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Mapping of rRNA genes and telomeric sequences in Danube salmon (*Hucho hucho*) chromosomes using primed in situ labeling technique (PRINS)

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Abstract In the current paper we described the application of primed in situ (PRINS) labeling approach for the chromosomal mapping of repetitive DNA sequences in Danube salmon (*Hucho hucho*) ($2n = 82$, NF = 112). PRINS was successfully performed with primers enabling amplification of 5S rRNA genes (minor rDNAs), NOR building DNA sequences (major rDNAs), and telomeric sequences. Two loci of 5S rRNA were observed on distinct chromosome pairs; the minor arrays were located interstitially on the long (*q*) arms of two large metacentrics (chromosomes No. 3) and the large clusters of 5S rDNAs were assigned to the short (*p*) arms of two subtelocentric chromosomes No. 18. Major rDNA clusters were observed on the *p*-arms of two submeta-subtelocentric chromosomes No. 10. These chromosomal areas were built with GC-rich chromatin what was proved in the course of chromomycin A_3 (CMA_3) staining performed sequentially. Major and minor rDNA families were not co-localized in the Danube salmon chromosomes.The distinct hybridization signals at the ends of all the chromosomes were provided in the course of PRINS with $(CCCTAA)_n$ primer. The chromosomal localization of rRNA genes and telomeric DNA sequences was discussed in the context of Salmonidae karyotype evolution.

Keywords Chromosomes · Danube salmon · *Hucho hucho* · PRINS · rDNA · Telomere

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Introduction

Although the autotetraploidization origin and the chromosomal evolution following that process make Salmonidae one of the best cytogenetically studied, karyological data about huchonines (*Hucho* and *Parahucho*) is rather poor (Phillips and Ráb 2001). Chromosomes of two subspecies of the Danube salmon (*Hucho hucho*): huchen (*H. hucho hucho*) and taimen (*H. hucho taimen*) have been investigated so far. The diploid chromosome number in huchen from Bosna and Hercegovina, Slovakia and Yugoslavia is $2n = 82$ (Sofradzhija 1979; Ráb and Liehman 1982) whereas in taimen diploid cells, 84 chromosomes are observed (Viktorovskij et al. 1985; Frolov and Frolova 2000). Despite the different chromosome number, chromosome arm value (NF) is similar in both subspecies what suggests that the Robertsonian fussions can trigger the chromosome reduction in huchen (Ráb et al. 1994). On the other hand, the Japanese huchen (*Parahucho perryi*) which belongs to a separate but sister genus to *Hucho*, shows substantially reduced diploid chromosome number of $2n = 62$ (Ráb et al. 1994; Fujiwara et al. 1998) and represents the more advanced karyotype in the context of rediploidization process following the entire genome duplication in the ancestor of Salmonids (Allendorf and Thorgaard 1984).

Contrary to Japanese huchen, Danube salmon chromosomes have so far been litle studied and the C-banding and AgNOR distribution patterns provided only (Ráb et al. 1994). In the current report, sequential use of molecular and traditional cytogenetic techniques was carried out for the better cytogenetic characteristics of Danube salmon. The chromosomal distribution of major and minor rDNA and telomeric DNA sequences was discussed in the context of Salmonidae karyotype evolution.

Materials and methods

Fish

Fifty-four one-year-old individuals of *Hucho hucho* (24 females and 30 males) were studied cytogenetically. Fish were obtained from the fish hatchery Lopuszna (Polish Anglers Association—PZW), Southern Poland.

Chromosome preparation

Metaphase plates were prepared from pooled cephalic kidney cells, using conventional air-drying technique (Ráb and Roth 1988). Briefly, fishes were injected with 0.1% colchicine solution (1 ml/100 g body weight) 60 min before sacrifice by overdose of anesthetic Phenoxyethanol (ICN, Biomedicals, Aurora, USA). Kidneys were removed, dissected in 0.075 M KCl and cell suspension free of tissue fragments was hypotonized for 60 min in 0.075 M KCl, fixed in 3:1 methanol : acetic acid fixative, washed twice in fixative, and finally spread onto slides.

