*Evolutionary history and population genetics of a cyprinid fish (*Iberochondrostoma olisiponensis*) endangered by introgression from a more abundant relative*

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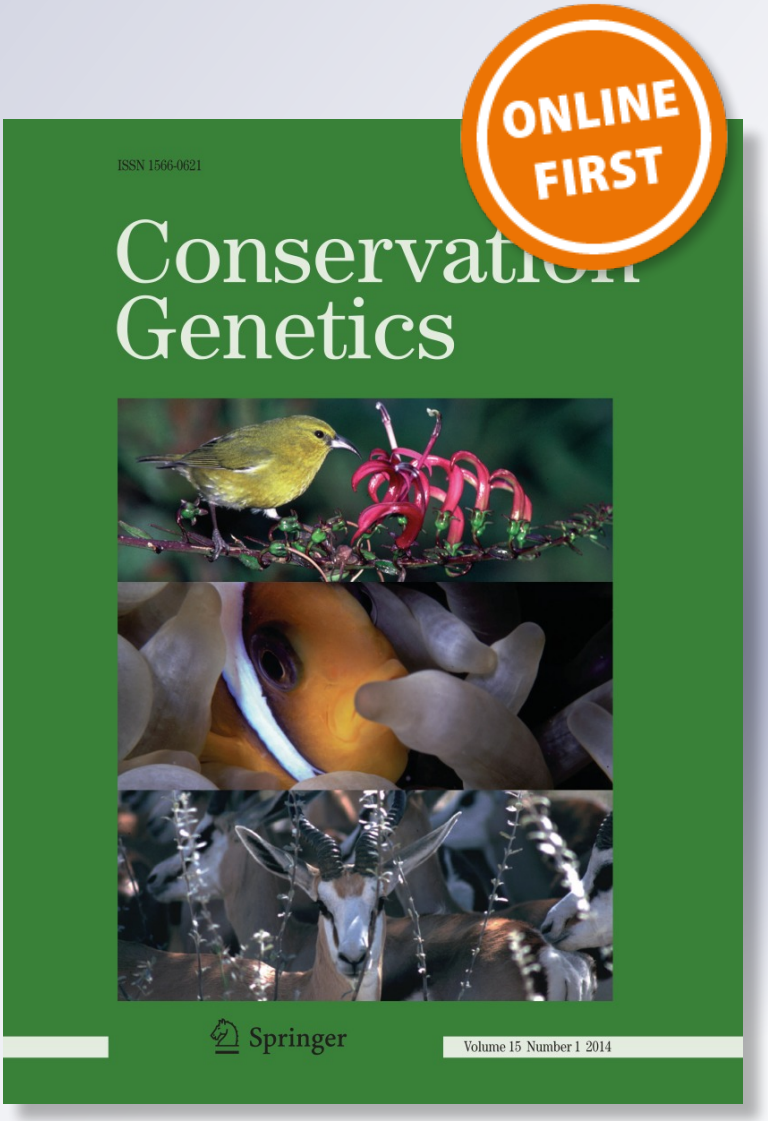
Conservation Genetics

ISSN 1566-0621

Conserv Genet
DOI 10.1007/s10592-014-0568-1



Springer

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RESEARCH ARTICLE

Evolutionary history and population genetics of a cyprinid fish (*Iberochondrostoma olisiponensis*) endangered by introgression from a more abundant relative

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Received: 10 July 2013 / Accepted: 10 January 2014

Abstract The use of molecular techniques has shown that hybridization and introgression have significant impacts in evolution, by means of transfer of genetic variation and formation of hybrid species. In this paper we use mitochondrial and nuclear sequence data to investigate the evolutionary history, levels of genetic diversity and population differentiation of a rare and endangered fish species. Our results suggest that a hybrid origin scenario of *Chondrostoma olisiponensis* is a likely explanation for the shared genetic and morphological traits with *Iberochondrostoma* and *Achondrostoma* + *Pseudochondrostoma*. The basal positioning of *C. olisiponensis* alleles in all loci analyzed indicates that hybridization events occurred before differentiation within each of these groups, most likely during Middle–Late Miocene. Originally described as *C. olisiponensis*, we suggest that this species should be placed in the genus *Iberochondrostoma* to avoid confusion with 'real' central European *Chondrostoma* and to (partially) reflect its evolutionary history. Analyses of levels of genetic diversity and patterns of population subdivision show that populations of the rare *Iberochondrostoma olisiponensis* are differentiated (high and significant φ_{ST} and F_{ST}) and genetically depauperate (very low S, π, and θ). *I. olisiponensis* is simultaneously imperiled by small population sizes and contemporary bidirectional hybridization with another critically endangered sympatric species (*Iberochondrostoma lusitanicum*). Urgent ex-situ conservation measures involving supportive breeding of *I. olisiponensis* are needed to preserve present genetic variation and eventually increase in situ population sizes, along with further studies focused on different life history and behavioral characteristics of this highly endangered species.

Keywords Intergeneric hybridization · Introgression · Hybrid speciation · Cyprinidae · Parental species displacement · Mito-nuclear discordance

Electronic supplementary material The online version of this article (doi:10.1007/s10592-014-0568-1) contains supplementary material, which is available to authorized users.

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Published online: 23 January 2014

Introduction

The application of molecular techniques in evolutionary studies has indicated that gene flow among many species of plants and animals is common and widespread (Mallet 2005). The pervasiveness of introgression of genetic material across many different organisms has slowly resulted in increased recognition of its creative role in the evolutionary process, including the transfer of neutral and adaptive variation and formation of hybrid (recombinant) species (Dowling and Secor 1997; Mallet 2005; Arnold 2006). On the other hand, while hybridization and introgression are the hidden side of the speciation process and have a clear impact in the evolution of species (Abbott et al. 2013), disrupting this balance can have negative consequences for the future of hybridizing species (Rhymer and Simberloff 1996; Seehausen 2006).

Among vertebrates, several traits make freshwater fish especially prone to hybridize, including external fertilization, weak behavioral isolating mechanisms, unequal abundance of the two hybridizing species, competition for limited spawning habitat, decreased habitat complexity, and susceptibility to secondary contact between recently evolved forms (reviewed by Scribner et al. 2001; Chávez and Turgeon 2007). Additionally, hybrids between distantly related fish species are frequently viable (e.g. Ünver and Erk'Akan 2005; Almodóvar et al. 2012) suggesting that fish appear to be less susceptible to severe developmental incompatibilities that affect interspecific hybrids in other vertebrates (Scribner et al. 2001). Accordingly, in the diverse freshwater Cyprinidae (minnows and carps), many of its members hybridize (e.g., Hubbs 1955; Smith 1992; Yakovlev et al. 2000). Iberian cyprinids are no exception, with numerous accounts of introgressive hybridization and species of hybrid origin with alternative modes of sexual reproduction (Almaça 1965; Doadrio 1980; Machordom et al. 1990; Alves et al. 1997, 2001b).

