

Description of three new species of the genus *Cobitis* L., 1758 (Actinopterygii, Cobitidae) in the Iberian Peninsula

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ABSTRACT

Description of three new species of the genus *Cobitis* L., 1758 (Actinopterygii, Cobitidae) in the Iberian Peninsula

Three new species, *Cobitis almadae* sp. nov., *Cobitis atlantica* sp. nov., and *Cobitis mellaria* sp. nov. are described on the basis of morphological and genetic traits. *Cobitis almadae* sp. nov. is restricted to the Sizandro Drainage in Portugal and can be distinguished from other *Cobitis* species through a combination of morphometric and genetic traits including large and low peduncle depth, lateral ethmoid (suborbital spine) well developed with long narrow mediocaudal, laterocaudal and mediorostral processes, an elongated and narrow frontoparietal fontanel and a wide third Gambetta's zone sprinkled with numerous black spots. Furthermore, two autapomorphies are found within its mitochondrial cytochrome b gene. *Cobitis atlantica* sp. nov. inhabits the northern Atlantic rivers of the Iberian Peninsula from the Minho Drainage to the Alcoa Drainage and can be differentiated from other *Cobitis* species through a set of morphometric and genetic traits including short and high peduncle depth, a well-developed lateral ethmoid (suborbital spine) with short and wide mediocaudal, laterocaudal and mediorostral processes, wide frontoparietal fontanel, and developed ventral pigmentation in adult individuals. In females, the Gambetta's fourth row has 10-16 blotches reaching the ventral pigmentation in the caudal region and the third Gambetta's zone is narrow with black spots. *Cobitis mellaria* sp. nov. inhabits the Valle Drainage in southern Spain and is distinguished from other *Cobitis* species through the following morphometric and genetic traits: low peduncle depth, lamina circularis with convex outer edge, lateral ethmoid (suborbital spine) with short laterocaudal process and large mediorostral process, elongated frontoparietal fontanel, no ventral pigmentation in adults. In females, Gambetta's fourth row has 10-15 blotches. One autapomorphy is found within the mitochondrial cytochrome b gene of *Cobitis mellaria* sp. nov.

Key words: taxonomy, Iberian Peninsula, *Cobitis*, Cypriniformes, Cobitidae, genetics, morphology

RESUMEN

Descripción de tres nuevas especies del género *Cobitis* L., 1758 (Actinopterygii, Cobitidae) en la península Ibérica

Se describen tres nuevas especies del género *Cobitis*: *Cobitis almadae* sp. nov., *Cobitis atlantica* sp. nov., y *Cobitis mellaria* sp. nov. mediante caracteres morfológicos y genéticos. *Cobitis almadae* sp. nov. está restringida a la cuenca del Sizandro en Portugal y se puede diferenciar de otras especies del género *Cobitis* a través de una combinación de caracteres morfométricos y genéticos que incluyen un pedúnculo largo y estrecho, etmoides lateral (espina suborbitaria) bien desarrollado con apófisis mediocaudales, laterocaudales y mediorostrales largas y estrechas, fontanela frontoparietal alargada y estrecha, la tercera zona de Gambetta es ancha y se encuentra salpicada de numerosas manchas negras. Además, tiene dos autapomorfias en su citocromo b. *Cobitis atlantica* sp. nov. vive en los ríos que vierten al Atlántico norte de la Península Ibérica, desde la cuenca del Miño hasta la cuenca del río Alcoa y se puede diferenciar de otras especies del género *Cobitis* a través de un conjunto

de caracteres morfométricos y genéticos que incluyen un pedúnculo corto y alto, etmoides lateral (espina suborbitaria) bien desarrollada con apófisis mediocaudales, laterocaudales y mediorostrales cortas y anchas, fontanela frontoparietal ancha, pigmentación ventral presente en los individuos adultos. En las hembras, la cuarta fila de Gambetta tiene 10-16 manchas que alcanzan la pigmentación ventral en la región caudal y la tercera zona de Gambetta es estrecha con algunas manchas negras. *Cobitis mellaria* sp. nov. vive en la cuenca del río Valle en el sur de España y se puede distinguir de otras especies del género *Cobitis* a través de una combinación de caracteres morfométricos y genéticos: pedúnculo caudal bajo, lámina circular con su borde exterior convexo, etmoides lateral (espina suborbital) con apófisis laterocaudal corta y apófisis mediorostral muy larga, fontanela frontoparietal alargada, sin pigmentación ventral en los adultos, en las hembras la cuarta fila de Gambetta tiene 10-15 manchas. *Cobitis mellaria* sp. nov. presenta una autapomorfía en el citocromo b.

Palabras clave: taxonomía, península ibérica, *Cobitis*, Cypriniformes, Cobitidae, genética, morfología

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INTRODUCTION

From a phylogenetic and biogeographical perspective, the freshwater ichthyofauna of the Iberian Peninsula should be considered as island fauna. This is due to its almost complete isolation from other fauna since the opening of the Strait of Gibraltar 5.33 MYA (Krijgsman et al., 2018). Only a few contacts between the fauna of southern France and northern Spain occurred during the drop in sea level caused by the Plio-Pleistocene glaciations (Corral-Lou et al., 2019). This is not surprising and for this reason the Iberian ichthyofauna is one of the most endemic in Europe and this endemism continues to be described with the development of genetic and morphological techniques.

The three most diversified families of freshwater fishes in the Iberian Peninsula are: Cyprinidae, Leuciscidae and Cobitidae. Although there are numerous taxonomic studies on Cyprinidae and Leuciscidae (e.g. Robalo et al., 2005; Doadrio & Carmona, 2003; Doadrio & Elvira, 2007; Doadrio et al., 2007), the Cobitidae family is still the least studied from a taxonomic perspective. This family Cobitidae is represented in the Iberian Peninsula by the genus *Cobitis*.

With around 96 species distributed mainly across Eurasia and North Africa, the genus *Cobitis* is one of the most diversified genera of freshwater fishes (Fricke et al., 2022). Molecular studies combined all of the Iberian and North African species of *Cobitis* in the *Iberocobitis* Bacescu, 1962 subgenus (Perdices & Doadrio, 2001). Within this subgenus the following four species are currently recognized: *Cobitis caldero-*

ni Bacescu, 1962; *C. maroccana* Pellegrin, 1929; *C. paludica* (de Buen, 1930) and *C. vettonica* Doadrio & Perdices, 1997 (Perdices & Doadrio, 2000, 2001; Doadrio & Perdices, 2005; Tang et al., 2008) (Fig 1).

Cobitis calderoni inhabits mainly the Douro and Ebro basins in the northern and central regions of the Iberian Peninsula with a single population confined in the Tagus basin (Doadrio, 1981; Doadrio 2002; Leunda et al., 2007; Doadrio et al., 2011; Perea et al., 2011). This is a singular species within the *Iberocobitis* lineage characterized by the absence of sexual dimorphism (Perdices & Doadrio, 1997; Tang et al., 2008). The separation of *C. calderoni* from other *Iberocobitis* species occurred around the Miocene 12-17 MYA when endorheic basins were present across the Iberian Peninsula (Doadrio & Perdices, 2005).

Cobitis maroccana is an endemic species to Morocco. The species inhabits the Loukos and Sebou drainages located along the Atlantic coast of the Strait of Gibraltar. Freshwater fish fauna interchange between North Africa and the Iberian Peninsula was interrupted by the opening of the Gibraltar Strait 5.33 MYA (Krijgsman et al., 2018) and for this reason the separation of *C. maroccana* from Iberian *Cobitis* species should have occurred at least since 5.3 MYA.

The species *Cobitis vettonica* inhabits a small region in western Spain including the Douro and Tagus drainages and in some small adjacent rivers in Portugal (Sousa-Santos et al., 2014; Perdices & Coelho, 2020). Unlike its sister species *C. paludica*, *C. vettonica* prefers upstream rivers and extensive hybridization between these two species

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has been reported along low stretch rivers of the Tagus basins (Corral-Lou et al., 2022). The divergence of *C. vettonica* and *C. paludica* was calibrated to 5.3 ± 1.6 MYA, i.e., which was the end of the Miocene and Pliocene (Perdices & Doadrio, 2005).

The fourth species belonging to the subgenus *Iberocobitis* is *Cobitis paludica*. This is a widespread species, native to the central and southern regions of the Iberian Peninsula and introduced by fisherman to bodies of water along northern and southeastern Spain (Muñoz, 1993; Doadrio et al., 2011; Perea et al., 2011; Verdiel-Cubedo et al., 2012; Márquez-Rodríguez, 2014; Sánchez-Hernández et al., 2018). This wide distribution area is uncommon in Iberian freshwater fishes and high morphology and genetic diversity has been recorded in all studies regarding *C. paludica* (Perdices et al., 1995; Doadrio & Perdices, 1997; Doadrio & Perdices, 2005; Corral-Lou et al., 2022).

Two other species of the genus *Cobitis* have been cited in the Iberian Peninsula: *Cobitis haasi* Klausewitz, 1955 and *Cobitis victoriae*.

