

# Reticulate evolution: ancient introgression of the Adriatic brown trout mtDNA in softmouth trout *Salmo obtusirostris* (Teleostei: Salmonidae)

S. SUŠNIK<sup>1</sup>, S. WEISS<sup>1</sup>, T. ODAK<sup>2</sup>, B. DELLING<sup>3</sup>, T. TREER<sup>2</sup> and A. SNOJ<sup>4\*</sup>

<sup>1</sup>Karl-Franzens Universität Graz, Institut für Zoologie, Universitätsplatz 2, A-8010 Graz, Austria

<sup>2</sup>University of Zagreb, Faculty of Agriculture, Department of Fisheries, Beekeeping and Special Zoology, Svetošimunska 25, 10000 Zagreb, Croatia

<sup>3</sup>Department of Vertebrate Zoology, Swedish Museum of Natural History, POB 50007, S-104 05 Stockholm, Sweden

<sup>4</sup>University of Ljubljana, Biotechnical Faculty, Department of Animal Science, Groblje 3, 1230 Domžale, Slovenia

Received 30 June 2005; accepted for publication 1 March 2006

Two populations of softmouth trout (*Salmo obtusirostris*) from the rivers Neretva (Bosnia and Herzegovina) and Jadro (Croatia), along with two neighbouring populations of brown trout (*Salmo trutta*) were analysed with a suite of genetic markers (two mtDNA genes, two nuclear genes, and nine microsatellites) as well as morphological characters. The Jadro softmouth trout were fixed for a brown trout mtDNA haplotype of the Adriatic lineage, which is 1.7% divergent from a previously described haplotype characteristic for the Neretva softmouth trout. All other genetic markers, as well as morphological analysis, supported the clear distinction of softmouth trout from the rivers Neretva and Jadro from brown trout in neighbouring populations, and thus a mtDNA capture event is assumed. Population specific microsatellite allele profiles, as well as a high number of private alleles for both populations of softmouth trout, support the hybridization between brown trout and the Jadro softmouth trout most likely being of ancient origin, thus leading to a reticulate evolutionary pattern of mtDNA in this taxon. © 2007 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2007, 90, 139–152.

ADDITIONAL KEYWORDS: Adriatic basin – introgressive hybridization – microsatellites – mtDNA capture.

## INTRODUCTION

Interspecific hybridization is a well accepted evolutionary force in plants (Rieseberg, 1997), but its importance in animal evolution has always been a subject of debate (Arnold, 1992; Seehausen, 2004). However, the widespread application of genetic markers has increasingly revealed patterns of reticulate evolution at the molecular level across many animal taxa, indicating that various modes of hybridization and degrees of introgression are common also in animals. Thus, the recognition that hybridization may be a major evolutionary mechanism in animals is increasing. Among vertebrates, hybridization is per-

haps most common among fishes (Hubbs, 1955; Scribner, Page & Bartron, 2001), presumably due to the lack of physical reproductive barriers in aquatic environments, but also due to increasing environmental disturbance and translocations (Seehausen, Alphen & White, 1997; Allendorf *et al.*, 2001). Dowling & Secor (1997) provide a review of putative hybrid animal taxa, in which fishes are the most common. One example is *Gila seminuda*, a riverine cyprinid from the south-western USA, which allegedly originated through introgressive hybridization between *Gila robusta robusta* and *Gila elegans* (DeMarais *et al.*, 1992; Dowling & DeMarais, 1993). Among the radiations of African cichlids, two recent studies demonstrate the role of interspecific hybridization and reticulate evolution in generating new diversity

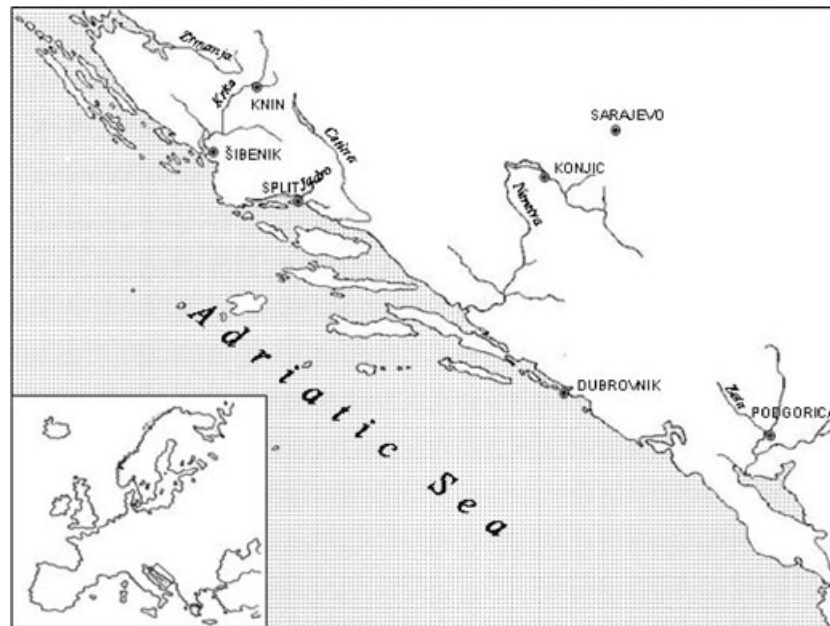
\*Corresponding author. E-mail: ales.snoj@bfro.uni-lj.si

(Salzburger, Baric & Sturmbauer, 2002; Smith, Konings & Kornfield, 2003).

Similar to the situation for Cyprinidae and Cichlidae, natural events of interspecific hybridization are common in Salmonidae, occurring frequently within the genus *Salvelinus* in north America (Bernatchez *et al.*, 1995; Baxter *et al.*, 1996; Redenbach & Taylor, 2002) and among coastal species of *Oncorhynchus* (Young *et al.*, 2001; Ostberg, Slatton & Rodriguez, 2004), and between brown trout and Atlantic salmon *Salmo salar* (Jansson *et al.*, 1991; Gephard, Moran & Garcia-Vazquez, 2000). Natural interspecific hybridization often occurs in so-called hybrid zones, where formerly allopatric lineages come into secondary contact. Such regions exist throughout Europe, but can be particularly plentiful within major peninsula refugia such as Iberia (Gomez & Lunt, 2006). Interestingly, compared with the other two major peninsulas (Iberian and Italian), the Balkan Peninsula has not received the same degree of attention in terms of molecular-based studies and, thus, suspected hybrid events are not well documented. However, the Balkan Peninsula represents one of the major glacial refugia and also one of the 17 biodiversity hotspots of the world (Conservation International, 2004). As such, the Balkans serve as an evolutionary arena, whereby evolutionary lineages repeatedly undergo periods of expansion and contraction, promoting zones of secondary contact, hybridization, and introgression across many taxa (Kryštufek & Reed, 2004). This is presumably the case for the genus *Salmo*, which is especially diverse in the Balkans, exhibiting both putative

archaic and derived forms. One particularly enigmatic taxon, the softmouth trout, is endemic to the Adriatic river system of Western Balkans and was first described from the rivers Zrmanja, Jadro, and Vrljika as *Salar obtusirostris* Heckel (1851). Subsequently, morphological differences gave rise to the description of three additional putative subspecies of *Salar obtusirostris* (Mrakovčić & Mišetić, 1990; Kottelat, 1997): *Salar oxyrhynchus* (Steindachner, 1882) from the River Neretva, Bosnia, and Herzegovina (Fig. 1), *Salar salonitana* from the rivers Jadro and Krka (Croatia), and *Salar krkensis*, also from the River Krka (Karaman, 1927). *Salmo zetensis* (Hadžišće, 1961) from the River Zeta (Montenegro) is also sometimes regarded as a subspecies of *S. obtusirostris* (Marić, 1995). (Phenotypic distinctness between the softmouth trout subspecies is available at: <http://www.balkan-trout.com>.) Historically softmouth trout has been considered as an archaic salmonid in the genus *Salmothymus* Berg, 1908. With the notable exception of Stearley & Smith (1993), more recent comparative studies place *S. obtusirostris* as close to or even within *Salmo* (Svetovidov, 1975; Dorofeyeva, 1999; Sanford, 2000) and, based on molecular data from material collected in the River Neretva, softmouth trout was recently re-classified as *Salmo obtusirostris* (Snoj *et al.*, 2002).

Initial field observations of *S. obtusirostris* from the rivers Jadro and Neretva, together with notes on their morphology (Karaman, 1927; Behnke, 1965), raised questions concerning the evolutionary origin of the Jadro softmouth trout because some characters (e.g.



**Figure 1.** The map of sampling locations.

coloration and mouth shape) appear intermediate between other softmouth populations and brown trout. Softmouth trout spawn in the spring (Karaman, 1927), but they have been shown to successfully hybridize with brown trout in captivity, when late spawning brown and early spawning softmouth trout are used (Kosorić & Vuković, 1969). Natural hybridization is also reported from the Neretva basin (Vuković, 1982). In light of these observations, we sought to evaluate the evolutionary origin of the softmouth trout from the River Jadro.

