Hydrobiologia (2017) 785:249–260
DOI 10.1007/s10750-016-2930-4

PRIMARY RESEARCH PAPER

Phylogeography of stream-dwelling trout in the Republic of Macedonia and a molecular genetic basis for revision of the taxonomy proposed by S. Karaman

Saša Marić · Simona Sušnik Bajec · Johannes Schöffmann · Vasil Kostov · Aleš Snoj

Received: 24 February 2016 / Revised: 22 July 2016 / Accepted: 27 July 2016 / Published online: 11 August 2016

Abstract To evaluate whether molecular data support the distinctiveness of *Salmo macedonicus* (Karaman, 1924) and *Salmo pelagonicus* Karaman, 1938, and to examine a possible impact of non-native trout translocated from the Drim drainage on the indigenous trout of the Vardar drainage, 187 individuals from 15 populations sampled across both drainages were studied by analysing the complete mitochondrial DNA control region and 12 microsatellite DNA loci. On the basis of both marker systems, the analysed populations were divided into two main genetic groups: I, native populations of the Drim drainage, along with some introduced populations of the Vardar drainage, and II, native Vardar populations, along with some populations exhibiting introgressed genotypes. The populations assigned to group I correspond taxonomically to *Salmo farioides* Karaman, 1938, among which those from the Drim were indigenous and those detected in the Vardar drainage were introduced. Four native Vardar populations from group II indicated two distinct clusters whose distribution matched the proposed range of *S. macedonicus* (two populations from the upper Vardar system) and *S. pelagonicus* (two populations from the Crna Reka system). Based upon the results of the study, some conservation genetic guidelines are proposed to help propagate and sustain the non-introgressed native trout populations.

Keywords Vardar drainage · Genetic diversity · mtDNA · Microsatellites · Translocations · Conservation

Introduction

The Balkan Peninsula is recognised as one of the three main Pleistocene refugia in Europe (Hewitt, 1999) and exhibits high levels of diversity and endemism in trout. A consequence of its history is the description of

Handling editor: Christian Sturmbauer

Electronic supplementary material The online version of this article (doi:10.1007/s10750-016-2930-4) contains supplementary material, which is available to authorized users.

S. Marić (✉)
Faculty of Biology, Institute of Zoology, University of Belgrade, Studentski trg 16, 11001 Belgrade, Serbia
e-mail: sasa@bio.bg.ac.rs

S. Sušnik Bajec · A. Snoj
Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domžale, Slovenia

J. Schöffmann
Finkenweg 18, 9300 St. Veit/Glan, Austria

V. Kostov
Institute of Animal Science, University in Skopje "Ss. Cyril and Methodius", Blvd. Ilinden 92A, 1000 Skopje, Republic of Macedonia

numerous species on the basis of morphology (reviewed in Kottelat & Freyhof, 2007). One of the tasks of modern conservation genetics is to re-evaluate such taxonomy and resolve uncertainties to identify units of conservation value. The first step in this direction is to check whether the proposed range of a certain taxon coincides with the distribution area of genetically homogenic and distinct units.

A relatively large portion of Balkan trout has already been studied in this regard, with the taxonomic status of several trout entities thoroughly examined and resolved, e.g. *Acantholingua ohridana* (Steindachner, 1892) (Ohrid belvica) and *Salmothymus obtusirostris* (Heckel, 1851) (softmouth trout) were placed into genus *Salmo* (Phillips et al., 2000; Snoj et al., 2002; Sušnik et al., 2006); the species status of *Salmo letnica* (Karaman, 1924) (letnica trout) was recommended to be retained (Sušnik et al., 2007a; Pustovrh et al., 2014); *Salmo dentex* (Heckel, 1851) (dentex trout) was recognised as polyphyletic entity comprising a particular life history form (Snoj et al., 2010); and *Salmo marmoratus* Cuvier, 1829 (marble trout) was classified on the basis of SNPs markers as a distinct cluster of the same or similar rank to that of *Salmo trutta* Linnaeus, 1758 and *S. obtusirostris* and thus supported as separate species (Pustovrh et al., 2014). The brook-dwelling trout populating streams from the Krka to the Drim–Skadar (Albanian: Drin–Shkodër) river systems, described as *Salmo farioides* Karaman, 1938, is another questionable species among the Balkan trouts: mtDNA analysis revealed that a core range of this taxon was strongly associated with the distribution of a distinct haplogroup (haplotypes Ad*Prz, AdN, AdRc and AD-C1) designated as the Balkan cluster (Snoj et al., 2009 and references herein). On the other hand, the status of some key trout populations from the Balkans has yet to be resolved. The territory of the Republic of Macedonia (Former Yugoslav Republic of Macedonia, FYROM) has been particularly understudied and the taxonomic status of its stream-dwelling trout not yet deliberated.

Freshwaters in FYROM drain into two main rivers: the Vardar River (Greek: Axiós), rising in the northwestern part of the country and emptying into the Aegean Sea in northern Greece, and the Crni Drim River (Albanian: Drin i Zi), which takes its source in Lake Ohrid and merges with the Beli Drim (Albanian: Drin i Bardhë) forming the Drim River (Albanian: Drin) in northern Albania and flowing into the Adriatic Sea. Both watersheds are populated with various types of trout.

In the first half of the last century, Karaman described two trout species for the Vardar drainage: *Salmo macedonicus* (Karaman, 1924) and *Salmo pelagonicus* Karaman, 1938. On the basis of molecular testing (Lo Brutto et al., 2010), the species status was not supported, simply confirming the existence of two distinct morphotypes: *S. trutta macedonicus* and *S. trutta pelagonicus*. However, the validity of these taxa remains questionable given that some misidentifications are apparent in that study (see below). *Salmo macedonicus* is considered to populate the upper Vardar drainage, apart from the Crna Reka system (Kottelat & Freyhof, 2007). Hereafter, we refer to this taxon also as the upper Vardar trout. Its type locality is the estuary of the Treska River (Karaman, 1924). Several other locations populated with these trout were later reported, e.g. the Babuna and Kadina rivers (Karaman, 1938; Ristovska et al., 2011), upper reaches of the Bregalnica River (Dimovski & Grupče, 1971; Kostov et al., 2010) and the Došnica River (Kottelat & Freyhof, 2007).

