DESCRIPTION OF TWO NEW SPECIES OF THE GENUS SQUALIUS BONAPARTE, 1837 (ACTINOPTERYGII, LEUCISCIDAE) IN THE IBERIAN PENINSULA

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ABSTRACT

Two new species, Squalius gaditanus sp. nov. and Squalius tartessicus sp. nov. are described on the basis of morphological and genetic traits. Squalius gaditanus is restricted to the Barbate, Jara and Miel drainages in the province of Cádiz (Southern Spain). Squalius gaditanus sp. nov. can be distinguished from other Squalius species from the Iberian Peninsula through a combination of morphometric, meristic and genetic characters: 36-40 (χ²=38) pored scales on the lateral line; 6-7 (χ²=6.7) scales above the lateral line; 2-3 (χ²=2.8) scales below the lateral line; vertebrae 37-39 (χ²=38); second infraorbital bone narrower than the third in adults; maxilla with reduced pointed anterior process; posterior process of the maxilla long and thin; lower branch of the pharyngeal bone short and robust; pharyngeal plate of basiocipital rounded and two autapomorphies in the mitochondrial cytochrome b gene. Squalius tartessicus sp. nov. inhabits the Almargem, Gilão, Odiel, Guadiana, Guadalquivir, Guadalete, Guadalhorce, Vélez, Guadafío and Segura drainages in the southern part of the Iberian Peninsula. Squalius tartessicus sp. nov. can be differentiated from other Squalius species from the Iberian Peninsula through a set of morphometric, meristic and genetic traits: 37-41 (χ²=38.8) pored scales on the lateral line; 6-7 (χ²=7) scales above the lateral line; 2-3 (χ²=2.9) scales below the lateral line; 37-39 (χ²=38) number of vertebrae; infraorbital bones unusually wide in adults; maxilla with discernable pointed anterior process; posterior process of the maxilla long and thin; lower branch of the pharyngeal bone short and robust; pharyngeal plate of basiocipital triangular in shape; posterior lamina of cleithrum expanding posteriorly.

Keywords. Taxonomy, Iberian Peninsula, Squalius, Cypriniformes, Leuciscidae, genetics, morphology.

RESUMEN

Descripción de dos especies nuevas del género Squalius Bonaparte, 1837 (Actinopterygii, Leuciscidae) en la Península Ibérica

Se describen dos nuevas especies, Squalius gaditanus sp. nov. y Squalius tartessicus sp. nov. sobre la base de caracteres morfológicos y genéticos. Squalius gaditanus está restringido a las cuencas de Barbate, Jara y Miel en la provincia de Cádiz (sur de España). Squalius gaditanus sp. nov. se puede distinguir de otras especies del género Squalius, de la Península Ibérica, a través de una combinación de caracteres morfométricos, merísticos y genéticos: 36-40 (χ²=38) escamas canalizadas en la línea lateral; 6-7 (χ²=6.7) escamas por encima de la línea lateral; 2-3 (χ²=2.8) escamas debajo de la línea lateral; vértebras 37-39 (χ²=38); en los ejemplares adultos el segundo infraorbitario es más estrecho que el tercero; maxilar con su proceso anterior poco puntiagudo; proceso posterior del maxilar largo y delgado; la rama inferior del hueso faríngeo es corta y robusta; placa faríngea del basiocipital redondeada y dos autapomorfías en el gen mitocondrial citocromo b. Squalius tartessicus sp. nov. vive en las cuencas de los ríos Almargem, Gilao, Odiel, Guadiana, Guadalquivir, Guadalete, Guadalhorce, Vélez, Guadafío y Segura en el sur de la Península Ibérica. Squalius tartessicus sp. nov. se puede distinguir de otras especies del género Squalius; de la Península Ibérica, a través de una combinación de caracteres morfométricos, merísticos y genéticos; 37-41 (χ²=38.8), escamas canalizadas en la línea lateral; 6-7 (χ²=7) escamas por encima de la línea lateral; 2-3 (χ²=2.9) escamas por debajo de la línea lateral; 37-39 (χ²=38) número de vértebras; en los adultos infraorbitarios excepcionalmente anchos; maxilar con la apófisis anterior puntiaguda; apófisis posterior del maxilar larga y delgada; la apófisis inferior del hueso faríngeo es corta y robusta; placa faríngea del basiocipital de forma triangular; lámina posterior de cleitro extendida.

Palabras clave. Taxonomía, Península Ibérica, Squalius, Cypriniformes, Leuciscidae, genética, morfología.
Introduction

The Iberian Peninsula presents an insular freshwater ichthyofauna as a consequence of its hydrological isolation since the formation of the unsurmountable barrier represented by the Pyrenees during the Oligocene and the opening of the Strait of Gibraltar in the Miocene 5.33 million years ago (Krijgsmann et al., 2018). This is especially noticeable for primary freshwater fish families, such as: Nemacheilidae, Cobitidae, Leuciscidae and Cyprinidae. That is, those fishes whose ancestors entered inland waters much earlier and cannot survive in seawater, being strictly confined to freshwater systems (Myers, 1938; Darlington, 1948).

The insularity of the Iberian freshwater ichthyofauna is manifested in its faunistic composition with few genera, due to the difficulty of colonizing the Peninsula by different lineages and by a high number of endemic species as a consequence of the several hydrological reorganizations that have occurred since the Oligocene within the Iberian Peninsula (Perea et al., 2016).

The genus *Squalius* Bonaparte, 1837 is one of the genera with a large number of endemic species in the Iberian Peninsula (Doadrio et al., 2011). This genus is currently represented in the Iberian Peninsula by two different lineages, one formed by ancient Mediterranean endemisms and the other by a lineage from Central and East Europe and the northern areas of the Mediterranean basin towards Minor Asia, described as Euroasiatic (Sanjur et al., 2003). Divergence between both Euroasiatic and Mediterranean lineages should have occurred in the Late Miocene, approximately 7 Ma (Sanjur et al., 2003). The Euroasiatic lineage is represented in Spain by an autochthonous single species, *Squalius laietanus* Doadrio, Kottelat & Sostoa, 2007, distributed next to the Pyrenees, in the northeast of the Iberian Peninsula and in the southwest of France.

The Mediterranean lineage is much more diverse in the Iberian Peninsula and some species have aroused the interest of evolutionary biologists in recent years. Currently, nine species are recognized, all of them endemic to the Peninsula: *Squalius albunroides* (Steindachner, 1866), *Squalius aradensis* (Coelho, Bogutskaya, Rodrigues & Collares-Pereira, 1998), *Squalius carolitertii* (Doadrio, 1988), *Squalius castellanus* Doadrio, Perea & Alonso, 2007, *Squalius malacitanus* Doadrio & Carmona, 2006, *Squalius palaciosi* (Doadrio, 1980), *Squalius pyrenaicus* (Günther, 1868), *Squalius torgalensis* (Coelho, Bogutskaya, Rodrigues & Collares-Pereira, 1998) and *Squalius valentinus* Doadrio & Carmona, 2006. One of these species, *S. albunroides* is unique within the European leuciscid fauna due to its hybrid origin, with specimens of different ploidies and reproduction by hybridogenesis (Carmona et al., 1997; Cunha et al., 2004, 2011). Probably *S. palaciosi*, of which specimens with different ploidies have also been described, was a species of hybrid origin but seems to have been extirpated since the end of the 20th century (Doadrio et al., 2011). The nomenclature of these two hybrid-origin species is controversial, as they result from ancient hybridizations between one species of the *Squalius* genus and species belonging to different lineages than *Squalius*. In this context, we follow the latest nomenclature revision of the group (Collares-Pereira & Coelho, 2010) to provide nomenclatural stability, but it requires further review.

