

SHORT COMMUNICATION

Extreme genetic differentiation among the remnant populations of marble trout (*Salmo marmoratus*) in Slovenia

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Abstract

Populations of the marble trout (*Salmo marmoratus*) have declined critically due to introgression by brown trout (*Salmo trutta*) strains. In order to define strategies for long-term conservation, we examined the genetic structure of the 8 known pure populations using 15 microsatellite loci. The analyses reveal extraordinarily strong genetic differentiation among populations separated by < 15 km, and extremely low levels of intrapopulation genetic variability. As natural recolonization seems highly unlikely, appropriate management and conservation strategies should comprise the reintroduction of pure populations from mixed stocks (translocation) to avoid further loss of genetic diversity.

Keywords: conservation genetics, hybridization, introgression, microsatellites, population structure, *Salmo marmoratus*

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Introduction

Hybridization and introgression, induced by anthropogenic activities such as the introduction of nonindigenous taxa and habitat modification, have led to the extinction of many native populations and species. Subsequent genetic mixing can lead to a loss of genotypically distinct populations, in particular when hybrids are fertile and mate both among themselves and with parental (nonhybrid) individuals, resulting in the creation of hybrid swarms (see Leary *et al.* 1995). The consequences of these phenomena can be particularly harmful for rare, threatened species coming into contact with more abundant ones. In fish, hybridization is especially common because of the extensive introduction of nonnative species for angling or commercial purposes, and because interspecific hybrids are often fertile (Rhymer & Simberloff 1996; Allendorf *et al.* 2001).

The marble trout (*Salmo marmoratus*) has a restricted native range within the Adriatic basin (northern Italy,

former Yugoslavia and Albania), and is characterized by a particular marbled colour and a very large size (up to 25 kg in weight). Molecular studies [mitochondrial DNA (mtDNA) sequence data] have shown that within the Po basin (Italy), all *marmoratus* populations form a distinct evolutionary lineage among the brown trout (*Salmo trutta*) population complex. Before massive stockings in recent years, brown and marble trout were parapatric, behaving as true species (Giuffra *et al.* 1994, 1996). Hybridization between marble and brown trout has been so extensive that nowadays morphologically typical *marmoratus* forms are very rare, and the species is considered to be one of the most endangered freshwater fish of the Adriatic basin (Povž 1995; Delling *et al.* 2000). Within the Slovenian distribution, introduction of nonindigenous brown trout started in 1906 and hybrids now dominate most rivers, with the exception of secluded headwater streams of the Soča River basin separated from downstream hybrid populations by impenetrable natural barriers (Povž *et al.* 1996). Recent allozyme and mtDNA investigations revealed within such isolated streams the existence of eight genetically pure *marmoratus* populations, according to diagnostic loci (Berrebi *et al.* 2000; Plesko & Budihna 2000; Snoj *et al.* 2000).

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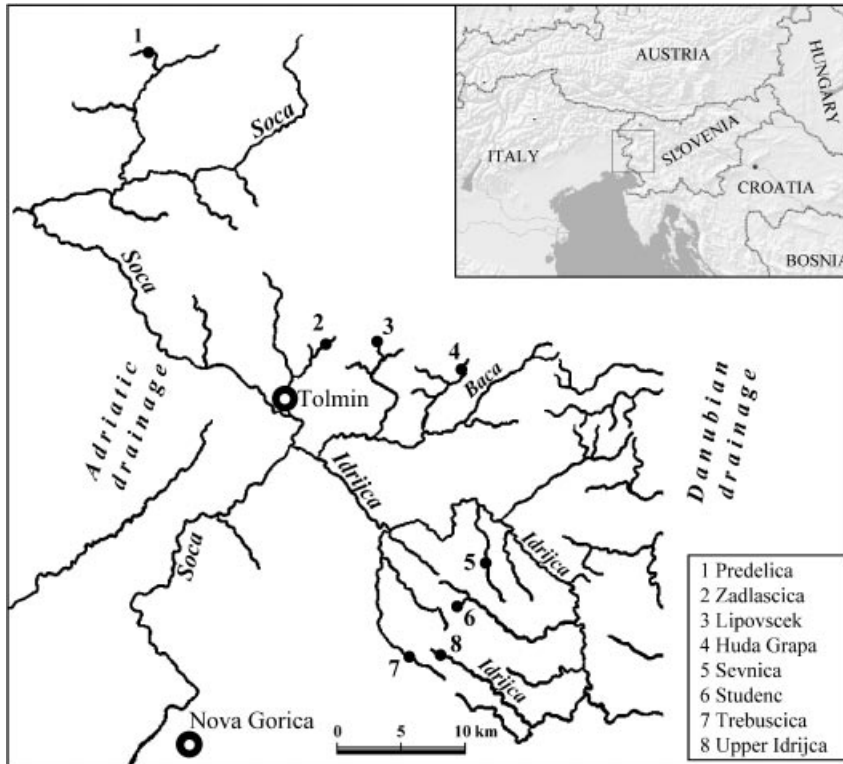


