

Do assemblages of *Coregonus* (Teleostei: Salmoniformes) in the Central Alpine region of Europe represent species flocks?

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Abstract

To examine models of evolution for *Coregonus* from the Central Alpine region of Europe, 20 populations from nine lakes were assessed for variation at six microsatellite DNA loci. Patterns of variation were tested against three evolutionary models: phenotypic plasticity, multiple invasions of lakes by divergent forms, and within-lake radiation of species flocks. All sympatric and all but one allopatric pairs of populations were significantly divergent in allele frequencies. Pairwise *F*-statistics indicated reduced gene flow among phenotypically divergent sympatric populations. These results reject the hypothesis that within-lake morphological and ecological diversity reflects phenotypic plasticity within a single gene pool. Genetic similarity was higher among forms within lakes than between populations of the same form in different lakes. Among-lake divergence was primarily a product of allele size differences. Mantel tests contrasting patterns of genetic divergence against patterns predicted from the multiple invasions and species flocks models indicated that the latter is the best explanation of the observed genetic variation. Thus, reproductively isolated species diverged within lakes, with similar patterns repeatedly emerging among lakes. While this study argues for a particular mode of evolution in Central Alpine *Coregonus*, the taxonomy of these forms remains unresolved.

Keywords: Central Alps, *Coregonus*, Mantel test, microsatellite DNA, population genetics, species flocks

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Introduction

Diversity is a fundamental aspect of biology (Wilson 1988), yet its quantification depends upon a firm taxonomic basis (May 1990). A common method for accomplishing this is enumeration of clades with independent evolutionary histories (Mayden & Wood 1995). However, the shallow evolutionary histories of recently evolved groups are often difficult to resolve because of methodological limitations.

Freshwater fishes of the northern hemisphere provide examples of recently evolved clades. They often display a number of divergent forms with uncertain taxonomic status that coexist in sympatry (Smith & Skúlason 1996). Many fulfil criteria for recognition as biological species

(Mayr 1963). This tendency towards extensive, taxonomically unrecognized diversity is perhaps greatest in salmoniforms (Behnke 1972). Virtually every family (as defined by Sanford 1990) has evolved 'sibling taxa': Salmonidae (Sandlund *et al.* 1992; Hindar & Jonsson 1993; Taylor *et al.* 1997), Osmeridae (Taylor & Bentzen 1993a; Bernatchez 1997) and Coregonidae (Bernatchez & Dodson 1990; Pigeon *et al.* 1997). Sympatric forms within lakes are often referred to as 'ecotypes' or 'morphs', in spite of reproductive isolation. The occurrence of morphologically different forms within lakes has been replicated among lakes, particularly within the Coregonidae, where the existence of multiple forms within and among lakes is especially common. Thus, the questions become not only 'Have these forms arisen in sympatry?' but also, 'How did they arise multiple times?' Local assemblages of coregonids have been referred to as single, but plastic species (Steinmann 1951), as products of multiple invasions by

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divergent forms (Bernatchez & Dodson 1990; Bernatchez *et al.* 1996), and as 'species flocks' (Smith & Todd 1984). Here, the term 'species flock' is used to accommodate the rapid evolution of Central Alpine *Coregonus*, and to define an aggregate in a single lake where forms are more closely related to one another than to phenotypically similar forms in other lakes. The latter would hence represent parallel (but separate) divergence.

In this study, coregonids in Central Alpine lakes of Europe are used in an assessment of the various models of evolution in this group. Most lakes in this relatively small region contain at least two coregonid forms, whereas several contain three or more (Steinmann 1950a; Kirchhofer 1996; Kottelat 1997), and one may have up to six coregonid forms (M. R. Douglas, unpublished). Sympatric forms differ in a variety of life-history and ecological traits (Steinmann 1950a; Kottelat 1997), which are often correlated with striking size differences (Douglas 1998). During the year, populations may intermix within the water column, but they segregate spatially and/or temporally at spawning time (Steinmann 1950a,b; Kirchhofer & Tschumi 1986). This indicates an affinity by individuals to a specific, reproductively isolated group.

Fish communities within Central Alpine lakes have been studied extensively from an ecological and fisheries perspective, but rarely from a systematic or evolutionary stance (Kottelat 1997). Understanding the evolutionary history of resident *Coregonus* forms would provide

insights into mechanisms and processes responsible for their variability. Namely, it is crucial to determine whether similarity among forms represents plasticity, homology or homoplasy. Conservation and management of diversity within *Coregonus* hinges upon understanding the evolutionary history of individual populations.

Microsatellite DNA loci were used to determine relatedness and test hypotheses of origin for 20 *Coregonus* populations distributed within and among nine lakes in the Central Alpine region of Europe. Microsatellite loci offer several advantages over other types of molecular markers in that they are abundant, highly variable and can be assayed from minute quantities of DNA (Ashley & Dow 1994). In salmonids, microsatellite loci are particularly appropriate for studying divergence over a microgeographical scale (Angers & Bernatchez 1998) and for detecting levels of genetic differentiation when other markers fail to do so (Brunner *et al.* 1998).

Patterns of genetic divergence were tested against three alternative evolutionary scenarios (Fig. 1): phenotypic plasticity in a single species (model I), multiple invasion of lakes by divergent species (model II) and occurrence of species flocks (model III). Model I predicts that morphologically or ecologically divergent forms of *Coregonus* reflect plasticity within a single gene pool (i.e. ecophenotypes). Phenotypic plasticity has been the favoured hypothesis to explain diversity among *Coregonus* forms from the Central Alpine region (Steinmann 1950a,b; 1951).