Salmo pelagonicus, which we refer to as Crna Reka trout, was reported to populate streams flowing into the Crna Reka, the longest right tributary to the Vardar River. The type specimens originate from the Bela, Zlokućanska and Dihovska rivers (Karaman, 1938). In the Crni Drim river system, the most well-known and studied trout are those endemic to Lake Ohrid: *S. ohridanus* and *S. letnica* (Karaman, 1924, 1927; Stanković, 1960) along with four distinct spawning forms of the latter (typicus and balcanicus, both winter spawners; aestivalis, a summer spawner; and lumi, a river spawner; Wilson, 2004; Sušnik et al., 2007a). Stream-dwelling trout, also common in the Crni Drim system, have been particularly abundant in the Radika River, right tributary to the Crni Drim River. According to Karaman (1957), its upper part (from source to the Bridge of Boško) is populated with *S. farioides*, hereafter referred to as the Drim trout. The lower part is populated with a different kind of trout, described as *Salmo montenigrinus* (Karaman, 1933), which, according to Karaman (1957), populates Lake Ohrid itself and tributaries to Lake Skadar. On the basis of one morphological study, *S. montenigrinus* was later tentatively described as a species also in the Neretva River (Delling, 2003), though subsequently recognised as a recent hybrid between softmouth trout and other Neretva trout (Razpet et al., 2007). Nevertheless, the molecular systematics of this taxon has yet to be studied and its species status remains questionable. *Salmo marmoratus* (for details see Pustovrh et al., 2011), also described in the Drim drainage (Delpino, 1935; Šorić, 1990), is a very rare and endangered trout in the Radika River with its most recent literature record dating from 1961 (Georgiev, 2011). The waters of Lake Prespa, another hydrological unit of the Drim drainage, discharge underground from the lake and emerge as springs flowing into Lake Ohrid. Trout in Lake Prespa and its tributaries were described by Karaman as *Salmo peristericus* Karaman, 1938. Recently, Berrebi et al. (2013) undertook a genetic analysis of this trout, denied its species status and, on the basis of microsatellite DNA, recognised it as an evolutionary significant unit.

Several trout transfers from the Adriatic watershed into the Aegean rivers are reported to have taken place in FYROM during the second half of the last century (Dimovski & Grupče, 1971; Simo Georgiev personal communication). The main source of the transfers was the fry produced in Mavrovska Akumulacija hatchery located on the Radika River. Sidorovski (1955, 1960) stated that broodstock individuals used for fry production were collected from the upper course of the Radika.

Several genetic studies have been performed on trout taxa of the Crni Drim river system (Sell & Spirkovski, 2004; Sušnik et al., 2006, 2007a; Snoj et al., 2009), mostly confirming the species denomination of the studied taxa. Analysis of CR mtDNA demonstrated that trout from some tributaries of the Drim belong to the Balkan cluster (Marić et al., 2006; Snoj et al., 2009).

Genetic research has been done also on trout from the Aegean watershed (Bernatchez, 2001; Marić et al., 2006; Apostolidis et al., 2008a, b; Kohout et al., 2013), though molecular data on trout from the Vardar drainage in FYROM are scarce and inferences incomplete. Marić et al. (2006) found a *S. farioides* genetic profile in trout of the Pčinja River (Aegean drainage) and thus considered this population non-native, though its exact origin has yet to be determined. Genetic divergence between *S. macedonicus* and *S. pelagonicus* has been investigated recently using mtDNA to test whether molecular data support their species status (Lo Brutto et al., 2010). However, that study was based on 15 individuals only and some population misidentifications are apparent (authors' personal observation), leading to questionable conclusions.

The aim of the present work was to undertake an extensive phylogeographic and population genetic study of populations of Vardar trout, which inhabit the distribution area of *S. macedonicus* and *S. pelagonicus*, in order to see if the distinctness of the two taxa proposed by Karaman is supported by genetic data. The study also examined the possible impact of non-native trout translocations on indigenous populations. In this context, special attention was paid to the genetic characterisation of previously unexamined trout from the Radika River, as these have frequently served as a source of translocations into the Vardar drainage. On the basis of the results obtained here, we aimed also to provide information useful for the conservation and management of trout in FYROM.

Materials and methods

Sampling and DNA isolation

A total of 187 trout individuals were collected from 15 locations in the Vardar and Drim drainages, from 2004 to 2014 (Fig. 1; Table 1). Fin clip samples were taken and stored in 96% ethanol. After capture, the individuals were returned to the water. DNA was isolated using the phenol–chloroform–isoamyl alcohol method (Sambrook et al., 1989).

Mitochondrial DNA

The complete mitochondrial control region (mtDNA CR, ca. 1100 bp) in 183 individuals was amplified (Table 1) using polymerase chain reaction (PCR) and primers LRBT-25 and LRBT-1195 (Uiblein et al., 2001), following the conditions described in Marić et al. (2012). Both-direction sequencing was carried out on an ABI Prism 3130xl DNA sequencer using the same primers. Sequences were aligned using the computer program Clustal X (Thompson et al., 1997) implemented in MEGA version 6 (Tamura et al., 2013). The relationships among haplotypes were presented as a Median-Joining network created from the program Network 4.6.1.3. (Bandelt et al., 1999).

Microsatellite DNA

Twelve microsatellite loci (Lerceteau-Köhler & Weiss, 2006) were amplified in 187 individuals

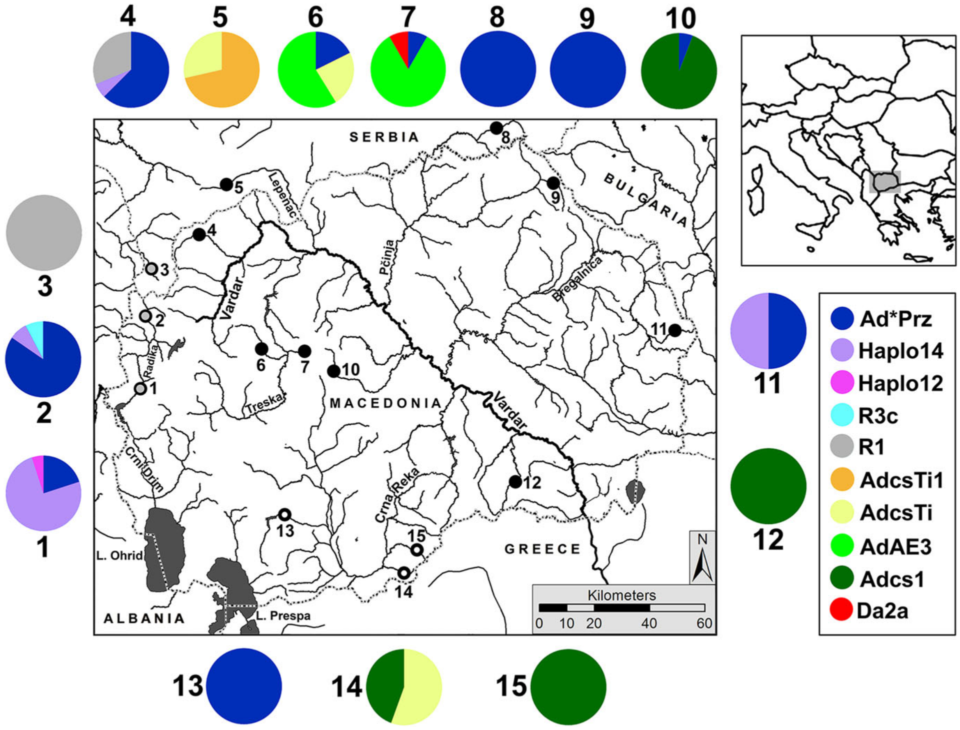

Fig. 1 Main diagram: Map of sampling locations. Names and codes of sample locations are reported in Table 1. *Grey circles* with *bold* border on the map represent populations analysed from the Drim drainage; *black circles*, the Vardar system; *white circles* with bold border, the Crna Reka system. *Coloured pie charts* around the map represent distribution and frequencies of the CR mtDNA haplotypes per population

(Table 1) using fluorescently labelled forward primers. PCR amplification and multiplex PCR conditions were as described in Lerceteau-Köhler & Weiss (2006). Aliquots of amplified fluorescently labelled DNA were mixed with formamide and GENESCAN-500 ROX Size Standard (Applied Biosystems) and genotyped on the ABI Prism 3130xl with GeneMapper® Software v4.0 (Applied Biosystems).

