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Cytogenetic Characteristics of Two Species of the Genus *Pseudophoxinus* Bleeker, 1860 (Cypriniformes: Leuciscidae) from Anatolia, Turkey

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Abstract: The karyotypes of two Anatolian endemic leuciscid species, *Pseudophoxinus alii* Küçük, 2007 and *P. elizavetae* Bogutskaya, Küçük & Atalay, 2007, were studied using conventional cytogenetic methods. The diploid chromosome numbers were invariably *2n* = 50. The chromosome morphology in the karyotypes was as follows: 18 metacentric, 24 submetacentric and eight subtelo-acrocentric chromosomes in *P. alii* and eight metacentric, 34 submetacentric and eight subtelo-acrocentric chromosomes in *P. elizavetae*. Heteromorphic sex chromosomes were not observed in the two species. The largest chromosome pair in the karyotypes of *P. alii* and *P. elizavetae* was a subtelo-acrocentric. C-bands were determined on the pericentromeric regions of the most of the chromosomes in *P. alii* and on the pericentromeric regions of some chromosomes in *P. elizavetae*. Multiple nucleolus organizer regions (NORs) were observed in the two species. A review of the data on the karyotypes of 13 species of the genus *Pseudophoxinus* occurring in Turkey is presented.

Key words: *Pseudophoxinus alii*, *Pseudophoxinus elizavetae*, fish cytotaxonomy, chromosome number, *2n* = 50, C-banding, Ag-NOR

Introduction

The genus *Pseudophoxinus* Bleeker, 1860 belongs to the family Leuciscidae and its subfamily Leuciscinae (Çiçek et al. 2020). The majority of the members of this genus have very small geographical ranges, occurring in streams or springs and are distributed in the inland waters of Anatolia (Freyhof & Özuluğ 2010). The genus *Pseudophoxinus* has the greatest endemicity among the leuciscids (Çiçek et al. 2018). This genus has 22 endemic species in the inland waters of Anatolia (Çiçek et al. 2018, Saç et al. 2019). Of these, *P. alii* is endemic to southern Anatolia and *P. elizavetae* is endemic to central Anatolia (Çiçek et al. 2018).

Besides the fish taxonomy and fish molecular studies, cytogenetic studies are also very common and useful. Fish cytogenetics has been developed since 1960 (Chiarelli & Capanna 1973). Obtaining chromosomes and karyotypes from fish species is often difficult. However, improvements in methodological approaches have contributed to chromosome analyses for evaluating fish karyotypic diversity (Bertollo et al. 2015). Chromosomal studies in fish are often limited to the determination of *2n*, fundamental number (Fn) and chromosome morphology.

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C-banding and silver-staining are also widely used methods in fish cytogenetics (Rábová et al. 2015). The first karyological study on the genus *Pseudophoxinus* focused on *Pseudophoxinus firati* Bogutskaya, Küçük & Atalay, 2006 (Karasu 2009). In this and in the subsequent studies, *2n*, chromosome morphologies and chromosomal banding properties of 11 species of this genus have been reported: *P. antalyae* Bogutskaya, 1992, *P. battalgilae* Bogutskaya, 1997, *P. burduricus* Küçük, Gülle, Güçlü, Çiftçi & Erdoğan, 2013, *P. crassus* (de Ladige, 1960), *P. egridiri* (M. S. Karaman, 1972), *P. evliyae* Freyhof & Özuluğ, 2010, *P. fahrettini* Freyhof & Özuluğ, 2010, *P. firati*, *P. hittitorum* Freyhof & Özuluğ, 2010, *P. maeandri* (de Ladige, 1960) and *P. zekayi* Bogutskaya, Küçük & Atalay, 2006 (see Ergene et al. 2010, Karasu et al. 2011, Unal et al. 2014, Ayata et al. 2016, Unal & Gaffaroğlu 2016). However, there is no information about Giemsa-stained karyotypes, C-banding and silver-staining properties of *P. alii* Küçük, 2007 and *P. elizavetae* Bogutskaya, Küçük & Atalay, 2007.

The aim of the present study is to determine the chromosomal characteristics of *P. alii* (from southern Anatolia, Turkey) and *P. elizavetae* (from central Anatolia, Turkey) through conventional cytogenetic methods (Giemsa staining, C-banding and silver-staining).

