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Microsatellite and mitochondrial DNA assessment of population structure and stocking effects in Arctic charr *Salvelinus alpinus* (Teleostei: Salmonidae) from central Alpine lakes

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Abstract

Despite geographical isolation and widespread phenotypic polymorphism, previous population genetic studies of Arctic charr, *Salvelinus alpinus*, have detected low levels of intra- and interpopulation variation. In this study, two approaches were used to test the generality of low genetic diversity among 15 Arctic charr populations from three major drainages of the central Alpine region of Europe. First, a representative subsample of each drainage was screened by PCR–RFLP analysis of mtDNA using 31 restriction enzymes. All individuals but one shared an identical haplotype. In contrast, microsatellite DNA variation revealed high levels of genetic diversity within and among populations. The number of alleles per locus ranged from six to 49, resulting in an overall expected heterozygosity from 0.72 ± 0.09 to 0.87 ± 0.04 depending on the locus. Despite evidence for fish transfers among Alpine charr populations over centuries, genetic diversity was substantially structured, as revealed by hierarchical Φ statistics. Eighteen per cent of total genetic variance was apportioned to substructuring among Rhône, Rhine, and Danube river systems, whereas 19% was due to partitioning among populations within each drainage. Cluster analyses corroborated these results by drainage-specific grouping of nonstocked populations, but also revealed damaging effects of stocking practices in others. However, these results suggest that long-term stocking practices did not generally alter natural genetic partitioning, and stress the importance of considering genetic diversity of Arctic charr in the Alpine region for sound management. The results also refute the general view of Arctic charr being a genetically depauperate species and show the potential usefulness of microsatellite DNAs in addressing evolutionary and conservation issues in this species.

Keywords: Arctic charr, conservation, microsatellite DNA, mtDNA, population genetics, *Salvelinus alpinus*

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Introduction

The distribution, diversity, and genetic structure of northern biota have been considerably influenced by the repeated coverage of northern Europe and central mountain ranges (e.g. Alps, Pyrenees) by Pleistocene ice sheets (Hantke 1978). These dramatic climate oscillations undoubtedly resulted in habitat and range alterations by forcing species into suitable refugia during colder conditions and allowing recolonization and dispersal during warmer interglacials. Hewitt (1996) considered these processes instrumental to Quarternary population divergence in northern temperate regions.

The Arctic charr, *Salvelinus alpinus* L. (Teleostei; Salmonidae), has long intrigued biologists as a prime example for population differentiation in northern fishes. This freshwater and anadromous fish has a Holarctic

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distribution (Behnke 1972; Johnson 1980), and exhibits a complex mosaic of variability in morphology, colouration, ecology, and life-history traits (Hindar & Jonsson 1982; Skúlason *et al*. 1989; Malmquist *et al*. 1992). Paradoxically, most population genetic studies of Arctic charr have failed to detect significant genetic differentiation among morphs, distant populations, and even subspecies, using allozymes (Kornfield *et al*. 1981; Andersson *et al*. 1983), restriction fragment length polymorphism (RFLP) and sequence analysis of mitochondrial DNA (mtDNA) (Danzmann *et al*. 1991; Hartley *et al*. 1992; Volpe & Ferguson 1996), and even minisatellite fingerprinting (Volpe & Ferguson 1996). Although Wilson *et al*. (1996) documented the existence of three mtDNA phylogeographic assemblages among North American populations, they similarly observed extremely low levels of mtDNA polymorphism. Generalized low levels of genetic variation within species have often been associated with recent and rapid recolonization from bottlenecked refugial populations (e.g. Sage & Wolff 1986; Billington & Hebert 1991; Zink & Dittman 1993).

The central European Alps are of particular interest to the evolutionary history of Arctic charr for several reasons. Many Alpine lakes shelter sympatric and allopatric morphs that differ strikingly in morphological features, growth rate, feeding and water depth preferences (Brenner 1980, and references therein). This region was also completely covered by ice during the last glaciation. As a result, all lakes within this area have similar ages of about 10 000–20 000 years (Hantke 1978), which enables comparisons of rates of population differentiation both among and within lakes. Finally, all lakes containing native Arctic charr populations belong to one of three unconnected major river systems which drain into separate sea basins. This provides a situation in which contemporary drainage subdivision and historical isolation can be inferred.

Genetic studies of Arctic charr from the central Alpine region of Europe have been very limited, and involved allozyme analysis (Ruhlé 1977; Hecht 1984). Consequently, one objective of this study was to perform a PCR–RFLP analysis to test the hypothesis that central Alpine charr populations are similar to those from other regions, in possessing low levels of mtDNA variation. A second objective was to assess the potential of microsatellite DNA analysis to detect genetic variation in Arctic charr from the European Alps by comparison among drainage systems, among lakes within drainages, and among sympatric populations within lakes. Using microsatellite DNA has been effective in detecting variation in other species with low levels of either allozyme or mtDNA polymorphism (e.g. Hughes & Queller 1993; Taylor *et al*. 1994), and has also been used to address issues related to population structure in other *Salvelinus* species (Angers *et al*. 1995; Angers & Bernatchez 1996). Finally, by obtaining high-resolution genetic markers for these populations, it was possible to examine the genetic impacts of past stocking practices by comparing relationships of stocked populations with native ones.

Materials and methods

Sampling

A total of 440 Arctic charr was collected from 12 lakes of the central Alpine region of Europe, representing 15 populations from the Rhine, Danube, and Rhône river drainages (Fig. 1). All fish were caught over traditional spawning sites to take into account possible substructures within lakes due to reproductively isolated populations. An additional population from the Lake Saimaa system, Finland, was included in the clustering analyses as an outgroup. On the basis of classical morphology and zoogeography Behnke (1980, 1984) recognized Arctic charr from northern Europe as *Salvelinus a. alpinus*, distinct from the central Alpine *S. a. salvelinus*. Liver samples were recovered from freshly killed or deep-frozen specimens and preserved in 95% ethanol. Total DNA was isolated from liver tissue as described in Bernatchez *et al*. (1992).

Mitochondrial DNA

Mitochondrial DNA variation was analysed by RFLP performed on products amplified via PCR (Saiki *et al*. 1988). Two adjacent segments were amplified; one encompassing the complete NADH dehydrogenase subunits 5 and 6, the other, the cytochrome *b* gene and the control region. Primers and PCR conditions were described in Bernatchez & Danzmann (1993) and Bernatchez *et al*. (1995).

Ten individuals from each of five populations representing the three drainage systems were screened by digesting pooled PCR aliquots of the two amplified segments with 31 restriction enzymes (Table 1). The resulting fragments were separated by horizontal electrophoresis in 1% agarose gel and sized by using *Hin*dIII/*Eco*RI-digested λ phage DNA as a molecular-weight standard. Mitochondrial DNA fragments were visualized and photographed under UV light after ethidium bromide staining.

Microsatellite DNA

Eighty fish representing 10 Arctic charr populations were first screened at 16 loci to examine the potential of corresponding primers originally developed for other salmonids. Six primers producing microsatellite bands that could be unambiguously determined were then selected for final analysis. A summary of all loci tested, primer sources, and annealing temperatures is provided in Table 2.

