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Polymorphism of the rDNA Chromosomal Regions in the Weatherfish *Misgurnus fossilis* (Teleostei: Cobitidae)

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The weatherfish, *Misgurnus fossilis* (Linnaeus, 1758), is native and an endangered species in Europe. Individuals with a chromosome number of 100 representing the most frequent cytotype seem to have a polyploid origin in comparison with rarely observed individuals of 50 chromosomes. This study cytogenetically characterized *M. fossilis* (five males, seven females) possessing 100 chromosomes to show the chromosomal distribution of major and minor ribosomal DNAs, and telomeric DNA repeats using fluorescence in situ hybridization with 28S and 5S rDNAs, and telomere peptide nucleic acid probes, respectively. Silver nitrate ($AgNO_3$) staining was used to show the activity of nucleolus organizer regions (Ag-NORs) and chromomycin A_3 (CMA_3) fluorochrome staining to detect the GC-rich chromosome regions. In all individuals size polymorphism of Ag-NORs and rDNA clusters was identified. Most of the studied individuals exhibited four 28S rDNA sites found in the short arms of the submetacentric and subtelo-acrocentric chromosomes, however in one female only two 28S rDNA sites were observed. Chromosome regions consisting of 28S rDNA corresponded to $AgNO_3$ positive and GC-rich chromatin. The 5S rDNA loci were located predominantly in a pericentromeric position of eight or six subtelo-acrocentric chromosomes and did not co-localize with 28S rDNA. The telomeric repeats were exclusively localized at the ends of all *M. fossilis* chromosomes. The presence of four NOR-bearing chromosomes may reflect the polyploid origin of *M. fossilis* with 100 chromosomes. In turn, reduction of the NOR sites observed in one specimen might be considered as part of the rediploidization process.

Key words: Cytogenetics, NORs, FISH, polyploidy, ribosomal genes, telomeres.

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The weatherfish, *M. fossilis* (Linnaeus, 1758), is the only *Misgurnus* species native to Europe but presently exhibiting a Eurasian distribution (KOTTELAT & FREYHOF 2007). It is a freshwater (or rarely brackish water) bottom-dwelling species considered endangered in Europe due to the loss of habitat (FREYHOF 2011).

Available karyological data for *Misgurnus* species revealed diploid chromosome numbers from 2n = 48 (LEE *et al.* 1987; KIM & PAK 1995) and 2n = 50 (VASIL'EV & VASIL'EVA 2008) to species of polyploid origin, *e.g. M. anguillicaudatus* and *M. fossilis* (BOROŃ 2000; ENE & SUCIU 2000; LI *et al.* 2010; ARAI & FUJIMOTO 2013).

Individuals of *M. fossilis* distributed throughout Europe rarely show a diploid number of 50 chromosomes (VUJOSEVIC *et al.* 1983) as the karyotype of 100 chromosomes is the most frequently observed cytotype (RAICU & TAISESCU 1972; BOROŃ 2000; ENE & SUCIU 2000). In two localities in the Czech Republic individuals of different ploidy level were reported (PALÍKOVÁ *et al.* 2007; DROZD *et al.* 2010) (Table 1).

M. anguillicaudatus individuals with 50 and 100 chromosomes are recognized as diploids and tetraploids, respectively (e.g. LI *et al.* 2010, 2013; ARAI & FUJIMOTO 2013). Similarly, it has been proposed that the *M. fossilis* lineage composed of individu-

als with 100 chromosomes appeared in the course of the ancestral polyploidization event (DROZD *et al.* 2010). Such a karyotype may still be arranged into 25 quadruplets (ENE & SUCIU 2000; PALÍKOVÁ *et al.* 2007) however such individuals behave as normal diploids showing regular gametogenesis (BOROŃ 2000). Moreover, induced gynogenetic progeny with only a maternal set of chromosomes (50) was inviable (ARAI & FUJIMOTO 2013), indicating a progressive process of genome diploidization following the polyploidization event (MABLE *et al.* 2011; WERTHEIM *et al.* 2013). In contrast to *M. fossilis*, induced gynogenetic progeny of *M. anguillicaudatus* with 50 chromosomes were viable indicating that individuals with 100 chromosomes are genetically true tetraploids (LI *et al*. 2012). Therefore *M. fossilis* with 100 chromosomes should be treated as functionally diploid (ARAI & FUJIMOTO 2013).

