Cytogenetic and Genome Research

Research Article

Cytogenet Genome Res 2024;164:243–256
DOI: 10.1159/000542804

Received: August 28, 2024
Accepted: November 22, 2024
Published online: December 2, 2024

A Detailed Karyological Investigation of three Endemic *Cobitis* Linnaeus, 1758 Species (Teleostei, Cobitidae) in Anatolia, Türkiye

Sevgi Unal Karakus[a] Muhammet Gaffaroğlu[b] Muradiye Karasu Ayata[c] Martin Knytl[d, e]

[a]Department of Molecular Biology and Genetics, Faculty of Science, Bartin University, Bartin, Turkey; [b]Department of Molecular Biology and Genetics, Faculty of Science and Arts, Kirsehir Ahi Evran University, Kirsehir, Turkey; [c]Department of Nutrition and Dietetics, Faculty of Health Sciences, Kirsehir Ahi Evran University, Kirsehir, Turkey; [d]Department of Cell Biology, Faculty of Science, Charles University, Prague, Czech Republic; [e]Department of Biology, McMaster University, Hamilton, ON, Canada

Keywords
Fish · C-banding · Ag-NORs · Spined loach · Centromeric index

Abstract

Introduction: Comparative cytogenetics is a vital approach for diagnosing chromosome abnormalities and identifying species-specific patterns. In this study, chromosomal analysis of three Anatolian endemic *Cobitis* species was performed: *Cobitis bilseli*, *C. fahireae*, and *C. turcica*. ***Methods:*** Conventional cytogenetic techniques such as Giemsa staining, C-banding, and Ag-NOR staining were applied, followed by measurements of chromosome arm lengths including analysis of the measured data. ***Results:*** The diploid chromosome number, $2n = 50$, was determined for all three species. The karyotype formulas were as follows: four pairs of metacentric, 5 pairs of submetacentric, and 16 pairs of subtelo-telocentric chromosomes in *C. bilseli*; 11 pairs of metacentric, 7 pairs of submetacentric, and 7 pairs of subtelo-telocentric chromosomes in *C. fahireae*; and 4 pairs of metacentric, 4 pairs of submetacentric, and 17 pairs of subtelo-telocentric chromosomes in *C. turcica*. Dark C-bands were observed on the pericentromeres of nearly all chromosomes in *C. bilseli* and *C. turcica*, whereas light C-bands appeared on the pericentromeres of some chromosomes in *C. fahireae*. Silver-stained metaphases revealed signals on the short arm of a submetacentric chromosome pair in *C. fahireae* (each homologous chromosome carries one signal), while in *C. bilseli* and *C. turcica*, Ag-NOR signals were detected on the long arm of a single metacentric chromosome (only one homologous chromosome carries the signal, and the signal-carrying chromosome is the largest chromosome in the karyotype). ***Conclusion:*** This study provides new cytogenetic data consistent with the phylogenetic distances between the studied species, indicating that pericentric inversions and/or translocations govern the formation of *Cobitis* karyotypes.

Introduction

The spined loaches Cobitidae are a species-rich group of small freshwater fishes [1]. Representatives of the genus *Cobitis* Linnaeus, 1758 show very little morphological variation, making it difficult to distinguish some species from others [2]. A comparative experimental

karger@karger.com
www.karger.com/cgr

Correspondence to:
Sevgi Unal Karakus, sunal@bartin.edu.tr
Martin Knytl, martin.knytl@natur.cuni.cz

taxonomy approach has been used in *Cobitis* loaches, applying genetic and molecular methods for identification of species [3]. Chromosome studies have shown that most *Cobitis* species have a diploid chromosome number of $2n = 50$ (with the only exception being *Cobitis taenia* $2n = 48$ [4]) but highly diversified karyotypes [3]. A karyotype is an arrangement of chromosomes into individual morphological categories based on the centromeric index (*i*). Morphological categories are generally divided into meta-, submeta-, subtelo-, acro-, and telocentric [5]. Differences that occurred during the karyotype evolution in Cobitidae play an important role in speciation [3]. Cytogenetics may provide a powerful tool in the complex systematics and taxonomy of spined loaches of the genus *Cobitis* [6]. In addition to the fish genus *Cobitis*, karyotype descriptions can reveal various chromosome rearrangements, such as pericentric or paracentric inversions, decay of an ancestral centromere followed by the establishment of a new one, fissions, and fusions. These rearrangements may be significant drivers of karyotype evolution [7–9]. The presence of chromosome rearrangements can serve as an important indicator of diversity between species as well as inter-population diversity within a species, even when the morphological approach fails [10–13].

The species diversity of the genus *Cobitis* in the Middle East was reviewed by Freyhof et al. [2], recognizing 25 species in Anatolia, Türkiye. Additionally, *C. indus* was recently recognized from Anatolia by Eagderi et al. [14]. Out of these 26 *Cobitis* species, most are endemic to Anatolia, Türkiye [2, 14]. Among these loaches, *Cobitis bilseli* Battalgil, 1942, is endemic to the Lake Beyşehir basin in Central Anatolia. *Cobitis fahireae* Erkakan, Atalay-Ekmekçi, and Nalbant, 1998, occurs as an endemic species in the eastern Aegean Sea basin in west Anatolia, while *Cobitis turcica* Hanko, 1924, is endemic to the wider Lake Tuz basin in Central Anatolia [2].

Contrary to the species richness of Cobitidae in Anatolia, karyological studies have been reported in only *C. elazigensis* [15], *C. phrygica*, *C. simplicispina* [16], and *C. bilseli* [17] from Türkiye. However, no chromosomal study has been performed on *C. fahireae* and *C. turcica*. The aim of this study was to process chromosomal measurement information and use conventional staining methods to provide detailed resolution of the karyotypes of three endemic *Cobitis* species (*C. bilseli*, *C. fahireae*, and *C. turcica*) from three different localities in Anatolia, Türkiye. Based on the measurement of chromosome arms and the number of cytogenetic markers, we discuss the possibilities of karyotype evolution.

Methods

One male of *C. bilseli* was caught from Sarıöz Stream, Beyşehir Lake, Konya prov., Türkiye (38°42′N, 31°41′E). Two females and five males of *C. fahireae* were captured in Gediz River, Salihli, Manisa prov., Türkiye (38°31′N, 28°08′E). Two females of *C. turcica* were caught from Selime Village, Melendiz Creek, Aksaray prov., Türkiye (38°18′N, 34°15′E). The collection locations of all three species studied are depicted in Figure 1a. The live specimens were carried to the laboratory. The process was approved by the Local Animal Ethics Committee of Türkiye (Protocol Number: 68429034/15/17). Species were identified based on morphological characteristics according to Freyhof et al. [2]. Chromosome slides were prepared according to the method of Bertollo et al. [18]. Each fish was injected intra-peritoneally with a colchicine solution (0.1%; 1 mL/100 g body weight). The fish were kept in a well-aerated aquarium and after 2 h, the cephalic kidney tissue was dissected out and placed in a hypotonic 0.56% KCl solution. After this step, the cellular suspension was centrifuged at 1,200 rpm for 10 min. The hypotonic solution was removed and the pellet was suspended and washed three times in methanol:glacial acetic acid (3:1). After centrifugation at 1,200 rpm for 10 min, the drops of cellular suspension were put on a clean slide. The slides were air-dried. Some of them were stained with 10% Giemsa solution in distilled water for 20 min. At least 10 slides were prepared from each specimen. Constitutive heterochromatin regions and Ag-NORs were detected by the following methods: Sumner [19] and Howel and Black [20], respectively.

