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Diversification within glacial refugia: tempo and mode of evolution of the polytypic fish *Barbus sclateri*

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Abstract

A diversity of evolutionary processes can be responsible for generating and maintaining biodiversity. Molecular markers were used to investigate the influence of Plio-Pleistocene climatic oscillations on the evolutionary history of taxa restricted to the freshwaters of a classical glacial refugium. Population genetic, phylogenetic and phylogeographical methods allowed the inference of temporal dynamics of cladogenesis and processes shaping present-day genetic constitution of *Barbus sclateri*, a polytypic taxon found in several independent river drainages in southern Iberian Peninsula. Results from different analyses consistently indicate several range expansions, high levels of allopatric fragmentation, and admixture following secondary contacts throughout its evolutionary history. Using a Bayesian demographical coalescent model on mitochondrial DNA sequences calibrated with fossil evidence, all cladogenetic events within *B. sclateri* are inferred to have occurred during the Pleistocene and were probably driven by environmental factors. Our results suggest that glaciation cycles did not inhibit cladogenesis and probably interacted with regional geomorphology to promote diversification. We conclude that this polytypic taxon is a species complex that recently diversified in allopatry, and that Pleistocene glaciation–deglaciation cycles probably contributed to the generation of biological diversity in a classical glacial refugium with high endemicity.

Keywords: Iberia, Pleistocene glaciation, refugia, river capture, speciation, vicariance

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Introduction

Speciation is a complex process, with a diversity of factors potentially involved. Given the significance of speciation to evolution, identification of the various factors involved in generating biological diversity is fundamental to gaining insight into evolution. Global climate change is thought to be among the major factors that contributed to species diversification, with latitudinal and altitudinal range shifts associated with climatic oscillations hypothesized to fuel evolutionary change (Hewitt 1996, 2000, 2004). The last series of glacial fluctuations began affecting world's climate in the Late Pliocene, *c.* 2.75 Ma (Ravelo *et al.* 2004), and became more severe during the Pleistocene (Bennett 1990). During successive glacial periods, it is thought that taxa became fragmented and isolated in refugia, which in turn promoted vicariance and increased speciation rates of the surviving isolates. While the importance of Pleistocene climatic changes in driving genetic divergence and speciation has been implicated in the diversification of many taxa (e.g. Knowles 2000; Clarke *et al.* 2001; Barraclough & Vogler 2002; Johnson & Cicero 2004; Near & Benard 2004; Ribera & Vogler 2004), controversial evidence for no effect or

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diminishing cladogenesis has been suggested for others (e.g. Klicka & Zink 1997, 1999; Near *et al.* 2003; Kadereit *et al.* 2004). One possible explanation for these disparate results could lie in the interplay between Pleistocene climate and the organisms' life history, emerging or disappearing ecological opportunities, and regional geomorphological history (e.g. see Weir 2006). Therefore, it is reasonable to expect that certain taxa and certain geographical areas are more likely to show increased levels of Pleistocene diversification than others.

Several temperate European taxa survived through glacial cycles in Pleistocene refugia, especially in southern peninsulas (Taberlet *et al.* 1998; Hewitt 1999, 2000). These refugia have been traditionally seen as homogeneous sources of colonizers of northern latitudes after glacial periods, yet recent studies suggest that these regions were more complex and heterogeneous than originally thought (reviewed in Hewitt 2004; Gómez & Lunt 2007). One of these refugia, the Iberian Peninsula, contains several taxa for which 'refugia-within-refugia' were postulated (Gómez & Lunt 2007). Although Iberia was not directly impacted by polar ice sheets, glaciers did form on the highest mountain ranges (Pérez Alberti *et al.* 2004) and local climatic conditions fluctuated dramatically during Plio-Pleistocene cycles (Hernández Fernández *et al.* 2007).

Pleistocene climatic oscillations were shown to be major determinants in structuring Central European ichthyofauna (Bernatchez & Wilson 1998), although up until now such information is lacking for southern peninsulas. It is conceivable that glacial cycles affected the evolution of freshwater-restricted species, both directly and indirectly. For instance, shifts in both baseline sea level and water balance of river catchments associated with glacial cycles are particularly relevant to aquatic organisms, as these can lead to complex changes in fluvial dynamics (Williams *et al.* 1998). Changes in temperature and precipitation regimes can directly affect the quality and availability of habitat for local freshwater populations. Thus, refugial freshwater-restricted species (like many fishes) were most probably affected by climatic changes during glacial periods, as shrinking aquatic habitat was embedded in expanding terrestrial environments. Even though fishes may be able to track suitable habitat along river stretches at different altitudes, one probable outcome would be the persistence of fragmented local populations and extinction of others. Given this pattern, freshwater fishes in these island-like habitats make excellent models for examination of Plio-Pleistocene effects on diversification of freshwater-restricted taxa.

To understand the influence of Plio-Pleistocene climatic oscillations on the cladogenesis and population structuring of refugial taxa restricted to freshwaters, we studied the evolutionary history of the polytypic fish *Barbus sclateri* Günther 1868 for its geographical distribution and biological characteristics. This tetraploid species complex inhabits a broad area along southern Iberia, across two ichthyogeographical regions, including the southwestern region, which is home to endemic *Chondrostoma* (*Iberochondrostoma*) and *Squalius* species with very restricted distributions and ancient origins (Fig. 1; Mesquita *et al.* 2007). Southern Iberia has high elevation mountain ranges where glaciers formed, although it has traditionally been seen as a more amenable climatic region during glacial periods when compared to higher latitudes. Furthermore, southern Iberian mountains are drained by several independent rivers,

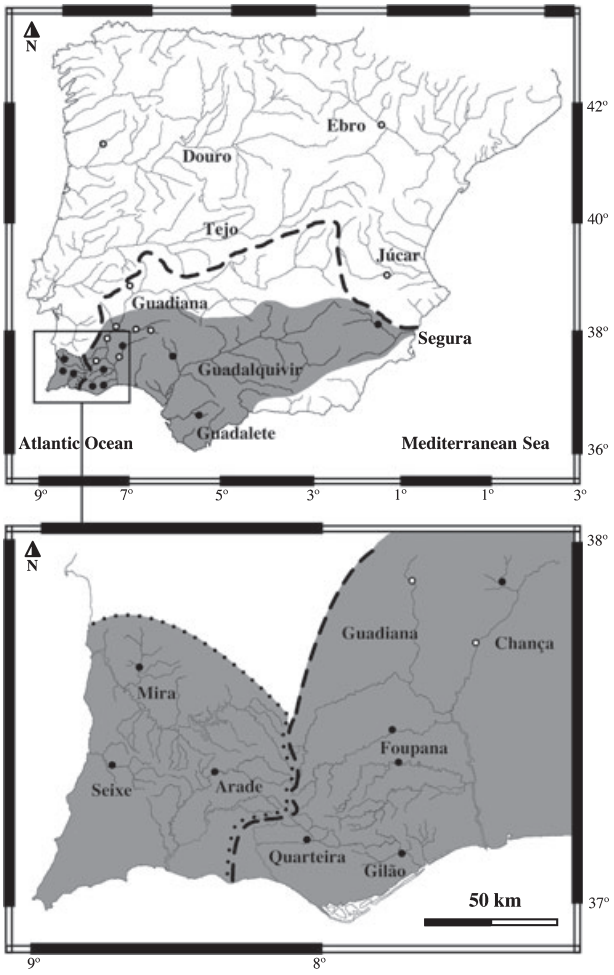

Fig. 1 Map of the Iberian Peninsula showing localities sampled for *Barbus sclateri* complex (●) and for other Iberian *Barbus* species (○; see Table 1). The grey-shaded area represents distribution area of *B. sclateri*. The broken line delimits southeastern (SE) region and dotted line delimits southwestern (SW) region (*sensu* Mesquita *et al.* 2007). Inset shows the limits between ichthyogeographical regions and sampling localities in more detail.

some with headwaters close to the ocean, where baseline sea level fluctuations could have had increased impacts on habitat quality for aquatic species. *Barbus sclateri* species complex shows high levels of osteological differentiation among populations (Doadrio 1990), including those of southwestern drainages (Gante, personal observation), contrasting with reduced levels of molecular differentiation (Machordom *et al.* 1995; Doadrio *et al*. 2002; Mesquita *et al.* 2007).

