

# The Alps as barrier to dispersal in cold-adapted freshwater fishes? Phylogeographic history and taxonomic status of the bullhead in the Adriatic freshwater drainage

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## Abstract

The freshwater faunas of the Italian peninsula are isolated from the rest of Europe by the geographic barrier of the Alps and consequently have developed many endemic forms and contain few non-endemic species. However, some 'non-endemics' may either represent recent invaders of the Adriatic basin or cryptic endemic species. To test these two hypotheses against each other, we studied the origin and phylogenetic relationships of bullheads, cold adapted freshwater fishes of the genus *Cottus*, from both sides of the Alps and Dinaric Mountains. From the Adriatic basin, *Cottus ferrugineus* (Heckel and Kner, 1858) was described as an endemic species, but the present analyses of sequences of the complete mitochondrial control region of 146 individuals from 43 localities showed no major differentiation between bullheads from both sides of the Alps. The very low diversification between representatives across the Alps suggests active transfers of haplotypes across this geographic barrier from the glacial cycles up to recent times. The transfers are most likely based on stream capture, since the cold-adapted bullhead is able to colonise the highest stretches of the water courses. No other freshwater fish in Europe is known to have experienced such an extensive gene flow across the highest European Mountains. In contrast, the Dinaric Mountains seem to have been a much more effective barrier between the Danube and the Adriatics. Our data reject the hypothesis of *C. ferrugineus* as an endemic species in the whole Adriatic drainage.

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## 1. Introduction

The cyclic changes of climate during the Pleistocene is considered to have had one of the most fundamental influence on the recent biogeography of Europe (Dynesius

and Jansson, 2000; Hewitt, 2000; Taberlet et al., 1998). Almost all taxa are considered to have undergone range expansions during the periods of favourable conditions while having been restricted to smaller 'refuge' areas during periods of unsuitable climate (Bernatchez and Wilson, 1998). The most common mode of these changes was that species colonised Central and Northern Europe during the relatively warm interglacial periods

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while outlasting the cold period in refuges in the South, especially in the Iberian, Balkan, and Italian peninsulas (Hewitt, 1999).

However, this scenario holds true only for terrestrial animals and plants, while the look on the freshwater fauna in Europe, the fishes in particular, reveals a different pattern. Southern Europe, here understood as the river systems draining into the Mediterranean Sea, hosts ichthyofaunas that are strikingly different from that of the rest of Europe: most of their species are endemic to small parts of the Mediterranean freshwater drainage, while most of the species native to the non-Mediterranean drainages of Europe (here understood as European river systems north of the Pyrenees, Alps, Dinaric, and Balkan Mountains) are missing in the Mediterranean freshwater drainages (Bănărescu, 1992; Bianco, 1990; Bohlen and Ráb, 2001; Economidis and Bănărescu, 1991). This incompatibility of fish species makes it highly unlikely that non-Mediterranean Europe was recolonised after Pleistocene glaciations from refuges in the Mediterranean, otherwise both regions were expected to share most species. Additional support for the independence of the Mediterranean subregions comes from molecular data: studies on various freshwater fish have shown that the species occurring in the different Mediterranean subregion are isolated from each other and from their non-Mediterranean relatives for a much longer time than it would be expected if the non-Mediterranean species stemmed recently from Mediterranean population (Durand et al., 1999; Perdices and Doadrio, 2001; Perdices et al., 2003; Salzburger et al., 2003).

The reason for the differences in recolonisation between aquatic and non-aquatic taxa has to be seen in the different possibilities to disperse. In contrast to terrestrial animals and plants, which often undergo a stage capable of migration by walking (e.g., mammals) or flying (e.g., plant seeds or insect imagoes), freshwater animals are restricted to pathways offered by hydrogeographic systems (Bănărescu, 1992; Bernatchez and Wilson, 1998). Especially watersheds make a difference: while mountain ridges are potentially passable for terrestrial animals and plants, they usually represent impassable barriers for aquatic animals. As a result, areas that are well surrounded by mountain ridges become isolated zoogeographic units according to their aquatic fauna. Within Europe, the Iberian, Balkan, and Italian peninsulas represent examples of such isolated zoogeographic units in Europe (Bănărescu, 1992; Bianco, 1990). Especially the northern Adriatic freshwater drainage is considered as one of the best-isolated zoogeographic units in Europe, since it is blocked from the rest of Europe by one of the highest European Mountains, the Alps.

However, there are some examples of freshwater fish species that do not fit completely into this picture and do occur in the Adriatic freshwater drainage as well as in

non-Mediterranean Europe. One of them is the bullhead, *Cottus gobio*, which is a cold-adapted freshwater fish that occurs mainly in fast flowing headwaters of rivers. Because of its isolated and stationary populations, in combination with low dispersal abilities, *Cottus* was used as a model to study phylogeographic questions in freshwater fish by various authors (Englbrecht et al., 2000; Hänfling and Brandl, 1998; Kontula and Väinölä, 2001; Volckaert et al., 2002). The species is widely distributed in non-Mediterranean Europe from the Pyrenees to the Ural Mountains including most of Scandinavia and the Danube basin (Bănărescu, 1992; Lelek, 1987). Seven major mtDNA lineages were identified across non-Mediterranean Europe (Englbrecht et al., 2000; Volckaert et al., 2002), and glacial refuges in Western Europe have been postulated for this species (Volckaert et al., 2002).

Additionally, populations of this species occur in the northern Adriatic freshwater drainage. Origin, phylogenetic relationships and taxonomic status of these populations have been poorly studied, leaving the genetic contribution of the Mediterranean populations as potential sources for the recolonisation of Europe untouched. Until now, two controversial hypotheses were under discussion: Heckel and Kner (1858) assumed the bullheads of the Adriatic drainage to form a homogeneous lineage distinct from the Central European *C. gobio* and described it as a separate species, *C. ferrugineus*. Their opinion was supported by later investigations on morphology of these fish by Băcescu and Băcescu-Meşter (1964). On the other hand, Bianco (1990, 1993) suggested that *Cottus* is a recent “trans-Alpine” invader from the Danubian drainage area and agrees with Koli (1969) in that the populations of the Adriatic freshwater drainage belong to the widespread species *C. gobio*. Most recently, Kottelat (1997) has noted a ‘lack of data’ regarding the phylogenetic relationships of the bullhead populations from the Adriatic freshwater drainage. However, the most important difference between the two competing hypotheses is the fact that the second hypothesis assumes the Alps not to have acted as an efficient barrier for the dispersal of this fish. Single cases were documented, in which genetically close bullhead populations lived on both sides of a watershed, suggesting its ability to disperse via stream-capture (Hänfling and Brandl, 1998; Riffel and Schreiber, 1995; Šlechtová, 2001). Nevertheless, these observations were done in Central Europe where watersheds are comparably low. In contrast, the colonisation of the Adriatic freshwater drainage from northern rivers would require crossing the Alps, an exceptional case of across-watershed dispersal in freshwater animals.

In the present study, the phylogeography of bullhead in the Adriatic freshwater drainage is inferred from mitochondrial DNA sequences. The aim of the study was to explain the origin of the populations from the Adriatic

drainage as well as their level of isolation with respect to populations from the surrounding parts of the distribution area. The conclusions should address the importance of major European mountain ridges on the postglacial recolonisation of Central Europe as well as the taxonomic status of bullheads from the Adriatic freshwater drainage.

## 2. Materials and methods

### 2.1. Sampling strategy

Altogether, 146 individuals from 18 localities inside the Adriatic freshwater drainage and from 25 localities outside this region (Rhône, Rhine, Elbe, Odra, Hérault, and Danube River basins) were included into the study. The sampling strategy rather focussed on a high density of localities across the investigated area than on a high number of specimens per locality to reveal the geographic structure of the observed clades with special emphasis on the Alps region. The sampling sites are depicted in Fig. 1 and corresponding detailed information about material analysed is listed in Table 1. Fish were caught in the years 2000–2003. Two specimens of *C. poecilopus* were included into the analyses as outgroup. Isolation of DNA from three syntype specimens of *C. ferrugineus*

from the collection of the Natural History Museum in Vienna failed.

