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BRIEF COMMUNICATION

Evidence of extensive mitochondrial introgression with nearly complete substitution of the typical *Squalius pyrenaicus*-like mtDNA of the *Squalius alburnoides* complex (Cyprinidae) in an independent Iberian drainage

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The first occurrence of massive mitochondrial introgression of *Squalius aradensis* genes in *Squalius alburnoides*, a hybridogenetic complex that usually carries mtDNA of its maternal ancestor (*Squalius pyrenaicus*) is reported. Possible implications of such introgressions for the history of the complex are discussed.

Key words: beta-actin; cytochrome *b*; hybridogenetic complex; mitochondrial introgression; *Squalius alburnoides*; *Squalius aradensis*.

Although still lacking a formal generic recognition, the cyprinid species of *Squalius*, previously included in the genus *Leuciscus*, form a very well defined monophyletic clade only distantly related to *Leuciscus leuciscus* (*L.*) (Briolay *et al.*, 1998), within which the Iberian species form a strongly supported monophyletic group (Zardoya & Doadrio, 1999; Sanjur *et al.*, 2003). The species of *Squalius* have attracted considerable attention from researchers studying fish hybridization since the group provided several instances of formation of hybridogenetic lineages [*e.g.* *Squalius alburnoides* (Steindachner) (Alves *et al.*, 1997*a*) and possibly *Squalius palaciosi* (Doadrio) (Zardoya & Doadrio, 1998)], extensive interspecific introgression (Durand *et al.*, 2000) and intergeneric hybridization (Freyhof *et al.*, 2005; Ünver & Erk'akan, 2005; Robalo *et al.*, 2006). Since its first description in the 1980s (Collares-Pereira, 1984, 1985), great advances have been made to understand the

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origin and maintenance of the *S. alburnoides* hybridogenetic complex, which comprises diploid (2n = 50), triploid (3n = 75) and tetraploid (4n = 100) hybrid forms (Alves *et al.*, 2001). This complex of widely distributed minnows seems to have resulted from unidirectional interspecific crosses between *Squalius pyrenaicus* (Günther) females (P genome) and males from an unknown (and possibly extinct) species, A genome (Alves *et al.*, 2001). The peculiar mechanisms of gamete formation in the complex also lead to the reconstitution of diploid 'nuclear nonhybrids' exhibiting *S. pyrenaicus*-like mtDNA and the nuclear genome of the missing paternal ancestor (Alves *et al.*, 2002). This non-hybrid form, constituted almost entirely by males (females are inexistent or extremely rare), has only been found in Tagus, Sado and Guadiana drainages (Cunha *et al.*, 2004; Pala & Coelho, 2005), numbered 3, 4 and 5, respectively, in Fig. 1. In the drainages to the north of the Tagus River, *S. pyrenaicus* is absent but *S. alburnoides* is sympatric with another *Squalius* species, *Squalius carolitertii* (Doadrio) (Fig. 1). In such river basins, the mtDNA of *S. alburnoides* is *S. pyrenaicus*-like (Alves *et al.*, 1997*b*, 2002, Cunha *et al.*, 2004, Pala & Coelho, 2005), with only one exception reported by Alves *et al.* (1997*b*): a specimen with *S. carolitertii*-like mtDNA. As *S. alburnoides* males are much less common than females, it has been assumed that the maintenance of the complex depends mainly on unidirectional crosses between *S. alburnoides* females and *S. pyrenaicus* and *S. carolitertii* males (Alves *et al.*, 2001; Cunha *et al.*, 2004; Pala & Coelho, 2005). The almost exclusive presence of *S. pyrenaicus* mtDNA in *S. alburnoide*s has been interpreted as indicating that crosses between *S. alburnoides* males and females of other species are absent or extremely uncommon, except at the origin of the complex (Alves *et al.*, 2001). Based on the pattern of variation of the

