

# Phylogenetic overview of the genus *Squalius* (Actinopterygii, Cyprinidae) in the Iberian Peninsula, with description of two new species

by

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**ABSTRACT.** - A more accurate assessment of the true diversity within the genus *Squalius* on the Iberian Peninsula was made using morphological and molecular characters. A phylogenetic analysis based on the complete sequence of the mitochondrial cytochrome *b* gene was used to reveal monophyletic groups that had the sufficient evolutionary significance to be regarded as new species. On the basis of genetic and morphological characters, Mediterranean populations from eastern and southern Spain that, in the past, were included in *Squalius pyrenaicus* are herein described as two new species. *Squalius valentinus* sp. nov., the new eastern Spanish species, inhabits the Mijares, Turia, Júcar, Serpis, Bullent, Gorgos, Guadalest, Monebre (Verde), and Vinalopo basins as well as the Albufera de Valencia Lagoon. It is distinguished from *S. pyrenaicus* by a combination of morphometric, meristic and genetic characters, such as a wide caudal peduncle, low number of scales (8-9/35-39/3), large fourth and fifth infraorbital bones, pointed anterior process of the maxilla, frontal bone wider in the middle, large and narrow urohyal, robust lower branch of the pharyngeal bone. Divergence distances of the cytochrome *b* in *S. valentinus* sp. nov. are  $p = 0.021-0.032$  with respect to *S. pyrenaicus*. In addition, *S. valentinus* sp. nov. has two diagnostic loci to nuclear molecular markers (IDHP-2 and IDHP-3). *Squalius malacitanus* sp. nov., the new southern Spanish species, inhabits the Guadalmina, Guadaiza and Guadiaro basins in Málaga Province, in southern Spain. *S. malacitanus* sp. nov. is distinguished from *S. pyrenaicus* by a combination of morphometric, meristic and genetic characters, such as narrow caudal peduncle, and high number of scales (7-8/39-43/3). The fourth and fifth infraorbitals are large and the anterior process of the maxilla is not pointed. Frontal bone is not wider in the middle. The urohyal is short and wide and the lower branch of the pharyngeal bone is thin. Divergence distances of the cytochrome *b* in *S. malacitanus* sp. nov. are  $p = 0.078-0.086$  with respect to *S. pyrenaicus*. The distribution range of *S. pyrenaicus* currently includes the Ebro, Júcar, Segura, Guadalfeo, Guadalhorce, Vélez, Guadalquivir, Tinto, Odiel, Piedras, Guadiana, Alportel, Sado, Sorraia, Tajo, Colares, Samarra, Sizandro, Grande, Lis and San Pedro basins.

**RÉSUMÉ.** - Révision phylogénétique du genre *Squalius* (Actinopterygii, Cyprinidae) en péninsule Ibérique, avec description de deux nouvelles espèces.

L'utilisation de caractères morphologiques et moléculaires a permis de réévaluer la diversité réelle du genre *Squalius* en péninsule Ibérique. Une analyse phylogénétique fondée sur l'étude de séquences complètes du gène mitochondrial cytochrome *b* nous a permis d'identifier des groupes monophylétiques présentant une signification évolutive suffisante pour être considérés comme de nouvelles espèces. Sur la base de leurs caractères morphologiques et génétiques, les populations méditerranéennes de l'Est et du Sud de l'Espagne qui, jusqu'à présent, étaient incluses dans *Squalius pyrenaicus* sont ici décrites comme deux nouvelles espèces. La nouvelle espèce de l'Est de l'Espagne, *Squalius valentinus*, vit dans les bassins des rivières Mijares, Turia, Serpis, Bullent, Gorgos, Guadalest, Monebre (Verde) et Vinalopó, ainsi que dans l'Albufera de Valencia. *S. valentinus* se différencie de *S. pyrenaicus* par une combinaison de caractères méristiques, génétiques et morphométriques comme un large pédoncule caudal, un nombre réduit d'écailles (8-9/35-39/3), des larges quatrième et cinquième os infraorbitaires, un processus antérieur de l'os maxillaire pointu, un os frontal élargi en son centre, un urohyal grand et étroit, une robuste branche inférieure de l'os pharyngien. La divergence des séquences du cytochrome *b* entre *S. valentinus* et *S. pyrenaicus* est égale à  $p = 0,021-0,032$ . De plus, *S. valentinus* présente deux loci diagnostiques pour des marqueurs nucléaires (IDHP-2 et IDPH-3). La nouvelle espèce du Sud de l'Espagne, *S. malacitanus* vit dans les bassins des rivières Guadalmina, Guadaiza et Guadiaro. *S. malacitanus* se différencie de *S. pyrenaicus* par une combinaison de caractères méristiques, génétiques et morphométriques comme un pédoncule caudal étroit et un nombre élevé d'écailles (7-8/39-43/3) ; des grands quatrième et cinquième infraorbitaires et un processus antérieur du maxillaire non pointu ; un os frontal non élargi en son centre ; un urohyal court et large et une fine branche inférieure de l'os pharyngien. La divergence entre cette nouvelle espèce et *S. pyrenaicus* est de  $p = 0,078-0,086$ . Actuellement, l'aire de répartition de *S. pyrenaicus* inclut les bassins des rivières Ebro, Júcar, Segura, Guadalfeo, Guadalhorce, Vélez, Guadalquivir, Tinto, Odiel, Piedras, Guadiana, Alportel, Sado, Sorraia, Tajo, Colares, Samarra, Sizandro, Grande, Lis et San Pedro.

Key words. - Cyprinidae - *Squalius* - Iberian Peninsula - Taxonomy - Molecular phylogeny - Cytochrome *b*.

The genus *Squalius* is widely distributed throughout European freshwater lakes and rivers, and shows a high level of diversity in the Mediterranean area (Zardoya and

Doadrio, 1999; Durand *et al.*, 2000; Doadrio and Carmona, 2003). Recent advances in molecular studies have identified three different lineages of this genus (Sanjur *et al.*, 2003):

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the "Mediterranean lineage" composed of the species *S. aradensis* (Coelho *et al.*, 1998), *S. carolitertii* (Doadrio, 1987), *S. pyrenaicus* (Günther, 1868) and *S. torgalensis* (Coelho *et al.*, 1998), from the Iberian Peninsula, *S. lucumonis* (Bianco, 1983) from Italy, *S. keadicus* (Stephanidis, 1971) from Greece and *S. illyricus* Heckel and Kner, 1858 and *S. zrmanjiae* Karaman, 1928 from the Balkan area. A second lineage is the "Euro-Asiatic" lineage, which is principally made up of large-sized taxa, such as *S. cephalus cephalus* (L., 1758), widely distributed throughout Central Europe and the Danube basin, *S. orientalis* (Nordmann, 1840) distributed in the Transcaucasian area, *S. lepidus* Heckel, 1843 from the Tigris basin, *S. peloponensis peloponensis* (Valenciennes, 1844), *S. peloponensis moreoticus* (Stephanidis, 1971), *S. cephalus vardarensis* Karaman, 1928 and *S. macedonicus* (Karaman, 1955) from Greece, *S. prespensis* (Fowler, 1977) from Prespa Lake and *S. cabeda* (Risso, 1827) from France and Italy. The third lineage is the "Paratethys lineage" that includes *S. borysthenicus* Kessler, 1859 and *S. smyrnaeus* (Boulenger, 1896) from the Black Sea basin and adjacent rivers in Turkey.

Complementary molecular studies found that two other taxa from the Iberian Peninsula, *Squalius alburnoides* (Steindachner, 1866) and *S. palaciosi* (Doadrio, 1980), were closely related to the species from the "Mediterranean Lineage" (Zardoya and Doadrio, 1998, 1999). These species were not analysed by Sanjur *et al.* (2003) because they formed part of a hybrid complex with *S. pyrenaicus*, which is the maternal ancestor of *S. alburnoides* (Alves *et al.*, 1997, 1999, 2001; Carmona *et al.*, 1997). Although both taxa *S. alburnoides* and *S. palaciosi* share modes of unisexual reproduction they likely have an important role in the evolutionary history of the genus *Squalius* (Carmona *et al.*, 1997; Alves *et al.*, 2001; Cunha *et al.*, 2004).

Durand *et al.* (2000) also recognized *Ladigesocypris ghiggi* (Gianferrari, 1927) as a member of the genus *Squalius*. Nevertheless, they described two very different and unrelated haplotypes for *L. ghiggi* and, therefore, the suggested relationship between this species and the *Squalius* lineage remains unclear.

Other species that share morphological characters similar to *Squalius* are *S. cephalus albus* (Bonaparte, 1838) from Lake Trasimeno in Italy, *S. agdamicus* Kamensky, 1901 from the Kura basin in Azerbaijan, *S. aphipsi* (Aleksandrov, 1927) from the Kuban River in Transcaucasia, *S. berak* Heckel, 1843 from Syria, *S. persidis* from Iran, *S. squalius culus* Kessler, 1872 from the Aral Sea basin, *S. svallize* Heckel and Kner, 1858 from Croatia, *S. turcicus* De Filippi, 1865 from Turkey and *S. ulanus* (Günther, 1899) from Iran. Unfortunately, given that these species have hardly been studied and no molecular studies are available, the phylogenetic relationships among these species are still largely unknown.

Within the distribution range of the genus *Squalius*, the Iberian Peninsula is singularly one of the most diverse areas. The Iberian Peninsula is inhabited by at least seven taxa belonging to the "Mediterranean" and "Euro-Asiatic" lineages (*S. alburnoides*, *S. aradensis*, *S. carolitertii*, *S. cephalus*, *S. palaciosi*, *S. pyrenaicus* and *S. torgalensis*). Except for *S. cephalus*, the remaining species are endemic to Iberia. Moreover, two fossil species described within the genus *Leuciscus*: *L. pachecoi* Royo, 1922 and *L. antunesi* Gaudant, 1977 from the Miocene of the Iberian Peninsula, are morphologically similar to living species of the genus *Squalius* (Doadrio, 2003). The first fossil record of the family Cyprinidae in Spain was dated to the Upper Oligocene by De la Peña (1995).

