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Evolutionary and biogeographical patterns within Iberian populations of the genus *Squalius* inferred from molecular data

Oris I. Sanjur,[a] José A. Carmona,[b] and Ignacio Doadrio[b,*]

[a] *Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Panama*
[b] *Museo Nacional de Ciencias Naturales, Departamento de Biodiversidad, C/José Gutiérrez Abascal, 2. 28006 Madrid, Spain*

Abstract

Origins and biogeography of the chub, genus *Squalius* (formerly within the genus *Leuciscus*), in the Iberian Peninsula were inferred from comparison between patterns of geographic distribution and phylogenetic relationships among populations belonging to 14 European *Squalius* species. The phylogeny recovered was based on the complete sequence of the mitochondrial cytochrome *b* gene. *Squalius* species were grouped into three major clades. The basal clade included species distributed across the ancient Paratethys Sea. The second clade included species from Central and East Europe and the northern areas of the Mediterranean basin towards Minor Asia. The third clade included species from the Mediterranean Peninsulas (Iberian, Italy, and Balkans). The Iberian *Squalius* species do not constitutes a monophyletic group. Our data indicate that the Iberian Peninsula was colonized at least twice by two different monophyletic lineages, a meridional group and a Central Europe group. The amount of species diversity found in the Iberian Peninsula and the phylogenetic relationships among these species, together with their geographic distribution, suggest that the Central Europe lineage colonized the Iberian Peninsula at a latter time. Our data indicate that the northeastern Iberian lineage is phylogenetically close to Greek populations of *Squalius cephalus*, while the second lineage formed a monophyletic group including *Squalius pyrenaicus*, *Squalius carolitertii*, *Squalius aradensis*, and *Squalius torgalensis*. The speciation process that generated these species and the geographic structure of their populations, principally in *S. pyrenaicus*, can be attributed to paleogeographical events like the ancient endorrheism and the development of hydrographic basins.
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1. Introduction

The Mediterranean basin has been identified as one of the 25 ''biodiversity hotspots'' where exist an exceptional concentration of endemic species, with an estimation of the 0.9% of endemic vertebrates and the 4.3% of endemic plants of the world (Myers et al., 2000). The main process that has originated such a high diversity is directly related to important geological and climatic changes that altered this area, specially during the Cenozoic period. The study of this diversity requires, besides geological studies, information concerning the distribution patterns and evolution of the biota in order to test different paleogeographical hypothesis.

It is commonly accepted that the evolution and distribution patterns of primary freshwater fishes reflect the paleogeographical complexity of a region, especially the development of hydrographic basins and their isolation and interconnection processes (Bermingham and Martin, 1998; Doadrio, 1988; Durand et al., 1999a). Therefore, historical biogeographical analyses of freshwater fishes allow to infer information regarding the biotic and geological evolution of a region (Lundberg, 1993).

The origin and diversity of endemic freshwater fishes in the Mediterranean peninsulas have been explained by three biogeographical hypotheses, all of them assuming the Asiatic origin of the ancestral European freshwater fish fauna (Banarescu, 1989, 1992). The first hypothesis considers that the dispersal of Asian freshwater fishes across central Europe via river connections might have been possible only during the Oligocene owing to the

* Corresponding author.
E-mail address: mcnd147@mncn.csic.es (I. Doadrio).

uplift of the Urals (Banarescu, 1992). The spread of cyprinids to the southern part of Europe and northern Africa across the Gibraltar strait was possible until the Pliocene and subsequent isolation of the Iberian Peninsula and the southern Greece would have been the responsible for the rich endemic fauna (Almaça, 1976; Banarescu, 1964, 1989, 1992). Nevertheless, the uniform cyprinid fauna of central Europe, depleted during the glacial periods, was replaced with Danubian cyprinids fauna during interglacial and postglacial periods (Banarescu, 1992)

The second hypothesis (Bianco, 1990) postulates a Mediterranean dispersal of an Asian ancestor through the lacustre Lago-Mare phase of the Mediterranean Sea during the Messinian Crisis in the Pliocene (5.5 MYA), when the Mediterranean basin almost dried up and was refilled with fresh water from the Sarmatic Sea (Parat-ethys) (Hsü et al., 1977). According to this hypothesis the Lago-Mare phase of the Mediterranean would be the responsible of a dispersal of cyprinids around the circum-Mediterranean region and the actual high level of endemics on the Iberian Peninsula and southern Greece (Bianco, 1990).

The third hypothesis suggests that dispersions occurred through intercontinental land-bridges during the formation of the actual North African coast in the Early Pliocene. The land-mass movements across the Mediterranean Sea would favored the South Mediterranean vicariance of cyprinids and the subsequent colonization of northern Africa and the Iberian Peninsula (Doadrio, 1990).

Cyprinids represent a model group to test these biogeographical hypotheses. The Cyprinidae is one of the most successful families of the European freshwater fish fauna with a wide distribution range and ancient origin. The two major subfamilies, the Cyprininae and the Leuciscinae are thought to have originated during the Oligocene (Cavender and Coburn, 1992; Chen et al., 1984; Zardoya and Doadrio, 1998). For instance, the Cyprininae may have originated in the mid-Oligocene (Gaudant, 1977) while the earliest cyprinid fossils recovered in the Iberian Peninsula correspond to a Leuciscinae from the Late Oligocene (Cabrera and Gaudant, 1985).

