

Article

Widespread Hybridization between Invasive Bleak (*Alburnus alburnus*) and Iberian Chub (*Squalius* spp.): A Neglected Conservation Threat

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Abstract: Hybridization between native and nonnative fish species is a major conservation issue, especially in ecosystems with high levels of endemism, such as Iberian streams. To date, hybridization with the invasive bleak *Alburnus alburnus* has been reported for the Iberian chub *Squalius alburnoides* and *S. pyrenaicus* and in scattered locations only. However, the bleak is spreading in the region, potentially increasing the risks of hybridization with other *Squalius* species. To gather a more comprehensive picture on the current geography of hybridization, we compiled records on hybrids between bleak and chub in Portugal and conducted genetical assessments of hybrids between bleak and *S. carolitertii*. We found that hybridization with bleak is widespread throughout Portuguese river basins and involves at least *S. alburnoides*, *S. pyrenaicus* and *S. carolitertii*. Hybridization with bleak may not only cause waste of reproductive effort and damage the genetic integrity of these endemic species but also promote shifts in the reproductive dynamics of the *S. alburnoides* hybrid complex, which includes individuals with various ploidy levels and combinations of parental genomes, reproducing sexually and asexually. We recommend that future studies characterize the fitness of bleak hybrids and their ecological and genetic interactions with native fish, in order to design effective conservation measures.

Keywords: biological invasions; introgression; reproductive effort; endemic species; freshwater diversity; genetic integrity

1. Introduction

Biological invasions threaten biodiversity and contribute to species loss through different mechanisms, including hybridization [1–4]. Interbreeding between nonnative species colonizing new areas and local native species increases the extinction risk of the latter, with conservation concerns even if hybrids are unviable or infertile due to wasted

reproduction effort [5–8]. Fertile hybrids may form homoploid or polyploid lineages, potentially displacing parental species and leading to admixture between native and nonnative genomes [8–11]. The introgression of nonnative genes into native species is irreversible, damaging genetic integrity and disrupting local adaptations through the introduction of maladaptive genes, possibly causing the extinction of native genotypes [5,12–14]. Conversely, nonnative species may benefit from introgression of genetic variability and locally adapted genes from the native species, alleviating the loss of genetic variation through the founder effect [5,12,15,16].

Hybridization between native and nonnative species is particularly concerning in Iberian streams, which are among the most biodiverse and invaded ecosystems in the world [17]. Fish in particular are highly prone to interbreeding, due to external fertilization, weak reproductive isolation, high interspecies genetic compatibility, and human-mediated decrease in habitat complexity [18–21]. The spread of nonnative alleles among the gene pools of Iberian fish through introgression may have serious consequences on the fitness, ecology, behavior, and likelihood of population persistence [5,7,12].

After being introduced in Iberian streams as a foraging species by anglers in 1992, the bleak (*Alburnus alburnus*) has been expanding through multiple intentional introductions and natural expansion, and is currently widespread and locally abundant across the region [22–24]. The invasion of *A. alburnus* is associated with impacts on native fish fauna related to trophic competition and hybridization [25]. The latter may be particularly prevalent, since *A. alburnus* hybridizes with many different species in their native range [26–31]. Hybridization between *A. alburnus* and the Iberian chub (*Squalius pyrenaicus*) and the allopolyploid complex *Squalius alburnoides* has been reported in a few localities [32,33], but the extent of interbreeding with these and potentially with other species of *Squalius* remains unknown. Clarification of this issue is particularly important because endemic *S. alburnoides* is a hybrid complex, including individuals with various ploidy levels and combinations of parental genomes, reproducing sexually and asexually [34,35], whose reproductive dynamics may be disrupted by the inclusion of another hybridizing species, and particularly of the invasive *A. alburnus*.

Here, we sought to assess the geographical extent of interbreeding between the invasive bleak and native chub across Portuguese river basins. We mapped the occurrence of hybrids identified through morphological characters in multiple river basins across the current distribution range of bleak and most widespread chub, building on records gathered during ongoing projects. The first records of hybridization between bleak and *Squalius carolitertii* were further confirmed by the molecular assessment of individuals with intermediate morphology, captured in areas of sympatry between the two species. The results provide a more comprehensive picture of the occurrence of hybridization associated with bleak invasion throughout the Portuguese river basins and open new perspectives on the consequences of hybridization associated with biological invasions.

