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Hybridization and invasive species in a threatened freshwater fish community under environmental pressures: Morphometric and molecular evidence

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Abstract

1. Mediterranean freshwater systems are under threat owing to increased drought driven by climate change, intensive human land uses and non-native species. This is causing increased fish hybridization in isolated watercourses.
2. The genetic and morphological characteristics of hybrids of sympatric native and non-native fish species were studied in four streams of the Mediterranean Guadalquivir basin (south-west Spain). Fish morphology was analysed using geometric morphometrics, and molecular determination of parenthood was inferred through one mitochondrial gene (*cytb*) and one nuclear gene (*Beta-actin*) for all hybrids and a subset of pure parental specimens.
3. Molecular analyses confirmed hybrids between the native *Squalius alburnoides* and non-native *Alburnus alburnus* in a stream with continuous flow. Haplotype analyses suggested that they originated from backcrossing of hybrid offspring. Intergeneric crosses between native species *S. alburnoides* and *Pseudochondrostoma willkommii*, and *S. alburnoides* and *Iberochondrostoma lemmingii* were detected in streams under reduced connectivity scenarios.
4. Morphometrics revealed that hybrid phenotypes were similar to *S. alburnoides*. In some cases, molecular markers uncovered hybridization events that were neither detected in the field nor by morphometric analyses, potentially supporting a backcrossing/introgression scenario.
5. Hybridization is likely to be increasing in Mediterranean rivers where *S. alburnoides* are present owing to increased fragmentation caused by summer drought exacerbated by climate change and human land uses and pressures. This can become a problem for these endemic vulnerable species if genetic diversity is lost, morphological homogenization occurs and hybrids cannot be easily detected in the field.

Begoña Martinez-Cruz and Richard P. Brown, are joint last authors.

6. The potential risks could be addressed by monitoring and eradication of non-native species and segregation from natives. To avoid native–native crosses, habitat quality and desiccation risk could be tackled by improved water quality and riparian reforestation to provide shade and reduce evapotranspiration. This would need increased coordination and intervention between the institutions that share conservation responsibilities in the area.

KEYWORDS

Alburnus alburnus, freshwater fish, hybrids, *Iberochondrostoma lemmingii*, Mediterranean, molecular introgression, morphometrics, non-native species, *Pseudochondrostoma willkommii*, *Squalius alburnoides*

1 | INTRODUCTION

The Mediterranean region is a crucial biodiversity hotspot for freshwater fish communities (Smith & Darwall, 2006; Reyjol et al., 2007), with high levels of endemicity as a result of its palaeobiogeographical history (Almaça, 1976; Bianco, 1990; Bănărescu, 1992; Zardoya & Doadrio, 1999). Fish in this region are threatened by numerous human impacts, such as pollution, habitat loss, fragmentation and the introduction of non-native species, which has increased exponentially in recent decades (Cobo et al., 2010; Anastácio et al., 2019). Invasive non-native species are currently considered one of the most important drivers of biodiversity loss worldwide (Clavero & Garcia-Berthou, 2005; Early et al., 2016; Pyšek et al., 2020). In rivers they can cause extinctions through predation, competition (Ribeiro & Leunda, 2012), and hybridization, leading to a loss of the genetic integrity of native species (e.g. Fitzpatrick et al., 2010; Todesco et al., 2016; Quilodrán, Currat & Montoya-Burgos, 2018).

Cyprinids are the dominant freshwater fish group in Europe, and they make up 80% of fish species in Iberian rivers (Doadrio et al., 2011; Freyhof & Brooks, 2011), where they have very high levels of endemicity (>80%, Freyhof & Brooks, 2011). More than 60% of cyprinids in the Iberian Peninsula are classified as Vulnerable, Endangered or Critically Endangered according to International Union for Conservation of Nature (IUCN) criteria (Doadrio et al., 2011; IUCN, 2022; Saez-Gomez & Prenda, 2022). Cyprinids are well known for their high rates of hybridization, at both the intra- and the inter-genera level (Scribner, Page & Bartron, 2000). Historical intra- and inter-genera hybridization is common in the cyprinid subfamily of Leuciscinae (Zardoya & Doadrio, 1999; Leunda, 2010; Tancioni et al., 2013), with evidence of modern occurrence in the allopolyploid hybrid complex *Squalius alburnoides* which arose from an intergeneric hybridization involving *Squalius pyrenaicus* females and males of *Anaecypris hispanica*-like species in the Pleistocene (Sousa-Santos, Collares-Pereira & Almada, 2007; Cunha et al., 2011; Morgado-Santos et al., 2016). Frequent hybridization can become a serious threat to native diversity if the hybrids can backcross with the original parental species by contamination of the parental genome (Ketmaier & Bianco, 2015; Quilodrán, Currat & Montoya-Burgos, 2018). Sometimes, hybrids with non-native invasive species have the potential to adapt quickly to degraded habitats and become dominant in local populations, with the risk of native parental exclusion from those areas (Hayden et al., 2011).

Watercourses in the Iberian Peninsula have a natural desiccation/flood regime with a severe summer drought that often interrupts river flow. During the summer drought, fish communities become isolated in pools in upstream reaches, resulting in temporally naturally fragmented populations (Gasith & Resh, 1999; Bernardo et al., 2003; Magalhães, Schlosser & Collares-Pereira, 2003; Magalhães et al., 2007). Native species are naturally adapted to this fluctuating water regime and populations recover with the autumn rains. However, climate change has already increased summer temperatures by 1.5°C at rates 20% above global means (Ali et al., 2022). Predictions suggest that drought periods will become longer and more intense, and headwater streams may dry out completely in the near future (a projected reduction of 12% of the expected mean rainfall under a 3°C temperature rise scenario; XIth IPCC Report, Ali et al., 2022). This will either confine native species to ever smaller isolated pools or force them down the river continuum to intermediate areas where they will meet the upward migration of non-native species from reservoirs, which are a main source of non-native species (Vinyoles et al., 2007; Clavero & Hermoso, 2011). There is concern that increased mixing with non-natives in these areas, and tightly packed natives in upstream pools, may promote the creation of novel hybrid zones (Hayden et al., 2011; Quilodrán, Currat & Montoya-Burgos, 2018) or increase the hybridization rate for these fish communities under already challenging conditions.

Hybridization between the invasive *Alburnus alburnus* (bleak) and native cyprinid species has already been reported in Mediterranean river basins using molecular methods and has been flagged as an increasing phenomenon that can threaten the vulnerable native populations (Almodóvar et al., 2012; Sousa-Santos et al., 2018a; Guadiana: review in Curto et al., 2022). In addition, hybrids between native species, including inter-genera crosses, can become more frequent under a reduced connectivity and intensified drought scenario (Vieira-Lanero et al., 2019). Hybridization within sympatric *Iberochondrostoma* species has already been identified as a threat to the rarer species in Portugal because of the risk of introgression (Sousa-Santos et al., 2014), but the prevalence of current inter-genera hybridization among native species has not been studied in detail in many Iberian Peninsula basins.

Hybridization studies have highlighted some evidence of unclear morphological characteristics when diagnosing hybrid specimens (Almodóvar et al., 2012; Curto et al., 2022). It is widely assumed that hybrids will be intermediate between parental species for useful characters such as the number of scales along the lateral line, but this may not be the case in all crosses (Sousa-Santos et al., 2018a). This, together with the high intraspecific morphological variability of Mediterranean fish (Manaresi, Mantovani & Zaccanti, 2001), introduces potential error in the identification of hybrids in the field. In some cases, hybrids may even be unnoticed during routine monitoring and biodiversity assessments.

The aim of this study was to explore the cytonuclear genetic and morphological characteristics of hybrids of sympatric native and non-native freshwater fish species. This was studied in small streams subjected to summer drought. An interdisciplinary analysis was used that included both morphometric data and molecular determination of ancestry through one mitochondrial gene and one nuclear gene. The detection of hybrids originating from both native–native and native–non-native crosses will shed light on whether the genetic integrity or viability of these threatened freshwater fish populations is at risk.