Within the Leuciscinae, the genus Squalius is an important ecological and evolutionary component in most aquatic communities of the Paleartic region. This genus, commonly known as chub, was formerly considered as a subgenus within the genus Leuciscus (Banarescu, 1964; Doadrio and Carmona, 1998) but current phylogenetic studies (Briolay et al., 1998; Gilles et al., 1998) demostrated that the Squalius species are not closely related to Leuciscus leuciscus, the type species for the genus. Squalius includes approximately 15 species (Doadrio and Carmona, 1998; Kottelat, 1997), two of them widely reported in central and North Europe while the rest (13 species) are only found in the Mediterranean basin.

The genus has received much attention from taxonomist and evolutionary biologist. Most of this attention has been focussed in Central Europe and in the Balkan area were the phylogenetic relationships seem to be well-resolved both at the family (Briolay et al., 1998; Zardoya and Doadrio, 1998, 1999; Zardoya et al., 1999) and generic level (Doadrio and Carmona, 1998; Durand et al., 1999a,b, 2000; Imsiridou et al., 1998). Nevertheless, the Iberian Peninsula has remained poorly studied. Molecular analysis on Portuguese populations of Squalius revealed a considerable population differentiation (Brito et al., 1997; Coelho et al., 1995), which led to the recent description of two new Squalius species (Coelho et al., 1998). Those results suggest that the diversity of this genus might be underrepresented, and that phylogenetic relationships among species and populations from the Iberian Peninsula needed to be revisited. The phylogenetic analyses constitute a previous step to any biogeographical study in which among-area relationships are intended to be resolved (Bermingham and Avise, 1986; Bermingham and Martin, 1998). Geological events such as the uplift of the Pyrinees Mountains and the opening of the Gibraltar Strait isolated the freshwater fish fauna of the Iberian Peninsula at least 5.3 MYA (Lourens et al., 1996). However, it is not clear if the genus Squalius colonized the Iberian Peninsula through the land connection with Europe before the uplift of the Pyrinees or during the Mediterranean "Lago-Mare phase." The ancient fossil record, together with the comparison between patterns of geographic distribution and phylogenetic relationships among species are useful tools to discriminate among the alternative hypothesis regarding the origins of the genus Squalius in the Iberian Peninsula.

In the present study we determined phylogenetic relationships among species of the genus Squalius from all Mediterranean peninsulas. The inferred phylogeny is then used to revise the systematic of the genus, and to test biogeographical hypotheses concerning the origins of the cyprinids in the Mediterranean areas. An especial analysis on the origins of the genus Squalius in the Iberian Peninsula was made. To do that we determined and analyzed the complete cytochrome b (1140 bp) of 39 Squalius samples from the Iberian Peninsula and Italy. In addition, 24 samples from other European Squalius species were included in the analyses and used to construct a phylogeny.

2. Materials and method

The cytochrome b sequences of 63 individuals representing 14 Squalius species were analyzed (Fig. 1, Table 1). Among these, 39 are new sequences from individuals representing populations of Squalius pyrenaicus, Squalius carolitertii, Squalius aradensis, and Squalius cephalus
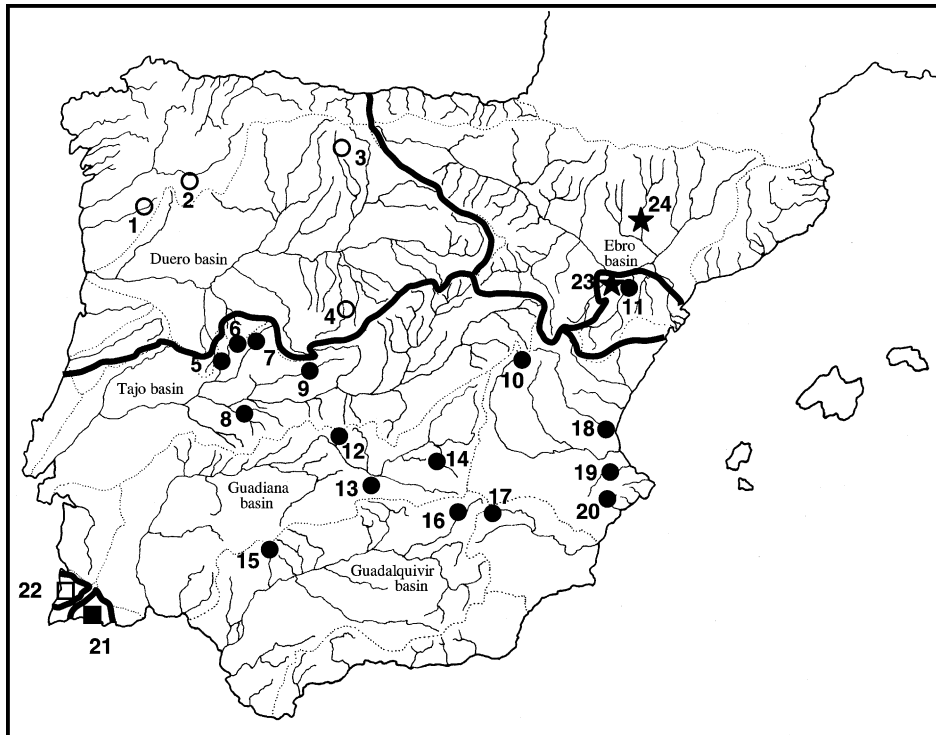
Fig. 1. Collection sites of the Iberian samples analyzed. *Squalius carolitertii* (open circles): 1, Salas; 2, Bibei; 3, Boedo; 4, Adaja; *Squalius pyrenaicus* (filled circles): 5, Pesquero; 6, Acebo; 7, Arrago; 8, Almonte; 9, Tiétar; 10, Uña Lagoon; 11, Matarraña; 12, Estena; 13, Azuer; 14, Ruidera Lagoons; 15, Montemayor; 16, Guadalmena; 17, Bogarda; 18, Turia; 19, Serpis; 20, Algar; *Squalius aradensis* (filled squares): 21, Arade; *Squalius torgalensis* (open squares): 22, Torgal; and *Squalius cephalus* (filled stars): 23, Matarraña; 24, Cinca. Thick lines show the distribution border among the five Iberian *Squalius* species.

