

Molecular and ecological features of the soft-muzzled trout *Salmo obtusirostris* (Heckel, 1852) in the Zeta River, Montenegro

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Abstract: This paper reports about the occurrence of soft-muzzled trout in Montenegro. It was found only in the Zeta River, at three localities, always in low abundance. Results of genetic investigation on twelve microsatellite loci (i.e., high F_{st} and high heterozygosity values) as well as lack of hybridization with the putative brown trout unequivocally confirm that the soft-muzzled trout from the Zeta River is a species distinct from the putative brown (*Salmo* cf. *farionides*) and marble (*Salmo marmoratus*) trout that live in sympatry with it. This paper also confirms high genetic diversity of Zeta River soft – muzzled trout population. Habitat and feeding analyses revealed that soft-muzzled trout reduce the competition with syntopic putative brown trout by displacing as the bottom-dweller in their common type of habitat, as well as by narrowing its feeding niche and feeding dominantly with the benthic macroinvertebrates, the *Gammarus* shrimps as their far dominant feeding item. Results also revealed that they did not share the same habitat with marble trout. Due to this population low abundance and their importance on species level, Zeta River soft-muzzled trout conservation needs are urgent in terms of *in situ* protection and repopulation measures.

Key words: *Salmo obtusirostris*; Zeta River; microsatellites; habitat preferences; food analysis

Introduction

Soft-muzzled trout *Salmo obtusirostris* (Heckel, 1852) is an endemic salmonid fish inhabiting the eastern coast of the Adriatic Sea basin in the area between the rivers Krka in the north and Morača in the south. Four different populations, considered as separate subspecies, are known: *Salmo obtusirostris krkensis* (Karaman, 1926) in the Krka River (Croatia), *Salmo obtusirostris oxyrhynchus* (Steindachner, 1882) in the Neretva River (Bosnia and Hercegovina), *Salmo obtusirostris saloni-tana* (Karaman, 1926) in the Jadro River and *Salmo obtusirostris zetensis* (Hadžišće, 1962) in the Zeta River (Montenegro).

Generic status of this species was several times changed. It was described as *Salar obtusirostris* (Heckel, 1852). Later Berg (1908) proposed a new genus, *Salmothymus* for this taxon. Hadžišće (1962) stated that *Salmothymus* was a valid generic name and that two species should be included in this genus – *S. obtusirostris* and *S. ohridanus*. Recently, Snoj et. al. (2002) revised this genus using DNA data and proposed that both species should be included into the genus *Salmo*.

Presence of *Salmo obtusirostris* in Montenegro was firstly discovered in the River Zeta by Karaman (1932) and he assigned it as *Salmo obtusirostris let-nica*. Hadžišće (1962) revised the taxonomic status and assigned the soft-muzzled trout from the River Zeta as *Salmothymus zetensis*.

In the Montenegrin part of the Adriatic Sea drainage area, *S. obtusirostris* inhabits the Zeta River while it was reported that occasionally enters into the Morača River and Skadar Lake, as well (Ivanović 1973). As soft-muzzled trout population number dramatically decreased in last two decades, this fish recently can be found only in the narrow limited area in the upper section of the lower Zeta River. Since 1960, when Hadžišće assigned the species status for this fish in the Zeta River, Ivanović (1973) reported about morphological and meristic data based on two samples of this fish and its living area. In following years, apart of the mentioning in the lists of fish species inhabiting the Skadar Lake drainage area (Knežević 1985; Drecun et al. 1985; Šorić 1990; Marić 1995; Crivelli, 1996; Ražnatović & Dhora, 2001), there was no research on this species in Montenegro so far.

According to IUCN Red List, the soft muzzled trout *S. obtusirostris* is considered Endangered B2ab(v) throughout its dispersal area (IUCN 2010), while in the national legislation of Montenegro this is the only protected fish species (Anonymous 1982). During 1990's, their population sizes dramatically decreased in the whole dispersal area and in few reports on monitoring of protected animal species in Montenegro it was reported that this species is the most probably extinct there (National Agency for Nature Protection of Montenegro, 2003, 2004 and 2005). However, following the rumors that there is still soft-muzzled trout in the Zeta River, we managed to find one population at the locality