Cobitis haasi was described in the Albufera of Valencia, in the Spanish Mediterranean coastline and synonymized with *C. paludica* with the support of allozyme studies (Perdices et al., 1995). Nevertheless, no further molecular studies have included this population until now. Morphological traits were done in Klausewitz (1955) and this species is characterized by the dotted pattern of pigmentation on the body and a very reduced caudal upper spot (Fig.1).

The taxonomic status of *Cobitis victoriae* was uncertain and we clarify its taxonomic position in the results and discussion section of this article.

Taxonomic problems in the subgenus *Iberocobitis* have already been reported by a previous molecular study by Doadrio & Perdices (2005). These authors found, on the one hand, that one population close to the Gibraltar Strait located in the Valle Drainage in Spain is phylogenetically closer to *C. maroccana* from Morocco than to the Iberian populations recognized as *C. paludica* within the Valle Drainage. On the other hand, *Cobitis* populations inhabiting drainages close to

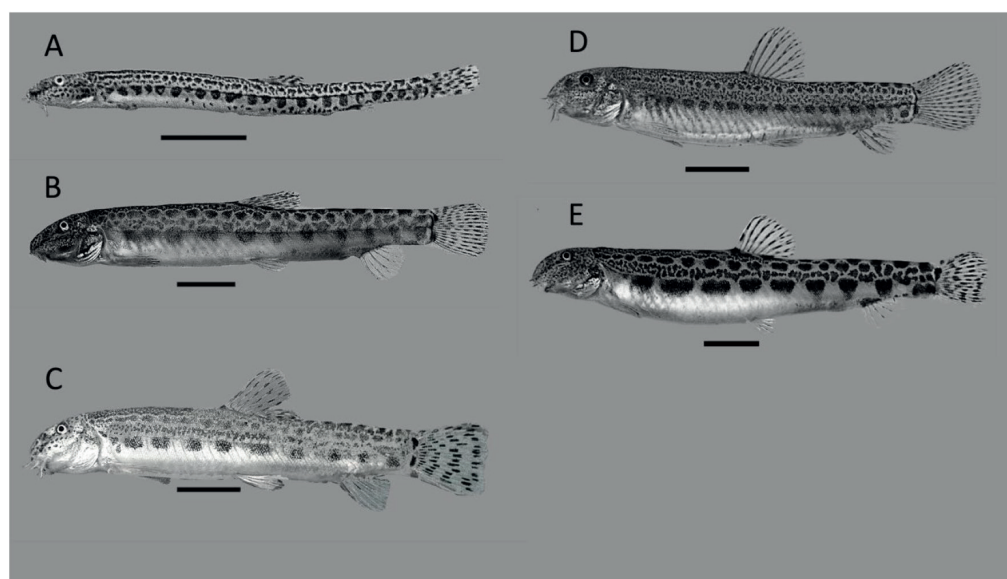


Figure 1. Described *Cobitis* species in the Iberian Peninsula and North Africa. A: *C. calderoni* from Sil River, Minho Drainage. B: *C. paludica* from Alardos River to 40 km of the type locality in Tagus Drainage. C: *C. maroccana* from Loukos River, Loukos Drainage. D: *C. haasii* from Serpis River, Serpis Drainage. E: *C. vettonica* from Angeles River, Tagus Drainage. Scale represents 10 mm. *Especies descritas del género Cobitis en la Península Ibérica y Norte de África. A: C. calderoni del río Sil, cuenca del Minho. B: C. paludica del río Alardos a 40 km de la localidad tipo en la cuenca del Tagus. C: C. maroccana del río Loukos, cuenca del Loukos. D: C. haasii del río Serpis, Cuenca del Serpis. E: C. vettonica del río Angeles, Cuenca del Tagus. La escala representa 10 mm.*

Lisbon were genetically more distant to *C. paludica* within the cytochrome b gene than to *C. marroccana* from Morocco and *C. paludica* from the Iberian Peninsula (Doadrio & Perdices, 2005).

In this paper, two new *Cobitis* species are described corresponding to populations previously recognized as *C. paludica* from the Valle Drainage in Gibraltar Strait and Lisbon drainages. Additionally, a description of the Northwestern Iberian Peninsula populations of *C. paludica* is provided, as well as a replacement of the name *Cobitis victoriae*. These three taxonomic entities are geographically distant, and display high morphological and genetic differentiation.

MATERIALS AND METHODS

The description of the three new *Cobitis* species was based on the analysis of 70 individuals: 18 from Valle River (Valle Drainage), Spain; 26 from the Sizandro River (Sizandro Drainage), Portugal; 11 from Alcoa River (Alcoa Drainage) Portugal; 9 from Tea River (Minho drainage) Spain and 6 from Macaco River (Limia Drainage), Spain.

Since no type specimens of *C. paludica* are known, for comparative morphometric purposes we examined 3 individuals collected in 1978 by the first author, from the small spring where this species was originally described (type locality): Fuente del Roble, Talayuela, Cáceres, Spain. No additional specimens could be studied because this species does not occur in this spring anymore, although this spring does not currently connect with the adjacent Tiétar River, it was probably able to do so during flooding periods of the Tiétar River. For this reason, we also studied 7 specimens from the adjacent Tiétar River (Tagus Drainage).

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

Morphology

Twenty-two morphometric measurements (in mm) and three meristic variables were recorded from digital photographs using TpsDig v.1.4 (Rohlf, 2003). The following abbreviations were used for morphometric and meristic characters: TL, total length; SL, standard length; HL, head

length; PrOL, preorbital length; ED, eye diameter; PsOL, postorbital length, IO, interorbital distance; PrDD, predorsal distance; PrPD, prepectoral distance; PrVD, preventral distance; PrAD, preanal distance; CPL, caudal peduncle length; APL, anal peduncle length; DHL, dorsal fin height; PFL, pectoral fin length; VFL, ventral fin length; AFL, anal fin length; AHL, anal fin height; CFL, caudal fin length; BD, body depth; BLD, body least depth. AVD, distance between anal and ventral fins; Vr, number of vertebrae. D, number of dorsal fin rays; A, number of anal fin rays;. Count of fin rays is shown in Appendix 1 (see Supplementary information available at <https://www.limnetica.net/en/limnetica>). 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 represent the shape in order to describe the morphology of the new species we calculated different ratios. For the morphology of the caudal peduncle, we used the ratios of APL/BLD and CPL/BLD to quantify the depth. Due to the head being the most complex structure we included different ratios that represent its relative size with respect to its body: SL/HL and BD/HL, and the length of preorbital and postorbital distances: PrOL/ED and PsOL/SL. Only the ratios of caudal peduncle depth were used as diagnostic traits and to construct the identification key for the Iberian species.

To identify the variables that contributed most to the variation among populations, one principal component analyses (PCA) were performed using the covariance matrix for morphometric characters. Statistical analyses were carried out using PAST software (Hammer *et al.*, 2001). The pigmentation pattern followed Gambetta (1934) who defined four different zones in the body of the species of the genus *Cobitis*. Each of these zones was characterized by having a different pattern in the shape and size of the spots, as is shown in Appendix 2. (see Supplementary information available at <https://www.limnetica.net/en/limnetica>).

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Due to the sexual dimorphism of *Cobitis paludica* (Doadrio et al., 2002; Doadrio et al., 2011), we removed fin size to subsequent morphological analyses.

Osteological characteristics were investigated through computer tomography (CT) scan and digital dissection using VGStudio MAX v2.2 (Volume Graphics, <http://www.volumegraphics.com>) and dry skeletons preserved in the MNCN_ ICTIO collections.

Institutional acronym: MNCN Museo Nacional de Ciencias Naturales, Madrid, Spain.

Genetic analyses

Two hundred and thirteen specimens from different populations of genus *Cobitis* were analysed (Appendix 3, (see Supplementary information available at <https://www.limnetica.net/en/limnetica>)). In these analyses, we examined sequences for the entire mitochondrial cytochrome b gene (*MT-CYB*), using previously published data deposited in GenBank and new sequences data obtained from the DNA and Tissue Collection and Biobank at the MNCN-CSIC. The new sequences were deposited in the GenBank data base under the Accession Numbers OQ872589 - OQ872768 (Appendix 3).

Total genomic DNA was extracted from fin-clip tissue using the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA), following the manufacturer's protocol.

For each specimen, the complete *MT-CYB* region (1140 bp) was amplified. Primers and protocols used for PCR for *MT-CYB* amplification followed Corral-Lou et al (2022). PCR products were purified with ExoSAP-IT (USB Cleveland, OH, USA) and then sequenced on a 3730xl DNA Analyzer by Macrogen Europe Inc. (<http://www.macrogen.com>). Two different phylogenetic analyses were performed using Bayesian inference (BI) implemented in MrBayes v. 3.2 (Ronquist et al., 2012) and Maximum Likelihood implemented in the IQ-tree online web server from the Vienna University (<http://iqtree.cibiv.univie.ac.at>; Trifinopoulos et al., 2016). Model-Finder, 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 5×10^7 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). One sequences of *Cobitis calderoni* was used as outgroup (Appendix 3). Patristic distances among *Cobitis* populations were calculated for the *MT-CYB* gene using Geneious 10.1.3 (<http://www.geneious.com>; Kearse et al. 2012). Only nodes with a posterior probability (pp) of 0.95 and Bootstrap of 70, or higher, in at least one analysis are considered as statistically supported.