2 | METHODS

2.1 | Study area and study species

The Guadalquivir River Basin is located in the south of the Iberian Peninsula. It rises in the Cazorla Mountains at approximately 1400 m above sea level and flows into the Gulf of Cádiz. The basin covers an area of 57,679 km^2 and the main river channel is 657 km long. The area is characterized by a Mediterranean climate with strong monthly variation in precipitation and a mean yearly temperature of 16.8°C (Expósito & Berbel, 2020). The river network comprises 80 main tributaries and 80 sub-catchments (Figure 1). The hydrological regime is extremely variable, with severe droughts and flooding. The flow is typically dependent on the seasonal distribution of rain (an average of 573 mm per annum), which is greatest between October and March. During the summer months, June to September, streams become reduced to isolated pools (until river connectivity is re-established by autumn rains) or may dry up entirely (Gasith & Resh, 1999; Alvarez-Cobelas, Rojo & Angeler, 2005).

The area is under several intense human pressures: 47% of the basin land cover is agriculture and the river flow is regulated by 65 large dams. These twentieth-century reservoir constructions led to the introduction of non-native IANS (introduced angling species). Downstream flow from these reservoirs has facilitated colonization of the Guadalquivir main river channel, which has become a source of IANS for the rest of the river network (Carpio et al., 2019). In addition, most river courses are under threat from pollution, including urban and industrial wastewater discharge, erosion, and nutrient and pesticide runoff from agricultural land (Confederación Hidrográfica del Guadalquivir, CHG, 2013). The present freshwater fish community in the Guadalquivir River Basin comprises 13 native and 14 non-native species, presenting different seasonal and spatial patterns (Fernández-Delgado et al., 2014). In this study, 'native' species refers to species of Mediterranean origin.

Like all river basins in Spain, the waters of the Guadalquivir basin are under the management of the Spanish central government, whereas biodiversity conservation responsibilities lie with regional governments. Most of the basin is located within the Andalusian region (90.2%), so the Andalusian regional government (Junta de Andalucia) has included

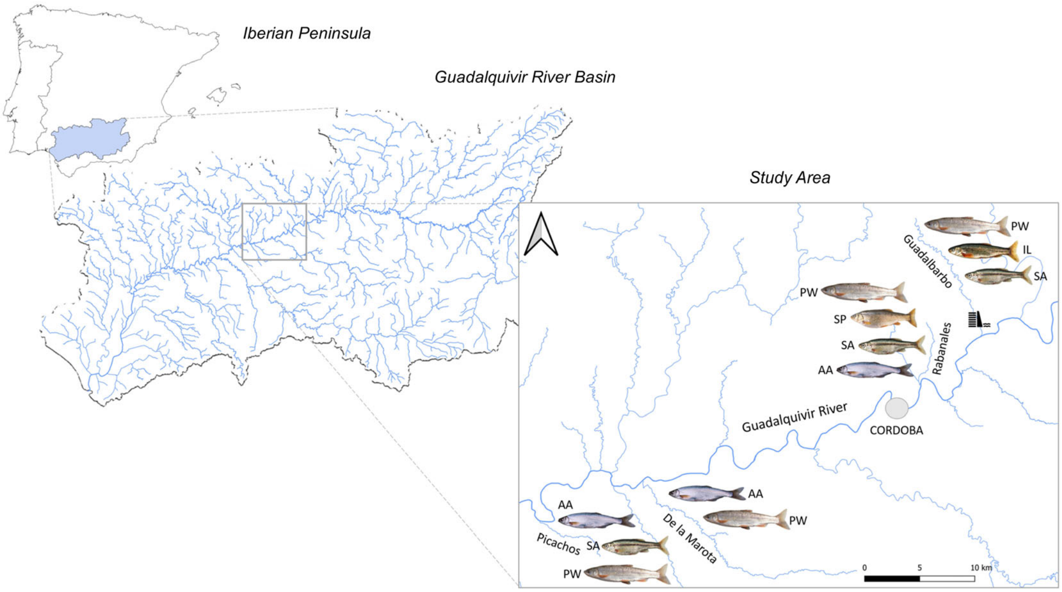

FIGURE 1 Location of the study area and streams within the Guadalquivir River basin, in southern Spain. Inset shows the streams where hybrids (Picachos and Rabanales) and parentals (De la Marota, Guadalbarbo) were captured. Fish pictured represent where specimens of target species were obtained. AA, *Alburnus alburnus* (non-native); IL, *Iberochondrostoma lemmingii* (native); PW, *Pseudochondrostoma willkommii* (native); SA, *Squalius alburnoides* (native); SP, *Squalius pyrenaicus* (native).

the Guadalquivir fish fauna within a general biodiversity conservation plan that spans all basins in the region (CSMAEA, 2015). Only species catalogued as Endangered have a specific conservation and monitoring plan. Other threatened or endemic species, such as those included in this study, are not considered in the conservation plan and there are no specific conservation measures that have them as targets.

2.1.1 | Target species

The native cyprinid species considered in this study are all endemic and of high conservation concern, as they are all classified as Vulnerable or Near Threatened in the IUCN Red List of Threatened Species (IUCN, 2022) and in the European Red List of Freshwater Fishes (Freyhof & Brooks, 2011). They are: southern straight-mouth nase, *Pseudochondrostoma willkommii*; Iberian arched-mouth nase, *Iberochondrostoma lemmingii*; and Iberian chub, *S. pyrenaicus* (and the allopolyploid complex) and *S. alburnoides*. (See Table S1 for details of each species and the meristic characters used for field identification.).

In contrast, the non-native bleak, *A. alburnus*, is a small cyprinid from Central Europe and Western Asia (Freyhof & Kottelat, 2008). Since 1990, it has been repeatedly introduced into Iberian rivers by anglers as a forage species (especially for black bass, *Micropterus* spp.; Elvira & Almodovar, 2001). It was introduced into the Guadalquivir River basin in 2006 (Latorre et al., 2018) and first observed in the study area in 2012 (R.J. de Miguel, personal observation). It is listed as a non-native species of concern in the Spanish Invasive Species List (Ministerio para la Transición Ecológica y el Reto Demográfico, MITECO, 2021) because of potential competition with native cyprinids for space and prey, as well as hybridization (Leunda, 2010; Almodóvar et al., 2012).

Alburnus alburnus can reach high densities and has a high invasive potential (Latorre et al., 2018), as it is currently the main non-native species with enough plasticity to colonize mid- and high-stream locations of the Guadalquivir (Vinyoles et al., 2007, Almeida et al., 2014; see Supporting Information Table S1 for further details).

2.1.2 | Target streams

All watercourses chosen for this study are direct tributaries of the Guadalquivir River, so there are no other external sources of

TABLE 1 Sampled streams, including coordinates, fish species composition, connectivity with the main Guadalquivir River channel and flow, and number and species of specimens caught and successfully processed for morphometric and molecular analyses. Streams are arranged from most connected/highest flow to least connected to the Guadalquivir and most fragmented. *(I)* indicates an invasive non-native species, as listed in the Spanish Invasive Species Catalogue (MAGRAMA, 2013).

