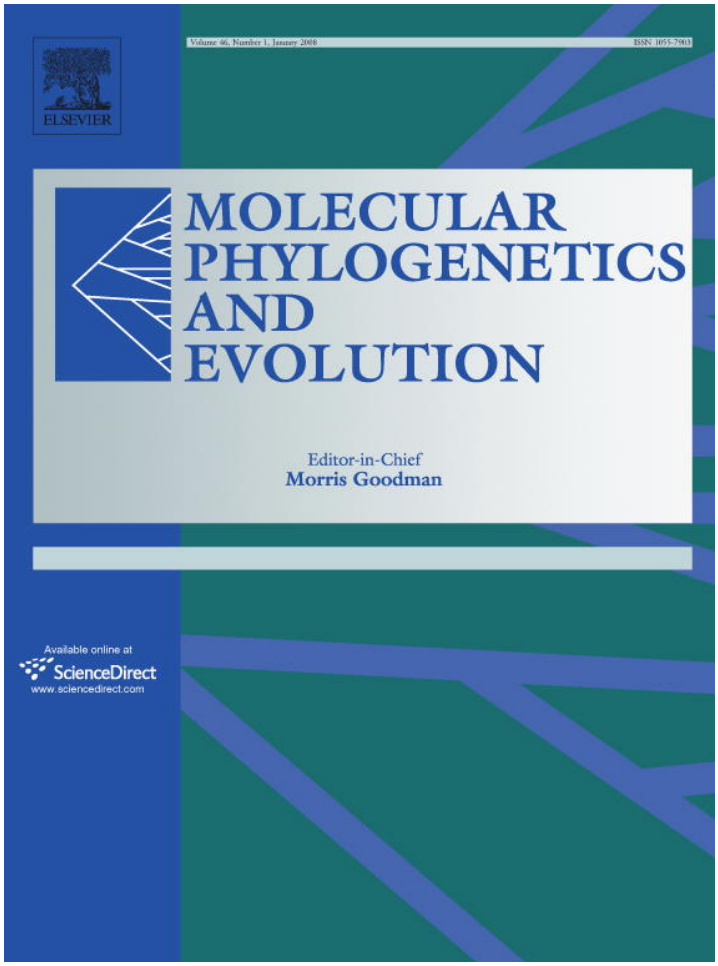

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Insights on speciation patterns in the genus *Iberochondrostoma* (Cyprinidae): Evidence from mitochondrial and nuclear data

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Abstract

In this paper, the patterns of cladogenesis in the cyprinid fish genus *Iberochondrostoma* were analysed using a mitochondrial (cytochrome *b*) and a nuclear (beta-actin) gene fragment. The two genes yielded discordant results. While the cytochrome *b* gene yielded a fully dichotomous tree, where all species of the genus are monophyletic, the much slower beta-actin gene yielded star-like relationships. However, when information from both genes was considered together, the data suggested the persistence of a very large central unit from which at least two peripheral clades arose at different times. This pattern which is akin to peripatric speciation was shown to be compatible with the paleogeographical information available. It is suggested that combining the techniques of phylogeny and phylogeography and the use of multiple markers varying in their rate of evolution may enrich our understanding of speciation and evolution of clades beyond species level.

Keywords: Endemic fish; Cyprinids; Peripatric speciation; SNP analysis; Portugal; Spain

1. Introduction

Although much progress has been achieved, a considerable amount of work is still needed to fully bridge the gap between studies of the genetic relationships among populations and those involving taxa beyond the species level.

In phylogeographic studies, emphasis has been placed on the relationships among populations, commonly represented by haplotype networks or star-like tree topologies. These patterns are largely caused by incomplete lineage sorting and absence of reciprocal monophyly (e.g. Avise, 2000). On the contrary, phylogenetic analysis, especially when it is based on the rapidly evolving mitochondrial genes tends to result in trees presenting dichotomous structures with monophyletic groups. Implicit in many phylogenetic analyses is the assumption that in speciation the ancestral species tend to originate two sister species not very different in size. In this perspective, polytomies are viewed as being usually soft, being due to insufficient sampling, either in the amount of DNA or in the number of taxa. Hard polytomies in turn, would result from a very rapid succession of speciation events which left no record in a given genetic marker (e.g. Page and Holmes, 1998; Poe and Chubb, 2004). Another scenario is however conceivable. Consider a species with a broad geographical distribution and with a large effective population size. Such a "large" species can in different occasions, give rise to new peripheral species with small geographical areas and effective sizes without substantial changes in its genetic structure. Indeed, the larger the effective population size the slower the lineage sorting will be and the split of a small subset like the formation of a new "small" peripheral

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species may be of little consequence for the genetic pool of the large population which gave rise to it. In such situations, "large" species may originate different descendent species at rather separated times.

This speciation pattern can be detected using two or more genetic markers with different rates of evolution and may represent one of the possible explanations for a phenomenon frequently reported in phylogenetic studies: the fact that in many cases, different genes yield different tree topologies (e.g. Russo et al., 1996; Mattern, 2004). The combination of phylogenetic and phylogeographic techniques using large DNA datasets and comprising genes with different rates of evolution may help to recognize the modes of speciation that took place in a clade, namely the hypothesis of peripatric speciation (Mayr, 1982). In this speciation pattern, we expect to find a species with a central position presenting a high level of genetic diversity and ancestral polymorphisms and a few species in its periphery each one with low genetic diversity, small effective population size and geographical area, but revealing much more complete lineage sorting. The same pattern is unlikely to occur if a species gives rise to two species of similar size.

In this paper, we try to test the hypothesis of peripatric speciation using the genus *Iberochondrostoma* as a model. This genus was recently described (Robalo et al., 2007a) and constitutes an excellent model because it is well characterized from the morphological and molecular point-of-view and has a compact distribution area that occupies the west, centre and south of the Iberian Peninsula, being present almost without exception in all Atlantic basins between Tagus and Guadalquivir. Three of the four species included in this genus are Critically Endangered (Doadrio, 2001; Doadrio and Carmona, 2003a; Cabral et al., 2005; see Fig. 1) thus the study of their genetic structures assumes a great importance for their conservation (Mesquita et al., 2001; Robalo et al., 2007b).

Iberochondrostoma includes the following species at the present moment: *I. lemmingii* (Steindachner, 1866); *I. lusitanicum* (Collares-Pereira, 1980); *I. oretanum* (Doadrio and Carmona, 2003a); *I. almacai* (Coelho et al., 2005) (for the species distribution see Fig. 1). At least an additional one is awaiting formal description (the populations of *I. lusitanicum* from the Lower Tagus, adjacent drainages, in this paper collectively named west, and Lagoa de Albufeira, Robalo et al., 2007b). The old reports of *I. lemmingii* in the Douro drainage have been shown to correspond to a different fish of a distinct genus (*Achondrostoma*, Robalo et al., 2007a) which is being formally described as a new species.

