Phylogeography and postglacial dispersion of the chub (*Leuciscus cephalus*) in Europe

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Abstract

A phylogeographic analysis of mitochondrial DNA variation was performed in order to test the hypothesis of a postglacial recolonization of mid- and north-European rivers from a Danubian refuge. Over 345 chub specimens from European rivers covering most of the species' native range were investigated using 600 bp of the cytochrome *b* gene. Chub in European rivers belong to four highly divergent mitochondrial groups (lineages) differing by mean divergence estimates from 5.2% to 7.89%. These four lineages have a largely allopatric distribution, implying four geographical sets: two Mediterranean, and two north-European sets. This pattern provided strong evidence for: (i) the eradication of this species from most of Europe during maximum ice extent; (ii) its survival in four refugia (Adriatic side of the Balkans, eastern Greece (Aegean Rivers), southern tributaries of the Danube, and periphery of Black and Caspian Seas); (iii) a differential postglacial recolonization of mid- and northern Europe from the last two refugia only; (iv) the occurrence of this recolonization in two steps for the Danubian (western) lineage that entered western Europe (Rhine–Rhone–Loire drainages) during the Riss–Würm interglacial period and survived the last glaciation there before colonizing Garonne, UK and German drainages up to the Elbe during the Holocene; and (v) the occurrence of this recolonization in a single step for the Ponto-Caspian (eastern) lineage that entered the Baltic area as far as the Oder in the Holocene. Both lineages came into contact in the River Elbe without evident mixing.

Keywords: Cyprinidae, *Leuciscus cephalus*, mitochondrial DNA, phylogeography, Pleistocene.

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Introduction

Pleistocene glaciations have a deeply modified distribution (Berg 1949; Thienemann 1950; Banarescu 1960; Wheeler 1977; Persat & Keith 1997) and genetic diversity (Hocutt & Wiley 1986; Bentzen *et al.* 1989; Billington & Hebert 1991; Bernatchez *et al.* 1992; Bernatchez & Osinov 1995; Dodson *et al.* 1995; Richardson & Gold 1995; Bernatchez 1997; Strange & Burr 1997; Bernatchez & Wilson 1998) of freshwater species. These modifications have not necessarily implied a reduction but sometimes an extension of their distribution area, both by sea-level lowering (Bermingham & Avise 1986; Wheeler 1977) and by changes in the watercourses (river capture) (Waters *et al.* 1994; Strange & Burr 1997). However, for temperate species, this extension has only been possible in areas far enough from the direct influence of the glaciers, that were usually located on mountain ranges of parts of the northern continents. Southern fish populations have reached new river basins during this climatic event and thus extended their genetic diversity (Durand *et al.* 1997). In contrast, northern populations were either removed or locked in restricted areas (refugia) where conditions were still adequate for survival (Banarescu 1992; Hewitt 1996; Taberlet *et al.* 1998). In Europe, few phylogeographical studies have considered fish postglacial recolonization and, thus, tried to locate refugia; as far as we are aware only the brown trout, *Salmo trutta* has been studied in this way (Bernatchez *et al.* 1992; Bernatchez & Osinov 1995) and

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to a lesser extend the perch, *Perca fluviatilis*, in Scandinavian rivers (Refseth *et al.* 1998).

The middle and lower part of the Danube river is usually considered as the refuge for the Danubian fresh-water species. It may have allowed alone the post-glacial recolonization of all northern rivers in spite of the existence of several Mediterranean refugia such as the Iberic, Italian, and Balkanic Peninsulas (Banarescu 1992). However, Bernatchez & Osinov (1995) indicated that brown trout populations from Atlantic and Danubian drainages have a different dispersal origin because they belong to different phylogenetic groups. Two different dispersal origins were also reported for Scandinavian populations of the perch, *Perca fluviatilis* (Refseth *et al.* 1998). Thus, freshwater fish recolonization history seems more complex than has previously been considered.

