Haplotype Diversity and Phylogenetic Relationships Among the Iberian Barbels (*Barbus,* Cyprinidae) Reveal Two Evolutionary Lineages

I. Doadrio, J. A. Carmona, and A. Machordom

The phylogenetic relationships and haplotype diversity of all Iberian barbels were examined by analyzing the complete mitochondrial cytochrome *b* gene sequence (1141 bp) of 72 specimens from 59 Iberian localities. Phylogenetic findings demonstrated a clear distinction between two mitochondrial lineages and confirmed the existence of two previously considered subgenera: *Barbus* and *Luciobarbus.* The first subgenus, *Barbus,* is represented on the Iberian Peninsula by *Barbus haasi* and *Barbus meridionalis.* The second subgenus, *Luciobarbus,* includes the remaining endemic Iberian species: *Barbus comizo, Barbus bocagei, Barbus microcephalus, Barbus sclateri, Barbus guiraonis,* and *Barbus graellsii.* Mean haplotype divergence between these subgenera was 10.40%, providing evidence of a clear subdivision within the Iberian barbels. Our results conflict with those reported in a recent study, based on 307 cytochrome *b* base pairs, that failed to identify any division within the genus *Barbus* in the Iberian Peninsula. The inclusion of nine further species belonging to this genus (used as outgroups) allowed us to establish a closer relationship of the Iberian species of the subgenus *Barbus* with other European taxa than with the Iberian *Luciobarbus,* which was found to cluster with North African, Caucasian, and Greek species. At the population level, no biogeographic structure was shown by specimens of each species (only 5.98% of the variation was attributable to differences among populations of each species). Given the discrete amount of divergence found among the *Luciobarbus* species, the formation of current hydrographic basins during the Plio-Pleistocene seems to have played a major role in their isolation and evolution.

The genus *Barbus* is known for its morphological plasticity and different levels of ploidy. The diploid and hexaploid species are distributed in Asia and Africa, while the tetraploid species inhabit Africa and mainly the Palearctic area including Europe, North Africa, and western Asia. The ploidy level of this genus has recently been shown to be homoplasic (Machordom and Doadrio 2001b) and precludes the possibility of clarifying its taxonomy according to this character.

Barbels are among the most widespread and diverse primary fishes (Myers 1938) in Europe, where only tetraploid species exist. Given its marked diversity, especially on the Iberian and Balkan peninsulas, this genus shows features that make it an ideal evolutive model for European freshwater fauna.

Two different monophyletic groups with different biogeographic histories have been postulated for the Palearctic area (Doadrio 1984, 1990, 1994; Tsigenopoulos and Berrebi 2000). One of these groups is represented by the subgenus *Barbus,* distributed over most of central Europe and the north of the southern peninsulas, as well as across some of Asia (see Berrebi 1995). The second clade is formed by the subgenus *Luciobarbus,* which occupies the Iberian Peninsula, southern Greece, the Near East, and North Africa. However, the existence of these two different phylogenetic lineages on the Iberian Peninsula has recently been questioned by Callejas and Ochando (2000), who consider that the Iberian Peninsula species correspond to a single lineage. According to these authors, the ancestor of these species would need to have arisen on the Iberian Peninsula before the isolation of the European lineage by the formation of the Pyrenees. This theory is in agreement with classic studies based on external morphological data (Almaça 1976; Banarescu 1960) which have been refuted by more recent works (Tsigenopoulos and Berrebi 2000; Zardoya and Doadrio 1998, 1999).

These classic and alternative theories

From the Museo Nacional de Ciencias Naturales, José Gutiérrez Abascal 2, 28006 Madrid, Spain. The authors wish to thank L. de Ambrosio, A. de Sostoa, A. Doadrio, I. Doadrio Jr., P. Garzón, F. Lerma, F. Morcillo, A. Perdices, and S. Schönhuth for their help in obtaining fish specimens. We appreciate the skillful technical assistance provided by L. Alcaraz. We also thank A. Burton for her conscientious revision of the English. A. Machordom and J. A. Carmona were sponsored by postdoctoral grants from the "Comunidad de Madrid." This study was funded by projects 07B/0019/1998, "Caracterización genética de las especies piscícolas de la cuenca del Tajo" (Comunidad de Madrid), and PB97-1178, "Relaciones filogenéticas y diversidad de la ictiofauna continental española" (DGES). Address correspondence to Annie Machordom at the address above or e-mail: annie@mncn.csic.es.

 93:140–147

mainly differ according to biogeographic concepts. Classic theories emphasize the origin and dispersion of the genus (Banarescu 1973), while the alternative hypothesis centers on the barriers that lead to speciation (Doadrio 1984). Moreover, the theories differ in the phylogenetic relationships that support them. While classic theories closely linked Iberian species to those from central Europe (Almaça 1976; Banarescu 1973), the alternative hypothesis considers that Iberian species are more closely related to North African taxa (Doadrio 1984, 1990). This last theory was reinterpreted as another dispersalist model, considering that it implies or suggests colonization of the Iberian Peninsula through a North African route (Berrebi 1995) (contrary to the classically proposed dispersion through Europe). However, this new point of view is simplistic, since the configuration of the areas involved during the Cenozoic era (when these putative colonizations would have occurred) was very different to the current setting (Doadrio 1994).

The present study was designed to estimate the speciation period for barbels inhabiting the Iberian Peninsula by testing two hypotheses: (1) that barriers present from the time of formation of the current (Plio-Pleistocene) basins were responsible for isolation and differentiation, or (2) that this differentiation occurred much before (in the Miocene) via Iberian endorheic basins. Due to the primary fish condition of barbels, it was envisaged that besides shedding light on this issue, findings might serve to establish the relationships of the Iberian barbels with those inhabiting the Palearctic region. To this end, we included other closely related taxa in our study.

The main aim was thus to test both hypotheses using a database similar to that presented by Callejas and Ochando (2000) derived from the cytochrome *b* sequences of the different populations. However, the present analysis was based on the complete gene sequence (1141 bp), instead of the 307 bp examined by Callejas and Ochando (2000). Indeed, Lydeard and Roe (1997) reported that the complete cytochrome *b* gene was useful for diagnosing relationships of representative actinopterygian fishes, contrary to the method used in previous studies based on only a portion of the gene. We also used a higher population number such that all taxa inhabiting the Iberian Peninsula were represented.

Given the diverse and widespread nature of the *Barbus* genus on the Iberian Peninsula, knowledge of its evolutive history may contribute toward a greater understanding of Iberian Peninsula paleohydrology. The hydrographic record is highly complex, since over most of the Tertiary the area was comprised of endorheic basins. Thus an additional aim was to explore the phylogeography of current basins and potential contacts among them.

