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Phylogenetic Analysis of Native *Salmo* Species in Türkiye Using Mitochondrial 12S rDNA, 16S rDNA, Cytochrome b Sequences, and Cytogenetics

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ABSTRACT

In this study, phylogenetic relationships and genetic diversity of *Salmo* spp. populations in different water bodies in Türkiye were investigated, focusing on morphologically similar specimens to *Salmo macrostigma*. Significant genetic variability and evolutionary differentiation within these populations have been identified using mitochondrial regions sequences (12S rDNA, 16S rDNA, and Cyt-b), morphological observations, and cytogenetic analysis. Samples collected from eight river basins revealed three main phylogenetic clades and local adaptations influenced by ecological pressures. Cytogenetic findings showed that they have chromosome numbers ranging from 78 to 80 (2n), consistent with known Salmo species. For this purpose, three mitochondrial DNA fragments encoding 12S rDNA, 16S rDNA, and Cyt-b regions were amplified by PCR, sequenced, and then used for phylogenetic analysis of the studied species. The study revealed the need for comprehensive integrative taxonomic approaches to clarify the classification of *Salmo* spp. with critical implications for conservation strategies in Türkiye biodiversity hotspots. In conclusion, this study not only advances our knowledge of *Salmo macrostigma* or similar *Salmo* spp., but also suggests innovative approaches for future Salmonid phylogenetics and cytogenetics studies. The findings have important implications for understanding the evolution, taxonomy, and conservation of these economically and ecologically important fish.

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Introduction

The family Salmonidae is a monophyletic lineage divided into three subfamilies, Coregoninae, Thymallinae, and Salmoninae, represented by 11 genera and about 226 species. The subfamily Salmoninae is considered the largest and most diverse lineage, containing five genera, *Brachymystax*, *Hucho*, *Salmo*, *Salvelinus*, and *Oncorhynchus*, represented by at least 100 species (Shedko, 2013; Houston & Macqueen, 2019). Among these, the genus Salmo contains the most important fish species in terms of their commercial and recreational value (Noakes, 2014). According to Fishbase (http://www.fishbase.org) (Froese & Pauly, 2014) records, at least 50 confirmed representatives of the genus have been discovered worldwide to date, 18 of which are found naturally in Türkiye's inland waters (Küçük et al., 2024). Species within the genus *Salmo* represent an evolutionarily interesting lineage whose taxonomy is controversial due to their complex, high phenotypic variability and low level of genetic differentiation. Inconsistencies between morphological and genetic data have led to contrasting taxonomic classifications for *Salmo trutta* Linnaeus, 1758. Molecular studies, exemplified by Machordom et al. (2000), Bernatchez (2001), and Cortey et al. (2004), support the identification of distinct geographic populations as lineages within *S. trutta*. Conversely, recent morphological studies tend to separate *S. trutta* into separate species using the traditional morphological species concept and integrating phylogenetic insights from molecular data (Delling, 2003; Kottelat & Freyhof, 2007).

According to recent phylogenetic data, more precisely based on mitochondrial DNA sequences, nine main brown trout evolutionary lineages of the *Salmo trutta* complex, namely Adriatic, Atlantic, Danube, Danube, Marmaratus, Mediterranean, Duero, Tigris, Dades and North Africa, have been identified and evolved during the Pleistocene (Bernatchez, 2001, Apostolidis et al., 1997; Suárez et al., 2001; Bardakci et al., 2006; Snoj et al., 2011; Tougard et

al., 2018; Turan et al., 2024). Moreover, at least 57 different subspecies or ecological forms have been reported within the *Salmo trutta* complex so far (Ferguson, 2004; Kocabas, 2009). To date, multiple ecotypes/subspecies of *Salmo trutta* have been distinguished in Turkish aquatic systems and are now considered as separate species (Caballero et al., 2006; Turan et al., 2012). Among these, *Salmo macrostigma*, also known as the red-spotted trout or rocky mountain trout, is a species of freshwater trout native to Algeria in northwestern Africa. The name *Salmo trutta macrostigma* (or *Salmo macrostigma*) was previously used for many populations in other areas around the Mediterranean, but in recent years these have been separated into separate local species (Tougard et al., 2018). It has also been described in many areas of Türkiye, which borders the Mediterranean (Okumuş et al., 2007). However, many species previously described under the name *Salmo macrostigma* around the Mediterranean region have been renamed. The best examples are *Salmo cettii* in Italy and *Salmo farioides* in the eastern Adriatic region (Kottelat, 1997). In Türkiye, the taxonomic situation is further complicated by the presence of multiple specimens formerly misidentified as *Salmo macrostigma*. These are *Salmo tigridis* (in the Tigris-Euphrates drainage, Persian Gulf basin), *Salmo labecula* (Ecemiş Creek in the Seyhan River basin and other Mediterranean coastal drainages), *Salmo opimus* (Tekir, Fırnız, and Göçüksu creeks in the lower Ceyhan River), *Salmo chilo* (in Akdere Creek in the Upper Ceyhan River), *Salmo okumusi* (in the West Euphrates basin) and *Salmo euphrataeus* (in the Northeast Euphrates basin) (Okumus et al., 2006; Turanet al., 2011; Turan et al., 2014; Turan et al., 2002; Ehrlich & Wilson, 1991). These misidentifications may have arisen due to limited genetic data in earlier taxonomic studies and high morphological variability in trout populations, which are often adapted to specific local conditions. Modern molecular methods have helped to clarify many of these distinctions. However, there are still gaps in the distinction of trout species. Many trout species in Türkiye have similar physical characteristics, making identification in the field difficult. Although modern research utilizing molecular phylogenetic and ecological data has significantly clarified the taxonomy of these trout populations, there are still gaps in the understanding of endemic trout species in regions such as Türkiye, which harbors a rich diversity of endemic trout species. The misidentification of endemic trout specimens in Türkiye has important consequences for both science and conservation. Particularly for the species *S. macrostigma*, although its existence has not been fully proven, the fact that previous studies have indicated the presence of this species raises the suspicion that the species is extinct or threatened with extinction in the region. Its population may have declined due to human activities such as the introduction of non-native species (Ehrlich & Wilson, 1991). Or they may have declined in Turkish waters due to increased competition and reproductive interactions with alien species introduced by human activities. In addition, water pollution, overfishing, and poaching may have affected their habitats and populations. From this point of view, if this species is to survive in Turkish waters, it is necessary to protect critical habitats, conduct more genetic studies in unexplored areas, and raise awareness about Türkiye unique trout diversity. In this respect, it is important to re-examine species such as *S. macrostigma* in regions where it was previously suspected.

