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Research Article

Karyoevolutive consideration of two sea fish species via the newest short-term culture method

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Abstract

This study successfully determined the chromosome numbers and karyotypes of two marine fish species from separate families using the short-term culture method (PB-MAX™ application). The culture incubation periods varied for each species. Kidney tissues from the fish samples were treated with PB-MAX™ for either 2.5 or 4 h. Results showed that diploid chromosome numbers, karyotypes, and arm numbers differ by species: Red Mullet, *Mullus barbatus*, has 2n – 44, with 3 pairs of metacentric, 7 pairs of submetacentric, 7 pairs of subtelocentric, and 5 pairs of acrocentric chromosomes (NF=64). Round Goby, *Neogobius melanostomus*, has 2n – 46, consisting of 46 pairs of acrocentric chromosomes (NF=46). C– and NOR–positive results were achieved through constitutive heterochromatin banding and silver nitrate staining for both species. The primary objective of this research is to demonstrate the applicability of short-term culture in producing successful cytogenetic results in marine fish. This study will support future research and facilitate chromosome studies on marine fish, making the process quick and practical.

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Introduction

Cytogenetics is a branch of genetics that studies hereditary changes by analyzing chromosomes. This field developed from the collaboration of cytology and genetics: applying chromosome analysis methods to genetics. As a result, the growth of this science has been closely linked to advances in chromosome staining and analysis techniques (Topaktas and Rencuzogullari, 2010). In fact, studying chromosome structure, number, and genome size in many vertebrate groups, along with mitochondrial and nuclear gene sequences, has helped overcome challenges in understanding fish biology, systematics, and evolution. However, fish, the most diverse group of vertebrates, have traditionally been classified based on higher taxonomic groupings, relying far less on cytogenetic data than on morphology and paleontology. This is partly because karyotypes can only be obtained from the tissues and cells of living specimens, which makes it difficult to study the karyotypes of fish that are hard to collect, such as deep-sea species. Today, scientists have documented chromosome structures in nearly 4,000 fish species (Arai, 2011) out of approximately 37288 known species (Fricke *et al.*, 2025). There are 552 marine and brackish water fish species along the coasts of Türkiye (Bilecenoglu, 2024) and 427 freshwater fish species in inland waters (Cicek *et al.*, 2023). Since Gul's first academic study in 1988, chromosomes have been examined in 103 fish species (Saygun, 2021). Although the number of scientists working in fish cytogenetics has decreased, chromosome analyses are performed annually on dozens of fish species, providing valuable insights into fish taxonomy.

In Mullidae, there are 107 family members in 6 genera, which are included in Syngnathiformes. Mullets or goatfish are widespread from the Atlantic Ocean to the Indian and Pacific Oceans but are rarely found in brackish waters. *Mullus barbatus* (Red mullet), one of the 5 species belonging to the *Mullus* and *Upeneus* genera, is widely found in Turkish waters. It is a species also found in the Mediterranean, including the Canary and Azores Islands, in the Sea of Marmara, in the Black Sea, and east of the North Atlantic Ocean from Norway to the British Isles, and its habitat extends from Scandinavia to Dakar and Senegal (Froese and Pauly, 2025; Fricke *et al.*, 2025). While 41 species are reported in the genus *Mullus*, only five of them are taxonomically valid: *M. argetinae*, *M. auratus*, *M. barbatus*, *M. ponticus,* and *M. surmuletus* (Froese and Pauly, 2025). A total of 12 cytogenetic studies were conducted for 8 species in 4 genera in the Mullidae family. Most chromosome studies have been conducted on the *Mullus barbatus* species. In addition to the diploid chromosome number of 46 reported by all researchers in *M. barbatus*, variations in both karyotype and some chromosome features (such as the C+ and NOR+ regions) were detected.

The order Gobiiformes includes 4656 species. More than 25% (1332 sp.) of these species are represented in the Gobiinae subfamily in the Gobiidae family (Fricke *et al.*, 2025). There are 34 species belonging to the *Neogobius* genus. Nevertheless, most of them belong to the *Ponticola* genus, and only four species (*N. caspius*, *N. fluviatilis*,

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N. melanostomus, and *N. pallasi*) are currently recognized as taxonomically valid species (Froese and Pauly, 2025). However, cytogenetic and karyological studies in this genus have focused on 11 known species (only two valid *Neogobius* species). These studies reported that all these species, which live in the Black Sea and surrounding freshwater basins, have chromosome numbers ranging from 30 to 46. The round goby, *N. melanostomus*, one of these four valid species, was examined in this study. The round goby is not an economically important species. It is distributed in the Sea of Azov, the Black Sea, and the Caspian basins. Unlike the other three studies conducted on *N. melanostomus*, this study identified chromosomal features such as NOR+ and C+ regions, which are important in terms of evolutionary development.

Progress in cytogenetics has advanced significantly, especially since the 1980s, with the widespread adoption of DNA gene markers and probes used in fluorescent staining techniques such as MM, DAPI, and CMA3, replacing traditional sequential staining methods. Pinkel *et al.* (1986) introduced a practical FISH staining technique with probes they designed for the 45S rDNA locus. Today, many variations of this method have been developed, offering valuable tools for collecting crucial data, particularly in medical genetics and fish evolution. For example, MonoFISH (mFISH) staining became popular in the early years, and now, in medical cytogenetics and zoology, molecular techniques such as dual-color FISH (D-FISH), M-FISH (multicolor spectral karyotyping), and other chromosome mapping methods can identify structural and numerical chromosome rearrangements across different species and populations. However, these advanced methods are still employed to detect sex chromosome variations, polymorphisms in active or inactive heterochromatin and NOR regions, and numerical differences in NORs, which serve as markers of morphological and cytogenetic similarities or differences among geographically separated groups of marine fish species. Additionally, these methods can provide deeper insights into gene regulatory systems that influence inheritance (Rossi, 2021). Within the *Neogobius* genus, only *N. melanostomus* has been studied for DNA gene regions such as telomeric repeats using fluorescence staining like FISH (Ocalewicz and Sapota, 2011). Similarly, GC-rich DNA regions were identified in *N. eurycephalus* (Ene, 2003).