We applied both maternally and bi-parentally inherited molecular markers to samples from the River Jadro, as well as softmouth trout from the Neretva basin, and samples from two brown trout populations from neighbouring locations. For additional nonmolecular support of our inferences, we also analysed the available morphological data from softmouth specimens from the rivers Jadro and Neretva, as well as brown trout from one sympatric (Neretva) and one nearby population (Krka). Because there is no rigid and reliable classification of softmouth and brown trout, we use informal names (e.g. 'Jadro softmouth trout' and 'Adriatic brown trout') throughout the text.

## MATERIAL AND METHODS

### SAMPLES AND DNA EXTRACTION

Sixty-seven fish were caught by electrofishing across four locations from 1999 to 2003. Nineteen individuals came from the River Jadro, assumed to contain only softmouth trout; 16 softmouth trout came from the River Neretva, and the remaining samples were brown trout from the rivers Krka ( $N = 20$ ) and Zrmanja ( $N = 12$ ) (Table 1, Fig. 1). Total DNA was isolated from fin clips, preserved in ethanol, using the Wizard Genomic DNA Purification Kit (Promega).

### MTDNA AMPLIFICATION AND SEQUENCING

Two mtDNA [control region and cytochrome *b* (*cyt b*)] and two nuclear gene regions (LDH-C1\* and ITS1), which were shown to be informative in differentiating softmouth and brown trout (Snoj *et al.*, 2002; Sušnik,

Schöffman & Snoj, 2004) were chosen for analysis. Polymerase chain reaction (PCR) amplification of an approximately 2400-bp mtDNA fragment spanning the entire *cyt b* gene and control region (CR) was performed using primers HN20 (Bernatchez & Danzmann, 1993) and C-Glu (Cronin, Spearman & Wilmot, 1993). The PCR conditions were: initial DNA denaturation (95 °C for 3 min) and 30 successive cycles of strand denaturation (94 °C for 45 s), primer annealing (52 °C for 45 s) and DNA extension (72 °C for 2 min). Primers Ldhxon3F and Ldhxon4R were used for amplifying approximately 440 bp of the LDH-C1\* gene, as previously described (McMeel, Hoey & Ferguson, 2001). Primers KP2 and 5.8S were used to amplify approximately 630 bp of the ITS1 region according to Phillips *et al.* (2000).

All DNA-amplifications were performed in a programmable thermocycler GeneAmp® PCR System 9700 (AB Applied Biosystems). A total volume of 30 µL contained 1 µM of each primer, 0.2 mM dNTP, 1.5 mM MgCl<sub>2</sub>, 1 × PCR buffer, 1 U of *Taq* polymerase (PE Applied biosystems) and 100 ng of genomic DNA. Amplified DNA fragments were run on a 1.5% agarose gel and were isolated from the gel using QIAEX II Gel Extraction Kit (Qiagen).

The analysis of gene fragments was done by either cycle sequencing using BigDye Terminator Ready Reaction Mix (PE Applied Biosystems) according to manufacturer's recommendations, or via the development of diagnostic restriction fragment length polymorphism (RFLP) protocols. For sequencing, primer 28RIBa (Snoj *et al.*, 2000) was used for the 5'-end of mtDNA CR, C-Glu for the 5'-end of the *cyt b* gene, Ldhxon3R for the LDH-C1\* fragment, and KP2 and 5.8S for the ribosomal ITS1. Termination PCR-s were performed under the conditions: 10 s denaturation at 96 °C, 5 s annealing at 50 °C and 4 min extension at 60 °C, repeated for 30 cycles. Sequences were analysed on an ABI PRISM 310 automated sequencer.

### MICROSATELLITES

Seven di-nucleotide and one tetra-nucleotide microsatellite loci isolated and characterized from other salmonid species were chosen for analysis. The loci

**Table 1.** Description of sampling localities, species name, number of individuals analysed ( $N$ ), number of sequenced samples ( $N_{\text{seq}}$ ), the year of sampling, and haplotype designation

Population	Location	Taxon	$N$	$N_{\text{seq}}$	Year of sample collection	MtDNA haplotypes
Jadro	Croatia	Softmouth trout	19	7	2003	Ad11
Neretva	Bosnia and Herzegovina	Softmouth trout	16	16	1999	Soxy
Krka	Croatia	Adriatic brown trout	20	10	2003	AdBoz
Zrmanja	Croatia	Adriatic brown trout	12	12	2002, 2003	Ad4, Ad12

names, literature reference and GenBank accession numbers are: BFRO001 (Snoj, Pohar & Dovec, 1997), U90327; BFRO002 (Sušnik *et al.*, 1997), AF005074; Ssa197 (O'Reilly *et al.*, 1996), U43694; Str24 and Str58 (Poteaux, Bonhomme & Berrebi, 1999), U60225 and U60223; Str591INRA (Presa & Guyomard, 1996), AB001064; Ssosl438 (Slettan, Olsaker & Lie, 1996), Z49134; and One $\mu$ 2: (Scribner, Gust & Fields, 1996), U56700. An additional locus, Str-LDH4 (GenBank no. AY365467), located in the fourth intron of the LDH-C1\* gene, was amplified using the primers Str-LDHF (5'-TCATCAAACACTCCCCCAACTGC-3') and LDH1R (McMeel, Hoey & Ferguson, 2001). All forward primers were fluorescently labelled. PCR were performed in 10  $\mu$ L reaction mixtures containing 0.5  $\mu$ M of each primer, 0.2 mM dNTP, 1.5 mM MgCl<sub>2</sub>, 1  $\times$  PCR buffer, 0.5 U of *Taq* polymerase (PE Applied biosystems), and 50 ng of genomic DNA. PCR profile comprised initial DNA denaturation (95 °C for 3 min), and 30 successive cycles of strand denaturation (94 °C for 45 s), primer annealing (52 °C for all loci, except 60 °C for BFRO002 and 62 °C for Str-LDH4; 15 s) and DNA extension (72 °C for 5 s), in programmable thermocycler GeneAmp PCR System 9700 (AB Applied Biosystems). Aliquots of fluorescently labelled amplified DNA were mixed with formamide and GENESCAN-350 (TAMRA or ROX) Size Standard (PE Applied Biosystems) and genotyped on the ABI Prism 310 Genetic Analyser using GeneScan Analysis Software, Version 2.1.

#### SEQUENCE ANALYSIS

Mitochondrial gene sequences were aligned using the computer program, Clustal X (Thompson, Higgins & Gibson, 1994). To depict the basic genetic relation of the combined CR and *cyt b* sequences, a statistical parsimony network (Templeton, Crandall & Sing, 1992) was constructed using the program TCS 1.3 (Clement, Posada & Crandall, 2000). To gain a broader picture of the relation of these haplotypes to published data, we also compared a portion of the CR sequence with several already described haplotypes from each of the five major mtDNA lineages (Atlantic, Danubian, Mediterranean, Adriatic, and *Salmo marmoratus*).

#### MICROSATELLITE DATA

Microsatellite allele frequencies (displayed with bubble graphs), the number of alleles per locus (A) and probability tests for deviations from Hardy–Weinberg equilibrium (HWE) and deviations from genotypic linkage equilibrium were performed using program GENETIX 4.04 (Belkhir *et al.*, 1996–2004). All tests were conducted using 10000 permutations. Program FSTAT 2.9.3.2 (Goudet, 2001) was used for calcula-

tion of allelic richness and pairwise  $F_{st}$  values. Corrections for multiple significance tests were made with a sequential Bonferroni-type correction (Rice, 1989).

Genetic relationships between all individuals were estimated based on the proportion of shared alleles at each locus (i.e.  $D_{AS}$  distances; Bowcock *et al.*, 1994). This distance assumes no explicit mutation model, and allows a relatively robust approach to evaluate individual relationships, without a priori designation of an individual to a specific population, or taxon. A matrix of  $D_{AS}$  distances was used to construct a tree of individuals using a Neighbour-joining algorithm. The distance matrix and corresponding tree was obtained using the program POPULATIONS (Langella, 2002). Statistical support estimates for major nodes in the tree were obtained with 1000 bootstrap replicates both across loci and individuals. Global values of  $D_{AS}$  distances are also reported within and between populations and taxa.

