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EVALUATION OF POTENTIAL HYBRIDIZATION BETWEEN NATIVE FISHES AND THE INVASIVE BLEAK, *ALBURNUS ALBURNUS* (ACTINOPTERYGII: CYPRINIFORMES: CYPRINIDAE)

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Background. Freshwater fishes are among the most threatened taxa worldwide. The proliferation of introduced species in the Iberian Peninsula is currently one of the main drivers of native fish declines. One of such species, the bleak, *Alburnus alburnus* (Linnaeus, 1758), has become widespread in the last 25 years and, due to its phylogenetic proximity, poses a high risk of hybridization with native species. The aim of this study was to improve the current knowledge on this issue by 1) evaluating the presence of hybrids with *Alburnus alburnus* in the wild, using molecular screening of individuals representing the intermediate morphology and 2) testing if artificial crosses between *Alburnus alburnus* and the highly threatened native cyprinid *Anaecypris hispanica* (Steindachner, 1866) resulted in viable hybrid offspring.

Material and methods. The genetic profile of *Alburnus alburnus* was established using the cytochrome *b* and beta-actin genes to allow comparisons with the profiles of the sympatric species with which it could potentially hybridize: *Anaecypris hispanica*; *Squalius alburnoides* (Steindachner, 1866); and *Squalius pyrenaicus* (Günther, 1868). This profile was further used to assess if fish with the intermediate morphological features were indeed hybrids. Finally, artificial crosses between *Alburnus alburnus* and *Anaecypris hispanica* were conducted to test the viability of the offspring.

Results. *Alburnus alburnus* individuals were genetically identical to the stocks introduced in Spain and the Czech Republic. A reference library composed of 15 species-specific nDNA loci was built and used to characterize wild fish showing intermediate morphological features. Results showed that *Alburnus alburnus* is currently hybridizing with males and females of *Squalius alburnoides* and that morphometric identification is insufficient for a reliable detection of the hybrids. Artificial crosses between the bleak and the highly threatened *Anaecypris hispanica* did not result in viable offspring.

Conclusion. Phylogenetic relatedness, traduced in the absence of pre- and post-zygotic barriers to reproduction, together with other factors related to the ecology and life history of the species involved are essential for hybridization to occur. As such, the proliferation of the bleak through the Iberian hydrographical network represents a serious additional threat for the already imperilled native *Squalius* and *Anaecypris* species.

Keywords: hybridization risk, invasive fish species, biodiversity loss, conservation

INTRODUCTION

Freshwater fish species are recognizably among the most threatened taxa worldwide, facing an increasingly risk of local, regional, or global extinction mostly due to habitat loss and degradation, invasive species proliferation, pollution, over-exploitation, and direct and indirect effects of climate change (Jenkins 2003, Xenopoulos et al. 2005, Arthington et al. 2016). In the Mediterranean Basin, one of the main hotspots for freshwater biodiversity at a global scale, holding over 200 Key Biodiversity Areas (Máiz-Tomé et al. 2017), the main drivers of native fish declines are water extraction, pollution, and invasive species proliferation (Clavero et al. 2010, Hermoso and Clavero 2011, Hermoso et al. 2012, Gozlan 2012). The Iberian

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Peninsula is not an exception to this scenario, with about 68% of its native cyprinid ichthyofauna imperilled due to similar threats (Sousa-Santos et al. 2016). In particular, the spread and establishment of introduced species poses a strong threat to the conservation of native fishes in highly fluctuating environments and with human-induced disturbances such as the temporary streams of southern Iberia (Hermoso et al. 2012, Ilhéu et al. 2014).

The spread of non-native fish species results in increasing competition (both for food and space) and predation of eggs, juveniles, and adults of the sympatric species, resulting in the disruption of the native community balance, and in a risk of hybridization and genetic introgression (Ribeiro and Leunda 2012, Bangs et al. 2018). Introduced species may also represent an input of new pathogens and parasites to which native species may be vulnerable (Ribeiro and Leunda 2012).

The common bleak, *Alburnus alburnus* (Linnaeus, 1758), is a small sized cyprinid from central Europe and western Asia which was repeatedly introduced in the Iberian Peninsula in the last 25 years by anglers as a forage species and quickly spread throughout almost all major Iberian water basins (reviewed by Vinyoles et al. 2007). The Iberian populations of this non-native species generally show high effective sizes due to their successful reproduction and recruitment (Carbonero Ciria et al. 2006, Vinyoles et al. 2007, Almeida et al. 2014), with obvious impact on native fish communities. The problem is aggravated by the fact that this species is known to hybridize with other cyprinids (Błachuta and Witkowski 1983/1984, Crivelli and Dupont 1987, Witkowski et al. 2015), especially with *Squalius* species (Wheeler 1978, Witkowski and Błachuta 1980, Kammerad and Wüstemann 1989, Almodóvar et al. 2012). The potential risk of hybridization between the common bleak and Iberian fish species, already emphasized by several authors (Robalo et al. 2006, Vinyoles et al. 2007, Perea et al. 2010) and already reported by Almodóvar et al. (2012) for the Tagus River basin, prompted a study on the impact of *Alburnus alburnus* on populations of the Spanish minnowcarp, *Anaecypris hispanica* (Steindachner, 1866), locally known as saramugo, from the Guadiana River basin, as part of a LIFE project targeting the conservation of this endemic species (project reference: LIFE13/NAT/PT/000786-Saramugo). *Anaecypris hispanica* is included in the Annexes II and IV of the European Union Habitats Directive* and in Appendix III of the Bern Convention** and is listed as "Endangered" and "Critically Endangered", respectively, in the IUCN Red List of Threatened Species (Crivelli 2006) and in the Portuguese Red data Book of Vertebrates (Cabral et al. 2005). This species currently faces a declining population trend, with local extinctions reported mainly due to pollution, habitat loss, water scarcity, damming, and proliferation of exotic species (Doadrio 2001, Sousa-Santos et al. 2014). Besides *Anaecypris hispanica*, the Guadiana River basin harbours six other endemic cyprinid species, five of them with high conservation status (Cabral et al. 2005): *Pseudochondrostoma willkommii* (Steindachner, 1866) and *Squalius alburnoides* (Steindachner, 1866) ("Vulnerable"); and *Luciobarbus sclateri* (Günther, 1868); *Luciobarbus comizo* (Steindachner, 1864); and *Iberochondrostoma lemmingii* (Steindachner, 1866) ("Endangered").

