

First Record of a Novel Mitochondrial DNA Haplotype of *marmoratus* Lineage of *Salmo trutta* in Turkey

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Abstract

A new record of a marble trout mtDNA haplotype known to be restricted to Adriatic basin (called *marmoratus* (MA) lineage within *Salmo trutta* complex) has been reported from Eşen Stream in the Aegean Sea basin of southwestern Turkey, based on sequence data of the mitochondrial DNA control region. The results of this study showed a single unique haplotype from this population, called MATR1. Phylogenetic analyses of this haplotype along with other haplotypes belonging to different mitochondrial DNA lineages of the *S. trutta* complex confirmed the existence of the *marmoratus* lineage in Turkey, suggesting a possible river capture between the Adriatic and Aegean Sea basins until the last (Würmian) marine regression.

Introduction

The brown trout (*Salmo trutta* Linnaeus, 1758) is the most widely distributed species native to Eurasia and North Africa. The species displays a considerable amount of morphological variation and plasticity in many aspects of its morphology, ecology and behaviour. Based on the sequence divergence in the two segments of the mtDNA control region, five major evolutionary lineages were proposed: Danubian (DA), Atlantic (AT), Mediterranean (ME), Adriatic (AD) and *marmoratus* (MA) (Bernatchez, 2001). In addition, a highly divergent lineage called Tigris (TI) (Bardakci *et al.*, 2006) from Turkey has been described (Susnik *et al.*, 2005). Moreover, Vera *et al.* (2010) have also reported another

lineage confined to the Duero River in the Iberian Peninsula.

Turkey is one of the most important geographical regions with high biodiversity and endemism due to its overlapping location with at least three biodiversity hotspot regions, Caucasus (northeastern Turkey), Irano-Anatolian (central and eastern Turkey) and Mediterranean basin (southern Turkey) (Conservation International, 2005; Mittermeier *et al.*, 2005). One of the most recent studies on freshwater fish diversity reported that 194 (47.4%) species of Turkish freshwater ichthyofauna are endemic, and 12 of them belong to the Salmonidae family (Çiçek *et al.*, 2018).

Considerable variation in the external morphology of brown trout across Turkey were reported in early

studies, leading to many taxonomic units (reviewed by Geldiay and Balik, 2007; Turan *et al.*, 2009; Turan *et al.* 2011;2012), though hybridization between different morph of the DA lineage in Turkey proposed a single taxonomic unit, *Salmo trutta* (Kalayci *et al.*, 2018). Molecular studies on brown trout in Turkey have also revealed that there is a large amount of genetic variation and genetic structuring in brown trout populations (Bernatchez, 2001; Bardakci *et al.*, 2006; Arslan and Bardakci, 2010; Ozen, 2013). Previous mtDNA analyses based on PCR-RFLP of mtDNA NADH 5/6, cytochrome *b* and control region showed that all Turkish brown trout populations were clustered into three distinct lineages, DA, AD and TI (Bardakci *et al.*, 2006). While the AD lineage is widespread in the Mediterranean and Adriatic basin, the DA lineage is predominantly seen in the Black/Caspian/Aral seas basins. TI lineage has only reported from a tributary of the Tigris River (Catak Stream) in the Persian Gulf basin.

Molecular phylogenetic analyses of brown trout based on analyses of mtDNA sequences showed that native range of a distinct *marmoratus* lineage within *S. trutta* complex was confined mainly to the Adriatic Sea basin, northern Italy, Slovenia, Croatia, Albania and Greece (Bernatchez, 2001; Apostolidis *et al.*, 1997). Moreover, extensive introgression has been reported between native marble trout (*S. marmoratus*) and brown trout (*S. trutta*) within the western Po basin (Giuffra *et al.*, 1996), Slovenia (Fumagalli *et al.*, 2002) and northern Italy (Meraner *et al.*, 2007).

In this study, the complete sequences of the mtDNA control region obtained from the *S. trutta* population in Eşen Stream in Turkey were analysed together with previously reported brown trout mtDNA haplotypes to determine the evolutionary relationships of this population within the *S. trutta* complex. Findings from this study might shed light on our understanding of the impact of major historical events on the distribution of species.

