

DIVERSITY OF BROWN TROUT, *SALMO TRUTTA* (ACTINOPTERYGII: SALMONIFORMES: SALMONIDAE), IN THE DANUBE RIVER BASIN OF CROATIA REVEALED BY MITOCHONDRIAL DNA

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Background. The molecular diversity of brown trout, *Salmo trutta* Linnaeus, 1758, has been poorly studied in Croatia. The control region of mitochondrial DNA (CR mtDNA) is in addition to other molecular markers a reliable for identifying phylogenetic lineages (haplogroups) and haplotypes of brown trout. Based on analyses of the control region of mitochondrial DNA several major brown trout phylogenetic lineages were identified of which the Danubian (DA) haplotypes, though not all, are considered native to Croatian rivers belonging to the Danube basin. The introduction of allochthonous haplotypes into natural streams seriously threatens the genetic diversity of this species. Therefore, the aim of this study was to map brown trout populations inhabiting Croatian rivers of the Danube River basin and to investigate their molecular diversity and phylogeographic patterns of the established haplotypes.

Materials and methods. Anal fin tissue was taken from 141 specimens of brown trout in 14 localities in the protected areas of Croatia, situated in the mountainous regions of Gorski Kotar, Žumberak, as well as Mountain Papuk in the western Slavonia. The total DNA was extracted and then the amplification of the mtDNA control region was carried out using primers Trutta-mt-F and HN20. Amplification of the 440 bp long region of the LDH-C1 gene locus was done using primers Ldhxon3F and Ldhxon4R. Amplified LDH-C* fragments were used for Restriction Fragment Length Polymorphism (RFLP) analysis using BseII restriction enzyme.

Results. Analysis of the CR mtDNA revealed the presence of two phylogenetic lineages, the DA and the Atlantic (AT). Haplotypes Da1, Da2, and Da22 were recorded within the DA lineage and At1 was recorded within the AT haplogroup. Two new haplotypes were described for the first time in this study and are named Da1f and Da1g. Restriction analysis of the lactate dehydrogenase gene locus revealed a high degree of hybridization between brown trout of DA and AT haplogroups.

Conclusion. The results of this study confirmed the complex molecular diversity of brown trout and the high degree of the introduction of non-native haplogroups into rivers of the Danube basin in Croatia. Conservation of native brown trout populations has become evident, as introduced allochthonous DA and AT haplogroups severely disrupt the indigenous brown trout stock.

Keywords: trout, Croatia, mtDNA, haplotype diversity, conservation

INTRODUCTION

The taxonomy of the brown trout, *Salmo trutta* Linnaeus, 1758, was described for the first time far back by Artedi (1738), and subsequently questioned by extensive synonymy of over 25 nominal taxa described (Kottelat 1997). From 25 described taxa, some are

described from rivers belonging to the Danube basin in Croatia, e.g., *Trutta likana* Karaman, 1932 from the Lička Jesenica River as terra typica. *Trutta likana* was recently considered a synonym of *Salmo taleri* (Karaman, 1933) described for the first time from the upper Zeta River in the south-western part of Montenegro (Karaman 1933).

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Recent approaches tend to untangle incongruent taxonomy of this complex taxa. Kalayci et al. (2018) suggested that all brown trout populations belonging to the Danubian (DA) lineage (sensu Bernatchez et al. 1992) should have nominotypical species status. This is in line with Berrebi et al. (2000) who appraised that diversification (i.e., genetic differentiation) between lineages should be sufficiently high for absolute reproductive isolation between them. The conservative morphology and low level of diversification between trout taxa from different phylogeographic lineages in continuous morphological characters (Simonović et al. 2007) likewise support their close evolutionary relations. The DA lineage is considered ancestral and hence, the most ancient compared to other brown trout lineages: Mediterranean (ME), Adriatic (AD), *marmoratus* (MA), Atlantic (AT) (Bernatchez 2001), Duero (DU) (Vera et al. 2010), and Tigris (TI) (Bardakci et al. 2006). Initially, the low genetic variability was observed within the DA lineage (Laikre et al. 1999), but, thanks to numerous studies that encompassed many populations, nowadays records show higher genetic diversity values (Marić et al. 2006, Snoj et al. 2011, Kohout et al. 2013, Tošić et al. 2014, Tougard et al. 2018). Simonović et al. (2017) inferred the most ancient character for brown trout stocks in the DA lineage for Da*Dž (#DQ318124.1) and Da*Vr (#DQ318125.1) haplotypes that Marić et al. (2006) described from the western part of Balkans. In contrast, Sušnik et al. (2005) inferred the most ancient Da26 haplotype (originally assigned #AY736130.1, currently replaced with the #AH014547.2) from the upper Tigris River basin.