from the Iberian Peninsula, and *Squalius lucumonis* and *S. c. cabeda* from Italy. These sequences were aligned by hand using as Ref. [24] published cytochrome *b* sequence data belonging to the seven *Squalius* species from Greece (Zardoya and Doadrio, 1999), *S. pyrenaicus*, *S. carolitertii*, and *S. cephalus* from Spain (Zardoya and Doadrio, 1998), *S. aradensis* and *Squalius torgalensis* from Portugal (Brito et al., 1997), *Squalius illyricus* and *Squalius zrmanjae* from Croatia (Durand et al., 2000), *Squalius lepidus*, and *Squalius smyrnaeus* from Turkey (Durand et al., 2000), and *Squalius c. cephalus* from France (Briolay et al., 1998) (see Table 1 for all GenBank Accession Nos.). *Rutilus rutilus*, *R. rutilus caspicus*, *Chondrostoma lusitanicum*, and *C. willkommii* (Zardoya and Doadrio, 1999) were used as outgroups in the phylogenetic analyses.

Total cellular DNA was extracted from a piece of 1–3 cc of dorsal muscle by phenol/chloroform extraction and ethanol precipitation (Towner, 1991). PCR amplification of the entire cytochrome *b* gene was carried out using the primers L14724 and H15915 designed by Schmidt and Gold (1993). Reactions were performed in a total volume of 25 µl containing approximately 67 mM Tris–HCl, pH 8.3, 1.5 mM MgCl$_2$, a 0.4-mM concentration of each dNTP, a 2.5-µM concentration of each primer, template DNA (10–100 ng), and Taq DNA polymerase (1 U; Promega). Double-stranded product was amplified using the following cycling profile: initial denaturation at 92 °C for 2 min; 35 cycles of denaturation at 92 °C for 1 min, annealing at 50 °C for 1.5 min, and extension at 72 °C for 6 min; and a final extension at 72 °C for 10 min.

PCR products were sequenced using the FS-Taq Dye Deoxy Terminator Cycle-sequencing Kit (Applied Biosystems) on an automated DNA sequencer ABI 377 (Applied Biosystems) following the manufacturer's instructions.

Alignment was based on the inferred amino acid sequence. No ambiguous alignments were found and no gaps were postulated. All codon positions were included in the phylogenetic analyses. Nucleotide saturation was assessed by plotting the number of transitions and transversions against patristic distance values (Fig. 2). Distance, Maximum-parsimony (MP), and Maximum-likelihood (ML) analyses were performed using PAUP* version 4.0b10 (Swofford, 2002). For MP, a heuristic search was conducted with 10 random stepwise additions of taxa, followed by branch swapping using the TBR routine (MULPARS option in effect). A transition/ transversion ratio was estimated from the data, and transversions (Tv) were given five times the weight of transitions (Ts).

