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Genetic admixture of burbot (Teleostei: *Lota lota*) in Lake Constance from two European glacial refugia

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Abstract

The burbot, *Lota lota*, is the only freshwater species of the codfish family and has a Holarctic distribution. Pleistocene glaciations caused significant geographical differentiation in the past, but its life history characterized by winter spawning migrations over large distances is likely to homogenize populations by contemporary gene flow. We investigated the population genetic structure of 541 burbots from Lake Constance and adjacent Rhine and Danube tributaries in Europe using the entire mitochondrial DNA (mtDNA) control region and 11 microsatellites. Microsatellites revealed considerable population divergence (F_{ST} = 0.26) and evidenced recent bottlenecks in two Central European rivers. In accordance to previous evidence two main phylogeographic lineages (Atlantic and Danubian) were found co-occurring at similar frequencies in Lake Constance, where they currently undergo random mating as indicated by microsatellites. The Danubian lineage contributed only a small proportion to the lake's mtDNA diversity, and probably expanded within the lake shortly after its formation ~10 000–15 000 BP. The larger Atlantic haplotype diversity suggested a population expansion older than the lake itself. Levels of admixture at microsatellite loci were less obvious due to their high variability, and coalescence methods were used to estimate past admixture proportions. Our results reinforce a model of a two-step colonization of Europe by burbot from an ancestral Danubian refuge, and confirm the persistence of a secondary Atlantic refuge, as proposed to exist for other freshwater fish. We conclude that the present-day burbot population in Lake Constance bears the genetic signature of both contemporary gene flow and historical separation events.

Keywords: freshwater fish, microsatellites, mitochondrial DNA, panmixia, population genetics, postglacial colonization

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Introduction

Geographical distribution and genetic structure of temperate species are well known to reflect the climatic fluctuations during Quaternary glacial periods (e.g. Taberlet *et al*. 1998; Avise 2000; Hewitt 2000). Most species experienced severe range contractions during the cold periods, surviving in climatically more favourable regions, so-called glacial refugia, from which subsequent range expansions took place (Hewitt 1996). Consequently, historical range expansions following the withdrawals of the glaciers are among the main factors determining the patterns of present-day population structure of many species in Central Europe (e.g. Hewitt 1996, 2004). Additionally, for aquatic organisms, reversal of river flows or temporary connections between different drainages following the retreat of the ice and warming after the last glacial period drastically affected potential dispersal routes (Arkhipov *et al*. 1995). Freshwater fishes are well suited to study the genetic signatures of postglacial recolonization, since their dispersal depends on water routes, and their phylogeographic distributions are therefore likely to reflect historical causes more closely than those of terrestrial species (Bernatchez & Wilson 1998).

Postglacial colonization of freshwater fishes in the Palearctic has been studied most extensively by the geographic distribution of mitochondrial DNA (mtDNA) haplotypes (e.g. Bernatchez & Osinov 1995; Durand *et al*. 1999; Nesbø *et al*. 1999; Englbrecht *et al*. 2000; Bernatchez 2001; Kotlík & Berrebi 2001; Weiss *et al*. 2002; Salzburger *et al*. 2003; Van Houdt *et al*. 2003, 2005; Gum *et al*. 2005). Only in some cases nuclear markers were included (e.g. Hänfling *et al*. 2002;

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Koskinen *et al*. 2002; Gum *et al*. 2005). Although the number of mtDNA lineages in Central Europe varies considerably between species, different fish show genetic signatures of common glacial refugia. The lower Danube appears as a major refuge area for Central European freshwater fishes (see Hewitt 2004; references therein), while additional northern refugia in proximity to the European glacial margins might have also existed (Stewart & Lister 2001), such as in central Germany and southern England for the cold-adapted bullhead (Hänfling *et al*. 2002), or a vaguely defined Atlantic refuge for brown trout, chub, barbel and grayling (Bernatchez & Osinov 1995; Durand *et al*. 1999; Kotlík & Berrebi 2001; Gum *et al*. 2005). Another feature common to most European fish species is that major lineages came into secondary contact in Central Europe forming suture or hybrid zones (Hewitt 2004; Gum *et al*. 2005). Admixture of mtDNA types is commonly found, although the admixture at the nuclear level in these contact zones still remains to be examined (but see Gum *et al*. 2005).

The region of Lake Constance in the alpine area of Central Europe is where the rivers Rhine and Danube come into closest contact, and has been proposed as a potential suture zone between Danubian and Atlantic elements (e.g. Nesbø *et al*. 1999; Bernatchez 2001; Behrmann-Godel *et al*. 2004; Gum *et al*. 2005). Geological data confirm that major connections between these two drainages have existed until the Riss/Saalian glaciation period (150 000–300 000 BP; Hantke 1993; Keller & Krayss 2000). Lake Constance is one of the largest (570 km²) and deepest (250 m) pre-alpine lakes in Central Europe, and its lakebed was formed by the Rhine glacier proceeding from the inner Alps. The earliest colonization of this lake by fish may have occurred during the retreat of the glacier at the beginning of the present warm period (10 000–15 000 BP; see Behrmann-Godel *et al*. 2004). Thus, Lake Constance fish populations are likely to have retained the genetic legacy of recent connections between those drainages.

The burbot (*Lota lota* L. 1758) is the only member of the ocean dwelling codfish family (Gadidae) with a nonmarine life cycle. It has a Holarctic distribution (Nelson 1994), and fossil evidence suggests that fishes of this genus already inhabited freshwater in Europe in the early Pliocene 5 million years ago (Pietschmann 1934). According to molecular and fossil evidence, burbot colonized North America from Europe in the early Pleistocene (Cumbaa *et al*. 1981; Van Houdt *et al*. 2003, 2005), where it differentiated into two major lineages, currently considered as two subspecies (*L. lota lota* and *L. lota maculosa* — the latter being endemic to North America) (Van Houdt *et al*. 2003, 2005). While Nearctic burbot might have survived the climatic oscillations of the glacial periods, there are indications from genetic studies that the Eurasian form had become temporarily extinct or was reduced to very small numbers (Van Houdt *et al*. 2003, 2005; see also Englbrecht *et al*. 2000). Van Houdt *et al*. (2003, 2005) identified three major phylogeographic lineages for northern and central Europe: Danubian, Scandinavian, and Atlantic. Two of these lineages, Danubian and Atlantic, have been reported to coexist in Lake Constance, but the extent of this polymorphism as well as genetic admixture levels have yet to be examined since Van Houdt *et al*. (2005) only included five individuals from Lake Constance in their study.

Burbot is a highly mobile species with great dispersal abilities, a characteristic that might have been retained from its marine ancestors. Adults inhabit deep, cold waters of lakes and rivers in which they prefer to be near the bottom in areas of low light intensity (usually in the deepest water available; Muus & Dahlström 1968). In contrast, burbot larvae are pelagic (Fischer 1999; Miler & Fischer 2004) as in most gadoid species, and the juveniles are bottom-dwellers in the littoral zone (Fischer & Eckmann 1997; Fischer 2000). In winter, burbot migrates over long distances to common spawning areas (Slavík & Bartos 2002), an interesting behaviour that should preclude genetic differentiation across broad spatial scales. Population subdivision of burbot within single water bodies could, however, occur through kin-biased distribution of juveniles or natal homing with regard to spawning site, as has been shown in other fishes (e.g. Stepien & Faber 1998; Gerlach *et al*. 2001). Hence, some characters of burbot biology would support the uniformity of populations within continuous habitats, while others may operate towards population divergence.

In the present study, we used both sequences of the mtDNA control region and 11 polymorphic microsatellite loci to investigate the population genetic structure of burbot in the Lake Constance area, putting our results into the larger picture of the colonization history of burbot across Europe. The specific goals of the study focused on two different scales. On the large scale, we aimed to understand the origin of the burbot populations that colonized Lake Constance after its formation by comparing them to populations from adjacent river systems in Europe, and to discuss whether our results fit the general idea of a two-step colonization of Europe by freshwater fish species. On the small scale, we aimed to test whether mtDNA lineages from different origins genetically admix within Lake Constance. Additionally, we tested the hypothesis of panmixia of the highly mobile burbot within the lake by comparing several sampling localities, as well as adult, juvenile and larval populations, in order to better understand the genetic implications of the life history of the species.