#### MORPHOLOGICAL ANALYSIS

Independent of our sampling for genetic tissue, preserved specimens (Museum material; See Supplementary Material) of softmouth trout from the rivers Jadro ( $N = 6$ ) and Neretva ( $N = 12$ ), as well as Adriatic brown trout from the rivers Krka ( $N = 12$ ) and Neretva ( $N = 21$ ) were available for morphological analysis (specimens were fixed in formalin, and thus DNA was not available). A total of 29 morphometric and 15 meristic characters were analysed on all specimens following the procedures outlined in Delling (2002). All morphometric characters are given in percent of standard length to correct for allometry. Means and standard deviations are reported for each character, and a Mann–Whitney  $U$ -test was applied to test for significant differences between each taxon. To depict the general relationship of the phenotype for all taxa, a principal components analysis (PCA) was carried out in two steps using a covariance matrix on log-transformed measurements and a correlation matrix on square-rooted counts. Extracted factors of the most informative principal components in each data set were plotted to visualize their effectiveness in delineating populations.

## RESULTS

### MTDNA

A total of 410 bp of the CR and 276 bp of the *cyt b* gene were resolved with sequence analysis in 45 individuals (seven softmouth from Jadro, 16 from Neretva, and 22 brown trout). For softmouth trout, two haplotypes were found: one in the River Jadro and one in the River Neretva. The Neretva haplotype corresponded

to that previously published for softmouth trout (Snoj *et al.*, 2002). However, in the River Jadro, a previously undescribed haplotype is revealed (Ad11; GenBank No. AY653218; following nomenclature in Bernatchez, 2001), which falls clearly within the group of brown trout specific haplotypes (Fig. 2). For the brown trout samples, three haplotypes were found, two in the River Zrmanja and one in the Krka (AdBoz; DQ318128). In the Zrmanja, one haplotype corresponds to Ad4 (Gene Bank No. AY260520), whereas the other is previously unpublished but bears close relation to other haplotypes of the Adriatic lineage and thus is given the name Ad12 (AY653216). New haplotypes differ from previously known Adriatic haplotypes by 2 bp in the CR; 95 and 280 (Ad12) and 194 and 278 (Ad11). By contrast, there was no variation found in the *cyt b* sequence, which corresponds to the previously described Ad3-4 haplotype (Acc. No. X76251). The haplotype found in the River Jadro differs from the Neretva softmouth haplotype by a total of seven substitutions and one indel in the CR, and seven substitutions in the *cyt b* sequence.

To analyse the additional samples ( $N = 22$ ) at the CR, we digested the CR-Cyt *b* PCR product with *Eco130I*, which cut the CR at position 278 and thus distinguishes the newly-described Jadro haplotype from all other brown trout haplotypes, as well as the previously published softmouth haplotype found in the Neretva basin. All individuals ( $N = 19$ ) from the River Jadro were characterized with this CR mutation and thus the population is considered to be fixed for the mtDNA haplotype Ad11.

#### RIBOSOMAL DNA ITS-1 AND LDH-C1\*

Sequencing of 22 softmouth trout (six from River Jadro and 16 from River Neretva) for the ITS-1 (574 bp) and LDH-C1\* (380 bp) genes revealed no variation, and a haplotype corresponding to that previously found in softmouth trout from the River Neretva (Snoj *et al.*, 2002; AF488540; Sušnik, Schöffman & Snoj, 2004, AY260509). Ten brown trout samples from the rivers Zrmanja ( $N = 4$ ) and Krka ( $N = 6$ ) were sequenced and characterized by haplotypes previously found in the Adriatic mtDNA lineage (Snoj *et al.*, 2002; Sušnik, Schöffman & Snoj, 2004; GenBank no. AF498756 and AY260507). One minor exception was the presence of one allele in one individual from the River Krka, differing only in the number of cytosines in the poly C stretch located in the LDH-C1\* intron. All other individuals were homozygous across both genes. A total of three substitutions and one indel in the LDH-C1\*, and eight substitutions in the ITS-1 region distinguish the softmouth haplotype and those of the Adriatic clade of brown trout. For the remaining 35 individuals, an RFLP protocol was developed

(*Hin1I* for ITS1 and *RsaI* for LDH) to cut two sites that were diagnostic for softmouth trout. Based on this protocol, all individuals from the rivers Jadro and Neretva carried the alleles characteristic for softmouth trout and this allele did not occur in the two brown trout populations sampled.

#### MICROSATELLITES

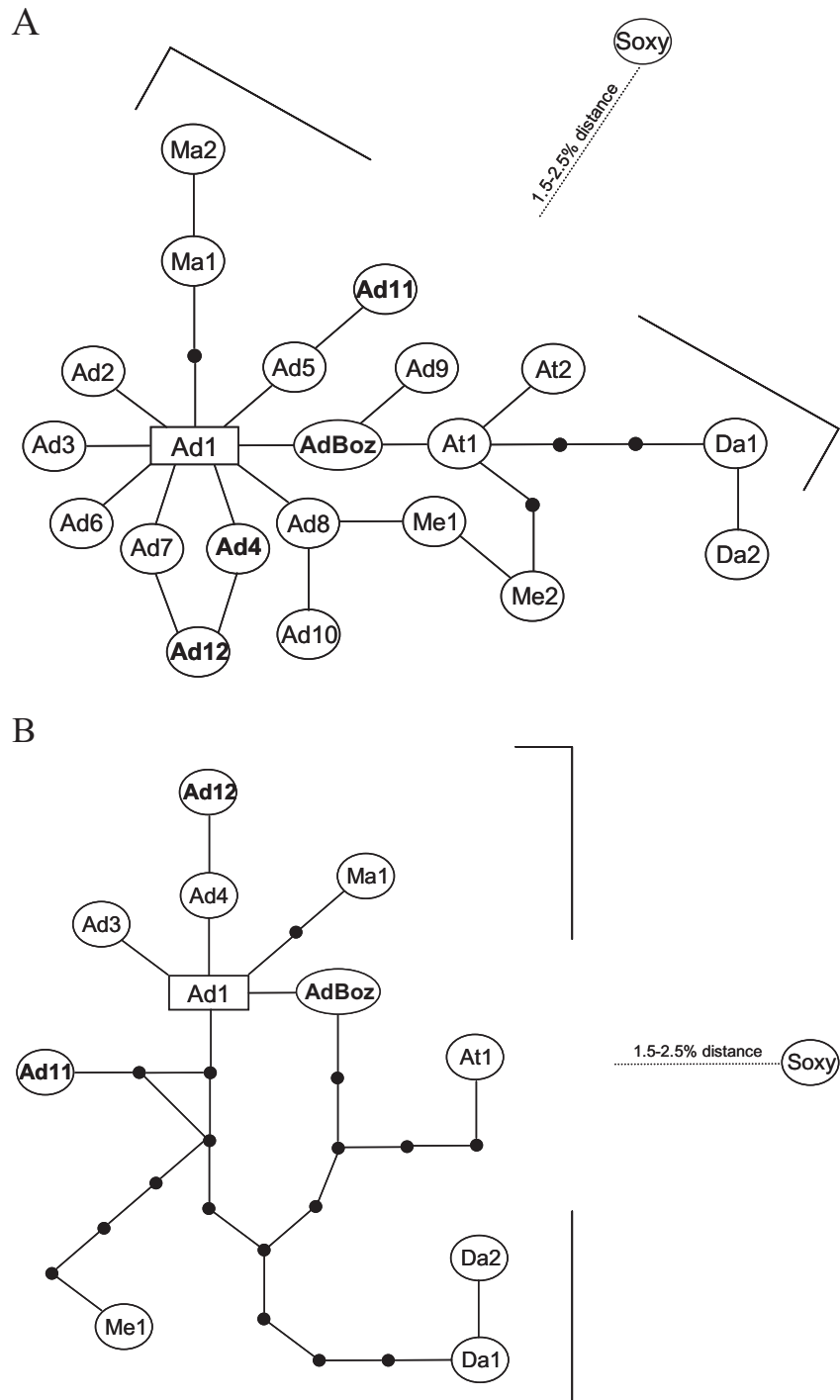
Allele number ( $A$ ), allele richness, observed and expected heterozygosity and departure from Hardy-Weinberg equilibrium are listed in the Supplementary Material. The softmouth trout population from the River Jadro exhibited a significant deviation from HWE at two loci but, over all loci, no deviation was detected. After correction for multiple tests, there was no evidence of significant linkage disequilibrium in the softmouth trout from the River Jadro

Non-overlapping allele size ranges between softmouth trout from the Neretva and brown trout (Zrmanja and Krka populations) are seen for four of the nine loci (BFRO001 & 002; Str58, Str-LDH4), and one additional locus would exhibit such a pattern except for the presence of a shared allele in a single individual (One $\mu$ 2) (Fig. 3). In terms of private alleles, the Neretva softmouth trout had 26 alleles not found in brown trout, and brown trout had 59 alleles not found in the Neretva softmouth. Although all Jadro softmouth trout carried a brown trout mtDNA haplotype, their microsatellite allele size ranges were often similar to the Neretva softmouth trout, although not across all loci. This is reflected in the wide range of locus-specific  $F_{st}$ -values (0.0063 for One $\mu$ 2 to 0.9178 for BFRO001) between the populations of the Jadro and Neretva softmouth trout (Table 2). The population