This study aimed to determine the chromosome structure and number in marine fish by applying short-term cell culture techniques *in vitro* on dead fish cells and various staining and banding preparations.

Materials and methods

Fish samples were obtained from professional and amateur fishermen at two points (41°03.51'N, 37°30.50'E and 41°02.45'N, 37°31.42'E) in Fatsa Bay, Ordu Province (Fig. 1). For this purpose, random sampling was carried out between February and November 2023, and chromosome structures were determined by taking samples from 3 species belonging to two different families. In this study, the methods of Aksiray (1987), Bat *et al.*

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(2008), Bilecenoglu *et al.* (2011), and Nelson *et al.* (2016) were used to determine the species. While the fish samples were fresh for at most four hours during the postmortem period, the tissues obtained were transported to the Fatsa Faculty of Marine Sciences Biochemistry Laboratory without delay and placed in a culture environment.

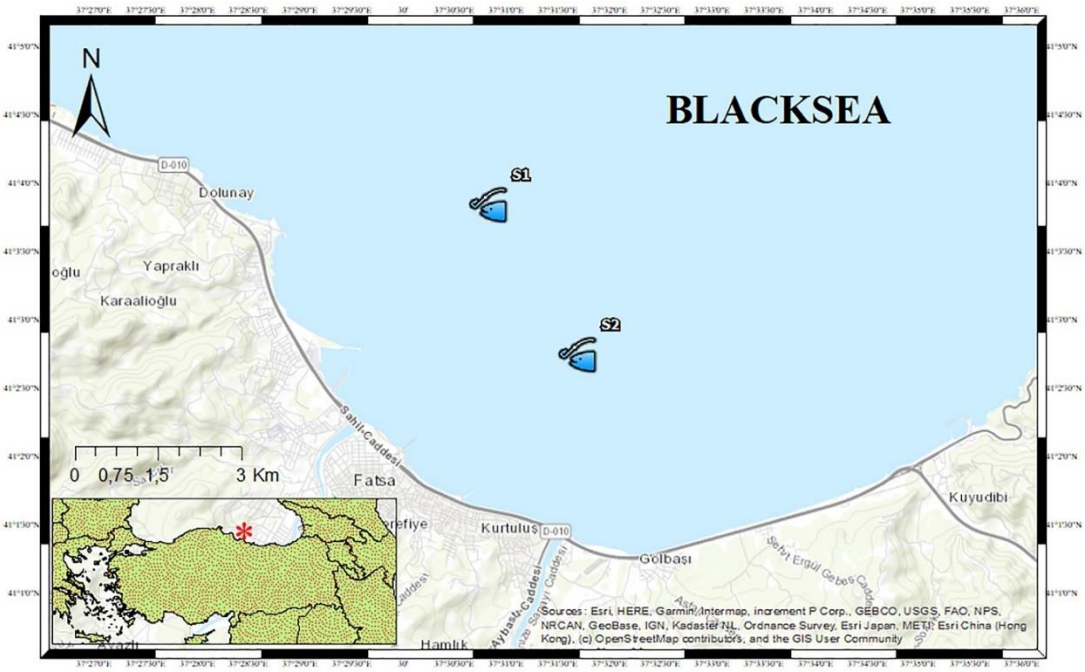

Figure 1: Areas where fish samples are taken randomly.

As noted in the literature (Araya-Jaime *et al*., 2021), tissues from the kidney, spleen, and gills yield the best results. In this study, we prepared kidney tissues for all other fish samples. To ensure sample sterility, the outer surfaces of the fish samples were wiped with alcohol before removing the kidney tissue, and 1 g of kidney tissue was placed into Falcon tubes. The mixture was incubated for at least 2.5–4 hours and up to 12 hours for each sample from each species at +4°C in the refrigerator. Slide preparations followed the methods of Kligerman and Bloom (1977) and the air-drying techniques outlined by Blanco *et al*. (2012) and Hedari Salkhordeh *et al*. (2016). The slides were first stained in 5% Giemsa solution (pH 6.8–7.1) for at least 10–15 min, rinsed with distilled water, and air-dried (Saberii *et al*., 2023). After drying, the slides were examined under microscopes (Trinocular Leica DM500, Germany, and Trinocular Nikon Eclipse™, Japan) to identify chromosome sites. Using Sumner's (1972) modified C-band method (adapted by Artoni *et al*., 2001), the preparations were processed with minor adjustments per the specified procedures. The ethanol used to wash the Giemsa stain on the slides was replaced with fresh ethanol at least twice. The slides were then destained by soaking in Carnoy's fixative (3:1 methanol: acetic acid) for at least 15 min., rinsed with distilled water, and left to dry. The slides were incubated in 0.2 N HCl solution at room temperature for 10–15

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min., then washed with distilled water. The samples were briefly immersed in a freshly prepared 5% $Ba(OH)_2$ solution for 15–20 seconds, followed by a few seconds in 0.2 N HCl, then rinsed again with distilled water and allowed to dry. Dried slides were placed in a Coplin jar filled with 2×SSC buffer and heated at 60°C for one hour. After gently rinsing with distilled water, the slides were air-dried. The preparations were stained again with 5% Giemsa (pH 6.8–7.1) for 10–15 min., then rinsed with distilled water to remove residual stain and left to dry. This study also employed various modifications of Howell and Black's (1980) method, which has been successfully used in fish, as adapted by Kavalco and Pazza (2004). C-banded slides were first destained with Giemsa stain, ethanol, and fixative. Then, two drops of 1% gelatin (comprising 1 g gelatin and 0.25 ml formic acid) and four drops of 25% silver nitrate (0.25 g/L mL) were placed on each slide. A 40×22 mm coverslip was applied, and the slide was placed in a microwave oven. The mixture was heated for 5 seconds, then removed and washed in tap water to eliminate excess stain and remove the coverslip. Finally, the slides were stained with 5% Giemsa solution (pH 6.8–7.1) for 10–30 seconds, rinsed with distilled water, and left to dry.