Materials and methods

Sample collection

We collected specimens of the burbot from all life stages (larvae, juveniles, and adults) in Lake Constance, north of

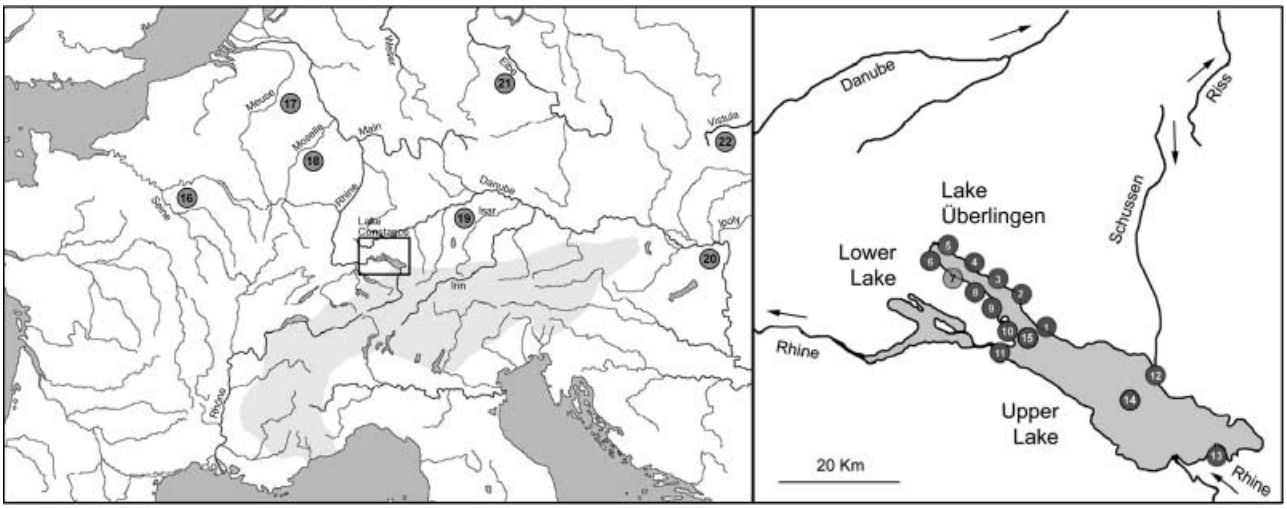

Fig. 1 Map of Central Europe showing the Alps (shadow area) and major river systems (left) and map of the area of Lake Constance (right) showing the sampling sites for burbot *Lota lota* in both areas (sites 1–15 in Lake Constance — no samples were found in site 7 — and sites 16–22 in Central European rivers).

Table 1 Sampling localities, number of individuals, mtDNA haplotypes and sequence accession numbers in GenBank for the burbot, *Lota lota*, fish studied in Lake Constance and adjacent river systems. Asterisks mark samples obtained from GenBank

	N	mtDNA haplotypes	GenBank Accession nos
Lake Constance:			
Adults	77	1, 4, 5, 7, 14, 17, 20, 42, 45	DQ630963, DQ630966–67, DQ630969, DQ630971, DQ630974, DQ630976, DQ630990, DQ630993
Juveniles	284	1–8, 14–17, 19, 20, 42–47	DQ630963–70, DQ630971–76, DQ630990–93
Larvae	72	1, 2, 4–7, 14, 15, 42	DQ630963–64, DQ630966–69, DQ630971–72, DQ630990
Atlantic lineage:			
Moselle River	30	14	DQ630971
Seine River*	10	14, 18	AY656912–13
Meuse River*	1	5	AY656895
Danubian lineage:			
Isar River	40	21, 23, 26, 27	DQ630977, DQ630979, DQ630981–82
Ipoly River	38	21–23, 25, 35–41	DQ630977, DQ630979, DQ630980, DQ630983–89
Elbe River*	9	21	AY656887
Vistula River*	6	21, 23, 34	AY656887, AY656914–15
Scandinavia*	46	9–13, 24, 28	AY656896, AY656900–01, AY656907–10
Russia*	13	29, 30, 32	AY656884, AY656888–89
North America*	24	30, 31, 33	AY656888, AY656890, AY656911

the Alps in Central Europe (Fig. 1, Table 1). Benthic juveniles (n = 284) were collected from stony-gravel substrate areas in the littoral of the lake with electric fishing gear during three consecutive years (2001–2003) and two seasons (December and June) in a combined sampling campaign for several littoral fish species in Lake Constance (see Barluenga & Meyer 2005). Several localities separated by a regular distance of approximately 4 km along the shoreline in the northwestern arm of Lake Constance, Lake Überlingen (sites 1–11, n = 20 per locality, but no samples were found on locality 7; see Fig. 1), and two additional sites in the northeastern part of the lake, the Upper Lake (sites 12, n = 50; 13, n = 36) were sampled. A maximum of 20 individuals per site for each sampling campaign were collected. Adults (n = 77) were collected with gill nets in 2001 from two localities in the open water column, one situated in Lake Überlingen, and another in the Upper Lake (sites 14, 15 n = 42; 15, n = 35; see Fig. 1). From each adult and juvenile collected a fin clip was preserved in 80% ethanol for laboratory analyses. Pelagic larvae (n = 72) were collected in the open water of Lake Überlingen (site 15; Fig. 1) between April–July in 2001 with a Hydrobios multiple

closing/opening net (for details see Miler & Fischer 2004). Larvae were preserved in ethanol and only the tail was used for laboratory analyses. We included specimens of a population from the lower Rhine system (Moselle River, France, $n = 30$; site 18), and two populations from the Danube system (Isar River, Germany, $n = 40$, site 19; Ipoly River, Hungary, $n = 38$, site 20). In total, 541 specimens were included in this study. In order to compare our results with previous studies on the large-scale phylogeography of burbot (Van Houdt *et al*. 2003, 2005), we included 109 already published sequences from Seine River (site 16), Meuse River (site 17), Elbe River (site 21), Vistula River (site 22) and additional samples from Scandinavia, Russia and North America (Table 1).

mtDNA amplification and sequencing

Total DNA was isolated using a proteinase *K* digestion followed by sodium chloride extraction and ethanol precipitation (Bruford *et al*. 1998). The complete mitochondrial control region (891 bp) was amplified using published primers and polymerase chain reaction (PCR) conditions (L-Pro, Meyer *et al*. 1994; H00651, Kocher *et al*. 1989) with a GeneAmp PCR System 9700 Thermocyler (Applied Biosystems). The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN), and sequenced in both directions with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequencing products were analysed with an ABI 3100 Automated Sequencer (Applied Biosystems). The mtDNA sequences of this study have been deposited in GenBank under Accession nos DQ630963–DQ631411.

Microsatellite analysis

Eleven nuclear microsatellite loci, *Llo1*, *Llo6*, *Llo7*, *Llo12*, *Llo13*, *Llo15*, *Llo16*, *Llo21*, *Llo26*, *Llo32*, and *Llo34*, designed for *L. lota* were genotyped. Primer sequences and amplification conditions for these loci have been reported elsewhere (Sanetra & Meyer 2005). Microsatellites were amplified with fluorescently labelled forward primers (FAM, HEX and TAMRA dyes) and fragment length was analysed with the internal size marker GENESCAN-500 ROX (Applied Biosystems) with an ABI 3100 Automated Sequencer (Applied Biosystems), and scored with GENOTYPER version 3.7 (Applied Biosystems) software package.

Phylogenetic and Statistical Analyses

mtDNA control region. Mitochondrial DNA sequences were aligned by eye and different haplotypes were identified with COLLAPSE version 1.2 (Posada 1999). All haplotypes found were plotted on an un-rooted haplotype network (Fig. 2a, b), according to the optimal tree obtained from a maximum likelihood analysis in PAUP version 4.0b10 (Swofford 2002). A model of sequence evolution was chosen using a nested series of likelihood ratio tests (Huelsenbeck & Crandall 1997) applying MODELTEST version 3.06 (Posada & Crandall 1998). MODELTEST revealed that the optimal model of molecular evolution for our data set was Hasegawa–Kishino–Yano (corrected with a gamma parameter of $\alpha = 0.69$ and a proportion of invariable sites of 0.92). The optimal maximum likelihood tree was translated into an unrooted network with maximum parsimony branch lengths in order to associate each branch with mutational steps. As additional evaluation of the haplotype network, the consistency index (CI; Kluge & Farris 1969) for each mutation estimated under maximum parsimony using PAUP was calculated (see Fig. 2b). Gaps were included in the definition of the haplotypes and the construction of the network.

A mismatch analysis was performed to study the demographic history of the species in the area (Fig. 2c). The fit of the observed pairwise mismatch distributions to a sudden expansion demographic model was tested using a generalized least square procedure and by computing the raggedness index of the observed distributions (Harpending 1994) as implemented in ARLEQUIN version 3.0 (Excoffier *et al*. 2005). The validity of a stepwise expansion model for the data was tested using Markov Chain Monte Carlo simulations (1000 steps) with ARLEQUIN. We computed the moment estimator of the age of the expansion (τ), and the mutation parameters θ_0 ($2\mu N_0$) and θ_1 ($2\mu N_1$) using a parametric bootstrap approach (1000 simulations), where μ is the mutation rate and N is the female effective population size. Tajima's D (Tajima 1996) and Fu's Fs (Fu 1997) analyses were performed with ARLEQUIN as additional tests of population expansion.

Genetic differences between seasons, years, life stages and localities were estimated with pairwise F-statistics (Weir & Cockerham 1984) as implemented in ARLEQUIN. To determine how genetic variation was partitioned among geographic regions (Lake Constance, Danube and Rhine), a hierarchical nesting of genetic diversity was estimated using the analysis of molecular variance (AMOVA) approach of Excoffier *et al*. (1992) with ARLEQUIN. Two different nesting approaches were performed: (i) among geographic regions — 1. Lake Constance: Lake Überlingen North, Lake Überlingen South, Upper Lake; 2. Danube system: Isar and Ipoly rivers; 3. Rhine system: Moselle River; and (ii) among mtDNA lineages — 1. Danubian lineage: Isar and Ipoly rivers and Danubian Lake Constance; 2. Atlantic lineage: Moselle River and Atlantic Lake Constance.

