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Research Article

Integrative taxonomy reveals non-native *Squalius* hybrids in the Iberian Peninsula

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Abstract

The introduction of non-native species poses a critical threat to biodiversity in Mediterranean freshwater ecosystems. This study documents the first record of non-native *Squalius* hybrids in the Ave River (Iberian Peninsula), combining morphological and molecular data to confirm their presence and assess their potential origin. The specimens exhibit hybrid characteristics, with most morphological and meristic traits overlapping those of both *S. cephalus* and *S. squalus*. Almost all individuals analysed showed an admixed genomic background for both species, suggesting a hybrid origin likely within the species' native range before their introduction into Portugal through human-mediated activities, likely related to recreational fishing. Furthermore, molecular data also supports hybridization with the native *S. carolitertii*, raising conservation concerns given the negative impacts associated with hybridization with non-native species. By combining data from social media and citizen science platforms, we were able to map the rapid expansion of this invasive chub in the Ave River from 2018 until now. Our findings emphasize the urgent need for population control measures, while enhancing monitoring, genetic studies, and increased public awareness to mitigate the impacts of invasive species on Iberian freshwater biodiversity.

Key words: Ave river, chub, hybridization, invasive species, morphology, Portugal, *Squalius cephalus*, *Squalius squalus*

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Introduction

The Mediterranean region is considered a freshwater biodiversity hotspot, characterised by high levels of endemic species shaped by its unique palaeogeographic history (Zardoya and Doadrio 1999). However, fish species face severe threats in this region, mainly caused by human pressures including pollution, habitat destruction, fragmentation, and the introduction of non-native species, with the rapid spread of these species in recent decades being particularly concerning (Clavero et al. 2010; Hermoso et al. 2011; Milardi et al. 2022). Addressing this trend

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requires the implementation of effective biosecurity strategies supported by active monitoring of new occurrences and research into their dispersal pathways and vectors (Ricciardi et al. 2021).

Within the Mediterranean region, the Iberian basins have some of the highest proportions of native species at risk of extinction (Costa et al. 2021), a situation that has been significantly exacerbated by the proliferation of non-native species (Clavero et al. 2013; Anastácio et al. 2019; Valerio et al. 2022). Most of the recently established species in the Iberian Peninsula originate from other parts of Europe (Anastácio et al. 2019). The northeastern Iberian basins are regarded as a key gateway for species introductions from France, facilitating a northeast-to-west spread across both Spanish and Portuguese basins (García-Berthou et al. 2005; Clavero and García-Berthou 2006), although some evidence suggests alternative introduction pathways (Ribeiro and Veríssimo 2014; Garcia-Raventós et al. 2020). There is, however, a consensual understanding that non-native fishes are primarily introduced for recreational fishing purposes, either as bait or as targeted species (Banha et al. 2017a; Anastácio et al. 2019), a trend that continues to grow (Banha et al. 2024).

In recent years, technological advancements and increased awareness of non-native species impacts have improved our capacity to monitor new species arrivals. Nevertheless, monitoring aquatic habitats remains challenging due to logistical constraints, including limited accessibility to remote locations, insufficient funding and equipment availability, and a lack of trained personnel. Additionally, the introduction of species that are morphologically similar or indistinguishable from other already established non-native species, such as *Carassius auratus* (Linnaeus, 1758) and *Carassius gibelio* (Block, 1782) (Ribeiro et al. 2015) or those resembling native species, presents significant challenges for accurate identification, monitoring, and management (e.g., Aparicio et al. 2013; Pérez-Bielsa et al. 2025). These morphological similarities can delay the identification of newly occurring species, allowing them to spread undetected for extended periods. In this context, species from highly diverse genera including minnows (Artaev et al. 2024), chubs (Buj et al. 2020) and barbels (Rossi et al. 2021), which share ambiguous morphological traits and have taxonomic classifications that remain actively debated, are particularly susceptible to these identification and monitoring challenges.

This is the case of the genus *Squalius* Bonaparte, 1837, which is present across European watersheds and comprises more than 50 species, many of which are endemic to specific drainages in the Meridional peninsulas (Kotellat and Freyhof 2007; Perea et al. 2020). Most of these species share morphological features and exhibit overlapping meristic traits. In the Iberian Peninsula, thirteen species of *Squalius* have been identified (Mendes et al. 2024), many of which have been described mainly based on molecular data, with subtle morphological differences (e.g., Doadrio and Carmona 2006; Doadrio et al. 2007). Until now, no non-native species from this genus has been recorded in Portugal. However, recent genetic analyses have identified a chub population of *Squalius cephalus* (Linnaeus, 1758) and evidence of its hybridization with the endemic *Squalius laietanus* Doadrio, Kottelat and de Sostoa, 2007 in Spain (Pérez-Bielsa et al. 2025). Furthermore, eDNA data from the Ave River detected the presence of *Squalius squalus* (Bonaparte, 1837), which needs to be confirmed (Curto et al. 2025). This study examines the occurrence of non-native *Squalius* hybrids in the Ave River using morphological and molecular analyses. Additionally, we used data mined from social media forum groups to map recent occurrences and to construct their current potential distribution in Portugal.

Materials and methods

Study area

This study was conducted in the Ave River, northern Portugal, which hosts a single native *Squalius* species: the Northern Iberian chub, *Squalius carolitertii* (Doadrio, 1988). This species rarely exceeds 25 cm, has a moderately compressed body, rounded snout with slightly subterminal mouth, and silvery-olive scales without dark margins. Its paired, anal, and caudal fins are hyaline to pale amber with no vivid pigmentation (Collares-Pereira et al. 2021). In contrast, non-native chubs in Portuguese basins, documented through angler photos and iNaturalist records, exceed 40 cm, have bulkier heads with terminal mouths, display orange-red or black ventral and anal fins, and show scales with dark edges forming a reticulate pattern. (Kottelat and Freyhof 2007). These differences in size, fin coloration, and scale pattern provide clear criteria for anglers and citizens to distinguish native *S. carolitertii* from non-native chubs.