Here we proposed to utilize the phylogeographic approach (Avise *et al.* 1987) to try to understand the recent history of European chub, and then to identify potential refugia and postglacial routes of recolonization. The chub is native from Europe and is an adequate model organism due to its wide distribution in Europe and its absence from European fish farms which could interfere with its natural genetic structure. Its natural range extends from southern Norway (but not Denmark) and northwestern Russia southwards to the Euphrates–Tigris Basin and nearly all north Mediterranean drainages (Berg 1949). From west to east, its range spans from the UK and the northern part of Spain (Doadrio & Lobon-Cervia 1985) to the Ural mountains (Berg 1949). This species has the widest distribution in Mediterranean districts although the fish fauna of these districts is rich in endemic species

(Bianco 1990). Chub genetic structure has been studied in Greece (Imsiridou *et al.* 1997, 1998; Doadrio & Carmona 1998; Durand *et al.* 1997, 1999). Three phylogenetic groups were observed: an Adriatic group, an Aegean group, and a Danubian–central Greece group.

In order to test the assumption of a single dispersal origin of north European chub (Banarescu 1992) we employed comparisons of mitochondrial cytochrome *b* sequences in populations from 21 European rivers. Mitochondrial sequence data reflect relatively recent as well as ancient evolutionary events and, by including the palaeohistory of the European river basins, different perspectives of chub phylogeographic history were highlighted.

Materials and methods

Populations sampled

A total of 161 chub belonging to 28 populations was collected from 21 European rivers (Fig. 1). Fish scales were used as a source of mitochondrial DNA (mtDNA) for the genetic analysis. Between 12 and 15 scales per fish were collected in the field and stored in 100% or 70% ethanol.

mtDNA amplification and sequencing

Total genomic DNA was extracted from scales following the protocol described by Kocher *et al.* (1989) with some simplifications (Briolay *et al.* 1998). Amplification conditions for the polymerase chain reaction (PCR) (Saiki *et al.* 1988) were 30 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 30 s and extension at 72 °C for
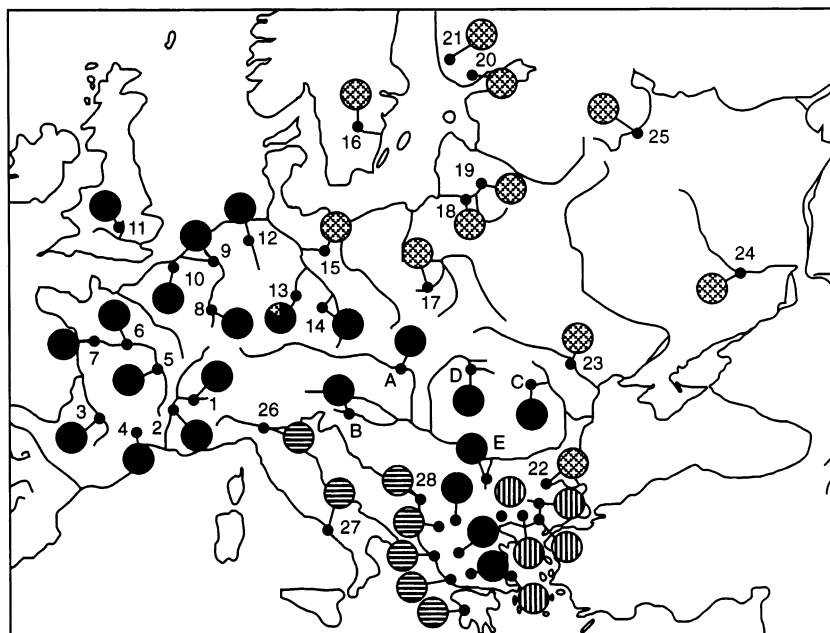

Fig. 1 Sampling sites and distributions of chub mtDNA lineages in European rivers. 1, 2, Rhône; 3, Garonne; 4, Hérault; 5, 6, 7, Loire; 8, 9, Rhine; 10, Meuse; 11, Thames; 12, Weser; 13, 14, 15, Elbe; 16, Eman; 17, Vistula; 18, 19, Nemunas; 20, Kokemäenjoki; 21, Kymijoki; 22, Kamtcha; 23, Dniester; 24, Don; 25, Volga; 26, Pô; 27, Alento; 28, Drin; A, B, C, D, E, Danube (in Durand *et al.* 1997, 1999). The circles indicate: Aegean (vertically hatched circles), Adriatic (horizontally hatched circles), Western (dark circles), and Eastern (grey circles) lineages. All populations sampled in Greece in previous studies (Durand *et al.* 1997, 1999) are not shown in order to not introduce redundant data into the figure.