Diploidization as the gradual conversion of polyploidy to diploidy takes place through chromosome rearrangements and retention or loss of the duplicated genes (LIEN *et al.* 2016). Such genome reorganization may also affect activity and distribution of the ribosomal genes and telomeric DNA sequences commonly used as very informative cytogenetic features (FUJIWARA *et al*. 1998; ARAI & FUJIMOTO 2013; GORNUNG 2013; OCALEWICZ *et al.* 2013; BISCOTTI *et al.* 2015). In higher eukaryotes, ribosomal RNA genes (rDNAs) are organized into the nucleolus forming major rDNA (45S) family composed of clusters of multiple copies of tandemly repeated units with coding regions for 18S, 5.8S and 28S rRNA genes and the non-nucleolus forming minor rDNA (5S) family (FUJIWARA *et al*. 1998).

Cytogenetic data for *M. fossilis* is restricted to conventional staining techniques (BOROŃ 2000; ENE & SUCIU 2000). Thus, in the present paper, we performed a molecular cytogenetic study on *M. fossilis* with 100 chromosomes to analyze the chromosomal distribution of ribosomal genes and telomeric DNA sequences by fluorescence in situ hybridization (FISH) with 28S rDNA, 5S rDNA and telomeric DNA repeats and to show the chromosomal distribution of active nucleolar organizer regions (Ag-NORs) and GC-rich sites using sequential staining with silver nitrate ($AgNO_3$) and chromomycin A_3 (CMA_3) fluorochrome. The results provided information about the functional structure of the chromosomes and are discussed in the context of tetraploidization and re-diploidization processes in the genome of this polyploid species.

Material and Methods

In total 12 individuals, five males and seven females of *M. fossilis* (average length of 170 mm and 195 mm, respectively) were collected from two sites in Poland. Seven of them (three males and four females) were collected from the Kortowka River tributary of Kortowskie Lake (53°45′43"N; 20°26′42"E), the Pregola River drainage (Baltic Sea basin). The other five specimens (two males and three females) were caught from the Siemianowka Dam Reservoir (52°55′34"N; 23°49′39"E) at the Narew River, Vistula River drainage (Baltic Sea basin). Taxonomic identification of individuals was established based on their external morphological features according to KOTTELAT and FREYHOF (2007).

Fish sampling and valid animal use protocols for experiments were done with the permission of the Polish Ministry of Environment (no. DOP-OZGiZ.6401.10.12.2011.ls) and the Local Ethics Commission from the University of Warmia and Mazury in Olsztyn, Poland (no. 20/2013/N). Voucher specimens were preserved frozen at the Department of Zoology, University of Warmia and Mazury in Olsztyn, Poland.

Chromosome preparation and conventional staining

Mitotic chromosome preparations were made from each individual following BOROŃ (2000). First, fish were injected with a dose of 1 ml of 0.05% colchicine solution per 100 g body weight to prevent microtubule formation during cell division to arrest the chromosomes in metaphase. Then, fish were anaesthetized using MS 222 prior to sacrifice. Mitotic chromosomes were obtained from kidney cell suspensions using the air-drying method. The kidney cells were exposed to a hypotonic solution (0.075 M KCl) for 30 min and fixed in methanol: acetic acid (3:1). After drying at room temperature, chromosomes were stained with a solution of 4% Giemsa (pH=6.8).

Chromosome slides of all studied individuals were sequentially stained with silver nitrate ($AgNO_3$) and chromomycin A_3 (CMA_3) fluorochrome, according to SOLA *et al.* (1992) to detect respectively, the active NOR (Ag-NORs) and the GC-rich chromosome regions.

