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Phylogenetic relationships among Spanish *Barbus* species (Pisces, Cyprinidae) shown by RAPD markers

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Random amplified polymorphic DNA (RAPD) markers were used to estimate the population structure and phylogenetic relationships among the eight species of the genus *Barbus* that inhabit the Iberian Peninsula. Ten random oligodecamers were used to amplify DNA from 232 fish sampled from 15 populations. A total of 270 markers were detected that revealed low levels of genetic variability. The conclusions of cluster analysis indicate two main branches and three well-differentiated groups: north-eastern, Mediterranean and Atlantic. This clustering mainly reflects the evolutionary history of the genus, which is closely related to the paleogeography of the Iberian Peninsula. The contribution and application of these results to the conservation of the species, to their taxonomic status and to the process of colonization of the Iberian Peninsula by the genus *Barbus* are discussed.
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Keywords: *Barbus*; RAPD; phylogeny; palaeogeography; colonization

Introduction

The genus *Barbus* Cuvier and Cloquet 1816, one of the most diversified of the family Cyprinidae, is widely distributed across the Old World (Africa, Europe and Asia) where it is the dominant component among cyprinid fauna. In the Iberian Peninsula, the genus is one of the most important of all freshwater fauna, showing the highest specific diversity among all fish genera (Encina and Granado, 1988). At the present time, eight species of *Barbus* are found in Spain: *Barbus meridionalis*, *B. haasi*, *B. bocagei*, *B. comizo*, *B. graellsii*, *B. guiraonis*, *B. sclateri* and *B. microcephalus*. All of these, except the first, are endemic. Most show an allopatric distribution (Doadrio *et al*, 1991). The high number of endemic species is due to mountain (the Pyrenees) and maritime barriers (the Straits of Gibraltar). These provide the area with its singular biogeographical characteristics and favour the maintenance of local fish fauna within the Iberian Peninsula (Granado-Lorencio, 1992).

The phylogenetic relationships and taxonomic identity of *Barbus* have been the subject of debate for decades. Prior to 1990, their study in Europe was based on morphological data and geographical distribution (Tsigenopoulos *et al*, 1999). Osteological characters have also been employed in some studies to determine the relationships among the different species (Doadrio, 1990). Nevertheless, due to the considerable morphological diversity of *Barbus* (mainly related to growth, biogeographical and ecological factors (Encina and Granado, 1988)), the interpretation of these data have sometimes been very difficult.

Studies based on morphology, osteology, ecology and, to a lesser extent, on genetics, have all made contributions to the present understanding of the *Barbus* genus (Encina and Granado-Lorencio, 1988, 1997; Collares-Pereira and Madeira, 1990; Torralva *et al*, 1997; Aparicio and Sostoa, 1998; Chenuil *et al*, 1998, 2000; Tsigenopoulos *et al*, 1999, Callejas and Ochando, 2000; Tsigenopoulos and Berrebi, 2000). However, basic questions on its evolutionary differentiation remain unsolved (see Tsigenopoulos and Berrebi, 2000 and references therein). These issues can be extended to the Iberian Peninsula where, although *Barbus* is the most speciose genus of all freshwater fish, only a few genetic studies have tried to answer questions on their phylogenetic relationships and taxonomic status (Machordom *et al*, 1990, 1995; Callejas and Ochando, 1998, 2000; Zardoya and Doadrio, 1998, 1999; Machordom and Doadrio, 2001). Much more information is needed for understanding the processes of evolution that has occurred in the genus *Barbus*, as well as for the development of efficient strategies in future conservation programmes.

The development of the random amplified polymorphic DNA technique (RAPD) (Williams *et al*, 1990; Welsh and McClelland, 1990) has provided a useful tool for research into genetic variability. It consists of the PCR amplification of small, inverted repeats scattered throughout the genome, using a single, short primer of arbitrary sequence. Thus, the genome can be scanned more randomly than with conventional techniques. The ability to examine genomic variation without previous sequence information (Williams *et al*, 1990), the relatively low cost of the technique, and the requirement of only nanograms of template DNA, are all advantages of RAPD in population and other genetic studies.

