Cytogenetic Variation of Geographically Isolated Four Populations of *Garra rufa* [(Heckel, 1843) (Pisces, Cyprinidae)] in Turkey

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Abstract —This paper describes the karyotype analysis of *Garra rufa* samples with G-, C- banding and Ag-NOR staining techniques from four distinct localities (Mersin, Hatay, Kahramanmaras and Sivas) in Turkey. The diploid chromosomes number was found as 50 in females of both Mersin and Sivas regions samples. The karyotype was determined as 26M + 10SM + 8ST + 6A chromosome and fundamental number (NF) 94 in Mersin and 28M + 14SM + 4ST + 4A chromosome (NF=96) in Sivas regions samples. Diploid chromosomes number both Hatay and Kahramanmaras regions male and female samples were described as 46. The karyotype of Hatay region female samples was included 22M + 12SM + 8ST + 4A (NF=88) chromosome and male 22M + 12SM + 7ST + 5A chromosome (NF=87). The karyotype of Kahramanmaras region female samples was included 32M + 6SM + 6ST + 2A chromosomes (NF=90) and male 31M + 6SM + 6ST + 3A chromosomes (NF=89). The male presented a pair of differentiated heteromorphic chromosomes, characterizing as XX/XY sex chromosome system of Kahramanmaras and Hatay region samples. The comparative analysis revealed intraspecific variability of NORs and fixed differences in their number in the four populations. The results are compared with data available for populations from native geographic ranges.

Key words: *Garra rufa,* G-, C-, Ag-NOR banding, geographic isolation, karyotype.

INTRODUCTION

Fish is the most primitive vertebrate group, may be found in several types of environments and show wide genetic variability both at the chromosomal and molecular levels, which makes them an interesting group for evolutionary and cytotaxonomic studies (Kosswig 1973). The investigation on the fish chromosomes number and morphology are said to be useful for the hybridization, classification and evolution (Freeman and Herron 1999; Campbell and Reece 2006).

Genus *Garra* is well known in the south of Turkey and it is represented by two species; *Garra variabilis* and *Garra rufa.* (Kuru 1971; Kuru 1979; Geldiay and Balik 1996). *G. rufa* is known as doctor fish. They live and breed in the outdoor pools of some Turkish spas where they feed on skin of patients with psoriasis. *G. rufa* is legally protected from commercial exploitation in Turkey due to concerns of over harvesting for export (Froese and Pauly 2005).

Ag-NOR, G- and C- banding techniques are using for determining karyological analyses in fishes and the other organisms. NOR regions are used as important chromosomal marker in fishes. The number and position of NORs on chromosomes changes according to genus, species and population. That's why NOR regions are used in phylogenetic works. The works on chromosomes show that the number of chromosomes can be change by centromeric fusion. This event is very common in fishes. The number and position of heterochromatin are very important on speciation (John and Miklos 1979).

The aim of this study is determine the karyological differences with between four populations of *G. rufa* uses Ag-NOR, G- and C- banding techniques.

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MATERIALS AND METHODS

G. rufa specimens were collected by seine from the Mezitli and Müftü stream in Mersin; Kesis stream in Kahramanmaras (which is connection with Ceyhan River); Büyükkaracay stream in Hatay (which is connection with Orontes River); Kangal fish spring in Sivas (Fig.1). Cytogenetic analyses were carried out on 108 specimens. Chromosome preparation followed the technique described by ERGENE *et al.* (1999). For the conventional karyotype, the preparations were stained for 20 min with

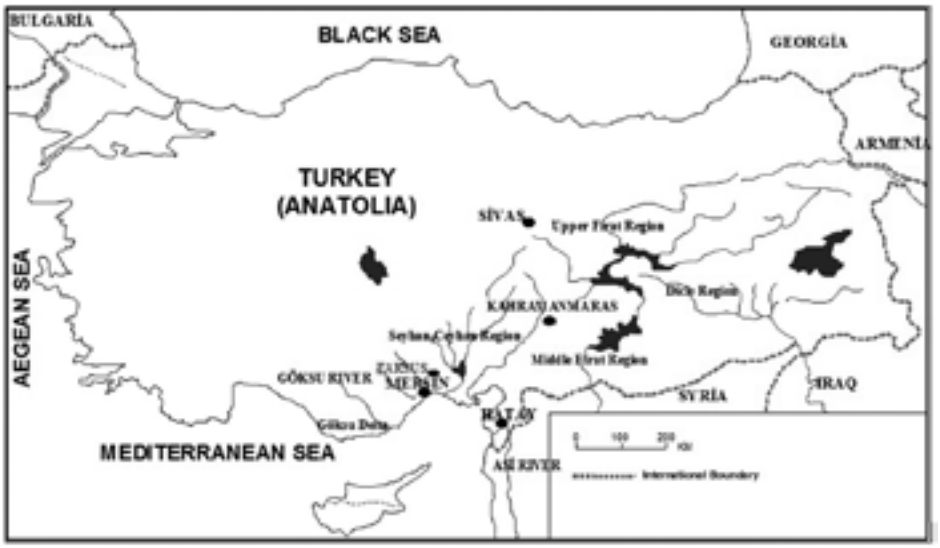

Fig. 1 — Collecting sites of *G. rufa* samples.

