

**EFFECT OF THE INTROGRESSION OF ATLANTIC BROWN TROUT, *SALMO TRUTTA*, INTO ADRIATIC TROUT, *SALMO FARIOIDES* IN A STREAM AT THE DRAINAGE AREA OF THE ADRIATIC SEA BASIN OF MONTENEGRO**

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**Background.** The diversity of wild trout stocks in Montenegro is compromised by insufficiently controlled stocking. Adriatic and Mediterranean areas show a high degree of endemism of the salmonid species, with numerous native *Salmo* taxa described. The invasive effect of brown trout, *Salmo trutta* Linnaeus, 1758, of the Atlantic haplogroup on the endemic Adriatic trout, *Salmo farioides* Karaman, 1938, in the drainage area of the Adriatic Sea basin is lower compared to the effect inflicted by *S. trutta* has on *Salmo labrax* Pallas, 1814 of the Danubian haplogroup. The presently reported study was intended to describe the genetic structure of the population of Adriatic trout, *Salmo farioides*, from the Mrtvica River, a right tributary of the Morača River, Montenegro, in order to get an insight into the spread of non-native strains and their introgression into the native Adriatic trout gene pool.

**Material and methods.** Trout specimens sampled from the lower section of the Mrtvica River from 2004 to 2007, and from its upper section in the spring of 2014, were analysed for their mtDNA haplotypes. Nuclear DNA markers (microsatellites, *LDH-C1\** gene) were also included in genetic analyses since mtDNA is inherited only maternally. After statistical analyses, relations between individuals from the upper and lower Mrtvica River were reconstructed.

**Results.** Using both *LDH-C1\** and seven microsatellite loci with several alleles exclusive to *S. trutta*, an introgression was detected only in the upper reaches of the Mrtvica River, where only stream-dwelling trout form occurs. In the lower reaches no introgression was detected at all, as revealed by the absence of both the Atlantic mitochondrial haplotype and microsatellite alleles exclusive to *S. trutta*.

**Conclusion.** The allelic frequency at seven microsatellite loci of *Salmo* spp. from the two river sections revealed very different population structure, as a consequence of a low rate of gene flow between them. In addition to the physical barrier that prevents *Salmo* sp. from the lower Mrtvica River to migrate to the upper river section, it seems that the opportunity to mate with the large pool of conspecifics, including lake-dwelling *S. farioides* (i.e., *Salmo* cf. *dentex*), prevents *Salmo trutta* from surviving at the lower reach and spread to the rest of the Morača River and Lake Skadar systems.

**Keywords:** trout, Adriatic Sea basin, stream- and lake-dwelling, mtDNA, microsatellites

## INTRODUCTION

The inconsistency of the taxonomy of trout (*Salmo* spp.) (see Kottelat 1997) resulted in the description of numerous nominal species (and subspecies). In order to resolve this taxonomic confusion, new techniques,

using various molecular markers, have been extensively employed in the last two decades. The first comprehensive study of the genetic variation of the mtDNA control region (CR) sequence among geographically distant trout *Salmo* spp. populations (Bernatchez et al. 1992) revealed five major

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phylogenetic assemblages (Danubian, Atlantic, Adriatic, Mediterranean, and marmoratus). The most widely adopted recent categorization of mitochondrial haplogroups in *Salmo* spp. was provided by Bernatchez (2001) and Suárez et al. (2001) who distinguished six main evolutionary lineages of trout: the Danubian (DA), Atlantic (AT), Adriatic (AD), Mediterranean (ME), marmoratus (MA), and Duero (DU). In the Adriatic region, only MA, AD, and ME phylogenetic lineages are considered autochthonous (Giuffra et al. 1994, Apostolidis et al. 1997, Snoj et al. 2000). The use of mitochondrial DNA (mtDNA) markers (Berg and Ferris 1984, Gyllensten and Wilson 1987, Bernatchez 2001, Suárez et al. 2001, Snoj et al. 2002, 2010, Sušnik et al. 2004, Cortey et al. 2004, Bardakci et al. 2006, Turan et al. 2011, Simonović et al. 2015) and nuclear markers (Ferguson and Fleming 1983, Antunes et al. 2002, Hashemzadeh Sagherloo et al. 2012, Pustovrh et al. 2014, Araguas et al. 2017) revealed that the genus *Salmo* is genetically a highly structured taxon and therefore, effective conservation strategies need to be focused on the population level. Genetic screening of variation in brown trout, *Salmo trutta* Linnaeus, 1758, has been largely applied towards evaluating introgression of hatchery strains into wild populations (Hansen and Loeschcke 1994, Arias et al. 1995, Largiadèr and Scholl 1995).