In a group of closely related Iberian cyprinids previously included in the genus *Chondrostoma s.l.* (before Robalo et al. 2007a), several reports of hybridizing species have been published (Almaça 1965; Collares-Pereira and Coelho 1983; Elvira et al. 1990; Gante et al. 2004, 2010; Aboim et al. 2010). One of these accounts refers to hybrids between the recently described "*Chondrostoma*" *olisiponensis* (*Chondrostoma s.l.*) and a close relative, *Iberochondrostoma lusitanicum* (Gante et al. 2010). The former is an extremely rare species, whose known distribution is restricted to localized areas in three small tributaries of the lower Tagus River (Portugal) and embedded in the wider range of the relatively more common *I. lusitanicum* (Gante et al. 2007, 2010). Additionally, *C. olisiponensis* is a relatively ancient species which split around 12.5–7.9 MY from its most recent ancestor (Gante et al. 2010), with a puzzling evolutionary history. For instance, it is monophyletic with *Iberochondrostoma* species at the mitochondrial cytochrome *b* and with *Achondrostoma* + *Pseudochondrostoma* species at the beta-actin nuclear gene and shares many diagnostic morphological characters with either genus (Gante et al. 2007). Interestingly, from a biogeographical perspective, the lower Tagus River is the only area co-inhabited by *Achondrostoma* and *Iberochondrostoma*, although they live in different tributaries and are not known to co-occur (Fig. 1). *I. lusitanicum* has shown drastic population crashes over the last decades (Sousa et al. 2008) and is listed as Critically Endangered both in the Portuguese Red Data book (Cabral et al. 2005) and in the IUCN Red List of Threatened Species (Crivelli 2006). Levels of genetic diversity and population fragmentation also followed these demographic trends, indicating a genetically depauperate, highly fragmented species (Alves and Coelho 1994; Robalo et al. 2007b; Sousa et al. 2008, 2012). The rarer and much less known *C. olisiponensis* is listed in the IUCN Red List of Threatened Species also as Critically Endangered (Gante et al. 2012). Given the conservation challenges faced by both species, the preliminary accounts of introgressive hybridization involving them (Gante et al. 2010) call for additional work. In this paper we expand the number of collection sites, samples and molecular markers to address the phylogenetic relationships and evolutionary history of *C. olisiponensis*. We also address the extent of introgression, genetic characteristics of extant populations and possible causes of breakdown of prezygotic isolation mechanisms, in particular those of the rarest and least known species (*C. olisiponensis*), since it is known that introgression usually affects the rarer of two hybridizing species the most (Levin et al. 1996).

Methods

Individuals of *C. olisiponensis* and *I. lusitanicum* were collected by electrofishing throughout the distribution ranges of these species in the lower Tagus River basin (Fig. 1). Different sampling efforts were applied and frequently directed to the rarest species (*C. olisiponensis*). When a sufficient sample size of the most abundant species (*I. lusitanicum*) was reached, individuals were returned to the river without being quantified to reduce manipulation impacts. Similarly, as *C. olisiponensis* is an extremely rare species, newly collected specimens were returned to the river course after the removal of fin clips for genetic analyses. These procedures prevented an accurate estimation of the population size of both species, although it was evident that *I. lusitanicum* significantly outnumbered *C. olisiponensis.*

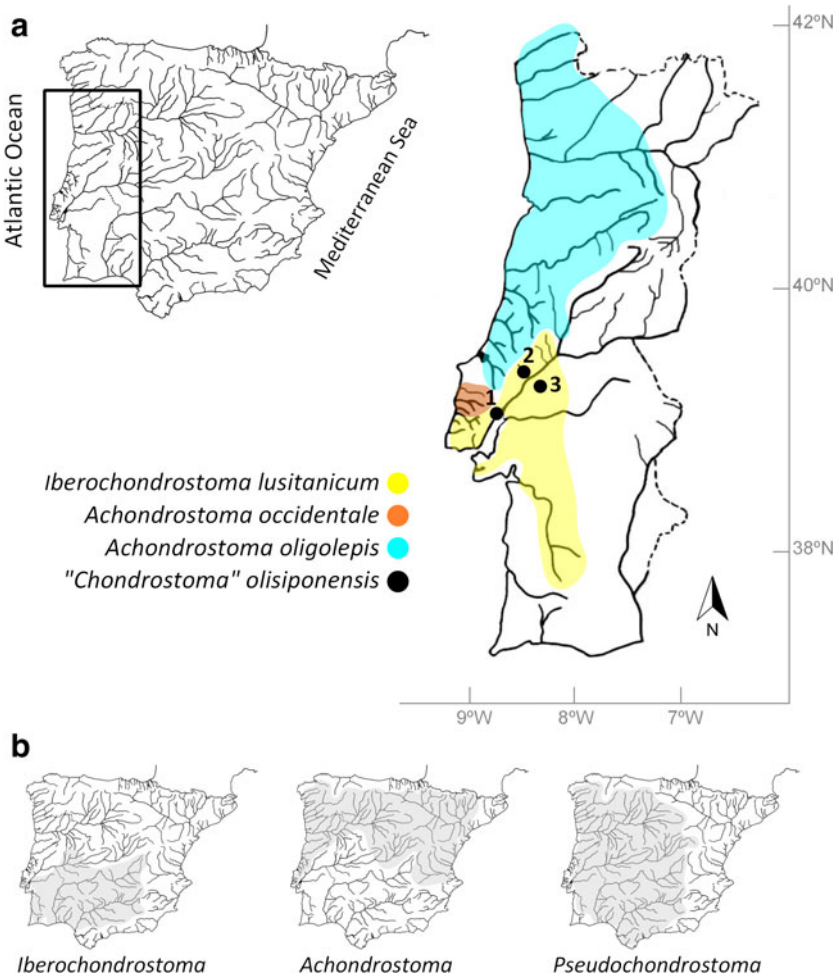

Fig. 1 **a** Sampling locations of *Chondrostoma olisiponensis* and distribution areas of *Iberochondrostoma lusitanicum, Achondrostom occidentale* and *Achondrostoma oligolepis* in the Iberian Peninsula. River basins: *1* Trancão, *2* Maior, *3* Muge. **b** Distribution areas of the *Iberochondrostoma*, *Achondrostoma* and *Pseudochondrostoma* genera