A genetic study based on nuclear and mitochondrial gene sequences on the populations from southern drainages in Spain found two well different lineages within *S. malacitanus* (Perea et al., 2016). These two lineages, one inhabiting the Atlantic slope and the other the Mediterranean slope, were geographically separated with the reorganization of the Iberian southern drainages which occurred during the opening of the Gibraltar Strait 5.33 Ma (Krijgsmann et al., 2018). Only the population from the Miel River, belonging to the Atlantic lineage, drains to the Mediterranean Sea as a consequence of secondary contacts during the Quaternary (Perea et al., 2016).

Divergence between these two lineages of *S. malacitanus* occurred in the Mio-Pliocene period around 4.9 Ma (2.1–8.5 Ma) (Perea et al., 2016). A posterior work extended the genetic study of the genus *Squalius* to all populations from the Iberian Peninsula and, by analyzing six nuclear genes, corroborated the existence of these two Atlantic and Mediterranean lineages within *S. malacitanus* (Perea et al., 2020, 2021). In addition, populations of *S. pyrenaicus* were not monophyletic based on nuclear markers and three independent lineages were recognized: a Northern clade distributed throughout the Tajo Drainage and ascribed to the nominal species *S. pyrenaicus*; a Sado clade inhabiting the Sado drainage in Portugal; and a Southern clade distributed in the remaining Iberian drainages (Perea et al. 2021). Genomic data also corroborated the existence of these three independent lineages (Mendes et al. 2021). Phyllogenetic
relationships among Squalius species, based on nuclear genes, have shown that S. pyrenaicus is not monophyletic and the Northern clade is a sister group of S. carolitertii and S. castellanus with respect to one group formed by the Southern clade, the Sado clade, S. valentinus and S. malacitanus (Perea et al. 2020, 2021). To determine whether the lineages of S. pyrenaicus from the southern Iberian Peninsula and Sado, and the two lineages of S. malacitanus (Atlantic and Mediterranean), could be identified as distinct species, Perea et al. (2020) conducted a Bayesian nuclear multilocus species delimitation analysis following the MSC framework (Yang & Rannala, 2010; Yang, 2015). They utilized 4871 base pairs obtained from six nuclear loci plus 1140 base pairs of one mitochondrial locus. The analysis yielded robust support, with a posterior probability of 1.00 for these lineages, endorsing the hypothesis that these lineages should indeed be considered as separate species.

In this paper we present a formal description for the two independent lineages inhabiting Spain: the Southern lineage of S. pyrenaicus and the Atlantic lineage of S. malacitanus.

**Material and methods**

**MORPHOLOGY**

The morphometric and meristic study of the genus Squalius was based on the analysis of 58 specimens belonging to the Mediterranean lineage of S. malacitanus, 42 specimens from the type locality (Table 1). Ninety-eight specimens of the Squalius population previously assigned to the Atlantic lineage of S. malacitanus (Perea et al. 2020, 2021). Eighty-one specimens belonging to the Northern lineage of S. pyrenaicus. One hundred twenty-five specimens of the Squalius population previously assigned to the Southern lineage of S. pyrenaicus (Perea et al. 2020, 2021) (Table 1).

Holotypes and paratype series of the two new species have been deposited in the Museo Nacional de Ciencias Naturales (MNCN-CSIC, Spain).

Twenty-four morphometric measurements (in mm) and eleven meristic variables were recorded from digital photographs using TpsDig ver. 1.4 (Rohlf, 2003). The following abbreviations were used for morphometric and meristic characteristics: TL, total length; SL, standard length; PrDD, predorsal distance; PrPD, prepectoral distance; PrVD, preanal distance; APL, anal peduncle length; CPL, caudal peduncle length; HL, head length to opercular; PrOL, preorbital length; ED, eye diameter; PsOL, postorbital length; NL, head length to nape; HH, head high; PmxL, premaxilla length; PFL, pectoral fin length; VFL, ventral fin length; DFL, dorsal fin length; DHL, dorsal fin height; AFL, anal fin length; AHH, anal fin height; CFL, caudal fin length; BD, body depth; BLD, body least depth; LLS, lateral line scale rows; SRA, scale rows above lateral line; SRB, scale rows below lateral line; D, dorsal fin rays; A, anal fin rays; P, pectoral fin rays; V, ventral fin rays; C, caudal fin rays; RPT, right pharyngeal teeth; LPT, left pharyngeal teeth; Vr, Vertebrae. After constructing the measurement matrix, Burnaby’s method was used to correct for size effect. The Burnaby method removes the effects of a within population size-factor from between-group morphometric analyses through an orthogonal projection procedure (Burnaby, 1966). All analyses were conducted with the corrected matrix. Morphometric and meristic characters were analysed independently. To identify the variables that contributed most to the variation among populations, one principal component analyses (PCA) was performed using the covariance matrix for morphometric characters. Row-wise bootstrapping was carried out to 100,000 replicates and 95% bootstrap confidence intervals are given to the eigenvalues. Statistical analyses were carried out using Past ver. 4.12 (Hammer et al., 2001), we used the option scree plot to indicate the number of significant components.

**OSTEOLOGY**

Osteological features were investigated through computer tomography (CT) scan and digital dissection using VGStudio MAX ver. 2.2 (Volume Graphics, http://www.volumegraphics.com) of the following localities:

- **Squalius pyrenaicus** of the Northern Lineage:
  MNCN_ICTIO 213943-52, 10 specimens from Almonte River, Jaraicejo (Cáceres); MNCN_ICTIO 267007-16, 10 specimens from Jerte River, Navaconcejo (Cáceres).
- **Squalius pyrenaicus** of the Southern Lineage:
  MNCN_ICTIO 272254-63, 10 specimens from Ciudadcej River, Las Navas de la Concepción (Sevilla); MNCN_ICTIO 272324-33, 10 specimens from Guadalete River, Puerto Serrano (Cádiz); MNCN_ICTIO 289456, 1 specimen from Estena River, Navas de Estena (Ciudad Real).
- **Squalius malacitanus** of the Mediterranean Lineage:
  MNCN_ICTIO 212280-89, 10 specimens (paratypes) from Guadalmina River, Benahavis (Málaga); MNCN_ICTIO 63647-56 10 specimens from Hozgarganta River, Jimena de la Frontera (Cádiz).
- **Squalius malacitanus** of the Atlantic Lineage:
  MNCN_ICTIO 29700-09, 10 specimens from Jara River, Tarifa (Cádiz); MNCN_ICTIO 296956-65, 10 specimens from Vega River, Tarifa (Cádiz).

Additionally dry skeletons preserved in the MNCN collections were studied from the following localities:
Table 1.– Material studied for morphometric analyses. Population Names, Collection Numbers, Sites (River, Drainages, and Coordinates), and Number of Individuals (before slash morphometry, after slash CT-Scan). * Material from the same locality as the holotype and paratypes.

