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Introgressive hybridization in Iberian cyprinid fishes: a cytogenomic approach to homoploid Leuciscinae

Carla Sofia Alves Pereira

DOUTORAMENTO EM BIOLOGIA
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Tese orientada pela Prof.ª Doutora Maria João Collares-Pereira e pelo Prof. Doutor Petr Ráb, especialmente elaborada para a obtenção do grau de doutor em Biologia (Biologia Evolutiva)

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Nota prévia

Na preparação da presente dissertação e, nos termos do nº 1 do Artigo 45 do regulamento de Estudos Pós-Graduados da Universidade de Lisboa, publicado em *Diário da República* nº 65, II Série de 30 de Março de 2012, apenas foram considerados integralmente artigos científicos originais publicados (2), em provas (1) ou submetidos a publicação (1) em revistas internacionais indexadas. A autora declara ainda que teve participação total na elaboração e execução dos trabalhos práticos, análise e interpretação de resultados, preparação e discussão dos manuscritos com primeira autoria. No caso de trabalhos de colaboração, como o *Chromosome studies of European cyprinid fishes: interspecific homology of leuciscine cytotaxonomic marker – the largest subtelocentric chromosome pair as revealed by cross-species painting* (**Apêndice III**) e o *Synaptonemal complexes in the hybridogenetic* Squalius alburnoides *fish complex: new insights on the gametogenesis of allopolyploids* (**Apêndice IV**), a autora executou parte do trabalho prático e participou na análise, interpretação e discussão de resultados, assim como na preparação dos manuscritos. Importante ainda de mencionar que a genotipagem dos indivíduos analisados ao longo deste trabalho foi levada a cabo pelo membro da equipa e co-autora de um dos trabalhos (**Capítulo 3.1**) Maria Ana Aboim.

Preliminary note

In the preparation of the present dissertation solely original scientific papers published (2), in press (uncorrected proofs) (1) or submitted (1) for publication in international indexed journals were considered. Moreover, the author declares full participation in the conception and execution of the experimental work, analysis and interpretation of the results, preparation and discussion of the manuscripts as first author. In case of collaborative works such as **Appendix III** and **Appendix IV**, the author of this dissertation executed part of the practical work, analysis and discussion of the results, as well as in the preparation of the manuscripts. Also important to mention, the genotyping performed throughout this work was performed by the team member and co-author of one publication (**Chapter 3.1**) Maria Ana Aboim.

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À minha família,

no seu sentido mais extenso.

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'Learn from yesterday, live for today, hope for tomorrow.

The important thing is not to stop questioning.'

by Albert Einstein

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Antes de mais a toda a minha família – incluindo a recém-integrada – que, mesmo não percebendo bem o trabalho que faço, acreditam em mim e me apoiam a 400 km de distância.

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Resumo

O processo de hibridação inter-específica é actualmente considerado também entre os animais como um promotor de biodiversidade, evolução e especiação, ao mesmo tempo que desafia a maioria dos conceitos de espécie mais reconhecidos. A hibridação é prevalente entre os peixes, particularmente os representantes da família Cyprinidae, que, assim, constituem bons modelos de estudo para (1) aceder aos padrões gerais de variabilidade genómica, (2) identificar a base genética e os processos evolutivos por detrás de adaptação e especiação naturais, (3) trabalhar a vários níveis, desde o genómico e citogenómico a vastas áreas geográficas e gradientes múltiplos de selecção. Tendo em conta que os híbridos são geralmente caracterizados por apresentarem sinais de instabilidade e de reestruturação genómica, o presente estudo pretendeu investigar estas questões com base na análise de híbridos homoploides entre *Achondrostoma oligolepis* e ou *Pseudochondrostoma duriense* ou a sus espécie-irmã *P. polylepis*, essencialmente através de técnicas de citogenética molecular, integradas com dados de morfologia e genética já descritos para as espécies parentais. O conjunto de sondas moleculares seleccionado incluiu DNAs ribossomais (rDNA), DNA genómico total, fracção de DNA altamente a moderadamente repetitivo (C_0t-1 DNA), retroelemento Rex3, e repetições teloméricas $(TTAGGG)_n$, as quais foram utilizadas em metodologias de hibridação *in situ* fluorescente (FISH), hibridação *in situ* genómica (GISH) e hibridação genómica comparativa (CGH).

Inicialmente, nove espécies Ibéricas de *Chondrostoma s.l.*, nomeadamente *Achondrostoma arcasii*, *A. occidentale*, *A. oligolepis*, *Iberochondrostoma almacai*, *I. lemmingii*, *I. lusitanicum*, *Pseudochondrostoma duriense*, *P. polylepis* e *P. willkommii*, foram caracterizadas para a distribuição das duas famílias de rDNA evidenciando uma variabilidade em número e localização de *clusters* superior ao que seria expectável tendo em conta a reconhecida uniformidade macroestrutural dos seus cariótipos e da subfamília (Leuciscinae) a que pertencem (**Capítulo 2**). Estes resultados apontavam já para um nível de diferenciação cariotípica, praticamente indetectável com técnicas de citogenética convencional e/ou de menor resolução. Numa destas espécies, melhor estudada em termos de genética populacional (i.e. *I. lemmingii*), foi possível traçar a história evolutiva dos genes de rDNA no seio do género a que pertence (*Iberochondrostoma*). Na fase seguinte deste estudo, e tendo já sido definidos marcadores específicos para cada uma das espécies parentais, procedeu-se à análise dos já mencionados híbridos naturais, amostrados em várias zonas híbridas independentes: Rio Sousa na bacia do Rio Douro, rios Caima e Serra na bacia do Rio Vouga, e rios Mortágua, Alva e Ceira na bacia do Mondego) (**Capítulo 3**). Neste capítulo foi evidenciada a importância da utilização de abordagens

múltiplas no estudo de híbridos e de zonas híbridas naturais, especialmente em casos de retrocruzamento extensivo e recorrente, onde os híbridos rapidamente se confundem numa observação superficial com as espécies parentais. Esta abordagem integrativa permitiu identificar padrões genéticos não-puros em todos os peixes seleccionados como possíveis híbridos. Os resultados foram, regra geral, idênticos em ambos os sistemas híbridos estudados (i.e. *A. oligolepis* x *P. duriense* e *A. oligolepis* x *P. polylepis*), tendo sido possível comprovar a existência de processos de retrocruzamento preferencial com *A. oligolepis*. Assim, *A. oligolepis* pôde ser considerada a espécie parental com uma maior contribuição genética para a constituição dos genomas híbridos analisados, surgindo como uma espécie mais permissiva à introgressão do que as outras duas. Adicionalmente, a espécie *A. oligolepis* não só aparentou um maior envolvimento como também se revelou mais afectada pelos recorrentes eventos de hibridação, uma vez que só foram encontradas translocações cromossómicas óbvias em híbridos de tipo-*A. oligolepis*. Não obstante, os híbridos apresentaram um extenso polimorfismo de rDNA, aparentemente ausente nas espécies parentais, mas dentro das possíveis combinações entre as contribuições parentais, com excepção de alguns fenótipos transgressivos inexplicáveis à luz de uma recombinação normal dos genomas parentais. Estes resultados dão provas de uma evolução e reestruturação genómica rápida nos híbridos, provavelmente responsável pela disponibilização de combinações genéticas distintas que permitirão uma melhor adaptação face a novas adversidades ambientais ou genómicas. Um dos mecanismos que poderia explicar esta rápida reorganização do genoma nos híbridos seria a re-activação de elementos transponíveis. Para testar esta hipótese, procedeu-se ao mapeamento de um dos elementos transponíveis mais comum entre os teleósteos: Rex3 (**Capítulo 3.2**). Para identificar a existência de um eventual processo de reactivação de Rex3 nos híbridos, foi previamente analisada a distribuição deste elemento no genoma das espécies parentais, como ponto de comparação pré- e pós-hibridação. Assim, o mapeamento físico do Rex3 foi efectuado pela primeira vez em espécies de ciprinídeos, tendo sido incluídas as espécies Ibéricas *Anaecypris hispanica*, *Iberochondrostoma lemmingii*, *I. lusitanicum*, *Pseudochondrostoma duriense*, *P. polylepis* e híbridos de *P. polylepis* x *A. oligolepis*. Em paralelo foram também mapeadas outras sequências repetitivas para as quais se pretendia determinar a possibilidade de associação com este retroelemento. O mapeamento do Rex3 mostrou um padrão de acumulação preferencialmente centromérico-telomérico, correlacionando-se com regiões de heterocromatina constitutiva mas não com nenhum dos rDNAs. O padrão observado foi genericamente idêntico e comparável em todas as espécies testadas, sugerindo uma presença muito antiga e anterior ao respectivo processo de diferenciação. Nos híbridos, o padrão de distribuição de Rex3 foi essencialmente do mesmo tipo, embora presente em mais pares de cromossomas e claramente

associado a *clusters* de rDNA, em particular *clusters* de 45S rDNA translocados (ver **Capítulo 3.1**). Estes resultados parecem evidenciar uma re-activação de mecanismos de transposição nos híbridos, surgindo portanto como uma das muitas consequências dos processos de hibridação inter-específica.

