Molecular Ecology (2001) **10**, 2177–2185

Phylogeography of the barbel (*Barbus barbus*) assessed by mitochondrial DNA variation

P. KOTLÍK*† and P. BERREBI*‡
*Laboratoire Génome, Populations, Interactions CNRS UMR 50003, Université Montpellier 2, Place E. Bataillon, 34095 Montpellier Cedex 5, France, †Laboratory of Fish Genetics, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, 277 21 Liběchov, Czech Republic

Abstract

Using the phylogeographic framework, we assessed the DNA sequence variation at the mitochondrial cytochrome *b* gene across the distribution range of the barbel *Barbus barbus*, a widely distributed European cyprinid. Reciprocal monophyly of non-Mediterranean European and Balkan/Anatolian populations is taken as evidence for a long-term barrier to gene flow, and interpreted as a consequence of survival of the species in two separate refugia during several later glacial cycles. Lack of profound genealogical divergence across Europe from western France to the northwestern Black Sea basin is consistent with recent colonization of this area from a single glacial refuge, which was probably located in the Danube River basin. This may have occurred in two steps: into the Western European river basins during the last interglacial, and throughout the Central European river basins after the last glacial. The populations from the Balkans and Anatolia apparently did not contribute mitochondrial DNA to the post-Pleistocene colonization of non-Mediterranean Europe. Lack of detectable variation within the Balkans/Anatolia is attributed mainly to recent expansion throughout these regions, facilitated by the freshwater conditions and seashore regression in the Black Sea during the Late Pleistocene and Early Holocene.

Keywords: *Barbus*, biogeography, gene genealogy, glaciation, mtDNA, phylogeography, Pleistocene

Received 1 December 2000; revision received 27 April 2001; accepted 9 May 2001

Introduction

The major global climatic and environmental changes of the Quaternary (i.e. glacials) greatly influenced evolution of organisms on the Earth (Dynesius & Jansson 2000; Hewitt 2000). Toward the end of the Pleistocene, many species went through successive range contractions and expansions, characterized by the species' extinction often from large northern parts of their ranges when temperatures decreased, and a subsequent re-colonization from one or more refugia when the climate became warmer (Hewitt 1999, 2000). There have been many studies on the effects of Quaternary events on European animal species based on their present phylogeographic DNA patterns. The vast majority of these have been conducted on terrestrial invertebrates and vertebrates (reviewed in Taberlet *et al*. 1998; Hewitt 1999, 2001). The phylogeographic structure and Quaternary history of the European freshwater fishes have been only recently addressed, and they still remain largely unknown (Bernatchez & Dodson 1994; Durand *et al*. 1999; Nesbø *et al*. 1999; Englbrecht *et al*. 2000; Weiss *et al*. 2000; Bernatchez 2001). Freshwater fish are well suited for phylogeographic studies, because they are dependent upon water routes for dispersal and their phylogeographies are therefore likely to reflect historical causes more closely than those of terrestrial species (Bernatchez & Wilson 1998).

The present paper examines phylogeography of the barbel *Barbus barbus*. The barbel is a riverine cyprinid distributed throughout Europe from southeastern England and France in the west to the Black Sea basin in the east; it is absent from most of southern Europe (Fig. 1). The barbel is a vagile species that occupies the middle stretches of rivers

Correspondence: P. Kotlík. †Laboratory of Fish Genetics, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, 277 21 Liběchov, Czech Republic. Fax: ++ 420 206 697 186; E-mail: kotlik@iapg.cas.cz
‡Present address: UMR 5119 'Ecosystèmes Lagunaires' CP 093, Université Montpellier 2, Place E. Bataillon, 34095 Montpellier Cedex 5, France.