Sequence data from *C. olisiponensis* ($n = 25$) and *I. lusitanicum* ($n = 81$) collected in the Tagus River, were compared with sequences of *I. lusitanicum* from other river basins [Jamor ($n = 2$), Lage ($n = 5$), Sado ($n = 1$) and Samarra ($n = 1$)] and with those of 13 other Iberian native species belonging to all clades within the formerly known genus *Chondrostoma s.l.*: genera *Iberochondrostoma*, *Pseudochondrostoma*, *Achondrostoma* and *Parachondrostoma*—Online Resource 1. Samples from the genus *Chondrostoma s. st.* from central and eastern Europe were also included—Online Resource 1. Genbank accession numbers (noncoded sequences) and sampling sites are presented in Online Resource 1.

DNA was extracted using Extract-N-Amp Tissue PCR Kits by Sigma-Aldrich (Lisbon, Portugal) and three loci (one mitochondrial and two nuclear) were amplified using the following primers and PCR conditions: LCB1-AAT GACTTGAAGAACCACCGT (Brito et al. 1997) and HA-CAACGATCTCCGGTTTACAAGAC (Schmidt and Gold 1993) for cytochrome *b* gene (35× [94 °C 1′ + 50 °C 1′ + 72 °C 2′]); BACTFOR-ATGGATGATGAAATTGC CGC and BACTREV-AGGATCTTCATGAGGTAGTC (Robalo et al. 2007a) for *β-actin* gene (35× [94 °C 30″ + 55 °C 40″ + 72 °C 1′30″]); and S7RPEX1F-TGGC CTCTTCCTTGGCCGTC and S7RPEX2R-AACTCGTCT GGCTTTTCGCC (Chow and Hazama 1998) for the first intron of the *S7* gene (35× [94 °C 30″ + 60 °C 1′ + 72 °C 2′]). The same primers were used for sequencing reactions and the PCR products were purified and sequenced at GATC (Germany) using Applied Biosystems 3730xl and 3,700 sequencers. The sequences obtained were trimmed at the 3′ end so they had the same length for all the individuals sampled.

Sequences were edited and aligned with CodonCode Aligner v4.0.4 (CodonCode Corp., USA). Concerning the nuclear genes, sequences of heterozygous individuals were manually phased following the procedures described in Sousa-Santos et al. (2005) before the alignment process.

Phylogenetic analyses

Phylogenies from independent loci were constructed to address evolutionary relationships of *C. olisiponensis*. Since ongoing hybridization is expected to homogenize

gene pools, phylogenies were also used to determine species-specific alleles at each locus.

Alignment gaps were responsible for much of the variability between nuclear sequences. For the calculation of genetic distances used in phylogenetic methods, it was assumed that contiguous gap positions arose from single insertion-deletion events (as argued by Barriel 1994; Simmons and Ochoterena 2000), except when intermediate haplotypes were found. Thus, to avoid that a block of contiguous alignment gaps was accounted as having as many mutations as the number of bases that constituted the block, the number of bases of the indel was manually reduced to one in edited contigs. Additionally, and in order to be suitable for distance and likelihood analyses, gaps were coded as a transversion for each particular locus.

Phylogenetic relationships were reconstructed using distance (minimum evolution, ME, and neighbour-joining, NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods, using PAUP 4.0 (Swofford 1998) and MrBayes (Ronquist and Huelsenbeck 2003). Likelihood settings from the best-fit model were selected using AIC criterion, as implemented in jModeltest 0.1.1 (Guindon and Gascuel 2003; Darriba et al. 2012). Branch support was assessed by bootstrap analyses using 500 (ML) or 1,000 (MP, ME and NJ) replicates. *Anaecypris hispanica*, *Rutilus rutilus* and *Squalius pyrenaicus* were used as outgroups. For the Bayesian analysis, four independent runs of four Metropolis-coupled chains of 1,000,000 generations each were performed to estimate the posterior probability distribution, using *R. rutilus* as outgroup. Samples from all the Iberian clades included in the formerly known *Chondrostoma* genus (Robalo et al. 2007a) were also included in the phylogenetic analyses.

Phylogenetic trees were edited for publication using Mesquite (Maddison and Maddison 2011). Networks of haplotypes were performed with TCS 1.21 (Clement et al. 2000), using statistical parsimony to connect haplotypes based on a 95 % confidence interval. This last procedure was applied to the nuclear genes only, because the much faster mutation rate of the mitochondrial cyt *b* yielded networks (not shown) that were less informative than the phylogenetic trees presented.

Levels of sequence polymorphism, population differentiation and admixture dynamics

Levels of mitochondrial and nuclear polymorphism were calculated in DnaSP v5.00.07 (Librado and Rozas 2009) independently for each population of *I. lusitanicum* and *C. olisiponensis*: number of segregating sites (S), pairwise nucleotide diversity (π), and Watterson's theta (θ). MtDNA and nuDNA pairwise fixation indices (φ_{ST} and F_{ST}, respectively) were calculated among populations within species to assess levels of differentiation. Likewise, proportion of nuclear DNA variation among individuals within populations (inbreeding coefficient—F_{IS}) was assessed using hierarchical analyses of molecular variance (AMOVAs) in Arlequin 3.5 (Excoffier et al. 2005). Given the high number of introgressed individuals found (see results below), we included only species-specific alleles identified by phylogenetic methods (above). Therefore, for each species we excluded introgressed alleles from these calculations to avoid parameter inflation, which we conservatively replaced with missing data.

To assess whether *I. lusitanicum* and *C. olisiponensis* freely interbreed in sympatry, deviations from the Hardy–Weinberg (H–W) principle were calculated for populations where these species co-occur. Significance of deviations was assessed with ADERSIM (Almada and Oliveira 1997). This program generates 1,000 simulations in which values are randomly assigned to the different classes with probabilities reflecting their expected frequencies. The number of times out of 1,000 that for each genotypic class the observed values are equal or greater, and equal or smaller, than the simulated values allows the assessment of the significance of deviations. In addition, the number of times the χ^2 is equal or exceeds the χ^2 computed for each simulation is also provided. This procedure has the advantage over the conventional χ^2 tests of being free of the assumptions of the χ^2 distributions, at the same time allowing the assessment of the significance of the deviations of individual classes.