Materials and Methods

We examined all the species of Iberian barbels described, including those of the *Barbus* (*Barbus haasi* and *Barbus meridionalis*) and *Luciobarbus* subgenera (*Barbus graellsii, Barbus guiraonis, Barbus sclateri, Barbus microcephalus, Barbus comizo,* and *Barbus bocagei*) (Figure 1 and Table 1). *B. comizo* was identified in the field on the basis of its last unbranched dorsal fin ray ossification and the number of scales, irrespective of the head elongation level. Thus we included both the morphotypes *B. comizo* and *Barbus steindachneri,* considered as two valid species (Almaça 1967, 1972) or as two morphotypes (Doadrio 1988; Karaman 1971). The following closely related taxa were also examined to establish phylogenetic relationships with the Iberian barbels: *Barbus barbus, Barbus caninus,* and *Barbus aff. petenyi* (from the subgenus *Barbus*), and *Barbus callensis, Barbus magniatlantis, Barbus moulouyensis, Barbus capito,* and *Barbus graecus* (from the subgenus *Luciobarbus*) (Table 1). To better establish the relationships between the two putative subgenera inhabiting the Iberian Peninsula, we included one hexaploid barbel as an outgroup: *Barbus (Labeobarbus) fritschii.* Several localities were investigated for each Iberian species to recover their possible diversity, including their *terrae typicae* to avoid the misidentification of each taxon. In total, 81 specimens were examined: 61 sequenced in the present study and 20 sequenced previously (Briolay et al. 1998; Machordom and Doadrio 2001a,b; Zardoya and Doadrio 1998, 1999).

All fish specimens were obtained by electrofishing. Muscle and liver tissues were kept in alcohol or liquid nitrogen before storage at −74°C. Voucher specimens were included in collections of the "Museo Nacional de Ciencias Naturales" (Spain).

Total DNA was extracted from approximately 0.1–0.2 g of tissue (preferentially muscle) according to the phenol/chloroform extraction procedure (Sambrook et al. 1989). Complete cytochrome *b* sequences were amplified by the polymerase chain reaction (PCR). The primers used were GluF 5′AACCACCGTTGTATTCAACTACAA3′ (Zardoya R, unpublished) and ThrR 5′ACCTCCGATCTTCGGATTACAAGACCG3′ (Zardoya R, unpublished). PCR mixtures were prepared under similar conditions in a final volume of 25 μl containing 10–100 ng DNA, 0.5 μM of each primer, 0.2 mM of each dNTP, 1.5mM $MgCl_2$, 1 U *Taq* DNA polymerase (Biotools), and the corresponding buffer plus ddH_2O. The amplification process was conducted as follows: 94°C (2 min), 35 cycles at 94°C (45 s), 48°C (1 min), 72°C (1 min 15 s), and a final extension phase at 72°C (5 min). PCR products were cloned using the pGEM-T vector (Promega) into *Escherichia coli* JM109 and sequenced using the FS-Taq Dye Deoxy Terminator Cycle-Sequencing kit (Applied Biosystems 377) according to the manufacturer's instructions. DNA sequences of both strands were obtained using M13 universal (forward and reverse) sequencing primers.

The sequences obtained were cleaned at the primer ends, aligned, and verified using forward and reverse overlap sequences (Sequencher program, Gene Code Corp.). Translation to proteins was also verified using this and the MacClade program (Maddison and Maddison 1992) and base codon positions designated.

Analyses were performed according to the principles of neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML). The best model of evolution that fitted our data was obtained using the program Model Test 2.1 (Posada and Crandall 1998). Thus the model's general time reversible (GTR) model (Lavane et al. 1984; Rodríguez et al. 1990) and HKY85 distances (Hasegawa et al. 1985) were used first. Given that the trees were better resolved according to this last model (HKY85), the results presented for NJ and ML were based on this model. The possibility of saturation for the transition and transversion changes was checked by plotting the absolute number of changes at each codon position against uncorrected percentage divergence values (*p*).

Parsimony analyses were performed by TBR branch swapping, MULTREES option, and random stepwise additions using the heuristic search algorithm. The ML analysis was performed by Quartet Puzzling (with 1000 pseudoreplications) using the PAUP* (Swofford 2000) package. Confidence for the analyses was estimated by bootstrapping (1000 repetitions for NJ and 500 for MP) (Felsenstein 1985) and decay

index values (Bremer 1988, 1994; and using the AutoDecay program; Eriksson 1998). The transition/transversion ratio was determined by a ML approach. To determine whether a particular tree topology corresponded to a significantly better or worse interpretation of the data than an alternative tree, we used both the Wilcoxon signed rank test (Templeton 1983) and Kishino–Hasegawa (Kishino and Hasegawa 1989) test as implemented in PAUP. The Arlequin program was used to obtain intraspecific diversity indices and to perform the MANOVA analyses (Schneider et al. 2000).

Results

The nucleotide variables characterizing the sequences analyzed are shown in Table 2. Of the 1141 characters obtained (corresponding to the complete cytochrome *b* sequence), 722 were constant (63.28%) and 266 (23.31%) were parsimony informative in comparisons of all the species analyzed. According to codon position, the most informative was the third (224 parsimony informative characters). The empirical percentages of the different nucleotides were A = 28.57, C = 30.15, G = 15.11, T = 26.16, with no differences shown among taxa ($P = 1.00$) and with a bias against G, which is usual for the mitochondrial DNA of fish. Estimated transition/transversion ratios ranged from 7.59 to 12.25. This variable was taken into account in the MP and ML analyses.

The results of the saturation tests of transition and transversion changes are shown in Figure 2. For all the changes considered together, the graph fitted a straight line. Nevertheless, there was an indication of transition saturation in the third positions starting from 15% divergence (thus only affecting relationships between the species used as the outgroup and remaining species).