<table>
<thead>
<tr>
<th>Species name</th>
<th>After Perea et al. (2020)</th>
<th>Current Study</th>
<th>Collection Number Morphometry</th>
<th>Collection Number. CT-Scan</th>
<th>Drainage</th>
<th>River/N° ind</th>
<th>Coordinates</th>
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<td>S. malacitanus</td>
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<td>MNCN_ICTIO 212280-89</td>
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<td>S. gaditanus sp. nov.</td>
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<td>MNCN_ICTIO 63647-56</td>
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<td>MNCN_ICTIO 297000-09</td>
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<td>Vega/45*/10</td>
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</tbody>
</table>
Two new species of the genus *Squalius*

- *Squalius pyrenaicus* of the Northern Lineage: MNCN_ICTIO 69458-62, 69463-68, 20671-73, 20680, 14 specimens from Alburriel River, Valencia de Alcántara (Cáceres); MNCN_ICTIO 69484-85, 2 specimens from Jarama River, Talamanca del Jarama (Madrid); MNCN_ICTIO 69470, 1 specimen from Tajo River, Villarreal de San Carlos (Cáceres); MNCN_ICTIO 69471, 1 specimen from Salor River, Membrió (Cáceres); MNCN_ICTIO 69477, 20674, 2 specimens from Guadalix River, San Agustín de Guadalix (Madrid); MNCN_ICTIO 20277, 20280, 20675, 20676, 4 specimens from Pinilla Reservoir, Pinilla (Madrid); MNCN_ICTIO 20278, 1 specimen, from Aurela River, Santiago de Alcántara (Cáceres).

- *Squalius pyrenaicus* of the Southern Lineage: MNCN_ICTIO 69469, 1 specimen from Gévora River, Alburquerque (Badajoz); MNCN_ICTIO 69472-74, 3 specimens from Záncara River, Zafra de Záncara (Cuenca). MNCN_ICTIO 69475, 69476, 69478, 3 specimens from Robledillo River, Solana del Pino (Ciudad Real).

Institutional acronyms: MNCN_ICTIO, Ichthyological Collection, Museo Nacional de Ciencias Naturales (Spain).

**GENETIC ANALYSES**

Genetic analyses of Iberian *Squalius* have been the focus of previous studies to resolve their phylogenetic relationships and biogeography with mitochondrial and nuclear genes (Sanjur *et al.*, 2003; Perea *et al.*, 2016, 2020, 2021). For this reason, the Iberian populations of the genus *Squalius* are well studied group from the phylogenetic point of view. In this study we reanalyse a data set of 268 sequences for the mitochondrial cytochrome *b* gene (*MT-CYB*, 924 bp) obtained from previously published sequences (see Appendix 1 for GenBank accession numbers). Two different phylogenetic analyses were performed using Bayesian inference (BI), implemented in MrBayes ver. 3.2 (Ronquist *et al.*, 2012), and Maximum Likelihood, carried out in the IQ-tree online web server from the Vienna University (http://iqtree.cibiv.univie.ac.at/); Trifinopoulus *et al.*, 2016). ModelFinder, implemented in the previous IQ-Tree web server (Kalyaanamoorthy *et al.*, 2017) and the Bayesian Information Criterion (Schwarz, 1978) were used to estimate the evolutionary model that best fitted the data. The selected evolutionary model was TNF+F+G4. The Bayesian analysis was performed with two simultaneous independent runs each with four Markov chain Monte Carlo (MCMC), which were run for 5 × 10⁷ generations. The first 25% of generations were removed as burn-in. Posterior probability (pp) values were used to assess the reliability of the phylogenetic hypothesis. The accuracy of the Maximum Likelihood phylogeny was evaluated with the UltraFast Bootstrap method (1000 replicates) (Minh *et al.*, 2013). For nuclear phylogeny, we derived a concatenated tree topology from the phylogenetic tree presented by Perea *et al.* (2020), based on the analysis of six nuclear genes and 4871 bp including gaps (Bayesian Inference and Maximum Likelihood analysis; GenBank Accession Numbers: *MT-CYB*: MT008486–MT008603; *RAG1*: MT008604–MT008704, *S7*: MT00855–MT008805, *EFA1*: MT008910–MT009018, *EGR2b*: MT051740–MT051843, *RHO*: MT008806–MT008909, *ACTB*: MT051635–MT051739). We also calculated the uncorrected p-distances and verify the presence of autapomorphies among *Squalius* populations studied for the *MT-CYB* gene using Mega X (Kumar *et al.*, 2018) to sequences download from the GeneBank data base (Appendix1).

**Results and discussion**

The principal component analysis to all populations and species divided the studied species of *Squalius* into four groups corresponding to populations of: a) *S. pyrenaicus* from the Northern Lineage (Tajo Drainage); b) *S. pyrenaicus* from the Southern Lineage (Guadalquivir, Guadalhorce and Vélez Drainages); c) *S. malacitanus* from the Mediterranean Lineage (Guadalmina and Guadiaro Drainages); and d) *S. malacitanus* from Atlantic Lineage (Jara drainage). All populations of *Squalius pyrenaicus* and all populations of *Squalius malacitanus* were considered in different groups without overlapping and the same occurred with the Northern and Southern lineages of *S. pyrenaicus* (Fig. 1). On the contrary, Atlantic and Mediterranean lineages of *S. malacitanus* showed a wide overlapping (Fig. 1). This overlapping between the Mediterranean and Atlantic lineages of *S. malacitanus* was similar to that found between populations from different rivers within the same species. A certain arrangement can be observed between the populations from the Almonte and Jerte Rivers, within the Northern Lineage, which live in rivers with different typologies. The Almonte River is a Mediterranean-like river influenced by severe water stress during the summer, with specimens of *S. pyrenaicus* surviving in disconnected pools. On the contrary, the Jerte is a mountain river with a permanent flow throughout the year. It can also be observed in the Southern Lineage a certain arrangement separating the populations from Vélez and Guadalquivir drainages.

The eigenvalues of the two first principal components, with the Burnaby-corrected matrix, explained most of the variance (Table 2). The highest values for eigenvectors, and consequently the variables that contributed most to the ordination in the PCA, were the measurements related...
with the different proportions of the head (Table 2) as occur in other Iberian species of the genus *Squalius* (Doadrio, 1988; Doadrio & Carmona, 2006; Doadrio et al., 2007a, 2007b). The length and height of the caudal peduncle was not as decisive in the ordination of the populations as in other morphological studies of *Squalius* species (Doadrio & Carmona, 2006).

To clarify the variables that in each species contribute most to the ordination of the PCA we conducted a separated PCA for the populations of each species. Populations of *S. pyrenaicus* were divided in Northern and Southern lineages (Appendix 2) and the eigenvalues of the two first principal components, with the Burnaby-corrected matrix, explained most of the variance (Appendix 3). This was a result of the different position of the eye on the head between the specimens of both lineages. In the southern lineage, the eye was displaced toward the snout resulting in a shorter preorbital lengths and a longer postorbital length. The PCA also shown a small differentiation between the populations from the Tajo drainage, as was explained previously. This was not so evident in the Southern lineage.