Fig. 1 Map showing sampling localities of *Salmo marmoratus* within the Soča River basin in Slovenia. The Soča River system, representing the single Slovenian drainage inhabited by *S. marmoratus*, lies in the northwestern part of Slovenia, and belongs to the Adriatic basin. Overall it covers ≈ 2300 km², but the study area where tributaries of the Soča River have been shown to still host pure *S. marmoratus* populations (see text for further details) covers ≈ 900 km². All sampling locations are separated downstream from the hybridization zone by several impassable barriers (waterfalls) preventing upstream immigration, and have presumably never been influenced by any fishing and stocking activities. Note that the natural Predelica population went extinct in 2000 due to an important landslide, however, a hatchery-reared population derived from wild Predelica still exists.

The isolated nature of these populations makes them extremely vulnerable to stochastic factors, and the probability of spontaneous colonization of new habitats seems extremely reduced. Furthermore, the small size of the remnant populations could also decrease their genetic diversity, potentially leading to fixation of deleterious alleles and to a lack of evolvability in response to environmental changes (Frankham *et al.* 2002). As the applicability of conservation strategies critically depends on prior knowledge of both within-population genetic diversities and between-population differentiation, we performed a genetic survey of all known pure populations of the marbled trout using 15 microsatellites.

Materials and Methods

A total of 159 samples of *Salmo marmoratus* were obtained from the headwaters of 8 tributaries of the Soča River by means of electrofishing (Fig. 1). DNA was extracted using the DNeasy Tissue kit (QIAGEN), following supplier's instructions. Twelve microsatellite loci isolated and characterized from other salmonid species (*Str15*, *Str60*, *Str73*: Estoup *et al.* 1993; *Str24*: Poteaux 1995; *MST85*, *MST591*: Presa & Guyomard 1996; *Ssa171*, *Ssa197*: O'Reilly *et al.* 1996; *BS131*, *T3-13*, *MST543ae*: Estoup *et al.* 1998; *Strutta-58*: Poteaux *et al.* 1999) and three from *S. marmoratus* (*BFRO 001*: Snoj *et al.* 1997; *BFRO 002*: Sušnik *et al.* 1997; *BFRO 003*: Snoj *et al.* 2000) were amplified and scored.

Polymerase chain reactions (PCR) were performed in 10 μ L reaction mixtures containing 25 ng DNA, 0.5 μ M of each primer, 1.5–2 mM MgCl₂, 200 μ M each dNTP, 1 \times reaction buffer and 0.25 units of *Taq* DNA polymerase (Applied Biosystems, Gibco BRL or QIAGEN). The PCR profile consisted of 30 iterations of 95 $^{\circ}$ C for 30 s, 48 $^{\circ}$ C (*locus BFRO 003*), 50 $^{\circ}$ C (*BS131*, *MST85*), 55 $^{\circ}$ C (*Ssa171*, *Str58*, *Str591*), 58 $^{\circ}$ C (*Str24*, *MST15*, *MST73*, *MST543ae*) or 60 $^{\circ}$ C (*T3-13*, *Str60*, *BFRO 001*, *BFRO 002*, *Ssa197*) for 30 s, 72 $^{\circ}$ C for 30 s. Amplified products were genotyped either with ABI PRISM 377 DNA Sequencer or ABI Prism 310 using GENESCAN ANALYSIS 2.1 software (Applied Biosystems).