We also used the Bayesian clustering program STRUCTURE v2.3.2 (Pritchard et al. 2000; Falush et al. 2003) to identify genetic clusters and assign individuals to K populations without a priori classification of pure-bred or hybrid individuals. STRUCTURE works by minimizing deviations from Hardy–Weinberg equilibrium within clusters and maximizing linkage disequilibrium between them, and detects gene flow (admixture) between clusters. Alleles of nuclear loci were numerically coded by species based on phylogenies (above). Twenty replicates were run for each value of K, from 1 to 6, consisting of 250,000 Markov Chain Monte Carlo (MCMC) iterations discarded as burn-in, followed by 500,000 MCMC replicates to estimate the posterior sample distribution, using the admixture and correlated allele frequencies model. To improve the accuracy of the inference and aid in identification of hybrids we used "learning samples" pre-defined as coming from each pure-bred species, using the USEPOPINFO model (Pritchard et al. 2000, 2010). Therefore, emphasis is placed in identifying admixed individuals, rather than in detecting intraspecific population structure. Consensus clustering across iterations for the best K was generated using the greedy algorithm in CLUMPP (Jakobsson and Rosenberg 2007) and visualized using the

program DISTRUCT (Rosenberg 2004). The best K identified by STRUCTURE was determined by comparing changes in LnP(D) values of consecutive K (Pritchard et al. 2010) and Evanno et al.'s (2005) ΔK, calculated using STRUCTURE HARVESTER (Earl and vonHoldt 2012).

It has been suggested that rare species are the most susceptible to extinction through demographic swamping and genetic assimilation by a more abundant relative (Levin et al. 1996). In this study, we assessed a possible relation between frequency of hybridization and population sizes in the rare *C. olisiponensis*. In the absence of direct demographic data, we used the measures of polymorphism calculated above as proxies for population size, since a positive linear relationship is expected between genetic diversity and effective population size (Wright 1931). As a consequence of their small population size, neutral population genetics theory predicts that less abundant species (populations) will be genetically less diverse than more abundant ones (Kimura 1983). In fact, it has been shown that genetic diversity and abundance are correlated in fish (McCusker and Bentzen 2010). Frequency of hybridization was estimated by a hybrid index for each population, calculated as the proportion of alien (*I. lusitanicum*) alleles in all individuals with at least one *C. olisiponensis* nuclear allele. We predict that if effective population size plays a role in frequency of introgressive hybridization differences across populations, then introgression should be higher in populations with lower genetic diversity.

Results

Phylogenetic relationships

The analysis of the haplotype networks shows that cyt *b* haplotypes of *C. olisiponensis* (C1–C3) share a common ancestor with those of *Iberochondrostoma*, being linked to it by 49 mutational steps (data not shown). Mitochondrial haplotype C1 is the most frequent in our samples, occurring in all populations where *C. olisiponensis* was sampled (Maior, Trancão and Muge) (Online Resource 1). Haplotype C2 is shared between Maior and Trancão populations and haplotype C3 is a singleton exclusive to the Muge population (Online Resource 1). *S7* alleles of *C. olisiponensis* (S1–S4) are also more closely related with those of *Iberochondrostoma*, differing only by 2–6 mutational steps from the putative common ancestor with *Iberochondrostoma* (Fig. 2). Allele S2 is present in all three populations, allele S1 is shared between Maior and Trancão populations and alleles S3 and S4 are singletons, respectively, of Muge and Trancão populations.

Contrastingly, regarding the *β-actin* gene, *C. olisiponensis* alleles (B2 and B3) differ by 4 and 6 mutational steps from a common ancestor with *Achondrostoma* + *Pseudochondrostoma*. B3 is the most frequent *-actin* allele and is present in all three populations, whilst allele B2 is shared by individuals from Muge and Trancão populations.

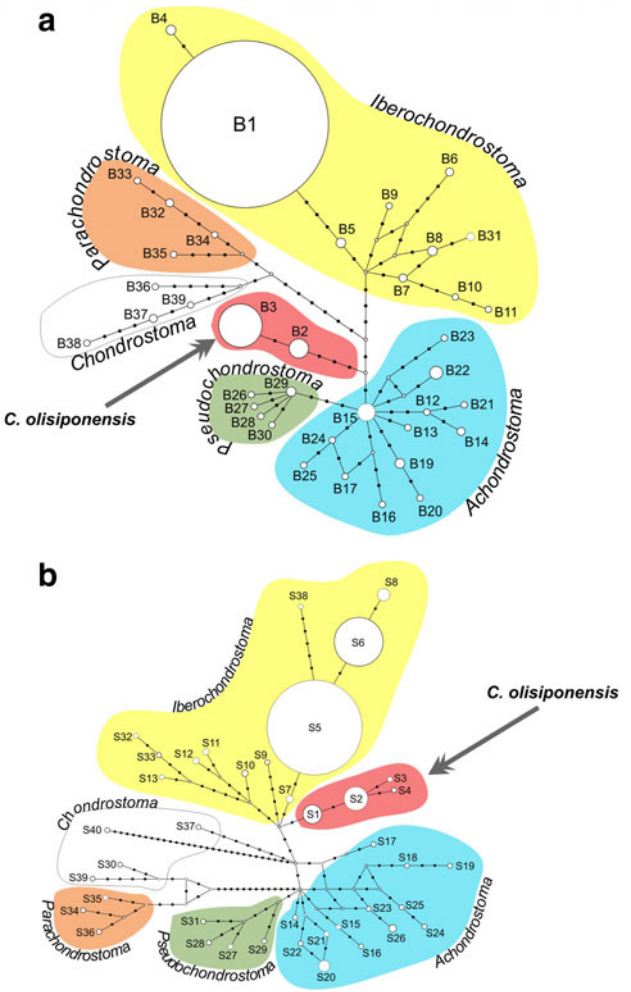

Fig. 2 Network of **a** *β-actin* (*above*) **b** and *S7* (*below*) haplotypes. Each haplotype is identified with its code label, listed in Table 2, and its dimension is proportional to the number of individuals bearing it. Mutational steps are represented by *small black dots* and missing ancestors as *small white dots*

Phylogenetic trees support the affinities described above for all loci studied: *C. olisiponensis* occupies a basal position to the differentiation of *Achondrostoma* + *Pseudochondrostoma* extant species at the *β-actin* gene level, while it is the extant species basal to *Iberochondrostoma* at the cyt *b* and *S7* genes level (Online Resource 2).