### 2.2. DNA isolation, PCR amplification, and sequencing

Genomic DNA was isolated from fin or muscle tissue following the standard phenol–chloroform method (Sambrook et al., 1989), or in some cases with DNeasy Tissue Kit (Qiagen). The mitochondrial control region was amplified using the pair of primers CotL1 and HN20. The reverse HN20 primer 5'-GTGTTATGCTT TAGTTAAGC (Bernatchez and Danzmann, 1993) was already successfully applied on *Cottus* by Engbrecht et al. (2000). As forward primer we used the new primer CotL1 (5'-CCGGAGGTTAAAATCCTCCC), which was designed particularly for *Cottus* mitochondrial control region sequence (Arne Nolte, pers. comm.).

PCR amplification was performed in 50  $\mu$ l reaction volumes containing 10 mM Tris–HCl, 50 mM  $(\text{HN}_4)_2\text{SO}_4$ , 0.1% Triton X-100, 1.2–1.8 mM  $\text{MgCl}_2$ , 2 mM TMA oxalate (PCR enhancer), 10 nmol of each nucleotide, 2.5 U *Taq* polymerase (all chemicals by Top–Bio), and 25 pmol of each primer. The PCR profile (carried out on MJ Research thermocycler) started with 2 min period of initial denaturation at 95 °C followed by (1) five cycles at 94 °C for 45 s, 48 °C for 45 s, and 72 °C for 90 s and (2) 29 cycles of 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 90 s. The PCR

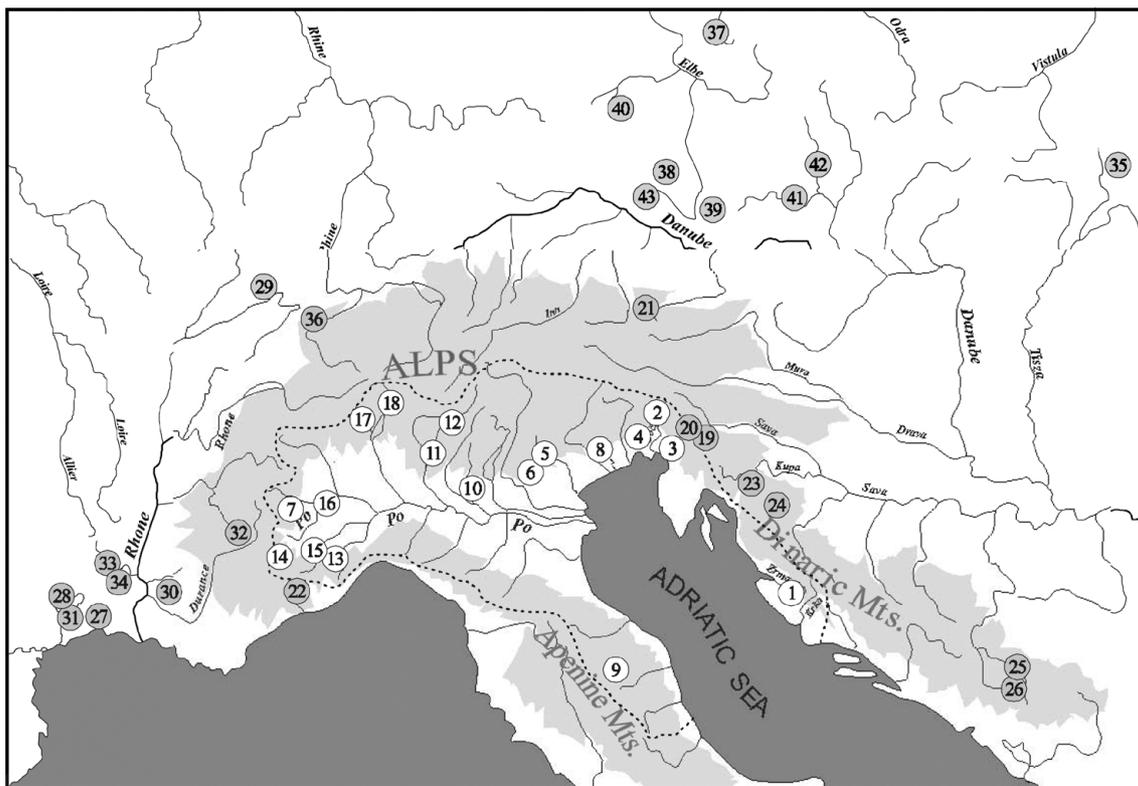


Fig. 1. The geographic distribution of our sampling sites. The sites within the Adriatic freshwater drainage are indicated by white circles, sites from outside by grey circles. Mountainous areas are marked with grey. Further information about the localities can be found in Table 1.

Table 1  
Locality information for the analysed bullhead samples

Locality No.	River	Name of locality	Drainage	Country	Coordinates	Sample size	Haplotypes found
<i>Localities inside the Adriatic freshwater drainage</i>							
1	Zrmanja		Zrmanja	HR	44°05'92"N, 16°00'43"E	7	Cot44 (5), Cot45, Cot46
2	Soča	Cez Soča	Soča	SLO	46°18'30"N, 13°36'19"E	5	Cot22 (5)
3	Vipava		Soča	I	45°53'55"N, 13°54'21"E	5	Cot26 (4), Cot27
4	Stella		Stella	I	45°52'58"N, 13°05'27"E	3	Cot28, Cot29, Cot30
5	Brenta	Cismon del Grappa	Brenta	I	45°56'25"N, 11°43'36"	3	Cot31, Cot32, Cot33
6	Bacchiglione	Lupia	Bacchiglione	I	45°38'12"N, 11°36'23"E	5	Cot23, Cot24 (3), Cot25
7	Pellice		Po	I	44°48'35"N, 07°15'54"E	5	Cot11 (3), Cot12 (2), Cot13
8	Livenza	Polcenigo	Livenza	I	46°02'13"N, 12°29'48"E	5	Cot24, Cot29 (2), Cot34 (2)
9	Campodónico	Campodónico	Potenza	I	43°13'23"N, 12°51'50"E	7	Cot58 (7)
10	Chiese	Barghe	Po	I	45°40'39"N, 10°24'29"E	6	Cot53, Cot54, Cot55 (2), Cot56, Cot57
11	Brembo	Zogno	Po	I	45°48'46"N, 9°40'294"E	2	Cot50 (2)
12	Adda	Berbenno di Valtellina	Po	I	46°09'40"N, 9°49'01"E	4	Cot48 (2), Cot49, Cot50
13	Corsaglia	Torre Mondovì	Po	I	44°20'55"N, 7°53'47"E	6	Cot51 (2), Cot52 (4)
14	Stura di Demonte	Festiona	Po	I	44°18'23.0"N, 7°20'42.9"E	5	Cot14 (5)
15	Maira	Solerette	Po	I	44°36'36.4"N, 7°37'20.6"E	4	Cot60, Cot61 (2), Cot62
16	Po	Cardè	Po	I	44°42'48.6"N, 7°27'38.1"E	3	Cot11, Cot13, Cot63
17	Toce	Beura	Po	I	46°06'06.3"N, 8°18'23.7"E	4	Cot64 (4)
18	Brenno (trib. of Ticino)	Biasca	Po	CH	46°21'36"N, 08°57'55"E	6	Cot67 (6)
<i>Localities outside the Adriatic freshwater drainage</i>							
19	Iska		Danube	SLO	45°57'23"N, 14°32'12"E	5	Cot35 (3), Cot36, Cot37
20	Iscica		Danube	SLO	46°01'23"N, 14°51'12"E	5	Cot38 (4), Cot39
21	Lammer	Voglaui	Danube	A	47°35'26"N, 13°19'58"E	3	Cot59 (3)
22	Roya	Gorges of Bergues	Roya	F	44°01'16"N, 7°34'11"E	2	Cot11, Cot14
23	Kolpa	Kuzelj	Danube	HR	45°27'58"N, 14°51'32"E	5	Cot20, Cot21 (4)
24	Tounjica	Tounj	Danube	HR	45°14'74"N, 15°13'49"E	5	Cot40, Cot41 (2), Cot42, Cot43
25	Tara	Kolasin	Danube	SIM	42°49'57"N, 19°31'38"E	2	Cot16, Cot17
26	Pcinja		Danube	SIM	42°48'22"N, 19°26'17"E	3	Cot15, Cot16, Cot17
27	Lez		Lez	F	43°42'00"N, 03°51'56"E	1	Cot10
28	Arre		Harault	F	43°58'02"N, 03°31'10"E	3	Cot47 (3)
29	Reigne		Rhône	F	47°40'10"N, 06°28'30"E	2	Cot5, Cot6
30	Sorgues Vaucluse		Rhône	F	43°55'48"N, 04°59'42"E	1	Cot6
31	Foux de Brissac		Harault	F	43°52'47"N, 03°42'09"E	3	Cot2, Cot3, Cot4
32	Drac Blanc (trib. of Isère)		Rhône	F	44°43'17"N, 06°15'42"E	2	Cot70 (2)
33	Chassezac		Rhône	F	44°25'37"N, 04°17'59"E	2	Cot71, Cot72
34	Ceze		Rhône	F	44°11'40"N, 04°31'13"E	4	Cot7 (2), Cot8, Cot9
35	Ulicka		Danube	S	22°29'32"N, 48°55'37"E	3	Cot18 (2), Cot19
36	La Scheulte (trib. of Birse)	Courchapoix	Rhine	CH	48°55'37"N, 07°27'47"E	2	Cot65, Cot66
37	Nisa	Liberec	Odra	CZ	50°49'31"N, 15°07'54"E	1	Cot1
38	Blanice	Husinec	Elbe	CZ	49°08'47"N, 13°42'24"E	1	Cot1
39	Dluhostský potok		Elbe	CZ	48°52'42"N, 14°47'22"E	1	Cot1
40	Tepla		Elbe	CZ	50°05'54"N, 12°42'58"E	1	Cot1
41	Dyje	Znojmo	Danube	CZ	48°50'23"N, 16°03'28"E	1	Cot68
42	Punkva		Danube	CZ	49°21'55"N, 16°42'34"E	2	Cot69 (2)
43	Debrník	Zelezna Ruda	Danube	CZ	49°08'10"N, 12°42'58"E	2	Cot68 (2)