5% Giemsa in phosphate buffer pH 6.8. Detection of the Nucleolus Organizer Regions (NORs) was done following the silver (Ag-NO_3) staining method of HOWELL and BLACK (1980). C-bands were obtained according to the method described by SUMNER (1972). Metaphase chromosomes were banded using the conventional Trypsin-Giemsa banding technique (SEABRIGHT 1971). G-banded, Ag-NOR stained and C-banded mitotic chromosomes were photographed using a digital camera and the images were digitally processed with Adobe Photoshop v. 7.0 software. The karyogram was constructed with chromosomes organized in order of decreasing size and the chromosomes classified according to LEVAN *et al.* (1964). Arm ratio was figure out according to Micro-Measure program. The fundamental number (NF) or arm number was determined by considering metacentric (M), submetacentric (SM) and subtelocentric (ST) chromosomes with two arms and acrocentric (A) with only one.

RESULTS

The diploid chromosome number of Mersin, Hatay, Kahramanmaras and Sivas population were determined as 50, 46, 46 and 50 respectively.

G. rufa from Mersin - The diploid metaphase complements consisted of 50 chromosomes of females. A karyotype formula was described as 26 M, 10 SM, 8 ST, 6 A chromosome and fundamental arm number (NF) 94. The karyotype was determined according to the arm measurement which consequence of C- band (Fig. 2a). It was seen that three band region on the first chromosome arm, two band region 2., 4., 6., 9., 13. chromosome arms, one band region 5., 7., 8., 11., 12., 14., 15., 16., 17., 18., 19., 20., 22., 23. chromosome arms. (Fig. 2b). Ag-NORs were observed on terminal region at 15. pair SM and 20. pair ST chromosome short arms (Figure. 2c, 7a). The karyotype of the Mersin region samples was given in Fig. 6a. The clear band regions schema was given in graphic with the consequence of Giemsa band at Figure 7a.

G. rufa from Hatay - The karyotype structure of these specimens consist of 2n=46 chromosome. The karyotype formula of females is 22M + 12SM + 8ST + 4A, NF = 88 and of males is 22M+12SM+7ST+5A, NF = 87. Thus, whereas the females presented a homomorphic karyotype, the males showed heteromorphic chromosomes. A single ST chromosome (X) and a small A chromosome (Y) were examined in all male individuals. The metaphase plate of C-band was given at

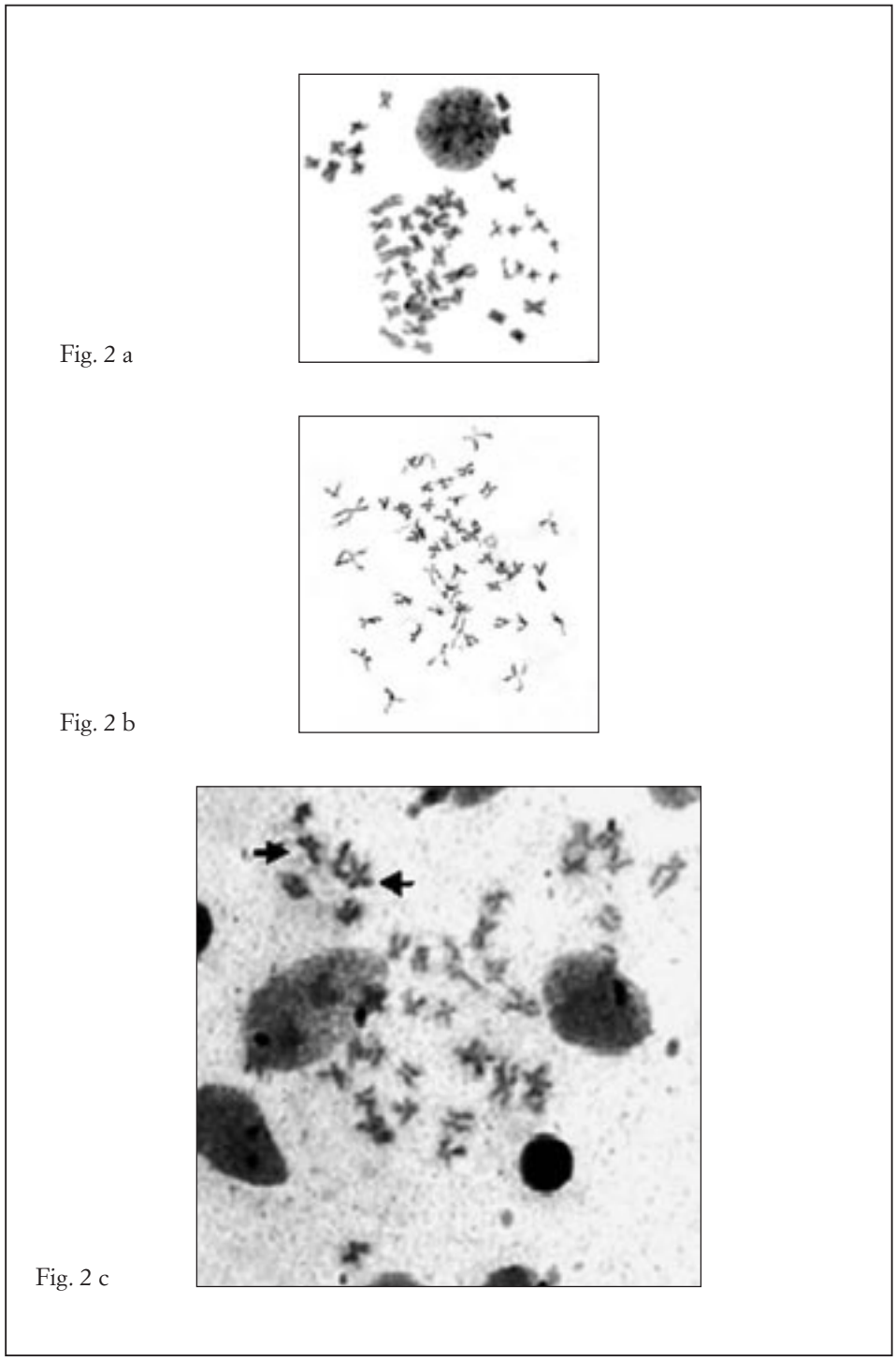

Fig. 2 — Metaphase images of Mersin *G. rufa* samples (female): a) C-banded; **b)** G-banded; **c)** Ag-NOR staining (arrow: Ag-NOR regions).