Mean sequence divergence values are provided in Table 3 and range from 1.32 to 16.67%. This last value corresponds to the divergence between the hexaploid species, *B. (Labeobarbus) fritschii* and *B. caninus* or *B. aff. petenyi*. Within the Iberian specimens, two levels of interspecific values were shown: between *B. haasi* or *B. meridionalis* and the rest (9.76–11.11%) and within the *Luciobarbus* species (1.32–6.06%). When the outgroups were considered, smaller interspecific divergence values were established for the Iberian *Luciobarbus* species with respect to the North African and Caucasian or Greek spe-

Table 1. Species, number in Figure 1 map, specimen collection reference, location data, and GenBank accession number

Species	Map	Reference	River	Basin	GenBank accession no.
B. bocagei	1	653 AT	Odra	Duero	AF334067
	2	649 AT	Arlanzón	Duero	AF334066
	3	1172 ES	Bañuelos	Duero	AF334060
	4	914 ES	Duratón	Duero	AY004728(1)
	4	913 ES	Duratón	Duero	AF045969(2)
	5	602 ES	Cega	Duero	AF334056
	6	857 ES	Moros	Duero	AF334057
	7	1997 ES	Huebra	Duero	AF334058
	8	1999 ES	Águeda	Duero	AF334059
	9	88 AT	Trevijana	Tagus	AF334061
	10	95	Árrago	Tagus	AF334065
	11	95 AT	Acebo	Tagus	AF334063
	12	570 AT	Caparro	Tagus	AF334062
	13	174 AT	Guadarrama	Tagus	AF334052
	14	502 AT	Jerte	Tagus	AF334064
	15	172 AT	Alberche	Tagus	AF334054
	16	3 BT	Tagus	Tagus	AY004727(1)
	17	909 AT	Uso	Tagus	AF334053
	18	G-1936	Almonte	Tagus	AF334051
	19	480 AT	Vid	Tagus	AF334055
B. comizo	16	2 BT	Tagus	Tagus	AY004735(1)
	18	G-1935	Almonte	Tagus	AF045967(2)
	20	417 AT	Tiétar	Tagus	AF334042
	21	G-1939	Almonte	Tagus	AF334044
	22	73 AT	Magasca	Tagus	AF334043
	23	527 AT	Alburrel	Tagus	AF334046
	23	531 AT	Alburrel	Tagus	AF334045
	24	8 AT	Gévora	Guadiana	AF334048
	25	115 AT	Albuera	Guadiana	AF334050
	26	1 BC	Quejigares	Guadiana	AF334049
	27	360 AT	Zújar	Guadiana	AF334047
B. microcephalus	27	362 AT	Zújar	Guadiana	AF334085
	28	2220 BM	Estena	Guadiana	AF045971(2)
	29	140 AT	Sillo	Guadiana	AF334084
B. sclateri	29	142 AT	Sillo	Guadiana	AF334072
	30	203 AT	Montemayor	Guadalquivir	AF334073
	31	240 AT	Molinos	Guadalquivir	AF334070
	32	223 AT	Vendoval	Guadalquivir	AF334068
	33	404 AT	Guadiato	Guadalquivir	AF334069
	34	962 AT	Manzano	Guadalquivir	AF334082
	35	750 AT	Salar	Salar (South)	AF334071
	36	26 GEB	Alhama	Guadalquivir	AF045970(2)
	37	761 AT	Genal	Guadiaro (South)	AF334077
	38	814 AT	Pereilas	Guadalhorce (South)	AF334075
	39	754 AT	Manilva	Manilva (South)	AF334076
	40	768 AT	Banahavís	Banahavís (South)	AF334078
	41	786 AT	Verde	Verde (South)	AF334079
	42	705	El Real	El Real (South)	AF334080
	43	808 AT	de las Posadas	(South)	AF334074
	44	752 AT	Vélez	Vélez (South)	AF334081
	45	1036 AT	Segura	Segura	AF334083
B. guiraonis	46	25 B	Bullent	Bullent	AF045972(2)
	47	37	Jardín	Júcar	AF334090
	48	999 AT	Magro	Júcar	AF334091
	48	1000 AT	Magro	Júcar	AF334092
	48	1001 AT	Magro	Júcar	AF334093
	49	1009 AT	Realillo	Turia	AF334094
	49	1010 AT	Realillo	Turia	AF334095
	50	1011 AT	Palancia	Palancia	AF334096
	50	1013 AT	Palancia	Palancia	AF334097
B. graellsii	51	980 AT	Mesa	Ebro	AF334089
	52	4	Cinca	Ebro	AF334087
	53	146 EBG	Gállego	Ebro	AF045973(2)
	54	2061 ES	Irati	Ebro	AF334088
	55	2119 ES	Araquil	Ebro	AF334086
B. haasi	51	988 AT	Mesa	Ebro	AF334101
	56	598 AT	Jalón	Ebro	AF334098
	57	621 AT	Alhama	Ebro	AF334100
	58	2006 ES	Esca	Ebro	AF045976(2)
	54	2053 ES	Irati	Ebro	AF334099
B. meridionalis	59	1 B	Tordera	Tordera	AF045977(2)
	59	2 B	Tordera	Tordera	AF334102
Outgroups					
B. barbus					Y10450(3)
B. callensis		104 AL	Kebir		AF045974(2)
B. caninus		1ital	Judrio		AF287424(4)
B. capito		207 MO	Terek		AF045975(2)
B. (L.) fritschii		103 M	Kasab		AF287430(4)

Table 1. Continued.

Species	Map	Reference	River	Basin	GenBank accession no.
B. graecus		660 G	Kifisos		AF090786(5)
B. magniatlantis		68 A	Oum Er Rbia		AY004734(1)
B. moulouyensis		116 A	Moulouya		AY004742(1)
B. aff. petenyi		330 G	Gallikos		AF287440(4)

Numbers between brackets correspond to 20 previously sequenced specimens: (1) Machordom and Doadrio (2001a); (2) Zardoya and Doadrio (1998); (3) Briolay et al. (1998); (4) Machordom and Doadrio (2001b); (5) Zardoya and Doadrio (1999).

cies (*B. capito* and *B. graecus,* respectively) than with respect to those also inhabiting the Iberian Peninsula but corresponding to the subgenus *Barbus.* In the same way, *B. haasi* and *B. meridionalis* showed smaller divergence values (6.59–9.03%) with respect to some of the European species (*B. barbus, B. caninus,* and *B. aff. petenyi*) than the rest of the Iberian species.

Of the 61 haplotypes established for species from the Iberian Peninsula, intraspecific nucleotide diversity values were greatest for *B. microcephalus* and *B. meridionalis* (Table 4). There was no positive correlation ($r = -0.34$) between intraspecific diversity and sample size, with the least number of specimens analyzed corresponding to these latter two species.