2. Materials and Methods

2.1. Occurrence Data and Mapping

Data on the occurrence of hybrids between *A. alburnus* and *Squalius* spp. were collected between 2015 and 2021 throughout the distribution range of *A. alburnus* in Portugal, covering areas of sympatry with *S. alburnoides*, *S. pyrenaicus* and *S. carolitertii*, the most widespread *Squalius* species in Portugal.

Records were obtained from several research projects (see Acknowledgments), covering seven basins and 503 sites (5 to 146 sites per basin), and involving electrofishing surveys. These surveys mainly targeted species other than *Squalius* sp. and *A. alburnus*, but whenever detected, hybrids were recorded. In all cases, hybrids were identified from morphological traits, focusing on meristic characters recommended by Almodóvar et al. [32], namely, number of: (a) lateral line scales; (b) transverse scales; (c) branched dorsal fin rays; (d) branched ventral fin rays; and (e) branched anal fin rays. Meristic counts are not informative in differentiating *Squalius* species that contributed to hybridization in areas

where multiple *Squalius* species coexist. Nevertheless, they do not overlap with meristic counts of hybrids involving other leuciscid species [32,36,37].

2.2. Molecular Analysis of Hybrids between *A. alburnus* and *S. carolitertii*

Records pointed for the first time to the occurrence of hybridization between *A. alburnus* and *S. carolitertii*, which required molecular assessment. Specifically, to this end, additional sampling was conducted in August 2020 and in June 2021 at two sites on Vizela River (Ave basin; 41°22'31.1" N 8°20'01.7" W and 41°22'30.7" N 8°15'53.8" W), where both species are abundant, using electrofishing (300 V, 1–2 A). Five putative hybrids were identified based on intermediate morphology as described above, and fin clips were collected for molecular analysis. Invasive *A. alburnus* and hybrids were euthanized with a lethal dose of anesthetic (MS-222) and stored in 4% formaldehyde for deposition in the collections of the National Museum of Natural History and Science, University of Lisbon. Native fish were returned to the river.

Total DNA was extracted from the fin clips with a commercial isolation kit (E.Z.N.A. Tissue DNA Kit, Omega Bio-Tek, Norcross, GA, USA) following manufacturer instructions, checked for purity and concentration (ng/μL) with NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA), and stored at −20 °C. One nuclear and one mitochondrial loci (beta-actin and COI, respectively) were amplified and sequenced for each putative hybrid individual to assess genomic composition and direction of hybridization.

Beta-actin was amplified using the primers described in Sousa-Santos et al. [38], and COI was amplified with a universal mix of four different primers, namely, VF2_t1, FishF2_t1, FishR2_t1, and FR1d_t1, with M13 tails to facilitate sequencing, as described in Ivanova et al. [39]. PCRs were performed with final concentrations of 5–15 ng/μL of DNA template, 2 mM of MgCl₂, 0.1–0.2 mM of each dNTP, and 0.03–0.05 U/μL of DNA polymerase, namely, GoTaq (Promega, Madison, WI, USA) or Taq PCR Mix with MgCl₂ (Abnova, Taipei, Taiwan). PCR conditions for both genes were as follows: 1 cycle of 95 °C for 5 min (initial denaturation), 35 cycles of 95 °C for 30 s (denaturation), 55 °C for 40 s (annealing) and 72 °C for 90 s (elongation), and 1 cycle of 72 °C for 10 min (final elongation). A sample of each PCR product was run in 3% agarose gel to check for successful amplification using EZ-Vision BlueLight DNA dye (VWR Life Sciences, Radnor, PA, USA), purified using ExoCleanUp FAST PCR clean-up reagent (VWR Life Sciences, Radnor, PA, USA), and sequenced at the forward direction in outsourcing at StabVida (Caparica, Portugal). Sequences were deposited in GenBank (see Table S1).

Sequences of beta-actin and COI from the parental species (*A. alburnus* and *S. carolitertii*) were obtained from GenBank (see Table S1), aligned in BioEdit, and used to create consensus sequences to compare SNPs between the putative hybrids and the parental species. The alleles of the beta-actin sequences of heterozygous individuals were extracted using Indigo (Gear Genomics, EMBL, Heidelberg, Germany), and COI sequences were blasted in BOLD (Barcode of Life Data System, Guelph, ON, Canada).