Relative lengths (μ) and arm lengths (μ) of chromosomes in the metaphase site of at least 25–30 of the most appropriate lengths for each sample were measured (Maneechot *et al.*, 2015) from photographs taken under the microscope AKAS Multispecies© (v.3.5.1.0; Argenit Smart Information, Tech. Ltd. Co., Istanbul) (Karasu Ayata *et al.*, 2016) and/or microscope-specific image analysis programs (LAS EZ© 3.4.0), and diploid (2n) chromosome numbers were used. However, in ordering the homologous chromosomes measured in the centromeric plane, extracting karyotypes, and preparing karyogamy, Levan *et al.* (1964) used the Adobe Photoshop© CC programs (Saberii *et al.*, 2023). The arm ratios ($r=q/p$) of the classified chromosomes were obtained by dividing the length of the long arm (q) by the length of the short arm (p). The number of arms of the chromosomes (NF=the number of fundamental) was determined by counting bi-armed chromosomes (metacentric and submetacentric with p and q arms) as two each and uni-armed chromosomes with q arms (subtelocentric, telocentric/acrocentric) as one (Thorgaard and Disney, 1990).

Results

In this study, the slides were prepared from kidney tissues from two red mullet fish samples with PB-MAX™ for three hours. Because of this preparation, many chromosome sites were detected in one sample, and diploid counts were made from digital photographs of these Giemsa-stained plates. The 2n–44 cytotype, the most common (65%) among the 14 different cytotypes identified, was used as the model diploid chromosome number for *M. barbatus* (Fig. 2).

In *M. barbatus*, 2n–44 diploid chromosomes are 3 pairs of metacentric chromosomes, 7 pairs of submetacentric chromosomes, 7 pairs of subtelocentric chromosomes, and 5 pairs of acrocentric chromosomes. When the NF chromosome arm number was formulated, it was found

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to be 64 (Fig. 3-B). In Fig. 2, the diagrammatic representation of n homologous chromosomes is given in the ideogram. As shown in Figure 4, constitutive heterochromatin – C+ region centromeres were located in two acrocentric chromosomes among the *M. barbatus* chromosomes.

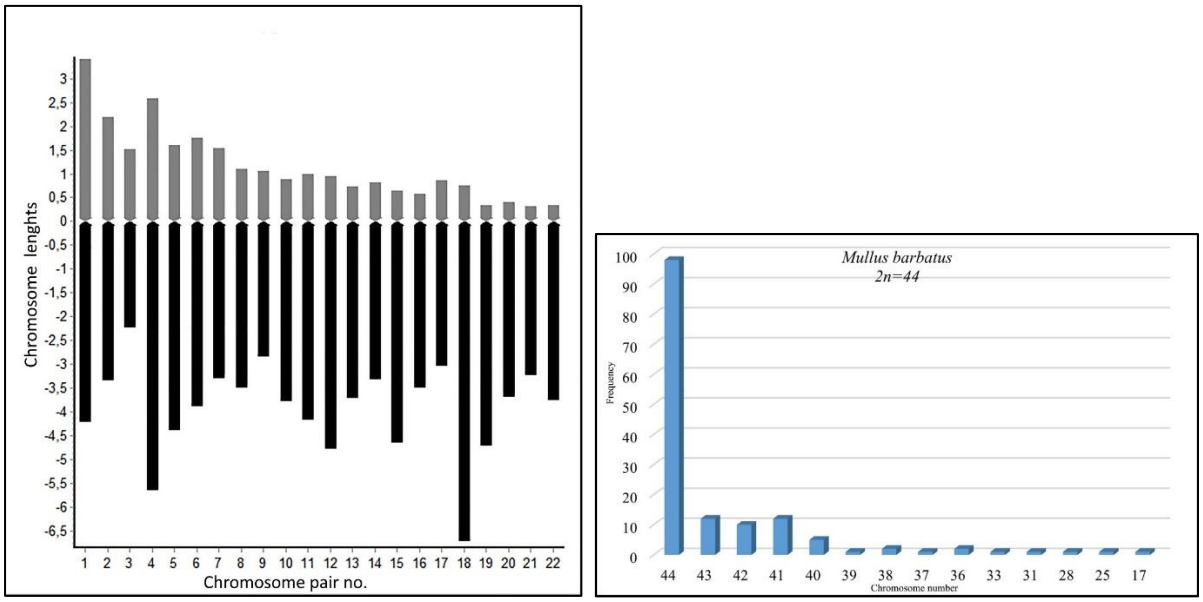

Figure 2: Idiogram and frequency distributions of cytotypes of *Mullus barbatus*.

