e-ISSN 1734-9168
http://www.isez.pan.krakow.pl/en/folia-biologica.html

Folia Biologica (Kraków), vol. 72 (2024), No 2
https://doi.org/10.3409/fb_72-2.06

Molecular organisation and chromosomal localisation of 5S rDNA tandem arrays in evolutionary polyploid cyprinids from the genera *Carassius* and *Barbus*

Aleksandra Szabelska, Lech Kirtiklis, Anna Przybył, Krzysztof Kukuła, Aneta Bylak, and Alicja Boroń

Accepted June 06, 2024 *Published online June 24, 2024* *Issue online June 28, 2024*

Original article

Szabelska A., Kirtiklis L., Przybył A., Kukuła K., Bylak A., Boroń A. 2024. Molecular organisation and chromosomal localisation of 5S rDNA tandem arrays in evolutionary polyploid cyprinids from the genera *Carassius* and *Barbus*. Folia Biologica (Kraków) **72**: 45-64.

Some evolutionary polyploid *Carassius* and *Barbus* species are poorly differentiated morphologically and hybridise to form viable intrageneric hybrids. 5S rDNA arrays and their chromosomal localisation were studied in *C. carassius*, *C. gibelio*, *B. barbus*, *B. carpathicus* and *B. waleckii* distributed throughout Poland, in order to contribute to the knowledge of their genetic and cytogenetic differences. Two 5S rDNA array classes in *C. carassius* (203 bp, 380 bp) and in three *Barbus* species (196 bp, 215 bp) were characterised by a highly-conserved coding region. The adjacent non-transcribed spacer (NTS) of *C. carassius* exhibited a relatively high level of variation due to base substitutions and insertions/deletions, whereas the NTS of the *Barbus* species were highly conserved. Fluorescence *in situ* hybridisation with 5S rDNA array classes as probes indicated their syntenic chromosomal location and the utility of these sites as suitable marker of the analysed *Carassius* taxa ploidy. 5S rDNAs in two chromosomes of the *Barbus* species was discovered. The observed low variability of 5S rDNA arrays makes them an inappropriate tool for an investigation of the *Barbus* species, whereas the suitable for investigation within karyologically varied in the *Carassius* taxa.

Key words: Barbel, chromosome mapping, coding sequence, Crucian carp, minor rDNA repeats, molecular evolution, NTS, polyploidy.

Aleksandra Szabelska, Lech Kirtiklis✉, Anna Przybył, Alicja Boroń – Department of Zoology, Faculty of Biology and Biotechnology, University of Warmia and Mazury, Olsztyn, Poland.
E-mail: aleksandra.szabelska@uwm.edu.pl (A.SZ.)
leo@uwm.edu.pl (L.K.)
anna.przybyl@uwm.edu.pl (A.P.)
alibo@uwm.edu.pl (A.Bo.)
Krzysztof Kukuła, Aneta Bylak – Department of Ecology and Environmental Monitoring, University of Rzeszów, Rzeszów, Poland.
E-mail: kkukula@ur.edu.pl (K.K.)
abylak@ur.edu.pl (A.By.)

Genome duplication is a significant process in the evolution of many taxa, including those from the species-richest freshwater family Cyprinidae (sensu Tan and Armbruster 2018) with very divergent chromosome patterns (reviewed by Arai 2011). This fish taxon consists of more than 3,130 species, and its largest subfamily – Cyprininae (more than 1,700 species) – contains around 400 closely-related polyploid species (Arai 2011; Yang *et al.* 2015, 2022; Froese & Pauly 2024). These latter species are evolutionary tetraploids (2n = ca. 100) or hexaploids (2n = ca. 150), which originated via allopolyploidisation and that have returned to a diploid state via rediploidisation. Most of the other species belonging to the family Cyprinidae are characterised by the diploid number of 50 chromosomes which is considered an ancestral state (Le Comber & Smith 2004).

Among others, the cyprinids of two genera, *Carassius* and *Barbus* are distributed in European inland waters (Kotlík *et al.* 2002; IUCN 2024). They belong to two out of 11 subfamilies (Tan & Armbruster 2018): Cyprininae and Barbinae, respectively. The crucian carp *C. carassius* (Linnaeus, 1758), possessing 2n=100 chromosomes, is a native species of Europe (Spóz *et al.* 2014; Knytl *et al.* 2018; IUCN 2024). It may hybridise with a relative of the gibel carp (the Prussian carp) *C. gibelio* (Bloch, 1782) with a diploid (2n=100) or triploid (3n=150 plus 1-14 microchromosomes) number of chromosomes (Boroń *et al.* 2011; Kalous *et al.* 2012). Among the potential mechanisms responsible for the presence of microchromosomes in the genome of *Carassius* hexaploids are macrochromosome fissions and post-polyploidisation segmental duplication in pericentromeric areas (Fornaini *et al.* 2024). The gibel carp, which probably originating from East Asia, is regarded as a separate species (as in the present study) or as one form of the polyploid *C. auratus* complex, which is represented by the diploids of both sexes reproducing sexually and the triploid females exhibiting unisexual modes of reproduction, via gynogenesis (Rylková & Kalous 2013; Šimková *et al.* 2015; Zhang *et al.* 2015; Knytl *et al.* 2018).

The genus *Barbus* sensu stricto is distributed within Eurasia and north-western Africa and includes 34 riverine species (Froese & Pauly 2024) characterised by 2n = 100 chromosomes (Ráb *et al.* 1996; Yang *et al.* 2015). Three of these species occur in Poland: the barbel *Barbus barbus* (Linnaeus, 1758), *B. carpathicus* (Kotlík, Tsigenopoulos, Ráb & Berrebi, 2002) and *B. waleckii* (Rolik, 1970) (Amirowicz & Kukuła 2005; Konopiński *et al.* 2007, 2013; Kotusz & De Wever 2019). However, the systematic status of *B. waleckii* seems to be far from settled, since it has been considered as a post F1 hybrid between *B. barbus* females and *B. carpathicus* males (Kotlík *et al.* 2002; IUCN 2024).

Ribosomal genes (rDNA) belong to the most informative repeats that, due to high rates of transcription and recombination, contribute to genome diversification (Symonová & Howell 2018) and are widely used to study polyploidisation (Cao *et al.* 2020). In higher eukaryotes, the 5S rDNA sequence is organised in repetitive units consisting of a highly-conserved coding sequence (CDS) of 120 base pairs (bp), which plays an important role in stabilising the ribosomal structure and enhancing the peptidyl transferase activity (Vierna *et al.* 2013; Castro *et al.* 2016). Each of these transcribed regions lies adjacent to a non-transcribed spacer (NTS). The NTSs have a higher mutation rate through insertions, deletions, mini-repeats and pseudo-genes, rather than through nucleotide substitution (Martins & Wasko 2004; Ferreira *et al.* 2007). The NTSs variations in length, nucleotide composition (Pinhal *et al.* 2011; Barman *et al.* 2016) and/or chromosomal location (Zhang *et al.* 2015; Qin *et al.* 2019) are used as valuable markers for the identification of interspecific and intergeneric wild and artificially induced diploid fish hybrids (Zhang *et al.* 2015; Wang *et al.* 2017), as well as those of a different ploidy level (Szabelska *et al.* 2017; Qin *et al.* 2019). Moreover, all the above mentioned 5S rDNA features are useful for clarifying the relationships between closely related species (Knytl *et al.* 2018; Teixeira *et al.* 2018). On the other hand, some nucleotide substitutions within the 120 bp CDS have also been reported among different 5S rDNA array classes (Martins & Galetti 2001; Gornung *et al.* 2007).

Different classes of the 5S rDNA array conditioned by changes in the structure of the NTSs have been identified in many freshwater and marine ray-finned fish species; among others, those belonging to the following orders: Characiformes (Martins & Galetti 2001; Teixeira *et al.* 2018), Gymnotiformes (Utsunomia *et al.* 2014), Gadiformes (Campo *et al.* 2009), Cypriniformes (He *et al.* 2013), Salmoniformes (Morán *et al.* 1996), Cyprinodontiformes (Tigano *et al.* 2004) and Perciformes (Barman *et al.* 2016), as well as some Chondrichthyes species (Pinhal *et al.* 2011). Such a dual 5S rDNA repeated unit system is regarded as an ancient condition in the fish genome (Campo *et al.* 2009). Analyses of the numbers and chromosomal locations of the 5S rDNA sites has been performed on several fish groups, including Cyprinidae (among others: Kumar *et al.* 2013; Han *et al.* 2015). However, only a few polyploid cyprinid taxa, such as *Carassius auratus* (Cao *et al.* 2020), *Cyprinus carpio* (Ye *et al.* 2017), *Tor* species (Mani *et al.* 2011), *Catlocarpio* and *Probarbus* species of tribe Probarbini (Saenjundaeng *et al.* 2018), have been examined for this characteristic. Most species possess two or more 5S rDNA array classes detected in co-localisation (in synteny) in the same chromosome pairs (Martins & Wasko 2004). In turn, the *Leporinus* species (Martins & Galetti 2001) and *Oreochromis niloticus* (Martins *et al.* 2002) are characterised by two 5S rDNA arrays with a distinct chromosome location.

We were prompted to undertake the study described in the present paper for two reasons. First was the successful use of the 5S rDNA as a research tool in the genome evolution and the dynamics of repetitive sequences, as well as it being an efficient genetic marker in many fish species, including cy-

prinids. Second was a real differentiation of the 5S rDNAs into two array classes previously described in *C. gibelio* (Szabelska *et al.* 2017), and the lack of such molecular cytogenetic data for the *Barbus* species. Thus, in the present study, the 5S rDNA arrays of the crucian carp *C. carassius* and three *Barbus* species have been amplified, sequenced and physically mapped (by the FISH technique – fluorescence *in situ* hybridisation) to their chromosomes, and tests were conducted on how polyploidy may have an influence on the molecular and cytogenetic faces of the 5S rDNAs. Moreover, two 5S rDNA array classes were verified as a potential species-specific molecular and cytogenetic marker for the taxonomic characterisation of these taxa, which are poorly differentiated from a morphological point of view.

Material and Methods

Ethics statement

Standard procedures used in aquaculture for catching fish were employed in the study. No *in vivo* experiments were performed on the animals. Fish sampling and valid protocols for animal use in experiments were performed with a sampling permit (No. RG-IX.7143.6.2017.MS) issued by the Marshal Office of the Podkarpackie Voivodeship, following approval by the Regional Directorate for Environmental Protection. The research was conducted under individual licenses (Nos. 1614/08, 1703/11) for the operation of electric shocking tools and No. 3736/2016 to perform animal investigations, according to the legislation on the protection of animals and the recommendations of the International Council for Laboratory Animal Science (ICLAS) and the Local Ethics Committee of the University of Warmia and Mazury in Olsztyn (no. 56/2017). No animals were sacrificed during this study.

The suspensions of the kidney cells used for chromosome slide preparations were obtained from previous studies (Spóz *et al.* 2014; Szabelska *et al.* 2017) and all the experimental procedures were approved according to Permit No. 20/2013/N of the Local Ethics Committee of the University of Warmia and Mazury in Olsztyn.

Sample collection

Fin clips were collected from 22 individuals of the crucian carp *C. carassius* (2n=100) sourced from the Siemianówka Reservoir (Poland) (52°55′N, 23°48′E), as well as of 25 *B. barbus* (2n=100), 26 *B. carpathicus* (2n=100) and 7 *B. waleckii* (2n=100) individuals from seven localities (49°11′29″N, 22°41′07″E; 49°29′17″N, 22°15′48″E; 49°30′07″N, 21°54′45″E; 49°52′03″N, 21°44′55″E; 49°47′12″N, 22°21′23″E; 49°39′17″N, 22°40′27″E; 49°38′49″N, 22°45′04″E) in the San River basin, a tributary of the Vistula River (the drainage basin of the Baltic Sea), Poland. Fin clips of three other individuals of *B. carpathicus* from the Strwiąż River (49°28′42″N, 22°41′18″E), were collected from the Dniester River basin (the drainage basin of the Black Sea), Poland (Table 1). The sexes of all the investigated individuals of *C. carassius* were determined according to the morphology of the gonads, whereas the sexes of the *Barbus* individuals were not determined.

Table 1

Details of the analysed fish; M – male, F – female, 2n – diploid (2n=100 chromosomes), 3n – triploid (3n=150-164 chromosomes)

Taxon	Number of individuals used in the analysis of 5S rDNA		Sampling locality
	Structure	Chromosome mapping	
C. carassius	10M, 12F	3M, 3F	Siemianówka Reservoir, Narew River, Baltic Sea basin
C. gibelio	–	(2n) 4M, 5F	
	–	(3n) 3M, 5F	
B. barbus	25	2M, 2F	San River basin, Vistula River, Baltic Sea basin
B. carpathicus	26	3M, 5F	
	3	–	Strwiąż River, Dniester River, Black Sea basin
B. waleckii	7	1M	San River basin, Vistula River, Baltic Sea basin

The *C. carassius* individuals were taxonomically identified by the following morphological traits: non-pigmented peritoneum, deep body, rounded dorsal fin, small serration on the last unbranched ray in the dorsal fin, and golden colour of the dorsal and lateral parts of the body (Spóz *et al.* 2014; IUCN 2024).

