KARYOLOGY OF TWO *SQUALIUS* (TELEOSTEI: LEUCISCIDAE) SPECIES DISTRIBUTED IN TURKIYE

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Abstract. The karyotype and chromosomal properties of *Squalius orpheus* Kottelat & Economidis, 2006 and *S. cappadocicus* Özuluğ & Freyhof, 2011 were examined using conventional cytogenetical methods involving Giemsa and silver staining and C-banding. The diploid chromosome numbers (2n) of *S. orpheus* and *S. cappadocicus* were 50, the fundamental arm numbers (FNs) were respectively 85 and 92, and the karyotypes displayed some differences from each other. Heteromorphic sex chromosomes were not detected. The largest chromosome pair in the karyotypes was a subtelo-acrocentric chromosome. C-bands were observed on the pericentromeric regions of most of the chromosomes in the two studied species. The nucleolus organizer regions were detected terminally on the short arms of a single submetacentric chromosome pair in both species. This study aims to improve the cytogenetic data of chubs.
Keywords: *chubs, cytogenetic, chromosomal banding, karyotype, Anatolia*

Introduction

Türkiye has the highest freshwater fish diversity compared to its neighbouring countries. The freshwater fishes of Türkiye is known to consist of 427 species belonging to 37 families. Among these, 215 species (50.4%) are considered to be endemic. At the family level, the Leuciscidae has the highest number of species (126 species; 29.8% of the total species) (Çiçek et al., 2023). The genus *Squalius* Bonaparte, 1837 belonging to family Leuciscidae commonly known as chubs, is represented by 23 species in the inland waters of Türkiye (Çiçek et al., 2023). From these chubs, *Squalius orpheus* –Thracian chub- distributes in streams and rivers of South-eastern Europe and Black Sea watersheds (Greece, Bulgaria and Türkiye) (Çiçek et al., 2023). *S. orpheus* was also found in the longest river named as Kızılırmak in Türkiye (Durand et al., 2000). Otherwise, endemic *S. cappadocicus* – Cappadocian chub- inhabits streams and waters on sand and gravel bottoms of Lake Tuz Basin of Türkiye (Çiçek et al., 2023).

Chromosomal studies are useful in aquaculture, conservation, cytotaxonomy and comparative genetics among fishes (Salvadori et al., 2015). Cytogenetic studies in fish species have not been as common as in other vertebrate groups because of the high number of small chromosomes (Arai, 2011). 2n, chromosome morphologies and chromosomal banding properties vary in fish species. Fish cytogenetic studies are important in obtaining chromosomal knowledge, karyoevolution and phylogenetic relationships among them (Unal and Gaffaroğlu, 2016).

The karyotypes and chromosomal bandings of representatives of the genus *Squalius* have been little studied. Cytogenetical studies of Anatolian representatives of the genus

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Squalius have started recently. *Squalius cephalus* (Pekol, 1999), *S. orientalis* (Kılıç-Demirok, 2000), *S. seyhanensis* (Unal and Gaffaroğlu, 2016), *S. carinus*, *S. fellowesii* (Karasu-Ayata, 2020), *S. anatolicus* (Ünal-Karakuş and Gaffaroğlu, 2021), *S. recurvirostris* (Doori and Arslan, 2022) and *S. cappadocicus* (Doori and Arslan, 2023) have been studied karyologically. In most of these above-mentioned studies, 2n, chromosome morphologies in the karyotypes, C-banding and Ag-NOR staining properties have been reported. This study aimed to reveal the karyotype and chromosomal characteristics of Anatolian two chubs (in *S. orpheus* for the first time) by means of sequential Giemsa staining, C-banding and silver staining.