Regarding *Squalius malacitanus*, the first two PCs arranged the specimens in Atlantic and Mediterranean lineages (Appendix 4). However, the variance was spread among most of the PCs. The four first PCA were significant and eigenvalues to the first two PCs only explained 38.99 % of the variance (Appendix 5). For these reasons, we interpret that the PCA was not successful and our morphometric variables do not adequately separate the two lineages of *S. malacitanus*. 

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**Fig. 1.–** Variables that most contributed to the PCA to all populations of the genus *Squalius*. Symbols: Fill Squares, Almonte River (Tajo Drainage), Northern Lineage of *S. pyrenaicus*. Stars, Jerte River (Tajo Drainage), Northern Lineage of *S. pyrenaicus*. Triangles, Mediterranean Lineage of *S. malacitanus*. Fill Triangles, Atlantic Lineage of *S. malacitanus*. Dots, Grande River (Guadalhorce drainage), Southern Lineage of *S. pyrenaicus*. Circles, Sabar and Cuevas Rivers (Vélez Drainage), Southern Lineage of *S. pyrenaicus*. Plus, Cala River (Guadalquivir Drainage), Southern Lineage of *S. pyrenaicus*. Diamonds, Ciudadela River (Guadalquivir Drainage), Southern Lineage of *S. pyrenaicus*. Abbreviations are defined in the Material and methods section.

**Fig. 1.–** Variables que más contribuyen al ordenamiento en el PCA para todas las poblaciones del género *Squalius*. Símbolos: Cuadrados, en negro río Almonte (Cuenca del Tajo), Linaje del Norte de *S. pyrenaicus*. Estrellas, río Jerte (Cuenca del Tajo), Linaje del Norte de *S. pyrenaicus*. Triángulos, Linaje Mediterráneo de *S. malacitanus*. Triángulos en negro, Linaje Atlántico de *S. malacitanus*. Puntos negros, río Grande (Cuenca del Guadalhorce), Linaje del Sur de *S. pyrenaicus*. Círculos, ríos Sabar y Cuevas (Cuenca del Vélez), Linaje del Sur de *S. pyrenaicus*. Más, río Cala (Cuenca del Guadalquivir), Linaje del Sur de *S. pyrenaicus*. Más, río Cala (Cuenca del Guadalquivir), Linaje del Sur de *S. pyrenaicus*. Las abreviaturas están descritas en el epígrafe de Material y métodos.
Two new species of the genus *Squalius*

The number of scales in the lateral line is very similar in all Mediterranean species of the genus *Squalius*, although some differences were observed in the studied populations (Fig. 2).

The number of scales in the lateral line was smaller in populations of the Atlantic lineage of *S. malacitanus* ($\bar{x} = 38, 36-40, n=98$) and in the Southern populations of *S. pyrenaicus* ($\bar{x} = 39, 37-41, n=125$). Populations of the Northern Lineage of *S. pyrenaicus* ($\bar{x} = 41, 39-43, n=81$) and of Mediterranean Lineage of *S. malacitanus* ($\bar{x} = 40, 39-43, n=58$) had highest number of scales in the lateral line.

The number of vertebrae was also smaller in the Atlantic Lineage of *S. malacitanus* ($\bar{x} = 37, 36-38, n=20$). The values greater were to the Northern Lineage of *S. pyrenaicus* ($\bar{x} = 39, 39-41, n=20$) and intermediate values were to the Mediterranean Lineage of *S. malacitanus* ($\bar{x} = 38, 37-39, n=20$) and Southern Lineage of *S. pyrenaicus* ($\bar{x} = 38, 37-39, n=20$) (Appendix 6).

In the case of *S. malacitanus* these differences cannot be explained by differences in environmental variables since both lineages inhabit adjacent rivers with identical typology.

### Osteology features

Infraorbital bones were large in all populations of *Squalius* except in the Mediterranean population of *S. malacitanus*, which had narrower infraorbital bones. This was more conspicuous on 2nd and 3rd infraorbitals (Fig. 3). All the examined adult specimens from the Southern population of the *S. pyrenaicus* had exceptionally wide infraorbital bones (Fig. 3).

The skull of the Southern population of *S. pyrenaicus* was wider than in other populations of the genus and with a wide ethmoids bone (Appendix 7). The maxilla of the Northern populations of *S. pyrenaicus* was very robust, with a posterior process stronger than in any other studied population (Appendix 8). The anterior process of the maxilla was more pointed in *S. pyrenaicus* populations, both Northern and Southern lineages, than in *S. malacitanus*, as was previously described (Doadrio & Carmona, 2006). The coronoid process was variable depending on the size of the specimen, but always was more perpendicular to the skull axis in *S. pyrenaicus* (northern and southern populations), whereas in *S. malacitanus* (Atlantic and Mediterranean populations)
The most divergent species based on the MT-CYB gene were the Portuguese *Squalius aradensis* and *S. torgalensis*, sister to the remaining Iberian *Squalius* species. Previous studies on the phylogenetic relationships of the Iberian *Squalius* showed three highly divergent nuclear and mitochondrial clades in the species *S. pyrenaicus*, as is showed in the phylogenetic tree of the Fig. 4A. These three mitochondrial lineages are constituted by Northern populations (Tagus Drainage), Southern populations (Guadiana and Guadalquivir Drainage) and Sado Drainage, and they were the sister group of *Squalius valentinus*. Nevertheless, the nuclear monophyly of these three lineages was not recovered (Fig. 4B): indeed, Northern populations of *S. pyrenaicus* were clustered in a polytomy together with *S. carolitertii* and *S. castellanus*, and they were not closely related with the other two *S. pyrenaicus* clades (Southern and Sado). The species *Squalius malacitanus* also exhibited highly mitochondrial and nuclear divergent clades (Fig. 4A and 4B), which encompassed the Atlantic and the Mediterranean populations separately. The species *Squalius carolitertii* was closely related with *Squalius castellanus*. The mitochondrial and GENETICS

The most divergent species based on the MT-CYB gene were the Portuguese *Squalius aradensis* and *S. torgalensis*, sister to the remaining Iberian *Squalius* species. Previous studies on the phylogenetic relationships of the Iberian *Squalius* showed three highly divergent nuclear and mitochondrial clades in the species *S. pyrenaicus*, as is showed in the phylogenetic tree of the Fig. 4A. These three mitochondrial lineages are constituted by Northern populations (Tagus Drainage), Southern populations (Guadiana and Guadalquivir Drainage) and Sado Drainage, and they were the sister group of *Squalius valentinus*. Nevertheless, the nuclear monophyly of these three lineages was not recovered (Fig. 4B): indeed, Northern populations of *S. pyrenaicus* were clustered in a polytomy together with *S. carolitertii* and *S. castellanus*, and they were not closely related with the other two *S. pyrenaicus* clades (Southern and Sado). The species *Squalius malacitanus* also exhibited highly mitochondrial and nuclear divergent clades (Fig. 4A and 4B), which encompassed the Atlantic and the Mediterranean populations separately. The species *Squalius carolitertii* was closely related with *Squalius castellanus*. The mitochondrial and
Two new species of the genus *Squalius*

Fig. 4.—A. Phylogenetic tree rendered by Maximum Likelihood and Bayesian Inference based on MT-CYB gene. B. Phylogenetic tree topology obtained from Perea et al., 2020, based on six nuclear genes. In both tree topologies terminal nodes are collapsed. Numbers on branches indicate posterior probability (before slash) and bootstrap (after slash) values.

