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PRIMARY RESEARCH PAPER

Natural and domestic introgressions in the marble trout population of Soča River (Slovenia)

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Abstract The endemic marble trout, *Salmo trutta marmoratus*, suffered massive stocking with brown trout in Slovenia and Italy. In the Soča River (Slovenia) long-term surveys have evidenced Danubian and Atlantic introgressions. The observation of discrepancies between introgression frequencies when using morphology, mitochondrial sequences, allozymes, or microsatellites markers led to reanalyze the hybrid population of the Soča River. New analyses on diachronic samples of the Soča hybrid populations was performed, genotyping partly the same individuals on one diagnostic allozyme (*LDH-C1**), eight microsatellite, and one mitochondrial loci. The confirmed discrepancies led to interpret the anomaly as the consequence of ancient natural immigration of Danubian trout by rivers captures together with stocking with Atlantic domestic trout. Management consequences are discussed because the natural Soča inhabitant which was thought to be a pure marble trout should be now changed into a natural hybrid lineage accepting few Danubian alleles.

Keywords Salmonids · Conservation · Stocking · Microsatellites · Lactate dehydrogenase

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Introduction

One of the serious dangers threatening the genetic integrity of natural taxa is hybridization with phylogenetically close forms introduced by man (Leary et al., 1995). By this way, higher is the number of hybridized populations in a given taxon, higher is the risk that the original taxon disappears, giving place to hybrid swarms (Wilde & Echelle, 1992; Leary et al., 1995; Hasselman et al., 2014), possibly having reduced fitness, a phenomenon well documented in salmonids (Hansen et al., 2002; McGinnity et al., 2004; Miller et al., 2004; Muhlfeld et al., 2009; Brockmark et al., 2010; Wollebaek et al., 2012).

The marble trout of Slovenia, *Salmo trutta marmoratus* Cuvier 1817, is a typical case. Literature tells us that this taxon (its status of species, subspecies, or geographical variety is still discussed) suffered massive

stocking with brown trout in Slovenia and Italy (respectively, Povz, 1995; Chiesa et al., 2016). Artificial propagation began probably in 1880. Records exist about stocking in 1906 from hatcheries of Bosnia-Herzegovina and from various origins including Denmark after the WW2 (Ocvirk, 2000; Sušnik Bajec et al., 2015). A detailed history reconstitution of the Soča stocking is given in Sušnik Bajec et al. (2015). Then the legal ban of brown trout stocking was established in 1996 in Slovenia after the first molecular investigations and the publication of a marble trout Action Plan (Povz et al., 1996).

In the present nomenclature, marble, Danubian, and Atlantic lineages are considered to belong to the *S. trutta* L. complex, within which neither reproductive isolation nor assortative mating is known. This compatibility was experimentally confirmed between Soča marble trout and commercial Atlantic domestic trout (Meldgaard et al., 2007). This situation led to a hybrid population in the main Soča River and several more or less isolated tributaries (Povz, 1995).

From the Soča spring (1,350 m elevation) to the Tolmin zone (150 m), Ocvirk (1989) determined, with morphological measurements, that the marble trout occupied 62% of the main population and the hybrids 24%, which would lead to an estimation of about 74% of marble forms between 1983 and 1985. In the Ocvirk publication, Atlantic and Danubian lineages were gathered into a brown trout class.

Molecular analyses began in 1993 (Berrebi et al., 2000). According to allozymes, the main stream hybrid population was estimated to be composed, in 1993–1994, of 42% of marble allozymic alleles (*LDH-C1*120*), 23% of Danubian (**100*), and 35% of Atlantic (**90*) as recalculated from Berrebi et al. (2000) on grouped samples in the zone of confluence of Soča River with its Volarja tributary, near Tolmin. In the Soča hybrid population, linkage disequilibrium (LD) has been evidenced between allozymes and meristic characters (Delling et al., 2000), showing that the hybrid population was not panmictic in 1998.

Mitochondrial DNA was analyzed on Soča and Tolminka rivers samples (the last is a short tributary of the former) in 1997–1998 (Snoj et al., 2000). The 21 individuals analyzed showed 52% of MA (marble), 19% of DA (Danubian), and 29% of AT (Atlantic) haplotypes, confirming the triple composition of the hybrid population, while giving a majority (instead of a third) to marble haplotypes.

Microsatellites confirmed the triple lineages composition of the hybrid population. However, Jug et al. (2005), by considering only diagnostic alleles at 5 microsatellite loci, found up to 78% of marble alleles in the Soča River in 1998, nearly no Danubian ones (3%), and 19% of Atlantic presence.

Recently, the study of Sušnik Bajec et al. (2015), using 36 SNPs and mtDNA control region (CR) through RFLP, explored the whole upper basin, together with lower parts and some Danubian tributaries. Confirming most of the former observations, this considerable survey completed the whole basin trout genetic composition. This survey provided several new results like the presence of Danubian populations with only 10–30% of marble introgression in two Idrijca tributaries. Concerning the central part of the hybridized population (Tolmin region), 95–100% of both MA haplotypes and marble trout SNP were found in 2008 in the Soča River and in the Tolminka River, its local tributary.