The most ancient fossil remnants of trout fish in Croatia was that of *Salmo immigratus* (Kramberger, 1891), discovered from the Upper Sarmatian (Miocene) deposits at the locality in the vicinity of Samobor (western Croatia) dated to 13 m.y. (Anđelković 1989). Contemporary reports of molecular diversity in modern brown trout stocks from the rivers draining the Danube basin in Croatia are scarce. Jadan et al. (2007) reported about the Da2 and At1 haplotypes in the Gacka River, sinking karst waterbody situated at the western slopes of the karst field in the Lika region (western Croatia) that flows underground to the Adriatic Sea. Simonović et al. (2017) included only a few Croatian samples from the streams draining the Sava River that belong to the Danube River basin. They established that Da1 is the most widely distributed native brown trout haplotype in the area. This haplotype was also recorded in other bodies of water in the western part of Balkans, e.g., headwaters of the Kupa River, and the sinking stream Lička Jesenica (draining Kupa River) both in Croatia. Furthermore, Da1 was also established in the upper Zeta and Nožica rivers, sinking streams in Northern Montenegro belonging to the Adriatic Sea basin. Brown trout of the AT haplotypes inhabit rivers of the Atlantic Basin. However, the anthropogenic factor is considered important for their spread beyond the boundaries of its natural habitat, primarily because of the long tradition of the stocking with the hatchery fish of AT origin that exists in many countries (Sanz 2018). Similarly, mostly

due to anglers' stocking activities, feral brown trout of AT lineage has been introduced into suitable environments in Croatia (Piria et al. 2020). The origin of brown trout for stocking in inland waters is not regulated by any legal act in Croatia, although the stocking with brown trout of the AT lineage outside the territory of the Republic of Croatia was recognized as the main cause of the loss of native genetic diversity of the genus *Salmo* spp. (Simonović et al. 2015, 2017).

A specific feature of the molecular diversity of Croatian populations of brown trout is the occurrence of the Da22 haplotype in the headwaters and tributaries of the Una River, a bordering river between Bosnia and Herzegovina, and Croatia (Škraba et al. 2017). That haplotype was considered native in the upper part of the Una River, being the only brown trout haplotype recorded in its headwaters that are cut-off from the downstream section of the river by two high waterfalls. The same haplotype was also recorded at a distant locality, in eastern Serbia (Marić et al. 2006). Such a disjunct distribution of the Da22 haplotype enabled Simonović et al. (2017) to hypothesize that brown trout populations still holding/possessing this haplotype are remnants of once widely distributed population probably from the Pontian–Messinian periods of Late Miocene and Early Pliocene. The same authors considered that the recent/contemporary molecular diversity of brown trout in the area is an outcome of their dynamic evolution.

In the middle section of the Una River, haplotype Da22 was accompanied by the haplotypes Da2 and At1, both considered non-native, probably being introduced into the population by stocking with the hatchery-reared brown trout (Simonović et al. 2007). This consideration is based on the literature sources claims (Gridelli 1936, Razpet et al. 2007), as well as on the fact that the current distribution of these two haplotypes, occurring in sympatry with the widely distributed native Da1 haplotype, is in the streams throughout the western part of Balkans, including all drainage basins, even occurring as the only haplotype in the sinking streams in the western part of Croatia and southern part of Herzegovina that once lacked brown trout (Jadan et al. 2007, Simonović et al. 2017).

Application of various molecular markers added to the better insight into the relations between brown trout nominal taxa. In addition to cytochrome b, LDH-C1* locus and microsatellite loci, the Control Region (CR), or D-loop of the mitochondrial DNA (mtDNA) as a molecular marker featuring a great variability provided the systematization of the recent molecular diversity in brown trout into lineages (i.e., haplogroups) and haplotypes. Hence, CR marker was widely employed for the same purpose elsewhere in the region (Marić et al. 2006, Kohout et al. 2013, Tošić et al. 2014, Simonović et al. 2017).

Recently, it became evident that there is a strong need for conservation of brown trout populations that feature native haplotypes (Simonović et al. 2015). For that purpose, a better insight into hitherto incompletely known molecular diversity in brown trout populations in Croatia is needed. The aim of this study was to map brown trout populations

in weakly explored and unexplored rivers in the Danube basin in Croatia, and to investigate their molecular diversity, gaining an insight into the phylogeographic patterns of haplotypes by employing various molecular markers.