In the column "haplotypes" the numbers in brackets represent the absolute frequencies of haplotypes in case of more than one individual.

was completed by a final elongation step of 5 min at 72°C. PCR products were purified by ethanol precipitation or using Microcon PCR Filter Units (Millipore) and subjected to cycle sequencing employing BigDye Terminator Cycle Sequencing Kit v.3.1 (PE Applied Biosystems) according to manufacturer's instructions. Sequencing products cleaned by ethanol precipitation or with DyeEx 2.0 Spin Kit (Qiagen) were resolved on ABI Prism 310 Genetic Analyser (Perkin–Elmer). Each sample was sequenced from both (3' and 5') ends of the fragment with the same primers as used for double strand PCR amplification.

### 2.3. Molecular data analysis

#### 2.3.1. Sequence alignment

The raw chromatograms were assembled and for potential mistakes checked by eye in the SeqMan II module of the DNASTar software package (<http://www.dnastar.com>). Edited sequences were aligned using the ClustalW algorithm in BioEdit (Biological sequence alignment editor v5.0.9, <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Gaps within the alignment were treated as indels.

#### 2.3.2. Population genetics and phylogenetic analysis

The population genetic statistics were calculated with the use of the program Arlequin 2.000 (Schneider et al., 2000). The average number of nucleotides per site called nucleotide diversity  $\pi$  was calculated (Nei, 1987). The fixation indices,  $\Phi$  (which are statistics analogous to Wright's (1951)  $F$  statistics) and the significance of regional grouping of *Cottus* were assessed using a hierarchical analysis of molecular variance (AMOVA) (Excoffier et al., 1992) with Kimura-2 parameter model (Kimura, 1980).

To determine the best fitting model of nucleotide substitution for the following phylogenetic analysis, the aligned sequences were tested by the program Modeltest 3.06 (Posada and Crandall, 1998). Under the Akaike information criterion (AIC), the General Time Reversible model (GTR +  $\Gamma$  + I) was chosen as the most appropriate for the present dataset. The model included unequal base frequencies (A = 0.3178, C = 0.2113, G = 0.1664, and T = 0.3045), six substitution categories (A–C = 1.0000, A–G = 13.9368, A–T = 2.2850, C–G = 2.2850, C–T = 13.9368, and G–T = 1.0000), a proportion of invariable sites (I) = 0.8307 and a rate heterogeneity among sites following a gamma distribution with value 0.5391.

The phylogenetic relationships were estimated from the aligned sequences using the methods of neighbour-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) in PAUP\* version 4.0b10 (Swofford, 2000) and MEGA 2.1 software (Kumar et al., 2001) and Bayesian analyses using the program MrBayes ver. 3.0 (Huelsenbeck and Ronquist, 2001).

The distance NJ tree of haplotypes was constructed basing on the GTR +  $\Gamma$  + I substitution model with the

estimated parameters. The reliability of branches was estimated by a nonparametric bootstrap resampling with 1000 replicates. For MP analysis a heuristic search was conducted with TBR branch-swapping algorithm. The node support was assessed by 1000 bootstrap replicates. The ML analysis was performed using the quartet puzzling method with estimated parameters. Likelihood tree was constructed also using Bayesian inference of phylogeny. Four Monte Carlo Markov Chains were running simultaneously for 1,000,000 generations. The likelihood scores reached the stability after ca. 900 generations. The remaining trees were used to build a majority-rule consensus tree and the posterior probabilities were used to indicate branch supports in the final tree. All presented trees were rooted with homologous sequences of the Siberian bullhead *C. poecilopus*.

Recent studies of Englbrecht et al. (2000) and Volckaert et al. (2002) have revealed six and seven major haplotype lineages of the populations of *C. gobio* in Europe, respectively. To integrate the present data into this framework and to reveal the phylogenetic position of the bullheads from the Adriatic freshwater drainage on the Europe-wide scale, the present data were combined with 11 haplotypes representing five major clades of *C. gobio* from the study of Volckaert et al. (2002) and NJ, MP, and ML analyses were performed. Since the length of sequences in Volckaert et al. (2002) was shorter than in our study, our data were shortened to 771 bp for the comparison.

The program TCS v. 1.13 (Clement et al., 2000) was used to construct a statistical parsimony network (SPN). Since the precise routes of dispersal across mountain barriers in our model animal were not traceable, the application of a nested clade analysis was hampered by the lack of reliable data for the historically correct geographic distance between populations, especially across mountain barriers.

## 3. Results

### 3.1. Sequence divergence

The analyses of sequences of mitochondrial control region from 146 specimens of *C. gobio* representing 43 populations revealed 72 haplotypes (see Table 1). The length of sequences ranged from 889 to 892 bp. The sequences have been deposited in the GenBank library under the Accession Nos. AY453695–AY453766 (see Table 2) as well as the sequences from two specimens of *C. poecilopus* (Accession Nos. AY453767 and AY453768).

The total length of the alignment including *C. poecilopus* sequences was 900 bp. Since the analysed sequences were closely related, no danger of misalignment was expected and the default settings of parameters were used. In spite of relatively large alignment span, the variability