Because of its diversity, this area has received much attention from taxonomists and evolutionary biologists. Hence, there have been an increasing number of publications focused on Iberian *Squalius* species in the last ten years. Studies were initially developed using allozyme electrophoresis (Coelho *et al.*, 1995; Alves *et al.*, 1997; Carmona *et al.*, 1997), later mitochondrial gene sequencing was introduced (Brito *et al.*, 1997; Zardoya and Doadrio, 1998; Doadrio and Carmona, 2003; Sanjur *et al.*, 2003) and most recently microsatellites are being used to study phylogeographical relationships (Cunha *et al.*, 2004). Interestingly, all of these approaches have revealed several genetic and geographically well-differentiated *Squalius* populations within the Iberian Peninsula, that still have not been recognized as different species. In particular, Sanjur *et al.* (2003) identified East Mediterranean populations of *S. pyrenaicus* as a monophyletic clade. Differences in the complete cytochrome *b* sequence between these populations and Atlantic populations of *S. pyrenaicus* ranged from 2.24 to 3.45%.

Within the Iberian *Squalius* species, the absence of high variability in the principal meristic characters used in taxonomic studies, such as the number of fin rays, number of scales, number of branchiospines and number of vertebrae, has hindered making an accurate estimation of the true diversity of this genus. Fortunately, molecular approaches can be applied in parallel to morphological analyses, which help mitigate the obstacles caused by the lack of phenotypic variability. For example, two new *Squalius* species *S. aradensis* and *S. torgalensis* were described in Portuguese rivers (Coelho *et al.*, 1998). Through allozyme and gene sequence analyses (Coelho *et al.*, 1995; Brito *et al.*, 1997) these species were found not to be *S. pyrenaicus* and were later, formally, described as new species on the basis of morphological characters (Coelho *et al.*, 1998). Similar procedures have also been utilised to identify species within other Iberian populations of the genera *Chondrostoma*, *Gobio*, *Cobitis* and *Aphanius* (Doadrio and Perdices, 1997; Doadrio *et al.*, 2002; Doadrio and Carmona, 2003; Doadrio and Madeira, 2004).

Studies have revealed that nucleotide substitution rates for the cytochrome *b* gene can be used to establish phylogenetic relationships among *Squalius* lineages at species and population levels (Brito *et al.*, 1997; Durand *et al.*, 1999; Zardoya and Doadrio, 1999; Zardoya *et al.*, 1999; Doadrio and Carmona, 2003; Sanjur *et al.*, 2003). Hence, analysis of this molecular marker can reveal evolutionary significant monophyletic groups.

The aim of this work is to analyse the phylogenetic relationships of the Iberian *Squalius* species, giving special attention to the widely distributed *S. pyrenaicus*, in order to determine whether there are genetically divergent populations that should be distinguished at the species level. Unequivocally differentiated populations are analysed on the basis of morphometric and osteological characters and described as new species.

**MATERIALS AND METHODS**

**Nucleotide sequence analysis**

A total of 54 samples from five species inhabiting the Iberian Peninsula were analysed. Because they were part of a hybrid complex, the species *Squalius alburnoides* and *S. palaciosi* were not included in this study. The new samples studied were collected in the wild from multiple locations. (Tab. I; Fig. 1). DNA was extracted from dorsal muscle, and 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. The cyprinid species *Rutilus rutilus* and *Squalius borys-thenicus* were used as outgroups.

Total cellular DNA was isolated from tissues using a

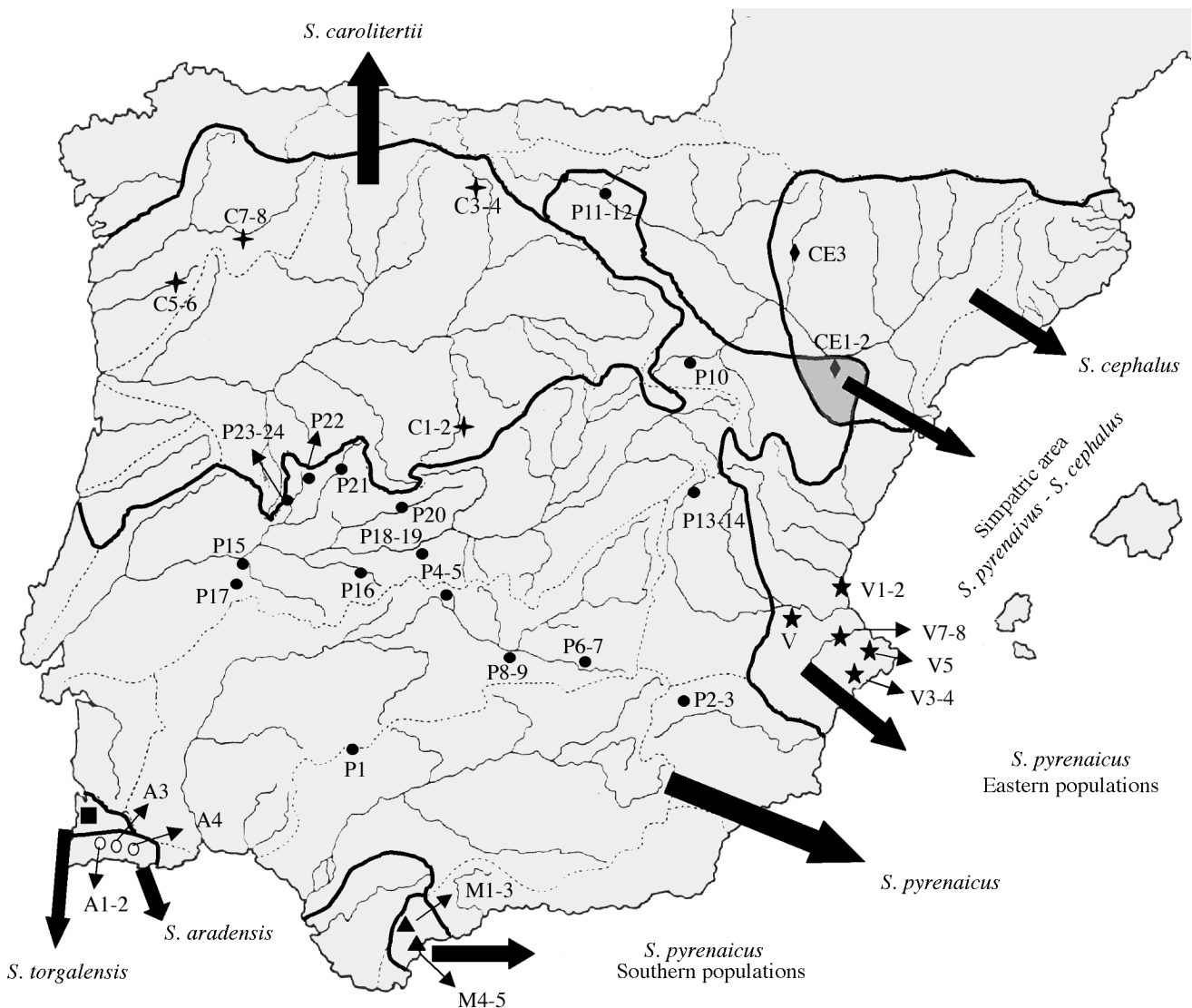


Figure 1. - Distribution of sampling localities for *Squalius* specimens. Corresponding rivers and basins are described in table I. [Localités de répartition des spécimens de *Squalius*. Les rivières et bassins correspondants sont décrits dans le tableau I.]

Table I. - Species analysed, location data, population designation (following figure 1) and GenBank accession number. Sequences retrieved from: a = Sanjur *et al.*, 2003; b = Zardoya and Doadrio, 1999; c = Brito *et al.*, 1997; d = Briolay *et al.*, 1988. [*Espèces analysées, données sur leur localisation, désignation de la population (d'après la figure 1) et numéro d'accès à la base de données GenBank.*]

Species	Basin	River	Locality (Pop. designation)	GenBank Accession N°
<i>S. aradensis</i>	Arade	Arade	Portugal (A1)	AF421824 <sup>a</sup>
	Arade	Arade	Portugal (A2)	AF421825 <sup>a</sup>
	Aljezur	Aljezur	Portugal (A3)	New sequence
	Bordeira	Bordeira	Portugal (A4)	New sequence
<i>S. caroliteriti</i>	Duero	Adaja	El Fresno (C1)	AF421799 <sup>a</sup>
	Duero	Adaja	El Fresno (C2)	AF421800 <sup>a</sup>
	Duero	Boedo	Bascones de Ojeda (C3)	AF421797 <sup>a</sup>
	Duero	Boedo	Bascones de Ojeda (C4)	AF421798 <sup>a</sup>
	Limia	Salas	Rubia delos Mixtos (C5)	AF421795 <sup>a</sup>
	Limia	Salas	Rubia delos Mixtos (C6)	AF421796 <sup>a</sup>
	Miño	Bibeí	San Agustín (C7)	AF421793 <sup>a</sup>
	Miño	Bibeí	San Agustín (C8)	AF421794 <sup>a</sup>
<i>S. cephalus</i>	Rhone	Rhone	France (CE1)	Y10446 <sup>d</sup>
	Ebro	Matarraña	Nonaspe (CE2)	AF421801 <sup>a</sup>
	Ebro	Matarraña	Nonaspe (CE3)	AF045995 <sup>b</sup>
	Ebro	Monzon	Cinca (CE4)	AF421803 <sup>a</sup>
<i>S. pyrenaicus</i>	Guadalquivir	Montemayor	Cañaverl de León (P1)	AF421790 <sup>a</sup>
	Segura	Bogarra	Las Mohedas (P2)	AF421820 <sup>a</sup>
	Segura	Bogarra	Las Mohedas (P3)	AF421821 <sup>a</sup>
	Guadiaro	Genal	Jubrique (M1)	New sequence
	Guadiaro	Genal	Jubrique (M2)	New sequence
	Guadiaro	Genal	Jubrique (M3)	New sequence
	Guadalmina	Guadalmina	Benahavis (M4)	New sequence
	Guadalmina	Guadalmina	Benahavis (M5)	New sequence
	A. de Valencia	Font del Barret	Sollana (V1)	AF421808 <sup>a</sup>
	A. de Valencia	Font del Barret	Sollana (V2)	AF421809 <sup>a</sup>
	Serpis	Serpis	Beniarres (V7)	AF421810 <sup>a</sup>
	Serpis	Serpis	Beniarres (V8)	AF421815 <sup>a</sup>
	Algar	Algar	Callosa d en Sarriá (V3)	AF421818 <sup>a</sup>
	Algar	Algar	Callosa d en Sarriá (V4)	AF421819 <sup>a</sup>
	Bullent	Bullent	Pego (V5)	New sequence
	Júcar	Bco. del Agua	Jarafuel (V6)	New sequence
	Guadiana	Estena	Navas de Estena (P4)	AF421813 <sup>a</sup>
	Guadiana	Estena	Navas de Estena (P5)	AF421814 <sup>a</sup>
	Guadiana	L. de Ruidera	Ossa de Montiel (P6)	AF421822 <sup>a</sup>
	Guadiana	L. de Ruidera	Ossa de Montiel (P7)	AF421823 <sup>a</sup>
	Guadiana	Azuer	Casa de Fuente Vieja (P8)	AF421804 <sup>a</sup>
	Guadiana	Azuer	Casa de Fuente Vieja (P9)	AF421805 <sup>a</sup>
	Ebro	Piedras	Cimballa (P10)	New sequence
	Ebro	Baias	Álava (P11)	New sequence
Ebro	Baias	Álava (P12)	New sequence	
Júcar	Laguna de Uña	Uña (P13)	AF421806 <sup>a</sup>	
Júcar	Laguna de Uña	Uña (P14)	AF421807 <sup>a</sup>	
Tajo	Alburrel	Valencia de Alcántara (P15)	New sequence	
Tajo	Almonte	Jaraicejo (P16)	AF421791 <sup>a</sup>	
Tajo	Aurela	Santiago de Alcántara (P17)	New sequence	
Tajo	Cedena	Navahermosa (P18)	New sequence	
Tajo	Cedena	Navahermosa (P19)	New sequence	
Tajo	Tietar	Talayuela (P20)	AF045993 <sup>b</sup>	
Tajo	Arrago	Hoyos (P21)	AF421826 <sup>a</sup>	
Tajo	Acebo	Cadalso de Gata (P22)	AF421827 <sup>a</sup>	
Tajo	Pesquero	Valverde del Fresno (P23)	AF421811 <sup>a</sup>	
Tajo	Pesquero	Valverde del Fresno (P24)	AF421812 <sup>a</sup>	
<i>S. torgalensis</i>	Mira	Mira	Portugal	X99742 <sup>c</sup>
<i>Rutilus rutilus</i>	Rhone	Saone	France	Y10440 <sup>d</sup>
<i>S. borysthenicus</i>	Filiouris	Filiouris	Greece	AF090759 <sup>b</sup>

