted with distilled water, 1:7,5), for 40 min [18-20]. They were then fixed in fresh and cold Carnoy (3:1) for 40 min. Staining was with 20 % Giemsa in Sorenson buffer solution for 7 min. The concentration of Giemsa may be reduced, but the treatment should then be longer [18].

C- and G-banding were performed according to Summer and Cano et al. respectively [21, 22]. Restriction endonucleases were employed according to Lloyd & Thorgaard, 1988; Hartley, 1991; Sanchez et al., 1990; Bron & Murray., 1975; Roberts et al., 1976; Gelınas et al., 1977, [14, 15, 23-26].

Observations and microphotograps were made with a Nikon light microscope. Chromosomes were classified on the basis of the arm- lenght ratio [27].

RESULTS

Relatively small and high number chromosomes were observed in *A. microlepis*. In 78 metaphases from the gill epithelial cells of fifteen *A. microlepis* specimens, the diploid number was found to be 2n=50 (Figs 1-3). Different chromosome number in a total of 12 metaphase cells was recorded ranging from 48 to 52 (Table 1).

	Chromosome number						karyotype (2n=50)			
Number of fish	48	49	50	51	52	total metaphases	m	sm	a	NF
1			4	1		5	16	14	20	80
2			7		1	8				
3		1	5			6				
4			2	1		3				
5	1		6			7				
6		1	6			7				
7			4	1		5				
8			3			3				
9			4			4				
10	1		3			4				
11		1	3		1	5				
12			5			5				
13			5			5				
14	1	1	5			7				
15			4			4				
Totals	3	4	66	3	2	78				

m: Metacentric sm: Submetacentric a: Acrocentric NF: Number of arms

Table 1. Chromosome complement of *A. microlepis.*

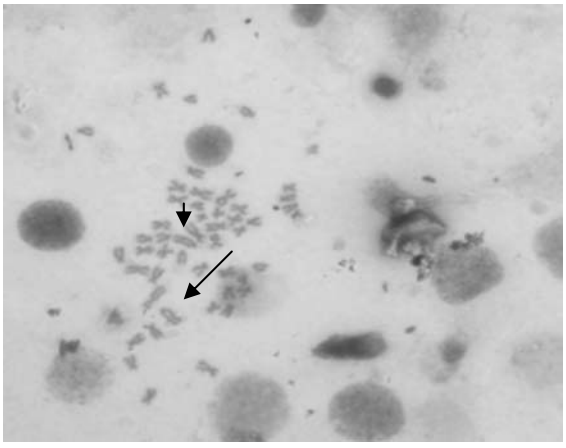

Fig. 1. Metaphase spread from gill epithelial tissue of *A. microlepis* from Kura-Aras river basin (Turkey).Largest acrocentric chromosome pairs, indicated by arrow. X 1.600. Bar, 5µm.

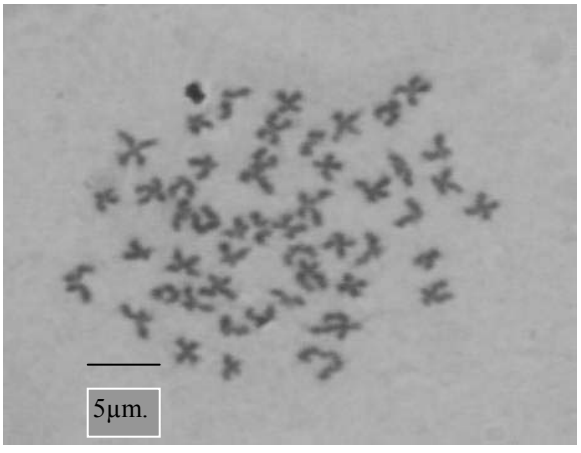

Fig. 2. Metaphase spread from gill epithelial tissue of *A. microlepis* from Kura-Aras river basin (Turkey). X 1.600. Bar, 5µm.

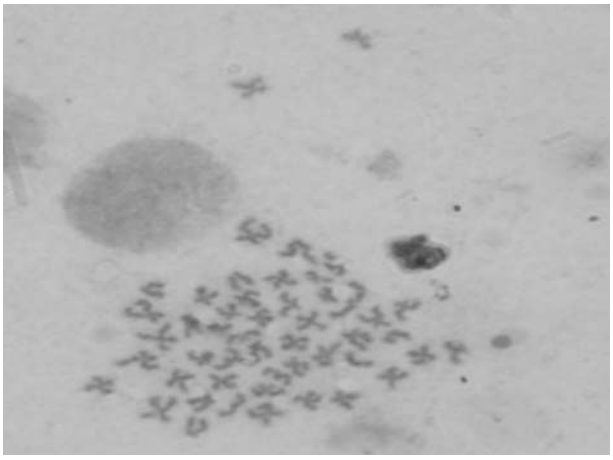

Fig. 3. Metaphase spread from gill epithelial tissue of *A. microlepis* from Kura-Aras river basin (Turkey). X 1.600.

