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Paleobiogeography of Two Iberian Endemic Cyprinid Fishes (*Chondrostoma arcasii-Chondrostoma macrolepidotus*) Inferred from Mitochondrial DNA Sequence Data

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Abstract

We tested different hypotheses related to the origin and evolution of the endemic Iberian fishes *Chondrostoma arcasii* and *Chondrostoma macrolepidotus* from northern and central regions of the Iberian Peninsula. We evaluated the monophyly of the populations within each species and sought to determine if diversification of the populations coincided in time with the formation of the Iberian drainages dating back to the upper Pliocene (2.5–1.8 million years ago). A molecular phylogenetic analysis of the mitochondrial cytochrome *b* gene showed that the different populations of the northern Iberian Peninsula are clustered into five phylogroups and do not fit into the dichotomy *C. arcasii-C. macrolepidotus*. We propose that species differentiation occurred prior to the upper Pliocene formation of the present hydrographic basins and that endorheic basins, a system of inland lakes found in Spain during the Mio-Pliocene, played an important role in this diversification and differentiation process.

In the Iberian Peninsula, as in other southern European peninsulas, the primary freshwater fish fauna is dominated by cyprinids and is characterized by a high level of endemism, a low number of genera, and a high number of species per genus (Doadrio 2001). The prevalence of endemic species and the low number of genera have been explained by the persistence of important barriers that strongly reduce the possibilities of colonization of Iberia by freshwater fishes. Surrounded by the Atlantic and the Mediterranean and connected to the bulk of Europe by a zone of very high mountains, the Pyrenees, Iberia was colonized by only a few lineages that succeeded in surmounting these barriers (e.g., Doadrio 2001).

This high level of diversity and endemism in the Iberian cyprinids resulted from cladogenetic processes that occurred within the peninsula after its colonization by these few lineages (Doadrio 2001). How to explain this high level of diversity of endemic cyprinids? The present-day hydrographic network of the peninsula is geologically recent. During the upper Miocene and lower Pliocene many of the modern rivers did not exist as such, and several endorheic (closed) basins drained the bulk of freshwater in Iberia (Andeweg et al. 1999). One way to explain the diversity of the fish fauna is to hypothesize that it originated at least as early as the Mio-Pliocene due to the isolation of the numerous inland lakes that then existed and the heterogeneity of the habitats provided by the lacustrine environments. Alternatively, the diversification of the fauna may be more recent, resulting from the separation of different watersheds as the modern river network was formed. These two hypotheses, although not being mutually exclusive, lead to distinct predictions. If speciation was associated with the formation of the modern rivers, we predict that (1) the separation of most sister species must be of Pliocene origin and their separation times must correlate well with the formation of the different river systems and (2) different rivers must harbor different sister species widely distributed along their basins.

If most of the diversification occurred in the isolated inland lakes of Mio-Pliocene, we predict that (1) the timing of the cladogenetic events must predate the formation of the modern exorheic drainage pattern and (2) sister species

may co-occur as the result of the connections between different ancient lakes, which took place after speciation.

In this paper, we assess the relative importance of these two hypotheses using a phylogenetic and biogeographic analysis of the sister species *Chondrostoma arcasii* (Steindachner 1866b) and *Chondrostoma macrolepidotus* (Steindachner 1866a), two cyprinid fishes endemic to the north central Iberian Peninsula. *C. macrolepidotus* is restricted to the western part of the Iberian Peninsula on the Atlantic slope of Portugal, while *C. arcasii* inhabits north and central Spain on both the Mediterranean and Atlantic slopes (Collares-Pereira 1983; Doadrio 2001). The two species are difficult to distinguish morphologically (Casado 1995; Collares-Pereira 1979, 1983), and Zardoya and Doadrio (1998) provided molecular evidence contradicting their monophyly, although showing that taken together they form a monophyletic clade. Thus, this paper also aims to contribute to a better understanding of their relationship. We used samples that cover, for the first time, the entire distribution area of the two species. Our analysis was based on the sequences of the mitochondrial cytochrome *b* gene.