Metaphases were photographed under a Leica DM 3000 microscope and photographs were taken using AKAS software. Both arms of each chromatid were measured in pixels using ImageJ, v 1.54f [21]. The short (p) and long (q) arm lengths were quantified as described in Knytl and Fornaini [22]. To identify each chromosome, we analyzed chromosomal length (*l*), centromeric index (*i*), and q/p arm ratio (r_2) [5]; for formulas, see Knytl et al. [23]. Data were analyzed in R software for statistical computing, R 4.3.1 [24], using ggplot2 and ggpubr packages. A one-way analysis of variance was performed to compare the mean values of *l* and *i* between the karyotypes of all three species. The analysis aimed to test the null hypothesis, which states that there is no difference in the means, against the alternative hypothesis, which suggests that the mean values of individual orthologous chromosomes significantly differ from those

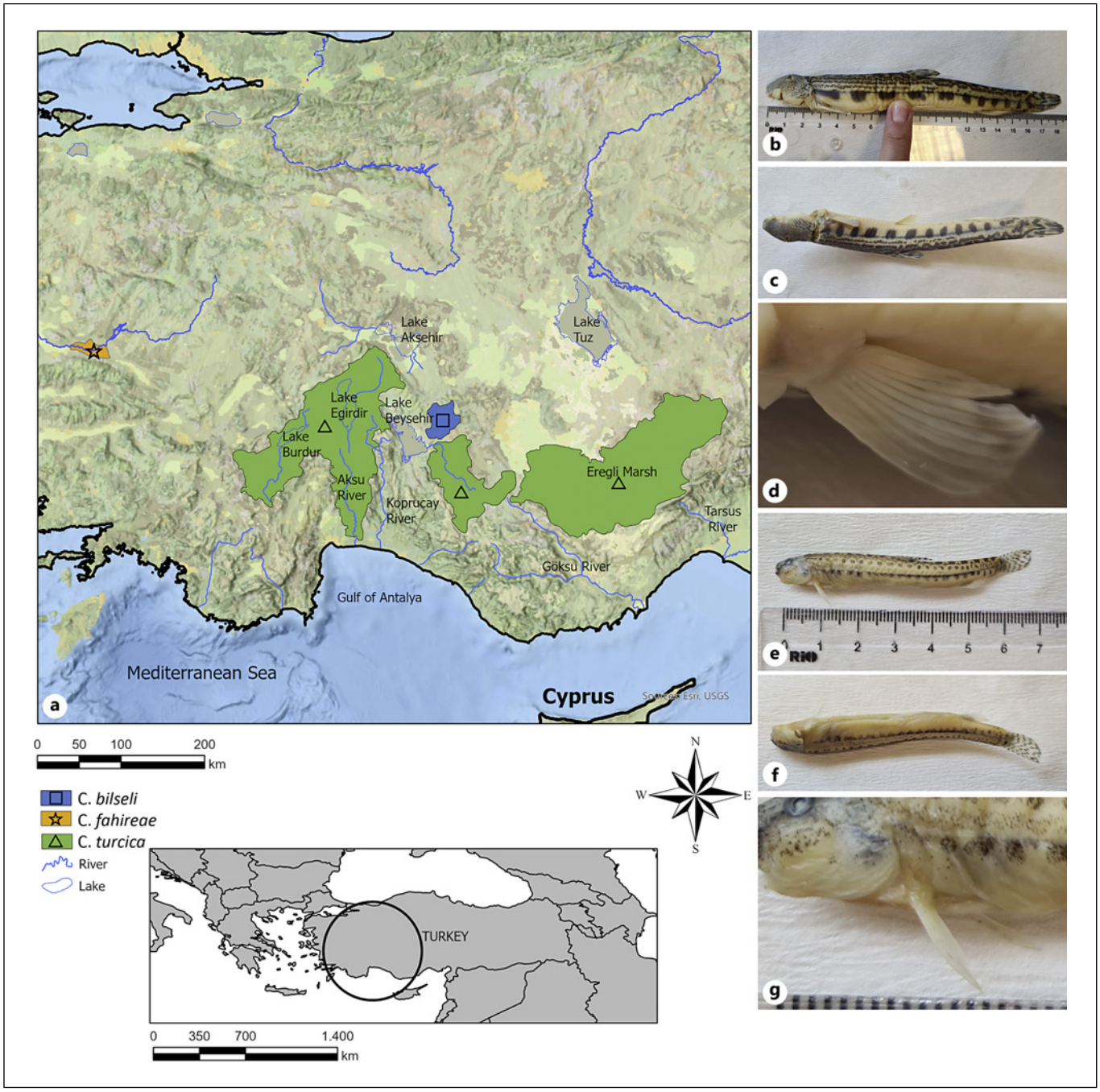
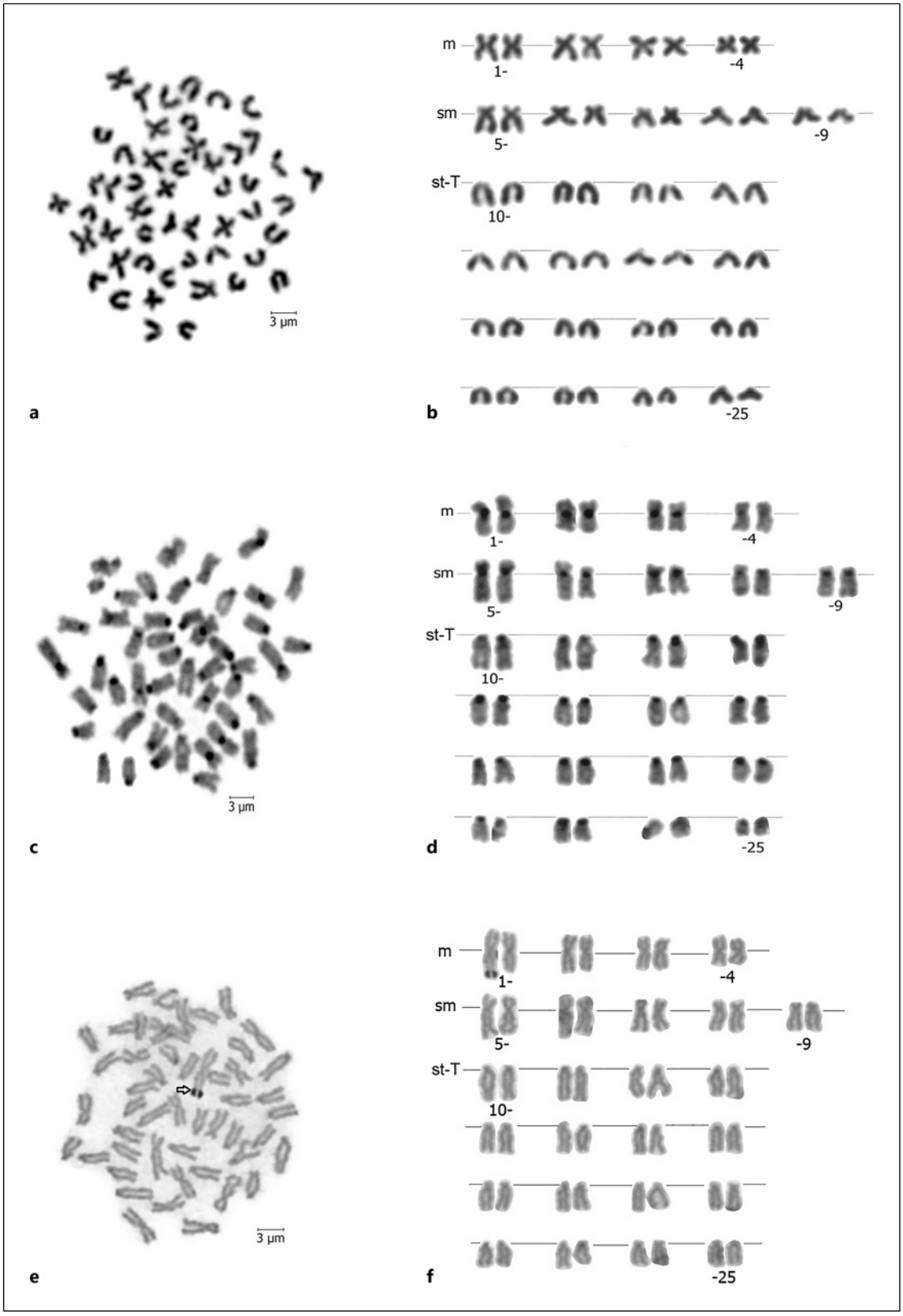


Fig. 1. a Geographical map of collection sites of Cobitidae specimens. Each location of the species has a different color and mark, i.e., *C. bilseli* square in blue, *C. fahireae* star in orange, *C. turcica* triangle in green. **b**–**d** Male of *C. bilseli* with one lamina circularis. **e**–**g** Male of *C. fahireae* with one lamina circularis.

of the orthologous chromosomes in the other species. We used and modified R scripts developed by Knytl and Fornaini [22]. Chromosome pairs were arranged according to their *l* and *i* values into individual morphological categories in the karyotypes. Chromosome categories and identical chromosome classification were taken from Levan et al. [5]. To determine the fundamental arm number (FN), meta- and submetacentrics were considered as biarmed and subtelo-, acro-, and telocentrics as uniarmed.



2

(For legend see next page.)