Anaecypris hispanica, as well as *Squalius pyrenaicus* (Günther, 1868) and *Squalius alburnoides*, are proven to be phylogenetically close to *Alburnus alburnus* (see Robalo et al. 2006, Levy et al. 2008), increasing the risk of intergeneric hybridization for these three native species occurring in the Guadiana River. Other potentiating factors are added to the phylogenetic relatedness, such as the partially overlapping breeding seasons (Ribeiro et al. 2000, Almeida et al. 2014), during which fish may be spatially confined (in summer, the Mediterranean-type Guadiana River is typically reduced to a series of more or less disconnected pools); identical habitat preferences (Ribeiro et al. 2000, Ilhéu et. al 2016, Matono et al. 2018); and the disproportionally higher local densities represented by the large dimension shoals of *Alburnus alburnus* (see Ilhéu et al. 2016) when compared with the generally depleted and fragmented native populations. The potential hybridization with *Alburnus alburnus* would result in the introgression of alien genes in the genetic pool of native species, resulting in the loss of their taxonomical integrity and other unpredictable consequences.

The hybridization between *Alburnus alburnus* and *Anaecypris hispanica* will undoubtedly raise major concerns if these conditions are met simultaneously: long-lasting sympatric occurrence, synchronic breeding seasons, compatible gametes, and fertile hybrid offspring. A proper evaluation of the new potential threat for the conservation of the highly endangered *Anaecypris hispanica* is thus needed as well as the assessment of natural occurring hybrids between other native fish species and the invasive *Alburnus alburnus*, in the Guadiana River basin. As such, the aim of this study was to improve the current knowledge by 1) evaluating the presence of hybrids with *Alburnus alburnus* in the wild, using molecular screening of individuals with intermediate morphologies and 2) testing if artificial crosses between *Alburnus alburnus* and *Anaecypris hispanica* resulted in viable hybrid offspring. To achieve these goals, the genetic profile of *Alburnus alburnus* occurring in the Guadiana and Sado rivers was established using two molecular markers (cytochrome *b*, latter referred to as cyt*b* and beta-actin genes) to allow comparisons with the profiles of the three sympatric species with which it could potentially hybridize (*Anaecypris hispanica, Squalius alburnoides*, and *Squalius pyrenaicus*). This profile was further used to assess if fish identified in the field as hybrids due to their intermediate morphological features were indeed hybrids. Finally, artificial crosses between *Alburnus alburnus* and *Anaecypris hispanica* were conducted to test the compatibility of their gametes and the viability of the offspring.

* *https://eur-lex.europa.eu/eli/dir/1992/43/oj.*
** *https://rm.coe.int/CoERMPublicCommonSearchServices/DisplayDCTMContent?documentId=0900001680304356.*

MATERIALS AND METHODS

Sampling. Field surveys were conducted by electrofishing in the Sado River (Alcáçovas sub-basin; 38º26′34.27″N, 8º05′47.69″W) and in the Guadiana River (Xévora and Caia sub-basins; 39º03′37.63″N, 7º00′21.50″W and 39º07′32.66″N, 7º16′51.38″W, respectively), under the scope of the LIFE Saramugo project (LIFE13/NAT/PT/000786), in 2015 and 2016. Permits for field work were given by the ICNF, the National Institute for the Conservation of Nature and Forests. Captured individuals, showing intermediate morphological features, were identified as potential hybrids (n = 26), measured for fork length (FL), and brought to the lab after being euthanized by freezing. The number of scales of the lateral line (SLL) of these intermediate specimens was counted to test if this meristic character, which is distinct among the potentially hybridizing species (38–44 for *Squalius alburnoides*, 59–71 for *Anaecypris hispanica*, and 47–52 for *Alburnus alburnus*) (Doadrio et al. 2011), could be used in the future for an expedite recognition of hybrids in the wild.

Fin clips were collected from potential hybrids and from 15 specimens of *Alburnus alburnus* preserved for diet studies under the above-mentioned project. Tissue samples were preserved in 96% ethanol and stored at the collection of the MARE-ISPA for subsequent DNA extraction, amplification and sequencing.

DNA analyses. Total genomic DNA was extracted from fin clips using REDExtract-N-Amp Tissue PCR kits (Sigma-Aldrich), following the manufacturer's instructions. The mitochondrial cytochrome *b* (cyt*b*) and the nuclear beta-actin genes were amplified using the primers and conditions described in Sousa-Santos et al. (2014). PCR products were purified and sequenced in the forward direction at Stabvida (FCT, Portugal). The obtained sequences were aligned using the CodonCode Aligner v4.0.4 software (CodonCode Corp., USA) and deposited in GenBank. The search for shared haplotypes and the listing of representative sequences was conducted by DNAcollapser (FaBox v.1.41*).

In order to build a reference library for each molecular marker, the newly obtained sequences of *Alburnus alburnus* from the Guadiana River (cyt*b*: n = 15; beta-actin: n = 7) were grouped with sequences of *Alburnus alburnus* from other Euro–Asian river basins (cyt*b*: n = 6; beta-actin: n = 1) and of the sympatric species from the Guadiana River with which *Alburnus alburnus* could potentially hybridize: *Anaecypris hispanica* (cyt*b*: n = 1; beta-actin: n = 159, 29 haplotypes), *Squalius pyrenaicus* (cyt*b*: n = 1; beta-actin: n = 9, six haplotypes), and *Squalius alburnoides* (cyt*b*: n = 1; beta-actin: n = 20, eight haplotypes) (Table 1). Accession numbers of all the sequences were included in Table 1.

Although the phylogeny of *Alburnus alburnus* was already described elsewhere (Levy et al. 2008, Perea et al. 2010), a phylogenetic tree was drawn to assess the phylogenetic positioning of the *A. alburnus* individuals from the Guadiana River, based on cyt*b* gene sequencing. Phylogenetic relations were reconstructed by maximum parsimony using PAUP 4.0 (Swofford 1998). Branch

Table 1

Sequences used to build the reference libraries for both molecular markers used (cyt*b* and beta-actin), with which the studied *Alburnus alburnus* individuals were compared

