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Introgressive hybridization between two Iberian endemic cyprinid fish: a comparison between two independent hybrid zones

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Abstract

Pseudochondrostoma duriense and *Achondrostoma oligolepis* are two Iberian endemic cyprinid fish species that occur in sympatry over most of their distribution range and that are suspected to hybridize in nature. Here, we employed a combination of mitochondrial and microsatellite markers to explore the extent of introgressive hybridization between these fishes. Two natural hybrid zones were identified in different river basins. Introgression was bi-directional and both hybrid zones consisted mostly of parental genotypes/phenotypes (i.e. bimodal hybrid zones). Yet, they appeared to differ in the extent and direction of introgression, which supports the view that they constitute independent outcomes of different hybridization processes probably influenced by environmental features. Several discordances were found between mtDNA and microsatellite results, suggesting that this hybridization process has complex consequences and illustrating the importance of using independent markers to define accurately the hybrid status of individuals in the presence of high levels of backcrossing.

Introduction

The traditional view that hybridization is a rare phenomenon in animals with little influence in speciation and evolution is increasingly being challenged (Dowling & Secor, 1997; Barton, 2001; Mallet, 2005). Hybridization is now well known in several animal groups and has also proven to have, through introgression, a key influence in several evolutionary phenomena such as the origin of evolutionary novelties, adaptive radiation and speciation (Dowling & Secor, 1997; Seehausen, 2004). It has also been one of the motivations behind the recent reappraisal of the debate about species concepts (Hewitt, 2001; Mallet, 2005). This revived interest in animal introgressive hybridization is partly due to the fact that modern molecular techniques and analyses allow more powerful identification of hybrids and introgressed species lineages (Mallet, 2005), but also the realization that anthropogenically driven changes on the spatial distribution of species are increasing the incidence of hybrid zones (Reusch & Wood, 2007).

In spite of this interest, the evolutionary role of introgressive hybridization in nature is controversial (Seehausen, 2004). Research in stable hybrid zones have often revealed various kinds of hybrid unfitness, which could drive hybrids to evolutionary 'dead ends', whereas, in other biological frameworks, hybridization may have the potential to generate genetic diversity and create opportunities for novel adaptive radiations in natural populations (e.g. Barton, 2001). Moreover, many studies indicate that hybrids between the same complex of species may show high variation in fitness (Arnold *et al.*, 2001) and provide evidence for genotype–environment interactions (e.g. Campbell & Waser, 2001). Finally, in some cases, the outcome of hybridization has been shown to vary dramatically with historical and

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geographical factors (Nolte *et al.*, 2009; Senn & Pemberton, 2009). It is therefore clear that, when possible, multiple independent hybrid zones between the same two species must be analysed to determine the relative importance of intrinsic (e.g. genetic) and extrinsic (e.g. ecological) factors on the evolution of hybrid zones (e.g. Morgan-Richards & Wallis, 2003).

In terrestrial environments, a large number of hybrid zones represent contacts between divergent lineages reunited after the extreme climate changes occurred during the Pleistocene, when glacial cycles forced range expansions and contractions upon the fauna and flora (Hewitt, 2001). This is particularly relevant in freshwater fishes, where the literature on the distribution and evolutionary history of some groups, especially in Europe, has widely relied not only on major geographical and climatic events (e.g. Briolay *et al.*, 1998; Doadrio & Carmona, 2004), but also on hybridization between divergent lineages (Alves *et al.*, 2001; Costedoat *et al.*, 2006; Nolte *et al.*, 2006). For example, cyprinids from southern European peninsulas such as Iberia are characterized by a high number of endemic species spread throughout independent river basins, a pattern that has been explained by the colonization of a restricted number of lineages that underwent subsequent cladogenetic processes after the formation of the Pyrenees and the isolation of the peninsula (Doadrio & Carmona, 2004). However, other ecological and evolutionary processes such as hybridization seem also responsible for the patterns of genetic diversity observed in several Iberian cyprinids in the genera *Chondrostoma* (e.g. Elvira *et al.*, 1990), *Barbus* (e.g. Almodóvar *et al.*, 2008) and *Squalius* (e.g. Alves *et al.*, 2001).

The genus *Chondrostoma* (Agassiz 1832) comprises several Iberian lineages that have been recently reconsidered as five different genera: *Pseudochondrostoma*, *Achondrostoma*, *Iberochondrostoma*, *Protochondrostoma* and *Parachondrostoma* (Robalo *et al.*, 2007). In particular, *Pseudochondrostoma duriense* and *Achondrostoma oligolepis* are believed to have diverged around 11 million years ago (MYA) (Doadrio & Carmona, 2004) and current records indicate extensive sympatry in northwestern Iberian basins. *Pseudochondrostoma duriense* is endemic of Galiza and north of Portugal with a southern distribution limit in Vouga (Kottelat & Freyhof, 2007; Aboim *et al.*, 2009), whereas *A. oligolepis* distribution is limited to river basins between Minho and Mondego and in Douro is restricted to tributaries in Portuguese territory, i.e. has an eastern delimitation near the boarder between Portugal and Spain (Robalo *et al.*, 2006; Kottelat & Freyhof, 2007) (Fig. 1).

Putative hybrids between the two species have been characterized morphologically by Collares-Pereira & Coelho (1983) in the Douro River basin. Morphological evidence has led to the suggestion that introgression is unidirectional, from *P. duriense* into *A. oligolepis*, whereas the absence of introgression at mtDNA markers has raised the interpretation that it is also sex biased (Gante *et al.*, 2004). However, the understanding of this hybridization case has been hampered by limited geographical coverage (i.e. only a particular hybrid zone, Távora River, Fig. 1) and trait analyses (i.e. only one type of marker, morphology or mtDNA), such that the results obtained might be biased and incomplete (Gante *et al.*, 2004).