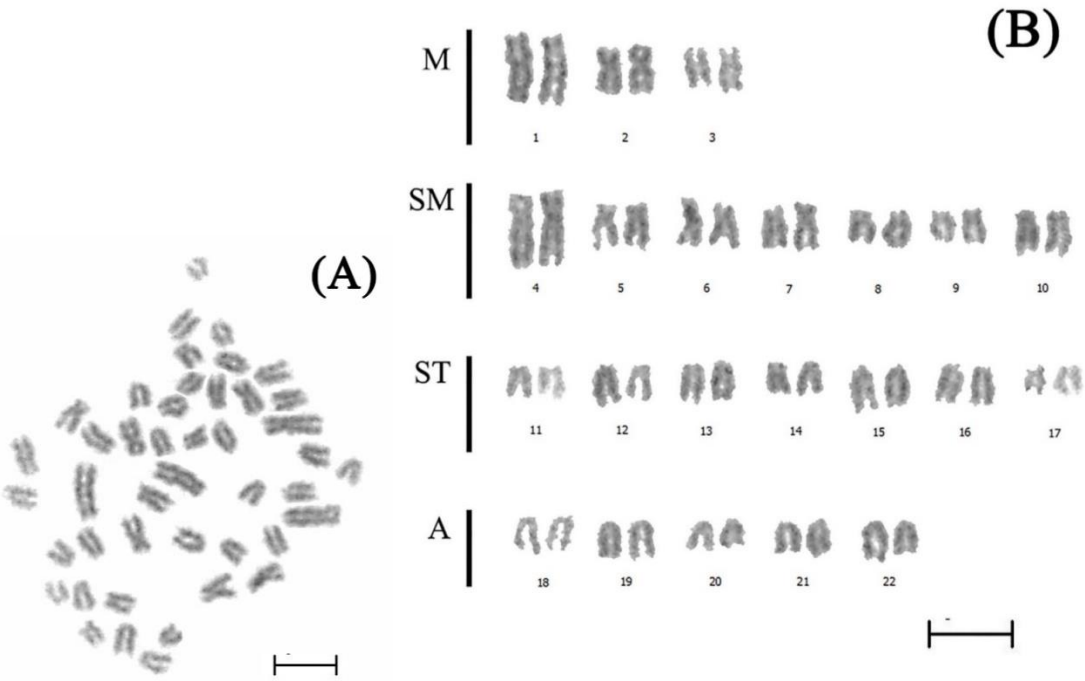

Figure 3: Metaphase (A) and karyotype (B) of *Mullus barbatus*, bar is 5µ.

The results of $Ag(NO)_3$ staining of *M. barbatus* are shown in Figure 5. NOR+ regions were found in 8 chromosomes of *M. barbatus*, and it was determined that they

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were in the telomere of two submetacentric chromosomes, in the interstitial (middle of the long or short arm) region of both arms of the other four acrocentric chromosomes, and a region close to the telomere of the right arm in two chromosomes.

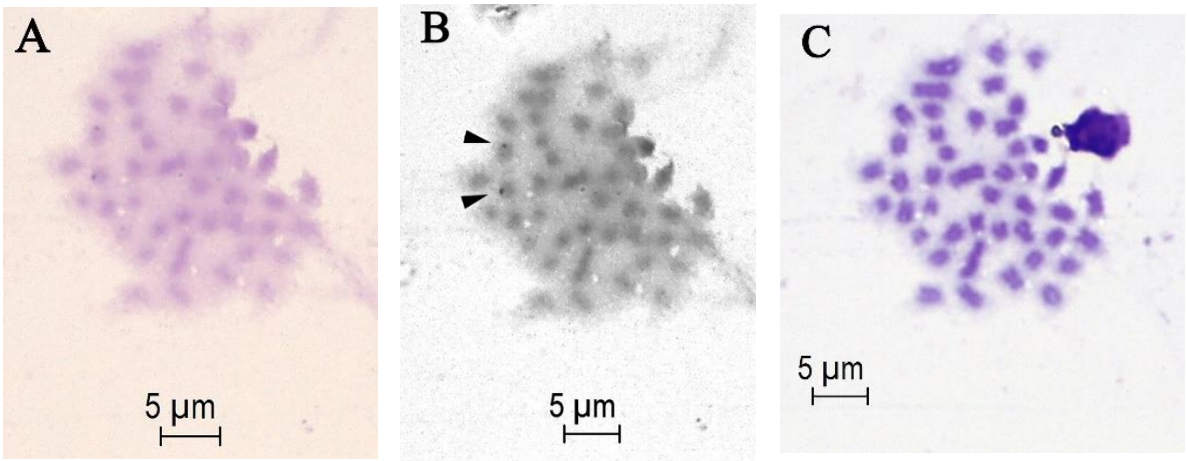

Figure 4: C+ banded chromosome regions in *Mullus barbatus.* A) C-banded metaphase, B) C+ positive regions (arrows), C) Giemsa-stained metaphase, bar is 5µ.

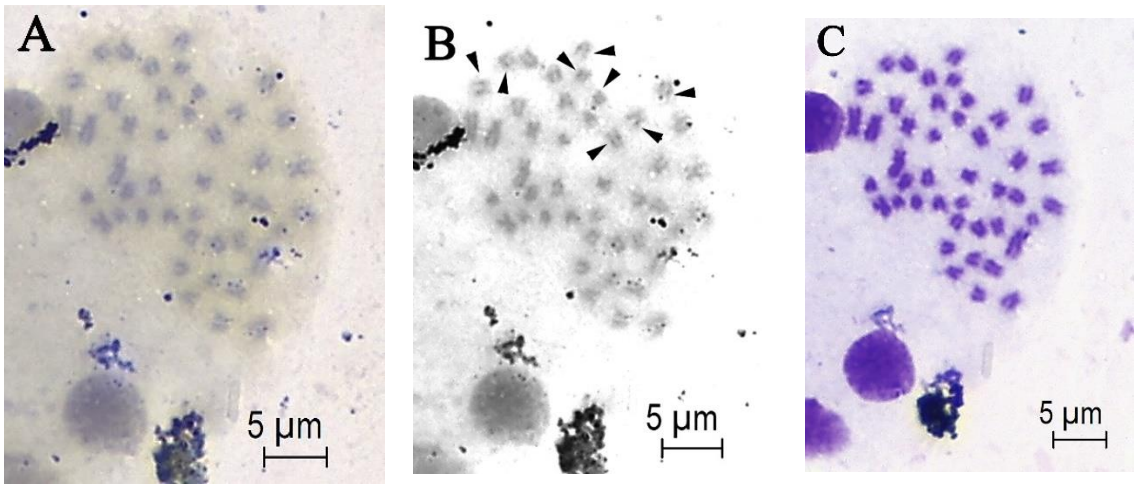

Figure 5: Ag(NO)$_3$-stained chromosome regions in *Mullus barbatus.* A) NOR-banded metaphase, B) NOR+ positive regions (arrows), C) Giemsa-stained metaphase.

Two of the round goby samples were treated with PB-MAX™ for different culture incubation periods ranging from 12 to 14 hours. However, no positive results were obtained, and no chromosomes were detected in the preparations. Additionally, suitable chromosome sites were detected in the preparations prepared after a short-term culture for three hours from the tissue samples from another sample. Fifteen different cytotypes from approximately 123 metaphase plates observed in the counting of these chromosome plates are graphically indicated in Figure 7. The highest percentage (65%) of 2n diploids for *Neogobius melanostomus* consisted of 46 chromosomes, and the lowest number of chromosomes was 2n–28. In this study, *N. melanostomus*, which was examined cytogenetically, had a karyotype consisting of 46 acrocentric chromosomes (Fig. 6-A), and the chromosome arm number was

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NF=46 (Fig. 7). In Figure 7, the classification of n number of homologous chromosomes is shown in the idiogram. The C-banding of preparations obtained from *Neogobius melanostomus* revealed constitutive heterochromatin regions in telomeric and centromeric positions in 8 chromosomes (Fig. 8).