Fig. 3a. It was seen that four band region on the first chromosome arms, two band region 2., 4., 6., 12., 18. chromosome arms and one band region 9., 10., 11., 13., 14., 21. chromosome arms consequence of G- band. Quite large heterochromatin region was seen on 13. SM chromosome long arms (Fig. 3b). Ag-NOR were observed on terminal region of short arm in ST 21. chromosome (Figure 3c). Karyotype of the Hatay region samples was given in Fig. 6b. Clear band region schema was

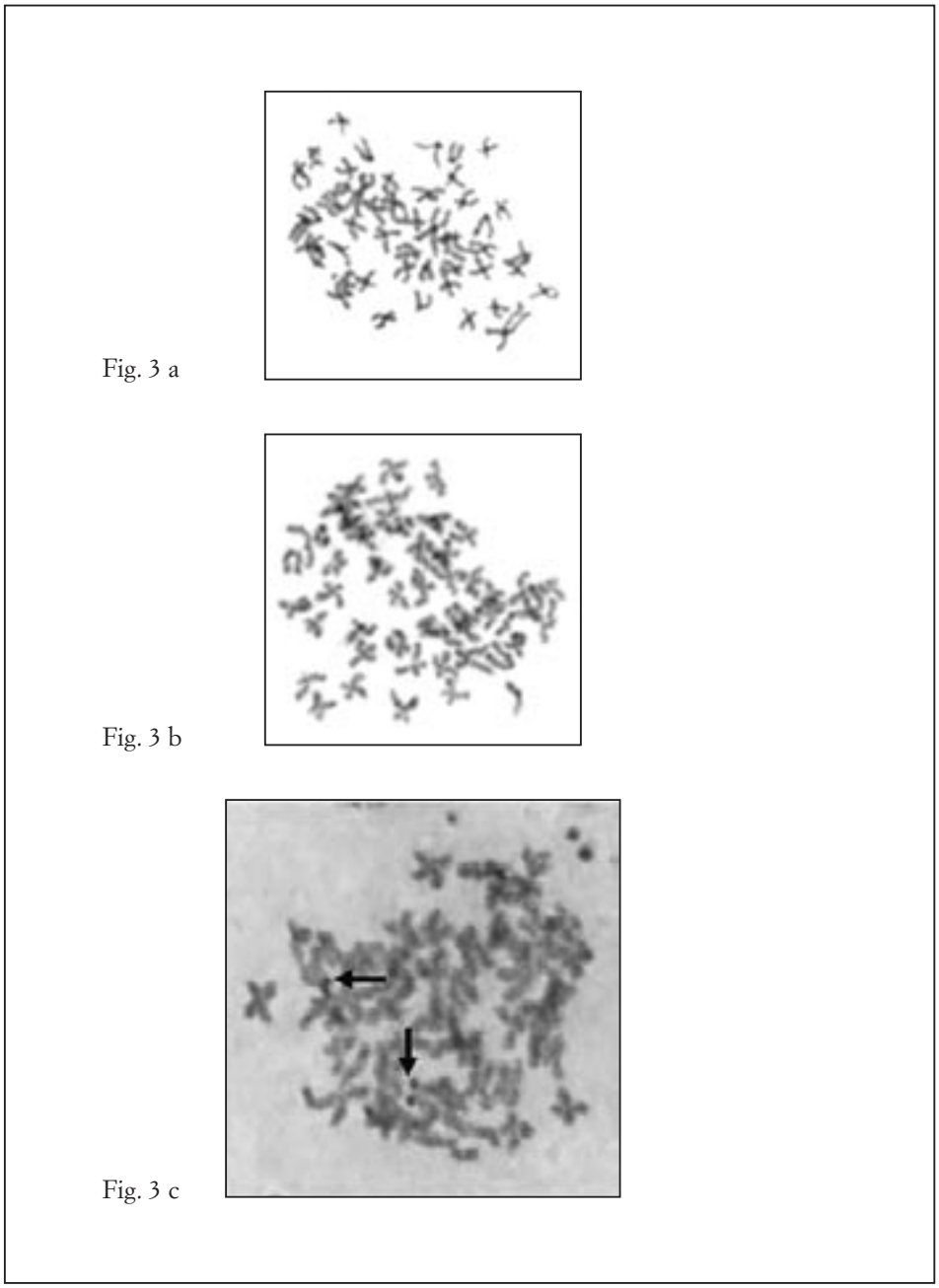

Fig. 3 a

Fig. 3 b

Fig. 3 c

Fig. 3 — Metaphase images of Hatay *G. rufa* samples*:* a**)** C-banded; **b)** G-banded; **c)** Ag-NOR staining (arrow: Ag-NOR regions).

given in graphic with the consequence of G- band at Fig. 7b.

