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# Novel cytogenetic data in two gudgeons from the genera *Gobio* and *Romanogobio* (Teleostei: Cypriniformes: Gobionidae): chromosomal mapping of rDNAs and telomeric repeats

Lech Kirtiklis, Konrad Ocalewicz, Michał Nowak, Marta Gwiazda-Orzoł and Alicja Boroń

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Original article

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The taxonomic status of gudgeons (Gobionidae) and the genetic relationships among them are still unclear. The current knowledge on the molecular cytogenetics of gudgeons is quite limited and concerns the basic parameters of their karyotype. Therefore, the main aim of this study was to obtain new data on the chromosomes of two gudgeons – *Gobio gobio* and *Romanogobio belingi* – which would allow for characterising these species in a more precise way. Chromosomes of the examined species were analysed by conventional Giemsa staining and, for the first time, by fluorescence *in situ* hybridisation (FISH) with 28S rDNA, 5S rDNA and telomeric probes. The diploid chromosome numbers in both species were the same and equalled 2n=50, while the chromosome arm number (NF) was 96 and 98 in *G. gobio* and *R. belingi*, respectively. Moreover, small interspecific differences concerning the chromosomal morphology were observed. Major 28S rDNA sites were mapped to the short (*p)* arms of two submetacentric chromosomes with a similar size in *G. gobio*, whereas in *R. belingi*, FISH signals were observed from the short and the long (*q*) arms of two different-sized submetacentric chromosomes. Minor 5S rDNA sites were found on the *p* arms of eight submetacentric chromosomes of different sizes in *G. gobio* and on five submetacentric chromosomes in *R. belingi*. Unique chromosomal co-locations of major and minor rDNA sites were observed in the karyotypes of both species. In the chromosomes of both species, telomeric DNA sequences were typically located at the ends of all chromosomes. The number of chromosome arms, as well as the number and location of 45S and 5S rDNAs, and the number of unexpected co-located major and minor ribosomal genes may be useful cytotaxonomic species-specific markers.

Key words: cytotaxonomic marker, FISH, karyotype, rDNA synteny location, telomeres.

*Lech Kirtiklis✉, Marta Gwiazda-Orzoł, Alicja Boroń, Department of Zoology, Faculty of Biology and Biotechnology, University of Warmia and Mazury, Olsztyn, Poland.*
*E-mail: leo@uwm.edu.pl (L.K.); alibo@uwm.edu.pl (A.B.)*
*Konrad Ocalewicz, Department of Marine Biology and Biotechnology, Faculty of Oceanography and Geography, University of Gdańsk, Gdynia, Poland.*
*E-mail: konrad.ocalewicz@ug.edu.pl*
*Michał Nowak, Department of Ichthyobiology and Fisheries, University of Agriculture in Kraków, Kraków, Poland; 4-Mazanów Fish Farm, Mazanów, Poland.*
*E-mail: mikhael.nowak@gmail.com*
*Marta Gwiazda-Orzoł, Department of Gamete and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Science, Olsztyn, Poland.*

*E-mail: m.gwiazda-orzol@pan.olsztyn.pl*

Gudgeons (Gobionidae) are small-bodied bottom-dwelling fishes inhabiting the freshwaters of Eurasia (Froese & Pauly 2024). For a long time, they were recognised as a subfamily (Gobioninae) within the Cyprinidae family. Recently, they have been recognised as a separate family Gobionidae (Stout *et al.* 2016; Tan & Armbruster 2018), which is a very diverse fish taxon consisting of 29 genera and 217 species (Froese & Pauly 2024). However, the taxonomic status of gudgeons and some genetic relationships

among the species within the family are still unclear and need to be revised (Friedrich *et al.* 2018; Karakus 2021; Martynova & Vasil'eva 2021). The high degree of morphological similarity among gudgeons and the limited availability of efficient species-specific molecular markers has significantly impeded their discrimination.