Stream	Longitude	Latitude	Native spp. present	Non-native spp.	Connectivity with Guadalquivir	Specimens used for morphometric analyses	Total processed for molecular analyses
Rabanales	37°54′11.1″N	4°43′55.5″W	*Luciobarbus sclateri* *Pseudochondrostoma willkommii* *Squalius alburnoides* *Squalius pyrenaicus* *Cobitis paludica*	*Alburnus alburnus (I)* *Lepomis gibbosus (I)* *Gambusia holbrooki (I)*	Continuous flow	26 hybrids 29 *A. alburnus* 1 *P. willkommii* 5 *S. alburnoides*	28 hybrids 29 *A.alburnus* 4 *S. alburnoides* 1 *P. willkommii*
De la Marota	37°47′17.3″N	5°00′36.8″W	*Luciobarbus sclateri* *Pseudochondrostoma willkommii* *C. paludica*	*A. alburnus (I)* *L. gibbosus (I)*	Isolated deep pools	29 *P. willkommii*	11 *P. willkommii*
Picachos	37°45′22.0″N	5°06′07.2″W	*Luciobarbus sclateri* *Pseudochondrostoma willkommii* *Squalius alburnoides* *C. paludica*	*A. alburnus (I)*	Isolated shallow pools	32 hybrids 1 *A. alburnus* 8 *P. willkommii*	32 hybrids 1 *A. alburnus* 7 *P. willkommii*
Guadalbarbo	37°56′36.1″N	4°41′10.4″W	*Luciobarbus sclateri* *Pseudochondrostoma willkommii Squalius alburnoides* *Iberochondrostoma lemmingii* *C. paludica*		Disconnected, with isolated deep pools	37 *I. lemmingii* 1 *P. willkommii* 27 *S. alburnoides*	16 *I. lemmingii* 1 *P. willkommii* 23 *S. alburnoides*
Total						196	153[a] (93 nDNA)

[a] A total of 153 + 5 *S. pyrenaicus* individuals were sequenced for Cytochrome b, (*cytb*, mtDNA). Of those, 93 + 4 *S. pyrenaicus* were successfully sequenced for *Beta-actin* (nDNA).

freshwater fish (Figure 1, Table 1). Rabanales and Picachos streams were chosen as likely sources of hybrids, as revealed in a previous Guadalquivir basin-wide study (Fernández-Delgado et al., 2014). Picachos stream dries out substantially during the summer drought, resulting in several isolated pools, but maintains some degree of connectivity to the main Guadalquivir River. Rabanales stream, however, maintains a continuous flow throughout the year, with just a moderate decrease in flow during the summer. De la Marota and Guadalbarbo were both used as a source of pure parental species. Deep pools remain in both streams during the summer, but Guadalbarbo is the most isolated, as it becomes completely disconnected from the main Guadalquivir River (Table 1).

2.2 | Sample collection and species identification

In September 2018 and October 2019, 196 fish of the target species were captured across the four tributaries by electrofishing, following the Spanish Ministerial official protocol ML-R-FI-2015 (Ministerio de Agricultura, Alimentación y Medio Ambiente, MAGRAMA, 2015). Fish were anaesthetized with clove oil and identified in the field using meristic characters (see Supporting Information Table S1). Those with intermediate characteristics were designated as potential hybrids. A fin clip (0.5–1 cm^2) of each fish caught was preserved in 96% ethanol and only native species were released back into the river. Non-native and hybrid specimens were euthanized by overdose of clove anaesthetic in accordance with Spanish legislation on non-native species (MAGRAMA, 2013). Sampling was conducted in accordance with ethical standards and Spanish legislation, LAW 32/200 of 7 November 2007 (BOE 8/11/2007) and EU Directive 2010/63/EU (https://eur-lex.europa.eu/eli/dir/2010/63/oj). Sampling was carried out under fishing permit 201799900546722-06/10/2017 by the Andalusian Environmental Agency. Five *S. pyrenaicus* tail fin clips obtained in 2008 as part of a Guadalquivir-wide study from nearby basins were also included to support the molecular analyses (Fernández-Delgado et al., 2014).

A combination of shape analyses and molecular determination was chosen to confirm the hybrids identified in the field and address their phenotypic characteristics and any potential for field misidentification. This multidisciplinary approach has been widely applied to identify hybrids in wild fish populations (Hayden et al., 2010; Geiger et al., 2016; Bradbeer et al., 2019). Combining geometric morphometrics with genetic data provides an insight into hybrid phenotypes (Stelkens et al., 2009) and information about variability or overlap with parental species (Valentin, Sévigny & Chanut, 2005; Shechonge et al., 2018).

2.3 | Geometric morphometric analyses

Photographs of 196 specimens in lateral view were taken with a digital camera in the field. Specimens were positioned on a lateral plane and next to a ruler, and the camera lens was placed parallel to the sagittal plane of each fish. All specimens were adults, and there was no distinction between male and female fish, as there is no strong sexual dimorphism in the study species.

The programme tpsDig (version 2.30, Rohlf, 2015) was used to collect morphological data from the photographs. The scale bar was set adjacent to the specimen (Zelditch, Swiderski & Sheets, 2012). Fourteen homologous morphological landmarks were digitized using *x*- and *y*-coordinates on each photograph, in the same order, to describe fish shape (Figure 2, Table S2). Landmarks were based on those used in fish studies that identified these traits as robust descriptors of fish body size and shape (Loy et al., 2000; Cunha et al., 2009; Tancioni et al., 2013). As the curved lateral line of fish has no discrete anatomical loci, semi-landmarks were placed along this region at 20 equal intervals, from landmarks 6 to 11, using the curve-tracing function in tpsDig (Figure 2) (Bookstein, 1997; Zelditch, Swiderski & Sheets, 2012).

Landmarking was carried out by a single operator, so any operator bias was avoided (Moccetti et al., 2023). Error measurement followed the protocol of Viscosi & Cardini (2011). The repeatability and precision of the landmark configuration was assessed by digitizing the landmarks twice with a 2 week interval between the first and the second digitization (Supporting Information Table S2). This was performed on a subsample of the photographs, consisting of four individuals of each species and four hybrids, to compute the differences between the replicates of each configuration. Measurement error was assessed using a Procrustes ANOVA, a method for assessing the relative amounts of shape variance explained by individuals and error (programme: MorphoJ v.1.06; Klingenberg, 2011). A survey based on 20 specimens landmarked twice yielded an error of less than 5% for both centroid size (CS) and shape, indicating good repeatability.

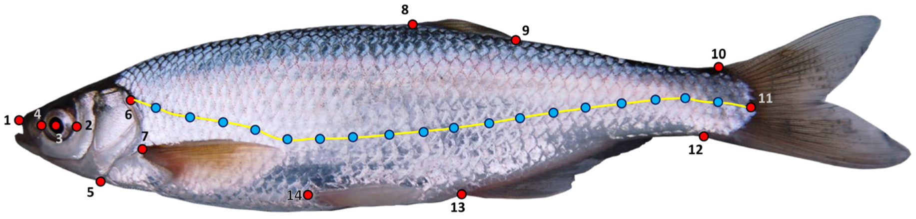

FIGURE 2 A specimen of Squalius alburnoides showing the 14 defined landmarks (●) used for geometric morphometric analysis of external shape, as described in Supporting Information Table S2. The curved line (●) along the entire lateral line on which 20 equally spaced semi-sliding landmarks were placed (– –) (later converted to landmarks) is shown. Digitized in tpsDig (Rohlf, 2006).

Shape data were obtained by Procrustes superimposition which involves: (i) the standardization of size; (ii) the removal of translational variation; and (iii) the minimization of rotational differences, according to the configuration's centroid. This transformed the original 2D landmark coordinates into shape (= Procrustes) coordinates. Size was extracted from each original landmark configuration using the CS defined as the square root of the summed squared distances between the centroid (centre of gravity) of each configuration and each landmark (Bookstein, 1997). A principal component analysis of Procrustes coordinates was used to assess and visualize shape variation across the two-dimensional landmark data, using MorphoJ v1.06 (Klingenberg, 2011).