The control region (CR) of mtDNA, nuclear lactate dehydrogenase (LDH) gene, and microsatellites have proved to be very good and widely applied molecular markers in resolving genetic and phylogenetic problems within the genus *Salmo*. Suitable nuclear genetic marker system used in the study on the population genetics and phylogeography of the *Salmo* spp. is *LDH-CI*\* gene, whose polymorphism enables discrimination between particular phylogeographic lineages. The two most common alleles are \*100 (found throughout the taxon range), and \*90 (naturally restricted to the populations from north-western Europe) (Hamilton et al. 1989). Those alleles represent the AT haplogroup within the genus *Salmo*. The majority of hatchery stocks generated from *Salmo* sp. of the AT lineage in Europe show a very high frequency of the \*90 allele. For that reason, the \*90 allele is a convenient marker for the success of stocked fish and subsequent introgression, especially when hatchery fish are stocked into the Mediterranean or Black Sea drainages, where \*90 does not occur naturally and where native populations have a high frequency of the \*100 allele (McMeel et al. 2001). Microsatellites are also very useful for detecting gene flow and admixture between indigenous, relocated, or domesticated populations (Hansen 2002, Jug et al. 2005), apart from their use in the analysis of the population structure.

The goal of this study was to describe the population and genetic structure of *Salmo farioides* Karaman, 1938 from the Mrtvica River, Montenegro. Mrdak et al. (2006) reported the finding of the MN87p haplotype (renamed to Ad-M1 in Sušnik et al. 2007, GenBank accession number DQ381566) of *S. farioides* from the lower reaches of the river. However, there was no information on the population from the upper part of the river, which is, due to the high elevation gradient and several cascades, separated from

the river's lower reaches. Therefore, the aim of our study was to describe the population and genetic structure of *S. farioides* population from the whole Mrtvica River, in order to determine if there are any differences between the two reaches of the stream (assigned the upper and lower Mrtvica River).

## MATERIALS AND METHODS

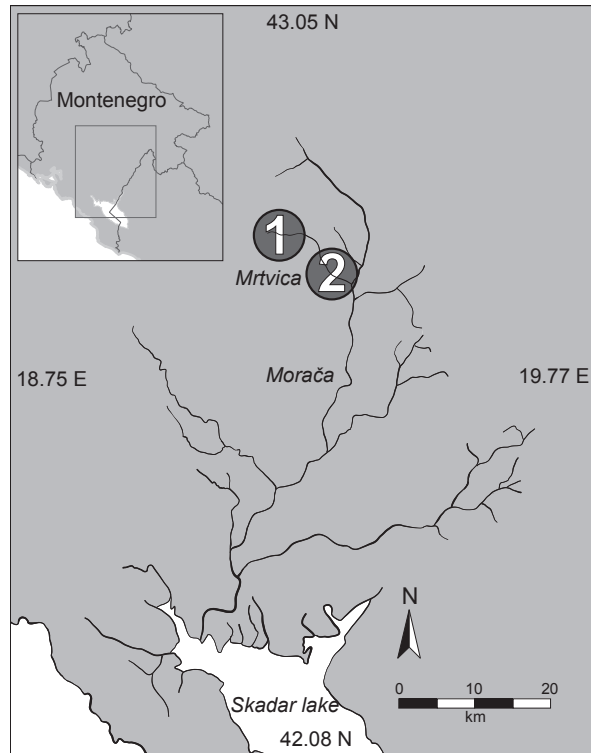
The Mrtvica River is the right tributary of the upper Morača River, Montenegro, approximately 8 km long, with the headwaters in the high mountain slopes that descend to a broad mountain plateau. The Morača River is the biggest tributary of the Lake Skadar, contributing with 62% of the total water draining into the lake (Lasca et al. 1981).

In the lower Mrtvica River, the sampling was conducted by fishing with hooks and line, from 2004 to 2007, while samples from the upper Mrtvica River were obtained by electrofishing, in the spring of 2014 (Fig. 1). Tissue samples (~4-mm<sup>2</sup> anal fin clips), collected from 11 trout from the lower reaches and 12 individuals from the upper reaches, were stored in 96% ethanol. After sampling, the fish were released into the water, DNA was extracted using the High Salt Extraction technique (Miller et al. 1988). Amplification of the control region (CR) of mtDNA was carried out using the forward primers 28Riba (Snoj et al. 2000) and *Trutta\_mt\_F* (5'-TGAATGAACCTGCCCTAGTAGC-3', designed by Miloš Brkušanin), and the reverse primer HN20 (Bernatchez and Danzmann 1993), following the protocol from Tošić et al. (2014). PCR products were purified and sequenced at Macrogen Europe. Sequencing reactions were performed in a DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD) using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the protocols supplied by the manufacturer by single-pass sequencing on each template using the forward (*Trutta\_mt\_F*) primer. Obtained sequences were aligned with those from the GenBank using program Mega 7.0.21 (Larkin et al. 2007).

