






## A new species of the genus *Squalius* (Leuciscidae, Actinopterygii) from the Sado River basin in Portugal

Sofia L. Mendes<sup>1\*</sup> , Silvia Perea<sup>2</sup> , Vitor C. Sousa<sup>1</sup> , Carla Sousa-Santos<sup>3</sup>  and Ignacio Doadrio<sup>2\*</sup> 

<sup>1</sup> cE3c - Centre for Ecology, Evolution and Environmental Changes & CHANGE - Global Change and Sustainability Institute, Departamento de Biología Animal, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal.

<sup>2</sup> Museo Nacional de Ciencias Naturales. José Gutiérrez Abascal 2. 28006 Madrid. Spain.

<sup>3</sup> MARE –Marine and Environmental Sciences Centre/ARNET - Aquatic Research Network, ISPA – Instituto Universitário de Ciências Psicológicas, Sociais e da Vida, Rua Jardim do Tabaco 34, 1149-041 Lisboa, Portugal.

\* Corresponding author: slmendes@ciencias.ulisboa.pt; doadrio@mncn.csic.es

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### ABSTRACT

#### A new species of the genus *Squalius* (Leuciscidae, Actinopterygii) from the Sado River basin in Portugal.

A new species, *Squalius caetobrigus* sp. nov., is described based on morphological and genetic traits. *Squalius caetobrigus* sp. nov. can be distinguished from other *Squalius* species through a combination of morphometric, meristic and genetic characteristics: 39-42 ( $\bar{X}=40.7$ ;  $\bar{X}=41$ ) canaliculate scales on the lateral line; 6-7 ( $\bar{X}=7$ ;  $\bar{X}=7$ ) scales above the lateral line; 3-4 ( $\bar{X}=3.1$ ;  $\bar{X}=3$ ) scales below the lateral line; short preorbital distance HL/PrOL is 4.2-6.2 ( $\bar{X}=5$ ); no contact between the fourth infraorbital bone and the preopercular; short dentary with a very developed coronoid process; the posterior process of the maxilla is long and thin as well as the lower branch of the pharyngeal bone; small pharyngeal plate of the pharyngeal bone and 5 autapomorphies in the mitochondrial cytochrome b gene. *Squalius caetobrigus* is restricted to the Sado River basin in southern Portugal.

**KEY WORDS:** taxonomy; Iberian Peninsula; *Squalius*; Cypriniformes; Leuciscidae; genetics; morphology.

### RESUMEN

#### Una nueva especie del género *Squalius* (Leuciscidae, Actinopterygii) de la cuenca del río Sado en Portugal.

Se describe una nueva especie, *Squalius caetobrigus* sp. nov., a partir de caracteres morfológicos y genéticos. *Squalius caetobrigus* sp. nov. se puede distinguir de otras especies del género *Squalius* mediante una combinación de caracteres morfométricos, merísticos y genéticos: 39-42 ( $\bar{X}=40.7$ ;  $\bar{X}=41$ ) escamas canaliculadas en la línea lateral; 6-7 ( $\bar{X}=7$ ;  $\bar{X}=7$ ) escamas por encima de la línea lateral; 3-4 ( $\bar{X}=3.1$ ;  $\bar{X}=3$ ) escamas debajo de la línea lateral; distancia preorbitaria corta HL/PrOL es 4.2-6.2 ( $\bar{X}=5$ ); cuarto infraorbitario sin contacto con el preopercular; dentario corto con una apófisis coronoidea muy desarrollada; apófisis posterior del maxilar larga y delgada; la rama inferior del hueso faríngeo es larga y delgada; la placa faríngea del hueso faríngeo es pequeña y 5 autapomorfias en el gen mitocondrial citocromo b. *Squalius caetobrigus* está restringida a la cuenca del río Sado en el sur de Portugal.

**PALABRAS CLAVE:** taxonomía; península ibérica; *Squalius*; Cypriniformes; Leuciscidae; genética; morfología.

## INTRODUCTION

The arrangement of the main hydrographic basins of the Iberian Peninsula is geographically oriented in an east-west or west-east direction, primarily because of the positioning of its mountain ranges. This disposition acts as an obstacle to the colonization of freshwater organisms from either the northern or southern regions, particularly those with restricted dispersal abilities like primary freshwater fish (Myers, 1938; Darlington, 1948). This has resulted in the isolation of the Iberian Peninsula from the primary European and African fish fauna during the Cenozoic period. This isolation was a key factor giving rise to the distinctive composition of the Iberian freshwater fish fauna, marked by a restricted array of genera and a notable prevalence of endemic species (Doadrio *et al.*, 2011). Nevertheless, the hydrographic basin of the Sado River is unique, as it follows a south-north direction along its 175-kilometer course, running approximately parallel to the coastline. This particular orientation of the Sado River basin should indeed correspond to a corridor for the freshwater fish fauna between the hydrographic basins of the central and southern Iberian Peninsula. This alignment is also consistent with previous paleogeographic explanations linking the origin of the Sado River to the lower course of the Tagus River (Pimentel, 2002) and with the presence, in both basins, of *Luciobarbus bocagei* (Steindachner, 1864), *Iberochondrostoma lusitanicum* (Collares-Pereira, 1980) and *Pseudochondrostoma polylepis* (Steindachner, 1864) (Sousa-Santos *et al.*, 2007; Almada & Sousa-Santos, 2010). However, this alignment does not completely coincide with molecular studies conducted on the freshwater fish fauna, particularly those focused on the fishes belonging to the Leuciscidae family. These studies reveal a noteworthy divergence between the populations of freshwater fishes present in the Sado River basin and those in the neighboring river basins which should be explained by an isolation of this basin during the Cenozoic (Robalo *et al.*, 2007; Sousa *et al.*, 2008; Almada & Sousa-Santos, 2010; Sousa-Santos *et al.*, 2019).

