Research paper

**Cenozoic tectonic and climatic events in southern Iberian Peninsula: implications
for the evolutionary history of freshwater fish of the genus *Squalius*
(Actinopterygii, Cyprinidae)**

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ABSTRACT

Southern Iberian freshwater ecosystems located at the border between the European and African plates represent a tectonically complex region spanning several geological ages, from the uplifting of the Betic Mountains in the Serravalian-Tortonian periods to the present. This area has also been subjected to the influence of changing climate conditions since the Middle-Upper Pliocene when seasonal weather patterns were established. Consequently, the ichthyofauna of southern Iberia is an interesting model system for analyzing the influence of Cenozoic tectonic and climatic events on its evolutionary history. The cyprinids *Squalius malacitanus* and *Squalius pyrenaicus* are allopatrically distributed in southern Iberia and their evolutionary history may have been defined by Cenozoic tectonic and climatic events. We analyzed *MT-CYB* (510 specimens) and *RAG1* (140 specimens) genes of both species to reconstruct phylogenetic relationships and to estimate divergence times and ancestral distribution ranges of the species and their populations. We also assessed their levels of genetic structure and diversity as well as the amount of gene flow between populations. To investigate recent paleogeographical and climatic factors in southern Iberia, we modeled changes-through-time in sea level from the LGM to the present. Phylogenetic, geographic and population structure analyses revealed two well-supported species (*S. malacitanus* and *S. pyrenaicus*) in southern Iberia and two subclades (Atlantic and Mediterranean) within *S. malacitanus*. The origin of *S. malacitanus* and the separation of its Atlantic and Mediterranean populations occurred during the Serravalian-Tortonian and Miocene-Pliocene periods, respectively. These divergence events occurred in the Middle Pliocene and Pleistocene in *S. pyrenaicus*. In both species, Atlantic basins possessed populations with higher genetic diversity than Mediterranean, which may be explained by the Janda Lagoon. The isolation of *S. malacitanus* was earlier and related

to the rising of the Betic Mountains. Divergence of its Atlantic and Mediterranean populations was associated with the creation of the freshwater systems of southern Iberia close to the Gibraltar Strait. The presence of *S. pyrenaicus* in southern Iberia may be the result of recent colonization associated with river capture, as demonstrated our biogeographic reconstruction.

Keywords: Ancestral areas reconstruction, Cenozoic, Phylogeny, southern Iberia, Molecular clock, *Squalius*

1. Introduction

The Betic Mountains and its foreland basin, the Guadalquivir River, which together bound the Hercynian Iberian Massif in its south and southeastern area, constitute the southern area of the Iberian Peninsula since the Miocene period. The Betics are part of the Gibraltar Arc along with the Moroccan Rif, which is located in the western end of the Alpine belt (Miller et al, 2013). The southern area of the Iberian Peninsula constitutes a differentiated biogeographical region on the basis of freshwater fishes that encompasses not only the large Guadalquivir Basin but also several smaller independent river drainages in southwestern Portugal and in areas close to the Gibraltar Strait in Spain (Figs. 1, 2) (Doadrio, 1988; Filipe et al., 2009). Several significant tectonic processes have formed the complex configuration of the hydrological network of southern Iberia, directly affecting the resident freshwater fauna, including the uplifting of the Betic Mountains in the Serravalian and Tortonian periods and events following the opening of the Gibraltar Strait at the Miocene-Pliocene boundary (Weijermars, 1991; Krijgsman et al., 1999; Braga et al., 2003), which have originated complex processes of speciation in a relatively small geographical region.

In addition to these macroevolutionary processes, more recent events could have influenced the evolutionary history of freshwater fishes in southern Iberia. The independent smaller basins have been affected since the Middle-Upper Pliocene by a heterogeneous annual hydrological cycle typical of the Circum-Mediterranean region and by catastrophic and erratic events (Mesquita et al., 2005, Benito et al., 2008; Lario et al., 2011). In these fluctuating systems, the drying up of large river sections in summer often results in a series of isolated ponds, leading to high fish mortality and subsequent population bottlenecks (Coelho et al., 1995, Magalhães, 2002). In addition, sea level fluctuations during Pleistocene glaciations may have impacted habitat of aquatic species and played an important role in the population structure and genetic diversity of freshwater ichthyofauna (Gante et al., 2009). Besides, current research on the Gibraltar Strait, supported by geological and geomorphological evidence, indicates that at least five tsunamis generated by strong earthquakes have affected this area during the past 7000 years, at a recurrence interval of 1200 to 1500 years (Lario et al., 2011). These tsunamis may have been catastrophic for primary freshwater fishes from small rivers in southern Iberia. The genetic structure and population diversity of fishes inhabiting this area also reflect these recent climatic events. These microevolutionary processes related to climatic factors must have also contributed to shape the speciation pattern followed by freshwater organisms through the presence or absence of genetic exchange. The freshwater ecosystems of southern Iberia around the Gibraltar Strait are considered together a peninsular glacial refuge (Gómez and Lunt, 2007; Gante et al., 2009).

To evaluate such events and their influence in the phylogenetic structure and speciation processes of aquatic communities, primary freshwater fish have been widely used as models (Doadrio et al., 2002b; Perdices et al., 2003; Böhlen et al., 2006; Lopes-

Cunha et al., 2012), since distribution of their lineages tends to reflect the history of river drainage systems rather than contemporary dispersal (Bernatchez and Wilson, 1998; Avise, 2000). The current hydrological network in the Iberian Peninsula was formed in the Upper Pliocene-Pleistocene periods (Calvo et al., 1993; Pais et al., 2012), and the general absence of connections among rivers has maintained the distribution of native fish, except in cases of human transfer, leading to vicariant speciation (Doadrio, 1988; Filipe et al., 2009).

