CompCytogen 8(4): 249–257 (2014)
doi: 10.3897/CompCytogen.v8i4.7623
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Karyotype, C-banding and AgNORs of two endemic leuciscine fish, *Pseudophoxinus crassus* (Ladiges, 1960) and *P. hittitorum* Freyhof & Özulug, 2010 (Teleostei, Cyprinidae)

Sevgi Unal[1], Muhammet Gaffaroğlu[2], Muradiye Karasu Ayata[2], Eşref Yüksel[1]

1 *Gazi University, Science Faculty, Department of Biology, Ankara, Turkey* **2** *Ahi Evran University, Science and Art Faculty, Department of Biology, Kırşehir, Turkey*

Corresponding author: *Sevgi Ünal* (sevgiunal@ymail.com)

Academic editor: *Natalia Golub* | Received 1 April 2014 | Accepted 8 October 2014 | Published 31 October 2014

http://zoobank.org/761AB8E5-0659-487B-A715-C465E2C6D08F

Citation: Unal S, Gaffaroğlu M, Ayata MK, 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. doi: 10.3897/CompCytogen.v8i4.7623

Abstract

The genus *Pseudophoxinus* Bleeker, 1860 is found in a wide range of habitats in central Anatolia, but it is not well known from a cytogenetic aspect. In this study the first karyotypic description of the spring minnows *Pseudophoxinus crassus* (Ladiges, 1960) and *P. hittitorum* Freyhof & Özulug, 2010 by means of conventional methods (Giemsa staining, C-banding, silver nitrate impregnation (Ag-NORs)) was performed. Both species are endemic and have restricted distributions in Central Anatolia. *P. crassus* and *P. hittitorum* have the same diploid chromosome number, 2n = 50, patterns of distribution of constitutive heterochromatin (CH), and localization of nucleolus organizer regions (NORs), but differ in their karyotypic formulae (KFs). The C-banding technique revealed clear pericentromeric blocks of CH in many chromosomes; Ag-NORs treatment revealed consistent positive signals at the end of the short arms of a submetacentric chromosome pair, likely homologous in both species. The karyotypic differences found between these species can be used for their taxonomical study.

Keywords

Karyotype, C-banding, NOR-phenotype, Leuciscinae, cytotaxonomy

Introduction

Spring minnows of the cyprinid genus *Pseudophoxinus* Bleeker, 1860 are distributed from Central Anatolia east to Azerbaijan and South to Israel (Freyhof and Özuluğ 2010). The genus belongs to the subfamily Leuciscinae, the major element of the Anatolia cyprinid fauna. Leuciscinae fishes include 54 species belonging to 17 genera in Anatolia, of which 26 species and subspecies are endemic. With 19 species recognized in Turkey, *Pseudophoxinus* is one of the most species-rich genera with a great number of the endemic species (Bogutskaya 1997, Freyhof and Özuluğ 2006, Bogutskaya et al. 2007, Karasu et al. 2011, Küçük et al. 2012, Küçük and Güçlü 2014). Species of this genus are found in a wide range of habitats in central Anatolia (Hrbek et al. 2004). According to IUCN, a significant point about the herein studied species is the fact that *P. crassus* and *P. hittitorum* are endangered (EN) species and their population trends are decreasing (IUCN 2014a; IUCN 2014b).

Karyotypic data for the genus are available only for *P. antalyae* Bogutskaya, 1992 and *P. firati* Bogutskaya, Küçük & Atalay, 2007 (Table 1). In both species a karyotype with 2n = 50 was revealed, indicating a conserved karyotypic evolution in relation to the diploid number (Ergene et al. 2010, Karasu et al. 2011). Thus, cytogenetic data for *Pseudophoxinus* are insufficient, and further study is needed to evaluate karyological characteristics of the genus, to improve the taxonomic identification of these fish, and to understand the evolutionary trends in this taxon (Yüksel and Gülkaç 1992).

The aim of this study is to describe the karyotypes of *P. crassus* and *P. hittitorum*, including identification of CH blocks and NORs by conventional cytogenetic techniques (Giemsa staining, C-banding, and Ag impregnation).