We used rapidly and slowly evolving molecular markers with different modes of inheritance [mitochondrial DNA (mtDNA) and allozymes] to explore evolutionary processes acting on *B. sclateri* at different timescales, and to assess the temporal dynamics of species distribution, impact of climate on vicariance and on its genetic make-up. Our results from population genetic, phylogenetic and phylogeographical analyses indicate a complex and dynamic evolutionary history of expansion, fragmentation and secondary contacts shaped by Plio-Pleistocene glaciation cycles.

Materials and methods

Specimen collection and sample processing

Specimens of *Barbus sclateri* ($n = 307$) were collected throughout its range. We specifically focused sampling on boundary basins separating different ichthyogeographical provinces (*sensu* Mesquita *et al.* 2007; Fig. 1; Table 1), including samples from three localities from the southwestern (SW) and seven from the southeastern (SE) ichthyogeographical regions. To provide perspective, samples representing other species the Iberian *Barbus* lineage, including *Barbus setivimensis* from Algeria (see Machordom & Doadrio 2001), were used in phylogenetic analyses. All samples were collected by electrofishing and immediately processed or frozen. Specimens were deposited in the ichthyological collections 'Museu Bocage' (MB) of Museu Nacional de História Natural, Portugal, and in Museo Nacional de Ciencias Naturales, Spain.

Characterization of mitochondrial DNA variation

Muscle tissue stored in absolute ethanol was digested using Proteinase K in an STE + SDS solution (0.1 M NaCl, 0.05 M Tris–HCl pH 7.5, 0.001 M EDTA disodium, 1.5% SDS) and proteins were precipitated using an ammonium acetate solution (5 M, pH 8.0). Total DNA was precipitated using cold isopropanol and washed with ethanol before re-suspension in ultra pure water (modified from Sambrook *et al.* 1989).

Mitochondrial DNA variation in *B. sclateri* was first assessed in all 307 specimens by characterization of single-stranded conformational polymorphisms (SSCPs) of a 275-bp fragment of the cytochrome *b* gene (cyt*b*; Table S1). The fragment was generated using internal primers LBB377 and HBB674 and analysed following Gante *et al.* (2008). A subsample of individuals (102 specimens, ~10 specimens/locality; Table S2) was sequenced for variation at a 1320-bp fragment containing the more rapidly evolving NADH dehydrogenase subunit 2 (ND2) (Jacobs *et al.* 1988) and flanking tRNAs (portions of tRNA-Gln and tRNA-Ala and the complete tRNA-Met and tRNA-Trp). This fragment was amplified and sequenced using primers ILE (5′-CCG GAT CAC TTT GAT AGA GT-3′) and ASN (5′-CGC GTT TAG CTG TTA ACT AA-3′) (G. J. P. Naylor, personal communication). PCR amplifications were carried out in 25 μL reactions containing 1× PCR buffer, 0.5 μM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 U *Taq* polymerase and ~50 ng of template DNA. Cycling profile for PCR amplifications was 3 min at 94 °C (one cycle), 30 s at 94 °C, 30 s at 52 °C and 60 s at 72 °C (30 cycles) and 4 min at 72 °C (one cycle). Fragments were sequenced on an ABI 3730 DNA Analyzer. Composite cyt*b* and ND2 + tRNAs haplotypes (1595 bp in length) were designated by letters and numbers respectively. Letter codes for cyt*b* follow Gante *et al.* (2008). One individual from each of six other species that belong to the Iberian *Barbus* lineage was also sequenced for these fragments.

Characterization of allozyme variation

Muscle and liver samples were stored in liquid nitrogen or in an ultracold freezer until analysed. Tissues were ground using an Ultra Turrax blender in Tris–EDTA pH 6.8 (Pasteur *et al.* 1987). Allozymes of all *B. sclateri* populations, plus *Barbus bocagei* and *Barbus comizo* (Table 1), were assayed on 10% w:v horizontal starch gels, using stain recipes modified from Pasteur *et al.* (1987) and Murphy *et al.* (1996). Seven systems yielding 13 presumptive loci were analysed for the purpose of this study: for muscle, using TC 6.7 buffer, adenylate kinase (*Ak*, EC 2.7.4.3), creatine kinase (*Ck-1* and *Ck-2*, EC 2.7.3.2) and malate dehydrogenase (*Mdh-1* and *Mdh-2*, EC 1.1.1.37); for liver, using TC 8.0 buffer, glucose-6-phosphate isomerase (*Gpi-1*, *Gpi-2* and *Gpi-3*, EC 5.3.1.9) and phosphogluconate dehydrogenase (*Pgdh-1* and *Pgdh-2*, EC 1.1.1.44), and using '*Poulik*' buffer, superoxide dismutase (*Sod-2*, EC 1.15.1.1) and phosphoglucomutase (*Pgm-1* and *Pgm-2*, EC 5.4.2.2) were assayed. Scoring accuracy was ensured by re-running representative samples from each population and species on the same gel. When null alleles were suspected, individual genotypes for that locus were coded according to the minimizing method of Berrebi *et al.* (1990). In

