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Probable direction of the postglacial colonization of rivers on northern slopes of the Carpathian Ridge by *Barbus carpathicus* (Teleostei: Cyprinidae) evidenced by cline of genetic variation

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A cline in microsatellite diversity appeared among populations of *Barbus carpathicus* in four adjacent river basins on the northern slopes of the Carpathian Ridge, with western populations more variable than eastern ones (H_S equals 0·45, 0·32, 0·23 and 0·13, respectively). Overall genetic variation was low, likely reflecting population bottlenecks during postglacial expansions through the Danube–Vistula watershed and eastward into Carpathian tributaries of the Vistula River. Genetic similarities between populations in the westernmost tributary of the Dniester (Strwiąż River) and those in the adjacent Vistula tributary (San River) suggest that at least the western part of the Dniester basin was colonized from the Vistula River system.

Key words: clinal variation; microsatellites; postglacial expansion; riverine fishes.

Barbus carpathicus Kotlík, Tsigenopoulos, Ráb & Berrebi, 2002 belongs to the monophyletic lineage of tetraploid species of western Palaearctic barbels (Doadrio, 1990; Berrebi *et al.*, 1996; Tsigenopoulos *et al.*, 1999; Tsigenopoulos & Berrebi, 2000; Machordom & Doadrio, 2001; Kotlík *et al.*, 2002). The major distributional range of *B. carpathicus* includes the drainage of the Tisza River, a northern tributary of the Danube. *Barbus carpathicus* also occurs in the tributaries of the Vistula (Baltic basin) and in the Dniester River drainage (Black Sea basin) (Kotlík *et al.*, 2002). The portion of the Vistula River basin in the Carpathian Mountains is the northernmost distribution of *B. carpathicus* and is farthest from the species' probable glacial refuge.

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Barbus carpathicus probably expanded into rivers on the northern slopes of the western Carpathian Mountains after the last glacial maximum, from populations in the Tisza River basin, as this river is the only portion of the Danube part of the species' distribution next to the Black Sea–Baltic watershed. This cool-water species survived glacial periods in southern areas of its former distribution and later colonized the Vistula River basin. Ancestral populations probably dispersed across the Danube–Vistula watershed by stream captures. This form of dispersal has been documented, for example, in several montane populations of bullhead (Riffel & Schreiber, 1995; Hänfling & Brandl, 1998; Šlechtová *et al.*, 2004). Postglacial expansions are often accompanied by a gradual loss of genetic diversity (Bernatchez & Wilson, 1998; Schmitt *et al.*, 2002; Hewitt, 2004), especially in species with patchy distributions, as barriers between habitats permit only rare migrations between populations. As a result of these isolations, genetic differences between populations of riverine fishes may occur over short distances (Wong *et al.*, 2004; Wofford *et al.*, 2005). Isolations are probably more pronounced for mountain fishes with distributions restricted to short reaches of a river.

The aim of this study was to assess the levels of genetic variation in *B. carpathicus* populations in Carpathian rivers of south-east Poland. Patterns of variation in this area may reflect pathways of postglacial expansion because *B. carpathicus* populations are not commercially harvested and have not been stocked or influenced by fishery management practices. Specific questions were: (a) whether populations in the tributaries of the Vistula watershed maintain connectivity with each other; (b) whether genetic structuring in the Vistula reflects multiple invasions or a single invasion and subsequent expansion through drainage and (c) whether the Dniester River basin was colonized by individuals from the Vistula basin.

Materials were sampled in three hydrologic units of the Vistula (Wisłoka, Wisłok and San rivers; populations V1, V2 and V3, respectively) and in the Dniester system (Strwiąż River; population Dn) (Fig. 1). Fish were caught using an IUP-1.2 backpack electroshocker (Radet, Poznań, Poland; 350 V pulsed DC, 3·5 A, pulse frequency 20–100) in September 2002 and from July to October 2003. A total of 44 specimens of *B. carpathicus* were collected at 12 locations (Fig. 1). About 0·5 cm^2 of fin was preserved in 5 ml of 96% ethanol and stored at −25° C. Immediately before extraction, fins were cut into small pieces and dried at 60° C overnight. DNA was isolated using a Nucleospin Tissue kit (Macherey & Nagel, Duren, Germany) following the manufacturer's instructions.