Between 2017 and 2025, we collected 37 unidentified chubs, using electrofishing in the Ave River (Table 1). The first specimen (FRISK3481) was captured in 2017 during a regular fish monitoring campaign. Upon examination, there was a suspicion that the specimen could be an unreported non-native species to Portuguese freshwaters and thus a tissue sample was kept for genetic analysis. For the same reason, in 2020, tissue samples were collected from seven additional individuals (PEI0456–PEI0462). The remaining 29 individuals were captured during subsequent monitoring campaigns between 2024 and 2025 at four distinct locations along the Ave River (Table 1). Fin clips were extracted from these specimens and preserved in absolute ethanol for genetic analyses. For these, the whole individuals were kept for morphological analysis. The one specimen captured in 2024 was transported frozen to the laboratory and stored at -20 °C for posterior morphological identification. The 28 specimens captured in 2025 were euthanized in the field using clove oil and immediately fixed in 10% formalin. Following fixation for at least three days, these specimens were gradually transitioned to a 70% ethanol solution for long-term preservation, involving exposure to progressively higher ethanol concentrations (30% for two days, followed by 50% for three days). Additionally, to serve as reference for the genetic analysis, tissue samples from six *S. cephalus* individuals from the native range (50°01'55.6"N, 14°59'35.0"E), captured in 2022, and four from *S. carolitertii*, captured in 2017 in the Ave River, were added.

Morphological analysis

In the laboratory, the specimens were photographed, and species identification was carried out using general identification keys (Doadrio and Carmona 2006; Kottelat and Freyhof 2007). Morphological measurements were conducted from point to point to the nearest 0.1 mm with vernier calipers on the left side of the specimen (Suppl. material 1), following the methodology described by Bogutskaya and Zupanič (1999; 2010). The standard length was measured from the tip of the snout to the end of the hypural complex. The length of the caudal peduncle was measured from the posterior edge of the base of the last anal fin ray to the end of the hypural complex, at the mid-height of the caudal fin base. Both head length and interorbital width were measured including the skin fold. Post-dorsal length was measured from the insertion point of the dorsal fin to the end of the hypural complex. The total lateral-line scale count included all pored scales, extending from the first scale

Table 1. Details of *Squalius* specimens collected for genetic analysis, including sample codes, number of individuals (n), geographic coordinates (latitude and longitude), and year of collection. Samples marked with an asterisk (*) were also examined for morphological and meristic characteristics.

Codes	Samples (*n*)	Coordinates	Year
FRISK3481	1	41°21'05.0"N, 8°40'54.6"W	2017
PEI0456–PEI0462	7	41°20'48.9"N, 8°35'48.0"W	2020
Scep_1_13*	1	41°22'22.7"N, 8°42'07.6"W	2024
Squa1-Squa10*	10	41°24'43.8"N, 8°22'46.2"W	2025
Squa11-Squa13*	3	41°28'18.1"N, 8°20'48.0"W	2025
Squa14-Squa28*	15	41°26'57.1"N, 8°21'00.2"W	2025

just posterior to the post temporal bone to the most posterior scale located at the base of the caudal-fin rays. Fin rays were counted separately as simple and branched rays (Bogutskaya and Zupanič 2010; Suppl. material 1). The last two branched rays articulating on a single pterygiophore in the dorsal and anal fins were noted as "1½". Prior to statistical analysis, all morphometric variables were standardized to remove the effects of size following Kottelat and Freyhof (2007). Head-related measurements were expressed as proportions of head length (HL), while all remaining body measurements were expressed as proportions of standard length (SL) or body depth. Differences in morphometric and meristic characteristics between the studied groups were assessed using independent two-sample (Student's) t-test, after confirming normality with Shapiro–Wilk tests (Zar 1996). All data processing and analyses were performed in R version 4.4.3 (R Development Core Team 2025). Specimens and tissues were deposited in the zoological collections "Museu Bocage" of the Museu Nacional de História Natural e da Ciência, Universidade de Lisboa, Portugal (MNHNC-MB05-004006 – MB05-004015) (Suppl. material 2).

Genetic analysis

DNA was extracted from fin clips or muscle tissue preserved in absolute ethanol using commercial kits. More specifically, the seven samples collected in 2020 were processed using EZ-10 Genomic DNA Kit (Bio Basic, Canada), while the remaining were processed using the E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek, Norcross, GA, USA). Both approaches followed the manufacturer's protocols. DNA quality was assessed through 0.8% agarose gel electrophoresis. Molecular assessment of specimen identity was based on variation at three loci. The mitochondrial cytochrome b (cyt b) gene was amplified through the primer set Glu-F and Thr-R (amplicon ~1140 bp, Zardoya and Doadrio 1998). Two nuclear genes were also targeted: early growth response 2b (egr 2b, ~747 bp; Waap et al. 2011) and beta-actin (actb, ~933 bp; Robalo et al. 2006). PCR reactions were carried out in a 10 µl volume containing 5 µl of MyTaq Mix (Meridian Bioscience, Cincinnati, Ohio, USA) and 0.4 µl of each primer (10 µM). The following temperature profile was implemented: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min (50 °C in the case of cyt b) and 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR success was evaluated through 2% agarose gel electrophoresis. The resulting amplicons were sequenced in both directions using Sanger sequencing, implemented by the molecular analysis services from STAB Vida (Caparica, Portugal).

The obtained chromatograms were manually aligned using Geneious Prime (Biomatters Ltd., New Zealand, v. 2025.1.2). For each locus, the sequences generated in this study were joined to publicly available sequences in NCBI for the

genus *Squalius* (Suppl. material 3). Up to 10 sequences per each species were downloaded with care to cover multiple drainages for widespread taxa; otherwise, 10 random sequences were downloaded. Additionally, three sequences from the genus *Iberochondrostoma* Robalo, Almada, Levy & Doadrio, 2007 were added as outgroups. Sequences were aligned using AliView (Larsson 2014) implementing the MUSCLE algorithm. For the nuclear markers (egr 2b and actb) haplotypes were phased in DnaSP v. 5.10.1 (Librado and Rozas 2009). Genetrees for each marker were estimated using the maximum likelihood method implemented in IQtree (Nguyen et al. 2015). The same program was used to select for the best substitution model considering partitions based on codon position. Node support was obtained through 1000 fast bootstraps. IQtree was run using the web server W-IQ-TREE (Trifinopoulos et al. 2016). The obtained tree was visualised and customised using FigTree ver. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Social media and citizen science data collection