The introduction and recent advances in molecular genetic and cytogenetic study techniques have enabled a better understanding of the evolutionary history and current genetic differentiation of many fish species and populations (Bernatchez et al., 1992; Giuffra et al., 1994; Bernatchez & Osinov, 1995). In recent years, molecular genetic studies have attracted significant attention in fisheries. Moreover, the use of molecular data in phylogenetic and taxonomic sampling has facilitated the identification of new species (Miya & Nishida, 2015). Cytotaxonomic studies, which are widely accepted as the basis of phylogenetic and taxonomic research, are essential for understanding genetic diversity and evolutionary relationships among species. However, in the context of fish species in Türkiye, it is noteworthy that the karyotypes of approximately 90% of these species have not yet been described. As a result, there is a significant gap in the chromosomal structure and genetic characteristics of these species. To fill this gap, we present the first study on the chromosome numbers of mountain trout *Salmo* spp. specimens in Türkiye, in other words, some specimens suspected to be *Salmo macrostigma*. Although sequence analysis is widely used in phylogenetic studies, it cannot fully explain evolutionary relationships due to mutations that cannot be prevented in PCR (Simon et al., 1994). From this perspective, in addition to phylogenetic studies involving multiple genes to explain both these mutations and their evolutionary relationships, determining chromosome numbers may also help address this issue. Such methods can identify evolutionary elements that are conserved across species (Mayrose and Lysak, 2021). Additionally, they can identify specific regions within sequences that are critical for organisms and potentially provide evolutionary information (Poretsky et al., 2014). Classifying fish species and identifying closely related species is very important for various scientific disciplines such as biology and ecology. However, distinguishing between closely related fish species, especially in the family Salmonidae, can be difficult due to similar morphological features. Although traditional molecular markers such as COI, D-Loop, Cyt-b, 12S and 16S ribosomal regions have difficulty in drawing a clear picture of species boundaries, they have been an important source of information in the identification of *Salmo lineages* and species so far (Küçük et al., 2024). These regions are molecular markers that show differences between species. Analyzing the differences in these regions can allow for accurate discrimination of closely related species. Especially Cytochrome b (Cyt-b) is one of the important regions in the mitochondrial genome (Martin & Palumbi, 1993). However, 12S and 16S ribosomal RNA (rRNA) regions used in classification are widely used for phylogenetic analysis in vertebrates (Simons & Mayden, 1998; Wang & Lee, 2002). However, a literature review has confirmed that there may be a lack of research on the phylogenetic relationships of these gene regions in fish. This lack prevents a complete knowledge of species discrimination and typing ability in this area. Therefore, research efforts in this area should be increased to better understand the evolution and biology of fish species.

In this study, we investigate the phylogenetic status of *Salmo* spp. more precisely *S. macrostigma*-like fish specimens from mountainous regions in Turkish waters and determine their relationship with other representatives of the genus *Salmo* in the region by applying selected mitochondrial DNA regions sequence analysis. It also presents the first cytotaxonomic survey of mountain trout samples within the geographical boundaries of Türkiye. This cytotaxonomic analysis aims to contribute to the understanding of the karyotype of this species and its potential phylogenetic implications.

Materials and Methods

Ethics Approval and Consent to Participate

This experiment was approved by Munzur University (Approval No:2022/22-06/15-02), and all applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All experimental procedures were carried out in accordance with the guidelines established by Munzur University. In addition, the study was conducted in accordance with ARRIVE guidelines.

Study Area and Fish Sampling

Fish were collected by electrofishing between February 2017 and March 2019 from river basins in Türkiye that drain into the Mediterranean Sea and the Persian Gulf (Figure 1). The location and coordinates where Salmonidae samples were collected are detailed in Table 1. A total of 147 samples were collected from the mountainous areas of the streams: Akdere (n=28), Gökpınar (n=14), Zindan (n=17), Aksu (n=24), Tohma (n=12), Munzur (n=18), Nergele (n=14) and Göksu (n=20). To confirm the species identity of the collected specimens, morphological observations were made following established protocols (Mearelli et al., 1995; Gibertoni et al., 1998; Kocabas, 2009; Iaffaldano, et al., 2016). The main morphological characters for the correct identification of *S. trutta* are presented in Figure 2. These characteristics include a large and prominent postorbital spot, fewer vertebrae than in other subspecies, and intense pigmentation with red spots surrounded by a white halo. Samples showing the highest similarity to mountain trout *Salmo macrostigma* in terms of morphological characteristics were used for DNA isolation. To avoid harming this endangered species, fish were quickly sampled and then released back into the habitat. Adipose fin clips were collected from each fish, placed in tubes containing 70% ethanol, and stored at -20 ºC until DNA isolation. In addition, ten fish samples were caught and brought to the laboratory in a cold chain for use in chromosome studies. Two samples were collected from the Munzur and Göksun locations, with one sample each from other locations.

Cytogenetic Analysis

Chromosome number determination was performed according to the protocol described by Netto et al. (2007) with minor modifications, using kidney and gill cells obtained from freshly euthanized fish to ensure the presence of viable dividing cells, following the approach of Saenjundaeng et al. (2018). Kidney and gill tissues were carefully excised and finely chopped. The tissues were washed in Roswell Park Memorial Institute culture medium (RPMI-1640; Life Technologies, USA) and transferred into centrifuge tubes containing a total volume of 10 mL RPMI-1640 medium. The tubes were incubated in RPMI-1640 medium supplemented with approximately 18 µg mL^{-1} phytohemagglutinin (PHA) as a mitogen, following Fujiwara et al. (2011), and maintained at 18–20 °C for 10–12 h to allow mitogenic stimulation and cell division. Subsequently, five drops of 0.05% (w/v) colchicine were added to the suspension to arrest cells in metaphase, and the tubes were incubated at room temperature for 30 min. After colchicine treatment, the samples were centrifuged at approximately 150 × g for 10 min, and the supernatant was discarded.

Table 1. Location and coordinates where *Salmonidae* samples were collected

Location (Sample Numbers)	Coordinates
Akdere (n=28)	37°44'37.0"N 37°21'40.3"E
Aksu (n=24)	37°06'25.1"N 30°52'54.6"E
Gokpinar (n=14)	38°39'22.5"N 37°18'17.5"E
Goksu (n=20)	37°48'12.4"N 37°50'10.7"E
Munzur (n=18)	39°09'59.5"N 39°27'49.1"E
Nergele (n=14)	38°01'02.5"N 37°09'20.8"E
Tohma (n=12)	38°37'27.4"N 37°28'45.8"E
Zindan (n=17)	37°48'42.4"N 31°05'06.3"E

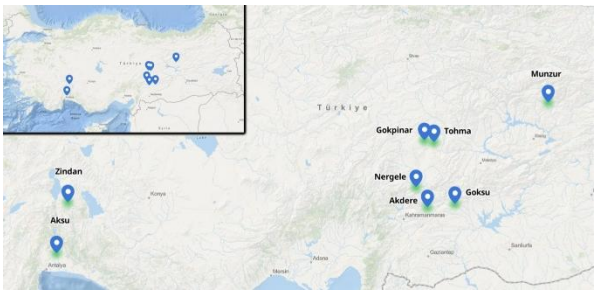


Figure 1. Sampling map in Türkiye

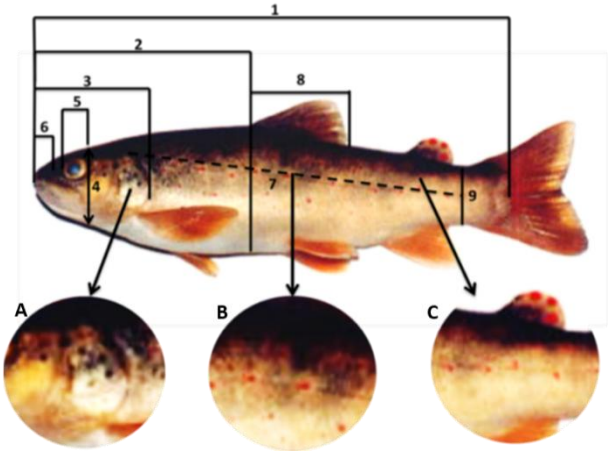


Figure 2. Main morphological characters of *Salmo* spp. and morphometric measurements used for species identification in the field during sampling.

