

Distribution of non-native trout in Slovenia and their introgression with native trout populations as observed through microsatellite DNA analysis

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Received 8 October 2004; received in revised form 20 November 2004; accepted 26 November 2004

Abstract

In Slovenia, the Adriatic basin inhabited by native marble trout (*S. marmoratus*), and the Danubian basin inhabited by native Danubian lineage of brown trout (*S. trutta*) have been intensively affected by stocking with non-native trout strains. In order to assess spread of non-native strains and their introgression with native trout, a population study based on five microsatellite loci was applied across ten marble and ten brown trout populations, ranging from allegedly non-introgressed to heavily managed. On the basis of correspondence analysis, which revealed three clear groupings consisting of the Danubian and Atlantic lineages of brown trout and the marble trout, the alleles, characteristic of each grouping were identified and used for estimating genetic composition of each population according to the three possible origins. Among the wild populations, five marble and one brown trout populations were found to be pure; all the others were introgressed with exotic alleles (Atlantic and *marmoratus* alleles in the Danubian basin and Atlantic and Danubian in the Adriatic basin) that markedly dominate in intensively managed populations. As revealed by non-significant F_{IS} values, panmixia between native and introduced fishes has for the most part already been reached. Our research showed that it is not only marble trout whose identity is endangered in Slovenia but also the existence of autochthonous Danubian brown trout is critically compromised, which is new information to be taken into account for local trout conservation.

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Keywords: Conservation; Hybridization; Stocking; *Salmo marmoratus*; *Salmo trutta*

1. Introduction

Anthropogenic pressure, such as pollution, habitat destruction, commercial fishing and angling are common causes of freshwater fish populations declines. But, concerning genetic integrity of local populations, the main threat is gene flow from non-native strains used for stocking (Leary et al., 1995; Huxel, 1999; Poteaux et al., 2001; Allendorf et al., 2001; Englbrecht et al.,

2002; Sušnik et al., 2004). Fish exploited for sport-fishing, such as salmonids are notably subjected to stock enhancement programs, which are based mainly on releases of hatchery-reared fish. Hatchery stocks, however, usually represent taxa that are different from the native ones, but if stocked strain corresponds with the native one, it exhibits only a minor part of the total genetic variability of the species and therefore threatens genetic diversity of the native strain. The brown trout (*Salmo trutta*), one of the most attractive recreative and managed salmonid species, shows strong genetic differentiation among local populations, and it is therefore highly vulnerable in this regard. Moreover, many wild

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populations of the brown trout have been heavily stocked with domestic strains, stemming from the Atlantic phylogeographic lineage of the brown trout (Bernatchez, 2001), that are genetically very similar (Aurelle et al., 2002). The effects of this stocking have been extensively studied (e.g., Hansen and Loeschcke, 1994; Largiader and Scholl, 1995; Poteaux et al., 1998a,b; Berrebi et al., 2000a,b; Mezzera and Largiader, 2001; Almodovar et al., 2001; Fritzner et al., 2001; Hansen, 2002) and it was observed that interbreeding of wild and domestic populations may result in a breakdown of local genetic population structure, loss of genetic variation among stocks and loss of specific genotype combinations of potential adaptive value.

Slovenia covers a part of the Adriatic and Danubian river system, harbouring two native trout species: the Danubian lineage of brown trout (*S. trutta*) and marble trout (*S. marmoratus*; nomenclature as in Kottelat, 1997).

In Slovenia, the marble trout was originally restricted to the Soča basin (the Adriatic river system; Povž et al., 1996), but according to former fishery management policy, several attempts were made to spread it out of its natural range into several adjacent tributaries of the Sava basin (the Danubian river system) in order “to increase growth capacity of the brown trout”.