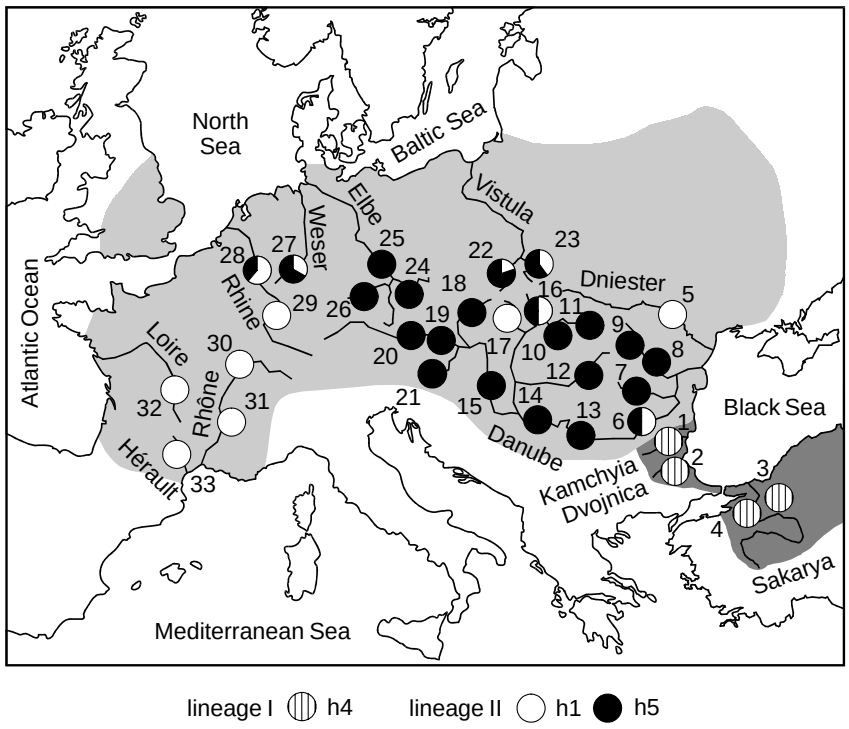

Fig. 1 Map illustrating the location of sampling sites within the geographical range of the barbel (shaded areas; after Lelek 1987), and the distribution and relative frequencies of the single haplotype of lineage I and the two most frequent haplotypes of lineage II. The distribution of haplotype h5 also includes haplotypes h9 to h11, which represented its rare variants. A detailed distribution of all haplotypes is described in Table 1. Data for sites 3, 4, and 31, were from Tsigenopoulos & Berrebi (2000). Dark grey shading refers to the subspecies *Barbus barbus escherichi* distributed throughout rivers of the Black Sea basin from eastern Bulgaria to northern Anatolia, perhaps as far as western Transcaucasia.

but also enters their lower parts (Lelek 1987). This may result in high dispersal ability with gene flow occurring throughout a river basin, while migration between river drainages through river captures of headwaters is likely to be limited (cf. Bănărescu 1998). Artificial breeding and stock transfers by humans has been limited in the barbel (Bohlen & Nolte 1998; Šlechtová *et al*. 1998), and they are, therefore, unlikely to confound the phylogeographic analysis.

We examine the genealogical relationships and geographical distribution of mitochondrial DNA (mtDNA) haplotypes throughout the majority of the barbel geographical range, and interpret the observed patterns within the phylogeographic framework. We present a hypothesis on the historical biogeography of the barbel to account for the current spatial distribution of its mtDNA variation.

Materials and methods

Sampling

We obtained 87 individual *Barbus barbus* samples from 30 sites throughout the barbel distribution range (Fig. 1, Table 1). As our goal was to describe the phylogeography of the species, we sought to increase the number of sites and geographical coverage at the expense of within population sample sizes. Among the criteria taken into account when selecting sampling sites were the inclusion of large European river basins, and of presumed Pleistocene refugial areas located within the Danube River basin and in the Balkans. We used published sequence data (Tsigenopoulos & Berrebi 2000) for a homologous *B. barbus* mtDNA segment from the Drôme River in France (GenBank accession no. AF112123) to complete the sample from the Rhône River basin, and for *B. barbus escherichi* from two sites within the Sakarya River basin in northern Anatolia (AF112432). The sequence of *B. tauricus* from the northern Caucasus (AF095605), which may be the nearest relative of *B. barbus* (Zardoya & Doadrio 1999), was used as an outgroup. Sequences of phylogenetically close species from Mediterranean Europe *B. plebejus* (AF112410), *B. tyberinus* (AF112411 and AF112412) and *B. macedonicus* (AF112129 and AF112426) (Tsigenopoulos & Berrebi 2000), were used for comparative purposes.

Molecular biological techniques

DNA was extracted from muscles or fins preserved with ethanol (95%) either by incubating a very small amount of the tissue in 400 µL of 5% (w/v) suspension of Chelex 100 resin (Biorad) and 15 µL of proteinase K (20 mg/mL) at 55 °C overnight and then boiling for 15 min, or by phenol–chloroform extraction (Sambrook *et al*. 1989). A 664-bp product that included a small part of the glutamic acid tRNA and 598 bp of 5′ extremity of cytochrome *b* (cyt*b*) gene was polymerase chain reaction (PCR) amplified with primers L15267 and H15891 from Briolay *et al*. (1998). The resulting PCR products were purified by ethanol

Table 1 Geographic origin of the barbel samples and absolute sample frequencies of the 11 mtDNA haplotypes

					Haplotype										
No.*	River	Country†	Drainage	*n*	h1	h2	h3	h4	h5	h6	h7	h8	h9	h10	h11
1	Kamchyia	BG	Kamchyia	3				3							
2	Dvojnica	BG	Dvojnica	7				7							
3	Kirmir‡	TY	Sakarya	3				3							
4	Karasu‡	TY	Sakarya	1				1							
5	Dniester	MO	Dniester	5	4	1									
6	Danube	BG	Danube	2	1				1						
7	Ialomiţa	RO	Danube	1					1						
8	Putna	RO	Danube	1					1						
9	Buzău	RO	Danube	1					1						
10	Lăpuş	RO	Danube	3					3						
11	Iza	RO	Danube	1									1		
12	Mureş	RO	Danube	3					3						
13	Archar	BG	Danube	3					3						
14	Danube	YU	Danube	2					1	1					
15	Danube	H	Danube	5					4			1			
16	Laborec	SK	Danube	2	1				1						
17	Torysa	SK	Danube	3	3										
18	Váh	SK	Danube	2					2						
19	Dyje	CZ	Danube	2					1		1				
20	Dyje	CZ	Danube	4			1		3						
21	Rabnitz	A	Danube	2					2						
22	Poprad	SK	Vistula	5	1				4						
23	San	PL	Vistula	5	2				3						
24	Sázava	CZ	Elbe	1					1						
25	Elbe	CZ	Elbe	2					2						
26	Berounka	CZ	Elbe	3					1					2	
27	Eder	D	Weser	3	1									1	1
28	Rhine	D	Rhine	3	2				1						
29	Rhine	D	Rhine	3	3										
30	Rhône	F	Rhône	5	5										
31	Drôme‡	F	Rhône	4	4										
32	Loire	F	Loire	3	3										
33	Lergue	F	Hérault	2	2										
Total Haplotype Count					32	1	1	14	39	1	1	1	1	3	1