Probes and fluorescence *in situ* hybridization (FISH)

Double-color FISH with human 28S and loach 5S rDNA probes was used according to FUJIWARA *et al.* (1998) and BOROŃ *et al.* (2009). The 28S rDNA probe was labeled with digoxigenin-11-dUTP using the DIG-Nick Translation Mix kit (Roche), while the 5S rDNA probe was labeled with biotin-16-dUTP using Biotin-Nick Translation Mix kit (Roche), according to the manufacturer's instruc-

tions. The chromosome slides were pre-treated with RNase for 60 min at 37°C in a moist chamber. After denaturation for 1 min in 70% formamide (FA)/2×SSC, chromosome slides were dehydrated in a cold (-20°C) ethanol series, 70% for 5 min and 80%, 90%, and 100% for 2 min each. Hybridization was performed at 37°C in a moist chamber with a mixture containing denatured rDNA probes, Bovine Serum Albumin (20 mg/ml), 50% dextran sulphate, 20×SSC, and double-deionized water. Post-hybridization washes were performed in 50% FA/2×SSC at 37 °C for 20 min, 2×SSC and 1×SSC for 20 min each, and 4×SSC for 5 min. 28S and 5S rDNA probes were detected with Anti- Digoxigenin-Rhodamine (Roche) and Avidin-Fluorescein (Roche), respectively, and chromosomes were counterstained with DAPI in Antifade solution (Vector Laboratories, USA).

Telomeric DNA repeats were detected by FISH using a telomere Peptide Nucleic Acid (PNA) probe conjugated with FITC (DAKO, Denmark) according to the manufacturer's protocol. Both chromosomal DNA and PNA probes were denatured at 85°C for 5 min simultaneously. Hybridization took place in darkness at room temperature for 60 min. Metaphase spreads were counterstained with DAPI in antifade solution Vectashield (Vector, Burlingame, USA).

Microscopy and Image Processing

At least 15-20 metaphase spreads from each individual were analysed using a Nikon Eclipse 90i fluorescence microscope equipped with ProgRes MFcool camera (Jenoptic) and supported by an appropriate filter set. The images were processed using Lucia software 2.0 (Laboratory Imaging).

All chromosomes were classified according to LEVAN *et al.* (1964), where metacentric (m) and submetacentric (sm) chromosomes were considered as biarmed, whereas subtelocentric and acrocentric (sta) as uniarmed elements.

Results

Karyotype and banding patterns

Figure 1a-d and Figure 2a-b present karyotype and banding patterns of *M. fossilis*. Analyzed individuals showed a diploid chromosome number of 100 in 168 (93.3%) out of 180 analyzed metaphase plates. In the remaining 6.7% of metaphases, 98 and 99 chromosomes were observed. The karyotype was composed of 16m + 20sm + 64st/a chromosomes (Fig. 1a-b) and thus a chromosome arm number (NF) of 136. The four submetacentric pairs nos. 9-12 were easily recognizable in all metaphase plates, being the largest elements in the chromosome complement (Fig. 1a-b). One of the homologues of the submetacentric chromosome pair no. 15 clearly evidenced a secondary structure along its short arm easily distinguishable after Giemsa staining while this structure was less evident in the other homologue (Fig. 1a-b, shown inboxed).

After Ag-NO_3 staining, four Ag-NORs were identified in the short (p) arms of two sm (pair no. 15) and two sta chromosomes (pair No. 35) in a subcentromeric position in 87 (83.7%) out of 104 analyzed metaphase plates in 11 out of 12 individuals of *M. fossilis* (Fig. 1c).Two (11.5%) and three (4.8%) NORs were observed in the remaining metaphases. Sequential $AgNO_3$ and CMA_3 staining confirmed the co-location of NORs and CMA_3-positive sites (Fig. 1c-d). Among analyzed metaphases, NORs exhibited length polymorphism related to variation in NOR size (both Ag- and CMA_3-positive sites) (Fig. 1c).