Materials and Methods

Twenty individuals of *P. alii* were collected from Aksu Stream, Antalya, Turkey (37°04′N, 30°54′E) and 15 individuals of *P. elizavetae* were collected from Sultan Swamps, Develi, Kayseri, Turkey (38°22′N, 35°21′E) (Fig. 1). Both areas had shallow, clean and slow-flowing waters. The individuals were transported alive to the laboratory and kept in well-aerated aquaria until analysis. The study was permitted by the Gazi University Ethics Committee for Animal Experiments (permit number 160-23299). The air-drying technique of Bertollo et al. (2015) was applied to the "head" kidney for chromosome preparations. Ten chromosome slides were prepared from each studied individual. Firstly, some of the chromosome slides were stained by 5% Giemsa. After chromosomal analysis, the individuals were deposited in 70% ethanol at the Cytogenetic Research Laboratory of the Faculty of Arts and Sciences of the Kırşehir Ahi Evran University, Kırşehir, Turkey, under collection numbers MG 300-335. The C-banding technique of Sumner (1972) was used for determining constitutive heterochromatin regions. The silver-staining technique of Howell & Black (1980) was used for visualization of NORs. The chromosome slides were observed under a Leica DM 3000 microscope (Leica Microsystems GmbH, Germany). The photographs of stained metaphases were captured with AKAS software (Argenit Mikrosistem, Turkey). At least 100 metaphase plaques were counted from each species to determine the *2n*.

The chromosomes were measured using a digital device. Karyotypes were arranged manually. The chromosomes were classified according to Levan et al. (1964). For calculating the Fn, metacentric and submetacentric chromosomes were classified as bi-armed, whereas subtelo-acrocentric chromosomes were classified as uniarmed. For determining constitutive heterochromatin regions, at least five banded metaphase plaques were observed from each individual (102 in *P. alii* and 78 in *P. elizavetae*). For de-

Fig. 1. Collecting sites (red circles) of *Pseudophoxinus alii* and *Pseudophoxinus elizavetae*. Map prepared using Google Earth Pro, 2021.

termining Ag-NORs, at least four banded metaphase plaques were observed from each individual (64 in *P. alii* and 60 in *P. elizavetae*). Image processing was performed in Adobe Photoshop CS6.

Results

The diploid chromosome numbers of *P. alii* and *P. elizavetae* were *2n* = 50 (Figs. 2a, 3a). We recorded the following chromosome morphologies in the karyotype of *P. alii*: 18 metacentric, 24 submetacentric and eight subtelo-acrocentric chromosomes (Fig. 2b). In the karyotype of *P. elizavetae*, we recorded eight metacentric, 34 submetacentric and eight subtelo-acrocentric chromosomes (Fig. 3b). No heteromorphic sex chromosomes were observed in the karyotypes of both *P. alii* and *P. elizavetae*. The largest chromosome pair in the karyotypes of *P. alii* and *P. elizavetae* was a subtelo-acrocentric. Fn was 92 in the two species. C-bands were detected on the pericentromeric regions of most of the chromosomes (except Nos. 5 metacentric, 13 and 19 submetacentric) in *P. alii* (Fig. 2c, 2d). On the other hand, C-bands were detected on the pericen-

