Analysis of the situation of bullhead (*Cottus gobio*) population in the Sense River and tributaries.

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Abstract

This project investigated the genetic population structure of bullhead (*Cottus gobio*) using 10 highly polymorphic microsatellites. As a study system we chose one river and its tributaries. We sampled fish at 22 two different sites. The sampled populations were either connected or they were disconnected through artificial barriers. This sampling allowed us to assess migration behavior as well as the influence of river fragmentation on this small bottom-dwelling fish. We found very high genetic differentiation between the populations at this small geographic scale. Additionally we found a strong isolation by distance pattern. These two results confirm the findings of earlier studies which testimonialize the bullhead as a rather resident species. Furthermore we observed reduced genetic diversity in the populations from the head reaches due to a strong asymmetrical migration in a downstream direction. We also found strong evidence for the influence of barriers leading to a greater genetic differentiation in disconnected populations and reduced genetic diversity in the above-barrier populations. In light of their limited migration, but also because of the high genetic differentiation between bullhead populations, we suggest more research to elucidate the relevant scales at which evolutionary significant units exist in bullheads.

1. Introduction

1.1 River management

Because of the worrying biodiversity declines in rivers in Switzerland but also all over the world, programs in river restoration have increased over the last few decades. One of these ventures was the Rhone-Thur-Project, founded by the WSL, EAWAG, ETH Zürich and EPF Lausanne. It was made to give an overview of the outcomes of restoration steps and the decisive factors which lead to these outcomes. As a follow-up project the same partners founded a new venture, called "integrated river management", to implement the results of the Rhone-Thur-project. Under the guiding theme "dynamic habitats and floodwater protection" several projects have been defined, which focus on two main issues:

- Interactions between constructive floodwater protection and habitat heterogeneity in watercourses (4 subprojects).
- Longitudinal and diagonal connectivity of watercourses (4 subprojects).

The subprojects will deal with the interaction between constructed, but naturally oriented floodwater protection, also in regards to natural habitat diversity. These subprojects are connected through questions about the recolonization dynamics of restored watercourses. This leads to the focus of water network dynamics and analyses of the longitudinal connectivity of waterbodies. A further aim is to increase cooperation between science and practice (rivermanagement 2009).

The following project focuses on the population situation of bullheads in the Sense River and its tributaries, with the goal of obtaining basic knowledge of the situation of this fish.

Humans have the habit to change their environment for their own benefit. This benefit is usually a big disadvantage for all species that share the same habitat. Many of the constructions that men build for their welfare result in habitat loss and fragmentation for the rest of the community. This often leads to discontinuities in environmental conditions and the distribution of critical resources (Segelbacher et al. 2010) and a reduction in connectivity among populations (Kindlmann and Burel 2008). The

outcome is a decline in population sizes and an increased isolation of populations (Frankel and Soule 1981). However, gene flow among populations is crucial to their long-term viability. This is because gene flow spreads potentially adaptive genes, and counteracts the potentially detrimental effects of genetic drift in small populations (Van Dyck and Baguette 2005; Segelbacher et al 2010). Migration between populations can also help small populations to avoid inbreeding due to the arrival of new individuals from other populations (Hastings and Harrison 1994). This led to the awareness that habitat loss and fragmentation are major reasons for population declines and species extinctions (Sala et al. 2000). In that sense estimation of gene flow is a key factor to understand and predict human impacts on natural populations (Hänfling & Weetman 2006) and genetic diversity is an important indicator for the fitness of populations (Knaepkens et al. 2002).

If a natural system provides services to mankind, it is often threatened by several human activities. Rivers are such systems. Dams are build to generate energy, river stretches become passable for ships by building locks. Furthermore, land use and flood protection measures lead to canalization and water regulation. Because of such activities, fragmentation is one of the major ecological concerns in rivers (Raeymaekers et al. 2009).

In Switzerland, a survey of weirs and dams with a height of more than 0.5 m has been carried out during 2009. The number of artificial barriers was estimated at around 101`000, which amounts to 1.6 artificial barriers per kilometer of a river (Zeh et al. 2009). Such barriers probably affect the migration behavior of many purely aquatic species. Especially upstream migration is prevented by fragmentation and leads to an isolation of populations in the head reaches of a river. In these populations, a decline of population size and a higher possibility for inbreeding may exist, which increases the risk of extinction (Frankham and Ralls 1998, Yamamoto et al. 2004). Studies of population genetics can give an insight in the dispersal abilities of aquatic animals, the effects of river fragmentations and might give important knowledge for their conservation and also suggestions for the improvement of river restauration.

1.2 Bullhead (*Cottus gobio*)

The bullhead (*Cottus gobio*) is a small freshwater fish, with almost no economical value. It therefore has relatively natural populations which are not influenced by human fishing or stocking (Englbrecht et al. 2000). Its maximum size is about 18 cm. Depending on the productivity of the water the bullhead needs one to four years to reach fertility. The species spawn between February and June. The mating system is polygynic where females choose the males by size and the males hatch and guard eggs from several females (Bisazza and Marconato 1988). The fish has a reduced swim bladder and lives in benthic habitat in streams and lakes. Bullheads are also morphologically adapted to the benthic lifestyle and from earlier studies this species is known as rather resident, only moving from 10 to several hundred meters a year (Downhower et al. 1990; Knaepkens et al. 2004). For the bullhead it is known that barriers with a height of 20 cm are not passable (Utzinger et al. 1998). Their food contains mainly macroinvertebrates (Gädtgens, 2004). The bullhead lives primarily in trout rivers and bigger streams and sometimes in lakes (Gaudin & Caillere 1990). The fish is common in Switzerland but the population has been on the decline for the past 50 years. This led to its classification as a potentially endangered species in 2003 (Zaugg et al. 2003). In a study from Utzinger et al. (1998) several papers were reviewed and 4 main reasons for the decline of bullheads have been identified, which are still supported. First, during the last decades an increase in chemical water pollution took place (Starmach 1965, Späh and Beisenherz 1984, Bucher et al. 1992, Waterstraat 1992). Secondly, newly constructed river obstructions changed, fragmented or destroyed habitats (Bless 1981, Barandun 1990, Bless 1990, Hofer and Bucher 1991, Jungwirth 1996, Knaepkens et al. 2004, Tudorache et al. 2008). Thirdly, after incidents that led to a die-out of fish, only fish species with economical value have been reintroduced (Barandun 1990, Hofer and Bucher 1991). Fourthly, bullheads were misleadingly known as predator of eggs and young trouts and were therefore hunted intensively (Adamicka 1979, 1984, Gaudin and Heland 1984, Späh and Beisenherz 1884, Gaudin 1985). An unhallowed interplay of these circumstances has lead to a population decline in this species. From a conservation perspective it is therefore crucial now establish knowledge of the biology and ecology of the bullhead (Zbinden et al. 2004).

1.3 Sense River and its tributaries

The Sense River is located between the canton Bern and canton Fribourg. It arises when the Kalte Sense and the Warme Sense flow together. The Kalte Sense has its source in the Gantrisch Mountains in an elevation of about 1500 m.a.s.l. and the Warme Sense emanates from a small lake called Schwarzsee in 1046 m.a.s.l. They flow together at a place called Zollhaus. The Sense then flows down until Laupen, where it ends up in the bigger Saane River. The Sense River has a very diversified morphology. The stretch called Warme Sense between Schwarzsee and Zollhaus is heavily fragmented by a total of 18 river bed drops. Thanks to a thorough documentation of the river obstruction history the appearance of each barrier can be accurately estimated. The first 5 barriers where built during 1917 very near to Zollhaus. By 1957 the slope of the lower reaches in the Warme Sense was diminished with 13 bed drops. By 1988 the head reaches were also obstructed with two drops whereas in the lower reaches two drops were removed (fig. 1 and 11). The Sense River itself between Zollhaus and Neuenegg is one of the most natural and unspoiled rivers in Switzerland. Furthermore one of the longest passable river stretches in Switzerland exists in that part. The lower reaches of the Sense River at Flamatt are heavily canalized and again interspersed with two bed drops and several ramps. This pattern makes the Sense River an ideal location for study of the effects of river habitat alterations of fish populations because differences between natural and artificial river stretches and also fragmented and connected habitats can be examined.

The Warme Sense has a lot of bed drops (pic. 1). Following the river downstream one passes to the long almost natural stretch where the river is braided and has a lot of gravel banks (pic. 2 Sense next to Rufenen and pic. 3 shows the huge natural canyon at Schwarzenburg). Through Flamatt the Sense is straightened and the river banks are stabilized by rip raps (pic. 4).

Picture 1; Bed drops in the Warme Sense, pic. 2; long natural stretch at Rufenen, pic. 3 Canyon at Schwarzenburg, pic. 4; degraded lower reaches at Flamatt.