1. Standard Length (SL); 2. Anterior Dorsal (AD); 3. Head Length (HL); 4. Head Depth (HD); 5. Eye Diameter (ED); 6. Nose Length (NL); 7. Body Depth (BD); 8. Dorsal Fin Length (DFL); 9. Caudal Peduncle Depth (CPD). (A) head region with dense melanophore spots, (B) mid-body lateral area with scattered red pigmentation along the lateral line, and (C) caudal peduncle region with reduced spotting and gradual dorsal–ventral color transition.

The cell pellet was then resuspended in 10 mL of hypotonic solution (0.075 M KCl) and incubated at room temperature for 50 min to promote chromosome spreading.

The suspension was gently mixed and allowed to stand for an additional 20–30 min to complete the hypotonic treatment. Following hypotonic treatment, five drops of ice-cold Carnoy's fixative (methanol:acetic acid, 3:1 v/v) were added while gently mixing. The suspension was centrifuged again at 150 × g for 10 min, and the supernatant was removed. The cell pellet was then resuspended in 6 mL of freshly prepared Carnoy's fixative at room temperature and centrifuged again at 150 × g for 10 min. This fixation step was repeated twice to ensure adequate fixation of chromosomal material. Finally, the fixed cell suspension was homogenized with Carnoy's fixative at a 1:1 (v/v) ratio and dropped onto clean microscope slides for chromosome spreading. The chromosomal preparations were stained with 5% Giemsa solution for 8–10 min, rinsed with distilled water, and air-dried. Chromosome spreads were examined and photographed using a photomicroscope (Olympus BX-51).

DNA Isolation, PCR Amplification and Sequencing

Genomic DNA was extracted from adipose fin clips using either the phenol-chloroform method (Sambrook et al., 1989) or a DNeasy Tissue Kit (QIAGEN), following the manufacturer's instructions. The concentration and purity of the isolated DNA were assessed using a NanoDrop ND-1000 spectrophotometer and verified by 1.5% agarose gel electrophoresis.

In this study, we targeted three mitochondrial regions to investigate the phylogenetic relationships of *Salmo* spp. samples: cytochrome b (Cyt-b), 12S rRNA, and 16S rRNA. These regions were selected because of their variability, which helps distinguish closely related species, and their conservation among fish taxa. These features make these regions suitable for large-scale analyses of fish diversity and evolution. We amplified and sequenced these regions to reconstruct the phylogenetic relationships. Primer sequences were designed based on sequences retrieved from mitochondrial genomes of different salmonid fish species, such as: *Oncorhynchus mykiss*, *S. trutta*, *S. salar,* and *Salvelinus fontinalis* deposited in NCBI GenBank (http://www.ncbi.nlm.nih.gov). The designed primers amplify mitochondrial genome regions of approximately 971bp (F: GCTAATGACGCACTAGTCGACC-R: CAGGTGAGGATAAGTATGTCTGCTAC) for Cyt-b, 888bp (F: ACTTACACATGCAAGTCTCCGC- R: CTTACCATGTTACGACTTGCCTC) for 12S, and 1444 bp (F:ATTTTTCCACCTTAGTAGGGGCG-R: CTCAGATCACGTAGGACTTTAATCGT) for 16S. The PCR reactions were performed in total volume of 40 µl, which consisted of 50 ng genomic DNA, 2.5 U Taq DNA polymerase, 5 µl of 10 X Taq buffer (100 mM Tris-HCl, 500 mM KCl pH-8.3), 200 µM dNTP, 10 pmol of each primer. Amplifications were performed in a Thermal cycler (BIO-RAD) under the following program: a) initial denaturation at 95°C for 5 minutes; b) 35 cycles consisting of denaturation at 94ºC for 1 minute, annealing at 65°C (12S), 66°C (16S), and 68°C (*Cyt-b*) for 1 minute and extension at 72ºC for 1 minute. c) the last stage of amplification was the final extension at 72ºC for 5 minutes. The relatively high annealing temperatures were selected based on the melting temperatures (Tm) of the primers, which were calculated to be approximately 68–72 °C, and were further empirically optimized using gradient PCR to ensure specific amplification and minimize nonspecific products. The selected annealing temperatures therefore represent approximately 3–6 °C below the primer Tm, which is consistent with standard PCR optimization guidelines. To check the quality of amplified products, the electrophoresis in 1.5% agarose gel containing ethidium bromide was carried out.

PCR products were sent to the commercial sequencing service Iontek Laboratories (Istanbul, Türkiye) for Sanger sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing was performed bi-directionally using the same primers employed for PCR amplification of the mitochondrial genome regions. Both forward and reverse sequences were obtained for all samples to ensure sequence accuracy. Raw sequence chromatograms were examined using ChromasPro V1.34, and sequence editing and assembly were conducted using Clone Manager 10. Forward and reverse reads were aligned to generate consensus sequences. In cases where minor discrepancies or ambiguous base calls occurred between the two reads, chromatograms were manually inspected and the consensus nucleotide was determined based on peak quality and signal intensity.

Genetic variability and Phylogenetic Analyses

In the genetic diversity analyses, the number of haplotypes, haplotype diversity, and nucleotide diversity values were determined using DnaSP v5 (Librado and Rozas, 2009). Phylogenetic relationships between haplotypes, intraspecific and interspecific genetic variation, and all measures of polymorphism were calculated by MEGA version X (Kumar et al., 2018). In addition, inter-individual distances calculated in MEGA X were transferred to Excel and minimum distances between species, maximum distances within species, and maximum and minimum distances between subspecies were calculated. The phylogenetic analyses were performed based on the sequences of *Cyt-b* obtained from the studied fish and the reference data retrieved from the NCBI GenBank for all available in the database representatives of the *Salmo* genus. Besides the sequences generated from this study, other reference taxa for our phylogenetic analysis were selected from GenBank (http://www.ncbi.nlm.nih.gov/), and the original publications of the phylogenetic analyses contributing the sequences were referenced in Table 2. Alignment of the analyzed nucleotide sequences of the 12S, 16S, and Cyt-b regions was performed using BioEdit v7.2.5 software (Hall, 1999) with the CLUSTAL-W algorithm (Thompson et al., 1994). Phylogenetic analyses were conducted using the MEGA X platform. Prior to tree reconstruction, the best-fit nucleotide substitution model for each dataset was determined using the model selection function implemented in MEGA X based on the Bayesian Information Criterion (BIC) (Kumar et al., 2018). The selected substitution model was subsequently applied in the Maximum Likelihood (ML) (Tamura & Nei 1993) and Bayesian Inference (BI) analyses. Evolutionary distances were calculated using the Maximum Composite

