

TESTING FRESHWATER LAGO MARE DISPERSAL THEORY ON THE PHYLOGENY RELATIONSHIPS OF IBERIAN CYPRINID GENERA *CHONDROSTOMA* AND *SQUALIUS* (CYPRINIFORMES, CYPRINIDAE)

I. Doadrio*¹ and J. A. Carmona*

ABSTRACT

A phylogeny of the species in the genera *Chondrostoma* and *Squalius* was constructed based on the complete mitochondrial cytochrome *b* gene (1140pb). The molecular phylogeny was used to test the effect of the Mediterranean Lago Mare dispersal theory on the processes of divergence and speciation of European freshwater fishes. Phylogenetic relationships among *Squalius* samples and the molecular clock revealed that the ancestor of the current Iberian *Squalius* species inhabited a wide geographic area in the central and southwestern part of the former Iberian Peninsula during the Miocene before the Lago Mare phase. Similarly, the four main Iberian lineages of the genus *Chondrostoma* originated in the Middle-Upper Miocene. Hence, the Lago Mare phase of the Mediterranean Sea seems to have been a too recent paleogeographic event to have had any major impact on the dispersion of *Squalius* and *Chondrostoma* species. However, the reduction of the water-bodies during the Tortonian and Messinian may have intensified the isolation of populations. The Operational Biogeographic Units recovered from the *Squalius* and *Chondrostoma* phylogenies also reject the Lago Mare dispersal theory and support the idea that the differentiation processes were due to both the formation of the current hydrographical basin during the Plio-Pleistocene as well as to an earlier endorrheism event that occurred prior to hydrographical configuration.

Key words: Cyprinids, *Squalius*, *Chondrostoma*, Biogeography, Mitochondrial DNA, Cytochrome *b*.

RESUMEN

Comprobando la Teoría dispersalista del Lago Mare en las relaciones filogenéticas de los géneros ibéricos de ciprinidos *Chondrostoma* y *Squalius* (Cypriniformes, Cyprinidae)

Realizamos una filogenia de los géneros *Chondrostoma* y *Squalius* mediante el estudio de la secuencia completa del gen mitocondrial citocromo *b* (1140pb). La filogenia molecular fue usada para comprobar el efecto que la teoría de dispersión del Mediterráneo Lago Mare ha tenido sobre los procesos de divergencia y especiación en los peces de agua dulce europeos. Las relaciones filogenéticas entre las muestras de *Squalius* y la aplicación del reloj molecular pusieron de manifiesto que el ancestro de las actuales especies ibéricas de *Squalius* habitaba una amplia área del centro y suroeste de la Península Ibérica durante el Mioceno antes de la fase Lago Mare. Del mismo modo, los cuatro grandes linajes ibéricos del género *Chondrostoma* tuvieron su origen en el Mioceno Medio-Superior. La fase Lago Mare del Mediterráneo parece un hecho demasiado reciente como para haber tenido un impacto importante en la dispersión de las especies de los géneros *Squalius* y *Chondrostoma*. No obstante, la reducción de los cuerpos de agua durante el Tortonense y Mesiniense pudo haber intensificado el aislamiento de las poblaciones. Las Unidades Biogeográficas Operativas obtenidas de las filogenias de los géneros *Squalius* y *Chondrostoma* también rechazan la teoría de la dispersión en la fase Lago Mare y sugieren que los procesos de

* Dept. Biodiversidad y Biología Evolutiva. Museo Nacional de Ciencias Naturales, CSIC. José Gutiérrez Abascal 2. 28006 Madrid.
¹ Corresponding author. E-mail: mcnd147@mmcn.csic.es

diferenciación de la fauna de peces de agua dulce se debió al aislamiento de las cuencas hidrográficas, y a fenómenos antiguos de endorreísmo que se produjeron antes de la configuración de las cuencas hidrográficas.

Palabras Claves: Ciprinidos, *Squalius*, *Chondrostoma*, Biogeografía, ADN mitocondrial, Citocromo *b*.

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es despertar al alma
de su sueño infinito*
José Bergamín

Introduction

Primary freshwater fishes intolerant to marine conditions (Myers, 1938) have been of interest to biogeographers because of their dispersal ability, which is mainly restricted to freshwater contacts among different basins, lakes or rivers. It is commonly accepted that current geographical distribution of primary freshwater fishes is determined by a summary of historical changes related to the geological evolution of the region (Lundberg, 1993). Therefore, analyses of freshwater fishes allow us to test paleogeographical hypotheses, especially those related to the development of hydrographic basins and to processes of isolation and interconnection (Doadrio, 1988; Bermingham & Martin, 1998).

Within primary freshwater fishes, cyprinids with more than 1,700 species, display a high degree of diversity. Their wide biological and ecological plasticity gives this group an important role in biogeographical theories (Zardoya & Doadrio, 1998, 1999; Durand *et al.*, 2003). Most theories regarding the paleobiogeography of European cyprinid fishes tried to explain which were the centre of origin and their dispersion routes (Banarescu & Coad, 1991), and only few were interested to know the barriers that explain the vicariant patterns observed in some cyprinid fishes (Howes, 1980, 1991; Doadrio, 1990, 1994).

Banarescu's ideas, espoused in several works, have dominated European freshwater biogeography for the past 40 years (see Banarescu, 1992 and references there in). According to Banarescu (1973), current distribution of freshwater fishes in Europe resulted from a dispersal through eastern Asia into Siberia towards Central Europe and the Mediterranean countries ("the north dispersal theory"). This migratory route was only plausible

when the Turgai Strait was closed 34 my ago and Europe and Asia were part of the same landmass (Briggs, 1987). The fossil record suggests a north and east Asiatic origin since the first known cyprinid fossil is from North Asia (Sytchevskaya, 1986) and until Oligocene, when the Ob Sea disappeared, no cyprinid fossils were found in Europe (Cavender, 1991).

However, it is not entirely possible to attribute the distribution of some Mediterranean taxa to northern dispersion. Because no geological evidence has been found to explain the connections between the northern and southern rivers of Italy, an alternative model (Bianco, 1990) was proposed. According to Banarescu's (1973, 1989), hypothesis Mediterranean Peninsulas were colonized via northern Europe, through river captures or through Parathethyan lakes (Economidis & Banarescu, 1992). In contrast Bianco's (1990) model suggests that the Mediterranean Peninsulas were colonized by crossing the Mediterranean Sea during the Messinian salinity crisis 5.3 MY; a time when the Mediterranean basin was almost completely dried up but which was subsequently refilled with fresh water from the Sarmatic Sea (Hsü *et al.*, 1977). Bianco (1990) hypothesized that the freshwater fish fauna of the Mediterranean Peninsulas originated from a Parathethys dispersal centre.

Both theories are not entirely in conflict, however Bianco (1990) contributed to Banarescu's theory by introducing new geological advances that explained an alternative dispersal route for the endemic species from the Mediterranean area. This southern dispersal route has widespread acceptance and has now been incorporated into Banarescu's north dispersal theory (Banarescu & Coad, 1991).

The current synthesis to explain the biogeography of European cyprinid fishes is based on two assumptions: 1) European cyprinids originated in Asia and 2) their subsequent dispersal occurred via two routes – a northern route (Banarescu's theory) and a southern route (also known as the Lago Mare theory). Central European taxa dispersed via the north while endemic freshwater fauna from the

Mediterranean area dispersed via the southern route. The northern route probably occurred in early Miocene, while the southern route in the late Miocene. Fish dispersed through water captures (rivers or lakes) and across the Mediterranean Sea, when it was oligohaline.

According to Popper's science demarcation dispersal theories can not be accepted because impossibility to be refuted (Croizat *et al.*, 1974). Dispersal, in explaining everything, really explains nothing (Wiley, 1981). However, dispersal theories have experienced a comeback due to developments in scientific philosophy and molecular sciences and to evidence that Popper's criteria has serious restrictions in its application to Biological Sciences (Mayr, 1998). Molecular studies allow us to estimate information concerning the history of populations such as the spread of a taxon and the presence of migrations (Templeton *et al.*, 1995).

In the last years, advances in molecular studies, particularly mitochondrial cytochrome *b* and *d-loop* genes sequences, have significantly increased our knowledge of the phylogenetic relationships of cyprinid fishes. As a result, the biogeographical hypothesis has been re-evaluated. Molecular phylogenies were used to compare current distribution patterns in order to define barriers of allopatric speciation. These barriers were dated on the basis of current geological knowledge and were then used to calibrate molecular clocks (Zardoya & Doadrio 1999; Perdices & Doadrio 2001; Machordom & Doadrio 2000; Sanjur *et al.*, 2003).

The principal vicariant model for Mediterranean freshwater fishes is based on evidence that the southern Iberian Peninsula and North Africa were part of the same land mass during almost all of the Miocene. Tectonic movement during the upper Miocene divided this land mass into two, separating and isolating populations of such genera as *Barbus* and *Cobitis* (Doadrio, 1994).

