

# Phylogeography and evolution of freshwater cottid fishes

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Academic dissertation

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## Original articles and manuscripts

The thesis is based on the following articles and manuscripts, which are referred to in the text by their Roman numerals:

- I Kontula T & Väinölä R (2001) Postglacial colonization of Northern Europe by distinct phylogeographic lineages of the bullhead, *Cottus gobio*. *Molecular Ecology*, **10**, 1983–2002.
- II Kontula T & Väinölä R (2003) Molecular and morphological analysis of secondary contact zones of *Cottus gobio* in Fennoscandia: geographical discordance of character transitions. *Biological Journal of the Linnean Society*, in press.
- III Kontula T & Väinölä R (2003) Comparative phylogeography of freshwater cottid fishes: continent-scale versus European affinities in the *Cottus poecilopus* and *C. gobio* complexes. Submitted.
- IV Kontula T & Väinölä R (2003) Relationships of Palearctic and Nearctic ‘glacial relict’ *Myoxocephalus* sculpins from mitochondrial DNA data. *Molecular Ecology*, in press.
- V Kontula T, Kirilchik SV & Väinölä R (2003) Endemic diversification of the monophyletic cottoid fish species flock in Lake Baikal explored with mtDNA sequencing. *Molecular Phylogenetics and Evolution*, **27**, 143–155.

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## Summary

### Introduction

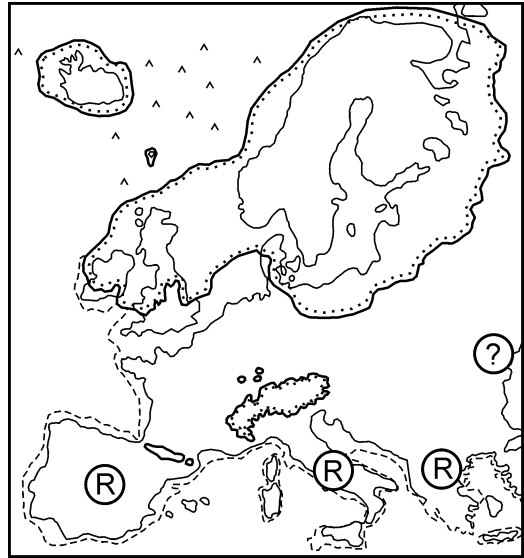
#### *Phylogeographic inference*

Phylogeography is a branch of biogeography concerned with geographic patterns of evolutionary subdivisions within species or among closely related species (Avise *et al.* 1987; Avise 2000). It is intimately related to the very core of life sciences, formation of new species. Speciation is usually a long-term process, which relates to decrease of gene flow between the differentiating taxa. Therefore, phylogeographic studies may offer snapshots of evolutionary processes, which lead to origin of new species.

Comparative approach to phylogeography of co-distributed taxa can reveal previously unrecognized biogeographic patterns in regional biota. In Europe, common subdivisions among terrestrial species have been interpreted in relation to the Pleistocene glacial events (Taberlet *et al.* 1998; Hewitt 1999). Relationships of extant populations have been examined particularly using mitochondrial DNA (mtDNA) data, and concordance in the intraspecific lineage distributions among different species have been explained by shared glacial refugia and routes of postglacial recolonization. Particularly, the Iberian Peninsula, Italy, the Balkans and southeastern Europe are recognized as the major postglacial dispersal centres of terrestrial biota in the western Palearctic (Fig. 1). On the scale of whole Eurasia, phylogeographic studies of terrestrial taxa are still few (e.g. Jaarola & Searle 2002; Brunhoff *et al.* 2003).

#### *European freshwater zoogeography*

A plain inspection of distributional patterns of European aquatic fauna shows an important difference compared to the terrestrial spe-



**Fig. 1** The main potential glacial refugia of terrestrial species in Europe, and the maximum ice cover during the last ice age according to Taberlet *et al.* (1998) and Hewitt (1999).

cies: The Iberian and Italian peninsulas, and central and southern Greece have not contributed much to the postglacial recolonization of European freshwaters (Banarescu 1990, 1992). This is hardly surprising, as no Pleistocene river connections are known across the Pyrenees or Alps. Instead of southern peninsulas, the Danube river basin in southeastern Europe appears to have been crucially important for the initial invasion of European freshwaters; almost all fishes living in the Atlantic and Baltic Sea basins are found also in the Danube (Banarescu 1990).

Among fishes, no comprehensive phylogeographic comparisons have been made in Europe this far, although mtDNA surveys have been published from several species (e.g. Bernatchez & Dodson 1994; Durand *et al.* 1999; Nesbø *et al.* 1999; Englbrecht *et al.* 2000; Koskinen *et al.* 2000; Bernatchez 2001; Brunner *et al.* 2001; Nilsson *et al.* 2001). Broad-scale studies have regularly shown subdivisions into several refugial lineages within species, but only rarely have obvious concordance in the lineage distributions

been noted among species. The level of observed intermixing between the differentiated units has also varied from almost complete allopatry (e.g., chub *Leuciscus cephalus*; Durand *et al.* 1999) to thorough intermixing across large areas (e.g. perch *Perca fluviatilis*; Nesbø *et al.* 1999). In any event, apart from the Danubian region, several studies have pointed also to more northern refugia, situated relatively close to the ice margin in Western and Central Europe (e.g. Durand *et al.* 1999; Nesbø *et al.* 1999; Hänfling *et al.* 2002).

In Northern Europe, the Baltic Sea has been the single most important dispersal route northwards for aquatic fauna during and after the deglaciation. The alternating fresh- and brackish water phases of the Baltic basin (Donner 1995) have variously enabled and disabled the postglacial immigration of freshwater species. Apart from Central European rivers in the south, the basin could have been reached from the east through the ice-lake systems that were situated along the margin of the Scandinavian ice-sheet (Kvasov 1979; Faustova 1984). The northeasterly ice-lakes have been proposed as the immediate source of several early invaders of the Baltic Sea, e.g., the “glacial relict” crustaceans (Segerstråle 1982; Väinölä *et al.* 1994). The same region has been regarded also as a glacial refugium for freshwater fishes, such as the Atlantic salmon *Salmo salar* (Koljonen *et al.* 1999) and perch (Nesbø *et al.* 1999).