Finally, upper isolated mountainous tributaries were shown to harbor the pure marble lineage, with 8 isolated populations detected (Berrebi et al., 2000; Fumagalli et al., 2002), while being genetically nearly monomorphic and quite differentiated each other.

According to the publications describing the hybrid population of the Tolmin zone of the Soča River, several unexpected results or contradictions need new exploration:

- The morphological determination of Ocvirk (1989) estimated the central hybrid population composed of about 74% of marble trout, morphological determination higher than any molecular estimation.
- While not discussed in their publication, the multidimensional diagram given in Fig. 2 by Jug et al. (2005) detected nearly no Danubian genotypes (3%) in the Soča hybridized population in 1998 using microsatellites. This is in contradiction with the values obtained 4–5 years before (1993–1994) by Berrebi et al. (2000). The same method gave the same anomaly in Fig. 3 of the Sušnik Bajec et al. (2015): using SNP, the Danubian lineage was nearly absent of the hybrid population in 2008.

The morphological divergence of several pure marble trout populations (presence of red spots in most pure marble trout), together with the discovery of

two Danubian populations led Sušnik Bajec et al. (2015) to hypothesize a natural origin of a part of the Danubian introgression of the hybrid population.

The aim of this essay is to analyze some samples with both allozymes and microsatellites, and CR mtDNA sequences (RFLP) in order to find an explanation to the contradictions described above, and to suggest new hypotheses on the origin of the different lineages hybridizing in the Soča River.

Materials and methods

Sampling

Genetic researches on the Soča trout hybridization is a long-term project beginning in 1993 (Crivelli et al., 2000). Since this time, numerous tissue samples have been constituted and collected at the Montpellier University between 1993 and 2010, mainly analyzed with nuclear markers.

Samplings for allozyme markers were done from 1993 to 2004 (Table 1). After electrofishing, trout subadults and adults were anesthetized with MS-222 (Tricaine S of Western Chemical) in the field until death and then dissected. Tissues were frozen immediately in liquid nitrogen.

Samplings for microsatellite genotyping were constituted from 2001 to 2010 (Table 1). Collected trout were anesthetized with phenoxy ethanol, fin clips of maximum 1 cm long were stored in 96° alcohol in the field, and the fish were released.

Geographic situations of sites of sampling are given in Fig. 1.

In order to investigate the relationships between allozyme and microsatellite genotypes in the hybrid populations, 402 individuals were analyzed using one, two, or three markers (Table 1).

In order to recognize the three lineages present in the watershed, the following references have been used: pure marble populations from Studenec, Lipovšček, and Trebuščica Rivers (Berrebi et al., 2000; Fumagalli et al., 2002), a Danubian population from Danube basin (Trižiška Bistrica River) while hybridized with 25% of domestic Atlantic allozymic alleles (Berrebi et al., 2000), two Danubian populations from the Soča basin (Cerknica and Peklenski Rivers: Sušnik Bajec et al., 2015), and an Atlantic domestic population from France (Isère hatchery: Bohling et al., 2016).

The Soča basin hybrid population has been sampled several times, with four samples of the Volarja River (1993, 2000, 2003 and 2004), a small left side tributary of the Soča River (Fig. 1). These samples constitute the central sampling of the present survey, genotyped with 1–29 allozyme loci (while only *LDH-C1** locus is exploited here) and 8 microsatellite loci.

Note that in order to reach larger sample sizes, the Studenec pure marble population has been augmented by the Plascak river replicate (pure marble population replicates are translocated trouts from a threatened valuable population to another stream for conservation), and the Lipovšček population by the replicates of the Kokosnjak and Mortinkov rivers. The replicate populations are not shown in the map (Fig. 1).

Allozyme genotyping

Among more than 50 allozymic loci currently analyzed in trout (Krieg & Guyomard, 1985; Laikre, 1999), 29 presumptive loci were tested on most of the samples involved in this study (Berrebi et al., 2000; unpublished data). However, *LDH-C1** locus (L-lactate dehydrogenase, E.C. 1.1.1.27) is used alone in the present survey because, among allozymes, it is the only diagnostic marker for marble, Danubian, and Atlantic lineages and because some samples have been genotyped at this unique diagnostic locus.

The diagnostic status of this marker needs some explanation. Allele *LDH-C1*100* of this marker is considered ancestral because it is present in related species like the Atlantic salmon and in basal trout lineages like the Mediterranean (Danubian, Adriatic and west-Mediterranean locations) and southern Atlantic (southern France and Iberian Peninsula) populations. It can be considered as partly diagnostic since it occurs in several trout lineages and in several species. In the Soča River trout populations, the local marble trout exhibits the **120* allele and the introduced commercial Atlantic trout, the allele **90*. The partly diagnostic **100* allele can be considered here as fully diagnostic for Danubian lineage because of the absence of ME lineage in the river (Sušnik Bajec et al., 2015 and other publications therein).