Several recent molecular studies supported the Lago Mare dispersion and have underscored the role of the Messinian salinity crisis in the speciation of cyprinid fishes (Durand *et al.*, 2002, 2003). Lago Mare dispersal is mainly supported by the large number of polytomies found in cyprinid phylogenetic relationships. Durand *et al.* (2003) considered that the northern dispersal theory, in its oldest form (Banarescu, 1960), was mainly based on gradual water captures. Because they colonized gradually, the phylogenetic tree of cyprinid fishes must show bifurcations. However, the evidence of hard polytomies in the phylogenetic relationships of the genera *Squalius* (Durand *et al.*, 2000) and

Chondrostoma (Durand *et al.*, 2003) are interpreted as the split of a common ancestral population that developed through cladogenesis into multiple lineages during a short period of time. The molecular clock employed by Durand *et al.* (2003), which dated the hard polytomies to the Messinian period, supports the Lago Mare Dispersal Theory.

In this work we revise the phylogenetic relationships within the cyprinid genera *Chondrostoma* and *Squalius* to test the role of the Messinian period in cyprinid speciation and their dispersal through the Mediterranean Sea during the Lago Mare phase. We also investigated both the biogeographical relationships among these genera and the principal barriers that existed for cyprinids during the Cenozoic.

Material and Methods

The genus *Chondrostoma* comprises of approximately 30 species of freshwater fishes that are distributed throughout Europe and Asia; extending from the Iberian Peninsula to the Ural Mountains, and in the Middle East, from the Anatolian Peninsula to Iran. Our study used cytochrome *b* sequence data belonging to 61 specimens (Table 1, Fig. 1). The outgroups used were *Rutilus rutilus*, and *Telestes souffia* (Zardoya & Doadrio, 1999).

The genus *Squalius* is also widely distributed throughout European and is highly diversified in the Mediterranean area. Recent studies separated three different lineages (Sanjur *et al.*, 2003). A "Mediterranean lineage" is composed of small species from southern Spain, Central Italy, Southern Greece and the Balkans. A "Euro-Asiatic" lineage is principally made up of large-size taxa that is widely distributed through Central Europe, Asia and North of Mediterranean area. The third lineage, the "Paratethys lineage", contains small species around the Black Sea basin and western Anatolia. Our study used cytochrome *b* sequence data for 47 specimens (Table 1, Fig. 1). The outgroups used were *Chondrostoma willkommii* and *Rutilus rutilus* (Zardoya & Doadrio, 1999).

For each genus we used the complete nucleotide sequences of Cytochrome *b* to recover the phylogenetic relationships among populations belonging to different species. Monophyletic groups of populations were employed to determine significant evolutionary units that were subsequently labelled: biogeographical units (OBU). We established phylogenetic relationships among OBUs in order to identify barriers and ancient speciation patterns.

Table 1.— Species analysed, location data, source of DNA sequences and population number (following the Fig. 1).

Species	River	Basin (Country)	Source	Number in the Fig. 1
<i>C. arrigonis</i>	Cabriel	Júcar (Spain)	Doadrio & Carmona (in Press)	59
<i>C. arcasii 1</i>	Trabaque	Tajo (Spain)	Robalo <i>et al.</i> (in press)	54
<i>C. arcasii 2</i>	Gévalo	Tajo (Spain)	Robalo <i>et al.</i> (in press)	41
<i>C. arcasii 3</i>	Arakil	Ebro (Spain)	Robalo <i>et al.</i> (in press)	47
<i>C. arcasii 4</i>	Jalón	Ebro (Spain)	Robalo <i>et al.</i> (in press)	50
<i>C. arcasii 5</i>	Mijares	Mijares (Spain)	Robalo <i>et al.</i> (in press)	55
<i>C. arcasii 6</i>	Palancia	Palancia (Spain)	Robalo <i>et al.</i> (in press)	57
<i>C. arcasii 7</i>	Riaza	Duero (Spain)	Robalo <i>et al.</i> (in press)	45
<i>C. arcasii 8</i>	Adaja	Duero (Spain)	Robalo <i>et al.</i> (in press)	44
<i>C. cyri*</i>	Araxes	Araxes (Turkey)	Durand <i>et al.</i> (2002)	90
<i>C. duriense 1</i>	Adaja	Duero (Spain)	Doadrio & Carmona (in press)	44
<i>C. duriense 2</i>	Ulla	Ulla (Spain)	Doadrio & Carmona (in press)	1
<i>C. genei 1</i>	Arno	Arno (Italy)	Doadrio & Carmona (in press)	68
<i>C. genei 2</i>	Arno	Arno (Italy)	Doadrio & Carmona (in press)	68
<i>C. holmwoodi</i>	Bekincay	(Turkey)	Durand <i>et al.</i> (2003)	85
<i>C. lemmingii 1</i>	Alburrel	Tajo (Spain)	Doadrio & Carmona (in press)	17
<i>C. lemmingii 2</i>	Tietar	Tajo (Spain)	Doadrio & Carmona (in press)	42
<i>C. lemmingii 3</i>	Alcarrache	Guadiana (Spain)	Doadrio & Carmona (in press)	19
<i>C. lemmingii 4</i>	Cigüela	Guadiana (Spain)	Doadrio & Carmona (in press)	39
<i>C. lemmingii 5</i>	Belmez	Guadalquivir (Spain)	Doadrio & Carmona (in press)	34
<i>C. lemmingii 6</i>	Ovejuna	Guadalquivir (Spain)	Doadrio & Carmona (in press)	26
<i>C. lusitanicum 1</i>	Samarra	Samarra (Portugal)	Robalo <i>et al.</i> (in press)	15
<i>C. lusitanicum 2</i>	Colares	Colares (Portugal)	Robalo <i>et al.</i> (in press)	16
<i>C. macrolepidotum 1</i>	Paiva	Duero (Portugal)	Robalo <i>et al.</i> (in press)	9
<i>C. macrolepidotum 2</i>	Sobral	Mondego (Portugal)	Robalo <i>et al.</i> (in press)	12
<i>C. macrolepidotum 3</i>	S. Pedro	S. Pedro (Portugal)	Robalo <i>et al.</i> (in press)	11
<i>C. macrolepidotum 4</i>	Lis	Lis (Portugal)	Robalo <i>et al.</i> (in press)	13
<i>C. meandrense</i>	Menderes	(Turkey)	Durand <i>et al.</i> (2003)	87
<i>C. miegii 1</i>	Jalón	Ebro (Spain)	Zardoya & Doadrio (1998)	50
<i>C. miegii 2</i>	Cinca	Ebro (Spain)	Doadrio & Carmona (in press)	48
<i>C. nasus 1</i>	-	Rhône (France)	Briolay <i>et al.</i> (1998)	64
<i>C. nasus 2</i>	-	Danube (Slovakia)	Durand <i>et al.</i> (2003)	71
<i>C. oxyrhinchum</i>	Samur	Samur (Russia)	Zardoya & Doadrio, 1999	91
<i>C. polylepis 1</i>	Acebo	Tajo (Spain)	Doadrio & Carmona (in press)	7
<i>C. polylepis 2</i>	Gallo	Tajo (Spain)	Doadrio & Carmona (in press)	53
<i>C. prespense</i>	Lake Prespa	Lake Prespa (Greece)	Zardoya <i>et al.</i> (1999)	74
<i>C. regium</i>	-	Tigris (Turkey)	Durand <i>et al.</i> (2002)	89
<i>C. soetta 1</i>	Po	Po (Italy)	Doadrio & Carmona (in press)	66
<i>C. soetta 2</i>	Po	Po (Italy)	Doadrio & Carmona (in press)	66
<i>C. sp. a1</i>	Tea	Miño (Spain)	Doadrio & Carmona (in press)	2
<i>C. sp. a2</i>	Ulla	Ulla (Spain)	Robalo <i>et al.</i> (in press)	1
<i>C. sp. a3</i>	Bernesga	Duero (Spain)	Zardoya & Doadrio (1998)	4
<i>C. sp. a4</i>	Tera	Duero (Spain)	Doadrio & Carmona (in press)	5
<i>C. sp. b1</i>	Sizandro	Sizandro (Portugal)	Robalo <i>et al.</i> (in press)	14
<i>C. sp. b2</i>	Sizandro	Sizandro (Portugal)	Robalo <i>et al.</i> (in press)	14
<i>C. sp. c1</i>	Robledillo	Guadalquivir (Spain)	Zardoya & Doadrio (1998)	36
<i>C. sp. c2</i>	Robledillo	Guadalquivir (Spain)	Zardoya & Doadrio (1998)	36
<i>C. sp. d1</i>	Boina	Arade (Portugal)	Zardoya & Doadrio (1998)	25
<i>C. sp. d2</i>	Mira	Mira (Portugal)	Doadrio & Carmona (in press)	21
<i>C. sp. e1</i>	Huebra	Duero (Spain)	Doadrio & Carmona (in press)	6
<i>C. sp. e2</i>	Turones	Duero (Spain)	Doadrio & Carmona (in press)	8
<i>C. toxostoma</i>	Rhône	Rhône (France)	Durand <i>et al.</i> (2003)	64
<i>C. toxostoma</i>	Nive	Nive (France)	Cunha <i>et al.</i> (2002)	63
<i>C. turiense 1</i>	Mijares	Mijares (Spain)	Doadrio & Carmona (in press)	55
<i>C. turiense 2</i>	Turia	Turia (Spain)	Doadrio & Carmona (in press)	58
<i>C. vardarensis 1</i>	Aoos	Aoos (Greece)	Zardoya <i>et al.</i> (1999)	75
<i>C. vardarensis 2</i>	Aoos	Aoos (Greece)	Zardoya <i>et al.</i> (1999)	75
<i>C. willkommii 1</i>	Jandula	Guadalquivir (Spain)	Zardoya & Doadrio (1998)	35
<i>C. willkommii 2</i>	Genal	Guadiaro (Spain)	Doadrio & Carmona (in press)	28
<i>C. willkommii 3</i>	Gévora	Guadiana (Spain)	Doadrio & Carmona (in press)	18
<i>C. willkommii 4</i>	Quejigares	Guadiana (Spain)	Doadrio & Carmona (in press)	37
<i>Rutilus rutilus</i>	Saone	Rhône (France)	Briolay <i>et al.</i> (1998)	65
<i>Telestes soufia</i>	Saone	Rhône (France)	Briolay <i>et al.</i> (1998)	65
<i>Telestes beoticus</i>	Kifissos	Kifissos (Greece)	Zardoya & Doadrio (1999)	80
<i>S. aradensis 1</i>	Cerca	Cerca (Portugal)	This paper	22
<i>S. aradensis 2</i>	Bordeira	Bordeira (Portugal)	This paper	23
<i>S. aradensis 3</i>	Arade	Arade (Portugal)	Sanjur <i>et al.</i> (2003)	24
<i>S. borysthenticus</i>	Filiouris	Filiouris (Greece)	Zardoya <i>et al.</i> (1999)	83
<i>S. cabeda 1</i>	Po	Po (Italy)	Sanjur <i>et al.</i> (2003)	66
<i>S. cabeda 2</i>	Arno	Arno (Italy)	This paper	68