A presente dissertação encontra-se estruturada em seis Capítulos e quatro Apêndices que, à excepção da Introdução (**Capítulo 1**), Discussão (**Capítulo 5**) e Considerações Finais incluindo novas perspectivas de estudo (**Capítulo 6**), resultam da compilação de publicações científicas em revistas internacionais indexadas (**Capítulos 2** a **4**). Em Apêndice foram compilados todos os dados de morfologia resultantes desta investigação (**Apêndice I**) e três publicações da co-autoria da autora desta dissertação (**Apêndices II** a **IV**) que, não sendo parte essencial deste trabalho, foram consideradas de interesse como complemento a alguns dos aspectos discutidos no **Capítulo 5**. No **Capítulo 1** são abrangidos os temas chave fundamentais para a integração dos capítulos seguintes. No **Capítulo 2** faz-se a caracterização das espécies parentais envolvidas nos dois sistemas híbridos abordados neste trabalho, *Achondrostoma oligolepis*, *Pseudochondrostoma duriense* e *P. polylepis*, dentre outras seis espécies Ibéricas que ocorrem em território nacional (nomeadamente, *A. arcasii*, *A. occidentale*, *Iberochondrostoma almacai*, *I. lemmingii*, *I. lusitanicum* e *P. willkommii*). O **Capítulo 3** trata da caracterização dos mencionados híbridos homoploides, tendo como base de comparação as espécies parentais previamente caracterizadas. Pela primeira vez, estes híbridos foram abordados de um modo multidisciplinar, combinando marcadores morfológicos, genéticos e citogenómicos. No **Capítulo 4** faz-se uma síntese dos avanços na citogenética de peixes, com particular ênfase para os leuciscíneos da Península Ibérica e para os híbridos homoploides. Esta investigação providenciou novos dados relativamente a zonas híbridas independentes, ajudando a ilustrar: (1) que os cariótipos das espécies-alvo não são tão conservados como se pensavam com base em estudos anteriores de nível macroestrutural; (2) a sua diferenciação ao nível da subfamília; (3) a interacção de genomas divergentes nas várias composições híbridas analisadas; e (4) a sua dinâmica e rápida reorganização após eventos recorrentes de hibridação. Contudo, várias questões permanecem ainda sem resposta, nomeadamente, acerca da *fitness* das várias composições híbridas em diferentes ambientes, do comportamento meiótico dos heterocariótipos híbridos, dos modos de hereditariedade e de compensação de dosagem de *loci* parentais divergentes. Em conclusão, os sistemas de peixes híbridos endémicos da Península Ibérica – homoploides e poliploides – podem ser considerados bons modelos para estudos de composição, plasticidade e dinâmica genómica, assim como de processos de relevância evolutiva como a hibridação, a adaptação ou a especiação, entre muitos outros.

PALAVRAS-CHAVE: *Chondrostoma s.l.*, ciprinídeos Ibéricos, citogenética de peixes, citogenómica, evolução do genoma, hibridação natural

Abstract

Hybridization is currently a well-recognized process amongst animals responsible for biodiversity, evolution and speciation processes while defying most species concepts. Hybridization is prevalent among fishes, particularly cyprinids, which therefore constitute good models of study (1) to access general patterns of genomic variation, (2) to identify the genetic basis and the evolutionary processes behind adaptation and speciation in the wild, (3) working at different levels, from genome-wide to large geographic ranges and multiple gradients of selection. Considering that hybrids are usually characterized by genome instability and restructuring, the aim of this dissertation was to understand some processes of genome dynamics while also characterizing natural homoploid hybrids between *Achondrostoma oligolepis* and either *Pseudochondrostoma duriense* or its sister-species *P. polylepis*, mainly by means of cytogenomics integrated with morphologic and genetic data sets. Molecular probes included ribosomal DNAs, whole genomic DNAs, highly-moderately repetitive DNA fraction (C_0t-1 DNA), retroelement Rex3, and telomeric $(TTAGGG)_n$ repeats, used to characterize nine Iberian species of *Chondrostoma s.l.* and the aforementioned hybrids using FISH, CGH and GISH procedures. This investigation provided new data on independent hybrid zones helping to better understand these not-so-highly conservative karyotypes as previously considered, their differentiation within the subfamily Leuciscinae, the interacting genomes in the hybrid compositions, and their post-hybridization dynamics and rapid reorganization. But many questions, including new ones, remain unanswered, namely, hybrids' fitness in different environments, meiotic behaviour of hybrid heterokaryotypes, modes of inheritance and dosage compensation of diverging loci from each parent; as well as continuing to refine karyotype differentiation against a virtually constant macrostructure in highly flexible genomes. In summary, the hybrid fish systems that occur in the Iberian Peninsula – homoploid or polyploid – are promising regarding unresolved questions related to genome composition, plasticity and dynamics, and to evolutionary relevant processes like hybridization, introgression, adaptation or speciation, amongst many other biologically relevant subjects.

KEY-WORDS: *Chondrostoma s.l.*, fish cytogenetics, genome evolution, Iberian cyprinids, cytogenomics, natural hybridization

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Abbreviations

2n	diploid number
AT	Adenine-Thymine
BIO	Biotin
bp	base pairs
BSA	bovine serum albumin
CBD	C-banding counterstained with DAPI
CBP	C-banding counterstained with propidium iodide
CGH	comparative genomic hybridization
DAPI	4'-6'-diamidino-2-fenilindole
DIG	digoxigenin
DOP-PCR	degenerated oligonucleotide primed–PCR
EST	expressed sequence tag
FACS	fluorescent activated chromosome flow sorting
FISH	fluorescent *in situ* hybridization
FITC	fluorescein isothiocyanate
GC	Guanine-Cytosine
GISH	genomic in situ hybridization
LINE	long interspersed nuclear element repeats
m, sm, st, a chromosomes	metacentric, submetacentric, subtelocentric, acrocentric chromosomes
my	million years
mya	million years ago
n	haploid number
NF	total number of chromosome arms or fundamental number
NGS	next generation sequencing
NOR	nucleolar organizer region
p	chromosome short arm
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
q	chromosome long arm
QTL	quantitative trait loci
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
TE	transposable element
WCP	Whole chromosome painting probe
ZOO-FISH	cross-species chromosome/genome fluorescent in situ hybridization

Species names' abbreviations

AHI	*Anaecypris hispanica*
AAR	*Achondrostoma arcasii*
AOC	*Achondrostoma occidentale*
AOL	*Achondrostoma oligolepis*
FCA	*Felis catus*
HSA	*Homo sapiens*
IAL	*Iberochondrostoma almacai*
ILE	*Iberochondrostoma lemmingii*
ILU	*Iberochondrostoma lusitanicum*
IOL	*Iberochondrostoma olisiponensis*
PDU	*Pseudochondrostoma duriense*
PPO	*Pseudochondrostoma polylepis*
PWI	*Pseudochondrostoma willkommii*
SAL	*Squalius alburnoides*
SAR	*Squalius aradensis*
SCA	*Squalius carolitertii*
SPY	*Squalius pyrenaicus*
STO	*Squalius torgalensis*

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Chapter 1

Introduction

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1. Introduction

HYBRIDIZATION[1] is currently a renowned process amongst animals and has already proven a noteworthy role in biodiversity, evolution and SPECIATION processes. The study of natural HYBRIDIZATION allied to innovative tools of EVOLUTIONARY ECOLOGICAL GENOMICS provides (1) access to general patterns of genomic variation, (2) the possibility of identifying the genetic basis and the evolutionary processes behind ADAPTATION and SPECIATION in the wild, while (3) working at different levels (genome-wide, large geographic range, multiple gradients of SELECTION) (Orsini et al. 2013).

HYBRIDIZATION is prevalent amongst fishes, particularly cyprinids. HYBRIDS – HOMOPLOID and POLYPLOID – were found to be generally characterized by genome instability, and HYBRIDIZATION alone is suspected to induce more substantial genomic rearrangements than POLYPLOIDY (e.g. Fontdevila 2005; Hegarty et al. 2006; Abbott et al. 2013). Some Iberian Leuciscinae are known to recurrently hybridize in nature (e.g. Collares-Pereira & Coelho 1983; Elvira 1990; Alves et al. 1997; Gante et al. 2004; Kalous et al. 2008; Aboim et al. 2010) producing fertile HOMOPLOID HYBRIDS, therefore constituting good models of study in this subject, in which this dissertation is mainly focused.

The present dissertation was targeted to the characterization of natural HOMOPLOID HYBRIDS within two systems involving the cyprinids *Achondrostoma oligolepis* (Robalo, Doadrio, Almada & Kottelat 2005) and either one of the sister-species *Pseudochondrostoma duriense* (Coelho 1985) or *P. polylepis* (Steindachner 1865). Based mainly on cytogenomic tools alongside with genetic and morphological data, this investigation addressed independent HYBRID ZONES involving these pairs of SPECIES. Altogether, these analyses aimed to contribute to better understand the interacting genomes and hopefully shed some light into the dynamics of such successful, persistent and apparently extensive process of INTROGRESSIVE HYBRIDIZATION.