Table 1 Sample information of analysed Iberian *Barbus* lineage

Species	Sample sizes	Basin, River	Locality (Country)	Region	Accession no.	
					Cyt*b*	ND2 + tRNAs
Barbus sclateri	31, 31, 13	Segura, Segura	Calasparra (ES)	SE	AM748078, AM748079	AM948081–AM948085
	34, 34, 10	Guadalquivir, Guadiamar	Gerena (ES)	SE	AM748079	AM948086–AM948090
	20, 20, 10	Guadalete, Majaceite	El Bosque (ES)	SE	AM748079, AM748081	AM948091–AM948093
	35, 35, 8	Guadiana, Chança	Diogo Martins (PT)	SE	AM748080	AM948094, AM948096, AM948098, AM948100
	28, 28, 12	Guadiana, Foupana	Pereiro (PT)	SE	AM748080	AM948094, AM948095, AM948097, AM948099
	48, 48, 12	Gilão, Séqua	Picota (PT)	SE	AM748080	AM948094, AM948100
	7, 7, 7	Quarteira, Algibre	Andrezes (PT)	SE	AM748080	AM948100
	31, 31, 10	Arade, Odelouca	São Marcos da Serra (PT)	SW	AM748080	AM948100
	28, 28, 10	Seixe, Seixe	Reguengo (PT)	SW	AM748080	AM948100
	45, 45, 10	Mira, Torgal	Fonte Boa (PT)	SW	AM748080	AM948100
Barbus bocagei	49, 1, 1	Douro, Tâmega	Gatão (PT)	AT	AM748072	AM948076
Barbus comizo	1, 0, 0	Guadiana, Caia	Arronches (PT)	SE	—	—
	5, 0, 0	Guadiana, Guadiana	Moura (PT)	SE	—	—
	1, 0, 0	Guadiana, Guadiana	Serpa (PT)	SE	—	—
	1, 0, 0	Guadiana, Murtéga	Barrancos (PT)	SE	—	—
	7, 0, 0	Guadiana, Ardila	Santo Amador (PT)	SE	—	—
	1, 0, 0	Guadiana, Chança	Corte do Pinto (PT)	SE	—	—
	1, 1, 1	Guadiana, Vascão	Giões (PT)	SE	AM748074	AM948077
Barbus microcephalus	1, 0, 1	Guadiana, Guadiana	Serpa (PT)	SE	—	AM948080
Barbus graellsii	1, 0, 1	Ebro, Mesa	Jaraba (ES)	ME	—	AM948078
Barbus guiraonis	1, 0, 1	Júcar, Cabriel	Venta de Contreras (ES)	ME	—	AM948079
Barbus setivimensis	1, 0, 1	Aissi	Azouz (DZ)	—	—	AM948101

Sample sizes refer to number of specimens screened for allozymes, SSCPs of cyt*b*, and sequencing of ND2 + tRNAs respectively.
Country code: PT, Portugal; ES, Spain; DZ, Algeria. Definition of Iberian ichthyogeographical regions follows Mesquita *et al.* (2007): SE, southeastern; SW, southwestern; AT, atlantic; ME, Mediterranean.
Cytochrome *b* sequences previously published in Gante *et al.* (2008).

populations polymorphic for null alleles, the incomplete genotype option implemented in SPAGeDI 1.2 (Hardy & Vekemans 2002) was used.

Levels of polymorphism and population genetic structure

For each population of *B. sclateri*, Watterson's theta (θ) and number of pairwise differences (π) of mtDNA haplotypes were calculated in Arlequin 3.11 (Excoffier *et al.* 2005), and allozyme heterozygosity (*H*) was calculated in SPAGeDI 1.2 (Hardy & Vekemans 2002). Hierarchical analyses of molecular variance (AMOVAS; Excoffier *et al.* 1992) using maximum-likelihood nucleotide distances for mtDNA data sets, and traditional *F*-statistics for the allozymes data set, tested for population structure and amount of molecular variation attributable to ichthyogeographical regions. This hierarchy yields three components of genetic variation: between SE and SW regions (F_{CT}), among populations within each region (F_{SC}), and overall divergence of populations (F_{ST}). Two mtDNA data sets were used for the AMOVAS: one included the cyt*b* haplotypes for the 307 samples assayed by SSCP and another included the composite cyt*b* + ND2 + tRNAs haplotypes for the 102 specimens sequenced. Calculations were performed as implemented in Arlequin 3.11, using 20 000 permutations. For allozymes, Nei's D_S was used to assess population differentiation calculated with SPAGeDI 1.2 (20 000 permutations of genes, individuals and populations).

Phylogenetic analyses

Nucleotide sequences of unique composite haplotypes were aligned in BIOEDIT v.5.0.6 (Hall 1999) and analysed in PAUP* 4.0b10 (Swofford 2002) using maximum likelihood (ML), maximum parsimony (MP) and neighbour-joining (NJ) methods. *Barbus bocagei* and *B. comizo* were

used as outgroups. ML and MP analyses were completed using 10 replicate heuristic searches, random sequence addition and tree-bisection reconnection branch swapping. The appropriate model of nucleotide sequence evolution was identified using ModelTest 3.7 (Posada & Crandall 1998). Following Posada & Buckley (2004), we used corrected Akaike and Bayesian Information Criteria (AICc and BICc respectively), with branch lengths as parameters at the 0.01 level. Model and estimated parameters were used in ML and NJ analyses (ML distance). Bootstrap replication was used to assess internode robustness (Felsenstein 1985). One-thousand pseudoreplicates were conducted in MP and NJ analyses using 10 sequence addition replicates, and 200 pseudoreplicates in ML analysis using five sequence addition replicates.

A population network was inferred using Nei's D_S standard genetic distances calculated from allozyme frequency data (Nei 1978) and the NJ algorithm in PAUP* 4.0b10. *Barbus bocagei* and *B. comizo* were used to root the resulting network.

Phylogeographical analyses

To infer the population history of *B. sclateri*, an mtDNA haplotype network was constructed using 102 composite cyt*b* + ND2 + tRNAs sequences with the statistical parsimony procedure (Templeton *et al.* 1992) implemented in TCS 1.21 (Clement *et al.* 2000). A nested clade phylogeographical analysis (NCPA) was conducted using 10 000 permutations in GEODIS 2.5 (Posada *et al.* 2000). As geographical coordinates do not adequately measure geographical distances in riverine species, the shortest linear geographical distances along rivers stretches among collection sites, within and between basins, were measured using a map wheel (Alvin No. 1112, Switzerland) on a 1:250 000 map, following Mesquita *et al.* (2005). We chose to measure the shortest distances through headwaters of neighbouring basins for multiple reasons. *Barbus* have limited tolerance to salinity (e.g. Kraiem & Pattee 1988), therefore, they cannot currently move between these basins through their mouths and along the coastline. In addition, there is no evidence for the confluence of distinct basins during low sea level periods from available geological data (e.g. Gutierrez-Mas *et al.* 1996; Dias *et al.* 2000), suggesting that dispersion of *B. sclateri* was unlikely to have occurred along coastal marine waters during those times. Biological inferences for clades showing significant geographical association were drawn using the updated inference key (11Nov05, available from http://darwin.uvigo.es/software/geodis.html; Templeton 2004). Additionally, to test the hypothesis of recent demographical expansion, and refute or support NCPA inferences, conformity of clades to a model of population equilibrium was tested using Fu's F_S (Fu 1997), using 1000 simulated samples in Arlequin 3.11. Fu's F_S is especially sensitive to an excess of rare haplotypes, for which it takes on negative values under scenarios of population expansions and background selection (Fu 1997; Ramos-Onsins & Rozas 2002).

Divergence time estimation

We used a Bayesian approach and fossil evidence to estimate time of divergence within *B. sclateri* for a data set consisting of ND2 + tRNAs sequences (1320 bp) of 102 *B. sclateri* specimens, irrespective of their haplotype. External perspective was provided by inclusion of one individual from all other six species that belong to the Iberian *Barbus* lineage. The best model of sequence evolution for this data set was selected using AICc and BICc, with branch lengths as parameters at the 0.01 level using ModelTest 3.7. Divergence times and their credibility intervals (highest posterior density: HPD) were estimated using a relaxed clock model in BEAST v1.4.6 (Drummond & Rambaut 2007), with branch rates drawn from an uncorrelated lognormal distribution to accommodate possible rate variation among lineages (Drummond *et al.* 2006). The root of the tree was calibrated at 6 Myr, which is the approximate age of the oldest known fossils of Iberian *Barbus*, found in the European Mammal Neogene reference assemblage MN13 (7–4.9 Ma; Doadrio & Casado 1989; Machordom & Doadrio 2001). As fossils provide the minimum age of clades, we calibrated the root of the Iberian assemblage with a lognormal prior (Ho 2007), in which the lower age (6 Ma) corresponds to a hard bound, and the upper age corresponds to a soft bound free to vary. Separate coalescent demographical models of exponential growth were applied to each of *B. sclateri* lineages, with population sizes and growth rates estimated individually. An uninformative tree prior was used for the basal branches connecting all the other species. Analysis was run for 10^7 generations, sampled every 50 000 with a subsequent burn-in of 501 trees using TREEANNOTATOR v1.4.6 (Rambaut & Drummond 2002). Conversion and stability of the estimated parameters were checked using TRACER v1.4 (Rambaut & Drummond 2003). The tree was visualized using FIGTREE v1.1.2 (Rambaut 2006).