A. microlepis has largest acrocentric chromosomes pairs, indicated by arrow (Fig. 1).

Cells not having normal values (2n=48-52) were probably caused by losses during preparation or additions from nearby cells. Results showed that in 84,6% of metaphases, the chromosome number of *A. microlepis* was 2n=50, comprising 8 pairs of metacentric and 7 pairs of submetacentric and 10 pairs of acrocentric chromosomes (Fig. 4). The number of chromosome arms were therefore determined to be NF=80.

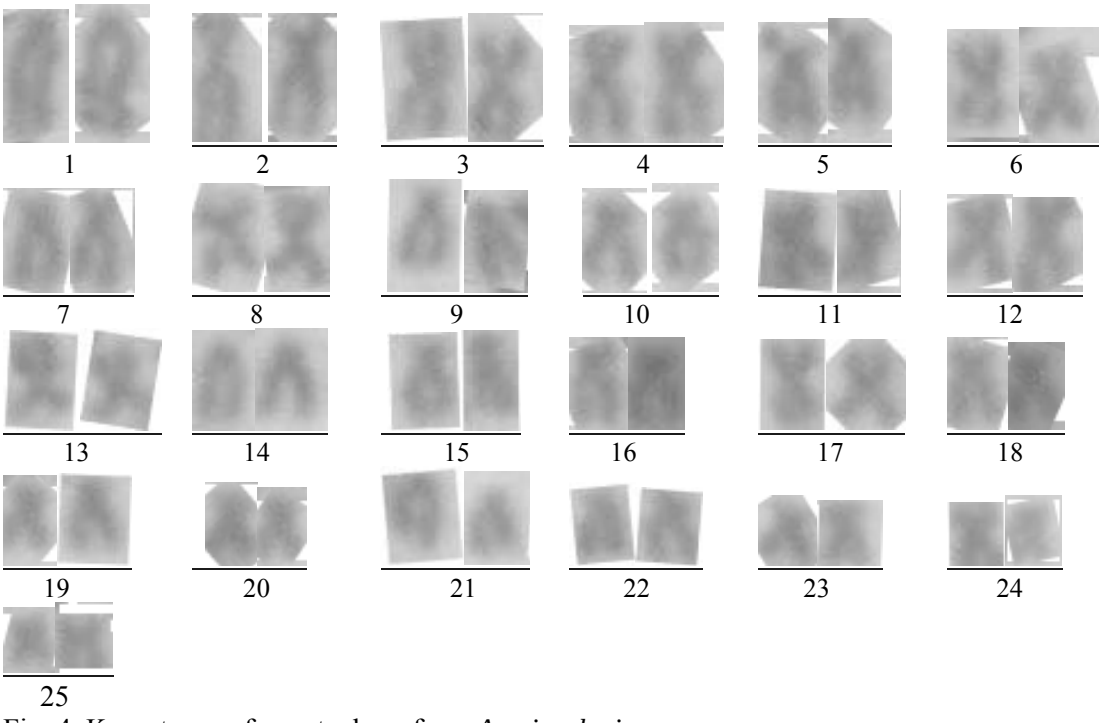

Fig. 4. Karyotypes of a metaphase from *A. microlepis*.

Alu I, which identifies and cleaves the DNA specific sequence AG/CT, produced a similar C-banding pattern in some chromosomes (Table 2). Some telomeres remained intact after enzyme treatment, while others were cleaved, therefore possesing cleavage sites for this enzyme. Since telemores are heterochromatic, this suggests that different types of heterochromatin occur in this population. When present, the B microchromosome was not digested by *Alu I*, and it remained stained. *Hae III*, which identifies and cleaves the DNA specific sequence GG/CC, *Hinf I* G/ANTC, *Nhe I* enzyme G/CTAGC and *Mbo I* cleaves the DNA specific sequence /GATC. The enzymes *Hae III, Hinf I, Nhe I* and *Mbo I* revealed banding patterns similar to the those G-bands (Table 2).

RE's	Specific DNA sequence	Banding pattern
Alu I	*5'.....AG↓CT.....3'* *3'.....TC↓GA.....5'*	C-banding
Hae III	*5'.....GG↓CC.....3'* *3'.....CC↓GG.....5'*	G-banding
Hinf I	*5'.....G↓ANTC.....3'* *3'.....CTNA↓G.....5'*	G-banding
Nhe I	*5'.....G↓CTAGC.....3'* *3'.....CGATC↓G…5'*	G-banding
Mbo I	*5'.....↓GATC.....3'* *3'.....CTAG↓...5'*	G-banding

Table 2. Banding pattern of *A. microlepis* chromosomes, treated with restriction endonucleases (*Alu I, Hae III, Hinf I, Nhe I, Mbo I*).

For the first time, it is this study that determined in detail the karyotype of the endemic cyprinid fish in the Kura-Aras river basin.