Likelihood method, and phylogenetic trees were reconstructed using the Neighbour-Joining (NJ) (Saitou and Nei, 1987), Maximum Likelihood (ML), and Maximum Parsimony (MP) approaches. Node support for NJ, ML, and MP analyses was evaluated using bootstrap analysis with 1,000 replicates (Felsenstein, 2004). To root the constructed phylogenetic trees, sequence data from *Salmo salar* (JX960834.1 and HQ167673.1) were set as outgroups. Bayesian phylogenetic analyses were performed using MrBayes (version 3.2) (Ronquist et al., 2012) under the selected substitution model. Two independent Markov Chain Monte Carlo (MCMC) runs were conducted to estimate posterior probabilities of phylogenetic relationships. Bootstrap and posterior probability values were reported on the resulting trees. In accordance with common practice in molecular phylogenetics, support values ≥70% were considered strong, whereas values below this threshold were interpreted cautiously (Coulson et al., 2006; Shen et al., 2011). Additionally, previously published phylogenetic datasets were incorporated and reconstructed following the approaches suggested by Küçük et al. (2024) and Tougard et al. (2018) to compare the obtained sequences with previously described Salmo lineages and museum specimens. For this purpose, Maximum Likelihood analyses were also conducted using PhyML (version 3.0), and Bayesian inference analyses were again performed using MrBayes. The resulting phylogenetic trees for the 12S, 16S, and Cyt-b datasets were visualized and edited using the Interactive Tree of Life (iTOL) web server (http://itol.embl.de/) (Letunic & Bork, 2019).

Table 2. Taxa used in this study and their GenBank accession numbers for the 12S, 16S, and cytb genes

Taxon	Origin	12S	16S	Cyt-b	N/R
Salmo abanticus	Endemic to Lake Abant, Türkiye	–	–	MH424338.1	–
Salmo akairos	Lake Ifni, Morocco	–	–	KT279177.1	–
Salmo araxensis	Arax River basin, E Türkiye	–	–	OR441091.1	–
Salmo ardahanensis	Kura–Aras basin, NE Türkiye	–	–	OR441083.1	–
Salmo baliki	Sakarya River, Türkiye	–	–	MW366856.1	–
Salmo brunoi	Guadalquivir River, Spain	–	–	–	–
Salmo chilo	Eastern Anatolia, Türkiye	–	–	MW366850.1	–
Salmo duhani	Kura River basin, Türkiye	–	–	–	–
Salmo ekmekciae	SE Türkiye	–	–	OR713904.1	–
Salmo kottelati	SE Türkiye	–	–	MT515724.1	–
Salmo labecula	Aegean basin, Greece	–	–	MW366847.1	–
Salmo marmoratus	Adriatic basin, Europe	–	–	JX960838.1	–
Salmo murathani	Murat River, Türkiye	–	–	OR441093.1	–
Salmo opimus	Black Sea basin, NE Türkiye	–	–	OR713908.1	–
Salmo carpio	Endemic to Lake Garda, Italy	–	X77560.1	–	–
Salmo caspius	S Caspian Sea basin	MT163681.1	–	MN815909.1	–
Salmo ciscaucasicus	Kuban River basin, Russia	–	–	MG029553.1	–
Salmo coruhensis	Çoruh River basin, NE Türkiye	–	–	MN815912.1	–
Salmo euphrataeus	Euphrates basin (TR–SY–IQ)	–	–	MN815911.1	–
Salmo fahrettini	Upper Euphrates basin, E Türkiye	–	–	MN815913.1	–
Salmo fibreni	Fibreno River, Italy	–	X77559.1	–	–
Salmo labrax	Black Sea basin, Eurasia	GU233802.1	–	MH424336.1	MGR
Salmo munzuricus	Munzur River, E Türkiye	–	–	MN815914.1	–
Salmo obtusirostris	Adriatic basin, SE Europe	–	–	JX960841.1	–
Salmo ohridanus	Lake Ohrid basin	–	–	JX960764.1	–
Salmo okumusi	Upper Euphrates basin, Türkiye	–	–	MN815915.1	–
Salmo platycephalus	SE Türkiye	–	–	JX960842.1	–
Salmo rizeensis	NE Türkiye streams	–	–	MN815910.1	–
Salmo salar	North Atlantic basin	HQ167667.1	HQ167673.1	JX960834.1	RS
Salmo thymus ohridana	Lake Ohrid basin	–	–	AF053590.1	–
Salmo tigridis	Tigris basin (TR–IQ–IR)	–	–	MN815916.1	–
Salmo trutta	Europe & W Asia	OD909581	OD909581	OD909581	–
Salmo trutta caspius	S Caspian Sea basin	–	–	FJ655773.1, MG029549.1	MS
Salmo trutta fario	Europe	GU233801.1	GU214390.1	JN995186.1	–
Salmo macrostigma	NW Africa	EU048341.1	–	LT617631.1	SM
Salmo trutta	Museum specimens	–	–	LT617630.1, LT617632.1	HS
Salmo trutta trutta	Europe & W Asia	–	–	EU492282.1	–
Reference cyt-b sequence	–	–	–	NC_024032.1	-
Outgroup (*Oncorhynchus* sp.)	Pacific salmon lineage	–	–	XXXXXXXX	US

N/R: Note / Reference; MGR: Multiple GenBank records; RS: Reference sequence; MS: Multiple sequences; SM: Syntype mitogenome; HS: Historical samples; US: Used for tree rooting

Results and Discussion

Considering the morphological characters (Figure 2) in previous studies on *Salmo* spp. samples (Figure 3), which are morphologically most similar to *Salmo macrostigma*, it was determined that the diploid chromosome number in metaphase could vary between 78-80 (2n = ~40) in the karyotype analysis of the tissue samples obtained from this fish species. In some preparations 39 (20%) and in some 40 (80%) were counted. More detailed analyses are required for discussion. For more detailed studies, fluorescence in situ hybridization (FISH) with DNA probes, i.e. FISH analyses with microsatellite motifs, can be used to determine significant genomic differences between species. As a consequence of the lack of an obvious difference in chromosome number, the difference in DNA regions was analyzed.

Genetic Diversity and Phylogenetic relationship within Salmo spp. in the studied area

The genomic regions of *Cyt-b*, 12S, and 16S ribosomal RNA (rRNA) were scrutinized across 147 *Salmo macrostigma*-like (Figure 3) *Salmo* spp. samples sourced from river basins in Türkiye, serving as tributaries to the Mediterranean Sea and the Indian Ocean. Sequencing efforts yielded segments of 971 base pairs (bp), 888 bp, and 1444 bp for the *Cyt-b*, 12S, and 16S regions, respectively. Within the 12S locus, a total of 121 mutations were identified, delineating 57 polymorphic, 4 monomorphic, and 55 parsimony informative positions, along with 17 singleton polymorphic positions. The 16S region displayed 105 mutations, including 62 polymorphic, 36 monomorphic, and 62 parsimony informative positions, with no singleton polymorphic positions observed. In the *Cyt-b* region, 55 mutations were detected, comprising 8 monomorphic, 53 polymorphic, and 53 parsimony informative positions, with no singleton polymorphic positions recorded. These findings provide insight into the genetic diversity and evolutionary dynamics of some *Salmo* spp. populations living in Turkish river systems.