Species	River basin, Country	Cyt*b* GenBank accession number	Beta-actin GenBank accession number	Reference
Anaecypris hispanica	Guadiana, Portugal	AJ427834	KM435483-KM435725	Alves et al. 2001b Sousa-Santos et al. 2014
Squalius alburnoides	Guadiana, Portugal	AJ427837	AA genomotype: DQ263227, DQ263229, EF458779-81, EF458783, EF458788-89, EF458792 PA genomotypes: DQ263230-31, EF458785 PAA genomotypes: DQ263228, DQ263232-33, EF458782, EF458784, EF458786-87, EF458797	Alves et al. 2001b Sousa-Santos et al. 2005, 2006
Squalius pyrenaicus	Guadiana, Portugal	DQ263236	DQ150279-80, DQ150327-32, DQ150323-24, DQ150272-73, AY943877	Sousa-Santos et al. 2006a, 2006b
Alburnus alburnus	Guadiana, Portugal	MH141602-616	MH141618-23	This study
Alburnus thessalicus	Pinios, Greece	AF090744	—	Zardoya and Doadrio 1999
Alburnus alburnus	Strymon, Greece	AF090745	—	Zardoya and Doadrio 1999
Alburnus alburnus	Croatia	DQ350254	MH141617	GenBank and this study
Alburnus thessalicus	Elbe, Czech Republic	HM560060	—	Perea et al. 2010
Alburnus alburnus	Sinhuka, Russia	HM560062	—	Perea et al. 2010
Alburnus alburnus	Jarama, Spain	JQ436541	—	Almodóvar et al. 2012

* *http://www.birc.au.dk/software/fabox.*

support was assessed by bootstrap analyses using 1000 replicates and *Cyprinus carpio* Linnaeus, 1758 as outgroup (GenBank accession number: DQ868875).

Concerning the beta-actin nuclear gene, which allows for the identification of hybrids as alleles inherited by both parents, sequences of heterozygous individuals were analysed according to the procedures described in Sousa-Santos et al. (2005) and single nucleotide polymorphisms were integrated in the reference library of haplotypes along with the sequences of *Squalius pyrenaicus*, *Anaecypris hispanica*, and *Squalius alburnoides*. As this latter species is in fact a polyploid complex which arose from intergeneric hybridization between *Squalius pyrenaicus* females (PP genome) and males from an already extinct *Anaecypris*-like species (AA genome), its beta-actin profile is hybrid, reflecting various combinations of the parental genomes (genomotypes): PAA, PPA, PPAA, PAAA, PA, and AA (Alves et al. 2001a, Morgado-Santos et al. 2016). As such, beta-actin sequences of the most common genomotypes occurring in the Guadiana River were included in the reference library: AA, PA, and PAA (Table 1). The method described by Sousa-Santos et al. (2005) and already used successfully to identify *Squalius alburnoides* genomotypes (Sousa-Santos et al. 2006a, 2006b, 2007) was also applied to assess the genome constitution of the hybrids involving this species. These results, combined with the information on the reproductive modes known for the *Squalius alburnoides* complex (Alves et al. 2001a), allowed the extrapolation of the genomotype of the *S. alburnoides* mother/father of the identified hybrid individuals.

In those cases where unambiguous calling the bases of both genetic complements was not possible, the nucleotide ambiguity code was used to map the mutations present in beta-actin haplotypes. Trimming the 3′ ends of all the sequences was adopted as a caution measure so that the portion of the fragment where typically a loss of signal occurs was not considered for a reliable identification of the called bases. Finally, the polymorphic sites of the beta-actin gene were resumed in a table of differences using the CodonCode Aligner software and used as species-diagnostic loci to allow for the detection of hybrids.

Fertilization experiments. Artificial crosses between *Alburnus alburnus* and *Anaecypris hispanica* were conducted using, respectively, individuals captured in the field during the breeding season (between May and July, in the rivers Caia and Degebe; Ilhéu et al. 2016) and individuals maintained at ex-situ conservation facilities located at the "Parque Natural do Vale do Guadiana". Fish were sorted according to their maturation stage, which was assessed by applying a slight pressure on the abdomen. Only the females which released gametes during this procedure were used for the artificial crosses to obviate the use of immature oocytes. Oocytes of one species were gently mixed with sperm from the other species, in distinct Petri dishes to which 10 mL of water tank was added. After 10 min, the eggs of each Petri dish were transferred into small tanks (1 L) with permanent aeration and methylene blue (~0.5 mL) to avoid fungal contamination. After 24 h, a small portion of the eggs was carefully removed from the tank and inspected under a binocular microscope to check for evidence of successful fertilization. Each day, eggs showing signs of fungal contamination were individually removed from the tank to avoid the contamination of the whole batch. This procedure was replicated in the four trials conducted during the reproductive season of 2015 and 2016. The number of males and females used in each trial differed according to the number of mature individuals available and the number of gametes released. All fish survived the procedure and were returned live to their respective maintenance tank.

Simple descriptive statistics were conducted using Microsoft Excel.

The presently reported study was carried on in accordance with the Portuguese state regulation and field-work permits were obtained from the National Institute for the Conservation of Nature and Forests (ICNF), Portugal.

RESULTS

DNA analyses. Sequencing of the mitochondrial cyt*b* and nuclear beta-actin genes resulted in aligned fragments of 991 and 405 bp, respectively. Cyt*b* gene sequencing of the *Alburnus alburnus* individuals (n = 15) yielded four haplotypes (ALBURN1-4), differing only by 1 to 5 mutations (Table 2), which accounts for 0.10% to 0.50% of pairwise divergence among sequences.

The phylogenetic tree, based on the analysed cyt*b* gene fragment (with 168 parsimony informative loci out of a total of 278 variable sites), showed that *Alburnus alburnus* is more closely related to *Anaecypris hispanica* than to the sympatric *Squalius* species (Fig. 1). One of the *Alburnus alburnus* haplotypes from the Guadiana River (ALBURNCYT3) is shared with the Jarama River (Spain). These and the remaining *Alburnus alburnus* haplotypes from Guadiana River group with one haplotype from the Czech Republic forming a well-supported clade, which is clearly distinct from that from Russia, Croatia, and Greece (Fig. 1).

Table 2

Relative frequencies (n) of cyt*b* haplotypes of *Alburnus alburnus* from the Guadiana River found and number of pairwise differences between each pair of haplotypes

	n	ALBURNCYT1	ALBURNCYT2	ALBURNCYT3
ALBURNCYT1	10			
ALBURNCYT2	1	1		
ALBURNCYT3	3	4	5	
ALBURNCYT4	1	3	4	1

Regarding beta-actin gene sequencing, 12 haplotypes were identified among sequences of *Alburnus alburnus* from the Guadiana River (n = 7) and Croatia (n = 1), with only three of them being found in homozygosity (Table 3). These haplotypes and the ones obtained for *Squalius alburnoides*, *Anaecypris hispanica*, and *Squalius pyrenaicus*, integrated the reference library built for the beta-actin gene (Table 3). The inspection of the reference library shows 15 species-diagnostic loci, i.e., point mutations which are shared by all the individuals of a given species, which were used to distinguish sympatric species from the Guadiana River at the nuclear DNA level.