Material and methods

Specimens were captured by electrofishing in two distinct localities during the summer-autumn, 2012 and spring-summer, 2013. Three males and two females of *P. crassus* were collected in Cihanbeyli-İnsuyu spring (38°42'N, 32°45'E) and four females and four males of *P. hittitorum* in Beyşehir-Eflatunpınarı spring (37°52'N, 31°34'E). Specimens were transported alive to the laboratory and kept in well-aerated aquaria until analysis was performed. Chromosome spreads were obtained using standard kidney protocol (Collares-Pereira 1992). Chromosomes were stained with 4% Giemsa solution (pH = 6.8). C-bands were obtained according to Sumner technique (Sumner 1972). Silver impregnation to detect NORs followed the method of Howell and Black (1980).

The chromosome slides were observed by 100× objective with immersion oil and photographed using a Leica DM 3000 research microscope. AKAS software was used to take pictures of the metaphase plates. Measurements of chromosomes were performed by digital caliper from each individual and karyotypes were prepared manually. Chromosomes were arranged in decreasing size order and classified according to their arm ratios (Levan et al. 1964) in three categories: metacentric (M), submetacentric

Table 1. Cytogenetic data available for the genus *Pseudophoxinus*.

Species	Locality	2n	Karyotypic formula	FN	NOR	C-band	Reference
P. antalyae	Berdan River	50	16M+14SM+12ST+8A	92	1 pair *st.* *p* terminal	several	Ergene et al. 2010
P. firati	Tohma Creek	50	38M-SM+12ST	88	2 pairs *sm-st.* *p* terminal	6 pairs	Karasu et. al. 2011
P. crassus	İnsuyu Spring	50	12M+30SM+8ST-A	92	1 pair *sm* *p* terminal	several	Present study
P. hittitorum	Beyşehir Spring	50	14M+26SM+10ST-A	90	1 pair *sm* *p* terminal	several	Present study

2n: diploid number; FN: fundamental number; NOR: nucleolus organizer regions type; M: metacentric; SM: submetacentric; ST: subtelocentric; A: acrocentric; *p* short arm.

(SM) and subtelocentric to acrocentric (ST-A). To determine the fundamental number (FN), M and SM chromosomes were considered as bi-armed whereas those of group ST/A as uni-armed.

Results

243 metaphase plates were examined for *P. crassus* and 266 metaphase plates – for *P. hittitorum*. For *P. crassus* the percentage of the finding of 50 chromosomes was 81.50%. Other percentages were: for 49 chromosomes – 14.45%, for 48 chromosomes – 2.70%, for 47 chromosomes – 1.35%. For *P. hittitorum* the percentage of the finding of 50 chromosomes was 80.00%. Other percentages were: for 49 chromosomes – 13.50%, for 48 chromosomes – 3.00%, for 47 chromosomes – 2.30% and for 46 chromosomes – 1.20%. Therefore it was considered that the analyzed individuals of *P. crassus* and *P. hittitorum* had the same diploid numbers 2n = 50, but differed in their karyotypic formulas (KFs), which were 12 M + 30 SM + 8 ST-A (FN = 92) for *P. crassus* and 14 M + 26 SM + 10 ST-A (FN = 90) for *P. hittitorum*, respectively (Fig. 1). No sex chromosomes were identified for either species.

C-banding revealed the presence of the blocks of constitutive heterochromatin at the pericentromeric regions of many chromosome pairs in both species (Fig. 2).

The NORs were localized near to the secondary constriction on the short arm of a SM chromosome pair in both species (Fig. 3).

Discussion

P. crassus and *P. hittitorum* karyotypes demonstrated the general pattern described for most Leuciscinae that have the chromosome number (2n = 50), but their KFs differed. This is consistent with most other species of the genus *Pseudophoxinus*, which share 2n = 50 and differ in their KFs (Ergene et al. 2010, Karasu et al. 2011). The chromosome