Phylogenetic and phylogeographic analyses on *Salmo* spp. populations from certain regions of Turkey (Table 3) reveal that three different mitochondrial gene regions (12S, 16S, and *Cyt-b)* are rich in genetic diversity. The 12S region is characterized by a significant haplotype diversity (Hd = 0.960) with a total of 40 different haplotypes, but the average number of nucleotide differences (k = 1.537) and nucleotide diversity (π = 0.0026) indicate a moderate level of genetic variation. In the 16S gene region, almost like the 12S region, 34 haplotypes were detected in all populations, and haplotype diversity was slightly higher than in 12S (Hd = 0.965). In particular, the average number of nucleotide differences (k) increased to 2,586 and was accompanied by a higher nucleotide diversity (π) of 0.0039, indicating a relatively higher genetic diversity compared to the 12S region. This was interpreted to suggest that the 16S region may better reflect population differentiation in *Salmo* spp. samples compared to the 12S region. In contrast, the *Cyt-b* region showed the highest haplotype diversity (Hd = 0.926) and an average number of nucleotide differences (k = 7.209) among the three regions, underlining a significant genetic diversity within populations. In parallel, nucleotide diversity (π = 0.0084) is significantly higher compared to both 12S and 16S regions, indicating significant genetic diversity. Overall, the *Cyt-b* marker emerges as the main reservoir of genetic diversity among the analyzed markers, followed by 16S and then 12S.



Figure 3. *Salmo* spp. samples used in morphological and chromosome studies

In the analysis of genetic distances within and between species, a clear difference was observed between the maximum intraspecific distances and the minimum interspecific distances (Table 4). This pattern generally indicates a distinct separation between intra- and interspecific variation. However, when comparisons involved sequences of *Salmo salar* (KF792729.1), the range of interspecific genetic distances overlapped slightly with the upper values of intraspecific distances, resulting in a reduced separation between these two categories. For the 12S, 16S, and *Cyt-b* sequences of Salmo spp. from 8 different sites, intraspecific and interspecific distances ranged from 0 to 0.8 for 12S, 0.020 to 0.33 for 16S, and 0 to 1.48 for Cyt-b, respectively. For the 12S and *Cyt-b* arrays, the maximum intraspecies distance (0.4 for 12S, 1.48 for Cyt-b) overlaps with the minimum interspecies distances (0 for both), meaning that there is no obvious barcoding gap. The 16S sequence showed slightly better segregation than the other marker regions, with minimal overlap (intraspecific max = 0.33 vs. interspecific min = 0.024). These distances suggest that the degree of genetic differentiation is said to increase gradually with increasing taxonomic levels of taxa (Wu et al., 2018). 16S appears to be more reliable for species identification among *Salmo* species, which is in line with previous studies (Wu et al., 2018).

Mitochondrial cytochrome b (*Cyt-b*) region sequences were acquired from 147 *Salmo* spp. originating from eight distinct springs (Akdere, Gökpınar, Zindan, Aksu, Tohma, Munzur, Nergile, and Göksu) within the study area. Utilizing an NJ/ML/MP modeled tree, we endeavored to assess intra- and inter-population genetic diversity (Figure 4). Notably, all three methods—NJ, ML, and MP—yielded concordant outcomes. While morphological traits among samples from varied regions remained homogeneous, genetic scrutiny through *Cyt-b* sequences unveiled a disparate trend, suggesting morphological characteristics may not reliably indicate genetic diversity within this population.

Table 3. Genetic polymorphism measurements based on 12S, 16S, and *Cyt-b* regions for *Salmo macrostigma*-like *Salmo* spp. in Türkiye

Marker	Population Code	N	S	n	h	Hd	k	π
12S	Akd	28	41	10	10	0.857	1.070	0.0018
	Aks	24	41	6	6	0.760	1.615	0.0028
	Gokp	14	37	5	5	0.769	1.465	0.0025
	Goksu	20	53	5	5	0.621	1.555	0.0026
	Mnz	18	38	5	5	0.705	0.838	0.0014
	Nrg	14	36	4	4	0.758	1.838	0.0031
	Thm	12	37	3	3	0.712	1.967	0.0034
	Zindan	17	37	5	5	0.845	1.305	0.0022
	Total	147	57	40	40	0.960	1.537	0.0026
16S	Akd	28	43	4	4	0.761	1.830	0.0027
	Aks	24	55	3	3	0.521	2.238	0.0033
	Gokp	14	56	4	4	0.780	2.554	0.0038
	Goksu	20	38	3	3	0.700	1.683	0.0025
	Mnz	18	37	6	6	0.862	1.753	0.0026
	Nrg	14	56	5	5	0.857	2.694	0.0040
	Thm	12	38	3	3	0.681	2.067	0.0031
	Zindan	17	56	6	6	0.845	2.842	0.0043
	Total	147	62	34	34	0.965	2.586	0.0039
Cyt-b	Akd	28	7	3	3	0.539	2.063	0.0024
	Aks	24	2	2	2	0.507	1.014	0.0011
	Gokp	14	0	1	1	0	0	0
	Goksu	20	5	2	2	0.505	2.526	0.0029
	Mnz	18	10	3	3	0.392	2.091	0.0024
	Nrg	14	0	1	1	0	0	0
	Thm	12	0	1	1	0	0	0
	Zindan	17	10	3	3	0.676	4.117	0.0048
	Total	147	53	16	16	0.926	7.209	0.0084

Abbreviations: *N*, sequence number; *S*, number of segregating sites, *n*, total number of mutations; *h*, haplotype number; Hd, haplotype diversity; *k*, the average number of nucleotide differences, *π*, nucleotide diversity.

Table 4. Intraspecific and interspecific genetic distances at various taxonomic levels for 12S, 16S, and *Cyt-b* sequences of *Salmo* spp. samples from 8 different regions. P distance represents the number of base differences per site.

Comparison with	12S sequence			16S sequence			Cyt-b sequence		
	Min.*	Mean	Max.*	Min.	Mean	Max.	Min.	Mean	Max.
Intraspecific	0	0.22	0.4	0.020	0.16	0.33	0	0.79	0.148
Interspecific	0	0.41	0.8	0.024	0.14	0.27	0	0.56	0.148

*Note: Min; Minimum and Max: Maximum.

Contrary to morphological homogeneity, DNA analysis (Table 2) indicated a potential for substantial genetic differentiation at the *Cyt-b* locus among *Salmo (trutta) macrostigma* populations. Pairwise sequence comparisons divulged a predominant level of genetic similarity exceeding 90% across populations, exemplified by samples from Tohma, Munzur, and Gökpınar streams. However, the Aksu River population manifested significant divergence, indicating a distinct evolutionary lineage (Figure 4).

Phylogenetic elucidation revealed three primary clades within the tree. Clade I delineated samples from Göksu, Munzur, Tohma, and Gökpınar streams as molecularly proximate, while Clade II clustered Zindan, Akdere, and Nergile samples in close association. Notably, specimens obtained from the Aksu stream formed a distinctly separate group, supported by a robust bootstrap value (96%). Despite inter-clade differences, samples from analogous streams exhibited close genetic relatedness.

