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# Genetic analysis of potential postglacial watershed crossings in Central Europe by the bullhead (*Cottus gobio* L.)

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Natural colonizations across watersheds have been frequently proposed to explain the present distributions of many freshwater fish species. However, detailed studies of such potential watershed crossings are still missing. Here, we investigated potential postglacial watershed crossings of the widely distributed European bullhead (*Cottus gobio* L.) in two different areas along the Rhine–Rhône watershed using detailed genetic analysis. The main advantage of studying bullheads vs. other freshwater fish species is that their distribution has been lightly influenced by human activities and as such, interpretations of colonization history are not confounded by artificial transplantations. The genetic analyses of eight microsatellite loci revealed strong genetic similarities between populations of both sides of the Rhine–Rhône watershed in the Lake Geneva area, giving strong evidence for a natural watershed crossing of bullheads from the upper Rhine drainage into the Rhône drainage in the Lake Geneva area likely facilitated by the retreat of the glaciers after the last glacial maximum some 20 000 years ago. Populations from the Lake Geneva basin were genetically more similar to populations from across the watershed in the upper Rhine drainage than to populations further downstream in the lower Rhône. In contrast, populations from Belfort, an area, which was not covered by ice during the last glacial maximum, showed strong genetic differentiation between populations of the upper Rhine and Rhône drainages. Based on our results on the bullhead, we propose that glacial retreat may have eased the dispersal of numerous European freshwater fish species across several geological boundaries.

*Keywords:* bullhead, colonization history, *Cottus gobio*, microsatellite, postglacial colonization, watershed

## Introduction

Watersheds between river systems represent impassable barriers for most freshwater fish species (Gyllensten 1985; Currens *et al.* 1990; Carvalho *et al.* 1991; Ward *et al.* 1994; Estoup *et al.* 1998). Nevertheless, natural crossings of watersheds have been frequently proposed to explain the present distributions of freshwater fish species (Hansen *et al.* 1999; Bernatchez 2001; Costello *et al.* 2003; Behrmann-Godel

*et al.* 2004). The recolonization history of European rivers and lakes by fish after glaciation cycles of the Quaternary using genetic markers has been addressed in many freshwater fish species including brown trout (*Salmo trutta*, Bernatchez & Osinov 1995; Aurelle & Berrebi 2001; Bernatchez 2001), grayling (*Thymallus thymallus*, Koskinen *et al.* 2002; Weiss *et al.* 2002), European perch (*Perca fluviatilis*, Nesbo *et al.* 1999), bullhead (*Cottus gobio*, Englbrecht *et al.* 2000; Kontula & Vainola 2001), chub (*Leuciscus cephalus*, Haenfling & Brandl 1998c) and vairone (*Leuciscus souffia*, Salzburger *et al.* 2003). Some studies argue that the European freshwater fish fauna was highly influenced by both climate

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and geological changes from the Quaternary glaciation (Waters *et al.* 2001; Hewitt 2004; Costedoat *et al.* 2006) and that fish migration could have been facilitated by ephemeral contacts between river systems like for example through temporal proglacial lakes (Behrmann-Godel *et al.* 2004) or even by changes in the flow direction of upper river stretches (Waters *et al.* 2001).

Because of its wide European distribution and trivial economic importance, bullheads have been lightly influenced by artificial introductions (Haenfling & Brandl 1998a; Englbrecht *et al.* 2000) and received considerable attention to study the colonization history of freshwater fish (Haenfling & Brandl 1998a; Englbrecht *et al.* 2000; Kontula & Vainola 2001; Haenfling *et al.* 2002; Volckaert *et al.* 2002; Slechtova *et al.* 2004). Previous genetic studies of bullheads used allozyme and mitochondrial sequence data to infer European Pliocene–Pleistocene colonization history (Riffel & Schreiber 1995; Haenfling & Brandl 1998b; Riffel & Schreiber 1998; Englbrecht *et al.* 2000; Kontula & Vainola 2001; Volckaert *et al.* 2002; Slechtova *et al.* 2004). All these studies reported closely related mtDNA haplotype families and distinct restriction fragment length polymorphisms among neighbouring drainages suggesting common ancestry of bullheads between the Danube, the Rhine and Adriatic drainages. This suggests that the crossing of watersheds by the bullhead occurred multiple times in different areas. It has been suggested that such crossings may have been mediated by the retreat of glaciers after the last glaciation maximum (LGM) (Slechtova *et al.* 2004). While mtDNA and allozyme markers proved to be highly informative for the reconstruction of the general recolonization scenario, their usefulness for fine-scale population genetic analyses – especially in the case of a Danubian lineage – was probably hampered by a continuous homogenization because of active haplotype transfers through river captures during the glacial cycles (Englbrecht *et al.* 2000; Slechtova *et al.* 2004).

Little is known about the colonization history of freshwater fish species within the Alpine region, an area where several major European drainages (Rhine, Rhône, Danube, Po) meet within a relatively small area. During the LGM some 20 000 years ago (Jäckli 1962; Hantke 1991), large parts of this Alpine region were covered by ice (Hantke 1991, Fig. 1b), and thus the present distribution of freshwater fish species in this area is most probably strongly influenced by the retreat of the glaciers after the LGM. In the Lake Geneva area of Switzerland, natural postglacial crossings of the watershed from the upper Rhine to the Rhône drainage have been suggested for several fish species including whitefish (Steinmann 1951), arctic charr (Rubin 1990) and brown trout (Largiader *et al.* 1996). However, artificial transplantations among the same watersheds have also been documented for those fish species (Largiader *et al.* 1996; Brunner *et al.* 2001; Douglas

& Brunner 2002; Nicod *et al.* 2004). Consequently, it is difficult today to distinguish, at the genetic level, between the effects of natural colonization and those of artificial introductions.

The aim of this study was to perform a fine-scale investigation of potential watershed crossings between the upper Rhine and Rhône drainages by bullheads. We used eight highly informative microsatellite markers to investigate the genetic relationship between populations of both sides of the Rhine–Rhône watershed at two potential watershed crossing routes: first, the Lake Geneva area, which was highly influenced by the last glaciation and second, the plain residing between the Jura Mountains and the Vosges Mountains in the Belfort region, which was not covered by ice during the LGM (Hantke 1991). In both areas, the watershed is shallow and thus fish migration between the two drainages could have been facilitated by short contacts between the river systems (Hantke 1991) or even by changes in the flow direction of upper river stretches (Waters *et al.* 2001). Low population differentiation on both sides of and across a watershed is expected, if transfer was primarily due to anthropogenic introductions. On the other hand, strong population differentiation at a similar level on both sides of and across a watershed is anticipated if watershed transfer was facilitated by glacial retreat. In case neither glaciation nor anthropogenic introduction had any significant effects on the population structure, a consistent pattern of high differentiation among populations across the watershed is expected because of genetic drift and recolonization from different glacial refugia. Here, we evaluate the extent to which both processes have mediated present bullhead distributions and hint to possible determinant mechanisms for the colonization of other European freshwater fish species.