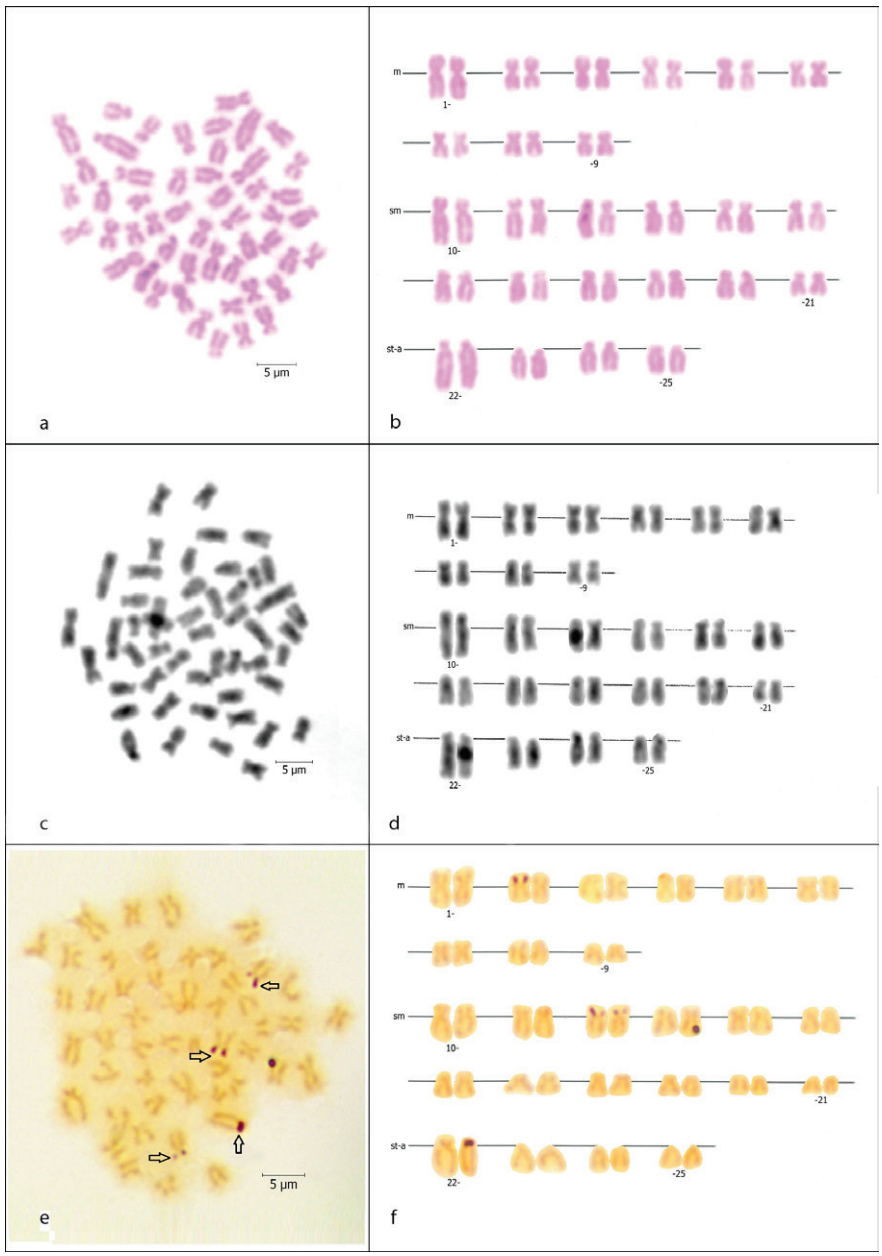

Fig. 2. Metaphases and karyotypes of *Pseudophoxinus alii*: a. Giemsa stained metaphase. b. Arranged karyotype of Giemsa stained metaphase. c. C-banded metaphase. d. Arranged karyotype of C-banded metaphase. e. Silver-stained metaphase (arrows indicate the Ag-NORs). f. Arranged karyotype of silver-stained metaphase. Scale bars: 5 µm. m: metacentric, sm: submetacentric, st-a: subtelo-acrocentric.

