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ORIGINAL ARTICLE

Introgressive hybridization as a promoter of genome reshuffling in natural homoploid fish hybrids (Cyprinidae, Leuciscinae)

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Understanding the mechanisms underlying diversification and speciation by introgressive hybridization is currently one of the major challenges in evolutionary biology. Here, the analysis of hybridization between two pairs of Iberian Leuciscinae provided new data on independent hybrid zones involving *Achondrostoma oligolepis* (AOL) and *Pseudochondrostoma duriense* (PDU), and confirmed the occurrence of hybrids between AOL and *Pseudochondrostoma polylepis* (PPO). A multilevel survey combining morphological, genetic and cytogenomic markers on a vast population screening successfully sorted the selected fishes as admixed. Results were similar in both AOL × PDU and AOL × PPO systems. Overall, hybrid morphotypes, cytogenomic data and genetic profiling indicated preferential backcrossing and suggested AOL as a major genomic contributor. Moreover, results implied AOL as more permissive to introgression than PDU or PPO. Although PDU- and PPO-like individuals appeared more resilient to genome modifications, AOL appeared to be more involved and affected by the ongoing hybridization events, as chromosomal translocations were only found in AOL-like individuals. All hybrids analysed evidenced extensive ribosomal DNA (rDNA) polymorphism that was not found in parental species, but usually seen falling within the range of possible parental combinations. Yet, transgressive phenotypes that cannot be explained by normal recombination, including more rDNA clusters than expected or the occurrence of syntenic rDNAs, were also detected. Present results proved rapid genomic evolution providing the genetic novelty for species to persist. In addition, although the ultimate consequences of such apparently extensive and recurrent events remain unknown, modern genome-wide methodologies are of great promise towards answering questions concerning the causes, dynamics and impacts of hybridization.
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INTRODUCTION

Introgressive hybridization acts as a promoter of diversification and speciation in animals (Dowling and Secor, 1997; Mallet, 2005), and it was suggested to potentially induce more substantial genomic rearrangements and rapid genome repatterning than gene and/or genome duplications (reviewed in Mable, 2013). Hybrids are seen as triggers of transposition that may account for the genetic instability and extensive genome repatterning following hybridization (Fontdevila, 2005; Abbott *et al.*, 2013). Chromosomes, allowing for a macro-examination of the genome, may afford for stronger evidences of hybridization and genome reorganization, given the right tools such as fluorescence *in situ* hybridization (FISH) with specific probes or comparative genomic hybridization (CGH) (Phillips and Reed, 1996; Dobigny and Yang, 2008).

Natural introgression is commonly found among freshwater fishes, particularly in Cyprinidae (Scribner *et al.*, 2001), where several cases have been comprehensively evaluated (Gerber *et al.*, 2001; Costedoat *et al.*, 2007; Aboim *et al.*, 2010; Broughton *et al.*, 2011). The different outcomes witnessed (reviewed in Abbott *et al.*, 2013) reinforce the opportunity of using cyprinid fish to search for genomic signatures of hybridization. Intergeneric hybrids involving the Iberian arched-mouth *Achondrostoma oligolepis* (AOL) and the straight-mouth nase *Pseudochondrostoma duriense* (PDU) were identified in the past (Steindachner, 1866; Almaça, 1965; Collares-Pereira and Coelho, 1983; but see Gante *et al.*, 2004). These once congeneric chondrostomines (Robalo *et al.*, 2007) are broadly sympatric (Figure 1) and seem to actively hybridize, contributing to the establishment and maintenance of several independent hybrid zones (HZs) with different levels of asymmetric bidirectional introgression (Gante *et al.*, 2004; Aboim *et al.*, 2010). In addition, hybridization between AOL and another straight-mouth nase *Pseudochondrostoma polylepis* (PPO) has long been suspected (Almaça, 1965; Collares-Pereira and Coelho, 1983), although it remained unexplored.

The pure parental species are believed to have diverged around 11 million years ago (Aboim *et al.*, 2010), representing distinct genetic and morphologic groups (Collares-Pereira and Coelho, 1983; Aboim *et al.*, 2010). Earliest cytogenetic characterization confirmed great karyotype similarity ($2n = 50$, 6–7 metacentric (m) + 15–16

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submetacentric (sm) + 3 subtelo-acrocentric (st/a) chromosome pairs) (Pereira *et al.*, 2009), and only recently diagnostic chromosome markers were designated for them (Pereira *et al.*, 2012). PDU and PPO were characterized by a small and stable number of ribosomal DNA (rDNA) clusters, contrasting with the polymorphisms observed in AOL (Pereira *et al.*, 2012). Still, there are few studies using in-depth molecular cytogenetic analyses on natural fish hybrids (Fujiwara *et al.*, 1997; Zhu *et al.*, 2006; Gromicho *et al.*, 2006b; Hashimoto *et al.*, 2012; Rampin *et al.*, 2012).

Given the heterogeneous patterns of introgression previously found on HZs comprising these species (Gante *et al.*, 2004; Aboim *et al.*, 2010), present work integrates, for the first time, cytogenetic data with morphological and robust genetic profiling on AOL × PDU and putative AOL × PPO natural hybrids, in order to better understand the impact of the successful intergeneric hybridization process on such genomes. Using a multidisciplinary approach, we widened and reinforced previous analyses based on one or few feebler markers (Collares-Pereira and Coelho, 1983; Gante *et al.*, 2004; Aboim *et al.*, 2010), while testing for their value in depicting genome introgression in similar situations. Moreover, obtained results showed rapid genome modifications and instability not always explained by normal recombination, emphasizing the role of admixture events in generating genetic novelties for species (old or new) to thrive under particular spatial contexts.