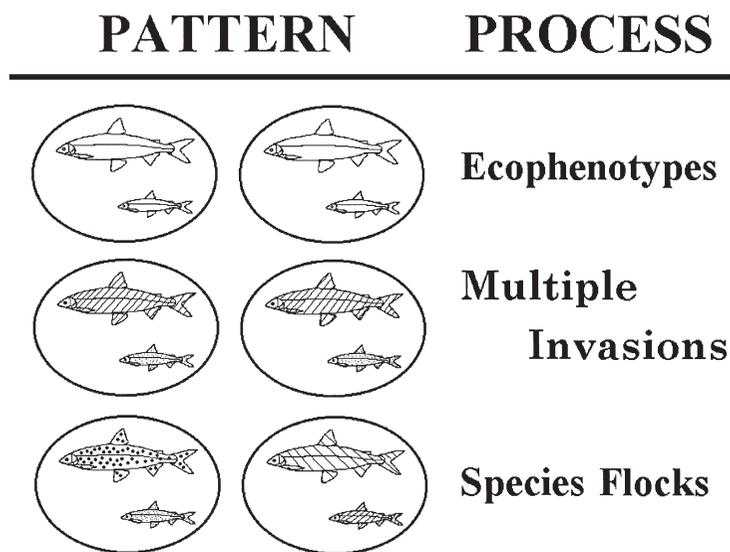


Fig. 1 Schematic depiction of three alternative evolutionary scenarios to explain diversity in Central Alpine *Coregonus* populations. 'Pattern' depicts the array of phenotypes found within lakes. 'Process' refers to the evolutionary mechanism that produced the pattern. Three hypotheses are listed beneath Pattern. 'Ecophenotypes' suggest forms are different with regard to morphology and ecology, but are genetically identical. They are the result of extrinsic factors acting upon a plastic phenotype. The 'multiple-invasion' hypothesis suggests that phenotypically equivalent forms are genetically most similar among lakes, while divergent forms are genetically most different. This pattern is produced by repeated invasions of lakes by lineages already differentiated. The 'species flock' hypothesis argues that forms within a lake are more closely related to one another genetically than are forms among lakes. Here, the pattern is produced by invasion of a lake by a single ancestral lineage that undergoes within-lake radiation.

Model II hypothesizes multiple invasions by already divergent forms that, for example, could have evolved in separate refugia during Pleistocene glaciations. Under this model, phenotypically similar forms in allopatry would be more closely related to one another than they are to phenotypically divergent forms in sympatry. This model has been supported in salmoniform fishes (Hynes *et al.* 1996; Bernatchez 1997), including coregonids (Bernatchez & Dodson 1990; Bernatchez *et al.* 1996). It also could explain diversity among Central Alpine *Coregonus* (Wagler 1950). Model III, the species flock hypothesis, predicts that each form within a lake is a distinct taxon whose closest relatives occur within the same lake, and that similar forms (species) evolved repeatedly among lakes (i.e. convergent evolution). There is increasing evidence that this may represent the predominant mode of sympatric-pair origins in northern temperate fishes; however, evidence supporting such a mode of evolution in *Coregonus* is rare (but see Bernatchez *et al.* 1996; Pigeon *et al.* 1997).

Materials and methods

Sampling

A total of 583 specimens of *Coregonus*, representing 20 populations, were collected from nine lakes of the Central Alpine region of Europe (Fig. 2). A sampling regimen was devised, with emphasis primarily upon endemics. Two to four forms of *Coregonus* were collected from each lake (except Lake Achen where only one form was obtained). Sampling locations, abbreviations of lakes and local name of forms are listed in Table 1.

Specimens were captured at spawning time over traditional spawning sites. Within-lake catches for a particular form were pooled as a single sample from several sites (as per Hillis *et al.* 1996: p. 22). Each individual was identified

with respect to form by spawning affinities and adult body size, which was later corroborated by phenotypic evaluation in the laboratory (Douglas 1998).

Whenever possible, 30 specimens were collected per population (Table 1). Liver tissue was sampled from freshly killed or deep-frozen specimens and preserved in 100% ethanol. Total DNA was isolated using the CTAB protocol (Hillis *et al.* 1996: p. 224).

Microsatellite markers

Microsatellite loci were amplified using primers originally developed for coregonids or salmonids. Initially, 30 primer sets were screened using a single specimen from each of 10 different populations. Six primers producing unambiguously determined bands were selected for further analyses (Table 2). The extent of polymorphism in these loci was assessed in 40 fish representing eight populations. Polymerase chain reaction (PCR) reactions were set up in 15- μ L volumes, with amplifications performed on a Perkin-Elmer thermal cycler (model 480), as previously described by Brunner *et al.* (1998). PCR products were separated on 5–6% acrylamide sequencing gels and autoradiographed. Alleles were compared with the standard M13 sequence to determine size differences.

Population genetic analyses

Samples were tested for departure from Hardy–Weinberg equilibrium (HWE) using GENEPOP 3.1a (Raymond & Rousset 1995a). In addition, samples were assessed for deviations from HWE under the alternative null hypothesis of heterozygote excess and under one of heterozygote deficit (Rousset & Raymond 1995). Significance values were computed by unbiased estimates of Fisher's exact

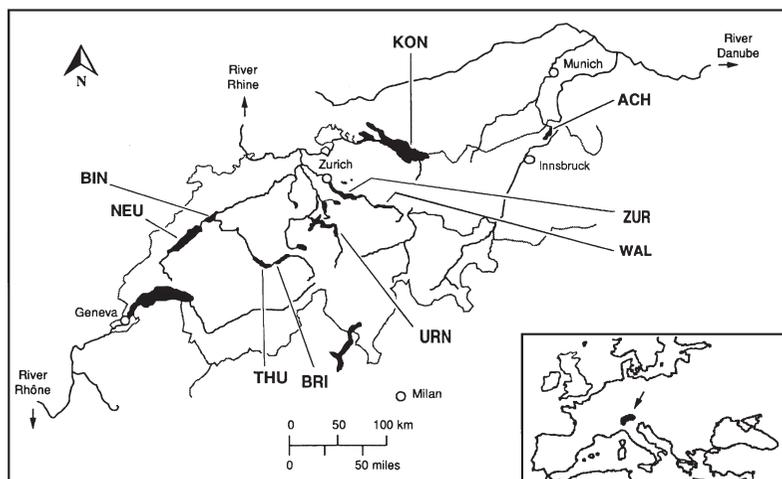


Fig. 2 Lakes from which *Coregonus* populations were sampled in the Central Alpine region of Europe. Insert indicates geographical location of the Central Alps within Europe. Names and locations of sampling sites are presented in Table 1.

