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New Mitochondrial DNA Haplotype of Brown Trout *Salmo trutta* L. from Crni Timok Drainage Area in Serbia

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Abstract

Brown trout *Salmo trutta* wild stocks sustain a remarkable angling and hatchery-reared fish stocking pressure in waters of Serbia, where four drainage-specific and indigenous mitochondrial DNA haplotypes were reported for the drainage area of the Danube River basin. One of these mitochondrial DNA haplotypes, Da23b, was exclusive for brown trout in headwaters of the Crni Timok River (Grand Timok River system, Eastern Serbia). After its discovery in 2003, brown trout stocking was completely halted and abandoned, and a strict Catch-and-Release fishing regime was issued. However, failure of this regime due to enforcement resulted in decline of brown trout fishery. On checking the aboriginality of brood fish sampled in three headwater forks for artificial brown trout propagation, a novel, drainage-specific mtDNA haplotype was recorded in 54% of brown trout. It was assigned Da23c, due to its similarity (substitutions at the two variable sites) to the hitherto known haplotypes of Da23 group. Its finding and absence of Da23b haplotype imply an oversight in earlier haplotype determination. Incorporation of the non-indigenous haplotypes Da2 and Da-s1, introduced in the area by stocking before 2003, into the gene pool of native brown trout and declining of the latter's stock imposes the need for the more stringently enforced conservational activities (genotyping of each brood fish involved in artificial propagation) and improved fisheries management (Catch-and-Release) in the area.

Keywords: D-loop, Da23c, Eastern Serbia, conservation, fisheries management.

Introduction

Complicated and incompletely described evolutionary history and the great variability in life history and significance as a fishery resource contribute to the wide interest in investigating the brown trout *Salmo trutta* L. 1758 species complex. This is especially evident in regions such as the Balkan Peninsula, harboring the most diverse phenotypic variation among *Salmo* spp. populations (Kottelat and Freyhof, 2007; Simonovic *et al.*, 2007), where numerous, though still debatable *Salmo* taxa have been described so far. In addition, most of the populations examined were genetically highly divergent (possessing private genotypes), indicating that they may represent distinct and potentially locally adapted gene pools (Apostolidis *et al.*, 2011). Molecular markers, such as mtDNA D-loop and cytochrome *b*, microsatellites, RFLP (Restriction Fagments Length Polymorphism) and AFLP (Amplified Fragments Length Polymorphism), were recently used in a variety of investigations regarding brown trout. These include evolutionary and population-genetic studies (Zhang and Hewitt, 2003), phylogeography (Bernatchez, 2001; Cortey *et al.*, 2002, 2004), effects of stocking, conservation and management of populations and species (Apostolidis *et al.*, 2008; Vera *et al.*, 2010b; Kohout, 2012, 2013), identification of migrating strains (Habibi *et al.*, 2013) and resolving of the taxonomy-regarding confusion (Apostolidis *et al.*, 2011; Mrdak *et al.*, 2012). Mitochondrial DNA (mtDNA) has a number of characteristics that makes it a valuable molecular marker for evolutionary and population-genetic structure studies (Zhang and Hewitt, 2003). mtDNA is inherited maternally without intermolecular recombination and it has a higher mutation rate (Avise, 2000), which is one of the reasons for its use in the majority of phylogeographic studies (Bernatchez, 2001; Corty *et al.*, 2004; Marić *et al.*, 2006; Vera *et al.*, 2010a; Kohout *et al.*, 2013). Bernatchez *et al.* (1992) proposed that five main evolutionary lineages of brown trout exist: The Danubian, Atlantic, Adriatic, Mediterranean and the form *marmoratus*, which was subsequently widely adopted. Studies of brown trout from the Iberian

Peninsula (Weiss *et al.,* 2000, Suarez *et al.*, 2001) have revealed the existence of substantial polymorphism within the Atlantic clade, so much that a new evolutionary lineage (Duero) has been proposed (Suarez *et al.*, 2001; Cortey *et al.*, 2002). Several ancestral character states found in the most basal haplotypes within the Danubian clade suggested that the Danubian populations might be the oldest fragmented lineage of the recent brown trout complex. Some extremely divergent Danubian haplotypes, such as the Da*Vr and Da*Dž from southern Serbia that hold intermediate position within the entire haplotype network (Marić *et al.*, 2006), as well as Da26 from upper Tigris area of south-eastern Turkey (Sušnik *et al.,* 2005) and Da24 from the upper Danube drainage area in northern Austria (Duftner *et al.*, 2001), additionally suggest the long existence of the Da clade in the evolution of the brown trout complex.