Materials and methods

Six specimens (three females and three males) of *S. orpheus* were collected from Delice Stream, Yerköy, Yozgat Prov., Türkiye (39°35′N, 34°33′E) and 10 (four females and six males) specimens of *S. cappadocicus* were collected from Melendiz Stream, Selimiye, Aksaray Prov., Türkiye (38°18′N, 34°15′E). Individuals of each species were transported alive to the laboratory and kept in well aerated aquaria until analysis. Studied individuals were deposited in 70% ethanol at the Kırşehir Ahi Evran University Genetics Research Laboratory, Türkiye under the collection numbers (MKA138-154). Chromosome preparations were carried out as described by Bertollo et al. (2015). Cell suspensions were obtained after injecting 0.1% colchicine intraperitoneally (0.01 ml g^{-1} body weight) 70 min before sacrificing. Kidneys were removed, homogenized and hypotonized by KCl 0.075 M for 5 min at 37°C. Suspensions were centrifuged at 1200 rpm for 30 min. Supernatant was removed and fixative solution (3 methanol: 1 glacial acetic acid) was added for fixation of cells. This process was repeated three or five times depending on density of cells. At least 10 slides for each species were stained for 15-25 min with 10% Giemsa. Constitutive heterochromatin regions were analysed with a modified C-banding technique (Sumner, 1972). Chromosome slides were treated with 0.2 N HCl for 65 min at room temperature and incubated in 5% $Ba(OH)_2$ for 20 min at 37°C in a waterbath, and incubated with 2 × SSC for 60 min at 60°C. Slides were stained by Giemsa for 45 min. Otherwise, Ag-NORs were detected with silver nitrate staining method according to the Howell and Black (1980). For staining of AgNORs, two drops of protective colloidal developer and four drops of the silver nitrate are pipetted onto chromosome slide. The slides were covered with a coverglass and stabilized at 70°C. The coverglass was removed from onto the slide and it was rinsed off under water. All metaphase images after the three techniques were photographed by a Leica DM 3000 microscope (Leica Microsystems GmbH, Germany) equipped with a camera and AKAS software (Argenit Microsystems, Istanbul, Türkiye). Chromosome morphologies were determined according to the ratio of the chromosome arms (Levan et al., 1964). Meta- and submetacentrics were taken as biarmed whereas subtelo-acrocentrics were taken as uniarmed.

Results

The diploid chromosome numbers of *S. orpheus* and *S. cappadocicus* were invariably 2n = 50 (*Figs. 1A* and *2A*). Karyotypes were as follows: 20 metacentric, 20 submetasentric and 10 subtelo-acrocentric in *S. orpheus* (*Fig. 1B*) and 18 metacentric, 24 submetasentric and eight subtelo-acrocentric chromosomes in *S. cappadocicus* (*Fig. 2B*).

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The largest chromosome pair of the complements was a subtelo-acrocentric chromosome in the two studied species. Heteromorphic sex chromosomes were not detected in the karyotypes of the two studied species. FNs were found as 90 in *S. orpheus* and as 92 in *S. cappadocicus*. C-bands were found on the pericentromeric regions of the most of the chromosomes in *S. orpheus* and *S. cappadocicus* (*Figs. 1C* and *2C*). In C-banded karyotypes, dark C-bands were in No. 1-6., 8., 21-23. and 25. chromosome pairs in *S. orpheus* (*Fig. 1D*), and No. 1-5. (except 3), 8., 11., 13., 15., 16., 19. and 21. chromosome pairs in *S. cappadocicus* (*Fig. 2D*). Also light C-bands were in No. 7., 9-11., 18., 19. and 24. chromosome pairs in *S. orpheus* (*Fig. 1D*) and No. 3., 6., 9., 10., 12. and 22-24. chromosome pairs in *S. cappadocicus* (*Fig. 2D*). Ag-NOR signals were observed terminally on the short arms of one submetacentric chromosome pair in the two species (*Figs. 1E* and *2E*). In the silver stained karyotypes, these Ag-NORs were located on the 4th submetacentric chromosome pair in *S. orpheus* (*Fig. 1F*) and on the 3th submetacentric chromosome pair in *S. cappadocicus* (*Fig. 2F*). Also, only one Ag-NOR signal was detected on the some silver stained metaphases of the two studied species (*Fig. 3A, B*).

