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The stability of telomereless chromosome fragments in adult androgenetic rainbow trout

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Summary

The study provides new data on the stability of gamma radiation-induced chromosome fragments of a putative maternal nuclear genome in an androgenetic vertebrate, rainbow trout (*Oncorhynchus mykiss* Walbaum). The fragments were found in five of 16 examined individuals and they were mostly centromeric parts of metacentric or subtelocentric chromosomes. Chromosome fragments were identical in all cells of a given androgenetic individual, indicating that segregation of chromosome fragments is active from the early cell divisions. Most of the fragments were telomereless, i.e. they had no telomeric sequences on their ends. This shows that telomeres are not necessary for stability of chromosomal structures in a vertebrate genome. In one individual, the interstitial telomeric sites were found in chromosomes, which could be the effect of joining chromosome fragments.

Key words: androgenesis, centromere, chromosome, fish, gamma radiation, telomere.

Introduction

Centromeres and telomeres assure stability of eukaryotic chromosomes that undergo multiple segregations during cell divisions (Blackburn and Szostak, 1984). Chromosome movement during mitosis and meiosis is regulated by a centromere, which exists in each chromosome. The protein complex assembled at each centromere, named the kinetochore, serves as an attachment site for spindle microtubules (Cleveland et al., 2003). Centromeres are heterochromatic in most organisms and the role of centromeric heterochromatin has been investigated in regard to sister-chromatid cohesion at metaphase (Bernard and Allshire, 2002). Heterochromatic pericentromeric regions are recognized by conventional cytogenetic techniques such as C-banding or restriction enzyme digestion. Centromeric regions of chromosomes in some teleosts consist of AT-rich chromatin that can be revealed by DAPI banding (Ocalewicz et al., 2003).

Ends of eukaryotic chromosomes are capped with nucleo-protein complexes named telomeres, which serve many functions, such as protecting chromosome ends from degradation during the cell cycle and preventing chromosome end-to-end fusions. The DNA component of a telomere is built of short, tandemly repeated G-rich sequences, such as TTAGGG in vertebrates (Blackburn, 2001). Telomeric probes are widely used to investigate chromosomal rearrangements because in many vertebrates they appear frequently at interstitial sites of chromosomes (Meyne et al., 1990). These interstitial telomeric sites (ITS) can be remnants of chromosome rearrangements (fusions or inversions) occurring in genome evolution (Go et al., 2000; Castiglia et al., 2002; Slijepcevic, 1998) or aberrations (Natarajan et al., 1996; Slijepcevic et al., 1997). Telomeres allow the repairing system to distinguish between natural chromosome ends and chromosomal breaks such as double strand breaks (DSB). These can occur spontaneously during DNA replication, formation of dicentric chromosomes followed by breakage during chromosome segregation or by recombination. Also, DSB can be induced experimentally by subjecting DNA to damaging agents, such as radiation, alkylating agents, certain antibiotics or endonucleases (Obe et al., 2002). The naked ends of chromosomes arrest the cell cycle, and the repairing process starts before chromosome aberrations become irreparable and kill the cell (Crompton, 1997).

Cellular repairing mechanisms can fix the breaks *de novo* by healing the chromosome end with a new telomere built by a

telomerase, addition of telomere-associated DNA sequences or through specific retrotransposons, or recombination (Biessmann and Mason, 1997). Fusion of chromosomes that have lost their telomeres or fusion during subsequent cell divisions, termed the breakage–fusion bridge (B/F/B) cycle, is another repair mechanism (McClintock, 1941).