2.3. Ethical Statement

All field and laboratory procedures followed the recommended ethical guidelines and legislation regarding animal capture, manipulation, and experimentation for scientific purposes, and were conducted under permits obtained from the Portuguese Nature Conservation Authority (ICNF—Instituto da Conservação da Natureza e das Florestas, Lisbon, Portugal).

3. Results

Hybrids were detected based on intermediate morphology in 33 of the 503 sites, but in all basins surveyed. These involved areas of sympatry between *A. alburnus* and *S. alburnoides*, *S. carolitertii*, and *S. pyrenaicus* in the Ave, Douro, Vouga, Mondego, Tagus, Sado, and Guadiana basins (Figure 1).

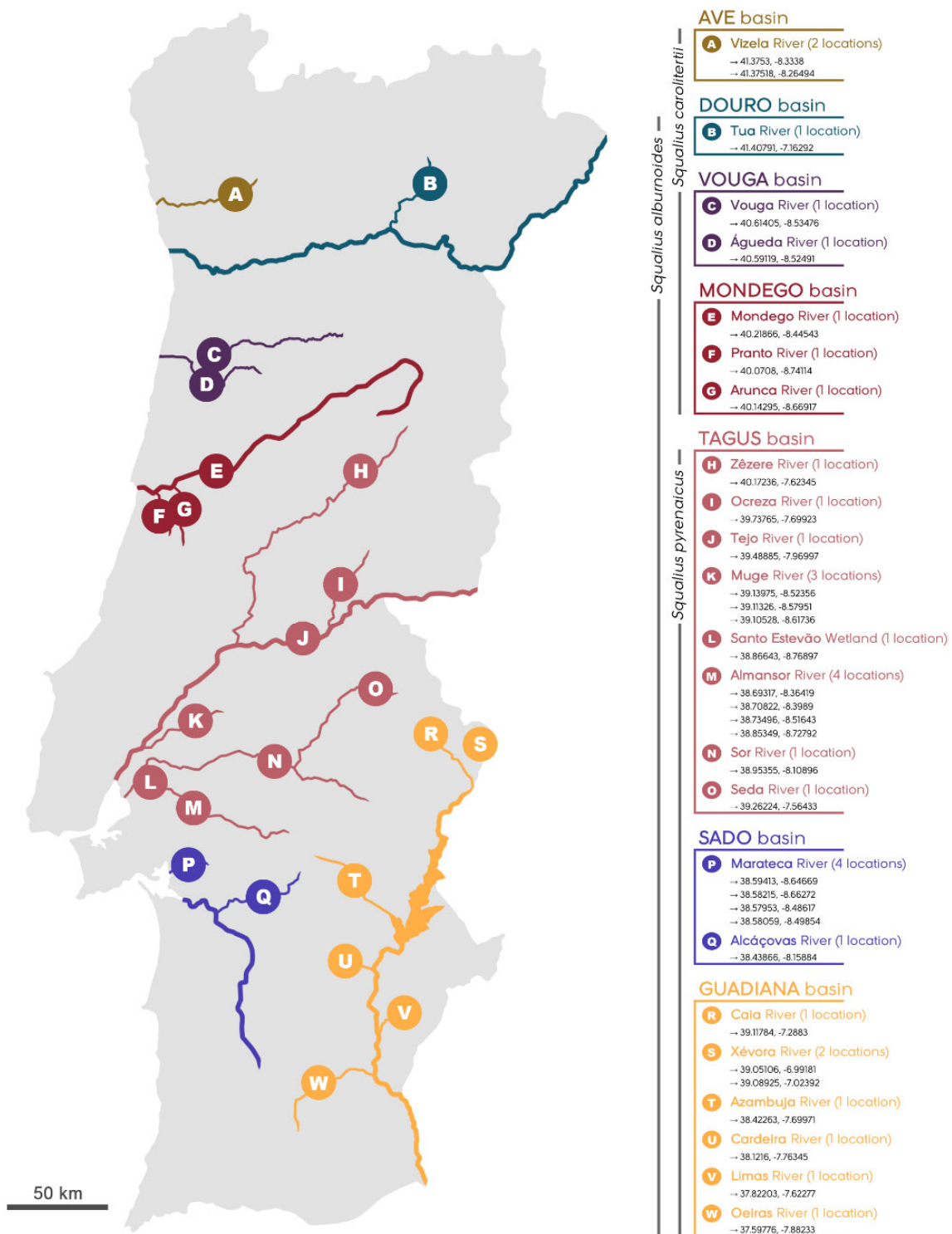


Figure 1. Map of hybrid records in Portuguese river basins, with indication of the geographical coordinates of localities (World Geodetic System (WGS84), in decimal degrees). Gray vertical lines on the right of the map indicate the areas of sympatry between *A. alburnus* and each *Squalius* species.