Here, we analyse variation at a set of molecular markers (mtDNA and microsatellites) to investigate the potential for introgressive hybridization between *A. oligolepis* and *P. duriense*. In particular, we perform a thorough characterization of individual hybrids to infer the extend, direction and dynamics of introgression between the two species, aiming to test the hypothesis of Gante *et al.* (2004), which states that introgression in

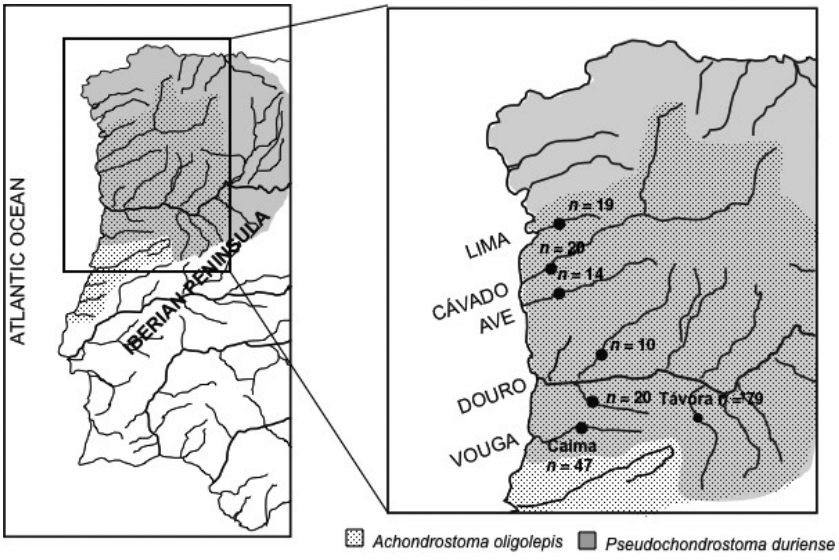

Fig. 1 Sampling Map. Distribution of *Pseudochondrostoma duriense* and *Achondrostoma oligolepis* in Portugal. The stippling/shading pattern represents areas of sympatry between the two species. Dots represent sampling sites and *n* = individuals sampled per site.

this case is unidirectional and sex biased. Besides, we also take advantage of the existence of two independent hybrid zones, on different river basins, to compare the outcome of hybridization on each zone and test the hypothesis that hybrid zones can evolve differently and/or result from independent hybridization processes (Landry & Aubin-Horth, 2007).

Material and methods

Sampling and DNA extraction

We sampled all the river systems encompassing the sympatric distribution range of the two species (Fig. 1). We sampled 45 individuals or more in localities where putative hybrids were suspected based on external morphology (see below). A total of 209 individuals were collected from rivers Lima, Cávado, Ave, Douro (Tâmega, Távora and Paiva) and Vouga (Caima) (Fig. 1). Fin clips from all captured individuals were preserved in absolute ethanol for subsequent molecular analysis. Most individuals were photographed and released alive into the water after recovery from anaesthesia (MS-222). Some specimens were fixed in formalin and preserved in 70% ethanol to integrate the Ichthyologic Collection at the Museu Nacional de História Natural, Lisboa. DNA was extracted following a standard phenol–chloroform protocol.

Genetic characterization of hybrids

Microsatellites

A pilot analysis was performed with 20 microsatellite loci isolated in *Squalius aradensis* and *Luxilus cornutus* but known to amplify across several cyprinid species (Mesquita *et al.*, 2003; Turner *et al.*, 2004; N. Mesquita, personal communication). Ten loci were chosen for further analysis based on amplification success and polymorphism in the studied species. All loci were screened in three different 10 μL multiplex PCR with 5 μL Multiplex (Qiagen, Hilden, Germany), 2 μL H_2O, 1 μL Primer Mix (2 mM each primer) and 1.5 μL template DNA (20–50 ng μL^{-1}) under the following amplification conditions: 95 °C 15 min, followed by 20 cycles of 94 °C 30 s, 57 °C 1.30 min, 72 °C 1 min and 72 °C 10 min. PCR products were resolved on a ABI Prism 310 Genetic Analyser (Applied Biosystems) with 500-LIZ as size standard. DNA fragments were analysed with GeneScan® ABIPrism Analysis Software 3.7, and genotypic data were generated with Genotyper® 2.1 ABIPrism.

To assess the power of microsatellites for identifying admixed individuals, we used the multilocus genotypes and HYBRIDLAB 1.0 (Nielsen *et al.*, 2006) to simulate four groups of hybrid individuals (F_1, F_2, backcross_*oligolepis*-B×$_{OLI}$ and backcross_*duriense*-B×$_{DUR}$). Twenty-five individuals of each parental group were randomly selected among individuals from areas where morphological hybrids were not detected to generate 25 of each hybrid type. The simulated genotypes were used to carry out admixture analyses with STRUCTURE 2.2 (Pritchard *et al.*, 2000) using $K = 2$ and 10^5 steps of the Markov Chain (preceded by a burn-in period of 10^5 steps), assuming an admixture model and independent allelic frequencies (Pritchard *et al.*, 2000). Results were evaluated aiming to assess the efficiency of admixture analyses and to compute the proportion of hybrid individuals that were correctly identified as such in the simulated data set. In our case, the threshold q-values were arbitrarily set up to $q > 0.9$ for Cluster I (*P. duriense*), $q < 0.1$ for Cluster II (*A. oligolepis*) and $0.1 < q < 0.9$ for hybrids (Vähä & Primmer, 2006). Simulated genotypes were also analysed with NEWHYBRIDS (Anderson & Thompson, 2002) to assess the efficiency of this Bayesian analysis in the correct identification of different hybrid classes. A burn-in of 10 000 steps followed by a sampling period of 10 000 steps was used and the threshold qi values of belonging to a certain hybrid class were arbitrarily set up to $qi > 0.5$ (see text below for more details).