To assess the phylogeographic structure among the haplotypes which are present in several separated basins, haplotype networks were reconstructed using the Median-joining algorithm (Bandelt et al., 1999) as implemented in the program PopArt (Leigh & Bryant, 2015).

RESULTS AND DISCUSSION

Taxonomic review of *Cobitis victoriae*

Cobitis victoriae was mentioned for the first time in 1996 in the unpublished proceedings of a symposium (without a publication identifier code) which referred to a single population from the Minho Drainage (Fernández de la Cigoña & García-Ferreira, 1996). For this reason and following the Code of Nomenclature [Code art. 9.9] we consider that this name constituted an unavailable name which was further indicated by other authors (Silva et al., 2010). Kottelat (2012) also considers the original description of the name as not available because the name *Cobitis victoriae* due to the fact that this name, *Cobitis vitoriae* (misspelling of *Cobitis victoriae*), was conditionally proposed after 1960 [Code art. 15.1]. However, Kottelat (2012) considers the name available because the references made by C. Antunes in the preface of a posterior book (Fernández de la Cigoña, 1999,



Figure 2. Morphological differences between autochthonous and allochthonous specimens of the genus *Cobitis* in the Minho Drainage. A: specimen autochthonous from Tea River, Minho Drainage. B: specimen allochthonous from Sil River, Minho Drainage. Pd: caudal peduncle. Vertical white bar: peduncle depth. Horizontal white bar: anal peduncle length. Scale represents 10 mm. *Diferencias morfológicas entre individuos autóctonos y alóctonos del género Cobitis en la Cuenca del Minho. A: individuo autóctono del río Tea, cuenca del Minho. B: individuo alóctono del río Sil en la cuenca del Minho. Pd: pedúnculo caudal. Barra blanca vertical. Altura del pedúnculo caudal. Barra blanca horizontal: longitud del pedúnculo caudal anal. La escala representa 10 mm.*

legal deposit Vg 729/99) in which Antunes indicates that Fernández de la Cigoña named *Cobites vicroriae* (misspelling of *Cobitis victoriana*), as the loaches of Galicia and further validated by the figure 15 represented on page 21 of Fernández de la Cigoña (1999). The description made by Fernández de la Cigoña (1999) does not permit the differentiation between *C. victoriana* and the other species belonging to the genus *Cobitis*. The traits indicated by Fernández de la Cigoña (1999) were: size, presence of six barbels, elongated body, and brown color, which cannot be considered a valid description of the species within the genus *Cobitis* because these are common traits for almost all of the *Cobitis* species [Code art. 15.1].

The type locality of *C. victoriana* was an artificial lagoon in Caldelas (Galicia) where the authors also cited the presence of *C. calderoni* (Fernández de la Cigoña & García-Ferreira, 1996). This environment in the Minho Drainage and in other areas of the Iberian Peninsula are common areas for the introduction of exotic species (Radinger & García-Berthou, 2020). Thus, the presence of *C. calderoni* in the Minho Drainage

was associated with one introduced population of *C. paludica* near to the Rozas Reservoir where both species could have been introduced artificially as bait for sport fishing (Perea *et al.*, 2011). In this way, the drawing depicted in Fernández-Cigoña (1999) showed the features of the southern populations of *C. paludica* with a longer and lower caudal peduncle and a less robust body than the native populations of the Minho Drainage (see features of the autochthonous Minho population in Results and Discussion of this manuscript and Fig. 2).

Subsequently, the distribution of *C. victoriana* in the Minho Drainage was extended (Fernández-Cigoña & Oujó, 1999, legal deposit Vg 943/99) to three more localities, once again without reference to its diagnostic characteristics, only size was referred to and one drawing along with two bad quality photos are shown. However, in the new localities cited the autochthonous population exists in the Minho Drainage, but the absence of diagnostic traits and of data related to the existence of preserved specimens do not permit the separation of the autochthonous from the

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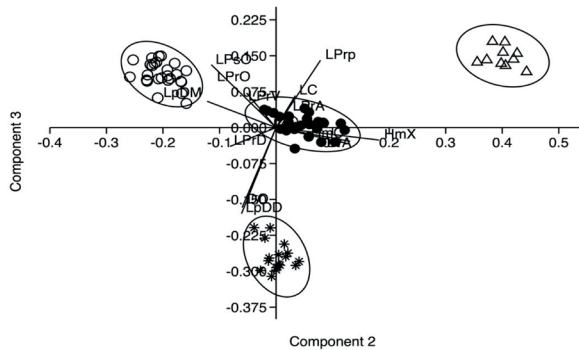


Figure 3. Variables that most contributed to the PCA analysis. Dots, the northwestern drainages populations from Iberian Peninsula. Triangles, Population of the type locality. Circles, Sizandro Drainage population. Stars, Valle River Drainage population. Variables that most contributed to the analysis of PCA. Puntos, poblaciones de las cuencas del noroeste de la Península Ibérica. Triángulos, Población de la localidad tipo. Círculos, población de la cuenca del Sizandro. Estrellas, Población de la cuenca del río Valle.

Table 1. Eigenvalues and eigenvectors for the first three principal components (PC1-PC3) of 14 morphometric variables for all *Cobitis* populations. Acronyms are defined in the Material and Methods section. In bold, variables with the highest eigenvectors for each principal component. Eigenvalores y eigenvectores para los tres primeros componentes principales (PC1-PC3) para 14 variables morfológicas de todas las poblaciones estudiadas del género *Cobitis*. Los acrónimos se describen en la sección de Material y Métodos. En negrita, se señalan las variables con los valores más altos para cada componente principal.

Variables	PCI	PCII	PCIII
Eigenvalue	0.6504	0.0400	0.0246
% variance	90.4	5.55	3.42
Eigenvectors			
SL	0.2925	-0.0396	-0.0056
HL	0.1311	0.0930	0.1891
PrOL	0.2210	-0.1425	0.2262
ED	0.2028	-0.1943	-0.4694
PsOL	0.2128	-0.2967	0.4146
PrDD	0.2855	-0.2375	-0.0970
PrPD	0.1476	0.2309	0.4058
PrVD	0.2971	0.0025	0.1930
PrAD	0.28521	0.0563	0.0951
CPL	0.3098	-0.1929	-0.5034
APL	0.4010	-0.3669	0.1335
BLD	0.3193	0.4392	-0.0878
BD	0.2726	0.4993	-0.0967
AVL	0.2368	0.3416	-0.1101

introduced specimens of the Minho Drainage in the studies done on *C. victoricae*.

For these reasons we consider *C. victoricae* not available and junior synonym of *C. paludica* as indicated in Kottelat & Freyhoff (2007).

Comparison of Morphology Among Populations

The principal components analysis divided the *Cobitis* populations into four groups corresponding to the populations of a) drainages of the northwest of the Iberian Peninsula, b) Sizandro Drainage, c) Valle Drainage, d) *Cobitis paludica* of the type locality (Fig. 3). The first principal component explained 90.4 % of the variance (I) but all the eigenvectors had similar values and the same sign that are explained by the different body size of the individuals in the different populations studied (Table 1). Therefore, the first principal component was not considered. Principal components two and three divided the populations into four groups with size corrected as shown by the values of the eigenvectors (Table 1). For the second principal component, the least body depth and the length of the anal peduncle and for the third principal component the length of the caudal peduncle and the orbital distance were the variables that best explained the partition of the variance in the principal component analyses (Table 1).

The northwestern population specimens had a body shorter and deep and APL/BLD was $\bar{x} = 1.4-0.7$. In the opposite side, the individuals from Sizandro Drainage had a longer and lower caudal peduncle therefore the index APL/BLD was higher $\bar{x} = 2.1-1.8$. Populations of Valle Drainage and type locality of *C. paludica* had intermediated values (Valle $\bar{x} = 1.4-1.2$ and Tiétar $\bar{x} = 1.5-1.4$).

Osteology Features

The osteological analyses revealed a clear differentiation of the population from northwestern of the Iberian Peninsula, that presented a wider interorbital distance and a shorter and wider frontoparietal fontanelle (Appendix 4, (see Supplementary information available at <https://www.limnetica.net/en/limnetica>)).