A different pattern with only two NORs located on the short arms of two sm chromosomes (pair No. 15) was found in most of the metaphases (92.3%) of one female from Kortowka River (Fig. 2a). This was detected by both Ag- and CMA_3-staining (Fig. 2a-b). However, the single pair of NORs of this female exhibited the same length polymorphism related to variation in NOR size (Fig. 2a-b). In the remaining 7.7% of the metaphases only one positive site was observed.

Major rDNA mapping

FISH with the 28S rDNA probe analyzed in 166 metaphase plates of six (out of seven) females and five males confirmed data from Ag-NO_3 and CMA_3 staining and revealed four loci in their chromosome complement in the majority of the metaphase plates (83.7%) (Fig. 1e-g). Positive signals were found in the short arms of two sm (pair No. 15) and two sta chromosomes (pair No. 35) (Fig. 1f-g). Positive hybridization on sm chromosomes were commonly intense and large, whereas the signals on the sta chromosome pair were smaller and weaker (Fig. 1e-g). In the remaining 16.3% of metaphase plates, the 28S rDNA loci were located in three (11.4%) or two (4.9%) chromosomes (data not shown). No variability of 28S rDNA location was found between males and females from either of the two localities. The size polymorphism observed in chromosome pair no. 15 after conventional staining was less visible after FISH (Fig. 1f-g).

Two distinct heteromorphic 28S rDNA hybridization signals on the short arms of two sm chromosomes (pair No. 15) were observed in 92.3% of the 26 metaphases examined in the single female from Kortowka River with only two NORs (Fig. 2c-e).