Lake	Latitude	Longitude	Population	<i>n</i>	Local name	Form
Neuchâtel	46°58'	7°00'	NEU1	30	Palée	Large
			NEU2	30	Bondelle	Small
Bienne	47°05'	7°10'	BIN1	26	Palée	Large
			BIN2	30	Bondelle	Small
Thun	46°40'	7°42'	THU1	30	Albock	Large
			THU2	30	Balchen	Large
			THU3	30	Kropfer	Large
			THU5	30	Sommerbrienzig	Small
Brienzi	46°43'	7°58'	BRI1	30	Albock	Large
			BRI2	30	Sommerbrienzig	Small
Urnersee	46°56'	8°36'	URN1	30	Albeli	Small
			URN2	30	Ballen	Large
Zürich	47°15'	8°42'	ZUR1	30	Albeli	Small
			ZUR2	17	Felchen	Large
Walensstad	47°07'	9°10'	WAL1	30	Albeli	Small
			WAL2	30	Felchen	Large
Konstanz	47°30'	9°30'	KON1	30	Gangfisch	Small
			KON2	30	Blaufelchen	Small
			KON3	30	Sandfelchen	Large
Achensee	47°25'	11°45'	ACH1	30	Felchen	Large

Listed for each lake are geographical location (latitude, longitude), population abbreviation, sample size of population (*n*), local name, and designated 'form'. The upper (larger) section of the table contains Rhine River populations, whilst the lower (smaller) section contains the single Danube River population.

test using the Markov chain method through 8000 iterations (Guo & Thompson 1992). Genotypic phase disequilibrium was also evaluated for all pairwise combinations of loci for each population, and over all populations, again using GENEPOP 3.1a. Genetic polymorphism in each population was calculated as number of alleles per locus (N_A), mean number of alleles (A), observed heterozygosity (H_O) and expected heterozygosity (H_E) from Hardy–Weinberg assumptions, using POPGENE 1.21 (Yeh *et al.* 1997).

Heterogeneity in allele frequencies was compared for all pairs of populations, again using GENEPOP 3.1a. Each locus

and population pair was examined using an unbiased estimate of Fisher's exact test (Raymond & Rousset 1995b). Differentiation of population pairs, based on a combination of all six loci, was calculated using the χ^2 -test (Fisher's method). Significance of results was evaluated with Bonferroni-adjusted *P*-values (Harris 1975: pp. 96–101).

Distribution of microsatellite DNA diversity was quantified using the analysis of molecular variance model (AMOVA) in ARLEQUIN 1.1 (Schneider *et al.* 1997). Genetic correlation measures were expressed as both *F* and Φ statistics (Excoffier *et al.* 1992; Michalakis & Excoffier 1996).

Locus	TA (°C)	Size range (bp)	Allele no.	Species	Primer reference
SFO-23	58	158–214	27	<i>Salvelinus fontinalis</i>	Angers <i>et al.</i> (1995)
BWF-1	60	208–234	14	<i>C. nasus</i>	Patton <i>et al.</i> (1997)
BWF-2	55	147–187	18	<i>C. nasus</i>	Patton <i>et al.</i> (1997)
PuPuPy-300	64	330–381	14	<i>Oncorhynchus mykiss</i>	Morris <i>et al.</i> (1996)
PuPuPy-600	64	587–641	19	<i>O. mykiss</i>	Morris <i>et al.</i> (1996)
Cocl-23	55	259–367	48	<i>C. clupeiiformis</i>	Bernatchez (1996)

Listed are locus, annealing temperature (TA), size range of alleles, no. of detected alleles, species for which the locus was originally developed and citation for the primer.

Table 1 Characteristics of 20 *Coregonus* populations from the Central Alpine Region of Europe

Table 2 Six microsatellite DNA loci used to evaluate *Coregonus* populations of the Central Alpine region of Europe

Components of genetic variance were computed at three hierarchical levels for two different population structures: within populations (Φ_{ST}); among populations within lakes or forms (Φ_{SC}); and among lakes or forms (Φ_{CT}). The resulting values were tested for significant departure from zero using permutations ($n = 1000$) of multilocus haplotypes within and among populations and lakes or forms. Pairwise estimates of population differentiation were also calculated using ARLEQUIN 1.1.

Estimates of population divergence

Methods to quantify population divergence, based on microsatellite polymorphism, are still under debate (Goodman 1997; Valsecchi *et al.* 1997). Alternative mutation models are employed and gauge population divergence through model-specific distance estimators (e.g. Goldstein *et al.* 1995; Takezaki & Nei 1996; Feldman *et al.* 1997). Recently, Paetkau *et al.* (1997) tested the performance of six different genetic distance measures at a number of timescales, ranging from recent divergence to that of several million years. While some measures (such as Nei's distance) performed well at the finest scale, others specifically designed to accommodate microsatellite mutational processes, such as $\delta\mu^2$, did not. Given this, it was elected in this study to estimate population divergence using chord distances (Cavalli-Sforza & Edwards 1967) and Nei's unbiased genetic distance (Nei 1978) generated in POPGENE 1.21.

Ordination and clustering of populations

A tree topology was first derived from a matrix of chord distances. Using PHYLIP 3.5c (Felsenstein 1989) a majority-rule consensus topology was generated (1000 bootstrap replications) and clustered with the algorithm implemented in the FITCH option.

Principal coordinate analysis (PCOORD; Sneath & Sokal 1973: p. 248) can be used to ordinate (i.e. derive principal components from) a distance matrix without access to either the raw, original data, or its variance-covariance structure. PCOORD was used to array the study forms along the first three principal components derived from Nei's genetic distances, using the multivariate analysis program NTSYSPC, version 2.01 (F. James Rohlf, Exeter Software, Setauket, NY). Several authors (cited in Legendre & Legendre 1984: p. 314) noted that benefits of both ordination and clustering can be obtained in a single analysis. For example, correct positioning of main clusters can be obtained using PCOORD, while finer relationships between most similar pairs can be produced using single linkage clustering. Here, a minimum-spanning tree (MST) was superimposed onto the PCOORD ordination, again using NTSYSPC.

Alternative evolutionary models and their testing

From the various evolutionary scenarios offered in the literature to explain discordant variation among and between *Coregonus* populations (Smith & Todd 1984), three alternative hypotheses were formulated (Fig. 1) and predicted patterns tested against genetic data. Model I represents an hypothesis of phenotypic plasticity among ecophenotypes and predicts no significant within-lake heterogeneity in allele frequencies.

Model II offers the hypothesis that divergent forms arose through multiple invasions. To develop this model, each population was compared with every other population in pairwise evaluations. If both were the same form (i.e. small vs. small, for example) then the corresponding cell of the matrix was scored as 0. If each was a different form, then the score = 1.