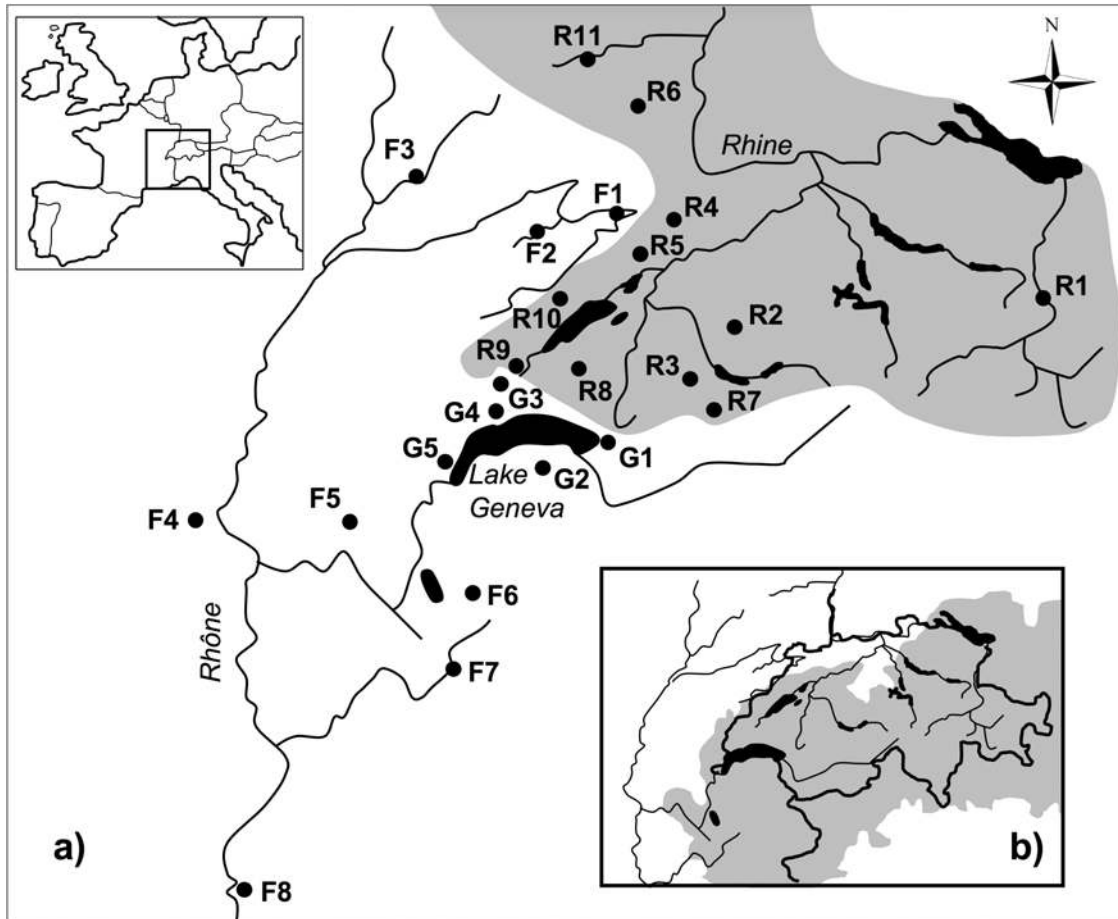
## Materials and methods

### Sampling

A total of 498 bullheads originating from 11 populations of the upper Rhine drainage and from 13 populations of the Rhône drainage were sampled from 1999 to 2004 (see Fig. 1a, Table 1). The sampled populations covered a wide geographical range with a fine sampling scheme in the area of the two potential watershed-crossing areas. All specimens were collected by electro-fishing and muscle or fin tissue was preserved in absolute ethanol.

### DNA extraction and microsatellite amplification

Total DNA was extracted using a standard phenol–chloroform/ethanol extraction method (Estoup & Martin 1996). All specimens were genotyped at eight highly informative microsatellite markers and forward primers



**Fig. 1** (a) Geographical distribution of bullhead (*Cottus gobio* L.) population samples collected in the upper Rhine and the Rhône drainages. The shaded area represents the Rhine drainage. A key to sample designations is given in Table 1. (b) Representation of the maximum glacial expansion during the Würm glaciation about 20 000 years BP in the Alps.

were labelled with the following fluorescent dyes (ABI): Cgo1033PBBE, Cgo33ZIM, and Cgo42ZIM with 6-FAM; Cgo310MEHU, Cgo18ZIM and Cgo34ZIM with VIC; Cgo56MEHU and Cgo1114PBBE with PET (Englbrecht *et al.* 1999). Because of overlapping allele size ranges of many microsatellite loci, two different sets of markers were created. The first set included locus Cgo1033PBBE, Cgo33ZIM, Cgo310MEHU, Cgo18ZIM, and Cgo56MEHU and the second set included Cgo42ZIM, Cgo34ZIM, and Cgo1114PBBE. QIAGEN Multiplex PCR kit for polymerase chain reaction (PCR) amplification was used according to the manufacturer's protocols. PCR was carried out in 10  $\mu$ L reaction volumes containing 5  $\mu$ L QIAGEN Multiplex PCR Master mix, 3  $\mu$ L ddH<sub>2</sub>O, 1  $\mu$ L DNA (20 ng/ $\mu$ L) and 1  $\mu$ L primer mix (2 pmol/ $\mu$ L each primer). The thermocycler profile started with an initial denaturation step at 95 °C for 15 min, followed by 30 cycles of 30 s at 94 °C, 90 s at  $T_A$ , 90 s at 72 °C and ended with a final extension of 10 min at 72 °C. A quantity of 1  $\mu$ L of a 1:3 dilution of the PCR was added to a buffer containing a LIZ 500 size standard ladder (ABI) and denatured fragments were

resolved on an automated DNA sequencer (ABI PRISM 3100). Genotypes were determined with the GENEMAPPER 3.0 (ABI) software and checked by eye.

#### Statistical analysis

Each locus in each population was tested for departure from Hardy–Weinberg Equilibrium (HWE) using GENEPOP version 3.2 (Raymond & Rousset 1995) with 10 000 dememorization steps, 100 batches and 5000 iterations per batch based on the approach by (Guo & Thompson 1992). For each sample,  $F_{IS}$  was calculated at each locus and tested for significant deviation from zero using FSTAT version 2.9.3 (Goudet 1995) while accounting for multiple comparisons using sequential Bonferroni corrections (Rice 1989). FSTAT was also used to calculate allelic richness indices ( $A_R$ ) (El Mousadik & Petit 1996) and both observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosities. Tests for deviations from genotypic equilibrium between all pairs of loci for each population, estimation of null-allele frequencies (Dempster *et al.* 1977; Excoffier & Slatkin 1995), computation

**Table 1** Geographical location of bullhead (*Cottus gobio* L.) sampling sites including drainage basin, geographical coordinates, number of analysed individuals ( $N$ ) and sample abbreviations. The following columns show the mean expected ( $H_E$ ) and observed heterozygosity ( $H_O$ ), mean allele numbers per locus ( $A_N$ ), mean allelic richness ( $A_R$ ) and the inbreeding coefficient ( $F_{IS}$ ) with corresponding  $P$  values

