

**A NEW SPECIES OF THE GENUS *SQUALIUS* BONAPARTE, 1837
(ACTINOPTERYGII, CYPRINIDAE) FROM THE
TAGUS RIVER BASIN (CENTRAL SPAIN)**

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ABSTRACT

A new species of *Squalius* is described on the basis of morphological and genetic characters from the Gallo River and its tributaries (Tagus Basin) in central Spain. The new species is distinguished from other Iberian *Squalius* species by a combination of morphometric, meristic and genetic characters, such as wide infraorbital bones that do not cover the adductor mandibulae muscle; seven scales on the upper transversal line; small number of lateral line scales, mean = 39.8 (39-42); a short and wide head, 4.3-4.9 (mean = 4.5) times the standard length; Short pectoral-ventral length, reaching 2.2-2.4 (mean = 2.3) times the standard length. The new species differs from its closer taxa in terms of genetic GTR distances, *S. carolitertii* and *S. pyrenaicus* at 20 fixed nucleotide positions (molecular autapomorphies), in the mitochondrial cytochrome *b* gene. Genetic divergence values for this new species and *S. carolitertii* and *S. pyrenaicus* are 3.8% and 6.1% respectively. The new species should be considered Critically Endangered (CR) according to the IUCN Red List Categories.

Key words: Cypriniformes, Cyprinidae, Taxonomy, *Squalius castellanus*, Gallo River, Tagus basin.

RESUMEN

**Una nueva especie del género *Squalius* Bonaparte, 1837 (Actinopterygii,
Cyprinidae) para la cuenca del Tajo (España Central)**

Se describe una nueva especie de *Squalius* en base a caracteres morfológicos y genéticos. Esta nueva especie está restringida al río Gallo (cuenca del Tajo) en el centro de España y difiere del resto de las especies ibéricas de *Squalius* en una serie de caracteres morfométricos, merísticos y genéticos, tales como huesos infraorbitarios anchos pero que no cubren totalmente el músculo adductor mandibulae; siete escamas en la línea transversal superior, menor número de escamas en la línea lateral media = 39.8 (39-42); cabeza corta y ancha, siendo 4.3-4.9 (media = 4.5) veces la longitud estándar; longitud ventral-pectoral corta alcanzando 2.2-2.4 (media = 2.3) veces la longitud estándar. La nueva especie difiere de sus taxones más cercanos, en término de distancias genéticas GTR, en 20 posiciones nucleótídicas fijadas (autopomorfías moleculares) en el gen

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mitochondrial citocromo *b*. La divergencia genética entre la nueva especie descrita respecto a *S. carolitertii* y *S. pyrenaicus* para el gen citocromo *b* fue de 3.8% y 6.1% respectivamente. Esta nueva especie debería ser considerada En Peligro Crítico (CR) de acuerdo con las Categorías de la Lista Roja de la IUCN.

Palabras clave: Cypriniformes, Cyprinidae, Taxonomía, *Squalius castellanus*, Río Gallo, cuenca del Tajo.

Introduction

The genus *Squalius* Bonaparte, 1837 is widely distributed throughout European freshwater lakes and rivers, and shows a high level of diversity in the Mediterranean area (Zardoya & Doadrio, 1999; Durand *et al.*, 2000; Doadrio & Carmona, 2003). This genus includes approximately 15 species (Kottelat 1997; Doadrio & Carmona 1998) distributed along the Mediterranean basin; two species are widely reported in Central and North Europe (Sanjur *et al.*, 2003).

In the Iberian Peninsula, the genus *Squalius* is represented by eight endemic species that belong to the “Mediterranean lineage” (Sanjur *et al.*, 2003): *Squalius alburnoides* (Steindachner, 1866), *S. aradensis* (Coelho, Bogutskaya, Rodrigues & Collares-Pereira, 1998), *S. carolitertii* (Doadrio, 1987), *S. malacitanus* Doadrio & Carmona, 2006, *S. palaciosi* (Doadrio, 1980), *S. pyrenaicus* (Günther, 1868), *S. torgalensis* (Coelho, Bogutskaya, Rodrigues & Collares-Pereira, 1998) and *S. valentinus* Doadrio & Carmona, 2006 and one species that belongs to the “Euro-Asiatic lineage”, *S. cephalus* (Linnaeus, 1758), which is widely distributed throughout Europe (Sanjur *et al.*, 2003). *Squalius alburnoides* and *S. palaciosi* are characterized as belonging to a different hybrid complex, and both species probably played an important role in the evolutionary history of *Squalius* (Carmona *et al.*, 1997; Alves *et al.*, 1997, 1999, 2001; Cunha *et al.*, 2004).

Nevertheless, the taxonomy of *Squalius* in the Iberian Peninsula has been controversial because of inconsistencies between analyses based on genetic and morphological characters. Phenotypic plasticity is high among the species and diagnostic characters are difficult to find, but genetic approaches have demonstrated a high degree of differentiation between Iberian *Squalius* populations based on allozymes (Coelho *et al.*, 1995; Alves *et al.*, 1997; Carmona *et al.*, 1997) and mitochondrial (mt) DNA sequences variation (Brito *et al.*, 1997; Sanjur *et al.*, 2003). These studies have focused mainly on populations of *S. pyrenaicus* and the high levels of

genetic variation between these populations have resulted in the diagnoses and identification of four new species from the southern and eastern Iberian Peninsula, *S. aradensis* and *S. torgalensis* from Portugal (Coelho *et al.*, 1998) and *S. malacitanus* and *S. valentinus* from Spain (Doadrio & Carmona, 2006). *Squalius carolitertii*, which is also widely distributed throughout the north-western Iberian Peninsula, has not been as thoroughly studied, and no comparable levels of genetic variation have been found in initial studies of this species (Sanjur *et al.*, 2003).

