

Genetic variation among trout in the River Neretva basin, Bosnia and Herzegovina

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Mitochondrial DNA haplotypes, characteristic of the Adriatic, Danubian and Atlantic lineages of brown trout *Salmo trutta* and of *Salmo obtusirostris* were found in trout inhabiting the River Neretva basin. With the exception of the one associated with softmouth trout, haplotypes were not correlated with operational taxonomic units based on phenotype. Nuclear DNA analysis identified four genetic assemblages corresponding to *S. obtusirostris*, different geographically confined autochthonous *S. trutta* populations, introduced *S. trutta* and a genetically heterogeneous group located between *S. obtusirostris* and *S. trutta* in the dendrogram of individuals, indicating the existence of hybrid swarms in the Neretva basin. Genetic assemblages corresponding to *Salmo marmoratus* and the recently proposed *Salmo cf. montenigrinus* were not detected. The presence of genetic intermediates indicates that the studied taxa are not completely reproductively isolated and that genetic stability has been either anthropogenically interrupted or not yet achieved among Neretva trout. This finding should be considered in management decisions since such an unstable community must be particularly susceptible to breakdown in genetic population structure as a result of hybridization between native and non-native introduced trout stocks.

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Key words: Adriatic drainage; brown trout; hybrids; introgression; marble trout; softmouth trout.

INTRODUCTION

The present coastal areas of the south-western Balkans were never glaciated during the Pleistocene Ice Ages and have as such served as major European refugia for organisms. The existence of such refugia has been confirmed by phylogeographic studies of several non-fish taxa (Hewitt, 1996, 1999; Taberlet *et al.*, 1998) and fish taxa (Bianco, 1990; Durand *et al.*, 1999; Kotlik & Berrebi, 2001), including *Salmo trutta* L. (Garcia-Marin *et al.*, 1999). The co-existing incipient and invading representatives have thus created an opportunity for hybridization and generation of hybrid swarms (Seehausen, 2004). Consequently, a high level of genetic diversity is expected in this region.

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The rivers of the south-western Balkans are grouped into three major catchments draining into the Black, Aegean and Adriatic seas. River Neretva and its tributaries represent the second largest (after River Drin) river system in the east Adriatic watershed and a central habitat for ichthyofauna in this area. Salmonids from the Neretva basin show considerable variation in many aspects of morphology, ecology and behaviour. It is therefore not surprising that on the basis of these characteristics, several species, *i.e.* *S. trutta* (brown trout), *Salmo marmoratus* Cuvier (marble trout), *Salmo obtusirostris* (Heckel) (softmouth trout), *Salmo farioides* (Karaman) and *Salmo dentex* (Heckel) were identified in earlier studies (Heckel, 1852; Karaman, 1937). The existence of *S. trutta* and *S. obtusirostris* in the Neretva basin has never been disputed (Karaman, 1926; Vuković, 1982; Kosorić *et al.*, 1983). Similarly, *S. marmoratus* has been widely recognized as occurring in the basin, but no genetic data have been presented to verify this taxon as synonymous with *S. marmoratus* of the northern Adriatic.

The status of *S. dentex* and *S. farioides* has always been questionable due to inadequate original descriptions, rare sightings and absence of type material for *S. farioides* (Kottelat, 1997). On the basis of a recent morphological study (Delling, 2003), another new species that 'in morphology appears intermediate between *S. trutta* and *S. obtusirostris*' has been spotted in River Neretva and tentatively designated *Salmo cf. montenigrinus* [referring to *Trutta montenigrina* (Karaman) from the River Morača, Montenegro]. For detailed chronology of descriptions of the Neretva trout and changes in their nomenclature, see Kottelat (1997) and Delling (2003).

Natural hybrids such as *S. obtusirostris* × *S. trutta* and *S. marmoratus* × *S. trutta* have been observed and reported for the Neretva basin (Vuković, 1982). The hybridization between autochthonous taxa was confirmed also in a hatchery experiment performed in the fish farm located in the River Buna, a tributary in the lower part of the River Neretva (Kosorić & Vuković, 1969). However, introduction of non-native brown trout has also been practised in River Neretva (Vuković, 1982). For instance, several transfers of brown trout from different locations in Slovenia into the River Neretva were carried out several times during the period of the former Yugoslavia (J. Ocvirk, pers. comm.). According to local fishermen, the stocking of River Neretva with trout of unknown lineage from various fish farms in Bosnia and Herzegovina and elsewhere is still practised (S. Mustafić, pers. comm.). Moreover, stocking activities have never been well documented, especially in the disturbed post-war years, and no official information is therefore available regarding the purpose of stocking and the origin or the numbers of stocked fish.