Model III depicts the evolutionary pattern that would result if a species-flock hypothesis was in operation. Again, each population was compared one against another. If both were from the same lake, they were scored as 0. If each population was of the same form but from a sister lake, then they were scored as 1. If each was from a different lake, then their score = 2.

Mantel (1967) devised a generalized, nonparametric regression approach to matrix comparison (described in Douglas *et al.* 1999). Because the test is nonparametric, distributional abnormalities associated with data collected from different sources (e.g. genetic, morphological, ecological, geographical, etc.) are avoided. In addition, potential problems with comparing continuous vs. categorical data (here, genetic vs. hypothesis matrices) are also negated (e.g. Douglas & Endler 1982; Douglas & Matthews 1992; Smouse & Long 1992).

Pairwise Mantel tests were used to evaluate a null hypothesis that assumes no significant covariation when genetic divergence among populations were compared against assumed relationships derived from model II and model III. The Bonferroni technique (Harris 1975: pp. 96–101) was applied to assign significance level and establish a probability level for making Type 1 errors (i.e. identifying a matrix comparison as significant when it is not; see also Douglas & Endler 1982; Douglas & Matthews 1992).

Results

Microsatellite DNA variation

Microsatellite loci were chosen by evaluating success of amplification and extent of polymorphism. Of 30 primer pairs, 25 (83%) successfully amplified loci in Central Alpine *Coregonus*. Eleven possessed one or two alleles, while 14 displayed moderate-to-high polymorphism. Five of the latter were selected for subsequent analysis (Table 2). One primer (PuPuPy) amplified two independent loci, both of

which were used for analysis (i.e. PuPuPy-300: 330–381 bp; PuPuPy-600: 587–641 bp). Both PuPuPy loci consisted of trinucleotide repeats, while the remaining four loci consisted of dinucleotide repeats. Genetic diversity was moderate to high at six loci (Appendix I). The number of alleles per locus averaged 8.7 ± 3.78 and overall mean H_E was 0.65 ± 0.16 . There was no evidence of deviation from HWE or linkage disequilibrium, hence confirming that samples indeed represented single populations and not a mixture of populations.

Within-population genetic diversity was similar among populations and/or forms, although some lake-specific characteristics were identified (Appendix I). ACH1 showed low numbers of alleles at most loci and a different size range of alleles at locus Cocl-23, when compared with Rhine River populations. Among the latter, THU2 and THU3 displayed low numbers of alleles, and KON populations had elevated numbers at Cocl-23, PuPuPy-600 and BWF-2. Thirty-two alleles were specific for a particular lake or lake aggregate (NEU/BIN = 6, THU/BRI = 6, URN = 1, ZUR/WAL = 7, KON = 10 and ACH = 2).

Population divergence

All sympatric forms were significantly divergent in allele frequencies, refuting the hypothesis of phenotypic plasticity within a single, lake-specific gene pool. Furthermore, all except one of the allopatric pairs of populations were sig-

nificantly divergent ($\chi^2 = \infty$, $P < 0.008$, Bonferroni-adjusted P -value). The nonsignificant comparison ($\chi^2 = 24.7$, $P = 0.015$ for WAL2/ZUR2) involved two ecologically similar forms in geographically proximate lakes. However, sample size was small for ZUR2 (Table 1). Similarly, population divergence was indicated by pairwise F_{ST} ; values ranged from 0.001 to 0.253 (mean 0.088) and were significantly different from zero in all but two pairs ($F_{ST} = 0.001$, $P = 0.28$ for BIN2/NEU2; $F_{ST} = 0.002$, $P = 0.22$ for BRI2/THU5). Again, nonsignificant cases involved ecologically similar forms in geographically proximate lakes.

Pairwise Φ_{ST} values ranged from -0.018 to 0.835 (mean 0.158). No significant divergence was found between four sympatric pairs of populations ($\Phi_{ST} = -0.007$, $P = 0.52$ for BIN1/BIN2; $\Phi_{ST} = 0.010$, $P = 0.22$ for THU2/THU5; $\Phi_{ST} = 0.023$, $P = 0.08$ for KON1/KON2; and $\Phi_{ST} = 0.012$, $P = 0.18$ for KON2/KON3) and eight allopatric pairs (data not provided).

AMOVA revealed that 19.1% of the total variance in allele frequencies was caused by among-lake heterogeneity ($\Phi_{CT} = 0.191$) and 4.8% was caused by differences among forms within lakes ($\Phi_{SC} = 0.059$; Table 3). The remainder (76.2%) was caused by variation within populations ($\Phi_{ST} = 0.238$). Considering only allele frequencies (regardless of size), 4.7% of the total variance was distributed among lakes, 4.7% within lakes, and 90.5% within populations (Table 3). These results indicate that mutation substantially contributed to genetic differentiation among lakes (but not within lakes). Excluding the

Source of variation	Variance component		Fixation indices	P
	V	%		
Among lakes	0.10	4.74	F_{CT} 0.047	<0.0000
	38.09	19.06	Φ_{CT} 0.191	<0.0000
Among populations/ within lakes	0.10	4.72	F_{SC} 0.049	<0.0000
	9.48	4.75	Φ_{SC} 0.059	<0.0000
Within populations	1.89	90.54	F_{ST} 0.095	<0.0000
	152.23	76.19	Φ_{ST} 0.238	<0.0000
Among forms	0.02	0.77	F_{CT} 0.008	0.0694
	-3.16	-1.65	Φ_{CT} -0.016	0.7214
Among populations/ within forms	0.17	8.32	F_{SC} 0.084	<0.0000
	43.00	22.39	Φ_{SC} 0.220	<0.0000
Within populations	1.89	90.91	F_{ST} 0.090	<0.0000
	152.23	79.26	Φ_{ST} 0.207	<0.0000

Listed are test statistics, including variance (V) and haplotypic correlation at corresponding level (F or Φ). Also listed is the probability (P) of a more extreme variance component than that observed. The upper section of table indicates results for population structure by lakes or lake aggregates (as per Appendix I) and the lower section of table provides results by forms (as per Table 1). For each statistic, upper values are based on simple allele frequencies, while lower values were derived by taking allele size into account.

