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Cytogenetic studies on six species of the leuciscine genus *Pseudophoxinus* Bleeker, 1860 (Teleostei, Cyprinidae)

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ABSTRACT

A study of the cytogenetics of *Pseudophoxinus battalgilae* Bogutskaya, 1997, *P. burduricus* Küçük, Gülle, Güçlü, Çiftçi & Erdoğan, 2013, *P. egridiri* (Karaman, 1972), *P. evliyae* Freyhof & Özuluğ, 2009, *P. fahrettini* Freyhof & Özuluğ, 2009 and *P. maeandri* (Ladiges, 1960) was conducted by means of Giemsa staining, C- and AgNOR-banding procedures. Diploid chromosome numbers of analyzed species were found to be the same ($2n = 50$) but their karyotypes formulae were different. For all species examined, the largest chromosome pair of the complements was subtelo-acrocentric. Heteromorphic sex chromosomes were not detected in any of the studied species. C-bands were found on the centromeres of several chromosomes in all studied species. NORs were detected in one pair of submetacentric chromosomes in *P. burduricus*, *P. egridiri* and *P. fahrettini*, and in two pairs of submetacentric chromosomes in *P. battalgilae*, *P. evliyae* and *P. maeandri*. Further, NOR polymorphisms were observed in some specimens of *P. battalgilae*, *P. burduricus*, *P. evliyae* and *P. fahrettini* for number, location and size. This study may contribute to other leuciscine cytogenetic studies.

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Leuciscinae; *Pseudophoxinus*; karyotype; constitutive heterochromatin; nucleolus organizer region; Anatolia

Introduction

The genus *Pseudophoxinus* Bleeker, 1860 belongs to the subfamily Leuciscinae. This genus has 29 species that are distributed in the Balkans, Anatolia, the Middle East, the Caspian Sea Basin and North Africa (Küçük et al. 2012; Eschmeyer 2015). The genus *Pseudophoxinus* is the fourth largest genus of Anatolian freshwater fishes, having 21 species in Anatolia (Kuru et al. 2014; Küçük and Güçlü 2014a; Ekmekçi et al. 2015). Of these species, 19 of them are endemic (Ekmekçi et al. 2015; Eschmeyer 2015). The species are small in size and are distributed in cold springs, small lakes and slow-flowing rivers. Due to their small size, taxonomic problems still exist in the species (Küçük et al. 2012, 2013). For this reason, some populations were re-examined and new species have been described from Anatolia, as mentioned in detail by Ekmekçi et al. (2015). Changes in the systematic position of some species were also made. In this regard, *P. fahirae* was placed in the genus *Chondrostoma* (Freyhof and Özuluğ 2009). In addition, most of the Anatolian *Pseudophoxinus* species are affected especially by pollution, water abstraction and climate change and are, therefore, assessed as endangered (IUCN 2015).

A cytogenetic study may be useful to reveal phylogenetic relationships among species (Boron et al. 2009). An apparent conservation of the diploid chromosome number ($2n = 50$) and chromosome morphology was observed among 25 leuciscine genera that were cytogenetically examined (Rossi et al. 2012). Cytogenetic studies of Anatolian freshwater fishes have been initiated by Çolak et al. (1985). With the exception of *Chondrostoma meandrense*, all Anatolian leuciscine species have the diploid number of 50 chromosomes; however, chromosome morphologies show some differences between the species (Uysal 2011; Arslan and Gündoğdu 2016). On the other hand, chromosomal band studies have been reported in fewer Anatolian leuciscine species and moderate variations were indicated in the distribution of C-bands and NORs (Arslan and Gündoğdu 2016).

Chromosomal studies in the genus *Pseudophoxinus* have been conducted in recent years (Ergene et al. 2010; Karasu et al. 2011; Gaffaroğlu et al. 2014; Ünal et al. 2014; Ünal 2015). However, these studies are considered inadequate in terms of richness of this genus. One study claims that conserved karyotypic evolution was observed in the genus *Pseudophoxinus* (Ünal et al. 2014). The aim of this study is to reveal the diploid chromosome number, chromosome morphology, C-band and NOR phenotypes of endemic leuciscins *P. battalgilae*, *P. burduricus*, *P. egridiri*, *P. evliyae*, *P. fahrettini* and *P. maeandri* from Anatolia.