Despite the apparent complexity and disputed taxonomy of *Salmo* in the system, no systematic study has been undertaken to verify the various operational taxonomic units (OTUs) in the Neretva basin. Specifically, no phylogeographic comparisons with the commonly accepted evolutionary lineages of brown trout (Bernatchez, 2001; Suarez *et al.*, 2001) have been performed and no studies have been undertaken in this river system in terms of possible interactions between native and introduced trout populations. Thus, it is clear that the status of Neretva trout is insufficiently determined. The main goal of the present study was, therefore, to answer the following questions. (1) Which mtDNA

lineages exist in the Neretva basin? (2) Are there any population substructures? (3) Is there congruence among mtDNA lineages and any genetically determined substructures and operational taxonomic groups based on previous phenotypic inferences (*i.e.* with proposed species names)? Four microsatellite loci, which emerged as informative in the previous studies of Balkan trout (unpubl. data), were chosen for analysis along with the mtDNA control region (CR) and two nuclear gene regions [part of lactate dehydrogenase LDH-C1* and the C intron of growth hormone 2 (GH2C)], which have already been used in similar studies (Oakley & Phillips, 1999; Snoj *et al.*, 2002; Phillips *et al.*, 2004) and which therefore offer a suitable comparative experimental system. On the basis of lineage-specific mtDNA haplotypes and nuclear DNA data processed with a Bayesian approach, the authors also aimed to distinguish between indigenous and introduced trout and thus examine the impact, if any, of stocking.

MATERIALS AND METHODS

SAMPLING, CLASSIFICATION OF OTUS AND DNA ISOLATION

In two sampling campaigns undertaken in October 1999 and May 2001, a total of 202, mostly adult, trout were collected in the Neretva basin using electrofishing (Table I). As a result of insufficient official information about stocking, the sampling strategy relied upon consultation with local anglers. The authors sampled both in remote and allegedly unstocked locations, including River Neretva near the village of Ocrkavlje (Ocr) and River Rakitnica (Rak), and in urban and allegedly stocked locations [River Neretva near Konjic (Kon) and Glavatičevo (Gla) and the rivers Ljuta (Lju) and Bukovica (Buk)], along with tributaries with a completely unknown management history [rivers Krupac (Kru) and Ladanica (Lađ)] (Fig. 1). Natural migration of fish between the sampling locations was theoretically possible, with the exception of River Rakitnica, where waterfalls in the lower part of the river prevent upstream fish migration.

TABLE I. Sampling sites, abbreviation, sample size (n), OTU (T, *Salmo trutta*; O, *Salmo obtusirostris*; M, *Salmo marmoratus*; X, OTUx) inferred from phenotypic differentiation among sampled fish, average number of alleles per locus (A), expected (H_e) and observed (H_o) heterozygosities at a particular sampling site and F_{IS} value for populations with $n \geq 30$. All F_{IS} values were calculated with strict Bonferroni corrections and are highly significant ($P < 0.01$)

Sampling site	Abbreviation	n	OTU				A	H_e	H_o	F_{IS}
			T	O	M	X				
Bukovica	Buk	9	9				3.00	0.29	0.31	
Krupac	Kru	11	11				4.17	0.34	0.27	
Ladanica	Lađ	5	5				3.17	0.39	0.43	
Ljuta	Lju	10	9			1	4.00	0.42	0.35	
Glavatičevo	Gla	60	22	22	7	9	9.83	0.69	0.51	0.27
Konjic	Kon	2		1	1		1.67	0.33	0.67	
Ocrkavlje	Ocr	60	57	1	1	1	6.83	0.38	0.30	0.21
Rakitnica	Rak	45	45				6.00	0.47	0.37	0.21

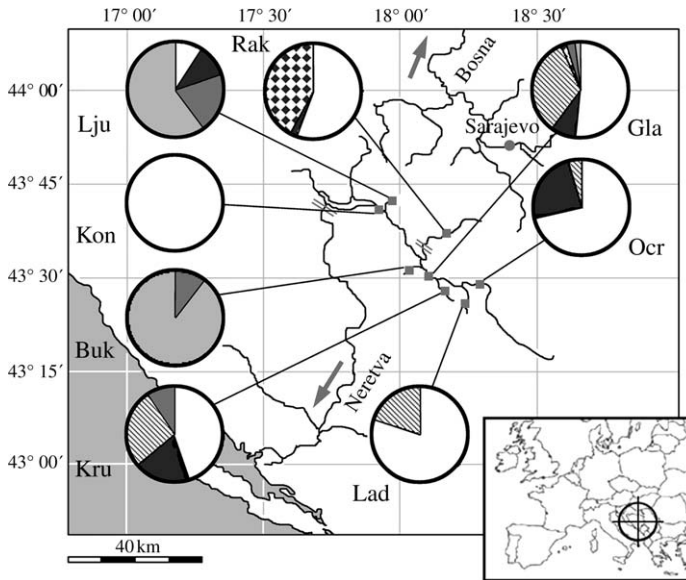


FIG. 1. Map of the River Neretva basin showing sampling locations. See Table I for abbreviations. Short parallel lines indicate dams and impassable barriers. Pies represent the frequency distribution of mtDNA haplotypes detected in a single location. □, AdN; ■, Ad-s3; ▨, Soxy; ▩, Da-s1; ▪, Da-s2; ▮, At-s1.

