MASARYKOVA UNIVERZITA V BRNĚ

Přírodovědecká fakulta
Ústav experimentální biologie
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Využití mtDNA pro charakterizaci populačních struktur druhů rodů *Gobio* a *Romanogobio*

Přílohy k disertační práci

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Brno, 2007

Soubor publikací

Příloha A

Mendel, J., Lusková, V., Halačka, K., Lusk S., & Vetešník, L. (2005). Genetic diversity of *Gobio gobio* populations in the Czech Republic and Slovakia, based on RAPD markers. *Folia Zoologica* 54 (1): 13-24.

Příloha B

Mendel, J., Lusk, S., Lusková, Koščo, J., V., Papoušek, I., Halačka, K., & Vetešník, L. (2006). Molekulárně-biologické analýzy hrouzka Kesslerova ve vodách České republiky a Slovenska. (Molecular biological analyses of Sand gudgeon (*Romanogobio kesslerii*) in the waters of the Czech Republic and Slovakia). *Biodiverzita ichtyofauny ČR* (VI): 95-101.

Příloha C

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Příloha D

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Příloha E

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Příloha A

Mendel, J., Lusková, V., Halačka, K., Lusk S., & Vetešník, L. (2005). Genetic diversity of *Gobio gobio* populations in the Czech Republic and Slovakia, based on RAPD markers. *Folia Zoologica* 54 (1): 13-24.

Folia Zool. – 54 (Suppl. 1): 13–24 (2005)

Genetic diversity of *Gobio gobio* populations in the Czech Republic and Slovakia, based on RAPD markers

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Received 12 November 2003; Accepted 3 March 2005

A b s t r a c t . The random amplified polymorphic DNA (RAPD) method was applied to eight *Gobio gobio* populations living in the rivers of the Czech Republic and Slovakia. The application of seven RAPD primers yielded eight fingerprint characteristics for the populations examined. Forty diagnostic markers have been identified, which can reliably identify the populations under study. Intrapopulation diversity varied between 0.26 and 0.38. A phenogram documented the close agreement of the particular populations with the geographic pertinence of their localities to the different sea basins.

Key words: RAPD, common gudgeon, diagnostic markers, intraspecific diversity

Introduction

Even though the common gudgeon *(Gobio gobio* Linnaeus, 1758) is widely distributed over Europe and Asia (B e r g 1949, B ă n ă r e s c u et al. 1999), knowledge of its genetic diversity is insufficient. In the past, attention was chiefly paid to the variability of its external morphological characters. Based on differences in some, largely plastic, characters and differences in geographic distribution, several subspecies or even morphs have been described within the species *G. gobio* (B e r g 1914, 1949, V l a d y k o v 1925, 1931, B ă n ă r e s c u 1961, 1962, 1964). Several studies aimed at the knowledge of the genetic diversity of this species have occurred only recently (S c h r e i b e r 2002, W o l t e r et al. 2003, C a l l e j a s et al. 2004, Š l e c h t o v á et al. 2005).

The common gudgeon is generally distributed over the waters of the Czech Republic and Slovakia (L u s k et al. 2005, K o š č o et al. 2005). It can find optimum environmental conditions in streams inhabited by fish communities of the barbel type, characterised by the predominance of cyprinids (L u s k et al. 1998). The hydrographic systems in the territories of the Czech Republic and Slovakia belong to three different sea basins, which fact allows one to presume marked interpopulation differences within the species. Therefore, the morphological variability of the gudgeon was studied in the past in different drainage areas in order to find out the subspecific pertinence of the gudgeon (A l b e r t o v á & S u c h o m e l o v á 1953, T o u š k o v á 1978, Z á v ě t a 1990). According to B ă n ă r e s c u (1961), the nominate subspecies, *G. gobio gobio* (Linnaeus, 1758), should inhabit the North Sea basin, and *G. gobio obtusirostris* (Cuvier et Valenciennes, 1842) the Danube drainage area (the Black Sea basin). A l b e r t o v á & S u c h o m e l o v á (1953) were unable to formulate an unequivocal evaluation of the populations studied by them, since their different characters corresponded to different subspecies. T o u š k o v á (1978) and Z á v ě t a (1990), however, included the common gudgeon populations inhabiting the Czech Republic and Slovakia in the nominate subspecies, *G. gobio gobio.* Having analysed certain morphological characters found in *G. gobio* sampled in nine localities in the drainage areas of the rivers Labe, Danube, Odra, and

Vistula, K u x & L i b o s v á r s k ý (1981) concluded that ecological factors determine the incidental differences. K o t t e l a t (1997) annulled the taxonomic value of subspecies connected with *Gobio gobio* in the European space, or elevated some of them to the species level (*G. benacensis*). Even though in some more recent papers (B ă n ă r e s c u et al. 1999) one does encounter with the subspecies category based on certain morphological differences as well as geographically different distribution, their value is considerably vague due to the ecological variability of external morphological characters.

The aim of the present paper was to obtain genetic markers for the identification of populations of the species *Gobio gobio* and to obtain knowledge of the intrapopulational and interpopulational diversity of this species.

Material and Methods

The examinations involved a total of 76 specimens of common gudgeon from 8 populations living in the streams belonging to the Black Sea, North Sea and Baltic Sea basins in the territories of the Czech Republic and Slovakia (Fig. 1, Table 1). Fish were obtained by electro-fishing and transported live to the laboratory. In fish killed with an overdose of the narcotisation solution (2-phenoxy-ethanol) we performed the basic measurements and took samples of ca 100 mg muscular tissue. Each sample was preserved in TNES-U buffer (10 mM Tris-HCl pH 8.0, 125 mM NaCl, 10 mM EDTA, 0.5 % SDS, 4 M Urea). Tissue was digested with 20 μl proteinase K (20mg/ml in 50 % glycerol) for 36 hours at 50 °C. DNA was extracted using the phenol-chloroform-isoamylalcohol method (S a m b r o o k et al. 1989) with minor modifications. The extraction was supplemented with a purification step with chloroform-isoamylalcohol (24:1). DNA obtained by EtOH precipitation, was resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

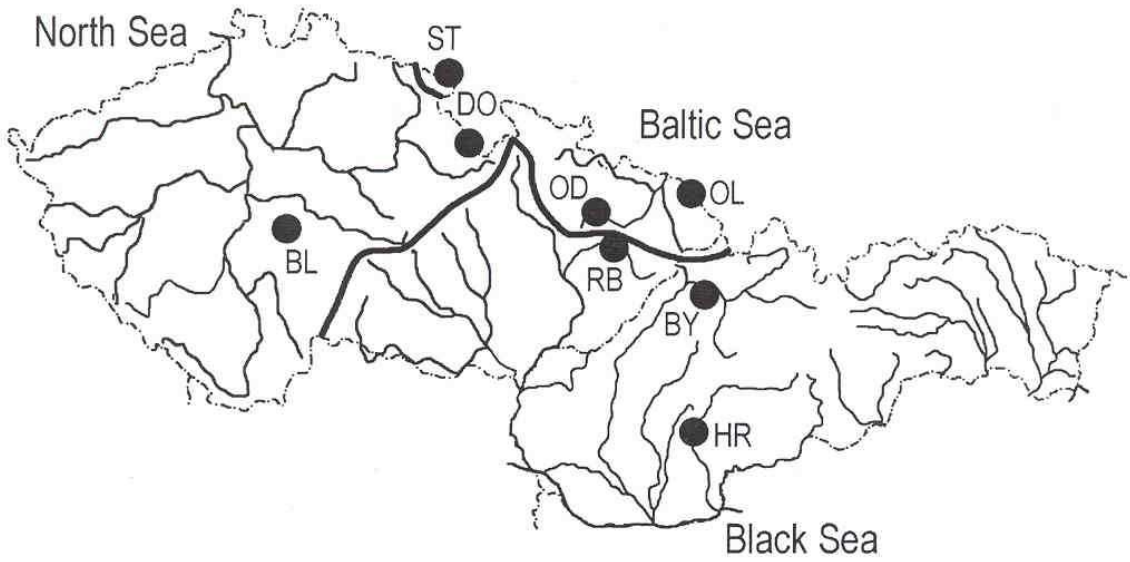

Fig. 1. Geographic location of *G. gobio* populations in the waters of the Czech Republic and Slovakia.

R A P D a n a l y s i s

Seven RAPD primers were used to identify the RAPD markers (Table 2). The primers were selected to contain 60–70 % GC nucleotides. Which criterion is closely connected with the

amount of amplified products (B e ž o et al. 2001). The RAPD-PCR was performed according to the conditions described by W i l l i a m s et al. (1990) and C a l l e j a s & O c h a n d o (2001) with minor modifications. The PCR mixture was mixed in a 25 μl volume and contained 45 ng DNA, 5 pmol primer, 0.1 mM dNTP mix, 1.5 mM MgCl2, 2.5 μl 10x reaction buffer, and 1.25 U Taq DNA polymerase. RAPD-PCR was performed in a TGRADIENT Thermocycler (Whatman Biometra). The amplification condition were programmed as follows: 94 °C for 5 minutes and then 45 cycles of denaturation step 94 °C for 1 min., anneling 36 °C for 1 min., elongation 72 °C for 6 min., followed by final elongation 72 °C for 6 minutes. A negative control, without template DNA, was also included to monitor any possible contamination of the reactions with non-target template DNA. The PCR products were separated at 60 V in 1x TBE buffer (10x TBE: 1 m Tris base, 900 mM boric acid, 1 mM EDTA) on 1.4 % agarose gel which, for the purpose of visualisation, contained ethidium bromide (10 mg.ml-1).

Table 1. Origin (river, sea basin) of the *G. gobio* populations under study and number of specimens examined (N).

Identification of population	River	Sea basin	N
BL	Blanice	North Sea	10
BY	Bystrička	Black Sea	10
DO	Divoká Orlice	North Sea	10
HR	Hron	Black Sea	10
OD	Odra	Baltic Sea	10
OL	Olše	Baltic Sea	10
RB	R. Bečva	Black Sea	6
ST	Stěnava	Baltic Sea	10

The gel was analysed by the documentation and analytic system GeneGenius (Trigon-Plus). To determine the size of the amplified fragments, we used pBR322 DNA/Bsu RI Marker,5 and Lambda DNA/Eco471(AvaII) Marker,13. The PCR products obtained were described by the name of the primer used and the fragment size (e.g. fragment labelled A09 514 denotes product length 514 bp amplified with A09 primer). Each sample was analyzed at least twice according to the same protocol, under the given conditions and types of chemical. The final comparison of genetic maps of the populations under study included marked, well separated bands only (excluding weak and equivocal ones). The "molecular phenotypes" were determined according to the presence or absence of those bands.

For statistical evaluation of intrapopulation variability, we used the GeneTools programme, using the similarity coefficient according to N e i & L i (1979). Interpopulation variability was analysed by the FreeTree software (P a v l í č e k et al. 1999), using the Neighbor-joining method (S a i t o u & N e i 1987). The dendrogram was compiled by the TreeView software (P a g e 1996).

Results

Seven RAPD primers were used to find out the genome organisation in the eight *G. gobio* populations examined. They revealed 212 distinct, well separated nDNA sections 172 – 2081 bp in size. The diagnostic bands, generated by the RAPD primers across the populations, varied from 7 to 25, averaging 13 bands per primer. Primers A08, A06, and A09 yielded 51 % of these DNA markers.

The RAPD method, selected for the identification of the populations under study, yielded a fingerprint characteristic for each of the populations, see Table 3a, 3b. An example of the RAPD patterns of five *G. gobio* populations obtained using primer A06, is given in Fig. 2.

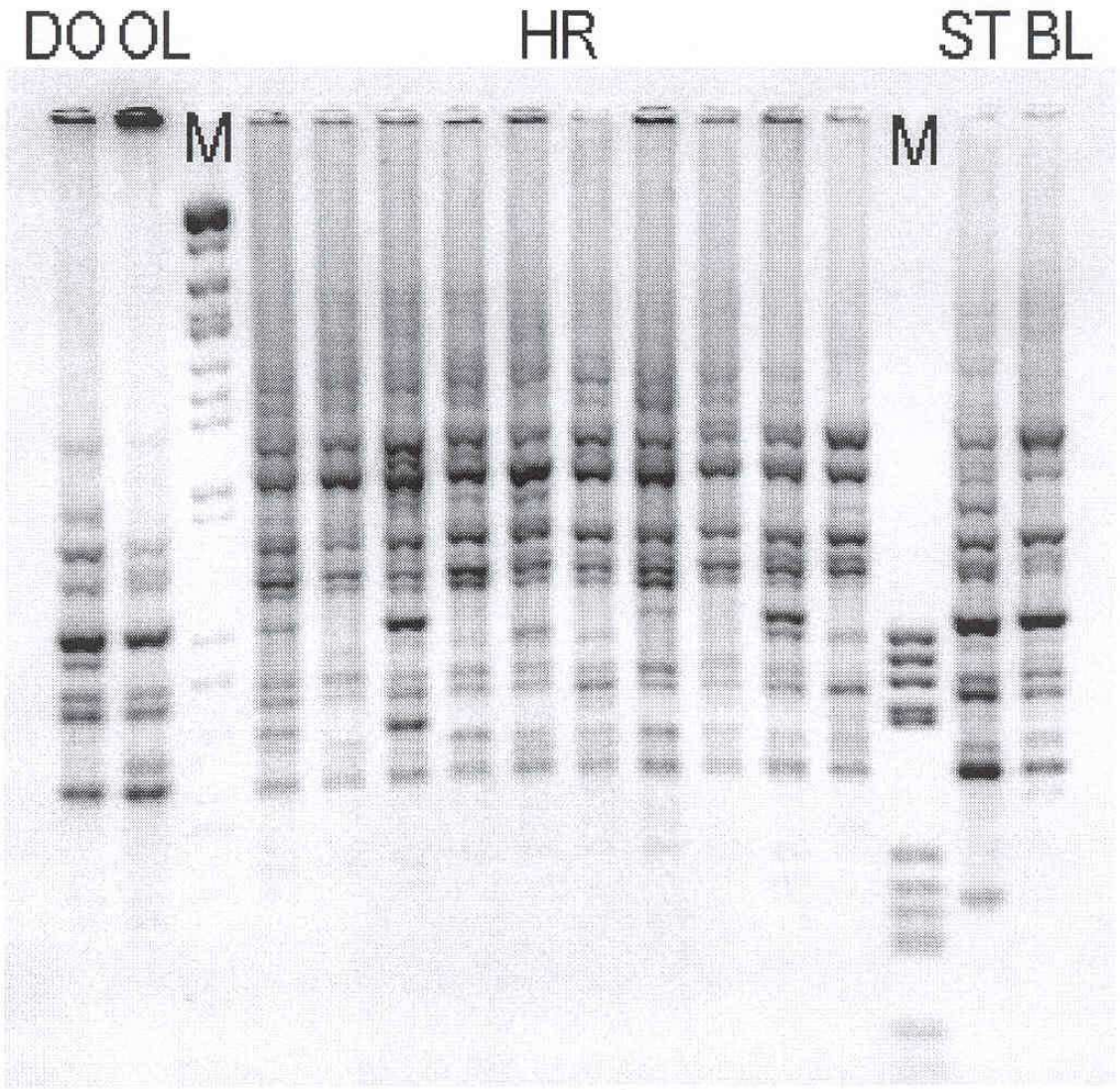

Fig. 2. RAPD patterns of *G. gobio* populations, obtained by using primer A06. M = DNA markers (bp).

Table 2. Description of the decamer primers of random sequence (RAPD oligonucleotides).

Primer	Sequence	(G + C)%
A03	GATGACCGCC	70
A04	GAACGGACTC	60
A06	TGGACCGGTG	70
A07	CTCACCGTCC	70
A08	AAAGCTGCGG	60
A09	GACGGATCAG	60
A10	CACACTCCAG	60

Table 3. RAPD fingerprints of *G. gobio*. Size given in base pairs (bp). Symbol +, marker presented at all specimens of population.

Primer/bp	BL	DO	HR	BY	OD	OL	RB	ST	Primer/bp	BL	DO	HR	BY	OD	OL	RB	ST
A03 465			+	+	+				A07 927					+			
A03 518					+				A07 1000					+			
A03 587								+	A07 1032			+					
A03 650					+				A07 1350					+			
A03 710			+				+		A08 172					+			
A03 737				+	+				A08 241					+			
A03 778							+		A08 341	+		+	+			+	
A03 860		+							A08 445				+				
A03 894			+		+				A08 469			+					
A03 911				+					A08 505							+	
A03 944			+						A08 553				+	+			
A03 1049					+		+		A08 570			+			+	+	+
A03 1103				+					A08 587				+			+	
A03 1255	+	+							A08 600			+					
A03 1350				+					A08 663							+	
A03 1402								+	A08 696			+	+	+		+	+
A03 1708								+	A08 709			+					
A04 236					+				A08 744							+	
A04 329					+				A08 829							+	
A04 390					+				A08 853			+					
A04 541							+		A08 878				+				
A04 564					+				A08 902							+	
A04 587	+						+		A08 975							+	
A04 603				+					A08 999	+		+			+		
A04 620							+		A08 1060				+			+	
A04 673					+				A08 1170							+	
A04 695	+						+		A08 1267				+			+	
A04 716					+				A08 1296			+	+				
A04 726	+								A08 1634							+	
A04 766					+				A09 255					+			
A04 784			+						A09 313					+			
A04 894	+						+		A09 350				+				
A04 950			+	+	+		+		A09 374					+			
A04 1350			+						A09 384						+		
A04 1450	+								A09 422	+							
A04 1980								+	A09 496					+		+	
A04 2071		+							A09 514			+					
A06 270				+					A09 530							+	
A06 351			+	+	+	+	+		A09 587				+			+	
A06 387							+		A09 600					+			
A06 408	+								A09 659					+			
A06 417			+	+					A09 709					+			
A06 493			+	+			+		A09 771	+				+			
A06 502					+				A09 866					+		+	
A06 521				+					A09 902						+		
A06 563							+		A09 1006					+		+	

Table 3. continued.

Primer/bp	BL	DO	HR	BY	OD	OL	RB	ST	Primer/bp	BL	DO	HR	BY	OD	OL	RB	ST
A06 589			+		+	+			A09 1036					+			
A06 602					+				A09 1108						+		
A06 617		+							A09 1234		+						
A06 695			+						A09 1284					+			
A06 722					+			+	A09 1342							+	
A06 734			+	+					A09 1689				+				
A06 789						+			A10 231						+		
A06 805			+		+				A10 475			+	+	+		+	
A06 860				+					A10 512			+					
A06 894					+				A10 536			+					
A06 996			+						A10 582			+		+			
A06 1033					+				A10 675			+	+	+	+		
A06 1157			+	+					A10 710			+					
A06 1179					+				A10 757			+					
A06 1440			+						A10 863			+					
A06 2081							+		A10 894			+	+				
A07 353	+	+	+	+					A10 927			+	+	+			
A07 410					+				A10 1109			+					
A07 535			+	+					A10 1210				+			+	
A07 594					+				A10 1310			+					
A07 647			+	+					A10 1340								+
A07 721					+				A10 1391			+	+				
A07 766			+	+					A10 1465				+				
A07 827				+				+	A10 1597					+			
A07 894			+		+		+		A10 1723					+			
									A10 2068			+					

Table 4. Population diagnostic RAPD markers of the *G. gobio* populations examined, primer and size of fragment analysed (bp).

Blanice	D. Orlice	Hron	Bystrička	Odra	Olše	R. Bečva	Stěnava
A04 1450	A04 2071	A08 600	A03 911	A04 236	A06 789	A04 541	A03 1402
A09 422	A09 1234	A08 709	A08 878	A04 564	A09 384	A06 2081	A03 1708
		A08 853	A06 270	A04 766		A08 663	A04 1980
		A09 514		A06 602		A08 744	A10 1340
		A10 710		A06 894		A08 975	
		A10 1109		A07 410		A08 1634	
		A10 2068		A07 594			
				A07 927			
				A07 1000			
				A07 1350			
				A08 241			
				A09 255			
				A09 374			
				A10 1597			

Forty population diagnostic markers were revealed (Table 4). The largest number (14) was found in the gudgeon population in the river Odra, the least number (2) in the populations in the rivers Blanice, Divoká Orlice, and Olše (see Table 4 for particulars). The identification markers were exclusively present in all or most (> 95 %) of the specimens of the respective populations.

The statistical evaluation is based on the mean diversity values determined for each population by all primers applied. The greatest intrapopulation diversity was found in gudgeon populations inhabiting the rivers Odra (ranging from 26 to 43 %, mean 38 %) and Bystrička (ranging from 34 to 47 %, mean 37 %). The least values of intrapopulation diversity, averaging less than 30 %, were found in populations inhabiting the rivers Olše (ranging between 17 and 38 %, mean 28 %) and Blanice (ranging from 23 to 34 %, mean 26 %). The mean intrapopulational diversity values were very close to one another in the rivers Hron (26–46 %, mean 33 %), Stěnava (26–42 %, mean 33 %), Rožnovská Bečva (22–37 %, mean 32 %), and Divoká Orlice (20–37 %, mean 31 %).

Table 5. Genetic distance among *G. gobio* populations examined.

River	Blanice	D. Orlice	Hron	Bystrička	Odra	Olše	R. Bečva	Stěnava
Blanice								
D. Orlice	0,700							
Hron	0,869	0,927						
Bystrička	0,887	0,915	0,545					
Odra	0,939	0,967	0,743	0,785				
Olše	0,833	0,889	0,797	0,882	0,875			
Rožnovská Bečva	0,843	1,000	0,767	0,718	0,780	0,918		
Stěnava	0,909	0,875	0,895	0,878	0,903	0,800	0,915	

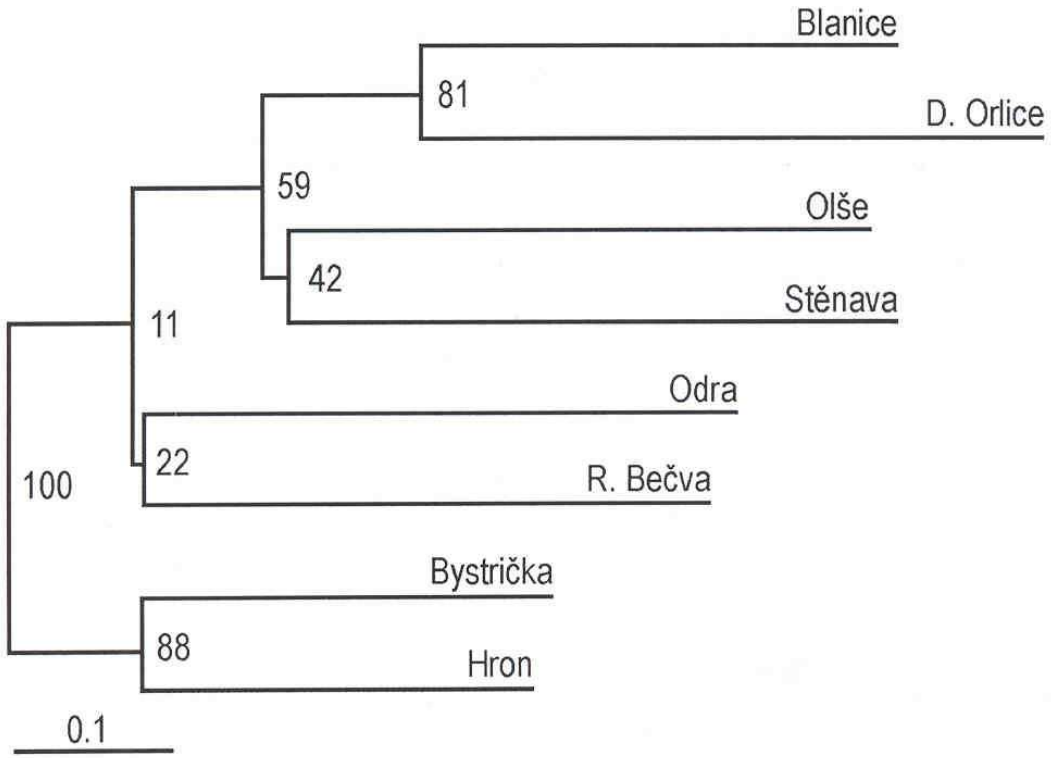

Fig. 3. Neighbor-joining (NJ) bootstrap consensus tree analyzed with FreeTree. Numbers at the nodes represent the percentage of 1000 bootstrap replications.

The Nei & Li genetic distance values of the populations examined are given in Table 5. The closeness or distance of the *G. gobio* populations are depicted by the phylogenetic tree in Fig. 3. The similarity of populations, concordant with the geography of the river networks in the particular sea basins, has been confirmed: the North Sea basin – populations in the rivers Blanice and Divoká Orlice (genetic distance 0.700); the Black Sea basin – populations in the rivers Bystrička, Rožnovská Bečva, and Hron (genetic distance 0.718 and 0.767); the Baltic Sea – populations in the rivers Olše and Stěnava (genetic distance 0.800). This rule was only disturbed by the population in the river Odra (Baltic Sea basin), which produced a fragile cluster (bootstrapping value 22) with the population in the river Rožnovská Bečva (Black Sea basin). The values of the Nei & Li coefficient suggest a closer distance of the population in the river Odra to those in the Black Sea basin rather than to those in the Baltic Sea (genetic distances 0.743, 0.780, and 0.785 vs. 0.875 and 0.903). The least distance was found for the populations in the river Hron and Bystrička (0.545) and those between the rivers Divoká Orlice and Blanice (0.700). The greatest distance was found for the populations in the rivers Divoká Orlice and Rožnovská Bečva (1.000) and for those in the rivers Divoká Orlice and Odra (0.967).

Discussion

Genetic analyses based on the random amplified polymorphic DNA have been successfully utilised in breeding programmes, genetic mapping, population genetics or phylogeography, and they yield individual DNA fingerprints of viruses, plants, animals and man (Welsh & McClelland 1990, Williams et al. 1993, Haig et al. 1994, van Oppen et al. 1994).

The method examines the whole nDNA and amplifies sequences mutually defined by RAPD primers. The very short size of the primer (10 bp) makes it possible to visualise a number of complementary sites at various places in the genome. DNA mutations as insertions, deletions, inversions and substitutions have an impact on RAPD fingerprint. Genetic analyses have demonstrated the sensitiveness of this method to various changes, such as the purity and concentration of DNA, primer, Mg2+ ions, the type of DNA polymerase in the PCR reaction, and even the type of apparatus employed in the PCR reaction or PCR test tubes, etc. All this accounts for the limited possibility to reproduce the results among laboratories. Nevertheless, this limitation can be partly eliminated by strict adhering to the protocols. Experiments testing the capability of different laboratories to obtain identical RAPD profiles have been described (Jones et al. 1997). The majority of recipient laboratories were able to amplify the same bands as the sender and to observet he same polymorphism but none reproduced the profile exactly (Jones et al. 1997). The low capability to reproduce the RAPD results is solely due to the PCR reactions, not to the phylogenetic studies themselves. If one and the same sample series is subject to RAPD analysis in two different laboratories, almost identical matrices of genetic distances are obtained even on the basis of different electrophoregrams, and thus also nearly identical phylogenetic trees. (Flegr 2003, oral communication).

In our analysis seven decanucleotide primers were used, which revealed 212 distinctly separated bands, reliably characterising eight *G. gobio* populations. Thus, it has been confirmed that the RAPD method is able to generate informative DNA fingerprints, as stated by Borovsky et al. (1995), Fritch & Rieseberg (1996) and Welsh & McClelland (1990). From the 212 bands we selected 40 that can be considered diagnostic for particular populations and can unequivocally differentiate among the populations under study.

The mean genetic intrapopulation variability varied from 0.26 to 0.38. Compared to other papers, obtained values of the genetical variability of *G. gobio* populations rank in the middle

among the values reported (Foo et al. 1995, Yoon & Kim 2001, Wolter et al. 2003). Foo et al. (1995) reported the values of genetic variability of two different (probably isolated) varieties of *Poecilia reticulata* (0.19 ± 0.08 in "3/4 Black" and 0.22 ± 0.10 in "Green Snakeskin"). On the other hand, having analysed the populations of Korean *Silurus asotus*, Yoon & Kim (2001) found a significantly higher genetic dissimilarity within two populations examined: 0.44 ± 0.08 and 0.41 ± 0.07. Wolter et al. (2003) reported a the mean level of genetic dissimilarity of 0.05 for the *G. gobio* population in the river Labe. This genetic dissimilarity was markedly higher in the populations evaluated in this study (0.26–0.38). The differences may be partly due to different procedures employed and the resulting different sensitivity (Kernodle et al. 1993, Papadopoulos 2000). If the RAPD® 10mer Kits Operon technologies, Alameda, CA are employed, the number of gene bands obtained differs from that obtained by a non-commercial procedure. The rather high diversity level in the *G. gobio* populations examined by ourselves (26–38 %) indicates their low uniformity and thus their high level of fitness.

The values of genetic distance between populations (Nei & Li 1979), expressed in the form of a phylogenetic tree, divided the *G. gobio* populations under study into two main branches (Fig. 3). Pairs of populations thus created agree with the pertinence of respective rivers to the sea basins, except for the pair of populations in the rivers Odra and Rožnovská Bečva. In this case the radiation phylogenetic tree indicates mutual proximity although weakly supported (Fig. 4). Here one can consider an ancient proximity or identical origin of the two

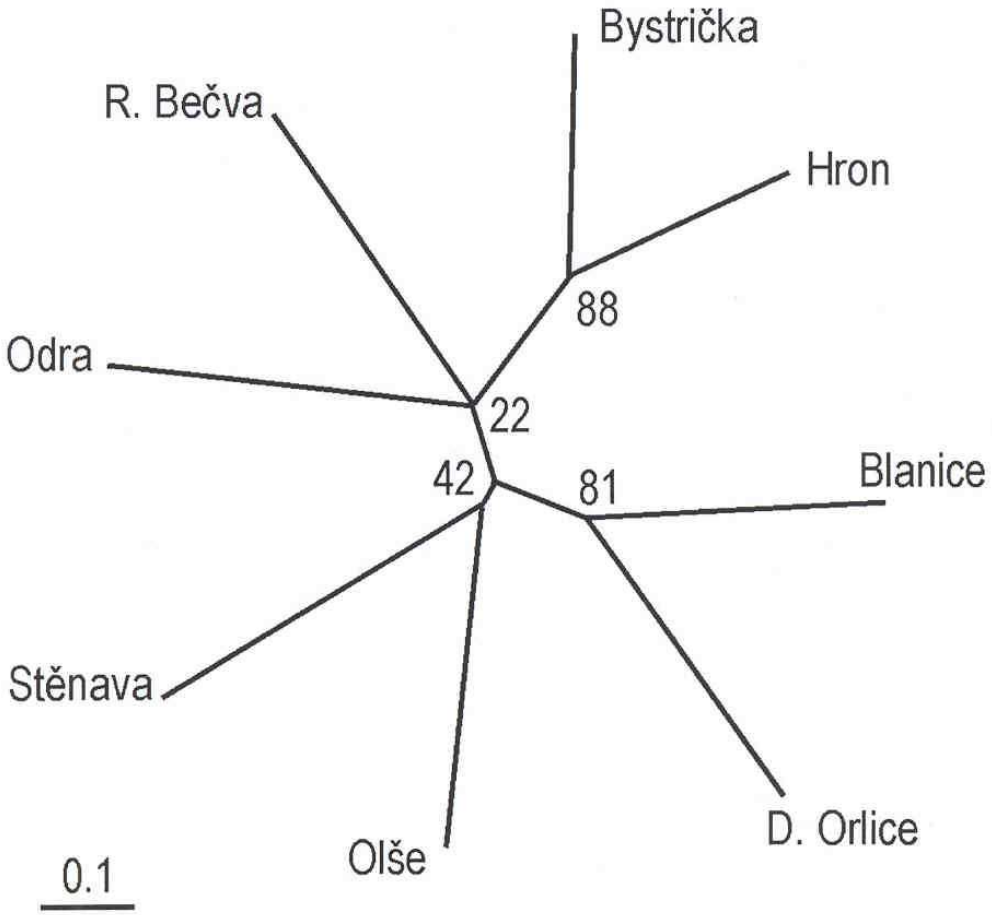

Fig. 4. Neighbor-joining (NJ) radiation tree of *G. gobio* populations.

populations, as during the Pleistocene (e.g. the Elster glaciation) the waters of the Odra flowed towards the south into the drainage area of the river Bečva (C z u d e k 1997). The values of genetic distances (N e i & L i 1979) indicate the proximity of the populations in the Rožnovská Bečva and those in Slovakia, particularly those inhabiting the river Bystrička (genetic distance 0.718), which fully agrees with the identical pertinence of these populations to the Black Sea basin.