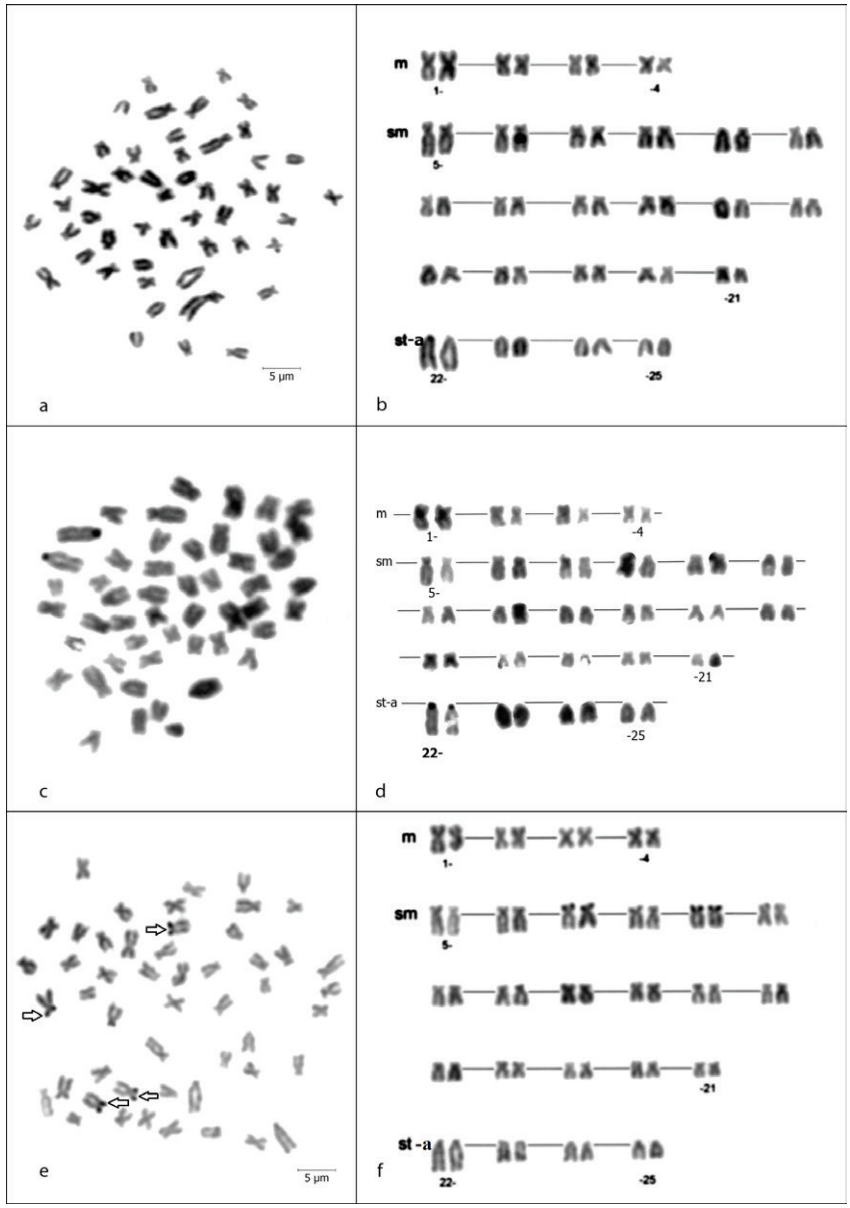

Fig. 3. Metaphases and karyotypes of *Pseudophoxinus elizavetae*: a. Giemsa stained metaphase. b. Arranged karyotype of Giemsa stained metaphase. c. C-banded metaphase. d. Arranged karyotype of C-banded metaphase; e. Silver-stained metaphase (arrows indicate the Ag-NORs). f. Arranged karyotype of silver-stained metaphase. Scale bars: 5 µm. m: metacentric, sm: submetacentric, st-a: subtelo-acrocentric.

Table 1. Ag-NOR number variations in *Pseudophoxinus alii* and *P. elizavetae*.

Number of Ag-NOR sites	**Ag-stained metaphase plate (%) of *P. alii***	**Ag-stained metaphase plate (%) of *P. elizavetae***
1	1.56	1.65
2	24.28	15
3	15.62	16.7
4	39.8	55
5	7.81	10
6	9.37	1.65
8	1.56	---

tromeric regions of some of the chromosomes (Nos. 1 and 2 metacentric, 17 submetacentric and 22 subtelo-acrocentric) in *P. elizavetae* (Fig. 3c, 3d). Multiple Ag-NORs were detected in both species, with the most common Ag-NOR number for both species being four (Fig. 2e, 3e). Ag-NORs were located on the terminal regions of the short arms of Nos. 2 (metacentric), 12 (submetacentric) and 22 (subtelo-acrocentric) chromosomes in *P. alii* (Fig. 2f). Ag-NORs were located on the terminal regions of the short arms of Nos. 7 (submetacentric) and 9 (submetacentric) chromosomes in *P. elizavetae* (Fig. 3f). The number of Ag-NOR sites varied from