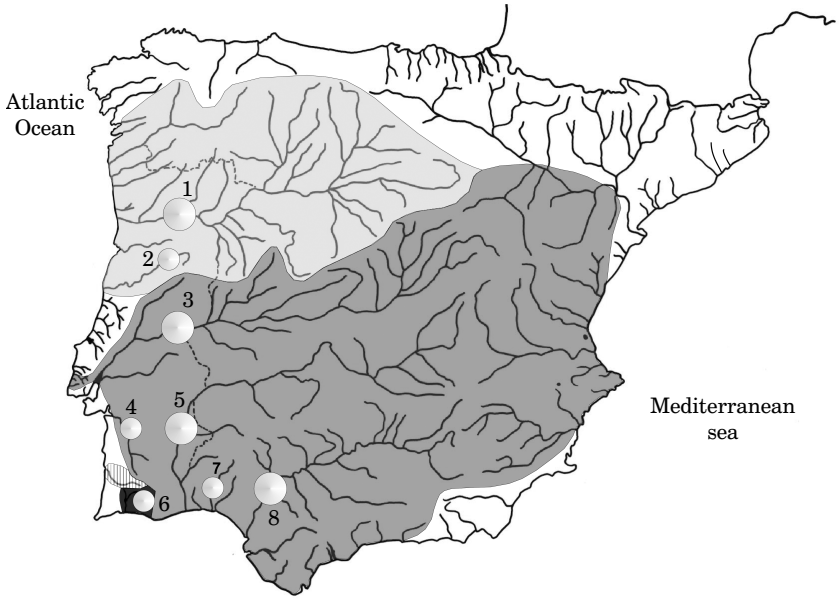


FIG. 1. Distribution areas of the Iberian endemic minnows *Squalius carolitertii* (□), *Squalius pyrenaicus* (▩), *Squalius torgalensis* (▥) and *Squalius aradensis* (■). The numbered circles represent the already analysed river basins for *Squalius alburnoides*: 1, Douro; 2, Mondego; 3, Tagus; 4, Sado; 5, Guadiana; 6, Quarteira; 7, Odiel; 8, Guadalquivir. River basins numbered 9 and 10 are, respectively, Arade and Mira.

cyt*b* gene, Alves *et al.* (1997*b*) postulated that the complex had at least two distinct hybridogenetic origins, one in the Sado drainage and the other in the Guadiana and Tagus drainages, and that it had dispersed from the Tagus into the northern drainages. More recently Cunha *et al.* (2004), using a broader data set, postulated five independent hybridization origins based on the fact that the mtDNA of *S. alburnoides* had a stronger affinity with that of *S. pyrenaicus* from the same river than with conspecifics from other drainages. In Quarteira, a small independent drainage in south-west Portugal, *S. alburnoides* is sympatric with another *Squalius* species, *Squalius aradensis* (Coelho, Bogutskaya, Rodrigues & Collares-Pereira), an endemic fish with a very restricted range (Fig. 1). Apart from the Quarteira drainage, it only occurs in a few distinct independent drainages, mainly in that of the Arade River (Fig. 1) which was recently considered as the evolutionary centre of origin of the species (Mesquita *et al.*, 2005). The Quarteira drainage represents the western limit of the geographical distribution of *S. alburnoides* and the eastern limit of *S. aradensis* (Mesquita & Coelho, 2002).

Live *S. alburnoides* individuals are easily distinguished in the field by the presence of a black line surrounding the base of the dorsal fin, which is absent in all other Iberian *Squalius* species (C. Sousa-Santos unpubl. obs.). This distinction was confirmed in *S. alburnoides* from all the drainages where it occurs, after comparing hundreds of fish with specimens of all other *Squalius* species of Portuguese fresh waters. In addition, *S. alburnoides* fish show a conspicuous longitudinal dark band above the lateral line (Collares-Pereira, 1984) that is absent in the remaining Iberian *Squalius*.

A small fin clip was taken from *S. alburnoides* and *S. aradensis* from Quarteira and the fish were safely returned to the water. Previous experience with all the cyprinids that underwent this procedure and were temporally kept in aquaria for other purposes demonstrated that the procedure described above does cause neither mortality nor diseases. Total genomic DNA was extracted from fin clips preserved in ethanol by a SDS/proteinase-k based protocol, precipitated with isopropanol and washed with ethanol before re-suspension in water (adapted from Sambrook *et al.*, 1989). The amplification process was conducted as follows: 35 cycles of 94° C (30 s), 55° C (40 s) and 72° C (90 s). Each sample was sequenced in both directions with the same primers used for PCR. Sequences were aligned with BioEdit® v.5.0.6. A total of 1123 bp of the cyt*b* gene of samples of 20 *S. alburnoides* (GenBank: DQ003238-42, DQ003246, DQ003253-55, DQ003259, DQ14179-88) and of 19 *S. aradensis* (GenBank: DQ003243-45, DQ003247-52, DQ003256-58, DQ14189-94, DQ145177) from Quarteira were amplified using the primers *LCB1* (Brito *et al.*, 1997) and *HA* (Schmidt & Gold, 1993). For comparisons of the cyt*b* gene with that of other *Squalius* species samples available in Genbank: *S. pyrenaicus* from Sado (Y10133), from Guadiana (Y10134, AF421822-23, AF421813-14, AF421804-05, AF045991), from Guadalquivir (AF421816-17, AF421790) and from Tagus (Y10131-132, AF421811-12, AF421826-27, AF421791, AF045993) were used. Additional samples of *S. aradensis* from Arade (AF421824-25) and *Squalius torgalensis* (Coelho, Bogutskaya, Rodrigues & Collares-Pereira) (Z75929) from Mira were also included (*S. alburnoides* is absent from both these drainages). A segment of 927 bp of the nuclear beta-actin gene was also amplified from samples of 20 *S. aradensis* (GenBank: DQ150307-22 and DQ150260-71) and 15 *S. alburnoides*