Materials and Methods

One hundred and two specimens were collected throughout the entire range of the *C. arcasii-C. macrolepidotus* species group. Voucher specimens were deposited in the collections of the Museo Nacional de Ciencias Naturales, Madrid, Spain. Total genomic DNA was extracted from a piece of fin clip or muscle by the standard proteinase K and phenol/chloroform extraction method (Sambrook et al. 1989). From each individual DNA sample, two overlapping fragments of the cytochrome *b* gene (total of 1,036 bp) were amplified via polymerase chain reaction (PCR). The primers used for cytochrome *b* were those mentioned in Machordom and Doadrio (2001). The amplification process was conducted as follows: 94°C (2 min), 35 cycles at 94°C (45 s), 48°C (1 min), 72°C (5 min). PCR mixtures were prepared in 25-μl volumes with a final concentration of 0.5 mM each primer, 0.2 mM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase (Perkin-Elmer, Boston, MA). After checking PCR products on 1.5% agarose gels, they were cloned using the pGEM-T vector (Promega, Madison, WI) into *Escherichia coli* JM109 and were sequenced using the FS-Taq Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems Inc., Foster City, CA). DNA sequences of both strands were obtained using M13 universal (forward and reverse) sequencing primers. All samples were sequenced on an Applied Biosystems 3700 DNA sequencer following the manufacturer's instructions. Chromatograms and alignments were visually checked and verified, and there were no gaps in the resulting DNA sequences.

Sequences have been deposited in GenBank. Accession numbers and capture localities are presented in Table 1.

Data Analysis

The aligned sequences were analyzed using distance [minimum evolution (ME)], maximum likelihood (ML), and maximum parsimony (MP) methods. Bayesian phylogenetic inference was also performed, using MRBAYES 3.0 (Huelsenbeck and Ronquist 2001) by simulating a Markov chain for 1,000,000 cycles. MP analysis was performed using heuristic searches with 10 random stepwise additions and tree bisection-reconnection branch swapping. Results were based on a 9:1 Ti/Tv weight, following the empirically determined Ti/Tv ratio for *C. arcasii-C. macrolepidotus*. To find the best model of evolution that fit our data for ML and ME analyses, we performed a hierarchical likelihood ratio test (LRT), using the program MODELTEST 3.04 (Posada and Crandall 1998). All phylogenetic analyses, except Bayesian inference, were performed using PAUP* 4.0 (Swofford 1998). Bootstrap analyses were used to assess the relative robustness of branches of the ML (1,000 replicates), ME (1,000 replicates), and MP (500 replicates) trees.

Sequences of *Chondrostoma lusitanicum* (from River Samarra, Portugal), *Telestes soufia*, and *Rutilus rutilus* (both from River Saone, France) (GenBank accession numbers AY254584, Y10439, and Y10440, respectively) were used as outgroups.

The saturation of transitions and transversions was checked by plotting the absolute number of changes of each codon position against uncorrected sequence divergence values (*p*). There was no evidence of saturation in the ingroup (graph not shown).

To examine whether the different *C. arcasii-C. macrolepidous* populations evolved at the same rate, we conducted an LRT with and without the molecular clock constraint, using PUZZLE (Strimmer and von Haeseler 1996). We also conducted a relative-rate test (Sarich and Wilson 1973; Wu and Li 1985) among the main clades, with *C. lusitanicum* as outgroup, using the program RRTREE 1.1 (Robinson-Rechavi and Huchon 2000). The PUZZLE program found no taxa with statistically longer branches, and the results of the relative-rate tests were not significant. Thus, the data were compatible with the use of a molecular clock.

Dowling et al. (2002) based on multiple fossil data and sequence comparisons estimated a divergence rate of about 0.53% per lineage per million years for the cytochrome *b* gene of cyprinids. This figure of about 1% of pairwise divergence per million years was adopted in other phylogenetic studies of cyprinids (Doadrio and Carmona 2004; Durand et al. 2003). We also used this calibration to estimate both divergence dates and the principal events that caused speciation in *C. arcasii* and *C. macrolepidotus* populations.