*Sample numbers refer to those used in Fig. 1.
†BG, Bulgaria; TY, Turkey; MO, Republic of Moldova; RO, Romania; YU, Yugoslavia; H, Hungary; SK, Slovakia; CZ, Czech Republic; A, Austria; PL, Poland; D, Germany; F, France.
‡Data from Sakarya River basin in Turkey, and from the Drôme River in France, are from Tsigenopoulos & Berrebi (2000).

precipitation in the presence of 5 M ammonium acetate, and both strands were directly cycle-sequenced with the same primers as those used for the initial amplification. The PCR amplification and sequencing with a Pharmacia automated sequencer followed methods given in Tsigenopoulos & Berrebi (2000). Sequences were aligned manually to each other and to the published complete cyt*b* sequence of *B. barbus* from the Drôme River in France (AF122123). For the majority of individuals ($n = 79$), sequences of both complementary strands were compared to eliminate ambiguities and to check polymorphic sites. All polymorphisms were additionally checked by visual inspection of automated sequencer chromatographs when encountered for the first time. Sequences determined in this study were deposited in the GenBank database under the accession numbers AY013480–AY013488.

Data analyses

Estimates of nucleotide divergence between different sequences were calculated with the PAUP* software package, version 4.0b7 (Swofford 1999), using a corrected distance based on the HKY85 model of sequence evolution accounting for uneven base composition and unequal

transversion / transition ratio, and assuming gamma distribution of among-site rate variation (Hasegawa *et al*. 1985; Yang *et al*. 1994). The HKY85 model was determined to be the appropriate model for our data set by the hierarchical likelihood ratio test of goodness of fit of 56 different nested models to the data, as implemented in the MODELTEST program, version 3.04b (Posada & Crandall 1998).

Phylogenetic relationships among the barbel haplotypes were reconstructed by maximum parsimony (MP) criterion using PAUP* (exhaustive search). To quantify the confidence in the applicability of MP criterion to the barbel data set, we used the method of Templeton *et al*. (1992) as implemented in TCS software, version 1.06 (Clement *et al*. 2000). The program outputs the maximum number of pairwise site differences in the data set, for which the estimated probability that none of the differences is due to more than one mutation is greater than 0.95. Analyses with the outgroup were performed using the MP criterion (branch-and-bound search) and (for comparative purposes) by analysing the HKY85 corrected distance matrix with the neighbour-joining (NJ) algorithm.

The distances were used to estimate the time to the most recent common ancestor (MRCA) of mtDNA lineages. A mutation rate in mitochondrial cyt*b* of 0.0076 per site per Myr was calibrated for the western Palearctic cyprinids (including *Barbus* species) by Zardoya & Doadrio (1999), and a very similar estimate of 0.0072– 0.0075 per site per Myr was obtained for the western Palearctic *Barbus* by C. S. Tsigenopoulos (personal communication). Hence, we employ the range of mutation rates of 0.0072–0.0076 per site per Myr to estimate the time to MRCA.

Results

Nucleotide polymorphism

We determined the complete nucleotide sequence for the first 594 bp of the mitochondrial cyt*b* gene for 87 *Barbus barbus* individuals. Of the sites analysed, 15 positions showed two nucleotide states while the remaining 579 sites were monomorphic in our sample. Combinations of nucleotide states at polymorphic sites defined 11 different haplotypes (Table 2). The haplotype described by Tsigenopoulos & Berrebi (2000) for four individuals from the Drôme River in France (the Rhône River basin) was identical with our haplotype h1. The haplotype from Sakarya River basin in northern Anatolia reported in Tsigenopoulos & Berrebi (2000) was identical to our haplotype h4. All the polymorphisms were single nucleotide changes, and all but one of them were transitions. The only transversion polymorphism was inferred to have arisen by a change from Adenine to Thymine at position 43, and it represented the only replacement polymorphism (asparagine to tyrosine) within the barbel data set. This mutation defines the origin of haplotype h11 from its precursor, h10. Haplotype h11 was a tip haplotype, identified in only one individual from the Weser River basin, where it coexisted with h10 (otherwise h10 was only detected at one site in the Elbe River basin), and with the widely distributed h1.