In this paper we reanalysed between-population genetic variation in *Squalius carolitertii* and *S. pyrenaicus* including a population from the upper Tagus Basin, which preliminary phenotypic examinations suggested as being distinct from both *S. caroliterii* and *S. pyrenaicus*. The purpose of this paper is to examine the phylogenetic relationships of this Tagus Basin population based on mitochondrial cytochrome *b* sequence data, and to provide diagnostic morphological characters and a description of the new species of *Squalius*.

Materials and methods

MORPHOMETRIC ANALYSES

The description of this new *Squalius* species is based on the study of 21 individuals from the Gallo River and its tributaries (Tagus Basin). Only 17 adult specimens were used for comparative morphometric analysis. The holotype and a series of paratypes have been deposited in the Museo Nacional de Ciencias Naturales, Spain (MNCN). For comparative purposes we studied the following material: *Squalius aradensis*: MNCN243553-86, 34 ex. Algibre River, Querença, Portugal, leg I. Doadrio, 25/3/02. *Squalius carolitertii*: MNCN 26052, holotype, Cega River, Rebollo, Segovia prov., Duero Basin, leg. D. García de Jalón, 1/5/82, MNCN24141-24169, paratypes, 29 ex. Duero River, Peleagonzalo, Zamora prov., Duero Basin leg I. Doadrio and B. Elvira 14/8/81. *Squalius malaci-*

Table 1.— Samples, collection locations, number of sampled populations in the tree and GenBank access numbers. GenBank Accession Numbers with * are new sequences obtained in this study.

Tabla 1.— Muestras, localidades, número de población en el árbol y número de acceso de GenBank. Los números de acceso de GenBank con * son secuencias nuevas obtenidas en este estudio.

Species	Locality	River	Basin (Population designation)	GenBank number Accession
<i>Squalius lucumonis</i>	Istia d'Ombrone. Italy	Ombrone	Ombrone	AF421828
<i>Squalius keadicus</i>	Sparta. Grecia	Evrotas	Evrotas	AF090760
<i>Squalius aradensis</i>	Portugal	Arade	Arade 1	AF421824
<i>S. aradensis</i>	Portugal	Arade	Arade 2	AF421825
<i>Squalius castellanus</i>	Ventosa. Spain	Gallo	Tajo	DQ521423*
<i>Squalius carolitertii</i>	Monzón de Campos. Spain	Pisuerga	Duero (C1)	DQ521424*
<i>S. carolitertii</i>	El Fresno. Spain	Adaja	Duero (C2)	AF421800
<i>S. carolitertii</i>	El Fresno. Spain	Adaja	Duero (C3)	AF045994
<i>S. carolitertii</i>	El Fresno. Spain	Adaja	Duero (C4)	AF421799
<i>S. carolitertii</i>	Bacones de Ojeda. Spain	Boedo	Duero (C5)	DQ521425*
<i>S. carolitertii</i>	Bacones de Ojeda. Spain	Boedo	Duero (C6)	AF421797
<i>S. carolitertii</i>	Bacones de Ojeda. Spain	Boedo	Duero (C7)	AF421798
<i>S. carolitertii</i>	Peñarrubias de Pirón. Spain	Pirón	Duero (C8)	DQ521427*
<i>S. carolitertii</i>	Villanueva de Argaño. Spain	Hormazuelas	Duero (C9)	DQ521430*
<i>S. carolitertii</i>	Santana. Spain	Valdivia	Duero (C10)	DQ521426*
<i>S. carolitertii</i>	Riaza. Spain	Riaza	Duero (C11)	DQ521428*
<i>S. carolitertii</i>	Cuéllar. Spain	Cega	Duero (C12)	DQ521429*
<i>S. carolitertii</i>	Portugal	Vouga	Vouga (C13)	DQ521432*
<i>S. carolitertii</i>	San Agustín. Spain	Bibeí	Miño (C14)	AF421793
<i>S. carolitertii</i>	San Agustín. Spain	Bibeí	Miño (C15)	AF421794
<i>S. carolitertii</i>	Rubia de Mixtos. Spain	Salas	Limia (C16)	AF421795
<i>S. carolitertii</i>	Rubia de Mixtos. Spain	Salas	Limia (C17)	AF421796
<i>S. carolitertii</i>	Portugal	Montego	Mondego (C18)	DQ521431*
<i>S. carolitertii</i>	Portugal	Montego	Mondego (C19)	DQ521433*
<i>Squalius malacitanus</i>	Guadaira. Málaga. Spain	Guadaira	(M1)	New sequence
<i>S. malacitanus</i>	Benaojan. Málaga. Spain	Guadiaro	(M2)	New sequence
<i>Squalius pyrenaicus</i>	Alcaraz. Spain	Guadalmina	Guadalquivir (P1)	AF421816
<i>S. pyrenaicus</i>	Alcaraz. Spain	Guadalmina	Guadalquivir (P2)	AF421817
<i>S. pyrenaicus</i>	Cañaverl de León. Spain	Montemayor	Guadalquivir (P3)	AF421790
<i>S. pyrenaicus</i>	Navas de Estena. Spain	Estena	Guadiana (P4)	AF421813
<i>S. pyrenaicus</i>	Casa de Fuente Vieja. Spain	Azuer	Guadiana (P5)	AF421804
<i>S. pyrenaicus</i>	Casa de Fuente Vieja. Spain	Azuer	Guadiana (P6)	AF421805
<i>S. pyrenaicus</i>	Ossa de Montiel. Spain	Lagunas de Ruidera	Guadiana (P7)	AF421822
<i>S. pyrenaicus</i>	Ossa de Montiel. Spain	Lagunas de Ruidera	Guadiana (P8)	AF421823
<i>S. pyrenaicus</i>	Uña. Spain	Laguna de Uña	Júcar (P9)	AF421806
<i>S. pyrenaicus</i>	Uña. Spain	Laguna de Uña	Júcar (P10)	AF421807
<i>S. pyrenaicus</i>	Álava. Spain	Río Baias	Ebro (P11)	DQ521434*
<i>S. pyrenaicus</i>	Álava. Spain	Río Baias	Ebro (P12)	DQ521435*
<i>S. pyrenaicus</i>	Álava. Spain	Río Baias	Ebro (P13)	DQ521436*
<i>S. pyrenaicus</i>	La Adrada. Spain	Tiétar	Tajo (P14)	DQ521437*
<i>S. pyrenaicus</i>	Jaraicejo. Spain	Almonte	Tajo (P15)	AF421791
<i>S. pyrenaicus</i>	Hoyos. Spain	Acebo	Tajo (P16)	AF421727
<i>S. pyrenaicus</i>	Cadalso de Gata. Spain	Arrago	Tajo (P17)	AF421726
<i>S. pyrenaicus</i>	Valverde del Fresno. Spain	Pesquero	Tajo (P18)	AF421811
<i>S. pyrenaicus</i>	Valverde del Fresno. Spain	Pesquero	Tajo (P19)	AF421712
<i>S. pyrenaicus</i>	Las Moedas. Spain	Bogarda	Segura (P20)	AF421820
<i>S. pyrenaicus</i>	Las Moedas. Spain	Bogarda	Segura (P21)	AF421821
<i>Squalius valentinus</i>	Sollana. Spain	Font de Barrent	Turia (V1)	AF421808
<i>S. valentinus</i>	Sollana. Spain	Font de Barrent	Turia (V2)	AF421809
<i>S. valentinus</i>	Callosa d'en Sarria. Spain	Algar	Algar (V3)	AF421818
<i>S. valentinus</i>	Callosa d'en Sarria. Spain	Algar	Algar (V4)	AF421819
<i>S. valentinus</i>	Beniarres. Spain	Serpis	Serpis (V5)	AF421810
<i>S. valentinus</i>	Beniarres. Spain	Serpis	Serpis (V6)	AF421815
<i>Squalius torgalensis</i>	Portugal	Torgal	Mira	DQ521438*