Subsequently, we used real multilocus genotypes and STRUCTURE 2.2 to estimate the contribution of both parental species to each individual's genome, using the same run parameters and q thresholds as described earlier. We separately assessed the average probability of membership to the two parental clusters and their 90% posterior probability intervals (CI). This analysis was performed on (i) the whole sample and (ii) on the localities where putative hybrids were detected (i.e. Távora and Caima, see Results), to overcome a possible bias of the results because of within-species population structuring. STRUCTURE 2.2 was run for each species in separate without imposed K, and the number of clusters was inferred with 20 repetitions of 10^5 steps of the Markov chain following a burning-period of 10^5 interactions, taking the value that maximized the posterior probability of the data, as advised by Evanno *et al.* (2005).

In addition, classification of putative hybrids was accomplished with the Bayesian methods implemented in NEWHYBRIDS (Anderson & Thompson, 2002). Whereas STRUCTURE 2.2 only calculates the posterior probability of admixture origin of each individual, NEWHYBRIDS uses allele frequencies of multilocus genotypes to estimate the posterior probability (qi) that individuals fall into each of a set of genotypic classes corresponding to hybrid categories (H_i): parental species (P_{DUR} or P_{OLI}), F_1, F_2 and backcrosses (B×$_{DUR}$ or B×$_{OLI}$). These probabilities can be converted into Q-values, which are arbitrarily defined as the proportion of an individual's genome to be of *A. oligolepis* origin, $Q = \sum qi \times P(H_i)$, where $P(H_i)$ is the expected proportion of *A. oligolepis* genome on each hybrid category [e.g. $P(F_1) = 0.5$]. This allows for the analysis of the introgression rate in terms of the quantification of a species' genome integration into the other. A Markov Chain Monte Carlo procedure, with a burn-in of 10 000 steps, followed by a sampling period

of 10 000 steps was used as recommended by the authors of NEWHYBRIDS. This analysis was performed exclusively for the populations where putative hybrids were detected (Távora and Caima).

mtDNA

In 25 μL PCR [1× buffer, 2 mM $MgCl_2$, 0.08 DNTP's, 0.4 mM Primers, 1 U Taq and 2 μL of template DNA (20–50 ng μL^{-1})], 957 bp of the mitochondrial cyt *b* gene were amplified using primers Glu-dl-c – 5′ TGA CTT GAA RAA CCA YCG TTG 3′ and H16460 – 5′ CGA YCT TCG GAT TAC AAG ACC G 3′ (Palumbi, 1996). PCR amplification conditions were: 94 °C 1 min, followed by 30 cycles of 94 °C 1 min, 54 °C 1 min, 72 °C 2 min, and a final extension of 72 °C 3 min. Purification of PCR products was performed with QIAquick (Qiagen) columns, and sequencing of both forward and reverse strands was performed on an ABI 377 DNA Analyser.

To develop a faster technique allowing the diagnostic distinction between the two species, 83 cyt *b* sequences (Accession numbers: FJ513355–FJ513369, EU045833) were screened for fixed nucleotide differences (revealing 64 fixed-nucleotide sites, *c.* 6.7% sequence divergence; Table S1). An *Alu*I restriction site was found at position 344 in *A. oligolepis* (hereafter O haplotypes) and positions 356 and 902 in *P. duriense* (hereafter D haplotypes), and subsequently used for identification of additional samples ($N = 112$).

Morphological identification

Both species exhibit marked differences in morphology and are easily recognizable at first sight (Collares-Pereira, 1979; Coelho, 1985): while *P. duriense* individuals present a ventral, straight mouth with a conspicuous corneous blade and numerous small scales in the lateral line (> 60); *A. oligolepis* individuals are recognized by a sub-terminal, arched mouth with no corneous blade and fewer lateral-line (30–45) bigger scales. Intermediate forms of these four diagnostic traits: (i) position of the mouth intermediate between sub-terminal and ventral, (ii) elliptical-arched mouth, (iii) presence of an inconspicuous mouth blade and (iv) 45–60 lateral line scales have already been described and considered to characterize putative hybrids (Collares-Pereira & Coelho, 1983). In order to assess whether if the different introgression levels revealed by genotypic data are reflected at the phenotypic level, in a way that can be detected in the field, we have considered individuals exhibiting all morphological characters correspondent to either parental species as morphologically pure forms, whereas individuals with an assortment of morphological characters of both parental species and/or intermediate forms were considered as putative hybrids. Depending on the general body appearance and main parental character contribution, putative hybrids are hereafter designated by *P. duriense*-like or *A. oligolepis*-like.

Results

Genetic characterization of hybrids

Microsatellites

All ten microsatellite loci were highly polymorphic and presented variation for nearly all the localities (Table S2). We detected in total 129 alleles across all loci, with numbers of alleles per locus ranging between 3 and 35. Although some samples had incomplete genotypes (i.e. less than ten loci scored), < 1% of individuals per locus are missing and there was never more than one missing locus per individual.

Admixture analysis of simulated hybridization revealed that most hybrids can be correctly identified as such both with STRUCTURE (94%) with *q* threshold values $0.1 < q < 0.90$ and NEWHYBRIDS (93%) with a *qi* baseline of 0.5 (Fig. S1). With STRUCTURE, only six simulated backcross individuals failed to be identified as admixed from the sample of 100 simulated hybrids, even though about 10% of the individuals randomly chosen as parentals did not have a $q < 0.1$ or $q > 0.90$ (the average *q*-value for each parental type were 0.07 for *A. oligolepis* and 0.95 for *P. duriense*) (Fig. S1). With NEWHYBRIDS, 96% of F_1s were correctly identified as such with an average *qi* value of 0.89, and only in more admixed genotypic classes, the power decreased to 72% (F2s, $\overline{q}i = 0.69$), 80% (B×Dur, $\overline{q}i = 0.71$) and 72% (B×Oli, $\overline{q}i = 0.61$).