#### *Fish diversity on Eurasian scale*

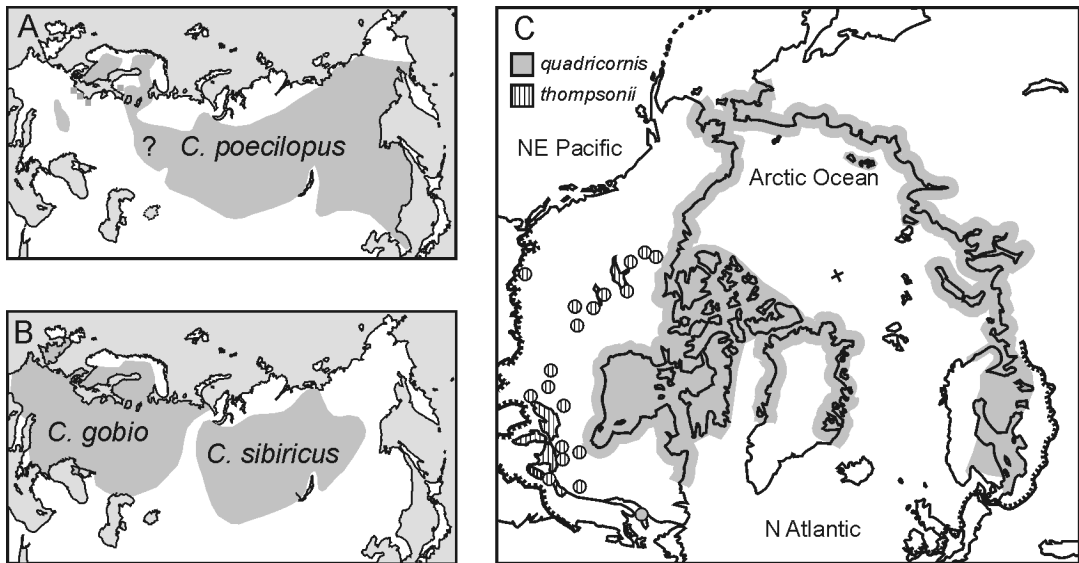
In the traditional freshwater zoogeography of northern Eurasia, the main division occurs between the Euro-Mediterranean and Siberian subregions (Banarescu 1992). However, the European vs. Siberian distinction does not appear to repeat itself on the level of intra-specific phylogeography, as shown by the few continent-scale surveys accomplished this far (Bernatchez & Dodson 1994; Brunner *et al.*

2001; Van Houdt *et al.* 2003). For instance, in the *Coregonus lavaretus* whitefish complex, the deepest split in the Holarctic mtDNA diversity separates the North American continental whitefish from the Eurasian + Beringian populations, and the main division within the Euro-Siberian region is found between a basically Alpine lineage and the others (Bernatchez & Dodson 1994).

The ancient Lake Baikal in East Siberia represents an environment very different from the rest of Eurasia, and in terms of aquatic fauna it is referred to a distinct biogeographical subregion (Banarescu 1992). Baikal is the deepest and oldest lake on earth; one or more lake basins have existed in the Baikal rift valley since the Oligocene (Mats 1993). It harbours more than 1000 endemic taxa, including several species flocks of invertebrates, such as amphipods, oligochaetes and mollusks (Timoshkin 1997; Kozhova & Izmet'eva 1998; Sherbakov 1999). As regards native fishes, the Baikalian fauna comprises Eurasian and Siberian species, and the endemics belonging to the suborder Cottoidei (Sideleva 2000). The non-endemic and endemic fishes occupy mainly separate habitats in Lake Baikal. The Eurasian and Siberian taxa live primarily in the shallow littoral zone, whereas the endemic cottoids inhabit deeper waters.

#### *Cottids, abyssocottids and comephorids - the fish of interest in the thesis*

This thesis is a step towards understanding evolution in a group of fishes during the last few million years: the history of the northern freshwater cottids and their relatives in Lake Baikal. The family Cottidae, and the Baikalian endemic families Abyssocottidae and Comephoridae belong to the suborder Cottoidei. In its entirety, Cottoidei comprises eleven families, and its distribution encompasses all oceans. The diversity of the family Cottidae (sculpins) is highest in the ma-



**Fig. 2** Distributions of *Cottus poecilopus* (A), *C. gobio* and *C. sibiricus* (B), and fishes of the *Myoxocephalus quadricornis* complex (C). The distributions of *Cottus* species are primarily according to Sideleva (2002), with modifications from our own data, and from Andreasson (1972) and Witkowski (1979). For *M. quadricornis* complex, the Eurasian range is according to Ekman (1953), and North American according to Parker (1988) and Houston (1990). The question mark in A denotes an uncertain part of *C. poecilopus* range. The approximate southern limits of the Late Wisconsinian / Late Weichselian continental glaciers in North America and northern Europe are indicated in C.

rine realm of the northern Pacific (c. 130 species; Froese & Pauly 2003). Freshwater cottids outside Lake Baikal are confined to four genera *Mesocottus*, *Trachidermus*, *Myoxocephalus*, and *Cottus*, which has 40 species in North America and Eurasia. A common anatomical feature for cottids, and also for Baikalian cottids, is the lack of swimming bladder, which basically determines their typically benthic life-style.

Only three species of *Cottus* are widespread in Eurasia. The Alpine bullhead *C. poecilopus* is a cold-adapted fish, which inhabits rivers and lakes from the Russian Far East to restricted areas of Northern and Eastern Europe (Fig. 2A). The European common bullhead *C. gobio* is also regarded as a cold-adapted species, but in mountain areas it normally lives downstream of the *C. poecilopus* range (e.g. Witkowski 1979). Both *C. poecilopus* and *C. gobio* thrive also in the brackish coastal waters of the Baltic

Sea (in salinities up to 6 and 7 p.p.t, respectively; Koli 1969; Andreasson 1972). East of the Ural Mountains, *C. gobio* is replaced by the similarly widespread Siberian *C. sibiricus* (Fig. 2B), and a close relative, *C. ricei*, inhabits freshwaters in North America. On the basis of head sensory canal pores, *C. gobio*, *C. sibiricus*, *C. ricei*, and three taxa only known from the Syr-Darya drainage, have been referred to the *gobio* group (Nybelin 1958; McAllister & Lindsey 1961; Koli 1969).

Fishes of the *Myoxocephalus quadricornis* complex have a circumpolar distribution primarily in Arctic coastal and estuarine waters (Fig. 2C). Yet, they also occur in deep lakes of the once-glaciated northern regions in Europe and North America, where they are regarded as a part of the “glacial relict” zoogeographical element (Seegerstråle 1962; Dadswell 1974). The North American continental *Myoxocephalus* populations are re-

ferred to the deepwater sculpin *M. q. thompsonii* or *M. thompsonii*, distinct from the Arctic and European fourhorn sculpin *M. q. quadricornis* or *M. quadricornis* (McAllister 1961; McAllister & Aniskowicz 1976; Eschmeyer 1998).

In comparison to the widespread Eurasian cottids, their Baikalian relatives show much more diverse morphologies, and also life-styles (Sideleva 1982, 2001). Therefore, they have been classified into three separate families of the Cottoidei: Abyssocottidae, Comephoridae and Cottidae. In Baikal, the evolution of cottoids has proceeded in two directions from the proposed ancestral shallow-water, benthic habitats (Sideleva 1994). They have invaded even the most abyssal environments deeper than 1500 m, and some species have shifted to a pelagial life-style. The pelagial adaptations of the Baikalian oilfishes *Comephorus* include a lowered body density through increased fat content (resulting in buoyancy and a translucent body) and viviparous reproduction in the water column. As regards the origin of the Baikalian cottoid diversity, interpretations of the morphological and molecular evidence have led to rather different age estimates, which altogether range from 1 Myr to Oligo-Miocene ages (e.g. Taliev 1955; Sideleva 1982; Hunt *et al.* 1997; Kiril'chik & Slobodyanyuk 1997).

The original papers of this thesis, **I–V**, examine the variation of freshwater cottoids on various spatial scales, and across the micro- and macroevolutionary time scales. In **II**, the focus is on regional variation and on local population structure in the contact zones between intraspecific lineages of *Cottus gobio*; **I+III** and **IV** offer insights to phylogeographic patterns on the Holarctic scale in the *C. gobio*, *C. poecilopus* and *Myoxocephalus quadricornis* species complexes; and in **V**, a molecular phylogeny is build for the endemic cottoid species flock of Lake Baikal.

