

Haplotype diversity of brown trout *Salmo trutta* (L.) in the broader Iron Gate area

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Abstract: Mitochondrial DNA (mtDNA) haplotypes of brown trout (*Salmo trutta* L.) populations in the area of Djerdap National Park were analyzed in order to determine their phylogeographic statuses. Sequencing the control region of mtDNA in 66 specimens from 9 streams in the broader Iron Gate area revealed that 7 brown trout from those streams carried haplotypes of Atlantic lineage, while the remaining 59 fish were of Danubian lineage, either of Da23c or Da1 haplotypes. Four streams were inhabited by populations of the indigenous Da23c mtDNA haplotype, while the population in one stream had the Da1 haplotype. In the remaining 4 streams, brown trout of exclusively Atlantic lineage were present in 3, while those of the Da23c haplotype were additionally present in the fourth. The significant fixation index F_{ST} in all populations, caused by a significantly large proportion of the among-population component of variance, revealed the fixation of particular haplotypes in the majority of populations. The size of a particular stream determines native brown trout abundance and limits their suitability for restorative purposes.

Key words: Brown trout, Atlantic lineage, indigenous haplotypes, Iron Gate, control region

1. Introduction

Iron Gate Gorge is in the central area of Djerdap National Park, located in northeastern Serbia on the border with Romania. The national park occupies 63,000 ha and has been protected since 1974 (Amidžić et al., 2011). Iron Gate is the longest and largest incising gorge in Europe. It consists of 4 smaller gorges: the Golubac, Gospodjin Vir, Great and Small Kazan, and Sip gorges, connected by the Ljupkovska, Donji Milanovac, and Orsova valleys. The most famous and narrowest gorge is the Great Kazan, which is the deepest point of the Danube River (up to 90 m).

The environmental issues of this area are of great scientific interest. Close to the border of the national park, in the Sip Gorge, the dam Iron Gate I was constructed in 1972. In accordance with the statement of Friedl and Wuest (2002) about the effects of damming on the water level regime, turbidity, thermal stratification, primary production, and oxygen and nutrient balance, Brilly (2010) reported that a sediment mass of $140 \times 10^6 \text{ m}^3$ was dredged from the impoundment space of the hydropower station for Iron Gate I between 1968 and 1990, due to drastically changed hydrologic conditions and sediment regime. Construction of dams additionally caused a 35-m rise in the water level of the river near the dam, submerging islands and cold karstic springs that flow into the Danube.

These changes likely affected fish stocks in local tributaries by decreasing communication between streams and increasing the isolation of populations.

The southern bank of the gorge is dissected by a number of streams flowing into the Danube River as tributaries. Their fish communities have so far very rarely and scarcely been investigated, and only in their furthest downstream inlet sections during the preparation of local fishery management plans (e.g., Simonović et al., 2010). Nothing has been published so far about their structure or about the occurrence of brown trout, *Salmo trutta*, in their upstream headwater sections.

Mitochondrial DNA as a marker is often used to identify strains and lineages of brown trout and many other organisms. The most widely adopted recent categorization of mitochondrial haplotypes in brown trout was proposed by Bernatchez (2001) and Suarez et al. (2001). Six main evolutionary lineages of brown trout were defined: the Danubian (Da), Atlantic (At), Adriatic (Ad), Mediterranean (Me), *marmoratus* (Ma), and Duero (DU). In addition to these, Bardakci et al. (2006) proposed a seventh, the Tigris lineage, from the Tigris River basin in southeastern Anatolia. Lerceteau-Kohler et al. (2013) questioned the allopatric origin of the Mediterranean lineage, suggesting the possibility of independent origin