The 'whitefin gudgeons' are a group of closely related species that have been historically classified as subspecies of a catch-in-all species, *Romanogobio albipinnatus* (Lukasch, 1933) (Naseka *et al.* 1999; Naseka 2001), of a usually allopatric distribution. *R. albipinnatus* sensu stricto occurs in the Volga River drainage, with *Romanogobio tanaiticus* (Naseka, 2001) in the Don River drainage, *Romanogobio vladykovi* (Fang, 1943) in the Danube River drainage, *Romanogobio ciscaucasicus* (Berg, 1932) in the Kuma River, and *Romanogobio parvus* (Naseka & Freyhof, 2004) occurring in the Kuban River. The last species, *Romanogobio belingi* (Slastenenko, 1934), has the largest distribution area, ranging from the Dnieper and Dniester Rivers in the east to the Elbe River in the west. The northern slopes of the Carpathians present a contact zone between *R. belingi* and *R. vladykovi* (Kottelat & Freyhof 2007). However, the whitefin gudgeons of the Vistula and Odra Rivers are commonly referred as *R. belingi* and the so-called 'Northern whitefin gudgeon' (Kottelat & Freyhof 2007). In turn, the European geographical range of the gudgeon *Gobio gobio* (Linnaeus, 1758) includes the Atlantic Ocean, North and Baltic Sea basins, ranging from Loire drainage eastward, eastern Great Britain, Rhône and Volga drainages, to the upper Danube and middle and upper Dniester and Dnieper drainages (Kottelat & Freyhof 2007). It also occurs in Finland, and this species has been introduced to the waters of eastern and northern Italy, Ireland, Wales and Scotland (Kottelat & Freyhof 2007). However, it is possible that a number of divergent phylogenetic lineages of gudgeons have become mixed within these geographic regions (Zangl *et al.* 2020; Takács *et al.* 2021, 2022).

In the past two decades, a noticeable range expansion of *R. belingi* has been observed (Naseka *et al.* 1999; Naseka 2001; Kottelat & Freyhof 2007) and both species were observed in the geographical range occupied so far by *G. gobio* (Nowak *et al.* 2013). As both species occur together in the same watersheds (potential hybrid zones), species-specific genetic markers should be provided for the efficient identification of *R. belingi* and *G. gobio*. Cytogenetic data including chromosome and chromosome arm numbers, chromosome banding patterns and the localisation of particular DNA sequences on the chromosomes have large taxonomic importance and may be used for both species and hybrid identification (Stace 2000). Variations in the chromosome numbers and the morphology and chromosomal distribution of the major 45S and minor 5S ribosomal genes observed sometimes in closely related cyprinid fish species (Gornung 2013; Rebordinos *et al.* 2013; Sochorová *et al.* 2018; Khensuwan *et al.* 2023) may facilitate the development of such markers.

In the cytogenetic literature, information on species from the genera *Gobio* and *Romanogobio* is relatively scarce and is usually limited to basic karyotype parameters, such as the chromosome number and the number of chromosomal arms based on Giemsa staining (Raicu *et al.* 1973; Klinkhardt *et al.* 1995; Vasil'eva *et al.* 2004, 2005; Arai 2011). Only in the cases of *G. gobio*, *G. fahrettini*, *G. gymnostethus*, *G. insuyanus* is some other chromosomal data known: the number and localisation of the nucleolus organiser regions (NORs) and the distribution of AT-rich regions in *G. gobio* (Kirtiklis *et al.* 2010), as well as and the number and localisation of NORs and the distribution of constitutive heterochromatin in the three remaining species (Karakus 2021).

Taking into account the fact that the current knowledge about gudgeons' chromosomes is quite limited and concerns only the basic parameters of their karyotype, two species of gudgeon – *G. gobio* and the Northern whitefin gudgeon *R. belingi* – were cytogenetically studied using conventional Giemsa staining and, for the first time, fluorescence *in situ* hybridisation (FISH) with 28S rDNA, 5S rDNA and the telomeric sequence as probes.

## Material and methods

### Sampling

A cytogenetic analysis was performed on nineteen specimens (six females, seven males and six individuals of an unidentified sex) of *G. gobio*, and nine specimens (two females, three males and four individuals of an unidentified sex) of *R. belingi* that originated from the Vistula River and were caught in the vicinity of the Kraków, between Łączany and Tyniec Villages, Poland (49°59′07.2″N 19°41′18.0″E). All individuals of *R. belingi* (formerly referred to as *R. albipinnatus*) were captured in accordance with the permission issued by the General Director of En-

vironmental Protection: DOP-OZ.6401.10.10.2013. ls. All the manipulations and the experimental procedures were provided according to the Positive Opinion No. 20/2013 of the Local Ethical Commission from The University of Warmia and Mazury in Olsztyn, Poland. The taxonomic status of all individuals used in the study was confirmed based on their external morphological features, according to Kottelat & Freyhof (2007). Voucher specimens were preserved with 96% ethanol and deposited in the fish collection of the Department of Ichthyobiology and Fisheries, University of Agriculture in Kraków, Poland.

