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# Phylogenetic relationships of Iberian cyprinids: systematic and biogeographical implications

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The phylogenetic relationships among all Iberian endemic cyprinids were inferred using the complete nucleotide sequence of the cytochrome *b* gene. The inferred molecular phylogeny included representatives from Central European, Asian and North African species, and is highly congruent with previous phylogenies based on osteological characters. Iberian cyprinids were grouped into only five, very speciose lineages (with the exception of the monotypic *Anaecypris*): *Barbus*, *Luciobarbus*, *Chondrostoma*, *Leuciscus* and *Anaecypris*. The existence of such a relatively small number of Iberian cyprinid lineages can be explained by the historical isolation of the Iberian Peninsula. North African and Asian barbels are the sister group of Iberian *Luciobarbus*, supporting a south-eastern route of colonization of the Iberian Peninsula for this subgenus. Within leuciscins, *Anaecypris hispanica* was considered a relict species as it could not be related to any other Iberian cyprinid. The phylogenetic relationships among the main lineages of Iberian cyprinids based on cytochrome *b* sequence data supported the traditional division of the Cyprinidae into two subfamilies: Cyprininae and Leuciscinae.

**Keywords:** Cyprinidae; cytochrome *b*; molecular phylogeny; Biogeography

## 1. INTRODUCTION

The Cyprinidae are one of the most successful families of fish, with more than 2000 species grouped in approximately 340 genera (Banarescu & Coad 1991). Cyprinid fishes have received much attention from evolutionary biologists, as they show a wide distribution around the world and occur in almost every freshwater environment. Nevertheless, ever since Cuvier (1817) established this family, its systematic relationships have remained contentious. Earlier classifications of cyprinids were based mainly on external features (e.g. the presence, type and number of barbels), as well as the structure and arrangement of the pharyngeal dentition (Howes 1991). More recently, osteological characters were also used to determine the phylogenetic relationships among different groups of cyprinids (e.g. *Barbus*; Doadrio 1990). Yet, the monophyly and relationships within the Cyprinidae and some of its subfamilies (e.g. Cyprininae, Leuciscinae and Rasborinae) have to be firmly established (Howes 1991).

Chen *et al*. (1984) provided the first seemingly cladistic analysis of cyprinid interrelationships. Cavender & Coburn (1992) reanalysed Chen *et al*.'s (1984) data and presented the most thorough hitherto phylogenetic analysis of the Cyprinidae. In their diagnoses of the phylogenetic relationships among North American cyprinids, Cavender & Coburn (1992) recognized two subfamilies: the Cyprininae, which contained the barbins, cyprinins and labeonins, and the Leuciscinae, which contained the tincins, gobionins, rasborins, leuciscins, cultrins, xenocyprins, acheilognathins and phoxinins (figure 1). However, despite these efforts, the interpretation of morphological data has proven to be difficult. The lack of valid and unambiguous morphological characters that define the different groups has prevented agreement among cyprinid systematists (Nelson 1994; Fink & Fink 1996).

Recently, allozyme markers have been employed to study hybridization and introgression processes, as well as population structure, and low-level phylogenetic relationships of European cyprinids (e.g. Coelho 1992; Berrebi *et al*. 1995; Coelho *et al*. 1995; Karakousis *et al*. 1995; Carmona *et al*. 1997; Alves *et al*. 1997*a*). However, phylogenetic assessment at higher taxonomic levels requires the use of a different type of molecular marker. In this respect, the collection of DNA sequence data to analyse the interrelationships among the main lineages of cyprinids is largely wanting (Berrebi *et al*. 1996).

The wide distribution of cyprinids raises very interesting biogeographical and evolutionary questions regarding the origin and further radiation of these fish. For instance, cyprinids within Europe show a particularly interesting distribution pattern with numerous endemic species in the Iberian Peninsula and southern Greece, and relatively small speciose genera in Central Europe (Banarescu 1973*b*). This characteristic distribution has been explained in terms of an ancient isolation of the Iberian Peninsula and southern Greece from the rest of the continent, which would have limited (as they are primary freshwater fish) the number of cyprinid genera able to colonize both regions. However, the precise scenario that led to the actual biogeographical distribution remains unsettled. Although some of the oldest cyprinid fossils are found in the Oligocene strata of Central Europe (Obrhelova 1971), it is generally accepted

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*Received* 18 March 1998 *Accepted* 1 April 1998

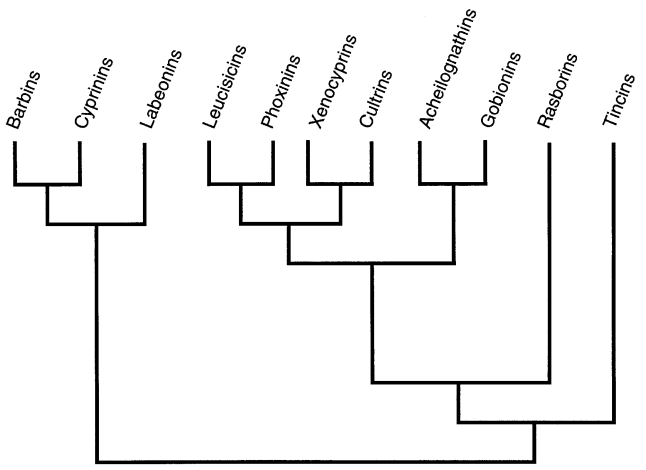


Figure 1. Phylogenetic relationships of the main lineages of Cyprinidae based on the cladistic analysis of osteological characters (Cavender & Coburn 1992).