Thus, our results support the hypothesis of the genetic similarity of populations originating from a single geographic region (K i r p i t c h n i k o v 1999, R e p o r t 2002). Moreover, the results presented here confirm that the RAPD method is a good tool for population studies aimed at analysing the genetic relationships within a species, particularly if the knowledge of the genome of the organism under analysis is minimal.

A c k n o w l e d g e m e n t

This study was carried out as a part of the research project reg. no. IAA6045005, supported by the Grant Agency of the Academy of Sciences of the Czech Republic, and the research project reg. no. S5045111 supported by the Academy of Sciences of the Czech Republic.

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MOLEKULÁRNĚ-BIOLOGICKÉ ANALÝZY HROUZKA KESSLEROVA VE VODÁCH ČESKÉ REPUBLIKY A SLOVENSKA. Molecular biological analyses of Sand gudgeon (*Romanogobio kesslerii*) in the waters of the Czech Republic and Slovakia

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Úvod

Nezbytnou součástí ochrany biodiverzity je vedle druhové ochrany i ochrana vnitrodruhová (genetická). Základním předpokladem je však její identifikace, i když pravidlo „předběžné opatrnosti“ by mělo být samozřejmostí (Lusk a kol. 2002). Zůstává realitou, že znalosti o vnitropopulační a mezipopulační diverzitě nativních druhů ryb v podmínkách České republiky a Slovenska se týkají prozatím pouze několika druhů (Lusková et al. 1997, 2004, Mendel et al. 2005, Šlechtová 1998). Zejména u druhů vzácných a chráněných, kde se objevují snahy převážně na úrovni „amatérské“ o obnovu jejich výskytu, či podporu početnosti populací vysazováním odchovaných násad nebo přesazováním jedinců z existujících početnějších populací, je determinace jejich genetické diverzity doslova prioritou. Poznatky o důsledcích tzv. podpůrného vysazování či obnově populací, které máme např. u pstruha obecného, nebo bolena dravého, ukazují, že tyto aktivity bez předchozího vyhodnocení a respektování původní genetické diverzity vedou k její unifikaci a destrukci (Lusk a kol. 1995, 2002, Lusková et al. 1995).

Hrouzek Kesslerův, *Romanogobio kesslerii* (Dybowski, 1862) je v České republice vzácným druhem, který se původně vyskytoval pouze v toku Bečvy a v roce 2003 byl zjištěn i v toku Moravy (Lusk et al. 2005, Merta, Lusk 2004). V současnosti je chráněn národní legislativou (vyhl. 395/1992 Sb.) jako kriticky ohrožený druh. Podstatná část úseků, kde se tento druh v toku Bečvy a Moravy vyskytuje, byla vyhlášena v rámci Natura 2000 jako evropsky významné lokality (nařízení vlády č. 132/2005 Sb.). Na Slovensku je hrouzek Kesslerův více rozšířen, ale v průběhu posledních 30 let z řady lokalit vymizel (Koščo et al. 2005).

V této studii předkládáme předběžné poznatky o genetické diverzitě hrouzka Kesslerova z několika populací z moravských a slovenských toků.

Materiál a metodika

Molekulárně genetické analýzy byly provedeny u 30 jedinců hrouzka Kesslerova, kteří byli odloveni z různých vodních toků v letech 2002-2005. Na území ČR se jednalo o řeku Bečvu – lokalita Rybáře (5 ks), řeku Moravy – lokality NPR Zástudánčí, Blata a Tovačov (10 jedinců). Na Slovensku byly vzorky sebrány v řece Laborec – lokalita Kochánovce (5 ks), řeka Topl'á - lokality Poliakovce a Nemcovce (5 ks) a řeka Ipel' – st. hranice (5 ks). Všem jedincům byl odebrán vzorek tkáně – část ploutvičky, který byl uchován v 96% alkoholu.

Celková genomová DNA byla vyizolována pomocí metody fenol-chloroform-isoamyl alkohol (Sambrook et al., 1989). Pro posouzení zkoumaných jedinců bylo využito sekvenční analýzy vybraného úseku mtDNA – control region a části prvního intronu jaderného genu S7. Část mitochondriální sekvence o velikosti 610 bp byla amplifikována pomocí sady čtyř PCR primerů: D-loop159L 5´- CCC AAA GCA AGT ACT AAC GTC - 3´, D-loop493U 5´- TTG GGT AAC GAG GAG TAT GTA - 3; D-loop439L 5'- AAA TGT TTT TCC CAC ACT TA - 3', D-loop851U 5'- TGC GAT GGC TAA CTC ATA C - 3'. Část prvního intronu r-proteinu o velikosti 372 bp byla vymezena sekvencí primerů: S7343F 5'- CGG CAT GCT AAG AAC CTA C - 3'; S7935U 5'- CGC GCT GGT ACT GAA C -3'.

PCR reakce byla provedena v přístroji TGRADIENT Thermocycler (Whatman Biometra) s následujícími podmínkami:

Control region: 95°C (1 min) a pak následovalo 33 cyklů 94°C (45 s), 52,6°C (30 s, první část sekvence) or 60,9°C (30 s, druhá část sekvence) a 72°C (45 s) , závěrečná elongace 72°C (5 min). S7: 95°C (1 min) a pak následovalo 29 cyklů 94°C (45 s), 52,4°C (30 s) a 72°C (25 s), závěrečná elongace 72°C (5 min).

PCR produkty byly purifikovány pomocí PEG/Mg/NaAc. Přečištěné produkty byly analyzovány na genetickém analyzátoru ABI PRISM 310 (Applied Biosystems). Pro kontrolu kvality byly testované úseky analyzovány z obou stran. Správnost získané sekvence byla potvrzena srovnáním s databázovými sekvencemi GenBank.

Pro vyhodnocení sekvenčních analýz vzorků hrouzka Kesslerova byly vzaty i sekvence druhu *Gobio gobio* z řeky Bečva (ev.č. 2341) a z řeky Topl'á (ev.č. 4023) a také druhu *Romanogobio albipinnatus* z řeky Morava (ev. č. 3674) a z řeky Topl'á (ev. č. 4041). Jako outgroup byly použity sekvence z GenBank s přístupovým číslem AF529882 *Vimba vimba* a

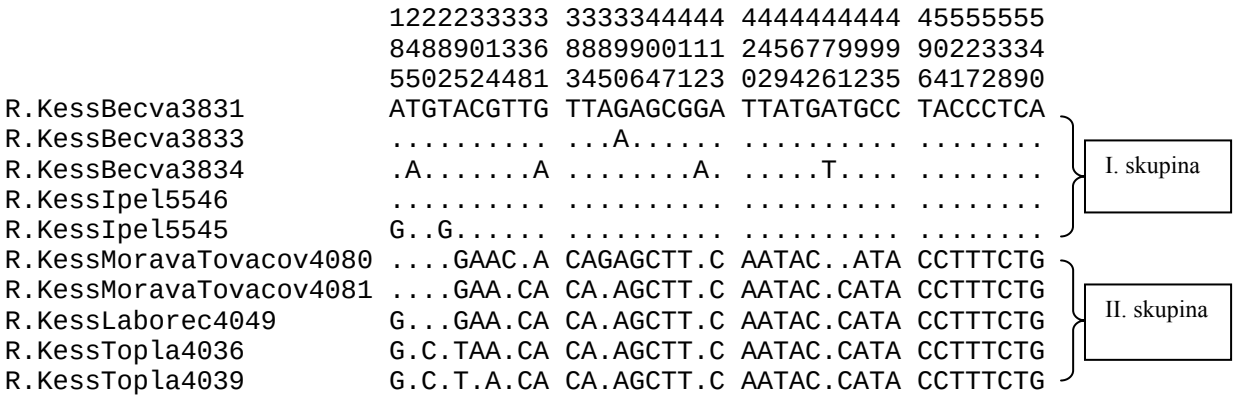
AY325789 *Rhodeus occelatus*. Všechny hodnocené sekvence byly uspořádány pomocí algoritmu Clustal W a upraveny použitím programu Lasergene 6 (DNASTAR, Inc.).

Molekulární analýzy byly hodnoceny pomocí MEGA version 3.1 (Kumar et al. 2004). Pro statistické vyhodnocení bylo využito dvou metod shlukové analýzy: neighbour-joining (NJ, metoda připojení souseda) a unweighted pair-group method using arithmetic averages (UPGMA, nevážená párová metoda aritmetických průměrů). Jako nejvhodnější substituční model byl zvolen Kimurův dvouparametrový model (K2P; Kimura 1980). Pro ověření spolehlivosti jednotlivých větví fylogenetického stromu bylo využito neparametrické techniky opakovaného výběru (resampling metody), tzv. bootstrap test, s 1000 pseudoreplikací. Fylogenetické stromy uváděné v naší studii jsou stromy konsensuální a byly sestrojeny ze všech kodonových pozic nukleotidové sekvence a s kritériem – d: transitions + transversions.

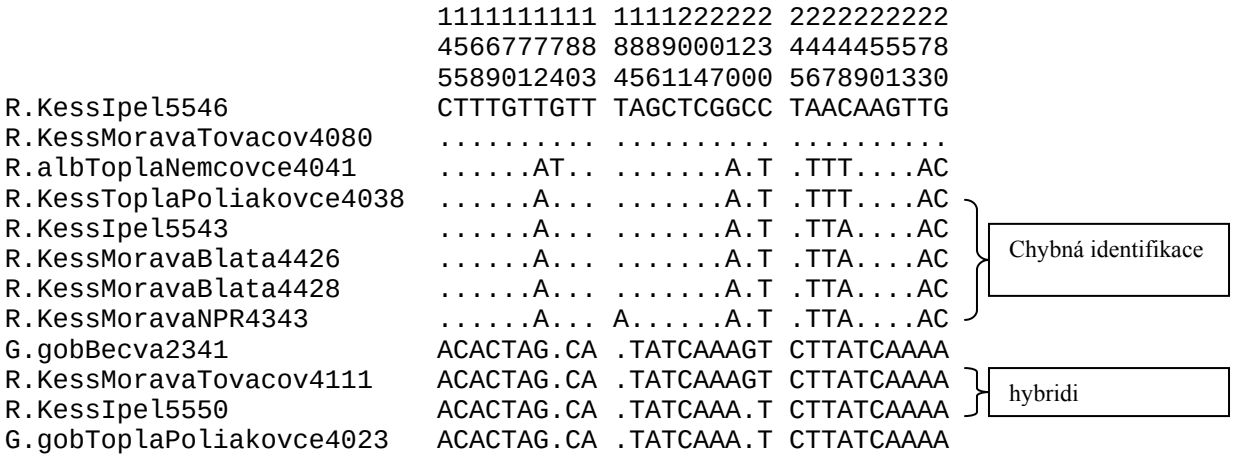
Výsledky a diskuse

Analýza všech jedinců hrouzka Kesslerova odhalila celkem 9 haplotypů. Nejvyšší genetickou variabilitou se vyznačoval vzorek populace z Bečvy, kde se vyskytovaly 3 haplotypy, po dvou haplotypech měly vzorky z řek Morava ‚Topl'a. Ipel' (zde jeden haplotyp shodný s Bečvou) a jediný haplotyp se vyskytoval u všech jedinců z řeky Laborec (Obr.1). Vnitropopulační diverzita na základě vzorků ze zkoumaných populací z výše uvedených toků nepřevyšovala hodnotu 0,7%. Mezipopulační diverzita uvnitř první skupiny (Bečva a Ipel') činila 0,4% a uvnitř druhé skupiny (Topl'a, Laborec, Morava) 0,5-1,0%.

Analýzy části mtDNA control regionu (610 bp) rozdělili vzorky hrouzka Kesslerova do dvou odlišných skupin (Obr. 1). První skupinu tvořily vzorky jedinců z řek Bečva a Ipel' a druhou tvořily vzorky populací z Topl'é, Laborce a Moravy. Obě skupiny se od sebe lišily maximálně v 38 nukleotidových pozicích, t.j v 6,2% z celkové analyzované sekvence (610 bp).



Obr. 1. Přehled rozdílných pozic mtDNA haplotypů control regionu *R. kesslerii*. Identické =symbol "."

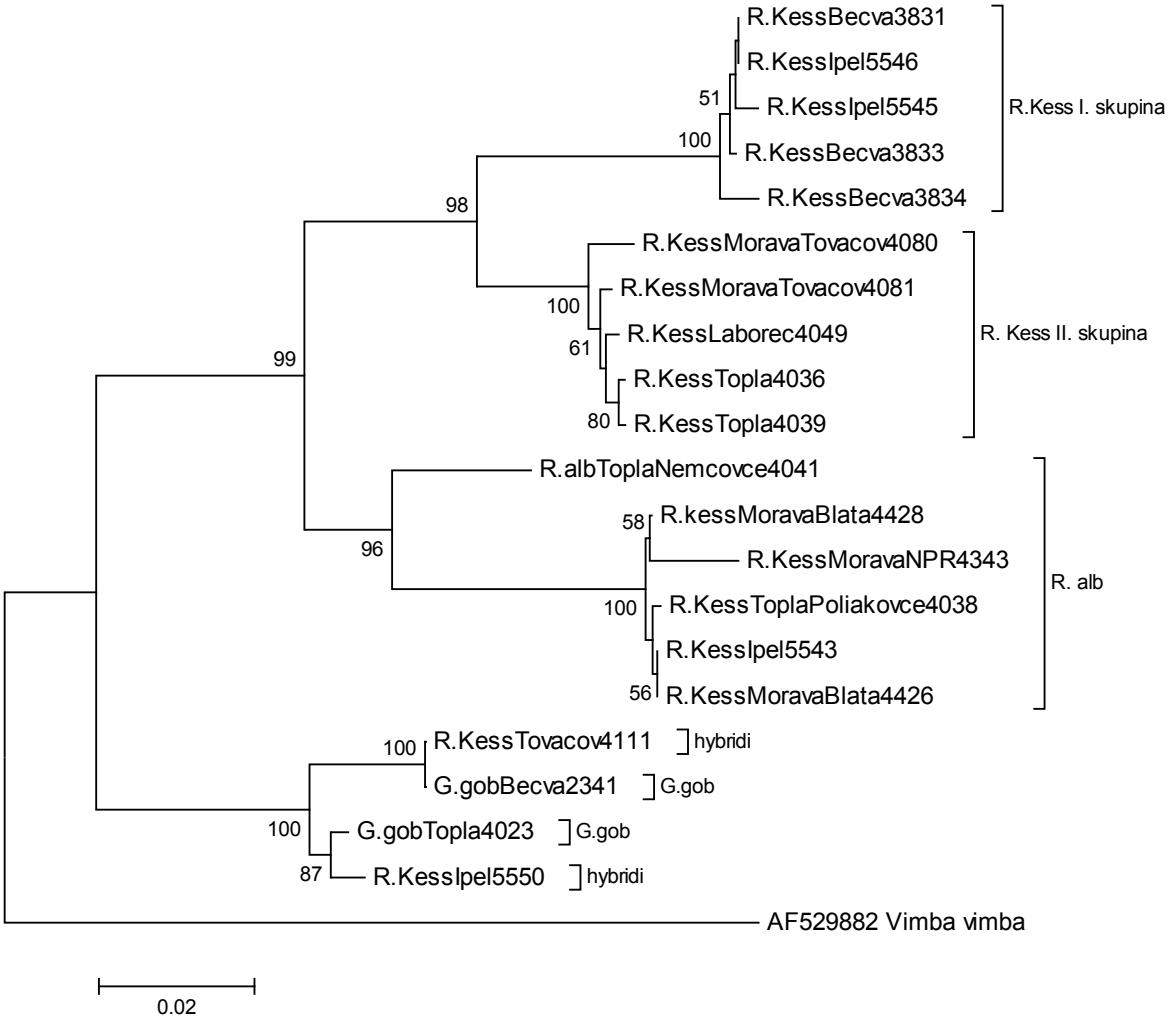


Obr. 2. Výběr variabilních pozic control regionu u hybridů a chybně identifikovaných jedinců *R. kesslerii* na území ČR a SR. Identické = symbol "."

Sekvenční analýza mitochondriálního markeru jednoho jedince z řeky Moravy (ev. č. 4111 z lokality Tovačov) a jednoho jedince z řeky Ipel' (ev. č. 5550 z lokality st. hranice) nepřímo poukázala na možnost, že se jedná o hybridy mezi druhy *R. kesslerii* a *G. gobio*. Testovaná část mitochondriálního genomu přiřazovala tyto jedince k druhu *G. gobio*, naproti tomu morfometrické charakteristiky naznačovaly příslušnost k druhu *R. kesslerii*. Proto jsme přistoupili k otestování druhého genomu – jaderného, jež byl reprezentován úsekem genu S7 r-proteinu o velikosti 372 bp a aplikaci analýzy alozymů. Sekvenční alignment prokázal příslušnost jedince z řeky Ipel' k druhu *R. kesslerii* (S7 alignment neuveden) podobně i alozymové analýzy prokázaly, že jedinec z řeky Moravy je mezidruhový hybrid. Aplikace dalších analýz potvrdila předpoklad, že se jedná opravdu o hybridy druhů *G. gobio* (maternální partner) a *G. kesslerii.* Na možný výskyt hybridů mezi druhy G. gobio x G. kesslerii poukázali v minulosti již Weisz, Kux (1962) a to ze slovenského toku Ondava, z Blhu - přítok Slané Kux, Weisz (1964) a z Torysy (lokalita Ploské) Kux (1964).

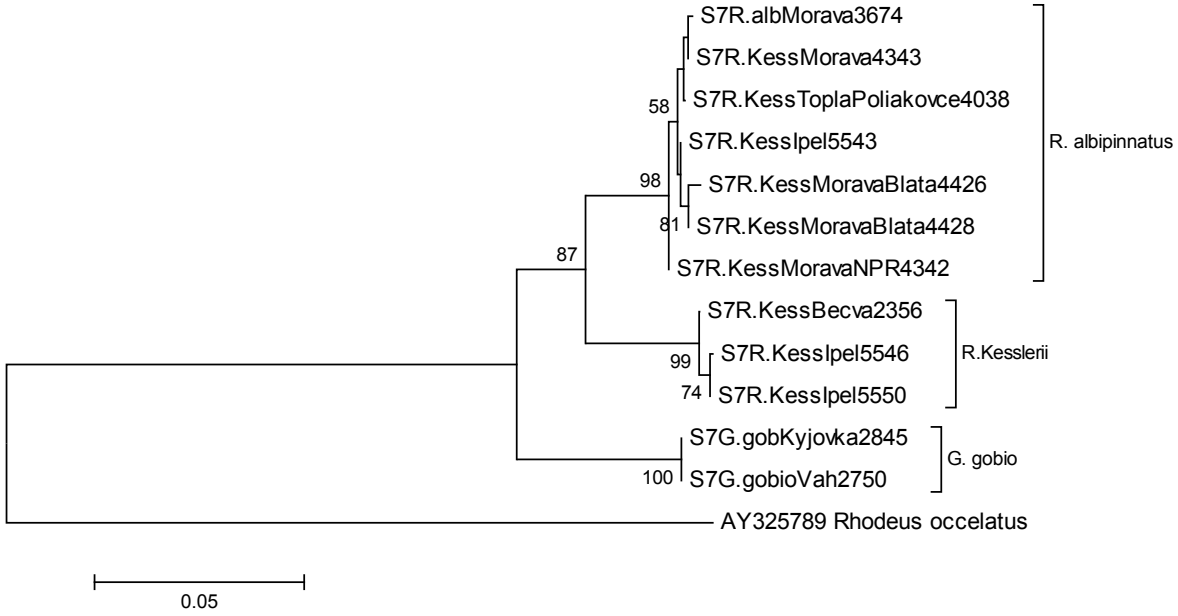
Rozlišení druhů *R. kesslerii* a *R. albipinnatus* na základě morfometrických znaků, zejména v případě společného výskytu je v některých případech obtížné. Na možnou záměnu obou druhů mezi sebou poukázali např. Kux, Weisz (1964). Využili jsme genetické analýzy výše zmiňovaných markerů k druhové identifikaci sporných jedinců, kteří mohli být hodnoceni jako *R. kesslerii*. Jednalo se o sedm jedinců z řek Moravy (Blata, ev. č. 4426-4428; NPR, 4342 a 4343), Ipel' (ev.č. 5543) a Topl'á (ev. č. 4038). Oba markery jednoznačně potvrdily příslušnost zmiňovaných jedinců k druhu *R. albipinnatus* (Obr. 2, 3 a 4).

Na podkladě sekvenčních dat control regionu a pomocí metod shlukové analýzy NJ a UPGMA (neuvedeno) byly sestrojen fylogenetický strom., viz. Obr. 3.



Obr. 3. Neighbour-joining bootstrap konsensuální strom analyzovaný na základě sekvence control regionu (610 bp). Čísla v uzlech stromu reprezentují procenta z 1000 bootstrap pseudoreplikací; pouze hodnoty nad 50% jsou zobrazeny.

Obě dvě distanční metody názorně ukazují existenci dvou skupin *R. kesslerii* a v kombinaci s fylogenetickým stromem sestrojeným pomocí jaderného markeru S7 i existenci obou hybridů a chybnou identifikaci výše zmiňovaných jedinců z řek Moravy, Ipel' a Topl'a, viz Obr. 3 a 4.



Obr. 4. Neighbour-joining bootstrap konsensuální strom analyzovaný na základě sekvence genu S7 r-proteinu (372 bp). Čísla v uzlech stromu reprezentují bootstrap podporu při 1000 opakování; pouze hodnoty nad 50% jsou zobrazeny.

Sekvenční analýzy vzorků z pěti populací hrouzka Kesslerova z moravských a slovenských řek poukázaly na existenci dvou skupin, jejichž nepodobnost je 5,6 - 6,0%. Naše výsledky genetické charakteristiky *R. kesslerii* zpochybnily původní předpoklad, že populace v Moravě vznikla teprve před několika lety na základě jedinců splavených z populace, která je známá z řeky Bečvy (Lusk et al. 2005). Bečva je přítok Moravy, který ústí do středu části Moravy, kde se vyskytuje tamní populace *R. kesslerii*. Bude proto nutné provést další analýzy většího počtu jedinců, které by potvrdily, že se jedná o rozdílné populace.

Analýza 30 jedinců odhalila dva hybridní jedince, kteří mitochondriálním genomem náleželi k druhu *G. gobio* a jaderným k druhu *R. kesslerii*. Jednalo se o jedince z řeky Morava a Ipel', tedy lokality se společným výskytem obou druhů.

Summary

Sand gudgeon (*Romanogobio kesslerii*) is among the critically endangered fish species which enjoy protection by the national legislatives and whose localities have been proclaimed localities of European significance within the NATURA 2000. In this paper, we present the first data on the genetic diversity of five populations of *R. kesslerii* from the Moravian and Slovakian rivers. The 30 specimens under study were subjects of sequential analysis of a selected mtDNA section, i.e., a control region and a part of the first intron of the nuclear gene

S7. The analysis revealed the presence of 9 haplotypes. The greatest genetic variability was found in a population sample taken from the Bečva River, containing three haplotypes. Population samples taken from the Morava, Topľa, and Ipeľ rivers showed two different haplotypes, and a single haplotype was found in all specimens taken from the Laborec River. In the above rivers, the intraspecific diversity did not exceed 0.7%. Interpopulation diversity inside the first group (the Bečva and Ipeľ r.) was 0.4%, that inside the second group (the Topľa, Laborec, and Morava r.) was 0.5 - 1.0%. Analyses of the mtDNA in the control region (610 bp) divided the samples of Sand gudgeon into two different groups. The first one comprised samples from the Bečva and Ipeľ rivers, the other one samples from the Topľa, Laborec, and Morava rivers. The two groups differed in 6.2%. Combinations of sequential analyse methods applied to mtDNA and nDNA sections, the same as alozyme analyses, confirmed the occurrence of hybrids of *G. gobio* and *R. kesslerii* in the Morava and Ipeľ rivers. In some cases, it is problematical to distinguish between *R. kesslerii* and *R. albipinnatus* on the basis of their morphometric characters. Therefore, identification molecular biological markers have been worked out that can unequivocally separate the two species mentioned.

P o ď ě k o v á n í

Studie byla zpracována v rámci řešení projektu r.č. VaV- MS/6/3/05 finančně podporovaného Ministerstvem životního prostředí ČR.

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Rybí osídlení a genetická diverzita populace hrouzka obecného z Divoké Orlice u Kostelce nad Orlicí

The fish community in the Divoká Orlice River near Kostelec nad Orlicí, and the genetic diversity of the local common gudgeon population

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A b s t r a c t: The fish community was investigated in two localities (r. km 46.7 and 49.1) on the Divoká Orlice River near Kostelec nad Orlicí. As regards species composition, this community bears the character of a mixed salmonid and cyprinid (barbel) type. Besides the species characteristic of salmonid waters (brown trout *Salmo trutta* m. *fario*, grayling *Thymalus thymallus*, minnow *Phoxinus phoxinus*, sculpin *Cottus gobio*) there are species typical of a barbel community (barbel *Barbus barbus*, chub *Leuciscus cephalus*, dace *L. leuciscus*, gudgeon *Gobio gobio*). The occurrence of *Lampetra planeri* has also been recorded. The abundance attained 4,294 and 6,612 exx..; the weight, 363.8 and 509.7 kg respectively, converted to 1 hectare of water surface. For a list of species and data on their respective numbers and biomass, see Tabs. 1 and 2.

The genetic diversity of the gudgeon *(Gobio gobio)* population was examined by means of allozyme and DNA analysis (the RAPD method and mitochondrial marker sequencing). The allozyme analysis has shown that the diversity of the population under study is average, compared to other populations examined in other streams (computed heterozygosity H = 0.106, expected heterozygosity H = 0.095, mean number of alleles per locus 1.6, polymorphous loci 29.4). Using the RAPD method, two identification markers have been found for the fish in the population examined. The genetic analysis of mtDNA provided a unique haplotype serving to differentiate the gudgeon population examined from those in other streams in the Czech Republic and Slovakia.

Úvod

Nezbytnou součástí aktivního přístupu k ochraně biologické rozmanitosti je i její poznání v maximálním rozsahu. Jedná se o poznávací proces, který není krátkodobí. U rybího osídlení vodních toků je potřebná znalost druhové pestrosti a to z hlediska odpovídajícího typu rybího společenstva. Tento aspekt biodiverzity je v posledních letech v rámci Společenství Orlice postupně doplňován v rámci ichtyologických výzkumů (Lohniský, Lusk 1998, Lusk a kol. 1997, Lusk a kol. 1998a, Lusk a kol. 2000) při nichž byly hodnoceny i jiné aspekty jako např. vliv extrémních povodní v letech 1997 a 1998 (Lusk a kol. 1997, Lusk aet al. 1998, 1999). Vyhodnoceno bylo i zatížení ryb v horní části toku Tiché Orlice (Korunová a kol. 1997, Lusková a kol. 1997). Druhá stránka biodiverzity, tj. oblast tzv. genetické diverzity jednotlivých druhů ryb prozatím patří k těm málo poznaným. V současnosti je zvýšená pozornost zaměřena na výzkum genetické diverzity u zvláště chráněných druhů. V naší studii jsme se pokusili o jakési modelové spojení obou stránek biodiverzity s tím, že vedle druhové pestrosti rybího osídlení toku Divoké Orlice u Kostelce nad Orlicí jsme se zabývali i genetickou charakteristikou populace hrouzka obecného.

Materiál a metodika

Pomocí elektrolovu byl proveden průzkum rybího osídlení dvou úseků Divoké Orlice v ř.km 46,7 a 49,1. První úsek se nachází asi 1 km pod Kostelce n. O. pod tamní ČOV. Tok je ve zkoumaném úseku (160 m, při šířce 17 m) neregulovaný, levý břeh zpevněný kamenným záhozem. Obsah kyslíku (106 % nasycení) stejně jako vodivost vody (278 µS.cm), stejně jako skladba rybího společenstva svědčily o vysoké účinnosti ČOV. Druhý úsek (ř.km 49,1, délka 86 m, šířka 11 m)) se nachází v příměstské části, koryto regulované, v pravém břehu v narušeném opevnění úkryty pro ryby. Nasycenost kyslíkem i vodivost shodná s předchozím úsekem. Zkoumané úseky byly proloveny dvakrát a odhad početnosti a biomasy proveden dle postupu Seber, LeCren (1967).

U vzorku 50 jedinců hrouzka obecného byla uplatněna metoda biochemické genetiky - alozymová analýza Byla zjišťována variabilita alozymových vzorů v 11 aktivních enzym/proteinových systémech zahrnujících 17 lokusů. Detailní popis metody je ve studii Lusková et al. (1997). U 10 jedinců byly souběžně provedeny molekulárně-biologické analýzy, za použití metody RAPD (random amplified polymorphic DNA) a metoda přímého sekvencování mitochondriálního markeru – control regionu. Podrobný popis aplikace metody RAPD je uveden ve studii Mendel et al. (2005).

Výsledky a diskuse

Rybí osídlení

Rybí osídlení Divoké Orlice v ř.km 46,7 mělo velmi pestrou druhovou skladbu, celkem zjištěno 18 druhů patřících k 9 čeledím (Tab. 1). Rybí společenstvo je smíšeného charakteru mezi typem salmonidním a cyprinidním (parmovým). Vedle druhů charakteristických pro salmonidní vody (pstruh obecný, lipan podhorní, střevle potoční, vranka obecná) jsou zastoupeny druhy charakteristické pro parmové společenstvo (parma obecná, jelec tloušť, jelec proudník, hrouzek obecný). Velmi početná je plotice obecná, výskyt ostatních druhů byl málo početný až ojedinělý, blíže viz Tab. 1. V úseku v jemným náplavech se vyskytovaly larvy mihule potoční v početnosti až 12 ks.m^2. Index diverzity činil 3,297 a ekvitabilita 0,791.