standard proteinase K and phenol/chloroform extraction method (Sambrook *et al.*, 1989). Two overlapping fragments of the cytochrome *b* gene (total of 1140 bp) were amplified via polymerase chain reaction (PCR) from each individual DNA sample. The primers used for cytochrome *b* were those mentioned in Machordom and Doadrio (2001).

The amplification process was conducted as follows: 94°C (2 mn), 35 cycles at 94°C (45 s), 48°C (1 mn), 72°C (90 s), 72°C (5 mn). PCR mixtures were prepared in 25 ml with a final concentration of 0.4 mM of each primer, 0.2 mM of each dNTP, 1.5 mM MgCl<sub>2</sub>, 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 the manufacturer's instructions. Chromatograms and alignments were visually checked and verified.

Nucleotide composition was examined, and the  $\chi^2$  homogeneity test of base frequencies was carried out in PAUP\* ver 4.0b10 (Swofford, 2002) for all positions. Saturation was analysed by plotting the absolute number of transitions and transversions against patristic distance values. The aligned data were analysed independently using maximum parsimony (MP), neighbour joining (NJ), maximum likelihood (ML), and Bayesian methods of phylogenetic inference. MP analyses were performed with PAUP\* using heuristic searches with 10 random stepwise additions and TBR branch swapping. Transversions (Tv) were weighed 10 times the transitions (Ti) following the empirically determined Tv/Ti ratio for *Squalius*. Different weighing schemes were also tried (6:1, 8:1, or equal weight). For NJ, ML and Bayesian inference analyses 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 and Crandall, 1998). NJ analyses were carried out with PAUP\*. ML analyses were performed with quartet puzzling as implemented in PAUP\* with 5,000 puzzling steps. Bootstrap analysis (500 replicates) was used to assess the relative robustness of branches of NJ and MP trees. A Bayesian inference of *Squalius* phylogeny was performed with MrBayes 3.0 (Huelsenbeck and Ronquist, 2001) by simulating a Markov chain for 1,000,000 cycles.

### Morphometric analysis

The description of the East-Mediterranean populations of *Squalius pyrenaicus* as a new *Squalius* species is based on 22 adults from the Algar River (Guadalest basin) in Callosa d'Ensarriá, Alicante, Eastern Spain and 17 adults from Turia River, Turró in Valencia, Spain. The description of the

South-western Mediterranean populations of *Squalius pyrenaicus* as a new *Squalius* species is based on 21 adults from the Guadalmina River in Benahavis, Málaga, Spain and four adults from Genal River (Guadiaro basin) in Jubrique, Málaga, Spain. The holotypes and the paratypes have been deposited in the Museo Nacional de Ciencias Naturales, Madrid, Spain.

The comparative material of *Squalius pyrenaicus* is shown in Appendix A.

Twenty-two morphometric variables were measured. All measurements are in millimetres on ethanol material. The following abbreviations were used for morphometric and meristic characters: SL, standard length; HL, head length; PrOL, preorbital length; ED, eye diameter; ID, interorbital distance; PrDD, predorsal distance; PrPD, prepectoral distance; PrVD, preventral distance; PrAD, preanal distance; CPL, caudal peduncle length; APL, anal peduncle length; PVL, pectoral-ventral length; VAL, ventral-anal length; DFL, dorsal fin length; DFH, dorsal fin height; PFL, pectoral fin length; VFL, ventral fin length; AFL, anal fin length; AFH, anal fin height; CFL, caudal fin length; BD body depth; BLD, body least depth; D, dorsal fin rays; A, anal fin rays; P, pectoral fin rays; V, ventral fin rays; C, caudal fin rays; LLS, lateral line scales; RSA, scale rows above lateral line; RSB, scale rows below lateral line; GR, gill rakers; PT, pharyngeal teeth; Abd. Vert., abdominal vertebrae; Cau. Vert., caudal vertebrae. Only branched fins rays were counted. The osteological characters were studied from cleared and stained specimens (Wassersug, 1976). Institutional acronym: MNCN Museo Nacional de Ciencias Naturales, Madrid, Spain.

### *SQUALIUS MALACITANUS* SP. NOV.

#### Holotype (Fig. 2, Tab. II)

MNCN243699, Guadalmina River, Benahavis, Málaga, leg. I. Doadrio, P. Garzón and R. Zardoya, 29 Mar. 2003.

#### Paratypes

MNCN212280-311, 32 specimens, Guadalmina River, Benahavis, Málaga, Guadalmina basin, leg. A. Doadrio, I. Doadrio, I. Doadrio Jr. and P. Garzón, 5 Mar. 2003. - MNCN243700-703, 4 spms, Guadalmina River, Benahavis, Málaga, Guadalmina basin, leg. I. Doadrio, P. Garzón and R. Zardoya, 29 Mar. 2003. - MNCN212057-61, 5 spms, Genal River, Jubrique, Málaga, Guadiaro basin, leg. I. Doadrio and P. Garzón, 5 Mar. 2000. - MNCN246678-680, 3 spms, Guadaiza River, Benahavis, Málaga, Guadaiza basin, leg Oscar Gavira, 13 Oct. 2003.

#### Diagnosis

Differs from all other known species of *Squalius* by the following combination of characters: seven or eight branched rays (9) in the dorsal fin, eight branched rays (7) in the anal

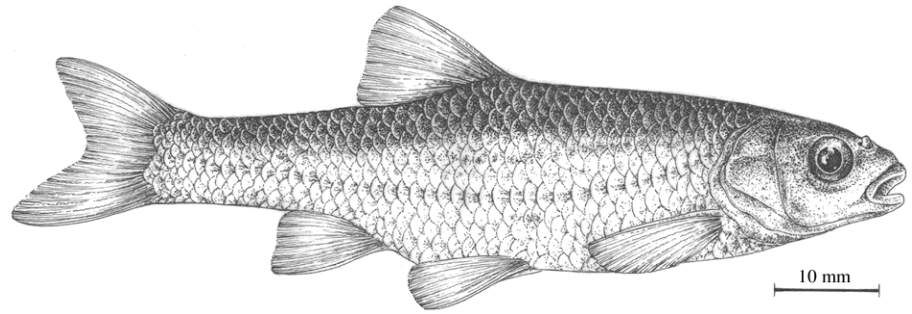


Figure 2. - *Squalius malacitanus* sp. nov. Holotype, MNCN243699, Guadalmina River, Guadalmina basin, Benahavis, Málaga, Spain.