Technically, trout eyes were homogenized with specific buffers and centrifuged. The supernatant was introduced in horizontal starch gels (11%) for protein electrophoreses. The staining method was developed by Pasteur et al. (1988), modified by Berrebi et al.

Table 1 Sampling details

River name	Basin	Type reference	Map sample code	Year	NLDH	NMi	NCR
Studenec	Kanomljica-Idrijca-Soča	Marble trout	lA	2001	0	12	0
Plascak	Studenec replicate	Marble trout	lB	2010	0	7	0
Lipovšček	Kneza-Baca-Idrijca-Soča	Marble trout	2A	2001	0	27	4
Kokosnjak	Lipovšček replicate	Marble trout	2B	2010	0	13	0
Mortinkov Poto	Lipovšček replicate	Marble trout	2C	2010	0	11	0
Trebuščica	Idrijca-Soča	Marble trout	3	1993	**28**	**30**	**5**
Isère hatchery	France	Hatchery French Atlantic domestic	4	2008	0	30	7
Podbreg	Volarja-Soča	River Italian Atlantic domestic	5	2004	**10**	**31**	**31**
Cerknica	Idrijca-Soča	Danubian domestic	6	2002	0	24	24
Peklenski potok	Idrijca-Soča	Danubian domestic	7	2009	0	25	25
Trižiška Bistrica	Sava-Danube	Danubian Sava	8	1994	**18**	**17**	**0**
Kamno	Soča	Hybrid population	9	2006	0	30	0
Volarja	Soča	Hybrid population	10A	1993	**25**	**27**	**10**
Volarja	Soča	Hybrid population	10B	2000	**32**	**31**	**0**
Volarja	Soča	Hybrid population	10C	2003	**28**	**30**	**10**
Volarja	Soča	Hybrid population	10D	2004	**33**	**57**	**36**

Basin succession of tributaries from the sampling location to the Soča River, *NLDH* number *LDH-C1** genotypes, *NMi* number of microsatellite multilocus genotypes, *NCR* number of CR haplotypes RFLP. Statistics in bold indicate the LDH/microsatellite/D-loop investigations on the same individuals. Number of haplotypes underlined = data extracted from Sušnik Bajec et al. (2015)

Fig. 1 Geographic situation of the sampled sites. The stations numbers are detailed in Table 1

(2000). The Shaklee et al. (1990) allozymic gene nomenclature conventions have been applied along this text.

Microsatellite genotyping

DNA was extracted from fin tissue samples by the Chelex/proteinase *K* protocol described by Estoup et al. (1996). The 8 microsatellite markers used in this study were adapted from various publications. Details of each locus are given in Table 2. For each marker, the 5′ ends of one of the two primers was covalently linked to fluorescein, Cy3, or Cy5 labels. Polymerase chain reactions (PCR) were performed in an Eppendorf Mastercycler programmable thermocycler with a 10 µL reaction volume containing 0.2 U of *Taq* polymerase (Sigma-Aldrich), 1.5–2 mM MgCl2 (see Table 2), 0.4 mM of each dNTP (Invitrogen), 1 µL 10× reaction buffer, and 3.75 µM of each primer (Eurofins MWG). The thermal cycling conditions were set as follows: initial denaturation (94°C, 3 min); 30 denaturation (94°C, 30 s), annealing (15 s at the temperatures given in Table 2 for each locus) and extension (72°C, 15 s) cycles; and then a final extension (72°C, 3 min). The PCR products were electrophoresed in 6% denaturing polyacrylamide gels (Bio-Rad) and visualized with a FMBIO-II fluorescent imaging system (Hitachi). Allele's sizes were determined based on a fluorescently labeled ladder 100–600 bp (Promega), with the FMBIO Analysis 8.0 image analyzer program (Hitachi). The genotype matrix was then constructed and used as a basis for all of the following statistical analyses.

Control region mtDNA RFLP

A part of the extracted DNA was rapidly characterized by RFLP using the method described by Sušnik Bajec et al. (2015), after amplification of the CR. Restriction enzymes were used to distinguish between MA and DA–AT lineages (AluI) and between AT and DA lineages (SatI).