Fig. 4.—A. Árbol filogenético generado por Máxima Verosimilitud e Inferencia Bayesiana a partir del gen *MT-CYB*. B. Árbol filogenético obtenido de Perea et al., 2020 a partir de seis genes nucleares. En ambas topologías los nodos terminales han sido colapsados. Los números en las ramas indican valores de probabilidad posterior (antes de la barra) y de bootstrap (después de la barra).
nuclear phylogenetic relationships inferred in this study were in concordance with previous studies of the genus (Doadrio & Carmona, 2003, 2006; Sanjur et al., 2003; Almada & Sousa-Santos, 2010; Perea et al., 2020, 2021; Mendes et al. 2021).

Uncorrected-p genetic distances based on \textit{MT-CYB} between all Iberian \textit{Squalius} species ranged from 1.6\% between Northern and Southern populations of \textit{S. pyrenaicus} and 12.1\% between \textit{S. valentinus} and \textit{S. torgalensis} (Appendix 12). Uncorrected-p genetic distances of Southern populations of \textit{S. pyrenaicus} relative to the remaining species were 2.7\% with \textit{S. valentinus}, 6, and 6.4\% with \textit{S. carolitertii} and \textit{S. castellanus}, 7.6 and 8.6\% with Mediterranean and Atlantic populations of \textit{S. malacitanus}, and finally 10.6 and 11.6\% with \textit{S. aradensis} and \textit{S. torgalensis}. In turn, uncorrected-p distances of Atlantic populations of \textit{S. malacitanus} relative to the other analyzed species were 4.2\% with Mediterranean populations of \textit{S. malacitanus}, from 7.9 to 8.7\% with the clade formed by \textit{S. valentinus} and the three divergent lineages of \textit{S. pyrenaicus}, and, finally, 10.9 and 11.4\% with \textit{S. aradensis} and \textit{S. torgalensis}.

Atlantic population of \textit{S. malacitanus} had two autapomorphies in the mitochondrial \textit{MT-CYB} gene, none of them were transversions (Appendix 13).

\textbf{TAXONOMY}

\textbf{DESCRIPTION OF THE SQUALIUS POPULATIONS}

The high degree of morphological and genetic differentiation of \textit{Squalius malacitanus} populations endemic to the Atlantic drainages and to the Miel drainage in the Mediterranean slope, and of the \textit{Squalius pyrenaicus} populations from Southern Iberian drainages justifies the consideration of these population as distinct species. No available names for these populations exist, and therefore, these are described as new species in the present study.

\textbf{Squalius gaditanus} Doadrio & Perea sp. nov.

\texttt{urn:lsid:zoobank.org:act:5FB352FD-3789-4707-B74F-8C924579ED0D}

Figs. 5–6, Table 3

\textbf{Holotype:} MNCN\_ICTIO 296955 89.3 mm SL, 104.2 mm TL; Vega River, Jara Drainage, Tarifa, Cádiz, Spain, 36.028230, -5.610120, 7 m.a.s.l., Leg. P. Garzón-Heydt, T. Nester, A. López Solano and I. Doadrio, 13.V.2022.


\textbf{Additional Material}


In turn, uncorrected-p distances of Atlantic populations of \textit{S. malacitanus} relative to the other analyzed species were 2.7\% with \textit{S. valentinus}, 6, and 6.4\% with \textit{S. carolitertii} and \textit{S. castellanus}, 7.6 and 8.6\% with Mediterranean and Atlantic populations of \textit{S. malacitanus}, and finally 10.6 and 11.6\% with \textit{S. aradensis} and \textit{S. torgalensis}. At any rate, uncorrected-p distances of Atlantic populations of \textit{S. malacitanus} had two autapomorphies in the mitochondrial \textit{MT-CYB} gene, none of them were transversions (Appendix 13).

Fig. 5.– Holotype of \textit{Squalius gaditanus} sp. nov. from the Vega River, Jara Drainage, Tarifa, Cádiz, Spain. MNCN\_ICTIO 296955 SL=89.3 mm. Scale bar = 5 mm.

Fig. 5.– Holotipo de \textit{Squalius gaditanus} sp. nov. del río Vega, cuenca del río Jara, Tarifa, Cádiz, España. MNCN\_ICTIO 296955. SL=89,3 mm. Escala = 5 mm.
Two new species of the genus *Squalius*


**Description.** *Squalius gaditanus* sp. nov. is a member of the Mediterranean clade of the Iberian species of the genus *Squalius* (Sanjur et al., 2003; Perea et al., 2020). *Squalius gaditanus* sp. nov. can be differentiated from all other known species of *Squalius* from the Iberian peninsula according to the following set of characters:

36-40 (χ = 38; X = 38; n = 98) pored scales on the lateral line; 6-7 (χ = 6.7; X = 7; n = 98) scales above the lateral line; 2-3 (χ = 2.8; X = 3; n = 98) scales below the lateral line; 36-38 (χ = 37; X = 37; n = 10) number of vertebrae. Second infraorbital bone narrower than the third in adults. Maxilla with reduced pointed anterior process. Dentary short with inclined coronoid process. Posterior process of the maxilla long and thin. The lower branch of the pharyngeal bone is short and robust. Pharyngeal plate of basioccipital rounded. *Squalius gaditanus* sp. nov. is distinguishable from *S. malacitanus*, the morphological and phylogenetically most related species by lesser number of pored scales on the lateral line χ = 38 36-40 vs χ = 41, 39-43; lesser number of vertebrae 36-38 (χ = 37) vs 37-39 (χ = 38); second infraorbital bone wide vs narrow; lower branch of the pharyngeal bone short and robust vs long and thin and pharyngeal teeth robust vs thin. Genetic distances from the other species of *Squalius* inferred from the mitochondrial MT-CYB gene were: 4.2% with respect to *S. malacitanus*; 8.5% with respect to *S. pyrenaicus* of Northern population; 8.6% with respect to *S. pyrenaicus* of Southern population; 8.6% with respect to *S. valentinus*; 8.7% with respect to *S. castellanus*; about 7.9% with respect to *S. carolitertii*, 11.4% with respect to *S. torgalensis* and 10.9% with respect to *S. aradensis*. The new species has two autapomorphies none of them transversions in the *MT-CYB* gene (positions 714 and 870; Appendix 9).

**Diagnosis.** *Squalius gaditanus* sp. nov. is a member of the Mediterranean clade of the Iberian species of the genus *Squalius* (Sanjur et al., 2003; Perea et al., 2020). *Squalius gaditanus* sp. nov. can be differentiated from all other known species of *Squalius* from the Iberian peninsula according to the following set of characters:

- The species name *gaditanus* is derived from the Phoenician name of the current Cádiz province where the species is distributed.

