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OXFORD

Review

Molecular insights into spined loaches (Cobitidae: *Cobitis*) reveal the complex evolutionary history of freshwater fishes in the Iberian Peninsula and North Africa

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ABSTRACT

Palaeogeographic and tectonic rearrangements of rivers have significantly influenced the phylogeography of Iberian and North African freshwater fishes, but their impact remains unclear. We revisit the phylogeographic relationships and diversification timelines of *Cobitis* (spined loaches, family Cobitidae) on both sides of the Strait of Gibraltar. Sampling 230 sites across 41 river basins, we analysed genetic variability using haplotype networks of two mitochondrial (*Cytb* and *COI*) and two nuclear (*S7* and *RAG-1*) genes. A time-calibrated multilocus phylogeny was constructed and compared with the evolution timeline of the Iberian hydrographic network. Our results indicate that Iberian and North African *Cobitis* are

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monophyletic at the mitochondrial, but not at the nuclear level. Three new mitochondrial lineages, potentially new species, were identified in Iberia (2) and Morocco (1). Most Iberian *Cobitis* show wide distributions and frequent sympatry in major basins. Diversification started in the Mid-Late Miocene (7.9–8.9 Mya), with major speciation during the Late Miocene–Pliocene transition (6.5–5.7 Mya). Palaeographic changes in river basins, from endorheic to exorheic regimes, probably affected *Cobitis* speciation. Tectonic strike-slip faults probably influenced isolation, genetic mixing, and incomplete lineage sorting, particularly along Atlantic margins. These results highlight the interplay of geological and evolutionary processes in shaping freshwater fish diversity.

Keywords: Strait of Gibraltar; phylogeography; mitochondrial and nuclear genes; tectonic strike-slip faults

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INTRODUCTION

The Mediterranean peninsulas are home to nearly half of the native European freshwater fishes, including at least 250 endemic species (Smith and Darwall 2006, Freyhof and Brooks 2011). This figure would increase by over 50% when accounting for the newly described endemic species over the past two decades, with more than 20 of them in the Iberian Peninsula alone (SIBIC 2017, http://www.cartapiscicola.es/#/home). Such a high level of endemism reflects the partial or complete isolation of river basins in the Mediterranean peninsulas from those in Central Europe and their highly fragmented hydrographic networks (Bănărescu 2001). The Iberian Peninsula has been isolated from the rest of Europe by the Pyrenees since the Miocene (Stange *et al.* 2016), and since then, primary freshwater fishes (hereafter FWF) that are strictly intolerant to salt water (Myers 1966) have been diverging in allopatry, resulting in a high proportion (over 80%) of endemic FWF species (Doadrio *et al.* 2011).

Most Iberian FWF species are restricted to one or a few adjacent basins (Doadrio 2001, Filipe *et al.* 2009). Their disjunct distributions are generally explained as a consequence of the endorheic origin of most Iberian basins during the Miocene (Sousa-Santos *et al.* 2019), further reinforced by historical barriers, such as basin boundaries, orogenic processes, and tectonic faulting (Gómez and Lunt 2008, Filipe *et al.* 2009). The shift to an exorheic regime, where basins become open and began draining to the sea in the Pliocene–Pleistocene, further contributed to these distribution patterns (Sousa-Santos *et al.* 2019). The natural co-occurrence of native FWF species across basins can be explained by two non-exclusive hypotheses. Under the first hypothesis, headwater stream captures, promoted by tectonic adjustments, connected independent basins, enabling FWF species that had previously diverged in allopatry to coexist. The second one proposes that past, climate-induced marine regression episodes promoted the connection of adjacent independent basins. Under both hypotheses, Iberian FWF would have been able to disperse across previously isolated basins, thus promoting species' exchange and the colonization of previously unconnected rivers (Dias *et al.* 2000, Schönhuth *et al.* 2015, Lima *et al.* 2017, Shelley *et al.* 2020).

Iberian primary FWF belong to the large and widespread order Cypriniforms, which includes only two genera occurring on both sides of the Strait of Gibraltar: *Luciobarbus*, consisting of more than 10 species, has a wide North African distribution (Morocco, Argelia, Tunisia), and *Cobitis*, which is represented by a single species (*Cobitis maroccana*), with a restricted range in northern Morocco. The trans-continental distribution of *Luciobarbus* and *Cobitis* probably resulted from the dispersal of lineages via connections between some southern Iberian hydrographic networks and North Africa during the Miocene (the Lago Mare phase) before the opening of the Strait of Gibraltar, approximately 5.3 million years ago (Mya) (Machordom and Doadrio 2001, Doadrio and Perdices 2005, Gante 2011). A more recent connection between the Iberian Peninsula and North Africa during the Late Pleistocene, after the opening of the Strait of Gibraltar, has been proposed to explain the distribution of other trans-continental (terrestrial) species, such as *Chamaleo chamaleon* and *Hyla meridionalis* (Harris *et al.* 2002, Carranza *et al.* 2004, 2006). However, evidence indicates that human-mediated translocations played a role in the present distributions of these species (Paulo *et al.* 2002, Recuero *et al.* 2007). The influence of the recent connection on the biogeography of more water-dependent organisms, such as FWF, remains unclear.

Spined loaches of the genus *Cobitis* are small in size, benthic, and have limited dispersal ability and a complex taxonomy (Doadrio and Perdices 2005, Kottelat 2012, Perdices *et al.* 2016). The genus is currently found throughout the Palaearctic, including the Mediterranean peninsulas and North Africa (Perdices *et al.* 1995), and frequent occurrences of hybrid complexes are known throughout its distribution range (Bohlen and Rab 2001). Based on previous analyses of mitochondrial DNA (mtDNA) cytochrome *b*, seven endemic species have been described for the Iberian Peninsula: *C. almadae*, *C. atlantica*, *C. calderoni*, *C. haasi*, *C. mellaria*, *C. paludica*, and *C. vettonica*; while only one endemic species, *C. maroccana*, has been documented in North Africa (Doadrio and Perdices 2005, Perdices *et al.* 2016, Doadrio *et al.* 2024). The distribution ranges of the Iberian spined loaches are highly heterogenous. *Cobitis calderoni* and *C. paludica* are found across the northern and central-southern Iberian basins, respectively, whereas *C. almadae*, *C. atlantica*, *C. haasi*, and *C. mellaria* are restricted to a single or a few adjacent basins (Doadrio *et al.* 2024). *Cobitis maroccana* is restricted to only two basins in northern Morocco (Azeroual *et al.* 2000). Except for *C. vettonica*, which is sympatric with *C. paludica* throughout its range (Doadrio *et al.* 2011, Perdices and Coelho 2020), the species are allopatric. Hybridization has been documented only between sympatric *C. paludica* and *C. vettonica* (Corral-Lou *et al.* 2022), although it has also been hypothesized for other species pairs, such as *C. paludica* and the southern lineage of *C. atlantica* (Doadrio *et al.* 2024), suggesting that allopatric diversification is not the only factor influencing the current distribution of *Cobitis* species (Doadrio and Perdices 2005).

In this study, we assess the phylogenetic status and phylogeography of the Iberian and Moroccan *Cobitis* species based on mitochondrial [cytochrome *b* (*Cytb*); cytochrome *c* oxidase subunit I

(*COI*)] and nuclear [recombination activation gene 1 (*RAG-1*); ribosomal protein s7 gene (*S7*)] genes to infer the biogeographical processes that drove their current distribution. To address historical connections, the analysis of the two closely related species *C. maroccana* and *C. mellaria* inhabiting either side of the Strait of Gibraltar will be particularly useful. In addition, we use updated information on species' distributions in the major Iberian basins to identify and evaluate past river connectivity and palaeobasin evolution, and to infer recent scenarios of river connectivity. Through the mitochondrial and nuclear gene analyses, we also delimit the distribution of the Iberian *Cobitis* lineages and identify areas with admixed individuals, constituting potential hybrid zones.

MATERIALS AND METHODS

Study area and sample collection

A total of 690 new specimens were analysed and assigned to one of the eight *Cobitis* species described for the Iberian Peninsula and Morocco: *C. almadae*, *C. atlantica*, *C. calderoni*, *C. haasi*, *C. maroccana*, *C. mellaria*, *C. paludica*, and *C. vettonica*. Individuals were collected from 230 sites distributed across 41 river basins covering the main Iberian basins and the two Moroccan river basins inhabited by *C. maroccana* (Fig. 1; Supporting Information, Table S1). Five specimens of *C. bilineata* from native Italian and introduced Iberian localities were sequenced and used as outgroups. Available *Cytb* sequences (140) and *COI* (8) from Iberian and Moroccan specimens from previous studies (Perdices and Coelho 2020, Perdices and Doadrio 2001, Doadrio and Perdices 2005, Clavero *et al.* 2023) were also included in the analyses, and for some of these specimens, the mitochondrial (*Cytb*, *COI*) and nuclear (*RAG-1*, *S7*) genes were newly sequenced and added to the dataset (Supporting Information, Table S1). A map of sampling sites was constructed using QGIS software v.3.0 (http://www.qgis.org) based on geographical coordinates recorded by GPS at the time of sampling or raw information from fieldwork notes was subsequently post-transferred to coordinates using Google Earth (Supporting Information, Table S1).

Spined loaches were sampled by electrofishing, hand, or fyke netting. Tissue samples were taken by clipping a small portion of the caudal or pectoral fins. Some of the collected specimens were euthanized with an overdose of the anaesthetic MS-222 (tricaine methanesulfonate), clove oil/eugenol, or 95% ethanol and immediately preserved in 95% ethanol in the field. The study was conducted under permits granted by the governmental administrations of Portugal, Spain, and Morocco, and conformed to international guidelines and national legislation regarding animal capture, handling, and experimentation for scientific purposes.