Cot35	....G.AT-	.....CGA	G.....T...	.....A..	...A-C....	.....-CT	..A...	19	AY453729
Cot36	..G.G.AT-	.....CGA	G.....T...	.....AT.	...-.....	.....-CT	..A...	19	AY453730
Cot37	..G...AT-	.....CGA	G.....T...	.....AT.	..T.TC....	.....-CT	..A...	19	AY453731
Cot38	.....-	.....G....	.....T...	.....A..	T.T.-.A...	.....-	..A...	20	AY453732
Cot39	.....-	.....G....	.....T...	.....A..	T.T.-.A...	.....-	..A..A	20	AY453733
Cot40	....G.A.-	..A.....G	....CCC...	...A..A..	...T-C.G..	....T.-.T	T.....	24	AY453734
Cot41	....G.A.-	..A.....G	....CT....	.ATA..A..	...T-C.G..	....T.-.T	T.....	24	AY453735
Cot42	....G.A.-	.....G	....CT....	.A.A..A..	...T-C.G..	....T.-.T	.....	24	AY453736
Cot43	....G.A.-	..A.....G	....CT....	.A.A..A..	...T-C.G..	....T.-.T	T.....	24	AY453737
Cot44	.....-	.....	...T..T...	.....A.G	..T.-.A...	.....-	..A...	1	AY453738
Cot45	.....-	.....	...T..T...	.....A.G	..T.-.A...	.....-	..A.G.	1	AY453739
Cot46	.....-	.....	...T..T...	.....A.G	...-CA...	.....-	..A...	1	AY453740
Cot47	....A.-	..A.C.A..	...C..T...	...T..A..	T...-A...	..AA...TC.	..A...	28	AY453741
Cot48	.....-	.....	....T...	.....	TGT.-.....	.....-	.....	12	AY453742
Cot49	.....-C	.....	....T...	.....	TGT.-.....	.....-	.....	12	AY453743
Cot50	.....-	.....	....T...	.....	.GT.-.....	.....-	..A...	11, 12	AY453744
Cot51	.....-	.....	....T...	.....A..	..T.-.A...	.....-	..A...	13	AY453745
Cot52	.....A.-	.....	....T...	G.....	..T.-.A...	.....-	..A...	13	AY453746
Cot53	....G.A.-	A.....GT	G.....T...	....A.A..	..TT-.A...	.....-T	T.....	10	AY453747
Cot54	....G.A.-	A....ACGT	G.....T...	....A.A..	..T.-.A...	.....-T	T.....	10	AY453748
Cot55	....G.A.-	A....T.GT	G.....T...	....A.A..	..TT-.A...	.....-T	T.....	10	AY453749
Cot56	....G.A.-	A.....GT	G.....T...	....A.A..	...-.A...	.....-T	T..A..	10	AY453750
Cot57	....G.A.-	A.....T	G.....T...	....A.A..	..T.-.A...	.....-T	T.....	10	AY453751
Cot58	....G.A.-	A.....	....T...	.....A..	..T.-.A...	.....-	.....	9	AY453752
Cot59	.....T-	A.....	.....	.....A..	..T.-.....	.....-	.....	21	AY453753
Cot60	..G..A.-T	.....	....T...	.....A..	...-C....	.....-	..A...	15	AY453754
Cot61	.G.....-T	.....	....T...	.....A..	...-.....	.....-	..A...	15	AY453755
Cot62	....A.-T	.....	....TC..	.....A..	...-.....	.....-	..A...	15	AY453756
Cot63	.....-T	.....	....T...	.....A..	T...-.....	.....-	..A...	16	AY453757
Cot64	.....-T	.....	....T...	.....A..	..T.-.A...	.....-	..A...	17	AY453758
Cot65	....A.-	A.....	.....	.....G	...-.....	.....C-	.....	36	AY453759
Cot66	....A.-	.....	....T...	....A...	T.T.-.....	..A.....	.....	36	AY453760
Cot67	.....-	.....	....T...	.....	.GT.-.....	.....-	.....	18	AY453761
Cot68	.....-	.....	.....	....A...	..T.-.....	.....-	.....	41, 43	AY453762
Cot69	.....-	A.....	.....	....A...	..T.-.....	.....-	.....	42	AY453763
Cot70	....A.-	....A...	....T...	.....A.G	T.TT-.....	A.AA...-C.	..A...	32	AY453764
Cot71	....A.-	..A..A...	....T...	....A.A..	T...-CA.T.	AAAA...-C.	.....	33	AY453765
Cot72	....A.-	..A..A...	....T...	....A.A..	T...-A.T.	AAAA...-C.	.....	33	AY453766

Each line represents one haplotype and corresponding GenBank accession number; the numbers above columns indicate site position in the alignment.

Table 3

Analyses of molecular variance (AMOVA) for the a priori grouping of Adriatics + non-Adriatics and grouping suggested by phylogenetic analyses showing the fixation indices ( $\Phi_{ST}$ ,  $\Phi_{CT}$ , and  $\Phi_{SC}$ ), percentage of total variation explained by given grouping (%) and its significance ( $P$ ) based on 1000 permutations

Grouping	$\Phi_{ST}$	$\Phi_{SC}$	$\Phi_{CT}$	%	$P$
Adriatics + non-Adriatic	0.85394	0.83861	0.09500	9.50	<0.001
French + Dinaric + “Danubian”	0.88643	0.79890	0.43523	43.52	<0.001
French + Dinaric + “Danubian” + Chiese + Iska	0.88815	0.77040	0.51284	51.28	<0.001

$\Phi_{ST}$ —Similarity of any 2 sequences from the same population in relation to the similarity of pair of sequences drawn from all the samples.

$\Phi_{CT}$ —Similarity of any 2 sequences from the same group of localities relative to any 2 sequences from all the sequences.

$\Phi_{SC}$ —Similarity of any 2 sequences from the same locality in relation to the similarity of any 2 sequences from the same group of localities.

revealed was rather low. The alignment consisting merely of *C. gobio* sequences has shown only 7.4% (66 of total 895) of variable and 5.7% (51 of 895) of parsimony informative sites. The 66 variable nucleotide positions within alignment are depicted in Table 2.

The overall nucleotide diversity,  $\pi$  (Nei, 1987), calculated from the sequences was 0.011949 (SD 0.006056). The results of AMOVA are shown in Table 3.

The comparison of genetic diversity within and between a priori created groups, i.e., Adriatics versus non-Adriatics, revealed a very low contribution of this grouping to the explanation of the observed overall diversity (only 9.5%). The incorrect postulation of this grouping is shown also by very low value of the relevant  $\Phi_{CT}$  parameter. In contrast, structure based on the grouping suggested by molecular phylogenetic analyses accounted for 51.2% of the overall diversity. Nevertheless, the  $\Phi_{ST}$  values show that the population-specific differences can be considered the main influencing factor.

### 3.2. Phylogeny reconstruction

The phylogenetic analyses have shown no significant differences between the Adriatic and non-Adriatic bullheads. Neither classical tree building methods (Fig. 2) nor the statistical parsimony network (Fig. 3) did support a potential monophyly and distinctness of the Adriatic bullheads in respect to non-Adriatic populations. The Adriatic populations were shown to be genetically very closely related to the populations from surrounding drainage areas.

Despite the close relatedness of fishes within the analysed dataset, several monophyletic haplotype groups were identified within the haplotype network: (1) the lineage of “Northeastern Italy” containing individuals from localities of rivers Vipava, Brenta, Stella, and Livenza—the localities of northeast Italy (NJ bootstrap support 88%, ML 53%, MP 83%, and MrBayes posterior probabilities 100%). Nevertheless, within the mentioned localities also more widespread haplotypes were found. (2) The second statistically supported lineage collects most haplotypes from southern France, i.e., all

samples from the River Herault, River Lez, and some populations of southern parts of River Rhône basin (NJ bootstrap support 66%, ML 50%, MP 65%, and MrBayes 99%). (3) Two separated groups of ‘Northern Dinaric’ populations: Tara and Pcinja from the eastern parts of Dinaric Mountains (Northern Dinarics A) and Kolpa and Tounjica from the western parts (Northern Dinarics B). In traditional phylogenetic analyses these two groups had tendencies to join together. Nevertheless, this grouping was statistically very poorly supported by NJ (58%) and MP (53%) analyses, but neither by ML nor MrBayesian analyses. (4) In two cases, single populations gained an exceptional position, these are the population Iska from upper Sava River basin (NJ bootstrap support 95%, MP 98%, ML 87%, and MrBayes 97%) and Chiese from Adriatic freshwater drainage (NJ bootstrap support 88%, MP 74%, and MrBayes 100%). The last and largest clade collected all remaining samples from Italy plus all samples from the rivers upper Rhine, Elbe, Odra, Roya, and Danube (except the samples from Iska and the Northern Dinaric clade) as well as two samples from the Rhône basin. Nevertheless, all phylogenetic analyses, which were applied on the dataset in the present study, failed to resolve the base of the tree.