1.4 Questions and hypotheses

Several studies have shown asymmetric migration downstream for aquatic species in river habitats (e.g. Müller 1954, Waters 1972). This has also been shown for bullheads (Hänfling et al. 2002, Hänfling and Weetman 2006). Such a process leads to a lower genetic diversity in upstream populations (Yamamoto et al. 2004, Caldera and Bolnick 2008). Furthermore, there are several studies that testimonialize *Cottus gobio* as a resident species. But this knowledge was based either on tagging experiments (Smyly 1957, Andreasson 1971, Lelek 1987) or the studies were genetically based but in systems with a very large geographical scale and with many impassable barriers (Nolte et al., 2005, Hänfling and Weetman 2006, Vonlanthen et al., 2007). However, compared to these studies we worked on a smaller scale in one River which is an ideal location for study of the effects of river habitat alterations of fish populations because differences between natural and artificial river stretches and also fragmented and connected habitats can be examined**.**

This allows us to address the question of whether differences in genetic differentiation are correlated simply with distance or also with degree of connectivity. If the barriers have an influence on the population genetics of bullheads one would expect larger genetic differences between disconnected populations than between connected ones. Furthermore, we would expect to observe a smaller allelic richness and a distinct allelic composition in the disconnected populations.

2. Methods and analysis

2.1 Sampling design

To get a good overview over the situation of bullhead populations, first we had to assess their distribution. We did electrofishing (backpack electro shocker: EFKO 1.5KW) at Sixteen sites in the Sense River. We also fished at two points in the Warme Sense and at two points in the Kalte Sense. Additionally we fished in seven tributaries. We caught bullheads at twenty-two sites from a total of thirty-two attempted sites (Fig. 1). At each site fin clips from fish were collected and stored in 100% ethanol. Until processing the clips were kept in a freezer at -21°C. Before releasing the fish, they were weighted and their size was measured. We also documented the conductivity and temperature of the water at each site. The sample size per site differs between three and thirty-seven individuals (Table 1).
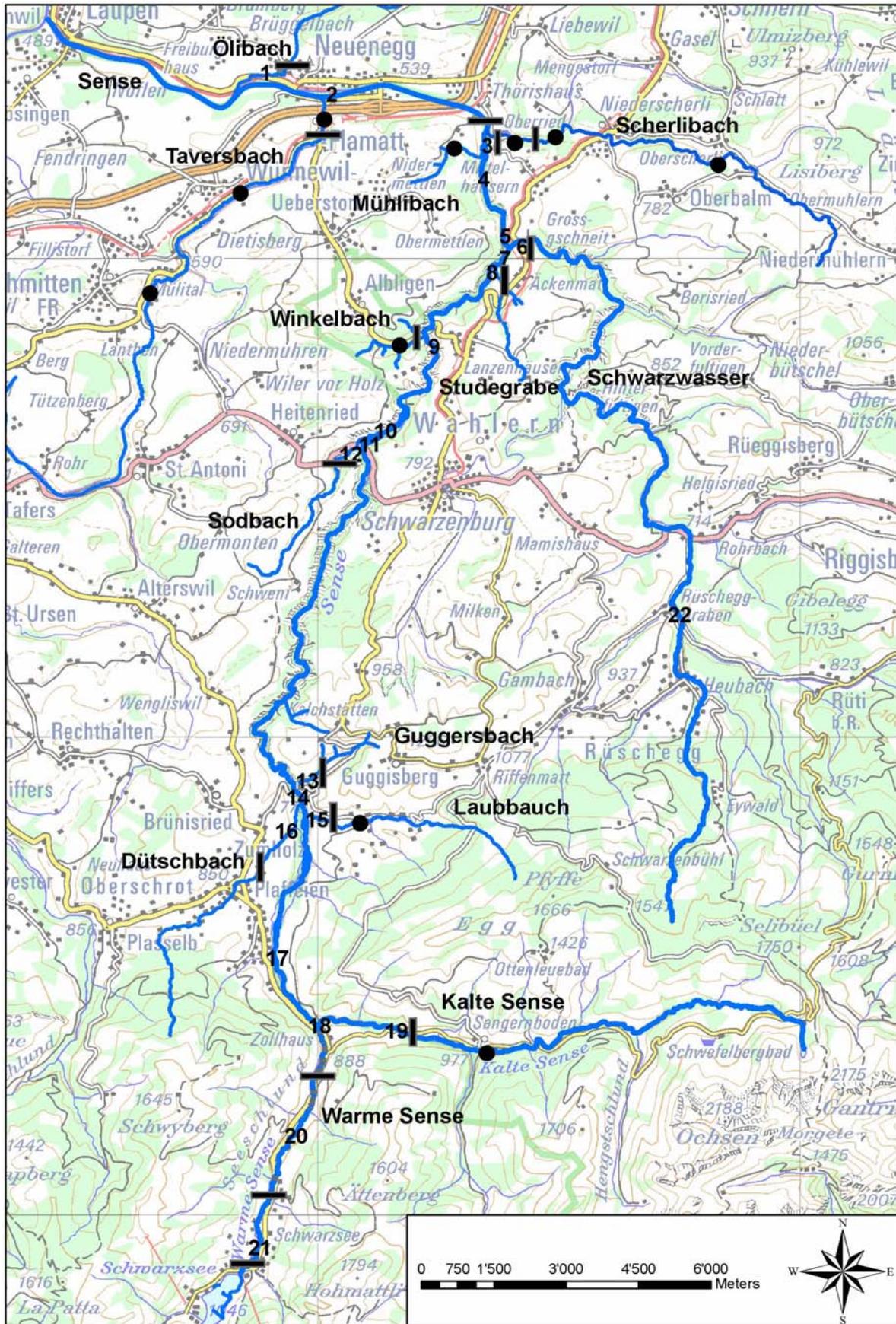
Fig. 1. Sampling sites in the Sense and tributaries. The numbers 1 to 22 represent places where we caught bullheads. The black dots show the places where we fished but no bullheads where caught. Black bars symbolize one or several impassable barriers.

2.2 Genotyping

The DNA was extracted with a Qiagen® Bio Sprint 96 extraction robot and each fish was genotyped at 10 microsatellite loci: Cgo33ZIM, Cgo18ZIM, Cgo1033PBBE, Cgo42ZIM, Cgo34ZIM, Cgo1114PBBE, Cgo56MEHU (Englbrecht et al., 1999), CottE10, Cott687, Cott179 (Nolte et al., 2005). The forward primers were labeled using three different colored fluorescent dyes. Because of different annealing temperatures and overlapping allele ranges the primers were divided into two multiplex primer sets. The first multiplex contained Cgo33ZIM, Cgo18ZIM, Cgo1033PBBE, Cgo42ZIM, Cgo34ZIM, Cgo1114PBBE. The primer mix contained 0.5 µl Cgo1033PBBE, 1 µl Cgo1114PBBE, 0.75 µl Cgo33ZIM, 0.75 µl Cgo42ZIM, 6 µl Cgo18ZIM, 1 µl Cgo34ZIM and 80 µl water. In the second multiplex Cgo56MEHU, CottE10, Cott687, Cott179 were used. The primer mix contained 1.5 µl Cgo56MEHU, 2.5 µl CottE10, 0.8 µl Cott687, 1 µl Cott179 and 282 µl water. The stock solution of all primers had a concentration of 100µM. The PCR amplification was accomplished with the Qiagen® Multiplex PCR Kit. The PCR mix per reaction contained 6.25 µl multiplex mastermix, 1.25 µl primer-mix, 4 µl water and 1 µl DNA. The PCR was performed with a Techne TC-412 thermocycler. The cycling protocol for the first multiplex was composed of an initial denaturation for 15 min at 95°C, followed by 35 cycles with 30 sec at 94°C, 90 sec at 58°C, 60 sec 72°C and a final extension of 10 min at 60°C. The second cycling protocol was of an initial denaturation for 15 min at 95°C, followed by 35 cycles with 30 sec at 94°C, 90 sec at 55°C, 60 sec 72°C and a final extension of 10 min at 60°C. The PCR product was diluted 1:20 for multiplex one and 1:50 for multiplex two. These dilutions were run on Beckman Coulter®, CEGTM 8000 following manufacturer's protocol. The alleles were scored with the program GeneMarker® Version 1.85 (Kellan 2002).

2.3 Analysis

GENEALEX 6® was used to test for deviations of Hardy-Weinberg equilibrium and to calculate the observed and expected heterozygosity (Peakall and Smouse 2006). Linkage disequilibrium and F_{ST}'s were calculated with the program ARLEQUIN 3.11 (Excoffier et al., 2005). To calculate the F_{IS} and allelic richness the program FSTAT version 2.9.3.2 was used (Goudet 1995).