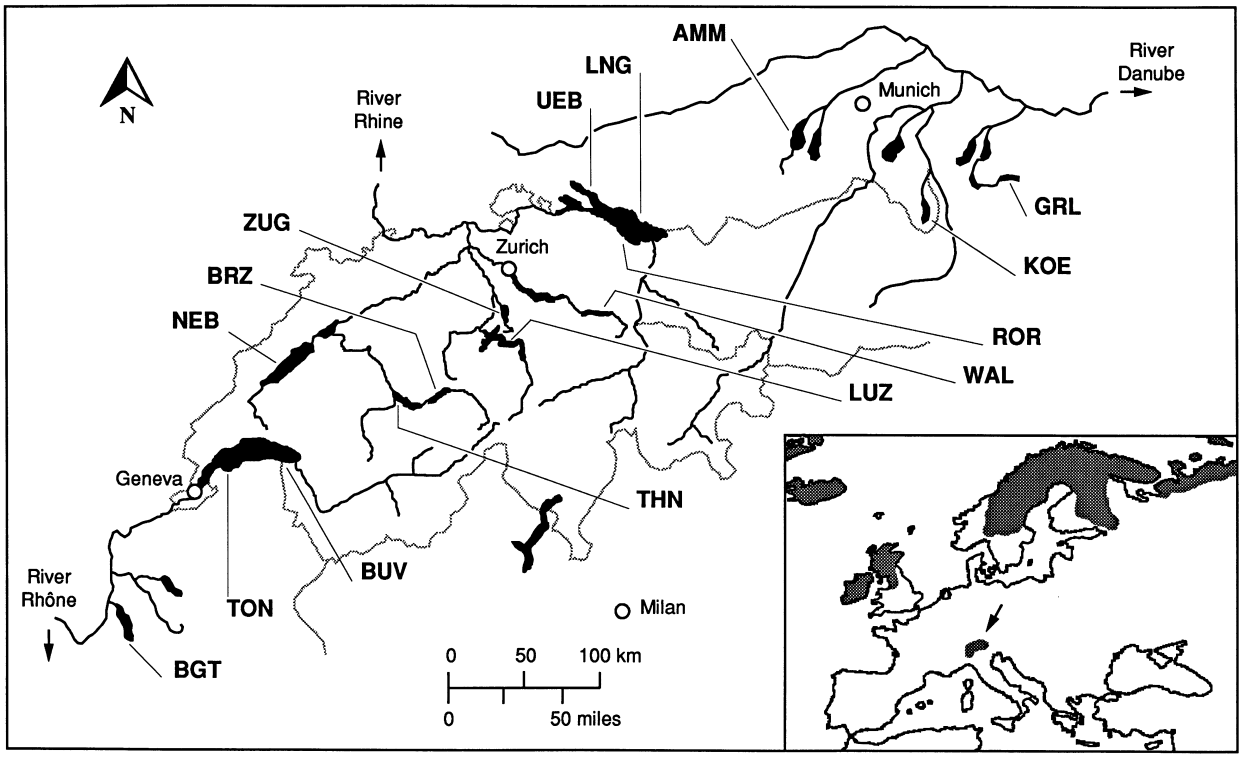


Fig. 1 Sampling localities of *Salvelinus a. salvelinus* in the central Alpine region, with insert of European range for the species. Names and locations of sampling sites are listed in Table 3. Dotted lines denote country borders.

PCR reactions were set up in 15-μL volumes, each containing 50–100 ng of total DNA, 10 pmol of each primer, 75 mM of each dNTP, 1.5 mM $MgCl_2$, 1× *Taq* buffer and 0.25 units of *Taq* polymerase. Analyses were carried out using either end-labelled primers [γ-^{32}P]-ATP or direct incorporation of [α-^{35}S]-ATP. Amplifications were performed in a Perkin Elmer thermal cycler (model 480), and consisted of an initial 3 min denaturation step at 95 °C, 15 cycles of 60 s at 94 °C, 45 s at the annealing temperature, and 10 s extension at 72 °C, followed by 20 additional cycles of 30 s at 94 °C, 30 s at the annealing temperature, and 10 s at 72 °C. PCR products were separated on a standard 6% acrylamide sequencing gel and autoradiographed. Alleles were sized by comparison with the standard M13 sequence.

Data analysis

Genetic polymorphism for each population was estimated as the mean number of alleles per locus (A), observed heterozygosity (H_O), and expected heterozygosity from Hardy–Weinberg assumptions (H_E), using the GENEPOP package version 2.0 (Raymond & Rousset 1995). An option implemented in the same package also allowed us to test specifically for heterozygote deficiency. Pairwise intra- and interdrainage differences in H_E and A were assessed by Wilcoxon's signed rank test (Snedecor & Cochran 1978). This test was performed after the number of alleles had been adjusted for a common sample size of 30/population and 150/drainage using equation 11 in Ewens (1972). All statistical pairwise comparisons of diversity were corrected by applying table-wide significance levels using a sequential Bonferroni test (Rice 1989).

GENEPOP was also used to assess heterogeneity in allele frequencies among pairwise comparisons of samples, and to test for deviations from Hardy–Weinberg equilibrium within population for every locus and over all loci. Significance values were estimated with a Markov chain method to obtain unbiased estimates of the exact Fisher test through 1000 iterations (Guo & Thompson 1992). The distribution of microsatellite DNA diversity was quantified using the analysis of molecular variance model (AMOVA) that expresses genetic correlation measures as Φ statistics (Excoffier *et al*. 1992). The Φ statistics are analogous to the F statistics of Cockerham (1969) but also incorporate variance in allele size between pairs of genes (Michalakis & Excoffier 1996). Components of genetic variance were computed at three hierarchical levels. Among drainage systems, Φ_{CT} is defined as the correlation of random haplotypes within a group of populations, relative to that of random pairs of haplotypes drawn from the whole species. Among populations

Table 1 PCR–RFLP analysis of mtDNA variation in Alpine *Salvelinus a. salvelinus*. Listed are restriction enzymes used to screen 50 fish from five populations, and generated fragment sizes (bp). Fragments less than 300 bp have not been considered. The *Ava*I B haplotype was only observed in one individual from Lake Walenstadt

Enzymes	Fragment sizes					
*Acc*I	2400	1600	520	420		
*Alu*I	1080	700	590	530	480	380
*Apa*I	2400	2200				
*Ava*IA	1880	840	660	520	500	
*Ava*IB	2400	–	840	660	–	500
*Ava*II	1880	530				
*Ban*I	2400	1780	470			
*Ban*II	1600	900	770	380		
*Bgl*I	2400	1250	1100			
*Bsp*1286I	890	740	540	420	320	
Bst EII	2400	2300				
*Cfo*I	900	840	740	480		
*Cla*I	2300	2200				
*Dde*I	680	600	560	380		
*Dra*I	2130	830	600	530		
*Eco*RV	2500	2300				
*Hae*II	1690	1210	1140	560		
*Hae*III	1080	620	560			
*Hin*cII	2180	960	880	530		
*Hin*dIII	2300	1320	1280			
*Hin*fI	1400	660	510	420		
*Mbo*I	1030	950	840	420	370	320
*Mbo*II	1120	870	650	400		
*Msp*I	1340	710	580			
*Nci*I	2500	1070	640			
*Nco*I	2500	2200				
*Nhe*I	2500	2300				
*Pst*I	2500	2300				
*Pvu*II	2300	1140	830	580		
*Rsa*I	1370	1020	860	510	450	
*Sma*I	2400	2300				
*Taq*I	1070	980	900	560	500	

within drainages, Φ_{SC} is the correlation of random haplotypes within populations, relative to that of random pairs of haplotypes from the drainage. Finally, for the within-populations analysis, Φ_{ST} is the correlation of random haplotypes within populations, relative to that of random pairs of haplotypes drawn from the whole species (Excoffier *et al*. 1992).