Levels of sequence polymorphism and population differentiation

Sequencing of the mitochondrial cyt *b*, and nuclear *β-actin* and *S7* loci resulted in aligned fragments of 769, 886 and 507 bp, respectively. All measures of sequence polymorphism (S, π,

Table 1 Genetic diversity measures for mtDNA and nuDNA across populations of both species from Tagus River

Species	Population	Mitochondrial				Nuclear			
		No.	S	π	θ	No.	S	π	θ
I. lusitanicum	Total	82	23	0.00438	0.00601	178	11	0.00068	0.00142
	Maior	45	6	0.00205	0.00178	104	11	0.00069	0.00158
	Alviela	6	1	0.00078	0.00057	12	0	0.00000	0.00000
	Trancão	19	2	0.00106	0.00074	38	1	0.00018	0.00018
	Seda	4	0	0.00000	0.00000	8	1	0.00018	0.00028
	Sor	8	3	0.00176	0.00150	16	8	0.00088	0.00178
C. olisiponensis	Total	24	2	0.00062	0.00070	52	10	0.00219	0.00169
	Maior	10	0	0.00000	0.00000	22	1	0.00020	0.00021
	Trancão	10	1	0.00072	0.00046	22	9	0.00248	0.00186
	Muge	4	2	0.00130	0.00142	8	8	0.00294	0.00227

Sample size (No.), number of segregating sites (S), pairwise nucleotide diversity (π), and Watterson's theta (θ)

Table 2 Pairwise F_{ST} and φ_{ST} estimates (above and below diagonal, respectively) for *I. lusitanicum* from Tagus River

I. lusitanicum	Maior	Alviela	Trancão	Seda	Sor
Maior	–	0.09544$^{\bullet}$	0.63313***	0.62785***	−0.01291^{ns}
Alviela	0.00542^{ns}	–	0.80444***	0.88599***	0.07197^{ns}
Trancão	0.61401***	0.74486***	–	−0.08076^{ns}	0.59948***
Seda	0.91912***	0.97588***	0.95525***	–	0.53265***
Sor	0.10951**	0.19625$^{\bullet}$	0.59189***	0.93741***	–

^{ns} $P > 0.1$; $^{\bullet}$ $P > 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 3 Pairwise F_{ST} and φ_{ST} estimates (above and below diagonal, respectively) for *C. olisiponensis* from Tagus River

C. olisiponensis	Maior	Trancão	Muge
Maior	–	0.66236***	0.70895***
Trancão	0.44444***	–	−0.03064^{ns}
Muge	0.42029$^{\bullet}$	−0.08108^{ns}	–

^{ns} $P > 0.1$; $^{\bullet}$ $P > 0.05$; *** $P < 0.001$

and θ) vary considerably among populations of each species for both mtDNA and nuDNA loci (Table 1). Irrespective of that variation, all measures indicate low levels of diversity. Both species show further evidence of small effective population sizes as indicated by high and significant inbreeding indices ($F_{IS} = 0.39193$, $P < 0.001$ in *I. lusitanicum*; $F_{IS} = 0.67897$, $P < 0.001$ in *C. olisiponensis*). Additionally, both types of markers show high levels of genetic structure and differentiation among most populations of each species as revealed by high levels of φ_{ST} and F_{ST} (Tables 2, 3).

Admixture dynamics

From the 106 *I. lusitanicum* and *C. olisiponensis* individuals from Tagus River, 80 individuals show only alleles typical of *I. lusitanicum*, 17 show only alleles typical of *C. olisiponensis*, while the remaining nine individuals have alleles of both origins in at least one locus (Table 4). Three times more hybrid individuals carried *C. olisiponensis* than *I. lusitanicum* mtDNA, although the numbers are overall small (Table 4).

Deviations from Hardy–Weinberg equilibrium indicate that *I. lusitanicum* and *C. olisiponensis* do not freely interbreed in sympatry. Results show an excess of pure (homozygous) individuals and a deficit of hybrids (heterozygous) individuals relative to what would be expected by random mating, in populations from Maior and Trancão where both species were simultaneously collected. The differences between observed and expected allele frequencies (Online Resource 3) are significant for the *β-actin* gene [$\chi^2(1df) = 51.6$ never greater or equal in 1,000 simulations, for Maior; and $\chi^2(1df) = 180.0$ never greater or equal in 1,000 simulations, for Trancão] and for the *S7* gene [$\chi^2(1df) = 302.8$ never greater or equal in 1,000 simulations, for Maior; and $\chi^2(1df) = 111.5$ never greater or equal in 1,000 simulations, for Trancão]. Even though our sampling does not completely reflect the relative abundances of *I. lusitanicum* and *C. olisiponensis* in each population, because several *I. lusitanicum* individuals were returned to the water without being sampled for genetic analyses, having included all additional *I. lusitanicum*

Table 4 Number of individuals with *lusitanicum* (L) or *olisiponensis* (O) alleles, for each population sampled and for each molecular marker sequenced: cytochrome *b* (CYT *b*), *β-actin* (*BACT*), and first intron of the *S7* ribosomal protein (*S7*)

Locus			Population						Total no. of individuals	
CYT *b*	*BACT*	*S7*	Maior	Alviela	Muge	Trancão	Seda	Sor		
O	OO	OO	3		4	10			17	Pure *olisiponensis*
O	LO	OO	6						6	
L	LL	LO	1			1			2	Hybrids
O	OO	LO	1						1	
L	LL	LL	44	6	[a]	18	4	8	80	Pure *lusitanicum*
			55	6	4	29	4	8	106	

[a] *I. lusitanicum* was not sampled in the Muge population, although it is known to occur in this sub-basin

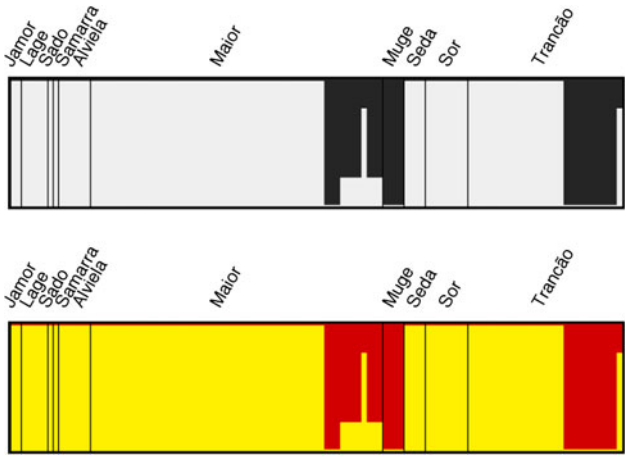

Fig. 3 STRUCTURE analysis of all *I. lusitanicum* and *C. olisiponensis* specimens (*light grey* and *dark grey*, respectively). Each individual is represented by a *vertical line*, and different colors refer to assignment to different groups