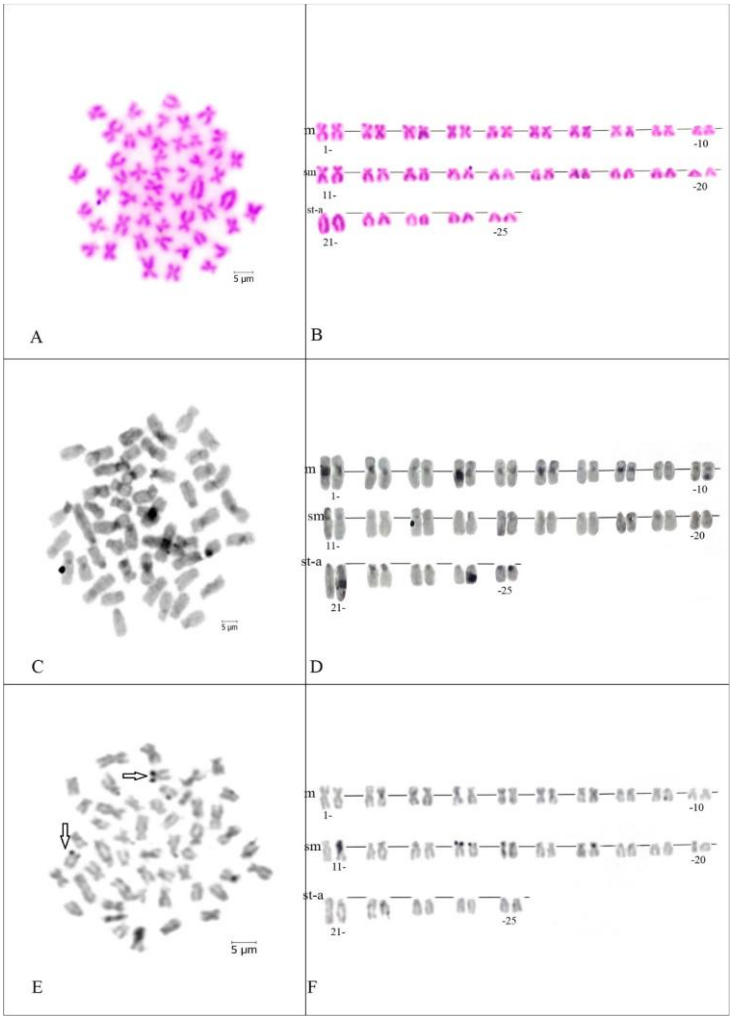

Figure 1. *Metaphases and karyotypes of Squalius orpheus: (A) Giemsa stained metaphase; (B) the arranged karyotype of Giemsa stained metaphase; (C) C-banded metaphase; (D) the arranged karyotype of C-banded metaphase; (E) silver-stained metaphase (arrow indicates the Ag-NOR); (F) the arranged karyotype of silver-stained metaphase. Scale bar = 5 µm. m: metacentric, sm: submetacentric, st-a: subtelo-acrocentric*