The phylogenetic relationships established also identified the splitting into two groups, corresponding to the two subgenera of the Iberian Peninsula. Similar topologies were recovered in all the analyses (Figure 3), even when the transition/transversion ratio was taken as 10 or the default value as 2. The monophyly of *Luciobarbus* was clearly established (bootstrap values for MP and NJ 100%, ML 96%, DI = 10), including the Iberian Peninsula endemisms (*B. bocagei, B. comizo, B. sclateri, B. microcephalus, B. graellsii,* and *B. guiraonis*), the North African taxa (*B. magniatlantis, B. callensis,* and *B. moulouyensis*), *B. capito* (from the Caucasus), and a Greek species (*B. graecus*). Further, the Iberian *Luciobarbus* lineage was strongly supported (bootstrap values for MP 70%, NJ 98%, ML 94%, DI = 1). The sister group of this assemblage was the *Barbus* subgenus, including the two Iberian populations of *B. haasi* and *B. meridionalis,* and the European *B. caninus, B. aff. petenyi,* and *B. barbus* species, which also formed a well-supported cluster (bootstrap values for MP 81%, NJ 96%, ML 98%, DI = 5).

Two clusters were also distinguished within the Iberian *Luciobarbus* lineage: one grouped *B. bocagei, B. comizo,* and *B. sclateri* together (Atlantic and southern drainage systems), and the other included *B. guiraonis, B. graellsii* (Mediterranean and east Cantabrian rivers), and *B. microcephalus* (from the Guadiana basin of the Atlantic slope) (bootstrap values for this last group MP and NJ 100%, ML 98%, DI = 9). Within the assemblage of Atlantic species (*B. bocagei, B. comizo,* and *B. sclateri*), the relationship between *B. comizo* and *B. bocagei* was strongly supported (MP 95%, NJ and ML 100%, DI = 7). In contrast, the grouping of *B. sclateri* with the previous two species (MP 50% and NJ 84%) was less well supported. The Templeton and Kishino–Hasegawa tests ($P = 0.04$) indicated that *B. sclateri* clusters significantly better with *B. comizo* and *B. bocagei* than with *B. guiraonis, B. graellsii,* and *B. microcephalus.* Nevertheless, a further equally parsimonious option was the consideration of the three groups in a polytomy: (1) *B. comizo* + *B. bocagei*; (2) *B. guiraonis* + *B. microcephalus* + *B. graellsii*; and (3) *B. sclateri.* This was also reflected in the null decay index value in the dichotomy between *B. bocagei* + *B. comizo* and *B. sclateri.*

The relationships among the different populations of Iberian species were not well resolved, mainly due to their great similarity. This similarity in species sequences was shown by a tree in which branch length represents divergence (Figure 4). The cluster grouping all the *B. sclateri* populations from the Guadalquivir River, for example, was not supported by bootstrap values (Figure 3). Nevertheless, each Iberian species represented by a different number of specimens always showed bootstrap values greater than 90%.

The MANOVA results for the Iberian specimen sequences also indicated that most diversity was due to interspecies differences (54.55% of the variation) relative to interpopulation variation (5.98%). These results were based on the structure defined by previous phylogenetic data. That is, three groups were defined: one for the *Barbus* subgenus (*B. haasi* and *B. meridionalis*) and two for *Luciobarbus* (*B. bocagei* + *B. comizo* + *B. sclateri* and *B. microcephalus* + *B. graellsii* + *B. guiraonis*).

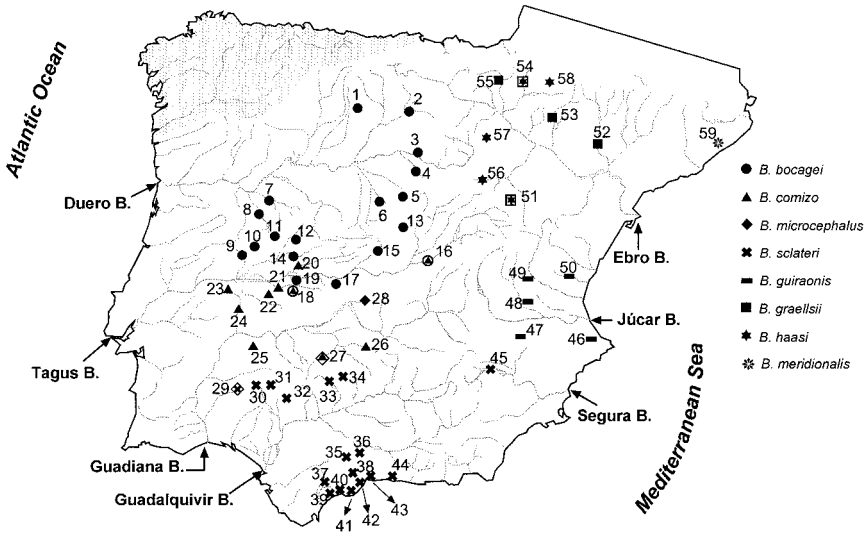

Figure 1. Sampling sites of Iberian specimens. When two specimens where found in sympatry; symbols corresponding to both species appear superimposed. Sample site numbers correspond to those shown in Table 1.

Discussion

Based on the present findings, the relationships between the two Iberian groups and other species examined would be in line with the idea of a double origin of lineages. One of these lineages, including the species considered as the subgenus *Barbus* (*B. meridionalis* and *B. haasi*), clearly showed a greater affinity to European species such as *B. barbus,* which is widely distributed. According to Banarescu (1992), this clade would be of Asian origin. The second Iberian lineage (*Luciobarbus* subgenus) was found to group with most circum-Mediterranean and Near Eastern species such as those from North Africa and

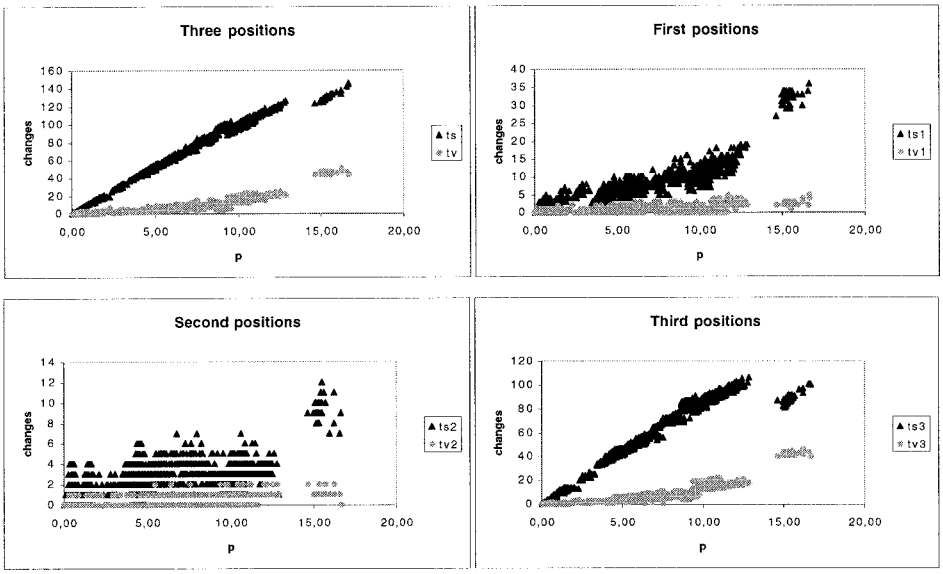

Figure 2. Relationship between uncorrected mean divergence (*p*) and the number of transversion and transition changes at different codon positions.