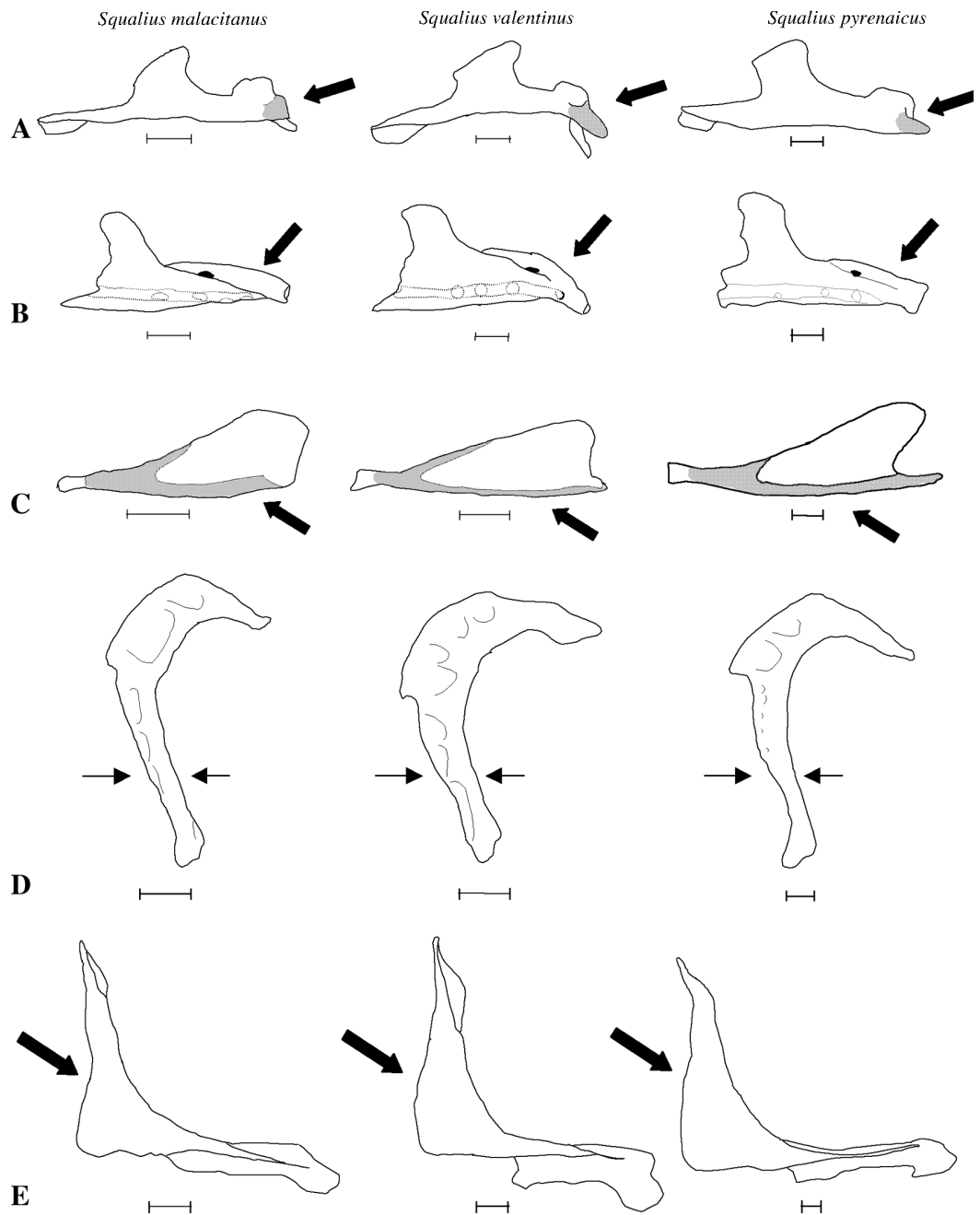


Figure 3. - Osteological characters in *Squalius pyrenaicus*, *S. malacitanus* sp. nov. and *S. valentinus* sp. nov. **A**: maxilla; **B**: dentary bone; **C**: urohyal; **D**: pharyngeal bone; **E**: cleithrum. Scale bars = 1 mm. [Caractères ostéologiques de *Squalius pyrenaicus*, *S. malacitanus* sp. nov. and *S. valentinus* sp. nov. **A** : maxillaire ; **B** : os dentaire ; **C** : os urohyal ; **D** : os du pharynx ; **E** : cleithrum. Échelles = 1 mm.]

<i>Squalius malacitanus</i> sp. nov. (n = 25)					
Variable	Holotype	Range	Mean	SD	Mediana
SL	59.2	43.9-76.1	56.1	9.4	54.6
HL	15.2 (3.9)	11.7-20 (3.6-4.1)	14.4 (3.9)	2.1 (0.1)	14.2 (3.8)
PrOL	3.6 (16.5)	3-5.3 (12.2-19.9)	3.9 (15.1)	0.7 (1.9)	3.7 (15.1)
ED	3.8 (15.5)	3.4-5.5 (11.0-15.5)	4.3 (13.2)	0.6 (1.1)	4.2 (13.2)
ID	5.1 (11.6)	4.1-7.2 (9.7-11.6)	5.3 (10.6)	0.9 (0.6)	5.2 (10.7)
PrDD	30.7 (1.9)	22.5-41.6 (1.8-2.1)	29.3 (1.9)	5.4 (0.1)	27.9 (1.9)
PrPD	15.2 (3.9)	10.3-18.9 (3.6-4.8)	13.8 (4.1)	2.4 (0.2)	13.2 (4.0)
PrPV	29.2 (2.0)	22-37 (1.9-2.2)	27.1 (2.1)	4.6 (0.1)	25.9 (2.1)
PrAD	40.3 (1.5)	28.3-51 (1.4-2.1)	36.6 (1.5)	6.1 (0.2)	34.8 (1.5)
CPL	22.6 (2.6)	16.2-29.6 (2.5-2.9)	21.2 (2.6)	3.9 (0.1)	19.7 (2.6)
APL	14.3 (4.1)	10.6-19.5 (3.6-4.2)	14.5 (3.9)	2.6 (0.2)	13.7 (3.9)
PVL	14.1 (4.2)	9.4-17.1 (4.0-5.2)	12.7 (4.4)	2.3 (0.3)	12 (4.4)
VAL	10.2 (5.8)	7.3-13.4 (5.2-6.5)	10 (5.6)	1.8 (0.3)	9.5 (5.6)
DFL	6 (9.9)	4.4-8.3 (8.3-10.4)	6 (9.7)	1.1 (0.6)	5.7 (9.4)
DFH	10.1 (5.9)	7.6-14.3 (4.2-6.0)	11.1 (5.1)	2.1 (0.5)	11.1 (5.1)
PFL	12.6 (4.7)	9.6-17.4 (4.1-5.4)	11.9 (4.7)	2.2 (0.3)	11.6 (4.7)
VFL	9.3 (6.3)	6.7-13.2 (5.5-7.2)	9.2 (6.1)	1.8 (0.5)	9 (5.9)
AFL	5 (11.9)	4-7.3 (9.5-11.9)	5.2 (10.9)	0.9 (0.7)	4.8 (11.1)
AFH	9.2 (6.5)	6.7-13.8 (5.0-7.4)	9.4 (6.0)	2 (0.6)	8.9 (5.9)
CFL	10.4 (5.7)	9-14.5 (4.4-7.0)	10.7 (5.3)	1.4 (0.6)	10.3 (5.1)
BD	14.4 (4.1)	9.6-18.3 (4.0-4.8)	12.6 (4.4)	2.3 (0.2)	12.2 (4.5)
BLD	5.9 (10.1)	4.2-7.6 (9.4-11.5)	5.5 (10.2)	1 (0.4)	5.2 (10.2)
LLS	41	39-43	41	1.4	41
SRA	8	7-8	7.6	0.5	8
SRB	3	3-3	3	0	3
D	8	7-9	8	0.3	8
A	8	7-8	7.8	0.4	8
GR	-	9-11	10	0.7	10

fin. Two rows of pharyngeal teeth on both sides with two and five teeth, respectively (2.5-5.2); low caudal peduncle. Number of gill rakers  $\bar{x}$  = 10 (range: 9-11). Number of scales in

Table II. - Statistical parameters for the morphometric characters of *Squalius malacitanus* sp. nov. All morphometric variables are showed as a percentage of the standard length (in brackets SL/-). Variables are described in Methods. (SD = Standard deviation). [Paramètres statistiques pour les caractères morphométriques de *Squalius malacitanus* sp. nov. Toutes les variables morphométriques sont données en pourcentage de la longueur standard (entre parenthèses SL/-). Les variables sont décrites dans les méthodes (SD = déviation standard).]

lateral line  $\bar{x}$  = 41 (range: 39-43). Number of scale rows above lateral line  $\bar{x}$  = 7.6 (range: 7-8). Three scale rows below lateral line. Thirty-eight vertebrae, 21 abdominal and 17 caudal vertebrae. Large fourth and fifth infraorbital bones. Maxilla without pointed anterior process. The middle part of the frontal bone is narrow. Narrow neurocranium bone. The lower branch of the pharyngeal bone is long and delicate. The inferior lamina of the urohyal bone is short and wide.

### Description

D II-III 7-9 ( $\bar{x}$  = 7.7), A II-III 7-8 ( $\bar{x}$  = 7.8), P I 14, V II 7, C 17, LLS 39-43 ( $\bar{x}$  = 41), SRA 7-8 ( $\bar{x}$  = 7.6). SRB 3, PT 2.5-5.2, GR 9-11 ( $\bar{x}$  = 10). Morphometric characters are given in tables II and IV. A small sized species that rarely reaches a standard length of 100 mm. Maximum body depth is 4-4.8 ( $\bar{x}$  = 4.5) times the standard length. Head is large and is 3.6-4.1 ( $\bar{x}$  = 3.9) times the standard length. The head length is greater than the maximum body depth. Preorbital distance is shorter than eye diameter, reaching 0.7-1.1 ( $\bar{x}$  = 0.9) times the eye diameter. Short interorbital distance, reaching 1-1.5 ( $\bar{x}$  = 1.2) times the eye diameter. Ventral fin is inserted at or slightly before the origin of the dorsal fin, on the same axis. Predorsal length is 1-1.1 ( $\bar{x}$  = 1.1) times the preventral length. Low caudal peduncle. Minimum body depth is 3.7-4.1 ( $\bar{x}$  = 3.9) times the caudal peduncle length and 2.3-2.9 ( $\bar{x}$  = 2.6) times the anal peduncle length (Tab. IV). Fin size moderately large, with a pectoral fin length of 1.1-1.3 ( $\bar{x}$  = 1.2) times the dorsal fin height.

### Pigmentation pattern

Silver body, darker dorsally. The colour is less bright than in *Squalius valentinus* sp. nov. The scales have one big black spot on the base and a series of small black spots on the distal border. There is a black spot at the base of the scales that appears to be divided by the lateral line pore into two separate spots. Silver peritoneum with a few small black spots. Scales are not deciduous.

### Osteology

Wide and short supraethmoid, parietal and frontal bones (Fig. 3). The posterior process of the pterotic is pointed. The lower branch of the pharyngeal bone is long and delicate. Short and wide urohyal. The anterior process of the maxilla is pointed. Fourth and fifth infraorbitals bones are wide but less expanded than in *Squalius valentinus* sp. nov. The pos-

Table IV. - Frequency distribution of individuals for Body Less Depth and Orbital indexes (-/BLD, -/ED) in *Squalius pyrenaicus*, *S. valentini* sp. nov. and *S. malacitanus* sp. nov. [Distribution de fréquences des individus pour la hauteur minimale des corps et les indices orbitaux (-/BLD, -/ED) chez *Squalius pyrenaicus*, *S. valentini* sp. nov. et *S. malacitanus* sp. nov.]