Microsatellites. The basic descriptive statistics: number of alleles, expected heterozygosity (H_E) and observed heterozygosity (H_O) were compiled using ARLEQUIN. Departure from Hardy–Weinberg equilibrium for each locus across and within populations was calculated using a test analogous

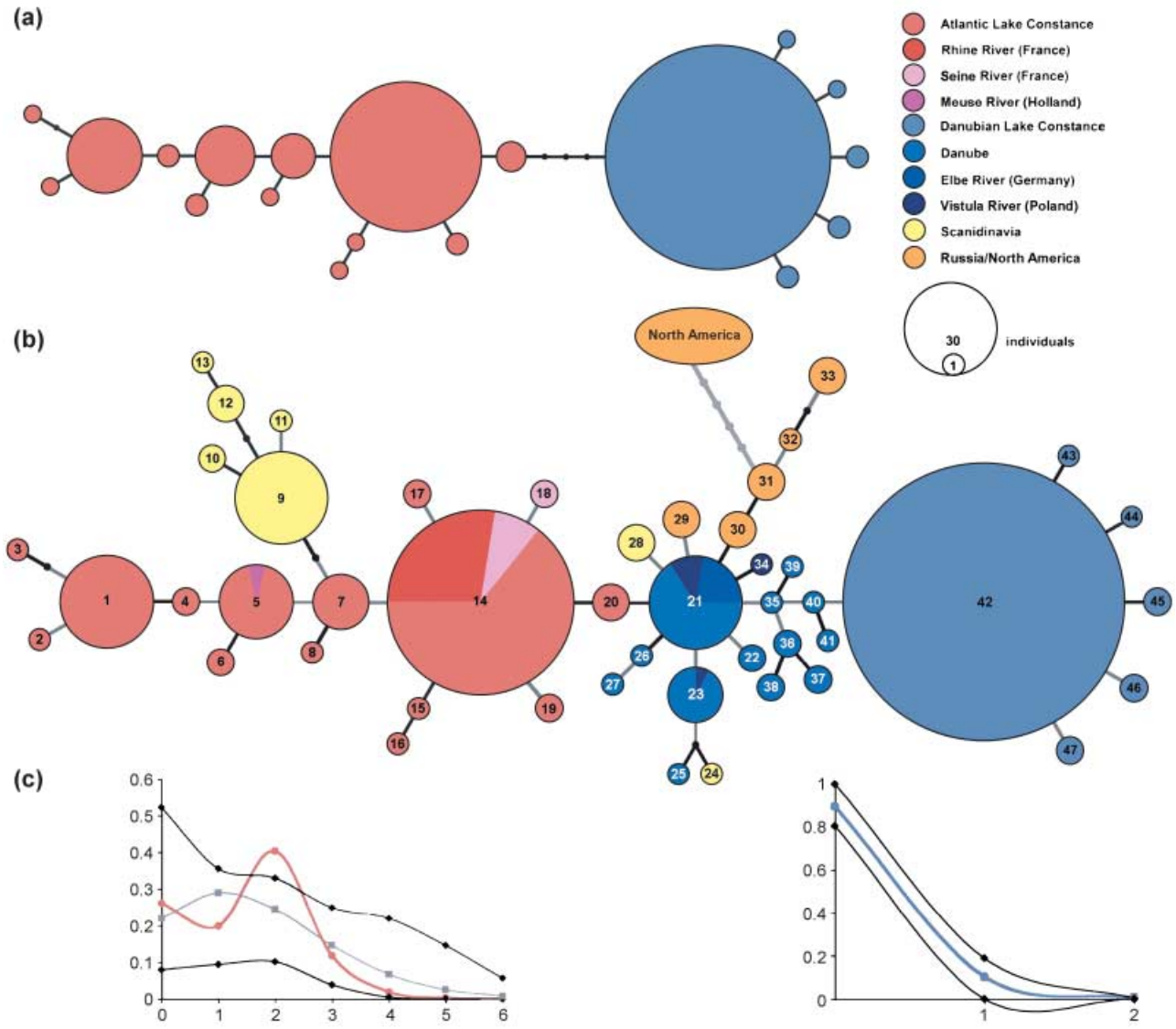

Fig. 2 Unrooted haplotype network of the complete mtDNA control region of (a) the 433 burbot samples collected in Lake Constance, and (b) all 590 burbot samples included in this study and collected in different European sites. Circles represent unique haplotypes and the size reflects the number of individuals sharing a particular haplotype (see scale). Colors refer to different geographical regions. Connections between haplotypes represent mutational steps, depicted in black those mutations with a consistency index (CI; Kluge & Farris 1969) = 1, and in grey mutations with CI < 1. (c) mismatch analysis showing the inferred demographic histories of individuals in Lake Constance belonging to either Atlantic — in red — or Danubian — in blue — lineages. Red and blue lines depict the observed data, grey lines represent the best-fit model, and in black are the upper and lower boundaries.

to Fisher's exact tests (Guo & Thompson 1992) estimated with a 100 000-step, 1000 iteration, Markov chain Monte Carlo series of permutations, as implemented in ARLEQUIN. Linkage disequilibrium was tested for all possible pairs of loci in each population and globally for each pair of loci across populations with ARLEQUIN. The effective number of alleles was calculated as $n_e = \sum 1/p_i^2$ (Hartl & Clark 1997). Differences between populations in diversity measures (H_E, n_A, n_e) were estimated with a Mann–Whitney *U*-test using STATISTICA (StatSoft, Inc. 2003). Shared alleles between pairs of populations were quantified across all loci combined.

Genetic structure between seasons, years, life stages and localities was analysed by Wright's *F*-statistics (Weir & Cockerham 1984) based on differences in allele frequencies, and by R_{ST} that assume a step mutation model, as implemented in ARLEQUIN. Additionally, for the study of the population genetic structure at the microgeographical level, we used exact tests on contingency tables of allele

distributions as a very sensitive tool for population differentiation. We used a hierarchical AMOVA to assess the distribution of microsatellite variation using ARLEQUIN. The same nesting approaches as for mtDNA were used. The probabilities that the molecular variances and fixation indices at different levels were significantly positive (indicating differentiation) were determined by permutation analysis using 1000 randomly permuted data sets.

Unrooted neighbour joining (NJ) clustering analysis was performed with a drift-based genetic distance, Cavalli-Sforza & Edwards (1967) chord distance, D_{CE}, and a mutation-based estimator, Goldstein *et al*. (1995) $(\delta\mu)^2$, using POPULATIONS version 1.2.14 (Langella 2001). Consistency of tree topology was assessed by bootstrapping over loci with replacement and 1000 replicates. Trees were visualized with TREEVIEW (Page 1996).

A factorial correspondence analysis (FCA) of individual diploid genotypes was performed using GENETIX version 4.05 (Belkhir *et al*. 1996–2002) to reveal the portion of the hyperspace of alleles from all considered loci occupied by each group of individuals. The Bayesian clustering method of STRUCTURE (Pritchard *et al*. 2000) was also used to assemble individuals into groups with a model that assumes admixture, but also with a model that does not assume admixture and with prior information on their sampling location. The number of clusters ($K = 2-8$) was determined by comparing log-likelihoods in multiple runs for values of K between one and five. Each run consisted of 100 000 iterations with a burn-in period of 10 000.

Genetic admixture proportions of Rhine and Danube lineages in Lake Constance were also estimated using a coalescence method as implemented in ADMIX version 2.0 (Dupanloup & Bertorelle 2001). This method is based on the average coalescence times between pairs of genes sampled within and between populations. To this end, Rhine and Danube were defined as parental populations and assumed to have evolved independently for approximately 3000–4000 generations (generation time = 3–4 years; Arndt & Hutchinson 2000; Eveson 2000) with a mutation rate of 10^{-3}. Additionally the admixture coefficient (mY) of Bertorelle & Excoffier (1998) was calculated, where the molecular divergence between alleles is estimated from the average squared difference in allele sizes under the assumption that microsatellites follow the stepwise mutation model. Standard deviations of these estimates were calculated with a parametric bootstrap approach.

To study the demographic history of the populations based on microsatellites an interlocus g-test was performed (Reich & Goldstein 1998; Reich *et al*. 1999). This method is based on the observation that the variance in allele sizes across loci is usually lower in an expanding population compared to a population that remains constant in size. In practice, the variances across loci derived from the data are compared to what is theoretically expected in an expanding population. The test statistic is $g = \mathrm{Var}[V_j]/(4/3V^2 + 1/6V)$, where V_j is the variance at the jth locus, and V is the average of all V_j (equation 3 of Reich & Goldstein 1998).

Past population reductions were evaluated with the test statistic M-ratio (Garza & Williamson 2001). The ratio $M = k/r$ between the number of alleles present at a given microsatellite locus, k, and the range in allele sizes in base pairs, r, was used to detect the occurrence of recent population bottlenecks. This ratio will be significantly reduced after a population bottleneck because rare alleles are lost by drift more often than common alleles during a population size reduction. We used the settings $N_e = 5000$, $\mu = 0.001$, $p_s = 0.9$, and $\Delta_g = 3.5$ (see Garza & Williamson 2001 for discussion). Statistical significance was estimated by simulating an equilibrium distribution of M using 1000 replicates, according to the method described in Garza & Williamson (2001), and ranking the calculated value relative to the equilibrium distribution. Using conventional criteria, there is evidence of a significant reduction in population size if less than 5% of the replicates are below the observed value.

