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# Genetic Diversity of Populations of the Red Mullet *Mullus barbatus* Linnaeus, 1758 (Mulliformes: Mullidae) along the Bulgarian Black Sea Coast as Evident by Microsatellite Analysis*

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**Abstract:** The red mullet *Mullus barbatus* holds significant ecological and economic value within the Black Sea fisheries. However, similar to numerous other species, the stocks of red mullet are currently being harvested at levels that are not sustainable. The knowledge of the genetic diversity is an essential prerequisite for maintenance of the resources of this species as well as for understanding its adaptation to various environmental pressures. In this case study, nine microsatellite markers were used to evaluate the genetic population structure of *M. barbatus* along the Bulgarian Black Sea coast. This investigation represents the initial assessment of such genetic patterns in this particular geographic area. Totally, 80 specimens were genotyped from two locations along the Bulgarian Black Sea coast, revealing 122 alleles. For the samples from both locations, the mean values of observed and expected heterozygosity were rather high (Ho = 0.628 and He = 0.696 for Balgarevo; Ho = 0.672 and He = 0.716 for Sveti Vlas), indicating a high level of genetic diversity. Our results showed a substantial level of migration occurring among populations of the studied species. The low Fst value (0.007) is primarily related to within-population variation (90.81%) and implies a low level of population differentiation. The Garza-Wiliamson index values, when combined with population size, show a decrease in genetic variation in the population studied because of a combination of high fishing pressure, habitat fragmentation and intrinsic fluctuations of the population size.

**Key words:** Black Sea, red mullet, microsatellite, genetic diversity

## Introduction

The red mullet *Mullus barbatus* Linnaeus, 1758 occurs in the Eastern Atlantic from Western Norway, English Channel (rare in North Sea) to Dakar, Senegal and the Canary Islands, including the Mediterranean and the Black Sea (Whitehead et al. 1986). This species can grow to a standard length up to 21 cm in the Black Sea. Larvae and juveniles differ from adults in their external features and ecologi-

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cal traits. At the age of 1–1.5 months, the species switch to a bottom habitat. It is carnivorous, the diet consisting mainly of polychaetes, bivalves and crustaceans. Red mullet typically reach sexual maturity at c. 1–2 years, with spawning occurring in June and September in the Black Sea. The species undergoes spawning and seasonal migrations. In the spring, it migrates from its wintering areas in the Bosphorus northward to the Bulgarian coast for spawning and feeding (Karapetkova & Zhivkov 2006).

The red mullet is a commercially important species in all Black Sea countries (FAO 2020). During 2019–2021, the status of the Black Sea red mullet stocks was described as overexploited (Mikeladze et al. 2023).

Overfishing, pollution, habitat degradation and the impacts of climate change are all threatening fish populations (Martinez et al. 2018). Overexploitation and habitat loss are the primary causes of marine species stock reduction and extinction (Dulvy et al. 2006). An increasing number of studies demonstrates that extensive size-selective fishing could result in evolutionary changes in life-history traits in the harvested populations, which can have major implications for populations, ecosystems and fisheries (Pukk et al. 2013). Stock identification and structure are critical components for effective fisheries management and stock assessment. Misidentification of population structure within and among stock complexes may contribute to genetic resource erosion by decreasing spawning components, even when the fishery is adequately managed (Ramšak et al. 2004).

Microsatellites, a type of highly polymorphic markers, have proven to be valuable in assessing population genetic structure, especially among organisms with low levels of differentiation such as marine fishes (Estoup et al. 1998). The aforementioned molecular techniques have been employed for indirect monitoring of demographic traits of shared fish stocks, yielding useful scientific data for adjustment of fisheries management strategies and conservation plans (Hauser et al. 2002). Genetic structure studies of red mullet have been carried out in the Mediterranean Sea, revealing highly structured metapopulations and a transition zone between eastern and western populations (Galarza et al. 2009). Adriatic populations of *M. barbatus*, previously considered panmictic and isolated from other Mediterranean regions (Maggio et al. 2009), showed population connectivity with the northern Ionian and Tyrrhenian Seas (Matić-Skoko et al. 2018). Genetic divergence and phylogenetic relationship of two *Mullus*