Results

Levels of polymorphism and population genetic structure

The 10 populations of *Barbus sclateri* surveyed yield four cyt*b* haplotypes identified by analysis of SSCPs

(n = 307), whereas direct sequencing of the ND2 + tRNAs fragment (n = 102) reveals 20 haplotypes. Combination of these two markers yields 21 composite haplotypes that show strong geographical concordance suggestive of fragmentation (Table S2). Of the 13 allozyme loci analysed, 10 loci exhibit mobility and activity variation among species and populations (Table S3). Of these, *Gpi-1*, *Gpi-2*, *Gpi-3*, *Mdh-2*, *Pgdh-1*, *Pgdh-2* and *Pgm-1* show fixed or substantial allelic frequency differences, or unique alleles in *B. sclateri* relative to the outgroup species. *Gpi-1* and *Gpi-3* loci are particularly informative relative to different *B. sclateri* populations: *Gpi-1* is likely a geographically localized duplication exclusive of *B. sclateri*, with an active electromorph fixed in all SW basins (Mira, Seixe and Arade), whereas *Gpi-3* locus exhibits a mobility variant fixed in all SW populations. These variants also occur in Quarteira and Gilão, at varying frequencies, admixed with alleles typical of SE populations. Gilão shows additional active *Gpi-1* alleles at lower frequencies (Fig. 2; Table S3).

Levels of mitochondrial polymorphism are higher and generally similar among SE populations, except for Segura, which has considerably higher levels of variation (Fig. 2; Table S4). SE populations to the west of Guadalquivir show reduced levels of mtDNA variability (Chança, Gilão and Quarteira). This trend is maintained in all SW populations (Mira, Seixe and Arade), which show no mtDNA variation. Levels of mean allozyme heterozygosity approximately follow this pattern, except that Gilão shows the highest mean heterozygosity of all populations (Fig. 2; Table S4).

AMOVAs reveal substantial levels of genetic fragmentation within *B. sclateri* at both mitochondrial and nuclear loci consistent with the above observations, as shown by F_{ST}-values around 0.7 (Table 2). The most relevant difference between the two types of markers is the amount of differentiation consistent with SE and SW ichthyogeographical regions (F_{CT}). Analysis of mtDNA does not identify substantial structure associated with the ichthyogeographical regions, as indicated by low and nonsignificant or marginally significant F_{CT}-values. Of the total mtDNA variation found, only 8.4% and 18.8% is associated with the ichthyogeographical regions, whereas 49.2% to 70.5% of the variation is found within regions (Table 2). On the other hand, 51.6% of allozymic variation is significantly partitioned across the ichthyogeographical divide, whereas only 18.7% is found within regions (Table 2).

Mitochondrial and nuclear phylogenies

The ingroup exhibits 39 variable and 21 parsimony informative characters for the 21 composite mitochondrial haplotypes identified. Sequence evolution is best characterized by a TrN model (Tamura & Nei 1993) with a proportion of invariable sites. The tree topologies recovered by ML, MP and NJ are highly concordant (Fig. 3), and they support the monophyly of *B. sclateri* and the existence of two divergent lineages (A and B). The deeper nodes are found to the east, whereas shallower nodes are found to the west, suggesting a westward expansion. Lineage A is represented by four haplotypes restricted to the Segura drainage; Lineage B is widely distributed and represented by 17 haplotypes that show further substructuring and strong geographical concordance, indicating allopatric fragmentation.

The population network based on Nei's D_S calculated from allozyme variation is also consistent with

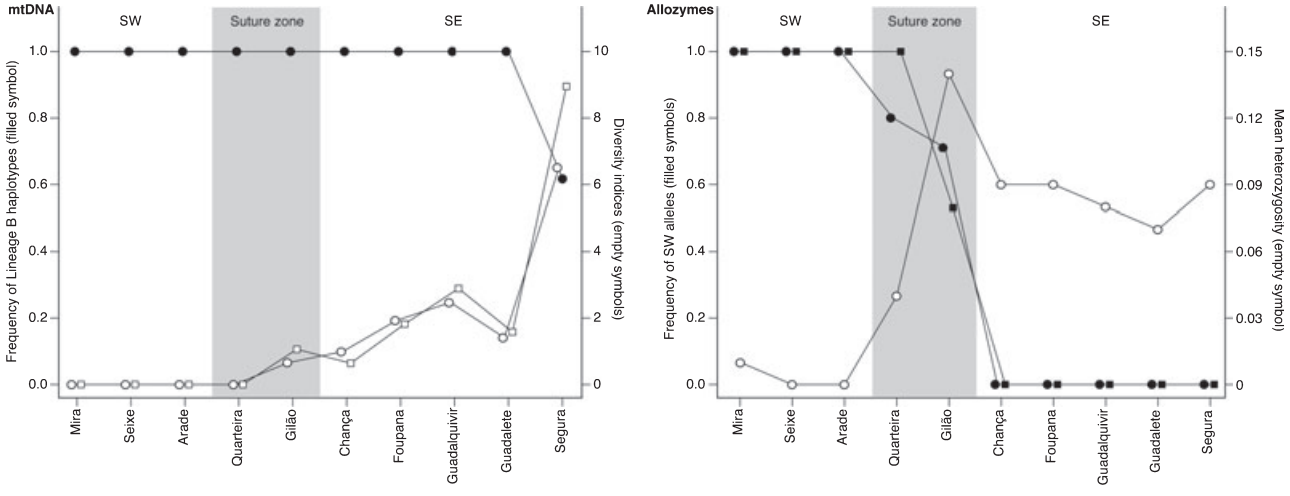

Fig. 2 Geographical distribution of mitochondrial and allozyme variation in *Barbus sclateri* complex. SW, southwestern; SE, southeastern. mtDNA: filled circles = frequency of Lineage B haplotypes, empty circles = θ, and empty squares = π. Allozymes: filled squares = frequency of *Gpi-1* active alleles, filled circles = frequency of *Gpi-3* SW allele, and empty circle = mean heterozygosity. Data in Tables S2–S4.