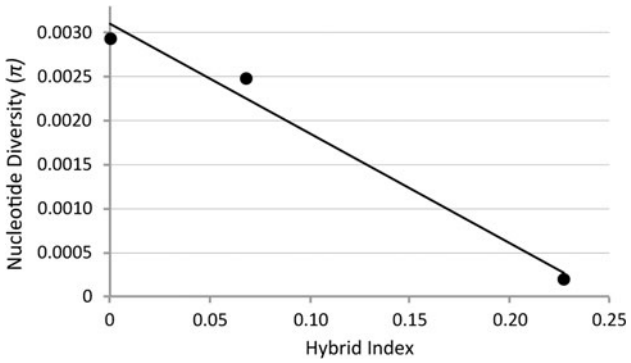

Fig. 4 Correlation between hybrid index (*hi*) and nucleotide diversity (π) across populations where *I. olisiponensis* was collected ($\pi = -0.0125hi + 0.0031$; $r^2 = 0.981$)

would make deviations from H–W expectations even more significant. In particular, results would show an extreme bias towards *I. lusitanicum* and fewer hybrids than would be expected by random mating. Interestingly, no hybrids heterozygous for the two nuclear markers were found (Table 4), indicating that we did not collect any F1 hybrid.

These results are corroborated by STRUCTURE analysis, which also uses deviations from H–W, in addition to linkage disequilibrium across loci to define genetic clusters. The results indicated K = 2 is the best solution with highest mean probability of the data (mean LnP(D) = −82.565000) and deltaK (10^6). Two groups of pure individuals can be recognized in every population, while individuals of admixed ancestry are found in populations from Maior and Trancão rivers (Fig. 3). Concerning the six distinct populations sampled (tributaries of both margins of the Tagus River), pure *C. olisiponensis* was only detected in Maior, Muge and Trancão populations (Table 4).

The results above indicate heterogeneous levels of gene flow between *I. lusitanicum* and *C. olisiponensis* in different populations (highest in Maior and lowest in Muge). We find a strong correlation between the amount of shared alleles (hybrid index) and levels of polymorphism at nuclear loci, indicating that effective population size and frequency of introgressive hybridization across populations are connected (Fig. 4).

Discussion

Origin by intergeneric hybridization or sorting of ancestral polymorphism?

Ancient intergeneric hybridization giving rise to new species/lineages has gone relatively undetected within European freshwater fish (but see Alves et al. 1997; Ráb et al. 2000; Bohlen and Ráb 2001; Robalo et al. 2006 for examples of asexual lineages). Nevertheless, recent intergeneric hybrids with sexual reproduction and originated by introgressive hybridization are quite common in many regions, e.g. the Iberian Peninsula (Collares-Pereira 1983;