Table 1.— Especies analizadas, datos de localización de las poblaciones, fuente de donde se obtuvo las secuencias de DNA y número de población (siguiendo la Fig. 1).

Species	River	Basin (Country)	Source	Number in the Fig. 1
<i>S. cabeda 3</i>	Adda	Po (Italy)	This paper	67
<i>S. cabeda 4</i>	Tevere	Tevere (Italy)	This paper	70
<i>S. cabeda 5</i>	Tevere	Tevere (Italy)	This paper	70
<i>S. carolitertii 1</i>	Bíbei	Miño (Spain)	Sanjur <i>et al.</i> (2003)	3
<i>S. carolitertii 2</i>	Hormazuela	Duero (Spain)	This paper	46
<i>S. carolitertii 3</i>	Mondego	Mondego (Spain)	This paper	10
<i>S. cephalus</i>	Saone	Rhône (France)	Briolay <i>et al.</i> (1998)	65
<i>S. c. vardarensis</i>	Sperchios	Sperchios (Greece)	Zardoya <i>et al.</i> (1999)	82
<i>S. illyricus</i>	Cetina	Cetina (Croatia)	Durand <i>et al.</i> (2000)	72
<i>S. keadicus</i>	Evrotas	Evrotas (Greece)	Zardoya <i>et al.</i> (1999)	78
<i>S. lepidus</i>	Lake Beyshir	Lake Beyshir (Turkey)	Durand <i>et al.</i> (2000)	88
<i>S. lucumonis</i>	Ombro	Ombro (Italy)	Sanjur <i>et al.</i> (2003)	69
<i>S. macedonicus 1</i>	Nestos	Nestos (Greece)	Zardoya <i>et al.</i> (1999)	84
<i>S. macedonicus 2</i>	Manikiotiko	Manikiotiko (Greece)	Zardoya <i>et al.</i> (1999)	81
<i>S. orientalis</i>	Rubas	Samur (Russia)	Zardoya & Doadrio (1999)	91
<i>S. peloponnensis 1</i>	Thyamis	Thyamis (Greece)	Zardoya <i>et al.</i> (1999)	76
<i>S. peloponnensis 2</i>	Alphios	Alphios (Greece)	Zardoya <i>et al.</i> (1999)	77
<i>S. p. moreoticus</i>	Lake Stymphalia	Lake Stymphalia (Greece)	Zardoya <i>et al.</i> (1999)	79
<i>S. prespensis</i>	Lake Prespa	Lake Prespa (Greece)	Zardoya <i>et al.</i> (1999)	74
<i>S. pyrenaicus 1</i>	Pereilas	Guadalhorca (Spain)	Doadrio & Carmona (in press)	32
<i>S. pyrenaicus 2</i>	Guadalfeo	Guadalfeo (Spain)	Doadrio & Carmona (in press)	33
<i>S. pyrenaicus 3</i>	Bogarda	Segura (Spain)	Sanjur <i>et al.</i> (2003)	62
<i>S. pyrenaicus 4</i>	Azuer	Guadiana (Spain)	Sanjur <i>et al.</i> (2003)	38
<i>S. pyrenaicus 5</i>	Viar	Guadalquivir (Spain)	This paper	26
<i>S. pyrenaicus 6</i>	Aberche	Tajo (Spain)	This paper	43
<i>S. pyrenaicus 7</i>	Laguna de Uña	Júcar (Spain)	Sanjur <i>et al.</i> (2003)	56
<i>S. pyrenaicus 8</i>	Matarrña	Ebro (Spain)	Sanjur <i>et al.</i> (2003)	52
<i>S. pyrenaicus 9</i>	Cimbaya	Ebro (Spain)	This paper	51
<i>S. pyrenaicus 10</i>	Huso	Tajo (Spain)	This paper	40
<i>S. pyrenaicus 11</i>	Algar	Algar (Spain)	Sanjur <i>et al.</i> (2003)	61
<i>S. pyrenaicus 12</i>	Serpis	Serpis (Spain)	Sanjur <i>et al.</i> (2003)	60
<i>S. pyrenaicus 13</i>	Sado	Sado (Spain)	This paper	20
<i>S. pyrenaicus 14</i>	Genal	Guadiaro (Spain)	Doadrio & Carmona (in press)	28
<i>S. pyrenaicus 15</i>	Guadalmina	Guadalmina (Spain)	Doadrio & Carmona (in press)	31
<i>S. pyrenaicus 16</i>	Miel	Sur (Spain)	This paper	29
<i>S. pyrenaicus 17</i>	Vega	Vega (Spain)	This paper	30
<i>S. smyrnaeus</i>	Gumuldur	Gumuldur (Turkey)	Durand <i>et al.</i> (2000)	86
<i>S. sp 1</i>	Matarrña	Ebro (Spain)	Zardoya & Doadrio (1998)	52
<i>S. sp 2</i>	Llobregat	Llobregat (Spain)	This paper	49
<i>S. torgalensis</i>	Torgal	Mira (Portugal)	This paper	21
<i>S. zrmanje</i>	Zrmanje	Zrmanje (Croatia)	Durand <i>et al.</i> (2000)	73
<i>Rutilus rutilus</i>	Saone	Rhône (France)	Briolay <i>et al.</i> (1998)	65

DNA was extracted from dorsal muscle and was preserved in liquid nitrogen or 70% ethanol. Voucher specimens for these species were deposited in the collections of the Museo Nacional de Ciencias Naturales, Madrid, Spain. Total cellular DNA was isolated from tissues by a standard proteinase K and phenol/chloroform extraction method (Sambrook *et al.*, 1989). Frozen or 70% ethanol preserved specimens usually gave the same quality and quantity of DNA. Two overlapping fragments of the cytochrome b gene (total of 1140 bp) were amplified via polymerase chain reaction (PCR). The primers used for cytochrome b in all species were those mentioned in Machordom & Doadrio (2001).

The amplification process was conducted as follows: 94°C (2 min), 35 cycles at 94°C (45 s), 48 °C (1 min), 72°C (90 s), 72°C (5 min). PCR mixtures

were prepared in 25 µl with a final concentration of 0.4 mM of each primer, 0.2 mM of each dNTP, 1.5 mM MgCl₂, and 1 unit of Taq DNA polymerase (Biotools). PCR products were checked on 1.5% agarose gels, and cloned using the pGEM-T vector (Promega) into *E. coli* JM109. Positive clones were sequenced using the Big Dye Deoxy Terminator cycle-sequencing kit (Applied Biosystems Inc.). DNA sequences of both strands were obtained using M13 universal (forward and reverse) sequencing primers. All samples were sequenced on an Applied Biosystems 3700 DNA sequencer following manufacturer's instructions. Chromatograms and alignments were visually checked and verified.