To calculate divergence times, we performed an ML analyses based on the HKY85 model (Hasegawa et al. 1985) with empirical Ts/Tv ratios and base frequencies using PUZZLE version 4.0.1 (Strimmer and von Haeseler 1996) with the clocklike option to obtain a clock-constrained tree (in which all root-to-tip distances have equal value).

Results

The 102 specimens yielded 57 distinct haplotypes. Among all the sequences studied, 299 sites were variable and 197 were parsimony informative. According to codon position, the

Table 1. Specimens of the *C. arcasii-C. macrolepidotus* complex included in this study

Water body	Drainage	Locality/country	Number of specimens	Number of haplotypes	Accession numbers
Alcabrichel	Alcabrichel	Lourinhã/P	4	4	AY254585–88
Lagoa do Canário	Mondego[a]	São Miguel, Azores/P	5	1	AY254589–90, AY254652–53
Ceira	Mondego	Góis/P	3	3	AY254648–50
Alva	Mondego	Sobral/P	1	1	AF045980
Alcoa	Alcoa	Alcobaça/P	4	2	AY254591–94
Cávado	Cávado	Barcelos/P	5	4	AY254595–99
Paiva	Duero	Castro D'Aire/P	1	1	AY254600
Távora	Duero	Vila da Ponte/P	5	1	AY254601–05
Araviana	Duero	Olvega/S	1	1	AY254606
Arlanza	Duero	Castillo de la Reina/S	1	1	AY254607
Aranzuelo	Duero	Arauzo de Torres/S	1	1	AY254608
Pisuerga	Duero	Salinas de Pisuerga/S	1	1	AY254609
Cubillo	Duero	Zael/S	1	1	AY254610
Adaja	Duero	Niharra/S	1	1	AY254611
Duraton	Duero	Saldaña de Ayllon/S	1	1	AY254612
Piron	Duero	Peñarrubias de Piron/S	1	1	AY254613
Sabor	Duero	Torre de Moncorvo/P	5	4	AY254614–18
Maçãs	Duero	—/P	1	1	AY254619
Bernesga	Duero	Beberino/S	2	2	AF045979, AY254620
Jalón	Ebro	Medinaceli/S	1	1	AY254621
Araquil	Ebro	Ciordi/S	1	1	AY254622
Júcar	Júcar	Gritos/S	1	1	AY254623
Estorãos	Lima	Ponte de Lima/P	4	1	AY254624–27
Lis	Lis	Porto de Mós/P	4	2	AY254628–31
Mijares	Mijares	Olba/S	1	1	AY254632
Valbona	Mijares	Valbona/S	2	2	AY254633–34
Coura	Minho	Vilar de Mouros/P	12	4	AY254635–46
Sil	Minho	Orallo/S	1	1	AY254647
Palancia	Palancia	Bejis/S	2	1	AY254654–55
Real	Real	Óbidos/P	4	2	AY254656–59
Safarujo	Safarujo	Mafra/P	6	1	AY254660–65
São Pedro	São Pedro	S. Pedro de Moel/P	3	2	AY254666–68
Sizandro	Sizandro	Torres Vedras/P	5	2	AY254669–73
Dulce	Tagus	Pelegrina/S	1	1	AY254674
Trabaque	Tagus	Villaconejos/S	1	1	AY254675
Gébalo	Tagus	Alcaudete de la Jara/S	2	2	AY254676–77
Lozoya	Tagus	Pinilla del Valle/S	2	2	AF045981, AY254678
Tornada	Tornada	Tornada/P	1	1	AY254679
Ulla	Ulla	Ponte Ulla/S	2	2	AY254680–81
Zela	Vouga	Vouzela/P	3	3	AY254682–84

Their water body, drainage, locality/country, number of specimens, number of haplotypes per locality, and GenBank accession number. P, Portugal; S, Spain.

[a] The specimens from Lagoa do Canário (Azores) came from a population of *C. macrolepidotus* that originated in the Mondego River and was introduced to São Miguel Island in the 19th century. We decided to include them because they represent material present in Mondego prior to the advent of sport fishing, which may have encouraged some fish transfers between basins.

most informative was the third (168 parsimony informative characters), followed by the first (21 characters).