The program Micro-Checker v. 2.2.3 (Van Oosterhout et al., 2004) was used to check for the presence of null alleles. The extent of genetic diversity was assessed from the levels of heterozygosity, average number of alleles per locus (Genetix 4.05; Belkhir et al., 1996–2004) and allelic richness (FSTAT 2.9.3.2; Goudet, 1995). The general picture of genetic differentiation among trout was obtained using *F* statistics (FSTAT 2.9.3.2), and factorial correspondence analysis (FCA; Genetix 4.05). All calculations were performed for only those populations comprising nine individuals or more in order to satisfy minimal statistical requirements of sampling.

The genetic structure of the whole sample set was assessed using hierarchical STRUCTURE analysis (Pritchard et al., 2000; Vähä et al., 2007). In STRUCTURE 2.3.4, the admixture model and correlated allele frequencies between populations were chosen. MCMC was run for 1,000,000 replicates subsequent to 250,000 burn-in generations. The number of clusters, *K*, in the dataset was explored from $K = 1$ to $K = 15$, and for each value seven run replicates were performed. The ΔK method (Evanno et al., 2005) was applied to estimate the most probable *K*. In hierarchical STRUCTURE, the most differentiated cluster was excluded from the analysis, allowing for more precise clustering of the remaining individuals. Each cluster was analysed separately to check for subpopulations. For this approach, 100,000 burn-in generations and 500,000 subsequent replicates were used.

Results

Mitochondrial DNA

Complete CR sequences were aligned in 183 trout individuals. Some sequences contained an 82-bp repeat towards the 3′-end of the CR. As the elongation model of this repeat is generally thought to be the result of intra-molecular processes (Buroker et al., 1990), only the first copy was kept in the analysis.

The aligned sequences collapsed into ten haplotypes: nine for the Adriatic lineage of brown trout, of

Table 1 Sample locations with a summary of mtDNA haplotype frequencies, diversity and microsatellite genetic diversity

					Haplotype frequency															
Location	**Drainage, basin**	**Species**	**Coordinates**	**mtDNA CR N**	**Ad*Prz**	**Haplo 14**	**Haplo 12**	**R3c**	**R1**	**AdcsTi1**	**AdcsTi**	**AdAE3**	**Da2a**	**ADcs1**	**Microsatellite DNA N**	**A**	**Ar**	**H_O**	**H_E**	**F_{IS}**
1. Radika lower course	Crni Drim, AD	*S. farioides*	41° 50' 53.4" N 20° 37' 21.2" E	20	4	15	1								20	7.75	3.78	0.554	0.590	0.086
2. Radika upper course	Crni Drim, AD	*S. farioides*	41° 33' 01.5" N 20° 36' 43.8" E	13	11	1		1							13	4.33	3.00	0.539	0.511	-0.015
3. Restelička Reka	Plavska Reka, Beli Drim, AD	*S. farioides*	41° 56' 29.3" N 20° 40' 31.9" E	3					3						3	-	-	-	-	-
4. Tetovska Reka	Vardar, AE	*S. macedonicus*	42° 03' 25.2" N 20° 52' 42.8" E	16	10	1			5						16	5.50	3.34	0.573	0.544	-0.022
5. Suva Reka	Lepenac, Vardar, AE	*S. macedonicus*	42° 12' 12.5" N 20° 59' 11.4" E	14						10	4				15	3.36	2.93	0.412	0.435	0.092
6. Mala Reka	Treska, Vardar, AE	*S. macedonicus*	41° 39' 23.2" N 21° 12' 57.0" E	17	3						4	10			17	6.36	4.83	0.626	0.590	-0.029
7. Belička Reka	Treska, Vardar, AE	*S. macedonicus*	41° 40' 46.5" N 21° 16' 49.8" E	12	1							10	1		12	5.50	3.43	0.500	0.552	0.145
8. Tripušnica	Pčinja, Vardar, AE	*S. macedonicus*	42° 24' 41.8" N 22° 16' 40.5" E	7	7										10	2.75	2.19	0.367	0.363	0.042
9. Kriva Reka	Pčinja, Vardar, AE	*S. macedonicus*	42° 11' 22.3" N 22° 27' 14.7" E	10	10										10	4.17	3.07	0.483	0.468	0.020
10. Babuna	Vardar, AE	*S. macedonicus*	41° 39' 22.3" N 21° 27' 12.4" E	17	1									16	17	5.00	3.09	0.562	0.573	0.051
11. Bregalnica	Vardar, AE	*S. macedonicus*	41° 43' 39.1" N 22° 59' 17.6" E	2	1	1									2	-	-	-	-	-
12. Došnica	Vardar, AE	*S. macedonicus*	41° 19' 07.0" N 22° 13' 42.5" E	11										11	11	2.42	2.02	0.326	0.329	0.059
13. Šemnica	Crna Reka, Vardar, AE	*S. pelagonicus*	41° 09' 11.1" N 21° 12' 48.9" E	12	12										12	2.75	2.28	0.316	0.400	0.253
14. Konjarska Reka	Crna Reka, Vardar, AE	*S. pelagonicus*	40° 58' 14.5" N 21° 42' 58.8" E	9							5			4	9	2.08	1.74	0.269	0.220	-0.163
15. Bela Reka	Crna Reka, Vardar, AE	*S. pelagonicus*	41° 02' 06.8" N 21° 48' 26.0" E	20										20	20	4.17	2.21	0.308	0.343	0.126
Σ				183	60	18	1	1	8	10	13	20	1	51	187					
%					33	10	1	1	4	5	7	11	1	28						

Details of samples: numerical code, sample location, river drainage and basin (AD, Adriatic; AE, Aegean), species, geographical coordinates, number of individuals analysed (N), summary of control region haplotype frequencies and genetic diversity indices of microsatellite marker data: *N* number of individuals; *A* average number of alleles; *Ar* allelic richness; H_O,. observed heterozygosity; H_E, expected heterozygosity in the population; F_{IS}, values showed no statistically significant deviations from HWE ($P < 0.001$)

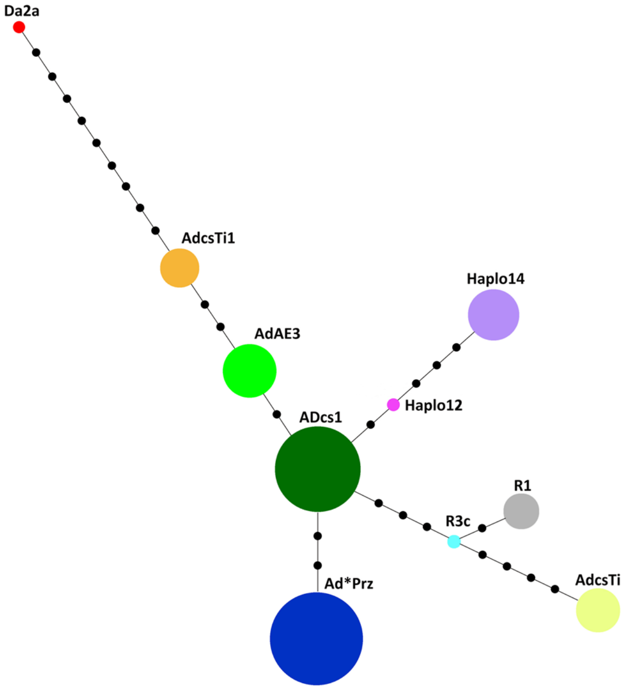

Fig. 2 Median-Joining network based on CR mtDNA sequences. Detected haplotypes are represented by *coloured circles*, the size of which is proportional to haplotype frequencies in the overall sample. Numbers of variable sites are indicated with *small black circles*. List of CR haplotypes used for network construction and GenBank accession numbers: Ad*Prz, DQ318129 and KU667310 (partial and complete sequence, respectively); Haplo 14, AY926571; Haplo 12, AY926570; R3C, KU667313; R1, KU667311; AdcsTi1, KU667314; AdcsTi, KU667312; AdAE3, GQ357910; Da2a, GQ284834; Adcs1, AY836330