Polymerase chain reaction (PCR) amplifications were performed in a total volume of 10 µl containing 20–30 ng of DNA, 1× Gold Buffer, 0·5 U of AmpliTaq Gold DNA polymerase (Applera, Norwalk, CT, U.S.A.), 200 µM deoxynucleotide triphosphates, 0·5 µM of each primer (Oligo, Warszawa, Poland), 2 mM or 3 mM of $MgCl_2$ (Table I) and 1 µg of Bovine Serum Albumin (Fermentas, Vilnius, Lithuania). Amplifications were carried out using a PTC-200 thermal cycler (MJ Research, Waltham, MA, U.S.A.) under the following conditions: 5 min at 95° C followed by 35 cycles for 40 s at 95° C, 45 s at appropriate annealing temperature (Table I), 50 s at 72° C and final elongation for 5 min at 72° C. Samples were denaturated with deionized formamide (1:1;

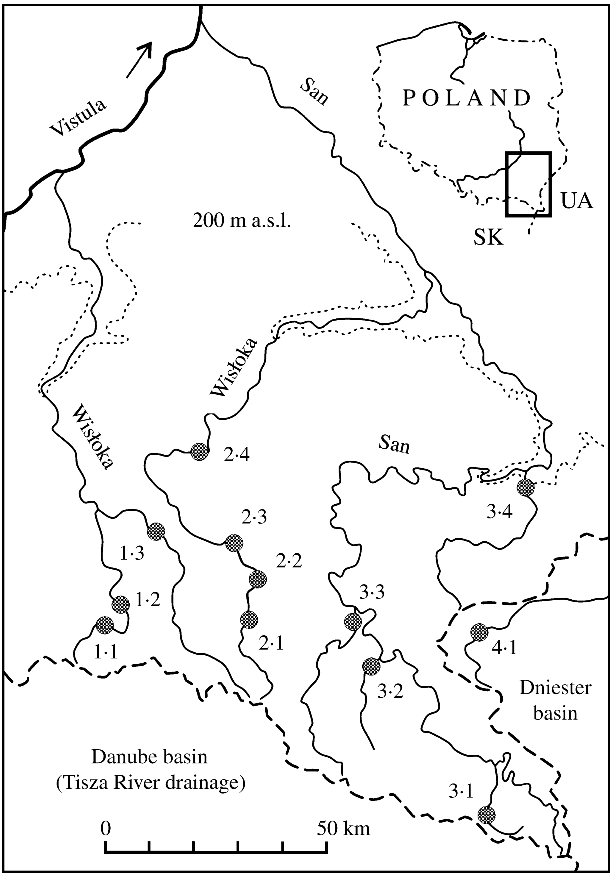

FIG. 1. Sampling localities for *Barbus carpathicus* in the Wisłoka (population V1), Wisłok (V2) and San (V3) rivers in the Vistula system and in the Strwiąż River (population Dn) in the Dniester system: 1.1, 1.2, Wisłoka River (subpopulation V1.1); 1.3, Jasiołka River (V1.2); 2.1–2.3, the upper course of Wisłok River (V2.1); 2.4, the middle course of Wisłok River (V2.2); 3.1, Wołosatka stream (V3.1); 3.2, Hoczewka stream; 3.3, Osława River (both V3.2); 3.4, Wiar River (V3.3); and 4.1, Strwiąż River (Dn). Dashed lines indicate major river basins. Dotted lines indicate the 200 m contour and the approximate downstream limit of the distribution of *B. carpathicus*. Country codes: SK, Slovakia, UA, Ukraine.

Applichem, Darmstadt, Germany) for 5 min at 90° C and separated with denaturating polyacrylamide gel electrophoresis (7 M urea, 8% of acrylamide:bisacrylamide 19:1, 0·5× TBE; Applichem, Germany). Gels were silver-stained on the glass plate according to standard protocol, and pictures were taken with a digital camera. Allele lengths were established with Gel-Pro 3.0 (Media Cybernetics) by comparison to a 10 bp DNA Ladder (Invitrogen).

PCR amplicons were examined in detail to avoid amplification of paralogous loci, as a tetraploidization occurred in the early evolution of barbels (Chenuil *et al.*, 1997, 1999). Two sets of microsatellite primers were tested (Chenuil *et al.*, 1999; Tong *et al.*, 2002). If necessary, PCR conditions were re-optimized to obtain amplicons for all samples (Table I). Ambiguous PCR products were

TABLE I. PCR conditions and summary statistics for microsatellite loci used in this study

