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# Evidence for independent origin of two spring-spawning ciscoes (Salmoniformes: Coregonidae) in Germany

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Combined analyses of mitochondrial DNA (mtDNA) and microsatellite loci were performed to assess the genetic differentiation of two spring-spawning ciscoes from each other and from sympatric *Coregonus albula* in two German lakes. Polymorphism was screened at six microsatellite loci and mtDNA for a total of 247 and 94 ciscoes, respectively. Microsatellite data showed a weak differentiation between spring-spawning *Coregonus fontanae* and sympatric *C. albula* in Lake Stechlin ($F_{ST}$ = 0–0·008), whereas a significant differentiation was observed between spring-spawning *Coregonus lucinensis* and sympatric *C. albula* in Lake Breiter Luzin ($F_{ST}$ = 0·013–0·039). A more pronounced genetic difference was observed between both spring-spawning species ($F_{ST}$ = 0·05–0·128). Shared mtDNA haplotypes among sympatric species within both Lake Stechlin and Lake Breiter Luzin were observed, whereas no haplotype was shared between *C. fontanae* and *C. lucinensis*. These results suggest an independent origin for spring-spawning ciscoes in each lake. Evidence is also provided for mtDNA introgression of *Coregonus sardinella* into *C. lucinensis* and *C. albula* in Lake Breiter Luzin. Postglacially, this species or at least a population which showed mtDNA introgression has colonized the Baltic Sea basin up to the glacial margin that was located between Lakes Stechlin and Breiter Luzin. 

Key Words: *Coregonus albula*; introgressive hybridization; sympatric speciation.

## INTRODUCTION

Because of their widespread distribution, commercial importance and high phenotypic diversity throughout the northern hemisphere, coregonids have been the subject of intensive research efforts for more than a century (Clark, 1885; Thienemann, 1933; Pravdin, 1936; van Oosten, 1942; Svärdson, 1979; Bernatchez, 2004).

Of particular interest is the occurrence of sympatric pairs and flocks of divergent and reproductively isolated populations (Taylor, 1999). This common phenomenon of sympatric pairs and flocks originates mainly from two

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evolutionary forces. First, fish populations were isolated by geographic barriers, in northern fish species mainly given by the alternating ice covers of glaciers in the past, and then came into secondary contacts (Taylor, 1999). Second, species pairs and flocks might evolve in sympatry (Wilson *et al.*, 2000). Recent molecular studies indicated that evolution of similar phenotypes in distinct evolutionary lineages might be a common phenomenon in coregonids (Bernatchez *et al.*, 1996; Pigeon *et al.*, 1997; Douglas *et al.*, 1999; Taylor, 1999).

Traditionally, all cisco populations from the Baltic Sea basin have been identified as vendace, *Coregonus albula* (L.). *Coregonus albula* usually spawns from mid-October to mid-December. The occurrence of other sympatric 'forms' of ciscoes that are obviously distinguished by differential spawning times or maturity size has long been reported from several lakes in Germany, Finland, Sweden and Russia (Günther, 1866; Mäklin, 1869; Smitt, 1886; Trybom, 1903; Ekman, 1909; Thienemann, 1933; Pravdin, 1936; Airaksinen, 1968; Svärdson, 1979; Vuorinen & Lankinen, 1978; Anwand *et al.*, 1997, Anwand, 1998). The co-occurring ciscoes spawn during winter or spring (mid-December to mid-February for winter-spawning; March to June for spring-spawning) (Vuorinen & Lankinen, 1978; Svärdson, 1979; Schulz & Freyhof, 2003). Svärdson (1979) described the spring-spawning cisco from Swedish Lake Ören as a distinct species: *Coregonus trybomi* Svärdson, also occurring in Swedish Lakes Hålsjön, Äsunden and Fegen. He considered *C. trybomi* to be similar to *C. albula* and he only considered the spawning time (linkage of spawning to increasing photoperiod) as diagnostic character. By studying enzyme gene variability of Finnish populations, Vuorinen *et al.* (1981) concluded that autumn-, winter- and spring-spawning populations do not form monophyletic units. Because their results support a polyphyletic evolution of spring-spawning ciscoes, Vuorinen *et al.* (1981) tentatively refused to consider *C. trybomi* as a valid species.

In German lakes, the existence of spring-spawning ciscoes different from *C. albula* was recently reviewed by Schulz & Freyhof (2003), who distinguished two species by morphological characters: *Coregonus lucinensis* Thienemann, endemic to Lake Breiter Luzin and *Coregonus fontanae* Schulz & Freyhof, endemic to Lake Stechlin. Characters used to distinguish species were scales in the lateral line, interorbital distance related to dorsal head length ($L_H$), head width, body depth and the length of the last gill raker in the upper branch of the first gill arch related to the length of outer gill filament and dorsal $L_H$ (Schulz & Freyhof, 2003). Both species co-occur with *C. albula*. While Vuorinen *et al.* (1981) studied ciscoes from geographically distant lakes, Lake Breiter Luzin and Lake Stechlin are only 30 km apart. Both lakes also belong to the same water drainage system (River Elbe) and it is therefore obvious that one species of spring-spawning ciscoes might have been able to migrate from one lake to the other or might even have been stocked accidentally. Therefore, it was the aim of the present study to test if both spring-spawning ciscoes evolved from one ancestor and subsequently came into secondary contact with autumn-spawning ciscoes in each lake, or if both spring-spawning ciscoes might have an independent origin.