DNA extraction, polymerase chain reaction amplification, and sequencing

DNA extraction and polymerase chain reaction (PCR) amplification of the four genes (*Cytb*, *COI*, *RAG-1*, and *S7*) were performed in three laboratories: Museo Nacional de Ciencias Naturales (MNCN, Madrid), Centro de Investigação em Biodiversidade e Recursos

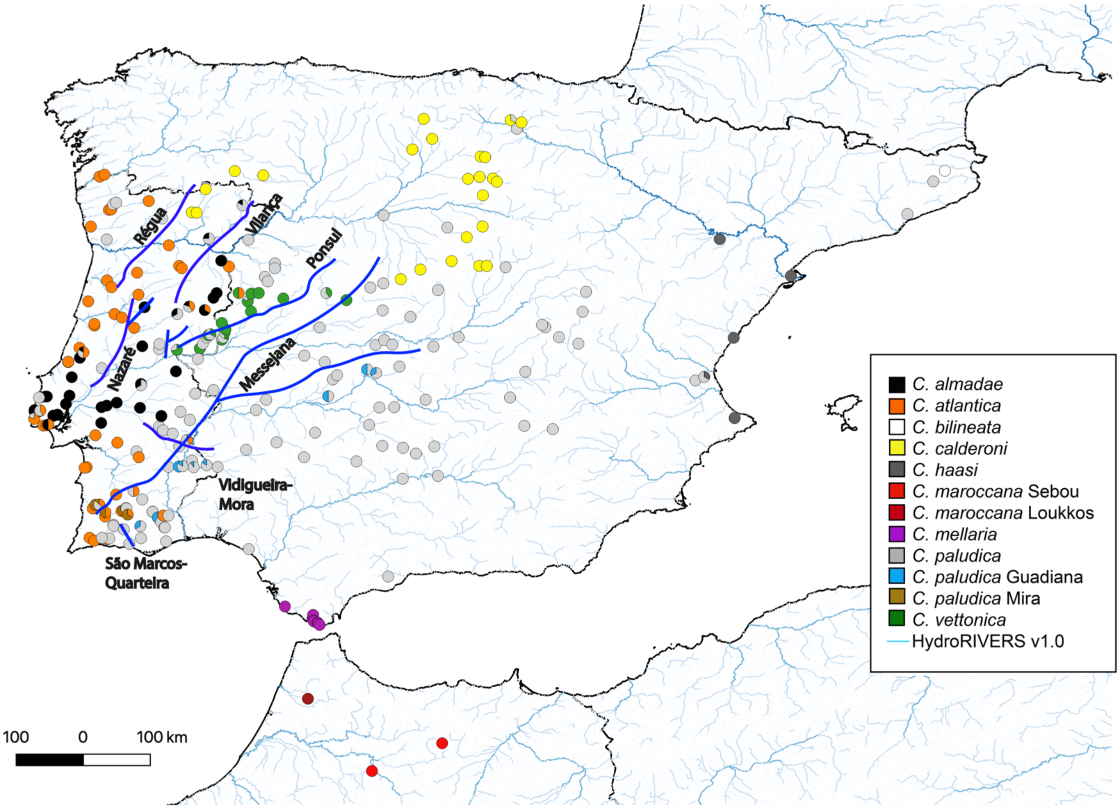

Figure 1. Map of the Iberian Peninsula and northern Morocco showing the main Atlantic faults (based on Pais *et al.* 2012) and hydrographical networks. Sampling locations and geographical distribution of the Iberian and Moroccan *Cobitis* species analysed, including the three new *Cobitis* lineages and the outgroup (*C. bilineata*), are indicated by the different colours (colour code corresponds across figures). Delineation of the distribution area of the *Cobitis* species or lineages is based on the *Cytb* analysis.

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Genéticos (CIBIO-InBIO, Porto), and Centre for Advanced Studies (CEAB, Blanes). Genomic DNA was extracted using the Charge Switch gDNA Microtissue Kit (Invitrogen, Inc.), EasySpin Genomic DNA Tissue Kit (Citomed, Lisbon, Portugal), or QIAGEN DNeasy Blood and Tissue Kit (QiagenTM, Hilden, Germany), according to the manufacturer's protocol (primers and PCR conditions provided in Supporting Information, Table S2). All PCR products were checked on 0.8%–1% or 2% agarose gels and cleaned with EXOSAP according to the manufacturer's protocol. In the case of *S7*, double bands were observed. The bands were gel excised from 1% low melting point agarose gels and then cleaned with EXOSAP. Sequencing was performed in-house using an Applied Biosystems 3130xl Genetic Analyzer (CIBIO-InBIO) or by the sequencing service, Macrogen, Inc. (Spain) or Secugen, Inc. (Spain) using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) and the forward and reverse primers of each marker. The fragment size sequenced was 1103 base pairs (bp) for *Cytb*, 605 bp for *COI*, 1373 bp for *RAG-1* (exon 3), and 564 bp (including gaps) for *S7*. The new sequences were deposited in GenBank (Accession Numbers PX552905-PX553004, PX553012-PX553891, PX579137-PX579456, PX617885-PX618015, PX618252-PX618438, PX63704-PX63708).

Phylogenetic and phylogeographic analyses

Chromatograms and alignments were verified using SEQUENCHER v.4.0 (Gene Codes Corporation, Inc.) or SEQSCAPE v.2.5 (Applied Biosystems). For each gene, nucleotide composition was examined for variable sites, and a $\chi 2$ homogeneity test of base frequencies for all positions was checked using PAUP_4.0a (Swofford 2002). No gaps were found for *Cytb*, *COI*, and *RAG-1* sequences; six gaps with 26 missing nucleotides were found in the *S7* sequences. A collapse haplotype dataset was obtained for the mitochondrial genes in DnaSP v.5.10.1 (Librado and Rozas 2009) for the phylogenetic and phylogeographic analyses. However, due to the low quality of some sequences at the beginning and end of the *Cytb*, two datasets were generated: a full-length dataset of fragments with the maximum size, which included haplotypes representative of all the lineages for the phylogenetic analysis (*Cytb*: 1103 bp, $N_{hap} = 225$; *COI*: 605 bp, $N_{hap} = 112$), and a short-length dataset consisting of the overall set of sequences for phylogeographic analysis (*Cytb*: 904 bp, $N_{seq} = 802$, $N_{hap} = 226$; *COI*: 605 bp, $N_{seq} = 310$, $N_{hap} = 101$). In the case of the nuclear genes (*RAG-1*: 1373 bp, $N_{hap} = 126$; *S7*: 564 bp, $N_{hap} = 162$), haplotypes were phased using the PHASE algorithm (Stephens and Donnelly 2003), as implemented in DnaSP v.5.10.1 (Librado and Rozas 2009), and following the default parameter settings.

Phylogenetic analyses were conducted using the full-length dataset of each of the four markers (*Cytb*, *COI*, *RAG-1*, and *S7*) and the two combined full-length datasets: (i) *Cytb* + *COI* (*N* = 263, 1708 bp) and (ii) *RAG-1* + *S7* (*N* = 314, 1937 bp). Maximum likelihood (ML) and Bayesian inference (BI) analyses were performed in PhyML 3.0 on the ATGC platform (Guindon *et al.* 2010) and MrBayes v.3.0 (Ronquist and Huelsenbeck 2003), respectively. For ML, we used PhyML with the BI criterion selection of the evolutionary model and 1000 bootstrap replicates to assess branch support. For BI, two runs of four Markov chains Monte Carlo (MCMC) were run simultaneously for 10–50 million generations, sampling every 1,000-5,000 generations. Log-likelihood stability was reached after 25,000 generations and the first 25% of trees were discarded as burn-in. The remaining trees were used to compute a 50% majority rule consensus tree. Posterior probability values were used to assess the robustness of the inferred tree. Majority-rule consensus trees were edited with FigTree v.1.4.4. Genetic distances (uncorrected *p*) among lineages were calculated for the *Cytb* full-length dataset using PAUP 4.0b1 (Swofford 2002) and SEQUENCER (provided by B. Kessing) for comparison with previous studies (Doadrio and Perdices 2005, Doadrio *et al.* 2024).

Haplotype network analyses of the mitochondrial (short-length datasets of *Cytb* and *COI*) and nuclear markers (*RAG-1* and *S7*) were performed in PopArt using a median-joining algorithm (Leigh and Bryant 2015), with ambiguities treated as missing data. Phylogeographic relationships of lineages were visualized using QGIS v.3.0 (www.qgis.org).

Divergence times

To estimate divergence time among *Cobitis* species and lineages, we used the mitochondrial *Cytb* and nuclear (*RAG-1* + *S7*) datasets and a relaxed molecular clock as implemented in BEAST2 v.2.7.4 (Bouckaert *et al.* 2014). The temporal origin of the mtDNA lineages was estimated using the *Cytb* haplotype matrix with 225 haplotypes (1103 bp), calibrating the molecular clock with a strong prior to the clock substitution rate of 0.0034 subs/site/Myr, following Doadrio and Perdices (2005). We then performed another analysis on the nuclear dataset (*RAG-1* + *S7*) (314 taxa, 1937 bp), setting a TMRCA prior on the clade comprising *C. maroccana* and *C. mellaria* (log-normal distribution with mean in real space, mean = 5, SD = 0.1, encompassing an interval from 4 to 6 Mya). This prior represents the hypothetical ancestral lineage that gave rise to the Iberian and North African groups after the opening of the Strait of Gibraltar and had no subsequent contact (Machordom and Doadrio 2001, Doadrio and Perdices 2005, Gante 2011). Additionally, species' tree analyses (startbeast3) were performed using the TMRCA prior, with the mtDNA, nuclear, and combined (mtDNA + nuclear) datasets. The latter failed to converge and is not shown in the results. For these analyses, we used the following specifications and priors: bModelTest module to average over substitution models during the search, optimized relaxed molecular clocks, birth and death model as the tree prior, and non-informative priors on substitution rates [either gamma distribution (0.1,1) or log-normal (1.0,0.2)] when calibrated with the TMRCA prior. We also ran haplotypic analyses (mtDNA and nDNA) with a Bayesian skyline coalescence prior to check for the effect of the tree prior on the final topology of the trees. For the haplotype analyses, simulations were run for 10^8 generations, sampling the MCMC every 10^4 generations; for the species' tree analyses, they were run for 5×10^7 generations. Convergence of the MCMC was visually assessed in TRACER v.1.7.2 (Rambaut *et al.* 2018), and the results of the analyses were synthesized and annotated in a maximum clade credibility (MCC) tree in TreeAnnotator v.2.7.4 (Drummond and Rambaut 2007).

RESULTS

Phylogenetic and phylogeographic analyses

The phylogenetic trees based on ML and BI approaches were largely congruent for each gene (Fig. 2; Supporting Information,

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Figs S1–S3). In general, the mitochondrial phylogenies showed greater resolution compared to the nuclear ones, with the *Cytb* one being the most resolved (Fig. 2) and the *RAG-1* one the least (Supporting Information, Fig. S2). In all cases, *C. calderoni* was the most differentiated taxon and the sister-species of all the remaining *Cobitis* species analysed, which formed a monophyletic group with strong support. We refer to this major group as the 'Ibero-Moroccan dimorphic *Cobitis* clade' due to their display of secondary sexual dimorphism, such as the presence of the scale of Canestrini on the male pectoral fins, whereas *C. calderoni* does not exhibit any secondary sexual character (Perdices and Doadrio 1997a, b).

Mitochondrial variation

The *COI* and *Cytb* phylogenies were congruent, though the latter was more resolved (Fig. 2; Supporting Information, Fig. S1). In these phylogenies, *C. maroccana*, which was subdivided into two lineages (*C. maroccana* from the Sebou basin, and *C. maroccana* from the Loukkos basin), with *C. mellaria* always recovered as sister-species. *Cobitis haasi* was recovered as a divergent *Cytb* lineage of *C. paludica*, though this finding was not well supported in the *COI* phylogeny. The remaining species were reciprocally monophyletic with high support but without clear sister-group relationships. Two highly divergent monophyletic lineages of *C. paludica*, *C. paludica* from the Guadiana basin and *C. paludica* from the Mira basin, were found making the species paraphyletic.