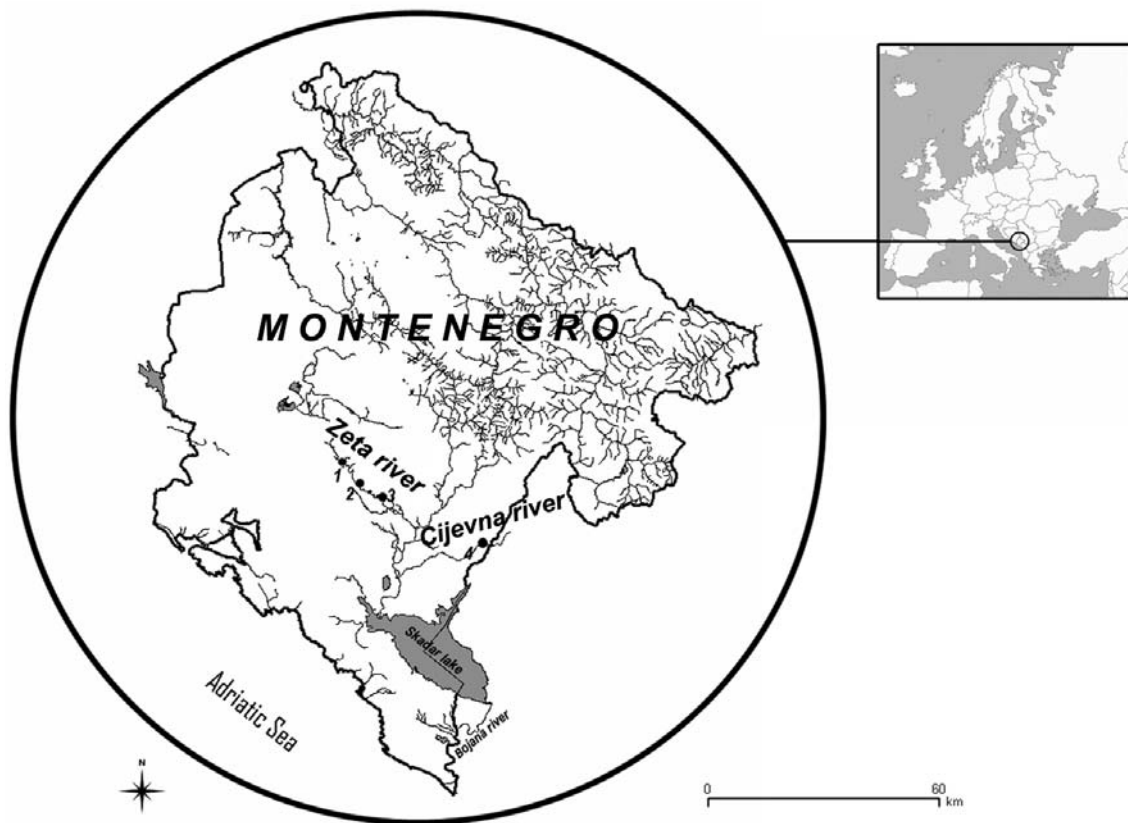


Fig. 1. Sampling localities: 1 – locality Tunjevo, 2 – locality Slap Zete, 3 – locality Slapci, 4 – locality Albanian border.

Table 1. Number of samples for each of three nominal trout species and both analyses, sampled from rivers in the Adriatic Sea drainage area of Montenegro.

Species	DNA Analysis				Food item analysis
	Locality 1 (Tunjevo)	Locality 2 (Slap Zete)	Locality 3 (Slapci)	Locality 4 (Albanian border)	Locality 2 (Slap Zete)
<i>Salmo cf. farioides</i>	28			13	21
<i>Salmo marmoratus</i>	7			9	
<i>Salmo obtusirostris</i>	4	4	6		12

of the village of Tunjevo (N 42°37'912"; E 019°01'016") that was still the only one known and with the ultimately small number of specimens (Sušnik et al. 2007).

Considering the low population number and ultimately limited dispersal area of the Zeta River soft-muzzled trout, it is very urgent to define its recent population status. By investigating nuclear DNA (12 microsatellites loci) of this fish and two other sympatric trout species, marble (*Salmo marmoratus* Cuvier, 1829) and putative brown (*Salmo farioides* Karaman, 1938) trouts, our intention was to research the genetic differentiation among them. In order to get the more thorough insight into this species biology, we analyzed Zeta River *S. obtusirostris* habitat and food preferential from newly discovered locality in comparison with sympatric and syntopic putative brown trout. Additional goal of this study was also to define Zeta River soft-muzzled trout conservation status.

Material and methods

Genetic analysis

Samples of soft-muzzled, putative brown and marble trout for DNA analysis were caught by angling and electrofishing in 2004–2008 period at sampling locations at the lower Zeta and Cijevna Rivers (Fig. 1): three sampling locations on Zeta River (Tunjevo, Slap Zete and Slapci) and one sampling location on Cijevna river (near the border with Albania). In total, samples were taken from 41 putative brown trout, 16 marble trout and 14 soft-muzzled trout (Table 1) of which 11 soft-muzzled, 7 marble and 18 brown trout are published here for the first time, whereas other data were already used in Sušnik et al. (2007). On each locality all species we sampled were in sympatry, while on localities Slap Zete and Slapci putative brown and soft-muzzled trout were in syntopy, too. A small piece of anal fin was taken from each fish, placed in separate 1.5 ml tube and preserved in 96% ethanol while fish was released. Total DNA was isolated from fin tissue preserved in ethanol, using a high-salt extraction technique (Miller et al. 1988).

Twelve microsatellite loci were chosen and 71 samples (41 samples of *S. trutta*, 14 samples of *S. obtusirostris* and 16 samples of *S. marmoratus*) were analyzed. PCR were optimized for amplification in two multiplex polymerase chain reactions (PCRs; 8- and 4-plex, Lerceteau-Köhler & Weiss 2006). Primers were fluorescently labelled and PCR was performed in 10 µl reaction mixtures containing from 0.0375 to 1 µM of each primer pair, 0.2 mM dNTP, 1.5 mM MgCl₂, 1X PCR buffer, 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems) and 50 ng of genomic DNA. PCR conditions were as follows: initial DNA denaturation at 94°C (3 min), and 35 successive cycles of strand denaturation at 94°C (45 s), primer annealing at 57°C (1.5 min), and DNA extension at 65°C (1 min). Aliquots of fluorescently labelled amplified DNA were mixed with formamide and GENESCAN-500 ROX Size Standard (Applied Biosystems) and genotyped on an ABI-3100 automated capillary sequencer using GeneScan™ Analysis Software 3.7. Microsatellite allele frequencies, the number of alleles per locus (*A*), observed and expected heterozygosity (*H_o* and *H_e*, respectively) were performed using the program GENETIX 4.04 (Belkhir et al. 1996). Allelic richness and pairwise *F_{st}* and *F_{is}* values were calculated in FSTAT 2.9.3.2 (Goudet 2001). Test on Hardy–Weinberg equilibrium was performed in ARLEQUIN 3.5.1.2 (Excoffier & Lischer 2010) software package. In order to estimate genetic relationships among individuals of the putative brown, marble and soft-muzzled trout, a matrix of *D_{AS}* distances (Bowcock et al. 1994) was used to construct a tree based on a Neighbor-Joining (NJ) algorithm using the program POPULATIONS 1.2.30. (Langella 2002). Statistical support estimates for major nodes in the tree were obtained with 1000 bootstrap replicates across both loci and individuals.