which four were newly described (i.e. R3c, R1, AdcsTi1 and AdcsTi; see caption to Fig. 2 for GenBank accession numbers of the sequences), and the haplotype Da2a characteristic of the Danubian lineage (Table 1). Ad*Prz was the most frequent haplotype (33%), found in the Crni Drim, upper Vardar and Crna Reka systems (Fig. 1; Table 1). ADcs1 was the second most frequent haplotype (28%), prevalent in the Crna Reka system, and observed also in the upper Vardar system (i.e. in Babuna and Došnica) but not in the Drim system. Other haplotypes were less frequent and ranged from 11% down to 1% (Table 1).

The populations of the Mala and Belička rivers (upper Vardar system) were dominated by the haplotype AdAE3, which was not found elsewhere. In the lower Radika, Haplo14 dominated, while in the upper Radika, haplotype Ad*Prz was the most frequent. In the Pčinja river system and in the Šemnica River, Ad*Prz was the only haplotype detected (Fig. 1; Table 1).

In the Median-Joining network (Fig. 2), haplotype ADcs1 took the central position, while other AD lineage haplotypes were from 1 to 7 mutation steps away. The number of variable sites among the AD lineage haplotypes ranged from 1 to 11, and between the Da2a and AD haplotypes, from 9 to 17.

Microsatellites

On the basis of the results obtained using Micro-Checker v. 2.2.3 (Van Oosterhout et al., 2004), no null alleles were detected apart from the population Šemnica at locus Sssp2213, implied by an excess of homozygotes.

None of the populations showed deviation from Hardy–Weinberg expectations. The average numbers of alleles per locus and allelic richness were heterogeneous among the populations (ranging from 2.08 to 7.75 and 1.74 to 4.83, respectively), while the expected heterozygosity ranged from 0.220 to 0.590 (Table 1).

The degree of differentiation among most of the analysed populations was found to be significant (Table 2) with pairwise F_{ST} values up to 0.658.

The FCA graph (Fig. 3) indicates three distinctive clusters: cluster I comprising all of the Drim populations along with presumably stocked populations from the upper Vardar (Tetovska and Bregalnica rivers and the Pčinja tributaries) and the Crna Reka system (Šemnica River), while cluster II was represented by other upper Vardar populations, and cluster III by the Crna Reka trout. In cluster II, two substructures emerged, one composed of Suva and Došnica trout, and another, Mala and Belička trout; Babuna individuals were also detected in the latter group. The specimen bearing the Da2a haplotype took a relatively independent position, which is even more evident if the third axis is also considered (not shown). Taking into account only the first axis, which describes the main structure on the graph, the Suva and Došnica trout are positioned to the right, towards cluster III, while the Mala, Belička and Babuna individuals are positioned on the left, towards cluster I.

From the STRUCTURE analysis (Fig. 4), the most probable number of groups in the dataset was $K = 2$ (Appendix 1 in Supplementary Material). In

Table 2 Pairwise values of F_{ST} above and significance values (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, non-significant after Bonferroni-type correction) below the diagonal for microsatellite marker data

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	Radika lower course		0.069	0.075	0.386	0.202	0.215	0.248	0.073	0.278	0.436	0.142	0.467	0.485
2	Radika upper course	***		0.108	0.449	0.232	0.246	0.277	0.108	0.312	0.495	0.178	0.512	0.537
3	Tetovska Reka	***	***		0.420	0.195	0.178	0.271	0.070	0.293	0.458	0.138	0.500	0.502
4	Suva Reka	***	**	**		0.256	0.283	0.504	0.468	0.245	0.358	0.491	0.581	0.543
5	Mala Reka	***	***	***	***		0.032	0.319	0.243	0.183	0.203	0.280	0.470	0.437
6	Belička Reka	**	*	*	***	NS		0.332	0.231	0.223	0.336	0.265	0.492	0.464
7	Tripušnica	***	**	***	**	***	NS		0.300	0.369	0.588	0.330	0.650	0.605
8	Kriva Reka	***	***	***	**	***	NS	***		0.336	0.529	0.090	0.564	0.567
9	Babuna	***	***	***	***	***	*	***	***		0.320	0.380	0.478	0.447
10	Došnica	***	**	***	**	***	NS	***	**	***		0.566	0.658	0.530
11	Šemnica	***	***	***	***	***	NS	***	**	***	***		0.596	0.597
12	Konjarska Reka	***	***	***	**	***	NS	**	**	***	**	**		0.554
13	Bela Reka	***	***	***	***	***	*	***	***	***	***	***	***	

For populations Suva Reka and Mala Reka calculations were made based on 11 microsatellite loci, since locus Ssa410 did not amplify in these two populations