Table 2. Number of characters analyzed, nucleotide proportions, and transition/transversion (ts/tv) ratios in the comparison of all the taxa analyzed, the species from the Iberian Peninsula, and only considering those of the subgenus *Luciobarbus*

	All the species analyzed	Iberian Peninsula	Iberian *Luciobarbus*
Characters			
Total	1141	1141	1141
Constant	722	884	968
Parsimony informative	266	189	105
1st positions	33	24	13
2nd positions	9	5	5
3rd positions	224	160	87
A %	28.57	28.60	28.68
C %	30.15	30.27	30.43
G %	15.11	15.09	15.01
T %	26.16	26.04	25.88
ts/tv ratio	7.59	9.77	12.25

the Caucasus or Greece. This genetic distinction of the two subgenera and their phylogenetic relationships has been previously established (e.g., Machordom and Doadrio 2001b; Tsigenopoulos and Berrebi 2000).

Refuting the so-called classic theories of the history of barbels, the present data confirm the findings of previous studies derived from morphological (Doadrio 1984, 1990; Miranda and Escala 2000) and molecular techniques (Machordom and Doadrio 2001a; Zardoya and Doadrio 1998) and provide new evidence for the existence of two lineages within the *Barbus* genus represented on the Iberian Peninsula. Indeed, Doadrio (1990) identified these two groups as two subgenera. According to this author, the subgenus *Barbus* is formed by one species endemic to the Iberian Peninsula, *B. haasi,* and one distributed throughout southern French and northeastern Spanish rivers, *B. meridionalis.* The second subgenus, *Luciobarbus,* includes the Iberian species: *B. comizo, B. bocagei, B. sclateri, B. graellsii, B. guiraonis,* and *B. microcephalus.*

Bianco (1998) later split the European barbels into two genera using morphological and ecological data: *Barbus* and *Messinobarbus.* These two groups mainly coincide with the subgenera previously designated *Barbus* and *Luciobarbus* (Doadrio 1994). Thus the genus *Messinobarbus* should probably be considered a *Luciobarbus* synonym. The only difference between *Messinobarbus* and *Luciobarbus* is the inclusion by Bianco (1998) of the species *B. haasi* in the genus *Messinobarbus,* which has been allocated by several authors to other lineages or to the subgenus *Barbus* (e.g., Machordom and Doadrio 2001a,b; Machordom et al. 1995; Miranda and Escala 2000; Zardoya and Doadrio 1998).

Callejas and Ochando (2000) reject the hypothesis of two subgenera or groups on the Iberian Peninsula by analyzing partial cytochrome *b* sequences and a low number of populations. However, data in support of this rejection were not presented. Their study lacked a *B. meridionalis* specimen, and the two specimens of *B. haasi* analyzed showed the same sequence as two other species (one of them identical to a sequence of *B. bocagei* and the other identical to one of *B. graellsii*) (note that here we report that *B. haasi* shows a mean nucleotide divergence of up to 10% with respect to the other two species, and that in the first 307 bp that these authors analyzed, we found 26–28 substitutions between *B. haasi* and *B. bocagei* specimens, and 29 changes between *B. haasi* and *B. graellsii* specimens). This was proposed by the authors to be attributable to hybridization. However, the presence of *B. bocagei* in the Ebro River has never been previously reported. Other causes, such as sampling error, would be more plausible than the theory of hybridization for areas in which the two species are not in sympatry. Thus we think that these authors have no real basis to deny the existence of two groups, especially since they failed to examine species from both of these groups.

On the Iberian Peninsula, home to barbels over most of its territory with the exception of its northwestern rivers, the *Barbus* subgenus was found to be confined to northeastern rivers. Correspondingly only species of the subgenus *Luciobarbus* were observed across the remaining distribution range. Both subgenera are considered to be sympatric in some Mediterranean rivers.

Two main groups within the subgenus *Luciobarbus* appeared on the Iberian Peninsula. The first one includes the species distributed in the south and west of the Iberian Peninsula: *B. bocagei, B. comizo,* and *B. sclateri.* The other cluster grouped the species inhabiting the eastern Iberian Peninsula and the Guadiana basin: *B. guiraonis, B. microcephalus,* and *B. graellsii.* These results are in accordance with published data derived from mitochondrial sequences (Machordom and Doadrio 2001a; Zardoya and Doadrio 1998, 1999). *B. microcephalus* and *B. guiraonis,* which presently live in sympatry in some of their distribution areas, were sister species with respect to the allopatrically distributed *B. graellsii.* A similar situation was shown by *B. comizo* and *B. bocagei,* which live in sympatry and clustered together with respect to *B. sclateri,* which only appears in sympatry with *B. comizo* in a few places. This phenomenon reveals an interesting problem related to the speciation models proposed for this group. It is known that this genus shows a high degree of speciation in lotic environments of Africa (i.e., Tana Lake) (Nagelkerke et al. 1994; De Graaf et al. 2000) and that the historic paleogeography of the Iberian Peninsula indicates the existence of large lakes during

Table 3. Interspecific uncorrected mean divergence between each pair of taxa analyzed