Therefore, the observed population divergence in the freshwater fishes of the Sado River basin is

better explained by the existence of the Alvalade paleobasin, a hydrographic basin that was isolated from the lower Tagus after the Miocene (Messinian), by a paleogeographic barrier of Paleozoic rocks named Senhor das Chagas-Valverde horst (Antunes & Pais, 1993; Albardeiro *et al.*, 2014; Perea *et al.*, 2021). The sedimentary deposits in this basin exhibit an alternation between marine and fluvial layers, and remnants of elasmobranch fossils have been unearthed from the lower Miocene (Antunes *et al.*, 1999; Antunes & Balbino, 2010; Fialho *et al.*, 2021). However, the most plausible hypothesis suggests that at some point during the Cenozoic Era, the Alvalade paleobasin likely transitioned to a freshwater environment, leading to the isolation of the Leuciscidae populations (Albardeiro *et al.*, 2014). This isolation is corroborated by molecular studies conducted on freshwater fishes inhabiting the Sado River basin (Sousa-Santos *et al.*, 2019, Perea *et al.*, 2020, 2021).

Thus, the formation and paleogeomorphological evolution of the Sado River (and, consequently, the biogeographic history of its freshwater fauna) appear to be intricate, including processes of isolation and connections between paleodrainages throughout the Cenozoic era, both northwards to the lower Tagus and southwards to the Guadiana River basin, through contacts with the of Moura paleobasin (Sousa-Santos *et al.*, 2019).

Among the leuciscids of the Iberian Peninsula, one of the most diversified genera is the genus *Squalius*, with twelve described species: *Squalius alburnoides* (Steindachner, 1866), *Squalius aradensis* (Coelho, Bogutskaya, Rodrigues & Collares-Pereira, 1998), *Squalius carolitertii* (Doadrio, 1988), *Squalius castellanus* Doadrio, Perea & Alonso, 2007, *Squalius gaditanus* Doadrio, Sousa-Santos & Perea, 2023, *Squalius laietanus* Doadrio, Kottelat & de Sostoa, 2007, *Squalius malacitanus* Doadrio & Carmona, 2006, *Squalius palaciosi* (Doadrio, 1980), *Squalius pyrenaicus* (Günther, 1868), *Squalius tartessicus* Doadrio, Sousa-Santos & Perea, 2023, *Squalius torgalensis* (Coelho, Bogutskaya, Rodrigues & Collares-Pereira, 1998) and *Squalius valentinus* Doadrio & Carmona, 2006. Out of these twelve species, one species, *S. laietanus*, occurs in the Iberian Peninsula and the southwest of France,

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aligning with the Euroasiatic lineage (Sanjur et al., 2003). The remaining eleven species are endemic to the Iberian Peninsula and they all belong to the Mediterranean clade (Sanjur et al., 2003). Within the Mediterranean clade, *S. alburnoides* and *S. palaciosi* are hybrid complexes (Carmona et al., 1997; Alves et al., 2001; Robalo et al., 2006; Cunha et al., 2008, 2011). The nomenclature of these two complexes of hybrid origin is controversial, as they result from ancient hybridizations between one species of the *Squalius* genus (maternal ancestor) and a second species belonging to a different genus. In this context, we follow the latest nomenclature revision of the group (Collares-Pereira & Coelho, 2010) to provide nomenclatural stability, but this issue requires further review.

Since the initiation of molecular studies on the *Squalius* genus, the population of *S. pyrenaicus* from the Sado River basin has been deemed highly divergent, regarding both nuclear and mitochondrial genes, and has been considered a potential new species (Coelho et al., 1995; Doadrio & Carmona, 2003; Sousa-Santos et al., 2007, 2019; Almada & Sousa-Santos, 2010; Waap et al., 2011; Perea et al., 2020, 2021). However, molecular studies also reveal discordance in the phylogenetic relationships of the Sado population. Indeed, while mitochondrial DNA studies place the Sado population in a clade comprising *S. pyrenaicus* from the Tagus River basin, *S. tartessicus* and *S. valentinus*, nuclear DNA studies link the Sado population to a clade composed only of *S. tartessicus* and *S. valentinus* (Waap et al., 2011; Perea et al., 2020; Doadrio et al., 2023). These disparities between mitochondrial and nuclear DNA were also reported for other species within the *Squalius* genus (Perea et al., 2016). As a consequence of this discordance, the isolation of the Sado population from other *Squalius* has been estimated to have occurred during the Upper Miocene–Lower Pleistocene (7.5 Ma, HPD 95%, 4.4–11.7 Ma) or in the Middle Miocene (12.3 Ma, HPD 95%, 8.0–18.0 Ma) using, respectively, mtDNA and nDNA for dating the events based on genetic data (Perea et al., 2021).

Despite the high genetic divergence observed between the Sado population and other *Squalius* populations from the Iberian Peninsula (Almada

and Sousa-Santos, 2010; Doadrio et al., 2023), with divergence levels that surpass those found among other *Squalius* species, a formal description of the Sado population as a new species has not been conducted so far, mainly due to the lack of morphological analyses of this population. In this study, we endeavor to address this knowledge gap by morphologically characterizing the Sado population and comparing it with *S. pyrenaicus* and *S. tartessicus*, its genetically closest species, inhabiting the adjacent hydrographic basins of the Tagus and Guadiana rivers, respectively.

## MATERIALS AND METHODS

### Sampling

For this study, 20 *Squalius* adult individuals from the Sado River basin were sampled in one of its tributaries, the Grândola stream (sampling location coordinates: 38.169591 -8.565635), using electrofishing with low-duration pulses to avoid juvenile mortality. The individuals were euthanized on-site with an overdose of tricaine mesylate (MS-222) according to permit 953/2023/CAPT issued by the Portuguese authority for species conservation (Instituto de Conservação da Natureza e das Florestas – ICNF) and preserved in 70% ethanol after fixation in a 10% formalin solution (4% formaldehyde). These specimens were added to the Museu Nacional de História Natural e da Ciência (MUHNAC, Portugal) collection (MNHNC.MB05:003765, MNHNC.MB05:003766). The remaining samples used for morphology and osteology in this study were already part of the Museo Nacional de Ciencias Naturales (MNCN-CSIC, Spain) collection. No additional sampling was carried out for the genetic analysis in this study, as we used either publicly available data or fin clips previously collected for other projects.

