Acta Aquatica Turcica
E-ISSN: 2651-5474 16(2), 283-289 (2020) DOI:https://doi.org/10.22392/actaquatr.667595

Chromomycin A_3 and DAPI Staining of Chromosomes of Three Endemic *Pseudophoxinus* Bleeker, 1860 (Teleostei: Leuciscidae) Species from Anatolia

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Research Article

Received 30 December 2019; Accepted 10 April 2020; Release date 01 June 2020.

How to Cite: Karasu Ayata, M. (2020). Chromomycin A_3 and DAPI staining of chromosomes of three endemic *Pseudophoxinus* Bleeker, 1860 (Teleostei: Leuciscidae) species from Anatolia. *Acta Aquatica Turcica*, *16*(2), 283-289. https://doi.org/10.22392/actaquatr.667595

Abstract

The karyotypes and other chromosomal markers of representatives of the genus *Pseudophoxinus* have been little studied. Therefore, this study documents chromosomal NOR phenotypes as revealed using Chromomycin A_3 (CMA_3) and DAPI chromosomal stainings in three species *Pseudophoxinus elizavetae* Bogutskaya, Küçük & Atalay, 2007, *P. firati* Bogutskaya, Küçük & Atalay, 2007 and *P. hittitorum* Freyhof & Özuluğ, 2010, endemic in Anatolia. Simple NOR phenotype with only two CMA_3 positive signals were observed in the karyotype of *P. firati* whereas derived NOR phenotype with four CMA_3 positive signals were observed in those of *P. elizavetae* and *P. hittitorum*, respectively. No DAPI positive signal was detected in karyotypes in all three species. This study described new NOR phenotypes, i.e. number and position of major rDNA genes, in genomes of three *Pseudophoxinus* species contributing thus to known diversity of NOR phenotypes in otherwise karyotypically highly conservative leuciscid fishes.

Keywords: Fish cytotaxonomy, chromosome banding, chromosomal markers, major rDNA genes

Anadolu'da Yaşayan Endemik Üç *Pseudophoxinus* Bleeker, 1860 (Teleostei: Leuciscidae) Türünün CMA_3 ve DAPI Boyalı Kromozomları

Özet

Pseudophoxinus cinsindeki balıkların kromozom ve diğer sitotaksonomik markerları konusundaki çalışma sayısı azdır. Bu çalışmada, Anadolu'ya endemik üç leuciscine türü *Pseudophoxinus elizavetae* Bogutskaya, Küçük & Atalay, 2007, *P. firati* Bogutskaya, Küçük & Atalay, 2007 ve *P. hittitorum* Freyhof & Özuluğ, 2010'da kromozomal NOR fenotipleri CMA_3 ve DAPI kromozomal boyama ile çalışılmıştır. *P. firati*'nin karyotipinde iki, *P. elizavetae* ve *P. hittitorum*'un karyotiplerinde ise dört CMA_3 pozitif sinyal gözlenmiştir. Üç türün karyotipinde de DAPI pozitif sinyal gözlenmemiştir. Bu çalışma üç *Pseudophoxinus* türünün genomunda majör rDNA genlerinin sayısı ve pozisyonu gibi yeni NOR fenotiplerini tanımlamıştır. Böylece karyotipik olarak yüksek derecede konservatif bilinen leuciscid NOR fenotip çeşitliliğine katkıda bulunur.

Anahtar kelimeler: Balık sitotaksonomisi, kromozomal bantlama, kromozom markerları, majör rDNA genleri

INTRODUCTION

The genus *Pseudophoxinus* Bleeker, 1860 belongs to family Leuciscidae and its subfamily Leuciscinae (Tan and Ambruster, 2018). The genus *Pseudophoxinus* contains at least 30 locally endemic species in Anatolia and other countries of Near and Middle East (Froese and Pauly, 2020). This genus has 21 endemic species in the freshwaters of Anatolia (Güçlü and Küçük, 2017; Saç et al., 2019). Karyological studies of representatives of this genus have started just recently (Karasu, 2009). In, the last decade, *Pseudophoxinus antalyae*, *P. battalgilae*, *P. burduricus*, *P. crassus*, *P. egridiri*, *P. elizavetae*, *P. evliyae*, *P. fahrettini*, *P. firati*, *P. hittitorum*, *P. maeandri* and *P. zekayi* have been studied cytogenetically (Table 1). In these studies, karyotypes, C-banding and Ag-NOR stainings have been performed. Also, G- and Q-banding patterns in the karyotype of *P. antalyae* have been reported (Ergene et al., 2010).