In the region of Serbia and Montenegro, fifteen Control Region (CR, or D-loop) mitochondrial DNA haplotypes were recorded so far from the three brown trout phylogenetic lineages, (Danubian (Da), Adriatic (Ad) and Atlantic (At)), with high level of indigenous haplotype polymorphism (eight Da and six Ad haplotypes). Marić *et al.* (2006) revealed that the most common haplotype in this region was Da-s1, whereas other Da haplotypes were both less frequent and limited in distribution as specific for a particular drainage. One of those less common and drainage-specific haplotypes, Da23b, originally reported by Duftner *et al.* (2003) for the Lohnbach River in Lower Austria, was also reported exclusively for the Radovanska stream, one of the headwaters of the Crni Timok River (in the Grand Timok River system, the easternmost tributary of the Danube River in Serbia), as well as for Vratna and Rečka streams out of that drainage area, but in its close vicinity. Da23b haplotype considered indigenous was accompanied there with the Da2 and Da-s1 haplotypes, introduced most likely by stocking until 2003. Immediately after the discovery of haplotype Da23b, the Fishery Management Plan (Simonović *et al.*, 2003) was updated by issuing a comparably strict Catch-and-Release brown trout fishing regime. However, despite the efforts, fishery management failed to keep the brown trout stock from declining, which lead to the drop in biomass and annual natural production, as revealed in the subsequent Fishery Management Plan (Simonović *et al.*, 2011). In order to determine the causes and the extent of decline in brown trout fishery, and protect indigenous brown trout populations in Eastern Serbia, conservational management strategies were proposed to the regulatory agencies. One of the most important components of these strategies, the artificial propagation and stocking of brown trout in depleted water bodies, was initiated in 2012 and included genetic analysis of existing brood fish. Consistent with the proposed management plan, this study aims to genetically analyze brown trout brood fish in headwaters of the Crni Timok River drainage area, utilizing mitochondrial DNA D-loop region to establish a genetic baseline data specific to brown trout in the area. Fishery management and conservation management implications of novel markers were also discussed.

Materials and Methods

Brown trout anal fin clip samples were collected from three locations across Crni Timok drainage basin area: seventeen from the Radovanska Stream (N 43°53’48.6"; E 21°47’04.0"), eight from the Mirovštica stream (N 43°48´37.1˝; E 21°53´32.5˝) and one from the Lukovo spring (N 43°48´50.9˝; E 21°51´39.4˝) by electrofishing in 2012 (Figure 1). Total DNA was extracted from the tissue samples of the size of approximately 4 mm^2 preserved in 96% ethanol, using the High Salt Extraction technique (Miller *et al.*, 1988), without quantification. Amplification of the CR was carried out using primers 28RIBa (5’CACCCTTAACTCCCAAAGCTAAG-3’) (Snoj *et al.*, 2000) and HN20 (5’-GTGTTATGCTTTAGTTAAGC-3’), (Bernatchez and Danzmann, 1993) under the following conditions: initial denaturation (95°C, 5 min) followed by 30 cycles of strand denaturation (94°C, 45 s), primer annealing (52°C, 45 s) and DNA extension (72°C, 2 min; the last extension prolonged to 5 min) in the programmable MultiGene® Thermo Cycler TC9600-G-230V™ (Labnet International, Inc. ®). Each PCR reaction in volume of 30 µl contained 10 µM of each primer (ThermoScientific®), 10 mM dNTP, 10X PCR buffer with $MgCl_2$ (Kapa Biosystems®), 1U of *Taq* polymerase (Kapa Biosystems®) and 100 ng (i.e., 1 µl) of genomic DNA. Amplified DNA fragments were run on a 1.5% agarose gel using Applichem® SYBR Green™ for visualization. Samples with PCR products were sequenced and purified at MACROGEN® Europe using modified Sanger sequencing method. 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. Single-pass sequencing was performed on each template using forward (28RIBa) primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with the BigDye XTerminator® Purification Kit (Applied Biosystems). The samples were injected to electrophoresis in an ABI 3730xl DNA Analyzer (Applied Biosystems). Sequences were aligned using program ClustalX2 (Larkin *et al.,* 2007) with particular CR sequences of Da haplotypes occurring in streams of Serbia after Marić *et al.* (2006) (e.g., Da2, Da23b) and those similar to them (e.g., Da23a, Da22, Da24) obtained from the GenBank (Accession Numbers AY185570.1,

