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Karyotypes of Danubian lineage brown trout and their hybrids

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Abstract. Cytogenetic analysis of brown trout, *Salmo trutta*, have been described for different populations and morphs; however, cytogenetic analysis of interspecific brown trout hybrids is unknown. Cultured kidney cells from four brown trout subspecies (*Salmo trutta abanticus*, *S.t. caspius*, *S.t. fario* and *S.t. labrax*) and their reciprocal hybrids were karyotyped using conventional staining, C-banding and Ag-NOR staining techniques. Chromosome number (2N) and chromosome arm number (NF) ranged from76 to 80 and 98 to 102, respectively. Silver staining revealed the presence of NOR sites on the short arm of the submetacentric chromosome. The size and number of NOR sites showed uniformity. The presence of heterochromatin on different chromosome arms was confirmed by C-banding. The presence and position of constitutive heterochromatin showed variability among individuals. Chromosome structures of purebred brown trout subspecies belonging to the Danubian linage and their hybrids were similar, and no distinctive characteristics were observed in any of the species. The results of this study are applicable to the development of improved conservation and management strategies for brown trout.

Keywords. Cytogenetic, Karyotype, *Salmo trutta*, Ag-NOR, C-banding.

INTRODUCTION

Brown trout, *Salmo trutta* (Linnaeus, 1758), is a polymorphic and widespread species. Its historic geographic range covers Europe, Western Asia and Northern Africa. During the past century, *Salmo trutta* have been introduced to different parts of the world, and the range of brown trout has been extended to all continents except Antarctica (Elliott, 1989). The systematic classification of *Salmo trutta* is plagued by many nomenclatural issues. *Salmo trutta* was once recognized as a polymorphic species with three morphs based on life-history variation: resident trout, lake trout and river trout (Ferguson, 2004). Mitochondrial DNA (mtDNA) sequence variation analysis revealed the existence of five major phylogenetic groups, which are believed to have been separated for some 500,000 to 2 million years (Bernatchez, 1995). Over the years, distinct species or nominal subspecies have

been described based on morphological and molecular analysis (Kottelat & Freyhof, 2007; Turan, Kottelat, & Engin, 2014). However, *S. trutta* subspecies such as *S.t. abanticus*, *S.t. caspius*, *S.t. fario* and *S.t. labrax* belonging to Danubian lineage have been proved to be a single biological species called *Salmo trutta*. Thus, it was recommended that strains should be named according to location, such as Abant, Caspian, Anatolian and Black Sea (Kalayci et al., 2018).

Inter- and intraspecific hybridization experiments in fish are often less concerned with identification of the genomic composition than with the evolution of performance and survival (Johnson & Wright, 1986). Morphology and variation in chromosome number have been proven useful in identifying fish populations (Phillips, 2005). Cytogenetically, the *Salmo trutta* complex is one of the best analyzed salmonid. The karyotype of *Salmo trutta* consists of 80 chromosomes with a fundamental arm number (NF) ranging from 98 to 102 (Amaro, Abuin, & Sanchez, 1996; Woznicki, Jankun, & Luczynski, 1998; Woznicki, Sanchez, Martinez, Pardo, & Jankun, 2000). Although *Salmo trutta* have been subjected to numerous cytogenetic analyses, and karyotypes have been described for different populations and morphs, (Caputo, Giovannotti, Cerioni, Splendiani, & Olmo, 2009; Jankun, 2000; Kalbassi, Dorafshan, Tavakolian, Khazab, & Abdolhay, 2006; Northland-Leppe, Lam, Jara-Seguel, & Capetillo-Arcos, 2009; Woznicki, Jankun, & Luczynski, 1997; Woznicki et al., 1998), the chromosome complement of interspecific brown trout hybrids seems to be comparatively less studied (Polonis, Fujimoto, Dobosz, Zalewski, & Ocalewicz, 2018; Ziomek, Debowska, Hliwa, & Ocalewicz, 2016). A cytogenetic characterization of hybrids and parental species would aid in a better understanding of their species status. Therefore, the aim of the present study was 1) to determine the chromosomal characteristics of Abant trout (*S.t. abanticus*), Black Sea trout (*S.t. labrax*), Caspian trout (*S.t. caspius*), Anatolian trout (*S.t. fario*) and their reciprocal hybrids and 2) to determine if the NF of chromosomes varies among purebred and hybrid trout.