## Material and methods

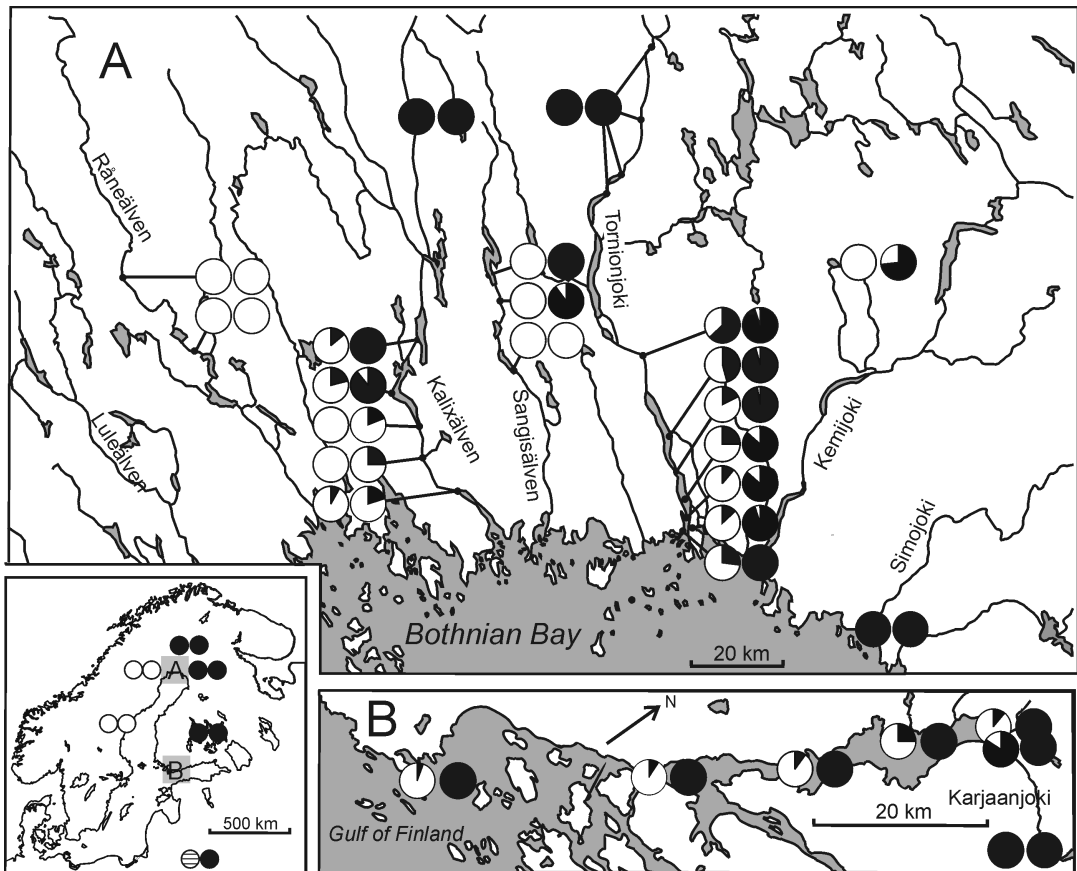
### Samples

To examine phylogeographic patterns among northern cottid fishes, samples from over 130 locations were studied for mitochondrial DNA, allozyme or morphological variation. The initial sampling of *C. gobio* was ample in Northern Europe (**I**). After the localization of the contact areas between the distinct *C. gobio* lineages sampling was further focused on two regions, one in southern Finland and the other in northern Finland and northern Sweden (Fig. 3; **II**). For the broad-scale phylogeographic study of the *C. gobio* and *C. poecilopus* species complexes (**III**), samples were obtained from ten countries across Eurasia, and from Canada. The relationships within the *Myoxocephalus quadricornis* species group were assessed using material from the Canadian Arctic coast, from the Baltic Sea, and from land-locked populations in North America and Northern Europe (**IV**). The material used in the phylogenetic study of the Baikalian cottoid species flock (**V**) included DNA samples of 22 of the 33 Baikalian species, and of 8 outgroup taxa from Japan, USA and Russia.

### Molecular and morphological analyses

In recent years, mitochondrial DNA (mtDNA) has been the most popular marker in phylogeographic studies (Avice 2000). Applicability of mtDNA markers is due to the almost exclusively maternal inheritance, lack or rarity of recombination and fairly fast mutation rate of the mitochondrial genome. In this thesis, sequencing or restriction fragment length polymorphism (RFLP) analyses were used to recognize distinct mtDNA lineages and to assess relationships among them. Complete or nearly complete mitochondrial cytochrome *b*, ATPase 8 and 6 genes, and non-coding control region were sequenced (in all 2833–2916 bp) to examine phylogeny





**Fig. 3** The areas studied in the analysis of the *C. gobio* contact zones, and distributions of the western and eastern mtDNA lineages and of *GPI-1* allele frequencies (left and right pie charts, respectively) north of the Baltic Sea (A) and in southern Finland (B). The map in the lower left corner shows the locations of the two study areas, and the corresponding data for the 6 reference populations representing the pure lineages (the hatched circle denotes the third, southeastern haplotype group).

in the *Cottus* species complexes (III), and in the Baikalian cottoid species flock (V); in other studies mtDNA analyses were based on shorter fragments.

Allozyme electrophoresis was used to assess gene frequency variation at nuclear loci in North European *C. gobio* (I–II). First, variation was scored at 25 loci to characterize differentiation between the main lineages (I), and the five most informative ones were used in the analysis of the contact zone populations (II).

In addition to molecular markers, four morphometric characters, previously found to differentiate the Fennoscandian lineages

of *C. gobio* (Koli 1969), were studied for more than 600 individuals, from the contact zone populations or from the “pure” lineages (II).

#### Data analyses

**Phylogenetic methods.** Phylogenetic analyses in I and in III–V applied the maximum-parsimony (MP), maximum-likelihood (ML) or minimum-evolution (ME) criteria. The MP and ML methods aim at inferring the nucleotide changes separately at each site along the sequence for a given topology, whereas ME applies genetic distances calculated from pairwise sequence comparisons (e.g. Nei &

Kumar 2000). In MP, the mutational changes are assumed to occur equally in all directions among the four nucleotides, and the topology (or topologies), which explains the whole genealogy of the studied sequences with smallest number of changes, is chosen to be the best. In ML, the preferred tree (topology and branch lengths) is the one, which maximizes the likelihood of obtaining the observed set of sequences under a specific substitution model. The MP analysis was applied as the exclusive or primary method for assessing the relationships among mtDNA haplotypes in I and IV, where the haplotypes of main interest were closely related. The analyses of broader species complexes (III and V) employed also ML. A hierarchical series of likelihood ratio tests were used to find the most appropriate substitution model for each data set (with MODELTEST program; Posada & Crandall 1998). In the models selected, the number of different substitution rates varied from two to six (HKY, Hasegawa *et al.* 1985; TrN, Tamura & Nei 1993; GTR, Yang 1994), and in each case, the models were modified to assume a proportion of invariable sites (I) and a gamma-distributed substitution rate variation among the remaining sites ( $\Gamma$ ). Genealogy of the mtDNA haplotypes was assessed with MP using NONA software version 1.5.1 (Goloboff 1993–1997) or with MP, ML or ME using PAUP\* versions 4.0b8–10 (Swofford 1998–2001).