In all groups, saturation was analysed by plotting the absolute number of transitions and transversions against patristic distance values (not

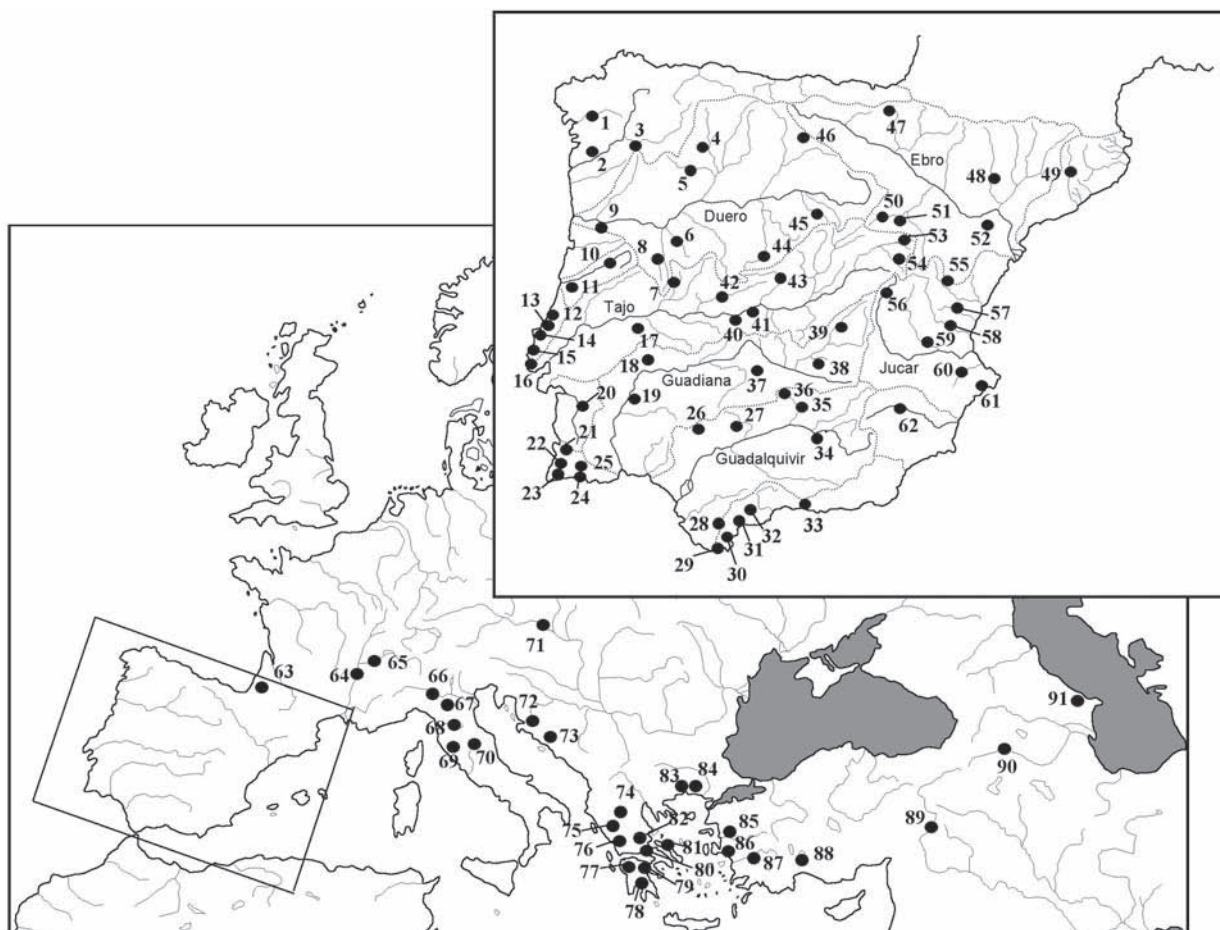


Fig. 1.— Distribution of sampling localities for *Squalius* and *Chondrostoma* specimens. Corresponding rivers and basins are described in Table 1.

Fig. 1.— Localidades de procedencia de las muestras de *Squalius* y *Chondrostoma*. En la Tabla 1 se describen los ríos y cuencas que se corresponden con estas localidades.

shown). There were not evidences of saturation at any codon position. A hierarchical likelihood ratio test (LRT) was performed to find the best model of evolution that fit our data using the program Model test 3.04 (Posada & Crandall, 1998). Bayesian inference was performed with MrBayes 3.0 (Huelsenbeck & Ronquist, 2001) by simulating a 1.000.000 cycles for 4 markov chains. Burnin parameters were 300 to *Squalius* and 450 to *Chondrostoma*. To determine whether a particular tree topology corresponded to a significantly better or worse interpretation of the data than an alternative tree, we used the Shimodaira-Hasegawa test (Shimodaira & Hasegawa, 1999), as implemented in PAUP* version 4.0b10 (Swofford, 2002).

To examine whether lineages within a particular genus evolved at equal rates (molecular clock), we conducted a Likelihood Ratio Test with and without molecular clock constraints using Puzzle version 4.0.1 (Strimmer & Von Haeseler, 1996). Since this test does not determine which lineages cause rate disparity, we also conducted a relative rates test (Sarich & Wilson, 1973; Wu & Li, 1985) among the main clades within a particular genus using the program RRTree v 1.1 (Robinson-Rechavi & Huchon, 2000). We compared total number of substitutions as well as Ks (number of synonymous substitutions per synonymous site), and Ka (number of non-synonymous substitutions per non synonymous site).

Results

Chondrostoma and *Squalius* data set included 422/418 variable sites, respectively. Third codon positions were the most informative (308/326 parsimony informative characters), followed by first codon positions (81/65 characters).

The general time reversible model with among-site rate heterogeneity GTRI + G + I (Yang, 1994; Gu *et al.*, 1995) was selected as the best that fit the *Chondrostoma* and *Squalius* data sets. Rate matrix parameters, to *Chondrostoma* and *Squalius* respectively, were $R(a) = 1.7363/1.044$, $R(b) = 43.275/46.401$, $R(c) = 0.6002/0.5181$, $R(d) = 1.7236/1.8642$, $R(e) = 12.674/9.6695$, $R(f) = 1/1$. Base frequencies were $A = 0.2666/0.2788$, $C = 0.2666/0.2964$, $G = 0.1591/0.1462$, $T = 0.2926/0.2786$. Among site rate variation was approximated with the gamma distribution shape parameter $\alpha = 0.54/1.23$. Proportion of invariable sites $I = 0.27/0.54$. These parameters were also used for subsequent phylogenetic analyses.

The analyses of cytochrome *b* sequence data on *Squalius* (47 specimens) and *Chondrostoma* (61 specimens) identified different mtDNA lineages within each genus. Phylogenetic analysis of the genus *Squalius* data set recovered the previously identified Mediterranean, Euro-Asiatic and Paratethys lineages (Sanjur *et al.*, 2003) (Fig. 2). Phylogenetic relationships of Iberian, Greek and Italo-Balkan species within the Mediterranean lineage were unresolved indicating that those species diverged in a brief period of time (Fig. 3).

Similarly, phylogenetic analysis of the genus *Chondrostoma* recovered two main lineages: Iberian and Euro-Asiatic. The two Italian species *C. soetta* and *C. genei* appeared to be independent lineages (Fig. 4). Phylogenetic relationships displayed in the bayesian tree showed two main polytomies within of the genus *Chondrostoma*. The first polytomy was found during the divergence process of the Italian and other Euro-Asiatic species. The second was found within the Iberian lineage and affected the four main species groups (or lineages) that inhabited the Iberian Peninsula. These groups were: *Toxostoma*, *Lemmingii*, *Arcasii* and *Polylepis* (Fig. 4).

Within the Iberian Peninsula, the recovered phylogenetic trees identified several phylogenetically significant units that we grouped geographically and called Operacional Biogeographic Units (OBU). Some of these OBUs were small because the mitochondrial lineage that defined them was present in only one or a few populations. Other

OBUs comprised more than a complete hydrographical basin and were composed of populations with closely related mitochondrial lineages belonging to widely distributed species.

GENUS *SQUALIUS*

The analysed Iberian *Squalius* species included *S. torgalensis* and *S. aradensis*, which are restricted to the extreme south-western part of Portugal, and *S. carolitertii* from the Mondego, Miño and Duero basins. These three species represent separate OBUs.

Populations of the fourth species, *S. pyrenaicus*, were widely distributed, and populations from the extreme South and from the Levantine rivers of the Mediterranean slope of Spain were the most differentiated. Among *S. pyrenaicus* specimens, five different OBUs were observed (Fig. 2). The first OBU included populations from the Guadiana, Guadalquivir, Segura and small independent basins from the southern part of the Iberian Peninsula. The second OBU was formed by populations from the Tajo and Júcar basins, in addition to isolated populations from the Ebro Basin. The third OBU consisted of a *S. pyrenaicus* population from the Sado Basin. The fourth OBU included populations from the Mediterranean slope (Algar and Serpis rivers). The fifth OBU was formed by small independent basins, located just near the Gibraltar strait, and were highly differentiated from the rest of *S. pyrenaicus* populations.

Squalius cephalus from Catalonia and Ebro basin belong to other different lineage and formed a different OBU.

Using the relative-rate test (Robinson *et al.*, 1998), we investigated whether the eight well-separated lineages, ("*S. aradensis*", "*S. torgalensis*", "*S. carolitertii*", "*S. pyrenaicus*", "Euro-Asiatic lineage", "Paratethys lineage" and "Italian and Balkanic species of the Mediterranean lineage"), had different substitution rates. Test results indicated that these clades did not exhibit significantly different evolutionary rates for synonymous and non-synonymous substitutions with Bonferroni correction (Robinson *et al.*, 1998).