One of the primary freshwater fish lineages currently inhabiting the Iberian Peninsula is *Squalius* Bonaparte, 1837. It is distributed throughout European freshwater lakes and rivers, and shows a high level of diversity in the Mediterranean area (Doadrio and Carmona, 1998; Zardoya and Doadrio, 1998; Durand et al., 2000; Doadrio and Carmona, 2003). Two main lineages, which diverged in the Miocene, are present in Europe: a group of large species, mainly occurring in central and northern Europe, and a second clade of small species present in the Mediterranean peninsulas with limited distribution ranges (Sanjur et al., 2003; Perea et al., 2010). In the Iberian Peninsula there are nine species of the genus *Squalius*, all of them endemic from different drainage systems and showing allopatric distribution ranges except for *S. alburnoides*, which co-occur with other species of *Squalius* in different basins due to their complex reproduction modes (Doadrio et al., 2011; Fig. 1). In southern Iberia, Guadalquivir Basin and the smaller independent basins around the Gibraltar Strait, *Squalius* is represented by two species, with a vicariant pattern, of the Mediterranean lineage (*sensu* Sanjur et al., 2003), *Squalius malacitanus* (Malaga chub) and *Squalius pyrenaicus* (Iberian chub). *Squalius malacitanus* is restricted to small rivers of southern Spain around the Gibraltar Strait (Doadrio and Carmona, 2006; Doadrio et al., 2011). The distribution range of *Squalius pyrenaicus* covers mainly the southern half of the Iberian

Peninsula, in particular the Tajo, Guadiana, Sado, Guadalquivir, Guadalete and Odiel basins, flowing into the Atlantic Ocean, and the Segura, Guadalhorce, Vélez, Guadalfeo, and occasionally Ebro, river basins flowing into the Mediterranean Sea, being the Guadalhorce, Vélez and Guadalfeo basins the southernmost part of its range (Figs. 1, 2). These southernmost Mediterranean populations along with the Atlantic Guadiana, Guadalquivir, Guadalete y Odiel basins have been considered as a monophyletic lineage relative to northern basins of Tajo, Sado and Ebro (Doadrio & Carmona, 2003).

It is assumed that both *S. malacitanus* and *S. pyrenaicus*, as a consequence of their status as primary freshwater fishes, should show clear phylogenetic and phylogeographical structure. For this reason they are suitable candidates for investigation of the effects of the complex tectonic and climatic changes occurred during the end of the Tertiary and the Quaternary geological eras in the southern Iberian biogeographical region on their speciation processes. Similar to in other Iberian cyprinids with distribution across several basins, a strong inter- and intraspecific phylogenetic structure is expected, associated with geographical events (Doadrio et al., 2002a,b; Doadrio and Carmona, 2003, 2004; Sousa-Santos et al., 2007; Sousa et al., 2008; Gante et al., 2009). However, atypical or erratic events, such as Mediterranean climate weather patterns and the recent tsunamis occurring around the Gibraltar Strait, may lead to more complex phylogenetic and phylogeographical patterns in this southern Iberian region. We hypothesize that the southern Iberian populations of the genus *Squalius* are sensitive to tectonic and climatic changes and that these changes are reflected in their current phylogenetic structure and genetic diversity. We suggest that larger drainage basins, particularly the Guadalquivir, should retain ancestral polymorphisms, whereas peripheral populations, mainly the ones of *S. malacitanus*

because of its restricted distribution range, will present lower genetic diversity and marginal haplotypes, due to high mortality resulting from catastrophic Cenozoic events. Indeed, the range of *S. malacitanus*, an Endangered species (Freyhof and Kottelat, 2008), may be drastically reduced in the future as a result of global climate change and the trend to an increasingly arid Mediterranean region (Seager et al., 2014).

The goals of this study were to use mitochondrial and nuclear DNA sequence data to 1) investigate phylogenetic patterns of *Squalius* species inhabiting southern Iberia; 2) evaluate the influence of Cenozoic tectonic and climatic changes on population genetic structure and diversity in those southern Iberian *Squalius* populations; 3) estimate divergence times among southern Iberian *Squalius* and 4) perform an ancestral area reconstruction analysis of southern Iberian *Squalius* populations in order to unravel the evolutionary history of this genus in southern Iberia.

2. Material and methods

2.1. Tissue and data collection

A total of 510 specimens of *S. malacitanus* and *S. pyrenaicus* from 13 drainage basins of southern Iberia were analyzed (Table 1, Fig. 2). Samples of *S. malacitanus* were collected from nearly its complete distribution range. Samples of *S. pyrenaicus* were mainly obtained from the southern distribution of the species in the Iberian Peninsula. Other Iberian populations of *S. pyrenaicus* as well as some individuals of all Iberian *Squalius* species but *S. alburnoides* were included in the analyses in order to resolve the phylogenetic position of southern Iberian populations of the genus *Squalius* (Table 1, Fig. 2). For phylogenetic purposes, *Squalius squalus* and *S. laietanus*, belonging to the Euroasiatic lineage *sensu* Sanjur et al. (2003), were included as

outgroups based on previous studies (Doadrio and Carmona, 2003, 2006). Fish were captured by electrofishing under local authority permission, fin clipped, and returned to the stream, with the exception of 2-3 specimens per locality that were preserved in formalin for morphological identification. Fin clips were preserved in 95% ethanol. All voucher samples were stored in the fish collection and at the DNA collection of the Museo Nacional de Ciencias Naturales, CSIC, Madrid, Spain.

2.2. DNA protocols

The complete mtDNA cytochrome *b* gene (*MT-CYB*; 1140 bp) of 510 individuals and a fragment of 1041 bp of the third exon of the nuclear Recombination Activating Gene 1 (*RAG1*) of 181 individuals, representing all southern *Squalius* populations, were amplified (Table 1). Primers and PCR protocols for both genes followed Perea *et al.* (2010). After checking PCR products on 1% agarose gels, they were purified by ExoSAP-IT (USB, Cleveland, OH, USA) and directly sequenced on MACROGEN Inc. (Seoul, Korea; <http://www.macrogen.com>). All new sequences (haplotypes and alleles) were deposited in the GenBank database (Accession Numbers: xxx-xxx).

Sequences were aligned using the default pairwise and multiple alignment parameters in Clustal W (Thompson *et al.*, 1994) implemented in MEGA software v.6.0 (Tamura *et al.*, 2013). Nuclear data was unphased with the PHASE algorithm in DNAsp v.5.0 (Librado and Rozas, 2009) using a probability threshold of 0.9 to resolve alleles. Recombination of the nuclear *RAG1* gene ($p = 0.55$) was tested using the PHI test in Splitstree v. 4.13 (Huson and Bryant, 2006).