Most of the data concerning chromosome fragmentation and cell response to this event were obtained through irradiating cell lines with mild radiation (Natarajan et al., 1996; Slijepcevic et al., 1996). Much higher irradiation doses are applied to inactivate nuclear DNA in fish gametes during induced artificial gynogenesis or androgenesis, i.e. in order to produce individuals possessing exclusively maternal or paternal nuclear DNA, respectively (Thorgaard, 1986). These manipulations include oocyte (androgenesis) or spermatozoa (gynogenesis) irradiation with UV, gamma rays or X rays, followed by fertilization and diploidization of a haploid zygote with thermal or hydrostatic pressure shock, which in turn results in retention of the second polar body (heterozygous gynogenesis) or blockage of the first mitotic division (homozygous gynogenesis and androgenesis) of the zygote (Thorgaard, 1986). Although the radiation used for induced gynogenesis or androgenesis is believed to eliminate paternal or maternal nuclear DNA from the subsequent zygote entirely, chromosome fragments have been found in some cells of androgenetic or gynogenetic embryos and larvae. They are supposed to be residues of the irradiated genome (Parsons and Thorgaard, 1985; Chourrout, 1986; Lin and Dabrowski, 1998); however, to our knowledge no thorough investigation on their characteristics has been reported so far. As gyno- and androgenetic individuals are viable, they seem to be a good *in vivo* model for investigating fate of chromosome fragments during cell division as well as the cell response to chromosome breaks and mechanism of replication of chromosome fragments. In the present study, we performed a cytogenetic analysis of chromosomal fragments, putative maternal remnants in the genome of androgenetic rainbow trout. Androgenetic individuals were adult and normally developed. This ensured that the replication process of chromosome fragments was active through multiple cell divisions. Using conventional and molecular cytogenetic techniques, we have identified telomereless chromosome fragments and investigated chromosomal rearrangements that occurred as a consequence of fusions of putatively maternal chromosome fragments and intact paternal chromosomes. Mechanisms of chromosome fragment stability and repair of the chromosome breaks are discussed.

Materials and methods

Androgenesis

Androgenetic development was induced in rainbow trout as described by Babiak et al. (2002a). Gametes were collected from donors kept at the Department of Salmonid Research, Inland Fisheries Institute in Olsztyn, Rutki, Poland. Oocytes originated from six silver-blue-coloured females of the Rutki strain, whereas spermatozoa were obtained from a single yellow-coloured male from a gynogenetic yellow strain (Babiak et al., 2002a). Yellow colouration is recessive to wild colouration, therefore it can serve as a colour marker for efficiency of androgenesis: androgenetic progenies are yellow-coloured whereas control half-siblings are wild-coloured. Oocytes were irradiated with gamma rays at the Clinic of Radiotherapy, Medical School in Gdansk, Poland, at a dose

Table 1. *Number of chromosomes, chromosome fragments and their characteristics*

Fish no.	Number of chromosomes	Number of chromosome fragments	Characteristic of chromosome fragments	
			DAPI banding	PRINS
1	58	–	–	–
2	58	1	Positive	No signals
3	58	–	–	–
4	60	–	–	–
5	62	–	–	–
6	60	–	–	–
7	58	–	–	–
8	60	–	–	–
9	58	–	–	–
10	60	1	Positive	No signals
11	60	–	–	–
12	58	2	All positive	No signals
13	62	–	–	–
14	62	1	Negative	No signals
15	62	3	All negative	Two fragments showed fluorescent signals at their ends
16	60	–	–	–

The number of chromosome arms was 104 in all examined karyotypes. Chromosome fragments were observed in all metaphases of a given individual. $(CCCTAA)_7$ primers were used for PRINS.

ranging from 35 000 to 36 382 R, in order to destroy maternal DNA. Hydrostatic pressure shock (48 265 or 62 055 kPa for 3–4 min) was applied 350 min after fertilization during egg incubation at 10°C in order to diploidize a haploid zygote during the first mitotic division. Androgenetic progenies were yellow-coloured and also homozygous at locus *str60*, a microsatellite marker (Estoup et al., 1993) used to prove the occurrence of androgenetic development. Control half-siblings were wild-coloured, and the founder father was heterozygous at the locus *str60* (Babiak et al., 2002a). The fish were reared