1.1. Introgressive Hybridization

SPECIES are the fundamental unit of virtually all subfields of biology, particularly systematics and evolutionary biology. Being essentially responsible for the study of the different kinds of organisms (biodiversity) and their relationships (PHYLOGENETICS), these disciplines rely utmost on the basic category of biological classification (SPECIES). The problem of SPECIES CONCEPTS in ichthyology have been

[1] more information on words written in SMALL CAPITALS throughout the text may be found in the Glossary section.

extensively debated in recent times (Lecointre 1994; Kotelatt 1997; Nelson & Hart 1999; Ruffing et al. 2002; Mooi & Gill 2010), all competing for acceptance. SPECIES are no longer seen as discrete units of biodiversity but as reservoirs in permanent GENE FLOW in a continuous hierarchy of biodiversity (Mallet 2005); it is more useful to think of SPECIES as gene complexes. The modern general SPECIES CONCEPT emphasizes SPECIES as separately evolving METAPOPULATION LINEAGES; interconnected populations forming an extended reproductive community and an unevenly distributed but unitary gene pool or field for gene RECOMBINATION (reviewed in Queiroz 2005). Telling apart one SPECIES from another should no longer be the critical focus of biological investigation, but rather the existence of barriers to RECOMBINATION and the factors affecting them, that control the accumulation of differences between populations and ultimately contribute to biodiversity (Ortíz-Barrientos et al. 2002).

INTROGRESSIVE HYBRIDIZATION is the exchange of genetic material between independent evolutionary lineages resulting in bisexual hybrid populations, as opposed to a more classic view of HYBRIDIZATION yielding exclusively unviable or infertile offspring (see Seehausen 2004). While botanists have long recognized the importance of 'genome invasion' (i.e. INTROGRESSION) in evolution as a diversity-generating mechanism, zoologists have traditionally considered it as unnatural and unusual, an exception to the rule (e.g. Dowling & Secor 1997; Epifanio & Nielsen 2001; Seehausen 2004; Mallet 2005; Soltis 2013). Nowadays, not only is it considered a commonplace phenomenon, but also assumed to play an important role in ADAPTATION, DIVERGENCE, evolution and SPECIATION (e.g. Dowling & Secor 1997; Shapiro 1999; Epifanio & Nielsen 2001; Kidwell 2002; Fontdevila 2005; Abbott et al. 2013; Soltis 2013). In fact, SPECIATION and ADAPTATION in a POPULATION GENETICS sense has a clear link to RECOMBINATION (**Figure 1.1**) (Ortíz-Barrientos et al. 2002). HYBRIDIZATION may occur in many different contexts, may be common and widespread, spatially or temporally localized or globally rare, and result in distinct outcomes (Abbott et al. 2013; Soltis 2013): (1) delay or reverse differentiation through homogenization (loss of biodiversity), sometimes even promoting invasiveness at the cost of the native SPECIES (e.g. Costedoat et al. 2005, 2007); (2) promote niche differentiation through increased genetic variation (ADAPTIVE DIVERGENCE of existing populations) while reinforcing barriers to GENE FLOW (REINFORCEMENT; increase in biodiversity) (e.g. Rieseberg 2001; Ortíz-Barrientos et al. 2002); (3) accelerate SPECIATION (increase in biodiversity) by either transferring ADAPTIVE traits via INTROGRESSION with the establishment of recombinant forms (HOMOPLOID HYBRID SPECIATION; e.g. Gerber et al. 2001; Mavárez & Linares 2008), or via ALLOPOLYPLOIDIZATION (instantaneous SPECIATION or nearly so; recently reviewed in Mable et al. 2011 and Collares-Pereira et al. 2013). The extent and the form of genetic novelty will vary with the spacio-temporal context and most certainly delineate the magnitude of the HYBRIDIZATION process.

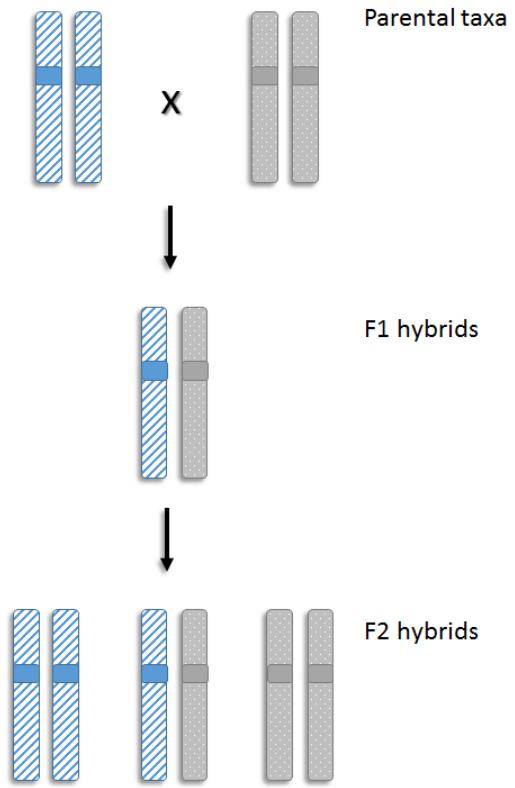

Figure 1.1 HYBRIDIZATION without RECOMBINATION. Assuming that F1 HYBRIDS are not infertile, without RECOMBINATION no genetic novelty would ever arise from HYBRIDIZATION. Only parental types or identical F1 HETEROKARYOTYPES would be produced in a second generation HYBRIDIZATION or BACKCROSSING. Adapted from Ortíz-Barrientos et al. 2002.

HYBRIDIZATION in general, as well as INTROGRESSION in particular, challenges the classic SPECIES CONCEPT as they break apart the required isolating mechanisms. For that, HYBRIDS have been stigmatized with concepts of low FITNESS or no evolutionary value at all. HYBRIDS may exhibit distinct FITNESS levels, either lower, equivalent or higher than in parental taxa (Arnold & Hodges 1995). In fact, overall HYBRIDS' FITNESS is not more inferior to their parents; in some cases, HYBRID FITNESS is higher in some environments where they can outcompete the parental SPECIES; occupation of new habitats by HYBRID genotypes is a common observation both in plants and animals (Fontdevila 2005). By these means, new genotypes with the ability to establish new evolutionary lineages are thus generated (e.g. Arnold & Hodges 1995; Dowling & Secor 1997; Gerber et al. 2001; Seehausen 2004; Mallet 2005; Abbott et al. 2013). Notwithstanding, HYBRIDIZATION complicates the delineation and identification of distinct evolutionary units in conservation genetics and, for example, conventions like the Endangered SPECIES Act (ESA) do not protect HYBRIDS between recognized endangered SPECIES (Allendorf et al. 2001; DeSalle & Amato 2004).

The resulting admixed populations may be sexual or asexual, HOMOPLOID or POLYPLOID (Abbott et al. 2013). HOMOPLOID HYBRID SPECIATION has been demonstrated but considered to be rare (e.g. Mallet 2007; Mavárez & Linares 2008), even though levels of INTROGRESSION have been underestimated in the past (e.g. Dowling & Secor 1997; Mallet 2005). More commonly, genetic exchanges between hybridizing taxa are not reciprocal resulting in the directional incorporation of genes of one SPECIES into the genome of another SPECIES (i.e., INTROGRESSIVE HYBRIDIZATION) (Scribner et al. 2001). The present availability of genome-wide data for a large number of SPECIES (including non-model ones) offers improved means for the identification of HYBRIDS and the opportunity for a thoughtful consideration on the genetic and evolutionary consequences of HYBRIDIZATION.

HYBRIDIZATION can result in different evolutionary outcomes, as previously stated. Understanding the genetic mechanisms underlying the different alternatives will allow understanding the impact of HYBRIDIZATION on ADAPTATION and SPECIATION processes, central questions in evolutionary biology. SPECIATION is the process by which RECOMBINATION between genomes of subpopulations is minimized over time either due to geographic barriers (strict ALLOPATRY), accumulation of genomic incompatibilities (through chromosomal rearrangements), or ADAPTIVE DIVERGENCE so that two gene pools can come into contact and yet maintain their distinctiveness (Ortíz-Barrientos et al. 2002). The rate of genome stabilization in HOMOPLOID HYBRID SPECIES is believed to be extremely rapid; few generations of RECOMBINATION between genes or chromosomal regions promoting isolation may be sufficient for the origin and success of a lineage with high FITNESS and REPRODUCTIVE ISOLATION. But genome stabilization is not a synonym of HYBRID SPECIATION. While segregating factors that contribute to initial ecological or intrinsic genetic isolation may become quickly stabilized, the remainder of the genome likely requires a longer time to become stable with RECOMBINATION and DRIFT dictating the contributions of each parental genome (Buerkle & Rieseberg 2008).

HYBRIDIZATION has been known at least since Linnaeus (Mallet 2005) and, thanks to the advances in genome-wide molecular methods, natural cases of INTROGRESSIVE HYBRIDIZATION among animal SPECIES have been increasingly reported (e.g. Dowling & Secor 1997; Seehausen 2004; Mallet 2005, 2007; Abbott et al. 2013). Progressively accepted as a biological process not restricted to plants, HYBRIDIZATION events are more often spotted among freshwater fishes than in any other group of vertebrates (Scribner et al. 2001). Interspecific HYBRIDIZATION occurs widely across a taxonomically diverse array of fish SPECIES for which human influences (aquacultures, SPECIES' introductions, and loss or alteration of habitats) have frequently been implicated as contributing factors (Dowling & Secor 1997; Allendorff 2001; Scribner et al. 2001; Costedoat et al. 2007). Family Cyprinidae, as the most speciose fish family, accounts for many HYBRID taxa, equally powered by anthropogenic or natural factors (Dowling & Secor 1997; Scribner et al. 2001). Some of the most scrutinized examples of HOMOPLOID HYBRIDIZATION within Cyprinidae are explored ahead in **Section 1.2.2**.