### Morphology

The following morphometric and meristic study was based on the analysis of a total of 135 specimens belonging to *Squalius* from the Tagus, Guadiana and Sado basins. Thirty-four specimens belonging to *S. tartessicus*: MNCN\_ICTIO

27225-242, 18 specimens from the type series in the Ciudadeja River (Guadalquivir River basin), Las Navas de la Concepción (Sevilla; Spain); MNCN\_ ICTIO 289456-471, 16 specimens from the Estena River (Gadriana River basin), Navas de Estena (Ciudad Real; Spain). Eighty-one specimens belonging to *S. pyrenaicus* from the Tagus River basin: MNCN\_ ICTIO 213943-72, MNCN\_ ICTIO 215791-816, 56 specimens from the Almonte River (Tagus River basin), Jaraicejo (Cáceres, Spain); MNCN\_ ICTIO 196609-23, MNCN\_ ICTIO 266495-504, 25 specimens from the Jerte River (Tagus River basin), Jerte (Cáceres, Spain). Finally, 20 specimens previously assigned to *S. pyrenaicus* from the Grândola stream (Sado River basin), Grândola (Portugal): MNHNC.MB05:003765, MNHNC.MB05:003766.

Holotypes and paratype series of the new species have been deposited in the Museu Nacional de História Natural e da Ciência (Portugal) and in the Museo Nacional de Ciencias Naturales (Spain).

Twenty-four morphometric measurements (in mm) and ten meristic variables were recorded from digital photographs using TpsDig v.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, preventral distance; PrAD, 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; AHL, 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. After constructing the measurement matrix, Burnaby's method was used to correct the effect of size. The Burnaby method removes the effects of a within population factor (size) from between-group morphometric analyses using

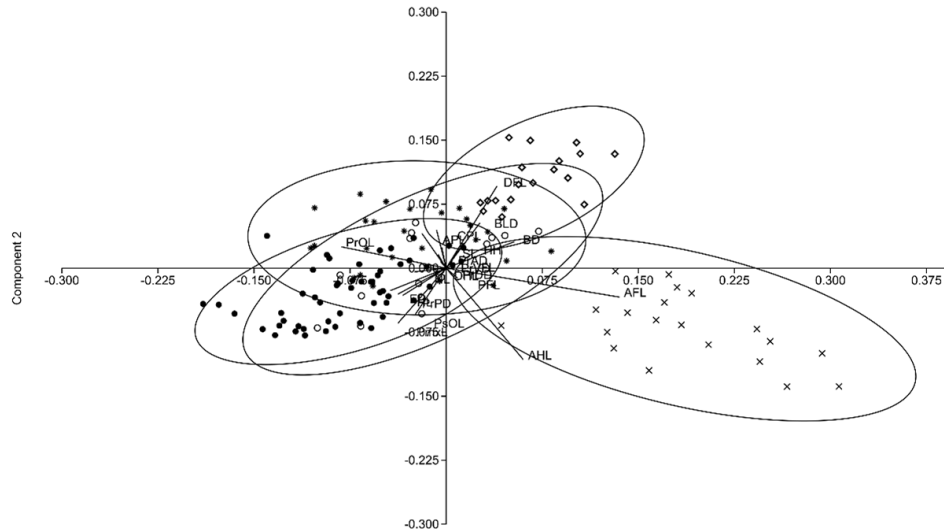
an orthogonal projection procedure (Burnaby, 1966). All analyses were conducted with the corrected matrix. Morphometric and meristic characters were analyzed independently. To identify the variables that contributed most to the variation among populations, a principal component analysis (PCA) was performed using the covariance matrix for morphometric characters. Row-wise bootstrapping was carried out using 100 000 replicates to estimate 95% bootstrap confidence intervals for eigenvalues. Statistical analyses were carried out using Past v. 4.12 (Hammer *et al.*, 2001), applying 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 v2.2 (Volume Graphics, <http://www.volumegraphics.com>) for the following samples: *S. pyrenaicus* of the Tagus River basin: MNCN\_ ICTIO 213943-52, 10 specimens from the Almonte River, Jaraicejo (Cáceres, Spain); MNCN\_ ICTIO 267007-16, 10 specimens from the Jerte River, Navaconcejo (Cáceres, Spain). Specimens of the Sado River basin previously assigned to *S. pyrenaicus*: MNHNC.MB05:003766 (individual labels MNHNC.MB85:019249, MNHNC.MB85:019252 and MNHNC.MB85:019258), 3 specimens from the Grândola stream (Sado River basin), Grândola (Portugal). *S. tartessicus*: MNCN\_ ICTIO 272254-63, 10 specimens from the Ciudadeja River, Las Navas de la Concepción (Sevilla, Spain); MNCN\_ ICTIO 289456, 1 specimen from the Estena River, Navas de Estena (Ciudad Real, Spain).

Additionally, dry skeletons preserved in the MNCN collections were studied for the following samples: *S. pyrenaicus* of the Tagus River basin: MNCN\_ ICTIO 69458-62, 69463-68, 20671-73, 20680, 14 specimens from the Alburrel River, Valencia de Alcántara (Cáceres, Spain); MNCN\_ ICTIO 69484-85, 2 specimens from the Jarama River, Talamanca del Jarama (Madrid, Spain); MNCN\_ ICTIO 69470, 1 specimen from the Tagus River, Villarreal de San Carlos (Cáceres, Spain); MNCN\_ ICTIO 69471, 1 specimen from the Salor

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**Figure 1.** Variables that most contributed to the PCA to all populations of the genus *Squalius*. Symbols: Dots, *S. pyrenaicus* from Almonte River (Tagus River basin). Stars, *S. pyrenaicus* from Jerte River (Tagus River basin). Cross mark (x), *S. pyrenaicus* from Grândola stream (Sado River basin). Diamonds, *S. tartessicus* from Ciudadaja River (Guadalquivir River basin). Circles, *S. tartessicus* from Estena River (Gadiana River basin). Abbreviations are defined in the Materials and Methods section. *Variables que más contribuyen al ordenamiento en el PCA para todas las poblaciones del género Squalius.* Símbolos: Puntos, *S. pyrenaicus* del río Almonte, (Cuenca del Tajo Drainage). Estrellas, *S. pyrenaicus* del río Jerte (Cuenca del Tajo). X, *S. pyrenaicus* del río Grândola (Cuenca del Sado). Diamantes, *S. tartessicus* del río Ciudadaja (Cuenca del Guadalquivir). Círculos, *S. tartessicus* del río Estena (Cuenca del Gadiana). Las abreviaturas se describen en la sección de material y métodos.