Phylogeny of haplotypes

The MP phylogenetic analysis of 11 unique *B. barbus* haplotypes yielded a single MP tree (Fig. 2). The maximum number of pairwise nucleotide differences between haplotypes, for which the use of the MP criterion is justified with 95% confidence (Templeton *et al*. 1992), was determined to be 10, which exceeds the maximum number of pairwise nucleotide differences in the barbel data set.

MP and NJ phylogenetic analyses with the outgroup consistently confirmed the monophyly of the barbel haplotypes, and identified the position of the root of the barbel intraspecific tree along its longest branch (Fig. 2). Two major mtDNA lineages were identified in the barbel genealogy, denoted here as lineage I and lineage II. Each of the two lineages is defined by synapomorphic character states.

Table 2 Haplotypes defined by 15 variable nucleotide positions in 594 bp of the barbel mitochondrial cyt*b* gene. Nucleotide position numbers correspond to the complete barbel mitochondrial cyt*b* sequence in GenBank (accession number AF112123; Tsigenopoulos & Berrebi 2000), which is identical in the 594 bp analysed here with haplotype h1. The sequence for h1 is shown at all variable sites, the bases in the other haplotypes are shown only where different from h1

	Nucleotide position														
Haplotype	43	48	102	115	159	198	201	243	309	324	405	459	501	507	588
h1	A	C	T	C	G	T	C	C	C	C	A	A	A	A	T
h2								T							
h3					A						G				
h4			C			C	T				G	G			C
h5														G	
h6				T										G	
h7													G	G	
h8									T					G	
h9										T				G	
h10		T												G	
h11	T	T												G	

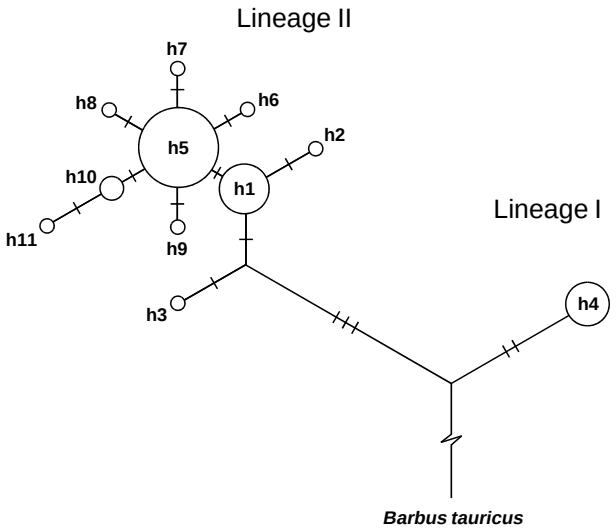

Fig. 2 A maximum parsimony tree for the barbel mtDNA haplotypes. Circle sizes are proportional to the observed haplotype frequencies. Branch lengths correspond to the observed number of nucleotide changes along each branch (also indicated by dashes across the branch). The tree is shown rooted using *Barbus tauricus* from the northern Caucasus as an outgroup.

Lineage I is formed by haplotype h4, lineage II is made up of the remaining 10 haplotypes. Haplotypes of lineage I and of lineage II differ by a minimum of six nucleotide changes and an average sequence divergence of 0.013.

Geographical structure of mtDNA variation

The geographical distribution of haplotypes was not random (Table 1). The two major lineages had strictly nonoverlapping geographical distributions (Fig. 1). Populations from Kamtchiya River and Dvojnica River in eastern Bulgaria (sites 1 and 2), and from the Sakarya River basin in northern Anatolia (sites 3 and 4) all belonged to lineage I, being fixed for h4. All individuals sampled in populations from Loire River in western France through Central Europe to Dniester River and lower Danube River of the Black Sea basin belonged to lineage II. From one to three haplotypes per population were observed in the lineage II. Haplotypes h1 and h5, differing from each other by a single nucleotide change, were the most frequent (frequencies in the lineage II populations of 39.5% and 48.1%, respectively). Haplotype h10 was the third most abundant lineage II haplotype occurring in three individuals (3.7%). All other lineage II haplotypes were singletons, each identified in only one individual (Table 1).

The two most frequent lineage II haplotypes exhibited a marked geographical distribution pattern (Fig. 1). Haplotype h1 was fixed in all our samples from the Rhône, Loire, and Hérault River basins (sites 30–33), as well as in Torysa River of the Danube River basin (site 17), and at one of the two sites in the Rhine River basin (site 29). It also dominated the sample from the Dniester River basin (site 5), where it coexisted with its single-mutational derivate, haplotype h2. Haplotype h1 was absent from our samples from all three sites in the Elbe River basin (sites 24–26), and from 13 out of 16 sites within the Danube River basin (sites 7–15, and 18–21), which were characterized by h5, except for site 11. The single fish from this site carried haplotype h9, a single-mutational derivate of h5. Haplotypes h1 and h5 coexisted at both sites in the Vistula River basin (sites 22 and 23), at one site in the Rhine River basin (site 28), and at two sites in the Danube River basin (sites 6 and 16). In the Weser River basin (site 27), h1 coexisted with descendants of h5, haplotypes h10 and h11.