Results

The diploid chromosome number for each of the three *Cobitis* species was determined to be $2n = 50$ (Figs. 2a, 3a, 4a). The karyotypes consist of 4 pairs of metacentric, 5 pairs of submetacentric, and 16 pairs of subtelo-telocentric chromosomes in *C. bilseli* (Fig. 2b); 11 pairs of metacentric, 7 pairs of submetacentric, and 7 pairs of subtelo-telocentric chromosomes in *C. fahireae* (Fig. 3b); and 4 pairs of metacentric, 4 pairs of submetacentric, and 17 pairs of subtelo-telocentric chromosomes in *C. turcica* (Fig. 4b). FN was calculated as 72 in *C. bilseli*; as 86 in *C. fahireae*; and as 66 in *C. turcica*. No heteromorphic sex chromosomes were found in any of the three karyotypes. Dark C-bands were found on the centromeres of almost all chromosomes in *C. bilseli* (Fig. 2c, d) and *C. turcica* (Fig. 4c, d). Otherwise, slightly stained C-bands were observed on the centromeres of most chromosomes in *C. fahireae*, for instance, metacentric pairs 1–6, 8, 9, and 11 (Fig. 3c, d). The Ag-NOR signals were detected in the terminal region of the p arm of the 6th submetacentric chromosome pair in *C. fahireae* (Fig. 3e, f) and in the terminal region of the q arm of only one chromosome from the 1st metacentric pair in *C. bilseli* (Fig. 2e, f) and *C. turcica* (Fig. 4e, f). In the latter two species, the chromosome carrying the signal is the largest chromosome of the karyotype. Regarding the exceptional pattern of single-chromosome Ag-NOR signal, no polymorphisms in the number of Ag-NORs were observed within a species or an individual.

Both p and q arms of each chromatid were measured from 10, 9, and 7 metaphase spreads for *C. bilseli*, *C. fahireae*, and *C. turcica*, respectively. Chromosomes were identified based on the calculated median values of *l* and *i*. The *l* value was quantified as a percentage of the sum of *l* for all chromosomes to account for variation in resolution and pixel size of our images. Each individual chromosome was also assigned a chromosomal category based on the *i* value. If the *i* value was equal to or greater than 37.5, the chromosome was categorized to be metacentric (m). If the *i* value was equal to or higher than 25 and lower than 37.5, the chromosome was categorized as submetacentric (sm). If the *i* value was equal to or greater than 12.5 and lower than 25, the chromosome was categorized as subtelocentric (st). If the *i* value was higher than 0 and lower than 12.5, the chromosome was classified as acrocentric (a). If the *i* value was equal to 0, the chromosome was identified as telocentric sensu stricto (T), i.e., without the p arm (Table 1). The m, sm, and T categories were present in all three species. In addition, we identified one pair of st and one pair of a chromosomes in *C. bilseli*.

The extreme T category was represented by 14, 7, and 17 chromosomes (homologous pairs) of *C. bilseli*, *C. fahireae*, and *C. turcica*, respectively (i.e., 14, 7, and 17 points lie on the dashed vertical line of interval 0 in Fig. 5). The T chromosomes had a relatively wide range of *l* values in all three species (most of the chromosomes from 1.5 to 2.1%). Another extreme M category (metacentric *sensu stricto*, $i = 50$, *sensu*; Levan et al. [5]) was not present in any of the species studied, but chromosome 1 in *C. bilseli* and *C. fahireae* had the *i* value close to 50, i.e., 49.44 and 49.45, respectively (see two points close to the dashed vertical line of interval 50) (Fig. 5). Four chromosomes belonged to the m category in *C. bilseli* and *C. turcica*, with *l* values ranging from 2.14 and 2.22 to 2.46 and 2.38%, respectively. *Cobitis fahireae* possessed 11 m chromosomes with the *l* values ranging from 1.97 to 2.23%. Apart from the four m chromosomes in *C. bilseli* and *C. turcica*, the number of other categories differed, e.g., five, seven, and four sm chromosomes in *C. bilseli*, *C. fahireae*, and *C. turcica*, respectively. The st and a chromosomes were present solely in *C. bilseli*, each of these two categories being represented by one chromosome.

Another analysis we performed concerned the intrachromosomal variation of *l* and *i* values. The *l* and *i* values were assigned to each chromosome 1–25 and plotted (Fig. 6, 7). A measure of statistical dispersion, the interquartile range (Q_1–Q_3), was used to evaluate the extent of morphological divergence of each orthologous pair between *Cobitis* species. Each box depicts a group of measured *l* (Fig. 6) and *i* (Fig. 7) values. Chromosomes were ordered according to the morphological categories from metacentric chromosomes on the left side of plots to telocentric chromosomes on the right side of plots. If the *i* value was equal, chromosomes were ordered decreasingly according to the *l* value (T chromosomes with $i = 0$). Within each category, chromosomes were ordered decreasingly according to the *l* value from the largest to the shortest chromosome. For instance, the largest metacentric chromosome for all three species was

Fig. 2. Metaphases and karyotypes of *C. bilseli*. **a** Giemsa-stained metaphase. **b** Arranged karyotype of the Giemsa-stained metaphase. **c** C-banded metaphase. **d** Arranged karyotype of the C-banded metaphase. **e** Silver-stained metaphase (arrow indicates the Ag-NOR). **f** Arranged karyotype of the silver-stained metaphase. Scale bar = 3 μm. m, metacentric; sm, submetacentric; st-T, subtelo-telocentric chromosomes.

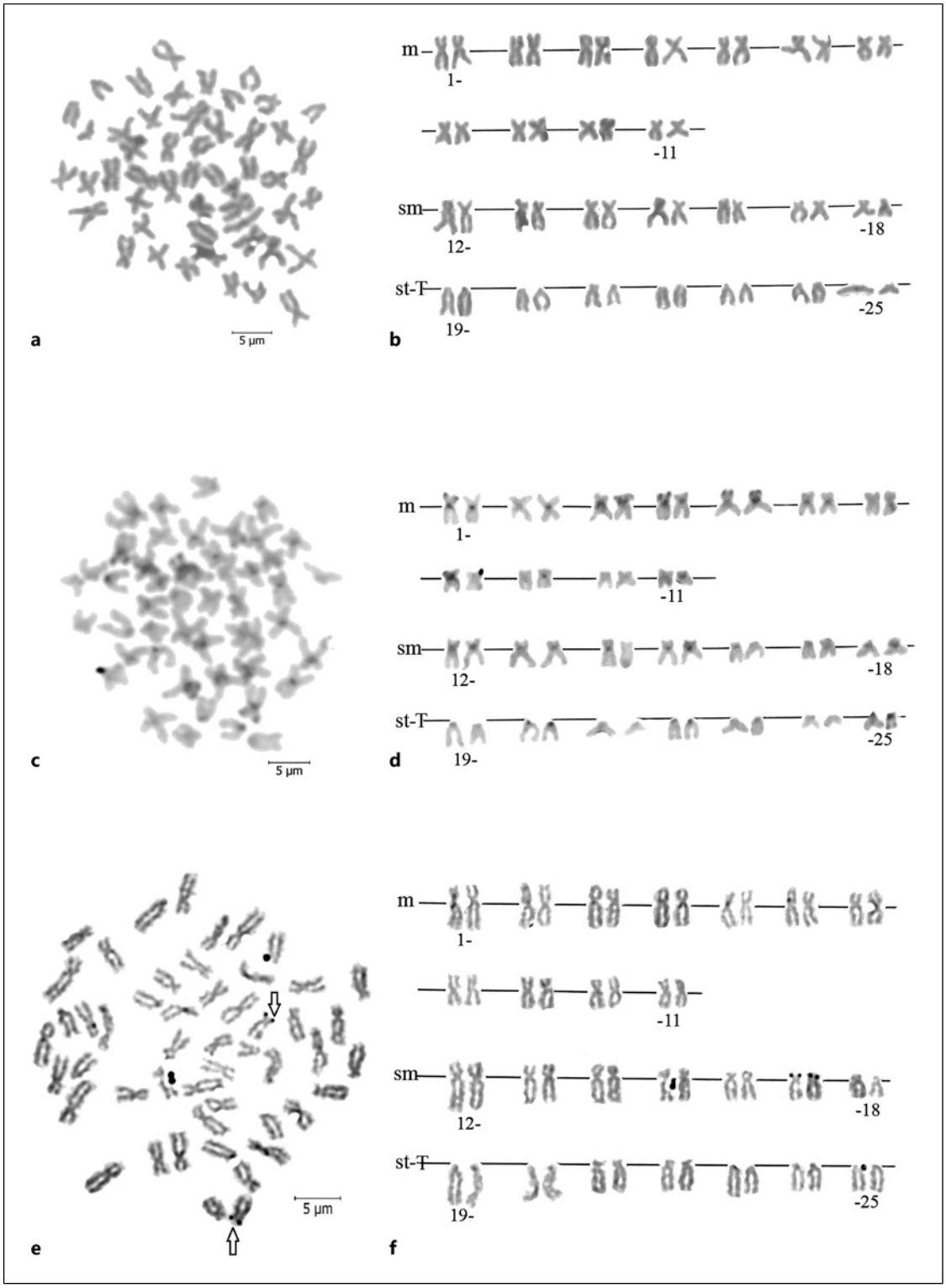


Fig. 3. Metaphases and karyotypes of *C. fahireae*. **a** Giemsa-stained metaphase. **b** Arranged karyotype of the Giemsa-stained metaphase. **c** C-banded metaphase. **d** Arranged karyotype of the C-banded metaphase. **e** Silver-stained metaphase (arrows indicate the Ag-NORs). **f** Arranged karyotype of the silver-stained metaphase. Scale bar = 5 µm. m, metacentric; sm, submetacentric; st-T, subtelo-telocentric chromosomes.