The use of social media information about invasive fishes has proven valuable for detecting and identifying new invasive fish (Ribeiro and Veríssimo 2014; Kalous et al. 2018), describing spatial invasion patterns (Banha et al. 2017b), and updating the distribution of recently arrived invasive species (Martelo et al. 2021). To update the distribution of alien chubs in the Ave River, extensive data collection was conducted using various sources of information as recommended by Tricarico (2022), including Facebook and citizen science platforms. Publicly available social media posts related to fishing for individuals belonging to the genus *Squalius* were collected from Facebook, a popular platform among anglers in Portugal (see Gago et al. 2016; Banha et al. 2017b). The data collection process adhered strictly to ethical guidelines for using social media in fisheries research, ensuring that no personal information was recorded and that no original user content was shared publicly without permission (Monkman et al. 2018). Searches were conducted within fishing-focused groups where catches predominantly occur in Portuguese waters following the guidelines described in Chowdhury et al. (2024). The time window of our search was from July 2019 (the creation date of the oldest fishing group) to December 2024. Keywords used to identify relevant posts included the species' common name and its variants, such as "chub", "escalo", "leucisco", "chevesne" and the nickname "trutas do Ave". Online searches were also conducted on BioDiversity4All (https://www.biodiversity4all.org/), a Portuguese biodiversity citizen science platform connected to the international project iNaturalist. Species identification was primarily based on the visual examination of photographs accompanying the posts, using criteria established by Gago et al. (2016). Only posts explicitly related to recreational fishing for fish belonging to the genus *Squalius* were analysed. These posts featured content such as videos and photographs of the fish specimens after being caught. For each post, the primary objective was to extract information on the date and location of the catch. Publications lacking a photographic record of the specimen were not considered for presence records. All posts were manually reviewed to confirm species identification and verify catch locations. Where possible, the background of the photographs was cross-referenced with satellite imagery available on Google Street View to increase the accuracy of the location data (following the georeferencing steps on Chowdhury et al. 2024). The information collected was compiled into a dataset listing locations and dates of the catches, serving as the basis for further analysis.

Results

Morphological analysis

After the visual examination of the 29 specimens, 10 were identified as *S. cephalus* and 19 as *S. squalus* (Bonaparte, 1837). The *S. cephalus* individuals had standard lengths (SL) of 107–169 mm and body masses of 20.5–87 g. Fig. 1 illustrates two *S. cephalus* individuals: the specimen in Fig. 1a (S_cep_1_13), collected in 2024, and the specimen in Fig. 1b (Squa13), collected in 2025. Both individuals (Fig. 1a, b) were streamlined, laterally compressed, and fusiform, lacking a ventral keel anterior to the anus. Each displayed a pointed head, a slightly arched dorsal profile, and a more convex ventral profile, forming a clear head-trunk discontinuity. Flanks showed a reticulate pattern from black pigment along scale margins. Ventral and anal fins were vivid orange-red in Fig. 1a but less intensely coloured in Fig. 1b, which showed only red-orange pigments in the ventral and pelvic fins.

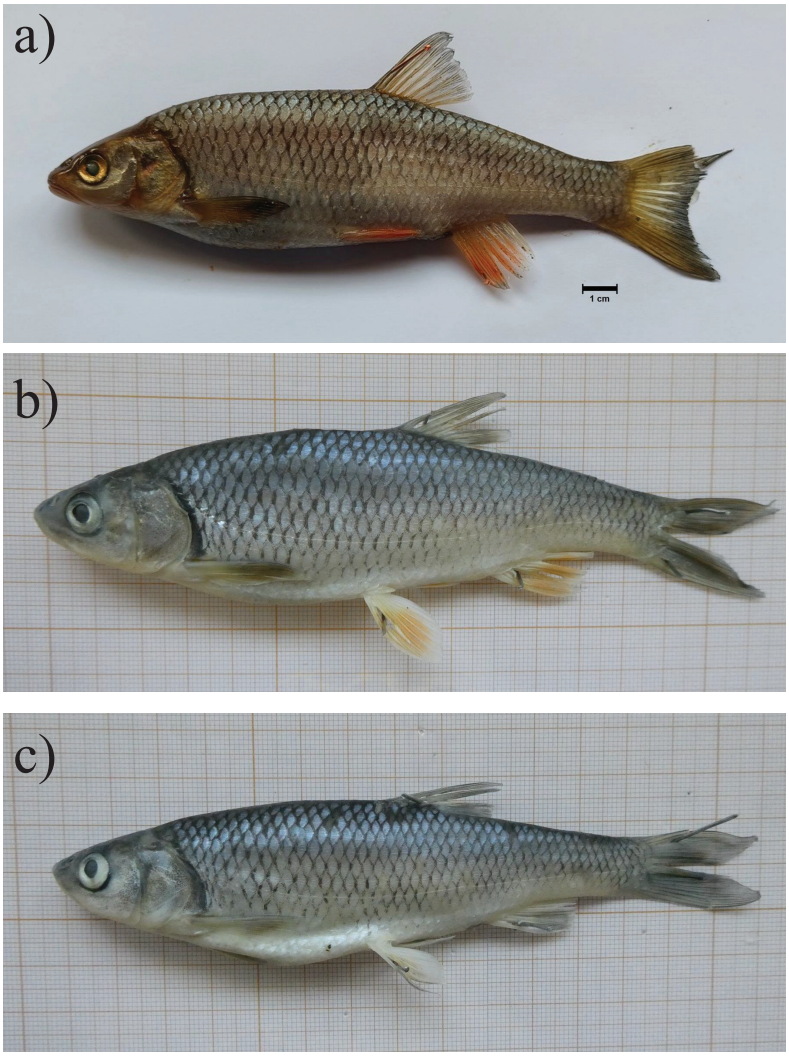

Figure 1. Representative hybrid specimens between *Squalius cephalus* and *S. squalus* captured in the Ave River, illustrating the observed morphological continuum. **a.** hybrid individual with predominantly *Squalius cephalus*-like morphological traits (169 mm total length, 87.0 g); **b.** hybrid individual with predominantly *Squalius cephalus*-like morphological traits (135 mm total length, 41.9 g); **c.** hybrid exhibiting more *Squalius squalus*-like traits (125 mm total length, 30.1 g). All three individuals were confirmed as hybrids by mitochondrial and nuclear markers and have been deposited in the Museu Bocage collection, Museu Nacional de História Natural e da Ciência (University of Lisbon, Portugal), under accession numbers MB05-004015, MB05-004008 and MB05-004012, respectively.