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in particular (e.g., Adriatic) mtDNA lineages with respect to both their broad dispersal range and phenotypic divergence, and emphasized that brown trout of the Danubian lineage exhibit extensive life-history diversity and stretch across the entire Ponto-Caspian basin, leaving some uncertainty about where the lineage actually arose as well as its significance related to evolutionary or taxonomic debate. In Serbia and the Balkans, the most common brown trout are those of the Da mtDNA lineage. For this reason, only brown trout with the Da haplotypes are considered indigenous in this region, while the occurrence of other lineages is considered a consequence of introduction (Bernatchez et al., 1992; Bernatchez, 2001; Duftner et al., 2003; Snoj, 2004; Marić et al., 2006). Although there are some disagreements about the modes of settlement of Atlantic populations in Austrian Danube drainages (Balon, 1968; Weiss et al., 2001; Lerceteanu-Kohler et al., 2013), there is no evidence of the natural occurrence of this lineage south and southeast of the Alps. This is supported by the fact that Romanian rivers are exclusively inhabited by brown trout of the Danubian lineage (Popa et al., 2013). Brown trout of the At lineage have so far been found scattered in Serbia, e.g., in the Gradac River, in the main bed of the Danube River near Veliko Gradište (1059th river kilometer), in the Brankovačka River (Marić et al., 2006), and in the Jerma River (Simonović et al., 2015). Brown trout in streams of the narrower Iron Gate area have never been investigated for their mtDNA haplotypes

thus far, only those from the Rečka and Vratna Rivers in the broader Iron Gate area (Marić et al., 2006).

2. Materials and methods

During the period from 2010 to 2014, brown trout anal fin clips were collected in 9 different Danube tributaries in the broader Iron Gate area (Figure 1). A total of 66 specimens were caught and analyzed, 11 of them in the Rečka River (44°31'10.82"N, 22°21'13.15"E), 10 in the Vratna River (44°22'57.07"N, 22°20'23.99"E), 6 in the Zamna River (44°17'32"N, 22°16'40"E), 15 in Kožica (44°36'04"N, 21°58'01"E), 6 in Mala Boljetinska (44°29'26"N, 22°01'10"E), 12 in Zlatica (44°28'00"N, 22°05'50"E), 2 in Brnjica (44°38'9.12"N, 21°45'9.70"E), and 1 specimen in the Dobrinjska River (44°38'46.1"N, 21°54'17.1"E). Sampling was carried out by electrofishing using the AquaTech device IG2001 (input 12 V per maximum 15 A DC, output 500 V, and frequency 65 P s⁻¹). All sampled fish were released alive into their native water immediately after the sampling. Fin clips of 3 brown trout from the Porečka River that were sudden and unexpected catches were obtained as frozen for over 3 months from a local angler.

Extraction, amplification, and sequencing procedures are given in detail by Tošić et al. (2014). Extraction was performed using the high-salt extraction technique (Miller et al., 1988), followed by amplification of the mtDNA control region (CR). Obtained PCR products were sent



Figure 1. Sampling sites on the streams Brnjica (1), Dobrinjska reka (2), Kožica (3), Mala Boljetinska reka (4), Zlatica (5), Porečka reka (6), Rečka reka (7), Vratna (8), and Zamna (9) in the broader Iron Gate area with the position in the Balkan region given in small figure in lower left.

to MACROGEN Europe for purification and sequencing services. Single-pass sequencing was performed on each template using forward primer 28RIBa (Snoj et al., 2000); i.e. the CR was sequenced from the 5' region up to the poly T block to the investigated sequence length of 560 bp.

Sequences were compared to a query sequence from GenBank using BLAST (Basic Local Alignment Search Tool) and aligned together using ClustalX2 (Larkin et al., 2007) with particular CR sequences of Da haplotypes occurring in the streams of Serbia (e.g., Da1, Da2, Da23b, and Da23c) after Marić et al. (2006) and Tošić et al. (2014), as well as the sequences of the most common haplotypes in other brown trout lineages (Ad1, At1c, and M1). For inferring relationships between the haplotypes found in the broader Iron Gate area, sequences were examined with the MEGA 6.0 package (Tamura et al., 2013) using both the maximum likelihood method (reconstruction search type was heuristic with the stepwise addition of branches using the nearest neighbor interchange method) and maximum parsimony method (the model of nucleotide substitution type was a Kimura 2-parameter model that included both transitions and transversions, assuming uniform rates among sites, as well as the number of bootstrap replicates, with the probabilities given in the consensus tree figure).