With purpose to define relationships among soft-muzzled and other two nominal trout species (putative brown and marble trout) in Montenegro, we used already published data of Sušnik et al. (2007) together with new data of putative brown trout from the Zeta River, putative brown trout from the Cijevna River, as well as data for marble and soft-muzzle trout from Zeta and Cijevna rivers, respectively (*n* = 71). For an assessment of their interspecific and intraspecific genetic distances, we organized groups of individuals according to their taxonomy, as well as by dividing putative brown trout individuals in two groups (Zeta and Cijevna rivers), in order to detect intraspecific distances between them.

Habitat and food analyses

Soft-muzzled trout were first detected at the localities of Slap Zete and Slapci by snorkeling and SCUBA diving during spring/summer period of 2008, 2009 and 2010. The precise positions of localities (GPS coordinates) were determined with the Garmin Oregon 3000 device. For habitat description, the Visual Census technique was used, while aquatic plants were collected and identified later in laboratory using Tutin et al. (1968, 1993).

Locality of Slap Zete was chosen for sampling because of the syntopy of putative brown and soft-muzzled trout occurring there. For the stomach contents analysis, fish were caught by electrofishing gear during the first half of July 2010 (12 soft-muzzled and 21 brown trout) and anesthetized using the MS-222 (Sandoz) in the aerated plastic pool of about 200 L in volume. For each fish, the total length (TL) and total weight (TW) were measured. Stomach content was washed out from each fish by pumping water directly into the stomach through slug. Each fish's stomach content was preserved with formaldehyde in 100 ml bottles.

Identification and quantification of food items was accomplished using the stereo binocular magnifier ZEISS Stemi 2000 C. Systematical identification of food items was performed at order, or higher systematic levels (e.g., Planaria, Gastropoda). Ephemeroptera, Trichoptera and Plecoptera prey groups were divided in adults marked with A and nymphs marked with N. Due to extremely low abundance of soft muzzled trout, it was impossible to accomplish more detail survey with more samples of this fish.

Niche overlapping level among sympatric soft-muzzled and putative brown trout was estimated by Pianka (1973) modification of MacArthur & Lewin (1967) methodology using the formula:

$$O_{jk} = \frac{\sum_i^n p_{ij}p_{ik}}{\sqrt{\sum_i^n p_{ij}^2 \sum_i^n p_{ik}^2}};$$

where *O_{jk}* is niche overlapping among species *j* and *k*, *p_{ij}* is proportion of resource *i* in all resources used by species *j*, *p_{ik}* is proportion of resource *i* in all resources used by species *k* and *n* is number of resources used by species *j* or *k*. Percentage of feeding niche overlapping was estimated using the following formula:

$$P_{jk} = \left[\sum_{i=1}^n (\min p_{ij}, p_{ik}) \right] 100;$$

where *P_{jk}* is percentage overlapping, *p_{ij}* is proportion of resource *i* in all resources used by species *j*, *p_{ik}* is proportion of resource *i* in all resources used by species *k* and *n* is number of resources used by species *j* and *k*.

Results

Genetics

All analyzed loci were polymorphic in analyzed populations. Loci Ssa410, Ssa408 and Ssa-71 appeared the most variable in our data set (Table 2). Overall expected (*H_e*) and observed (*H_o*) heterozygosity per population for all loci varied from 0.4585 to 0.6664 and from 0.4153 to 0.6234, respectively while the highest values for both expected and observed heterozygosity were detected for soft-muzzled trout population (Table 2). Mean allelic diversity per population was similar in all trout samples and varied between 4.58 in putative brown trout to 6.58 in marble trout. For significance threshold level of *P* < 0.05, *F_{st}* values among populations were significant, except among putative brown trout populations from Zeta and Cijevna Rivers (Table 3) where it was *P* = 0.061 suggesting only moderate genetic differentiation (Wright 1978). For soft-muzzled trout we detected highest segregation from other three populations with highest *F_{st}* values. Marble trout also appeared different from other analyzed species with significantly (*P* < 0.05) high *F_{st}*. *F_{is}* index (range −1.00 to + 1.00) was from −0.053 to 0.385, respectively (Table 4). In Hardy–Weinberg equilibrium test (HWE), all

Table 2. Sample size (N), number of alleles per population (A_n), expected heterozygosity (H_e) values, observed heterozygosity (H_o) values, number of detected alleles per locus (A_{nl}) and mean allelic diversity per population ($\bar{A}_n$).

Locus	<i>S. cf. farioides</i> Lower Zeta River	<i>S. marmoratus</i> Lower Zeta and Cijevna rivers	<i>S. cf. farioides</i> Cijevna River	<i>S. obtusirostris</i> Lower Zeta River
Str60				
(N)	28	16	13	14
A_n	1	2	1	3
H_e	0	0.1172	0	0.6046
H_o	0	0.1210	0	0.6270
A_{nl}	5			
Ssosl438				
(N)	28	16	13	14
A_n	1	4	2	3
H_e	0	0.6191	0.2604	0.4005
H_o	0	0.2500	0.3077	0.2143
A_{nl}	7			
Ssa85				
(N)	28	16	13	14
A_n	2	2	2	2
H_e	0.4928	0.4922	0.3550	0.4770
H_o	0.4643	0.5000	0.3077	0.6429
A_{nl}	5			
SSsp2216				
(N)	28	16	13	14
A_n	3	5	3	5
H_e	0.1346	0.6113	0.1450	0.7781
H_o	0.1428	0.4375	0.1538	0.7857
A_{nl}	12			
Str73				
(N)	28	16	13	14
A_n	2	4	2	2
H_e	0.4904	0.7012	0.4882	0.4362
H_o	0.5714	0.3750	0.5385	0.3571
A_{nl}	5			
Ssosl417				
(N)	28	16	13	14
A_n	1	2	1	NA
H_e	0	0.1975	0	NA
H_o	0	0.1325	0	NA
A_{nl}	2			
Ssa410				
(N)	28	14	13	14
A_n	10	14	10	8
H_e	0.8316	0.8827	0.8785	0.8163
H_o	0.8571	0.5714	0.8333	0.7143
A_{nl}	25			
Ssa408				
(N)	28	15	13	14
A_n	6	13	8	10
H_e	0.6199	0.8022	0.8432	0.8469
H_o	0.6071	0.6000	0.9231	0.7857
A_{nl}	24			
Ssa-D190				
(N)	26	16	13	14
A_n	5	5	5	3
H_e	0.7078	0.7559	0.7130	0.6122
H_o	0.6923	0.6875	0.6154	0.5714