Phylogenetic position of Salmo spp. within the genus Salmo

Maximum-likelihood (ML) or Bayesian inference (BI) phylogenetic analyses yielded trees with a topology similar to that of Tougard et al. (2018) (Figure 5). This tree, obtained using *Cyt-b* sequences, reveals a well-supported phylogeny of *Salmo trutta* lineages, with strong support for the monophyly of each lineage and clear geographic clades. The reconstructed phylogenetic tree has clear support values that can help in lineage separation corresponding to geographical and ecological regions. The resulting phylogenetic trees were highly concordant and the bootstrap values obtained were supported by bootstrap percentages ($51\% < BP < 100\%$) and posterior probability ($0.68 < PP < 1.00$) values for most nodes (Figure 5). The inclusion of Zindan, Aksu, Akdere, Göksu, Nergele, Munzur, Gökpınar, and Tohma samples in the trees of Tougard et al. (2018) adds resolution to the pedigrees of Adriatic and Turkish lineages by revealing previously undetected local genetic structure, especially in Zindan and Aksu samples.

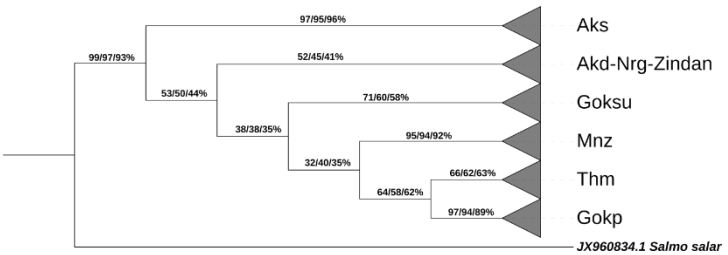


Figure 4. Phylogenetic tree obtained by NJ/ML-TN93+G/MP models of *Cyt-b* region of *Salmo trutta macrostigma* samples obtained from different water sources (Similarity %).

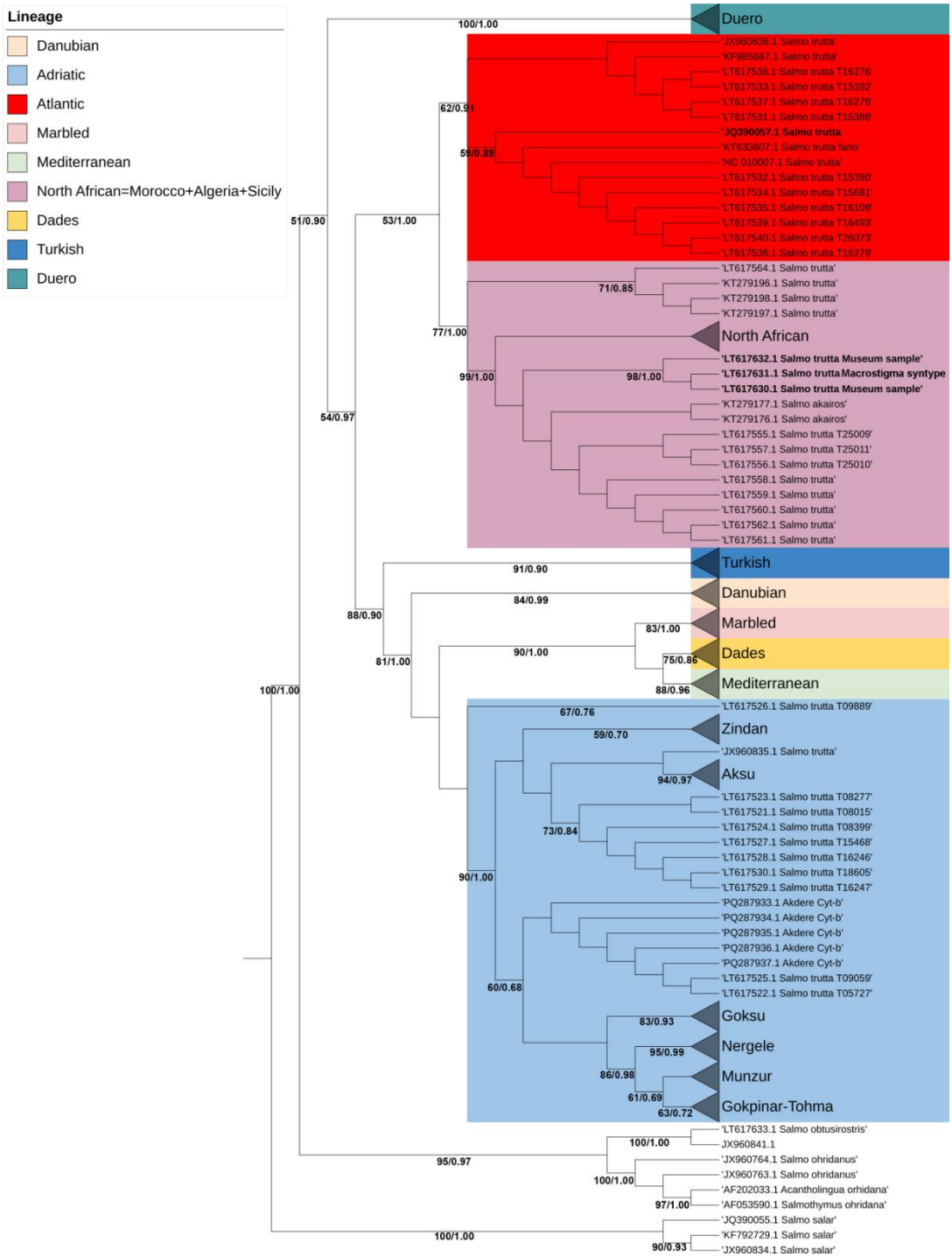


Figure 5. In addition to the study by Tougard et al. (2018), a maximum likelihood (ML) tree (visualized with iTOL) was reconstructed by merging *Cyt-b* sites.

The results of the Bayesian and ML analyses are consistent with each other. Macrostigma MNHN sytype (LT617631) and museum specimens (LT617630 and LT617632) are in bold. Numbers in nodes are for bootstrap percentages (≥50%) and posterior probabilities (≥0.80). Surnames are visualized according to the color scale on the left.