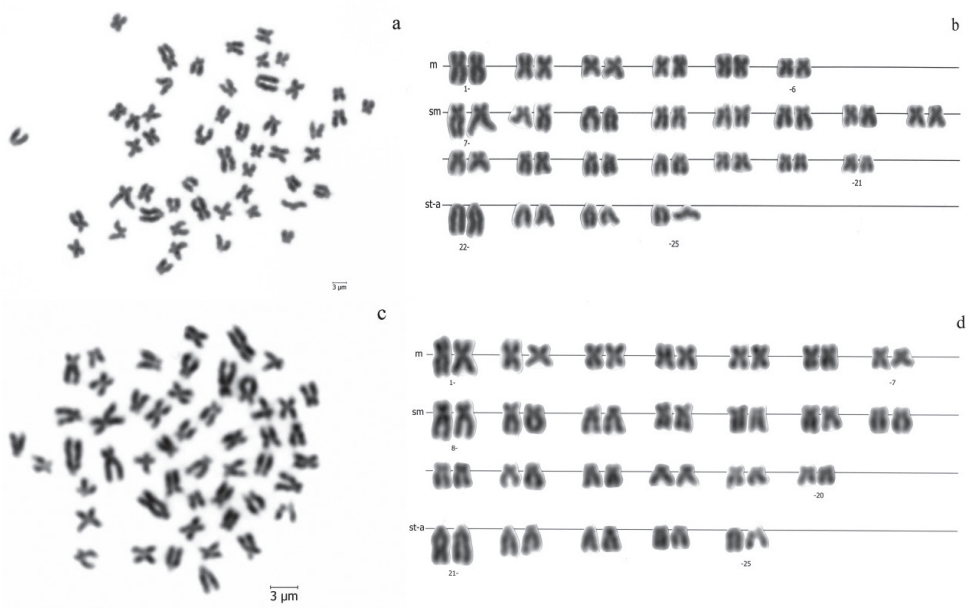

Figure 1. a Giemsa stained metaphase and **b** corresponding karyotype of *P. crassus* from Cihanbeyli stream **c** Giemsa stained metaphase and **d** karyotype of *P. hittitorum* from Beyşehir drainage. Scale bar = 3 µm.

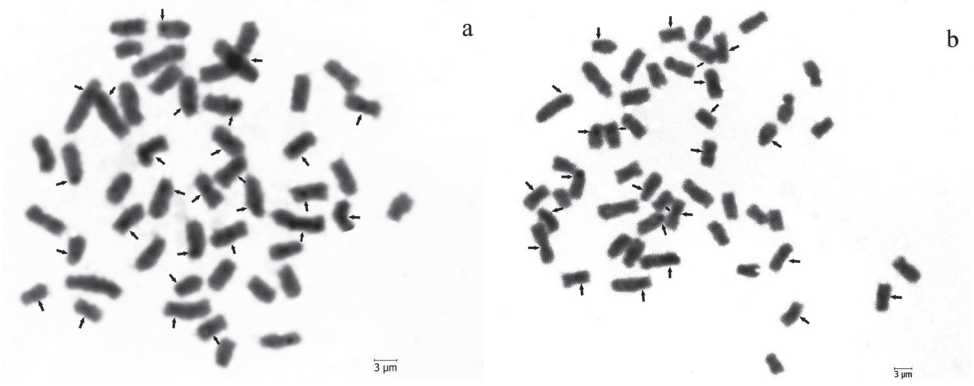

Figure 2. Metaphase spreads of (**a**) *P. crassus* and (**b**) *P. hittitorum* with C-banding. Arrows show CH regions. Scale bar = 3 µm.

sets of leuciscine cyprinids are characterized mainly by bi-armed (meta- and submetacentric) compared to the uni-armed (subtelo- and acrocentric) elements as observed in *P. crassus* and *P. hittitorum*. A large subtelocentric/acrocentric chromosome pair is considered as a cytotaxonomic marker for the subfamily Leuciscinae (Rab and Collares-Pereira 1995, Rab et al. 2008) and it is also present in both analysed species. However, cyprinid sex chromosomes appear to have remained morphologically undifferentiated (Sola and Gornung 2001). *P. crassus* and *P. hittitorum* also display the cyprinid characteristics mentioned above.

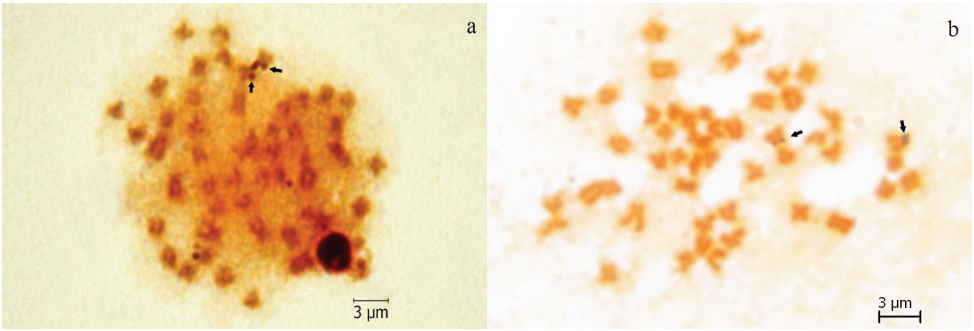

Figure 3. Metaphase spreads of (**a**) *P. crassus* and (**b**) *P. hittitorum* with Ag-NOR treatmets. Arrows show NORs. Scale bar = 3 µm.