Divergence from other European Barbus *species*

The average pairwise sequence divergence of *B. barbus* from three phylogenetically close *Barbus* species from southern Europe ranged between 0.045 (lineage I from *B. plebejus*) and 0.054 (lineage I from *B. macedonicus*). The average pairwise sequence divergence of *B. barbus* from *B. tauricus* was 0.043 for lineage I and 0.046 for lineage II.

Coalescence time estimates

The time to the MRCA of haplotypes from two populations that show reciprocal monophyly can be estimated from average sequence divergence between them (Hoelzer *et al*. 1998). With this approach, the barbel haplotypes of lineage I would coalesce with those of lineage II at ≈ 0.84–0.89 Myr, when using range of mutation rates of 0.0072–0.0076 per site per Myr. Applying these rates, the divergence between *B. barbus* and phylogenetically close species from Mediterranean Europe would date back approximately 2.97–3.74 Myr.

Discussion

This study has revealed a remarkable level of phylogeographic structuring in the mitochondrial cyt*b* gene of the barbel. In the following discussion, we interpret the observed phylogeographic patterns, and propose a historical biogeography hypothesis to explain them.

Intraspecific haplotype tree

We found a single MP tree for the 11 barbel haplotypes detected in this study. The MP method of phylogenetic inference is expected to give a correct solution under the assumption that nucleotide changes are rare, which implies no or low homoplasy (Felsenstein 1983, 1988; Smouse 1998). This assumption seems to be met by our data. The overall nucleotide divergence between sequences

was low (0.002–0.015), and the barbel data set fitted the infinite-site model of sequence evolution (no nucleotide site was inferred to have undergone more than one change), which resulted in the absence of homoplasy. These properties of the data set are reflected by the result of the test of Templeton *et al*. (1992), which justified the use of the MP criterion for the entire barbel data set with 95% confidence. Therefore, we consider the MP tree a credible representation of the phylogenetic relationships among the barbel mtDNA haplotypes.

Refugial origin of two major mtDNA lineages

The deep phylogenetic break between lineages I and II suggests that there has been a long-term barrier to the gene flow between populations from eastern Bulgaria and northern Anatolia on the one hand, and populations from the river basins of Western and Central Europe on the other. The time to the MRCA of haplotypes from the two regions was estimated to be ≈ 0.84–0.89 Myr, which falls within the Middle Pleistocene. The error associated with both the calibration and the application of a molecular clock is usually considerable (Nei 1992), and this time estimate should therefore be considered provisional. Despite the potential inaccuracy, the average sequence divergence of 0.013 would be consistent with the Pleistocene divergence of populations (≈ 0.01–2 Myr) using a wide range of plausible substitution rate calibrations (Avise *et al*. 1998). Evolution in the absence of gene flow since within the Pleistocene would imply that the two lineages descended from two separate allopatric refugia, where they probably survived over several later glacial maxima.

Superficial phylogeographic structure across Europe north of the Mediterranean region

It is likely that the barbel distribution during the Pleistocene would have been different from its present-day distribution because of the unsuitability of most of the contemporary river basins at that time (Bănărescu 1991). Bănărescu (1991) claimed that the barbel colonized the part of Europe now encompassed by mtDNA lineage II from a glacial refuge located in the Danube River basin. The absence of pronounced genealogical differentiation between the populations of lineage II appears consistent with this scenario. The Danube River basin is now dominated by h5, but the majority of the lineage II haplotypes were found in this basin, indicating an absence of recent severe bottlenecks. In the Vistula River and Elbe River basins, h5 was also frequent. Barbel from the Danubian refuge probably dispersed throughout these river basins following the retreat of ice and warming at the end of the last (Würm) glacial (i.e. approximately 10 000 years ago; Adams *et al*. 1999) when temporary connections between rivers probably existed, e.g. through glacial meltwater or periglacial rivers (Arkhipov *et al*. 1995). Populations sampled in France have most likely been derived from the Danubian refuge also. However, these samples were fixed for h1, the direct ancestor of the dominant Danubian haplotype h5, which itself was missing in French rivers (Fig. 1). This suggests that the colonization of the Western Europe may have taken place before the Würm glacial. The possible scenario appears to be colonization of Western European rivers such as Rhine and Rhône from the Danube River during the last interglacial (Riss/Würm, i.e. approximately 130–115 kyr ago), and subsequent reduction in effective size of these populations during the Würm glacial that would account for the absence of detectable polymorphism in the French samples. Rivers of the Atlantic (Loire River) and Mediterranean basins (Hérault River) may have been colonized during the dispersion of these populations at the end of Würm. This would parallel the two-step expansion scenario from the Danubian refuge suggested for the chub (Durand *et al*. 1999).