As autochthonism of the brown trout in the Sava basin is not questionable, it is disputable whether the brown trout have been native to the Soča basin or not (Gridelli, 1936; Behnke, 1968; Guiffra et al., 1996). Since the beginning of the last century, brown trout of various non-native sources (e.g., from Bosnia and Italy) were massively stocked into the Soča basin (Gridelli, 1936) but later the stocking has continued with brown trout of unknown origin. Intense stocking of the Sava basin with brown trout has also been frequently performed in Slovenia, however there are insufficient records regarding origin and quantity of stocked fish. Nevertheless, allozyme marker LDH-5* 90, diagnostic for the Atlantic phylogeographic lineage of the brown trout (Hamilton et al., 1989; McMeel et al., 2001), was detected in both river systems in Slovenia (Berrebi et al., 2000b), suggesting that stocking with non-native strains of the brown trout has been widespread also in the Sava basin.

Introgression between Slovenian native and introduced lineages of trout has been poorly studied and mainly limited to the marble trout (Berrebi et al., 2000b; Snoj et al., 2000; Jug et al., 2004). Eight genetically marble pure (i.e., not-introgressed) populations have been found in the upper part of the Soča basin (Fumagalli et al., 2002), while all other populations were, using allozyme and mtDNA markers, found to be introgressed with non-native trout of the Danubian and Atlantic origin (Berrebi et al., 2000b; Snoj et al., 2000). In the Sava basin, brown trout from only two riv-

ers were analysed, revealing highly mixed populations composed of native and introduced Atlantic stocks (Berrebi et al., 2000b).

The aim of this study is to get the first estimation of dissemination range of non-native trout in the Soča and Sava basins in Slovenia and to assess a level of their introgression with native stocks. Since the existent knowledge about trout introgression in Slovenia is based on allozyme markers, which are more or less subjected to selection pressure, and exhibit only a limited amount of genetic variability, we applied microsatellite markers that may, due to their neutrality and higher level of variation, give a more reliable picture of actual introgression in the zones where hybridisation takes place. To test diagnostic quality of the microsatellite markers used in this study, we added to the analysis several populations that were, using allozymes and mtDNA, previously determined as pure.

2. Materials and methods

2.1. Sampling

A total of 358 fish was investigated (Table 1). 117 originated from seven rivers of the Sava basin (the Danubian river system), 216 from 10 rivers of the Adriatic drainage, 14 from the main Slovenian fish-farm Povodje, eight from the Adriatic Sea and three from a commercial fish-farm Mount Lassen, CA, USA, representing in this study a reference group of the Atlantic lineage of the brown trout (Table 1 and Fig. 1). Populations marked with asterisk in Table 1 were on the basis of our previous studies using allozymes (Berrebi et al., 2000b) and mtDNA markers (Snoj et al., 2000) considered as pure, whereas those marked with cross were due to remoteness and isolation of their origin locations presumed to be pure. The origin of the brown trout from the Adriatic Sea has been previously determined using mtDNA and nuclear DNA markers, and was used here only as a reference population (for details, see Snoj et al., 2002). The remaining unmarked populations originated from heavily stocked locations.

2.2. PCR analysis

Genomic DNA was isolated from the red blood cells according to the protocol described by Medrano et al. (1990).

Five microsatellite loci (BFRO001: Snoj et al., 1997; BFRO002: Sušnik et al., 1997; BFRO003: Snoj, 1997; Str85INRA: Presa and Guyomard, 1996; Ssa197: O'Riilly et al., 1996) were amplified and scored.

PCR amplification was carried out in a 10 µl reaction volume containing 50 ng genomic DNA, 0.5 µM each

Table 1
Description of the populations analysed

Lineage/origin	Location	Status	Year of sampling	Sample size	
Danubian	Iška	+	Wild	1995	6
Danubian	Mahnečica	+	Wild	1995	10
Danubian	Krka		Wild	1998	35
Danubian	Sovpot	+	Wild	1998	20
Danubian	Ribnica	*	Wild	1998	21
Danubian	Kozji jarek	+	Wild	1998	7
Danubian	Obrh		Wild	1998	18
Atlantic	Adriatic Sea		Wild	1998	8
Atlantic	Povodje		Fish-farm	1995	14
Atlantic	Mount Lassen	*	Fish-farm	1995	3
Marble	Tolminka		Wild	1997	11
Marble	Soča		Wild	1998	10
Marble	Predelica	*	Wild	1998	22
Marble	Zadlaščica A/B	*	Wild/fish-farm	1995/1993	16/20
Marble	Trebuščica A/B	*	Wild/fish-farm	1995/1993	16/20
Marble	Huda grapa	*	Wild	1994	20
Marble	Lipovšček	*	Wild	1994	20
Marble	Studenc	*	Wild	2000	20
Marble	Sevnica	*	Wild	2000	20
Marble	Idrija	*	Wild	2001	21

Asterisks indicate populations considered pure and crosses populations presumed to be pure.