Detection of hybrids with *Alburnus alburnus* in the wild. A total of 26 potential hybrids were identified in the field based on intermediate morphological features, in the following sub-basins of the Guadiana and Sado river basins: Xévora (n = 13), Caia (n = 10), and Alcáçovas (n = 3). These individuals were primarily identified as *Squalius alburnoides* but with atypical coloration and head and pelvic fin profiles and represented 26% and 50% of the total number of *S. alburnoides* captured in these sampling sites from the Guadiana River basin (n = 50 in Xévora and n = 20 in Caia, respectively). The total number of *Squalius alburnoides* captured in the Alcáçovas sub-basin was not recorded. The mean fork length (±standard deviation) was of 84.42 ± 12.15 mm (64–113 mm) and the number of scales of the lateral line (SLL) was 49.27 ± 2.95 (44–57).

The cyt*b* gene sequencing results showed that 23 individuals had *Alburnus alburnus* mtDNA while the remaining three had *Squalius pyrenaicus* mtDNA (Table 4). Beta-actin gene sequencing was not possible for four individuals (likely due to the degradation of the nuclear DNA of these samples) but out of the 22 individuals sequenced, six (27%) were hybrids between *Squalius alburnoides* and *Alburnus alburnus* and the remaining 16 individuals were *A. alburnus*, homozygous (n = 11) or heterozygous (n = 5) for the considered gene fragment (Table 4). Photos from the hybrids and from pure *Squalius alburnoides* and *Alburnus alburnus* are shown in Fig. 2.

Combining the results of both markers and looking to the nature of the maternally inherited sequence, it is possible to identify that hybrids resulted from crosses between *Squalius alburnoides* females and *Alburnus alburnus* males (n = 3; two from Caia and one from Xévora) and from the reciprocal cross, i.e., between *Squalius alburnoides* males and *A. alburnus* females (n = 3; two from Caia and one from Alcáçovas) (Table 4). Considering the beta-actin profiles of the hybrids and the reproductive modes known for the *Squalius alburnoides* complex, it was possible to predict the parental genomotypes: all the *S. alburnoides* females involved in the hybrid crosses with *Alburnus alburnus* had a SAA genomotype, while all the males involved in the reciprocal hybrid crosses were *Squalius alburnoides* with a AA genomotype (Table 4). Regarding the number of scales of the lateral line, these hybrids had 47.3 ± 2.1 scales (mean ± SD) (44–50), while the non-hybrids had 49.4 ± 2.2 scales (mean ± SD) (47–54); for the values obtained for each individual see Table 4.

Artificial crosses between *Alburnus alburnus* and *Anaecypris hispanica*. Four trials of crosses between *Anaecypris hispanica* females and *Alburnus alburnus* males and three trials of the reciprocal cross were conducted, involving a total of 69 gamete-donor fish (Table 5).

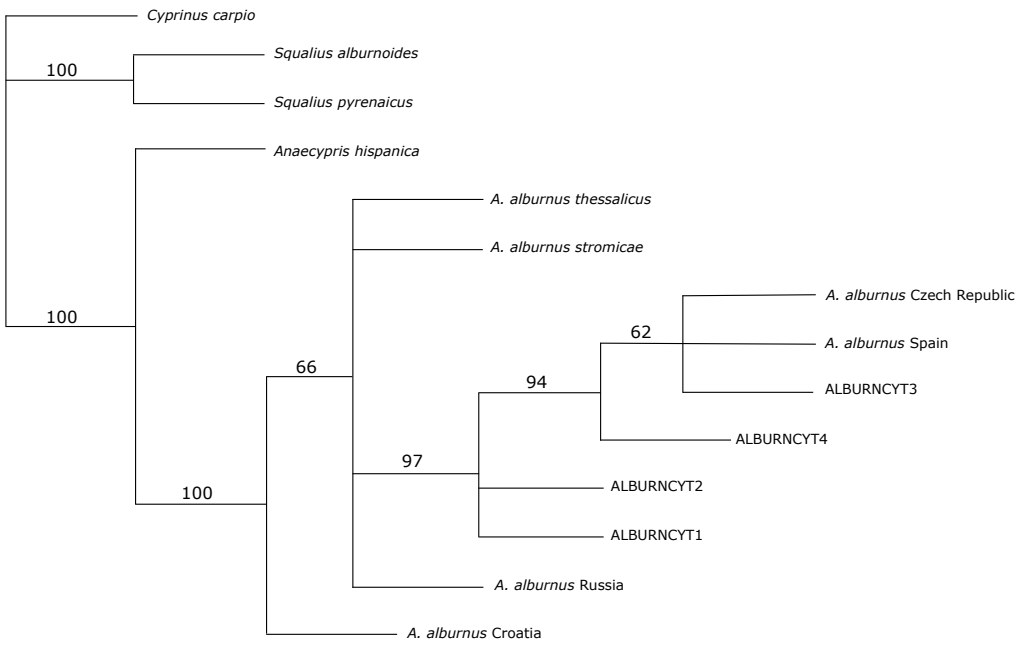

Fig. 1. Schematic representation of the parsimony tree illustrating the phylogenetic relations among sampled *Alburnus alburnus* based on cyt*b* gene sequences; bootstrap values are indicated for each well supported node (60% majority rule); sequences of *Anaecypris hispanica*, *Squalius alburnoides*, *Squalius pyrenaicus*, and *Cyprinus carpio* (outgroup) were also included in the tree

Table 3

Reference library built from beta-actin gene sequences, containing the diagnostic loci for sympatric *Alburnus alburnus*, *Anaecypris hispanica*, *Squalius alburnoides* (AA, PA, and PAA genomotypes), and *Squalius pyrenaicus* from the Guadiana River