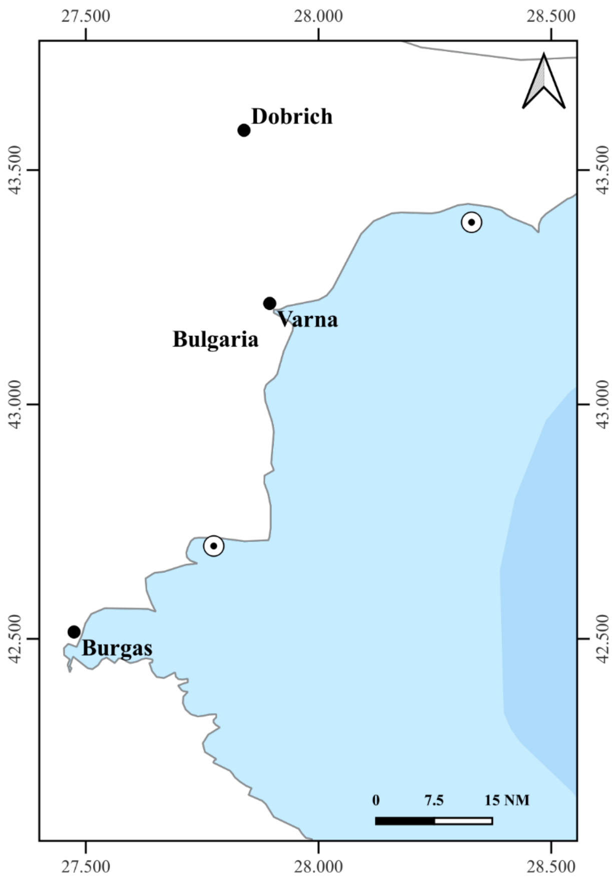


**Fig. 1.** Sampling sites (white circle) along the Bulgarian Black Sea coast.

*barbatus* populations from the western part of Black Sea (Varna) and *M. surmuletus* from the Mediterranean Sea (Thessaloniki) were investigated based on allozyme markers (Ivanova et al. 2014). With the exception of this research, data on genetic diversity of *M. barbatus* throughout the Bulgarian Black Sea coast is lacking.

The objective of this study was to assess genetic diversity of red mullet along the Bulgarian Black Sea coast for the first time using nine microsatellite markers as well as to detect population structure and contemporary connectivity as a tool for stock assessment and rational exploitation of the species. Based on the ecology and behaviour of red mullet and short geographical distances between the investigated regions, this study aimed to assess the hypothesis that there is no genetic structuring of the analysed populations.

# Materials and Methods

## Sample collection and DNA extraction

Eighty red mullet specimens were collected in summer 2022 along the Bulgarian Black Sea coast (Balgarevo – 40 and Sveti Vlas – 40; Fig. 1). Tis-

**Table 1.** Characterization of microsatellite loci for Black Sea red mullet genotyping.

| GenBank N | Locus | Repeat motif | Primer sequences | No. of alleles | Size range |
|---|---|---|---|---|---|
| DQ473548 | Mbar3 | (CA)24 | F: PET-GCTCCCCCGACACACTGTCT<br>R: ACCTTGGCCCTTCTTACGTC | 16 | 111-149 |
| DQ473549 | Mbar11 | (GT)10GC(GT)10 | F: VIC-TGACTGTCAGCACTTGCATT<br>R: CTGAGGAGAGTCATGAGT | 9 | 156-179 |
| DQ473547 | Mbar55 | (CA)7CG(CA)3TA(CA)6 | F: NED-TACACACAAACACTCACCCA<br>R: CGCAACCAATAGCACACTAC | 12 | 146–176 |
| DQ473553 | Mbar63 | (AC)10AT(AC)8 | F: VIC-AACCAGCAGGTCTCACA<br>R: TTCATGCTCCTTTTGTTCC | 11 | 301–337 |
| DQ473550 | Mbar130 | (AC)10 | F: NED-GAGGGTAGATTTGGTTGCAG<br>R: AGAGTATTGCATTTTTCGCC | 8 | 185–209 |
| DQ473556 | Mbar132 | (GT)10 | F: FAM-GGAGCAAGGAAGAGGAGA<br>R: CTCTGCAGACCTGCTCAA | 10 | 112–132 |
| DQ473554 | Mbar133 | (CA)14CG(CA)5 | F: PET-CTCGGCACATCACAGAAAC<br>R: CCTCCCAAATTACACACATC | 11 | 226–266 |
| EF675632 | Mbarb-002 | (AC)13ag(AC)3 | F: GGCCGTGACCACATATCAT<br>R:CCAATCTCTTCCTCTCCGC | 9 | 208–232 |
| EF675635 | Mbarb-051 | (CA)4cg(CA)9 | F:AACGAGGACCACTGAGACATTA<br>R:GGAACGATGACAACAGAGCA | 11 | 171–201 |
| n/a | Mbarb-064 | | F: ACAGTTCTGACTGCCCTCG<br>R: CGTCAATACCAGAATGCATCA | 6 | 129–143 |