The *S. squalus* specimens ranged from 46–134 mm in SL and weighed between 1.27 and 40.12 g. Fig. 1c shows a representative *S. squalus* (Squa19), which exhibited an elongated body lacking the pronounced dorsal discontinuity characteristic of *S. cephalus*. A row of dense black pigment dots along the outer margins of the scales on the back and flanks produced a regular reticulate pattern. Body coloration ranged from bronze to brown, while the ventral and anal fins were typically black to hyaline, without the distinctive red pigmentation seen in *S. cephalus*.

Although all 39 morphometric variables exhibited overlapping values between *S. cephalus* and *S. squalus*, a subset of 12 traits was found to significantly differentiate the body shape and proportional anatomy of the two species (Suppl. material 1). *Squalius cephalus* exhibited a significantly longer postorbital distance (47.3–51.7% HL vs. 41.9–49.1% HL; $p < 0.001$) compared to *S. squalus*. The head morphology further differed with *S. cephalus* having greater head length relative to standard length (24.9–27.9% SL vs. 24.2–26.8% SL; $p < 0.05$) and greater head length as a proportion of predorsal distance (45.7–52.2% vs. 44.7–48.9%; $p < 0.05$). *Squalius cephalus* exhibited a significantly deeper body profile at the dorsal fin origin (23.4–26.2% SL vs. 20.6–25.2% SL; $p < 0.01$) and a greater distance between the pectoral and pelvic fin origins (25.2–29.9% SL vs. 23.7–29.6% SL; $p < 0.05$). In contrast, *S. squalus* displayed a thicker caudal peduncle relative to body depth (40.0–46.4% vs. 35.3–43.2%; $p < 0.01$). Regarding fin morphology, *S. squalus* exhibited significantly greater dorsal and anal fin depths relative to body depth (dorsal fin: 80.1–95.9% vs. 78.2–89.2%, $p < 0.01$; anal fin: 63.5–89.9% vs. 51.1–73.8%, $p < 0.01$), as well as a larger anal fin depth relative to standard length (15.5–19.1% SL vs. 12.8–17.6% SL; $p < 0.05$). *Squalius squalus* had significantly larger eyes relative to both standard length and head length (6.2–8.4% SL vs. 4.9–6.9% SL; 23.3–29.9% HL vs. 19.8–26.8% HL; $p < 0.01$ for both). It also exhibited greater head depth at the nape compared to *S. cephalus* (68.3–79.3% HL vs. 67.4–75.2% HL; $p < 0.01$). The number of branched rays in the anal fin differed significantly, with *S. squalus* showing mostly 9½ rays (rarely 8½) and *S. cephalus* 8½ (Suppl. material 1). Other meristic traits did not differ significantly.

Genetic analysis

Sequence information (in GenBank as PV686259–PV686389) was successfully retrieved for all samples for cyt b and egr 2b, while for actb sequencing failed for two individuals (Table 2, Suppl. material 2). These were added into a final alignment containing additional 217, 44, and 62 sequences from 37, 9, and 11 *Squalius* species for cyt b, egr 2b, and actb, respectively. The final matrix of the cyt b sequences consisted of 1,156 characters, containing 453 variable characters with 401 parsimony informative sites. The alignments of egr 2b and actb genes comprised 638 and 859 characters, respectively, with 20 and 69 variable sites, of which 17 and 64 were informative. For cyt b, the samples of the unidentified *Squalius* clustered into three groups with relatively high support values (>80%). Six individuals clustered with *S. cephalus*, one with the group containing all *S. carolitertii* sequences, while the remaining individuals grouped with *S. squalus* (Fig. 2). This last group also includes the Adriatic-Hellenic *Squalius* clade, comprising *S. zrmanjae* Karaman, 1928, *S. lucumis* (Bianco, 1983) and *S. prespensis* (Fowler, 1977). Unknown *Squalius* sequences for the nuclear genes were also clustered into three groups (Fig. 2), the main difference being that the *S. squalus* group forms a polytomy at the

Table 2. Summary of morphological and molecular results. Morphological species identification was based on identification keys (Doadrio and Carmona 2006; Kottelat and Freyhof 2007). Measurements were rounded to the nearest 0.1 mm with vernier callipers. Molecular analyses include results for the mitochondrial cytochrome b gene ("cytb"; amplicon ~1140 bp, Zardoya and Doadrio 1998), and haplotypes, labelled as "H1" and "H2", for the two nuclear genes, the early growth response 2b gene ("egr 2b"; ~747 bp, Waap et al. 2011), and β-actin gene ("Beta actin" ; ~933 bp, Robalo et al. 2006).