Species	*n*	Loci														
		22–23	33	39	58	60	71	75–79	83	97	102	109	128	135	141	405
Squalius alburnoides AA genomotype	9	—	G	T	T	G	T	TTTTA	A	C	C	A	T	T	—	A
Squalius alburnoides PA genomotype	3	RS	A	W	G	R	A	TTWAA	K	R	Y	T	S	T	M	R
Squalius alburnoides PAA genomotype	8	RS	A	W	G	R	A	TTWAA	K	R	Y	T	S	T	A	R
Anaecypris hispanica	159	AG	G	A	T	G	A	TTTTA	—	C	T	A	T	T	—	C
Squalius pyrenaicus	9	AG	A	A	G	G	A	TTT-A	A	C	C	G	T	T	T	C
Alburnus alburnus HMZ	1	AG	A	A	G	G	A	TTTTA	A	C	C	A	T	T	—	C
	1	AG	A	A	T	G	A	TTTTA	A	—	C	A	T	T	—	T
	1	AG	A	A	G	G	A	TTT-A	A	C	C	A	T	T	—	C
Alburnus alburnus HTZ	0.5	AG	A	A	T	A	A	ATTTA	A	—	C	A	T	T	—	T
	0.5	AG	A	A	T	A	A	ATTTA	A	—	C	A	T	A	—	T
	0.5	AG	A	A	G	G	A	TTT-A	A	C	C	A	T	T	—	Not clear
	0.5	AG	R	A	G	G	A	TTT-A	A	C	C	A	T	T	—	C
	0.5	AG	A	A	G	G	A	TTTAA	A	—	C	A	T	T	—	Not clear
	0.5	AG	R	A	G	G	A	TTTAA	A	C	C	A	T	T	—	C
	0.5	AG	A	A	G	G	A	TTTTA	—	C	C	A	T	T	—	C
	0.5	AG	A	A	G	G	A	TTTTA	A	—	M	C	T	T	—	C
	0.5	AG	A	A	T	G	A	TTTTA	A	—	C	A	T	T	—	T
	0.5	AG	G	A	G	G	A	TTTTA	A	C	M	T	T	T	—	C

Species-diagnostic loci are highlighted grey; the nucleotide ambiguity code was used to map the mutations present in *S. alburnoides* hybrid genomotypes; *n* = number of individuals; HMZ = haplotypes retrieved from homozygous individuals; HTZ = haplotypes retrieved from heterozygous individuals, according with the method described by Sousa-Santos et al. (2005); in this latter case, the presented number of samples is equivalent to the number of gene complements for which the haplotype was found (e.g., *n* = 0.5 means that the haplotype was found in one gene complement of a heterozygous individual).

Table 4

Characterization of the potential hybrids identified, based on the mitochondrial and nuclear markers analysed (cyt*b* and beta-actin), including the nucleotide present in each diagnostic loci

SLL	FL [mm]	cyt*b*	beta-actin	Identification	Loci														
					22–23	33	39	58	60	71	75–79	83	97	102	109	128	135	141	405
49	95	AAL	AAL homozygous	AAL	AG	A	A	T	G	A	TTTTA	A	—	C	A	T	T	—	T
47	99	AAL	AAL homozygous	AAL	AG	A	A	G	G	A	TTTTA	A	C	C	A	C	T	—	C
47	113	AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTTTA	A	C	C	A	T	T	—	C
		AAL	AAL heterozygous	AAL	AG	A	A	T	G	A	TTTTA	A	—	C	A	T	T	—	T
48	89	AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTTTA	A	C	C	A	C	T	—	C
		AAL	AAL heterozygous	AAL	AG	A	A	T	G	A	TTTTA	A	—	T	A	T	T	—	T
49	107	AAL	AAL heterozygous	AAL	AG	A	A	T	G	A	TTT-A	A	C	C	A	T	T	—	C
		AAL	AAL heterozygous	AAL	AG	A	A	T	G	A	TTTTA	A	—	C	A	T	T	—	T
52	81	AAL	AAL heterozygous	AAL	AG	G	A	T	G	A	TTTTA	A	—	C	A	T	T	—	C
		AAL	AAL heterozygous	AAL	AG	A	A	T	G	A	TTTTA	A	—	C	A	T	T	—	T
49	78	AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTT-A	A	C	C	A	T	T	—	C
		AAL	AAL heterozygous	AAL	AG	A	A	T	G	A	TTTTA	A	—	C	A	T	T	—	T
47	81	AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTTTA	A	—	C	A	T	T	—	T
		AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTTTA	A	C	C	A	C	T	—	C
54	86	AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTT-A	A	C	C	A	T	T	—	C
		AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTTTA	A	C	C	A	C	T	—	C
50	100	AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTTTA	A	C	C	A	T	T	—	C
		AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTTTA	A	—	C	A	T	T	—	T
51	74	AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTT-A	A	C	C	A	T	T	—	C
		AAL	AAL heterozygous	AAL	AG	A	A	T	G	A	TTTTA	A	—	C	A	T	T	—	C
47	80	AAL	AAL heterozygous	AAL	AG	A	A	T	G	A	TTTTA	A	—	C	A	T	T	—	T
50	81	AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTTTA	A	C	C	A	C	T	—	C
		AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTT-A	A	C	C	A	T	T	—	C
50	76	AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTTTA	A	C	C	A	C	T	—	C
		AAL	AAL heterozygous	AAL	AG	A	A	T	G	A	TTTTA	A	—	C	A	T	T	—	T
48	84	AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTTTA	A	C	C	A	T	T	—	C
53	94	AAL	AAL heterozygous	AAL	AG	A	A	G	G	A	TTTTA	A	C	C	A	T	T	—	C
44	99	SP	SAA × AAL	SAlb × AAL	RS	A	W	G	R	A	TTWWA	W	S	Y	W	Y	T	A	R
48	86	SP	SAA × AAL	SAlb × AAL	RS	A	W	G	A	A	TYWAA	W	S	T	W	Y	T	A	R
48	78	SP	SAA × AAL	SAlb × AAL	RS	A	A	G	R	A	TTWWA	W	S	Y	W	C	T	A	R
46	75	AAL	AA × AAL	AAL × SAlb	RS	A	W	G	R	A	TTWWA	W	S	C	W	Y	T	A	R
50	72	AAL	AA × AAL	AAL × SAlb	RS	A	W	K	R	A	TTWWA	W	R	Y	T	S	T	M	R
48	78	AAL	AA × AAL	AAL × SAlb	RS	A	W	G	R	A	TTWWA	W	S	Y	W	Y	T	A	R
52	85	AAL	—	—															
46	64	AAL	—	—															
48	66	AAL	—	—															
53	74	AAL	—	—															

SLL = number of scales of the lateral line, FL = fork length (measured to the nearest mm), AAL = *Alburnus alburnus*, SP = *Squalius pyrenaicus*, SAlb – *Squalius alburnoides*, SAA = *S. alburnoides* SAA genomotype, AA = *S. alburnoides* AA genomotype.