Materials and Methods

Sampling and DNA isolation

We have analyzed six specimens from Eşen Stream in the Aegean Sea basin of southwestern Turkey (Figure 1). Brown trout specimens were caught by electrofishing and preserved in 95% (v/v) ethanol. Total genomic DNA from muscle tissue at the bottom of dorsal fin of specimens was isolated following the method described in Bardakci & Skibinski (1994) with a modification using two steps of phenol-chloroform (25 phenols: 24 chloroform: 1 isoamyl alcohol) before. Precipitated DNA was re-suspended in distilled water and quantified with a spectrophotometer (Nanodrop1000; NanoDrop Technologies, Wilmington, DE, USA) at a wavelength of 260 nm.

DNA amplification and sequencing

Approximately 1012 bp of the mtDNA control region were amplified using primers, PST (5'-CCAAAGCTAAAATTCTAAAT-3') and FST (5'-GCTTTAGTTAAGCTACGC-3') (Cortey *et al.*, 2004). The temperature profile for the 30 cycles of amplification reaction was as follows: initial denaturation at 94°C for 1 min, denaturation at 94°C for 30 s, annealing at 52°C for 1 min, extension at 72°C for 1 min, repeated for 35 cycles and a final extension step at 72°C for 1 min. To monitor the quality of the products, two microliters of each purified product were examined after electrophoresis in 1% agarose gel. PCR products were visualized in agarose gel stained with ethidium bromide and visualized under UV light and purified with the GenElute PCR Clean-Up Kit® (Sigma-Aldrich, Germany). Purified PCR products were sequenced by Macrogen Europe B.V. (Amsterdam, Netherlands) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3730XL capillary sequencer.

Sequence alignment and phylogenetic analysis

A BLAST search was performed at NCBI to determine sequence homology of the mtDNA control region of trout samples from Eşen Stream named MATR1 (GenBank Accession no. JN543996.1) with those previously deposited in the GenBank. The results of this search showed the highest homology with sequences of the mtDNA control region of *marmoratus* strain followed by other strains of *S. trutta*, proving MATR1 is a new mtDNA haplotype, first identified in this study.

These sequences showing the highest homology with sequences of the mtDNA control region of different *S. trutta* strains deposited in the GenBank, and the sequences of *S. salar* mtDNA control region as an outgroup, were combined and aligned with MATR1 sequences to assess its phylogenetic position within the *S. trutta* complex. As a result, the 990 bp of mtDNA D-loop region was successfully aligned using the Clustal W software (Thompson *et al.*, 1994) in BioEdit version 7.1.3.0 (Hall, 1999).

The Neighbor-Joining method (Saitou and Nei, 1987) was used to construct a phylogenetic tree in MEGA X (Kumar *et al.*, 2018). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980), which was the best-fit model to our data, as inferred from MEGA X software, following pairwise-deletion model with 1000 bootstrap replicates. Estimates of sequence divergences (Kimura 2-parameter distances) were also performed in MEGA X software.

Results

Sequencing of mtDNA control region has revealed a single haplotype, designated MATR1 (Accession no:

JN543996) from six individuals from the Eşen Stream. A BLAST search was performed to determine the homology of this haplotype with the sequences from GenBank, and results showed that MATR1 is a unique distinct haplotype with close similarity to other known haplotypes of mtDNA control region of *marmoratus* lineage within *S. trutta* complex. The nucleotide differences between these sequences are given in Table 1. Estimated nucleotide sequence divergence values between these sequences showed that the pairwise sequence divergence of MATR1 haplotype with those known haplotypes of *marmoratus* lineage ranged from 0.205% to 0.411%.