MATERIALS AND METHODS

Fish

Abant, Anatolian, Black Sea and Caspian trout were crossed to each other to produce the F1 generation of all possible reciprocal crossing combinations (16 cross-types) (Table 1). After fertilization, each family was separately incubated in a vertical incubator and transferred to a separate flow-through indoor tank after hatching. This study was approved by the Institutional Animal Care and Use Committee at Karadeniz Technical University (approval #14/2013).

Chromosome Preparation

Five fish from each cross-type were used in chromosome analysis (Table 1). Fish were anaesthetized with ice, and their anterior kidney tissue was sampled on ice. Tissue was cut into small pieces and incubated in 1.5 ml of RPMI media supplemented with penicillin G (75 U/ml), fungizone (1.5 µg/ml), gentamycin sulphate (30 µg/ml) and streptomycin sulphate (75 µg/ml) for 24 h at room temperature. Supplementing the culture media with antibiotics eliminated any growth of fungi, yeasts, mycoplasma and Gram-positive and Gram-negative bacteria. After incubation of the tissue with colchicine (0.1%) for 1 h, samples were centrifuged at 1000 x *g* for 10 min, and the supernatant was removed. Pellets were resuspended in 3 ml ice-cold 0.075 mol/l KCl solution, incubated at 4°C for 30 min and then four drops of ice-cold Carnoy fixative (methanol: acetic acid, 3:1) were added. Samples were centrifuged at 1000 x *g* for 10 min, and the supernatant was removed. After that, 5 ml of fixative was added to the sample, which was then centrifuged at 1000 x *g* for 10 min. This step was repeated three times to wash the cells. Tissues were transferred to a petri dish with one milliliter of fixative and then cut into small pieces with a surgery blade. Slides were placed over boiled

Table 1. Cross-types of fish and their abbreviation, mean length and weight.

Crosses (female X male)	Family Abbreviation	Mean Length (cm)	Mean Weight (gr)
S.t labrax X *S.t. labrax*	LL	18.63±1.41	69.18±5.25
S.t. labrax X *S.t. abanticus*	LA	19.70±1.50	71.51±5.31
S.t. labrax X *S.t. caspius*	LC	24.36±1.81	156.0±10.12
S.t. abanticus X *S.t. abanticus*	AA	17.37±1.28	38.84±3.00
S.t. abanticus X *S.t. labrax*	LL	16.45±1.11	48.58±3.41
S.t. abanticus X *S.t. caspius*	LA	15.20±1.08	34.78±2.04
S.t. caspius X *S.t. labrax*	LC	15.88±1.12	41.70±3.06
S.t. caspius X *S.t. abanticus*	AA	11.62±0.84	13.67±0.07
S.t. caspius X *S.t. caspius*	LL	12.54±0.92	18.30±1.025
S.t. fario X *S.t. fario*	FF	7.15±0.41	5.11±1.01
S.t. fario X *S.t. abanticus*	FA	6.01±0.28	5.09±0.09
S.t. fario X *S.t. caspius*	FC	5.12±0.17	4.81±0.41
S.t. fario X *S.t. labrax*	FL	6.57±0.65	4.51±0.46
S.t. abanticus X *S.t. fario*	AF	7.24±0.47	5.11±1.06
S.t. caspius X *S.t. fario*	CF	5.03±0.21	4.24±0.38
S.t. labrax X *S.t. fario*	LF	7.31±0.58	5.19±0.91

water steam, and three drops of cell suspension were dropped onto slides from a height of 30–40 cm. For each fish species, a total of 15 slides were prepared and air dried, and 5 of them were stained with 10% Giemsa. The remaining 10 were used for C-banding (5 slides) and Ag-NORs analysis as explained below.

C-banding was performed according to the method described by Sumner (1972), with slight modifications. Slides containing the chromosome preparation were treated with 0.2 mol/l HCl solution at 37°C for 1 h and rinsed with distilled water. Washed slides were incubated in 2X SSC (pH 7.0) at 60°C for 1 h, rinsed with distilled water and finally stained with 10% Giemsa for 20 min.