2.3. Phylogeny, and population structure

For phylogenetic analysis of independent genes, sequences of *MT-CYB* and *RAG1*

were collapsed to haplotypes using the web-based program ALTER (González-Peña et al., 2010). For concatenated phylogeny, the dataset including specimens amplified for both genes was used. The best-fit model of molecular evolution for each gene and codon position was estimated via AIC in PartitionFinder v. 1.1.1 (Lanfear et al., 2012). Best partition schemes used in different phylogenetic analyses based on the selected evolutionary models are presented in Table S1. Bayesian inference (BI) was performed using MrBayes v.3.2 (Ronquist et al., 2012). Two simultaneous analyses were run for 10^7 generations, each with four MCMC chains sampling every 100 generations. Convergence was checked with Tracer v.1.6. (Rambaut and Drummond, 2007). After discarding the first 10% of generations as burn-in, the 50% majority rule consensus tree and posterior probabilities were obtained. Phylogenetic analyses of both genes, independently and concatenated, were conducted using Maximum Likelihood (ML) implemented in the on-line version of software implemented in the Trex-online server (Stamakis, 2006) using the substitution model GTRGAMMAI and the rapid bootstrap algorithm (Stamakis et al., 2007) for both *MT-CYB* and concatenated analyses. Node confidence in ML analyses was estimated by rapid bootstrapping using a random seed (1000 replicates). Uncorrected *p*-distances for both genes were estimated in MEGA v.6.0 (Tamura et al., 2013) in order to quantify genetic differences between species and among their populations. To assess the phylogeographic history of *Squalius* in southern Iberia, we reconstructed two independent networks for *MT-CYB* and *RAG1* sequences using HaploViewer (available at <http://www.cibiv.at/~%20greg/haploviewer>).

We estimated levels of genetic diversity based on *MT-CYB*, including number of haplotypes (H), haplotype diversity (H_d) and nucleotide diversity (π), using DnaSP v. 5.0 (Librado and Rozas, 2009) for southern Iberian populations of the genus *Squalius*. To assess the relative contribution of genetic variation to structure within and between

populations in *S. malacitanus* and southern populations of *S. pyrenaicus*, we performed several analyses of molecular variance (AMOVA) implemented in Arlequin v. 3.5.1.2. (Excoffier and Lischer, 2010), testing for genetic structure between groups of populations (Atlantic and Mediterranean) and among basins in both species. Global genetic structure of *S. malacitanus* and *S. pyrenaicus* considering their geographic location was also investigated using a Spatial Analysis of Molecular Variance (SAMOVA) (Dupanloup et al., 2002). We ran SAMOVA from $k=2$ to $k=13$ (all southern Iberian basins) with 500 and 1000 simulated annealing processes. Each run was repeated three times to check consistency. The genetic differentiation among populations was addressed through *MT-CYB* Φ_{ST} pairwise comparisons (Hudson et al., 1992) and estimates of gene flow as virtual number of migrants (Nm) exchanged among populations per generation (Slatkin and Barton, 1989), using Arlequin v. 3.5.1.2. (Excoffier and Lischer, 2010). Significant deviations from the null hypothesis of no differentiation were assessed with 10 000 permutation tests. As multiple paired tests were performed, p -values were adjusted by Bonferroni's correction (Rice, 1989).

2.4. Species tree and divergence time estimates

A multilocus species tree analysis of Iberian populations of the genus *Squalius* belonging to the monophyletic clade *S. malacitanus* + *S. pyrenaicus* + *S. valentinus* (Doadrio and Carmona, 2006; Perea et al., 2010; results from this study) was carried out using the concatenated matrix (*MT-CYB* + *RAG1*) and the multispecies coalescent method *BEAST implemented in BEAST v.1.7 software (Heled and Drummond, 2010; Drummond et al., 2012). This analysis allows incorporating information of population to the species tree analyses through the coalescent theory and requires predefined groups to be tested, therefore, we considered the monophyletic clades obtained in

previous performed phylogenetic and network analyses as different clusters. This analysis was also used to estimate divergence time among southern Iberian *Squalius* using a lognormal relaxed molecular clock and a Yule speciation model. We calibrated the molecular clock, incorporating as normal prior the evolutionary rate of 0.4% divergence per lineage per million years for the *MT-CYB* gene proposed for cyprinids of the subfamily Leuciscinae, which is based on fossil calibrations (Perea et al., 2010) and estimated the evolutionary rate of the nuclear *RAG1* gene relative to the *MT-CYB*. MCMC analyses were run for 50 million generations, with parameters logged every 10 000 generations. The remaining parameters were used as default. Output from BEAST was examined in Tracer 1.6, and the results were summarized using TreeAnnotator 1.7 (Drummond and Rambaut, 2007).

2.5. Ancestral area reconstruction

We reconstructed the ancestral states of the distribution ranges of the southern Iberian populations of the genus *Squalius* using the Dispersal-Extinction-Cladogenesis (DEC) model of LAGRANGE (Ree et al., 2005; Ree et al., 2008) and the statistical DIVA (S-DIVA) method (Yu et al., 2010) implemented in RASP v.3.1 software (Yu et al., 2015). The ultrametric and dichotomous tree obtained for the *MT-CYB* gene in *BEAST analysis (containing all populations of southern Iberian *Squalius*) was used as the tree topology on which mapping ancestral areas. Tips of the ultrametric tree were code by areas (basins) in order to reconstruct ancestral states.

2.6. Sea level patterns

We employed a geographic information system to visualize changes in sea level from the LGM, when sea level was 135 m lower than currently (Miller et al., 2005), to the

present. Datasets used to generate a sea level change map were obtained from the Digital Chart of the World (ESRI, 1993) and manipulated in ArcMap v. 10 (ESRI, Redlands, CA). Bathymetric data were obtained from a 30 arc-second (ca. 1 km) dataset, GEBCO 08 (www.gebco.org; September 2010 release) and manipulated to produce bathymetric contours and sea-level drainage patterns using the hydrological tools in ArcInfo.

3. Results

3.1. Phylogeny and population structure

The phylogenetic analysis based on mitochondrial (*MT-CYB*) and concatenated (*MT-CYB+RAG1*; 2181bp) genes and both phylogenetic methods performed (BI and ML) were congruent and relationships among Iberian *Squalius* species belonging to the Mediterranean lineage (Sanjur et al., 2003) were highly supported and consistent with previous published studies (Doadrio and Carmona, 2006; Perea et al., 2010; Sousa-Santos and Almada, 2010). All Iberian species of the genus *Squalius* were monophyletic. From the root of the phylogenetic tree to the tips, southwestern Portuguese species of the genus, *S. aradensis* and *S. torgalensis*, appeared as the sister group of the rest of Iberian species; the second divergent clade was that constituted by *S. carolitertii* and *S. castellanus*, and finally, *S. malacitanus* was the sister group of a monophyletic clade formed by *S. pyrenaicus* and *S. valentinus* (Figs. 3 and S1).

