Journal of FISH BIOLOGY
Published for the Fisheries Society of the British Isles

Journal of Fish Biology (2010) **76**, 965–974
doi:10.1111/j.1095-8649.2010.02536.x, available online at www.interscience.wiley.com

Phylogenetic relationships of the newly described species *Chondrostoma olisiponensis* (Teleostei: Cyprinidae)

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(Received 13 January 2009, Accepted 19 November 2009)

Phylogenies were generated using mitochondrial cytochrome *b* and nuclear ß-actin gene DNA sequences to infer the phylogenetic relationships of the newly described *Chondrostoma olisiponensis*. Results indicate that the species is monophyletic with species of the *lemmingii*-group in mtDNA phylogenies, while it is monophyletic with species of the *arcasii*-group in the nuclear ß-actin trees. This is in agreement with the morphological resemblance of *C. olisiponensis* to both species groups. Results from nuclear but not mitochondrial DNA indicate that one population could be currently hybridizing with sympatric *Chondrostoma lusitanicum*. Based on a relaxed clock calibration of cytochrome *b*, it is estimated that *C. olisiponensis* split 12·5–7·9 million years ago (middle–upper Miocene) from its most recent ancestor, which coincides with a period of endorrheism in the Iberian Peninsula.

Key words: *Achondrostoma*; cytochrome *b*; *Iberochondrostoma*; molecular phylogeny; nuclear ß-actin gene.

INTRODUCTION

The genus *Chondrostoma* Agassiz is composed of small to medium-sized cyprinids distributed in the northern Mediterranean drainages across Europe, western Asia and Middle East (Elvira, 1987, 1991, 1997; Durand *et al*., 2003). Phylogenetic analyses suggest that *Chondrostoma* is a monophyletic taxon composed of seven monophyletic lineages or groups of species with and without a horny blade on the lower lip (Coelho *et al*., 1997; Zardoya & Doadrio, 1998; Doadrio & Carmona, 2003, 2004), hereafter referred to as *Chondrostoma s.l.* The genus is particularly diverse in the Iberian Peninsula, where over one-third of the known species are found. Recently, Robalo *et al*. (2007) suggested the species groups are in agreement with morphological criteria and proposed their elevation to different genera. According to this new classification, *Chondrostoma s.st*. consists only of the *nasus* and *soetta*-species groups, while the *arcasii*, *genei*, *lemmingii*, *polylepis* and *toxostoma*-species groups

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were each elevated to the generic level (respectively, *Achondrostoma*, *Protochondrostoma*, *Iberochondrostoma*, *Pseudochondrostoma* and *Parachondrostoma*).

Subsequent to this splitting, a new bladeless species was described from the lower Tejo basin in Portugal. The Lisbon arched-mouth nase *Chondrostoma olisiponensis* Gante, Santos & Alves is a small species that lacks a horny blade on the lower lip, a characteristic that makes it more similar to *Achondrostoma* and *Iberochondrostoma* (the *arcasii* and *lemmingii*-species groups, respectively). Nevertheless, the new species has a combination of characters that does not fit in the proposed genera and breaks the combinations of characters deemed diagnostic of these two new genera (Gante *et al*., 2007). These facts led the authors to include the new species in *Chondrostoma s.l.* before proceeding to further changes that could lead to instability in the taxonomy of the group.

The present study aims to assess the phylogenetic relationships of *C. olisiponensis* using mitochondrial and nuclear sequences, and to provide an estimate for time of divergence using a molecular clock calibration.

MATERIALS AND METHODS

Muscle and fin tissues from 17 specimens of *C. olisiponensis* deposited in the tissue collection Museu Bocage (MB) of the Museu Nacional de História Natural, Portugal (accession numbers MB55-0610, MB55-4678, MB55-4679, MB55-4756, MB55-4757, MB55-4833 through to 4840, MB55-5825, MB55-5826, MB55-5840 and MB55-5967) were used to extract total genomic DNA, using standard phenol–chloroform protocols (Sambrook *et al*., 1989). Specimens originated from Trancão, Maior and Muge River basins and include the type series (Tejo River basin, Portugal; Fig. 1).