sue samples from the dorsal fin were cut and preserved in 96% ethanol at 4°C for genetic analysis. The DNeasy Blood & Tissue Kit (QIAGEN®) was used to extract the genomic DNA. The DNA quality of the DNA samples was assessed by gel electrophoresis.

### Microsatellite genotyping

Nine high polymorphic microsatellite loci (*Mbarb002, Mbarb3, Mbarb11, Mbarb051, Mbarb63, Mbarb064, Mbarb130, Mbarb132* and *Mbarb133*) were amplified and analysed (Table 1). The primers utilised were identical to those developed by Matić-Skoko et al. (2018) for the same species in the Mediterranean Sea. All DNA samples were subjected to genetic analysis via multiplex reactions, and the primer dies were cautiously arranged to prevent overlap in the sizes of similar alleles. The polymerase chain reaction (PCR) was performed in a reaction volume of 50 µL containing 1 µL (0.2 µM) of each primer, 25 µL of Mastermix (MyTaq™ HS Mix, Meridian Bioscience®) and 2 µL (~100 ng) of the target DNA.

The PCR amplification was conducted under the following conditions: an initial denaturation step at 95°C for 5 min, followed by 30 cycles of: denaturation at 95°C – 30 s, annealing at 56.5°C – 30 s, and extension at 72°C for 30 s. The fragment analysis was carried out by using Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher Scientific). The fragments size was determined by means of GeneMapper 4.0 (Thermo Fisher Scientific) software package.

### Statistical analyses

The Hardy-Weinberg equilibrium (HWE) exact tests and loci combinations for linkage disequilibrium with the Markov Chain methods, number of different alleles (Na), number of effective alleles (Ne), Shannon's information index (I), observed (Ho), expected (He) and unbiased expected (uHe) heterozygosity, fixation index (Fst), inbreeding coefficient (Fis), number of migrants (Nm), private alleles (alleles observed in only one of the populations) and the allelic richness estimations were conducted using GenAlEx 6.5 (Peakall & Smouse 2012). Polymorphic information content (PIC) was calculated using DEMoMa tool (Hanci, 2022) and Garza and Wil-liamson index were computed using Arlequin ver 3.5.2.2 (Excoffier & Lischer 2010).GenAlEx 6.5 (Peakall & Smouse 2012) software was used for calculation of variations among and between populations, genetic diversity and separation values between locations. The program NeEstimator v2.1 (Do et al. 2014) was used to estimate contemporary effective population size using the single-sample linkage disequilibrium method for each of the examined populations.

**Table 2.** Descriptive statistics of *Mullus barbatus* for nine loci samples from two different populations along Bulgarian Black Sea coast.