We tested the hypothesis that upstream populations had fewer alleles using allelic richness values. We first measured the distance from the confluence of the Sense River to each sample site in Google Earth. With this data a linear correlation in SPSS was calculated. Since the allelic richness is calculated by rarefaction and takes the smallest sample size as a reference, the sites with the fewest individuals were excluded (Leberg 2002). These are the populations 1, 3, 8, 13. Doing this the smallest sample size amounts twelve individuals. We conducted a second linear regression leaving out all the disconnected populations (2, 20, 21 and 22), with the aim of omitting the influence of barriers on the loss of alleles by focusing on the long connected stretch in the middle of the river.

To determine if there are several genetically distinguishable clusters the data was run on STRUCTURE (Pritchard et al., 2000). To find the best number of clusters the following we ran the program for each value of K from 2 to 7, iterating each value of K ten times. The burn-in was set as 10'000 and the run time as 100'000. To select the optimal K the calculated posterior probability was plotted against K=2 to K=7 as recommended by Pritchard (2000). In addition, we conducted a principal component analysis using GENALEX 6 (Peakall and Smouse 2006) on the raw microsatellite data as an additional test for genetic clustering among sampled populations. We built a phylogenetic tree from microsatellite data using PHYLIP 3.65 (Felsenstein 1993), in order to visualize the genetic relationships between the populations. To build the tree we first calculated gene frequencies using CONVERT 131 (Glaubitz 2004), and used this same program create the input file for the PHYLIP program SEQBOOT. Afterwards the data was run in the PHYLIP programs GENDIST, NEIGHBOUR and CONSENSUS TREE. For drawing the tree, the program TREE VIEW (Page 1996) was used. The consensus tree was calculated using Cavalli-Sforza chord distances D_c (Cavalli-Sforza and Edwards 1967) and bootstrapping was done with 1000 replicates. In order to obtain branch lengths for the consensus bootstrap tree, we used the Phylip programs GENEDIST and NEIGHBOUR, keeping nodes which were supported in at least 80% of bootstrap replicates in the consensus tree.

To test for isolation by distance (IBD) a simple mantel test was done (Mantel 1967)..This used a matrix of pairwise F_{ST} values, and a second matrix of pairwise geographic distance between all the sample sites. These distances were measured by drawing a line following the course of the river from one site to the next in Google Earth, and calculating the distance of these lines. To test the influence of barriers on

pairwise F_{ST} values, another matrix was calculated including the number of barriers between all sites. With this matrix and the matrix of the F_{ST} a second simple Mantel test was performed. Since there is a significant correlation between the number of barriers and the length of the geographic distance also partial Mantel tests have been done. The partial Mantel test, tests for a correlation between two variables given other explanatory variables (Goslee and Urban 2007). According to this a partial Mantel test for the correlation between F_{ST} and numbers of barriers given geographic distance was done and in a second partial mantel test the correlation between F_{ST} and geographic distance given the number of barriers was done. Since a river can be regarded under the two dimensional stepping stone population genetic model, Rousset suggested that using $F_{ST}/(1 - F_{ST})$ in isolation-by-distance tests is appropriate (Rousset 1997). We repeated simple and partial mantel tests using these calculated values. All the Mantel tests were done with the ECODIST package in R, performing 10000 permutations (Goslee and Urban 2007, Rousset 1997, Mantel 1967).

The lack of migration between populations which lead to a genetic differentiation can be due to geographic distance or because of impassable barriers. Our next approach had the goal to exclude the influence of geographic distance on genetic differences just counting for the effect of barriers. Therefore we conducted individual assignment tests using the program GENECLASS 2 (Piry et al., 2004). This task calculates probabilities for individuals belonging to different populations. The frequency based method of Patkeau was chosen by simulating 10000 individuals (Patkeau et al. 1995). For this approach just the populations 18, 19 and 20 were used for the calculations. This was done because of the presence of impassable barriers between populations 18 and 20 but not between 18 and 19.

We also used the program BAYESASS 3.1 to calculate recent migration rates and the direction of migration between populations. This program works poor if the genetic differences between populations are small and it performs better with larger sample sizes (Wilson and Rannala 2003). For these reasons populations were pooled together and calculations were done with just two of these population pools. Using this approach we increased population samples sizes enough to allow the program to converge since the chosen populations were sufficiently genetically differentiated. Several approaches were then used using BAYESASS calculations. To get an idea about long distance migration in the connected stretch, populations 10

and 11 and populations 18 and 19 were pooled together and movements between these sites were investigated. The migration between populations 15 and 18 was calculated to get an overview for a smaller scale. And also the migration rates between the populations 3-5 and 6 were calculated to have again even a smaller scale. Furthermore, a measurement of migration rates between the populations 17/18/19 and 20/21 were done to test for migration between the fragmented populations. To reach convergence the following settings were chosen: 3000000 iterations, of which 999999 where burn-in. During post burn-in every 2000 iteration was sampled. The allele frequency (delta p) was set as 0.1. The migration rate (delta m) ranged from 0.025 to 0.05 and the inbreeding (delta F) was set as 0.15.

3. Results

In total there where 11 cases, where a locus deviates significantly from Hardy-Weinberg. These cases were divided over 9 of 22 populations. The locus Cgo56MEHU is the most suspicious, since it appears significant in 6 populations. This locus is between 238-250 bp and has some stutter peaks. One explanation could therefore be errors in scoring at this locus. All other loci that deviate significantly from Hardy-Weinberg occurred at most in two populations but just in 2 populations was a significant linkage disequilibrium observed (table 1).

Table 1 Sampling sites, number of individuals and genetic diversity

Sampling site	n	Ho	He	A_R	A_N	F_{IS}	nHWD	nLD
1. Ölibach	7	0.457	0.527	.	3.800	0.207	.	.
2. Taverna	14	0.636	0.578	4.865	5.100	-0.063	1	.
3. Mittelhäusern	3	0.533	0.450	.	2.500	0.015	.	.
4. Sense Beach	26	0.562	0.565	4.427	5.300	0.025	.	.
5. Sense Unt Schw	12	0.533	0.542	4.3	4.300	0.06	1	.
6. Schwarzwasser Brücke	30	0.500	0.504	3.947	4.700	0.024	2	.
7. Sense. Ob. Schw.	15	0.573	0.530	4.677	5.200	-0.047	.	.
8. Studegrabe	3	0.433	0.450	.	2.600	0.235	.	.
9. Winkelbach	27	0.568	0.573	4.354	5.600	0.028	1	2
10. SenseSodbach	23	0.571	0.566	4.513	5.400	0.012	.	.
11. SodbachIrene	23	0.574	0.565	4.704	5.900	0.007	.	.
12. Sodbach	26	0.615	0.590	4.889	6.000	-0.023	1	.
13. Guggersbach	6	0.610	0.543	.	3.600	-0.027	.	1
14. Zumholz	31	0.571	0.543	4.439	5.700	-0.034	1	.
15. Laubbach	30	0.563	0.553	4.49	5.700	-0.001	.	.
16. Dütschbach	13	0.569	0.553	4.297	4.400	0.01	.	.
17. Rufenen	37	0.516	0.512	4.178	5.700	0.006	1	.
18. Zollhaus	26	0.437	0.474	3.903	5.000	0.097	1	.
19. Hoflandbrücke	26	0.453	0.484	3.936	5.100	0.084	2	.
20. Eispaläste	28	0.357	0.376	2.992	3.500	0.068	.	.
21. Schwarzsee	30	0.407	0.393	2.8	3.200	-0.018	.	.
22. Rüschegg	26	0.466	0.464	3.6	4.500	0.016	.	.

Notes: n, number of individuals; Ho, mean observed heterozygosity over Loci; He, mean expected heterozygosity over loci; A_R, allelic richness; A_N, mean number of alleles per locus and population; F_{IS}, inbreeding coefficient; nHWD, number of loci which showing a significant deviation from HWE after Bonferroni correction ($\alpha = 0.005$); nLD, number of pairs of loci with a significant genotypic linkage disequilibrium with $p < 0.001$.

The F_{ST} values found in this rather small geographic scale are relatively high (table 2).

Tabel 2. Strong genetic divergence among 22 populations within one river and it's tributaries. F_{ST} values are calculated using 10 microsatellite loci.