River, Membrío (Cáceres, Spain); MNCN\_ ICTIO 69477, 20674, 2 specimens from the Guadalix River, San Agustín de Guadalix (Madrid, Spain); MNCN\_ ICTIO 20277, 20280, 20675, 20676, 4 specimens from the Pinilla Reservoir, Pinilla del Valle (Madrid, Spain); MNCN\_ ICTIO 20278, 1 specimen, from the Aurela River, Santiago de Alcántara (Cáceres, Spain). *S. tartessicus*: MNCN\_ ICTIO 69469, 1 specimen from the Gévora River, Alburquerque (Badajoz, Spain); MNCN\_ ICTIO 69472-74, 3 specimens from the Záncara River, Zafra de Záncara (Cuenca, Spain). MNCN\_ ICTIO 69475, 69476, 69478, 3 specimens from the Robledillo River, Solana del Pino (Ciudad Real, Spain).

### Genetic analyses

Several previous studies have used nuclear and mitochondrial markers to study the Iberian *Squalius* populations from a phylogenetic and biogeographical perspective (Sanjur et al., 2003; Almada & Sousa-Santos, 2010; Sousa-Santos et al., 2019; Perea et al., 2020, 2021). Thus, the relationship

between the different Iberian *Squalius* species has been relatively well studied.

We obtained the sequence of the mitochondrial MT-CYB gene for a total of 133 individuals from 9 Iberian *Squalius* species and the Sado lineage (Appendix 1, see supplementary information, available at <https://www.limnetica.net/en/limnetica>). This dataset comprises both newly generated sequences for 12 of the Sado individuals, as well as previously published data for the remaining individuals of the Sado and the other species, retrieved from Genbank. DNA extraction, amplification and sequencing protocols to obtain the new sequences were carried out according to Sousa-Santos et al. (2016). For the Sado lineage, we focused on obtaining data for as many of the Sado sub-basins as possible – Campilhas, Corona, Grândola, Odivelas, São Martinho and Marateca –, thus covering the distribution of this lineage. Regarding the other Iberian species, we aimed to obtain a larger representation from *Squalius pyrenaicus* (36 individuals) and *Squalius tartessicus* (43 individuals) when compared with the remaining Iberian *Squalius* species (Appendix 1). This is

**Table 1.** Eigenvalues and eigenvectors for the first three principal components (PCI-PCIII) of 22 morphometric variables. Acronyms are defined in the Material and Methods section. In bold variables with the highest eigenvectors for each PC. *Eigenvalores y eigenvectores para los tres primeros componentes principales (PCI-PCIII) de 22 variables morfológicas. Las abreviaturas están descritas en el epígrafe de Material y Métodos. En negrita variables con los eigenvectores más altos para cada CP.*

	PC 1	PC 2	PC 3
Eigenvalue	0.0107	0.0040	0.0016
% Variance	47.29	17.90	7.33
Eig. 2.5%	40.98	14.38	5.32
Eigenvectors			
Eig. 97.5%	52.66	21.11	9.42
SL	-0.02	-0.12	0.02
PrDD	-0.01	0.00	0.05
PrPD	-0.16	-0.16	-0.11
PrVD	-0.02	0.04	-0.04
PrAD	-0.03	0.08	-0.02
APL	-0.09	0.20	0.11
CPL	-0.04	0.22	0.08
HL	-0.18	-0.15	-0.08
PrOL	<b>-0.40</b>	0.12	-0.31
ED	-0.21	-0.13	0.26
PsOL	-0.12	-0.27	-0.15
NL	-0.12	-0.02	-0.03
HH	0.06	0.14	-0.16
PmxL	-0.18	<b>-0.31</b>	<b>-0.35</b>
PFL	0.04	-0.06	0.21
VFL	0.02	0.04	0.42
DFL	0.19	0.47	-0.11
DHL	-0.05	-0.00	0.20
AFL	<b>0.66</b>	-0.17	-0.45
AHL	0.29	<b>-0.52</b>	<b>0.33</b>
BD	0.26	0.15	0.17
BLD	0.13	-0.26	-0.00

because these are also the species used for morphological and osteological comparison with the Sado specimens. Moreover, along with the Sado

lineage, these were all previously classified under *Squalius pyrenaicus* until recently (Doadrio *et al.*, 2023).

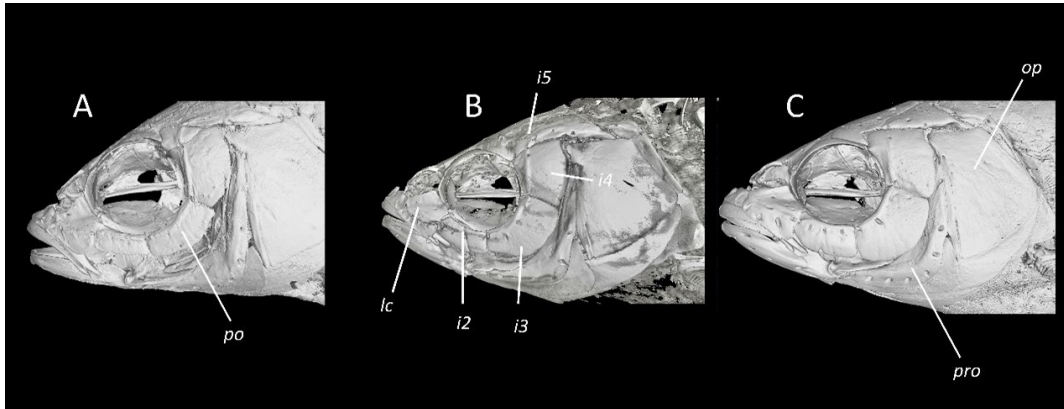
We used this dataset to construct a maximum likelihood phylogeny using IQ-TREE v2.1.2 (Nguyen *et al.*, 2015). We used ModelFinder (Kalyaanamoorthy *et al.*, 2017) implemented on IQ-TREE to determine the best substitution model. According to the corrected Akaike Information Criterion (AIC), the best substitution model was TIM2+F+G4, which we used to construct a phylogeny with 50 000 ultrafast bootstrap replicates (Minh *et al.*, 2013). The resulting best tree was visualized with midpoint-rooting in FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). We also calculated uncorrected *p*-distances and investigated the presence of autapomorphies using a subset of 100 individuals (Appendix 1) using MEGA11 (Tamura *et al.*, 2021). For detecting autapomorphies 33 individuals were removed since they had a sequence length shorter than 1140bp.