MATERIALS AND METHODS

Fish sampling

Information regarding the specimens used in this study is summarized in Table 1 (see also Figure 1 and Supplementary Material). A total of 200 individuals were captured by electrofishing in years 2007 and 2008 (see Supplementary Material). Genetic and morphological profiling was performed to retrieve potential hybrids for further analyses (see Supplementary Material). The successful cytogenetic characterization dictated sample size and selection of a total of 13 hybrid individuals from Douro (4), Vouga (6) and Mondego (3) basins (Table 1). Specimens were killed by an overdose of anesthetic MS-222, fixed in formalin and preserved in 70% ethanol for deposition in the Ichthyological Collection at MNHNC (Lisbon, Portugal). All manipulations were performed in accordance with Portuguese guidelines and regulations regarding animal welfare and experimentation.

Morphological analysis

Upon recurrent backcrossing, hybrids tend to become indistinguishable from parental forms (Mallet, 2005). Representatives of *Achondrostoma* and *Pseudochondrostoma* genera exhibit clear morphological differences that are easily recognizable at first sight (Collares-Pereira, 1979; Coelho, 1985; see Supplementary Material), and intermediate forms of diagnostic traits can be used to distinguish hybrid morphotypes (Collares-Pereira and Coelho, 1983). Therefore, a specific set of morphological characters (shape and position of the mouth, presence of a corneous lower lip, number of scales in the lateral line and number of gill-rakers) were used in a primary approach of classification (see Supplementary Material). Specimens with an assortment of both parent-specific morphological characters and/or intermediate patterns were assigned as putative hybrids (Supplementary Table S1). Depending on their general phenotype and main parental characters' contribution, hybrids were classified and hereafter designated as AOL-like, PDU-like or PPO-like.

Genetic profiling

A considerable fraction of these hybrids cannot be reliably distinguished from pure parental species based on morphology alone (Aboim *et al.*, 2010). For this reason, a genetic analysis on all 200 individuals was also included (see Supplementary Material), based on molecular markers previously developed for the parental species—mtDNA *cyt b* gene, 10 microsatellite loci (Aboim *et al.*, 2010) and the single-copy *RAG-1* nuclear gene (recombination activating gene 1) (Aboim *et al.*, 2009). Representative sample sizes of each parental species per population/river/basin were considered, as well as the independent

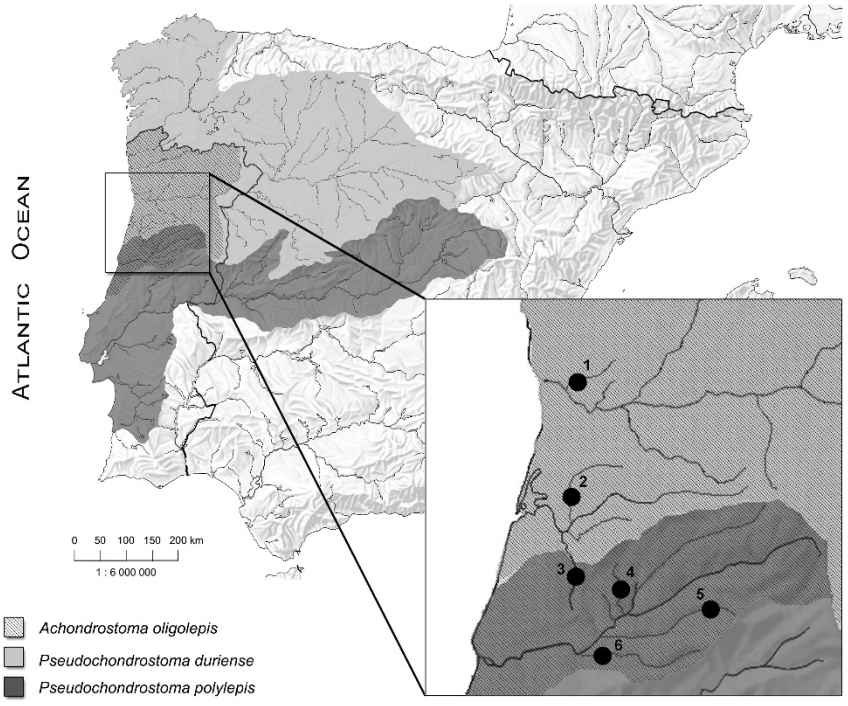

Figure 1 Sampling locations ● across the distribution ranges of AOL, PDU and PPO in the Iberian Peninsula. Sampling points: 1—Sousa River (Douro basin), 2—Caima River (Vouga basin) representing AOL × PDU HZs; 3—Serra River (Vouga basin), 4—Mortágua River, 5—Alva River and 6—Ceira River (Mondego basin) representing AOL × PPO HZs.

and heterogeneous nature of the already known HZs. Individuals from Caima River were characterized earlier by Aboim *et al.* (2010); the remaining ones were profiled using the same methods, and all of them were classified into one of the following classes: pure parental species, F_1, F_2, $B_{\times AOL}$, $B_{\times PDU}$ or $B_{\times PPO}$ backcrosses (Supplementary Table S2; see also Supplementary Material).