The individuals of three *Barbus* species were diagnosed according to Rolik (1970; 1971a, b) (Kotlík *et al.* 2002; IUCN 2024) by the following characters typical for: a) *B. barbus*: lower lip thick with a median swollen pad (clearly adnate a median lobe by Rolik (1970; 1971a, 1971b)), pointed tip of the dorsal fin, posterior margin of the dorsal concave, clearly visible serration along the entire posterior edge on the last simple spinous dorsal ray (only palpable in smaller individuals), strongly embedded scales with free posterior part pointed, and sides of the body without spots (all the individuals were mature); b) *B. carpathicus*: lower lip thick with non-adnate a median lobe, last simple dorsal ray weak not serrated posteriorly and segmented on its entire length, plain top of the head, cheek and opercula unspotted or with very few small spots, paired fins unspotted, with rays unpigmented or with few dark pigments not forming dark bars, plain body with faint dots and blotches slightly darker than the background, and caudal fin unspotted or with spots smaller than the pupil diameter, with weakly embedded scales; and c) *B. waleckii*, with intermediate morphological traits of *B. barbus* and *B. carpathicus*: lower lip thick, with a non-adnate median lobe, last simple dorsal ray slightly thickened, serrated posteriorly to the middle, but with the serration rather delicate, sometimes weakly palpable, medium or weakly embedded scales with the free posterior part rounded and without epithelial crests, and the base of the pelvic fin slightly-to-clearly behind the base of the dorsal fin.

The fin clips of each individual were preserved in 96% ethanol and were stored at -20°C for further DNA extraction. For sequencing, 14 individuals of *C. carassius* and each of the 10 individuals of *B. barbus* and *B. carpathicus*, as well as seven individuals of *B. waleckii* were used. As *C. gibelio* has been described formerly in respect to the 5S rDNA structure (Szabelska *et al.* 2017), in the present paper only the mapping of its two classes of 5S rDNA in the karyotype was investigated.

The chromosome slides were made from fixed and preserved kidney cell suspensions of *C. carassius* (six individuals) and *C. gibelio* (nine diploid and eight triploid individuals) collected from the Siemianówka Reservoir, as well as of *B. barbus* (four individuals), *B. carpathicus* (eight individuals) and males of *B. waleckii* that were collected from the San River basin (Table 1).

Analysis of the 5S rDNA array sequence

Genomic DNA was extracted from the fin clips using the Chelex 100 method, as described by Walsh *et al.* (1991) with some modifications (Kirtiklis *et al.* 2011). The quality of the obtained DNA was assessed by electrophoretic separation and a spectrophotometric analysis.

A set of primers: 5S-1 (5'-TAC GCC CGA TCT CGT CCG ATC – 3' and 5S-2 (5' – CAG GCT GGT ATG GCC GTA AGC – 3') by Pendás *et al.* (1994) was used for the amplification of the 5S rDNA. PCR amplification was performed using a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, USA). The PCR cycling conditions were as follows: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds, and primer extension at 72°C for 30 seconds with a final extension at 72°C for 7 minutes.

The amplified products were visualised by electrophoresis (at 60V for 70 minutes) in an ethidium bromide-stained 1.5% agarose gel. A longer electrophoresis (at 60V for 110 minutes) in 2% agarose gel was conducted in order to get a better separation of the bands visible in the gel. All amplicons were extracted from the gel using Gel-Out kit (A&A Biotechnology, Poland) and were then sequenced on both strands using a commercial service (Genomed, Poland).

Sequence alignment and evaluation, phylogenetic analysis

The forward and reverse sequences of each individual were edited and assembled using BioEdit Sequence Alignment Editor v.7.2.5 (Hall 1999) to build a consensus sequence, and were then compared with the nucleotide sequences available in the GenBank database using BLAST (Altschul *et al.* 1997). The reference sequences of other polyploid cyprinids were taken from GenBank (NCBI). A multiple alignment of the analysed sequences was performed using Clustal W (Thompson *et al.* 1994). The phylogenetic analysis was carried out using MEGA 11 software (Tamura *et al.* 2021) by the maximum likelihood (ML) method with 1,000 bootstrap replicates. The best model of the nucleotide substitution (T92+G) was selected using the Find Best DNA/Protein Model tool integrated into MEGA software. Evolutionary distances were computed using the p-distance method.

Preparing the chromosome slides and fluorescence *in situ* hybridisation (FISH)

Chromosome slides were made from the suspension of kidney cells, which were obtained using a conventional hypotonic treatment and methanol-acetic fixation following the standard air-drying technique, as shown in detail by Boroń *et al.* (2011). The karyotype (not presented) was adopted based on our previous research as follows: a) *C. carassius* (2n=100; 20 metacentric (m) + 36 submetacentric (sm) + 44 subtelo-acrocentric (sta) chromosomes (Spóz *et al.* 2014); b) *C. gibelio* (2n=100, 26m+38sm+36sta; 3n=150-156, 34-36m+54-58sm+60-64sta plus from four to six microchromosomes (Boroń *et al.* 2011; Przybył *et al.* 2020); and c) *B. barbus*, *B. carpathicus*, *B. waleckii* (2n=100, 20m+34sm+46sta) (Spóz *et al.* 2015). In the present study, only the chromosome number was verified by counting at least 15 metaphase plates of each individual.

The double-colour FISH with two array classes of the 5S rDNA sequences of *C. carassius* and three *Barbus* species obtained in the current study was performed according to Fujiwara *et al.* (1998) and Boroń *et al.* (2006). For *C. gibelio*, FISHs with two sequences (340 bp and 470 bp) obtained and described formerly by Szabelska *et al.* (2017) were conducted.

Species-specific 5S rDNA probes were obtained by PCR amplification under the conditions described by Szabelska *et al.* (2017), then separated by electrophoresis and extracted from the agarose gel using the Gel-Out Concentrator kit (A&A Biotechnology, Poland). A proper DNA concentration of each probe was performed in a Centrifugal Concentrator DNA Integrated System (Genevac, UK). The 5S rDNA probes (corresponding to two rDNA array classes) were labelled with biotin-16-dUTP using the Biotin-Nick Translation Mix kit (Roche, Germany) and digoxigenin-11-dUTP using the DIG-Nick Translation Mix kit (Roche, Germany), respectively.

The labelled probes were denatured at 80°C for 10 min and were then stored on ice for 10 min to stop the reaction. The chromosome slides were initially hardened at 65°C for 3h, and were then incubated with rNase for 60 min at 37°C in a moist chamber. Afterwards, the chromosome preparations were denatured at 72°C for 1 min in 70% formamide (FA)/2×SSC. After dehydration at room temperature in an ethanol series – 70% for 5 min and 80%, 90%, and 100% for 2 min – hybridisation with a mixture containing denatured rDNA probes, Bovine Serum Albumin (Roche, Germany), 50% dextran sulphate, 4×SSC and double-deionised water was performed at 37°C overnight in a moist chamber. Post-hybridisation 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. Subsequent to the post-hybridisation washing in a low stringency condition (37°C, 20 min), the chromosome slides were subjected to detection with avidin-FITC (Roche, Germany) and anti-digoxigenin-rhodamine (Roche, Germany) for 5S rDNAs, with array Class I and Class II as probes, respectively. The chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in VECTASHIELD® Antifade Mounting Medium (Vector Laboratories, USA). At least 15 metaphase plates per each individual were observed under a Nikon Eclipse 90i fluorescence microscope (Nikon, Japan). The images were processed using Lucia 2.0 software (Laboratory Imaging, Czech Republic).

Results

5S rDNA array sequences of *C. carassius*

PCR amplification of the 5S rDNA generated two major bands, approximately 200 and 380 bp in length, for *C. carassius* (Cc) and several discreet bands with a length above 500 bp, visible in all the analysed individuals (Figure 1). In this study, we focused on two main bands: 200 and 380 bp, named Class I and Class II.

Two fragments of 5S rDNA corresponding to both main bands with sizes about 200 bp and 380 bp were sequenced, and a consensus sequence of the repeat unit (coding sequence plus NTS) was obtained for each of them. However, sequences of a similar length were obtained for both rDNA array Classes I and II (Figure 2). The nucleotide sequences of the 5S rDNA coding regions in *C. carassius* shared a highly conserved region (99% identity), 120 bp (99 bp and 21 bp of the encoding regions) in length, containing three internal regulatory elements, i.e. Box A, Box Cand IE, which function as a promoter of the gene (Figure 2). Only one difference between both classes of 5S rDNA arrays among Box A (at +38 bp) was revealed. The nucleotide compositions of Box A, Box C and IE are: AGCTAAGCAGGTTTG (location: +27 bp to +41 bp), TGGATGGGAGACCGCCTG (location: +57 bp to +74 bp) and CCTGGT (location: +44 bp to +49 bp), respectively. A T-rich tail (TTTT) (location: +96 bp to +99 bp) sequence acting as a transcription termination signal was identified at the 3′ end of the 5S rRNA coding sequence. The NTS region, identified to be 82 bp long in both the

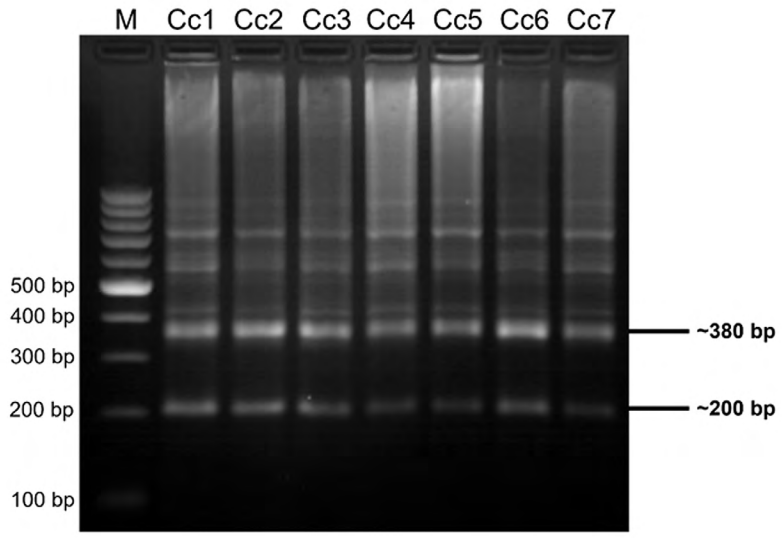

Fig. 1. Electrophoresis of the 5S rDNA amplicons in 1.5% agarose gel: *C. carassius* females (Cc1-Cc4) and males (Cc5-Cc7); M – Molecular weight DNA marker.

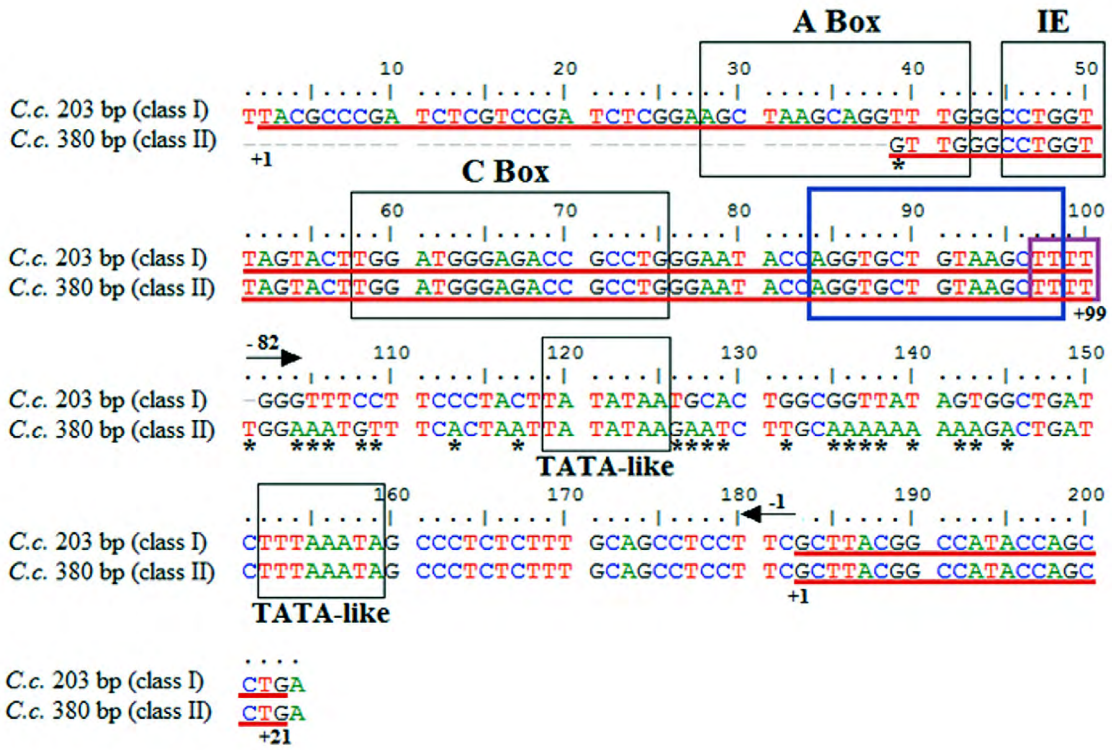

Fig. 2 Sequence alignment of the two 5S rDNA classes identified in *C. carassius*. The coding region of both 5S rDNA classes are red underlined with the conserved Box A, Box C and IE included in black boxes. AT-rich terminal region is evidenced in the purple box, whereas a 3`end-coding region is evidenced in the blue box. TATA-like elements characteristic for NTS are included in black boxes. Asterisks indicate the different nucleotides.