The general time reversible model with among-site rate heterogeneity GTRI+G+I (Lanave et al. 1984; Yang 1994) was selected by MODELTEST as the best fit to the data. The rate matrix parameters estimated were $R(a) = 7.27$, $R(b) = 1.59$, $R(c) = 3.57$, $R(d) = 7.47$, and $R(e) = 4.98$. Base frequencies were $A = 0.274$, $C = 0.274$, $G = 0.155$, and $T = 0.292$. Among-site rate variation was approximated with the gamma distribution shape parameter alpha = 3.02. The proportion of invariable sites was $I = 0.61$.

MP analysis resulted in a single most parsimonious tree of 576 steps. The results of the four phylogenetic inference methods are summarized in Figure 1, together with the geographic representation of the groups obtained. The topologies recovered by the four methods shared most features. The ML tree was taken as the base for Figure 1, where bootstrap values for the ML, MP, and ME analyses and Bayesian inference with posterior probabilities are shown. Phylogenetic reconstructions for all inference methods showed two monophyletic lineages supported by high bootstrap and posterior probability values, separated by an average HKY85 distance of $d = 6.5\%$. Contrary to what could be expected from the current taxonomy of the group, none of these lineages correspond to *C. arcasii* or *C. macrolepidotus*. Indeed, *C. macrolepidotus* and most populations of *C. arcasii* are