Table 1. Cytogenetic data for the representatives of the genus *Pseudophoxinus* from Anatolia, Turkey

Species	2n	Chromosome morphology	FN	Ag-NOR number	References
P. antalyae	50	16m+14sm+12st+8a	92	2	Ergene et al., 2010
P. firati	50	38m-sm+12st	88	4	Karasu et al., 2011
P. elizavetae	50	8m+34sm+8st	92	4	Gaffaroğlu et al., 2014
P. crassus	50	12m+30sm+8st-a	92	2	Ünal et al., 2014
P. hittitorum	50	14m+26sm+10st-a	90	2	Ünal et al., 2014
P. battalgilae	50	16m+28sm+6st-a	94	4	Ayata et al., 2016
P. burduricus	50	18m+26sm+6st-a	94	2	Ayata et al., 2016
P. egridiri	50	14m+28sm+8st-a	92	2	Ayata et al., 2016
P. evliyae	50	14m+30sm+6st-a	94	4	Ayata et al., 2016
P. fahrettini	50	16m+26sm+8st-a	92	2	Ayata et al., 2016
P. maeandri	50	10m+32sm+8st-a	92	4	Ayata et al., 2016
P. zekayi	50	16m+26sm+8st-a	92	2	Ünal and Gaffaroğlu, 2016

2n: diploid chromosome number, FN: fundamental number, m: metacentric, sm: submetacentric, st-a: subtelo-acrocentric

The number and locations of NORs have been used as a cytotaxonomic character in fish cytotaxonomy (Rábová et al., 2015). Ag-NOR staining is a very common cytogenetic technique (Rábová et al., 2015). However, this method identifies only transcriptionally active rDNA genes. Also, the number and location of NORs have been determined using CMA_3 staining (Gornung, 2013; Rábová et al., 2015). This staining is based on GC-specific fluorochrome and detects both active and inactive NORs in fish (Gornung, 2013). Both Ag- and CMA_3 stainings have been done in many fish species (Arai, 2011; Gornung, 2013). Fluorescence staining with 4',6-diamidino-2-phenyl-indole (DAPI) dyes detect chromosomal regions that contain AT-rich DNA (Kapuscinski, 1995). This method is studied on many fish species too (Mayr et al., 1986; Valic et al., 2010).

A presence of four Ag-NORs on submetacentric (sm) chromosomes in karyotype of *P. elizavetae* (Gaffaroğlu et al., 2014) and four Ag-NORs on sm-subtelocentric (st) chromosomes in that of *P. firati* (Karasu et al., 2011) have previously been reported. Additionally, Ünal et al. (2014) observed two Ag-NORs on sm chromosome pair in karyotype of *P. hittitorum*. The present study was carried out to determine NOR phenotypes using CMA_3 staining and DAPI stainings in *P. elizavetae*, *P. firati* and *P. hittitorum*, endemic in Anatolia.

MATERIALS and METHODS

The analyzed individuals of *P. elizavetae*, *P. firati* and *P. hittitorum* were collected by electrofishing from Anatolia, Turkey (Table 2, Figure 1). The specimens were carried alive to the laboratory. The chromosomes were obtained from head kidney cells according to the air drying technique of Bertollo et al. (2015). Slides were stained with CMA_3/DAPI stainings according to Rábová et al. (2015). For this technique, slides were treated with an ice-cold ethanol series (70%, 90%, 100%, 2 minutes in each). After this step, work continued in the dark. Slides were incubated in McIlvaine buffer with $MgCL_2$ for 10 minutes at room temperature. Then stained with CMA_3 in a wet chamber for 15 minutes, at room temperature. Slides were covered with a coverslip and washed up shortly in McIlvaine buffer and allowed the coverslip to detach. After this step, slides were incubated in McIlvaine buffer with Methyl Green for 15 minutes at room temperature. Slides were washed shortly in McIlvaine buffer again. Then, slides were stained with DAPI in a wet chamber for 20 minutes at room temperature. Slides were washed shortly in McIlvaine buffer and drops were removed by touching slide, mounted with antifade (glycerol-propylgalate), and sealed the margins with nail polish. Sealed slides were placed on a tray covered by aluminum foil and kept in the fridge several days before the examination. Slides were screened in an Olympus BX 60 epifluorescence microscope equipped with a DP50 Olympus CCD camera. At least 10 metaphases were examined per individual. Karyotypes from CMA_3 stained metaphases were arranged manually.