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There is now increasing evidence that the RAPD technique, which has been used in different fields, can detect nuclear variation in fish (Borowski *et al*, 1995; Naish *et al*, 1995; Sultmann *et al*, 1995; Bielawski and Pumo, 1997; Caccone *et al*, 1997; Callejas and Ochando, 1998; Mamuris *et al*, 1999; Allendorf and Seeb, 2000). These studies have shown that RAPD is an extremely sensitive method for detecting DNA variation and for establishing genetic relationships in closely related organisms. In the present work, RAPD markers were used to infer phylogenetic relationships among the Spanish species of genus *Barbus*.

Materials and methods

Sample collection and DNA extraction

Fifteen samples from the eight Spanish species of barbel were collected from 14 separated sites in the Iberian Peninsula. Table 1 contains the collection sites, the species collected and the number of individuals sampled ($n = 232$). Figure 1 is a map showing the collection sites. Specimens were dissected *in situ*. Liver tissue was shipped to the laboratory in liquid nitrogen and maintained at –80°C until DNA extraction.

Genomic DNA was extracted from 2 g of liver according to Benito *et al* (1993), with minor modifications. Samples were treated with SDS and genomic DNA isolated using phenol:chloroform extraction and isopropanol precipitation. The resulting pellets were washed in 70% ethanol, dried and resuspended in 10 mM Tris (pH 8.0), 1 mM EDTA.

RAPD-PCR analysis

Ten oligodecamers from Operon Technologies were used (C-02, C-04, C-05, C-06, C-07, C-08, C-09, C-11, C-15 and C-16). DNA amplifications were based on conditions reported by Williams *et al* (1990), with slight modifications.

Table 1 Barbel populations, sample size (No.) and collection sites. In the last column, numbers indicate the sampling localities as shown in Figure 1

Taxon	*Code*	*No.*	*Sampling site*	*SL*
B. bocagei	CEA	20	River Cea (Duero basin)	1
B. bocagei	MAN	20	River Manzanarea (Tajo basin)	2
B. bocagei	IBO	13	River Ibor (Tajo basin)	3
B. comizo	CIJ	20	Cijara dam (River Guadiana)	4
B. comizo	GUA	20	River Guadiana	5
B. graellsii	GAL	20	River Gállego (Ebro basin)	6
B. graellsii	ARA	20	River Aragón (Ebro basin)	7
B. graellsii	NOG	12	River Noguera Ribagorçana (Ebro basin)	8
B. guiraonis	JUC	5	Alarcón dam (River Júcar)	9
B. haasi	BAS	11	Basa Stream (Ebro basin)	10
B. meridionalis	SER	11	River Set (Fluviá basin)	11
B. microcephalus	MIC	3	River Guadiana	5
B. sclateri	MON	20	Montoro dam (River Jándula, Guadalquivir basin)	12
B. sclateri	MUN	20	River Mundo (Segura basin)	13
B. sclateri	SEG	17	River Segura	14

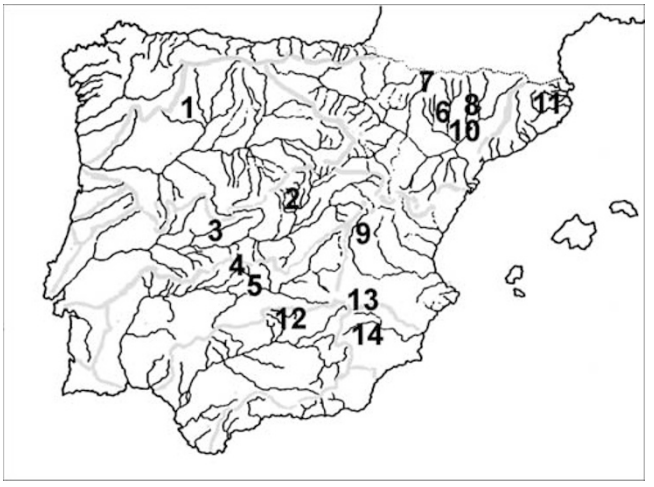

Figure 1 Location of sampling sites of *Barbus* spp. in the Iberian Peninsula.

RAPD-PCR was performed in 25 μl reaction volumes containing 25 ng barbel DNA, 5 pmoles of primer, 0.1 mM of each dNTP, 4 mM $MgCl_2$, 2.5 μl Stoffel buffer 10× (Perkin-Elmer) and 1.2 units of Stoffel Fragment DNA polymerase (Perkin-Elmer). DNA was amplified by a PTC-100 MJ Research Thermocycler programmed to provide a first denaturation of 5 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at 36°C and 6 min at 72°C, and, finally, one cycle at 72°C for 6 min.