## MATERIALS AND METHODS

### STUDY SITES AND SAMPLING OF THE BIOLOGICAL MATERIAL

Lake Stechlin (Germany, Brandenburg, 53°10′ N; 13°02′ E) and Lake Breiter Luzin (Germany, Mecklenburg-Vorpommern, 53°21′ N; 13°27′ E) are medium sized lakes (4·25 and 2·68 $km^2$, respectively). Both are deep (Lake Stechlin: maximum depth = 68·5 m, mean depth = 23 m, Lake Breiter Luzin: maximum depth = 58·5 m, mean depth = 21·3 m) and dimictic stratified lakes. Lake Stechlin is oligotrophic, whereas Lake Breiter Luzin is mesotrophic (Koschel *et al*., 1983, 2002). Both lakes were formed following the Weichselian glaciation *c*. 12 000 years ago (Marcinek & Nitz, 1973). There is no recent connection between the lakes although a historical connection during the last deglaciation cannot be ruled out.

Individuals of *C. albula* from both lakes were caught using gillnets (12 and 15 mm knot to knot) in December 2000, just prior to spawning. Muscle and fin tissue were individually preserved in 98% ethanol. *Coregonus albula* were unambiguously distinguished from *C. fontanae* and *C. lucinensis* by the state of maturity following Nikolskii (1963); *C. albula* were fully mature in mid-December whereas *C. fontanae* and *C. lucinensis* were at stage 4. The difference in stage of sexual maturation was also used when collecting *C. fontanae* and *C. lucinensis* in April 2001. Those individuals were now in a stage of maturity of 5 to 6 whereas the *C. albula* were at stage 3. Only females were included in the data analysis. Between 43 and 48 individuals of each of the four populations were caught (Table I). Two populations of *C. albula* from Finland (Lake Onkamo: 62°20′ N; 30°00′ E and Lake Kuohijärvi: 61°12′ N; 24°53′ E) were also included in the study to gauge the extant of differentiation between sympatric species relative to those from geographically remote populations.

### GENETIC ANALYSES

DNA was obtained from pectoral fin tissue using a standard phenol-chloroform extraction method (Bernatchez *et al*., 1992). Four microsatellite markers developed for *Coregonus artedi* Lesueur (*Cisco90, Cisco126, Cisco157* and *Cisco200*; Turgeon *et al*., 1999) and two from *Coregonus nasus* (Pallas) (*BWF1* and *BWF2*; Patton *et al*., 1997) were used. All polymerase chain reactions (PCR) were performed individually for each locus. Between 0·2 and 1·0 μl of the PCR product for each locus and 2 μl blue formamide containing 10% of GS 350 internal size standard (GeneScan TAMRA 350 bp ABI) was loaded on an ABI 377 automated sequencer. Fragments were measured in reference to the standard using GeneScan version 2.1 and Genotyper 2.0.

### MITOCHONDRIAL DNA SEQUENCE ANALYSES

Mitochondrial DNA (mtDNA) variation was analysed by directly sequencing PCR amplified products. A 241 bp fragment of the ND-3 region was amplified using primers ND-3-FOR (5′-CATCACCATCGCACTATCCA-3′) and ND-3-REW (5′-CCTCCTTGGGTTCACTCGTA-3′) developed by K. Østbye (unpubl. data). Amplifications were performed in 25 μl reaction volume with 50–75 ng of genomic DNA; 150 μM CTP, GTP, TTP and ATP; 2·5 ml 10X buffer (5·2 mM MgCl), 840 nM of each primer and 0·25 units Taq DNA polymerase. PCR cycles were as follows: 1 × 15 min at 95° C, 25–30 × (1 min at 95° C, 45 s at 48° C, 1 min at 72° C), 1 × 10 min at 72° C. PCR products were purified using the commercial kit QIA-quick (Qiagen, Hilden, Germany). DNA sequences (forward direction) were generated using a 3100 Applied Biosystems (Forster City, CA, U.S.A.) automated sequencer. Sequences were aligned using the multiple sequence editor ALIGN (Hepperle, 2003). A 328 bp fragment of the control region was also sequenced for one representative of each haplotype defined for the ND-3 region. Primers used to sequence the control region

TABLE I. Sample location, population characteristics and sample size for genetic analyses of *Coregonus* species

| Waterbody | Country | Latitude | Longitude | Population | Spawning time | Sample size µsat | ND-3 | D-loop |
|---|---|---|---|---|---|---|---|---|
| Lake Stechlin | Germany | 53°10′ N | 13°02′ E | *C. albula* | Autumn | 48 | 19 | 2 |
| | | | | *C. fontanae* | Spring | 46 | 17 | 5 |
| Lake Breiter Luzin | Germany | 53°21′ N | 13°27′ E | *C. albula* | Autumn | 48 | 20 | 3 |
| | | | | *C. lucinensis* | Spring | 43 | 10 | 4 |
| Lake Onkamo | Finland | 62°20′ N | 30°00′ E | *C. albula* | Autumn | 35 | 9 | 3 |
| Lake Kuohijärvi | Finland | 61°12′ N | 24°53′ E | *C. albula* | Autumn | 29 | 4 | 1 |
| Great Slave Lake | Canada | 61°23′ N | 115°38′ W | *C. sardinella* | Autumn | – | 1 | 1 |
| Avak River | U.S.A. | 71°13′ N | 156°36′ W | *C. sardinella* | Autumn | – | 1 | 1 |
| Shingle Point | Canada | 68°59′ N | 137°23′ W | *C. sardinella* | Autumn | – | 3 | 3 |

were LN20 (5′-CCACTAGCTCCCAAAGCTA-3′) and H2 (5′-ACTTTCTAGGG-TCCATC-3′) of Bernatchez *et al.* (1992). PCR amplifications were performed in a total reaction volume of 12·5 μl with 50–100 ng of genomic DNA, 75 μM CTP, GTP, TTP and ATP, buffer 10X (2·5 mM MgCl), 400 nM of each primer and 0·2 units *Taq*-polymerase. PCR cycles were: 1 × 15 min at 95° C, 25–30 × (1 min at 95° C, 45 s at 45° C, 1 min at 72° C), and 1 × 10 min at 72° C.