Simulations of population genetic dynamics were performed with the software EASYPOP version 1.8 (Balloux 2001) to estimate the divergence of populations and the number of private alleles that can arise after a certain number of generations. The parameters used for two populations with interrupted gene flow were $N_e = 5000$, $\mu = 10^{-3}$ and 10^{-4} (corresponding to the known microsatellite mutation rates in fish, see e.g. Jones *et al*. 1999; Shimoda *et al*. 1999), random mating, no migration, 4000 generations, and minimum variability as the starting point. A mixed mutation model of single-step mutation with a proportion of double or multistep mutation events set to 0.2 was applied for 20 loci with 10 replicates. Allele frequency distributions were further analysed with GENEPOP and the average number of private alleles per population determined. Differences to the observed number in Lake Constance were evaluated using t-tests.

Significance levels in all analyses were corrected for multiple testing following the sequential Bonferroni procedure (Rice 1989).

Relatedness estimation

To investigate whether related individuals stay together in the larval or juvenile stage, genetic relatedness among individuals within sampling sites was estimated from microsatellite genotypes using RELATEDNESS version 5.0.5 (Goodnight 2000). This algorithm uses the methods described by Queller & Goodnight (1989) calculating the regression relatedness (b) on the basis of average population allele frequencies. Groups were weighted equally and standard errors were estimated by jackknifing over groups. Ninety five percent confidence intervals were used to examine the statistical significance of the relatedness values.

Table 2 Mismatch analysis estimated parameters. τ is the moment estimator of the age of the expansion (in parentheses, the 95% confidence intervals calculated for $\alpha = 0.05$); θ_0 and θ_1 are the mutation parameters before and after the expansion, respectively; SSD is the test of the validity of a stepwise expansion model based on the sum of square deviations between the observed and the expected mismatch, and the significance of the test is estimated with a parametric bootstrap approach, and the same method is used to test the significance of the Raggedness index (probability values: *$P < 0.05$, **$P < 0.001$, NS, nonsignificant)

	Mean no. of differences	τ	θ_0	θ_1	SSD	Raggedness index
Danubian	0.11	0.44 (0.01–0.44)	0.00	0.12	0.000^{NS}	0.635^{NS}
Atlantic	1.77	1.88 (0.48–3.94)	0.00	8.03	0.039**	0.138*
All	3.463	4.89 (1.97–8.62)	0.00	8.15	0.014^{NS}	0.043^{NS}

Results

mtDNA variation and demographic analyses

Forty-seven different mtDNA haplotypes were found among the 650 DNA sequences included in this study (541 new individuals plus 109 sequences obtained from GenBank; Table 1). Haplotypes differed from each other by a maximum of 13 mutations. A haplotype network of the haplotypes existing in Lake Constance (Fig. 2a), and a network containing all recovered haplotypes (Fig. 2b) were reconstructed to show the evolutionary relationships among haplotypes. The samples from Lake Constance contained the largest amount of diversity with 20 haplotypes (among which the maximum distance was 12 mutations; Fig. 2a), whereas only 13 and relatively closely related haplotypes (1–3 mutations) were found at the two locations in the Danube (Ipoly and Isar rivers), and only a single haplotype in the samples from the Rhine (Moselle River) (Fig. 2b). Haplotypes from Lake Constance appeared in two different parts of the network, which were separated by four mutational steps (Fig. 2a). One larger cluster with 14 haplotypes also included the single Rhine haplotype and the rest of the Western European samples (Seine and Meuse rivers — France and Holland, respectively), while the other cluster comprising six haplotypes was associated with samples from the Danube and other Central European rivers (Elbe and Vistula rivers — Germany and Poland, respectively) (Fig. 2b). We found in Lake Constance almost equal numbers of individuals from each of the two lineages. The samples from Scandinavia comprised seven haplotypes, five of which included most samples (haplotypes 9–13) and were connected to Western European haplotypes, while two others (24, 28) were connected to Central European haplotypes. Burbot from Russia and North America represented five additional haplotypes (29–33) connected to Danube haplotypes (Fig. 2b). Scandinavia appears to have been colonized by the Atlantic lineage, although some haplotypes are included in the Danubian lineage, while Russia/North America appears to have been colonized by the Danubian lineage.

A mismatch analysis was performed to determine the demography of the two burbot lineages in Lake Constance (Fig. 2b). The Danubian lineage of burbot in Lake Constance follows a model of sudden expansion (Table 2) and shows the signature of a very recent population expansion. This result is further corroborated by significant Tajima's D and Fu's Fs statistics ($D = -1.50$, $P = 0.02$; $Fs = -12.15$, $P < 0.001$). In contrast, the Atlantic burbot lineage in Lake Constance is more diverse and does not follow a model of sudden expansion (Table 2).

Microsatellite diversity and nuclear demographic analyses

Mean expected heterozygosities (H_E) in Lake Constance, Moselle River (Rhine), Ipoly and Isar rivers (Danube) were 0.72, 0.60, 0.85 and 0.49, respectively. The most variable loci were *Llo16* ($H_E = 0.80$) and *Llo32* (0.81), while the least variable was *Llo15* (0.40). The total number of alleles per locus ranged from 6 to 36 (mean 21), considering all populations combined (see allele distribution and frequencies in Fig. 3). Details on the genetic diversity and effective number of alleles of individual populations are shown in Table 3. Interestingly, the Lake Constance population displayed a large absolute number of alleles across loci (15) while having a low effective number of alleles (4.28) due to many low frequency alleles. The largest genetic diversity both in terms of H_E and n_e was found in the Ipoly, which is closest to the presumed Danube refuge. Populations from Moselle and Isar had significantly lower genetic variability as reflected by most of the diversity measures (n_A: $U_{11} = 114.5–135.5$, $P < 0.001$; n_e: $U_{11} = 104–115$, $P < 0.003$; H_E: $U_{11} = 100.5–115$, $P < 0.001$) except for H_E and n_e between Lake Constance and Moselle River ($U_{11} = 89.5$, $P = 0.06$; $U_{11} = 92.0$, $P = 0.04$). Genotype distributions were generally in accord with expected Hardy–Weinberg proportions, and only four out of 44 population–locus combinations showed significant deviations. The number of genotypic disequilibrium tests between microsatellite locus pairs that remained significant was small (three out of 202) and showed no consistent pattern, indicating that independence of loci could be assumed. These results were similar to previous estimates (Sanetra & Meyer 2005).