Amplification products were resolved by electrophoresis in 2% agarose gels with TAE buffer (40 mM Tris-Acetate, 1 mM EDTA pH 8.0) containing ethidium bromide. A 100 bp ladder marker was used as a molecular size standard.

The RAPD-PCR method has some limitations, such as sensitivity to reaction conditions, occasionally non-reproducible amplification products, and possible co-migration of amplified fragments. However, these problems can be solved by following a strict protocol with standardised conditions, repeating the amplification reactions twice in order to score clearly reproducible bands, and increasing the resolution of band separation (Hadrys *et al*, 1992). In the present work, amplification results were routinely repeatable.

Statistical analysis

Amplified bands were scored for their presence (1) or absence (0). Mean bandsharing similarity indices were calculated within (S_i) and between populations (S_{ij}) for all possible comparisons, according to Lynch (1990): $S_{ij} = 1 + S'_{ij} - 0.5\ (S_i + S_j)$ where S_i is the average similarity of individuals within population i, and S'_{ij} is the average similarity between random pairs of individuals across populations i and j. When S'_{ij} equals the mean similarity in the two populations, $S_{ij} = 1$ indicating that the populations are homogeneous.

Genetic differentiation among the samples was examined by clustering and multivariate analysis. Genetic distances were obtained from both bandsharing and frequency data. Nei's (1972) genetic distance values were calculated from frequencies of markers using the NTSYS-PC programme (Rohlf, 1990). Lynch's (1991) analogue of Nei's genetic distance was calculated from mean simi-

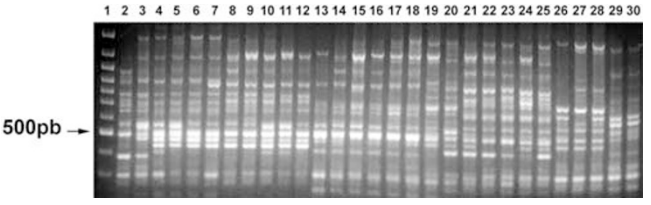

Figure 2 RAPD profiles of Spanish barbels with the primer C08. The first lane contains a 100 bp molecular weight marker. Lanes 2 and 3: *Barbus microcephalus*. Lanes 4–7: *B. comizo*. Lanes 8–12: *B. sclateri*. Lanes 13–18: *B. bocagei*. Lanes 19 and 20: *B. guiraonis*. Lanes 21–25: *B. graellsii*. Lanes 26–28: *B. haasi*. Lanes 29 and 30: *B. meridionalis*.

larity within (S_i) and between population (S_{ij}) samples. Both sets of distance values were used to construct dendrograms with the unweighted pair-group (UPGMA, Sneath and Sokal, 1973) and neighbour-joining (NJ, Saitou and Nei, 1987) methods of analysis, employing NTSYS software. Correlations between genetic distances produced using both methods were compared using a randomised test for matrix correspondence – the Mantel test (Mantel, 1967). The reliability of the trees was evaluated using 1000 bootstrap replicates with the RAPDDIST 1.0 programme from the RAPD-PCR software package (Black IV, 1997). Finally, a principal components analysis (PCA) (Sneath and Sokal, 1973) was performed using NTSYS software (Rohlf, 1990).

Results

In the 232 specimens analysed, a total of 270 scorable bands were observed for the eight species of *Barbus*, ranging in size from 2000 to 300 bp. Only 17 (6.30%) were monomorphic for all species. Differences among the eight species were detected with all 10 primers. Figure 2 shows the RAPD profiles of the different species with the primer C08. As observed in Table 2, RAPD analysis also revealed intraspecific polymorphism in barbel populations, ranging from 0% in *B. meridionalis*, where all specimens analysed showed identical patterns, to 29.36% in *B. comizo* (GUA population). Frequencies of polymorphic bands were also included for species comparisons.