Differences in size between species were assessed using one-way ANOVA on log-transformed CS. Owing to variances not being homogeneous (Levene test, $F_{4,188} = 3.643$, $p = 0.007$) a *post hoc* Dunnett T3 test was performed on any size differences. Discriminant function analysis (DFA) was applied to identify the contribution of both size and shape in species discrimination and hybrid classification. The DFA included all specimens and each of the potential parental species and hybrids as a grouping variable (five groups). This DFA allowed testing of whether hybrid specimens could be discriminated from other species. The DFA was performed with a stepwise variable selection (entry F probability $= 0.05$, removal F probability $= 0.10$) using principal component vector scores as well as natural log-transformed CS as independent variables. A leave-one-out cross-validation method (number of permutation runs = 1,000) was applied to assess the reliability of classifications. This process has been successfully applied to geometric morphometric data (Meloro, 2011; Meloro, Hudson & Rook, 2015) and it allows the selection of only relevant shape or size variables to maximize discrimination and reduce ‘overfitting’ (Kovarovic et al., 2011). A second DFA was computed with the four parental species as grouping variables and with the hybrid specimens input as unclassified individuals. This second DFA was used to assess the morphological similarities of hybrids with their potential parental species according to which parental species they were assigned to.

2.4 | Molecular analyses

Total DNA was extracted from fin clips using the ChargeSwitch® gDNA Micro Tissue Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. A mitochondrial DNA fragment corresponding to the cytochrome b (*cytb*) gene and a nuclear locus corresponding to the *Beta-actin* gene were selected for PCR amplification and sequencing. The mtDNA gene was used to identify maternal lineage while the nuclear gene provided information on biparental inheritance and hence hybridization. The *cytb* fragment was selected as it appears to show considerable diversity at low taxonomic levels relative to other regions of the mtDNA, particularly in cyprinid fish (Tang et al., 2006). The *Beta-actin* locus was selected because primers had been designed and its utility for testing hybridization had been thoroughly tested in *S. alburnoides* (and the other study species) previously (Sousa-Santos et al., 2005). This was important because potential allele drop-out in an untested locus could invalidate any findings, despite the limitation that the use of one nuclear locus only allows determination of F_1 hybrids but does not guarantee detection of F_2 and subsequent generation hybrids (which would require the use of many unlinked nuclear loci). Nonetheless, it provided a rapid and effective assessment of recent hybridization.

For *cytb*, the 1107 bp sequence was PCR amplified using LCB1 (Brito et al., 1997) and HA primers (Schmidt & Gold, 1993) or with L14724 and H15915 (He, Chen & Tsuneo, 2001). A 751 bp fragment of the nuclear *Beta-actin* gene was PCR amplified using primers BactFor_new (5′ GGATGATGAAATTGCCGCACTGG3′) and BactRev_new (5′AGGATCTTCATGAGGTAGTCTGT3′) (Sousa_Santos pers. com.; Sousa-Santos et al., 2018b). All PCRs were carried out in 25 μl volumes using conditions described in Table S3. The *cytb* PCR products were purified with ExoSAP-IT™ PCR product cleanup reagent (Thermo Fisher). For *Beta-actin*, the PCR volume was reduced by half in a spinning vacuum, checked in a 1.5% Tris–acetate low-melting-point agarose gel. Double bands were excised and 900–1,000 bp bands were sequenced. Both genes were sequenced with the same set of PCR primers at Macrogen (Madrid, Spain). Chromatograms and alignments were verified with Sequencher ver. 4.0 (Gene Codes Corporation, Inc.).

In total, 156 sequences/individuals were obtained for *cytb*, while 97 were obtained for *Beta-actin*. This was due to problems with *Beta-actin* amplification caused by multiple peaks, especially in hybrids and *S. alburnoides* individuals. Therefore, full molecular parentage was obtained from 62% of individuals. This could be an important restriction in studies with a limited number of specimens. Sequences were deposited in GenBank (see Appendix S1 for accession numbers).

All *cytb* and *Beta-actin* sequences were aligned with cyprinid species downloaded from GenBank for phylogenetic reconstruction: *A. alburnus*, *S. alburnoides*, *S. pyrenaicus*, *P. willkommii*, *I. lemmingii* and *A. hispanica* (Appendix S2). For the nuclear *Beta-actin* gene and to detect interspecific hybrids, all nuclear genomotypes of *S. alburnoides* complex found by a previous study (Sousa-Santos, Collares-Pereira & Almada, 2007) as well as sequences from all potential parental species present in the area or in geographically close river drainages were also downloaded from GenBank (Appendix S1). *Rhodeus notatus* was used as an outgroup.

Phylogenetic trees were obtained for the *cytb* (1107 bp) and *Beta-actin* (774 bp alignment including indels) datasets independently using maximum likelihood (ML) (programme: PhyML 3.0; Guindon et al., 2010) and Bayesian inference (BI) (programme: Mr. Bayes v3.2.6; Ronquist & Huelsenbeck, 2003). For ML analyses, automatic model selection under the Bayesian information criterion was used. Support for tree nodes was assessed by bootstrapping (b) (1,000 replicates) for ML analyses and from posterior probabilities (pp) for BI analyses. For ML analyses, discovery of ML trees for each bootstrap sample was achieved using heuristic searches (1,000 runs) under the favoured TN93 + I (0.601) model for the *cytb* BI. Four simultaneous Monte Carlo Markov Chains were run for 4 million generations with a sample interval of 100 generations. Log-likelihood stationarity was attained after 10,000 generations but a much larger proportion of trees (i.e. the first 10%) were discarded as burn-in. The remaining trees were used to compute a 50% majority rule consensus tree using PAUP* v. 4.0a (Swofford, 2002). Disparity between phylogenetic trees based on mitochondrial or nuclear data was used to assess the existence of interspecific crosses.

Minimum spanning networks were obtained for *cytb* (1107 bp) and *Beta-actin* (774 bp) haplotypes using PopArt (http://popart.otago.ac.nz). For the *Beta-actin* gene networks, haplotype datasets were first reconstructed by resolving phases of segregating sites using DNAsp v.5 (Librado & Rozas, 2009). This programme was also used to calculate haplotype and nucleotide diversity and polymorphic sites. Shared haplotypes between species were assumed to show crosses between the target species. Range haplotypic diversity within parental species was calculated and used as the maximum haplotypic diversity for each parental species. If hybrid haplotypic values fell within a particular parental range, hybrids were assigned to this parental species.

3 | RESULTS

3.1 | Morphometric results

The first 11 vectors of the principal component analysis explained 95.44% of shape variation. The plot of the two principal components, PC1 (56.31% of variance) and PC2 (13.90%), showed a strong differentiation between parental species with hybrids generally overlapping with *S. alburnoides* or *A. alburnus* (Figure 3). Along PC1, *I. lemmingii* and *P. willkommii* are characterized by a relatively wider body shape with dorsal fin positioned more anteriorly than in *A. alburnus*, which has positive axis scores, indicating a slender body (Figure 3).

PC2 separated *P. willkommii* (positive scores) from *I. lemmingii* (negative scores) and describes changes in relative body width as well as curvature of the lateral line. Their lateral line is positioned more in the middle of the fish body and the relative distance between the pelvic and anal fins is wider. The curve is also projected more inferiorly in *I. lemmingii* on negative PC2 scores when compared with *P. willkommii* and *A. alburnus*, whose lateral line is much straighter.

The analysis of body size (here represented by the natural log transformation of the variable CS) also showed significant differences between species (ANOVA $F_{4,188} = 95.683$, $p < 0.001$). Dunnett's T3 tests showed that all pairs of parental taxa were significantly distinct ($p < 0.001$ in all cases); however, no difference in size occurred between hybrids and *S. alburnoides* (mean difference $= 0.10$, $p = 1.000$; Supporting Information Figure S1).