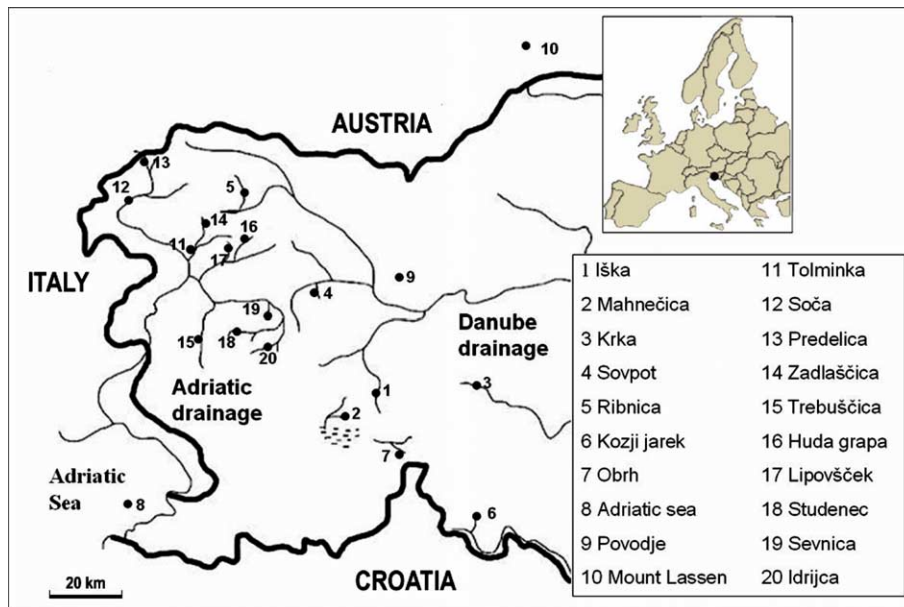


Fig. 1. A map of Slovenia showing sampling locations.

primer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 10 mM Tris-HCl, 50 mM KCl and 0.1 U of *AmpliTaq* DNA polymerase. Thermal cycling reaction was performed in the PTC-100™ Programmable Thermal Controller (MJ Research, Inc.) by initial denaturation at 94 °C for 3 min followed by 35 cycles of 45 s at 94 °C, 25 s at appropriate annealing temperatures (48 °C for BFRO03, Str85INRA and Ssa197 and 60 °C for BFRO001 and BFRO002) and DNA extension for 5 min at 72 °C. Aliquots of fluorescently labelled amplified DNA were mixed with formamide and GENSCAN-350 (TAMRA)

Size Standard (PE Applied Biosystems) and scored on the ABI Prism 310 Genetic Analyser using the GeneScan™ Analysis Software 2.1.

2.3. Statistical analyses

The relationships between all the individuals were described by the factorial correspondence analysis (CA). For this, individuals are first coded according to the presence of the different alleles with values 0 (allele absent), 1 (heterozygote), 2 (homozygote for the allele).

The computation then aims at finding composite axes which are a combination of the variables and which optimise the differences between the analysed individuals. This computation is not completely independent from differentiation parameters, as Guinand (1996) has shown that the inertia values (i.e., the proportion of the total information explained by an axis, given in percent) along each axis are equivalent to linear combinations of the monolocus F_{ST} values. The relationships among individuals can be visualised on two axes, which is very useful for analysing the genetic homogeneity of a given sample when hybridisation occurs.