Table 2 Details of the microsatellite loci used in this study

Locus	$MgCl_2$ (mM)	Annealing temp. (°C)	Marble trout allele sizes (bp) in this study	Atlantic trout allele sizes (bp) in this study	Danubian trout allele sizes (bp) in this study	Hybrid population trout allele sizes (bp)	Original references
Mst543	1.5	52	130–150	122–52	130–152	120–180	Presa et al. (1994)
Mst85	2	52	167–181	147–173	147–179	147–191	Presa & Guyomard (1996)
Omm1105	2	53	198–278	238–458	114–318	134–388	Rexroad et al. (2002)
Omy21Dias	2	58	106–114	102–128	106–120	90–128	Holm & Bendixen (2000)
Oneμ9	1.5	60	185–201	197–207	197–201	185–211	Scribner et al. (1996)
Sfo1	2	59	108–150	110–150	118–166	110–154	Angers et al. (1995)
Ssa197	2	53	155–201	123–143	135–195	123–195	O'Reilly et al. (1996)
SsoSL311	2	57	130–204	126–166	128–198	114–200	Slettan et al. (1995)

Multidimensional analyses

A Factorial Correspondence Analysis (FCA, Benzécri, 1973), implemented within the GENETIX 4.04 program, using the 3D option (Belkhir et al., 2004), provided the overall genetic structure of the samples. This method is well adapted to genotype data. The matrix necessary for the runs must be constituted of disruptive data, i.e., presence or absence in classes. Naturally, the classes can be alleles and so the correspondences which are calculated are the co-occurrence of two given alleles in the same individual or in a group of individuals. Clusters (clouds) detected on the diagram correspond to homogeneous genetic lineages, independent of the fish geographic origin. Each of the 402 trouts was positioned in a hyperspace, structured by the reference sample representatives of the three lineages hybridizing in the river. The inertia values (i.e., the proportion of the total information contained by an axis, expressed as a percentage) along each axis were shown to be equivalent to linear combinations of the monolocus fixation index (F_{st}) values (Guinand, 1996). Mathematical details of the method are friendly given in She et al. (1987).

Assignment analyses

Assignment tests were carried out in order to detect differentiated subgroups. This method, using here the Bayesian STRUCTURE 2.1 program (Pritchard et al., 2000), subdivided the whole sample into $K = 2$–10 subgroups characterized by the best genetic equilibrium in terms of panmixia and lower linkage. The admixture ancestry model and correlated allele frequencies were chosen. A burn-in of 200,000 iterations followed by 400,000 additional Markov Chain Monte Carlo iterations was performed, in order to stabilize assignments among runs. The estimations of the true K value (number of biological subgroups in the entire sample), using the Evanno et al. (2005) method through STRUCTURE HARVESTER (Earl & von Holdt, 2012), were applied on 20 runs for each K value. This automatic estimation was supplemented by the "higher K which makes sense" method (Berrebi et al., 2013). This precaution was taken because, as explained by Pritchard et al. (2007) and more precisely said by Gilbert et al. (2012), "selecting the optimal K can be quite a subjective procedure and is best inferred when the biology and history of the organism are taken into account." Levels of K higher than the more significant one (suggested by the Delta K method) have been explored, following the example of Betto-Colliard et al. (2015).

Panmixia and linkage disequilibria

Panmixia was estimated through F_{is} parameter. For this, the estimator f of Weir & Cockerham (1984) was calculated, together with its significance through 5,000 permutations performed with the GENETIX software.

Another potentially important statistical calculation is the LD. This estimation can indicate a recent introduction of foreign lineage (Walhund effect: Sinnock, 1975). It can also indicate differential behavior between markers kinds. The Black & Krafsur (1985) method is implemented in GENETIX software, followed by an estimation of the significance with 5,000 permutations. Sequential Bonferroni corrections have been performed (Rice, 1989).

Results

Lineages detected in the sampling

The first objective was to compare the diagrams of FCA provided in Jug et al. (2005) and Sušnik Bajec et al. (2015) with that constituted with the present sampling. Figure 2, based on the whole sampling (402 individuals) and 8 microsatellite loci, reproduces the typical triangular disposition due to the presence of three main entities in the sampling: marble, Danubian, and Atlantic lineages. Like in the 2005 and 2015 studies, the Soča hybrid trouts are not in the middle of the triangle but mainly between the marble and Atlantic corners.

Numbering the lineages occurrences

Using the simple count of allozymic alleles, *LDH-C1** confirms the existence of three lineages: the marble, Danubian, and Atlantic ones (Table 3), as already observed by Berrebi et al. (2000).

Concerning microsatellites, assignment test is the method chosen for lineages delineation. Delta K test gave $K = 2$ as the best partition. Figure 3 explores other partitions. From $k = 4$, the assignment test