In addition to European cisco samples, both of the above mentioned mtDNA regions of five individuals of the least cisco *Coregonus sardinella* Valenciennes, closely related to *C. albula* (Bernatchez *et al.*, 1991) were also sequenced, to use this species as outgroup for phylogenetic analyses. Those samples were collected in Canada (Great Slave Lake, North-west Territories, 61°23′ N; 115°38′ W, Shingle Point, North-west Territories, 68°59′ N; 137°23′ W) and U.S.A. (Avak River, Alaska, 71°13′ N; 156°36′ W) (Turgeon & Bernatchez, 2003).

## DATA ANALYSES

Genetic polymorphism at microsatellites was quantified by the number of alleles per locus ($A$), observed heterozygosity ($H_o$), and unbiased gene diversity ($H_e$) using the GENETIX software, version 4.02 (Belkhir, 2001). Samples were tested for conformity to the Hardy-Weinberg equilibrium under the alternative hypotheses of heterozygote excess or heterozygote deficiency using the score test ($U$-test) (Raymond & Rousset, 1995). Global tests across loci and across samples were also performed using GENEPOP version 3.1d (Raymond & Rousset, 1995). Initial tests for conformity to the Hardy-Weinberg equilibrium revealed pronounced heterozygote deficiencies. This may be caused by non-amplification of the largest alleles or, at least in part, by hybridization between spring- and autumn-spawners. The most likely cause, however, is the occurrence of null alleles given that all loci used were developed from other coregonine species. Consequently, the method of Brookfield (1996) was applied to estimate the proportion of null alleles in all samples where significant deficits were detected for a given locus. Then, allele frequencies were corrected by including a null allele in the raw data with observed frequency = r, and all the following calculations were based on this correction. The null hypothesis of no difference in allelic frequency distribution at each locus between all sample pairs was tested using the Markov chain method to obtain unbiased estimates of Fisher's exact test through 1000 iterations (Guo & Thompson, 1992) available in GENEPOP. Probability values over all loci were obtained by the Fisher method (Sokal & Rohlf, 1995). The extent of genetic differentiation between samples was estimated from the estimator θ (Weir & Cockerham, 1984) of $F_{ST}$ using $F$-stat 1.2 (Goudet, 1996). The 95% CI were estimated by bootstrapping over loci (1000 replicates) using the same programme. Statistical significance was adjusted for multiple comparisons using sequential Bonferroni adjustments (Rice, 1989). Hierarchical patterning of population structuring among populations from the two German lakes was assessed by performing two analyses of molecular variance (AMOVA; Excoffier *et al.*, 1992), using Arlequin (v.2.000) (Schneider *et al.*, 2000). Components of genetic variance were computed, first using lakes as the top level of grouping, and then using forms as the top level of grouping. Significance was assessed using the permutation procedure (n = 1000).

For mtDNA data, pair-wise $F_{ST}$ was estimated based on variance in haplotype frequency as well $\Phi_{ST}$, which integrates mutational information using Arlequin. Statistical significance for $F_{ST}$ and $\Phi_{ST}$ was estimated by permutation procedures (1000 permutations). The hierarchical pattern of mtDNA population structuring was also assessed using the AMOVA, as given in detail for microsatellites. The tests of differences in haplotype frequency as well as the AMOVA were based on ND-3 variances alone. For the phylogenetic analysis, however, mtDNA sequence data from both the ND-3 and D-loop segments were combined and a neighbour-joining tree (maximum parsimony analysis, neighbour-joining method with absolute distance) constructed using PAUP (v. 4.0 beta version) (Swofford, 2002). Confidence in tree topology was assessed by

performing 1000 full-heuristic bootstrap replicates (Felsenstein, 1985). TREEVIEW (v. 1.6.6) (Page, 1996) was used to visualize the tree.

## RESULTS

### GENETIC DIFFERENTIATION AT MICROSATELLITE LOCI

Following the correction for the occurrence of null alleles, departures from the Hardy-Weinberg equilibrium were found for only two loci (*BWF2* and *Cisco200*) that each showed a slight deficit in heterozygotes in a single population (Table II; uncorrected data available at: http://www.igb-berlin.de/abt4/mitarbeiter/Mehner/Appendix_SchulzJFB2006.pdf). All populations differed in one or more descriptive statistics, including the most common allele ($A_C$), the range of the allele size ($A_R$), and the frequencies of the most common alleles ($F_C$) (Table II). Consequently, homogeneity tests of allele frequency distributions following sequential Bonferroni corrections revealed significant differences in all pair-wise sample comparisons. Yet, the most striking differences in allelic composition were observed between *C. fontanae* and *C. lucinensis* (Table II). Heterogeneity in allele frequency translated into significant $\theta$ estimates in all pair-wise comparisons, except between both ciscoes in Lake Stechlin, where difference in $\theta$ was nevertheless near significance ($P = 0{\cdot}07 \pm 0{\cdot}01$) (Table III). Ongoing gene flow or, alternatively, very recent divergence of species, was suggested by the fact that $\theta$ estimates between sympatric forms in each lake were lower than any other pair-wise comparisons (Table III). Another salient observation stemming from Table III is that pair-wise comparisons of $\theta$ estimates involving *C. lucinensis* were significantly higher than any other comparisons (including comparisons with the two populations from Finland), indicating that it was the most genetically distinct population in this study. For example, the mean $\theta$ estimate (0·081) involving that population was significantly higher than the mean estimate of comparisons (0·05) involving the two Finnish populations *v.* the German populations (Mann–Whitney *U*-test, $P < 0{\cdot}05$).