Table 2. General information regarding collected individuals

Species	Locality	Coordinates	Date	Number
P. elizavetae	Sultan Swamps, Develi, Kayseri	38°22'N, 35°21'E	2010	15
P. firati	Tohma Creek, Gürün, Sivas	38°47′N, 36°57′E	2009	20
P. hittitorum	Spring Eflatun, Beyşehir, Konya	37°52'N, 31°34'E	2010	8

Figure 1. The map of Turkey with three collection sites of *Pseudophoxinus* species. The map was created using the software Google Earth Pro

RESULTS and DISCUSSION

Karyotypes of examined species corresponded to previous reports, i.e. 2n = 50 and karyotypes composed of 8 m chromosomes, 34 sm chromosomes and 8 st chromosomes in *P. elizavetae*; 38 m-sm chromosomes and 12 st chromosomes in *P. firati*; 14 m chromosomes, 26 sm chromosomes, and 10 st-a chromosomes in *P. hittitorum* (Karasu et al., 2011; Gaffaroğlu et al., 2014; Ünal et al., 2014).

Four CMA_3 positive signals were observed in chromosomes of *P. elizavetae* (Figure 2A) and those of *P. hittitorum* (Figure 2E) whereas two CMA_3 positive signals were observed in the genome of *P. firati* (Figure 2C). CMA_3 staining revealed their location in the whole short (p) arms of medium-sized sm chromosome pair in karyotypes of all three species. No DAPI positive signal was detected in the genomes of examined species (Figures 2B, D, F).

Molecular methods are a highly efficient tool for providing species-specific markers in many areas of fish biology. As a result, the comprehensive use of both cytogenetic and molecular methods increase the level of certainty also in fish taxonomy (Kirtiklis et al., 2010; Gornung, 2013). Among the cytogenetic markers, NOR phenotypes, i.e. number and position of major rDNA sites on chromosomes, have played an important role in fish comparative cytogenetics. The number and localization of NORs have mainly been studied using silver nitrate ($AgNO_3$), Mithramycin (MM), or CMA_3 staining (e.g. Mani et al., 2009; Gornung, 2013; Rábová et al., 2015).

This study is the first attempt to characterize the CMA_3 and DAPI characteristics in Anatolian freshwater fishes. The result of CMA_3 staining confirmed Ag-NOR phenotype observed in *P. elizavetae* (Gaffaroğlu et al., 2014). In this study, CMA_3 positive signals were located in no. 7 and no. 9 in karyotype of *P. elizavetae* (Figure 3A). However, two more positive NOR sites were detected in chromosomes of *P. hittitorum* by CMA_3 staining. CMA_3 positive signals were located in no. 9 and no. 13 in karyotype of *P. hittitorum* (Figure 3C). This polymorphism could be the result of translocation of rDNA sites. Only two CMA_3 positive signals were observed in no. 5 in genome of *P. firati* (Figure 3B). In this sense, four Ag-NORs that reported by Karasu et al. (2011) should be a polymorphism in this species. Consequently, our data suggest that major rDNA genes have been conserved in sm chromosomes in the genomes of three endemic species of the genus *Pseudophoxinus*.

Characteristics of CMA_3 staining in *P. firati* resembles that in other leuciscins for instance *Leuciscus borysthenicus* (Ráb et al., 1996), *Pachychilon macedonicum* (Ráb et al., 2000), *Vimba vimba* and *V. elongata* (Rábova et al., 2003), some *Rutilus* and *Scardinius* species (Bianco et al.,

2004), *Squalius pyrenaicus* (Gromicho and Collares-Pereira, 2004), *S. aradensis* and *S. torgalensis* (Nabais et al. 2013).

Moreover, results of CMA_3 staining in *P. elizavetae* and *P. hittitorum* resembles that of *Leuciscus idus*, *L. cephalus* and *L. leuciscus* (Boron et al., 2009), *Iberochondrostoma almacai* and *I. lusitanicum* (Monteiro et al., 2009) and *Telestes ukliva* (Valic et al., 2010).