5S rDNA array classes, contained two conserved AT rich regions (TATA-like boxes) (location: +119 bp to +125 bp and +152 bp to +159 bp, respectively). The high dynamism of the NTS of both the 5S rDNA unit classes in *C. carassius* was indicated by the presence of 20 nucleotide substitutions (Figure 2). The obtained consensus sequences were deposited in GenBank under the following accession numbers: MG550276 (~200 bp fragment of *C. carassius*) and MG550277 (~380 bp fragment of *C. carassius*).

5S rDNA array sequences of the *Barbus* species

Since the PCR amplification of the 5S rDNA array generated two major products for all three *Barbus* species (Figure 3), with two major conspicuous forms of the 5S rDNA array in *B. barbus*, *B. carpathicus* and *B. waleckii*, one with about 195 bp (array Class I) and the other with about 215 bp (array Class II) were found. Moreover, in the *B. carpathicus* from both

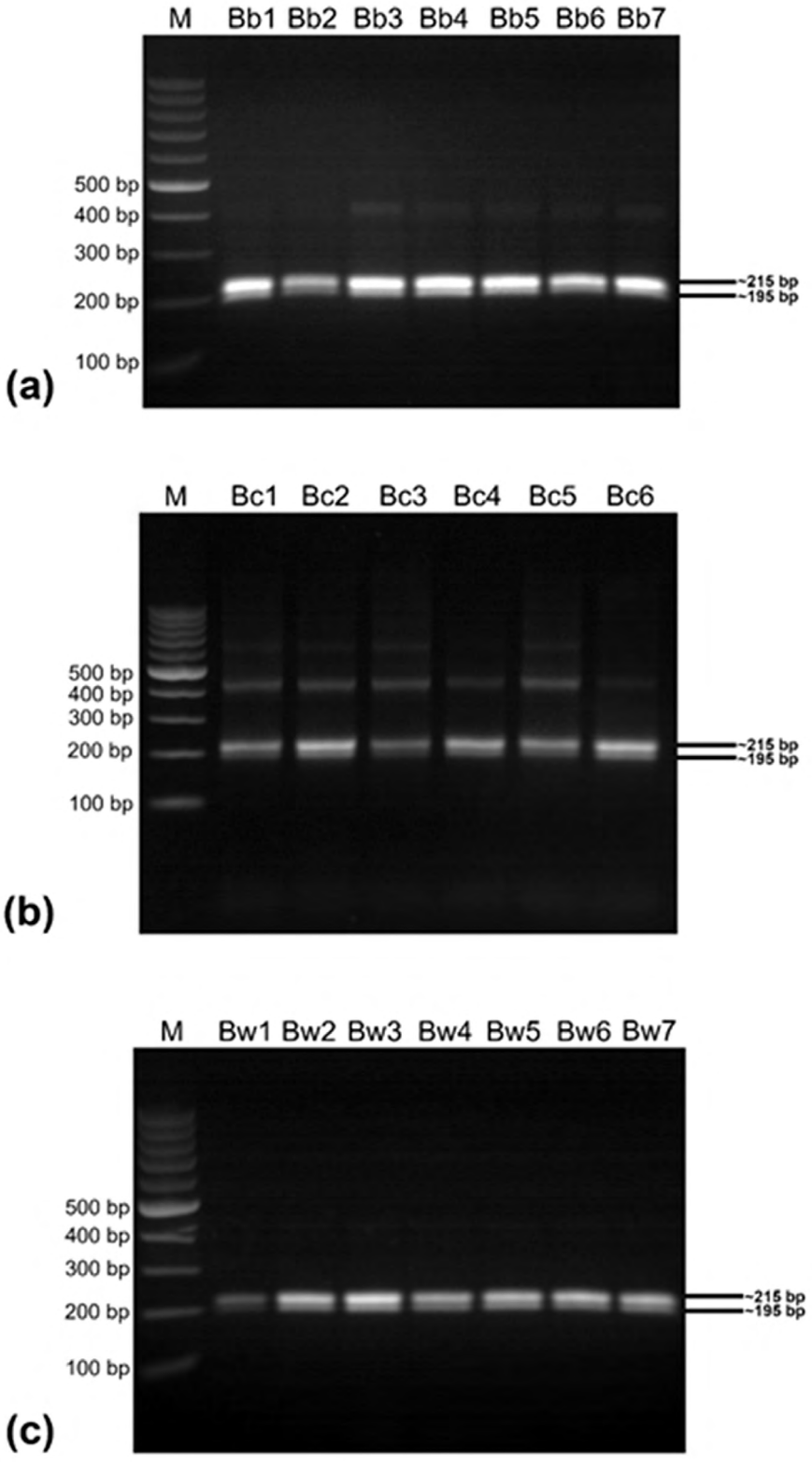

Fig. 3. Electrophoresis of the 5S rDNA amplicons in a 1.5% agarose gel: a) *B. barbus* (Bb1-Bb7); b) *B. carpathicus* (Bc1-Bc6); and c) *B. waleckii* (Bw1-Bw7). M – Molecular weight DNA marker.

sampled localities, one band approximately 460 bp in length with a lower intensity was visible (Figure 3b).

The analysis of the 5S rDNA fragment revealed few differences relating to individual substitutions within the 196 bp class, and also in the 215 bp class, among the three investigated *Barbus* species regardless of the sampled locality. Additionally, in the course of the comparative analysis of both array classes, single nucleotide differences in terms of substitutions in position +128 bp and +129 bp and an insertion-deletion in the positions from +131 bp to +149 bp were found within the NTS. The nucleotide sequences of the 5S rDNA coding regions among the three species shared a highly-conserved region (100% identity) of 120 bp (99 bp and 21 bp of the encoding regions), as well as a length containing three internal regulatory elements, i.e. Box A, Box C and IE (Figure 4). The nucleotide compositions of

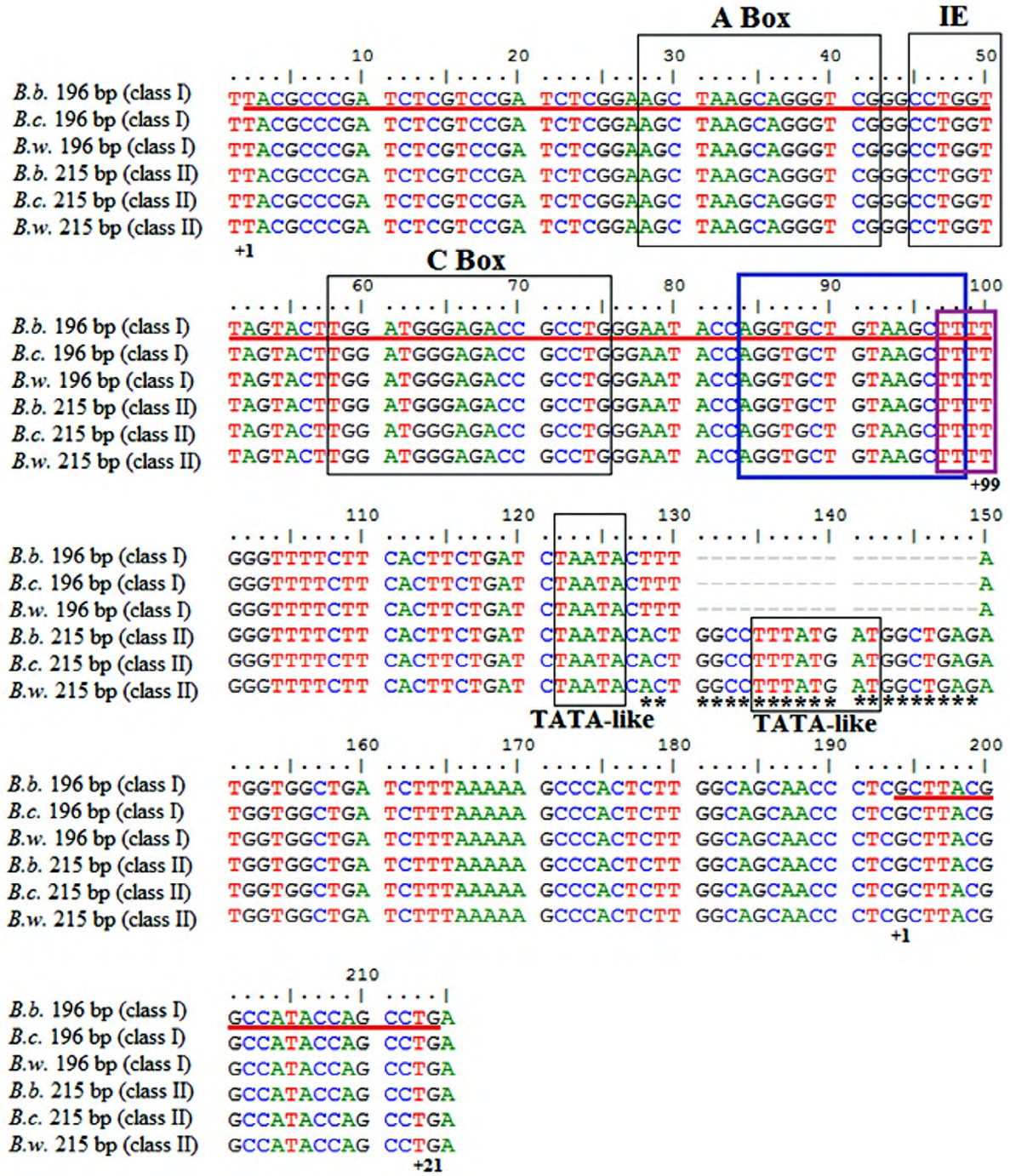

Fig. 4. Aligned consensus sequences of Class I and Class II of the 5S rDNA identified in *B. barbus*, *B. carpathicus* and *B. waleckii*. The coding region of both 5S rDNA classes are red underlined with the conserved Box A, Box C and IE included in black boxes. A T-rich terminal region is evidenced in the purple box and a 3`end-coding region is evidenced in the blue box. TATA-like elements characteristic for NTS are included in black boxes. Asterisks indicate the nucleotide differences.

Box A, Box C and IE were AGCTAAGCAGGGTCG (location: +27 bp to +41 bp), TGGATGGGAGAC-CGCCTG (location: +57 bp to +74 bp) and CCTGGT (location: +44 bp to +49 bp), respectively. The NTS region in array Class I was identified to be 74 bp long, while the NTS region in array Class II was identified to be 93 bp long in all the *Barbus* species (Figure 4). Both classes of the 5S rDNA sequences contained one TATA-like element within the NTS at positions +122 bp to +126 bp. Class II contained an additional TATA-like box element within the NTS at positions +135 bp to +142 bp.

In addition, the presence of one putative pseudogene in *B. carpathicus* about 460 bp in length containing a CT repeat in Box A was also observed (Figure 5). The sequence alignment of all three classes of 5S rDNA in *B. carpathicus* revealed some substitutions in IE and one insertion-deletion in position +65 bp in Box C (Figure 5). No differences were detected in the analysed 5S rDNAs between *B. carpathicus* individuals collected from the two localities. All the obtained sequences, apart from a 462 bp putative pseudogene, were deposited in the GenBank under the following accession numbers: MH212374 (196 bp fragment of *B. barbus*), MH212375 (215 bp fragment of *B. barbus*), MH212376 (196 bp fragment of *B. carpathicus*), MH212377 (215 bp fragment of *B. carpathicus*), MH212378 (196 bp fragment of *B. waleckii*) and MH212379 (215 bp fragment of *B. waleckii*).