Table 3 Hierarchical analysis of molecular variance based on six microsatellite DNA loci in 20 populations of Central Alpine Coregonus

Danube River population (ACH1) and evaluating only the 19 Rhine River populations, distribution of allele size-based variance changed to 9.1% among lakes ($\Phi_{CT} = 0.093$), 5.1% within lakes ($\Phi_{SC} = 0.056$) and 85.6% within populations ($\Phi_{ST} = 0.144$). This suggests that patterns of genetic structuring are mirrored within and among drainages.

Partitioning genetic structure based on forms, rather than lakes, indicated that only 0.8% of total genetic variation was distributed among forms, 8.3% occurred within forms and 90.9% occurred within populations (Table 3).

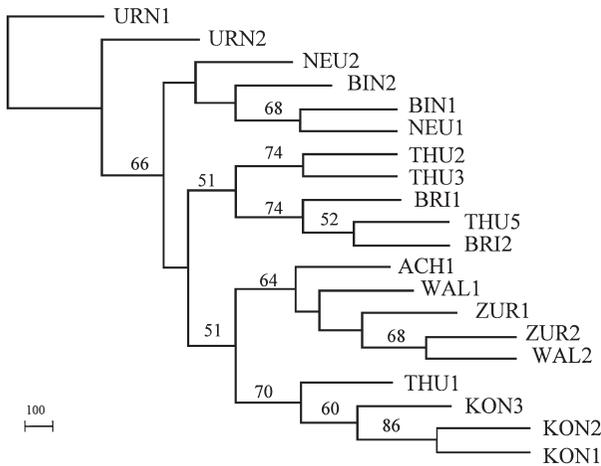


Fig. 3 Unrooted, majority-rule consensus tree representing 1000 bootstrap replicates of chord distance data clustered with the FITCH algorithm. Values on nodes indicate percentage of occurrence of this particular node; values smaller than 50% are not shown. Abbreviations for populations and their lakes of origin are listed in Table 1.

Again, if allele size was taken into account, variation changed to - 1.7% for among forms, 22.4% for within forms and 79.3% for within populations (Table 3). Thus, genetic structuring was more pronounced among lakes rather than among forms.

Ordination and clustering of populations

A majority-rule consensus tree (based on 1000 bootstrap-replicated chord distance matrices) grouped populations by lake or sister lake rather than by forms (Fig. 3). Most internal nodes were consistent (i.e. bootstrap values > 70%). However, populations within these lake groups were labile, such that no percentage occurrence was greater than 50% (bootstrap values not indicated). Ecologically equivalent forms often formed subclusters within these sister lake assemblages. THU1 clustered with the KON populations, but not, as expected, with THU/BRI populations (see Note added in proof). Also, ACH1 clustered with ZUR/WAL populations, rather than separately from all Rhine River populations.

Major relationships among lakes and populations were confirmed by PCOORD of Nei's distances, as were finer relationships among populations by superimposed MST (Fig. 4). Again, sympatric populations were generally close, with equivalent forms from sister lakes closest. However, PCOORD clearly positioned ACH1 distantly from all Rhine populations, and KON populations were also separate from all others. MST connected all five populations from THU/BRI, although THU1 was linked equidistant between THU2 and KON2 and seemingly clustered in three-dimensional space close to KON3. Furthermore, although placed in proximity by PCOORD,

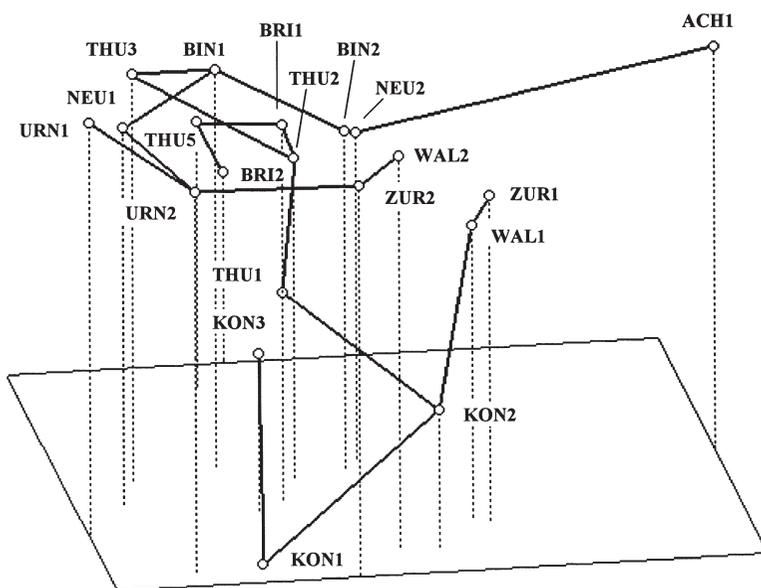


Fig. 4 Single-linkage cluster analysis of 20 Central Alpine *Coregonus* populations superimposed upon the first three axes of a principal coordinates (PCOORD) ordination of Nei's genetic distances for the same populations. Abbreviations for populations and their lakes of origin are listed in Table 1.

MST did not connect the small and large forms of lakes ZUR/WAL. This demonstrated the utility of superimposing single-linkage and eigenvector analyses.

Mantel tests

The results presented in Table 4 indicated that matrices of Nei's and chord distances were significantly associated with scores for model III. The association between Nei's distances, chord distances and scores derived from model II were small and nonsignificant (Table 4). The two alternative models were themselves uncorrelated, whereas those for chord and Nei's distances were significantly associated. These results support the hypothesis that forms within lakes (or sister lakes) comprise species flocks.

Discussion

Central Alpine *Coregonus* provide an excellent opportunity for investigating evolutionary divergence within and among lakes because populations are land-locked and date back to postglacial colonization. Divergence would thus represent *in situ* mutation, drift and/or selection. Surprisingly, genetic studies on these forms are scarce and those that have been performed were of limited scope. One study failed to compare sympatric populations (Luczynski & Ritterbusch-Nauwerck 1995), and several others re-evaluated simply the same sympatric pair from Lake Konstanz (i.e. populations KON1 and KON2). Among the latter populations, two studies did not find differences (Löffler 1985; Luczynski *et al.* 1995), while two others (Vuorinen *et al.* 1986; Hecht *et al.* 1987) detected slight differentiation. Genetic differences were elusive in these studies because markers were utilized with resolving power insufficient for the evolutionary time frame under investigation. Some evolutionary events (such as postglacial colonization of

northern habitats, or radiation of species flocks in temperate freshwater lakes), have not been of sufficient duration to produce allozyme or mitochondrial DNA (mtDNA) variants among populations (Avisé *et al.* 1984). Thus, phylogeography cannot be inferred using these markers (Meyer *et al.* 1990; Brunner *et al.* 1998). Unfortunately, rather than taking temporal limitations into account, most studies on Central Alpine *Coregonus* instead argued that forms under consideration did not represent distinct gene pools (e.g. Löffler 1985), in spite of trenchant ecological and morphological differences (Ruhlé 1986).