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Table 1. General information regarding collected specimens.

Species	Locality	Coordinates	Date	Number and sex of specimens
P. battalgilae	Kuğulupark, Seydişehir, Konya	37°23′N, 31°50′E	2013	3 ♀, 8 ♂
P. burduricus	Düğer Village, Burdur	37°34′N, 30°01′E	2012	4 ♀, 6 ♂
P. egridiri	Eğirdir Lake, Isparta	38°07′N, 30°54′E	2012	1 ♀, 7 ♂
P. evliyae	Kırkpınar Village, Korkuteli, Antalya	37°08′N, 29°55′E	2013	11 ♀, 10 ♂
P. fahrettini	Köprüçay River, Pazarköy, Isparta	37°44′N, 31°01′E	2012	1 ♀, 5 ♂
P. maeandri	Işıklı Spring, Çivril, Denizli	38°19′N, 29°51′E	2013	4 ♀, 3 ♂

Materials and methods

Fish sampling

In total 63 specimens of *P. battalgilae*, *P. burduricus*, *P. egridiri*, *P. evliyae*, *P. fahrettini* and *P. maeandri* were collected by electrofishing from Turkey in 2012 and 2013 (Table 1). Specimens were transported alive to the laboratory and kept in well-aerated aquaria until analysis. After analysis, the specimens were deposited in 70% ethanol at the Cytogenetics Laboratory of the Department of Biology, Faculty of Arts and Sciences of the Ahi Evran University, Kırşehir, Turkey (M. K. A. 30-93).

Chromosome preparations

Chromosome preparations were from the head kidney by using the air drying technique of Collares-Pereira (1992). The fish were injected intraperitoneally with 0.1% colchicine solution (1 ml per 100 g body weight) and kept in aerated aquaria for 2 h. Then the head kidneys of the specimens were removed and placed in hypotonic KCl solution (0.075 M) for 40 min at 37 °C. After this step, the cell suspension was centrifuged for 10 min at 1200 rpm, after which the supernatant was discarded. The cells were fixed with 5 ml fixative solution (3:1, methanol:glacial acetic acid) for 30 min at 4 °C. Then the cells were centrifuged and supernatant was discarded again. These last two steps were repeated two to three times. The cell suspensions were then dropped onto cleaned and cold slides. Air-dried slides were stained by 10% Giemsa for 20 min. Then slides were rinsed with distilled water and allowed to dry at room temperature. 10 to 20 slides were prepared from each specimen.

Bandings

C-banding technique of Sumner (1972) with some modifications was used for determining constitutive heterochromatin regions and the Ag-staining technique of Howell and Black (1980) was used for determining NORs. For C-banding, slides were treated with 0.2 N HCl for 30 min at room temperature, then rinsed with distilled water and air-dried. The slides were then incubated with 5% $Ba(OH)_2$ for 15–20 min at 37 °C, followed by rinsing and drying. Slides were incubated with 2 × SSC for 2 h at 70 °C and rinsed and dried once again. Then slides were stained by 10% Giemsa for 30 min. For Ag-staining, two drops of colloidal developer and four drops of 50% $AgNO_3$ solution were added onto the slides. The coverslip was used to cover the slide and then placed in an incubator at 70 °C. When the slide color changed to golden brown, the coverslip was removed. Then slide was rinsed and dried.

Microscopy, image processing and karyotyping

All preparations were scanned with a Leica DM 3000 microscope (Leica Microsystems GmbH, Germany) and photographs of good metaphase plaques were taken with AKAS software (Argenit Mikrosistem, Turkey). At least 100 metaphase plaques were counted from each species to determine the diploid chromosome number. Chromosomes were classified according to Levan et al. (1964) and karyotypes were prepared manually. For calculating fundamental arm number (FN), metacentric (m) and submetacentric (sm) chromosomes were taken as biarmed, while subtelo-acrocentric (st-a) chromosomes were taken as uniarmed.