With regards to southern Iberia populations of the genus *Squalius*, BI and ML analyses of *MT-CYB* and *MT-CYB+RAG1* revealed two main clades: one containing samples attributed to *S. malacitanus* and the other to *S. pyrenaicus*, being this later group sister to the Spanish Levantine species *S. valentinus* (Fig. 3, S1). Nuclear phylogeny also separated *S. malacitanus* and *S. pyrenaicus* (Fig. S2). Mitochondrial and

nuclear phylogenies supported the structure of *S. malacitanus* into two subclades (Figs. 3, S1), one comprising the Mediterranean populations (Guadiaro, Guadalmina and Guadaiza basins) and the other Atlantic populations from the Celemín (Barbate Basin), Jara, Vega, and Miel river basins. Miel Basin is located near the Gibraltar Strait and currently drains into the Mediterranean Sea at its junction with the Atlantic Ocean. Accordingly, although this river flows on the Mediterranean slope, we assigned its populations to the Atlantic, due to its geographic proximity to, and common history with, those populations (Fig. 2). *Squalius pyrenaicus* did not present a geographically well-supported phylogenetic structure, and Mediterranean populations (Guadalhorca, Vélez and Guadalfeo) clustered in the phylogenetic tree together with haplotypes observed in some tributaries of the middle part of the Guadalquivir Basin and a tributary of the Upper Guadalete Basin on the Atlantic slope (Figs. 3, S1).

Overall uncorrected-*p* genetic distances for the *MT-CYB* gene varied from 0.01% observed in specific pairwise comparisons of Mediterranean populations of *S. pyrenaicus* to 8.4% between Mediterranean populations of the two species (Table S2). Within *S. malacitanus*, genetic distances between Atlantic and Mediterranean populations for the *MT-CYB* gene ranged from 4.1 % to 4.5 %, while this range was 0.6-1.0% between Atlantic and Mediterranean basins in southern Iberian populations of *S. pyrenaicus*. Uncorrected-*p* genetic distances for the nuclear *RAG1* gene ranged from 0.4% to 0.8% between *S. malacitanus* and southern Iberian populations of *S. pyrenaicus*, from 0.1 to 0.2% in *S. malacitanus* and from 0.1 to 0.4% in *S. pyrenaicus* (Table S2).

Mitochondrial and nuclear network analyses of both *Squalius* species (Fig. 4) matched the well-supported lineages recovered in the phylogenetic tree: one group for *S. pyrenaicus* and two groups for *S. malacitanus* (Mediterranean and Atlantic

populations). In *S. malacitanus*, no mitochondrial haplotype or nuclear allele was shared between the Atlantic (Jara, Vega, Miel and Celemín) and Mediterranean (Guadiaro, Guadalmina and Guadaiza) populations, demonstrating high genetic and geographic separation. In *S. pyrenaicus*, no mitochondrial haplotypes were shared between Atlantic (Guadalquivir, Odiel and Guadalete) and Mediterranean (Guadalhorce, Vélez and Guadalfeo) basins, however, sharing of nuclear alleles among populations was common (Fig. 4).

Genetic diversity values were lower for analyzed *S. pyrenaicus* populations than for *S. malacitanus* (Table S3). Haplotype and nucleotide diversity of Atlantic populations of *S. malacitanus* was higher than in Mediterranean populations. Within Mediterranean populations, values of genetic diversity were high only in the Guadiaro Basin (Table S3). *Squalius pyrenaicus* also showed a similar pattern of higher genetic diversity values in Atlantic populations relative to Mediterranean populations; these values were higher in the larger basin, the Guadalquivir (Table S3).

AMOVA analyses supported high and significant genetic differentiation among and within populations (Atlantic vs Mediterranean) of both species (Table S4). In *S. malacitanus*, higher percentages of genetic differentiation were found among basins than within basins. In *S. pyrenaicus*, the within-basin genetic variation was higher than among basins, especially in the larger basin (Guadalquivir). SAMOVA analysis of both species revealed the highest differentiation among groups and the smallest percentage of variation within groups for $K=4$, corresponding these four groups to Atlantic and Mediterranean populations in both species, which proved to be the best partition of the genetic variation hypothesis according to geographic criteria (Table 2).

Pairwise Φ_{ST} values are presented in Table S5. In both species, the lowest differentiation values were found between basins belonging to the same slope, whereas

the highest pairwise values involved basins on different slopes. An exception was the Miel Basin, geographically belonging to the Mediterranean slope but showing lower Φ_{st} values in pairwise comparisons that placed this basin with Atlantic populations. These results of genetic differentiation were congruent with the restricted or absent gene flow found between Atlantic and Mediterranean populations of both species, as was reflected by Nm values lower than 1 (Table S5). Nevertheless, evidence of gene flow ($Nm > 1$) was found among rivers of the same slope in both species, especially among Mediterranean populations, although gene flow was also significant among Atlantic populations of *S. pyrenaicus*.

3.2. Species tree and divergence time estimates

Species tree analysis using *BEAST and both *MT-CYB* and *RAG1* genes recovered with high support ($pp=1$) Atlantic and Mediterranean populations of *Squalius malacitanus* as different groups. The split between *S. malacitanus* and the clade comprised of *S. pyrenaicus* + *S. valentinus* was estimated to take place 11.4 Ma (6.8-18.6 Ma), at the boundary of the Serravalian and Tortonian periods. Nevertheless, although posterior probability isolating Atlantic and Mediterranean populations in *S. pyrenaicus* was also high ($pp=1$) divergence between these two groups of populations was very recent (Fig. 5). The divergence of *S. pyrenaicus* from its sister species *S. valentinus* was estimated to have occurred during the Middle Pliocene 3.9 Ma (1.3-8.1 Ma). The separation between the Mediterranean and Atlantic populations of *S. malacitanus* was estimated to be 4.9 Ma (2.1-8.5 Ma), at the Miocene-Pliocene boundary, while the isolation of the Atlantic and Mediterranean populations of *S. pyrenaicus* took place 0.6 Ma (0.3-1.0 Ma), in the Pleistocene period (Fig. 5).

3.3. Ancestral area reconstruction

Ancestral area reconstructions using LAGRANGE (DEC) and S-DIVA methods showed a complex biogeographical history followed by southern Iberian *Squalius* populations belonging to both species, *S. malacitanus* and *S. pyrenaicus*, in which dispersal and vicariant events were frequent (Fig. 6, 7). In general, DEC analysis estimated more dispersal events than S-DIVA for the whole phylogenetic tree and some of the ancestral nodes could not be reconstructed by these methods with high support, such is the case of the ancestral area explaining the separation of *S. malacitanus* from the *S. pyrenaicus* + *S. valentinus* clade. Nonetheless, biogeographical routes followed by Atlantic and Mediterranean populations of both species were highly congruent between both biogeographical reconstructions, especially in those nodes highly supported by phylogenetic analyses. These biogeographical routes are explained below in the Discussion section.