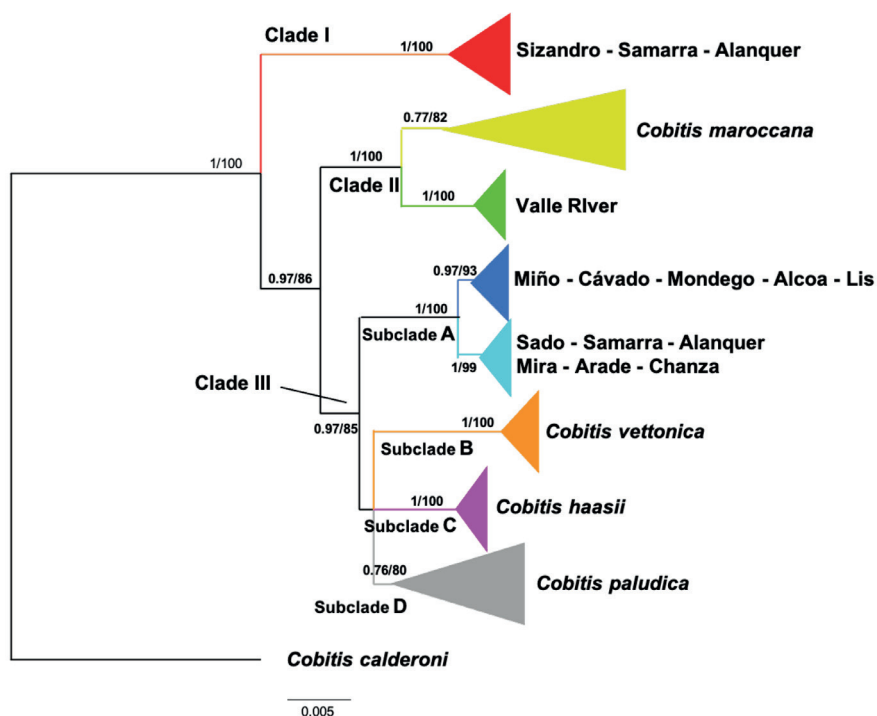


Figure 4. Phylogenetic tree rendered by Maximum Likelihood and Bayesian Inference based on *MT-CYB* gene, with terminal nodes collapsed. Numbers on branches indicate posterior probability (before slash) and bootstrap (after slash) values. *Árbol filogenético generado por Máxima Verosimilitud e Inferencia Bayesiana a partir del gen MT-CYB con los nodos terminales colapsados. Los números en las ramas indican valores de probabilidad posterior (antes de la barra) y de bootstrap (después de la barra).*

Additionally, the lateral ethmoid (suborbital spine) was also more robust in northwestern population with a mediocaudal and mediorostral processes clearly wider than in the remaining populations studied (Appendix 5, (see Supplementary information available at <https://www.limnetica.net/en/limnetica>). The population from Valle Drainage had a lateral caudal process very small and long and narrow mediorostral process (Appendix 5).

The *lamina circularis* of the males was different in the four populations studied (Appendix 6, (see Supplementary information available at <https://www.limnetica.net/en/limnetica>)). In the males from Sizandro Drainage an elongated and large *lamina circularis* with large and narrow handle was present. Northwestern population males had a smaller *lamina circularis* than those from Sizandro, with a widest handle. Proportionally to the *lamina circularis* the population from Valle Drainage had the largest handle.

The number of vertebrae was more numerous in Sizandro population (4+36-38+1) than in the other populations (4+35-36+1).

Genetics

Phylogenetic analyses based on the *MT-CYB* gene were congruent with previous studies (Doadrio & Perdices 2005) and supported three main groups in the phylogenetic tree (Fig. 4, Appendix 7, (see Supplementary information available at <https://www.limnetica.net/en/limnetica>)).

Clade I corresponded to the population of the Sizandro drainage together with the geographically adjacent populations of the Samarra Drainage and Alanquer River a small tributary at the mouth of the Tagus Drainage. Clade II grouped the populations of *C. maroccana* from North Africa and the population from the Valle Drainage located on the European side of the Gibraltar Strait. Clade III

Three new species of the genus *Cobitis*

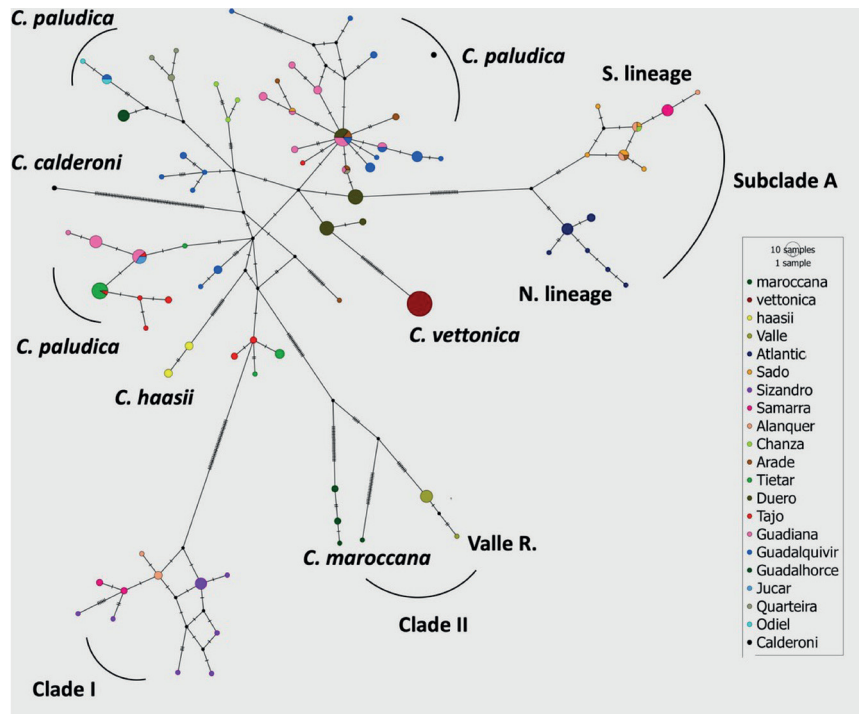


Figure 5. Haplotype network of *Cobitis* populations based on *MT-CYB* gene. Bars reflect mutational steps between haplotypes. Each circle represents a haplotype, and its size is proportional to its frequency. *Red de haplotipos de las poblaciones del género Cobitis basada en el gen MT-CYB. Las barras reflejan los pasos mutacionales entre haplotipos. Cada círculo representa un haplotipo, y su tamaño es proporcional a su frecuencia.*

clustered the remaining populations. Within Clade III four subclades were supported which relationships were not resolved. Subclade A grouped populations of the Atlantic basin from the Iberian Peninsula from the Minho Drainage in the north to the Arade Drainage in the south. Two lineages can be defined in Subclade A: the northern lineage from the Minho to the Alcoa Drainage in Portugal and the southern lineage that includes populations from the Sado to the Arade Drainage and haplotypes from other populations belonging to other clades as the Samarra Drainage, Alanquer River (Tagus Drainage) and some rivers of the lower basin of the Guadiana Drainage. Subclade B was composed by individuals assigned to *C. vettonica*. Subclade C grouped the populations of the Mediterranean basins (Turia and Serpis) where *Cobitis haasii* was described (Klausewitz, 1955). Previous genetic studies of *C. haasii* were carried out on allozymes and failed to differentiate the

populations of *C. haasii* from those of *C. paludica*. However, our phylogenetic study points to the validity of *C. haasii*.

Subclade D clustered all the populations previously assigned to *C. paludica* included those from the type locality.

Patristic distances (Appendix 8, (see Supplementary information available at <https://www.limnetica.net/en/limnetica>)) between populations belonging to Clade I (Sizandro – Alanquer – Samarra) and the rest of populations ranged from 4.2 % (*C. paludica* and population from Sado Drainage) to 6.1 % (*C. maroccana*). The same distances between populations from the Valle Drainage and the remaining populations ranged from 3.5 % (*C. paludica* and population from Sado Drainage) to 5.2 % (Clade I). When involving comparisons between populations of northern subclade A and the rest of populations distances ranged from 2.4 % (*C. paludica* and Sado Drainage) to 5 % (*C.*

maroccana). In the case of *C. haasii* from Turia and Serpis drainages the range of genetic distances was 1.9 % (*C. paludica* and Sado Drainage) to 4.8 % (Clade I and *C. maroccana*).

The haplotype network (Fig. 5), revealed that the populations from the Sizandro and Valle drainages only had haplotypes from their own clades. The same is true for the haplogroup grouping the populations from the Subclade A (Minho - Cávado - Mondego - Alcoa - Lis drainages). In contrast, recurrently populations at the edge of the distribution of each clade shared haplotypes

with other different clades. This is the case of Samarra and Arade drainages; Chanza River, in the lower Guadiana Drainage, and Alanquer River in the lower Tagus Drainage.

TAXONOMY

DESCRIPTION OF *COBITIS* POPULATIONS

The high degree of morphological and genetic differentiation of *Cobitis* populations endemic to Sizandro and Valle drainages and from Western

Table 2. Statistical variables used to define the morphometric and meristic characters of *Cobitis almadae* sp. nov. type series. Variables as described in Methods (SD = standard deviation). *Variables utilizadas para definir los caracteres morfométricos y merísticos de la serie tipo de Cobitis almadae* sp. nov. Las variables son descritas en la sección de métodos (SD = desviación estándar).