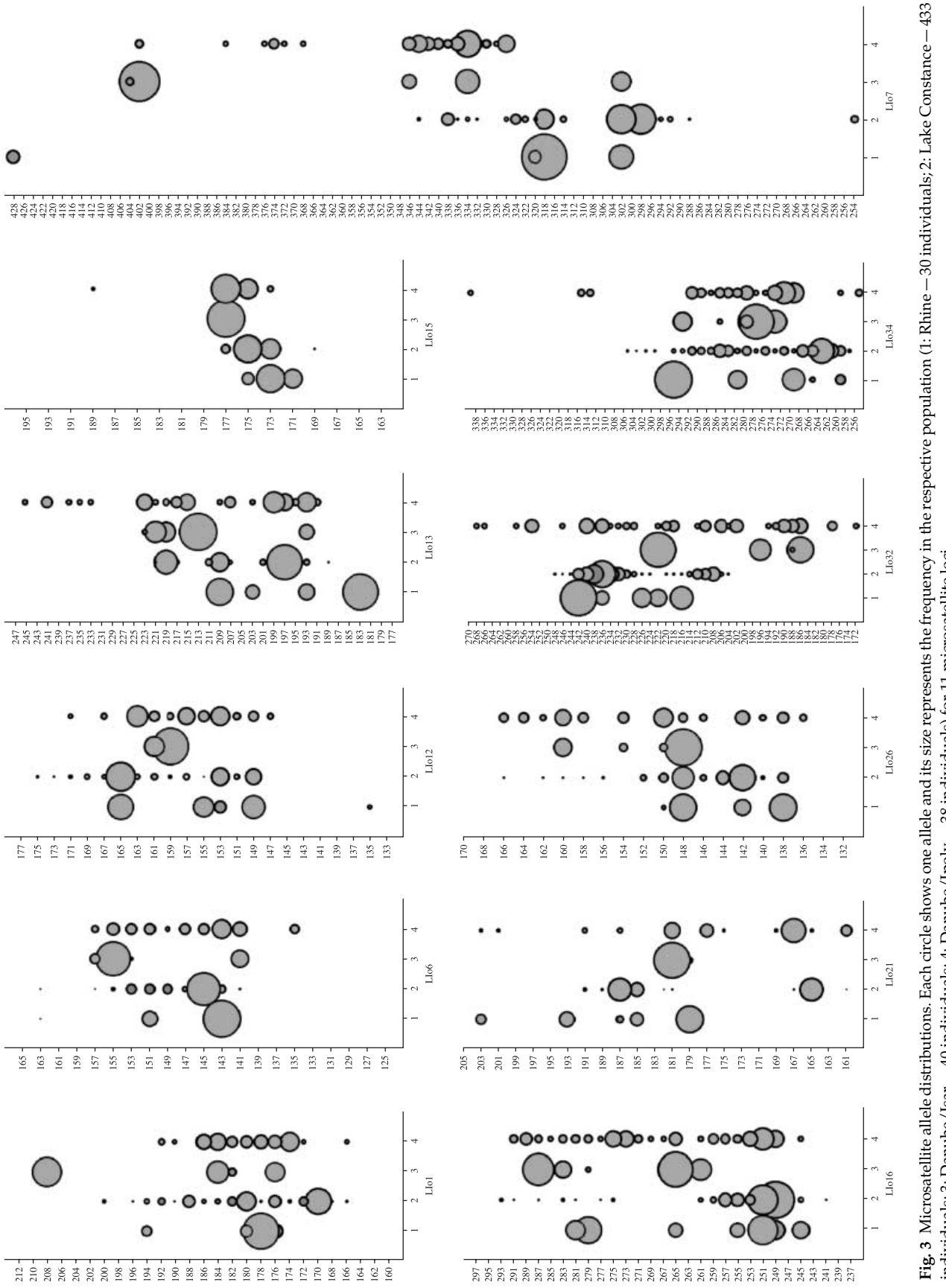

Fig. 3 Microsatellite allele distributions. Each circle shows one allele and its size represents the frequency in the respective population (1: Rhine — 30 individuals; 2: Lake Constance — 433 individuals; 3: Danube/Isar — 40 individuals; 4: Danube/Ipoly — 38 individuals) for 11 microsatellite loci.

Table 3 Observed number of alleles (N_A), effective number of alleles (N_e) and expected heterozygosity (H_E) for the microsatellite loci in the four a posteriori-defined major populations of *Lota lota*

	Llo1	*Llo6*	*Llo7*	*Lo12*	*Llo13*	*Llo15*	*Llo16*	*Llo21*	*Llo26*	*Lo32*	*Llo34*	Mean
Lake												
N_A	17	10	20	14	12	4	17	10	13	22	26	**15.00**
N_e	4.89	2.09	4.66	3.42	3.25	2.15	3.42	2.91	3.92	7.70	8.67	**4.28**
H_E	0.80	0.52	0.79	0.71	0.69	0.54	0.71	0.66	0.75	0.87	0.89	**0.72**
Moselle												
N_A	4	2	3	5	4	3	7	5	4	5	5	**4.27**
N_e	1.88	1.34	1.75	3.55	2.76	2.20	5.44	2.85	2.70	3.37	2.54	**2.76**
H_E	0.48	0.26	0.44	0.73	0.65	0.55	0.83	0.66	0.64	0.72	0.62	**0.60**
Ipoly												
N_A	11	10	17	11	18	4	22	11	13	26	17	**14.55**
N_e	7.18	7.57	8.15	6.16	9.66	2.00	12.60	3.91	9.04	17.39	8.67	**8.39**
H_E	0.87	0.88	0.89	0.85	0.91	0.51	0.93	0.75	0.90	0.96	0.90	**0.85**
Isar												
N_A	4	4	5	2	5	1	6	2	4	5	5	**3.91**
N_e	2.90	1.80	2.68	1.56	2.83	1	3.42	1.09	1.76	3.40	2.84	**2.30**
H_E	0.66	0.45	0.63	0.37	0.66	0.00	0.72	0.08	0.44	0.71	0.66	**0.49**
Mean	0.70	0.53	0.69	0.66	0.73	0.40	0.80	0.54	0.68	0.81	0.76	**0.66**

Two statistical tests were used to detect population demography from microsatellite data. The interlocus g-test was used to reveal whether populations experienced recent demographic expansions. The ratios of the observed and the predicted variances (g) in Lake Constance, Moselle River (Rhine), Ipoly and Isar rivers (Danube) were 2.64, 2.04, 0.92, and 3.21, respectively. None of them revealed a significant signature of population expansion, which is reached when g is smaller than 0.19 at the 0.05 level for 10 loci, sample size 20, according to Table 1 in Reich *et al*. (1999).

We found strong evidence for past demographic reduction in population size for two of the studied populations, Moselle River ($M = 0.40$, $P = 0.00$) and Isar ($M = 0.53$, $P = 0.00$), as shown by the M-ratio test (Garza & Williamson 2001). Lake Constance and Ipoly populations showed no significant signs of population bottlenecks ($M = 0.80$, $P = 0.27$; $M = 0.65$, $P = 0.07$, respectively). This pattern is also evident from the diversity measures in Table 3 and the allele frequency distribution (Fig. 3).

Population genetic differentiation

Macrogeographical scale. The four geographically distant locations included in this study, Lake Constance, Moselle, Isar and Ipoly rivers, were strongly differentiated with both types of molecular markers (average mtDNA $\Phi_{ST} = 0.28$; microsatellites $F_{ST} = 0.26$). All pairwise F-statistic comparisons were highly significant ($P < 0.0001$; Table 4). The three-dimensional plot derived from the factorial correspondence analysis also showed high levels of microsatellite differentiation and the spread of the variance for each population (Fig. 4). The AMOVA comparing geographical regions also showed a large fraction of variance among regions — Lake Constance, Rhine and Danube — (mtDNA 26.3%; microsatellites 19.5%), whereas the between-population within-region variance component was relatively small (mtDNA 1.5%; microsatellites 4.75%). The largest portion of the variance was contained within populations (mtDNA 72.2%; microsatellites 75.75%).

Table 4 Estimates of F-statistics for mitochondrial DNA (below the diagonal) and F-statistics/R-statistics for microsatellite loci (above the diagonal) values between the four major geographical groups of *Lota lota*

	Lake Constance	Moselle River	Ipoly River	Isar River
Lake Constance	—	0.24/0.28	0.16/0.48	0.34/0.73
Moselle River	0.34	—	0.22/0.25	0.44/0.48
Ipoly River	0.15	0.75	—	0.23/0.23
Isar River	0.26	0.87	0.26	—

Regrouping individuals into mtDNA lineage groups (Danubian tributaries and Danubian Lake Constance vs. Rhine tributary and Atlantic Lake Constance), we obtained the maximum between-group variance with mitochondrial data (62.3%) with smaller among-population within-group (24.19%) and within-population (13.52%) values. When applying the same grouping scheme to the microsatellite data, the AMOVA analysis yielded a negative between-group value (–10.82%), larger among-population within-group (27.3%) and largest within-population (83.52%) values. Thus, the observed patterns would indicate that microsatellite alleles are not linked to mtDNA lineages.

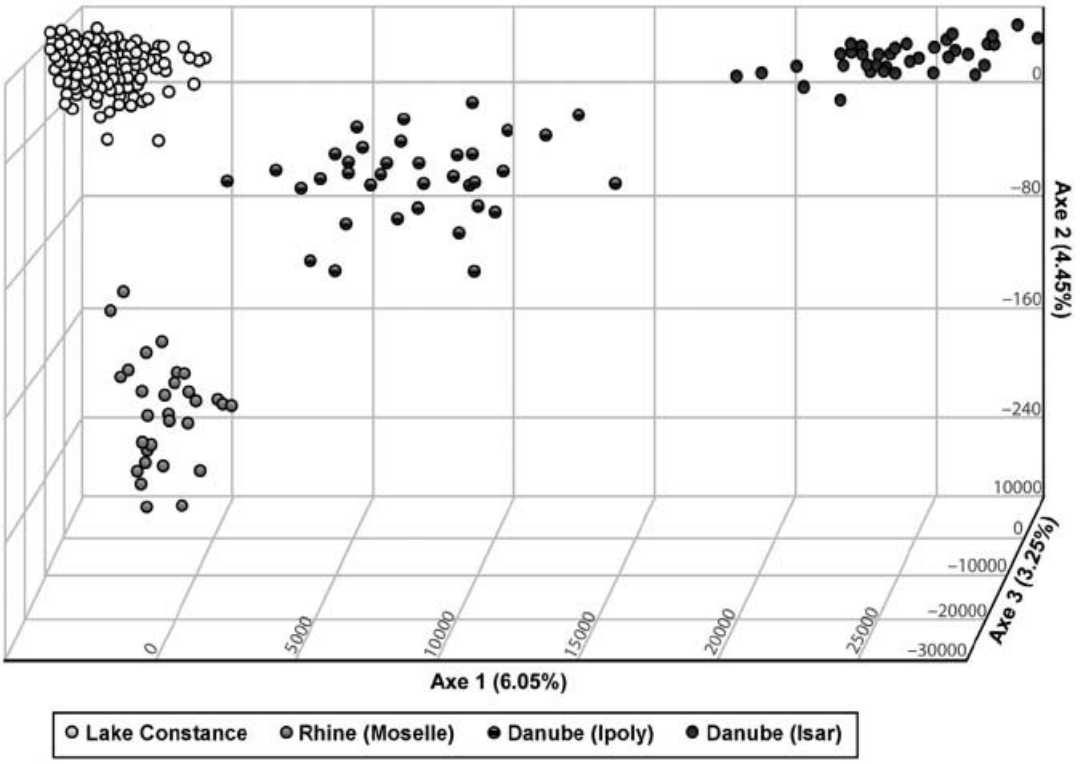

Fig. 4 Three-dimensional plot of microsatellite allele composition derived from a factorial correspondence analysis as implemented in GENETIX 4.5 graphically showing the degree of genetic separation of the individual burbot populations.