G. rufa from Kahramanmaras - This region samples karyotype is composed of 2n = 46 chromosome both males and females. However, differential karyotype was observed among males and females, a fact that appears to be characteristic for this population. The females have 32M + 6SM + 6ST + 2A chromosome (NF=90) while males have 31M + 6SM + 6ST + 3A chromosome (NF=89), indicating a sex chromosome system of the XX/XY type. The X chromosome was represented by the M pair in females and the single element of this type in males. In addition that, male has a small A chromosome (Y). Karyotype was determined according to the arm measurement of C-band chromosomes. The C-band positive heterochromatic regions were distributed in centromeric and

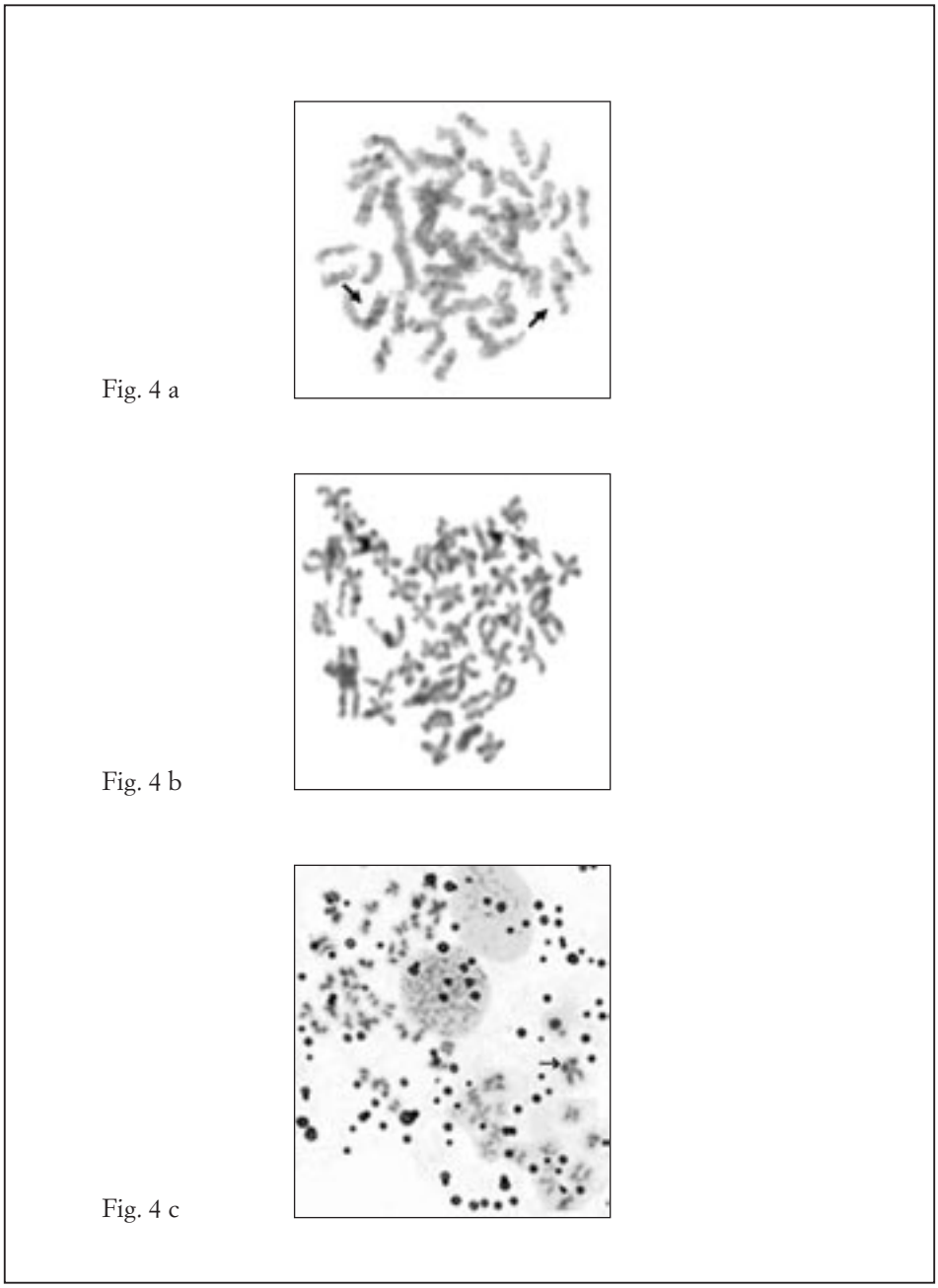

Fig. 4 — Metaphase images of Kahramanmaras *G. rufa* samples. **a)** C-banded (arrows show that heterochromatin region that is out of centromer); **b)** G-banded; **c)** Ag-NOR staining (arrow: Ag-NOR region).

pericentromeric positions. Clear C-band regions were seen at centromeric regions of many chromosomes and at interstitial regions of 1. and 13. M chromosomes long arms (Fig. 4a). It was seen that four band regions on the first chromosome arms, three-band region 17. chromosome arms; two band region 3., 5., 6., 8., 9., 10.,11.,12.,13., 22. chromosome arms; one band region 4., 7., 14., 18., 20., 21. chromosome arms consequent of Giemsa banding. The largest heterochromatin region was seen on M 14. chromosome (Fig. 4b). Ag-NORs were observed on the terminal region of short arm at M X chromosome (Fig. 4c). The karyotype of the Kahramanmaras region was given in Fig**.** 6c. The clear band region schema was given in graphic with the consequence of G- band at Figure 7c.

