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First records of the Vettonian spined loach *Cobitis vettonica* in Portugal with update on its Iberian distribution

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SUMMARY

The Iberian Peninsula represents a Mediterranean reserve for native freshwater fishes. Isolated from Central European drainages since the Miocene, approximately 80% of the native Iberian ichthyofauna is endemic. In this study, we report the Vettonian spined loach, *Cobitis vettonica* Doadrio & Perdices, 1997, also known as the Alagón spined loach, for the first time in Portugal. During an ichthyological survey of major Portuguese drainages, we found *C. vettonica* specimens in ten localities along three right tributaries of the Tagus River in Portugal: Erges, Aravil and Ponsul. All three Portuguese rivers are adjacent to the Alagón River in Spain (the type locality of *C. vettonica*). We found *C. vettonica* in sympatry with the related species *C. paludica* in six of the ten localities. *Cobitis vettonica* is listed as Endangered on the Spanish Red List, making it crucial to define its status in Portuguese waters.

Keywords: Alagón spined loach, distribution, endangered species, endemic species, Iberian Peninsula

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INTRODUCTION

The freshwater fish fauna of the European Mediterranean has a high number of endemic species (>250 species), of which more than 56% are threatened (Smith & Darwall, 2006). Endemism is particularly high in the Mediterranean peninsulas that have been isolated by mountain chains from main Central European rivers (Bănărescu, 1992), such as the Iberian Peninsula. Iberian drainages were isolated from Central Europe during the Miocene, when Pyrenees formed, creating a geographical barrier (Stange et al., 2016), and their biotas have been evolving independently since then. Approximately 80% of the native Iberian freshwater fish species are endemic, representing the highest number of endemics in European waters (Elvira, 1995; Clavero et al., 2004; Smith & Darwall, 2006). Water resources in the Mediterranean region have become more limited and their misuse has driven fish extinctions or reductions in their distribution areas (Doadrio et al., 2011). Current threats in the Iberian Peninsula are mostly related to human-mediated changes in water systems, e.g. ponds, dams, extraction, pollution, and habitat destruction, which, in general, have favoured alien species introductions and settlement (Ribeiro et al., 2008, 2009; Clavero et al., 2013).

Current efforts to preserve the Iberian endemic ichthyofauna focus primarily on increasing basic knowledge of species through monitoring, collection, distribution and biological data. Taken together, these data allow species to be catalogued on Red Lists, which would ideally (and hopefully) support their threatened status for conservation purposes. Although current knowledge of the distributions of Iberian freshwater fishes has been updated (Cabral, 2005; Doadrio, 2001; Doadrio et al., 2011), species with restricted distribution ranges living mainly in places of difficult access are more susceptible to being overlooked. This situation becomes particularly critical for potentially threatened endemic species whose status should be included on national Red Lists (Veríssimo et al., 2018). More than 15 new Iberian endemic freshwater fish species have been described or reassigned since 2001 (Leunda et al., 2009; Doadrio et al., 2011; Mateus et al., 2013; CPE, 2017) and half of them meet criteria to be considered threatened (Doadrio, 2001; Cabral, 2005).

Iberian spined loaches are freshwater fishes of the Palearctic genus *Cobitis*. Their distribution is patchy, and they are often in middle courses of rivers with sandy bottoms. There are only three native Iberian spined loaches species: *Cobitis calderoni* Bacescu, 1962; *Cobitis paludica* De Buen, 1930 and *Cobitis vettonica* Doadrio & Perdices, 1997. *Cobitis calderoni* is considered threatened in both Portugal and Spain, while *C. paludica* and *C. vettonica* are currently only considered threatened in Spain (Doadrio, 2001; Cabral, 2005).

Previous molecular studies have shown that *C. paludica* does not constitute a monophyletic group, but rather has four distinct mitochondrial lineages, identified as IIa, IId, IIe and IIf, which should have equivalent taxonomic status to that of *C. maroccana* and *C. vettonica* (see Figure 2 in Doadrio & Perdices, 2005). The Vettonian spined loach, *Cobitis vettonica*, morphologically resembles *C. paludica*, although it has smaller barbels and fins. Both are benthic species that have a fusiform body covered with minute scales, a coloration pattern organized according to the Gambetta's four zones (Z1–Z4) (Gambetta, 1934) and morphological sexual dimorphism. These similarities in morphology may complicate the identification of Iberian *Cobitis* species when only morphological characters are used.

The distribution of *C. paludica* and *C. vettonica* have recently been updated for Spain (Doadrio et al., 2011; Sánchez-Hernández et al., 2018); however, the distribution of *C. vettonica* in Portugal remains unknown despite indications of it is presence in the country (Doadrio et al., 2011; Perdices et al. 2018). *Cobitis vettonica* was originally described from the Alagón River, a right tributary of the Tagus in Spain (Doadrio & Perdices, 1997). It was also found in the Águeda River, a left tributary of the Duero drainage in Spain (Doadrio & Perdices, 2005; Doadrio et al., 2011; CPE, 2017).

To improve our knowledge about the current distribution of freshwater fishes in Portugal, we surveyed major Portuguese drainages, focusing on areas with endemic species, namely cobitids and cyprinids. We used molecular tools to avoid potential misidentifications and to assess cobitid diversity in Portugal. We formally report the first record of the endemic spined loach species *C. vettonica* in Portugal. The current distribution range of *C. vettonica* has been updated to include some Tagus tributaries in Portugal, all of which run near to the Spanish tributaries inhabited by the species

(Doadrio, 2001; Doadrio et al., 2011). We also provide an update on the distribution of known Iberian populations of *C. vettonica*.