Table 2. (continued)

Locus	<i>S. cf. farioides</i> Lower Zeta River	<i>S. marmoratus</i> Lower Zeta and Cijevna rivers	<i>S. cf. farioides</i> Cijevna River	<i>S. obtusirostris</i> Lower Zeta River
A_{nl}	9			
SSsp2213 (N)	27	16	12	14
A_n	5	7	7	5
H_e	0.7071	0.7324	0.7778	0.7474
H_o	0.8889	0.4375	0.6667	0.6429
A_{nl}	15			
Ssa-D71 (N)	25	16	13	14
A_n	15	11	9	7
H_e	0.8896	0.7871	0.7959	0.7857
H_o	0.8800	0.5625	0.5385	0.7827
A_{nl}	34			
OMM1064 (N)	27	16	13	14
A_n	3	10	5	8
H_e	0.6372	0.8340	0.7308	0.8265
H_o	0.7778	0.5625	0.8462	0.7857
A_{nl}	19			
OVERAL				
$\bar{A}_n$	4.58	6.58	4.58	5.09
H_e	0.4585	0.6277	0.4990	0.6665
H_o	0.4902	0.4153	0.4776	0.6234

Table 3. F_{st} values (* significant, $P < 0.05$) based on 12 microsatellites loci.

	<i>S. cf. farioides</i> Lower Zeta River	<i>S. obtusirostris</i> Lower Zeta River	<i>S. marmoratus</i> Lower Zeta and Cijevna rivers	<i>S. cf. farioides</i> Cijevna River
<i>S. cf. farioides</i> lower Zeta River	0.000	0.404*	0.254*	0.061
<i>S. obtusirostris</i> lower Zeta River		0.000	0.305*	0.363*
<i>S. marmoratus</i> lower Zeta and Cijevna rivers			0.000	0.183*
<i>S. cf. farioides</i> Cijevna River				0.000

loci showed departure in some of the tested populations (Appendix I, Tables 1–4). According to P -values (probability that researched differences in H_e and H_o are matter of chance), in putative brown trout from Zeta and Cijevna rivers 7 loci and their alleles were in HWE (or statistically in HWE), respectively. For marble trout population, only one locus was in HWE. For soft-muzzled trout we found 4 of 12 analyzed loci either in, or close to HWE.

Neighbour-Joining Tree of individuals showed strong soft-muzzled genotype grouping among all samples and no hybrids among soft-muzzled and other trout genotypes (Fig. 2). Marble trout, especially ones from the Zeta River, also showed segregations that corresponded to their specific genotype. In the Cijevna River, some individuals of marble trout appeared close to the putative brown trout group, suggesting hybridization. Putative brown trout individuals from both Zeta and Cijevna Rivers clustered together as one group (Fig. 2).

Table 4. F_{is} values per population and per each locus (pop1 – *Salmo cf. farioides* from Zeta River; pop2 – *Salmo obtusirostris*; pop3 – *Salmo marmoratus*; pop4 – *Salmo cf. farioides* from Cijevna River).

Locus	pop1	pop2	pop3	pop4
Str60	NA	–0.005	NA	NA
Ssos14	NA	0.494	0.617	–0.143
Ssa85	0.059	–0.315	0.016	0.172
SSsp22	–0.043	0.027	0.314	–0.021
Str73	–0.180	0.217	0.490	–0.063
Ssos14	NA	NA	1.000	NA
Ssa410	–0.013	0.161	0.385	0.074
Ssa408	0.039	0.109	0.284	–0.055
Ssa-D1	0.042	0.103	0.122	0.176
SSsp22	–0.239	0.176	0.429	0.136
Ssa-D7	0.031	0.037	0.315	0.359
OMM106	–0.203	0.086	0.354	–0.119
All loci F_{is}	–0.053	0.094	0.358	0.074