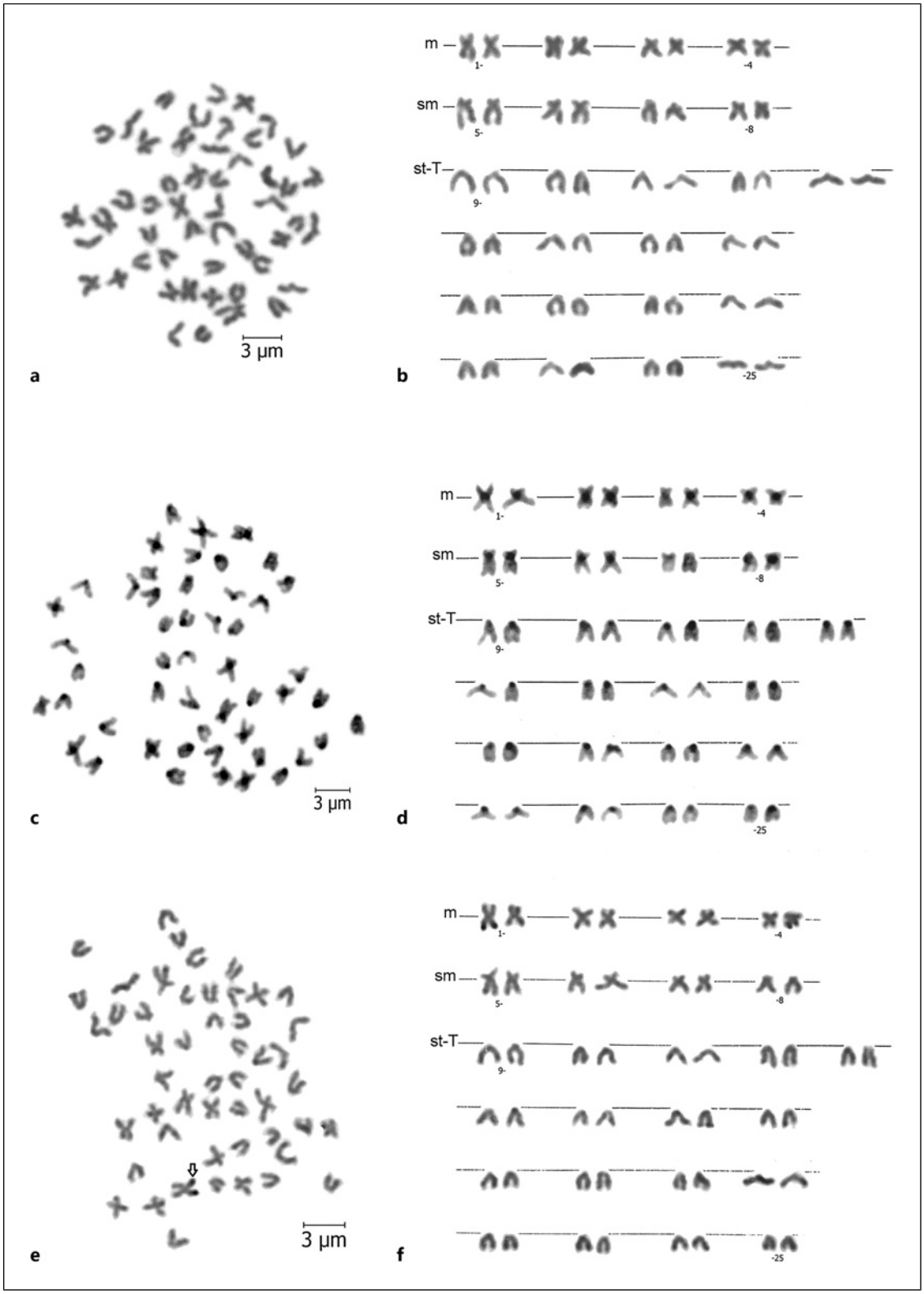


Fig. 4. Metaphases and karyotypes of *C. turcica*. **a** Giemsa-stained metaphase. **b** Arranged karyotype of the Giemsa-stained metaphase. **c** C-banded metaphase. **d** Arranged karyotype of the C-banded metaphase. **e** Silver-stained metaphase (arrow indicates the Ag-NOR). **f** Arranged karyotype of the silver-stained metaphase. Scale bar = 3 μm. m, metacentric; sm, submetacentric; st-T, subtelo-telocentric chromosomes.

Table 1. Characteristics of chromosome measurements

Cobitis bilseli				*Cobitis fahireae*			*Cobitis turcica*			ANOVA test					
chromosome	*l* (%)	*i*	category	*l* (%)	*i*	category	*l* (%)	*i*	category	cbi × cfa		cbi × ctu		cfa × ctu	
										l	*i*	*l*	*i*	*l*	*i*
1	2.46	44.71	m	2.32	37.79	m	2.38	42.68	m			[a]	*		*
2	2.27	46.61	m	2.30	49.45	m	2.36	48.61	m	[a]	*		*		**
3	2.25	41.37	m	2.26	38.58	m	2.27	45.66	m	*	**		*		***
4	2.14	49.44	m	2.26	39.55	m	2.22	41.01	m		***				***
5	2.59	32.51	sm	2.25	46.86	m	2.43	34.63	sm	*	***			*	***
6	2.41	36.59	sm	2.18	41.21	m	2.40	33.54	sm		***				***
7	2.23	35.01	sm	2.17	42.55	m	2.31	30.41	sm	**	***				***
8	2.08	28.32	sm	2.11	41.72	m	2.24	36.68	sm		***				***
9	2.02	25.16	sm	2.11	47.74	m	2.36	0	T	*	***	***	***		***
10	1.88	15.38	st	2.03	45.14	m	2.15	0	T	***	***	**	***		***
11	2.08	9.20	a	1.97	44.45	m	2.10	0	T	**	***	[a]	***	[a]	***
12	2.08	0	T	2.36	37.10	sm	2.02	0	T	[a]	***		**		***
13	2.04	0	T	2.23	33.23	sm	2.00	0	T		***				***
14	1.99	0	T	2.07	31.88	sm	1.98	0	T		***				***
15	1.95	0	T	2.06	30.39	sm	1.93	0	T	*	***	*		**	***
16	1.90	0	T	2.01	36.01	sm	1.91	0	T	*	***			*	***
17	1.87	0	T	1.95	34.83	sm	1.87	0	T	*	***			***	***
18	1.82	0	T	1.71	25.31	sm	1.81	0	T		***			***	***
19	1.79	0	T	1.92	0	T	1.75	0	T		***	*		*	**
20	1.77	0	T	1.84	0	T	1.69	0	T		*	**		*	*
21	1.72	0	T	1.75	0	T	1.64	0	T			***		[a]	
22	1.70	0	T	1.68	0	T	1.52	0	T			***			
23	1.65	0	T	1.63	0	T	1.40	0	T	[a]		***			
24	1.60	0	T	1.51	0	T	1.34	0	T	**		***			
25	1.52	0	T	1.19	0	T	1.29	0	T	***		***			

Chromosome measurements of *C. bilseli*, *C. fahireae*, and *C. turcica* are shown in median length (*l*, in %) and centromeric index (*i*). Chromosomal categories correspond to m, metacentric; sm, submetacentric; st, subtelocentric; a, acrocentric; and T, telocentric chromosomes. ANOVA test cbi x cfa, ANOVA test for *l* and *i* values of orthologous chromosomes in *C. bilseli* compared to those in *C. fahireae*. Similarly, *C. bilseli* and *C. fahireae* orthologs were compared to those in *C. turcica*, respectively (cbi x ctu, cfa x ctu). $*p < 0.05$. $**p < 0.01$. $***p < 0.001$. $^{a}p < 0.1$.