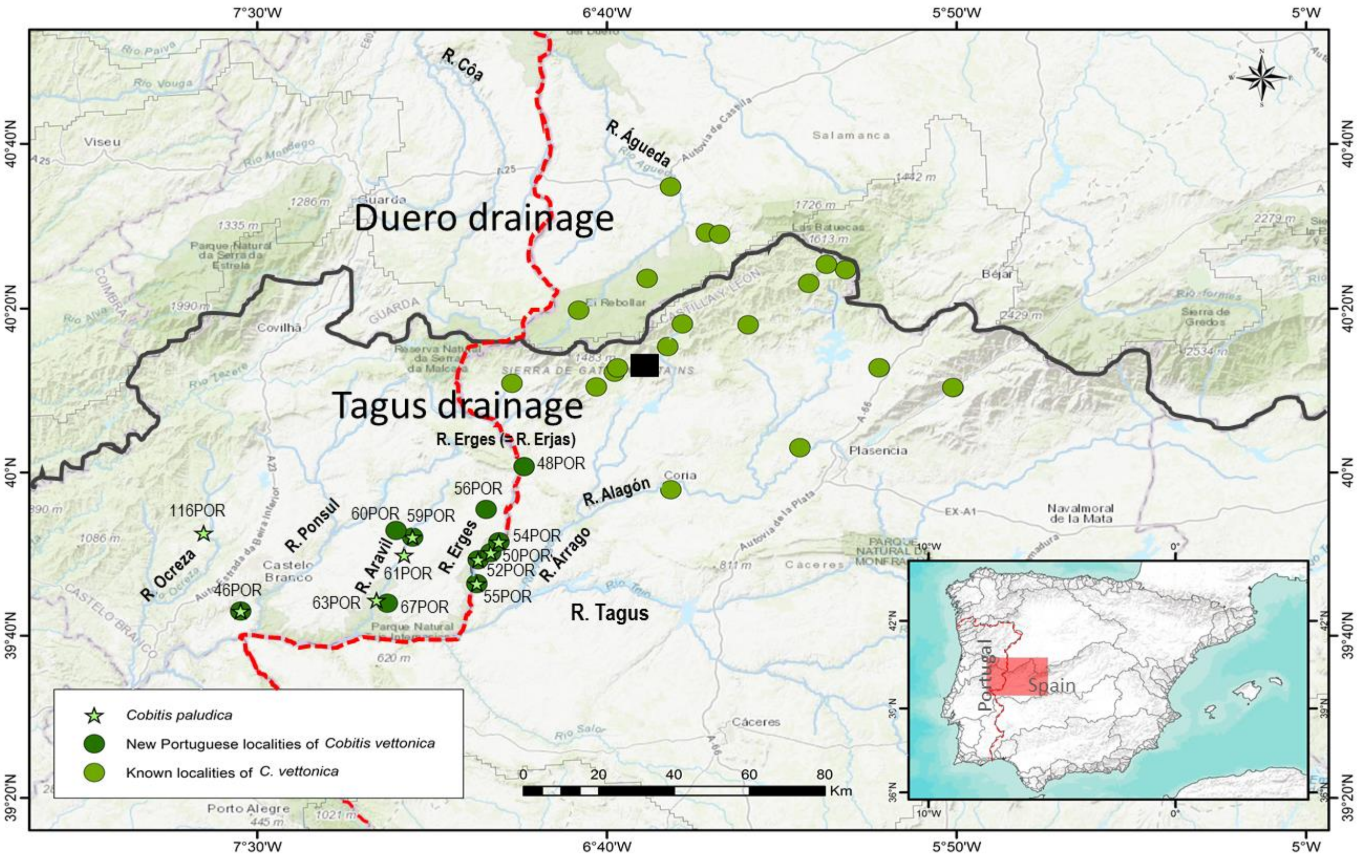

Figure 1. Distribution of *Cobitis vettonica*. Inset map shows the major hydrographical divisions in the Iberian Peninsula and the area enlarged in the detailed map, which shows all known localities of *C. vettonica* (detailed information on localities provided in Table 1): dark green circles = new localities of *C. vettonica* in Portugal; light green circles = previously known localities of *C. vettonica*; stars = presence of *C. paludica*. Black square represents the type locality of *C. vettonica*. Dashed red line indicates the border between Spain and Portugal; dark line delineates the Duero and Tagus drainages.

MATERIAL AND METHODS

A total of 131 localities were sampled in Portugal. Of these, 32 were located along four right tributaries of the Tagus in Portugal (Figure 1): Erges, Aravil, Ponsul and Ocreza. These tributaries are adjacent to the Alagón tributary in Spain, the type locality of *C. vettonica* (Doadrio & Perdices, 1997). Electrofishing was primarily used to collect specimens, although hand nets were used in some places. We used a HANS GRASSL EL62II to capture live fishes in an approximately 100-m section of river using a single pass without net blocking. Fin or muscle tissue samples were collected in the field and preserved in 96% ethanol. Voucher specimens were individually tagged and fixed in 10% formalin or preserved in 96% ethanol.

A total of 42 *Cobitis* individuals were analyzed from 13 localities along the four Tagus tributaries in Portugal (Figures 1 and 2, Table 1). *Cobitis* specimens were not identified to the species level to avoid potential species misidentification on the basis of morphology. When possible, several *Cobitis* specimens were analysed from the same locality to account for morphological variation within populations (Figure 2). For molecular analyses, the complete mitochondrial cytochrome b (cyt b) gene was used. Cyt b is a variable gene that is routinely used for fish identifications at the species level (Schönhuth et al., 2014; Perdices et al., 2018). In order to analyse all known Iberian and North African spined loach lineages (Perdices & Doadrio, 2001; Doadrio & Perdices, 2005; Perdices et al., 2016, 2018), cyt b sequences of previously analysed specimens available from GenBank were also included in the analyses: 16 *C. paludica*, 8 *C. vettonica*, 2 *C. maroccana*, 2 *C. calderoni*, and, for the outgroup, 2 *C. bilineata* (Table 1). These sequences were analysed together with those of the 42 newly collected specimens.