The highest mean percentage of divergence for *Cytb* (uncorrected *p*-distances, Table 1) was found between *C. calderoni* and the other *Cobitis* lineages, with values ranging from 8.04% to 10%. Within the Ibero-Moroccan dimorphic clade, the highest distance was observed between *C. maroccana* from the Loukkos basin and *C. almadae* (6.44% ± 0.13), and the lowest distances, between *C. paludica* and *C. haasi* (2.17% ± 0.24), *C. atlantica* (2.61% ± 0.31), and *C. vettonica* (2.78% ± 0.30). The divergence between the monophyletic lineages *C. paludica* from the Guadiana basin and *C. paludica* from the Mira basin and the remaining Ibero-Moroccan *Cobitis* ranged between 3.01% ± 0.21 (*C. paludica* from the Mira basin vs. *C. paludica*) and 5.50% ± 0.29 (*C. paludica* from the Guadiana basin vs. *C. maroccana* from the Loukkos basin).

The median-joining haplotype networks for both mitochondrial gene results were consistent with the phylogenetic trees (Figs 3–4), showing a clear segregation of *C. calderoni* from the other species and lineages. Notably, all species exhibited a high number of specific *Cytb* and *COI* haplotypes, with *C. paludica* being the most diverse.

In the *Cytb* network (Fig. 3), a total of 226 haplotypes were observed. *Cobitis paludica* had a central position in this network, with haplotypes differing by 18 to 29 mutations from those of the other species and lineages of the Ibero-Moroccan dimorphic *Cobitis* clade. The network structure within of *C. calderoni*, *C. almadae*, *C. atlantica*, *C. mellaria*, *C. vettonica*, and *C. paludica* from the Mira basin showed between one and four most common haplotypes, with other con-specific haplotypes linked by 1 to 15 mutations. *Cobitis paludica* from the Guadiana basin and *C. maroccana* from the Sebou and Loukkos basins showed a decentralized configuration, with almost every individual having a unique haplotype, differing by 1 to 10 mutations from the others. For *C. maroccana*, the network was split into two branches (*C. maroccana* from the Sebou basin and *C. maroccana* from the Loukkos basin), consistent with the phylogenetic results. For *C. almadae*, two ancestral *Cytb* haplotypes (H26, H28) were found dispersed across the Duero, Tagus (especially the Sorraia sub-basin), Mondego, Lis, and Samarra basins. Other derived, less frequent haplotypes were found in the Sizandro and other Tagus sub-basins. For *C. atlantica*, four common haplotypes (H30, H33, H34, H35) were distributed throughout the Atlantic basins of Minho, Cávado, Duero, Vouga, Mondego, Lis, Alcoa, Lizandro, Tagus (Jamor, Maior, Laje, Barcarena, Zézere), Sado, Melides, Mira, and Lagos. Other less common haplotypes were found in the Junqueira, Arade, Guadiana, and Alvor basins. For *C. paludica*, the ancestral *Cytb* haplotype (H10) was widespread across the major basins of the Duero, Tagus, Guadiana, and Guadalquivir, as well as medium-minor

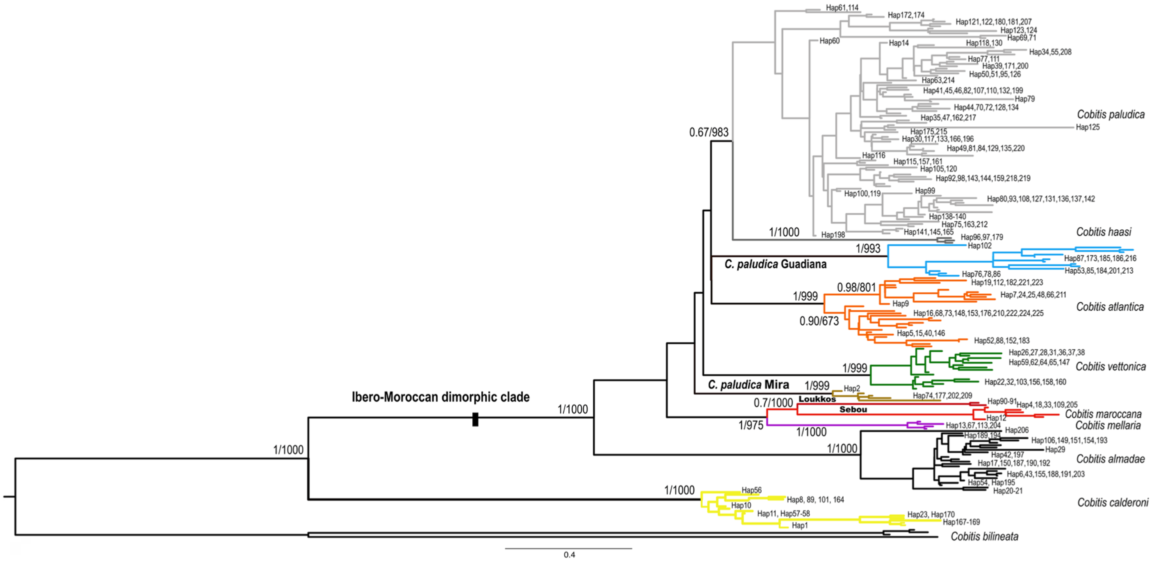

Figure 2. Phylogenetic relationships among Iberian and Moroccan *Cobitis* based on the *Cytb* gene and ML inference. Posterior probabilities and bootstrap values are indicated above branches. The colour of the branches corresponds to the colours shown in Figure 1.

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Table 1. Mitochondrial *Cytb* uncorrected *p*-distances between *Cobitis* species, including the new *Cobitis* lineages.

	Within species	*C. almadae*	*C. atlantica*	*C. calderoni*	*C. haasi*	*C. maroccana Sebou*	*C. maroccana Loukkos*	*C. mellaria*	*C. paludica*	*C. vettonica*	*C. paludica Guadiana*	*C. paludica Mira*
C. almadae	0.24 ± 0.30 (0–2.45)		5.49 ± 0.21 (4.08–6.35)	9.33 ± 0.17 (8.68–10.34)	5.39 ± 0.17 (4.78–6.22)	6.0 ± 0.16 (5.62–6.72)	**6.44 ± 0.13** **(6.17–6.98)**	5.25 ± 0.15 (4.99–6.17)	4.35 ± 0.25 (3.63–6.07)	5.28 ± 0.15 (4.99–6.26)	5.42 ± 0.24 (4.90–6.26)	5.03 ± 0.12 (4.71–5.53)
C. atlantica	0.47 ± 0.41 (0–2.99)			9.11 ± 0.29 (7.22–9.97)	3.36 ± 0.15 (1.91–3.86)	4.97 ± 0.13 (4.26–5.36)	5.42 ± 0.13 (4.44–5.71)	3.89 ± 0.13 (3.26–4.44)	2.61 ± 0.31 (0–4.26)	3.83 ± 0.14 (2.45–4.26)	3.66 ± 0.25 (2.90–4.49)	3.33 ± 0.11 (2.90–3.72)
C. calderoni	0.26 ± 0.32 (0–1.09)				9.0 ± 0.38 (8.25–9.72)	10 ± 0.13 (9.73–10.52)	9.44 ± 0.12 (9.03–9.79)	9.14 ± 0.13 (8.89–9.79)	8.04 ± 0.31 (6.79–9.88)	8.81 ± 0.17 (8.16–9.43)	9.13 ± 0.23 (8.26–10.06)	8.81 ± 0.14 (8.16–9.25)
C. haasi	0.51 ± 0.47 (0–1.38)					5.10 ± 0.17 (4.57–5.57)	5.17 ± 0.14 (4.8–5.41)	3.97 ± 0.13 (3.72–4.67)	**2.17 ± 0.24** **(1.48–3.64)**	3.75 ± 0.14 (3.36–4.18)	3.92 ± 0.28 (3.29–4.56)	3.38 ± 0.16 (2.87–3.57)
C. maroccana Sebou	0.30 ± 0.17 (0–0.73)						3.97 ± 0.14 (3.72–4.27)	3.61 ± 0.13 (3.45–4.18)	4.44 ± 0.25 (3.99–6.18)	5.48 ± 0.14 (5.08–5.09)	5.16 ± 0.30 (4.45–5.90)	4.96 ± 0.11 (4.71–5.27)
C. maroccana Loukkos	0.05 ± 0.05 (0–0.09)							3.12 ± 0.08 (2.99–3.35)	4.66 ± 0.26 (4.04–6.44)	5.72 ± 0.11 (5.44–5.98)	5.50 ± 0.29 (5.08–6.17)	5.35 ± 0.29 (5.08–5.33)
C. mellaria	0.09 ± 0.12 (0–0.63)								3.46 ± 0.22 (3.08–5.17)	4.72 ± 0.14 (4.53–5.35)	3.98 ± 0.28 (3.54–4.99)	4.0 ± 0.12 (3.81–4.53)
C. paludica	0.50 ± 0.43 (0–3.63)									2.78 ± 0.30 (2.08–4.35)	3.24 ± 0.31 (2.45–4.90)	3.01 ± 0.21 (2.59–4.26)
C. vettonica	0.55 ± 0.50 (0–1.45)										4.13 ± 0.28 (3.45–4.99)	4.06 ± 0.11 (3.73–4.35)
C. paludica Guadiana	1.16 ± 0.56 (0–2.72)											4.10 ± 0.28 (3.45–4.53)
C. paludica Mira	0.12 ± 0.08 (0–0.27)											–

± Standard deviation (SD), (range). First column, *p*-distances within species or lineages. The highest and lowest *p*-distances, excluding *C. calderoni*, are indicated in bold.

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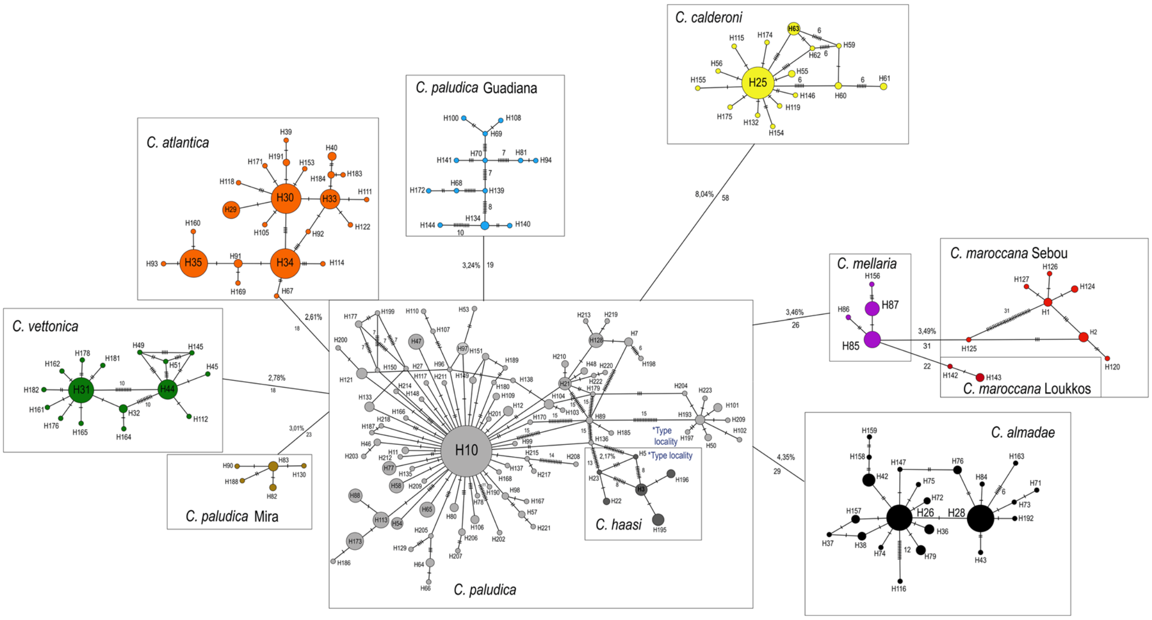