On the other hand, staining with DAPI did not show any positive signals along *P. elizavetae*, *P. firati* and *P. hittitorum* chromosomes. The same results were observed in chromosomes of other cyprinoid species (Mayr et al., 1986; Valic et al., 2010). Valic et al. (2010) reported that that this could be another common feature for genomes of cyprinids.

Also, FISH experiments have to be included in future studies to determine the exact number and position of NORs of the studied species. In conclusion, this study revealed the details of the NOR phenotypes in the three endemic species using CMA_3 and DAPI stainings.

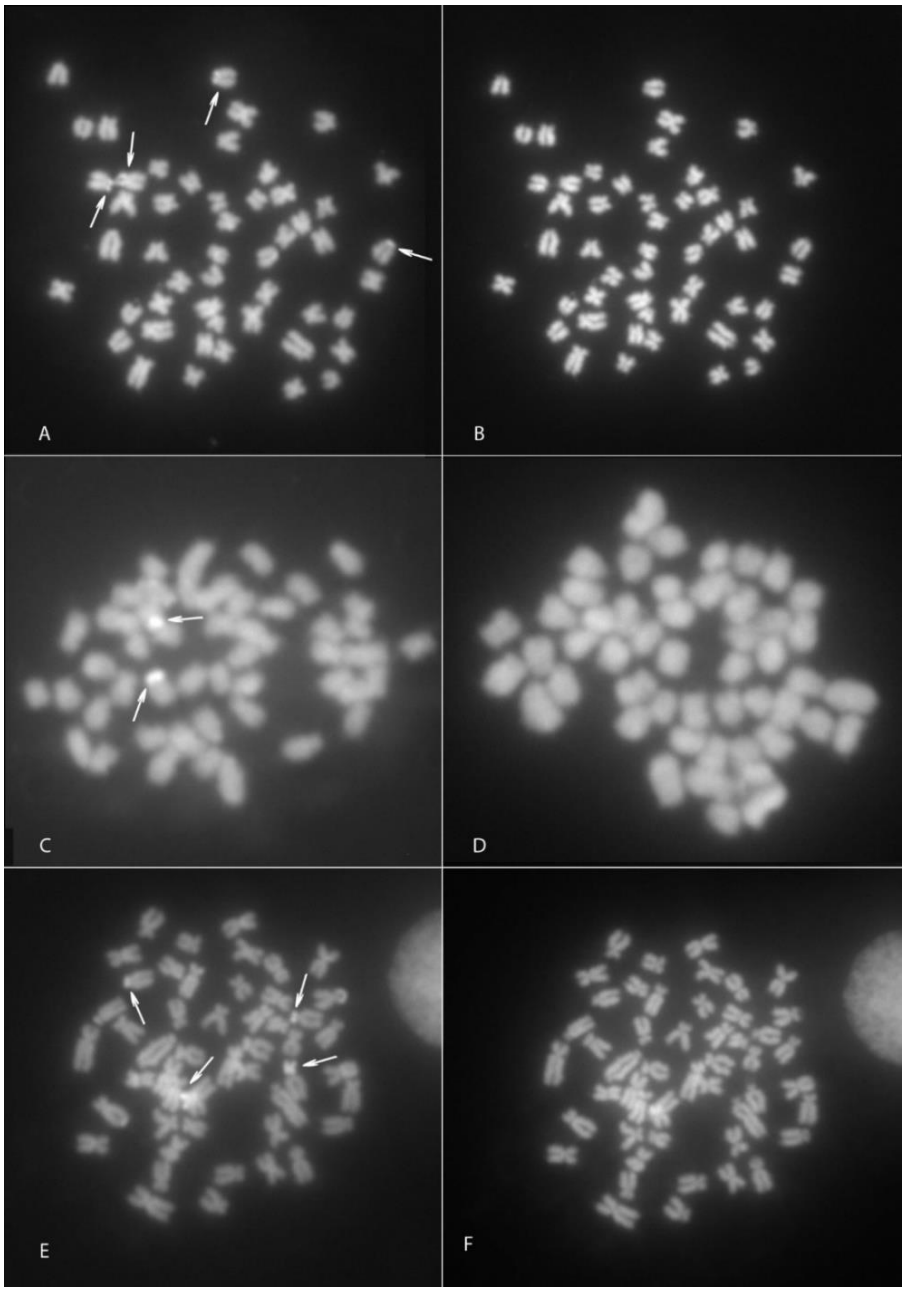

Figure 2. Metaphase of (A) *P. elizavetae*, (C) *P. firati*, (E) *P. hittitorum* after CMA_3 staining. Arrows indicate the CMA_3 positive signals. Metaphases of (B) *P. elizavetae*, (D) *P. firati*, (F) *P. hittitorum* after DAPI staining