Figure 2. Specimens of *Cobitis vettonica* and *Cobitis paludica* from different Tagus localities: a) 2279PT *C. paludica* male from LOC ID 52POR (Erges River); 50,92 cm standard length (SL) (GenBank ID MN583213), b) 2013PT *C. vettonica* male from LOC ID 52POR (Erges River); 46,65 cm SL (GenBank ID MN583198), and c) 2057PT *C. vettonica* female from LOC ID 56POR (Erges River); 73,43 cm SL (GenBank ID MN583204).

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Table 1. Information *Cobitis* specimens analysed in this study, including geographic location, voucher number (when available) and GenBank accession numbers. *Cobitis vettonica* specimens for which the GenBank ID is not provided were those used in the reconstruction of the updated distribution map shown in Figure 1. Locality Identifications (LOC ID) are those shown in Figure 1. * denotes specimen from the type locality. Country codes: IT= Italy, MA= Morocco, PT= Portugal, SP= Spain. Refs: 1- this study; 2- Perdices et al. (2016); 3- Doadrio & Perdices (2005); 4- Perdices & Doadrio (2001); 5- Perdices et al. (2018); 6- Doadrio et al. (2011); 7- Doadrio & Perdices (1997); 8- Confederación Hidrográfica Duero (2013).

Cobitis species	Drainage	Tributary/River	Locality	Country	Longitude	Latitude	LOC ID	Voucher	IDGENBank	Ref.
paludica	Tagus	Erges/Erges	Azenha do Roque	PT	39°49'19.29"	06°58'23.11"	50POR	1982PT	MN583212	1
paludica	Tagus	Erges/Erges	Serrinha	PT	39°46'23.27"	06°58'35.59"	52POR	2279PT	MN583213	1
paludica	Tagus	Erges/Erges	Cabeça Queimado	PT	39°50'12.61"	06°56'34.85"	54POR	2037PT	MN583214	1
paludica	Tagus	Erges/Erges	Salvaterra do Extremo	PT	39º51'32.11"	06º55'26.45"	55POR	2307PT	MN583215	1
paludica	Tagus	Aravil/ Rib. Toula	_	PT	39º52'06.60"	07º07'46.16"	59POR	2100PT	MN583216	1
paludica	Tagus	Aravil/Aravil	Zebreira	PT	39º49'54.27"	07º08'59.03"	61POR	2126PT	MN583217	1
paludica	Tagus	Aravil/Aravil	_	PT	39º44'22.85"	07º12'55.52"	67POR	2167PT	MN583218	1
paludica	Tagus	Ponsul/Alfrividas	Alfrividas	PT	39°43'01.59"	07°32'21.90"	46POR	2294PT	MN583219	1
paludica	Tagus	Ocreza/Rib. Alvito	Monte Gordo	PT	39°47'57.36"	07°45'39.53"	116POR	3187PT	MN583220	1
paludica	Tagus	Ocreza/Rib. Alvito	Monte Gordo	PT	39°47'57.36"	07°45'39.53"	116POR	3188PT	MN583221	1
paludica	Tagus	Ocreza/Rib. Alvito	Monte Gordo	PT	39°47'57.36"	07°45'39.53"	116POR	3190PT	MN583222	1
paludica	Tagus	Ocreza/Rib. Alvito	Monte Gordo	PT	39°47'57.36"	07°45'39.53"	116POR	3191PT	MN583223	1
vettonica	Duero	Águeda/Ar. Malena	_	SP	40°29'14.56"	06°25'45.50"	_	158CP	KP161123	2
vettonica	Duero	Águeda/Águeda	La Herguijuela	SP	40°23'31.74"	05°15'14.39"	_	168CP	AY860181	3
vettonica	Tagus	Alagón/Árrago	Cadalso de Gata	SP	40°15'23.40"	06°31'17.20"	_	1ARR	AF263072*	4
vettonica	Tagus	Alagón/Árrago	Cadalso de Gata	SP	40°15'23.40"	06°31'17.20"	_	2ARR	AF263073*	4
vettonica	Tagus	Alagón/Árrago	Cadalso de Gata	SP	40°15'23.40"	06°31'17.20"	_	5ARR	KP161122	3
vettonica	Tagus	Alagón/Ladrillar	La Rebollosa	SP	40°24'45.10"	06°05'49.63"	_	575A	AY860182	3
vettonica	Tagus	Erges/Erges	Valverde del Fresno	SP	40°10'54.57"	06°53'33.14"	_	268CP	AY860183	3
vettonica	Tagus	Erges/Erges	Termas de Monfortinho	PT	40°00'44.00"	06°51'48.87"	48POR	1945PT	MN583187	1
vettonica	Tagus	Erges/Erges	Termas de Monfortinho	PT	40°00'44.00"	06°51'48.87"	48POR	1946PT	MN583188	1

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Table 1. (continued).