<i>Cobitis almadae</i> sp. nov.							
Variable	Holotype	Paratypes	Males	N=10	Paratypes	Females	N=15
		Range	Mean	SD	Range	Mean	SD
SL	68.2	54.1-33.5	46.2	7.7	70-32.9	45.5	11.7
HL	13.3	9.8-6.9	8.7	1	11.5-6.5	8.9	2.2
PrOL	5.5	4-2.7	3.5	0.4	5.8-2.4	3.6	1
ED	2.4	2.1-1.4	1.8	0.2	2.4-1.3	1.8	0.4
PsOL	7.2	5.3-3.6	4.7	0.6	6.9-3.6	4.8	1.1
PrDD	34.8	28.7-17.5	24.3	3.8	37.6-17.4	24.2	6.2
PrPD	14.9	11.8-7.6	10.3	1.5	15.1-7.5	10.3	2.3
PrVD	35.1	29.7-18.1	24.9	4.2	38.9-17.2	24.5	6.5
PrAD	54.4	45.1-27.2	38	6.5	57.2-26.3	37	9.7
CPL	24.7	20.1-11.9	16.5	2.9	24.6-11.8	16.3	4.2
APL	12.9	10.9-7.3	9.4	1.2	12.8-7.1	9.1	1.8
PFL	9.6	10.7-5.5	8.9	1.8	10.7-4.5	6.9	1.8
VFL	5.8	7.8-3.9	6.4	1.4	8.4-3.7	5.4	1.5
AFL	1.7	4.3-1.7	3.2	0.9	3.8-1.6	2.5	0.7
AHL	6.8	6.6-4.1	5.4	0.8	6.8-3.6	5	1.1
DFL	7.9	6.5-3.4	5.2	1	7.1-3.4	4.7	1.2
DHL	7.3	8.5-5.5	7.4	1.2	8.8-4.9	6.6	1.3
CFL	10.2	10 5.8-	8	1.4	10.5-4.9	7.4	1.8
BLD	7	5.2-3.1	4.5	0.8	7.2-2.9	4.4	1.3
BD	10.3	9.1-4.9	7.4	1.5	12.2-4.6	7	2.3
AVD	19.1	15.5-8.8	12.9	2.4	21-8.4	12.5	3.7
D	7	7	7	0	7	7	0
A	5	5	5	0	5	5	0

Three new species of the genus *Cobitis*

Iberian Peninsula 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.

***Cobitis almadae* sp. nov.** Doadrio, Sousa-Santos & Robalo (Fig. 6 Table 2)
urn:lsid:zoobank.org:act:107818E8-E28A-4014-87CE-CD8C14075E40

HOLOTYPE: MNCN ICTIO29821 68.2 mm SL, 78.4 mm TL; female, Sizandro River, Sizandro Drainage, Runa, Portugal, 39° 04' 04.9" N 9° 12' 24.8" W, 42 m a.s.l., Leg. C. Sousa Santos, 4.II.2022.

PARATYPES: MNCN ICTIO29800-29809, MNCN ICTIO29811, MNCN ICTIO29813-29820, MNCN ICTIO29822-29828, 25 individuals, Sizandro River, Sizandro Drainage, Runa, Portugal, 39° 04' 04.9" N 9° 12' 24.8" W, 42 m a.s.l., Leg. C. Sousa Santos, 4.II.2022. MNCN ICTIO256704-56723, 20 individuals, Sizandro River, Sizandro Drainage, Gozundeira, Portugal, 39° 00' 30.0" N 9° 10' 53.2" W, 42 m a.s.l., Leg. I. Doadrio, 2.V.2003.

DIAGNOSIS: *Cobitis almadae* sp. nov. is a member of the *Iberocobitis* subgenus (Bacescu, 1962) with typical scales elongated with reduced focus and well-developed *lamina circularis*. *Cobitis (Iberocobitis) almadae* sp. nov. can be differentiated from all other known species of the subgenus *Iberocobitis* according to the following set of characters: Large and low peduncle depth (CPL/BLD > 3.4; APL/BLD > 1.8). Males with a long knife-shaped *lamina circularis* (Canestrini scale) at the base of the second pectoral fin ray and with a narrow handle. Lateral ethmoid (suborbital spine) well developed with long narrow mediocaudal, laterocaudal and mediorostral processes. Narrow skull with elongated and narrow frontoparietal fontanel. As occur in *C. paludica* the pigmentation pattern is highly variable, but *C. almadae* has smaller and more numerous spots on the body of females than *C. haasii*, *C. vettonica*, *C. paludica* and *C. maroccana*. *Cobitis almadae* has a well-developed pigmentation in the four



Figure 6. Holotype of *Cobitis almadae* sp. nov. from the Sizandro River, Sizandro Drainage, Portugal. MNCN ICTIO29821. SL = 68.2 mm. Scale is 1 cm. *Holotipo de Cobitis almadae* sp. nov. del río Sizandro, cuenca del Sizandro, Portugal. MNCN ICTIO29821. SL = 68.2 mm. La escala es 1 cm.

Gambetta's zones (Gambetta, 1934) and a poorly developed ventral pigmentation in adult individuals, frequently interrupted in the middle of the body. In females, Gambetta's fourth row has 12-21 small blotches (Median = 17.5). Third Gambetta's zone is wide and sprinkled with numerous black spots. In males, Gambetta's second and fourth zone spots fused to form two black stripes as in other *Iberocobitis* species.

Cobitis almadae sp. nov. is distinguishable from *C. calderoni* by the presence in the male of a *lamina circularis* at the base of the second pectoral ray. *Cobitis almadae* sp. nov. is distinguishable from *Cobitis haasii* by the presence of a large upper caudal spot. *Cobitis almadae* sp. nov. is distinguishable from *Cobitis paludica* and *Cobitis vettonica* by a third Gambetta's zone wide and covered with numerous black spots vs third Gambetta's zone narrow and covered with few black spots.

The new species presents two autapomorphies, both transversions, in the *MT-CYB* gene (positions 714 and 870; Appendix 9, (see Supplementary information available at <https://www.limnetica.net/en/limnetica>).

DESCRIPTION: D III 7, A III 5, Vr = 4+36-38+1). Morphometric characters of the type material are given in Table 2.

A medium-sized species of the genus *Cobitis* that rarely reaches 100 mm of total length. The head is large and SL/HL was 4.7-5.8 (\bar{x} = 5.2). The maximum body depth is low and the head length is larger than maximum body depth and HL/BD is 1-1.5 (\bar{x} = 1.3). The preorbital distance is short PrOL/ED is 1.5-2.4 (\bar{x} = 2). The postorbi-

tal distance is large but similar to the one found in the population of the type locality of *C. paludica* and PsOL/SL is 0.09-0.12 ($\bar{x} = 0.1$). The ventral fins are inserted in the same vertical to the origin of the dorsal fin. Predorsal length is 0.9-1.1 ($\bar{x} = 1$) times preventral length. The caudal peduncle is low and minimum body depth is 3.4-4.1 ($\bar{x} = 3.7$) times the length of the caudal peduncle and 1.8-2.3 ($\bar{x} = 2$) times the length of the anal peduncle. Caudal fin is short, and the head length is 0.9-1.3 ($\bar{x} = 1.2$) times the length of the caudal fin. Greater number of vertebrae than other species studied. Maxilla elongated mainly by the long and narrow posterior process. The pectoral girdle has a ventral cleithral lamina elongated and larger than in other species.

PIGMENTATION PATTERN: As in *C. paludica* the pattern of pigmentation is very variable more than in most species of the genus. Following Gambetta's nomenclature (Gambetta, 1934), the third zone is wider than in most species belonging to the *Iberocobitis* subgenus, and this zone, like the first, is dotted with numerous black spots. The second area is characterized by small round black spots, sometimes are fused. The number of spots in the second area varies from 14 to 28, Median = 21. The fourth area is covered by 12-21 small blotches and the Median = 17.5. In males, blotches of second and fourth Gambetta's zones are fused in marked dark stripes. Ventral body pigmentation is poorly marked in adult specimens. Dorsal body pigmentation conserve black blotches in adults, mainly in caudal area. Caudal fin base with two dark spots, dorsal spot smaller and more intense. Head sprinkled with many black spots (Fig. 6).

ETYMOLOGY: The name of the species *almadae* derives from the researcher Vitor Almada who, with an iron will and overcoming his birth blindness, dedicated his life to the study and conservation of the rivers and fauna of Portugal, where *C. almadae* lives.

DISTRIBUTION: *Cobitis almadae* is endemic to the Sizandro Drainage in the Lisbon district in Portugal. Sizandro River is a small river with a drainage area of 336.6 km² (Fig. 7). Haplotypes of *C. almadae* occur in Samarra Drainage and in



Figure 7. Distribution range of *Cobitis almadae* sp. nov. Área de distribución de *Cobitis almadae* sp. nov.

a small river of the Tagus Drainage (Alanquer River) all of them around of Sizandro Drainage. Genetic introgression between North Atlantic populations and *C. almadae* was detected in the drainages closest to Sizandro Drainage. For this reason and awaiting further studies, the distribution area of *C. almadae* is considered restricted to the Sizandro Drainage. In Sizandro Drainage *Cobitis almadae* overlaps its distribution with *Achondrostoma occidentale* Robalo, Almada, Sousa Santos, Moreira & Doadrio, 2005, although the latter species is also distributed in Alcabrichel and Safarujo drainages, two other small drainages of the same Lisbon district where the genus *Cobitis* is not present. Other than *C. almadae* and *A. occidentale* no other native freshwater fishes have been found in the Sizandro Drainage.