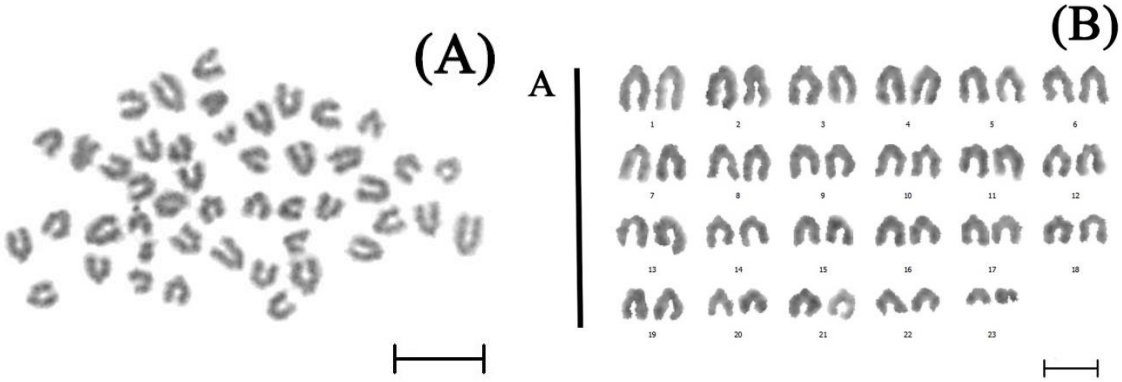

Figure 6: Metaphase (A) and karyotype (B) of *Neogobius melanostomus*, bar is 5µ.

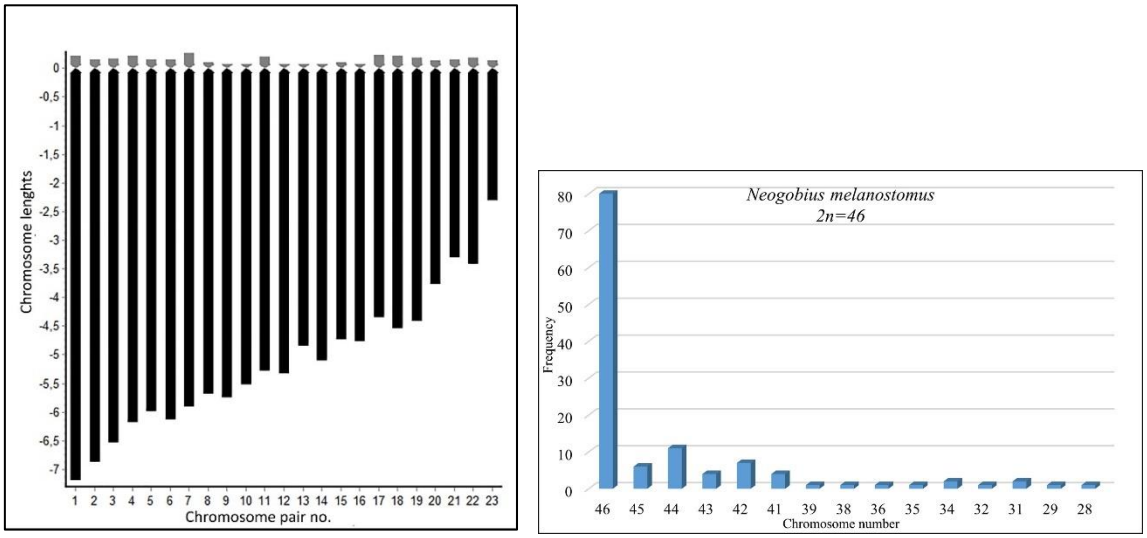

Figure 7: Idiogram and frequency distribution of cytotypes of *Neogobius melanostomus*.

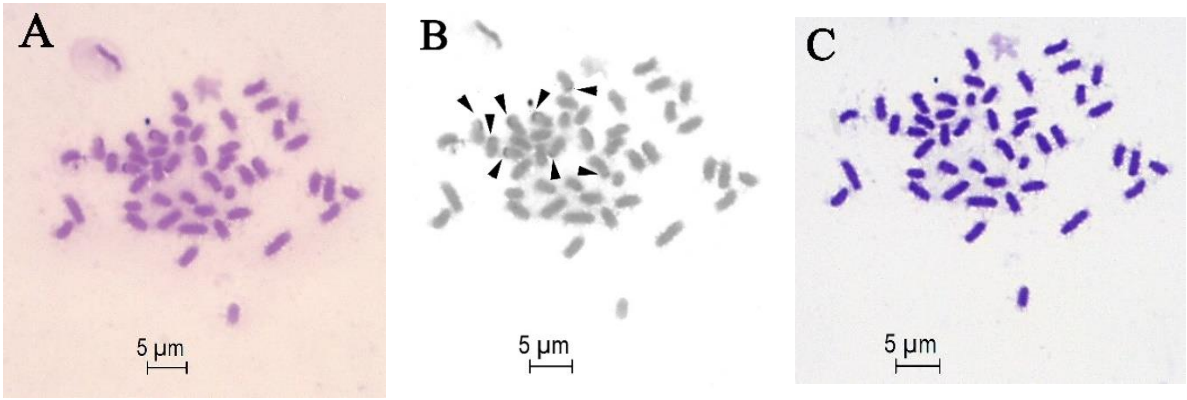

Figure 8: Chromosome regions with constitutive heterochromatin in *Neogobius melanostomus*. A) C-banded metaphase, B) C+ positive regions (arrows), C) Giemsa-stained metaphase.