Furthermore, the sequences of 27 haplotypes of mtDNA control region of known mtDNA lineages of brown trout (Bernatchez, 2001; Weiss *et al.*, 2001; Duftner *et al.*, 2003; Cortey *et al.*, 2004; Pujolar *et al.*, 2011) were retrieved from the GenBank database and analyzed together with MATR1 haplotype to confirm phylogenetic position of this new haplotype within *S.*

trutta complex. Phylogenetic analyzes distinguished five *S. trutta* lineages proposed by Bernatchez (2001), and TI lineage by Bardakci *et al.* (2006). NJ tree (Figure 2) confirmed phylogenetic position of MATR1 haplotype as a *marmoratus* lineage. The sequence divergence of this haplotype with other haplotypes of trout lineages ranged from 0.205% to 1.198%. Overall results showed that a single unique haplotype identified from Eşen population clustered with the *marmoratus* haplotypes with a high bootstrap value (Figure 2), suggesting the presence of a *marmoratus* lineage within *S. trutta* complex in Turkey. In addition, Eşen population does not have a typical marble trout (*S. marmoratus*) color pattern as reported in previous studies (Pustovrh *et al.*, 2011; Dellng *et al.*, 2020) but has some distinct phenotypic characteristics such as black spots with no white circles around on the most upper part of flanks and red ones on both upper, middle and lower part of flanks also with no white circles around, and a greater eye diameter (Figure 3).

Table 1. Variable nucleotide sites of MATR1 haplotype and other *marmoratus* haplotypes deposited in the GenBank.

Haplotype	Variable sites							
	105	254	255	540	555	725	788	953
Ma2a	A	A	T	C	-	T	C	T
Ma2b	A	A	T	C	-	C	C	T
Ma1a	A	A	C	C	-	C	C	T
MAcs4	A	G	C	C	T	C	C	T
MATR1	A	A	T	C	-	C	T	C
MAcs5	G	A	T	C	T	C	C	T
MAcs1	A	A	C	C	-	C	C	T
MaK1	A	A	C	T	-	C	T	C