MATERIALS AND METHODS

A total of 141 samples were collected by electro- and fly fishing from 14 localities, with the permission of the Ministry of Agriculture and Ministry of Environmental Protection and Energy for 2017 and 2018. All studied bodies of water were situated in the protected areas in the mountainous regions of Gorski Kotar, Žumberak, and on the slopes of the Mt. Papuk in the western Slavonia. Ninety-five individuals were sampled during 2017 at eight sites (sites 1–8, Fig. 1) in the continental part of western Croatia, while 46 were sampled in 2018 on six sites (sites 9–14, Fig. 1) in the Slavonia region in the eastern part of Croatia (Fig. 1). After anal fin clipping, 49 individuals were returned alive into the water, and 92 were sacrificed for morphological analysis. The fin tissue was stored in 96% ethanol, ready for molecular analysis. The total length of sacrificed specimens was between 10.0 and 30.3 cm and mass from 10.19 to 323.52 g (Piria et al. 2019).

Prior to isolation, tissue was digested by proteinase K (Applied Biosystems®, USA). The total DNA was isolated using Quick-gDNA™ MiniPrep extraction kit according to the manufacturer's instructions (Zymo Research Corporation, USA). Amplification of the mtDNA control region was carried out using primers Trutta-mt-F (5'-TGAATGAACCTGCCCTAGTAGC-3') and HN20 (5'-GTGTTATGCTTTAGTTAAGC-3') (Bernatchez and Danzmann 1993). Amplified mtDNA fragments were run on a 1% agarose

- gel, using SYBR Green for visualization. Samples with PCR products were purified and
- sequenced in both directions in MACROGEN®.
- Amplification of 440 bp long region of the LDH-C1 gene locus was done using primers Ldhxon3F

(5'-GGCAGCCTCTTCCTCAAAACGCCCAA-3') and Ldhxon4R (5'-CAACCTGCTCTCCCTCTGCTGACGAA-3') (McMeel et al. 2001). Amplified fragments were used for Restriction Fragment Length Polymorphism (RFLP) analysis. RFLP was performed using restriction enzyme BseII to determine potential hybridization between individuals of different haplogroups. The effectiveness of the enzyme was checked on 1.5% agarose gel.

The sequences obtained from MACROGEN® were compared with each other and with the most common and expected sequences of CR haplogroups on those localities using ClustalX2 (Larkin et al. 2007). The relation between the sequences was further analyzed in the Mega-X program (Kumar et al. 2018), using Maximum Likelihood and Neighbor-Joining methods, both based on the Kimura 2-parameter model and bootstrap consensus tree inferred from 1000 replicates. The pairwise distances were calculated using the Maximum Composite Likelihood model, as well.

The Arlequin program (Excoffier and Lischer 2010) was used for the analyses of molecular variance (AMOVA), the genetic diversity between the pairs of populations (pairwise F_{ST}) and the F_{ST} fixation index (using a pairwise difference method), as well as for assessing the composition and diversity of nucleotides. All samples were grouped according to the river basin/drainage they belong to.

RESULTS

The analysis of mitochondrial DNA showed that the collected samples belong to two main haplogroups; 110 individuals belonged to the Danubian (DA) and 31 to the Atlantic (AT) haplogroup (Table 1). Haplotypes Da1, Da2, and Da22 were recorded within the DA haplogroup, while only one haplotype, At-H3 (At1), was recorded within the AT haplogroup (Table 1). The most frequent haplotype was Da1 (Fig. 2), recorded in 73 individuals. Of these, 65 were registered as Da1a subtype. The

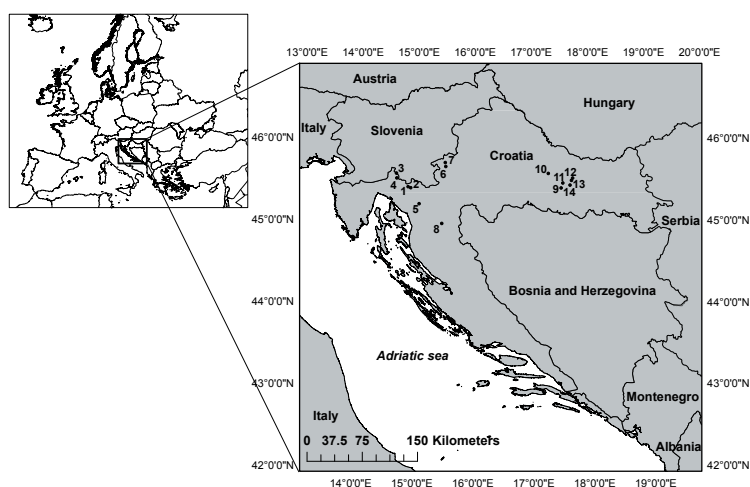


Fig. 1. Map of sampling sites of brown trout, *Salmo trutta*, in the Danube River basin of Croatia; 1 = Mala Lešnica, 2 = Curak (Zeleni vir), 3 = Čabranka, 4 = Bresni potok, 5 = Jasenak, 6 = Kupčina (and Vrabac fish farm), 7 = Slapnica, 8 = Lička Jesenica, 9 = Orjava, 10 = Toplica, 11 = Brzaja, 12 = Jankovački potok, 13 = Jankovačko jezero, 14 = Veličanka

remaining eight did not belong to any subtype of the Da1 haplotype described so far: Da1a (Duftner et al. 2003), Da1b (Duftner et al. 2003), Da1c (Baric et al. 2010), Da1d (Baric et al. 2010) or Da1e (Wetjen and Schmidt 2015).