Silver staining of nucleus organizer regions (Ag-NORs) were performed according to the method described by Howell and Black (1980). Two drops of colloidal developer and a single drop of aqueous silver nitrate were dropped onto a slide on which the chromosome preparation was mounted and covered with a cover glass. The slide was incubated at 70°C until the silver-staining mixture turned a golden-brownish color. The slides were then rinsed with distilled water, air dried and stained with 10% Giemsa.

Metaphase cells were screened with a fully automated karyotyping software system (CytoVision ver. 3.92) connected to an Olympus light microscope. Metaphase cell photos were captured at 100x magnification for further analysis. Ten high-quality metaphase spreads from each slide were used in chromosome analysis. Image-Pro Premier (Media Cybernetics), SmartType 3.1.0.43 (Digital Scientific, Cambridge, UK) and tpsDig2 v2.26 (New York State University, Stony Brook, USA) were used in karyotyping. The NF value was estimated by counting biarmed (metacentric and submetacentric) and unarmed (acrocentric and subtelocentric) chromosomes and calculated according to the formula given by Naran (1997).

RESULTS

The chromosome numbers and structures of four subspecies of brown trout and their cross-types (n = 16) were successfully determined. Furthermore, karyogram and chromosome measurement tables were generated. About 500 metaphase plates from 80 individuals were examined. Cross-types were karyotyped based on the representative chromosome image (Fig. 1) and chromosome arm scale (Table 2). Diploid chromosome numbers (2N) of all examined cross-types ranged from 76 to 80, but the majority of cross-types had 2N = 80 chromosomes (Table 3). The pure breed LL (see Table 1 for abbreviation) and the hybrid CA had 76 chromosomes, while CL had 78 chromosomes The NF varied from 96 to 102, the lowest being obtained from CL (96) followed by CC, LL and CA (98) (Table 3). Metacentric (M), submetacentric (SM) and acrocentric/telocentric (A/T) chromosome numbers varied from 14 to 18, 4 to 8, 2 to 14 and 46 to 56, respectively, among cross-types (Table 3).

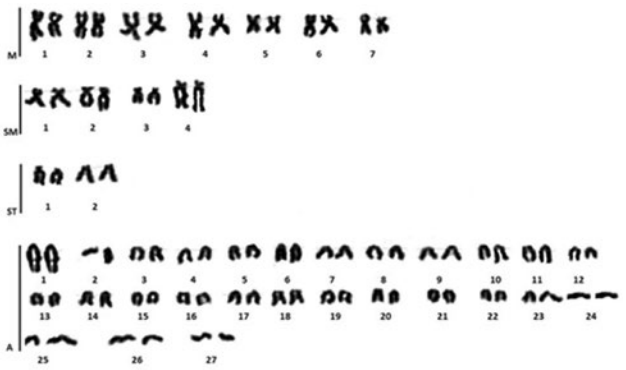

Figure 1. Karyotype of Abant trout *Salmo t. abanticus (2N=80)* stained conventionally with Giemsa. Metacentric (M), submetacentric (SM), subtelocentric (ST), acrocentric and telocentric chromosome (A/T) of cross-types.

Ag-NOR staining revealed the presence of one pair of NOR sites on the short arm of the SM chromosome in all the analyzed specimens (Fig 2). C-banding showed constitutive heterochromatin at the centromeres and arms of most of the chromosomes (Fig. 3) and the presence and position of constitutive heterochromatin within cross-types were variable even in pure breeds (Fig. 3). C-banding was not discriminative for brown trout subspecies.

DISCUSSION

Several cytogenetic methods of chromosome isolation have been developed. The main objective of all such methods is to obtain cells at the metaphase stage by disrupting the cell spindle (Pack, 2002). Solid tissues and cultured cells, together with colchicine treatment, are the most common sources of samples for the preparation of slides of fish chromosomes. Spleen, kidney, liver, gills and scales are the preferred sources of chromosomes. To prepare chromosomes, we first used the solid-tissue technique by harvesting various fish tissues and then empirically tested the colchicine concentration, exposure method (injection and bath) and fixation duration to obtain the most efficient means of chromosome preparation. Despite our efforts, we were unable to prepare metaphase plates for all but a couple of samples. With

Table 2. Relative arm lenght (μ), total lenght (μ), arm ratio (p/q) and chromosome type of Abant trout.