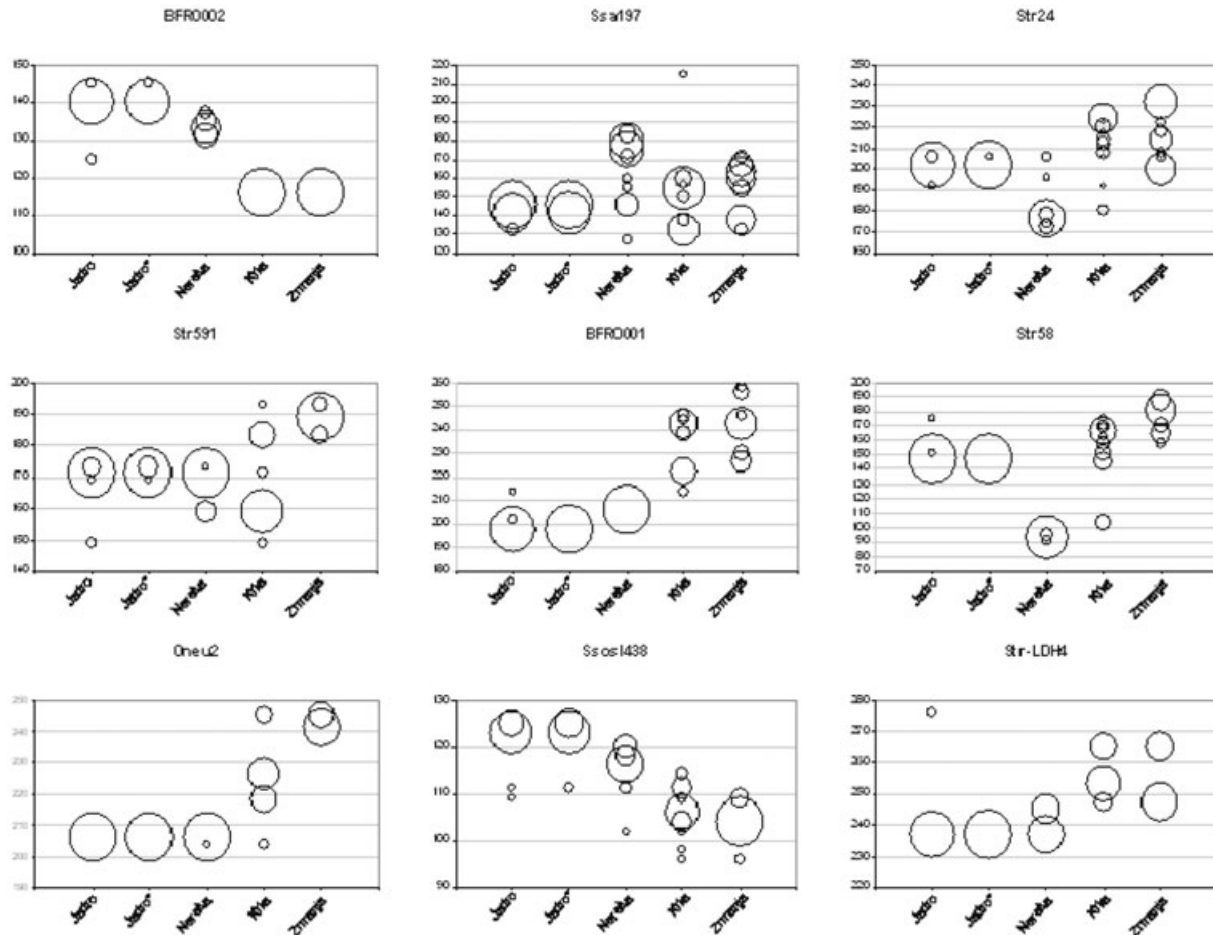
**Table 2.** Locus specific pairwise and overall  $F_{st}$  values

Locus	Pairwise comparison of Jadro population with:			
	Neretva	Krka	Zrmanja	Overall $F_{st}$
BFRO002	0.5273	0.8937	0.8655	0.717
Ssa197	0.2616	0.3537	0.3120	0.257
Str24	0.5958	0.4891	0.5772	0.415
Str591INRA	0.0421	0.4981	0.6137	0.480
BFRO001	0.9178	0.5513	0.6153	0.612
Str58	0.8068	0.5320	0.6581	0.531
One $\mu$ 2	0.0063	0.6679	0.8171	0.635
Ssos1438	0.4058	0.3649	0.5732	0.396
Str-Ldh4	0.1911	0.6165	0.7114	0.504
Overall	0.5359	0.5521	0.6392	

Pairwise  $F_{st}$  values correspond to comparison of the Jadro softmouth with the other analysed taxa/populations.



**Figure 2.** Haplotype network relating (A) new haplotypes [297 bp of mtDNA (CR) control region] with some *Salmo trutta* haplotypes from Bernatchez (2001) and (B) haplotypes with available data for mtDNA CR and cytochrome *b* (686 bp). Circles represent the haplotypes, and solid lines connect each haplotype (regardless of their length) and represent, in theory, single mutational events. Small black circles represent missing or theoretical haplotypes. The acronym ‘Soxy’ corresponds to the haplotype, characteristic for the River Neretva softmouth trout (*Salmo obtusirostris oxyrhynchus*). The Soxy haplotype is beyond the parsimony limit of the network, and thus is depicted only in terms of its percent differentiation (pairwise minimum and maximum) from brown trout haplotypes.



**Figure 3.** Graphical representation of allele size and frequency distributions of the nine microsatellite loci in the Jadro and Neretva softmouth trout and the Adriatic brown trout from the rivers Krka and Zrmanja. In the population denoted with an asterisk, the three introgressed specimens (JAD13, 15, and 17) are not taken into account. The bubble area corresponds to the frequencies of the respective alleles in each population.

**Table 3.** Mean individual pairwise  $D_{AS}$  distance values within (\*) and between populations

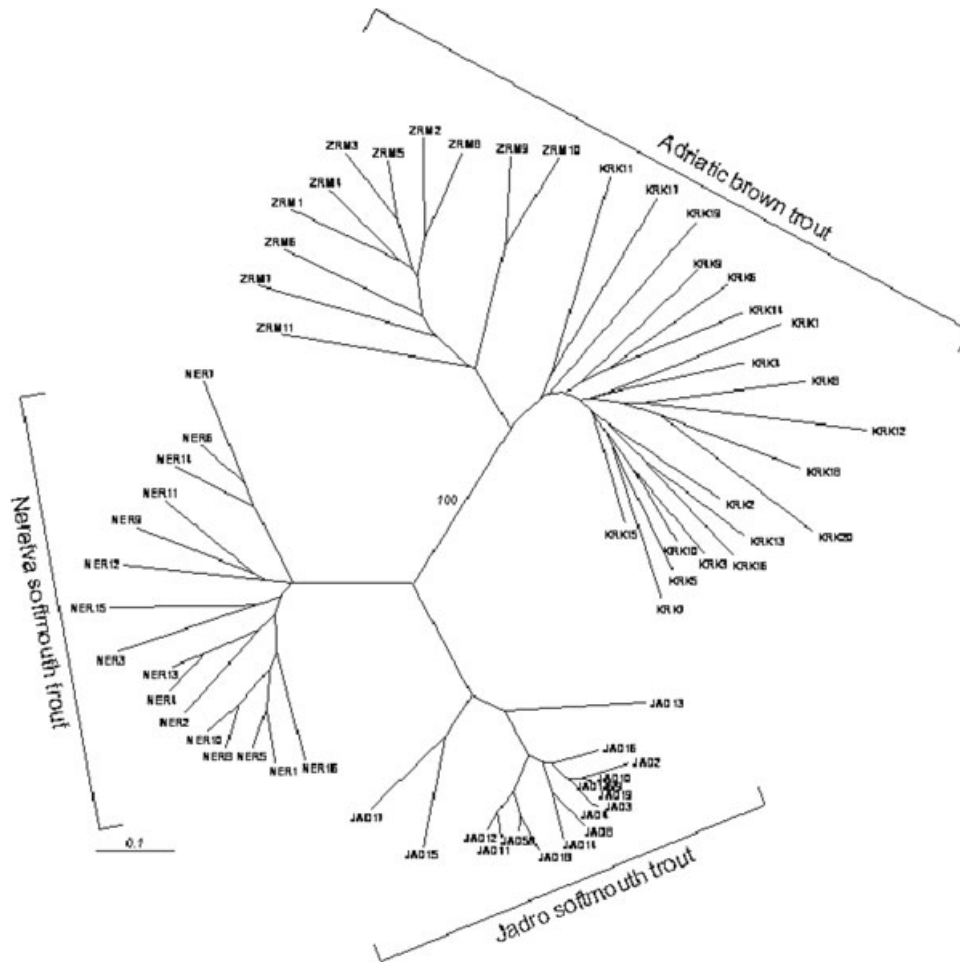
	Jadro softmouth trout	Neretva softmouth trout	Krka brown trout	Zrmanja brown trout
Jadro softmouth trout	0.2084*			
Neretva softmouth trout	0.7358	0.3894*		
Krka brown trout	0.9877	0.9730	0.5426*	
Zrmanja brown trout	0.9958	0.9966	0.7526	0.4343*

of the Jadro softmouth trout was itself distinct, exhibiting 12 private alleles (nine excluding samples JAD13, 15, and 17) in the global data set and 16 private alleles in comparison with brown trout. Nonetheless, there was strong support for the clustering of the Jadro and Neretva softmouth trout in the Neighbour-joining tree of individuals based on  $D_{AS}$  distances (Fig. 4), and differences both within and between soft-

mouth subspecies are much smaller than between softmouth and brown trout (Table 3).