C-bands identify regions of constitutive heterochromatin, which contain transcriptionally inactive highly repetitive DNA sequences (Gold et al. 1990). The difference in heterochromatin localization can be used as cytogenetic marker for the differentiation of species and for the reconstruction of chromosome evolution in the taxa (Gaffaroğlu and Yüksel 2009). In *P. crassus* and *P. hittitorum* C-positive blocks were pericentromeric, as in the *P. antalyae* (Ergene et al. 2010) and *P. firati* (Karasu et al. 2011). It was shown, that other studied Leuciscinae species as *Acanthobrama marmid* Heckel, 1843 (Gaffaroğlu and Yüksel 2009), *Squalius anatolicus* (Bogutskaya, 1997) (Ünal 2011) and *S. lucumonis* (Bianco, 1983) (Rossi et al. 2012) also have CH blocks on the pericentromeric regions. This pattern is conserved in Neotelostei as a whole, and also in all the Leuciscine genera examined to date (Collares-Pereira and Rab 1999, Boron et al. 2009, Rossi et al. 2012).

The number and location of NORs have been used as chromosome markers in fish cytotaxonomy (Pereira et al. 2012, Rossi et al. 2012, Nabais et al. 2013). The NORs located on a medium-sized SM chromosome pair corresponds to those observed in many of the leuciscines analyzed (Bianco et al. 2004). In spite of the many exceptions reported in Leuciscinae species from both Eurasia and North America (Pereira et al. 2009, Rossi et al. 2012), a single pair of NOR-carrying chromosome is considered as an ancestral character in this lineage (Rab and Collares-Pereira 1995, Rab et al. 2007). Within the genus *Pseudophoxinus*, a single NOR-bearing chromosome pair as in *P. crassus* and *P. hittitorum*, was observed in *P. antalyae* (Ergene et al. 2010) whereas multiple NOR-carrying chromosomes were detected in *P. firati* (Karasu et al. 2011). Although NORs are usually located on the short arms of chromosomes, sometimes they can be seen on the long arms of metacentric and acrocentric chromosomes (Rab and Collares-Pereira 1995, Rab et al. 1996). Furthermore, NORs can be seen between telomeres and centromeres (Amemiya and Gold 1988). Generally, the NOR-phenotype is observed at the terminal on short arms of mid-sized A-ST chromosomes (Takai and Ojima 1992), and rarely at the terminal on short arms of mid-sized SM chromosomes (Gold et al. 1988, Magtoon and Arai 1993) like in *P. crassus* and *P. hittitorum*. Conversely to what was reported for some others leuciscin cyprinids (Ünal

2011), no NOR polymorphism was observed in the specimens from our study. Further, there is no report of any variation in NORs' phenotype in all analyzed individuals of the genus *Pseudophoxinus* (Ergene et al. 2010, Karasu et al. 2011). Thus the karyotypes of these species conserved plesiomorphic condition that is confirmed by present study.

In conclusion, the karyotypic differences and CH and NOR localizations found in the two *Pseudophoxinus* species studied herein can be used as a cytogenetic comparison data.

Acknowledgements

We wish to thank Jörg Freyhof and Müfit Özuluğ for literature support. This research was funded by TÜBİTAK (112T730).

References

Amemiya CT, Gold JR (1988) Chromosomal NORs as taxonomic and systematic characters in North American cyprinid fishes. Genetica 76(2): 81–90. doi: 10.1007/BF00058806

Bianco PG, Aprea G, Balletto E, Capriglione T, Fulgione D, Odierna DG (2004) The karyology of the cyprinid genera *Scardinius* and *Rutilus* in southern Europe. Ichthyological Research 51(3): 274–278. doi: 10.1007/s10228-004-0221-y

Bogutskaya NG (1997) Contribution to the knowledge of leuciscine fishes of Asia Minor. Part 2. An annotated check-list of leuciscine fishes (Leuciscinae, Cyprinidae) of Turkey with descriptions of a new species and two new subspecies. Mitteilungen aus dem Hamburgischen Zoologischen Museum und Institut 94: 161–186. http://www.fishbase.org/references/FBRefSummary.php?id=33550&speccode=268

Bogutskaya NG, Küçük F, Atalay MA (2007) A description of three new species of the genus *Pseudophoxinus* from Turkey (Teleostei: Cyprinidae: Leuciscinae). Zoosystematica Rossica 15(2): 335–341. http://elibrary.ru/item.asp?id=11803973

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(2): 199–207. doi: 10.1007/s10709-008-9269-3

Collares-Pereira MJ (1992) *in vivo* Direct Chromosome Preparation (Air Drying Technique). 1st International Workshop on Fish Cytogenetic Techniques. France, September 14–24, 1990, 15–9.