## RESULTS AND DISCUSSION

The principal component analysis (PCA) distinguished the *S. pyrenaicus* population from the Sado River basin from the populations of *S. tartessicus* and from the Tagus River basin populations of *S. pyrenaicus* (Fig. 1). The eigenvalues of the first two principal components, computed with the Burnaby-corrected matrix, accounted for the majority of the variance (Table 1). The eigenvectors with the highest values, and consequently the variables that exerted the most significant influence on the ordination in the PCA, were the preorbital length, the size of the anal fin (anal fin length and anal fin height) and the premaxillary length. The latter measurement was strongly influenced by the preorbital length. Therefore, the population from the Sado River basin exhibited smaller preorbital and premaxillary lengths compared to other studied populations; conversely, the size of the anal fin was greater in the Sado population.

The number of caniculate scales along the lateral line in the Sado population (LL:  $\bar{X}=41$ , 39-42;  $n=20$ ), scale rows above lateral line (SRA:  $\bar{X}=7$ , 6-7;  $n=20$ ) and scale rows below lateral line

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**Figure 2.** Infraorbital bones of *Squalius* populations under study: A, Sado population; B, *S. pyrenaicus* from Tagus drainage; and C, *S. tartessicus* from Type locality (Ciudadaja river, Guadalquivir drainage, Sevilla, Spain). po, preopercle. op, opercle. pro, preoperculum. lc, lacrimal. i2 2nd infraorbital. i3 3rd infraorbital. i4, 4th infraorbital. i5, 5th infraorbital. *Huesos infraorbitarios de las poblaciones estudiadas del género Squalius: A, población del río Sado. B, población de la cuenca del Tajo de S. pyrenaicus y C, población de S. tartessicus de la localidad tipo (River Ciudadaja, Guadalquivir Drainage, Sevilla, Spain). po, preopercular. op, opérculo. pro, preopérculo. lc, lagrimal. i2, segundo infraorbitario. i3, tercer infraorbitario. i4, cuarto infraorbitario. i5, quinto infraorbitario.*

(SRB:  $\bar{X}$ =3, 3-4; n=20) was the same as that of *S. pyrenaicus* from the Tagus River basin (LL:  $\bar{X}$ =41, 39-43; SRA:  $\bar{X}$  =7, 6-7; SRB:  $\bar{X}$  =3, 2-4; n=81). The number of scales was also similar to that of *S. tartessicus*, with the exception of the number of caniculate scales along the lateral line which was greater in the Sado population than that of *S. tartessicus* (LL:  $\bar{X}$ =39, 37-41, SRA:  $\bar{X}$  =7, 6-7; SRB:  $\bar{X}$ =3, 2-3; n=125).

### Osteological Features

The population of the Sado River basin presented wide infraorbital bones as in *S. pyrenaicus* and *S. tartessicus*. However, our examination of specimens from the Sado River basin revealed that their 2nd and 3rd infraorbital bones were slightly narrower compared to those of *S. pyrenaicus* specimens of same size, and distinctly narrower than those of *S. tartessicus*. The 3rd infraorbital, in medium-sized specimens, did not make contact with the preopercular bone in the Sado population, whereas it did in *S. pyrenaicus* from the Tagus River basin and in *S. tartessicus*. The 5th infraorbital was larger in the Sado population than in the other populations, extending nearly across the entire width of the 4th (Fig. 2).

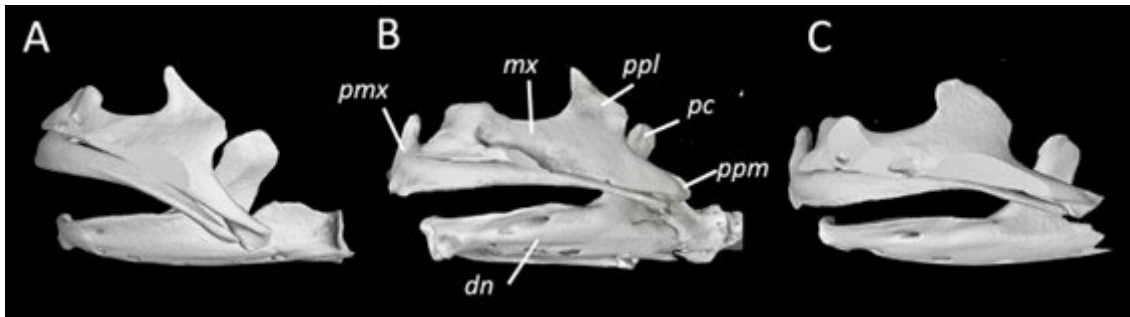
The ethmoid bone was similar to that of *S. pyrenaicus* from the Tagus River basin but narrower than in *S. tartessicus* (Appendix 2, see supple-

mentary information, available at <https://www.limnetica.net/en/limnetica>). This feature resulted in the head of *S. tartessicus* being wider and more robust than in the Sado population and *S. pyrenaicus* from the Tagus.

The maxilla exhibited the characteristic pointed anterior process, similar to *S. pyrenaicus* from the Tagus River and *S. tartessicus*, unlike other species in the genus *Squalius*, such as *S. malacitanus* and *S. gaditanus*. The palatine process of the maxilla in the Sado population was well-developed and its posterior process was narrow, as in *S. tartessicus*. The dentary bone had a short anterior process and a well-developed coronoid process. In general, the jaws of the Sado population differ from those of *S. pyrenaicus* from Tagus and *S. tartessicus* due to their shorter maxilla and dentary and with proportionally more developed coronoid process in the dentary (Fig. 3).