GENUS *CHONDROSTOMA*

Within *Chondrostoma*, two independent, evolutionary lineages were found. The first lineage corresponded to Iberian and eastern France species, while the second group contained the rest of the European and Asiatic species. (Fig. 4 and 5).

Within Iberian and French species four different lineages with high support values can be distinguished. We named these groups of lineages:

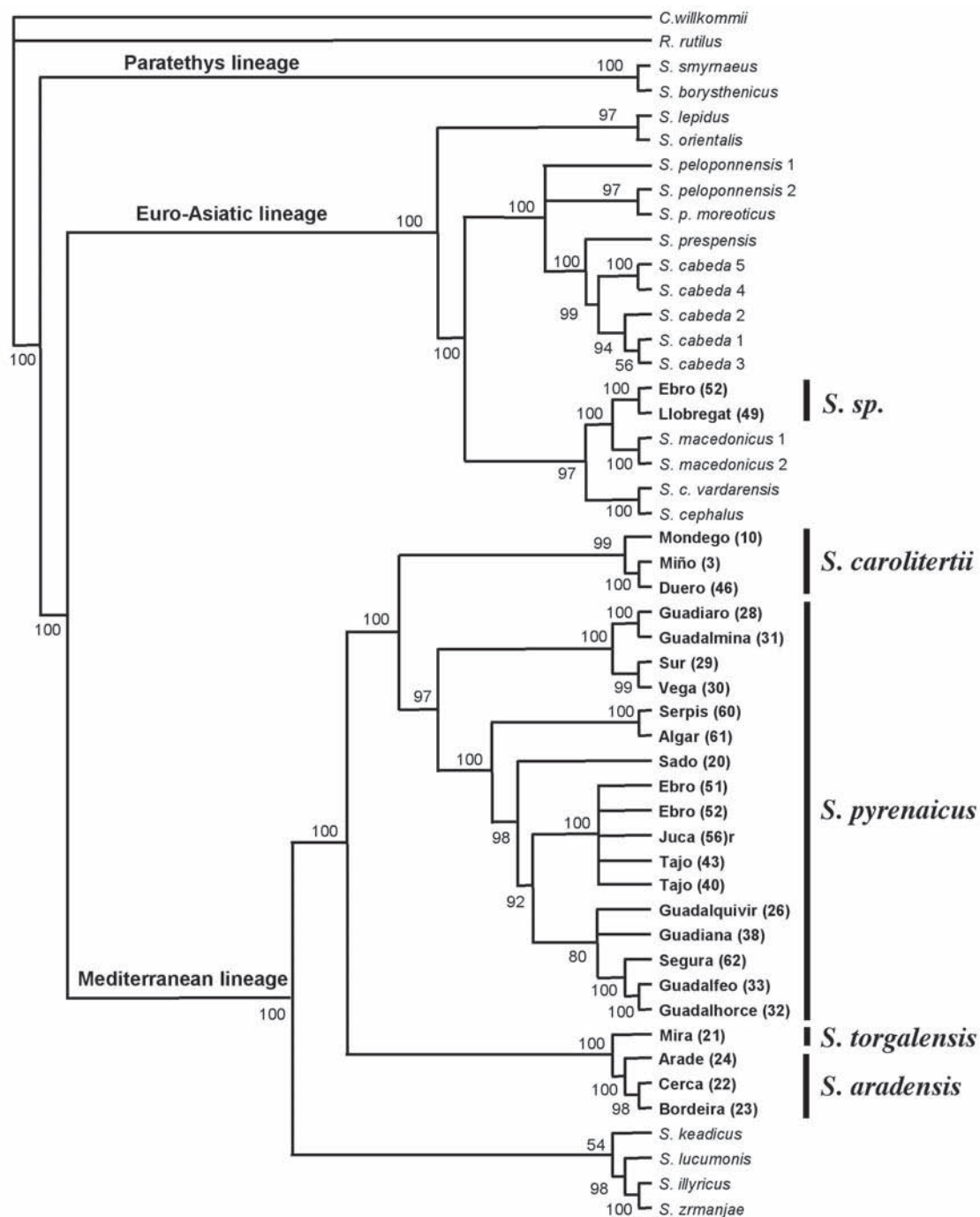


Fig. 2.— Phylogenetic relationships among *Squalius* populations, based on cytochrome *b* gene sequences. Numbers above branches represent the support for Bayesian inference. Nodes with posterior probabilities values below 50% were forced to collapse and yield polytomies. Population number (in brackets) following Fig. 1 and Table 1. Different specimens of the same species were identified with numbers (without brackets) following Table 1. Iberian populations in bold.

Fig. 2.— Relaciones filogenéticas entre poblaciones de *Squalius*, basadas en las secuencias del gen citocromo *b*. Los números en el árbol representan el soporte del análisis Bayesiano. Los nodos que muestran un soporte inferior al 50% se colapsaron formando una politomía. Se indica los números de las poblaciones (entre paréntesis) siguiendo la Fig. 1 y la Tabla 1. Los distintos ejemplares analizados de la misma especie se identificaron con números (sin paréntesis) como en la Tabla 1. Poblaciones ibéricas en negrita.

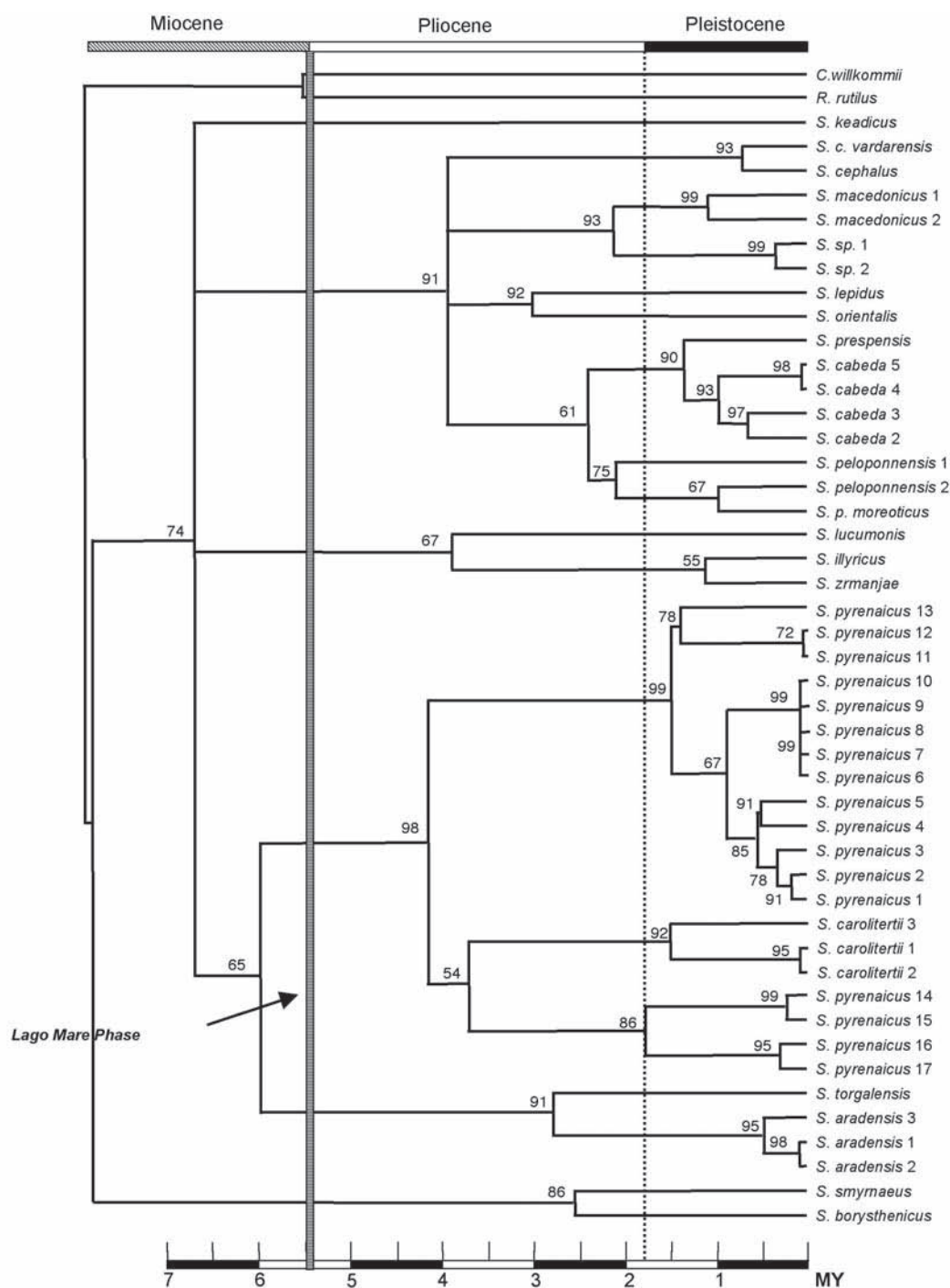


Fig. 3.— Puzzle ML tree showing the major cladogenetic events in the history of the genus *Squalius*. The scale bar below the tree shows the time scale resulting from the application of a molecular clock based on 1.05% divergence between taxa per MY. Numbers in nodes are support values based on 1,000,000 pseudo-replications