Cobitis species	*Drainage*	*Tributary/River*	*Locality*	*Country*	*Longitude*	*Latitude*	*LOC ID*	*Voucher*	*IDGENBank*	*Ref.*
vettonica	Tagus	Erges/Erges	Termas de Monfortinho	PT	40°00'44.00"	06°51'48.87"	48POR	2274PT	MN583189	1
vettonica	Tagus	Erges/Erges	Termas de Monfortinho	PT	40°00'44.00"	06°51'48.87"	48POR	2275PT	MN583190	1
vettonica	Tagus	Erges/Erges	Termas de Monfortinho	PT	40°00'44.00"	06°51'48.87"	48POR	2276PT	MN583191	1
vettonica	Tagus	Erges/Erges	Termas de Monfortinho	PT	40°00'44.00"	06°51'48.87"	48POR	2277PT	MN583192	1
vettonica	Tagus	Erges/Erges	Azenha do Roque	PT	39°49'19.29"	06°58'23.11"	50POR	1983PT	MN583193	1
vettonica	Tagus	Erges/Erges	Azenha do Roque	PT	39°49'19.29"	06°58'23.11"	50POR	2304PT	MN583194	1
vettonica	Tagus	Erges/Erges	Azenha do Roque	PT	39°49'19.29"	06°58'23.11"	50POR	2305PT	MN583195	1
vettonica	Tagus	Erges/Erges	Azenha do Roque	PT	39°49'19.29"	06°58'23.11"	50POR	2306PT	MN583196	1
vettonica	Tagus	Erges/Erges	Serrinha	PT	39°46'23.27"	06°58'35.59"	52POR	2012PT	MN583197	1
vettonica	Tagus	Erges/Erges	Serrinha	PT	39°46'23.27"	06°58'35.59"	52POR	2013PT	MN583198	1
vettonica	Tagus	Erges/Erges	Serrinha	PT	39°46'23.27"	06°58'35.59"	52POR	2278PT	MN583199	1
vettonica	Tagus	Erges/Erges	Cabeça Queimado	PT	39°50'12.61"	06°56'34.85"	54POR	2039PT	MN583200	1
vettonica	Tagus	Erges/Rib Arades	Salvaterra do Extremo	PT	39º51'32.11"	06º55'26.45"	55POR	2044PT	MN583201	1
vettonica	Tagus	Erges/Rib Arades	Salvaterra do Extremo	PT	39º51'32.11"	06º55'26.45"	55POR	2045PT	MN583202	1
vettonica	Tagus	Erges/Rib Arades	Salvaterra do Extremo	PT	39º55'29.67"	06º57'15.45"	56POR	2056PT	MN583203	1
vettonica	Tagus	Erges/Rib Arades	Salvaterra do Extremo	PT	39º55'29.67"	06º57'15.45"	56POR	2057PT	MN583204	1
vettonica	Tagus	Aravil/Rib. Toula	_	PT	39º52'06.60"	07º07'46.16"	59POR	2283PT	MN583205	1
vettonica	Tagus	Aravil/Aravil	Alcafozes	PT	39º52'19.81"	07º08'19.43"	60POR	2108PT	MN583206	1
vettonica	Tagus	Aravil/Aravil	Cegonhas Novas	PT	39º43'59.50"	07º11'23.58"	63POR	2284PT	MH842916	5
vettonica	Tagus	Aravil/Aravil	Cegonhas Novas	PT	39º43'59.50"	07º11'23.58"	63POR	2131PT	MN583207	1
vettonica	Tagus	Aravil/Aravil	Cegonhas Novas	PT	39º43'59.50"	07º11'23.58"	63POR	2285PT	MN583208	1
vettonica	Tagus	Aravil/Aravil	Cegonhas Novas	PT	39º43'59.50"	07º11'23.58"	63POR	2286PT	MN583209	1
vettonica	Tagus	Aravil/Aravil	Cegonhas Novas	PT	39º43'59.50"	07º11'23.58"	63POR	2287PT	MN583210	1

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Table 1. (continued).

Cobitis species	*Drainage*	*Tributary/River*	*Locality*	*Country*	*Longitude*	*Latitude*	*LOC ID*	*Voucher*	*IDGENBank*	*Ref.*
vettonica	Tagus	Aravil/Aravil	Cegonhas Novas	PT	39º43'59.50"	07º11'23.58"	63POR	2288PT	MN583211	1
vettonica	Tagus	Ponsul/Alfrividas	Alfrividas	PT	39°43'01.59"	07°32'21.90"	46POR	1910PT	MN583182	1
vettonica	Tagus	Ponsul/Alfrividas	Alfrividas	PT	39°43'01.59"	07°32'21.90"	46POR	2291PT	MN583183	1
vettonica	Tagus	Ponsul/Alfrividas	Alfrividas	PT	39°43'01.59"	07°32'21.90"	46POR	2292PT	MN583184	1
vettonica	Tagus	Ponsul/Alfrividas	Alfrividas	PT	39°43'01.59"	07°32'21.90"	46POR	2293PT	MN583185	1
vettonica	Tagus	Ponsul/Alfrividas	Alfrividas	PT	39°43'01.59"	07°32'21.90"	46POR	2295PT	MN583186	1
vettonica	Tagus	Alagón/Ambroz-Caparro	Zarza de Granadilla	SP	40°12'47.75"	06°01'03.20"	_	_	_	6
vettonica	Tagus	Alagón/Ambroz-Caparro	Zarza de Granadilla	SP	40°12'48.19"	06°01'03.82"	_	_	_	6
vettonica	Tagus	Alagón/Jerte	Navaconcejo	SP	40°10'23.02"	05°50'28.39"	_	_	_	6
vettonica	Tagus	Alagón/Jerte	Navaconcejo	SP	40°10'24.29"	05°50'29.45"	_	_	_	6
vettonica	Tagus	Alagón/Árrago	Descargamaría	SP	40°18'08.44"	06°29'11.57"	_	_	_	7
vettonica	Tagus	Alagón/Los Ángeles	Pinofranqueado	SP	40°18'02.38"	06°19'47.59"	_	_	_	7
vettonica	Tagus	Alagón/Acebo	Hoyos	SP	40°10'27.48"	06°41'30.03"	_	_	_	7
vettonica	Tagus	Alagón/Ladrillar	Las Mestas	SP	40°25'23.69"	06°08'34.51"	_	_	_	7
vettonica	Tagus	Alagón/Alagón	Torre de Don Miguel	SP	40°13'02.42"	06°34'34.72"	_	_	_	7
vettonica	Tagus	Alagón/Morcillo	Coria	SP	39°59'03.91"	06°30'23.05"	_	_	_	7
vettonica	Tagus	Alagón/Alagón	Carcaboso	SP	40°03'03.13"	06°12'22.23"	_	_	_	7
vettonica	Tagus	Alagón/Gata	Gata	SP	40°12'15.94"	06°38'54.34"	_	_	_	7
vettonica	Tagus	Alagón/Hurdano	Vegas de Coria	SP	40°23'04.88"	06°11'06.44"	_	_	_	6
vettonica	Tagus	Alagón/San Blas	Gata	SP	40°12'47.04"	06°38'27.72"	_	_	_	6
vettonica	Duero	Águeda/Mayas	El Sahugo	SP	40°23'41.41"	06°34'14.82"	_	_	_	6
vettonica	Duero	Águeda/Agadón	Agallas	SP	40°29'03.19"	06°23'52.08"	_	_	_	8
vettonica	Duero	Águeda/Águeda	Ciudad Rodrigo	SP	40°34'48.75"	06°30'53.55"	_	_	_	8
vettonica	Duero	Águeda/Águeda	Villar de Flores	SP	40°19'48.22"	06°44'02.31"	_	_	_	8

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Table 1. (continued).