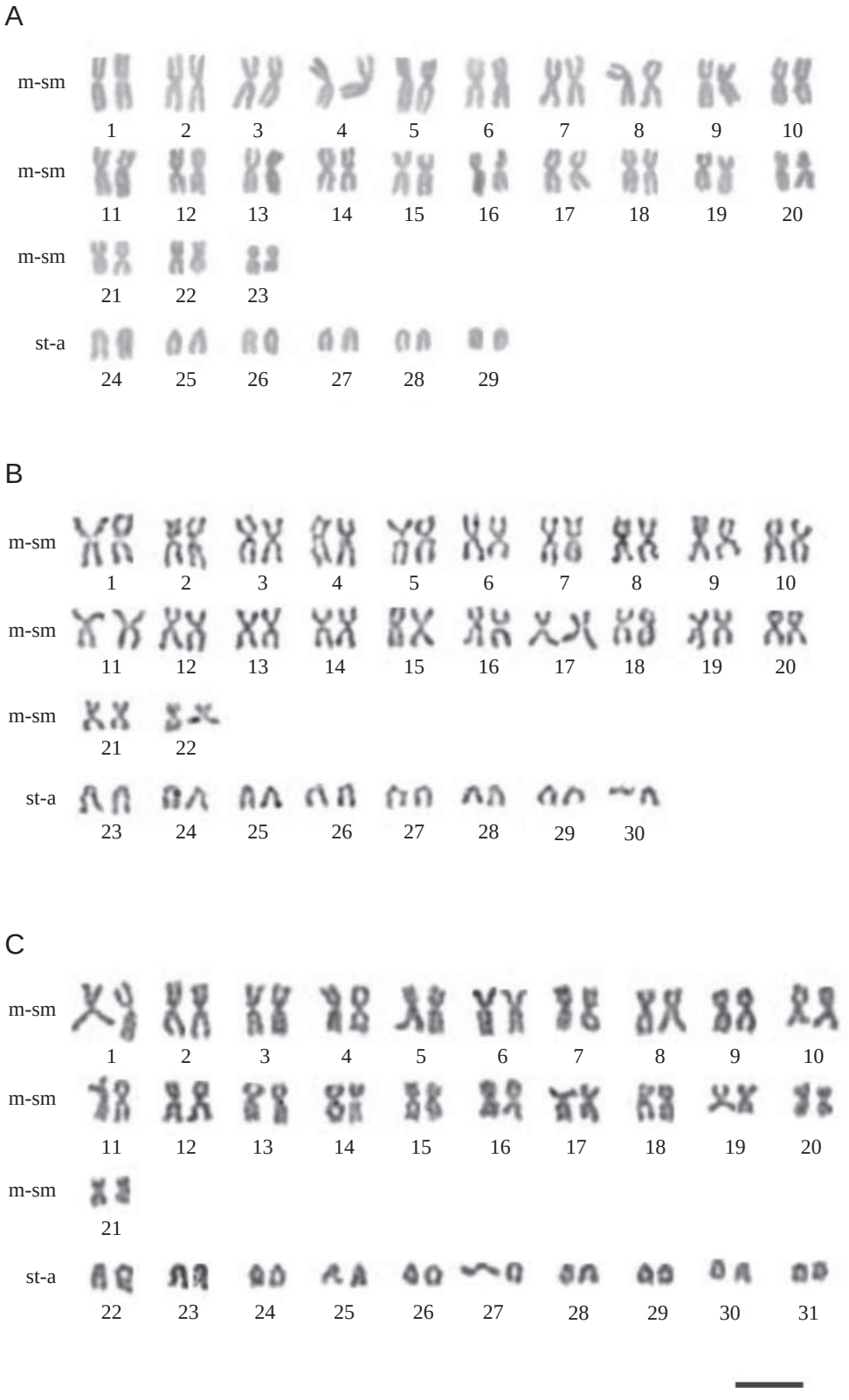

Fig. 1. Three karyotype patterns observed in androgenetic rainbow trout progeny from a single father: (A) 2n=58, (B) 2n=60 and (C) 2n=62. Scale bar, 10 μm.

until their maturity. Karyology was conducted on the material derived from 16 adult, healthy, morphologically normal 3-year-old individuals. As a control, five untreated normal fish were randomly chosen from one of the selection families.