Table 1 Specimens analysed in this study

Hybrid system	*Basin*	*Tributary*	*Sampling point* (Figure 1)	*ID code*	*Sex*	*Capture date*
AOLxPDU	Douro	Sousa River	1	cs3	♀	2008
				cs20	♂	
				cs19	♀	
				cs24	♂	
	Vouga	Caima River	2	cv23	n.d.	2007
				cv26	n.d.	
				cv14	♂	
				cv21	♂	
				cv16	♂	
AOLxPPO		Serra River	3	cv39	♂	2008
		Mortágua River	4	zd20	♀	
	Mondego	Alva River	5	zd52	♀	2007
		Ceira River	6	zd61	♀	
			Total	13		

Abbreviations: AOL, *Achondrostoma oligolepis*; ♀, female; ♂, male; n.d., not determined; PDU, *Pseudochondrostoma duriense*; PPO, *Pseudochondrostoma polylepis*.
Information on origin, sex and date of collection is given.

Cytogenetic analysis

Chromosome suspensions were obtained from standard kidney preparations and stored at −20 °C until further processing. Slides were thermally aged, digested with pepsin and RNase and immediately used for the following procedures. FISH experiments used 5S and 45S rDNA sequences (Pereira *et al.*, 2012) and the $(TTAGGG)_n$ pool of sequences (Ijdo *et al.*, 1991) as probes in either dual-colour or independent procedures. In addition, genomic DNA of AOL and PDU were used in the proportion of 1:1 for the CGH protocol. All probes were differentially labelled by nick translation with biotin-16-dUTP or digoxigenin-11-dUTP according to the manufacturer's recommendations. All *in situ* hybridizations were performed as detailed by Pereira *et al.* (2012), with the exception that CGH probes were allowed to pre-anneal (37 °C, 1 h). Slides selected for sequential analysis of constitutive heterochromatin (after FISH) were washed in 4 × SSC/0.01% Tween 20 (pH 7.0) and dehydrated in ethanol series. C-banding improved with DAPI counterstaining followed Pereira *et al.* (2009).

Chromosomes were observed under the fluorescence-equipped microscope Olympus AX70 coupled to a DP30VW (Olympus, Center Valley, PA, USA) CCD camera. Digital greyscale images were processed for contrast manipulation, over-layering and pseudo-coloration, affecting the whole image as one. At least 10 complete metaphases were analysed per individual. Karyotypes were arranged with chromosomes organized by decreasing sizes in three categories according to centromere position: m, sm and st/a (Levan *et al.*, 1964).

RESULTS

The combination of distinct methods for hybrid identification and classification detected signatures of introgression in all the 13 cytogenetically analysed individuals (Table 2 and Supplementary Material). All specimens presented diploid chromosome numbers of $2n = 50$ and grossly similar karyotypes. Molecular cytogenetic data were overall able to retrieve the non-pure origin of the individuals

Table 2 Summary table with the characterization of the AOL × PDU and AOL × PPO hybrids included in this study

ID code	*Phenotype*[a]	*Genotype*[b]	*Cytogenetic characterization*						*Corresponding figures*
			45S rDNA sites		*5S rDNA sites*		*Syntenic units*		
			Total	*Chromosome distribution*	*Total*	*Chromosome distribution*	*Total*	*Chromosome distribution*	
cs3	**AOL-like**	**Admixed**	**4**	3 sm + **1 st/a**	7	4 st/a + 2 sm + 1 m	**1**	1 st/a	Figure 4
cs20	AOL	**Admixed**	6	4 sm + 2 i sm	8	4 st/a + 2 sm + 2 m	0		Figure 2a
cs19	**AOL-like**	**Admixed**	6	6 sm	8	4 st/a + 2 sm + 2 m	0		Figure 2b
cs24	AOL	**Admixed**	**7**	4 sm + 1 i sm + **2 m**	8	4 st/a + 2 sm + 2 m	**2**	2 m	Figure 2c
cv23	**PDU-like**	PDU	**4**	4 sm	4	4 st/a	0		Figure 3a
cv26	**PDU-like**	**Admixed**	**4**	4 sm	6	4 st/a + 1 sm + 1m	0		Figure 3b
cv14	**PDU-like**	**Admixed**	**4**	4 sm	8	4 st/a + 2 sm + 2 m	0		Figures 3c and 5
cv21	**AOL-like**	**Admixed**	6	5 sm + **1 st/a**	6	4 st/a + 1 sm + 1 m	**1**	1 st/a	Figure 3d
cv39	AOL	AOL	6	3 sm + **1 m** + **2 st/a**	8	4 st/a + 2 sm + 2 m	**3**	1 m + 2 st/a	Figure 3e
cv16	**AOL-like**	**Admixed**	**8**	6 sm + **1 m** + **1 st/a**	8	4 st/a + 2 sm + 2 m	**2**	1 m + 1 st/a	Figure 3f
zd20	**PPO-like**	PPO	**5**	4 sm + 1 i sm	**5**	4 st/a + 1 m	0		Figure 2d
zd52	AOL	AOL	6	3 sm + 2 i sm + **1 m**	8	4 st/a + 2 sm + 2 m	**1**	1 m	Figure 2e
zd61	**AOL-like**	AOL	6	4 sm + **1 m** + **1 st/a**	8	4 st/a + 2 sm + 2 m	**2**	1 m + 1 st/a	Figure 2f
Parental species	AOL	AOL	6	4 sm + 2 i sm	6–8	4 st/a + (1–2) sm + (1–2) m	0		Pereira *et al.*, 2012
	PDU	PDU	3	3 sm	4	4 st/a	0		
	PPO	PPO	4	4 sm	4	4 st/a	0		