V druhém úseku (ř.km 49,1) bylo zjištěno celkem 14 druhů ryb patřících k 7 čeledím (Tab. 2). Jedná se opět o rybí společenstvo smíšeného typu, kde se mísí lososovité ryby s rybami parmového společenstva. Zajímavý v tomto úseku je početný výskyt mníka jednovousého (582 ks a 77,17 kg na ha), což se projevilo téměř úplnou absencí plůdku (důsledek predace tohoto druhu). Index diverzity činil 2,981 a ekvitabilita měla hodnotu 0,783.

Genetická diverzita hrouzka obecného

Hrouzek obecný patří k obecně rozšířeným druhům v části „spojené Orlice“ a vyskytuje se i v navazujících částech obou zdrojnic tj. Tiché Orlice a Divoké Orlice.

Průměrný počet alel na locus byl u zkoumané populace z Divoké Orlice 1,6, procento polymorfních locusů 29,4. Vypočítaná heterozygosita (H_o) byla 0,106 a očekávaná heterozygosita (H-W H_e) 0,095. Tyto hodnoty ve srovnání s hodnotami u řady dalších populací hrouzka obecného v České republice i na Slovensku lze hodnotit jako průměrné (Šlechtová et al. 2005).

Pro zkoumanou populaci byly pomocí metody RAPD zjištěny dva identifikační markery (A04 2071 a A09 1234), které se vyskytují u všech jedinců. Ty se nevyskytují u dalších populací, které byly námi zkoumané (Mendel et al. 2005).

Tab. 1. Rybí osídlení Divoké Orlice v ř.km 46,7 (početnost (N(ks)) a hmotnost (W(kg) přepočtena na vodní plochu 1 ha – Fish community inhabiting the Divoká Orlice River in r.km 46.7. Abundance (N, exx.) and weight (W, kg) transformed to l hectare of water surface.

Druh - Species	**N (ks)**	**N(ks) - %**	**W (kg)**	**W (kg) - %**
Úhoř říční *Anguilla anguilla*	15	0,58	3,39	1,59
Plotice obecná *Rutilus rutilus*	504	19,97	52,19	24,38
Jelec proudník *Leuciscus leuciscus*	334	13,21	23,01	10,75
Jelec tloušť *Leuciscus cephalus*	337	13,34	61,59	28,77
Střevle potoční *Phoxinus phoxinus*	288	11,41	1,80	0,84
Lín obecný *Tinca tinca*	15	0,58	2,65	1,24
Hrouzek obecný *Gobio gobio*	301	11,90	5,79	2,71
Parma obecná *Barbus barbus*	33	1,31	4,15	1,94
Podoustev říční *Vimba vimba*	15	0,508	0,44	0,21
Mřenka mramorovaná *Barbatula barbatula*	192	7,61	1,47	0,69
Štika obecná *Esox lucius*	4	0,15	0,66	0,31
Pstruh obecný *Salmo trutta* m. *fario*	76	2,99	17,26	8,06
Pstruh duhový *Oncorhynchus mykiss*	17	0,65	2,78	1,30
Siven americký *Salvelinus fontinalis*	4	0,15	1,29	0,60
Lipan podhorní *Thymallus thymallus*	108	4,29	21,05	9,84
Mník jednovousý *Lota lota*	4	0,15	0,96	0,45
Vranka obecná *Cottus gobio*	251	9,93	3,04	1,42
Okoun říční *Perca fluviatilis*	31	1,21	10,52	4,92
C e l k e m Total	4294	100,00	363,87	100,00

Tab. 2. Rybí osídlení Divoké Orlice v ř.km 49,1 (početnost (N(ks)) a hmotnost (W(kg) přepočtena na vodní plochu 1 ha – Fish community inhabiting the Divoká Orlice River in r.km. 49.1. Abundance (N, exx.) and weight (W, kg) transformed to 1 hectare of water surface.

Druh – Species	**N (ks)**	**N(ks) - %**	**W (kg)**	**W (kg) - %**
Úhoř říční *Anguilla anguilla*	382	5,77	38,85	7,62
Plotice obecná *Rutilus rutilus*	1086	16,42	43,60	8,55
Jelec proudník *Leuciscus leuciscus*	2328	35,21	78,86	15,47
Jelec tloušť *Leuciscus cephalus*	746	11,28	64,83	12,72
Lín obecný *Tinca tinca*	42	0,64	8,48	1,66
Hrouzek obecný *Gobio gobio*	207	3,13	4,80	0,94
Parma obecná *Barbus barbus*	76	1,15	8,93	1,75
Pstruh obecný *Salmo trutta* m. *fario*	259	3,92	36,18	7,10
Pstruh duhový *Oncorhynchus mykiss*	95	1,44	23,82	4,67
Siven americký *Salvelinus fontinalis*	88	1,33	24,09	4,73
Lipan podhorní *Thymallus thymallus*	465	7,03	86,06	16,88
Mník jednovousý *Lota lota*	582	8,80	77,17	15,14
Vranka obecná *Cottus gobio*	129	1,96	2,17	0,43
Okoun říční *Perca fluviatilis*	127	1,92	11,91	2,34
C e l k e m Total	6612	100,00	509,75	100,00

Statistické vyhodnocení intrapopulační variability jsme provedli pomocí programu GeneTools, kde jsme použili koeficient similarity podle Nei & Li (1979). U populace z Divoké Orlice byla zjištěna vnitropopulační diverzita v rozmezí 20 - 37%, průměrná hodnota činila 31%. Obdobně jak populace z jiných řek (Mendel et al. 2005) ani populace z Divoké Orlice nevykazovala velké procento identických „molekulárních fenotypů“, které by byly typické pro vysoce uniformní populaci.

Genetická analýza 726 bp mt úseku u pěti jedinců poskytla jedinečný haplotyp, kterým se populace z Divoké Orlice odlišovala od ostatních analyzovaných populací. Analýza mitochondriálního markeru dále potvrdila (podobně jako RAPD analýza) geografickou příslušnost populace z Divoké Orlice k populacím ze Severního moře a neučinila výrazné vyčlenění vůči populacím z Baltu. Naproti tomu potvrdila jednoznačnou odlišnost od populací hrouzka obecného z úmoří Černého moře.

P o d ě k o v á n í
Studie byla realizována v rámci výzkumných aktivit podporovaných Akademií věd ČR v regionu „Orlicko“.

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Molecular phylogeny of the genus *Romanogobio* (Cyprinidae, Pisces) and its contribution to taxonomy

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Abstract

Phylogenetic relations of 13 European nominal species and subspecies of the genus *Romanogobio* were investigated on the basis of the mitochondrial and nuclear genome sequencing. Molecular analyses confirm the separate generic status of *Gobio* and *Romanogobio* defining *Rheogobio* as a junior synonym of the latter. Valid species status was confirmed for nine studied taxa, and the taxonomic isolation were shown for Danubian and non-Danubian populations of the monophyletic group *´uranoscopus`*. At the same time molecular data suppose *R. tanaiticus* as a younger synonym of *R. albipinnatus* and suggest thorough morphological and genetic re-evaluation of *R. parvus* and elucidation of its relations with *R. albipinnatus* and *R. ciscaucasicus*. The analysis of the nuclear marker S7 revealed gudgeon populations genetically identical to *R. albipinnatus* in Transcaucasia and Turkey that shifted the boundary of its occurrence as far as the region of Fore-Caucasus.

Keywords: Romanogobio; Taxonomy; Control region; Cytochrome *b*; S7 ribosomal protein gene; Intron

1. Introduction

During the last 10-15 years an important shift in ichthyological research has taken place. Expanded research efforts have been focusing on the area of taxonomy, informed by recent insights gained from molecular phylogenetic and phylogeographic studies. This shift is caused particularly by more emphasis being placed upon mapping and conservation of biodiversity and by a radical increase in the application of novel molecular methods. These methods have brought forth new knowledge leading to changes in existing taxonomic categories. The revision of the structure of European fish made by Kottelat (1997) has led to a gradual decline in use of the 'subspecies' category, and has given the green light to both the reclassification of many existing subspecies to the category of a species and also to the establishment of new species. In spite of the fact that the necessity for comprehensive evaluation of the datasets as the basis for a respectable taxonomic study (Kottelat 1998) is being emphasized, to date only restricted sets of data (morphology, osteology, etc.) have normally been used for taxonomic conclusions. Furthermore, the use of different species concepts or, on the contrary, of one's own subjective criterion, has caused the onset of "the Tower of Babel" within European ichthyological taxonomy. This chaos resulted in 1933 different names being proposed for 358 European native freshwater fish species, an average of 5.4 names per species (Kottelat 1998).
This brief description of the fish taxonomy reflects the situation in the taxonomic development of the genus *Gobio* in its original classical scope. A clearly organized, revised structure of the genus *Gobio* with regard to time development of knowledge was given by Berg (1949). Some years later, Bănărescu (1961) described two additional subgenera within the genus *Gobio* (Cuvier, 1816), namely *Rheogobio* (Bănărescu, 1961; type species: *Cyprinus uranoscopus*) and *Romanogobio* (Bănărescu, 1961; type species: *Gobio kesslerii*). The latter was re-evaluated as an independent genus by Naseka (1996). More recently, most authors include gudgeons of the groups '*albipinnatus'* and '*kesslerii'* in genus *Romanogobio*, whereas others still use the original generic name *Gobio* (Bănărescu, 1999a; Naseka et al., 1999b; Koščo et al., 2005; Mustafič et al., 2005). Subgenus *Rheogobio* initially included two species: *Gobio uranoscopus* and *G. ciscaucasicus* Berg, 1932 (Bănărescu, 1992), but Naseka (1996) classified the latter species as a member of genus *Romanogobio*. On rare occasions monotypical *Rheogobio* has been considered to be an independent genus (Nalbant et al., 2004). Normally, the common distribution and identical or very similar ecological/biological characteristics of the individual forms of gudgeons, considerable visual similarity, as well as the existence of hybrids have lead to this complicated situation. In addition not only minimal morphometric differences in the evaluated forms but also different hydrogeographic

distribution were taken into consideration in the past (Berg, 1949; Bănărescu, 1964; and others). Therefore, there are a lot of different perspectives concerning the determination of their taxa (at the generic and subgeneric level).

Recently a number of studies dealing with the taxonomy of gudgeons have emerged, in which morphological, karyologic and genetic data have been utilized to describe new species or regrade existing subspecies to the species level (Doadrio and Madeira, 2004; Naseka and Freyhof, 2004; Vasileva et al., 2004; Freyhof and Naseka, 2005; Naseka et al., 2006). However, some of these studies seem to be prepared without a comprehensive approach. We believe that a certain caution should be exercised when announcing new species. Mapping of fish diversity and its immediate conservation should be closely linked to academic responsibility and precision. Therefore the working term "species-in-waiting" proposed by a group associated with Professor Herbert (Hebert et al., 2004; Hebert and Barrett, 2005) for cases in which new species are announced without a comprehensive approach seems to be a suitable solution which does not inhibit the process of rapid biodiversity mapping and at the same time provides additional time for its comprehensive revision. It is necessary to launch a serious and wide discussion about these problems that would bring more mandatory conclusions necessary both for correct identification of individual species and evaluation of their real conservation status. Taxonomic chaos causes great problems in the area of conservation legislation, which can frustrate or prevent the implementation of appropriate conservation management strategies (Kottelat, 1998; Lusk and Šlechta, 2005).

Gudgeons from genus *Romanogobio* inhabit one of eight defined ecozones, specifically the western region of the Palaearctic zone. This genus includes small cyprinid fishes which do not undertake periodic migrations and do not have any commercial value. However, the involved species are mostly endemic with different degrees of threat. In this study, we are trying to make a broader evaluation of some representatives of the genus *Romanogobio* through utilization of molecular data and with respect to existing descriptions of the species (subspecies).

2. Materials and methods

2.1. *Sample collection*

In the period from 2000 - 2005, 127 specimens were collected from 26 localities (Table 1). These samples cover the main distribution ranges of the investigated species and subspecies of the genus *Romanogobio* (Fig. 1). On the basis of present knowledge of phylogenetic

relationships among fishes within the family Cyprinidae and the subfamily Gobioninae (Briolay et al., 1998; Cunha et al., 2002; Yang et al., 2006) *Rhodeus ocellatus* and *Sarcocheilichthys microoculus* specimens were selected as an outgroup (Table 1). Voucher specimens are deposited in the collections of the Department of Ichthyology of the Institute of Vertebrate Biology (Brno, Czech Republic).

2.2. *DNA extraction, PCR amplification and sequencing*

Total genomic DNA was extracted from a small piece of the pectoral fin by proteinase K digestion followed by phenol-chloroform-isoamylalcohol purification and ethanol precipitation (Sambrook et al., 1989). Sequences of the control region (CR), cytochrome *b* (Cyt *b*) and the first intron of the S7 r-protein (S7) were amplified by a polymerase chain reaction with primers specified in Table 2. PCRs were performed in 50 μl volume containing 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM each primer, 2.5 U *Taq* DNA polymerase (Top-Bio) and approximately 100 – 200 ng genomic DNA. Reactions were performed in a TGRADIENT Thermocycler (Whatman Biometra) under the following conditions – CR: 95 °C for 1 min, followed by 37 cycles of 94 °C for 45 s, annealing at 52.6 °C (the first fragment) and 52.2 – 61.2 °C (the second fragment; gudgeons of the groups '*kesslerii'* – 52.2 °C, '*uranoscopus'* and *Gobio sp*. – 54.8 °C, '*albipinnatus'* – 59.0 and 61.2 °C) for 30 s, and an extension temperature of 72 °C for 45 s , followed by a final extension at 72 °C for 5 min. The other representatives of gudgeons: CR_STIR: 95 °C for 3 min, followed by 34 cycles of 95 °C for 30 s, annealing at 55.0 °C for 30 s, and an extension temperature of 72 °C for 1 min, and a final extension at 72 °C for 5 min. Cyt *b*: 94 °C for 2 min, followed by 4 cycles of 94 °C for 45 s, annealing at 53 °C for 45 s, and an extension temperature of 72 °C for 1.5 min; followed by: 29 cycles of 94 °C for 45 s, annealing at 58 °C for 45 s, and an extension temperature of 72 °C for 1.5 min, and a final extension at 72 °C for 7 min. S7: 95 °C for 1 min, followed by 34 cycles of 94 °C for 45 s, annealing at 52.4 °C for 30 s, and an extension temperature of 72 °C for 25 s, and a final extension at 72 °C for 5 min. The PCR products were visualized by mini-gel electrophoresis using ethidium bromide staining and 1.7% agarose gels. The PCR products were purified by means of precipitation PEG/Mg/NaAc (26% Polyethylene glycol, 6.5 mM $MgCl_2$. $6H_2O$, 0.6 M NaAc.$3H_2O$). Direct sequencing of purified PCR products was performed with BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v. 1.1 (Applied Biosystems) according to manufacturer's instructions and purified by EtOH/EDTA precipitation. The sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Each PCR

amplicons were multiply sequenced from both directions to ensure high quality reads. The DNA sequences were edited and aligned using the Seqman module within Lasergene v 6.0 (DNASTAR Inc.) and also checked manually. The accuracy of the sequence was confirmed by comparison with the NCBI database.

2.3. *Phylogenetic analyses*

Haplotype (Hd) and nucleotide diversity (π) (Nei, 1987) was computed using DNASP 4.0 (Rozas et al., 2003).

The web-based ModelTest 3.8 program was used to ascertain the best-fit model of nucleotide substitution for separate nuclear and mitochondrial regions (Posada, 2006). Phylogenetic relationships among the three gene sequences were examined using the neighbor-joining (NJ) algorithm, the criteria of optimality: maximum parsimony (MP) and maximum-likelihood (ML), and also using Bayesian inference (BI). The sequences were imported into PAUP* 4.0B.10 (Swofford, 2002) and MrBayes 3.1.2 (Ronquist et al., 2005) for phylogenetic analysis. Congruence among tree topologies generated for combined data (CR, Cyt *b* and S7 sequences) was tested with the incongruence length difference test (ILD) as implemented in the partition homogeneity test in PAUP* (Farris et al., 1994; Mickevich and Farris, 1981). For NJ analysis, the DNA distances were calculated. Non-parametric bootstrap analyses with 1000 pseudo-replicates were performed to obtain estimates of support for each node of the NJ trees. For MP tree construction unweighted parsimony analysis using a branch-and-bound search was used. The confidence levels in the resulting relationship were assessed using the bootstrap procedure with 1000 replications. ML search was performed under the best-fit model with the branch-and-bound algorithm on 100 bootstrap replicates. Bayesian analysis was performed using MrBayes 3.1.2. Starting from a random tree, four Markov chains were run for 1 x 10^6 generations (for Cyt *b* 3 x 10^6) with sampling frequency of 100. The best-fit models were specified. The combined data set was treated as three partitions with different models accounting for their heterogeneity. We utilized the "unlink" command in MrBayes 3.1.2. to unlink the following parameters: "unlink shape=(all) statefreq=(all) revmat=(all)". The application Tracer 1.2 (Rambaut and Drummond, 2003) was used to view the output of the *sump* file generated by MrBayes. The trees generated prior to reaching stationarity were discarded as burn-in. We then took the resulting 50% majority rule consensus tree.

A haplotype network was constructed to estimate the genealogical intraspecific relationships employing the statistical parsimony (Templeton et al., 1992) implemented in the TCS 1.21

program (Clement et al., 2000). A 95% connection limit was calculated, meaning that the haplotypes were disconnected when more than eight mutational steps divided them.

3. Results

3.1. *Sequence characteristics*

Sequence data was deposited in the GenBank database under accession numbers (CR: EF427385-EF427397, EF427425-EF427446; Cyt *b*: EF427398-EF427407; S7: EF427408-EF427424; Table 1). Up to 1703 bp of analyzable sequence data were obtained from the nuclear and mitochondrial genome fragments for each specimen. Fragments of the control region (709-713 bp and 357 bp), cytochrome *b* (622 bp) and the first intron of the S7 r-protein (338-368 bp) were analyzed both separately and in combination. As some sequences from the same taxa were identical, those taxa were reduced to one representative per taxon in all subsequent analyses. Nucleotide base composition showed a low level of G in Cyt *b* protein-coding sequence (15.6% across all sites/all taxa), which is characteristic for the mitochondrial genome (Johns and Avise, 1998). High values of AT pairs (A=31.4% and T=31.5%) were detected in the case of the CR sequence. A similar composition had been noted for Cypriniformes (Liu et al., 2002; 2003). In case of the sequence of the first intron of the S7 gene, low values of GC pairs were found (16.8% and 19.1%, respectively) which is typical for noncoding regions of the genome. Measures of molecular diversity for the main CR lineages together with statistical tests of neutrality (Tajima's D test; Tajima, 1989) are shown in Table 3.

3.2. *Phylogenetic analyses*

For both NJ and Bayesian analyses the best-fit model under Akaike information criterion (AIC; Akaike, 1974) was determined using the software ModelTest 3.8, see Table 4: for control region the HKY+Γ model; for cytochrome b the TrN+Γ model; for S7 r-protein the K81uf model. The genetic distances within and among *Romanogobio* lineages are shown in Table 5. The summary statistics of MP analyses for the separate and combined data sets for each gene are shown in Table 4. For ML analyses, the likelihood settings of the best-fit model for CR based on the hierarchical likelihood ratio tests (hLRTs) were as follows: base frequencies (A = 0.3136, C = 0.2134, G = 0.1582 and T = 0.3148); ti/tv ratio = 1.7032; and the shape parameter of the gamma distribution 0.4488. Likelihood settings of the best-fit

model for Cyt *b*: base frequencies (A = 0.2870, C = 0.2851, G = 0.1559 and T = 0.2720); ti/tv ratio = 8.5243; and the shape parameter of the gamma distribution 0.1649. Likelihood settings of the best-fit model for S7: base frequencies (A = 0.3621, C = 0.1906, G = 0.1679 and T = 0.2794); ti/tv ratio = 0.8326.

To examine the possible differences among the three markers, an incongruity length difference (ILD) test was employed. The resultant P value, computed from 1000 replicates, was greater than 0.01 (P = 0.65), indicating that combining the data improved, or at least did not reduce, phylogenetic accuracy.

Our phylogenetic analyses have shown that the analyzed species or subspecies of the genus *Romanogobio* have clustered into seven major monophyletic lineages with strong bootstrap supports (BS) and significant posterior probabilities (PP; Figs. 3 - 6). Within the genus *Romanogobio* the individual lineages clustered into two groups, A and B, with strong statistical support. The A group was formed by three lineages (I, II and VII), lineages II and VII forming a common cluster with a strong support. The compactness of the A group has a weak support, lineage I seems to be rather separated from lineages II and VII and together they represent basal taxa within this genus. The B group consisted of lineages III – VI, during further division lineages III and VI separated with a strong support.

3.3. Haplotype richness and haplotype network

A total of 32 haplotypes of the control region, representative of 74 sequences were used for the construction of the haplotype network. A detailed list of all studied taxa, their haplotype assignments and sampling localities and the GenBank accession numbers are given in Table 1. The schematic diagram of the statistical parsimony network (Fig. 2) shows a complex pattern of relationships within the genus *Romanogobio*. Sequence haplotypes of the control region included 8 species or subspecies of the genus *Romanogobio* mentioned in literature (according to Naseka and Freyhof (2004) this genus includes 15 valid species in Europe and West Asia). The haplotype network revealed 6 disconnected groups. The first group includes haplotypes 1 and 2 founded in *R. pentatrichus* from the Kuban' River and it corresponds to lineage I in the phylogenetic tree (the tree based on combined data; Fig. 3). The second group includes only haplotype 3 revealed in gudgeon from the Dniester River, which was identified as *R. kesslerii kesslerii* based on literature data and the sequence analysis of markers of Cyt *b* and CR (Figs. 3 - 5; lineage VII). The third group incorporates two subgroups which belong - with minimally one subgroup according to Cyt *b* - to *R. kesslerii banaticus*. The specimens with haplotypes 4 – 8 come from the rivers Bečva and Ipel' (the Danube River basin) and the

specimens with haplotypes 9 – 16 come from the rivers Topl'á, Laborec (the Tisza River basin) and Morava (the Danube River basin). Five mutational steps separated both subgroups. This haplotype group forms lineage II into the phylogenetic tree (Figs. 3; 5) and combines with specimens from the middle Nera River in Romania (the middle Danube River basin) based on Cyt *b* analysis. The fourth haplotype group combines haplotypes 17 – 20 and comes from populations from the Hornád R., the Laborec R. and the Ulička R. (the Tisza River basin). On the basis of literature (Bănărescu, 1953; 1962; 1964; 1992; Bănărescu and Nalbant, 1973) and the geographic affiliation it was classified as subspecies *Gobio* (*Rheogobio) uranoscopus frici*. This group forms a common cluster with the gudgeons from the middle Nera River in Romania (the middle Danube River basin) in the phylogram constructed on the basis of Cyt *b* data (Fig. 4). It forms lineage III in the phylogram (Figs. 3; 5). The fifth haplotype group is represented by a single haplotype, 21, and combines two taxa from different regions: *Romanogobio albipinnatus albipinnatus* from the Volga River and *R. parvus* from the Kuban River. It forms lineage VI in the phylogenetic tree (Fig. 5). The sixth and final group consisted of two subgroups which have been classified in literature as subspecies *R. albipinnatus belingi* and *R. a. vladykovi*. The first subgroup includes haplotypes 28 – 32 found in the rivers Dnieper, Dniester and Elbe. The second subgroup consists of specimens with haplotypes 22 – 27 from the rivers Dyje, Ipel' and Morava (the Danube River basin), as well as from the channel Revištia, the Topl'á R. and the Uh R. (the Tisza River basin). Three mutational steps separated both subgroups. They form lineages IV and V in the phylogenetic tree (Fig. 5). The third (lineage II) and sixth (lineages IV and V) groups incorporate two subgroups with similar haplotype profiles: each subgroup possesses the main, widely distributed haplotype occurring in specimens from different populations and subordinate haplotypes splitting off the main one. These subordinate haplotypes are population specific and mutually diverging. The numbers of haplotypes forming the individual lineages are summarized in Table 3.

4. Discussion

4.1. Phylogeny of Romanogobio, overview

Neither the traditional classification of gudgeons into one genus *Gobio*, nor the existence of two subgenera *Romanogobio* and *Rheogobio* detached from this genus (Bănărescu, 1961), was confirmed by our data. All our phylogenetic results strongly indicated the existence of two sister-groups *Gobio* and *Romanogobio*, which is consistent with the opinions of Naseka

(1996), Zardoya and Doadrio (1999) (with certain reservations - see below) and Yang et al. (2006). All our phylogenetic analyses based on testing of both the mitochondrial and nuclear genomes have shown that the analyzed species or subspecies of the genus *Romanogobio* have clustered into seven major monophyletic lineages with strong supports.

The taxonomy of gudgeons is very confusing at present. Numerous subspecies and species, and even 'nations' and 'forms' have been described over a long period of ichthyological study. Most of these were identified based on several morphological characteristics, which is why the validity of some taxa remains questionable. Hereafter, we deal with the individual representatives of the genus *Romanogobio* independently and to avoid confusion we adhere to the original names of taxa.

R. pentatrichus (Naseka et Bogutskaya, 1998)

This species described by Naseka and Bogutskaya in 1998 and specified as the Kuban long-barbelled gudgeon is probably endemic to the Kuban River basin. Its morphological and osteological characters are described in Naseka et al. (2002). On the basis of molecular analyses of both the mitochondrial and nuclear markers it would appear to be one of two basal lineages within the genus *Romanogobio*. In our study it is described as lineage I (Fig. 3), and shows sequence patterns significantly differing (see the haplotype network, Fig. 2) from the other species. Divergence of sequences in both markers, S7 and control region, ranged between 5.81 – 6.46%, and 7.52 – 13.12%, respectively (Table 5). This species appears to be very distinct within the genus *Romanogobio* (Naseka et al., 2005).

R. kesslerii (Dybowski, 1862) *and its subspecies*

In this species, six subspecies are recognised, which were originally proposed for *R. kesslerii* populations from various geographic regions (Bănărescu, 1999b). The population in the Dniester River in Ukraine is classified as *R. k. kesslerii* (Dybowski, 1862); the population in the upper Tisza River system of the Carpathian Ukraine as *R. k. carpathorossicus* (Vladykov, 1925); the population in southern tributaries of the lower Danube in northern Bulgaria (the Osam R. as a restricted type locality, Bănărescu and Nalbant, 1973) as *R. k. similis*; the population from the Timis R., near Timisoara, in the Banat, Romania, as *R. k. banaticus* (Bănărescu, 1953); the population from the Danube delta near Sulina as *R. k. antipai* (Bănărescu, 1953); the population from the Vardar R. in Macedonia as *R. k. banarescui* (Dimovski et Grupče, 1974). Except for *R. k. kesslerii* and *R. k. banarescui* the remaining four subspecies were described from the Danube R. system. In our study we examined phylogenetic relationships of gudgeons related to three of the six above mentioned subspecies.

Strong bootstrap support, as well as significant posterior probabilities (1.00; Figs. 3 - 5) clearly confirms the taxonomic independence of two taxa, *R. k. kesslerii* and *R. k. banaticus*. Our results demonstrate that a sequence (GenBank: AF090751) regarded by Zardoya and Doadrio (1999) and Cunha et al. (2002) to be from *G. banarescui* (= *R. kesslerii banarescui*), is wrongly identified by these authors. It is apparently a sequence from *R. uranoscopus elimeius* (Kattoulas, Stephanidis et Economidis, 1973). This subspecies actually occurs in the Aliakmon River basin (the same catching locality). Further information about this subspecies is represented in the paragraph "*R. uranoscopus and its subspecies"*. The investigated populations of *R. kesslerii* s. lato from the Dniester R. and the Danube River system (the Bečva R., Ipel' R., Laborec R., Morava R. and Topl'á R.) revealed their own significantly different control region patterns (lineage VII and lineage II, Fig. 2). Their position within the phylogenetic tree constructed on the basis of mtDNA showed similar characteristics as in case of *R. pentatrichus*, meaning that they represent the second and the third basal lineages (Figs. 3 - 5). *R. k. kesslerii* and *R. k. banaticus* significantly differ in the control region (357 bp) and Cyt *b* (622bp) characters by 4.68% and 8.58%, respectively. Their monophyly was confirmed on the basis of mitochondrial analyses. Additionally, the populations of *R. k. banaticus* clustered into two subgroups with main haplotypes No. 5 and 9 (Fig. 2). One of these subgroups included the populations from the Tisza R. basin and the Morava R. (the Danube R. basin) and the other from populations in the rivers Bečva and Ipel' (the Danube R. basin). The first subgroup could possibly be related to *R. k. carpathorossicus*, whose validity was questioned by Bănărescu (1999a), because morphometric analyses showed small differences that did not confirm the subspecies status. Our molecular analyses identified certain differences in one marker (CR), and at present, additional analyses of the remaining two markers are being performed to clarify the validity of this subspecies.

R. uranoscopus (=*Gobio (Rheogobio) uranoscopus*; Agassiz, 1828) *and its subspecies*

Three valid subspecies of *R. uranoscopus* are recognized in literature (Bănărescu et al., 1999a). The population from the Isar R. in Bavaria, the western Danube River basin, is classified as *R. u. uranoscopus*; the populations from central and eastern parts of the Danube R. basin (including the tributaries of the Tisza R., the lower Danube R.) in southern and eastern Romania and Bulgaria, and the rivers of the Banat, are related to *R. u. frici* (Vladykov, 1925); and the populations from the rivers south of the Danube (including the Vardar R., the Aliakmon R., etc.) belong to *R. u. elimeius* (Kattoulas, Stephanidis et Economidis, 1973). The fourth nominal subspecies *R. u. stankoi* (Dimovski and Grupce, 1976) from the Vardar River is concluded to be invalid based on insignificant morphometric differences (Bănărescu et al.,

1999a). We then studied two final valid subspecies. In the Bayesian tree built on the basis of molecular analyses of the three tested markers, lineage III, represented by haplotypes from *R. u. frici*, clearly joined the group of species of the genus *Romanogobio* with a significant support (Figs. 3-6). Thus, our findings clearly pointed out the erroneous views (Bănărescu, 1961; Naseka, 1996), which placed this species into the subgenera *Rheogobio* or *Gobio*, respectively. Moreover, CR haplotype network of the populations from the Tisza R. basin, as well as phylogenetic trees built on the basis of Cyt *b* and S7 markers confirmed high divergence of *R. uranoscopus*, represented by subspecies *R. u. frici* (Figs. 2-6). In addition, the analysis of Cyt *b*, in comparison with the database specimens from the Romanian rivers Nera in the Banat (AY952331) and Valsân R., a tributary of the upper Arges, (AY426593), demonstrated a significant difference of the individual from the Valsân R. and similarity of the individual from the Nera River with a good support (Fig. 4). The sequence difference were 2.8% and 1.7%, respectively. The two specimens from the database were differing between each other by 1.2%. Further analysis will be necessary to determine whether there could possibly be a greater haplotype variability of *R. u. frici* or the existence of another subspecies (nominotypical subspecies or cryptic subspecies). The stated erroneous identification of "*G. banarescui"*) by Zardoya and Doadrio (1999) provided us with the Cyt *b* sequence for *R. uranoscopus elimeius* (AF090751), which was included in the common cluster. The Bayesian tree indicated a strong support and PP = 1.00 for this statement (Fig. 4). The above mentioned Aliakmon R. belongs to type area of this subspecies, thus our results confirm its validity.