On the basis of phenotype, the individual fish were sorted into four OTUs corresponding to the species *S. obtusirostris* and *S. trutta*, to the trout with various degrees of marbled colour pattern (marbling) on their bodies, henceforth denoted as marmorated trout, and to a group of phenotypically similar fish (hereafter referred to as OTUx) that did not correspond to any of the other OTUs (Table I). Adult *S. obtusirostris* were easily distinguished phenotypically from all the other OTUs (for a detailed description, see Snoj *et al.*, 2002). Large variation was observed in marbling of marmorated trout and they did not all fulfil the phenotypic description valid for marble trout from the northern Adriatic drainage (Povž *et al.*, 1996; Berrebi *et al.*, 2000; Delling, 2000). The most striking morphometric characteristics of OTUx were, in comparison with *S. trutta*, the short jaw (upper jaw bone reaching to part to half of eye), with deep maxilla and deep body at origin of anal fin; *S. obtusirostris* differed from this group by an even shorter maxilla and subterminal snout. The phenotypic appearance of all the OTUs is available at <http://www.balkan-trout.com>.

Eight brown trout representing the Danubian lineage, two representing the Atlantic lineage and two the *marmoratus* lineage, all originating from different locations across Slovenia, were also included in the investigation as a reference group for GH2C genotyping. The identity of each lineage had been previously determined using mtDNA (Snoj, 2004) and nuclear markers (Jug *et al.*, 2005).

Fin clips were taken from all the individuals and stored in 96% ethanol. All the fish were released back into the river. Total DNA was extracted from the preserved fin tissue using the DNeasy Tissue kit (QIAGEN, Hilden, Germany), following the supplier's instructions.

DNA AMPLIFICATION AND SEQUENCING

MtDNA CR was amplified using primers 28RIBa (Snoj *et al.*, 2000) and HN20 (Bernatchez & Danzmann, 1993). Polymerase chain reaction (PCR) conditions were: 45 s denaturation at 95° C, 45 s of primer annealing at 52° C and 2 min of DNA

extension at 72° C, repeated for 32 cycles. Primers used to amplify *c.* 440 bp of the LDH-C1* gene were designated Ldhxon3F and Ldhxon4R (McMeel *et al.*, 2001) and those for GH2C (421 bp) were GH2CFB and GH2CR (Oakley & Phillips, 1999). PCRs were performed as described by McMeel *et al.* (2001) and Oakley & Phillips (1999).

Amplified DNA fragments were run on 1.5% agarose and isolated from the gel using the QIAEX II Gel Extraction kit (QIAGEN).

Analysis of PCR fragments was undertaken either by cycle sequencing using BigDye Terminator Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA) or by diagnostic restriction fragment length polymorphism (RFLP) protocols using restriction enzymes. The amplified DNA (*c.* 100 ng of purified PCR product) was sequenced using both forward and reverse PCR primers following ABI PRISM BigDye Terminator protocols.

Four microsatellite loci, Ssa197 (O'Reilly *et al.*, 1996), Str24, Str591 (Poteaux, 1995) and BFRO002 (Sušnik *et al.*, 1997), were amplified using fluorescently labelled forward primers. PCR was performed in 10 µl reaction mixtures containing 0.5 µM of each primer, 0.2 mM dNTP, 1.5 mM MgCl₂, 1 × PCR buffer, 0.5 U of *Taq* polymerase (AB Applied Biosystems) and 50 ng of genomic DNA. The PCR profile comprised initial DNA denaturation (95° C, 3 min) and 33 successive cycles of strand denaturation (94° C, 45 s), primer annealing (53° C for Ssa197, Str24, Str591, 20 s, and 60° C for BFRO002, 25 s) and DNA extension (72° C, 10 s). Aliquots of amplified DNA were mixed with formamide and GeneScan-350 ROX Size Standard (Applied Biosystems) and genotyped on an ABI Prism 310 Genetic Analyser using GeneScan™ Analysis Software 2.1.

MTDNA DATA ANALYSIS

DNA sequences were aligned using the computer programme ClustalX (Thompson *et al.*, 1997). Basic phylogenetic relationships between the CR sequences were presented as a network using the statistical parsimony criterion (Templeton *et al.*, 1992) in the programme TCS 1.3 (Clement *et al.*, 2000). To gain a broader picture of the relationship of these haplotypes to published data, the authors also compared a portion of the CR sequence to several already described haplotypes from the brown trout major mtDNA lineages (Atlantic, Danubian, Mediterranean, Adriatic and *marmoratus*).