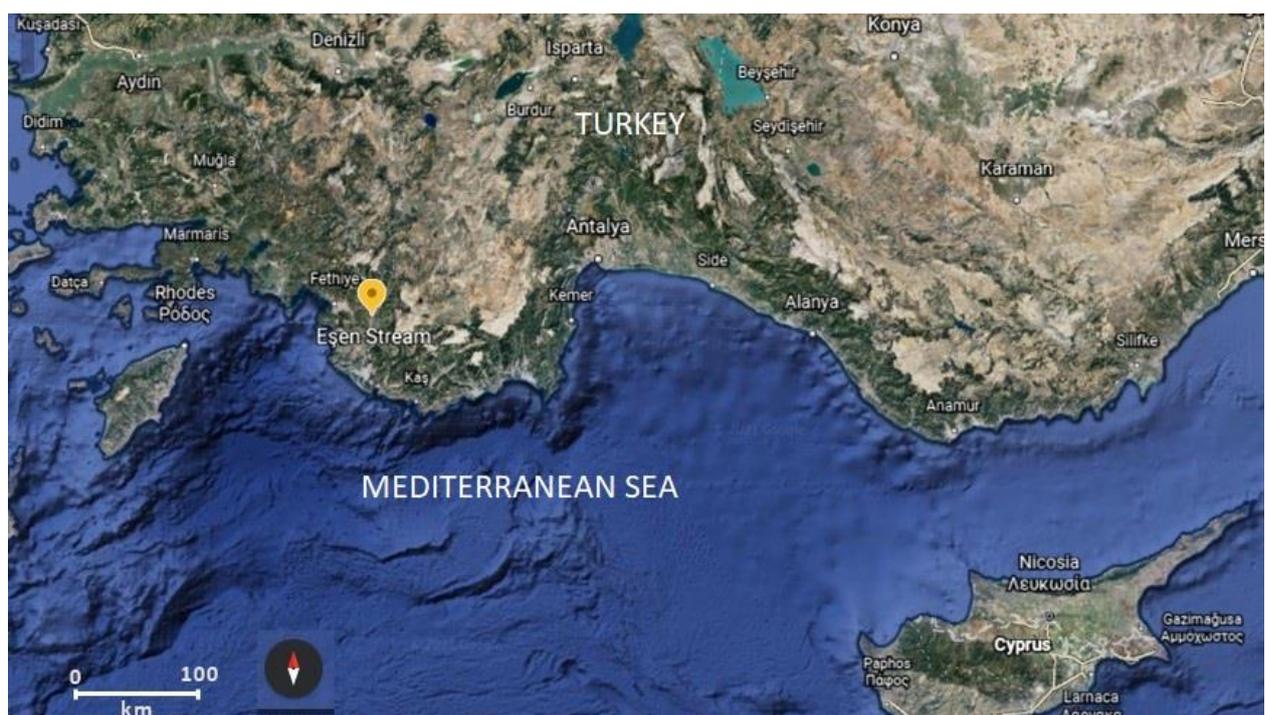


Figure 1. Sampling locality of *marmoratus* strain of *S. trutta* from the Eşen Stream (Coordinate: 36° 59'49.2"N 29°36'26.6"E)

Discussion

Extensive hybridization between brown trout and marble trout (*S. marmoratus*) has been reported in Slovenia and Italy (Fumagalli *et al.*, 2002; Splendiani *et al.*, 2006; Meraner *et al.*, 2007). To our knowledge, there is no existence, stocking or promotion of the marmoratus lineage of *S. trutta* and marble trout in Turkey. The genotypes found in populations throughout

the Mediterranean basin, from Spain to Turkey belong to one of the three major phylogenetic groups, AD, ME, MA. A dissertation on the population genetics and phylogenetics of brown trout in Turkey, based on the sequence data of mtDNA control region, has put forward the presence DA, AD, TI as well as MA mtDNA lineages (Ozen, 2013). The tree presented here suggest that the ME and AT lineages do not exist in Turkey. Splendiani *et al.* (2006) noted that the presence of

Table 2. Estimates of nucleotide divergence between sequences of *marmoratus* haplotypes using the Kimura 2-parameter model.

Haplotypes	Ma2a	Ma2b	Ma1a	MAcs5	MATR1	MAcs5	MAcs1
Ma2a							
Ma2b	0.00102						
Ma1a	0.00205	0.00102					
MAcs4	0.00308	0.00205	0.00102				
MATR1	0.00308	0.00205	0.00308	0.00411			
MAcs5	0.00205	0.00102	0.00205	0.00308	0.00308		
MAcs1	0.00205	0.00102	0.00000	0.00102	0.00308	0.00205	
MaK1	0.00514	0.00411	0.00308	0.00411	0.00205	0.00515	0.00308