Table 1

Species	Locality	River	Basin (population designation)	GenBank Accession No.
S. carolitertii	Rubia de los Mixtos (Spain)	Salas	Limia (1)	AF421795[a]
	Rubia de los Mixtos (Spain)	Salas	Limia (1)	AF421796[a]
	San Agustin (Spain)	Bibei	Miño (2)	AF421793[a]
	San Agustin (Spain)	Bibei	Miño (2)	AF421794[a]
	Bacones de Ojeda (Spain)	Boedo	Duero (3)	AF421797[a]
	Bacones de Ojeda (Spain)	Boedo	Duero (3)	AF421798[a]
	El Fresno (Spain)	Adaja	Duero (4)	AF421799[a]
	El Fresno (Spain)	Adaja	Duero (4)	AF421800[a]
	El Fresno (Spain)	Adaja	Duero (4)	AF045994
S. pyrenaicus	Valverde del Fresno (Spain)	Pesquero	Tajo (5)	AF421811[a]
	Valverde del Fresno (Spain)	Pesquero	Tajo (5)	AF421812[a]
	Hoyos (Spain)	Acebo	Tajo (6)	AF421827[a]
	Cadalso de Gata (Spain)	Arrago	Tajo (7)	AF421826[a]
	Jaraicejo (Spain)	Almonte	Tajo (8)	AF421791[a]
	Talayuela (Spain)	Tiétar	Tajo (9)	AF045993
	Una (Spain)	Laguna de Una	Jucar (10)	AF421806[a]
	Una (Spain)	Laguna de Una	Jucar (10)	AF421807[a]
	Nonaspe (Spain)	Matarrana	Ebro (11)	AF421802[a]
	Navas de Estena (Spain)	Estena	Guadiana (12)	AF421813[a]
	Navas de Estena (Spain)	Estena	Guadiana (12)	AF421814[a]
	Navas de Estena (Spain)	Estena	Guadiana (12)	AF045991
	Casa de Fuente Vieja (Spain)	Azuer	Guadiana (13)	AF421804[a]
	Casa de Fuente Vieja (Spain)	Azuer	Guadiana (13)	AF421805[a]
	Ossa de Montiel (Spain)	Lagunas de Ruidera	Guadiana (14)	AF421822[a]
	Ossa de Montiel (Spain)	Lagunas de Ruidera	Guadiana (14)	AF421823[a]
	Cañaveral de León (Spain)	Montemayor	Guadalquivir (15)	AF421790[a]
	Alcaraz (Spain)	Guadalmena	Guadalquivir (16)	AF421816[a]
	Alcaraz (Spain)	Guadalmena	Guadalquivir (16)	AF421817[a]
	Las Moedas (Spain)	Bogarda	Segura (17)	AF421820[a]
	Las Moedas (Spain)	Bogarda	Segura (17)	AF421821[a]
	Sollana (Spain)	Font de Barret	Turia (18)	AF421808[a]
	Sollana (Spain)	Font de Barret	Turia (18)	AF421809[a]
	Beniarres (Spain)	Serpis	Serpis (19)	AF421810[a]
	Beniarres (Spain)	Serpis	Serpis (19)	AF421815[a]
	Callosa d'en Sarria (Spain)	Algar	Algar (20)	AF421818[a]
	Callosa d'en Sarria (Spain)	Algar	Algar (20)	AF421819[a]
S. aradensis	Portugal	Arade	Arade (21)	X99743
	Portugal	Arade	Arade (21)	X99744
	Portugal	Arade	Arade (21)	X99745
	Portugal	Arade	Arade (21)	AF421824[a]
	Portugal	Arade	Arade (21)	AF421825[a]
S. torgalensis	Portugal	Torgal	Mira (22)	X99742
	Portugal	Torgal	Mira (22)	Z75929
S. cephalus	Nonaspe (Spain)	Matarrana	Ebro (23)	AF421801[a]
	NonaSpaine (Spain)	Matarrana	Ebro (23)	AF045995
	Monzón (Spain)	Cinca	Ebro (24)	AF421803[a]
	France	Rhône	Rhône	Y10446
S. c. macedonicus	Paranesti (Greece)	Nestos	Nestos	AF090752
	Oxilothos (Greece)	Manikiotiko	Manikiotiko	AF090755
S. c. cabeda	Carmagnola (Italy)	Po	Po	AF421792[a]
S. lucumonis	Istia d'Ombrone (Italy)	Ombrone	Ombrone	AF421828[a]
S. c. vardarensis	Lamia (Greece)	Sperchios	Sperchios	AF090754
S. c. orientalis	Cirtich (Daguestan)	Rubas	Samur	AF095609
S. prespensis	Psarades (Greece)	Lake Prespa	Lake Prespa	AF090753
S. peloponnensis	Igoumenitsa (Greece)	Thyamis	Thyamis	AF090756
	Karitana (Greece)	Alphios	Alfphos	AF090757
S. p. moreoticus	Stymphalia (Greece)	Lake Stymphalia	Lake Stymphalia	AF090758
S. keadicus	Sparta (Greece)	Evrotas	Evrotas	AF090760
S. borysthenicus	Fotolivos (Greece)	Filiouris	Filiouris	AF090759
S. lepidus	Turkey	Lake Beyshir	Lake Beyshir	AJ252812
S. smyrnaeus	Turkey	Gumuldur	Gumuldur	AJ252814
S. illyricus	Croatia	Cetina	Cetina	AJ251094
S. zrmanjae	Croatia	Zrmanje	Zrmanje	AJ251093
Chondrostoma willkommii	Spain	Guadalquivir	Guadalquivir	AF045984

Table 1 (continued)

Species	Locality	River	Basin (population designation)	GenBank Accession No.
Chondrostoma lusitanicum	Portugal	Arade	Arade	AF045986
Rutilus rutilus	France	Saone	Saone	Y10440
Rutilus r. caspicus	Daguestan	Rubas	Samur	AF095610