G. rufa from Sivas - The diploid metaphase com-

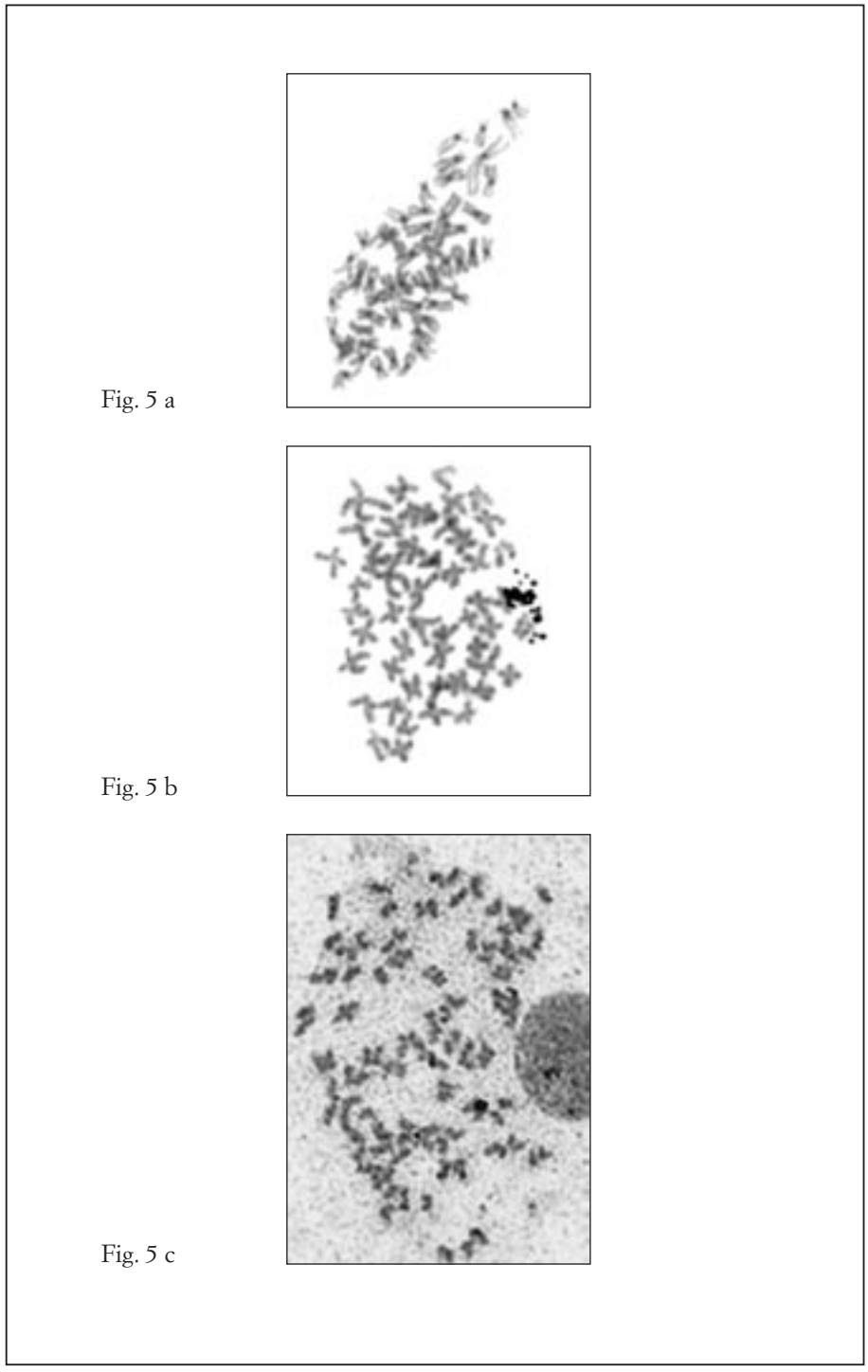

Fig. 5 a

Fig. 5 b

Fig. 5 c

Fig. 5 — Metaphase images of Sivas *G. rufa* samples (female): **a)** C-banded; **b)** G-banded; **c)** Ag-NOR staining.

plement consisted of 50 chromosome of female. A karyotype formula is 28M + 14SM + 4ST + 4A chromosome and NF = 96. Karyotype was determined according to the arm measurement which consequence of C- band (Fig. 5a). The C-band positive heterochromatic regions were distributed in centromeric positions. It is seen that three band region on first chromosome arms; two band region on 2., 3., 4., 7., 9., 12.,13., 15., 16., 17. chromosome arms; one band region on 6., 10., 11., 18., 19., 22., 23., 24. chromosome arms consequent of Giemsa banding (Fig. 5b). Ag-NOR's