Abbr.	River	Drainage	Coordinates		$N$	$H_E$	$H_O$	$A_R$	$A_N$	$F_{IS}$	$P$ value
			Longitude	Latitude							
R1	Saar	Rhine	09°27'41.1"	47°01'25.5"	20	0.56	0.53	4.50	4.75	0.031	0.316
R2	Emme	Rhine	07°44'50.5"	46°56'40.7"	20	0.50	0.48	3.73	4.00	0.004	0.519
R3	Simme	Rhine	07°37'58.3"	46°40'40.5"	19	0.40	0.38	3.36	3.63	-0.023	0.414
R4	Birse	Rhine	07°22'40.0"	47°16'48.2"	20	0.30	0.25	1.87	1.88	0.134	0.108
R5	Schüss	Rhine	07°12'50.9"	47°11'16.7"	20	0.25	0.20	1.89	2.00	0.135	0.107
R6	Largue	Rhine	07°10'31.0"	47°32'13.0"	20	0.59	0.55	3.75	3.88	0.045	0.232
R7	Torneresse	Rhine	07°05'10.6"	46°27'31.5"	20	0.33	0.33	2.40	2.50	-0.082	0.187
R8	Grenet	Rhine	06°48'41.5"	46°34'04.6"	20	0.43	0.42	3.39	3.63	-0.002	0.533
R9	Orbe	Rhine	06°31'01.1"	46°43'35.5"	20	0.37	0.36	2.22	2.38	-0.020	0.456
R10	Areuse	Rhine	06°46'27.8"	46°57'31.8"	20	0.34	0.30	2.24	2.25	0.039	0.313
R11	Doller	Rhine	06°59'47.0"	46°57'31.8"	20	0.49	0.44	4.86	5.50	0.089	0.049
G1	Grand Eau	Rhône	06°58'25.6"	46°19' 06.0"	20	0.43	0.40	2.81	3.00	-0.018	0.453
G2	Chevenne	Rhône	06°47'55.3"	46°18'29.2"	20	0.26	0.25	1.87	1.88	-0.002	0.510
G3	Venoge	Rhône	06°27'45.9"	46°36'35.8"	20	0.43	0.39	3.31	3.63	0.049	0.274
G4	Boiron	Rhône	06°28'17.7"	46°29'46.7"	20	0.31	0.27	1.96	2.00	0.046	0.339
G5	R. de Roulave	Rhône	05°59'32.1"	46°12'11.4"	20	0.38	0.32	2.75	3.00	0.106	0.094
F1	Doubs	Rhône	07°08'40.7"	47°21'35.4"	20	0.42	0.31	3.48	3.88	0.211	0.002*
F2	Dessoubre	Rhône	06°44'07.0"	47°16'48.0"	20	0.55	0.54	4.73	5.38	0.012	0.394
F3	Reigne	Rhône	06°28'30.0"	47°40'10.0"	20	0.68	0.60	5.76	6.38	0.103	0.015*
F4	Azergues	Rhône	04°30' 00.0"	45°49' 00.0"	20	0.27	0.22	2.26	2.38	0.119	0.120
F5	Ain	Rhône	05°13'00.0"	45°49' 00.0"	20	0.59	0.57	6.94	8.00	0.017	0.384
F6	Laysse	Rhône	05°58'42.0"	45°25'02.0"	20	0.17	0.17	1.49	1.50	-0.038	0.454
F7	Gelon	Rhône	06°08'54.0"	45°29'58.0"	20	0.29	0.23	2.62	2.75	0.143	0.030
F8	Sorgues	Rhône	04°59'42.0"	43°55'48.0"	19	0.55	0.55	5.07	5.50	-0.028	0.323

of pairwise multilocus  $F_{ST}$  values (Weir & Cockerham 1984) among samples and hierarchical analysis of molecular variance (Excoffier *et al.* 1992) were performed using ARLEQUIN version 3.11 (Excoffier *et al.* 2005). A neighbour-joining (NJ) tree (Saitou & Nei 1987) among populations was generated based on Cavalli-Sforza & Edwards Chord Distances ( $D_C$ ) (Cavalli-Sforza & Edwards 1967), which were calculated in PHYLIP version 3.65 (Felsenstein 1989). Following the recommendation of Van Dongen (1995) with respect to relatively low number of loci, the 1000 bootstrap pseudoreplicates were performed over individuals rather than loci. Bootstrap replicates were generated using the program MATHEMATICA version 5.0 (Wolfram Research) and processed with PHYLIP to obtain the distances and to assess the bootstrap support for individual nodes of the NJ tree.

To ordinate populations based on allelic frequencies, a principal component analysis (PCA) was performed using PCAGEN (Goudet 1999). We applied the Monmonier's maximum difference algorithm (Monmonier 1973) implemented in the program BARRIERS version 2.2 (Manni *et al.* 2004) on  $D_C$  and geographical distance matrices to identify genetic barriers, namely the zones where differences between

pairs of populations are largest. The significance of barriers is expected to decrease with their rank. Statistical support for individual segments of the genetic barriers was assessed using the same set of 1000 bootstrap pseudoreplicates of distance matrices as for the NJ tree. Genetic diversity indices ( $H_E$  and  $H_O$ ) were compared among population groups using Bartlett's test for homogeneity of variances and a two-tailed  $F$ -test (Sokal & Rohlf 1995). In addition, using the program JUP IN 4.0 (SAS Inc.), a one-way analysis of variance (ANOVA) was performed to test for differences in the allelic richness ( $A_R$ ) between the same population groups. Finally, we conducted a spatial AMOVA (SAMOVA) as described in Dupanloup *et al.* (2002) to identify groups of populations, which are geographically homogeneous and maximally differentiated.

## Results

### Genetic diversity within populations

Significant departures from HWE were observed only at one locus Cgo42ZIM and in only two populations, the Doubs (F1;  $P < 0.00001$ ) and Reigne (F3;  $P < 0.0025$ ). This

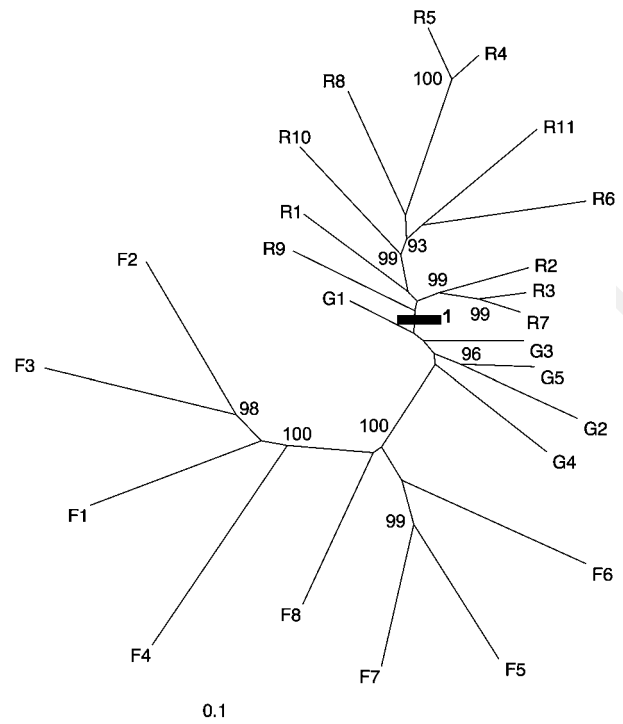
locus also demonstrated significant heterozygote deficiency as indicated by the positive and significant  $F_{IS}$  values ( $P < 0.00625$ ; Table 1). Deviation from HWE in these two populations at this locus was most likely attributable to null alleles as some individuals in both populations showed substantial problems in amplification for this locus only. Null alleles can either occur because of incomplete amplification or the presence of mutations in the primer region of a locus (Pemberton *et al.* 1995). The estimated frequency of null alleles was  $0.282 \pm 0.079$  SD and  $0.146 \pm 0.040$  for the Doubs (F1) and Reigne (F3) populations, respectively. As HWE at the Cgo42ZIM locus was rejected in only 2 out of 24 populations, it was not excluded from further analyses. When testing for deviations from linkage equilibrium, 63 out of 415 tests were significant at the 0.05 and 26 at the 0.01 level, respectively. Because these tests involved different pairs of loci in different samples, we concluded that significant results were likely the consequence of type I errors and of random genetic drift in small populations rather than of actual physical linkage between particular loci (Ohta 1982). This is in agreement with a genetic map of *Cottus gobio* reported by Stenshorn *et al.* (2005) including five (Cgo1033PBBE, Cgo33ZIM, Cgo42ZIM, Cgo18ZIM, and Cgo56MEHU) of the eight loci of the present study, which mapped on different linkage groups or in one case, on distant locations of a single linkage group.