Cytogenetic analysis

Metaphase chromosomes were obtained according to the standard air-dry procedure (Rab and Roth, 1988) or short-term culture of blood cells (Martinez et al., 1991). To estimate the chromosome number, metaphase plates were stained with 10% Giemsa solution (Sigma, Poznan, Poland) for 10 min. Chromosomes were stained with 4′,6-diamidino-2-phenylindole (DAPI) to identify AT-rich regions (Ocalewicz et al., 2003). Three drops of antifade solution (Vectashield; Vector Laboratories, Burlingame, USA) containing DAPI (1.5 μg ml^{-1}) were dropped onto a slide and covered with a coverslip. Above 30 metaphases per individual were examined.

Primed *in situ* labelling (PRINS) was performed using two systems: rhodamine PRINS reaction set kit (Roche, Mannheim, Germany; Ocalewicz and Babiak, 2003) or PRINS reaction with FITC-labelled dUTP. In both cases, $(CCCTAA)_7$ primers were used. The first procedure followed manufacturer's protocol. In the latter procedure, chromosomes were denatured by incubation in 70% formamide in 2×SSC at 70°C for 3 min and quenched in ice-cold 2×SSC and 70% ethanol. Then, the slides were dehydrated using an ice-cold ethanol series (70%, 80%, 96%), for 2 min each, and air dried. The PRINS reaction mixture consisted of dATP, dGTP, dCTP and FITC-dUTP (Roche; 0.5 μl each), 2.5 μl of glycerol (Sigma), 5 μl of Taq polymerase buffer (Promega, Madison, USA), 3 μl of $(CCCTAA)_7$ telomere primer (100 pmol μl^{-1}), 0.5 μl of Taq polymerase (Promega; 5 U μl^{-1}) and 37 μl of dH_2O. The mixture was dropped on a slide and covered with a coverslip. Slides were placed in a humid chamber at 65°C for 35 min in order to anneal primers and extend the new, labelled DNA strand. After reaction, coverslips were gently removed and the slides were transferred to stop buffer (50 mmol l^{-1} EDTA, pH 8) heated to 60°C. After 5 min of incubation in stop buffer, slides were washed three times for 5 min each in a washing buffer [4×SSC/0.05% Tween 20 (ICN Biomedicals, Aurora, USA), pH=7] and once in phosphate-buffered saline at room temperature. Chromosomes were air