Fig. 3.— Árbol de máxima verosimilitud (Puzzle) que muestra los principales eventos cladogenéticos en la historia evolutiva del género *Squalius*. La escala bajo el árbol es una escala temporal resultante de la aplicación de un reloj molecular basado en 1.05% de divergencia entre taxones por millón de años. Los números en los nodos indican su soporte basándose en 1.000.000 de pseudoreplicaciones

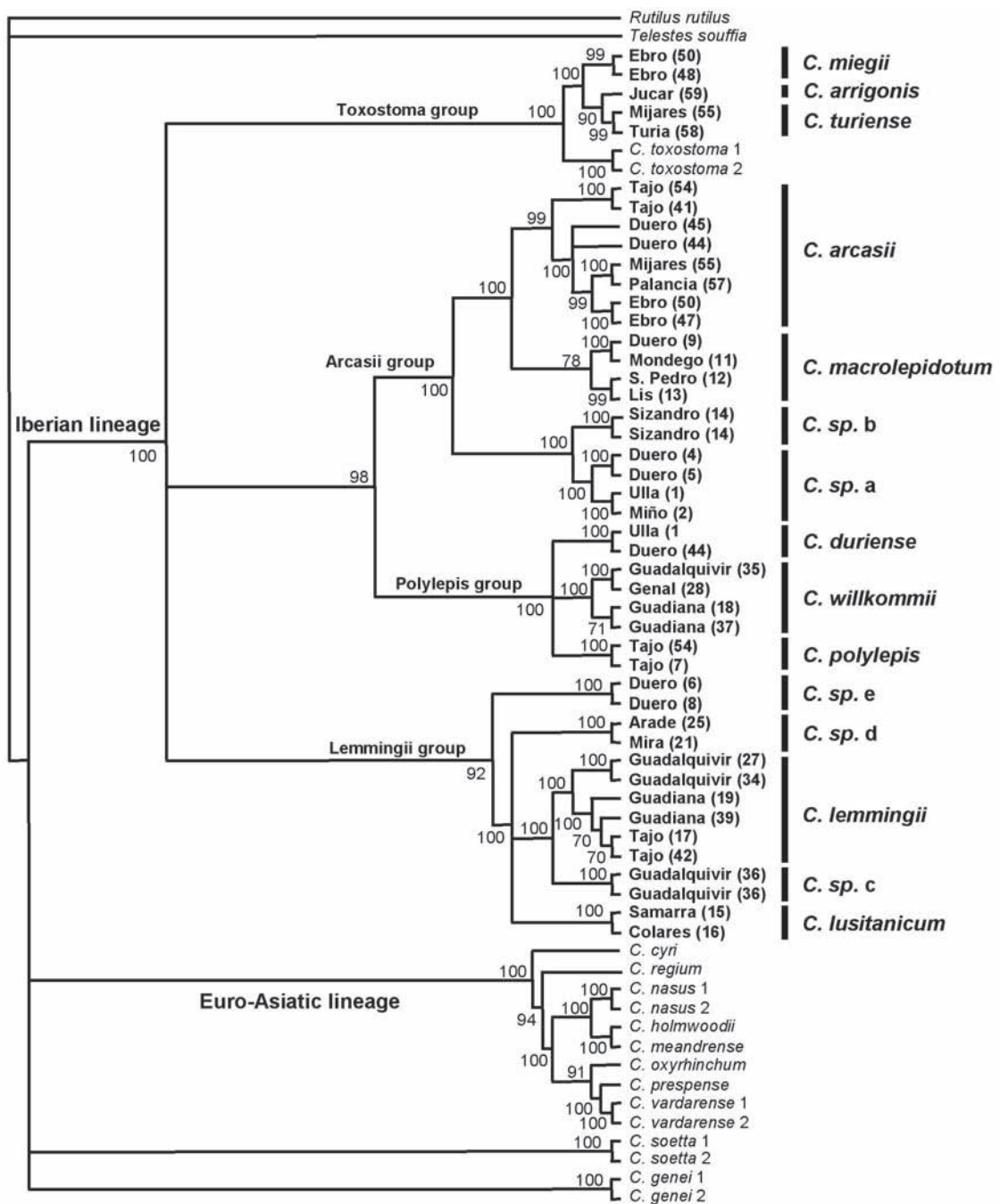


Fig. 4.— Phylogenetic relationships among *Chondrostoma* populations, based on cytochrome *b* gene sequences. Numbers above branches represent the support for Bayesian inference. Nodes with posterior probabilities values below 50% were forced to collapse and yield polytomies. Population number (in brackets) following Table 1. Different specimens of the same species were identified with numbers (without brackets) following Table 1.

Fig. 4.— Relaciones filogenéticas entre poblaciones de *Chondrostoma*, basadas en las secuencias del gen citocromo *b*. Los números en el árbol representan el soporte del análisis Bayesiano. Los nodos que muestran un soporte inferior al 50% se colapsaron formando una politomía. Se indica los números de las poblaciones (entre paréntesis) siguiendo la Tabla 1. Los distintos ejemplares analizados de la misma especie se identificaron con números (sin paréntesis) como en la Tabla 1.

“Arcasii”, “Toxostoma”, “Polylepis” and “Lemmingii”. Moreover, within the Euro-Asiatic lineage the two endemic species from Italy *C. genei* and *C. soetta* conformed well separated independent lineages (Fig. 4).

In our study the Arcasii group was composed of two different clades (Fig. 4). The first clade was formed by the species *C. arcasii* and *C. macrolepidotum*. The second included two undescribed species from the western part of Iberian Peninsula. Two different OBUs were detected within *C. arcasii*. The first included the populations from the upper Tajo basin while the second OBU consisted of the populations from the upper Duero basin and the Ebro, Mijares and Palancia basins from the Mediterranean slope of Spain. Based on populations from the Portuguese Duero, Mondego Basin and other small basins from the North of Portugal, *C. macrolepidotum*, represents an independent OBU. Moreover, the undescribed species *C. sp. a* was composed of populations grouped into two OBUs: the Galician Rivers (Ulla and Miño) and the northwestern part of the Duero basin. The population from the Sizandro basin, which belonged to *C. sp. b*, was considered as a separate OBU.

The Polylepis group comprised three species: *C. duriense*, *C. polylepis* and *C. willkommii*. Relationships among these species were unresolved. *C. duriense* populations from the Duero and Galician Basins represent one OBU, while *C. polylepis* from the Tajo, Mondego, Sorraia and Sado Basins defined another OBU. Populations of *C. willkommii* from the Gadiana River, which constituted one independent OBU, were sister groups of other populations from southern Spain that represented a different OBU.

The Toxostoma group included four species that formed four respective OBUs: *C. arrigonis* endemic to Júcar River in Spain, *C. turiense* endemic to the Turia and Mijares Rivers in Spain, *C. miegii* endemic to northeastern Spain and *C. toxostoma* endemic to France. *C. arrigonis* and *C. turiense* were sister group taxa to *C. toxostoma*.

Finally, the Lemmingii group was comprised of at least five species. Three of them corresponded to populations of an undescribed species, which formed three respective OBUs: the Robledillo River in the Guadalquivir basin (*C. sp. c*), the Mira and Arade Basins in Portugal (*C. sp. d*) and the southwestern Spanish Duero (*C. sp. e*). The Sado Basin and lower Tajo Basin from Portugal defined the OBU for *C. lusitanicum* populations. Finally, we distinguished two OBUs among *C. lemmingii*

populations. The first corresponded to the Guadalquivir Basin and the second to the Gadiana and Tajo Basins.

Using the relative-rate test (Robinson *et al.*, 1998), we investigated whether the seven clades (“Arcasii”, “Toxostoma”, “Polylepis”, “Lemmingii”, “Euro-Asiatic”, *C. genei* and *C. soetta*, which had well-separated lineages, had different substitution rates. The results of the test indicated that these clades did not exhibit significantly different evolutionary rates for synonymous and non-synonymous substitutions with Bonferroni correction (Robinson *et al.*, 1998).

Discussion

Different authors have calibrated a molecular clock for Cyprinidae using fossil or geological data. Zardoya & Doadrio (1999) calibrate a molecular clock in 0.73% divergence per lineage per million years using the opening of the Gibraltar strait 5.3 million years ago and the formation of the Corinth strait in Greece. Posteriorly, on the basis of this geological event Machordom & Doadrio (2001) estimated a molecular clock of 1.3% of divergence per pairwise comparison per million years (0.65 per lineage per million years). These molecular clocks were highly congruent with the estimate of 1.05% divergence per million years (0.53 per lineage per million years) calculate by Dowling *et al.* (2002) with fossil data. We used this latter molecular clock to estimate both divergence dates as well as principal events that caused speciation in *Squalius* and *Chondrostoma*. Durand *et al.* (2003) used the same molecular clock to date the divergence time of the genus *Chondrostoma*.

GENUS *SQUALIUS*

Regarding the origins of the Iberian *Squalius* species, our results showed that they do not constitute 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 it is to any other species on the Iberian Peninsula. Species of the second lineage found within the Iberian Peninsula, *S. pyrenaicus*, *S. carolitertii*, *S. aradensis* and *S. torgalensis*, form a monophyletic group with eight well-recognised OBUs.