All eight microsatellite loci showed considerable variability, with 4–43 alleles across all populations (allelic frequencies are reported in Table S1, Supplementary material). Over all populations and across all loci, a total number of 167 alleles were observed. Genetic variability was high among populations. Most notably, the mean number of alleles per locus ranged from 1.5 to 8,  $H_E$  ranged from 0.17 to 0.68, and  $A_R$  ranged from 1.49 to 6.94 (see Table 1).

Following the results of the population structuring (see below), we subdivided the populations of the Rhône into two groups, the lower Rhône group (F1–F8) and the Lake Geneva basin (G1–G5). Genetic diversity was highest in the Rhône populations (F1–F8) with a mean  $H_E$  of 0.44 and values ranging between 0.17 and 0.68, while those in the upper Rhine (R1–R11) showed a mean  $H_E$  of 0.41 with values ranging from 0.25 to 0.59 (Table 1). Lower  $H_E$  values were found among Lake Geneva populations (G1–G5) with a mean of 0.36 and a range of 0.26–0.43 (Table 1).

### Population differentiation

We found a high degree of overall genetic differentiation among populations with a multilocus  $F_{ST}$  of 0.484 (range: 0.10–0.76,  $P$ -values  $< 0.001$ ; Table 2). On the other hand, pairwise  $F_{ST}$  values among populations across the Rhine–Rhône watershed in the Lake Geneva area were generally



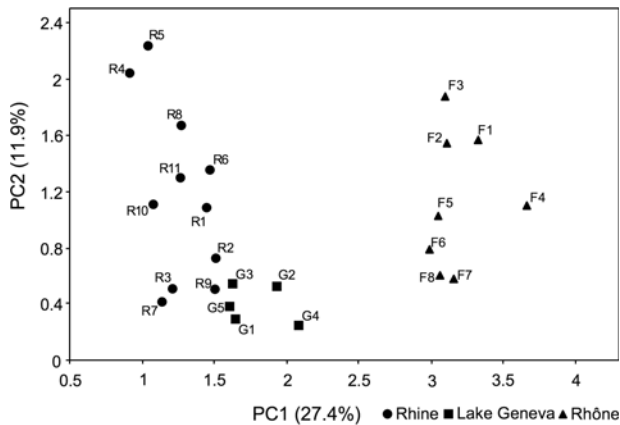
**Fig. 2** NJ tree of bullhead (*Cottus gobio* L.) populations based on chord distances  $D_C$  (Cavalli-Sforza & Edwards 1967) derived from eight microsatellite loci. Bootstrap values  $> 90\%$  based on 1000 pseudoreplicates are indicated at the respective nodes. The black bar and the adjacent number (1) correspond to the watershed between Rhine and the Rhône. A key to sample designations is given in Table 1.

lower; indicating a closer relationship among populations, than those found across the watershed in the Belfort region (Table 2). The lower Rhône populations (F1–F8) were most genetically structured with a global  $F_{ST}$  of 0.457, followed by the populations of the Lake Geneva basin ( $G1-G5$ ,  $F_{ST} = 0.366$ ) and the populations of the upper Rhine (R1–R11,  $F_{ST} = 0.347$ ).

Based on pairwise Cavalli-Sforza & Edwards Chord Distance ( $D_C$ ), two distinct clusters are visible in the derived NJ tree (Fig. 2). The first cluster is formed by the samples from the lower Rhône drainage (F1–F8) and is supported by a bootstrap value of 100%. The second cluster is formed by the populations of the Lake Geneva basin (G1–G5) and the populations from the upper Rhine drainage (R1–R11). Within this second cluster, the populations of the Lake Geneva basin cluster together and lay between the populations of the lower Rhône and those of the upper Rhine, but are clearly closer to the upper Rhine populations. The topology of the NJ tree agrees well with the results of the PCA computed to separate populations based on allelic frequencies (Fig. 3). Again, Lake Geneva populations (G1–G5) cluster closer to those from the upper Rhine (R1–R11) than to those from the Rhône (F1–F8). The

**Table 2** Multilocus pairwise  $F_{ST}$  estimated between pairs of samples (below the diagonal) and chord distance  $D_C$  (Cavalli-Sforza & Edwards 1967) between pairs of samples (above the diagonal) over all samples. All multilocus pairwise  $F_{ST}$  estimates were highly significant with all  $P$  values  $< 0.001$

		$D_C$																							
		R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	G1	G2	G3	G4	G5	F1	F2	F3	F4	F5	F6	F7	F8
$F_{ST}$	R1	—	0.06	0.06	0.09	0.09	0.08	0.07	0.09	0.07	0.08	0.08	0.06	0.11	0.08	0.10	0.09	0.15	0.16	0.17	0.17	0.12	0.13	0.13	0.11
	R2	0.20	—	0.04	0.08	0.09	0.09	0.05	0.08	0.07	0.09	0.08	0.05	0.10	0.06	0.07	0.07	0.15	0.14	0.16	0.16	0.14	0.13	0.14	0.11
	R3	0.22	0.11	—	0.09	0.10	0.10	0.02	0.09	0.07	0.08	0.09	0.05	0.10	0.06	0.09	0.07	0.16	0.16	0.17	0.17	0.15	0.14	0.15	0.12
	R4	0.36	0.39	0.45	—	0.02	0.09	0.10	0.08	0.11	0.08	0.08	0.11	0.09	0.09	0.12	0.11	0.18	0.19	0.18	0.20	0.17	0.15	0.17	0.17
	R5	0.37	0.44	0.54	0.25	—	0.10	0.11	0.08	0.12	0.11	0.09	0.12	0.13	0.11	0.13	0.12	0.17	0.19	0.17	0.20	0.17	0.15	0.17	0.17
	R6	0.19	0.25	0.32	0.34	0.40	—	0.09	0.08	0.10	0.09	0.08	0.10	0.12	0.10	0.11	0.09	0.17	0.17	0.16	0.19	0.15	0.15	0.16	0.14
	R7	0.28	0.21	0.10	0.50	0.58	0.34	—	0.09	0.05	0.07	0.10	0.06	0.07	0.06	0.09	0.06	0.16	0.16	0.18	0.17	0.16	0.13	0.14	0.12
	R8	0.27	0.32	0.36	0.36	0.43	0.26	0.41	—	0.09	0.08	0.10	0.08	0.10	0.09	0.12	0.08	0.17	0.17	0.15	0.18	0.15	0.15	0.15	0.14
	R9	0.28	0.30	0.32	0.52	0.58	0.35	0.33	0.42	—	0.08	0.10	0.05	0.08	0.07	0.10	0.07	0.15	0.14	0.17	0.16	0.15	0.12	0.13	0.11
	R10	0.28	0.36	0.36	0.42	0.54	0.28	0.37	0.35	0.40	—	0.09	0.08	0.08	0.07	0.12	0.08	0.16	0.17	0.17	0.16	0.16	0.16	0.14	0.15
	R11	0.23	0.29	0.33	0.34	0.42	0.18	0.37	0.33	0.39	0.30	—	0.09	0.12	0.09	0.10	0.10	0.17	0.18	0.17	0.18	0.15	0.15	0.16	0.15
	G1	0.21	0.22	0.26	0.51	0.54	0.32	0.32	0.40	0.29	0.40	0.35	—	0.08	0.04	0.06	0.05	0.14	0.14	0.16	0.15	0.13	0.13	0.12	0.09
	G2	0.46	0.48	0.53	0.58	0.66	0.40	0.50	0.53	0.47	0.49	0.47	0.47	—	0.06	0.08	0.05	0.13	0.16	0.16	0.14	0.13	0.12	0.12	0.10
	G3	0.27	0.28	0.30	0.45	0.52	0.29	0.33	0.40	0.39	0.34	0.29	0.23	0.36	—	0.07	0.07	0.14	0.15	0.16	0.16	0.14	0.10	0.13	0.10
	G4	0.40	0.37	0.47	0.60	0.65	0.40	0.52	0.55	0.53	0.55	0.41	0.35	0.50	0.33	—	0.06	0.14	0.15	0.16	0.14	0.12	0.13	0.13	0.10
	G5	0.32	0.29	0.35	0.54	0.58	0.29	0.35	0.42	0.39	0.41	0.33	0.27	0.41	0.34	0.37	—	0.15	0.15	0.17	0.14	0.14	0.14	0.14	0.11
	F1	0.49	0.53	0.60	0.65	0.67	0.49	0.63	0.58	0.59	0.60	0.53	0.53	0.59	0.51	0.57	0.57	—	0.10	0.10	0.11	0.13	0.14	0.14	0.11
	F2	0.43	0.42	0.49	0.58	0.61	0.43	0.54	0.50	0.49	0.55	0.49	0.46	0.57	0.49	0.54	0.50	0.39	—	0.09	0.14	0.14	0.16	0.15	0.12
	F3	0.37	0.40	0.47	0.50	0.53	0.34	0.50	0.40	0.47	0.49	0.43	0.43	0.49	0.41	0.47	0.46	0.33	0.25	—	0.13	0.13	0.17	0.16	0.12
	F4	0.58	0.58	0.65	0.73	0.76	0.58	0.69	0.66	0.67	0.69	0.62	0.63	0.68	0.62	0.66	0.63	0.54	0.52	0.44	—	0.12	0.15	0.12	0.13
	F5	0.32	0.36	0.45	0.51	0.54	0.33	0.49	0.42	0.44	0.48	0.40	0.39	0.45	0.38	0.41	0.43	0.41	0.38	0.27	0.43	—	0.10	0.08	0.09
	F6	0.56	0.59	0.67	0.73	0.76	0.55	0.70	0.65	0.65	0.72	0.62	0.64	0.69	0.58	0.68	0.66	0.66	0.61	0.54	0.72	0.39	—	0.10	0.14
	F7	0.50	0.52	0.60	0.69	0.72	0.51	0.64	0.60	0.61	0.65	0.58	0.56	0.64	0.56	0.61	0.58	0.62	0.55	0.47	0.62	0.28	0.62	—	0.09
	F8	0.36	0.33	0.42	0.59	0.62	0.40	0.48	0.48	0.44	0.54	0.46	0.33	0.52	0.38	0.42	0.43	0.41	0.32	0.27	0.48	0.26	0.57	0.41	—