Methods to quantify population differences based on microsatellite DNA polymorphism are still debated. Therefore, relatedness among populations was estimated comparing several genetic distances. Nei's genetic distance based on the infinite isoalleles neutral mutation model (IAM; Nei 1972) and chord distance (Cavalli-Sforza & Edwards 1967) assuming pure genetic drift were computed for all pairwise comparisons of populations using PHYLIP version 3.57c for the Macintosh (Felsenstein 1995). The $(\delta\mu)^2$ genetic distance (Goldstein *et al*. 1995) was computed using MICROSAT 1.5 (Minch 1996). This distance takes into account size differences between alleles and fits linearity with time better than IAM-based distance measures when microsatellite DNA follows a strict stepwise mutation model (SSM). All resulting distances were clustered using the UPGMA algorithm. Significance of the correlation between different distance matrixes was assessed using a Mantel test (Mantel 1967). Interpopulation relationships were also estimated by character-based analysis using the presence–absence matrix of alleles to construct phylogenetic trees under the parsimony criterion with the branch-and-bound option of PAUP 3.0 (Swofford 1990). Robustness of tree topologies based on all distances and character approaches was assessed through 1000 bootstrap replications. To test for stocking impact, these relationships were computed separately for all 15 populations, and for 12 populations after exclusion of stocked Rhine drainage populations NEB, WAL, and ZUG.

Results

Mitochondrial DNA variation

Extremely low polymorphism was detected by the PCR–RFLP analysis of mtDNA variation. Twenty-three of the 31 tested enzymes generated restriction fragments with a cumulative average of 63 sites recognized per fish, and representing approximately 2.5% of the total mitochondrial genome. The cytochrome *b*/*D*-loop segment was monomorphic for all enzymes, whereas the ND5/6 region was polymorphic for *Ava*I only, resulting in the identification of two haplotypes. All the specimens surveyed shared the same haplotype, except for one individual from population WAL (Table 1).

Genetic variability of microsatellite DNA

All 16 microsatellite DNA primers originally developed for other salmonid species successfully amplified apparently homologous loci (Table 2). Four of these were monomorphic while the others displayed moderate to very high levels of polymorphism. Six primers amplifying microsatellite alleles that could be unambiguously determined were then selected for subsequent analysis (Table 2). All Alpine populations were polymorphic at these loci, with the exception of GRL and the *Salvelinus a. alpinus* population which were both fixed for allele PuPuPy-486 (Fig. 2). Total number of alleles per locus ranged from six (locus PuPuPy) to 49 (locus Sfo-23), and allelic diversity (*A*) varied from 9.00 ± 2.07 to 16.17 ± 3.34 in the Alpine populations.

No significant differences in adjusted allelic diversity were observed among populations from the three differ-

Table 2 Salmonid microsatellite DNA loci examined for amplification of homologous fragments in *Salvelinus alpinus*. Loci in italics were choosen for final analysis of all populations

Locus	Annealing temperature (°C)	Allele number	Allele size range (bp)	Species	Primer reference
μ-60	60	Polymorphic *	170–240	*Salmo trutta*	Estoup *et al*. (1993)
μ-73	58	1	150	*S. trutta*	Estoup *et al*. (1993)
MST-85	55	41	186–276	*S. trutta*	Presa & Guyomard (1996)
Sfo-8	60	38	222–318	*Salvelinus fontinalis*	Angers *et al*. (1995)
Sfo-12	60	1	225	*S. fontinalis*	Angers *et al*. (1995)
Sfo-18	60	5	159–167	*S. fontinalis*	Angers *et al*. (1995)
Sfo-23	60	49	133–233	*S. fontinalis*	Angers *et al*. (1995)
SSOSL-85	55	22	216–262	*Salmo salar*	Slettan *et al*. (1995)
SSOSL-417	52	1	165	*S. salar*	Slettan *et al*. (1995)
Ssa-85	60	26	161–211	*S. salar*	O'Reilly *et al*. (1996)
Ssa-197	58	4	121–135	*S. salar*	O'Reilly *et al*. (1996)
Cocl-1	60	3	211–225	*Coregonus clupeaformis*	Bernatchez (1996)
Cocl-3	50	37	209–281	*C. clupeaformis*	Bernatchez (1996)
Cocl-21	52	Polymorphic *	200–224	*C. clupeaformis*	Bernatchez (1996)
Cocl-22	52	1	100	*C. clupeaformis*	Bernatchez (1996)
PuPuPy	60	6	456–495	*Oncorhynchus mykiss*	Morris *et al*. (1996)

*, Allelic diversity not further investigated.

ent Alpine drainages, although Rhône and Danube populations had lower values when compared to Rhine populations (Table 3). No significant differences were also found in pairwise population comparisons within these drainage systems. Values of expected heterozygosity (H_E) averaged across loci were also generally high, ranging from 0.72 ± 0.09 to 0.87 ± 0.04 (Table 3). Heterozygosity values were not significantly different among the Rhône and Danube drainages, but both had lower heterozygosity when compared to the Rhine system (P = 0.025 and P = 0.046, respectively; α = 0.05). No within-drainage heterozygosity differences were found for pairwise population comparisons after sequential Bonferroni correction.