PRINS

Minor and major rDNA sequences were localized using primed in situ labeling (PRINS) method with primers enabling amplification of related fragments of 5S (5S A: 5′-TACGCCCGATCTCGTCCGATC-3′ and 5S B: 5′-CA GGCTGGTATGGCCGTAAGC-3′) (Martins and Galetti 1999), 18S (18S A: 5′-GTAGTCATATGCTTGTCTC-3′ and 18S B:

5′-TCCGCAGGTTCACCTACGGA-3′) (Hatanaka and Galetti 2004) and 28S rDNA sequences (28S A: 5′-AAAC TCTGGTGGAGGTCCGT-3′and 28S B: 5′-CTTACCAAA AGTGGCCCACTA-3′) (Zhang et al. 2000). $(CCCTAA)_7$ primer was utlized to localize telomeric sequences.

PRINS method was carried out according to Koch et al. (1989) with some modifications. Freshly prepared slides (up to 3 days) with mitotic spreads were placed on a 96°C hotplate for 1 min, then 50 µl of reaction mixture was added, slides were covered with coverslips, left for 3 min, and transferred to a humid chamber in which they remained for 30 min (5S rDNA and telomeric PRINS reactions) and 60 min (18S/28S rDNA PRINS reaction) to anneal the primers and extend new, labeled DNA stretch. The temperatures of the primers annealing and DNA strand extension were 56°C for 18S, 28S and 5S rDNA sequences and 60°C for telomeric sequences. The PRINS reaction mixture consisted of dATP, dGTP, dCTP and tetramethyl-rhodamine-5-dUTP or fluorescein-12-dUTP (Roche, Mannheim, Germany) (0.5 µl each), 2.5 µl of glycerol (Sigma), 5 µl of Taq polymerase buffer (Promega, MD, USA), 1.5 µl of each of the primer (100 pmol/µl), 0.5 µl of Taq polymerase (Promega, MD, USA) (5 U/µl) and 37 µl of dH_2O. After reaction, coverslips were gently removed and the slides were transferred to a stop buffer (50 mM EDTA, 50 mM, pH = 8) heated up to 60°C. Chromosomes were air dried in dark, at room temperature and counter-stained with 10 µl of antifade solution (Vectashield) containing 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) (Vector, Burlingame, USA). Slides used for 5S, 18S and 28S rDNA sites localization were stained sequentially with chromomycin A_3 (CMA_3) fluorochrome.

Microscopy processing

Chromosomes were analyzed under a Nikon Optiphot microscope equipped with epifluorescence and a digital camera. Chromosomes were scored under fluorescent light and filters: FITC/rhodamine/DAPI (rhodamin), UV-1A (DAPI) and G-2A (CMA_3).

Results and discussion

The chromosome number of Danube salmon (*Hucho hucho*) individuals analyzed in the present paper was $2n = 82$ (NF = 112) what was in agreement with previous observations (Sofradzhija 1979; Ráb and Liehman 1982). The karyotype of Danube salmon was consisted of 26 metacentrics, 4 submetacentrics, 12 subtelocentrics and 40 acrocentrics and morphology of chromosomes from the analyzed individuals displayed so called the hucho marker chromosomes—four pairs of small metacentrics (chromosomes 10, 11, 12 and 13) (Fig. 1a). The DAPI staining approach was able to identify small but clear DAPI positive bands in the paracentromeric regions of all Danube salmon chromosomes. Moreover, interstitial DAPI positive sites were observed on *p* arm (chromosome 8), *q* arms (chromosome 13, 15, 17, 18, 23, 24, 26, 27, and 29) or on both arms (chromosome 3). Additionally, discrete DAPI bands were seen in the telomeric positions on chromosoms 2 (*q*) and 9 (*p*) (Fig. 1a). The morphology and length of the chromosomes together with DAPI banding pattern enabled identification of the homologous chromosomes in Danube salmon and arrange them into the karyotype (Fig. 1a).

The identification of the chromosomes bearing DNA sequences of our interest was contributed by the simultaneously performed PRINS and DAPI staining technique.