Bernatchez & Dodson (1994) concluded from mtDNA haplotypes that Central Alpine *Coregonus* formed a lineage that was phylogenetically distinct from North European populations. Thus, the possibility that all European populations should be consolidated into *C. lavaretus* (Steinmann 1950a,b) must be rejected (Kottelat 1997: pp. 101–102).

In this study, microsatellite DNA analysis identified high levels of genetic variation in Central Alpine *Coregonus* populations. Furthermore, the pattern of genetic diversity best fits a species-flock hypothesis and was significantly different from a model that defines forms as product of phenotypic plasticity within a single gene pool (i.e. ecophenotypes).

Genetic differentiation within populations

Conservation of microsatellite loci (or their flanking region) has been reported for a number of fish taxa (Rico *et al.* 1996; Zardoya *et al.* 1996), including the Salmonidae (Angers & Bernatchez 1996; Presa & Guyomard 1996). Although primers in this study were originally developed for other salmoniforms (Table 2), their origin did not affect the level of variation detectable (as noted by Moore *et al.* 1991). Polymorphism at the six microsatellite loci was comparable with that detected in *Salvelinus fontinalis* (Angers *et al.* 1995) and *S. alpinus* (Brunner *et al.* 1998), but higher than reported for *Salmo salar* (Tessier *et al.* 1995, 1997). At locus Sfo-23, Angers & Bernatchez (1996) detected 16 distinct alleles in *S. fontinalis* populations, and reported levels of H_E ranging from 0.45 to 0.71. Using the same primers, Brunner *et al.* (1998) detected 49 different alleles in Central Alpine *S. alpinus*, with H_E values ranging from 0.59 to 0.95. In this study, 27 different alleles were observed, with H_E values between 0.49 and 0.93 (Appendix I).

Levels of genetic diversity were similar among populations (Appendix I), albeit with some deviations. All three populations from Lake Konstanz (the largest lake in the study; see Dill 1990) had elevated levels of genetic diversity. A possible explanation might be that Lake Konstanz sustains larger populations and is therefore less susceptible to bottlenecks. Low genetic diversity was detected in two populations from Lake Thun. One (THU2) repre-

Table 4 Results of Mantel tests comparing the four test matrices in this study

Comparison	NEI	CHORD	MODEL II	MODEL III
NEI	–	6.577*	1.475	6.480*
CHORD	0.803	–	0.001	7.162*
Model II	0.089	0.001	–	0.529
Model III	0.454	0.501	0.006	–

NEI, matrix of Nei's unbiased genetic distance.

CHORD, matrix of Cavalli-Sforza and Edwards' chord measure; MODEL II, matrix representing multiple invasions of lakes; MODEL III, matrix representing lakes as species flocks.

Values above the diagonal represent pairwise student *t* scores, while values below the diagonal represent matrix correlation coefficients.

Significance at Bonferroni-adjusted value ($P < 0.017$) is indicated by an asterisk (*).

sented a controversial form not recognized as distinct by some authors (Roth & Geiger 1972; Kirchhofer & Tschumi 1986) and now presumably extinct via hybridization (Rufli 1978). In this instance, lower genetic diversity might reflect bottlenecking in a discrete gene pool rather than hybridization with another form (Hartl & Clark 1989). The second population (THU3) is considered morphologically and ecologically distinct, but rare (Kirchhofer & Tschumi 1986). The only population from the Danube River system (ACH1) had slightly lower genetic diversity and showed aberrant allele size distribution at two loci (BWF-2 and Cocl-23) when compared with the 19 Rhine River populations (Appendix I). However, conclusions for the entire drainage should not be based on results from a single population. Preliminary results indicate that other populations in the Danube drainage are more variable than ACH1 (M. R. Douglas, unpublished results).

Genetic differentiation among populations

All pairs of populations were significantly differentiated based on allele frequencies (except for one pair). Similar results were also indicated by pairwise F_{ST} , although two pairs were not significantly different. Marginally significant F -values occurred between morphologically and/or ecologically equivalent forms in geographically proximate sister lakes. From an evolutionary perspective, these lakes might have originally formed a single body of water that only recently divided into separate basins (Steinmann 1951). Thus, populations from each lake would be separated for a much shorter duration of time, expanding the potential for relatively recent gene flow events among them. From a conservation standpoint, it is important to note that all three sister lakes experienced historic alterations in hydrology (Bär 1976: p. 37; Kirchhofer 1996). Today, all are connected by channels, which either 'improved' existing hydrological connections between lakes or were constructed as a novelty (Vischer 1986). Nevertheless, it would be safe to say, given the underestimation of F_{ST} -based results (Slatkin 1995), that these forms are reproductively isolated populations. Significant differences found among populations argue against contemporary gene flow and clearly indicate that the 20 populations are discrete units. The hypothesis that sympatric forms reflect phenotypic plasticity within a single gene pool (model I) can therefore be rejected for Central Alpine populations.

The a priori assignment of specimens to particular populations was corroborated by tests of HWE, which showed no evidence of sampling across populations or population subdivision (i.e. heterozygote deficiency; Hartl & Clark 1989). Segregation during reproduction is a characteristic evolutionary behaviour in salmoniform fishes (Behnke 1972) and could be a driving force in the intralacustrine diversification of this group (Mayr 1984).