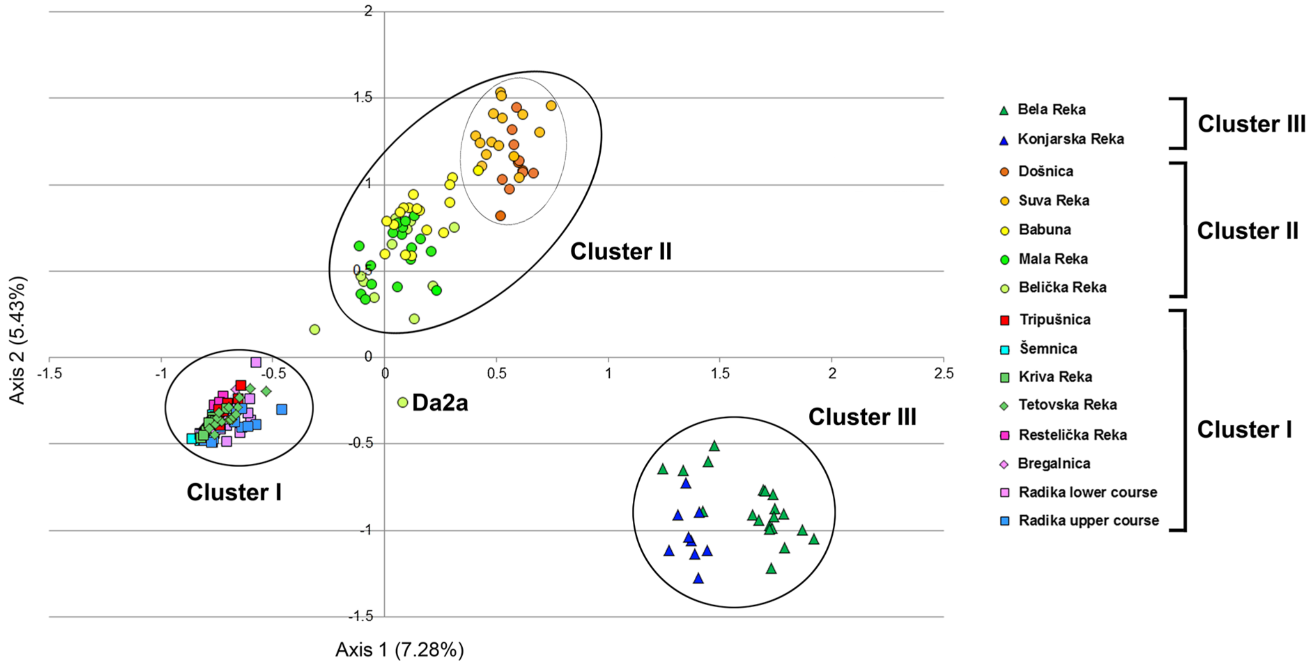

Fig. 3 Two-dimensional plot of factorial correspondence analysis for all trout specimens from Drim and Vardar drainages based on 12 microsatellite loci

the first hierarchical step, trouts were separated into two groups: group A, represented by the Drim populations and presumably stocked populations from the upper Vardar and Crna Reka systems (FCA cluster I), and group B, representing other populations from the upper Vardar and Crna Reka systems (FCA clusters II and III). In group B, considerable introgression of genes from group A was evident in the Belička, Mala and Babuna populations.

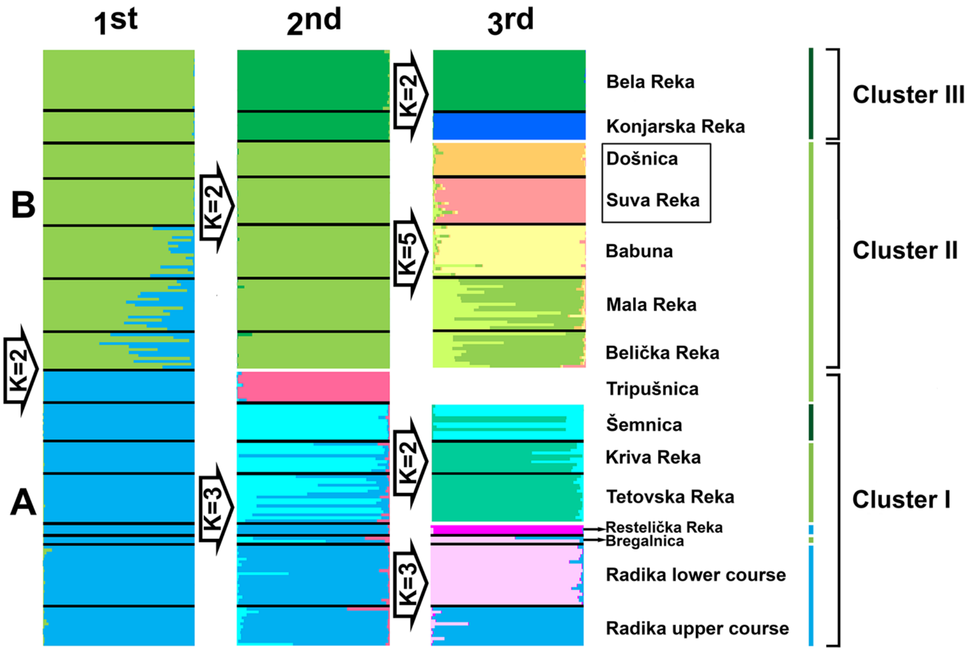

Fig. 4 Population structure as inferred by hierarchical STRUCTURE analysis of microsatellite data. *Black lines* separate the sampling sites. Affiliation of analysed population to a particular river system is indicated by the *vertical coloured lines* to the *right*: *light blue*, Drim drainage; *light green*, Vardar system; *dark green*, Crna Reka system

In the second hierarchical step with group A, trout from the Drim and Tripušnica populations emerged as genetically unified units, while all the others formed a third unit that exhibited much introgression of genes from the Drim trout. In group B, trout from the Crna Reka (in the Bela and Konjarska rivers; FCA cluster III) were distinguished from the other Vardar populations (FCA cluster II).

In the third step, many populations appeared as genetically distinct with the exception of the presumably stocked populations (e.g. in the Bregalnica, Kriva and Šemnica rivers) and some others (e.g. Belička and Mala rivers), all exhibiting relatively high proportions of admixed genotypes. No additional structuring within these populations was revealed when further steps of hierarchical Structure analysis were performed.

Discussion

Molecular data obtained from the present study show that trout in FYROM can be divided roughly into two main genetic groups. The first group is represented by the Drim trout (Adriatic watershed), along with some apparently introduced Adriatic populations found in the Vardar drainage (Figs. 3, 4), while the second group is more heterogeneous and represented by the native Vardar populations composed of the Crna Reka trout (cluster III in FCA) and the upper Vardar trout (cluster II in FCA). As clearly seen from the STRUCTURE graph (Fig. 4), Belička Reka, Mala Reka and Babuna populations, constituting the left part of cluster II (Fig. 3), exhibit admixed genotypes and very likely represent hybrids between native and introduced individuals. This genetic division is generally supported also by mtDNA data providing haplotypes that were found only in the Drim river system and in supposedly stocked locations in the upper Vardar river system, and the haplotypes found only in presumably native Vardar trout (Fig. 1). For example, the Drim trout are characterised by the haplotype Ad*Prz, previously found in the Drim–Skadar drainage (Marić et al., 2006; Sušnik et al., 2007a; Snoj et al., 2009; present study) along with a few other related haplotypes observed in the Neretva and Skadar river systems (AdN, AdRc and AD-C1; Razpet et al., 2007; Sušnik et al., 2007a). This haplogroup, named the Balkan cluster, represents a well-resolved and highly distinct clade within a more or less fuzzy assemblage of other widespread haplotypes of Mediterranean–Adriatic trout lineage (see Snoj et al., 2009 and Introduction for details). Despite an apparent association of these haplotypes with the very restricted range that Karaman (1938) proposed for *S. farioides*, Ad*Prz has been recently detected also in the upper Vardar river system in the Tripušnica River (Marić et al., 2006; present study), where this taxon had not been described previously. The same haplotype was detected also in the present study in many other sampling sites of the upper Vardar river system, though it was not found elsewhere in this or other Aegean river systems (Apostolidis et al., 2011; Kohout et al., 2013; authors' unpublished data). The same holds true for the haplotype Haplo14, which is a member of a distinct haplogroup characterising Ohrid trout (*S. letnica*; Sušnik et al., 2007a) and found also in some Drim tributaries (Snoj et al., 2009) and in the lower part of the Radika river (present study); this haplotype was detected also in some upper Vardar tributaries but not elsewhere in the Aegean river system.