tanus: MNCN243699, holotype, Guadalmina River, Benahavis, Málaga prov., leg I. Doadrio, P. Garzón and R. Zardoya, 29/3/03, MNCN212280-311, paratypes, 32 ex., Guadalmina River, Benahavis, Málaga prov., Guadalmina Basin, leg. A. Doadrio, I. Doadrio jr., P. Garzón and I. Doadrio, 5/3/03. *Squalius pyrenaicus*: MNCN 24785, lectotype, Sintra, Portugal, leg. Günther MNCN189911-27, MNCN187359-70 29 ex., Montemayor River, Cañaverale de León, Huelva prov., Guadalquivir Basin, leg. I. Doadrio, 5/5/99. MNCN25716-18, 3 ex. Lozoya River River, Pontón de la Oliva, Madrid prov., Tajo Basin leg. I. Doadrio. MNCN69638-53, MNCN69685-89 20 ex., Gévora River, Albuquerque, Badajoz prov., Guadiana Basin, leg I. Doadrio, 8/5/87. *Squalius torgalensis*: MNCN243478-243486, 9 ex. Mira River, Odemina, Portugal, leg I. Doadrio, 27/3/02. *Squalius valentinus*: holotype, Turia River, Tajar, Valencia prov., Turia Basin, leg. P. Risueño and J. Jiménez, 13/3/03, MNCN217795-98, MNCN 126706-23, 22 ex., paratypes, Algar River, Callosa D'Ensarriá, Alicante prov., Guadalest basin. Leg. CAE. 3/11/93.

Twenty-two morphometric variables were measured following Elvira (1986). All measurements are in millimetres and were log-transformed for morphometric analysis. The following abbreviations were used for morphometric and meristic characters: SL, standard length; HL, head length; PrOL, preorbital length; ED, eye diameter; AIO, Interorbital wide; 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; DFH dorsal fin height; DFL dorsal fin length; 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; LLS, lateral line scales; LTU, upper transversal scales; LTL, lower transversal scales.

The osteological characters were studied from both cleared-and-stained specimens (Wassersug, 1976). Differences in body shape among populations were analysed using Principal Component Analysis (PCA), which was performed with the statistical package NTSYS-pc (Rohlf, 1998). Burnaby's method was used to correct for size effects (Burnaby, 1966).