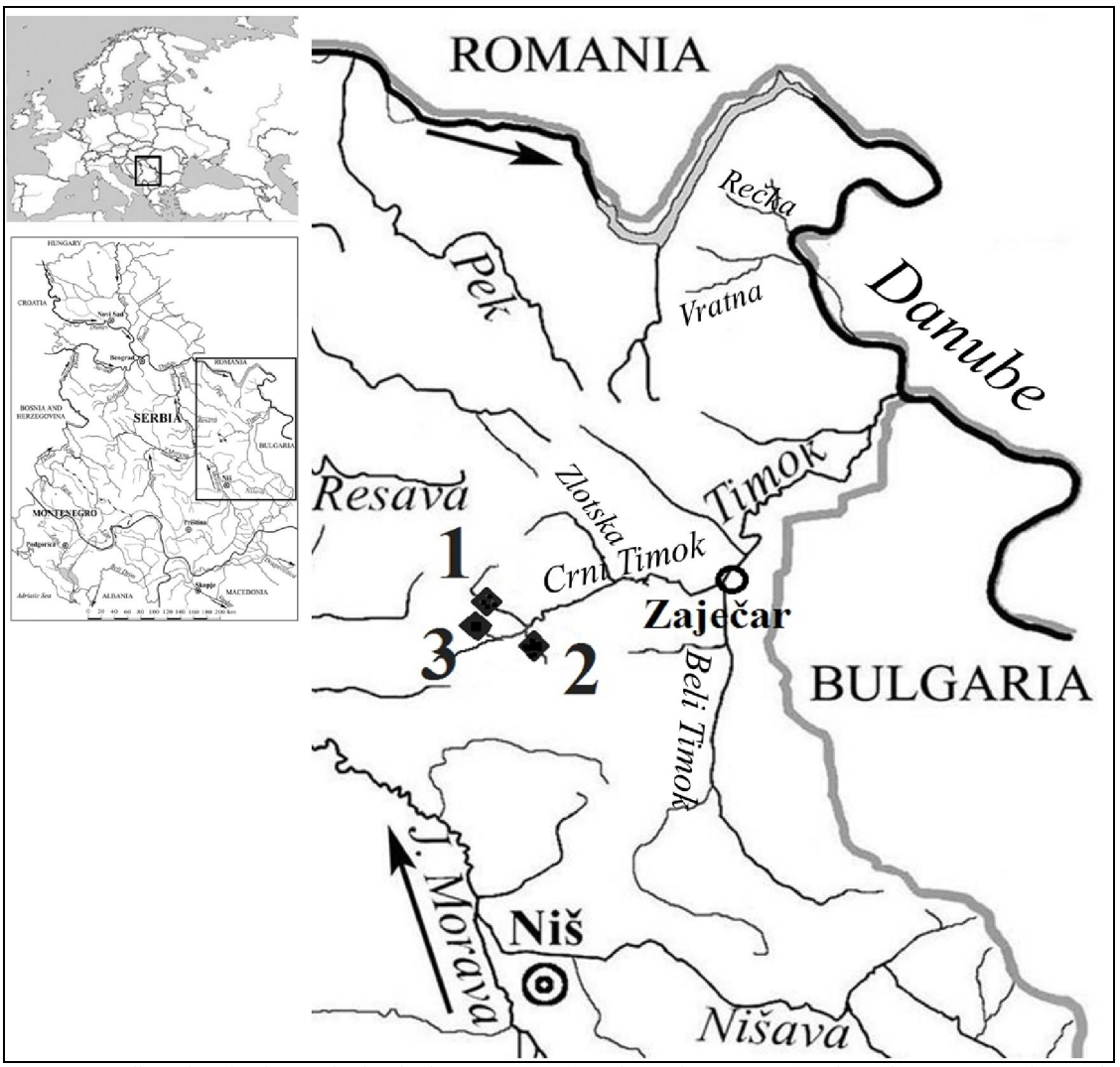

Figure 1. Sampling sites in the Crni Timok river system: 1) Radovanska stream 2) Mirovštica stream and 3) Lukovo spring.