CPL/BLD range	2.71-2.9	2.91-3.2	3.21-3.4	3.41-3.6	3.61-3.8	3.81-4	4.01-4.3			
<i>S. valentinus</i> sp. nov. (n = 39)	8	21	4	6						
<i>S. malacitanus</i> sp. nov. (n = 25)					8	12	5			
<i>S. pyrenaicus</i> (n = 95)			1	36	37	16	5			
APL/BLD range	1.61-1.8	1.81-2	2.01-2.2	2.21-2.4	2.41-2.6	2.61-2.8	2.81-3	3.01-3.2		
<i>S. valentinus</i> sp. nov. (n = 39)	14	14	11							
<i>S. malacitanus</i> sp. nov. (n = 25)				2	9	11	3			
<i>S. pyrenaicus</i> (n = 95)			19	41	29	6				
PrOL/ED range	0.71-0.8	0.81-0.9	0.91-1	1.01-1.1	1.11-1.2	1.21-1.3	1.31-1.4	1.41-1.5		
<i>S. valentinus</i> sp. nov. (n = 39)	1	9	13	6	4	6				
<i>S. malacitanus</i> sp. nov. (n = 25)	5	6	10	4						
<i>S. pyrenaicus</i> (n = 95)				2	22	32	28	11		
ID/ED range	1.01-1.1	1.11-1.2	1.21-1.3	1.31-1.4	1.41-1.5	1.51-1.6	1.61-1.7	1.71-1.8		
<i>S. valentinus</i> sp. nov. (n = 39)			7	16	7	7	2			
<i>S. malacitanus</i> sp. nov. (n = 25)		5	9	8	2	1				
<i>S. pyrenaicus</i> (n = 95)			11	18	18	22	16	10		
LLS	35	36	37	38	39	40	41	42	43	44
<i>S. valentinus</i> sp. nov. (n = 39)	4	12	9	11	3					
<i>S. malacitanus</i> sp. nov. (n = 25)					4	6	5	6	4	
<i>S. pyrenaicus</i> (n = 95)					2	16	30	33	10	4

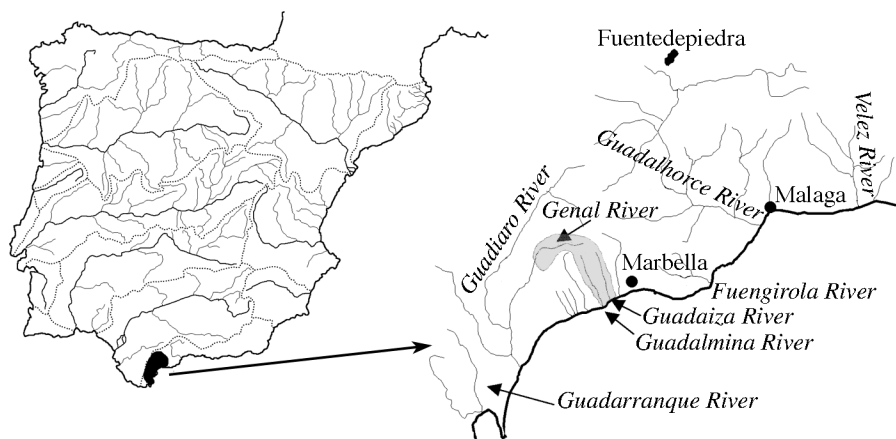


Figure 4. - Distribution area of *Squalius malacitanus* sp. nov. [Distribution géographique de *Squalius malacitanus* sp. nov.]

terior lamina of the cleithrum is not expanded. Short dentary, with a well-developed coronoid process. All these characters are marked with arrows in figure 3.

**Distribution**

We found *Squalius malacitanus* sp. nov. in the Guadalmina River, a short river that drains directly into the Mediterranean Sea, and in the Genal River, a tributary of the neighbouring Guadiaro River (Fig. 4). It is probably also present in other rivers of the Guadiaro basin.

**Etymology**

The species name “malacitanus” is derived from the name of the province of Malaga, the Spanish region the species inhabits.

**Remarks**

The species typically inhabits streams with clear waters and gravel bottoms and prefers moderate flowing stretches. In the Guadalmina River it is the only autochthonous fish. In the Genal River the species occurs in sympatry with the other native cyprinids *Barbus sclateri* and *Chondrostoma willkommii*, and also with *Anguilla anguilla* and *Atherina boyeri*.

**Conservation**

*Squalius malacitanus* sp. nov. is rare and has a restricted distribution range. In the last decade its habitat has been reduced by receding water levels due to the proliferation of golf fields. Consequently, the population is currently declining and should be considered critically endangered (CR B1



+ 2ce) according to the IUCN Red List Categories (Doadrio, 2003).

### *SQUALIUS VALENTINUS* SP. NOV.

#### **Holotype** (Fig. 5, Tab. III)

MNCN167, 77 mm SL, Turia River, Tuejar, Valencia, Turia basin, Spain. Leg. P. Risueño and J. Jimenez, 13 Mar. 2003.

#### **Paratypes**

MNCN239083-239134, 51 specimens, Barranco del Agua, Jarafuel, Valencia, Júcar basin, leg. J.A. Carmona and I. Doadrio. - MNCN217795-98, MNCN126706-23, 22 spms, Algar River, Callosa D'en Sarriá, Alicante, Guadalest basin, leg. I. Doadrio, 3 Nov. 1996. - MNCN126456-60, MNCN126453-55, 8 spms, Beniardá River, Beniardá, Alicante, Guadalest basin, leg. Centro de Acuicultura Experimental, 3 Nov. 1993. - MNCN124462-63, 2 spms, Font del Barret, Sollana, Valencia, Albufera de Valencia Lagoon, leg. I. Doadrio, A. Perdices and A. Machordón, 29 Apr. 1992. - MNCN118141-79, 39 spms, Gorgos River, Jalón, Alicante, Gorgos basin, leg. Universidad de Alicante, 13 Oct. 1973. - MNCN118186-97, 12 spms, Hondo de Elche Lagoon, Elche, Alicante, Vinalopo basin, leg. Universidad de Alicante, 13 Oct. 1973. - MNCN212768-70, 3 spms, Mijares River, Olba, Castellón, Mijares basin, leg. J.A. Carmona, 13 Aug. 2000. - MNCN24316973, 5 spms, Mijares River, Olba, Mijares basin, leg. J.A. Carmona and A. Corcuera, 6 Nov. 2001. - MNCN215385-89, 5 spms, Realillo River, Sort de Cuera, Valencia, Turia basin, leg. J.A. Carmona, 13 Aug. 2000. - MNCN124903-29, 27 spms, Mundo Lagoon, Elche, Alicante, Vinalopo basin, leg. Universidad de Alicante, 10 Jan. 1973. - MNCN124815-23, 9 spms, Serpis River, Alicante, Alicante, Serpis basin, leg. S. Peiró, 15 Jul. 1995. - MNCN24978-80, MNCN25518-25, 11 spms, Serpis River, Villalonga, Valencia, Serpis basin, leg. I. Doadrio, 27 Sept. 1978. - MNCN24772-77, MNCN24797, MNCN24830-31, MNCN24893-900, MNCN34273-77 MNCN34284-86, 25 spms, Tibi Dam (Verde River), Tibi, Alicante, Monebre basin, leg. I. Doadrio, 14 Oct. 1979. - MNCN196003-12, 9 spms, Turia River, Chulilla, Valencia, Turia basin, leg. B. Elvira and A. Bustamante, 6 Dec. 1983. - MNCN196780-98, 19 spms, Turia River, Pedralba, Valencia, Turia basin, leg. B. Elvira and A. Bustamante, 6 Dec. 1983. - MNCN244166, MNCN244168-182, 16 spms, 6 Dec. 1983, Turia River, Tuejar, Valencia, Turia basin, leg. P. Risueño and J. Jimenez, 13 Mar. 2003. - MNCN196552, MNCN24770-71, 3 spms, 6 Dec. 1983, Turia River, Villamarchante, Valencia, Turia basin, leg. I. Doadrio and P. Garzón, 20 Jul. 1978.

#### **Diagnosis**

Differentiated from all other known species of *Squalius* by the following combination of characters: eight branched rays (7) in the dorsal fin, eight branched rays (7-9) in the anal fin. Two rows of pharyngeal teeth on both sides with two and five teeth, respectively (2.5-5.2). Wide caudal peduncle. Number of gill rakers  $\bar{x} = 9.5$  (8-11). Number of scales in lateral line  $\bar{x} = 36.9$  (35-39). Number of scale rows above lateral line  $\bar{x} = 7.7$  (7-8). Three scale rows below lateral line. Thirty-nine vertebrae (38), twenty-two abdominal (21) and seventeen caudal. Large fourth and fifth infraorbitals bones. Maxilla with a very marked and pointed anterior process. Frontal bone expanded at its middle. Wide neurocranium bone. The lower branch of the pharyngeal bone is robust. Large and narrow urohyal. Presence of two diagnostic loci in allozymes (IDHP-2 and IDHP-3).

#### **Description**

D II-III 7-8 ( $\bar{x} = 7.9$ ), A II-III 7-9 ( $\bar{x} = 8$ ), P I 15, V II 7, C 19, LLS 35-39 ( $\bar{x} = 36.9$ ), SRA 7-8 ( $\bar{x} = 7,7$ ), SRB 3, PT 2.5(6)-5.2, GR 8-11 ( $\bar{x} = 9.5$ ). A medium size species that rarely reaches a standard length of 200 mm. Morphometric characters are given in tables III and IV. Maximum body depth is 3.5-4.2 ( $\bar{x} = 3.9$ ) times the standard length. Large head that is 3.5-4.1 ( $\bar{x} = 3.9$ ) times the standard length. The head length is the same as the maximum body depth. Preorbital distance is similar to eye diameter, reaching 0.8-1.4 ( $\bar{x} = 1$ ) times the eye diameter. Long interorbital distance, reaching 1.2-1.8 ( $\bar{x} = 1.4$ ) times the eye diameter. Ventral fin is inserted at or slightly before the origin of the dorsal fin, on the same axis. Predorsal length is 1-1.2 ( $\bar{x} = 1.1$ ) times the preventral length. High caudal peduncle. Minimum body depth is 2.7-3.5 ( $\bar{x} = 3.1$ ) times the length of the caudal peduncle and 1.6-2.1 ( $\bar{x} = 1.9$ ) times the length of the anal peduncle. Fin size moderately large, with pectoral fin length 1-1.5 ( $\bar{x} = 1.3$ ) times dorsal fin height.

#### **Pigmentation pattern**

Silver body, darker dorsally. The scales have one big black spot at the base and a series of small black spots on the distal border. There is a black spot at the base of the scales that appears to be divided by the lateral line pore into two

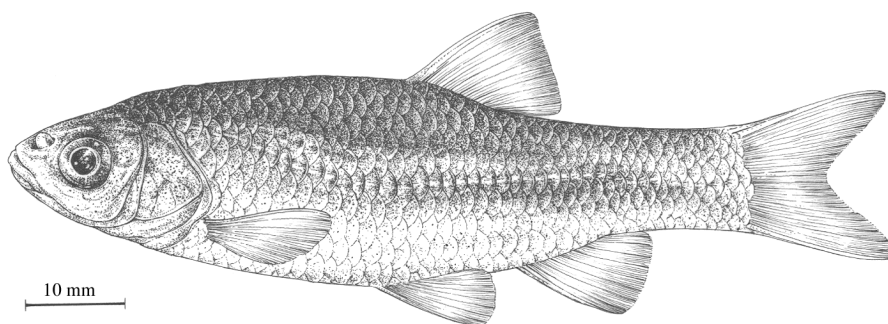


Figure 5. - *Squalius valentinus* sp. nov. Holotype, MNCN244167, Turia River, Turia Basin, Tuejar, Valencia, Spain.