MORPHOLOGY

Eleven of the 16 meristic characters were significantly different between softmouth and brown trout, with most of these being highly significant ( $P < 0.001$ )



**Figure 4.** Neighbour-joining tree of individuals based on  $D_{AS}$  distances and nine microsatellite loci.

(Table 4). Nonetheless, only the number of scales from the base of adipose fin to lateral line (softmouth, 12–14; brown trout, 15–19) and the number of gill rakers on the lower limb of first arch (softmouth, 14–21; brown trout, 10–13) displayed non-overlapping ranges between the species. Five characters also showed significant differences between the Jadro and Neretva softmouth trout (Mann–Whitney  $U$ -test: adipose-to-lateral line scales,  $P = 0.010$ ; left-branchiostegal rays,  $P = 0.032$ ; gills rakers on the lower arch,  $P = 0.001$ ; gill rakers on the upper arch,  $P < 0.001$ ; and dorsal fin position,  $P = 0.024$ ).

Nineteen of 28 morphometric characters showed statistically significant differences between brown trout and softmouth trout. Of these, four of the 19 body characters, and six of the nine head characters were highly significant. Only three characters (jaw and gill raker measurements), showed non-overlapping ranges between the species (Table 5). For the PCA, the first factor in the meristic data set explained 43% of the variance in the data, whereby

over 90% of the variance in the morphometric data set was explained by the first extracted factor. A bivariate plot of the first meristic factor and the second morphological factor delineated the two species extremely well (Fig. 5), showing non-overlapping values on both axes.

## DISCUSSION

All genetic data from the nuclear genome (microsatellites and two coding gene regions), as well as the morphological data (morphometric and meristic characters), reveal an unambiguous distinction between softmouth trout from the rivers Neretva and Jadro, and brown trout from either sympatric or neighbouring populations. However, the mtDNA control region sequence from the Jadro softmouth trout reflects a reticulate evolutionary pathway, differing by 1.7% from a previously described softmouth trout haplotype from the River Neretva, and only a single nucleotide divergent (considering an abbreviated sequence of

**Table 4.** Number of individuals, means and standard deviation for the 15 meristic characters analysed in the four populations

Meristic characters	Taxon group				Mann–Whitney <i>U</i>	
	Softmouth trout		Brown trout		<i>Z</i>	<i>P</i>
	Jadro ( <i>N</i> = 6)	Neretva ( <i>N</i> = 12)	Neretva ( <i>N</i> = 21)	Krka ( <i>N</i> = 12)		
Scales: adipose-to lateral line	12.50 ± 0.55	13.50 ± 0.52	17.19 ± 1.12	16.75 ± 1.14	-5.929	< 0.001
Left branchiostegal rays	10.67 ± 0.82	11.58 ± 0.51	10.71 ± 0.72	11.42 ± 0.67	-1.362	0.173
Right branchiostegal rays	10.67 ± 0.52	11.25 ± 0.45	10.33 ± 0.73	10.75 ± 0.45	-2.860	0.004
Gill rakers on lower arch	15.50 ± 1.05	17.92 ± 1.31	10.81 ± 0.75	11.75 ± 0.75	-5.956	< 0.001
Gill rakers upper arch	9.00 ± 0.63	10.83 ± 0.83	7.24 ± 0.44	6.75 ± 0.45	-6.195	< 0.001
Total vertebrae	57.33 ± 0.82	57.75 ± 0.45	57.38 ± 0.59	57.08 ± 0.67	-2.130	0.033
Abdominal vertebrae	35.83 ± 0.41	35.67 ± 0.49	34.05 ± 0.80	34.08 ± 0.51	-5.735	< 0.001
Caudal vertebrae	21.50 ± 0.55	22.08 ± 0.29	23.33 ± 0.58	23.00 ± 0.60	-5.508	< 0.001
Dorsal fin position	14.50 ± 0.55	15.33 ± 0.49	13.90 ± 0.62	14.00 ± 0.74	-4.573	< 0.001
Anal fin position	34.33 ± 0.52	34.00 ± 0.60	32.43 ± 0.68	32.50 ± 0.80	-5.602	< 0.001
Dorsal fin pterygiophores	13.00 ± 0.00	13.58 ± 0.51	13.52 ± 0.68	13.00 ± 0.60	-0.256	0.798
Anal fin pterygiophores	11.00 ± 0.00	10.67 ± 0.65	10.48 ± 0.75	10.33 ± 0.65	-1.690	0.091
Caudal fin upper procurrent rays	11.67 ± 0.52	12.33 ± 0.65	13.76 ± 0.77	13.67 ± 0.89	-5.151	< 0.001
Caudal fin lower procurrent rays	10.50 ± 0.84	11.17 ± 0.39	12.33 ± 0.80	11.83 ± 0.58	-4.912	< 0.001
Expanded neural spines	4.50 ± 0.55	4.75 ± 0.45	4.48 ± 0.51	4.42 ± 0.51	-1.436	0.151
Vertebrae having expanded neural spine	4.17 ± 0.75	4.50 ± 0.52	4.05 ± 0.59	4.17 ± 0.58	-1.731	0.084

Data are the results of Mann–Whitney *U*-test (*Z*-statistic and *P*-value) between the two groups (softmouth trout and brown trout) for each of 15 meristic characters.

297 bp) from a previously published Adriatic brown trout haplotype (Ad5; Fig. 2A). Thus, the mitochondrial genome of the Jadro softmouth trout appears to stem from hybridization with Adriatic basin brown trout.

The age of this hybridization event is not easy to determine, especially considering that the population is very small and found in a single 4 km-long stream. It is not only fixed for a single mtDNA haplotype but also displays a limited number of microsatellite alleles (one to four alleles per locus). Although stocking of brown trout has occurred in the past, there are presently none in the system and the temperature regime is presumably not optimal for reproduction (Behnke, 1965). Nonetheless, evidence of more recent hybridization is seen in the multilocus genotype of three individuals (JAD13, 15, and 17), which, in the microsatellite tree (Fig. 4) are markedly divergent from the rest of the population, and whose removal results in HWE for the remaining individuals. Because portions of the stream’s habitat have been destroyed through river-bed engineering measures and pollution, it must be assumed that recent bottlenecks have occurred. However, such recent events may not be related to the historical demography of the population, nor the fixation of brown trout mtDNA.

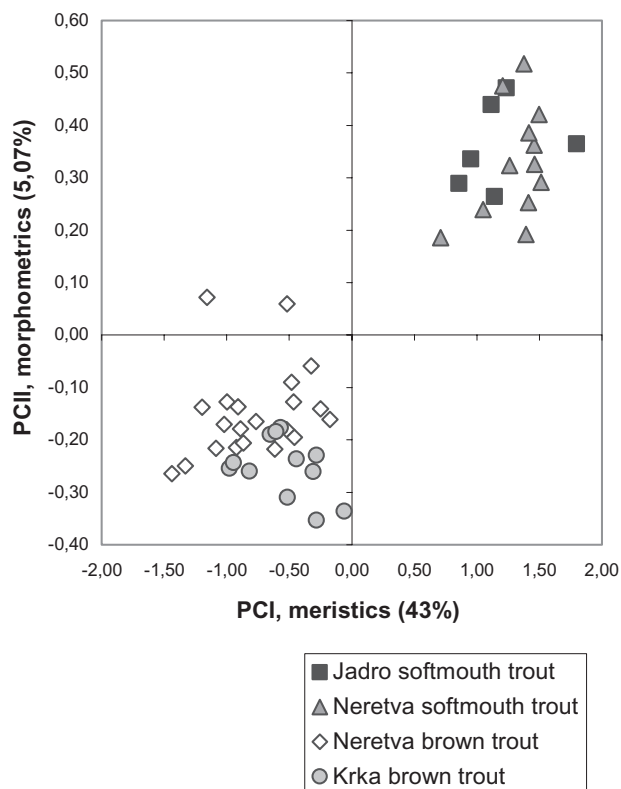
Strong support for a more ancient origin of this introgression is the fact that, despite the limited allelic diversity, 12 of the 26 (or nine of 16 excluding samples JAD13, 15, and 17) microsatellite alleles are private to the global data set, suggesting that this population has been in isolation long enough for mutation and drift to generate a highly unique allelic spectrum. This observation is also reflected in high pairwise *F<sub>ST</sub>* values between the Jadro softmouth and brown trout as well as between the Jadro and Neretva softmouth for some loci (Table 2).