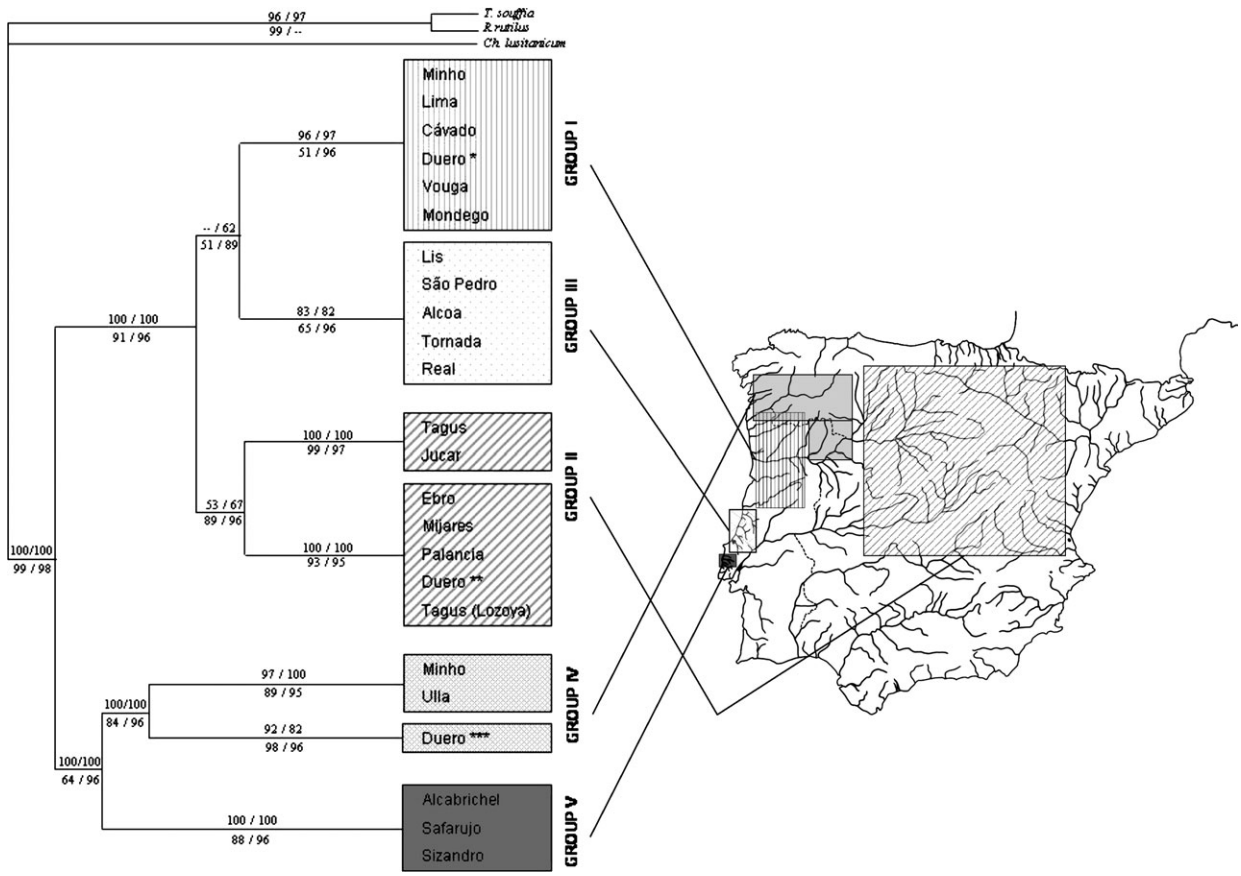

Figure 1. Figure based on the ML tree. Phylogenetic relationships among the populations analyzed based on cytochrome *b* sequences (left) and schematic map of Iberia with the distributions of the phylogroups recovered by the phylogenetic analysis (right). Numbers above branches represent the bootstrap values obtained for ME and ML; numbers below branches indicate those for MP and the posterior probabilities for Bayesian inference. Statistics for the parsimony analysis are tree length = 576 steps, consistency index (CI) = 0.632, retention index = 0.936, homoplasy index (HI) = 0.368, rescaled consistency index = 0.592, and F-ratio = 0.137 (without informative characters: CI = 0.565 and HI = 0.435). Most topological features were recovered by the four inference methods. When a particular branch was not recovered by a certain method, one hyphen replaces the corresponding bootstrap value. Each branch tip represents all the individuals sharing the same haplotype. In the boxes, the names of the rivers where the respective group of haplotypes was found are listed. Only one fish per haplotype was included in the figure, although the analysis was based on all the specimens. Duero*, lower section of the basin (in Portugal, rivers Paiva and Távora); Duero**, upper part of the basin (Spain, all tributaries upstream Bernesga); Duero***, tributaries around the border between Spain and Portugal (rivers Bernesga and Maçãs e Sabor).

included in the first of the two major lineages evidenced by the analysis (Groups I–III in Figure 1). *C. macrolepidotus* shows a clear geographic structure: Group I includes all the northern populations south to Mondego, while the remaining populations of the same species south of Mondego form Group III. Bayesian analysis suggests that these two groups form a monophyletic clade, sister to Group II that includes the majority of populations of *C. arcasii*. Interestingly, fishes from some Mediterranean rivers are grouped with those of Duero, while a few others are grouped with those of the Tagus. The Lozoya River is often not grouped with other tributaries of the Tagus basin because it tends to contain faunistic elements that are more representative of the Duero River, such as the species *Cobitis calderoni* (Doadrio 2001).

Surprisingly, the second major lineage includes populations that are geographically very distant, some located in northwestern Spain and Portugal (Group IV, traditionally ascribed to *C. arcasii*), while others (Group V, analyzed here for the first time) are in the extreme southwest of the entire distribution area. Both are separated by many hundreds of kilometers and show an average HKY85 distance of d = 4.18%. The HKY85 distances among the five major phylogroups recovered in the analyses are presented in Table 2, together with the nucleotide diversity values for each phylogroup.

It is important to note that except in the Minho drainage all the phylogroups described here were not found in sympatry, as can be seen in Figure 1.

Table 2. HKY85 distances among the five major phylogroups recovered in the analyses

	Group I	Group II	Group III	Group IV	Group V
Group I	0.0028 ± 0.0017	1.81%–3.48%	1.14%–1.81%	6.33%–6.97%	6.23%–7.40%
Group II	2.32%	0.0120 ± 0.006	1.61%–3.48%	6.42%–8.14%	6.64%–8.36%
Group III	1.33%	2.52%	0.0033 ± 0.002	6.01%–6.75%	6.12%–7.19%
Group IV	6.67%	7.31%	6.38%	0.0033 ± 0.002	3.88%–4.99%
Group V	6.64%	7.27%	6.50%	4.39%	0.0024 ± 0.0015

Average values are shown in the lower half, while the corresponding ranges are shown in the upper half. The nucleotide diversity values for each phylogroups are presented in the diagonal.