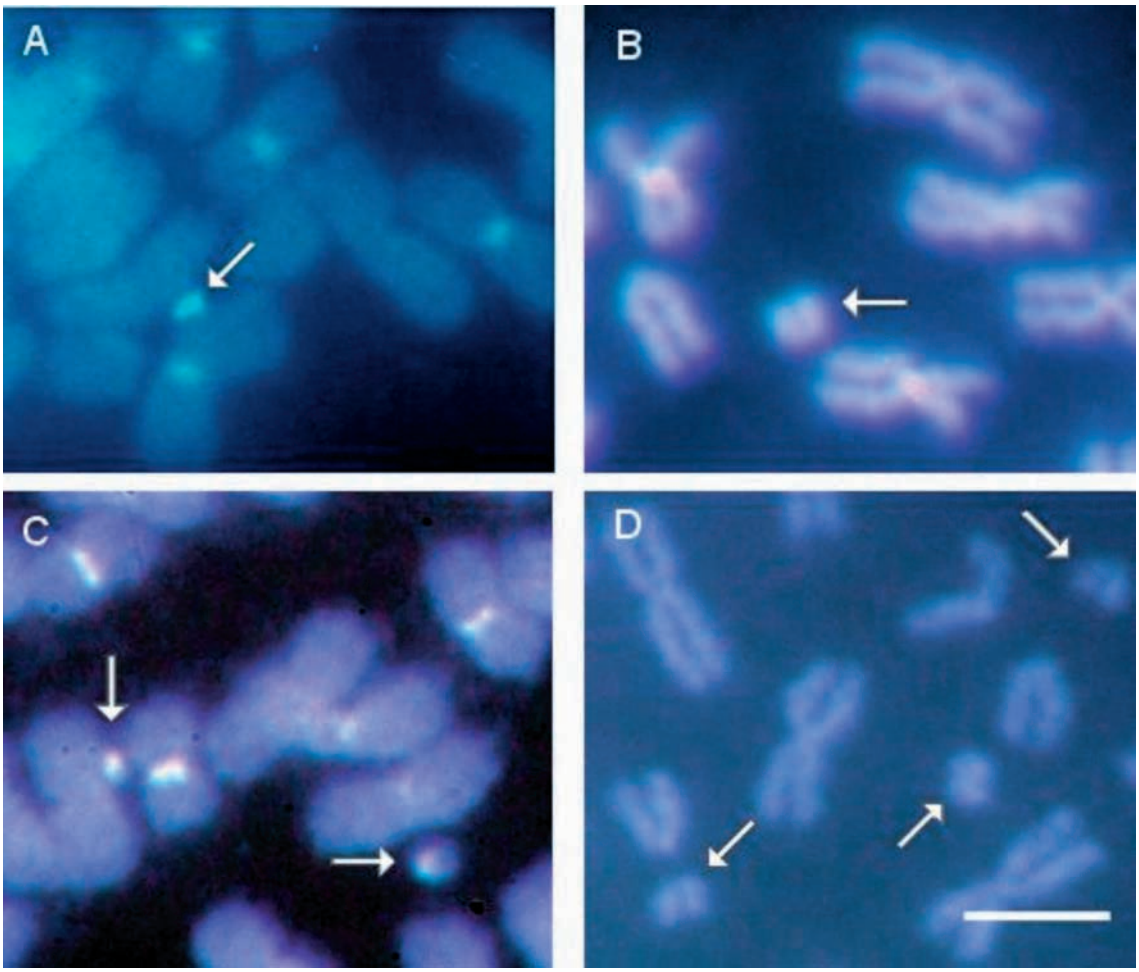

Fig. 2. Chromosomes of androgenetic rainbow trout (partial metaphase spreads) after DAPI staining. Arrows indicate chromosome fragments: (A) one DAPI-positive fragment (fish No. 10), (B) one DAPI-negative fragment (fish No. 14), (C) two DAPI-positive fragments (fish No. 12) and (D) three DAPI-negative fragments (fish No. 15). Scale bar, 10 μm.

dried in the dark at room temperature and counterstained with 10 µl of antifade solution (Vectashield) containing propidium iodide (PI; Vector Laboratories).

Metaphase plates were analyzed under a Nikon Optiphot microscope equipped with a fluorescent lamp and digital camera. Chromosomes were scored under fluorescent light and filters: UV-1A (DAPI), B-2A (FITC/PI), FITC/rhodamine/DAPI filter (rhodamin).

Results

Chromosome numbers were 2n=58 (6 specimens), 60 (6 specimens) or 62 (4 specimens; Table 1; Fig. 1). In all studied specimens, the number of chromosome arms was 104.

Chromosome fragments were found in cells of five individuals (31%), and their number ranged from 1 to 3 per specimen. These fragments were smaller than the smallest rainbow trout acrocentric chromosomes (Table 1; Fig. 2), but in individuals 14 and 15, chromosome fragments were bigger than chromosome fragments in other fish (Fig. 2). DAPI banding showed distinct bright, fluorescent patterns of AT-rich chromatin on intact chromosomes as well as fluorescent spots on some of the chromosome fragments (Table 1; Fig. 2). Specifically, all chromosome fragments in individuals 2, 10 and 12 were stained bright (Fig. 2A,C), whereas DAPI-negative chromosome fragments were found in individuals 14 and 15 (Fig. 2B,D). PRINS signals were observed on both ends in all intact chromosomes but only in some chromosome fragments, namely in two of three fragments in individual 15 (Fig. 3). In specimen 12, ITS were found in a paracentromeric position on four metacentric chromosomes (Fig. 4A,C). No ITS were found in chromosomes of control individuals (Fig. 4B).