Table 2 AMOVA table showing *F*-statistics and percentage of total variation explained by each hierarchical level and its significance

Loci	Source of variation	*F*-statistic	Percentage of variation	*P*-value
Mitochondrial (cyt*b* haplotypes)	Between ichthyogeographical regions (F_{CT})	0.0835	8.35	0.2682
	Among populations within regions (F_{SC})	0.7688	70.47	<0.0001
	Total among population variation (F_{ST})	0.7881	78.82	<0.0001
Mitochondrial (composite haplotypes)	Between ichthyogeographical regions (F_{CT})	0.1878	18.78	0.0653
	Among populations within regions (F_{SC})	0.6059	49.22	<0.0001
	Total among population variation (F_{ST})	0.6799	68.00	<0.0001
Allozymes	Between ichthyogeographical regions (F_{CT})	0.5156	51.56	<0.0001
	Among populations within regions (F_{SC})	0.3866	18.73	<0.0001
	Total among population variation (F_{ST})	0.7028	70.29	0.0092

Definition of Iberian ichthyogeographical regions follows Mesquita *et al.* (2007).

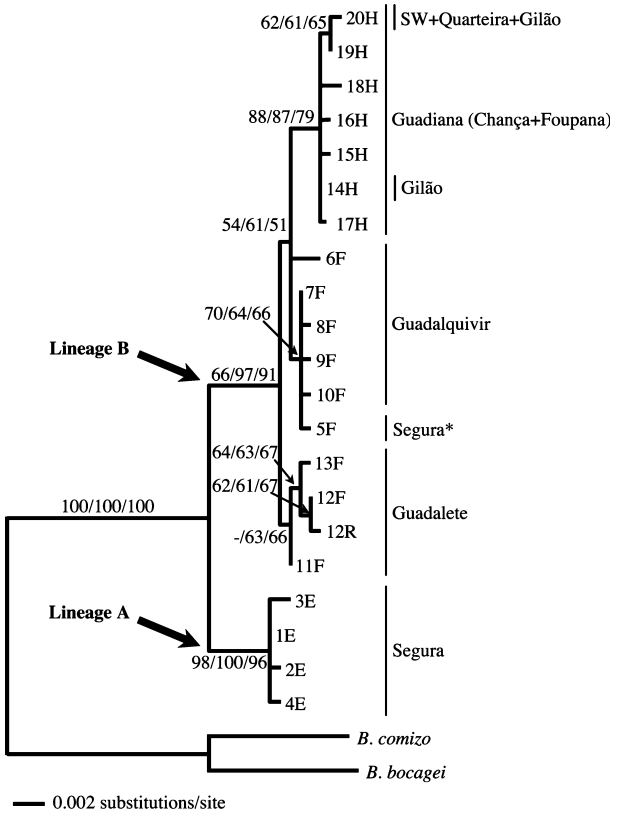

Fig. 3 Maximum-likelihood tree of composite mitochondrial cyt*b* + ND2 + tRNAs haplotypes of *Barbus sclateri* complex. Values above branches represent ML, MP and NJ bootstrap support respectively. Asterisk indicates the haplotype from Segura that was more closely related to haplotypes from the Guadalquivir.

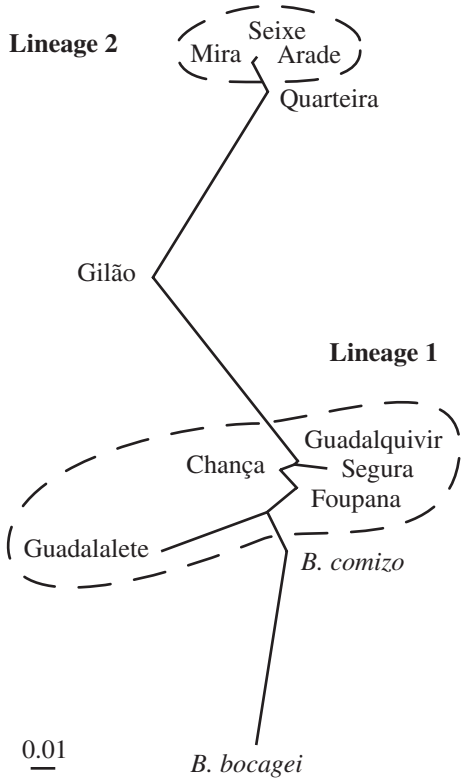

Fig. 4 Neighbour-joining network of Nei's D_S genetic distances among populations of *Barbus sclateri* complex, *Barbus bocagei* and *Barbus comizo* based on allozyme data.

allopatric fragmentation, as it identifies two main groups approximately consistent with the ichthyogeographical divide (Fig. 4): one group of populations from the SE region, which appear to be more similar to *Barbus bocagei* and *Barbus comizo* (Segura, Guadalquivir, Guadalete, Chança and Foupana—Lineage 1), and a second group of populations all from the SW region (Mira, Seixe and Arade—Lineage 2). Remaining populations (Quarteira and Gilão) show varying degrees of intermediacy relative to all others, with admixture of alleles from Lineages 1 and 2 (Figs 2 and 4). Furthermore, the sample from Gilão also shows the mtDNA haplotype typical of SW populations (20H) and the one most commonly found in Foupana and Chança (14H; Table S2).

Phylogeographical analyses

The statistical parsimony network (Fig. 5) suggests the same relationships among the mitochondrial haplotypes as the phylogenetic methods. As above, NCPA provides evidence for fragmentation and expansion acting at different levels and timescales in *B. sclateri*. In addition to two more inclusive clades (3-2 and 3-3) that show precise concordance with geography—evidence for allopatric fragmentation—four other clades exhibit haplotype variation with significant geographical association (Table 3): (i) clade 1-5 indicates fragmentation between haplotype 5F found in Segura and all other clade members found in Guadalquivir; (ii) clade 2-1, which includes populations from Guadiana (Chança and Foupana) and west of it, provides evidence of past range expansion followed by fragmentation; (iii) clade 3-1, which includes clade 2-1, all individuals from Guadalquivir and individuals from Segura with haplotype 5F, shows signs of allopatric fragmentation; and (iv) clade 4-1 (total cladogram) provides evidence for past fragmentation and range expansion. In agreement with NCPA, results from Fu's F_S support evidence of recent population expansion for clades 1-1 and 1-2, which include populations from Guadiana to the west (Table 4).

Table 3 Inference chain for NCPA of *Barbus sclateri* mtDNA

Clade	Chain of inference	Inferred historic event
1-5	1-2-3-4-9-10-No	Fragmentation
2-1	1-2-3-5-15-21-No	Past gradual range expansion followed by fragmentation
3-1	1-2-3-4-9-No	Allopatric fragmentation
4-1	1-2-11-12-13-Yes	Past fragmentation and range expansion

Only clades for which statistically significant patterns were found are shown.

Table 4 Fu's test of population expansion for different *Barbus sclateri* complex mtDNA clades identified by NCPA and their significance

Clade	Fu's F_S	*P*-value
1-1	−1.6022	0.0290
1-2	−2.1353	0.0060
1-5	−1.4465	0.0950
1-7	0.2007	0.3700
1-9	−0.6579	0.1560
2-1	−1.4456	0.2170
2-2	0.0621	0.5700
2-3	0.7232	0.6010
2-5	−1.1953	0.0930
3-1	−1.2196	0.3620
3-2	0.0779	0.4820
4-1	−2.2713	0.2660

Timing of divergence events

For ND2 + tRNAs data set of the Iberian *Barbus* assemblage, TrN with Γ distribution shape parameter is the best model of sequence evolution. Based on our fossil calibration, the mean rate of substitution is 0.0082 substitutions/site/lineage/MY (95% HPD: 0.0054–0.0120), with a mean coefficient of variation of 0.506 (95% HPD: 0.005–1.143) across branches. Mean estimated ages and confidence intervals for cladogenesis of the Iberian *Barbus* assemblage are shown in Fig. 6. The estimated age of the most recent common ancestor of the Iberian assemblage is 7.31 Ma (95% HPD: 6.17–8.59 Ma) and that leading to *Barbus setivimensis* is 4.84 Ma (95% HPD: 2.87–7.18 Ma). The initial split within *B. sclateri* is estimated to have occurred 0.9 Ma (95% HPD: 0.16–1.81 Ma), falling in the Pleistocene. Cladogenetic events within Lineage B are all recent, having occurred within the last 0.44 Ma. In particular, the haplotype typical of SW region (20H) is likely younger (0.1 Ma).