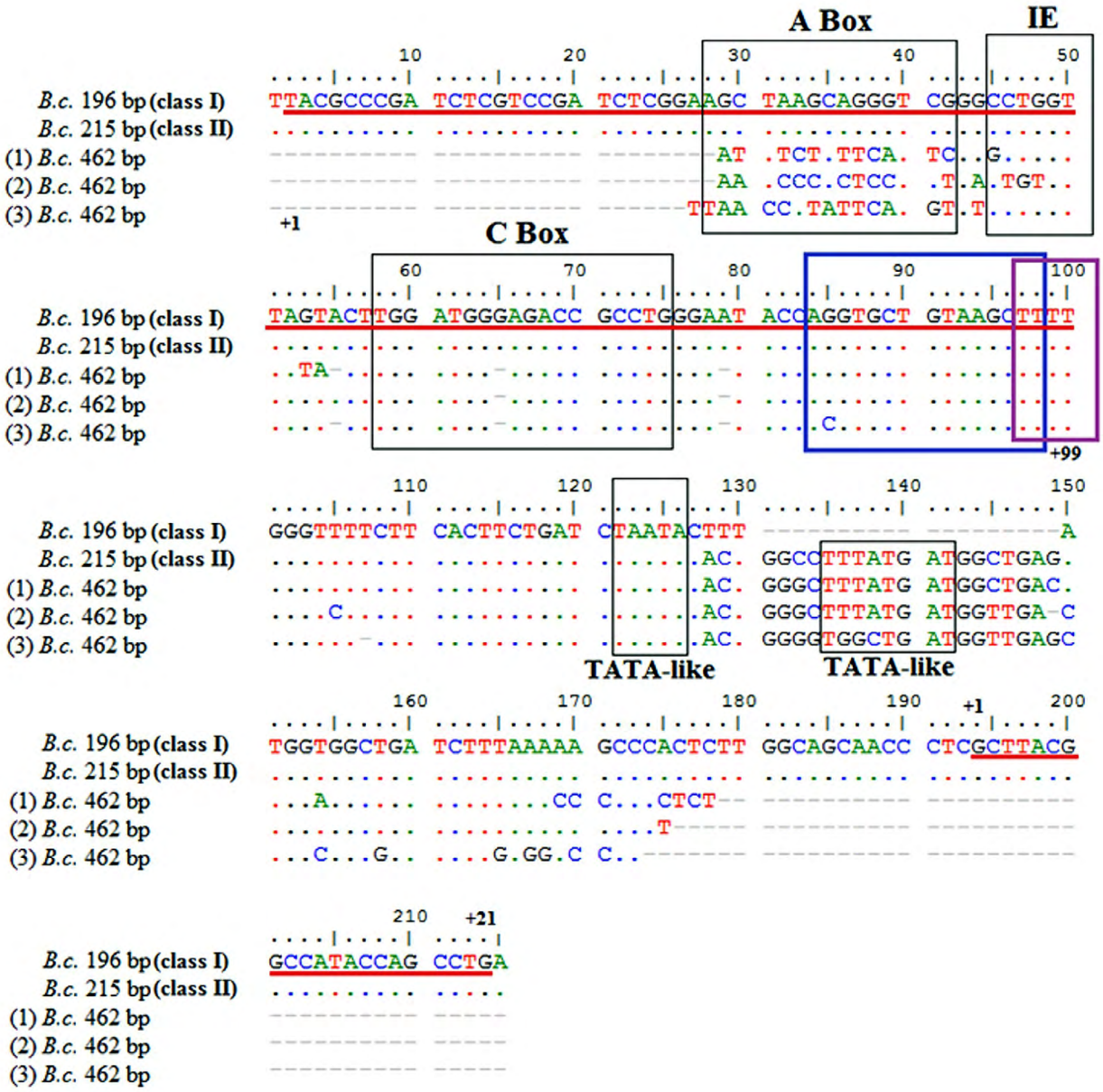

Fig. 5. Sequence alignment of two 5S rDNA classes and one putative pseudogene 462 bp in the length identified in *B. carpathicus*. The coding sequence of all 5S rDNA aligned sequences are underlined with the conserved Box A, Box C and IE included in black boxes. A T-rich terminal region is evidenced in the purple box and a 3′end-coding region is evidenced in the blue box. TATA-like elements characteristic for NTS are included in black boxes.

Phylogenetic analysis of 5S rDNA in polyploid Cyprininae

The phylogenetic survey of 5S rDNA array sequences of polyploid species from the subfamily Cyprininae verified that all genera, except for the genus *Tor*, were clustered in clearly separate clades (Figure 6). The ML tree showing three major clusters included the genera studied in this work – *Barbus* and *Carassius* as, well as the genera *Cyprinus*. However, *C. carassius* and *C. gibelio*, in contrast to the genus *Barbus*, formed distinct subclades (appropriate for the array Classes I and II) within the *Carassius* clade, what indicates a lower similarity between both 5S rDNA array classes in these both species. The separated cluster of the ML tree included only three species from the genus *Tor* and one from the genus *Neolissochilus* (Figure 6).

Chromosomal location of two 5S rDNA classes

The FISH with 5S rDNA array probes analysed in 120 metaphase plates of *C. carassius* females and males possessing 2n=100 chromosomes (Figure 7a) revealed a modal number of ten loci (in 76.6% of the analysed metaphases) within a range from 8 to 12. Both 5S rDNA sequences, 203 bp (array Class I) and 380 bp (array Class II) were always located

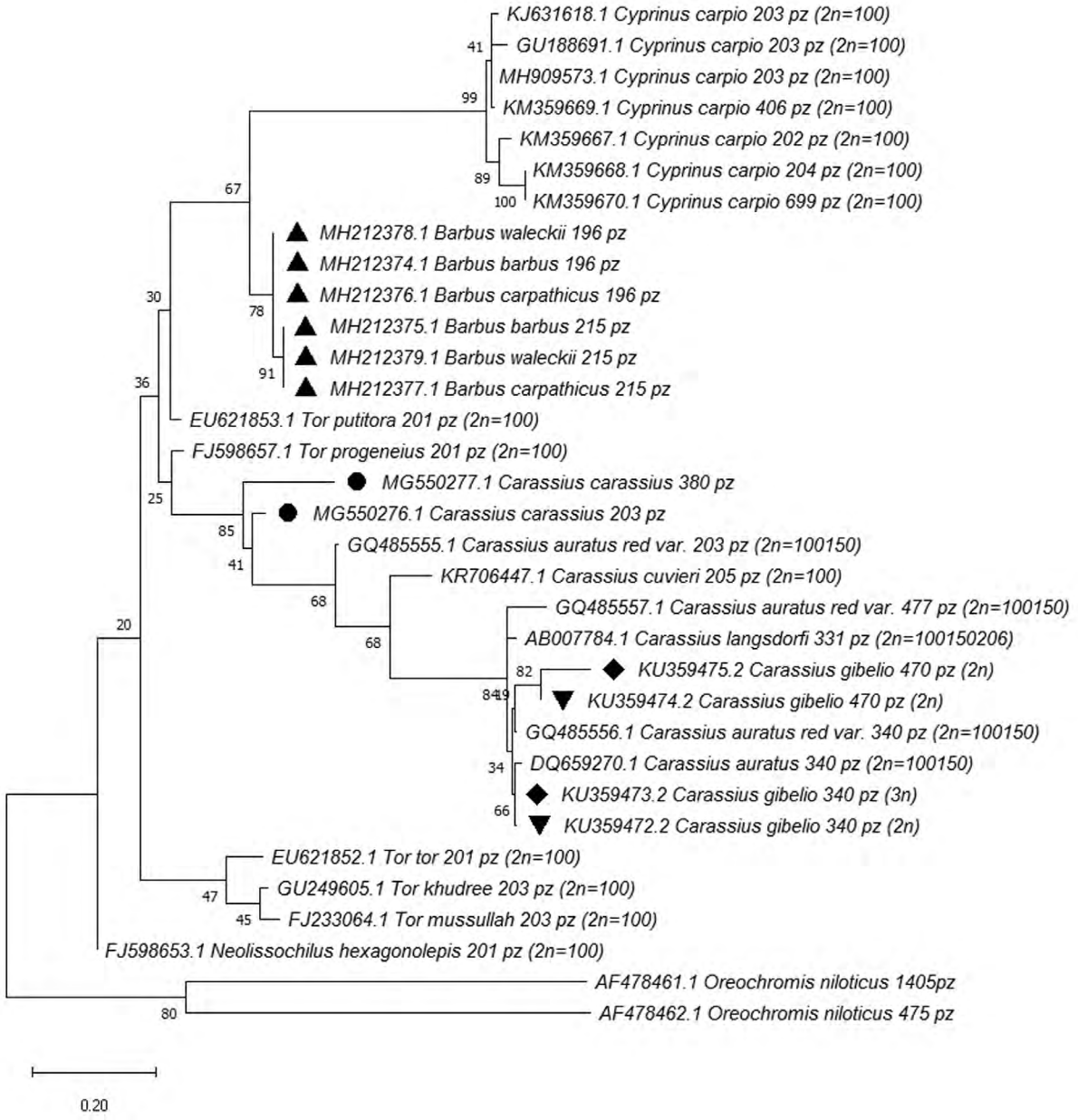

Fig. 6. The phylogenetic tree based on the maximum likelihood method analysis of two 5S rDNA classes of diploid (▼) and triploid (♦) *C. gibelio*, *C. carassius* (●), *B. barbus*, *B. carpathicus*, *B. waleckii* (▲) and reference sequences from other polyploid species of Cyprinidae from the subfamily Cyprininae. All sequences are available in the GenBank (NCBI) under the accession numbers mentioned on the graph. The bootstrap analysis was replicated 1000 times. The scale bar indicates the nucleotide diversity between sequences.

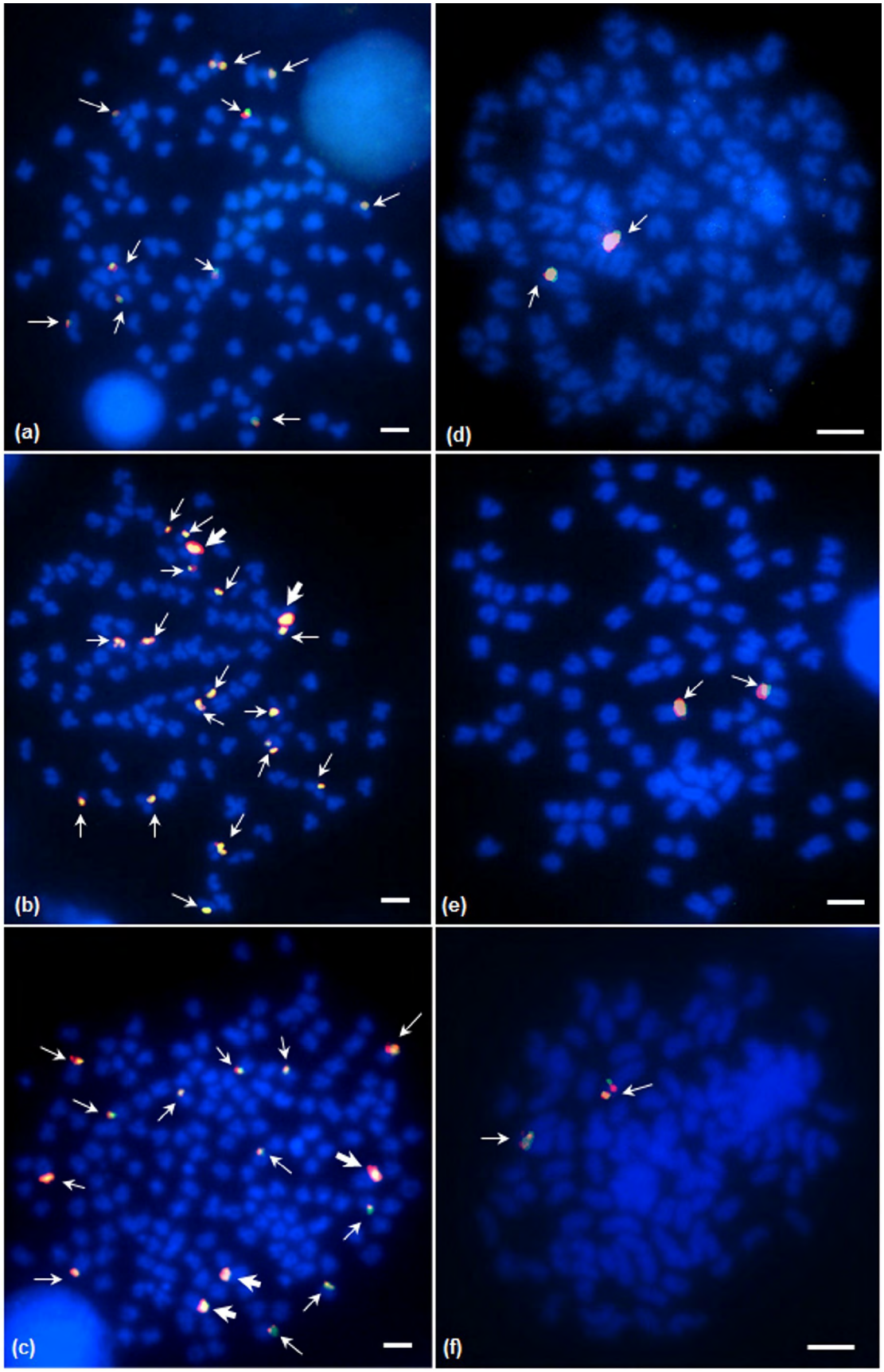

Fig. 7. Representative mitotic metaphase after FISH with: (a) *C. carassius* 5S rDNAs of 203 bp (green signals) and 380 bp (red signals); (b) diploid (2n=100); and (c) triploid (3n=160) *C. gibelio* 5S rDNA of 340 bp (green signals) and 470 bp (red signals) – and representative mitotic metaphase stained using FISH with: (d) *B. barbus*; (e) *B. carpathicus*; and (f) *B. waleckii* 5S rDNAs of 196 bp (green signals) and 215 bp (red signals). Arrows indicate chromosomes bearing both co-localised rDNA probes (thick and thin arrows represent strong and weak signals, accordingly). Scale bar = 10 μm.

in co-localisation in the same chromosomes (Figure 7a). The obtained hybridisation signals had different intensities on various chromosomes and could be classified as strong and weak. The 5S rDNA signals located on the short arm of two sm chromosomes were stronger than the other six to 10 signals on the sm and sta chromosomes (Figure 7a). Among 15% and 8.4% of the rest of metaphase plates, the 5S rDNAs were located, respectively, in 8 and 12 chromosomes.