R. albipinnatus (Lukasch, 1933) *and its subspecies*

More or less, pronounced morphological differences between populations of the white-finned gudgeon led to the identification of four its subspecies (Bănărescu, 1946; 1961; Bănărescu and Nalbant, 1973; Naseka, 2001). The populations in the Volga River system are identified as *R. a. albipinnatus* (Lukasch, 1933); the populations from the Danube River system as *R. a. vladykovi* (Fang, 1943); the population from the Dnieper and Dniester River systems as *R. a. belingi* (Slastenenko, 1934); the populations from the Don R. drainage area as *R. a. tanaiticus* (Naseka, 2001). We are examining all of the subspecies mentioned above. Phylogenetic analysis based on the combination of the data concerning all three markers clearly shows the monophyly of the group consisting of lineages IV to VI with strong BP and PP (1.00; Fig. 3). It has also clearly shown the monophyly of the cluster consisting of lineages IV and V, supported by an algorithmic method (NJ), the optimality criterion methods (MP, ML) and also BI. *R. a. albipinnatus* revealed a sequence pattern significantly different from the other subspecies, both in the case of mitochondrial markers and in the case of nuclear intron (data not provided). Concerning the intron S7 it is interesting that in studied populations from the

Klyaz'ma R., Malaya Tsiviľ R., Sura R. and Moksha R. (all of them from the Volga R. basin) an absolutely identical sequence motive was found. The average pairwise sequence divergence in the markers we used (CR/Cyt *b*/S7) were 2.24/7.57/1.39% in comparison with *R. a. belingi*, and 2.74/7.55/1.10% in comparison with *R. a. vladykovi*. The nuclear analysis incorporated all three mentioned subspecies into the cluster of *'albipinnatus'* with a strong BP and also significant PP (Fig. 6). The mitochondrial analysis singled out the nominative subspecies *R. a. albipinnatus* as an independent one (Figs. 4; 5).

The complicated history of taxonomic changes in some local forms of the white-finned gudgeon was presented by Naseka et al. (1999b). In this section we will deal with several facts from this history. Mahen (1930) found that gudgeons from the Jihlava River (the Danube R. basin) differed from *Gobio gobio* and *G. uranoscopus*. He described these specimens and classified them as *G. uranoscopus.* Vladykov (1931) described the specimens from the Danube R. basin as "hybrids" (congeneric, *G. gobio carpathicus* x *G. persa carpathorossicus* = *R. kesslerii*), which were able to breed (because he found both sexes). Fang (1943) regarded these "hybrids" to be a new valid species *Gobio vladykovi*. Bănărescu (1946) believed *G. belingi* and *G. vladykovi* to be synonyms. Berg (1949) maintained that *G. belingi* is a junior synonym of *G. albipinnatus* and regarded *G. vladykovi* as invalid hybrid form. Oliva (1950; 1951) compared morphological characteristics of mentioned subspecies and declared them to be identical to nominative subspecies of the white-finned gudgeon.

Our phylogenetic results clearly confirmed the validity of *R. a. belingi* and *R. a. vladykovi* with a strong support. At the same time they demonstrated that their mutual relationship is closer than that with *R. a. albipinnatus* from the Volga River (Figs. 3-6). CR haplotype network provided an overview of more frequent haplotypes and their derivatives (Fig. 2). The average pairwise sequence divergence of the CR/Cyt *b*/S7 markers were 1.62/2.25/1.53% between both the subspecies (Table 5).

The fourth subspecies, *R. a. tanaiticus*, was described by Naseka (2001) from the Don River based on weak non-significant differences in some morphometric characters. We analyzed two specimens from the Don River basin, specifically from the Khoper River. Sequence alignment of S7 showed identity with the sequences of *R. a. albipinnatus* from the Volga River. Using mt marker of Cyt *b* certain point mutations were identified. Sequence difference ranged up to 1.00%. Thus, molecular analyses do not confirm the validity of *R. a. tanaiticus* and the supposed interpopulation divergence between white-finned gudgeons from the Volga and Don Rivers.

Comparison with other species of the genus Romanogobio from the database and Gobio sp.

The phylogenetic tree constructed from Cyt *b* sequence data enabled mutual comparison also with other specimens from the GenBank database (Fig. 4). We included in our analysis two species, *R. ciscaucasicus* (Berg, 1932) from the Kuma River in southern Russia and *R. macropterus* (Kamensky, 1901) from the Aras River in Turkey. That both species formed a common cluster with *R. a. albipinnatus, R. a. tanaiticus* and *R. parvus*. Monophyly of *R. macropterus* (= *G. persus macropterus*; Naseka et al., 1999a) was confirmed with a strong support. *R. ciscaucasicus* formed a monophyletic group with *R. a. tanaiticus* from the Khoper R. (the Don R. basin) and *R. parvus* (Naseka and Freyhof, 2004) from the Kuban R. with a strong support only according to the BI and NJ methods (Fig. 4). Sequence differences between *R. ciscaucasicus* and *R. macropterus* and the other members (*R. a. albipinnatus, R. a. tanaiticus* and *R. parvus*) are (in the above given order) in average 1.58/1.51/1.30% and 2.08/2.83/3.33%, respectively. It is noteworthy that this group of five members contains three nominate species from the Ponto-Caspian region, which was one of the most important glacial refuges (Bănărescu, 1991).

It should be mentioned that *R. parvus* was separated from *R. ciscaucasicus* s. *lato*, as determined by the differences in relative skull width, eye diameter and presence/absence of connection between supraorbital and infraorbital sensory cephalic canals (Naseka and Freyhof, 2004). This last characteristic was found to be highly subjected to individual variability in related taxa (for example, in *R. albipinnatus* and its subspecies (Naseka, 2001), whereas skull width and eye diameter belong to size-variable characteristics, and thus the noted features seem to be sample-specific but not diagnostic. At the same time *R. parvus* is very similar to *R. albipinnatus* and its subspecies, and Naseka and Freyhof (2004) distinguished these based on the number of scales around the caudal peduncle mainly. According to these authors *R. albipinnatus* and its subspecies have 14-16 scales, whereas *R. parvus* have 10-12, but these values look very unusual, because previously Naseka and Bogutskaya (1999) characterized *R. ciscaucasicus* s. *lato* by the presence of 12-16 circum-peduncle scales with the mode 14. The other differences are demonstrated (Naseka and Freyhof, 2004) in average values of a few morphometric characteristics in individual comparisons between different nominal white-finned subspecies. Thus our molecular results confirm low divergence among the three mentioned nominal species. Moreover, our analysis of the nuclear marker S7 revealed that *R. parvus* possesses the same sequence pattern as *R. a. albipinnatus* from the Volga R.

For the sake of comparison the presented study also deals with three representatives of the genus *Gobio*. We called them *Gobio sp.* in general due to their vague taxonomic status and running analyses – both molecular and morphological. Therefore we are going to deal with

this genus only marginally. The above mentioned specimens come from different geographic regions belonging to two sea drainage areas – the North Sea (the Elbe R. and the Rhone R.) and the Black Sea (the Váh R., the Danube River basin). The very strong similarity of the noted specimens from the North Sea is apparent, as well as the considerable dissimilarity among the specimens from different sea drainage areas, accompanied by a significant PP (1.00; Fig. 4). According to our previous studies (Vasil'eva et al., 2004) the specimens from the North Sea drainage area should represent the species *Gobio gobio* (Linnaeus, 1758) s. stricto (=*G. g. gobio*, Bănărescu et al., 1999b) however the specimens from the Danube River basin could represent *G. obtusirostris* (Valenciennes, 1842). Considering the sequence patterns of both species there are apparent significant differences especially in the nuclear S7 marker, when *G. gobio* differed above all in two long 12 nt and 18 nt deletions (in addition to numerous substitutions). In case of mitochondrial sequences of the control region and cytochrome b it differed on the basis of many substitutions.

4.2. Surprising findings and new area of R. a. albipinnatus

Extensive sequence database obtained during the last four years with the help of our colleagues from other countries enabled us to compare the individual species and subspecies from different geographic areas. Although the comparison is still going on, some surprising findings have already become apparent. On the basis of analyses of the nuclear marker S7 it is obvious that the two nominal subspecies of *Gobio gobio* from Turkey, i. e., *G. g. gymnostethus* (Ladiges, 1960) from the locality Melendiz-Nigde in North Anatolia and *G. g. intermedius* (Battalgil, 1944) from Aksehir Lake (Central Anatolia) have the same sequence pattern as *R. a. albipinnatus* from the Volga R. The same is true for the non-specified *Gobio sp.* from the Psezuapse R. in Russia and specimens from the locality Camtur-Gerede in Turkey (North Anatolia), originally classified as *G. gobio*. This study has resulted in the discovery of a new area of distribution of *R. a. albipinnatus,* shifting of the boundaries of its occurrence as far as the Fore-Caucasus, and also the discovery of *R. a. albipinnatus* in the territory of Turkey (Fig. 1).

4.3. Taxonomic implications

At present there are as many as 26 different species concepts, which try to define a species (Mallet, 2005; 2007; Wilkins, 2006). Each concept has its advantages and disadvantages, and there is no a universal criteria which is accepted by everyone which would globally and

definitively resolve the dynamics of the evolution of animals. A major revolution in zoological taxonomy occurred around 1900 and during the following years, geographic varieties were given subspecies names within polytypic species. These changes were incorporated into the International Code of Zoological Nomenclature and are used up to this day. But recently developed Phylogenetic Species Concept (PSC) or Evolutionary Species Concept (ESC), which are equivalent for practical purposes (Kottelat, 1997; 1998), completely reject subspecies as taxonomic unit. Despite extensive debate between the co-authors, no consensus has been achieved concerning this dilemma, which is similar to the situation within the whole scientific ichthyological community. In the interest of compliance with the plurality of views among the co-authors, we therefore present both views in the text. We used the subspecies names as the original designations of the taxa in the previous paragraphs and now, in accordance with new scientific approaches we will use the definition of a species based on PSC or ESC in further discussion. The taxa in the tables and figures are also interpreted using these concepts.

The phylogenetic analyses we performed allow no doubts concerning the validity of two sister-groups of the genera *Gobio* and *Romanogobio* with the name *Rheogobio* as a junior synonym of the latter. Neither is the validity of the species *R. pentatrichus* from the Kuban River (monophyletic lineage I) questioned. Concerning the monophyletic group '*kesslerii*', the existence of two allopatric species *R. kesslerii* (lineage VII) from the Dniester River and *R. banaticus* from the Danube River basin (lineage II), both represented by high-diverged monophyletic lines, has been confirmed. Whereas taxonomic relations among Danube populations, including discussed species *R. carpathorossicus* from the Tisza River system, require further analyses ("species-in-waiting"). Concerning the monophyletic group '*uranoscopus*', the taxonomic isolation of allopatric Danubian *R. frici* (lineage III) and non-Danubian *R. elimeius* is obvious, but their relationship with *R. uranoscopus* from type locality and interrelations among different Danubian populations needs further investigations. Concerning the monophyletic group '*albipinnatus*' we confirmed the validity of three species represented by high-diverged phylogenetic lines: *R. albipinnatus* from the Volga River basin (lineage VI), *R. belingi* from the rivers Dniester, Dnieper and Elbe (lineage IV), and *R. vladykovi* from the Danube River basin (lineage V). Our results will require re-evaluation of the taxonomic status of *R. tanaiticus* due to its genetic identity and significant morphological similarity with *R. albipinnatus* s. stricto. According to genetic and morphological data, populations of the white-finned gudgeon from the Don and Volga River basins should be considered conspecific with proper definition of *R. tanaiticus* as a junior synonym of *R. albipinnatus*. We believe that it is necessary to focus on achieving a better understanding of

the relationships within the monophyletic group including *R. parvus*, *R. ciscaucasicus*, and *R. tanaiticus* = *R. albipinnatus* indicated by a grey line in the Cyt *b* tree (Fig 4). We suggest a more comprehensive morphological and genetic revision of the nominal species *R. parvus* and its relationship with mentioned species, because according to our preliminary results R. parvus seems to be species in question. We also suggest a comprehensive revision of two subspecies of the genus *Gobio*, namely of *G. g. gymnostethus* and *G. g. intermedius*, which are suspect of being conspecific with *R. albipinnatus*, or possibly its hybrid form. Summary Table 6 shows an overview of the original taxonomy of studied gudgeons and of the newest facts described in this phylogenetic study.

Acknowledgments

The study was carried out within the framework of the research project no. VaV-SM/6/3/05 supported by the Grant from the Ministry of Environment of the Czech Republic and by the Grant project no. IAA6045005 from the Grant Agency of the Academy of Sciences of the Czech Republic. Our investigations in Russia were partially supported by RAS Programs "Biodiversity" and "Biological resources of Russia". We are grateful to J. Taggart for his helpful advice and comments. We are thankful to C. S. Özeren, N. G. Bogutskaya and M. Przybylski for their help with the obtaining of samples. We thank G. Speas for English correction.

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Table 1
Species examined in this study, source of tissue sample used, and GenBank Accession numbers

Species/subspecies	[Locality Nos.] River, drainage, country (NHCR/NS)	Accession Nos.
Outgroups		
Gobio sp.	Rhone basin, FR*; R. Elbe at Neratovice, Elbe, CZ; R. Váh at Hlohovec, Danube, SK	AJ388392, Y10452, EF427406, EF427421, EF427397, EF427407, EF427420
*R. ocellatus**	unknown	AY017149, AF051876, AY325789
*S. microoculus**	unknown	NC_004694, NC_004694
Ingroup taxa		
R. albipinnatus	[1]R. Moksha-Mordovia, Volga, RUS (21/1); [2]R. Malaya Tsivil' at Shichazany, Volga, RUS; [3]R. Klyaz'ma at Vladimir, Volga, RUS	EF427390, EF427401, EF427408
R. tanaiticus	[4]R. Khoper-Penza district, Don, RUS	EF427402, EF427409
R. banaticus	[5]R. Bečva at Rybáře, Danube, CZ (4/1, 5/9, 7/1); [6]R. Ipel' at st. border, Danube, SK (6/1, 8/1)	EF427393, EF427435, EF427436, EF427405, EF427417, EF427445, EF427394, EF427416
"R. carpathorossicus" ("species-in-waiting")	[7]R. Laborec at Kochánovce, Tisza, SK (9/4, 15/1); [8]R. Morava at Tovačov, Danube, CZ (9/1, 12/1, 13/2, 14/1); [9]R. Topl'a at Poliakovce and Nemcovce, Tisza, SK (9/1, 10/1, 11/1, 16/1)	EF427441, EF427437, EF427443, EF427442,EF427444, EF427438, EF427439, EF427440
R. belingi	[10]R. Sluch, Dnieper, UA (28/4, 32/1); [11]R. Dniester, Dniester, UA (28/1, 29/1, 31/1); [12]R. Elbe at Střekov, Elbe, CZ (28/2, 30/1)	EF427425, EF427387, EF427398, EF427413, EF427388, EF427426, EF427431
R. frici	[14]R. Hornád at Košice, Tisza, SK (17/3, 19/2); [7]R. Laborec at Kochánovce, Tisza, SK (20/5); [15]R. Terešva at Krive, Tisza, UA; [16] R. Ulička at Ulič, Tisza, SK (18/2)	EF427392, EF427414, EF427433, EF427434, EF427415, EF427432, EF427404, EF427391
R. kesslerii	[11]R. Dniester, Dniester, UA (3/2)	EF427446, AY952328
R. parvus	[13]R. Kuban' at Armavir and Kropotkin, Kuban, RUS (21/1)	EF427389, EF427403, EF427410
R. pentatrichus	[13]R. Kuban' at Armavir and Kropotkin, Kuban, RUS (1/1, 2/1)	EF427395, EF427396, EF427418, EF427419
R. vladykovi	[17]R. Dyje at Břeclav and Podhradí, Danube, CZ (23/5, 25/1); [6]R. Ipel' at st. border, Danube, SK (24/1); [8]R. Morava - Blata, Danube, CZ (27/2); [18]Reviština channel, Tisza, SK (22/5); [9]R. Topl'a at Nemcovce, Tisza, SK (22/3); [19]R. Ublianka at Ubl'a, Tisza, SK; [20]R. Uh at Lehárovce, Tisza, SK (26/1); [21]R. Váh at Hlohovec, Danube, SK; [22]R. Warta - 1.5 km below Jeziorska Reservoir, Odra, PL	EF427428, EF427385, EF427412, EF427411, EF427429, EF427430, EF427400, EF427427, EF427399, EF427386, EF427424
Surprising findings		
"G. g. gymnostethus"	[23]Melendiz-Nigde, North Anatolia, TR	EF427422
"G. g. intermedius"	[24]Aksehir Lake, Central Anatolia, TR	EF427423
"G. gobio"	[25]Camtur-Gerede, North Anatolia, TR	EF427408
"Gobio sp."	[26]R. Psezuapse - Krasnodar district, RUS	EF427408
Additional Cyt b sequences for construction of the phylogenetic tree		
R. banarescui†*	R. Aliakmon, GR	AF090751
*R. banaticus**	R. Nera, RO	AY952330
*R. ciscaucasicus**	R. Kuma, RUS	AF095607
*R. kesslerii**	R. Dniester, UA	AY952328
*R. macropterus**	R. Aras, TR	AY952332
*R. uranoscopus**	R. Valsan, RO; R. Nera, RO	AY426593, AY952331

NHCR/NS = number of control region haplotype/number of analyzed specimens; * indicates sequences from the GenBank; † indicates misidentification; " " indicates discussed status identified by material collectors. CZ, Czech Republic; FR, France; GR, Greece; PL, Poland; RO, Romania; RUS, Russia; SK, Slovakia; TR, Turkey; UA, Ukraine.

Table 2
List of primers used in this study

Gene	Primer	Sequences (5´- 3´)	Reference
CR	CR159	CCC AAA GCA AGT ACT AAC GTC	This study
	CR439	AAC TGT TTT TCC CAC ACT TA	This study
	CR493	TTG GGT AAC GAG GAG TAT GTA	This study
	CR851	TGC GAT GGC TAA CTC ATA C	This study
CR_STIR	Carp-Pro	AAC TCT CAC CCC TGG CTA CCA AAG	Thai et al. 2004
	Carp-Phe	CTA GGA CTC ATC TTA GCA TCT TCA GTG	Thai et al. 2004
CYT B	GluDG.L	TGA CTT GAA RAA CCA YCG TTG	Palumbi 1996
	H16460	CGA YCT TCG GAT TAA CAA GAC CG	Bermingham pers. com.
S7	S7univL	ACA ATT GTA AGT CGG AGA TG	This study
	S7univP	CCC ACA AAA TAA GAT ATT AGG	This study

Table 3
Molecular diversity in the main control region lineages of genus *Romanogobio*

mtDNA lineage	N	NH	$\pi \pm$ SD	Hd ± SD	Tajima's D
Lineage I (*R. pentatrichus*)	2	2	0.003 ± 0.001	1.000 ± 0.500	x
Lineage II (*R. banaticus*)	27	13	0.011 ± <0.001	0.852 ± 0.053	0.126 NS
Lineage III (*R. frici*)	12	4	0.002 ± <0.001	0.682 ± 0.079	0.755 NS
Lineage IV (*R. belingi*)	11	5	0.003 ± 0.001	0.618 ± 0.164	-0.542 NS
Lineage V (*R. vladykovi*)	18	6	0.001 ± <0.001	0.386 ± 0.128	-0.438 NS
Lineage VI (*R. albipinnatus*)	2	1	0.000 ± 0.000	0.000 ± 0.000	x
Lineage VII (*R. kesslerii*)	2	1	0.000 ± 0.000	0.000 ± 0.000	x
Overall	74	32	0.062 ± 0.002	0.930 ± 0.015	

N - the number of specimens; NH - the number of haplotypes; π – nucleotide diversity; Hd - haplotype diversity; Tajima's D test (1989): NS = departure from neutrality - not significant, P>0.10.

Table 4

Analysed fragments of both genomes, their characteristics resulting from the MP analysis and the appropriate models selected by Modeltest

Partition	No. characters (pars. inf.)	TL	CI	RI	Model
CR	709-713 (121)	631	0.7434	0.8060	HKY+Γ
CYT B	622 (157)	392	0.6034	0.7491	TrN+Γ
S7	368 (53)	214	0.8462	0.8909	K81uf
All combined data	1703 (331)	828	0.6957	0.7485	Mixed model

CI, consistency index (excluding uninformative characters); Γ, gamma; pars. inf., number of parsimony informative characters; RI, retention index; TL, tree length.

Table 5

Mutual comparison of the representatives of the genus *Romanogobio* and their genetic distances obtained by analysis of both mitochondrial (CR) and nuclear (S7) genomes

Species	Lineage I *R. pentatrichus*	Lineage II *R. banaticus*	Lineage III *R. frici*	Lineage IV *R. belingi*	Lineage V *R. vladykovi*	Lineage VI *R. albipinnatus*	Lineage VII *R. kesslerii*
Lineage I *R. pentatrichus*	**0.25 ± 0.09**	13.12 ± 1.20	9.14 ± 0.98	7.52 ± 0.88	7.68 ± 0.95	7.87 ± 0.95	11.05 ± 1.80
Lineage II *R. banaticus*	6.46 ± 1.27	**1.14 ± 0.41**	11.94 ± 1.13	12.05 ± 1.15	12.23 ± 1.17	12.89 ± 1.19	4.68 ± 1.01
Lineage III *R. frici*	4.79 ± 1.10	4.10 ± 1.07	**0.24 ± 0.10**	2.65 ± 0.57	2.83 ± 0.58	2.82 ± 0.58	10.72 ± 1.81
Lineage IV *R. belingi*	6.44 ± 1.27	6.06 ± 1.26	3.23 ± 0.86	**0.34 ± 0.11**	1.62 ± 0.47	2.24 ± 0.48	10.43 ± 1.71
Lineage V *R. vladykovi*	6.42 ± 1.27	5.72 ± 1.17	2.93 ± 0.77	1.53 ± 0.55	**0.14 ± 0.05**	2.74 ± 0.58	9.95 ± 1.66
Lineage VI *R. albipinnatus*	5.81 ± 1.18	5.12 ± 1.16	2.35 ± 0.68	1.39 ± 0.55	1.10 ± 0.48	—	11.51 ± 1.82
Lineage VII *R. kesslerii*	x	x	x	x	x	x	**0.00 ± 0.00**

The mean sequence differences in percents ± SD above the diagonal for control region, under the diagonal there are the values for the intron S7 r-protein; values on the diagonal (in bold) indicate within-CR lineage divergences; x - no sequence capable of evaluation was obtained.

Table 6

The original taxonomy of selected species of the genera *Romanogobio* and *Gobio* in the light of new phylogenetic information

Original taxonomy		New knowledge	
Genus	Species/subspecies	Genus	Taxonomic status
Romanogobio	*R. pentatrichus*	*Romanogobio*	*R. pentatrichus*
Gobio	*G. k. kesslerii*	*Romanogobio*	*R. kesslerii*
Gobio	*G. k. banaticus*	*Romanogobio*	*R. banaticus*
Gobio	*?G. k. carpathorossicus?*	*Romanogobio*	*?R. carpathorossicus?**
Gobio	*G. a. albipinnatus*	*Romanogobio*	*R. albipinnatus*
Gobio	*G. a. belingi*	*Romanogobio*	*R. belingi*
Gobio	*G. a. vladykovi*	*Romanogobio*	*R. vladykovi*
Romanogobio	*R. a. tanaiticus*	*Romanogobio*	*R. albipinnatus*
Rheogobio/Gobio	*G. u. frici*	*Romanogobio*	*R. frici*
Rheogobio/Gobio	*G. u. elimeius*	*Romanogobio*	*R. elimeius*
Romanogobio	*?R. parvus?*	*Romanogobio*	*?R. albipinnatus?*
Rheogobio/Gobio	*?G. ciscaucasicus?*	*Romanogobio*	*?R. ciscaucasicus?*
Gobio	*G. persus macropterus*	*Romanogobio*	*R. macropterus*
Gobio	*?G. g. gymnostethus?*	*Romanogobio*	*?R. albipinnatus* or its hybrid form*?*
Gobio	*?G. g. intermedius?*	*Romanogobio*	*?R. albipinnatus* or its hybrid form?

"?" We suggest for a comprehensive revision; * indicates "species-in-waiting"

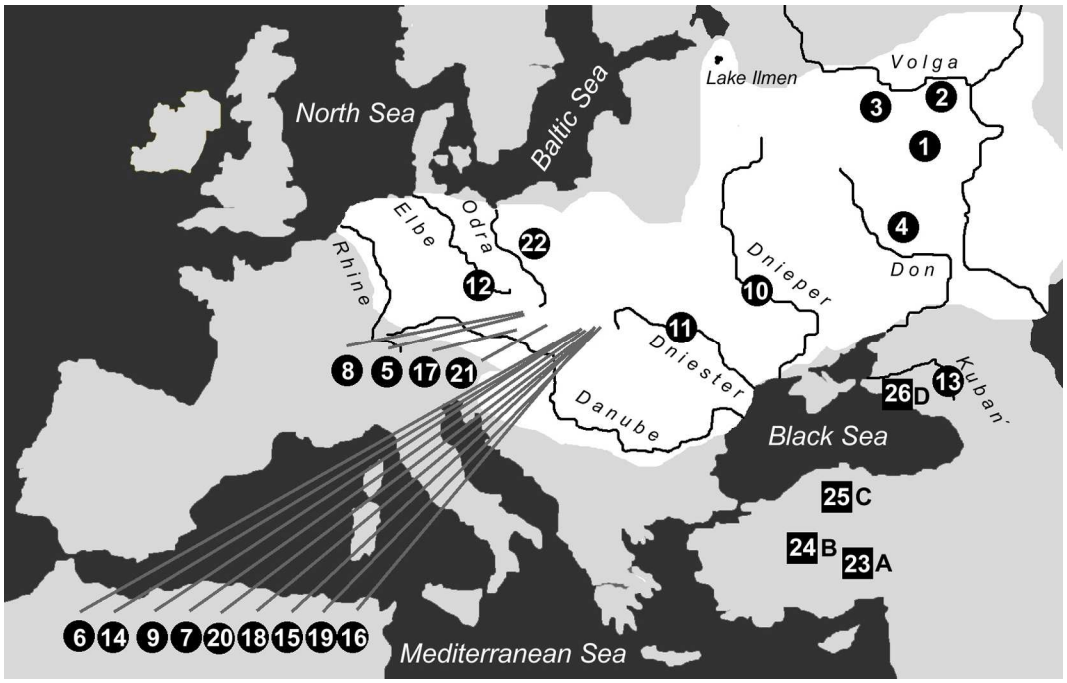

Fig. 1. Geographical origin of samples. Locality numbers correspond to the locality numbers in Table 1. The distribution area of the group '*albipinnatus*' is indicated in white (Freyhof et al., 2000; Naseka et al., 1999b; Scholten, 2000); records of sequences typical to *R. albipinnatus* but found in gudgeons traditionally related to genus *Gobio* and occurred outside the area of white-finned gudgeon (□): A – Melendiz-Nigde, B – Aksehir Lake, C – Gerede-Camtur, D – Psezuapse R.

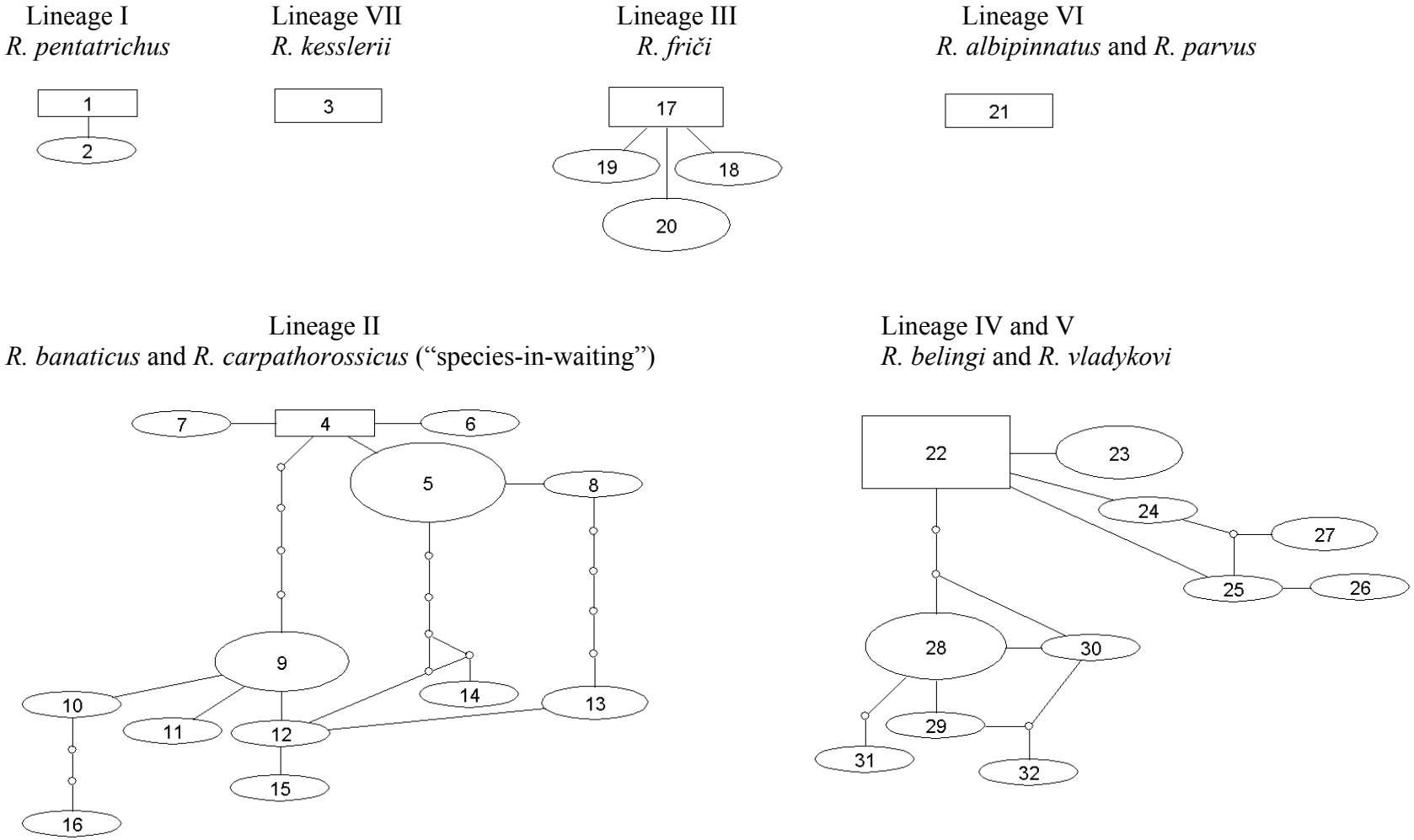

Fig. 2. Unrooted haplotype network based on the sequences of the control region of certain representatives of the genus *Romanogobio*. The haplotype numbers refer to the numbers in Table 1. The node sizes are proportional to the haplotype frequency (see Table 1).