Abbreviations: AOL, *Achondrostoma oligolepis*; i, interstitial; m, metacentric; PDU, *Pseudochondrostoma duriense*; PPO, *Pseudochondrostoma polylepis*; rDNA, ribosomal DNA; sm, submetacentric; st/a, subtelo-acrocentric.
Information on phenotype, genotype, number and chromosome location of rDNA clusters (chromosome type: m, sm and st/a; chromosome region: i = interstitial, otherwise terminal in the p arm) is given. General characterization of the respective parental species is included in the last rows. Discrete indicators of non-pure origin are highlighted in bold; the presence of at least one of these markers was considered enough to suspect admixture.
[a]After detailed morphological analysis.
[b]After detailed genetic profiling (see Supplementary Material).

under analysis, even in cases for which morphology and/or genetic profiling were not proficient in detecting introgression (cv23, cv39, zd20, zd52 and zd61), with two exceptions (cs19 and cs20) (Table 2).

The simultaneous mapping of both rDNAs demonstrated new rDNA profiles and noticeable chromosome rearrangements when compared with parental genomes (Pereira *et al.*, 2012). An assortment of combinations (number of clusters and chromosome location) was identified throughout the sample (Table 2; Figures 2–4a and b). Although 5S rDNA was mostly associated with pericentromeric regions, interstitial signals of 45S rDNA were often difficult to discriminate (Figures 2a and c–e). Numeric polymorphisms of both rDNA clusters ranged from four or five to eight FISH-positive signals, in agreement with parental profiles (Pereira *et al.*, 2012). In general, PDU- (Figures 3a and c) and PPO-like genomes (Figure 2d) exhibited a lower number of signals, namely concerning 45S rDNA clusters. Genomes of AOL-like individuals revealed rDNA phenotypes with higher number of positive signals for both probes (Figures 2a–c, e, f, 3d–f and 4a, b), representing the most common combination of six clusters of 45S rDNA and eight of 5S rDNA (6/8). This profile was found in both AOL × PDU and AOL × PPO systems, even though it had not been observed in individuals from Caima River (Vouga basin). In addition, one AOL-like specimen from Sousa River (cs24) and another from Caima River (cv16) HZs evidenced unexpected rDNA phenotypes with seven (Figure 2c) and eight (Figure 3f) positive signals of 45S rDNA, respectively.

The first hints of genome reorganization were evidenced by the translocation of a big cluster of 45S rDNA to chromosome number 23 (Figures 2f, 3d–f and 4b) and/or by the presence of other syntenic associations of both 5S and 45S gene clusters (Figures 2c, e, f and 3e and f). Such translocations or associations were not observed in the parental species despite a relatively good sample coverage ($N = 15$; Pereira *et al.*, 2012), nor in PDU- or PPO-like specimens, but only in AOL-like hybrids (Table 2). Distinct syntenic combinations could be consistently recognized in one or two large st/a chromosomes and/or in a pair of small m chromosomes, although never exceeding three syntenic units per metaphase (Figure 3e). In addition, no rDNA profile or the incidence of translocations seemed to correlate with any particular genomic composition or with sex.

Telomeric $(TTAGGG)_n$ repeats were mostly located at the terminal regions of each chromatid (Figure 4c) as expected. Interstitial telomeric sites, which have been documented as a 'footprint' of recent chromosomal rearrangements (Phillips and Reed, 1996), were rarely detected but seemed to be present beneath the bigger units of 45S rDNA (numbers 10 and 23 in Figure 4c).

Similarly to the pure parental species (Pereira *et al.*, 2009), constitutive heterochromatin (C-bands) was confined to most centromeres and very few other non-centromeric regions (Figure 5) that appeared as AT-rich DAPI-positive regions (Figures 2 and 3). Centromeric bands were also more pronounced in some pairs of chromosomes from each category (about 3 to 4 m, 10 sm and 2 st/a pairs) (Figure 5). After sequential analysis, C-bands were found to be intimately associated with 5S rDNA clusters but scarcely present in