Microgeographical scale. No temporal differences were found in the juvenile burbot samples from the different sampling localities throughout the lake ($P > 0.1$). Therefore, all samples within localities collected in different seasons and years were pooled for further analyses. To examine whether Lake Constance harbours a single panmictic population of burbot or whether it contains multiple spawning sites, we clustered individuals according to their spatial distribution within the lake, including Lake Überlingen North, Lake Überlingen South, and Upper Lake (Fig. 1; Table 1). We observed a random distribution of mtDNA types and microsatellite alleles throughout the lake in adult and juvenile burbot throughout Lake Constance as shown by *F*-statistics ($P > 0.05$). Even the very sensitive exact tests on contingency tables of allele distributions failed to reveal any differentiation among the three regions ($P > 0.05$). Benthic juveniles in each sampling locality (relatedness; $b = 0.0018 \pm 0.01$) and larvae from the putative spawning site in Lake Überlingen ($b = 0.0066 \pm 0.02$) formed no kin aggregations ($P > 0.05$), and did not differ genetically from the adult population through the lake.

Bayesian clustering. We estimated the number of distinct populations contained in our burbot sample applying a Bayesian model-based clustering algorithm to the microsatellite data as implemented in the program STRUCTURE (Pritchard *et al.* 2000). The trend in log-likelihood probabilities [log $Pr(X \mid K)$] for each model of K ($K = 2$–8) indicated differentiated groups at $K = 4$–5 by these showing the lowest negative values of log-likelihoods with all the models implemented in STRUCTURE (results for the admixture model: –7645.2, –6993.3, –6373.9, –6462.7, –6576.3, –7071.2, –7303.2). Following the conservative approach suggested by Pritchard *et al.* (2000), which proposes that in cases of such 'likelihood plateaus' one should choose the smallest number of K, we take this as corroborating evidence for the presence of four distinct populations according to geography (Moselle, Ipoly and Isar rivers, and Lake Constance) in the microsatellite data. This analysis provided no traces of further substructuring within Lake Constance, also when only the samples for the lake where included in the analysis, with $k = 1$ showing the lowest negative values of log-likelihood ($K = 1$–5; log-likelihood = –10698.6, –10792.6, –10961.3, –11044.4, –11688.3, respectively).

Ancestral population histories and genetic admixture

The Lake Constance mtDNA haplotypes are derived from populations that inhabited the Rhine and Danube tributaries in the past and diverged allopatrically (Fig. 2a, b; see also Van Houdt *et al.* 2003, 2005). We suggest that these two lineages colonized Lake Constance soon after

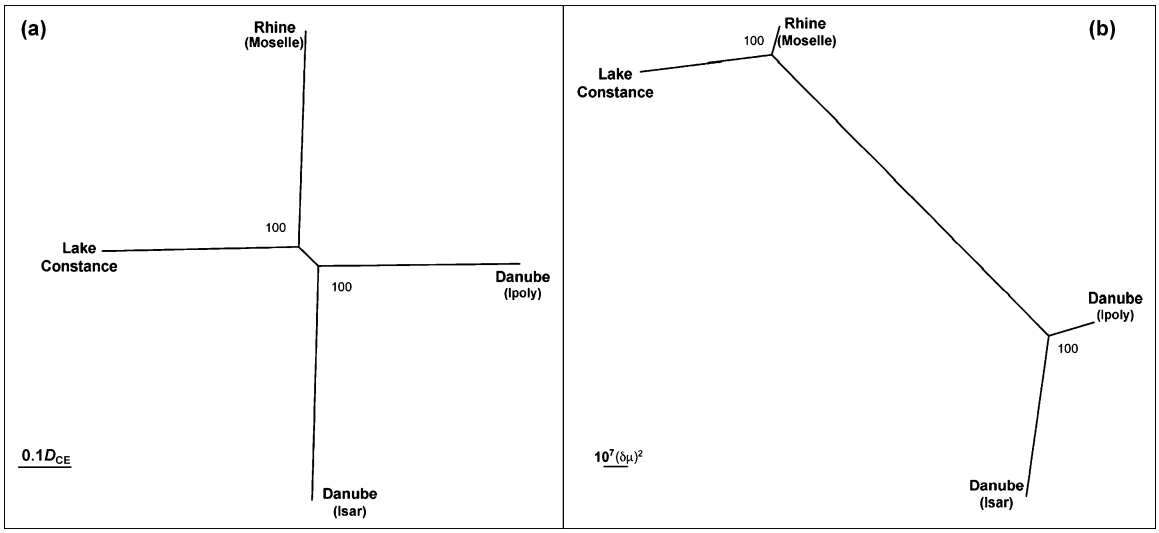

Fig. 5 Unrooted neighbour-joining trees of microsatellite data estimated with D_{CE} (a) and $(\delta\mu)^2$ (b) distances illustrating genetic relationships among burbot populations in Lake Constance and Danube and Rhine tributaries. Consistency tree topology was assessed by bootstrapping over loci with 1000 replicates, and bootstrap resampling percentages are depicted at branch points.

its formation and originated there a zone of secondary contact. Shared allele counts indicated that from a total of 231 alleles detected at the 11 loci combined, 165 occurred in Lake Constance, 167 in the Danube (Ipoly and Isar rivers) and 47 in the Rhine (Moselle River). Twenty-seven alleles were common to all regions. Sixty-four percent of the Danubian alleles were also found in the lake (106 alleles), and 77% of the alleles from the Rhine co-occurred in the lake (36 alleles). Thus there was considerable overlap of alleles between these populations. Several alleles were diagnostic of the Atlantic and the Danubian lineages (unique to the respective regions), approximately 60% of which were shared with the lake (9 Atlantic and 79 Danubian; see Fig. S1, Supplementary material). Still 30% of Lake Constance alleles (50 alleles, on average 4.5 per locus) were private and not detected in the other populations.

To evaluate the possibility that all these private alleles could have arisen *de novo* in the lake, we performed population genetic simulations with different mutation rates and mutation models assuming interrupted gene flow between populations. After 4000 generations, populations were in mutation-drift equilibrium, as indicated by the quantities H_O, H_S, H_T, and F_{IS}. The mean numbers of private alleles per locus estimated for a mixed model of single-step mutations with 0.2% double-step mutation events and mutation rates 0.001 and 0.0001 were 3.80 (± SD = 0.57) and 0.47 (± SD = 0.12), respectively. Under the assumption of 0.2% multistep mutation events (as 29% of mutations reported in zebrafish involved more than 5 repeat units (Shimoda *et al*. 1999)), simulations for mutation rates 0.001 and for 0.0001 yielded 8.22 (± SD = 0.95) and 2.56 (± SD = 0.37) private alleles per locus, respectively. The observed value of 4.5 was significantly larger than all simulated two tailed *t*-tests values ($P < 0.0001$) when the lower mutation rates of 0.0001 were used. With the higher mutation rate of 10^{-3} and the possibility of multistep mutations, the simulated value was significantly larger than the observed one ($P = 0.0012$). The only estimates that did not produce significantly different results were with mutation rate 10^{-3} and 0.2% double-step mutations ($P = 0.23$). F_{ST} values in the simulation studies ranged from 0.01 to 0.04 for mutation rates 10^{-3} and from 0.06 to 0.10 for mutation rates 10^{-4}.

Unrooted NJ trees based on microsatellites revealed similar distances among the four major populations when the drift-based chord distance, D_{CE}, was used (Fig. 5a). By contrast, closer relationship between Lake Constance and the Rhine system (Moselle River) was found using the mutation-based estimator $(\delta\mu)^2$. Also, in this tree the two Danube populations (Ipoly and Isar rivers) were grouped together with 100% bootstrap support (Fig. 5b). The second approach appears more appropriate for microsatellite loci considering their high mutation rates.

Since both the Bayesian model-based clustering algorithm and the factorial correspondence analysis failed to detect significant signs of admixture in our microsatellite data, which would be expected for recent hybridization events, we used a coalescence approach. Coalescence methods are particularly intended to extract information in cases when admixture events are relatively old, and present-day admixture proportions are likely to differ from those at the time of hybridization. Genetic admixture proportions of Rhine and Danube lineages in Lake Constance were estimated using a coalescence method as implemented in ADMIX version 2.0. We assumed a simple hybridization

model for the secondary contact zone in Lake Constance, in which the two parental populations (Western European and Danubian lineages) expanded initially, and after a number of generations gave rise to a single panmictic population (see Choisy *et al*. 2004). We consider that the burbot population in Lake Constance was established ~10 000–15 000 BP. Conventional admixture proportion estimates (those based on allele frequencies only not taking into account the molecular divergence between alleles) using the program ADMIX, revealed almost equal contribution of the putative parental populations following the historical admixture event for Moselle, Ipoly and Isar rivers of 0.32 (± SD = 0.002), 0.31 (± 0.001), and 0.37 (± 0.002), respectively. Calculating the admixture coefficient (*mY*) as introduced by Bertorelle & Excoffier (1998), which considers the degree of molecular divergence between alleles in the form of $(\delta\mu)^2$, we obtained fairly different results indicating a three-quarter bias in the genetic contribution towards the Rhine tributary (Moselle River $mY = 0.75 \pm 0.077$). The parental contributions of the Danube populations were estimated for Ipoly and Isar, $mY = 0.17 \pm 0.227$ and 0.08 ± 0.165, respectively. This result is consistent with the NJ tree using a mutation-based distance showing a closer relationship of the Rhine system to Lake Constance populations.