Results

Diploid chromosome number and karyotypes

Diploid chromosome numbers of *P. battalgilae*, *P. burduricus*, *P. egridiri*, *P. evliyae*, *P. fahrettini* and *P. maeandri* were determined as $2n = 50$. Karyotypes were as follows: eight pairs of m, 14 pairs of sm and three pairs of st-a for *P. battalgilae*; nine pairs of m, 13 pairs of sm and three pairs of st-a for *P. burduricus*; seven pairs of m, 14 pairs of sm and four pairs of st-a for *P. egridiri*; seven pairs of m, 15 pairs of sm and three pairs of st-a for *P. evliyae*; eight pairs of m, 13 pairs of sm and four pairs of st-a for *P. fahrettini*; five pairs of m, 16 pairs of sm and four pairs of st-a chromosomes for *P. maeandri* (Figure 1). The largest chromosome pair of the complements was st-a in all studied species. FN was calculated as 92 in *P. egridiri*, *P. fahrettini* and *P. maeandri*, and 94 in *P. battalgilae*, *P. burduricus* and *P. evliyae*. Heteromorphic sex chromosomes were not detected in any of the studied species.

Constitutive heterochromatin regions

Constitutive heterochromatin regions were found on the centromeres of several chromosomes in *P. battalgilae*, *P. burduricus*, *P. egridiri*, *P. evliyae*, *P. fahrettini* and *P. maeandri*. Additionally, heterochromatic blocks were determined in the pericentromeres of some chromosome pairs in *P. egridiri* and *P. fahrettini* (Figure 2).