1 min. The PCR was performed in a 50-μL reaction volume containing 12.5 nM MgCl$_2$, 10 nM of each of the four deoxynucleotides, 1 μL of each 10 mM primer, 10× PCR buffer II (Perkin-Elmer Cetus) and 2 units of *Amplitaq* (Promega) in a programmable thermal cycler (Perkin-Elmer Cetus, Model 9600). A fragment of 650 bp of the 5′ cytochrome *b* extremity of all 161 samples was amplified. PCR primers (Briolay *et al.* 1998) used were L15267 and H15891. Primer names indicate the light (L) or heavy (H) DNA strand and numbers represent the position of the 3′ base of the oligonucleotide in the complete mitochondrial sequence of the carp (Accession no. X61010). Double-stranded PCR products were purified with Qiaquick (QIAGEN) columns.

Direct sequencing (Sanger *et al.* 1977) was carried out using T7 DNA polymerase kits (Pharmacia). The primers used for sequencing were: L15639 (Durand *et al.* 1999), and L15267, H15512, H15149 (Briolay *et al.* 1998).

A total of 600 bp was sequenced for each 161 samples.

Sequence alignment, phylogeny and population genetic analyses

Sequences were aligned using the SEAVIEW program (Galtier *et al.* 1996). Relationships between genotypes were determined by distance methods with the PHYLO_WIN program (Galtier *et al.* 1996). Distance trees were estimated according to the neighbour-joining method of Saitou & Nei (1987), with Kimura's (1980) two-parameter model and Tajima & Nei's (1984) distance. The bootstrap resampling technique was used to assess the statistical significance of internal nodes. Because of the close relationship between *Leuciscus pyrenaicus* and *L. cephalus* (Briolay *et al.* 1998), three cytochrome *b* sequences of *L. pyrenaicus* (Brito *et al.* 1997) were used as outgroups to root the trees.

Spatial distribution of mitochondrial variation was tested, using analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) from the ARLEQUIN package.

Results

Sequence variation and diversity of mtDNA haplotypes

Among the 161 chub analysed we identified 18 genotypes defined by 70 variable sites, including 61 informative sites. Sequences have been deposited in the European Database (EMBL) under Accession nos AJ006887–AJ006902. The nucleotide composition of the 600 bp cytochrome *b* segment sequenced was globally G deficient (17.0%), whereas similar frequencies were observed for the other three nucleotides (A: 25.8%; C: 28.6%; T: 28.6%). Such a nucleotide composition pattern has been reported in several other fish studies (Cantatore *et al.* 1994; Brito *et al.* 1997). Sequence variation was predominantly due to

transitions, followed by transversions (transition/transversion ratio: 9.653), whereas no insertions or deletions were observed. The number of substitutions between the 18 chub genotypes varied from 1 to 46, corresponding to Tajima & Nei's (1984) distance values of 0.2% and 8.6%, respectively.

Phylogenetic relationships between chub genotypes

Additional sequences (34 genotypes from Danubian and Greek chub populations) from Durand *et al.* (1999), deposited in the European Database (EMBL) under Accession nos AJ002319–AJ002352, were used for phylogenetic reconstructions and genetic analysis (AMOVA). The phylogenetic tree obtained with the neighbour-joining approach revealed four lineages (Fig. 2) including two southern ones (Adriatic and Aegean) and two mostly (with the exception of the French and central Greek populations) northern ones (east and west Europe). The bootstrap values for these divisions ranged between 100% and 93% (Fig. 2). Only the east European lineage had not been observed in previous chub studies (Durand *et al.* 1997, 1999) while no genotypes belonging to the Aegean lineage were observed in populations sampled in this study. Average sequence divergences (Tajima & Nei's (1984) distance) among genotypes of the east European populations and those of the other three lineages were 7.89 ± 0.48% (east European/Adriatic lineages), 7.67 ± 0.4% (east European/Aegean lineages), and 7.51 ± 0.37% (east/west European lineages), which are higher than between genotypes of the west European and Adriatic lineages (5.2 ± 0.5%), between genotypes of the west European and Aegean lineages (5.64 ± 0.42%) and between genotypes of the Adriatic and Aegean lineages (6.24 ± 0.54%). Within both northern lineages average sequence divergences were similar (Table 1), whereas the sequence divergence among genotype Volg (observed in the Volga) and other eastern haplotypes was strongly supported by a bootstrap value of 85% (Fig. 2). In contrast, both southern (Mediterranean) lineages had higher average sequence divergences (Table 1). Within the Adriatic lineage two divisions were observed (Fig. 2): an Italian–Drin–Prespa group and a southwestern Greek group (bootstrap values 76% and 70%, respectively).