**Divergence time estimation.** In the estimation of divergence times from mtDNA data, the molecular clock assumption has been extensively used also in phylogeographical studies. However, as lineage-specific substitution rate variation appears to be a rather common phenomenon (e.g. Bromham *et al.* 1996), the divergence times inferred from DNA should be interpreted with caution (I, III–V). In most divergence time estimations

the rate range used was 0.5–1.3 % / Myr, as suggested for teleostean mtDNA in general or for particular protein coding genes (for references, see V).

In addition to the overall divergence rate variation among lineages, another confounding feature in the divergence time estimation is the varying ratio of non-synonymous to synonymous substitutions observed, e.g., among Baikalian cottoids (Kiril'chik & Slobodyanyuk 1997). As the molecular evolution at the synonymous sites is assumed to be more neutral, i.e., not subject to varying strengths of purifying selection, the putatively more comparable synonymous PBL distances (Li 1993; Pamilo & Bianchi 1993) were also used for the evaluation of divergence times in the study of the Baikalian cottoids (V).

**Other analyses.** Patterns of allele frequency variation in the North European *C. gobio* were examined with principal component analysis (PCA) with SYSTAT version 8.0 (I). In the study of interlineage contact zone of *C. gobio* (see Fig. 3), the differences in four morphometric characters were first tested among and between reference samples representing the “pure” eastern, western and southeastern lineages using ANOVA and Tukey's test (II). Covariance analyses were used to assess the dependence of these variables on body length, and dependent variables were either transformed or excluded from further statistical analyses.

The general patterns of molecular and morphological variation *among reference and contact zone populations* of *C. gobio* were summarized with PCA, based on the sample means in three morphological characters, and on the frequencies of six molecular characters. The clines within each river in the northern study area and along the transect in the southern study area (Fig. 3) were examined by plotting the scaled char-

acter means against geographical distance (sample means equal to the value of the W reference sample were scaled to 0.0, and those equal to the value of the E reference sample to 1.0). In the northern study area, the coincidence and concordance of the clines in different characters were also examined and compared with a transect-independent approach: Scaled character means were plotted against an average over characters (a mean hybrid index, PC1) instead of geographical distance (Kruuk 1997; Rohwer *et al.* 2001).

Several measures were used to examine associations of characters *within populations* in the *C. gobio* contact zones. The deviation of allozyme genotype frequencies from HWE was tested within samples or subsamples by heterozygote deficiency tests, using GENEPOP version 3.1a (Raymond & Rousset 1995) and GDA version 1.1 (Lewis & Zaykin 2001). The genotypic associations between pairs of loci were estimated in terms of linkage disequilibrium  $D$ , and a standardized disequilibrium  $R$  was calculated from

$$R = D / \sqrt{pquv},$$

where  $p$ ,  $q$ ,  $u$  and  $v$  are the allele frequencies at the two loci (Szymura & Barton 1986). In addition, variances of morphological characters, and Spearman correlation coefficients  $r_s$  among all characters were estimated within populations. To see whether the measures of non-random association between characters were highest in the central hybrid zone populations, the estimates of  $R$ , heterozygote deficit  $F_{IS}$ , variance and  $r_s$  were plotted against PC1 value in the northern study area or against distance in the southern study area. For further evaluation of possible trends, quadratic polynomials were fitted in the data, and the significance of quadratic terms was tested.

## Results and discussion

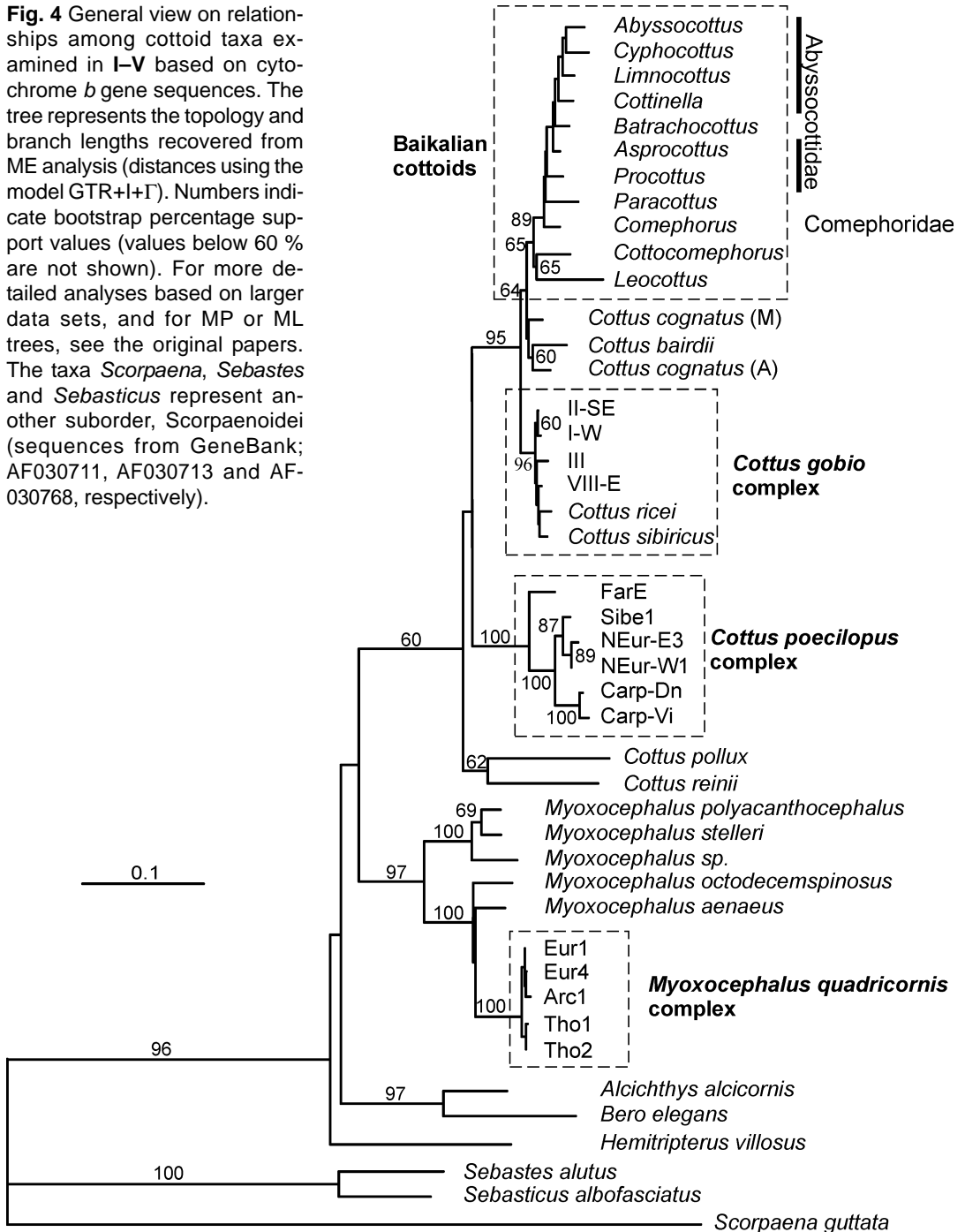
### *Continent-scale phylogeography in northern cottid fishes*