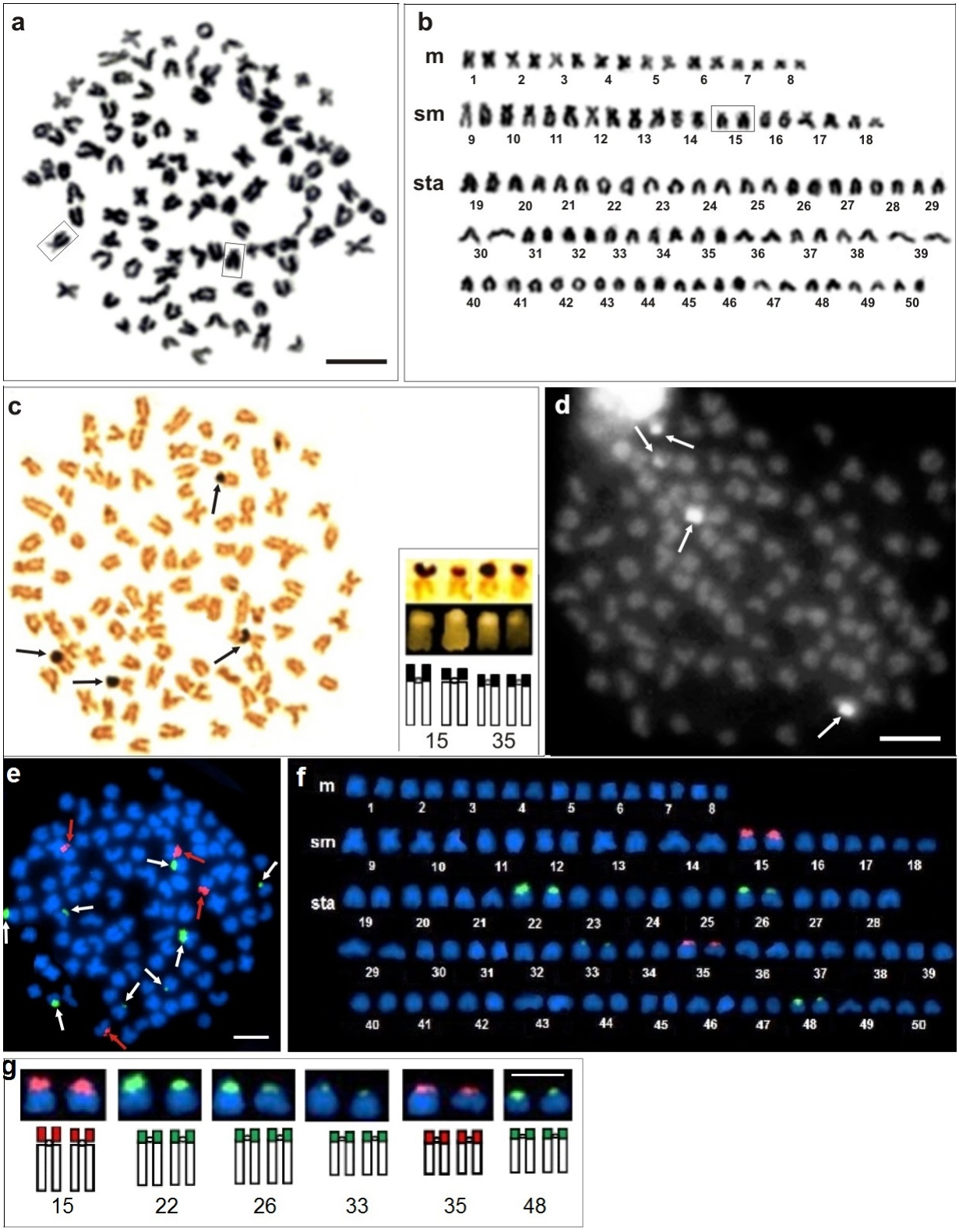

Fig. 1 Giemsa-stained metaphase spread (a) and respective karyotype (b) with in-boxed pair evidencing NOR secondary structure. The most common cytotype of *Misgurnus fossilis* from Poland evidenced by silver-stained metaphase (c) and CMA_3-stained metaphase (d), FISH with 28S (red signals, red arrows) and 5S (green signals and arrows) rDNA probes shown in a metaphase plate (e), assembled karyotype (f) and ideogram (g); two pairs of chromosomes with Ag-NORs (*top row*) sequentially chromomycin A_3-stained (*middle row*) and their ideogram are shown in the *inset* in c. Bar = 10 μm.