Sample code	First screening	Origin	Morphological ID	cytb	egr 2b		Beta actin		Molecular ID
					H1	H2	H1	H2	
Squa1	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus*	*S. squalus*	*S. squalus* x *cephalus*
Squa2	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. cephalus*	*S. cephalus*	*S. cephalus*	*S. cephalus*	*S. squalus* x *cephalus*
Squa3	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	Fail	Fail	*S. squalus* x *cephalus*
Squa4	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus*	*S. squalus*	*S. squalus* x *cephalus*
Squa5	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus*	*S. squalus*	S. *squalus* x *cephalus*
Squa6	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. cephalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
Squa7	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
Squa8	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. cephalus*	*S. cephalus*	*S. squalus*	*S. squalus*	*S. squalus* x *cephalus*
Squa9	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. cephalus*	*S. cephalus*	*S. squalus*	*S. squalus*	*S. squalus* x *cephalus*
Squa10	Unknown *Squalius* sp.	Ave River	*S. cephalus*	*S. cephalus*	*S. cephalus*	*S. cephalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
Squa11	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. cephalus*	Iberian lineage	*S. squalus*	Iberian lineage	*S. squalus* x *cephalus* x *carolitertii*
Squa12	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. cephalus*	*S. squalus*	*S. cephalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
Squa13	Unknown *Squalius* sp.	Ave River	*S. cephalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. cephalus*	S. squalus x *cephalus*
Squa14	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*
Squa15	Unknown *Squalius* sp.	Ave River	*S. cephalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. cephalus*	*S. cephalus*	*S. squalus* x *cephalus*
Squa16	Unknown *Squalius* sp.	Ave River	*S. cephalus*	*S. carolitertii*	*S. cephalus*	*S. cephalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus* x *carolitertii*
Squa17	Unknown *Squalius* sp.	Ave River	*S. cephalus*	*S. cephalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
Squa18	Unknown *Squalius* sp.	Ave River	*S. cephalus*	*S. cephalus*	*S. squalus*	*S. cephalus*	*S. squalus*	*S. squalus*	*S. squalus* x *cephalus*
Squa19	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
Squa20	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus*	*S. squalus*	*S. squalus* x *cephalus*
Squa21	Unknown *Squalius* sp.	Ave River	*S. cephalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
Squa22	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. cephalus*	*S. cephalus*	*S. squalus*	*S. squalus*	*S. squalus* x *cephalus*
Squa23	Unknown *Squalius* sp.	Ave River	*S. cephalus*	*S. carolitertii*	*S. squalus*	*S. cephalus*	*S. squalus*	*S. squalus*	*S. squalus* x *cephalus* x *carolitertii*
Squa24	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. cephalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
Squa25	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*
Squa26	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
Squa27	Unknown *Squalius* sp.	Ave River	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	Fail	Fail	*S. squalus*
Squa28	Unknown *Squalius* sp.	Ave River	*S. cephalus*	*S. cephalus*	*S. cephalus*	*S. cephalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
S_cep__1_13	Unknown *Squalius* sp.	Ave River	*S. cephalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
FRISK3481	Unknown *Squalius* sp.	Ave River	Not available	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
PEI0456	Unknown *Squalius* sp.	Ave River	Not available	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*
PEI0457	Unknown *Squalius* sp.	Ave River	Not available	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
PEI0458	Unknown *Squalius* sp.	Ave River	Not available	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*
PEI0459	Unknown *Squalius* sp.	Ave River	Not available	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	*S. squalus* x *cephalus*
PEI0460	Unknown *Squalius* sp.	Ave River	Not available	*S. squalus*	*S. squalus*	*S. squalus*	*S. squalus*	*S. cephalus*	S. squalus x *cephalus*
PEI0461	Unknown *Squalius* sp.	Ave River	Not available	*S. squalus*	*S. squalus*	*S. squalus*	Fail	Fail	*S. squalus*
FRISK481	*S. carolitertii*	Ave River (native)	Not available	*S. carolitertii*	*S. carolitertii*	*S. carolitertii*	Fail	Fail	*S. carolitertii*
FRISK 477	*S. carolitertii*	Ave River (native)	Not available	*S. carolitertii*	*S. carolitertii*	*S. carolitertii*	Fail	Fail	*S. carolitertii*
FRISK 478	*S. carolitertii*	Ave River (native)	Not available	*S. carolitertii*	*S. carolitertii*	*S. carolitertii*	Fail	Fail	*S. carolitertii*
FRISK 480	*S. carolitertii*	Ave River (native)	Not available	*S. carolitertii*	*S. carolitertii*	*S. carolitertii*	Fail	Fail	*S. carolitertii*
SC133 5L	*S. cephalus*	Czechia (native)	Not available	Fail	*S. cephalus*	*S. cephalus*	Fail	Fail	*S. cephalus*
SC73 5L	*S. cephalus*	Czechia (native)	Not available	Fail	*S. cephalus*	*S. cephalus*	*S. cephalus*	*S. cephalus*	*S. cephalus*
SC77 5L	*S. cephalus*	Czechia (native)	Not available	*S. cephalus*	*S. cephalus*	*S. cephalus*	*S. cephalus*	*S. cephalus*	*S. cephalus*
SC82 5L	*S. cephalus*	Czechia (native)	Not available	*S. cephalus*	*S. cephalus*	*S. cephalus*	Fail	Fail	*S. cephalus*
SC67 5L	*S. cephalus*	Czechia (native)	Not available	*S. cephalus*	*S. cephalus*	*S. cephalus*	Fail	Fail	*S. cephalus*
SC72 5L	*S. cephalus*	Czechia (native)	Not available	Fail	*S. cephalus*	*S. cephalus*	Fail	Fail	*S. cephalus*

base of *S. cephalus* and *S. carolitertii* for egr 2b, and *S. cephalus* is paraphyletic to *S. squalus* for actb. Furthermore, in both genetrees *S. carolitertii* sequences do not form a monophyletic group being intermingled with other Iberian *Squalius*