The post-Pleistocene dispersion of the barbel would therefore have taken place from two hypothesized refugia, the more eastern Danubian and the more western one, possibly located in southern France (Persat & Berrebi 1990). The fact that populations derived from the two refugia do not show reciprocal monophyly nor fixed differences would be concordant with them becoming isolated during the last glacial (Bernatchez & Dodson 1991). The coexistence of h1 with h5 or its variants in the Rhine River and the Weser River may be a consequence of post-Pleistocene secondary contact between descendants from the western and eastern refuge, respectively (Durand *et al*. 1999). The absence of h5 from the Dniester River indicates that this population has not been in contact with the Danubian in the postglacial times, perhaps as long as since the last interglacial. However, the complex glacial history of the northern Black Sea basin (Arkhipov *et al*. 1995) precludes the formulation of a detailed scenario.

Lack of polymorphism in the Balkans and Anatolia

Although the amount of variation detected is dependent on sample size, the lack of detectable variation throughout Eastern Bulgaria and northern Anatolia (all populations fixed for the lineage I haplotype h4) is somehow unexpected for populations from southern regions less directly affected by glaciations (Bernatchez & Wilson 1998; Hewitt 1999, 2000). A plausible explanation for the absence of differentiation between the populations from the eastern Balkan and Anatolian rivers of the Black Sea seems to be a recent expansion throughout these regions. The Balkans and Anatolia had an arid, steppe or semidesert climate during the last glacial maximum (Adams & Faure 1997). It

is therefore likely that habitat suitable for the barbel was more restricted than it is now. The freshwater conditions and the water level drop of more than 100 m below the present level in the Black Sea during the last glacial to Early Holocene (Ryan *et al*. 1997; Winguth *et al*. 2000) would have facilitated the dispersal between the rivers emptying into the Black sea in close geographical proximity to one another. Smaller contemporary effective population sizes supported by comparably smaller rivers in the eastern Balkans and northern Anatolia seem likely to account for the lack of detectable young polymorphisms at cyt*b* within these populations.

Postglacial colonization of non-Mediterranean Europe

Barbus barbus is absent from the Mediterranean peninsulas of Iberia and Italy, and from most of the Balkans. It is replaced by the parapatrically distributed, phylogenetically close species *B. macedonicus* in the central Balkans (Kottelat 1997), and *B. plebejus* and *B. tyberinus* in Italy (Bianco 1995); no phylogenetically close species live in the Iberian Peninsula (Doadrio 1990). The estimated divergence of these species from *B. barbus* (≈ 2.97–3.74 Myr) indicates that their mtDNA genomes have not contributed to the *B. barbus* gene pool since the Pliocene (≈ 2–5 Ma). The distribution range of *B. barbus* only reaches the eastern Balkans along the southwestern Black Sea coast, and it is present in northern Anatolia. The populations from these regions belong to the mtDNA lineage I, which apparently does not extend further north than the Balkans (delimited northward by the Stara Planina or Balkan Mts.), indicating that the Balkan and Anatolian populations did not contribute mtDNA to the post-Pleistocene colonization of non-Mediterranean Europe.

Phylogeographic studies on several other freshwater fishes showed the importance of major river basins on the unglaciated parts of Western, Central and Eastern Europe and Asia in the post-Pleistocene colonization of Europe (Bernatchez & Dodson 1994; Durand *et al*. 1999; Nesbø *et al*. 1999; Englbrecht *et al*. 2000; Koskinen *et al*. 2000; Bernatchez 2001). Hence, the non-Mediterranean refugia were an important source for European colonization in fishes. Indeed, the importance of Central and Eastern European refugia as opposed to the Mediterranean ones (Taberlet *et al*. 1998; Hewitt 1999) is also evident from phylogeographies of amphibians (Wallis & Arntzen 1989) and small mammals (Bilton *et al*. 1998).

Subspecies differentiation of B. barbus escherichi

The relatively long-term (≈ 0.84–0.89 Myr) absence of mtDNA gene flow between the barbel populations occupying the eastern Balkans and northern Anatolia and those from the rest of Europe implies the existence of reproductive barriers between these two population groups. On the contrary, mtDNA genealogical continuity between populations of each of these groups suggests recent reproductive ties within the groups (Avise & Walker 1999). A geographically concordant divergence pattern is displayed by protein-coding nuclear loci (Dobrovolov 1996). On the basis of genetic data, it therefore seems reasonable to recognize the two population groups as different taxa. Barbel of the southern population group was originally described as *Barbus lacerta* var. *escherichi*. This taxon was later raised to the subspecies level and successively considered conspecific with other species, namely *B. tauricus*, *B. barbus*, and *B. plebejus* (reviewed in Karaman 1971). The three times lower mtDNA differentiation of eastern Bulgarian and northern Anatolian populations from Western and Central European *B. barbus* relative to the other species shows that this form is genetically close to *B. barbus*. Nevertheless, mtDNA represents only a small part of the genome and this should be kept in mind when making evolutionary inferences about populations and species (Avise & Wollenberg 1997). Given this limitation, the low genetic differentiation from *B. barbus*, and the sister relationship and parapatric distribution of the southern form and the Western and Central European *B. barbus*, we propose keeping subspecies status for the southern form and classifying it as a subspecies of *B. barbus*.