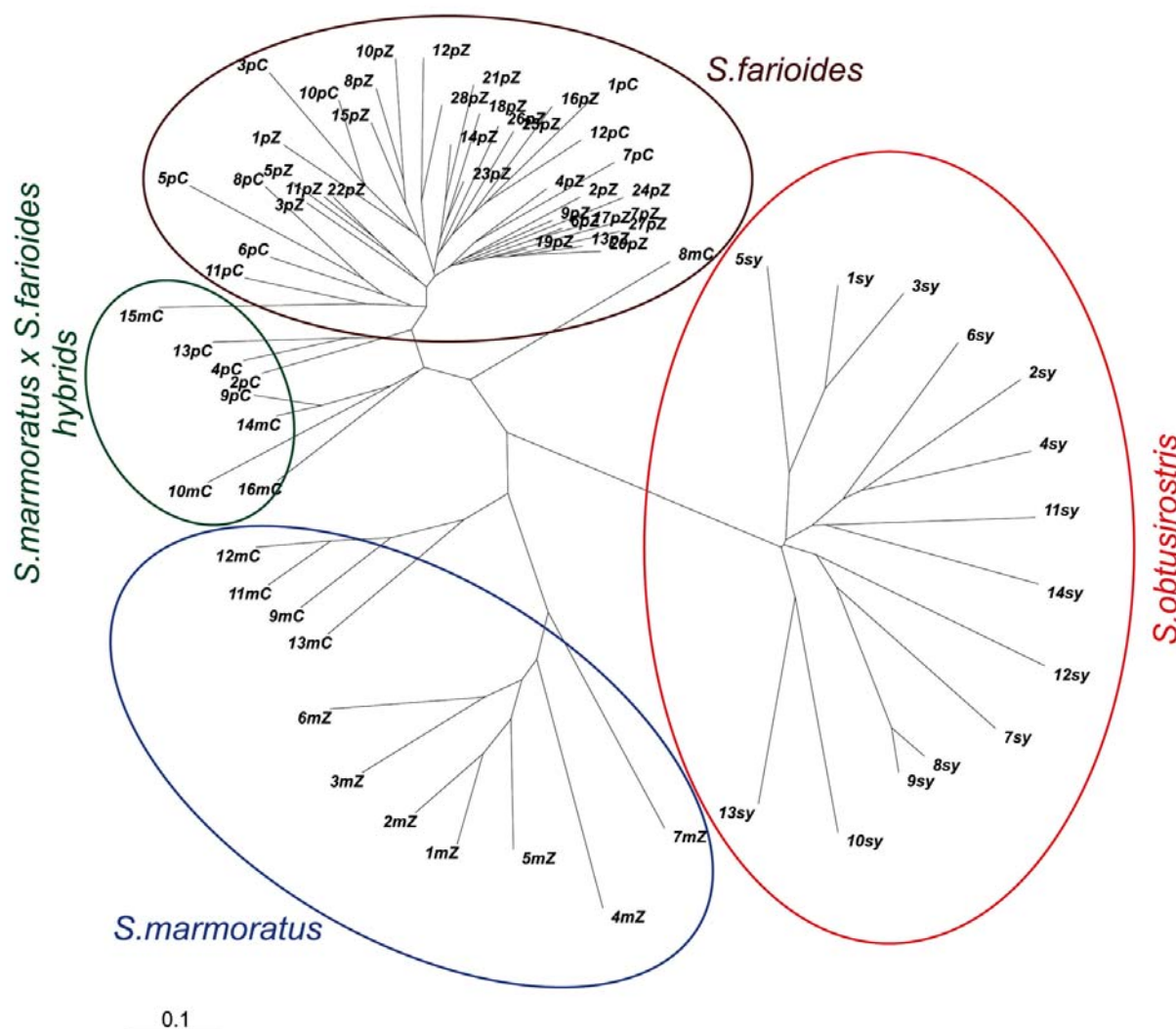


Fig. 2. Neighbour-Joining tree of individuals for the entire data set constructed using D_{AS} distances between them assessed on analysis of 12 microsatellite loci (abbreviations: sy – *S. obtusirostris*, mC – *S. marmoratus* from Cijevna River, mZ – *S. marmoratus* from Zeta River, pC – *S. cf. farioides* from Cijevna River, pZ – *S. cf. farioides* from Zeta River).

Habitat and feeding

In addition to the locality Tunjevo (N 42°37'912"; E 19°01'016") where soft-muzzled trout were already found in summer 2004, two more locations on the Zeta River with this fish were discovered: Slap Zete below the dam (N 42°35'990"; E 19°03'907") and Slapci (N 42°34'572"; E 19°04'902"). Soft-muzzled trout were gregarious (2–7 individuals in each school) at all three localities and positioned in the central, vegetation-free, deepest parts of large pools (15–50 m wide and 3–7 m deep) on the sandy-stony bottom type. The shallow, inshore parts of pools were densely covered with submerged macrophyte vegetation that surrounds pools in circle-shaped zone, where those fish hid when they were disturbed. Dominant species of plant community in all three habitats were *Ranunculus trichophyllus* (Chaix) and *Berula erecta* (Huds.) Coville. The other accompanied plant species were not significantly important in term of this habitat description. Vegetation goes up to 2 m in depth and occupies in average 36.5% of pools. All three localities were under strong impact of karst wells and springs rich in water occurring there. The

syntopic putative brown trout individuals were either near the surface (15–50 cm depth) in faster current of same pools, or near stones at the bottom in shallow parts, while marble trout were not seen in this type of habitat. Juveniles of marble trout (size 20–35 cm) were detected in parts with the faster (comparing to those in pools) water current and less in depth (up to 2 m) toward river bank beneath the rock or fallen timber. Any adults of marble trout were not noticed, at all. In the same habitat, in addition to soft-muzzled and putative brown trout, we detect eel (*Anguilla anguilla* L., 1758), chub (*Squalius* sp.), minnow (*Phoxinus* sp.), nase (*Chondrostoma* sp.) and bleak (*Alburnus* sp.), as well.

Stomachs of all analyzed individuals, 21 putative brown trout and 12 soft-muzzled trout were full. Prey items were grouped in 12 categories and 11 of them were recorded in putative brown trout, while 7 were detected in soft-muzzled trout (Tables 5, 6). One food item (*Planaria*) was detected only in the stomach contents of soft-muzzled trout. In the pooled stomach content of all soft-muzzled trout samples, *Gammarus* shrimps were by far the predominant food item (Ta-

Table 5. Total length (in cm) and weight (in g) of 12 *Salmo obtusirostris* from the Slap Zete locality and their stomach content composition (in frequencies and percentages of occurrence; mark A – adults, mark N – nymphs of particular insect group).

<i>Salmo obtusirostris</i>												Total	
Size (TL) (cm)	Weight (g)	Ephemeroptera		Trichoptera		Plecoptera		Diptera	Planaria	Gastropoda	<i>Gammarus</i>		Fish
		A	N	A	N	A	N						
39.5	1024		3		0		1	1	0	0	42	0	47
35.5	852		1		0		0	0	1	0	29	0	31
30.5	512		2		0		1	0	0	0	29	0	32
29.5	548		0		0		3	0	0	0	23	0	26
21.5	249		0		1		0	2	0	0	37	0	40
37.5	875		2		0		2	1	1	0	25	1	32
33.5	540		2		0		0	2	0	0	32	0	36
20.5	198		0		2		4	0	0	0	29	0	35
19.5	185		0		0		2	0	0	0	21	0	23
28	467		1		2		1	0	0	0	42	0	46
31	480		0		0		4	1	2	0	29	0	36
28.5	395		0		0		3	0	0	0	12	0	15
Total			11		5		21	7	4	0	350	1	399
Percentages (%)			2.8		1.3		5.2	1.7	1.0		87.7	0.3	100.0

Table 6. Total length (in cm) and weight (in g) of 21 *Salmo cf. farioides* individuals from the Slap Zete locality and their stomach content composition (in frequencies and percentages of occurrence; mark A – adults, mark N – nymphs of particular insect group).