Cobitis species	*Drainage*	*Tributary/River*	*Locality*	*Country*	*Longitude*	*Latitude*	*LOC ID*	*Voucher*	*IDGENBank*	*Ref.*
bilineata	Ter	Bañolas Lake	Bañolas	SP	_	_	_	B1	AY860114	3
bilineata	Arno	Arno	Arezzo	IT	_	_	_	318TA	AY860113	3
calderoni	Duero	Arlanzón	Burgos	SP	_	_	_	91CC	AY860115*	3
calderoni	Duero	Riaza	Saldana de Ayllón	SP	_	_	_	678	AY860117	3
maroccana	Kherrouba	Kherrouba	_	MA	_	_	_	21CMKHE	AF263075	4
maroccana	Kherrouba	Kherrouba	_	MA	_	_	_	1CMKHE	AF263076	4
paludica	Cavado	Cavado	Braga	PT	_	_	_	1742AT	AY860185	3
paludica	Duero	Duratón	Carrascal del Río	SP	_	_	_	919CP	AY860129	3
paludica	Guadiana	Valparaíso	Casas de Villaba Perea	SP	_	_	_	23A	AY860161	3
paludica	Guadiana	Gargáligas	Obando	SP	_	_	_	401ES	AY860145	3
paludica	Guadiana	Peralosa	Piedrabuena	SP	_	_	_	2399	AY860153	3
paludica	Guadiana	Jarero	Berlanga	SP	_	_	_	288	AY860148	3
paludica	Mondego	Arunca	Saure	PT	_	_	_	1538AT	AY860194	3
paludica	Samarra	Samarra	Samarra	PT	_	_	_	P2	AY860165	3
paludica	Sizandro	Sizandro	Torres Vedras	PT	_	_	_	1547AT	AY860166	3
paludica	Tagus	Pusa	San Martin de Pesa	SP	_	_	_	879AT	AY860176	3
paludica	Tagus	Fuente del Roble	Talayuela	SP	_	_	_	584CP	AF263074*	3
paludica	Tagus	Rib. Aurela	Santiago de Alcántara	SP	_	_	_	539AT	AY860171	3
paludica	Tagus	Cuervo	Santa María del Val	SP	_	_	_	990AT	AY860172	3
paludica	Tagus	Sangrera	San Bartolomé de las Abiertas	SP	_	_	_	885	AY860177	3
paludica	Tagus	Torcón	Humanes	SP	_	_	_	858AT	AY860179	3
paludica	Tagus	Guajaraz	Layos	SP	_	_	_	854AT	AY860174	3

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Genomic DNA was extracted from the new specimens using the ChargeSwitch gDNA Micro Tissue Kit (Invitrogen) and the gene was amplified using the primers GluDGL and H16460 in 25µl PCR reactions as previously described by Perdices et al. (2008, 2016). Purified PCR products were sequenced directly using the same primers used for PCR amplifications. (GenBank accession numbers: MN583182-MN583223).

For the gene tree reconstructions, we analysed the complete cyt b dataset using maximum likelihood (ML) and Bayesian Inference (BI) approaches. We analysed a total of 72 complete *Cobitis* cyt b sequences. All sequences were revised with Sequencher ver. 4.10 (Gene Codes Inc.), and none of the sequences had gaps or stop codons. The ML trees were calculated in RAxML through its graphical interface RAxMLGUI 1.3 using the GTR+I+G model of evolution with 1,000 bootstrap (BS) replicates to assess robustness. The BI analyses were implemented in Mr. Bayes v3.2.6 (Ronquist et al., 2012). For this analysis, four Markov chains Monte Carlo (MCMC) were run simultaneously for 2 million generations and sampled every 100 generations. Log-likelihood stability was attained after 10,000 generations, and the first 10% of trees were discarded as burn-in. The remaining trees were used to compute a 50% majority rule consensus tree. Robustness of the inferred tree was assessed by posterior probability (pb). Neighbor joining trees were also calculated. Kimura 2-parameter (K2P) genetic distances, estimated using PAUP*, were directly compared against those reported for *Cobitis* species in the COI barcode reference database, which were used by Geiger et al. (2014) in their study of Mediterranean freshwater fishes.