COMMON NAMES: Almada Spined loach

***Cobitis atlantica* sp. nov.** Doadrio, Sousa-Santos, Robalo & Perea (Fig. 8. Table 3)
urn:lsid:zoobank.org:act:1A57BE8D-1E8E-4E64-BE5A-4C9E6132F6A

Three new species of the genus *Cobitis*

Table 3. Statistical variables used to define the morphometric and meristic characters of *Cobitis atlantica* sp. nov. type series. Variables as described in Methods (SD = standard deviation). *Variables utilizadas para definir los caracteres morfométricos y merísticos de la serie tipo de Cobitis atlantica sp. nov. Las variables son descritas en la sección de métodos (SD = desviación estándar).*

<i>Cobitis atlantica</i> sp. nov.							
Variable	Holotype	Paratypes	Males	N=9	Paratypes	Females	N=15
		Range	Mean	SD	Range	Mean	SD
SL	75.6	75.6-51.7	62.6	7.4	87-45	70.5	12.2
HL	13.1	13.2-9.3	11.6	1.4	17.5-8.4	13.1	2.8
PrOL	5.9	5.9-4.8	5.4	0.4	8.6-3.4	6.1	1.5
ED	2.8	2.9-2.3	2.6	0.2	3.5-1.8	2.7	0.5
PsoL	6.5	6.5-4.4	5.6	0.7	8.7-4.1	6.2	1.2
PrDD	38.3	38.2-26.4	31.4	3.8	45.5-23.1	36.3	6.4
PrPD	15.1	15.1-10.4	13.0	1.6	17.8-8.2	14.7	2.7
PrVD	39.7	39.7-27.6	33.7	4.1	46.6-23.8	37.8	6.8
PrAD	60.4	60.4-41.5	51.0	6.2	69.5-35.6	57.4	10.3
CPL	29.5	29.5-18.6	23.0	3.2	31.8-16.4	26.1	4.3
APL	6.4	8.6-5.7	7.1	1.0	10.7-5.8	7.9	1.5
PFL	11.9	12-10.3	11.2	0.7	12.6-7.3	10.0	1.5
VFL	9.4	9.4-6.9	8.7	0.8	11-5.8	8.3	1.4
AFL	4.7	5.3-1.2	3.5	2.2	6.2-1.5	3.2	1.7
AHL	7.2	7.7-5.6	6.6	0.7	9.1-4.9	7.2	1.3
DFL	7.6	7.6-5.7	6.9	0.6	8.7-4.4	7.0	1.3
DHL	10.1	10.1-7.7	8.6	0.7	12.2-5.7	9.6	1.7
CFL	11.6	11.7-8.9	9.9	1.1	11.9-7.4	10.7	1.2
BLD	8.8	8.8-5.8	7.2	0.9	9.6-4.9	7.9	1.4
BD	13.7	13.7-8.9	11.2	1.6	16.8-7.4	12.6	2.8
AVD	20.7	20.7-13.9	17.8	2.4	26.3-11.2	20.0	4.0
D	7	7	7	0	7	7	0
A	5	5	5	0	5	5	0

HOLOTYPE: MNCN_ ICTIO29839 75.6 mm SL, 87.2 mm TL; female, Alcoa River, Alcoa Drainage, Fervença, Portugal, 39° 34' 01.3" N 8° 59' 21.2" W, 86 m a.s.l., Leg. C. Sousa- Santos, 29.I.2022.

PARATYPES: MNCN_ ICTIO29840-29849, 10 individuals, Alcoa River, Alcoa Drainage, Fervença, Portugal, 39° 34' 01.3" N 8° 59' 21.2" W, 86 m a.s.l., Leg. C. Sousa-Santos, 29.I.2022. MNCN_ ICTIO245591-245596, 6 individuals, Macaco River, Limia Drainage, Lobios,

Ourense, Spain, 41° 54' 36.7" N 8° 04' 39.5" W, 387 m.a.s.l., Leg. I. Doadrio, P. Garzón Heydt & J. L. González, 1.VII.2010. MNCN_ ICTIO455610-455612 MNCN_ ICTIO45562-45564, MNCN_ ICTIO45567-45569, 9 individuals, Tea River, Salvaterra do Minho, Pontevedra, Spain, 42° 05' 06.7" N 8° 31' 05.1" W, Leg. I. Doadrio, 7.VI.2003. MNCN_ ICTIO277726-27, 2 individuals, Tea River, Salvaterra do Minho, Pontevedra, Spain, 42° 05' 06.7" N 8° 31' 05.1" W, Leg. I. Doadrio, P. Garzón & J. L. González, 24.VII.2009.

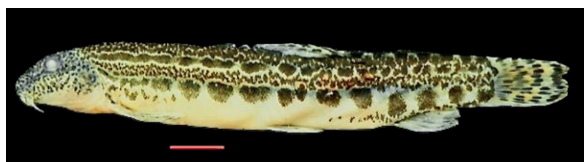


Figure 8. Holotype of *Cobitis atlantica* sp. nov. from the Alcoa River, Alcoa Drainage, Portugal. MNCN ICTIO29839. SL = 75.6 mm. Scale is 1 cm. *Holotipo de Cobitis atlantica* sp. nov. del río Alcoa, Cuenca del Alcoa, Portugal. MNCN ICTIO29839. SL = 75.6 mm. La escala es 1 cm.

ADDITIONAL MATERIAL: MNCN ICTIO256636-665, 30 individuals, Arunca River, Mondego Drainage, Soure, Coimbra, Portugal, 40° 04' 06.5" N 8° 37' 52.7" W, Leg. I. Doadrio, 2.V.2003. MNCN ICTIO53370-53776, 7 individuals, Cávado River, Cávado Drainage, Prados, Portugal, 41° 36' 05.5" N 8° 27' 00.3" W, Leg. I. Doadrio, Y. Bernat & J. Cubo, 8. IV. 1989. MNCN ICTIO1400-1420, 21 individuals, Cávado River, Cávado Drainage, Cabanelas, Coimbra, Portugal, 41° 34' 11.8" N 8° 29' 30.9" W. MNCN ICTIO257356 1 individual, Tributary of Cávado River, Braga, Coimbra, Portugal, 41° 35' 59.5" N 8° 27' 04.5" W, Leg. C. Sousa-Santos & J. Robalo, 6.VI.2003. MNCN ICTIOAT18011-18021, 11 individuals, Tea River, Salvaterra do Minho, Pontevedra, Spain, 42° 05' 06.7" N 8° 31' 05.1" W, Leg. I. Doadrio, P. Garzón & J. L. González. 1.VII.2010. MNCN ICTIO45077-45078, 2 individuals, Ave River, Ave Drainage, Gilhofrei, Portugal, 41° 34' 52.0" N 8° 06' 52.5" W, Leg. P. Santos, 30.IV.1988. MNCN ICTIO50027-50081 55 individuals, Frades River, Ribeira de Frades, Portugal, 40° 12' 56.4" N 8° 29' 17.0" W, Leg. I. Doadrio, 31. VIII.1987.

DIAGNOSIS: *Cobitis atlantica* sp. nov. is a member of the *Iberocobitis* subgenus (Bacescu, 1962). *Cobitis (Iberocobitis) atlantica* sp. nov. can be differentiated from all other known species of subgenus *Iberocobitis* according to the following set of characters: short and high peduncle depth (CPL/BLD < 3.6; APL/BLD < 1.4). Males with a long knife-shaped *lamina circularis* (Canestrini scale) at the base of the second pectoral fin ray and with a wide handle. Lateral ethmoid

(suborbital spine) well developed with short and wide mediocaudal, laterocaudal and mediorostral processes. Wide skull with short and wide frontoparietal fontanel. As occurs in *C. paludica* and *C. almadae* the pigmentation pattern is variable but with larger spots than *C. almadae* and similar to *C. paludica*. *Cobitis atlantica* has a well-developed pigmentation in the four Gambetta's zones (Gambetta, 1934) and a developed ventral pigmentation in adult individuals, frequently interrupted in the middle of the body. In females, Gambetta's fourth row has 10-16 blotches (Median = 13), these blotches reach the ventral pigmentation in the caudal region. The third Gambetta's zone is narrow with black spots. In males, Gambetta's second and fourth zone spots fused to form two black stripes as in other *Iberocobitis* species. Caudal fin base spots with the superior one reduced to lesser than eye diameter.

Cobitis atlantica sp. nov. is distinguishable from *C. calderoni* by the presence in the male of a *lamina circularis* at the base of the second pectoral ray. *Cobitis atlantica* sp. nov. is distinguishable from all the Iberian species of the same size by a high and compressed body: CPL/BLD < 3.6; APL/BLD < 1.4, vs CPL/BLD > 3.5; APL/BLD > 1.6.

DESCRIPTION: D III 7, A III 5, Vr = 4 + 35-36 + 1. Morphometric characters of the type material are given in Table 3.

A medium-sized species of the genus *Cobitis* that rarely reaches 150 mm of total length. Short head and SL/HL is 4.9-6 (\bar{x} = 5.4) as consequence of a small postorbital distance and PsOL/SL is 0.08-0.1 (\bar{x} = 0.09). The preorbital distance is larger than *C. almadae* and PrOL/ED is 1.9-2.5 (\bar{x} = 2.2). The maximum body depth is high, and the maximum body depth was similar to the head length and HL/BD was 1-1.2 (\bar{x} = 1.1). The ventral fins are inserted in the nearby area of the same vertical to the origin of the dorsal fin. Predorsal length is 0.9-1 (\bar{x} = 0.95) times preventral length. The caudal peduncle is high and minimum body depth is 3.1-3.6 (\bar{x} = 3.3) times the length of the caudal peduncle and 0.7-1.4 (\bar{x} = 1) time the length of the anal peduncle. Caudal fin is long, and the head length is only 0.7-1.1 (\bar{x} = 0.9) times the length of the caudal fin.