Locus	$MgCl_2$ (mM)	Annealing temperature (° C)	A	H_O	H_E	F_{IS} (global)
Barb79	3	46	2	0·159	0·221	0·278
MFW1b	2	52	3	0·114	0·110	−0·039
MFW17a	2	52	2	0·159	0·185	0·283
MFW19	2	52	6	0·614	0·713	0·136

not used in the analyses. Only Barb79, a primer designed for genus *Barbus* (Chenuil *et al.*, 1999), performed equally well in all samples. Three primer sets, MFW1, MFW17 and MFW19, were optimized. These primers had been developed for common carp (Crooijmans *et al.*, 1997) and used successfully in other cyprinid species (Tong *et al.*, 2002). Two of these primers, MFW1 and MFW17, produced up to three bands per individual with similar stutter bands and product intensities, indicating amplification of more than one locus. The duplicated products were described as 'a' (longer) and 'b' (shorter). In *B. carpathicus*, allele lengths of duplicated loci did not overlap and allowed for unambiguous allele scoring. MFW1a and MFW17b were monomorphic in *B. carpathicus*. A length polymorphism was found in these loci in the sympatric species *Barbus barbus* (L.), which supports their repetitive character (unpubl. data).

Linkage disequilibrium was tested with a routine in ARLEQUIN 3.01 (Excoffier *et al.*, 2005). Tests of pooled samples may be biased by population subdivision, whereas tests of small population samples may lack statistical power to detect disequilibria. Thus, linkage disequilibrium was tested at both levels. Only the three largest sample sets, excluding the Stwiąż River sample ($n = 4$), were used for these tests. Deviations from Hardy–Weinberg expectations were tested with the Guo & Thompson (1992) method in Arlequin with 1000 dememorization steps and 10^5 Markov chain steps. Global indices of genetic variation were calculated with FSTAT 2.9.3.2 (Goudet, 1995) and included Nei's unbiased estimator of gene diversity (H_S; Nei, 1987) and allelic richness (R_S) *sensu* El Mousadik & Petit (1996). Allelic richness was adjusted by the smallest sample size ($n = 4$). These indices were tested for correlation with the mean longitude of sampling locations, since the sampled rivers flow to the north. Differences in gene diversity and allelic richness between samples were tested with paired Student's *t*-test. Genetic differentiation between populations was estimated with Cavalli-Sforza's chord distance (D_C, Cavalli-Sforza & Edwards, 1967) and F_{ST} (Weir & Cockerham, 1984) along with its significance based on 10^5 permutations of alleles among samples were calculated with MSA 4.0 (Dieringer & Schlötterer, 2003). Because F_{ST} is prone to high variance when the genetic variation and sample sizes are small, D_C was used to test for isolation by distance with Mantel's (1967) test.

A total of 13 alleles appeared among the four polymorphic loci. Tests for linkage disequilibria did not yield any significant results in the 24 comparisons between all pairs of loci within populations and in pooled samples (P-values ranging from 0·054 to 1·000). Physical linkage between loci is unlikely to occur in Palaearctic tetraploid barbels because of a large number of chromosomes in these species ($2n = 100$; Machordom & Doadrio, 2001; Leggatt & Iwama, 2003). No significant deviations from Hardy–Weinberg expectations were found in any locus or sample. This supports correct allele scoring, as well as the lack of null alleles.

The overall level of variation was low among samples (Table I). Two of the polymorphic loci were diallelic (MFW17a and Barb79), one locus contained three alleles (MFW1b) and one locus contained six alleles (MFW19; Fig. 2). Expected and observed heterozygosities in the first three loci were low due to the occurrence of a single common allele and one or two rare alleles. A bimodal allele-size distribution occurred in MFW19, which had three alleles between 193 and 197 bp and two larger alleles between 223 and 225 bp (Fig. 2).

Western populations were more variable than eastern ones in every pair-wise comparison. The easternmost population in the Strwiąż River (the Dniester tributary) was homozygous in three of four loci. A strong gradient appeared between genetic diversity and longitude (Table II and Fig. 3). Large significant regressions appeared between longitude and H_S ($r^2 = 0{\cdot}972$, $P = 0{\cdot}014$) and between longitude and R_S ($r^2 = 0{\cdot}970$, $P = 0{\cdot}015$) (Fig. 3). Despite these significant gradients, both global and pair-wise F_{ST} values were small and not significant (global $F_{ST} = 0{\cdot}037$, Table III). The Dniester basin sample was also not significantly different from the Vistula samples in F_{ST}.