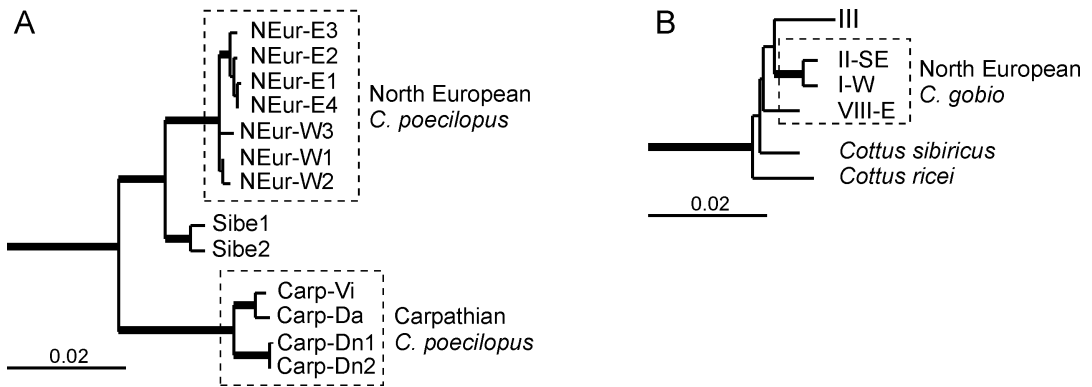
On the continental scale, the phylogeographic patterns emerging from the mtDNA data were rather different among the three northern cottid complexes studied but some subdivisions were familiar from other fish or even from terrestrial species (III and IV). In the *C. poecilopus* complex, the composite mtDNA haplotypes fell into four main lineages (Fig. 4). Apart from a deeply divergent basal Far Eastern branch (6 % sequence divergence), the most prominent division was observed within Europe (Fig. 5A). The North European and Siberian mtDNA lineages formed a widely distributed Euro-Siberian clade that differed from the Carpathian (Central Europe) lineage by over 4 % sequence divergence (III).

The mtDNA haplotypes of the European *C. gobio*, Siberian *C. sibiricus* and North American *C. ricei* clustered into a well-supported monophyletic group in the molecular phylogeny (Fig. 4). Thus, the mtDNA data agree with morphological evidence, which has recognized the *gobio* group among Holarctic *Cottus* (Nybelin 1958; McAllister & Lindsey 1961; Koli 1969). In contrast to the well-defined hierarchy of *C. poecilopus* lineages, the branching order within the *C. gobio* group, however, remained practically unresolved. Moreover, the Siberian and American lineages appeared inseparable from a bunch of European *C. gobio* diversity (III), which comprises seven deep mtDNA lineages identified in earlier studies (Englbrecht *et al.* 2000; Volckaert *et al.* 2002).

The divergences between the North American and Eurasian lineages of the *C. gobio* group were only about one third of those observed across the Eurasian range in

**Fig. 4** General view on relationships among cottoid taxa examined in I–V based on cytochrome *b* gene sequences. The tree represents the topology and branch lengths recovered from ME analysis (distances using the model GTR+I+ $\Gamma$ ). Numbers indicate bootstrap percentage support values (values below 60 % are not shown). For more detailed analyses based on larger data sets, and for MP or ML trees, see the original papers. The taxa *Scorpaena*, *Sebastes* and *Sebasticus* represent another suborder, Scorpaenoidei (sequences from GeneBank; AF030711, AF030713 and AF030768, respectively).





**Fig. 5** The mtDNA lineage relationships in the *Cottus poecilopus* (A) and *C. gobio* (B) complexes, with a focus on European diversity. The trees are based on combined data set of cytochrome *b* and ATPase 8 and 6 genes and represent parts of the mtDNA phylogeny presented in the comparative phylogeographic study of the northern cottids (III). Branches with strong support (BP = 90 % or higher) are indicated by thicker line.

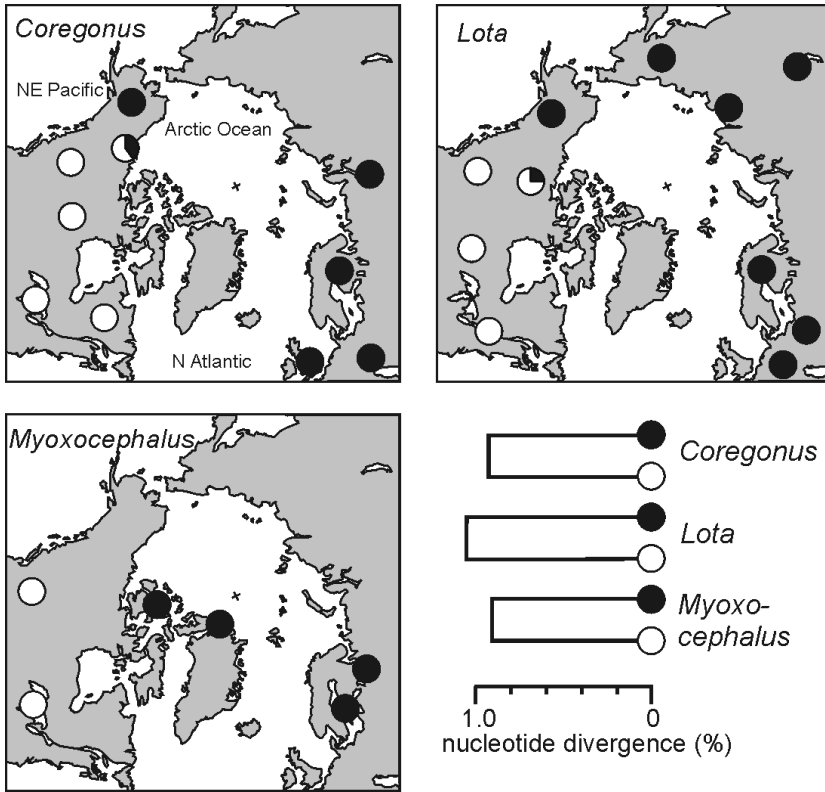
the *C. poecilopus* complex. Yet, the Euro-Siberian divergences were notably similar in both *Cottus* complexes (1–2 %; Fig. 5), and indicated isolation since the Pliocene or Middle Pleistocene (III). In terms of sequence divergence, the observed Euro-Siberian relationships have actually been closer in other studied fishes. For instance, the divergence between the genetically closest European and Siberian haplotypes was only 0–0.5 % in the ruffe *Gymnocephalus cernuus* (Stepien *et al.* 1998), perch (Nesbø *et al.* 1999) and Arctic charr *Salvelinus alpinus* (Brunner *et al.* 2001). The difference between cottids and these species may reflect weaker dispersal capacities of cottids, or a higher potential of surviving in local refugia. In all, still only a fraction of potential diversity of Asian *Cottus* can be considered examined; as indicated by a study of similarly distributed *Thymallus*, the great Asian rivers may harbour much unnoticed phylogeographic diversity (Koskinen *et al.* 2003).

The principal split in the mtDNA genealogy of the *Myoxocephalus quadricornis* complex separated the North American lacustrine deepwater sculpin (*M. q. thompsonii*) from the Arctic marine and North European landlocked populations of the fourhorn sculpin

(*M. q. quadricornis*; Fig. 4; IV). This finding, both in respect of the pattern and of the genetic divergence, is comparable to the Eurasian-Beringian vs. continental North American divisions e.g. in the *Coregonus lavaretus* whitefish complex (Bernatchez & Dodson 1994), and in the burbot *Lota lota* (Van Houdt *et al.* 2003) (Fig. 6). In the *M. quadricornis* complex, the North American continental invasion appears to have taken place several glaciation cycles ago in the Early to Middle Pleistocene. In all, such estimates of *Myoxocephalus* divergence are well in line with traditional views on the origin of the glacial relict element (e.g. Segerstråle 1962; Dadswell 1974), but deviate from the recent, molecular-based results on glacial relict crustaceans, which have shown much deeper subdivisions (Väinölä *et al.* 1994; 2001; Väinölä & Varvio 1989).