Collares-Pereira MJ, Rab P (1999) NOR polymorphism in the Iberian species *Chondrostoma lusitanicum* (Pisces: Cyprinidae) reexamination by FISH. Genetica 105(3): 301–303. doi: 10.1023/A:1003885922023

Ergene S, Karahan A, Kuru M (2010) Cytogenetic Analysis of *Pseudophoxinus antalyae* Bogutskaya, 1992 (Pisces: Cyprinidae) from the Eastern Mediterranean River Basin, Turkey. Turkish Journal of Zoology 34(1): 111–117. doi: 10.3906/zoo-0807-33

Freyhof J, Özuluğ M (2006) *Pseudophoxinus ninae*, a new species from Central Anatolia, Turkey (Teleostei: Cyprinidae). Ichthyological Exploration of Freshwaters 17(3): 255–259. http://www.pfeil-verlag.de/04biol/pdf/ief17_3_07.pdf

Freyhof J, Özuluğ M (2010) *Pseudophoxinus hittitorum*, a new species of spring minnow from Central Anatolia (Teleostei: Cyprinidae). Ichthyological Exploration of Freshwaters 21(3): 239–245. http://www.pfeil-verlag.de/04biol/pdf/ief21_3_06.pdf

Gaffaroğlu M, Yüksel E (2009) Constitutive heterochromatin in *Acanthobrama marmid* and *Cyprinion macrostomus* (Osteichthyes, Cyprinidae). Kafkas Üniversitesi Veteriner Fakültesi Dergisi 15(2): 169–172. http://vetdergi.kafkas.edu.tr/extdocs/2009_2/169_172.pdf

Gold JR, Zoch PK, Amemiya CT (1988) Cytogenetic studies in North American minnows (Cyprinidae). XIV. Chromosomal NOR phenotypes of eight species from the genus Notropis. Cytobios 54: 137–147. doi: 10.1139/z91-398

Gold JR, Li YC, Shipley NS, Powers PK (1990) Improved methods for working with fish chromosomes with a review of metaphase chromosome banding. Journal of Fish Biology 37(4): 563–575. doi: 10.1111/j.1095-8649.1990.tb05889.x

Gromicho M, Ozouf-Costaz C, Collares-Pereira MJ (2005) Lack of correspondence between CMA_3-, Ag-positive signals and 28S rDNA loci in two Iberian minnows (Teleostei, Cyprinidae) evidenced by sequential banding. Cytogenetic and Genome Research 109: 507–511. doi: 10.1159/000084211

Howell WM, Black DA (1980) Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36(8):1014-1015. doi: 10.1007/BF01953855

Hrbek T, Stölting KN, Bardakcı F, Küçük F, Wildekamp RH, Meyera A (2004) Plate tectonics and biogeographical patterns of the *Pseudophoxinus* (Pisces: Cypriniformes) species complex of central Anatolia, Turkey. Molecular Phylogenetics and Evolution 32(1): 297–308. doi: 10.1016/j.ympev.2003.12.017

IUCN (2014a) *Pseudophoxinus crassus*. http://www.iucnredlist.org/details/60751/0 [accessed 10 April 2014]

IUCN (2014b) *Pseudophoxinus hittitorum*. http://www.iucnredlist.org/details/19449272/0 [accessed 10 April 2014]

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. doi: 10.3906/zoo-0912-129

Küçük F, Atalay MA, Güçlü SS, Gülle İ (2012) Türkiye'de Yayılış Gösteren *Pseudophoxinus* (Teleostei:Cyprinidae) Türlerinin Bazı Morfolojik Özellikleri ve Zoocoğrafik Dağılımları. Eğirdir Su Ürünleri Fakültesi Dergisi 8(2): 1–9. http://sdu.edu.tr/edergi/index.php/esufd/article/viewFile/3827/3473

