



Trout *Salmo* spp. (Salmoniformes: Salmonidae) Molecular Diversity in Streams on the Southern Slopes of the Stara Planina Mts. in Serbia

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Abstract: Brown trout *Salmo trutta* and Macedonian trout *Salmo macedonicus* molecular diversity in the streams draining on the southern slopes of the Stara Planina Mts. was assessed from mitochondrial DNA control region (CR) extracted from fin clip samples collected during the last ten years. It revealed an occurrence of seven haplotypes from three phylogeographic lineages (Danubian, Adriatic and Atlantic) in total. Only the two haplotypes, Da1a and Da-s6, can be inferred for certain as the native ones, owing to their occurrence in the headwaters of streams hitherto void of introgression of brown trout from downstream sections, due to unsurmountable waterfalls. The conservation status of brown trout that have the very common Da22 haplotype is not certain yet. They are very abundant in streams that were subject to massive stockings in the last ten years, occurring both in the headwaters and in the downstream sections, without the physical barrier between them. The rest of haplotypes belong either to the Atlantic or Adriatic haplogroups and they are most likely non-native in this area.

Key words: haplotypes, originality, conservation status, threats, introgression

Introduction

The majority of brown trout *Salmo trutta* L., 1758 and Macedonian trout *S. macedonicus* (Karaman, 1924) stocks of Serbia has been mapped for their molecular diversity so far (MARIĆ et al. 2006, TOŠIĆ et al. 2014, 2016, SIMONOVIĆ et al. 2017). The westernmost part of the Stara Planina Mts. in the Eastern Serbia that is shared with the Western Bulgaria still remained scarcely investigated, although particular records were published when the risk assessment of the non-native trout strains was analysed (SIMONOVIĆ et al. 2015). In 1997, the whole Stara Planina Mts. in Serbia was declared a natural protected area (Nature Park

“Stara Planina”) and assigned to the Public Enterprise „Srbijašume“ to manage it (ANONYMOUS 2009).

The aim of this study was to analyse the collection of material sampled in the last ten years from streams situated on the southern slopes of the Stara Planina Mts. in order to assess the molecular diversity of trout in that area.

Materials and Methods

Collection of material sampled in the last ten years from streams situated on the southern slopes of the Stara Planina Mts. (Fig. 1) has been analysed in order to assess the molecular diversity in that

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area. Brown trout samples were taken either by fly fishing or electrofishing. Clips of pectoral fin were preserved in microtubes filled with 95% ethanol for subsequent analysis. Details of DNA extraction, amplification, purification, sequencing, alignment and identification of Control region (CR) haplotypes of the mitochondrial DNA (mtDNA) are according to Tošić et al. (2014, 2016). For 73 individuals out of 76 in total, the complete CR was sequenced. They were aligned using the Clustal W option of the MegaX software (Build: 10200331_x86_64 for iOS Version 10.1.8) (KUMAR et al. 2018, TAMURA et al 2020) and compared with the haplotypes from GenBank (<https://www.ncbi.nlm.nih.gov/>). Streams were compared for the occurrence of three presumably native DA haplotypes (Da1a, Da-s6 and Da22) using the sum of the binary distances between them for each haplotype. After the binary coding (presence-1, absence-0) and calculating the distance between streams, the addition of three distance matrices (one for Da1a, Da-s6 and Da22 each) and hierarchical clustering using the Complete Linkage method were accomplished in R version 3.4.3 (2017-11-30) – “Kite-Eating Tree” (R Core Team 2017) on the R.app GUI 1.70 (7463) x86_64-apple-darwin15.6.0 platform.

Results

In total, seven CR haplotypes belonging to three phylogeographic lineages *sensu* BERNATCHEZ (2001) were found (Table 1). In the Danubian (DA) haplogroup, two of three haplotypes in total are the native ones:

Da1a (GenBank accession number # AY185568) and Da-s6 (NEW SUBMISSION, #MW589188).