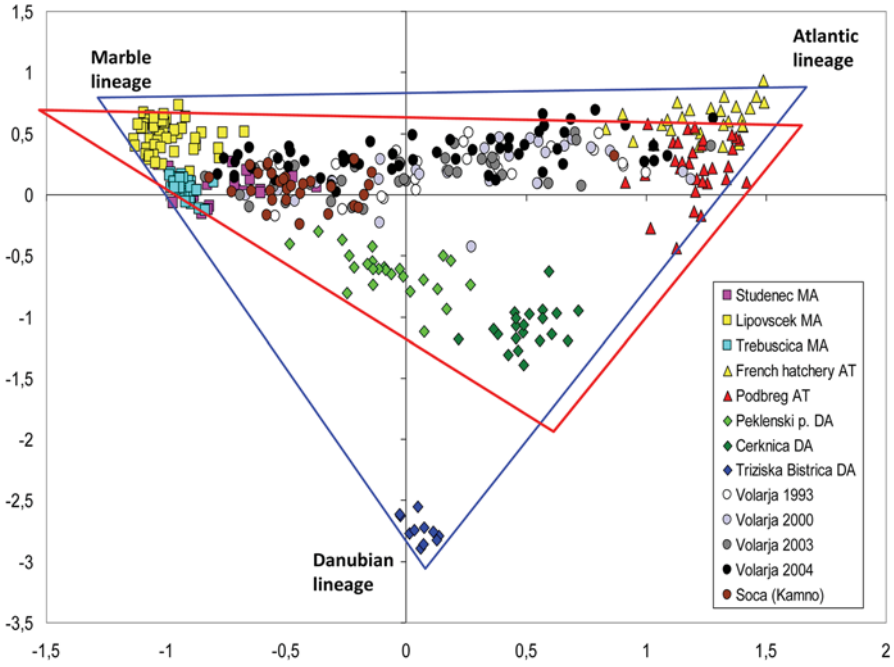

Fig. 2 FCA based on microsatellite data only. Each dot represents a trout according to its multilocus genotype. The *triangles* are joining the marble trout (*top-left*), the Atlantic lineage (here the domestic strains, *top-right*), and the Danubian forms (*center-bottom*). *Blue triangle* the whole data; *red triangle* only Soča data. The Danubian lineage (inside or outside the Soča basin) do not participate to the hybrid populations

placed the pure upstream isolated marble trout populations and the Soča ones in different lineages. Table 3 cumulates marble and Soča lineages frequencies, given their close relative position in Fig. 2, in order to compare them with the allozymic scores. According to assignment tests, the Danubian lineage is nearly absent in the main Soča hybrid population, which is another important result confirming the FCA analysis (Fig. 2).

Panmixia

F_{is} calculations are given in Table 4. With microsatellites, all hybrid population samples are in deep disequilibrium, far from Hardy–Weinberg equilibrium, with heterozygote deficiencies. Less expected, the pure marble populations, Studenec and Lipovšček, show also a clear heterozygote missing. Finally, one of the pure Danubian populations is in a significant excess of heterozygotes.

Linkage analysis

The linkage test concerns only the samples analyzed with both allozymes and microsatellites, i.e., the Trebuščica 1993, Podbreg 2004, Tržiška Bistrica 1994, and Volarja 1993, 2000, 2003, and 2004 samples. In the first step, the significant tests were counted after sequential Bonferroni correction (Table 5). The mean number of significant LD is 2.21 between microsatellite loci and 0.87 between LDH and microsatellite loci, i.e., 2.5 more disequilibria among microsatellite loci than between the two sorts of loci. Supplementary Fig. S1 suggests a link between LD and H.

Discussion

Discrepancy between allozymic and microsatellite structures

According to allozymic diversity already analyzed, partly on the same samples, by Berrebi et al. (2000) and the STRUCTURE output considering $k = 4$, trout living around the confluence of the Volarja tributary with the main Soča river (Tolmin region) belong to three main lineages: the marble, Danubian, and Atlantic ones. These three lineages are considered as geographic entities belonging to the *S. trutta* complex (Bernatchez, 2001), together with the Mediterranean and Adriatic ones not observed in the present sampling. Here, the marble lineage is considered as *S. trutta marmoratus*, waiting for consensus taxonomy.

Table 3 Relative proportion of the three lineages (marble, Danubian, Atlantic) in the 16 analyzed samples

River name	Type reference	Allozymes			Microsatellites			DLoop RFLP		
		Marble (*LDH-C1*120*)	Danubian (*LDH-C1*100*)	Atlantic (*LDH-C1*90*)	Marble lineage	Danubian lineage	Atlantic lineage	MA	DA	AT
Studenec	Marble trout	–	–	–	0.96	0.03	0.01	–	–	–
Plascak	Marble trout	–	–	–	0.95	0.04	0.01	–	–	–
Lipovšček	Marble trout	–	–	–	0.99	0	0.01	1	0	0
Kokosnjak	Marble trout	–	–	–	0.99	0	0	–	–	–
Mortinkov Potok	Marble trout	–	–	–	0.99	0	0	–	–	–
Trebuščica	Marble trout	0.96	0.04	0	0.99	0	0	1	0	0
Isère hatchery	Atlantic domestic	-	–	–	0.01	0	0.99	–	–	–
Podbreg	Atlantic domestic	0	0.05	0.95	0.01	0	0.99	0	0	1
Cerknica	Danubian Soča	–	–	–	0.06	0.93	0	0.04	0.96	0
Peklenski potok	Danubian Soča	–	–	–	0.01	0.98	0	0	1	0
Trižiška Bistrica	Danubian Sava	0	0.75	0.25	0.2	0.80	0			
Kamno	hybrid population	–	–	–	0.97	0.01	0.03	–	–	–
Volarja 1993	Hybrid population	0.29	0.35	0.35	0.66	0.02	0.32	0.60	0.20	0.20
Volarja 2000	Hybrid population	0.33	0.2	0.47	0.57	0.02	0.41	–	–	–
Volarja 2003	Hybrid population	0.59	0.16	0.25	0.72	0.02	0.27	0.60	0	0.40
Volarja 2004	Hybrid population	0.18	0.36	0.45	0.61	0.01	0.38	0.50	0	0.50