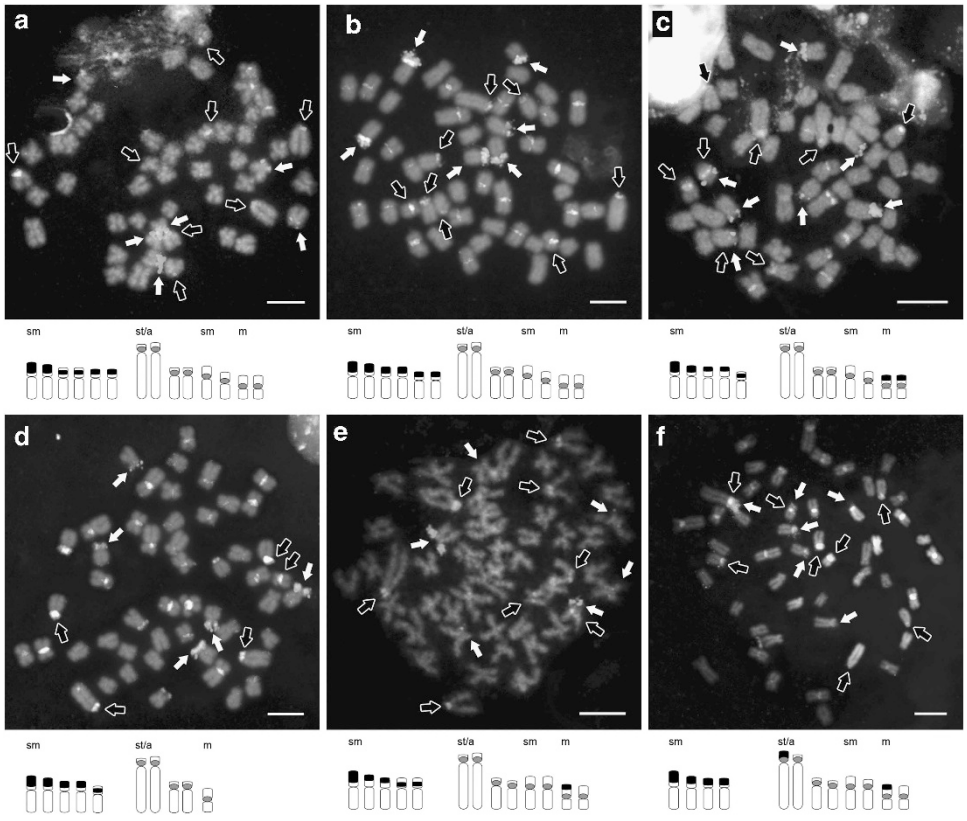

Figure 2 Simultaneous mapping of 5S (red probe, hollow arrows) and 45S rDNA (green probe, white arrows) clusters in specimens with admixed origin from (**a**–**c**) Douro (AOL-like) and (**d**–**f**) Mondego (one PPO- and two AOL-like) basins. Below each metaphase is a partial (unscaled) ideogram of the chromosomes bearing 5S (grey circle ●) and 45S rDNA (black box ■) gene clusters. Note the strong DAPI-positive bands at centromeres and other heterochromatic regions (white). Bar = 5 μm . A full color version of this figure is available at the *Heredity* journal online.

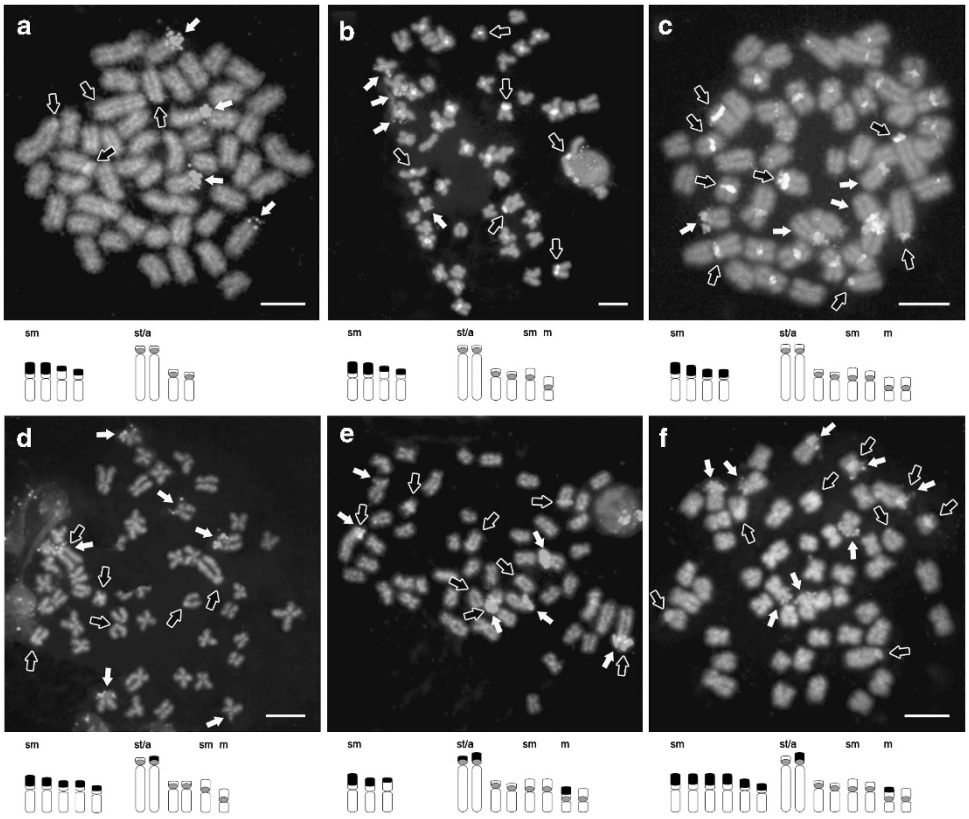

Figure 3 Dual-colour FISH with specific 5S (red probe, hollow arrows) and 45S rDNA probes (green probe, white arrows) in metaphases of (**a–c**) PDU-like and (**d–f**) AOL-like specimens from Vouga basin. Below each metaphase is an (unscaled) ideogram of the rDNA-bearing chromosomes (grey circle ● = 5S, black box ■ = 45S). Note the strong centromeric DAPI-positive bands (white). Bar = 5 μm. A full color version of this figure is available at the *Heredity* journal online.

some of the 45S rDNA-bearing chromosomes (for example, number 12 in Figure 5). In addition, 45S rDNA chromosome regions appear as DAPI-negative and were hardly detected after C-banding (numbers 10 and 12 in Figure 5).