that European cyprinids are of Asian origin (Banarescu 1989, 1992). Dispersal of Asian cyprinids to Europe would have been possible only during the Oligocene (Banarescu 1989), owing to the uplift of the Urals (Rögl & Steininger 1983), and finished once these mountains were formed. It is still unclear whether all of Europe was colonized through migration across the nascent Urals or whether some cyprinids (particularly the genus *Barbus*) reached the Iberian Peninsula through northern Africa (Doadrio 1990).

To further understand the phylogenetic relationships among representative cyprinid lineages, and their biogeographical origins, we have sequenced the complete mitochondrial cytochrome *b* gene for all Iberian cyprinid species (Elvira 1990, 1995), as well as several related Central European, African and Asian cyprinids. The cytochrome *b* gene has, in the past, provided complementary and informative sequence data sets for determining phylogenetic relationships between morphologically similar species. Therefore, it was expected to be a suitable phylogenetic marker to address the questions posed in this study (Zardoya & Meyer 1996).

## 2. MATERIAL AND METHODS

### (a) *DNA sources and extraction*

Total cellular DNA was extracted (Towner 1991) from the muscle of the Iberian, Central European, Asian and African cyprinid species (one specimen per species) listed in table 1. Samples of cyprinid species that live in sympatry in some rivers were selected from those rivers in which only one of the species lives to avoid introgression processes. The complete cytochrome *b* nucleotide sequences of *Crossostoma lacustre* (M91245), *Cyprinus carpio* (X61010), *Tinca tinca* (Y10451), *Barbus barbus* (Y10450), *Telestes souffia* (Y10439), *Rutilus rutilus* (Y10440), *Phoxinus phoxinus* (Y10448) and *Lythrurus roseipinnis* (X66456) were retrieved from GenBank and included in the phylogenetic analyses. In addition, the cytochrome *b* sequence of one characid (*Astyanax fasciatus*) was obtained to use as an outgroup. Sequences determined here have been deposited at the EMBL/GenBank data libraries under the accession numbers AF045966–AF045997.

### (b) *PCR amplification, cloning and sequencing*

A combination of two sets of versatile primers (Glu-F, 5′-GAAGAACCACCGTTGTTATTCAA-3′; Cytb-R, 5′-TCTTT-ATATGAGAARTANGGGTG-3′; Cytb-F, 5′-CACGARACRG-GRTCNAAYAA-3′; Thr-R, 5′-ACCTCCRATCTYCGGATTA-CA-3′) was designed based on highly conserved fish mitochondrial DNA sequences around and within cytochrome *b*. They were used to amplify, via polymerase chain reaction (PCR), two contiguous and overlapping fragments (660 and 521 base pairs (bp)) that covered the entire cytochrome *b* gene. These primers are expected to be of high versatility and are likely to successfully amplify mitochondrial cytochrome *b* in other non-cyprinid fish species (R. Zardoya and I. Doadrio, unpublished data). Cycles (35–40) of PCR (denaturing at 94 °C for 60 s, annealing at 45–50 °C for 60 s and extending at 72 °C for 60–105 s) were performed in 25 µl reactions containing 67 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.4 mM of each dNTP, 2.5 µM of each primer, template DNA (10–100 ng) and Taq DNA polymerase (1 unit, Promega).

PCR products were cloned using the pGEM-T vector (Promega) into *E. coli* JM109, and sequenced using the FS-Taq Dye Deoxy Terminator cycle-sequencing kit (Applied Biosystems Inc.) on an automated DNA sequencer (Applied Biosystems 377) following the manufacturer's instructions. DNA sequences of both strands were obtained using M13 universal (forward and reverse) sequencing primers.

### (c) *Phylogenetic analyses*

DNA sequences were aligned based on the inferred cytochrome *b* amino acid sequence. No ambiguous alignments were found, and no gaps had to be postulated. All codon positions were included in the phylogenetic analyses. Transitions (Ts) and transversions (Tv) were given either equal weight or Tv triple the weight of Ts. The cytochrome *b* data set was subjected to the maximum parsimony (MP) method (PAUP* v. d60 (Swofford 1997)), using heuristic searches (TBR branch swapping; MULPARS option in effect) with ten random stepwise additions of taxa to find the most parsimonious trees. Neighbour-joining (NJ) analyses (Saitou & Nei 1987) (based on HKY85 corrected-distance matrices with empirical Ts/Tv ratios, and base frequencies) of the sequences were performed with PAUP* v. d60. Robustness of the inferred trees was tested by bootstrapping (Felsenstein 1985) (as implemented in PAUP* with 100 pseudo-replications each).