Geographic distribution of mtDNA genotypes and genetic population structure (AMOVA)

Geographic distribution of genotypes according to their phylogenetic lineages (Fig. 1) defined four unbroken geographical areas: Adriatic, Aegean, Western and eastern Europe. Except in the Elbe where genotypes belonged to east and west European lineages (Fig. 1), there was no evident mixture of genotypes. Within Adriatic and
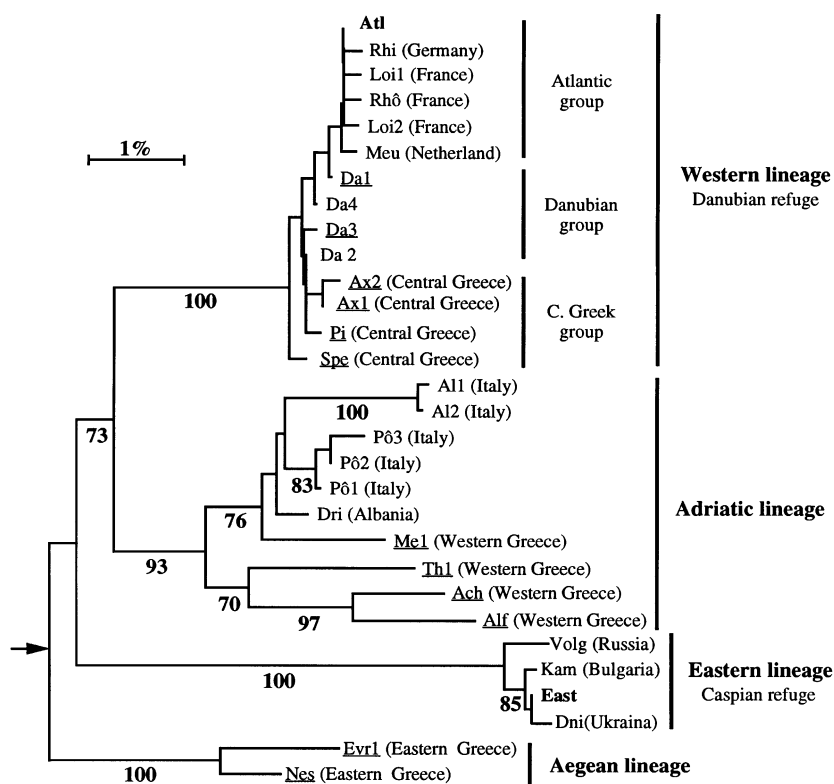
Fig. 2 Neighbour-joining tree based on Tajima & Nei's (1984) distance among 28 cytchrome *b* genotypes. The numbers represent percentage support of each branch in 500 bootstrap replications (only values above 70% have been noted). Haplotype designation is given in Table 2, underlined haplotypes (not shown in Table 2) are additional genotypes from Durand *et al.* (1999). Note that groups within the western lineage are not defined phylogenetically but in relation to geographical distribution of common western genotypes.