**DISTRIBUTION.** This new species is endemic to three small drainages of southern Spain: Jara, Barbate that drain on the Atlantic slope and Miel on the Mediterranean slope around the Gibraltar Strait. Probably *S. gaditanus* was widely distributed in the ancient Janda lagoon but this was dried up in the 20th century (Perea et al., 2016) (Fig. 6).

**COMMON NAME.** Cachuelo gaditano.

**REMARKS.** The species typically inhabits rivers with a Mediterranean typology conditioned by severe water stress during the summer, with specimens of *S. gaditanus* surviving in disconnected pools. During the autumn these rivers can have large discharges that considerably increase the flow of the river, sometimes causing disasters in human infrastructures. The drying up of the Janda Lagoon (Finlayson et al., 1997), with its 50 km², eliminated an important refuge for *S. gaditanus* in the face of the great discharges of autumn and the summer droughts. Reservoirs have drastically transformed the habitat of *S. gaditanus* in the Barbate drainage and have been a source of introduction and proliferation of invasive species. In this basin, the species is distributed

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**Table 3.** Morphological variables used to define the morphometric and meristic characters of *S. gaditanus* sp. nov. type series. Variables as described in the Material and methods section (SD = standard deviation).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Holotype</th>
<th>Paratypes (n = 68)</th>
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</thead>
<tbody>
<tr>
<td><strong>Range</strong></td>
<td><strong>Mean</strong></td>
<td><strong>SD</strong></td>
</tr>
<tr>
<td><strong>TL</strong></td>
<td>104.2</td>
<td>39.5-139.9</td>
</tr>
<tr>
<td><strong>SL</strong></td>
<td>89.3</td>
<td>33.3-120</td>
</tr>
<tr>
<td><strong>PDD</strong></td>
<td>50</td>
<td>179-62.7</td>
</tr>
<tr>
<td><strong>PFD</strong></td>
<td>22.9</td>
<td>8-27.9</td>
</tr>
<tr>
<td><strong>PVD</strong></td>
<td>45.7</td>
<td>16.1-59.5</td>
</tr>
<tr>
<td><strong>PAD</strong></td>
<td>64.8</td>
<td>22.6-83.9</td>
</tr>
<tr>
<td><strong>APL</strong></td>
<td>21.9</td>
<td>7.9-29.4</td>
</tr>
<tr>
<td><strong>CPL</strong></td>
<td>35</td>
<td>12.5-44.7</td>
</tr>
<tr>
<td><strong>HL</strong></td>
<td>23.3</td>
<td>7.8-28.5</td>
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<td><strong>POL</strong></td>
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<td>1.7-6.4</td>
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<tr>
<td><strong>ED</strong></td>
<td>5.9</td>
<td>2.4-7.6</td>
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<tr>
<td><strong>PrOL</strong></td>
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<td>3.9-14.3</td>
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<tr>
<td><strong>NL</strong></td>
<td>17.7</td>
<td>6.3-21.5</td>
</tr>
<tr>
<td><strong>HH</strong></td>
<td>17.5</td>
<td>6.1-21.6</td>
</tr>
<tr>
<td><strong>PmL</strong></td>
<td>7.8</td>
<td>2.5-8.6</td>
</tr>
<tr>
<td><strong>PFL</strong></td>
<td>14.9</td>
<td>5.9-19.9</td>
</tr>
<tr>
<td><strong>VFL</strong></td>
<td>13.7</td>
<td>5.1-16.8</td>
</tr>
<tr>
<td><strong>DFL</strong></td>
<td>11.7</td>
<td>3.5-15.5</td>
</tr>
<tr>
<td><strong>DHL</strong></td>
<td>16</td>
<td>6.2-21.6</td>
</tr>
<tr>
<td><strong>AFL</strong></td>
<td>9.8</td>
<td>3.7-12.7</td>
</tr>
<tr>
<td><strong>AHL</strong></td>
<td>13.7</td>
<td>4.7-17.7</td>
</tr>
<tr>
<td><strong>CFL</strong></td>
<td>18.2</td>
<td>7.23</td>
</tr>
<tr>
<td><strong>BLD</strong></td>
<td>10.5</td>
<td>3.9-13.7</td>
</tr>
<tr>
<td><strong>BD</strong></td>
<td>23.7</td>
<td>8.9-31.9</td>
</tr>
<tr>
<td><strong>LLS</strong></td>
<td>38</td>
<td>36-39</td>
</tr>
<tr>
<td><strong>SRA</strong></td>
<td>7</td>
<td>6-7</td>
</tr>
<tr>
<td><strong>SRB</strong></td>
<td>3</td>
<td>2-3</td>
</tr>
<tr>
<td><strong>D</strong></td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><strong>A</strong></td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

---

**Fig. 6.** – Distribution range of *S. gaditanus*. Dots: localities with *S. gaditanus* specimens in the MNCN_ICTIO collection
Two new species of the genus *Squalius*

almost exclusively in the headwaters of the rivers. The species should be considered Critically Endangered according to the IUCN red list criteria due to the extent of its occurrence being less than 100 km² and to the fragmentation of its populations.

*Squalius tartessicus* sp. nov.

urn:lsid:zoobank.org:act:E78C1F26-5FAC-4976-8148-CC89B9597E1D

Figs. 7–8, Table 4.

**Holotype:** MNCN IICTIO 272254 112.2 mm SL, 128 mm TL; Ciudadaleja River, Guadalquivir Drainage, Las Navas de la Concepción, Sevilla, Spain, 37.917956, -5.480368, 434 m.a.s.l., Leg. P. Garzón-Heydt, J. L. González, B. Prieto and E. Herrero, 05.V.2007.


**Additional material.** See Appendix 14.

**Diagnosis.** *Squalius tartessicus* sp. nov. is a member of the Mediterranean clade of the genus *Squalius* (Sanjur et al., 2003; Perea et al., 2020). *Squalius tartessicus* sp. nov. can be differentiated from all other known species of *Squalius* from Iberian peninsula according to the following set of characters: 37-41 (χ=38.8, X=39, n=125) pored scales on the lateral line; 6-7 (χ=7, X=7, n=125) scales above the lateral line; 2-3 (γ=2.9; X=3; n=125) scales below the lateral line; 37-39 (γ=38; X=38; n=20) number of vertebrae. Infraorbital bones unusually wide in adults. Maxilla with discernable pointed anterior process. Dentary short, not inclined. Posterior process of the maxilla long and thin. The lower branch of the pharyngeal bone is short and robust. Pharyngeal plate of basioccipital triangular in shape. Posterior lamina of cleithrum expanding posteriorly.

*Squalius tartessicus* sp. nov. is distinguishable from *S. pyrenaicus*, by lesser number of pored scales on the lateral line 37-41 (χ=38.8) vs 39-43 (χ=40); short preorbital length vs long preorbital length; mouth subterminal vs terminal mouth; in adults specimens 2nd infraorbital bone as wide as 3rd vs 2nd infraorbital narrower than 3rd; ethmoid bone wide vs narrow; in adults lamina of cleithrum expanding posteriorly vs scarcely expanding posteriorly.

Genetic distances from the other species of *Squalius*, inferred from the mitochondrial *MT-CYB* gene sequences, were: 7.6% with respect to *S. malacitanus*; 1.6% with respect to *S. pyrenaicus* of Northern population; 8.6% with respect to *S. gaditanus*; 2.7% with respect to *S. valentinus*; 6.4% with respect to *S. castellanus*; 6% with respect to *S. carolitertii*; 11.6% with respect to *S. torgalensis* and 10.6% with respect to *S. aradensis*.