The genetic structure of populations was analyzed for nucleotide composition and diversity in each population, as well as for the haplotype/gene diversity, population pairwise F_{ST} , fixation index (F_{ST}), and analysis of molecular variance (AMOVA) using the distance matrix (i.e. pairwise difference method) option of Arlequin software (Excoffier et al., 2005).

3. Results

Three different CR haplotypes were found in the broader Iron Gate area: Da23c, Da1, and At. Seven brown trout specimens out of 66 analyzed (10.61%) from this area carried the Atlantic clade haplotypes (Table 1). The other 59 samples were assigned as either the Da23c (GenBank No. KC 630984, Tošić et al., 2014) or the Da1 haplotype of the Danubian lineage. In the Kožica, Mala Boljetinska, Zlatica, and Zamna streams, the only haplotype that was observed was Da23c. Therefore, it is considered indigenous for this area. Seven analyzed samples in the Vratna River also carried the Da23c haplotype, while the others were within the Atlantic haplogroup. All 11 samples from the Rečka River carried the Da1 haplotype. Two sequences of brown trout from the Porečka River were unreadable, probably due to their poor preservation; they were excluded from analysis.

The maximum parsimony method of tree construction found the 7 most parsimonious trees from which the consensus tree was calculated. Trees constructed using both maximum parsimony and maximum likelihood methods revealed the same topology of the clade with Danubian haplotypes (Figure 2), where Da1 and Da23c were in a sister relationship. The only difference between these 2 haplotypes was at position 145, with Da1 holding G and Da23c A bases.

The mean number of alleles of 3 haplotypes present in 9 streams was 1.002 ± 0.015 . Considering that only the Vratna River had more than one haplotype, gene diversity of this river was 0.4667 ± 0.1318 . Average nucleotide diversity was detected only in the Vratna River; it was 0.008333 ± 0.005044 . Among 10 polymorphic sites,

Table 1. Brown trout CR mtDNA haplotypes in the Danube River drainage system over the broader Iron Gate area.

Streams and rivers	Lineage	Haplotype	Number of individuals	River km (mouth)
Brnjica	At	At	2 (3.03%)	1033
Dobrinjska	At	At	1 (1.52%)	1021
Kožica	Da	Da23c	15 (22.73%)	1016.8
Mala Boljetinska	Da	Da23c	6 (9.09%)	1003.2
Zlatica	Da	Da23c	12 (18.18%)	992.8
Porečka	At ?	H3 Unreadable	1 (1.51%) 2 (3.03%)	988.2
Rečka	Da	Da1	11 (16.66%)	884
Vratna	Da At	Da23c, At	7 (10.6%) 3 (4.54%)	879
Zamna	Da	Da23c	6 (9.09%)	871