Salmo cf. farioides												Total	
Size (TL) (cm)	Weight (g)	Ephemeroptera		Trichoptera		Plecoptera		Diptera	Planaria	Gastropoda	Gammarus		Fish
		A	N	A	N	A	N						
30.5	390	2	17	2	5	2	8	0	0	1	5	1	43
40.5	956	3	2	5	9	3	5	0	0	0	2	2	31
29.5	320	1	14	2	12	2	0	0	0	0	11	1	43
27.5	295	2	9	1	13	1	2	0	0	0	2	0	30
33	398	0	18	4	8	4	3	0	0	0	1	0	38
38	612	1	11	1	9	2	0	0	0	2	7	2	35
32	354	2	9	0	18	1	3	0	1	1	0	0	35
31.5	382	0	10	1	2	0	3	1	0	0	15	0	32
32	376	0	15	3	8	0	9	0	2	0	4	0	41
33	408	2	9	4	12	0	3	0	0	0	2	0	32
29	398	0	8	2	8	2	5	1	0	3	0	0	29
30	389	0	11	0	11	0	4	5	1	2	0	0	34
28	324	0	10	0	0	4	4	2	3	4	5	0	32
29.5	365	0	7	2	0	0	5	0	0	1	7	0	22
28.5	370	1	19	1	3	1	8	3	0	3	9	0	48
32.5	395	4	6	2	12	0	9	0	0	6	5	2	46
33	423	0	19	3	11	0	4	0	0	4	5	0	46
32.5	448	0	4	5	8	0	2	3	0	3	9	1	35
30.5	408	4	1	3	15	2	1	0	5	2	11	0	44
31	397	2	5	2	11	4	2	0	1	2	12	1	42
26.5	287	3	8	1	17	2	2	0	0	4	0	1	38
Total		27	212	44	192	30	82	15	13	38	112	11	776
Percentages (%)		3.5	27.3	5.7	24.7	3.9	10.6	1.9	1.7	4.9	14.4	1.4	100.0

ble 5). The fish prey that was found in the stomach content of only one soft-muzzled trout was almost entirely digested, making taxonomical identification impossible. In contrast to that, the most abundant prey items of putative brown trout were caddis flies, stoneflies and mayflies (Trichoptera, Plecoptera and Ephemeroptera, respectively), mostly their aquatic larvae, i.e., nymphs, whereas the adult stages of those insect groups were less

abundant (Table 6). Proportion of fish (whose taxonomical identification was impossible due to the digestion in progress) as a prey was far greater in the stomach content of putative brown trout samples than in that of soft-muzzled trout.

The niche overlapping among sympatric soft-muzzled and putative brown trout in the first half of July was $O = 0.357$, indicating the low level of sharing

mutual food resources. Moreover, the calculated percentage of feeding niche overlapping of $P = 19.28\%$ also supports the low level of competition among those two sympatric and syntopic species and their high differentiation in term of food resources utilization.

Discussion

Genetics

F_{st} pairwise values based on 12 microsatellites loci suggest high genetic differentiation occurring between all three nominal and sympatric trout species from Adriatic drainage area of Montenegro (Table 3, Fig. 2). Those values indicate genetic specificity and segregation of each nominal taxon and match with groups formed on their phenotype characteristics, but not on geographical occurrence (two groups of putative brown trout from two different rivers, Zeta and Cijevna, had lowest F_{st} , Table 3). Although we expected low overall heterozygosity (H_o) in soft-muzzled trout from the Zeta River due to their small abundance (i.e., population size) and restricted dispersal area, this population appeared the most diverse one, with the highest overall H_o among all three investigated taxa (Table 2). Soft-muzzled trout population from the Zeta River was polymorphic for all of 11 amplified microsatellites loci, while Snoj et al. (2008) for the same species in Neretva River and same sets of 11 amplified microsatellites (the vary same Ssosl417 locus was not amplified) reported 10 polymorphic loci. Other analyzed soft-muzzled populations (Vrljika River and Jadro River) were polymorphic for only 4 and 7 loci respectively (Snoj et al. 2008). Same authors in their research of Adriatic region soft-muzzled trout also found similar overall heterozygosity ($H_o = 0.594$) of Neretva River specimens like we did for Zeta River, while for other populations of *S. obtusirostris* (Vrljika and Jadro Rivers) the overall H_o was significantly lower (0.167 and 0.285, respectively). For *S. obtusirostris* in river Žrnovnica, Snoj et al. (2007) reported only expected heterozygosity, not the observed one, but even this value was much lower than one we found in the Zeta River population. Those low values of overall H_o could be a consequence of relatively recent bottleneck event or, what is more likely, of geographical isolation followed by small numbered population and significant inbreeding, which we didn't detect for soft-muzzled trout in the Zeta River. Such unexpectedly high genetic diversity of the Zeta River soft-muzzled trout could be explained by retention of intrapopulation genetic diversity of the original population from the past, when this species was far more abundant. Furthermore, Snoj et al. (2008) reported F_{st} value (0.3223) among soft-muzzled trout from Neretva River population and their sympatric brown trout population which is less than we found for same sympatric species in the Zeta River (0.404; Table 3), which additionally supports the distinctiveness of this soft-muzzled trout population.

Analysis we carried out didn't show any hybrids between soft-muzzled and other two trout species,

but we detected hybrids between marble and putative brown trout in the Cijevna River (Fig. 2). Although we analyzed 14 individuals of the Zeta River soft-muzzled trout, absence of observed hybrids indicates stronger reproductive isolation between soft-muzzled trout and other sympatric trout species than the one occurring between marble and putative brown trout. Having in sight features of reproductive biology of salmonid fish with the external fertilization of their eggs in pits on the bottom, the detected reproductive isolation could be explained either by different spawning time of soft-muzzled trout, or by their using of different spawning grounds in compare to the two other sympatric trout species. For soft-muzzled trout in the Morača River drainage area Drecun et al. (1985) pointed out that spawning of this species occurs during March, April and May, while sympatric and syntopic putative brown trout spawn in winter period (October – January). Janković (1961) listed the same months (March, April and May) as spawning ones for soft-muzzled trout in the Buna River. Both statements suggest that reproductive isolation among those two trout species is most probably caused by their different spawning times.