The AMOVA performed on the two German lakes populations revealed that genetic structuring was more pronounced between ciscoes of the same spawning form from different lakes than between populations of the different form within the same lake. For microsatellites, when lakes were used as the top level of grouping, 5·18% of total genetic variance was caused by interlake heterogeneity compared to 1·34% of the variance explained by differences between forms within lakes (Table IV). Partitioning of genetic variance using forms as the top level of grouping indicated that no genetic variance (−2·57%) was explained by differences between forms, whereas 6·63% of the variance was explained by population differences among lakes within forms (Table IV).

### MITOCHONDRIAL DNA POPULATION STRUCTURE ON ND-3 VARIATION

A total of 241 nucleotides of the ND-3 region were sequenced for each of the 89 cisco individuals analysed (Table V). All variation observed was in the form

TABLE II. Allelic variability at six microsatellite loci (*BWF1*, *BWF2*, *Cisco 90*, *Cisco 126*, *Cisco 157* and *Cisco 200*; corrected data) in cisco populations of Lake Stechlin and Lake Breiter Luzin as well as in two populations from Finland

| Location | Species | | *BWF 1* | *BWF 2* | *Cisco 90* | *Cisco 126* | *Cisco157* | *Cisco 200* | $A_M$ | $H_M$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Lake Stechlin | *Coregonus* | $N$ | 48 | 48 | 48 | 48 | 48 | 48 | | |
| | *albula* | $A$ | 13 | 23 | 14 | 4 | 5 | 30 | 15 | |
| | | $A_C$ | 225 | 187 | 138 | 203 | 119 | 256 | | |
| | | $F_C$ | 0·365 | 0·13 | 0·271 | 0·448 | 0·883 | 0·09 | | |
| | | $A_R$ | 205–269 | 151–219 | 116–148 | 203–209 | 119–151 | 232–320 | | |
| | | $H_E$ | 0·79 | 0·93 | 0·85 | 0·74 | 0·32 | 0·96 | | 0·77 |
| | | $H_O$ | 0·69 | 0·9 | 0·85 | 0·77 | 0·31 | 0·88 | | 0·73 |
| | *Coregonus* | $N$ | 46 | 46 | 46 | 45 | 46 | 45 | | |
| | *fontanae* | $A$ | 11 | 21 | 15 | 5 | 6 | 21 | 13 | |
| | | $A_C$ | 211 | 163 | 138 | 203 | 119 | 244 | | |
| | | $F_C$ | 0·37 | 0·14 | 0·25 | 0·34 | 0·80 | 0·13 | | |
| | | $A_R$ | 211–283 | 151–209 | 114–142 | 203–211 | 119–153 | 232–316 | | |
| | | $H_E$ | 0·74 | 0·92 | 0·87 | 0·78 | 0·35 | 0·94 | | 0·77 |
| | | $H_O$ | 0·78 | 0·91 | 0·98 | 0·82 | 0·39 | 0·87 | | 0·79 |
| Lake Breiter | *Coregonus* | $N$ | 48 | 48 | 48 | 48 | 48 | 47 | | |
| Luzin | *albula* | $A$ | 18 | 18 | 12 | 4 | 6 | 33 | 15 | |
| | | $A_C$ | 225 | 155 | 134 | 203 | 119 | 256 | | |
| | | $F_C$ | 0·292 | 0·4 | 0·212 | 0·521 | 0·771 | 0·1 | | |
| | | $A_R$ | 205–287 | 151–219 | 116–138 | 203–209 | 119–159 | 230–322 | | |
| | | $H_E$ | 0·86 | 0·81 | 0·87 | 0·66 | 0·39 | 0·96 | | 0·76 |
| | | $H_O$ | 0·85 | 0·69 | 0·85 | 0·63 | 0·44 | 0·94 | | 0·73 |