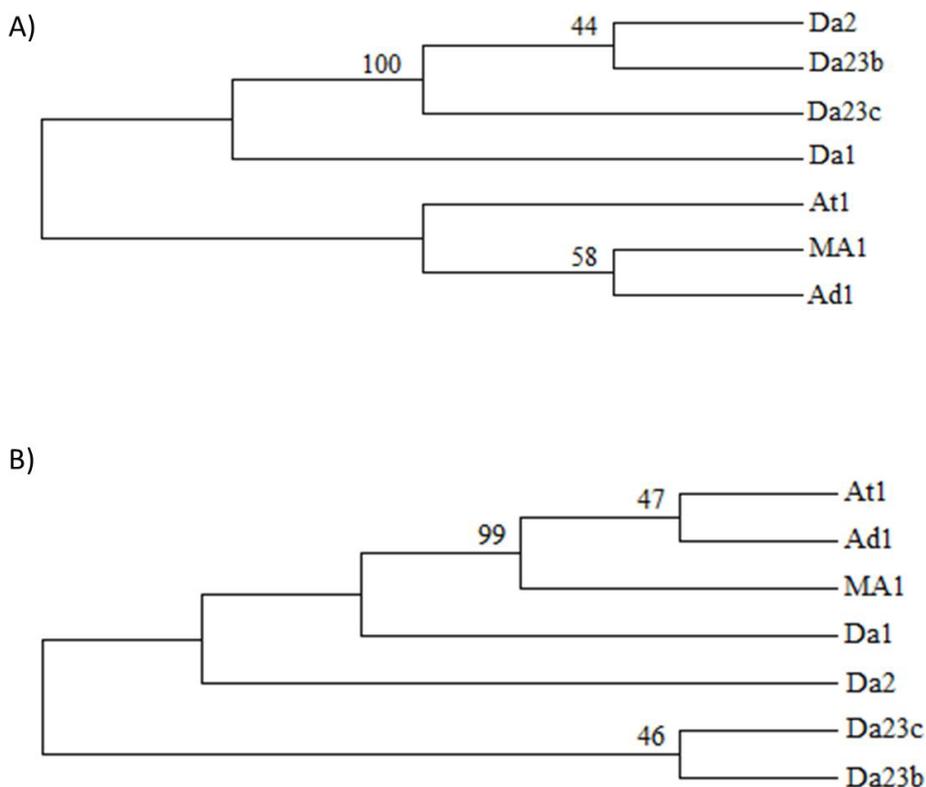


Figure 2. Relationships between CR haplotypes of brown trout populations in the broader Iron Gate area constructed using maximum likelihood (A) and maximum parsimony (B) methods (numbers at particular branches represent bootstrap probabilities; bootstrap values under 40% are not represented).

there were 7 observed transitions, 3 transversions, 10 substitutions, and no observed indels.

AMOVA results (Table 2) revealed that the majority of variance in all 9 streams was its component occurring among populations (68.75%), with the value of fixation index of $F_{ST} = 0.6875$.

Significant differences based on haplotype frequencies were observed between most of the streams. The Rečka River was significantly different from the greatest number of populations (7), while the Dobrinjska and Porečka streams were not significantly different compared to any other streams. According to F_{ST} values, the pairwise differences (Table 3) were significant between Rečka on one side and the Kožica, Mala Boljetinska, Zlatica, and Zamna streams ($1.00, P < 0.01$) on the other, with no significant difference between the Porečka River and all other streams.

For each of the polymorphic loci, fixation index was $F_{ST} = 0.6518$, with the same proportion of variation among the populations (65.18%) and within them (34.82%), except for locus 145, for which $F_{ST} = 0.8374$, with a proportion of variation among the populations of 83.74% and within them of 16.26%. All F_{ST} values were significant ($P < 0.01$).

4. Discussion

Due to the refugial role of the Iron Gate Gorge for ancient animal and plant species during the Pleistocene glaciation period, Djerdap National Park is an important conservation area for its indigenous flora and fauna. Brown trout populations in the Kožica, Zlatica, Mala Boljetinska, and Zamna streams were fixed for the indigenous Da23c haplotype. The discovery of brown trout populations that hold the Da23c haplotype in these streams contributes to the knowledge on the distribution and population structure of this new and unique brown trout strain, initially found in the Crni Timok River drainage area in the adjacent region of eastern Serbia, as described by Tošić et al. (2014).

The Porečka, Dobrinjska, Brnjička, and Vratna rivers revealed the presence of Atlantic haplotypes. In one specimen from the Porečka River, the whole CR sequence (1080 bp in length) was obtained and determined as H3 (At1c in Duftner et al., 2003), known as 1 of the 4 At haplotypes identified in Austrian hatcheries (Duftner et al., 2003). Although there is a possibility that natural postglacial colonization of the upper Danube River area

Table 2. Analysis of molecular variance (AMOVA) within and among populations. Degrees of freedom (d.f.), sums of squares (SS), proportion of variance (% Var.), probability (P).

	d.f.	SS	Variance components	% Var.	P
Among populations	8	48.0470	0.8400	68.75	<0.01
Within populations	55	21.0000	0.3818	31.25	
Total	63	69.0470	1.2218		
Fixation index F_{ST}	0.6875				<0.01

Table 3. Table of significant differences between streams (1- Brnjica, 2- Dobrinjska, 3- Kožica, 4- Mala Boljetinska, 5- Zlatica, 6- Porečka, 7- Recčka, 8- Vratna, 9- Zamna). Lower diagonal: pairwise F_{ST} values based on distance method. Upper diagonal: significance of these values, haplotype diversity (h), nucleotide diversity (π), *P < 0.05, ** P < 0.01.