When the STRUCTURE analysis was run over all samples with imposed $K = 2$ and using the earlier mentioned *q* threshold values, the results suggested that most fish were in fact purebred (*A. oligolepis* or *P. duriense*). Of the 202 samples successfully screened for microsatellite loci, 73 individuals were assigned to cluster *P. duriense* ($q > 0.90$) and 78 individuals were assigned to cluster *A. oligolepis* ($q < 0.1$). Assuming that values of $0.1 < q < 0.90$ indicate admixture, there were 51 (25%) individuals that showed signals of hybridization (i.e. individuals with partial assignment to both clusters). All these putative hybrid individuals were collected in populations Távora ($n = 28$) and Caima ($n = 23$) (Fig. 2a).

However, although most of the 90% CI values for individuals in cluster *P. duriense* appear relatively narrow, individuals in cluster *A. oligolepis* and putative introgressed individuals presented broader 90% CI values (Fig. 2b). Such differences cannot be attributed to missing data and instead more likely reflect genetic differences among the groups considered. Further inspection of STRUCTURE results indicates some within-species population structure among individuals of cluster *A. oligolepis*. ΔK values indicated that $K = 3$ represented the optimal clustering for *A. oligolepis*: northern Rivers (Lima + Ave + Cávado), Douro and Vouga (Fig. S2). A similar analysis in *P. duriense* also shows $K = 3$ as the optimal clustering, although with different geographical grouping and weaker population structuring (Fig. S2).

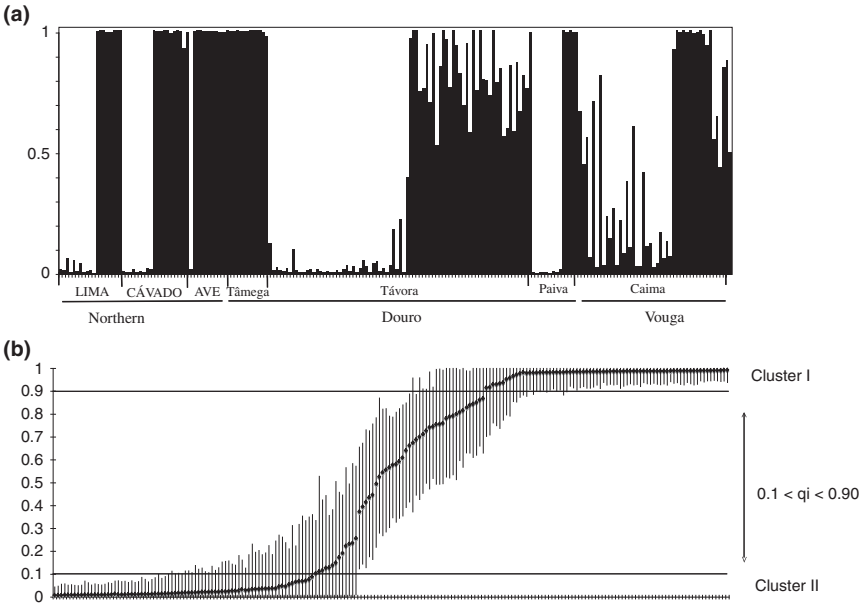

Fig. 2 (a) Bar plot representation of admixture analyses on the totality of specimens from all sampling localities computed by STRUCTURE with $K = 2$. Each individual is represented as a vertical bar partitioned into two segments whose length is proportional to the estimated membership in the two clusters. (b) Plots of individual admixture proportions (q) including 90% probability intervals. Individuals are ranked from lowest to highest q-value. q-values close to zero and one denote clusters *Achondrostoma oligolepis* and *Pseudochondrostoma duriense*, respectively.

Therefore, to avoid any possible bias of population structure in the assessment of hybridization, only admixture analyses performed exclusively in the two hybrid zones will hereafter be presented and discussed. Reanalysis of the hybrid zones using STRUCTURE with imposed $K = 2$ showed again that the majority of individuals in them are pure (78.5% in Távora, 63.8% in Caima). Yet, 17 (21.5%) and 17 (36.2%) individuals presented an admixed multilocus genotype in Távora and Caima, respectively (Fig. 3a,b and Table 1).

NEWHYBRIDS analysis also revealed that most fish in the hybrid zones appeared as pure parental forms.