[a] New sequences obtained in this study.
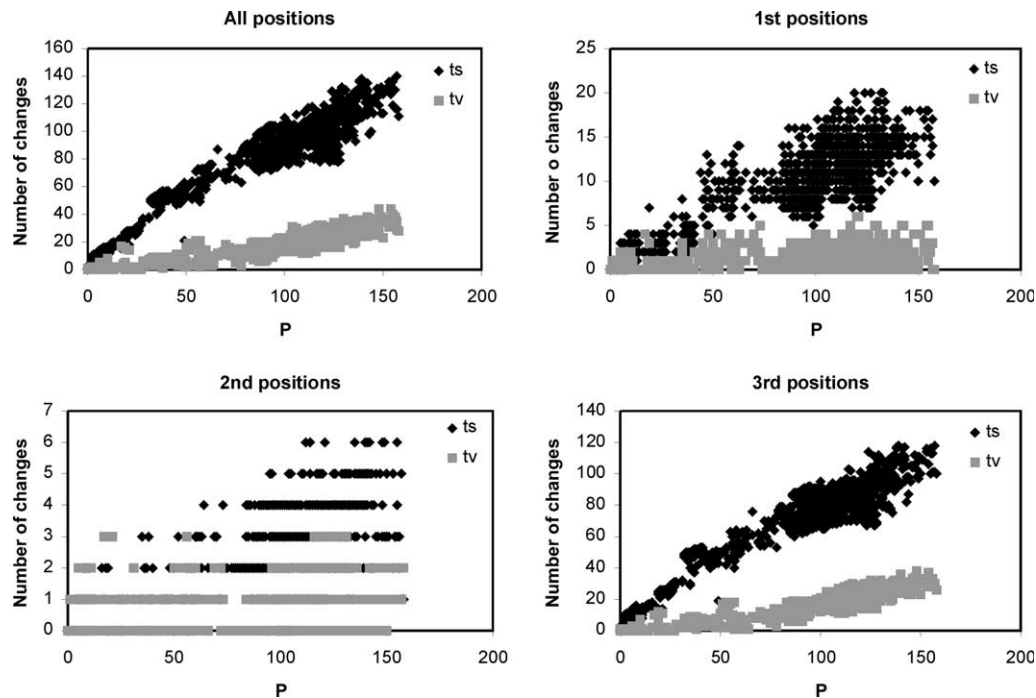
Fig. 2. Saturation processes of cytochrome *b* gene sequence. Transitions (Ts) and transversions (Tv) at different codon positions were plotted against patristic distances.

Neighbor-joining (NJ) (Saitou and Nei, 1987) analyses based on general time reversible (GTR) distance matrices were performed with PAUP* version 4.0b10 (Swofford, 2002). Maximum-likelihood (ML) analyses were performed using the Quartet Puzzling method (Strimmer and Von Haeseler, 1996) (implemented with PAUP*) and the fastDNAml 1.0.6 program (Olsen et al., 1994). Previously, the Modeltest 3.04 program (Posada and Crandall, 1998) was implemented for the ML analyses to find the best model of evolution that fit our data. As result, the GTR + G + I model with empirical base frequencies, proportion of invariable sites (0.54), and gamma distribution ($\Gamma = 1.45$) was employed. Robustness of the inferred MP, NJ, and ML trees was tested by bootstrap analysis (Felsenstein, 1985) with 500, 1000, and 1000 pseudoreplications, respectively.
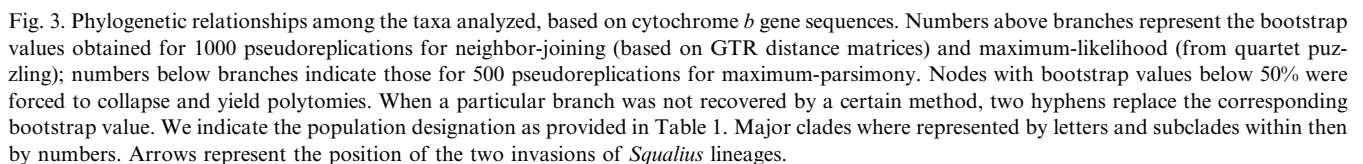
In order to determine if cytochrome *b* sequences of *Squalius* species are evolving at the same rate, we tested the hypothesis of a molecular clock using the likelihood ratio test (Page and Holmes, 1998). If the hypothesis of a molecular clock is accepted, then we can estimate divergence time using a calibrated molecular clock for cyprinids. We also performed a relative rate test using the CODRATES program (Muse and Gaut, 1994) to further identify the taxa that had significantly different rates of evolution.

To analyze the percentage of genetic variance due to variation within and among different hydrographical basins for *S. carolitertii* and *S. pyrenaicus* we performed the analysis of molecular variance procedure (Excoffier et al., 1992) in the AMOVA option of ARLEQUIN v2000 (Schneider et al., 2000).

3. Results

The complete nucleotide sequence of the mitochondrial cytochrome *b* gene was determined in 39 European specimens of the genus *Squalius*. A total of 1140 positions were analyzed, of which 713 were constant sites, and 345 were phylogenetically informative sites under the parsimony criterion. An overall Ts/Tv ratio of 5.59 was estimated for this data set, although different weighting scenarios recovered similar and congruent

Fig. 3. Phylogenetic relationships among the taxa analyzed, based on cytochrome *b* gene sequences. Numbers above branches represent the bootstrap values obtained for 1000 pseudoreplications for neighbor-joining (based on GTR distance matrices) and maximum-likelihood (from quartet puzzling); numbers below branches indicate those for 500 pseudoreplications for maximum-parsimony. Nodes with bootstrap values below 50% were forced to collapse and yield polytomies. When a particular branch was not recovered by a certain method, two hyphens replace the corresponding bootstrap value. We indicate the population designation as provided in Table 1. Major clades where represented by letters and subclades within then by numbers. Arrows represent the position of the two invasions of *Squalius* lineages.

topologies. Pairwise sequence divergence among ingroup taxa varied from 2.16 to 16.26%. Variation among sequences was mainly detected in third codon positions. Nonetheless, third positions were not saturated (Fig. 2).