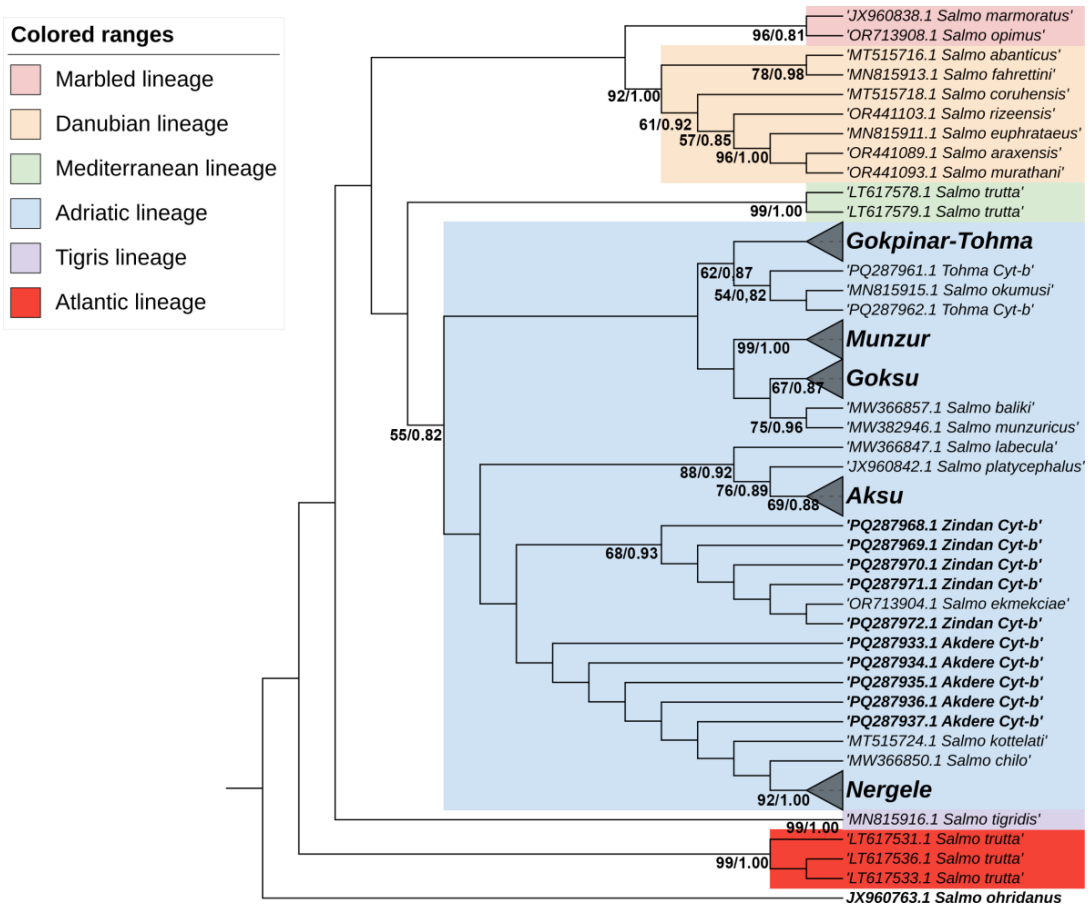


Figure 6. Maximum likelihood (ML) tree was reconstructed by merging *Cyt-b* regions (visualized with iTOL) in addition to the study by Küçük et al. (2024).

The results of Bayesian and ML analyses are consistent with each other. Numbers in nodes are for bootstrap percentages (≥50%) and posterior probabilities (≥0.80). Representative examples of lineages are shown in color on the left. Examples in the study are in bold.

Sub-clades including Zindan, Aksu, Akdere, and Goksu have moderate to high support values (PP = 0.95, BP = 84%). The Goksu samples in particular show the presence of local genetic differentiation, indicating the role of an important microsanctuary. The Nergele, Munzur, Tohma, and Gökpınar samples cluster into a distinct Turkish subclade with moderate support values (PP = 0.93, BP = 72%). These subclades may be useful for understanding the microevolutionary processes of *Salmo* spp. in some mountainous areas in Türkiye. In conclusion, the Zindan, Aksu, Akdere, Göksu, Nergele, Munzur, Gökpınar, and Tohma branches represent a small part of the evolutionary complexity of the *Salmo trutta* complex. The results obtained in this study suggest that trout populations in Turkey may exhibit unique genetic characteristics and that there are still new species waiting to be identified.

The phylogenetic tree constructed to make sense of the phylogenetic relationships in the genus *Salmo* based on Bayesian and ML analyses in parallel with the data used by Küçük et al. (2024) to identify *Salmo ekmekciae* as a new species in Turkey is shown in Figure 6 The phylogenetic tree indicates the existence of 6 different lineages (Marble, Danube, Mediterranean, Adriatic, Tigris and Atlantic). Each lineage is supported by bootstrap values (≥50%) and posterior probabilities (≥0.80). All samples in the study cluster within the Adriatic lineage. The Gökpınar-Tohma samples formed a subclade with posterior probabilities of 0.82 and bootstrap values of 54%, which included *Salmo okumusi* (MN815915). Munzur and Goksu samples clustered into a subclade containing *Salmo munzuricus* (MW382946) and *Salmo platycephalus* (JX960842), strongly supported by bootstrap values of 75% and posterior probabilities of 0.96. The Aksu samples strongly formed a sub-branch with *Salmo labecula* (MW366847) and *Salmo platycephalus* (JX960842) with bootstrap values of 88% and posterior probabilities of 0.92. While Zindan samples were in the same branch with *Salmo ekmekciae* (OR713904), Akdere and Nergele samples formed another sub-branch with *Salmo chilo* (MW366850) and *Salmo kottelati* (MT515724). These sub-branches are an important indicator of the regional adaptation and evolutionary differentiation of *Salmo* spp. within the river systems of Türkiye. The specimens in the study were grouped with other previously described species from Türkiye. This indicates that they have a common ancestor. The fact that they share the same cluster is the clearest indication that they have fewer genetic differences compared to other species.

Members of the *Salmo* genus are known to play a variety of diverse phenotypic traits, which could be easily influenced by environmental factors (Skaala & Solberg, 1997). As a result, traditional morphological-based taxonomic studies on the *Salmo* genus experience a lot of difficulties. The introduction of molecular techniques enabled for distinction between genetic structures resulting from intraspecific variation and introgression versus those resulting from speciation, allowing sometimes for the deep

taxonomic reassessment of evolutionary units in difficult taxa, as well as reconciling morphological and molecular taxonomies in integrative studies (Baumsteigeret et al., 2017). Large-scale integrative taxonomic studies of the genus *Salmo* have yet to be conducted, as there is not enough morphological and genomic data to evaluate (Englmaier & ark., 2024). Consideration of type specimens remains absolutely critical (Schultz et al., 2015). By combining the extensive DNA sequence data available to date, our study represents a pioneering effort in the phylogenetics of specimens resembling *Salmo macrostigma*, which has been shown to be distributed in Turkey in previous studies. In addition, our study revealed that the chromosome number in morphologically *Salmo macrostigma*-like specimens distributed in Turkey may vary between 78-80 (2n) (39 (20%) in some preparations and 40 (80%) in others). *S. trutta* has a chromosome number ranging between 38-42 (Hansen et al., 2021).