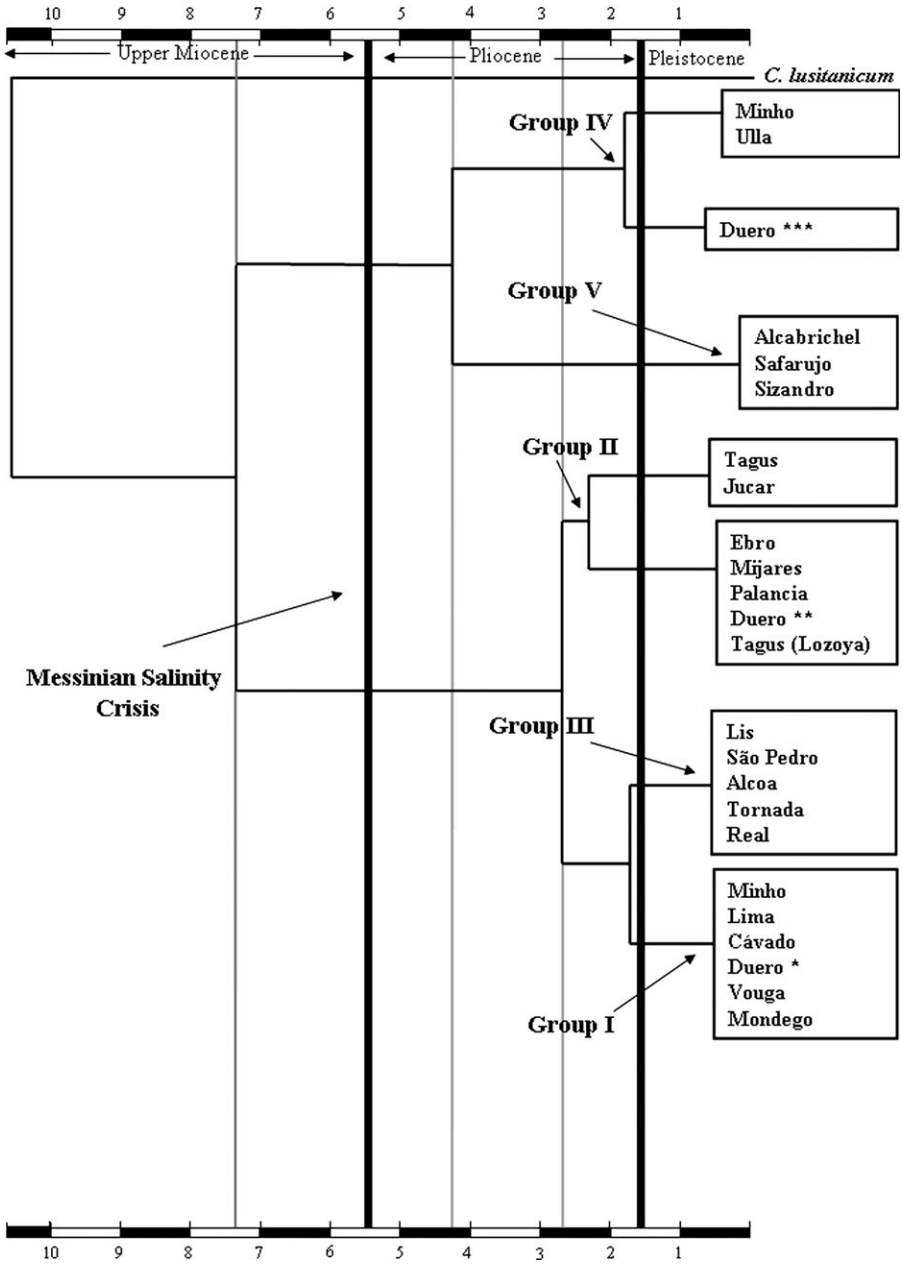

Figure 2. Clock-constrained ML tree showing the major cladogenetic events in the history of Iberian cyprinids. The scale bars show the time scale resulting from a calibration of the molecular clock (1% per MYA). For the explanation of the boxes at the tip of the branches, see Figure 1. Duero*, lower section of the basin (in Portugal rivers Paiva and Távora); Duero**, upper part of the basin (Spain, all tributaries upstream Bernesga); Duero***, tributaries around the border between Spain and Portugal (rivers Bernesga, Maçãs, and Sabor).