Ecophenotypes/ecotypes

Diversity in taxa below the species level has been described in a number of ways, using vaguely defined terms such as ecophenotype, ecotype, or geographical race, with obscure distinction between them (Douglas *et al.* 1999). Ecotypy is defined as accumulation of genetic differences between individuals of the same species living at different points in an ecological gradient, such that individuals differ from one another in aspects of appearance or life history. An ecophenotype, on the other hand, also represents a distinct form of a species occurring in a different habitat, but differs from an ecotype in that a genetic component is not involved (Mayr 1942). For the purpose of the discussion below, the two terms will be used synonymously (authors often do not distinguish between the two and just apply one or the other.) Ecotypy was defined by Turresson (1922) and first mentioned in the ichthyological literature by Hubbs (1940). It was subsequently referred to by a number of authors (Douglas *et al.* 1999 and references therein).

Steinmann (1950a,b, 1951) was thus contemporary when he applied the concept of ecotypy to explain diversity in Central Alpine *Coregonus*. While he captured quite well the variability of these forms through a comprehensive study of all lakes known to contain coregonids at that time, he ultimately failed in his holistic approach by forcing all forms into just several described 'ecotypes' (see Kottelat 1997). Some lakes contained forms that just did not fit his model. Although he mentioned sympatric divergence as the mechanism producing different forms within lakes, he considered all as a single polytypic species. Therefore, in each lake, the same ecotypes had to emerge. Steinmann could not test homoplasy or homology of traits, for he investigated morphological and ecological characters that were labile.

Relatedness of populations

Results presented herein suggest relatedness within rather than among lakes. Lake-specific assemblages (including sister-lake groupings) were distinct in both the PCOORD analysis with superimposed MST (Fig. 4) and the cluster analysis based on chord distances (Fig. 3). While linkage was often weak between large groups of sister lakes, the nodes within these groupings were relatively stable (Fig. 3). In situations where similar forms from sister lakes grouped together, they could indeed represent the same species, as suggested by Kottelat (1997). Deviant clustering of population THU1 in the consensus tree of chord distances (Fig. 3) was clarified by PCOORD analysis (Fig. 4) and is probably caused by reduction of a three-dimensional eigen analysis into a two-dimensional clustering.

AMOVA (Table 3) revealed that genetic variation in Central Alpine *Coregonus* is largely attributable to among-lake differences in allele size. The latter contributes little to divergence within lakes. In other words, alleles are of similar size among sympatric populations, but show lake-specific differences. Such a pattern is congruent with genetic divergence following postglacial recolonization when gene flow among lakes was greatly reduced compared to that within lakes or sister lakes (i.e. model III). Furthermore, AMOVA did not detect any trace of among-lake genetic structuring based on form (Table 3). An alternative hypothesis for within-lake similarity (i.e. extensive gene flow among sympatric populations that originated from different lineages; model II) seems therefore less plausible.

Mantel tests revealed that patterns of genetic relatedness and divergence among populations are best explained by a species-flock hypothesis (model III). Matrices of either chord or Nei's distances were not significantly associated with relationships predicted by the multiple-invasion hypothesis (model II). Moreover, matrices representing the two alternative models were themselves not correlated (Table 4). This is important in that it shows the two models clearly explain distinct patterns of population divergence, with no conflict or interference. Therefore, genetic diversity among populations of Central Alpine *Coregonus* is best explained by repeated, within-lake divergence of distinct forms, paralleled among lakes.

The paucity of molecular studies on Central Alpine *Coregonus* prevents comparison of results presented herein. In a mtDNA analysis, Bernatchez & Dodson (1994) found evidence of form-specific rather than lake-specific relationships among populations, but small sample sizes ($n = 12$) limit interpretations (for further discussion see Kottelat 1997: pp. 100–103). While this did not impact the global study of interest to Bernatchez & Dodson, it would affect resolution on a microgeographical scale. In addition, mtDNA is a marker with limited resolution for the Central Alpine situation (see above). Microsatellite markers provide more detailed insight into recent evolutionary events, and their patterns were quantitatively tested. Both aspects give weight to findings herein. The possibility that both within-lake radiation and secondary contact were responsible for similarity among populations within lakes or sister lakes, cannot be ruled out completely and remains to be tested with additional markers (M. R. Douglas, manuscript in preparation).

Coregonid radiation and Pleistocene glaciation

How do the results presented herein juxtapose with the known evolutionary history of the Coregonidae? Radiation and diversification of this group are intimately linked with Pleistocene events (Behnke 1972). Conditions prevalent during glacial retreat must have provided

numerous habitats for cold-water fishes to thrive. Indeed, estimates of mtDNA sequence divergence among North American coregonids (Bernatchez & Dodson 1991; Bodaly *et al.* 1992) suggest that radiation occurred relatively recently, and that origin of putative taxa spanned recent Pleistocene glaciations. A similar time frame was also estimated for radiation of European coregonids (Bernatchez & Dodson 1994).

Invasion of the Central Alpine region occurred along the retreating ice sheets. Presumably, habitat changes were quite dynamic, with lakes forming and disappearing in a rapidly changing environment that favoured a quick response to new conditions. Behnke (1972) suggested that colonization of recently deglaciated waters would allow radiation into unoccupied niches and result in rapid evolution of species over short temporal and spatial scales. In contrast, Hewitt (1996) argued that repeated geographical contraction and expansion of populations during Pleistocene climate fluctuations would allow adaptations and differences to accumulate slowly over time, protected from mixing zones where hybridization could occur.

Several studies examined these scenarios in the context of coregonid radiation (for example, Smith & Todd 1984; Pigeon *et al.* 1997). While Hewitt's scenario was the one most frequently supported (e.g. Bernatchez & Dodson 1990; Bernatchez *et al.* 1996), some evidence did point to postglacial, sympatric radiation (Bodaly *et al.* 1992; Bernatchez *et al.* 1996; Pigeon *et al.* 1997). Regardless of which scenario is true, Central Alpine coregonid evolution clearly took place rather rapidly. Hence, if assemblages of Central Alpine *Coregonus* represent species flocks, they must have evolved in the past 10 000 years, which would represent a remarkable rate of diversification.

Evolution of species flocks

Classic examples of lacustrine species flocks are the African lake cichlids and the cyprinids of lake Lanao, Philippines (see Echelle & Kornfield 1984). Greenwood (1984, p.18) reviewed definitions and concept of a species flock, and stated controversy and problems: 'Clearly then, the central issue in any attempt to formulate a species-flock concept and identify species flocks must hinge on the monophyly of its members and the level of universality at which that monophyly is determined. In other words, an aggregate of several species should be identified as a flock only if its members are endemic to the geographically circumscribed area under consideration and are each others' closest relatives.'