	1	2	3	4	5	6	7	8	9	H	π
1		0.9910	*	*	**	0.9910	**	0.1351	0.0540	0	0
2	0		*	0.1351	0.1171	0.9910	0.0811	0.3513	0.1351	0	0
3	1	1		0.9910	0.9910	0.9910	**	*	0.9910	0	0
4	1	1	0		0.9910	0.9910	**	0.3153	0.9910	0	0
5	1	1	0	0		0.9910	**	*	0.9910	0	0
6	0	0	1	1	1		0.0811	0.3243	0.1802	0	0
7	1	1	1	1	1	1		**	**	0	0
8	0.4643	0.3333	0.2905	0.1429	0.2520	0.3333	0.3300		0.1982	0.4667	0.0083
9	1	1	0	0	0	1	1	0.1429		0	0

by Atlantic trout haplotypes could have occurred (Balon, 1968; Osinov and Bernatchez, 1996; Weiss et al., 2001), this assumption is unlikely for the Danube River drainage area in its middle section (Weiss, 2001; Lerceteau-Kohler et al., 2013). There is no evidence of their natural occurrence south and southeast of the Alps; regardless, they predominate as native in the headwaters of the upper Danube in Bavaria and in numerous drainages of the Austrian Danube. One possible explanation for the presence of At haplotypes could be uncontrolled stocking with allochthonous, hatchery-reared brown trout of the Atlantic clade, which were introduced in this way into many streams and rivers throughout the Balkans (Marić et al., 2006; Jadan et al., 2010; Simonović et al., 2015) and Europe (Largiadèr and Scholl, 1996; Osinov and Bernatchez, 1996; Hansen et al., 2000; Weiss et al., 2000, 2001). Two specimens of the same H3 haplotype were recorded in 2010 in the Danube riverbed by Marić et al. (2012) near the city of Veliko Gradište at river kilometer 1059. There is a possibility that these fish could have acclimatized and survived in the main Danube riverbed due to the submerged karstic springs that feed into this section of the Danube River, bringing cold, oxygen-rich

water. The possibility of brown trout survival in the main riverbed of the Danube River in areas with cold springs raises the question of whether any settled population in the Danube River or in local streams draining into it could use cold springs as a spawning area. Their presence in the streams is undoubtedly confirmed, but they have been sampled only in late summer and early autumn. It is still uncertain whether they spawn in those streams, i.e. if they have already naturalized in the areas of karstic springs and established spawning migratory behavior. If they have, there is a legitimate concern about potential cross-breeding with indigenous brown trout and introgression into their gene pool in streams in Djerdap National Park, as has already happened in the Gradac River (Simonović et al., 2014). Considering that both the Gradac River and Iron Gate Gorge are protected natural areas, there are the same important ecological, scientific, economic, cultural, and moral/spiritual reasons (Bosse, 2004) for the conservation of the native brown trout in them.