Intra-population genetic diversity inferred from observed (H_{obs}) and expected (H_{exp}) heterozygosity was estimated following Nei (1972). Wright's fixation indices for intra-population deviation from random mating due to heterozygote disequilibrium (F_{IS}) were estimated following Weir and Cockerham (1984). Reported significant levels of F_{IS} values were, after strict Bonferroni corrections, based on the indicative adjusted P -values. All statistics mentioned above were computed using GENETIX software (Belkhir et al., 1998).

Lineage characteristic alleles were extracted out of a CA diagram. Frequencies of characteristic alleles in each population were also calculated. The alleles, characteristic for particular trout lineages allowed us to assess the degree of their introgression in each population. The introgression frequency was calculated as a proportion of native and non-native alleles.

3. Results

3.1. Heterozygosity and F_{IS} values

Overall, 72 alleles were found across the five loci tested and the mean number of alleles per locus ranged from 1.2 to 8.2 (Table 2). Expected heterozygosities (H_{exp}) ranged from 0.06 to 0.72 and observed heterozygosity (H_{obs}) values varied from 0.12 to 0.65 (Table 2).

Significant deviation from random mating was observed in the three analyzed populations from the River Krka ($F_{IS} = 0.14$; $P < 0.01$), Lipovšček ($F_{IS} = -0.26$; $P < 0.05$) and Idrija ($F_{IS} = 0.32$; $P < 0.01$) giving rise to statistically significant F_{IS} values (Table 2). All three F_{IS} values were significant after strict Bonferroni correction.

3.2. Correspondence analysis

The diagram of correspondence analysis showed a triangular plot of analysed individuals revealing three main groups (Fig. 2): the group at the positive end of the first axis (11.5% inertia) represented pure marble trout populations (*S. marmoratus*), whereas the other two, clustered at the negative end of this axis, characterized pure Danubian and Atlantic individuals, i.e., brown trout (*S. trutta*). Explained by the first axis, the differentiation between the two species is the main structure expressed by the genotypes matrix. The Danubian and

Table 2
Genetic variability of 22 populations of marble and brown trout

Population	Mean no. of alleles/locus	H_{exp}	H_{obs}	F_{IS}	Bonferroni correction
Iška	3.6	0.4063	0.4500	0.0357	n.s.
Mahnečica	1.8	0.1650	0.1600	0.0828	n.s.
Krka	8.2	0.7224	0.6332	0.1386	**
Sovpot	2.2	0.2230	0.2211	0.0357	n.s.
Ribnica	2.2	0.1803	0.2000	-0.0842	n.s.
Kozji jarek	2.8	0.4265	0.4000	0.1385	n.s.
Obrh	5.2	0.6242	0.5943	0.0853	n.s.
Adriatic Sea	4.2	0.5913	0.6143	0.0542	n.s.
Povodje	5.8	0.6347	0.6429	0.0242	n.s.
Mount Lassen	1.8	0.3222	0.6000	-0.8000	n.s.
Tolminka	6.2	0.6975	0.6364	0.1347	n.s.
Soča	6	0.6218	0.6533	0.0237	n.s.
Predelica	2.2	0.3332	0.3445	-0.0099	n.s.
Zadlaščica A	1.8	0.1893	0.2462	-0.2632	n.s.
Zadlaščica B	2.2	0.2319	0.2021	0.1541	n.s.
Trebuščica A	2	0.3204	0.2286	0.3557	n.s.
Trebuščica B	2.2	0.1734	0.1895	-0.0658	n.s.
Huda grapa	1.2	0.0640	0.0800	-0.2258	n.s.
Lipovšček	2	0.2487	0.3200	-0.2667	*
Studenc	1.8	0.2110	0.2200	-0.0170	n.s.
Sevnica	1.4	0.1015	0.1200	-0.1574	n.s.
Idrija	2.8	0.2161	0.1524	0.3185	**

Reported significant levels of F_{IS} were calculated after strict Bonferroni corrections: n.s., non-significant.