TABLE II. Continued

| Location | Species | | *BWF 1* | *BWF 2* | *Cisco 90* | *Cisco 126* | *Cisco157* | *Cisco 200* | $A_M$ | $H_M$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Lake Breiter | *Coregonus* | $N$ | 43 | 43 | 43 | 43 | 43 | 43 | | |
| Luzin | *lucinensis* | $A$ | 12 | 15 | 10 | 4 | 7 | 21 | 12 | |
| | | $A_C$ | 225 | 155 | 122 | 203 | 119 | 252 | | |
| | | $F_C$ | 0·593 | 0·54 | 0·384 | 0·442 | 0·663 | 0·151 | | |
| | | $A_R$ | 213–287 | 147–217 | 116–138 | 203–209 | 119–155 | 224–340 | | |
| | | $H_E$ | 0·70 | 0·73 | 0·78 | 0·59 | 0·5 | 0·93 | | 0·71 |
| | | $H_O$ | 0·67 | 0·69δ | 0·84 | 0·47 | 0·49 | 0·93 | | 0·68 |
| Finland Lake | *Coregonus* | $N$ | 35 | 35 | 35 | 35 | 35 | 35 | | |
| Onkamo | *albula* | $A$ | 10 | 16 | 13 | 4 | 9 | 21 | 12 | |
| | | $A_C$ | 213 | 159/167/169 | 124 | 207 | 119 | 238 | | |
| | | $F_C$ | 0·4 | 0·143 | 0·171 | 0·5 | 0·69 | 0·143 | | |
| | | $A_R$ | 205–263 | 151–223 | 114–138 | 203–209 | 119–159 | 220–340 | | |
| | | $H_E$ | 0·77 | 0·92 | 0·91 | 0·74 | 0·49 | 0·93 | | 0·79 |
| | | $H_O$ | 0·54 | 0·91 | 0·86 | 0·71 | 0·51 | 0·94 | | 0·74 |
| Finland Lake | *Coregonus* | $N$ | 29 | 29 | 29 | 28 | 29 | 29 | | |
| Kuohijärvi | *albula* | $A$ | 11 | 21 | 12 | 4 | 6 | 27 | 14 | |
| | | $A_C$ | 221 | 167 | 124 | 205 | 119 | 246/252 | | |
| | | $F_C$ | 0·379 | 0·207 | 0·207 | 0·375 | 0·828 | 0·09 | | |
| | | $A_R$ | 209–289 | 151–221 | 116–140 | 203–209 | 119–157 | 228–332 | | |
| | | $H_E$ | 0·82 | 0·9 | 0·9 | 0·78 | 0·31 | 0·96 | | 0·78 |
| | | $H_O$ | 0·86 | 0·83 | 0·9 | 0·82 | 0·34 | 0·86δ | | 0·77 |

$N$, number of samples successfully used for genetic analysis; $A$, number of alleles at each locus; $A_M$, mean number of alleles at six loci; $A_C$, most common allele (base pairs); $F_C$, frequencies of the most common alleles; $A_R$, range of allele size; $H_O$, observed heterozygosity; $H_E$, gene diversity at each locus; $H_M$, mean within-population heterozygosity at six loci. δ, significant heterozygote deficit following the sequential Bonferroni correction ($\alpha = 0{\cdot}005$, $k = 36$).

TABLE III. Above diagonal: $F_{ST}$ values between pairs of samples, based on six microsatellite loci with 95% CI in parentheses. Except for population pair Lake Stechlin *Coregonus albula* and *Coregonus fontanae* (*), all comparisons were significant at $P < 0{\cdot}05$. Below diagonal: $F_{ST}$ and $\Phi_{ST}$ values estimated from ND-3 sequence variation. All $F_{ST}$ *P*-values were $<0{\cdot}05$, and all $\Phi_{ST}$ *P*-values were $<0{\cdot}05$, except comparison between Lake Stechlin *C. albula* and *C. fontanae* (**) and between Lake Onkamo and Lake Kuohijärvi (#)

| Population | Lake Stechlin *C. albula* | Lake Stechlin *C. fontanae* | Lake Breiter Luzin *C. albula* | Lake Breiter Luzin *C. lucinensis* | Lake Onkamo *C. albula* | Lake Kuohijärvi *C. albula* |
|---|---|---|---|---|---|---|
| Lake Stechlin *Coregonus albula* | | 0·004* (0·000–0·080) | 0·043 (0·016–0·068) | 0·075 (0·041–0·118) | 0·051 (0·025–0·084) | 0·052 (0·015–0·096) |
| Lake Stechlin *Coregonus fontanae* | $F_{ST}$ 0·507 $\Phi_{ST}$ 0·084** | | 0·059 (0·027–0·084) | 0·089 (0·05–0·128) | 0·059 (0·024–0·107) | 0·053 (0·006–0·115) |
| Lake Breiter Luzin *Coregonus albula* | $F_{ST}$ 0·614 $\Phi_{ST}$ 0·493 | $F_{ST}$ 0·533 $\Phi_{ST}$ 0·665 | | 0·025 (0·013–0·039) | 0·051 (0·025–0·073) | 0·069 (0·026–0·104) |
| Lake Breiter Luzin *Coregonus lucinensis* | $F_{ST}$ 0·512 $\Phi_{ST}$ 0·474 | $F_{ST}$ 0·428 $\Phi_{ST}$ 0·582 | $F_{ST}$ 0·536 $\Phi_{ST}$ 0·349 | | 0·09 (0·038–0·149) | 0·124 (0·06–0·182) |
| Lake Onkamo *Coregonus albula* | $F_{ST}$ 0·534 $\Phi_{ST}$ 0·600 | $F_{ST}$ 0·429 $\Phi_{ST}$ 0·652 | $F_{ST}$ 0·565 $\Phi_{ST}$ 0·680 | $F_{ST}$ 0·439 $\Phi_{ST}$ 0·556 | | 0·054 (0·017–0·101) |
| Lake Kuohijärvi *Coregonus albula* | $F_{ST}$ 0·682 $\Phi_{ST}$ 0·682 | $F_{ST}$ 0·571 $\Phi_{ST}$ 0·750 | $F_{ST}$ 0·713 $\Phi_{ST}$ 0·744 | $F_{ST}$ 0·575 $\Phi_{ST}$ 0·589 | $F_{ST}$ 0·627 $\Phi_{ST}$ −0·061# | |

 