Sequencing of the beta-actin and COI confirmed the hybrid identity of five putative hybrids between *A. alburnus* and *S. carolitertii* collected in the Ave river basin (Vizela River) (Table 1). All were mothered by *S. carolitertii* and fathered by *A. alburnus*, as indicated through direct analyses of diagnostic mitochondrial SNPs and by blasting COI sequences in BOLD databases.

Table 1. Results of SNP analyses of beta-actin nuclear gene and of BOLD blast of COI mitochondrial gene, discriminating the parental species involved and the direction of hybridization for each hybrid analyzed (ID codes V2H1, V4H1, V4H1_21, V4H2, V4H3_21). Low and top percentages of similarity in BOLD blasts are shown under each COI identification. Ellipsis (...) represent sequence portions upstream and downstream Beta-actin of the diagnostic SNP.

ID	Nuclear Genome	Mitochondrial Genome	Parentage	
	Beta-actin Gene SNP	COI BOLD Systems Blast	Mother	Father
<i>Squalius carolitertii</i> (consensus sequence)	... TAAACGTTTTA TAAACGTTTTA ...	-	-	-
<i>Alburnus alburnus</i> (consensus sequence)	... TAAACATTTTA TAAACATTTTA ...	-	-	-
V2H1	... TAAACGTTTTA TAAACATTTTA ...	<i>S. carolitertii</i> (95.12–100%)	<i>Squalius carolitertii</i>	<i>Alburnus alburnus</i>
V4H1	... TAAACGTTTTA TAAACATTTTA ...	<i>S. carolitertii</i> (94.91–100%)	<i>Squalius carolitertii</i>	<i>Alburnus alburnus</i>
V4H1_21	... TAAACGTTTTA TAAACATTTTA ...	<i>S. carolitertii</i> (95.17–100%)	<i>Squalius carolitertii</i>	<i>Alburnus alburnus</i>
V4H2	... TAAACGTTTTA TAAACATTTTA ...	<i>S. carolitertii</i> (94.91–100%)	<i>Squalius carolitertii</i>	<i>Alburnus alburnus</i>
V4H3_21	... TAAACGTTTTA TAAACATTTTA ...	<i>S. carolitertii</i> (94.97–100%)	<i>Squalius carolitertii</i>	<i>Alburnus alburnus</i>

4. Discussion

Records gathered in the current study show that hybridization between *A. alburnus* and endemic *Squalius* occurs in seven of the major river basins in Portugal, indicating that hybridization may be geographically widespread. This is consistent with previous studies suggesting that interbreeding between nonnative and native species often leads to viable offspring [12,40–43]. The extent of hybridization is likely higher than derived herein, given that surveys were mostly directed to species with habitat requirements that may differ significantly from those of hybrids (e.g., *Lampetra* spp.). This should thus require further analysis based on an adequate sampling design.

Hybridization with *A. alburnus* is more extensive among *Squalius* spp. than previously thought. Besides interbreeding with *S. alburnoides* and *S. pyrenaicus*, as previously reported for the Sado, Guadiana [33], and Tagus basins [32], *A. alburnus* also interbreeds and produces viable hybrids with *S. carolitertii* in the Ave, Douro, Vouga, and Mondego river basins. This apparent incomplete reproductive isolation between Iberian chub and invasive *A. alburnus* raises significant conservation concerns. Indeed, as *A. alburnus* increasingly spreads across the Iberian Peninsula, it is possible that it may also interbreed with other critically endangered and endemic chub with very restricted distributions, namely *S. aradensis*, *S. castellanus*, *S. laietanus*, *S. malacitanus*, *S. palaciosi*, *S. torgalensis* and *S. valentinus* [44,45], which are likely to be severely impacted by hybridization. For example, *A. alburnus* has already invaded the Mira river basin (Portugal), and it is urgent to assess whether hybridization is also occurring with local *S. torgalensis*.