These results suggest the common origin of the populations examined in this study. F_{ST} strongly depends on overall genetic variation, and thus, it is possible that its low values result from low overall variation in the analysed loci. The clinal pattern of genetic variation found in *B. carpathicus* populations on the northern slopes of the Carpathian Mountains probably reflects the expansion of this species after the last glacial maximum. The direction of the gradient of genetic variation in the sampled populations of *B. carpathicus* suggests that the postglacial dispersal of this species to the northern slopes of the Carpathians across the Danube–Vistula watershed took place west of the studied area and its later expansion was directed eastwards. Because this rheophilic (preferring fast flowing rivers) species occurs in only the upper courses of rivers in montane and submontane areas at altitudes of 250–300 m and above (Kukuła, 1999, 2003), its distribution is limited by both mountain watersheds and lowland

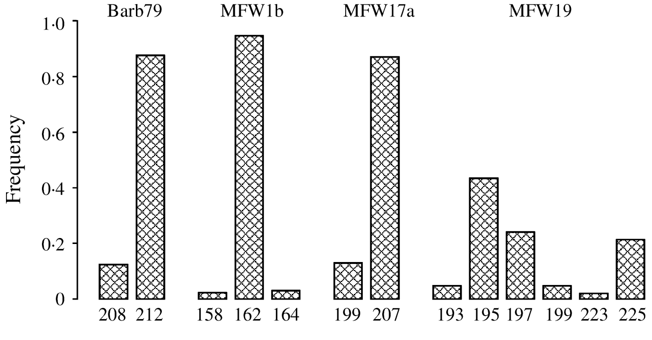

FIG. 2. Allele frequencies for four microsatellite loci in *Barbus carpathicus*.

TABLE II. Indices of the microsatellite variation in samples of *Barbus carpathicus*

Population	Mean eastern longitude	*n*	Barb79	MFW1b	MFW19	MFW17a	Mean
Gene diversity (H_S)							
V1	21°31′00′	10	0·395	0·195	0·442	0·774	0·4515
V2	21°54′00′	16	0·226	0·123	0·226	0·716	0·3228
V3	22°13′20′	14	0·138	0·071	0·071	0·640	0·2300
Dn	22°41′15′	4	0·000	0·000	0·000	0·536	0·1340
Allelic Richness (R_S)							
V1	21°31′00′	10	1·800	1·949	1·976	3·999	2·4310
V2	21°54′00′	16	1·705	1·500	1·705	3·522	2·1080
V3	22°13′20′	14	1·497	1·286	1·286	3·062	1·7828
Dn	22°41′15′	4	1·000	1·000	1·000	2·000	1·2500

segments of river networks, which form strong dispersal barriers (Fig. 1). Even within the same river system, populations in different hydrologic units may be effectively isolated. Postglacial dispersals probably involved a limited number of individuals each time. Also a difference between the populations V2 and V3 suggests the occurrence of additional bottlenecks in the founding of population V3. Low levels of diversity in the Strwiąż River (Dn) in the Dniester basin, along with a low genetic distance between San (V3) and Strwiąż populations (Table III), suggest that founders of at least some populations of the Dniester drainage probably originated in the Vistula rather than the Tisza basin.

The microsatellite loci examined in this study are very polymorphic in *B. barbus* (unpubl. data) and in other species (Crooijmans *et al.*, 1997; Chenuil *et al.*,

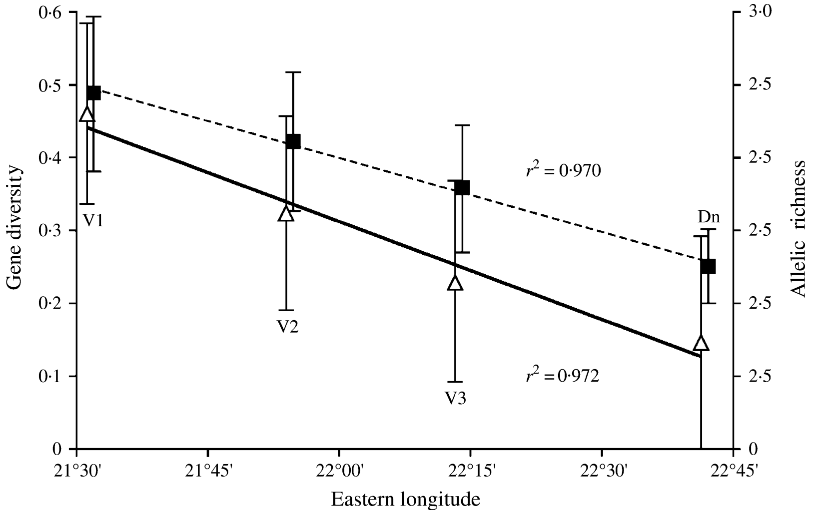

FIG. 3. Correlation between longitude and gene diversity (H_S, triangles and solid line) and allelic richness (R_S, squares and dashed line) in populations of *Barbus carpathicus* (Table I). Vertical bars represent standard errors.