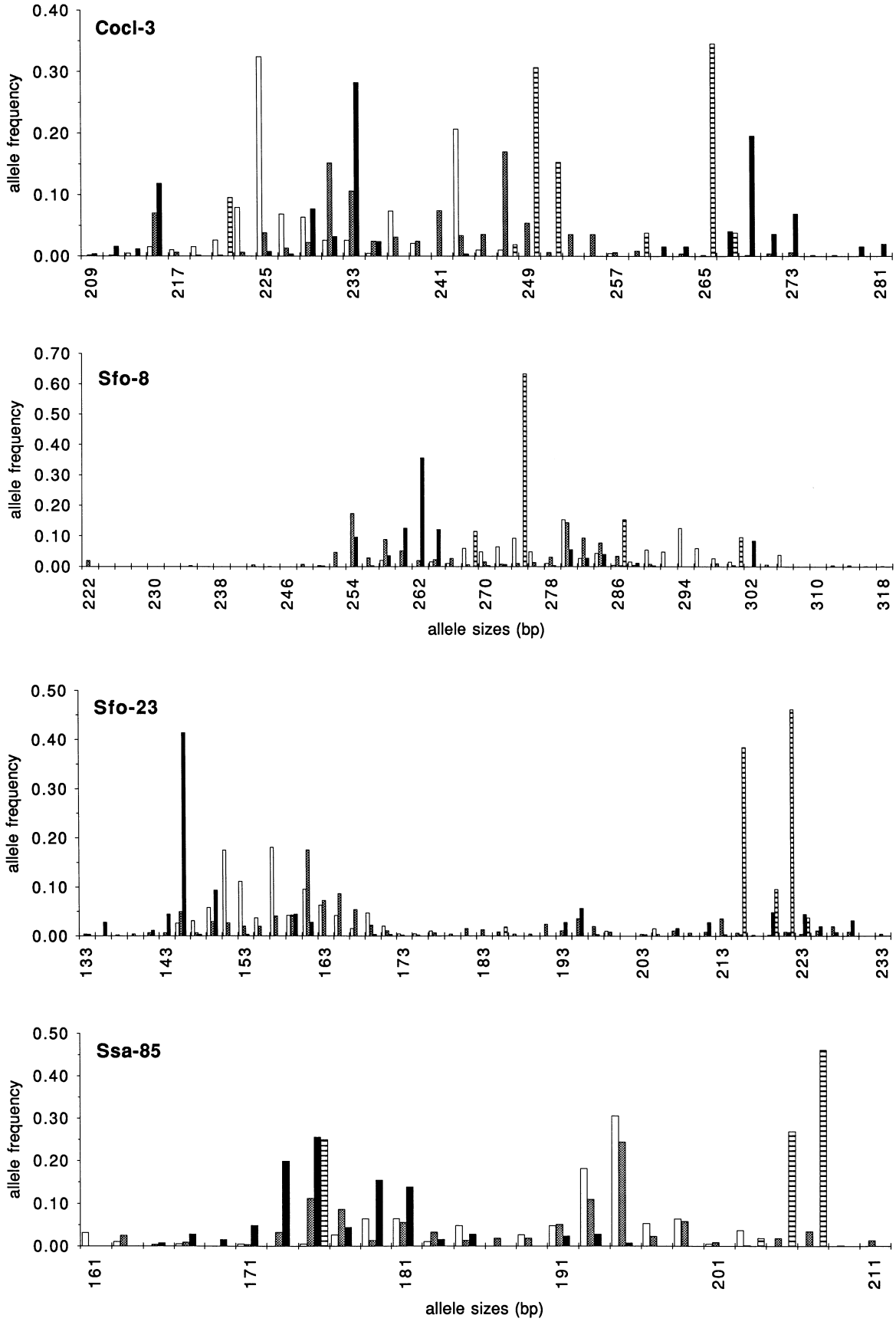
Hardy–Weinberg expectations for genotype frequences in the Alpine populations were rejected at the 5% level in five out of 90 cases, a number comparable to that expected by chance alone (Table 4). These departures were not clustered by locus or population, and the multilocus statistics were not significant ($P > 0.10$) for any population, indicating an overall absence of intra-individual allelic correlation.

Allelic diversity and H_E values for the Finnish *S. a. alpinus* population were significantly lower compared to the Alpine populations (Table 3). Two departures from Hardy-Weinberg expectations for this population were detected, but multilocus statistics were not significant (Table 4).

Population structure

The UPGMA phenograms grouped nonstocked populations by drainage (not shown). Inclusion of the stocked populations in the analysis did not affect the topology of the dendrogram with respect to the nonstocked populations (Fig. 3), but it generally weakend bootstrap supports on branching patterns. Relationships among the major cluster are therefore difficult to establish unambiguously for deeper branching. For instance, grouping of the Rhine population WAL with the Danube populations resulted in a major cluster that is no longer supported by more than 50% bootstrap values. In contrast, inclusion of the Rhine population NEB in the Rhône cluster did not affect the highly significant (97% and 100%, respectively) bootstrap support (Fig. 3). The phenograms built from Nei's and chord distances had identical topology (matrix correlation r = 0.846, probability P = 1.000). Population clustering was not improved by using the $(\delta\mu)^2$ genetic distance (not shown). No cluster was supported by high bootstrap values, as all but one were less than 70%. The three Danube populations did not group together any more, as population GRL clustered with the Rhône-group. However, this relationship is not significantly different, given the absence of bootstrap support and highly significant correlations with Nei's (r = 0.512, P = 0.999) or chord distances (r = 0.764, P = 1.000). Thus, all three clustering algorithms yielded similar results, and differences in population clustering can potentially be attributed to drawbacks of hierarchical clustering techniques such as UPGMA (Lessa 1990).

Note that Fig. 3 is an unrooted tree. Thus, the most basal branching of *S. a. alpinus* supported by maximal bootstrap values indicates a significantly divergent microsatellite composition compared to the Alpine *S. a. salvelinus* populations. Given that only one population-


Cocl-3
allele frequency
0.40
0.30
0.20
0.10
0.00
209
217
225
233
241
249
257
265
273
281
Sfo-8
allele frequency
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
222
230
238
246
254
262
270
278
286
294
302
310
318
allele sizes (bp)
Sfo-23
allele frequency
0.50
0.40
0.30
0.20
0.10
0.00
133
143
153
163
173
183
193
203
213
223
233
Ssa-85
allele frequency
0.50
0.40
0.30
0.20
0.10
0.00
161
171
181
191
201
211
allele sizes (bp)