| | *Mbarb 002* | *Mbarb 3* | *Mbarb 11* | *Mbarb 051* | *Mbarb 63* | *Mbarb 064* | *Mbarb 130* | *Mbarb 132* | *Mbarb 133* |
|---|---|---|---|---|---|---|---|---|---|
| **Balgarevo** | | | | | | | | | |
| N | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 |
| Na | 7.000 | 24.000 | 9.000 | 7.000 | 14.000 | 5.000 | 8.000 | 9.000 | 14.000 |
| Ne | 3.187 | 14.815 | 3.358 | 2.010 | 6.452 | 2.109 | 2.591 | 3.430 | 6.987 |
| I | 1.381 | 2.908 | 1.558 | 1.100 | 2.162 | 1.001 | 1.301 | 1.595 | 2.217 |
| Ho | 0.675 | 0.850 | 0.750 | 0.450 | 0.650 | 0.500 | 0.250 | 0.750 | 0.825 |
| He | 0.686 | 0.933 | 0.702 | 0.503 | 0.845 | 0.526 | 0.614 | 0.708 | 0.857 |
| uHe | 0.695 | 0.944 | 0.711 | 0.509 | 0.856 | 0.533 | 0.622 | 0.717 | 0.868 |
| PIC | 0.64 | 0.93 | 0.67 | 0.48 | 0.83 | 0.48 | 0.55 | 0.68 | 0.76 |
| **Sveti Vlas** | | | | | | | | | |
| N | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 |
| Na | 8.000 | 16.000 | 10.000 | 10.000 | 13.000 | 7.000 | 8.000 | 10.000 | 14.000 |
| Ne | 3.536 | 8.226 | 3.119 | 1.921 | 6.142 | 2.496 | 2.591 | 4.552 | 6.987 |
| I | 1.504 | 2.378 | 1.517 | 1.172 | 2.085 | 1.227 | 1.301 | 1.770 | 2.217 |
| Ho | 0.750 | 0.725 | 0.725 | 0.525 | 0.775 | 0.675 | 0.250 | 0.800 | 0.825 |
| He | 0.717 | 0.878 | 0.679 | 0.479 | 0.837 | 0.599 | 0.614 | 0.780 | 0.857 |
| uHe | 0.726 | 0.890 | 0.688 | 0.485 | 0.848 | 0.607 | 0.622 | 0.790 | 0.868 |
| PIC | 0.67 | 0.87 | 0.65 | 0.47 | 0.82 | 0.56 | 0.57 | 0.75 | 0.84 |

N – number of samples, Na – number of different alleles, Ne – number of effective alleles, I – Shannon's information index, Ho – observed heterozygosity, He – expected heterozygosity, uHe – unbiased expected heterozygosity, PIC – Polymorphic Information Content.

**Table 3.** F-statistics and estimate of the number of migrants (Nm) for all study populations by loci.

| | *Mbarb 3* | *Mbarb 133* | *Mbarb 130* | *Mbarb 132* | *Mbarb 051* | *Mbarb 11* | *Mbarb 63* | *Mbarb 002* | *Mbarb 064* | *Mean± SE* |
|---|---|---|---|---|---|---|---|---|---|---|
| **Fis** | 0.130 | 0.055 | 0.539 | -0.041 | 0.007 | -0.068 | 0.153 | -0.015 | -0.044 | **0.080**±0.063 |
| **Fit** | 0.138 | 0.064 | 0.543 | -0.026 | 0.010 | -0.065 | 0.158 | -0.011 | -0.042 | **0.085**±0.063 |
| **Fst** | 0.009 | 0.009 | 0.009 | 0.014 | 0.003 | 0.003 | 0.006 | 0.004 | 0.002 | **0.007**±0.001 |
| **Nm** | 27.595 | 26.235 | 28.924 | 17.137 | 82.684 | 85.019 | 44.123 | 56.137 | 100.028 | **51.99**±10.16 |

Fst – fixation index, Fis – inbreeding coefficient, Nm – number of migrants.

# Results

### Genetic variability of microsatellite loci

This study represents the first application of these markers to investigate the genetic variability of red mullet populations along the Bulgarian Black Sea coast. All analysed microsatellite markers proved to be polymorphic. A total of 122 alleles were discovered across the nine loci. The length of the identified alleles in the examined loci ranged between 105 and 345 base pairs (bp). The number of alleles ranged from 7 in locus *Mbarb064* to 25 in locus *Mbarb3.* The mean number of alleles observed was found to be 10.78, whereas the effective number of alleles was determined to be 4.99 (Table 2). The analysis of allelic frequencies by loci demonstrated that the minimum allelic frequency (0.013) was observed at all loci examined, while the maximum frequency (0.713) was specifically observed at locus *Mbarb051*.