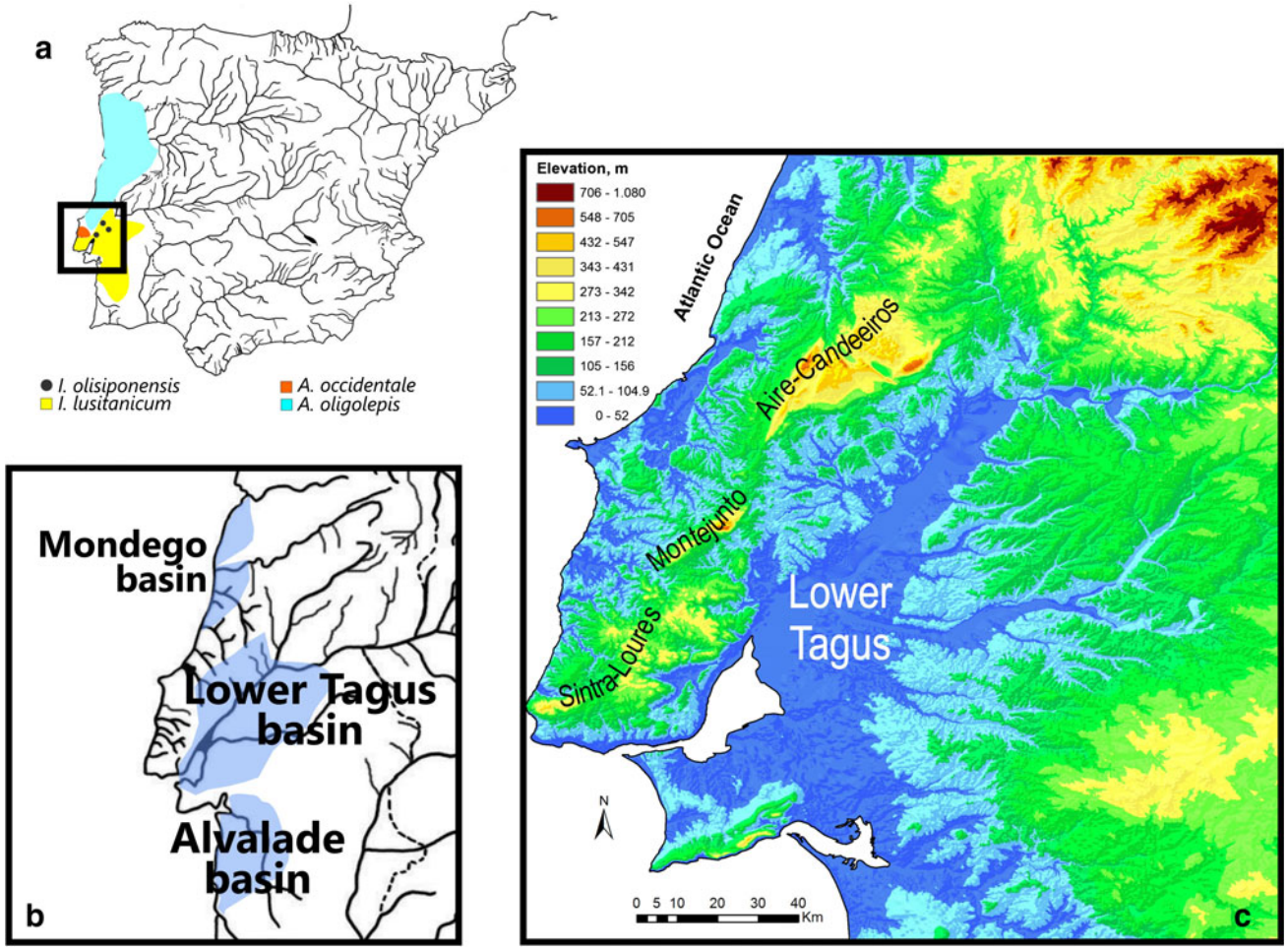

Fig. 5 **a** Schematic representation of Fig. 1, with the present distribution areas of *I. lusitanicum, I. olisiponensis, A. occidentale* and *A. oligolepis*. **b** Late Miocene endorheic basins at the study area (adapted from Pais et al. 2012). According to the hybrid origin scenario proposed, ancient *I. olisiponensis* may have been isolated in a small endorheic basin in the Lower Tagus until 4 MY and posteriorly established contacts with *I. lusitanicum* most probably coming from the Alvalade basin. **c** Digital terrain model showing the elevation and the main geomorphological units of the study area about 2 MY ago. Montejunto and Aires-Candeeiros mountain chains most probably prevented westward dispersals of *I. lusitanicum* coming from the Lower Tagus basin

Gante et al. 2004; Aboim et al. 2010; Almodóvar et al. 2012). While molecular markers have helped detect interspecific gene flow, identification of homoploid hybrid species is difficult due to variable genomic contributions from each parent and because recent origin of stabilized hybrid lineages is difficult to distinguish from recent or contemporary introgression (Mallet 2005). Our results show that *C. olisiponensis* individuals have unique alleles at all loci surveyed, with no suspected recombination, which is consistent with an old Middle–Late Miocene origin proposed for the species, perhaps in the Lower Tagus Cenozoic basin (Gante et al. 2010). These alleles share common ancestors with *Iberochondrostoma* or with *Achondrostoma* + *Pseudochondrostoma*, depending on the locus considered. Such patterns could be the result of ancestral hybridization or differential sorting of alleles between these two groups of species (Gante et al. 2010). Since *C. olisiponensis* shares many morphological characters with either *Achondrostoma* or *Iberochondrostoma*, while others are intermediate (Gante et al. 2007), a hybrid origin scenario is a likely explanation for the observed patterns. The consistent presence of *Iberochondrostoma*-like cyt*b* gene sequences in *C. olisiponensis* individuals strongly suggests that a hypothetical ancient hybridization event either involved females of *Iberochondrostoma* and males of the other clade, or by backcrossing with *Iberochondrostoma* females. Any demographic or preference differences between the hybridizing groups could result in a complete replacement of the mitochondrial genome in the ancient *C. olisiponensis* (Chan and Levin 2005). Such patterns of mitochondrial replacement have been detected in other taxa, including fish (Carson and Dowling 2006; Good et al. 2008; Barbanera et al. 2009; Nevado et al. 2009; Tang et al. 2012). The nuclear loci examined here

indicate that different genomic regions share different common ancestors, as would be expected from a species of hybrid origin (Mallet 2005).

Furthermore, the Lower Tagus River is a contact region between *Achondrostoma* and *Iberochondrostoma* (Fig. 5a), which adds biogeographical relevance to a scenario that would explain the hypothetical hybrid origin of *C. olisiponensis*: extant *Achondrostoma* are found in one sub-basin of the Lower Tagus basin (Nabão) and in several independent river basins to the north and west of the Tagus River, while *Iberochondrostoma* are found in Tagus and to the south, suggesting that ancestral representatives of both *Iberochondrostoma* and *Achondrostoma* may have established contact in the Lower Tagus basin (Fig. 5b). The basal positioning of *C. olisiponensis* alleles in all loci analyzed indicates that possible hybridization events occurred early in the differentiation of these two groups, most likely during Middle–Late Miocene (12.5–7.9 MY, Gante et al. 2010), and before differentiation within each of these groups (5.55 MY). Geological data indicate that between ca. 9.5 and 4 MY, the Lower Tagus basin became a region with several endorheic small basins that were isolated from the Alvalade basin (located towards SW and draining to the Atlantic Ocean—Fig. 5b) by an extensive terrestrial barrier (Pais et al. 2012). This series of events took place within the abovementioned time frame (12.5–7.9 to 5.55 MY) calculated for the origin of *C. olisiponensis*. Thus, it is plausible that geological events may have promoted contacts between ancestral representatives of *Iberochondrostoma* and *Achondrostoma* in a very restricted area, perhaps in one of the small endorheic basins that were formed in Late Miocene. This scenario would explain the very restricted distribution range of *C. olisiponensis*.

From ca. 4–2 MY, the hot climate was very humid and extensive drainages developed (Pais et al. 2012). During this process, all the formerly isolated sub-basins of the Lower Tagus basin became progressively connected with the Alvalade basin (Fig. 5b) and, consequently, fish formerly isolated became sympatric. The present distribution of *I. lusitanicum* (Fig. 5a) suggests that this species may have dispersed from the Alvalade basin to the north and to the west. Further colonizations were probably prevented by the existence of insurmountable geographical barriers represented by the Sintra, Montejunto and Aires-Candeeiros mountain chains (Fig. 5c), that isolated the rivers were *Achondrostoma* species occur (*A. occidentale* and *A. oligolepis*) from contacts with the headwaters of right bank tributaries of the Tagus River where *I. lusitanicum* are found.

Thus, several sources of evidence indicate that an ancient hybrid origin could explain the observed patterns of molecular and morphological variation of *C. olisiponensis*. Nevertheless, an alternative scenario of differential sorting of ancestral polymorphism between *Iberochondrostoma* and *Achondrostoma* + *Pseudochondrostoma* manifested in *C. olisiponensis* cannot be completely discarded, and would require the analysis of several additional nuclear loci.

Taxonomic considerations

The incongruence of the phylogenetic results (detected in Gante et al. 2010 and in the present study), poses serious questions on the assignment of taxonomically valid names and on the prevalent use of maternally inherited mitochondrial markers in studies describing new/cryptic species (e.g. April et al. 2011; Toews and Brelsford 2012). This incongruence is also manifested at the morphological level, which lead to the placement of *C. olisiponensis* in *Chondrostoma s.l.* in the original description (Gante et al. 2007). Nevertheless, this situation might create confusion with central European *Chondrostoma*. Given that taxonomic names should be stable over time and ideally reflect evolutionary relationships, we suggest that *C. olisiponensis* be designated as *Iberochondrostoma olisiponensis* based on its mitochondrial DNA. Even though this molecule only reflects its maternal ancestry, placement in *Iberochondrostoma* should lead to the stability of the species nomenclature. This has been done previously by different authors, although without contrasting different sources of information or providing objective criteria for doing so (Leunda et al. 2009; Perea et al. 2010).