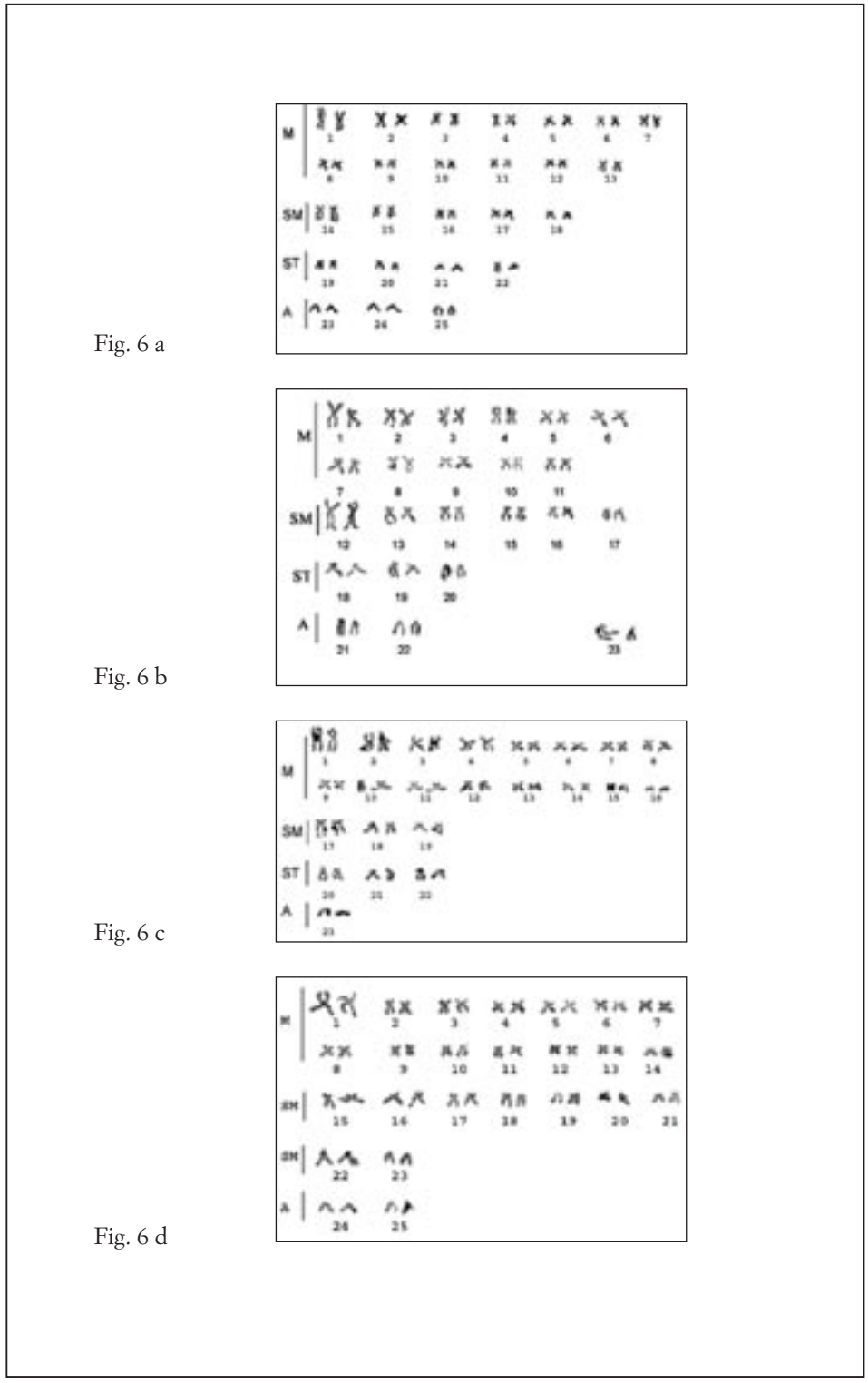

Fig. 6 a

Fig. 6 b

Fig. 6 c

Fig. 6 d

Fig. 6 — **A)** Karyotype of Mersin *G. rufa* female; **B)** Karyotype of Hatay *G. rufa* male; **C)** Giemsa banding karyotype of Kahramanmaras *G. rufa* female; **D)** Giemsa banding karyotype of Sivas *G. rufa* female.

were observed terminal region in M 3., 4., 5., 9. and SM 17. chromosomes' short arms (Fig. 5c). The karyotype of Sivas region was given at Fig. 6d. Clear band regions' schema was given in Fig. 7d. which was drawn according to G- banding.