The pharyngeal teeth were arranged in two rows, the first with five teeth and the second with two smaller teeth, similar to most species in the genus *Squalius*. The shape of the teeth resembled that of *S. pyrenaicus* and *S. tartessicus*, with strong denticulations, a terminal hook, and a narrow masticatory surface. We found that the lower branch of the pharyngeal bone was longer and less robust in the Sado population than in *S. tartessicus* but identical to that of *S. pyrenaicus*. Nevertheless, all examined specimens from the





**Figure 3.** Oral jaws of *Squalius* populations under study: A, Sado population; B, *S. pyrenaicus* from Tagus River basin; and C, *S. tartessicus* from Type locality (Ciudadreja river, Guadalquivir River basin, Sevilla, Spain). mx, maxilla. pmx, premaxilla. dn, dentary. ppl, palatine process. pc, coronoid process. ppm, posterior process of the dentary. *Aparato mandibular anterior de las poblaciones estudiadas del género Squalius: A, población del río Sado. B, población de la cuenca del Tajo de S. pyrenaicus y C, población de S. tartessicus de la localidad tipo (Ciudadreja river, cuenca del Guadalquivir, Sevilla, Spain). mx, maxilar. pmx, premaxilar. dn, dentario. ppl, proceso palatino. pc, apófisis coronoidea. ppm, apófisis posterior del dentario.*

Sado population had a smaller pharyngeal lamina compared to the Tagus population of *S. pyrenaicus* and *S. tartessicus* (Appendix 3, see supplementary information, available at <https://www.limnetica.net/en/limnetica>).

### Genetic analyses

The phylogeny obtained for the mitochondrial MT-CYB gene (Fig. 4) is congruent with those obtained in previous studies for the same gene. It consists of two main clades: a) one comprising *S. torgalensis* and *S. aradensis* and b) a second clade that includes the remaining Iberian *Squalius* species. The second clade is further subdivided into two main groups: one formed by *S. carolitertii* and *S. castellanus* and another including *S. pyrenaicus*, *S. tartessicus*, *S. valentinus*, *S. gaditanus* and *S. malacitanus*. Within this clade, the highly divergent Sado lineage is placed as a sister lineage to *S. pyrenaicus* and *S. tartessicus*. Although previous studies performed using concatenated datasets of six or seven nuclear genes obtained different placements for the Sado lineage (often as a sister lineage to *S. tartessicus*, *S. valentinus*, *S. malacitanus* and *S. gaditanus*), the high differentiation of the Sado lineage is always recovered (Sousa-Santos *et al.*, 2019; Perea *et al.*, 2020, 2021). To address this mitonuclear discordance, we suggest that studies using whole genome data should be performed. Nevertheless, the high levels of differentiation of the Sado lineage are supported by both mitochondrial and nuclear markers

and its current classification within *S. pyrenaicus* is not supported by genetic data.

The uncorrected p-distances calculated based on the mitochondrial MT-CYB gene for the Iberian *Squalius* ranged from 0.013 to 0.119 (Appendix 4, see supplementary information, available at <https://www.limnetica.net/en/limnetica>). The overall patterns of differentiation obtained are consistent with those from previous studies: the highest levels of differentiation were found between *S. aradensis* and *S. torgalensis* and the remaining species (Almada & Sousa-Santos, 2010). Regarding the Sado lineage, the lowest levels of differentiation were found between the Sado lineage and *S. pyrenaicus* and *S. tartessicus* (0.025 and 0.024, respectively), followed by *S. valentinus* (0.032), which is consistent with the placement of the Sado lineage on the phylogenetic tree (Fig. 4). The results also showed intermediate levels of genetic differentiation between the Sado lineage and the species pairs *S. carolitertii*-*S. castellanus* (0.059 and 0.066, respectively) and *S. malacitanus*-*S. gaditanus* (0.089 and 0.079, respectively).

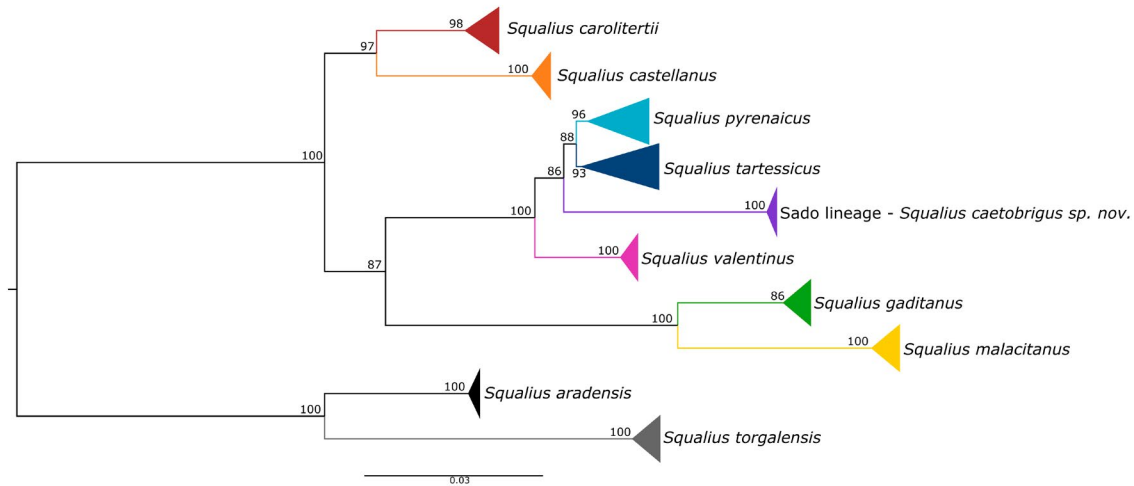
The Sado lineage had five autapomorphies in the mitochondrial MT-CYB gene, three of which are transversions (Appendix 5, see supplementary information, available at <https://www.limnetica.net/en/limnetica>).