MOLECULAR ANALYSIS

Fifty-two samples from seven species of *Squalius* from the Iberian Peninsula were analysed. *Squalius alburnoides* and *S. palaciosi* were

Table 2.— Statistical parameters for the morphometric and meristic characters of *Squalius castellanus* sp. nov. type series. Variables are described in Methods (SD = Standard deviation).

Tabla 2.— Parámetros estadísticos para los caracteres morfológicos y merísticos de la serie tipo de *Squalius castellanus* sp. nov. Las variables son descritas en los Métodos (SD = desviación típica).

Variable	<i>Squalius castellanus</i>			
	Holotype	Paratypes (n = 20)		
		Range	Mean	SD
SL	79.13	44.68-176.88	112.98	47.26
HL	18.19	9.17-40.51	25.29	11.24
PrOL	5.7	3.53-12.43	8.47	3.27
ED	4.11	2.75-7.82	5.81	1.83
PrDD	20.65	24.53-96.55	61.67	25.55
PrPD	40.33	12.39-40.79	27.95	10.62
PrVD	55.34	22.68-84.79	55.63	22.61
PrAD	42.79	30.71-120.07	77.82	31.98
CPL	26.23	14.82-60.4	37.34	15.77
APL	14.94	9.2-35.58	21.94	9.14
PVL	17.61	10.66-41.17	26.90	11.35
VAL	13.94	8.55-34.28	21.25	9.20
DFL	10.18	4.27-21.51	13.38	5.561
DFH	16.72	8.75-29.4	20.66	7.53
PFL	15.07	7.86-27.44	18.64	7.06
VFL	14.64	5.46-25.79	16.93	6.32
AFL	8.68	4.32-20.72	11.67	5.11
AFH	13.67	7.62-24.78	15.65	5.75
CFL	16.54	8.12-31.78	20.48	7.74
BD	19.25	10.81-46.36	28.11	12.05
BLD	8.89	5.07-19.01	12.17	4.98
AIO	7.35	4.51-15.65	10.74	4.13
LLS	40	39-42	39.82	1.01
LTU	7	7-7	7	0
LTL	3	3-3	3	0
D	8	8-8	8	0
A	7	7-8	7.53	0.51

not examined because these species are part of a hybridogenetic complex (Carmona *et al.*, 1997; Alves *et al.*, 1997, 1999, 2001; Cunha *et al.*, 2004). *Squalius lucumonis* and *S. keadicus* from the "Mediterranean lineage" (Sanjur *et al.*, 2003) were used as outgroup taxa. New samples examined were collected in the wild from multiple locations (Table 1). DNA was extracted from dorsal muscle, which was preserved in liquid nitrogen or 70% ethanol. Because in the last ten years only one specimen of *Squalius* from the Gallo River was captured (1999), we only included one specimen from this population in the molecular analysis. Posterior exhaustive samplings in this area have not reported



Fig. 1.— *Squalius castellanus* sp. nov. Holotype MNCN 212727, Gallo River, Tagus basin, Ventosa. Spain.

Fig. 1.— *Squalius castellanus* sp. nov. Holotype MNCN 212727, Río Gallo, Cuenca del Tajo, Ventosa. España.

new samples. Voucher specimens for this species were deposited in the collections of the Museo Nacional de Ciencias Naturales, Madrid, Spain.

Total cellular DNA was extracted from tissues by a standard proteinase K and phenol/chloroform extraction method (Sambrook *et al.*, 1989) and ethanol purification (Towner, 1991). Two overlapping fragments of the mitochondrial cytochrome *b* gene (total of 1140 bp) were amplified via polymerase chain reaction (PCR) from each individual DNA sample. The primers used for amplification of the cytochrome *b* were from Machordom & Doadrio (2001). Thermal cycle parameters were as follows: initial denaturation at 92°C (2 min), 35 cycles of denaturation at 94°C (1 min), annealing at 48°C (1 min), and extension at 72°C (5 min), and a final extension at 72°C for 10 min. PCR mixtures were prepared in 25 ml volumes with a final concentration of 0.5 ml each primer, 0.2 mM each dNTP, 1.5 mM MgCl₂, and 1 unit of Taq DNA polymerase (Biotools). After checking PCR products on 1.5 % agarose gels, they were cloned using the pGEM-T vector (Promega) into *E. coli* JM109, and 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 and there were no gaps in the resulting DNA sequences. Nucleotide saturation was analysed by plotting absolute number of tran-

sitions and transversions against patristic distance values (not shown). The aligned data were analysed by Bayesian inference performed with MrBayes 3.1 (Huelsenbeck & Ronquist, 2001) by simulating a Markov chain for 1,000,000 cycles. Model Test vers. 3.7 (Posada & Crandall, 1998) indicated that the best-fit model was the GTR (Rodríguez *et al.*, 1990) + I model of evolution. Not burnin parameters were necessary.

Results and discussion

The high degree of genetic and morphological divergence of specimens from the Gallo River (Tagus Basin) to other species of *Squalius* of the Iberian Peninsula warrants consideration of the Gallo River population as a different species. No available name could be applied to this population, and therefore, a new species was described.

Squalius castellanus sp. nov.