Gene diversities comprising observed (H_O), expected within-subpopulation (H_S) and expected overall heterozygosities (H_T) were estimated following Nei & Chesser (1983). Genotypic disequilibrium between loci in each sample and deviations from Hardy–Weinberg equilibrium (HWE) within samples were tested based on 14 560 permutations and 2000 randomizations, respectively. Wright's fixation indices for within-subpopulation deviation from random mating (F_{IS}), as well as pairwise subpopulation differentiation (F_{ST}), were estimated following Weir & Cockerham (1984). Deviation from random mating within populations (F_{IS}) per locus and sample were computed with a bootstrap procedure (2000 randomizations). Statistical support for pairwise population differentiation was obtained through exact *G*-tests on allelic frequencies as described by Goudet *et al.* (1996) with 2000 randomizations.

	<i>N</i>	Proportion of polymorphic loci	Mean no. of alleles/locus	H_O	H_S
Predelica	22	0.57	2.00	0.233	0.219
Zadlaščica	20	0.43	1.57	0.149	0.162
Lipovšček	20	0.36	1.50	0.191	0.156
Huda Grapa	20	0.14	1.14	0.046	0.039
Sevnica	20	0.29	1.29	0.097	0.088
Studenc	20	0.29	1.71	0.136	0.150
Trebusčica	19	0.29	1.43	0.103	0.094
U. Idrija	18	0.36	1.57	0.119	0.155
Overall	159	0.64	3.50	0.135	0.133

Table 1 Genetic variability as determined from 14 microsatellite loci for eight remnant populations of *Salmo marmoratus* in Slovenia

Reported significance levels are after strict Bonferroni corrections based on the indicative adjusted P -value. All summary statistics and tests mentioned above have been computed using FSTAT Version 2.9.3.2 (Goudet 1995). We further performed a factorial correspondence analysis (CA) using GENETIX software (Belkhir *et al.* 1998).

Results

Tests for HWE indicated that locus *Str24* was characterized by a significant heterozygote deficiency ($P = 0.0005$), suggesting the presence of null alleles, and was removed from further analyses. All other loci tested were at HWE and in genotypic equilibrium ($P < 0.05$). For the remaining 14 microsatellite loci, the number of alleles per locus ranged from 1 (5 loci were homozygous and monomorphic across the 8 populations) to 12 (average = 3.5), with a total of 49 alleles across 14 loci. Expected heterozygosities per locus within samples (H_S) ranged from 0 to 0.51, with an average of 0.13, whereas expected overall heterozygosity (H_T) averaged 0.36 (range per locus: 0–0.85). Observed heterozygosity (H_O) values varied from 0 to 0.56, with an average of 0.135. We found 16 population-specific (private) alleles (representing 33% of the total number of alleles sampled). Genetic diversities for the eight populations of *Salmo marmoratus* are summarized in Table 1.

There was evidence for significant deviation from random mating in none of the eight analysed populations (global $F_{IS} = -0.014$), suggesting the absence of a within-sample substructure. However, between-population genetic subdivision was very strong pointing to an extreme value of $F_{ST} = 0.66$ ($P < 0.001$). Furthermore, all pairwise population differentiation estimates (pairwise F_{ST}) were high, ranging from 0.31 to 0.88, with all being significant ($P < 0.01$) after strict Bonferroni correction for multiple tests.

Figure 2 illustrates the position of the eight populations on the main factorial plane described by the two main axes of the CA analysis. In this diagram, five groups of

populations are clearly discriminated: Huda Grapa and Zadlaščica placed at the positive end of the first axis are in fact discriminated by the third axis (not shown, inertia 12.9%), and successively Lipovšček, Predelica and a group of four populations at the negative end of the first axis and along the second axis. This last group of populations (all located within the Idrija drainage) is composed of largely overlapping individuals.

