

Natural hybridization with invasive bleak *Alburnus alburnus* threatens the survival of Iberian endemic calandino *Squalius alburnoides* complex and Southern Iberian chub *Squalius pyrenaicus*

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Abstract Hybrids of invasive bleak *Alburnus alburnus* and native endemic calandino *Squalius alburnoides* complex and Southern Iberian chub *Squalius pyrenaicus* were found in the River Jarama (Tagus River basin, central Spain). Morphological analyses show that hybrids are intermediate in shape between the parent species. Molecular mitochondrial and nuclear analyses evidence that hybridization was different in terms of the direction of gene introgression, with hybrids ♀ *A. alburnus* × ♂ *S. pyrenaicus* and ♀ *S. alburnoides* complex × ♂ *A. alburnus*. As far as we know, this is the first evidence of interspecific hybridization between a fish hybrid complex and an invasive exotic fish. The hybridization occurred at least during 3 years in a short time period after the arrival of exotic bleak. The spread of bleak in the Iberian Peninsula can be a serious threat for the survival of endemic *Squalius* species, as well as other

close Leuciscinae such as the endangered jarabugo *Anaocypris hispanica*.

Keywords Fish hybrids · Hybrid complex · Iberian cyprinids · Introgression

Introduction

There is growing appreciation that hybridization can facilitate invasions or lead to the evolution of invasiveness. Hence, many empirical examples of contemporary evolution take place from artificial propagation and introgression among populations or species, which eventually may lead to the loss of native genetic diversity and locally adapted populations (Rhymer and Simberloff 1996). New invasive hybrid lineages can outcompete with native parentals through vigorous hybrids, thus enhancing invasion success (Hänfling 2007). Further, novel adaptations in hybrids could be favoured by habitat disturbance and changes in ecological conditions due to human pressure.

The introduction of closely related fish species, together with habitat disturbance, has led to increased incidence of interspecific hybridization and the establishment of hybrid zones where the likelihood for interbreeding between native and exotic species is seriously augmented (Costedoat et al. 2005). Scribner et al. (2001) found that introductions and habitat loss were responsible for nearly 50 % of hybridization events in freshwater fishes.

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Extensive hybridization and introgression commonly occurs in fishes, more than in other vertebrates of comparable levels of genetic divergence (Epifanio and Nielsen 2001). Scribner et al. (2001) reviewed the hybridization events in freshwater fishes and found that cyprinid species accounted for 40 % of all hybrids represented, more than any other family (50 species and 37 species pairs). Specifically, the bleak *Alburnus alburnus* is one of the cyprinid species with the highest number of hybridization events, which have already been reported with species of genera *Abramis*, *Blicca*, *Leuciscus*, *Rutilus* and *Squalius* (Scribner et al. 2001).

Iberian native freshwater fish have suffered multiple and recurrent introductions during the last century, so that acclimatization of exotic fish is probably the main negative factor affecting the survival of native, mostly endemic, species (Elvira and Almodóvar 2001; Ribeiro et al. 2008). The bleak is a small cyprinid with a wide distribution in Europe, from the eastern slopes of the Pyrenees to the Urals. In the Iberian Peninsula it was collected for the first time in the Ebro basin in 1992. Since then, it has quickly spread to almost all the Iberian basins (Vinyoles et al. 2007). The danger of introgression of allochthonous genes of the bleak into the Iberian endemic Leuciscinae was predicted by some of us (Vinyoles et al. 2007); in particular, with its very close relative jarabugo *Anaocypris hispanica* (Perea et al. 2010) and with the calandino *S. alburnoides* complex, derived in part from an *Anaocypris*-like ancestor (Cunha et al. 2011). Both species are currently threatened; *S. alburnoides* complex is considered Vulnerable (A3ce) and *A. hispanica* Endangered (A2ace) by the IUCN.

In this work, we describe a case of recent natural hybridization between invasive bleak and endemic *Squalius* species following a short period of contact. This study deals with the morphological and genetic characterization of the hybrids and its distinction and similarities with the parental species.

Materials and methods

Hybrids and reference samples of *A. alburnus*, Southern Iberian chub *Squalius pyrenaicus* and *S. alburnoides* complex were collected in the River Jarama (a tributary to the River Tagus, central Spain) during annual qualitative samplings between summer 2007 and autumn 2010, and consequently abundance of

hybrids or parents could not be accurately determined. The native fish assemblage consisted of five endemic species: Iberian barbel *Barbus bocagei* (common), Iberian straight-mouth nase *Pseudochondrostoma polylepis* (scarce), *S. alburnoides* complex (scarce), *S. pyrenaicus* (scarce) and Southern Iberian spined-loach *Cobitis paludica* (scarce). Likewise, six exotic species were established in the area: *A. alburnus* (common), Pyrenean gudgeon *Gobio lozanoi* (common), goldfish *Carassius auratus* (scarce), black bullhead *Ameiurus melas* (rather common), Eastern mosquitofish *Gambusia holbrooki* (rather common) and pumpkinseed *Lepomis gibbosus* (common).