The matrix of the estimated values of within and between population similarity is shown in Table 3. Intrapopulation similarity indices (diagonal, Table 3) were high, ranging from 95.36% (MAN population, *B. bocagei*) to 100% (SER population, *B. meridionalis*). Interpopulation similarity values (above diagonal) were lower, ranging from 59.30% to 97.81%. The similarities between populations of the same species were clearly greater than those between populations of different species. The lowest values of between-population similarity were obtained for all comparisons of *B. haasi* (BAS population) and *B. meridionalis* (SER population) with the rest of the Spanish populations. Both species, *B. haasi* and *B. meridionalis*, inhabit the northeast of Spain. The mean similarity interpopulation value for all Spanish populations of *Barbus* was 75.85% ± 8.17, increasing to 80.34 ± 3.85 when *B. haasi* and *B. meridionalis* were excluded.

Genetic distance values obtained from bandsharing indices (Lynch, 1991) and marker frequencies (Nei, 1972) were also calculated. The Mantel test showed that genetic distances between populations, calculated using both methods, correlated significantly across comparisons ($r = 0.9762$, $t = 5.4261$, the one-tailed probability that observed Z was greater than random Z in 100 permutations was 0.0100). Table 3 (below diagonal) shows the values of Nei's genetic distances. The highest intraspecific distances were found in *B. bocagei* (range 0.045–0.025); the lowest in *B. comizo* (0.006). Between any two samples, interspecific values of distance ranged from 0.115 to 0.508.

Based on Nei (1972) and Lynch (1991) genetic distances, the four dendrograms generated by the UPGMA and NJ methods showed the same topology. Therefore, Figure 3 depicts only one of them, the UPGMA dendrogram based on Nei's genetic distances. Bootstrap values are displayed. The dendrogram exhibits two main branches: one contains the species *B. haasi* and *B. meridionalis*, both present in north-eastern Spain; the other includes the remaining Spanish species. This second

Table 2 Summary of band data and frequencies for the populations of *Barbus* analysed

Population	*Number of bands*				*Band frequency*				
	M	*P*	*T*	*%P*	*0–20%*	*20–40%*	*40–60%*	*60–80%*	*80–99%*
CEA (B.b)	92	23	115	20.00	5	6	3	6	3
IBO (B.b)	96	25	121	20.66	9	8	3	2	3
MAN (B.b)	90	34	124	27.42	8	10	8	6	2
CIJ (B.c)	88	33	121	27.27	13	7	4	3	6
GUA (B.c)	89	37	126	29.36	14	10	4	3	6
ARA (B.g)	89	28	117	23.93	7	7	4	3	7
GAL (B.g)	88	27	115	23.48	4	4	3	8	8
NOG (B.g)	91	25	116	21.55	7	3	4	5	6
JUC (B.guir)	95	23	118	19.49	9	8	3	2	3
BAS (B.h)	97	10	107	9.34	2	1	1	–	6
SER (B.mer)	84	–	–	0	–	–	–	–	–
MIC (B.mic)	94	9	103	8.74	–	5	–	4	–
MON (B.s)	91	29	120	24.17	8	7	5	5	4
MUN (B.s)	100	10	110	9.10	2	3	1	2	2
SEG (B.s)	99	15	114	13.16	7	5	1	2	–

B.b = *B. bocagei*; B.c = *B. comizo*; B.g = *B. graellsii*; B. guir = *B. guiraonis*; B.h = *B. haasi*; B.mer = *B. meridionalis*; B.mic = *B. microcephalus*; B.s = *B. sclateri*; M = Monomorphic bands; P = Polymorphic bands; T = total; %P = polymorphism index.

Table 3 Nei's genetic distances (below diagonal). Intrapopulation similarity (on diagonal) and interpopulation similarity indices (above diagonal) for *Barbus* samples of the Iberian Peninsula