### Chromosome slides and karyotype preparation

Metaphase chromosome slides were made from the cephalic kidney by the standard air-drying technique, according to Ráb & Roth (1988) with some modifications. A conventional 5% Giemsa solution was used for the karyotype determination. Only high-quality metaphase spreads (countable and not overlapping chromosomes) were analysed. The chromosomes were classified according to Levan *et al*. (1964). Subtelocentric and acrocentric chromosomes were considered to be uni-armed elements. At least 20 metaphase spreads from each individual were analysed for each technique.

### rDNAs and telomeric repeats mapping

The FISH protocol with 150 ng of each 28S and 5S rDNA sequences as probes (Fujiwara *et al*. 1998) was performed for mapping both rDNA sites on the chromosomes. rDNA probes were obtained via PCR with following two sets of primers: F8-28S: 5′-TGAAATACCACTACTCTTATCGTT-3′ and R8-28S: 5′-GGATTCTGACTTAGAGGCGTTCAG-3′ for 28S rDNA sites (Zardoya & Meyer 1996), with 5S-1: 5′-TACGCCCGATCTCGTCCGATC-3′ and 5S-2: 5′-CAGGCTGGTATG GCCGTAAGC-3′ for 5S rDNA sites (Pendas *et al*. 1994). The PCR products were confirmed by direct sequencing using the Sanger method with PCR primers. The 28S and 5S rDNA probes were labelled with FITC and Rhodamine (respectively) by nick-translation (Roche, Germany). Hybridisation of 150 ng of the rDNA probe (per slide) was performed with RNase-pretreated (37°C, 60 min) and formamide-denaturated (70%, 72°C, 1-2 min) chromosome slides. Subsequent to post-hybridisation washing (50% formamide) at a moderate stringency (37°C, 20 min), the chromosome slides were counterstained with 30 µl Vectashield with DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories, USA).

Telomeric DNA sequences were detected in the chromosomes using a Telomere PNA (peptide nucleic acid) FISH Kit/FITC (DAKO, Denmark), according to the manufacturer's protocol with some modifications (9). In brief, microscopic slides with the metaphase spreads were washed with TBS (Tris-buffered saline, pH 7.5), immersed in 3.7% formaldehyde, treated with Proteinase K and then washed again with TBS. Afterwards, the preparations were dehydrated through a cold ethanol series and air-dried. The chromosomal DNA was denatured at 85 °C for 5 min under the cover slip in the presence of the PNA probe. The hybridisation reaction took place in darkness at room temperature for 90 min. After hybridisation, the coverslips were gently removed and slides were washed in the Wash Solution (DAKO), dehydrated through a series of cold ethanol washes (70%, 85%, 100%) and air-dried. Fifteen minutes before the microscopic analysis, the cells were counterstained with DAPI in Vectashield antifade solution (Vector Laboratories, USA).

Hybridisation signals only on high-quality chromosome spreads were observed under a Nikon Eclipse 90i fluorescence microscope (Nikon, Japan) using a filter set for a multichannel colour FISH, and the images were captured with ProgRes MFcool camera (Jenoptic, Germany). The FISH images were processed using Lucia software ver. 2.0 (Laboratory Imaging, Czech Republic). CorelDRAW Graphics Suite 11 (Corel Corporation, Canada) was used for the post-processing elaboration of the figures.

## Results

Both species exhibited the same chromosome number 2n=50, whereas the chromosome arm numbers (Fundamental Number) were NF=96 and 98, in *G. gobio* and *R. belingi*, respectively. The karyotype of *G. gobio* was composed of 22 metacentric, 24 submetacentric, 2 subtelocetric and 2 acrocentric chromosomes; while 24 metacentric, 24 submetacentric and 2 acrocentric chromosomes were observed in *R. belingi* (Figs 1a, b).