chromosome 1 (hereafter chr1). The largest submetacentric chromosome was chr5 in *C. bilseli* and *C. turcica*. We compared both *l* and *i* values of each orthologous chromosome within three *Cobitis* species (between *C. bilseli* and *C. fahireae*, *C. bilseli* and *C. turcica*, and *C. fahireae* and *C. turcica*, Table 1) with the aim to determine whether significant variation occurs or whether chromosomes are morphologically similar. ANOVA test revealed significant variation in the *i* value in the most chromosomes of *C. fahireae* even when grouped within the same morphological categories (Table 1). The highest variation in *i* values is clearly visible for chrs10–12 in *C. bilseli*, chrs19–20 in *C. fahireae*, and chr9 in *C. turcica*. The Q_1–Q_3 interval within these

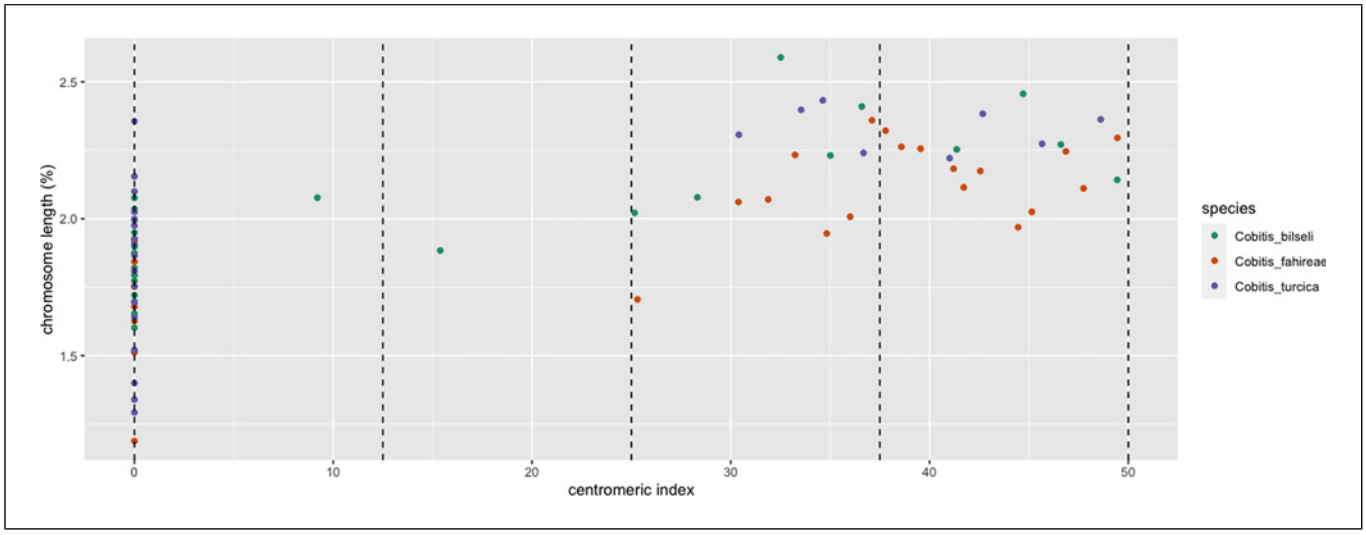


Fig. 5. Relationship between centromeric index (*i*), *x* axis, and chromosome length (*l*), *y* axis. Chromosome complements of *C. bilseli*, *C. fahireae*, and *C. turcica* are depicted in green, red, and dark blue, respectively. Black dashed vertical lines define intervals 0–12.5, 12.5–25, 25–37.5, and 37.5–50, which correspond to acrocentric, subtelocentric, submetacentric, and metacentric chromosomes, respectively. The dashed vertical line at $i = 0$ defines telocentric chromosomes. Plotted values of *i* and *l* are medians for each chromosome. The relative *l* value is given as a percentage of the overall length of all chromosomes.

variable chromosomes covers *i* values from 0 to 15 (Fig. 7). The order of chromosomes on karyotypes (Figs. 2–4) corresponds exactly to the order of chromosomes on box plots.

Taken together, we determined standardized karyotypes for *C. bilseli* (Fig. 2), *C. fahireae* (Fig. 3), and *C. turcica* (Fig. 4). Although we found all three karyotypes to be identical in terms of chromosome number, differences between karyotypes were found in NOR and C-band localization and chromosome structure (morphology, *l* and *i* values).

Discussion

Until recently, cytogenetic data were available for only three *Cobitis* species that have been distributed in Türkiye – *C. elazigensis*, *C. phrygica*, and *C. simplicispina* [15, 16]. Here we describe for the first time the karyotypic properties of two other Anatolian endemic *Cobitis* species – *C. fahireae* and *C. turcica*. The *C. bilseli* karyotype [17] was published during the review process of our study. Accordingly, we now also report the karyotype of *C. bilseli*. The present study reveals conserved diploid (2*n*) chromosome numbers but variable karyotype formulas. Pericentric inversions, the extinction of an old centromere and the origin of a new one, and/or translocations are likely to play an important role in the karyotype evolution of this species, as has already been stated for the other *Cobitis* species *C. elazigensis*, *C. phrygica*, and *C. simplicispina* [16]. Based on a detailed analysis of the *i* values, the karyotype composition of *C. bilseli* and *C. turcica* appeared to be more similar to each other than to the karyotype of either of these two species to that of *C. fahireae* (Table 1). All three species in this study share the 2*n* number with all the *Cobitis* species karyotyped so far from Türkiye (Table 2). Given the equal number of chromosomes in our studied species, we can exclude fissions and fusions that could have shaped the karyotype evolution because these rearrangements affect the number of chromosomes (e.g., Sember et al. [25]). In the Anatolian spined loaches, intrachromosomal rearrangements likely occurred, leading to spatial shifts in the position of the centromere without changes in the chromosome number. The most probable responsible mechanisms should be pericentric inversions, as reported recently in the other groups of fish [26, 27]. Some representatives of Anatolian Cobitidae species possess a karyotype composed of more uniarmed chromosomes than biarmed chromosomes, e.g., *C. elazigensis*, *C. phrygica*, *C. bilseli*, and *C. turcica* (Table 2). Contrary to this phenomenon, *C. simplicispina* and *C. fahireae* have more biarmed chromosomes in their karyotypes than uniarmed ones (Table 2). According to these differences, FNs in *C. simplicispina* and *C. fahireae* are higher than FNs in *C. elazigensis*, *C. phrygica*, *C. bilseli*, and *C. turcica*.