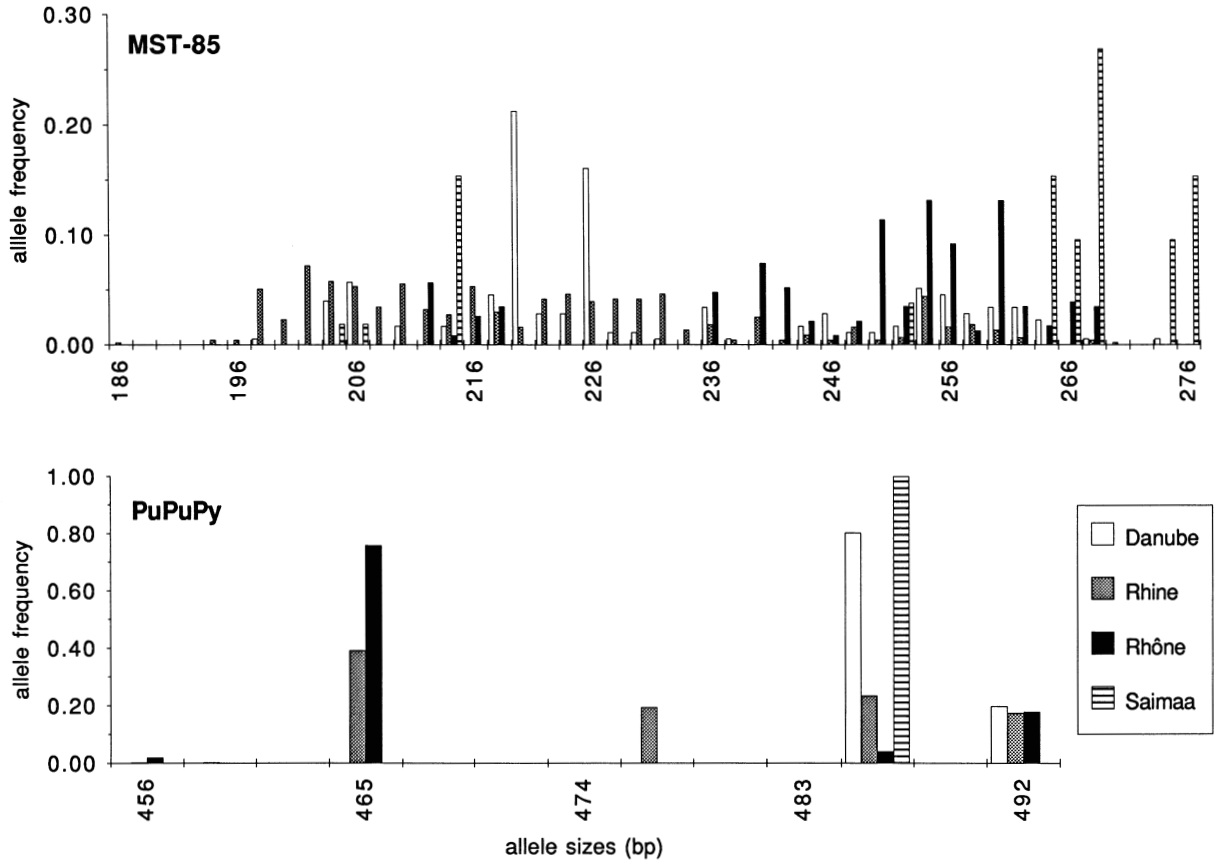


Fig. 2 Combined frequency histograms of allele sizes for six microsatellite DNA loci in Alpine *Salvelinus a. salvelinus* populations from the Danube (white), Rhine (grey), and Rhône (black) drainages, and one Finnish *S. a. alpinus* population from the Saimaa drainage (striped). Figures on vertical axes indicate frequencies, figures on the horizontal axis give allele sizes in bp. Steps between detected allele sizes are 2 bp with the exception of locus PuPuPy where they are 3 bp.

specific allele was detected in *S. a. alpinus*, these differences are based largely on frequency heterogeneity of shared alleles (Fig. 2). Caution is warranted when interpreting these results. Whether the microsatellite composition or the basal position reflect a true phylogenetic signal is speculative, given the high mutation rate for microsatellite loci. Clearly, questions of relationship among members of the Arctic charr 'species complex' need to be addressed in more detail, including more populations over a wider range. Thus, any conclusions with respect to the potential of microsatellite loci as phylogenetic markers in Arctic charr are premature.

The pattern of drainage specific grouping of non-stocked populations was less obvious in the parsimony analysis based on presence/absence of alleles (Fig. 4). The Rhône populations still composed a significantly supported monophyletic group, whereas relationships in the Danube group were not resolved. A major difference was observed among Rhine populations, as populations THN and BRZ were separated from the others and now clustered with the Rhône group.

The three Alpine drainages were partly characterized by alleles that tended to group by size for a given locus (Fig. 2). For instance, at Cocl-3, the allelic modal group varying from 261 bp to 281 bp mainly dominated in the Rhône drainage. Likewise, drainage-specific alleles PuPuPy-477 and Sfo23–135 were diagnostic for Rhine and Danube populations, respectively. This size-based heterogeneity in allele frequencies contributed to a substantial proportion of the total genetic variance attributable to interdrainage differences (Table 5). Thus, the AMOVA revealed that nearly 18% (averaged across loci) of the total microsatellite DNA diversity was explained by variance among the three drainages. However, interdrainage genetic variance (Φ_{CT}) varied considerabely among loci, ranging from 0.068 (Cocl-3) to 0.321 (PuPuPy).

The AMOVA also revealed that a high portion (19.20%) of the total variance (averaged across loci) was attributable to interpopulation differences within Alpine drainages. The remnant portion (63.04%) was partitioned within populations (Table 5). Interpopulation genetic variance (Φ_{SC}) varied considerably among loci, ranging

Table 3 Analysis of six microsatellite loci in 15 Alpine *Salvelinus a. salvelinus* populations from three drainage systems, and one *S. a. alpinus* population from Finland. Shown are geographic location of populations (latitude, longitude), sample sizes (*N*), mean number of alleles detected per locus (*A*), average observed (H_O) and expected Hardy–Weinberg (H_E) heterozygosity with standard errors in brackets. Values are shown before being adjusted for common sample size for statistic analyses

Drainage system/ Population (Lake; L.)	Latitude	Longitude	*N*	*A*	H_O	H_E
Danube						
GRL (Grundelsee)	47° 35'	13° 50'	32	9.00 (2.07)	0.85 (0.04)	0.79 (0.05)
KOE (Königssee)	47° 30'	12° 57'	30	10.00 (1.69)	0.84 (0.06)	0.76 (0.06)
AMM (Ammersee)	48° 00'	11° 05'	31	9.67 (2.29)	0.77 (0.11)	0.72 (0.09)
Combined			93	18.00 (3.57)	0.76 (0.10)	0.79 (0.10)
Rhine						
ROR (L. Constance)	47°30'	9° 30'	30	14.83 (2.60)	0.89 (0.04)	0.85 (0.04)
LNG (L. Constance)	47° 35'	9° 35'	25	13.50 (2.20)	0.88 (0.08)	0.86 (0.04)
UEB (L. Constance)	47° 45'	9° 10'	15	10.50 (1.48)	0.90 (0.07)	0.87 (0.04)
WAL (L. Walenstadt)	47° 07'	9° 10'	33	9.17 (1.58)	0.77 (0.07)	0.74 (0.06)
ZUG (L. Zug)	47° 05'	8° 30'	32	16.17 (3.34)	0.90 (0.04)	0.87 (0.05)
LUZ (L. Luzern)	47° 02'	8° 25'	30	13.00 (1.95)	0.85 (0.06)	0.80 (0.05)
BRZ (L. Brienz)	46° 43'	7° 58'	28	12.00 (2.31)	0.83 (0.04)	0.77 (0.03)
THN (L. Thun)	46° 40'	7° 45'	31	14.00 (2.57)	0.87 (0.03)	0.86 (0.03)
NEB (L. Neuchâtel)	46° 58'	7° 00'	30	11.83 (1.94)	0.79 (0.11)	0.75 (0.09)
Combined			254	30.17 (5.85)	0.85 (0.05)	0.89 (0.04)
Rhône						
BUV (L. Geneva)	46° 25'	6° 50'	31	9.33 (1.45)	0.74 (0.07)	0.74 (0.06)
TON (L. Geneva)	46° 23'	6° 30'	30	9.83 (1.66)	0.84 (0.07)	0.75 (0.07)
BGT (L. Bourget)	45° 45'	5° 53'	32	11.55 (1.75)	0.77 (0.11)	0.76 (0.09)
Combined			93	16.33 (2.95)	0.78 (0.08)	0.77 (0.07)
Saimaa						
S. a. alpinus	62 °00'	23 °00'	26	5.00 (2.75)	0.35 (0.05)	0.35 (0.06)

from 0.018 (Sfo-23) to 0.557 (MST-85). Interpopulation genetic diversity was also evidenced by differences in allele frequency distribution among pairwise comparisons within each lake of the three drainages. These differences were based not on allelic size distribution but rather on frequency heterogeneity of specific alleles (Fig. 2). For instance, the range of relative allele frequencies of Cocl3–243 and µ85–220 was 0.00–0.56 and 0.00–0.57 among Danube populations. Numerous population-specific alleles were detected for all loci (Fig. 2). Their proportion was highest within Danube populations, ranging from 25.9% to 40.7%, followed by the Rhône and Rhine populations.

Among sympatric populations from either Lake Constance (ROR, LNG, UEB) or Lake Geneva (BUV, TON) significant ($P < 0.01$) differences in allele frequency distribution were observed. All these populations were characterized by a relatively high percentage of population-specific alleles, ranging from 17% to 25%.

The allele size distribution of the *S. a. alpinus* population from Finland overlapped that of the Alpine populations with the exception of locus MST-85. The largest allele at this locus was also the only population-specific allele (Fig. 2).