The observed levels of expected heterozygosity (He) exhibited variation across different populations and loci. For instance, in the Sveti Vlas population, locus *Mbarb051* showed an anticipated heterozygosity of 0.479, whereas in the Balgarevo population, locus *Mbarb3* exhibited a greater expected heterozygosity of 0.933. On average, across all populations, the expected heterozygosity was calculated to be 0.712. The locus *Mbarb3* exhibited the highest observed heterozygosity (Ho) value of 0.850 in the Sveti Vlas population, while the locus

*Mbarb130* displayed the lowest value of 0.25 in both populations. The mean observed heterozygosity values of 0.633 in Balgarevo and 0.672 in Sveti Vlas suggest a relatively significant level of genetic variability among these populations. Unbiased mean expected heterozygosity ranged from 0.509 to 0.944 (mean 0.717) for Balgarevo, and from 0.485 to 0.890 (mean 0.725) for Sveti Vlas (Table 2). The observed heterozygosity displayed a lower average value compared to the expected heterozygosity at six loci in the Balgarevo samples and at four loci in the Sveti Vlas samples, suggesting a minor heterozygous deficiency. The polymorphic information content (PIC) exhibited a spectrum of values, with locus *Mbarb051* having a PIC of 0.47 and the marker *Mbarb3* having a PIC of 0.93. The average PIC value across all loci was calculated to be 0.68.

The observed heterozygosity was found to be lower than the expected values in both populations. Although the findings of the current investigation demonstrate the presence of a specific heterozygous deficiency, overall, a significant level of genetic diversity was detected in the examined populations, with an average value of 0.706±0.032. The Fis coefficient exhibited a range of values spanning from -1 to +1. Positive values of the coefficient indicate the presence of heterozygous deficiency within the particular locus, as well as within the overall population. The coefficient of inbreeding (Fis) exhibited positive values, with a mean of 0.080, serving as an indicator of inbreeding within the population (Table 3). The value of Fst (0.007) is an indicator of a small degree of genetic differentiation in the population and, respectively, a large gene flow. The estimates of single-locus Fst varied from 0.002 (*Mbarb064*) to 0.014 (*Mbarb132*). The effective population size of the Balgarevo population is estimated to be 219.9 (with a lower bound of 98.2 and an upper bound of infinity), nonetheless for the Sveti Vlas population, the estimated values converge to infinity (with a lower bound of 190.8 and an upper bound of infinity).

## Discussion

The impact of human activities on natural populations, such as fishing and habitat degradation, frequently leads to substantial decreases in population size, known as demographic bottlenecks, as well as a reduction in genetic diversity, referred to as genetic bottlenecks (Weber et al. 2004). The evolutionary and adaptive capacity of a population may be shaped by two factors: heterozygosity and the overall number of alleles within the population (Frankham et al. 2014).

The microsatellite loci of the red mullet exhibited significant and consistent genetic variability across the samples collected along the Bulgarian Black Sea coast. However, there was a minor overall trend indicating a deficiency of heterozygous genotypes. Numerous fish species have been documented to exhibit a deficiency in heterozygote genotypes (Waldman & McKinnon 1993). The expected heterozygosity (He) is considered the most reliable measure of genetic diversity within a population, which in our study is close to those reported by other authors (Galarza et al. 2009, Matić-Skoko et al. 2018) for the same loci in red mullet populations in the Atlantic Ocean and the Mediterranean Sea (mean value He = 0.760 and 0.763). The genetic differentiation observed among the populations under study, as determined by microsatellite analysis, indicated an average Fst value of 0.007. This result primarily reflects the variation within populations (90.81%), with a smaller proportion attributed to between-population variation (9.19%). The calculated mean value for Fst indicated a low level of genetic differentiation between the two red mullet populations. The Mediterranean, Adriatic and Aegean Seas have shown comparably low values for the same species (Garoia et al. 2004, Maggio et al. 2009, Matić-Skoko et al. 2018). The observed high migration coefficient (mean Nm = 51.987) could potentially be a significant contributing element to the observed low level of differentiation.