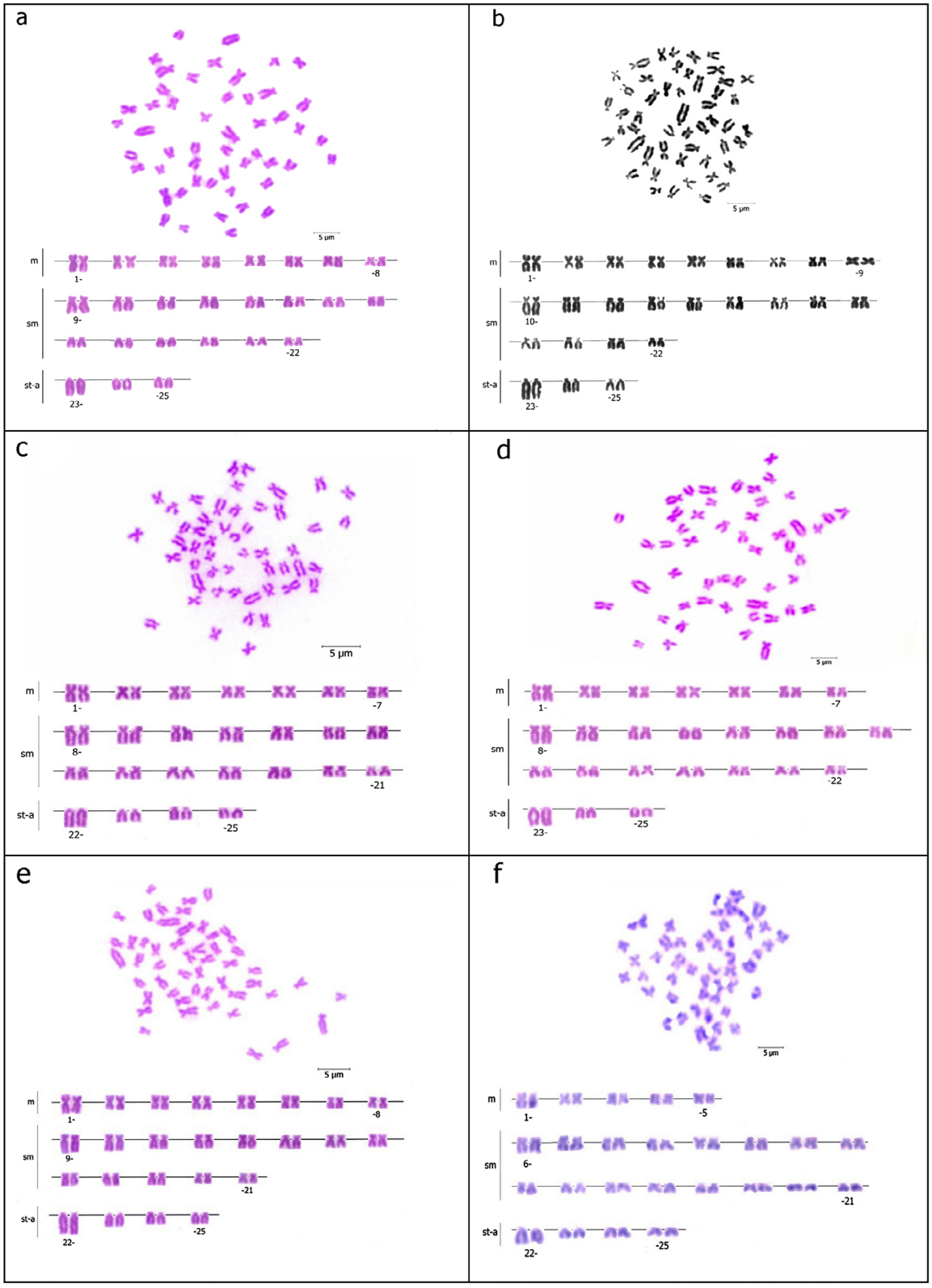

Figure 1. Giemsa stained metaphases and arranged karyotypes of (a) *P. battalgilae*; (b) *P. burduricus*; (c) *P. egridiri*; (d) *P. evliyae*; (e) *P. fahrettini*; (f) *P. maeandri*. Scale bar = 5 µm.

NOR phenotypes

NORs were detected in the telomeres of the short arms of one pair of middle sized sm chromosomes in *P. burduricus*, *P. egridiri* and *P. fahrettini*, and in the telomeres of the short arms of two pairs of middle sized sm chromosomes in *P. battalgilae*, *P. evliyae* and *P. maeandri* (Figure 3). Also NOR polymorphisms were observed on some specimens of *P. battalgilae*, *P. burduricus*, *P. evliyae* and *P. fahrettini* for number, location and size (Table 2, Figure 4).