The largest chromosome pair of the comple-

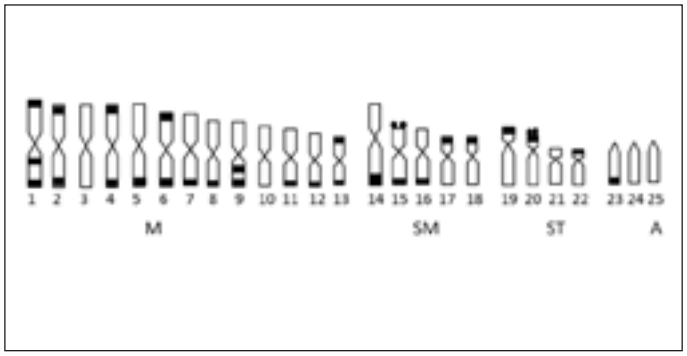

Fig. 7 a

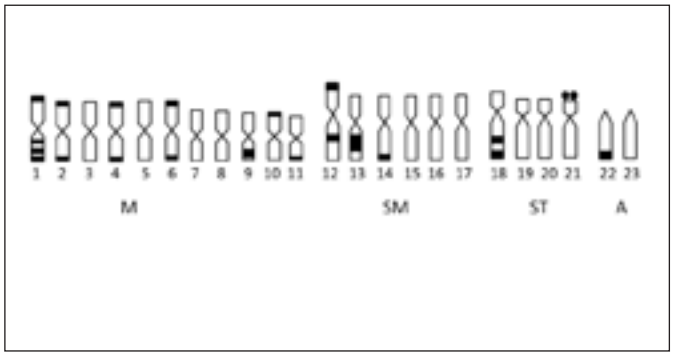

Fig. 7 b

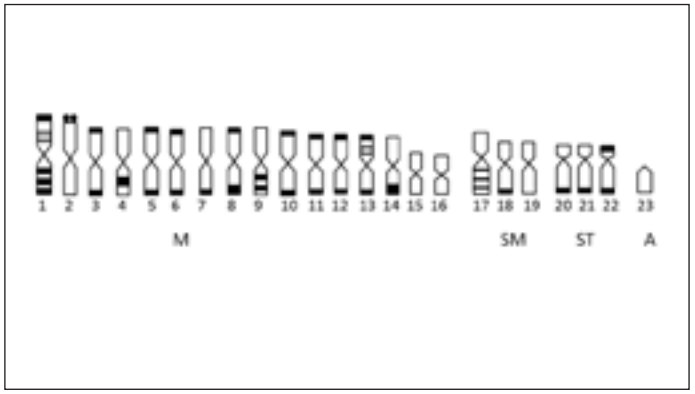

Fig. 7 c

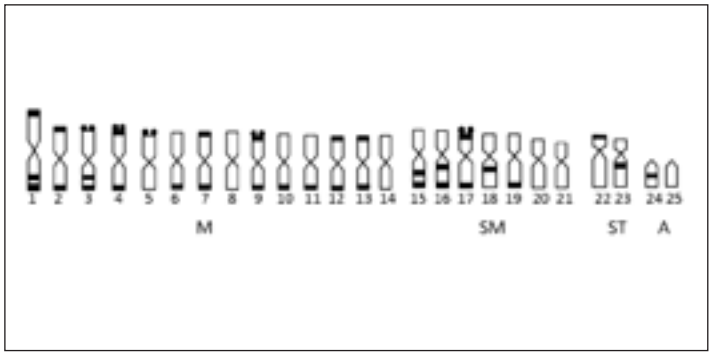

Fig. 7 d

Fig. 7 — Ideograms of *G. rufa* **a)** Mersin♀; **b)** Hatay ♀; **c)** Kahramanmaras ♀; **d)** Sivas ♀ regions. ■ Giemsa band regions, heterochromatin regions that is outside the centromer.

ments were characteristically SM in both Mersin and Kahramanmaras populations (Fig. 7a, 7b); M in both Sivas and Hatay population (Fig 7c, 7d).

DISCUSSION

The chromosome number of *G. rufa* populations has been notified as 2n=46-50 in the present study. In the previous studies chromosome numbers were given as 44-52. The chromosome numbers and karyotypes of *Garra* genus, which have been studied up to now, were given at Table 1.

Heterochromatin has played an important role in the karyotypic diversification of Cyprinidae fish (TAKAI and OJIMA 1988), where the karyotypic macrostructure is relatively constant but with differences related to the heterochromatin among the species (GALETTI *et al.* 1991). It has also shown to be efficient in the identification of polymorphisms, characterizing intrapopulation variability in some species (MANTOVANI *et al.* 2000). In this study, a quite large heterochromatin region was seen on 13. SM chromosome of Hatay and 14. M chromosome of Kahramanmaras samples (Fig. 7b, 7c). These heterochromatin regions have not been seen at the other populations. These chro-

Table1 — Cytogenetical data of fishes under *Garra* Genus.

No	Species	Locality	2n	FN	Chromosome formula	Sex chromosomes	References
1	*G. cambodgiensis*	Unspecified	52				VASIL'EV, 1980
2	*G. dembeensis*	Unspecified	50	82			KRYSANOV and GOLUBTSOV, 1993
3	*G. gotyla gotyla*	India Ooty, TN.	50	90	14m+26sm+10t,a		BARAT, 1985
4	*G. gotyla gotyla*	India Jammu, J&K	50	74	14sm+10sm+10st+16t,a	No heteromorphic sex chromosomes in male.	KHUDA-BUKHSH, 1984
5	*G. gotyla gotyla*	India, Itanagar, A.P	50	70	12m+8sm+8st+22T		KUMARI SAHOO *et al.*, 2007
6	*G. gotyla gotyla*	India, India	50	90	14m+26sm+10t,a		BARAT, 1985
7	*G. kempi*	India, Itanagar, A.P	50	78	14m+14sm+10st+12t		KUMARI SAHOO *et al.*, 2007
8	*G. lissorhynchus*	India, Itanagar, A.P	50	82	16m+16sm+6st+12t		KUMARI SAHOO *et al.*, 2007
9	*G. mullya*	India Chalakkudy river, Kerala	50	82	18m+14sm+10st+8t		NAGPURE *et al.*, 2006
10	*G. imberba*	Unspecified	50				ARKHIPCHUK, 1999
11	*G. lamta*	India Bihar	50	86	6m+18sm+12st+14 t,a		ARKHIPCHUK, 1999
12	*G. lamta*	India Simlipal Hills, Orissa	50	86	12m+24sm+2st+12t,a	Female,ZW Male WW	BARAT, 1985
13	*G. lamta*	Unspecified	50	74	6m+18sm+12st+14t,a		KHUDA-BUKHSH *et al.*, 1980
14	*G. makiensis*	Unspecified	50	84	34m,sm+16 t,a		KRYSANOV and GOLUBTSOV, 1993
15	*G. quadrimaculata*	Ethiopia	50	88	38m,sm+12 t,a		KRYSANOV and GOLUBTSOV, 1993
16	*G. rufa*	Unspecified	44-50				POST, 1965
17	*G. rufa*	Unspecified	44-52				VASIL'EV, 1980
18	*G. rufa*	Turkey, Mersin	44	85	22m+20sm+2a		ERGENE GOZUKARA and CAVAS, 2004
19	*G. rufa*	Turkey, Dicle River	44	87	16m+26 sm+1st+1a		KILIC-DEMIROK, 2000
20	*G. rufa*	Turkey, Mersin	50	94	26m+10sm+8st+6a	Female	Present study.
21	*G. rufa*	Turkey, Hatay	46	88 87	22m+12sm+8st+4a 22m+12sm+7st+5a	Female XX Male XY	Present study.
22	*G. rufa*	Turkey, Kahramanmaras	46	90 89	32m+6sm+6st+2a 31m+6sm+6st+3a	Female XX Male XY	Present study.
23	*G. rufa*	Turkey, Sivas	50	96	28m+14sm+4st+4a	Female	Present study.

mosomes can define the relation of between two regions samples.