(GenBank: DQ150335-61, DQ128102, AY943867 and AY943865) from Quarteira (primers are given in Sousa-Santos *et al*., 2005). This fragment is homologous to a region of the beta-actin gene of *Cyprinus carpio* L. (GenBank: M24113) between the positions 1622 and 2550, including introns B and C and three exons. The methods used for recovering the parental sequences of nuclear genes of hybrids are described elsewhere (Sousa-Santos *et al*., 2005). As there were no sequences of the beta-actin gene available in GenBank, DNA was sequenced also from samples of 20 *S. pyrenaicus* (GenBank: AY943877-79, DQ150272-83 and DQ150323-332), four nuclear non-hybrid *S. alburnoides* from Guadiana (GenBank: AY943892-93, AY943864, DQ010337), four nuclear non-hybrid *S. alburnoides* from Tagus (GenBank: AY943863, AY943894-96), eight *S. aradensis* from Arade (DQ150291-306) and eight *S. torgalensis* from Mira (GenBank: DQ150284-290 and DQ150333-34) (Fig. 1). Note that there are more accession numbers than individuals since some fishes are heterozygous for the analysed fragment of the beta-actin gene and each strand has its own accession number. For a description of a procedure that allows the identification of the two different sequences involved in heterozygotes and hybrids when analysing nuclear gene sequences see Bhangale *et al*. (2005).

The sequencing of beta-actin gene yielded three groups of haplotypes that showed characteristic patterns of mutations and indels that were sufficiently distinct to be identifiable when hybrid genomes were analysed. Based on these characteristic patterns, each individual fish could be classified as showing A, Q or P genome or other possible combinations: Q + A, P + A or P + Q (this notation does not denote the ploidy of the fish but merely what types of genomes were present) (Table I). 'Q haplotypes' were found in the fish morphologically diagnosed as *S. aradensis* from Quarteira, in the specimens of *S. aradensis* from Arade and in the specimens of *S. torgalensis* from Mira; 'P haplotypes' were observed in the samples of *S. pyrenaicus* from Guadiana and in one *S. alburnoides* individual from Quarteira; 'A haplotypes' were found in homozygosity in specimens morphologically diagnosed as *S. alburnoides* non-hybrids (five from Quarteira, four from Tagus and four from Guadiana). All other fishes from Quarteira morphologically diagnosed as *S. alburnoides* hybrids showed hybrid genomes with a combination of A and P and Q haplotypes.

The mean ± S.D. percentage of divergence between all six Q haplotypes found in fishes from Quarteira ($N = 28$), Arade ($N = 8$) and Mira ($N = 8$) was $0{\cdot}21 \pm 0{\cdot}08$. Of these six Q haplotypes, three were identified in fishes from Quarteira, one being specific to that drainage and the other two being shared with Arade. The average percentage of divergence between the seven distinct A haplotypes that were found in fishes from Quarteira ($N = 15$), Guadiana ($N = 4$) and Tejo ($N = 4$) was $0{\cdot}26 \pm 0{\cdot}13$. *Squalius alburnoides* from Quarteira exhibited four of these six A haplotypes (two of them being specific to that drainage, one shared with Guadiana and the other shared with Guadiana and Tejo). Nine different P haplotypes were recovered from fishes from Quarteira ($N = 1$) and Guadiana ($N = 20$), with an average percentage of divergence of $0{\cdot}35 \pm 0{\cdot}16$ between them. One triploid *S. alburnoides* from Quarteira showed two distinct P haplotypes, one of them exclusive of that river and the other shared with Guadiana. The average percentage of divergence