The DFA that treated hybrids as a distinct group (DFA1) confirmed that both size and shape contribute to the discrimination between the analysed species of fish and hybrids as distinct groups. Twenty variables were selected by the DFA, under the stepwise criteria (‘*F* to enter’). DF1 (67.2% of variance, Wilk's $\lambda = 0.001$,

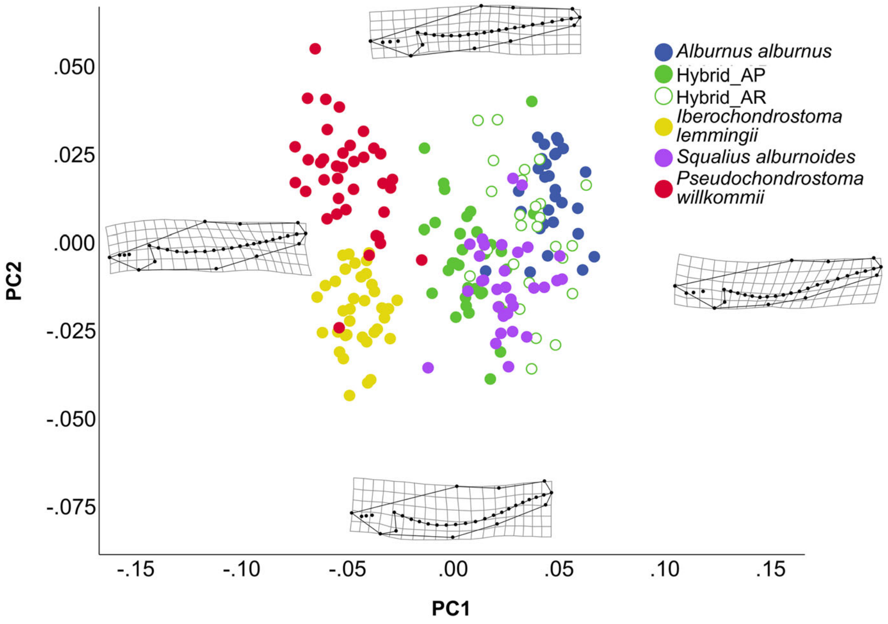

FIGURE 3 Plot of the first two principal component analysis vectors of fish shape for four fish species and hybrids. The different source streams for hybrids are shown: Rabanales stream (AR) connected to the Guadalquivir River, and Picachos stream (AP) disconnected from the Guadalquivir main river course. Three outliers from Guadalbarbo stream (RG) have been labelled – one *P. willkommii* and two *S. alburnoides* – while from Rabanales an individual of *P. willkommii* (PW038_AR) clearly clusters with the hybrid group from Picachos stream. Hybrids generally overlap with *S. alburnoides* or *A. alburnus* in PC1 and PC2 and they are found in the middle of the morphospace. When separated by stream of origin, hybrids from Picachos stream (AP) are in the central part of the morphospace, closer to *P. willkommii* and IL, but mainly overlap with *S. alburnoides* only. In contrast, hybrids from Rabanales stream (AR) appear to the right of the morphospace and overlap mainly with *A. alburnus* and *S. alburnoides*, suggesting an intermediate morphology between those two species.

$\chi^2 = 12.51311$, d.f. = 80, $p < 0.001$) was positively loaded by PC1 and negatively by size while the DF2 (19.1% of variance, Wilk's $\lambda = 0.020$, $\chi^2 = 75.119$, d.f. = 57, $p < 0.001$) was positively loaded by PC2 and PC20 and negatively by PC5. The cross-validated percentage of correctly classified cases is quite high for all species, showing *I. lemmingii* to be the most distinct taxon (100% of correctly classified cases), while most of the misclassified hybrid specimens were allocated to *S. alburnoides* (Supporting Information Table S4a).

For DFA2, which treated hybrid specimens as unclassified, the stepwise procedure selected 21 variables combined into three vectors. DF1 (71.1% of variance, Wilk's $\lambda = 0.0004$, $\chi^2 = 943.025$, d.f. = 63, $p < 0.001$) was positively loaded on PC1 while DF2 (19.7% of variance, Wilk's $\lambda = 0.015$, $\chi^2 = 504.285$, d.f. = 40, $p < 0.001$) was positively loaded on PC2, PC8, PC9 and PC12 and negatively on PC10, PC13, PC5 and PC19. Size showed a negative correlation with DF3 ($r = -0.298$), which explained 9.3% of variance in the DFA model. In this DFA all parental species were correctly classified after cross-validation in 100% of the cases except for *P. willkommii*, with 97.3% owing to PW_037 being classified as *I. lemmingii* (Supporting Information Table S4b) (Figure 3). Of the 32 hybrids from Picachos, three were assigned to *P. willkommii*, one to *A. alburnus* and the remainder (28) to *S. alburnoides*. For Rabanales, 26% (8) of the hybrids were assigned to *A. alburnus* and 74% (23) to *S. alburnoides* (Supporting Information Table S5). The percentage of cross-validated cases is high for all species. Hybrids are located centrally within the morphospace and most likely represent a separate group showing a high level of overlap, especially with *S. alburnoides* and partially with *A. alburnus* (Figure 3). Examples of parental and hybrid specimens can be found in Supporting Information Figures S4–S17.

3.2 | Molecular results

In total, 55 mitochondrial haplotypes (H1–H55) were detected within the 58 *cytb* sequences corresponding to all morphological parental species identified in the field: 7 from *A. alburnus*, 7 from *I. lemmingii*, 14 from *P. willkommii*, 6 from *S. pyrenaicus*, 7 from *S. alburnoides* and 2 from *Anaecypris*. A summary of the diversity indices and genetic structure is shown in Table 2. These relationships were confirmed by the mtDNA phylogenetic tree, which showed that all species correspond to distinct mtDNA lineages, which was supported by high boostrap (>98) and posterior probability values (>0.92). The only exception is *S. alburnoides* and *S. pyrenaicus*, with the latter forming a monophyletic mtDNA group nested within the *S. alburnoides* mtDNA clade (Supporting Information Figure S2).

The *cytb* network showed that many hybrids from Picachos (93.7%) had *P. willkommii* haplotypes (H9, H10, H14, H15, H16, H19 and H20) and most were included in the *P. willkommii*

TABLE 2 Haplotype diversity for *cytb* and *Beta-actin* genes from hybrids and parental specimens from the sampled Guadalquivir tributaries.

	Sample size (*N*)	Number of haplotypes (Nhap)	Haplotype (gene) diversity (HD)	Variance of haplotype diversity	Standard deviation of haplotype diversity	Nucleotide diversity	Average number of nucleotide differences (*K*)
Cytochrome b gene							
Hybrids Picachos	32	16	0.935	0.001	0.023	0.017	13.14
Hybrids Rabanales	24	11	0.884	0.002	0.045	0.072	52.29
Squalius pyrenaicus	11	6	0.727	0.021	0.144	0.005	5.13
Squalius alburnoides	28	7	0.675	0.004	0.062	0.062	68.78
Pseudochondrostoma willkommii	22	6	0.699	0.008	0.090	0.005	3.83
Iberochondrostoma lemmingii	18	7	0.722	0.009	0.096	0.005	4.79
Alburnus alburnus	32	7	0.714	0.003	0.055	0.002	2.43
Anaecypris hispanica	2	2	1	0.250	0.500	0.008	6.00
Beta-actin gene							
Hybrids Picachos	44	43	0.999	0.00003	0.005	0.053	40.234
Hybrids Rabanales	40	35	0.992	0.00006	0.008	0.027	20.572
Squalius pyrenaicus	14	4	0.692	0.00900	0.094	0.002	1.374
Squalius alburnoides	50	25	0.931	0.00045	0.021	0.030	22.284
Pseudochondrostoma willkommii	28	10	0.593	0.01200	0.110	0.008	5.976
Iberochondrostoma lemmingii	44	11	0.725	0.00200	0.048	0.003	2.469
A. alburnus	40	22	0.950	0.00037	0.019	0.014	8.865
A. hispanica	8	3	0.714	0.01500	0.123	0.003	2.286