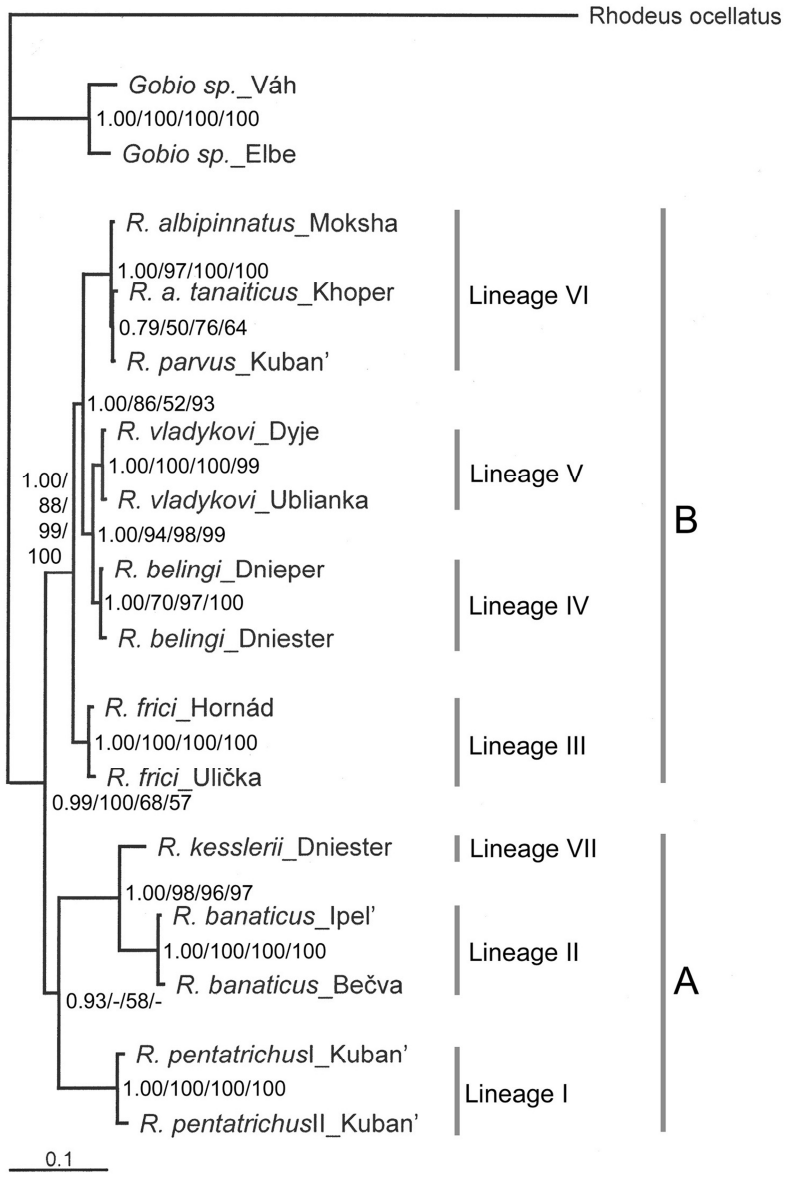

Fig. 3. Bayesian consensus tree resulting from analysis of combined S7 r-protein gene, Cyt *b* and control region data in studied gudgeon taxa, with Bayesian posterior probabilities/NJ bootstrap/MP bootstrap/ML bootstrap values listed near the nodes. Only values ≥ 50% are shown. Highlighted are the seven lineages categorized into two groups. The nominal species name is followed by the name of the locality, as in subsequent figures.

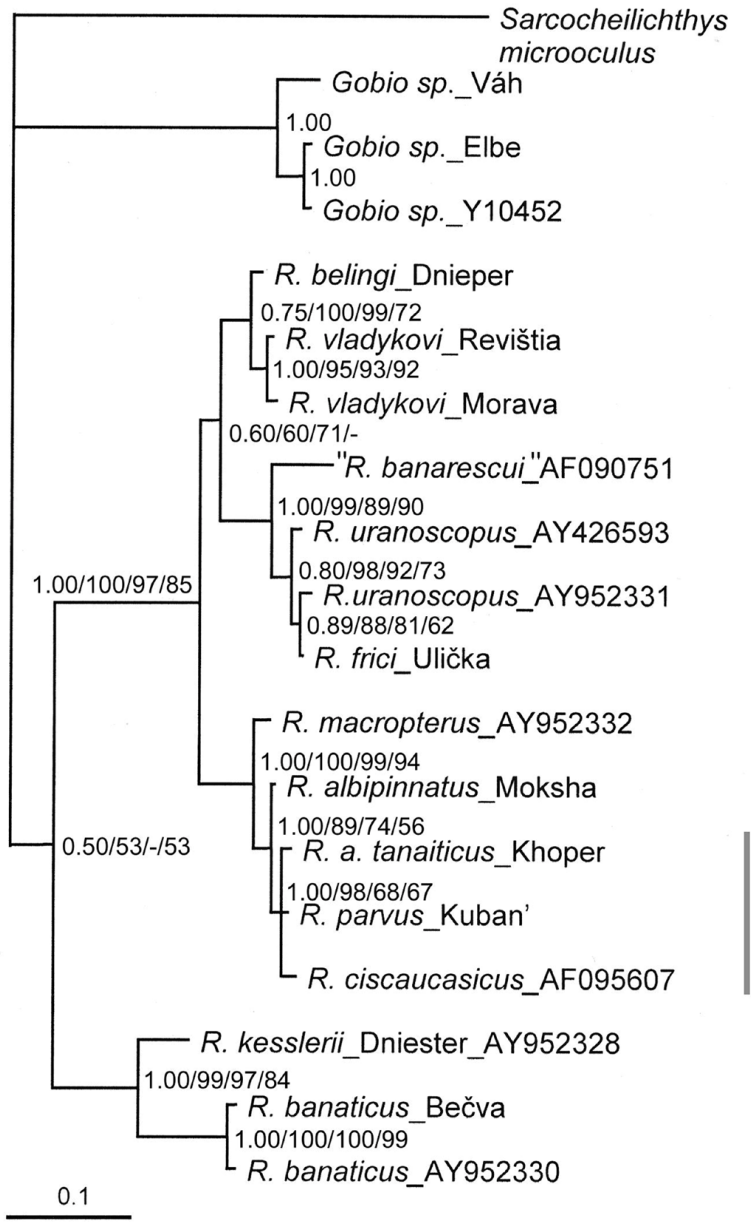

Fig. 4. Bayesian consensus tree resulting from the analysis of 622 bp sequence of the Cyt *b* gene, with Bayesian posterior probabilities/NJ bootstrap/MP bootstrap/ML bootstrap values listed near the nodes. Only values ≥ 50% are shown. Grey line – identification of a group for further revision.

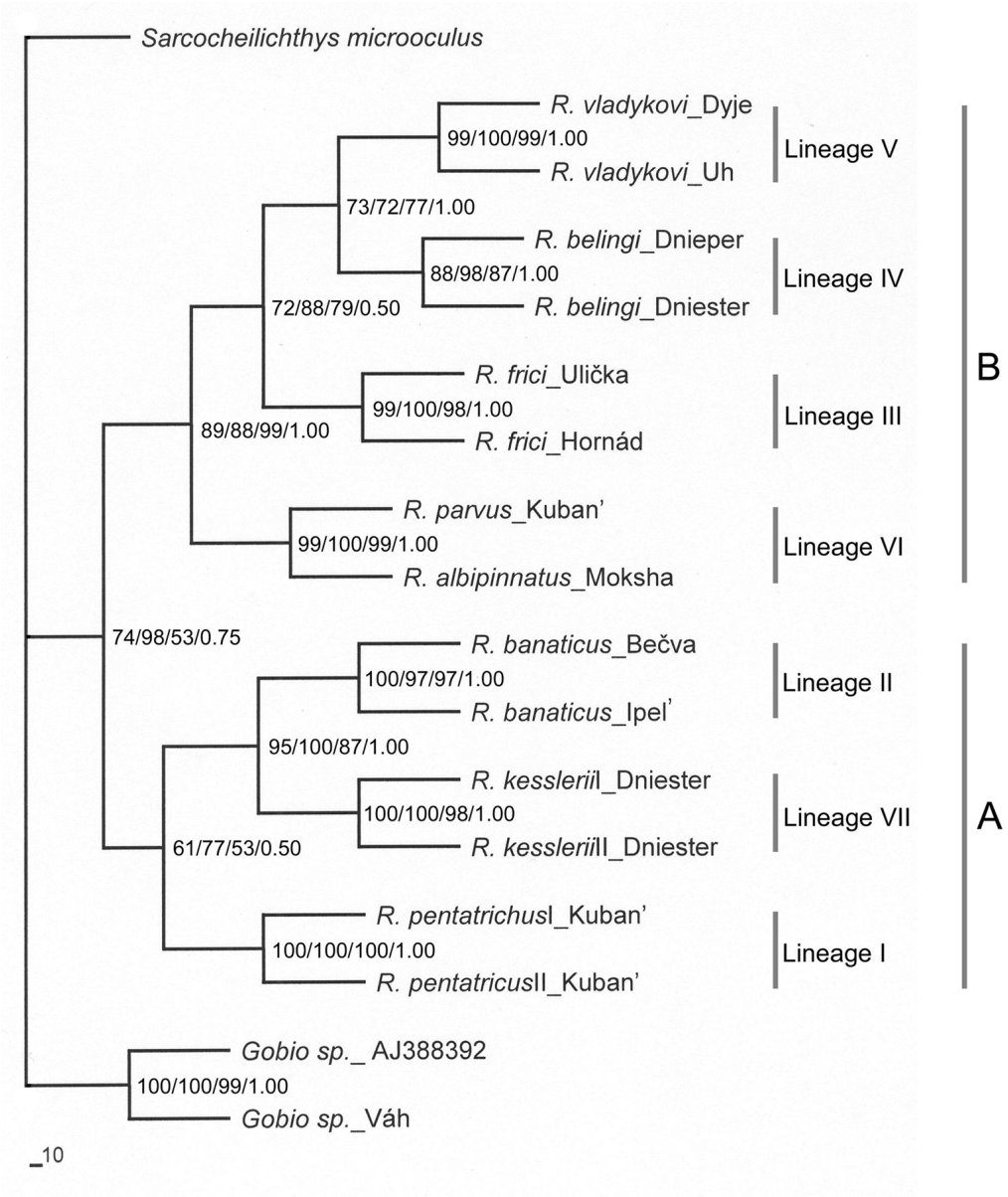

Fig. 5. Maximum parsimony tree inferred from the control region sequences (709 - 713 bp). Bootstrap values for MP/NJ/ML and also Bayesian posterior probabilities are listed near the nodes. Only values ≥ 50% are shown. Highlighted are the seven lineages categorized into two groups.

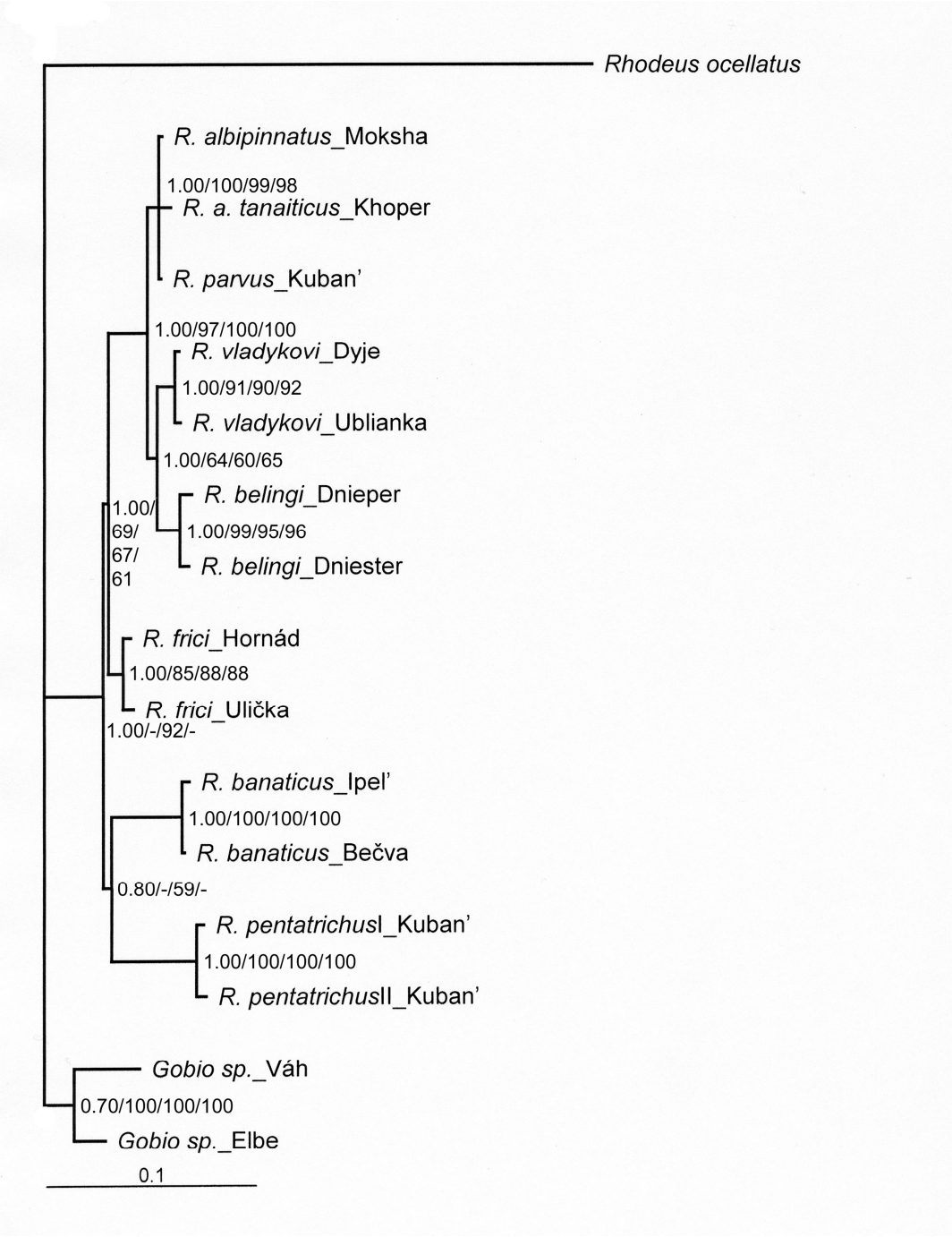

Fig. 6. Bayesian consensus tree resulting from the analysis of 338 - 368 bp intron sequence of the S7 ribosomal protein gene, with Bayesian posterior probabilities/NJ bootstrap/MP bootstrap/ML bootstrap values listed near the nodes. Only values ≥ 50% are shown.

Příloha E

Mendel, J., Lusk, S., Vasil'eva, E. D., Vasil'ev, V. P., Lusková, V., Erk'akan, F., Ruchin, A., Koščo, J., Vetešník L., Halačka, K., Šanda, R., Pashkov, A. N., & Reshetnikov, S. I. (2007). Molecular phylogeny of the genus *Gobio* (Cyprinidae, Pisces) and its contribution to taxonomy. *Mol. Phylogenet. Evol.* (submitted 8/2007).

Molecular phylogeny of the genus *Gobio* (Cyprinidae, Pisces) and its contribution to taxonomy

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Abstract

The phylogenetic relations among gudgeons that represent most nominal taxa within *Gobio gobio* sensu lato were examined by mitochondrial and nuclear genome sequencing. The molecular analyses confirmed the separate generic status of *Gobio* as a monophyletic group and revealed 15 Eurasian lineages divided into two main clades, the Northern European and the Ponto-Caspian. The validity of eleven nominal taxa as distinct species was confirmed, gudgeons from the Volga River basin were described as a new species *G. volgensis*, and three revealed phylogenetic lineages were submitted for a comprehensive revision as "species-in-waiting". The species *G. gobio* showed a wide range extending from the British Isles to the Black Sea coast and overlapped the areas of several other species. Four pure lineages were detected in the middle Danube River basin. As a whole, two hybrid zones were detected in the Upper Tisza River basin and in the Crimean Peninsula. The latter zone will require a special investigation to define species participating in hybridization events, and to establish further steps for the conservation of endemic native gudgeon species. A simple diagnostic method, based on different lengths of the PCR products, called "S7indel diagnostics" is presented for further taxonomic investigations in the genus *Gobio*.

Keywords: Control region; S7 ribosomal protein gene; Intron; Cytonuclear disequilibrium; S7indel diagnostics; Taxonomy; New species; Phylogeography; *Gobio*

1. Introduction

The genus *Gobio* Cuvier, 1816 belongs to the subfamily Gobioninae, which is part of the large family Cyprinidae. Its distribution reaches from Spain and the British Isles to the Far East and Northern China, and its representatives live in all types of waters, i.e., in standing and flowing waters, in freshwaters and in some cases found in brackish waters. The Palaearctic gudgeon *G. gobio* (Linnaeus, 1758) sensu lato is a complicated species especially in terms of taxonomy, due to its exceptional phenotypical diversity, and is therefore considered one of the most variable fish species in Europe (Bănărescu et al., 1999). *G. gobio* sensu lato includes many subspecies and local forms described in the past, whose validity is under extensive discussion now. For example, Naseka et al. (2006) argue that most of the designations attached to these fish do not have a real basis, because they apparently arose as artifacts due to the combination of inadequate material, discrepancies in the use of different species concepts, language barriers and an insufficient attention to detailed morphological studies (analogous to Kottelat, 1997; Kottelat and Persat, 2005). Moreover, we have noticed several descriptions of newer species of the genus *Gobio* from different geograghical areas, which were derived from incomplete information, being based solely on morphological data (Freyhof and Naseka, 2005; Kottelat and Persat, 2005; Naseka et al., 2006). On the other hand, several genetic investigations show signs of a more comprehensive approach to gudgeon phylogeny and taxonomy (Doadrio and Madeira, 2004 along with Madeira et al., 2005; Bianco and Ketmaier, 2005).

This study is an attempt to also adopt a comprehensive approach, as it is based on morphologically defined specimens of several nominal *Gobio* taxa from type localities or their close surroundings, which were subjected to analysis by molecular markers of both mitochondrial and nuclear genomes.

The main aim of this study was to evaluate the validity of particular species and subspecies of the genus *Gobio* and to estimate phylogenetic relations between them, and thus, gain a clear view of the taxonomy of gudgeons, to identify "species-in-waiting," and to propose that several previously described species/subspecies should be subject to a comprehensive revision. Furthermore, we wanted to introduce new information in the genetic field of this genus, including diagnostic markers.

2. Materials and methods

2.1. *Sample collection*

In the period from 2000 – 2006, 149 gudgeon specimens were collected from 44 localities (see Table 1), which represented areas of the most nominal taxa included in *Gobio gobio* s. lato (Fig. 1). The species *Rhodeus ocellatus* and *Sarcocheilichthys microoculus* were selected as outgroups (Table 1) based on recent knowledge of phylogenetic relationships among cyprinid fishes (Briolay et al., 1998; Cunha et al., 2002; Yang et al., 2006). Two species of the genus *Romanogobio* (*R. albipinnatus* and *R. frici*) were also used for comparison. Voucher specimens are deposited in the collections of the Department of Ichthyology of the Institute of Vertebrate Biology, v.v.i. (Brno, Czech Republic) and Zoological Museum of the Moscow State University (ZMMU).

2.2. *DNA extraction, PCR amplification and sequencing*

Total genomic DNA was extracted from a small piece of the pectoral fin by proteinase K digestion followed by phenol-chloroform-isoamylalcohol purification and ethanol precipitation (Sambrook et al., 1989). Sequences of the control region (CR), and the first intron of the S7 r-protein (S7) were amplified by polymerase chain reaction (PCR) with primers specified in Table 2. PCRs were performed in 50 μl volume containing 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM each primer, 2.5 U *Taq* DNA polymerase (Top-Bio) and approximately 100 - 500 ng of genomic DNA. Reactions were performed in TGRADIENT Thermocycler (Whatman Biometra) under the following conditions: CR: 95 °C for 1 min, followed by 37 cycles of 94 °C for 45 s, annealing at 52.6 °C (the first fragment) and 54.8 °C (the second fragment) for 30 s, and an extension temperature of 72 °C for 45 s, followed by a final extension at 72 °C for 5 min. In some cases, other pairs of primers (CR_STIR) were used under the following conditions: 95 °C for 3 min, followed by 34 cycles of 95 °C for 30 s, annealing at 55.0 °C for 30 s, with an extension temperature of 72 °C for 1 min, and a final extension at 72 °C for 5 min. S7: 95 °C for 1 min, followed by 30 cycles of 94 °C for 45 s, annealing at 52.4 °C for 30 s, and an extension temperature of 72 °C for 25 s, with a final extension at 72 °C for 5 min. The PCR products were visualized by mini-gel electrophoresis using ethidium bromide staining and 1.7% agarose gels. The PCR products were purified by means of precipitation PEG/Mg/NaAc (26% Polyethylene glycol, 6.5 mM $MgCl_2.6H_2O$, 0.6 M $NaAc.3H_2O$). Direct sequencing of purified PCR products was performed with the BigDye™ Terminator Cycle Sequencing Ready

Reaction Kit version 1.1 (Applied Biosystems) according to the manufacturer's instructions, and purified with EtOH/EDTA precipitation. The sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). All PCR amplicons were multiple sequenced from both directions to ensure high quality reads. The DNA sequences were edited and aligned using the Seqman module within Lasergene v. 6.0 (DNASTAR Inc.) and also checked manually. The accuracy of the sequence was confirmed by comparison with the NCBI database.

2.3. *Phylogenetic analyses*

Haplotype (Hd) and nucleotide diversity (π) (Nei, 1987) were computed using DNASP 4.0 (Rozas et al., 2003).

The web-based ModelTest 3.8 program was used to ascertain the best-fit model of nucleotide substitution for separate nuclear and mitochondrial regions (Posada, 2006). Phylogenetic relationships among the two gene sequences were examined using the neighbour-joining (NJ) algorithm, the criteria of optimality: maximum parsimony (MP) and maximum-likelihood (ML), as well as using Bayesian inference (BI). The sequences were imported into PAUP* 4.0B.10 (Swofford, 2002) and MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2005) for phylogenetic analysis. For NJ analysis, DNA distances were calculated. Non-parametric bootstrap analyses with 1000 pseudo-replicates were performed to obtain supporting estimates for each node of the NJ trees. For MP tree construction, unweighted parsimony analysis using a branch-and-bound search was used. The confidence levels in the resulting relationship were assessed using the bootstrap procedure with 1000 replications. ML search was performed under the best-fit model with the branch-and-bound algorithm on 100 bootstrap replicates. Bayesian analysis was performed using MrBayes 3.1.2. Starting from a random tree, four Markov chains were run for 1 x 10^6 generations with a sampling frequency of 100. The best-fit models were then specified. The combined data set was treated as two partitions with different models accounting for their heterogeneity. We utilized the "unlink" command in MrBayes 3.1.2. to unlink the following parameters: "unlink shape=(all) statefreq=(all) revmat=(all)". The application Tracer 1.2 (Rambaut and Drummond, 2003) was used to view the output of the *sump* file generated by MrBayes. The trees generated prior to reaching stationarity were discarded as burn-in. We then took the resulting 50% majority rule consensus tree. Congruence among tree topologies generated for the combined data (CR and S7 sequences) was tested with the incongruence length difference

test (ILD) as implemented in the partition homogeneity test in PAUP* (Farris et al., 1994; Mickevich and Farris, 1981).

As for treating the gaps as phylogentic characters, three types of analyses were compared during the process of phylogenetic inference from the sequence of nuclear marker (S7): 1) gaps as missing data, 2) gaps as the fifth state character (Barriel, 1994) and 3) gaps as a separate binary character (Simmons and Ochoterena, 2000). The best elaboration for dealing with indels (insertion/deletion) together with their incorporation to phylogenetic analyses was described by Simmons and Ochoterena (2000). They determined that there were two ways of coding gaps: a) the procedure "simple indel coding" (SIC) which is used mostly in present studies due to its simplicity, and b) the procedure "complex indel coding" (CIC), which is rarely used in scientific literature, (Simmons et al., 2001; Löhne and Borsch, 2005). Müller (2006) described a third approach of dealing with indels: "modified complex indel coding" (MCIC). The coding of indels was provided by the SeqState program (Müller, 2005) containing the implemented program IndelCoder. All of these approaches and methods were applied in this study. Instead of the CIC procedure, we used its newer, modified version MCIC.

Haplotype and nucleotype networks were constructed to estimate the genealogical intraspecific relationships employing the statistical parsimony (Templeton et al., 1992) implemented into the TCS 1.21 program (Clement et al., 2000). Indels were coded as the fifth state characters. A 95% connection limit was then calculated, meaning that the haplotypes were disconnected when more than ten mutational steps divided them.

3. Results and discussion

With regard to the extent of the submitted study, which gives the taxonomic and systematic overview of more than two thirds of the valid representatives of the genus *Gobio*, and in an effort to provide a clearer perspective, and as well as to save space, we have reorganised this part of the paper by connecting our results with the discussion.

3.1. *Sequence characteristics*

Sequence data was deposited in the GenBank database under accession numbers (CR: EU131542 - EU131588; S7: EU131589 - EU131626; Table 1). Up to 1097 bp of analyzable sequence data was obtained from the nuclear and mitochondrial genome fragments for each

specimen. Fragments of the control region (713 bp alignment) and the first intron of the S7 r-protein (384 bp alignment) were analyzed both separately and in combination. As some sequences from the same taxa were identical, these taxa were reduced to one representative per taxon in all subsequent analyses, exluding construction of the mtDNA and nDNA networks. Nucleotide base composition showed high values of AT pairs (A=33.4% and T=31.6% across all sites/all taxa) in control region sequences. A similar composition has been noted for Cypriniformes (Liu et al., 2002; 2003). In the case of the first intron sequences of the S7 gene, low values of GC pairs were found (16.7% and 19.3%, respectively) which is typical for noncoding regions of the genome. At the same time, AT-rich intron sequences, which are common in fishes, (Orti et al., 1996) were found. Measurements of molecular diversity for the main CR lineages together with statistical tests of neutrality (Tajima's D test; Tajima, 1989a) are shown in Table 3. The strong negativity of the Tajima's D value registered in Lineage_I shows a stastitically significant deviation from neutrality, which indicates its expanding character (Tajima, 1989b).

3.2. Haplotype and nucleotype richness – haplotype and nucleotype networks

Within the haplotype network, a total of 47 CR haplotypes representing 119 sequences were detected. 71 sequences were used for the construction of the nucleotype network and altogether 36 S7 nucleotypes were identified. A detailed list of all studied taxa, their haplotype and nucleotype assignment, sampling localities, haplotype and nucleotype frequency, and the GenBank accession numbers are given in Table 1. The schematic diagram of the statistical parsimony network shows a complex pattern of relationships within the genus G*obio*. CR haplotype network revealed five disconnected groups, and the S7 nucleotype network revealed eleven, consisting of 13 and 15 lineages, respectively (Figs. 2, 3).

3.3. Phylogenetic analyses

For both NJ and Bayesian analyses, the best-fit model under Akaike information criterion, (AIC; Akaike, 1974) was determined using the software ModelTest 3.8, see Table 4. The levels of divergence within and among G*obio* lineages are shown in Table 5. The summary statistics of MP analyses for the separate and combined data sets for each gene are shown in Table 4. For ML analyses, the likelihood settings of the best-fit model for CR based on the

hierarchical likelihood ratio tests (hLRTs) were as follows: base frequencies (A = 0.3337, C = 0.2132, G = 0.1372 and T = 0.3158); ti/tv ratio = 1.6559; and the shape parameter of the gamma distribution 0.3362. Likelihood settings of the best-fit model for S7 were: base frequencies (A = 0.2968, C = 0.1931, G = 0.1667 and T = 0.3433).

The three most common approaches of treating gaps in multiple sequence alignment S7 within a parsimony framework were used in this study. In the case of gap coding as separate characters, the results of two indel coding methods have been compared. The SIC and MCIC methods provided the same results in terms of topological accuracy and similar values of bootstrap support (data not shown).

Comparing different approaches of treating gaps:

i) In terms of the topological accuracy view:

Significant differences between topology trees obtained by coding gaps as "missing" (GM) characters and by the other two approaches – coding gaps as the fifth state character (G5) and coding gaps as separate present/absent characters (GS) – were found. Certain taxa were clustered without apparent logic when indels were exluded from the phylogenetic analysis. No significant differences were found between the results obtained by G5 and GS analyses (data not shown).

ii) In terms of the bootstrap support view:

The results of branch support based on GM coding differed in comparison with the results obtained by G5 and GS methods (data not shown). In the case of comparing nodal supports by G5 and GS analyses, there was a significant inrease in bootstrap values obtained by G5 method, which was apparent in the inner and terminal nodes (Fig. 4). Similar findings were reached by Simmons et al. (2001) who compared the results of 38 published sequence-based matrices, and by Ogden and Rosenberg (2007) in the study comparing the above mentioned methods using simulation. Hence, while building phylograms and also nucleotype and haplotype networks, we used indels as an additional phylogenetic signal beside substitutions, and in addition, gaps were treated as the fifth state character.

The data obtained by analysing the mitochondrial and nuclear markers was first analysed singularly, and then in combination. In terms of the combined dataset, the ILD test revealed significant incongruencies between the two analyzed loci. The P values were computed from 1000 replicates, and when S7 gaps were treated as the "additional state", the resultant value was P = 0.001. When gaps were treated as "missing", the resultant value was P = 0.004. Both values are very similar, which points out the fact that indels are not the reason for the reduction of phylogenetic accuracy. We then made a visual comparison of particular trees

obtained from both markers (S7 and CR). The aim was to determine that these results did not represent the case described by various authors (Barker and Lutzoni, 2002; Darlu and Lecointre, 2002) when the incongruence length difference test failed. The comparison revealed certain differences, but only in terms of the topology of trees. In contrast, the case of the bootstrap values or Bayesian posterior probabilities of terminal taxa, an increase in resolution and support was found (Figs. 4, 5). The topological diversity was apparent when certain lineages were separated differently into two main clades, whereas the results obtained by mtDNA analyses appeared to be more logical. On the basis of the above mentioned facts, we concluded that we would use information from both markers, including combined analysis for the evaluation of the taxonomic state of the studied representatives of the genus *Gobio* (the terminal nodes of the tree) similar to that reported by Gatesy et al. (1999) and Lavoué et al. (2003). However, in the systematics of the genus *Gobio* we used the phylogenetic signal only from the mitochondrial marker.