Discussion

Colonization of Lake Constance from two European glacial refugia

Several mtDNA phylogeographic lineages have been described in European burbot, whose origin predates the last glacial period (Van Houdt *et al*. 2003, 2005), similar to many other European freshwater fish studied so far (e.g. Durand *et al*. 1999; Nesbø *et al*. 1999; Englbrecht *et al*. 2000; Kotlík & Berrebi 2001; Salzburger *et al*. 2003; Gum *et al*. 2005). In Lake Constance, we found the co-occurrence of two clearly differentiated mtDNA lineages, which are separated by four mutations, and lack intermediate haplotypes between them. Those two lineages correspond to two of the lineages proposed by Van Houdt *et al*. (2005), Western European and Eurasian, which confirms the colonization of the lake by fish from two different geographical regions. This dual geographical and genetic origin of the Lake Constance burbot is reflected in the surprisingly high genetic diversity (almost 50% of the total European diversity described, see Fig. 2), considering the young age of the lake itself (~10 000–15 000 BP). This genetic diversity could, for the most part, not have evolved *in situ* in the lake. Interestingly, Danube mtDNA haplotypes sit in the centre of the network, linking the two Lake Constance lineages (Fig. 2b), suggesting that the Danube might have acted as the most ancestral refuge and reservoir of genetic diversity. Also Van Houdt *et al*. (2005) reported the most diverse populations of burbot in the Danubian area. These findings are in accordance with the previously reported importance of the Danubian refuge for the survival of the European freshwater fauna during the glacial periods (e.g. Nesbø *et al*. 1999; Bernatchez 2001; Kotlík & Berrebi 2001; Salzburger *et al*. 2003; Gum *et al*. 2005; Van Houdt *et al*. 2005).

Based on the genealogical relationships of mtDNA sequences, one of the two Lake Constance burbot lineages appears to be directly derived from Danubian ancestors, while the second one is associated with samples from Western Europe (Atlantic lineage). The Danubian lineage in Lake Constance displays rather low genetic diversity (one central haplotype shared by most individuals and five one-step haplotypes found in very few individuals; Fig. 2a), a pattern that is indicative of a recent population expansion as shown also by a coalescence-based mismatch analysis (Fig. 2c). Therefore, it seems likely that all the diversity of this lineage (on average, less than one mutation) originated after the colonization of Lake Constance within the last ~10 000–15 000 years. These estimations suggest that the upper Danube was only briefly connected to Lake Constance at the end of the last glacial period, probably through periglacial temporal ponds, which permitted the mtDNA contribution of the Danubian lineage to the current burbot population in Lake Constance.

In contrast, the Atlantic lineage in Lake Constance is surprisingly diverse in mtDNA haplotypes, particularly when compared to burbot samples from Western Europe (Rhine, Seine and Meuse rivers; France, Holland), which are genetically homogeneous. This homogeneity could be an artefact caused by insufficient sampling or by a selective sweep, but bottlenecks, such as those detected in the Rhine and Isar rivers from microsatellite data, would suggest a generalized reduction of genetic diversity in Western European rivers. Similar patterns of low genetic diversity were observed in previous studies of burbot from additional Western European sites (Italy, Switzerland, France, the Netherlands and Denmark; Van Houdt *et al*. 2005), although for these samples only half of the control region is available rendering difficult direct comparisons. Given that the maximum distance among Atlantic haplotypes in Lake Constance is seven mutations, this diversity is unlikely to have evolved in the short time period since the appearance of the lake (10 000–15 000 BP). A demographic analysis suggests that this lineage experienced a major expansion about two mutations ago that could correspond to the last interglacial maximum about 130 000 BP. Demographic analyses based on nuclear markers failed to find evidence of population expansion in the lake. This result could be the consequence of burbot being of mixed ancestry in the lake and the extensive admixture of the two original lineages. It is further known that variation in the mutation rates across loci negatively affects the power of this kind of analysis (see Discussion in Reich *et al*. 1999; Goldstein *et al*. 1999).

The above considerations imply that an Atlantic glacial refuge for burbot existed in addition to the well-supported Danubian refuge. Thus, the present Atlantic lineage was able to survive isolated from the Danubian populations throughout the last glacial period. This inference is supported by the observation that there is no overlap in all of Europe between Danubian and Atlantic haplotypes except for the unique secondary contact zone in Lake Constance. Considerable overlap of microsatellite alleles also corroborates the idea that burbot from Danubian and Atlantic tributaries constituted the source populations of Lake Constance. An alternative hypothesis would be that a polymorphic ancestor sharing the genetic composition from both present-day Atlantic and Danubian populations would have colonized Lake Constance. This would require extensive loss of mtDNA haplotypes and microsatellite alleles in the lake, and most likely also in the locality of origin, since no such polymorphic population has been found so far, neither in the Danube nor in the Atlantic area.

In recent years, there has been growing evidence favouring the existence of generalized northern glacial refugia in Europe in addition to those on the Mediterranean peninsulas (Stewart & Lister 2001; Hewitt 2004), particularly for freshwater fish (Durand *et al*. 1999; Nesbø *et al*. 1999; Englbrecht *et al*. 2000; Bernatchez 2001; Kotlík & Berrebi 2001; Hänfling *et al*. 2002; Weiss *et al*. 2002; Salzburger *et al*. 2003; Van Houdt *et al*. 2003, 2005; Barluenga & Meyer 2005; Gum *et al*. 2005). However, the exact locations of Atlantic refuge areas are far from being thoroughly understood. The Western European refuge of burbot could have been located not far from the Alpine area (perhaps in southern France), given the large Atlantic diversity we find in Lake Constance. Alternatively, burbot could have taken refuge in a brackish sea in the Atlantic Ocean, considering that at present, some burbot populations inhabit brackish areas in the Baltic Sea, staying there for their whole lifespan (Pulliainen *et al*. 1992). Only a more exhaustive sampling of Central and Western Europe could confirm these hypotheses.

A two-step colonization of Europe

The most ancient European fish populations have repeatedly been found in association to the Danubian area (Durand *et al*. 1999; Nesbø *et al*. 1999; Englbrecht *et al*. 2000; Bernatchez 2001; Kotlík & Berrebi 2001; Salzburger *et al*. 2003; Gum *et al*. 2005), confirming the general idea that the Danube constituted the most ancestral glacial refuge for European freshwater fish. It is widely accepted that fish from the Danubian refuge dispersed along Central, West and North Europe following the retreat of the ice during the warm periods (e.g. Durand *et al*. 1999; Nesbø *et al*. 1999; Englbrecht *et al*. 2000; Bernatchez 2001). The generalized absence of pronounced genealogical differentiation between populations throughout Europe (usually sequence divergence below 0.5%) agrees with this scenario. But for many species, geographical patterns within Europe are more complex and indicate multiple vicariance events (e.g. Durand *et al*. 1999). In particular, for many species an Atlantic and a Danubian region can be delimited on the basis of differentiated mtDNA haplotype composition (e.g. Durand *et al*. 1999; Bernatchez 2001; Kotlík & Berrebi 2001; Gum *et al*. 2005). According to these observations Durand *et al*. (1999) proposed a two-step expansion hypothesis for the chub, suggesting that the Danubian lineage extensively colonized Central and Western Europe during the Riss-Würm interglacial (~100.000 BP), and survived the next glacial period in Western European rivers, such as Rhine and Rhone. At the end of the Würm period (~10 000 BP), range expansions took place both from the western stocks into all Atlantic drainages, and from the Danube refuge into the rest of Europe, thus permitting secondary contact among refugial lineages. The genetic patterns described in this study (e.g. demographic population histories, levels of polymorphism) and by Van Houdt *et al*. (2003, 2005) in European burbot populations support the validity of the two-step colonization hypothesis also for this fish species. The main Central European rivers (Vistula and Elbe) appear to have been colonized from the Danube refuge in the most recent expansion given the large genetic similarity between these river systems. Russia and later North America could have been colonized also from the Danube (Fig. 2b).

Geographical areas that have been only recently colonized and did not serve as long-term refugia are typically genetically less diverse (e.g. Hewitt 1996, 2004), which applies to many Atlantic and Scandinavian populations of European freshwater fishes (e.g. Durand *et al*. 1999; Kotlík & Berrebi 2001). In this regard, the observed high levels of polymorphism of the Atlantic lineage in Lake Constance appears to be an exception, but again stresses that northern refugia contributed genetically to present Central European populations. Part of this polymorphism could have disappeared only in recent years from the Atlantic area due to bottlenecks, as measured in the Rhine River with nuclear markers, caused by pollution and alterations of the river beds. Scandinavian populations are good examples of the postglacial expansion from northern refugia in several fish species (burbot, present study; grayling and bullhead; Hänfling *et al*. 2002). Interestingly, our results indicate that Scandinavia was colonized by burbot mainly from an Atlantic refuge, although the Danube refuge appears to have contributed as well to its present population, which is contrary to previous analyses (Van Houdt *et al*. 2003, 2005; Fig. 2b).