The hypothesis that the evolution of this genus may have been characterized by the persistence of a large central species which gave rise to several peripheral small daughter species at different times is suggested by the present geographic distribution of the members of the genus (Fig. 1). In addition, it is known that, in the Miocene, many Iberian waters of the area where *Iberochondrostoma* species live drained to a number of inland lakes, the modern river system being very recent (Plio-Pleistocene) (Friend and Dabrio, 1996; Andeweg, 2002). Thus, the disappearance of the ancient lakes and the association of their remnants to different river systems is compatible with the pattern of speciation outlined above.

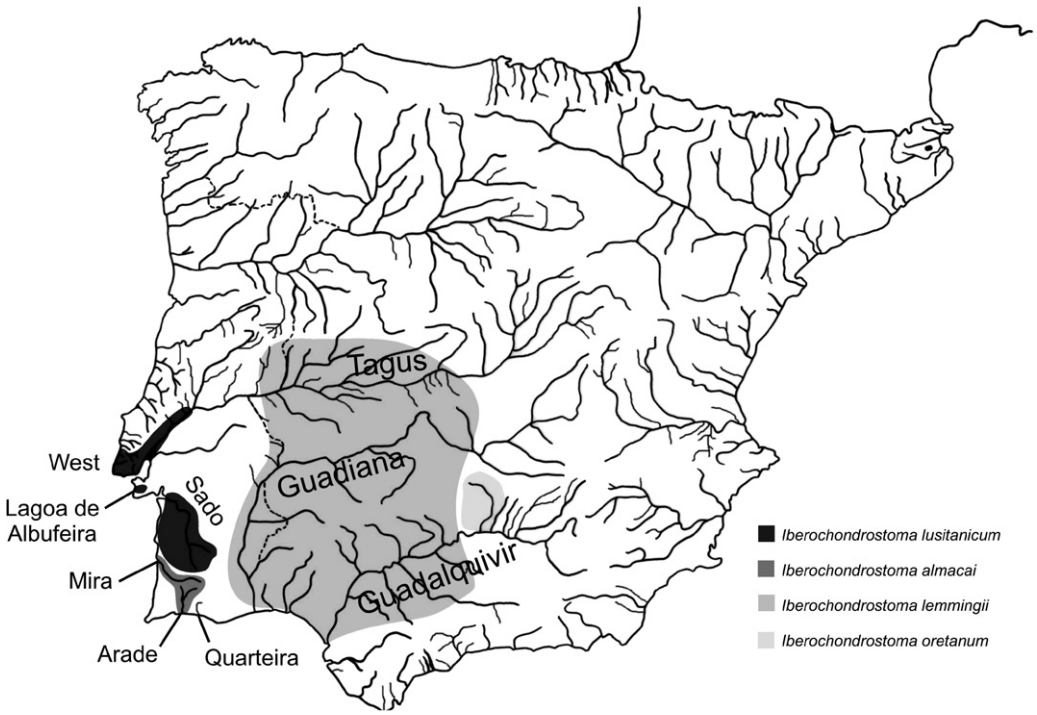

Fig. 1. Map of the Iberian Peninsula with the distribution of the *Iberochondrostoma* species.

This paper aims to describe the phylogeography of the entire genus *Iberochondrostoma*, using two molecular markers, with different rates of evolution. The mitochondrial cytochrome *b* (cyt *b* gene) was selected as a rapidly evolving gene, very commonly used in phylogenetic (e.g. Zardoya and Doadrio, 1998, 1999) and phylogeographic studies (Salzburger et al., 2003; Robalo et al., 2006a, 2007b). On the contrary, the nuclear beta-actin gene was selected due to its slow evolution rate (Robalo et al., 2006b, 2007a).

The hypothesis of speciation by peripheral isolation leads to the following predictions: (1) the presence of a central species of large size with several small species surrounding it; (2) more complete lineage sorting in the peripheral species than in the central one, promoted by their smaller sizes; (3) a much higher level of diversity and the persistence of ancestral polymorphisms in the central species.

2. Methods

2.1. Taxon sampling

The taxa analysed in this study, their collection sites and their corresponding GenBank sequence Accession Nos. are listed in Table 1 (for geographical origin of the samples see Fig. 1). Except in the few cases when not enough biological material was available, the DNA used for PCR and sequencing of both fragments came from the same individual. Voucher specimens are preserved in the fish collections of Museo Nacional de Ciencias Naturales (MNCN) and the Unidade de Investigação em Eco-Etologia, Instituto Superior de Psicologia Aplicada (UIEE/ISPA).

2.2. DNA analysis

Total genomic DNA was extracted from fin clips preserved in ethanol by an SDS/proteinase-k based protocol (adapted from Sambrook et al., 1989).

For the cyt *b* gene, a total of 738 bp was amplified using the primers LCB1–5′-AATGACTTGAAGAACCACC GT-3′ (Brito et al., 1997) and HA–5′-CAACGATCTC CGGTTTACAAGAC-3′ (Schmidt and Gold, 1993). PCR conditions followed those in Cunha et al. (2004). The amplification process was conducted as follows: 25 cycles of [94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min].

For the beta-actin gene, a total of 891 bp was amplified using the primers BactFor–5′-ATGGATGATGAAATTG CCGC-3′ and BactRev–5′-AGGATCTTCATGAGGTAG TC-3′ (Robalo et al., 2006b). PCR conditions followed those in Robalo et al. (2006b). The amplification process was conducted as follows:

35 cycles of [94 °C for 30 s, 55 °C for 40 s, and 72 °C for 1 min 30 s]. The amplified fragment is homologous to a region of the beta-actin gene of *Cyprinus carpio* (GenBank: M24113), including introns B and C and three exons.

Sequencing reactions were performed by Macrogen Inc. in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer.

2.3. Data analysis

Sequences were aligned with Clustal X (Thompson et al., 1997). Phylogenetic analyses were performed with PAUP 4.0 (Swofford, 1998) using maximum parsimony (MP) and distance (neighbour-joining, NJ). For the cyt *b* gene, the molecular evolution model was selected using AIC criterion, as implemented in Modeltest version 3.6. (Posada and Crandall, 1998). For the beta-actin gene, the uncorrected *p* distance was used instead of selecting a distance with Modeltest because it makes no sense to use a complex model to characterize relationships among very closely related species when using a gene with a very slow mutation rate. For instance, *Squalius pyrenaicus* and *Squalius carolitertii*, two sister species that according to cyt *b* data diverged about 4–6 MYA (Sanjur et al., 2003) did not suffer lineage sorting for beta-actin sharing haplotypes (Robalo et al., 2006b).