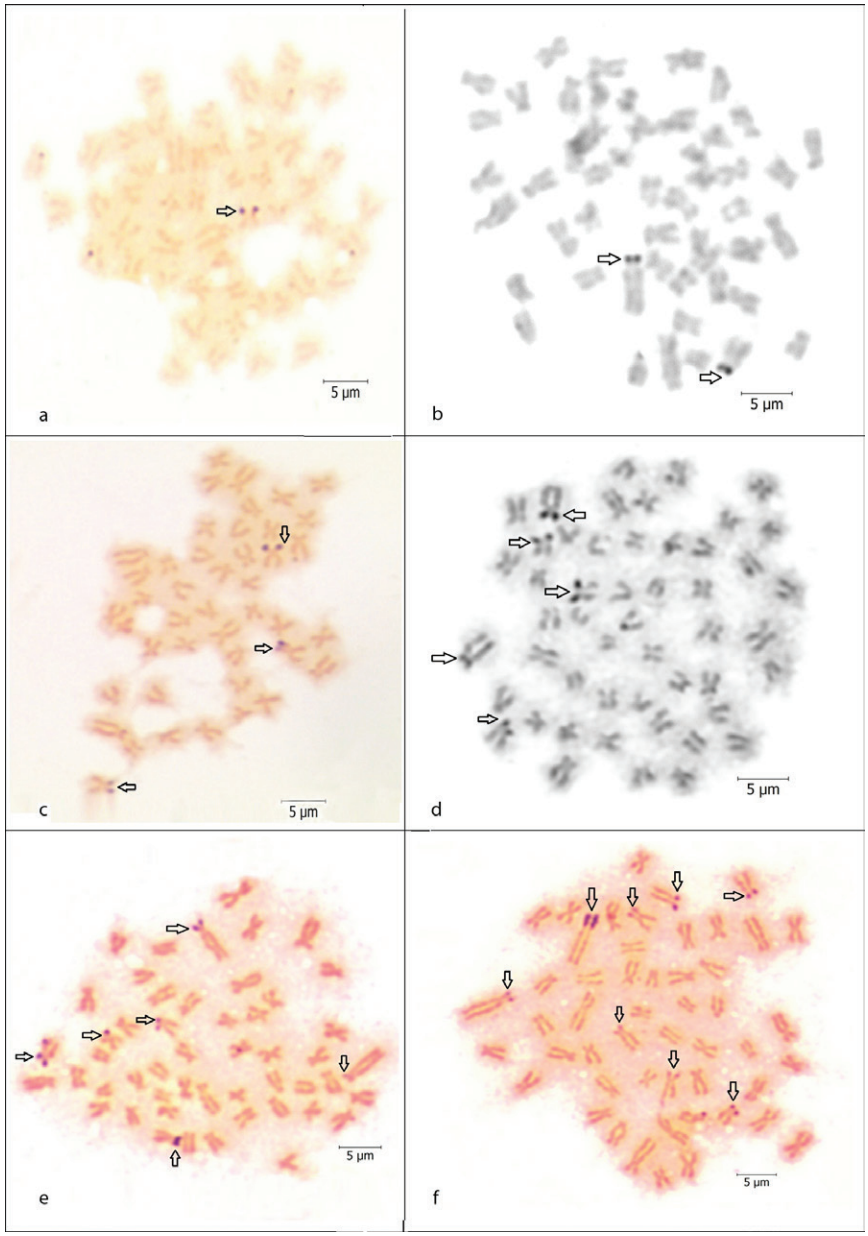

Fig. 4. Ag-stained metaphases of *Pseudophoxinus alii*: a. One Ag-NOR indicated by arrow. b. Two Ag-NORs indicated by arrows. c. Three Ag-NORs indicated by arrows. d. Five Ag-NORs indicated by arrows. e. Six Ag-NORs indicated by arrows. f. Eight Ag-NORs indicated by arrows. Scale bars: 5 µm.

one to eight in the 64 scored Ag-NOR stained metaphase plates in *P. alii* (Table 1, Fig. 4a, 4b, 4c, 4d, 4e and 4f). These Ag-NORs were localised on the terminal regions of metacentric, submetacentric and subtelo-acrocentric chromosomes of *P. alii* (Fig. 4a, 4b, 4c, 4d, 4e and 4f). The number of Ag-NOR sites in *P. elizavetae* varied from one to six in the 60 scored Ag-NOR stained metaphase plates (Table 1, Fig. 5a, 5b, 5c, 5d and 5e). These Ag-NORs were localised on the terminal regions of submetacentric chromosomes of *P. elizavetae* (Fig. 5a, 5b, 5c, 5d and 5e).