Pop	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	0																					
2	0.02	0																				
3	0	0	0																			
4	0	0.01	0	0																		
5	0	0.03*	0	0	0																	
6	0.02	0.05*	0	0.01*	0.02	0																
7	0.03	0	0.04	0.02*	0.04*	0.08*	0															
8	0.02	0.01	0	0.01	0.01	0.05	0.02	0														
9	0	0	0.01	0	0.01	0.05*	0	0.03	0													
10	0	0	0.03	0.01*	0.03*	0.07*	0	0.02	0	0												
11	0	0	0	0	0.01	0.05*	0	0.01	0	0	0											
12	0.02	0	0.02	0.01*	0.03*	0.07*	0	0.04	0	0	0	0										
13	0	0	0.03	0	0.03	0.06*	0.01	0.01	0	0	0	0	0									
14	0.02	0.01*	0.07*	0.03*	0.05*	0.10*	0.02*	0.04	0.01*	0	0.01*	0.01	0	0								
15	0.01	0.02*	0.07*	0.02*	0.05*	0.08*	0.01	0.03	0.01*	0	0.01*	0.01*	0	0	0							
16	0.03	0.03*	0.10*	0.04*	0.06*	0.12*	0.02*	0.06	0.02*	0.02	0.03*	0.02*	0.01	0.02*	0.01	0						
17	0.03*	0.03*	0.10*	0.05*	0.07*	0.12*	0.01*	0.07*	0.02*	0.01	0.03*	0.02*	0	0	0	0.02*	0					
18	0.04*	0.06*	0.15*	0.07*	0.10*	0.15*	0.03*	0.12*	0.04*	0.03*	0.04*	0.04*	0.03	0.02*	0.01*	0.02	0	0				
19	0.03	0.07*	0.15*	0.07*	0.09*	0.15*	0.06*	0.09*	0.05*	0.05*	0.06*	0.06*	0.02	0.02*	0.02*	0.04*	0.01*	0.01	0			
20	0.15*	0.16*	0.30*	0.17*	0.21*	0.27*	0.12*	0.23*	0.14*	0.12*	0.15*	0.13*	0.12*	0.08*	0.08*	0.1*	0.04*	0.03*	0.04*	0		
21	0.13*	0.14*	0.26*	0.14*	0.19*	0.24*	0.10*	0.21*	0.12*	0.09*	0.11*	0.1*	0.08*	0.06*	0.05*	0.09*	0.03*	0.02*	0.04*	0	0	
22	0.07*	0.10*	0.01	0.05*	0.06*	0.01	0.13*	0.09*	0.10*	0.12*	0.09*	0.12*	0.11*	0.14*	0.13*	0.17*	0.18*	0.21*	0.2*	0.32*	0.3*	0

Note: * significant diverences (p<0.05)
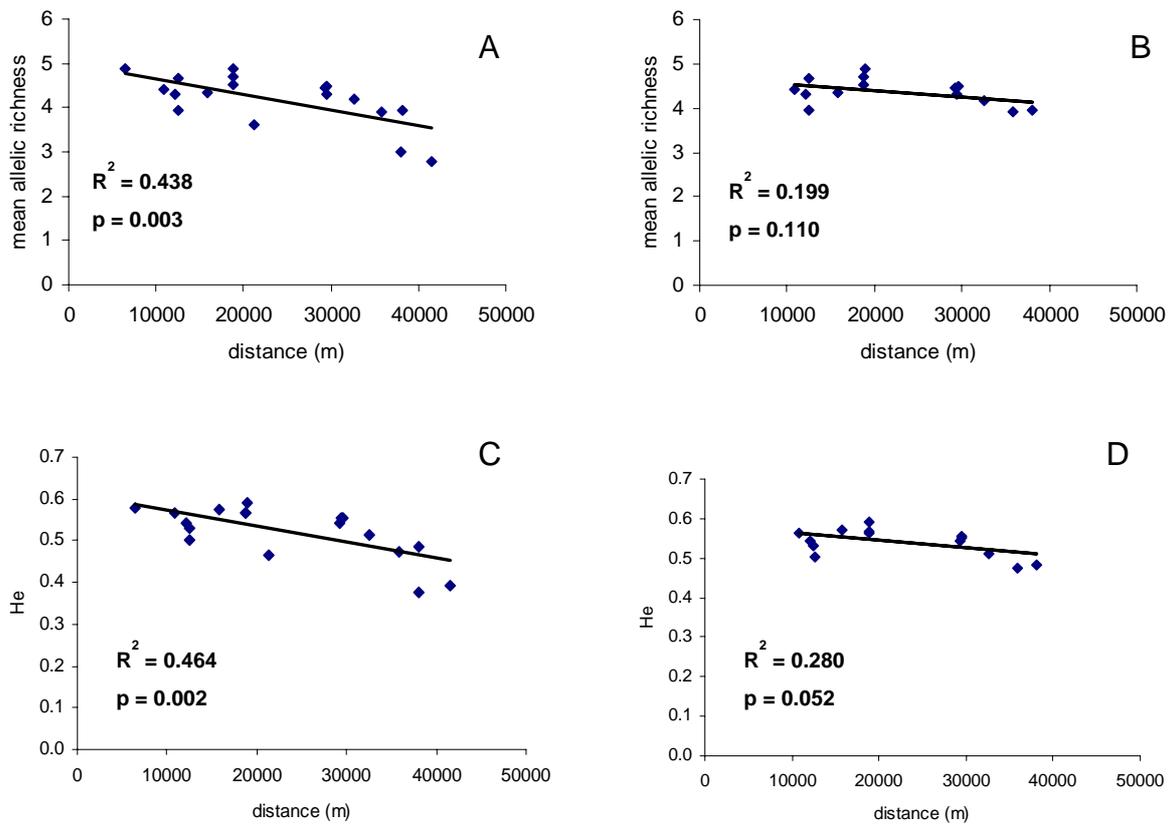
Fig. 2 Mean allelic richness and expected heterozygosity (He) as a function of distance to the confluence of the Sense river with 18 populations (A, C) or 14 populations (B, D). Allelic richness and He decrease with distance to the confluence.