The partial LDH gene was amplified using *Ldhxon3F* and *Ldhxon4R* primers (McMeel et al. 2001) and polymorphisms of the amplified fragments were detected using *SatI* endonuclease for CR mtDNA and *BseLI* for LDH, under the conditions given in Marić et al. (2010).

Seven microsatellite loci (Table 1) were included in this analysis: *Ssa85* (O'Reilly et al. 1996), *Str73INRA* (Estoup et al. 1993), *SSsp2216* (Paterson et al. 2004), *Ssa410Uos* (Cairney et al. 2000), *SsaD190*, *SsaD71* (King et al. 2005), and *OMM1064* (Rexroad et al. 2002). They were combined in three duplex and one single reactions, with forward primer labelled with a fluorescent dye (FAM or NED) (Table 1). Fragment analysis was performed using GeneScan 500 LIZ Size Standard (Applied Biosystems, USA) on an ABI-3130 Genetic Analyzer (Applied Biosystems, USA). The analysis was done using GeneMapper ID v3.2.1 (Applied Biosystems, USA). Microsatellite allele frequencies, observed and expected heterozygosity ( $H_o$ ,  $H_e$ ), and the number of alleles per locus were obtained using Genetix 4.05\* software (Tables 2 and 3). Allelic richness,

pairwise values  $F_{is}$  and  $F_{st}$ , and test on Hardy–Weinberg equilibrium were performed in Arlequin 3.5.1.2 (Excoffier and Lischer 2010) and Fstat 2.9.3.2\*\* software packages (Tables 2–5). Difference in frequencies of alleles between the *Salmo* spp. in the upper and lower Mrtvica River was



**Fig. 1.** Sampling sites at the Mrtvica River, Montenegro; 1 = upper reach, 2 = lower reach

examined by Structure 2.3.4 software (Falush et al. 2007), with the length of the burning period of  $10^4$  and  $10^5$  Markov chain Monte Carlo (MCMC) repeats for arrangements of clusters, using the Admixture Model, allele frequencies set as correlated, number of clusters  $K = 5$  and seven iterations. The best estimated  $K$  by its  $\Delta K$  value was assessed using the Structure Harvester-Taylor0! (Earl and vonHoldt 2012). Relations between individuals from the upper and lower Mrtvica River, inferred using allele frequencies of their microsatellite loci, were revealed by Neighbour-Joining clustering method, using the Shared Allele Distances (Chakraborty and Jin 1993). Calculation of Shared Allelic Distances was worked out in the Populations 1.2.31\*\*\*.

## RESULTS

The final alignment of the 700 bp long sequence of the CR mtDNA, obtained using the new *Trutta\_mt\_F* primer, indicated the presence of two main haplogroups of *Salmo* spp. in the Mrtvica River, namely AD (twenty individuals) and AT (three individuals). Fish sampled in the upper section belonged to both AD and AT lineages; *Salmo farioides* revealed one haplotype, ADcs11 (GenBank accession number AY836340, Cortey et al. 2004), while *Salmo trutta* (individuals Mr1, Mr4, and Mr7) revealed one haplotype, A17, from the AT lineage (GenBank accession number HQ848368, Kohout et al. 2012). All trout sampled in the lower section of the river belonged to the AD haplogroup; nine individuals had the ADcs11 haplotype, one individual had the Ad+Prz haplotype (GenBank accession number DQ318129, Marić et al. 2006), and one individual had the AD-M1 haplotype.

Restriction fragment length polymorphism (RFLP) of nuclear LDH gene was used to check for interbreeding

**Table 1**

Analyzed microsatellite loci, their structure, expected PCR products length, and primers used for their amplification with fluorescent label (FAM or NED)

Reaction	Locus	Repeated motive	Colour	Primer sequence	Reference	Expected product length [bp]
FDR	Str73INRA	(GT) × TTATCT(GT)3	FAM	5'-CCTGGAGATCCTCCAGCAGGA-3' 5'-CTATTCTGCTTGTAAGTACCTA-3'	Estoup et al. 1993	138–144
FDR	Ssa410Uos	(GACA) ×	FAM	5'-GGAAAATAATCAATGCTGCTGGTT-3' 5'-CTACAATCTGGACTATCTTCTCA-3'	Cairney et al. 2000	173–310
SDR	SsaD190	(TAGA) ×	FAM	5'-GGCATTGGAGGTAAGGACAC-3' 5'-CCAGACCACTGAACCTTCTCATC-3'	King et al. 2005	115–157
SDR	SsaD71	(TAGA) ×	FAM	5'-AACGTGAAACATAAATCGATGG-3' 5'-TTAAGAATGGGTTGCCTATGAG-3'	King et al. 2005	183–239
TDR	Ssa85	(GT)14	FAM	5'-AGGTGGGTCTCCTCAAGCTAC-3' 5'-ACCCGCTCCTCACTTAATC-3'	O'Reilly et al. 1996	101–113
TDR	SSsp2216	(GTTA)25	NED	5'-GGCCCAGACAGATAAACAAACACC-3' 5'-GCCAACAGCAGCATCTACACCCAG-3'	Paterson et al. 2004	133–215
FSR	OMM1064	(GATA)19	NED	5'-AGAATGCTACTGGTGGCTGTATTGTA-3' 5'-TCTGAAAGACAGGTGGATGGTTCC-3'	Rexroad et al. 2002	163–286

FDR = first duplex reaction, SDR = second duplex reaction, TDR = third duplex reaction, FSR = Fourth single reaction.