Table 1 Genetic diversity within the four chub mtDNA lineages observed in European populations with the number of populations and individuals (*N*), the number of haplotypes (*n*) and the mean sequence divergence (dmean)

Lineage	Group	N pop-indiv	n haplotypes	dmean ± SE
Adriatic		13–82	19	2.9 ± 1.38%
Aegean		8–54	13	1.2 ± 0.68%
Eastern Europe		11–61	4	0.5 ± 0.3%
Western Europe		27–148	14	0.53 ± 0.26%
	Central Greek	8–48	4	0.45 ± 0.18%
	Danubian	6–25	4	0.28 ± 0.12%
	Atlantic	13–75	6	0.27 ± 0.05%

Aegean areas, each genotype observed in chub populations was restricted to one river (Table 2 and Durand *et al.* 1999). In contrast, in western and eastern Europe, genotypes Atl and East, respectively, were widespread and very common in chub populations (Table 2). However, within Danubian and central Greek populations, which belong in the western Europe lineage, the genotype Atl was not observed (Fig. 3), although 21 and 48 chub were analysed in the Danube and Central Greece, respectively (Table 1). Both regions presented their own genotypes (Durand *et al.* 1999) even if genotypes Da2 and Da4 were found in one population of the Rhine and the Elbe (Table 2 and Fig. 3). All other genotypes (Rhô, Loi1, Loi2, Rhi, Meu), which occurred in sympatry with the genotype Atl were local genotypes without any broad distribution (Table 2). Within eastern Europe, genotypes Kam and Dni were also local genotypes, whereas genotype

Volg was abundant in the population of the Volga and observed in the only chub analysed from the Don (Table 2). The majority of these local haplotypes differed from common haplotypes by a single mutation and occurred as single or very few individuals (Table 2).

The significance of the genetic heterogeneity among populations and regions was assessed with AMOVA (Excoffier *et al.* 1992) using two predictive models of regional partitioning to test phylogeographic structure. Populations were partitioned on the basis of: (i) of three recognized refugia (Adriatic, Aegean, and Danubian including all northern populations) based on earlier ichthyological district hypotheses (Bianco 1990; Economidis & Banarescu 1991); and (ii) our phylogenetic results (Figs 1 and 2) assuming four refugium areas (Adriatic, Aegean, western and eastern Europe). Both models indicated significant geographical structuring ($P < 0.001$) within and

Table 2 Absolute frequency of chub cytochrome *b* genotypes from 28 locations in 21 different rivers

| Basin	Population	Sites	Da2	Da4	Atl	Rhô	Loi1	Loi2	Rhi	Meu	East	Dni	Kam	Volg	Pô1	Pô2	Pô3	Al1	Al2	Dri	*n*
Rhone	Lake Bourget	1			8	1															
	Doux	2			1																10
Garonne		3			10																10
Herault		4			4																4
Loire	Dompierre	5			2		1														
	Chinon	6			1																
	St Laurent	7			4			1													9
Rhine	Fessenheim	8		1	4																
	Rhur	9			4				1												10
Meuse		10			5					1											6
Thames		11			9																9
Weser		12			9																9
Elbe	Saale	13			7						1										
	Eger	14	2	2																	
	Spree	15									10										22
Eman		16									1										1
Vistula		17									7										7
Nemunas	Nemunas	18									9										
	Buka	19									2										11
Kokemäenjoki		20									4										4
Kymijoki		21									1										1
Kamtcha		22									4		2								6
Dniester		23									7	3									10
Don		24												1							1
Volga		25									2			8							10
Po		26													7	2	1				10
Alento		27																7	3		10
Drin		28																		1	1
Total Frequency			2	3	67	1	1	1	1	1	48	3	2	10	7	2	1	7	3	1	161
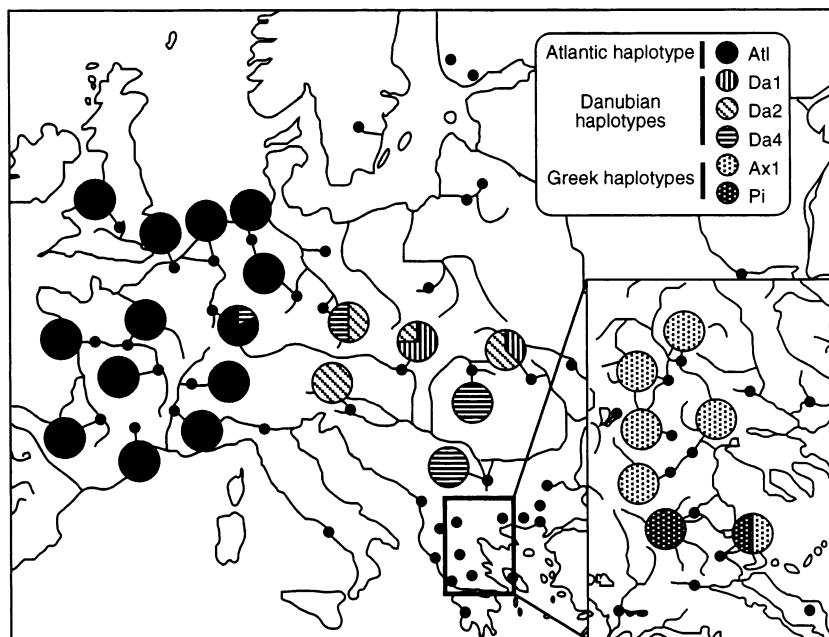
Fig. 3 Distribution of common haplotypes of the western lineage. Black dots indicate sites from which common western haplotypes are absent. Circles indicate relative and not absolute proportions of these haplotypes; presences/abundances of other haplotypes are not shown. Haplotypes designation is given in Table 2.