Fish were sampled by electrofishing using a 2,200 W DC generator. The examined fish were killed by lethal dose of anaesthetic (MS-222) in accordance to the recommended ethical guidelines and immediately frozen on dry ice. Fish were stored at -80°C until DNA extraction, fixed in 70 % ethanol and deposited at the Museum of Comparative Morphology and Anatomy of Vertebrates, Complutense University of Madrid. The analysed materials include 6 specimens of *A. alburnus*, 4 specimens of *S. alburnoides* complex, 4 specimens of *S. pyrenaicus*, and the only 3 specimens suspected to be hybrids, collected on 25 October 2007 (specimens H1 and H2) and 25 November 2010 (specimen H3).

Fishes were measured (standard length, SL) to the nearest millimetre and sexed by visual inspection of gonads. Seven meristic characters were counted: lateral line scales (LLS), transverse scale count (TSC), branched dorsal fin rays (BDFr), branched ventral fin rays (BVFr), branched anal fin rays (BAFr), pharyngeal teeth (PhT) and gill rakers (GR). Procedures follow Buj et al. (2010). Furthermore, the eventual development of a ventral keel between the pelvic fins and the anus was observed. Age was determined by scale inspection.

Total DNA was extracted from muscle tissue using DNeasy Tissue Kit (QIAGEN, IZASA, Spain) and then stored at -20°C . The quality and concentration of DNA was determined by spectrophotometry and was verified by 0.8 % agarose gel electrophoresis. In order to identify the parental species mitochondrial and nuclear genes were amplified and sequenced: (1) two regions of mtDNA, complete cytochrome b gene (cyt b) and partial 16S rRNA gene; (2) two regions of nDNA, complete internal transcribed spacer 1 of rRNA gene (ITS1) and partial beta-actin gene. The

Table 1 PCR primers used in the present study to amplify and sequence the cyt b gene, partial 16S rRNA gene, ITS1 region and partial beta-actin gene

Region	Primer	Sequence (5'-3')	Size (bp)	Annealing temperature (°C)	References
Cyt b	Glu-F	GAAGAACCACCGTTGTTATTCAA	1,141	45	Zardoya and Doadrio (1998)
	Thr-R	ACCTCCRATCTYCGGATTACA			
16S	16Sar-L	CGCCTGTTTATCAAAAACAT	595–622	52	Palumbi (1996)
	16Sbr-H	CCGGTCTGAACTCAGATCACGT			
ITS1	kp2	AAAAAGCTTCCGTAGGTGAACCTGCG	336–397	60	Phillips et al. (1995)
	5.8S	AGCTTGCTGCGTTCTTCATCGA			
Beta-actin	For	ATGGATGATGAAATTGCCGC	898–936	55	Sousa-Santos et al. (2005)
	Rev	AGGATCTTCATGAGGTAGTC			

amplified regions and primers sequences are shown in Table 1. For each fragment, polymerase chain reaction (PCR) was performed in a final volume of 50 µl containing 1.5–2.5 mM MgCl₂, 1× Mg-free PCR buffer, 200 µM of dNTPs, 0.4 µM of each primer, 1 U of Biotools HotSplit DNA polymerase (Biotools, Spain) and 100–200 ng genomic DNA template. The amplifications were performed using the following conditions depending on the primers and/or the samples: 95 °C 5 min, 30–35 cycles of 95 °C 30–60 s, 45 °C up to 60 °C (T_a, Table 1) 30–60 s and 72 °C 1 min, with a final extension at 72 °C for 5–10 min. PCR products were resolved by 1–1.5 % agarose gel electrophoresis, visualized by ethidium bromide fluorescence and purified using the Speedtools PCR Clean-Up kit (Biotools, Spain). For each amplified region, sequencing was performed twice in both directions on an ABI PRISM 3730 sequencer (Applied Biosystems). Sequences were then compared with known species sequences in GenBank using the BLAST search algorithm and aligned with CLUSTALX v2.1 (Larkin et al. 2007). Sequences were deposited in the GenBank database under the accession numbers JQ436540–JQ436555.

Results and discussion

Ages of the only three found hybrids were 1+ (H2 and H3) and 2+ (H1), so they belonged to the cohorts of 2005, 2006 and 2009. Then, we infer that hybridization can be considered to be scarce in number but rather regular in time. Both sexes were found in the hybrids, female (H1) and male (H2 and H3).