## 3. RESULTS

### (a) *Phylogenetic relationships of the Cyprinidae based on the analyses of cytochrome b nucleotide sequences*

The mitochondrial cytochrome *b* gene was successfully amplified by PCR, and sequenced in all cyprinid species tested, as well as in the outgroup characid species. A total of 1140 bp were aligned for all 40 taxa (including several cyprinid species retrieved from GenBank) of which 582 were constant sites, and 453 were phylogenetically informative sites using the parsimony criterion. Pairwise sequence divergence between taxa varied from 0.3 to 25%. An overall Ts/Tv ratio of 3.37 was calculated for this data set. Most of the variability among sequences was detected in third codon positions. Substitutions in third codon positions showed some saturation only for distantly related taxa (between 40 and 55% sequence divergence),

Table 1. *Species and sampling localities*

| species | basin[a] | river | MNCN collection number |
|---|---|---|---|
| *Anaecypris hispanica* | Guadiana (Sp) | Estena | 2675 ES |
| *Astyanax fasciatus* | Coatzacoalcos (Mx) | Grande | 457 MEX |
| *Barbus bocagei* | Duero (Sp) | Duratón | 913 ES |
| *Barbus callensis* | Kebir (Al) | Kebir | 104 AL |
| *Barbus capito* | Terek (Rs) | Terek | 207 MO |
| *Barbus comizo* | Tajo (Sp) | Almonte | 1935 B |
| *Barbus steindachneri* | Guadiana (Sp) | Quejigares | BC1 |
| *Barbus graellsii* | Ebro (Sp) | Gallego | 146 EBE |
| *Barbus guiraonis* | Buyent (Sp) | Buyent | 25 B |
| *Barbus haasi* | Ebro (Sp) | Esca | 2006 ES |
| *Barbus meridionalis* | Tordera (Sp) | Tordera | 1 B |
| *Barbus microcephalus* | Guadiana (Sp) | Estena | 2220 BM |
| *Barbus sclateri* | Guadalquivir (Sp) | Alhama | 26 G EB |
| *Carassius auratus* | pet store | — | — |
| *Chondrostoma lusitanicum* | Arade (Po) | Boina | 12 Po |
| *Chondrostoma polylepis* | Tajo (Sp) | Lozoya | 2 Chp |
| *Chondrostoma duriensis* | Duero (Sp) | Rubagón | 2410 CH |
| *Chondrostoma willkommii* | Guadalquivir (Sp) | Jándula | 12 CH |
| *Chondrostoma toxostoma* | Ebro (Sp) | Jalón | 1963 CH |
| *Gobio gobio* | Tajo (Sp) | Lozoya | 1 Gg |
| *Iberocypris palaciosi* | Guadalquivir (Sp) | Jándula | 4 IB |
| *Leuciscus carolitertii* | Duero (Sp) | Adaja | 507 ES |
| *Leuciscus cephalus* | Ebro (Sp) | Matarraña | 28 EBE |
| *Leuciscus pyrenaicus* | Guadiana (Sp) | Estena | 2247 LEU |
| *Leuciscus pyrenaicus* | Tajo (Sp) | Tietar | 12 TI |
| *Rutilus arcasi* | Duero (Sp) | Bernesga | 2620 ES |
| *Rutilus arcasi* | Tajo (Sp) | Lozoya | 1 Ra |
| *Rutilus lemmingii* | Guadiana (Sp) | Maillo | 201 ES |
| *Rutilus lemmingii* | Guadiana (Sp) | Estenilla | 231 ES |
| *Rutilus lemmingii* | Guadalquivir (Sp) | Robledillo | 2043 ES |
| *Rutilus macrolepidotus* | Mondego (Po) | Sobral | RM 1 |
| *Tropidophoxinellus alburnoides* | Guadiana (Sp) | Estena | 2235 ES |

[a] Country codes: Sp, Spain; Po, Portugal; Mx, Mexico; Al, Algeria; Rs, Russia.

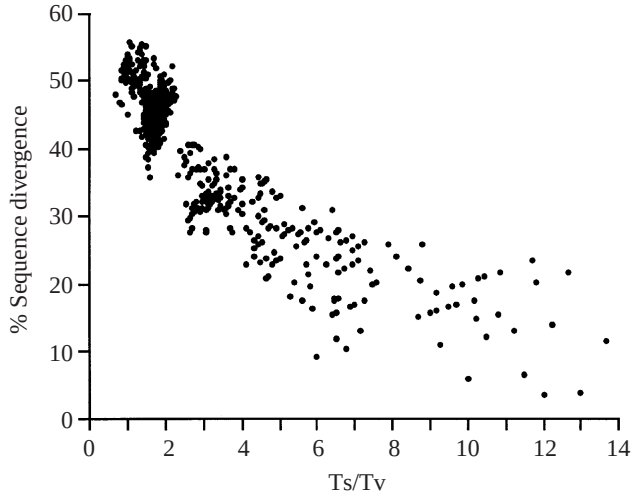


Figure 2. Phylogenetic utility of third codon positions of cytochrome *b* gene sequence. Sequence divergences were plotted against Ts/Tv ratios for third positions of the cyprinid cytochrome *b* data set. According to the graph, third codon positions showed some saturation only between 40 and 55% sequence divergence. At that level, phylogenetic information is contained in first and second positions.