In Danube salmon we identified two loci of 5S rRNA (Fig. 1b). The minor signals were assigned interstitially on the q-arms of the the large metacentric chromosomes No. 3 whereas the large hybridization signals covered almost the

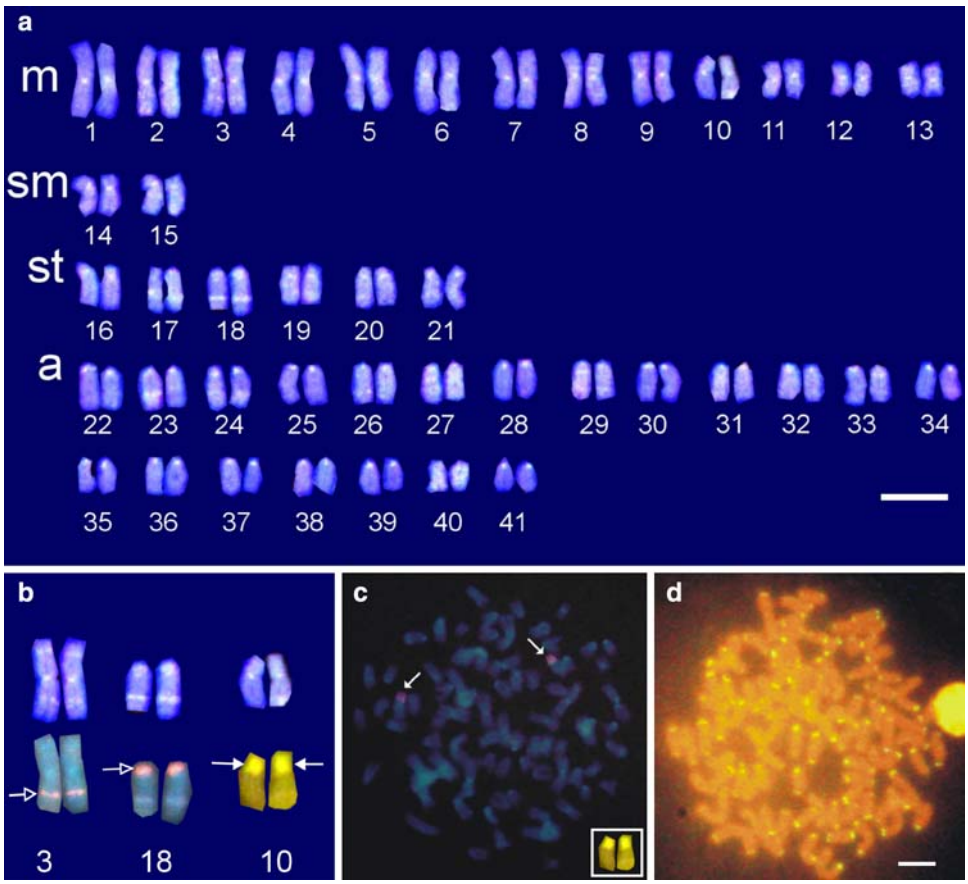

Fig. 1 Karyotype of Danube salmon (*Hucho hucho*) stained with DAPI (**a**). Enlarged partial karyotype (**b**) showing 5S rDNA bearing chromosomes Nos. 3 and 18 (*arrows* indicate 5S rDNA region) and the pair of chromosomes No. 10 with CMA_3 positive regions on the short arms (*arrows*). Chromosomal localization of 28S rDNA sequences (*arrows*) corresponding to CMA_3 positive sites (*inset*) (**c**). Distribution of telomeric sequences (**d**). Scale bar, 10 μm