Three new species of the genus *Cobitis*

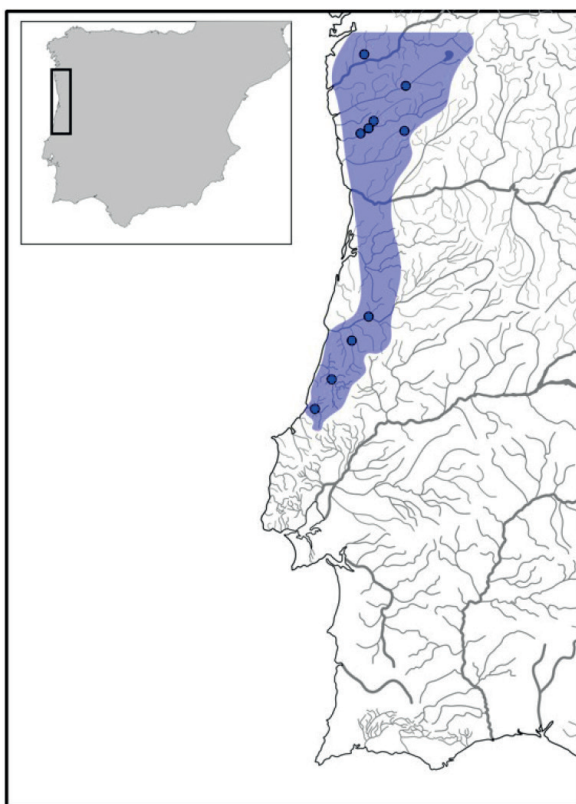


Figure 9. Distribution range of *Cobitis atlantica* sp. nov. Área de distribución de *Cobitis atlantica* sp. nov.

PIGMENTATION PATTERN: The pattern of pigmentation is very variable more than in most species of the genus. The third Gambetta's zone is narrow, and this zone, like the first, is dotted with black spots larger than in *C. almadae*. The second area is characterized by small round black spots that sometimes are fused. The number of spots in the second area range from 14 to 21, Median = 18.4. The fourth area is covered by 10-16 (Median = 13) large rectangular blotches, transversally elongated and reaching ventral pigmentation in caudal area. In males, blotches of second and fourth Gambetta's zones are fused in marked dark stripes. Ventral body pigmentation poorly marked in adult specimens. Dorsal body pigmentation conserve black blotches in adults, mainly in caudal area. Caudal fin base with two small dark spots, the superior smaller than eye diameter. Head sprinkled with many black spots (Fig. 8).

ETYMOLOGY: The name of the species *atlantica* derives from the Atlantic drainages in the Iberian Peninsula, from Minho to Alcoa where *C. atlantica* inhabits.

DISTRIBUTION: *Cobitis atlantica* is distributed in the northern Atlantic drainages of the Iberian Peninsula from Minho Drainage to Alcoa Drainage (Fig. 9). We found this species in the drainages of: Minho, Limia, Cávado, Ave, Mondego, Lis and Alcoa. The distribution of *C. atlantica* is similar to the one of *Achondrostoma oligolepis* Robalo, Doadrio, Almada & Kottelat, 2007 a small leuciscid fish species endemic from Portugal.

COMMON NAMES: Atlantic Spined loach

REMARKS: In this study, *Cobitis atlantica* is attributed only to the northern genetic lineage. The southern lineage, from the Sado Drainage to the Mira Drainage, possibly belongs to an undescribed species due to the genetic and morphological differences found in the Sado Drainage population (Fig. 10). Adult individuals from the Sado Drainage have highly developed ventral pigmentation and Gambetta's fourth zone reaches ventral pigmentation. In addition, the males have two black stripes wider than any other population of the subgenus *Iberocobitis*. However, the presence of haplotypes from populations located in other drainages within the southern lineage, as

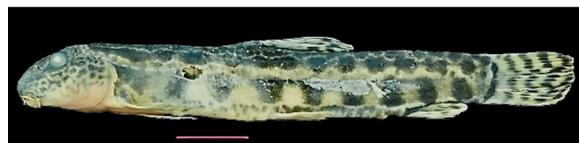


Figure 10. Female specimen of *Cobitis paludica* from Sado Drainage (MNCN ICTIOAT29774) shown the characteristic pigmentation pattern formed by larger blotches than other populations, reaching of ventral pigmentation. Third Gambetta zone very reduced due to second and fourth Gambetta zones very pigmented. Scale is 1 cm. Ejemplar hembra de *Cobitis paludica* de la Cuenca del río Sado (MNCN ICTIOAT29774) mostrando su característico patrón de pigmentación formado por manchas más grandes que en otras poblaciones alcanzando la pigmentación ventral. Tercera zona de Gambetta muy reducida debido a que la segunda y cuarta zona de Gambetta están muy pigmentadas. La escala es de 1 cm.

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Table 4. Statistical variables used to define the morphometric and meristic characters of *Cobitis mellaria* sp. nov. type series. Variables as described in Methods (SD = standard deviation). *Variables utilizadas para definir los caracteres morfométricos y merísticos de la serie tipo de Cobitis mellaria* sp. nov. Las variables son descritas en la sección de métodos (SD = desviación estándar).

<i>Cobitis mellaria</i> sp. nov.							
Variable	Holotype	Paratypes	Males	N=5	Paratypes	Females	N=4
		Range	Mean	SD	Range	Mean	SD
SL	45.2	62.4-44.7	49.6	8.3	85.2-58.7	71	13.9
HL	8.7	11.8-8.7	9.8	1.4	16.4-11.3	13.7	2.5
PrOL	3.3	5.3-3.2	3.9	0.9	6.8-5	5.8	0.9
ED	1.8	2.4-1.7	2	0.3	2.8-2.3	2.5	0.3
PsOL	4.3	8.8-4.6	5.7	2	8.9-5.5	7	1.7
PrDD	23.5	30.9-22.4	25.4	3.8	44.7-29.3	36.3	7.6
PrPD	9.8	13.5-10.1	11.2	1.5	18.5-12.5	15.4	2.6
PrVD	23.4	34.3-23.6	27	5	47.4-32.7	39.3	7.4
PrAD	36.1	51.3-35.3	40	7.6	69.6-46.4	57.1	11.4
CPL	17.8	23.1-17.9	19.6	2.3	31-23.1	27.2	4.2
APL	6.9	8.5-6.4	7.2	0.9	11.2-8.2	9.6	1.5
PFL	9.1	11.9-8.7	9.6	1.3	14-8.4	11.7	2.7
VFL	7.3	9.1-6.6	7.4	1.1	11-7.7	9.6	1.6
AFL	3.7	4.7-3.5	3.9	0.5	6.6-4.3	5.4	1.1
AHL	5.7	7.3-5.7	6.3	0.8	10.2-7.3	8.7	1.5
DFL	4.8	7.3-5.1	5.7	1	10.2-6.3	8.1	2.1
DHL	6.1	9.6-6.8	7.8	1.2	14.2-9	11.3	2.6
CFL	6.9	10.4-7.1	8.2	1.6	13.4-9.2	11.1	2.0
BLD	4.8	6.7-4.7	5.2	0.9	9.0-5.9	7.4	1.7
BD	7.4	11.9-8.4	9.4	1.6	17.5-11.1	14.3	2.8
AVD	12.3	17.9-12.3	14.2	2.6	23.2-16.1	19.1	3.6
D	7	7	7	0	7	7	0
A	5	5	5	0	5	5	0

a consequence of possible hybridization, requires further molecular research before proceeding to a formal description.

***Cobitis mellaria* sp. nov. Doadrio & Perea** (Fig. 11 Table 4)

urn:lsid:zoobank.org:act:27EFCE0D-655D-4A34-9E5C-B5D781AF5448

HOLOTYPE: MNCN_ICTIO272860, 45.2 mm SL, mm; male, Valle River, Valle Drainage, Tarifa, Cádiz, Spain, 36° 05' 53.0" N 5° 42' 02.6" W,

9 m a.s.l., Leg. I. Doadrio, P. Garzón-Heydt, & J.L. González, 25.VI.2010.

PARATYPES: MNCN_ICTIOAT30249-30256, 9 individuals, Valle River, Valle Drainage, Tarifa, Cádiz, Spain, 36° 05' 53.0" N 5° 42' 02.6" W, 9 m a.s.l., Leg. A. López, T. Nester, P. Garzón and I. Doadrio. 13.V.2022.

ADDITIONAL MATERIAL: MNCN_ICTIO1313-MNCN_ICTIO1314, 2 individuals, Valle River, Valle Drainage, Facinas, Cádiz,

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Figure 11. Holotype of *Cobitis mellaria* sp. nov. from the Valle River, Valle Drainage. Spain. MNCN ICTIO272860. SL = 45.2 mm. Scale is 1 cm. *Holotipo de Cobitis mellaria sp. nov. del río Valle, cuenca del Valle. España. MNCN ICTIO272860. SL = 45.2 mm. La escala representa 1 cm.*



Figure 12. Alive female of *Cobitis mellaria* with typical pigmentation pattern. Scale is 1 cm. *Ejemplar hembra viva de Cobitis mellaria con la pigmentación típica. La escala representa 1 cm.*

Spain, 36° 06' 25.8" N 5° 42' 23.7" W, 10 m.a.s.l. leg. P. Garzón & I. Doadrio, 6.VI.2001. MNCN ICTIO17517-MNCN ICTIO17530 14 individuals, Valle River, Valle Drainage, Tarifa, Spain, 36° 05' 53.0" N 5° 42' 02.6" W, 9 m a.s.l., Leg. I. Doadrio, P. Garzón-Heydt & J.L. González, 25.VI.2010.