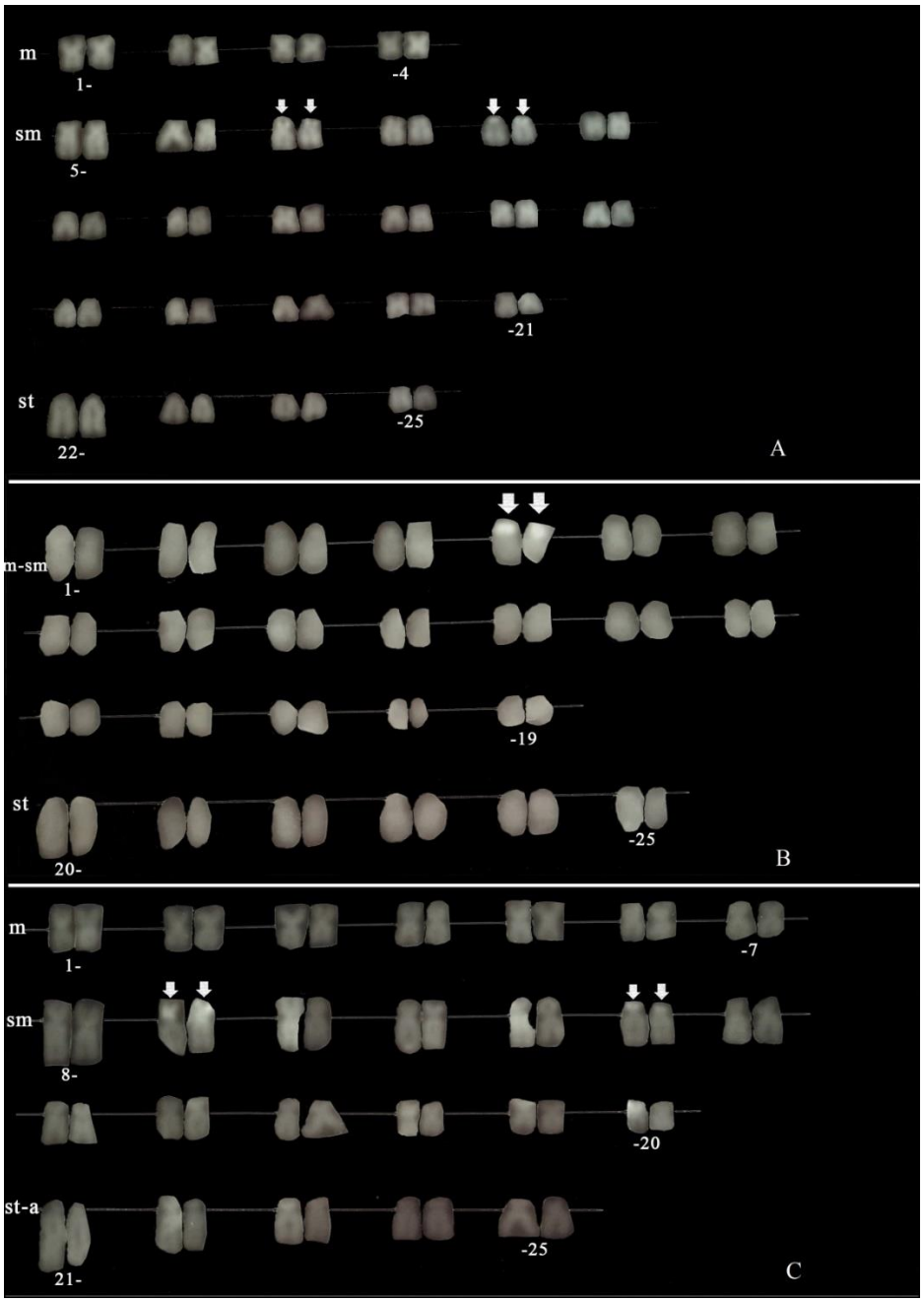

Figure 3. Arranged karyotypes of (A) *P. elizavetae*, (B) *P. firati*, (C) *P. hittitorum* after CMA_3 staining. Arrows indicate the CMA_3 positive signals

Acknowledgments: This study was supported by Kırşehir Ahi Evran University Scientific Research Projects (Project code: SYO.A4.18.001). The author is thankful to Prof. Muhammet Gaffaroğlu (Kırşehir Ahi Evran University) and Dr. Sevgi Ünal (Bartın University) for helping in the field and to Prof. Petr Ráb (Laboratory of Fish Genetics, Czechia) for his laboratory support.