\* <http://www.genetix.univmontp2.fr/genetix/intro.htm>.

\*\* <http://www2.unil.ch/popgen/software/fstat.htm>.

\*\*\* <http://bioinformatics.org/populations>.

between AD and AT haplogroups from the upper Mrtvica River. Two out of 12 individuals (Mr1 and Mr4) with the A17 haplotype were heterozygotes (*LDH-C\*90/100*), as well as two individuals (Mr8 and Mr9) with the ADcs11 haplotype inherited from mothers hybridized with males with the A17 haplotype. Remaining individuals were homozygotes (*LDH-C\*100/100*) and they belonged to either ADcs11 (Mr2, Mr3, Mr5, Mr6, Mr10, Mr11, and Mr12), or A17 (Mr7) haplotypes (Fig. 2).

As for the microsatellites in the Mrtvica River trout stock, several alleles exclusive to AD and AT lineages were detected (Table 6).

The mean ( $\pm$  standard deviation) gene diversity of the *Salmo* spp. in the upper Mrtvica River was  $0.503106 \pm 0.295779$ , and in the lower section of the river, it was  $0.620284 \pm 0.355738$ . A deviation from the Hardy–Weinberg equilibrium was detected only for the locus SsaD71 in *Salmo* spp. from the upper Mrtvica River ( $H_o = 0.16667$ ,  $H_e = 0.72826$ ,  $P < 0.001$ ) and for the locus Ssa410Uos from the lower Mrtvica River ( $H_o = 0.81818$ ,  $H_e = 0.94372$ ,  $P < 0.05$ ). The mean  $H_o$  values calculated using all seven microsatellite loci for the upper and lower Mrtvica River were  $0.50000 \pm 0.23570$  and  $0.58442 \pm 0.25636$ , and mean  $H_e$  values were  $0.50311 \pm 0.16790$  and  $0.62028 \pm 0.25987$ , respectively (Tables 2 and 3). *Salmo farioides* from the lower Mrtvica River revealed greater

allelic richness for SsaD71 and Ssa410Uos microsatellite loci (Table 5). A significant pairwise linkage disequilibrium between loci Str73INRA, Ssa85 and OMM1064 was detected in *Salmo* spp. from the upper Mrtvica River, whereas in the lower Mrtvica River it occurred between loci Ssa190 and SsaD71. AMOVA revealed that variation among populations ( $V_a = 0.55380$ , 22.06% of total variation) was much lower than within populations ( $V_b = 1.95661$ , 77.97%), giving the Fixation Index value of  $F_{ST} = 0.22060$  ( $P < 0.001$ ). Fstat calculated for all seven loci  $F_{IS} = 0.035$  and  $F_{IT} = 0.247$ . For the entire river, low variation among population,  $V_a = -0.00681$ , and  $F_{ST} = -0.04321$  values ( $P = 1.00000$ ), were detected only for the locus Ssa85; all other loci revealed significant genetic differentiation among *Salmo* spp. with  $F_{ST}$  values varying from 0.10152 to 0.40536. The negative values of  $F_{IS}$  for the majority of microsatellite loci in *Salmo* spp. from the upper Mrtvica River suggest the strong inbreeding between them, in contrast to positive  $F_{IS}$  values in the *S. farioides* from the lower Mrtvica River (Table 5).

*Salmo* spp. from the upper and lower reaches of the Mrtvica River were grouped in distinct clusters ( $K = 2$ ), with the  $\Delta K = 39.321459$  (Fig. 3), with the mean distances, i.e., expected heterozygosities of 0.6977 and 0.5002 between individuals in each of them, respectively. The memberships of each pre-defined population in each

Table 2

Test on Hardy–Weinberg equilibrium for *Salmo* sp. population from the upper reach of the Mrtvica River, Montenegro

Locus	$H_o$	$H_e$	$P$	SD
1	0.58333	0.53986	0.59551	0.00111
2	0.16667	0.72826	0.00000	0.00000
3	0.41667	0.35870	1.00000	0.00000
4	0.75000	0.63768	0.14874	0.00115
5	0.41667	0.34420	1.00000	0.00000
6	0.83333	0.61232	0.57182	0.00126
7	0.33333	0.30072	1.00000	0.00000

$H_o$  = observed heterozygosity,  $H_e$  = expected heterozygosity, SD = standard deviation.