Brown trout that held the third haplotype of the DA haplogroup, Da22 (#AY185573), were found in the very high proportion in streams throughout the area (Fig. 2) but Da22 was never the only haplotype in any stream (Table 1). It was accompanied with the Da1a haplotype in all streams (e.g., rivers Dojkinačka, Rosomačka and Vodenička) draining to the River Visočica as well as in the River Jerma; how-

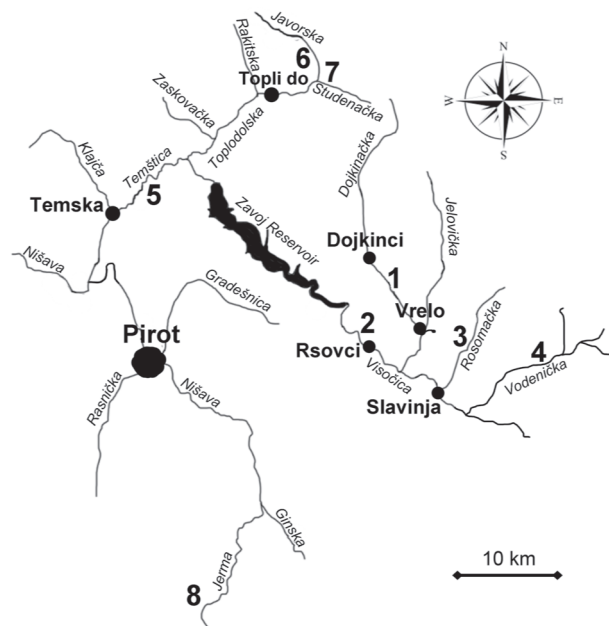


Fig. 1. Map of the southern slopes of the Stara Planina Mts., with the streams and approximate locations of samplings (1–8).

Table 1. Haplotypes of brown trout in streams at the southern slopes of the Stara Planina Mts. (number in the brackets after each stream’s name denotes the approximate position of the sampling spots given in the Fig. 1; #, accession number of haplotype in the GenBank n, number of haplotypes in brown trout in each stream; N, number of individuals in each stream’s sample; Σ , number of brown and Macedonian trout haplotypes in all streams and number of individuals holding each of haplotypes in all streams in the area of research).

		Haplotypes							N
		Da1a	Da-s6	Da22	At-cs1	At1f	Adcs1	AdAE1	
		# AY185568	MW589188	AY185573	AF321990	DQ841193	AY836330	GQ357908	
Streams	n								
Dojkinačka (1)	2	5		5					10
Visočica (2)	2			4	2				6
Rosomačka (3)	2	4		3					7
Vodenička (4)	2	2		16					18
Temštica (5)	1	1							1
Javorska (6)	2	12	6						18
Studenačka (7)	2	5	2						7
Jerma (8)	5	2		1		2	1	3	9
Σ	7	31	8	29	2	2	1	3	76

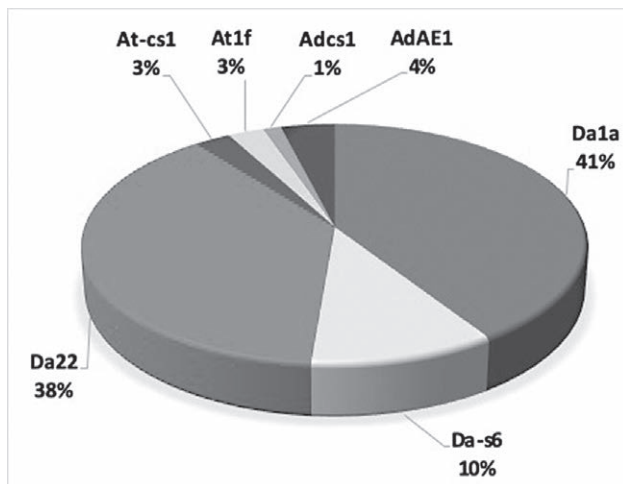


Fig. 2. Proportion of haplotypes of brown trout in the area of the Stara Planina Mts.