This sharing of geographically very restricted haplotypes may be genetic evidence of trout transfer from the Drim into the Vardar drainage. Indeed, there are reports of such an introduction of trout performed during the second half of the last century. These introductions depended primarily on translocations of fry originating from the Adriatic watershed (Dimovski & Grupče, 1971); e.g. one well-known centre of trout distribution was the fish farm on the upper Radika River (Sidorovski, 1955, 1960; Simo Georgiev, personal communication). Furthermore, records exist from 1978 of trout transfer from an unknown Adriatic source into the Tripušnica River (upper Vardar system), which was troutless prior to that introduction (Trgovište Anglers Association, personal communication).

This apparent phylogeographic discrepancy could be explained by ancestral polymorphisms and incomplete lineage sorting. However, such a scenario would be more likely if more widespread haplotypes, e.g. ADcs1 (Cortey et al., 2004), were involved in haplotype sharing. In the present case, however, these are locally specific haplotypes, which were found outside the Drim tributaries only in the adjacent upper Vardar river system.

Following this line of argument, we suggest that the presence of the Drim haplotypes in the upper Vardar is less likely to be a consequence of haplotype variation that arose as a result of ancestral polymorphism than as a consequence of stocking.

This stocking hypothesis is supported also by the observation that in some of the supposedly stocked locations (e.g. Tripušnica, Kriva, Tetovska, Bregalnica, Šemnica rivers) only individuals with non-introgressed Drim microsatellite genotypes were found (Figs. 3, 4, 1st step). Considering the lack of native genes, the stocking is likely to have been rather intense, or otherwise some of these rivers must have been troutless prior to stocking, as documented for Tripušnica River (Marić et al., 2006). In some other sampling sites in the upper Vardar system, populations with much smaller proportions of the Ad*Prz haplotype, and various levels of introgression with nuclear genes from the Crni Drim, were observed (i.e. Babuna, Mala and Belička rivers; Figs. 3, 4, 1st step). This observation suggests the presence of hybrid populations originating from Crni Drim and Vardar trout and thus the initial presence of native trout in these rivers.

There is no straightforward explanation for the presence in the Belička Reka of the Danubian haplotype (Da2a), which is clearly not native there. An exotic origin of the specimen bearing this haplotype is suggested also by its relatively independent position on the FCA graph. A few small commercial trout farms are situated along this river, rearing mostly rainbow trout (*Oncorhynchus mykiss*) but in very small quantities also brown trout (*S. trutta*) of unknown origin (a fish farm manager, personal communication). It is possible that escapees from these farms have contributed to the exotic genetic profiles.

It could be concluded that the populations assigned to the Balkan mtDNA cluster correspond taxonomically to *S. farioides* sensu Karaman, while those from the Drim are indigenous and those detected in the Vardar drainage are introduced. Further population fragmentation exhibited in the STRUCTURE graph (Fig. 4, 2nd and 3rd steps) could be a consequence of a genetically polymorphic broodstock material from the Drim drainage used for stocking of the Vardar drainage along with random genetic drift in newly established populations.

Aside from the Vardar populations affected by the introduction of trout from the Drim drainage, the remaining Vardar populations (from the Suva, Došnica, Bela and Konjarska rivers) showed no introgression with Drim trout. Clear genetic differentiation, shown from the microsatellite analysis (Figs. 3, 4, 2nd step), was observed within this group and indicates two distinct clusters: Suva and Došnica populations, to be found in the Vardar system, and Bela and Konjarska populations, found in the Crna Reka system. The distinct distribution of these two clusters matches the proposed range for *S. macedonicus* for the former two populations and *S. pelagonicus* (see Karaman, 1938) for the latter two. Clearly, grouping characters exist at the molecular level enabling a distinction to be made between these two hydrologically separated clusters, which were described by Karaman (1938) as separate species. However, the differences in microsatellite allele distribution between the two taxa might simply be a consequence of restricted gene flow and random genetic drift typical of many small and isolated populations (e.g. Fumagalli et al., 2002) that are described as units of a rank much lower than species.

On the basis of mtDNA analysis, we were unable to demonstrate any dichotomy, as two out of the four haplotypes native to the upper Vardar system (ADcs1, AdAE3, AdcsTi and AdcsTi1; Table 1) were shared between the two clusters. However, a lack of private haplotypes cannot preclude the presence of evolutionary units, including species delineation, because past population history can be blurred by incomplete lineage sorting or introgression that makes mtDNA phylogeny different from that of species (or nDNA; Renoult et al., 2009). Phylogenetic inconsistencies between mtDNA and nDNA have been observed also in Balkan trout (Sušnik et al., 2007b; Pustovrh et al., 2011) and represent a potential cause of misleading taxonomic interpretations.

In contrast to our inability to demonstrate an association of upper Vardar trout or Crna Reka trout with specific mtDNA lineages, Lo Brutto et al. (2010) highlight that "the degree of nucleotide variation in the cyt b + control region haplotypes allowed the discrimination of some populations and supported the existence of a morphotype in the River Došnica, *Salmo trutta macedonicus*, and another in the River Šemnica in Pelister National Park, *Salmo trutta pelagonicus*". It is worth mentioning that, based on the mtDNA and microsatellite data reported here, the Šemnica River is populated by Drim trout (a stock from the Radika) rather than by Crna Reka trout, and thus the results reported by Lo Brutto et al. (2010) should be treated with a certain degree of caution.

The split of Radika trout into two different populations, first reported by Karaman (1957), was noted also from the assignment test using microsatellites (Fig. 4, 3rd step) and on the basis of the distribution of the different haplotypes. While in the upper part the Ad*Prz haplotype was prevalent (Table 1), the most common haplotype in the lower part was Haplo14, which belongs to a group of haplotypes typical for letnica trout from Lake Ohrid (Sušnik et al., 2007a; see above for details). This observation is in line with Karaman (1957), who postulated that the upper Radika was populated "with *S. farioides*, and the lower with *S. montenigrinus*". However, additional sampling would be needed in the Radika to draw any statistically support for the possible existence there of two taxonomic units.

Recently, a local initiative to protect and increase the populations of upper Vardar trout in the waters in FYROM has arisen, including the establishment of broodstock for production of juveniles to be released into the wild. Since introgression with non-native genes was detected in many of the populations analysed in the Aegean basin in FYROM, proper selection of breeding animals is crucial to maintaining the genetic identity of the native trout. Successful preservation of such trout will only be achieved if broodstock are selected on the basis of genetic testing. So far, only trout from the Došnica and Suva rivers are recommended for repopulation and reintroduction. In addition to the genetic strain, genetic diversity should also be taken into consideration in broodstock creation. Low levels of genetic diversity were observed in some of the studied Vardar and Crna Reka populations (Table 1), especially in non-introgressed native populations, i.e., Došnica, and Konjarska rivers. Because this property is a crucial requirement for populations to cope with environmental changes (Frankham et al., 2004), it is questionable whether the observed levels of genetic diversity would be sufficient for stocked offspring to adapt to various different environments after release. To extend any adaptation capability of stocking material, a greater range of genetic diversity should be incorporated into the broodstocks, either by finding new non-introgressed native populations or by selecting genetically appropriate individuals from mixed populations.

The results of the present study indicate that the proposed ranges of *S. macedonicus* and *S. pelagonicus* coincide with the distribution areas of two genetically homogenic and distinct units. Nevertheless, the inconsistency between the data provided by mtDNA and those from microsatellites imply that any final judgment on the taxonomic status of these two units should be reserved until nDNA SNP and additional morphological analysis are completed. We also suggest that ecological characteristics of the habitats populated by each taxon are analysed, as this might provide information on possible environmental constraints of selection and the presence of alleles associated with adaptation to specific environments. In the meantime, we recommend that both taxa be treated as evolutionary significant units, which must be a consideration for future management activities.