3.4. Sea level change patterns

The bathymetric reconstruction showed a possible downstream connection only between Jara and Vega Basins during the LGM. On the other hand, headwaters of most small basins on the Atlantic slope were in the proximity of the ancient basin of the Janda Lagoon (Fig. 2).

4. Discussion

Southern Iberia represents one of the more complex areas to carry out biogeographic and diversification studies due to early orogenic and tectonic events that led to diversification processes during the Miocene, forming old endemic Mediterranean lineages in various taxonomic groups (Martínez-Solano et al., 2004; Miraldo et al.,

2011). The Miocene separation of the European-African continental margin and the reopening of the Gibraltar Strait in the Miocene-Pliocene boundary played a key role in shaping genetic diversity and biogeography of Western Mediterranean species, including fishes (Machordom and Doadrio, 2001; Doadrio and Perdices, 2005).

4.1. Interspecific diversification of southern Iberian Squalius

We demonstrate that the evolutionary history of *S. malacitanus* and southern Iberian populations of *S. pyrenaicus*, the two *Squalius* species occurring in southern Iberia in the region close to the Gibraltar Strait, displayed similar geographical divergence patterns, including a primary separation between Atlantic and Mediterranean populations of both species, most conspicuously in *S. malacitanus*, due to the close relationship of Mediterranean populations of *S. pyrenaicus* with some of the tributaries of the middle-upper part of the main Atlantic basins (Guadalquivir and Guadalete). However, the time, and consequently, the level, of divergence of Atlantic from Mediterranean populations differ. Thus it can be assumed that distinct geological or climatic events at different temporal scales were responsible for the phylogenetic and biogeographic patterns of Atlantic and Mediterranean populations of both species. The endemic local species, *S. malacitanus*, presented a deeper divergence of Atlantic from Mediterranean populations compared to southern populations of *S. pyrenaicus* close to the Gibraltar Strait, probably as a consequence of its ancient origin in the southern Iberian area (Figs. 5).

Our molecular clock dates the separation between *S. malacitanus* and the clade grouping *S. pyrenaicus* and *S. valentinus* at *ca* 11.5 Ma, close to the Serravalian-Tortonian boundary in the Upper Miocene (11.6 Ma). Similar divergence times have been found in other Iberian and North African vertebrate species around the Gibraltar

Strait (Carranza et al., 2006; Carretero, 2008). For amphibians and reptiles inhabiting southern Iberia, the Tortonian has been considered an important period for isolation and diversification, driven by seaways opening within the Betic and Rifian areas (Fromhage et al., 2004; Albert et al., 2007). Among freshwater fish species around the Gibraltar Strait, the geographical pattern and the temporal scale of the separation between *S. malacitanus* and the *S. pyrenaicus* + *S. valentinus* clade is unusual, since splitting of these species occurred between the Iberian area of the Gibraltar Strait and other Iberian basins and not between the southern Iberian Peninsula and North Africa. In other known freshwater fish species, the most common divergence pattern is the vicariance between southern Iberian and northern Moroccan populations associated with the opening of the Gibraltar Strait in more recent times than our estimate (Doadrio, 1990; Perdices et al., 1995; Machordom and Doadrio, 2001; Perdices et al., 2001; Doadrio and Perdices, 2005; Almada et al., 2009).

The estimated time of divergence of the analyzed *Squalius* species of southern Iberia predates the refilling of the Mediterranean Sea by the opening of Gibraltar Strait through the Zanclean flood (5.3 Ma), hence some older vicariant events must be involved (Esteban et al., 1996; Krijgsman et al., 1999; García-Castellanos et al., 2009). Both ancestral area reconstructions were not able to resolve the state of the node separating *S. malacitanus* from the *S. pyrenaicus* + *S. valentinus* clade, although the most probable route in DEC analyses estimated a dispersal towards southern Iberia and a posterior vicariance among distribution ranges; however, marginal probability supporting this biogeographical route was low (DEC=0.005; S-DIVA=0.125). The most plausible hypothesis to explain the divergence of *S. malacitanus* from the *S. pyrenaicus* + *S. valentinus* clade is its separation by the orogenic uplifting and tectonic movements related to the formation of the Betic Mountains during the Tortonian period and its

associated foreland, the Guadalquivir Basin, along with the formation of the southern hydrological network as uplifting and tectonic movements occurred. This resulted in the emergence of islands separated by seaways and the reconfiguration of the freshwater basins in the region when the seaways were closed, isolating *S. malacitanus* from other Iberian *Squalius* species and favoring its speciation process (Betzler et al., 2006; Martin et al., 2009, 2014).

In contrast to the limited distribution range of *S. malacitanus*, confined to rivers around the Gibraltar Strait, *S. pyrenaicus* is widespread throughout the central and southern Iberian Peninsula (Fig. 2). According to our molecular clock, *S. pyrenaicus* diverged from its sister species *S. valentinus* ca. 4 Ma during the Middle-Upper Pliocene, related to the transformation of the large Iberian Miocene endorheic lagoons into the current exorheic basins, which began in this period and culminated in the Pleistocene associated with tectonic reactivation of the region and resulting in tilting and local deformation of Neogene formations (Calvo et al., 1993; Pais et al., 2012). This timing of divergence and diversification during the Middle-Upper Pliocene is concordant with that reported for other *Squalius* species in the Iberian Peninsula, including *S. valentinus* (Doadrio and Carmona, 2006; Perea and Doadrio, 2015), *S. castellanus* (Doadrio et al., 2007b) and *S. laietanus* (Doadrio et al., 2007a). The Plio-Pleistocene has been considered an important period of diversification for Iberian cyprinids in general (Zardoya and Doadrio, 1998; Robalo et al., 2006; Sousa-Santos et al., 2007; 2014; Almada and Sousa-Santos, 2010), as well as other freshwater fauna (Ribera et al., 2004, 2011). The ancestral area reconstruction based on DEC and S-DIVA estimated a vicariance between the Spanish Levantine area, distribution range of *S. valentinus*, and the Guadalquivir Basin, in which inhabits *S. pyrenaicus*, as the most probable event isolating both species (marginal probability DEC=0.2; marginal probability S-

DIVA=1.0; Fig. 7). This vicariant event may be associated to the abovementioned formation of Iberian exorheic basins (Calvo et al., 1993; Pais et al., 2012).