Table 3

Test on Hardy–Weinberg equilibrium for *Salmo* sp. population from the lower reach of the Mrtvica River, Montenegro

Locus	$H_o$	$H_e$	$P$	SD
1	0.45455	0.64502	0.05552	0.00076
2	0.81818	0.93939	0.29345	0.00048
3	0.81818	0.50649	0.06515	0.00069
4	0.81818	0.94372	0.02523	0.00025
5	0.18182	0.31169	0.27950	0.00140
6	0.36364	0.32468	1.00000	0.00000
7	0.63636	0.67100	0.75932	0.00134

$H_o$  = observed heterozygosity,  $H_e$  = expected heterozygosity, SD = standard deviation.

Table 4

Allelic richness per locus in *Salmo* spp. from the Mrtvica River, Montenegro

Microsatellite locus	Upper Mrtvica River	Lower Mrtvica River	Upper and lower Mrtvica River
SsaD190	4.830	4.000	5.594
SsaD71	6.822	13.000	11.339
Str73INRA	2.917	2.000	3.413
Ssa410Uos	4.830	12.000	9.887
Ssa85	2.000	2.000	1.999
SSsp2216	4.830	3.000	5.020
OMM1064	2.917	3.000	4.338

Table 5

$F_{IS}$  values for each microsatellite locus and per all loci in *Salmo* spp. from the Mrtvica River, Montenegro

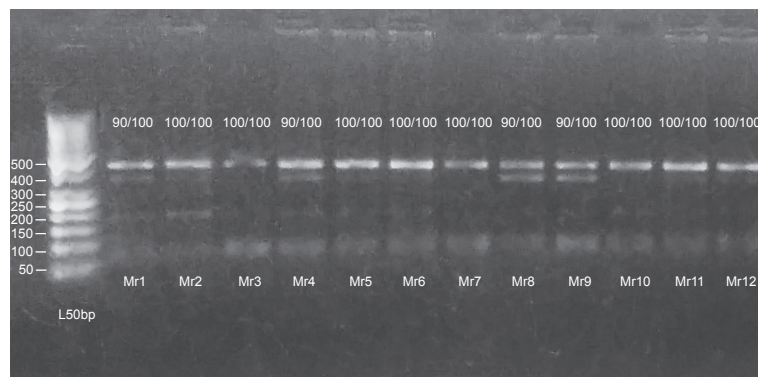
Microsatellite locus	Upper Mrtvica River	Lower Mrtvica River
SsaD190	-0.085	0.306
SsaD71	0.779	0.135
Str73INRA	-0.170	-0.667
Ssa410Uos	-0.186	0.139
Ssa85	-0.222	0.429
SSsp2216	-0.384	-0.127
OMM1064	-0.114	0.054
All loci	0.006	0.054

of the two clusters  $K$  were 87.5% and 94.9%, respectively, and the Net nucleotide distance between them was 0.1087. Mean  $F_{ST}$  values of 0.0051 for *Salmo* spp. at the upper reach and 0.2971 for those in the lower reach characterized their genetic differentiation as very low and high, respectively. Neighbour-Joining clustering of individuals by Shared Allelic Distances between them confirmed that *Salmo* spp. from the two Mrtvica River reaches belong to the two distinct clusters (Fig. 4), i.e., populations, with the very limited gene flow  $N_m = 0.442$  between them. Relation between *Salmo* spp. individuals in the population at the upper Mrtvica River of AT lineage (uRM1, uRM4,

and uRM7) and heterozygous for LDH-C (uRM1, uRM4, uRM8, and uRM9) gene were close, indicating gene flow and hybridization between them in this river sector.

## DISCUSSION

The occurrence of three AD haplotypes in the native *Salmo farioides* at both reaches of the Mrtvica River suggests their dynamic and complex evolutionary history. Given the isolation of *Salmo* spp. at the upper Mrtvica River from those at the lower section of the river, the ADcs11 haplotype seems ancestral to AD-M1 haplotype that occurs only in the lower reaches, which is consistent



**Fig. 2.** RFLP reaction of the partial *LDH\*CI* gene in *Salmo* sp. from the upper reach of the Mrtvica River, Montenegro; fragment sizes are shown alongside a 50 bp ladder (on the left)

**Table 6**

Alleles at seven microsatellite loci of 23 trout samples from the Mrtvica River, Montenegro