The 5S rDNAs of 340 bp (array Class I) and 470 bp (array Class II) used as probes in the FISH were observed in 190 metaphases of *C. gibelio* diploid males and females (Figure 7b), as well as in 120 metaphases *C. gibelio* triploids of both sexes (Figure 7c). Both sequences of the 5S rDNA always occurred in a co-localisation in the same loci of the chromosomes in all individuals (Figure 7b, c). Most frequently, the diploid individuals were characterised by two stronger hybridisation signals on the short arm of the sm chromosomes and 16 weaker and smaller signals on the sm and sta chromosomes (Figure 7b). A variable number, ranging from 14 to 18, of smaller and weaker signals in the chromosomes of the *C. gibelio* diploids was observed. The triploids of *C. gibelio*, both males and females, possessed an unexpectedly similar number of 5S rDNA loci, ranging from 12 to 18 (Figure 7c). A modal number of 15 signals of 5S rDNA hybridisation was observed, with three of them distinctly stronger than the other 12 (Figure 7c). The large signals were found in the short arms of the sm chromosomes, whereas others were located on the sm and sta chromosomes. No sex-dependent variability in the analysed cytogenetic features among *Carassius* taxa was found.

Both the 5S rDNA arrays of 196 bp (Class I) and 215 bp (Class II) used as probes were observed in 60 metaphase plates of the four *B. barbus*, in 120 metaphase plates of eight *B. carpathicus* and in 15 metaphases of one *B. waleckii* male. In all analysed metaphases of three the *Barbus* species, two submetacentric chromosomes with the hybridisation signals of both 5S rDNAs in co-localisation on the short arms were detected (Figure 7d-f). No sex-dependent variability in the analysed cytogenetic features of *Barbus* species was found.

Discussion

Structure of the 5S rDNAs

The existence of two classes of 5S rDNA arrays documented in the *Carassius* and *Barbus* taxa (Szabelska *et al.* 2017; present study) has been described for many animals, including other fish (Martins & Galetti 2001; Gornung *et al.* 2007; Kumar *et al.* 2013), and seems to correspond to the ancestral pattern of their genome (Pasolini *et al.* 2006). However, the number of 5S rDNA repeated unit classes is not typical, either for related species clustered in one genus or for polyploid species. For example, the evolutionary polyploid *Tor* species are characterised by one or two such classes (Mani *et al.* 2011). As usual, the presence of two or more 5S rDNA clusters reflects variations in the NTS region, as is shown in the analysed *Carassius* and *Barbus* taxa that differ mainly because of base substitutions and insertions/deletions in the NTS region. This may indicate that this region is evolving rapidly and has experienced recent evolution events, and can thus be used as a species-population genetic marker contributing to the knowledge of the biology of species in a broad sense (Ferreira *et al.* 2007; Qin *et al.* 2010; He *et al.* 2012).

The smallest NTS sizes described so far in fishes are 62 bp (Santos *et al.* 2006), 57-68 bp (Fujiwara *et al.* 2009), 71 bp of three cyprinid species (Kumar *et al.* 2013), 82 bp in the *Carassius* and *Cyprinus* taxa (Ye *et al.* 2017; present study), and 74 and 93 bp in the *Barbus* taxa (this study). In turn, a relatively larger NTS indicated as 220, 357 bp was found in *C. auratus* (Qin *et al.* 2019) and from 190 to 613 bp in the *Channa* species (Barman *et al.* 2016).

The 5S rDNA array sequences of the investigated taxa showed more than an 80% average similarity with other polyploid Cyprinini and Barbini taxa listed in the GenBank database and used for the phylogenetic comparison. The nucleotide sequences of the 5S rDNA coding region among the two *Carassius* and three *Barbus* species shared a highly conserved gene identity of 120 bp long (99% and 100%, respectively). The nucleotide variability between the NTS region of the array Class I and II in the *Carassius* and *Barbus* taxa was indicated as 25.6% and 22.6%, respectively. However, no differences in the length of the NTS fragment in both unit classes of *C. carassius* were observed. Two amplicons (approx. 350 and 700 bp) of 5S rDNA in two species of the genus *Brycon* were described as the monomer and dimer of that rDNA array, respectively (Wasko *et al.* 2001). One similar length of the NTS in both analysed fragments seemed to indicate the occurrence of different-sized NTS sequences in the *Brycon* genome, which were not discovered by the Sanger sequencing method. Therefore, these results may point to the need for continued research, e.g. using New Generation Sequencing (NGS).

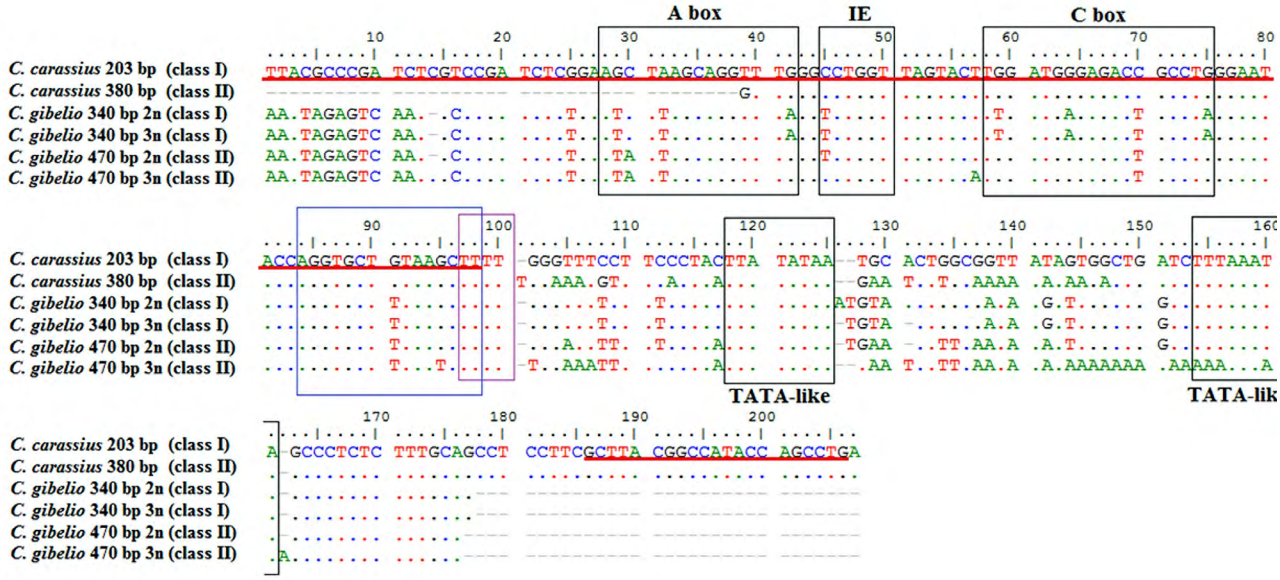

Fig. 8. Aligned nucleotide sequences of the two 5S rDNA classes identified in *C. carassius* (present study) and in the diploid and triploid *C. gibelio* (Szabelska *et al.* 2017). The CDS of all 5S rDNA aligned sequences are underlined with the conserved Box A, Box C and IE included in black boxes. A T-rich terminal region is evidenced in the purple box and a 3′end-coding region is evidenced in the blue box. TATA-like elements characteristic for NTS are included in black boxes.

The analysed 5S rDNA sequences from *C. carassius* and *C. gibelio* of a different ploidy (Figure 8) as well as the three *Barbus* species might be functional, as they possess all the necessary elements for stable secondary folding and transcription (Barman *et al.* 2016). The 5S rRNA is transcribed alone by RNA polymerase III with the participation of the transcription factor TFIIIA and the three elements of the internal control region (ICR) as Box A, Box C and IE (Pieler *et al.* 1987), shown in the present study. Box A is a general ICR sequence for RNA polymerase III, whereas the intermediate element (IE) and Box C are specific to the 5S rRNA transcription and work as binding sites for the transcription factor TFIIIA (Pieler *et al.* 1987), which is the first step in the formation of the transcription initiation complex (Veldhoen *et al.* 1994; Barman *et al.* 2016). Typically found in the presumed-functional 5S rRNA genes is a T-rich (TTTT) terminator region, identified at the 3`end of 5S rDNA in *Carassius* and *Barbus* species (this study) as well as in *Oreochromis niloticus* (Martins *et al.* 2000), and the 5` end of the NTS region in *Engystomops* spp. (Rodrigues *et al.* 2012). The conserved TATA-like control motifs detected in the NTS region in all taxa in the present study, as well as in this region of *Tor* sp. (Mani *et al.* 2011), *Lebias fasciata* (Tigano *et al.* 2004), some *Merluccius* species (Campo *et al.* 2009), *C. auratus* (Murakami & Fujitani 1998) and *C. carpio* (Inafuku *et al.* 2000), *Channa* species (Barman *et al.* 2016), along with RNA polymerase II-like transcriptional factors, plays a crucial role in RNA polymerase III transcription. Thus, these TATA-like motif sequences may be an important element in the regulation of the expression of 5S rRNA (Barman *et al.* 2016).

Most often, species belonging to the same genus are characterised by a quite conserved CDS of the 5S rDNA as some of the *Tor* species (Mani *et al.* 2011), *Leporinus* (Ferreira *et al.* 2007), three out of 12 species of the genus *Merluccius* (Campo *et al.* 2009) and *Barbus* (this study), or with only a few base substitutions among them, as in six species of the genus *Brycon*, six species belonging to Mugilidae (Wasko *et al.* 2001; Gornung *et al.* 2007) and the *Carassius* species (Szabelska *et al.* 2017; Qin *et al.* 2019; this study) showing, at the same time, a high degree of variation as nucleotide substitutions and/or insertion-deletion polymorphism in NTS (Kumar *et al.* 2013; Barman *et al.* 2016). NTS is successfully used as a molecular marker to identify different fish species, but the diversity of this sequence is not always species-specific. Small differences in the 5S rDNAs in the *Barbus* species (this study) also occurred in the sequence of some *Merluccius* species characterised by NTSs containing conserved blocks and a low number of nucleotide substitutions, whereas other species of the same genus differed significantly in the sequence length (Campo *et al.* 2009). On the other hand, the *C. carassius* (present study), diploids (2n=100) and triploids (3n=150-162) of *C. gibelio*,

formerly described by Szabelska *et al.* (2017), differed significantly both within the CDS and NTS regions of two 5S rDNA array classes, due to single base differences (Figure 8). The differences in the form of indel changes of between both pf the species were identified within the 120 bp CDS: in the Box A, IE, Box C and 3` end-coding regions. Moreover, two common TATA-like elements among the NTS were identified for all the aligned 5S rDNA sequences of both *Carassius* species.

Two out of three array classes of 5S rDNA detected in *C. auratus* red. var. (He *et al.* 2012; Qin *et al.* 2019; Zhao *et al.* 2021) with a length of 203 bp (Class I) and 340 bp (Class II) correspond with the Class I found, respectively, in *C. carassius* under the present study and in *C. gibelio* (Szabelska *et al.* 2017), whereas the length of the Class III (477 bp) was similar to the length of the Class II (470 bp) of *C. gibelio*. The array class of 340 bp, composed of the 120 bp coding region with 220 bp NTS, were cloned and sequenced in *C. a. gibelio* (3n=162) and *C. auratus* (2n=100) (Zhu *et al.* 2006; Qin *et al.* 2019). The above-cited data may indicate that at least two 5S rDNA array classes of about 340 and 470 bp seem to be specific in the *C. auratus* complex. Additionally, approx. 200-bp-long fragments may be typical for *Carassius* (Qin *et al.* 2019; Szabelska *et al.* 2017; present study), *Cyprinus carpio* (Inafuku *et al.* 2000; He *et al.* 2012) and for other polyploid cyprinids, such as some of the *Tor* species (Mani *et al.* 2011).