NUCLEAR DNA DATA ANALYSIS

A Bayesian-clustering iterative method (programme Structure 2.1; Pritchard *et al.*, 2000; Falush *et al.*, 2003) was applied for nuclear DNA data, assuming no *a priori* assignment of individuals to populations or OTUs. This method uses genotypes to assign individuals statistically, based upon Hardy–Weinberg expectations (HWE), to a user-defined number of anonymous genetic clusters (*K*). In order to determine the number of genetically distinct groups within the sample set, 20 independent analyses of microsatellite and allele-coding single nucleotide polymorphism (SNP) data, using simulations of 100 000 data iterations and 20 000 burn-in iterations, were performed to assign individuals into one to 10 groups (*K* = 1–10). The most probable number of groups was estimated on the basis of the value of lnPr (*X*/*K*) and applying Δ*K* as proposed by Evanno *et al.* (2005).

Microsatellite and allele-coding SNP data were also used for constructing an individual neighbour-joining tree based on Nei's minimum distance (Takezaki & Nei, 1996) using the programme Populations (Langella, 2002).

Microsatellite allele frequency estimates, average number of alleles per locus (*A*), exact probability tests for deviation from HWE and Wright's fixation indices for intra-population deviation from random mating due to heterozygote disequilibrium (*F*_{IS}; Weir & Cockerham, 1984) were performed using the programme GENETIX 4.04 (Belkhir *et al.*, 1996–2004). All tests were conducted using 10 000 permutations. HWE and

F_{IS} testing were performed on selected groups comprising >30 individuals, *i.e.* the populations from Rak (brown trout only), Ocr (predominantly brown trout with a few individuals from other OTUs) and Gla (mixture of OTUs) (Table I).

RESULTS

MITOCHONDRIAL DNA

A nucleotide sequence of 298 bp of mtDNA CR was determined for 81 samples representing the majority of marmorated trout, softmouth trout and OTUx, and randomly chosen samples of the brown trout (Fig. 1). A new haplotype was found within the brown trout samples and, in accordance with the river name (Neretva), designated AdN (accession number DQ297172). Additionally, four haplotypes, Ad-s3 (M97967), At-s1 (M97969), Da-s1 (M97973) and Da-s2 (M97974), already known to be characteristic of the Adriatic, Atlantic and Danubian lineages of brown trout (Bernatchez, 2001), and the haplotype characteristic of *Salmo obtusirostris oxyrhynchus* (Steindachner) (Snoj *et al.*, 2002), herein referred to as Soxy (AF488535), were detected. Phylogenetic analysis placed the haplotype AdN in the Adriatic cluster (Fig. 2). This haplotype differs from the co-existing haplotype Ad-s3 by three autapomorphic characters (T at position 141, C at 259 and T at 278; site numbers in accordance with Bernatchez, 2001) and a nucleotide change at position 59.

The rest of the samples ($n = 121$) were haplotyped using appropriate restriction enzymes (*AluI*, *SatI*, *NdeI* and *SmaI*), which enabled discrimination among the haplotypes obtained by sequencing. By far the most common haplotype found in this study was AdN; present in almost 55% of all samples analysed. The other haplotypes were less common. However, the proportion of the different haplotypes varied according to sampling location (Fig. 1). In the uppermost part of the Neretva (Ocr), only AdN, Ad-s3 and Soxy haplotypes were found, while further downstream (*e.g.* Buk and Lju), Danubian and Atlantic haplotypes were also detected. In River Rakitnica, a mixture of AdN and Da-s1 was detected, along with only a single specimen of Ad-s3.

NUCLEAR DNA MARKERS

Microsatellites

In all, 69 alleles were found across the four microsatellite loci tested (average $A = 17.25$). All four loci were found to be highly polymorphic, exhibiting from eight (BFRO002) to 28 (Str24) alleles. None of the alleles were found to be specifically associated with (private to) any OTU. Some alleles or allele size ranges clearly predominated in *S. obtusirostris* (170 at Str591, 131–137 at BFRO002 and 171–183 at Ssa197). These alleles were, though to a much lesser extent, found also in OTUx and *S. trutta*.

All the populations considered for HWE testing (*i.e.* from Rak, Ocr and Gla) showed highly significant deviation from random mating ($P < 0.001$). Heterozygosity values ranging from 0.30 to 0.51 and highly significant positive F_{IS} values were observed in each of these samples (Table I). These results suggest that the three populations do not represent genetically unified assemblages.