**Fig. 3** PCA performed on allele frequencies at eight microsatellite loci of 24 bullhead populations distributed in geographical proximity along the Rhine–Rhône watershed. Shown are the first two principal component axes (PC1 and PC2) with the corresponding inertia percentage.

first principal component (PC1) axis explains 27.4% of the total genetic variance corresponding to a global  $F_{ST}$  estimate of 0.130 while that of the second PC explains 11.9% corresponding to a global  $F_{ST}$  estimate of 0.057. A third PC (not shown) explains 10.2% of the total genetic variance and corresponds to a global  $F_{ST}$  estimate of 0.048. The majority of the differences between the upper Rhine (R1–R11 & G1–G5) and the Rhône (F1–F8) populations occur along the first PC whereas the second and the third PCs (not shown) mainly separate populations within groups (Fig. 3).

### Genetic population structure

Results from a hierarchical AMOVA with a priori groupings of the Lake Geneva populations with either the upper Rhine or the lower Rhône populations demonstrated that the Lake Geneva populations were genetically more similar to those in the upper Rhine drainage than those from the lower Rhône (Table 3; Groupings 1 & 2). Lower  $F_{CT}$  and

$F_{ST}$  values were found when comparing populations from the upper Rhine drainage and from the Lake Geneva area combined with the lower Rhône drainage. Conversely, when defining Lake Geneva populations within the upper Rhine drainage and comparing it to the lower Rhône populations,  $F_{CT}$  and  $F_{ST}$  values were higher, regardless of the fact that the lower Rhône and Lake Geneva population belong in the same drainage. Thus, Lake Geneva populations are more closely related to upper Rhine population across the watershed than to the lower Rhône population within the same river drainage system.

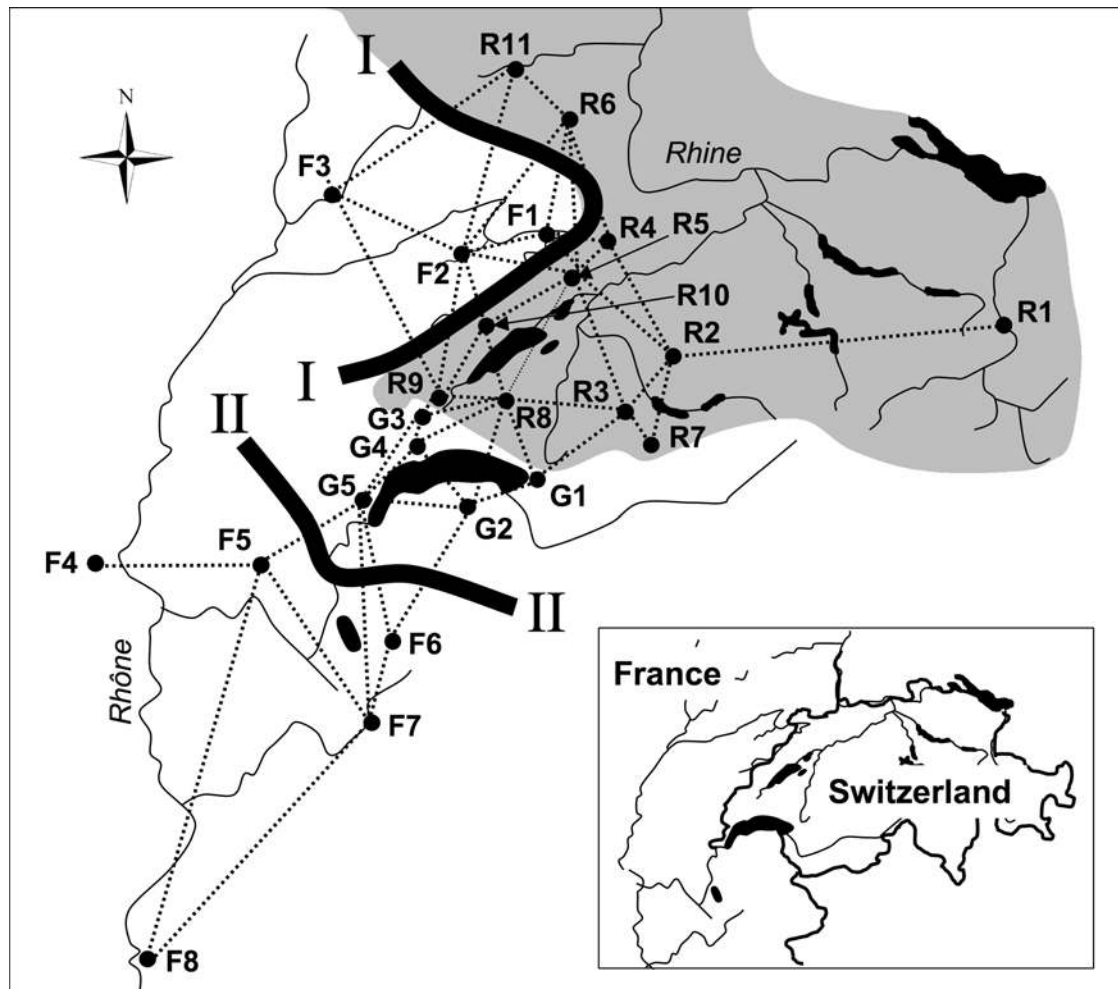
In contrast to the Lake Geneva region, in the Belfort region, a scenario consistent with upper Rhine populations crossing the watershed, demonstrated lower  $F_{CT}$  and  $F_{ST}$  values than when the populations from each drainage were considered separately. This scenario consists in grouping the Rhône (F1–F3) and the upper Rhine (R4–6, R10 & R11) populations together and comparing them to the remaining Rhône populations further south (F4–F8) (Table 3; groupings 3 and 4). Other groupings with populations from the upper Rhône drainage pooled together with upper Rhine populations as one group against remaining Rhône populations were assessed (data not shown). But in each case, these comparisons explained less genetic variability between groups of populations than strict Rhine–Rhône comparisons. Consequently, in the Belfort area, populations are genetically more related within drainages than between them.