Phylogenetic relationships of *C. olisiponensis* were investigated using sequences of two genes, the nuclear ß-actin gene and the mitochondrial cytochrome *b* (cyt *b*) gene. Amplification of the cyt *b* gene was accomplished using primers LCB1 (Brito *et al*., 1997) and HA (Schmidt & Gold, 1993) and the conditions of Mesquita *et al*. (2001). For amplification of the nuclear ß-actin gene fragment, conditions and primers in Robalo *et al*. (2006) were followed. Sequences were obtained by direct sequencing on an Applied Biosystems 3700 DNA sequencer (www.appliedbiosystems.com) following manufacturer's instructions. Chromatograms were visually inspected for sequencing errors, and sequences were manually aligned and trimmed with BioEdit v.5.0.6 (Hall, 1999). Sequences representing the most common allele of each locus within *C. olisiponensis* were used in the phylogenetic analyses. In addition to *C. olisiponensis*, several *Chondrostoma* and out-group species for which sequence data of ß-actin and cyt *b* are available were used (Table I).

Bayesian phylogenies were constructed in BEAST v.1.4.8 (Drummond & Rambaut, 2007), and maximum likelihood (ML) and maximum parsimony (MP) phylogenies were constructed in PAUP* 4.0b10 (Swofford, 2002). Substitution models employed in Bayesian and ML analyses were selected using the corrected Akaike information criterion (AIC) implemented in Modeltest 3.07 (Posada & Crandall, 1998), following Posada & Buckley (2004). Bayesian analyses were run for 10 000 000 generations, sampled every 1000 generations, with a subsequent burn-in of 2501 trees (*c*. 25%) using TreeAnnotator v.1.4.8 (Rambaut & Drummond, 2002). Conversion and stability of the estimated parameters were checked using Tracer v.1.4 (Rambaut & Drummond, 2003). Phylogenetic trees were visualized using FigTree v.1.1.2 (Rambaut, 2006). ML and MP analyses were conducted using heuristic tree searches (10 replicates, random sequence addition and tree bisection-reconnection (TBR) branch swapping, and gaps were treated as a fifth state). Bootstrap support was estimated using 1000 (MP) and 100 (ML) pseudoreplicates (10 and five sequence addition replicates, respectively, for MP and ML).

The time of divergence of *Chondrostoma* species and their credibility intervals (highest posterior density, HPD) were calculated using a relaxed clock model implemented in BEAST

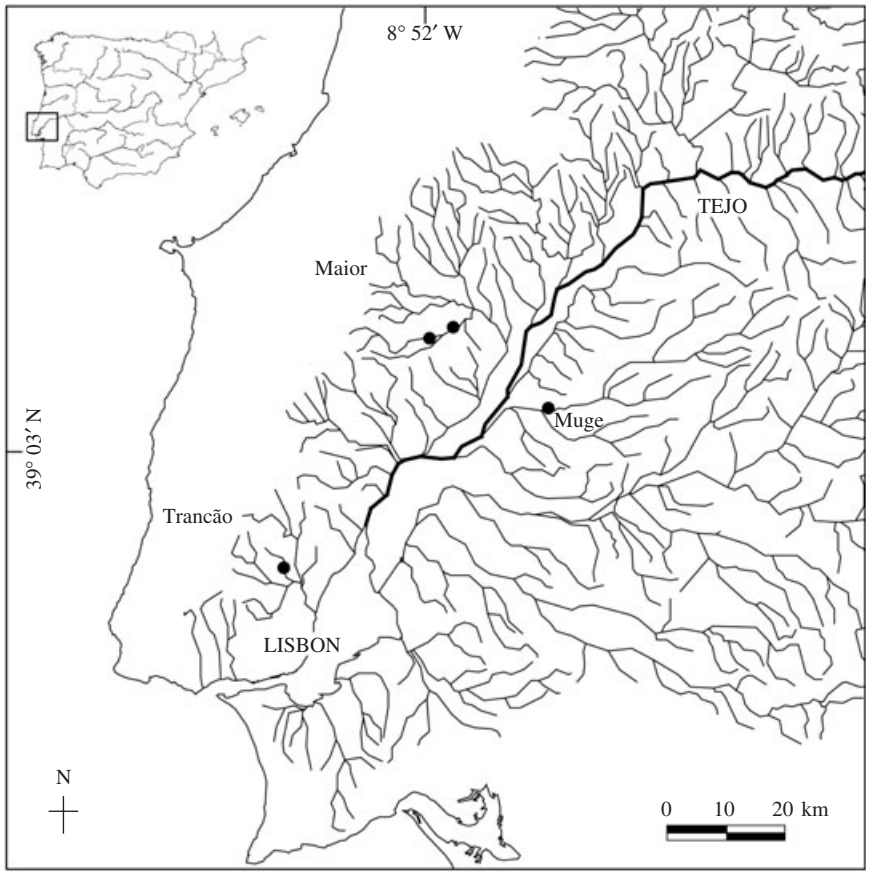

FIG. 1. Localities sampled in the lower Tejo River basin for *Chondrostoma olisiponensis*.