Based on the consistency of all nDNA markers, as well as morphological data in grouping the Jadro and Neretva softmouth trout populations together, there is no evidence for nDNA from brown trout in the Jadro population. Thus, the presence of brown trout mtDNA in the Jadro softmouth trout could be classified as so-called ‘mitochondrial DNA capture’ or nuclear replacement (a purely semantical difference). This mechanism, generating a reticulate evolutionary pathway for the mitochondrial genome, is known from diverse vertebrates such as pocket gophers (Ruedi, Smith & Patton, 1997), tree frogs (Lamb & Avise, 1986), sparrows (Weckstein *et al.*, 2001), and salamanders (Garcia-Paris *et al.*, 2003), as well as fishes (Bernatchez *et al.*, 1995; Ludwig *et al.*, 2003).

**Table 5.** Number of individuals, means and standard deviation for the 28 morphometric characters analysed in the four populations

	Adriatic brown trout												Softmouth trout												Mann-Whitney <i>U</i>			
	Krka						Neretva						Jadro						Neretva									
	<i>N</i>	Min	Max	Mean	SD	<i>N</i>	Min	Max	Mean	SD	<i>N</i>	Min	Max	Mean	SD	<i>N</i>	Min	Max	Mean	SD	<i>N</i>	Min	Max	Mean	SD	<i>Z</i>	<i>P</i>	
Standard length (mm)	12	124.2	264.0	170.2	48.10	21	128.0	269.0	170.2	38.52	6	173.2	236.0	206.6	21.36	12	107.1	226.0	166.6	34.65								
In percent of standard length																												
Prenasal length	12	74.3	79.3	76.6	1.77	21	74.0	78.4	75.6	1.11	6	76.7	79.8	77.7	1.16	12	75.3	79.6	77.2	1.36								
Prepelvic length	12	54.1	59.3	56.6	1.84	21	52.6	56.3	54.2	0.91	6	52.5	56.9	55.2	1.71	12	53.0	58.3	54.9	1.55								
Predorsal length	12	44.4	48.3	46.3	1.14	21	45.1	48.9	46.8	0.93	6	47.1	49.3	48.0	0.92	12	46.0	50.3	48.0	1.22								
Head length	12	23.8	27.5	25.7	1.22	21	23.4	26.6	24.8	0.90	6	22.1	26.2	24.0	1.49	12	20.9	25.4	23.4	1.27								
Premaxilla to preoperculum length	12	16.0	19.2	17.6	1.03	21	15.1	18.1	17.0	0.72	6	14.5	17.9	16.4	1.12	12	14.7	17.4	15.9	0.75								
Caudal peduncle length	12	16.6	18.6	17.5	0.65	21	16.7	19.3	18.1	0.75	6	15.0	17.0	15.7	0.74	12	15.5	18.1	16.9	0.75								
Caudal peduncle depth	12	9.4	10.8	10.1	0.42	21	10.1	11.5	10.8	0.36	6	9.5	10.3	10.0	0.32	12	9.5	10.4	9.9	0.31								
Length of upper caudal fin lobe	12	18.2	21.3	19.9	1.08	21	18.3	21.7	20.2	1.00	6	18.0	20.4	19.5	0.93	12	19.0	21.7	20.3	0.85								
Length of lower caudal fin lobe	12	17.8	22.5	20.0	1.43	21	18.6	21.7	20.1	0.98	6	18.4	21.3	19.8	1.08	12	19.8	22.9	21.0	1.17								
Length of middle caudal fin ray	12	11.9	14.0	12.8	0.71	21	12.3	15.1	13.7	0.71	6	11.6	13.9	12.8	0.84	12	10.5	14.3	12.5	1.01								
Height of dorsal fin	12	14.5	19.2	16.0	1.40	21	15.3	18.8	16.6	0.96	6	14.4	17.5	16.1	1.13	12	13.9	16.7	15.3	0.78								
Length of pectoral fin	12	16.8	21.0	19.3	1.30	21	17.4	21.2	19.7	0.91	6	16.5	19.3	18.0	1.06	12	16.1	21.8	18.3	1.69								
Length of pelvic fin	12	12.9	17.3	15.1	1.17	21	14.2	16.6	15.3	0.76	6	13.9	16.3	14.9	0.95	12	13.1	15.7	14.5	0.82								
Length of adipose fin	12	7.2	9.9	8.4	0.83	21	7.6	11.2	9.7	0.95	6	6.7	8.2	7.5	0.57	12	5.8	9.6	8.3	0.94								
Length of anal fin	12	15.0	17.5	16.4	0.82	21	14.9	17.8	16.6	0.85	6	15.8	18.2	16.8	0.89	12	14.5	17.7	16.1	0.94								
Width of the body	12	9.9	14.2	11.7	1.24	21	12.6	15.1	14.0	0.62	6	10.0	12.0	10.9	0.70	12	9.1	17.5	13.6	2.93								
Depth of the body at origin of dorsal fin	12	23.7	26.0	25.0	0.77	21	23.7	28.3	26.1	0.98	6	24.3	27.0	25.8	0.96	12	23.4	26.7	24.9	1.00								
Depth of the body at origin of anal fin	12	17.8	19.7	18.6	0.53	21	17.9	20.8	19.6	0.77	6	18.0	19.3	18.9	0.48	12	17.1	19.4	18.1	0.76								
Depth of the head	12	13.0	15.3	14.0	0.63	21	12.9	15.0	13.9	0.49	6	12.3	15.5	13.9	1.20	12	11.0	14.3	13.0	0.88								
In percent of head length diameter	12	23.0	28.7	26.4	1.69	21	21.9	29.0	25.4	1.98	6	24.6	30.7	28.0	2.10	12	24.5	31.1	27.7	2.41								
Vertical orbit diameter	12	19.6	24.9	22.6	1.79	21	19.6	26.6	23.1	2.16	6	19.6	26.3	23.1	2.62	12	20.9	28.1	23.7	1.82								
Interorbital width	12	23.8	28.4	26.5	1.26	21	26.9	31.2	29.3	1.35	6	28.3	33.4	30.3	2.07	12	26.2	30.8	29.0	1.40								
Length of snout	12	21.5	27.7	24.6	1.72	21	24.0	30.0	26.3	1.54	6	28.9	32.1	30.0	1.18	12	25.7	32.7	28.2	1.97								
Length of upper jaw	12	47.4	52.3	49.6	1.71	21	49.5	54.5	51.7	1.55	6	39.3	45.5	42.7	2.68	12	36.5	43.8	40.6	2.20								
Length of maxilla	12	36.4	42.8	39.9	1.87	21	36.7	42.2	40.5	1.35	6	31.2	35.1	33.6	1.41	12	28.6	35.0	31.4	1.80								
Height of maxilla	12	10.8	13.0	11.8	0.72	21	10.9	14.0	12.4	0.80	6	13.0	14.9	13.8	0.73	12	12.7	15.3	14.2	0.93								
Length of lower jaw	12	54.8	61.0	57.8	2.14	21	50.3	65.0	59.5	3.24	6	44.4	53.9	49.0	3.42	12	41.4	50.0	46.3	2.82								
Length of gill raker	12	8.0	10.0	8.9	0.69	21	5.9	9.2	7.9	0.95	6	5.6	6.7	6.1	0.41	11	5.6	7.8	6.4	0.56								

Data are the results of Mann-Whitney *U*-test (*Z*-statistic and *P*-value) between the two groups (softmouth trout and brown trout) for each of 28 meristic characters.



**Figure 5.** Bivariate scatterplot of the first principal component derived from principal components analysis (PCA) of 15 meristic characters, and the second principal component derived from PCA of 29 morphometric characters.

The genetic architecture of the Jadro softmouth trout could have developed through uni-directional hybridization (male softmouth  $\times$  female brown trout) followed by repeated backcrossing with only softmouth trout (for a schematic depicting this principle of mtDNA capture, see Weckstein *et al.*, 2001). Due to the disparate spawning times of the two species and the more protracted period of reproductive ability in males, the most likely scenario would involve invading male softmouth trout (or sneak spawners) introgressing into a very small population of brown trout. The chance that stocked brown trout played a role in this event can not be excluded, but appears less likely because it would involve fixation of the female specific mtDNA genome, with no success of stocked males' introgressing the population.