Although both populations of putative brown trout did not show big deviation from HWE (Appendix 1), marble and soft-muzzled trout were not in equilibrium. This especially refers to marble trout, where only one of the 12 tested loci was in HWE, while others were far from it (Appendix 1). We also found relatively high overall F_{is} value (0.358; Table 4) in marble trout, indicating inbreeding that could be, together with detected hybridization, the most likely explanation of strong disequilibrium. Putative brown trout also had the lowest overall F_{is} , which corresponds to their concordance with HWE. The population of soft-muzzled trout deviated from HWE, with four loci being in HWE and others being out, but this disequilibrium was less extreme than one we detected for marble trout population (Appendix 1). The overall F_{is} value for the Zeta river soft-muzzled trout was also low (0.094), suggesting almost no inbreeding, while similar F_{is} was found for Jadro and Žrnovnica population (Snoj et al. 2007).

Our data sets indicate that, population of soft-muzzled trout in Zeta River still preserved high intrapopulation variability with almost no inbreeding, regardless of their low abundance. Detected deviation from HWE as a consequence of reduction of some allele frequency in population seems to be caused by decrease in abundance that occurred in last 20 years. Genetic distinctiveness of soft-muzzled trout from the other two sympatric trout species in the Zeta River could be compared only with similar distinctiveness of soft-muzzled trout population in the Neretva River, while other populations of soft-muzzled trout were not compared with their sympatric brown trout population. In difference to genetically variable populations of soft-muzzled trout in Neretva and Zeta rivers, soft-muzzled trout in Jadro, Vrljika and Žrnovnica rivers showed significant loss of their genetic variability (Snoj et al. 2007, 2008).



Fig. 3. *Salmo obtusirostris* from the Zeta River revealing its silverish color.

Habitat and feeding

Salmonid fish are very diverse in the way they use their habitat and highly flexible to seasonal variation and habitat availability (Grossman & Freeman 1987; Heggenes & Saltveit 1989; Vondracek & Longanecker 1993). Among them, brown trout show incredible potential to use not only diverse riverine, but also lake and marine habitats. In the Zeta River, individuals of putative brown trout were present in almost all habitats (in terms of water velocity, water depth and type of bottom substrate), while sympatric and syntopic soft-muzzled trout were restricted to deeper pools strongly impacted by wells and springs. Occurrence of soft-muzzled trout individuals only in those habitats could be considered a consequence of either their recent extremely low abundance, or seasonal (i.e., early summer) dispersal pattern in habitat use. But, during past years when this fish was far more abundant, their predominant catching sites were such pools, as well as the mainstream of the Zeta River (dr Djoko Drecun, pers. comm.). That proved our findings about the habitat preference of soft-muzzled trout adults throughout the year. It is also possible that soft-muzzled trout shift to various habitats during ontogeny, as well as they shift to specific spawning grounds during the spawning season, but there are yet no records about that.

The habitat sharing between putative brown and soft-muzzled trout in Adriatic karst rivers looks very similar to that occurring between brown trout and European grayling *Thymallus thymallus* (L., 1758) living in sympatry in streams and rivers of their native North Sea and Atlantic Ocean drainage areas. Grayling prefer there deeper habitats with smaller diameter of substrate particles than brown trout (Greenberg et al. 1996; Haugen & Rygg 1996; Riley et al. 2006). Moreover, it seems that grayling and soft-muzzled trout have similar morphological features due to their similar ecological niche e.g., smaller (in compare to brown trout) and subterminal mouth, smaller head, higher body, larger scales and remarkable silverish body color (Fig. 3). However, that similarity is to be further investigated.

Food partitioning between syntopic putative brown and soft-muzzled trout in the Zeta River showed high segregation in their feeding niche. The nymphs of cad-

disflies, stoneflies and mayflies, as well as the *Gammarus* shrimp and dipterans (in total six food items) were mutual food resource of those two trout, but with different frequency of occurrence. The bottom living prey groups represent 79% of all food items in putative brown trout, while in stomach contents of soft-muzzled trout they represented 99%. That characterizes soft-muzzled trout the exclusive bottom feeders. Even more, the *Gammarus* shrimps participated with almost 88% in all prey items of stomach content in soft-muzzled trout and it seems that they are highly specialized for benthic macroinvertebrates, while putative brown trout did not show such a strong preference. The subterminal position of mouth in soft-muzzled trout is a morphological adaptation that corresponds well to the bottom feeding niche. Similar findings were already stated by Janković (1961) for soft-muzzled trout population in the Buna River. She found that 90.57% of total stomach content in spring were benthic larvae of Chironomidae, suggesting their strong preference for bottom feeding niche. Although the differences found in feeding habits of soft-muzzled and putative brown trout could be considered variable with season and prey availability (López-Álvarez 1984; Giller & Malmqvist 1998; Fochetti et al. 2003), several authors (Hunt & Jones 1972; López-Álvarez 1984; García de Jalón & Barceló 1987) showed that certain trout species can change their diet from specialist to generalist, independently of the prey composition, as well as that feeding items of trout can vary with population (García de Jalón & Barceló 1987; Kara & Alp 2005). Diets vary also with individual size (Neveu & Thibault 1977; López-Álvarez 1984; Montori et al. 2006) and even with sex due to differences in behavior during the spawning period (Johnsson et al. 2001; Montori et al. 2006). This ecological plasticity of brown trout is in high concordance with their diverse preferential for different habitats in rivers and lakes. On the other hand, soft-muzzled trout in the Zeta River show narrow specialization in terms of both food items (*Gammarus* shrimp) and habitat preferential, in order to reduce the competition with sympatric and syntopic putative brown trout. That is supported by calculated niche overlapping which indicates low level of mutual utilization of food item resource. As *Gammarus* shrimps are one of the most abundant groups

in the benthic community of rivers, especially where strongly impacted by karst wells and springs (Bonettini & Cantonati 1996; Barquin & Death 2004; Dumnicka et al. 2007; Šundić & Pešić 2007; Von Fumetti 2008), it seems that soft-muzzled trout specialized in using them as the main food resource.