TABLE III. F_{ST} values between populations of *Barbus carpathicus* (below diagonal) and statistical significance (above diagonal)

	V1	V2	V3	Dn
V1		NS	NS	NS
V2	0·0182		NS	NS
V3	0·0630	0·0145		NS
Dn	0·1117	0·1107	0·0446	

NS, not significant.

1999; Tong *et al.*, 2002). The low diversities of Barb79, MFW1b and MFW17 may be due to a population bottleneck during postglacial expansions into the Carpathian Ridge. Also allele length distribution in locus MFW19 may be a result of a bottleneck during the expansion to the Vistula drainage; however, size constraints often observed in microsatellite loci can produce a similar outcome (Li *et al.*, 2002). The hypothesis of the ancient bottleneck during expansion to the northern slopes of Carpathians requires further confirmation by analysis of the genetic diversity in the Tisza drainage.

The distribution of *B. carpathicus* suggests that the northernmost populations originated by river captures between the Danube basin and the Vistula and Dniester basins. Because the species exists in all these three river basins, it had to cross the watershed at least twice. In general, the Carpathian Mountains are an important barrier to freshwater fishes; however, they are not as efficient as the Dinaric Mountains (Šlechtová *et al.*, 2004). The analysis of river systems suggests that stream captures occurred in the past and are likely to occur in the future. Wrzosek (1968) described three pre-capture situations along a 406 km reach of the Vistula–Danube watershed in the Western Carpathians.

The mechanisms of later expansions on the northern slopes of the Carpathians are uncertain. If expansions took place along the river network, distances between populations should be correlated with the distances along the river courses of the drainage. However, montane and submontane populations are separated by long segments of lowland rivers, which impede the dispersal of *B. carpathicus* to neighbouring river systems. Other mechanisms, such as subsequent river captures, might be the driving force of population expansion. These alternative mechanisms of expansion were tested with two Mantel's tests (Mantel, 1967). In the first test, water distances between the midpoints of sampling locations along a river were used. In the second test, straight-line distances between the midpoints were used. Samples were divided between seven subpopulations because no clear correlation appeared between the three major Vistula populations (V1, Wisłoka River; V2, Wisłok River and V3, San River) (Fig. 1). Genetic and geographic distances within rivers were not included because they were small and may obscure a correlation. Genetic distances were positively correlated with the geographic distances in the river system (Mantel's $r = 0{\cdot}482$, $P = 0{\cdot}024$), whereas no correlation appeared with straight-line distances ($r = -0{\cdot}200$, $P > 0{\cdot}3$; Fig. 4). These results support the hypothesis that populations expanded into the adjacent Carpathian tributaries of the Vistula along the lowland segments of the river system.

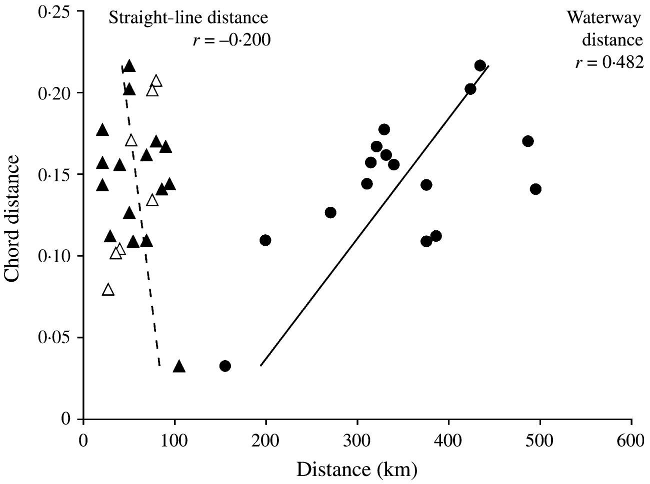

FIG. 4. Correlation between Cavalli-Sforza chord distances and geographic distances along rivers within the Vistula drainage (circles and solid line) and straight-line distances between populations. Distances between population Dn and V1, V2 and V3 populations are indicated by open triangles.

The results of this study show a substantial reduction in genetic variation in *B. carpathicus* over short distances within a river system. If similar genetic shifts occur in other Carpathian fishes, stockings and translocations carried out without previous genetic analyses species' diversity may severely affect the genetic variation of the target population, even if source populations are large and have not experienced demographic bottlenecks. In general, this pattern additionally supports the need for performing genetic analyses in riverine fishes prior to conservation actions (Antunes *et al.*, 2001, Walsh *et al.*, 2001).

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