DIAGNOSIS: *Cobitis mellaria* sp. nov. is a member of the *Iberocobitis* subgenus (Bacescu, 1962). *Cobitis mellaria* sp. nov. can be differentiated from all other known species of *Iberocobitis* by the following set of characters. Low peduncle depth (CPL/BLD > 3.5; APL/BLD 1.2-1.7). *Lamina circularis* at the base of the second pectoral fin ray and with convex outer edge. Well-developed lateral ethmoid (suborbital spine) with short laterocaudal process. Narrow skull with elongated frontoparietal fontanel. Pigmentation pattern less variable than in *C. paludica*, *C. almadae* and *C. atlantica*. *Cobitis mellaria* has a well-developed pigmentation in the four Gambetta's zones (Gambetta, 1934) and no ventral pigmentation in

adults. In females, Gambetta's fourth row has 10-15 blotches, Median = 11. Caudal fin base with two spots.

Cobitis mellaria sp. nov. is distinguishable from *C. calderoni* by the presence in the male of a *lamina circularis* at the base of the second pectoral ray. *Cobitis mellaria* sp. nov. is distinguishable from *Cobitis haasii* by the presence of a large upper caudal spot. Mature specimens of *Cobitis mellaria* sp. nov. are distinguishable from all Iberian species by fourth Gambetta's zone without ventral pigmentation.

The new species presents one autapomorphy (non-transversion) in the *MT-CYB* gene (position 693; Appendix 9).

DESCRIPTION: D III 7, A III 5, Vr = 4+35-36+1). Morphometric characters of the type material are given in Table 4.

A medium-sized species of the genus *Cobitis* that rarely reaches 150 mm of total length. Large head similar to *C. almadae* and SL/HL was 4.7-5.9 (\bar{x} = 5.1). However, the preorbital distance of *C. mellaria* is larger than *C. almadae* PrOL/ED is 1.7-2.8 (\bar{x} = 2.2). The maximum body depth is low and the maximum body depth is similar to the head length as occurs in *C. atlantica* and HL/BD was 0.9-1.2 (\bar{x} = 1.). The ventral fins are inserted in the nearby area of the same vertical to the origin of the dorsal fin. Predorsal length is 0.9-1.1 (\bar{x} = 1) times preventral length. The caudal peduncle is low and minimum body depth is 3.5-4.2 (\bar{x} = 3.8) times the length of the caudal peduncle and 1.2-1.7 (\bar{x} = 1.4) times the length of the anal peduncle. Caudal fin is long, and the head length is only 1.1-1.4 (\bar{x} = 1.2) times the length of the caudal fin.

PIGMENTATION PATTERN: The pattern of pigmentation is less variable than in *C. atlantica*, *C. almadae* and *C. paludica*. Unmarked ventral body pigmentation (Fig. 12). Dorsal pigmentation pattern with small black spots anterior to dorsal fin and large blotches behind dorsal fin. The second Gambetta's zone is characterized by small round black spots. The number of spots in the second area range from 17 to 26, Median = 20.5. The fourth area is covered by 10-15 (Median = 11) large rectangular blotches, only

transversally elongated in caudal area. In males, blotches of second and four Gambetta's zones are fused in marked dark stripes. Caudal fin base with two large dark spots, the upper one more conspicuous (Fig. 12).

COMMON NAMES: Gibraltar Spined loach.

ETYMOLOGY: The name of the species *mellaria* derives from roman site placed on the eastern bank of the mouth of the Valle River. This site was a fish salting factory during Punic-Roman period. The settlement was probably dedicated also to the honey trade by Hispanic people or Roman.

DISTRIBUTION: *Cobitis mellaria* is distributed only in the Valle Drainage and its area of occupation is less than 5 km², this being the smallest known distribution area for an Iberian fish species. The basins of the rivers adjacent to the Valle Drainage were intensively sampled and only *C. paludica* was captured (Fig. 13). Distribution of the all Iberian species is in Fig. 14.



Figure 13. Distribution range of *Cobitis mellaria* sp. nov. Área de distribución de *Cobitis mellaria* sp. nov.

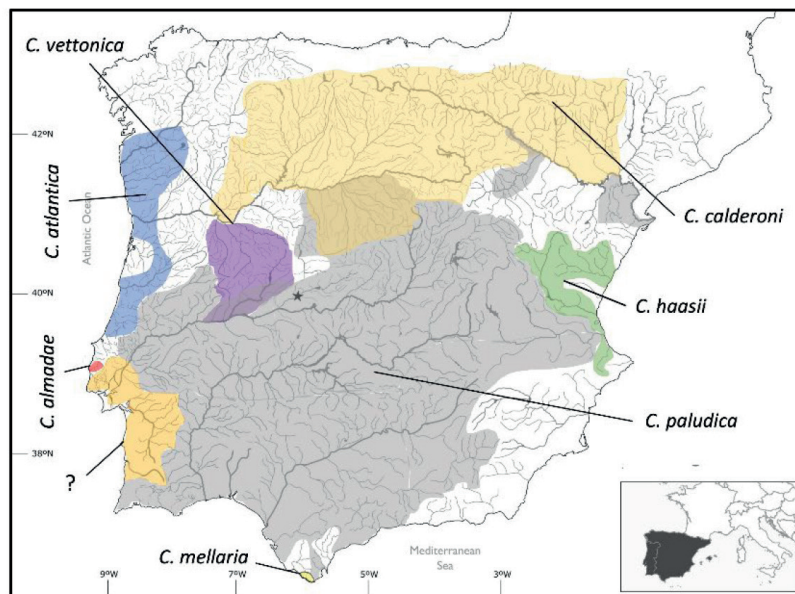


Figure 14. Distribution range of all Iberian *Cobitis* species. *C. calderoni*, light orange. *C. paludica*, grey. *C. haasii*, green. *C. atlantica*, blue. *C. almadae*, red. *C. mellaria*, yellow. Sado population, orange. Orange dark show the sympatry area between *C. calderoni* and *C. paludica*. Área de distribución de todas las especies ibéricas del género *Cobitis*. *C. calderoni*, naranja claro. *C. paludica*, gris. *C. haasii*, verde. *C. atlantica*, azul. *C. almadae*, rojo. *C. mellaria*, amarillo. Población del Sado, naranja. El naranja oscuro muestra la zona de simpatría entre *C. calderoni* and *C. paludica*.

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REMARKS: *Cobitis mellaria* is the only one native freshwater fish species present in the Valle Drainage. However, other fishes as *Atherina boyeri* Risso, 1810, *Pomatoschistus microps* (Kroyer, 1838), *Anguilla anguilla* (Linnaeus, 1758) and species of the Mugilidae family are present. The Gibraltar Strait Natural Park protects a large part of the territory around the Valle River, but only its mouth, where *C. mellaria* does not exist, is within the Natural Park. Therefore, the distribution of *Cobitis mellaria* is outside the area protected by the Gibraltar Strait Natural Park. There are some threats to the species, such as overstocking, water pollution from nearby campsites, and water extraction for both livestock and small horticultural crops. The invasive red swamp crayfish (*Procambarus clarkii* Girard, 1852) is also present in the Valle River. The species has already disappeared in areas where it was present in 2010. Therefore, following the IUCN criteria, *C. mellaria* should be considered Critically Endangered following the B2ab criteria. In 2022, the species was still present in the Valle River, which can be considered an extraordinary fact given its small distribution area and the existing threats to the species: presence of numerous invasive species mainly piscivorous, pollution, water extraction for irrigation and livestock and transformation of the channel at the mouth of the river.

Due to its scarcity between 2001 and 2022, only 10 specimens have been preserved in the MNCN ICTIO collections, which constitute the typical series, the rest of the specimens captured, after photographing them, were released alive to the river.

Species key in the Iberian Peninsula

- 1.1 Males without *lamina circularis*.... *calderoni*
- 1.2 Males with a single *lamina circularis* at the base of the second pectoral ray.....2
- 2.1 Very reduced upper caudal spot.....*haasi*
- 2.2 Caudal peduncle with two large caudal spots.....3
- 3.1 High and compressed body. CPL/BLD < 3.6; APL/BLD < 1.4 *atlantica*
- 3.2 Long body. CPL/BLD > 3.5; APL/BLD > 1.6.....4

- 4.1 Very small barbels and fins *vettonica*
- 4.2 Barbels and fins relatively large.....5
- 5.1 Fourth Gambetta's zone without ventral pigmentation *mellaria*
- 5.2 Fourth Gambetta's zone with ventral pigmentation in adults.....6
- 6.1 Third Gambetta's zone is wide and covered with numerous black spots..... *almadae*
- 6.2 Third Gambetta's zone is narrow and covered with few black spots.....*paludica*

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