The populations show a significant loss in allelic richness and a significant decrease in expected heterozygosity from downstream to upstream populations. The decrease is strong and highly significant in A and C where the most separated but also most upstream populations were taken into account for the calculations. In B and D just the populations from the 28 km long connected stretch were included. These results are not significant anymore but a tendency can still be seen.
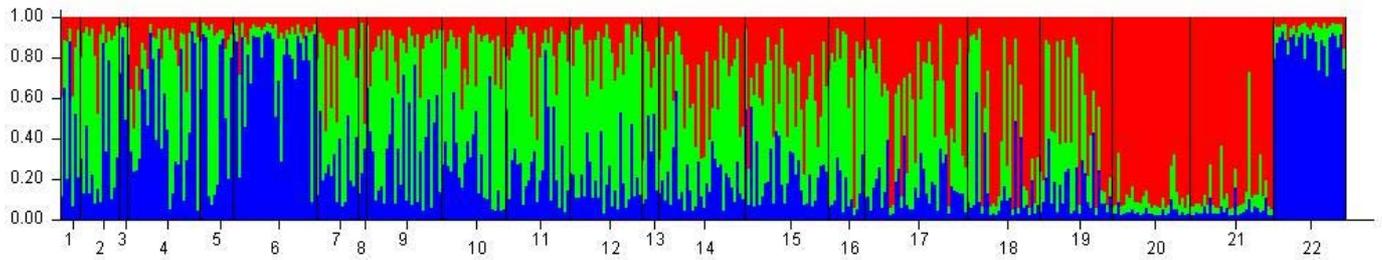
The Bayesian analysis performed in STRUCTURE assigns the individuals clearly into three clusters (Fig. 3). These clusters match perfectly with the geographic locations in the river (Fig. 4). The red cluster is very dominant in the Warme sense. Following the river downstream the green cluster becomes prevalent. The blue cluster belongs to the populations in the Schwarzwasser and below the stretch where the Schwarzwasser opens out into the Sense river the populations are intermediates between the blue and the green cluster.
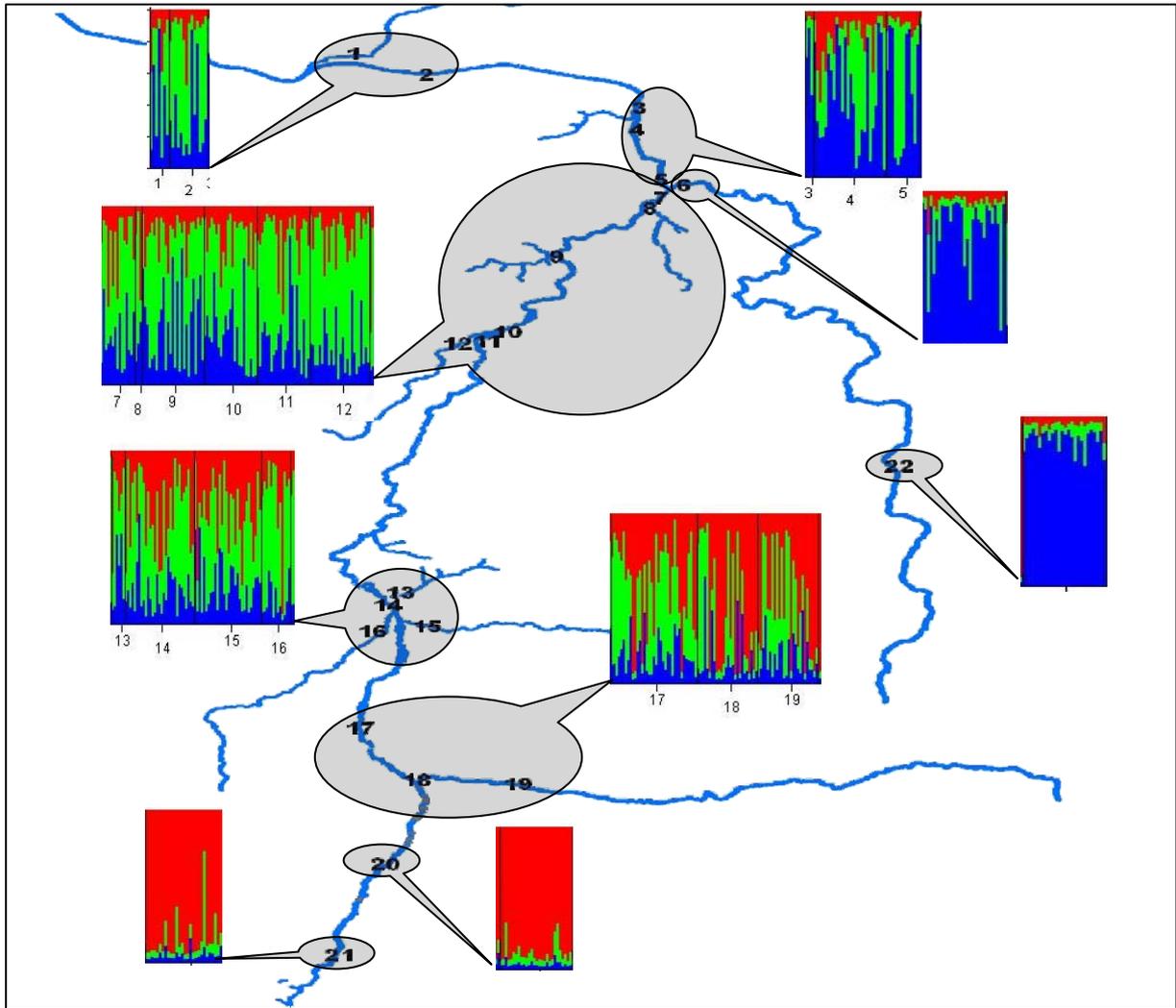
Fig. 4. The populations in their geographic locations displayed as the bar plots of the STRUCTURE output.
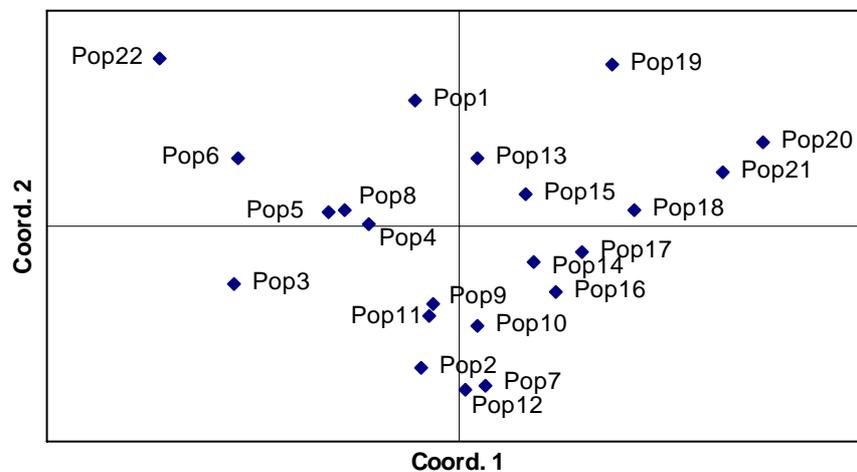
Fig. 5. Principal Component Analysis on the raw data. The first axis explains 76.22, together with the second it explains 86.42 and three axes together explain 92.11 of the variability in the data.

The principal component analysis attempts to explain variability in data (fig. 5). It finds the same population clusters as STRUCTURE does. The populations from the Warme Sense (20 and 21) are very close and next to them are the samples from the Kalte Sense (19) and the ones further downstream (17 and 18). The other populations that are close together are the ones from Schwarzwasser (6 and 22) and below the confluence of this tributary in the Sense River (3, 4 and 5). Population 8 is probably influenced by the small sample size and should not be taken into account. The next clustered populations are the ones from the long connected middle part of the Sense River (7, 9, 10, 11, 12, 14 and 16). The neighbour-joining tree using Cavalli-Sforza chord distances shows a pattern that reflects again the geographic situation of the different populations (Fig. 6). There are 3 nodes which are supported in more than 80% of the bootstrap replicates. The branch that was supported in 90% of all simulated trees separates the Schwarzwasser populations from the Sense populations. In the area where the Kalte Sense and Warme Sense flow together a next highly supported node is displayed. It splits the the Kalte Sense population (19) away from the population at the junction to the Warme Sense (18) and it is in 87% of all cases performed. A node that was in all trees displayed is the one that separates the disconnected populations (20 and 21) in the Warme Sense from the rest of the populations.
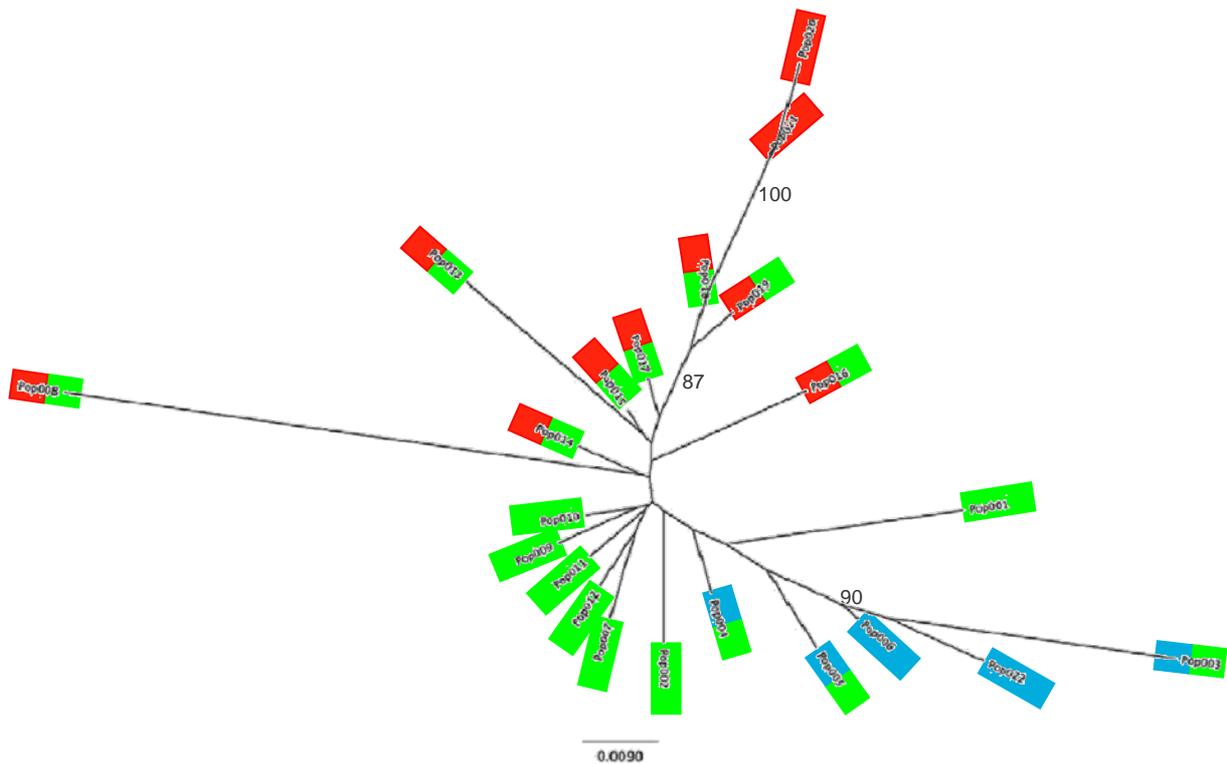
Fig. 6. A phylogeny estimated from Cavalli-Sforza chord distances (D_C), displaying bootstrap support for the nodes, if they where higher than 80%. The populations are highlighted with the assigned color of the STRUCTURE plot.