AY185574, AY185575, AY185573.1, AY185576.1). In addition, the CR sequence of the MAcs1 haplotype (Accession Number AY836365) was used as an alignment aid, due to a deletion occurring at the position 113, as well as an outgroup OTU in rooting of the 50% Majority Rule consensus tree that was constructed using PAUP 4.0b10 (Swofford, 2001). Relationship with other haplotypes (applying NJ method and Kimura 2-parameter model) was assessed using MEGA 3.1 (Kumar *et al.,* 2004).

Results

Based on sequence alignments of the CR sequences (Table 1) from the Radovanska and Mirovštica stream, and Lukovo spring, two different haplotypes were recorded. One of the haplotypes, Da2, was previously recorded at these locations. The other CR sequence (GenBank Accession Number KC630984) is a novel haplotype, hitherto unknown for brown trout from the Radovanska stream, or from elsewhere. It is similar to the Da23 haplotype group (a and b) (with the distances 0.5% and 0.4%, respectively), since it holds a nucleotide A at the position 146 (all other Da haplotypes hold a G at this position). However, the new haplotype differs from both Da23 haplotypes by two substitutions at positions 234 (a G, instead of an A) and 235 (an A, instead of a G). For another variable position, 545, the new haplotype shares a T nucleotide with haplotype Da23b, in contrast to a G nucleotide present at the same location in in Da23a. Based on sequence similarity between the novel haplotype with Da23 haplotype group, we designated the new haplotype as Da23c. In regard to Da2, Da22 and Da24 haplotypes, Da23c differs from them by two, two and six nucleotide substitutions, with the distances 0.4%, 0.5% and 1.1%, respectively. The new haplotype holds the greatest difference (1.6%) from the haplotype MAcs1, as revealed by both 50% Majority Rule Consensus Tree and Bootstrap analyses (Figure 2).

In regard to particular streams, eight out of seventeen brown trout from the Radovanska stream,

Table 1. Variable base positions in brown trout *Salmo trutta* mtDNA CR haplotype sequences based on 562 base-pair sequence

Haplotype	Position in the CR sequence														
	2	26	113	146	178	234	235	236	262	403	530	542	543	545	548
Da2	C	A	/	G	T	G	G	G	G	T	C	A	C	T	T
Da22	-	-	/	-	-	-	T	-	-	-	-	-	-	-	-
Da23a	-	-	/	A	-	A	-	-	-	-	-	-	-	G	-
Da23b	-	-	/	A	-	A	-	-	-	-	-	-	-	-	-
Da23c*	-	-	/	A	-	-	A	-	-	-	-	-	-	-	-
Da24	-	-	/	-	C	-	A	-	-	-	T	G	G	-	C
Macs1	T	C	A	-	-	-	A	T	A	C	-	G	G	-	C