among populations and regions. The model assuming two northern refugia was more effective than the ichthyological district model in partitioning variance components so that variance among regions was maximized (Va = 14.48 (80.7%) vs. 5.9 (37.1%)) and variance within regions was minimized (Vb = 3 (17%) vs. 9.6 (60.2%)). This result indicates that the model of two northern refugia for northern populations best explained the geographical patterns of genetic variation.

Discussion

Intraspecific divergence

Low levels of diversity within both northern mtDNA lineages (< 1%) (Table 1) are congruent with data from other northern freshwater species (Bernatchez & Wilson 1998; Wilson & Hebert 1998 and references therein). These are probably due to repeated glacial disturbances causing reduced mtDNA diversity through habitat loss, displacement, and persistence in suboptimal habitats during glacial events (Avise *et al.* 1984). In contrast, both southern chub mtDNA lineages show high levels of diversity (Table 1) which are also congruent with data from other fresh-water species living in nonglaciated areas (Bermingham & Avise 1986; Billington & Hebert 1991 and references therein). The deep divergence among lineages and the high diversity within both southern lineages suggest a pre-Pleistocene origin for each lineage, perhaps during the Pliocene according to the evolution rate of Orti *et al.* (1994) (2.8% sequence divergence per million years).

Refugial origins and postglacial dispersion

The geographical distributions of the four chub mtDNA lineages strongly suggest their survival in separate refugia and supports the previous multiple-refugia assumption (Banarescu 1992). However, the hypothesis of a single refugium responsible for the recreation of the north European ichthyofauna is not supported by our data because two different mtDNA lineages were observed in north European chub populations (Fig. 1). Chub dispersion from both southern refugia was restricted to the Mediterranean area as no southern haplotype was observed in northern populations (Fig. 1). Despite the low number of fish/populations analysed this conclusion is likely as there are strong dispersion barriers such as the Alps, the Pinde and the Rhodop mountains around these Mediterranean areas.

The geographic pattern within the west European area was complex and suggests two vicariant disruptions because three regions can be delimited on the basis of common haplotypes: Atl (for the Atlantic group), Da1, Da2, Da4 (for the Danubian group) and Ax1 and Pi (for the central Greece group) (Fig. 3). The presence of west European haplotypes in central Greece chub populations was interpreted as a genetic introgression (Durand *et al.* 1997) following an invasion by Danubian chub via a river connection between the Danube and Axios in the Vardar valley (Economidis & Banarescu 1991; Karakousis *et al.* 1995; Economidis & Nalbant 1996). Indeed, Axios colonization by Danubian populations seems more likely than the reverse as the central Greece chub populations showed a poorer genetic diversity than all other southern chub lineages (Table 1).

