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).

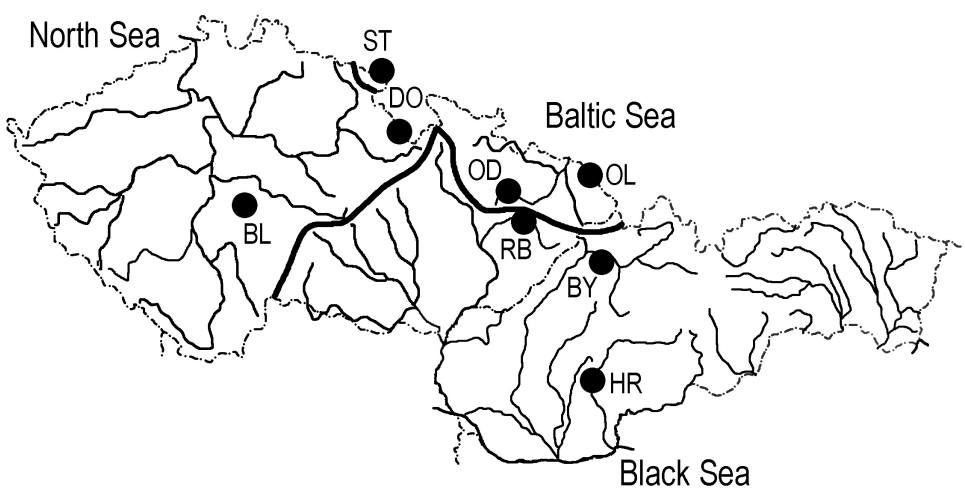

Fig. 1. Geographic location of *G. gobio* populations in the waters of the Czech Republic and Slovakia.

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: l 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.

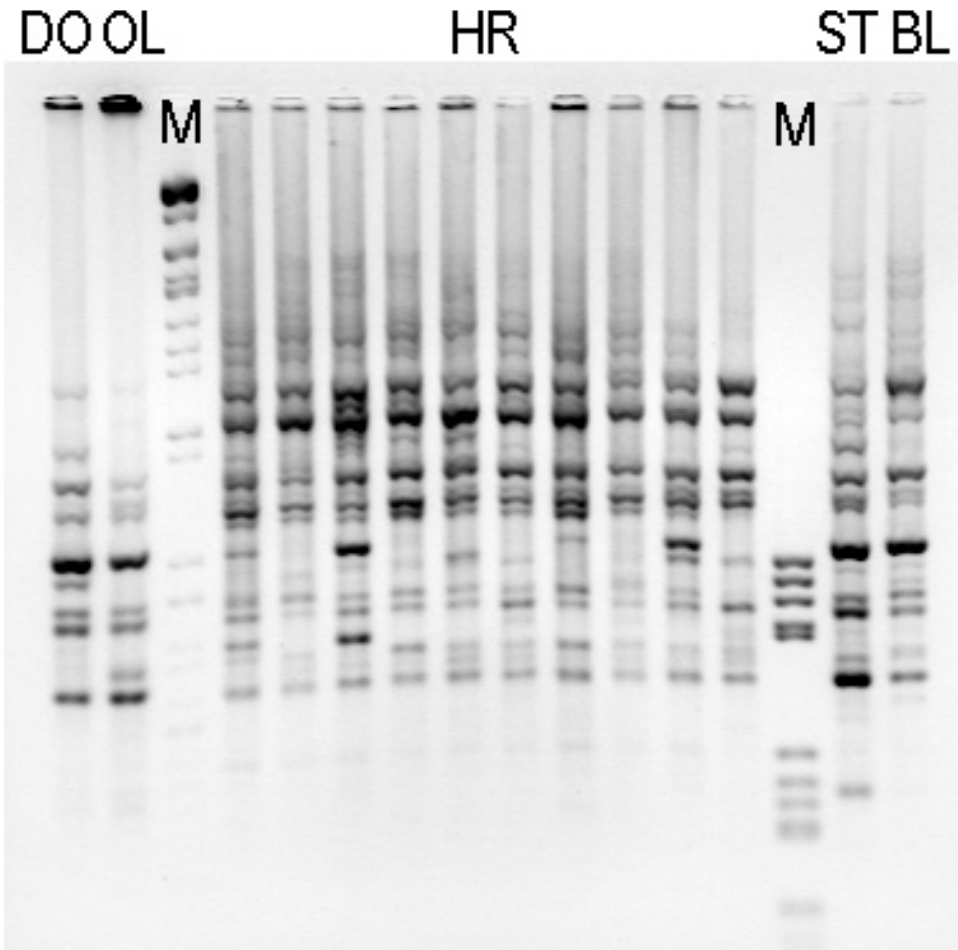

Fig. 2. RAPD patterns of *G. gobio* populations, obtained by using primer A06. M = DNA markers (bp).