Sample	K	SsaD190	SsaD71	Str73INRA	Ssa410Uos	Ssa85	SSsp2216	OMM1064	mtDNA							
Mr01	1	<b>116</b>	144	444	452	140	140	<b>200</b>	284	106	106	156	168	200	200	A17
Mr02	1	144	164	200	200	140	<i>142</i>	228	284	106	<i>110</i>	156	168	200	200	ADcs11
Mr03	1	144	164	200	200	140	144	<i>236</i>	<i>280</i>	106	<i>110</i>	168	168	200	256	ADcs11
Mr04	1	<b>152</b>	164	200	200	140	144	228	284	106	106	140	168	200	200	A17
Mr05	1	164	164	168	168	140	144	284	284	106	<i>110</i>	168	168	200	256	ADcs11
Mr06	1	164	164	452	<i>452</i>	140	144	284	284	106	<i>110</i>	140	168	200	256	ADcs11
Mr07	1	<b>152</b>	164	<b>192</b>	<b>192</b>	140	140	284	284	106	106	<b>152</b>	168	200	200	A17
Mr08	1	164	164	<i>196</i>	<i>196</i>	140	140	228	284	106	106	156	168	200	200	ADcs11
Mr09	1	<i>136</i>	164	200	200	140	140	228	<i>280</i>	106	106	168	<i>184</i>	200	<i>216</i>	ADcs11
Mr10	1	144	164	200	200	140	140	228	284	106	<i>110</i>	156	168	200	200	ADcs11
Mr11	1	164	164	200	200	140	140	228	284	106	106	156	168	200	200	ADcs11
Mr12	1	164	164	<i>448</i>	452	140	140	228	284	106	106	156	168	200	200	ADcs11
Mr76	2	<i>136</i>	<i>168</i>	<i>196</i>	<i>500</i>	<i>138</i>	140	<i>236</i>	<i>300</i>	<i>110</i>	<i>110</i>	168	168	200	208	ADcs11
Mr78	2	<i>168</i>	<i>168</i>	<i>240</i>	<i>496</i>	<i>138</i>	140	280	284	106	<i>110</i>	168	<i>172</i>	200	<i>204</i>	ADcs11
Mr79	2	<i>168</i>	<i>168</i>	<i>244</i>	452	<i>138</i>	140	276	292	106	106	168	<i>172</i>	<i>204</i>	<i>208</i>	ADcs11
Mr80	2	164	<i>168</i>	256	256	<i>138</i>	140	276	<i>300</i>	106	106	168	168	<i>208</i>	<i>208</i>	ADcs11
Mr82	2	168	168	196	<i>196</i>	<i>138</i>	<i>138</i>	256	256	106	106	168	168	<i>204</i>	<i>208</i>	ADcs11
Mr83	2	164	164	452	<i>464</i>	<i>138</i>	140	<i>264</i>	284	106	<i>110</i>	168	168	200	<i>208</i>	ADcs11
Mr84	2	<i>168</i>	<i>168</i>	<i>244</i>	<i>496</i>	<i>138</i>	140	284	288	106	106	168	168	<i>208</i>	<i>208</i>	ADcs11
Mr85	2	164	164	308	312	<i>138</i>	140	260	280	106	106	168	168	204	204	Ad+Prz
Mr86	2	<i>136</i>	<i>168</i>	<i>144</i>	<i>244</i>	<i>138</i>	140	260	280	106	106	<i>164</i>	168	200	<i>204</i>	ADcs11
Mr87	2	<i>136</i>	<i>168</i>	<i>196</i>	452	<i>138</i>	140	248	252	106	106	168	<i>172</i>	<i>208</i>	<i>208</i>	AD-M1
Mr88	2	136	140	444	<i>504</i>	<i>138</i>	<i>138</i>	252	252	106	106	168	168	200	<i>204</i>	ADcs11

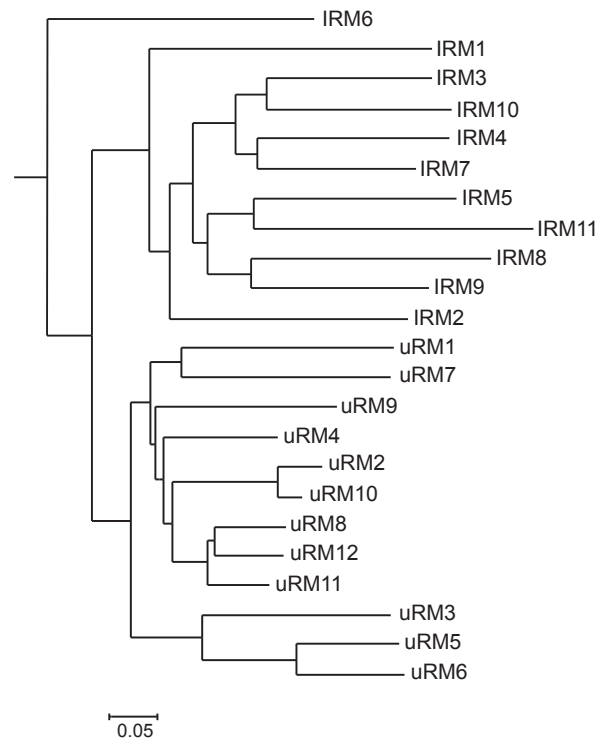
K = population, 1 = upper reach, 2 = lower reach; mtDNA = CR haplotype; alleles exclusive for AD lineage are italicized and those exclusive for AT lineage are bolded.

with the findings of Mrdak et al. (2006). According to Sušnik et al. (2007), Ad+Prz is a sister clade of the lineage containing Adcs11 and AD-M1 haplotypes, both being within the AD haplogroup. The occurrence of the AD+Prz haplotype only in the lower Mrtvica River implies that it introgressed there after the isolation of the upper Mrtvica River when the single transversion at the new polymorphic nucleotide site 256 in the ADcs11 has already occurred to derive the AD-M1 haplotype.