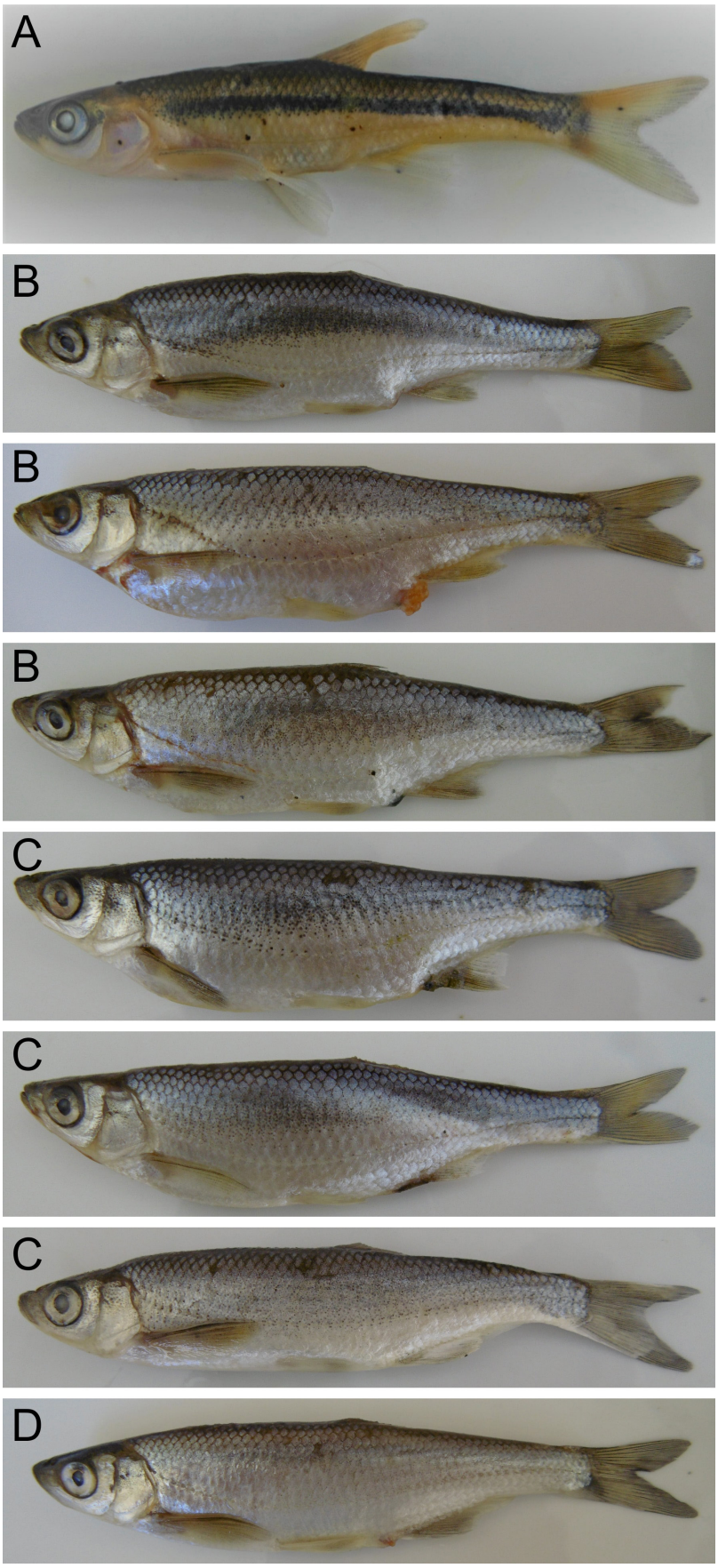

Fig. 2. Photographs of *Squalius alburnoides*, *Alburnus alburnus*, and their hybrids; *S. alburnoides* (**A**), *S. alburnoides** × *A. alburnus* hybrids (**B**), *A. alburnus** × *S. alburnoides* hybrids (**C**), *A. alburnus* (**D**); the asterisk (*) indicates the mother species of the hybrid, based on mitochondrial DNA results

Table 5

Experimental crosses between *Alburnus alburnus* and *Anaecypris hispanica* made with the artificial fertilization of eggs from one species with the sperm of the other species

Artificial cross	Date	*n* females	*n* males	*n* eggs
A. hispanica females × *A. alburnus* males	Jun 2015	6	2	7
	25 May 2016	5	1	68
	8 Jun 2016	4	11	11
	20 Jun 2016	5	5	107
A. alburnus females × *A. hispanica* males	25 May 2016	1	3	40 VE
	8 Jun 2016	1	10	50 VE
	20 Jun 2016	1	14	100 VE
	Total	23	46	>376

n = number of specimens, VE = visual estimate.

In total, over 186 oocytes of 20 *Anaecypris hispanica* females and over 190 oocytes of three *Alburnus alburnus* females were fertilized by sperm from19 *Alburnus alburnus* and 27 *Anaecypris hispanica* males, respectively. Although the clear distinction between the core and peripheral areas of the oocytes indicated the occurrence of fertilization and of the first divisions of the egg, all eggs were considered unviable 24–48 h after fertilization.

DISCUSSION

The high incidence of hybridization in fish seems to be a result of several contributing factors: external fertilization, weak behavioural isolating mechanisms, unequal abundance of the parental species, competition for limited adequate spawning habitat, decreasing habitat complexity, and susceptibility to secondary contact between recently evolved forms (reviewed by Scribner et al. 2001, Hernández Chávez and Turgeon 2007). Hybrids between distantly related fish species are also frequently viable since fish appear to be less susceptible to severe developmental incompatibilities that affect interspecific hybrids between other vertebrate taxa (Scribner et al. 2001). The risk of interspecific hybridization and the assessment of its extent is often an underappreciated problem but the more frequent use of molecular tools in biological assessments may contribute to change this reality (Rhymer and Simberloff 1996).