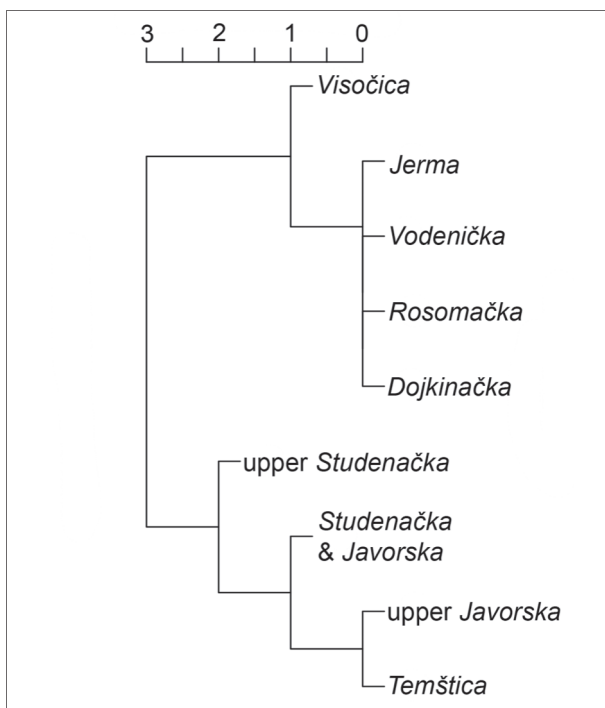


Fig. 3. Complete Linkage clustering of brown trout from streams on the southern slopes of the Stara Planina Mts. assessed using the binary distance between them derived from presence and absence of three indigenous haplotypes belonging to the DA haplogroup.

ever, it was the only haplotype of the DA haplogroup in the River Visočica itself. There are no permanent obstacles for brown trout in the drainage area of the River Visočica to disperse into tributaries.

Owing to small number of samples in particular streams as well as to introduction of haplotypes suspected as non-indigenous into them, only the preliminary analysis of similarity between streams



Fig. 4. Reservoir-dwelling, smoltified brown trout female, caught in the River Visočica on 12 June 2010 at the village Vrelo (bar size denotes 10 cm).

was accomplished by considering the occurrence of DA haplotypes in them. It revealed a clear distinction between brown trout in the drainages of the River Visočica and River Temštica, with the brown trout of the DA haplogroup in the River Jerma similar to those in streams of the River Visočica's drainage (Fig. 3).

The finding of brown trout of the two different AT haplotypes in the rivers Visočica and Jerma (Table 1) implies the independent stocking occasions when they were introduced. While in the River Jerma brown trout of the At1f (#DQ841193) haplotype were not distinguishable by bare eye from other brown trout, at least some of them of the Atcs1 haplotype (#AF321990) in the River Visočica were clearly different (Fig. 4).

Macedonian trout of the AD haplogroup were recorded exclusively in the River Jerma (Table 1), which strongly suggests their allochthonous character. There is no additional information, though finding two different AD haplotypes, Adcs1 (#AY836330) and AdAE1 (#GQ357908), suggests a possibility of two stocking events.

Discussion

The originality of the Da1a haplotype was inferred from its occurrence in brown trout throughout the area but the key clue was the occurrence of seven brown trout that held exclusively that haplotype in the upper section of the River Javorska. Da1a was the only haplotype in brown trout upstream of the high, impassable waterfall, situated about 2 km upstream of the joining with the River Studenačka. In the River Studenačka, brown trout that hold both Da1a and Da-s6 haplotypes were found. The main

source of brown trout of the Da-s6 haplotype hence is the River Studenačka in its upstream, headwater section isolated by several high waterfalls and void of brown trout of the Da1a haplotype.

MARIĆ et al. (2006) reported the occurrence of Da22 haplotype in brown trout from River Rosomačka, which might support the native character of that haplotype. DUFTNER et al. (2003) reported the Da22 haplotype for brown trout in the streams Longbach and Daglesbach in Austria, while Škraba JURLINA et al. (2017) recorded it for brown trout in the upper River Una in the Western Bosnia and continental Croatia. SIMONOVIĆ et al. (2017) stated that the recent vicariant pattern of an occurrence of the Da22 and Da-s6 haplotypes only at the few isolated spots might implicate to different periods and areas where they diversified and spread prior to subsequent reduction of their historical dispersal range. The herewith revealed dispersal and isolation of those two haplotypes in different streams of the Stara Planina Mts. declares this area as the easternmost and westernmost locations of brown trout of the Da22 and Da-s6 haplotypes, respectively. The phylogenetic relationships inferred for Da-s6 and Da22 haplotypes in MARIĆ et al. (2006) and especially in SIMONOVIĆ et al. (2017), assigning their more ancestral and very derived positions, respectively, also suggest their allochry of advancing and retreating in dispersal.