**Description.** D III 8; A III 8; P I 114-15; V I 8; C 17; LLS 39 (37-41); SRA 6-7; SRB 2-3; RPT 5.2 LPT 5.2; Vr= 38 (37-39). Morphometric and meristic characters of the type material are presented in Table 4; measurements used in the morphometric study are listed in Appendix 12.

The head length is shorter than the height maxima of the body.
Doadrio et al. pigmentation pattern. The body is silver to brownish but without the characteristic dorsal portion dark grey of S. malacitanus and S. gaditanus. The scales have one big black spot on the base and a series of small black spots on the distal border. The basis of pectoral fins is brown or orange. With small nuptial tubercles in males.

**Etymology.** The species name tartessicus is derived from Tartessos, a culture that for about 400 years (8th-5th centuries BC) was present in the southwest of the Iberian Peninsula where S. tartessicus sp. nov. is currently distributed.

**Distribution.** This new species is distributed throughout the Atlantic drainages of the southern Iberian Peninsula from Almargem in Portugal to Guadalete in Spain, including the main drainages of Guadiana and Guadalquivir. In the Mediterranean...
Table 5.– Diagnostic morphological characters of the four populations studied.

<table>
<thead>
<tr>
<th>Character</th>
<th>S. malacitanus</th>
<th>S. gaditanus</th>
<th>S. pyrenaicus</th>
<th>S. tartessicus</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLS</td>
<td>χ = 40 39-43</td>
<td>χ = 38 36-40</td>
<td>χ = 41 39-43</td>
<td>χ = 39 37-41</td>
</tr>
<tr>
<td>Vr</td>
<td>χ = 38, 37-39</td>
<td>χ = 37, 38-36</td>
<td>χ = 39, 41-39</td>
<td>χ = 38, 37-39</td>
</tr>
<tr>
<td>Infraorbitals 2nd, 3rd</td>
<td>Narrow</td>
<td>wide</td>
<td>wide</td>
<td>Unusually wide</td>
</tr>
<tr>
<td>Maxilla</td>
<td>Scarce pointed</td>
<td>Scarce pointed</td>
<td>Very pointed</td>
<td>Very pointed</td>
</tr>
<tr>
<td>Anterior process</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maxilla Posterior process</td>
<td>Short, Robust</td>
<td>Long, Thin</td>
<td>Long, Thin</td>
<td>Long, Thin</td>
</tr>
<tr>
<td>Pharyngeal Plate</td>
<td>Rounded</td>
<td>Rounded</td>
<td>Triangular</td>
<td>Triangular</td>
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<tr>
<td>Pharyngeal teeth</td>
<td>Denticulated</td>
<td>Denticulated</td>
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<td>Strongly Denticulated</td>
</tr>
<tr>
<td>Pharyngeal Bone</td>
<td>Long, Thin</td>
<td>Short, Robust</td>
<td>Short, Robust</td>
<td>Short, Robust</td>
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<tr>
<td>Cleithrum lamina posterior</td>
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<td>Not extended</td>
<td>Not extended</td>
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<tr>
<td>Pigmentation</td>
<td>Dorsally contrasted</td>
<td>Dorsally contrasted</td>
<td>Dorsally not contrasted</td>
<td>Dorsally not contrasted</td>
</tr>
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**Fig. 8.– Distribution range of S. tartessicus. Dots: localities with S. tartessicus specimens in the MNCN_ICTIO collection.**

**Fig. 8.– Área de distribución de S. tartessicus. Puntos localizaciones con ejemplares de S. tartessicus en la colección del MNCN_ICTIO.**

The species is distributed in Guadalhorce Vélez, Guadalfeo and Segura drainages (Fig. 6).

**Common Name.** Cachuelo meridional.

**Remarks.** The species lives in very different types of habitats from mountain rivers with a permanent flow throughout the year to Mediterranean-like rivers conditioned by severe water stress during the summer, where surviving specimens are found in disconnected pools. This species prefers deep pools within rivers and it forms a well-studied hybrid complex in many of the basins with S. alburnoides (Cunha et al., 2004). The species should be considered Vulnerable according to the IUCN red list criteria due to the decreasing of its distribution area and population number mainly by increasing of reservoirs, presence of invasive species and pollution by agriculture.

**References**


APPENDICES

Appendix 1.– GenBank samples used in the Squalius MT-CYB phylogenetic analyses

Apéndice 1.– Muestras provenientes del GenBank estudiadas en el análisis filogénético del MT-CYB para el género Squalius.

<table>
<thead>
<tr>
<th>Species Name</th>
<th>After Perea et al. (2020)</th>
<th>Current Study</th>
<th>River. Drainage. Country</th>
<th>GenBank Numbers</th>
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</thead>
<tbody>
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<td>Squalius aradensis</td>
<td>Squalius aradensis</td>
<td>Squalius aradensis</td>
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<td>AJ698711</td>
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<tr>
<td>Squalius carolitertii</td>
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<td>Squalius carolitertii</td>
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<td>Squalius carolitertii</td>
<td>Squalius carolitertii</td>
<td>Agueda. Vouga. Portugal</td>
<td>MT008596</td>
</tr>
<tr>
<td>Squalius carolitertii</td>
<td>Squalius carolitertii</td>
<td>Squalius carolitertii</td>
<td>Vouga. Vouga. Portugal</td>
<td>AJ698455</td>
</tr>
<tr>
<td>Squalius carolitertii</td>
<td>Squalius carolitertii</td>
<td>Squalius carolitertii</td>
<td>Cávado. Cávado. Portugal</td>
<td>MT008594</td>
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<tr>
<td>Squalius carolitertii</td>
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<td>Squalius carolitertii</td>
<td>Corvo. Mondego. Portugal</td>
<td>MT008589</td>
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<td>Squalius carolitertii</td>
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<td>MT008587</td>
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<td>Squalius carolitertii</td>
<td>Squalius carolitertii</td>
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Appendix 2.– Variables that most contributed to the PCA to S. pyrenaicus populations. Symbols: Fill Squares, Almonte River (Tajo Drainage), Northern Lineage of S. pyrenaicus. Stars, Jerte River (Tajo Drainage), Northern Lineage. Dots, Grande River (Guadalhorce drainage), Southern Lineage. Circles, Sabar and Cuevas Rivers (Vélez Drainage), Southern Lineage. Plus, Cala River (Guadalquivir Drainage), Southern Lineage. Diamonds Ciudadela River (Guadalquivir Drainage), Southern Lineage. Abbreviations are defined in Materials and Methods.

Appendix 3.– Eigenvalues and eigenvectors for the first two principal components (PCI-PCII) of 23 morphometric variables for all the Squalius pyrenaicus populations. Acronyms are defined in the Material and methods section. In bold, variables with the highest eigenvectors for each PC.