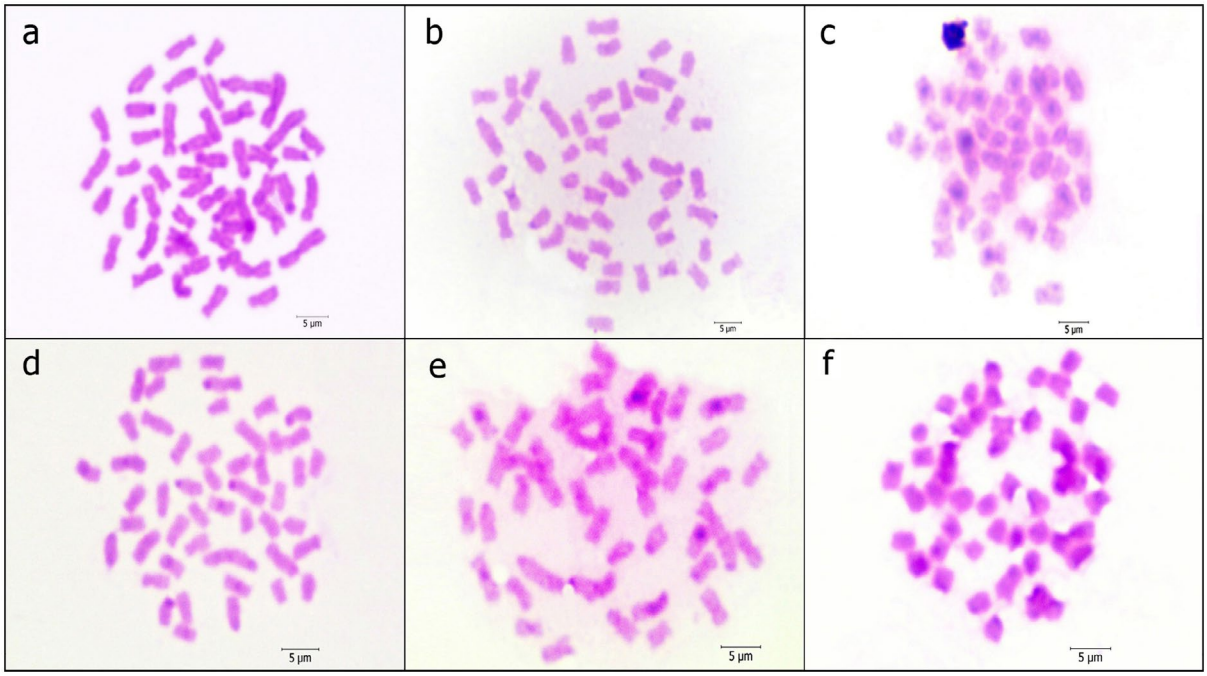

Figure 2. C-banded metaphases of (a) *P. battalgilae*; (b) *P. burduricus*; (c) *P. egridiri*; (d) *P. evliyae*; (e) *P. fahrettini*; (f) *P. maeandri*. Scale bar = 5 µm.

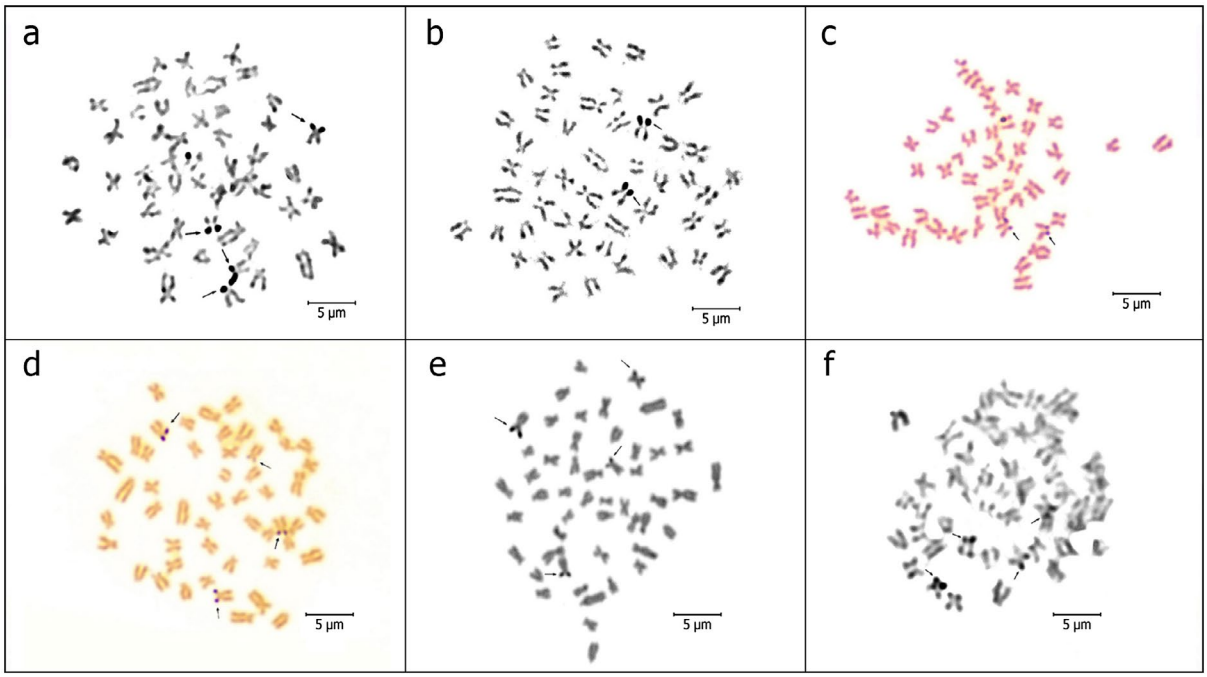

Figure 3. Ag-stained metaphases of (a) *P. battalgilae*; (b) *P. burduricus*; (c) *P. egridiri*; (d) *P. evliyae*; (e) *P. fahrettini*; (f) *P. maeandri*. Arrows indicate the NORs. Scale bar = 5 µm.

Table 2. NOR number polymorphism of the four studied species.