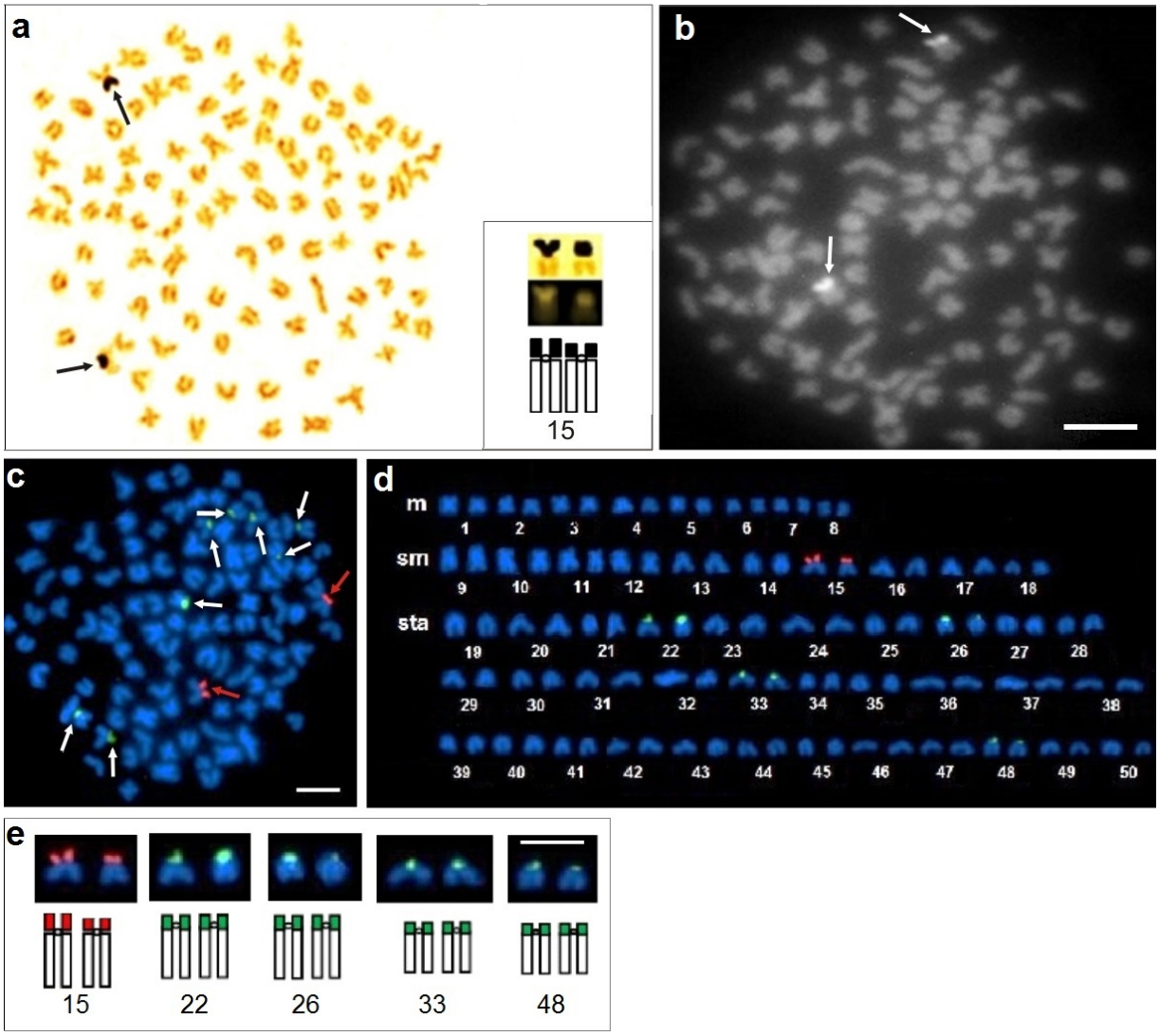

Fig. 2 Metaphase spread of the single female individual of *Misgurnus fossilis* from Kortowka River with a different cytotype evidenced by silver-stained metaphase (a), CMA_3-stained metaphase (b) and FISH with 28S (red signals, red arrows) and 5S (green signals and arrows) rDNA probes shown in a metaphase plate (c), assembled karyotype (d) and ideogram (e); one pair of chromosomes with Ag-NORs (*top row*) sequentially chromomycin A_3-stained (*middle row*) and its ideogram (*bottom row*) is shown in the *inset* in a. Bar = 10 µm.