Brown trout of the Atlantic haplotype in the Vratna River is probably not from the same source as that of the Brnjica, Dobra, and Porečka rivers. This could be a consequence of stocking, since it is unlikely that brown

trout could inhabit Vratna but skip the Rečka Stream, which is closer to the Iron Gate than the Vratna River. In addition, it is evident that brown trout of the Atlantic lineage do not reside permanently in other streams, as they do in the Vratna River. They were the only trout caught there, and only in the early autumn season, in contrast to brown trout of At lineage in the Vratna River sampled in early summer, together with other brown trout of the indigenous Da23c haplotype. It seems that the absence of indigenous Danubian brown trout from the Brnjica, Dobra, and Porečka rivers is somehow of significance for the introgression of Atlantic brown trout into these rivers. However, this is still to be investigated. All analyzed specimens in the headwaters of the Rečka River were of the Da1 CR mtDNA haplotype. This haplotype is indigenous for the western Balkan region (Marić et al., 2006). No specimen of the Da23c haplotype indigenous to the broader Iron Gate area was found there, although Marić et al. (2006) reported brown trout belonging to the Da23 haplotype group (most likely Da23c) in 1 of 3 sampled individuals from the second fork of this same stream. This might suggest that brown trout of the Da1 haplotype, which were introduced by stocking, overpopulated and supplanted the original trout of the Da23c haplotype. However, brown trout from the fork sampled on this occasion were exclusively from the headwater section situated upstream of the high, impassable waterfall. Both methods (maximum likelihood and maximum parsimony) suggested the sister-clade relationship between the Da1 and Da23c haplotypes (Figure 2), without the possibility of drawing conclusions on the ancestry of any of them. Using the results from brown trout populations from the Mediterranean and Euphrates River basins, Arslan and Bardakci (2010) also inferred that phylogenetic conclusions based on mtDNA analysis alone should be viewed with caution. Therefore, it is possible that brown trout of the Da1 haplotype in the headwater section of this stream upstream of the waterfall form an isolated native population that has remained there, without the introgression of brown trout of the more derived Da23c haplotype. This ambiguity in assessment of the true status of aboriginality emphasizes the strong need for consequent implementation of conservational measures in trout fishery management in the area, as given by Simonović et al. (2014).

For 10 polymorphic sites of all populations, only one (145) had a different F_{ST} value, as the only site with different bases in 2 Danubian haplotypes (Da1 and Da23c). The significance of fixation index F_{ST} is an outcome of the significantly large proportion of the among-population component of the molecular variance in comparison with the within-population component (Table 2), since most of the streams are fixed for one haplotype, which varies

in particular populations. F_{ST} pairwise differences (Table 3) showed similar results as haplotype frequency-based differences. The highest F_{ST} values between brown trout in the Rečka Stream population on one hand and the Kožica, Mala Boljetinska, Zlatica, and Zamna streams on the other hand is a consequence of different haplotype (Da1 and Da23c, respectively) fixation in them. The significant difference (Table 3) based on the haplotype frequency (Table 1) between the brown trout population from the Rečka River and other populations also came from its Da1 haplotype, unique in this area. Dobrinjska and Porečka streams had no significant differences, considering that they produced only one specimen each.

Indigenous brown trout stock in the Crni Timok River drainage area is already compromised by introduction of the stocked brown trout of the nonnative Da2 mtDNA haplotype. The finding of pure native brown trout populations out of the Crni Timok River drainage area gives an opportunity for restoration activities, which might be limited by the size of those populations. Some of the streams (e.g., Zlatica and Mala Boljetinska) holding the native brown trout are very small and suffer from great variation in water discharge, i.e. completely drying up during dry years. Despite its larger size, even the Zamna River occasionally dries up completely (most recently in 2013). This variation in water flow rate determines the size of the brown trout population, estimated by their relative abundance (at most up to 10 individuals per kilometer). Out of these streams, the Kožica Stream is the only one of larger size and stable water regime, with the brown trout population size in it estimated to be up to 100 individuals per kilometer. The generally low abundance in the majority of those streams puts additional limitations on their use for restorative purposes. It is likely that occasional unfavorable environmental conditions in these rivers have had an adverse effect on the brown trout populations. As a consequence of low abundance and isolation of trout populations, the fixation of the indigenous haplotype in them could have been facilitated by frequent bottleneck events. This assumption remains to be tested on microsatellite loci.

This research revealed that native brown trout stocks in streams that flow directly into the Danube River in the broader Iron Gate area add greatly to its conservational importance, which is already threatened by the introduction of alien brown trout strains.

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