Recently, numerous published papers on the structure and chromosomal location of the 5S rDNA array sequence in different fish species (Ferreira *et al.* 2007; Han *et al.* 2015; Teixeira *et al.* 2018) and interspecific diploid and/or polyploid fish hybrids (He *et al.* 2012; Wang *et al.* 2017; Zhao *et al.* 2021) have provided data on the mechanisms of inheritance and the evolution of these sequences. According to Kotlík *et al.* (2002), *B. waleckii* may be a F_1 hybrid between *B. barbus* and *B. carpathicus*. In practice, the small variation of the 5S rDNA repeated unit sequence documented in the present study among the *Barbus* species cannot be used as a potential marker indicating the proposed hybridisation leading to the formation of *B. waleckii*. The inheritance pattern of 5S rDNAs in hybrids varies. The two 5S rDNA array classes of *C. auratus red var.* and *C. carpio* were inherited from both the parental taxa independently and without a homoeologous recombination and interlocus gene conversion (Ye *et al.* 2017). Otherwise, a chimeric 5S rDNA array was detected in the hybrid genome of *Ctenopharyngodon idella* and *Megalobrama amblycephala* (He *et al.* 2013), and in two *Epinephelus* species allowing for the randomly repeated units of the parental 5S rRNA genes to be exchanged with each other during hybridisation (Huang *et al.* 2016). In turn, signs of a homoeologous recombination would be expected in the event that the 5S rDNA sequences of the parental species were located on homoeologous chromosomes (Ye *et al.* 2017), as it is hypothetically in the *Barbus* species. According to Ye *et al.* (2017), tandem repeat insertion events and an interlocus gene conversion appeared during the evolution of 5S rDNAs in allotetraploid fish. The tetraploid hybrids partially inherited the NTS part from the parental female and obtained a novel NTS sequence (He *et al.* 2012). The multiple 5S rDNA sites detected in the present and other studies (Spóz *et al.* 2017; Knytl *et al.* 2018) may suggest that rapid chromosome rearrangements still occurred in the postpolyploidy genomes of *Carassius* species. An example of the intense dynamic evolution of the 5S rDNA array sequence seems to be visible in the genome of *Oreochromis niloticus*, characterised by the presence of pseudogenes and inverted genes (Martins *et al.* 2002).

Evolution of the 5S rDNA array

According to the concerted evolution process, all members of a multi-gene family evolve in a concerted manner and variations due to mutations in a repeated unit extent to all the member genes through gene conversion and an unequal crossing over (Barman *et al.* 2016; Nei & Rooney 2005). Thus, the sequence homology is higher within a species than between related species (Nei *et al.* 1997), as was found between two *Carassius* species (this study). In turn, some data, including that found in teleost fishes, has shown that the high level of sequence divergence between ribosomal genes or exclusively NTS regions may be explained by a birth-and-death model of evolution (Eirín-López *et al.* 2012; Barman *et al.* 2016; Cao *et al.* 2020). New gene variants are generated by gene duplication events and, therefore, few duplicated genes remain functional, while others become pseudogenes. The pseudogenisation exhibited by a different degree of base substitutions was detected in the sequences of 5S rDNA coding regions in the diploid and autotriploid of the *C. auratus* complex of the Dongting water system (Cao *et al.* 2020). It is generally believed that pseudogenes are most often derived from the array Class I (shorter class) of the 5S rDNA (Pinhal *et al.* 2011). In contrast to mammals, pseudogenes in fish are rather rare and until now they have only been described in some taxa, e.g. for species belonging to the genera *Coregonus*, *Merluccius*, *Oreochromis* or *Carassius* (Murakami

& Fujitani 1998; Sajdak *et al.* 1998; Martins *et al.* 2000; Campo *et al.* 2009). The presence of a putative pseudogene in *B. carpathicus* may suggest that the 5S rDNA array family in the genus *Barbus* evolved by a birth-and-death process (Barman *et al.* 2016), but this path of evolution does not explain the high level of sequence similarity within the non- and coding regions of the 5S rDNA array among the analysed species of this genus. Interestingly, the mixed mechanism of concerted and birth-and-death evolution, as well as horizontal transference were indicated in the evolution of that rDNA unit among freshwater stingrays (Pinhal *et al.* 2011) and Sparidae species (Merlo *et al.* 2013). This is consistent with the findings that the evolutionary patterns of the 5S rDNA array in animals are complex, and the birth-and-death processes, selection, homogenising mechanisms typically involved in concerted evolution and horizontal gene transfer events seem to be responsible for the diversity of this multigene family in metazoans (Vierna *et al.* 2013).

The phylogenetic tree constructed from the 5S rDNA arrays of the investigated and some other polyploid Cyprininae taxa revealed that the *C. gibelio* from Poland are clustered together with the same taxa from East Asia, whereas *C. carassius* is closer to the *Barbus* species. This relationship seems to support the hypothesis of an Asian origin of the *C. gibelio* distributed in Europe. The phylogenetic relations obtained from the analysis of 5S rDNA repeated unit sequences of the two *Carassius* and three *Barbus* species is similar to the phylogeny constructed from the mitogenome and the recombination-activating gene 1 (RAG1) datasets (Yang *et al.* 2015), as well as from the combined data set (Zheng *et al.* 2016). These results support the validity of using the 5S rDNA array sequence in evolutionary studies of polyploid cyprinid species.

Chromosomal location of the 5S rDNA arrays

The karyotype of diploid *C. carassius* and *C. gibelio* as well as of *C. gibelio* triploids, adopted in the present study, corresponds to that described by Knytl (2013; 2018). In turn, the karyotypes of the *Barbus* species are similar with other data (Ráb *et al.* 1996; Roman 2013) concerning only *B. barbus* and *B. carpathicus*. By contrast, the karyotype of *B. barbus* containing 2n = 96 chromosomes was clearly different (Luca *et al.* 2010).

In this study, the dual colour FISH localised both the two 5S rDNA array classes in synteny on the chromosomes of three *Carassius* and three *Barbus* taxa. This is the first data that has clearly reporting a stable localisation of the 5S rDNA units at the short arms of two submetacentric chromosomes of *B. barbus*, *B. carpathicus* and *B. waleckii*. In turn, the *Carassius* taxa in the present study were characterised by multiple fluorescence sites of their two co-localised 5S rDNA array classes. The modal number of ten 5S rDNA hybridisation sites detected at the short arms of two submetacentric and eight subtelo-acrocentric chromosomes in the karyotype of *C. carassius* correspond with the formerly published data (Spóz *et al.* 2014; Knytl *et al.* 2018). Strong signals localised, respectively, on two and three submetacentrics of *C. gibelio* diploids and triploids (together with numerous additional weaker ones) have been reported in other *C. auratus* taxa (Zhu *et al.* 2006; Knytl *et al.* 2018). The two and three abovementioned strong hybridisation signals observed in the *C. gibelio* chromosomes clearly indicated diploid and triploid individuals, respectively. This feature, together with chromosome painting, was used to indicate the ploidy of the *C. auratus* taxa (Zhu *et al.* 2006). In turn, three strong and twenty-four additional weak signals of the 5S rDNA probe were observed in triploid (3n=156) hybrids possessing two genomes of *C. gibelio* and one genome of *C. carassius* (Knytl *et al.* 2018). This finding supports the conclusion that the number of strong signals of the 5S rDNA array probe detected in the karyotypes of the analysed in this study and the abovementioned taxa of the genus *Carassius* may indicate the level of their ploidy (Knytl *et al.* 2018; present study). In turn, in some *Carassius* hybrids, clusters of 5S rDNA were lost (Zhao *et al.* 2021). Therefore, the number of strong signals of 5S rDNA cannot be a reliable polyploidy marker in all *Carassius* taxa. A multiple signal pattern of hybridisation with the 5S rDNA probe (from four to eight strong and several additional weak signals) is also present in *Cyprinus carpio*, a polyploid cyprinid which is related to the *Carassius* species (Inafuku *et al.* 2000).

The heteromorphism in signal intensity observed between homologous chromosomes of the *Carassius* and *Cyprinus* taxa may be caused by a variety of mechanisms, such as unequal crossing over, transposition, tandem amplification and other rearrangements involving homologous segments causing structural modifications in rDNA repeated unit sequences (Qin *et al.* 2016). However, homologous chromosomes in karyotypes of the *Carassius* taxa could be identified after chromosome painting. It is a possible that the variation in clusters of 5S rDNA with an adjusted NTS are associated mostly with the number of repeated monomers.

Some differences in the intensity between hybridisation signals involving 5S rDNAs detected in the

karyotype of *C. auratus* (strong signals) and *C. carpio* (weak signals) indicated that the hybrids inherited only one cluster from one of the parents (Ye *et al.* 2017). Thus, the number of 5S rDNA loci in the parental species does not add up in the case of their hybrids. The current study confirmed that *C. gibelio* triploids contained a similar 5S rDNA loci as the diploids. This may be due to the degradation (Huang *et al.* 2016) and other events triggered by polyploidy, which influences the organisation and evolution of the 5S rRNA multigene array family in the fish genome (Qin *et al.* 2019). From this point of view, 5S rDNAs are useful in clarifying some aspects of vertebrate genome evolution (Saenjundaeng *et al.* 2018).

One pair of chromosomes bearing 5S rDNAs presented as the plesiomorphic state in fish (Martins & Galetti 1999) may be suggested as a permanent feature of the genus *Barbus*. However, it is not certain that, as is shown in the species of the other cyprinid genus *Tor* (2n = 100), some of them possess one pair of 5S rDNA-bearing chromosomes (Singh *et al.* 2009), while others have two or even four such pairs (Mani *et al.* 2011). Our data and the data published so far suggest a variable pattern ranging from two (one pair) to eight (four pairs) of 5S rDNA sites observed in species from the family Cyprinidae (Kirtiklis *et al.* 2010; Rossi *et al.* 2012, among others). The polyploid species investigated to date belonging to the subfamily Cyprininae are characterised by two 5S rDNA loci (one pair), as well as two out of three *Tor* (Singh *et al.* 2009; Mani *et al.* 2011), *Barbus* (this study) and *Catlocarpio siamensis* (Saenjundaeng *et al.* 2018); and multiple 5S rDNA loci (two and more pairs) *Carassius* (Zhu *et al.* 2006; Spóz *et al.* 2014), *Cyprinus* (Inafuku *et al.* 2000; Ye *et al.* 2017), some *Tor* species (Singh *et al.* 2009; Mani *et al.* 2011) and *Probarbus jullieni* (Saenjundaeng *et al.* 2018). In turn, one pair of chromosomes bearing 5S rDNAs has been documented in some diploid (2n=50) Cyprininae species such as *Onychostoma* (Han *et al.* 2015), three species of the tribe Labeonini (Yang *et al.* 2015; Kumar *et al.* 2013) and *Ctenopharyngodon idella* (He *et al.* 2013); whereas in the karyotype of the cyprinid species of the subfamily Leuciscinae possessing 2n=50 chromosomes, a single, as well as multiple chromosomal localisations of the 5S rDNA loci was observed (Kirtiklis *et al.* 2010; Pereira *et al.* 2012; Vierna *et al.* 2013). The current data (including this study) does not indicate that the number of 5S rDNA sites localised in the chromosomes of polyploid (2n=100) species is more numerous than in diploid (2n=50) species, which is consistent with the results of other authors (Mani *et al.* 2011; Saenjundaeng *et al.* 2018, among others).

Both classes of 5S rDNA array sequences of *Carassius* and *Barbus* taxa were located in synteny, terminally in the short chromosome arm, in a similar manner as in other cyprinids in which a pericentromeric position of the 5S rDNA loci was also reported (Pereira *et al.* 2012; Rossi *et al.* 2012). In contrast to this, an interstitial position of the 5S rDNA clusters has been suggested as common among fishes and is considered optimal in relation to the organisation of these genes in the vertebrate genome (Martins *et al.* 2002). Several fish such as *Oreochromis niloticus*, *Leporinus* species and *C. auratus red. var.* and its polyploid hybrids possess two or three classes of 5S rDNA arrays located in distinct chromosome loci, which reflects the absence of a non-homologous chromosome exchange between the chromosome pairs bearing 5S rDNA clusters and seems to be associated with the rapid rate of the evolution of these DNA fragments (Martins & Galetti 2001; Martins *et al.* 2002; Qin *et al.* 2015).

Conclusions

In the present study, variations in the number of FISH signals (two in *Barbus* and multiple in *Carassius*) and their position in the karyotype, as well as variations in the nucleotide sequences of the 5S rDNA array family have been documented for three *Carassius* and *Barbus* taxa. These results reflect the lack of a correlation between the chromosome number and the number of 5S rDNA loci. Moreover, it was detected that triploids of *C. gibelio* contained fewer 5S rDNA loci than diploid ones. The present findings suggest that the observed low variability of 5S rDNAs is not an appropriate tool for the investigation of karyotype differentiation in karyologically-conservative *Barbus* species, but seems to be a suitable tool for such an investigation in karyologically-varied *Carassius* diploids and polyploids, including hybrids. The existence of different 5S rDNA array classes seems to be a rule for fish. The presented results for the *Carassius* and *Barbus* species, as well as other Cyprinidae diploids and polyploids, will contribute to an elucidation of the level complexity of the 5S rDNA repeated unit organisation and evolution in this fish group.

Author Contributions

Research concept and design: A.Sz., L.K., A.Bo.; Funding acquisition: A.Bo.; Collection and/or assembly of data: A.Sz., K.K., A.By., A.Bo.; Data analysis and interpretation: A.Sz., L.K., A.P., K.K., A.By.,

A.Bo.; Writing the article: A.Sz.; Critical revision of the article: L.K., A.Bo.; Final approval of article: A.Bo.

Funding

This research was carried out within Project No. 12.610.006-300 and No. 12.620.042-300 of the University of Warmia and Mazury in Olsztyn, financed by the Ministry of Science and Higher Education, Poland.

Acknowledgments

We express our special thanks to the Polish Angling Association in Białystok and Mr Andrzej Filinowicz for help in collecting the fish.

Conflict of Interest

The authors declare no conflict of interest.