Bootstrap analysis was used to assess the relative robustness of branches of the MP (1000 replicates) and the NJ (100 replicates) (Felsenstein, 1985). Whenever for a given gene a haplotype was shared by several fish it was entered only once in the phylogenetic analysis. *Achondrostoma oligolepis* was used as outgroup in all analyses (GenBank Accession No.: AY254679 for cyt *b* gene and DQ447713 for beta-actin gene). The following methods were used only in the cyt *b* dataset due to the low mutation rate of the beta-actin gene. Relationships among haplotypes were analysed with a parsimony network estimated by the software TCS version 1.18 (Clement et al., 2000).

ARLEQUIN software package version 3.01 (Schneider et al., 2000) was used to estimate the genetic diversity in the species studied, to access population differentiation and to perform neutrality tests. Analysis of molecular variance (AMOVA; Excoffier et al., 1992) and pairwise *F*ST were performed whenever sample sizes were adequate. Fu's *F*s (Fu, 1997) and Tajima's *D* (Tajima, 1983) tests were performed to test for possible bottlenecks and population expansion. Mismatch analysis (Rogers and Harpending, 1992; Rogers, 1995) was not attempted because only for the Guadiana did the model fit the data so we felt unjustified to explore them in this direction.

Mean numbers of pairwise differences were corrected by subtracting the average within population differences for the populations of each pair, as implemented in ARLEQUIN. Subsequently, these values were transformed in percent sequence divergence after dividing the number of pairwise differences by the length of the DNA fragment analysed.

Table 1
Samples analysed in this study, their collection sites and their corresponding GenBank Accession Nos. for cytochrome *b* and beta-actin genes

Species	Basin	River/locality/country	Cyt *b* Number of samples/ GenBank Accession Nos.	Beta-actin Number of samples/ GenBank Accession Nos.
I. lemmingii	Quarteira	Quarteira/Paderne/Portugal	3/EF520187; EF520188; EF520190	5/EF520237; EF520239; EF520240; EF520242; EF520223
	Guadiana	—/— /Portugal	3/X99423*; EF520189; EF520191	
		—/— /Spain	1/AF045987	
		Jabalón/Bazan/Spain	3/EF524514; EF520147; EF520148	3/EF520224; EF520226; EF520225
		Esteras/Saceruela/Spain	7/EF520136; EF520142; EF520144; EF520145; EF520149; EF520181; EF520192	5/EF520230; EF520251; EF520252; EF520253; EF520257
		Gargantiel/Almadenejos/Spain	3/EF520162; EF520166; EF520179	1/DQ061940*
		Estomiza/Gamonoso/Spain	1/DQ089654*	1/EF520241
		Siruela/Tamurejo/Spain	4/EF520160; EF520175; EF520182; EF520198	1/EF520244
		Queijigares/Fontanosas/Spain	7/EF520127; EF520129; EF520134; EF520161; EF520196; EF520201; EF520214	3/EF520256; EF520250; EF520258
		Alcarrache/Higuera de Vargas/Spain	1/AY568605*	
		Guadalmez/La Bienvenida/Spain	1/EF520159	
		Valdeazogues/Almadenejos/Spain	1/EF520163	
		Navalatienda/Valdemanco de Esteras/Spain	1/EF520165	
		Guadamez/Valle/Spain	2/EF520168; EF520177	
		Alcudia/El Alamillo/Spain	1/EF520184	
		Maillo/Navas de Estena/Spain	3/EF520193; EF520194; AF045988	
		Sillo/Cumbres de San Bartolomé/Spain	3/EF520195; EF520202; EF520217	
		Estenilla/Gamonoso/Spain	2/EF520203; EF520219	
		Gévora/Alburquerque/Spain	1/EF520213	
		Higuerón/Cañaveral de León/Spain	1/EF520218	
	Tagus	Magasca/Trujillo/Spain	13/EF520128; EF520130; EF520178; EF520172; EF520131; EF520135; EF520169; EF520170; EF520171; EF520183; EF520173; EF520180; EF520200	9/EF520227; EF520246; EF520245; EF520229; EF520233; EF520234; EF520238; EF520249; EF520254
		Almonte/Jaraicejo/Spain	2/EF520156; DQ447733*	2/DQ447716*; EF520247
		Pesquero/Valverde del Fresno/Spain	6/EF520153; EF520154; EF520155; EF520157; EF520185; EF520186	2/EF520228; EF520248
		Tietar/Talayuela/Spain	2/AY568604*; EF520158	
		Alburrel/Valencia de Alcantara/Spain	1/AY568603*	
		Huso/Aldeanueva de San Bartolomé/Spain	1/EF520199	
		Albuera/Almendral/Spain	1/EF520206	
		Aurela/Santiago de Alcantara/Spain	2/EF520215; EF520216	
		Vid/Torrejón el Rubio/Spain	1/EF520221	
	Guadalquivir	Arenoso/Montoro/Spain		1/EF520222
		Matapuercas/Adamuz/Spain	4/EF520133; EF520138; EF520146; EF520174	2/EF520231; EF520255
		Tablillas/Brazatortas/Spain	8/EF520137; EF520139; EF520140; EF520141; EF520143; EF520164; EF520176; EF520197	3/EF520232; EF520235; EF520236
		Ovejuna/—/Spain	3/AY568608*; EF520204; EF520205	
		Belmez/Albardado/Spain	6/AY568607*; EF520208; EF520209; EF520210; EF520211; EF520212	
		—/—/Spain	1/EF520167	
		Montemayor/Cañaveral de León/Spain	1/EF520220	
		Molinos/Llerena/Spain	1/EF520207	