4.2. Intraspecific diversification of *Squalius malacitanus*

The species *Squalius malacitanus* showed intraspecific phylogenetic and geographical structure on the basis of the mitochondrial and nuclear genes analyzed. Therefore, all analyses performed in this study supported the subdivision of *S. malacitanus* into two groups, Atlantic and Mediterranean populations. Our molecular clock showed that the separation of Atlantic from Mediterranean populations dated from *ca.* 5 Ma, close to the Miocene-Pliocene boundary. The same divergence pattern and timing of divergence between Atlantic (southwestern clade) and Mediterranean (central clade) populations is found in the reptile genus *Blanus* in southern Iberia (Albert et al., 2007). Subsequent to the opening of the Gibraltar Strait during the Pliocene, the areas nearest the present-day coast of southern Iberia remained marine basins, but were progressively filled by Neogene sediment with the uplift of the Betic Mountains throughout this period (Braga et al., 2003). This event would lead to the development of new freshwater habitats in the southernmost area of the Iberian Peninsula, close to the Gulf of Cádiz and the Gibraltar Strait, and the progressive isolation of Atlantic populations of *S. malacitanus* from the Mediterranean populations. Indeed, both DEC and S-DIVA reconstructions estimated a common ancestral area shared by Guadiaro and Miel basins (Mediterranean slope) and Jara basins (Atlantic slope) as the most probable for both Atlantic and Mediterranean populations, and a posterior vicariant event isolating Mediterranean populations (Guadiaro) from Atlantic populations (Miel and Jara) (marginal probability DEC=0.09; marginal probability S-DIVA=1.0; Fig. 6).

The high genetic diversity and low genetic structure found in *S. malacitanus* Atlantic

populations, as demonstrated the predominance of dispersal events among these Atlantic basins in both DEC and S-DIVA reconstructions (Fig. 6), can not be explained by downstream connections among basins except for Jara and Vega basins, as shown by our bathymetric reconstruction (Fig. 2), which shared a common ancestral area (marginal probability DEC=0.4; marginal probability S-DIVA=1.0; Fig. 6). A possible explanation for this genetic pattern (high genetic diversity and low genetic structure) is the presence of the Janda Lagoon during the Quaternary (Fig. 2). This lagoon, which in periods of heavy rainfall reached an area up to 50 km², was drained in the mid-twentieth century for pasture and agriculture (Recio, 2007). This large lagoon is likely to have played an important role in genetic diversity and structure of *S. malacitanus* and other freshwater fish species inhabiting the Iberian slope of Gibraltar Strait during the Pleistocene.

The Janda Lagoon has a tectonic origin related to the collision of the European and African plates, but it was not until the Middle-Upper Pleistocene that it acquired the form of an endorheic freshwater lagoon fed by the Barbate Basin, from which Celemín River is its main tributary, and adjacent basins that did not flow into the sea. In the LGM period, the lagoon was connected to the sea during the Flandrian transgression 15 000 years ago, when the Barbate Basin was open to the sea. Fluvial sediments in the narrow strait that linked the lagoon with the sea blocked the lagoon-sea connection again 3800 years BP (Goy et al., 1995; Luque et al., 1999). The lagoon allowed connection and gene flow among Atlantic populations of species such as *S. malacitanus*, supporting the sharing of mitochondrial haplotypes (low genetic structure; Table S2, Fig. 4) and high genetic diversity (Table S3). The lagoon likely harbored high effective populations of freshwater fish populations, acting as a reservoir of freshwater organisms.

Within Mediterranean populations of *S. malacitanus*, Guadiaro and Guadalmina basins did not share mitochondrial haplotypes, as evidenced the limited gene flow estimated between these two basins. Nevertheless, gene flow between Guadalmina and Guadaiza, two adjacent basins, was high (Table S5). DEC and S-DIVA reconstructions estimated a colonization from Guadiaro to Guadalmina (marginal probability DEC=0.26; marginal probability S-DIVA=1.0; Fig. 6) and a second colonization from either an ancestral area shared by Guadiaro and Guadalmina to Guadaiza (marginal probability DEC=0.25; Fig. 6) or from Guadalmina to Guadaiza (marginal probability S-DIVA=1; Fig. 6). This biogeographical relationships were also supported by the presence of one highly frequent nuclear allele shared by these three basins, which it has not been sort yet as consequence of the recent colonization of the Guadalmina and Guadaiza basins (Fig. 4).

Our bathymetric reconstruction showed that mouths of these Mediterranean basins were widely separated during the LGM (Fig. 2). The continental shelf at the Mediterranean slope of the Gibraltar Strait is extremely narrow as a consequence of old deep canyons formed by regressive erosion caused by an east-flowing river during the time that the strait was opening (Blanc, 2002; Loget and Driessche, 2006; García-Castellanos et al., 2009). Therefore, continental shelf in this Mediterranean area has been similar in width since the Miocene-Pliocene boundary, not allowing downstream connections among river basins. Guadiaro Basin has an arched shape and headwaters of some of its tributaries are very close to the headwaters of Guadalmina and Guadaiza, being the area of connection among these basins (Fig. 2). In addition, these Mediterranean basins flow through mountainous areas before reaching the sea. This complex geomorphology promoted vicariant events after the colonization of the different basins of southern Mediterranean basins by *S. malacitanus* leading to the

isolation of river basins and the presence of isolated and highly diverse mitochondrial lineages (Guadiaro and Guadalmina + Guadaiza), as well as the presence of low frequent private nuclear alleles in these three Mediterranean populations of *S. malacitanus*.

4.2. Intraspecific diversification of southern Iberian populations of *Squalius pyrenaicus*

The divergence of Atlantic from Mediterranean populations of *S. pyrenaicus* is dated at the Pleistocene, *ca.* 0.6 Ma, when the hydrographic network of the Iberian Peninsula was already well established (Calvo et al., 1993; Pais et al., 2012). Similar intraspecific divergences during the Pleistocene are reported in other Iberian *Squalius* species inhabiting areas near the Gulf of Cadiz in southwestern Portugal, such as *Squalius aradensis* (Mesquita et al., 2005). The shallow divergence time, the close mitochondrial relationship of Atlantic populations of southern Iberia with other northern basins, such the Guadiana, and the sharing of nuclear alleles among Atlantic and Mediterranean populations of this species in southern Iberia probably as consequence of incomplete lineage sorting (Maddison, 1997; Maddison and Knowles, 2006), suggest recent colonization of the southern Iberian region by this species. DEC and S-DIVA reconstructions supported this hypothesis of colonization for both Atlantic and Mediterranean populations (Fig. 7).