The non-native population of *Alburnus alburnus*, introduced to Portugal, in the Guadiana River basin, around 12 years ago (Vinyoles et al. 2007), presents some haplotype diversity for the mitochondrial cyt*b* gene, with haplotypes that closely resemble those found in *Alburnus alburnus* from Spain and the Czech Republic. Moreover, a shared haplotype with an individual from the Spanish Tagus (Jarama River) suggests that the stock introduced in the Guadiana and Tagus River basins could have had the same provenience. Our results corroborate the close phylogenetic proximity between *Alburnus alburnus* and *Anaecypris hispanica* already described by other authors, as well as a wider distance to the sympatric *Squalius* species: *S. pyrenaicus* and *S. alburnoides* (see Levy et al. 2008, Perea et al. 2010). Despite the closest phylogenetic affinity between *Alburnus alburnus* and *Anaecypris hispanica*, natural occurring hybrids between these two species were yet not detected and the artificial crosses conducted during this study did not result in viable offspring. Contrastingly, the less phylogenetically related *Squalius alburnoides* hybridizes in the wild with *Alburnus alburnus* in at least three Iberian river basins: Sado, Guadiana, and Tagus (Almodóvar et al. 2012 and this study). As such, it seems clear that phylogenetic relatedness, if traduced in the absence of pre- and post-zygotic barriers to reproduction, is essential for hybridization. However, as shown in the presently reported study, phylogenetic relatedness by itself does not necessarily implies the occurrence of hybridization between the bleak and all the closely related native cyprinids with which it becomes sympatric along its invasive path. Other characteristics such as the preference for similar spawning grounds, synchronous spawning seasons, responsiveness to the same courting behaviours, and similar body size of adults are most likely the main factors that are essential for hybridization to occur. One should also expect that the magnitude of the hybridization events, which may range from the occurrence of rare and punctually-located hybrid individuals to a massive production of hybrids which may widespread and replace their parental species at a river basin scale, will likely depend on the similarity of the previously listed essential factors. Importantly to note, however, is that both the frequency and the magnitude of the hybridization events will most likely vary in time and space in response to changes potentially occurring in the factors which promote them. This means that if there are no reproductive barriers to hybridization between two species, the absence of naturally occurring hybrids at some point in time does not necessarily means that they will never occur nor that, once detected, their abundance will always show stabilized values. For instance, the preferred spawning grounds may shift in response to habitat destruction or degradation; the spawning seasons may become more synchronous due to climate change; and previously not attractive courting behaviours may later trigger positive responses as a result of the natural evolution process of the species. Thus, it becomes clear that the occurrence of *Alburnus alburnus* × *Squalius alburnoides* hybrids in low frequencies and the apparent absence of hybridization with *Anaecypris hispanica* should not be considered as immutable facts.

Regarding the highly endangered *Anaecypris hispanica*, it is important to conduct further studies to test the absence of barriers to reproduction with *Alburnus alburnus*. Indeed, it seems that these species are not hybridizing in the wild, at least so far, likely due to the absence of spatial sympatry (they do not coexist yet, after prospections made between 2015 and 2016 in more than 50 sampling sites; Ilhéu et al. unpublished data) and to the extremely low densities of *Anaecypris hispanica* populations (Cardoso et al. 2015). Eggs or larvae were also not found in the tanks where *Alburnus alburnus* and *Anaecypris hispanica* were kept for 3–4 months, over two consecutive reproductive seasons (May through July 2015 and April through July 2016), to study their behaviour in sympatry (da Silva et al. unpublished*). These observations were made during a preliminary study involving three groups of 15 *Alburnus alburnus* and 30 *Anaecypris hispanica* kept in three aerated outdoor tanks supplied with clay tiles for sheltering fishes.

Still, in the case of disruption of the above suggested constraints to sympatry in the wild, our results point to a likely unviability of the hybridization between *Alburnus alburnus* and *Anaecypris hispanica*: although there was evidence of fertilization, the further development of about 400 eggs was not accomplished in all the interspecific cross trials conducted. This result may be explained by one of the following hypotheses: 1) a chromosomal incompatibility between the two species prevents the normal development of the egg after its first mitotic divisions; 2) methodological aspects and the conditions under which fertilization and egg maintenance were made were inadequate; and 3) immature oocytes were used instead of spontaneous laid ones. Thus, the null hypothesis of crosses between *Alburnus alburnus* and *Anaecypris hispanica* do not result in the production of viable offspring may not be fully discarded.

Concerning the hybrids detected in the wild, the developed protocol using a combination of 15 diagnostic nuclear loci and mtDNA sequencing, showed not only that *Alburnus alburnus* is already hybridizing in the wild with a sympatric native species, *Squalius alburnoides*, but also that these hybridization events are bidirectional and give rise to viable hybrid adults with at least 2–3 years old (considering the fork length of the sampled individuals). The participation of females of both species in the interspecific crosses explains the detection of *Alburnus alburnus* and *Squalius pyrenaicus* mtDNA in the hybrid individuals (Fig. 3).

It was also possible to determine that the *Squalius alburnoides* genomotypes which hybridized with *Alburnus alburnus* were PAA females and AA males, the most common genomotypes found in the Guadiana River (Alves et al. 2001a). In both cases, the produced gametes are haploid (by normal meiosis in the case of AA males and by hybridogenetic meiosis in the case of the PAA females, which exclude the P-genome prior to the meiosis; Alves et al. 2001a), thus the resulting hybrids with *Alburnus alburnus* are diploid individuals with a AB genomotype (Fig. 3).

It is worth mentioning that from the 22 individuals captured in the Guadiana and Sado rivers, identified as potential hybrids between *Squalius alburnoides* and *Alburnus alburnus* due to their intermediate morphological features and sequenced for both molecular markers, only six (27.3%) were indeed hybrids between those two species. The number of scales of the lateral line (SLL) of the analysed hybrids (44–50) was intermediate between the SLL of the parental species (38–44 for *Squalius alburnoides* and 47–52 for *Alburnus alburnus*; Doadrio et al. 2011) although some superimposition of ranges was detected. Thus, despite being considered one of the main diagnostic characters of *Alburnus* × *Squalius* hybrids (Almodóvar et al. 2012), the use of SLL, which could be an easily used featured in the field, does not allow for a reliable identification of *Alburnus alburnus* and *Squalius alburnoides* hybrids, at least in the sampled river basins. Our results clearly demonstrate the need to rely on molecular rather than in morphological identifications,