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In microscopic and digital imaging analyses of the $Ag(NO)_3$ staining results, 2 NOR+ regions were identified at the centromeric and pericentromeric positions of two chromosomes of *N. melanostomus* (Fig. 9).

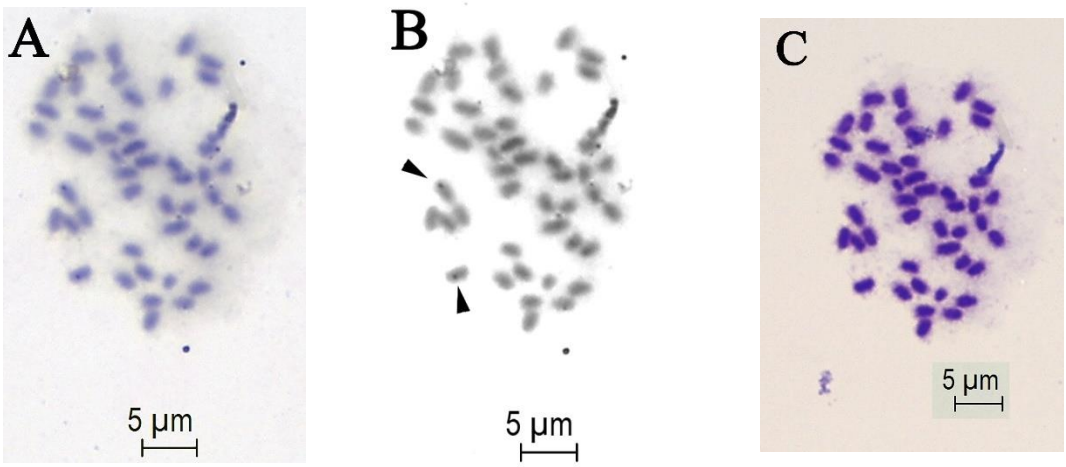

Figure 9: $Ag(NO)_3$-stained chromosome regions in *Neogobius melanostomus.* A) NOR-banded metaphase, B) NOR+ positive regions (arrows), C) Giemsa-stained metaphase, bar is 5µ.

Discussion

According to the study conducted by Araya–Jaime *et al.* (2021), who used short-term culture PB-MAX™ cell culture medium and introduced the method, the number of quality metaphases obtained from a drop of cell suspension of approximately 15 µL (0.015 mL) of each preparation varied from species to species but included 8–15 metaphases. Similarly, in this study, where 10 preparations were prepared from each sample, the number of quality metaphases varied from species to species. When a minimum of 0.15 mL (150 µL) of cell suspension was used in preparation, 1 and 30 metaphases were obtained. Netto *et al.* (2007) reported that incubation of fish samples known to be in the postmortem period for at least 20 min. and a maximum of two and a half hours in RPMI 1640 culture medium (PB-MAX™ was developed from this medium) for up to 12 hours would result in good cytogenetic performance. Although there is a difference in the number of metaphases between the studies conducted and this study, there are no species from which sufficient (at least 10) quality metaphases could be obtained for the karyotype. This technique has proven to be more advantageous than traditional *in vivo* methods, because obtaining chromosomes from marine fish has not always been possible or has been achieved with very little success. The disadvantage of this method is that the freshness of the fish sample and the time elapsed after death must be known exactly (at most 4 hours).

In this study, positive results in terms of quality metaphases were obtained in four out of six samples of both species by applying PB-MAX™ for 3 hours as a culture period. Araya-Jaime *et al.* (2021) reported that suitable metaphases could be obtained in up to 15 hours of incubation in culture, whereas in this study, PB-MAX™

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was tested between 10, 12, and 14 hours, but successful results were not obtained. In this study, no comparison was made between short-term and long-term tissue cultures.

Of the 109 valid species of the Mullidae family, only eight species have been cytogenetically investigated in a total of 12 studies. The largest number of (or the majority of) chromosome studies have been conducted on *M. barbatus*, *M. surmuletus*, and *M. argentinae* because Mullus is the genus with the fewest number of species (Froese and Pauly, 2025). In terms of traditional banding and staining, the C+ and NOR+ regions were also determined for the first time in this study. In terms of chromosome number, a diploid number of 44 was determined for this species , which is consistent with other studies (Laliberte *et al.*, 1979; Vitturi *et al.*, 1992; Saygun *et al.*, 2006; Prazdnikov, 2016). Table 1 summarizes the differences between the karyotypes of *Mullus barbatus* in previous studies. The karyotype determined in this study also highlights a difference. The number of bi-armed chromosomes was determined to vary between 4 and 8, and in our study, it was determined to be 20 (m+sm). Different results were obtained in this study because AKAS was used as an image analysis program. AKAS can automatically measure and sort digital photographs, in contrast to the methods of karyotyping performed manually in other studies of *M. barbatus*.

Table 1: Results of cytotaxonomic studies on the Mullidae family.

Species	2n	Karyotype	NF	Location	Reference
Mulloidichthys flavolineatus	48	48a	48	Japan	Ojima and Yamamoto (1990)
Mullus argentinae	44	2sm+42a	46	Brazil	Brum (1996)
M. barbatus	44	4m/sm+40a	48	Monaco	Laliberte *et al.* (1979)
	44	6m/sm+38a	50	Monaco	Laliberte *et al.* (1979)
	44	6m/sm+16st+22a	50	Italy (Palermo)	Vitturi *et al.* (1992)
	44	6m/sm+16st+11a	44	Turkey (Sinop -Black Sea)	Saygun *et al.* (2006)
	44	8sm+36st/a	52	Russia (Black Sea- Taman Peninsula)	Prazdnikov (2016)
	44	6m+14sm+14st+10a	64	Turkey (Ordu, Black Sea)	present study
M. surmuletus	48	-	50	Spain (Malaga)	Cano *et al.* (1982)
	44	8m/sm+16st+20a	52	Italy (Palermo)	Vitturi *et al.* (1992)
Paraupeneus spilurus	44	8m+8sm+28st/a	60	Japan (Chiba)	Arai and Koike (1979)
Upeneus parvus	44	8m/sm+38st/a	52	Brazil (Rio De Janeiro)	Pauls *et al.* (1996)
U. tragula	50	50st/a	50	India (Andaman Island)	Rishi (1973)
U. mossulensis	44	2m+2st+40a	46	Turkey (Mediterranean Sea)	Karahan (2016)