TABLE I. Morphological identification, mitochondrial and nuclear haplotypes of the *Squalius* specimens analysed from River Quarteira

Morphological identification	Mitochondrial haplotype	Nuclear haplotype
Squalius alburnoides hybrid 1	*S. aradensis*	Q + A
S. alburnoides hybrid 2	*S. aradensis*	Q + A
S. alburnoides hybrid 3	*S. aradensis*	(not sequenced)
S. alburnoides hybrid 4	*S. aradensis*	(not sequenced)
S. alburnoides hybrid 5	*S. aradensis*	(not sequenced)
S. alburnoides hybrid 6	*S. aradensis*	(not sequenced)
S. alburnoides hybrid 7	*S. aradensis*	Q + A
S. alburnoides hybrid 8	*S. aradensis*	P + A
S. alburnoides hybrid 9	*Squalius pyrenaicus*	Q + A
S. alburnoides hybrid 10	*S. aradensis*	(not sequenced)
S. alburnoides hybrid 11	*S. aradensis*	Q + A
S. alburnoides hybrid 12	*S. aradensis*	Q + A
S. alburnoides hybrid 13	*S. aradensis*	Q + A
S. alburnoides hybrid 14	*S. aradensis*	Q + A
S. alburnoides hybrid 15	*S. aradensis*	Q + A
S. alburnoides non-hybrid 16	*S. aradensis*	A
S. alburnoides non-hybrid 17	*S. aradensis*	A
S. alburnoides non-hybrid 18	*S. aradensis*	A
S. alburnoides non-hybrid 19	*S. aradensis*	A
S. alburnoides non-hybrid 20	*S. aradensis*	A
Squalius aradensis 1	*S. aradensis*	Q
S. aradensis 2	*S. aradensis*	Q
S. aradensis 3	*S. aradensis*	Q
S. aradensis 4	*S. aradensis*	Q
S. aradensis 5	*S. aradensis*	Q
S. aradensis 6	(not sequenced)	Q
S. aradensis 7	*S. aradensis*	Q
S. aradensis 8	*S. aradensis*	Q
S. aradensis 9	*S. aradensis*	Q
S. aradensis 10	*S. aradensis*	Q
S. aradensis 11	*S. aradensis*	Q
S. aradensis 12	*S. aradensis*	Q
S. aradensis 13	*S. aradensis*	Q
S. aradensis 14	*S. aradensis*	P + Q
S. aradensis 15	*S. aradensis*	Q
S. aradensis 16	*S. aradensis*	Q
S. aradensis 17	*S. aradensis*	Q
S. aradensis 18	*S. aradensis*	Q
S. aradensis 19	*S. aradensis*	Q
S. aradensis 20	*S. aradensis*	Q

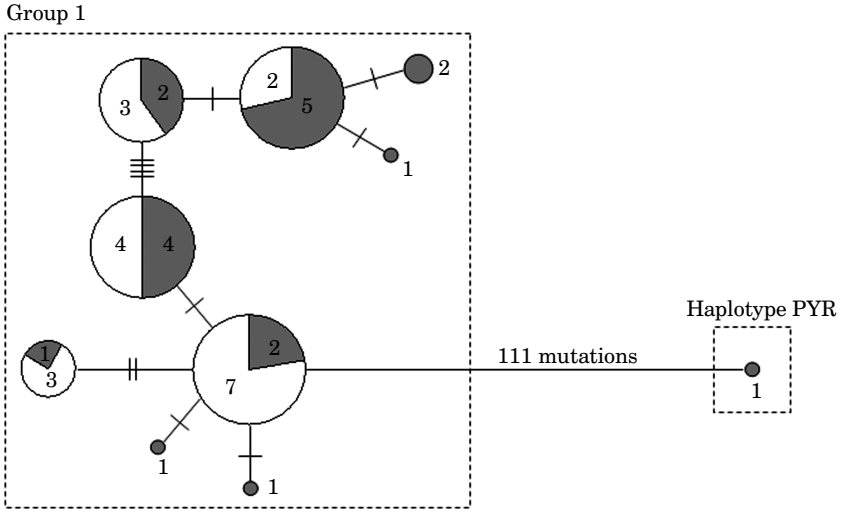
between the three types of nuclear haplotypes was: $0{\cdot}73 \pm 0{\cdot}16$ between P and Q, $2{\cdot}81 \pm 0{\cdot}13$ between Q and A and $3{\cdot}03 \pm 0{\cdot}77$ between P and A.