References

Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**: 3389-3402. https://doi.org/10.1093/nar/25.17.3389

Amirowicz A., Kukuła K. 2005. Stream habitat conditions and fish fauna within the occurrence range of Wałecki barbel, *Barbus cyclolepis waleckii* Rolik, 1970 (Teleostei: Cyprinidae) in Polish part of the Carpathian Mts. Pol. J. Ecol. **53**: 503-522.

Arai R. 2011. Fish Karyotypes. A Check List. Springer: Tokyo, Japan. pp. 340. https://doi.org/10.1007/978-4-431-53877-6

Barman A.S., Singh M., Singh R.K., Lal K.K. 2016. Evidence of birth-and-death evolution of 5S rRNA gene in *Channa* species (Teleostei, Perciformes). Genetica **144**: 723-732. https://doi.org/10.1007/s10709-016-9938-6

Boroń A., Ozouf-Costaz O., Coutanceau J.P., Woroniecka K. 2006. Gene mapping of 28S and 5S rDNA sites in the spined loach *Cobitis taenia* (Pisces, Cobitidae) from a diploid population and a diploid-tetraploid population. Genetica **128**: 71-79. https://doi.org/10.1007/s10709-005-5536-8

Boroń A., Szlachciak J., Juchno D., Grabowska A., Jagusztyn B., Porycka K. 2011. Karyotype, morphology, and reproduction ability of the Prussian carp, *Carassius gibelio* (Actinopterygii: Cypriniformes: Cyprinidae), from unisexual and bisexual populations in Poland. Acta Ichthyol. Piscat. **41**: 19-28. https://doi.org/10.3750/AIP2011.41.1.04

Campo D., Machado-Schiaffino G., Horreo J.L., Garcia-Vazquez E. 2009. Molecular organization and evolution of 5S rDNA in the genus *Merluccius* and their phylogenetic implications. J. Mol. Evol. **68**: 208-216. https://doi.org/10.1007/s00239-009-9207-8

Cao L., Zhao C., Wang C., Qin H., Qin Q., Tao M., Zhang C., Zhao R., Liu S. 2020. Evolutionary dynamics of 18S and 5S rDNA in autotriploid *Carassius auratus*. Gene **737**: 144433. https://doi.org/10.1016/j.gene.2020.144433

Castro S.I., Hleap J.S., Cárdenas H., Blouin Ch. 2016. Molecular organization of the 5S rDNA gene type II in elasmobranchs. RNA Biol. **13**: 391-399. Evolutionary dynamics of 18S and 5S rDNA in autotriploid *Carassius* https://doi.org/10.1080/15476286.2015.1100796

Eirín-López J.M., Rebordinos L., Rooney A.P., Rozas J. 2012. The birth-and-death evolution of multigene families revisited. Genome Dyn. **7**: 170-196. https://doi.org/10.1159/000337119

Ferreira I.A., Oliveira C., Venere P.C., Galetti P.M. Jr., Martins C. 2007. 5S rDNA variation and its phylogenetic inference in the genus *Leporinus* (Characiformes: Anostomidae). Genetica **129**: 253-257. https://doi.org/10.1007/s10709-006-0005-6

Fornaini N.R., Černohorská H., do Vale Martins L., Knytl M. 2024. Cytogenetic analysis of the fish genus *Carassius* indicates divergence, fission, and segmental duplication as drivers of tandem repeat and microchromosome evolution. Gene. Biol. Evol. **16**(3): evae028. https://doi.org/10.1093/gbe/evae028

Froese R., Pauly D. 2024. FishBase. World Wide Web electronic publication. http://www.fishbase.org (last accessed on 07 March 2024).

Fujiwara A., Abe S., Yamaha E., Yamazaki F., Yoshida M.C. 1998. Chromosomal localization and heterochromatin association of ribosomal RNA gene loci and silver-stained nucleolar organizer regions in salmonid fishes. Chromosome Res. **6**: 463-471. https://doi.org/10.1023/A:1009200428369

Fujiwara M., Inafuku J., Takeda A., Watanabe A., Fujiwara A., Kohno S-I., Kubota S. 2009. Molecular organization of 5S rDNA in bitterlings (Cyprinidae). Genetica **135**: 355–365. https://doi.org/10.1007/s10709-008-9294-2

Gornung E., Colangelo P., Annesi F. 2007. 5S ribosomal RNA genes in six species of Mediterranean grey mullets: genomic organization and phylogenetic inference. Genome **50**: 787-795. https://doi.org/10.1139/g07-058

Hall T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **49**: 95-98.

Han C-C., Yen T-B., Chen N-C., Tseng M-C. 2015. Comparative studies of 5S rDNA profiles and Cyt *b* sequences in two *Onychostoma* species (Cyprinidae). Int. J. Mol. Sci. **16**: 29663-29672. https://doi.org/10.3390/ijms161226193

He W., Qin Q., Liu S., Li T., Wang J., Xiao J., Xie L., Zhang C. Liu Y. 2012. Organization and variation analysis of 5S rDNA in different ploidy - level hybrids of red crucian carp x topmouth culter. PLoS ONE **7**: e38976. https://doi.org/10.1371/journal.pone.0038976

He W., Xie L., Li T., Liu S., Xiao J., Hu J., Wang J., Qin Q., Liu Y. 2013. The formation of diploid and triploid hybrids of female grass carp x male blunt snout bream and their 5S rDNA analysis. BMC Genet. **14**: 110. https://doi.org/10.1186/1471-2156-14-110

Huang W., Qin Q., Yang H., Li S., Hu C., Wang Y., Zhang Y., Liu S., Lin H. 2016. Formation of diploid and triploid hybrid groupers (hybridization of *Epinephelus coioides* ♀ × *Epinephelus lanceolatus* ♂) and their 5S gene analysis. BMC Genet. **17**(1): 136. https://doi.org/10.1186/s12863-016-0443-9

Inafuku J., Nabeyama M., Kikuma Y., Saitoh J., Kubota S., Kohno S. 2000. Chromosomal location and nucleotide sequences of 5S ribosomal DNA of two cyprinid species (Osteichthyes, Pisces). Chromosome Res. **8**: 193-199. https://doi.org/10.1023/A:1009292610618

IUCN. 2024. The IUCN Red List of Threatened Species. http://www.iucnredlist.org (last accessed on 18 April 2024).

Kalous L., Bohlen J., Rylková K., Petrtýl M. 2012. Hidden diversity within the Prussian carp and designation of a neotype for *Carassius gibelio* (Teleostei: Cyprinidae). Ichthyol. Explor. Freshw. **23**: 11-18.

Kirtiklis L., Boroń A., Ptasznik P., Lusková V., Lusk S. 2011. Molecular differentiation of three loach species (Pisces, Cobitidae) based on the nuclear 5S rDNA marker. Folia Biol. (Kraków) **59**, 141-145. https://doi.org/10.3409/fb59_3-4.141-145

Kirtiklis L., Porycka K., Boroń A., Coutanceau J.P., Dettai A. 2010. Use of the chromosomal co-location of the minor 5S and the major 28S rDNA as a cytogenetic marker within the genus *Leuciscus* (Pisces, Cyprinidae). Folia Biol. (Kraków) **58**: 245-249. https://doi.org/10.3409/fb58_3-4.245-249

Knytl M., Kalous L., Ráb P. 2013. Karyotype and chromosome banding of endangered crucian carp, *Carassius carassius* (Linnaeus, 1758) (Teleostei, Cyprinidae). Comp. Cytogenet. **7**: 205-215. https://doi.org/10.3897/CompCytogen.v7i3.5411

Knytl M., Kalous L., Rylková K., Choleva L., Merilä J., Ráb P. 2018. Morphologically indistinguishable hybrid *Carassius* female with 156 chromosomes: A threat for the threatened crucian carp, *C. carassius*, L. PLoS ONE **13**: e0190924. https://doi.org/10.1371/journal.pone.0190924

Konopiński M.K., Amirowicz A., Kotlík P., Kukuła K., Bylak A., Pekarik L., Šediva A. 2013. Back from the Brink: The holocene history of the Carpathian barbel *Barbus carpathicus*. PLoS ONE **8**: e82464. https://doi.org/10.1371/journal.pone.0082464

Konopiński M.K., Amirowicz A., Kukuła K. 2007. Probable direction of the postglacial colonization of rivers on northern slopes of the Carpathian Ridge by *Barbus carpathicus* (Teleostei: Cyprinidae) evidenced by cline of genetic variation. J. Fish Biol. **70**: 406-415. https://doi.org/10.1111/j.1095-8649.2007.01479.x

Kotlík P., Tsigenopoulos C.S., Ráb P., Berrebi P. 2002. Two new *Barbus* species from the Danube River basin, with redescription of *B. petenyi* (Teleostei: Cyprinidae). Folia Zool. **51**: 227-240.

Kotusz J., De Wever A. 2019. Freshwater fishes of Poland. Version 2.2. BioFresh. Occurrence dataset. https://doi.org/10.13148/bfcf8 (last accessed on 20 June 2019).

Kumar R., Kushwaha B., Nagpure N.S. 2013. Characterization and physical mapping of 18S and 5S ribosomal genes in Indian major carps (Pisces, Cyprinidae). Micron **49**: 40-45. https://doi.org/10.1016/j.micron.2013.03.001

Le Comber S.C., Smith C. 2004. Polyploidy in fishes: Patterns and processes. Biol. J. Linn. Soc. **82**: 431-442. https://doi.org/10.1111/j.1095-8312.2004.00330.x

Luca C., Suciu R., Costache M. 2010. Comparative karyotype in different lineages of Cyprinid fish (Teleostei: Cypriniformes: Cyprinidae). Studia Univ. VG **20**: 37-41.

Mani I., Kumar R., Singh M., Nagpure N.S., Kushwaha B., Srivastava P.K., Krishna Rao D.S., Lakra W.S. 2011. Nucleotide variation and physical mapping of ribosomal genes using FISH in genus *Tor* (Pisces, Cyprinidae). Mol. Biol. Rep. **38**: 2637-2647. https://doi.org/10.1007/s11033-010-0405-7

Martins C., Galetti P.M. Jr. 2001. Organization of 5S rDNA in species of the fish *Leporinus*: two different genomic locations are characterized by distinct nontranscribed spacers. Genome **44**: 903-10. https://doi.org/10.1139/gen-44-5-903

Martins C., Galetti P.M. Jr. 1999. Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). Chromosome Res. **7**: 363-367. https://doi.org/10.1023/A:1009216030316

Martins C., Wasko A.P. 2004. Organization and evolution of 5S ribosomal DNA in the fish genome. (In: Focus on Genome Research. C.R. Williams ed. Nova Biomedical Books, New York): 335-363.

Martins C., Wasko A.P., Oliveira C., Porto-Foresti F., Parise-Maltempi P.P., Wright J.M., Foresti F. 2002. Dynamics of 5S rDNA in the tilapia (*Oreochromis niloticus*) genome: repeat units, inverted sequences, pseudogenes and chromosome loci. Cytogenet. Genome Res. **98**: 78-85. https://doi.org/10.1159/000068542

Martins C., Wasko A.P., Oliveir, C., Wright J.M. 2000. Nucleotide sequence of 5S rDNA and localization of the ribosomal RNA genes to metaphase chromosomes of the tilapiine cichlid fish, *Oreochromis niloticus*. Hereditas **133**: 39-46. https://doi.org/10.1111/j.1601-5223.2000.00039.x

Merlo M.A., Cross I., Manchado M., Cárdenas S., Rebordinos L. 2013. The 5S rDNA high dynamism in *Diplodus sargus* is a transposon-mediated mechanism. Comparison with other multigene families and Sparidae species. J. Mol. Evol. **76**: 83-97. https://doi.org/10.1007/s00239-013-9541-8

Morán P., Martinez J.L., Garcia-Vazquez E., Pendás A.M. 1996. Sex chromosome linkage of 5S rDNA in rainbow trout (*Oncorhynchus mykiss*). Cytogenet. Cell Genet. **75**: 145-150. https://doi.org/10.1159/000134466

Murakami M., Fujitani H. 1998. Characterization of repetitive DNA sequences carrying 5S rDNA of the triploid ginbuna (Japanese silver crucian carp, *Carassius auratus langsdorfi*).