as established by comparing pairwise sequence divergence and Ts/Tv ratios (figure 2). Maximum parsimony analysis of the sequence data arrived at two equally most parsimonious trees of 3728 steps when a 3:1 Tv:Ts weighting was assumed, and *Astyanax fasciatus* (Characidae) and *Crossostoma lacustre* (Balitoridae) were used as outgroup taxa. Both MP trees differed only in the relative position of *Chondrostoma polylepis* with respect to *Chondrostoma duriensis* and *Chondrostoma willkommii*. The NJ analysis arrived at a similar and congruent tree. The robustness of the MP and NJ trees was confirmed by bootstrapping (figure 3). As expected, differences between MP and NJ trees were concentrated in the relative position of those taxa not supported by high bootstrap values. Similar results were obtained when MP was performed without weighting or when Tv were given double the weight of Ts in third codon positions. Two major assemblages could be distinguished within the Cyprinidae based on the results. One clade, the Cyprininae, included the carp, the goldfish and the *Barbus* species, whereas the other, the Leuciscinae, included *Tinca*, *Gobio*, *Phoxinus*, American cyprinid, *Leuciscus*, *Rutilus* and *Chondrostoma* species.

### (b) *The Cyprininae subfamily*

The barbin lineages formed a monophyletic group with *Cyprinus*, and *Carassius* as sister group species. Within the