Comparing the sequences of Da1 haplotype obtained in this study with previously obtained sequences it was revealed that three individuals from the Jankovački potok and five individuals from the Toplica River differed and are considered novel (Table 2). New haplotypes differ from other subtypes of Da1 haplotype in the position 853 for individuals from the Jankovački potok, where instead of C, T was recorded, and in the position 662 for individuals from the Toplica River, where instead of T, C was established. Based on similarities in other parts of the sequence with other Da haplotypes, we named the new haplotypes Da1f (GenBank Accession Number MK675073) and Da1g (GenBank Accession Number MK675074). The relation between novel and other Da haplotypes detected in sampling sites (Fig. 3) is weakly supported, as revealed by low bootstrap values in both Neighbor-Joining and Maximum Likelihood phylogenetic trees. Both trees, as well as the pairwise distance values (Table 3), also show grouping of the Da1b subtype of haplotype Da1 with the Da2c subtype of haplotype Da2, which is surprising since Da1b and Da2c belong to two different haplotypes.

The results of the analysis of molecular variance AMOVA (Table 4) for populations grouped into corresponding drainages showed the highest percentage of variability within the analyzed populations (83.36%), while the percentage of variability among the populations was 16.64%, with the corresponding value of the fixation index $F_{ST} = 0.16644$. From the pairwise values of genetic distance (Table 3) and nucleotide diversity (Table 4), population pairwise F_{ST} results (Table 5) revealed that the highest F_{ST} value was recorded for the populations' pair

Lička Jesenica and Kupa (Table 5). All F_{ST} values were statistically significant ($P < 0.05$).

The restriction analysis of the nuclear locus LDH-C* showed a high degree of hybridization between brown trout of the DA and AT haplogroups at all localities. Combining the results of the mtDNA and RFLP analysis for the LDH-C* locus, for 65 samples, LDH-C* was found to be in the homozygous state, indicating an origin from parents of the same haplogroup. Further, 76 samples showed that they originate from both DA and AT haplogroups; 21 of these appointed by CR mtDNA analysis to DA haplogroup were homozygous for LDH-C*90 allele featuring brown trout of the AT haplogroup, while 6 of them that were appointed by CR mtDNA analysis to the AT haplogroup were homozygous for LDHC*100 characteristic of DA haplogroup. Also, 49 samples were heterozygotes for LDH-C* gene locus carrying both *90 and *100 alleles (Fig. 4).

DISCUSSION

The newly discovered mtDNA haplotypes, Da1f and Da1g, revealed hitherto undescribed diversity of the brown trout in Croatia. Although there are clear

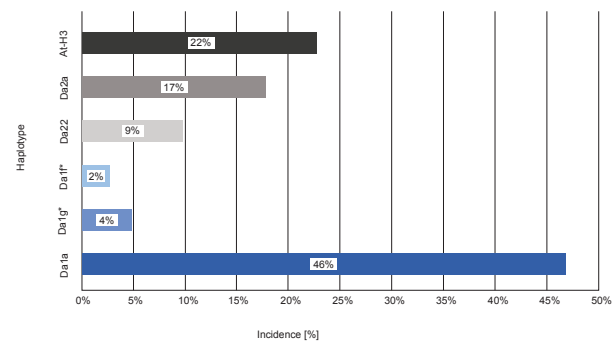


Fig. 2. The incidence of detected haplotypes of brown trout, *Salmo trutta*, in the waterbodies of Croatia (New haplotypes marked by an asterisk)

Table 1

Haplotypes of brown trout, *Salmo trutta*, in the waterbodies of Croatia based on mtDNA analysis

Water body	Drainage/basin	n	DA			AT
			Da1	Da2	Da22	At-H3
Curak	Kupa	12	10			2
Čabranka	Kupa	11	9	2		
Bresni potok	Kupa	11		2		9
Jasenak	Kupa	6	4			2
Mala lešnica	Kupa	12	9			3
Kupčina + VFF	Kupa	14 + 10	13 + 0			1 + 10
Slapnica	Kupa	10	8	2		
Lička Jesenica	Lička Jesenica	9	1	8		
Veličanka	Sava	6	3		1	2
Orljava	Sava	5			5	
Brzaja	Sava	9		1	7	1
Toplica	Drava	13	10	2		1
Jankovački potok	Drava	5	5			
Jankovačko jezero	Drava	8	1	7		
Total		141	73	24	13	31

n = sample size; DA = Danubian haplogroup; AT = Atlantic haplogroup; Da1, Da2, Da22, At-H3 = haplotypes; VFF = Vrabac fish farm.