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	

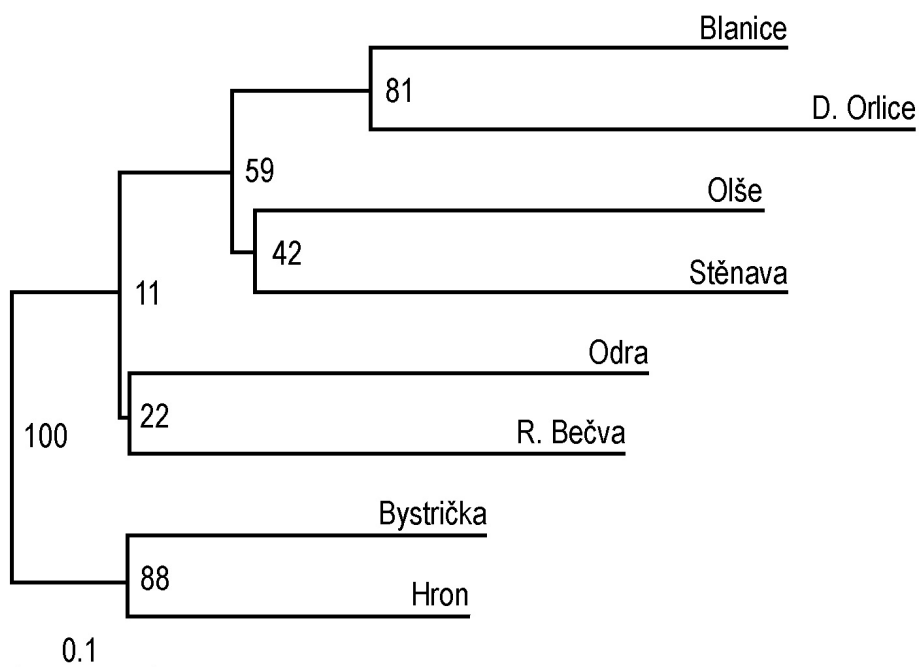

Fig. 3. Neighbor-joining (NJ) bootstrap consensus tree analyzed with FreeTree. Numbers at the nodes represent the percentage of 1000 bootstrap replications.

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

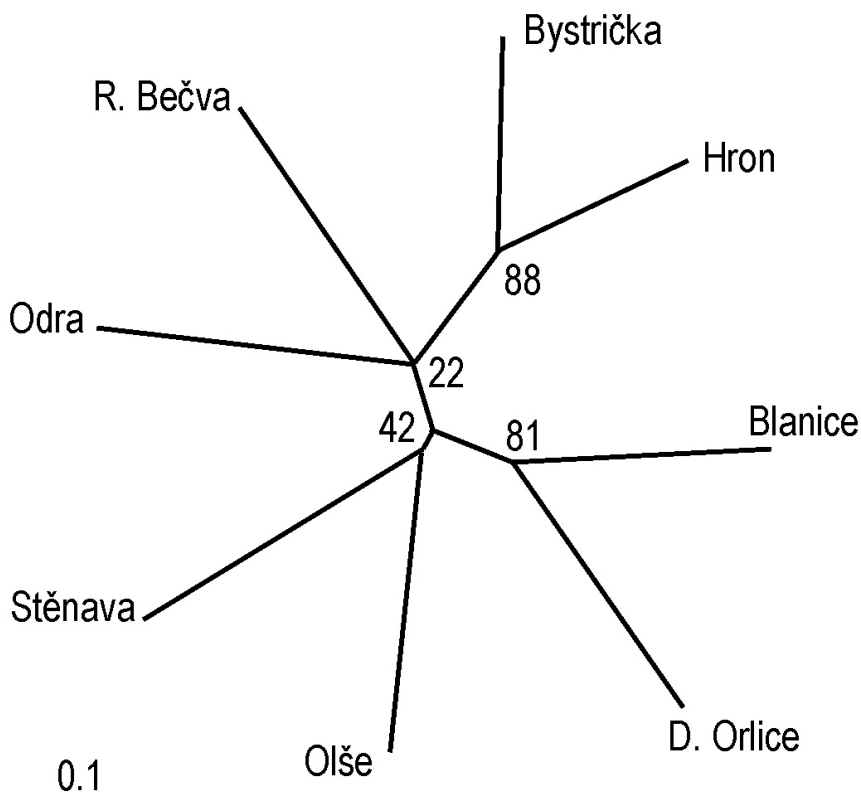

Fig. 4. Neighbor-joining (NJ) radiation tree of *G. gobio* populations.

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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