Acknowledgements

This study was made possible by the generosity of many people who helped with obtaining the barbel samples, namely E. Mikschi and H. Wellendorf from Austria, A. Cepov, I. S. Dobrovolov and M. Zhivkov from Bulgaria, S. Lusk, P. Ráb, V. Šlechta and M. Švátora from the Czech Republic, D. Aurelle and A. Chenuil from France, J. Freyhof and A. Schreiber from Germany, Z. Sallai from Hungary, A. Boroń from Poland, A. Moshu from the Republic of Moldova, P. Bănărescu, N. Crăciun, Mrs D. Hanganu, Mr D. Hanganu and G. Veress from Romania, and P. Simonović from Yugoslavia. We are grateful to J. W. Arntzen, N. Galtier, P. Ráb, J. Zima, L. Bernatchez and an anonymous reviewer for their constructive comments on the manuscript, and to C. S. Tsigenopoulos for numerous inspiring discussions. Members of the Laboratoire Génome, Populations, Interactions provided valuable laboratory assistance and advice. P. K. was supported by the French 'Reseau formation recherche du Ministère de la Recherche', and by the Ministry of Education, Youth and Sports of the Czech Republic (grant number VS97102).

References

Adams JM, Faure H (1997) Preliminary vegetation maps of the world since the last glacial maximum: an aid to archaeological understanding. *Journal of Archaeological Science*, **24**, 623–647.

Adams J, Maslin M, Thomas E (1999) Sudden climate transitions during the Quaternary. *Progress in Physical Geography*, **23**, 1–36.

Arkhipov SA, Ehlers J, Johnson RG, Wright HE Jr (1995) Glacial drainages towards the Mediterranean during middle and late Pleistocene. *Boreas*, **24**, 196–206.

Avise JC, Walker D (1999) Species realities and numbers in sexual vertebrates: Perspectives from an asexually transmitted genome. *Proceedings of the National Academy of Sciences of the USA*, **96**, 992–995.

Avise JC, Walker D, Johns GC (1998) Speciation durations and Pleistocene effects on vertebrate phylogeography. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **265**, 1707–1712.

Avise JC, Wollenberg K (1997) Phylogenetics and the origin of species. *Proceedings of the National Academy of Sciences of the USA*, **94**, 7748–7755.

Bănărescu P (1991) *Zoogeography of Fresh Waters, 2: Distribution and Dispersal of Fresh Water Animals in North America and Eurasia*. AULA-Verlag, Wiesbaden.

Bănărescu P (1998) On the relations between hydrography and the ranges of freshwater fish species and subspecies. *Italian Journal of Zoology*, **65** (Suppl.), 87–93.

Bernatchez L (2001) The evolutionary history of brown trout (*Salmo trutta*) inferred from phylogeographic, nested clade and mismatch analyses of mitochondrial DNA variation. *Evolution*, **55**, 351–379.

Bernatchez L, Dodson JJ (1991) Phylogeographic structure in mitochondrial DNA of the lake whitefish (*Coregonus clupeaformis*) and its relation to Pleistocene glaciations. *Evolution*, **45**, 1016–1035.

Bernatchez L, Dodson JJ (1994) Phylogenetic relationships among Palearctic and Nearctic whitefish (*Coregonus* sp.) populations as revealed by mitochondrial DNA variation. *Canadian Journal of Fisheries and Aquatic Sciences*, **51**, 240–251.

Bernatchez L, Wilson CC (1998) Comparative phylogeography of Nearctic and Palearctic fishes. *Molecular Ecology*, **7**, 431–452.

Bianco PG (1995) A revision of the Italian *Barbus* species (Cypriniformes: Cyprinidae). *Ichthyological Exploration of Freshwaters*, **6**, 305–324.

Bilton DT, Mirol PM, Mascherreti S *et al.* (1998) Mediterranean Europe as an area of endemism for small mammals rather than a source for northwards postglacial colonization. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **265**, 1219–1226.

Bohlen J, Nolte A (1998) Die barbe, *Barbus barbus* (Linné, 1758), in der Hunte. *Drosera*, **98**, 79–82.

Briolay J, Galtier N, Brito RM, Bouvet Y (1998) Molecular phylogeny of Cyprinidae inferred from cytochrome *b* DNA sequences. *Molecular Phylogenetics and Evolution*, **9**, 100–108.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1660.

Doadrio I (1990) Phylogenetic relationships and classification of western Palearctic species of the genus *Barbus* (Osteichthyes, Cyprinidae). *Aquatic Living Resources*, **3**, 265–282.

Dobrovolov IS (1996) Biochemical genetics characteristics of barbel (*Barbus* Cuvier genus) from Bulgarian rivers. *Folia Zoologica*, **45**, 59–65.

Durand JD, Persat H, Bouvet Y (1999) Phylogeography and postglacial dispersion of the chub (*Leuciscus cephalus*) in Europe. *Molecular Ecology*, **8**, 989–997.

Dynesius M, Jansson R (2000) Evolutionary consequences of changes in species' geographical distributions driven by Milankovitch climate oscillations. *Proceedings of the National Academy of Sciences of the USA*, **97**, 9115–9120.