The simple mantel test to test for isolation by distance shows a highly significant correlation between genetic and geographic distance (Fig. 7). There was also a strong significance in the mantel test between the genetic difference and the number of barriers between the populations (Fig. 8). But because of a correlation between geographic distance and number of barriers (mantel r = 0.38, mantel p = 0.0001), two other approaches have been tested. First one was to exclude the influence of barriers on geographic distance. To do this, we did a simple mantel test for genetic and geographic distance excluding all populations with barriers in between them (including only populations 3 to 19). Although the populations with the largest distance in between could then not be used, a strong significance is still observable (Fig. 9).
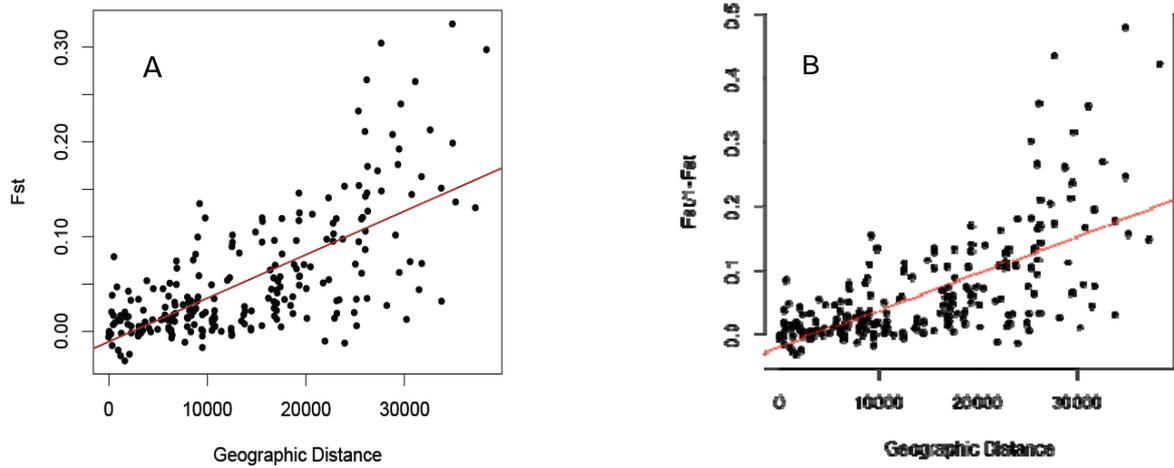
Fig. 7. Isolation by distance in the whole study system. The correlation between F_{ST} and genetic distance is shown in A, with a mantel r = 0.68 and a mantel p = 0.0001. In B the correlation between the corrected F_{ST} after Rousset against geographic distance is plotted. It is still significant with a mantel r = 0.53 and a mantel p = 0.0008.
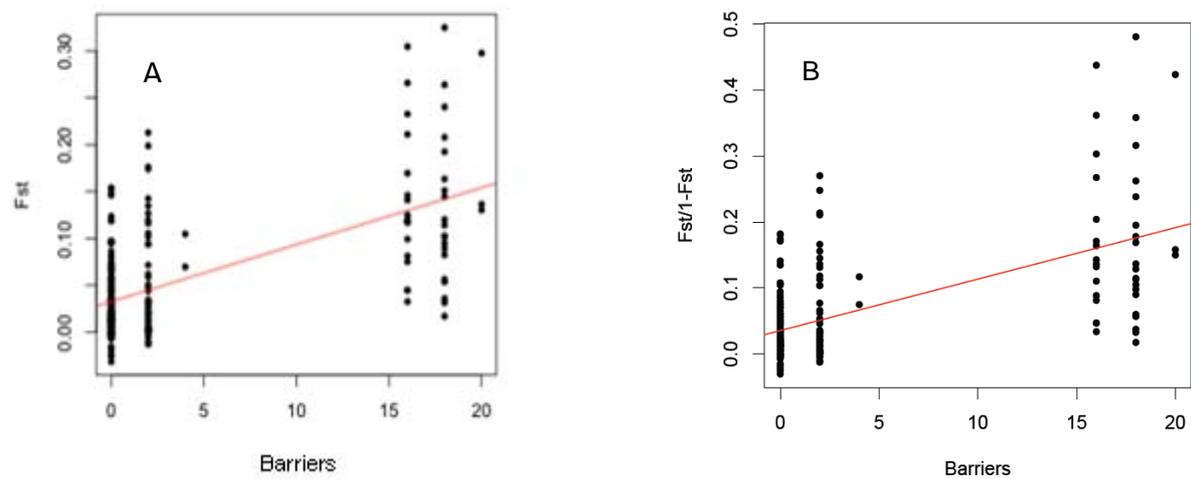
Fig. 8. Isolation by barriers with a mantel r = 0.60 and a mantel p = 0.0008 for A and mantel r = 0.59, mantel p =0.0005 for B.
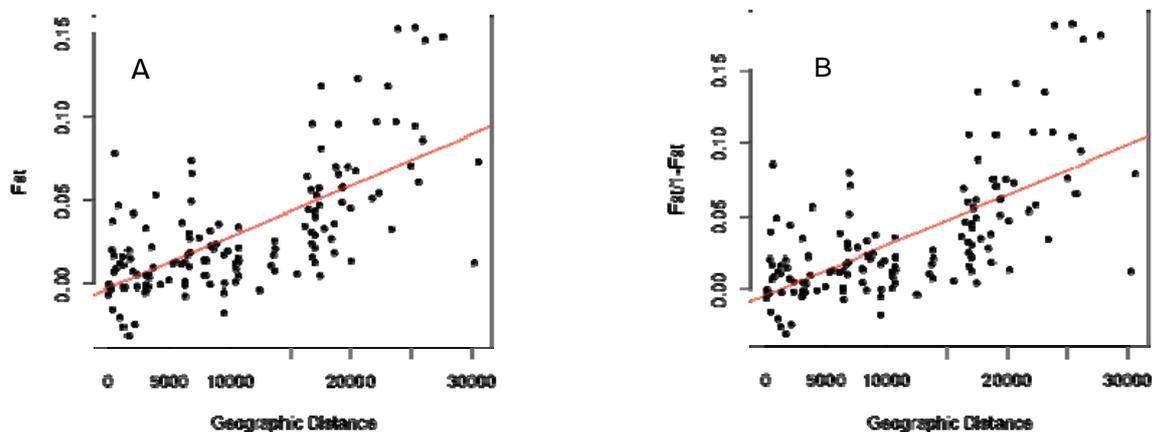
Fig. 9. IBD between populations of the long connected stretch. For A with a mantel r = 0.67 and a mantel p = 0.0001. In B there is a mantel r = 0.66 and mantel p = 0.0001.

The second approach to disentangle the influence of barriers and geographic distance was performed using partial mantel tests. This implementation allows the use of all populations in the system. The results of these tests show a significant influence on the bullhead populations caused by the barriers but also because of the geographic distance. Anyway in this study system with long passable stretches it seems that geographic isolation is more influential than barriers are (table 3).

Table 3 Partial mantel tests performed in the ecodist package in R

	mantel r	mantel p
F_{ST} ~ barriers + distance	0.50	0.0046
$F_{ST}/(1-F_{ST})$~ barriers + distance	0.50	0.0040
F_{ST} ~ distance + barriers	0.61	0.0001
$F_{ST}/(1-F_{ST})$~ distance + barriers	0.58	0.0001

The next approach had the goal of minimizing the influence of geographic distance and trying to focus just on the influence which barriers might have on the bullheads. Because of this we chose populations 18, 19 and 20 as our focus (Fig. 1 and Fig. 11). These populations build an angle with the population 18 as angular point and the populations 19 in the Kalte Sense and the population 20 in the Warme Sense representing the two arms. But whereas the population at Zollhaus (18) and the population from Hoflandbrücke (19) are connected, no free migration is possible between the populations Zollhaus and Eispaläste (20). To show the influence of these barriers an application for individual assignment was performed in GENECLASS 2. This application calculates probabilities for a given individual belonging to a population due to his genetic background. These probabilities for belonging to one or another population can then be plotted (Fig. 10). What one can see is that the program struggles to assign individuals from Zollhaus and Hoflandbrücke clearly into their populations, as would be expected if gene flow is going on. Compared to that, the individuals from Eispaläste and Zollaus show a very different pattern. The probability for "Eispaläste individuals" belonging to their own population or to Zollhaus is almost the same, whereas a large amount of the "Zollhaus individuals" have zero or just a very small probability to be part of the Eispaläste population. The distribution of alleles may explain this pattern. In that sense there are 11 alleles that exist in population Zollhaus but not in population