From the taxonomic point of view, we identified 13 separate pure monophyletic lineages of the genus *Gobio* (Figs. 4 - 6). Two gudgeon lineages, Lineage_XII or XIII (see below) and Lineage_XV were designated only by the S7 marker, and the mitochondrial marker revealed a hybrid origin for the analysed individuals (Figs. 2 - 5). The phylogenetic analyses based on both markers, CR and S7, showed *G. cynocephalus* to be the most divergent species (Lineage_XI). The systematics of the genus *Gobio* based on the analysis of the mitochondrial control region revealed a clustering of the mentioned lineages into two main clades with a strong bootstrap support (BP) and a significant Bayesian posterior probability (PP), exluding Lineages_XII and XIII (Fig. 5). The first major clade we designated Northern European, and according to the BI analysis with PP = 1.00, we subdivided it into two subclades: the Northwestern European (A) and the Northeastern European (B). The second major clade is designated Ponto-Caspian. We also subdivided this into two subclades; the Southern Ponto-Caspian (C) and the Northern Ponto-Caspian (D), being supported by a significant Bayesian posterior probability (PP = 1.00). However, the subdivision of the Northern European clade into subclades A and B is not supported by the other three statistical methods (MP, ML, NJ; data not shown) and the subclades C and D received only moderate support. In conclusion, without the division into subclades, the Northern European major clade was formed by the nominotypical Lineage_I, Lineage_II, Lineage_III, Lineage_IV, Lineage_V, Lineage_VI, Lineage_X, and Lineage_XIV. The Ponto-Caspian major clade was composed of the Transcaucasian Lineage_VII, two Turkey Lineages_VIII and IX, and two Northern Pontic Lineages_XII and XIII. Localities of common appearances of more lineages were revealed

and two hybrid zones were defined (Figs. 1, 7 and Table 1). Hybrid zone 1 is formed by the area of the upper Tisza tributaries (Belžanský stream, Ida River, Revištia River, Ulička River). Hybrid zone 2 is restricted to the Northern part of the Black Sea (the Southwestern part of the Crimean Peninsula and the Don River basin). The situation concerning hybridization of the gudgeons of the genus *Gobio* is very complicated and will therefore be specifically addressed in a subsequent article.

3.4 The first intron of the gene coding S7 r-protein as a diagnostic marker – S7indel diagnostics

The above presented results reveal the usefulness of the intron sequence for the evaluation of gudgeons taxonomy of the genus *Gobio*. In a more detailed comparison of nuclear sequences in the individual species we found the first intron S7 to be a suitable diagnostic marker. We found that this unencoding nuclear region contains numerous deletions and insertions (indels), which are responsible for the different lengths of amplified PCR products. Partial S7 alignment of regions related to gaps only, is shown in Figure 8. It documents the finding of 15 indels of different lengths, which always appear in at least two specimens of each lineage, except for the specimens from the Crimean Peninsula (the Chernaya River – 4549 and 4550). In this lineage only one specimen containing 7nt (seven nucleotide) insertion (gap-6) was detected. The combination of the presence or absence of gaps led to the differentiation of certain species and to the discovery of a fast, simple and cheap PCR diagnostic method – the "S7indel diagnostics". Different lengths of the PCR products in different species/subspecies are listed in Table 6. Individual diagnostic indels and their significance are displayed in the phylogenetic tree generated by the MP method (Fig. 4). The general benefit of this diagnostic method could be evaluated and verified only after the completion of the not yet examined species of the genus *Gobio*. If necessary, this technique can be further developed on the basis of primer position or RFLP method.

3.5. Phylogeny of Gobio, overview

The taxonomy of gudgeons in the Palaearctic zone still classified as the common gudgeon *G. gobio* by most authors (Bănărescu et al., 1999; Tsepkin, 2002; Golubtsov and Malkov, 2007) is an issue of great importance which is currently undergoing constant development. This is documented by numerous new species described over the last four years (Doadrio and

Madeira, 2004; Vasil'eva et al., 2004, 2005; Kottelat and Persat, 2005; Freyhof and Naseka, 2005; Naseka et al., 2006). In the past, Bănărescu and Nalbant (1973) recognized 19 subspecies in polytypical species *G. gobio* s. lato, and Bănărescu (1992a) documented only 17. In total, 149 nominal names were proposed for these fishes in scientific literature; the majority of them were later reevaluated as synonyms. Thus, aproximately 20 are now considered to be valid species at the present time (Froese and Pauly, 2007). In an effort to maintain transparency we will treat the individual analysed gudgeon taxa separately.

Gobio cynocephalus Dybowski, 1869

According to our molecular analyses, gudgeon specimens from the Zeya River (the Middle Amur River drainage) represent the most divergent group among the studied species/subspecies of the genus *Gobio*. The Amur River basin is inhabited by the Amur gudgeon *G. cynocephalus* Dybowski, 1869 s. stricto, described as a variety of the common gudgeon from the Onon and Ingoda rivers, belonging to the Upper Amur. Some authors consider the Amur gudgeon to be a subspecies of *G. gobio* distributed from Siberia to the Far East (Berg, 1949; Naseka, 1998; Tsepkin, 2002). In the phylogenetic tree (Figs. 4 - 6) the Amur gudgeon represents Lineage_XI in which one CR haplotype H_41 and one S7 nucleotype N_32 were detected (Figs. 2, 3). The sequence divergency towards the other analysed gudgeons ranged between 4.13 - 10.27% (mtDNA control region) and 3.63 - 6.12% (nDNA S7 intron). The actual values in comparison to the other lineages are listed in Table 5. The divergency of Lineage_XI is based mainly on substitutions, as it does not have any specific indel in the intron sequence of S7. In terms of the "S7indel diagnostics" Lineage_XI has the longest PCR product (371bp) and is therefore distinguishable from many other species (Table 6). Thus, our data supports the status of the Amur gudgeon as an independent species, and its relationship to gudgeons from Siberia needs further investigation.

Gudgeons of the major clade I - Northern European

Gobio gobio (Linnaeus, 1758) sensu stricto

The common gudgeon, *G. gobio* s. lato was claimed to have a large distribution area from the Iberian Peninsula and the British Islands to the Far East rivers (Berg, 1949; Bănărescu et al., 1999). The type specimen of *Cyprinus gobio* Linnaeus, 1758 is still unknown, and the type locality of the nominotypical subspecies of the common gudgeon, *G. gobio gobi*o, was supposedly located in the southeastern part of England (Bănărescu et al., 1999). Vasil'eva et

al. (2004) restricted the distribution of *G. gobio* s. stricto as a separate species to the Northeastern and Central Europe (from England to the Volga River basin in Russia), but Kottelat and Persat (2005) redescribed *G. gobio* and designated its neotype from the stream Sieg at Eitorf (Rhine River drainage in Germany). On the basis of our molecular analyses and comparison with the representatives from the Lark River in southeastern England (Table 1) we can prove that *G. gobio* s. stricto is widely distributed in the following river basins: Rhone (the Mediterranean Sea drainage area), Rhine (Lahn R.) and Elbe (Blanice R., D. Orlice R., Elbe R.) (the North Sea drainage area), Odra (Odra R., Stěnava R.; the Baltic Sea drainage area), Danube (Bečva R., Haná R.), Tisza (Ida R., Revištia channel), and Southern Bug (the Black Sea drainage area). Within the haplotype and nucleotype networks, nine mitochondrial haplotypes H_1 - 9 and four nucleotypes N_1 - 4 were distinguished (Figs. 2, 3). Haplotype H_1 is widespread. The representatives of this species form Lineage_I in the phylogram and they are part of subclade A – Northwestern European (Fig. 5). The common gudgeon showed a sequence pattern significantly different from other species/subspecies of the genus *Gobio.* This was shown by substitution within the mtDNA and by indels within the nDNA analyses (Figs. 4, 8 and Table 6). The important diagnostic feature of this species is the 18nt deletion (gap-12), which is present in this species only. The interspecific divergence of sequences in both markers, control region and S7, ranged between 1.34 - 8.30% and 1.42 - 7.18%, respectively (Table 5). The intraspecific divergence of sequences in both markers did not exceed 0.37%.

Besides the aforementioned four nucleotypes we found in this lineage two hybrid specimens from the Odra R. (2263, 2279) and one specimen from the Ida R. (2975) with nucleotypes closely related to Lineage_V (Figs. 2, 3). The hybrid origin of the other specimens highlighted in both networks (Bel'bek, Reviština; Figs. 2, 3 and Table 1) was proven, and is discussed below.

Gudgeons from the Danube River basin

The situation with the gudgeons from the Danube River basin is complicated due to the presence of a large number of lineages and also to reciprocal hybridzation. We found four pure lineages (L_I - IV) in this area. The first of them is L_I representing *G. gobio* s. stricto and is discussed above. Lineages III and IV are formed by Danubian samples only, whereas L_II is also represented in the Baltic Sea drainage. Three gudgeon taxa were described from

the Danube River basin; each of them is discussed further as available names for Lineages II – IV.

Gobio obtusirostris Valenciennes, 1842

This species was described from the Isar R. at Munich (München), Germany (the upper Danube R. basin). Vladykov (1925; 1931) considered it to be a valid subspecies of the common gudgeon and extended its distribution also to the middle and lower part of the Danube River basin, excluding the Tisza R. populated by another subspecies. Bănărescu (1961) presumed *G. gobio obtusirostris* to be the only subspecies of the common gudgeon occurring in the Danube River basin, but Bănărescu et al. (1999) began to doubt its validity. Freyhof and Naseka (2005) considered it a valid species. Our phylogenetic analyses confirmed the validity of the Danubian gudgeon being widely distributed in the Danube River basin (Bečva R., Bystrička R., Dyje R., Haná R., Ipel' R., Jevišovka R., Váh R.) and also occurring in the Odra River basin (Odra R.; Fig. 1 and Table 1). One nucleotype N_5 and five haplotypes H_10 – 14 were found for these populations, of which H_10 and H_13 occured most frequently (Figs. 2, 3). In the phylogram based on mtDNA analysis it is differentiated as monophyletic Lineage_II with strong support belonging to subclade B – Northeastern European (Fig. 5). The intraspecific sequence differences on both markers did not exceed 0.31% (Table 5). At the same time this lineage demonstrated high genetic differences in comparison to the common gudgeon lineage (L_I). Both sequence patterns on tested markers were significantly different, which resulted in high values of genetic divergence: 2.94% (CR) and 5.08% (S7) (Table 5). Moreover, Lineage_I and Lineage_II differed significantly in the nuclear marker, namely by numerous substitutions and especially by six indels with different length (Fig. 8). This means, in summary, that the S7 PCR product is 338 bp long in Lineage_I, and 364 bp long in Lineage_II (Table 6), which amounts to a difference of 26 nucleotides, and can easily be determined by electrophoresis. Finally, the areas of both lineages demonstrate significant overlapping (Fig. 1), but no hybridization between them was noted. This data leads us to conclude that the Danubian gudgeon is a separate valid species with the available name *G. obtusirostris*.

Gobio gobio carpathicus Vladykov, 1925, *G. gobio muresia* Jaszfalusi, 1951 and/or *Gobio* sp. 1

G. gobio carpathicus was described as a subspecies of the common gudgeon occurring in the Upper Tisza basin (middle Danube R. drainage) but differed from it and the Danubian subspecies *G. gobio obtusirostris* in several morphological characters (Vladykov, 1925). Berg

(1949) presumed that this subspecies may also occur in the Lower Danube. The type locality of another subspecies *G. gobio muresia* was designated at the Mures River near Gödemesterháza and at the confluence with the creeks Zebrak and Göde in Romania (Kottelat, 1997; the lower part of the Tisza River basin). Bănărescu (1961) concluded that both were synonyms of *G. g. obtusirostris*, whereas Freyhof and Naseka (2005) classified gudgeons from the Tisza and Mures rivers as *G. carpathicus.* Our phylogenetic analyses revealed two sympatric lineages in the Upper Tisza drainage: Lineage_III and Lineage_IV (Figs. 4 - 6 and Table 1), both included in the B – Northeastern European subclade in the CR phylogram (Fig. 5). Their genetic differences (Table 5) reach 1.69% (CR) and 2.70% (S7) concerning substitutions, and in terms of nDNA they differ in three indels (gap-2, gap-4, gap-11; Figs. 4, 8). However, the lengh of the S7 PCR products remains the same (Table 6). Of course, these two lineages are easily distinguishable from the nominotypical Lineage_I using "S7 diagnostics". We identified four haplotypes H_15 - 18 and one nucleotype N_6 of Lineage_III in the rivers Laborec and Top'lá, the Belžanský stream and four haplotypes H_19 - 22 and three nucleotypes N_7 - 9 of Lineage_IV in the rivers Dyje, Laborec, Tereshva, Ublianka and the Revištia channel (Figs. 2, 3). The intraspecific diversity in both lineages did not exceed 0.30%. Their ranges overlap significantly (Fig. 1), however, no hybrid individual of either Lineages_III × IV was observed up to the present time, even in the area of their sympatry (Fig. 7). At the same time the hybrid zone 1 was defined in the area of the Upper Tisza R. tributaries (Fig. 7) and the hybrid origin was proven for several individuals highlighted in the haplotype network. We found hybrid specimens between the following lineages: L_I × L_III, L_I × L_IV, L_III × L_V and L_IV × L_V (Figs. 2, 3 and Table 1).

Thus, our data can lead us to assume the presence of two independent species in the Tisza R. basin, but their relationship to the aforementioned nominal names needs further investigation, including the genetic and morphological studies of type specimens. Since the location of the type specimens of *G. gobio muresia* is still unknown (Kottelat, 1997), its validity or conspecificity with *G. carpathicus* will remain problematic. These conclusions from the genetic analysis of the type specimens of *G. carpathicus* seem plausible, as well as the comparative morphological study of these syntypes and voucher specimens for both lineages L_III and L_IV. Our study should prove the availability of the name *carpathicus* for one of the two lineages. In this work we will initially designate it for L_IV in concordance with the study of Freyhof and Naseka (2005), who describe the occurence of this species in the same localities. L_III is designated as *Gobio* sp. 1 – "species-in-waiting".

Gudgeons from the Volga River basin

Previously, gudgeons from the Volga R. basin were classified as the common gudgeon, *G. gobio* or its nominotypical subspecies (Berg, 1949; Bănărescu, 1961; Naseka, 1998; Bănărescu et al., 1999; Tsepkin, 2002; Ruchin and Naseka, 2003; Vasil'eva et al., 2004; Vasil'eva and Kuga, 2005; Freyhof and Naseka, 2005). In the phylogenetic tree (Figs. 4 - 6), the specimens from the Volga River basin form the monophyletic Lineage_VI, belonging to subclade B – Northeastern European. We identified one haplotype H_24 and two nucleotypes N_18 – 19 in specimens from the rivers Bol'shaya Lašva, Chardym, Malaya Tsivil', Moskva and Sura (Table 1 and Figs. 2, 3). The interpopulation divergency did not exceed 0.60% on the S7 marker. The interspecific divergency of the control region and S7 sequences ranged between 0.94 - 9.00% and 3.00 - 7.68%, respectively (Table 5). This lineage is separated from other lineages predominately by substitutions. The "S7indel diagnostics" is able to distinguish it from several gudgeon lineages, including Lineage_I, representing the common gudgeon (Table 6 and Fig. 8). Our results prove that gudgeons from the Volga R. basin should be classified as a separate species. According to a previous study (Vasil'eva et al., 2004) this species is very similar to the common gudgeon in external morphology, and thus should be considered as a cryptic species. We did not find any available name for it in previous publications and consequently describe it as a new species in this paper (see appendix).

Gudgeons from the Ohrid-Drim-Skadar hydrologic system (the Adriatic Sea drainage)

Two local gudgeon forms and one subspecies were described from this largest hydrologic system in the western Balkan zoogeographic region (Bănărescu, 1992b): *G. gobio* var. *ohridana* Karamanm 1924 from Ohrid Lake, *G. gobio lepidolaemus* form *skadarensis* Karaman, 1936 from Skadar Lake, and *G. gobio albanicus* Oliva, 1961 from the Kiri R. (the Drin R. system) in Albania. Futher comparative morphological and meristic analyses showed that all gudgeons within the Ohrid-Drim-Skadar system are conspecific (Grupče and Dimovski, 1975; Šorić and Ilić, 1988). Some observed differences related to different ecological conditions in each locality, but did not exceed the range of interpopulation variability (Šorić, 1990). Therefore, most authors consider *G. gobio ohridanus* as the only valid subspecies of the common gudgeon in this area (Grupče and Dimovski, 1975; Šorić and Ilić, 1988; Šorić, 1990; Bănărescu, 1992a; Bănărescu et al., 1999). In constrast to this opinion, Šanda et al. (2005) consider *G. g. ohridanus* to be a junior synonym of *G. gobio gobio* based

on the results from allozyme analysis. We studied gudgeon populations related to both the *ohridanus* and *skadarensis* nominal names.

Gobio ohridanus Karaman, 1924

The Ohrid Lake is the type locality of this taxon. Our phylogenetic analyses proved its validity and extended the range: besides the Ohrid Lake we also noted its occurrence in the Albanian Mat River (Table 1 and Fig. 1). In the phylogram, this species is marked as the monophyletic Lineage_X (Figs. 4 - 6), which is part of subclade B – Northeastern European, and contains four haplotypes H_37 - 40 and one nucleotype N_31 (Figs. 2, 3). The intraspecific divergence did not exceed 0.48%. The divergence from other gudgeon taxa for both markers, control region and S7, ranged between 1.31 - 8.07% and 0.93 - 5.95%, respectively, with the lowest values compared with Lineage_XIV (Table 5). This lineage can be distinguished from the other gudgeon lineages entirely on the basis of substitutions and 3nt deletion (gap-7), which can be also found in Lineage_I (Figs. 4, 8) representing the common gudgeon. Despite this common genetic feature, L_X demonstrates the high level of sequence divergence from L_I in both markers - 2.98% (CR) and 5.33% (S7) (Table 5) and can be clearly differentiated from *G. gobio* s. stricto and several other species by "S7indel diagnostics" (Table 6).

Gobio skadarensis Karaman, 1936

The local gudgeon form *skadarensis* was described from the Skutari or Skadar Lake (the Drim River basin) in present Montenegro and Albania. Bănărescu et al. (1999) noted that this form should be considered a synonym of *G. g. ohridanus*, but its "true taxonomic status" needs additional investigation. Furthermore, they listed morphological differences between populations from the Ohrid and Skadar systems, but noted that the biological differences were greater than the meristic ones. Namely, gudgeons from the Skadar Lake spawn in fast running streams flowing into the lake, whereas gudgeons from the Ohrid Lake always remain in a lacustrine habitat. We studied specimens from the Zeta River, the largest tributary of the Morača River, the main tributary of the Skadar Lake flowing into its northwestern section. These specimens are included into subclade B – Northeastern European – and form the monophyletic Lineage_XIV (Fig. 5), in which four haplotypes H_45 - 47 and two nucleotypes N_36 - 37 were found (Figs. 2, 3). In terms of the common alignment of individuals from the area of the Ohrid-Drim-Skadar system, the identical sequence pattern was also found in the Albanian Mat River where gudgeons from the Ohrid Lake (Lineage_X) were noted (Table 1

and Fig. 1). Nevertheless no hybridization between L_XIV and L_X was observed in the zone of sympatry. In comparison with the common gudgeon, (L_I) a more distinctive sequence divergence was found in the S7 intron (5.23%) and in CR (1.85%). The intraspecific variability in both markers did not exceed 0.27% (Table 5). The 3nt deletion is characteristic (gap-13; Figs. 4, 8) for the gudgeons from the Skadar Lake (gap-13; Figs. 4, 8) and they can be easily discriminated from the nominotypical Lineage_I by "S7indel diagnostics". However, the length of their PCR products is identical with the gudgeons from the Ohrid Lake (Table 6). All these results prove *G. skadarensis* to be a valid species.

Gudgeons of the major clade II – Ponto-Caspian, and Turkish gudgeons with European relations

Gudgeons from Turkey

The Turkish area is of great interest since it belongs to one of the most important glacial refuges, the Ponto-Caspian (Bănărescu, 1991), and represents a region with a high variability of gudgeon populations resulting in the identification of eight species/subspecies of the genus *Gobio* (Erk'akan et al., 2005; Naseka et al., 2006). Our analyses revealed three different monophyletic lineages from this area with strong support. On the basis of mitochondrial analysis, one of the lineages (Lineage_V) was assigned to subclade A – Northwestern European, and two lineages (Lineage_VIII and Lineage_IX) were assigned to subclade C – Southern Ponto-Caspian (Fig. 2). They can be distinguished from each other by substitutions in both markers, as well as by indels in the nuclear marker S7 (Figs. 4, 8). Sequence differences are shown in Table 5.

Gobio sp. 2

Specimens of Lineage_V (Figs. 4 - 6) come from Northwest Anatolia (Bakacak deresi, Biga; Fig. 1). Erk'akan et al. (2005) presumed this area to be populated by the nominotypical species *G. gobio*. One haplotype H_23 and two nucleotypes N_14, 15 were identified within this lineage. Surprisingly, some closely related nucleotypes were also found in individuals from the Tisza River basin (Ida R., Ulička R. and Belžan stream; N_10 - 13) and also the Odra River (N_16, 17). However, no pure Lineage_V was found, but hybridization between lineages L_I × V, L_IV × V, L_III × V and L_I × V, respectively, was proved for these specimens (Figs. 2, 3, 7 and Table 1). The intraspecific sequence divergence in Lineage_V did not exceed 0.45%. This lineage shows certain similarity with the nominotypical Lineage_I

(CR: 1.34% and S7: 1.70%; Table 5) and belongs to the same subclade A (Fig. 5) as well, but two deletions (gap-7 and gap-12), which are typical for the common gudgeon were not found in the Turkish lineage (Figs. 4, 8). Therefore Lineages_I and V are easy distinguishable by "S7 diagnostics" (Table 6) and it is evident that they are not conspecific. Freyhof and Naseka (2005) classified gudgeons from the Meria R. in the European part of Turkey as *G. bulgaricus* Drensky, 1926 that were originally described from the Maritza River (Southern Bulgaria). Since gudgeon populations from the Maritza R. basin were not studied genetically, we refrain from any conclusion on the availability of the name *bulgaricus* for gudgeons from our Anatolian Lineage_V and designate it as *Gobio* sp. 2, a species which needs a comprehensive revision. Moreover, it is neccessary to pay greater attention to the extent of the distribution of this species with regard to the hybridization found between individuals in the middle Danube River basin and the Odra River.

Gobio insuyanus Ladiges, 1960

The specimens of the second distinct Turkish Lineage_VIII originate from Central Anatolia, specifically from three localities, the Ayranci Dam Lake, the Sugla River, and the Insuyu Stream (Fig. 1 and Table 1). The last of these is the type locality of *G. gobio insuyanus* (Erk'akan et al., 2005; Naseka et al. 2006). Seven haplotypes H_29 - 35 and three nucleotypes N_26 - 28 were found in this lineage (Figs. 2, 3). The intraspecific diversity did not exceed 0.30%, the values of genetic divergence from L_V were high for both markers (Table 5). Figures 4, 8 and Table 6 show the existence of the typical gap-9 together with the "S7 diagnostics". These results prove the specific status of *G. insuyanus* and also extend its occurence outside of the type locality.

Gobio sp. 3

Gudgeons from the locality Bilecik in northwestern Anatolia, form the third Turkish monophyletic Lineage_IX with haplotype H_36 and nucleotypes N_29 - 30 (Table 1 and Figs. 1 - 6) with an intraspecific diversity about 0.30% (Table 5). The aforementioned locality belongs to the area of the Sakarya River basin, where the occurrence of the subspecies *G. g. obtusirostris* was previously recorded (Erk'akan et al., 2005). However, Lineage_IX significantly differs from Lineage_II in both molecular markers: by 9.53% (CR) and 2.81% (S7). Moreover, due to the "S7 diagnostics," L_IX is easily distinguishable, as the PCR products from this lineage are longer by 5 nucleotides (Table 6). Thus Lineages_II and IX

obviously represent different species, and we have designated the latter as *Gobio* sp. 3, "species-in-waiting" until a comprehensive revision can be undertaken.

Gudgeons from the northeastern coast of the Black Sea and the Crimean Peninsula

Phylogenetic analyses revealed four separate lineages of different origin among samples collected in this area. Only one of them, Lineage_VII, represents an unquestionably pure lineage with its own mitochondrial and nuclear patterns. On the contrary, lineages L_XII and L_XIII demonstrated an ambiguous situation with an indefinite origin of the CR pattern. Therefore, we used the label L_XII/XIII in Table 1 and Figs. 2, 5. The fourth lineage L_XV includes specimens demonstrated to be of hybrid origin.

Gobio caucasicus Kamensky, 1901

The specimens examined from the northeastern coast of the Black Sea (Fig. 1) form the monophyletic Transcaucasian Lineage_VII in the phylogram (Figs. 4 - 6), which belongs to subclade D – Northern Ponto-Caspian (Fig. 4). Four haplotypes H_25 - 28 and four nucleotypes N_20 - 23 were determined for this group. The intraspecific diversity on both markers did not exceed 0.55%, and the lowest values of genetic dissimilarity by S7 marker were observed with L_XIII and L_I (Table 5). However, Lineage_VII significantly differs from both of these lineages with the absence of certain specific indels inluding gaps 14, 7 and 12 which specify L_ XIII and L_I, respectively (Figs. 4, 8). "S7indel diagnostics" differentiates these lineages within subclade D (excluding Lineage_XV), as well as from other gudgeon lineages (Figs. 4, 8 and Table 6). These results demonstrate gudgeons from Lineage VII to be a distinct species. Freyhof and Naseka (2005) stated that rivers from the Black Sea basin in Krasnodar province (including rivers presented in this study) are populated by the Caucasian gudgeon *G. caucasicus* which is described as a variety *caucasica* of the Central Asian gudgeon *G. lepidolaemus* Kessler, 1872 from both the Caspian Sea (Podkumok and Sulak rivers) and the Black Sea (Rioni R. system) basins (see Kottelat, 1997). We are not sure of the conspecificity of the Caspian and Black Sea populations, but we presume to use *G. caucasicus* as an available name for gudgeon species represented by the phylogenetic Lineage _VII.

Gobio brevicirris Fowler, 1976

Freyhof and Naseka (2005) classified gudgeons from the Don River drainage as a distinct species *G. brevicirris.* This name became available after Fowler (1976) concluded *G. gobio* morpha *brevicirris* Berg, 1932 to be a valid subspecies *G. gobio brevicirris* distributed in Ukraine and Russia (see Kottelat, 1997). The specimens from the Don River basin (Sosna R.; Fig. 1) subjected to molecular analyses represent the second Northern Pontic lineage (subclade D; Fig. 5) designated as Lineage_XIII in the phylogram (Figs. 4, 6). This lineage is characterised by the haplotype H_44 and unique nucleotypes N_34 - 35 (Table 1 and Figs. 2, 3) which differ from each other by 0.26%, but at the same time demonstrate significant divergence from nucleotypes of other gudgeon groups due to substitutions and a significant long 22nt deletion not found in any other lineages (gap-14; Figs. 4, 8). "S7indel diagnostics" enables us to distinguish this lineage and Lineage XII specifically, (Table 6) despite their ambiguous species status of CR patterns. These results confirm the validity of the Don gudgeon *G. brevicirris*. Further analyses will be neccessary to resolve the lineage classification of the haplotype H_44 (L_XII/XIII; Fig. 2 and Table 1).

Gobio tauricus Vasil'eva, 2005 and *Gobio delyamurei* Freyhof & Naseka, 2005

The molecular analysis of specimens from the Chernaya River raises complicated questions about the taxonomic status of gudgeon populations in the western part of the Crimean Peninsula. The specimen labeled as Chernaya4549 had haplotype H_42 and a unique nucleotype N_33, whereas the specimen labeled as Chernaya4550 had a similar haplotype H_43 but a different nucleotype N_25, which is related to nucleotypes of Lineage_VII (Table 1 and Figs. 2, 3). Thus, the sequence divergences between these specimens reached 0.81% and 1.74% on mtDNA and nDNA markers respectively, and the percentage sequence divergence on the last marker reached the same value from both Ponto-Caspian lineages VII and XIII (Table 5). This increased "interindividual" difference results from the presence of 7nt insertion (gap-6; Figs. 4, 8) in the nucleotype N_33, not found in other gudgeon lineages. In accordance to the aforementioned differences, the specimen Chernaya4549 represents a distinct Lineage XII in the phylograms (Figs. 4 - 6) and is easy distinguishable by "S7indel diagnostics" from the specimen Chernaya4550, in which the PCR product has the same length as the PCR product of the Lineage_VII (Table 6). We will refrain from classifying the latter case and leave this question open for the present (Table 1 and Figs. 2, 3, 5 - 7). The above mentioned data indicates the genetic and taxonomic heterogeneity of gudgeons from the Chernaya River. This conclusion agrees with obvious morphological heterogeneity observed in samples used by Freyhof and Naseka (2005) in their description of *G. delyamurei*, and also

with our new materials collected from the same river this year. In accordance with the molecular data we surmise that the present gudgeon population in the Chernaya River results from hybridization between native species (Lineage XII) and from species having penetrated into the river during recent years as a result of acclimatization and irrigation activity in the Crimean Peninsula. The native species was subjected to karyological and craniological analyses based on specimens collected in the Chernaya River in 1981. The karyological and craniological peculiarities of these gudgeons are the main grounds for the description of a new species *G. tauricus* and were presented as its main diagnostic characters (Vasil'eva et al., 2005). Thus we classify the specimen Chernaya4549 as *G. tauricus,* despite the hybrid origin of several type specimens identified by molecular analyses (ICZN, 1999, art. 17). At the same time, the morphological characters of the holotype of *G. delyamurei* presented by Freyhof and Naseka (2005) allow consideration of this specimen to not be conspecific to native gudgeons that have been distributed in the Chernaya R. in the past. This situation with gudgeons in the Chernaya R. is very complicated and also needs further investigation.

Gobio krymensis Bănărescu & Nalbant, 1973

In contrast to previous Ponto-Caspian lineages, the molecular analyses of gudgeons from the Bel'bek River in the Steppe Crimea (Fig. 1) labeled as Bel'bek4605 and Bel'bek4607 revealed a specifically mixed origin. Bel'bek4607 had haplotype H_9 closely related to haplotypes from Lineage_I, and nucleotype N_38 was devoid of any characteristic insertions or deletions, but differed from others by numerous substitutions (Table 1 and Figs. 2, 3, 8). Therefore this specimen forms (based on S7 marker analysis) a separate Lineage_XV in phylograms (Figs. 4, 6). According to obtained data, we concluded that this specimen most probably represents a hybrid between a female from L_I and a male of another species with nucleotype N_38 distributed in the Bel'bek River. The other specimen from this sample (Bel'bek4605) had haplotype H_1 and nucleotype N_24 and should be considered as a hybrid between a female from Lineage_I and a male from another phylogenetic lineage with CR haplotypes most related to the ones of Lineage_VII (similar to the aforementioned hybrid specimen from the Chernaya River, 4550). The S7 sequence variability of both the representatives from the Bel'bek River was 2.24%. Their differentiation is based mainly on substitutions and can therefore not be detected by "S7indel diagnostics" (Table 6).

We refrain from the classification of males having participated in the aforementioned hybridization as members of Lineage_VII representing *G. caucasicus* since the hybridization between this species and Crimean gudgeons seems impossible due to their geographic

isolation. At the same time, arrangements of acclimatization and also irrigation in the Crimean Peninsula indicate there may have been an accidental introduction (and further distribution) of gudgeons from the Dnieper River basin, which were not subject to molecular studies. Therefore it is quite possible that at least one of the nucleotypes N_24 or N_25 belong to *G. sarmaticus* Berg, 1949 distributed in the Dnieper R. basin. As to an available name for the pure Lineage_XV distributed in the Steppe Crimea, we are inclined towards Freyhof and Naseka (2005), who classify these gudgeons as independent species *G. krymensis*. High levels of genetic divergence between populations from neighbouring river systems revealed in the recent study lead us to assume the validity of the mentioned species. Thus, the above stated data indicates that the situation with gudgeons from the Crimean Peninsula is extremely complicated and requires more exhaustive analyses, both morphological and genetic.