I. oretanum	Guadalquivir	Robledillo/Solana del Pino/Spain	5/DQ447737*; EF520150; EF520151; EF520152; AF045989	1/DQ447722*
		—/—/Spain	1/EF520132	1/EF520243
I. lusitanicum	Sado	Sado/Canal Caveira/Portugal	14/DQ845549*–DQ845562*	9/DQ845592*–DQ845600*
	West	Trancão/S. Julião do Tojal/Portugal	3/DQ845523*; DQ845532*; DQ845542*	2/DQ845579*; DQ845580*
		Maior/—/Portugal	5/DQ898232*–DQ898236*	5/DQ898237*–DQ898241*
		Jamor/Oeiras/Portugal	4/DQ898223*–DQ898226*	3/DQ898246*–DQ898248*
		Lage/—/Portugal	5/DQ898227*–DQ898231*	4/DQ898242*–DQ898245*
		Ossos/Rinchoa/Portugal	3/DQ845524*; DQ845544*	2/DQ845588*; DQ845590*;
		Colares/Colares/Portugal	7/DQ845514*; DQ845525*; DQ845527*; DQ845530*; DQ845535*; DQ845537*; DQ845538*; DQ845546*	4/DQ845581*–DQ845583*; DQ845589*
		Samarra/Praia da Samarra/Portugal	9/DQ845522*; DQ845526*; DQ845528*; DQ845529*; DQ845531*; DQ845536*; DQ845539*; DQ845541*; DQ845543*	3/DQ845578*; DQ845584*; DQ845591*
		Lizandro/Cheleiros/Portugal	4/DQ845533*; DQ845534*; DQ845540*; DQ845545*	3/DQ845585*–DQ845587*
		Lagoa de Albufeira/Herdade da Apostiça/Portugal	12/DQ845511*–DQ845513*; DQ845515*–DQ845521*; DQ845547*–DQ845548*	7/DQ845564*–DQ845570*
I. almacai		Mira/Santa Luzia/Portugal	18/DQ845489*–DQ845494*; DQ845496*–DQ845498*; DQ845500*; DQ845502*–DQ845504*; DQ845506*–DQ845510*	5/DQ845571*–DQ845573*; DQ845576*–DQ845577*
		Arade	5/DQ845488*; DQ845495*; DQ845499*; DQ845501*; DQ845505*	2/DQ845574*; DQ845575*

Accession Numbers marked with * were retrieved from GenBank.

The analysis of the Single Nucleotide Polymorphisms (SNPs) of the beta-actin was made by mapping, in the aligned sequences, all mutations present (fixed or in heterozygozity) in all haplotypes. The synapomorphic states were inferred by comparing the sequences with those of two outgroups: *A. oligolepis* (GenBank Accession No.: DQ447713; representing the sister clade of *Iberochondrostoma*, Robalo et al., 2007a) and *Protochondrostoma genei* (GenBank Accession No.: DQ061938, representing the most ancestral genus of the ones formerly comprised in *Chondrostoma*, Robalo et al., 2007a,b).

3. Results

For the cyt *b* gene, 167 sites were variable and 10 were parsimony informative. Seventy haplotypes were found in the 197 samples studied. MP analysis resulted in 100 trees retained, with a consensus tree of 249 steps (Consistency index = 0.66; Homoplasy index = 0.34; Retention index = 0.92; CI excluding uninformative characters = 0.55; HI excluding uninformative characters = 0.45; Rescaled consistency index = 0.61) (Fig. 2). The MP and NJ trees for cyt *b* had the same basic topology. The model selected by Modeltest corresponded to GTR + G + I with the following assumed nucleotide frequencies: A = 0.2655; C = 0.2673; G = 0.1609; T = 0.3063. The assumed proportion of invariable sites was 0.5154 and the distribution of rates at variable sites was equal to the gamma (continuous) distribution with shape parameter (alpha) = 1.5260.

For the beta-actin gene, 16 sites were variable and five were parsimony informative. Twenty-one haplotypes were found in the 86 samples studied. MP analysis resulted in 100 trees retained, with a consensus tree of 23 steps (Consistency index = 0.70; Homoplasy index = 0.30; Retention index = 0.46; CI excluding uninformative characters = 0.42; HI excluding uninformative characters = 0.58; Rescaled consistency index = 0.32) (tree not shown).

Only the cyt *b* tree resulted in a clear dichotomous topology at least at species level (Fig. 2). In this tree, the monophyly of *Iberochondrostoma* was confirmed with *I. almacai* in a basal position. Its sister clade splits in two well supported monophyletic groups, one comprising *I. lusitanicum* from Sado and its geographical neighbour *I. lusitanicum* from the west and Lagoa de Albufeira, while the other comprises *I. lemmingii* and *I. oretanum*. In the populations of *I. lemmingii* we find, however, some geographical differentiation. All haplotypes (except one) from the Rivers Belmez and Ovejuna (Spain, Guadalquivir basin) are included in a well supported separated clade, suggesting the presence of different haplotypes in the same basin. The remaining haplotypes of *I. lemmingii* (including those from other Guadalquivir drainages) do not show any relevant geographical association.

For the cyt *b*, dataset AMOVA was performed considering a single group with seven populations: *I. lemmingii* (Tagus), *I. lemmingii* (Guadiana), *I. lemmingii* (Guadalquivir), *I. lusitanicum* (west), *I. lusitanicum* (Lagoa de