Hoflandbrücke and 12 alleles that exist in Hoflandbrücke but not in Zollhaus. On the other hand there are 20 alleles that exist in Zollhaus alone but just 4 alleles that occur in the Eispaläste population but not in Zollhaus. This uneven distribution between the populations with barriers in between shows exactly what one would expect. Namely that there is some gene flow downstream from the Eispaläste towards Zollhaus but it is not possible for the bullheads to go upstream in the other direction. Like this the Eispaläste population shares alleles with Zollhaus individuals but not the opposite around. The Zollhaus population receives additionally alleles from populations in the long connected stretch, which gives another explanation for the large amount of private alleles in Zollhaus.
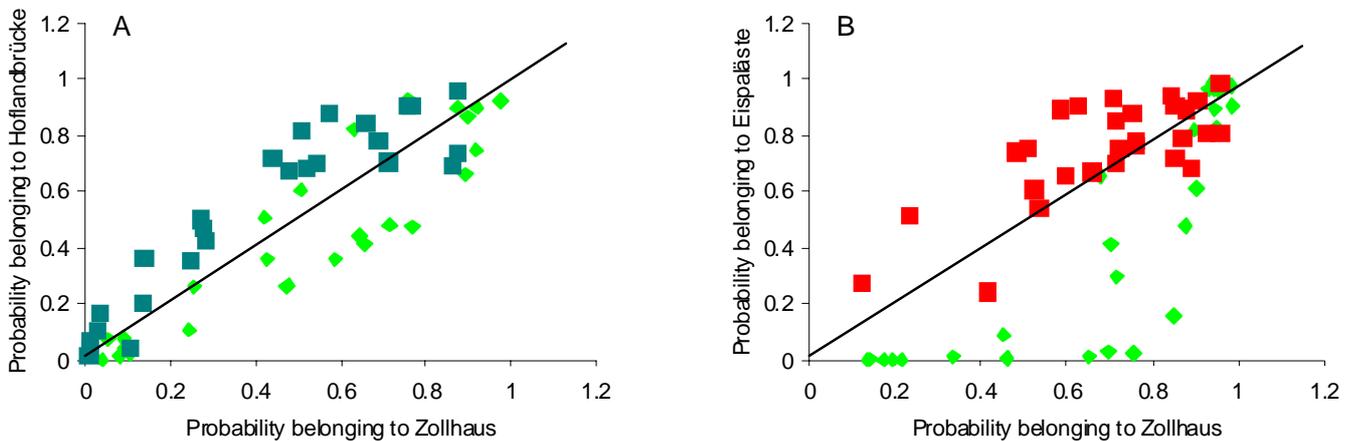
Fig. 10. Plot A shows the assignment for individuals of Zollhaus (light green) and Hoflandbrücke (dark green), whereas B shows the assignment again for Zollhaus (light green) but with the Individuals of the Eispaläste (red).

The genetic results in the Warme Sense and Kalte Sense and at the junction where they flow together go along with the situation of obstructions in the Warme Sense (Fig. 11). There is no genetic difference between the population at Zollhaus and Hoflandbrücke and also Structure clusters these two populations. Compared to that, there is a significant genetic difference between these two populations and the populations in the Warme Sense where migration is not possible. The similarity of the Eispaläste and Schwarzsee populations may be explained the rather short period of existence of the two barriers in between.

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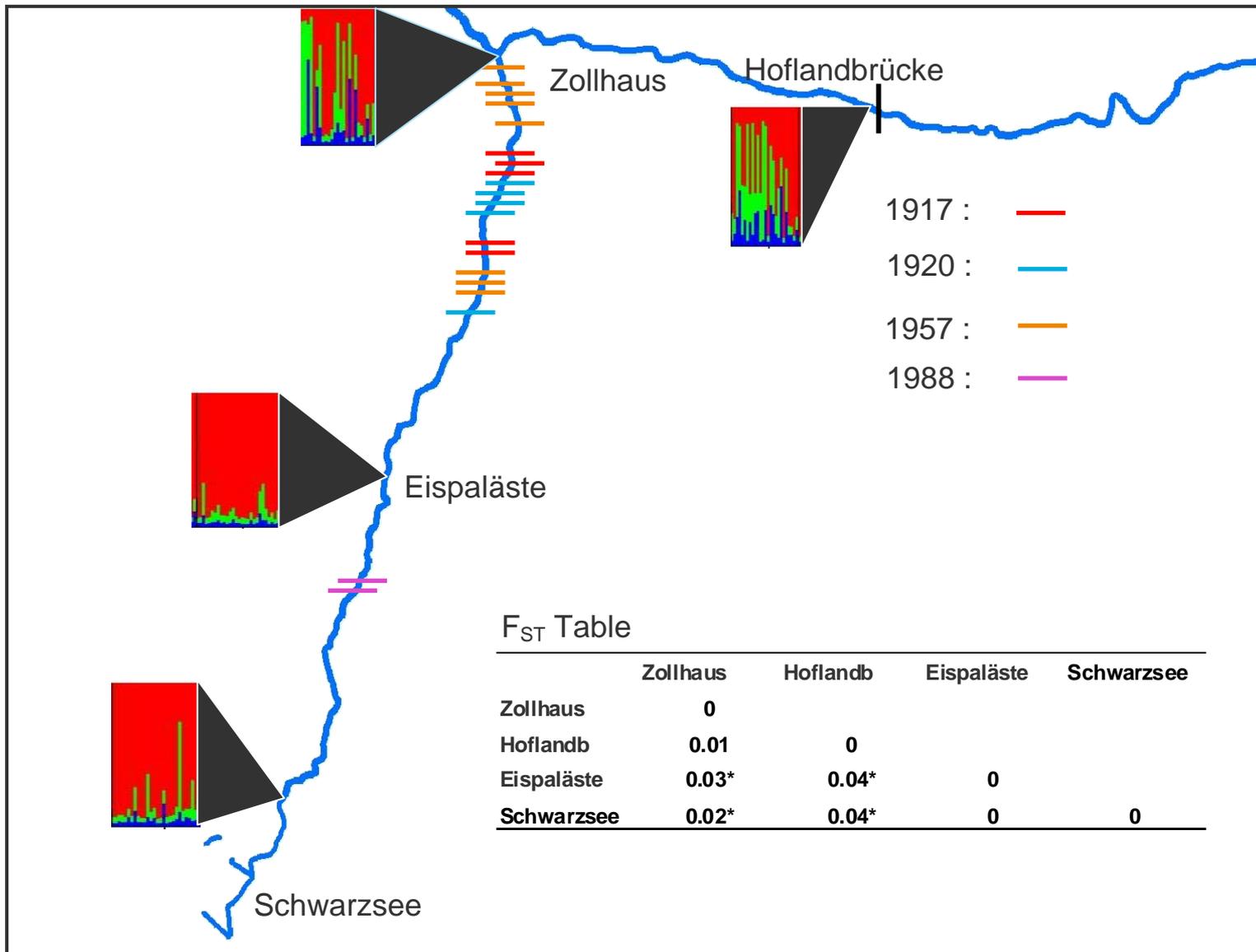
Fig. 11. STRUCTURE result and FST-values of the four sampling sites in the Warme Sense, Kalte Sense and their confluence. The bars represent the bed drops and the different colors show the year, when they appear for the first time on the maps.

The results of the BAYESASS analysis are shown in table 4. The first column shows where the individuals are coming from and the values in the rows show the migration rate into another population. In a study were all populations where calculated within one run the numbers in italics along the diagonal would represent the proportions of individuals derived from the source population per generation (Wilson and Rannala 2003). But to get these values one has to run all populations together since the calculations are in relations to other populations. Like already mentioned earlier the populations in this study were not always enough differentiated and therefore not all populations could have been run together. Because of that the values in italics show

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the proportion of individuals derived from the source populations as if there were no other populations living in the river. This is not true and there will be immigration from other populations, which the program can not count for. But the received values are still good to have an idea about the direction of migration and source sink relationships.

What our results suggest is that there is strong downstream migration and low upstream migration. This makes the upstream populations to source populations and the further downstream populations to receivers. Furthermore it seems like the downstream migration is not very strong influenced by distance, whereas the upstream migration decreases strongly with distance. In that sense one can see a stronger migration rate upstream between the population 3-5 and 6 where at least the populations 5 and 6 are just a few 100 meters apart from each other. Between the populations 15 and 18 lies a larger distance and the upstream migration also decreases. Even smaller is the upstream movement between the populations 10/11 and 18/19. But the smallest upstream movement is measured between the populations 17-19 and 20/21 which are separated by barriers, whereas the downstream migration seems to not to be influenced.