Discussion

Mitochondrial DNA variation

Three major centres of Arctic charr evolution are currently recognized over its Holarctic range: the widespread and diverse *Salvelinus a. erythrinus* group occurring in Asiatic and North American drainages, a north European *S. a. alpinus*, and a central European *S. a. salvelinus* group (Behnke 1984). The former two were previously examined for mtDNA RFLP variability (Grewe *et al*. 1990; Danzmann *et al*. 1991; Wilson *et al*. 1996). In the present study, the almost complete lack of mtDNA variation observed in Arctic charr of the central European Alps, even among samples collected from different drainages, is fully consistent with the reduced level of genetic variation reported previously. For example, mtDNA analysis of

Table 4 Summary table of variation detected at six microsatellite loci from 15 Alpine *S. a. salvelinus* populations, and one *S. a. alpinus* population from the Saimaa system, Finland. Listed are number of different alleles (A), observed (H_O) and calculated expected heterozygosity (H_E) by locus/population, and combined overall populations from the same drainage system. Number of specimens scored for each population is indicated in parentheses

	Danube populations				Rhine populations										Rhône populations				Saimaa
Locus	GRL (32)	KOE (30)	AMM (31)	Comb (93)	ROR (30)	LNG (25)	UEB (15)	WAL (33)	ZUG (32)	LUZ (30)	BRZ (28)	THN (31)	NEB (30)	Comb (254)	BUV (31)	TON (30)	BGT (32)	Comb (93)	*Saa* (26)
Cocl-3																			
A	7	13	8	18	17	14	12	8	16	14	12	14	14	26	9	9	14	17	7
H_O	0.69	0.93	0.62	0.73	0.93	1.00	0.81	0.88	0.94	0.90	0.82	0.88	0.93	0.90	0.77	0.83	0.81	0.80	0.58
H_E	0.63	0.85	0.70	0.83	0.88	0.93	0.87	0.85	0.90	0.85	0.76	0.86	0.87	0.92	0.76	0.81	0.83	0.84	0.76
P	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	*
Sfo-8																			
A	11	13	12	21	16	17	11	11	12	16	11	11	10	24	10	10	11	16	4
H_O	0.88	0.97	1.00	0.95	0.90	0.95	1.00	0.70	0.94	0.80	0.86	0.88	0.83	0.86	0.87	0.93	0.97	0.92	0.69
H_E	0.82	0.90	0.90	0.93	0.78	0.92	0.90	0.78	0.83	0.75	0.74	0.83	0.78	0.92	0.80	0.78	0.81	0.83	0.56
P	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	*
Sfo-23																			
A	13	10	16	19	21	20	15	11	24	19	13	22	17	35	8	14	14	22	5
H_O	0.88	0.90	1.00	0.93	1.00	0.95	1.00	0.64	0.79	0.93	0.79	0.87	0.87	0.85	0.60	0.87	0.78	0.75	0.69
H_E	0.86	0.81	0.92	0.90	0.93	0.92	0.95	0.59	0.94	0.92	0.76	0.93	0.82	0.94	0.70	0.79	0.85	0.79	0.64
P	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns
Ssa-85																			
A	7	12	15	18	11	13	10	13	17	11	9	14	11	23	10	9	10	12	4
H_O	0.84	0.87	0.94	0.88	0.90	0.95	1.00	0.94	1.00	0.93	0.82	0.97	0.93	0.93	0.90	0.90	0.97	0.92	0.73
H_E	0.70	0.85	0.90	0.85	0.83	0.90	0.87	0.87	0.92	0.80	0.75	0.90	0.76	0.91	0.78	0.79	0.84	0.82	0.66
P	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
MST-85																			
A	15	10	5	28	20	13	11	10	23	13	22	19	15	33	15	14	16	17	9
H_O	0.94	0.78	0.69	0.80	0.87	0.91	1.00	0.90	0.94	0.93	0.96	0.91	0.92	0.92	0.83	0.93	0.80	0.86	0.85
H_E	0.92	0.71	0.63	0.91	0.94	0.90	0.94	0.82	0.95	0.89	0.92	0.93	0.93	0.97	0.90	0.91	0.92	0.90	0.85
P	ns	ns	ns	ns	P	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns
PuPuPy																			
A	1	2	2	2	4	4	4	3	3	5	5	4	4	4	4	3	3	4	1
H_O	–	0.59	0.36	0.29	0.73	0.50	0.60	0.55	0.77	0.59	0.70	0.72	0.27	0.61	0.47	0.57	0.27	0.43	–
H_E	–	0.51	0.31	0.32	0.72	0.68	0.66	0.50	0.66	0.59	0.67	0.73	0.32	0.70	0.48	0.42	0.34	0.42	–
P	–	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	–

ns, not significant; *$P < 0.05$; **$P < 0.01$.

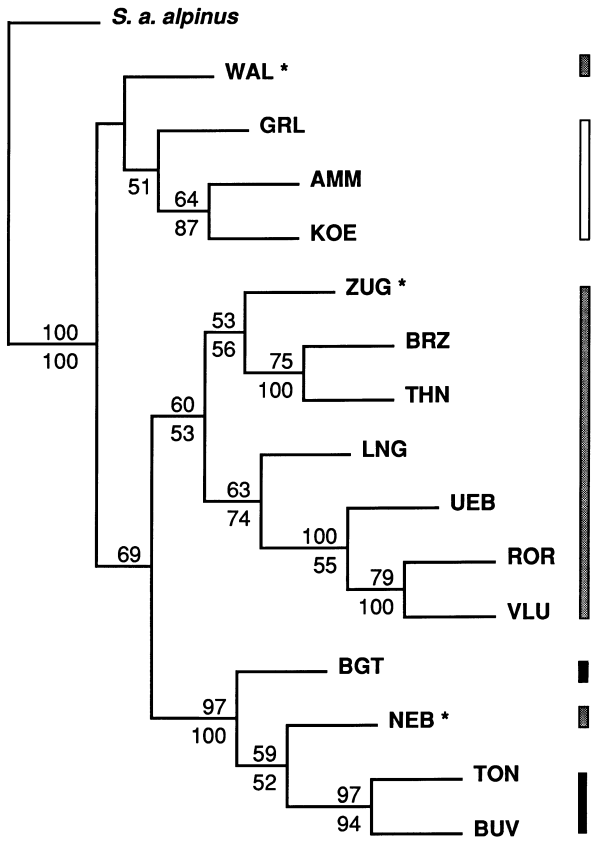


Fig. 3 UPGMA phenogram computed from allelic diversity at microsatellite DNA loci among stocked (indicated by asterisks) and nonstocked Alpine *Salvelinus a. salvelinus* populations and one *S. a. alpinus* population from Finland. Clustering on Nei's and chord distance produced a tree with identical topology. Bootstrap probability values (1000 replications) are given in per cent values above branches for Nei's distance, and below branches for chord distance. Unnumbered nodes were found in less than 50% of bootstrap samples. Black bars indicate populations from the Rhône, grey bars populations from the Rhine, and white bars populations from the Danube system, respectively.

seven allopatric Scottish populations detected only one polymorphic restriction enzyme (*Hin*dIII) among 19 tested (Hartley *et al*. 1992).