The detection of possible genetic barriers among population groups conducted with SAMOVA and BARRIER based on pairwise  $F_{ST}$  and  $D_C$  could not recover barriers separating populations from either side of the watershed in the Lake Geneva area. The spatial analysis of molecular variance (SAMOVA) did not find distinct population groups at all, which is most probably due to very high pairwise  $F_{ST}$  values among populations (results not shown). However, the first genetic barrier identified by the analysis with BARRIER suggested a strong separation of populations in the Belfort area with a bootstrap support of 100% and followed the watershed division between the Rhine and the Rhône river systems (Fig. 4). The second barrier

**Table 3** AMOVA analyses of bullhead populations. Shown are the a priori groupings of populations used for the calculations and the calculated  $F_{ST}$  and  $F_{CT}$  values with corresponding  $P$  values. In groupings 1 and 4, the populations were assigned to groups according to their drainage system. On the other hand, for the groupings 2 and 3, populations were assigned into two groups assuming a watershed crossing by *Cottus gobio*. A key to sample designations is given in Table 1

Grouping no.	Group 1	Group 2	Overall $F_{ST}$	$P$ value	$F_{CT}$	$P$ value
1	F1–F8 + G1–G5	R1–R11	0.509	0.000	0.137	0.000
2	F1–F8	R1–R11 + G1–G5	0.532	0.000	0.205	0.000
3	F4–F8	R4, R5, R6, R10, R11 + F1–F3	0.544	0.000	0.146	0.007
4	F1–F8	R4, R5, R6, R10, R11	0.569	0.000	0.252	0.000





**Fig. 4** Spatial analysis based on Monmonier's maximum difference algorithm for detecting genetic barriers among 24 bullhead populations. The simulation of potential barriers was based on chord distances  $D_C$  (Cavalli-Sforza & Edwards 1967). The black lined triangles represent the Delaunay triangulation, which define potential neighbours. The first and second barrier are represented in thick solid lines and are labelled I and II, respectively. Bootstrapping over individuals supported individual segments of barrier I in 100% and barrier II in 96% of the 1000 pseudoreplicates.

divided the French Rhône population into two groups, the French (F4–F8) and the Lake Geneva populations. This second barrier was also strongly supported by bootstrap values of around 96% for all segments (Fig. 4). All additionally calculated barriers of lower rank did not follow any watershed division between the two drainages and were much less supported by bootstrap analysis (data not shown).

The ANOVA analysis estimating differences in allelic richness between groups of populations showed no significant differences between the three population groups; the upper Rhine (samples R1–R11), Lake Geneva (samples G1–G5), and Rhône (F1–F8) (all  $P > 0.186$ ). A Bartlett's test and an  $F$ -test revealed no significant differences at the level of genetic variability between any two of the groups mentioned above (all  $P$  values  $> 0.05$ ).

## Discussion

### *Evidence for natural watershed crossing*

The fine-scale genetic analysis of bullhead populations revealed strong evidence for natural watershed crossings in the Lake Geneva area and it is likely that other freshwater fish species used the same connection across the watershed. All genetic analyses of the presented data reveal bullhead populations from the Lake Geneva area to be more closely related to neighbouring populations from the upper Rhine drainage than to those from the Rhône, suggesting that the Lake Geneva basin was not colonized by a simple upstream migration scenario from the lower Rhône, but by an immigration from fish from across the watershed of the upper Rhine drainage. The Lake Geneva

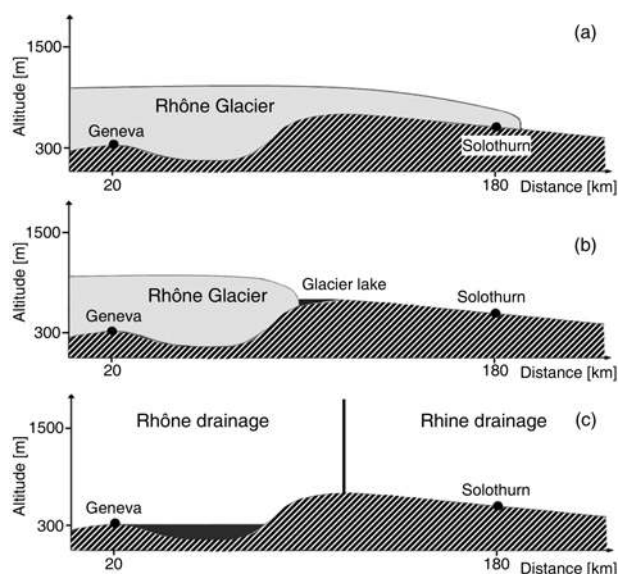
area was completely covered by the Rhône glacier during the LGM whereas this was not the case for the Belfort region (Hantke 1991). Consequently, the Lake Geneva area was most likely recolonized by fish after the retreat of this glacier while fish populations in the Belfort region were likely able to survive with minimum displacement during the same period. This would also support the previous hypotheses of such a crossing into Lake Geneva by other fish species like whitefish, brown trout, and arctic charr (Steinmann 1951; Rubin 1990; Largiader *et al.* 1996).

An alternative hypothesis is that bullheads migrated into the Lake Geneva area through the canal of Enteroche, which connected Lake Neuchâtel (Rhine drainage) with the Venoge (Rhône drainage) from 1638 to 1759 (Amberger *et al.* 1976). However, this scenario is doubtful, especially considering the high  $F_{ST}$  value (0.39) observed between the Venoge (G3) and the Orbe (R19) populations, as they are located precisely where the canal connected the drainages. Additionally, similar  $F_{ST}$  values within both the Rhine River and the Lake Geneva populations suggest that populations are of comparable age on either side of the watershed. Moreover, the ‘Rhône au Rhin’ canal which also connects the two drainages in the Belfort region since 1837 is likely to be a more efficient pathway for fish migration as compared to the canal of Enteroche. However, as the genetic differentiation between populations of the two drainages in this area is very high in all our analyses, a recent exchange between populations from the two drainages in the Belfort region seems to be very unlikely. Thus, it is reasonable to assume that the canal of Enteroche was not a major factor influencing bullhead colonization in the Lake Geneva region.