Table 4 Movements of the bullheads and distances

		Migration into		
		pop 6	**pops 3-5**	distance between sites in km
Migration from	**pop 6**	*0.69*	0.22	1.4
	pops 3-5	0.31	*0.78*	
		pop 18	**pop 15**	
	pop 18	*0.94*	0.31	6.3
	pop 15	0.05	*0.68*	
		pops 18/19	**pops 10/11**	
	pops 18/19	*0.98*	0.31	18.1
	pops 10/11	0.02	*0.70*	
		Pops 20/21	**Pops 17-19**	
	Pops 20/21	*0.99*	0.31	5.8
	Pops 17-19	0.01	*0.68*	

Notes: The rows show the migration into populations or the proportion of individuals that come from the source population (values in italics). All standard deviations were < 0.05.

4. Discussion

From our study three main findings appear: First, the bullhead is a rather resident species. Second, there is stronger downstream migration than upstream migration in bullheads. Third, barriers that prevent migration lead to a clear increase in genetic differentiation between populations.

4.1 *Cottus gobio* a rather resident species

Cottus gobio is known as a philopatric species (Downhower et al. 1990; Knaepkens et al. 2004). These findings from tagging experiments are confirmed by several genetically based results in this study. First, our F_{ST}'s were high for this small geographic scale. The strongest genetic differentiation was observed between the populations from Schwarzwasser and the Warme Sense. These populations have a large geographical distance inbetween and the F_{ST}'s reach 31%. These high values are additionally influenced by factors other than distance like barriers and changes in water flow, since movements between these sites contain downstream but also upstream migration. To exclude these factors we can just consider the populations in the long connected stretch and even there a F_{ST} of 7% on 28 kilometers is reached (fig. 1 and table 1). The highly significant IBD pattern that is shown by the different mantel tests again speak for a limited movement between populations (fig.9 and table 3). The findings of three clusters with STRUCTURE, in this small-scale study system, speak for a rather low movement for the bullhead species as well (fig. 3). Even if one follows the river downstream within the connected stretch one can see how the dominant color in the cluster detected with STRUCTURE changes. . According to the program, the red pattern is dominant in the head waters then the colors change from red to green and becomes blue where the Schwarzwasser opens out in the Sense River (fig. 4). Also the phylogeny put populations that are geographically close next to each other in the tree and shows in this way a very clear spatial pattern, which again is an indicator of less gene flow between populations as distance increases. Considering the results gained from BAYESASS, it seems that especially the low upstream migration is responsible for this high genetic distinction. Since the results suggest that especially the upstream migration is low and becomes

even lower with distance whereas the downstream migration remains constant on a higher level (table 4).

4.2 Downstream migration is more dominant than upstream migration

Many studies have investigated the symmetry of gene flow within rivers, with the result that migration along the direction of the water flow is generally stronger than against it. This has been shown for bullheads (Hänfling et al. 2006) but also for other species (e.g. Tatarenkov et al. 2010, Waters 1972). If one considers the plots, from the populations around the confluence of the Schwarzwasser and the Sense River from STRUCTURE, a clear sign of downstream migration can be observed (fig. 4). Namely the populations 3, 4 and 5 are a mix of Sense alleles (green) and Schwarzwasser alleles (blue). This pattern could be explained if bullheads from above the confluence either from the Schwarzwasser or from the Sense move downstream into the Sense River below the confluence and become resident further downstream rather than swim upstream again. The F_{ST} values of these populations go along with the STRUCTURE result (table 5 for F_{ST}). There is little gene flow between populations 6 and 7, where a fish first has to swim downstream into the other river and there change its direction, swimming upstream to arrive in either population 6 or 7. Thus there is a high F_{ST} of 8% between these two populations despite their close proximity. Comparing the F_{ST}'s between the populations from downstream of the confluence with the ones from upstream of the confluence, one can see that the downstream populations are more closely related to the upstream populations than they are to either population 6 from the Schwarzwasser or to population 7 in the Sense River (table 5).

Table 5 F_{ST} values from the populations around the Schwarzwasser confluence

	Sense below confluence		Schwarzwasser	Sense above confluence
	population 4	population 5	population 6	population 7
population 4	0			
population 5	0	0		
population 6	1*	2	0	
population 7	2*	4*	8*	0

Note: * indicates values significantly different from zero.

BAYESASS gives conflicting results. Since it shows not just strong downstream migration between the Schwarzwasser population into the Sense River population. Instead it shows also a very strong upstream migration from Sense fish into Schwarzwasser. On one hand, one would expect more migration between populations at this small geographic scale, perhaps including upstream movement. On the other hand, according to the color pattern gained in STRUCTURE, one would expect much stronger downstream migration than upstream migration between these populations. Perhaps the demographic admixt populations at the confluence have an impact on BAYESASS resulting in too high migration rates.

4.3 Barriers have a significant influence on gene flow

All tests conducted here show evidence that barriers in the river system have an influence on population structure of bullheads. The decline in allelic richness and heterozygosity in more upstream populations can be expected due to colonization history and stronger downstream migration in species which live in rivers (Hänfling et al., 2002). This pattern can also be observed in this system. But the decrease is highly significant if one takes populations from the disconnected parts of the river into account. The populations from the connected stretch show a decrease as well, although it is not as strong as when comparing all populations (fig. 2). This is a strong sign for the influence of the barriers. Furthermore mantel test and the partial mantel test, both showed significant correlation between the number of barriers and the genetic distance between the populations (fig. 8 and table 3). Also the result of GENECLASS 2 showed a distinct pattern when comparing results from the

disconnected populations Zollhaus and Eispaläste and the connected populations Zollhaus and Hoflandbrücke (fig. 10).

In a study of the beetle *Abax parallelepipedus* it has been shown that if a small population becomes separated from the large main population in a way that no gene flow is possible, F_{ST} values of 3.6 % can be expected after the duration of 30 generations of separation (Keller et al. 2004). The first bed drops in the Warme Sense were built ca. 90 years ago. The generation time of bullheads in this river stretch, with a high altitude and therefore with rather low temperatures, is around 2-3 years (Elliot 1981). This indicates that between the beginning of the first obstructions and now a similar number of generations passed as in the simulations of Keller et al. The F_{ST} between the populations 18 and 20 of 3% comes also very close to the one in the Keller study. A difference between these projects lies in the fact that the migration of bullheads is prevented just in upstream direction but downstream it is possible, whereas the result obtained within the beetles was based on a simulation with no immigration at all. Furthermore, the strength of genetic drift is inversely correlated with the effective population size. The simulations in the Keller study were performed, assuming an effective population size of 200 individuals in the smaller population. The number of individuals in the Warme Sense at the time of the first obstructions is not known. But populations of species with a polygynic mating system have a much higher census population size than an effective population size anyway (Frankham 1995). However, the results of this project suggest that at least part of the genetic differences is because of the barriers. This is also supported by the BAYESASS results which show that the upstream migration does almost not exist between disconnected populations with a small geographic distance in between, whereas between populations in the connected stretch, with a similar distance, at least some upstream migration is taking place (Table 4).

Genetic variance between populations is influenced by several factors. Colonization history, historical processes and still ongoing processes like gene flow all contribute to observed patterns of population genetic variation. But the conclusions may also reflect the spatial scale of the study system (Caldera and Bolnick 2008). Several population genetic studies have been done on bullheads, working on a large geographic scale or at least within entire watershed regions (Vonlanthen et al. 2007, Nolte et al. 2005, Hänfling and Weetman 2006). All of these studies share the finding

of very strong genetic differentiation between populations indicated by high F_{ST} values of up to 70%. Compared to the above mentioned analyses, this study was performed on a rather small spatial scale where all sampling sites lie within one river or its tributaries. But even here the differentiation between populations is already very high also compared to other species. For example the brown trout (*Salmo trutta*) sampled all over Switzerland showed considerably lower levels of genetic differentiation (Keller et al., submitted manuscript), than the bullheads from our study. This leads to the obvious conclusion that in bullhead genetically distinct subgroups exist, which should be treated as independent evolutionarily significant units (ESU) in conservation. We therefore suggest more detailed research leading in the direction of ESUs or even subspecies in bullheads. This is important because of the specific local adaptation to the different habitats, which potentially happened over time in these populations. In addition this study is in agreement with other studies showing that populations from the headwaters have already a lower genetic diversity due to colonization history and asymmetrical migration rates (Hänfling et al., 2002, Yamamoto et al. 2004, Caldera and Bolnick 2008, Raeymaekers et al. 2009). In addition the more upstream the fragmentation is the smaller becomes the habitat above the barriers leading to even smaller populations (Yamamoto et al. 2004). Since the upstream migration is prevented completely through the barriers the fragmentation in higher altitude might have an even more pronounced effect on populations in the headwaters. This should be taken into account for river restauration.

Acknowledgement

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