entire p-arms of two medium-sized subtelocentric chromosomes No. 18 (Fig. 1b). Presumably, the differences in the copy numbers of 5S rDNA repeats in two clusters on Danube salmon chromosome triggered the intensity of the hybridization signals. Moreover, it is very likely that these two loci represent two independently evolving variants of the 5S rDNA sequences (Martins and Galetti 2001a). In Neotropical fish species Martins and Galetti (2001b) observed 5S rDNA repeats organized in two distinct classes showing length differences in non-transcribed spacer (NTS) sequences and these two classes of 5S rDNA clusters are located on different chromosomes. On top of that, two separately located 5S rDNA repeats can reflect the dual system of independent regulation of minor rRNA genes expression in somatic and oocyte cells described in fish as well (Komiya et al. 1986). Several patterns of 5S rDNA distribution on Salmonidae chromosomes related to the number of the sites and their location have been described. Single loci of minor rDNA has been reported among others in the Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*) and coregonid species (Pendas et al. 1993; Pendas et al. 1994; Jankun et al. 2003a; Phillips et al. 2002; Ocalewicz et al. 2004). Apart from the Danube salmon, multiple chromosomal distribution of 5S rDNA forming minor and major clusters located terminally or interstitially is observed in some of *Oncorhynchus* sp and *Salvelinus* sp, European grayling (*Thymallus thymallus*) and Japanese huchen (Pendas et al. 1994; Fujiwara et al. 1998; Stein et al. 2001; Jankun et al. 2003b).

PRINS approach with both sets of primers-enabling amplification of NOR building DNA sequences in fish was able to show chromosomal regions overlapped by the major rDNA sequences in Danube salmon. Fluorescent spots were captured on the *p* arms of two submeta- subtelocentric chromosomes No. 10 (Fig. 1c). Similarly, one pair of chromosomes bearing rDNA clusters is observed among other salmonids; in the Atlantic salmon (Pendas et al. 1993), rainbow trout (*Oncorhynchus mykiss*), masu salmon (*Oncorhynchus masou*) (Fujiwara et al. 1998), the Alaskan char (*Salvelinus malma*) or chum salmon (*Oncorhynchus keta*) (Alonso et al. 1999). On the other hand, multiple major rDNA sites are observed in brown trout (*Salmo trutta*) (Pendas et al. 1993; Woznicki et al. 2000), brook trout, Japanese huchen (Fujiwara et al. 1998), the European grayling (Jankun et al. 2003b) and coregonid species (Jankun et al. 2001). The existence of a single locus of major rDNA could suggest the decrease of rDNA clusters through the accumulation process accompanying the chromosome number reduction observed in salmonids. On the other hand, the evolution of rDNA loci can be

performed bidirectionally in closely related species. Although, *Salvelinus sp* show similar chromosome numbers, both single and multiple rDNA sites are observed among them (Fujiwara et al. 1998; Alonso et al. 1999). Diversified patterns of major rDNA distribution in closely relative species can be triggered by the interspecies and interindividual differences in the amount of rDNAs (Schmidtke et al. 1976).

Chromosomal segments consisted of the major rDNA arrays in Danube salmon were positively stained with chromomycin A_3 (CMA_3) (Fig. 1c) what proved that these regions were composed of GC-rich chromatin as CMA_3 interacts with such clusters preferentially (Amemiya and Gold 1986). Although, in most of the teleost fish species studied under this regard so far, major rDNA sites are built with GC-rich and CMA_3 positive chromatin it is not a general rule (Gromicho et al. 2005). Moreover, CMA_3 staining performed sequentially on the slides after 5S PRINS proved that major and minor rRNA gene clusters were not syntenic (Fig. 1b, c). The divergent location of 45S and 5S rRNA gene clusters is the most frequent condition observed among vertebrates (DeLuccini et al. 1993; Suzuki et al. 1996) however in rainbow trout, the minor locus of 5S rRNA is located in the vicinity of NOR (Moran et al. 1996). The association of 45S and 5S RNA genes has been proved in the Atlantic salmon as well (Pendas et al. 1994).

The chromosome rearrangements during the process of chromosomal number reduction accompanying the rediploidization process following the genome duplication in the ancestor of Salmonidae, accumulation and dispersion of 45S or 5S rDNA clusters could have left some kind of "footprints" in the form of interstitialy located telomeric sites (ITS) for example. So far, ITSs have been shown in rainbow trout (Abuin et al. 1996), lake trout (Sal*velinus namaycush*) (Reed and Phillips 1995) and brook trout (Ocalewicz et al. 2004). In Danube salmon individuals from the current report, telomeric sequences were confined to the ends of the chromosomes only (Fig. 1d). The lack of the interstitially located hybridisation signals may be related to the complete loss of such telomeric arrays after the chromosome fussions or the internally located $(TTAGGG)_n$ sites could have been too small to be visualized by PRINS approach.

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