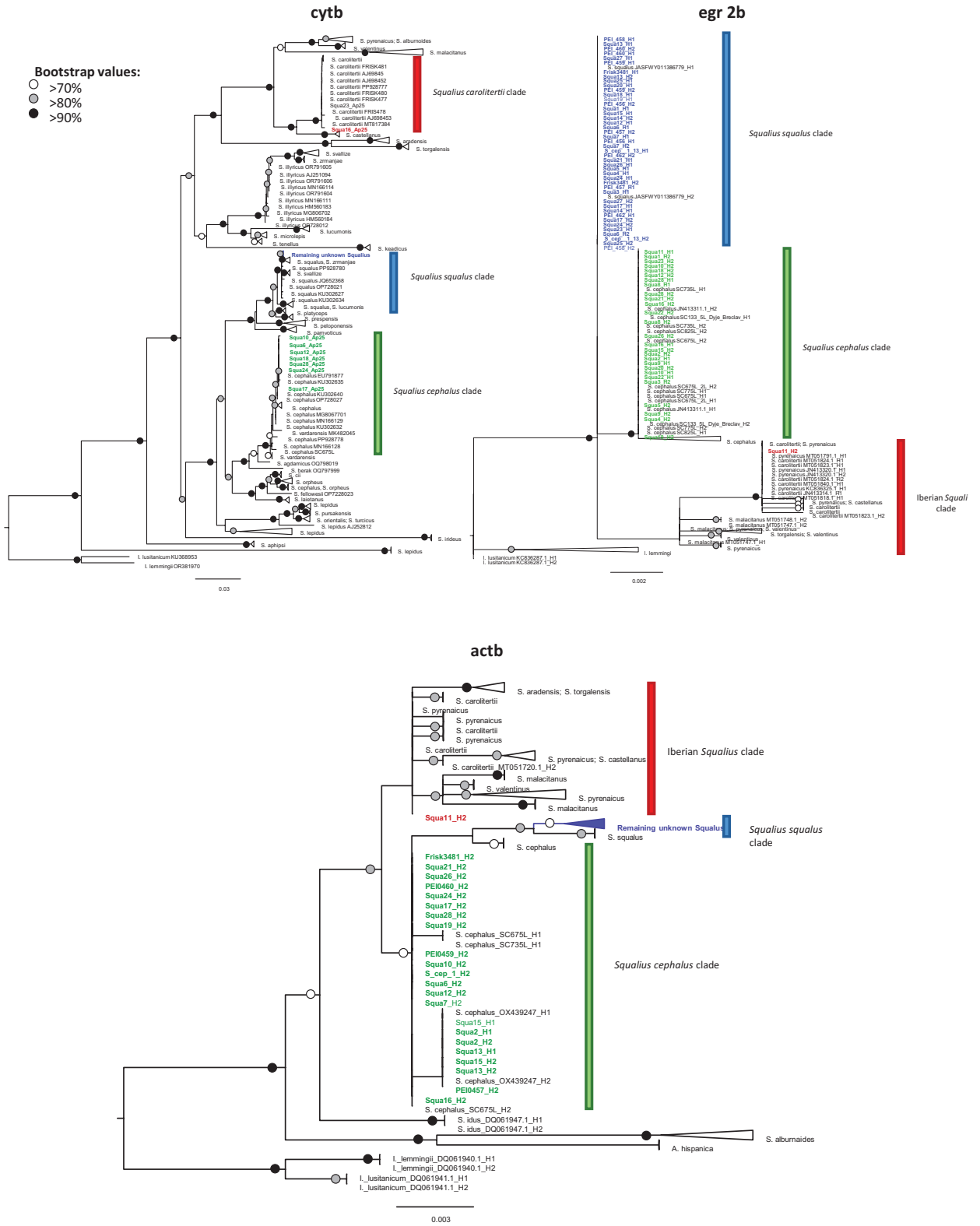

Figure 2. Reconstructed phylogeny of the cytochrome b (cyt b), early growth response 2b (egr 2b), and beta-actin (actb) sequences of *Squalius* samples included in present study together with sequences available in NCBI. The new sequences were colored based on the groupings they form: red *S. carolitertii* / *Iberian Squalius lineage*, blue *S. squalus*, and green *S. cephalus*.

species. Interestingly, several individuals carried heterozygous genotypes for haplotypes grouping with different species (Suppl. material 3). These included 13 individuals for egr 2b and 17 for actb, with the most common combination involving

S. cephalus and *S. squalus*. One of these (Squa11) had one of the haplotypes grouping with the Iberian *Squalius* group (Table 2). Only seven individuals had sequences consistently grouping with the same species, *S. squalus*. The remaining individuals show admixed origins involving the following combinations: 28 *S. cephalus* x *S. squalus* hybrids, with mitochondrial DNA from both species represented, and three tri-species hybrids, *S. cephalus* x *S. squalus* x *S. carolitertii*/ Iberian lineage, of which two individuals showed Iberian mtDNA and one had *S. squalus* mtDNA.

Online-based reports

We gathered 97 online records of non-native *Squalius*, comprising 95 angler records from social media and 2 citizen science records from Biodiversity4All/iNaturalist platform, demonstrating a broad distribution and rapid expansion of this species in the Ave River drainage (Suppl. material 4). The first citizen science record dated to 2018 and was located close to the site where we collected the first chub specimen, in the lower Ave River (Santo Tirso). Online fishing groups' posts of chub occurrences began appearing in 2020, with the highest number of records documented in 2023 (46 occurrences), followed by 2024 (26 occurrences) and 2021 (13 occurrences) (Fig. 3). Online records generally originated from the mainstem of the river and upstream of the locations where we collected specimens.

Discussion

This study provides robust evidence for the presence of the non-native *Squalius squalus* in the Ave River, confirming previous eDNA-based detection by Curto et al. (2025). More importantly, the combined use of morphological and multi-locus