Major rDNA sites were mapped to the terminal or interstitial positions on the gudgeons' short (*p*) arm of two submetacentric chromosomes showing a similar size (Figs 2 a-c), while in the Northern whitefin gudgeon, 28S rRNA genes were mapped in the interstitial position on *p* and the long (*q*) arms of two submetacentric chromosomes that exhibited size differences (Figs 2 d-f). In the case of the 5S rDNA sites

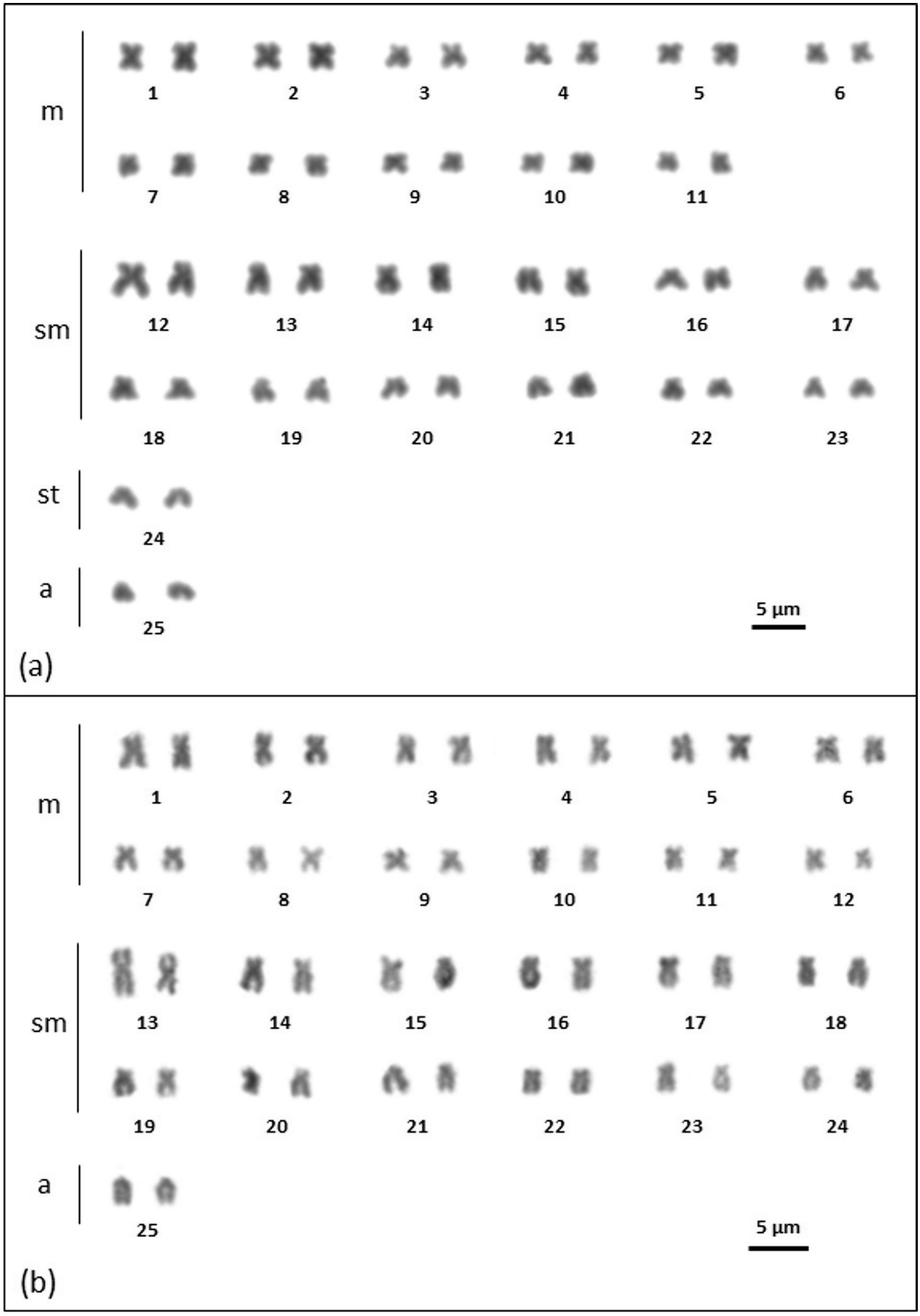


Fig. 1. Karyotypes of the gudgeon *Gobio gobio* (a) and the Northern whitefin gudgeon *Romanogobio belingi* (b) after Giemsa staining; m – metacentric chromosomes, sm – submetacentric chromosomes, st – subtelocentric chromosomes, a – acrocentric chromosomes.