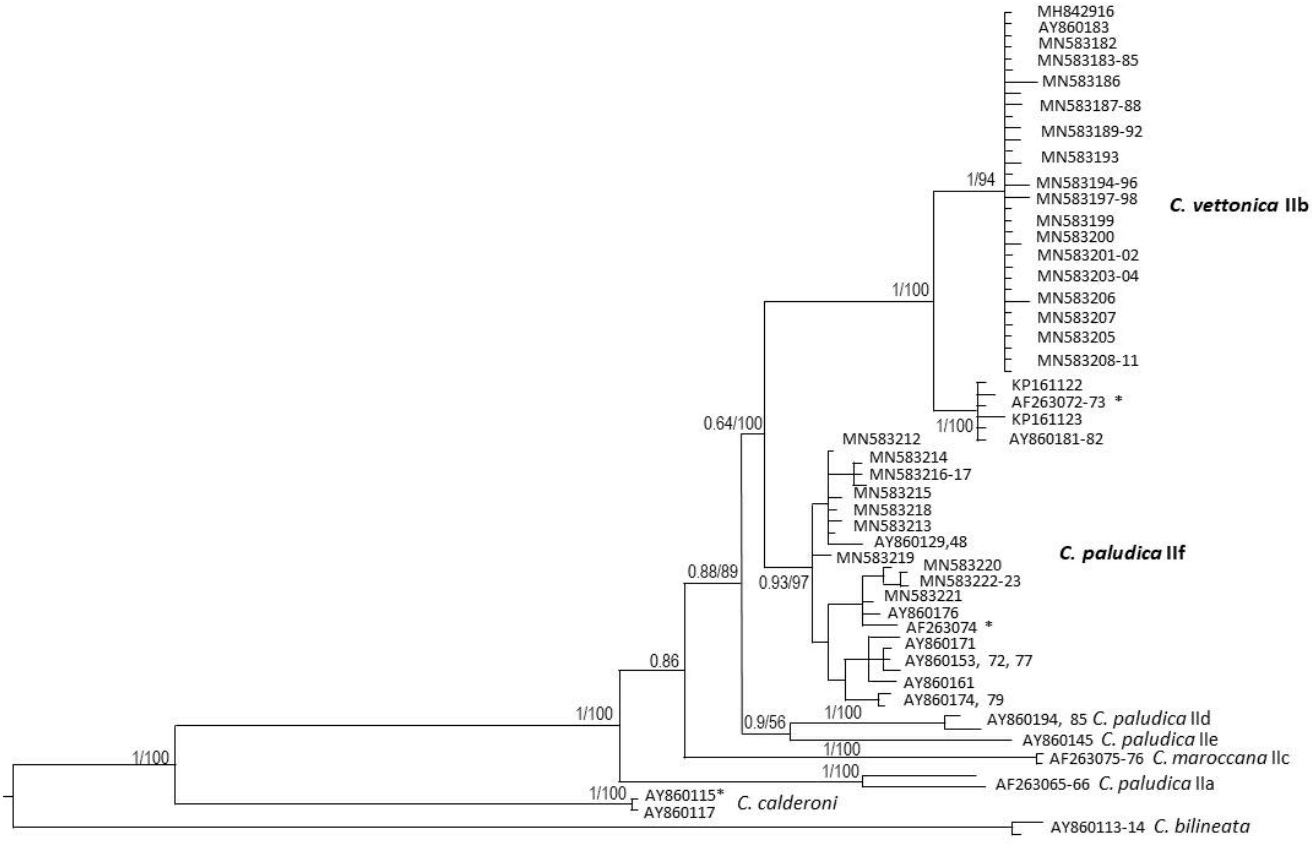

Figure 3. Phylogenetic relationships of the Iberian *Cobitis* specimens based on the mitochondrial cyt b gene. Bayesian tree (50% majority rule consensus) with values on branches corresponding to Bayesian posterior probabilities and maximum likelihood bootstrap values. * denotes specimens from the type locality.

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To further assess the genetic relationships among the *C. vettonica* individuals from different populations, a haplotype network was constructed using a minimum spanning algorithm in PopART (http://popart.otago.ac.nz) and visualized in Figtreev1.4.2. (http://tree.bio.ed.ac.uk).

RESULTS AND DISCUSSION

We analysed a total of 72 complete cyt b sequences: of the 1,140 base pairs (bp), 867 characters were constant. Base frequencies were homogeneous across all variable sites and did not differ significantly among sequences (X^2= 11.25, df= 213, P= 1). Cytochrome b sequences showed an anti-G bias (G= 14.9%, A= 27.2%, C= 26.1%, T= 31.8%), characteristic of mitochondrial genes.

The results of the ML and BI analyses were congruent. *Cobitis calderoni* resolved as the most differentiated endemic Iberian species with its haplotypes differing by a mean of 9.7 ± 0.3% (K2P range: 8.34 - 10.47%). All of the other Iberian *Cobitis* species grouped together in a strongly supported clade (100 BS, 1 pb) (Figure 3). Within this clade, six divergent lineages could be identified. Mean sequence divergence among lineages was 3.51 ± 1.1% (K2P range: 2.33 - 6.62%) (Table 2), while the mean within group distance was 0.43 ± 0.42% (K2P range: 0 - 2.1%), which is consistent with the intraspecific COI divergence estimated for Mediterranean *Cobitis* species (0.82%) (Geiger et al., 2014).

The six lineages identified in our study correspond to the six mitochondrial lineages (IIa–IIf) previously observed by Doadrio & Perdices (2005) and confirm, with high support (97 - 100 BS, 0.93 -1 pb), that *C. paludica* is not a monophyletic group comprised of four lineages (IIa, IId, IIe and IIf), with *C. vettonica* (IIb) and *C. maroccana* (IIc) comprising the remaining two lineages. Of the 42 new specimens analysed from the Tagus drainage in Portugal, 30 clustered within the *C. vettonica* clade and 12 in *C. paludica* lineage IIf. We found two sublineages within the *C. vettonica* clade: one corresponding to a group of specimens from the Duero and Alagón tributaries and the other, which included all of the *C. vettonica* haplotypes found in this study, to specimens from the other Tagus tributaries (Figure 3).

Table 2. K2P genetic distances among major lineages (IIa-IIe) of the Iberian *Cobitis* species based on the mitochondrial cyt b: C.p., *C. paludica* (IIa, IId-IIf); C. v., *C. vettonica* (IIb); and C. m., *C. maroccana* (IIc). Mean, ± standard deviation, (range).