* $P < 0.05$

** $P < 0.01$.

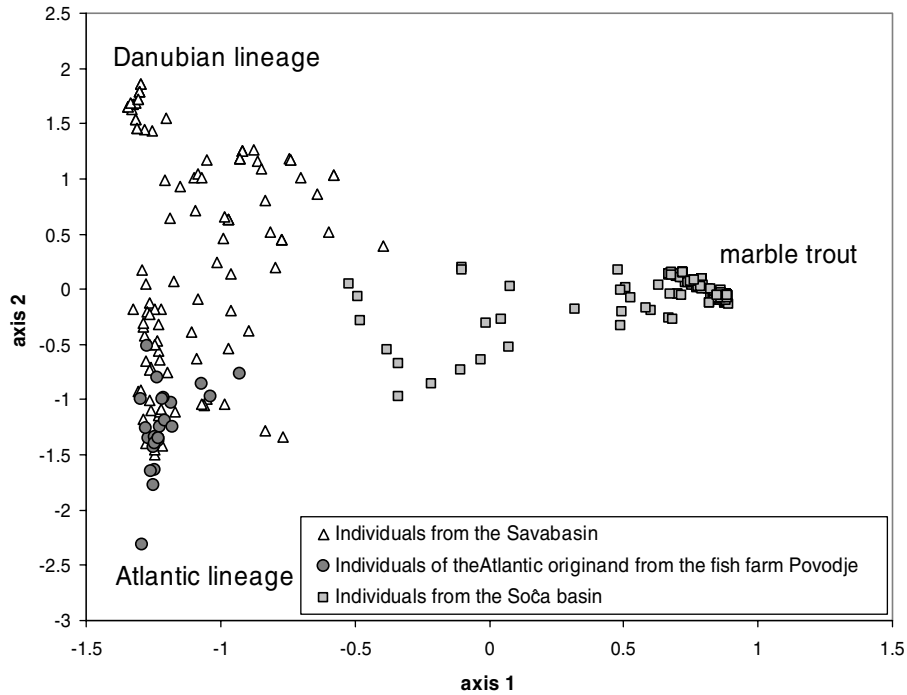


Fig. 2. A plot of individuals on axes 1 and 2 of the correspondence analysis.

Atlantic group were discriminated regarding the second axis (7.04% inertia) being placed at its positive and negative end, respectively, which represents the second strong structure included in the data.

Between the three main groupings, a complex continuous distribution of individuals originating mainly from stocked locations but to lesser extent also from presumably pure Danubian populations was revealed.

3.3. Assessment of lineage specific alleles and their proportions in lineages

By taking into consideration only the individuals plotted at the exterior edge of each group, the correspondence analysis identified the alleles that were characteristic of each of the lineages.

Forty-one lineage-specific alleles were found (Table 3). Fifteen of them were characteristic of the marble trout, eight of the Danubian and 18 of the Atlantic

lineage of brown trout. Using the frequencies of only these diagnostic alleles, we estimated the genetic composition of each population according to the three possible origins: marble, Danubian and Atlantic brown trout (Table 4).

Out of 20 populations, seven were found to be pure. Pure Danubian lineage of brown trout was found only in the river Ribnica, pure marble trout in the rivers Zadlaščica, Trebuščica, Huda grapa, Studenec and Sevnica and pure Atlantic lineage of brown trout was found only in the reference population from the hatchery in CA, USA. Other populations were introgressed to different degrees. Some of them expressed very small proportion of introgressed alleles (populations from the rivers Predelica, Lipovšček and Idrijca involved 1% or 2% of non-native alleles), while some of them were strongly hybridised (populations from the rivers Krka and Obrh, where non-native alleles prevailed).

Table 3
Characteristic alleles for marble and brown trout

Locus	Characteristic alleles		
	Marble trout	Danubian lineage	Atlantic lineage
BFRO001	200, 202, 210	206, 228, 230, 232, 234	212, 216, 218, 222, 224
BFRO002	124	120	116
BFRO003	124	122	136, 138, 142, 144, 148
Str85INRA	173, 181, 183		161, 167, 169
Ssa197	165, 181, 185, 193, 197, 201, 205	149	127, 131, 135, 143

The lineage-diagnostic alleles were generated by recording the alleles found at the exterior edge of each group, at the three tops of the triangle formed by the CA. Diagnostic quality of the alleles was checked by watching their occurrence in the pure populations (see Table 1).