v.1.4.8 (Drummond & Rambaut, 2007). Branch rates were drawn from an uncorrelated log-normal distribution (Drummond *et al*., 2006), and a Yule speciation prior was applied. The pair-wise rate of evolution of 1·05% per million years (Dowling *et al*., 2002), often applied to *Chondrostoma* cyt *b* data (Doadrio & Carmona, 2003, 2004; Robalo *et al*., 2006), was used.

RESULTS

The 17 specimens of *C. olisiponensis* originating from three localities in the lower Tejo River yield three ß-actin alleles. Allele A is found in all sampled populations, allele B occurs in both Trancão and Muge Rivers and allele C is exclusively found in Maior River (Table II). Alleles A and B differ from each other by only one mutation, and C differs from B and A by 11 and 12 mutations, respectively. The entire nuclear data set consists of 912 sites (453 exonic and 459 intronic) of which 88 are variable and 30 are parsimony informative. The most common allele of *C. olisiponensis* (allele A) is closest to *Chondrostoma oligolepis* (Robalo, Doadrio, Almada & Kottelat) with a genetic distance of 0·34%, and to *Chondrostoma arcasii* (Steindachner)

TABLE I. Taxa included in the phylogenetic analyses and sequence database accession numbers

Taxon	Cyt[b]	ß-actin	Taxon	Cyt[b]	ß-actin
Chondrostoma almacai	AF045986[a]	DQ447717[b]	*Chondrostoma oretanum*	AY582741[c]	DQ447722[b]
Chondrostoma arcasii	AY254611[d]	DQ447711[b]	*Chondrostoma oxyrhynchum*	AF095606[e]	DQ447721[b]
Chondrostoma arrigonis	AY568598[c]	DQ447714[b]	*Chondrostoma phoxinus*	AY494741[f]	DQ447723[b]
Chondrostoma duriensis	AF045983[a]	DQ447715[b]	*Chondrostoma polylepis*	AF045982[a]	DQ061945[b]
Chondrostoma genei	AF533766[g]	DQ061938[b]	*Chondrostoma prespensis*	AF090747[e]	DQ061944[b]
Chondrostoma knerii	DQ447739[b]	DQ447724[b]	*Chondrostoma soetta*	AY568623[c]	DQ061936[b]
Chondrostoma lemmingii	AF045987[a]	DQ447716[b]	*Chondrostoma salmantinum*	AY568612[c]	DQ447712[b]
Chondrostoma lusitanicum	AY254584[d]	DQ447718[b]	*Chondrostoma turiensis*	AY568619[c]	DQ061936[b]
Chondrostoma miegii	AY568609[c]	DQ455049[b]	*Chondrostoma vardarensis*	AF090749[c]	DQ447719[b]
Chondrostoma nasus	AY026402[h]	DQ447726[b]	*Chondrostoma willkommii*	AF045984[a]	DQ447725[b]
Chondrostoma occidentale	AY254585[d]	DQ447720[b]	*Anaecypris*	AJ427814[i]	DQ061936[b]
Chondrostoma oligolepis	AY254679[d]	DQ447713[b]	*Rutilus*	Y10440[j]	DQ061948[b]
Chondrostoma olisiponensis	AM886164[k]	AM886165[k]	*Telestes*	AY838934[l]	DQ061950[b]

[a]Zardoya & Doadrio (1998); [b]Robalo *et al*. (2007); [c]Doadrio & Carmona (2004); [d]Robalo *et al*. (2006); [e]Zardoya & Doadrio (1999); [f]Hrbek *et al*. (2004); [g]Durand *et al*. (2003); [h]Durand *et al*. (2002); [i]Alves *et al*. (2001); [j]Briolay *et al*. (1998); [k]Present study and [l]Freyhof *et al*. (2006).

and *Chondrostoma occidentale* Robalo, Almada, Sousa-Santos, Moreira & Doadrio with a genetic distance of 0·45%, all of which belong to the *arcasii*-species group (*i.e. Achondrostoma*). Allele C is identical to the typical *Chondrostoma lusitanicum* Collares-Pereira allele used in this study and highly divergent (0·91%) relative to the other alleles found in *C. olisiponensis*.