Table 2

Variable base positions in brown trout, *Salmo trutta*, mtDNA CR haplotype sequences

	2	26	145	177	233	234	235	388	389	529	541	542	544	547	662	838	853	877	893	899	905	906	924	926
Da1a	C	A	G	T	G	A	G	T	C	C	A	C	T	T	T	A	C	T	A	G	A	C	A	A
Da1b	T	.	.
Da1c	C
Da1d
Da1f*	T
Da1g*	C
Da2a	G	/	.	/	.
Da2b	.	.	.	C	.	G
Da2c	G	T	.	.
Da23a	.	.	A	.	A	G	G
Da23b	.	.	A	.	A	G	C
Da23c	.	.	A	T
Da22	T
Da21	T	G	.	.	G
At-H3	T	T	T	C	T	T	G	G	.	C	A

* new haplotypes

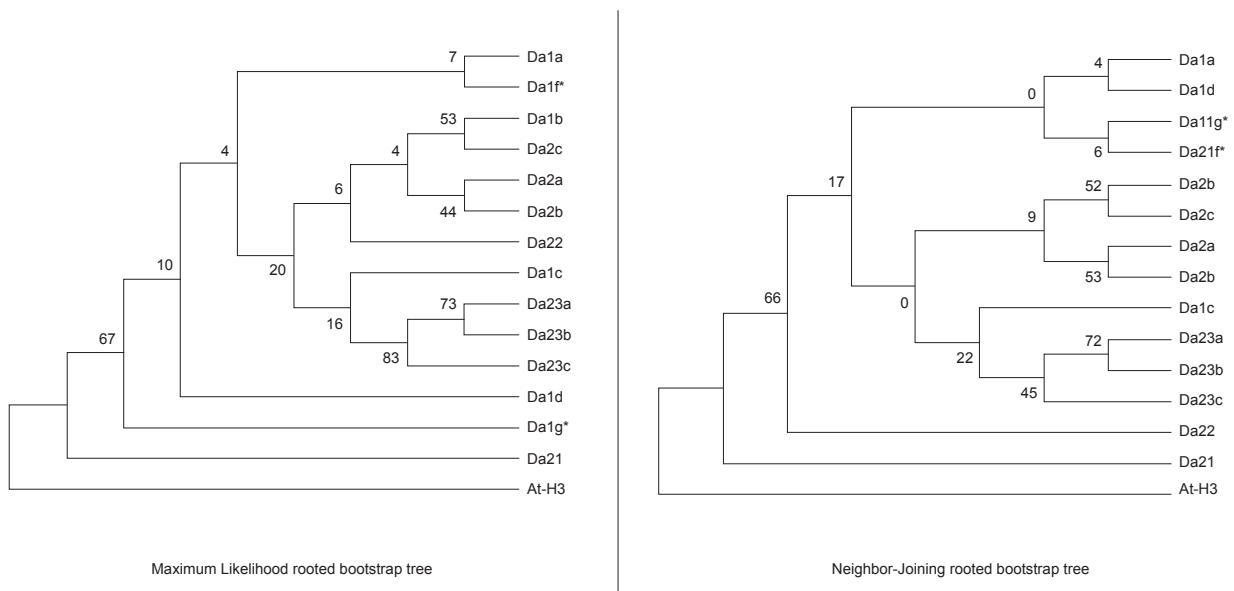


Fig. 3. The relation between the new haplotypes and other CR mtDNA haplotypes of brown trout, *Salmo trutta*; numbers at nodes present bootstrap values; asterisk denotes the novel haplotypes

differences between novel haplotypes and those already described (Table 2), the low bootstrap values supporting their inferred relations with the rest of the Da1 haplotypes and different positioning in the two phylogenetic trees (Fig. 3) suggest the low information content that they hold for reliable inferring on their phylogenetic positions.