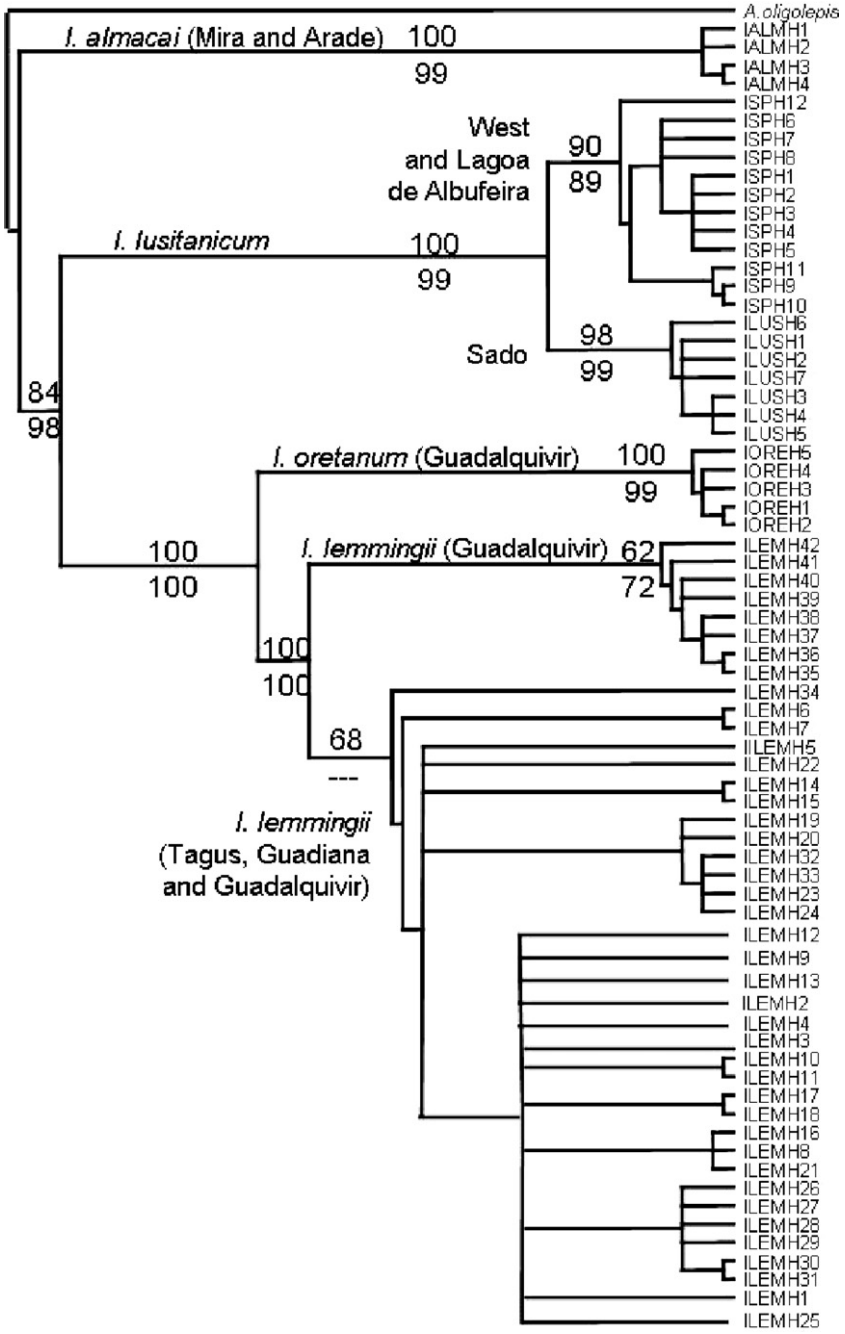

Fig. 2. Tree of cyt *b* based on MP analysis. Each haplotype was included only once in the tree, regardless of the number of fish that presented it. Numbers above branches represent bootstrap values for MP (1000 replicates) and numbers below branches represent bootstrap values for NJ (100 replicates). Haplotypes are the same considered in TCS (for haplotype labels see the legend of Fig. 3).

Albufeira), *I. lusitanicum* (Sado), and *I. almacai*. Populations of *I. lemmingii* from the Quarteira drainage and from the *I. oretanum* species were not included in this analysis due to their low number of samples. The results showed that among population variation explained 91.87% of the variance and was highly significant ($p < 0.001$; FST = 0.92). AMOVA was also performed considering three groups, corresponding to the species *I. lemmingii*, *I. lusitanicum*, and *I. almacai*. The results showed that among group variation explained 85.54% of the variance, among populations within groups 8.45% and within populations 6.02% ($p < 0.001$; FSC = 0.58; FST = 0.94; FCT = 0.86).

All comparisons involving pairs of populations were significant at the same *p* level (Table 2).

Concerning the molecular diversity indices the highest ones are generally found in the southeast in the populations of *I. lemmingii* from Guadiana and Guadalquivir and in *I. oretanum*. The smallest ones are found in the populations of *I. lusitanicum* from west and in *I. almacai* (Table 3).

Table 2
FSTs for pairs of populations

	I. lemmingii Tagus basin	*I. lemmingii* Guadiana basin	*I. lemmingii* Guadalquivir basin	*I. lusitanicum* west	*I. lusitanicum* Lagoa de Albufeira	*I. lusitanicum* Sado basin	*I. almacai*
I. lemmingii Tagus basin	0.00000						
I. lemmingii Guadiana basin	0.14917	0.00000					
I. lemmingii Guadalquivir basin	0.22064	0.18993	0.00000				
I. lusitanicum west	0.94839	0.94796	0.91399	0.00000			
I lusitanicum Lagoa de Albufeira	0.93104	0.93583	0.87120	0.38752	0.00000		
I. lusitanicum Sado basin	0.93844	0.94118	0.88308	0.91670	0.88812	0.00000	
I. almacai	0.96075	0.95998	0.92357	0.97703	0.97537	0.98077	0.00000

The values of Tajima *D* and Fu *Fs* only suggest population growth in the populations of *I. lemmingii* from Guadiana and *I. lusitanicum* from Sado (Table 3).

The results of TCS applied to the cyt *b* haplotypes (using a 95% confidence interval, Fig. 3) show that each species has its own network. In *I. lemmingii* the main haplotype, inferred as the ancestral one, is well represented in all basins where the species occurs. The Guadalquivir seems to represent an ancient centre of diversification, with haplotypes similar to the ancestral one but also others very different in the populations of Belmez and Ovejuna. The vast majority of haplotypes from Guadiana are very close to the presumed ancestral one. *I. lemmingii* haplotypes from Tagus are also in general close to the ancestral, differing in a maximum of six mutations.

The analysis of the beta-actin SNPs (Table 4) showed that heterozygosity is only present in haplotypes of the *I. lemmingii* populations (i.e. all mutations present are fixed in *I. lusitanicum*, *I. almacai*, and *I. oretanum*). Of the 21 mutation sites mapped, seven corresponded to fixed mutations, two to insertions and one to a deletion. Twelve sites were polymorphic and showed some degree of heterozygosity. From these, seven were present in haplotypes representing only one specimen with no geographical meaning and five represented ancestral polymorphisms (i.e. are present in individuals of the different populations of *I. lemmingii*). Concerning the meaning of the fixed mutations, we can find a derived mutation that gathers all *Iberochondrostoma*, another that groups *I. almacai* and haplotypes from the River Jabalón in the upper Guadiana basin, and still another characteristic of *I. lusitanicum*. As referred by Robalo et al. (2007b), the haplotypes of *I. lusitanicum* from west and Lagoa de Albufeira and *I. lusitanicum* from Sado differ in two fixed mutations, each being synapomorphic of each of the clades. *I. oretanum* also has a derived and fixed characteristic mutation, but the same mutation appears in a few *I. lemmingii* from the same basin (Guadalquivir), although only in heterozygosity, a situation that may have resulted from a possible hybridization event or may indicate that the mutation occurred before the separation between *I. lemmingii* and *I. oretanum*. Although a fixed mutation was found that groups *I. lusitanicum*, *I. almacai*, and the haplotypes from the River Jabalón in the Guadiana basin it is important to point out that the fixed allele shared by these fish corresponded to the primitive state. For the same locus, all the remaining *I. lemmingii* and *I. oretanum* shared the same derived allele.

4. Discussion

The results obtained with the cyt *b* gene confirm the monophyly of *Iberochondrostoma* as well as the monophyly of each species included in this genus.