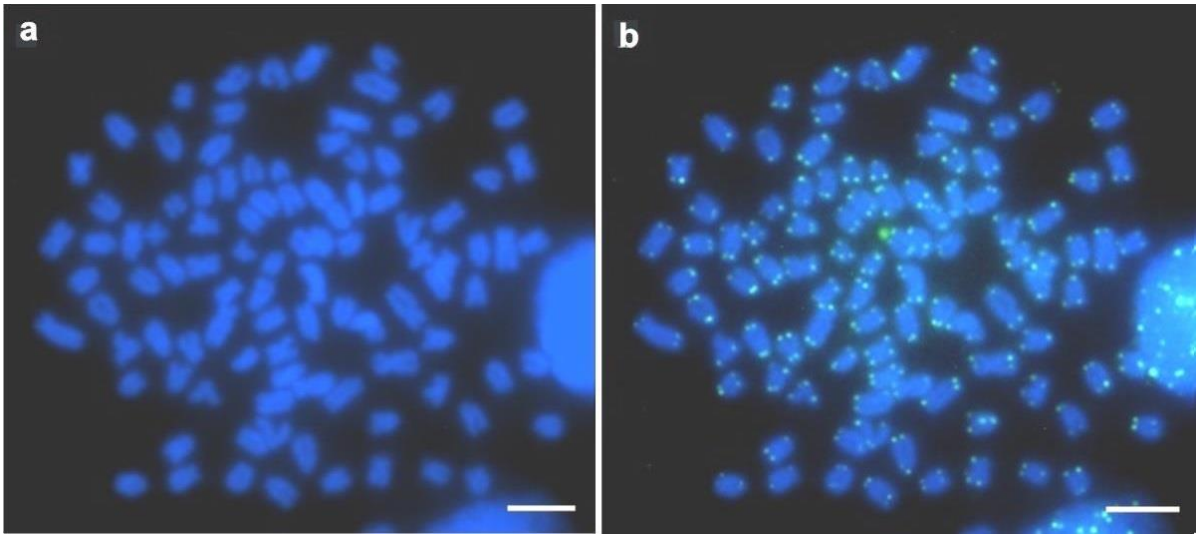

Fig. 3. Metaphase spread of *M. fossilis* showing the chromosomes after DAPI staining (a) and PNA-FISH with telomeric probe (b). Bar = 10 µm.

In the remaining metaphases (7.7%) a single 28S rDNA cluster was observed. In this female however, the length polymorphism of the NOR was also conspicuous after FISH (Fig. 2d-e).

Minor rDNA mapping

FISH with 5S rDNA probe revealed eight (52.1%) (Fig. 1e-g) and six (35.8%) positive signals in all individuals analyzed. Minor rDNA clusters were located at the pericentromeric positions of sta chromosomes of pairs Nos. 22, 26, 33 and 48 (Fig. 1f-g). The remaining metaphases (23 out of 190) revealed four (3.7%) or seven (8.4%) 5S rDNA loci with intra-individual variation. Hybridization signals had different intensities on various chromosomes and could be classified as strong or weak. Usually, in the metaphase plates with eight 5S rDNA signals, four signals were stronger (pairs nos. 22 and 26 in Fig. 1f-g) than the others (pairs nos. 33 and 48 in Fig. 1f-g). These weak signals were not visible in the metaphase plates with six hybridization sites of 5S rDNA (data not shown).

The *M. fossilis* female characterized by only two NORs revealed the same distribution pattern and number of 5S rDNA loci as all other individuals analyzed (Fig. 2c-e).

Both classes of rDNA probes were always located on different chromosomes (Fig. 1e-g and Fig. 2c-e). No variability in the 28S and 5S rDNA hybridization signals between individuals from the two localities as well as no sex-dependent variability in the cytogenetic features was found.

Mapping of telomeric sequences

The telomeric repeats were exclusively localized at the ends of all *M. fossilis* chromosomes in all the individuals. No interstitial signals were observed (Fig. 3) and no differences in the distribution of the telomere signals between specimens or between males and females from the two localities were found.

Discussion

The physical mapping of 28S rDNA revealed for the first time that the karyotype of *M. fossilis* from the lineage with 100 chromosomes is characterized by four independent major rDNA clusters. Their distribution corresponded to Ag- and CMA_3-positive sites (BOROŃ 2000; ENE & SUCIU 2000; present study). Taking into account that a single pair of NORs is considered as the plesiomorphic condition in fishes (MARTINS & WASKO 2004; NAKAJIMA *et al.* 2012; GORNUNG 2013), two pairs of NOR chromosomes may confirm the ancestral genome duplication event in this lineage of *M. fossilis*. Similar studies performed on individuals with 50 chromosomes would be useful for comparison, but they seem to be rare (Table 1) and we did not have such samples.