Also basing on the haplotype network (Fig. 3) constructed in the TCS program, all the 72 haplotypes were closely related to each other, no number of nucleotide differences exceeded 95% parsimony connection limit of 13 steps and all the haplotypes joined into a single network. Two of the haplotypes (Cot11 and Cot14) were shared across the watershed in the westernmost part of Adriatic freshwater drainage. In another case (haplotypes Cot22 and Cot38) the haplotypes from neighbouring watersheds differed in only one mutation step. Only four mutation steps across the watershed were traced between (1) haplotypes Cot48 and Cot68, (2) Cot48 and Cot66, and (3) Cot5, Cot6 and Cot58, Cot51. A distance of five mutation steps separated Cot5, Cot6, and Cot50. As in the linear trees, southern French haplotypes, the Northern Dinaric clade and the populations from Chiese and Iska formed the more distinct groups within the network.

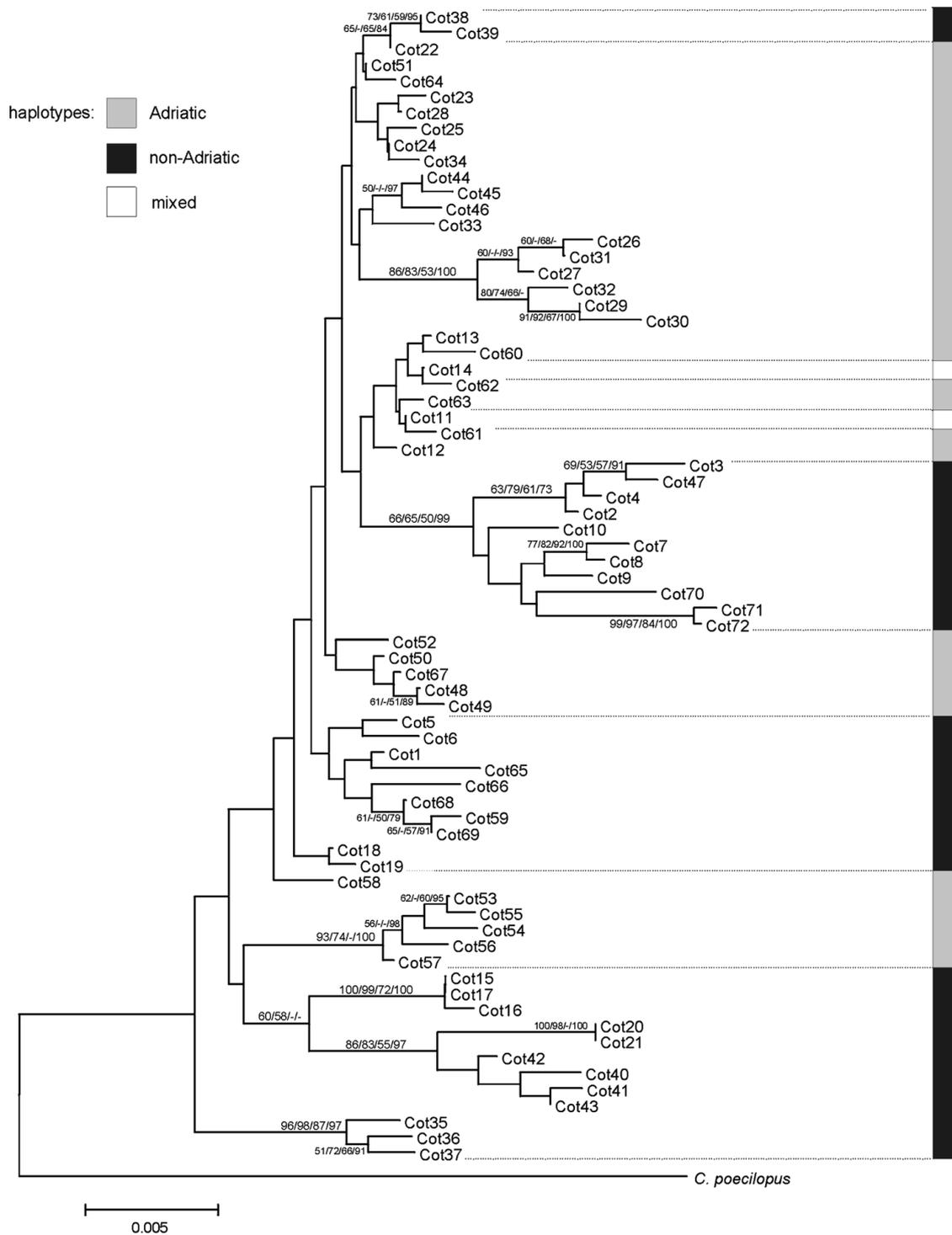


Fig. 2. Phylogenetic tree showing the relationships among 72 haplotypes of all analysed Adriatic and non-Adriatic *Cottus* rooted with *C. poecilopus*. Numbers at the branches represent the bootstrap support for NJ, MP, ML and Bayesian analyses, respectively. Branching pattern and branch lengths follow the NJ analysis. Bootstrap values are given for all the branches with NJ bootstrap support above 50%. For the branches not supported by some of the other methods, a corresponding bootstrap value is replaced by a dash.

Interesting results were yielded by the analysis of dataset combining the presented sequences and the haplotypes from the study of Volckaert et al., 2002 (Fig. 4). The scatter of haplotypes belonging to the clade I in the above mentioned study proved that all but one popula-

tions from the Adriatic freshwater drainage belong to the clade I established by Engbrecht et al. (2000) and Volckaert et al. (2002). However, including the haplotypes of the clades II, III, IV, and VII changed the topology of the phylogenetic tree by separating both groups of