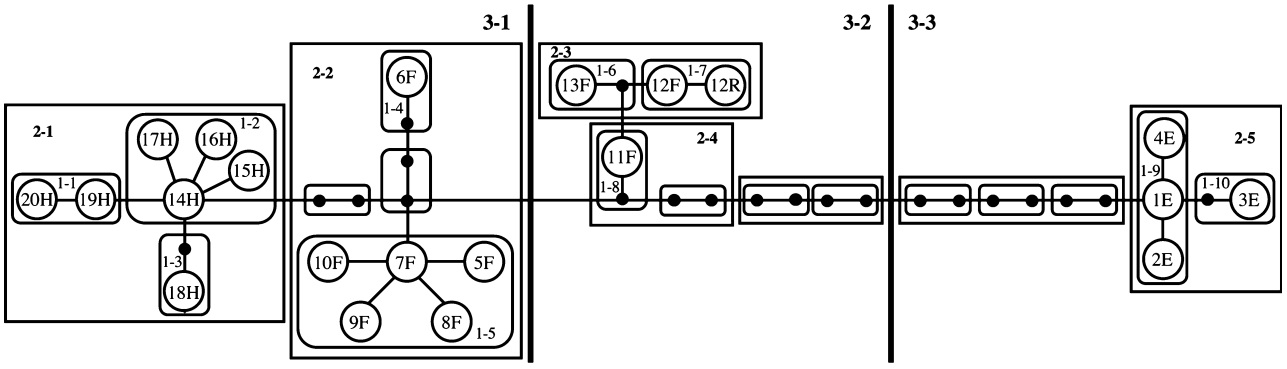

Fig. 5 Network of composite mitochondrial cyt*b* + ND2 + tRNAs haplotypes of *Barbus sclateri* complex. Small black circles represent inferred, undetected internal haplotypes, so that each line represents one mutational change. Rounded boxes define 1-step clades, square-angled boxes define 2-step clades and thick lines define 3-step clades.

Node	Divergence time (MY)	95% HPD	Posterior probability
1	0.050	0.010–0.101	0.82
2	0.094	0.020–0.176	1
3	0.063	0.005–0.130	0.99
4	0.239	0.035–0.438	1
5	0.074	0.009–0.161	1
6	0.069	0.010–0.153	1
7	0.896	0.157–1.805	1
8	1.914	0.458–3.740	1
9	1.787	0.541–3.240	0.75
10	2.655	1.160–4.854	1
11	4.841	2.870–7.184	0.84
12	7.305	6.166–8.588	1

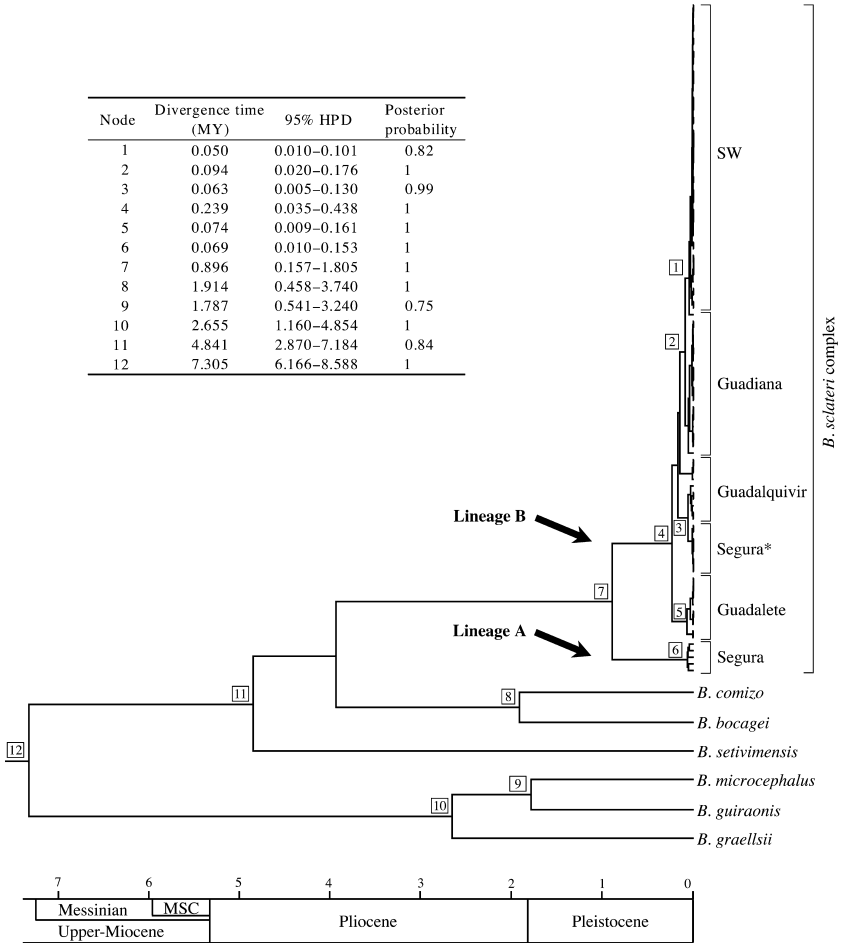

Fig. 6 Bayesian coalescent analysis of 108 mitochondrial ND2 + tRNAs sequences from Iberian *Barbus* calibrated using fossil evidence. Specific nodes numbered in boxes. Asterisk indicates haplotype from Segura more closely related to haplotypes from Guadalquivir. MSC, Messinian salinity crisis. Timescale: Ma.

Discussion

Our results from population genetic, phylogenetic and phylogeographical analyses of mtDNA and allozymes uncovered a complex evolutionary history of expansion, fragmentation and secondary contacts within the polytypic *Barbus sclateri*, shaped by Pleistocene glaciation–deglaciation cycles. All methods agree that allozymic and mitochondrial variation are strongly structured among populations or groups of populations, many of which are of recent origin. Discrepancies between the two types of markers used highlight their idiosyncratic responses to operating mechanisms and timescales involved.

Geographical, molecular and morphological concordance within B. sclateri *complex*

The combined use of fast-evolving mtDNA and slowly evolving allozyme loci across all populations allowed the identification of synapomorphic nucleotide substitutions and unique allozyme alleles for each population or group of populations. Molecular data are so highly concordant with geography that most populations are diagnosable with mtDNA sequences (Fig. 3), with allozymes providing additional differences among SE and SW populations (Fig. 4). The few instances where paraphyly or polyphyly are observed appear to result from secondary contact.