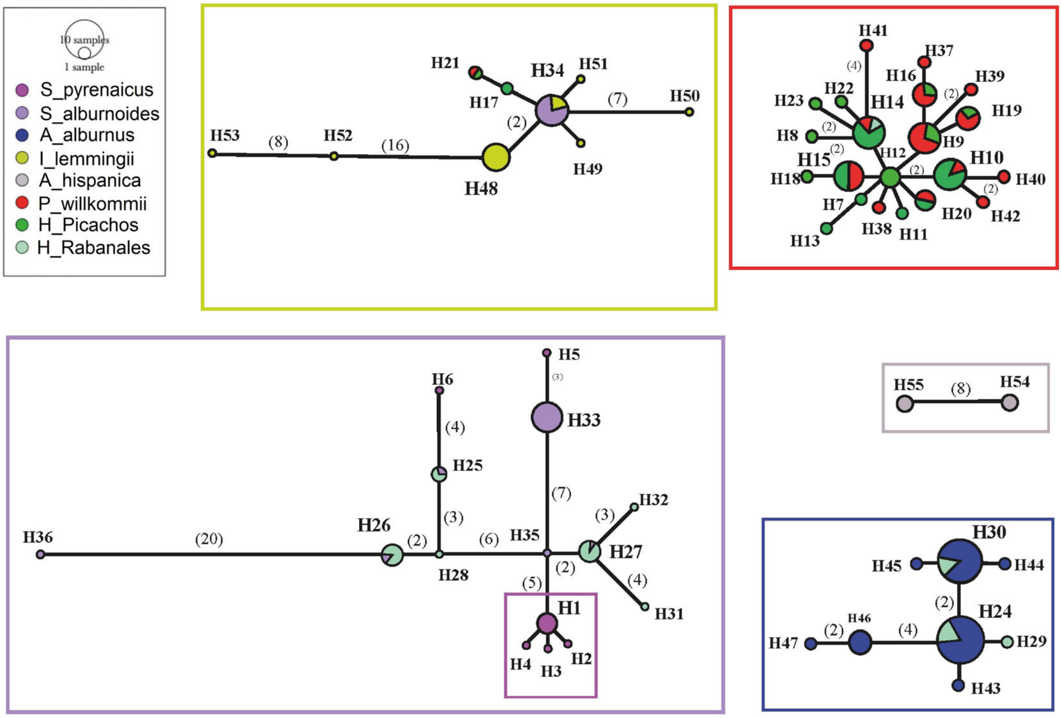

FIGURE 4 Haplotype network for the mitochondrial *Cytochrome b* (*cytb*) gene with mutational steps above lines linking haplotypes. Circles are proportional to the number of individuals sharing haplotypes. Colours correspond to the different species. See Table 2 for the haplotype number correspondence and Appendix S2 for individual haplotypes of each specimen. H_Picachos, hybrids from Picachos stream; H_Rabanales, hybrids from Rabanales stream.

mtDNA group (Figure 4). However, two (H17 and H21) were found to group with *I. lemmingii* haplotypes, although this cluster also contained one 'pure' *P. willkommii* (Pw034, H21) from Picachos stream and 10 *S. alburnoides* from Guadalbarbo stream for which the field assignment was 'pure *S. alburnoides*'. These 10 specimens all shared the *I. lemmingii* haplotype (H34) for *cytb* (Figure 4).

Rabanales hybrids showed a more diverse mtDNA pattern, with 25.0% (six) of them showing *A. alburnus* haplotypes (H24 and H30). Three of these (HR04, HR05 and Hy040) clustered with *A. alburnus* in the morphometric discriminant function analysis DFA2. The remaining 18 hybrids (70.8%) shared haplotypes with *S. alburnoides*, except for Hy033, which is included in one of the *P. willkommii* haplotypes (H14) (Supporting Information Figure S2).

In general, individuals identified in the field as *A. alburnoides*, *P. willkommii*, *I. lemmingii*, *S. alburnoides* and *S. pyrenaicus* presented the expected mtDNA haplotype and their mtDNA clustered with those from the same species, with some exceptions: one *P. willkommii* (Pw034) had a *I. lemmingii* haplotype (Hy027) and seven *S. alburnoides* had *I. lemmingii* haplotypes (Figure 4). In all mitochondrial analyses, hybrids from Picachos grouped with *A. alburnoides*, *P. willkommii* and *I. lemmingii*, whereas hybrids from Rabanales stream shared haplotypes and/or clustered with *A. alburnus* and *S. alburnoides* haplotypes (Figure 4).

Beta-actin gene sequences were obtained for 93 individuals. All individuals classified as *A. alburnoides*, *P. willkommii*, *I. lemmingii* and *S. pyrenaicus* in the field exhibited a single-parent haplotype for the *Beta-actin* gene, but a high number of *S. alburnoides* and hybrids were heterozygous. This was confirmed by the phylogenetic analyses on the *Beta-actin* gene, which recovered monophyletic lineages with moderate to high support (posterior probabilities, 0.56–1.00; bootstrap support, 69–100) for all four species. No hybrids were included within any pure parental nuclear lineages; all hybrids and *S. alburnoides* were independently recovered (Supporting Information Figure S3). One *A. alburnus* exhibited *S. pyrenaicus*/*S. pyrenaicus Beta-actin* sequence (Hy001, Appendix S2).

The reconstructed *Beta-actin* haplotype network (based on the phased sequences) showed a complex pattern of subnetworks. In the *S. alburnoides* network an ancestral haplotype (H46) was found, corresponding to *S. alburnoides* GenBank individuals and hybrids from Rabanales (HR03, HR06, HR09 and HR015) and Picachos (Hy033 and Hy034) (Figure 5). In general, hybrids shared haplotypes with *A. alburnus*, *S. alburnoides* and *S. pyrenaicus*. In Picachos, some heterozygous specimens (13) were found, all situated close to *S. alburnoides* and *P. willkommii*, but which could not be assigned to *P. willkommii* owing to large numbers of mutational steps that separated them, which greatly exceeded the variability observed within *P. willkommii* (30).

Many heterozygous individuals were found, all corresponding to field-identified hybrids and *S. alburnoides*, showing many exclusive haplotypes and few shared haplotypes (H9, H47, H54 and H55). These heterozygous individuals may have resulted from backcrosses between parental species, especially *S. alburnoides* and *A. alburnus*