TABLE IV. Hierarchical AMOVA based on six microsatellite loci and on mtDNA sequences (ND-3-region) in populations of Lake Stechlin and Lake Breiter Luzin. The upper section of the table indicates results obtained by using lakes as the top grouping level and the lower section provides results obtained by using forms (spawning-time) as the top grouping level. Given are test statistics, including absolute variance ($V$) and correlation at corresponding level ($F$). Also given is the probability ($P$) of a more extreme variance component than that observed. For each statistic, upper values are based on microsatellite loci, while lower values are based on mtDNA sequence variation

| | Variance component | | | | |
|---|---|---|---|---|---|
| Source of variation | $V$ | % | Fixation indices | | $P$ |
| Among lakes | 0·1231 | 5·18 | $F_{CT}$ | 0·052 | <0·000 |
| | 0·0009 | 0·18 | $F_{CT}$ | 0·002 | <0·000 |
| Among forms within lakes | 0·03 | 1·34 | $F_{SC}$ | 0·014 | <0·001 |
| | 0·261 | 52·22 | $F_{SC}$ | 0·523 | <0·001 |
| Among forms | −0·06 | −2·57 | $F_{CT}$ | −0·026 | 0·33 |
| | −0·0004 | −0·08 | $F_{CT}$ | −0·0008 | 0·34 |
| Among lakes within forms | 0·1536 | 6·63 | $F_{SC}$ | 0·065 | <0·000 |
| | 0·262 | 52·44 | $F_{SC}$ | 0·524 | <0·000 |

of single base-pair substitutions. Twelve nucleotide positions (3·6%) were polymorphic (nine transitions, two transversions), and these defined 11 *C. albula* haplotypes (*Coal-x*) for the ND-3 region. In addition, four haplotypes were defined among the *C. sardinella* samples (*Cosa-x*). A strong and highly significant pattern of heterogeneity in haplotype distribution was observed among all samples ($X^2$, d.f. = 64, $P = 0{\cdot}00001$). A highly significant difference was found between both species in Lake Stechlin ($X^2$, d.f. = 10, $P = 0{\cdot}004$), as well as between both species in Lake Breiter Luzin ($X^2$, d.f. = 8, $P = 0{\cdot}00001$). Both lakes also differed strikingly in their haplotype composition. The most frequent haplotype (*Coal-1*) in Lake Stechlin (69%) was observed in only two individuals (5%) in Lake Breiter Luzin. Conversely, the most abundant haplotype (*Coal-2*) in Lake Breiter Luzin (60%) was observed in only five individuals (14%) in Lake Stechlin. No haplotype was shared between *C. fontanae* and *C. lucinensis*. Thus, the most abundant haplotype (*Coal-3*) in *C. lucinensis* was not observed in any of the other populations from these two lakes. Heterogeneity in haplotype frequency distribution translated into strong and statistically significant $F_{ST}$ values in all pair-wise comparisons, with values ranging between 0·428 and 0·713 (Table III). In particular, there were pronounced differences between *C. albula* and *C. fontanae* in Lake Stechlin ($F_{ST} = 0{\cdot}507$) and also between *C. albula* and *C. lucinensis* in Lake Breiter Luzin ($F_{ST} = 0{\cdot}536$). The $\Phi_{ST}$ values were overall similar to $F_{ST}$, except in comparisons between the two Lake Stechlin species and between the two Finnish populations. Compared to the results given by the microsatellite loci, the AMOVA performed on mtDNA data revealed similar extents of genetic differentiation between populations of the same spawning type from different lakes and between the two cisco species within the same lake (Table IV).

TABLE V. Variable nucleotides for the ND-3-region and the D-loop mitochondrial segments (241 and 328 nucleotides) relative to the sequence Coal-1 deposited in genebank (accession numbers: AY277999 and AY277976). Accession numbers for all following haplotypes are AY278000–21 and AY277977–98, respectively. Numbers refer to nucleotide position in each segment. Populations are labelled as follows: L. Stechlin F/A, *C. fontanae* (F) and *C. albula*; L. B. Luzin L/A, *C. lucinensis* (L) and *C. albula* (A); L. Onka., Lake Onkamo *C. albula*, (A); L. Kuoh., Lake Kuohijärvi *C. albula* (A); G. S. Lake, *C. sardinella* (*Cosa*) from Great Slave Lake; Avak R., *C. sardinella* (*Cosa*) from Avak River; S. Point, *C. sardinella* (*Cosa*) population from Shingle Point