The observed polyphyly in Segura is likely the result of introgression from the adjacent Guadalquivir population, as suggested by the higher values of θ and π (Fig. 2) and the presence of one derived mtDNA haplotype (5F) that is most closely related to those in the Guadalquivir (Figs 3 and 5). Haplotypes of the two very well-supported mitochondrial Lineages A and B found in Segura coalesce 0.9 Ma, whereas Lineage A haplotypes coalesce 0.07 Ma (Fig. 6). In fact, the frequency spectrum of mitochondrial pairwise differences in the Segura population has the typical bimodal shape of a structured population with migration and not that of a single panmictic population (Hudson 1990; not

shown). Further phylogeographical evidence, such as the close paraphyly between Guadalquivir and Segura populations of co-occurring *Squalius pyrenaicus* (Sanjur *et al.* 2003), supports a scenario of secondary contact and admixture. Alternatively, this pattern could reflect human-mediated transfer among drainages. However, one would not expect human introductions to have such a significant impact over such a relatively short period of time, given the high frequency of 5F in Segura (Table S2) and the local abundance of *B. sclateri*. Additional focused sampling would be necessary to discriminate among these alternatives.

In the western limit of the SE region, allozyme data also strongly suggest the existence of secondary contacts and admixture, as populations from Gilão and Quarteira carry allozyme variants diagnostic of populations from the SW region (Fig. 2). One of these populations, Gilão, has the highest mean allozyme heterozygosity of all populations surveyed and has additional active *Gpi-1* alleles, which are possibly hybrizymes sometimes observed in hybrid zones (e.g. Woodruff 1989; Hoffmann & Brown 1995; Steinmetz *et al.* 2004; Godinho *et al.* 2006). Concurrently, the two mtDNA haplotypes found in this basin (14H and 20H) are the most abundant in adjacent populations to the east and west, respectively, further strengthening our interpretation (Table S2). Phylogeographical data from other co-occurring fish species support a secondary contact scenario, as suggested by Late Pleistocene contacts from Arade to Quarteira involving *Squalius aradensis* (Mesquita *et al.* 2005; Sousa-Santos *et al.* 2007) and from Guadiana to Quarteira involving *S. alburnoides* (Sousa-Santos *et al.* 2007), possibly through Gilão.

The geographical concordance of molecular variation found in this study is particularly relevant to the understanding of evolution within polytypic *B. sclateri*, as different populations have the levels of morphological differentiation comparable to those found among different species (Doadrio 1990). Morphological differences found across populations of this complex are concentrated in the head and mouth (Doadrio 1990; Gante, personal observation), which likely have an adaptive significance in food acquisition and niche occupancy. Ecological data also point to life-history differences among populations (e.g. Herrera & Fernández-Delgado 1992; Torralva *et al.* 1997). Our various results indicate that populations of this species complex have acquired these morphological and ecological differences very recently and are evolving along separate trajectories.

Timing and abiotic influences on cladogenesis of B. sclateri *complex*

Our results based on a lognormal coalescent prior with an age of 6 Myr, indicate that cladogenesis within the *B. sclateri* complex most probably occurred during the Pleistocene (Fig. 6). Substitution rates of ND2 + tRNAs estimated in this study for Iberian *Barbus*, are similar to those of previous independent estimates for cyt*b* based on biogeographical scenarios (see Mesquita *et al.* 2007, for a discussion of rate estimates). Additionally, time estimates presented here are consistent with geomorphological and stratigraphical evidence. Several marine corridors between Iberia and Northern Africa started becoming restricted by ~7.8 Ma, until the complete separation of the Atlantic and Mediterranean occurred at ~5.6 Ma with the establishment of a land bridge between Iberia and Africa (Garcés *et al.* 1998, 2001; Krijgsman *et al.* 1999a; Martín *et al.* 2001; van Assen *et al.* 2006). Mammalian fossil records suggest that the first faunal exchange between Iberia and Northern Africa occurred at 6.1–6.2 Ma, before the onset of Messinian Salinity Crisis of the Mediterranean (Pickford *et al.* 1993, 1995; Benammi *et al.* 1995, 1996; Garcés *et al.* 1998; Agustí *et al.* 2006). A second faunal exchange occurred between 5.9 and 5.3 Ma, which relates to Mediterranean sea level drop following the Messinian Salinity Crisis and before the opening of the Strait of Gibraltar (Krijgsman *et al.* 1999b; Agustí *et al.* 2006). Our results are consistent with the colonization of Iberia by *Barbus* from Northern Africa during the first period, with *Barbus setivimensis* originating through a return to Africa in the second (Fig. 6).

Most relevant to the understanding of evolution of freshwater-restricted organisms in glacial refugia, our data support that cladogenesis and morphological differentiation within *B. sclateri* complex occurred during the Pleistocene, and indicate that glaciation–deglaciation cycles could have promoted geographical expansion and differentiation through river rearrangement mechanisms (Bishop 1995). The time of split between mtDNA Lineages A and B is estimated to have occurred around 0.9 Ma (95% HPD: 0.16–1.81 Ma). Cladogenetic events within Lineage B are of much younger ages (all in the last 440 000 years), in particular in the SW region (in the last 100 000 years). These results indicate that such events have occurred in short intervals and were likely not concentrated in any one glacial–interglacial period. As elsewhere, the Pleistocene in the Iberian Peninsula was characterized by sharp thermal and precipitation variation. These climatic conditions led to a nearly complete replacement of mammalian fauna around 1 Ma after a thermal minimum (Hernández Fernández *et al.* 2007), which is consistent with the timing of initial cladogenesis within *B. sclateri* complex. Pleistocene climatic changes in association with tectonic activity were suggested to have promoted vicariance and divergence in several fish taxa through river capture/reversal (e.g. Waters *et al.* 2001, 2006; Burridge *et al.* 2006, 2007) and

by coastal confluence of independent basins during periods of low sea levels (e.g. Near *et al.* 2003; Swartz *et al.* 2007; Waters *et al.* 2007). It is well established that climate-mediated changes in surface water circulation and baseline sea level, and tectonic activity ultimately cause changes in configuration of rivers at various scales (e.g. Hattingh 1996; Williams *et al.* 1998; Harvey 2002; Craw & Waters 2007; Santisteban & Schulte 2007). In Iberia, tectonic uplift alone does not explain drainage patterns and requires a tight interplay with erosion and deposition of sediments (Cloetingh *et al.* 2002). Several documented cases of river captures in Southern Iberia point to a climatic influence, in particular during increased levels of erosion during interglacial periods (Wenzens & Wenzens 1997; Mather 2000; Mather *et al.* 2002, 2003; Candy *et al.* 2004; Azañón *et al.* 2005), and significant fluvial incision coincident with ~100 kyr Pleistocene eccentricity cycles (Santisteban & Schulte 2007). In fact, it has been shown that current configurations of Guadalquivir, Guadalete and Guadiana river systems are of Pleistocene age, and involved several episodes of river captures among them (Feio 1952; Rodriguez Vidal *et al.* 1993 and references therein). Hence, these multiple river captures have created favourable conditions for vicariance of freshwater-restricted organisms differentiated during previous glacial periods, including *B. sclateri*. These findings add to a growing body of evidence suggesting a Pleistocene influence in genetic structuring and diversification of several Iberian species associated but not restricted to the aquatic medium (Ribera & Vogler 2004; Martínez-Solano *et al.* 2006; Godinho *et al.* 2008; Sequeira *et al.* 2008) and land species (Melo-Ferreira *et al.* 2005; Vila *et al.* 2005; Geraldes *et al.* 2006).