		Ag-positive signals (%)					
Species	Number of Ag-stained metaphases	1	2	3	4	5	6
P. battalgilae	55	3.63	32.72	23.63	38.18	1.84	
P. burduricus	65	32.30	53.84	9.23	4.63		
P. evliyae	121		10.74	38.84	40.49	7.43	2.50
P. fahrettini	46	2.17	89.13	1.69	7.01		

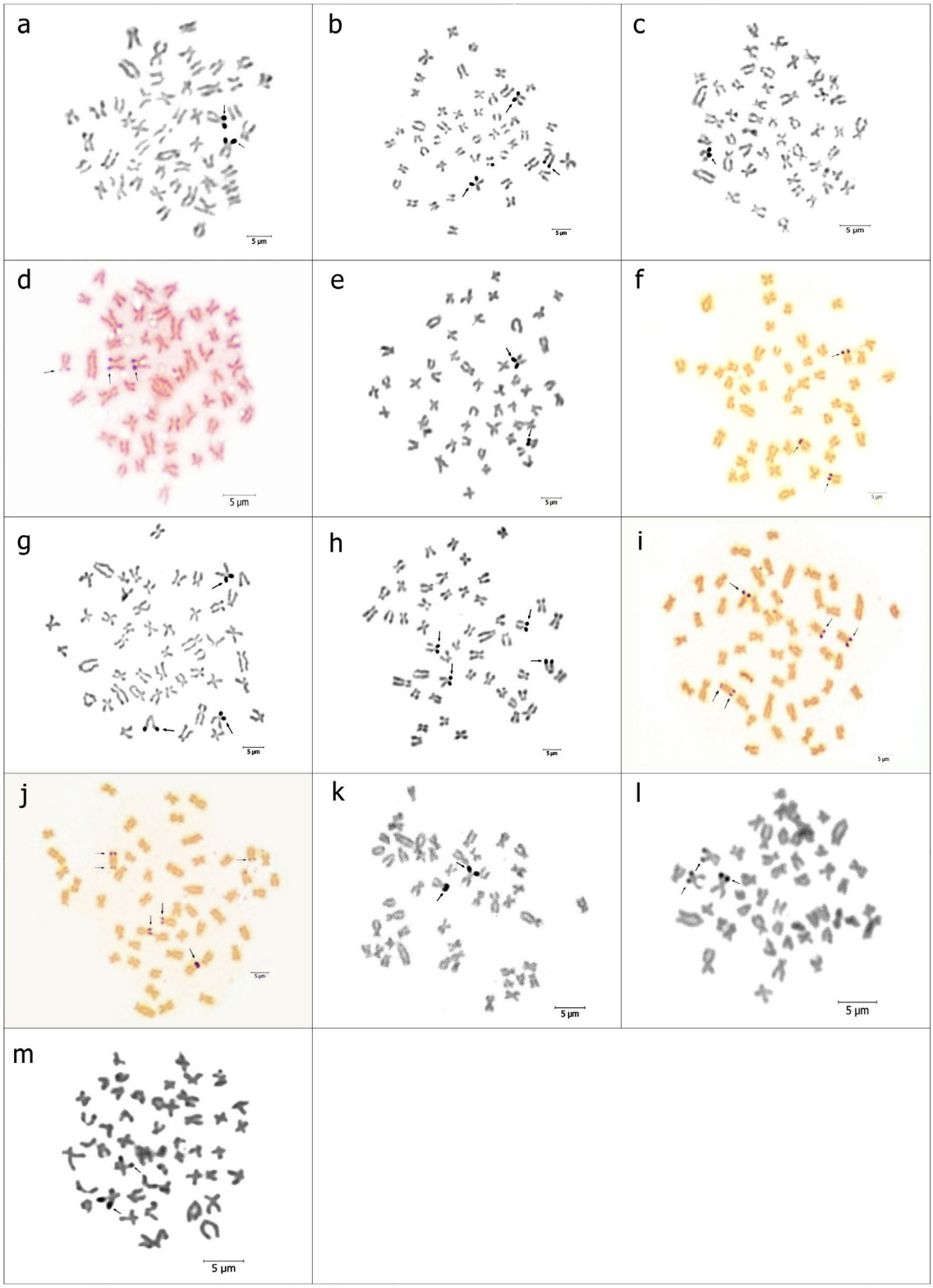

Figure 4. Ag-stained metaphases with number and/or location polymorphisms of (a, b) *P. battalgilae* showing two and three Ag-positive signals; (c, d) *P. burduricus* showing one and three Ag-positive signals; (e–j) *P. evliyae* showing from two to six Ag-positive signals; (k, l) *P. fahrettini* showing two and three Ag-positive signals; (m) *P. fahrettini* with size polymorphism. Arrows indicate the NORs. Scale bar = 5 µm.