Four cyt *b* haplotypes, differing by not more than three nucleotide substitutions, are found in the 17 specimens analysed. The most common haplotype (A) is found in the three populations sampled, haplotype B is absent from Maior River, while haplotypes C and D are found exclusively in Trancão and Muge Rivers, respectively (Table II). The entire mitochondrial data set (1140 bp) consists of 386 variable sites of which 272 are parsimony informative. The most common haplotype of *C. olisiponensis* (allele A) is closest to *C. lusitanicum* with a genetic distance of 7·11%, followed by *Chondrostoma lemmingii* (Steindachner) with a distance of 7·81%, both species belonging to the *lemmingii*-species group (*i.e. Iberochondrostoma*).

TABLE II. Geographic distribution (see Fig. 1) of different haplotypes found in *Chondrostoma olisiponensis* specimens

Locality	Cyt *b*	ß-actin
Maior ($n = 3$)	A = 3	A = 3
		C = 3
Trancão ($n = 10$)	A = 5	A = 14
	B = 3	B = 6
	C = 2	
Muge ($n = 4$)	A = 2	A = 5
	B = 1	B = 3
	D = 1	

The most common allele at each locus (allele A; Table II) was used to reconstruct phylogenetic relationships. The different tree-building methods give largely congruent results for each gene fragment; when a particular node is retrieved by one method, it is also retrieved with equivalent support by the other methods as well (Figs 2 and 3). *Chondrostoma olisiponensis* is monophyletic with the *lemmingii*-species group in cyt *b* trees, while it is monophyletic with the *arcasii* and *polylepis*-species groups in ß-actin phylogenies. In both phylogenies, *C. olisiponensis* shares a common ancestor with the entirety of these groups.

According to the relaxed clock calibration, divergence within *Chondrostoma* initiated *c*. 15·1 million years before present (B.P.) (95% HPD: 17·8–12·4 M B.P.) during the lower–middle Miocene (late Burdigalian–Serravalian). In particular, the time of divergence leading to *C. olisiponensis* is estimated to have occurred *c*. 10·1 M B.P. (95% HPD: 12·5–7·9 M B.P.), during the middle–upper Miocene (Serravalian–Late Tortonian; Fig. 2).

DISCUSSION

Chondrostoma olisiponensis was recently described for the lower Tejo River basin in Portugal (Gante *et al*., 2007). Since the species shares many morphological characters deemed diagnostic of *Achondrostoma* and *Iberochondrostoma*, while others fit none of the two newly designated genera, molecular data could shed light on this taxonomic hurdle. This conflict, however, is also evident in the molecular data, since *C. olisiponensis* is placed in either of these species groups, depending on the marker. *Chondrostoma olisiponensis* is recovered as monophyletic with the *lemmingii*-species group with high support by different methods in the mitochondrial trees (Fig. 2). Conversely, *C. olisiponensis* is recovered monophyletic with the *arcasii* and *polylepis*-species groups in the nuclear trees with high posterior probability (Fig. 3). This type of incongruence is usually due to incomplete lineage sorting of the nuclear gene fragment, biases associated with any of the markers or hybridization between two ancestral forms (Funk & Omland, 2003). Given the extensive contemporary hybridization between species belonging to different lineages of *Chondrostoma s.l.* (Collares-Pereira & Coelho, 1983; Elvira *et al*., 1990; Gante *et al*., 2004), it is very likely that hybridization and introgression have occurred in the past,