Discussion

Our results clarify the unexpected findings of Zardoya and Doadrio (1998), who questioned the monophyly of *C. arcasii* and *C. macrolepidotus*. Indeed, it becomes apparent that *C. arcasii* is a polyphyletic group, including the two major lineages identified (Groups II and IV). At the same time, our study also provides evidence that in the southwest of the distribution area of the species group there are populations that do not fit either in *C. arcasii* or in *C. macrolepidotus* (Group V). Group V populations emerge farther south of the distribution range of *C. macrolepidotus*, in the small coastal rivers of Alcabrichel, Sizandro, and Safarujo. This peculiar distribution (with populations in the extreme northwest and southwest limits of the group range) strongly suggests that the lineage must have had a much wider distribution in the past and that the populations represent relic pockets that were separated vicariantly.

In other freshwater fishes, phylogroups detected with molecular methods have subsequently been ascribed to different species (Hendry et al. 2000). It is probable that fishes belonging to Groups IV and V also correspond to distinct but yet undescribed species. A formal taxonomic revision of the entire group is urgently needed.

The divergence time that separates the two main lineages identified in this study of more than 7 million years, clearly suggests that this cladogenetic event took place in the Miocene, while the separation of phylogroups IV and V took place more than 4 million years ago (MYA), in the lower Pliocene (Figure 2). These events predate the formation of the present hydrographic network (Andeweg et al. 1999). In addition, as predicted by our second hypothesis, members of the two main lineages co-occur in the same basin, namely, in the Duero basin. Thus, our results indicate that a substantial proportion of the diversification of the group occurred before the present river drainages were formed, probably in the inland lakes that drained Iberian waters during the Mio-Pliocene. Our results also identified more recent events like the separation of the phylogroups II and I–III that occurred less than 3 MYA. Thus, the high diversity presently found in these Iberian cyprinids is likely the result of old cladogenetic events that took place in Miocene times and more recent events of Pliocene and possibly Pleistocene origin. If our conclusions are correct, the two scenarios presented in this paper both contributed to the present level of diversity.

We are aware that all the interpretations presented above depend critically on the validity of our calibration of the molecular clock. Different authors have calibrated molecular clocks for the cytochrome *b* gene of cyprinids using fossil or geological data. Zardoya and Doadrio (1999) calibrated a molecular clock of 0.76% divergence per lineage per million years, using the opening of the Gibraltar strait (5.5 MYA, Krijsman et al. 1999) and the formation of the Korinthos strait in Greece. Subsequently, on the basis of the opening of the Gibraltar strait and the genetic divergence between populations of the genus *Barbus* from Africa and Iberian Peninsula, Machordom and Doadrio (2001) estimated a molecular clock of 0.66% per lineage per million years. Dowling et al. (2002) and Smith et al. (2002) provided a calibration of approximately 0.5% divergence per lineage per million years for the cyt *b* gene, based on a series of comparisons for which divergence rates and fossil ages are available. A comparison involving North African and Iberian species of the genus *Cobitis* (which belongs to a family closely related to the cyprinids) yielded a divergence rate of 0.42% per lineage per million years (Perdices and Doadrio 2001). Thus, although older estimates of the divergence rates between lineages of cyprinids for the cyt *b* gene were higher (e.g., 0.76% per lineage per million years; Zardoya and Doadrio 1999), the more recent estimates converge on a divergence rate of around 1% sequence divergence per pairwise comparison per million years or 0.5% divergence within lineage per million years, the figure that we adopted in this study. However, even if we adopted the more conservative calibration of 0.76% per lineage per million years of Zardoya and Doadrio (1999), the separation of the two main lineages would still be of late Miocene-lower Pliocene origin, a timing that would not invalidate our conclusions.

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