While 28S rDNA sequences in *M. fossilis* of 100 chromosomes were exclusively mapped to the short arms of two sm and two st chromosomes, those in *M. anguillicaudatus* were detected in two and four homologous chromosomes in diploids (2n =50) and tetraploids (4n = 100), respectively (LI *et al.* 2010). This distribution of NORs suggests the duplication of the entire *M. anguillicaudatus* genome from diploid to tetraploid status (LI *et al.* 2010, 2011). Two morphologically different pairs of NOR-bearing chromosomes in *M. fossilis* may be connected with its allotetraploid origin, but the formation of tetraploidy still requires many studies (LI *et al.* 2010).

Although two pairs of NOR bearing chromosomes seem to be the typical pattern for *M. fossilis*, the number of active NORs (Ag-NORs) varied from two to four (BOROŃ 2000; present work) and from three to four (ENE & SUCIU 2000) consistently with different expression activity of the rRNA genes in the preceding interphase (REEDER 1990).

Only two clusters of 28S rDNA in one female suggests that the other two clusters putatively present at the subtelocentric chromosomes were composed of copy numbers too low to be detected. The different intensity of FISH signals between sm and sta and NOR size polymorphism observed in the karyotype of *M. fossilis* agree with the results exhibited in the tetraploid *M. anquillicaudatus* and are presumably also related to different copy numbers of the rDNAs as a result of unequal crossing over (LI *et al.* 2010).

On the other hand we cannot exclude that these sites of major rDNAs/NORs were eliminated by the re-duplication process to re-establish the diploid-like condition. Examination of fishes of tetraploid revealed that both duplicated gene copies may be maintained and retain original functions (1), one of the copies may attain a new function (2) or the duplicated gene may be suppressed and lost (3) (LI *et al.* 2015; LIEN *et al.* 2016). If so, the single NOR locus found in one of the examined *M. fossilis* could suggest a loss of the duplicated NOR.

Minor rRNA genes of *M. fossilis* showed great variability in terms of cluster size and genomic distribution. Some other Cypriniformes species also demonstrate multiple locations of 5S rDNA clusters, *e.g. C. taenia* (BOROŃ *et al.* 2006) and *Carassius carassius* (SPOZ *et al.* 2014). A single pair of 5S rDNAs is considered as the ancestral condition in fishes (MARTINS & WASKO 2004), so multiple 5S rRNA gene clusters observed in *M. fossilis* seems to be

a derived state. The DNA sequences encoding 5S rRNAs were located in the distal position in the chromosomes of *M. fossilis,* however, minor ribosomal genes have also been observed at internal chromosomal locations (ROSSI *et al.* 2012; among others).

Large variation in number and size of 5S rDNA clusters may be explained by high dynamism of the ribosomal genes that results from frequent linkage with other multigene families including transposable elements (REBORDINOS *et al.* 2013). The size polymorphism of the 5S rDNAs may also result from the presence of other genomic elements including LINEs, SINEs and microsatellite DNA in the non-transcribed spacers (NTS) that separate 5S transcriptional elements (GORNUNG *et al.* 2007; REBORDINOS *et al.* 2013).

The present work documented for the first time that clusters of major and minor rDNA sequences were located on different chromosomes in *M. fossilis* as in other Cypriniformes species (BOROŃ *et al.* 2009; ROSSI *et al.* 2012; among others).

In fish, as in other vertebrates, internally located telomeric sequences are residues of the chromosomal rearrangements including fusions and inversions (MOTA-VELASCO *et al.* 2010; OCALEWICZ *et al.* 2013). However, rearrangements related to rDNA and NORs observed in *M. fossilis* were not followed by the internal insertion of telomeric repeats.

In conclusion, the presence of two morphologically different pairs of NORs in the karyotype of *M. fossilis* studied here may reflect the polyploid origin of the lineage exhibiting 100 chromosomes. Doubled NORs may undergo further rearrangements leading to reduction as observed in one of the specimens studied herein. Moreover, intraspecific variation in the number and size of the 5S rDNA regions confirmed the dynamic nature of regions composed of these repeats in *M. fossilis*.

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