Whether the prevailing mechanisms behind HYBRIDS' success are 'genetic incompatibilities' or 'evolutionary novelties' both assume the appearance of potentially FITNESS-related phenotypic traits in HYBRIDS lying outside the parental distributions (Seehausen 2004; Reusch & Wood 2007; Abbott et al. 2013). Such TRANSGRESSIVE HYBRIDIZATION may speed up adaptive evolution. Novel HYBRID phenotypes may include genome restructuring, duplications/deletions, alterations in timing and levels of gene expression, epigenetic effects and TRANSPOSON activation (reviewed in Abbott et al. 2013). HYBRIDIZATION creates such variation instantaneously and simultaneously in several functional traits (Seehausen

2004). The immediate causes of extensive phenotypic novelty in HYBRIDS lie in differences between the contributing genomes that, when combined, have novel effects (Abbott et al. 2013). Heritable genetic variation mediates the rate and the direction of a population's response to SELECTION, crucial for natural populations to adapt to new environmental regimes (Orsini et al. 2013). The extent of novel expression patterns in the first few generations following HYBRIDIZATION often exceed what can be expected from simple reshuffling of pairwise epistatic interactions (see Seehausen 2004). In particular, regulatory genes are fast-evolving and evolve in a compensatory fashion within complex networks, increasing the probability of epistatic effects after HYBRIDIZATION (Abbott et al. 2013). Thus DIVERGENCE may occur quickly after isolation.

Genomic structural variation between SPECIES (chromosomal organization, gene duplication or loss and TRANSPOSABLE ELEMENT distribution) may induce further restructuring upon HYBRIDIZATION with possible phenotypic consequences directly affecting RECOMBINATION rates and reproductive compatibility with parental SPECIES (Rieseberg, 2001; Abbott et al. 2013). Nonetheless, the emergence of genetic novelties and variation is likely to be an ongoing process, with different phenotypes being exposed to NATURAL SELECTION over successive generations. Structural changes are expected to contribute primarily to barriers to GENE FLOW, while functional changes can have a wide array of effects, important in generating FITNESS-enhancing evolutionary novelty (Abbott et al. 2013). HYBRID ZONES provide excellent settings to study microevolutionary processes while cyprinid fish offer a good opportunity to search for genomic signatures of HYBRIDIZATION. Given the ancestral position of fishes in the phylogeny of vertebrates, the investigation of fish genomes is able to provide useful information for understanding structure, function and evolution of genes and genomes in vertebrates (see e.g. Jaillon et al. 2004).

1.2. Hybridization within Iberian *Chondrostoma s.l.* (Teleostei, Cyprinidae, Leuciscinae)

Most of the fish fauna in Iberian Peninsula – and in the Mediterranean Basin in general – is characterized by a high number of endemic SPECIES belonging to Cyprinidae, the largest family of freshwater fishes and one of the largest vertebrate families (Nelson 2006). The genus *Chondrostoma sensu lato* (Agassiz 1835), currently comprising 35 SPECIES – hereafter often referred to as chondrostomines, is a good representative of the Leuciscinae subfamily in the Iberian Peninsula, where records of high levels of diversity (biological, morphological and molecular) were already described (e.g. Doadrio & Carmona 2004; Robalo et al. 2007; Leunda et al. 2009; Perea et al. 2010; Doadrio et al. 2011).

As such a variable group, leuciscines' taxonomy has obviously been reformulated several times (see e.g. Bogutskaya 1994). By integrating distinct data such as molecular (both mitochondrial and nuclear) and BIOGEOGRAPHIC data, Perea et al. (2010) presented a robust model for the current distribution of Circum-Mediterranean leuciscines. Similarly, Robalo et al. (2007) proposed a revised taxonomy for the genus *Chondrostoma s.l.* based on morphologic and molecular markers, recognizing five additional new genera: *Achondrostoma*, *Iberochondrostoma*, *Pseudochondrostoma, Parachondrostoma* and *Protochondrostoma*, four of which Iberian and the first three exclusively exist in Portugal.

The diversification of Iberian leuciscines is highly related to the formation of the main Iberian river basins, though shaped by particular punctuated local events in some cases (i.e. incomplete river separation, recent river captures, and/or even more recent anthropogenic interventions) (e.g. Robalo et al. 2007; Perea et al. 2010; Aboim et al. 2013). Thus the main Iberian lineages *sensu* Robalo et al. (2007) are defined as follows, from more basal to more divergent: *toxostoma* with three SPECIES, *lemmingii* with four SPECIES, *arcasii* and *polylepis* both with three SPECIES each. Regarding their distribution throughout Portugal (**Figure 1.2**): (1) *Iberochondrostoma* genus comprises the *lemmingii* lineage and occurs in the large southern drainages of Tejo, Guadiana, and Guadalquivir, as well as in some small coastal Atlantic drainages (Gante et al. 2007; Robalo et al. 2007; Sousa et al. 2008; Monteiro et al. 2009; Lopes-Cunha et al. 2012). *Iberochondrostoma lemmingii* (ILE) is considered the central evolutionary unit of the genus with high levels of genetic diversity and ANCESTRAL POLYMORPHISMS retained in the larger populations (Lopes-Cunha et al. 2012) (**Figure 1.2a**); (2) the *Achondrostoma* genus comprising the *arcasii* lineage is found in the central-northern drainages of the Peninsula (Robalo et al. 2006, 2007). *A. oligolepis* (AOL) distribution is limited to river basins between Minho and Mondego basins, being restricted to Douro tributaries in Portugal, *A. arcasii* (AAR) distribution goes from Douro drainages in Portugal close to the border to being mostly spread across northern Spain, and *A. occidentale* (AOC) is a Portuguese endemic restricted to few small coastal Atlantic drainages (**Figure 1.2b**); (3) the widespread *Pseudochondrostoma* represents the *polylepis* lineage with SPECIES ALLOPATRICALLY distributed along the whole Portuguese territory (Aboim et al. 2009, 2013; Leunda et al. 2009). From north to south, *P. duriense* (PDU) occurs in Galiza and north of Portugal with a southern limit in Vouga basin, *P. polylepis* (PPO) can be found throughout Tejo and Mondego basins, and *P. willkommii* (PWI) in Portugal is only found in Guadiana basin (**Figure 1.2c**).

Iberian cyprinids, namely leuciscines, are particularly useful for studying HYBRIDIZATION events since they comprise several closely related SPECIES living in sympatry and several cases of extensive INTROGRESSIVE HYBRIDIZATION have been described or suspected to occur (e.g. Collares-Pereira & Coelho 1983; Elvira 1990; Alves et al. 1997; Gante et al. 2004; Kalous et al. 2008; Aboim et al. 2010).

Interestingly, cases of natural HYBRIDIZATION have been reported between *polylepis* x *arcasii* or between *toxostoma* x *arcasii* lineages (*sensu* Robalo et al. 2007), but never between *toxostoma* x *polylepis* lineages when they occur in sympatry (Robalo et al. 2007; Doadrio et al. 2011). In particular, natural HYBRIDS between AOL and PDU were first referenced by Steindachner (1866) but only later characterized on biometrical grounds by Collares-Pereira & Coelho (1983). In fact, the confirmation of the parental SPECIES involved was formerly done by Gante et al. (2004) and later by Aboim et al. (2010), who also used combined analysis of several markers.

Back in 1983, the frequencies of individuals with intermediate characters were found higher than expected for an occasional HYBRIDIZATION event, and the vast phenotypic variability towards the most abundant species pointed to the possibility of INTROGRESSION (Collares-Pereira & Coelho 1983). Later on, HYBRIDIZATION was found recurrent and extensively contributing for several independent HYBRID