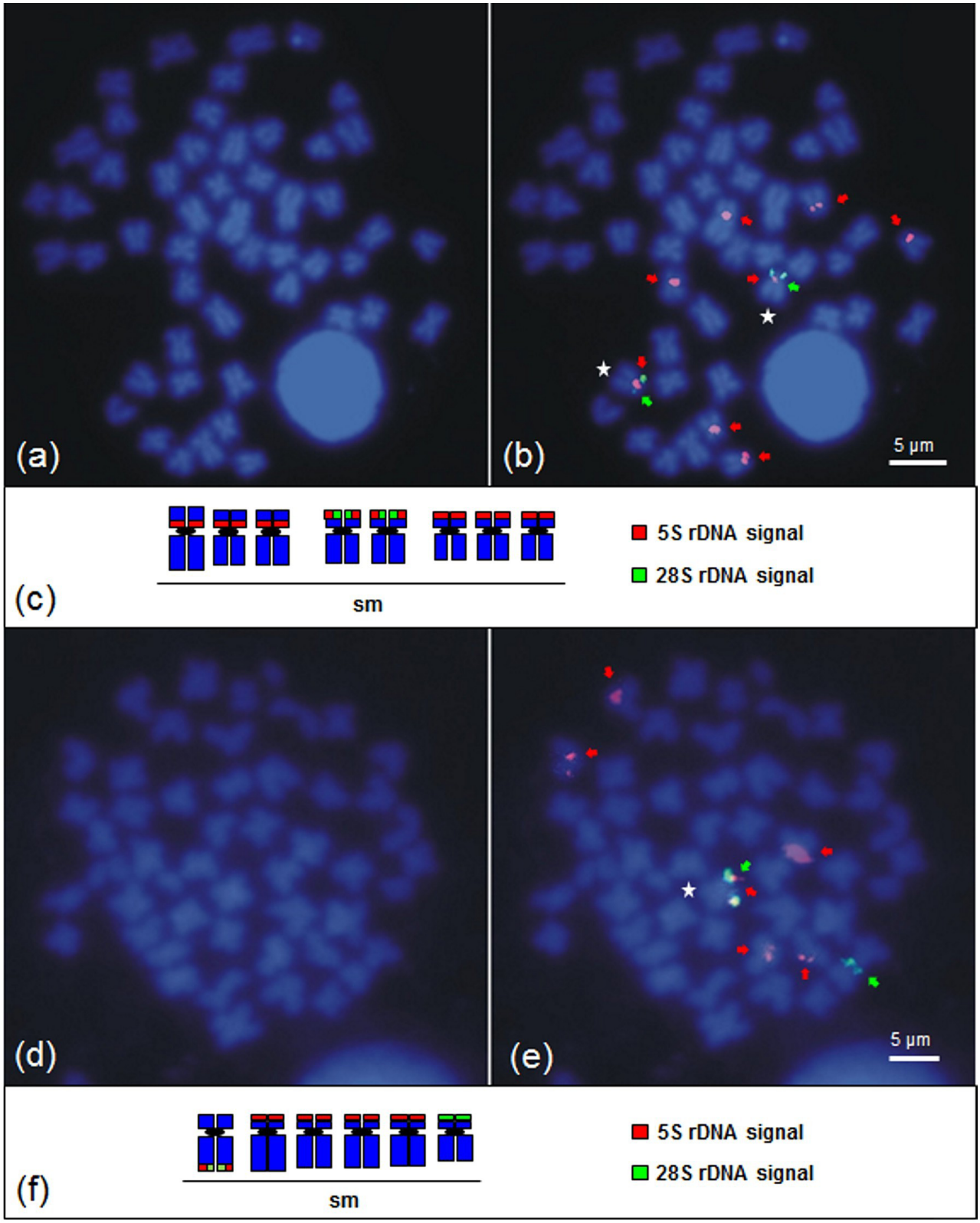


Fig. 2. Chromosomes of the gudgeon *Gobio gobio* after DAPI (a) and FISH with 28S rDNA and 5S rDNA as probes (b), and chromosomes of the Northern whitefin gudgeon *Romanogobio belingi* after DAPI (d) and FISH with 28S rDNA and 5S rDNA as probes (e), schematic representation of rDNA bearing-chromosomes of the gudgeon *Gobio gobio* (c) and the Northern whitefin gudgeon *Romanogobio belingi* (f); green and red arrows indicate hybridisation sites with 28S and 5S rDNA as probes, respectively; white asterisks indicate the co-location of major and minor rDNAs; sm – submetacentric chromosomes.