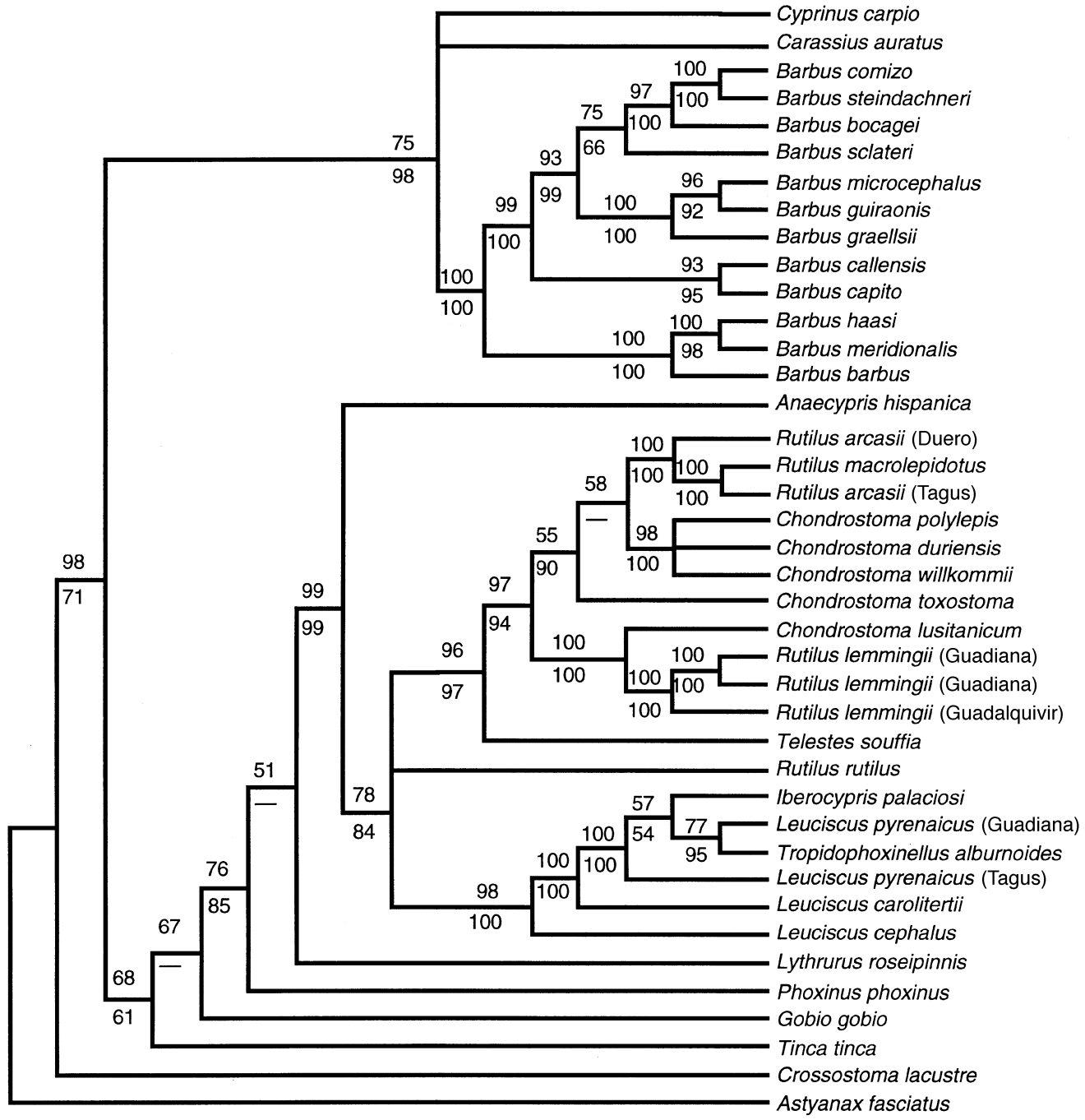


Figure 3. Phylogenetic relationships among the main lineages of European cyprinids with particular emphasis on Iberian species based on cytochrome *b* sequence data. The 50% majority-rule consensus bootstrap trees obtained with MP (values above branches; Tv:Ts weighting was 3:1) and NJ (values below branches; HKY85, empirical base frequencies and Ts/Tv ratio) analyses, based on 100 pseudoreplications, are shown. In the case of the NJ bootstrap values, three are missing (—) because those nodes were recovered by MP but not by NJ. Nodes with bootstrap values below 50% were forced to collapse and yield polytomies.

barbin subset, two major clades could be recognized: one comprising a Central European (*B. barbus*) and two north-eastern Iberian (*B. haasi* and *B. meridionalis*) *Barbus*; the other containing most of the Iberian *Barbus* (*B. comizo*, *B. steindachneri*, *B. bocagei*, *B. microcephalus*, *B. guiraonis*, *B. graellsii* and *B. sclateri*), and, as sister group, the Asian (*B. capito*) and African (*B. callensis*) *Barbus* (figure 3). Interestingly, Iberian barbels from Mediterranean river basins (*B. guiraonis* and *B. graellsii*) were clearly differentiated from those living in Atlantic rivers (*B. comizo*, *B. steindachneri*, *B. bocagei* and *B. sclateri*). Furthermore, barbels that were morphologically diverse but living in the same river (e.g. *B. bocagei*–*B. comizo*, and *B. guiraonis*–*B. microcephalus*) appeared to be more closely related than those that were morphologically similar (e.g. *B. bocagei*–*B. sclateri* and *B. graellsii*–*B. guiraonis*) but from different river drainages. *B. comizo* and *B. steindachneri* showed the least sequence divergence found within

Iberian cyprinids. Sequence divergence (below 1%) between these two barbel species suggests that their species status needs to be revisited (Karaman 1971).

### (c) *The Leuciscinae subfamily*

A second major group that comprised the Leuciscinae was found within the Cyprinidae. *Tinca tinca* showed a basal position in this group, followed by *Gobio gobio*, *Phoxinus phoxinus* and the North American minnow, *Lythrurus roseipinnis*. Iberian species could be grouped into two major clades: one included *Leuciscus* species, and *Iberocypris palaciosi*, and the other, *Rutilus* and *Chondrostoma* species gathered together with *Anaecypris hispanica* in a basal position. According to the results, *Rutilus* is a paraphyletic genus. Interestingly, *Chondrostoma lusitanicum* from Portugal is the sister group of *Rutilus lemmingii*, and not of other Iberian *Chondrostoma*. The hierarchical taxonomic status of different river basin populations of the same species was also analysed. Specimens of *Rutilus arcasii* from the Duero and Tagus river basins, *Rutilus lemmingii* from the Guadiana and Guadalquivir river basins, as well as *Leuciscus pyrenaicus* from Guadiana and Tagus river basins, were sampled. In addition, one specimen of *Chondrostoma polylepis*, *Chondrostoma duriensis* (also described as *C. polylepis duriensis*) and *Chondrostoma willkommii* (also described as *C. polylepis willkommii*) from the Tagus, Duero and Guadalquivir river basins, respectively, were analysed. The Portuguese *Rutilus macrolepidotus* from the Duero river basin was grouped with *R. arcasii* from the Tagus River, making *R. arcasii* paraphyletic. Finally, *Tropidophoxinellus alburnoides*, a hybridogenetic species (Alves *et al*. 1997*b*; Carmona *et al*. 1997), and *Iberocypris palaciosi* showed clear phylogenetic affinities to *Leuciscus pyrenaicus*. Moreover, the phylogenetic position of *L. carolitertii* as sister group of *L. pyrenaicus* was confirmed (Brito *et al*. 1997), and the widely distributed species *Leuciscus cephalus* was placed basal to Iberian *Leuciscus* endemics.