REFERENCES

Arai, R. (2011). Fish karyotypes, A Check List. Springer, Japan.

Ayata, M.K., Yüksel, E., & Gaffaroğlu, M. (2016). Cytogenetic studies on six species of the leuciscine genus *Pseudophoxinus* Bleeker, 1860 (Teleostei, Cyprinidae). *Caryologia, 69*(3), 215-222.

Bertollo, L.A.C., Cioffi, M.B., & Moreira-Filho, O. (2015). Direct chromosome preparation from freshwater teleost fishes. In: Fish cytogenetic techniques, ray-fin fishes and chondrichthyans. Eds: Ozouf-Costaz, C., Pisano, E., Foresti, F., & Toledo, L.F.A. CRC Press, USA, pp: 21-26.

Bianco, P.G, Aprea, G., Balletto, E., Capriglione, T., Fulgione, D., & Odierna, G. (2004). The karyology of the cyprinid genera *Scardinius* and *Rutilus* in southern Europe. *Ichthyological Research, 51*, 274-278.

Boron, A., Porycka, K., Ito, D., Abe, S., & Kirtiklis, L. (2009). Comparative molecular cytogenetic analysis of three *Leuciscus* species (Pisces, Cyprinidae) using chromosome banding and FISH with rDNA. *Genetica, 135*, 199-207.

Ergene, S., Karahan, A., & Kuru, M. (2010). Cytogenetic analysis of *Pseudophoxinus antalyae*, Bogustkaya, 1992 (Pisces: Cyprinidae) from the Eastern Mediterranean River Basin. Turkey. *Turkish Journal of Zoology, 34*(1), 111-117.

Froese, R., & Pauly, D. (2020). FishBase [Website]. Retrieved from https://URLwww.fishbase.de/Nomenclature/ValidNameList.php?syng=Pseudophoxinus&syns=&vtitle=Scientific+Names+where+Genus+Equals+%3Ci%3EPseudophoxinus%3C%2Fi%3E&crit2=CONTAINS&crit1=EQUAL.

Gaffaroğlu, M., Ayata, M.K., Ünal, S., & Yüksel, E. (2014). Chromosomal analysis of *Pseudophoxinus elizavetae* Bogutskaya, Kücük and Atalay, 2007 (Teleostei, Cyprinidae) from Anatolia. FABA2014: International Symposium on Fisheries and Aquatic Science; 25-27 September; Trabzon.

Gornung, E. (2013). Twenty years of physical mapping of major ribosomal RNA genes across the teleosts: a review of research. *Cytogenetic Genome Research, 141*, 90-102.

Gromicho, M., & Collares-Pereira, M.J. (2004). Polymorphism of major ribosomal gene chromosomal sites (NOR phenotypes) in the hybridogenetic fish *Squalius alburnoides* complex (Cyprinidae) assessed through crossing experiments. *Genetica, 122*, 291-302.

Güçlü, S.S., & Küçük, F. (2017). Endemic species of the genus *Pseudophoxinus* (Teleostei: Cyprinidae) in Anatolia and distribution areas. *First International Symposium on Limnology and Freshwater Fisheries*. 4-6 October, 2017, Eğirdir, Turkey.

Kapuscinski, J. (1995). DAPI: a DNA-specific fluorescent probe. *Biotechnic and Histochemistry, 70*(5), 220-33.

Karasu, M. (2009). *Pseudophoxinus firati (Pisces:Cyprinidae)'nin karyotip özellikleri*. Gazi Üniversitesi Fen Bilimleri Enstitüsü, Yüksek Lisans Tezi, Ankara.