(minor rDNAs), the hybridisation pattern of *G. gobio* was observed on eight submetacentric chromosomes that differed in size (in the pericentromeric position on the *p* arms of three chromosomes and in the interstitial position on the *p* arms of five other chromosomes) (Figs 2 a-c). In turn, 5S rDNA sequ-

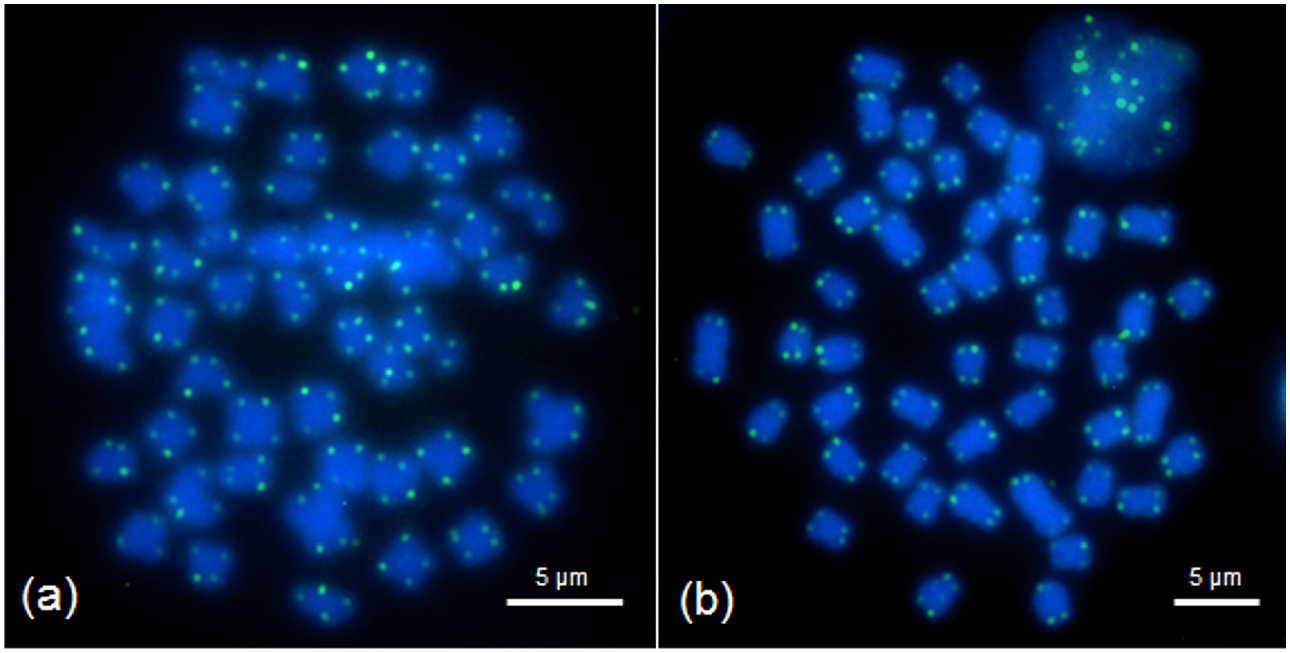


Fig. 3. Chromosomes of the gudgeon *Gobio gobio* (a) and the Northern whitefin gudgeon *Romanogobio belingi* (b) after PNA-FISH with a telomeric probe.

ences in the Northern whitefin gudgeon were found in the interstitial position on the *q* arms of one chromosome and in the interstitial position on the *p* arms of four other chromosomes (Figs 3 d-f). The co-location of major and minor rDNA sites was observed on the *p* arms of two submetacentric chromosomes in the gudgeons, and on the *q* arms of one submetacentric chromosome in the Northern whitefin gudgeon (Figs 3 a-f). Fluorescent signals after the PNA FISH with a telomeric probe were observed exclusively on the terminal regions of all the chromosomes in both species studied.

No sex-related differences were observed.