The NOR is certain indicator for rewiring chromosomal polymorphism in species and between species in lots of fish groups and it is notified that this variety can affect the position on the chromosome, size and active number of NOR's in the whole genome (OZOUF-COSTAZ and FORESTI 1992). Although the NOR is especially being seen at the short arm of chromosome, sometimes it can be seen on the long arm terminal of M and A chromosomes (SOLA *et al*. 1993; COLARES-PEREIRA 1995 and RAB *et al*. 1996). Furthermore, these can be seen between telomere and centromer, near centromer (GALETTI *et al.* 1984; JANKUN *et al*. 1998). Also NOR can exist rarely in sex chromosomes (BERTOLLO and CAVALLARO 1992). In this study, one NOR region was determined on M X chromosomes of Kahramanmaras (Fig. 4c, 7c); one NOR terminal region in the SM chromosome short arms (Fig. 3c, 7b) of Hatay; terminal region at 15. pair SM chromosome short arms and 20. pairs ST chromosome of Mersin (2c, 7a); terminal region at M 2., 4., 5., 9. and SM 17. pairs chromosome short arms of Sivas region samples (5c, 7d).

The diploid chromosome number of both Mersin and Sivas regions populations were different from Hatay and Kahramanmaras regions populations. Four regions chromosome formulas are variable. This indicates that Robertsonian rearrangements as well as pericentric inversions were the main rearrangements related with the karyotype diversification of these *G. rufa'*s. It was also observed that the fundamental arm number (NF) of Mersin and Sivas regions samples are genetically close to each other from Hatay and Kahramanmaras regions samples. These are forming two distinct clusters, one consisted of Mersin-Sivas regions populations and the other consisted of Hatay and Kahramanmaras regions populations.

The obtained chromosome number and morphology from chromosomal analyses are used to identify species easily and define the relationship and differences between different species. The chromosome number and morphology can differ among fish species. This variation can be used in the search of evolutional relationship between interpopulations and inpopulations (THORGARD and DISNEY 1990). *G. rufa*'s chromosomes number has changed from 2n=50 to 2n=46. It is estimated that centromeric fusion can have been effective during this differentiated period.

C- and Ag-NOR banding analyses were proved useful for the investigation of the karyotype evolution in bitterlings. However, the composition of the heterochromatin has to be better investigated for clarifying the karyotype evolution in this fish group (UEDA *et al*. 2001). In general, the patterns of C-band distribution of many fishes are simple. However, C-band distribution in the Cyprinidae is great interest. Cyprinidae seems to be an interesting group to study the distribution of heterochromatin and its relation to chromosome evolution and species differentiation (TAKAI and OJIMA 1988). In the present study, interstitial C-bands, which were revealed in the 1. and 13. pairs M chromosome in Kahramanmaras region samples, can be formed with tandem fusions (Fig 4a, 7c). Similar examples have been reported in Balistids (KITAYAMA and OJIMA 1984) and some Salmonids (UEDA and OJIMA 1983; 1984). This large C-banded heterochromatic region could have been formed by tandem duplication of heterochromatic DNA. C- band patterns in Kahramanmaras region samples were very different, this can be identified as a marker of this region.

G. rufa entered into Anatolia from Mesopotamia via Dicle and Firat River. When the ice age started, some species, which originated from Mesopotamia, migrated to south via the Firat and Dicle River systems to get preserved from cold climate. After the ice age, many species returned to the East Anatolia but they could not spear inside of Anatolia. Many genera could not reach the River in the western part of Tarsus region. During the ice age of Pleistosen, Mediterranean sea level was quite low, so that Orontes (Hatay) and Seyhan-Ceyhan Rivers connected each other with freshwater. Finally, some species, which were originated from Mesopotamia passed Hatay region *(DEMIRSOY 2002)*.

Hatay population was formatted after last ice age and at first, this population was initially in contact with Kahramanmaras population owing to Seyhan and Ceyhan Rivers. Then it was completely isolated and started to differentiate. Nowadays these four regions (Mersin, Hatay, Kahramanmaras and Sivas) have not connected each other with river basin. These regions samples are isolated with each other geographically. Differences which are between Hatay and Kahramanmaras populations are lower than the other areas because the geographically isolation between them formed lately.

Chromosomal distribution of heterochromatin regions and rDNA sites in *Garra* samples, which was collected from four different regions in Turkey, was analyzed using the C-,G- banding and Ag-NOR staining technique. These species showed various patterns of these band distributions. Many Cyprinid karyotypes are rich accord-

ing to m and sm chromosomes and *G. rufa* show the general Cyprinid karyotype. The chromosomal constitution of Mersin, Hatay, Kahramanmaras and Sivas *G. rufa*'s are different from each other. When four regions *G. rufa* samples were compared with each other, it is clear that the relation between Hatay and Kahramanmaras samples are more than the Mersin and Sivas regions samples.

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