Küçük F, Güçlü SS (2014) A new *Pseudophoxinus* (Teleostei, Cyprinidae) species from Asi River Drainage (Turkey). ZooKeys 411: 57–66. doi: 10.3897/zookeys.411.6833

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52(2): 201–220. doi: 10.1111/j.1601-5223.1964.tb01953.x

Magtoon W, Arai R (1993) Karyotypes and distribution of nucleolus organizer regions in cyprinid fishes from Thailand. Japanese Journal of Ichthyology 40(1): 77–85. http://www.wdc-jp.biz/pdf_store/isj/publication/pdf/40/401/40111.pdf

Nabais C, Rampin M, Collares-Pereira MJ (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. doi: 10.3897/CompCytogen.v7i1.4672

Pereira C, Neto A, Collares-Pereira MJ (2009) Cytogenetic survey of species of two distinct genera of Iberian nases (Cyprinidae, Leuciscinae) that hybridize extensively in nature. I. Evidence of similar and conserved chromosome pattern with some few species-specific markers at macro-structural level. Genetica 137(3): 285–291. doi: 10.1007/s10709-009-9379-6

Pereira CS, Rab P, Collares-Pereira MJ (2012) Chromosomes of European cyprinid fishes: comparative cytogenetics and chromosomal characteristics of ribosomal DNAs in nine Iberian chondrostomine species (Leuciscinae). Genetica 140(10–12): 485–495. doi: 10.1007/s10709-013-9697-6

Rab P, Collares-Pereira MJ (1995) Chromosomes of European Cyprinid fishes (Cyprinidae, Cypriniformes): A review. Folia Zoological 44(3): 193–214. http://serials.unibo.it/cgi-ser/start/en/spogli/dfs.tcl?prog_art=3256190&language=ENGLISH&view=articoli

Rab P, Karakousis Y, Rabova M (1996) Karyotype, NOR phenotype and C-banding study of *Barbus cyclolepis* from Greece. Folia Zoologica 45: 77–83. http://eurekamag.com/research/038/362/038362965.php#close

Rab P, Bohlen J, Rabova M, Flajshans M, Kalous L (2007) Cytogenetic as a tool in fish conservation: the present situation in Europe. In: Pisano E, Ozouf-Costaz C, Foresti F, Kappor BG, Enfield NH (Eds) Fish cytogenetics. 2.1. Science Publisher Inc., USA, 215–240.

Rab P, Rabova M, Pereira CS, Collares-Pereira MJ, Pelikanova S (2008) Chromosome studies of European cyprinid fishes: interspecific homology of leuciscine cytotaxonomic marker the largest subtelocentric chromosome pair as revealed by crossspecies painting. Chromosome Research 16(6): 863–873. doi: 10.1007/s10709-013-9697-6

Rossi AR, Milana V, Hett AK, Tancioni L (2012) Molecular cytogenetic analysis of the Appenine endemic cyprinid fish *Squalius lucumonis* and three other Italian leuciscines using chromosome banding and FISH with rDNA probes. Genetica 140(10–12): 469–476. doi: 10.1007/s10709-012-9695-0

Sola L, Gornung E (2001) Classical and molecular cytogenetics of the zebrafish, *Danio rerio* (Cyprinidae, Cypriniformes): an overview. Genetica 111(1–3): 397–412. doi: 10.1023/A:1013776323077

Souza ACP, Nagamachi CY, Milhomem SSR, Feldberg E, Pieczarka JC (2009) Cytogenetics analysis in catfish species of the genus *Peckoltia* Miranda Ribeiro, 1912 (Teleostei: Siluriformes: Loricariidae). Comparative Cytogenetics 3(2): 103–109. doi: 10.3897/compcytogen.v3i2.17

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75(1): 304–6. doi: 10.1016/0014-4827(72)90558-7

Takai A, Ojima Y (1992) Chromosomal distribution of nucleolus organizer regions in Japanese Cyprinid fish. Cytobios 71(284): 7–17. http://eurekamag.com/research/002/322/002322876.php

Ünal S (2011) *Squalius anatolicus* (Bogutskaya, 1997) (Pisces, Cyprinidae)'un Sitogenetik Analizi. MSc Dissertation. Science Institute, Ahi Evran University, Kırşehir, Turkey, 61 pp. [in Turkish]

Yüksel E, Gülkaç MD (1992) On the karyotypes in some populations of the subterranean mole rats in the lower Euphrates-basin, Turkey. Caryologia 45(2): 175–190. doi: 10.1080/00087114.1992.10797221