### Taxonomy

The high degree of morphological and genetic



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**Figure 4.** Maximum likelihood phylogeny obtained for the MT-CYB gene. Bootstrap support is indicated above each branch. Terminal nodes are collapsed. *Filogenia de máxima verosimilitud obtenida para el gen MT-CYB. El soporte de bootstrap se indica encima de cada rama. Los nodos terminales están colapsados.*

differentiation of the *Squalius pyrenaicus* populations from the Sado River basin supports the hypothesis of considering this population as a distinct species and, therefore, it is newly described in the present study.

*Squalius caetobrigus* sp. nov. (Fig. 5, Table 2) urn:lsid:zoobank.org:act:C962DCE3-D1F5-4E33-9154-9A41250F0DEA

**HOLOTYPE:** MNHNC.MB05:003765. 104 mm SL, 119 mm TL; Grândola Stream, Sado River basin, Grândola, Setúbal, Portugal, 38.169591,-8.565635, 102 m.a.s.l., Leg. C. Sousa-Santos & S. L. Mendes, 3.IV.2023.

**PARATYPES:** MNHNC.MB05:003766, 19 specimens, Grândola Stream, Sado River basin, Setúbal, Portugal, 38.169591,-8.565635, 102 m.a.s.l., Leg. C. Sousa-Santos & S. L. Mendes, 3/IV/2023. MNCN\_ ICTIO 25412-15. 4 specimens, Xarrama River, Sado River basin, Alcáçovas, Portugal, 38.360452, -8.067796, 170 m.a.s.l., Leg. C. Almaça & M. J. Collares-Pereira, 9.IV.1979.

**DIAGNOSIS:** *Squalius caetobrigus* sp. nov. is a member of the Mediterranean clade of the genus *Squalius* (Sanjur et al., 2003; Perea et al., 2020). *Squalius caetobrigus* sp. nov. can be differentiated from all other known species of *Squalius* according to the following set of characters: 39-42 ( $\bar{X}$ =40.7;  $\tilde{X}$ =41; n=24) canaliculate scales on the lateral line; 6-7 ( $\bar{X}$ =7;  $\tilde{X}$ =7; n=24)

scales above the lateral line; 3-4 ( $\bar{X}$ =3.1;  $\tilde{X}$ =3; n=24) scales below the lateral line. Short preorbital distance short HL/PrOL is 4.2-6.2 ( $\bar{X}$ = 5). No contact between the fourth infraorbital bone and the preopercular. Short dentary with a very developed coronoid process. Posterior process of the maxilla is long and thin. The lower branch of the pharyngeal bone is long and thin. Small pharyngeal plate of the pharyngeal bone. *Squalius caetobrigus* sp. nov. is distinguishable from *S. tartessicus* by a higher number of canaliculate scales on the lateral line 39-42 ( $\bar{X}$ =41) vs 37-41 ( $\bar{X}$ =38,8); longer preorbital length 4.2-6.2 ( $\bar{X}$ = 5) vs 3.8-4.8 ( $\bar{X}$ = 4.3); no contact between third infraorbital bone and the preopercle vs contacting with the preopercle; long and thin lower branch of the pharyngeal bone vs short and robust; very reduced pharyngeal plate and not extended laterally vs extended laterally; short anterior process of the dentary vs large; narrow ethmoid bone vs wide; the darker dorsal pigmentation is reduced to one-third of the body and always above the lateral line vs extending halfway down the body, often reaching the lateral line. *Squalius caetobrigus* sp. nov. is distinguishable from *S. pyrenaicus* by a slightly longer preorbital length 4.2-6.2 ( $\bar{X}$ = 5) vs 4.2-6.7 ( $\bar{X}$ = 4.4); no contact between the third infraorbital bone and the preopercle vs contact with the preopercle; very reduced pharyngeal plate and not extended laterally vs extend-



**Figure 5.** Holotype of *Squalius caetobrigus* sp. nov. from the Grândola stream, Sado River basin, Grândola, Portugal. MNHNC. MB05:003765, SL=104 mm. Scale is 10 mm. *Holotipo de Squalius caetobrigus* sp. nov. del río Sado, cuenca del río Grândola, Cuenca del Sado, Grândola, Portugal. MNHNC.MB05:003765, SL=104 mm. Escala es 10 mm.

**Table 2.** Morphological variables used to define the morphometric and meristic characters of *S. caetobrigus* sp. nov. type series. Variables as described in Materials and Methods (SD = standard deviation). *Variables morfológicas utilizadas para definir los caracteres morfométricos y merísticos de la serie tipo de S. caetobrigus* sp. nov. Las variables son descritas en la sección de Material y Métodos (SD = desviación típica).

<i>Squalius caetobrigus</i> sp. nov.				
Variable	Holotype	Paratypes (n = 23)		
		Range	Mean	SD
TL	136.7	158.3-70	105.4	22.7
SL	119.3	133.4-59.5	90.6	19.6
PrDD	67.7	72.9-35.1	51.2	10.8
PrPD	31.4	32.8-15.3	22.6	4.4
PrVD	61.4	65.8-31.2	45.8	9.6
PrAD	84.2	90.7-42.4	62.8	13.5
APL	29.2	31.4-12.7	20.8	5.2
CPL	43.7	50.2-21	32.6	7.4
HL	31.0	30.4-13.8	22.4	4.5
PrOL	7.1	6.9-2.5	4.6	1.3
ED	7.5	7.4-4.1	5.7	0.9
PsOL	17.0	16.6-7.8	12.2	2.6
NL	24.1	24.2-10.9	16.9	3.4
HH	22.1	23.3-11.4	16.5	3.2
PmxL	10.7	10.2-3.9	7.1	1.6
PFL	19.5	22.2-9.8	16.5	3.4
VFL	20.0	20.3-8.8	14.4	3.3
DFL	11.9	14.7-6.3	10.1	2.4
DHL	21.3	22-10.5	15.3	3.1
AFL	11.8	14.2-6.7	10.1	2.0
AHL	15.3	17.1-8.1	12.4	2.4
CFL	19.5	27.1-11.8	17.0	3.8
BLD	13.0	15.3-6.8	10.4	2.3
BD	34.5	37.6-16	24.7	5.7
LLS	41	42-39	40.7	0.7
SRA	7	7-6	7	0.2
SRB	3	4-3	3.1	0.3
D	8	8	8	0
A	8	8	8	0