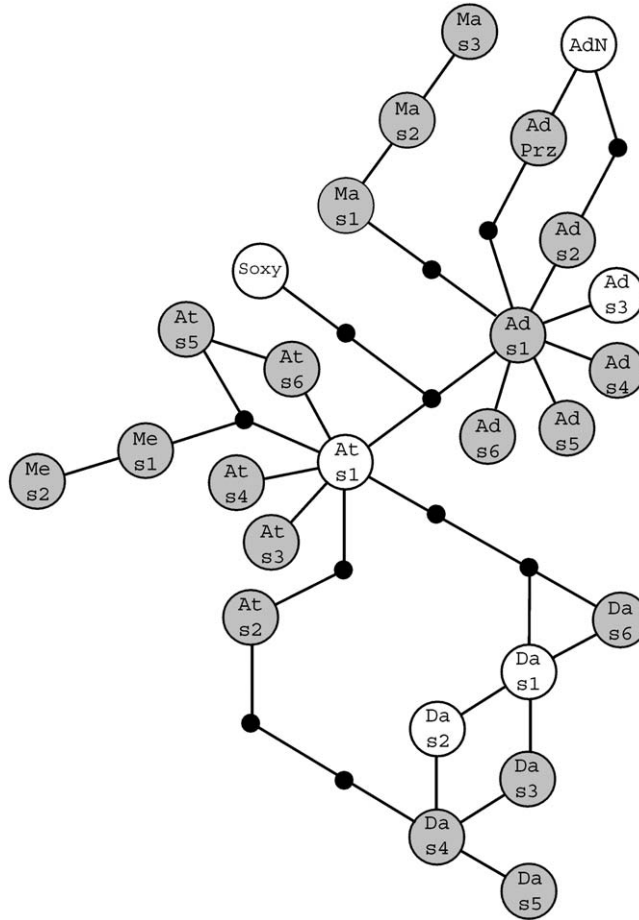


Fig. 2. Network of mtDNA haplotypes (298 bp of CR) found in the River Neretva (white) in relation to selected reference haplotypes (grey). For GenBank accession numbers, see Bernatchez (2001) and Cortey *et al.* (2004).

Lactate dehydrogenase

Based on the published LDH sequences of *S. obtusirostris* (AF488540) and *S. trutta* (AF488538, AF488541 and AF488539) and considering also the LDH sequences of nine Neretva brown trout from this study, an informative SNP at position 259, characterized by nucleotide C (allele LDH-C) in *S. obtusirostris* and by G (allele LDH-G) in *S. trutta*, was detected. An RFLP protocol using *RsaI*, a restriction enzyme that cuts allele LDH-C, was developed and applied to the genotype of the remaining samples.

The allele LDH-G predominated in almost all OTUs, being the most abundant in *S. trutta* (97%). The exception was *S. obtusirostris*, in which allele LDH-C clearly prevailed (Fig. 3).

No particular geographic distribution of the alleles LDH-C and LDH-G was observed across the sampling locations with the exception of Rak, where the former of these alleles was completely absent.

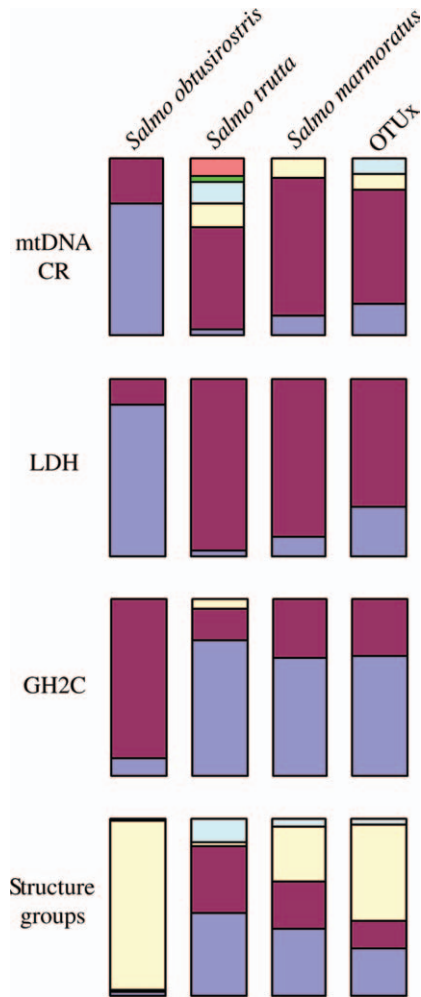


FIG. 3. Distribution of mtDNA haplotypes of LDH and GH2C alleles and of the groups defined by Structure. ■, Soxy; ■, AdN; ■, Ad-s3; ■, Da-s1; ■, Da-s2; ■, At-s1; ■, LDH-C; ■, LDH-G; ■, GH-CC; ■, GH-GC; ■, GH-GA; ■, I; ■, II; ■, III; ■, IV.

C intron of growth hormone 2

Sequencing of 12 individuals chosen randomly from the Neretva samples and 12 reference samples (see Materials and Methods) for GH2C revealed two SNPs located at positions 111 (G/C) and 437 (C/A; numbering as in AF005912) and exhibiting three composite alleles GH-GC, GH-CC and GH-GA. The remaining 190 samples were genotyped using diagnostic RFLP protocols: the first SNP of allele GH-CC was detected using restriction enzyme *Bse*GI, and the second using primer-introduced restriction analysis PCR (PIRA-PCR) with primer GH-CC-PIRA (5'-GCTTCAGGACCTGTGTGTGTAGATCTA-3', where C replaces the normal G) and restriction enzyme *Sfi*I that specifically cuts allele GH-GA.