Atlantic populations of *S. pyrenaicus*, as same as *S. malacitanus*, presented higher genetic diversity than did Mediterranean (Table S3). Some of the Atlantic populations of *S. pyrenaicus* share mitochondrial haplotypes, such is the case of the Guadalquivir Basin with both Guadalete and Odiel basins, as also demonstrated the significant gene flow found among these Atlantic populations (Fig. 4; Table S5). Our DEC and S-DIVA reconstructions supported two different colonization events of the Guadalete Basin

coming from the Guadalquivir, being one of these dispersal events followed by a vicariance between both basins (marginal probability DEC=0.6 and 0.54; marginal probability S-DIVA=1.0 and 1.0; Fig. 7), which promoted the formation of an isolated mitochondrial lineage in the Guadalete Basin. Sea level change cycles during the Pleistocene affected Atlantic basins (Hernández-Molina et al., 1994; Mediavilla et al., 2004; Santisteban and Schulte, 2007). Nevertheless, no evidence of downstream connections between the Guadalquivir and Guadalete basins has been reported, as was borne out by our bathymetric reconstruction for the region (Fig. 2). Palaeogeographic reconstructions of the Guadalete Basin based on fluvial sediments indicate that this river has flowed through a separate incised valley since the Middle-Upper Pleistocene (Dabrio et al., 2000; Mediavilla et al., 2004), for this reason, other paleogeographical events must be invoked to explain the connection between Guadalquivir and Guadalete basins, as we discuss below. Similar events of dispersal from Guadalquivir Basin to Odiel Basin and posterior vicariance between these two basins were also estimated by DEC and S-DIVA reconstructions (marginal probability DEC=1.0; marginal probability S-DIVA=1.0; Fig. 7).

Mediterranean populations of *S. pyrenaicus* in southern Iberia constituted two mitochondrial isolated lineages, Guadalhorce + Vélez basins, between which gene flow was high, and Guadalfeo Basin, closely related to Guadalquivir Basin (Fig. 3, S1). However, Bayesian phylogeny based on concatenated data clustered together all Mediterranean populations of this species with high support (pp=0.9) (Fig. 3). Lower genetic diversity values and scarce genetic structure of Mediterranean populations of *S. pyrenaicus* are probably the consequence of recent founder effects, supported by the shallow divergence times estimated (Fig. 5). DEC analysis estimated a dispersal event from Guadalquivir to Vélez (marginal probability = 0.22; Fig. 7) and a second dispersal

from the ancestral area shared by Guadalquivir and Vélez to Guadalfeo, on one side, and to Guadalhorce on the other side (marginal probability = 0.30; Fig. 7). Posterior vicariance isolated Mediterranean populations from Guadalquivir. S-DIVA analysis showed, although with low support, a colonization of *S. pyrenaicus* from Guadalquivir (Atlantic slope) to Mediterranean populations (marginal probability = 0.05; Fig. 7) and a posterior vicariance between these two areas (marginal probability = 0.17; Fig. 7).

Since no evidence of downstream connections may be invoked nor in Atlantic neither in Mediterranean populations due to the wide separation of their river mouths and the narrow continental shelf in the region (Fig. 2), colonization of southern Iberia by *S. pyrenaicus* may have taken place through headwater capture involving river basins in the Betic Mountains, a region with a complex orography and geology (Weijermars, 1991). Evidence of stream piracy during the Pleistocene is common in the eastern regions of this mountain range and in other southeastern Iberian watersheds (Wenzens and Wenzens, 1995; Calvache and Viseras, 1997; Pais et al., 2012), and similar piracy patterns may have occurred in the Betic area involving the Guadalhorce and Vélez basins, in the Mediterranean slope, and the Guadalete and Odiel basins, in the Atlantic slope, which would explain the presence of common mitochondrial haplotypes in these two basins and their tributaries (Fig. 4). Fluvial captures have also been postulated in headwaters of other southwestern Iberian basins, such as the Arade River watershed, to explain the intraspecific structure of *S. aradensis* (Mesquita et al., 2005).

5. Conclusions

Our results demonstrate the influence of Cenozoic tectonic and climatic events in shaping the evolutionary history of freshwater fish fauna of the southern Iberian Peninsula (Fig. 8). We found two evolutionary patterns in two species of the cyprinid

genus *Squalius* inhabiting a common area, the southern Iberian basins, mainly shaped by the time of colonization of southern Iberia. *Squalius malacitanus* appeared in the region earlier and was affected by two major tectonic processes, the uplifting of the Betic Mountains, which isolated it from other Iberian *Squalius* species, and the opening of the Gibraltar Strait, which led to the divergence of the Atlantic and Mediterranean populations.

The presence of *S. pyrenaicus* in southern Iberia is more recent than the presence of *S. malacitanus*, as a consequence of recent multiple colonization of southern Iberia from the Guadalquivir Basin through river captures since the present configuration of the hydrological network was formed, as no evidence of downstream connections among basins was found, which is congruent with the low genetic structure found between its Atlantic and Mediterranean populations.

Our study suggests a key role of the Janda Lagoon for freshwater communities, supported by the higher genetic diversity of Atlantic populations of *S. malacitanus*, contrary to our previous expectations, as well as the lack of genetic structure in Atlantic populations of this species compared to the Mediterranean populations. The Janda Lagoon likely played an important role on genetic diversity and structure of freshwater fish species inhabiting the Iberian slope of Gibraltar Strait during the Quaternary.

Acknowledgements

The authors thank to people that have helped in field sampling trips, among them P. Garzón, J.L. González, B. Prieto, C.P. Ornelas, C. Pedraza and J. Manzano. We also thank to L. Alcaraz for her help in laboratory work and to F. Morcillo for her valuable help with bathymetric reconstruction. The Lucidus Consultancy contributed to improve

the manuscript with its English edition. The Spanish Ministry of Science and Innovation has supported this study through the project CGL2010-15231BOS.

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Table 1

Data of analyzed *S. malacitanus* and *S. pyrenaicus* populations and GenBank Accesion

Numbers. Sample size refers to number of specimens analyzed for *MT-CYB/RAG-I*

genes.