Western European populations originate from the Danubian refuge, as the genetic structure of the Atlantic group is much simpler than the genetic structure in the Danubian group (Fig. 3). However, the extension of the Danubian chub into the Atlantic area might be more ancient than the last glaciation as no Atl haplotype was observed in Danubian populations. The most likely scenario explaining our results is a colonization during the Riss–Würm interglacial period (i.e. about 100 000 years ago) of rivers close to the Danube such as the Rhine or the Rhone and then a rapid range expansion in all Atlantic drainages including French Mediterranean drainages (Rhone and Herault) at the end of the Würm (10 000 years ago). The extension to southern France may have been made easier by the sea-level drop during the Würm glaciation. Indeed, chub is known from Magdalenian layers (18 000–16 000 years ago) in the Garonne drainage (Le Gall 1984; Keith 1998). With many other fish, chub certainly entered eastern UK and Elbe drainages from the Rhine at the Boreal time (i.e. about 7000 years ago), when these drainages converged across the North Sea bottom during low sea level (Gibbard 1988). The occurrence of one full Danubian haplotype in the Rhine drainage must be related to more recent events such as limited postglacial connections.

Dispersion of the east European lineage probably started from a Caspian refuge as the population of the Volga presents two significantly divergent haplotypes (East and Volg; Fig. 2) which testify the great age of this population. Dispersal of the chub from the Caspian refuge probably began via fluvial connections between the Don and Volga (Fortunatov 1979) and coastal exchanges through the Black Sea under low-salinity conditions during glaciations (Grosswald 1980; Ryan *et al*. 1997). Glacial retreat and ensuing dispersal opportunities began later around the Baltic Sea. The quite recent character of the recolonization of the Baltic drainage is ascertained by the lack of any variability in these populations (sites 21, 20, 19, 18, 17, and 16, Table 2). The more probable route for this extension was straying water in the badly defined watershed between Upper Dniepr (Pripiat) and Neman rivers in the late glacial time, then dispersal along the nearly fresh Baltic coasts and/or through the Polish marsh plain following the former east–west riverbed of the circumglacial Mega–Vistula (Grosswald 1980; Starkel 1991). Another path may have been the Upper Volga towards the Gulf of Finland and Baltic Sea.

Secondary contact among refugial lineages

The most striking result of this study is the lack of evident contact between any of the four mtDNA lineages. If the absence of contact with any southern lineages may be explained by strong dispersal barriers, contact between both northern lineages was expected, in particular in rivers around the Black Sea. This was not observed in our data but this may be due to the low number of individuals and sites sampled. However, the chub *Leuciscus cephalus* is at present absent from the terminal part of the Danube river where it is replaced by *L. borysthenicus* (Banarescu 1964), which suggests that the lowland and delta environments of the Danube may constitute a dispersal barrier for it.

The only evident contact zone observed in our study was in the Elbe drainage where haplotypes of both northern lineages are present. Furthermore, the haplotypes of the western Europe lineage present in the Elbe belong to two different groups: a Danubian (site 14) and an Atlantic (site 13) group. Finally, within populations of the Elbe basin as well as larger geographical scales there is not an obvious mixture of lineages. A similar genetic pattern has already been observed in a freshwater fish: the lake trout, *Salvelinus namaycush* (Wilson & Hebert 1998). This result was interpreted as due to a rapid expansion by former colonists in newly formed habitats, which would have placed newcomers at a numerical disadvantage. This assumption is based on the probability that persistence of mtDNA haplotypes within populations is proportional to their initial abundances (Birky *et al*. 1983). However, once again more individuals should be analysed per populations to thoroughly test this assumption.

Postglacial European freshwater fish history

The only freshwater species in Europe that has been studied in a similar manner to the present study is the brown trout, *Salmo trutta* (Bernatchez *et al*. 1992; Bernatchez & Osinov 1995). However, a comparison is quite difficult because this species may have taken migration routes very different from those of a stenohaline fish, such as coastal brackish waters or fully saline conditions. Few other genetic data illustrating fish dispersal in mid-Europe are available in the literature, especially concerning cyprinids. Some investigations on nase, *Chondrostoma nasus*, stressed the homogeneity, i.e. the quite recent extension of this species from Danube to Rhine and other French drainages (Gollmann *et al*. 1997). Common barbel, *Barbus barbus*, populations are also quite homogeneous over this area (Persat & Berrebi 1990; Berrebi *et al*. 1993; MacHordom *et al*. 1995; Tsigenopoulos *et al*., in press). However, it is quite difficult to generalize the dispersal pattern of chub in Europe to any other cyprinids because no any studies have been done over a wide geographical range.

Acknowledgements

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