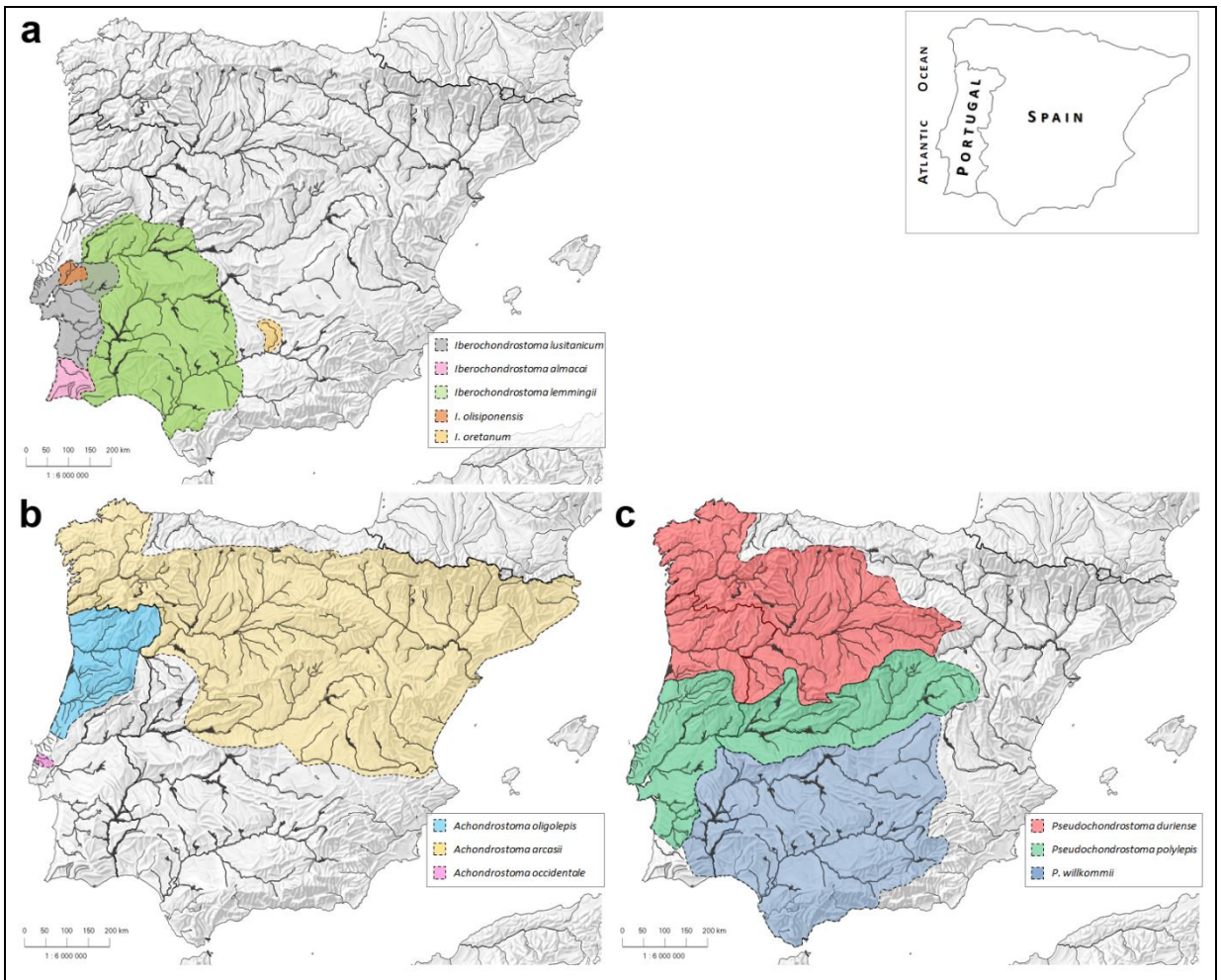

Figure 1.2 Distribution of Iberian *Chondrostoma s.l.* lineages occurring in Portugal: (**a**) *Iberochondrostoma*, (**b**) *Achondrostoma*, and (**c**) *Pseudochondrostoma*.

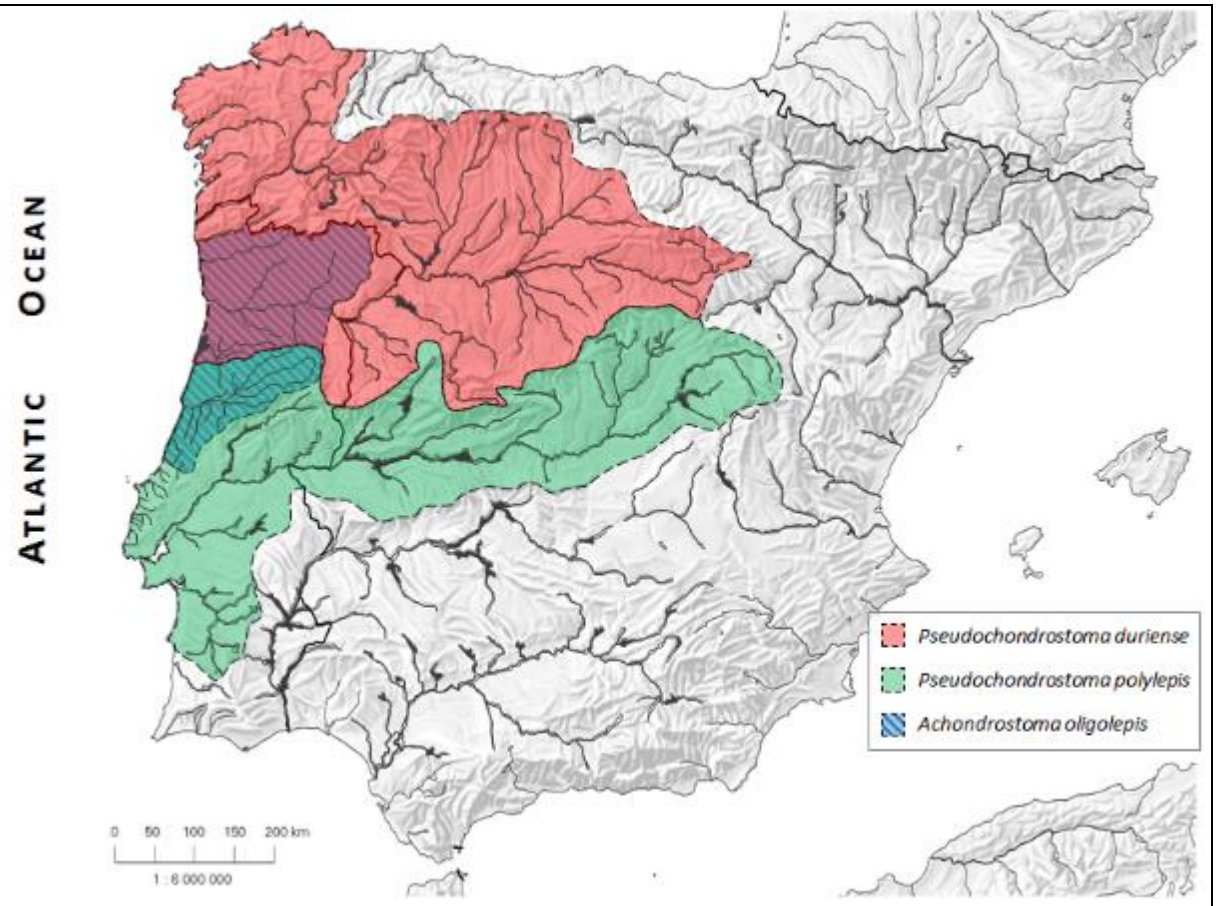

Figure 1.3 Range distribution of hybridizing Iberian *Chondrostoma s.l.* *Achondrostoma oligolepis*, *Pseudochondrostoma duriense* and *P. polylepis* accounting for extensive sympatric areas between both genera .

ZONES (HZs) (Aboim et al. 2010). Genome invasion was determined as heterogeneous and asymmetric (Aboim et al. 2010) with both morphological and molecular markers at gene and chromosomal level evidencing complex patterns of genome INTROGRESSION (Gante et al. 2004, Aboim et al. 2010; present dissertation). Additionally, the long suspected case of HYBRIDIZATION between AOLxPPO (e.g. Almaça 1965) was only confirmed in the frame of the present dissertation. AOL, PDU and PPO are broadly SYMPATRIC across the AOL geographic distribution (Robalo et al. 2006; Aboim et al. 2009; **Figure 1.3**). *Achondrostoma* and *Pseudochondrostoma* SPECIES are morphologically (Collares-Pereira 1979; Coelho 1985) (**Figure 1.4**; **Table 1.1**) and genetically well characterized, being definitely distinctive (e.g. Doadrio & Carmona 2004; Aboim et al. 2010). Therefore, HYBRID individuals are expected to exhibit intermediate MORPHOTYPES or an assortment of parental-specific traits (e.g. Collares-Pereira & Coelho 1983). However, HYBRIDS of first generation (F1) – expected to be strictly intermediate – were never found in the surveyed HZs to date (e.g. Collares-Pereira & Coelho 1983; Gante et al. 2004; Aboim et al.

2010; present work) probably as a consequence of the strong assortative mating shown by closely related SPECIES living in sympatry (Mallet 2005). F1 HYBRIDS are often the most difficult to originate, but once established (i.e. viable and fertile) BACKCROSSING and endorsing GENE FLOW between parental SPECIES is then possible (Mallet 2005), as it seems to be the case (Aboim et al. 2010; present dissertation).

1.2.1. Morphological Markers

Historically, meristic and morphometric measurements were the primary tools of identifying naturally occurring HYBRIDS (reviewed in Scribner et al. 2001). With no doubt, HYBRIDS between brightly coloured SPECIES (e.g. birds, butterflies) are easier to identify – and more commonly recorded – than within uniform groups. HYBRIDS between morphologically similar SPECIES are largely cryptic and only experts are able to distinguish them, also contributing to the still existing underestimation of natural HYBRIDS (Mallet 2005).