Meristic characters show that hybrids are intermediate in shape between parental species, bleak and both *Squalius* (Table 2). The most clear intermediate characters of hybrids are those related to numbers of scales along the LLS, BAFr and GR. However, the small number of hybrids prevented the development of statistical analyses. The hybrids do not bear any ventral keel between the pelvic fins and the anus, and only bleak specimens have this character.

Mitochondrial and nuclear molecular analyses were conducted to establish the pattern of introgression of bleak with the genus *Squalius*. The *S. alburnoides* complex exhibits distinctive reproductive modes (for example, hybridogenesis, meiotic hybridogenesis, production of unreduced gametes, gynogenesis) depending on the sex, genome constitution and ploidy (reviewed in Alves et al. 2001). In the Tagus basin, the mtDNA of specimens from *S. alburnoides* complex is *S. pyrenaicus*-like, showing that this species was the maternal ancestor of the complex (Alves et al. 2001; Sousa-Santos et al. 2007). Likewise, the southern Iberian populations of *S. alburnoides* complex resulted from a hybridization process between *S. pyrenaicus* (the P genome) and an extinct species closely related to *A. hispanica* (the A genome) (Alves et al. 2001; Sousa-Santos et al. 2007). The absence of recombination between A and P genomes means that by the combined use of mitochondrial and nuclear markers is possible to infer the parentage of each form of the complex.

In our case, mitochondrial DNA analyses (cyt b and 16S) indicated that hybrids H1 and H2 were of bleak maternal origin (haplotype Alb1) whereas the hybrid H3 presented a *S. pyrenaicus*-like mitochondrial genome (Sq5) (Table 3). Both mtDNA haplotypes

Table 2 Size (SL in mm) and meristic characters of hybrids and comparative material

Specimen	SL	LLS	TSC	BDFr	BVFr	BAFr	PhT	GR
H1	120	45	8–1–2½	8½	8	12½	5–2/2–5	14
H2	94	45	8–1–2	7½	8	10½	5–1/1–5	15
H3	73	46	8½–1–2	7½	8	11	5–0/0–4	15
B1	125	47	8–1–3	8½	8	18½	4–1/2–5	23
B2	120	49	9–1–3	8½	8	19½	5–1/2–4	20
B3	111	49	9–1–2½	8½	8	18½	5–1/2–5	22
B4	98	48	8–1–3	8½	8	19½	5–2/2–5	20
B5	97	49	9–1–3	8½	8	16½	5–2/2–5	17
B6	111	49	8–1–3	8½	8	17½	5–2/2–5	18
SIc1	127	42	8–1–3	8½	8	8½	5–2/2–5	11
SIc2	105	42	7½–1–3½	8	8	8½	5–2/2–5	8
SIc3	127	41	8–1–3	8½	8	8½	5–2/2–5	11
SIc4	103	41	7½–1–2½	8½	8	8½	5–2/2–5	9
C1	79	40	7–1–3	6½	7	7½	5–1/0–4	12
C2	82	43	8–1–3	6½	7	8½	5–1/1–5	14
C3	77	41	7–1–2½	7	6	8½	5–2/1–5	12
C4	75	41	8–1–2	7½	7	8½	5–1/1–5	13

Hybrid (H1–H3), bleak *Alburnus alburnus* (B1–B6), Southern Iberian chub *Squalius pyrenaicus* (SIc1–SIc4) and calandino *Squalius alburnoides* complex (C1–C4). For abbreviations see “[Materials and methods](#)”

and ITS1 sequences showed the same results, suggesting that ITS1 regions were of maternal type. Similarly, the paternal ITS1 region of rDNA was eliminated in the first generation of interspecific hybridization between the Leuciscinae bream *Abramis brama* and roach *Rutilus rutilus* (Slynko and Stolbu-nova 2010).

With regard to beta-actin gene, our results showed that all hybrids sequences had a double peak generated by two different parental sequences, bleak and *Squalius*. However, the hybrids were remarkably different in terms of the direction of gene introgression. Hence, the identification of the A genome in the hybrid H3 (A4) made possible to assign its maternal origin to the *S. alburnoides* complex while the other sequence (Alb2) proved the paternal origin of bleak (Table 3). On the other hand, hybrids H1 and H2 showed a P genome for beta-actin sequence (P11) which could be likely attributed to a *S. pyrenaicus* paternal origin. That is due to only haploid gametes with A genome from *S. alburnoides* complex have been observed in both wild populations and breeding experiments (Alves et al. 2001), and consequently the P genome of hybrids H1 and H2 ought to come from *S. pyrenaicus* sperm. The other sequence (Alb1) confirmed the bleak maternal origin (Table 3).

As far as we know, this is the first evidence of interspecific hybridization between a fish hybrid complex and an invasive exotic fish. This is also the first case of intergeneric hybridization between endemic and exotic fish species in Iberian rivers. Previous studies have only documented the introgression of exotic genes into native brown trout populations (e.g. Almodóvar et al. 2006). Further, hybridization took place in a short time scale, because bleak occurrence was first documented in the Tagus River basin in 2004.