Figure 3. *Cytb* haplotype network. Transversal dashes on the linkage lines indicate the number of mutational steps between haplotypes. The number is indicated in the cases with more than six steps. The *p*-distances (%) and number of mutational steps are shown on the lines linking *C. paludica* to the other taxa. Circles are proportional to the number of individuals sharing the haplotype. *Haplotypes found in the type locality.

basins like the Besòs, Ter, Júcar, Cávado, Sado, and Mira. Other less frequent haplotypes were found across the distribution range of the species. Remarkably, the haplotype found in the type locality of *C. paludica* (H197) was highly divergent from the other ones of the species. Three frequent and exclusive haplotypes (H3, H195, H196) dispersed across the Ebro (Matarraña and Júcar), along with some other exclusive ones, characterize the haplotype configuration of *C. haasi*. The ancestral haplotypes of *C. vettonica* (H31, H44) were found in the Tagus (Alagón, Erges, Aravil, Ponsul sub-basins) and Duero (Águeda and Tormes sub-basins), with 10 mutational steps between them, co-occurring with less frequent haplotypes. The ancestral *Cytb* haplotype (H85) of *C. mellaria* and other less frequent haplotypes were exclusive to the species and dispersed across the small southern basins of Salado, Barbate, Valle, Jara, and de la Vega. The haplotypes of the new lineage *C. paludica* from the Guadiana basin were distributed in the Guadiana mainstem and some sub-basins, including the Ardila, Estena, Ruecas, and Vascão, while those of *C. paludica* from the Mira basin were exclusive to the Mira basin, including the Torgal and Telhares sub-basins.

In the *COI* network (Fig. 4), a total of 101 haplotypes were identified, with new lineage *C. paludica* from the Guadiana basin occupying a central position and differing by 11 to 17 mutations from the other species and lineages (Fig. 4). Haplotypic diversity was low, with no haplotypes shared across species or lineages, and, as in the *Cytb* network, *C. paludica* exhibited the greatest diversity. The network configuration for *C. calderoni*, *C. almadae*, *C. atlantica*, *C. mellaria*, *C. vettonica*, and *C. paludica* from the Mira basin showed few common haplotypes, with haplotypes linked by less than six mutations. A decentralized haplotype network was observed for *C. maroccana* and *C. paludica* from the Guadiana basin, with almost every individual presenting an exclusive single haplotype (Fig. 4).

Nuclear variation

In the nuclear phylogenies based on the phased haplotypes, only *C. calderoni* was recovered as a monophyletic group, while the other groups were unresolved (Supporting Information, Figs S2, S3). For the Ibero-Moroccan dimorphic clade, individuals exhibiting nuclear phased haplotypes (*RAG-1* and *S7*) of two mitochondrial species were often found, and most species could not be differentiated at the nuclear level (Supporting Information, Figs S2, S3).

The nuclear networks based on the phased haplotypes showed a clear segregation of *C. calderoni*, *C. paludica* from the Guadiana basin, *C. maroccana*, and *C. mellaria* (Figs 5, 6), with the last two species sharing alleles in the *RAG-1* network (Fig. 5). All other species of the Ibero-Moroccan dimorphic clade showed a high proportion of haplotype mixture, with individuals showing alleles from diverse mitochondrial species. For most of the species and lineages, the *S7* and *RAG-1* network configurations showed between one and four common haplotypes, with other haplotypes linked by a few mutational steps (<6). *Cobitis vettonica* and *C. paludica* from the Mira basin were the exceptions, showing undefined haplotype networks mainly related to *C. paludica* haplotypes. For both nuclear genes, *C. paludica* showed the highest number of haplotypes and the highest degree of haplotype mixture with the other species. Specifically, *C. paludica* shared *RAG-1* and *S7* alleles with *C. vettonica*, *C. atlantica*, *C. almadae*, *Cobitis* from the Mira basin, and *Cobitis* from the Guadiana. *Cobitis almadae* also shared *RAG-1* and *S7* alleles with *C. atlantica* and *C. paludica*.

Divergence time

Estimates of divergence time were consistent among analyses, whether using the calibration point of the opening of the Strait of Gibraltar (4–6 Mya) or the 0.0034% *Cytb* divergence estimated between *C. maroccana* and *C. mellaria*. Generally, similar results

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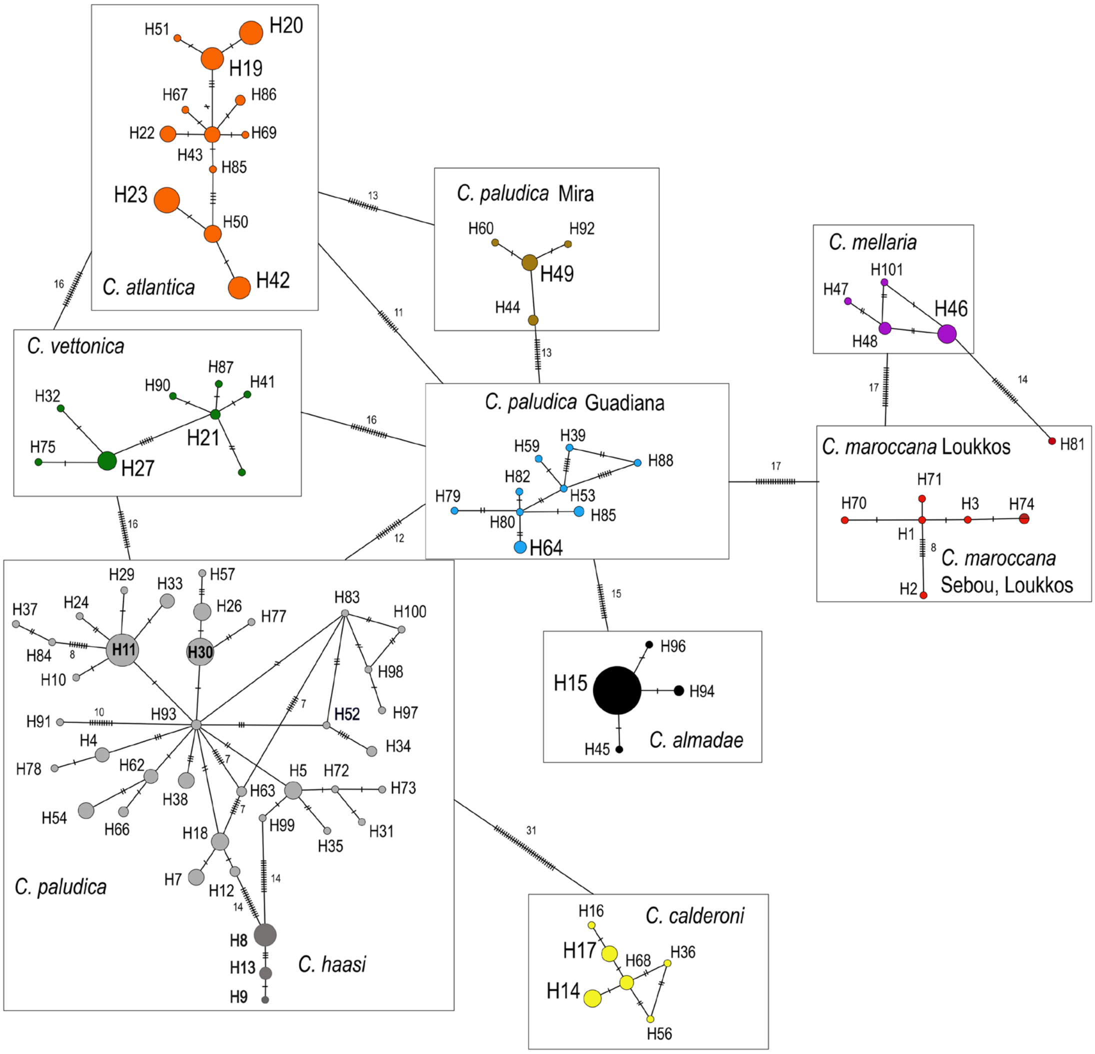

Figure 4. *COI* haplotype network. Transversal dashes on the linkage lines indicate the number of mutational steps between haplotypes. The number is indicated in the cases with more than six steps. The number of mutational steps is shown on the lines linking taxa. Circles are proportional to the number of individuals sharing the haplotype.

were observed with both the nuclear and mtDNA datasets, and the high probability density (95% HPD) intervals were very large (Figs 7, 8).

The divergence time estimated between *C. calderoni* and other Ibero-Moroccan *Cobitis* was between 15.69 and 16.95 Mya, according to the *Cytb* and nuclear genes (*RAG-1* and *S7*) analyses, respectively (Figs 7, 8). The TMRCA for *C. maroccana* and *C. mellaria* was set at 5.3 Mya, indicating that the separation of the Ibero-Moroccan dimorphic *Cobitis* clade occurred 7.48–8.45 Mya. According to the mitochondrial *Cytb* gene analysis, *C. paludica* from the Guadiana basin and *C. paludica* from the Mira basin were among the first Iberian lineages to diverge within the clade. In contrast, in the nuclear analysis estimated the divergence of *C. paludica* from the Mira basin to be more recent, at 0.11 Mya. As the relationships among *Cobitis* species were not well resolved at the nuclear level, this result should be interpreted with caution. *Cobitis haasi* diverged approximately 2.42–3.73 Mya, whereas *C. almadae, C. atlantica, C. paludica,* and *C. vettonica* diverged more recently, approximately 0.54–0.6 Mya (Figs 7, 8).

DISCUSSION

The systematics of Iberian and Moroccan *Cobitis* has traditionally relied on the phylogenetic relationships derived from mitochondrial data (Perdices and Doadrio 2001, Doadrio and Perdices 2005, Doadrio *et al.* 2024, Ouassal *et al.* 2024). This study significantly advances the understanding of this group by integrating both mitochondrial and nuclear genes, together with an extensive geographical sampling. Although the nuclear genes provided limited phylogenetic information, they were useful in uncovering phylogeographic patterns and revealing genetic admixture among *Cobitis* species. This combined approach not only enhances the accuracy of species delineation, but also highlights previously unrecognized evolutionary dynamics, offering a more robust framework for understanding the diversification and biogeography of Iberian and Moroccan *Cobitis*.