Allozymic estimations are the simple scoring of alleles, while microsatellite estimations are produced by assignment, with marble and Soča frequencies cumulated

When observing the allozymic and microsatellite composition of the samples analyzed (Table 3), a good agreement between the estimations of allozymic and microsatellite nuclear markers occurs for (i) the sample of pure marble trout of the Trebuščica River (96 and 99% of marble lineage, according to allozymes and microsatellites, respectively); (ii) the Podbreg ancient stocked population (95 and 99% of Atlantic lineage); and (iii) in the Danubian tributary (75 and 79% of Danubian lineage). This concordance confirms the diagnostic quality of *LDH-C1** correlated with the substantially different allelic frequencies of the microsatellite markers.

When applying these discriminant markers to the hybrid population (Volarja River) sampled in four diachronic occasions, large discrepancies appear. Calculating the mean proportions in the 4 samples, allozymes detect 35–27–38% of marble–Danubian–

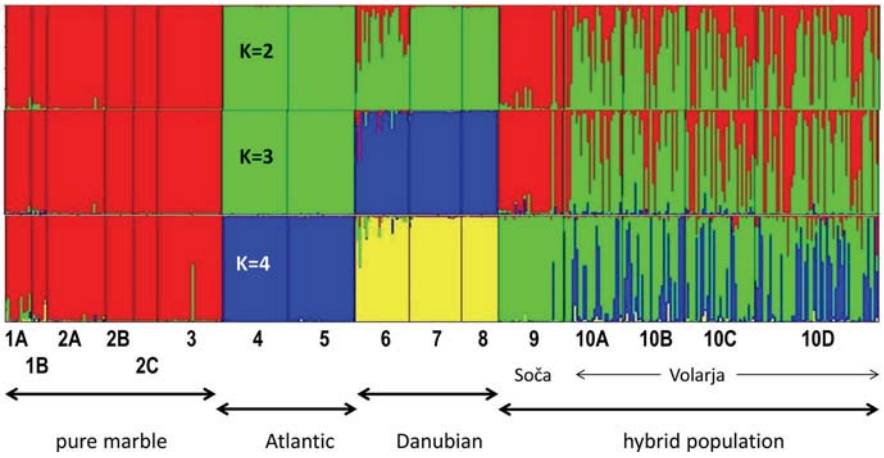

Fig. 3 Assignment analysis histogram representing several K values. According to Evanno et al. (2005) method, the best partition is for $K = 2$

Atlantic alleles, respectively, in the Volarja River between 1993 and 2004, but microsatellite assignment estimations detect 64–2–34% of the same lineages. Apparently, allozymes "see" the Danubian lineage but microsatellites do not.

Sušnik Bajec et al. (2015) analyzed the Cerknica and Peklenski potok rivers samples using SNPs and mtDNA RFLP and concluded that they were nearly pure Danubian populations. In the present survey, using the same individuals (by exchange of samples between laboratories), microsatellites arrived to the same deduction (respectively, 93 and 98% of Danubian lineage). Sušnik Bajec et al. (2015) suggested that these two populations naturally entered the watershed.

In the present survey, we suggest the same hypothesis, according to molecular arguments applied to the main hybrid populations. Allozymes allow detecting 27% of Danubian origin in the Volarja River because this marker evolves slowly and maintains the *LDH-C1*100* allele diagnostic of the Danubian lineage.

Microsatellites are hypervariable markers, which means that the variation in the number of repeats is rapid (each 10,000 copies) and saturation rapidly occurs. Allozymes are slow evolution markers and mtDNA provides clonal evolution markers. With these two last markers, the Danubian origin of a population or an individual cannot be lost, with microsatellites, yes. In the present study, the Danubian reference is double: one in Sava (Danube) River (Tržiška Bistrica) and two in Idrijca (Soča) River (Cerknica and Peklenski tributaries) probably after migration into the Soča basin (naturally or by men). Their history of isolation explains the allelic differences between all these samples. If the hypothesis of natural immigration of Danubian trout in the Soča basin during orogenic periods is true, their microsatellites alleles length evolved during a long time and because of saturation are no more similar to the original Danubian alleles. For microsatellites (and in FCA or assignment analyses), the Danubian alleles are now natural component of the hybrid Soča population and different from the core population alleles (in Danube River). Only slow and clonal markers can recognize their Danubian origin. Microsatellites cannot distinguish marble and Danubian lineages which hybridize naturally since several thousands of years and are so both natural. For the hypervariable microsatellites, only two categories exist: the natural (marble and Danubian) and the domestic (Atlantic) ones. That is why the estimation of Atlantic participation is similar (38 and 34%, respectively, with allozymes and microsatellites).