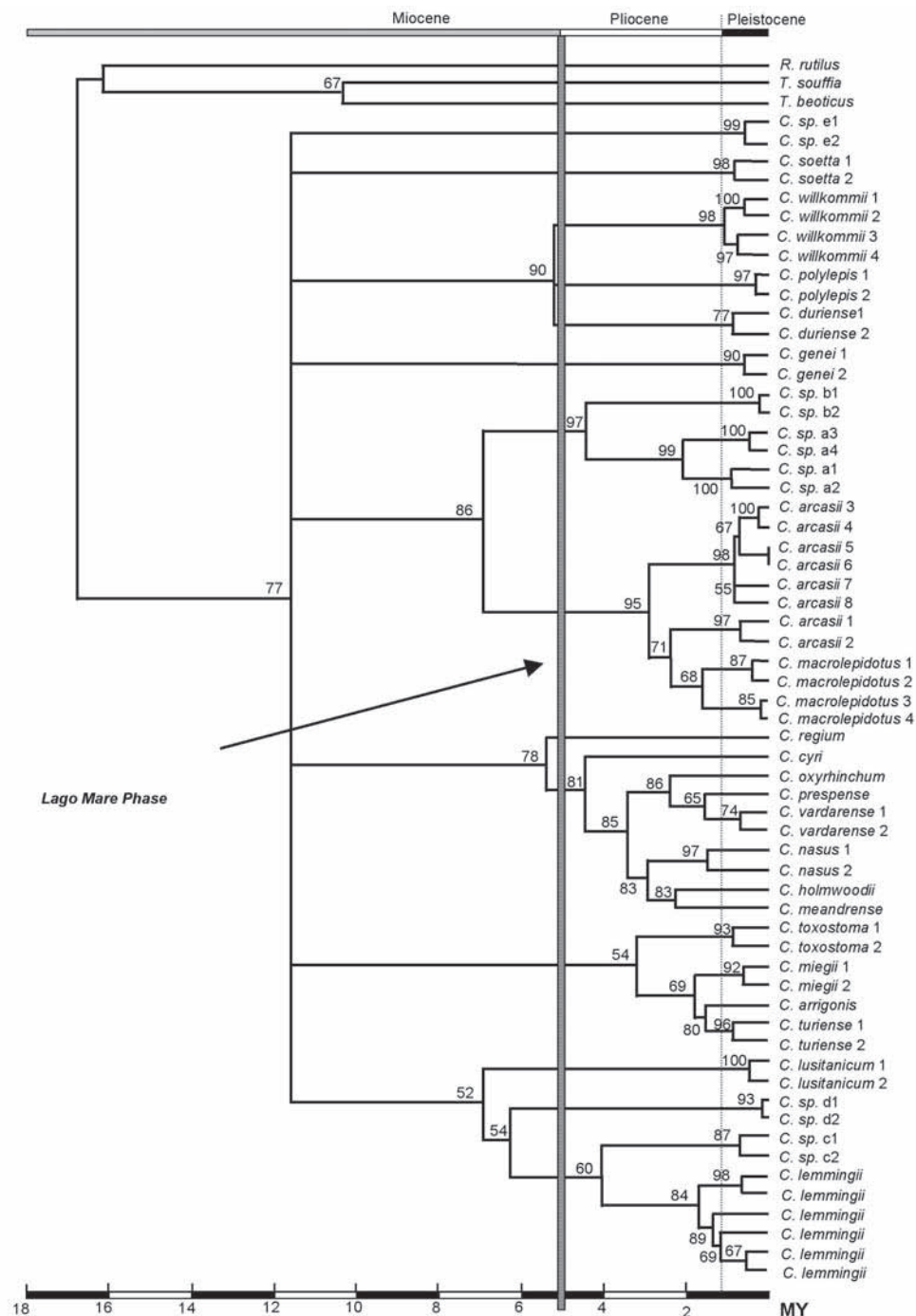


Fig. 5.— Puzzle ML tree showing the major cladogenetic events in the history of the genus *Chondrostoma*. The scale bar below the tree shows the time scale resulted from the application of a molecular clock based on 1.05% divergence between taxa per MY. Numbers in nodes are support values based on 1,000,000 pseudo-replications

Fig. 5.— Árbol de máxima verosimilitud (Puzzle) que muestra los principales eventos cladogenéticos en la historia evolutiva del género *Chondrostoma*. La escala bajo el árbol es una escala temporal resultante de la aplicación de un reloj molecular basado en 1.05% de divergencia entre taxones por millón de años. Los números en los nodos indican su soporte basándose en 1.000.000 de pseudoreplicaciones

Squalius aradensis and *S. torgalensis* were grouped together and appeared basal to *S. pyrenaicus* and *S. carolitertii* indicating an early differentiation of this biogeographical area, probably during the Messinian period (6 MYA). In this period, it is likely that many basins almost dried up, isolating the southwestern part of the Iberian Peninsula. This result supports previous studies based on allozymes and partial cytochrome *b* sequences (Coelho *et al.*, 1995; Brito *et al.*, 1997).

The speciation process that gave rise to *S. carolitertii* can be attributed to the earlier formation of the Duero Basin, which was an endorheic lagoon in the Miocene period (López-Martínez, 1989; De la Peña, 1995). The rest of the Iberian rivers were configured during Plio-Pleistocene and almost all of them have been isolated from one another since this time (Fig. 3).

Among *S. pyrenaicus* populations from the extreme South of Spain were clustered in a separate and basal group, and showed higher genetic divergence than the remaining populations of *S. pyrenaicus* (Fig. 2). Interestingly, the Levantine populations (Serpis and Algar basins) also displayed high genetic differentiation, which suggests that these populations are not connected to the remaining populations of *S. pyrenaicus*.

Populations from the Tajo basin are phylogenetically close to those from the Júcar and Ebro. The affinities between Tajo and Júcar basins must be interpreted as an historical event of interconnection since their headstreams are located very close to each other. Similarly, *C. arcasii* populations in the Tajo, Júcar, Ebro and Levantine Basins represent a single OBU. In addition, the inclusion in this OBU of *S. pyrenaicus* populations from the Matarraña and Cimbaya Rivers from the Ebro Basin is rather interesting since a similar pattern has been reported for *Cobitis paludica*, which inhabits the Tajo Basin and is sympatric with *C. calderoni* in the Ebro basin (Doadrio *et al.*, 1991).

The phylogeny of Iberian *Squalius* species detected two different monophyletic lineages, colonized at two different times on the Iberian Peninsula: the Meridional group and the Central Europe group. The level of species diversity found in the Central Europe lineage, together with their phylogenetic relationships and the geographic distribution of their OBUs, suggest that this lineage colonized the Iberian Peninsula after the Meridional group.

The molecular clock confirms that the ancestor of the Mediterranean group seems to have inhabited a wide geographic area in the central and south-

western part of the former Iberian Peninsula during the Miocene before the Lago Mare phase. Hence, the Lago Mare phase of the Mediterranean Sea seems to have been a recent paleogeographic event to have had any major impact on species dispersion of the genus *Squalius*. The effect of the lacustre Lago Mare phase may have been limited to local dispersal events. However, the reduction of the water-bodies during the Tortonian and Messinian may have intensified the isolation of populations (Doadrio, 2001).

GENUS *CHONDROSTOMA*

Cytochrome *b* sequence data did not clearly resolve the phylogenetic relationships among the four Iberian lineages.

The main *Chondrostoma* lineages were differentiated in the Middle-Upper Miocene (Fig. 5). Unlike most of Europe which was experiencing a dry period during this time, the Iberian Peninsula was wet, a condition which facilitated the formation of the main endorheic lagoons on the Peninsula. This period would coincide with the alleged origin of the genus *Squalius* (Sanjurjo *et al.*, 2003). However, the highest diversification of the family would have occurred during the Pliocene. This high diversification during the Pliocene agrees with previous works (Banarescu, 1989; Zardoya & Doadrio, 1999).

Regarding the distribution pattern of the four Iberian lineages, two significant features must be pointed out. First, *Polylepis* and *Toxostoma* lineages showed OBUs that, respectively, comprised complete hydrographical basins whereas *Arcasii* and *Lemmingii* lineages showed more complex geographical structures and were represented by several OBUs that consisted only of portions of current hydrographical basins (Fig. 6). Such differential distribution patterns are supposedly related to reproductive migrations developed by *Polylepis* and *Toxostoma* lineages, which include large-sized species. On the contrary, reproductive migrations are not present in species belonging to *Arcasii* and *Lemmingii* lineages, which include species of small size.

Secondly, it is worth noting that *Polylepis* and *Toxostoma* lineages are allopatric, as are the *Arcasii* and *Lemmingii* lineages (Fig. 6).