As another alternative, the colonization of Lake Geneva may have occurred by migrants from both the Rhine and the lower Rhône drainages merging in Lake Geneva as the glacier retreated. If this was the case, we would have expected to find at least indications for possible hybridization (i.e. a greater number of alleles in Lake Geneva samples compared to populations from either the upper Rhine or Rhône drainages), but no such signs were found. More so, the number of alleles in the Lake Geneva populations was lower than those reported in the surrounding upper Rhine and Rhône drainage samples.

### Colonization scenarios

Given the geological evidence indicating that part of the Rhône glacier crossed the watershed between the upper Rhine and Rhône and drained into the Rhine (Hantke 1991), colonization of Lake Geneva from Rhine bullhead migrants may have been facilitated by the retreating glacier during the Würm glaciation (20 000–10 000 BP). The potential for the formation of small ephemeral lakes and/or the swelling of connecting rivers and creeks at the



**Fig. 5** Retreat of the Rhône glacier after the last glaciation maximum. (a) shows the Würm glacial maximum of the Rhône glacier (20 000 years BP), (b) shows the hypothetical formation of a glacier lake at the edge of the glacier during his retreat over the watershed approximately 15 000 years BP, and (c) shows the present situation of the Rhine-Rhône watershed in Swiss Midlands.

retreating edge of the glacier (Fig. 5) may have allowed watershed crossing and drainage switching by freshwater fish (Waters *et al.* 2001). This however, implies that bullheads must have rapidly colonized large river stretches in order to be at the right time at the right place to make use of the very short-timed connection between the two river systems. Indeed, the deglaciation of the Swiss Midlands at the end of the LGM must have occurred in a relatively short period of time, given similar age ranges for pioneer vegetation in the Midlands and in Alpine pass areas (Welten 1982; Schlüchter 1988). Thus, a possible connection between Lake Geneva and the Atlantic drainage system may have existed for less than 100 years. This implies that bullhead colonization and dispersal abilities may be very dynamic and adaptable under a variety of circumstances. However, in ecological studies bullheads have been described as a nonmigratory fish with low dispersal ability compared to other species such as salmonids (Smyly 1957; Andreasson 1971; Knaepkens *et al.* 2005). This widely accepted view contradicts the wide distribution of bullheads in Europe, with the results presented herein, and the recently reported rapid colonization of the Rhine by a new *Cottus* hybrid species (Nolte *et al.* 2005).

The survival of bullheads in Europe during the fast changing climates and habitat alterations associated with glacial retreat along with its relatively rapid recolonization of the entire range demonstrates considerable migration ability. Such a conclusion implies that reliable predictions

of bullhead colonization and dispersal ability in changing environments cannot be inferred from their dispersal rates in their typical habitat. Future experiments need to be carried out in order to quantify migration ability of bullheads and their capacity to colonize new environments eventually with the help of computer simulations based on genetic data.

The exact nature of what prevented the colonization of Lake Geneva by bullheads from the Rhône after glacial retreat remains conjectural but two potential scenarios can be taken into account: first, the Rhône glacier may have retreated from the upper Rhine drainage more quickly than from the lower Rhône drainage, resulting in substantial ice barriers in the lower part of the Rhône while the upper Rhine became ice free. This may have allowed the colonization and establishment of Rhine bullheads in Lake Geneva more easily (Chapron 1999). Second, upstream migration from the Rhône after deglaciation may have been impeded by the presence of a series of waterfalls that existed in the Rhône River, downstream of Lake Geneva, which is presently hidden by an artificial lake (Lake Genissiat) created for hydro-electrical use (Bravard 1987). The presence of large waterfalls and other geological obstacles could very well prevent migration, which is supported by the fact that other studies report limited to nonexistent dispersal of freshwater species in a variety of systems (Forel 1892–1904; Currens *et al.* 1990; Preziosi & Fairbairn 1992; Costello *et al.* 2003).

#### *Comparison with mtDNA marker based studies*

Results presented herein appear to contradict previous bullhead colonization patterns for the Alpine region inferred from mitochondrial DNA, in which populations from the upper Rhine group together with those from the lower Rhône and Adriatic samples into a single phylogenetic clade (Riffel & Schreiber 1995; Haenfling & Brandl 1998b; Riffel & Schreiber 1998; Englbrecht *et al.* 2000; Kontula & Vainola 2001; Volckaert *et al.* 2002; Slechtova *et al.* 2004). In contrast, our data suggest that the upper Rhine (including the Lake Geneva area) and Rhône were colonized by bullhead from two different glacial refugia after the LGM. This interpretation is supported by an allozyme-based study (Riffel & Schreiber 1998), which reports that Rhône populations form a distinct clade compared to the Rhine and Danube bullhead populations. The different genetic patterns observed from the two different markers are not surprising as the phylogenetic results based on mtDNA rely on a single locus that is more strongly influenced by genetic drift than nuclear markers (Avisé 2004). This could have led to a massive loss of mtDNA genetic variability during the Pliocene–Pleistocene glaciation cycles, making mtDNA less suitable for small-scale population genetic analyses in particular

areas. Thus, our and other studies (Haenfling *et al.* 2002; Knaepkens *et al.* 2004; Nolte *et al.* 2005; Haenfling & Weetman 2006; Nolte *et al.* 2006) clearly show that microsatellite markers, which report a high allelic diversity because of a higher mutation rate (Paetkau *et al.* 1995) and to smaller effects of genetic drift as compared to mtDNA (Avisé 2004), are more suitable markers than mtDNA markers for studies addressing fine-scale phylogeographic processes during the LGM in the bullhead and in organisms with a similar population structure.

## Conclusions

We found genetic evidence for a natural crossing of a watershed by the bullheads between the upper Rhine and Rhône drainage in the Lake Geneva area. We hypothesize that this watershed crossing was assisted by the retreat of the Rhône glacier from across the watershed, a mechanism, which may have eased the dispersal of numerous European freshwater fish species across several geological boundaries. The evidence presented here demonstrates that a connection between the Rhine-Rhône drainages existed and that a potential for mass scale migration of many species across the watershed is not improbable. Results presented here also indicate that bullheads, which are considered as nonmigratory species with low dispersal ability, must have rapidly colonized large river stretches in order to be at the right time at the right place to make use of the very short-timed connection between the two river systems, implying that bullhead colonization and dispersal abilities may be very dynamic and adaptable under a variety of circumstances.

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107	-	-	-	-	-	0.1 3	-	-	-	0.2 0	-	-	-	-	0.0 5	-	-	-	-	0.0 3	-	-	0.0 5
109	-	-	-	-	-	-	-	-	-	-	-	-	0.0 3	-	0.0 3	-	-	-	-	-	-	-	0.7 0
113	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0 5	-
115	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2 8	-	-
117	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1 6
119	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1 5	-	-
121	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0 3	-	0.0 3
129	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0 3
131	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0 3

Cgo18ZIM

Fragment length (bp)	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	G1	G2	G3	G4	G5	F1	F2	F3	F4	F5	F6	F7	F8
228	-	-	-	-	-	-	-	-	-	-	-	-	0.1 0	-	-	-	-	-	-	-	-	-	-	-
232	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0 8	-	-	-	-	-	-	-	-	-
234	-	-	0.1 6	-	-	-	0.1 8	-	-	-	-	-	-	-	-	-	-	-	0.1 5	-	-	-	-	-
236	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6 3	-	-	-	-
238	-	-	-	-	-	-	-	-	-	-	-	0.0 5	-	-	-	-	-	0.0 3	-	-	-	-	-	-
240	-	-	0.0 8	-	-	-	-	-	-	-	0.1 0	-	-	-	-	-	-	-	-	-	-	-	-	-
242	0.5 8	0.3 3	0.4 2	0.5 0	0.7 8	0.3 8	0.7 0	0.4 3	0.5 8	1.0 0	0.7 3	0.9 5	0.9 0	0.9 5	0.9 3	1.0 0	0.8 0	-	-	-	-	-	0.0 8	0.0 9
244	-	-	-	-	-	-	-	0.5 8	-	-	-	-	-	0.0 5	-	-	0.1 0	0.2 0	0.2 0	-	-	-	-	-
246	0.0 5	0.0 5	0.0 3	-	-	0.3 0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2 8
248	-	-	-	0.5	0.2	0.2	-	-	0.4	-	-	-	-	-	-	-	-	-	0.0	-	-	-	-	0.0