Discussion

The karyotypes of leuciscids are characterised by the presence of small chromosomes, with their centromere position ranging gradually from median to nearly terminal (Ráb 1991) as observed in this study. Leuciscids possess an invariant $2n = 50$, with a uniform karyotype comprising dominantly biarmed chromosomes and only two to four pairs of uniarmed chromosomes (Ráb et al. 2008). The abovementioned properties have been observed also in *P. alii* and *P. elizavetae*. Thirteen out of 22 Anatolian endemic species of the genus *Pseudophoxinus* have

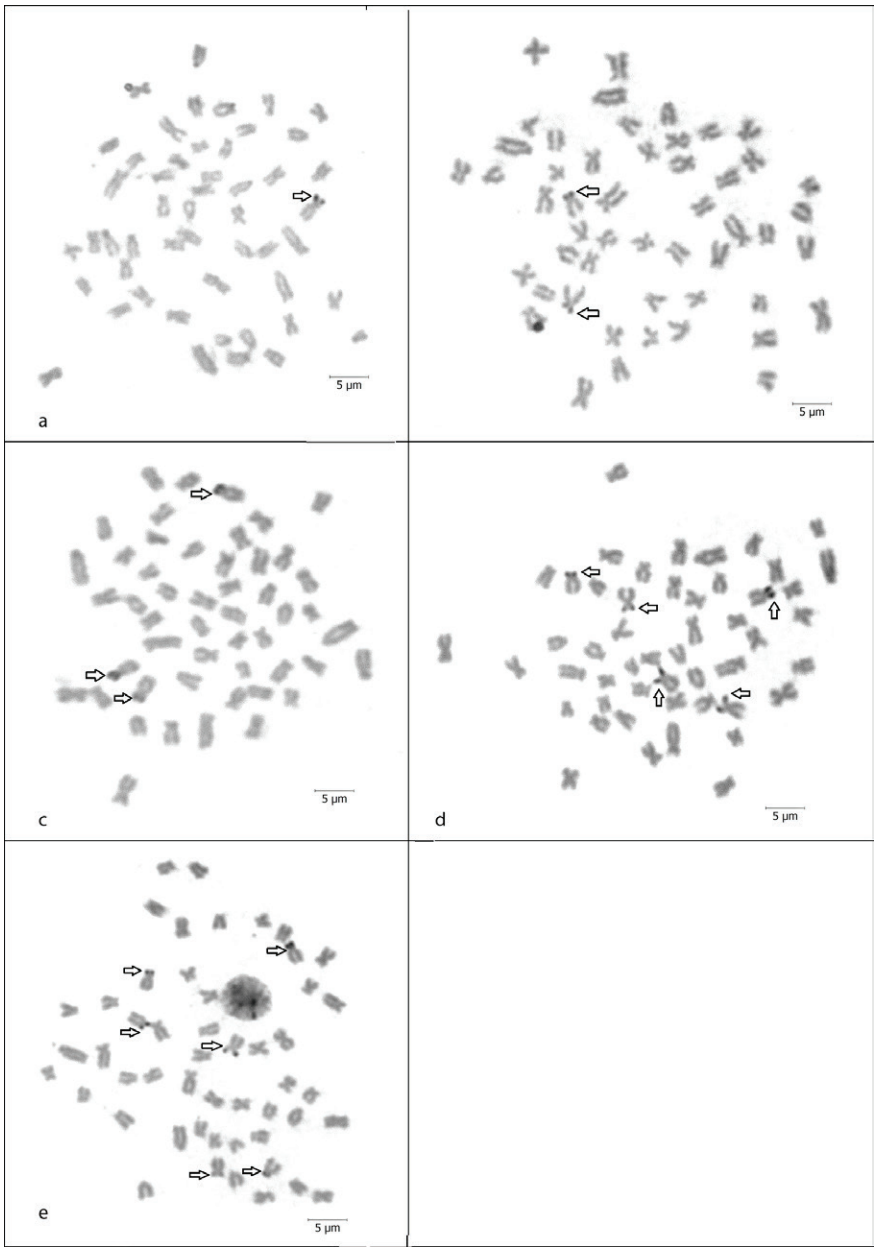

Fig. 5. Ag-stained metaphases of *Pseudophoxinus elizavetae*: a. One Ag-NOR indicated by arrow. b. Two Ag-NORs indicated by arrows. c. Three Ag-NORs indicated by arrows. d. Five Ag-NORs indicated by arrows. e. Six Ag-NORs indicated by arrows. Scale bars: 5 µm.