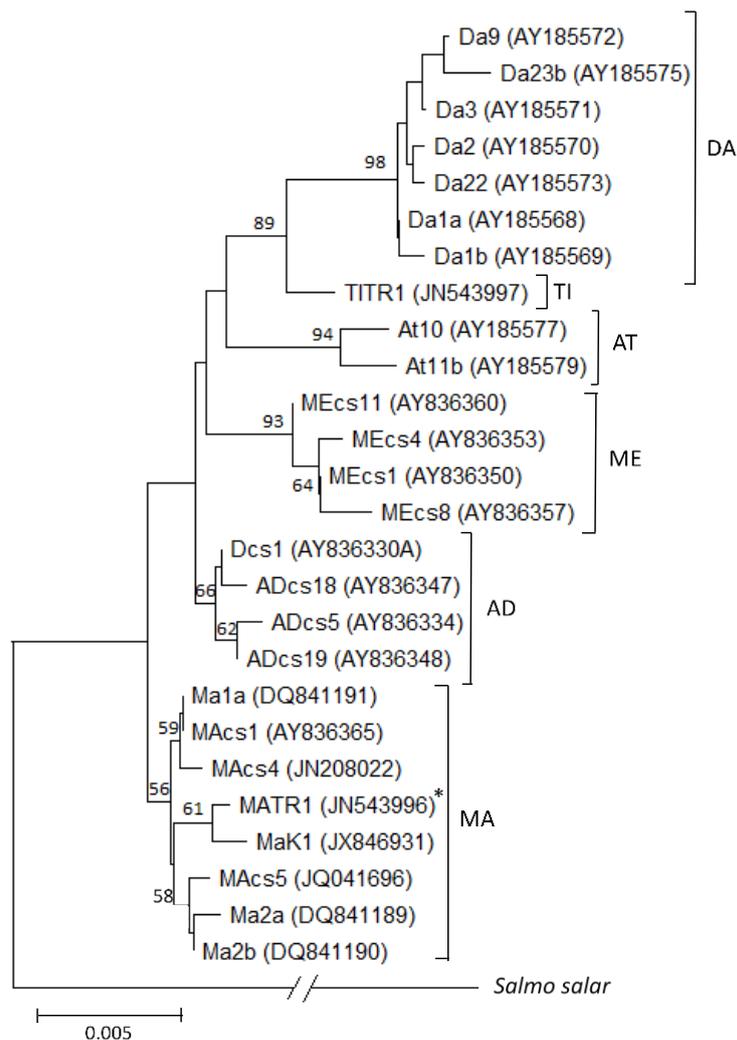


Figure 2. Neighbor-Joining tree of new MATR1 identified in this study (marked with an asterisk) and other mtDNA haplotypes of *Salmo trutta* lineages based on mtDNA control region. Bootstrap values (1000 replicates) (>50 %) are shown above branches. Accession numbers of sequences are given in brackets. (AD, Adriatic; ME, Mediterranean; AT, Atlantic; MA, *marmoratus*, TI, Tigris and DA, Danubian).



Figure 3. Photograph of a *Salmo trutta* individual from the Eşen Stream (Courtesy of Dr Yilmaz Ciftci).

marmoratus lineage in central Italy and in Greece might represent a remnant of an ancient, southernmost distribution as hypothesized by Antunes *et al.* (2002) based on analysis of transferrin gene. The net nucleotide divergence between MATR1 and other *marmoratus* haplotypes ranged from 0.205% to 0.411% (Table 2). Applying a mutation rate of 1-2% per million years, the time of diversification for this lineage would coincide with the Holocene (Bernatchez, 2001). Historical geography of Anatolia shows that there was a connection between the Southwestern Anatolian mainland and Greece (Perissoratis *et al.*, 2018). In the late Pleistocene, sea level around the Mediterranean, including Adriatic and Aegean, was regularly lower than the current levels (approx. 100-130 m), resulting in the formation of land bridges. Approximately 21,500 years ago, there were extensive shelf areas in the northern Aegean Sea and in parts of the Ionian and the eastern Aegean seas. Land protrusions on either side of the Aegean Sea, separated locally by sea channels, formed a kind of land bridge connecting Asia Minor and Greece. At 11,800 years before present, the shelf was considerably diminished due to the last (Würmian) marine regression. Approximately 7,000-8,000 years ago, the sea gradually intruded the lowlands and coastal configuration much like today's was formed (Perissoratis *et al.*, 2018). Bianco (1995) suggested that the current distribution of endemic freshwater fishes in ichthyogeographic regions of western Europe (from Tuscano Latium to Aegeo-Macedo-Anatolian) was affected by river isolation that occurred during the last marine regression (about 15,000-18,000 years ago). Marine regression, which caused the rivers to diverge in the Late Pleistocene, played an important role in the diversification of fish species such as chub (*Squalius cephalus*) (Durand *et al.*, 1999), and those reviewed in Bianco (1995).

In conclusion, these findings suggest that ancient river capture represents a strong vicariant event that might succeed a pattern of past fragmentation and genetic divergence of brown trout in the Mediterranean basin. It also supports the hypothesis of Antunes *et al.* (2002) suggested that the origin of MA lineage might be

south of the current occurrence based on the genealogy of the transferring gene. This population deserves conservation, and further analysis of both mtDNA and nuclear DNA sequences from various parts of Anatolia would be useful to infer the phylogeographic relationships among the *S. trutta* complex as the area is considered a hotspot for biodiversity (Bardakci *et al.*, 2006).

Ethical Statement

All listed co-authors declare that the present study was conducted in an ethical, professional and responsible manner.

Funding Information

There is no financial support for this project.

Author Contribution

F.B. conceived of the presented idea, supervised the findings, analyzed the data and wrote the manuscript. N.A. performed the experiments. T.A. provided fish tissue samples and reviewed the manuscript. R.B. conceptualization, provided critical comments and final approval of the manuscript

Conflict of Interest

The authors declare that they have no conflict of interest.

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