The differentiation of species belonging to the *Polylepis* lineage, distributed throughout the Atlantic slope of Iberian Peninsula, can be explained by the configuration of three main OBUs separated by the Central Cordillera and Toledo Mountains. The molecular clock employed here indicated that the diver-

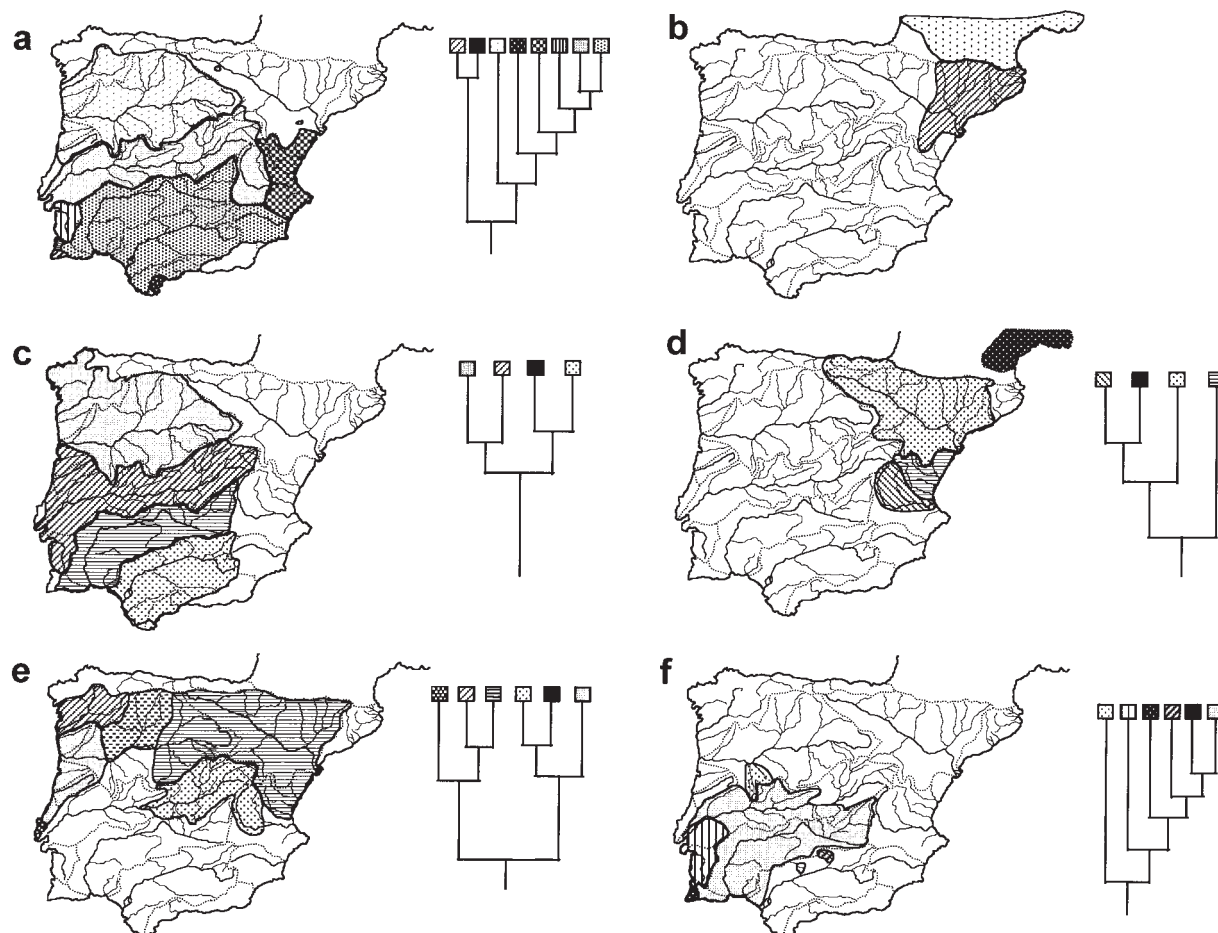


Fig. 6.— Main OBU and their phylogenetic relationships for Iberian species of *Squalius* (a-b) and *Chondrostoma* (c-f): a.- Iberian *Squalius* species of the Mediterranean lineage, b.- Iberian *Squalius* species of the Euro-Asiatic lineage, c.- Polylepis group, d.- Toxostoma group, e.- Arcasii group and f.- Lemmingii group.

Fig. 6.— Principales OBU y sus relaciones filogenéticas obtenidos para las especies Ibéricas de *Squalius* (a-b) y *Chondrostoma* (c-f): a.- especies Ibéricas de *Squalius* pertenecientes al linaje Mediterráneo, b.- especies Ibéricas de *Squalius* pertenecientes al linaje Euroasiático, c.- grupo Polylepis, d.- grupo Toxostoma, e.- grupo Arcasii y f.- grupo Lemmingii.

gence among *C. duriense*, *C. polylepis* and *C. willkommii* took place during the Messinian, which coincided with the Mediterranean transgression.

The Toxostoma lineage species diverged later, about 3 MY, in the Pliocene. No geological data are available to explain the connection between the Iberian basins and the Rhône basin during the Pliocene. This connexion between the Rhône and the Ebro Rivers could only have been possible during the Messinian 5.3-7 MYA when the Rhône discharged into the Mediterranean basin.

Within the Arcasii and Lemingii lineages the speciation process took place in the Miocene, about 7.5 MY, during the Tortonian. Consequently, the geographical structure created by their OBU is more complex and reflects the influence of the earlier endorrheism. During this period the early Duero and Ebro endorrheic lagoons were still connected, as were the upper Tago and Júcar basins.

The ancient endorrheism seems to be highly related with cladogenesis within the Iberian *Chondrostoma* clade.

THE LAGO MARE PHASE AND THE WESTERN PART OF THE MEDITERRANEAN AREA

The four main lineages of the genus *Chondrostoma* that inhabited the Iberian Peninsula originated before the Messinian salinity crisis (Fig. 5). Our result, reject an extensively colonized the Iberian Peninsula through a freshwater Mediterranean Lake, as has been proposed for other freshwater fishes (Bianco, 1990). Penzo *et al.* (1998) and Zardoya & Doadrio (1999) considered unrealistic this extensive dispersion in gobiids and cyprinids. However, a general dispersion model has been proposed for the genus *Chondrostoma* during the Lago Mare Phase (Durand *et al.*, 2003).

No geological arguments exist to support that the Lago Mare phase affected the western part of the Mediterranean area. The presence in the upper evaporates of a non-evaporitic subdivision was interpreted by Hsü *et al.* (1978) as a Lago Mare phase. However, the fact that the seismic register of Upper Evaporites in West-Mediterranean (Montandert *et al.*, 1978) recognized an average interval velocity of 3.5 Km/s, similar to the overlying Pliocene sediments, could be the result of lesser freshwater input in the western Mediterranean area than in eastern areas (Griffin, 2002). Important changes in the salinity seem that particularly affected the Cyrenaica Lake in the eastern Mediterranean area but not the western Mediterranean basin. Paleontological evidence also does not support the theory that cyprinids in the Iberian Peninsula originated during the Messinian crisis because of the presence of cyprinid fossils, including, the genus *Squalius*, in Iberian deposits, since Upper-Oligocene (De la Peña, 1995).

Molecular phylogenies that find a basal polytomy among different genera have been the principal argument used to support dispersion through an extensive freshwater lake. The polytomy found in the work of Durand *et al.* (2000) on the genus *Squalius* was resolved by Sanjur *et al.* (2003) just by increasing the number of taxa analyzed.

Bianco's (1990) dispersal theory that freshwater fishes originated in Paratethyan Sea was recently modified by Durand *et al.* (2003) after introducing new geological evidence. Durand *et al.* (2003) considered that freshwater fishes scattered through out the margins of the Paratethys Sea during the short time that the Mediterranean Sea was fresh water. It is predicted that colonization and speciation occurred quickly and in a short period of time displaying a model of hard polytomies in the molecular phylogenetic trees. We have found no support for this model with our analyse on the genera *Squalius*

or *Chondrostoma* because the polytomies in our trees are found before the Lago Mare phase. The differences dating the politomies between Durand *et al.* (2003) and our own results could be due to the sampling. The radiation showed before the Lago Mare phase could be an artefact as consequence of the type of molecular marker used (Cytochrome b). Other molecular markers should be used to verify if the radiation shown really existed.

Dispersion due to river captures could have been possible during the Messinian because the drawdown of Mediterranean sea caused the headwaters of the main European and African rivers to become incised into the bedrock during the Mediterranean regression. Moreover, because of wet periods and the monsoon influence (Griffin, 2002), dispersion of freshwater fish fauna by sea was possible in the southeastern area of the Mediterranean. In the western Mediterranean basin the situation was probably different. The Iberian Peninsula had a dry climate during the Messinian, causing the endorrheic lakes to shrink in size and probably fragment. Hence, the principal effect of the dry Messinian period in the Iberian Peninsula would have been the isolation and differentiation of populations. This would have then been followed by speciation in allopatry rather than a quick radiation after a dispersal event.

Finally, the serious differences in the distribution pattern displayed by *Squalius* and *Chondrostoma* species do not support a dispersal scenario like that proposed by the Lago Mare hypothesis. Because both the dry as well as the wet periods during the Cenozoic lasted for long periods of time, the dispersion and speciation of freshwater fishes in the Mediterranean region was more gradual than the Lago mare hypothesis predicts.

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