Allele GH-CC was not specifically linked to any OTU and neither was it detected in the reference group. However, allele GH-GC, which was detected in all OTUs, was prevalent in *S. obtusirostris* (Fig. 3) and was also present in the reference samples representing *S. marmoratus* and the Atlantic lineage of *S. trutta*. Allele GH-GA was detected in only a few *S. trutta* from the Neretva basin, while it was fixed in all reference samples representing the Danubian lineage of *S. trutta*. Allele GH-CC was common at all sampling sites apart from River Bukovica.

DEFINING GENETICALLY HOMOGENEOUS GROUPS

The two approaches that the authors applied in trying to detect genetically homogeneous groups resulted in the following findings: (1) after assigning individuals to $K = 1-10$ groups using the programme Structure, the most probable number of groups in the data set were estimated to be $K = 6$. The same number of groups were also identified by using the *ad hoc* statistic ΔK (Evanno *et al.*, 2005). Nevertheless, at $K = 4$ (groups I-IV; Fig. 3), the genetic variation was already clustered such that it illustrated a basic differentiation between softmouth trout and brown trout and also differentiation within brown trout samples. With $K > 4$, the analysis served only to subdivide further the admixed variation within group I (*i.e.* the one within *S. trutta*; see below) and did not elucidate any new genetic structure as the other groups remained unchanged. (2) An unrooted tree of individuals based on microsatellite and SNP data revealed three terminal clades along with some samples that took an intermediate position in the tree (Fig. 4).

CONGRUENCE BETWEEN OTUS, GENETIC ASSEMBLAGES, MTDNA LINEAGES AND SAMPLING SITES

The brown trout phenotypes predominantly corresponded to Structure groups I, II and IV, and the softmouth trout phenotypes were found mostly in group III (Fig. 3). No genetic assemblages corresponding to marmorated trout or OTUx were revealed, even when $K = 10$ was applied; the individuals of both groups were more or less evenly distributed among groups I, II and III (Fig. 3).

When the clusters were arranged by order of sampling site (Fig. 5), the samples from Buk and Lju corresponded to group IV, those from Rak were found to be associated with group II, while most of the samples from Ocr corresponded to group I. The trout population from Gla turned out to be the most structured, being represented by a relatively high proportion of all the detected groups.

Groups I and II were characterized by the Adriatic mtDNA haplotypes, while group IV exhibited mainly At-s1. Soxy was the most numerous haplotype found in group III (Fig. 3)

When the results of Structure analysis were plotted onto a tree (Fig. 4), most of the individuals of unambiguous groups (*i.e.* those individuals with $q \geq 0.95$) mapped to terminal clades in the tree, while the samples exhibiting the most 'admixed' genotypes took intermediate positions. The four individuals splitting



FIG. 4. Unrooted neighbour-joining tree of individuals based on Nei's minimum distance inferred from SNP and microsatellite data. The individuals corresponding to the particular groups ($q \geq 0.95$) defined by Structure are specifically coloured. Orange, group I; red, group II; blue, group III; green, group IV. The remaining individuals, mainly occupying intermediate positions on the tree, exhibit the most 'admixed' genotypes.

off from the clade corresponding to group IV were phenotypically designated to marmorated trout ($n = 2$), *S. obtusirostris* ($n = 1$) and *S. trutta* ($n = 1$). All these individuals possessed the AdN haplotype and a high proportion (0.7–0.9) of their genetic variation belonged to group III. A closer look at their genotypes revealed four microsatellite alleles (BFRO002 128, Ssa197 222, Str24 186 and Str591 154) common to all of them, while these alleles were extremely rare in the rest of the examined sample set.

DISCUSSION

CONGRUENCE BETWEEN OTU AND GENETIC ASSEMBLAGE

Genetic analysis confirms that the trout of the River Neretva are heterogeneous and structured, supporting the previous findings of phenotypic observations (Heckel, 1852; Karaman, 1937). However, there appears to be little association between the phenotype of an individual Neretva trout and its genetic make-up, with the single exception of members of the species *S. obtusirostris*, which has its own distinct set of mtDNA (haplotype Soxy) and nuclear

DNA markers (Fig. 3). As this species is so phenotypically distinct from *S. trutta*, such a result is to be expected and is in congruence with previous observations (Snoj *et al.*, 2002; Sušnik *et al.*, 2007b).

Salmo trutta in the Neretva basin do not form a genetically unified assemblage. The substructures are not correlated with phenotype but rather with sampling location. Thus, the most distinctive brown trout associations were observed in relation to either remote (supposedly unstocked) or more urban (supposedly stocked) locations (Fig. 5). It is worth noting that the former were strongly associated with the Atlantic haplotype, and the latter with the Adriatic haplotypes. Since Ad-s3 and AdN belong to the Adriatic phylogeographic lineage and At-s1 is common to north Atlantic brown trout and prevalent in commercially available hatchery strains, it is logical to assume that the first two are native to the Neretva basin, while the last is not. This assumption is supported by the fact that the samples bearing the Atlantic haplotype form a distinct group in the Structure (Fig. 5) and a separate cluster in the tree (Fig. 4). From the findings reported here, it can be concluded that remote locations of the Neretva basin are still inhabited by trout that are native, while the supposedly managed stretches are also populated with non-native brown trout. Among the native trout, allele GH-CC was predominant, while it was detected neither in the Danubian and Atlantic reference samples nor in stocked locations in the Neretva basin. This observation leads us to consider this allele as Neretva specific and suitable for discriminating between its native and non-native strains of *S. trutta*.