The NJ, MP, and ML analyses arrived at similar and congruent trees. An exception is made for the relative position of *S. c. orientalis* and *S. lepidus* which show a basal position respect to other Central European species in the MP tree. The robustness of the NJ, MP, and ML trees was supported with bootstrap analysis. *Squalius* species were grouped into three major clades (Fig. 3). The basal clade (A) included the samples of *S. borysthenicus* and *S. smyrnaeus*.

The clade B included specimens of *S. cephalus*, *Squalius peloponnensis*, *Squalius prespensis*, and *S. lepidus* from Central Europe, northern Spain, northern Italy, northern and Central Greece, Turkey, and Minor Asia. This clade was formed by four different groups. The group B1 included *S. c. orientalis* and *S. lepidus* from east Europe and Asia which are in a basal position. The group B2 consisted of *S. cephalus* populations from France and northwestern Greece (*S. c. cephalus* and *S. c. vardarensis*). The group B3 included the northeastern Spanish population (Ebro basin) of *S. cephalus* together with *S. c. macedonicus* from North Greece and Euboea Island. The group B4 included samples from Italy and south and east Greece including *S. c. cabeda*, *S. prespensis*, *S. peloponnensis*, and *S. p. moreoticus*.

The clade C included samples from the Iberian Peninsula, Italy, Croatia and the southern part of Greece, belonging to *S. carolitertii*, *S. pyrenaicus*, *S. aradensis*, *S. torgalensis*, *S. lucumonis*, *S. illyricus*, *S. zrmanjae*, and *S. keadicus* (Fig. 3). Several groups can be recognized within this clade. The group C1 was formed by *S. lucumonis* from Italy and *S. illyricus* and *S. zrmanjae* from Croatia while group C2 included *S. keadicus* from Greece. Phylogenetic relationships between both groups remain unresolved but seen to be basal to the four endemic Iberian species that conformed the remaining three groups (Fig. 3). The group C3 included *S. torgalensis* and *S. aradensis*, which are restricted to the southwestern extreme of Portugal. The group C4 included all samples of *S. carolitertii* and the group C5 consisted of the *S. pyrenaicus* populations in which the samples from Levantine rivers of the Mediterranean slope of Spain were the most differentiated.

The *S. pyrenaicus* populations (group C5) showed high levels of pairwise sequence divergence (uncorrected *p* distances) ranging from 0 to 3.45%, while *S. carolitertii* (group C4) showed a sequence divergence ranging from 0 to 0.35%. Within *S. carolitertii*, differentiation among populations from the Duero and the Galician (Limia and Miño) basins is suggested by bootstrap values. Among *S. pyrenaicus* specimens, four different groups were observed. The first group included populations from the Guadiana Basins and lower Guadalquivir basin. The second group included populations from Guadalquivir and Segura Basins. The third group was formed by populations from the Tajo, Jucar and Ebro basins. Interestingly, populations from the northwestern part of the Tajo basin were highly differentiated. The fourth group (supported with high bootstrap values) included populations from the Mediterranean slope (Algar, Turia, and Serpis rivers), which were highly differentiated from the rest of *S. pyrenaicus* populations (uncorrected *p* distances ranging from 2.24 to 3.45%).

According to the AMOVA analysis, nearly 41% of the variation detected in *S. carolitertii* was due to differences among populations from the Duero and the Galician basins, whereas most of the variation was due to within-population differences. For *S. pyrenaicus*, more than 73% of the variation detected was explained by differences among the populations belonging to the four groups described above within the subclade C5, whereas 4.9% of the variation was due to among-population differences within each group.

Results from the relative rate test indicated that *S. aradensis* and *S. torgalensis* are evolving significantly faster than the rest of the *Squalius* species.

4. Discussion

Nucleotide substitution rate for the cytochrome *b* gene resulted appropriate in establishing the phylogenetic relationships among *Squalius* lineages at species and population level. The phylogenetic relationships inferred by the molecular data are congruent with those previously obtained for the genus *Squalius* (Briolay et al., 1998; Durand et al., 1999a; Zardoya and Doadrio, 1999; Zardoya et al., 1999).

Three main clades were detected for the analyzed *Squalius* species with *S. borysthenicus* and *S. smyrnaeus* (clade A) basal to the rest of the species. This supports the hypothesis of a paratethyan origin of this genus (Doadrio and Carmona, 1998; Zardoya et al., 1999) since these two latter species are distributed around the ancient Parathetys Sea. Previous studies (Economidis and Banarescu, 1991) pointed out the close relationship between the Eastern Balkan and the Anatolian fish fauna, where at least seven endemic species can be found. The role played by the Parathetys Sea in this area became very important (Banarescu and Coad, 1991; Economidis and Banarescu, 1991) since promoted the isolation of this region from Central and Eastern Europe until the Late Miocene. Nevertheless, the Parathetys Sea developed into a complex of freshened lakes, favoring the current diversity in rivers belonging to the Black, Caspian, and Aral seas and the northern slopes of the Aegean Sea (Banarescu and Coad, 1991).