Conservational considerations

The geographical isolation of the Zeta River soft-muzzled trout and its expected genetic divergence from other West-Balkans soft-muzzled trout makes it even more important as an unique genetic resource, whose recent low abundance in compare to the original one is to be considered seriously. It is of vital importance to investigate further details of biology and life-history of the Zeta River soft-muzzled trout, in order to preserve this unique European trout and to implement the appropriate conservation measures for this population. Although we suspected the loss of genetic variability in soft-muzzled trout in Montenegro due to inbreeding and hybridization with other two sympatric trout species, we fortunately did not detect it. In terms that the most urgent conservation measure is to modify the present Fishery Management Plan for the Zeta River by issuing the protection of all three localities where soft-muzzled trout is known to occur. This only gregarious trout in the Adriatic Sea drainage area of Montenegro is of the yet unknown potential for fish farming, which is necessary to investigate. Repopulation measures are also important and they seem feasible, due to preserved genetic diversity and gregarious behavior of the Zeta River soft-muzzled trout.

Conclusions

Genetic analysis of 12 microsatellites loci, habitat preference and feeding ecology revealed that soft-muzzled trout *Salmo obtusirostris* from the lower Zeta River is definitively a trout species different from sympatric and syntopic putative brown trout *Salmo* cf. *farioides* of the Montenegrin Adriatic Sea drainage area. Soft-muzzled trout occur in several populations in several rivers in karst of the Adriatic Sea drainage area. In compare to soft-muzzled trout populations from Neretva, Jadro, Žrnovnica and Vrljika rivers, that from the Zeta River appeared the most diverse one for its genetic features. For more detailed insight into the genetic structure of soft-muzzled trout, further research that would test genetic distances between them should be carried out. According to research accomplished so far, we can expect that future investigations will reveal that soft-muzzled trout from Zeta and Neretva rivers will reveal strong differentiation with the still maintained genetic diversity confined to them, whereas soft-muzzled trout from other Dalmatian rivers will be positioned closer to soft-muzzled trout from the Neretva River.

Ecological segregation among putative brown trout occupying inshore and shallow water area and deeper bottom-dwelling soft-muzzled trout in the Zeta River where they live in both sympatry and syntopy resembles

to that occurring between European grayling *Thymallus thymallus* and brown trout in streams and rivers of Atlantic Ocean and North Sea drainage areas. It seems, therefore, we can consider soft-muzzled trout an ecological equivalent of grayling in streams and rivers of the Adriatic Sea drainage area.

In addition to the quick changes of the Fisheries Management Plan for the Zeta River that should issue protection measures of locations where soft-muzzled trout were recorded, it is necessary to investigate their farming potential, in order to start with its repopulation, while there is still sufficient genetic variability for that, in order to preserve the valuable and unique genetic resource in this endemic West-Balkans trout species.

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Appendix 1

Table 1. Test on Hardy–Weinberg equilibrium for *Salmo cf. farionides*, River Zeta population.

Locus	Obs.Het.	Exp.Het.	P-value
1	This locus is monomorphic: no test done.		
2	This locus is monomorphic: no test done.		
3	0.46429	0.49286	1.00000
4	0.14286	0.13701	1.00000
5	0.57143	0.48571	0.43879
6	This locus is monomorphic: no test done.		
7	0.85714	0.84675	0.75772
8	0.60714	0.63117	0.79271
9	0.69231	0.72172	0.78714
10	0.88889	0.72048	0.06598
11	0.88000	0.90776	0.13761
12	0.77778	0.64920	0.46816

Table 2. Test on Hardy–Weinberg equilibrium for *Salmo obtusirostris* population.

Locus	Obs.Het.	Exp.Het.	P-value
1	0.57143	0.62698	0.07370
2	0.21429	0.41534	0.01977
3	0.64286	0.49471	0.31994
4	0.78571	0.80688	0.02430
5	0.35714	0.45238	0.55477
6	This locus is monomorphic: no test done.		
7	0.71429	0.84656	0.40172
8	0.78571	0.87831	0.10594
9	0.57143	0.63492	0.85942
10	0.64286	0.77513	0.78113
11	0.78571	0.81481	0.97395
12	0.78571	0.85979	0.02933

Table 3. Test on Hardy–Weinberg equilibrium for *Salmo marmoratus* population.

Locus	Obs.Het.	Exp.Het.	P-value
1	This locus is monomorphic: no test done.		
2	0.25000	0.63911	0.00131
3	0.50000	0.50806	1.00000
4	0.43750	0.63105	0.16932
5	0.37500	0.72379	0.00000
6	0.00000	0.20915	0.05847
7	0.57143	0.91534	0.00000
8	0.60000	0.82989	0.01250
9	0.68750	0.78024	0.05378
10	0.43750	0.75605	0.00595
11	0.56250	0.81250	0.00347
12	0.56250	0.86089	0.00335

Table 4. Test on Hardy – Weinberg equilibrium for *Salmo cf. farionides* River Cijevna population.

Locus	Obs.Het.	Exp.Het.	P-value
1	This locus is monomorphic: no test done.		
2	0.30769	0.2707	1.00000
3	0.30769	0.36923	0.52229
4	0.15385	0.15077	1.00000
5	0.53846	0.50769	1.00000
6	This locus is monomorphic: no test done.		
7	0.84615	0.91077	0.74628
8	0.92308	0.87692	0.29963
9	0.61538	0.74154	0.63590
10	0.69231	0.79692	0.30430
11	0.53846	0.82769	0.0136
12	0.84615	0.76000	0.56835