been already studied karyologically (Table 2). There is no difference between the *2n* numbers of 13 species of *Pseudophoxinus* (Table 2). Unal et al. (2014) suggested that in all species with *2n* = 50 having the general leuciscine chromosome number, a conserved karyotypic evolution is observed. However, some differences in the chromosome morphology have been observed (Table 2). The Fn varies from 88 to 94 in the members of *Pseudophoxinus* (Table 2). The Fn's values of *P. alii* and *P. elizavetae* are higher than those of *P. firati* (Karasu et al. 2011) and *P. hittitorum* (Unal et al. 2014) and further lower in *P. battalgilae*, *P. burduricus* and *P. evliyae* (Ayata et al. 2016). The differences in the chromosome morphologies and Fn should be a result of some chromosomal rearrangements (e.g., pericentromeric inversions and/or translocations involving centromeres) as discussed for six species of *Pseudophoxinus* (see Ayata et al. 2016) and also for two species of *Squalius* (see Ayata 2020).

Pseudophoxinus alii and *P. elizavetae* have 42 biarmed and eight uniarmed chromosomes similarly to *P. crassus* (Unal et al. 2014), *P. egridiri*, *P. fahrettini*, *P. maeandri* (Ayata et al. 2016) and *P. zekayi* (Unal & Gaffaroğlu 2016). However, *P. battal-*

Table 2. Karyological data for species of the genus *Pseudophoxinus* from Turkey. Legend: *2n*: diploid chromosome number, Fn: fundamental number, m: metacentric, sm: submetacentric, st-a: subtelo-acrocentric

Species	2n	Chromosome morphology	Fn	References
P. antalyae	50	16m+14sm+12st+8a	92	Ergene et al. (2010)
P. firati	50	38m-sm+12st	88	Karasu et al. (2011)
P. crassus	50	12m+30sm+8st-a	92	Unal et al. (2014)
P. hittitorum	50	14m+26sm+10st-a	90	Unal et al. (2014)
P. battalgilae	50	16m+28sm+6st-a	94	Ayata et al. (2016)
P. burduricus	50	18m+26sm+6st-a	94	Ayata et al. (2016)
P. egridiri	50	14m+28sm+8st-a	92	Ayata et al. (2016)
P. evliyae	50	14m+30sm+6st-a	94	Ayata et al. (2016)
P. fahrettini	50	16m+26sm+8st-a	92	Ayata et al. (2016)
P. maeandri	50	10m+32sm+8st-a	92	Ayata et al. (2016)
P. zekayi	50	16m+26sm+8st-a	92	Unal & Gaffaroğlu (2016)
P. alii	50	18m+24sm+8st-a	92	This study
P. elizavetae	50	8m+34sm+8st-a	92	This study

gilae, *P. burduricus* and *P. evliyae* have 44 biarmed and six uniarmed chromosomes (Ayata et al. 2016). Another group of species consisting of *P. antalyae* (Ergene et al. 2010), *P. firati* (Karasu et al. 2011) and *P. hittitorum* (Unal et al. 2014) have less biarmed chromosomes than all of the above-mentioned species. According to Ganai et al. (2011), the presence of more biarmed chromosomes than uniarmed chromosomes represents a case of derived karyotype evolution. According to the number of biarmed chromosomes, *P. alii* and *P. elizavetae* should have a derived karyotype, similarly to *P. crassus* (Unal et al. 2014), *P. egridiri*, *P. fahrettini*, *P. maeandri* (Ayata et al. 2016) and *P. zekayi* (Unal & Gaffaroğlu 2016). In contrast, *P. antalyae* (Ergene et al. 2010), *P. firati* (Karasu et al. 2011) and *P. hittitorum* (Unal et al. 2014) should have less-evolved karyotypes on the basis of this character. Based on the afore-mentioned characteristic, *P. battalgilae*, *P. burduricus* and *P. evliyae* (Ayata et al. 2016) should display derived karyotypes as opposed to *P. alii*, *P. elizavetae* and all other species of *Pseudophoxinus* (Table 2). The largest chromosome pair is characteristically subtelo-acrocentric, which is a leuciscid marker chromosome in cytotaxonomy (Ráb et al. 2008). This marker was observed in the karyotypes of *P. alii* and *P. elizavetae*, similarly to all the other species of *Pseudophoxinus* (Karasu et al. 2011, Unal et al. 2014, Ayata et al. 2016, Unal & Gaffaroğlu 2016).