	1																
1. *B. bocagei*	—	2															
2. *B. comizo*	1.32	—	3														
3. *B. sclateri*	3.83	4.34	—	4													
4. *B. graellsii*	4.57	4.72	5.38	—	5												
5. *B. guiraonis*	4.67	4.35	5.56	2.69	—	6											
6. *B. microcephalus*	4.93	4.64	6.06	2.78	1.78	—	**7**										
7. *B. haasi*	**10.19**	**10.33**	**9.76**	**10.45**	**10.71**	**10.68**	**—**	**8**									
8. *B. meridionalis*	**9.78**	**10.29**	**10.27**	**10.54**	**11.11**	**10.71**	**4.84**	**—**	9								
9. *B. callensis*	7.24	7.51	8.40	8.15	8.24	8.41	**10.18**	**9.95**	—	10							
10. *B. moulouyensis*	7.58	7.82	7.63	8.24	8.13	8.56	**10.52**	**11.09**	5.70	—	11						
11. *B. magniatlantis*	8.90	8.81	9.19	9.02	8.94	9.23	**11.06**	**11.35**	5.43	5.87	—	12					
12. *B. graecus*	5.82	6.01	7.01	6.43	6.16	6.11	**9.83**	**9.86**	6.40	6.84	7.54	—	13				
13. *B. capito*	6.42	6.65	7.74	7.01	7.13	7.30	**10.53**	**10.65**	6.05	6.92	7.45	5.70	—	14			
14. *B. barbus*	9.48	9.43	10.28	10.03	10.37	10.40	**6.59**	**7.98**	10.69	10.52	10.60	9.47	10.69	—	15		
15. *B. caninus*	10.88	11.16	11.82	11.94	12.17	12.33	**8.34**	**8.68**	11.74	12.09	12.45	11.66	12.01	9.82	—	16	
16. *B. aff. petenyi*	11.05	11.34	12.00	12.38	12.44	12.65	**8.61**	**9.03**	11.98	12.45	12.88	11.66	12.18	9.38	1.40	—	17
17. *B. (L.) fritschii*	15.14	15.37	15.16	15.37	15.38	15.65	**15.27**	**15.49**	16.23	15.97	16.58	15.53	16.23	14.65	16.67	16.67	—

Among the 8 Iberian Peninsula taxa, those corresponding to the subgenus *Barbus* are in bold characters.

the Tertiary (López-Martínez 1989). This scenario would resemble the current setting of Tana Lake.

Nevertheless, most Iberian catchments are inhabited by only one species of the subgenus *Luciobarbus,* although exceptions to this rule include the Tagus basin, where *B. bocagei* and *B. comizo* are sympatric, and the Guadiana basin, which was found to contain up to four species: *B. comizo, B. sclateri, B. microcephalus* (exclusive to the Guadiana), and *B. guiraonis.* It should be noted that no specimens of this last species from this basin were examined in the present study.

The paleogeographic history of the Guadiana basin seems to include several river Pleistocene–Holocene capture episodes, such as upstream with the Júcar basin, allowing the passage of *B. guiraonis.* A further example of Guadiana–Mediterranean river captures is that of the Guadiana–Júcar illustrated by the distribution of *Salaria fluviatilis* (Perdices et al. 2000). Our results also suggest the possibility of contacts downstream with the Guadalquivir basin (Betic rivers), which may have led to colonization by *B. sclateri.*

From the distribution pattern of *Luciob-*

Table 4. Intraspecific percentage of uncorrected mean divergence within the Iberian species (*p*%)

Species	N1	N2	*p*%	N3
B. bocagei	20	15	0.23	2.67
B. comizo	11	10	0.42	4.82
B. sclateri	17	16	0.64	7.26
B. microcephalus	3	3	0.81	9.33
B. graellsii	5	4	0.39	4.40
B. guiraonis	9	7	0.35	4.00
B. haasi	5	4	0.19	2.20
B. meridionalis	2	2	0.70	8.00

N1 = number of specimens analyzed, N2 = number of haplotypes found, N3 = mean number of absolute pairwise differences.

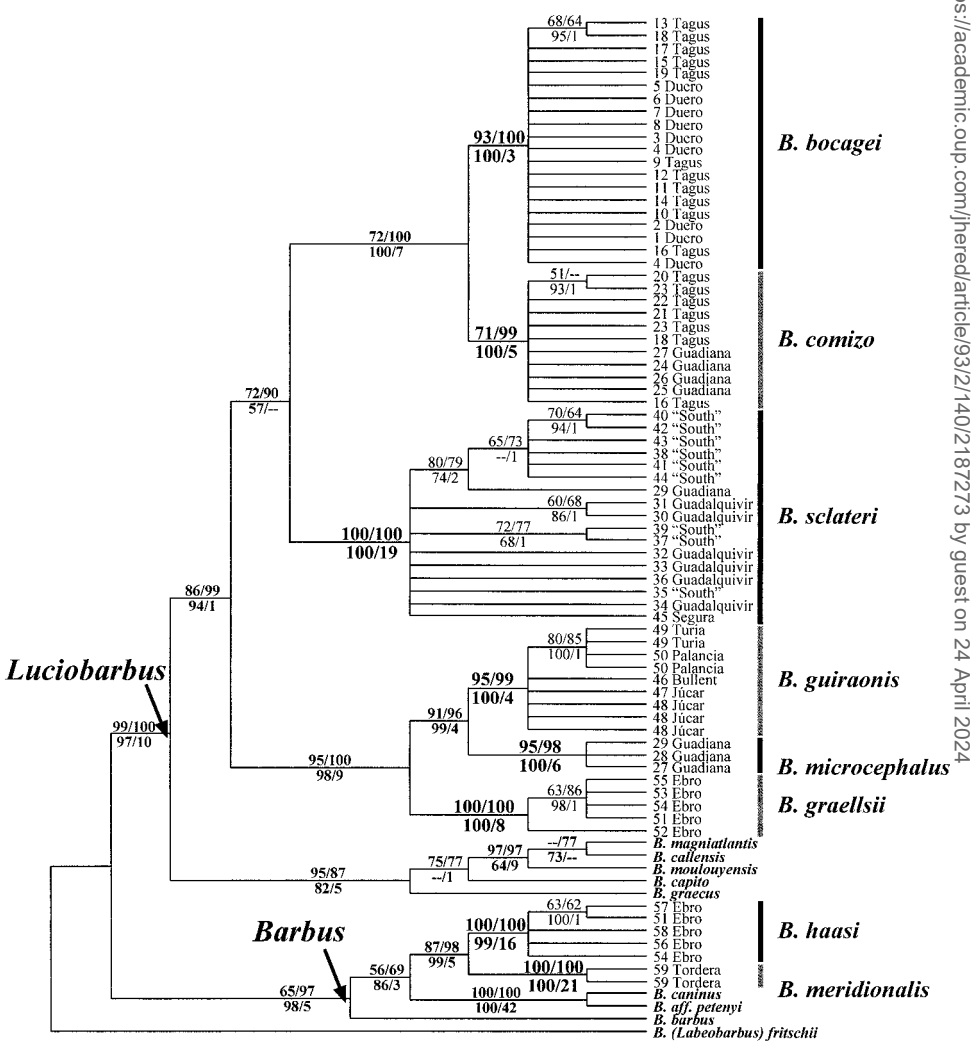

Figure 3. Maximum parsimony tree based on complete cytochrome *b* sequences. Numbers above branches represent the bootstrap values obtained for MP and NJ; numbers below branches indicate those of ML and decay indices. When a particular branch was not recovered by a certain method, two hyphens replace the corresponding value. Each specimen is designated by the number indicated in Table 1 and its basin of origin.