| | Nucleotid position ND-3 | | | | | | | | | | | | L. Stechlin | | L. B. Luzin | | L. Onka. | L. Kuoh. | Great S. L. | Avak R. | S. Point | Nucleotid position d-loop | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype number | 034 | 050 | 052 | 094 | 145 | 148 | 157 | 165 | 173 | 187 | 211 | 227 | F | A | L | A | A | A | *Cosa* | *Cosa* | *Cosa* | 024 | 100 | 105 | 115 | 125 | 208 | 241 | 243 | 289 | 297 |
| *Coal-1* | A | T | A | T | T | A | C | C | A | T | G | A | 11 | 14 | | 2 | 1 | | | | | G | T | A | G | C | T | C | A | A | A |
| *Coal-2* | . | C | . | . | . | . | . | G | . | . | . | . | | 5 | 8 | 16 | | | | | | . | . | . | . | . | . | . | . | . | . |
| *Coal-3* | . | C | . | C | . | . | . | . | . | . | . | . | | | 11 | | | | | | | A | C | . | . | . | C | A | G | | G |
| *Coal-4* | . | C | . | C | . | . | . | . | . | . | . | . | | | | 1 | | | | | | A | C | . | . | . | C | A | G | . | . |
| *Coal-5* | . | . | . | . | C | . | . | . | . | . | . | . | 2 | | | | | | | | | . | . | . | . | . | . | . | . | . | . |
| *Coal-6* | . | . | . | . | . | . | T | . | . | . | . | . | 1 | | | | | | | | | . | . | . | . | . | . | . | . | . | . |
| *Coal-7* | . | C | . | . | . | . | . | . | . | . | . | . | 2 | | | | | | | | | . | . | . | A | . | . | . | . | . | . |
| *Coal-8* | . | C | G | C | . | . | . | . | . | . | . | . | | | 1 | | | | | | | A | C | . | . | . | C | A | G | . | . |
| *Coal-9* | . | C | . | . | . | . | . | G | . | C | . | . | | | | 1 | | | | | | . | . | . | . | . | . | . | . | . | . |
| *Coal-10* | G | C | . | . | . | . | . | . | . | . | . | . | | | | | 6 | 4 | | | | . | . | . | . | . | . | . | . | . | . |
| *Coal-11* | G | C | . | . | . | . | . | . | G | . | . | . | | | | | 2 | | | | | . | . | . | . | . | . | . | . | . | . |
| *Coal-12* | . | . | . | . | . | . | . | . | G | . | . | . | 1 | | | | | | | | | . | . | . | . | . | . | . | . | . | . |
| *Cosa-1* | . | C | . | C | . | . | . | . | . | . | . | . | | | | | | | | 1 | | A | C | . | . | . | . | . | . | . | . |
| *Cosa-2* | . | C | . | C | . | G | . | . | G | . | . | . | | | | | | | 1 | | | A | C | C | . | A | . | . | . | . | . |
| *Cosa-3* | . | C | . | C | . | G | . | . | G | . | . | . | | | | | | | | | 1 | A | C | . | . | . | . | . | . | . | . |
| *Cosa-4* | . | C | . | C | . | . | . | . | . | . | A | . | | | | | | | | | 1 | A | C | . | . | . | . | . | . | C | . |
| *Cosa-5* | . | C | . | C | | G | . | . | . | . | . | G | | | | | | | | | 1 | A | C | . | . | A | . | . | . | . | . |
| | Number of analysed individuals | | | | | | | | | | | | 17 | 19 | 20 | 20 | 9 | 4 | 1 | 1 | 3 | | | | | | | | | | |

 

## PHYLOGENETIC ANALYSES COMBINING ND-3 AND D-LOOP POLYMORPHISM

A total of 10 polymorphic sites (six transitions, four transversions) out of 328 (3%) were observed for the D-loop segment that was sequenced for representatives of each haplotype identified by the analysis of the ND-3 segment (Table V). A total of 17 haplotypes were resolved by combining ND-3 and D-loop sequence polymorphism, that is 12 in *C. albula* (*Coal-x*) and five in *C. sardinella* (*Cosa-x*). Pair-wise sequence divergence between them varied from 0·18 to 1·78% (mean = 0·99%). The neighbour-joining phylogram clearly showed that mtDNA haplotypes are not monophyletic relative to *C. sardinella*. Thus, haplotypes *Coal-3*, *Coal-4* and *Coal-8* share three synapomorphic sites with *C. sardinella* haplotypes (*ND-3* 94, *D-loop 24* and *100*) and consequently cluster distinctively with them (Fig. 1). Twelve out of 14 ciscoes characterized by this clade belong to the Lake Breiter Luzin spring-spawning population, the other individual belongs to the Lake Breiter Luzin autumn-spawning population. Heterogeneity in distribution was observed for additional haplotype groupings. The clade formed by *Coal-10* and *Coal-11* was only observed in the two Finnish populations. Although not conclusively supported, additional haplotype groupings were suggested by their nucleotide composition. Namely, *Coal-1, Coal-5, Coal-6* and *Coal-12* group together and share a thymine at position 50 (*ND-3*), whereas all other haplotypes are characterized by a cytosine at that position. A total of 31 fish are characterized by this mtDNA haplotype group and most of them (29 or 94%) are from Lake Stechlin, the remaining two belonging to the population of Lake Breiter Luzin *C. albula*.

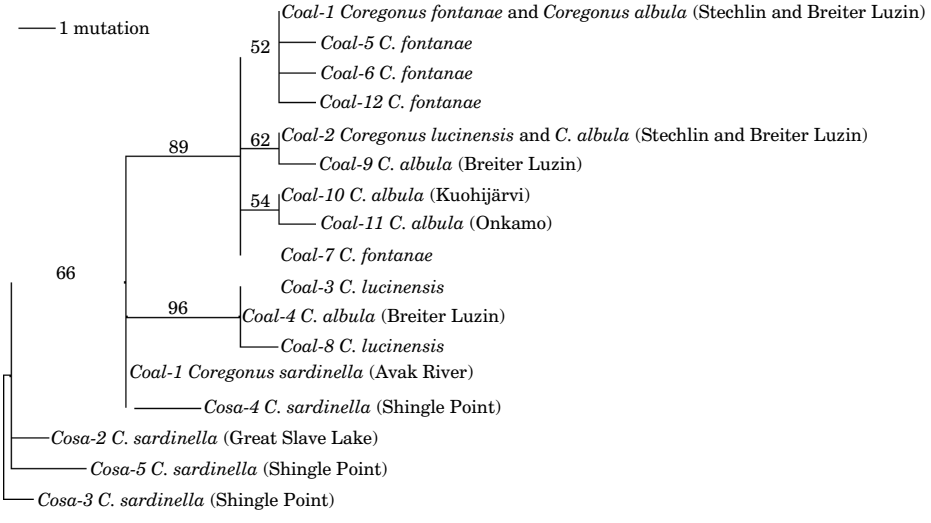


FIG. 1. Neighbour-joining 50% majority-rule consensus tree (phylogram) of 17 mtDNA haplotypes combining *ND*-3 and *D-loop* sequences. Only bootstrap values >50% are shown. *C. sardinella* (*Cosa*-3, Shingle Point, Canada) was used as outgroup. Haplotype codes as in Table V.