Opposition was less against the concept of 'species flock' itself, but more against a plausible underlying speciation process. Particularly, the term 'sympatric sister species' became tangled with the controversial issue of 'sympatric speciation' (Greenwood 1984). Rejection of the

latter concept inhibited recognition of the former. Recently, sympatric divergence has become an acceptable scenario as a mode of animal speciation (Schluter & McPhail 1992; Taylor & Bentzen 1993b; Bush 1994), and with it the recognition of 'sympatric sister species' (Knowlton & Jackson 1994).

Conclusions

The driving forces behind radiation of *Coregonus* cannot be tested. However, the signature remnant from this process can be. This study showed that the various forms found in lakes of the Central Alpine region represent distinct gene pools and that their morphological and ecological diversity is not a product of phenotypic plasticity. Patterns of genetic diversity were quantitatively tested against two models representing alternative evolutionary scenarios. A species-flock model was most congruent with extant genetic pattern, whereas a scenario assuming multiple invasions by lineages already differentiated was not. While results presented herein provide insight into pattern and process of Central Alpine *Coregonus* evolution, they do not define taxonomic status of the forms under study. This is because taxonomic decision should not be based on levels of molecular divergence alone (Avice 1976: p. 115). There is evidence that within some groups, such as *Cyprinodon* (Miller 1961) or *Gasterosteus* (Schluter & McPhail 1992), radiation and speciation can occur rather rapidly even though accumulation of genetic differences is much slower (Turner 1974; Schluter 1996).

Hence, the next steps are as follows. First, to determine if genetic relationships and mode of speciation (as determined herein) covary with morphological aspects of these forms and their geographical relationships. Second, the connection between endemics and known introductions must be clarified, and a plan established to conserve and manage these groups. Finally, their taxonomic history must be reviewed and (with data in hand), a final decision made with regard to their status. If the different forms are judged as discrete entities (as suggested by Kottelat 1997), then formal descriptions must be completed (M. R. Douglas, manuscript in preparation).

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Note added in proof

The origin of population THU1 may actually be Lake Konstanz (KON), as depicted in Figs 3 and 4. M.R.D. presented this study in a seminar at the University of Zurich on 8 January 1999. Dr Peter Friedli (Fisheries Chief of Canton Bern) attended the seminar and was intrigued by the results. There are no records of coregonid stocking from Lake Konstanz into Lake Thun, but Dr Friedli asked older fishermen about the possibility of undocumented stocking activities. Fishermen (and sons of fishermen) informed him that in the late 1920s or early 1930s, when no hatchery was yet present at Lake Thun, experimental introductions of fertilized eggs from Lake Konstanz had occurred. Understandably, there was some ambiguity regarding numbers of incidents and whom was involved. Since 1946, Prof. H. Roth (Dr. Friedli's predecessor) banned non-indigenous stocking of coregonids into Lake Thun, and the latter perspective is foremost in people's minds (i.e. Lake Thun has never been stocked). This oral history underscores two points: conservation genetics has the potential to elucidate incidents for which historical records are lacking, and involvement of resource managers (or individuals close to the resource) is imperative for studies such as this.

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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 Marlis Douglas' PhD research on systematics and evolutionary biology of Central Alpine *Coregonus*. Patrick Brunner's PhD studies focused on similar questions in *Salvelinus alpinus*. The work was carried out whilst visiting the laboratory of Louis Bernatchez whose major research interests centre on the understanding of patterns and processes of molecular and organismal evolution, as well as their significance for conservation.

Appendix I Summary of variation detected at six microsatellite loci from 20 *Coregonus* populations from the Central Alpine region of Europe

Lakes	Neuchâtel & Biènné*			Thun & Brienz*			Urnersee*			Zürich & Walenstadt*			Konstanz*			Achen*				
	NEU1 (30)	NEU2 (30)	BIN1 (26)	BIN2 (30)	THU1 (30)	THU2 (30)	THU3 (30)	THU5 (30)	BRI1 (30)	BRI2 (30)	URN1 (30)	URN2 (30)	ZUR1 (30)	ZUR2 (17)	WAL1 (30)	WAL2 (30)	KON1 (30)	KON2 (30)	KON3 (30)	ACHI (30)
SFO-23	14	15	10	11	15	9	15	14	8	12	15	12	10	11	9	11	15	10	14	7
N _A	0.90	0.93	0.73	0.80	0.80	0.60	0.87	0.76	0.51	0.75	0.67	0.70	0.73	0.88	0.70	0.89	0.87	0.80	0.90	0.77
H _O	0.90	0.85	0.78	0.72	0.79	0.62	0.82	0.70	0.49	0.66	0.75	0.78	0.70	0.80	0.63	0.86	0.87	0.72	0.90	0.69
H _E	166	158	166	170	162	166	166	166	164	160	162	170	166	158	166	158	166	166	166	166
Min (bp)	202	198	202	204	206	202	214	214	190	198	200	202	196	190	196	198	208	198	198	200
Max (bp)	170	170	170	170	170	170	170	170	170	170	170	170	170	170	170	170	170	170	170	170
Mode (bp)	6	8	10	8	7	5	4	5	6	7	7	6	7	5	9	9	8	7	8	4
BWF-1	0.57	0.80	0.73	0.71	0.67	0.59	0.10	0.67	0.87	0.60	0.47	0.20	0.72	0.47	0.83	0.63	0.73	0.62	0.80	0.48
N _A	0.53	0.73	0.68	0.70	0.65	0.47	0.10	0.70	0.79	0.68	0.47	0.22	0.74	0.45	0.73	0.60	0.76	0.67	0.75	0.62
H _O	208	208	208	210	214	214	220	214	214	218	214	218	216	218	214	214	214	214	216	214
H _E	226	230	228	226	228	226	226	226	226	230	228	228	230	228	230	234	228	228	228	224
Min (bp)	224	220	224	220	224	224	224	226	224	226	224	224	224	224	224	224	224	224	224	224
Max (bp)	224	220	224	220	224	224	224	226	224	226	224	224	224	224	224	224	224	224	224	224
Mode (bp)	224	220	224	220	224	224	224	226	224	226	224	224	224	224	224	224	224	224	224	224