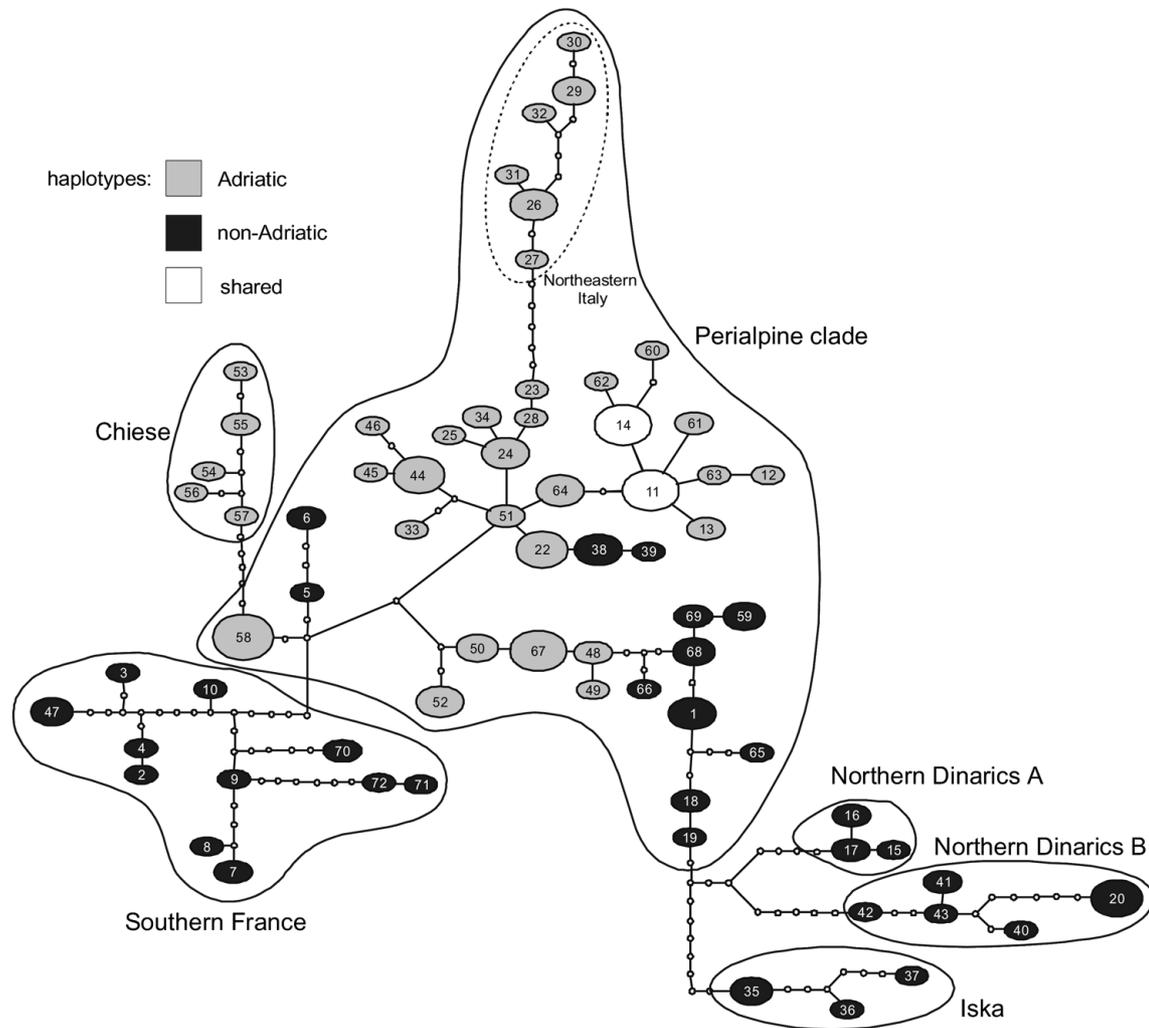


Fig. 3. Unrooted haplotype network for D-loop sequences of all Adriatic and non-Adriatic *Cottus* generated in TCS. The haplotype numbers refer to Cot-numbers in Table 1. The sizes of the ovals resemble the number of individuals per haplotype, the empty circles represent missing hypothetical haplotypes. The more distinct groups of haplotypes are encircled by black lines and labelled according to the names given in the text. The dashed line indicates the Northeastern Italian subclade, which is included into the “Perialpine” lineage.

‘Northern Dinarics’ and the two distinct populations Iska and Chiese from the haplotypes of European clade I. While this result strongly supports the distinctness of the considered populations, the base of tree still remains unresolved and poorly supported by bootstrap values.

#### 4. Discussion

##### 4.1. Origin of *Cottus* in the Adriatic freshwater drainage

Our analyses show the *Cottus* from both sides of the Alps to be closely related, no significant genetic differences and geographical sorting were observed (Fig. 5). In other cases of freshwater fishes studied, clear differences between Adriatic and non-Adriatic populations were found, like in *Squalius* (formerly *Leuciscus*) (Durand et al., 1999); *Cobitis* (Ludwig et al., 2001;

Perdices and Doadrio, 2001), *Thymallus* (Susnik et al., 2001), *Sabanejewia* (Perdices et al., 2003) or *Telestes* (formerly *Leuciscus*) (Salzburger et al., 2003), and the overall divergence between bullheads from the Adriatic and Danubian basins was much lower than in the above mentioned freshwater fishes. Such low differentiation does not permit the application of a molecular clock due to the danger of great error, but it becomes likely that in bullhead the last contact between Adriatic and non-Adriatic populations was much more recent than in other species of freshwater fishes. Moreover, the analyses of the present data in combination with the data of Volckaert et al. (2002) revealed that almost all *Cottus* from the Adriatic freshwater drainage belong, together with populations from the upper Danube, Elbe, and Upper Rhine and two populations of the Rhône River basin, to the most diverse and most widespread European clade (clade I in Englbrecht et al., 2000 and Volckaert



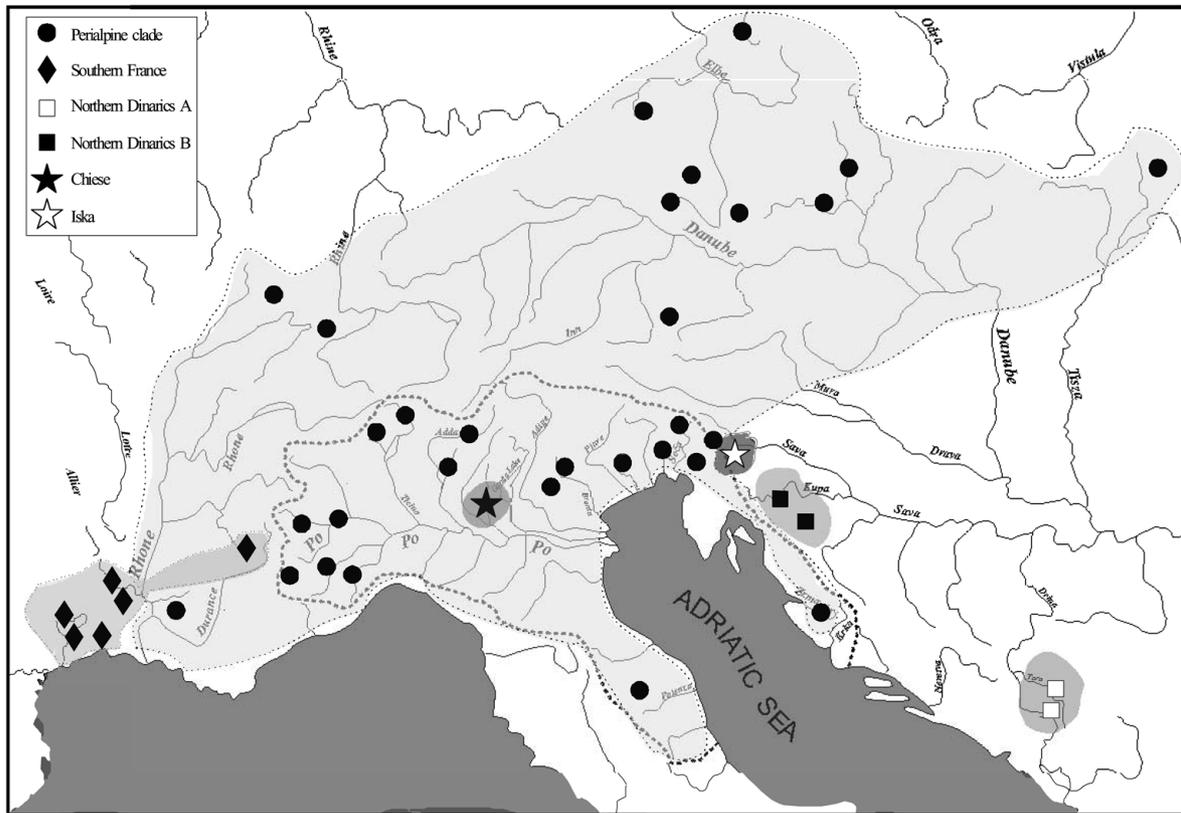


Fig. 5. Geographical distribution of the identified lineages of *Cottus* haplotypes. Names of clades correspond to Fig. 4.

drainage. Nevertheless, the high overall diversity observed (72 haplotypes within 146 individuals) makes it unlikely that the colonisation of the Adriatic freshwater drainage was the result of a single evolutionary event, which should result in a low genetic variability within the settled area (Hewitt, 1996). Consequently, we suppose rather multiple origins of bullhead in the Adriatic freshwater drainage as well as multiple exchanges of genetic information with the neighbouring drainages. It can thus be postulated that bullheads were still able to exchange genetic information across the Alps while this enormous barrier already prevented gene flow in most other freshwater fish species.

#### 4.2. The colonisation scenario

Multiple colonisation events have not necessarily taken place in one area or a short period, but our data predict several cases of contact across the Alpine watershed at different times.

The first case regards the populations with identical haplotypes across watersheds. Fishes from the Roya River (locality 22), which drains into the Ligurian Sea, shared identical haplotypes with three localities in the south-western Po basin, namely in the localities 7, 14, and 16 (Pellice, Stura, and Po, respectively). A similar close genetic relation was found between populations

of *Telestes muticellus* from the basins of Roya and the Po (Salzburger et al., 2003). According to Bianco (1990), all primary freshwater species are introduced in the River Roya and other ligurian rivers. Our data support this assumption, alternatively one would have to consider a very recent case of river capture, since no genetic differences between the populations from Roya and Po were fixed.