Karasu, M., Yüksel, E., & Gaffaroğlu, M. (2011). Karyotype, NORs, and C-banding analysis of *Pseudophoxinus firati* Bogutskaya, Küçük, & Atalay, 2007 (Actinopterygii, Cyprinidae) in the Euphrates River. Turkey. *Turkish Journal of Zoology, 35*(6), 865-868.

Kirtiklis, L., Porycka, K., Boroñ, A., Coutanceau, J.P., & Dettai, A. (2010). Use of the chromosomal co-location of the minor 5S and the major 28S rDNA as a cytogenetic marker within the genus *Leuciscus* (Pisces, Cyprinidae). *Folia biologica (Kraków), 58*, 3-4.

Mani, I., Kumar, R., Singh, M., Kushwaha, B., Nagpure, N.S., Srivastava, P.K., Murmu, K., Rao, D.S.K., & Lakra, W.S. (2009). Karyotypic diversity and evolution of seven mahseer species (Cyprinidae) from India. *Journal of Fish Biology, 75*, 1079-1091.

Mayr, B., Rab, P., & Kalat, M. (1986). NORs and counterstain-enhanced fluorescence studies in Cyprinidae of different ploidy level. *Genetica, 69*(2), 111-118.

Monteiro, R., Carvalho, C., & Collares-Pereira, M.J. (2009). Karyotype and genome size of *Iberochondrostoma almacai* (Teleostei, Cyprinidae) and comparison with the sister species *I. lusitanicum*. *Genetics and Molecular Biology, 32*(2), 268-275.

Nabais, C., Rampin, M., & Collares-Pereira, M.J. (2013). Comparative cytogenetics of two endangered leuciscine fish, *Squalius aradensis* and *S. torgalensis* (Teleostei, Cyprinidae), from the Iberian Peninsula. *Comparative Cytogenetics, 7*(1), 33-42.

Ráb, P., Rábová, M., Economidis, P.S., & Triantaphyllidis, C. (2000). Banded karyotype of the Greek endemic cyprinid fish. *Pachychilon macedonicum*. *Ichthyological Reserarch, 47*(1), 107-110.

Ráb, P., Karakousis, M.Y., Rábová, M., & Economidis, P.S. (1996). Banded karyotype of the cyprinid fish *Leuciscus borysthenicus*. *Ichthyological Research, 43*(4), 463-468.

Rábová, M., Ráb, P., Ozouf-Costaz, C., Ene, C., & Wanzebock, J. (2003). Comparative cytogenetics and chromosomal characteristics of ribosomal DNA in the fish genus *Vimba* (Cyprinidae). *Genetica, 118*, 83-91.

Rábová, M., Volker, M., Pelikánová, Š., & Ráb, P. (2015). Sequential chromosome bandings in fishes. In: Fish cytogenetic techniques, ray-fin fishes and chondrichthyans. Eds: Ozouf-Costaz, C., Pisano, E., Foresti, F., & Toledo, L.F.A. CRC Press, USA, pp: 66-73.

Saç, G., Özuluğ, M., Elp, M., Gaffaroğlu, M., Ünal, S., Karasu Ayata, M., Kaya, C., & Freyhof, J. (2019). New records of *Pseudophoxinus firati* from Turkey (Teleostei: Leuciscidae). *Journal of Applied Ichthyology, 1*, 1-6.

Tan M., & Armbruster J.W. (2018). Phylogenetic classification of extant genera of fishes of the order Cypriniformes (Teleostei, Ostariophysi). *Zootaxa, 4476*(1), 006-039.

Ünal, S., & Gaffaroğlu, M. (2016). Karyology of six cyprinid fishes from Seyhan and Ceyhan rivers in Anatolia. *Caryologia, 69*(4), 362-369.

Ünal, S., Gaffaroğlu, M., Ayata, M.K., & Yüksel, E. (2014). Karyotype, C-banding and AgNORs of two endemic leuciscine fish, *Pseudophoxinus crassus* (Ladiges, 1960) and *P. hittitorum* Freyhof & Özulug, 2010 (Teleostei, Cyprinidae). *Comparative Cytogenetics, 8*(4), 249-257.

Valic, D., Kapetanovıć, D., Zanella, D., Mrakovčić, M., Teskeredžıć, E., Besendorfer, V., Rabova, M., & Rab, P. (2010). The karyotype and NOR phenotype of *Telestes ukliva* (Cyprinidae). *Folia Zoologica, 59*(2), 169-173.