Discussion

Our study reveals extraordinarily strong genetic differentiation among populations on a restricted geographical range. For instance, populations of Lipovšček and Huda Grapa, separated by a waterway of < 15 km, showed an average pairwise F_{ST} value of 0.79, the extreme level being found between Trebuščica and Huda Grapa (0.88), separated by 28.5 km (Fig. 1). These extremely high F_{ST} estimates exceed by far the highest values reported between fish populations at a micro- or macrogeographical scale and are to the best of our knowledge the highest differentiation ever recorded with microsatellites at an intraspecific level. For example, levels of differentiation reported in other salmonids on a larger geographical scale are much lower (average F_{ST} value of 0.33 in Taylor *et al.* 2001; 0.36 in Koskinen *et al.* 2001; 0.37 in Angers & Bernatchez 1998). The remnant marble trout populations are thus completely isolated from each other and from the main river system as immigration from downstream is precluded by impassable barriers. Even in the complete absence of genetic exchanges, F_{ST} values are generally much lower for microsatellites because of their high mutation rates increasing within-population genetic variability, and thus avoiding within-population fixation of alleles (Balloux & Lugon-Moulin 2002). However, in these populations, microsatellite allele numbers and heterozygosity estimates were remarkably low compared with genetic variation at microsatellite loci commonly observed in other freshwater fish species (DeWoody &

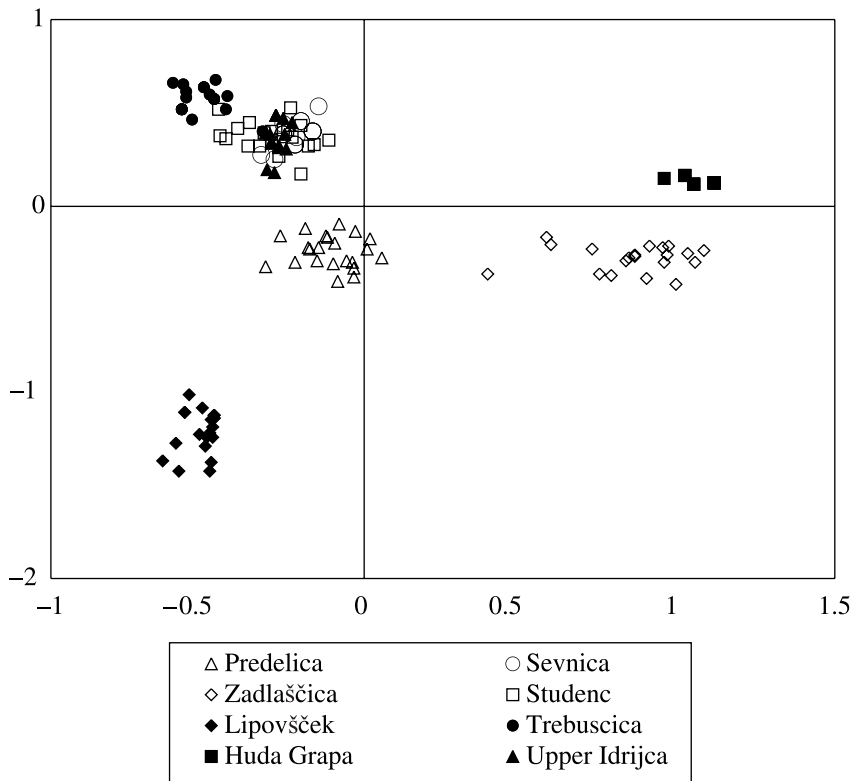


Fig. 2 Projection diagram of the first two axes from the factorial analysis based on the genotypes of 14 microsatellite loci from 8 marble trout populations from the Soča River system, Slovenia. The first axis accounts for 15.9% of the total inertia contained in the dataset and the second axis for 14.3% (cumulative inertia is thus 30% with 47 axes). Without structure, these axes should gather only $\approx 4\%$.

Avise 2000). This implies a strong impact of local genetic drift and points to low effective population sizes due to the absence of immigration.

Patterns of population structure reflect both current and historical levels of gene flow. Despite the extreme genetic structuration demonstrated by the microsatellite analysis, the presence of a single *marmoratus* mtDNA control region haplotype (Ma1) in Northern Italy (Giuffra *et al.* 1994) and in three pure Slovenian populations analysed so far (Snoj *et al.* 2000) indicates relatively recent connectivity prior to the fragmentation of habitats. Several lines of evidence support the idea that the pure populations within the Idrijca drainage (Sevnica, Studenc, Trebuscica and Upper Idrijca; see Fig. 1) belonged to an independent river system. They clustered together in the CA analysis (Fig. 2) and generally exhibit lower reciprocal levels of genetic structuring (mean = 0.49). Geological events during the Pliocene and climate fluctuations during the Pleistocene had a substantial impact on the evolution of the Idrijca drainage. Originally, its upper part flowed into the Danubian river system from northwest to southeast. Later, by the process of retrograde erosion, it became connected with what is today the modern Idrijca drainage, which flows in the opposite direction southeast to northwest (Janež *et al.* 1997). During the climate fluctuations of Pleistocene, the Idrijca drainage has never been covered by ice, in contrast to the upper Soča Valley, and some interconnections with the Danubian river system may have occurred (Melik 1963;