The observed hybridization event is highly problematic for other Iberian endemic Leuciscinae species that are currently in contact with bleak. The Iberian Peninsula holds a high number of endemic cyprinids and hybridization contributes to some extent to the observed patterns of genetic diversity in several genera, such as *Achondrostoma* and *Pseudochondrostoma* (e.g. Elvira et al. 1990), *Barbus* (e.g. Almodóvar et al. 2008) and *Squalius* (e.g. Alves et al. 2001). The destruction of reproductive habitat by human activities probably makes easier the simultaneous spawning of endemic and exotic related cyprinids.

The abundant introduced bleak can produce irreversible genetic swamping of scarce endemic species. Hybrids are not usually as ecologically fit as parental

Table 3 Identification of species or hybrids based on genetic analysis: hybrid (H), bleak *Alburnus alburnus* (B, *Aa*), Southern Iberian chub *Squalius pyrenaicus* (Sic, *Sp*) and calandino *Squalius alburnoides* complex (C, *Sac*)

Specimen	mtDNA		nDNA		Identification
	Cyt b	16S	ITS1	Beta-actin	
H1	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	H (Alb1 × P11)	♀ <i>A. alburnus</i> × ♂ <i>S. pyrenaicus</i>
H2	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	H (Alb1 × P11)	♀ <i>A. alburnus</i> × ♂ <i>S. pyrenaicus</i>
H3	<i>Sp</i> (Sq5)	<i>Sp</i> (Sq5)	<i>Sp</i> (Sq3)	H (A4 × Alb2)	♀ <i>S. alburnoides</i> complex × ♂ <i>A. alburnus</i>
B1	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	–	<i>A. alburnus</i>
B2	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	–	<i>A. alburnus</i>
B3	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	–	<i>A. alburnus</i>
B4	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1 × Alb2)	<i>A. alburnus</i>
B5	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1 × Alb2)	<i>A. alburnus</i>
B6	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1)	<i>Aa</i> (Alb1 × Alb2)	<i>A. alburnus</i>
Sic1	<i>Sp</i> (Sq2)	<i>Sp</i> (Sq2)	–	–	<i>S. pyrenaicus</i>
Sic2	<i>Sp</i> (Sq1)	<i>Sp</i> (Sq1)	<i>Sp</i> (Sq2)	<i>Sp</i> (P1 × P11)	<i>S. pyrenaicus</i>
Sic3	<i>Sp</i> (Sq1)	<i>Sp</i> (Sq1)	<i>Sp</i> (Sq2)	<i>Sp</i> (P1 × P11)	<i>S. pyrenaicus</i>
Sic4	<i>Sp</i> (Sq1)	<i>Sp</i> (Sq1)	<i>Sp</i> (Sq3)	<i>Sp</i> (P1 × P11)	<i>S. pyrenaicus</i>
C1	<i>Sp</i> (Sq3)	<i>Sp</i> (Sq3)	<i>Sp</i> (Sq1)	<i>Sac</i> (P1 × P11 × A4)	<i>S. alburnoides</i> complex
C2	<i>Sp</i> (Sq1)	<i>Sp</i> (Sq1)	<i>Sp</i> (Sq2)	<i>Sac</i> (P11 × A4)	<i>S. alburnoides</i> complex
C3	<i>Sp</i> (Sq1)	<i>Sp</i> (Sq1)	<i>Sp</i> (Sq3)	<i>Sac</i> (P2 × P2 × A4)	<i>S. alburnoides</i> complex
C4	<i>Sp</i> (Sq4)	<i>Sp</i> (Sq4)	<i>Sp</i> (Sq1)	<i>Sac</i> (P2 × P1 × A4)	<i>S. alburnoides</i> complex

The mitochondrial (mtDNA) and nuclear (nDNA) haplotypes are indicated between brackets. All new sequences have been deposited in GenBank (accession numbers JQ436540–JQ436555). Concerning the beta-actin gene, reconstruction of the parental sequences, information on the ploidy and genome constitution, were performed as reported in Sousa-Santos et al. (2005). The beta-actin haplotypes derived from the *S. alburnoides* complex were assigned to the haplotypes described in Sousa-Santos et al. (2007)

taxa; however, there is increasing evidence which supports the theory that hybridization can lead to adaptation through the establishment of novel genotypes and morphologies. Further, hybrids with phenotypic traits outside the phenotypic range of parental species may exploit new ecological niches by transgressive segregation (Rieseberg et al. 1999). It is clear that the long-term conservation of *Squalius* species and other endemics closely related to bleak (e.g. *A. hispanica*) requires to reliably identify the parental hybrid taxa and to know the current extent and distribution of gene flow between endemic species and bleak.

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