The morphological characters traditionally used to define *Chondrostoma s.l.* were considered feeble, having probably evolved several times in distinct lineages (Robalo et al. 2007; Corse et al. 2012). Morphological traits often used to identify taxa may represent ADAPTATIONS to specific environments even in the face of INTROGRESSION (e.g. Gerber et al. 2001). Nonetheless, and considering that Iberian

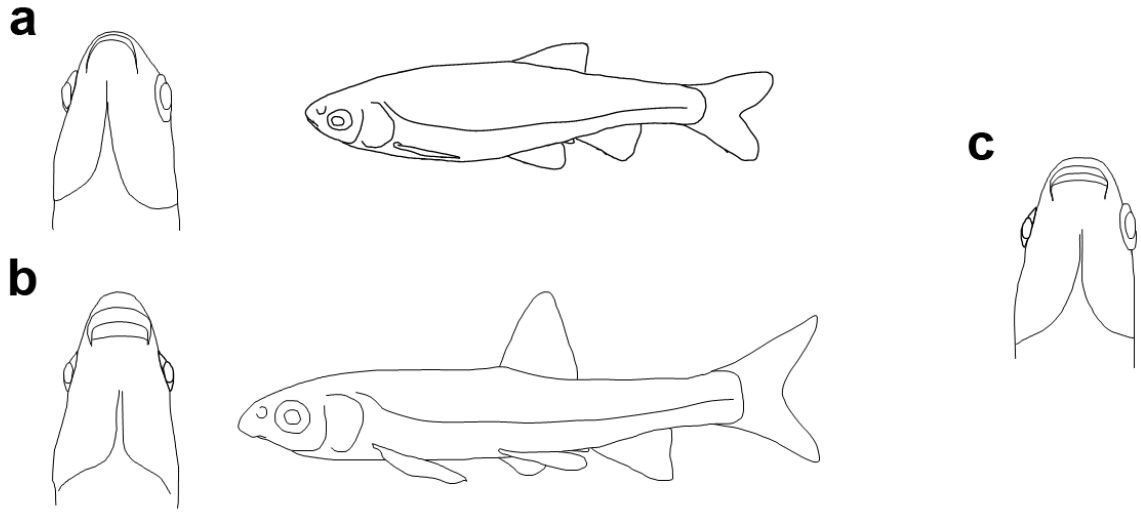

Figure 1.4 Morphological representation of (**a**) *Achondrostoma*, (**b**) *Pseudochondrostoma* and (**c**) their intergeneric HYBRIDS. Main diagnostic characters rely on: (1) shape and position of the mouth; (**a**) arched and terminal in *Achondrostoma*, (**b**) straight and ventral in *Pseudochondrostoma*, (**c**) elliptic and subterminal in the HYBRID; and (2) on the presence/absence of a corneous lower lip; (**a**) absent in *Achondrostoma*, (**b**) but characteristic of *Pseudochondrostoma*, and (**c**) modestly present in the HYBRIDS.

sister-species are usually ALLOPATRICALLY distributed, representatives of *Achondrostoma* and *Pseudochondrostoma* genera exhibit clear morphological differences easily recognizable at first glance (Collares-Pereira 1979; Coelho 1985; **Figure 1.4a-b; Table 1.1**), as already mentioned. Amongst the diagnostic traits, the most effortless identifiable include the shape and position of the mouth, and the presence/absence of a corneous lower lip (**Figure 1.4**); with a little more work, scales count in the lateral line and gill-rakers count are also valuable tools (**Table 1.1**). Intermediate forms or an assortment of parent-specific characters have shown to differentiate HYBRID MORPHOTYPES (Collares-Pereira & Coelho 1983; **Figure 1.4c**; **Table 1.1**) and contribute as a primary method of identification/classification. In this dissertation, depending on their general phenotype and main parental character contribution, HYBRIDS were classified and designated as AOL-*like*, PDU-*like* or PPO-*like*. All data and information regarding the specimens addressed for their morphological characterization during the present investigation can be found in **Appendix I**.

However, identifying HYBRIDS based on morphological characters alone may be challenging due to ANCESTRAL POLYMORPHISMS (**Figure 1.5**) or especially due to recurrent BACKCROSSING so that HYBRIDS become indistinguishable from their parental taxa (Mallet 2005), as it seems to be the case (Aboim et al. 2010; this dissertation).

Table 1.1 Morphological set of diagnostic characters used to identify the specimens included in this study.

	mouth			no. scales in lateral line	no. gill rakers	classification
	position	shape	corneous lip			
parental species	terminal	arched	absent	30-45	12	*Achondrostoma*
	ventral	straight	present	>60	21	*Pseudochondrostoma*
hybrids	sub-terminal	elliptic	inconspicuous	45-60	12-21	AOL-, PDU- or PPO-*like*

1.2.2. The Genetics of Introgression

As previously referred, in such cyprinid group, a considerable fraction of these intergeneric HYBRIDS cannot be reliably identified based on morphology alone (Aboim et al. 2010). Genetic markers and POPULATION GENETICS theory provide valuable tools for studying fish biodiversity and HYBRIDIZATION (Scribner et al. 2001). In contrast to morphological traits, most molecular markers are assumed to be

neutral allowing them to freely move among hybridizing taxa. Cyto-nuclear genealogical discordances (i.e. mitochondrial vs. nuclear genes) have been used in several studies to portray cases of INTROGRESSIVE HYBRIDIZATION (Avise 2001), even though this discordance may not always be obvious (see Seehausen 2004) (**Figure 1.5**). In particular, the quantification of associations between nuclear and cytoplasmic alleles can be used to: (1) formulate hypotheses regarding HYBRID formation in specific ecological settings, (2) determine the rate and direction of evolutionary change within each HYBRID ZONE (Avise 2001; Scribner et al. 2001), (3) deduce the frequency of HYBRIDIZATION and INTROGRESSION in nature, (4) define the behavioural and ecological factors controlling the genetic architecture of HYBRID populations, (5) identify the degree of consistency in genetic outcomes across multiple HYBRID ZONES, and (6) assess the contribution of environmental changes to the incidence of HYBRIDIZATION (Avise 2001). In any case, caution is required to distinguish between INTROGRESSION and incomplete LINEAGE SORTING (**Figure 1.5**), stressing the importance of obtaining data from multiple independent genetic sources (e.g. Gerber et al. 2001; Scribner et al. 2001; Gante et al. 2004; Mallet 2005; Gromicho & Collares-Pereira 2007; Mavárez & Linares 2008; Lajbner et al. 2009; Aboim et al. 2010; Waap et al. 2011; Twyford & Ennos 2012; Choleva et al. in press).

The viability of an interspecific cross is negatively affected by the genetic distance between two SPECIES (Mallet 2005). On the other hand, the longer the time since SPECIES DIVERGENCE, the probable effect of incomplete lineage sorting decreases; the probability of encountering an ANCESTRAL POLYMORPHISM is negligible after a DIVERGENCE time of ~1 my (Shedlock et al. 2004). The parental SPECIES of the HYBRID systems considered in this study are thought to have diverged around 11 mya (Doadrio & Carmona 2004; Aboim et al. 2010; Perea et al. 2010) for which incomplete lineage sorting can in principle be ruled out. However, more complex patterns may emerge and discordance between gene trees and SPECIES phylogeny might be found with higher probability (Shedlock et al. 2004).

The North American *Gila robusta* complex comprises seven taxa endemic to distinct habitats (Dowling & DeMarais 1993; Gerber et al. 2001) of which only three live in sympatry at the moment, displaying simultaneously different patterns of HYBRIDIZATION and local ADAPTATION (Gerber et al. 2001). In ALLOPATRY: (1) *Gila robusta* and *G. elegans* were readily characterized by distinctive HAPLOTYPES, while (2) *G. cypha* revealed some traces of admixture with *G. elegans*. In sympatry: (1) all parental taxa evidenced *G. cypha*'s HAPLOTYPES regardless of the MORPHOTYPE exhibited, proving for extensive INTROGRESSION and total replacement of *G. robusta*'s mtDNA for *G. cypha*'s mtDNA; (2) *G. seminuda* had been inferred and further confirmed of ancient HYBRID origin between *G. robusta* x *G. elegans*; while (3) *G. jordani* seems to have resulted from HYBRIDIZATION between *G. robusta* x *G. cypha* instead.

The case of the North American *Cyprinella lutrensis* complex encompasses three SPECIES with overlapping ranges and signatures of past and ongoing HYBRIDIZATION events (Broughton et al. 2011). Analyses based on mtDNA and nuclear genes revealed: (1) multiple HZs between *C. lutrensis* x *C. venusta* where parental SPECIES co-occur with their HYBRIDS and HYBRIDIZATION seems reciprocal, widespread with possibly recurrent BACKCROSSING; (2) historical HYBRIDIZATION between *C. lutrensis* x *C. lepida* in a single population, with complete mtDNA replacement for *C. lutrensis* and no known HYBRID ZONES at the present time.