HOLOTYPE: (Fig. 1, Tables 2 and 3). MNCN212727 Male, 79.13 mm SL. Gallo River, Ventosa, Guadalajara. leg. I. Doadrio, 30/10/99.

PARATYPES: (Tables 2 and 3). MNCN136311-13, 3 ex. Arandilla River, Cuevas Labradas, Guadalajara, Gallo Basin, 13/7/44. MNCN136320-21, 2 ex. Gallo River, Molina de Aragón, Guadalajara, 13/7/44. MNCN34147-148, 2 ex. Gallo River, Cuevas Labradas, Guadalajara, leg. J. Lobón, 30/10/86. MNCN138857, 1 ex. Arandilla River, Cuevas Labradas, Guadalajara, Gallo Basin, 11/10/44. MNCN137042-45, 4 ex. Gallo River, Ventosa, Guadalajara, 13/7/44. MNCN42066-73, 8 ex. Gallo River, Cuevas Labradas, Guadalajara, leg. J. Lobón, 30/10/86.

Table 3.— Statistical parameters for the morphometric characters of *Squalius castellanus* sp. nov. Each variable is divided by standard length and multiplied by ten. Variables are described in Methods (SD = Standard deviation).

Tabla 3.— Parámetros estadísticos para los caracteres morfo-métricos de *Squalius castellanus* sp. nov. Cada variable está dividida por la longitud estándar y multiplicada por diez. Las variables son descritas en los Métodos (SD = desviación típica).

<i>Squalius castellanus</i> nov. sp.				
Variable	Holotype	Paratypes (n = 20)		
		Range	Mean	SD
SL	79.13	44.68-176.88	112.98	47.26
HL/SL	2.3	2.1-2.3	2.2	0.01
PrOL/SL	0.7	0.6-0.8	0.7	0.005
ED/SL	0.5	0.4-0.7	0.5	0.008
PrDD/SL	2.6	2.3-2.9	2.5	0.02
PrPD/SL	5.1	4.7-5.2	4.9	0.01
PrVD/SL	7.0	6.7-7.2	6.9	0.02
PrAD/SL	5.4	5.2-5.6	5.5	0.01
CPL/SL	3.3	3.2-3.4	3.2	0.007
APL/SL	1.9	1.8-2.1	1.9	0.009
PVL/SL	2.2	2.2-2.5	2.4	0.007
VAL/SL	1.8	1.7-2.0	1.9	0.009
DFL/SL	1.9	0.9-1.3	1.2	0.01
DFH/SL	2.1	1.6-2.2	1.9	0.02
PFL/SL	1.9	1.4-1.9	1.7	0.01
VFL/SL	1.8	1.2-1.8	1.5	0.01
AFL/SL	1.1	0.8-1.2	1	0.01
AFH/SL	1.7	1.3-1.9	1.5	0.02
CFL/SL	2.1	1.7-2.1	1.8	0.01
BD/SL	2.4	2.3-2.6	2.5	0.009
BLD/SL	1.1	1-1.1	1.1	0.003
AIO/SL	0.9	0.9-1.1	1	0.007

DIAGNOSIS: Differs from all other known species of *Squalius* by the following combination of characters: Seven to eight branched rays on the anal fin; A short and wide head, 4.3-4.9 (mean = 4.5) times the standard length; Short pectoral-ventral length, reaching 2.2-2.4 (mean = 2.3) times the stan-

dard length; Number of scales on lateral line mean = 39.8 (39-42); Number of scales on upper transversal line mean = 7. Three scales on lower transversal line. Large infraorbitals bones that do not cover the adductor mandibulae muscle. Twenty autapomorphies in the mitochondrial cytochrome *b* gene.

DESCRIPTION: D II-III 8, A II-III 7-8 (mean = 7.5), P I 13, V II 7, C 18, LLS 39-42 (mean = 39.8), A medium sized species that rarely reaches a standard length of 200 mm. Morphometric characters are given in Tables 2 and 3. Maximum body depth is 3.8-4.2 (mean = 4) times the standard length. Head short and is 4.3-4.9 (mean = 4.5) times the standard length. Head length is lesser than maximum body depth. Preorbital distance is longer than eye diameter, reaching 1.2-1.6 (mean = 1.4) times the eye diameter. There is a long interorbital distance, reaching 1.6-2 (mean = 1.8) times the eye diameter. Ventral fin is inserted slightly before origin of dorsal fin. Predorsal length 1.1-1.2 (mean = 1.1) times pre-ventral length. High caudal peduncle. The ventral-anal distance is long, with an anal index (VAL/PRAD) of 2.4-2.9 (mean = 2.7). Minimum body depth is 2.9-3.2 (mean = 3) times length of caudal peduncle and 1.7-1.9 (mean = 1.8) times length of anal peduncle. The pectoral-ventral distance is short, reaching 2.2-2.4 (mean = 2.3). Fin size is moderately large.

PIGMENTATION PATTERN: Silver body, darker dorsally. 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. The peritoneum is silver with small black spots.

DISTRIBUTION: This new species is endemic to the Gallo River and its tributaries in the upper Tagus Basin (Central Spain) (Fig. 2).

ETYMOLOGY: The species name “*castellanus*” derives from the name of the Spanish region –Castille– inhabited by this species. An adjective.

Table 4.— Autapomorphies in cytochrome *b* gene in *Squalius castellanus*. Transversions are indicated with *.