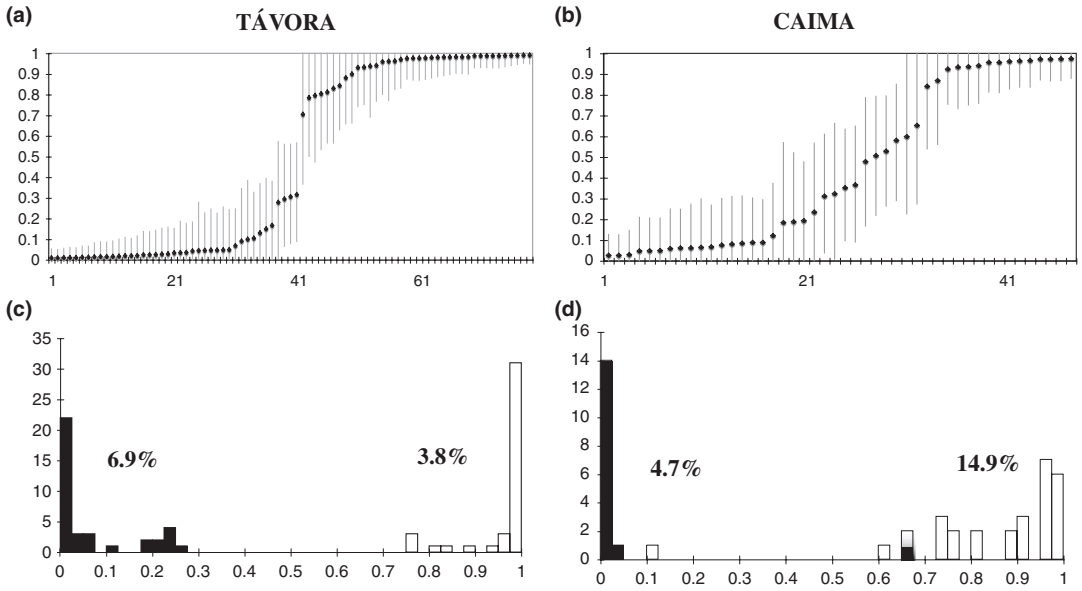

Fig. 3 (a and b) Plots of individual admixture proportions (q) including 90% probability intervals calculated with STRUCTURE for each hybrid zone. Individuals are ranked from lowest to highest q-value. q-values close to zero and one denote clusters *Achondrostoma oligolepis* and *Pseudochondrostoma duriense*, respectively. (c and d) Frequency distribution of Q-values, i.e. the proportion of an individual's genome that comes from *A. oligolepis* (Q-value = 0) or *P. duriense* (Q-value = 1) calculated with NEWHYBRIDS. Black and white bars represent individuals morphologically identified as *A. oligolepis*/*A. oligolepis*-like and *P. duriense*/*P. duriense*-like, respectively. Individuals with a mixed colour bar in Caima have a *P. duriense* morphology but appear as *A. oligolepis* backcrosses.

Table 1 Individual specimens showing evidence of introgression as diagnosed using: morphology, mtDNA cyt *b* and microsatellite genotypic class assignments using STRUCTURE and NEWHYBRIDS for each hybrid zone.

Population	Specimen	Morphology	MtDNA	STRUCTURE	NEWHYBRIDS – *qi* values
Douro					
Távora River *N* = 79	27DOc	OLI	OLI	ADM	P_{OLI} = 0.967
	35DOc	*A. oligolepis*-like	DUR	ADM	Bx_{OLI} = 0.677; P_{OLI} = 0.321
	38DOc	OLI	OLI	ADM	P_{OLI} = 0.869; Bx_{OLI} = 0.13
	44DOc	*A. oligolepis*-like	OLI	ADM	Bx_{OLI} = 0.957
	46DOc	*A. oligolepis*-like	OLI	ADM	Bx_{OLI} = 0.788; P_{OLI} = 0.124
	1001DOc	OLI	OLI	ADM	Bx_{OLI} = 0.911
	1019DOc	*A. oligolepis*-like	OLI	ADM	P_{OLI} = 0.584; Bx_{OLI} = 0.414
	1020DOc	OLI	OLI	ADM	Bx_{OLI} = 0.721; P_{OLI} = 0.245
	1DOc	DUR	DUR	ADM	Bx_{DUR} = 0.945
	4DOc	DUR	OLI	ADM	Bx_{DUR} = 0.944
	8DOc	DUR	OLI	P_{DUR}	P_{DUR} = 0.949
	11DOc	DUR	DUR	ADM	Bx_{DUR} = 0.984
	12DOc	DUR	OLI	P_{DUR}	P_{DUR} = 0.990
	15DOc	*P. duriense*-like	DUR	P_{DUR}	Bx_{DUR} = 0.876; P_{DUR} = 0.123
	16DOc	*P. duriense*-like	DUR	ADM	Bx_{DUR} = 0.813; P_{OLI} = 0.163
	17DOc	*P. duriense*-like	OLI	ADM	Bx_{DUR} = 0.770; P_{DUR} = 0.216
	18DOc	DUR	OLI	P_{DUR}	P_{DUR} = 0.970
	19DOc	DUR	OLI	P_{DUR}	P_{DUR} = 0.864; Bx_{DUR} = 0.135
	21DOc	DUR	DUR	ADM	Bx_{DUR} = 0.925
	22DOc	DUR	DUR	ADM	P_{DUR} = 0.751; Bx_{DUR} = 0.243
	24DOc	DUR	DUR	P_{DUR}	Bx_{DUR} = 0.688; P_{DUR} = 0.305
	48DOc	*P. duriense*-like	DUR	ADM	Bx_{DUR} = 0.866; P_{DUR} = 0.048
	1008DOc	DUR	OLI	P_{DUR}	P_{DUR} = 0.911
	1012DOc	*P. duriense*-like	OLI	P_{DUR}	P_{DUR} = 0.992
	1016DOc	*P. duriense*-like	OLI	P_{DUR}	P_{DUR} = 0.997
	1022DOc	DUR	DUR	ADM	P_{DUR} = 0.960
N_{HYB} = 26		10	10	17	14 (Bx_{OLI} = 5 + Bx_{DUR} = 9)
Vouga					
Caima River *N* = 47	178VOa	*A. oligolepis*-like	DUR	ADM	Bx_{OLI} = 0.656; F_2 = 0.153
	179VOa	OLI	OLI	ADM	Bx_{OLI} = 0.728; F_2 = 0.142
	180VOa	*A. oligolepis*-like	DUR	ADM	Bx_{OLI} = 0.878; F_2 = 0.101
	182VOa	*A. oligolepis*-like	OLI	ADM	Bx_{OLI} = 0.533; F_2 = 0.251
	183VOa	*A. oligolepis*-like	DUR	P_{OLI}	P_{OLI} = 0.947
	184VOa	*A. oligolepis*-like	DUR	ADM	P_{DUR} = 0.710; Bx_{DUR} = 0.113
	186VOa	*A. oligolepis*-like	OLI	ADM	P_{OLI} = 0.851; Bx_{OLI} = 0.144
	187VOa	OLI	DUR	ADM	P_{OLI} = 0.609; Bx_{OLI} = 0.376
	188VOa	*A. oligolepis*-like	OLI	ADM	Bx_{OLI} = 0.887; P_{OLI} = 0.098
	cv3VOa	OLI	OLI	ADM	Bx_{OLI} = 0.737; P_{OLI} = 0.247
	cv4VOa	*A. oligolepis*-like	OLI	P_{OLI}	P_{OLI} = 0.630; Bx_{OLI} = 0.368
	cv6VOa	*A. oligolepis*-like	OLI	ADM	Bx_{OLI} = 0.727; P_{OLI} = 0.150
	cv9VOa	*A. oligolepis*-like	DUR	ADM	Bx_{OLI} = 0.731; F_2 = 0.217
	cv16VOa	OLI	OLI	ADM	Bx_{OLI} = 0.673; P_{OLI} = 0.276
	cv21VOa	*A. oligolepis*-like	DUR	P_{OLI}	P_{OLI} = 0.865; Bx_{OLI} = 0.133
	cv26VOa	*P. duriense*-like	DUR	ADM	Bx_{OLI} = 0.935
	cv14VOa	*P. duriense*-like	OLI	ADM	Bx_{OLI} = 0.903
	cv15VOa	DUR	OLI	ADM	Bx_{OLI} = 0.702; F_2 = 0.264
	cv19VOa	*P. duriense*-like	DUR	ADM	Bx_{OLI} = 0.874; F_2 = 0.116
	cv23VOa	DUR	DUR	ADM	P_{DUR} = 0.932
N_{HYB} = 20		14	8	17	13 (Bx_{OLI} = 13)
Total of introgressed		24	18	34	27