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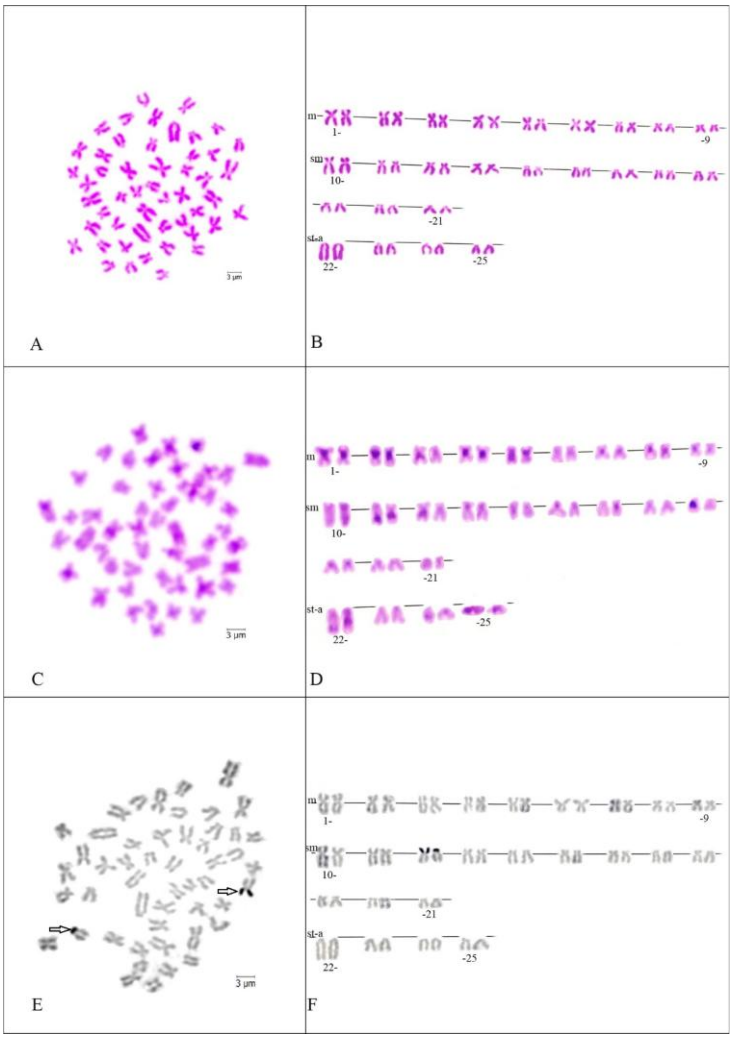

Figure 2. *Metaphases and karyotypes of Squalius cappadocicus: (A) Giemsa stained metaphase; (B) the arranged karyotype of Giemsa stained metaphase; (C) C-banded metaphase; (D) the arranged karyotype of C-banded metaphase; (E) silver-stained metaphase (arrow indicates the Ag-NOR); (F) the arranged karyotype of silver-stained metaphase. Scale bar = 3 µm. m: metacentric, sm: submetacentric, st-a: subtelo-acrocentric*

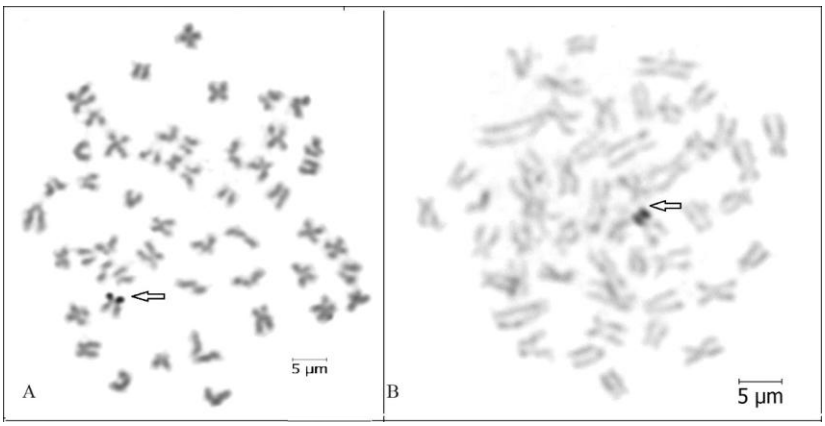

Figure 3. *Ag-NOR number polymorphisms in the silver stained metaphases of S. cappadocicus (A) and S. orpheus (B). Arrows indicate the Ag-NOR. Scale bar = 5 µm*