CGH analyses provided good results (that is, hybridization patterns comparable between metaphases) for a single AOL-like specimen from Sousa River (Douro basin; cs3). Genomic DNAs of both pure parental species (AOL and PDU) were simultaneously hybridized on chromosomes of the hybrids, trying to quantify each specific contribution to the resultant genome. Hybridization signals were not uniform along the chromosomes, being strongest at specific regions where probes overlapped at least partially (Figure 4d), and seemed to match bands of constitutive heterochromatin (Figure 5). This result suggests that some of these bands may in fact be shared by both parental species (generally centromeric), most likely resultant from shared dispersed repetitive sequences and/or denoting common ancestry to some extent. Painted chromosomes could not be exclusively assigned to one or the other parental species; however, overall, chromosomes were mostly evidenced by the AOL genomic probe, suggesting a greater genomic identity to AOL, which is consistent with its morphotype and genotype (Table 2). However, it was possible to recognize a small number of species-specific bands from each parental species (single probe colour) either colocalized to the same chromosome and/or only present in one of the chromosome homologues (Figure 4d), revealing intergenomic recombination with PDU. Besides the obvious translocation of an rDNA segment to one of the large st/a chromosomes (Figure 4b), CGH results allowed the identification of additional possible translocations easily identifiable at the most heteromorphic pairs—perceptible in pair numbers 8, 10 and 11 (Figure 4d).

DISCUSSION

Multilevel characterization of HZs

This investigation provided a multilevel survey on natural homoploid hybrids. New data on independent HZs involving AOL × PDU were endowed, while confirming the occurrence of AOL × PPO hybrids (Figure 1). Previous works strongly expressed the necessity of using multiple approaches when studying natural hybrids (Gerber *et al.*, 2001; Scribner *et al.*, 2001; Gante *et al.*, 2004; Gromicho and Collares-Pereira, 2007; Mavárez and Linares, 2008; Lajbner *et al.*, 2009), especially when second-generation crossing might be occurring, as it seems to be the case (Aboim *et al.*, 2010). The combination of three different identification methods and a vast population screening data set allowed to successfully sort all the selected 13 individuals as admixed, whereas, if using each method independently, some hybrids were probably untraceable (Table 2 and see also Supplementary Material), as observed in Serra R. where hybrids were undetected by genetic markers (Supplementary Figure S1). Identifying hybrids on the basis of morphology alone may be challenging owing to ancestral polymorphisms, preferential backcrossing and intraspecific plasticity (Dowling and Secor, 1997; Mallet, 2005; Mavárez and Linares, 2008). Genetics might be more precise, but its resolution power is limited by the number of diagnostic markers available, their genomic coverage and also by intraspecific population structure. Chromosomes, on the other hand, provided herein solid evidences of hybridization, particularly significant given the gross