DISCUSSION

The karyotype, characterised by chromosome number, size and morphology, is a definitive and constant character of each species. The number, shape and banding of chromosomes can be determined using various dissecting and staining techniques. Chromosomal taxonomy can be quite useful, both in determining the phylogenetic relationships of the taxa, as well as in the segregation of sibling or cryptic species [28].

Karyotypes are prepared from metaphases with well spread chromosomes. The major difficulty encountered is the morphological variation existing even between homologous chromosomes in the same nucleus [18, 29]. Sometimes it could happen that some chromosomes are more contracted than others, so chromosome measurements are very difficult, and especially in fish, which have very small chromosomes compared to those of man and mammals. Another problem is that fish karyotypes are not identical as in human or in other animal species, so for fish we cannot have a standart karyotype because differences not only exist between species, but polymorphism often occurs within one fish species [18].

Banding patterns following the treatment of fixed metaphase chromosomes with restriction endonucleases have been described for several species of vertebrates [9-11] and for *Drosophila* [12]. We have found that *A. microlepis* chromosomes show bands similar to C- and G-bands after treatment with restriction enzymes. Five enzymes were tested and *Alu I* produced a pattern similar to the C-banding pattern produced with $Ba(OH)_2$ but the bands were more distinct and reproducible (Table 2). Conventional C-banding methods are known to stain areas containing heterochromatin which is located in the centromeres and telomeres of *A. microlepis*.

The factors proposed to be responsible for the differential staining of metaphase chromosomes following restriction endonuclease treatment are: [13] differences in base sequence along the metaphase chromosomes, making some DNA more susceptible to enzyme digestion [10, 30, 31], and [18] differences in chromatin structure, making DNA more available for enzyme digestion in some regions [32]. The correlation between the extraction of DNA and the decrease in chromosomal staining has been proposed as direct evidence that differences in sequence of DNA being removed play a major role in the mechanism of restriction endonuclease banding [30, 31]. Since after treatment with certain enzymes there is a G-banding pattern in chromosomes stained with Giemsa and not in chromosomes stained with ethidium bromide (a DNA-specific dye), Mezzanotte & Ferruccı (1984) [32] concluded that differences in chromatin structure are important in restriction endonuclease banding.

Carvalho, Giuliano-Caetano and Dias, utilized *Alu I* enzyme in *Iheringichthys labrosus* from the Tibagi River, in which it was also possible to see a banding pattern similar to C-banding, in various chromosomes of the complement [33]. Swarça et al. (1999) (2001) [34, 35], also obtained banding patterns similar to C-banding with *Alu I* in *Pinirampus*

pirinampu and *Pimelodus maculatus*, as did Swarça (2003) in *Steindachneridion sp* and *S. Scripta* [36].

Several incomplete metaphases were encountered in the preparation that probably have resulted from hypotonic overtreatment [37].

The majority of authors classify uni-armed and bi-armed chromosomes according to the guidelines of levan et al. (1964) [27]. Where differences in the number of chromosome arms have been reported for the same species, this is usually result of a difference in the scoring of subtelocentric chromosomes by different authors [5].

The majority of cyprinid species have 2n=50 chromosomes [18], gross karyotypic change in North American cyprinids appears to have been minimal: over 90 % of all species assayed (including all *Notropis* species examined) possess diploid chromosome numbersof 50 (range=48-52) and (estimated) diploid chromosome arm numbers between 92 and 100 (range=80-100) [38, 39], karyotypes have been described for specimens of *R. aula, S. erythrophthalmus*, *R. rubilio, O. angorae* and *P. Persidis, Acanthobrama marmid* all possessing 2n=50 chromosomes [40-44], while *Cyprinus carpio* has 2n=98-100 [45], the polyploid *Barbus species* from Southern Africa have 2n= 148 or 150 [46], *Capoeta trutta* and *C. capoeta umbla* have 2n=150 chromosomes [47]. Heteromorphic sex chromosomes have been identified in *Coregonus sardinella, Oncorhynchus mykiss*, *Oncorhynchus nerka* and *Salvelinus namaycush*. There is an XY/XX system in *S. namaycush* and *O. mykiss*, and an XYY system in *C. sardinella*. The formation of heteromorphic sex chromosomes often involves heterochromatin addition, as in other animals, and this appears to be the case in *S. namaycush* and *O. mykiss*. There was no evidence of sexual dimorphism of the chromosomes in *A. microlepis*. Similar results were also observed in most fish species [5, 48].

The chromosomes of fishes have been difficult to study since no method has consistently produced detailed linear banding. In *A. microlepis* only a few pairs of homologs can be identified by morphological characteristics and the results of C-banding, replication banding or NOR staining. This study has shown that some restriction endonucleases produce better defined and more reproducible bands than the conventional C-banding methods. In addition, smaller bands not seen with the conventional C-banding methods were sometimes observed. This new technique should provide additional information for chromosome identification in fishes.

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