	MIC *Bmic*	GUA *Bc*	CIJ *Bc*	MON *Bs*	SEG *Bs*	MUN *Bs*	CEA *Bb*	MAN *Bb*	IBO *Bb*	JUC *Bguir*	ARA *Bg*	GAL *Bg*	NOG *Bg*	BAS *Bh*	SER *Bmer*
MIC	0.9777	0.8158	0.8203	0.8110	0.7953	0.8033	0.7606	0.7621	0.7685	0.8029	0.7815	0.7764	0.7754	0.6626	0.6162
GUA	0.1982 ± 0.0285	0.9579	0.9406	0.8683	0.8551	0.8586	0.8469	0.8457	0.8381	0.8109	0.8347	0.7820	0.7666	0.6698	0.6312
CIJ	0.2001 ± 0.0286	0.0066 ± 0.0001	0.9616	0.8829	0.8660	0.8693	0.8564	0.8546	0.8477	0.8228	0.7916	0.7898	0.7729	0.6674	0.6230
MON	0.2357 ± 0.0314	0.1190 ± 0.0216	0.1150 ± 0.0212	0.9645	0.9435	0.9451	0.8095	0.8038	0.8151	0.7914	0.7804	0.7720	0.7615	0.6485	0.6119
SEG	0.2455 ± 0.0333	0.1395 ± 0.0235	0.1301 ± 0.0112	0.0333 ± 0.0112	0.9833	0.9781	0.8092	0.8030	0.8100	0.7741	0.7787	0.7701	0.7576	0.6544	0.6010
MUN	0.2385 ± 0.0316	0.1319 ± 0.0229	0.1288 ± 0.0225	0.0335 ± 0.0111	0.0083 ± 0.0002	0.9806	0.8065	0.8013	0.8092	0.7741	0.7726	0.7637	0.7518	0.6417	0.5930
CEA	0.2854 ± 0.0350	0.1437 ± 0.0239	0.1432 ± 0.0239	0.2028 ± 0.0287	0.2041 ± 0.0289	0.2064 ± 0.0282	0.9715	0.9698	0.9670	0.8276	0.7580	0.7524	0.7366	0.6598	0.6156
MAN	0.2840 ± 0.0349	0.1407 ± 0.0235	0.1419 ± 0.0236	0.2096 ± 0.3001	0.2130 ± 0.0279	0.2173 ± 0.0298	0.0255 ± 0.0009	0.9536	0.9519	0.8381	0.7676	0.7622	0.7476	0.6534	0.6254
IBO	0.2799 ± 0.0346	0.1606 ± 0.0254	0.1599 ± 0.0253	0.2020 ± 0.0297	0.2084 ± 0.0290	0.2109 ± 0.0295	0.0405 ± 0.0124	0.0401 ± 0.0123	0.9689	0.8573	0.7897	0.7847	0.7736	0.6689	0.6219
JUC	0.2466 ± 0.0322	0.1973 ± 0.0284	0.1927 ± 0.0279	0.2443 ± 0.0327	0.2600 ± 0.0331	0.2645 ± 0.0334	0.2006 ± 0.0287	0.1814 ± 0.0271	0.1607 ± 0.0254	0.9561	0.8801	0.8738	0.8681	0.6501	0.6134
ARA	0.2578 ± 0.0330	0.2204 ± 0.0302	0.2092 ± 0.0290	0.2410 ± 0.0318	0.2477 ± 0.0325	0.2510 ± 0.0321	0.2669 ± 0.0341	0.2456 ± 0.0333	0.2263 ± 0.0307	0.1302 ± 0.0227	0.9639	0.9620	0.9610	0.6979	0.6280
GAL	0.2621 ± 0.0333	0.2281 ± 0.0309	0.2151 ± 0.0298	0.2568 ± 0.0329	0.2646 ± 0.0339	0.2684 ± 0.0341	0.2732 ± 0.0342	0.2576 ± 0.0330	0.2392 ± 0.0317	0.1422 ± 0.0238	0.0127 ± 0.0067	0.9665	0.9635	0.6981	0.6227
NOG	0.2715 ± 0.0340	0.2517 ± 0.0321	0.2398 ± 0.0318	0.2767 ± 0.0347	0.2685 ± 0.0351	0.2898 ± 0.0353	0.3063 ± 0.0364	0.2885 ± 0.0351	0.2580 ± 0.0321	0.1475 ± 0.0242	0.0255 ± 0.0090	0.0259 ± 0.0091	0.9704	0.6801	0.6219
BAS	0.4387 ± 0.0452	0.4179 ± 0.0438	0.4106 ± 0.0434	0.4445 ± 0.0455	0.4558 ± 0.0461	0.4589 ± 0.0467	0.4388 ± 0.0452	0.4627 ± 0.0470	0.4255 ± 0.0439	0.4467 ± 0.0458	0.3863 ± 0.0428	0.3714 ± 0.0416	0.4070 ± 0.0428	0.9830	0.8000
SER	0.4973 ± 0.0488	0.4449 ± 0.0455	0.4326 ± 0.0447	0.4796 ± 0.0477	0.5064 ± 0.0494	0.5085 ± 0.0496	0.4766 ± 0.0472	0.4824 ± 0.0481	0.4704 ± 0.0468	0.4940 ± 0.0487	0.4663 ± 0.0472	0.4464 ± 0.0457	0.4685 ± 0.0457	0.1954 ± 0.0280	1.0000