Inspection of Table I clearly shows that all fish morphologically diagnosed as *S. alburnoides* exhibited either pure A or hybrid beta-actin genomes. All fishes

morphologically diagnosed as *S. aradensis* showed Q genomes, except one with a P + Q genome, indicative of a history of hybridization between *S. aradensis* and *S. pyrenaicus*. The *S. aradensis* from Arade showed Q genomes that helped to confirm the identification of *S. aradensis* from Quarteira. Thus, the morphological distinction between *S. alburnoides* and *S. aradensis* was fully supported by the beta-actin data. Of the 10 hybrid *S. alburnoides*, nine showed Q + A combinations indicating that they had a history of hybridization between *S. alburnoides* and *S. aradensis*, while one showed a P + A constitution indicative of the incorporation of a *S. pyrenaicus* haplotype in its ancestry. These results clearly showed that in the Quarteira drainage the DNA of *S. aradensis* introgressed massively into *S. alburnoides*.

The sequencing of the cyt*b* gene of *S. alburnoides* (both hybrids and non-hybrids) and of *S. aradensis* from Quarteira yielded nine *S. aradensis*-like mitochondrial haplotypes distinguished by one to seven mutations (group I), and one *S. pyrenaicus* mtDNA haplotype found in one *S. alburnoides* which differed from the others by 111–116 mutational steps (haplotype PYR; see Fig. 2) (haplotype

(a)



(b)

		Group I ($N = 38$)	Haplotype PYR ($N = 1$)
Squalius pyrenaicus	$N = 20$	10·16 ± 0·27	1·40 ± 0·59
Squalius aradensis	$N = 2$	0·91 ± 0·14	9·93 ± 0·06
Squalius torgalensis	$N = 1$	6·25 ± 0·12	10·51

FIG. 2. (a) Haplotype network of *Squalius alburnoides* and *Squalius aradensis* ($N = 39$) from the River Quarteira. Each haplotype is represented by a circle, proportional to the number of individuals of *S. alburnoides* (●) and *S. aradensis* (○) that share that haplotype (number of individuals indicated). The number of mutations between haplotypes is represented by the number of small lines perpendicular to the branches linking haplotypes. (b) Mean ± S.D. percentage of divergence ("p" distance) between pairs of haplotypes from Quarteira and from *Squalius pyrenaicus*, *Squalius torgalensis* and *S. aradensis*.

network performed using TCS 1.21®, Clement *et al.*, 2000). When compared with the cyt*b* sequences of other *Squalius* species (Fig. 2), the haplotypes included in group I were phylogenetically closer to *S. aradensis*, while the haplotype PYR had more affinities with *S. pyrenaicus* being closer to haplotypes from Guadiana (mean ± S.D. percentage of divergence between pairs of haplotypes of 0·83 ± 0·19 for Guadiana, 1·48 ± 0·45 for Guadalquivir, 1·77 ± 0·29 for Tagus and 2·67 for Sado specimens). The haplotype network (Fig. 2) strongly suggests that in the history of the *S. alburnoides* population of Quarteira several hybridization events involving females of *S. aradensis* took place. Indeed, if a single cross had taken place at the origin of the complex, with no subsequent involvement of *S. aradensis* females, a very different haplotype network was to be expected, with the haplotypes of *S. alburnoides* and *S. aradensis* clustering in two well differentiated clades. The analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992), performed using Arlequin 2.0® (Schneider *et al.*, 1999), showed no clear distinction between the cyt*b* gene sequences of all *S. aradensis* and *S. alburnoides* from Quarteira: 1·09% of variation among species and 98·91% of variation within species. Neither AMOVA nor the exact test of population differentiation based on haplotype frequencies yielded significant results ($F_{ST} = 0{\cdot}0109$ with $P = 0{\cdot}1896 \pm 0{\cdot}0116$, and $P = 0{\cdot}2003 \pm 0{\cdot}0064$, respectively). The AMOVA results were also consistent with the hypothesis of a history with multiple hybridization events involving *S. aradensis* females and *S. alburnoides* males. Finally, the finding that the five *S. alburnoides* with pure A nuclear genomes have *S. aradensis* mtDNA suggests that they were reconstituted from hybrids and that crosses involving *S. aradensis* females occurred in their ancestry. The beta-actin sequences of these fishes were identical to the sequences found in other nuclear non-hybrid males of *S. alburnoides*: mean ± S.D. percentage of divergence between pairs of haplotypes of 0·19 ± 0·16 and 0·29 ± 0·15 for fishes with pure A nuclear genomes from Guadiana and from Tagus drainages, respectively.