Acknowledgments This study was supported by the Slovenian Research Agency, and the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 173045). Many thanks go to Simo Georgiev who provided very useful information about trout translocations in Republic of Macedonia, and Iain F. Wilson for English revision of the manuscript.

References

Apostolidis, A. P., D. Loukovitis & C. S. Tsigenopoulos, 2008a. Genetic characterization of brown trout (*Salmo trutta*) populations from the Southern Balkans using mtDNA sequencing and RFLP analysis. Hydrobiologia 600: 169–176.

Apostolidis, A. P., M.-J. Madeira, M. M. Hansen & A. Machodrom, 2008b. Genetic structure and demographic history of brown trout (*Salmo trutta*) populations from the southern Balkans. Freshwater Biology 53: 1555–1566.

Apostolidis, A. P., M. Th. Stoumboudi, E. Kalogianni, G. Cote & L. Bernatchez, 2011. Genetic divergence among native trout Salmo trutta populations from southern Balkans based on mitochondrial DNA and microsatellite variation. Journal of Fish Biology 79: 1950–1960.

Bandelt, H. J., P. Forster & A. Röhl, 1999. Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution 16(1): 37–48.

Belkhir, K., P. Borsa, L. Chikhi, N. Raufaste & F. Bonhomme, 1996–2004. GENETIX v. 4.04, Logiciel sous WindowsTM pour la Génétique des Populations. Université Montpellier 2, Laboratoire Génome et Population, Montpellier.

Berrebi, P., C. Tougard, S. Dubois, Zh Shao, I. Koutseri, S. Petkovski & A. J. Crivelli, 2013. Genetic diversity and conservation of the Prespa trout in the Balkans. International Journal of Molecular Sciences 14: 23454–23470.

Bernatchez, L., 2001. The evolutionary history of brown trout (*Salmo trutta* L.) inferred from phylogeographic, nested clade, and mismatch analyses of mitochondrial DNA variation. Evolution 55: 351–379.

Buroker, N. E., J. R. Brown, T. A. Gilbert, P. J. O'Hara, A. T. Beckenbach, W. K. Thomas & M. J. Smith, 1990. Length heteroplasmy of sturgeon mitochondrial DNA: an illegitimate elongation model. Genetics 124: 157–163.

Cortey, M., C. Pla & J. L. García-Marín, 2004. Historical biogeography of Mediterranean trout. Molecular Phylogenetics and Evolution 33: 831–844.

Delpino, I., 1935. La diffusione e la distribuzione in Italia della *Trutta genivittata* (Heckel-Kner 1858). Bollettino di Pesca Piscicoltura e Idrobiologia Roma 11: 196–210.

Delling, B., 2003. Species diversity and phylogeny of *Salmo* with emphasis on southern trouts (Teleostei, Salmonidae). Unpublished D. Phil. Thesis, Stockholm University.

Dimovski, A. & R. Grupče, 1971. L'Ichtyofaune de la riviere Brégalnitsa. Izdanija, Institut de Pisciculture de la RS de Macedonie. Skopje IV(7): 1–37.

Evanno, G., S. Regnaut & J. Goudet, 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14: 2611–2620.

Frankham, R., J. D. Ballou & D. A. Briscoe, 2004. A primer of conservation genetics, 1st ed. University Press, Cambridge.

Fumagalli, L., A. Snoj, D. Jesenšek, F. Balloux, T. Jug, O. Duron, F. Brossier, A. Crivelli & P. Berrebi, 2002. Extreme genetic differentiation among the remnant populations of marble trout (*Salmo marmoratus*) in Slovenia. Molecular Ecology 11(12): 2711–2716.

Georgiev, S., 2011. Study of *Salmo* genus (Pisces, Salmonidae) on the Balkan Peninsula. Ribarstvo 69: 11–20.

Goudet, J., 1995. FSTAT (Version 1.2): a computer program to calculate F-statistics. Journal of Heredity 86: 485–486.

Hewitt, G. M., 1999. Post-glacial re-colonization of European biota. Biological Journal of the Linnean Society 68: 87–112.

Karaman, S., 1924. Pisces Macedoniae. Hrvatska Stamparija, Split: 90.

Karaman, S., 1927. Salmonidi Balkana. Glasnik Skopskog Naučnog Društva 2: 253–268.

Karaman, S., 1938. Beitrag zur Kenntnis der Süsswasserfische Jugoslawiens. Bulletein de la Société Scientifique de Skoplje 18: 131–139.

Karaman, S., 1957. The Radika river trouts. Folia Balcanica 10: 57–70.

Kohout, J., A. Šedivá, A. Apostolou, T. Stefanov, S. Marić, M. Gaffaroğlu & V. Šlechta, 2013. Genetic diversity and phylogenetic origin of brown trout *Salmo trutta* populations in eastern Balkans. Biologia 68(6): 1229–1237.

Kostov, V., K. Rebok, V. Slavevska-Stamenković & M. Ristovska, 2010: Fish fauna of River Bregalnica (R. Macedonia). In BALWOIS, Proceedings of the Fourth Conference on Water Observation and Information System for Decision Support. Ohrid, 1–8.

Kottelat, M. & J. Freyhof, 2007. Handbook of European Freshwater Fishes. Kottelat, Cornol, Switzerland & Freyhof, Berlin: 646 pp.

Lerceteau-Kohler, E. & S. Weiss, 2006. Development of multiplex PCR microsatellite assay in brown trout *Salmo trutta*, and its potential application for the genus. Aquaculture 258: 641–645.

Lo Brutto, S., N. Hristovski & M. Arculeo, 2010. Genetic divergence between morphological forms of brown trout *Salmo trutta* L. in the Balkan region of Macedonia. Journal of Fish Biology 76: 1220–1227.

Marić, S., S. Sušnik, P. Simonović & A. Snoj, 2006. Phylogeographic study of brown trout from Serbia, based on mitochondrial DNA control region analysis. Genetics Selection Evolution 38: 411–430.

Marić, S., B. Kalamujić, A. Snoj, A. Razpet, L. Lukić-Bilela, N. Pojskić & S. Sušnik Bajec, 2012. Genetic variation of

European grayling (*Thymallus thymallus*) populations in the Western Balkans. Hydrobiologia 691: 225–237.

Phillips, R. B., M. P. Matsuoka, I. Konon & K. M. Reed, 2000. Phylogenetic analysis of mitochondrial and nuclear sequences supports inclusion of *Acantholingua ohridana* in the genus *Salmo*. Copeia 2: 546–550.

Pritchard, J. K., M. Stephens & P. Donnelly, 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945–959.

Pustovrh, G., S. Sušnik Bajec & A. Snoj, 2011. Evolutionary relationship between marble trout of the northern and southern Adriatic basin. Molecular Phylogenetics and Evolution 59: 761–766.

Pustovrh, G., A. Snoj & S. Sušnik Bajec, 2014. Molecular phylogeny of *Salmo* of the western Balkans, based upon multiple nuclear loci. Genetics Selection Evolution 46: 7.