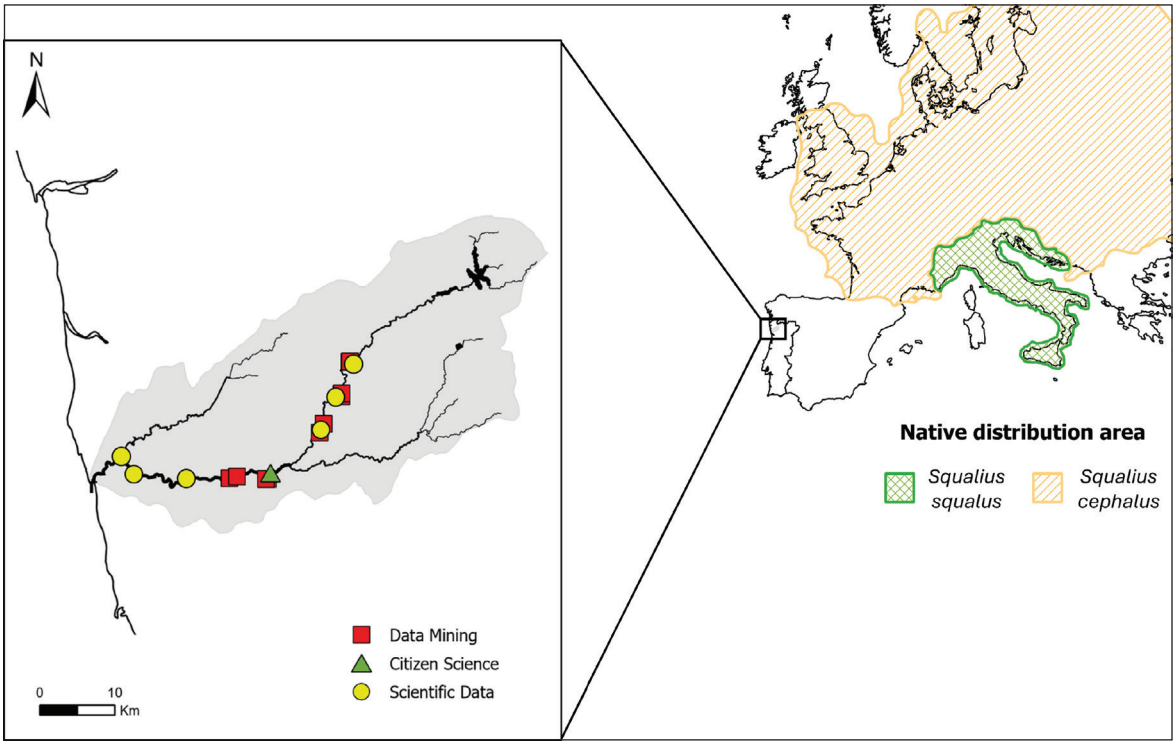

Figure 3. Map of the analysed *Squalius* samples in the Portuguese part of the Ave River. Records depict scientific (yellow circle), data mining records (red square), and citizen science (green triangle). Native distribution areas adapted from Kottelat and Freyhof (2007).

molecular data, complemented with citizen science information, revealed the widespread occurrence of non-native hybrids across the basin. Our findings indicate that these hybrids have been present in the Ave River since 2017 and are currently occupying approximately 50 km of a river stretch. The detection of *S. squalus* increases the number of established non-native freshwater fishes in mainland Portugal to 22 (see Anastácio et al. 2019; Brandão et al. 2025). The present study further highlights illegal introductions for recreational fishing from central Europe as the primary vector for new fish introductions in the region (Ribeiro et al. 2009a; Ribeiro and Veríssimo 2014; Garcia-Raventós et al. 2020). The addition of molecular analysis to traditional morphological identification of the collected specimens was essential to show that these specimens are hybrids between *S. cephalus and S. squalus*, given its admixed molecular assignment to both species. This underscores the well-documented difficulty of diagnosing pure individuals when these taxa come into secondary contact and hybridise (Gouskov and Vorburger 2016). Most morphometric and meristic traits showed extensive overlap, allowing hybrids to fall within the phenotypic range of either species and escape detection without genetic confirmation. In our material, the characteristics that contributed most strongly to the differentiation between species were the coloration of the fins (red in *S. cephalus*, black in *S. squalus*), a longer head and thicker dorsal trunk in *S. cephalus*, a thicker caudal peduncle, larger eyes, and larger dorsal and anal fins in *S. squalus*, as well as a higher number of branched anal fin rays in *S. squalus* (Bogutskaya and Zupančič 2010). Consequently, reliance on morphology alone would have misclassified these specimens as pure species and masked the occurrence of hybrids in the Ave River, highlighting the necessity for molecular diagnostics in field assessments, conservation planning and monitoring programmes.

Both species hybridize in their native range, in contact-areas (Gouskov and Vorburger 2016), which may be the source of the individuals found in the Ave River. This hypothesis contrasts with a scenario involving independent introductions of both species and is supported by three lines of evidence. First, no individual was exclusively assigned to the *S. cephalus* lineage, which would be expected if "pure" individuals of this species had been introduced. Second, most individuals exhibited some degree of hybridization, consistent with the source to be already a hybrid population. Third, most hybrid individuals showed a predominant genetic assignment to one of the two species, indicating that they are likely multi-generational hybrids. The individuals not showing any signal of admixture were assigned to *S. squalus* by both molecular and morphological analysis. However, due to the limited number of markers we cannot rule out the hypothesis that also these have some degree of admixture in their genome. It is thus necessary to increase the marker density to confirm if this is the case. In any case, it is evident that these hybrids constitute a new occurrence for the Iberian Peninsula as it does not relate to any known Iberian native fish species.

In the Iberian Peninsula, earlier studies have shown that the main entryway for non-native fish is the north-eastern part of Iberia, such as Boadella reservoir, Lake Bañoles and the Lower Ebro (García-Berthou et al. 2005; Gkenas et al. 2015; Merciai et al. 2018), from which secondary introductions spread to the main Iberian drainages (Ribeiro et al. 2009b; Gago et al. 2016). However, *S. squalus* has not been recorded in the Catalonia region and *S. cephalus* was only recorded in 2021 (Pérez-Bielsa et al. 2025), with no other known records in central and western Iberia. This new record in the northwestern part of Iberia highlights the existence of different patterns of introduction across Iberia, with Portuguese drainages also receiving direct introductions from outside of Iberia. Ribeiro and Veríssimo (2014) first proposed the

hypothesis of a possible direct introduction of the roach, *Rutilus rutilus* (Linnaeus, 1758) from France to northwestern Iberia (Ave River). Similarly, Garcia-Raventós et al. (2020) documented the occurrence of a French minnow (*Phoxinus* sp.) in an isolated western drainage in Portugal (Douro River), with genetic analyses revealing that this population was distinct from other minnow populations across Iberia, further supporting the idea of multiple routes of introduction in the peninsula.

The occurrence of this new non-native hybrid in the Ave River drainage, together with previous first records of invasive fish for Portugal (Barros et al. 2000; Ribeiro and Veríssimo 2014), reinforces that this region serves as an entryway for new non-native fish species in mainland Portugal. This underscores the urgent need to strengthen law enforcement efforts and develop a comprehensive fisheries management plan for the Northern region of Portugal. Such a plan should involve not only outreach to angler groups but also the stocking of native fish species to reduce the risk of new introductions or the spread of existing non-natives. Moreover, we emphasize the importance of including this new non-native hybrid in the Portuguese Invasive Species List (Decree-Law No. 92/2019, Annex II). Implementing this measure promptly would prohibit the transport and possession of this non-native fish by anglers and reduce the likelihood of its secondary introduction into other Iberian basins. Additionally, considering the rumours of this hybrid population already in nearby river basins, but still without scientific confirmation, it is urgent to implement a monitoring network around this river basin and immediately initiate population control actions for this hybrid population while it is still spatially restricted.