OLI, *Achondrostoma oligolepis*; DUR, *Pseudochondrostoma duriense*; P_{DUR}, pure *P. duriense*; P_{OLI}, pure *A. oligolepis*; F_1, first generation hybrid; F_2, second generation hybrid; Bx_{DUR}, F_1 – backcross with *P. duriense*; Bx_{OLI}, F_1 – backcross with *A. oligolepis*; ADM, admixture nature.

However, a comparable, albeit slightly smaller number of hybrids were detected in this analysis compared to STRUCTURE. Here, 27 individuals (21.4% of the total sample) were classified as backcrosses using the posterior probability threshold of 0.5 in both sampling sites (14 in Távora and 13 in Caima). No F_1 or F_2 categories were

found in any of the localities (Table 1). However, NEWHYBRIDS also revealed an interesting pattern in terms of the actual amount of introgression between the two species in the hybrid zones despite the high standard deviation values found. Thus, in the Távora River, the overall proportion of *A. oligolepis* nuclear genome introgressed into *P. duriense* was $Q = 6.9\%$ (SD = 9.2%), whereas introgression in the reciprocal direction was about one-half as much (3.8%, SD = 7.2%) (Fig. 3c). In the Caima River, the asymmetrical pattern was reversed and even more pronounced, with introgression of *A. oligolepis* genome into *P. duriense* in a proportion of $Q = 4.8\%$ (SD = 16.7%), and $Q = 14.9\%$ (SD = 18.6%) in the opposite direction (Fig. 3d).

mtDNA

The mtDNA genotype of 195 individuals was resolved with either sequencing or restriction analysis (Table S1). Mitochondrial introgression was found in both species but exclusively in rivers Távora (12.7% of the sample, 38.5% of hybrids) and Caima (12.8% of the sample, 30% of hybrids) (Table S1). However, despite their high similarity in overall levels of mtDNA introgression, both hybrid zones are remarkably different in terms of the direction of introgression. In the two hybrid zones, mtDNA of both species was found in individuals genetically identified as hybrids (based on microsatellite loci): in Távora, 57% of the introgressed individuals presented characteristic *P. duriense* haplotypes and 42% characteristic of *A. oligolepis*; in Caima, 38% presented *P. duriense* haplotypes and 62% *A. oligolepis*' ones. More specifically, in Távora, mtDNA is almost exclusively flowing from *A. oligolepis* towards *P. duriense*, because nine individuals with characteristic mtDNA haplotypes of *A. oligolepis* presented multilocus genotypes of *P. duriense* or $B\times_{DUR}$ hybrids, whereas only one individual was found in the opposite situation. On the other hand, in Caima, the flow of mtDNA introgression appears completely reversed, with six individuals with a *P. duriense* mtDNA haplotype presenting nuclear genotypes typical of *A. oligolepis* or $B\times_{OLI}$ hybrids. No individual was found in the opposite situation.

Genotype/phenotype correspondences

Under the adopted morphological characterization scheme (individuals exhibiting all morphological characters correspondent to either parental species are pure forms and individuals with an assortment of morphological characters of both parental species and/or intermediate forms are putative hybrids), pure individuals represented 88.5% of total sample and included all individuals from rivers Lima, Cávado, Ave, Paiva and Tâmega as well as the majority of individuals from Távora and Caima. Putative hybrids represented 11.5% of the total sample and were only found in Távora (12.6% of the local sample) and Caima (29.8% of the local sample) (Table 1). With a single exception, all individuals morphologically identified as putative hybrids (*P. duriense*-like or *A. oligolepis*-like) were confirmed as such genetically, either by discordance between markers (e.g. mtDNA vs. morphology/microsatellites), admixture at microsatellite multilocus genotypes or both (Table 1 and see Fig. S3 for a better visualization). Only one putative morphological hybrid (from Caima) did not show evidence of nuclear or mitochondrial introgression (cv4VOa, Table 1), although the support for its status as a genetically pure *A. oligolepis* was not very strong ($P_{OLI} = 0.630$). On the other hand, eleven individuals that presented an admixed genetic nature, i.e. a backcross multilocus genotype, did not show an evident morphological signature of that admixture, representing 26.9% and 20.0% of hybrids in Távora and Caima, respectively (Table 1). Furthermore, five hybrid individuals, all from Caima, show conflicting information about their hybrid status, with a multilocus genotype typical or mainly of one species but a morphology characteristic of the other (inds. 184VOa, cv26VOa, cv14VOa, cv15VOa, cv19VOa, Table 1). Finally, another striking discordance was found between mitochondrial and both microsatellites and morphological markers. Six individuals (five in Távora, one in Caima) show morphological and microsatellite evidence of being pure members of one species but a mtDNA haplotype characteristic of the other (inds. 8DOc, 12DOc, 18DOc, 19DOc, 1008DOc and 187VOa, Table 1).