The close relation between Da1b and Da2c haplotypes in both reconstructions of phylogenetic relations (Fig. 3) questions the current nomenclature of CR haplotypes in brown trout. It is supported by their synapomorphy of T nucleotide at the 908 position in the Control Region, in contrast to other haplotypes' subtypes that hold C nucleotide at that position (Table 3). Neither Dufner et al. (2003), nor Baric et al. (2010), considered this on naming the haplotypes they discovered. The consistency of tree

topology in the section that holds these two haplotypes supports the reconsideration of these two haplotypes' nomenclature, but for final inferring on this issue the more detailed analysis of CR haplotypes is needed.

In this research, for the first time, the composition and distribution of various phylogenetic lineages in the western and eastern parts of Croatia were analyzed. The analysis of the mtDNA control region revealed the existence of the two main phylogenetic lineages of brown trout, DA and AT, in the investigated area. Trout belonging to the DA lineage were more frequent. The DA lineage was presented by three haplotypes (Da1, Da2, and Da22), while AT lineage was presented only by At1 haplotype.

Half of the total sample (52%) were individuals carrying the Da1 haplotype, which has so far been

Table 3
Pairwise distance values between novel haplotypes and other characteristic CR mtDNA haplotypes of brown trout, *Salmo trutta*

	Da1a	Da1b	Da1c	Da1d	Da1f*	Da1g*	Da2a	Da2b	Da2c	Da21	Da22	Da23a	Da23b	Da23c	At-H3
Da1a															
Da1b	0.00101														
Da1c	0.00101	0.00202													
Da1d	0.00101	0.00202	0.00202												
Da1f*	0.00101	0.00202	0.00202	0.00202											
Da1g*	0.00101	0.00202	0.00202	0.00202	0.00202										
Da2a	0.00101	0.00202	0.00202	0.00202	0.00202	0.00202									
Da2b	0.00202	0.00303	0.00303	0.00303	0.00303	0.00303	0.00101								
Da2c	0.00202	0.00101	0.00303	0.00303	0.00303	0.00303	0.00101	0.00202							
Da21	0.00303	0.00405	0.00405	0.00405	0.00405	0.00405	0.00405	0.00507	0.00507						
Da22	0.00101	0.00202	0.00202	0.00202	0.00202	0.00202	0.00101	0.00202	0.00202	0.00405					
Da23a	0.00405	0.00506	0.00506	0.00506	0.00506	0.00506	0.00303	0.00405	0.00405	0.00710	0.00404				
Da23b	0.00405	0.00507	0.00303	0.00507	0.00507	0.00507	0.00303	0.00405	0.00405	0.00711	0.00405	0.00202			
Da23c	0.00404	0.00506	0.00506	0.00506	0.00506	0.00506	0.00506	0.00608	0.00608	0.00710	0.00506	0.00607	0.00608		
At-H3	0.01119	0.01222	0.01222	0.01222	0.01222	0.01222	0.01222	0.01325	0.01325	0.01222	0.01221	0.01530	0.01531	0.01530	

The bold number indicates the grouping of the Da1b haplotype with the Da2c haplotype.

considered autochthonous for the waters of the Danube basin (Bernatches et al. 1992, Marić et al. 2006, Tošić et al. 2016, Simonović et al. 2017). Only in the Jankovački potok (five individuals), Da1 was a single haplotype, while in the remaining 13 localities this haplotype was present along with other detected haplotypes (Da2, Da22, and/or At1) (Table1). Simonović et al. (2017) considered Da1 as a unique haplotype in completely isolated headwater sections of sinking streams such as the Lička Jesenica River. At three localities: Bresni potok, Orłjava River, and Brzaja River, Da1 haplotype was completely absent, indicating the non-native character of haplotypes introduced into those rivers (Table 1).

However, in this study, only one individual in the Lička Jesenica carried Da1 haplotype, while all the others carried Da2 haplotype. On the other hand, Jadan et al. (2007) described the presence of both Da2 and At1 haplotypes in the Gacka River. Although the Gacka River belongs to the Adriatic Sea basin, the complete absence of Ad (Adriatic) haplotypes, whose presence was expected, was recorded. Jadan et al. (2007) concluded that it is possible that the Da2 haplotype is native to this river because, in the geological past, the Gacka River belonged to the Danube River Basin. The same authors also reported data on the stocking of the Gacka River with trout from Italian, Bosnian, and Croatian fish farms since 1970, which gives a greater probability of Da2 haplotype being introduced.

Da2 haplotype is considered native to the streams and rivers in southern Germany (Bernatches 2001) and streams belonging to the Austrian part of the Danube drainage (Weiss et al. 2001). It is known that during the 19th century, the fish from southern Germany (then the Austro-Hungarian Empire) were introduced into various parts of Europe (Kohout et al. 2012), possibly also into nowadays Croatia. It has already been revealed that Da2 is not native to the rivers of the Danube River basin of the Balkan Peninsula, as shown in the Serbian rivers (Marić et al. 2006, Simonović et al. 2015, Tošić et al. 2016), in Bosnia and Herzegovina (Mrdak et al. 2012, Škraba et al. 2017), and in Montenegro (Mrdak et al. 2012). The results of the presently reported study suggest the same for the Croatian rivers.