Englbrecht CC, Freyhof J, Nolte A *et al.* (2000) Phylogeography of the bullhead *Cottus gobio* (Pisces: Teleostei: Cottidae) suggests a pre-Pleistocene origin of the major central European populations. *Molecular Ecology*, **9**, 709–722.

Felsenstein J (1983) Parsimony in systematics: Biological and statistical issues. *Annual Review of Ecology and Systematics*, **14**, 313–333.

Felsenstein J (1988) Phylogenies from molecular sequences: inference and reliability. *Annual Review of Genetics*, **22**, 521–565.

Hasegawa M, Kishino H, Yano T (1985) Dating the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, **22**, 160–174.

Hewitt GM (1999) Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society*, **68**, 87–112.

Hewitt GM (2000) The genetic legacy of the Quaternary ice ages. *Nature*, **405**, 907–913.

Hewitt GM (2001) Speciation, hybrid zones and phylogeography — or seeing genes in space and time. *MolecularEcology*, **10**, 537–549.

Hoelzer GA, Wallman J, Melnick DJ (1998) The effects of social structure, geographical structure, and population size on the evolution of mitochondrial DNA: II. molecular clocks and the lineage sorting period. *Journal of Molecular Evolution*, **47**, 21–31.

Karaman SM (1971) Süsswasserfische der Türkei. 8. Teil: Revision der Barben Europas, Vordenasien und Nordafrikas. *Mitteilungen Aus Dem Hamburgischen Zoologischen Museum und Institut*, **67**, 175–254.

Koskinen MT, Ranta E, Piironen J *et al.* (2000) Genetic lineages and postglacial colonization of grayling (*Thymallus thymallus*, Salmonidae) in Europe, as revealed by mitochondrial DNA analyses. *Molecular Ecology*, **9**, 1609–1624.

Kottelat M (1997) European freshwater fishes. An heuristic checklist of the freshwater fishes of Europe (exclusive of former USSR), with an introduction for non-systematists and comments on nomenclature and conservation. *Biologia, Bratislava*, **5**, 1–271.

Lelek A (1987) *The Freshwater Fishes of Europe, Vol. 9: Threatened Fishes of Europe*. AULA-Verlag, Wiesbanden.

Nei M (1992) Age of the common ancestor of human mitochondrial DNA. *Molecular Biology and Evolution*, **9**, 1176–1178.

Nesbø CL, Fossheim T, Vøllestad LA, Jakobsen KS (1999) Genetic divergence and phylogeographic relationships among European perch (*Perca fluviatilis*) populations reflect glacial refugia and postglacial colonization. *Molecular Ecology*, **8**, 1387–1404.

Persat H, Berrebi P (1990) Relative ages of present populations of *Barbus barbus* and *Barbus meridionalis* (Cyprinidae) in southern France: preliminary consideration. *Aquatic Living Resources*, **3**, 253–263.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Ryan WBF, Pitman III WC, Major CO *et al.* (1997) An abrupt drowning of the Black Sea shelf. *Marine Geology*, **138**, 119–126.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Šlechtová V, Šlechta V, Lusková V, Lusk S, Berrebi P (1998) Genetic variability of common barbel, *Barbus barbus* populations in the Czech Republic. *Folia Zoologica*, **47** (Suppl.1), 21–33.

Smouse PE (1998) To tree or not to tree. *Molecular Ecology*, **7**, 399–412.

Swofford DL (1999) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0*. Sinauer Associates, Sunderland, Massachusetts.

Taberlet P, Fumagalli L, Wust-Saucy AG, Cosson JF (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology*, **7**, 453–464.

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic association with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics*, **132**, 619–633.

Tsigenopoulos CS, Berrebi P (2000) Molecular phylogeny of North Mediterranean freshwater barbs (genus *Barbus*: Cyprinidae) inferred from cytochrome *b* sequences: biogeographic and systematic implications. *Molecular Phylogenetics and Evolution*, **14**, 165–179.

Wallis GP, Arntzen JW (1989) Mitochondrial-DNA variation in the crested newt superspecies: limited cytoplasmatic gene flow among species. *Evolution*, **43**, 88–104.

Weiss S, Antunes A, Schlötterer C, Alexandrino P (2000) Mitochondrial haplotype diversity among Portuguese brown trout *Salmo trutta* L. populations: relevance to the post-Pleistocene recolonization of northern Europe. *Molecular Ecology*, **9**, 691–698.

Winguth C, Wong HK, Panin N *et al*. (2000) Upper Quaternary water level history and sedimentation in the northwestern Black Sea. *Marine Geology*, **167**, 127–146.

Yang Z, Goldman N, Friday A (1994) Comparison of models for nucleotide substitution used in maximum-likelihood phylogenetic estimation. *Molecular Biology and Evolution*, **11**, 316–324.

Zardoya R, Doadrio I (1999) Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. *Journal of Molecular Evolution*, **49**, 227–237.

This work is part of the PhD thesis research of Petr Kotlík on the evolutionary biology and systematics of the Central European freshwater fish of the genus *Barbus*. Patrick Berrebi is interested in *Barbus* evolution, polyploidy and hybridization; he is also interested in conservation genetics and stocking of salmonids in Europe.