Hybridization with *A. alburnus* is likely to cause impacts on native chub even if hybrids are sterile, due to the wasted reproductive effort. *Alburnus alburnus* is generally abundant throughout its invasive range [22–24], and it may be a competitor for reproductive resources of *Squalius*, with which it shares early maturation and multiple spawning, among other traits [46–50]. Hybridization with the invasive bleak will be particularly concerning for *S. pyrenaicus* and *S. carolitertii* species, which are already sexually parasitized by the allopolyploid *S. alburnoides* [34]. In contrast to previous studies reporting hybridization with *S. alburnoides* and *S. pyrenaicus* to be bidirectional [32,33], only hybrids between *S. carolitertii* females and *A. alburnus* males were found, suggesting the process may be unidirectional. This needs further analysis, given our small sample size (N = 5), but if confirmed, it will imply that parental contributions to hybrids may vary and warrants understanding of the behavioral and molecular mechanisms involved.

Interbreeding with the invasive bleak may affect the intricate reproductive dynamics of the *S. alburnoides* allopolyploid complex. *Squalius alburnoides* includes males and females with different ploidy and combinations of the parental genomes (i.e., genotypes) that are fertile, able to breed with each other and with parental species, and to produce offspring [34]. This reproductive network, upheld by crosses among genotypes with variable frequencies, may maintain populations at a stable hybrid state (i.e., triploid-dominated) or route towards hybrid speciation (i.e., allotetraploidy) [51]. The interbreeding with invasive bleak may cause imbalances in the genotype composition of populations and changes in gamete availability, which may ultimately increase extinction risk. Shifts in reproductive dynamics due to nonnative species have already been reported for the *Pelophylax* hybrid system [11], which shares many traits with the *S. alburnoides* complex.

Hybridization with *A. alburnus* may also lead to introgression of nonnative genes into native species genes, resulting in the deterioration of their genetic resources. Introgression may be particularly detrimental for *S. alburnoides*, which includes a genome of an extinct species that currently is expressed without recombining with other native genomes [34], but is phylogenetically close to the genome of *A. alburnus* [52], which may favor meiotic recombination. Besides disrupting local adaptations by introducing maladaptive genes, admixture may affect female mate choice, which is determined by the genetic integrity of their own genomes and those of mates [35].

Finally, we highlight the importance of early detection of hybrids especially for threatened species because introgression may occur quickly and even be favored by natural selection [14,53,54]. Here, we used a combination of meristic characters and sequences of two diagnostic loci to identify hybrids. Including molecular data overcame some of the ambiguities of the meristic approach, namely, in identifying parental *Squalius* species and highlighting the need of implementing an integrative approach for hybrid detection. The combination of mitochondrial information with the beta-actin marker may effectively identify early-generation hybrids, but largely misdiagnose individuals introgressed via backcrossing [55], thereby underestimating the extent of introgressive hybridization. Such limitations can be overcome by complementing morphological analysis with genome-wide molecular approaches, such as RAD sequencing [56], SNP panels [57], or SSR-GBS [58], tools that are becoming increasingly available. Including these methods in further studies will be critical to clarify whether hybrids are fertile and reproduce sexually, and to evaluate the possible impacts of ongoing hybridization on the genetic integrity of native fish fauna.

5. Conclusions

Biological invasions have received increasing attention in the last few decades, but the inconspicuous and silent consequences of hybridization between native and nonnative species remain poorly addressed. The occurrence of hybrids between invasive *A. alburnus* and several *Squalius* species across Portugal suggests that hybridization may be widespread and have potential impacts on endangered endemic chub. Interbreeding with the invader may waste reproductive effort of individual species and interfere with several aspects of the reproductive dynamics of the *S. alburnoides* complex. Finally, if hybrids are fertile and reproduce sexually, native and nonnative genomes may admix, irreversibly altering the genetic composition of native species. Given these potentially serious impacts and the continued expansion of *A. alburnus*, we recommend that future studies integrate molecular tools and characterize hybrid fitness and their ecological and genetic interactions with native *Squalius* species to elucidate effective conservation measures for the latter.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fishes7050247/s1>, Table S1: List of GenBank accession numbers of all sequences produced.

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Institutional Review Board Statement: According to Portuguese law, it is forbidden to release exotic species, which includes the bleak, and therefore it was not necessary an approval from an ethic comity. However, all animals were capture with the approval of the Portuguese institute of nature conservation and forests (ICNF) through several fishing licenses (e.g., 367 / 2022 / CAPT).

Data Availability Statement: All sequence data have been deposited in GenBank (see Table S1).

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Conflicts of Interest: The authors declare no conflict of interest.

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