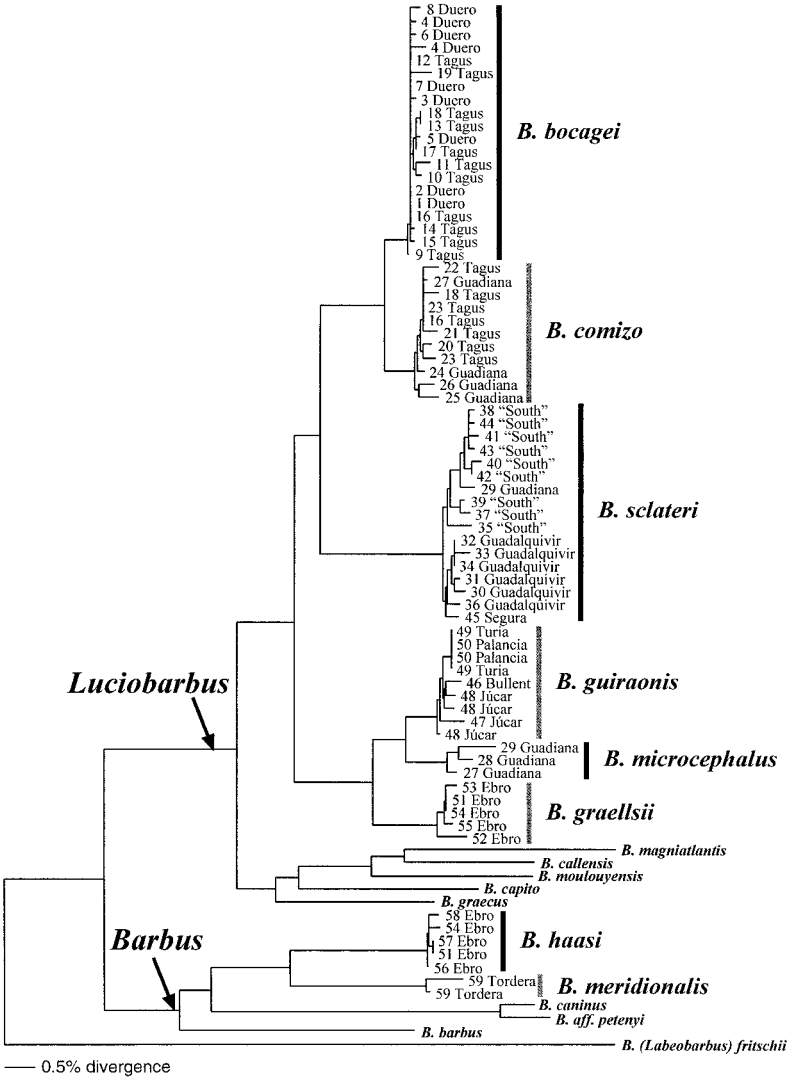

Figure 4. Neighbor-joining tree representing the relationships among the 81 specimens analyzed. Branch length is proportional to divergence. Each specimen is designated by the number indicated in Table 1 and its basin of origin.

arbus on the Iberian Peninsula, the relationship between the formation of the different basins and the isolation and speciation of the different species may be inferred. The formation of the current basins dates back to the Plio-Pleistocene period. The species examined here would have had to diversify during this period, a time of incipient formation of the fluvial basins. This presumption is based on calibrations of the proposed molecular clocks of Zardoya and Doadrio (1999) for cyprinids (1.52%/million years) or of Machordom and Doadrio (2001a) for *Luciobarbus* (1.32%/million years). Both fall within the general range considered for mitochondrial genes (1–2%) (Bermingham et al. 1997; Brown et al. 1982; Moritz et al. 1987).

The least amount of divergence was shown between *B. comizo* and *B. bocagei* (between 800,000 and 1 million years), two species living in sympatry in the Tagus basin. Hybridization between these two species has been postulated (Callejas and Ochando 2000), but was not based on definite evidence. Even if we consider this possibility, which is not rare in fishes, the morphological plasticity of *B. comizo* would no doubt pose specimen misidentification problems. If we take into account our data, we would also have to rule out the existence of two species (*B. comizo* and *B. steindachneri*), since they are impossible to distinguish using genetic tools (Machordom et al. 1995; Zardoya and Doadrio 1998).

Even if we were to investigate the putative hybridization phenomenon using nuclear markers, differentiation estimates based on mitochondrial data may serve to unequivocally discriminate between Iberian species. The splitting into two groups was well established here, as were the relationships of each group with barbels of different origin. These findings are also supported by several other published reports (Machordom et al. 1995; Tsigenopoulos and Berrebi 2000; Zardoya and Doadrio 1998, 1999). It should be emphasized that the only genetic analysis that disputes this hypothesis (Callejas and Ochando 2000) did not include specimens of both groups.

The substantial diversity found among the Iberian specimens analyzed (61 haplotypes of 72 specimens) did not correspond to an intraspecific biogeographic structure. Only *B. sclateri* from the Guadalquivir basin was grouped in an independent cluster, but this result was not supported by bootstrap repetitions. The probable recent origin of these species might explain the lack of differentiation between barbels from the current isolated rivers, at least according to the results obtained using this mitochondrial marker.

References

Almaça C, 1967. Estudo das populaçoes portuguesas do Gen. *Barbus* Cuvier, 1817 (Pisces, Cyprinidae). Rev Fac Ciénc Lisboa 14:151–400.

Almaça C, 1972. Sur la systématique des barbeaux (genre et sous-genre *Barbus*) de la Péninsule Ibérique et de l'Afrique du Nord. Arq Mus Boc 2ª ser 3:319–330.

Almaça C, 1976. La spéciation chez les Cyprinidae de la Péninsule Ibérique. Rev Trav Inst Pêches Marit 40: 399–411.

Banarescu P, 1960. Einige Fragen zur Herkunft und Verbreitung des Sübwasserfischfauna der europäisch-mediterranen Unterregion. Arch Hydrobiol 57:16–134.

Banarescu P, 1973. Origin and affinities of the freshwater fish fauna of Europe. Ichthyologia 5:1–8.

Banarescu P, 1992. Distribution and dispersal of freshwater animals in North America and Eurasia, vol. 2. Wiesbaden, Germany: Aula.