## 4. DISCUSSION

Cyprinid interrelationships were addressed with cytochrome *b* nucleotide sequence data. The substitution rate for the cytochrome *b* gene proved to be adequate in establishing the phylogenetic relationships among cyprinid lineages at the genus and species level. The inferred molecular phylogeny was highly congruent with previous phylogenies based on osteological data (Doadrio 1990; Cavender & Coburn 1992), and was useful in determining which osteological characters are phylogenetically more informative (table 2). According to our results, the family Cyprinidae is divided into two major groups, the Cyprininae and the Leuciscinae. These two major groups were also recognized by Chen *et al*. (1984) and Cavender & Coburn (1992) based on osteological characters, and ranked as subfamilies within the Cyprinidae.

Within the Cyprininae, *Barbus* species appeared as the sister group of carps and goldfish. This result remains tentative because no labeonins were included in the study (Cavender & Coburn 1992) (see figure 1). The split of Iberian *Barbus* into two lineages is supported by cytochrome *b* sequence data. With the exception of *B. haasi* and *B. meridionalis* from Catalonia (Machordom *et al*. 1995), which are related to Central European *Barbus* species (e.g. *B. barbus*), the rest of the Iberian members of the genus *Barbus* form a monophyletic group, with the North African and Asian *Barbus* as sister groups. Similar results were reported by Doadrio (1990) based on osteological characters. However, cytochrome *b* evidence contradicts traditional hypotheses based on morphological characters, which supported a closer relationship of Iberian and North African barbels to Central European species to the exclusion of Asian barbels (Banarescu 1960, 1973*a*; Almaça 1988). Our results also contradict a previous hypothesis based on allozyme data, which proposed a closer relationship between Iberian and Central European species to the exclusion of North African and Asian barbels (El Gharbi *et al*. 1993; see Berrebi (1995) for a review).

Following Doadrio (1990), the two barbel groups were defined as different subgenera: *Barbus* (Central European species, *B. haasi* and *B. meridionalis*) and *Luciobarbus* (North African, Asian and Iberian species). Based on these results, and assuming an Asian origin for the genus *Barbus* (Banarescu 1960, 1973*a*; see Doadrio (1990) for a review), only two hypotheses can explain the actual distribution of the Iberian species of this genus: (i) *Luciobarbus* subgenus species colonized the Iberian Peninsula through southern Spain, whereas the *Barbus* subgenus species radiated via Central Europe; or (ii) *Luciobarbus* spread through Central Europe to the Iberian Peninsula and North Africa, and afterwards, a second invasion of *Barbus* from Asia colonized Central Europe, replacing *Luciobarbus*, but not the Iberian Peninsula. The absence of a fossil record for *Luciobarbus* species in Central Europe strongly supports the southern Spain dispersal hypothesis.

So far, the phylogenetic position of the monotypic genus *Tinca* has been highly controversial. It has been placed either in a separate subfamily (e.g. Bogustkaya 1986), as a member of the Cyprininae (e.g. Dumitrescu & Bananarescu 1979; Chen *et al*. 1984), or as the most basal member of the Leuciscinae (e.g. Cavender & Coburn 1992). Our results clearly support the latter phylogenetic position. The second most basal group of the Leuciscinae is represented by *Gobio*, which traditionally has been placed in a separate subfamily (Howes 1991). *Phoxinus* is the only cyprinid genus that is found both in Eurasia and North America (Howes 1991). This characteristic distribution supports the immediate basal position of *Phoxinus* with respect to North American minnows (e.g. *Lythrurus*).

Endemic Leuciscinae species of the Iberian Peninsula form a monophyletic group with *Anaecypris hispanica* in a basal position. The phylogenetic relationships of the monotypic genus *Anaecypris* have been considered difficult due to the atypical morphology of these fish (Collares-Pereira 1983). The most recent morphological analysis of this species proposed that their closest sister group was *Chondrostoma* (Bogutskaya & Collares-Pereira 1997). Cytochrome *b* evidence clearly rejects this relationship, and establishes that *Anaecypris* conforms as an independent lineage not intimately related to any other Iberian cyprinid.

The rest of the Iberian Leuciscinae are divided into two different clades. One group comprises *Leuciscus*, *Iberocypris* and *Tropidophoxinellus* genera. The other group includes *Chondrostoma* and *Rutilus* species. Sequence

Table 2. *Morphological characters phylogenetically informative and congruent with the cytochrome b phylogeny depicted in figure 3*