Table III. - Statistical parameters for the morphometric characters of *Squalius valentinus* sp. nov. All morphometric variables are showed as a percentage of the standard length (in brackets SL/-). Variables are described in Methods. (SD = Standard deviation). [Paramètres statistiques pour les caractères morphométriques de *Squalius valentinus* sp. nov. Toutes les variables morphométriques sont données en pourcentages de la longueur standard (entre parenthèses SL/-). Les variables sont décrites dans les méthodes (SD = déviation standard).]

separate spots. Silver peritoneum with a few small black spots. Deciduous scales on juveniles give body a characteristic brightness.

### Osteology

Wide and short supraethmoid, parietal and frontal bones (Fig. 3). The posterior process of the pterotic is wide and robust. Lower branch of the pharyngeal bone is very robust. Large and thin urohyal. The anterior process of the maxilla is pointed. Fourth and fifth infraorbitals bones are very wide. The posterior lamina of the cleithrum is widened. Short dentary, with a well-developed coronoid process. Third, fourth and fifth pharyngeal teeth of the external row are very small or lack masticatory area. All these characters are marked with arrows in figure 3.

### Distribution

*Squalius valentinus* sp. nov. inhabits rivers in the Mediterranean slope of Spain between the Mijares and Vinalopo basins (Fig. 6). The Júcar basin is inhabited by *S. pyrenaicus* and *S. valentinus* sp. nov., although both species are in allopatry. The former inhabits the upper basin while the latter is distributed through out rivers of the lower basin. We found *S. valentinus* sp. nov. in the following basins: Mijares, Turia, Júcar, Serpis, Bullent, Gorgos, Guadalest, Monebre or Verde and Vinalopo Rivers and in the Albufera de Valencia Lagoon.

### Etymology

The species name “*valentinus*” is derived from the roman name of Valencia, the Spanish region the species inhabits.

### Common names

We propose to use the name Levantine bagra.

### Remarks

The species typically inhabits streams, with clear waters and gravel bottom and prefers moderate flowing stretches. In short Mediterranean streams such as the Gorgos Rivers it is the only autochthonous fish. In the Turia and Mijares basins the species occurs in sympatry with other native cyprinids *Barbus guiraonis*, *Chondrostoma turiense*, and *Chondrostoma arcasii*. *Squalius valentinus* sp. nov. inhabits the Júcar, Serpis and Vinalopo basins along with *B. guiraonis*.

<i>Squalius valentinus</i> sp. nov. (n = 39)					
Variable	Holotype	Range	Mean	SD	Mediana
SL	77.0	51.4-131.4	89.3	23.9	91.1
HL	20.5 (3.8)	13.6-32.7 (3.5-4.1)	23 (3.9)	5.5 (0.2)	23.1 (3.9)
PrOL	5.1 (15.0)	3.6-8.9 (13-17.4)	6.1 (14.7)	1.6 (0.9)	6.6 (14.7)
ED	5.6 (13.8)	4.6-8.2 (11.1-20.4)	5.9 (14.8)	0.9 (2.4)	5.8 (14.2)
ID	7.7 (10.0)	5.1-11.8 (9.1-11.7)	8.5 (10.4)	1.9 (0.7)	8.5 (10.3)
PrDD	41.4 (1.9)	27.6-70.8 (1.7-2.0)	47.8 (1.9)	12.5 (0.0)	49.7 (1.9)
PrPD	20.1 (3.8)	13.1-33.9 (3.6-4.2)	23.4 (3.8)	6.2 (0.1)	23.4 (3.8)
PrVD	38.2 (2.0)	24.6-68.7 (1.9-2.1)	45.2 (2.0)	12.6 (0.1)	45 (2.0)
PrAD	53.0 (1.5)	35.4-94.5 (1.4-1.5)	63.1 (1.4)	18.2 (0.0)	63.9 (1.4)
CPL	27.3 (2.8)	19.0-47 (2.6-3.0)	32.5 (2.8)	8.8 (0.1)	33.7 (2.7)
APL	17.3 (4.4)	11.7-27.5 (4.2-5.0)	19.7 (4.5)	5.0 (0.2)	19.7 (4.5)
PVL	17.6 (4.4)	11.3-31.9 (3.7-5.0)	21.2 (4.3)	6.2 (0.3)	21.4 (4.3)
VAL	12.5 (6.2)	7.2-29.4 (4.0-7.7)	16.8 (5.6)	6.5 (0.9)	18.2 (5.5)
DFL	9.2 (8.4)	6.5-15 (6.9-11.2)	10.1 (8.8)	2.3 (1.0)	9.7 (8.8)
DFH	12.8 (6.0)	9.7-23.6 (4.5-6.7)	16.7 (5.4)	4.1 (0.5)	18.6 (5.3)
PFL	14.1 (5.5)	10.2-29.6 (3.9-5.8)	17.8 (5.1)	4.8 (0.4)	18.4 (5.1)
VFL	12.8 (6.0)	8.3-21.6 (5.3-6.9)	14.8 (6.0)	3.8 (0.4)	16.1 (6.0)
AFL	8.4 (9.1)	5.2-12.9 (7.4-13.6)	8.8 (10.1)	1.8 (1.4)	8.6 (9.9)
AFH	12.2 (6.3)	8.8-19.9 (4.7-7.2)	14.4 (6.2)	3.1 (0.6)	14.6 (6.1)
CFL	12.7 (6.0)	10.5-22.7 (4.4-6.3)	16.8 (5.3)	3.9 (0.5)	18.2 (5.3)
BD	20.7 (3.7)	12.5-34.2 (3.5-4.3)	23.2 (3.9)	6.9 (0.2)	24 (3.9)
BLD	9.9 (7.8)	5.4-15.6 (7.8-9.6)	10.5 (8.6)	3.1 (0.6)	11 (8.5)
LLS	37	35-39	36.9	1.2	37
RSA	8	7-8	7.7	0.5	8
RSB	3	3-3	3	0	3
D	8	7-8	7.9	0.3	8
A	8	7-9	8	0.2	8
GR	-	8-11	9.5	0.8	9

### Conservation

*Squalius valentinus* sp. nov. is frequently the dominant species in small rivers but in wide rivers the species is local

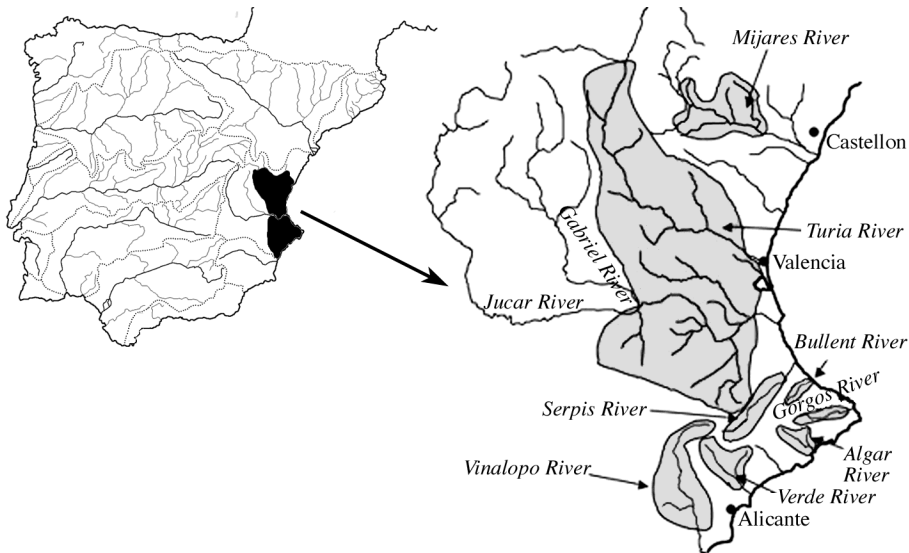


Figure 6. - Distribution area of *Squalius valentinus* sp. nov. [Distribution géographique de *Squalius valentinus* sp. nov.]

and populations are rare. In the last decade its habitat has been reduced by: 1) receding water levels due to extensive farming, 2) the construction of dams and by 3) the introduction of exotic fishes. Consequently, the population is currently declining and should be considered endangered (EN B1 + 2c) according to the IUCN Red List Categories. (Doadrio, 2003).

## RESULTS

In the analysed data set, 617 sites were variable, and 512 were parsimony informative. Third codon positions were the most informative (356 parsimony informative characters), followed by first codon positions (110 characters). Maximum parsimony analysis of the *Squalius* cytochrome *b* sequence data resulted in 310 most parsimonious trees of 5409 steps when a 10:1 Tv:Ti weighting scheme was used (Consistence Index = 0.4304; Retention Index = 0.7882). MP trees obtained using 6:1, 8:1, or equal Tv/Ti weights reconstructed similar and congruent trees (not shown). Saturation of transition and transversion changes were checked by plotting the absolute number of changes of each codon position against patristic distances. There was no evidence of saturation within the ingroup at any of the three positions (not shown). The general time reversible model with among-site rate heterogeneity GTRI + G + I (Lanave *et al.*, 1984; Yang, 1994; Gu *et al.*, 1995) was selected as the best fit to the *Squalius* data set. Rate matrix parameters were  $R(a) = 0.79$ ,  $R(b) = 37.82$ ,  $R(c) = 0.41$ ,  $R(d) = 1.71$ ,  $R(e) = 8.51$ . Base frequencies were  $A = 0.28$ ,  $C = 0.29$ ,  $G = 0.15$ ,  $T = 0.28$ . Among site rate variation was approximated with the gamma distribution shape parameter  $\alpha = 2.87$  and proportion of invariable sites  $I = 0.6$ . These parameters were used for subsequent phylogenetic analyses.

Table V. - Support values of nodes in figure 7 when the data were analysed independently with Maximum Parsimony, Bayesian and Maximum Likelihood methods of phylogenetic inference. [Valeurs des nœuds de la figure 7, données analysées de façon indépendante grâce aux méthodes d'inférence phylogénétique : Maximum de vraisemblance, Bayésien et Maximum de parsimonie.]