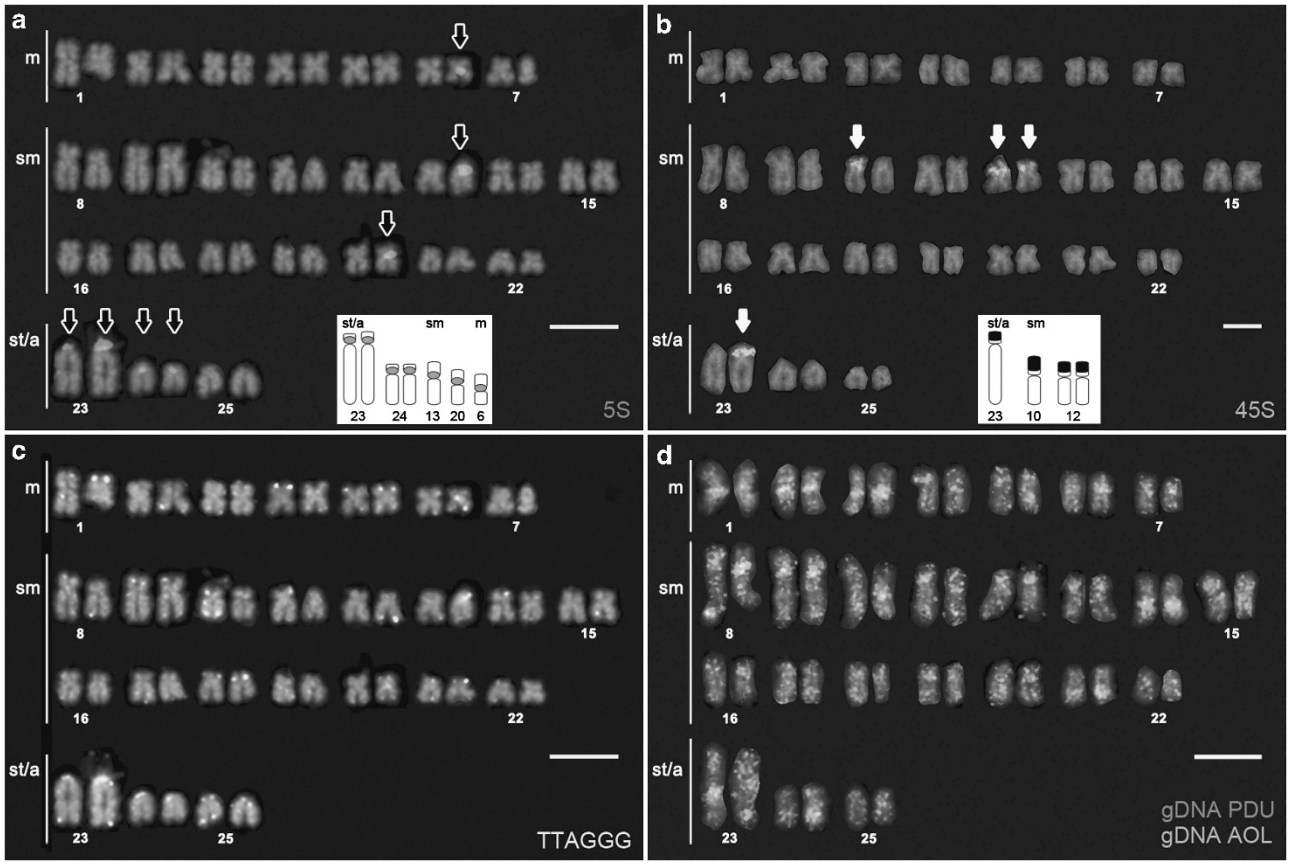

Figure 4 Karyotype of a female AOL-like hybrid from Douro basin after (**a**) FISH with 5S (red probe, hollow arrows) and (**b**) 45S rDNA (green probe, white arrows) sequences, both including inset ideograms of the rDNA-bearing chromosomes (grey circle ● = 5S, black box ■ = 45S); (**c**) FISH with telomeric $(TTAGGG)_n$ repeats and (**d**) CGH using AOL and PDU genomic DNA as probes. Genetic admixture can be retrieved from non-matching bands between homologue pairs after CGH, particularly in marker chromosomes numbers 8, 10 and 23. Bar = 5 μm. A full color version of this figure is available at the *Heredity* journal online.

chromosome homeology empirically estimated. In fact, observed translocations were crucial in distinguishing hybrid rDNA profiles that seemed to overlap those of pure parental species (Figures 2e, f, and 3d, e).

The extent of hybridization

Hybridization may have multiple effects at different stages and spatial contexts (Gerber *et al.*, 2001; Costedoat *et al.*, 2007; Broughton *et al.*, 2011; Abbott *et al.*, 2013). The already surveyed HZs of AOL × PDU seem to behave differently (Aboim *et al.*, 2010) with character displacement happening towards one or the other parental species (Collares-Pereira and Coelho, 1983; Gante *et al.*, 2004; Aboim *et al.*, 2010). Morphological similarities to one of the parental species are presumed to reflect its major genomic/genetic contribution during the introgression process (Mallet, 2005; Mavárez and Linares, 2008), and present data allude indeed to that presumption. Specifically, altogether, hybrid morphotypes (Supplementary Table S1), molecular cytogenetic data (Figures 2–4) and the genetic profiles (Supplementary Figure S1 and Supplementary Table S2) point to preferential backcrossing, as initially suspected (Gante *et al.*, 2004). This may be correlated to both genetic attractiveness/compatibility and/or to the relative densities of the parental species within each HZ.

The conservative and widespread karyotype pattern of leuciscines (Ráb and Collares-Pereira, 1995) does not allow determining the directionality of introgression (but see Gante *et al.*, 2004), nor discerning parental contributions to the hybrid genomes by means

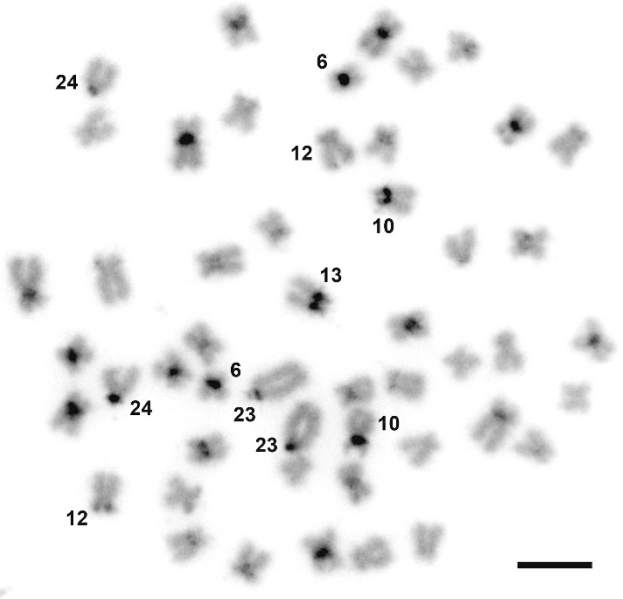

Figure 5 C-banding improved with DAPI counterstaining on a metaphase of a male PDU-like hybrid from Vouga basin evidencing mostly centromeric bands of constitutive heterochromatin. Chromosome pairs numbers 6, 10, 12, 13, 23 and 24 are numbered for better paralleling with sequential banding results. Bar = 5 μm.

of conventional cytogenetic markers (that is, 2*n*, NF, karyotypes). Not even the major macrostructural differences between parental species (Pereira *et al.*, 2009) could be unambiguously correlated to any hybrid morphotype or genotype. On the other hand, molecular cytogenetic results imply AOL to be more permissive to introgression—either more vulnerable or flexible (that is, prone to hybridize)—than each of the co-occurring and more widespread sister-species PDU or PPO. Irrespective of the hybrid system it engages, not only does AOL appear to be largely involved but also greatly affected by the ongoing hybridization events, as chromosomal translocations were only found in AOL-like individuals. Conversely, PDU- and PPO-like individuals appear to be more resilient to genome modifications, apparently retaining the original genome organization while simply inheriting rDNA-bearing chromosomes from each parent (Table 2). Backcrosses with PDU/PPO were also less frequently detected by the genetic assessment (Supplementary Figure S1 and Supplementary Table S2). Similarly, AOL-like morphotypes are strikingly more evident at first sight than PDU- or PPO-like morphotypes, owing to the detection of a corneous lower lip otherwise absent in *Achondrostoma sp.* These results seem conflicting with previous works on hybridizing cyprinids where distinct HZs represented different outcomes of independent hybridization processes (Costedoat *et al.*, 2007; Aboim *et al.*, 2010; Broughton *et al.*, 2011), which means that either the environmental pressure is identical in the HZs considered here (unlikely) or that the endogenous (genetic) selection forces are in fact the strongest in action.