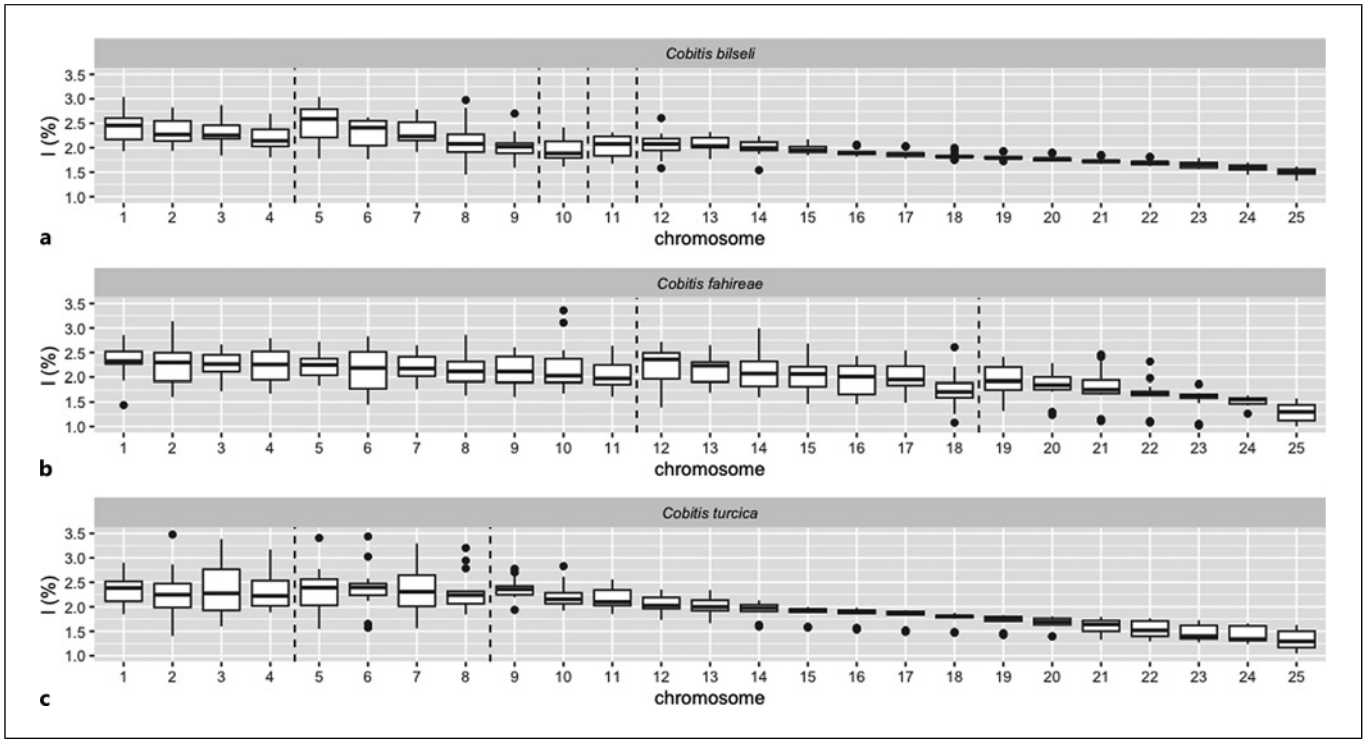


Fig. 6. Intrachromosomal variability of *l* value (*y* axis) is displayed for the haploid complement of 25 chromosomes of *C. bilseli* (**a**), *C. fahireae* (**b**), and *C. turcica* (**c**) (*x* axis). Each box defines interquartile range (Q_1–Q_3) with the median value indicated by black lines. Upper and lower whiskers show the maximum and minimum values of the data set, respectively. Outliers are drawn as black points. Black dashed vertical lines delineate the chromosome categories as follows: in *C. bilseli*, from the left to right: metacentric, submetacentric, subtelocentric, acrocentric, and telocentric chromosomes; in *C. fahireae* and *C. turcica*: metacentric, submetacentric, and telocentric chromosomes.

Otherwise, *C. taenia* from Europe [4] has a different 2*n* number from *C. bilseli*, *C. fahireae*, and *C. turcica*. *Cobitis taenia*'s karyotype contains a remarkable large metacentric chromosome, which is considered to be the result of a centric fusion of two acrocentric chromosomes [28]. Moreover, spined loaches from different countries named *C. calderoni*, *C. maroccana* [29], *C. vardarensis* [6], *C. taurica* [30], *C. lutheri*, *C. choii*, *C. melanoleuca* [31], *C. linea* [32], *C. strumicae* [33], *C. derzhavini*, *C. saniae* [34], and *C. satunini* [1] have the same diploid chromosome number, which is consistent with this study. However, the karyotype composition of the abovementioned species differs from *C. bilseli*, *C. fahireae*, and *C. turcica*. Majtánová et al. [3] observed a large variability in the karyotype structure and chromosomal markers in European *C. elongatoides*, *C. taenia*, and *C. tanaitica* and their hybrids. Most *Cobitis* species differ in their karyotype, which is routinely defined by variation in number, size, and morphology of chromosomes, as revealed in this study. Homomorphic, i.e., morphologically undifferentiated, sex chromosomes are a common feature among *Cobitis* loaches [15, 16, 32], and we also did not find any structural differences in male and female karyotypes.

Another significant difference between the karyotypes of *C. bilseli*, *C. fahireae*, and *C. turcica* is the variation in the number and location of Ag-NOR signals and C-banding patterns. These differences may be the result of specific chromosomal rearrangements such as inversions and translocations, which have occurred during their evolutionary history, as reported in the other *Cobitis* species [35]. The number and location of ribosomal DNA (rDNA) sites are essentially species specific and provide a useful karyotypic marker in fish cytotaxonomy [22, 26, 36]. The most of rDNAs are associated with nucleolus [37]; thus, their location on chromosomes is identical to that of NORs and is consistent with our analysis of silver staining. Cobitidae karyotypes have variable numbers and locations of NORs, ranging from single to complex multiple NOR phenotypes [6]. These variations do not consistently correlate with phylogenetic distance between Cobitidae species [38]. The NOR phenotypes of *C. bilseli* and *C. turcica* are the same. However, *C. fahireae* has a different NOR phenotype than the former two species. The number and location of Ag-NORs on submetacentric chromosomes in *C. fahireae* show similarity to *C.*

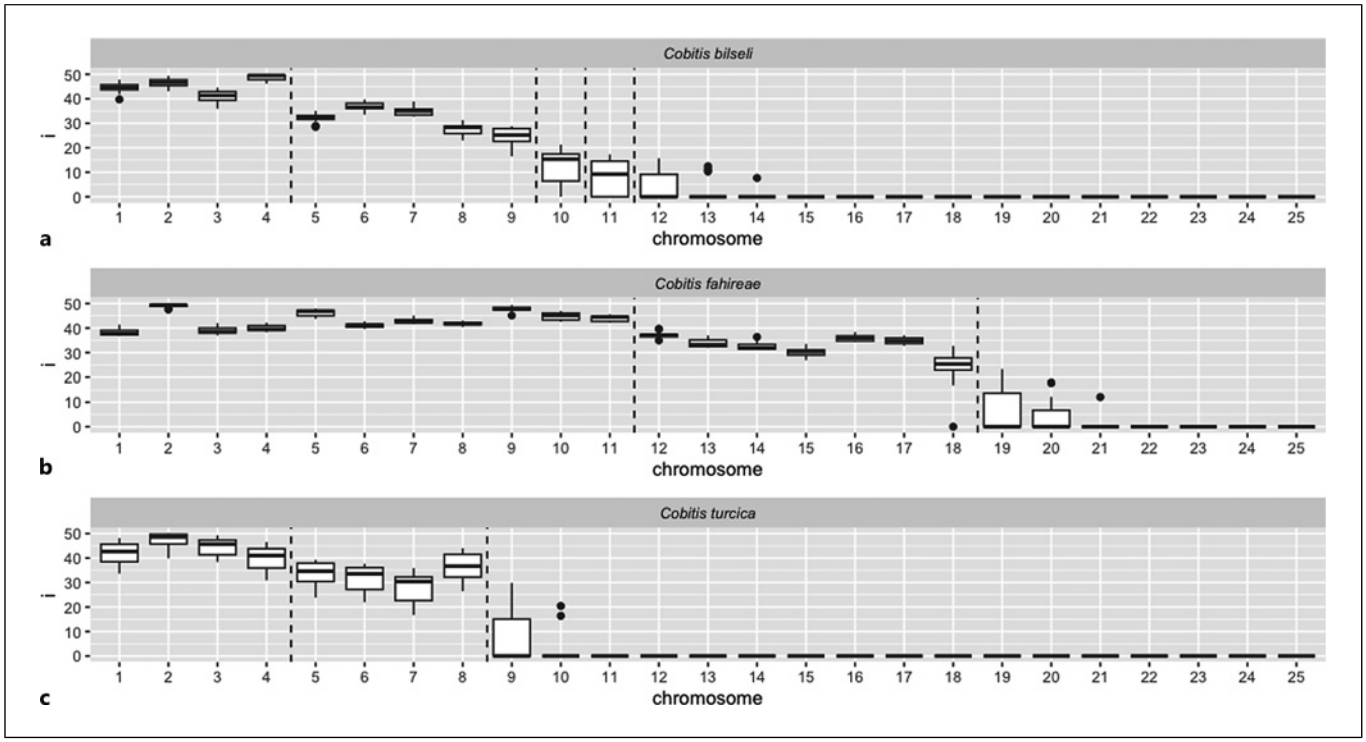


Fig. 7. Intrachromosomal variability of *i* value (*y* axis) is displayed for the haploid complement of 25 chromosomes of *C. bilseli* (**a**), *C. fahireae* (**b**), and *C. turcica* (**c**) (*x* axis). Each box defines interquartile range (Q_1–Q_3) with the median value indicated by black lines. Upper and lower whiskers show the maximum and minimum values of the data set, respectively. Outliers are drawn as black points. Black dashed vertical lines delineate the chromosome categories as follows: in *C. bilseli*, from the left to right: metacentric, submetacentric, subtelocentric, acrocentric, and telocentric chromosomes; in *C. fahireae* and *C. turcica*: metacentric, submetacentric, and telocentric chromosomes.