Razpet, A., S. Sušnik, T. Jug & A. Snoj, 2007. Genetic variation among trout in the River Neretva basin, Bosnia and Herzegovina. Journal of Fish Biology 70: 94–110.

Ristovska, M., V. Kostov, D. Prelic, V. Slavevska-Stamenković & J. Arsovska, 2011. Fish community structure and water quality assessment of Babuna River. Journal of International Environmental Application & Science 6(4): 508–517.

Sambrook, J., E. F. Fritsch & T. Maniatis, 1989. Molecular cloning. A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sell, J. & Z. Spirkovski, 2004. Mitochondrial DNA differentiation between two forms of trout *Salmo letnica*, endemic to the Balkan Lake Ohrid, reflects their reproductive isolation. Molecular Ecology 13: 3633–3644.

Sidorovski, M., 1955. Nekoi Morfološki karakteri na pastrmkite od Mavrovskoto ezero i reka Radika. Izdanija, Institut de Pisciculture de la RP Macedoine 1(5): 135–147. (in Macedonian).

Sidorovski, M., 1960. Plodnost pastrmki iz Mavrovskog Jezera i reke Radike. Publikacije stručnog udruženja za unapređenje slatkovodnog ribarstva Jugoslavije 1: 60. (in Serbian).

Snoj, A., E. Melkič, S. Sušnik, S. Muhamedagić & P. Dovč, 2002. DNA phylogeny supports revised classification of *Salmothymus obtusirostris*. Biological Journal of the Linnean Society 77(3): 399–411.

Snoj, A., S. Marić, P. Berrebi, A. Crivelli, S. Shumka & S. Sušnik, 2009. Genetic architecture of trout from Albania as revealed by mtDNA control region variation. Genetics Selection Evolution 41: 22.

Snoj, A., B. Glamuzina, A. Razpet, J. Zablocki, I. Bogut, E. Lerceteau-Köhler, N. Pojskić & S. Sušnik, 2010. Resolving taxonomic uncertainties using molecular systematics: *Salmo dentex* and the Balkan trout community. Hydrobiologia 651(1): 199–212.

Stanković, S., 1960. The Balkan Lake Ohrid and its living world. Monographiae Biologicae, Vol. IX. Uitgeverij Dr. W. Junk, Den Haag.

Sušnik, S., I. Knizhin, A. Snoj & S. Weiss, 2006. Genetic and morphological characterization of a Lake Ohrid endemic, *Salmo* (*Acantholina*) *ohridanus* with a comparison to sympatric *Salmo trutta*. Journal of Fish Biology 68(A): 2–23.

Sušnik, S., A. Snoj, I. F. Wilson, D. Mrdak & S. Weiss, 2007a. Historical demography of brown trout (*Salmo trutta*) in the Adriatic drainage including the putative *S. letnica* endemic to Lake Ohrid. Molecular Phylogenetics and Evolution 44: 63–76.

Sušnik, S., S. Weiss, T. Odak, B. Delling, T. Treer & A. Snoj, 2007b. Reticulate evolution: ancient introgression of the Adriatic brown trout mtDNA in softmouth trout *Salmo obtusirostris* (Teleostei: Salmonide). Biological Journal of the Linnean Society 90: 139–152.

Šorić, V., 1990. Salmonids in the Ohrid–Drim–Skadar system. Acta Societatis Zoologicae Bohemoslovacaei 54: 305–319.

Tamura, K., G. Stecher, D. Peterson, A. Filipski & S. Kumar, 2013. MEGA6: molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution 30: 2725–2729.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin & D. G. Higgins, 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876–4882.

Uiblein, F., A. Jagsch, W. Honsig-Erlenburg & S. Weiss, 2001. Status, habitat use, and vulnerability of the European grayling in Austrian waters. Journal of Fish Biology 59: 223–247.

Vähä, J. P., J. Erkinaro, E. Niemelä & C. R. Primmer, 2007. Life-history and habitat features influence the within-river genetic structure of Atlantic salmon. Molecular Ecology 16: 2638–2654.

Van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills & P. Shipley, 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4: 535–538.

Wilson, I., 2004. Low genetic variability in the summer Koran (*Salmo letnica aestivalis* Stefanovic) of Lake Ohrid. The Albanian Journal of Natural & Technical Sciences 16: 3–13.

Terms and Conditions

Springer Nature journal content, brought to you courtesy of Springer Nature Customer Service Center GmbH (“Springer Nature”).

Springer Nature supports a reasonable amount of sharing of research papers by authors, subscribers and authorised users (“Users”), for small-scale personal, non-commercial use provided that all copyright, trade and service marks and other proprietary notices are maintained. By accessing, sharing, receiving or otherwise using the Springer Nature journal content you agree to these terms of use (“Terms”). For these purposes, Springer Nature considers academic use (by researchers and students) to be non-commercial.

These Terms are supplementary and will apply in addition to any applicable website terms and conditions, a relevant site licence or a personal subscription. These Terms will prevail over any conflict or ambiguity with regards to the relevant terms, a site licence or a personal subscription (to the extent of the conflict or ambiguity only). For Creative Commons-licensed articles, the terms of the Creative Commons license used will apply.

We collect and use personal data to provide access to the Springer Nature journal content. We may also use these personal data internally within ResearchGate and Springer Nature and as agreed share it, in an anonymised way, for purposes of tracking, analysis and reporting. We will not otherwise disclose your personal data outside the ResearchGate or the Springer Nature group of companies unless we have your permission as detailed in the Privacy Policy.

While Users may use the Springer Nature journal content for small scale, personal non-commercial use, it is important to note that Users may not:

1. use such content for the purpose of providing other users with access on a regular or large scale basis or as a means to circumvent access control;
2. use such content where to do so would be considered a criminal or statutory offence in any jurisdiction, or gives rise to civil liability, or is otherwise unlawful;
3. falsely or misleadingly imply or suggest endorsement, approval , sponsorship, or association unless explicitly agreed to by Springer Nature in writing;
4. use bots or other automated methods to access the content or redirect messages
5. override any security feature or exclusionary protocol; or
6. share the content in order to create substitute for Springer Nature products or services or a systematic database of Springer Nature journal content.

In line with the restriction against commercial use, Springer Nature does not permit the creation of a product or service that creates revenue, royalties, rent or income from our content or its inclusion as part of a paid for service or for other commercial gain. Springer Nature journal content cannot be used for inter-library loans and librarians may not upload Springer Nature journal content on a large scale into their, or any other, institutional repository.

These terms of use are reviewed regularly and may be amended at any time. Springer Nature is not obligated to publish any information or content on this website and may remove it or features or functionality at our sole discretion, at any time with or without notice. Springer Nature may revoke this licence to you at any time and remove access to any copies of the Springer Nature journal content which have been saved.

To the fullest extent permitted by law, Springer Nature makes no warranties, representations or guarantees to Users, either express or implied with respect to the Springer nature journal content and all parties disclaim and waive any implied warranties or warranties imposed by law, including merchantability or fitness for any particular purpose.

Please note that these rights do not automatically extend to content, data or other material published by Springer Nature that may be licensed from third parties.

If you would like to use or distribute our Springer Nature journal content to a wider audience or on a regular basis or in any other manner not expressly permitted by these Terms, please contact Springer Nature at

onlineservice@springernature.com