According to the two-step expansion hypothesis, areas of secondary contact of the major phylogeographic fish lineages are found in the same Central European areas. Similarly to burbot, western and eastern lineages of perch, brown trout, barbel and grayling met in the area of Lake Constance (Nesbø *et al*. 1999; Bernatchez 2001; Kotlík &

Berrebi 2001; Behrmann-Godel *et al*. 2004; Gum *et al*. 2005). Additional suture zones where chub, barbel and grayling lineages converge are the Weser and Elbe rivers (Durand *et al*. 1999; Kotlík & Berrebi 2001; Gum *et al*. 2005).

Common areas of secondary contact of distinct glacial races of fish have also been reported for the Nearctic region (e.g. Danzmann *et al*. 1998; Turgeon & Bernatchez 2001; Fraser & Bernatchez 2005; Gagnon & Angers 2006). In eastern North America, the freshwater systems in area of Québec have been extensively studied, and appear to have been colonized from multiple refugia by several fish species, following postglacial river flow reversals and temporary water connections (Wilson & Hebert 1998; Turgeon & Bernatchez 2001; Fraser & Bernatchez 2005; Gagnon & Angers 2006). Mitochondrial polymorphisms clearly revealed different glacial origins in *Coregonus artedi* (Turgeon & Bernatchez 2001) and *Perca flavescens* (Gagnon & Angers 2006). In other fish species like *Salvelinus fontinalis* mitochondrial races were not polymorphic (Danzmann *et al*. 1998; Fraser & Bernatchez 2005), although microsatellites confirmed the different refugial origin (Fraser & Bernatchez 2005). For both species, microsatellites proved extensive admixture between the mitochondrial lineages (Turgeon & Bernatchez 2001; Fraser & Bernatchez 2005). Aquatic contact zones between divergent lineages have also been reported in western North America, where major glacial refugia have been identified (see e.g. Redenbach & Taylor 2002).

Genetic admixture of Atlantic and Danubian populations in Lake Constance

The large correspondence between Lake Constance and the Atlantic and Danubian mtDNA haplotypes, which clearly shows that the latter regions harbour the most likely source populations, is not entirely corroborated by the nuclear composition. This is because the microsatellite allele contribution was not intermediate between the two regions but rather distinct (Figs 4 and 5). The discrepancy between the two molecular markers indicates that the admixture between the two burbot lineages in the lake is relatively old, and hence, the mixing signature is relatively weak. The three populations have evolved separately for thousands of generations and have consequently accumulated a notable amount of genetic differences which is more pronounced at the microsatellite loci due to their relatively higher mutation rates compared to mtDNA (Frankham *et al*. 2002). After the initially allopatric populations came into secondary contact, gene flow and recombination diluted the allelic Danubian and Atlantic signature along with the evolution of new alleles (given the large proportion of private alleles we found in the lake), which resulted in the previously divergent lineages looking more similar within the contact zone than in surrounding areas. Individuals in Lake Constance with mtDNA haplotypes of Atlantic and Danubian ancestry showed no differentiation at the nuclear genome, at least measured by microsatellites, which could only be explained by free interbreeding between the two lineages.

Simulations of the dynamics of microsatellites show that after 4000 generations of isolation (the age of the lake) and with a mutation rate of 10^{-3}, an estimate of the number of private alleles per locus could be produced that was not significantly different from the one observed in Lake Constance. However, using different mutation models and rates, these values varied considerably with those simulations that allowed for multistep mutations yielding the largest numbers of private alleles. Bearing in mind that multistep changes have been recorded in zebrafish in almost 30% of microsatellite mutations (Shimoda *et al*. 1999), the role of mutation for postglacial differentiation needs to be considered carefully. Other factors can also increase the number of private alleles in a population, such as sudden expansions and bottlenecks, which could not be included in the simulation studies. Some of the private alleles in Lake Constance might have existed in the Rhine and other Atlantic populations, although they have disappeared recently due to the demonstrated bottlenecks. This would explain the substantially lower F_{ST} (0.01–0.10) in the simulation study, which was generated under constant population size. Another possibility would be that those rare alleles have not been found in the present samples, but further sampling of both Atlantic and Danubian populations would reveal them.

Two different estimates for admixture proportions of the Atlantic and Danubian lineage as genetic sources of the Lake Constance population were obtained from the microsatellite data. While the nuclear parental contributions of the two were very similar using conventional admixture proportions based on allele frequencies, the coalescence approach, particularly designed to extract information when present-day admixture estimates differ to those at the time of hybridization, was more in accordance with the mtDNA data. With the latter method, the proportion of nuclear genes at the time of admixture coming from the Danubian lineage was estimated to be about three times smaller than that from the Atlantic lineage, as shown by the admixture coefficient (mY). The distance-based reconstruction of population inter-relationships from microsatellites also indicated that the burbot population of Lake Constance is genetically closer to the Rhine than to the Danube system (Fig. 5b). This result is consistent with the above-mentioned hypothesis that the upper Danube and Lake Constance were only shortly connected at the beginning of the present warm period. The larger genetic introgression of the Atlantic lineage into the Lake Constance population points to a more prolonged connection. At present, the Rhine is flowing through Lake Constance, although the lake has become disconnected from the lower Rhine tributary by a large waterfall after the last glaciation, thereby preventing upstream migration of fish (see also Paragamian *et al*. 1999 for the

effect of waterfalls on the dispersal of burbot). However, the maintenance of a long-term connection between Rhine and Lake Constance might have existed when large masses of melt water filled the upper Rhine valley only slowly draining northwards into the lower Rhine valley. It is noteworthy that the successful colonization of Lake Constance by Atlantic burbot stands in contrast to the hypothesized colonization by Eurasian perch mainly (or exclusively) through the Danube connection (see Behrmann-Godel *et al*. 2004). Only a more comprehensive approach including samples of different species from the same sites throughout Central Europe would help to reconstruct the postglacial flow of genetic lineages.

Widespread panmixia of burbot in Lake Constance

Lake Constance harbours a single panmictic population of burbot, as indicated by the random distribution of mitochondrial haplotypes and microsatellite alleles throughout all geographical sites included in this study in both adults and juveniles. Moreover, we found no association between particular mitochondrial and nuclear types that would evidence preferential matings within the lake. All fish apparently move freely in the lake without experiencing barriers to dispersal leading to assortative mating.

Adult burbot are bottom-dwellers in deep areas, and the analysis of population genetic structure detected no differentiation between geographically distant sampling sites. This implies that no major barriers exist for the dispersal of adult burbot in this environment. In winter, burbot aggregate in common spawning areas, often migrating long distances (see Slavík & Bartos 2002). In Lake Constance, at least one spawning site has been identified (Fischer 1999; Miller & Fischer 2004), and larvae collected from this site contained the entire genetic diversity found throughout the lake, and showed low levels of relatedness. This result shows that within the lake, either all individuals meet in one single spawning site and interbreed freely, or, alternatively, that a genetically random proportion of individuals meet in different spawning sites. Both scenarios would result in complete admixture. Finally, juvenile burbot, which are concentrated in the shallow areas of the lake associated to stony littoral habitats (Fischer 2000), are not significantly related and do not form kin aggregations, and geographically distant juvenile populations do not show any genetic substructuring within Lake Constance.

None of the life-history characters considered here upholds the divergence of burbot populations within Lake Constance, but rather support their uniformity. The only effective barrier for gene flow described so far for burbot populations within a single water system is a physical obstacle, the waterfalls in the Kootenai River basin, North America, which separates two genetically differentiated populations (Paragamian *et al*. 1999). However, population structure of burbot could also come from the coexistence of populations with different life history strategies. Related to the winter migration to common spawning sites, four different strategies have been described in burbot: (i) *lacustrine*, fish that stay their whole life within a lake and migrate for spawning within the lake (Bailey 1972; Bernard *et al*. 1993; Carl 1995; Paragamian *et al*. 1999); (ii) *fluvial*, fish that reside in a river their whole life and migrate within the river or associated tributaries for spawning (Bresser *et al*. 1988; Paragamian *et al*. 1999); (iii) *brackish*, fish that inhabit sea environments and migrate within this habitat for spawning (Pulliainen *et al*. 1992); and (iv) *adfluvial*, fish that dwell in lacustrine or brackish habitats and migrate into rivers for spawning (Sorokin 1971; Müller & Berg 1982; Paragamian *et al*. 1999). The presence of populations with different life history strategies in single geographical areas (see Paragamian *et al*. 1999) might result in differentiated populations. In Lake Constance, adfluvial populations associated to some rivers draining into the lake could be genetically differentiated from the typical lacustrine form.

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Supplementary material

The supplementary material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC3045/MEC3045sm.htm

Fig. S1 Diagram showing the number (and proportion from the total) of microsatellite alleles that are unique to each of the studied populations, and shared among them. Lake Constance has a total of 165 alleles: 50 of them unique to the lake, 27 common to all three regions (lake, Rhine and Danube rivers), 9 Rhine alleles (unique to the lake and Rhine river), and 79 Danubian alleles (unique to the lake and Danube tributaries).

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