(Characters: (A) 2–3 vertebral centra†: (1) fused, (2) separated. (B) Postcleitrum size†: (1) developed, (2) reduced. (C) 5th ceratobranchial morphology, morphotypes*: (1) *Cyprinus*, (2) *Carassius*, (3) *Luciobarbus*, (4) *Barbus*, (5) *Anaecypris*, (6) *Chondrostoma*, (7) *Telestes*, (8) *Rutilus*, (9) *Squalius*, (10) *Phoxinus*, (11) *Gobio*, (12) *Tinca*. (D) Teeth morphology, morphotypes*: (1) *Cyprinus*, (2) *Carassius*, (3) *Luciobarbus*, (4) *Barbus*, (5) *Anaecypris*, (6) *Chondrostoma*, (7) *Telestes*, (8) *Rutilus*, (9) *Squalius*, (10) *Phoxinus*, (11) *Gobio*, (12) *Tinca*. (E) Pharyngeal process of the basioccipital: (1) the posterior process is laterally compressed, (2) the posterior process is dorsoventrally compressed. (F) Supraorbital canal†: (1) connected to infraorbital canal, (2) disconnected from infraorbital canal. (G) Pterotic size†: (1) reduced, (2) elongated. (H) Crest or blade of the neural complex†: (1) divided dorsally, (2) not divided dorsally. (I) Pseudobranchial and suprabranchial arteries†: (1) not connected, (2) connected. (J) Interorbital septum†: (1) formed by orbitosphenoid and parasphenoid, (2) formed only by orbitosphenoid. Characters preceded by † are listed in Cavender & Coburn (1992). *Ceratobranchial and teeth morphotypes are from Rutte (1962), except those of *Anaecypris* (this study). Character polarities are not shown.)

| | characters | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| taxon | A | B | C | D | E | F | G | H | I | J |
| *C. carpio* | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *C. auratus* | 1 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 |
| *B. comizo* | 1 | 1 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 |
| *B. steindachneri* | 1 | 1 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 |
| *B. bocagei* | 1 | 1 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 |
| *B. sclateri* | 1 | 1 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 |
| *B. microcephalus* | 1 | 1 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 |
| *B. guiraonis* | 1 | 1 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 |
| *B. graellsii* | 1 | 1 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 |
| *B. callensis* | 1 | 1 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 |
| *B. capito* | 1 | 1 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 |
| *B. haasii* | 1 | 1 | 4 | 4 | 1 | 1 | 1 | 1 | 1 | 1 |
| *B. meridionalis* | 1 | 1 | 4 | 4 | 1 | 1 | 1 | 1 | 1 | 1 |
| *B. barbus* | 1 | 1 | 4 | 4 | 1 | 1 | 1 | 1 | 1 | 1 |
| *A. hispanica* | 2 | 2 | 5 | 5 | 1 | 2 | 2 | 2 | 2 | 2 |
| *Ch. polylepis* | 2 | 2 | 6 | 6 | 2 | 2 | 2 | 2 | 2 | 2 |
| *Ch. duriensis* | 2 | 2 | 6 | 6 | 2 | 2 | 2 | 2 | 2 | 2 |
| *Ch. willkommii* | 2 | 2 | 6 | 6 | 2 | 2 | 2 | 2 | 2 | 2 |
| *Ch. toxostoma* | 2 | 2 | 6 | 6 | 2 | 2 | 2 | 2 | 2 | 2 |
| *R. arcasii* | 2 | 2 | 6 | 6 | 2 | 2 | 2 | 2 | 2 | 2 |
| *R. macrolepidotus* | 2 | 2 | 6 | 6 | 2 | 2 | 2 | 2 | 2 | 2 |
| *R. lemmingii* | 2 | 2 | 6 | 6 | 2 | 2 | 2 | 2 | 2 | 2 |
| *Ch. lusitanicum* | 2 | 2 | 6 | 6 | 2 | 2 | 2 | 2 | 2 | 2 |
| *T. souffia* | 2 | 2 | 7 | 7 | 1 | 2 | 2 | 2 | 2 | 2 |
| *R. rutilus* | 2 | 2 | 8 | 8 | 1 | 2 | 2 | 2 | 2 | 2 |
| *I. palaciosi* | 2 | 2 | 9 | 9 | 1 | 2 | 2 | 2 | 2 | 2 |
| *L. pyrenaicus* | 2 | 2 | 9 | 9 | 1 | 2 | 2 | 2 | 2 | 2 |
| *T. alburnoides* | 2 | 2 | 9 | 9 | 1 | 2 | 2 | 2 | 2 | 2 |
| *L. carolitertii* | 2 | 2 | 9 | 9 | 1 | 2 | 2 | 2 | 2 | 2 |
| *L. cephalus* | 2 | 2 | 9 | 9 | 1 | 2 | 2 | 2 | 2 | 2 |
| *L. roseipinnis* | 2 | ? | ? | ? | ? | 2 | 2 | 2 | 2 | 2 |
| *P. phoxinus* | 2 | 1 | 10 | 10 | 1 | 2 | 2 | 2 | 2 | 2 |
| *G. gobio* | 1 | 1 | 11 | 11 | 1 | 2 | 1 | 2 | 2 | 2 |
| *T. tinca* | 1 | 1 | 12 | 12 | 1 | 2 | 1 | 1 | 1 | 1 |

divergence (below 1%) between *Leuciscus pyrenaicus*, *Tropidophoxinellus alburnoides* and *Iberocypris palaciosi* strongly indicates their close relationships, and does not support their current generic status. According to our results, *Tropidophoxinellus alburnoides* (initially described as *Leuciscus alburnoides* by Steindachner (1866*a*)) and *Iberocypris palaciosi* should be considered within the genus *Leuciscus*. Recently, it has been demonstrated that *Tropidophoxinellus alburnoides* (=*Leuciscus alburnoides*) is part of a hybridogenetic complex (Carmona *et al*. 1997), and very closely related to *Leuciscus pyrenaicus* (Alves *et al*. 1997*a*,*b*; Carmona *et al*. 1997). *Iberocypris palaciosi* (=*Leuciscus palaciosi*) shows different ploidy levels (data not shown), and it is likely that it may be involved also in a unisexual complex with *Leuciscus pyrenaicus*.