Discussion

Despite the high diversity and endemism of Anatolian leuciscins only about 19% have been karyologically studied (Pekol 1999; Gaffaroğlu et al. 2006; Nur 2006; Kaya 2009; Uysal 2011; Ünal 2011; Arslan and Gündoğdu 2016; references in Table 3). The diploid chromosome numbers and chromosome morphologies obtained for the six endemic species are considered to reflect the characteristic leuciscine pattern ($2n$ = 50: dominance of meta- and submetacentrics) (Pereira et al. 2009). The

Table 3. Karyological studies in the genus *Pseudophoxinus*.

Species	2*n*	Karyotype	FN	Reference
P. antalyae	50	16M+14SM+12ST+8A	92	Ergene et al. 2010
P. firati	50	38M-SM+12ST	88	Karasu et al. 2011
P. elizavetae	50	8M+34SM+8ST	92	Gaffaroğlu et al. 2014
P. crassus	50	12M+30SM+8ST-A	92	Ünal et al. 2014
P. hittitorum	50	14M+26SM+10ST-A	90	Ünal et al. 2014
P. zekayi	50	16M+26SM+8ST-A	92	Ünal 2015
P. battalgilae	50	16M+28SM+6ST-A	94	Current study
P. burduricus	50	18M+26SM+6ST-A	94	Current study
P. egridiri	50	14M+28SM+8ST-A	92	Current study
P. evliyae	50	14M+30SM+6ST-A	94	Current study
P. fahrettini	50	16M+26SM+8ST-A	92	Current study
P. maeandri	50	10M+32SM+8ST-A	92	Current study

obtained data indicate very low karyotypic variation with the chromosome sets of the species undertaken in this research. Other karyologically studied species of the genus *Pseudophoxinus* also have 2*n* = 50 chromosomes (Table 3). However, their chromosome morphologies show little difference with the species in this study (except *P. zekayi* and *P. fahrettini*). In accordance with this discrepancy, the FNs of the some species are found to be different. Distinct FNs of these species may be the result of pericentromeric inversions (and/or translocations involving centromeres) as reported by Pereira et al. (2012). This situation may be considered valid for the studied species in this research which have the same FNs but different chromosome morphologies. Further, the largest chromosome pair (st-a) of the complements are suggested to be a cytotaxonomic marker for leuciscins (Rab et al. 2008), as observed in this study. In regard of this marker chromosome, the *Pseudophoxinus* species in this study resemble *P. crassus*, *P. elizavetae*, *P. firati*, *P. hittitorum* and *P. zekayi* but differ to *P. antalyae* (references in Table 3). In addition to the recognizable marker chromosome, the largest m pair and largest two sm pairs can be easily recognized in all metaphase plaques of six species such as Iberian leuciscine species (Pereira et al. 2012). On the other hand, no heteromorphic sex chromosomes are observed in this study as reported for *P. firati* (Karasu et al. 2011), *P. crassus*, *P. hittitorum* (Ünal et al. 2014) and *P. zekayi* (Ünal 2015).

One of the most common techniques successfully applied to fish chromosomes is C-banding. By using this technique, constitutive heterochromatin regions can be identified and this region is a useful tool for cytotaxonomy of cyprinids (Ren et al. 1992). In this study, constitutive heterochromatin regions were found to be associated to centromeric and pericentromeric regions as in the other *Pseudophoxinus* species (Ergene et al. 2010; Karasu et al. 2011; Gaffaroğlu et al. 2014; Ünal et al. 2014; Ünal 2015) and as in the other leuciscine genera such as *Abramis* (Ocalewicz et al. 2004), *Acanthobrama* (Gaffaroğlu and Yüksel 2009), *Chondrostoma* (Arslan and Gündoğdu 2016), *Leuciscus* (Boron et al. 2009) and *Squalius* (Ünal 2011; Rossi et al. 2012). Additionally, the heterochromatic blocks in *P. egridiri* and *P. fahrettini* were reported in *P. antalyae* (Ergene et al. 2010), *Acanthobrama marmid* (Gaffaroğlu and Yüksel 2009) and also in the other representatives of European leuciscins (Boron 2001). It is possible to form these heterochromatic blocks after pericentric inversions (Boron 1995).