Map site	Sample size	River/Basin	Latitude	Longitude	GenBank Numbers
<u><i>Squalius pyrenaicus</i></u>					
1	8/1	Odiel/Odiel	36.69	-6.66	xxx
2	3/0	Rivera de Huelva/ Guadalquivir	37.95	-6.62	xxx
3	33/6	Rivera de Cala/Guadalquivir	37.91	-6.23	xxx
4	3/0	Viar/Guadalquivir	38.09	-6.14	xxx
5	9/0	Huéznar/Guadalquivir	37.86	-5.74	xxx
6	9/2	Rivera de Ciudadeja/ Guadalquivir	37.90	-5.42	xxx
7	4/3	Cabrillas/Guadalquivir	37.98	-5.06	xxx
8	6/4	Yeguas/Guadalquivir	38.32	-4.24	xxx
9	13/4	Cabrera/Guadalquivir	38.27	-4.09	xxx
10	28/6	Robledillo/Guadalquivir	38.42	-4.04	xxx
11	9/4	Montoro/Guadalquivir	38.50	-4.29	xxx

12	4/0	Fresneda/Guadalquivir	38.57	-3.77	xxx
13	24/7	Guadalete/Guadalete	36.84	-5.39	xxx
14	4/3	Guadalete tributary/Guadalete	36.92	-5.54	xxx
25	26/6	Grande/Guadalhorce	36.73	-4.86	xxx
26	8/0	Pereilas/Guadalhorce	36.66	-4.76	xxx
27	23/6	Turón/Guadalhorce	36.79	-4.95	xxx
28	30/7	Cuevas/Vélez	36.91	-4.29	xxx
28	19/6	Sabar/Vélez	36.94	-4.23	xxx
30	3/1	Guadalfeo	36.88	-3.42	xxx
-	8/	Guadalquivir Basin	-	-	627290- 627293; 627298; AF421790; AF421816- AF421817
-	30/0	Guadiana Basin	-	-	CG1-CG2; CG74-CG75; AF421805- AF421806; AF481813- AF421814;

AF421822-
AF421823;

627295-
627306;
263234-
263239;
045991

Squalius malacitanus

15	23/7	Celemín/Barbate	36.34	-5.81	xxx
16	20/10	Vega/Vega	36.07	-5.66	xxx
17	18/6	Miel/Miel	36.13	-5.45	xxx
18	16/8	Jara/Jara	36.01	-5.6	xxx
19	21/7	Hozgarganta/Guadiaro	36.43	-5.45	xxx
20	15/6	Guadiaro/Guadiaro	36.65	-5.28	xxx
21	18/8	Guadalevín/Guadiaro	36.74	-5.17	xxx
22	35/7	Genal/Guadiaro	36.56	-5.21	xxx
23	9/5	Guadaiza/Guadaiza	37.91	-6.23	xxx
24	33/6	Guadalmina/Guadalmina	36.52	-5.05	xxx

Other *Squalius* species

Squalius aradensis

AJ698711, AJ852503 (MT-CYB)

<i>Squalius castellanus</i>	DQ521423 (<i>MT-CYB</i>); HM560575 (<i>RAG1</i>)
<i>Squalius carolitertii</i>	AJ698453-AJ698456 (<i>MT-CYB</i>); HM560446 (<i>RAG1</i>)
<i>Squalius laietanus</i>	HM560187-HM560188 (<i>MT-CYB</i>)
<i>Squalius squalus</i>	HM560204-HM560205 (<i>MT-CYB</i>)
<i>Squalius torgalensis</i>	HM560210 (<i>MT-CYB</i>); HM560460 (<i>RAG1</i>)
<i>Squalius valentinus</i>	KR871719-KR871767 (<i>MT-CYB</i>); KR871768-KR871795 (<i>RAG1</i>)

Table 2

SAMOVA analysis of *MT-CYB* data for $k = 4$ (Atlantic and Mediterranean populations of *S. malacitanus* and Atlantic and Mediterranean populations of *S. pyrenaicus*). df = degrees of freedom; SSD = sum of squared deviation.

Source of variation	df	SSD	Variance components	Percentage variation
Among groups	3	7625.760	34.23596	95.17
Among populations				
Within groups	13	227.095	0.93613	2.60
Within populations	299	239.458	0.80086	2.23
Total	315	8092.313	35.97295	

FIGURE LEGENDS

Fig. 1. Distribution range of Iberian *Squalius* species and location of Betic Mountains.

Fig. 2. Sampled river basins and bathymetric contours for the LGM and present-day sea levels.

Fig. 3. Phylogenetic tree of *S. malacitanus* and *S. pyrenaicus* populations analyzed in this study derived from the BI and ML analyses of the concatenated dataset (*MT-CYB* + *RAG1*). Posterior probability values for BI and bootstrap values for ML (1000 replicates) are shown before and after slash respectively. Labels indicate river systems.

Fig. 4. **A.** Mitochondrial DNA haplotype network. **B.** Nuclear DNA haplotype network. The size of the circles is proportional to the frequencies of the haplotypes. Colors represent river systems. Numbers between haplotypes are the mutational steps.

Fig. 5. Species tree and divergence times for *S. malacitanus* and southern Iberian populations of *S. pyrenaicus* estimated by *BEAST software. Analysis is based on concatenated *MT-CYB* and *RAG1* genes, a relaxed molecular clock, and an evolutionary rate of 0.4% per lineage per million years. Bayesian probability >0.95 (*). Bayesian probability >0.99 (**).

Fig. 6. Ancestral area reconstruction of *S. malacitanus* using the Dispersal-Extinction-Cladogenesis (LAGRANGE) and the Statistical-DIVA (S-DIVA) models implemented in RASP software. * denotes vicariant events. Arrows indicates dispersal events.

Fig. 7. Ancestral area reconstruction of *S. pyrenaicus* using the Dispersal-Extinction-Cladogenesis (LAGRANGE) and the Statistical-DIVA (S-DIVA) models implemented in RASP software. * denotes vicariant events. Arrows indicates dispersal events.

Fig. 8. Divergence and biogeographical events in *S. malacitanus* and *S. pyrenaicus*.

Figure 1

Figure 2

ACCEPTED MANUSCRIPT

Figure 4

A) *Squalius malacitanus*

B) *Squalius pyrenaicus*

Figure 5

Figure 6

Figure 7

Figure 8

Graphical abstract

Highlights

- The origin of *Squalius malacitanus* dated from Serravalian-tortonian period
- Divergence between Atlantic and Mediterranean populations of *S. malacitanus* is associated to the creation of the Gibraltar Strait
- Squalius pyrenaicus* colonized southern Iberian from the Guadalquivir Basin during Pleistocene likely through river piracy
- The Iberian Janda lagoon acted as a reservoir for freshwater fish populations during Pleistocene