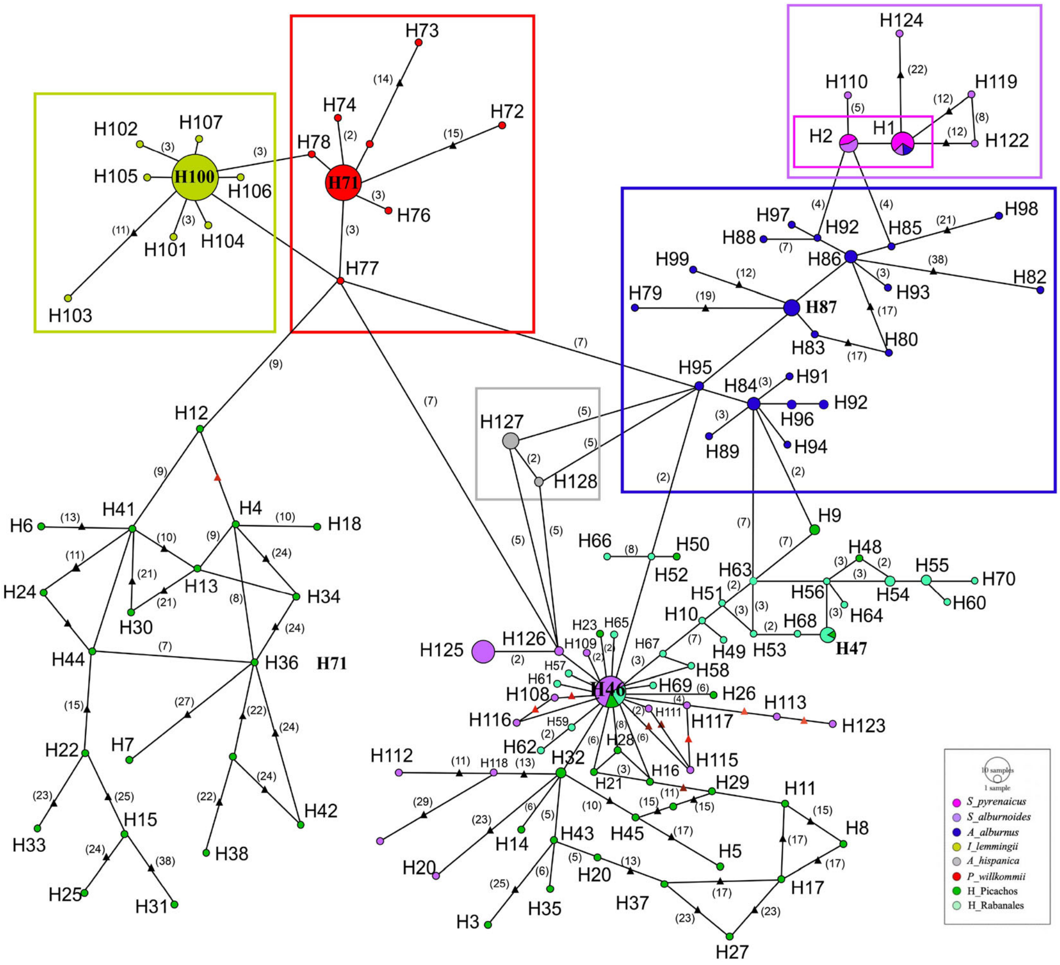

FIGURE 5 Haplotype (H) network based on the phase of segregating sites for the nuclear *Beta-actin* gene. The numbers of mutational steps that separate haplotype sequences are proportional to the lengths of the connecting edges, with values provided above each edge. Nodes (circles) correspond to observed haplotypes and their sizes are proportional to the number of individuals sharing a particular haplotype. Boxes with different outline colours show the correspondence between haplotype clusters and each species. Haplotype labels (e.g. H124) correspond to those in Table 2 and Appendix S2, where additional information on each haplotype is provided.

in Rabanales, and *S. alburnoides* with *P. willkommii* in Picachos stream (Hy004, Hy006, Hy011, Hy012, Hy014 and Hy030; see Appendix S2).

In summary, most hybrids from Picachos stream showed a nuclear combination that involved *S. alburnoides* and *P. willkommii* as a parental species, with two of them exhibiting a maternal *I. lemmingii* haplotype and one with *A. alburnus* paternal haplotype, but they all resembled *S. alburnoides* morphologically, as shown by the DFA2 analysis (Appendix S2). All hybrids from Rabanales stream showed a nuclear combination that involved the non-native *A. alburnus*. Most specimens showed maternal *S. alburnoides* mtDNA and *A. alburnus*/*S. alburnoides* combined ncDNA (*A. alburnus*/*S. alburnoides*, 54,1%). Of these, >76% were morphologically similar to *S. alburnoides* by DFA2 and the remainder resembled *A. alburnus*. Six Rabanales hybrids had *A. alburnus* mtDNA: three of them *A. alburnus*/*S. alburnoides* ncDNA and two *A. alburnus*/*A. alburnus* (no nc data for one specimen) Of these, three were most morphologically similar to *S. alburnoides* and three to *A.alburnus*.

There were 10 *S. alburnoides* specimens that were discovered to exhibit *I. lemmingii* mtDNA, sharing cytb haplotype H34 with IL, and confirmed as having *S. alburnoides* ncDNA or *S. alburnoides*/*S. pyrenaicus*, so they were in fact hybrids. Morphologically they fully resembled *S. alburnoides* and were thus classified by both the DFA1 and DFA2 analyses.

Finally, two Rabanales specimens (HR04, HR05) had both mtDNA and ncDNA of *A. alburnus* and were classified as *A. alburnus* by the DFA2 analysis. This showed that our initial field assessment of these as hybrids was incorrect. Another Rabanales hybrid (Hy033) had *P. willkommii* mtDNA and ncDNA *S. alburnoides*/*A. alburnus* and was classified by the morphometric discriminant function as *S. alburnoides*.

4 | DISCUSSION

This study has revealed the existence of hybrids between fish from the *S. alburnoides* complex and both native and non-native cyprinids in tributaries of the Guadalquivir River. Crosses with the non-natives were more abundant in streams that are directly connected to the Guadalquivir River, whereas crosses between native species were found in the more isolated streams, which stay fragmented in shallow pools during most of the year. The dominance of *S. alburnoides*-like morphology in hybrids and haplotype analyses suggests that a large proportion of hybrids may be backcrosses with the parental species.

The propensity of *S. alburnoides* to hybridize is well known (Alves et al., 1997; Carmona et al., 1997; Alves, Coelho & Collares-Pereira, 2001; Cunha et al., 2004; Cunha et al., 2011). Nineteen (1 Picachos, 18 Rabanales) hybrids of *S. alburnoides* with the non-native species *A. alburnus* were identified. *Alburnus alburnus* is a potadromous species that has been repeatedly introduced into Guadalquivir basin reservoirs and can now be found throughout the main course of the Guadalquivir River (Vinyoles et al., 2007). Rabanales stream is a direct tributary of the Guadalquivir River and maintains moderate but constant flow, enabling colonizing *A. alburnus* individuals from the Guadalquivir to reach upstream stretches during their reproductive migration. Fernández-Delgado et al. (2014) identified *A. alburnus* as a potential threat in this area and there have been recent concerns about the widespread occurrence of *A. alburnus* hybrids in Iberian rivers (Curto et al., 2022). Apart from the risk of genetic dilution or introgression, both *A. alburnus* specimens and hybrids may outcompete the native fish species for food resources (Almeida et al., 2017) and by having a greater tolerance to the severe human disturbances in the area (R.J. de Miguel, personal observation; Morales-Mata, Curros-Ruiz & de Miguel Rubio, 2020).

Hybridization between native species was also confirmed at Picachos stream, predominantly between females of *P. willkommii* and males of *S. alburnoides*, with these hybrids being correctly identified in the field. This parental combination was unexpected because the most widely reported hybridization mechanism for *S. alburnoides* is sperm parasitism by females, using sperm of other sympatric male cyprinids (*S. pyrenaicus*, *Squalius aradensis* and *Squalius carolitertii*; Sousa-Santos, Collares-Pereira & Almada, 2007). There is ample evidence that the mtDNA of *S. alburnoides* individuals is *S. pyrenaicus*-like, after their maternal ancestor, and there have been previous reports of maternal introgression from other *Squalius* species (Alves et al., 1997; Sousa-Santos, Collares-Pereira & Almada, 2007). However, reports of *S. alburnoides* hybridization with native species of different genera are scarce (but see Sousa-Santos et al., 2014). At Picachos, winter rains facilitate the migration of *P. willkommii* to the fast-flowing riffles within the upper stretch of the stream. This is also where *S. alburnoides* prefers to spawn (Scribner, Page & Bartron, 2000). In recent years, earlier and more severe summer drought conditions convert the stream into a series of isolated pools as early as spring or early summer. Our hypothesis is that this reduces the space available for spawning and consequently increases the probability of interspecific gamete exchange, potentially explaining the current high numbers of hybrids in this stream compared with those of surveys from just over a decade ago (Fernández-Delgado et al., 2014).

A similar phenomenon could be happening at Guadalbarbo stream. This stream is separated from the main Guadalquivir River channel by a 2 m weir. This leads to some isolation of upstream areas with reduced influx of downstream species. In these reaches, flow is maintained in winter, but only a few deep pools persist in the summer, and these are becoming smaller each year (R.J. de Miguel, *personal observation*). This double isolation may favour unexpected native crosses such as those observed between *I. lemmingii* and *S. alburnoides*. These hybrid individuals were initially assigned as 'pure' *S. alburnoides* according to meristic characters in the field and morphometric analyses (Supporting Information Table S1), but the molecular analyses revealed that they have maternal *I. lemmingii* ancestry.