NOR phenotype (number and location) is another useful tool which is generally used in cyprinid cytotaxonomy (Valic et al. 2010). The most studied Anatolian leuciscine species – *P. crassus*, *P. hittitorum* (Ünal et al. 2014), *Squalius anatolicus* (Ünal 2011), *P. zekayi*, *Squalius seyhanensis* (Ünal 2015) and *Chondrostoma beysehirense* (Arslan and Gündoğdu 2016) – have a single NOR in the sm pair, like *P. burduricus*, *P. egridiri* and *P. fahrettini*. This phenotype is observed in most leuciscins from Europe and considered as an ancestral character (Rab et al. 2000; Bianco et al. 2004; Rossi et al. 2012; Nabais et al. 2013). Further, differences in the location of single NORs have been reported in Anatolian and European leuciscins (Pekol 1999; Kalous et al. 2008; Ergene et al. 2010). In addition, multiple NORs are considered as a derived character (Valic et al. 2010). These NORs have been observed in some Anatolian leuciscine species (*A. marmid* (Gaffaroğlu et al. 2006), *P. firati* (Karasu et al. 2011) and *P. elizavetae* (Gaffaroğlu et al. 2014)) and also in several European leuciscine species (Boron 2001; Monteiro et al. 2009; Pereira et al. 2009) such as *P. battalgilae*, *P. evliyae* and *P. maeandri*.

In most metaphases, *P. burduricus* and *P. fahrettini* have a pair of NOR-bearing chromosomes; while *P. battalgilae* and *P. evliyae* have two pairs of NOR-bearing chromosomes. However, NOR number polymorphism was detected within and among the specimens of the so-mentioned species. Similar results have been reported for only *P. zekayi* (Ünal 2015) from the studied Anatolian leuciscine species and for several European leuciscins (Collares-Pereira and Rab 1999; Boron 2001; Boron et al. 2009; Monteiro et al. 2009; Pereira et al. 2009). Besides these, NORs were mainly located in the telomeres of the short arms of sm chromosomes in *P. battalgilae*, *P. evliyae* and *P. fahrettini*, while they were observed in the telomeres of the long arms of sm chromosomes on some metaphases. This condition has been detected in Eurasian leuciscins such as *S. cephalus* (Pekol 1999) and *P. phoxinus* (Boron 2001). Additionally, NOR size

polymorphism that was observed in *P. fahrettini* has been reported in *S. cephalus* (Pekol 1999) and *S. anatolicus* (Ünal 2011) of the Anatolian leuciscins and in European leuciscins such as *Chondrostoma lusitanicum* (Collares-Pereira and Rab 1999), *Rutilus aula* (Bianco et al. 2004), *Squalius lucumonis* (Rossi et al. 2012) and the three species of the genus *Vimba* (Rabova et al. 2003). The observed NOR polymorphisms in this study may be considered as the result of rearrangements (unequal crossing over, duplication, translocation, amplification, etc.) of rDNA sites, as stated by other researchers (Collares-Pereira and Rab 1999; Gromicho and Collares-Pereira 2004). It was also suggested that complex NOR phenotypes could be either a hybrid situation or the possible involvement of an association between mobile elements and rDNA loci (Kalous et al. 2008; Silva et al. 2013). Thus, CMA_3-staining and FISH with rDNA probes may be useful in understanding the detailed NOR phenotypes of the studied species.

This study showed that the karyological properties of the six species proved to be significantly similar at the macrostructural level. This suggests a close phylogenetic relationship between the species. Further, karyotypes with more biarmed chromosomes are regarded to represent a derived condition (Ganai et al. 2011). According to number of biarmed chromosomes and FNs, *P. egridiri*, *P. fahrettini* and *P. maeandri* can be considered to be primitive fishes when compared to *P. battalgilae*, *P. burduricus* and *P. evliyae*, which display a more derived karyotype.

As discussed in a study by Küçük and Güçlü (2014b), the taxonomy of the genus *Pseudophoxinus* remains complex in light of results of morphological and molecular studies. Based on the findings of the study, it is considered that this study shall contribute to the cytotaxonomy of Anatolian *Pseudophoxinus* species.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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