Biogeography of Iberian and Moroccan *Cobitis*

The formation of the current Iberian hydrographic basins, promoted by massive landscape reconfigurations from the Eocene to

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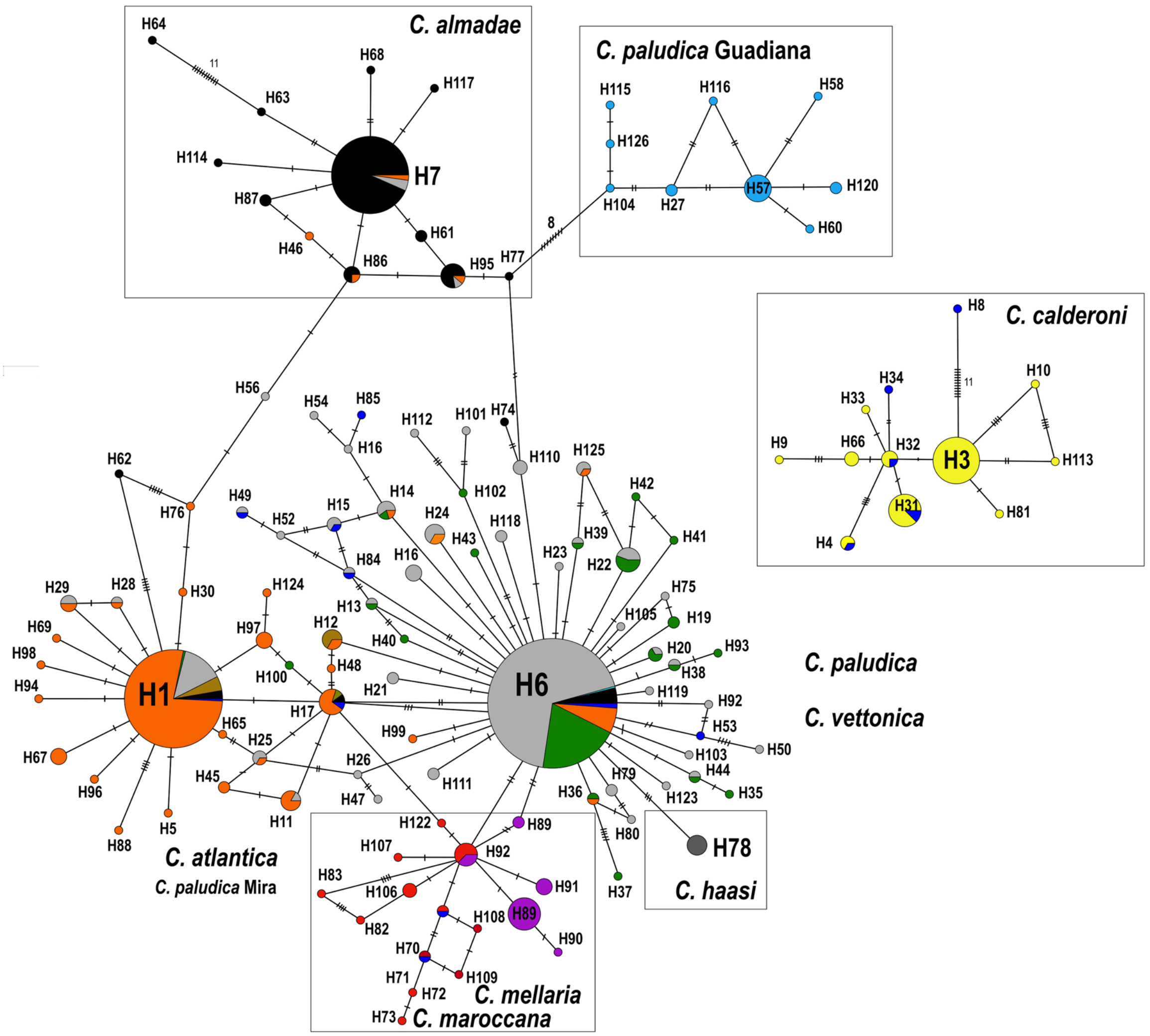

Figure 5. *RAG-1* haplotype network based on phased sequences. Transversal dashes on the linkage lines indicate the number of mutational steps between haplotypes. The number is indicated in the cases with more than six steps. The number of mutational steps is shown on the lines linking C. *almadae* and C. *paludica* from the Guadiana basin. Circles are proportional to the number of individuals sharing the haplotype. In dark blue, the individuals without data for the *Cytb*.

the Quaternary (De Vicente *et al.* 2011, Pais *et al.* 2012), probably has had significant evolutionary implications for *Cobitis*, as has also been inferred for other Iberian FWF (Perea *et al.* 2010, Sousa-Santos *et al.* 2019). During the Cenozoic (66 Mya–present), the Iberian Peninsula underwent a northern compression caused by the collision of the African and Eurasian plates, which resulted in the uplift of major mountain ranges, and led to the formation of basins, including the lower Tagus, Guadiana, and Mondego (Pais *et al.* 2012). The uplift of the Pyrenean Mountains, promoted by this northward movement against the Eurasian plate, isolated the Iberian Peninsula from the rest of Europe during the Middle Miocene. *Cobitis* is currently distributed throughout the Palaearctic, including the Mediterranean peninsulas and North Africa, and, therefore, it is likely that the genus was broadly distributed throughout the Iberian Peninsula before its isolation from northern and central Europe (via the Pyrenean uplift) and North Africa (via the opening of the Strait of Gibraltar). Our mitochondrial phylogenetic results suggest that Iberian and Moroccan *Cobitis* descended from a single ancestral lineage that inhabited areas north of the Pyrenees, migrated to the Iberian Peninsula through a French river connection, and then diversified. A similar scenario has been proposed for the Iberian leuciscid genus *Squalius* (Sousa-Santos *et al.* 2007, 2019, Perea *et al.* 2010). In south-eastern Iberia and North Africa, *Cobitis* probably used the Betic–Rifian Massif (including the Kabilian Mountains) connection, which was open during the Late Miocene, to expand into North Africa. Movement through this route is thought to account for the current phylogenetic pattern of other genera, including *Luciobarbus* (Machordom and Doadrio 2001) and *Podarcis* lizards (Kaliontzopoulou *et al.* 2011). The connection closed after the opening of the Strait of Gibraltar around 5.3 Mya (Krijgsman *et al.* 1999), and no further river connections are known to have occurred thereafter. Although another connection of both sides of the Strait of Gibraltar has been proposed to explain the distribution of several less water-dependent organisms (Harris *et al.* 2002, Carranza *et al.* 2004, 2006), this hypothesis has been contested, and human-mediated introductions are now considered the most likely explanation for their current distributions (Paulo *et al.* 2002,

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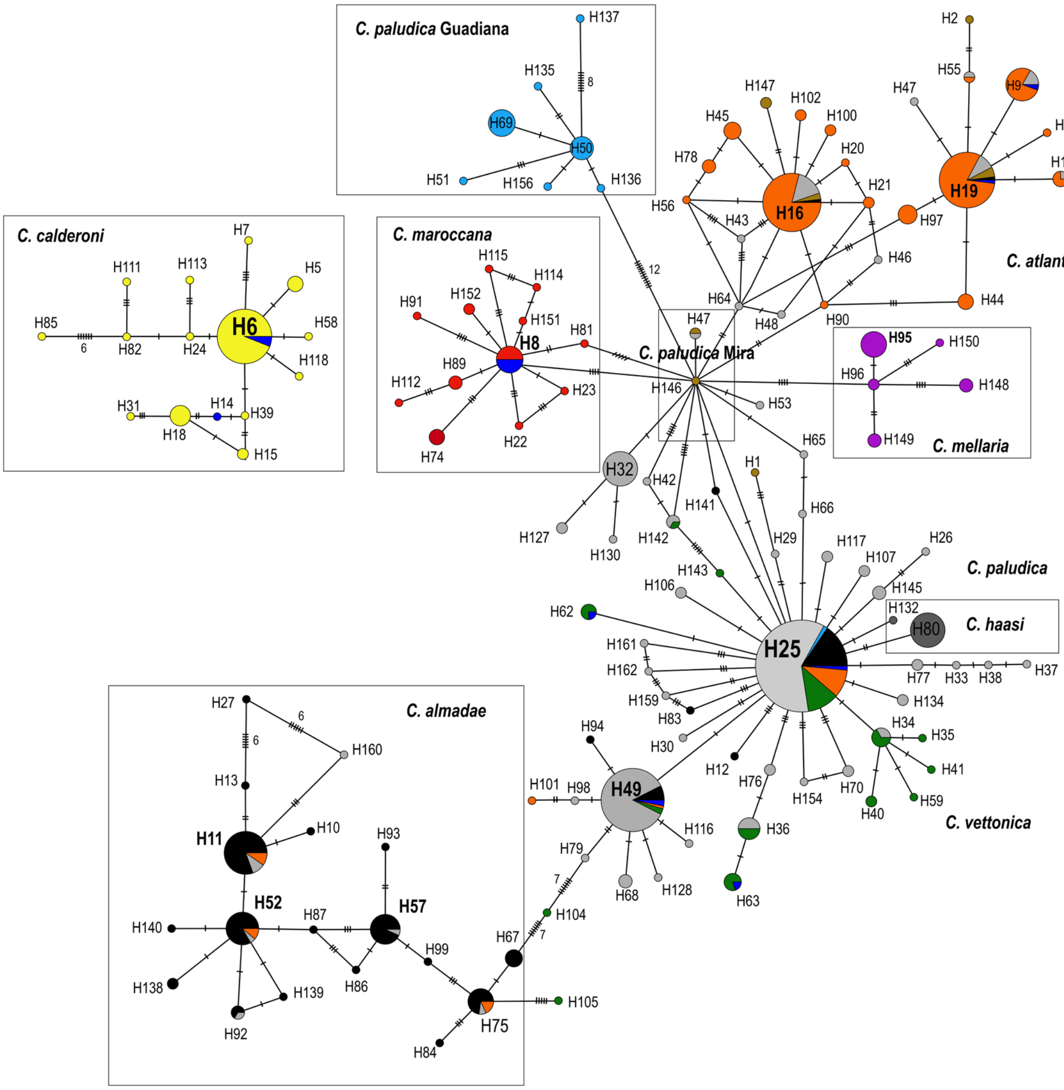

Figure 6. *S7* haplotype network based on phased sequences. Transversal dashes on the linkage lines indicate the number of mutational steps between haplotypes. The number is indicated in the cases with more than six steps. The number of mutational steps is shown on the lines linking *C. almadae* and *C. paludica* from the Guadiana basin. Circles are proportional to the number of individuals sharing the haplotype. In dark blue, the individuals without data for the *Cytb*.

Recuero *et al.* 2007). Given this context, the isolation of the Iberian Peninsula probably allowed Iberian *Cobitis* to evolve independently, and in allopatry, from both European and North African counterparts.

The oldest *Cobitis* fossil record in Europe dates back to 24 Mya (Böhme and Ig 2003). Our study indicates that *C. calderoni* is the oldest Iberian species, having diverged approximately 15.69–16.95 Mya. Currently, this species is restricted to the northern basins of the Duero and Ebro, and to a few localities in the Tagus basin in Spain. This is in line with geological evidence indicating that the Duero and Ebro were a single basin during the formation of the endorheic Pyrenean foreland basin in the Eocene to Early Miocene (De Vicente *et al.* 2011, Pais *et al.* 2012), and points to a Tagus–Duero stream capture.