On the other hand, the occurrence of several trout hatcheries and farms in Western Bosnia (e.g., those in Martinbrod, Klokot and Kruščica) capable of producing brown trout from local brood fish could implicate the non-native character of Da22 haplotype for brown trout in streams of the Stara Planina Mts. The import of the stocking material from that area into Serbia after 2000 was not unlikely at all. There are no reliable records about stockings with brown trout before the year 2000 but many fishermen personally communicated stockings with the brown trout yolked larvae from the only active hatchery in the village of Braduljica near Ivanjica in Western Serbia, where the breeding material of brown trout originated from the local streams that homed wild fish of the Da1 haplotype (MARIĆ et al. 2006). It is not likely that brown trout stocked then into streams at the Stara Planina Mts. were of any other haplotype but Da1. Afterwards, following the Fishery Management Plans, the Public Enterprise „Srbijašume“ stocked the rivers Temštica and Visočica almost every year. Therefore, it is not currently possible to determine the character of the Da22 haplotype until the more detailed analyses on other molecular markers such as microsatellites

would be accomplished, which might help in resolving this matter.

In contrast to brown trout of the AT haplogroup indistinguishable from the other ones in the River Jerma, some of them were clearly different in the River Visočica. Fishermen recorded every year catches of large (over 40 cm in standard length) and reservoir-dwelling brown trout, predominantly females (Fig. 4), resembling strongly smoltified brown trout. They migrated from the Zavoj Reservoir upstream the River Visočica to the village of Vrelo, where some proportion of them managed to spawn and return to the reservoir. This implicates to the partial migratory behaviour inherent to brown trout of the AT haplogroup (FERGUSON et al. 2019), making those migrants highly resilient and adaptable to harsh conditions in the scarcity of resources in their environment. Škraba JURLINA et al. (2020) revealed the supremacy of migratory, lake-dwelling Adriatic trout *Salmo farioides* over the resident brown trout. However, they also reported the strong introgression of brown trout of the AT haplogroup into the resident native Adriatic trout of the AD haplogroup in the lower and upper stretches of the River Mrtvica in Montenegro, respectively, as well as the strong introgression of the resident brown trout of the AT haplogroup into stream-dwelling brown trout of the DA haplogroup and migrating of the reservoir-dwelling brown trout of the AT haplogroup into streams in the broader Iron Gate (Đerdap) Gorge area homing the native resident brown trout of the DA haplogroup. That confirmed the evaluation of SIMONOVIĆ et al. (2015) assessed using the FISK screening tool for brown trout of the AT haplogroup as of the high risk *sensu stricto* invasiveness. In the research that will follow, the degree of introgression of brown trout of the AT haplogroup into the native ones in streams of the Stara Planina Mts. will be worked out. However, this preliminary report already warns about the urgent need for the cease of stocking practice and undertaking of conservation and fishery management measures (SIMONOVIĆ et al. 2015, Škraba JURLINA et al. 2020) that would prevent spread of migrating brown trout further upstream in the River Visočica and admixing with the resident brown trout of the DA haplogroup from tributaries.

Macedonian trout of the AD haplogroup were recorded exclusively in the River Jerma (Table 1), which strongly suggests their allochthonous character. Only personal information (Aleksandar Panić, pers. comm.) is testifying that stocking materials from Bulgaria have been used. MARIĆ et al. (2006) reported the Adcs1 haplotype also in Macedonian trout from the River Dragovištica drainage area

as well as in the admixed brown trout from River Toplodolska, a headwater of the River Vrla in the River Danube drainage area, situated very close to the divide between the watersheds of the Black and Aegean Seas in the vicinity of the Vlasina Reservoir. That implies their domestication in hatcheries situated on trout streams in the River Struma drainage area of Bulgaria and stocking across the region. The current ecosystem status of the Macedonian trout in the River Jerma is that it naturalised and revealed the strong invasive character (SIMONOVIĆ et al. 2015) by spreading in the River Nišava downstream and entering into large tributaries such as the River Temštica (personal observations), with the occasional reports of catches even in the city of Niš, more than 100 km downstream. That spreading was facilitated by the tailwater effect of cold water that comes through the tunnel and pipes from the Zavoj Reservoir to the turbines of the hydropower plant in the city of Pirot, to be released into the River Nišava.

Conclusion

In total, brown and Macedonian trout in the streams from the south-western slopes of the Stara Planina Mts. feature seven haplotypes that belong to three haplogroups. Da1a and Da-s6 are undoubtedly the native ones, whereas the character of the Da22 haplotype is still uncertain. The rest of haplotypes comprising the two of the AD (Adcs1 and AdAE1) and AT (Atcs1 and At1f) haplogroups are most likely non-native, introduced by stocking. Brown trout holding them already pose a strong invasive effect.

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