Marine species often exhibit low levels of population differentiation as indicated by low Fst values. Our study revealed that high effective population size could contribute to reduced genetic differentiation in neutral markers, such as microsatellites.

The low levels of genetic differentiation observed among geographic regions may be attributed to the increased likelihood of dispersal during the planktonic egg, larval or adult phases, coupled with the absence of physical barriers restricting their movement across basins or adjacent continental boundaries (Grant & Bowen 1998, Hewitt 2000). Hence, larval interchange and gene flow are predominantly influenced by the dispersion capacity of the species (Rossi et al. 2014).

Larval dispersal in marine species serves as a mechanism for connecting local populations, hence contributing to the formation of large marine metapopulations (Kritzer & Sale 2004). The long larval pelagic phase (larvae and 0-group fish at the age of 1.5–2 months) have been described for red mullet in the Black Sea (Melnikova & Kuzminova 2020). Drifting eggs and larvae of red mullet could be transported along the current system, homogenizing neutral allele frequencies into a single gene pool within this region and leading to lack of genetic differentiation. Adult *M. barbatus* inhabit a wide range of depths and biotopes, and they can probably move over large areas and cover long distances. The geographical distance between the analysed populations is to short − 100 km. Consequently, the distribution pattern of red mullet can be one of continuous admixture of distinct subpopulations during seasonal migrations, resulting in a sampling bias and a Wahlund effect (Maggio et al. 2009). Moreover, due to the significant level of population connectivity observed in marine ecosystems, it may not always be feasible to sample discrete populations. In such cases, sampling cohorts of recruits originating from multiple populations could be a viable approach to assess genetic diversity (Martinez et al. 2018).

Studies of red mullet populations are characterized by high values of genetic diversity, according to the Shannon index (I), which is consistent with our results 1.691 and 1.686 for Balgarevo and Sveti Vlas respectively (Table 2). The effective population size is widely recognised as a crucial parameter in management strategies due to its direct influence on the rates of genetic drift and on the potential loss of genetic variation (Hare et al. 2011). The Ne serves as an accurate indication of population viability, and it is crucial to maintain a minimum threshold of 50, as evidenced by our research findings, in order to prevent population extinction resulting from the negative effects of inbreeding depression.

The genetic diversity data received for red mullet populations along the Bulgarian Black Sea coast support the hypothesis for genetic flow among different units (subpopulations) with high connections between them and a lack of genetic structuring.

The Garza-Wiliamson (M) index was employed for the identification of the bottleneck effect. The average value obtained for the populations under study was 0.3299, with a range of 0.163 to 0.469. This finding suggests a consistent decline in genetic diversity within the population, most likely a result of founder effect and/or bottleneck (Kaczmarczyk & Wolnicki 2016). It is important to acknowledge that between populations examined in the current study, the M coefficients derived for all genetic markers were found to be below 0.68; this indicates a recent decline in population size, possibly linked to the effects of overfishing. Values in the range of several hundreds and an M-factor below the established critical M threshold values for the red mullet in the Mediterranean (G.W index 0.73) were considered indicative of recent population changes resulting from a combination of overexploitation, fragmentation of habitats and inherent fluctuations in population size (Matić-Skoko et al. 2018). The adverse impact of fishing-induced population fluctuations on effective population size results in heightened selection and genetic drift effects, ultimately leading to lower genetic diversity levels.

The prevailing consensus is that there is currently a lack of an efficient fishery management strategy for the entire Mediterranean basin, namely for demersal stocks and more specifically for red mullets (Morat et al. 2012). This is also valid for the Black Sea red mullet stocks. The determination of genetic structures and diversities within fish populations holds significant importance in ensuring the maintenance of fish populations and the implementation of sustainable fisheries management (Maggio et al. 2009, Tuncay et al. 2014).

# Conclusions

The results obtained emphasize the significance of assessing the genetic structure of red mullet populations as a means of enhancing stock assessment and management programs, mitigating the adverse impact of fishing on the effective population size and preserving genetic diversity. This study has the potential to make a valuable contribution to the sustainable management of red mullet in the coastal areas of Bulgarian Black Sea region, benefiting both the local and regional fisheries. Furthermore, it can facilitate the shift towards sustainable fisheries practices.

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