## Discussion

The diploid chromosome number of fish species from the order Cypriniformes is most often 2n=48 or 2n=50 (Singh *et al*. 2009; Arai 2011; Sember *et al*. 2015; Buasriyot *et al*. 2024). Diploid cells of *Gobio* and *Romanogobio* species, except for *G. uranoscopus* (2n=52) (Ráb & Collares-Pereira 1995), are characterised by 50 chromosomes. which indicates a high degree of conservatism in this feature among gudgeons. Individuals of both the *G. gobio* and *R. belingi* studied in this work exhibited the same chromosome number, but the morphology of the chromosomes showed interspecies variation concerning slight differences within the submeta- and subtelocentric chromosomes that affect the karyotype composition (different numbers of the chromosome arms, NF) (Table 1). The abovementioned karyotype differences among other gudgeon species of the genera *Gobio* and *Romanogobio* have been reported by other authors (for a review, see Klinkhardt *et al*. 1995; Arai 2011; Karakus 2021). Referring to the ancestral karyotype of Teleostei with 48 uni-armed chromosomes (NF=48) (Ohno 1970; Parey *et al*. 2022), it can be assumed that the karyotype of these gudgeons when compared to the ancestral one was presumably subjected to rearrangements regarding the centromeric positions that included pericentric inversions, fission that may be followed by the reposition of centromeres (Rocchi *et al*. 2012), amplification/deletion of the regions built with repetitive DNAs and some interchromosomal translocations.

The location of the 45S rRNA genes on one pair of chromosomes is considered to be an ancestral feature of the karyotype, which is observed in many fish species (Gornung 2013; Sochorová *et al*. 2018). This type of 45S rRNA gene distribution was described (based on data from Ag-NOR and $CMA_3$) for *G. gobio*, where the sequences encoding these genes are located on the *p* arms of one pair of the submetacentric chromosomes (Kirtiklis *et al*. 2005; present paper). This pattern suggests a high stability of the gudgeon chromosomal regions composed of major rDNA sequences. The location of major ribosomal

Table 1

Summary of the cytogenetic data for the gudgeon *Gobio gobio* and Northern whitefin gudgeon *Romanogobio belingi*; m – metacentric chromosomes, sm – submetacentric chromosomes, st – subtelocentric chromosomes, a – acrocentric chromosomes, TS – telomeric sites

| | Diploid chromosome number (2n) | Chromosome arm number (NF) | Karyotype formulae | Ag-NOR | $CMA_3$ | Major rDNA (28S) | Minor rDNA (5S) | Telomeric DNA sequences | References |
|---|---|---|---|---|---|---|---|---|---|
| *Gobio gobio* | 50 | 96 | 22m+24sm+2st+2a | – | – | – | – | – | Raicu et al. 1973 |
| | 50 | 88 | 38m/sm + 12st/a | – | – | – | – | – | Sofradžija & Berberović 1975 |
| | 50 | 98 | 22m + 26sm/st +2a | – | – | – | – | – | Hafez et al. 1978a, 1978b |
| | 50 | 96 | 20m+26sm+2st+2a | – | – | – | – | – | Vasil'eva at al. 2004 |
| | 50 | 98 | 24m + 24sm + 2st/a | – | – | – | – | – | Vasil'eva at al. 2004 |
| | 50 | 98 | 22m + 26sm/st + 2a | 2sm | 2sm | – | – | – | Kirtiklis et al. 2005 |
| | 50 | 96 | 22m+24sm+2st+2a | – | – | 2sm | 8sm | TS | present paper |
| *Romanogobio belingi* (formerly *Gobio albipinnatus vladykovi*) | 50 | 98 | 28m + 20sm +2a | – | – | – | – | – | Raicu et al. 1973 |
| | 50 | 98 | 24m + 24sm + 2a | – | – | 2sm | 8sm | TS | present paper |

genes on the *p* and *q* arms of two *R. belingi* submetacentric chromosomes that differed in size suggests a translocation of the NOR-related rDNAs.