	C. p. IIa	C. v. IIb	C. m. IIc	C. p. IId	C. p. IIe	C. p. IIf
C. p. IIa	2.1±0.0 (-)					
C. v. IIb	6.1±0.1 (5.8-6.4)	0.4±0.4 (0.0-1.4)				
C. m. IIc	6.6±0.1 (6.5-6.6)	5.6±0.1 (5.3-5.9)	0.0±0.0 (-)			
C. p. IId	5.5±0.3 (5.1-5.8)	3.9±0.1 (3.6-4.3)	5.0±0.1 (5.0 -5.15)	0.3±0.0 (-)		
C. p. IIe	5.7±0.1 (5.6-5.7)	4.2±0.1 (4.0-4.5)	5.5±0.0 (-)	3.5±0.1 (3.4-3.6)	0.0±0.0 (-)	
C. p. IIf	4.6±0.2 (4.2-5.0)	2.9±0.3 (2.3-3.5)	4.5±0.1 (4.3-4.7)	2.7±0.2 (2.3-3.1)	3.4±0.2 (3.1-3.6)	0.6±0.3 (0.0-1.1)

A total of 14 haplotypes was detected in *C. vettonica* (H1–H14) (Table 3). The most frequent haplotype was H1, which was found in 21 of the new specimens collected from the Erges, Aravil and Ponsul populations. Haplotype H1 was also found in two other specimens whose sequences were available in GenBank (both form the Tagus drainage: AY860183 from the Erjas River in Spain and MH842916 from the Aravil River in Portugal), but H1 was absent in specimens from the other populations analysed in this study (i. e., the Duero and Alagón). Three haplotypes (H2–H4), identified from the cyt b sequences available in GenBank, were private to the Duero and Alagón populations (Table 3). The haplotype network analysis revealed the 14 *C. vettonica* haplotypes structured into two major groups (Figure 4). The first group contained the common haplotype (H1) plus 10 others (H5–H14) present from the Tagus tributaries in Portugal and the Erjas River (= Erges) in Spain. The second group contained the three haplotypes (H2–H4) exclusive to specimens from the Alagón tributary (of the Tagus drainage) and the Duero drainage.

We found *C. vettonica* and C. *paludica* in sympatry in six of the new Tagus localities in Portugal (Table 1 and Figure 1). Our phylogenetic analysis confirmed the presence of *C. vettonica* in at least three Tagus tributaries in Portugal, namely Aravil, Ponsul and Erges. Although we found *C. paludica* (IIf) in the Ocreza River in Portugal, the westernmost Tagus tributary analysed in our study, which runs adjacent to the three other tributaries surveyed, we did not find *C. vettonica* in this locality. However, its absence should be confirmed with more detailed sampling as we only analysed four specimens from the locality. Likewise, given the confirmed presence of *C. vettonica* in the Águeda River, detailed sampling of the adjacent Portuguese Duero tributary Côa should be conducted.

Table 3. Haplotype diversity in *Cobitis vettonica* specimens (H1-H14). * denotes specimens from the type locality

Drainage/ Tributary	N	Haplotype number	Individual ID
Duero/ Águeda	2	H2	KP161123, AY860181
Tagus/Alagón	2	H2	KP16112, AF263073*
	1	H3	AF263072*
	1	H4	AY860182
Tagus/Erges (=Erjas or Eljas)	10	H1	AY860183, MN583197-98, MN5831202-04, MN583189, MN583192-94
	1	H6	MN583187
	1	H7	MN583188
	2	H8	MN583201, MN583190
	1	H9	MN583191
	1	H10	MN583193
	1	H11	MN583195
	1	H12	MN583196
	1	H13	MN583200
Tagus/Aravil	7	H1	MH842916, MN583205, MN5832207-11
	1	H14	MN583206
Tagus/Ponsul	4	H1	MN583182-85
	1	H5	MN583186