## DISCUSSION

The combined results of both microsatellite and mtDNA analyses suggest that *C. lucinensis* and *C. fontanae* remain genetically differentiated and reproductively isolated from sympatric *C. albula* in each lake. The present results are not consistent with the hypothesis that all ciscoes in each lake belong to one panmictic population and that recognisable 'forms' are the result of individual phenotypic plasticity. Furthermore, the results did not support the hypothesis that both lakes were invaded by two already separated species, which already were or later evolved into autumn-and spring-spawners in both lakes. No single haplotype is shared between spring-spawning *C. fontanae* and *C. lucinensis*. In particular, the extent of genetic differentiation was consistently less pronounced among species within each lake than among populations with the same spawning time from different lakes. Of course, phylogenetic relations detected by mtDNA analyses may fail when mitochondrial introgression cannot be ruled out (Ballard & Whitlock, 2004). Indeed, haplotypes *Coal3, Coal4* and *Coal8* strongly suggest mitochondrial introgression of *C. sardinella* into *C. lucinensis* and *C. albula* populations from Lake Breiter Luzin. But even if the introgressed mutations on position 94 of *ND-3* and on positions 24 and 100 of the *D-loop* region where ignored, no haplotype was shared between *C. lucinensis* and *C. fontanae*. Therefore, the hypothesis of *C. lucinensis* inhabiting the two geographically very close lakes has to be rejected. In contrast, it can be suspected, that the spring-spawning ciscoes evolved by sympatric speciation from *C. albula* in each lake.

Summarizing results of genetic studies in coregonid fish species, Bernatchez (2004) showed that ecological opportunity stemming for depurate fish diversity in new postglacial and favourable habitats have contributed to an elevated rate of speciation in freshwater fishes at northern latitudes, particularly in coregonids. Of course, glacial expansions and retreats created conditions for the occurrence of parapatric populations. Yet, sympatric divergence has also contributed to the diversification of 'forms' which share similarities in general patterns of morphological, behavioural and life-history variation in both lakes. Overall, spring- and autumn-spawning ciscoes from Lake Stechlin and Lake Breiter Luzin adds to the bulk of evidence supporting the fact that evolution of ecologically similar forms is a common phenomenon in coregonids. Thus far, this phenomenon has been reported in European cisco *C. albula* from Scandinavia (Vuorinen *et al.*, 1981), lake whitefish *C. clupeaformis* (Mitchill) from various locations in North America (Bernatchez *et al.*, 1996, Pigeon *et al.*, 1997), European whitefish *Coregonus lavaretus* (L.) complex (Douglas *et al.*, 1999), as well as in the North American cisco complex (*C. artedi*) (Turgeon & Bernatchez, 2003). Sympatric speciation processes have also been hypothesized for explaining the diversification observed in other lacustrine fish species groups (Schliewen *et al.*, 1994, 2001; Higashi *et al.*, 1999; Doorn & Weissing, 2001).

Despite the fact that ciscoes in Lake Breiter Luzin and Lake Stechlin did not originate from a common postglacial ancestor, their evolutionary and biogeographic history remains uncertain. It cannot be excluded that two populations of ciscoes already present in one glacial refuge invaded each lake. In this case, two glacial refuges must have existed, one providing the source for ciscoes of Lake

Breiter Luzin and one for ciscoes of Lake Stechlin. Also, each lake might have been colonized two times from the same glacial refuge leading to the evolution of spring-spawning behaviour by character displacement by one of the invaders. Of course, it is also not possible to rule out that genetic similarities within each lake are at least partly imputable to past or even ongoing intralacustrine gene flow and thus ciscoes might have been more divergent, when invading the lakes, than today. Due to the fact that the lakes are not connected, currently interlacustrine gene flow lakes is impossible.

Each lake was characterized by the occurrence of distinct mtDNA clades. This is most consistent with the hypothesis that both lakes were colonized independently of two different glacial refuges. There is indirect evidence that both lakes have a distinct postglacial colonization history that could be related to the southwestern glacial borderline of colonization routes from eastern refuge areas, located between Lakes Stechlin and Breiter Luzin. This assumption is particularly supported by the occurrence of the opossum shrimp *Mysis relicta* Lovén and the sculpin *Cottus poecilopus* Heckel (Thienemann, 1925, 1928; Waterstraat, 1988) in Lake Breiter Luzin. These species have never been observed in Lake Stechlin (Casper, 1985) despite comparable ecological conditions (Koschel *et al.*, 1983, 2002). Like in Lake Stechlin, oxygen concentrations are adequate for deepwater adapted species in Lake Breiter Luzin too, even when it became mesotrophic during the last 50 years (Koschel *et al.*, 1983, 2002). In addition, this study provided evidence for introgression of *C. sardinella* mtDNA into both ciscoes of Lake Breiter Luzin but not in Lake Stechlin. This also supports the view of two different sources of biota of both lakes. At least, mtDNA of *C. sardinella* has postglacially reached the Baltic basin to the glacial margin that was located between Lake Stechlin and Lake Breiter Luzin. It is not possible, however, to assess whether the introgressive hybridisation event occurred in Lake Breiter Luzin or in the source population and if hybridization occurred before or after spring- and autumn-spawning ciscoes diverged, or if this had an incidence on the divergence process in that lake.

In summary, despite the fact that both lakes are geographically very close and belong to the same river drainage, the combined analyses of microsatellite and mtDNA markers strongly suggests the independent origin of *C. fontanae* and *C. lucinensis*.

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