The clade B, formed by *S. cephalus*, *S. prespensis*, *S. peloponnensis*, and *S. lepidus* populations from Spain, France, Greece, Russia, and Turkey, is congruent with previously published data (Durand et al., 2000; Zardoya et al., 1999). This clade can be subdivided into two groups: the *S. cephalus* group, which is distributed across Central and East Europe (groups B2 and B3 in Fig. 3); and a North Mediterranean group, which includes the rest of species (groups B1 and B4 in Fig. 3). A similar scenario has been reported for the genus *Barbus* (Zardoya and Doadrio, 1999), in which the subgenus *Barbus* was differentiated into two subgroups, a Central European lineage (*B. barbus* and related species) and a North Mediterranean lineage (*B. meridionalis* and related species). Interestingly *S. cephalus cabeda* did not group with other *S. cephalus* populations, suggesting a taxonomic and nomenclatural discrepancy that is also obvious for *S. cephalus orientalis*.

The clade C is composed by the Iberian *S. pyrenaicus*, *S. carolitertii*, *S. aradensis*, and *S. torgalensis* with *S. keadicus* from the southern extreme of Greece, *S. lucumonis* form Italy and *S. illyricus* and *S. zrmanjae* from Croatia as sister taxa. The geographical connection among Minor Asia, Greece, Balkans, Italy, and the Iberian Peninsula during the Miocene, well documented for mammals (Barbadillo et al., 1997), may have provided the conditions for the origin of the meridional lineage (Fig. 4). When such wide connection broke down, allopatric isolation may have originated the diversity that we currently appreciate (Doadrio, 2001).

The present distribution and the phylogenetic relationships among *Squalius* species indicate that the ancestors of clades B and C diverged just before they colonized the Mediterranean basin. Following the calibration of a molecular clock of 0.76% sequence divergence per million years for European cyprinids (Zardoya and Doadrio, 1998) the divergence between both northern and meridional lineages should took place in the Late Miocene (7 MYA) (Fig. 4). Additional evidence for this argument is given by *Squalius* species from Italy,
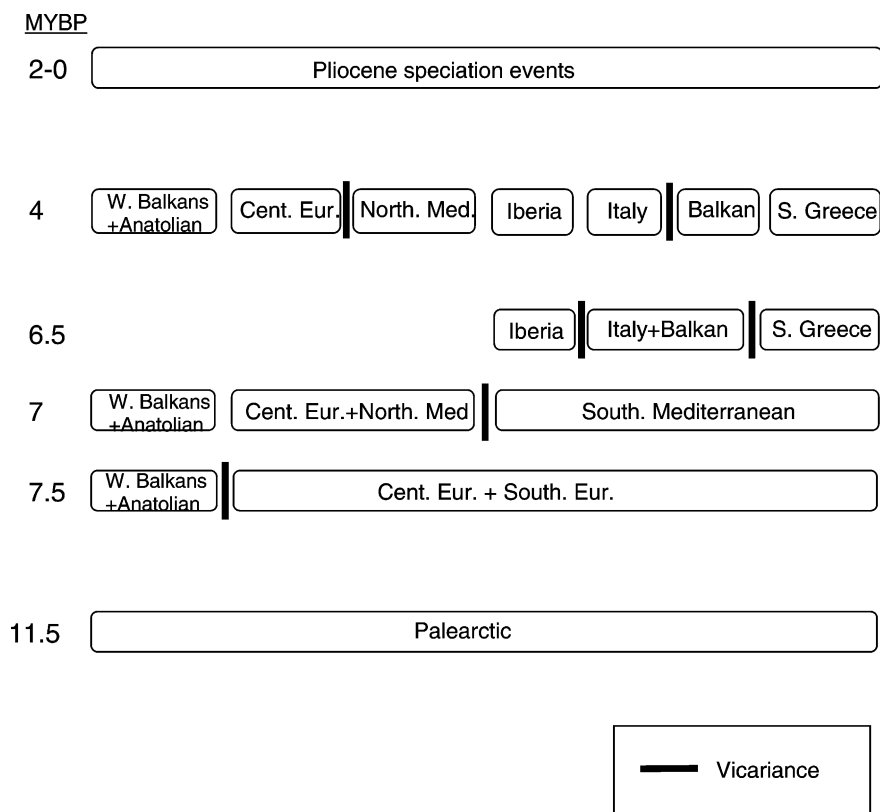

Fig. 4. Paleogeographical hypothesis of *Squalius* evolution showing major vicariance events. Following the calibration of a molecular clock of 0.76% sequence divergence per million years for European cyprinids (Zardoya and Doadrio, 1998) *Squalius* are supposed to appear during the Miocene (11.5 MYA). Data show an early radiation of *Squalius* species during the Late Miocene. The first vicariant split of *Squalius* species could have occurred in western Balkans and Anatolian favouring the differentiation of *S. borysthenicus* and *S. smyrnaeus* (probably at the ancient Paratethys that evolved into a brackish water lake) (clade A on Fig. 3). The second vicariant event promoted the isolation of species which inhabited Central Europe + northern Mediterranean and southern Mediterranean (clade B vs clade C on Fig. 3). A third vicariant event allowed the differentiation of the Iberian *S. aradensis*, *S. carolitertii*, *S. pyrenaicus*, and *S. torgalensis* in the Southwestern Mediterranean and *S. keadicus* in the southeastern Mediterranean. During the Early Pliocene new events split the *S. cephalus* group in Central Europe and the rest of the northern Mediterranean species. The establishment of the actual hidrographical systems led to a major speciation event in *Squalius*.

Croatia, and Greece (i.e., *S. lucumonis* from Italy, is more closely related to Greek and Iberian *Squalius* than to other Italian congeners).