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Apéndice 3.– Eigenvalores y eigenvectores para los dos primeros componentes principales (PCI-PCII) de 23 variables morfométricas para las poblaciones de Squalius pyrenaicus. Las abreviaturas están descritas en el epígrafe de Material y Métodos. En negrita variables con los eigenvectores más altos para cada CP.
Appendix 4.– Variables that most contributed to the PCA to populations of *Squalius malacitanus*. Triangles, Mediterranean Lineage. Fill Triangles, Atlantic Lineage. Abbreviations are defined in Material and methods.

Apéndice 4.– Variables que más contribuyen al ordenamiento en el PCA para las poblaciones de *Squalius malacitanus*. Símbolos: Triángulos, Linaje Mediterráneo. Triángulos en negro, Linaje Atlántico de *S. malacitanus*. Las abreviaturas están descritas en el epígrafe de Material y métodos.

Appendix 5.– Eigenvalues and eigenvectors for the first two principal components (PCI-PCII) of 23 morphometric variables for *Squalius malacitanus*. Acronyms are defined in the Material and methods section. In bold variables with the highest eigenvectors for each PC.

Apéndice 5.– Eigenvalores y eigenvectores para los dos primeros componentes principales (PCI-PCII) de 23 variables morfométricas para las poblaciones de *Squalius malacitanus*. Las abreviaturas están descritas en el epígrafe de Material y métodos. En negrita variables con los eigenvectores más altos para cada CP.

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<th>PCIV</th>
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<td>0.002</td>
<td>0.001</td>
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<tr>
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<td>12.08</td>
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<tr>
<td>Eig. 2.5%</td>
<td>20.02</td>
<td>13.68</td>
<td>8.04</td>
<td>6.14</td>
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<tr>
<td>Eig. 97.5%</td>
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<td>19.02</td>
<td>14.03</td>
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<td>PmxL</td>
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Two new species of the genus *Squalius*

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Appendix 6.– Number of vertebrae in the different populations: 1, Mediterranean Lineage of *S. malacitanus*; 2, Atlantic Lineage of *S. malacitanus*; 3, Southern Lineage of *S. pyrenaicus* and 4, Northern lineage of *S. pyrenaicus*.

Apéndice 6.– Número de vertebrales para las diferentes poblaciones: 1, Linaje Mediterráneo de *S. malacitanus*; 2, Linaje Atlántico de *S. malacitanus*; 3, Linaje del sur de *S. pyrenaicus* y 4, Linaje del Norte de *S. pyrenaicus*. 
Appendix 7. – Ethmoid bone of *Squalius* populations under study: A, Mediterranean population of *S. malacitanus* from type locality; B, Atlantic population of *S. malacitanus*; C, Northern population of *S. pyrenaicus* and D, Southern population of *S. pyrenaicus*. Abbreviations: fr = frontal, et = ethmoid, pm = premaxilla.

Two new species of the genus *Squalius*

**Appendix 8.** Oral jaws of *Squalius* populations under study. A. Mediterranean population of *S. malacitanus* from type locality; B. Atlantic population of *S. malacitanus*; C. Northern population of *S. pyrenaicus* and D. Southern population of *S. pyrenaicus*. Abbreviations: *dn* = dentary, *mx* = maxilla, *pc* = coronoid process, *pmx* = premaxilla, *ppl* = palatine process, *ppm* = posterior process of the dentary.


**Appendix 9.** Basioccipital of *Squalius* populations under study. A. Mediterranean populations of *S. malacitanus* from type locality; B. Atlantic populations of *S. malacitanus*; C. Northern populations of *S. pyrenaicus* and D. Southern populations of *S. pyrenaicus*. Abbreviations: *fv* = lateral processes of the first vertebra, *mx* = maxilla, *ppl* = pharyngeal plate, *pps* = posterior process of the basioccipital, *sv* = lateral processes of the second vertebra, *tr* = tripod.

Appendix 10.– Pharyngeal teeth of *Squalius* populations under study. A, Mediterranean populations of *S. malacitanus* from type locality; B, Atlantic populations of *S. malacitanus*; C, Northern populations of *S. pyrenaicus* and D, Southern populations of *S. pyrenaicus*. Abbreviations: *dn* = denticulations, *lp* = lower process of pharyngeal bone, *ms* = masticatory surface, *rt* = replacement teeth, *up* = upper process of pharyngeal bone.


Appendix 11.– Cleithrum of *Squalius* populations under study. A, Mediterranean population of *S. malacitanus* from type locality; B, Atlantic population of *S. malacitanus*; C, Northern population of *S. pyrenaicus* and D, Southern population of *S. pyrenaicus*. Abbreviation: *plc*, posterior lamina of the cleithrum.

Appendix 12.– Uncorrected-p genetic distances between species and populations for the genus *Squalius* from the Iberian Peninsula.

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<tr>
<th></th>
<th><em>S. aradensis</em></th>
<th><em>S. pyrenaicus</em> Northern populations</th>
<th><em>S. pyrenaicus</em> Sado</th>
<th><em>S. pyrenaicus</em> Southern populations</th>
<th><em>S. carolitertii</em></th>
<th><em>S. castellanus</em></th>
<th><em>S. malacitanus</em> Atlantic populations</th>
<th><em>S. malacitanus</em> Mediterranean populations</th>
<th><em>S. valentinus</em></th>
<th><em>S. torgalensis</em></th>
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<td>0.7</td>
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<td>7.3</td>
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<td><em>S. valentinus</em></td>
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<td>11.9</td>
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Appendix 13.– Autapomorphies in the mitochondrial cytochrome *b* gene detected for the genus *Squalius*. Transversions are indicated with *.

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<td>A</td>
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<td>A</td>
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Appendix 14.– Localities of Squalius tartessicus in MNCN_ICTIO Collection.

GUADALquivIR DRAINAGE

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<td>A</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>S. torgalensis</td>
<td>C</td>
<td>G</td>
<td>A/G</td>
<td>A</td>
<td>T</td>
<td>G</td>
<td>C</td>
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</table>

Appendix 14.– Localities of Squalius tartessicus in the Colección de Ictiología del MNCN.
Two new species of the genus Squalius

GUADIANA DRAINAGE

Graellsia

Doadrio, I.; Elvira, B., 8.5.1987. MNCN_ICTIO 212588-
Albarragena, Badajoz, Spain, 39.045590, -6.906453, Leg.,
MNCN_ICTIO 45272, Zapatón River, Botoa, Puente
Doadrio, I.; González, J.A.; Perdices, A., 3.3.1992. Zafra de Záncara, Cuenca, Spain, 39.895067, -2.560379,
18.2.1996. MNCN_ICTIO 79313-79329, Zancara River,
Guadalmez, Cuidad Real, Spain, 39.702857, -4.920903,
12.6.1996. MNCN_ICTIO 211435-211443, Vélez River,
Carrascosa del Campo, Cuenca, Spain, 38.085164, -0.946064.

Odeñal Drainage


Segura Drainage

MNCN ICTIO 157696-157704, de Bogarra River, Las Mohedas, Albacete, Spain, 38.603804,-2.252087, Leg., González, J.A.; Ambrosio, L., 31.10.1996. MNCN ICTIO 25650-25654, Segura River, Orihuela, Alicante, Spain, 38.085164,-0.946064.

Vélez Drainage


Guadalhorce Drainage


**GUADALETE DRAINAGE**


**GUADALFEO DRAINAGE**