Bermingham E, McCafferty SS, and Martin AP, 1997. Fish biogeography and molecular clocks: perspectives from the Panamanian isthmus. In: Molecular systematics of fishes (Kocher TD and Stepien CA, eds). San Diego: Academic Press; 113–126.

Berrebi P, 1995. Speciation of the genus *Barbus* in the north Mediterranean basin: recent advances from biochemical genetics. Biol Conser 72:237–249.

Bianco PG, 1998. Diversity of Barbinae fishes in southern Europe with description of a new genus and a new species (Cyprinidae). Ital J Zool 65:125–136.

Bremer K, 1988. The limits of amino-acid sequence data in angiosperm phylogenetic reconstruction. Evolution 42:795–803.

Bremer K, 1994. Branch support and tree stability. Cladistics 10:295–304.

Briolay J, Galtier N, Brito RM, and Bouvet Y, 1998. Molecular phylogeny of Cyprinidae inferred from cytochrome *b* DNA sequences. Mol Phylogenet Evol 9:100–108.

Brown WM, Prager EM, Wang A, and Wilson AC, 1982.

Mitochondrial DNA sequences of primates: tempo and mode of evolution. J Mol Evol 18:225–239.

Callejas C and Ochando MD, 2000. Recent radiation of Iberian barbel fish (Teleostei, Cyprinidae) inferred from cytochrome *b* genes. J Hered 91:283–288.

De Graaf M, Dejen E, Sibbling FA, and Osse JWM, 2000. The piscivorous barbs of Lake Tana (Ethiopia): major questions on their evolution and exploitation. Neth J Zool 50:215–223.

Doadrio I, 1984. Relaciones filogenéticas y biogeográficas de los barbos (*Barbus,* Cyprinidae) de la Península Ibérica y aportes corológicos y biogeográficos a su ictiofauna continental (PhD dissertation). Madrid: University Complutense de Madrid.

Doadrio I, 1988. Sobre la taxonomía de *Barbus comiza* Steindachner, 1865 (Ostariophysi: Cyprinidae). Doñana Acta Vert 15:19–28.

Doadrio I, 1990. Phylogenetic relationships and classification of west Palaeartic species of the genus *Barbus* (Osteichthyes, Cyprinidae). Aquat Living Resour 3:265–282.

Doadrio I, 1994. Freshwater fish fauna of North Africa and its biogeography. Ann Mus R Afr Centr Zool 275: 21–34.

Eriksson T, 1998. AutoDecay version 4.0. Stockholm: Department of Botany, Stockholm University.

Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Hasegawa M, Kishino H, and Yano T, 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 21:160–174.

Karaman M, 1971. Süsswasserfische der Türkei. Revision der barden Europas Vordderasiens und Nordafrikas. Mitt Hamburg Zool Mus Inst 67:175–254.

Kishino H and Hasegawa M, 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J Mol Evol 29:350–353.

Lavane C, Preparata G, Sacone C, and Serio G, 1984. A new method for calculating evolutionary substitution rates. J Mol Evol 20:86–93.

López-Martínez N, 1989. Tendencias en Paleobiogeografía. El futuro de la biogeografía del pasado. In: Paleontología, Nuevas Tendencias (Aguirre E, ed). Madrid: CSIC; 271–296.

Lydeard C and Roe KJ, 1997. The phylogenetic utility of the mitochondrial cytochrome *b* gene for inferring relationships among Actinopterygian fishes. In: Molecular systematics of fishes (Kocher TD and Stepien CA, eds). San Diego: Academic Press; 285–303.

Machordom A, Doadrio I, and Berrebi P, 1995. Phylogeny and evolution of the genus *Barbus* (Osteichthyes: Cyprinidae) in the Iberian Peninsula as revealed by allozyme electrophoresis. J Fish Biol 47:211–236.

Machordom A and Doadrio I, 2001a. Evidence of a Cenozoic Betic-Kabilian connection based on freshwater fish phylogeography (*Luciobarbus,* Cyprinidae). Mol Phylogenet Evol 18:252–263.

Machordom A and Doadrio I, 2001b. Evolutionary history and speciation modes in the cyprinid genus *Barbus.* Proc R Soc Lond B 268:1297–1306.

Maddison WP and Maddison DR, 1992. MacClade: analysis of phylogeny and character evolution, version 3.05. Sunderland, MA: Sinauer.

Miranda R and Escala MC, 2000. Morphological and biometric comparison of the scales of the barbels (*Barbus* Cuvier) of Spain. J Morphol 245:196–205.

Moritz C, Dowling TE, and Brown WM, 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Annu Rev Ecol Syst 18:269–292.

Myers GS, 1938. Fresh-water fishes and West Indian zoogeography. Smithsonian Rep 1937:339–364.

Nagelkerke LAJ, Sibbling FA, Van den Boogaart JGM, Lammens EHRR, and Osse JWM, 1994. The barbs (*Barbus* spp.) of Lake Tana: a forgotten species flock? Environ Biol Fish 39:1–22.

Perdices A, Doadrio I, Côté IM, Machordom A, Economidis P, and Reynolds JD, 2000. Genetic divergence and origin of Mediterranean populations of the river blenny *Salaria fluviatilis* (Teleostei: Blenniidae). Copeia 3:723–731.

Posada D and Crandall KA, 1998. Model test: testing the model of DNA substitution. Bioinformatics 14:817–818.

Rodríguez R, Oliver JL, Marín A, and Medina JR, 1990. The general stochastic model of nucleotide substitution. J Theor Biol 142:485–501.

Sambrook J, Fritsch EF, and Maniatis T, 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schneider S, Roessli D, and Excoffier L, 2000. Arlequin version 2000. A software for population genetic data analysis. Geneva: University of Geneva.

Swofford DL, 2000. PAUP*: phylogeny analysis using parsimony (*and other methods), version 4.0b3. Sunderland, MA: Sinauer.

Templeton AR, 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. Evolution 37:221–244.

Tsigenopoulos CS and Berrebi P, 2000. Molecular phylogeny of north Mediterranean freshwater barbs (genus *Barbus*: Cyprinidae) inferred from cytochrome *b* sequences: biogeographic and systematic implications. Mol Phylogenet Evol 14:165–179.

Zardoya R and Doadrio I, 1998. Phylogenetic relationships of Iberian cyprinids: systematic and biogeographical implications. Proc R Soc Lond B 265:1365–1372.

Zardoya R and Doadrio I, 1999. Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. J Mol Evol 49:227–237.

Received January 23, 2001
Accepted December 31, 2001

Corresponding Editor: Lisa Seeb