Table 4
Proportions of all characteristic alleles for marble and brown trout (the Danubian and Atlantic lineages) for each population

Population	No. of characteristic alleles	Proportion of marble trout	Proportion of the Danubian lineage	Proportion of the Atlantic lineage
Iška	18 (60)	0.44	0.50	0.06
Mahnečica	42 (100)	0.29	0.69	0.02
Krka	206 (350)	0.06	0.23	0.71
Sovpot	97 (200)	0.18	0.82	0
Ribnica	142 (210)	0	1	0
Kozji jarek	36 (70)	0	0.75	0.25
Obrh	94 (180)	0	0.19	0.81
Adriatic Sea	53 (80)	0	0.08	0.92
Povodje	93 (140)	0.01	0.11	0.88
Mount Lassen	21 (30)	0	0	1
Tolminka	79 (110)	0.63	0.09	0.28
Soča	59 (100)	0.78	0.03	0.19
Predelica	175 (220)	0.99	0	0.01
Zadlaščica	291 (360)	1	0	0
Trebuščica	208 (360)	1	0	0
Huda grapa	200 (200)	1	0	0
Lipovšček	174 (200)	0.98	0	0.02
Studenc	175 (200)	1	0	0
Sevnica	188 (200)	1	0	0
Idrijca	166 (210)	0.99	0	0.01

The numbers of all characteristic alleles found across the five microsatellite loci in each population are also presented. The numbers of all the alleles are in brackets.

4. Discussion

Our results confirm preliminary findings and pre-suppositions that in Slovenia, both river systems are contaminated with non-native trout lineages. In the Sava basin, these are the Atlantic lineage of *S. trutta* and the marble trout, whereas in the Soča basin, the non-natives are the Danubian and Atlantic brown trout lineages. The purity of the populations previously determined by allozyme and mtDNA markers has been in general confirmed in this study. Foreign alleles markedly dominated in currently stocked populations, however, few non-characteristic alleles were found in populations that were considered to have preserved their original identity. The most common non-native alleles were found to be the Atlantic ones. They vastly predominated in the brown trout from the fish-farm Povodje. This fish-farm is the main breeding and distributing centre of the brown trout in Slovenia and has been officially bound to propagate autochthonous fishes. We showed, however that brown trout of Atlantic origin have been reared in this fish-farm as well, and it is not surprising therefore that the Atlantic alleles were abundant in the rivers, where sport fishing is popular and intense stocking is going on. Good examples are well known fly-fishing rivers like the Rivers Krka and Obrh from the Sava basin, and the Rivers Soča and Tolminka from the Soča basin, where the frequency of the Atlantic alleles is approaching to the frequency of the native ones or even exceeding it.

As shown by CA in stocked populations, gene flow has already been going on between native and introduced strains. This fact was confirmed also by non-sig-

nificant F_{IS} values and $H-W$ equilibrium, which indicates that there are no population substructures in the zones of intermixing and that in most mixed populations, panmixia has already been reached. This suggests that no perturbation in these populations due to stocking activities has occurred recently. However, there are some exceptions, like for instance, in the River Krka, where $H-W$ and F_{IS} value disequilibria with high heterozygosity were observed indicating the existence of population substructures in the Krka that might be a consequence of intense and ongoing stocking of trout mainly composed of the Atlantic lineage. Aside from numerous Atlantic alleles found in the River Krka, characteristic alleles of the marble trout were also detected, giving possible evidence of unrecorded introduction of the marble trout into this river. Disequilibrium in F_{IS} characterized either by heterozygote deficiency or overabundance was found in genetically pure marble trout population from the River Idrijca and Lipovšček, respectively. It is interesting that Berrebi et al. (2000b) have found a bias of heterozygote proportions in the same populations using allozymes. The absence of panmixia can be explained by an organization in age classes or selection. Similar observations have been noted in other trout populations and several hypotheses proposed (Aurelle et al., 2002).