branch shows that *B. comizo*, *B. sclateri* and *B. bocagei*, species of the Atlantic basin, cluster together, while *B. graellsii* and *B. guiraonis*, both species of the Mediterranean side of the Peninsula, group together in their own cluster. *B. microcephalus* (River Guadiana) is placed in the terminal node of this second main branch.

In order to visualise the gathering of populations, PCA was used to examine the variability assessed by RAPD. The first three axes covered a large portion of the total variation (89.5%), with three main groups observed (Figure 4). This PCA topology is congruent with the distribution of the groups as revealed by the dendrogram.

Discussion

Bands that were monomorphic in one species (and therefore its diagnostic markers) or some species, but absent in the rest, are those mainly responsible for the greater inter- rather than intraspecific polymorphism detected (Callejas and Ochando, 2001). Levels of intraspecific polymorphism ranged between 0 and 29.36% (Table 2). The higher polymorphism of some populations, such as CIJ, GUA and MAN, are due mainly to the presence of a high number of bands in low frequencies (Table 2). In general, levels of polymorphism are low compared with those seen in other fish RAPD studies (Bielawski and Pumo, 1997; Caccone *et al*, 1997; Gomes *et al*, 1998; Mamuris *et al*, 1999).

Table 3 shows the similarity values within- (on the diagonal) and between-populations (above the diagonal). Within-population indices were very high, ranging from 95.36% to 100%, and greater than those found among other species of fish using RAPD analysis (Bardakci and Skibinski, 1994; Foo *et al*, 1995; Bielawsky and Pumo, 1997). Likewise, the genetic similarity between populations of the same species was smaller than the intrapopulation similarities. This implies that individuals within each population are genetically more similar to each other than to individuals from any other population of the same species. In general, similarity values ranged from 94.06% between populations of *B. comizo*, to 97.81% between populations of *B. sclateri*, and were higher than interpopulation similarity values reported for other species of fish (Bielaswky and Pumo, 1997). Thus, although there is much genetic similarity between these populations, some geographical species differentiation appears to exist.

These parameters allow quantification of genetic diversity, and indicate levels of genetic variability in barbel populations to be rather low. Diversity was low within populations and was mainly found between populations of different species. Previous surveys in several species of European barbels, using allozymes, also showed their genetic variability to be low (Berrebi and Cattaneo-Berrebi, 1993; Machordom *et al*, 1995). Nowadays, although barbel populations are larger in numbers when compared with other cyprinids species, they are in decline due to environmental changes such as drought, freshwater contamination, river dredging, excessive water supply for human, agricultural or industrial purposes and the introduction of exotic species (García-Berthou and Moreno-Amich, 2000; Penczak and Kruk, 2000; Vila-Gispert *et al*, 2000; Doadrio, 2001). Thus, the low genetic variability of *Barbus* spp. is probably attributable, at least in part, to genetic drift owing to small population sizes arising as a consequence of habitat alteration. However, we cannot forget another important process that must be implicated in such low variability, ie founder effect in the evolutionary history of the genus due to its recent radiation in the Iberian Peninsula (Callejas and Ochando, 2000).

An understanding of the inter- and intraspecific distribution of genetic variation within *Barbus* is essential for the development of appropriate conservation strategies. At present, conservation programmes are being promoted to protect *Barbus* species (Economidis, 1998). The results of the present RAPD study reveal that nuclear DNA variation in *Barbus* Spanish species is low, and moreover this kind of study can provide important information for the management of future conservation strategies.