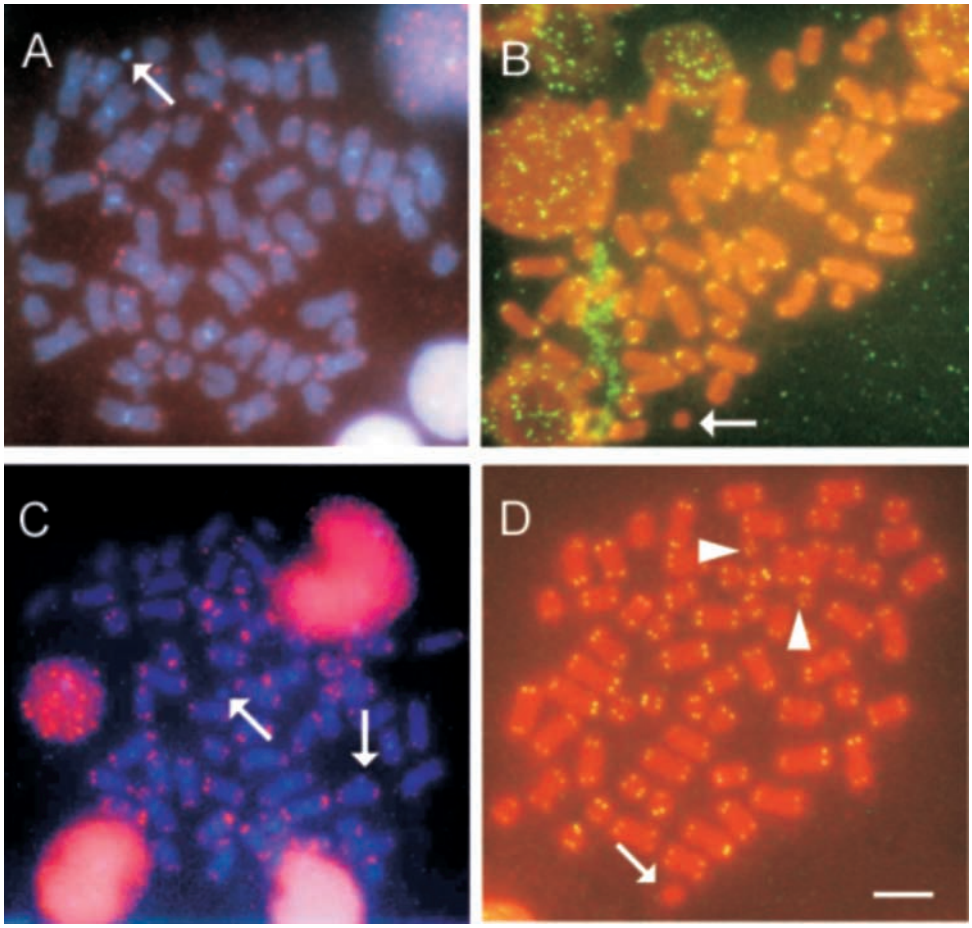

Fig. 3. Chromosomes of androgenetic rainbow trout after PRINS with telomere primers. Arrows indicate chromosome fragments in specimens No. 10 (A), 14 (B), 12 (C) and 15 (D) showing no hybridization signals. Arrowheads point to two out of three chromosome fragments in individual No. 15 having telomeres. Scale bar, 10 µm.

Discussion

The existence of male subgenomic DNA in the form of chromosome fragments called microchromosomes was shown in Amazon Molly (*Poecilia formosa*), a species reproducing through sperm-induced parthenogenesis. The microchromosomes are unstable components of the Amazon Molly genome and only some of them are transmitted to the offspring (Schartl et al., 1995). In chromosome-mediated transgenic rainbow trout, fragments of brook trout (*Salvelinus fontinalis*) chromosomes show high, mosaic stability in adults; moreover, they can be transcriptionally active and, as minichromosomes, transmitted to the next generations (Disney et al., 1987, 1988; Peek et al., 1997). Mechanisms of their stability are not elucidated yet (Disney et al., 1988). Contrary to chromosome-mediated gene transfer, the purpose of induced gynogenesis or androgenesis is to eliminate the entire paternal or maternal nuclear DNA, respectively, from the zygote. The confirmation of successful induction of gynogenesis or androgenesis is based on molecular (allozymes, DNA) or phenotypical (colour, scales) markers (Pandian and Koteeswaran, 1998). However, this does not exclude paternal and maternal residues in the resulting individuals. In the present study, examination of lengths of the smallest acrocentric chromosomes and lengths of chromosome fragments in fishes 2, 10, 12, 14 and 15 indicated that these structures were not paternal