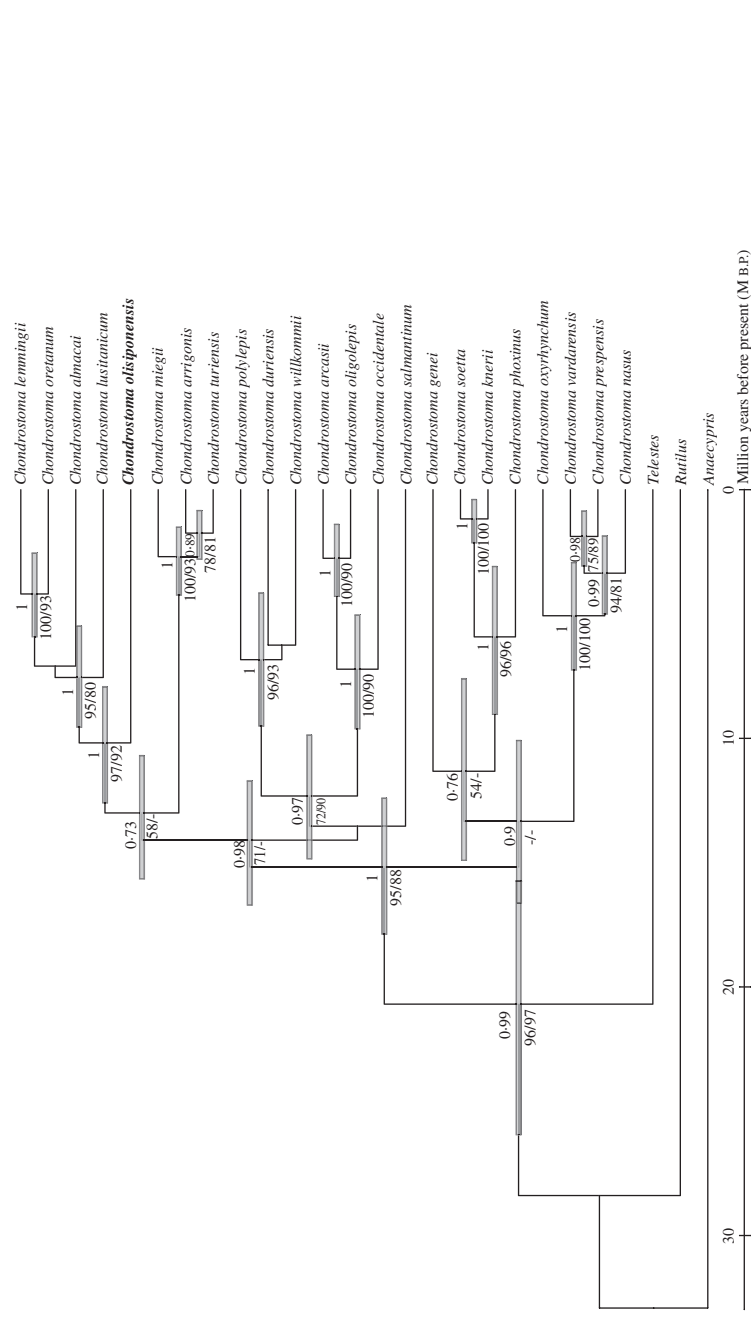

FIG. 2. Bayesian phylogenetic tree showing relationships among *Chondrostoma s.l.* species based on cyt *b* sequences. Values above branches refer to posterior probabilities. Values below branches refer to maximum likelihood (ML) and maximum parsimony (MP) bootstrap values, respectively. Grey bars represent the 95% highest posterior density (HPD) for each estimated age based on a relaxed molecular clock. The most common haplotype found in *C. olisiponensis* was used (haplotype A; see Table II).