With respect to genetic distances (Table 3, below diagonal), intraspecific values varied from 0.0066 to 0.0405, and fall within the range reported by other authors for local populations of the same species (Ayala, 1975; Singh, 1989; Ward *et al*, 1992) Conversely, interspecific distance values found among the present species (except for all comparisons of *B. haasi* (BAS) and *B. meridionalis* (SER) with the remaining Spanish populations) are lower than values commonly reported among species within a genus. Rather, they are more in accordance with genetic distance data detected for subspecies (Ayala, 1975; Nei, 1976; Avise and Smith, 1977; Avise, 1994). Consequently, RAPD data can also provide insights into taxonomic status. The debate over whether these different groups represent species or subspecies is still open. According to the biological species concept (BSC), species are 'groups of interbreeding natural populations that are reproductively isolated from other such groups' (Mayr, 1970). Yet from the genetic point of view, most of the Iberian barbel groups might be better considered as different subspecies with recent radiation. This idea is supported by the finding of different hybrid barbels (Encina and Granado, 1988; Berrebi and Cattaneo-Berrebi, 1993; Almaça, 1996; Crespin and Berrebi, 1999; Callejas and Ochando, 2000).

The dendrogram and PCA elaborated from RAPD data, both highly congruent, are shown in Figures 3 and 4 respectively. The robustness of the tree was shown by the high bootstrap values. As a whole, cluster analysis of both bandsharing- and frequency-based distances indi-

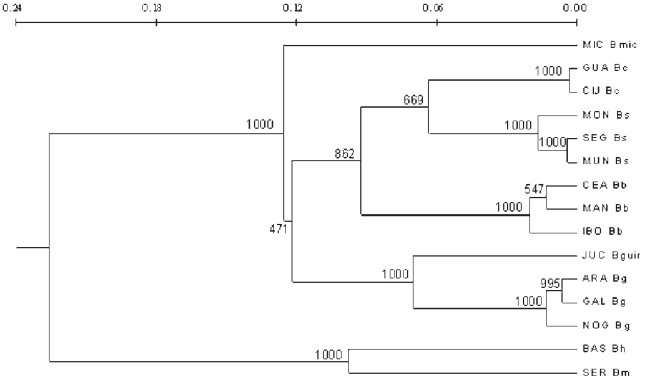

Figure 3 UPGMA dendrogram using Nei's genetic distances inferred from RAPD. Bootstrap values based on 1000 replications are presented.

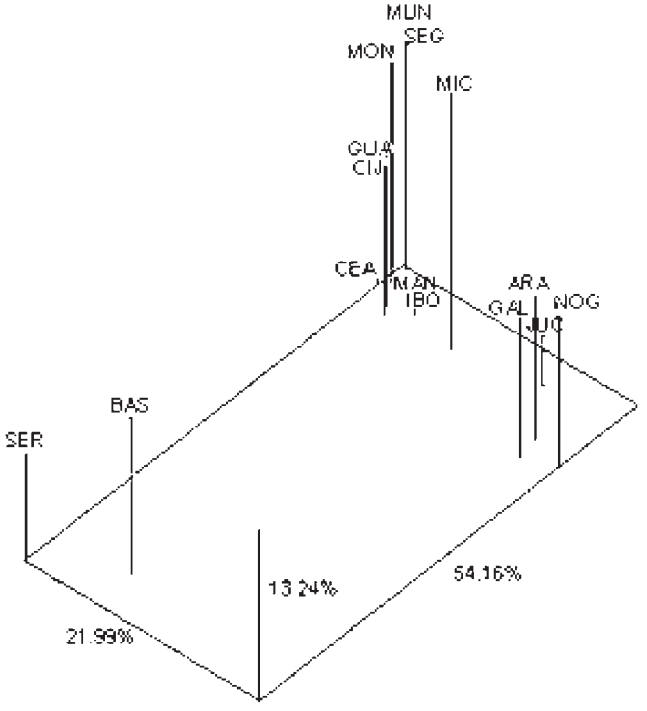

Figure 4 Plot of the first three components in a principal component analysis of RAPD data from the fifteen populations of *Barbus* sampled. See Table 1 for abbreviations of names of populations.

cates that *B. haasi* and *B. meridionalis* (both inhabitants of north-eastern Spain) are differentiated from the remaining Spanish *Barbus* species. At the same time, the *Barbus* species of the Atlantic basin and the south of the Iberian Peninsula (*B. bocagei*, *B. comizo* and *B. sclateri*) are clearly differentiated from *Barbus* species of the Mediterranean basin (*B. graellsii* and *B. guiraonis*). This pattern of differentiation is corroborated by the PCA results (Figure 4), and indicates that clustering of the populations reflects their geographical distribution. Four molecular studies have been performed on Iberian populations of *Barbus* in order to assess their relationships and to attempt to answer some taxonomic questions. Based on allozymes (Machordom *et al*, 1995), on cytochrome b sequences (Zardoya and Doadrio, 1998; Callejas and Ochando, 2000) and on *ATPase 6* and *8* genes (Machordom and Doadrio, 2001), north Mediterranean species were differentiated from the remaining Iberian barbel species as were Mediterranean samples from their Atlantic counterparts.