chromosome fragments despite the fact that androgenetic nuclear genome putatively consists of paternal DNA only. Chromosomal fragments were not found in normal rainbow trout in the present study (controls) nor during previous studies (Ocalewicz, 2002; Ocalewicz and Babiak, 2003; Ocalewicz et al., 2003). This indicates that the observed fragments were residues of maternal chromosomes in androgenetic genomes. The presence of stable maternal DNA residues, detected by microsatellite DNA analysis, has been already found in interspecific androgenetic Atlantic salmon (*Salmo salar*) embryos developing in gamma ray-enucleated oocytes of brown trout (*Salmo trutta*) (Babiak et al., 2002b). The present study shows that maternal residues are physically chromosomal fragments, which have persisted through multiple cell divisions and are very stable in cells of adult rainbow trout.

Some of the observed fragments showed bright DAPI-positive signals (Fig. 2) similar to centromeres in normal rainbow trout metacentric or X chromosomes (Ocalewicz, 2002). Function of kinetochores could be retained in centromeres of the chromosome fragments. The DAPI-negative fragments in fishes 14 and 15 could be centromeric remnants of chromosomes with no AT-rich chromatin in centromeric regions, similar to those observed in acrocentric chromosomes (Ocalewicz et al., 2003). Centromeres are heterochromatic structures and are less sensitive to damage caused by irradiation (Blackburn and Szostak, 1984), therefore higher resistance to the radiation in observed fragments could be attributed to their centromeric origin.

Different diploid chromosome numbers in androgenetic individuals result from Robertsonian rearrangements, which are common in rainbow trout. Fish from the Rutki strain show