Instable allelic composition in diachronic survey

The allelic composition of the Volarja River population, sampled during four campaigns, is far to be stable. The diachronic analysis shows a huge variation in lineages proportion both with allozymes (18–59% for the marble lineage) and microsatellites (57–72%). Because the sampling site in the Volarja tributary is only 400 m from the confluence with main Soča River, the sampled population Volarja cannot be independent of the big hybrid population of the Soča River.

This Soča population is not homogeneous downstream–upstream and important movements have been observed, due in part to well-known periodic severe flood events (Vincenzi et al., 2012, 2014), especially in October 2004. Moreover, because marble and

Table 4 F_{is} calculated according to the Weir & Cokerham (1984) estimator *f*

Ri ver name	ONEU9	MST85	SSOSL311	OMY21DIA	MST543	SFO1	SSA197	OMM1105	Total microsatellites	*LDH-C1**
Studenec	0.497**	0.397**	0.032	0.047	1.000***	0.896***	0.242*	0.647***	0.416***	na
Lipovšček	−0.081	−0.112	−0.272**	−0.318***	0.504***	0.850***	0.625***	−0.062	0.168***	na
Trebuščica	m	−0.094	−0.272**	0.113	0.165	0.155	0.043	0.179	0.097	m
Isère hatchery	0.243**	−0.031	−0.092	0.181*	0.015	−0.090	0.056	0.113*	0.040	na
Podbreg	−0.338***	0.076	0.106	−0.088	0.017	0.028	−0.008	0.241**	0.011	m
Cerknica	m	−0.371	−0.119	−0.120	0.623***	0.035	−0.184	0.045	−0.006	na
Peklenski potok	−0.571**	0.0442	−0.140	−0.383***	−0.317	−0.154	−0.125	0.335**	−0.170***	na
Trižiška Bistrica	m	m	−0.280	m	m	0.568***	0.272*	−0.422**	−0.044	−0.308
Kamno	0.169	0.092	−0.033	−0.110	0.126	0.379**	0.141*	0.133**	0.109**	na
Volarja 1993	0.077	0.201**	0.195**	0.092	0.425***	−0.124	0.088	0.072*	0.128***	0.142
Volarja 2000	0.245**	0.021	0.568***	0.023	0.244***	0.101*	0.104*	0.247***	0.199***	−0.199
Volarja 2003	0.260**	0.317***	0.234**	0.178**	0.273***	0.094	0.329***	0.122**	0.224***	0.069
Volarja 2004	0.209**	0.134**	−0.046	0.150***	0.579***	0.034	0.013	0.055*	0.135***	0.481***

The two last columns give the global F_{is} for microsatellites and the F_{is} for *LDH-C1** alone

m monomorphic locus, *na* not analyzed

*, **, and *** are the three levels of significance at 95, 99, and 99.9%

Table 5 Matrix crossing the 8 microsatellite and LDH loci

ONEU9	MST85	SSOSL311	OMY21DIAS	MST543	SFO1	SSA197	OMM115	LDH5	
	$A+B$	B	C	$B+C+D$	C	–	B	–	ONEU9
		$A+B$	D	$B+C+D$	$C+D$	D	$B+C$	D	MST85
			$C+D$	$A+B+C+D$	$B+C$	$B+C$	$A+B+C$	D	SSOSL311
				$3+A+C+D$	$B+C$	$B+C+D$	$B+D$	3	OMY21DIAS
					$C+D$	$A+C+D$	$A+B+C+D$	$3+A+D$	MST543
						$B+C+D$	$8+B$	–	SFO1
							$A+B+C+D$	D	SSA197
								–	OMM115
									LDH5

The letters in each cell correspond to the map numbers of the sample (given Table 1) where the two loci are correlated ($A = 10$A, $B = 10$B, $C = 10$C, $D = 10$D)

Atlantic trout have different aptitudes to adapt according to the slope and the flood (Meldgaard et al., 2007), it is probable that periodic floods can explain sudden variation of the Soča hybrid population passing in front of the Volarja–Soča confluence and influencing its trout population.