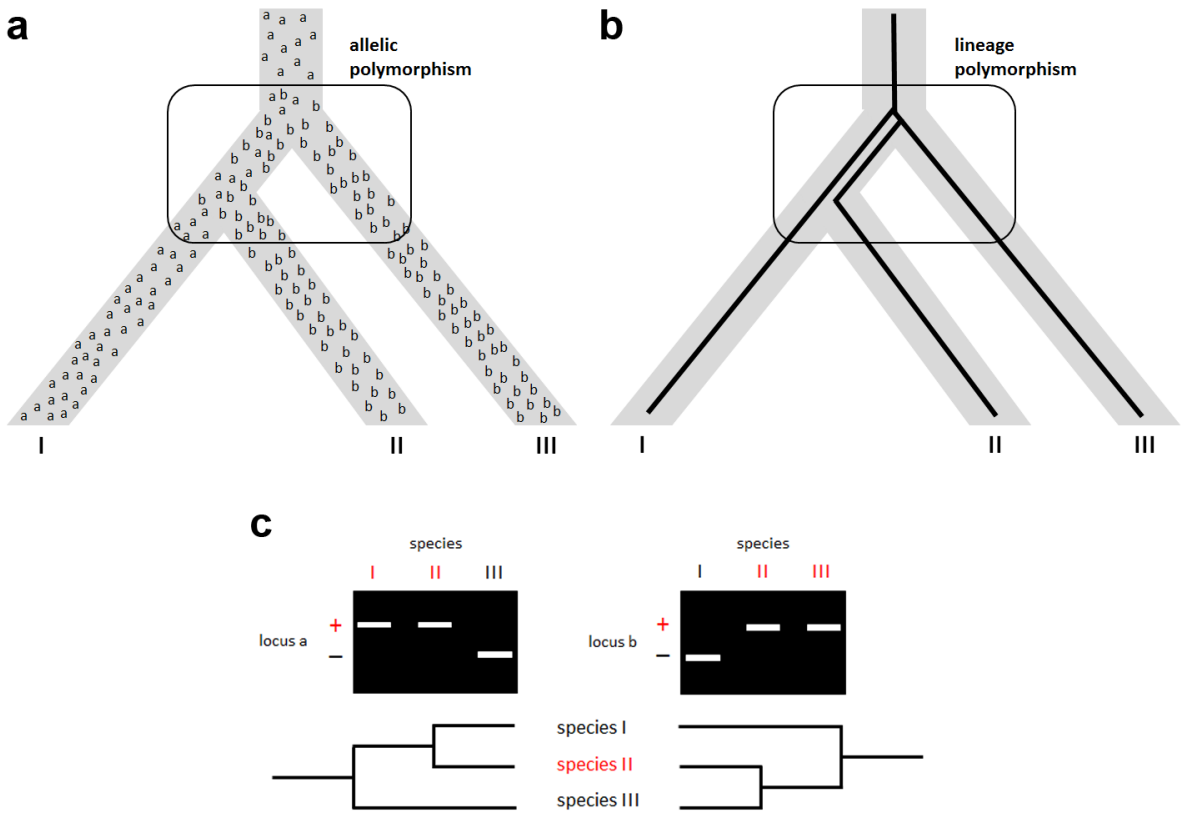

Figure 1.5 Schematic representations of HEMIPLASY and incomplete LINEAGE SORTING. The distributions of a genic or chromosomal POLYMORPHISM (**a**) and a set of genealogical lineages (**b**) traversing successive SPECIATION nodes in an organismal phylogeny (broad grey branches) and becoming fixed by LINEAGE SORTING in descendant SPECIES in a pattern discordant with the SPECIES PHYLOGENY. SPECIES II and III have the homologous and derived character 'b' so the gene tree indicate these SPECIES as the more closely related. However, in truth SPECIES I and II are sister taxa, despite the fact that SPECIES I alone retains the ancestral genetic condition 'a'. Unfixed POLYMORPHISMS might be evident by PCR (**c**) and represent ongoing sorting of alleles towards the eventual loss or fixation in younger SPECIES. Fixed loci in older SPECIES might provide discordant patterns but accurately reflect gene genealogies in the absence of parallel evolution. Genetic discordance is most likely explained by the random fixation of alleles in different lineages that underwent rapid, successive SPECIATION in the distant past or, alternatively, by interspecific HYBRIDIZATION. Adapted from Shedlock et al. 2004 and Robinson et al. 2008.

The French case-study of *Chondrostoma nasus* x *C. toxostoma* relates to a recent case of bidirectional INTROGRESSIVE HYBRIDIZATION powered by human activity, in which the genetic assimilation of the rarer SPECIES (native and endangered *C. toxostoma*) by the invasive *C. nasus* seems to be in place, seriously jeopardizing the endemic SPECIES (Costedoat et al. 2005). No continuum (or HYBRID gradient) could be found linking the distinct HYBRID ZONES, and morphological convergence seems to be occurring towards the native SPECIES even though most HYBRIDS present mtDNA of *C. nasus* (Costedoat et al. 2005; Corse et al. 2012).

In the present investigation, a thorough genetic profiling of all individuals (parental and putative HYBRIDS) was included, based on molecular markers previously developed for the parental SPECIES – mtDNA *cyt b* gene, ten microsatellite loci (Aboim et al. 2010), and the single-copy *RAG-1* nuclear gene (recombination activating gene 1) (Aboim et al. 2009) (see for example **Chapter 3.1**). Earlier analyses have demonstrated that independent HYBRID ZONES seem to behave differently regarding levels and direction of INTROGRESSION: (1) in Távora HYBRID ZONE (Douro basin) INTROGRESSION of mtDNA was asymmetric towards PDU (Aboim et al. 2010) in contrast with previous findings where no mtDNA INTROGRESSION was found (Gante et al. 2004) or morphological trait INTROGRESSION seemed to happen in the direction of AOL (Collares-Pereira & Coelho 1983); (2) in Caima HYBRID ZONE (Vouga basin) INTROGRESSION was found bidirectional but highly asymmetric towards AOL (Aboim et al. 2010). In this study, other HYBRID ZONES were further identified and also characterized by means of the same genetic markers in addition to the cytogenetic assessment (**Chapter 3.1**).

1.3. Cytogenomics

Above all, HYBRIDIZATION provides an extraordinary opportunity to study divergent genomes interacting in a completely novel context. Chromosomes, on the other hand, offer a chance for the macro-examination of the genome and may therefore afford for stronger evidences of HYBRIDIZATION and reorganization. Despite some controversy, chromosome breaking points/regions seem rather non-random and conservative (e.g. Mallet 2005; Longo et al. 2009; Brown et al. 2012; Robinson & Yang 2012) allowing to establish a correlation between the genomic stress imposed by HYBRIDIZATION and the probable rearrangements found in the new genomic (HYBRID) compositions.

Few studies have used karyology/chromosomes to identify HYBRIDS mainly due to the great effort of fish cytogenetics compared to other genetic approaches and also because congeneric SPECIES (most of the HYBRIDIZING fish SPECIES) often have the same standard KARYOTYPE (reviewed in Scribner et al. 2001), as it is the case (Pereira et al. 2009; **Appendix II**). In this study, by integrating for the first time

cytogenetic data with morphological and robust genetic profiling on AOLxPDU and AOLxPPO HYBRIDS (**Chapter 3**), we aimed to better understand the impact of the ongoing inter-generic HYBRIDIZATION process on the genomes of these SPECIES, especially considering the heterogeneous patterns of INTROGRESSION previously found on distinct HZs (Gante et al. 2004; Aboim et al. 2010)

One of the major challenges in EVOLUTIONARY ECOLOGICAL GENOMICS is the management and comprehensive analysis of the huge data volumes generated. After identifying the signature of SELECTION underlying relevant phenotypes, the functional association to genotypes must be validated (e.g. Phillips et al. 2006) by methods not always available for non-model SPECIES, such as LINKAGE and CHROMOSOMAL MAPS (Orsini et al. 2013) PHYSICAL MAPS are indispensable tools that form the intermediate layer between local (gene) sequences, genetic maps, and whole genome sequences (Oeveren et al. 2011). In contrast to GENETIC MAPS, CHROMOSOMAL MAPS can lead to the identification of the genomic mechanisms underlying adaptive phenotypes even in the absence of a genome sequence (Orsini et al. 2013). Far from feared, cytogenetics was not placed aside in the genomics era, but yet greatly boosted by the thriving technical advances. Cytogenomics (or modern cytogenetics) became a multidisciplinary science relying now on a combined set of approaches of conventional (banding) and molecular tools (chromosome painting, gene mapping and sequencing) broadening the perspectives in the study of KARYOTYPES and chromosomes.

Despite rarely used for that purpose, chromosomes are undoubtedly powerful characters for inferring PHYLOGENETIC relationships (with inherent limitations; Dobigny et al. 2004; Robinson & Yang 2012). KARYOTYPE structural variations were originally considered potential effective barriers to GENE FLOW between hybridizing taxa; either through FITNESS reduction in the HYBRIDS (e.g. meiotic impairments of the HETEROKARYOTYPES), by reducing the RECOMBINATION rates in rearranged areas of the genome (Rieseberg 2001; Dobigny et al. 2004; Kawakami et al. 2011), or alternatively by accelerating DIVERGENCE between populations through the spread of locally adapted alleles or protecting combinations of genes associated with REPRODUCTIVE ISOLATION (suppressed-RECOMBINATION; Kawakami et al. 2011). The perspective of suppressed-RECOMBINATION has significant implications for both SPECIATION models and for the outcome of contact between neo-SPECIES and parental SPECIES (Rieseberg 2001). However, the meiotic impact of the different chromosomal changes may vary and many researchers simply consider chromosomal rearrangements as incidental by-products of SPECIATION processes.