More cases of river contacts are observed in the close relationship between the populations from the rivers Brembo and Adda (localities 11 and 12) and the sample from the Swiss Rhine basin (locality 36) and Austrian Danubian haplotype (locality 21) (distinct by four mutation steps), suggesting a gene flow between Danubian and Adriatic freshwater drainages across the northern border of the Adriatic drainage. A connection across the western border is suggested by the close relation between two populations from the Rhône basin with populations in the western Po drainage (four mutation steps). Since the related haplotypes in these cases differed from each other in several mutation steps, the corresponding contact events were older than in the above mentioned connection between the Roya and Italian rivers. An interesting case is the finding of 'Adriatic' haplotypes in the upper Sava basin (locality 20). The two haplotypes in this Danubian population are tip-haplotypes derived from the most common haplotype in the

upper Soča basin (locality 2), which is geographically very close, but drains into the Adriatic Sea. Here, we suggest a dispersal of bullheads from the Adriatic into the Danubian basin. A similar flow of genetic information has been already suggested by Kotlík and Berrebi (2002) and Tsigenopoulos et al. (2002) for *Barbus* (Cyprinidae). In *Barbus* as well as in bullheads (present data) the difference between haplotypes from Soča (Adriatic freshwater drainage) and Sava (Danube River basin) was only a single substitution.

Connections of the described type can be traced in the present dataset also between the Rhine and the Danube basins: the population from the La Scheulte stream (locality 36) bears haplotypes that are very closely related to several populations from the Danube basin (localities 21, 37–43). Connections between the hydrologic systems of the upper parts of the rivers Rhône, Rhine, Po, and Danube are well documented by the distribution of freshwater fishes of the genera *Zingel*, *Telestes*, and *Sabanejewia* (Ladiges and Vogt, 1979) and were observed in studies on the genetic structure of freshwater fishes, namely *Telestes* (Salzburger et al., 2003), *Cobitis* (Ludwig et al., 2001; Perdices and Doadrio, 2001), *Sabanejewia* (Perdices et al., 2003), *Barbus* (Tsigenopoulos et al., 2002), and *Squalius* (Durand et al., 1999). In all these recorded cases, however, the populations in the Adriatic drainage represent a distinct species than the populations in the neighbouring drainages.

Volckaert et al. (2002) considered mountains as suited interglacial refuges for bullheads and lowlands as suited habitat during the glacial times. According to this assumption, populations from neighbouring tributaries would meet in the lower altitudes during glacial times, leading to geneflow within river systems. When the climate became warmer, bullheads moved with the shift of suited habitat into higher altitudes where the melting glacier increased the run-off of water and the erosion. This could enable bullheads to cross watersheds due to an increased probability of river captures. Repeated shifts of climate happened during the whole Pleistocene, leading to multiple contacts between bullhead populations and to a genetic homogenisation of populations around the Alps. This perialpine group of closely related haplotypes today is reflected in the European clade I and was the source for the present haplotype distribution over parts of Danube, Rhine, Rhône, and Adriatic basins as well. For the populations of bullheads in the lower and middle Rhine basin, refuges in western Europe were postulated by Volckaert et al. (2002), while the populations in southern France could have had their refuge right there.

#### 4.3. Dispersal within the Adriatic freshwater drainage

Within the Adriatic freshwater drainage, the poor geographic structuring and close relatedness of most

haplotypes point on extensive contacts of neighbouring populations in most of the Po basin. One factor that should have promoted distribution and geneflow of bullheads was the lowering of the sea level during glacial maxima. The global fixation of huge water masses in glacial ice lowered the water level of the Adriatic Sea by 100–200 m below today's level and let fall dry the northern part of the Adriatic Sea. The extended Po River drained then the rivers of the northern Adriatic from the Vomano River in Italy to the Krka River in Croatia (Bianco, 1990). Under such circumstances, a dispersal of bullheads through the lower parts of rivers was possible, especially in the generally cold climate during glacial maxima. This dispersal hypothesis, which is well accepted for other freshwater fishes and lampreys (Bianco, 1990), is supported by: (1) the occurrence of bullhead in comparably short lowland rivers in north-eastern Italy that nowadays drain directly into the Adriatic Sea (e.g., Brenta, Stella, and Vomano), (2) the lack of bullheads in all Adriatic rivers south of the Vomano and Zrmanja Rivers, and (3) the close relationship of the population from the Zrmanja River with the populations from north-eastern Italy.

The colonisation of the Zrmanja River from north-eastern Italy implies that bullheads must have been present in north-eastern Italy at the last glacial maximum to provide the founder specimens. The presence of a 'north-east Italian' subclade further points on the reduced contact of the populations in this area with other populations most likely during the last interglacial periods, but the further development of a distinct evolutionary unit was disturbed by occasional, and probably recent, contacts with other populations in the Po basin as indicated by shared haplotypes.

A slightly differing example may be seen in the case of the population 10, the Chiese River. This population turned out to be genetically diverse (6 analysed specimens revealed 5 different haplotypes), but all observed haplotypes settled outside of the European clade I, separated by 11 mutation steps from the geographically most close haplotype. This population may either be the remnant of a colonisation event that left no genetic traces in other populations or the product of a strongly increased genetic drift within this population. However, both explanations would require a long-term geographic isolation of this population, but no geologic indication of such isolation is known to us.

Among the investigated samples, a high number of haplotypes occurred, most of them very closely related and often geographically widespread. According to Avise et al. (1987), such distribution of haplotypes points at relatively extensive and recent historical gene flow, pronouncing that the Alps have not represented an efficient barrier to the dispersal of bullhead. The evidences for close relationships and recent contact among populations from different water drainages leads to the

conclusion that the bullhead populations in the Adriatic freshwater drainage were object of contact events that appear to be more recent and numerous than in any other freshwater fish studied so far.

#### 4.4. The dinaric barrier

Unlike the incomplete separation of bullhead populations by the Alps, no indication of geneflow across the Dinaric Mountains was found. This eastern prolongation of the Alps is less high than the main ridges of the Alps and headwaters of Danubian tributaries on the northern slopes come in geographically close to headwaters of Adriatic rivers on the southern slopes. Bullhead is widely distributed in the Danubian drainage of the Dinaric Mountains and occur only few kilometres from the watershed to an Adriatic river (about 5 km in the case of our locality 26). Nevertheless, in the Adriatic drainage of the Dinaric Mountains, bullhead was reported exclusively from Zrmanja River, although a number of Dalmatian rivers offer suitable ecological conditions. However, the present data show the population from Zrmanja River to be much closer related to the samples from northern Adriatic rivers than to populations in the Danubian drainage of the Dinaric Mountains. These findings together with the general lack of bullheads in most rivers of the Dalmatian slopes of Dinaric Mountains indicate that these mountains represent an efficient barrier for the dispersal of bullhead. The most likely reason is the absence of ice caps in the Dinaric Mountains during the glacial maxima (Hewitt, 1999). The resulting lack of a strong water runoff during the deglaciation periods made stream captures across the mountains unlikely to happen. The efficiency of the Dinaric barrier is documented also by the general lack of Danubian freshwater fish species on the Adriatic slope of Dinaric Mountains.

#### 4.5. Taxonomic implications

The present data show a non-monophyletic arrangement of the bullhead populations in the Adriatic freshwater drainage and indicate a recent or ongoing geneflow with populations from the surrounding basins of Rhône and Danube Rivers. Both findings are very uncommon among freshwater fishes of the Adriatic drainage, an area characterised by a high rate of endemism. Consequently, the concept of Heckel and Kner (1858) about a single endemic species *C. ferrugineus* in the whole Adriatic freshwater drainage has to be rejected. The recent or ongoing geneflow shows the populations in the Adriatic freshwater drainage not to be reproductively isolated and makes the existence of a single endemic species of *Cottus* in the Adriatic freshwater drainage unlikely. Hypothetically, one of the genetic lineages or subclades within the samples from the

Adriatic freshwater drainage could represent a recently established endemic species. It was shown that endemic species of bullhead may express only slight genetic distance to neighbouring populations (Epepe et al., 1999), but in this case molecular data cannot identify this species.

## 5. Conclusions

The present data show *C. gobio* to have probably crossed several times the highest mountain range in Europe, which was formerly shown to provide a complete barrier for geneflow in several freshwater fish species. Consequently, the often generalised assumption of mountains as impassable barriers for freshwater animals has to be checked more carefully in the future. These results imply that the bullhead populations in the Adriatic freshwater drainage cannot be considered as a distinct species (*C. ferrugineus*) in the sense of Heckel and Kner (1858).