In both the *G. gobio* and *R. belingi* analysed in this study, a multichromosomal distribution of the 5S rRNA gene was described. The 5S rRNA gene location on one pair of chromosomes is also considered to be an ancestral condition (Martins & Wasko 2004; Sochorová *et al*. 2018). Such a pattern of distribution of the minor ribosomal gene has been described in various fish species, e.g. in zebrafish *Danio rerio* (Phillips & Reed 2000) as well as burbot *Lota lota* (Kirtiklis *et al*. 2017). However, unlike mammals, the location of the 5S rDNA in fish can also be multichromosomal, as has been observed, e.g. in the examined gudgeons, Nile tilapia *Oreochromis niloticus* (Martins *et al*. 2002), Northern pike *Esox lucius* (Symonová *et al*. 2017), European bitterling *Rhodeus amarus* (Kirtiklis *et al*. 2014), golden zebra loach *Sinibotia pulchra* (Sember *et al*. 2018) and crucian carp *C. carassius* (Szabelska *et al*. 2024). This redistribution of the minor rDNAs (as well as major rDNAs) may be caused by an ectopic (non-homologous) recombination, the extrachromosomal circular rDNA or could be driven by the transposable elements if integrated with rDNA arrays (TEs may carry rDNA fragments) (Pérez-González & Eickbush 2001; Nguyen *et al*. 2010; Mansisidor *et al*. 2018).

The occurrence of major and minor ribosomal genes on separate chromosomes is present in most of the fish species studied to date and it is considered to be a plesiomorphic feature. The explanation for this may be related to the different transcription sites of specific rRNA genes in the eukaryotic cell and to the different enzymes catalysing this process (nucleolar enzyme RNA polymerase I in the case of 45S, with non-nucleolar RNA polymerase III in the case of 5S) (Martins & Wasko 2004). However, a rather infrequent chromosomal co-location of the abovementioned genes (syntenic location) was also observed and has been also described in cyprinid fishes (Inafuku *et al*. 2000; Gromicho *et al*. 2006; Kirtiklis *et al*. 2010; Rossi *et al*. 2012; Grabowska *et al*. 2020; Buasriyot *et al*. 2024). In the case of the *G. gobio* analysed in this study, such a co-location was observed within the *p* arms of two submetacentric chromosomes, while in the *R. belingi* it was observed on the *q* arms of one submetacentric chromosome. Previously, differences in the number of chromosomes with co-localised 45S and 5S rDNA sites have been de-

scribed in fish of the genus *Leuciscus* (*L. leuciscus*, *L. idus* and *L. cephalus*) (Kirtiklis *et al*. 2010), where this characteristic was unique and allowed the species to be distinguished. Similarly, within both of the gudgeon species analysed in this study, it is possible to distinguish them based on the number and location of the 28S and 5S rDNA that show a species-specific pattern. The previously-mentioned mechanisms responsible for the redistribution of major and minor rDNAs may also be related to their co-localisation (Pérez-González & Eickbush 2001; Nguyen *et al*. 2010; Mansisidor *et al*. 2018).

Chromosome rearrangements are preceded by doubled strand breaks (DSBs) that, if occurring within the telomeric region(s), may result in the interstitial location of the telomeric sequences (ITSs) observed at the fusion sites. ITSs are observed on the fish chromosomes that experienced fusion(s), inversion(s) (Ocalewicz *et al*. 2013) and a redistribution of the genomic regions built with 45 S rDNA (Reed & Phillips 1995; Pomianowski *et al*. 2012). Despite the gudgeon karyotype being shaped by at least some of the abovementioned rearrangements, no ITSs on the chromosomes of either *G. gobio* or *R. belingi* were observed. The lack of internally located telomeric sites may suggest that the DSBs were out of the telomeric regions prior to the rearrangements, or the DSBs were within the telomeres and ITSs had formed, however such sites were too small to be microscopically observed or degenerated and were not able to hybridise with the FISH probe (Bolzán 2012).

In conclusion, the observed differences in the karyotypes of the studied fish species suggests a higher rate of chromosomal rearrangements in the *R. belingi* than in the *G. gobio* genomes. Moreover, the observed number of chromosome arms, as well as the number and location of the 45S and 5S rDNAs, and the number of co-located major and minor ribosomal genes seem to be useful chromosomal species-specific taxonomic markers for gudgeons.

## Author contributions

Research concept and design: L.K., K.O., M.N.; Collection and/or assembly of data: L.K, M.N; Data analysis and interpretation: L.K, K.O., M.N., A.B., M.G-O.; Writing the article: L.K, K.O., M.N.; Critical re-vision of the article: L.K, K.O., M.N., A.B.; Final approval of the article: L.K., K.O., M.N.

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## Conflict of Interest

The authors declare no conflict of interest.

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