Chromosome number (2n)	Short arm length (p)	Long arm length(q)	Total Lenght	Arm ratio (q/p)	Chromosome Type
1	0.12	0.12	0.24	1.00	M
2	0.12	0.12	0.24	1.00	M
3	0.12	0.12	0.24	1.00	M
4	0.12	0.12	0.24	1.00	M
5	0.90	0.90	1.80	1.00	M
6	0.10	0.10	0.20	1.00	M
7	0.80	0.80	1.70	0.89	M
8	0.05	0.12	0.17	2.40	SM
9	0.07	0.13	0.20	1.86	SM
10	0.05	0.10	0.15	2.00	SM
11	0.03	0.12	0.15	4.00	ST
12	0.06	0.19	0.25	3.17	ST
13	0.02	0.13	0.15	6.50	ST
14	0.00	0.22	0.22	∞	A
15	0.00	0.09	0.09	∞	A
16	0.00	0.14	0.14	∞	A
17	0.00	0.12	0.12	∞	A
18	0.00	0.14	0.14	∞	A
19	0.00	0.15	0.15	∞	A
20	0.00	0.11	0.11	∞	A
21	0.00	0.11	0.11	∞	A
22	0.00	0.11	0.11	∞	A
23	0.00	0.11	0.11	∞	A
24	0.00	0.11	0.11	∞	A
25	0.00	0.12	0.12	∞	A
26	0.00	0.10	0.10	∞	A
27	0.00	0.11	0.11	∞	A
28	0.00	0.10	0.10	∞	A
29	0.00	0.08	0.08	∞	A
30	0.00	0.10	0.10	∞	A
31	0.00	0.12	0.12	∞	A
32	0.00	0.12	0.12	∞	A
33	0.00	0.11	0.11	∞	A
34	0.00	0.11	0.11	∞	A
35	0.00	0.07	0.07	∞	A
36	0.00	0.08	0.08	∞	A
37	0.00	0.08	0.08	∞	A
38	0.00	0.10	0.10	∞	A
39	0.00	0.08	0.08	∞	A
40	0.00	0.13	0.13	∞	A

Table 3. Chromosome number (N) fundamental number (NF) and structure [metacentric (M), submetacentric (SM), subtelocentric (ST), acrocentric and telocentric chromosome (A/T)] of cross-types.

Cross-type	M	SM	ST	A/T	N	NF
AA	14	8	2	56	80	102
CC	14	4	4	58	80	98
LL	16	6	4	50	76	98
FF	14	6	4	56	80	100
AC	16	4	8	52	80	100
AL	16	6	2	56	80	102
CA	16	6	6	48	76	98
CL	14	4	8	52	78	96
LA	16	4	14	46	80	100
LC	18	4	2	56	80	102
AF	18	4	2	56	80	102
FA	16	4	4	56	80	100
FC	18	4	4	54	80	102
CF	16	4	6	54	80	100
LF	16	6	4	54	80	102
FL	16	4	6	54	80	100

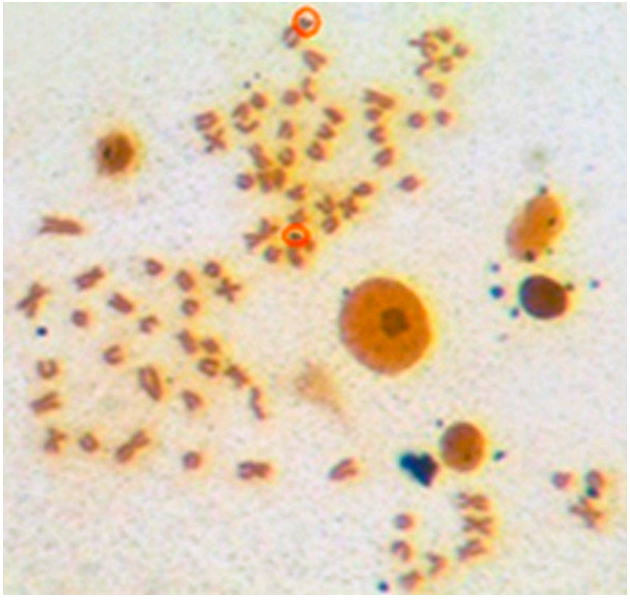

Figure 2. Karyotype of Abant trout *Salmo t. abanticus* with silver staining. Presence of NOR sites on the short arm of the submetacentric chromosome indicated with red ring.

the cell culture technique as described in the Materials and Methods section, we were able to obtain numerous well-spread metaphase chromosomes. The solid-tissue technique is applicable to various eukaryotic organisms (Kligerman & Bloom, 1977), but we favor the culture technique when working with salmonid fish, especially *Salmo trutta*.