Concerning the phylogeography of *I. lemmingii* the first conclusion is that, based on the present results, there is poor geographical differentiation (with many haplotypes shared among drainages) between Tagus, Guadiana, and Guadalquivir, with the exception of the fishes from the Rivers Belmez and Ovejuna from the Guadalquivir basin (Figs. 2 and 3). Their position in relation to the remaining *I. lemmingii* will need further evaluation because they were not present in the beta-actin dataset due to lack of samples. In *I. lemmingii*, the Guadalquivir tributaries Belmez and Ovejuna are the only ones to present distinctive haplotypes. All other basins and rivers share several haplotypes and present low corrected pairwise differences among populations, providing at the same time evidence for ancestral polymorphisms and indicating that the events that severed the last connections among these three basins (Tagus, Guadiana, and Guadalquivir) must have been very recent. Assuming a molecular clock of 1% between lineages per MY (e.g. Dowling et al., 2002; Doadrio and Carmona, 2004; Robalo et al., 2007a) the populations of *I. lemmingii* of Tagus and Guadiana were connected until about 80,000 years ago.

The basin of the Guadalquivir seems to be a major diversification centre at least at some of its drainages. Apart from the already mentioned populations of the Rivers Belmez and Ovejuna, *I. oretanum* also had its origin in the Guadalquivir basin.

Taken together, the results derived from cyt *b* gene show a very broadly distributed species, *I. lemmingii*, present in the upper part of the Tagus, Guadiana, and Guadalquivir, from which a small albeit genetically diverse *I. oretanum*, splited about 2 MYA.

The populations of *I. lemmingii* are clearly more diverse genetically than those of the remaining species of the genus, except perhaps *I. oretanum*. The number of samples of

Table 3
Number of samples sequenced for cyt *b* gene, number of haplotypes found in each population, molecular diversity indices, Tagima's D and Fu's *Fs* values and their significance levels

	N	Number of haplotypes	Number of polymorphic sites	Gene diversity	Mean number of pairwise differences	Nucleotide diversity	Tajima's *D*	Tajima's *D p*	Fu *Fs*	Fu *Fs p*
I. lemmingii Tagus basin	29	11	17	0.7167 ± 0.0839	3.192118 ± 1.698584	0.004403 ± 0.002608	−0.90454	0.19400	−2.12922	0.16300
I. lemmingii Guadiana basin	46	21	38	0.8647 ± 0.0438	2.825121 ± 1.517649	0.003897 ± 0.002324	−2.31028	0.00000	−12.94177	0.00000
I. lemmingii Quarteira	3	1	0	0.0000 ± 0.0000	0.000000 ± 0.000000	0.000000 ± 0.000000	0.00000	1.00000	n.a.	n.a.
I. lemmingii Guadalquivir basin	24	14	31	0.8841 ± 0.0564	7.148551 ± 3.473630	0.009860 ± 0.005341	−0.52481	0.32900	−1.97370	0.21800
I. oretanum	6	5	7	0.9333 ± 0.1217	2.800000 ± 1.714643	0.003862 ± 0.002731	−0.50439	0.36000	−1.41795	0.09500
I. lusitanicum west	40	8	12	0.4013 ± 0.0979	1.305128 ± 0.832867	0.001795 ± 0.001273	−0.99364	0.16000	−2.31000	0.09000
I. lusitanicum Lagoa de Albufeira	12	4	7	0.6364 ± 0.1277	2.136364 ± 1.275780	0.002947 ± 0.001981	0.40482	0.43600	1.08500	0.72400
I. lusitanicum Sado basin	14	7	7	0.8242 ± 0.0781	1.395604 ± 0.909935	0.001925 ± 0.001409	0.09026	0.08800	−3.26000	0.00500
I. almacai	23	4	3	0.4862 ± 0.1053	0.577075 ± 0.483719	0.000796 ± 0.000744	−0.74000	0.21200	−1.11000	0.12000

I. oretanum is however too low to draw firm conclusions on this issue. The more peripheral *I. almacai* and *I. lusitanicum* from west and Lagoa de Albufeira show the lowest diversity indices.

I. lemmingii retained several ancestral polymorphisms with several haplotypes shared among major basins.

I. almacai and *I. lusitanicum* correspond to much older splits in *Iberochondrostoma*. The timing of separation of *I. almacai* from the remaining fish is about 6/7 MYA and the split of *I. lusitanicum* from *I. lemmingii* took place about 4, 5/5, 5 MYA. These old phylogroups are all peripheral in geographical location (occurring in the southwest and west of Iberia, respectively), all occupy areas that are much smaller than that of *I. lemmingii* and present low levels of genetic diversity when compared with those of *I. lemmingii*. This overall pattern is consistent with a scenario of peripatric speciation (Mayr, 1982) with a broadly distributed species located well inside the Iberian Peninsula, originating small peripheral species at different times. Indeed, all three predictions presented in the Section 1 were matched by the results presented above.

This picture is also supported by the analysis of the SNPs in the beta-actin gene. As stated in the Section 3, there is not a single synapomorphic allele specific to *I. lemmingii*. On the contrary, all the small phylogroups or species (*I. lusitanicum*, *I. almacai*, and *I. oretanum*) have fixed derived alleles, some of which occur in heterozygosity in *I. lemmingii.* This pattern is the one we would expect if we had a very large population or set of populations with frequent connections in which lineage sorting would proceed slowly and a number of "daughter" peripheral populations in which lineage sorting and allele substitution would proceed much faster (Kimura, 1983). We suggest that the SNP that reflects the oldest split is the one that unites *I. almacai* and *I. lusitanicum*. Indeed, it is fixed in all these species and is found in *I. lemmingii*, although with very low frequency, in the River Jábalon (upper Guadiana). An ancestral polymorphism in the ancient populations that gave rise to what is now *I. lemmingii* would have become fixed in the colonists that dispersed to the west and southwest of the Iberian Peninsula. This interpretation is consistent with what we know on the geology of Iberia. The present-day river system is very recent, being of Plio-Pleistocene age and took its modern shape and exorheic condition after the peninsula tilted to the west, causing many of its water sheds to drain into the Atlantic. As stated above, in the Miocene, most freshwaters of Iberia drained to several inland lakes. Rivers like Douro, Tagus, and Guadiana did not exist in their present configuration and each of them comprised several drainage systems feeding the inland several lakes (e.g. Andeweg, 2002). For instance, the upper Tagus was connected to the upper Guadiana up to the Pleistocene (Moya-Palomares, 2002) but was separated from the lower Tagus in several occasions during the Mio–Pliocene (Cunha et al., 1993). The lower Tagus, in turn, had a southward extension that approached the south of Portugal in the area that presently corresponds