Such homogeneity in mtDNA variation can potentially be explained by severe bottlenecks experienced by the species throughout its range. Given mutation rate estimates reported for mtDNA ($\approx 10^{-9}$–10^{-8} mutation/site/generation), the virtually nonexisting mtDNA diversity in central Alpine *S. alpinus* suggests a recent divergence of populations. It is probable that populations dispersed after the last glaciation some 15 000 years ago, possibly from a single ancestral group. Similar scenarios have been invoked to explain reduced levels of mtDNA diversity in other northern fishes, such as whitefish (*Coregonus* sp.) (Bernatchez & Dodson 1994). Mitochondrial DNA data from this and previous studies on other European salmonids support the hypothesis of a single postglacial origin for the Alpine *S. alpinus* populations. On the basis of a dichotomous distribution of two major mtDNA phylogeographic groups in whitefish, Bernatchez & Dodson (1994) concluded that central Alpine lakes have been postglacially recolonized by a single race, distinct from a second that recolonized northern Europe. Interestingly, this observation is congruent with the two distinct evolutionary lineages in Arctic charr phylogeny (*S. a. alpinus* and *S. a. salvelinus*) proposed for these regions based on traditional meristic and morphological characters (Behnke 1984).

An alternative scenario to explain similar homogeneity of mtDNA among populations would involve recolonization of the area by more than one glacial race, followed by substantial secondary intergradation and retention of a

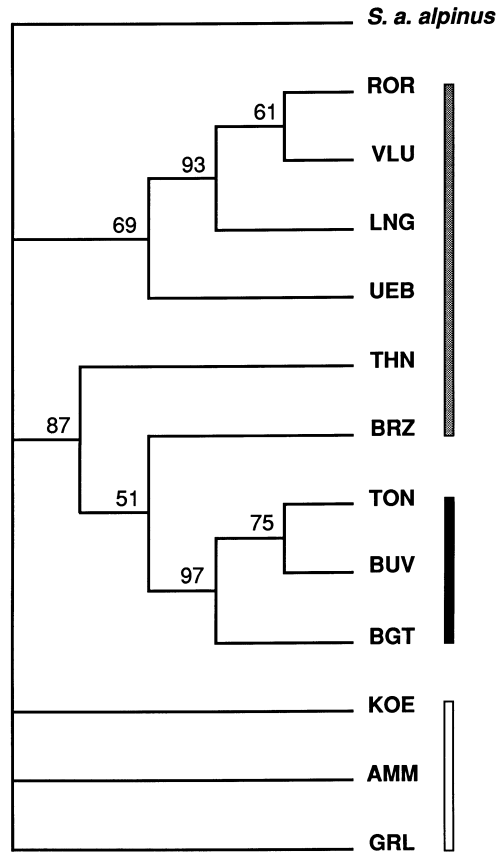


Fig. 4 PAUP majority rule consensus tree based on a cladistic analysis of an allele presence–absence matrix from the same nonstocked populations as in Fig. 3. Bootstrap probability values (1000 replications) are given in per cent above branches. Un-numbered nodes were found in less than 50% of bootstrap samples. Tree length = 472, CI excluding uninformative characters = 0.457, rescaled consistency index = 0.488. Black bars indicate populations from the Rhône, grey bars populations from the Rhine, and white bars populations from the Danube system, respectively.

Table 5 Hierarchical analysis of molecular variance based on six microsatellite DNA loci of Alpine *Salvelinus a. salvelinus V*, variance and Φ, haplotypic correlation at corresponding levels. See the Materials and methods section for definition of each statistic. *P*, probability of a more extreme variance component than that observed by chance alone

	Among drainage systems				Among populations/ within drainage systems				Within populations			
Locus	*V*	%	*P*	Φ_{CT}	*V*	%	*P*	Φ_{SC}	*V*	%	*P*	Φ_{ST}
Cocl-3	4.401	6.75	0.139	0.068	13.808	21.19	<0.001	0.227	46.951	72.05	<0.001	0.279
Sfo-8	11.989	17.89	0.029	0.179	12.756	19.04	<0.001	0.232	42.256	63.07	<0.001	0.369
Sfo-23	10.734	7.01	<0.001	0.070	2.525	1.65	0.045	0.018	139.861	91.34	<0.001	0.087
Ssa-85	6.418	20.21	0.045	0.202	5.554	17.49	<0.001	0.219	19.779	62.30	<0.001	0.377
MST-85	27.399	22.60	0.089	0.226	52.222	43.08	<0.001	0.557	41.609	34.32	<0.001	0.657
PuPuPy	5.351	32.12	0.010	0.321	2.121	12.73	<0.001	0.188	9.189	55.15	<0.001	0.448
Combined/												
Mean	11.049	17.76		0.178	14.831	19.20		0.240	49.941	63.04		0.370
SE	3.495	3.96		0.040	7.751	5.56		0.071	18.968	7.68		0.077

single haplotype among populations. This situation would have been facilitated by formation and fusion of postglacial lakes and changing river drainage connections in Europe. Pleistocene connections between the ancient Rhine and Danube rivers, and a pre-Quaternary Ice Age connection between the Danube and the Rhône are well documented (Hantke 1978).

Homologous microsatellite DNAs in S. alpinus

In sharp contrast to the mtDNA data, high levels of polymorphism were detected at microsatellite DNA loci. This clearly contradicts the general view that Arctic charr is genetically depauperate, and stresses the importance of making such inference only in terms relative to the genetic marker used. Moore *et al*. (1991) suspected that levels of polymorphism at microsatellite DNA loci may decrease with phylogenetic distance from the species used to make the genomic library. In this study, all PCR primers used to amplify microsatellite DNA were originally developed for other salmonids. Origin of microsatellite DNA primers apparently did not affect level of variation detected in Arctic charr. In fact, the heterozygosities depicted here rank among the highest reported in fish thus far. For example, Angers *et al*. (1995) reported levels of H_E ranging from 0.45 to 0.71, and detected 16 distinct alleles in *S. fontinalis* populations at locus Sfo-23, whereas in this study H_E for the same locus varied from 0.59 to 0.95 and a total of 49 alleles was observed. Similarly, in Atlantic salmon (*Salmo salar*) Tessier *et al*. (1995) detected low levels of heterozygosity (0.00–0.30) and only two alleles at locus MST-85. These are clearly lower than our findings (Tables 2 and 4).

Heterozygosity estimates at microsatellite DNA loci exceeded those reported for mtDNA or allozyme studies in Arctic charr. Average heterozygosity of the six microsatellite DNA loci reported here was $H = 0.82$ compared with $H = 0.03$ reported for allozymes (Kornfield *et al*. 1981) and an equivalent measure for mitochondrial DNA nucleon diversity, $h = 0.40$ (Danzmann *et al*. 1991). The discrepancy between findings for microsatellite and mitochondrial DNA can be partly explained by a weak correlation between mitochondrial and nuclear diversity, based on independent demographic processes imposed by historical events (e.g. Avise *et al*. 1984; DeSalle & Templeton 1988). A nonexclusive second explanation for these discrepancies may be related to differential mutation rates. This is expected to be higher in microsatellite DNAs (e.g. Moritz *et al*. 1987; Weber & Wong 1993), promoting the establishment of higher effective number of alleles at mutation-drift equilibrium for a given population size. Consequently, the argument that the species is still relatively genetically depauperate at the great majority of loci can not generally be refuted. Estimating possible levels of genetic divergence since a bottleneck-type event are highly speculative, because reliable estimates of microsatellite mutation rates in fish are not yet available. However, given the poor genetic diversity observed in previous studies, our results emphasize the importance of addressing this issue relative to a specific marker, rather than to assume a generally genetically depauperate species.