"*N.*" and "*U.*" terms are abbreviations for the genera *Neogobius* and *Upeneus.*

Table 1 shows the difference in the karyotypes compared with those of the Mullid species. The differences in NF are affected by different types and numbers of chromosome arrangements (Prazdnikov, 2016; Karahan, 2016). As shown in Figure 10, compared with our study, Vitturi *et al.* (1992) reported that in addition to the C+

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regions detected in the subtelocentric chromosomes of *Mullus barbatus*, NOR+ regions of different sizes are present in two pairs of chromosomes. In this study, the constitutive heterochromatin regions (C+ regions) were detected in the centromere of two subtelocentric chromosomes. NOR regions were found on four different chromosomes (Fig. 10).

However, since *M. surmuletus* and *M. barbatus* are highly similar species, a cytogenetic study revealed that they carry similar chromosomes and similar numbers of NORs, which refutes the hypothesis that NOR polymorphisms can be used as a taxonomic characteristic to distinguish species (Vitturi *et al.*, 1992). In our study, constitutive heterochromatin - C+ regions were found in centromeres of two acrocentric chromosomes and NOR+ regions were found in 8 chromosomes: these regions were recorded in the telomeres of one pair of submetacentric chromosomes, in the interstitial (middle of the long or short arms) regions of both arms of the other 2 pairs of acrocentric chromosomes, and a region close to the telomere of the right arm in 1 pair of chromosomes. Unlike the other study, the presence of NOR regions on bi-armed chromosomes may also be due to rearrangements in acrocentric chromosomes. Considering the populations residing in two different regions, FISH staining to reveal polymorphisms in the 18S rDNA genes, which are indicators of numerical and regional polymorphisms in NORs seen in *M. barbatus*, should be examined in more detail.

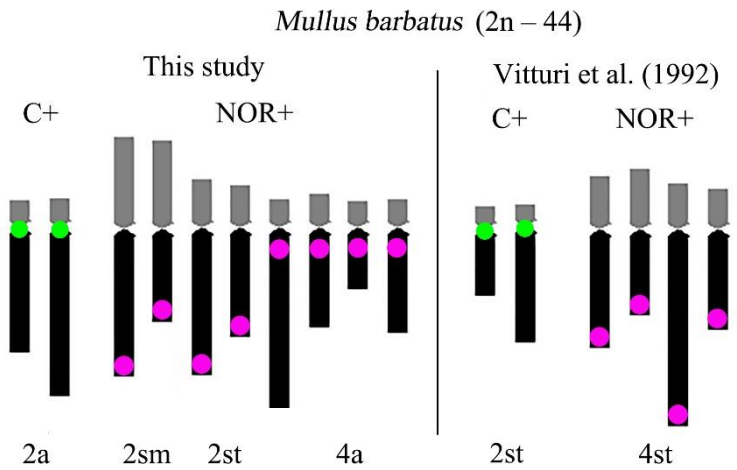

Figure 10: Graphical representation of C+ (green points) and NOR+ (pink points) results of *Mullus barbatus* in this study compared with other studies.

Table 2 summarizes cytotaxonomic studies that were carried out on 11 species of the *Neogobius* genus. Vasil'ev and Grigoryan (1994) revealed that the chromosome morphology of *N. melanostomus* has 46 acrocentric chromosomes. In addition to the common model chromosome type of the Neogobiid species being 46 acrocentric (uni-armed), the *Neogobius constructor* has been reported to have 42 to 44 chromosome numbers and 2 to 4 m-sm chromosomes (Vasil'ev and Grigoryan, 1990; 1993; Vasil'eva and Vasil'ev, 1995). *N. eurycephalus*, which has a large number of

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meta- and submetacentric (bi-armed) chromosomes (13–14 m+sm), has 3 different cytotypes depending on the sampling region, such as 2n – 30, 31, and 32 chromosomes. Whereas *N. kessleri* has 16-17 m-sm (Vasil'ev and Vasil'eva, 1992) as shown in Table 2.

The serial staining performed by Ocalewicz and Sapota (2011) on *Neogobius melanostomus* revealed that it had a karyotype.