Genes Genet. Syst. **73**: 9-20. https://doi.org/10.1266/ggs.73.9

Nei M., Gu X., Sitnikova T. 1997. Evolution by the birth-and-death process in multigene families of the vertebrate immune system. Proc. Natl. Acad. Sci. USA **94**: 7799-7806. https://doi.org/10.1073/pnas.94.15.7799

Nei M., Rooney A.P. 2005. Concerted and birth-and-death evolution of multigene families. Annu. Rev. Genet. **39**: 121-152. https://doi.org/10.1146/annurev.genet.39.073003.112240

Pasolini P., Costagliola D., Rocco L., Tinti F. 2006. Molecular organization of 5S rDNA in Rajidae (Chondrichthyes): structural features and evolution of piscine 5S rRNA genes and non-transcribed intergenic spacers. J. Mol. Evol. **62**: 564-574. https://doi.org/10.1007/s00239-005-0118-z

Pendás A.M., Morán P., Freije J.P., Garcia-Vazquez E. 1994. Chromosomal mapping and nucleotide sequence of two tandem repeats of Atlantic salmon 5S rDNA. Cytogenet. Cell Genet. **67**: 31-36. https://doi.org/10.1159/000133792

Pereira C.S.A., Ráb P., Collares-Pereira M.J. 2012. Chromosomes of European cyprinid fishes: comparative cytogenetics and chromosomal characteristics of ribosomal DNAs in nine Iberian chondrostomine species (Leuciscinae). Genetica **140**: 485-495. https://doi.org/10.1007/s10709-013-9697-6

Pieler T., Hamm J., Roeder R.G. 1987. The 5S gene internal control region is composed of three distinct sequence elements, organized as two functional domains with variable spacing. Cell **48**: 91-100 https://doi.org/10.1016/0092-8674(87)90359-X

Pinhal D., Yoshimura T.S., Araki C.S., Martins C. 2011. The 5S rDNA family evolves through concerted and birth-and-death evolution in fish genomes: An example from freshwater stingrays. BMC Evol. Biol. **11**: 151. https://doi.org/10.1186/1471-2148-11-151

Przybył A., Przybylski M., Spóz A., Juchno D., Szabelska A., Kowalewska K., Boroń A. 2020. Sex, size and ploidy ratios of *Carassius gibelio* from Poland. Aquat. Invasions **15**: 335-354. https://doi.org/10.3391/ai.2020.15.2.08

Qin Q., He W., Liu S., Wang J., Xiao J., Liu Y. 2010. Analysis of 5S rDNA organization and variation in polyploid hybrids from crosses of different fish subfamilies. J. Exp. Zool. B **314**: 403-411. https://doi.org/10.1002/jez.b.21346

Qin Q., Wang J., Hu M., Huang S., Liu S. 2016. Autotriploid origin of *Carassius auratus* as revealed by chromosomal locus analysis. Sci. China Life Sci. **59**: 622-626. https://doi.org/10.1007/s11427-016-5040-7

Qin Q., Wang J., Wang Y., Liu Y., Liu S. 2015. Organization and variation analysis of 5S rDNA in gynogenetic offspring of *Carassius auratus* red var. (♀) × *Megalobrama amblycephala* (♂). BMC Genet. **16**: 26. https://doi.org/10.1186/s12863-015-0186-z

Qin Q, Liu Q., Wang C., Cao L., Zhou Y., Qin H., Zhao C., Liu S. 2019. Molecular organization and chromosomal localization analysis of 5S rDNA clusters in autotetraploids derived from *Carassius auratus* Red Var. (♀) × *Megalobrama amblycephala* (♂). Front. Genet. **10**: 437 https://doi.org/10.3389/fgene.2019.00437

Ráb P., Karakousis Y., Rabova M. 1996. Karyotype, NOR phenotype and C-banding study of *Barbus cyclolepis* from Greece. Fol. Zool. **45**: 77-83.

Rodrigues D.S., Rivera M., Lourenço L.B. 2012. Molecular organization and chromosomal localization of 5S rDNA in Amazonian *Engystomops* (Anura, Leiuperidae). BMC Genet. **13**: 17. https://doi.org/10.1186/1471-2156-13-17

Rolik H. 1970. *Barbus cyclolepsis waleckii* ssp. n. – a new subspieces of *B. cyklolepsis* Heckel, 1840, from the Vistula and Dniestr basins (Pisces, Cyprinidae). Bull. Acad. Pol. Sci**. 18**: 401-404.

Rolik H. 1971a. Fishes of upper and middle San River and its tributaries (in Polish with English summary). Fragm. faun. **17**: 559-584.

Rolik H. 1971b. Studies on three *Barbus* species (Pisces, Cyprinidae) in San and Wisłoka basins in Poland. (in Polish with English summary). Ann. Zool. **28**: 257-330.

Roman A.M. 2013. Karyotypes of the three taxons of genus *Barbus* (Cypriniformes, Cyprinidae) from the reservoirs of Ukraine (in Russian). Zbirn. Prats Zool. Mus. **44**: 70-76.

Rossi A.R., Milana V., Hett A.K. 2012. Tancioni, L. Molecular cytogenetic analysis of the Appenine endemic cyprinid fish *Squalius lucumonis* and three other Italian leuciscines using chromosome banding and FISH with rDNA probes. Genetica **140**: 469-476. https://doi.org/10.1007/s10709-012-9695-0

Rylková K., Kalous L. 2013. Genetic diversity in the genus *Carassius* (Teleostei: Cyprinidae) in the Czech Republic. Acta Soc. Zool. Bohem. **77**: 73-79.

Saenjundaeng P., Cioffi M.B., Oliveira E.A., Tanomtong A., Supiwong W., Phimphan S., Collares-Pereira M.J., Sember A., Bertollo L.A.C., Liehr T., *et al.* 2018. Chromosomes of Asian cyprinid fishes: cytogenetic analysis of two representatives of small paleotetraploid tribe Probarbini. Mol. Cytogenet. **11**: 51. https://doi.org/10.1186/s13039-018-0399-8

Sajdak S.L., Reed K.M., Phillips R.B. 1998. Intraindividual and interspecies variation in the 5S rDNA of coregonid fish. J. Mol. Evol. **46**: 680-688. https://doi.org/10.1007/PL00006348

Santos L.V.R., Foresti F., Wasko A.P., Oliveira C., Martins C. 2006. Nucleotide sequence, genomic organization and chromosome localization of 5S rDNA in two species of Curimatidae (Teleostei, Characiformes). Genet. Mol. Biol. **29**: 251-256. https://doi.org/10.1590/S1415-47572006000200009

Šimková A., Hyršl P., Halačka K., Vetešník L. 2015. Physiological and condition-related traits in the gynogenetic-sexual *Carassius auratus* complex: different investments promoting the coexistence of two reproductive forms? BMC Evol. Biol. **15**: 154. https://doi.org/10.1186/s12862-015-0438-6

Singh M., Kumar R., Nagpure N.S., Kushwaha B., Gond I., Lakra W.S. 2009. Chromosomal localization of 18S and 5S rDNA using FISH in the genus *Tor* (Pisces, Cyprinidae). Genetica **137**: 245-252. https://doi.org/10.1007/s10709-009-9367-x

Spóz A., Boroń A., Ocalewicz K., Kirtiklis L. 2017. Polymorphism of the rDNA chromosomal regions in the weatherfish *Misgurnus fossilis* (Teleostei: Cobitidae). Folia Biol. (Kraków) **65**: 63-70. https://doi.org/10.3409/fb65_1.63

Spóz A., Boroń A., Porycka K., Karolewska M., Ito D., Abe S., Kirtiklis L., Juchno D. 2014. Molecular cytogenetic analysis of the crucian carp, *Carassius carassius* (Linnaeus, 1758) (Teleostei, Cyprinidae), using chromosome staining and fluorescence *in situ* hybridisation with rDNA probes. Comp. Cytogenet. **8**: 233-248. https://doi.org/10.3897/CompCytogen.v8i3.7718

Spóz A., Grabowska A., Boroń A., Juchno D., Kotusz J. 2015. Gene mapping of 28S and 5S rDNA sites in chromosomes of two *Barbus* species and their F1 hybrids (Teleostei, Cyprinidae). Front. Mar. Sci. Conference Abstract, XV European Congress of Ichthyology, Porto (Portugal), 7-11 September 2015. https://doi.org/10.3389/conf.FMARS.2015.03.00186

Symonová R., Howell W.M. 2018. Vertebrate genome evolution in the light of fish cytogenomics and rDNAomics. Genes **9**: 96. https://doi.org/10.3390/genes9020096

Szabelska A., Kirtiklis L., Przybył A., Boroń A. 2017. 5S rDNA sequence shows differences between diploid and triploid Prussian carp *Carassius gibelio* (Teleostei, Cyprinidae). Turk. J. Fish. Aquat. Sci. **17**: 1127-1133. https://doi.org/10.4194/1303-2712-v17_6_06

Tamura K., Stecher G., Kumar S. 2021. MEGA 11: Molecular evolutionary genetics analysis version 11. Mol. Biol. Evol. **28**: 3022-3027. https://doi.org/10.1093/molbev/msab120

Tan M., Armbruster J.W. 2018. Phylogenetic classification of extant genera of fishes of the order Cypriniformes (Teleostei: Ostariophysi). Zootaxa **4476**: 6-39. https://doi.org/10.11646/zootaxa.4476.1.4

Teixeira T.K.S.S., Venere P.C., Ferreira D.C., Mariotto S., Castro J.P., Artoni R.F., Centofante L. 2018. Comparative cytogenetics of *Astynax* (Teleostei, Characidae) from the upper Paraguway basin. Neotrop. Ichthyol. **16**: e170092. https://doi.org/10.1590/1982-0224-20170092

Thompson J.D., Higgins D.G., Gibson T.J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**: 4673-4680. https://doi.org/10.1093/nar/22.22.4673

Tigano C., Rocco L., Ferrito V., Costagliola D., Pappalardo A.M., Stingo V. 2004. Chromosomal mapping and molecular characterization of ribosomal RNA genes in *Lebias fasciata* (Teleostei, Cyprinodontidae). Genetica **121**: 95-100. https://doi.org/10.1023/B:GENE.0000019931.89458.dc

Utsunomia R., Scacchetti P.C., Pansonato-Alves J.C., Oliveira C., Foresti F. 2014. Comparative chromosome mapping of U2 snRNA and 5S rRNA genes in *Gymnotus* species (Gymnotiformes, Gymnotidae): evolutionary dynamics and sex chromosome linkage in *G. pantanal*. Cytogenet. Genome Res. **142**: 286-292. https://doi.org/10.1159/000362258

Veldhoen N., You Q., Setzer D.R., Romaniuk P. J. 1994. Contribution of individual base pairs to the interaction of TFIIIA with the *Xenopus* 5S RNA gene. Biochemistry **33**: 7568-7575. https://doi.org/10.1021/bi00190a009

Vierna J., Wehner S., Höner zu Siederdissen C., Martínez-Lage A., Marz M. 2013. Systematic analysis and evolution of 5S ribosomal DNA in metazoans. Heredity **111**: 410-421. https://doi.org/10.1038/hdy.2013.63

Walsh P.S., Metzger D.A., Higuchi R. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques **10**: 506-513. https://doi.org/10.2144/000114018

Wang S., Ye X., Wang Y., Chen Y., Lin B., Yi Z., Mao Z., Hu F., Zhao R., Wang J., *et.al.* 2017. A new type of homodiploid fish derived from the interspecific hybridization of female common carp x male blunt snout bream. Sci. Rep. **7**: 4189. https://doi.org/10.1038/s41598-017-04582-z

Wasko A.P., Martins C., Wright J.M., Galetti P.M. Jr. 2001. Molecular organization of 5S rDNA in fishes of the genus *Brycon*. Genome **44**: 893-902. https://doi.org/10.1139/gen-44-5-893

Yang L., Naylor G.J.P., Mayden R.L. 2022. Deciphering reticulate evolution of the largest group of polyploid vertebrates, the subfamily cyprininae (Teleostei: Cypriniformes). Mol. Phylogenet. Evol. **166**: 107323. https://doi.org/10.1016/j.ympev.2021.107323

Yang L., Sado T., Hirt M.V., Pasco-Viel E., Arunachalam M., Li J., Wang X., Freyhof J., Saitoh K., Simons A.M., Miya M., He S., Mayden R.L. 2015. Phylogeny and polyploidy: resolving the classification of cyprinine fishes (Teleostei: Cypriniformes). Mol. Phylogenet. Evol. **85**: 97-116. https://doi.org/10.1016/j.ympev.2015.01.014

Ye L., Zhang C., Tang X., Chen Y., Liu S. 2017. Variations in 5S rDNAs in diploid and tetraploid offspring of red crucian carp × common carp. BMC Genet. **18**: 75. https://doi.org/10.1186/s12863-017-0542-2

Zhang C., Ye L., Chen Y., Xiao J., Wu Y., Tao M., Xiao Y., Liu S. 2015. The chromosomal constitution of fish hybrid lineage revealed by 5S rDNA FISH. BMC Genet. **16**: 140. https://doi.org/10.1186/s12863-015-0295-8

Zhao C., Zhang Y., Qin H., Wang C., Huang X., Yang L., Yu T., Xu X., Luo X., Qin Q., Liu S. 2021. Organization and expression analysis of 5S and 45S ribosomal DNA clusters in autotetraploid *Carassius auratus*. BMC Ecol. Evo. **21**: 201. https://doi.org/10.1186/s12862-021-01918-2

Zheng C., Voorrips R.E., Jansen J., Hackett C.A., Ho J., Bink M.C.A.M. 2016. Probabilistic multilocus haplotype reconstruction in outcrossing tetraploids. Genetics **203**: 119-131. https://doi.org/10.1534/genetics.115.185579

Zhu H.P., Ma D.M., Gui J.F. 2006. Triploid origin of the gibel carp as revealed by 5S rDNA localization and chromosome painting. Chromosome Res. **14**: 767-776. https://doi.org/10.1007/s10577-006-1083-0