3.6. Phylogeography, genetic aspects and taxonomic implications

The aim of this phylogenetic study is to bring up new findings in the field of genetics and the phylogeography of the genus *Gobio* and to throw light on the current and rather complicated taxonomy and systematics of this genus. We attempted a more comprehensive molecular approach based on combinations of both mitochondrial and nuclear genomic markers. On the basis of sequence analyses of the material collected at type localities or in their close surroundings and on the background of the data from literature, we have arrived at the following findings and conclusions. The gudgeons of the genus *Gobio* show a large scale of haplo- and nucleotype patterns, which also exhibit a large distribution spectrum ranging from small areas (*G. ohridanus*, *G. skadarensis*, some Turkish gudgeons, etc.) to vast territories covering thousands of kilometres, e.g. extending from the British Isles to the Black Sea as in the case of the nominotypical species *G. gobio* s. stricto. Localities represented by several monophyletic lineages (Bečva R., Dyje R., Haná R., Odra R., Revištia ch., Bel'bek R., etc.; Table 1 and Fig. 1) demonstrate the sympatry of several different species (*G. gobio* and *G. obtusirostris*, gudgeons from the Tisza River basin, etc.), which leads to problems in their identification, especially due to observed hybridization events (Fig. 7). Our analysis reveals that the cytonuclear disequilibrium is a common phenomenon among gudgeons of the genus *Gobio* (Table 1). Altogether, 11 specific cases were found (Table 1 and Figs. 2, 3). The phylogeography of different species, including the zones of their sympatry, as well as two revealed zones of hybridization are shown in Figs. 1, 7. We surmise that the existence of many closely related species (without an apparent reproductive barrier) living together in the

same proximity presents the likelihood of frequent hybridization or introgresive hybridization. We consider this phenomen to be one of the principal reasons for the wide variability of gudgeons, mentioned by most previous authors. The question whether or not specimens of hybrid origin form a numerous, viable and spawning lineage can be answered only after further investigation.

The above-mentioned results, leads also to the conclusion that data resulting from phylogenetic studies based on mtDNA analyses only, is not sufficient for taxonomical reconstructions. The application of a suitable nDNA locus in combination with the mtDNA marker provides more useful tools to answer systematic questions. In addition to employed molecular methods we discovered a new and promising method called "S7indel diagnostics" which is based on different lengths of the PCR products in most studied gudgeon lineages and therefore allows for a more simple identification of species of the genus *Gobio* undistinguishable by traditional morphological characters; for example, *G. gobio* and the new species *G. volgensis*. We presume that further investigations of gudgeons from different parts of the generic distribution will illustrate the convenience of this method with regard to the taxonomy.

The phylogentic analyses based on both mtDNA and nDNA markers confirm the validity of the genus *Gobio* as a monophyletic group with strong support, similarly mentioned by Yang et al. (2006) and Mendel et al. (2007). Altogether 15 gudgeon lineages are distinguishable in this genus, most of them identified as pure species. The phylogenetic relations obtained by control region analysis and applied statistical methods (NJ, MP, ML, BI) demonstrate these lineages are divided into two main clades, namely, the Northern European and the Ponto-Caspian. According to the BI analysis, the first clade was subdivided into two subclades – Northwestern European (A) and Northeastern European (B). The second main clade was subdivided into the Southern Ponto-Caspian (C) and the Northern Ponto-Caspian (D). These results agree with previous zoogeographic data.

The molecular analyses confirmed the validity of 11 taxa as independent species of the genus *Gobio*, namely *G. gobio*, *G. obtusirostris*, *G. carpathicus*, *G. caucasicus*, *G. insuyanus*, *G. ohridanus*, *G. skadarensis*, *G. cynocephalus*, *G. brevicirris, G. tauricus*, and G. *krymensis*. Their genetic diagnostic characters were revealed, as well as the nucleotype expected for *G. sarmaticus*. Based on these studies, gudgeons from the Volga River basin were separated from *G. gobio* s. stricto and described as a new species *G. volgensis*. Moreover, three phylogenetic lineages designated as *Gobio* sp. 1 - 3 were submitted for a comprehensive revision owing to their description/redescription as separate species. Thus, this study opens a

new page for the recent taxonomy of the genus. At the same time, the complicated situation concerning gudgeons from the Crimean Peninsula, in which specimens showed the hybridization between four phylogenetic lineages (L_I, L_XII/XIII, L_XV, and L_?; Table 1), needs a more detailed investigation to define species participating in hybridization events and to establish further steps for the conservation of endemic native gudgeon species.

Acknowledgments

The study was carried out within the framework of the research project no. VaV-SM/6/3/05 supported by the Grant from the Ministry of Environment of the Czech Republic and by the Grant project no. IAA6045005 from the Grant Agency of the Academy of Sciences of the Czech Republic. Our investigations in Russia were partially supported by RAS Programs “Biodiversity” and “Biological resources of Russia”. The investigations in Albania and Montenegro were supported by the Grant project no. MK00002327201 of the Czech Ministry of Culture. We are thankful to C. S. Özeren, G. Ekmekci, J. Taggart, N. G. Bogutskaya for their help with the obtaining of samples. We thank G. Speas for English correction.

Appendix. Description of new species

***Gobio volgensis* Vasil'eva, Mendel, Vasil'ev, Lusk, Lusková sp. nova**

Cyprinus gobio (not of Linnaeus, 1758): Pallas, 1814: 295 (part.: Volga R. basin).

Gobio fluviatilis (not of Fleming, 1828): Cuvier in Cuvier, Valenciennes, 1842: 300 (Europe - part.); Kessler, 1877: 251 (part.: Eastern Europe - part.).

Gobio gobio (not of Linnaeus, 1758): Berg, 1914: 428 (part.: Europe - part.); Berg, 1916: 218 (part.: Europe - part.); Lukash, 1923: 174, 1976 (Vychegda); Lukash, 1933: 56 (rivers Vyatka, Voya, Iryuk); Lukash, 1940: 26 (Vyatka and Kama basins); Berg, 1949: 640 (part.: Europe - part.); Bănărescu, 1992a: 317 (part.: Caspian Sea basin); Naseka, 1998: 82 (part.: European part of Russia - part.); Bănărescu et al., 1999: 81 (part.: Europe - part.); Tsepkin, 2002: 249 (part.: Europe - part.); Ruchin and Naseka, 2003: 334-335 (Sura R.); Vasil'eva et al., 2004: 772 (part.: Volga R. basin). Freyhof and Naseka, 2005: 336 (part.: Sura, Volga).

Gobio gobio gobio (not of Linnaeus, 1758): Bănărescu et al., 1999: 109 (part.); Ruchin and Naseka, 2003: 334 (Volga).

Holotype. ZMMU P-21861, SL 91.5 mm, TL 109.0 mm, the Moskva River at Staraya Ruza City, Moskovskaya District; collector V.P. Vasil'ev, 2004, August 21.

Paratypes. ZMMU P-21865, 4 spec., SL 64.2 - 86.0 mm, TL 76.5 - 103.0 mm the Moskva River at Zvenigorod City, Moskovskaya District; collector V.P. Vasil'ev, 2005, June 14; P-21910, 4 spec., SL 46.5 - 66.0 mm, TL 57.0 - 78.0 mm the Moskva R. at Zvenigorod City, collector V.P. Vasil'ev, 2004, June 05, voucher specimens for this molecular study.

Additional materials. The Moskva R. basin: P-442 (5 spec.), P-2705 (1 spec.), P-16229 (4 spec.), P-16819 (1 spec.), P-17966 (2 spec.), P-21235 (9 spec.), P-21413 (1 spec.), P-21422 (10 spec.), P-21426 (5 spec.). The Volga R. basin: P-3441 (Moksha R., 45 spec.), P-4164 (Oka R., 3 spec.), P-9561 (Ozerna R., 1 spec.), P-21040 (Sura R., 1 spec.), P-21234 (Kobra R., 7 spec.), P-21206 (Vytebet' R., 1 spec.), P-21236 (Mytets R., 5 spec.), P-21844 (Sura R., 3 spec.).

Comparative materials on *Gobio gobio* s. stricto. England: P-9423 (Thames R. at Reading, 1 spec.). The Baltic Sea basin: P-2762 (Neman R., Lithuania, 4 spec.), P-13033 (pound Vira at Třeboň, Southern Bohemia, 1 spec.), P-19034 (Ahja R., South Estonia, 1 spec.).

Diagnosis. *D* (II) III (7) 8; *A* II (III) 6 - 7; *V* I (II) 7(8); *P* I (14) 15-16; *l.l.* 40 - 43, usually 42 - 43; the body and the caudal peduncle are moderately compressed; the minimum body depth is somewhat smaller than the width of the caudal peduncle at the level of the last anal ray in larger specimens and somewhat greater in smaller fishes; the anus is closer to the insertion of the anal fin than to the origin of the pelvic fins; there are no epithelial crests on the dorsal scales and there are also barbellike prolongations at the corners of the mouth; barbels are moderately long: they usually extend beyond the anterior edge of the eye (only rarely do they not reach anterior eye edge), sometimes reaching up to the middle of the eye, but never reach to its posterior edge; the barbel length varies from 15 to 28 % of the head length with modal values between 21 - 22 %; paired fins are moderately long: pectoral fins never reach the pelvic fin insertion, and their average length varies from 74.7 to 84.8 % of the distance between the base of paired fins; ventral fins never reach the anal fin insertion, and their average length varies from 72.8 to 75.7 % of the distance between ventral and anal fin bases; there are large, more or less rounded, dark spots located along the lateral line and several rows of small dark spots on the dorsal and caudal fins; the eye is large with a diameter greater than ¾ of the interorbital distance; the breast in front of the level of the rear extent of the pelvic fin insertions usually lacks scales; the lateral branch of the supraorbital cephalic sensory canal (*CSO*) is connected with the infraorbital canal behind the eye; there are usually 7 pores in the fronto-parietal area of CSO and 5 pores in the pteroticum; both supra- and infraorbital bones are wide: the average width of the supraorbital bone exceeds 40 % of its length, and the average width of the last infraorbitals comes to more than half of the bone length; 2n=50 (24 meta-, 24 submeta-, 2 subtelo-acrocentric chromosomes), NF=98; the total number of vertebrae (according to Naseka, 2001) – 40 (caudal – 19, preanal caudal – 2, abdominal – 21).

Other morphological features. Morphometric characters of gudgeons from the Sura River have been presented earlier by Ruchin and Naseka (2003). The variability of the relative length of paired fins among different populations from the Volga River basin, as well as the karyotype of specimens from the Yakot' River (Volga R. basin) were described by Vasil'eva et al. (2004). The craniological features and indices were demonstrated for gudgeons from the Yakot' R. (Vasil'eva et al., 2004; Vasil'eva and Kuga, 2005).

Distribution. According to our molecular data we have restricted the range of this species to the Volga River basin only. Its occurrence in neighbouring river systems needs further investigation.

Etymology. The name *volgensis* refers to the range of the species.

Comparative remarks. *G. volgensis* differs from most of the species previously included in *G. gobio* s. lato with the complex of features presented in the diagnosis, but, as mentioned previously, this species is very similar to *G. gobio* s. stricto in its external morphology. According to our preliminary study *G. gobio* (we examined several specimens of this species for comparison) differs due to the smaller average number of lateral line pored scales. The analysis of karyotypes presented by different authors for *G. gobio* s. lato reveals that the karyotype of *G. volgensis* obviously differed from karyotypes of such species as *G. tauricus* Vasil'eva, 2005 and *G. kubanicus* Vasil'eva et Vasil'ev, 2004, but was quite similar to karyotypes obtained from gudgeons from the Odra R. basin, Lower Danube R. and Garonna R. (Hafez et al., 1978; Vujošević et al., 1983; Raicu et al., 1996; Kirtiklis et al., 2005). This result indicates karyological similarity between *G. gobio* and *G. volgensis*. Thus, the last taxon represents a cryptic species distinguishing from *G. gobio* only by molecular analysis and "S7indel diagnostics".

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Table 1
Species examined in this study, source of tissue samples used, number of haplotype/nucleotype and GenBank Accession numbers

Species/lineage	[Locality Nos.] River, drainage, country (NCRH/NS, NS7N/NS)	Accession Nos.
Outgroups		
R. albipinnatus	R. Moksha-Mordovia, Volga, RUS	EF427390, EF427408
R. frici	R. Hornád at Košice, Tisza, SK	EF427392, EF427414
*R. ocellatus**	unknown	AY017149, AY325789
*S. microoculus**	unknown	NC_004694
Ingroup taxa		
Lineage_I *G. gobio* s. stricto	[1]R. Bečva at Rybáře, Danube, CZ (H1/1, H2/2); [2]R. Blanice at Vlašim and Vodňany, Elbe, CZ (H1/5); [3]R. D. Orlice at Kostelec n. Orlicí, Elbe, CZ (H1/3, H4/1, H5/1); [4]R. Elbe at Neratovice and Srnojedy, Elbe, CZ (H1/3, N1/1, N2/2, N3/1, N4/1); [5]R. Haná at Vyškov, Danube, CZ (H1/2); [6]R. Lahn, Rhine, D (H1/3, H6/2); [7]R. Lark near Isleham, Great Ouse, UK (H1/2, N2/1); [8]R. Odra at Odry, Odra, CZ (H2/1, H3/1); [9]Revištia channel, Tisza, SK (H1/2, N2/1); [10]Rhone basin, FR* (H1/1); [11]R. S. Bug, UA (H1/3, H7/1, H8/1); [12]R. Stěnava at Broumov, Odra, CZ (H1/3)	EU131542, EU131543, EU131544, EU131545, EU131546, EU131547, EU131548, EU131550, EU131589, EU131590, EU131591, EU131592
Lineage_II *G. obtusirostris*	[1]R. Bečva at Rybáře, Danube, CZ (H13/1, H14/1); [13]R. Bystrička at Martin, Danube, SK (H10/2, H11/1, H13/2); [14]R. Dyje at Soutok, Danube, CZ (H10/2, N5/2); [5]R. Haná at Vyškov, Danube, CZ (H10/1, H13/2); [15]R.Ipel' at st. border, Danube, SK (H10/4, H12/1); [16]R. Jevišovka at Božice, Danube, CZ (H10/1); [8]R.Odra at Odry, Odra, CZ (H13/1); [17]R. Váh at Hlohovec, Danube, SK (H13/5, N5/2)	EU131554, EU131555, EU131556, EU131557, EU131558, EU131607
Lineage_III *Gobio* sp. 1	[18]Belžan Stream, Tisza, SK (H15/2, H18/2); [19]R.Laborec at Kochánovce, Tisza, SK (H16/1); [20]R. Topl'a at Poliakovce, Tisza, SK (H16/5, N6/1)	EU131563, EU131564, EU131565, EU131603
Lineage_IV *G. carpathicus*	[14]R. Dyje at Soutok, Danube, CZ (H19/1); [19]R. Laborec at Kochánovce, Tisza, SK (H19/3, N8/1); [9]Revištia channel, Tisza, SK (H19/1); [21]R. Tereshva at Krive, Tisza, UA (H21/1, N7/1); [22]R. Ublianka at Ubl'a, Tisza, SK (H22/1, N7/1, N9/1);	EU131559, EU131560, EU131561, EU131604, EU131605, EU131606
Lineage_V *Gobio* sp. 2	[24]Bakacak deresi - Biga, TR (H23/1, N14/1, N15/2)	EU131551, EU131593, EU131594
Lineage_VI *G. volgensis*	[25]R. Bol'shaya Lašva, Perm District, Volga, RUS (N18/1, N19/1); [26]R.Chardym at Aryash, Volga, RUS (N18/2); [27]R. Malaya Tsivil' at Shichazany, Volga, RUS (N18/1); [28]R. Moskva at Zvenigorod, Volga, RUS (H24/1, N18/1); [29]R. Sura at Nikolaevka, Volga, RUS (N18/3)	EU131566, EU131613, EU131614
Lineage_VII *G. caucasicus*	[30]R. Ashe, Sochi region, Karsnodar district, RUS (H27/1, N20/1, N23/1); [31]R. Mzymta at Adler, Krasnodar district, RUS (H25/1, H26/1, N21/1); [32]R. Shakhe, Sochi region, Krasnodar district, RUS (H28/1, N20/2, N22/1)	EU131584, EU131585, EU131586, EU131587, EU131615, EU131616, EU131617, EU131618
Lineage_VIII *G. insuyanus*	[33]Ayranci Dam Lake at Karaman, TR (H33/1, H34/1, H35/1, N26/2, N27/1); [34]R. Insuyu at Cihanbeyli, Tuz Lake, TR (H29/2, H30/1, H31/1, H32/1, N27/5); [35]R. Sugla at Seydisheir, TR (N26/1, N28/1)	EU131574, EU131575, EU131576, EU131577, EU131578, EU131579, EU131580, EU131621, EU131622, EU131623
Lineage_IX *Gobio* sp. 3	[36]Bilecik, TR (H36/1, N29/1, N30/1)	EU131581, EU131624, EU131625
Lineage X *G. ohridanus*	[37]R. Mat at Milot, AL (H39/1); [38]Lake Ohrid, Drin, AL (H37/1, H38/1, H40/1, N31/3)	EU131570, EU131571, EU131572, EU131573, EU131626
Lineage_XI *G. cynocephalus*	[39]R. Zeya at Blagoveshchensk, Amur, RUS (H41/1, N32/1)	EU131582, EU131608
Lineage_XIV *G. skadarensis*	[37]R. Mat at Milot, AL (H45/1, H47/1); [42]R. Zeta at Danilovgrad, Morača, AL (H45/4, H46/1, N36/2, N/37/2)	EU131567, EU131568, EU131569, EU131601, EU131602
Ingroup taxa - lineages discriminated by the nuclear marker S7		
Lineage_XII *G. tauricus*	[40]R. Chernaya at Sevastopol, Crimean Peninsula, UA, 4549 (N33/1)	EU131609
Lineage_XIII *G. brevicirris*	[41]R. Sosna at Elets, Don, RUS, 4568 (N34/1), 4570 (N35/1)	EU131611, EU131612
Lineage_XV *G. krymensis*	[43]R. Bel'bek at Lyubimovka village, Crimean Peninsula, UA, 4607 (N38/1)	EU131620
Ingroup taxa - unambiguous hybrids (mixed lineages; mtDNA × nDNA)		
Hybrid L_III × L_ I	[9]Revištia channel, Tisza, SK, 3783 (H17/1, N2/1)	EU131562, EU131589
Hybrid L_IV × L_I	[9]Revištia channel, Tisza, SK, 3785 (H20/1, N2/1)	EU131561, EU131589
Hybrid L_I × L_V	[8]R.Odra at Odry, Odra, CZ, 2279 (H1/1, N16/1); 2263 (H3/1, N17/1); [44]R. Ida at Buzica, Tisza, SK, 2975 (H1/1, N12/1)	EU131542, EU131596; EU131545, EU131595; EU131542, EU131600
Hybrid L_III × L_ V	[18]Belžan Stream, Tisza, SK, 2954 (H15/1, N11/1)	EU131564, EU131597
Hybrid L_IV × L_V	[23] R. Ulička at Ulič, Tisza, SK, 3543 (H19/1, N10/1); 3544 (H19/1, N13/1)	EU131552, EU131598, EU131599
Hybrid L_I × L_XV	[43]R. Bel'bek at Lyubimovka village, Crimean Peninsula, UA, 4607 (H9/1, N38/1)	EU131549, EU131620
Ingroup taxa - submitted specimens for further investigation**		
Pure L_XII or Hybrid L_XIII × L_XII	[40]R. Chernaya at Sevastopol, Crimean Peninsula, UA, 4549-B (H42/1, N33/1)	EU131583, EU131609
Pure L_? or Hybrid_?	[40]R. Chernaya at Sevastopol, Crimean Peninsula, UA; 4550-C (H43/1, N25/1)	EU131553, EU131610
Hybrid L_I × L_?	[43]R. Bel'bek at Lyubimovka village, Crimean Peninsula, UA; 4605 (H1/1, N24/1)	EU131542, EU131619
Pure L_XIII or Hybrid L_XII × L_XIII	[41]R. Sosna at Elets, Don, RUS, 4570-A (H44/1, N35/1)	EU131588, EU131612

* indicates sequences from the GenBank; NCRH/NS = number of the control region haplotype/number of analyzed specimens, NS7N/NS = number of S7 nucleotype/number of analyzed specimens; ** explained in the text; 4549 = identification number; A - C see Fig. 7. AL, Albania; CZ, Czech Republic; D, Germany; RUS, Russia; SK, Slovakia; TR, Turkey; UA, Ukraine; UK, United Kingdom.

Table 2
List of primers used in this study

Gene	Primer	Sequences (5´- 3´)	Reference
CR	CR159	CCC AAA GCA AGT ACT AAC GTC	This study
	CR439	AAC TGT TTT TCC CAC ACT TA	This study
	CR493	TTG GGT AAC GAG GAG TAT GTA	This study
	CR851	TGC GAT GGC TAA CTC ATA C	This study
STIR_CR	Carp-Pro	AAC TCT CAC CCC TGG CTA CCA AAG	Thai et al. 2004
STIR_CR	Carp-Phe	CTA GGA CTC ATC TTA GCA TCT TCA GTG	Thai et al. 2004
S7	S7univL	ACA ATT GTA AGT CGG AGA TG	This study
	S7univP	CCC ACA AAA TAA GAT ATT AGG	This study

Table 3
Molecular diversity in the main control region and S7 lineages of genus G*obio*

mtDNA lineage	$N_{cr/s7}$	$NHN_{cr/s7}$	$\pi_{cr/s7}$ ± SD	$Hd_{cr/s7}$ ± SD	Tajima's $D_{cr/s7}$
Lineage_I	43/9	9/4	0.001 ± <0.001/0.004 ± 0.002	0.480 ± 0.093/0.583 ± 0.183	-2.083°/0.078 NS
Lineage_II	24/4	5/1	0.003 ± <0.001/0.000 ± 0.000	0.638 ± 0.061/0.000 ± 0.000	0.040/x NS
Lineage_III	12/1	4/1	0.002 ± <0.001/x	0.712 ± 0.105/x	0.872/x NS
Lineage_IV	10/4	4/3	0.002 ± <0.001/0.003 ± <0.001	0.533 ± 0.180/0.833 ± 0.222	-0.521/-0.710 NS
Lineage_V	1/12	1/8	x/0.005 ± 0.001	x/0.924 ± 0.057	x/-0.440 NS
Lineage_VI	1/9	1/2	x/0.006 ± 0.002	x/0.600 ± 0.154	x/-0.106 NS
Lineage_VII	4/8	4/4	0.006 ± 0.002/0.005 ± 0.002	1.000 ± 0.177/0.800 ± 0.172	-0.069/-1.337 NS
Lineage_VIII	8/10	7/3	0.003 ± <0.001/0.002 ± <0.001	0.964 ± 0.077/0.533 ± 0.095	-0.345/1.303 NS
Lineage_IX	1/2	1/2	x/0.003 ± 0.001	x/1.000 ± 0.500	x/x
Lineage_X	4/3	4/1	0.005 ± 0.001/0.000 ± 0.000	1.000 ± 0.177/0.000 ± 0.000	0.039/x NS
Lineage_XI	1/1	1/1	x/x	x/x	x/x
Lineage_XII	2*/1	2*/1	0.008 ± 0.003/x	1.000 ± 0.272/x	x/x
Lineage_XIII	1*/2	1*/2	0.008 ± 0.003/0.003 ± 0.001	1.000 ± 0.272/1.000 ± 0.500	x/x
Lineage_XIV	7/4	3/2	0.003 ± <0.001/0.002 ± <0.001	0.709 ± 0.099/0.667 ± 0.204	0.413/1.633 NS
Lineage_XV	*/1	*/1	x/x	x/x	x/x
Overall	119/71	47/36	0.025 ± 0.002/0.036± 0.002	0.912 ± 0.019/0.964 ± 0.009	

N - the number of specimens; NHN - the number of haplotypes/nucleotypes; π – nucleotide diversity; Hd - haplotype diversity; Tajima's D test (1989): NS = departure from neutrality - not significant (P>0.10), ° statistical significance (P<0.05); * explained in the text.

Table 4

Analysed fragments of both genomes, their characteristics resulting from the MP analysis and the appropriate models selected by Modeltest

Partition	No. characters (pars. inf.)	TL	CI	RI	Model
CR	713 (80)	375	0.5551	0.7183	HKY+Γ
S7	384 (62) + 15 gaps	276	0.8840	0.9516	K81uf
All combined data	1097 (142) + 15 gaps	819	0.7082	0.8446	Mixed model

CI, consistency index (excluding uninformative characters); Γ, gamma; pars. inf., number of parsimony informative characters; RI, retention index; TL, tree length.

Table 5
Mutual comparison of the representatives of the genus *Gobio* and their sequence divergences obtained by analysis of both mitochondrial (CR) and nuclear (S7) genomes

	Lineage I	Lineage II	Lineage III	Lineage IV	Lineage V	Lineage VI	Lineage VII	Lineage VIII	Lineage IX	Lineage X	Lineage XI	Lineage XII	Lineage XIII	Lineage XIV	Lineage XV
Lineage_I	**0.14 ± 0.00**	2.94 ± 0.60	1.62 ± 0.41	2.83 ± 0.58	1.34 ± 0.38	1.93 ± 0.47	6.06 ± 0.75	4.88 ± 0.70	8.30 ± 0.91	2.98 ± 0.60	4.93 ± 0.74	5.79 ± 0.75	5.33 ± 0.71	1.85 ± 0.46	—
Lineage_II	5.08 ± 0.85	**0.34 ± 0.09**	1.87 ± 0.45	2.09 ± 0.55	2.47 ± 0.54	2.51 ± 0.55	6.69 ± 0.79	5.33 ± 0.75	9.53 ± 0.89	2.74 ± 0.58	5.57 ± 0.77	6.50 ± 0.79	6.01 ± 0.78	2.26 ± 0.55	—
Lineage_III	5.91 ± 0.95	2.72 ± 0.65	**0.20 ± 0.07**	1.69 ± 0.47	2.08 ± 0.52	1.78 ± 0.45	6.22 ± 0.77	4.17 ± 0.66	8.12 ± 0.90	2.33 ± 0.55	4.76 ± 0.70	5.63 ± 0.75	5.17 ± 0.69	1.24 ± 0.38	—
Lineage_IV	5.05 ± 0.85	1.07 ± 0.39	2.70 ± 0.65	**0.23 ± 0.08**	2.08 ± 0.47	1.71 ± 0.45	6.10 ± 0.76	4.95 ± 0.71	9.52 ± 0.89	2.57 ± 0.57	4.84 ± 0.75	5.91 ± 0.78	5.44 ± 0.75	1.47 ± 0.43	—
Lineage_V	1.70 ± 0.45	5.37 ± 0.91	5.91 ± 0.95	5.34 ± 0.91	—	2.56 ± 0.58	4.70 ± 0.68	5.04 ± 0.69	9.62 ± 0.89	2.77 ± 0.58	5.28 ± 0.76	4.87 ± 0.75	4.42 ± 0.68	2.00 ± 0.47	—
Lineage_VI	3.72 ± 0.71	5.98 ± 0.95	6.77 ± 1.01	6.22 ± 0.97	3.00 ± 0.69	—	6.61 ± 0.80	4.70 ± 0.68	9.00 ± 0.90	2.18 ± 0.53	4.22 ± 0.66	6.01 ± 0.75	5.53 ± 0.75	0.94 ± 0.35	—
Lineage_VII	1.42 ± 0.45	5.22 ± 0.90	6.34 ± 0.98	5.19 ± 0.86	1.48 ± 0.46	3.41 ± 0.70	**0.55 ± 0.17**	6.47 ± 0.78	9.70 ± 0.92	5.90 ± 0.78	8.38 ± 0.88	1.63 ± 0.47	1.25 ± 0.37	6.11 ± 0.76	—
Lineage_VIII	7.18 ± 1.12	2.96 ± 0.68	4.08 ± 0.80	2.95 ± 0.68	6.76 ± 1.01	7.68 ± 1.19	7.21 ± 1.12	**0.28 ± 0.08**	4.72 ± 0.68	3.57 ± 0.59	6.33 ± 0.78	5.90 ± 0.78	5.42 ± 0.75	3.89 ± 0.66	—
Lineage_IX	7.00 ± 1.10	2.81 ± 0.66	3.91 ± 0.79	2.80 ± 0.66	6.57 ± 1.00	7.49 ± 1.16	7.03 ± 1.10	1.99 ± 0.50	—	8.07 ± 0.89	10.27 ± 0.93	9.86 ± 0.95	9.39 ± 0.88	8.13 ± 0.89	—
Lineage_X	5.33 ± 0.91	1.34 ± 0.43	2.43 ± 0.60	1.34 ± 0.43	5.07 ± 0.85	5.95 ± 0.95	5.50 ± 0.92	2.68 ± 0.65	2.54 ± 0.62	**0.48 ± 0.18**	5.19 ± 0.75	5.25 ± 0.75	4.87 ± 0.71	1.31 ± 0.38	—
Lineage_XI	3.75 ± 0.71	4.34 ± 0.82	5.17 ± 0.87	4.31 ± 0.82	3.83 ± 0.79	4.68 ± 0.83	3.70 ± 0.71	6.12 ± 0.96	6.08 ± 0.96	4.60 ± 0.83	—	7.95 ± 0.85	7.44 ± 0.84	4.13 ± 0.66	—
Lineage_XII	1.79 ± 0.46	5.51 ± 0.92	6.63 ± 1.00	5.48 ± 0.92	1.76 ± 0.46	3.70 ± 0.71	1.62 ± 0.45	7.52 ± 1.16	7.32 ± 1.14	5.80 ± 0.94	3.98 ± 0.80	**0.81 ± 0.28**	0.73 ± 0.18	5.53 ± 0.76	—
Lineage_XIII	1.68 ± 0.45	5.56 ± 0.92	6.46 ± 0.99	5.52 ± 0.92	1.56 ± 0.45	3.61 ± 0.71	1.42 ± 0.45	7.69 ± 1.19	7.48 ± 1.16	5.85 ± 0.94	3.63 ± 0.71	1.42 ± 0.45	—	5.06 ± 0.74	—
Lineage_XIV	5.23 ± 0.91	1.08 ± 0.39	2.84 ± 0.66	1.21 ± 0.41	5.50 ± 0.92	6.40 ± 0.98	5.35 ± 0.91	3.09 ± 0.69	2.94 ± 0.68	0.93 ± 0.38	4.46 ± 0.82	5.65 ± 0.93	5.69 ± 0.93	**0.27 ± 0.08**	—
Lineage_XV	2.57 ± 0.62	6.26 ± 0.98	7.38 ± 1.14	6.22 ± 0.98	2.44 ± 0.58	4.40 ± 0.68	2.31 ± 0.56	8.26 ± 1.20	8.07 ± 1.19	6.53 ± 0.98	4.69 ± 0.83	2.31 ± 0.56	2.42 ± 0.58	6.39 ± 0.98	**0.14 ± 0.00**

The mean DNA distance in percents ± SD above the diagonal is for control region, under the diagonal there are the values for the intron S7 r-protein; values on the diagonal (in bold) indicate within-CR lineage divergences.