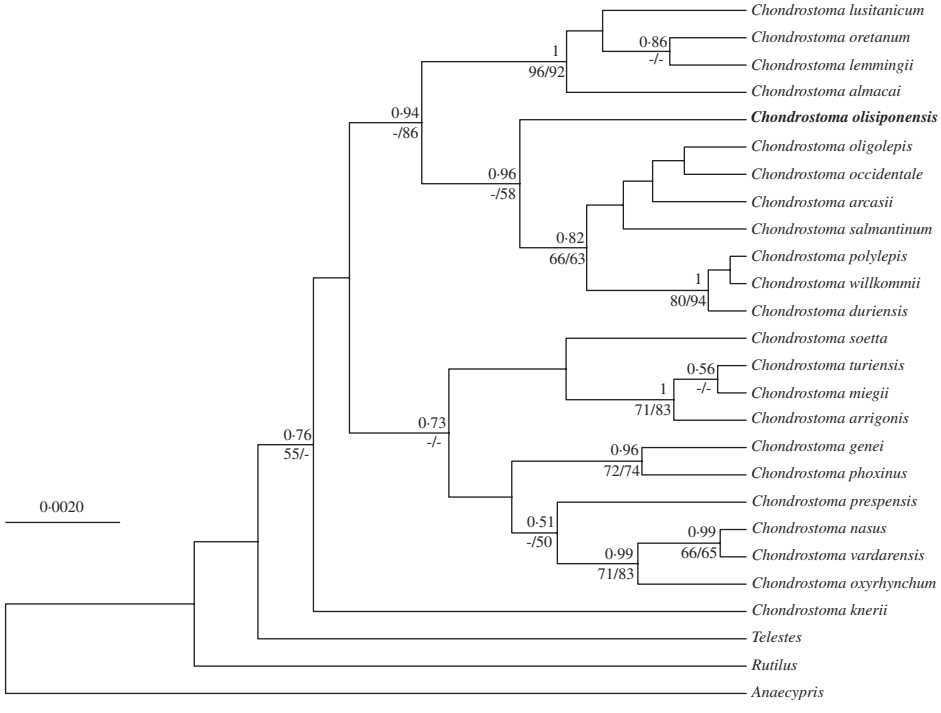

FIG. 3. Bayesian phylogenetic tree showing relationships among *Chondrostoma s.l.* species based on ß-actin sequences. Values above branches refer to posterior probabilities. Values below branches refer to maximum likelihood (ML) and maximum parsimony (MP) bootstrap values, respectively. The most common allele found in *C. olisiponensis* was used (allele A). Allele C found only in Maior River is identical to the typical *C. lusitanicum* allele (Table II).

potentially with persisting effects in the phylogeny (Smith, 1992), and causing the observed conflicting phylogenetic signals. Testing of these hypotheses will require additional sequences of ß-actin from each species and further analysis of unlinked nuclear markers.

Evidence for contemporary hybridization and introgression is also present in this study. ß-actin allele C, found in heterozygosity in all *C. olisiponensis* specimens from Maior River (Table II), is typical of the sympatric *C. lusitanicum* (Fig. 3). This pattern of allele sharing suggests the occurrence of gene flow between these species. Breakage of reproductive barriers could be associated with small population sizes of *C. olisiponensis* and environmental disturbance of its habitats (Gante *et al.*, 2007).

The mean estimated ages of splitting of *Chondrostoma* species derived in the present study, using a relaxed molecular clock with a mean pair-wise rate of 1·05% per million years, are generally slightly older than that reported by Doadrio & Carmona (2003, 2004) using a strict clock calibration (Fig. 2). Nevertheless, many of these later estimates fall within the present 95% HPD. According to the present results, initial cladogenesis within *Chondrostoma* started between 17·8 and 12·4 M B.P. and that of *C. olisiponensis* between 12·5 and 7·9 M B.P., which was the first species to split from the ancestor of the *lemmingii*-species group. This timing of speciation for *C. olisiponensis* coincides with a period of endorrheism in the

Iberian Peninsula (Ribeiro *et al*., 1979), suggesting that speciation of *C. olisiponensis* occurred in Lower Tejo Tertiary basin. The influence of Tertiary endorrheic basins in the evolution of Iberian *Chondrostoma* and other freshwater fishes has been recognized by several authors (Carmona *et al*., 2000; Doadrio & Carmona, 2003, 2004; Sanjur *et al*., 2003; Doadrio & Perdices, 2005; Robalo *et al*., 2006, 2008), since many speciation events coincide with this period. As noted by Doadrio & Carmona (2003, 2004) for other bladeless species of the *arcasii* and *lemmingii*-species groups, the distribution area of *C. olisiponensis* is restricted to a portion of the present-day hydrographic basin, implying its reduced vagility.

The molecular data presented in this study do not allow unequivocal placement of *C. olisiponensis* in either of the new designated genera, which is in agreement with the previous morphological results (Gante *et al*., 2007). In particular, *C. olisiponensis* has morphological traits deemed diagnostic of *Achondrostoma* and *Iberochondrostoma*, as well as molecular markers distinctive of each of these genera. This pattern could have originated either by incomplete sorting in a form ancestral to both groups or by ancient hybridization and deserves further investigation. In addition, extent and directionality of contemporary introgression between *C. olisiponensis* and *C. lusitanicum* should be investigated, as both species are rare and threatened.

The authors would like to thank M. Arruda for providing fin tissue from Maior River and M. Drago for laboratory support. This work was partially funded by the project grant PTDC/BIA-BDE/66519/2006 (Fundação para a Ciência e a Tecnologia, co-participated by FEDER) to M.J.A.

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