A group that deserves a special attention includes the populations, which were, before this study, regarded as non-introgressed, but were nevertheless found to exhibit non-native alleles. We found two such populations in the Soča basin sampled in the Rivers Predelica and Lipovšček. Both populations are separated from down-

stream by impassable barriers, and, using allozymes and mtDNA were previously recognized as pure. However, a non-characteristic allele BFRO002 (116) was detected in both of the two populations. This allele has been 100% present in all specimens representing the Atlantic lineage of the brown trout in Slovenia, however it was recently found in a high proportion in the brown trout from the southwest Balkans (Razpet et al., unpublished data). Because this Atlantic allele is introgressing the Predelica and Lipovšček populations alone, it is therefore plausible that this allele represents a part of ancestral polymorphism, or, on the other hand, that it occurred due to homoplasy. In any case, the diagnostic value of this allele is questionable and cannot be decisive in defining the purity of marble trout in the Rivers Predelica and Lipovšček.

In the Sava basin, the alleles BFRO001 (202) and BFRO002 (124), characteristic for the marble trout, were in high frequencies found in the Rivers Sovpot and Mahnečica. The sampling location of the River Sovpot is remote and isolated from downstream, but its upper part neighbours the springs of the Idrijca basin (a part of the Soča basin). Temporary interconnection between the adjacent streams of the Idrijca and Sava basins are believed to have occurred during the Pleistocene (Melik, 1963; Bavec and Tulaczyk, 2002), which may have promote gene flow between neighbouring trout lineages and contributed to their introgression. Up to now, only one directional gene flow (from the Idrijca to the Sava basin) is suspected. Evidences of reverse introgression can only be implied from phenotypic observation: the Idrijca marble trout is characterized by red spots that are absent in all other marble trout populations. This phenotypic trait may represent a remnant of ancient introgression of brown trout genes into the marble trout genome.

The presence of the allele BFRO002 (124) in the River Mahnečica is difficult to explain since the river is remote and isolated and there is no evidence of any paleogeographic connection with the Soča basin. On the other hand, introgression due to anthropogenic introduction does not seem likely either since relatively recent hybridisation would probably cause the introgression of foreign alleles in more than one locus only. So, homoplasy or ancestral polymorphism seems to be the most probable explanation for the presence of allele 124 in *S. trutta*.

The River Iška feeds the River Ljubljana that used to be stocked with the marble trout (Povž et al., 1996), and the River Kozji jarek discharges into the River Kolpa, currently stocked with the Atlantic lineage of brown trout. At their lower part, the Iška and Kozji jarek go underground, which should prevent fish immigration. Nevertheless, transitory surface connection may occur during rainy seasons, which seems to be sufficient factor for gene flow between neighbouring trout populations

and introgression of foreign genes into the native genomes.

It would be interesting to establish whether the observed introgression proportions (Table 4) depend on successful reproductive performances of introduced trout or are simply a consequence of a high number of stocked fish. Since no accurate records about stocking intensity and wild fish concentration in stocked areas are available, no such inferences can be made. Nonetheless, our research clearly showed that despite many demonstrations of poor performances and low fitness of stocked trout in the wild (reviewed in Hansen, 2002), gene-flow between native and introduced stocks can readily take place under inappropriate management. Although genetically pure marble and Danubian brown trout do still exist in Slovenian rivers, the majority of populations originating from the main watercourses are seriously polluted with non-native genes. It is clear though that it is not only marble trout whose identity is endangered in Slovenia but also the existence of autochthonous Danubian brown trout as well is critically compromised. For this reason, it is crucial that the indigenous and non-introgressed populations of marble and Danubian brown trout in Slovenia, representing a valuable source for the species rehabilitation, are preserved and strictly protected from any stocking activity.

Acknowledgements

The authors thank P. Dovč, the Head of the Genetic Laboratory of the Department of Animal Science, University of Ljubljana, for supervising, assistance and moral support during the project. Thanks also to D. Jensenšek, N. Budihna and S. Pleško for organizing sampling campaigns.

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