Hybrid rDNA polymorphisms—genetic novelties

In general, all hybrids evidenced extensive rDNA polymorphism that was not found in parental species (Table 2). Species-specific rDNA polymorphisms were recently characterized in European leuciscines (Gromicho *et al.*, 2006a,b; Kirtiklis *et al.*, 2010; Pereira *et al.*, 2012; Rossi *et al.*, 2012), as well as in artificially reared hybrids of the *Squalius alburnoides* complex (Gromicho *et al.*, 2006b). In the present study, each hybrid stood for a distinct rDNA profile, usually falling within the range of possible combinations of parental contributions (Supplementary Table S3) and reflecting their admixed nature. Exceptions included (i) two AOL-like individuals with more 45S rDNA clusters than expected (Figures 2c and 3f) and (ii) the occurrence of syntenic rDNAs. Both can be interpreted as transgressive—exceeding parental phenotypic values in either a negative or positive direction. Although less reported in animals, transgressive phenotypes are more common in interspecific hybrids than expected as a consequence of recombination between parental lines (Rieseberg *et al.*, 1999). Still, present results document genetic novelties that cannot be explained by normal recombination between parental genomes. For example, translocations did not implicate homeologous chromosomes, as it would be expected in normal recombination. Instead, it involved chromosomes of even different classes (that is, usually, 45S rDNA-bearing chromosomes are sm, but after translocations occurred m and st/a chromosomes were also found carrying such clusters).

Considering the possible syntenic combinations observed in these hybrids, present results propose (i) higher 'mobility' of 45S rDNA sequences, (ii) preferential 'jumping' to chromosomes already bearing 5S rDNA with (iii) higher flexibility for incorporation (probably involving the non-transcribed spacers), resulting in (iv) repeatable rearrangements, and possibly in (v) erroneous pairing of the homeologous chromosomes inherited from each parent, as observed by Santos *et al.* (2002). Although the high number of 5S rDNA clusters was likely inherited from AOL (Pereira *et al.*, 2012), the association of these clusters to heterochromatin (Figure 5) can eventually be correlated to mobile DNAs, which may as well account for their original dispersion throughout *Achondrostoma spp.* genomes (Pereira *et al.*, 2012).

The number of studies in cyprinids using dual-FISH with rDNA probes is frankly growing, and colocalized ribosomal genes in leuciscines were described within *Anaecypris*, *Leuciscus* and *Squalius* genera (Gromicho *et al.*, 2006a,b; Kirtiklis *et al.*, 2010; Rossi *et al.*, 2012) but never in chondrostomine species (for example, parental species under focus; Pereira *et al.*, 2012). The novel syntenic associations (Figures 2–4) are distinct evidences of genome instability and reshuffling, most likely driven by the hybridization events (Fontdevila, 2005; Abbott *et al.*, 2013). It is rather probable that other regions of the genome might be experiencing the same processes as suggested by the CGH results (Figure 4d), even though no key interstitial telomeric sites were detected (Figure 4c). The 'repeatability' of syntenic blocks, that is, different individuals bearing comparable 45S–5S rDNA associations, was earlier observed in both natural and synthetized hybrids of *Helianthus sp.*, thus interpreted as a consequence of endogenous rather than ecological (exogenous) selection forces (reviewed in Fontdevila, 2005). This might also explain the 'abnormal' recombination patterns previously discussed.

Final remarks

The genomic instability herein documented proved rapid and traceable genomic evolution. Genetic novelty is thus generated for selection to operate and for species to persist under newly imposed stressful conditions (environmental and/or genomic) (Dowling and Secor, 1997; Gerber *et al.*, 2001; Seehausen, 2004; Mallet, 2005; Abbott *et al.*, 2013; Mable, 2013). In this case study, ecological barriers between hybrids and parental species might be altered and/or degraded, so that gene flow back to pure species never actually ceases. However, the ultimate consequences of such apparently extensive and recurrent events to these populations and species remain unknown, and the probability of fixation of the resulting rearrangements depends on the demography of the hybrid swarms (Gerber *et al.*, 2001; Seehausen, 2004; Costedoat *et al.*, 2007; Broughton *et al.*, 2011; Abbott *et al.*, 2013). Usually disregarded (reviewed in Costedoat *et al.*, 2007), these interactions might ultimately result in speciation or extinction faster than expected. Coupling the study of these hybrid complexes with modern genome-wide approaches (Twyford and Ennos, 2012) will allow for the exploration of evolutionarily relevant processes and answering questions inherent to hybridization, its causes, dynamics and impacts.

DATA ARCHIVING

Data deposited in the Dryad repository: doi:10.5061/dryad.5m121.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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