* Denotes the new haplotype

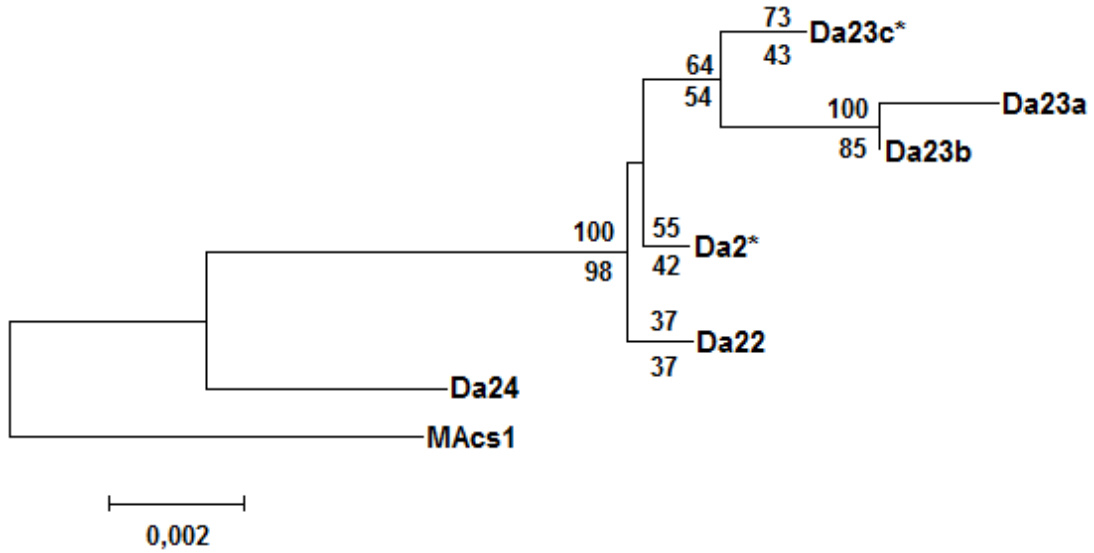

Figure 2. Relationship between the new Da23c haplotype and other CR mtDNA haplotypes of brown trout, as revealed by 50% Majority Rule Consensus tree derived from the 10 most parsimonious trees of equal length and Bootstrap analysis. Haplotypes Da2 and Da23c from the Crni Timok River drainage area reported here are marked with an asterisk (upper number denotes the Consensus Tree probability, lower number the Bootstrap probability for each clade).

five out of eight brown trout from the Mirovštica stream and one out of one brown trout from the Lukovo spring were of Da23c haplotype.

Discussion

Finding of the new mtDNA haplotype Da23c that unequivocally belongs to indigenous brown trout populations implies that the status of Da23b haplotype in brown trout populations from Radovanska, Vratna and Rečka streams Marić *et al.* (2006) reported needs to be re-examined, in order to ascertain the true status of specified brown trout stock with regard to this marker. It is certain that fish stockings which were accomplished before 2003 introduced the hatchery reared brown trout with Da2 haplotype into the Crni Timok River drainage area, which was halted immediately after the discovery of the drainage-specific brown trout haplotype was reported as Da23b. Considering both localities of sampling in the headwaters of the Crni Timok River and number of samples, it is unlikely that none of 26 brown trout under investigation would be carrying haplotype Da23b. Therefore, annotation of haplotype Da23b of Marić *et al.* (2006) as an indigenous haplotype for brown trout could be a simple oversight perhaps due to erroneous determination of the Control Region sequence, and requires full investigation.

There are very important conservational and fishery management implications of the discovery of a new, drainage-exclusive mtDNA haplotype. Since 2003, when the stocking activities with the hachery-reared brown trout ceased, the fishery managers failed to implement an efficient enforcement of the Catch-and-Release fly fishing there, which lead to the permanent decline of the standing stock from 27,261 kg km^{-1} to 9,270 kg km^{-1} and to the decline of the annual natural production from 12,000 kg km^{-1} to 2,053 kg km^{-1} in 2003 and 2008, respectively (Simonović *et al.,* 2003; 2011). Hence, it was necessary to perpetuate the artificial spawning of indigenous brown trout with the drainage-specific haplotype for the stocking. That spawning activities will provide an even better insight into the status of brown trout, especially when these fish are used as brood fish from the area. It appears that there are no impassable obstacles for brown trout to migrate from the upper section of the Crni Timok River upstream

into tributaries (e.g. Mirovo, Lukovo, Radovanska and Zlot streams). Marić *et al.* (2006) reported the non-indigenous Da-s1 haplotype together with the tentatively indigenous Da23b for the Rečka and Vratna streams in the vicinity of the Crni Timok River drainage area. Therefore, it appears that currently there is no pure, indigenous brown trout stock in the area. Finding of the new haplotype Da23c in this study imposes a much more stringent genetic screening of brown trout populations in entire Crni Timok River drainage area and Vratna and Rečka streams for sufficient data gathering needed for sound decision-making with respect to conservational and management activities in order to maintain the autochthonous genetic diversity of brown trout in this area.

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