The presence of the Danubian haplotype in the River Rakitnica is more difficult to explain. This population has been considered indigenous and unaffected by stocking or hybridization with downstream fish owing to waterfalls that prevent upstream migration. The presence of the Danubian haplotype here could be explained by capture in one of the neighbouring rivers belonging to the Bosna River system of the Danubian basin (Fig. 1). As Danubian haplotypes have been detected everywhere in the Danubian part in the Balkans studied so far (Marić *et al.*, 2006), it is probable that they have been occurring also in the Bosna River system. River captures were not uncommon during the Pleistocene (Bănărescu, 2004) and may have caused trout migrations from one drainage into another (Sušnik *et al.*, 2005). However, it is important to note that this population was not in HWE and that an excess of heterozygotes

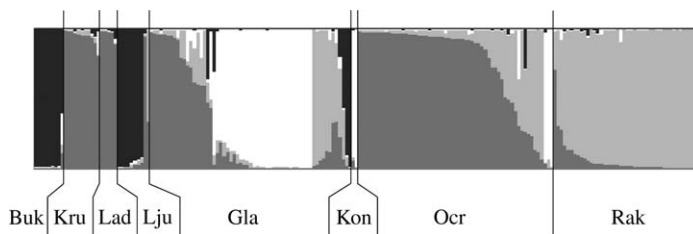


FIG. 5. Structure plot in which each sample is represented by a vertical line and partitioned according to the estimated membership fractions into $K = 4$ groups. Samples are arranged by order of sampling site. See Table I for abbreviations. ■, I; ▒, II; □, III; ■, IV.

along with a significant positive F_{IS} value was found there, indicating the existence of a structured population in which panmixia has not yet been reached. This finding supports the view of a recent and anthropogenic arrival of the Danubian haplotype into the River Rakitnica. The presence of heterozygotes with alleles GH-CC and GH-GA (the latter characteristic of the Danubian reference samples) in the River Rakitnica indicates that the introgression with the introduced brown trout alleles has already occurred. This observation is supported by the fact that after removing individuals holding Danubian haplotypes, the remaining data set still does not fit HWE (data not shown).

No genetic assemblages corresponding to marmorated trout were detected nor were any DNA markers characteristic of marble trout populations of Slovenia and Italy (Ma haplotypes). It is possible that the northern Adriatic marble trout and the marmorated trout from the Neretva basin have evolved a common feature (marbling) separately and they do not share other unique distinctive characters. This is in congruence with a report on a distinct race of brown trout inhabiting the River Otra in Norway that exhibits a marbled colour pattern (Skaala & Solberg, 1997) but that is not monophyletic with northern Adriatic *S. marmoratus* (Delling, 2000). Ma haplotypes have been found in trout inhabiting the rivers to the north and south of the Neretva basin, and in the rivers Krka in Croatia (Bernatchez, 2001) and in Acheloos and Mornos in Greece (Apostolidis *et al.*, 1997), but no marbling was reported for those samples, indicating that this pattern and Ma haplotypes are not correlated. Given the fact that the marmorated individuals were scattered across the different groups identified by Structure analysis, it is possible that they were hybrids between *S. marmoratus* and other OTUs, as a result of which markers diagnostic for *S. marmoratus* might have been missed and the real position of Neretva *S. marmoratus* not revealed. Yet, this presumption seems unlikely given that no genetic markers diagnostic for *S. marmoratus* have been detected in marmorated trout found in the Adriatic part of the Montenegrin river system (Sušnik *et al.*, 2007a).

Special consideration should be given to the individual fish grouped under OTUx. Phenotypically, most correspond to specimens tentatively proposed recently as a new species, *S. cf. montenigrinus* (Delling, 2003). However, these individuals did not form a separate genetic cluster in the dendrogram (Fig. 4) and Structure analysis distributed them among all the inferred groups, mostly with *S. obtusirostris* (Fig. 3). When these samples were analysed for alleles diagnostic of *S. obtusirostris* (e.g. LDH-C, BFRO002 131–137 and Ssa197 171–186), most appeared in a heterozygous state with those characteristic of the other genetic assemblages. The numerous haplotypes (AdN, Soxy, Ad-s3 and Da-s1; Fig. 3) found in OTUx highlighted their large genetic heterogeneity. No genetic uniformity or monophyly was revealed within OTUx, and thus the members of this group cannot be regarded as representatives of a single species or form of species, at least as far as the samples from the River Neretva basin are concerned. Considering also the fact that natural hybrids have already been recorded for the Neretva basin (Vuković, 1982), it seems plausible that these phenotypically similar fish have evolved as a consequence of hybridization between members of the different genetic assemblages. The same may hold true for the four specimens clustered as a sub-branch in the clade corresponding to Structure group IV in the tree of individuals (Fig. 4).