Genetic diversity and contemporary introgression of *I. lusitanicum* genes: implications for conservation of *I. olisiponensis*

All measures of sequence polymorphism (S, π, and θ) indicate low levels of diversity, while high levels of φ_{ST} and F_{ST} indicate significant genetic structure and differentiation among most populations within species, i.e. low connectivity between populations. Both species show additional evidence of small effective population sizes as indicated by high and significant inbreeding indices. These results are qualitatively concordant with previous findings in *I. lusitanicum* indicating a genetically depauperate, highly fragmented species that experienced severe demographic crashes in historical times (Alves and Coelho 1994; Robalo et al. 2007b; Sousa et al. 2008, 2012). Relative to previous studies we found higher levels of population differentiation in both nuclear and mitochondrial markers, perhaps due to the use of sequence data instead of microsatellites from additional populations. Likewise, levels of mitochondrial polymorphism (π) are lower than previously reported. Compared to its relative, *I. olisiponensis* has one

order of magnitude less polymorphism at the mitochondrial level, while similar or even higher levels of polymorphism at the nuclear level. Taken together, these results indicate that *I. olisiponensis* is experiencing similarly negative population trends. Compared to other endangered freshwater fishes from Iberia or elsewhere, *I. olisiponensis* shows extremely low levels of genetic polymorphism and high population subdivision (e.g. Alves et al. 2001a; Salgueiro et al. 2003; Mesquita et al. 2005; Sousa et al. 2010; Dowling et al. 2012; Lopes-Cunha et al. 2012; Osborne et al. 2012; Sterling et al. 2012; Chen et al. 2013). Furthermore, in addition to the suspected old hybridization events discussed above, we present clear evidence that *I. olisiponensis* is currently being subjected to strong genetic assimilation through hybridization with the sympatric *I. lusitanicum* (Gante et al. 2010 and present study). We detected signs of introgression from *I. lusitanicum* in a fair number of individuals in most populations where *I. olisiponensis* was collected. Our genetic data indicate that all admixed individuals sampled are late generation hybrids and not F1s. Most of these individuals have *I. olisiponensis* mtDNA and morphology, indicating a majority of backcrosses in that direction, although introgression into *I. lusitanicum* was also observed. Collares-Pereira (1983) had already reported the occasional occurrence of hybrids between *I. lusitanicum* and other native sympatric species, namely, *Squalius alburnoides*, *S. pyrenaicus* and *Pseudochondrostoma polylepis*.

The amount of introgressed alleles observed in each population is highly correlated with local measures of diversity in *I. olisiponensis*, used as a proxy for its effective population sizes. These results are in line with expectations that rare species are more likely to experience demographic swamping and genetic assimilation by a more abundant relative, and hence are the most susceptible to extinction (Levin et al. 1996; Burgess et al. 2005; Lepais et al. 2009; Taylor et al. 2012). We also found roughly three times more admixed individuals with *I. olisiponensis* than with *I. lusitanicum* mitochondrial DNA. Although our sample sizes are necessarily small due to the rarity of the species studied, they further indicate that female *I. olisiponensis* might be having difficulties in finding conspecific mates, since, as suggested by Wirtz (1999), rarest species have a greater chance to mate with heterospecifics because of the shear differences in numbers. These patterns also indicate that a significant threat to the survival of *I. olisiponensis* might lie in its low numbers, perhaps more than in its low genetic diversity, and come from another highly endangered sympatric species.

Taken together with the extremely low abundances of *I. olisiponensis*, these genetic results indicate that *I. olisiponensis* needs special protection measures if its long-term existence is to be assured. Reasons for its low abundances and genetic diversity are not clear but are likely of anthropogenic origin such in many other endangered freshwater taxa showing historical demographic and genetic collapses (e.g. Sousa et al. 2008, 2010). At the present stage of knowledge it is not possible to design proper conservation policies because water quality of the natal rivers is quite low. However, we strongly recommend the establishment of an experimental program to be conducted ex-situ to investigate breeding behavior and possible isolation mechanisms.

In conclusion, present genetic data and hypothetical scenarios supported by geological and morphological data, indicate that *I. olisiponensis* is probably an ancient species of hybrid origin that currently hybridizes with another endangered sympatric species (*I. lusitanicum*). Gene flow is somewhat restricted in at least three localities where pure *I. olisiponensis* individuals were collected. However, the relative prevalence of *I. lusitanicum* throughout its distribution range and the occurrence of fertile hybrids contribute to an extremely precarious survival of *I. olisiponensis*. We suggest that national 'critically endangered' status should urgently be given to this species so that proper conservation measures can be taken. Further studies should be conducted to clarify the phylogenetic placement of this species in more detail, using additional suitable unlinked nuclear loci. New sampling campaigns should also be conducted to allow for more precise quantification of relative abundances of hybrids and parental species and to test for possible spatial segregation between them and different habitat requirements. At the same time, it is important to describe the reproductive behavior of *I. olisiponensis per se* and in a sympatric context with *I. lusitanicum* for a full evaluation of the prospects of the future of the taxon. Any ex-situ conservation measures involving supportive breeding of these species must be preceded by a carefully designed genetic study for a proper selection of breeders.

Acknowledgments Professor Vítor Almada passed away during the manuscript revision process of this paper. He was the coordinator of the UIEE-ISPA and he devoted much of his work to the preservation of native freshwater fish species in Portugal. CS-S and JR will be forever grateful for his guidance. We thank Ignacio Doadrio (MNCN-CSIC, Madrid, Spain) and Jöerg Freyhof (IGB, Berlin, Germany) for providing samples for genetic analyses. We also thank G. Lemos, C. Lima, P. Coelho and F. Ronenberg for their help during sampling, and M. Drago for help in the lab. The AFN and ICNB Portuguese agencies provided the necessary permits for fielwork. This study was financed by the European Fund for Economic and Regional Development (FEDER) through the Program Operational Factors of Competitiveness (COMPETE) and National Funds through the FCT—Portuguese Foundation of Science and Technology, under the Pluriannual Program UI&D 331/94 and 329/2011, and the projects PTDC/AAC-CLI/103110/2008 and PTDC/BIA-BDE/66519/2006. C. Sousa-Santos was supported by a Post-doctoral grant from FCT (SFRH/BPD/29774/2006).

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