Tabla 4.— Autopomorfías en el gen citocromo *b* en *Squalius castellanus*. Las transversiones se indican con *.

Cytochrome <i>b</i> position	101	179	302	332	434	464	494	497	503	616	722	747	755	861	878	896	905	938	1088	1100
<i>Squalius castellanus</i>	C*	T*	C	G	G	T	A	G*	T	C*	C	T	T	T	G	A	G	G	A*	G
<i>Squalius carolitertii</i>	G	G	T	A	A	C	G	T	C	A	T	C	C	C	A	G	A	A	C	A
<i>Squalius pyrenaicus</i>	A	A	T	A	A	C	G	A	C	A	T	C	C	C	A	G	A	A	C	A

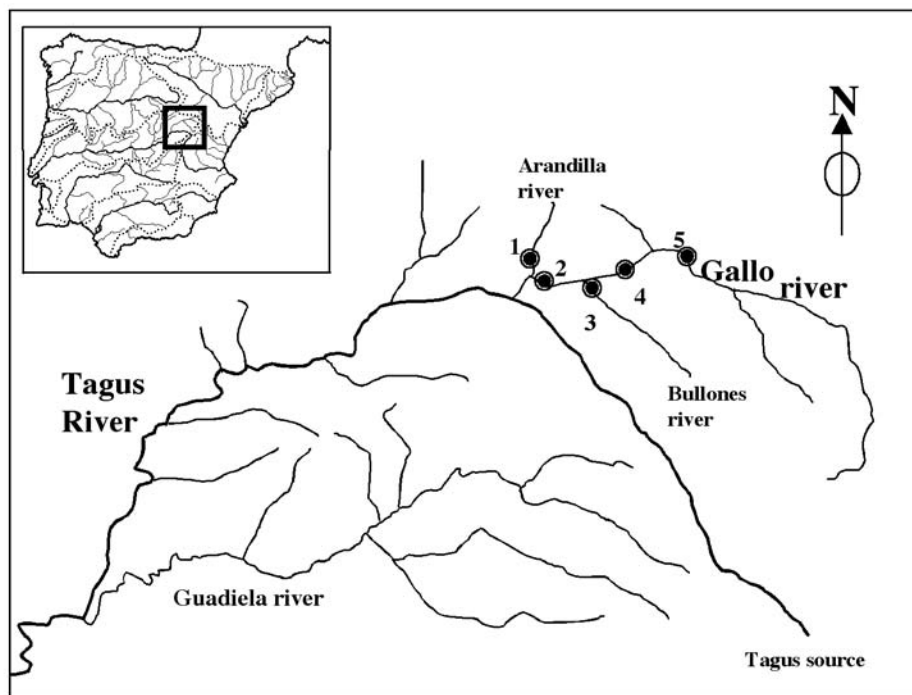


Fig. 2.— Locations sites of *Squalius castellanus*. 1. Arandilla river (Cuevas Labradas); 2. Gallo river (Cuevas Labradas); 3. Bullones river (Torete); 4. Gallo river (Ventosa); 5. Gallo river (Molina de Aragón).

Fig. 2.— Localidades de *Squalius castellanus*. 1. Río Arandilla (Cuevas Labradas); 2. Río Gallo (Cuevas Labradas); 3. Río Bullones (Torete); 4. Río Gallo (Ventosa); 5. Río Gallo (Molina de Aragón).

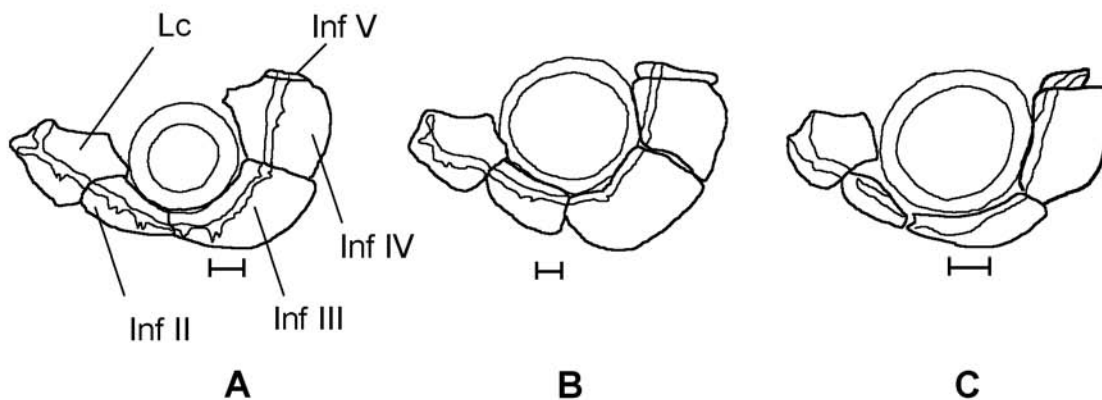


Fig. 3.— Infraorbital bones of Iberian *Squalius* species. Lc: Lacrimal bone; Inf II: Second infraorbital bone; Inf III: Third infraorbital bone; Inf IV: Fourth infraorbital bone; Inf V: Fifth infraorbital bone. A: *Squalius castellanus*; B: *Squalius pyrenaicus*; C: *Squalius carolitertii*.