Node	Maximum Parsimony	Bayesian	Maximum Likelihood
1	94	99	99
2	86	100	98
3	100	100	100
4	-	79	-
5	94	99	93
6	91	99	90
7	100	100	98
8	87	90	90
9	100	100	99
10	88	96	78
11	100	100	90
12	100	99	92
13	71	70	79
14	91	99	-
15	77	79	-
16	92	99	-
17	97	100	50
18	-	75	-
19	58	99	-
20	85	90	65
21	93	100	86
22	97	90	70
23	100	100	98
24	100	100	99
25	99	100	72

Within *Squalius pyrenaicus* populations, those from the extreme South of Spain (Guadalmina, Guadaiza and Guadiaro basins) were clustered in a separate and basal mono-

phyletic group, showing greater genetic divergence than the remaining populations of *S. pyrenaicus* (Fig. 7). Similarly, the Levantine populations (Serpis, Bullent, Guadalest and Albufera de Valencia basins) were also clustered separately, and displayed a high genetic differentiation.

Hence, within the analysed Iberian *Squalius* species, seven strongly supported evolutionary independent lineages were found, corresponding to the species: *S. aradensis*, *S. carolitertii*, *S. cephalus*, *S. pyrenaicus*, *S. torgalensis*, *S. malacitanus* sp. nov. and *S. valentinus* sp. nov. (Tab. V; Figs 7, 8). Because *Squalius cephalus* belongs to the Euro-Asiatic lineage it was the most divergent in all the analyses. Nevertheless, the Spanish populations of *S. cephalus* were clearly differentiated from the French populations ( $p = 5\% \pm 0.2$ ).

*Squalius aradensis* and *S. torgalensis* were a sister group and form the most divergent clade within the Mediterranean lineage. When maximum parsimony, maximum likelihood or Bayesian inferences were applied, *S. pyrenaicus* and *S. valentinus* sp. nov. were sister groups, while *S. malacitanus* sp. nov. was the sister species to the clade *S. pyrenaicus*-*S. valentinus* sp. nov. and *S. carolitertii* was basal to all of them (Fig. 7). On the other hand, the neighbour joining approach (Fig. 8) found *S. carolitertii* to be the sister species of the clade formed by *S. pyrenaicus*-*S. valentinus* sp. nov., and *S. malacitanus* sp. nov. was basal.

Morphometric analyses revealed that the peduncle caudal was diagnostic in *Squalius malacitanus* sp. nov. (Tab. IV). The preorbital distance and the wide size of the neurocranium are different also in *S. malacitanus* sp. nov. and *S. valentinus* sp. nov. with respect to *S. pyrenaicus*. Preorbital distance and neurocranium width differ among *S. malacitanus* sp. nov., *S. valentinus* sp. nov. and *S. pyrenaicus*. The number of scales on the lateral line is a diagnostic character in *S. valentinus* sp. nov. (Tab. IV). This low number of scales is only found in *S. torgalensis* within the genus *Squalius* from the Iberian Peninsula. Regarding osteological characters, the anterior process of the maxilla and the shape of the urohyal are different in *S. malacitanus* sp. nov. with respect to the remaining *Squalius* species (Fig 3). Similarly, *S. valentinus* sp. nov. can be distinguished by the overall shape and larger width of the neurocranium, and by a more robust pharyngeal bone.

## DISCUSSION

### Taxonomic considerations

We recognized seven phylogroups within the analysed *Squalius* data set (*S. alburnoides* and *S. palaciosi* were not analysed here). All of them were monophyletic and clearly differentiated by cytochrome *b* gene sequence comparisons. Our findings are consistent with previous molecular studies (Doadrio and Carmona, 2003; Sanjur *et al.*, 2003) that have

suggested a high degree of molecular and morphological diversity on the Iberian Peninsula.

All phylogenetic approaches with the exception of neighbour joining recovered similar and congruent relationships among the Iberian *Squalius* species. Discrepancies found in the NJ tree (Fig. 8) were restricted to the relative position of *S. malacitanus* sp. nov. and *S. carolitertii* and were likely a consequence of a long branch attraction phenomenon.

The Northeastern Iberian population of *Squalius* is phylogenetically much closer to European populations of *S. cephalus* than it is to any other species on the Iberian Peninsula (Fig. 7). Likewise, it appears clearly differentiated from the closest French population of *S. cephalus*, an indication that it evolved independently of this group. In fact, Sanjur *et al.* (2003) found a close relationship between the Iberian and northern Greek *S. cephalus* populations.

*Squalius aradensis* and *S. torgalensis* were grouped together and appeared basal to *S. pyrenaicus* and *S. carolitertii* (Brito *et al.*, 1997; Zardoya and Doadrio, 1998) indicating an early differentiation of this biogeographical area, probably during the Messinian period (Doadrio and Carmona, 2003).

The speciation process that gave rise to *S. carolitertii* can be attributed to the formation of the Duero basin, which was an endorheic lagoon in the Miocene period (López-Martínez, 1989; De la Peña, 1995). Although Sanjur *et al.* (2003), suggested that populations from the Duero and the Galician (Limia and Miño) were different. The low levels of pairwise sequence divergence (uncorrected *p* distances ranging from 0 to 0.35%) they obtained seem to indicate a more recent isolation.

However, within *Squalius pyrenaicus* populations, our results suggest that those from the extreme South of Spain and the Levantine populations have had little or no connection with the other populations of *S. pyrenaicus*. The greatest genetic distances were found between *S. pyrenaicus* and *S. malacitanus* sp. nov. (7.8 to 8.6%). These distances were higher than those between other Iberian cyprinid fishes such as *Chondrostoma arcasii* and *C. macrolepidotum* or *C. willkommii* and *C. polylepis* or *Barbus bocagei* and *B. comizo*. No available names can be applied to the South and Levantine populations, and therefore, we choose to designate these populations as *Squalius malacitanus* sp. nov. and *S. valentinus* sp. nov., respectively.

Currently, nine *Squalius* species are present in the Iberian Peninsula (Fig. 1). *S. cephalus* inhabits the north-eastern part of Spain between the Ebro and Muga basins. Interestingly, in the Matarraña River (Ebro basin) *S. cephalus* lives in sympatry with *S. pyrenaicus* but *S. cephalus* is the dominant species. *S. carolitertii* inhabits the north-western part of Spain between the Mondego and Umia basins. Genetic

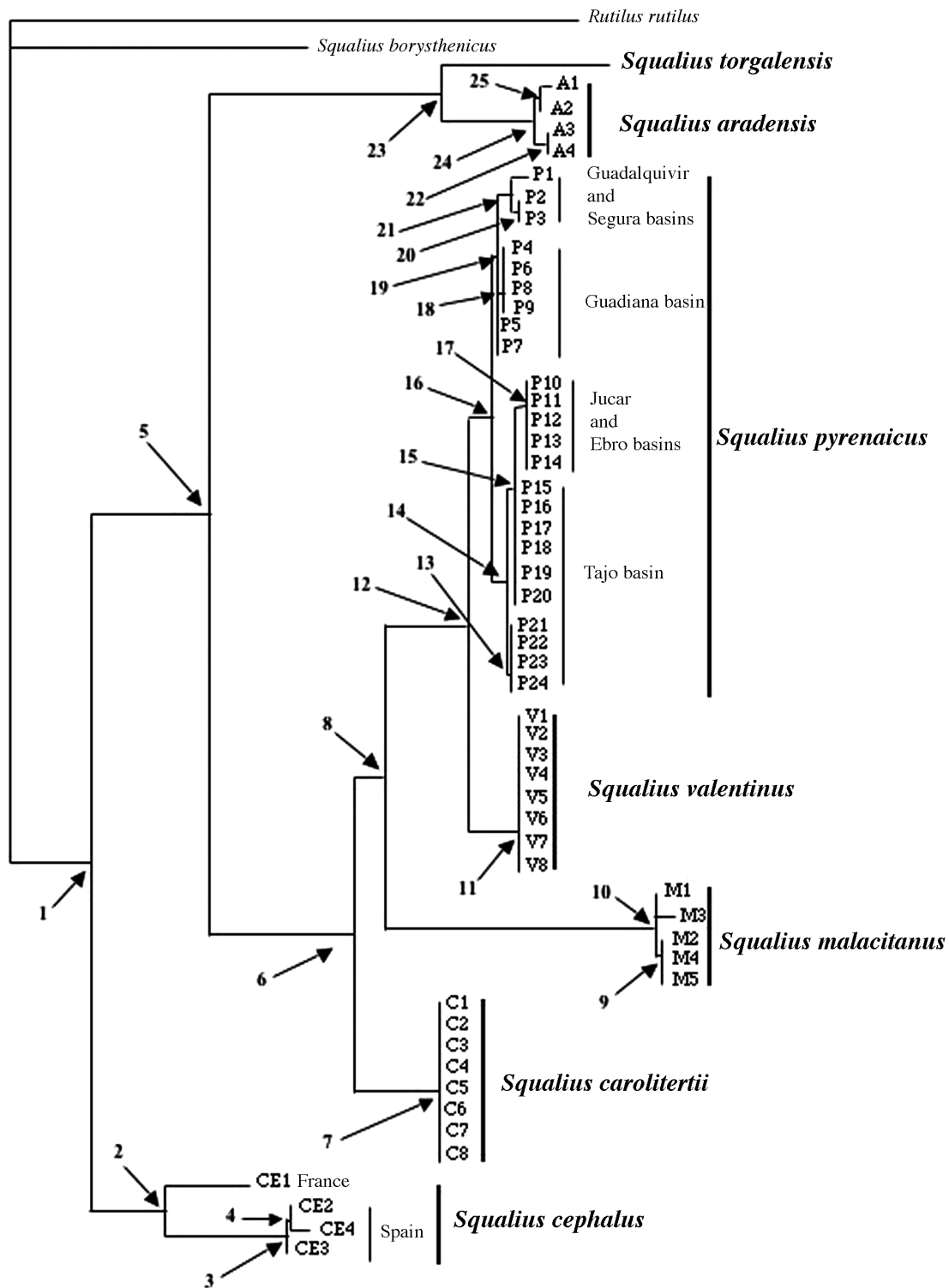


Figure 7. - Phylogenetic relationships among populations of the Iberian *Squalius* species, based on cytochrome *b* gene sequences. A Puzzle ML tree is depicted showing code-numbers above nodes. These code-numbers are listed in table II and represent the support values of nodes when the data were analysed independently with, Maximum Likelihood, Bayesian and Maximum Parsimony methods of phylogenetic inference. [Relations phylogénétiques des populations des espèces ibériques de *Squalius*, fondées sur des séquences du gène du cytochrome *b*. L'arbre Puzzle ML donne les numéros codés sur les nœuds. Ces codes sont répertoriés dans le tableau II et représentent les valeurs d'appui de nœuds pour les données analysées de façon indépendante grâce aux méthodes de Maximum de vraisemblance, Bayésien et Maximum de parsimonie.]