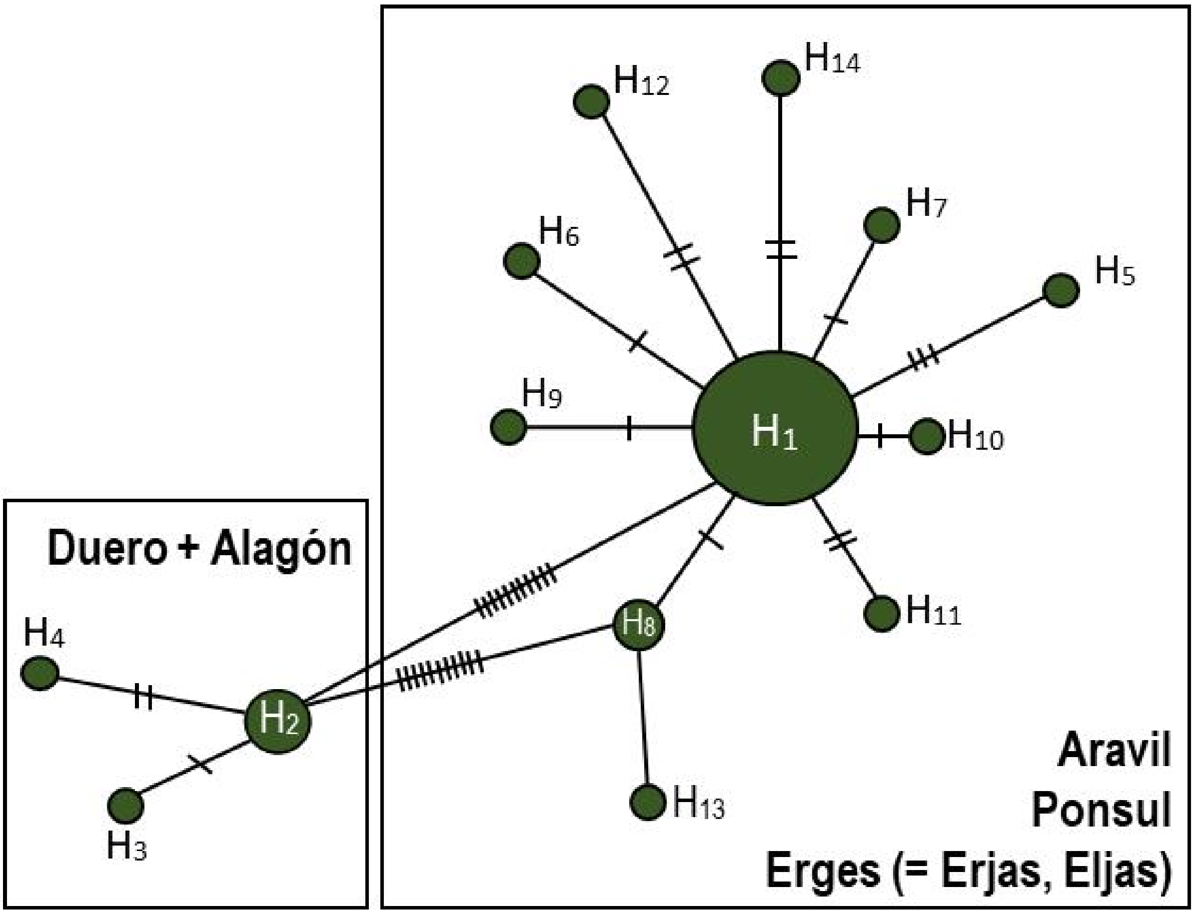

Figure 4. A median-joining haplotype network showing the 14 haplotypes found in *C. vettonica* specimens. Haplotypes H2-H4, identified from GenBank sequences, were found in the specimens from the Duero drainage and the Alagón River (Tagus drainage) in Spain. Haplotypes H1 and H5-H14 correspond to the ones found in the *C. vettonica* specimens analysed from the Tagus drainage in Portugal. Haplotype H1 was also found in two other specimens whose sequences were available in GenBank (AY860183 and MH842916, both from the Tagus drainage).

In terms of its habitat in Portugal, *C. vettonica* was collected from shallow waters that had either no or a slow current at the time of collection (spring to summer) and from sandy or stone substrates (Figure 5). This is consistent with its habitat type in Spain. Overall, our data extend the current known distribution of *C. vettonica* (IIb) to include Portugal. Its current Iberian range now includes four Iberian tributaries of the Tagus drainage (Alagón River and Eljas or Erjas River in Spain, and Aravil, Ponsul, and Erges =Eljas, Erjas rivers in Portugal) and the Duero drainage (Águeda River in Spain) (Figure 1 and Table 1). The current distribution of *C. vettonica* spans through rivers of two major Iberian drainages (Tagus and Duero) that are currently isolated but share a common paleographical history. The Alagón River was originally a tributary of the Duero that was captured by the Tagus basin (Casas-Sainz & De Vicente, 2009). The current distribution of *C. vettonica* is a biotic documentation of that historical geographic connection, further supporting to the numerous freshwater fish particularities already known for this area (Doadrio et al., 2011, LIFE Cipriber, 2013).

The high genetic diversity found in *C. vettonica* populations, its haplotype distribution and its geographic range support

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the natural distribution of *C. vettonica* in Portugal. The species may have passed undetected in the country due to morphological similarities with *C. paludica*. Although our study has enlarged the distribution range of *C. vettonica* in the Iberian Peninsula, its restricted range in Portugal suggests a threatened status, similar to its critical situation in Spain. The species is currently on the Spanish Red List of Threatened Species and, given the data presented here, it should also be added to the Portuguese Red List of Vertebrates. In addition, more expansive monitoring studies are needed to complement the findings presented here and to further increase our knowledge of *C. vettonica* in the Iberian Peninsula for its conservation.

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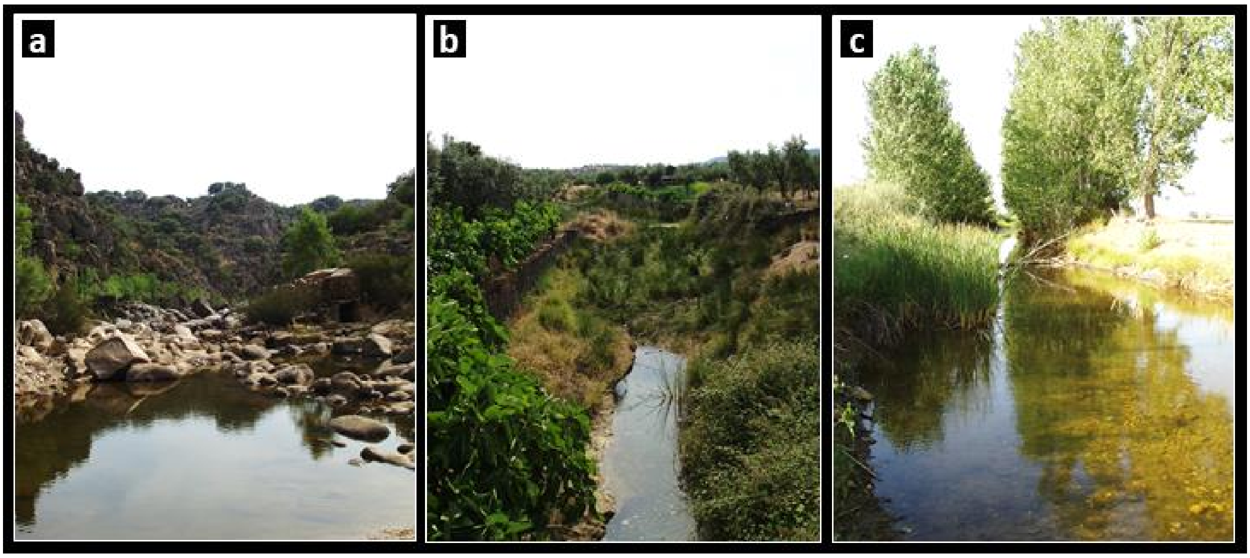

Figure 5. Sampling localities in which *Cobitis vettonica* and *Cobitis paludica* were found living in sympatry. Habitat types include areas characterized primarily by stones: a) Erges River (=Erjas or Eljas) (LOC ID 50POR), and others by sand and stone: b) Ponsul River (LOC ID 46 POR) and c) Aravil River (LOC ID 60POR) (locality details found in Table 1).

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