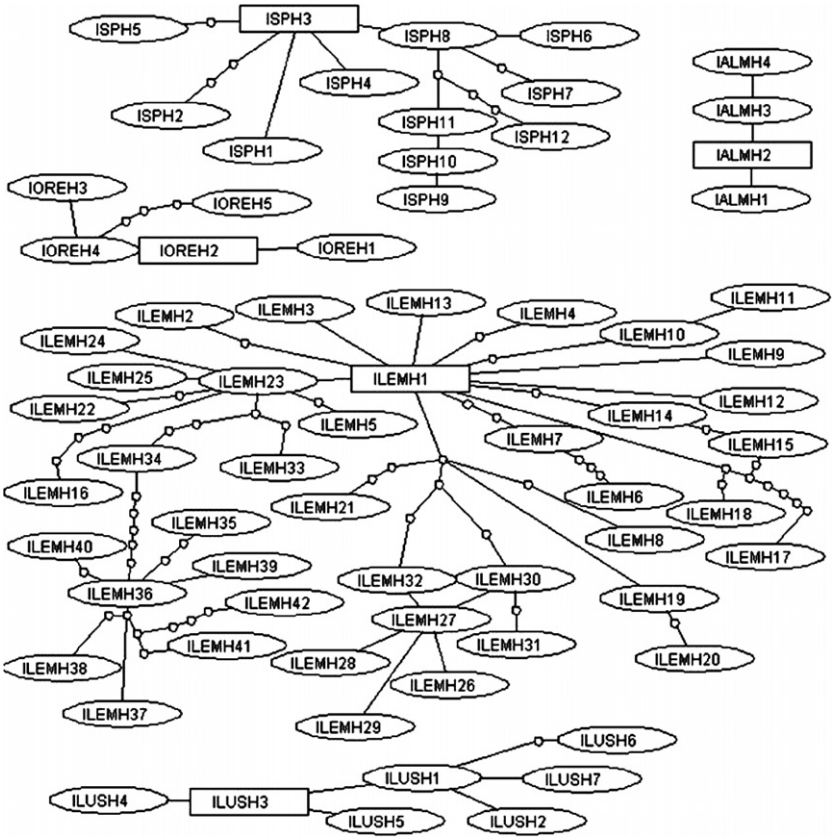

Fig. 3. TCS network. Labels: ILEM = *I. lemmingii*; ILUS = *I. lusitanicum* (Sado); ISP = *I. lusitanicum* (West and Lagoa de Albufeira); IORE = *I. oretanum*; IALM = *I. almacai.* ILEMH1 = 42 fish, Tagus, Guadiana, Quarteira, Guadalquivir; ILEMH2 = 1 fish, Guadiana; ILEMH3 = 2 fish, Guadiana; ILEMH4 = 1 fish, Guadiana; ILEM5 = 2 fish, Guadiana; ILEMH6 = 1 fish, Guadiana; ILEMH7 = 1 fish, Guadiana; ILEMH8 = 1 fish, Tagus; ILEMH9 = 1 fish, Guadiana; ILEMH10 = 2 fish, Guadiana; ILEMH11 = 2 fish, Guadalquivir; ILEMH12 = 2 fish, Guadiana; ILEMH13 = 1 fish, Guadiana; ILEMH14 = 1 fish, Guadiana; ILEMH15 = 1 fish, Tagus; ILEMH16 = 1 fish, Guadiana; ILEMH17 = 1 fish, Guadiana; ILEMH18 = 1 fish, Guadiana; ILEMH19 = 2 fish, Guadiana; ILEMH20 = 1 fish, Guadalquivir; ILEMH21 = 1 fish, Guadalquivir; ILEMH22 = 1 fish, Guadiana; ILEMH23 = 9 fish, Guadiana, Guadalquivir; ILEMH24 = 1 fish, Guadiana; ILEMH25 = 1 fish, Guadiana; ILEMH26 = 2 fish, Guadiana, Tagus; ILEMH27 = 1 fish, Tagus; ILEMH28 = 1 fish, Tagus; ILEMH29 = 1 fish, Tagus; ILEMH30 = 5 fish, Tagus; ILEMH31 = 1 fish, Tagus; ILEMH32 = 1 fish, Guadiana; ILEMH33 = 1 fish, Tagus; ILEMH34 = 1 fish, Guadalquivir; ILEMH35 = 1 fish, Guadalquivir; ILEMH36 = 1 fish, Guadalquivir; ILEMH37 = 1 fish, Guadalquivir; ILEMH38 = 1 fish, Guadalquivir; ILEMH39 = 1 fish, Guadalquivir; ILEMH40 = 1 fish, Guadalquivir; ILEMH41 = 1 fish, Guadalquivir; ILEMH42 = 1 fish, Guadalquivir; IOREH1 = 2 fish, Guadalquivir; IOREH2 = 1 fish, Guadalquivir; IOREH3 = 1 fish, Guadalquivir; IOREH4 = 1 fish, Guadalquivir; IOREH5 = 1 fish, Guadalquivir; ISPH1 = 1 fish, Tagus and adjacent drainages; ISPH2 = 1 fish, Tagus and adjacent drainages; ISPH3 = 31 fish, Tagus and adjacent drainages, Lagoa de Albufeira; ISPH4 = 1 fish, Tagus and adjacent drainages; ISPH5 = 1 fish, Tagus and adjacent drainages; ISPH6 = 1 fish, Lagoa de Albufeira; ISPH7 = 1 fish, Lagoa de Albufeira; ISPH8 = 7 fish, Lagoa de Albufeira; ISPH9 = 2 fish, Tagus; ISPH10 = 2 fish, Tagus; ISPH11 = 1 fish, Tagus; ISPH12 = 3 fish, Lagoa de Albufeira; ILUSH1 = 4 fish, Sado; ILUSH2 = 1 fish, Sado; ILUSH3 = 5 fish, Sado; ILUSH4 = 1 fish, Sado; ILUSH5 = 1 fish, Sado; ILUSH6 = 1 fish, Sado; ILUSH7 = 1 fish, Sado; IALMH1 = 1 fish, Arade; IALMH2 = 16 fish, Arade, Mira; IALMH3 = 5 fish, Mira; IALMH4 = 1 fish, Mira.

to the Sado basin (Teresa Azevedo, personal communication). We suggest that in the upper Miocene (judging from the cyt *b* data) fish from the upper Tagus, upper Guadiana, and belonging to the stock that gave rise to *I. lemmingii* manage to reach the lower Tagus. From the lower Tagus/Sado, this ancestral stock would have spread to the southwest of Portugal where it originated *I. almacai*. It is interesting to note that fossils of *Squalius*, another cyprinid genus, are known from the Miocene of the lower Tagus (Póvoa de Santarém, Gaudant, 1977) and in *Squalius*, there are also species with very restricted ranges in the southwest of Portugal (*S. torgalensis* and *S. aradensis*; Coelho et al., 1998) implying a connection of the lower Tagus/Sado with the bulk of the Peninsula and a corridor that allowed the dispersal of fish to southwest Portugal. The separation of the *Squalius* species of southwest Portugal points to a similar timing as the separation of *I. almacai* (around 6 MYA, Sanjur et al., 2003; Doadrio and Carmona, 2003b). The differentiation of the Tagus and Sado during the Pliocene promoted the speciation of *I. lusitanicum* from West/Lagoa de Albufeira and *I. lusitanicum*, respectively. There is ample geological evidence that the lower Tagus and Sado were connected at some periods and both rivers suffered very pronounced marine transgressions (Pimentel, 1997; Andeweg, 2002) that would have pushed their populations of *Iberochondrostoma* to the respective headwaters, accelerating lineage sorting and genetic differentiation. In the Pliocene, the connection between upper and lower Tagus was