Iberian *Chondrostoma* and *Rutilus* form a monophyletic group with *Telestes souffia* as a sister group. The phylogenetic position of *T. souffia* has been debated (Kottelat 1997). Traditionally, it has been described also as *Leuciscus souffia* (Risso 1826), being related to other *Leuciscus*. However, our results support a closer phylogenetic association of *T. souffia* to *Chondrostoma*. Iberian species included in the genus *Rutilus* are paraphyletic, and closely related to *Chondrostoma*. Moreover, they are unrelated to *Rutilus rutilus*, the type species of the genus, which inhabits Central Europe. Therefore, to facilitate taxonomic and phylogenetic studies, we propose to redefine the taxonomic status of Iberian *Rutilus*, and use *Chondrostoma* to designate Iberian species formerly known as *Rutilus* (but see Elvira 1997) (indeed, *R. lemmingii* was

redescribed as *C. lemmingii* by Steindachner (1866*b*)). Interestingly, *C. arcasii* is closer to *Chondrostoma sensu stricto* than *C. lemmingii* and *C. lusitanicum*. The latter were proposed to be the sister group to *Chondrostoma* due to their morphological affinities (Collares-Pereira 1980). However, it has been shown that *C. arcasii* naturally hybridizes with *C. polylepis* (Collares-Pereira & Coelho 1983), supporting our conclusion. According to our results, *C. arcasii* from the Tagus basin is closer to *Rutilus macrolepidotus* (=*Chondrostoma macrolepidotus*) than to *C. arcasii* from the Duero drainage. Therefore, cytochrome *b* evidence suggests that *C. macrolepidotus* is not restricted to Portugal but also found in the upper Tagus (Spain). Similarly, sequence divergence (below 5%) among *C. polylepis*, *C. duriensis* and *C. willkommii*, suggests that these species may simply be considered different subspecies of *C. polylepis*, as some authors have previously proposed (reviewed in Elvira (1997)). However, taxonomic changes suggested here are tentative and will require further support from similar studies with additional European cyprinid taxa (Zardoya & Doadrio 1998) before being implemented.

In conclusion, cytochrome *b* sequence data were used successfully to resolve the phylogenetic relationships among different cyprinid genera, with particular emphasis on Iberian species. The numerous endemic cyprinid species of the Iberian Peninsula were classified in only five independent monophyletic lineages, i.e. *Barbus*, *Luciobarbus*, *Chondrostoma*, *Leuciscus* and *Anaecypris*. The molecular phylogeny presented here was highly congruent with phylogenies based on osteological characters. Nevertheless, the taxonomic status of several genera and species was revised based on sequence divergence data and the inferred phylogenies. Cytochrome *b* evidence was also helpful in tracking the radiation processes that led to the actual biogeographical distribution of some of the Iberian cyprinids. Future studies on the phylogenetic relationships of European cyprinids should also incorporate cytochrome *b* sequences from Central European species, as well as endemic cyprinids from Greece (Zardoya & Doadrio 1998).

After this manuscript was submitted, Briolay *et al.* (1998) reported a study related to ours. In their study, the phylogenetic relationships among several cyprinid species from Central Europe were analysed using cytochrome *b* sequence data. Their results are basically congruent with ours (particularly with respect to the relative position of the *Cyprinus*, *Barbus*, *Gobio*, *Phoxinus*, *Lythrurus*, *Leuciscus* and *Chondrostoma* genera). However, in contrast to our findings, Briolay *et al.*'s (1998) analysis failed to support with strong confidence the traditional subdivision of the Cyprinidae into two subfamilies (Chen *et al.* 1984; Howes 1991; Cavender & Coburn 1992). The stronger support for the Cyprininae and Leuciscinae clades in our study is most likely due to the inclusion of additional barbel species, the 3:1 Tv:Ts weighting applied to account for the observed transitional bias and the use of a closer outgroup taxon (one Characidae species) instead of the less-related rainbow trout (Salmonidae).

We thank Guillermo Ortí, Ehab Abouheif, and two anonymous reviewers for providing helpful suggestions on a previous version of the manuscript. Lourdes Alcaraz assisted in the DNA extraction and cloning. David Swofford granted permission to publish results based on the test version of his PAUP* program. R.Z. was sponsored by a postdoctoral contract of the Ministerio de Educación y Ciencia of Spain. This work received partial financial support from a grant from the Ministerio de Educación y Ciencia PB-920025 to I.D.

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