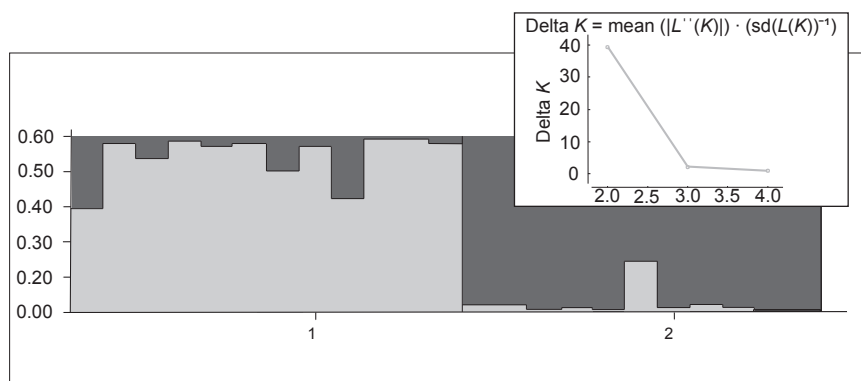
The finding of *Salmo* sp. of the allochthonous AT haplogroup in the Mrtvica River was unexpected, particularly in individuals having the A17 haplotype. Their occurrence in the highly elevated streams of the Adriatic Sea basin in Montenegro is most likely the consequence of stocking. Numerous native *Salmo* taxa have been described within the Adriatic and Mediterranean areas, which is characterized by a high degree of endemism of the salmonid species (Behnke 1973), as well as high phenotypic diversity among trout populations (Bernatchez 2001). Apostolidis et al. (2011) concluded that it is not clear whether these taxa are phenotypic variants or distinct species. In addition to the marble trout, *Salmo marmoratus* Cuvier, 1829, and the softmouth trout, *Salmo zetensis* (Hadzisce, 1960), who are clearly distinct, three other *Salmo* species were reported for the Morača River system: Adriatic trout *Salmo farioides*, *Salmo montenigrinus* Karaman, 1933, and “strun” *Salmo dentex* (Heckel, 1851) (see Drecun 1962, Ivanović 1973, Šorić 1990, Maric 1995). Based on mtDNA control region and exon 13 of transferrin gene, as well as information obtained through field investigations and observations, Mrdak et al. (2006) and Snoj et al. (2010) concluded that in Montenegro, *Salmo* cf. *dentex* is an ecomorph of Adriatic trout *S. farioides* from Lake Skadar, which migrate upstream to the spawning grounds in the upper Morača River. According to Marić and Milošević (2011), only one trout species, the Adriatic trout (*S. farioides*), occurs in the Morača River system.

The presently reported A17 haplotype constitutes the first record of this haplotype in the Balkans and the

Adriatic Sea basin and it suggests a single stocking event of the Mrtvica River with *Salmo trutta*, as no other allochthonous haplotypes were recorded there. Recently reported occurrence of the Atcs1 haplotype in brown trout in many streams attractive for angling throughout the western Balkans (Snoj 2004, Marić et al. 2006, 2012, Jadan et al. 2007, Simonović et al. 2015, Mrdak unpublished\*), as well as the hatchery origin of the brown trout reported by Marić et al. (2010), raise a question about the period of domestication and stocking of *S. trutta* of the otherwise



**Fig. 4.** Relations between *Salmo* spp. individuals from the upper (u) and lower (l) Mrtvica River (RM), Montenegro as revealed by Neighbour-Joining clustering method and Shared Allelic Distances between them, calculated from the allelic structure on seven microsatellite loci



**Fig. 3.** Visualization of the structure of genotypes in *Salmo* spp. from the upper (1) and lower (2) Mrtvica River, Montenegro, as revealed by allelic structure on the seven microsatellite loci (larger picture) and the best number of clusters  $K$  estimated by  $\Delta K$  value (smaller picture)

\* Mrdak D. 2011. Pastrmke (*Salmo* L., 1758) rijeka Crne Gore – diverzitet, taksonomski status i filogenetski odnosi. [Trout (*Salmo* L., 1758) of streams in Montenegro—Diversity, taxonomic status, and phylogenetic relations.] Doctoral Dissertation, University of Belgrade, Belgrade, Serbia. [In Serbo-Croatian.]

uncommon A17 haplotype, that far from their reported native range (Kohout et al. 2012).