The divergence of the Ibero-Moroccan dimorphic *Cobitis* clade was probably the start of a new phase of diversification of *Cobitis* more than 7–9 Mya later, in the Lower Miocene (7.48–8.45 Mya). During this period, the Iberian Plateau was covered by endorheic lakes, some of which may have been connected, thus facilitating the dispersal of the *Cobitis* ancestor. These lakes then changed from endorheic to exorheic, opening and draining to the Atlantic Ocean and Mediterranean Sea (De Vicente *et al.* 2011). Our divergence-time estimates point to some cladogenetic events at the Miocene–Pliocene transition (4.29–5.89 Mya) that may have led to the isolation of the two geographically restricted lineages, *C. paludica* from the Guadiana basin and *C. paludica* from the Mira basin, respectively. These lineages subsequently differentiated and became peripheral local endemics characterized by a complete mitochondrial differentiation and a partial nuclear distinctiveness. However, the divergence-time estimate based on the nuclear genes (*S7* + *RAG-1*) suggests that *C. paludica* from the Mira basin remained in contact with other Iberian *Cobitis* until recently, and only diverged approximately 0.11 Mya. At present, the nuclear differentiation of this lineage from the other Iberian *Cobitis* is still low, indicating incomplete lineage sorting of their nuclear genomes. Currently, the Mira basin also harbours *C. atlantica*,

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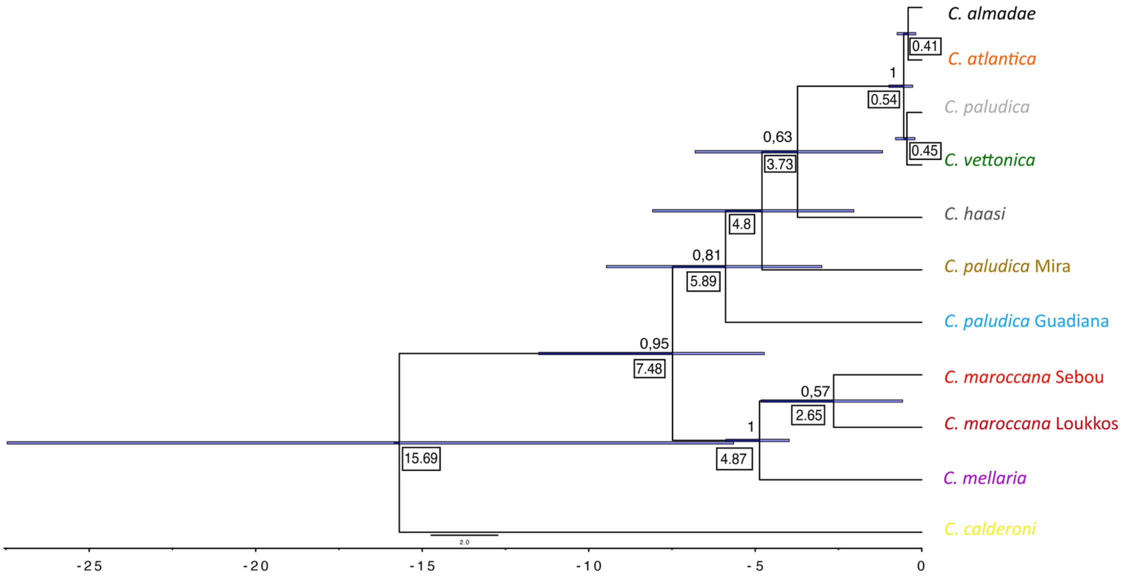

Figure 7. Species tree based on *Cytb* divergence. Divergence times are shown in the boxes, and posterior probability values, above branches.

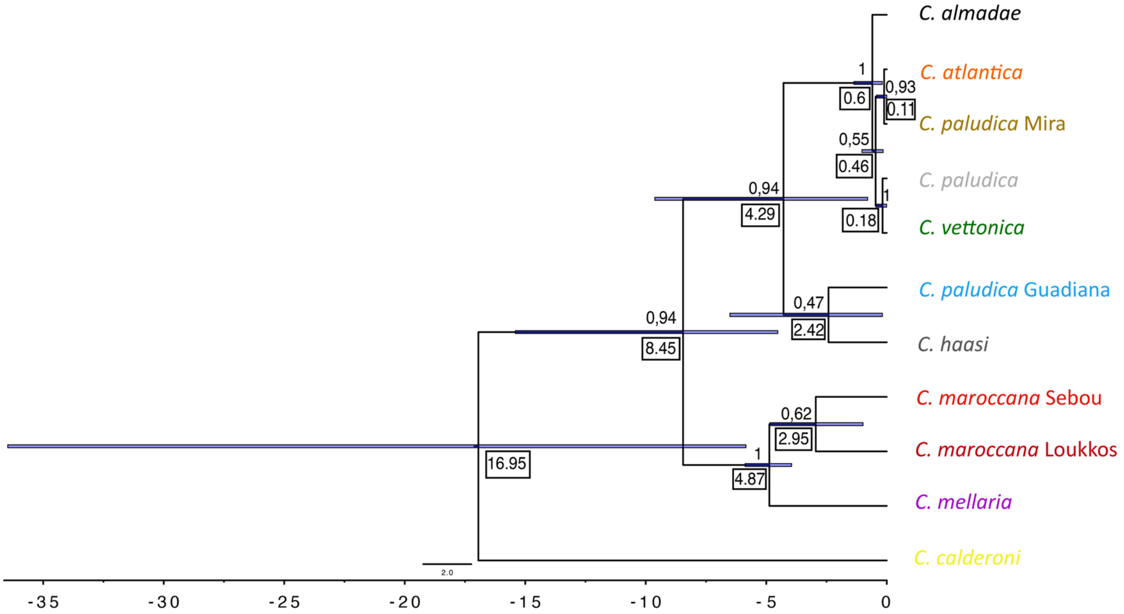

Figure 8. Species tree based on *RAG-1* + *S7* divergence. Divergence times are shown in the boxes, and posterior probability values, above branches.

which diverged more recently (0.11–0.41 Mya) and has a widespread distribution along the Atlantic coast, implying that two species evolved in sympatry when the basin was already configured, a situation not observed for local endemic leuciscids, such as the genera *Iberochondrostoma* or *Squalius*, which are just represented by a single species (Robalo *et al.* 2008, Magalhães *et al.* 2023). Although no river captures have been identified for the Mira basin, it is possible that river mouth connections during low sea-level periods might have enabled dispersal across isolated coastal palaeobasins.

Along the North Atlantic margin, several tectonic strike-slip faults, almost parallel to the coast, were activated in western Iberia during the Miocene–Pliocene transition (Messinian, 5.3 Mya), and some, such as the Vilariça in the Duero and the Ponsul in the Tagus, and their tectonic adjustments, may have played an important role in the isolation and mixture of *Cobitis* species (De Vicente *et al.* 2011). Although the temporal framework of main Iberian Cenozoic basins is not well delineated and is still under study (De Vicente *et al.* 2011), we found a temporal gap between these events and the divergence times of *Cobitis* species, suggesting that they should be associated with more recent geological events in the region (Rockwell *et al.* 2009). According to our estimates, *C. almadae* and *C. vettonica* were in contact as recently as the Quaternary (0.54–0.6 Mya), possibly mixing with *C. paludica*, and thus showing incomplete nuclear differentiation. The Pliocene–Pleistocene transition was another period of change from an endorheic to an exorheic regime, facilitating westward dispersal through basins opening along the Atlantic margin.

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During this period, the lower Tagus basin may have acted as a littoral corridor for the north to south dispersal of *C. paludica*, *C. atlantica*, and *C. almadae*, which was also probably related to the strike-slip faults found in the area (Fig.1).

Along the Mediterranean margin, the divergence of *C. haasi* from *C. paludica*, as well as from other species of the Ibero-Moroccan dimorphic clade, was estimated to have occurred around 2.42–3.73 Mya as the divergence of other Levantine endemics, such as the leuciscid *Squalius valentinus*. The transformation of the Miocene endorheic lakes and lagoons into exorheic basins has been proposed to account for the leuciscid divergence (Perea *et al.* 2016). Our time results, along with the predominantly coastal distribution of *C. haasi*, suggest geographical differentiation of this peripheral species, followed by limited gene flow with other *Cobitis* species.

Species' diversity and distribution patterns

Our phylogeographic and phylogenetic results provide a basis for reviewing the diversity and current distribution of *Cobitis* across the Iberian Peninsula and North Africa. Our findings reveal that the Iberian and North African *Cobitis* exhibit high diversification, with clear mitochondrial differentiation greater than previously described. In addition to the eight previously recognized species, our analyses identified three new lineages, two Iberian and one Moroccan, suggesting the existence of three as-yet-undescribed species. The Iberian *Cobitis* species are distributed across most of the Iberian basins, except for the northern Cantabrian rivers (N Spain), with translocated populations of *C. paludica* occurring in the lower Nalón basin (N Spain) (Rodríguez Muñoz 1993, Elvira and Almodóvar 2001), as well as in some Catalonian basins (NE Spain) (Clavero *et al.* 2023). Our study provides updated information on the species' distributions (see Supporting Information, Fig. S4), including the following occurrences: (i) *C. calderoni* in north-eastern Iberia (Duero and Ebro basins with isolated localities in the upper Tagus); (ii) *C. almadae* in the Atlantic Duero, Mondego, Lis, Sizandro, Samarra, and Tagus basins; (iii) *C. atlantica* in the Atlantic basins of the Minho, Cávado, Ave, Duero, Junqueira, Vouga, Mondego, Lis, Alcoa, Lizandro, Colares, Tagus, Sado, Melides, Mira, Alvor, Arade, Lagos, and Guadiana; (iv) *C. haasi* in the Mediterranean rivers of the Ebro, Albufera Valencia, and Júcar basins; (v) *C. mellaria* in the Salado, Barbate, Valle, Jara, and De la Vega basins in southern Spain; (vi) *C. paludica* in most of the Iberian basins on the Atlantic and Mediterranean slopes; (vii) *C. vettonica* in the Tagus (Árrago, Aravil, Erges and Ponsul sub-basins) and Duero (Águeda and Tormes sub-basins); and (viii) *C. maroccana* in the Sebou basin in northern Morocco. The three new *Cobitis* lineages were found to have restricted distributions, with *C. paludica* from the Mira basin occurring in the Mira basin in the Atlantic slope of Portugal, *C. paludica* from the Guadiana in the middle and lower Guadiana basin (Estena, Ruecas, Ardila, and Vascão sub-basins) and the Guadiana River, and *C. maroccana* in the Loukkos basin in Morocco.

Notably, our study provides evidence of sympatry among most *Cobitis* species, contrasting with the previously suggested allopatric distributions (Doadrio *et al.* 2024). Most major Iberian basins appear to harbour several *Cobitis* lineages, with the exception of the small Atlantic rivers and the left tributaries of the lower Tagus in Portugal. For example, *C. atlantica* is the only species of the genus found in the Minho, Alcoa, Junqueira, Vouga, Alvor, Lagos, and Melides basins; and *C. almadae* is the only species in the Samarra basin, and the most widespread species in the Sorraia sub-basin of the lower Tagus.