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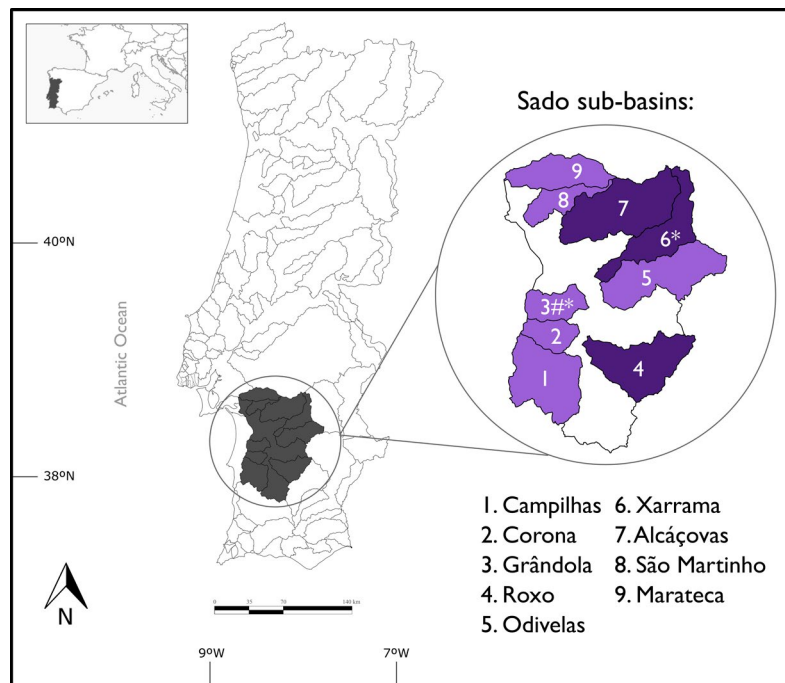
ed laterally; short anterior process of dentary vs large; wide coronoid process of the dentary bone vs thin; a conspicuous darker dorsal pigmentation on one-third of the body and always above the lateral line vs absence of a conspicuous darker dorsal pigmentation. Genetic distances from the other species of *Squalius* inferred from the mitochondrial MT-CYB gene were: 2.5% with respect to *S. pyrenaicus* and 2.4% with respect to *S. tartessicus* (Appendix 4). The new species has 5 autapomorphies, 3 of which are transversions in the MT-CYB gene (positions 336, 522 and 810; Appendix 5).

**DESCRIPTION:** D III (II) = 8; A III (II) = 8; P I 14; V I 8; C= 17; LLS= 41 (39-42); SRA= 7 (6-7); SRB= 3.1 (4-3); RPT 5.2 LPT 5.2. Morphometric and meristic characters of the type material are given in Table 2. A medium-sized species that rarely reaches 200 mm of standard length. The head is short with the mouth terminal or subterminal and SL/HL is 3.7-4.4 ( $\bar{X}$  = 4).

The head length is similar to the height maxima of the body and BD/HL is 0.9-1.2 ( $\bar{X}$ = 1.1). The preorbital distance is short, smaller than in *S. tartessicus* and *S. pyrenaicus* and HL/PrOL is 4.2-6.2 ( $\bar{X}$ = 5). The caudal peduncle is low and CPL/BLD is 2.7-3.6 ( $\bar{X}$ = 3.2). The minimum body depth is 2.2-2.7 ( $\bar{X}$ = 2.4) times smaller than the maximum body depth. The ventral fins are inserted approximately at the same level of the origin of the dorsal fin and PrDD/PrVD is 1-1.2 ( $\bar{X}$  = 1.1). Fins are large. Small nuptial tubercles present in males.

**PIGMENTATION PATTERN:** The body is silver with a dark grey coloration in the dorsal portion, clearly visible on one-third of the body and always above the lateral line in all specimens. The scales have one big black spot on the base and a series of small black spots on the distal border. Fin rays are dark grey.

**ETYMOLOGY:** The species name caetobrigus is derived from the Roman name of the the present-day city of Setúbal (Caetobriga), the most



**Figure 6.** Distribution of *Squalius caetobrigus* sp. nov. from the Sado River basin, Portugal. Sub-basins of the Sado coloured in a lighter shade correspond to those where the individuals for the genetic analysis were sampled. # indicates the sampling location of the holotype. \* indicates the sampling locations of the paratypes. *Distribución de Squalius caetobrigus* sp. nov. de la cuenca del río Sado, Portugal. Las subcuencas del Sado coloreadas en un tono más claro corresponden a aquellas en las que se tomaron muestras de los individuos para el análisis genético. # indica el lugar de muestreo del holotipo. \* indica el lugar de muestreo de los paratipos.

important locality in the Sado River basin.

**DISTRIBUTION:** This new species is endemic of Sado River basin in Portugal (Fig. 6).

**COMMON NAMES:** Escalo do Sado, Sado Chub.

**REMARKS:** The species typically inhabits rivers with a Mediterranean typology conditioned by severe water stress during the summer, with specimens of *S. caetobrigus* surviving in disconnected pools until the rainy season. Anthropogenic threats to this species include poor water quality due to pollution and habitat degradation, as well as the introduction and proliferation of invasive species. Severe and prolonged droughts due to climate change, coupled with water abstraction for agriculture, also pose a challenge to the survival of the species, as the disconnected pools where it survives throughout the increasingly long-lasting summers become smaller and further apart, with some drying out completely during more severe droughts. The species is sympatric with other fish species including *Iberochondrostoma lusitanicum*, *Luciobarbus bocagei*, *Pseudochondrostoma polylepis* and *Lampetra lusitanica*, among others, which face similar environmental and anthropogenic pressures in the Sado River basin.

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## AUTHOR CONTRIBUTIONS

SLM: Data curation; Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – Reviewing and Editing; SP: Methodology, Writing – Reviewing and Editing; VCS: Methodology, Writing – Reviewing and Editing, Funding acquisition; CSS: Conceptualization, Data curation; Visualization, Writing – Reviewing and Editing, Funding acquisition; ID: Conceptualization, Data curation, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – Reviewing and Editing, Funding acquisition.

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