The results of the presently reported study impose the question as to why trout with the A17 mtDNA marker could not migrate downstream since they are not recorded in the lowermost section of the Mrtvica River and in the Morača River system (Sušnik et al. 2007). We hypothesize that there are three possible scenarios that are not necessarily exclusive:

- There is a stronger fishing pressure in that, easily accessible reach of the stream, so that trout are fished out quickly
- Trout with the A17 haplotype disappear due to stronger selection pressure at the spawning sites they share with the migratory form of Adriatic trout *Salmo farioides* (formerly denoted *Salmo cf. dentex*) (Mrdak et al. 2006), so they fail to survive or reproduce there
- The number of *Salmo trutta* with the A17 haplotype in the upper section is too low to allow successful gene flow ( $N_m = 0.442$ ) into the gene pool of *S. farioides* in the lower Mrtvica River

All three scenarios are supported by the lack of microsatellite alleles exclusive for *Salmo* sp. of the A17 haplotype in *S. farioides* at the lower Mrtvica River and by high genetic differentiation between *Salmo* sp. from the two river sections (Fig. 4).

Negative  $F_{IS}$  values for almost all microsatellite loci in samples from the upper Mrtvica River indicate inbreeding rates higher than expected and, therefore, low population number of trout in that river section. Such a situation favours hybridization because a lower number of *Salmo* sp. of the AT lineage can have a stronger genetic impact on the original *Salmo farioides* gene pool. This was supported by relations reconstructed from microsatellite loci (Fig. 4), as well as by the occurrence of deviation from the Hardy–Weinberg equilibrium ( $H_o$  and  $H_e$  values), implying a lack of panmixia in this river part. The population in the lower Mrtvica River was far more diverse than that in the upstream section, as indicated by  $F_{ST}$  and  $F_{IS}$  values, and allelic richness.

Generally, many native *Salmo* spp. populations are threatened by anthropogenic activities, such as environmental degradation, harvesting, and stocking, that may swamp indigenous gene pools and lower the fitness of local populations (Hansen 2002). Introduction of domesticated, hatchery-reared *Salmo* sp. (commonly of AT haplogroup, hence *Salmo trutta*) was detected throughout the western Balkans (Simonović et al. 2017), where its invasive effect of introgression into the native gene pool of recipient, native trout stocks of the DA lineage, was revealed (Marić et al. 2012), and the risk of their invasiveness had already been assessed as high in Serbia (Simonović et al. 2015). Fortunately, the situation concerning the impact of *S. trutta* on *S. farioides* in the Mrtvica River seems somewhat different.

The introgression of *Salmo trutta* into the gene pool of the *Salmo farioides* was detected only in the upper Mrtvica River, where the two stream-dwelling *Salmo* spp. were sympatric. The upper Mrtvica River is a

headwater only few meters wide, extremely limited in resources. In the past, older and larger individuals of the stream-dwelling *S. farioides* from the upper Mrtvica River probably had migrated downstream, which has maintained the integrity of population in the whole stream. However, the extreme reduction in their number in the upper Mrtvica River, that has occurred most likely due to poaching, caused a decline in the number of migrants. The stocking of non-native *S. trutta* in the upper Mrtvica River could have led to population structure changes and further differentiation from the population in the lower Mrtvica River. In that way, the genetic structure of the native *S. farioides* population in the fragile, improperly managed mountain stream could have been rapidly changed in a short-time period.

The lack of introgression, i.e., successful establishment of *Salmo trutta* of the AT haplogroup in the lower Mrtvica River (despite their introgression into the gene pool of the native *Salmo farioides* at the upper Mrtvica River), could be a consequence of difference in the population structure, i.e., the existence of two life-history forms there. In addition to the resident, stream-dwelling *S. farioides* in the lower Mrtvica River that are free to move to the large Morača River and to meet many conspecifics at their common spawning grounds, that river section is also visited by *S. cf. dentex*, the lake form of *S. farioides*, who migrate during the spawning season far upstream the Morača River from the Lake Skadar, to meet stream-dwelling conspecifics. Mrdak (2011) sampled there two, and in the Morača River additional 11 *Salmo cf. dentex*, who had similar allele structure at microsatellite loci like stream-dwelling *S. farioides*, suggesting high interbreeding between the stream- and lake-dwelling forms. This is supported by occurrence of the Hardy–Weinberg equilibrium in the six out of seven microsatellite loci of *S. farioides* from the lower Mrtvica River, which implies panmixia. It is a fortunate outcome for the status of original diversity that *S. farioides* in the lower Mrtvica River and in the Morača River system so far still can cope with the alien *Salmo trutta* introduction.

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