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Discussion

Cytogenetic studies on *Squalius* species are reported in eight species from Türkiye (*Table 1*). Eight from 23 Anatolian species of the genus *Squalius* that karyologically studied showed an invariable 2n = 50 (*Table 1*). Their karyotypes were dominated by biarmed chromosomes (*Table 1*). The two species examined herein also have 2n = 50 chromosomes and karyotypes dominated by biarmed chromosomes. However, their karyotypes showed some differences compared to the previous reports (*Table 1*). This karyotype differentiation should be the result of chromosomal rearrangements in the evolution of this genus, such as pericentric inversions, and or translocations as reported before for leuciscins (Karasu-Ayata, 2020). Anatolian species of the genus *Squalius* are karyotypically highly conservative leuciscid fishes. The diploid chromosome numbers within leuciscins are usually 2n = 50 (Ráb and Collares-Pereira, 1995) as this study. In addition, they reported that leuciscin karyotypes are composed of six to eight pairs of metacentric, 12 to 14 pairs of submetacentric, and two to four pairs of subtelo-acrocentric chromosomes (Ráb and Collares-Pereira, 1995). The karyotypes of two *Squalius* species in this study show similarity to these karyotype content about having 20 to 21 biarmed chromosomes and four to five pairs of subtelo-acrocentric chromosomes. Otherwise, the karyotype complement of two populations of *S. cappadocicus* have differences. Doori and Arslan (2023) reported 20 subtelo- and acrocentric chromosomes whereas we detected eight subtelo-acrocentric chromosomes in *S. cappadocicus*. Otherwise, the largest chromosome pair of the leuciscinae sets is a subtelo-acrocentric chromosome pair and this is a cytotaxonomic marker chromosome (Ráb et al., 2008) as seen in the studied two species.

Table 1. *Karyological reports in the genus Squalius in Türkiye*

Species	2n/FN	Chromosomes	References
S. cephalus	50/80	18m + 12sm + 20st-a	Pekol, 1999
S. cephalus	50/82	20m + 12sm + 18st-a	Pekol, 1999
S. orientalis	50/84	14m + 20sm + 16st-a	Kılıç-Demirok, 2000
S. seyhanensis	50/94	16m + 28sm + 6st-a	Unal and Gaffaroğlu, 2016
S. carinus	50/94	24m + 20sm + 6st-a	Karasu-Ayata, 2020
S. fellowesii	50/90	20m + 20sm + 10st-a	Karasu-Ayata, 2020
S. anatolicus	50/82	10m + 22sm + 10st + 8a	Ünal-Karakuş and Gaffaroğlu, 2021
S. recurvirostris	50/90	12m + 18sm + 11st + 9a	Doori and Arslan, 2022
S. cappadocicus	50/90	14m + 16sm + 10st + 10a	Doori and Arslan, 2023
S. orpheus	50/90	20m + 20sm + 10st-a	This study
S. cappadocicus	50/92	18m + 24sm + 8st-a	This study

Buasriyot et al. (2024) reported that heteromorphic sex chromosomes should be present in fish karyotypes however, at an early stage of differentiation they cannot be detected by classical cytogenetic analyses. These chromosomes on the karyotypes are known only in a limited fish species (Arai, 2011). Differentiated sex chromosomes are not observed in this study as not reported for other Anatolian species of the genus *Squalius* (Unal and Gaffaroğlu, 2016; Karasu-Ayata, 2020; Ünal-Karakuş and Gaffaroğlu, 2021; Doori and Arslan, 2023) except *S. recurvirostris* (Doori and Arslan, 2022).