Our phylogeographic analyses based on *Cytb* showed that *C. paludica* is the central, most widespread and genetically variable species of the Iberian-Moroccan dimorphic group. This species occupies the central-southern Atlantic and Mediterranean basins, showing connections to peripheral and geographically restricted species that inhabit the lower reaches of major basins and of small to medium-sized Atlantic basins. Individuals from the Duero in Portugal exhibited the ancestral and most common *Cytb* haplotype (H10), which is present in most Iberian basins and is related, by a few mutational steps, to other haplotypes exclusive to the Duero populations. The connectivity between the Duero *Cytb* haplotypes of *C. paludica* in Portugal supports its natural occurrence in the region, as previously indicated in the Red List of Portugal (Magalhães *et al.* 2023). On the other side of the Iberian Peninsula, the Ebro River is the natural north-eastern limit of *C. paludica*, with recent records of the species in the coastal rivers of northern Catalonia being due to human-mediated translocation (Clavero *et al.* 2023).

At the nuclear level, however, most of the species were recovered as non-monophyletic, with the exceptions of *C. paludica* from the Guadiana basin, *C. mellaria*, and *C. maroccana*. These results indicate that most species of the Ibero-Moroccan dimorphic clade share nuclear, but not mitochondrial, information. This mito-nuclear discordance can result from incomplete lineage sorting or molecular introgression as a consequence of secondary contact following river re-arrangements in the Quaternary, as previously suggested for *C. vettonica* and *C. paludica* (Corral-Lou *et al.* 2022). Nuclear haplotypes of *C. paludica* are present in *C. vettonica*, as well as in *C. almadae* and *C. atlantica*, suggesting that sympatric distributions favoured contact and genetic exchange. Introgression is often found in sister-species that have experienced secondary contact during their evolutionary histories, especially when speciation is incipient (Harrison and Larson 2014). The *RAG-1* and *S7* allele network, with allele sharing across species, suggests incomplete lineage sorting between most of the Ibero-Moroccan dimorphic species (namely, *C. paludica*, *C. vettonica*, *C. atlantica*, *C. almadae*, *C. paludica* from the Mira basin and *C. paludica* from the Guadiana basin). Other species, such as *C. mellaria* and *C. maroccana* from the Sebou basin, shared some exclusive *RAG-1* alleles between them.

The evolutionary patterns found in the mitochondrial and nuclear phylogenies of Iberian and Moroccan *Cobitis* differs in some important points from the morphological and mitochondrial identification recently published by Doadrio *et al.* (2024). Specifically, our results do not support the restriction of *C. almadae* and *C. mellaria* to two small Atlantic basins, or the allopatry between *C. almadae* and other *Cobitis* species. Our results indicate that these species have a wider distribution than previously thought, with *C. almadae* being sympatric with *C. paludica* and *C. atlantica* in the Sizandro and Maior river basins and in the Zêzere River in the Tagus basin. Additionally, our mtDNA data do not support the comparable evolutionary significance of *C. haasi* with respect to other Iberian *Cobitis*. We identified both *C. haasi* and *C. paludica* in the type locality of *C. haasi* in Valencia (Fig. 1).

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However, based on the present data, it remains unclear whether the presence of *C. paludica* is the result from an introduction or a recent colonization. Moreover, our findings suggest that *C. haasi* would be the sister-species of *C. paludica*, instead of *C. vettonica*, as previously suggested by Corral-Lou *et al.* (2022).

Our results have allowed us to infer the biogeographical processes that occurred on both sides of the Strait of Gibraltar and provide new insights on past and present scenarios of river connectivity and palaeobasin evolution in the region. Finally, the advances gained from our analyses of the genetic patterns and species' delimitation of the Iberian and Moroccan *Cobitis* species are important for biodiversity assessments and conservation efforts in the regions where the species occur.

CONCLUSION

In summary, our results confirm that *C. calderoni*, a species lacking secondary sexual dimorphism, is a clearly differentiated species that is phylogenetically related, but not included, in the Ibero-Moroccan dimorphic clade, in line with the results of a previous study (Perdices *et al.* 2018). The remaining Iberian and North African species are phylogenetically closely related and difficult to distinguish morphologically, and together form the Ibero-Moroccan dimorphic clade. Among these, six species are of equivalent evolutionary status, showing reciprocal monophyly: *C. almadae*, *C. atlantica*, *C. maroccana* from the Sebou basin, *C. mellaria*, *C. paludica*, and *C. vettonica*. Our study confirms the paraphyly of *C. paludica* and identifies two new lineages: *C. paludica* in the Guadiana basin and *C. paludica* in the Mira basin. The phylogenies, high genetic divergence, and nuclear haplotype networks support the hypothesis that these divergent lineages represent new species. Additionally, the phylogenetic subdivision and high genetic distances found among populations of *C. maroccana* also indicate the presence of an undescribed species, *C. maroccana* in Morocco in the Loukkos basin, in accordance with the *Cytb* genetic diversity reported in previous works (Keltoum 2022, Ouassal *et al.* 2024). Further investigation, including a morphological analysis of the new *C. paludica* and *C. maroccana* lineages, is needed to confirm whether they should be elevated to species and formally described, which would increase our knowledge of *Cobitis* diversity.

Overall, our findings, based on a geographically extensive sampling across the Iberian Peninsula and Morocco, shed light on longstanding taxonomic uncertainties within *Cobitis* and provide a robust framework for understanding species' diversity and distribution. These insights are critical for supporting effective conservation strategies and prioritizing management efforts, and highlight the need for a comprehensive re-evaluation of the conservation status of all *Cobitis* species from the Iberian Peninsula and Morocco.

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AUTHOR CONTRIBUTIONS

Anabel Perdices, João Queirós, Maria Judite Alves, Maria Filomena Magalhães, Manuel Lopes-Lima, and Maria Manuela Coelho (study conception and design). Anabel Perdices and João Queirós (draft manuscript preparation). Anabel Perdices, João Queirós, and Jongmo Suh (laboratory work). Anabel Perdices, David Buckley, Manuel Lopes-Lima, João Queirós, Jongmo Suh, and Joana Veríssimo (molecular analyses). Anabel Perdices, João Queirós, Pedro Beja, Miguel Clavero, Elsa Froufe, Joana Garrido Nogueira, João Manuel Oliveira, Quim Pou-Rovira, Ronaldo Sousa, Amílcar Teixeira, Simone Varandas, Marc Ventura, Ana Filipa Filipe, and Manuel Lopes-Lima (fieldwork). All authors contributed to the interpretation of the results and the final version of the manuscript.

SUPPLEMENTARY DATA

Supplementary data is available at *Zoological Journal of the Linnean Society* online.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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DATA AVAILABILITY

The data underlying this article are available at the GenBank Nucleotide Database and can be accessed with PX552905-PX553004, PX553012-PX553891, PX579137-PX579456, PX617885-PX618015, PX618252-PX618438, PX63704-PX63708 Accession Numbers.

REFERENCES

Azeroual A, Crivelli AJ, Yahyaoui A *et al.* L'ichthyofaune des eaux continentales du Maroc. *Cybium* 2000;**24**(Suppl):17–22.

Bănărescu P. *Zoogeography of Fresh Waters. Vol. 2. Distribution and Dispersal of Freshwater Animals in North America and Eurasia*. Wiesbaden: AULA-Verlag, 2001.

Bohlen J, Rab P. Species and hybrid richness in spined loaches of the genus *Cobitis* (Teleostei: Cobitidae), with a checklist of European forms and suggestions for conservation. *Journal of Fish Biology* 2001;**59**:75–89. https://doi.org/10.1111/j.1095-8649.2001.tb01380.x

Böhme M, Ilg A. FosFARbase. 2003. https://www.fosfarbase.org/ (29 May 2024, data last accessed).

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Bouckaert R, Heled J, Kühnert D *et al.* BEAST 2: a software platform for Bayesian evolutionary analysis. *PLoS Computational Biology* 2014;**10**:e1003537. https://doi.org/10.1371/journal.pcbi.1003537

Carranza S, Arnold EN, Wade E *et al.* Phylogeography of the false smooth snakes, *Macroprotodon* (Serpentes, Colubridae): mitochondrial DNA sequences show European populations arrived recently from Northwest Africa. *Molecular Phylogenetics and Evolution* 2004;**33**:523–32. https://doi.org/10.1016/j.ympev.2004.07.009

Carranza S, Harris DJ, Arnold EN *et al.* Phylogeography of the lacertid lizard, *Psammodromus algirus*, in Iberia and across the Strait of Gibraltar. *Journal of Biogeography* 2006;**33**:1279–88. https://doi.org/10.1111/j.1365-2699.2006.01491.x

Chow S, Hazama K. Universal PCR primers for S7 ribosomal protein gene introns in fish. *Molecular Ecology* 1998;**7**:1255–6.

Clavero M, Suh J, Franch N *et al.* Invaders they are changing: a recent, unexpected surge of invasive loaches in Catalonia. *Freshwater Biology* 2023;**68**:621–31. https://doi.org/10.1111/fwb.14051

Corral-Lou A, Perea S, Perdices A *et al.* Quaternary geomorphological and climatic changes associated with the diversification of Iberian freshwater fishes: the case of the genus *Cobitis* (Cypriniformes, Cobitidae). *Ecology and Evolution* 2022;**12**:e8635. https://doi.org/10.1002/ece3.8635

De Vicente G, Cloetingh S, Van Wees JD *et al.* Tectonic classification of Cenozoic Iberian foreland basins. *Tectonophysics* 2011;**502**:38–61. https://doi.org/10.1016/j.tecto.2011.02.007

Dias JMA, Boski T, Rodrigues A *et al.* Coast line evolution in Portugal since the Last Glacial Maximum until present—a synthesis. *Marine Geology* 2000;**170**:177–86. https://doi.org/10.1016/S0025-3227(00)00073-6

I Doadrio (ed.). *Atlas y Libro Rojo de los Peces Continentales de España*. Madrid: Ministerio de Medio Ambiente, CSIC, 2001.

Doadrio I, Perdices A. Phylogenetic relationships among the Ibero-African cobitids (*Cobitis*, Cobitidae) based on cytochrome b sequence data. *Molecular Phylogenetics and Evolution* 2005;**37**:484–93. https://doi.org/10.1016/j.ympev.2005.07.009

Doadrio I, Perea S, Garzón-Heydt P *et al. Ictiofauna Continental Española. Bases para su Seguimiento*. Madrid: MARM, 2011.

Doadrio I, Sousa-Santos C, Robalo J *et al.* Description of three new species of the genus *Cobitis* L., 1758 (Actinopterygii, Cobitidae) in the Iberian Peninsula. *Limnetica* 2024;**43**:1–198. https://doi.org/10.23818/limn.43.12

Drummond A, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* 2007;**7**:214. https://doi.org/10.1186/1471-2148-7-214

Elvira B, Almodóvar A. Freshwater fish introductions in Spain: facts and figures at the beginning of the 21st century. *Journal of Fish Biology* 2001;**59**(Suppl A):323–1.

Filipe AF, Araújo MB, Doadrio I *et al.* Biogeography of Iberian freshwater fishes revisited: the roles of historical versus contemporary constraints. *Journal of Biogeography* 2009;**36**:2096–110. https://doi.org/10.1111/j.1365-2699.2009.02154.x

Folmer O, Black M, Hoeh W *et al.* DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 1994;**3**:294–9.