Table 6
S7indel diagnostics

Lineage	PCR product (bp)
Lineage_I	338
Lineage_II	364
Lineage_III	366
Lineage_IV	366
Lineage_V	364
Lineage_VI	360
Lineage_VII	359
Lineage_VIII	367
Lineage_IX	369
Lineage_X	366
Lineage_XI	371
Lineage_XII	366
Lineage_XIII	337
Lineage_XIV	366
Lineage_XV	359
Total alignment	384

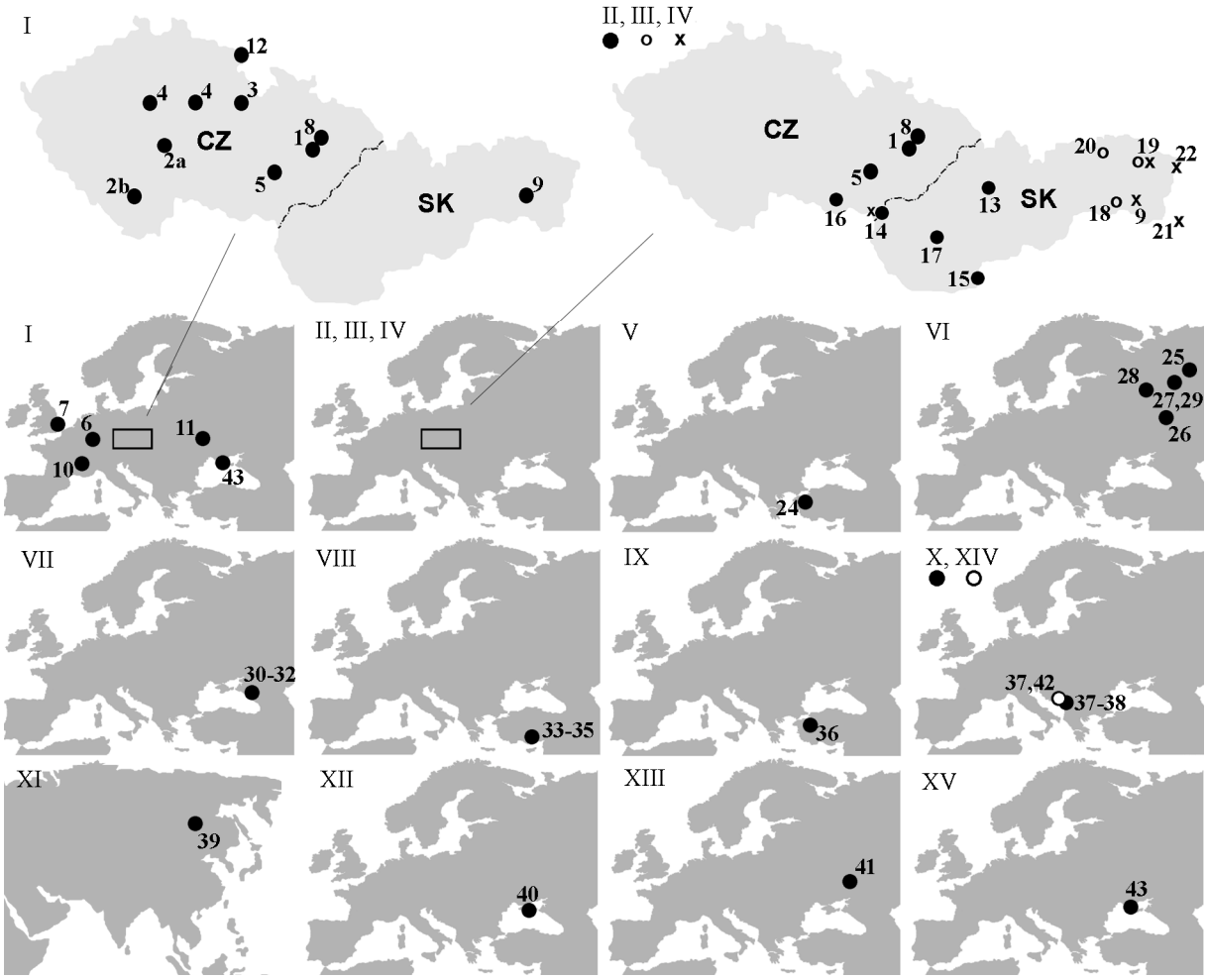

Fig. 1. Geographical origins of the fifteen lineages of the genus *Gobio*. Locality numbers correspond to the locality numbers in Table 1. In cases of the existence of more lineages, they are designated by various symbols. The rectangle demarcates the areas of large concentrations of collecting localities and these are displayed in the larger map. CZ = Czech Republic, SK = Slovakia. The dotted line indicates the border between countries.

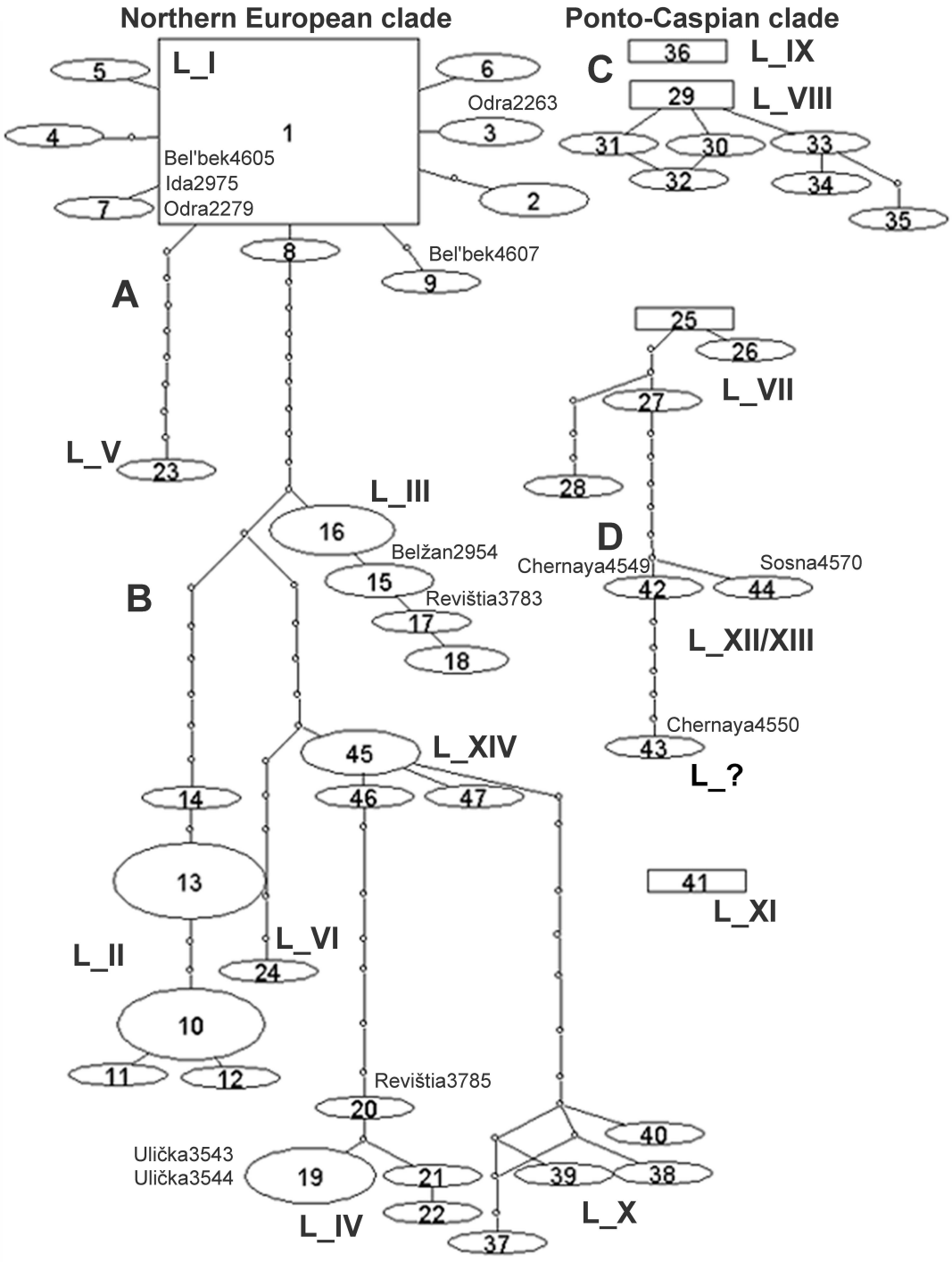

Fig. 2. The unrooted haplotype network based on sequences of the control region of certain representatives of the genus *Gobio*. The haplotype numbers refer to the numbers in Table 1. The node sizes are proportional to the haplotype frequency (see Tab. 1). The pertinence of individual haplotypes to the lineages (e.g. L_I) and subclades (A, B, C, D) is marked. Marked individuals of certain lineages show the hybrid origin. The names in the network indicate the collection locality followed by the identification number of the individual, (also in Fig. 3).

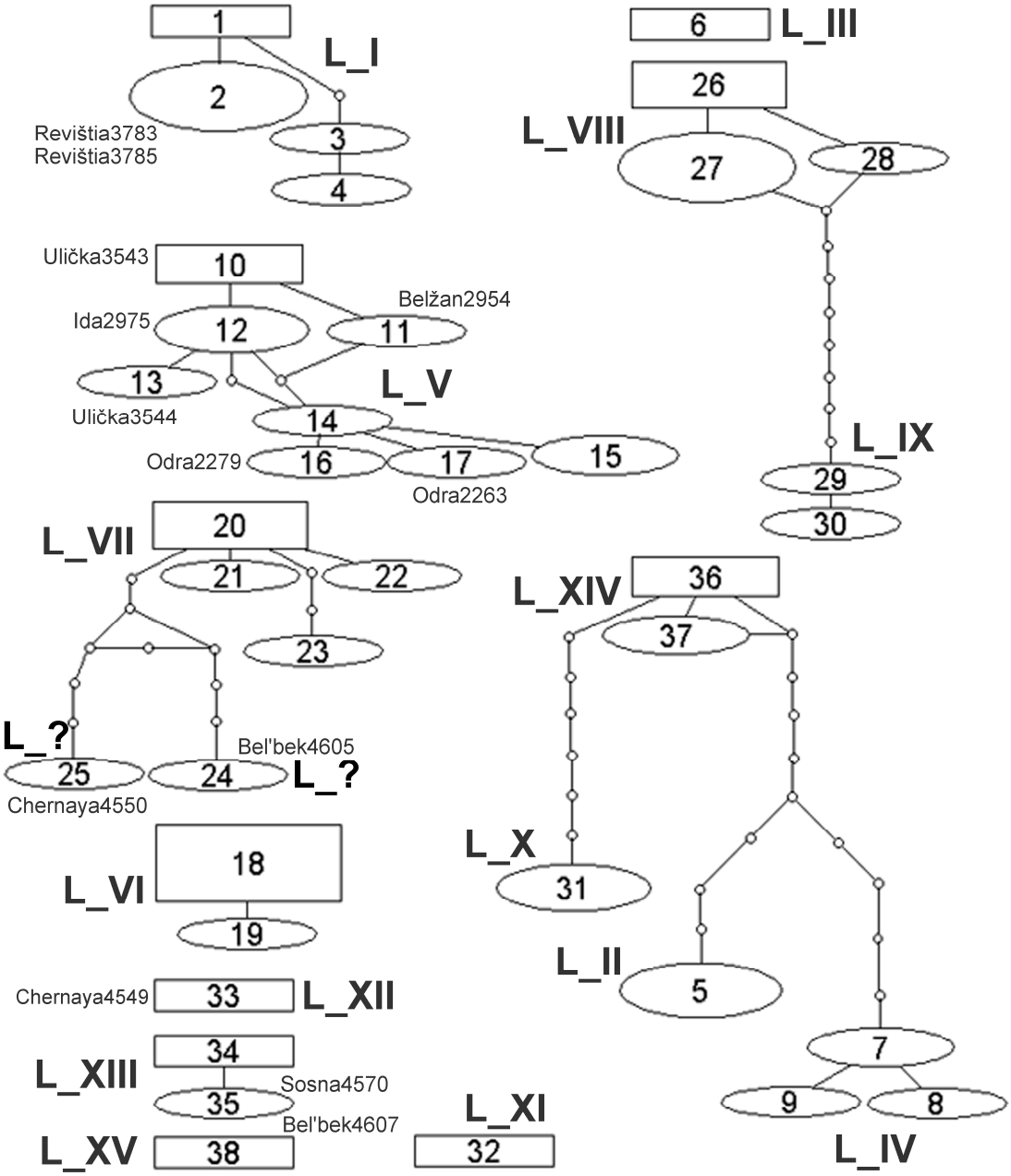

Fig. 3. The unrooted S7 nucleotype network of certain representatives of the genus G*obio*. The nucleotype numbers refer to the numbers in Table 1. The node sizes are proportional to the haplotype frequency (see Tab. 1). The marked individuals of certain lineages show their hybrid origin in comparison with the mitochondrial marker.

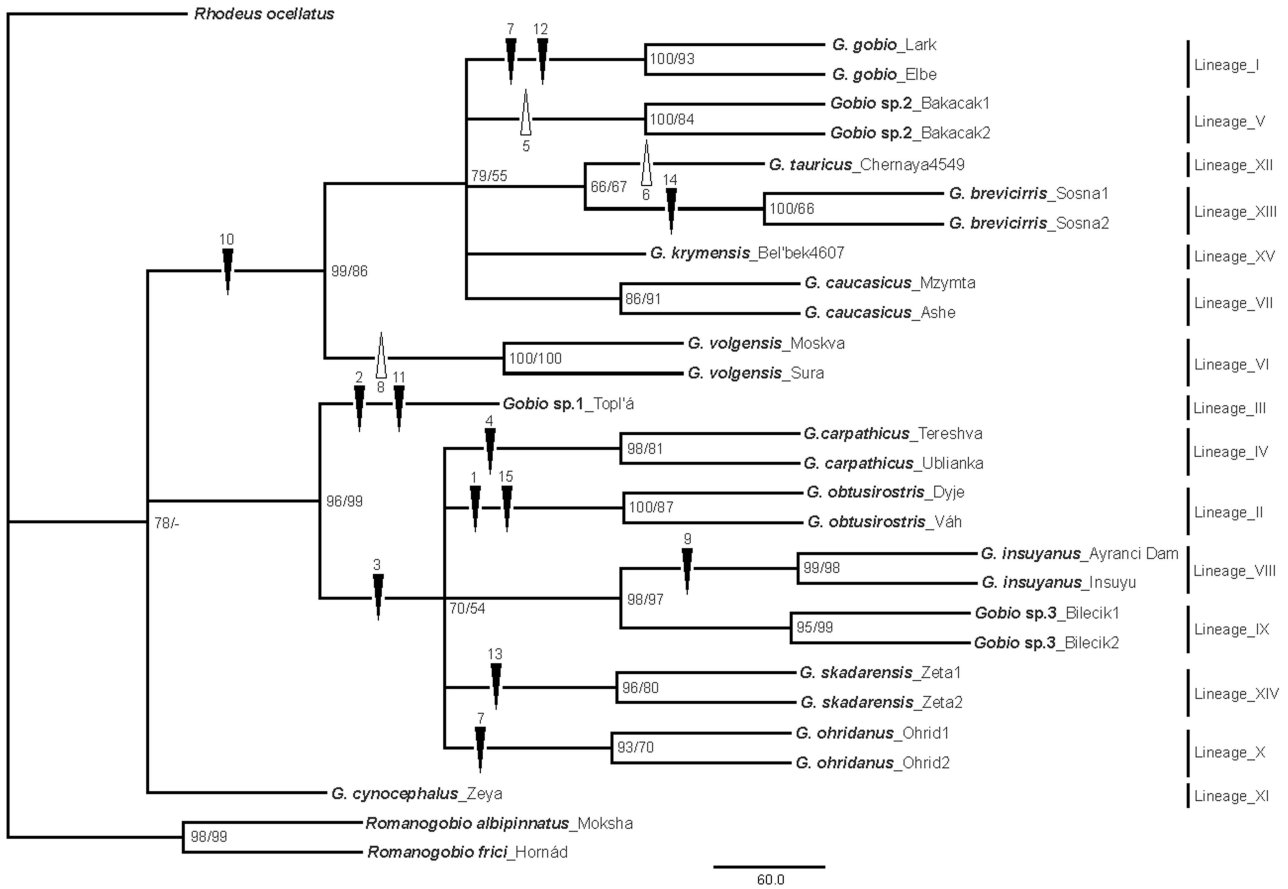

Fig. 4. Maximum parsimony tree inferred from the S7 sequences based on equal weighing of each character. Nodal support is assessed by bootstrap values (1000 replicates; shown only when >50%). The number preceeding the slash describes the value of support based on the G5 method; the number after the slash describes the value of support based on the GS method. *G. cynocephalus* was displayed based on the GS method as the third outgroup excluding *Rhodeus ocellatus* and the species of the genus *Romanogobio*. Up and down arrows represent insertions and deletions respectively. Numbers on the arrows correspond to the gap codes in Figure 8. The nominal species name is followed by the name of the locality, as well as in the subsequent three phylograms.

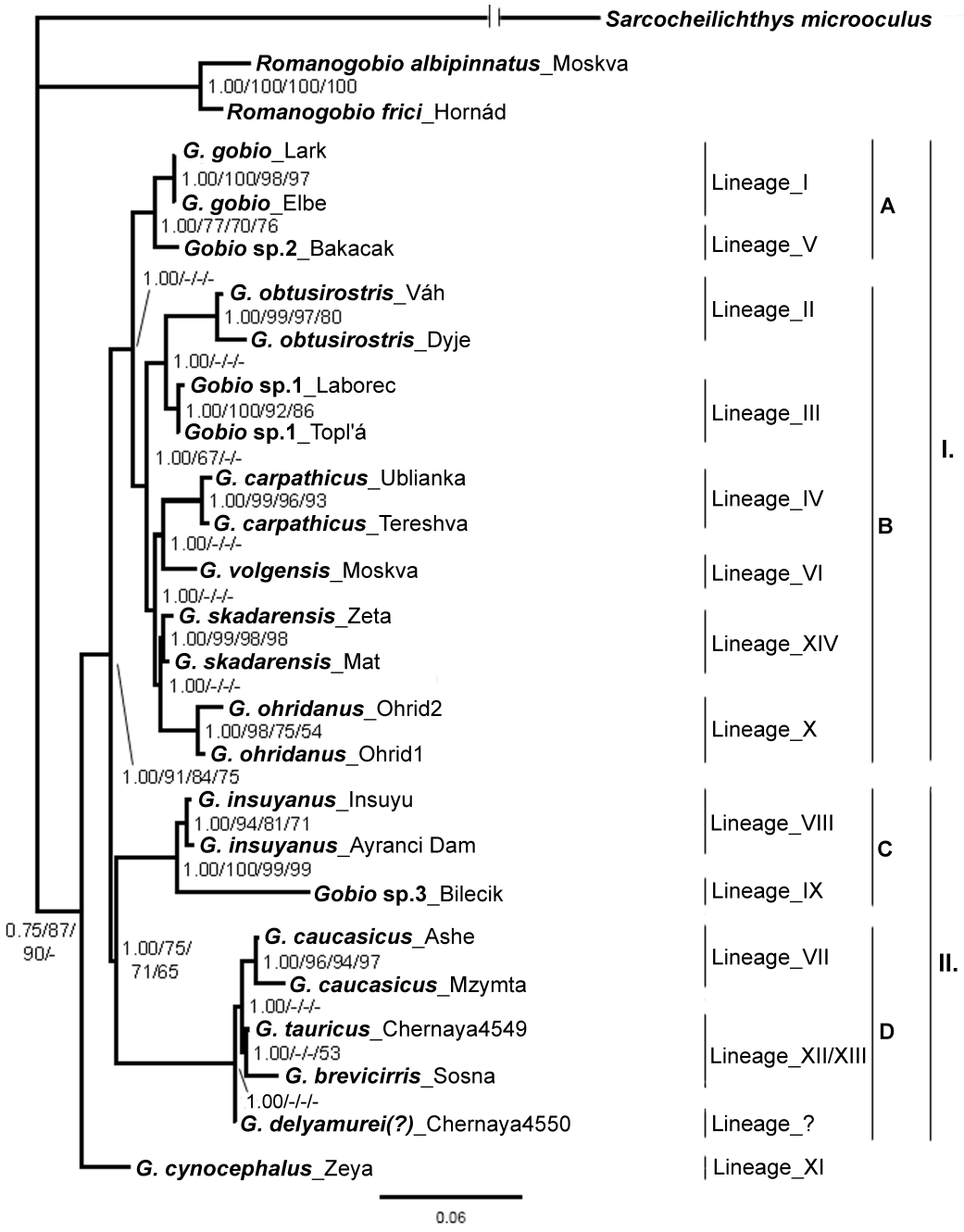

Fig. 5. Bayesian consensus tree resulting from the analysis of the control region data in studied gudgeon taxa, with Bayesian posterior probabilities/NJ bootstrap/MP bootstrap/ML bootstrap values listed near the nodes. Only values ≥ 50% are shown. The species *G. cynocephalus* was associated with the three outgroups based on the ML method. The fourteen highlighted lineages are categorized into four subclades and two major clades.

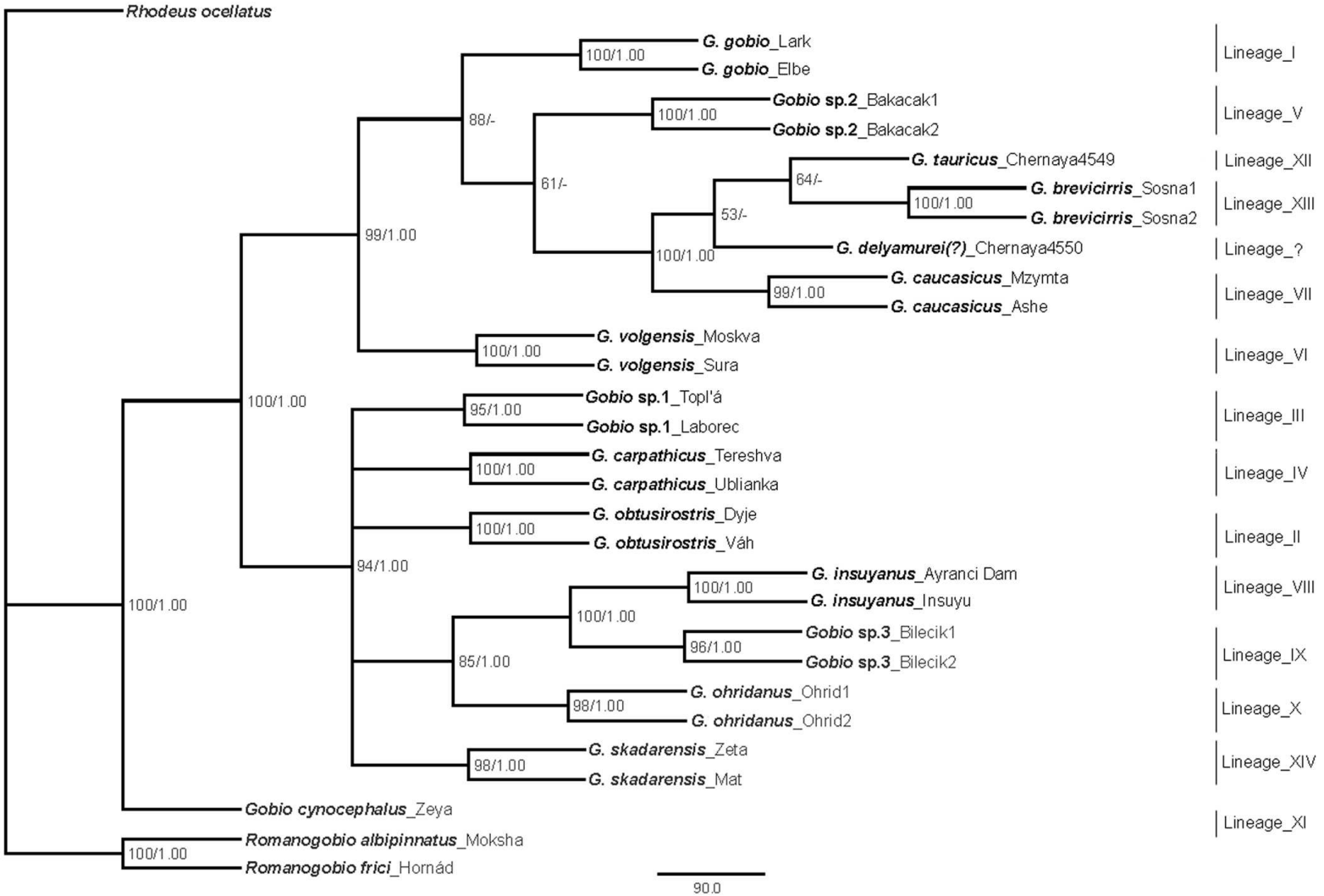

Fig. 6. Maximum parsimony tree inferred from combined data (CR and S7). Bootstrap values for MP and Bayesian posterior probabilities are listed near the nodes. Only values ≥ 50% are shown. The fifteen lineages are highlighted in the phylogram.

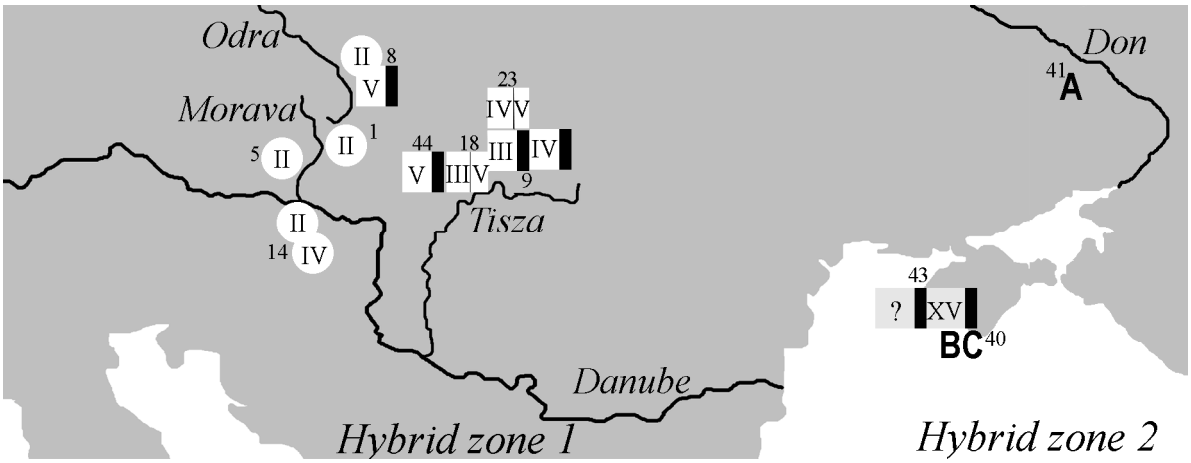

Fig. 7. Overview of the common occurrence of pure lineages in certain localities of the Danube River basin and the demarcation of two hybrid zones (1 and 2). Lineage_I appears within both hybrid zones (not shown) and forms hybrids with marked lineages (if not designated otherwise). The number of the locality corresponds with the number of the locality in Table 1. The indistinct total status of certain individuals: A= pure L_XIII or hybrid L_XII × L_XIII, B= pure L_XII or hybrid L_XIII × L_XII, C= pure L_? or hybrid_? (explained in the text and Table 1);

◯ = pure individual of one lineage; ◨ = hybrids with Lineages_I;

◫ = hybrids between different lineages, exluding Lineage_I.

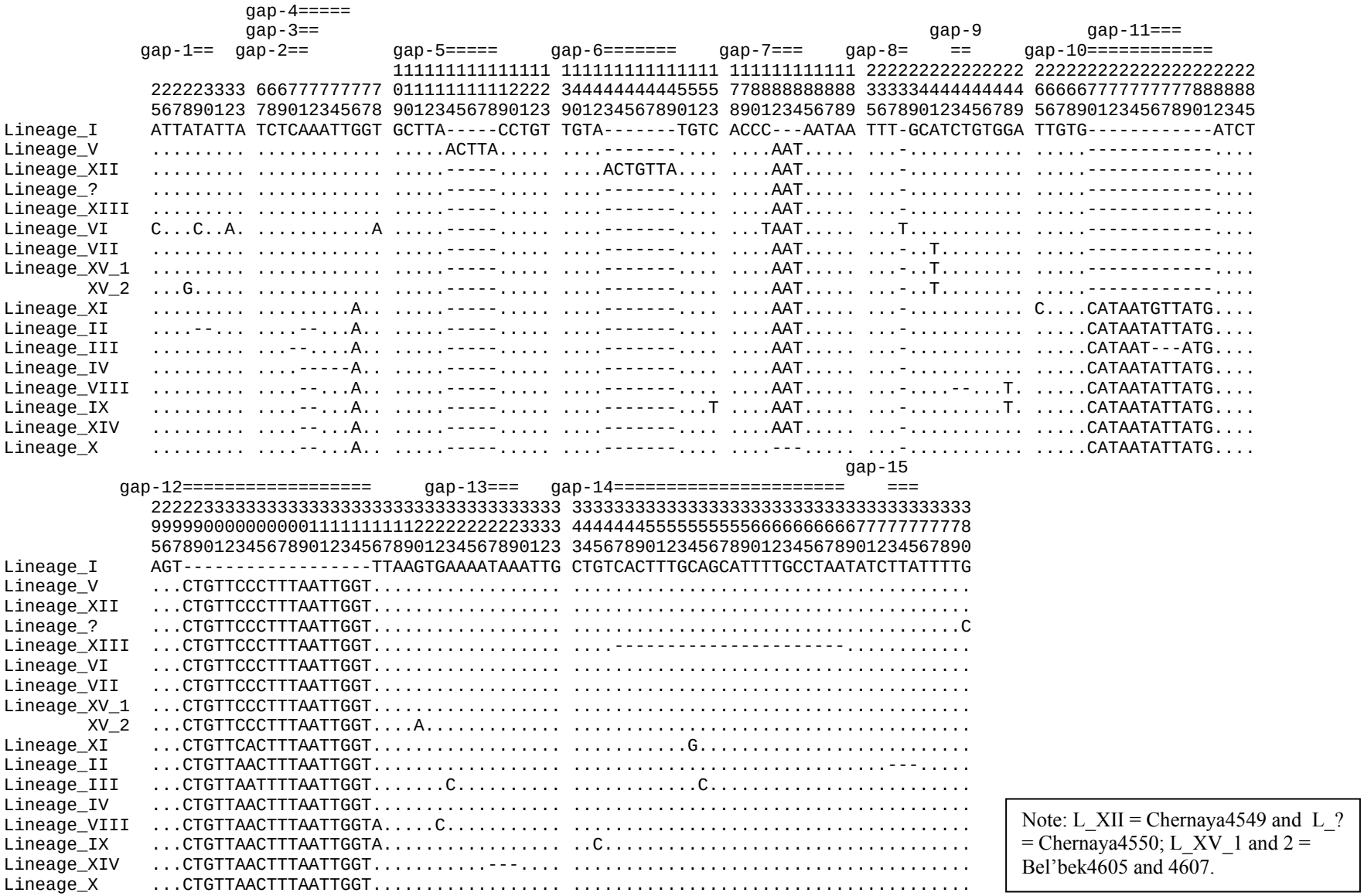

Fig. 8. Partial S7 alignment of the region containing gaps in selected representatives of fifteen lineages of the genus *Gobio*. Parsimony-informative gaps, treated as single indel mutations, are indicated by = = =.