Our initial investigation of the Jadro softmouth trout reveals that it is not only fixed for brown trout mtDNA, but also shows some morphological differences to the Neretva softmouth trout. Statistically significant differences between the two softmouth taxa were seen at five meristic and seven morphometric characters (data not shown). Variation in snout shape, the basis for the description of a long-snouted, 'oxy-

*rhynchus*' softmouth trout from the River Neretva is also observed in this material. However, regarding relative snout length, the material appears to approximately fall into two classes (i.e. short and long snout). Both types are present in both rivers, with the long snout being more common in the River Neretva. It is also noticed, across all population pairwise comparisons, that the largest differences (in number of characters) occurs between Neretva softmouth and Neretva brown trout, a pattern evoking character displacement, although the sample sizes are too low for these comparisons to make firm conclusions (data not shown). Nevertheless, from a multivariate perspective, neither softmouth population appears to be more similar morphologically to brown trout overall (Fig. 5).

Differentiation between the two populations of softmouth was detected also at highly variable microsatellite loci. Clearly, from a conservation perspective, the Jadro softmouth trout must be considered vulnerable, but also so unique that it should under no circumstances be interbred with other softmouth populations, regardless of the taxon's rarity.

Although we have no evidence of introgression of nDNA, it must have occurred when the two species hybridized, and we cannot exclude residual genetic influence of the Adriatic brown trout genome on Jadro softmouth trout, possibly demonstrated by some morphological characters (e.g. gill raker counts and snout length). Because several more putative *Salmo* taxa in the Balkans (including softmouth subspecies) have yet to be genetically investigated, it also remains to be seen whether reticulate evolution is more widespread in the genus across the region. Recently, an ancient hybrid origin has been hypothesized for marble trout (*S. marmoratus*) based on signals of recombination in a nuclear gene (Templeton, 2004), and it is proposed that phylogenetic analysis without consideration of reticulate evolution may be inadequate for revealing the historical development of a taxon. Clearly, vertebrate systematists, similar to their botanical counterparts, are becoming increasingly challenged with systematic revision involving complex evolutionary pathways. Such challenges, often related to conservation interests, will further require, or benefit from, multidisciplinary approaches, including the application of diverse genetic markers with both uni- and bi-parental inheritance (Roca, Georgioudis & O'Brien, 2005).

#### ACKNOWLEDGEMENTS

We would like to thank R. Safner, I. Aničić, and the Anglers' Society of Knin for organizing sampling campaigns, and J. Schöffmann and R. Šanda for providing samples from the River Zrmanja. Research was supported by Ministry of Education, Science and Sport

(Grant No. SLO-HRV 33/01-03). B. D. thanks C. Weber (MHNG), G. Schulze (ZMH), N. Bailly, and P. Pruvost (MNHN), E. Dorofeyeva (ZISP), and H. Wellendorf (NMW) for hospitality and kind help during his visits. Expenses related to visits and research at MNHN was granted by PARSYST program (ERBFMGE-CT98-0132). The work was partially supported by Marie Curie Intra European Fellowship (MEIF-CT-2003-501446) granted to S. S.

## REFERENCES

- Allendorf FW, Leary RF, Spruell P, Wenburg JK. 2001.** The problems with hybrids: setting conservation guidelines. *Trends in Ecology and Evolution* **16**: 613–622.
- Arnold ML. 1992.** Natural hybridization as an evolutionary process. *Annual Review of Ecology and Systematics* **23**: 237–261.
- Baxter JS, Taylor EB, Devlin RH, Hagen J, McPhail JD. 1996.** Evidence for natural hybridization between Dolly Varden (*Salvelinus malma*) and bull trout (*Salvelinus confluentus*) in a northcentral British Columbia watershed. *Canadian Journal of Fisheries and Aquatic Sciences* **54**: 421–429.
- Behnke RJ. 1965.** A systematic study of the family Salmonidae with special reference to the genus *Salmo*. PhD Thesis, University of California.
- Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F. 1996–2004.** GENETIX 4.04, logiciel sous Windows TM pour la genetique des populations. Montpellier: Laboratoire Genome, Populations, Interactions, Universite de Montpellier.
- 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.
- Bernatchez L, Danzmann RG. 1993.** Congruence in control-region sequence and restriction-site variation in mitochondrial DNA of brook charr (*Salvelinus fontinalis* Mitchell). *Molecular Biology and Evolution* **10**: 1002–1014.
- Bernatchez L, Glemet H, Wilson CC, Danzmann RG. 1995.** Introgression and fixation of Arctic char (*Salvelinus alpinus*) mitochondrial genome in an allopatric population of brook trout (*Salvelinus fontinalis*). *Canadian Journal of Fisheries and Aquatic Sciences* **52**: 179–185.
- Bowcock AM, Ruiz-Linares A, Tomfohrde J, Minch E, Kidd JR, Cavalli-Sforza LL. 1994.** High resolution of human evolutionary trees with polymorphic microsatellites. *Nature* **31**: 455–457.
- Clement M, Posada D, Crandall KA. 2000.** TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**: 1657–1659.
- Conservation International. 2004.** WWW document. Available at <http://www.conservation.org/xp/CIWEB/home>.
- Cronin MA, Spearman WJ, Wilmot RL. 1993.** Mitochondrial variation in Chinook (*Oncorhynchus tshawytscha*) and Chum salmon (*O. keta*) detected by restriction enzyme analysis of Polymerase Chain Reaction (PCR) products. *Canadian Journal of Fisheries and Aquatic Sciences* **50**: 708–715.
- Delling B. 2002.** Morphological distinction of the marble trout, *Salmo marmoratus*, in comparison to marbled *Salmo trutta* from River Otra, Norway. *Cybiurn* **26**: 283–300.
- DeMarais BD, Dowling TE, Douglas ME, Minckley WL, Marsh PC. 1992.** Origin of *Gila seminuda* (Teleostei: Cyprinidae) through introgressive hybridization: implications for evolution and conservation. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 2747–2751.
- Dorofeyeva EA. 1999.** Salmons and trouts of Eurasia: morphology, classification and relationships. Dissertation Thesis, St Petersburg State Universit.
- Dowling TE, DeMarais BD. 1993.** Evolutionary significance of introgressive hybridization in cyprinid fishes. *Nature* **362**: 444–446.
- Dowling TE, Secor C. 1997.** The role of hybridization and introgression in the diversification of animals. *Annual Review of Ecology and Systematics* **28**: 593–619.
- Garcia-Paris M, Alcobendas M, Buclkey D, Wake DB. 2003.** Dispersal of viviparity across contact zones in Iberian populations of fire salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. *Evolution* **57**: 129–143.
- Gephard S, Moran P, Garcia-Vazquez E. 2000.** Evidence of successful natural reproduction between brown trout and mature male Atlantic salmon parr. *Transactions of American Fisheries Society* **129**: 301–306.
- Gomez A, Lunt DH. 2006.** Current perspectives in phylogeography and the significance of South European refugia in the creation and maintenance of European biodiversity. In: Weiss S, Ferrand N, eds. *Phylogeography of Southern European Refugia*. Dordrecht: Kluwer Academic Publishers, 155–188.
- Goudet J. 2001.** FSTAT, version 2.9.3: a program to estimate and test gene diversities and fixation indices. Available at <http://www.unil.ch/izea/software/fstat.html>.
- Hadžišće S. 1961.** Zur Kenntnis des *Salmothymus ohridanus* (Steindachner) (Pisces, Salmonidae). *Internationale Vereinigung für Theoretische und Angewandte Limnologie Verhandlungen* **14**: 785–791.
- Heckel JJ. 1851.** Bericht einer ichtyologischen Reise. II. Beiträge zu den Gattungen *Salmo*, *Fario*, *Salar*, *Coregonus*, *Chondrostoma* und *Telestes*. Wien: Sitzungsberichte der Akademie der Wissenschaften, 347–390.
- Hubbs CL. 1955.** Hybridization between fish species in nature. *Systematic Zoology* **4**: 1–20.
- Jansson H, Holmgren I, Wedin K, Andersson T. 1991.** High frequency of natural hybrids between Atlantic salmon (*Salmo salar* L.) and brown trout (*S. trutta*) in a Swedish River. *Journal of Fish Biology* **39** (Suppl. A): 343–348.
- Karaman S. 1927.** Les salmonidés des Balkans. *Bulletin de la Société Scientifique de Skoplje* **2**: 253–268.
- Kosorić Đ, Vuković T. 1969.** Research of possibilities of hybridisation of Salmonidae species of the Neretva River confluence. *Ichthyologia* **1**: 57–67.