Discussion

The study of natural hybrid zones provides a powerful means to further our understanding and predicting the consequences of gene flow between two species, namely, because they can contain a wide variety of genotypes that result from many generations of recombination (Barton, 2001). Yet, detecting introgressive hybridization can turn to be a very difficult task, especially when hybridizing taxa are closely related (i.e. lack of diagnostic polymorphisms) and when rates of hybridization are low. However, in this case, these difficulties appear less important because the deep divergence between the two species (i.e. about 11 MYA) provides a very convenient set of diagnostic morphological, mitochondrial and nuclear markers, and because hybrid individuals represent a large fraction of the hybrid zones (around 37%), suggesting extensive rates of introgressive hybridization.

However, the use of highly polymorphic and sensitive markers, such as microsatellites, should consider the resolution power of markers/analyses and the influence of within-species population structure, especially in freshwater fishes that present high rates of population differentiation between river basins (e.g. Mesquita *et al.*, 2005; Sousa *et al.*, 2008). Both issues have been raised in this study; by using HYBRIDLAB to find out the best cut-off values to distinguish parental and hybrid classes and

by comparing analyses performed at different geographical scales to detect any influence of intra-specific population structure. The second issue is of particular importance given the confounding effect it can have on the identification of hybrid individuals (Pritchard *et al.*, 2000; Anderson & Thompson, 2002). For instance, in this study, STRUCTURE analysis of multilocus data detects 51 admixed individuals in the hybrid zones when all samples are taken in consideration, but just 34 when only samples from Távora and Caima are included, revealing that intra-specific population structure can influence the assessment of hybrids status. This was overcome by performing our analyses per hybrid zone.

Also, the estimation of the actual rate of hybridization proved to depend highly on the markers used. For instance, although 46 hybrids are detected in the whole sample using all the evidence, only 18 (39.1% of 46) (mtDNA), 24 (52.2%) (morphology), 27 (58.7%) (NEWHYBRIDS) and 34 (73.9%) (STRUCTURE) hybrids were detected using each marker or analysis, representing almost a two-fold difference between the extreme estimations.

Similar disagreements among markers have been found in the fish hybridization literature and have raised a caution flag for the necessity of multi-marker approaches to the study of hybridization (reviewed in Scribner *et al.*, 2001). However, marker discordance can be informative about ecological and genetic processes. For instance, large differences between nuclear and mitochondrial markers usually provide clues about sex-related differences on the rate of introgressive hybridization (e.g. Prugnolle & de Meeus, 2002). Likewise, large differences between morphological and genetic estimations can sometimes be used to infer selective processes in nature (e.g. Arias *et al.*, 2008; Gay *et al.*, 2008).

General patterns of introgression

Pseudochondrostoma duriense and *A. oligolepis* introgressively hybridize in both Douro (Távora) and Vouga (Caima) river basins. The classification of individuals in these hybrid zones shows that they belong to either parental or backcross classes, without evidence of F_1 or F_2 hybrids. As simulations showed that F_1 and F_2 individuals could be easily detected, their absence in the hybrid zones is not an artefact of the study and must reflect an underlying biological phenomenon. Lack of first generation hybrids has been described in several hybrid zones and is often associated with selection against hybrids (e.g. Saavedra *et al.*, 1996). The proportion of F_1s is expected to be reduced given that this class is the most distant from both parentals and usually suffers more from fitness reductions associated with behavioural (e.g. Bridle *et al.*, 2006; Svedin *et al.*, 2008), genetic (i.e. Haldane's rule; Turelli & Orr, 1995) and ecological factors (e.g. Sambatti *et al.*, 2008). In fact, our results fit a growing body of empirical data suggesting an ample opportunity for introgression despite a lack of evidence for F_1 hybrids (e.g. Goodman *et al.*, 1999; Berthier *et al.*, 2006; Arias *et al.*, 2008).

The distribution of parental and hybrid genotypes indicates then that this hybrid zones correspond to the bimodal type, i.e. the majority of individuals belong to distinct genetic clusters associated with the two parental or parental-like genotypes (Jiggins & Mallet, 2000). Among freshwater fishes, bimodal hybrid zones are believed to be the consequence of strong, although incomplete, prezygotic isolation including the occupation of distinct microhabitats (e.g. Bierne *et al.*, 2002; Crespin *et al.*, 2002), reproductive behaviour (Wirtz, 1999; McDonald *et al.*, 2001; Costedoat *et al.*, 2007) and gamete recognition (Palumbi, 2009); intrinsic or extrinsic postzygotic isolation (e.g. Rogers & Bernatchez, 2006) or both. The presence of backcrosses with q-values close to the thresholds (i.e. second generation backcrosses) in the *A. oligolepis* × *P. duriense* hybrid zones indicates that F_1 and first generation backcrosses hybrids must be, at least, partially viable and fertile. This suggests that reproductive isolation in this case must be associated mainly with ecological factors causing prezygotic or extrinsic postzygotic barriers to reproduction. Potential sources for such barriers between these two species can include microhabitat specialization and/or spawning asynchrony. For instance, *P. duriense* individuals are known to feed on the stony bottoms in the middle of the channels and perform upstream migrations during the breeding season, whereas *A. oligolepis* are found mostly under riparian vegetation on banks and present slightly different maturation timings (Collares-Pereira & Coelho, 1983). Whether these differences are important enough to drive prezygotic isolation between these fishes is at present unknown but should be further explored because similar features have been shown to contribute to isolation in other fish species (e.g. salmonids; Taylor, 2004).