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A common cytogenetic technique in fish species is Ag-NOR staining. This method identifies transcriptionally active rDNA genes (Rábová et al., 2015). These genes are responsible for ribosome synthesis (18S, 5.8S, and 28S ribosomal RNA) (Buasriyot et al., 2024). Among the cytogenetic markers, NOR phenotypes, number and localization of rDNA sites on chromosomes have played an important role in fish cytogenetics and fish cytotaxonomy (Rábová et al., 2015). Leuciscids possess a single Ag-NOR and this is a common feature in most European leuciscine fishes as an ancestral character (Ráb et al., 2008) as seen in this study. About having a single pair of Ag-NOR-bearing submetacentric chromosomes in *S. orpheus* and *S. cappadocicus* shows similarity to *S. seyhanensis* (Unal and Gaffaroğlu, 2016), *S. carinus, S. fellowesii* (Karasu-Ayata, 2020) and *S. anatolicus* (Ünal-Karakuş and Gaffaroğlu, 2021) from Anatolia. Otherwise, the number of Ag-NORs in *S. cephalus* (Pekol and Arslan, 2015) is similar to *S. orpheus* and *S. cappadocicus* whereas the localization in subtelo-acrocentric chromosome pair is different from this study. Doori and Arslan (2022) reported that Ag-NORs were hemizygous and detected in two different submetacentric chromosomes in *S. recurvirostris* (Doori and Arslan, 2022) unlike *S. orpheus* and *S. cappadocicus*. Ag-NOR number observed in this study is different from Doori and Arslan (2023)'s study. They reported three hemizygous Ag-NOR in *S. cappadocicus* whereas two of them were in submetacentric chromosomes that were not homologues and one of them was on the acrocentric chromosome (Doori and Arslan, 2023). The differences of Ag-NOR number and location in *S. cappadocicus* with Doori and Arslan (2023)'s study should be the result of differences among populations. Ag-NORs in subtelo-acrocentric chromosomes were only reported in *S. cephalus* (Pekol and Arslan, 2015; Boron et al., 2009) and *S. cappadocicus* (Doori and Arslan, 2023) contrary to this study. Otherwise, the most common Ag-NOR phenotype among European leuciscids is a single Ag-NOR that especially localize on the short arms of submetacentric chromosomes (Boron et al., 2009). This feature have been reported in European chubs like *Squalius alburnoides, S. pyrenaicus* (Gromicho and Collares-Pereira, 2004), *S. aradensis, S. torgalensis* (Nabais et al., 2013) and *S. lucumonis* (Rossi et al., 2012) as observed in this study. Moreover, the size heteromorphism of Ag-NOR was reported in *S. anatolicus* (Ünal-Karakuş and Gaffaroğlu, 2021) from Anatolian chubs. However, number polymorphism of Ag-NOR has been reported only in *S. cephalus* (Pekol and Arslan, 2015) from Anatolia as observed in this study. These one Ag-NOR signal should be the result of deletion of Ag-NOR sites or by unequal crossing-over and or other chromosomal rearrangements as reported before by Boron et al. (2009) in leucisicins. It was reported that intraspecific and intraindividual variability in the number and location of Ag-NOR among leuciscid species seem to be more numerous and complicated (Boron et al., 2009).

The C-banding technique shows the regions of constitutive heterochromatin. These regions contain highly and moderately repetitive DNA. These C- bands usually were located on pericentromeres of chromosomes and telomeric and sometimes intercalary regions. C-bands should be different in many fish species and in addition among individuals and populations of the same species (Salvadori et al., 2015). C-bands in Anatolian chubs like *S. seyhanensis* (Unal and Gaffaroğlu, 2016), *S. carinus, S. fellowesii* (Karasu-Ayata, 2020), *S. anatolicus* (Ünal-Karakuş and Gaffaroğlu, 2021) and *S. recurvirostris* (Doori and Arslan, 2022) were observed in the pericentromeres of the most of the chromosomes as this study. The C-banding properties of *S. orpheus* and *S. cappadocicus* are similar to Doori and Arslan (2023)'s report. Altogether, this study

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have pericentromeric heterochromatin on almost all chromosome pairs like *S. lucumonis* (Rossi et al., 2012) and *S. cephalus* (Boron et al., 2009) from European localities.

In conclusion, fish cytogenetics is an important tool for the detection of biodiversity and genetic relationships (Buasriyot et al., 2024; Kirtiklis et al., 2024). Especially small chromosomal differences about the morphology are usually observed between the species of the related fish genus (Kirtiklis et al., 2024). Many fish cytogenetic studies showed the evolution of the macro and micro karyotype structures of different groups (Buasriyot et al., 2024). This study has determined *S. orpheus* and *S. cappadocicus* by classical cytogenetics. Diploid chromosome number and the other chromosomal features are similar in the two studied *Squalius* species with a remarkable $2n = 50$ chromosomes as the other Anatolian chubs whereas different karyotypes were observed among them.

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