#### European cottid phylogeography

In Europe, our analyses on *Cottus poecilopus* and *C. gobio* revealed various phylogeographic subdivisions, some of which were congruent among species. The deepest mtDNA divergences were found within *C. poecilopus*. The North European and Carpathian lineages, which represent the disjunct



**Fig. 6** The principal phylogeographic subdivisions observed on a Holarctic scale in the *Coregonus lavaretus* whitefish complex (Bernatchez & Dodson 1994), in the burbot *Lota lota* (Van Houdt *et al.* 2003), and in the *Myoxocephalus quadricornis* complex (IV). The mtDNA divergence between the major lineages is close to 1 % in all three taxa.

sub-ranges (Fig. 2A), appear to have diverged already in the Pliocene or even Late Miocene (3–8 Myr ago; **III**). Such “intraspecific” divergences are rare, but not totally unparalleled among other European fishes (cf. Durand *et al.* 1999). It should be noted that at the time of this population split the ancestral *C. poecilopus* may not have been living anywhere near the Carpathians or Northern Europe. If the ancestral *C. poecilopus* was cold-adapted as the modern one, the Pliocene climate of Central Europe may have been too warm for it (Haywood *et al.* 2000).

The mtDNA data also showed finer phylogeographic structuring, probably of Pleistocene origin, within both European

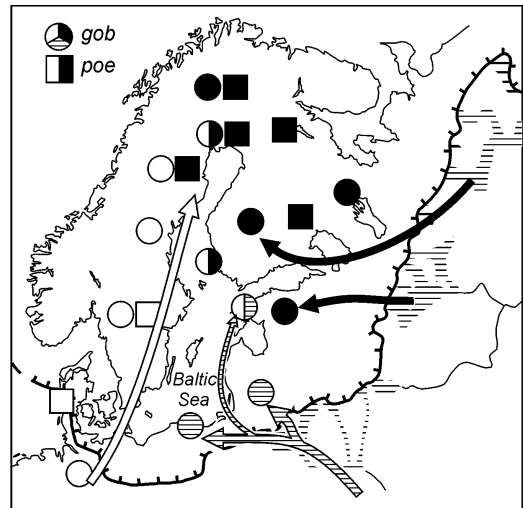
lineages of *C. poecilopus* (Fig. 5A; **III**). In the Carpathians, the haplotype relationships implied a more recent connection over the mountain range (i.e. Danube + Vistula vs. Dneestr) than through the lower reaches of the rivers draining to the Black Sea. In Northern Europe, on the other hand, the North Fennoscandian haplotypes made a cluster distinct from those found in Southern Fennoscandia and Denmark (Figs. 5A and 7, see below).

The genetic diversity and population history of the bullhead *C. gobio* has been thoroughly studied using allozymes, mtDNA and microsatellites (Englbrecht *et al.* 2000; Hänfling *et al.* 2002; Volckaert *et al.* 2002;

I–III). The mtDNA analyses have identified seven main lineages, labelled I–VII. The identity of the main lineage found in eastern North Europe (E lineage of study I, in Finland, NE Russia and Estonia), however, has not been unambiguously resolved in the earlier studies (cf. Englbrecht *et al.* 2000 and Volckaert *et al.* 2002). It was now named VIII to highlight its distinctive status (III). It is not clear whether the major split within the European *C. gobio* divides the eastern lineage VIII from the other lineages (Figs. 4 and 5B; III), or whether the principal division is between the cluster I + VIII (Danubian + eastern North European lineages) and the more western lineages (Volckaert *et al.* 2002). In any event, the first subdivision probably dates back to Early Pleistocene – Late Pliocene ages, and is of the same magnitude as observed among *C. gobio*, *C. sibiricus* and *C. ricei* on much larger geographical scale (Fig. 5B).

#### Postglacial colonization of Northern Europe

Northern Europe was entirely glaciated until approximately 15 000 years ago, and the aquatic and terrestrial habitats were recolonized postglacially. As to *C. gobio*, most of the Fennoscandian freshwater range seems to have been invaded during the Ancylus Lake stage of the Baltic basin. The current distribution roughly corresponds to the drainages accessible from that freshwater lake (Koli 1969; Nybelin 1969). Northern Europe was actually colonized from three different directions by the distinct western, southeastern and eastern lineages (Fig. 7 and D). The North European western (I-W) and southeastern (II-SE) haplogroups of *C. gobio* represent the Danubian lineage (lineage I), whereas the lineage VIII probably stems from a stock that survived in or near the eastern ice-lakes in NW Russia (I, III). The principal pattern in Fennoscandia is a strong east-west subdivision, which has even previously been recognized in morphology (e.g. Koli



**Fig. 7** Mitochondrial DNA haplogroup distributions of *C. gobio* and *C. poecilopus* in Northern Europe (circles and squares, respectively; I and III). The arrows represent proposed routes of postglacial recolonization for *C. gobio*. The hatched line shows the maximum extent of the Late Weichselian glaciation (c. 18 kya) (proglacial lake systems and rivers according to Kvasov (1979) and Arkhipov *et al.* (1986)).

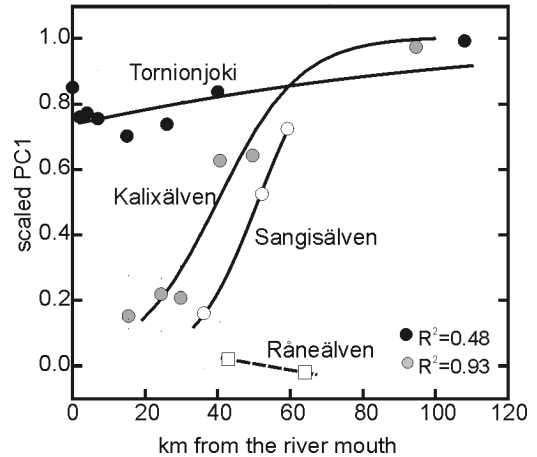
1969; Nybelin 1969). This distinction represents a common biogeographic pattern in Fennoscandia; molecular data indicate distinct eastern and western origins in the Atlantic salmon (Koljonen *et al.* 1999), grayling *Thymallus thymallus* (Koskinen *et al.* 2000), a freshwater amphipod *Gammarus lacustris* (Vainio & Väinölä 2003), and in several mammals (Taberlet & Bouvet 1994; Jaarola *et al.* 1999).

In North European *C. poecilopus*, the northern and eastern haplotypes also clustered into an eastern mtDNA sub-lineage (NEur-E in Fig. 5A). Apart from indicating two sources of postglacial colonization (see also Andreasson 1972), this pattern also suggests glacial survival in northern Central Europe, conceivably even between the Scandinavian ice sheet and the northern Carpathian mountain slopes (cf. Hänfling *et al.* 2002).