ADMIXTURE BETWEEN *S. TRUTTA* AND *S. OBTUSIROSTRIS*

Although recognized as separate species, confirmed here using molecular markers, the two trout assemblages *S. obtusirostris* and *S. trutta* found in the Neretva basin appear to hybridize and introgress: genetic intermediates were often identified, exhibiting different proportions of the alleles associated with either one or the other assemblage. Interestingly, hybrids were detected in unmanaged locations (e.g. Ocr), indicating that non-native individuals used for stocking are not required for there to be an exchange of genetic material; hybridization in the Neretva should be considered to be a natural process. The natural capability of the two species to introgress had indirectly been proved previously by demonstrating the capture of the Adriatic brown trout mtDNA in *S. obtusirostris* ssp. *salonitana*, originating from the River Jadro in Croatia (Sušnik *et al.*, 2007b). It is interesting how, despite hybridization and admixture, the integrity of the different assemblages is maintained in the Neretva. A possible explanation is that the frequency of hybridization is not high enough to break down the species barrier, as reported for *Salmo salar* L. and *S. trutta* (Scribner *et al.*, 2001).

EVOLUTIONARY HISTORY CONSIDERATIONS

Structured genetic composition along with high levels of morphological variation and low haplotype diversity within the native Neretva trout (*S. obtusirostris* is excluded from this consideration) may indicate relatively recent evolution of the detected assemblages. The maximum time of their independent evolution is connected with the proposed population expansion of the Adriatic lineage that probably took place c. 155 000 years ago in the western Mediterranean as a response to dynamic climate and sea level fluctuations (Cortey *et al.*, 2004; Sušnik *et al.*, 2007a). Although the haplotypes, AdN and Ad-s3 belong to the same evolutionary lineage, it is evident from the haplotype network (Fig. 2) that they do not represent contiguous haplotypes on the haplotype network. The absence of intermediate haplotypes and the prevalence of apomorphy in AdN additionally refute a common ancestry and rather indicate unique arrivals into the Neretva basin, probably as a consequence of successive colonization events. A bimodal mismatch distribution based on mtDNA CR haplotypes has been observed for the Adriatic drainage (Sušnik *et al.*, 2007a), supporting the notion that the diversity of brown trout in the whole Adriatic drainage has been influenced by two colonization events. As the divergence between AdN and Ad-s3 exceeds even the net divergence between the Adriatic and Mediterranean clades (Cortey *et al.*, 2004), it is assumed that the two haplotypes diverged in the initial phase of the Adriatic lineage formation. The haplotype AdN strongly outnumbers Ad-s3 and is more distant from the central haplotype on the network. It is therefore plausible that AdN represents a relict from the first wave of colonization, which followed the expansion within the Adriatic lineage, with Ad-s3 reaching the Neretva basin at a later stage. Wide distribution of the haplotype Ad-s3 throughout the Mediterranean [from the Adriatic rivers in Slovenia (Snoj, 2004) to the islands of Corsica and Sardinia (Bernatchez, 2001)] additionally supports the hypothesis of a more recent dispersal of brown

trout bearing this haplotype. However, the indigenous state of haplotype AdN to the territory of the south-western Balkans has been supported by a recent finding of closely related haplotypes (AdPrz, DQ318129, Marić *et al.*, 2006, Fig. 2; and AD-C1, DQ381567, Sušnik *et al.*, 2007a) present in a neighbouring part of the Adriatic drainage. Since hybrids often exhibit novel or extreme characters compared with parental taxa (Rieseberg *et al.*, 1999; Seehausen, 2004), hybridization between successively invading lineages may be one of the important factors that have contributed to the heterogeneous phenotypes of trout in the Neretva basin.

CONSERVATION ASPECTS

The results presented here clearly show that stocking of non-native trout lineages has been performed in the River Neretva basin; moreover, there are indications that introgression with the introduced brown trout alleles has already occurred. From a conservation point of view, this is an important issue as balance between the different sympatric genetic assemblages present in the basin must be, as a result of establishment of only weak reproductive barriers, very unstable and sensitive. Any abundant introductions, reflected in introgression of exotic alleles into indigenous genomes, may create a bridge for hybridization with native trout resulting in overall panmixia and extinction of the extant genetic assemblages. The harmful effects of such hybridization have already been demonstrated in Adriatic rivers, in the Soča basin in Slovenia and Italy, where *S. marmoratus* and Adriatic grayling (*Thymallus thymallus* L.) have almost been replaced by exotic brown trout and grayling introduced from River Sava (Berrebi *et al.*, 2000; Sušnik *et al.*, 2004). Since the trout genealogy and demography in the Neretva basin are far more complicated than those in the River Soča, extreme caution in management decisions is recommended and any introductions of non-native fishes are to be emphatically discouraged.

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