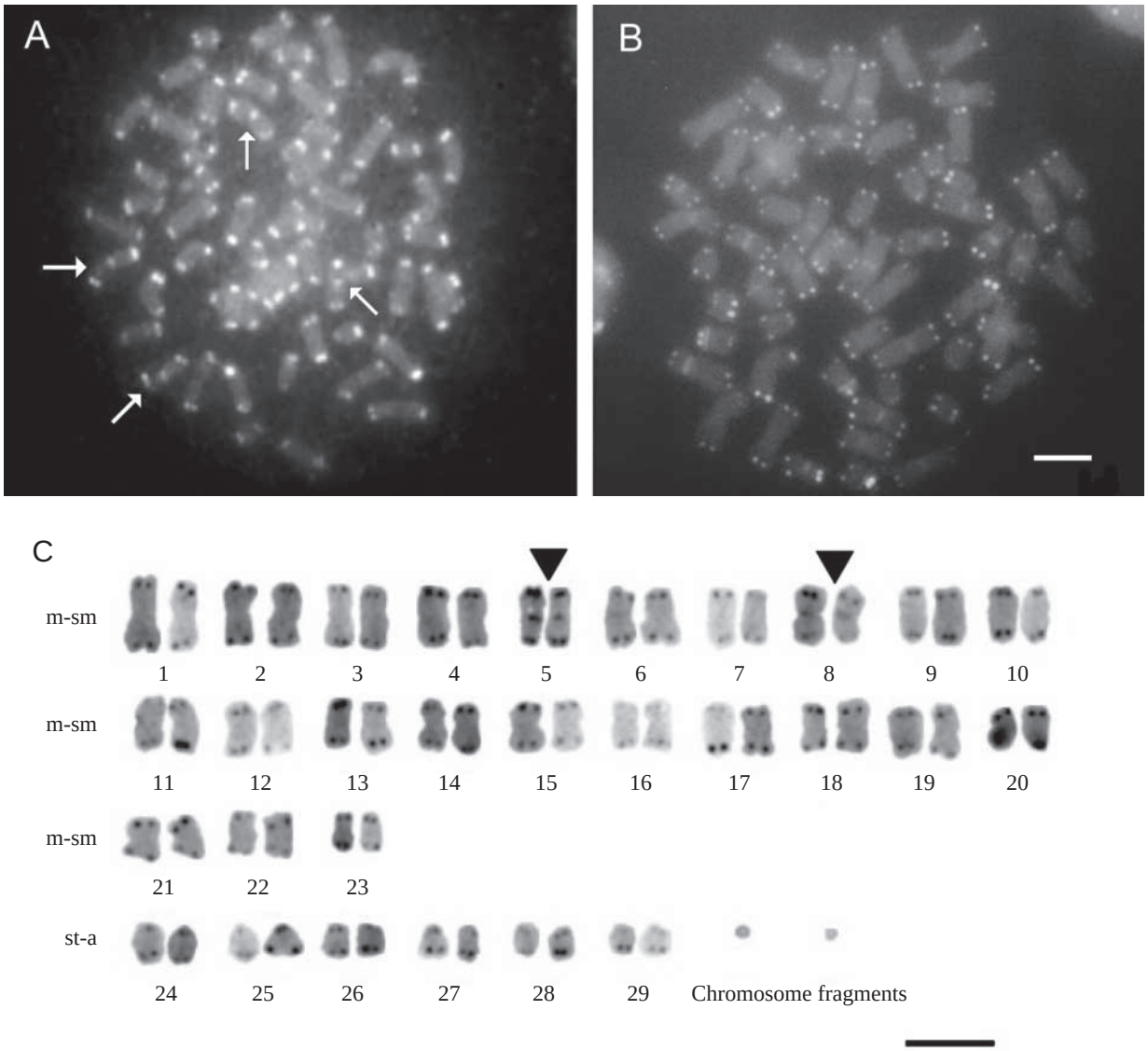

Fig. 4. Chromosomes of (A) androgenetic and (B) normal rainbow trout after PRINS with telomere primers. Arrows indicate chromosomes possessing interstitial telomeric sites (ITS). (C) Karyotype of individual No. 12 (2n=58) with signals derived from telomeric sequences. Two pairs of chromosomes with ITS are shown by arrowheads. Scale bar, 10 μm.

Robertsonian polymorphism in chromosome number, which varies from 59 to 62 (Ocalewicz, 2002). The father of examined individuals probably possessed 59 chromosomes. His haploid spermatozoa contained either 29 or 30 chromosomes. After diploidization in the course of androgenesis, resulting progeny should have 58 or 60 chromosomes. Specimens having 62 chromosomes differ from this simple segregation model, however. It might be because of abnormal meiotic disjunctions in testes of the father. Such anomalies in males having odd numbers of chromosomes (2n=59, 61) were reported by Nakayama and Chourrout (1993).

It is hypothesized that centromeres, telomeres and replication origins are necessary for retaining chromosomal functionality during the cell cycle (Blackburn and Szostak, 1984). Our results indicate that telomereless chromosome fragments having centromeric regions are as stable as regular chromosomes. These fragments apparently do not disturb the cell cycle and function. Also, the fragments do not trigger repairing mechanisms to rebuild the telomeric structures on their naked ends. In yeast, ends of artificially broken chromosome arrest the cell cycle in the absence of functioning repair mechanisms, although some cells eventually re-enter the cell cycle (Sandel and Zakian, 1993). Our study demonstrates for the first time the stability of telomereless fragments in a vertebrate.

The fusion of chromosome fragments as revealed by ITS appearance is frequently observed in irradiated mammalian somatic cells (Slijepcevic et al., 1997). In the present study, the chromosome fragments missing centromeres might have to undergo a process of DNA incorporation into intact chromosomes. ITS found on metacentric chromosomes (fish No. 12) could be the remnants of such a process (Fig. 4).

In conclusion, the lack of telomere sequences on chromosome fragments in androgenetic rainbow trout did not disturb the replication process and segregation of the fragments into daughter cells. We hypothesize that the stability of such fragments is possible because of the presence of active centromeres. Fragments lacking functional centromeres could incorporate into intact chromosomes, forming ITS.

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