Table 2: Results of cytotaxonomic studies conducted on the genus *Neogobius.**

Species	2n	Karyotype	NF	Location	Reference
Neogobius (*Ponticola*) *cephalargoides*	46	46a	46	Black Sea	Vasil'ev and Grigoryan (1990)
N. (*Ponticola*) *constructor*	44	2m+2sm+40a	48	Black Sea Basin	Vasil'ev and Grigoryan (1990)
	42	4m/sm+38a	46	Georgia (Tbilisi R.)	Vasil'ev and Grigoryan (1993)
	44	2m+42a	48	Black Sea Basin	Vasil'eva and Vasil'ev (1995)
N. (*Ponticola*) *cyrius*	37	9m/sm+2st+26a	46	Georgia (Kura R.)	Vasil'ev and Grigoryan (1993)
	38	8m/sm+30a	46	Georgia (Tbilisi R.)	Vasil'eva and Vasil'ev (1995)
	40	6m/sm+34a	46	Georgia (Kura R.)	Vasil'ev and Grigoryan (1993)
	41	5m/sm+1st+35a	46	Georgia (Kura R.)	Vasil'ev and Grigoryan (1993)
N. (*Ponticola*) *eurycephalus*	30	14m+2sm+14a	46	Danube Delta System	Ene (2003)
	31	13m+2sm+16a	46	Danube Delta System	Ene (2003)
	32	12m+2sm+18a	46	Danube Delta System	Ene (2003)
N. (*Ponticola*) *eurycephalus odessicus*	46	46a	46	Sasyk Lake (Black Sea)	Vasil'eva *et al.* (2011)
N. fluviatilis	46	46a	46	Turkey (Bilecik)	Unal Karakus *et al.* (2023)
	46	46a	46	Russia (Don R.)	Grigoryan and Vasil'ev (1993a)
N. (*Ponticola*) *gorlab*	46	46st/a	46	Caspian Sea Basin	Vasil'ev and Vasil'eva (1992)
	43-46	3m+3st+37a-46a	46	Russia (Bolshoy Uzen) Russia (Cheboksary Reservoir)	Prazdnikov *et al.* (2013)
N. (*Babka*) *gymnotracheilus*	46	46a	46	Black Sea Basin	Grigoryan and Vasil'ev (1993b)
	46	2sm/st+44a	-	Black Sea Basin	Grigoryan and Vasil'ev (1993b)
	46	1m+1sm+44a	48	Black Sea Basin	Grigoryan and Vasil'ev (1993b)
N. (*Ponticola*) *kessleri*♀	30	14m+2sm+14st/a	46	Black Sea Basin	Vasil'ev and Vasil'eva (1992)
	29	15m+2sm+12st/a	46	Black Sea Basin	Vasil'ev and Vasil'eva (1992)
	30	14m+2sm+14a	46	Black Sea Basin	Grigoryan and Vasil'ev (1993b)
	29	17m/sm+12a	46	Black Sea Basin	Grigoryan and Vasil'ev (1993b)
	46	46t/a	46	Caspian Sea	Esmaily and Kalbassi (2008)
N. melanostomus	46	46a	46	Azov Sea	Vasil'ev and Grigoryan (1994)
	46	46st-a	46	Gulf of Gdansk, Baltic Sea, Poland	Ocalewicz and Sapota (2011)
	46	46a	46	Turkey (Ordu, Black Sea)	present study
N. (*Ponticola*) *rhodioni*	46	46a	46	Black Sea Basin	Vasil'ev and Grigoryan (1994) Vasil'eva and Vasil'ev (1995)
N. (*Ponticola*) *syrman*	32	10m+4sm+18a	46	Sasyk Lake (Black Sea)	Vasil'eva *et al.* (2011)

*All terms in parentheses refer to new genus names currently valid for the genus *Neogobius* (Froese and Pauly, 2025; Fricke *et al.*, 2025). "*N.*" terms are abbreviations for the genus *Neogobius.*

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As can be seen from the comparative illustration in Figure 11, restriction endonuclease -RE banding, DAPI, CMA3, and FISH fluorescence staining confirmed the status of these NORs. In their study, Ocalewicz and Sapota (2011) obtained the following results after banding with RE, DAPI, and NOR: *Alu*I and *Dde*I restriction endonuclease (which recognize and cut different DNA sequences: CT+AG and AG+CT, respectively) banding, DAPI– and CMA_3– (negative) regions banding results were found to be the same as NOR+ regions bearing in terminal and pericentromeric regions (interstitial positions) of four chromosomes, as in the illustrative Figure 11.

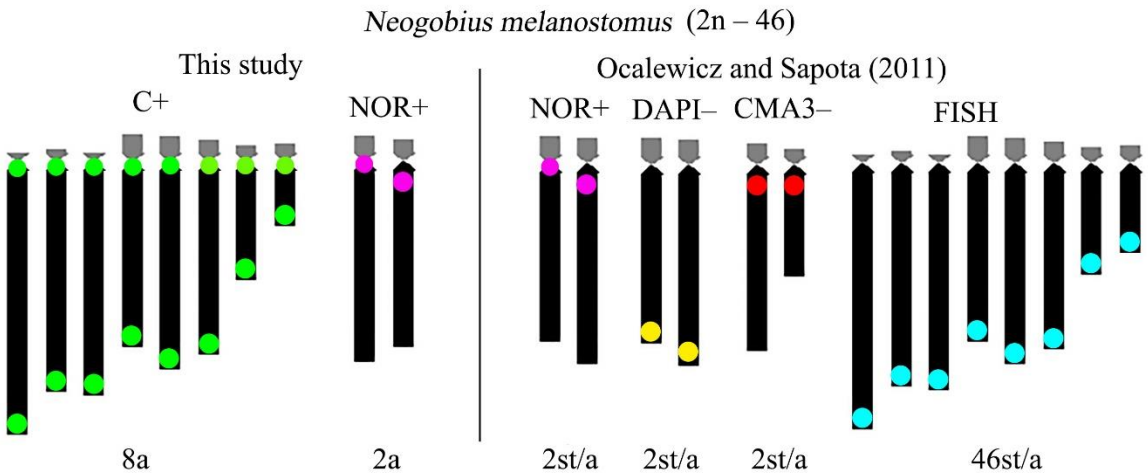

Figure 11: Graphical representation of C+ (green points) and NOR+ (pink points) results of *Neogobius melanostomus* in this study compared with other studies.

Our study revealed close similarity to the chromosome number and morphology with others conducted with *Neogobius melanostomus* living in the Black Sea Basin (Vasil'ev and Grigoryan, 1994). In the other studies, the intraspecific chromosome differences observed in Neogobiids were also revealed. For example, 11 different cytotypes of *Neogobius gorlab*, an endemic species of the Caspian Sea, were detected in samples taken from freshwater sources feeding this sea. In the study, these cytotypes were reported to have chromosome numbers ranging from 43 to 46. These authors suggested that chromosomal changes in these different populations were caused by Robertsonian-type translocations (Prazdnikov *et al.*, 2013; Bigaliev *et al.*, 2014).

Conclusions

As a result, in our study, both species presented less or more variation according to the karyotype and chromosomal features and NOR+ and C+ positive regions, as stated in the literature. These variations may be due to research conditions, research techniques, and chromosome image analysis techniques. Given these observations, further investigations employing advanced molecular cytogenetic techniques—such as multicolor fluorescence *in situ* hybridization (M-FISH), dual fluorescence *in situ* hybridization (D-FISH), and genomic *in*

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situ hybridization (GISH)—are necessary to achieve a more comprehensive understanding of the chromosomal organization and evolutionary relationships within these species. By utilizing these high-resolution cytogenetic tools, future studies can provide more precise insights into the mechanisms underlying chromosomal variation and contribute to broader genetic and evolutionary research.

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