Bavec & Tulaczyk 2002). Altogether, these complex and recurrent events of river isolation and interconnection may also have contributed to the particular situation of local genetic pattern, by separating and/or maintaining some of the pure *marmoratus* populations in contact. Further evidence suggesting distinct drainage patterns stems from the distribution of other species. For instance, the stone crayfish (*Austropotamobius torrentium*) inhabits the Danubian system but also the upper part of the Idrijca and Baca drainages, whereas the rest of the Idrijca drainage and the Soča River are occupied by the white-clawed crayfish (*A. pallipes*) (Budihna 2001).

Since the discovery of new pure populations in Slovenia seems unlikely (Crivelli *et al.* 2000), management efforts should focus on maintaining and expanding these remaining populations. Their geographical isolation protects them from natural hybridization, hence the presence of hybrids within the main river does not represent a threat *per se* for their survival. Similarly, excessive fishing pressure or stocking with domestic brown trout strains does not seem particularly worrying, as access to most of these streams (often located within narrow valleys and canyons) is generally very arduous, and stocking has been banned legally since 1996. Nevertheless, it is important to give highest protection priority to these genetically distinct populations (management units within a single evolutionary significant unit, *sensu* Moritz 1994), which are among the very few nonintrogressed populations documented

within the entire species' range. As the populations are extremely isolated and small, and because there is no potential of spontaneous colonization of new habitats through dispersal, the risk of local extinction due to natural factors (e.g. diseases, catastrophes) is very high. In particular, the Soča River area is tectonically very active and frequently affected by severe landslides. Recent overflowing of the Predelica River valley subsequent to an earthquake, which led to the extinction of its pure *marmoratus* population, conveys urgency to conservation management.

A rehabilitation programme for the species started in 1993 (Povž *et al.* 1996; Crivelli *et al.* 2000). Its purpose is the establishment of a captive breeding programme with wild genitor stocks from pure populations, in order to release genetically pure *marmoratus* individuals into currently fishless streams isolated from the hybridization zone, thus creating new pure populations and repopulating the Soča River basin. To date, five new fishless rivers have been stocked with farm-bred fish all originating from a single pure population (but one from a mixture of two populations), and both genetic and demographic monitoring is undertaken annually. Given the extremely low levels of genetic variability detected in the populations analysed, consideration should be given to mixing different remnant populations for farm-breeding and subsequent translocation to new sites, in order to maximize diversity in reintroduced populations. Whether to augment local population genetic diversities in the short-term by reciprocal mixing is a difficult decision to take as it urgently requires further research, including potential evidence for inbreeding depression and impact on the viability of source populations.

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The work presented here is part of a collaboration between different laboratories on marble trout genetics and conservation. L. Fumagalli is responsible for the Conservation Biology Laboratory at the University of Lausanne (<http://www.unil.ch/lbc>); his research interests range from conservation genetics to phylogeography and molecular evolution. A. Snoj is a research associate at the University of Ljubljana, particularly engaged in conservation genetics and phylogeography of salmonids of the Adriatic drainage. D. Jesenšek is a production manager of the Tolmin hatchery, occupied with aquaculture of endangered endemic fish species. F. Balloux is interested in various aspects of evolutionary genetics. O. Duron, F. Brossier and T. Jug are preparing for a master's degree. A.J. Crivelli is a researcher in fish ecology; he is the project leader of the rehabilitation of the marble trout in the upper Soča River basin, Slovenia. P. Berrebi is a senior researcher interested mainly in the evolution of fishes (speciation, introgression, polyploidization); he is now developing researches on adaptation of brackish water fish.