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## References

- Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A., Saunders, N.C., 1987. Intraspecific phylogeography—the mitochondrial DNA bridge between population genetics and systematics. *Annu. Rev. Ecol. Syst.* 18, 489–522.
- Băcescu, M., Băcescu-Meşter, L., 1964. *Cottus petiti* sp. n., un chabot nouveau récolté en France. Considérations zoogéographiques et données comparatives sur d'autres *Cottus* de Europe. *View Milieu* 1 (Suppl. 17), 431–446.
- Bănărescu, P., 1992. Zoogeography of Fresh Waters. Vol. 2. Distribution and Dispersal of Freshwater Animals in North America and Eurasia. Aula Verlag, Wiesbaden.
- Bernatchez, L., Danzmann, R.G., 1993. Congruence in control-region sequence and restriction-site variation in mitochondrial DNA of brook charr (*Salvelinus fontinalis* Mitchell). *Mol. Biol. Evol.* 10, 1002–1014.
- Bernatchez, L., Wilson, C.C., 1998. Comparative phylogeography of nearctic and palaearctic fishes. *Mol. Ecol.* 7, 431–452.

- Bianco, P.G., 1990. Potential role of the paleohistory of the Mediterranean and Paratethys basins on the early dispersal of Euro-Mediterranean freshwater fishes. *Ichthyol. Explor. Freshw.* 1, 167–184.
- Bianco, P.G., 1993. L'Ittiofauna continentale dell'Appennino umbromarchigiano, barriera semipermeabile allo scambio di componenti dell'Italia centrale. *Biogeographia* 17, 427–485.
- Bohlen, J., Ráb, P., 2001. Species and hybrid richness in spined loaches of the genus *Cobitis* L. (Teleostei: Cobitidae), with a checklist of European forms and suggestions for their conservation. *J. Fish Biol.* 59A, 75–89.
- Clement, M., Posada, D., Crandall, K.A., 2000. TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* 9, 1657–1659.
- Durand, J.D., Persat, H., Bouvet, Y., 1999. Phylogeography and postglacial dispersion of the chub (*Leuciscus cephalus*) in Europe. *Mol. Ecol.* 8, 989–997.
- Dynesius, M., Jansson, R., 2000. Evolutionary consequences of changes in species' geographical distributions driven by Milankovitch climate oscillations. *Proc. Natl. Acad. Sci.* 97, 9115–9120.
- Economidis, P.S., Bănărescu, P., 1991. The distribution and origin of freshwater fishes in the Balkan peninsula, especially in Greece. *Int. Rev. Ges. Hydrobiol.* 76, 257–283.
- Englbrecht, C.C., Freyhof, J., Nolte, A., Rassmann, K., Schliewen, U., Tautz, D., 2000. Phylogeography of the bullhead *Cottus gobio* (Pisces: Teleostei: Cottidae) suggests a pre-Pleistocene origin of the major central European populations. *Mol. Ecol.* 9, 709–722.
- Eppe, R., Persat, H., Beaudou, D., Berrebi, P., 1999. Genetic variability in sculpin (genus *Cottus*) from southern France, with reference to the taxonomic status of an endemic species, *C. petiti*. *Heredity* 83, 533–540.
- Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131, 479–491.
- Hänfling, B., Brandl, R., 1998. Genetic differentiation of the bullhead *Cottus gobio* L. across the watersheds in Central Europe: evidence for two taxa. *Heredity* 80, 100–117.
- Heckel, J., Kner, R., 1858. Die Süßwasserfische der Österreichischen Monarchie mit Rücksicht auf die angränzenden Länder. Verlag Wilhelm Engelmann, Leipzig.
- Hewitt, G.M., 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biol. J. Linn. Soc.* 58, 247–276.
- Hewitt, G.M., 1999. Post-glacial re-colonization of European biota. *Biol. J. Linn. Soc.* 68, 87–112.
- Hewitt, G.M., 2000. The genetic legacy of the quaternary ice ages. *Nature* 405, 907–913.
- Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17, 754–755.
- Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16, 111–120.
- Koli, L., 1969. Geographic variation of *Cottus gobio* L. (Pisces, Cottidae) in northern Europe. *Ann. Zool. Fenn.* 6, 353–390.
- Kontula, T., Väinölä, R., 2001. Postglacial colonization of Northern Europe by distinct phylogeographic lineages of the bullhead, *Cottus gobio*. *Mol. Ecol.* 10, 1983–2002.
- Kotlík, P., Berrebi, P., 2002. Genetic subdivision and biogeography of the Danubian rheophilic barb *Barbus petenyi* inferred from phylogenetic analysis of mitochondrial DNA variation. *Mol. Phylogenet. Evol.* 24, 10–18.
- Kottelat, M., 1997. European freshwater fishes. *Biologia* 52 (Suppl. 5), 1–271.
- Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Arizona State University, Tempe, Arizona, USA.
- Ladiges, W., Vogt, D., 1979. Die Süßwasserfische Europas, second ed. Verlag Paul Parey, Hamburg, Berlin.
- Lelek, A., 1987. Freshwater fishes of Europe. Vol. 9. Threatened Fishes of Europe. Aula Verlag, Wiesbaden.
- Ludwig, A., Bohlen, J., Wolter, C., Pitra, C., 2001. Phylogenetic relationships and historical biogeography of spined loaches (Cobitidae, *Cobitis* and *Sabanejewia*) as indicated by variability of mitochondrial DNA. *Zool. J. Linn. Soc.* 131, 381–392.
- Nei, M., 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York.
- Perdices, A., Doadrio, I., 2001. The molecular systematics and biogeography of the European Cobitids based on mitochondrial DNA sequences. *Mol. Phylogenet. Evol.* 19, 468–478.
- Perdices, A., Doadrio, I., Economidis, P.S., Bohlen, J., Banareescu, P., 2003. Pleistocene effects on the European freshwater fish fauna: double origin of the cobitid genus *Sabanejewia* in the Danube basin (Osteichthyes: Cobitidae). *Mol. Phylogenet. Evol.* 26, 289–299.
- Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Riffel, M., Schreiber, A., 1995. Coarse-grained population structure in Central European sculpin (*Cottus gobio* L.): secondary contact or ongoing genetic drift? *J. Zool. Syst. Evol. Res.* 33, 173–184.
- Salzburger, W., Brandstätter, A., Gilles, A., Parson, W., Hempel, M., Sturmbauer, C., Meyer, A., 2003. Phylogeography of the vairone (*Leuciscus souffia*, Risso 1826) in Central Europe. *Mol. Ecol.* 12, 2371–2386.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning. A Laboratory Manual*, second ed. Cold Spring Harbor Laboratory, Cold Spring Harbor.
- Schneider, S., Roessli, D., Excoffier, L., 2000. Arlequin ver. 2.000: A Software for Population Genetics Data Analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.
- Susnik, S., Snoj, A., Dovc, P., 2001. Evolutionary distinctness of grayling (*Thymallus thymallus*) inhabiting the Adriatic river system, as based on mtDNA variation. *Biol. J. Linn. Soc.* 74, 375–385.
- Swofford, D.L., 2000. PAUP. Phylogenetic Analysis using Parsimony (and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.
- Šlechtová, V., 2001. Genetická struktura populací vranky obecné (*Cottus gobio* L.) na území České republiky: I. Alozymové mapování. BS Thesis. Faculty of Biological Sciences, University of South Bohemia at České Budějovice, Czech Republic.
- Taberlet, P., Fumagalli, L., Wust-Saucy, A.G., Cosson, J.F., 1998. Comparative phylogeography and postglacial colonization routes in Europe. *Mol. Ecol.* 7, 453–464.
- Tsigenopoulos, C.S., Kotlík, P., Berrebi, P., 2002. Biogeography and pattern of gene flow among *Barbus* species (Teleostei: Cyprinidae) inhabiting the Italian Peninsula and neighbouring Adriatic drainages as revealed by allozyme and mitochondrial sequence data. *Biol. J. Linn. Soc.* 75, 83–99.
- Volckaert, F.A.M., Hänfling, B., Hellemans, B., Carvalho, G.R., 2002. Timing of the populations dynamics of bullhead *Cottus gobio* (Teleostei: Cottidae) during the Pleistocene. *J. Evol. Biol.* 15, 930–944.
- Wright, S., 1951. The genetical structure of populations. *Annu. Eugenics* 15, 323–354.