Freyhof J, Brooks E. *European Red List of Freshwater Fishes*. Luxembourg: Publications Office of the European Union, 2011.

Gante H. Diversification of circum-Mediterranean barbels. In: O Grillo, G Venora (eds), *Changing Diversity in Changing Environment*. Rijeka: University Campus SteP Ri, 2011, 283–98. https://doi.org/10.5772/24639

Gómez A, Lunt DH. Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. In: S Weiss, N Ferrand (eds), *Phylogeography of Southern European Refugia*. Amsterdam: Springer-Verlag, 2008, 155–18.

Guindon S, Dufayard JF, Lefort V *et al.* New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* 2010;**59**:307–21. https://doi.org/10.1093/sysbio/syq010

Harris DJ, Carranza S, Arnold EN *et al.* Complex biogeographical distribution of genetic variation within *Podarcis* wall lizards across the Strait of Gibraltar. *Journal of Biogeography* 2002;**29**:1257–62. https://doi.org/10.1046/j.1365-2699.2002.00744.x

Harrison RG, Larson EL. Hybridization, introgression, and the nature of species boundaries. *The Journal of Heredity* 2014;**105**(Suppl 1):795–809. https://doi.org/10.1093/jhered/esu033

Kaliontzopoulou A, Pinho C, Harris DJ *et al.* When cryptic diversity blurs the picture: a cautionary tale from Iberian and North African *Podarcis* wall lizards. *Biological Journal of the Linnean Society* 2011;**103**:779–800. https://doi.org/10.1111/j.1095-8312.2011.01703.x

Keltoum O. Diversité génétique mitochondriale, phylogéographie et histoire démographique des poissons indigènes des eaux continentales du Maroc: Cas des genres *Luciobarbus*, *Cobitis* et *Coptodon*. PhD Thesis, University of Rabat, 2022.

Krijgsman W, Hilgen FJ, Raffi I *et al.* Chronology, causes and progression of the Messinian salinity crisis. *Nature* 1999;**400**:652–5. https://doi.org/10.1038/23231

Kottelat M. Conspectus Cobitidum: an inventory of the loaches of the world (Teleostei: Cypriniformes: Cobitoidei). *The Raffles Bulletin of Zoology* 2012;**26**:1–199.

Leigh JW, Bryant D. PopART: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* 2015;**6**:1110–6. https://doi.org/10.1111/2041-210X.12410

Lima SMQ, Berbel-Filho WM, Araújo TFP *et al.* Headwater capture evidenced by paleo-rivers reconstruction and population genetic structure of the armored catfish (*Pareiorhaphis garbei*) in the Serra do Mar Mountains of Southeastern Brazil. *Frontiers in Genetics* 2017;**8**:199. https://doi.org/10.3389/fgene.2017.00199

Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics (Oxford, England)* 2009;**25**:1451–2. https://doi.org/10.1093/bioinformatics/btp187

Machordom A, Doadrio I. Evidence of a Cenozoic Betic–Kabilian connection based on freshwater fish phylogeography (*Luciobarbus*, Cyprinidae). *Molecular Phylogenetics and Evolution* 2001;**18**:252–63.

Magalhães MF, Amaral SD, Sousa M *et al. Livro Vermelho dos Peixes Dulceaquícolas e Diádromos de Portugal Continental*. Lisboa: Fciências. ID and ICNF IP, 2023.

Myers GS. Fresh-water fishes and West Indian zoogeography. Smithsonian Report for 1937. Publication No. 3465: 1938, 1966.

Ouassal K, Doadrio I, Yahyaoui A *et al.* Cytochrome b mitochondrial gene revealed a strong genetic differentiation between two populations of the endemic freshwater fish Moroccan loach *Cobitis maroccana* Pellegrin, 1929. *Turkish Journal of Fisheries and Aquatic Sciences* 2024;**24**:TRJFAS24858. https://doi.org/10.4194/TRJFAS24858.

Pais J, Cunha PP, Pereira D *et al. The Paleogene and Neogene of West Iberia (Portugal). A Cenozoic Record in European Atlantic Domain*. Berlin, Heidelberg: Springer Berlin Heidelberg, 2012. https://doi.org/doi 10.1007/978-3-642-22401-0

Palumbi SR. Nucleid acids II: the polymerase chain reaction. In: DM Hillis, C Moritz, MK Mable (eds), *Molecular Systematics*, 2nd edn. Sunderland, MA: Sinauer Associates Inc, 1996, 205–47.

Paulo OS, Pinto I, Bruford MW *et al.* The double origin of Iberian peninsular chameleons. *Biological Journal of the Linnean Society* 2002;**75**:1–7. https://doi.org/10.1046/j.1095-8312.2002.00002.x

Perdices A, Coelho MM. First records of the vettonian spined loach *Cobitis vettonica* in Portugal with update on its Iberian distribution. *Fishes in Mediterranean Environments* 2020;15. http://doi.org/10.29094/fishmed.2020.002

Perdices A, Doadrio I. Threatened fishes of the world: *Cobitis paludica* (De Buen, 1930) (Cobitidae). *Environmental Biology of Fishes* 1997a;**49**:360.

Perdices A, Doadrio I. Threatened fishes of the world: *Cobitis calderoni* Bacescu, 1961 (Cobitidae). *Environmental Biology of Fishes* 1997b;**50**:148.

Perdices A, Doadrio I. The molecular systematics and biogeography of the European cobitids based on mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* 2001;**19**:468–78. https://doi.org/10.1006/mpev.2000.0900

Perdices A, Machordom A, Doadrio I. Allozyme variation of African and Iberian populations of the genus *Cobitis*. *Journal of Fish Biology* 1995;**47**:707–18. https://doi.org/10.1111/j.1095-8649.1995.tb01936.x

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Perdices A, Bohlen J, Šlechtova V *et al.* Molecular evidence for multiple origins of the European spined loaches (Teleostei, Cobitidae). *PLoS One* 2016;**11**:e0144628. https://doi.org/10.1371/journal.pone.0144628

Perdices A, Ozeren CS, Erkakan F *et al.* Diversity of spined loaches from Asia Minor in a phylogenetic context (Teleostei: Cobitidae). *PLoS One* 2018;**13**:e0205678. https://doi.org/10.1371/journal.pone.0205678

Perea S, Böhme M, Zupančič P *et al.* Phylogenetic relationships and biogeographical patterns in circum-Mediterranean subfamily Leuciscinae (Teleostei, Cyprinidae) inferred from both mitochondrial and nuclear data. *BMC Evolutionary Biology* 2010;**10**:265. http://www.biomedcentral.com/1471-2148/10/265

Perea S, Cobo-Simon M, Doadrio I. Cenozoic tectonic and climatic events in southern Iberian Peninsula: implications for the evolutionary history of freshwater fish of the genys *Squalius* (Actinopterygii, Cyprinidae). *Molecular Phylogenetics and Evolution* 2016;**97**:155–69. https://doi.org/10.1016/j.ympev.2016.01.007

Quenouille B, Bermingham E, Planes S. Molecular systematics of the damselfishes (Teleostei: Pomacentridae): Bayesian phylogenetic analyses of mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution* 2004;**31**:66–88. https://doi.org/10.1016/S1055-7903(03)00278-1

Rambaut A, Drummond AJ, Xie D *et al.* Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology* 2018;**67**:901–4. https://doi.org/10.1093/sysbio/syy032

Recuero E, Iraola A, Rubio X *et al.* Mitochondrial differentiation and biogeography of *Hyla meridionalis* (Anura: Hylidae): an unusual phylogeographic pattern. *Journal of Biogeography* 2007;**34**:1207–19. https://doi.org/10.1111/j.1365-2699.2007.01688.x

Robalo JI, Doadrio I, Valente A *et al.* Insights on speciation patterns in the genus *Iberochondrostoma* (Cyprinidae): evidence from mitochondrial and nuclear data. *Molecular Phylogenetics and Evolution* 2008;**46**:155–66. https://doi.org/10.1016/j.ympev.2007.07.010

Rockwell T, Fonseca J, Madden C *et al.* Palaeosismology of the Vilariça segment of the Manteigas-Bragança fault in northeastern Portugal. *Geological Society, London, Special Publications* 2009;**316**:237–58. https://doi.org/10.1144/SP316.15

Rodríguez Muñoz R. Captura de *Cobitis maroccana* Pellegrin, 1929 (Osteichthyes, Cobitidae) en el río Nalón (Cuenca del Norte de España). *Miscellania Zoologica* 1993;**17**:286–7.

Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics (Oxford, England)* 2003;**19**:1572–4. https://doi.org/10.1093/bioinformatics/btg180

Shelley JJ, Swearer SE, Dempster T *et al.* Plio-Pleistocene sea-level changes drive speciation of freshwater fishes in north-western Australia. *Journal of Biogeography* 2020;**47**:1727–38. https://doi.org/10.1111/jbi.13856

Schönhuth S, Lozano-Vilano L, Perdices A *et al.* Phylogeny, genetic and phylogeography of the genus *Codoma* (Teleostei, Cyprinidae). *Zoologica Scripta* 2015;**44**:11–28. https://doi.org/10.1111/zsc.12083

SIBIC. Carta Piscícola Española. Electronic publication (v.02/2017), 2017. http://www.cartapiscicola.es/#/home (July 2024, data last accessed).

Šlechtová V, Bohlen J, Tan HH. Families of Cobitoidea (Teleostei; Cypriniformes) as revealed from nuclear genetic data and the position of the mysterious genera *Barbucca*, *Psilorhynchus*, *Serpenticobitis* and *Vaillantella*. *Molecular Phylogenetics and Evolution* 2007;**44**:1358–65. https://doi.org/10.1016/j.ympev.2007.02.019

Smith KG, Darwall WRT. *The Status and Distribution of Freshwater Fish Endemic to the Mediterranean Basin*. Gland, Switzerland and Cambridge, UK: IUCN, 2006.

Sousa-Santos C, Collares-Pereira MJ, Almada A. Reading the history of a hybrid fish complex from its molecular record. *Molecular Phylogenetics and Evolution* 2007;**45**:981–96. https://doi.org/10.1016/j.ympev.2007.05.011

Sousa-Santos C, Jesus TF, Fernandes C *et al.* Fish diversification at the pace of geomorphological changes: evolutionary history of western Iberia Leuciscinae (Teleostei: Leuciscidae) inferred from multilocus sequence data. *Molecular Phylogenetics and Evolution* 2019;**133**:263–85. https://doi.org/10.1016/j.ympev.2018.12.020

Stange KM, Van Balen RT, Garcia-Castellanos D *et al.* Numerical modelling of Quaternary terrace staircase formation in the Ebro foreland basin, southern Pyrenees, NE Iberia. *Basin Research* 2016;**28**:124–46. https://doi.org/10.1111/bre.12103

Stephens M, Donnelly P. A comparison of Bayesian methods for haplotype reconstruction from population genotype data. *American Journal of Human Genetics* 2003;**73**:1162–9.

Swofford DL. *Phylogenetic Analysis Using Parsimony (PAUP), v. 4.0 b10*. Champaign: Illinois Natural History Survey, 2002.

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