Fig. 3.— Huesos infraorbitarios de las especies ibéricas de *Squalius*. Lc: hueso lacrimal; Inf II: Segundo hueso infraorbitario; Inf III: Tercer hueso infraorbitario; Inf IV: Cuarto hueso infraorbitario; Inf V: Quinto hueso infraorbitario. A: *Squalius castellanus*; B: *Squalius pyrenaicus*; C: *Squalius carolitertii*.

Table 5.— Summary of diagnostic morphological and meristic characters of *Squalius castellanus* and differences with *S. carolitertii* and *S. pyrenaicus*.

Tabla 5.— Resumen de los caracteres morfológicos y merísticos diagnósticos de *Squalius castellanus* y diferencias con *S. carolitertii* y *S. pyrenaicus*.

Characters	<i>S. castellanus</i> sp nov	<i>S. carolitertii</i>	<i>S. pyrenaicus</i>
Number of scales in lateral line	39-42 (mean = 39.8)	40-43 (mean = 41.6)	38-44 (mean = 41.3)
Number of scales in upper transversal line	7	8	8-9
Head	Short and wide Cephalic index (HL/SL)= 2.0-2.3	Long and narrow Cephalic index (HL/SL)= 2.3-2.5	Long and narrow Cephalic index (HL/SL)= 2.6-2.8
Infraorbital bone	Wide Cover partially the muscle adductor mandibulae	Narrow	Wide Cover totally the muscle adductor mandibulae
Pectoral-Ventral length	Short Ventral index (PVL/PRDD)= 4.1-4.5	Long Ventral index (PVL/PRDD)= 4.6-5	Long Ventral index (PVL/PRDD)= 3.3-4.9
Ventral-Anal length	Short Ventral-Anal index (VAL/SL)= 1.7-1.9	Long Ventral-Anal index (VAL/SL)= 1.8-2.1	Short Ventral-Anal index (VAL/SL)= 1.2-2.0

COMMON NAMES: We propose using the common name “bordallo del Gallo” in Spanish, and “Gallo chub” in English.

CONSERVATION: *Squalius castellanus* is only known from small populations along the Gallo River (Tagus basin) and its main tributaries, both Bullones and Arandilla Rivers. In the last ten years only one individual has been collected in the Gallo River. The main threat to the species is pollution from the city of Molina de Aragon. Consequently, the population is currently declining and should be considered Critically Endangered (CR B1 + 2ce) according to the IUCN Red List Categories.

GENETICS: The genetic distance to the other Iberian species was the following: 3.7-4.2 (mean_{GTR}=3.8) to *S. carolitertii*, 5.7-6.6 (mean_{GTR}=6.1) to *S. pyrenaicus*, 6.6 to *S. valentinus*, 9.2 to *S. malacitanus*, 11.7-11.9 (mean_{GTR}=11.8) to *S. aradensis* and 12.9 to *S. torgalensis*. *Squalius castellanus* has twenty autapomorphies in the cytochrome *b* gene, five of which are transversions (Table 4).

INTERESPECIFIC COMPARISONS: Specimens of *Squalius* from the Gallo River (Tagus basin) show many differences in both genetic and morphological characters with respect to *S. carolitertii* and *S. pyrenaicus*. Thus, this population deserves recognition as a new species, *Squalius castellanus*. On Table 5 there is a summary of the main differences between the new species *S. castellanus* and both *S.*

Table 6.— Eigenvectors for the first three principal components Burnaby transformed for 21 variables.

Tabla 6.— Eigenvectors para los tres primeros ejes principales de las 21 variables transformadas mediante Burnaby.

	C1	C2	C3
SL	0.0049	0.0041	0.0041
HL	0.0295	0.0113	0.0131
PRO	-0.0206	0.0034	0.0094
ED	0.0259	0.0064	0.0041
PrDD	0.0033	0.0004	-0.0034
PrPD	0.0044	0.0030	0.0047
PrPV	0.0018	0.0019	-0.0015
PrAD	0.0009	0.0036	0.0005
CPL	0.0278	0.0047	-0.0007
APL	0.0294	0.0129	-0.0007
PVL	-0.0212	-0.0002	-0.0087
VAL	-0.0404	0.0313	-0.0039
DFL	-0.0059	-0.0158	0.0207
DHL	-0.0140	-0.0167	0.0115
PFL	0.0041	-0.0154	-0.0152
VFL	0.0046	-0.0126	-0.0039
AFL	-0.0064	-0.0133	-0.0165
AHL	-0.0027	-0.0191	-0.0016
BD	0.0079	-0.0026	-0.0057
BLD	-0.0066	0.0009	-0.0005
AIO	-0.0105	0.0034	0.0005
Eigenvalues	0.113 (79.5%)	0.018 (13.1%)	0.001 (1.1%)