In general, freshwater fish have limited means of dispersal, and geological events are important factors in their evolution (Borowsky *et al*, 1995). The present RAPD results, concordant with those mentioned above, indicate, as might be expected, that the phylogenetic relationships among Spanish barbels (Figure 3) are closely related to the formation of fluvial basins and the palaeobiogeography of the Iberian Peninsula (López-Martínez, 1989). A special case is *B. microcephalus*, which is placed on the terminal node of a cluster that includes Mediterranean and Atlantic barbels (the lowest value of bootstrap). This species also has an intermediate position between both groups in the PCA. *Barbus microcephalus*, now inhabiting the Guadiana basin, would have a common ancestor with *B. graellsii* and *B. guiraonis* as can be inferred from the number of RAPD bands they share and from its cluster position in other studies (Machordom *et al*, 1995; Zardoya and Doadrio, 1998; Callejas and Ochando, 2000; Machordom and Doadrio, 2001). There is faunistic evidence that a fluvial capture process occurred between the Guadiana and Jucar Rivers (Doadrio, 1984; Perdices *et al*, 2000) and the ancestor of *B. microcephalus* would have migrated from the eastern river basins to the Guadiana basin.

On the basis of ecological traits, barbels in Europe belong to two ecophenotypes: reophilic and fluvio-lacustrine barbels. Molecular studies have been performed on barbel samples using different markers (Tsigenopoulos *et al*, 1999; Tsigenopoulos and Berrebi, 2000) in an attempt to clarify whether there is one lineage for each different ecophenotype or whether groups of *Barbus* species represent clusters of morphologically convergent taxa. The results have provided contradictory conclusions in this respect. Therefore, the clear differentiation of *B. meridionalis* and *B. haasi* (north-east of Spain) from the remaining Iberian species might be explained by a longer isolation period, or as a consequence of adaptation to different environmental conditions (given that both species are mountain barbels), or by possible interactions with other European *Barbus* species.

At present there are some controversies surrounding barbel organisation in the northern Mediterranean area. The classical hypothesis of freshwater fish migrations (Darlington, 1948; Banarescu, 1973), established that a lineage of *Barbus* came from Asia to Europe and finally colonised the Iberian Peninsula and North Africa. An alternative hypothesis (Doadrio, 1990) based on osteological characters and the fossil record, proposed two different lineages. The lineage *Barbus* colonised the whole of Europe, including north-east Spain, from Asia, while a second lineage, *Luciobarbus*, migrated from Asia through the Middle East and North Africa, occupying the Iberian Peninsula from the south. Since then, the results of this group seem to support this idea (Machordom *et al*, 1995; Zardoya and Doadrio, 1998, 1999; Machordom and Doadrio, 2001). In contrast, other authors, based on morphological, parasitological and genetic studies, agree with the classical hypothesis of barbel dispersion (for further details, see Berrebi, 1995; Berrebi *et al*, 1996).

Regardless of how the directions of colonization have taken place, differences between the species of north-eastern Spain (*B. haasi* and *B. meridionalis*) and the remaining Spanish *Barbus* species were observed as well as a Mediterranean and Atlantic separation of the Iberian species of *Barbus*.

In summary, the results demonstrate that the RAPD technique is a very advantageous and useful tool for studying the genetic structure and phylogenetics of the species and population levels of *Barbus*. The study of the genetic structure of populations is essential in order to devise conservation programme strategies, which can efficiently maintain genetic variability. The RAPD markers revealed generally low levels of intra- and interpopulation variation, and a north-eastern, Mediterranean and Atlantic differentiation of the Iberian species. Their dispersion and divergence during a recent period, together with an adaptation to local habitats, might indicate that most of Iberian groups are still probably under speciation process from a genetic point of view.

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