This also adds support for the potential occurrence of backcrosses and introgression in these streams. This is particularly true for *A. alburnus* × *S. alburnoides* hybrids, found at Rabanales stream, and *P. willkommii* × *S. alburnoides* hybrids found at Picachos stream, where maternal introgression by *P. willkommii* cannot be ruled out. However, the use of only one nuclear molecular marker limited our ability to detect backcrossed individuals. Future genome-level analyses will provide greater insights into the extent of backcrossing and its impact on the diversity of the forms observed.

Recent studies and unpublished surveys suggest that the relative abundance of both non-native and native inter-genera hybrids driven by *S. alburnoides* in these streams is increasing (Fernández-Delgado et al., 2014; Curto et al., 2022; R.J. de Miguel, personal observation). Although the present study is biased because hybrids were specifically targeted, the high numbers of both expected and unexpected hybrids suggest that there might be a higher prevalence of intergeneric hybridization than previously thought. These findings are specific to the four sampled streams and collected individuals, but the connectivity scenarios that they represent are widespread within the Guadalquivir River basin. Therefore, we hypothesize that these hybridization events are widespread within the basin and could also be happening in other Mediterranean rivers. This needs to be confirmed at larger geographical scales using more comprehensive analyses of genomic divergence. It will also be important to establish whether native inter-genera hybridization prevalence has a historical phylogenetic origin and 'expected' frequencies, or whether our suspicions of it being an increasing phenomenon driven by recent changes in conditions are confirmed.

If future studies reveal widespread introgression through reproductively viable hybrids, there will be additional risks to potential gene-pool dilution and homogenization (Curto et al., 2022). Hybridization with introgression is a severe risk to endangered species, as the original pure forms can be gradually lost and replaced by hybrid individuals. There is also a risk that *S. alburnoides* could have a negative influence on other native species via both genetic dilution and morphological homogenization. This would be a serious threat in areas where native communities are naturally species-poor – and thus more vulnerable to the loss of species and genetic diversity – and under other human threats such as pollution and disruptive land uses. This is even more important given the endemic nature and high threat categories of most cyprinids in these rivers, so the preservation of their genetic diversity is paramount.

4.1 | Conservation implications and management recommendations

Most of the sub-basins in the Guadalquivir River basin are under high desiccation threats in their upper reaches owing to excessive water extraction and increased drought conditions as a result of climate change (Fernández-Delgado et al., 2014). In addition, non-native species have been expanding and colonizing the middle reaches of these streams extensively since the second decade of this century (De Miguel et al., 2016). Under a climate change scenario, where more intense summer droughts decrease water availability in upstream areas, native species will be forced to share the scarce remaining pools (which is likely to increase the risk of native inter-genera hybridization) or move downstream to find larger water bodies in the middle section of the river. However, in this middle section they will encounter a greater abundance of non-native species that swim up from the main Guadalquivir River channel and reservoir sources in neighbouring basins. This is especially relevant for *A. alburnus*, which is currently the main non-native species that is able to swim upstream and reach those middle sections where natives are being forced to descend owing to desiccation. In addition, these middle stretches are often sites of intensified human impacts and lower water quality (Fernández-Delgado et al., 2014). However, this may favour *A. alburnus* which is quite tolerant of degraded habitats and poor water quality (Maceda-Veiga & de Sostoa, 2011), reaching very high local population densities (Ilhéu et al., 2016) and further increasing the risk of hybridization or niche competition between native cyprinids and this exotic invasive non-native species.

The sum of upstream and downstream pressures may be creating a ‘lethal sandwich’ that forces native species into ever smaller stretches and pools, compromising the survival of the few remaining native fish communities in the basin. The net effect of these pressures may vary depending on stream connectivity with the main Guadalquivir River channel: hybridization and/or competitive exclusion by invasive non-native species in well-connected streams, and native inter-genera hybridization with gene dilution and morphological homogenization in fragmented watercourses. Large-scale quantitative studies that include genome-wide analyses should be carried out to find out the extent and geographical distribution of different hybrids (i.e. what proportion of fish are hybrids and which rivers are most affected). It is important to confirm or refute the hypothesis that inter-genera native hybrids are more abundant in courses prone to desiccation or with certain environmental characteristics. These studies should potentially be extended to other river systems in the Iberian Peninsula, and in a more generalized manner, to the Mediterranean area.

Managing the potential multiple threats faced by fish in Mediterranean basins is likely to be challenging in the future. The influx of non-native species can be tackled by comprehensive surveys and the creation of a risk assessment map that identifies areas that are at greatest risk of interactions between *A. alburnus* and native cyprinids or high concentrations of native cyprinids in very small, isolated pools (Matono, Da Silva & Ilhéu, 2018). In these areas, invasive non-natives should be eradicated. Targeted elimination of invasive individuals can be coordinated by regional or local governments and carried out by local volunteers, which would also help in the dissemination and education of the local communities about this issue (LIFE Saramugo, 2019). As a preventive measure, segregation of native from non-native species using small barriers such as eel-ladders has been recommended (Starrs et al., 2017; Sanz-Ronda et al., 2019) and shown to be effective as a short-term measure (Clarkson, Marsh & Dowling, 2012). This might appear counterintuitive for the preservation of river connectivity and integrity. However, under this ‘lethal sandwich’ scenario the selective isolation of upper, well-conserved reaches could be an emergency measure that could temporarily ease this threat. In addition, a top-down approach by including non-native invasive species as part of the biological quality indicators in the European Water Framework Directive (Council of the European Communities, 2000) would force national and regional governments to address this issue seriously.

If native inter-genera hybridization is confirmed as a threat to species integrity, small pools caused by desiccation and habitat destruction will be the key aspects to address.

Native fish populations would benefit from a reduction of organic pollution and an increase in riparian reforestation efforts to increase canopy shade that will reduce evaporation and reduce the risk of complete desiccation of isolated pools (LIFE Saramugo, 2019). Similar habitat management actions have been successful for other species (Sayer et al., 2020). Under this scenario, the close collaboration of national and regional governments to increase conservation efforts for endemic cyprinid species is essential, so water quality improvement efforts can be coupled with adequate riparian restoration.

As an additional challenge, morphological homogenization among hybrids is of concern for monitoring and management efforts. Future surveying teams should take into account that some hybrids may be cryptic with regards to one of their parental species, with potentially the need to identify a new meristic characteristic that can be observed in the field and assist in the strategy of eliminating hybrids.

AUTHOR CONTRIBUTIONS

Lucia Galvez-Bravo: Conceptualization (equal); funding acquisition (equal); project administration (lead); supervision (supporting); writing—original draft (lead). **Anabel Perdices**: Investigation (molecular lead); formal analyses (equal); methodology (equal); resources (supporting); supervision (colead); visualization (colead); writing—original draft (equal). **Ramon de Miguel**: Conceptualization (equal); investigation (fieldwork lead); resources (supporting); writing—review and editing (supporting). **Angela Lambea-Camblor**: Investigation (supporting); visualization (supporting); writing—review and editing (supporting). **Carlo Meloro**: Investigation (morphometrics lead); formal analysis (equal); methodology (equal); supervision (colead); visualization (colead); writing—original draft (equal). **Charlotte Penney**: Investigation (supporting); formal analysis (supporting); visualization (supporting); writing—original draft (supporting). **Begoña Martinez-Cruz**: Conceptualization (equal); writing—original draft (supporting);

writing—review and editing (supporting). **Richard Brown**: Conceptualization (equal); funding acquisition (colead); writing—review and editing (supporting).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The molecular sequence data for each individual are available on GenBank (see accession numbers for each individual in Appendix S1). Morphometric coordinates and photographs of each individual fish are available from the correspondence author upon request.

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