4.1. The Iberan Squalius species

Regarding the origins of the Iberian *Squalius* species, our results showed that they do not constitutes a monophyletic group. Two different lineages were detected in the Iberian Peninsula, suggesting that the colonization of this area was not a single event, but probably the result of at least two invasions. One of the two lineages, corresponding to the northeastern Iberian population of *Squalius*, is phylogenetically much closer to the northern Greek and Central Europe populations of *S. cephalus* than to any other species in the Iberian Peninsula. The second lineage found within the Iberian Peninsula included *S. pyrenaicus*, *S. carolitertii*, *S. aradensis*, and *S. torgalensis*, these species forming a monophyletic group. These results were congruent with those of Brito et al. (1997) since corroborated that all Portuguese *Squalius* species belong to a single group, but the larger list of *Squalius* species analyzed in our work allowed to extend the conclusions rejecting that this lineage was originated from northern European *S. cephalus*. *S. aradensis* and *S. torgalensis* grouped together and appeared basal to *S. pyrenaicus* and *S. carolitertii* indicating an early differentiation, probably during the Messinian period (6 MYA). In this period, it is likely that many basins almost dried up and the southwestern part of the Iberian Peninsula became isolated. This result supports previous studies based on allozymes and partial cytochrome *b* sequences (Brito et al., 1997; Coelho et al., 1995).

The speciation process that generated *S. carolitertii* and *S. pyrenaicus* and the geographic structure of their populations can be attributed to the earlier split of the Duero basin in relation to the remaining Iberian drainages, which conform an ancient endorrheic lagoon in the Miocene period (De la Peña, 1995; López-Martínez, 1989). The rest of the Iberian rivers started to get their configuration during Pliocene and almost all of them have been isolated from one another since this time.

Although little variation was found within *S. carolitertii*, populations from the Duero basin appeared lightly differentiated from that belonging to Limia and Miño basins in the northwestern part of the Iberian Peninsula. In contrast, *S. pyrenaicus* showed high level of genetic variation. Genetic differentiation patterns based in hydrographical catchments were also detected among the *S. pyrenaicus* populations. Populations from the Levantine rivers Algar, Serpis, and Turia were clustered as a separate and basal group respect to the remaining populations of *S. pyrenaicus* (Fig. 3). Levels of genetic divergence between these and the other

Squalius populations were the highest, suggesting that these populations have had very little or no connections with the remaining populations of *S. pyrenaicus*. Allozymic and morphological data (Doadrio and Carmona, unpublished data) also support the differentiation of these populations.

Interestingly, *S. pyrenaicus* populations from the Tajo basin did not form a monophyletic group. Populations from the Almonte and Tiétar rives in the Tajo basin are phylogenetically closer to that from Jucar and Ebro populations than those from the Alagón river, a main tributary of the southwest Tajo basin. The affinities between Tajo and Jucar basins must be interpreted as an historical event of interconnection since their headstreams are placed less than 5 km. Supporting this hypothesis is the well analyzed relationship between the populations of *Chondrostoma arcasii* from the Júcar and Tajo rivers (Casado, 1995). In addition, the inclusion in this group of *S. pyrenaicus* population from Matarraña river in the Ebro Basin, is rather interesting since this is the only known population of the species from the northeast of the Iberian Peninsula and is a unique case of simpatry with *S. cephalus*. A similar pattern has been reported for *Cobitis paludica*, which inhabits the same Ebro basin and is simpatric with *C. calderoni* (Doadrio et al., 1991). The genetic differentiation showed by the populations from the Alagón river (lower Tajo basin) has been also observed in other freshwater fishes, suggesting that this tributary constitutes a biogeographical unit isolated from the rest of the basin (Carmona et al., 1998; Doadrio and Perdices, 1997).

Despite the general pattern of differentiation of populations among basins, supported by the AMOVA analysis, we can observe both small interdrainage genetic divergences in some neighbor basins, and high intradrainage genetic divergences in some basins like Tajo and Guadalquivir with a more complex paleogeographical history (Carmona et al., 2000). Those results show that the influence of ancient endorrheic lagoons that originated some of these main rivers can still be detected. The impact of these paleogeographical events in the generation of genetic diversity has also been observed in populations of *Chondrostoma lemmingii* (Carmona et al., 2000) for which the Duero basin population constitutes a new species. The population from the upper Guadalquivir should started to diverge in the south of the Iberian Peninsula prior to the complete isolation of Guadiana basin.

In conclusion, our data indicates that the Iberian Peninsula was colonized at two different times by two different monophyletic lineages, the meridional group and the Central Europe group. The amount of species diversity found in the Iberian Peninsula and the phylogenetic relationships among these species, together with their geographic distribution, suggest that the Central

Europe lineage colonized the Iberian peninsula at a latter time.

The Messinian Crisis of the Mediterranean Sea in the Pliocene seem to be a paleogeographic event too recent to have had any major impact in the dispersion of species in the genus *Squalius*. The effect of the lacustre Lago-Mare phase may have been reduced to local dispersion events. However, the reduction of the waterbodies during the Messinian may have intensified the isolation of populations (Doadrio, 2001).

Future studies in other fish groups with similar patterns of geographic distribution may provide additional insights, not only on the history and evolutionary relationships among fish species, but also on the events that may have caused speciation and dispersion in this area.

Acknowledgments

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