The new record reported here is also important for guiding future risk analyses, i.e., the evaluation of the likelihood and impacts of the introduction and establishment of non-native species (Roy et al. 2018). Previous risk assessment studies have included several European species, including some of the leuciscids. In fact, representatives of this family have recently been introduced to the Iberian Peninsula, such as the asp *Leuciscus aspius* (Linnaeus, 1758) (Merciai et al. 2018) or the currently widespread bleak *Alburnus alburnus* (Linnaeus, 1758) (Martelo et al. 2021). Earlier studies conducting risk analyses (Almeida et al. 2013) and horizon scanning exercises (Cano-Barbacil et al. 2023; Oficialdegui et al. 2023) for the Iberian Peninsula have not included the cavedano chub (*S. squalus*), possibly because it was previously considered to have a negligible likelihood of introduction (Banha et al. 2024). In contrast, the European chub (*S. cephalus*) has been evaluated in several studies, though it was classified as having a low risk of arrival in the Iberian Peninsula (Cano-Barbacil et al. 2023; Oficialdegui et al. 2023) and a medium risk of invasiveness (Almeida et al. 2013), but now is occurring in north eastern Spain and hybridising with native catalonian chub (Pérez-Bielsa et al. 2025).

We found evidence of hybridization with the native chub species, namely with *S. carolitertii*. This was evident by three individuals sharing either mitochondrial or nuclear lineages with *S. carolitertii*. These individuals possessed both native and non-native mitochondrial lineages, suggesting that hybridization can occur with both male and female *S. carolitertii*. This increases the likelihood of hybridization and, consequently, raises conservation concerns. Hybridization with non-native species is a major conservation concern since backcrosses can disrupt the genetic integrity of native species, while favoring the invasive species with higher genetic diversity and locally adapted genes (Verhoeven et al. 2011). Hybridization is frequent within the genus *Squalius* (Durand et al. 2000; Buj et al. 2020) and even intergeneric hybrids (Almodóvar et al. 2012; Curto et al. 2022; Galvez-Bravo et

al. 2024). Indeed, Pérez-Bielsa et al. (2025) described the putative hybridization of the invasive *S. cephalus* with the endemic Catalan chub, *Squalius laietanus* Doadrio, Kottelat and de Sostoa, 2007 in northeastern Iberia, and highlighted the importance of genetic screening of the *Squalius* populations in this region. However, *S. carolitertii* may be of particular concern since it is already being impacted by hybridization with the invasive bleak (Curto et al. 2022; Galvez-Bravo et al. 2024). Thus, the negative effects of hybridization with non- native species can be further intensified by having two independent sources of intraspecific introgression.

One significant concern is the depletion of native fish populations caused by food competition and predation by the European chub (Caffrey et al. 2018). Previous studies conducted in the native range of the chub have demonstrated that this species undergoes an ontogenetic dietary shift, becoming piscivorous once individuals reach approximately 15 cm in fork length (FL), after which the proportion of fish in their diet increases progressively with length (Mann 1976; Ünver and Erk'akan 2011). Given the absence of native predators in Portuguese watersheds, Iberian fishes may exhibit some naiveness towards this fish, making them more susceptible to predation, as has been observed with other recent invaders (Ferreira et al. 2019; Ribeiro et al. 2021).

Finally, the introduction of diseases and parasites through spill-over from non-native species poses a significant risk. Recent evidence suggests that 50% of species introductions are accompanied by parasite co-introduction (Bernery et al. 2022). The likelihood of parasite transmission is further heightened by close evolutionary relationships between host species (Ortega et al. 2022), making this a particular concern for endemic Iberian chubs (*S. carolitertii*) as well as other leuciscids in Portuguese watersheds. In the Iberian Peninsula, there is limited knowledge about the putative co-introduction of non-native parasites and their effects on native fish fauna (Ribeiro and Leunda 2012).

Conclusion

A population of non-native *Squalius* hybrids is now established in Iberian watersheds and is likely to continue spreading throughout the Ave River, with a high probability of being introduced into nearby watersheds. Thus, it is highly recommended that water authorities implement population control actions towards this species as soon as possible to prevent the widespread dispersal of another non-native fish in Portugal. Moreover, our study underscores the need for further research to clarify its origin. Comprehensive genetic analyses incorporating genomic tools and a broader geographic scope are necessary to unravel the full history of these introductions, including identifying the source populations to improve management and outreach. Additionally, ecological monitoring of non-native *Squalius* hybrids is critical to assess its impacts on native fish communities, particularly given their potential for competition and broad hybridization cases. Management strategies should prioritize early detection and prevention of future introductions, with a focus on regulating angling practices. Public awareness campaigns targeting recreational fishermen and collaborative international efforts are essential to mitigate the spread of non-native species in Iberian freshwater ecosystems.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

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Author contributions

Conceptualization: CG, MC, MLL, FR. Investigation: CG, SN, MC, DD, DR, RR, JGN, MLL, MJA, FR. Methodology: CG, SN, MC, DD, DR, RR, JGN, MLL, FR. Funding acqui-sition: FR. Supervision: CG, FR. Visualization: MC, DD, DR. Writing – original draft: CG, SN. Writing – review and editing: MC, DD, DR, RR, JGN, MLL, MJA, FR.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Results of Student's t-tests comparing morphometric and meristic variables between hybrid specimens from the Ave River with predominantly *Squalius cephalus*-like traits and those with predominantly *Squalius squalus*-like traits

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Data type: xlsx

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Supplementary material 2

Voucher specimen information and GenBank accession numbers

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Supplementary material 3

Sequences downloaded from NCBI to complement molecular analysis

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Supplementary material 4

Location of records for the non-native *Squalius* hybrids in the Ave River, derived from social media and citizen science platforms

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