The third haplotype of the Danube phylogenetic lineage, Da22, was the least numerous (Table 1). It was present as a single haplotype in the Orłjava River, which occurred together with Da1 and At1 in the Veličanka River, and with Da2 and At1 in the Brzaja River. The Da22 haplotype was so far considered to be native only in the drainage area of the Una River (Škraba et al. 2017) and in the Lohnbach and Daglesbach streams in Austria (Duftner et al. 2003). Discovery of Da22 haplotype as a single one in the Orłjava River, situated in the mid-Sava River drainage at the southern slopes of the Mt. Papuk, indicated that either this haplotype is native there, or it was introduced by stocking in the short-extending headwater section of this predominantly lowland river, previously completely devoid of brown trout. Future similar findings of a single occurrence of Da22 in isolated headwater sections of other streams, like in the Una River, will additionally clear the evolutionary history of this haplotype, and resolve the

Table 4

Analysis of molecular variance (AMOVA) within and among populations of brown trout, *Salmo trutta*, from different drainages

Variation source	df	Sum of squares	Variance components	Percentage of variation
Among population	3	37.011	0.38810 V_a	16.64
Within population	137	266.280	1.94365 V_b	83.36
Total	140	303.291	2.33175	
F_{ST}	0.16644			

df = the degrees of freedom in the source, F_{ST} = fixation index, V_a = among population variance, V_b = within population variance.

Table 5

Significant differences between populations of brown trout, *Salmo trutta*, representing different drainages

	KU	LJ	S	D	G	π
KU	*	0.01802 ± 0.0121	0.03604 ± 0.0201	0.00901 ± 0.0091	0.5228 ± 0.0391	0.000224 ± 0.000329
LJ	0.24634	*	0.00000 ± 0.0000	0.00901 ± 0.0091	0.2222 ± 0.1662	0.000224 ± 0.000329
S	0.10769	0.20029	*	0.00000 ± 0.0000	0.5579 ± 0.1131	0.003491 ± 0.002076
D	0.16835	0.14752	0.13338	*	0.7631 ± 0.0429	0.001867 ± 0.001232

KU = Kupa, LJ = Lička Jesenica, S = Sava, D = Drava; F_{ST} = fixation index; F_{ST} values are given under diagonal, P values are given above diagonal ($P < 0.05$), G = gene diversity, π = nucleotide diversity.