The results of the present study demonstrate that in the Quarteira drainage *S. alburnoides* suffered massive introgression from both nuclear and mitochondrial genes of *S. aradensis* and suggest that probably many crossings involving *S. aradensis* females occurred in the history of this population. An ancient connection between the Quarteira and Guadiana river basins would have allowed the passage of *S. pyrenaicus*-like mtDNA carriers and their subsequent crossing with *S. aradensis*. This massive introgression could have been promoted by a disproportionately low number of colonizers and by behavioural and ecological preferences that favoured crosses with *S. aradensis* females. This does not exclude the possibility of other crosses, namely between *S. alburnoides* females (which tend to be the most abundant sex in the populations; Alves *et al.*, 2001) and males of other *Squalius* species. The maternal inheritance of mtDNA means, however, that a massive introgression of mtDNA of *S. aradensis* in *S. alburnoides* of Quarteira must have been achieved through crosses between males of *S. alburnoides* and females of *S. aradensis*. As in the Guadiana the great majority of *S. alburnoides* males are nuclear non-hybrids (Alves *et al.*, 2001) it is probable that these were the *S. alburnoides* males most frequently involved in the crosses with *S. aradensis* females. It is also interesting to note that the reconstituted nuclear non-hybrids seem to be inexistent or nearly absent in the

northern rivers (Pala & Coelho, 2005) but were detected in Quarteira with a considerable frequency (five out of 15 individuals). These non-hybrid individuals, almost always males, may be the key to understand the synthesis of new hybrid lineages through crosses with *S. aradensis* females. Observations on the reproductive behaviour in captivity (C. Sousa-Santos, unpubl. obs.) showed that: 1) these non-hybrid males assume a sneaking behaviour in the presence of courting pairs of *S. pyrenaicus* and 2) actively spawned and fertilized the eggs of females of *S. torgalensis* (a species closely related to *S. aradensis*; Brito *et al.*, 1997; Sanjur *et al.*, 2003; Mesquita *et al.*, 2005) in the absence of conspecific males. The presence of non-hybrid males in Quarteira might have, therefore, promoted the expansion of the complex, contributing to a rapid replacement of the typical *S. pyrenaicus* mtDNA lineage. An independent origin of the complex in Quarteira (assuming that the paternal ancestor existed in this drainage and went extinct), as postulated by Cunha *et al.* (2004), although not disproved by this study seems unlikely since the presence of one individual with a *S. pyrenaicus*-like mtDNA and one individual with *S. pyrenaicus*-like *beta-actin* sequences although possessing *S. aradensis* mtDNA were detected. Cunha *et al.* (2004) found that in several drainages the mtDNA of *S. alburnoides* is more similar to the mtDNA of other sympatric *Squalius* species than to the mtDNA of *S. alburnoides* from other drainages. It is interesting to note that this phenomenon is only apparent in the drainages where nuclear non-hybrid males have been reported: Tagus, Sado and Guadiana. Conversely to their interpretation and postulation of multiple independent origins for *S. alburnoides*, these similarities in mtDNA between *S. alburnoides* and *S. pyrenaicus* may have been promoted by frequent crosses between *S. alburnoides* nuclear non-hybrid males and local *S. pyrenaicus* females. Indeed, when there are no such males in the populations, as apparently happens in Douro and Mondego (Alves *et al.*, 2001; Pala & Coelho, 2005), it is expected that the most frequent crosses involve triploid *S. alburnoides* females and the most abundant males (either *S. carolitertii* or diploid hybrid *S. alburnoides*), explaining the high frequency of individuals with *S. pyrenaicus*-like mtDNA in these drainages (Pala & Coelho, 2005). The available evidence is consistent with a single origin for the *S. alburnoides* complex, whose dispersal caused by historical changes of the Iberian hydrographical network would have allowed multiple crosses with the different *Squalius* species found in the newly colonized drainages. This situation is more parsimonious than admitting the coexistence of the ancestors of the complex in multiple river basins, the independent synthesis of the complex in each of those basins, and the subsequent extinction of one or both the ancestors depending on the river basin considered. Plausibility is not equivalent to definitive proof, however, and further studies are needed to a more accurate evaluation of the different hypotheses proposed to explain the history of this interesting complex.

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