Table 2. Karyological data of the genus *Cobitis* from Türkiye

Species studied	2*n*	Karyotype formula	FN	References
C. elazigensis	50	18m-sm + 32a	68	[15]
C. phrygica	50	8m + 8sm + 34st-a	66	[16]
C. simplicispina	50	16m + 16m + 18st-a	82	[16]
C. bilseli	50	8m + 6sm + 6st + 30a	70	[17]
C. bilseli	50	8m + 10sm + 32st-T	72	This study
C. fahireae	50	22m + 14sm + 14st-T	86	This study
C. turcica	50	8m + 8sm + 34st-T	66	This study

2*n*, diploid chromosome number; FN, fundamental number; m, metacentric; sm, submetacentric; st-a, subtelo-acrocentric; st-T, subtelo-telocentric chromosomes (*sensu stricto*).

elazigensis [15], *C. phrygica*, *C. simplicispina* [16] from Türkiye and *C. strumicae* from Europe [33]. The Ag-NORs on metacentric chromosomes have been reported in *C. vardarensis* from Europe [6], as well as in *C. bilseli* and *C. turcica* from Türkiye (this study, [16]). *Cobitis taenia* was characterized by one pair of Ag-NOR located on subtelocentric chromosomes [4]. Intraindividual polymorphism in the number of Ag-NORs that was reported in *C. simplicispina* [16] is not observed in any of the species in this study.

Interestingly, we found Ag-NORs on only one chromosome of the largest homologous metacentric pair in both *C. bilseli* and *C. turcica*. This heterozygosity in both species confirms their close evolutionary relationship and more divergent relationship with *C. fahireae*, which has Ag-NOR on both homologous chromosomes of the submetacentric category. Contrary to our finding, Doori and Arslan [17] identified Ag-NOR on the p arm (not on the q arm as we did) of both chromosomes of the largest metacentric pair in *C. bilseli*. They did not measure the length of chromosome arms, and thus the NOR position may indeed be on the q arm, as we designated by accurate measurements. Also, slight differences in the karyotype formulas of *C. bilseli* in our study and the study of Doori and Arslan [17] were found. An explanation for the presence of the heterozygous Ag-NOR position may be the inactivity of NOR in the previous interphase since Ag binds NOR-associated proteins. If NORs were not active, rRNA synthesis would not occur and therefore Ag-NORs would not be detectable by silver staining technique [23, 39]. One future direction could be FISH with an rDNA probe (e.g., [40]) that hybridizes with a specific genome sequence associated with the NOR (e.g., 28S rDNA), thereby revealing the true NORs [41]. Even though the geographical distribution of *C. bilseli* and *C. turcica* is close (Fig. 1a), we assume that the similarity in their karyotype structure is a consequence of evolutionary history and timing of divergence rather than hybridization or gene introgression. Due to the polymorphism in number and location of NOR [4, 15, 16], interspecies hybridizations [3], and incomplete NOR mapping in *Cobitis* species, it is difficult to estimate the ancestral position of NOR in the entire *Cobitis* genus.

C-bands indicate the presence of constitutive heterochromatin regions [19]. These regions contain transcriptionally inactive and highly repetitive DNA sequences. The distribution of C-bands could be applied to identify homologous chromosomes within and orthologous chromosomes between species and improve chromosome classification [4, 42]. Numbers and positions of C-bands in *C. bilseli* and *C. turcica* are similar to *C. elazigensis* [15], *C. phrygica*, *C. simplicispina* [16] from Türkiye and *C. vardarensis* [6] and *C. taenia* [4] from Europe. A low C-banding content distinguishes *C. fahireae* from the above species, while this pattern is similar to that of *C. strumicae* from Europe [33]. Another explanation for the low intensity of C-bands in *C. fahireae* could be the low efficacy and thus non-specificity of the technique. Notably, relatively close phylogenetic relationships between *C. fahireae* and *C. strumicae*, as well as among *C. bilseli*, *C. turcica*, *C. elazigensis*, *C. phrygica*, and *C. simplicispina*, reflect their similar C-banding patterns and indicate a true specificity of C-bands.

Overall, the obtained results contributed to a better understanding of the karyotype structure and chromosomal evolution of Anatolian spined loaches. The cytogenetic findings, which showed a higher degree of similarity in *C. bilseli* and *C. turcica* and a higher degree of divergence in *C. fahireae*, are consistent with phylogenetic relationships of the genus *Cobitis* [38]. Also, our results indicate that main drivers of karyotype evolution are pericentric inversions, translocations, and the decay of old centromeres followed by the establishment of new ones, resulting in centromere repositioning. However, more detailed cytogenetic studies in other Anatolian *Cobitis* species are needed to solve the puzzle of chromosomal evolution and to discover the precise mechanisms driving changes in karyotype structure. In addition, we improved the R scripts previously developed for the determination of a standardized karyotype [22] and applied them in the *Cobitis* species.

Acknowledgment

We thank two anonymous reviewers for their constructive comments on early versions of the manuscript. The karyotype of *C. bilseli* was presented as an oral presentation in II. International Turkish World Engineering and Science Congress (2019) and the summary of the work was included in the abstract book of the congress.

Statement of Ethics

The process was approved by the Local Animal Ethics Committee of Türkiye (Protocol Number: 68429034/15/17).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This study was supported by Bartın University Scientific Research Projects, project code: 2019-FEN-B-006 (S.U.K.), Kırşehir Ahi Evran University Scientific Research Projects, project code: SYO.A4.21.006 (M.K.A.), and the P JAC project MSCA Fellowships CZ-UK, CZ.02.01.01/00/22_010/0002902 (M.K.).

Author Contributions

All authors contributed to the study conception and design and project administration. Material preparation, data collection, and investigation were performed by S.U.K., M.G.,

and M.K.A. Funding was secured by S.U.K., M.K.A., and M.K. Formal analysis, software, and supervision were provided by M.K. The first draft of the manuscript was written by S.U.K. and M.K.A. All authors commented on previous versions of the manuscript, and read and approved the final manuscript.

Data Availability Statement

All metaphase images and scripts within exported R files, including steps outlining how the measured values were calculated and processed into data frames and plots, are available at https://github.com/martinknytl/2023_Cobitis_karyotype.

References

1 Vasil'eva ED, Solovyeva EN, Vasil'ev VP. Phylogenetic relationships, taxonomy and diagnostics of spined loaches (Cobitidae: *Cobitis*, Sabanejewia) of the Caspian Sea basin. Proceedings of the 9th National and 1st International Iranian Conference of Ichthyology. 2021. p. 79–90.

2 Freyhof J, BayÇelebİ E, Geiger M. Review of the genus *Cobitis* in the Middle East, with the description of eight new species (Teleostei: Cobitidae). Zootaxa. 2018;4535(1):1–75. https://doi.org/10.11646/zootaxa.4535.1.1

3 Majtánová Z, Choleva L, Symonová R, Ráb P, Kotusz J, Pekárik L, et al. Asexual reproduction does not apparently increase the rate of chromosomal evolution: karyotype stability in diploid and triploid clonal hybrid fish (*Cobitis*, Cypriniformes, Teleostei). PLoS One. 2016;11(1):e0146872. https://doi.org/10.1371/journal.pone.0146872

4 Boroń A. Banded karyotype of spined loach *Cobitis taenia* and triploid *Cobitis* from Poland. Genetica. 1999;105(3):293–300. https://doi.org/10.1023/a:1003939813878

5 Levan A, Fredga K, Sandberg AA. Nomenclature for centromeric position on chromosomes. Hereditas. 2009;52(2):201–20. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

6 Rábová M, Ráb P, Ozouf-Costaz C. Extensive polymorphism and chromosomal characteristics of ribosomal DNA in a loach fish, *Cobitis vardarensis* (Ostariophysi, Cobitidae) detected by different banding techniques and fluorescence in situ hybridization (FISH). Genetica. 2001;111(1–3):413–22. https://doi.org/10.1023/a:1013763903513

7 Fornaini NR, Černohorská H, do Vale Martins L, Knytl M. Cytogenetic analysis of the fish genus *Carassius* indicates divergence, fission and segmental duplication as drivers of tandem repeat and microchromosome evolution. Genome Biol Evol. 2024;16(3):1–13. https://doi.org/10.1093/gbe/evae028

8 Gvoždík V, Knytl M, Zassi-Boulou A-G, Fornaini NR, Bergelová B. Tetraploidy in the Boettger's dwarf clawed frog (Pipidae: *Hymenochirus boettgeri*) from the Congo indicates non-conspecificity with the captive population. Zool J Linn Soc. 2024;200(4):1034–47. https://doi.org/10.1093/zoolinnean/zlad119

9 Schubert I. What is behind "centromere repositioning". Chromosoma. 2018;127(2):229–34. https://doi.org/10.1007/s00412-018-0672-y

10 Knytl M, Tlapakova T, Vankova T, Krylov V. *Silurana* chromosomal evolution: a new piece to the puzzle. Cytogenet Genome Res. 2018;156(4):223–8. https://doi.org/10.1159/000494708

11 Knytl M, Forsythe A, Kalous L. A fish of multiple faces, which show us enigmatic and incredible phenomena in nature: biology and cytogenetics of the genus *Carassius*. Int J Mol Sci. 2022;23(15):8095. https://doi.org/10.3390/ijms23158095