### Secondary contact zones between *Cottus gobio* lineages

North of the Baltic Sea, the current distributions of the western and eastern *C. gobio* genomes show a clear-cut west-east distinction in the upstream parts of the rivers (Fig. 3 and II). During the postglacial recolonization of northern waters, the E lineage (VIII) has obviously followed the eastern coast of the Baltic basin (Ancyclus Lake) and colonized the freshwaters as far as River Kalixälven. Correspondingly, the W lineage (I-W) has immigrated along the western coastline and reached River Råneälven on the west coast of the Bothnian Bay. At some point after the initial contact in the north, the W lineage appears to have continued more successfully east than the E lineage towards west, as detected from the mixed composition of populations along the northeastern Baltic coasts (Koli 1969; I). Koli (1969) suggested that the W lineage was more tolerant of saltwater and therefore got an advantage in recolonization of Baltic habitats after the most saline Litorina Sea stage of the Baltic basin.

As a result of the W-lineage-biased dispersal, the intergradation between the lineages is seen also within rivers in a south-north, or downstream-upstream direction in the northern study area (Figs. 3 and 8). We studied contact zone populations for characters, which were known to differ between the W and E lineages of *C. gobio*, and compared the clines in each of the four morphometric and four genetic characters to the average character transition, or the mean hybrid index PC1. The clines in mtDNA and at the allozyme locus *GPI-1* appeared displaced from the transition in the PC1 hybrid index (Fig. 9). The mtDNA clines were generally found upstream and to the east from the centre of the other transitions, and the *GPI-1* cline was mostly shifted downstream in the northern rivers and west of the other transi-

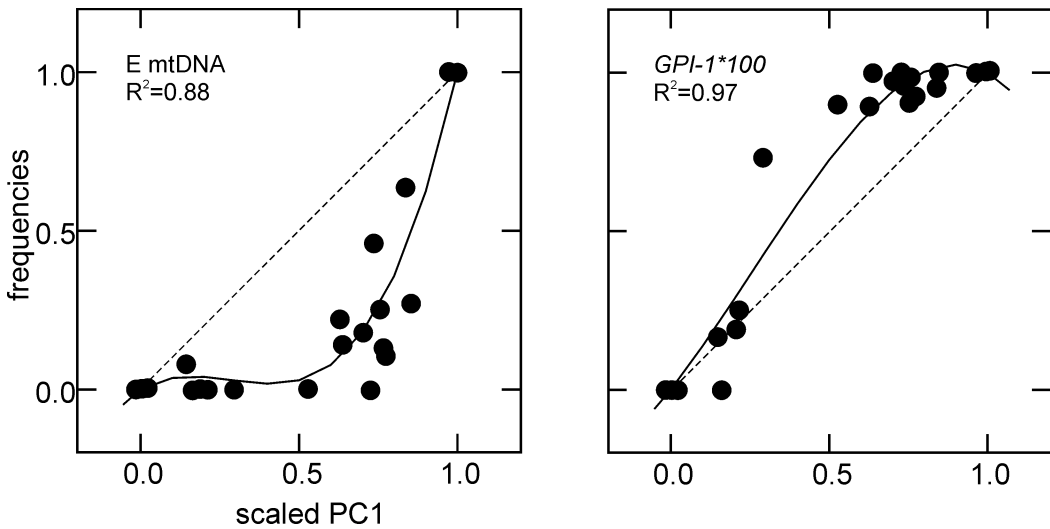


**Fig. 8** The average character transitions in four northern rivers in the *C. gobio* contact zone plotted against distance from the river mouth. The first principal component score (PC1) of each population was estimated based on variation in three morphometric and four genetic characters, and the PC1 axis was defined with reference samples from the pure western and eastern lineages of *C. gobio* (scaled PC1 value 0.0 and 1.0, respectively). The fitted curves are logistic regressions  $y=1/(1+e^{-4(x-m)/w})$ , where  $x$ =geographic distance from the river mouth,  $m$ =midpoint of the cline and  $w$ =width of the cline (see Cahan *et al.* 1998). The goodness-of-fit  $R^2$  values of the fitted regressions are given for rivers Tornionjoki and Kalixälven.

tions on the broader scale of the Baltic Sea. These cline displacements may be related to selection against deleterious cytonuclear character combinations (e.g., Jaarola *et al.* 1997). In this scenario, the combination of the eastern mtDNA with a western nuclear background would be disadvantageous compared to the reciprocal case, and the clines would have shifted apart so as to reduce the frequency of deleterious combinations (Barton 1993). As regards mtDNA, the cline non-coincidence could also result from dispersal being overall asymmetric (predominantly downstream) and sex-biased (stronger in males). Further evaluation of these explanations remains an issue for future studies.

In another coastal transition area in southern Finland (Fig. 3), clinal patterns resem-





**Fig. 9** Differences in character transitions in the northern contact zone (populations from all studied rivers) between the eastern and western lineages of *C. gobio* (II). Frequencies of the eastern variants in mtDNA and at *GPI-1* allozyme locus are plotted against scaled PC1 scores, which represent an average character transition. The lines are fitted cubic polynomials  $y=x+2\alpha x(1-x)+2\beta x(3x-2x^2-1)$  forced to pass through the points (0,0) and (1,1). Here,  $\alpha$  represents twice the shift in the position of the transition, and  $\beta$  is the decrease in the cline width below the average, both in units of average cline width (see Szymura & Barton 1986). The goodness-of-fit  $R^2$  values are given for the curves.

bled those in the northern contact zone. However, the southern area could not be interpreted as a parallel of the northern one, as population compositions could be explained neither by *in situ* mixing of the eastern and western lineages (I-W and VIII), nor by mixing of the whole set of the three putatively pure North European lineages (I-W, II-SE or VIII).

#### *Evolution of the Baikalian cottoid fishes*

On the basis of mtDNA sequence data, we found, for the first time, strong support for the monophyly of the whole Baikalian cottoid diversity (bootstrap support values in Fig. 4 based on *cyt-b* only are fairly low, but for an analysis with a larger data set, see V). In the molecular phylogeny, the Baikalian cottoids were deeply nested within the Holarctic freshwater *Cottus*, making the family Cottidae paraphyletic (see below). All but one of the current Baikalian genera formed well-supported monophyletic groups, but the

branching order among them could not be resolved completely. The short basal branches in the phylogeny indicate rapid diversification early in the history of the species flock – a feature, which also compromised the inference about the major ecological leaps in the Baikalian cottoid evolution, i.e., about the transitions to pelagial and abyssal lifestyles. In any event, the rate of morphological evolution has been fast in Baikalian cottoids (see also Kiril'chik *et al.* 1995; Kiril'chik & Slobodyanyuk 1997). The molecular divergences between Baikalian taxa are comparable to those observed within the Eurasian *Cottus* species complexes (Fig. 4) but the morphological and ecological diversification has achieved quite different levels, as indicated by the assignment of Baikalian cottoids into three different families (Sideleva 2001). If the molecular calibrations are reliable, the diversification of the Baikalian cottoids seems to have started at the earliest in the Middle-to-Late Pliocene. As regards other

studied species flocks of Lake Baikal, the molluscan family Baicaliidae may be of similar age with cottoids, whereas the flocks of the gammarid crustaceans and of the pulmonate *Choanomphalus* appear much older (for review, see Sherbakov 1999).