The lack of strong and distinct signaling in mitochondrial data has been the primary cause of ongoing uncertainty in Salmonidae molecular phylogenetics (Crespi & Fulton 2004). As many native species and populations in Turkish waters are currently threatened by human activities (e.g. increased water use, environmental degradation due to deforestation) and mixing with domestic *Salmo* species (Tougard et al., 2018), biodiversity conservation should be prioritized, taking into account phylogenetic adaptations, especially taking into account species identification by the mtDNA analysis (Freudenstein et al., 2017). *Salmo macrostigma* is commonly used for phenotypic identification of trout specimens around the Mediterranean from the Middle East, North Africa, Southern Europe, and Türkiye. However, a limited number of phylogenetic studies have focused on phenotype and genotype. Because morphological and meristic traits that vary considerably within the *S. trutta* complex are wrongly employed to identify an illegitimate taxon, its designation as a genuine species can be taxonomically useful. The results showed that the specimens, identified as *S. macrostigma* as in previous studies, were different species by molecular analysis (Turan et al., 2024, Turan et al., 2014). It seems unlikely that the 8 Salmo spp. trout specimens with morphological similarity to *Salmo macrostigma* considered in this study are (sub)species covering this very large geographical area, because except for Aks, the other specimens were not clustered (Figure 4). Considering that the range of *Salmo macrostigma* specimens is limited to Morocco, Algeria, and the Mediterranean Sea (Turan et al., 2024; Zouakh, 2009; Doadrio et al., 2015), it is not surprising that the specimens studied in this region are found in different branches. In addition, recent studies do not accept the view that this fish species is widely distributed (Turan et al., 2024). Genetic data are needed to address the suitability of *Salmo macrostigma* or other described Salmo species with morphological similarity as valid species.

The genetic heritage of *Salmo trutta* has been considerably studied over the past many years, and its phytogeographic shape has been nicely established. Recently, a consensus has already been reached that the *S. trutta* complex consists of five major phylogenetic lineages (Ad, At, Da, Ma, and Me) and that further subdivision into similarly deep divergences is unlikely (Cortey et al., 2004). *Salmo* species distributed in Türkiye (*S. coruhensis, S. rizeensis, S. abanticus, S. caspius, S. tigridis*, *S. euphrataeus, S. okumusi, S. chilo, S. opimus, S. platycephalus, S. labecula, S. kottelati, S. cf. coruhensis, S. cf. rizeensis*) were determined in previous studies. It was shown that the species identified were previously defined as *Salmo macrostigma*, but together with the morphological and molecular data, they are a new species (Turan et al., 2014). The phylogenetic tree reconstructed using the *Cyt-b* sequences obtained in this study (Figure 5) provided important information about the evolutionary relationships in the genus *Salmo*, especially the taxonomic status of *Salmo macrostigma*. This tree confirms the findings of Tougard et al. (2018) that *Salmo macrostigma* trout in Türkiye, North Africa, and Sicily do not represent a separate species. Rather than a new species, these populations appear to cluster within the *Salmo trutta* complex, specifically as part of a North African mtDNA lineage close to the Atlantic lineage of *S. trutta* (Tougard et al., 2018). In particular, the syntype (LT617631.1) and museum specimens (LT617630-32) are consistent with a North African lineage, suggesting geographic origins. This supports the hypothesis that *S. macrostigma* is a geographical lineage rather than a distinct species. The lack of monophyly in the Balkans, Türkiye, and North Africa, which were shown to be the putative range of *S. macrostigma* in previous studies, supports the theory that these specimens may be subspecies or new species rather than a species. The heterogeneous topography of Anatolia and the advantages of climate change have made it a known biodiversity center for trout populations. The populations of Zindan, Aksu, Akdere, Göksu, Nergele, Munzur, Gökpınar, and Tohma used in this study are distributed within the Adriatic lineage. This distribution was interpreted as being influenced by local ecological pressures. Species with high rates of translocation, such as trout, may be due to anthropogenic influences in river systems. It is also thought to be due to low genetic distances between groups, indicating ongoing or recent gene flow between branches in these populations.

The obtained DNA sequence analyses showed that the specimens of *Salmo* spp. are closely related. This observation raises the question of their phylogenetic status within the genus, especially in relation to the current phytogeographic lineages of *Salmo* spp. Based on the various phylogenetic analyses conducted on the DNA sequences of known *Salmo* species, including Neighbor-joining-Maximum Likelihood (Jukes and Cantor 1969-JC69 and Tamura and Nei 1993-TN93+G) and Maximum Parsimony (Bootstrap:1000), none of the specimens from the Zindan, Nrg, Akd, and Goksun populations clustered within any of the lineages of *S. trutta*. Instead, they consistently formed a distinct clade as sister taxa (Fig. 6). Genetic data suggest that the specimens in this study evolved from a common ancestor with the previously identified *Salmo platycephalus* (JX960842), *Salmo munzuricus* (MN815914), and *Salmo okumusi* (MN815915). The similarity of the specimens of Zindan, Nrg, and Akd with other *Salmo* species suggests that it is a monophyletic group and possibly a new species, however; to prove this, it needs to be confirmed by mtDNA genomic study along with morphological data. Other evidence, in addition to the genetic data, points to their monophyletic

origin. Given their close geographic distribution and the assumption that all three specimens are unique to this location, we may infer that their evolution took place in a spatially homogeneous area (Berg, 1908). It would not be wrong, then, to regard these specimens, found in three different rivers, as a new species. However, to prove this, it needs to be confirmed by mtDNA genomic study along with morphological data. The phylogenetic analyses presented in Figures 5 and 6 highlight the significant genetic diversity and phylogeographic structuring of Salmo species in the Munzur, Göksu, Aksu, Gökpınar, Tohma, Akdere, Zindan and Nergele regions. They were identified as distinct groups forming monophyletic subclades, each well supported in both maximum likelihood (ML) and Bayesian analyses. Consistent with the Adriatic lineage, the Munzur, Göksu, Aksu, Gökpınar, Tohma, Akdere, Zindan, and Nergele populations exhibit the mixing of broader lineages into local populations while maintaining strong phylogenetic distinctions. They are represented within the Adriatic lineage within the wider *Salmo trutta* complex (Figure 5). They were found to have strong genetic differentiation, supported by high bootstrap values and posterior probabilities, reflecting the influence of local ecological adaptations.

Conclusion

According to the findings, haplotype diversity, nucleotide diversity and mean nucleotide differences among mitochondrial genes indicate that there are different levels of genetic diversity and evolutionary characteristics among *Salmo* spp. populations in Turkey. This study revealed that there is a significant difference between morphological characteristics and genetic diversity in *Salmo* spp. populations across the study area. *Cyt-b* sequence analysis is the most obvious evidence of significant genetic differentiation between populations, and the Aksu River population emerges as a distinct evolutionary lineage. Certain RNA sequences, such as 12S rRNA, 16S rRNA, and *Cyt-b* regions, can be used as useful genetic markers to distinguish between species and to study evolutionary relationships. Furthermore, sequences of RNA can provide additional taxonomic information, especially in the case of closely related species. The results of the study revealed that it is important to carefully select and study specimens for this species, which has a controversial classification. In order to resolve the correspondence between *Salmo* species, the phylogenetic relationship needs to be fully clarified. To clarify this situation, it is necessary to complete all mtDNA genome types. This will greatly facilitate the identification of Salmo spp. both in Turkey and in other parts of the world.

Declarations

Ethical Approval Certificate

This experiment was approved by the Ethics Committee of Munzur University (Approval No: 2022/22-06/15-02). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All experimental procedures were carried out in accordance with the guidelines established by the Ethics Committee of Munzur University. Necessary permits for the collection of specimens were obtained from the provincial agriculture directorate. In addition, the study was conducted in accordance with the ARRIVE guidelines.

Author Contribution Statement

All authors contributed equally to the study.

Fund Statement

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Conflict of Interest

The author declares that this study complies with Research and Publication Ethics.

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