12 Song X-Y, Furman BLS, Premachandra T, Knytl M, Cauret CMS, Wasonga DV, et al. Sex chromosome degeneration, turnover, and sex-biased expression of sex-linked transcripts in African clawed frogs (*Xenopus*). Philos Trans R Soc Lond B Biol Sci. 2021;376(1832):20200095. https://doi.org/10.1098/rstb.2020.0095

13 Evans BJ, Mudd AB, Bredeson JV, Furman BLS, Wasonga DV, Lyons JB, et al. New insights into *Xenopus* sex chromosome genomics from the Marsabit clawed frog *X. borealis*. J Evol Biol. 2022;35(12):1777–90. https://doi.org/10.1111/jeb.14078

14 Eagderi S, Secer B, Freyhof J. *Cobitis indus*, a new spined loach from the Dalaman River in the Eastern Aegean Sea basin (Teleostei: Cobitidae). Zootaxa. 2022;5162(4):410–20. https://doi.org/10.11646/zootaxa.5162.4.5

15 Değer D. The karyological investigations of some types from Cobitoidea from river system Tigris and Euphrates; 2011.

16 Ayata MK, Unal S, Gaffaroğlu M. Chromosomal analyses of *Cobitis phrygica* Battalgazi, 1944 and *C. simplicispina* Hanko, 1925 (Teleostei, Cobitidae). Cytologia. 2018;83(3):295–9. https://doi.org/10.1508/cytologia.83.295

17 Doori ASJ, Arslan A. Karyological analysis of endemic loach *Cobitis bilseli* Battalgil, 1942 (Cobitidae) in Turkey. Cytologia. 2024;89(1):47–51. https://doi.org/10.1508/cytologia.89.47

18 Bertollo LAC, Cioffi MB, Moreira-Filho O. Direct chromosome preparation from freshwater teleost fishes. In: Ozouf-Costaz C, Pisano E, Foresti F, de Almeida Toledo LF, eds. Fish cytogenetic techniques: ray-fin fishes and chondrichthyans. Boca Raton: CRC Press; 2015. p. 21–6.

19 Sumner AT. A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res. 1972;75(1):304–6. https://doi.org/10.1016/0014-4827(72)90558-7

20 Howell WM, Black DA. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia. 1980;36(8):1014–5. https://doi.org/10.1007/BF01953855

21 Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9(7):671–5. https://doi.org/10.1038/nmeth.2089

22 Knytl M, Fornaini NR. Measurement of chromosomal arms and FISH reveal complex genome architecture and standardized karyotype of model fish, genus *Carassius*. Cells. 2021;10(9):2343. https://doi.org/10.3390/cells10092343

23 Knytl M, Fornaini NR, Bergelová B, Gvoždík V, Černohorská H, Kubíčková S, et al. Divergent subgenome evolution in the allotetraploid frog *Xenopus calcaratus*. Gene. 2023;851:146974. https://doi.org/10.1016/j.gene.2022.146974

24 R Core Team. R: a Language and environment for statistical computing; 2020. Available from: https://www.r-project.org/

25 Sember A, Bohlen J, Šlechtová V, Altmanová M, Pelikánová Š, Ráb P. Dynamics of tandemly repeated DNA sequences during evolution of diploid and tetraploid botiid loaches (Teleostei: Cobitoidea: Botiidae). PLoS One. 2018;13(3):e0195054. https://doi.org/10.1371/journal.pone.0195054

26 Sember A, Pelikánová Š, de Bello Cioffi M, Šlechtová V, Hatanaka T, Do Doan H, et al. Taxonomic diversity not associated with gross karyotype differentiation: the case of bighead carps, genus *Hypophthalmichthys* (Teleostei, Cypriniformes, Xenocyprididae). Genes. 2020;11(5):479. https://doi.org/10.3390/genes11050479

27 Krysanov EY, Nagy B, Watters BR, Sember A, Simanovsky SA. Karyotype differentiation in the *Nothobranchius ugandensis* species group (Teleostei, Cyprinodontiformes), seasonal fishes from the east African inland plateau, in the context of phylogeny and biogeography. Comp Cytogenet. 2023;17(1):13–29. https://doi.org/10.3897/compcytogen.v7.i1.97165

28 Ráb P, Hnátková E, Majtánová Z, Šlechtová VB, Bohlen J. Karyotype record for the morphologically derived, rarely collected, freshwater fish *Ellopostoma mystax* (Cypriniformes, Cobitoidea, Ellopostomatidae). Ichthyol Herpetol. 2021;109(4):998–1001. https://doi.org/10.1643/i2020032

29 Madeira JM, Collares-Pereira MJ, Elvira B. Cytotaxonomy of Iberian loaches with some remarks on the karyological evolution of both families (Pisces, Cobitidae, Homalopteridae). Caryologia. 1992;45(3–4):273–81. https://doi.org/10.1080/00087114.1992.10797231
30 Janko K, Vasil'ev VP, Ráb P, Rábová M, Šlechtová V, Vasil'eva ED. Genetic and morphological analyses of 50-chromosome spined loaches (*Cobitis*, Cobitidae, Pisces) from the Black Sea basin that are morphologically similar to *C. taenia*, with the description of a new species. Folia Zool Brno. 2005;54(4):405–20.
31 Vasil'ev VP, Vasil'eva ED. Comparative karyological analysis of mud loach and spined loach species (genera *Misgurnus* and *Cobitis*) from the far east region of Russia. Folia Zool Brno. 2008;57(1–2):51–9.
32 Esmaeili HR, Pirvar Z, Ebrahimi M, F Geiger M. Karyological and molecular analysis of three endemic loaches (Actinopterygii: cobitoidea) from Kor River basin, Iran. Mol Biol Res Commun. 2015;4(1):1–13.
33 Hnátková E, Triantaphyllidis C, Ozouf-Costaz C, Choleva L, Majtánová Z, Bohlen J, et al. Karyotype and chromosomal characteristics of rDNA of *Cobitis strumicae* Karaman, 1955 (Teleostei, Cobitidae) from Lake Volvi, Greece. Comp Cytogenet. 2018;12(4):483–91. https://doi.org/10.3897/CompCytogen.v12i4.28068
34 Vasil'eva ED, Solovyeva EN, Levin BA, Vasil'ev VP. *Cobitis derzhavini* sp. nova—a new spined loach species (Teleostei: Cobitidae) discovered in the Transcaucasia. J Ichthyol. 2020;60(2):135–53. https://doi.org/10.1134/s0032945220020198
35 Marta A, Dedukh D, Bartoš O, Majtánová Z, Janko K. Cytogenetic characterization of seven novel satDNA markers in two species of spined loaches (*Cobitis*) and their clonal hybrids. Genes. 2020;11(6):617. https://doi.org/10.3390/genes11060617
36 Knytl M, Kalous L, Rylková K, Choleva L, Merilä J, Ráb P. Morphologically indistinguishable hybrid *Carassius* female with 156 chromosomes: a threat for the threatened crucian carp, *C. carassius*, L. PLoS One. 2018;13(1):e0190924. https://doi.org/10.1371/journal.pone.0190924
37 Symonová R, Howell WM. Vertebrate genome evolution in the light of fish cytogenomics and rDNAomics. Genes. 2018;9(2):96–27. https://doi.org/10.3390/genes9020096
38 Perdices A, Ozeren CS, Erkakan F, Freyhof J. Diversity of spined loaches from Asia Minor in a phylogenetic context (Teleostei: Cobitidae). PLoS One. 2018;13(10):e0205678. https://doi.org/10.1371/journal.pone.0205678
39 Schmid M. Chromosome banding in Amphibia. Chromosoma. 1982;87(3):327–44. https://doi.org/10.1007/bf00327634
40 Dias S, Souza RC, Vasconcelos EV, Vasconcelos S, da Silva Oliveira AR, do Vale Martins L, et al. Cytomolecular diversity among *Vigna* Savi (Leguminosae) subgenera. Protoplasma. 2024;261(5):859–75. https://doi.org/10.1007/s00709-024-01944-z
41 Dobigny G, Ozouf-Costaz C, Bonillo C, Volobouev V. "Ag-NORs" are not always true NORs: new evidence in mammals. Cytogenet Genome Res. 2002;98(1):75–7. https://doi.org/10.1159/000068541
42 Knytl M, Kalous L, Rab P. Karyotype and chromosome banding of endangered crucian carp, *Carassius carassius* (Linnaeus, 1758)(Teleostei, Cyprinidae). Comp Cytogenet. 2013;7(3):205–15. https://doi.org/10.3897/CompCytogen.v7i3.5411