Heteromorphic sex chromosomes determined on the karyotypes are known only in a restricted group of fish species (Arai 2011). No heteromorphic sex chromosomes were observed in 11 species of *Pseudophoxinus* from Anatolia (Ergene et al. 2010, Karasu et al. 2011, Unal et al. 2014, Ayata et al. 2016, Unal & Gaffaroğlu 2016) as *P. alii* and *P. elizavetae*.

Chromosomal banding properties like C-banding and silver-staining are usually studied in fish cytogenetics and contribute to fish cytotaxonomy (Valic et al. 2010). In the C-banded metaphases, *P. alii* has more C-bands than *P. elizavetae*. In our study, C-bands were observed on the pericentromeric regions as in *P. antalyae* (Ergene et al. 2010), *P. firati* (Karasu et al. 2011), *P. crassus*, *P. hittitorum* (Unal et al. 2014), *P. battalgilae*, *P. burduricus*, *P. egridiri*, *P. evliyae*, *P. fahrettini*, *P. maeandri* (Ayata et al. 2016) and *P. zekayi* (Unal & Gaffaroğlu 2016). However, heterochromatic blocks that had been determined in *P. antalyae* (Ergene et al. 2010), *P. egridiri* and *P. fahrettini* (Ayata et al. 2016) were not observed in the C-banded metaphases of *P. alii* and *P. elizavetae*. It is assumed that single Ag-NOR is a common feature in most European leuciscine fishes and it has been reported as an ancestral character (Ráb et al. 2000, Valic et al. 2010, Rossi et al. 2012, Nabais et al. 2013). Single Ag-NOR was reported in *P. antalyae* (Ergene et al. 2010), *P. crassus*, *P. hittitorum* (Unal et al. 2014), *P. egridiri* (Ayata et al. 2016) and *P. zekayi* (Unal & Gaffaroğlu 2016). *Pseudophoxinus alii* and *P. elizavetae* are different from the above-mentioned species based on the number of Ag-NORs. In most Ag-stained metaphases, *P. alii* and *P. elizavetae* have four Ag-NORs like *P. firati* (Karasu et al. 2011), *P. battalgilae*, *P. evliyae* and *P. maeandri* (Ayata et al. 2016). However, multiple Ag-NORs are suggested as a derived character (Valic et al. 2010). Also, it seems that Ag-NORs variability is a common feature in *P. alii* and *P. elizavetae* as well as in *P. battalgilae*, *P. burduricus*, *P. evliyae*, *P. fahrettini* (Aya-

ta et al. 2016) and *P. zekayi* (Unal & Gaffaroğlu 2016). This variability should be based on some rDNA rearrangements like duplication, translocation and amplification (Ayata et al. 2016). Previously, Ag-NOR locations were reported in *P. crassus*, *P. hittitorum* (Unal et al. 2014), *P. battalgilae*, *P. burduricus*, *P. egridiri*, *P. evliyae*, *P. fahrettini*, *P. maeandri* (Ayata et al. 2016) and *P. zekayi* (Unal & Gaffaroğlu 2016). Ag-NOR locations were on subtelo-acrocentric chromosomes in some silver-stained metaphases of *P. alii*, similarly to *P. antalyae* (Ergene et al. 2010) and *P. firati* (Karasu et al. 2011). The Ag-NORs were localised invariably on submetacentric chromosomes in *P. elizavetae*; in contrast, they were on metacentric, submetacentric and subtelo-acrocentric chromosomes in *P. alii*. Therefore, this differentiation should be a marker for the two species.

This study has determined the karyomorphology of two endemic species of the genus *Pseudophoxinus* from Anatolia. In general, the karyological characteristics of the two species are similar. With the present study, we believe that the necessary data for the cytotaxonomic assessment of the species of the genus *Pseudophoxinus* are completed.

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