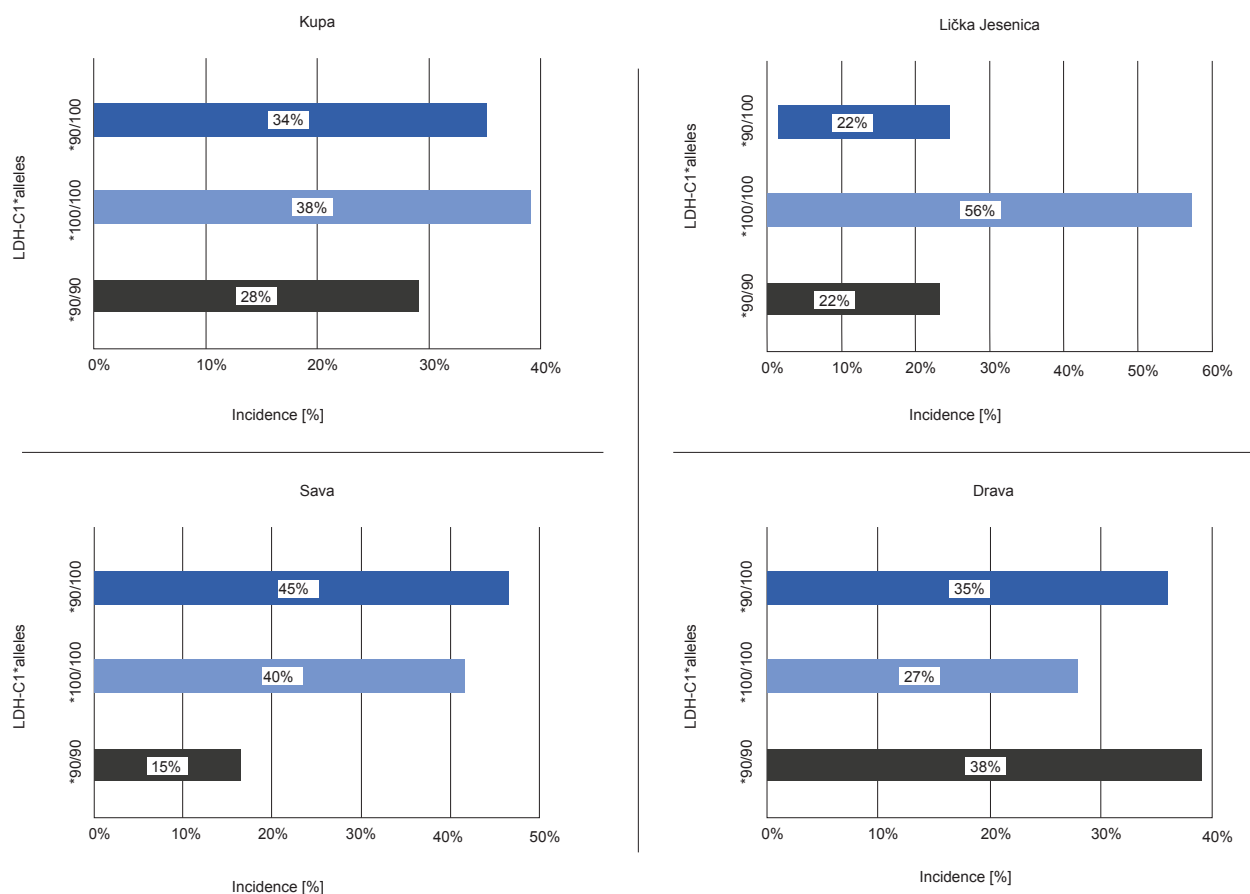


Fig. 4. The incidence of the introduction of the AT haplogroup of brown trout, *Salmo trutta*, in four river drainages in Croatia

dilemma about its character. Currently, findings of Da22 in combination with other non-native haplotypes in the area covered by this study, as well as in other localities in the western part of Balkans, e.g., in streams of south-eastern Serbia (Simonović et al. 2015), strongly indicates to their hatchery origin.

The presence of brown trout of the AT lineage was recorded at eight sites and only in the Vrabac fish farm, it was a single haplogroup. The AT lineage is not autochthonous for the predominant area of the Black Sea basin, and it is considered introduced by uncontrolled stocking from hatcheries (Marić et al. 2006, Jadan et al. 2007, Simonović et al. 2015, Tošić

et al. 2016). Together with non-native DA haplotypes, AT haplogroup is a serious problem in maintaining the autochthonous character of native brown trout. The adverse effect of the introduction of alien strains has been recognized as a major driver in loss of brown trout native genetic origin (Simonović et al. 2017). Based on presented results seems that in the majority of the Croatian streams native genetic diversity has been lost. It would be necessary to genotype remaining brown trout stock from inland waters, as well as to set the national legislation in a way that would introduce the mandatory genotyping of brown trout stocks from hatcheries and to mark and register native brood fish, which is recently in Croatia still weakly enforced.

The only sampling site in which the results of the CR mtDNA and RFLP analyses were congruent was the Vrabac fish farm, showing that all analyzed individuals by both maternal and paternal inheritance originated from brown trout of the AT haplogroup. At all other sites, hybridization occurred between brown trout of the DA and AT haplogroups. Even in the sinking, isolated Lička Jesenica River, which was considered intact from the introgression of non-native brown trout, the restriction analysis revealed that two individuals were homozygous for the LDH-C*90 allele and two were LDH-C* heterozygotes. Only one individual was the “pure” Da1 for its CR mtDNA haplotype and homozygous for its LDH-C*100 alleles while being accompanied by eight brown trout of the Da2 haplotype (Table 1). It is difficult to make a sound inference about the originality of any of the two DA haplotypes that were recorded there, but the recent pieces of evidence of introgression imply a long history of stocking with brown trout of the hatchery origin.

CONCLUSIONS AND FUTURE PERSPECTIVES

Due to specific characteristics of brown trout that have already been highlighted, such as the ability to adapt to variable environmental conditions, as well as their complex genetic structure, brown trout conservation issues should be seriously considered. This research reaffirms that the native character of brown trout natural populations is disturbed and indicates the need to take control measures for the preservation and protection of this species genetic diversity. Uncontrolled stocking with non-native haplogroups has already affected many unique and “pure”, i.e., native populations of this species, and seriously threatens those which still exist, but could easily disappear. Therefore, it is necessary to perform detailed genotyping of brown trout stock in Croatia, protect identified localities with the native brown trout strains from further stocking with alien strains and to issue the fishing regime that balances fishermen’s pressure with the fishing pressure that fisheries sustain, keeping them sustainable in both fishery and conservational sense.

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