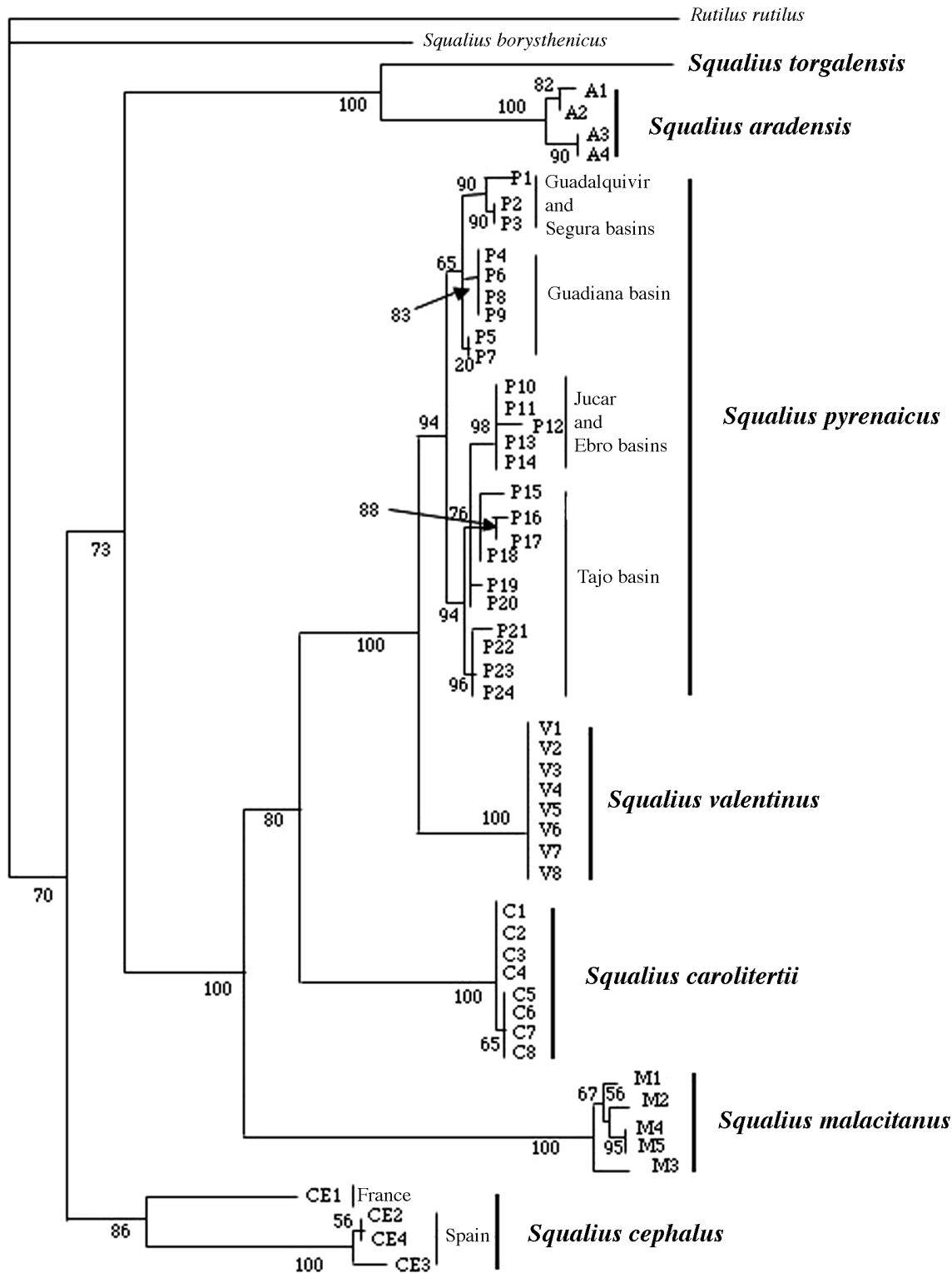


Figure 8. - Phylogenetic relationships among populations of the Iberian *Squalius* species based on 50% majority-rule consensus bootstrap tree obtained with neighbour joining, based on 500 pseudoreplications. *Rutilus rutilus* and *S. borysthenticus* were used as outgroup. Nodes with bootstrap value below 50% were forced to collapse and yield polytomies. [Relations phylogénétiques entre populations d'espèces ibériques du genre *Squalius* fondées sur le consensus majoritaire (à 50%) des arbres Neighbor-joining issus d'un bootstrap à 500 répliquats des données. Les nœuds à valeur de bootstrap inférieures à 50% ont disparu et forment des polytomies.]

introgression with *S. pyrenaicus* has been observed in the Zezere River (Tajo basin) (Coelho *et al.*, 1995). *Squalius pyrenaicus* is now restricted to the central and southern rivers of the Iberian Peninsula (Fig. 1), between the San Pedro River (North Portugal) and the Guadalquivir basins in southwest Spain. This area is shared by *S. alburnoides* since this species strongly depends on *S. pyrenaicus* as a hybridogenetic reproduction mechanism (Alves *et al.*, 1997; Carmona *et al.*, 1997). In the eastern and southern parts of Spain their distribution is restricted to isolated areas and they have only been observed in three rivers in the Ebro basin (Doadrio, 2003) as well as in some localities from the upper Júcar, Guadalfeo, Guadalhorca and Vélez basins. Moreover, *S. torgalensis* is endemic to the Mira basin (Coelho *et al.*, 1998) and *S. aradensis* is present in the Aljezur, Bordeira, Cerca, Arade and Alportel basins in Portugal. The endangered *S. palaciosi* is an endemic species whose distribution is restricted to the Jándula River (Guadalquivir basin). Finally, *Squalius malacitanus* sp. nov. is endemic to the Gudaiza, Guadalmina and Guadiaro basins while *Squalius valentinus* sp. nov. is an endemic species from the Mediterranean slope, inhabiting the area between the Mijares and Vinalopo basins.

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## Appendix A

MNCN24785, lectotype of *Leuciscus pyrenaicus*, Sintra, Portugal, leg. Günther. **Guadalfeo Basin**. - MNCN195309-10, MNCN24901-5, MNCN25088-185, MNCN34279-83, MNCN34287-89, 112 specimens, Guadalfeo River, Órgiva, Granada, leg. I. Doadrio and P. Garzón, 03 Oct. 1978. - MNCN195961, MNCN25711-16, 8 spms, Guadalfeo River, Vélez de Benaudalla, Granada, leg. I. Doadrio and P. Garzón, 21 Oct. 1978. **Guadalhorce Basin**. - MNCN212312, 1 spm, Fahala River, Alaurín el Grande, Málaga, leg. I. Doadrio and P. Garzón, 06 Mar. 2000. - MNCN196388-9, MNCN25586, 3 spms, Burgo River, El Burgo, Malaga, leg. I. Doadrio and P. Garzón, 24 Oct. 1978. - MNCN213882, 16 spms, Pereilas River, Coín, Málaga, leg. I. Doadrio and P. Garzón, 06 Mar. 2000. **Guadalquivir Basin**. - MNCN196156-75, 20 spms, Hueznar River, El Pedroso, Sevilla, leg. I. Doadrio, 08 Dec. 1979. - MNCN53479-96, 18 spms, Castril River, Cortes de Baza, Granada, leg. Y. Bernat and J. Cubo, 14 Jun. 1989. - MNCN54480-89, 10 spms, Guadalbarbo River, Obejo, Córdoba, leg. I. Doadrio and J. Cubo, 19 Apr. 1989. - MNCN189911-27, MNCN187359-67, 29 spms, Montemayor River, Cañaveral de León, Huelva, leg. I. Doadrio, 05 May 1999. **Guadiana Basin**. - MNCN25013-67, 55 spms, Aljucén River, Aljucén, Badajoz, leg. I. Doadrio, 21 Jul. 1984. - MNCN24331-40, 10 spms, Guadalemar River, Fuenlabrada de los Montes, Badajoz, leg. P. Barrachina and C. Sunyer, 16 Jul. 1984. - MNCN24600-10,

11 spms, Matachel River, Hornachos, Badajoz, leg. P. Barrachina and C. Sunyer, 28 Dec. 1984. - MNCN24459-88, 30 spms, Retín River, Llera, Badajoz, leg. P. Barrachina and C. Sunyer, 26 Jul. 1984. **Júcar Basin**. - MNCN73265-70, 6 spms, Júcar River, Uña, Cuenca, Leg. J.A. Carmona, I. Doadrio and A. Perdices, 24 May 1993. - MNCN126124-26, 3 spms, Júcar River, Villalba de la Sierra, Cuenca, leg. F. Alonso, 30 Apr. 1996. **Odiel Basin**. - MNCN196102, 1 spm, Odiel River, Campofrío, Huelva, leg. I. Doadrio, 09 Dec. 1979. - MNCN24809, MNCN24927-36, MNCN25079-87, 21 spms, Cascabelero River, Villanueva de las Cruces, Huelva, leg. I. Doadrio, 12 Apr. 1979. - MNCN725282-97, 16 spms, Tamujoso River, Calañas, Huelva, leg. I. Doadrio, 12 Apr. 1979. **Segura Basin**. - MNCN25187-91, 5 spms, Segura River, Murcia, leg. Lozano and Cusí. - MNCN25650-54, 5 spms, Segura River, Orihuela, Alicante. **Tagus Basin**. - MNCN215791-835, 45 spms, Almonte River, Jaraicejo, Cáceres, leg. I. Doadrio and F. Morcillo, 20 Sept. 1999. - MNCN211295-314, 20 spms, Ambroz River, Zarza de Granadilla, Cáceres, leg. I. Doadrio, 03 Jul. 1998. - MNCN25716-801, 86 spms, Jarama River, Patones, Madrid, leg. I. Doadrio, 07 Nov. 1980. - MNCN214444-75, 32 spms, Ladrillar River, La Rebollosa, Cáceres, leg. I. Doadrio, F. Morcillo and P. Garzón, 03 Oct. 1999. - MNCN53006-15, 53033, 53082-121, 52 spms, Zézere River, Valhelhas, Portugal, leg. Y. Bernat and J. Cubo, 11 Apr. 1989. **Tinto Basin**. - MNCN83736-39, 4 spms, Berrocal, Huelva, leg. L. Domínguez, 26 Jan. 1991. **Velez Basin**. - MNCN196548-51, MNCN25416-17, 6 spms, Salia-Velez River, Viñuela, Málaga, leg. I. Doadrio and P. Garzón, 22 Oct. 1978.