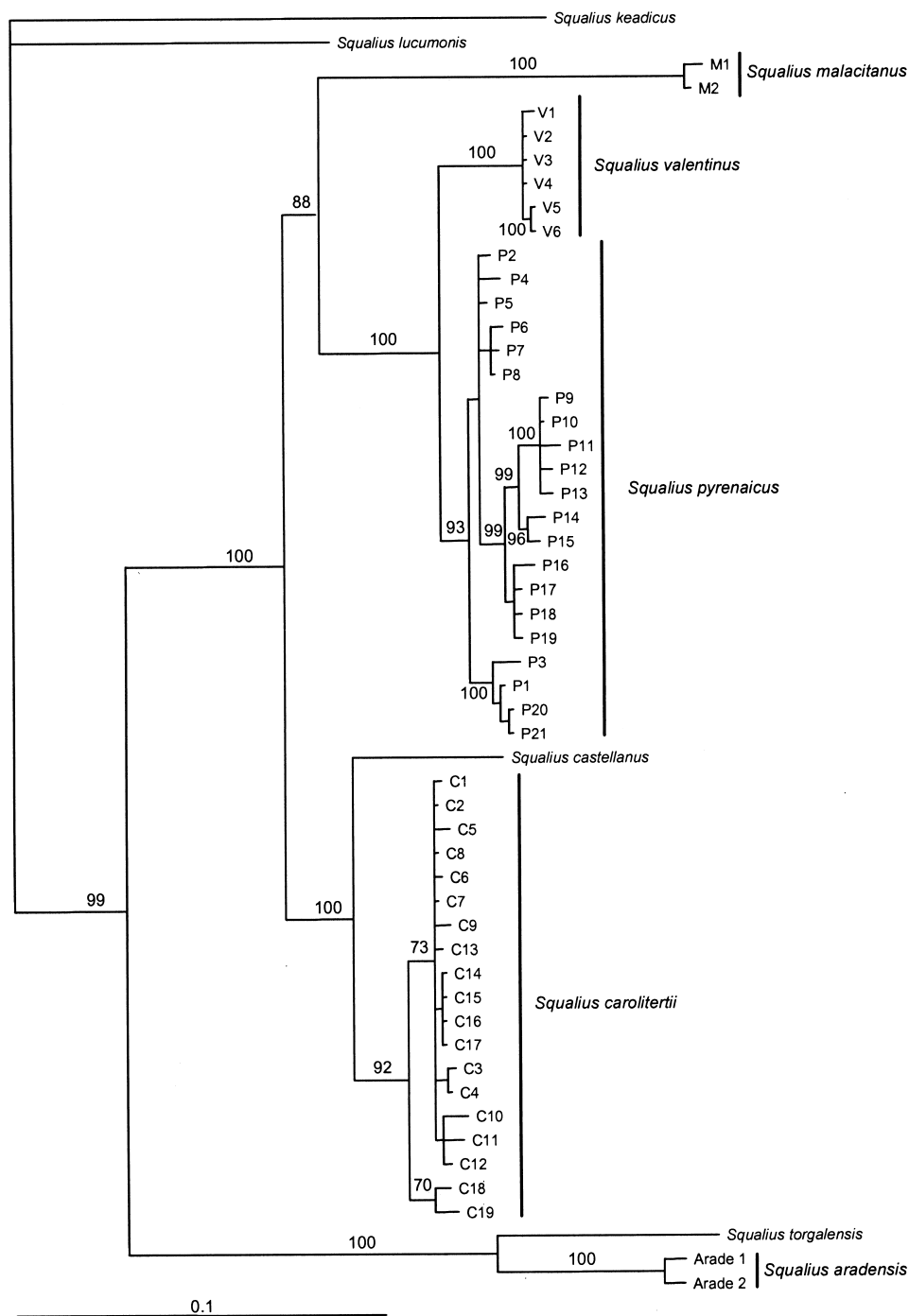


Fig. 5.— Phylogenetic tree of 52 analysed specimens of genus *Squalius* recovered from cytochrome *b* sequences according to the bayesian method. Branch lengths are proportional to the estimated mean number of substitutions per site. Numbers are posterior probabilities of bayesian analysis.

Fig. 5.— Árbol filogenético de 52 especímenes del género *Squalius* obtenido a partir de secuencias de citocromo *b* analizadas mediante inferencia Bayesiana. Las longitudes de las ramas son proporcionales al número medio de sustituciones por sitio. Los números indican las probabilidades posteriores del análisis bayesiano.

carolitertii (mean_{GTR}=3.8, 3.7-4.2) than to *S. pyrenaicus* (mean_{GTR}=6.1, 5.7-6.6). In respect of *S. aradensis* and *S. torgalensis*, the genetic distances found between these species and *S. castellanus* were the highest (mean_{GTR}=11.8, 11.7-11.9 and mean_{GTR}=12.9 respectively). If a molecular clock with a 1% rate of mutation per 1 My is assumed for the mt cytochrome *b* gene (Dowling *et al.*, 2002; Doadrio & Carmona, 2004), the Iberian *Squalius* species belonging to the “Mediterranean lineage” diverged from other European species in the upper Miocene period (13 MYA). This divergence was possible when Asia Minor, Greece, the Balkans, Italy and the Iberian Peninsula were geographically connected. The break up of this geographical connection may have provided the conditions for the origin of Iberian *Squalius* species that subsequently became isolated populations that experienced further allopatric speciation (Sanjur *et al.*, 2003). The complexity of the Iberian hydrographic basins in the Tertiary period contributed to allopatric speciation within the Iberian Peninsula, and led to the current diversity of Iberian endemic species. According to the molecular clock for cytochrome *b* (Dowling *et al.*, 2002; Doadrio & Carmona, 2004), the separation between the new species described, *S. castellanus*, and the species genetically closer to it, *S. carolitertii*, is coincident with the lower Pliocene (3.8 MYA). Not geological events during the Pliocene could be claimed to explain the colonization and posterior speciation of *S. castellanus*.

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