#### *Taxonomic remarks*

Our analyses on *Cottus* and *Myoxocephalus*, and on Baikalian cottoids have shown several cases, where molecular data disagree with the conventional morphology-based classification, or otherwise indicate a need for systematic revision. In the Eurasian *Cottus poecilopus* complex, the divergence of the Far Eastern mtDNA lineage is exceptionally high for an intraspecific comparison (c. 6 %), and probably deserves taxonomic recognition (III). However, the previously suggested taxonomic units do not concur with observed mtDNA clades (Fig. 4). For instance, Banareescu (1992) referred the populations of Far Eastern and Siberian “*poecilopus*” to *C. szanaga* Dybowski, but the mtDNA phylogeny showed a clade comprising the Siberian + North European *C. poecilopus*.

In European *C. poecilopus*, Witkowski (1984) has documented consistent morphological differences between the northern and southern population groups, and he interpreted these as a recent adaptive response to the lake vs. river habitats of the populations. As shown by the deep mtDNA divergence within European *C. poecilopus* (Fig. 5A), this morphological distinction may, however, be related to remarkably old (c. 3 – 8 Myr) lineages warranting taxonomic recognition (III).

In the *Cottus gobio* complex, the distribution of the lineage VIII largely seems to correspond to that of a controversial East European bullhead (sub)species *C. g. koshewnikowi* Gratzianow, which differs

from the typical form in the length of the lateral line and in the prickling of skin (e.g. Berg 1949; Nybelin 1958). Yet, as populations of the contact area in Northern Europe show all levels of intergradation and genetic mixing between the eastern bullhead and the typical form, a specific status has been denied, and even subspecific treatment is difficult in the broad overlap area (Koli 1969; I and II). In a wider framework, the mtDNA divergence of this unit is still similar to the conventionally recognized vicariant species further east, *C. sibiricus* of Siberia and *C. ricei* of North America (III). On the basis of mtDNA data, a higher than subspecies distinction for the latter taxa does not seem warranted either.

As regards *Myoxocephalus quadricornis* complex, the morphological and genetic data for geographically vicarious fourhorn (*M. q. quadricornis*) and deepwater (*M. q. thompsonii*) sculpins are congruent (Figs. 2C and 6), and the recognition of the taxa in itself should not be challenged (IV). Yet, a full specific rank for these taxa, suggested e.g. by McAllister (1961; 1990), Bailey & Smith (1981), Parker (1988) and Eschmeyer (1998), would deviate from the practice recently promoted for similarly structured diversity in other Holarctic fish complexes (Bernatchez & Dodson 1994; Van Houdt *et al.* 2003). Therefore, we have preferred a subspecies rather than species distinction, in accord with McPhail & Lindsey (1970) and McAllister & Aniskowicz (1976). The generic affinities of the *M. quadricornis* complex also deserve a comment. Several recent authors have referred the fourhorn and deepwater sculpins into their own genus *Triglopsis* Girard, 1852 (e.g. Fedorov 1986; Eschmeyer 1998). In the mtDNA phylogeny, the *M. quadricornis* complex is deeply nested within the *Myoxocephalus* genus (Fig. 4 and IV), and accepting *Triglopsis* thus would make *Myoxocephalus* paraphyletic. Following McAllister

(1961), Page & Burr (1991) and Kottelat (1997), we suggest that also the fourhorn and deepwater sculpins should be retained in *Myoxocephalus*.

In the flock of Baikalian cottoids, the incongruence between the molecular and systematic subdivisions was also obvious. First, the suggested endemic families Abyssocottidae and Comephoridae (e.g. Berg 1949; Taliev 1955; Sideleva 1982) were completely nested within Cottidae, or more specifically within the genus *Cottus* in the molecular phylogeny (Fig. 4 and V). Under phylogenetic principles this indicates either a need for further splitting of the family Cottidae or recognition of the Baikalian diversity on lower taxonomic level. Second, as to the groupings themselves, the genus *Batrachocottus* has been placed within the subfamily Cottocomephorinae in Cottidae, whereas in the mtDNA phylogeny it rather shows affinity with the taxa referred as abyssocottids.

## Conclusions

In this thesis I studied the diversity and evolution of freshwater fishes in the suborder Cottoidei, using mtDNA sequence data, allozymes or morphology, and material from more than 40 taxa. Some important results and new, or at least rarely emphasized ideas emerging from the data are discussed below.

Much of the observed molecular diversity within cottid taxa appear to have its background in the Pleistocene, probably in population subdivisions related to glacial events. However, this 'genetic legacy of the Quaternary ice ages' (Hewitt 2000) does not account for all phylogeographic structuring observed within the northern cottid species complexes; the mtDNA analyses revealed also ancient, previously unnoticed subdivisions that appear older than the Pleistocene (III).

It is also worth emphasizing that the effects of Quaternary ice ages on biota apparently were more diverse than just devastation of northern populations, or splitting the populations into isolated glacial refugia. For cold-adapted species, such as the Alpine sculpin *Cottus poecilopus*, the glacial periods could also have represented times of range expansions (Thienemann 1950, see also Volckaert *et al.* 2002). Furthermore, glacial periods created freshwater connections across present watersheds, e.g., vast ice-dammed lakes in central northern Russia (Arkhipov *et al.* 1995; Mangerud *et al.* 2001). Such, currently non-existing, connections across central and western Palearctic probably partly explain why subdivisions in many species are deeper within Europe than between Asian lineages and their European relatives. For instance, the European *C. gobio* "is one of the most highly structured freshwater fish species" (Hänfling & Brandl 1998), but on the Holarctic scale, the total divergence within the *C. gobio* complex appears no greater than between neighbouring lineages in Europe (III).

The phylogeographic patterns among the studied cottids are only partly congruent with each other, but most of the identified evolutionary units find counterparts from other fish taxa. Starting from the broadest scale, the Euro-Siberian clades of the *C. gobio* and *C. poecilopus* complexes deserve a notion (III). Similarly widespread, although somewhat younger clades have been observed in various taxa from fish to rodents (e.g. Nesbø *et al.* 1999; Jaarola & Searle 2002). In Europe, one of the most commonly repeated phylogeographic patterns appears to be the east-west subdivision around the Baltic Sea (Taberlet *et al.* 1998; Jaarola *et al.* 1999; Koskinen *et al.* 2000; I–III). The suture zone recognized in northern Sweden on the basis of terrestrial phylogeography is notably close

also to the observed contact zone in *C. gobio*. Yet, the role of the Baltic Sea is not adequately described as just a divider of post-glacial immigration routes. The large basin has also enabled contact and mixing of differentiated units, and thus brought about new, and exceptionally high intraspecific genetic diversity in these northern aquatic habitats.

The whole endemic diversity of the Baikalian cottoid species flock appears monophyletic from the mtDNA data (V). In Baikal, the cottoid radiation has produced 33 currently recognized species with a wide spectrum of morphologies. At similar time scales, the evolution of cottids in other Eurasian freshwater habitats could be rather described as morphological stasis; for instance, the Far Eastern and North European *C. poecilopus* have gained no obvious differences despite estimated divergence of more than 5 Myr. Such distinction in evolutionary rates reflects on one hand the power of adaptive radiation related to invasions of new habitats (the oxygenated abyssal of Baikal), and on the other hand, primarily stabilizing selection acting in the cottid populations that inhabit the shallow-water environments of Eurasia. It is also noteworthy that the fish species radiation involved particularly cottoids, not other fishes of ancient Baikalian faunas (e.g. *Perca* and *Rutilus*; Sideleva 2000). The crucial difference between an ancestral cottoid and other fishes may have been the lack of swimming bladder in cottoids, which could be seen as a preadaptation for life in high-pressure environments.

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