On another hand, our mitochondrial results show that there is a significant amount of bidirectional mtDNA introgression between the two species. This result is in direct opposition with Gante *et al.* (2004), who found no evidence of mitochondrial introgression on a sample of ten fish collected from Távora. The difference between our study and Gante *et al.* (2004) can very probably be attributed to the small sample size of the latter. In fact, assuming that the proportion of mtDNA hybrids in Távora has not changed in the last 5 years, the probability of *not* finding a hybrid in a random sample of ten individuals from this zone is 0.26. This highlights once again the necessity for appropriate sample sizes when conducting population genetic studies.

Moreover, our results also show that more than a third of the mtDNA introgressed hybrids did not present any evidence of microsatellite introgression, suggesting that mitochondrial markers from one species can sometimes

be transferred into another as a result of hybridization events that can no longer be detected by nuclear markers. The presence of individuals with this kind of nuclear-mtDNA discordance suggests therefore that hybrid females do not suffer from strong fitness reductions. For instance, after just three generations of female backcrossing to one species, an average of 6.25% of the nuclear genome of hybrids will have an origin in the other, yet their mtDNA will be foreign (e.g. Goodman *et al.*, 1999). This can lead to extreme cases, like a 'capture' of mtDNA of another species, as in the brook trout (*Salvelinus fontinalis*) from Lake Alain (Québec, Canada) where Bernatchez *et al.* (1995) have found that all individuals possessed the mtDNA of *Salvelinus alpinus* (Arctic char) or a case of mtDNA 'inheritance from the rare species' phenomena as proposed by Costedoat *et al.* (2007) for *Chondrostoma toxostoma* × *Chondrostoma nasus* hybrid zones.

It is also worth mentioning that this study shows that only a fraction of the individuals genetically identified as hybrids show indeed some assortment of the parental and/or intermediate traits we use to identify these species in the field and can therefore undoubtedly be morphologically considered as hybrids. The remaining individuals exhibit morphological variation that overlaps considerably with either pure species. Such a pattern is strongly indicative of significant rates of introgressive hybridization and it is expected as a result of high levels of backcrossing between pure and introgressed individuals (Campton, 1987). However, as a consequence, a substantial fraction of hybrid individuals cannot be reliably distinguished from pure fishes on the basis of external morphological traits alone. This highlights the necessity for a genetic identification of 'pure' *A. oligolepis* and *P. duriense* specimens before they can be used in any sort of studies in which a precise classification or the genetic integrity of individuals is important.

Different patterns of introgression in two independent hybrid zones

Despite the bidirectional and asymmetric nature of the introgressive hybridization found in both hybrid zones they presented distinct patterns of introgression. In the Távora hybrid zone, molecular markers suggested a higher introgression rate of *A. oligolepis* into *P. duriense* and cyto-nuclear discordances almost invariably involve *P. duriense* or *P. duriense*-like individuals with an *A. oligolepis* mtDNA, suggesting that mtDNA introgression is highly asymmetric. On another hand, in the Caima zone, all backcross hybrids were in the direction of *A. oligolepis*, whereas mtDNA of both species was found in virtually the same proportions among *A. oligolepis/A. oligolepis*-like and *P. duriense/P. duriense*-like individuals. In general, these asymmetries in hybrid zones are considered to be the consequences of intrinsic attributes of the whole species that rarely or never depend on the geographical set-up such as partial hybrid sterility, biased survival of hybrids (Chenouil *et al.*, 2004) or biased assortative mating and sexual selection (Meyer *et al.*, 2006). However, more recently, several features of hybrid zones have been shown to vary geographically, which suggests that some of the underlying processes somehow depend or interact with varying features of the environment (e.g. Nolte *et al.*, 2009). The two species studied here apparently exhibit different ecological adaptations to local environmental conditions, but nothing is known about the response of these fishes, or their hybrids, to changes in the environment. Iberian river habitats can vary dramatically at the local level, probably inducing heterogeneous adaptation patterns of populations (Sousa *et al.*, 2008). Such changes in habitat conditions have been shown to be important motors for the dynamics of hybrid zones and be responsible for evolutionary changes at both spatial and temporal scales (e.g. *Gila*, DeMarais *et al.*, 1992; *Cottus*, Nolte *et al.*, 2006). Moreover, this study shows that intra-specific population structure between river basins exists in these fishes, particularly for *A. oligolepis*. Genetic differences between populations as a result of historical processes are also known to influence introgressive hybridization (Dowling *et al.*, 1997) and can be especially important in the case of multiple, independent areas of hybridization, like in different river basins, each not only with its own distinctive environmental features but also with their unique evolutionary history (Nolte *et al.*, 2009).

Conclusions

This study revealed that *P. duriense* and *A. oligolepis* introgressively hybridize more extensively then previously believed and with an outcome that depends on the specific set-up of the independent hybrid zones. Although at the moment, we cannot fully explain the causes and consequences of these processes, these results open new questions for future studies and experimental work. Departures from expected genotype frequencies show this is a good system to study adaptation and the different patterns of hybridization found raise questions related to the reproductive isolation mechanisms and their action between these two species.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Admixture analysis of simulated hybrids.

Figure S2 Intraspecific population structure analysis.

Figure S3 Visualisation of genotype/phenotype correspondences.

Table S1 MtDNA variability.

Table S2 Microsatellite loci characterization.

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