Genetic variability among drainages and populations

A salient feature of this study is evidence for a considerable proportion of total genetic variance attributable to differences among the three drainages (Table 5). Two alternative hypotheses account for such geographical partitioning of microsatellite DNA diversity: (i) distinct

origin of charr populations in each drainage; or (ii) a common postglacial origin with subsequent differentiation by genetic drift and mutation. The possibility for multiple origins of charr in different drainages is supported by the fact that watersheds of several major European drainages (Adige, Danube, Pô, Rhine and Rhône) meet within the area, and that genetic diversity in other fish can best be explained through recolonization by distinct glacial races. For instance, diagnostic genetic differentiation in mtDNA and several protein loci suggests that brown trout (*Salmo trutta*) populations in Switzerland originated from the Danube, Mediterranean, and Atlantic basins (Guyomard 1989; Bernatchez *et al*. 1992; Largiadèr *et al*. 1996). A corollary of the multiple-origin hypothesis is that populations belonging to the same drainage should share unique apomorphies (i.e. alleles), or exhibit distinct allele frequencies compared to other drainages. According to a stepwise mutation model proposed for the evolution of microsatellite DNA loci (e.g. Valdes *et al*. 1993), one would expect development of modal allele size distributions among groups that have been isolated for relatively long periods of time. This was apparently the case in central Alpine *S. alpinus*, as several loci, at least qualitatively, showed frequency shifts between drainages (e.g. locus Cocl-3 in Fig. 2). Additional support for distinct origin of charr populations in each drainage is provided by the occurrence of drainage-specific alleles.

The second hypothesis of a single postglacial origin for charr from all three drainages is supported by mtDNA data (see discussion above), and the evidence of a unique origin for other species, such as whitefish. Accordingly, the putative glacial refuge may have been in the Atlantic basin, and Arctic charr would have invaded the Alpine region through the Rhine system. Invasion of the Danube and Rhône by small founder populations would explain the significantly reduced allelic diversity and heterozygosity found in those drainages when compared to the Rhine populations. Subsequent genetic drift, postglacial genesis of drainage-specific alleles by mutation, and restricted gene flow would all have contributed to the observed genetic partitioning among drainage systems and populations. Further insight into the origins of charr populations by microsatellite DNA analysis may eventually be obtained once reliable estimates of mutation rates become available, thus allowing tentative estimates of divergence from common ancestry (e.g. Goldstein *et al*. 1995).

Analysed specimens were collected from different traditional spawning sites and population status was assigned to each. This *a priori* assignment appears to be justified, as tests for Hardy–Weinberg equilibrium and heterozygote deficiency showed no evidence of a Wahlund effect among sympatric and allopatric populations. It has to be noted, however, that tests may have limited power due to sample sizes that may be too small, given the high number of alleles detected per locus. Nevertheless, our results suggests that site fidelity and phylopatry, as in other salmonid fishes, are potentially evolutionary important behaviours in Arctic charr. They reduce gene flow between spawning sites and promote genetic population differentiation within lakes. Sound evidence for restricted gene flow between populations is also provided by a high proportion of population-specific alleles, and significant differences in allele frequencies, resulting in a substantial proportion of molecular variance imputable to among-lake differences. This supports the general observation that the populations examined are strictly lacustrine, and implies that rivers, although potential avenues of dispersal, represent effective barriers to gene flow between lakes. Furthermore, samples from two spawning sites from Lake Geneva and three from Lake Constance showed high proportions of specific alleles, and highly significant differences in allele frequencies. Microsatellite DNA results thus strongly support the assumption that each represents a genetically distinct population. Previous studies based on allozyme differentiation failed to detect genetic population partitioning within Alpine lakes (Ruhlé 1977; Hecht 1984). Similarly, molecular approaches with increased genetic resolution, such as direct sequencing of mtDNA and multilocus minisatellite analysis, have failed thus far to clarify whether ecologically segregated Arctic charr morphs from Iceland represent distinct gene pools (Danzmann *et al*. 1991; Volpe & Ferguson 1996).

Conservation relevance and conclusion

European charr populations have been extensively stocked and mixed over centuries (Pechlaner 1984), thereby decreasing the possibility of detecting phylogenetic signals among populations. The application of microsatellite DNA analysis allowed to estimate previously unknown genetic impacts of fishery management. The Swiss Government recently passed a fisheries law (Bundesgesetz vom 21. Juni 1991, über die Fischerei, BGF) to protect indigenous populations. It prohibits use of non-native material for stocking and for exchange across watersheds within the country. Results show a significant proportion of genetic variance among drainages or lakes, and demonstrate that, despite a long history of stocking, original genetic diversity of most populations has not substantially been eroded (Figs 3 and 4). This justifies the new legislation and stresses its importance in future management of native *S. alpinus*.

On the other hand, detectable stocking effects varied considerably depending on lakes. For example, a negative effect of fishery management is evident in population NEB (Fig. 3). This Rhine system lake was stocked with progeny

from Lake Geneva (Rhône system) after the presumed extinction of its native population (Rubin & Büttiker 1987). The introduction of non-native genetic material is evidenced by numerous alleles found in populations of the Rhône system only, and the absence of allele PuPuPy-477, a diagnostic allele for Rhine populations. However, also alleles typical for Rhine populations were detected in NEB. This suggests that its native population was not entirely extinct, and that stocking strongly introgressed a remnant population. In contrast, documented introduction of fish from the Danube system into Lake Zug (Ruhlé 1977) apparently had little impact on the genetic structure of this population (Fig. 3). This could be explained by genetic adaptation of native charr to a specific local environment, and/or selection against stocked individuals. Alternatively, if the number of introduced fish is small relative to the native population, an existing genetic input may not be detected in our analysis. This can not be quantified, given the assumed long stocking history and incomplete documentation of number (and origin) of introduced fish in Alpine lakes. However, the detection of substantial genetic partitioning that can be attributed to historic geological events and ecological separation of sympatric populations supports long-term management and conservation of natural biodiversity through detailed knowledge of genetic diversity within and between populations.

To conclude, the discrepancy between results of mtDNA RFLP and microsatellite DNA suggests that estimates of population subdivision using currently available molecular methods should be interpreted with caution. With the analysis of microsatellite DNA data, we were able to demonstrate strong genetic differentiation among Alpine *S. a. salvelinus* populations, and among- and within-drainage structuring. These findings clearly refute the general belief that Arctic charr are genetically depauperate. On the contrary, by the standards of microsatellite DNA loci, *S. alpinus* must be ranked among the most variable species. Our results demonstrate that Arctic charr populations examined were genetically distinct, and represent unique gene pools that warrant individual recognition for conservation and management.

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This paper is the result of a Swiss–Canadian collaboration, and is part of a continuing series of studies on phylogenetic and population genetic questions in salmonid fishes. The study reported is part of Patrick Brunner's PhD research applying molecular genetic techniques and morphometric analysis to the study of polymorphism in Arctic charr. Marlis Douglas is a doctoral candidate in systematics and evolutionary biology studying similar questions in the genus *Coregonus*. The work was carried out whilst visiting the laboratory of Louis Bernatchez whose major research interests are in the understanding of patterns and processes of molecular and organismal evolution, as well as their significance to conservation.