Population disequilibria

LD should confirm this hypothesis. Composite populations conserve genetic linkage long after Hardy–Weinberg equilibrium is reached due to numerous generations of crossing. Because of the different discriminant capacities of allozymes and microsatellites, linkage should be occurring among microsatellite loci but less between microsatellite and *LDH-C1** ones. That is the case with a mean number of 2.21 significant linkages (after Bonferroni correction) among microsatellite loci, but 0.87 between microsatellite and allozyme locus. However, it is known that linkage depends on the allele distribution and especially the diversity of each locus. A clear correlation links the number of LD and the different H (Ho, He, Hnb) and A parameters, considering the whole sampling or simply the Volarja samples (data not shown). The diagram presented in Supplementary Fig. S1 is an example plotting the number of significant linkage between a given locus and all others (see Table 5) with the locus diversity (here the observed heterozygosity Ho). The effect of diversity differences between loci on LD is then too dominant to allow detection of other causes of linkage. This LD parameter thus provides no useful information about the correlations between allozyme and microsatellite markers.

Panmixia is not always confirmed. First, a high heterozygote deficit is observed in the hybrid population (Kamno and Volarja samples, Table 4). Microsatellite loci Oneμ9, Mst543, and OMM1105 are mainly involved. This is expected in populations currently introgressed. Here, we can suspect the effect of domestic marble trout intensive stocking. More surprising is the disequilibrium observed in two pure marble populations without easy explanation except for a family structure due to small population sizes.

The mtDNA marker

Table 3 gives the haplotypes frequencies for the 8 samples analyzed with this marker, according to the

three lineages which are easy to distinguish (Bernatchez, 2001). Two pure marble samples, one Atlantic and two Danubian, confirmed the perfect discrimination ability of this marker. Applied to three samples from Volarja River, the central hybrid population, marble haplotypes are in majority (1993 and 2003) or equality (2004) with the Atlantic haplotypes. Danubian haplotypes were detected only in 1993 (sample size: 10 haplotypes), but not in 2003–2004 (46 haplotypes). However, in 1997–1998, Snoj et al. (2000) detected 19% of DA haplotypes among 21 trout in the Tolmin region of the Soča River.

Origin of the Danubian lineage in Soča River

Using red spots and demographic arguments, Sušnik Bajec et al. (2015) suggested that the two Cerknica and Peklenski populations (Idrijca subbasin) have naturally migrated into the Soča basin, using the opportunity of river captures linked to the high orogenic activity of the region. This hypothesis can be discussed, but does not interfere with the hybrid population molecular hypothesis. In the Soča hybrid population (represented here by Kamno and Volarja samples), naturally entered Danubian lineage is deduced from fast/slow evolving markers' discrepancy but their origin should be the same: river captures allowing exchanges by the top of Danube and Soča watersheds. River captures have been well documented in New Zealand (Waters et al., 1994, 2001; Waters & Wallis, 2000). Long distance migration of strictly freshwater species can be explained only by this capture phenomenon, as for example in the *Barbus* model (Durand et al., 2002; Tsigenopoulos et al., 2002b, 2010; Tweddle & Skelton, 2008).

In the studied region, natural exchanges between Danubian and Soča Rivers are well known. It was frequently observed that the distribution of monophyletic clades, according to reconstructed molecular tree structures, crossed the watershed boundaries and did not correspond to expected distribution. The vairone, *Telestes* (previously *Leuciscus*) *souffia*, freshwater cyprinid, is suggested to have crossed the limits between Danube and Soča (Machordom et al., 1999; Salzburger et al., 2003). *Phoxinus phoxinus/lumarieul* clade 2 extends across both the Adriatic and Danube watersheds (Palandačić et al., 2015). *Barbus caninus* and *B. petenyi/balcanicus* intraspecific close relationships are explained by Sava and Soča drainage exchanges (Kotlik & Berrebi, 2002; Kotlik et al., 2002; Tsigenopoulos et al., 2002a). The stone crayfish *Austropotamobius torrentium* is another example of cross watersheds homogeneous lineage (Trontelj et al., 2005).

Management consequences

Management and rehabilitation of the marble trout in Slovenia began more than 20 years ago. The first investigations tried to find pure marble trout populations in order to provide genitors to fish farms (Berrebi et al., 2000; Fumagalli et al., 2002) allowing the description of 8 not hybridized populations.

In the present days, intensive stocking is performed by the Tolmin Angling Association, using captive marble wild genitors. The choice of the genitors was first phenotypic (the external aspect of the fish should be perfectly of marble morphology). The selected wild genitors were then genetically checked, using D-loop RFLP and 4 diagnostic SNPs until 2010 and now 16 SNPs among those used in Sušnik Bajec et al. (2015).

According to the present hypothesis, the natural inhabitant of the Soča basin is a natural hybrid between marble and Danubian lineages. Probably, more populations able to provide pure wild genitors can be found for management, perhaps without the disadvantage of a very low polymorphism (Fumagalli et al., 2002) because Danubian alleles become acceptable for restocking.

Practically, any population showing only the wild lineage when analyzed with microsatellites can be considered as the natural Soča trout. Their genitors can be used for restocking endangered subpopulations. Analyzed with allozymes or D-loop markers, they should express Danubian alleles and haplotypes but not Atlantic ones.

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