Mode and polarity of cladogenesis in B. sclateri *complex*

Cladogenesis within the *B. sclateri* complex involved a series of range expansions, allopatric fragmentation, and admixture between differentiated populations, a pattern expected if climatic oscillations were responsible for generating divergence. Phylogenetic methods, NCPA and AMOVAS all agree that allozyme and mitochondrial variation are strongly structured among populations or groups of populations, which also consistently vary for morphological traits (Doadrio 1990; Gante, personal observation). Using the information generated, it is possible to establish a series of events responsible for the observed variation. One of the methods used, NCPA, is currently under close scrutiny as it has been shown to lead to false positives in simulation studies (reviewed in Knowles 2008; but see Garrick *et al.* 2008 and Templeton 2008). Nevertheless, under simulated panmixia the most commonly inferred false positives were 'isolation by distance' and 'contiguous range expansion', whereas 'fragmentation' (our most common inference) was only incorrectly inferred in a negligible proportion of simulated data sets (Panchal & Beaumont 2007). Therefore, for our data set, NCPA provided statistical results consistent with results from other analyses, strengthening our confidence in the accuracy of results.

Using all lines of evidence, it is possible to understand the processes generating observed variation and to infer the pattern of differentiation. Divergence of Iberian *Barbus* occurred 6.17–8.59 Ma, and initial cladogenesis within the *B. sclateri* complex probably occurred in south-eastern Iberian Peninsula around 0.9 Ma, an area exhibiting high levels of Plio-Pleistocene crustal uplift (Cloetingh *et al.* 2002 and references therein) and glacier accumulation during cooler periods (Pérez Alberti *et al.* 2004). After a period of differentiation, vicariant populations became established in the Guadalete and Guadiana through river captures (Rodriguez Vidal *et al.* 1993) <0.44 Ma. Subsequently, in the last 100 000 years, the population from the Guadiana River colonized the SW region. These western populations (Guadiana, Gilão, Quarteira and SW) represent the front wave of expansion, as indicated by NCPA and Fu's F_S (clades 1-1, 1-2 and 2-1; Tables 3 and 4). More recent secondary contacts and transfer of mtDNA occurred from Guadalquivir to Segura (around 63 000 years ago), and admixture of mtDNA and/or allozyme alleles typical of Guadiana and SW rivers in Quarteira and Gilão (see above). In light of these and previous findings from other taxa (Mesquita *et al.* 2005; Sousa-Santos *et al.* 2007), Quarteira and Gilão might represent a suture (hybrid) zone between SE (i.e. Guadiana drainage) and SW ichthyogeographical areas, and differentiation observed in Segura might warrant further subdivision of the SE ichthyogeographical region.

Pleistocene climatic oscillations would have had more than an indirect impact on freshwater organisms through influence on fluvial geomorphological processes; they would have also cyclically affected established populations. The relatively low levels of intra-population divergence and star-like phylogenies observed in *B. sclateri* complex are not consistent with large and stable long-term effective population sizes, but are more consistent with recent demographical expansions reflecting recovery from suboptimal glacial conditions, as previously suggested for other Iberian refugial taxa (e.g. Martínez-Solano *et al.* 2006; Godinho *et al.* 2008).

Given the recent founding of SW populations and their low levels of molecular variation, the high level of allozyme differentiation observed is rather striking, as mtDNA evolves more rapidly (Figs 2–4; Tables 2 and S4). Increased sample sizes of composite

cyt*b* + ND2 + tRNAs could identify significant differences among ichthyogeographical regions. However, the reduced level of mtDNA variation across the ichthyogeographical divide, relative to that of allozymes, is not likely to change appreciably. These results could either mean that previous mtDNA history of SW populations has been erased by recent events, while nuclear variation persisted, or that an unprecedented high rate of allozyme evolution has occurred. The first hypothesis (hybrid speciation) implies pre-existing nuclear variation in SW rivers, followed by admixture and complete replacement of a resident species' mtDNA by colonizing specimens of Guadiana origin, whereas the second hypothesis (founder effect speciation) implies the fixation of a common mtDNA haplotype and generation of new allozyme alleles in a small population with Guadiana origin. A combination of these hypotheses is also possible, via reinvasion from Guadiana after an initial period of differentiation. Further analysis using nuclear DNA sequences is required for formal testing of these hypotheses. Nevertheless, both scenarios highlight the dynamic nature of evolution and the recency of processes operating on freshwater species in this classical glacial refugium.

Conclusions

This study highlights the importance of Pleistocene climatic cycles in driving genetic divergence and speciation, as implicated in the diversification of many taxa (e.g. Knowles 2000; Clarke *et al.* 2001; Barraclough & Vogler 2002; Johnson & Cicero 2004; Near & Benard 2004). The interplay between climatic oscillations, regional geomorphological history and adaptability of taxa to new ecological opportunities is likely responsible for the Pleistocene radiation as observed in the *B. sclateri* complex.

Pleistocene climatic oscillations were previously shown to be major determinants in structuring Central European ichthyofauna (Bernatchez & Wilson 1998) and to affect fish distribution in the Adriatic region (Tsigenopoulos *et al.* 2002 and references therein). The present work extends their influence to Iberia, traditionally considered a more stable glacial refugium, and suggests that they played a significant role in population structure and diversification of freshwater-restricted species. This study adds to the growing body of evidence suggesting a complex evolutionary history of refugial Iberian populations (e.g. Suárez *et al.* 2001; Melo-Ferreira *et al.* 2005; Martínez-Solano *et al.* 2006; Espanhol *et al.* 2007; Godinho *et al.* 2008; Sequeira *et al.* 2008). Not only did species survive in different Iberian refugia during successive glacial periods (Gómez & Lunt 2007), they also diversified genetically, morphologically, and ecologically during those events, contributing to high endemicity. These new data for freshwater-restricted species are consistent with recent evidence for speciation in water-associated (Ribera & Vogler 2004) and land animals (Vila *et al.* 2005; Geraldes *et al.* 2006) in Iberia, and suggest a rather dynamic scenario in which glaciation–deglaciation cycles probably promoted vicariance, admixture and allopatric speciation in glacial refugia.

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This article is part of HFG's PhD thesis on the evolution of Iberian *Barbus*. HFG's is interested in understanding the

genetic basis of speciation, the evolutionary consequences of hybridization and adaptive evolution. JM has an interest in multidisciplinary approaches (biology, ecology, population genetics) in developing conservation strategies in aquatic systems. FJO-P's research interests include studies on diversity, biology and ecology of cyprinids from Mediterranean areas. ID is interested in the biogeography, systematics, taxonomy and evolution of freshwater fishes. TED is an evolutionary biologist interested in examining the processes that generate and maintain biodiversity and takes an active role in conservation and management of endangered fishes. MJA is interested in the study of the evolutionary processes responsible for generating and maintaining genetic diversity within and among populations, and driving speciation in freshwater and diadromous fishes.

Supporting information

Additional supporting Information may be found in the online version of this article:

Table S1 Distribution of cytb haplotype frequencies at each sampled locality

Table S2 Distribution of composite mitochondrial haplotype frequencies at each sampled locality

Table S3 Distribution of allozyme alleles (italicized) found in populations of *Barbus sclateri*, *Barbus bocagei* and *Barbus comizo*

Table S4 Summary of *Barbus sclateri* polymorphisms at mitochondrial and allozyme loci

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