The typical karyotypes of all three ecological forms of *Salmo trutta* (2N = 80 and NF = 100 – 102) were found, in agreement with numerous other studies

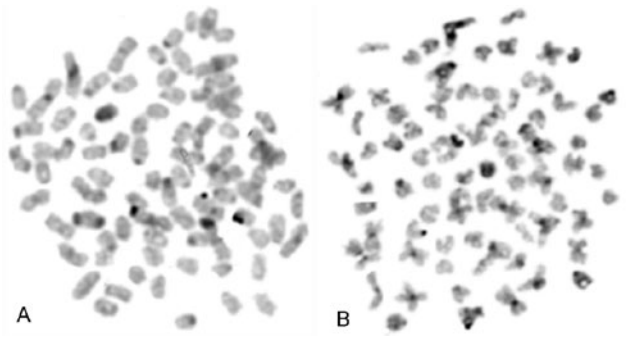

Figure 3. C-banded karyotype of Abant trout *Salmo t. abanticus.* Constitutive heterochromatin at the centromeres and arms of most of the chromosomes.

(Woznicki et al., 1998). This study documented slight karyotype variation among cross-types, with a diploid chromosome number and NF ranging from 76 to 80 and 98 to 102, respectively, while the majority of the cross-types exhibited 2N = 80, in agreement with previous reports (Woznicki et al., 1998). Intra-specific variation in both chromosome number and NF was previously documented among different fish species, including brown trout (Gjedrem, Eggum, & Refstie, 1977). Intra-specific variation in chromosome numbers in these trout forms and their hybrids suggest centric fusion between acrocentric chromosome pairs during the karyotype evolution of Robertsonian translocation. Loss of chromosome number due to counting errors and chromosome loss during preparation of slides is within the bounds of possibility (Gold & Gall, 1975; Zenzes & Voiculescu, 1975). Allopolyploids have genomes from different species; therefore, it is associated with hybridization. Allopolyploidy can be occurred in the nature as a results of interspecific or intergeneric hybridizations and offspring holds two different diploid chromosome sets (Zhou & Gui, 2017). Consequence of interhomolog recombination in genomic rearrangements can cause gene losses, and gametic aneuploidy (Hollister, 2015).

Polymorphic NOR size is common in fish and particularly in salmonids (Gold, 1984; Woznicki & Jankun, 1994). The NORs are commonly located on chromosome pair number 11 in *Salmo trutta,* but multichromosomal NOR-site polymorphism and variation in NOR size has also been reported (Sanchez, Martinez, Vinas, & Bouza, 1990; Schmid et al., 1995; Zhuo, Reed, & Phillips, 1995). In our study, the positions of NORs showed remarkable uniformity among individuals and cross-types. We could not detect any variation in the size and number of NORs.

Chromosomal characteristics of brown trout hybrids were studied for the first time in the present study. Chromosome structures of purebred brown trout subspecies (*S.t. abanticus*, *S.t. caspius*, *S.t. fario* and *S.t. labrax*) belonging to the Danubian linage and their hybrids were similar, and no distinctive characteristic was observed in any of the species. Therefore, they should be the same species but different strains. This statement was confirmed by Kalayci et al. (2018). They found that *S.t. abanticus*, *S.t. caspius*, *S.t. fario* and *S.t. labrax* are single biological species which should be called *Salmo trutta*. The results of this study are applicable to the development of improved conservation and management strategies for brown trout. Brown trout population in the nature is very low and governmental fisheries agencies are releasing hatchery reared brown trout to the stream or rivers to restore the population. Therefore, extra precaution should be should be taken in order to protect local brown trout population genetics

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DISCLOSURE STATEMENT

The authors declare that they have no conflict of interest

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