				0	0	3			3								3			3	
					0.0	0.1				0.0							0.0	0.0		0.0	
250	-	-	-	-	3	0	-	-	-	5	-	-	-	-	-	-	8	-	3	-	6
		0.1								0.0											
252	-	8	-	-	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	
																	0.0	0.1	0.1		
254	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8	8	5	-	
																	0.0	0.0			
256	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	5	-	
																	0.0	0.1			
258	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	8	-	-	
										0.0					0.0	0.3	0.0				
260	-	-	-	-	-	-	-	-	-	3	-	-	-	-	3	3	8	-	-	-	
										0.0					0.0	0.0					
262	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-	8	3	-	3	-	
															0.0						
264	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	0	-	
																			0.1	0.0	
266	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	5	-	
															0.0				0.0	0.0	
268	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	5	-	0	-	
																			0.1		
270	0.0	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	0.8	
																			5	0.7	
272	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	
																			0.1	0.1	
274	0.0	5	-	-	-	-	-	-	-	5	-	-	-	-	-	-	-	-	3	0.1	
																			3	0.1	
276	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	
																			0.0		
278	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	
																			0.0		
280	0.2	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
																			0.1		
282	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8	-	-	-	
																			-	0.0	
284	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	3	-	-	-	
																			0.0		
286	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	5	-	
																			-	0.0	
288	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-	







## Cgo34ZIM

Fragment length (bp)	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	G1	G2	G3	G4	G5	F1	F2	F3	F4	F5	F6	F7	F8
279	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	-	-	-
295	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.0	-	-	-	-
297	-	-	-	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-
299	-	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-	-
301	-	-	-	-	0.0	-	-	-	-	-	-	-	-	-	-	-	0.7	-	0.1	0.4	1.0	0.7	-	-
303	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	3	-	-	5	-	-	-	-
305	-	-	-	-	-	-	-	0.2	0.0	-	0.0	0.2	-	-	0.0	0.3	-	-	-	0.0	-	-	-	-
307	-	-	-	-	-	-	-	0.1	-	-	-	0.0	-	0.0	-	-	-	-	-	-	-	0.0	-	-
309	-	0.0	0.0	-	-	-	-	0.0	0.0	0.1	0.4	0.0	0.1	0.0	0.3	0.6	-	-	0.0	0.6	-	-	-	-
311	0.2	0.4	0.8	0.7	0.2	-	1.0	0.6	0.7	0.8	-	0.2	0.8	0.6	0.0	-	-	-	8	7	-	-	-	-
313	8	5	7	0	8	-	0	3	5	4	-	0	5	3	-	5	-	-	-	-	-	0	-	-
315	0.0	0.1	0.0	0.3	0.7	0.1	-	-	0.0	-	0.1	0.0	-	0.2	0.2	-	0.0	0.3	0.1	-	0.0	0.6	-	-
317	5	5	3	0	0	5	-	-	3	-	0	3	-	8	3	-	3	3	0	-	3	5	-	-
319	0.0	0.2	0.0	-	-	-	-	-	0.1	-	0.1	-	-	0.0	-	-	-	0.0	-	-	0.0	-	-	-
321	3	3	8	-	-	-	-	-	8	-	8	-	-	3	-	-	-	3	-	-	3	-	-	-
323	-	-	-	-	-	0.3	-	-	-	-	0.2	-	-	-	-	-	-	0.0	-	-	0.0	-	-	-
325	-	0.1	-	-	-	0.0	-	-	-	-	-	-	-	-	-	-	0.1	0.5	0.1	-	-	-	-	-
327	0.5	0.0	-	-	-	0.1	-	-	-	-	-	0.4	-	0.3	-	-	0.0	-	-	-	0.1	-	-	-
329	5	5	-	-	-	3	-	-	-	-	-	8	-	8	-	8	-	-	-	-	8	-	-	-
323	0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	-	-	0.0	-	-	-
325	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	5	-	-	-
327	0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	0.0	-	-	-
329	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	3	-	-	-
327	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	0.0	-	-	-	-	-
329	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	-	0.2	-	-	-	-

335	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	0.0	
337	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	0.0
																									3
																									0.0
																									5

Cgo42ZIM

Fragment Length (bp)	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	G1	G2	G3	G4	G5	F1	F2	F3	F4	F5	F6	F7	F8	
208	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	6	-	-	-	-	-	-	-
214	-	-	-	-	-	-	-	-	-	0.3	-	-	0.6	-	-	-	0.3	-	0.1	1.0	0.1	-	-	-	-
216	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-
218	-	-	-	-	-	-	-	0.0	-	-	-	0.0	0.1	0.0	-	0.2	-	-	-	-	5	-	-	-	-
220	-	-	-	0.0	0.4	-	-	8	-	-	-	3	8	3	-	3	-	-	-	0.2	8	-	0	9	-
222	-	-	-	8	3	-	-	8	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-
224	0.0	-	-	0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0
226	5	-	-	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
228	-	-	-	-	-	0.1	-	0.0	-	-	-	0.0	-	-	-	-	-	-	-	0.0	-	-	-	-	0.1
230	0.1	0.0	0.1	-	-	0	-	5	-	-	-	3	-	-	-	-	-	-	-	3	-	-	-	-	3
232	3	3	1	-	-	-	-	-	-	-	-	8	3	3	-	3	4	-	-	-	5	-	-	-	0.5
234	-	0.2	-	-	-	0.2	-	0.2	-	-	-	-	-	-	0.1	0.3	-	-	-	-	0.0	-	-	-	0
236	0.2	-	-	-	-	0.0	-	0.0	0.7	-	0.4	-	-	-	0.9	-	-	-	-	-	3	-	-	-	0.0
238	5	-	-	-	-	3	-	3	3	-	5	-	-	-	0	-	-	-	-	-	-	-	-	-	3
240	0.2	0.6	0.1	0.3	0.5	0.2	0.2	0.0	-	-	0.0	-	-	-	-	0.0	0.1	-	-	-	-	-	-	-	-
242	5	8	6	3	8	5	0	8	-	-	8	-	-	-	-	3	4	-	-	-	-	-	-	-	-
	0.0	0.0	0.1	-	-	-	-	-	0.2	-	0.0	0.1	-	-	-	-	0.0	0.3	0.0	-	-	-	-	-	-
	8	3	8	-	-	-	-	-	5	-	3	3	-	-	-	-	7	4	8	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	0.0	-	0.0	-	-	-	-	-	-	-	-	-	0.0	-	-	-	-
	-	-	-	-	-	0.0	-	-	-	-	0.0	-	-	-	-	-	-	-	-	0.0	3	-	-	-	-
	-	-	-	-	-	3	-	-	-	-	3	-	-	-	-	-	-	-	3	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	0.2	-	-	0.0	-	0.0	0.0	0.0
	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	1	-	-	5	-	5	6	6