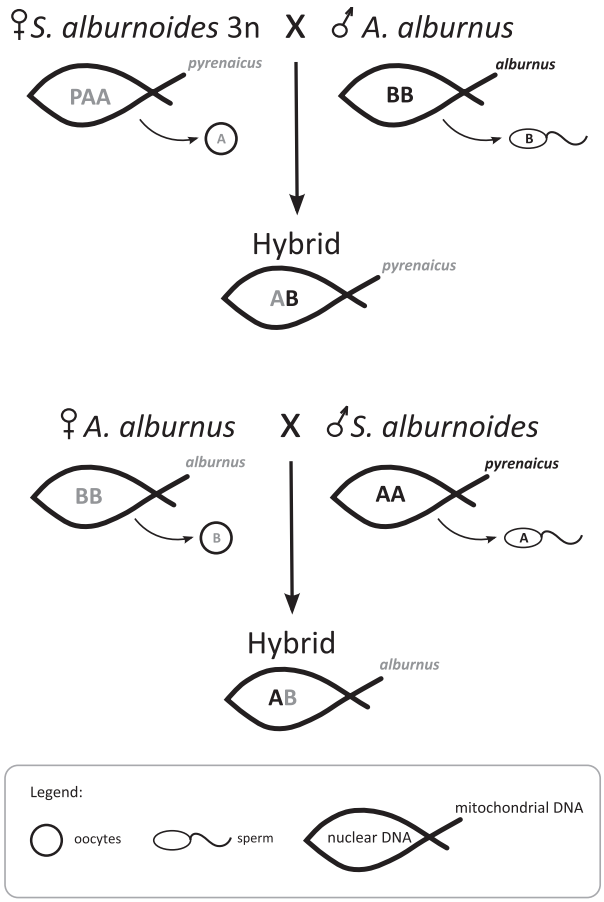

Fig. 3. Schematic representation of the hybridization between *Squalius alburnoides* and *Alburnus alburnus*, described in this study, with the identification of the mitochondrial DNA and nuclear genomotypes of the hybrid individuals sequenced; legend of the genotypic complements: P = *S. pyrenaicus*, A = *S. alburnoides*, B = *A. alburnus*

* da Silva J., Matono P., Barata E.N., Bernardo J.M., Costa A.M., Ilhéu M. Behavioural interactions between the endangered native fish saramugo, *Anaecypris hispanica,* and the invasive bleak, *Alburnus alburnus*.

using at least one mitochondrial and one nuclear marker. This approach was already suggested for the cryptic but frequent intrageneric hybrids of *Luciobarbus* from Guadiana River (Sousa-Santos et al. 2018).

If the proliferation of the bleak progresses towards the last refugia of the critically endangered *Anaecypris hispanica* (now occurring in extremely low densities in very small areas scattered along five Guadiana River tributaries; Sousa-Santos et al. 2014), a scenario of sympatry between *Alburnus alburnus*, *Anaecypris hispanica*, and *Squalius alburnoides* would be probable. Under this hypothetical scenario, hybridization with *Squalius alburnoides* would be expectedly privileged due to the high density of their populations (when compared to the rarefied populations of *Anaecypris hispanica*) and to the more similar body size (*Anaecypris hispanica* rarely reaches a maximum body length of 10 cm, in contrast to the 13 cm of *Squalius alburnoides* and 15 cm of *Alburnus alburnus*; Doadrio et al. 2011). However, if the hybridization between *Alburnus alburnus* and *Anaecypris hispanica* becomes viable in the wild (the absence of reproductive isolation, as mentioned above, could not be fully discarded in the presently reported study), this could likely result in a serious aggravation of the declining trend or even local extinctions of the native species. This expectation in supported by 1) the probable shifts in the environmental conditions leading to a higher confinement of both species in space and time, during the reproductive season, due to the intensification of summer droughts in temporary Mediterranean streams (Lehner et al. 2006, Anonymous 2012, Robson et al. 2013); and 2) the fact that ethological observations of mixed groups in the same experimental tanks, showed that *Anaecypris hispanica* individuals do not react to *Alburnus alburnus* with avoidance behaviours but rather with a higher activity and a wider occupation of the available space (Ilhéu et al. 2016, da Silva et al. unpublished[*]). Thus, in the case of a strong confinement in summer pools, the presence of a high number of *Alburnus alburnus* reproductive males would likely result in a lower probability of *Anaecypris hispanica* females crossing with conspecifics and in the consequent lower recruitment of the native species. If this situation continued for some generations, it could lead to the short-term extinction of *Anaecypris hispanica*. Also, if the hybrid offspring were fertile and ecologically successful, *Anaecypris hispanica* could be displaced or eliminated by the resultant hybrid complex, as reported for other hybridizing fish species (e.g., Konishi and Takata 2004, Rosenfield et al. 2004). This outcome was also predicted to explain the extinction of the paternal ancestor of the hybridogenetic complex *Squalius alburnoides*: the maternal species of the complex (*Squalius pyrenaicus*) likely hybridized massively with the paternal species, generating hybrid individuals with larger body size, with higher fecundity and ecologically more adapted than their paternal ancestors (Robalo et al. 2006, Sousa-Santos et al. 2007). The fundamental difference between this scenario and the one hypothesized for an eventual massive hybridization event between *Alburnus alburnus* and *Anaecypris hispanica* is that the *Squalius alburnoides* complex was most likely originated as a result of a contact between previously allopatric populations due to rearrangements of Iberian paleobasins (Sousa-Santos et al. 2007) and not due to an anthropogenic-mediated event.

In conclusion, all the above expectations require deeper investigations which fall beyond the scope of the present paper. However, the conducted study raised awareness about the impact that the proliferation of an invasive species may impose to the conservation of phylogenetically related native species, by threatening their taxonomical integrity which may, ultimately, lead to its displacement or extinction by the resultant hybrids—a process which may be achieved in a small timescale (Epifanio and Philipp 2000). A higher risk of hybridization with invasive species is potentially linked with climate change, as recently reported by Muhlfed et al. (2014). These authors showed that decreases in spring precipitation and increases in summer stream temperature over a 30-year period had promoted the upstream expansion of the non-native rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), and the consequent increase of hybridization with the endangered native westslope cutthroat trout, *Oncorhynchus clarkii clarkii* (Richardson, 1836), in North America, highlighting the fact that climate warming may indeed exacerbate hybridization between native and non-native species, which can result in the extinction of many species. Thus, in order to effectively contribute to the conservation of native fish species in temporary river systems invaded by exotic species, it is crucial to invest in standardized and continued ecological and genetic monitoring which can produce reliable data regarding the identification of cryptic hybrids and the detection of eventual shifts in environmental conditions and life history traits that promote hybridization events. In parallel, there is an obvious need to implement management measures that result in an effective mitigation of invasive species proliferation or, ideally, in its complete eradication and to invest in the rehabilitation of summer refugia for native fish, contributing to the preservation of the Mediterranean as one of the main hotspots for global biodiversity.

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[*] See footnote on page 118.

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