Table 4
SNPs found and analysed in the beta-actin gene

Haplotypes\bp	73	101	108	114	122	138	204	296	385	424	447	455	482	518	520	528	604	608	611	677	720–721
IlembactH1	A		A	C	T	C	G	A	C	G	T	C		T	T	C	C	A	C	G	CT
IorebactH1	A		A	C	T	C	G	A	C	G	T	C		A	T	C	C	A	C	G	CT
IlembactH2	A		A	C	T	C	G	A	C	G	Y	C		T	T	C	C	A	C	G	CT
IlembactH3	A		A	C	T	C	G	A	C	G	Y	C		W	T	C	C	A	C	G	CT
IlembactH4	A		A	C	T	C	R	R	C	G	C	C		T	T	Y	C	A	Y	G	CT
IlembactH5	A		A	C	T	C	G	A	C	G	C	C		T	K	C	C	A	C	G	CT
IlembactH6	A		A	C	T	C	G	A	C	G	C	C		T	T	C	C	A	C	G	CT
IspbactH1	G		A	C	G	C	G	A	C	G	C	C		T	T	C	A	A	C	G	CT
IlusbactH1	G		A	C	G	C	G	A	C	G	C	C		T	T	C	C	A	C	G	
IlembactH7	A	T	A	C	G	C	G	A	C	G	C	C		T	T	C	C	A	C	G	CT
IlembactH8	A	T	A	S	G	C	G	A	C	G	C	C		T	T	C	C	A	C	G	CT
IlembactH9	A	T	A	C	G	C	G	A	C	A	C	C		T	T	C	C	A	C	G	CT
IalmbactH1	A	T	A	C	G	C	G	A	C	G	T	T	T	T	T	C	C	G	C	G	CT
IlembactH10	A		T	C	T	G	G	A	C	G	C	C		T	T	C	C	A	C	K	CT
IlembactH11	A		T	C	T	G	G	A	C	G	C	C		T	T	C	C	A	C	T	CT
IlembactH12	A		T	C	T	G	G	A	C	G	C	C		T	T	C	C	A	C	G	CT
IlembactH13	A		W	C	T	S	G	A	C	G	Y	C		W	T	C	C	A	C	G	CT
IlembactH14	A		W	C	T	S	G	A	Y	G	Y	C		K	T	C	C	A	C	G	CT
IlembactH15	A		W	C	T	S	G	A	C	G	Y	C		T	T	C	C	A	C	G	CT
IlembactH16	A		W	C	T	S	G	A	C	G	C	C		T	T	C	C	A	C	G	CT
IlembactH17	A		W	C	T	S	G	A	C	G	C	C		T	T	C	C	A	C	K	CT
A. oligolepis	A		A	T	G	C	G	A	C	G	C	C		T	T	C	C	A	C	G	CT
P. genei	A		A	T	G	C	G	A	C	G	C	C		T	T	C	C	A	C	G	CT

Ilembact = haplotypes for *I. lemmingii*; Iorebact = haplotypes for *I. oretanum*; Ispbact = haplotypes for *I. lusitanicum* (west and Lagoa de Albufeira); Ilusbact = haplotypes for *I. lusitanicum* (Sado); Ialmbact = haplotypes for *I. almacai*; IlembactH1 = 3 individuals; Tagus, Guadalquivir; IorebactH1 = 1 fish, Guadalquivir; IlembactH2 = 2 fish, Tagus, Guadalquivir; IlembactH3 = 1 fish, Guadalquivir; IlembactH4 = 1 fish, Tagus; IlembactH5 = 1 fish, Tagus; IlembactH6 = 3 fish, Tagus, Guadiana; Ispbact H1 = 40 fish, Tagus and adjacent basins, Lagoa de Albufeira; IlusbactH1 = 9 fish, Sado; IlembactH7 = 1 fish, Guadiana; IlembactH8 = 1 fish, Guadiana; IlembactH9 = 1 fish, Guadiana; IalmbactH1 = 7 fish, Mira, Arade; IlembactH10 = 1 fish, Tagus; IlembactH11 = 1 fish, Guadalquivir; IlembactH12 = 10 fish, Quarteira, Guadiana, Guadalquivir; IlembactH13 = 1 fish, Guadalquivir; IlembactH14 = 1 fish, Guadiana; IlembactH15 = 1 fish, Tagus; IlembactH16 = 8 fish, Tagus, Guadiana; IlembactH17 = 1 fish, Tagus.

severed (Cunha et al., 1993; Andeweg, 2002) avoiding that new waves of *I. lemmingii* reinvaded the lower reaches of the Tagus. Even nowadays, with the Tagus being a continuous river, *I. lemmingii* is restricted to the Spanish part of the Tagus and a few tributaries near the Portuguese boarder, while *I. lusitanicum* from west and Lagoa de Albufeira is restricted to the tributaries closest to the estuary.

Overall, the paleobiogeographical information available is compatible with the scenario of a large species giving rise to two smaller ones at different periods and further fragmentation of one of these smaller entities.

Two notes of caution are needed:

(1) This sequence of events assumes that the few SNPs involved did not suffer recurrent mutations that would cause homoplasy. The low number of polymorphic sites is in our view, consistent with this possibility.
(2) The low genetic diversity of the peripheral species may have also resulted from bottlenecks that occurred after their differentiation, namely strong marine transgressions in the western rivers, causing drastic reductions in population size.

The use of other nuclear markers will help to test the validity of the model presented in this paper.

In conclusion, we note that what seemed to be a well resolved dichotomous tree inferred from cyt *b* did not correspond to the picture obtained with a slowly evolving nuclear gene. The slow rate of evolution of this fragment and its fourfold higher effective population size (Zhang and Hewitt, 2003) apparently allowed the preservation of old signatures, of past events that the rapid lineage sorting, that probably took place in the cyt *b* gene, did not preserve.

We suggest that phylogeographic reconstruction, in particular using SNPs applied to slowly evolving nuclear genes, in combination with phylogenetic analysis, may help us to get a better understanding of the evolutionary history of clades beyond the species level, an essential step to study speciation.

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