- Kottelat M. 1997.** European freshwater fishes; An heuristic checklist of the freshwater fishes of Europe (exclusive of former USSR), with an introduction for non-systematists and comments on nomenclature and conservation. *Biologia* **52** (Suppl. 5): 1–271.
- Kryštufek B, Reed JM. 2004.** Pattern and process in Balkan biodiversity – an overview. In: Griffiths HI, Kryštufek B, Reed JM, eds. *Balkan biodiversity pattern and process in the European hotspot*. Dordrecht: Kluwer Academic Publishers, 203–217.
- Lamb T, Avise JC. 1986.** Directional introgression of mitochondrial DNA in a hybrid population of tree frogs: the influence of mating behavior. *Proceedings of the National Academy of Sciences of the United States of America* **83**: 2526–2530.
- Langella O. 2002.** *Populations, 1.2.28 (12/5/2002)*. Copyright 1999, Olivier Langella. Gif-sur-Yvette: CNRS UPR9034.
- Ludwig A, Congiu L, Pitra C, Fickel J, Gessner J, Fontana F, Patarnello T, Zane L. 2003.** Nonconcordant evolutionary history of maternal and paternal lineages in Adriatic sturgeon. *Molecular Ecology* **12**: 3253–3264.
- Marić D. 1995.** Endemic fish species of Montenegro. *Biological Conservation* **72**: 187–194.
- McMeel OM, Hoey EM, Ferguson A. 2001.** Partial nucleotide sequences, and routine typing by polymerase chain reaction-restriction fragment length polymorphism, of the brown trout (*Salmo trutta*) lactate dehydrogenase, LDH-C1\*90 and \*100 alleles. *Molecular Ecology* **10**: 29–34.
- Mrakovčić M, Mišetić S. 1990.** Status, distribution and conservation of the salmonid, *Salmothymus obtusirostris* (Heckel) and cyprinid, *Aulopyge hugely* (Heckel) in Yugoslavia. *Journal of Fish Biology* **37** (Suppl.): 241–242.
- O'Reilly PT, Hamilton LC, McConnell SK, Wright JM. 1996.** Rapid analysis of genetic variation in Atlantic salmon (*Salmo salar*) by PCR multiplexing of dinucleotide and tetranucleotide microsatellites. *Canadian Journal of Fisheries and Aquatic Sciences* **53**: 2292–2298.
- Ostberg CO, Slatton SL, Rodriguez RJ. 2004.** Spatial partitioning and asymmetric hybridization among sympatric coastal steelhead trout (*Oncorhynchus mykiss irideus*), coastal cutthroat trout (*O. clarki clarki*) and interspecific hybrids. *Molecular Ecology* **13**: 2773–2788.
- Phillips RP, Matsuoka MP, Konon I, Reed KM. 2000.** Phylogenetic analysis of mitochondrial and nuclear sequences supports inclusion of *Acantholingua ohridana* in the genus *Salmo*. *Copeia* **2**: 546–550.
- Poteaux C, Bonhomme F, Berrebi P. 1999.** Microsatellite polymorphism and genetic impact of restocking in Mediterranean brown trout (*Salmo trutta* L.). *Heredity* **82**: 645–653.
- Presca P, Guyomard R. 1996.** Conservation of microsatellites in three species of salmonids. *Journal of Fish Biology* **49**: 1326–1329.
- Redenbach Z, Taylor EB. 2002.** Evidence for historical introgression along a contact zone between two species of char (Pisces: Salmonidae) in northwestern North America. *Evolution* **56**: 1021–1035.
- Rice WR. 1989.** Analysing tables of statistical tests. *Evolution* **43**: 223–225.
- Rieseberg LH. 1997.** Hybrid origins of plant species. *Annual Review of Ecology and Systematics* **28**: 359–389.
- Roca AL, Georgioudis N, O'Brien SJ. 2005.** Cytonuclear genomic dissociation in African elephant species. *Nature Genetics* **37**: 96–100.
- Ruedi M, Smith MF, Patton JL. 1997.** Phylogenetic evidence of mitochondrial introgression among pocket gophers in New Mexico (family Geomyidae). *Molecular Ecology* **6**: 453–462.
- Salzburger W, Baric S, Sturmbauer C. 2002.** Speciation via introgressive hybridization in east African cichlids? *Molecular Ecology* **11**: 619–625.
- Sanford CPJ. 2000.** Salmonid fish osteology and phylogeny (Teleostei: Salmonidei). *Theses Zoologicae* **33**: 1–264.
- Scribner KT, Gust JR, Fields RL. 1996.** Isolation and characterization of novel microsatellite loci: cross-species amplification and population genetic applications. *Canadian Journal of Fisheries and Aquatic Sciences* **53**: 833–841.
- Scribner KT, Page KS, Bartron ML. 2001.** Hybridization in freshwater fishes: a review of case studies and cytonuclear methods of biological inference. *Reviews in Fish Biology and Fisheries* **10**: 293–323.
- Seehausen O. 2004.** Hybridization and adaptive radiation. *Trends in Ecology and Evolution* **19**: 198–207.
- Seehausen O, Alphen JJ, Witte F. 1997.** Cichlid fish diversity threatened by eutrophication that curbs sexual selection. *Science* **277**: 1808–1811.
- Slettan A, Olsaker I, Lie O. 1996.** Polymorphic Atlantic salmon, *Salmo salar* L., microsatellites at the SSOSL438, SSOSL439 and SSOSL444 loci. *Animal Genetics* **27**: 57–58.
- Smith PF, Konings A, Kornfield I. 2003.** Hybrid origin of a cichlid population in Lake Malawi: implications for genetic variation and species diversity. *Molecular Ecology* **12**: 2497–2504.
- Snoj A, Jug T, Melkić E, Sušnik S, Jesenšek D, Budihna N, Pohar J, Dovc P. 2000.** Mitochondrial and microsatellite DNA analysis of marble trout in Slovenia. *Journal of Freshwater Biology (Quaderni ETP)* **29**: 5–11.
- Snoj A, Melkić E, Sušnik S, Muhamedagić S, Dovc P. 2002.** DNA phylogeny supports revised classification of *Salmothymus obtusirostris*. *Biological Journal of the Linnean Society* **77**: 399–411.
- Snoj A, Pohar J, Dovc P. 1997.** The first microsatellite DNA marker BFRO001 for Marble trout. *Journal of Animal Science* **75**: 1983.
- Stearley RF, Smith GR. 1993.** Phylogeny of the Pacific trouts and salmonids (*Oncorhynchus*) and genera of the family Salmonidae. *Transactions of the American Fisheries Society* **122**: 1–33.
- Steindachner F. 1882.** *Ichthyologische Beiträge (XII)*. Wien: Sitzungsber Akademie Wiss, 61–82.
- Sušnik S, Schöffman J, Snoj A. 2004.** Phylogenetic position of *Salmo* (*Platysalmo*) *platycephalus* Behne 1968 from South-Central Turkey, evidenced by genetic data. *Journal of Fish Biology* **64**: 947–960.
- Sušnik S, Snoj A, Pohar J, Dovc P. 1997.** The microsatellite marker (BFRO 002) characteristic for different geographi-

- cally remote brown trout, *Salmo trutta* L., populations. *Animal Genetics* **28**: 372.
- Svetovidov A. 1975.** Comparative osteological study of the Balkan endemic genus *Salmothymus* in relation to its classification. *Zoologicheskii Zhurn* **54**: 1174–1190.
- Templeton AR. 2004.** Using haplotype trees for phylogeographic and species inference in fish populations. *Environmental Biology of Fishes* **69**: 7–20.
- Templeton AR, Crandall KA, Sing CF. 1992.** A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**: 619–633.
- Thompson JD, Higgins DG, Gibson TJ. 1994.** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4637–4680.
- Vuković T. 1982.** Prirodni hibridi u otvorenim vodama. In: Habekovic D, ed. *Slatkovodno ribarstvo*. Zagreb: Jugoslovenska Medicinska Naklada, 166–168.
- Weckstein JD, Zink RM, Blackwell-Rago C, Nelson DA. 2001.** Anomalous variation in mitochondrial genomes of white-crowned (*Zonotrichia leucophrys*) and golden-crowned (*Z. atricapilla*) sparrows: pseudogenes, hybridization, or incomplete lineage sorting? *Auk* **118**: 231–236.
- Young WP, Ostberg CO, Keim P, Thorgaard GH. 2001.** Genetic characterization of hybridization and introgression between anadromous rainbow trout (*Oncorhynchus mykiss irideus*) and coastal cutthroat trout (*O. clarki clarki*). *Molecular Ecology* **10**: 921–930.

### SUPPLEMENTARY MATERIAL

The following material is available for this article online:

**Table S1.** Number of alleles, allelic richness, expected heterozygosity and non biased heterozygosity, and  $F_{is}$  values of four analyzed populations.

**Appendix S1.** Morphologically examined material.

This material is available as part of the online article from:

<http://www.blackwell-synergy.com/doi/abs/10.1111/j.1095-8312.2007.00717.x>

(This link will take you to the article abstract).

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.