Syntenic blocks involving entire chromosomes, chromosomal arms, or large chromosomal segments are sometimes shared across even distantly-related SPECIES (e.g. mammals; Graphodatsky et al. 2011; Ruiz-Herrera et al. 2012). They are considered and used as powerful PHYLOGENETIC markers, since

independent convergent evolution of the syntenic blocks seems highly unlikely. Nowadays, chromosomal rearrangements are known to reuse breakpoints usually rich in segmental duplications, repetitive elements, and fragile sites, and also involve an epigenetic component (e.g. Longo et al. 2009; Brown et al. 2012; Robinson & Yang 2012). On the other hand, HYBRIDIZATION is known to result in genomic, transcriptomic and epigenetic shocks; new HYBRID phenotypes may include genome restructuring, duplications/deletions, alterations in timing and levels of gene expression, epigenetic effects and TRANSPOSON activation, readily available after the HYBRIDIZATION event(s) (e.g. Seehausen 2004; Fontdevila 2005; Hegarty et al. 2006; Czypionka et al. 2012; Abbott et al. 2013). Structural alterations result from primary or secondary chromosome rearrangements. Primary rearrangements (insertion, deletion or duplication, peri- or paracentric inversion, and intra- or interchromosomal reciprocal translocation) are the outcome of illegitimate RECOMBINATION between homologous sequences. Secondary rearrangements can occur in organisms with double heterozygosity for two primary rearrangements involving a single chromosome and thus originate gametes with a new KARYOTYPE (Schubert & Lysak 2011). From this point of view, chromosome rearrangements provide satisfactory explanation for KARYOTYPE EVOLUTION. In theory, genome modifications strongly affecting FITNESS can only become fixed through DRIFT in small, inbred populations (Rieseberg 2001; Kawakami et al. 2011). On the other hand, if rearrangements are neutral or weakly UNDERDOMINANT the conditions for their fixation are relaxed (Rieseberg 2001).

In **Chapter 4** of this dissertation, a short revision on fish cytogenetics is given, with special emphasis on Iberian Leuciscinae and the HOMOPLOID HYBRIDS under focus. The major advance may yet be considered the employment of molecular cytogenetic techniques to fish chromosomes, helping to overcome the limitations of conventional banding (e.g. Phillips & Reed 1996; Phillips 2001; Dobigny et al. 2004). Fluorescent *in situ* hybridization (FISH) is a powerful complementary tool to genome analysis, useful for correlating genetic maps with specific chromosomes, or for quickly generating such maps for SPECIES that do not have them and for which performing standard crossing experiments may be a problem (Phillips 2001). One of the primary reasons for the central role of FISH in developing PHYSICAL MAPS is the remarkable resolution it provides over all other approaches (~5 Mb in metaphase FISH to ~5 kb by fiber-FISH, or even few-bp-long if highly repetitive; Raudsepp & Chowdhary 2008). On the other hand, FISH is particularly important for the study of the distribution of repeated sequences, which are frequently excluded from shotgun genome assemblies (Fischer et al. 2004; Oeveren et al. 2011).

Moreover, the demand for studies integrating cytogenetics with population genetics, morphological and systematic data is growing (Oliveira et al. 2009), not only to better understand the importance of

karyotypic changes in SPECIATION and evolution, but also as an additional tool to trace inter-specific HYBRIDIZATION processes.

1.3.1. Repetitive DNAs as Probes

Heterochromatin is characterized by a complex arrangement of various types of repetitive sequences often responsible for lineage-specific patterns (amount and distribution). The correlation between repetitive sequences and chromosomal rearrangements has been extensively documented, for which modifications in this genomic fraction might result in effective reproductive barriers. Investigations using repetitive sequences proved useful in revealing the evolutionary forces driving the huge diversity found in fishes and in improving our comprehension on genome structure and evolution (e.g. reviews of Cabral-de-Mello & Martins 2010 and Cioffi & Bertollo 2012).

Repetitive DNAs are a major structural component of most eukaryotic genomes (Charlesworth et al. 1994; Cioffi & Bertollo 2012) comprising tandem repeats (multigene families like rDNAs, micro- and minisatellites) and mobile (TRANSPOSABLE) ELEMENTS (TEs). The molecular organization and cytogenetic location of repetitive DNAs are amongst the most investigated sequences in fish genomes (e.g. Phillips & Reed 1996; Phillips 2001; Cabral-de-Mello & Martins 2010; Cioffi & Bertollo 2012). Some TRANSPOSABLE ELEMENTS (TEs) and tandem repeats belong also to the main forces driving gene and genome evolution (e.g. Kidwell 2002; Volff 2005) (discussed ahead in detail). In fishes, in particular, these studies have demonstrated the enormous potential that the investigation of repetitive DNAs offers toward extending our knowledge on KARYOTYPE differentiation or the evolution of sex chromosomes and B chromosomes, for example (reviewed in Cioffi & Bertollo 2012). Repetitive sequences isolated from various fish SPECIES have been localized to centromeres, telomeres, and sex chromosomes; some of them now used as SPECIES-specific, chromosome-specific or sex-specific probes (e.g. Phillips & Reed 1996; Cabral-de-Mello & Martins 2010).

1.3.1.1. Highly & Moderately Repetitive DNAs – C_0t-1 DNA

The C_0t-1 DNA is a fraction of genomic DNA enriched for highly and moderately repeated DNAs, obtained by the application of the principles of the re-association kinetics of DNA strands (Zwick et al. 1997; Ferreira & Martins 2008). In general, the regions labelled by this genomic fraction correspond to the heterochromatic areas, such as centromeres and telomeres. Despite the disadvantage of this method being the isolation of a large number of unknown sequences, it is rather inexpensive and easy to use without the necessity for cloning or sequence analysis. Its use, although still restricted, has

shown promising in studies of comparative analysis and genomic organization of chromosomes also in fish SPECIES (e.g. Ferreira & Martins 2008; Fantinati et al. 2011).

In the present investigation, the potential of C_0t-1 DNA mapping was not fully explored. Yet it was isolated and used as HOMOSPECIFIC probe in PDU and PPO metaphases revealing strong hybridization signals to some centromeres, telomeres and very few interstitial bands, overall congruent with C-banding results (see **Chapter 3.2**).

$$C_0t = 1 = mol/L \times T_s$$

DNA re-association kinetics. Calculating the time needed for a reannealing reaction. Initial concentration (C_0) is calculated in moles of nucleotides (mol) per litre (L) and time is in seconds (T_s), assuming an average molecular weight for a deoxynucleotide monophosphate to be 339 g/mol (if %GC ~50%) (Zwick et al. 1997).

1.3.1.2. Centromeric & Telomeric repeats

The telomeric repeat sequence $(TTAGGG)_n$ is conserved in all vertebrates (Meyne et al. 1989) but centromeric and subtelomeric sequences are often SPECIES-specific and/or even chromosome specific (e.g. Jabbs & Perisco 1987). The study of telomeric repeats has been successfully employed in the analysis of chromosomal rearrangements underlying KARYOTYPE differentiation in a variety of organisms (Lin & Yan 2008). The occurrence of interstitial telomeric sites (ITS) has been critical for identifying chromosome rearrangements (e.g. Cioffi et al. 2010) even though some studies have shown that, instead of remnants of true telomeres, ITS might as well represent heterochromatic derivation (telomere-like sequences) or be part of satellite DNAs (e.g. Garrido-Ramos et al. 1998), and also originate from differential crossing-over or repair of double-strand breaks (Lin & Yan 2008). In any case, one of the most interesting aspects of ITS is its usefulness in detecting genetic instability, hotspots for chromosome breakage and amplification sites (Lin & Yan 2008), even if in many cases ITS may not be detected due to loss or drastic telomere reduction during the process (reviewed in Cioffi & Bertollo 2012). Moreover, taking into consideration physical chromosomal constraints (Shubert & Lysak 2011), reciprocal translocations – that do not require the improbable interaction of telomeres with break-ends – are more likely to be the most common type of chromosomal rearrangements in KARYOTYPE EVOLUTION; and therefore lacking the interstitial telomeric sites as signatures of recent rearrangements.

Centromeric and telomeric probes have been applied to fish genomes to survey intraspecific chromosome rearrangements, such as the transposition of rDNAs (e.g. Phillips & Reed 1996; Caputo

et al. 2009). Similarly, these probes can be used as genome markers for examining interspecific HYBRIDS and expected genome rearrangements. Centromeres contain several classes of repeated sequences some of them highly conservative even between distantly related SPECIES (functional role). By means of endonuclease digestion of the genomic DNA, centromeric sequences have been isolated and characterized in several fish SPECIES (e.g. Capriglione et al. 1994; Garrido-Ramos et al. 1998; Canapa et al. 2002; Viñas et al. 2003; Azevedo et al. 2005). Fish genomes inspected for telomeric repeats evidenced, as expected, positive hybridization to the telomeres of all chromosomes (e.g. Meyne et al. 1989; Gornung et al. 1998; Fischer et al. 2000; Cross et al. 2006; Gromicho et al. 2006) and in some cases additional non-telomeric sites were also found (e.g. Phillips & Reed 1996; Sola & Gornung 2001, Rocco et al. 2001, and references therein; Sola et al. 2003; Caputo et al. 2009; Cioffi et al. 2010).

1.3.1.3. Tandem Repeats

Multigene families like the ribosomal DNAs (rDNAs) and the histone gene families represent a common structural element of eukaryotic genomes and are composed of hundreds to thousands of clustered gene copies. The majority of tandem repeats investigated in fish genomes were localized at the centromeres of most chromosomes (e.g. Sola & Gornung 2001). The two classes of ribosomal DNAs (**Figure 1.6**) have been extensively used as DNA probes in cytogenetic studies of fish SPECIES (e.g. Cabral-de-Mello & Martins 2010). The multiple copies of rDNA found at the nucleolar organizing regions (NORs) can also be visualized in lower vertebrates by conventional cytogenetic techniques (staining with silver nitrate – AgNORs, and chromomycin A_3) or by molecular cytogenetics (FISH with specific probes). Silver-staining only detects active copies of rDNA while chromomycin A_3 (CMA_3) and FISH detect rDNAs regardless of activity; and despite the great correlation between these banding results, incongruences have been reported (e.g. Gromicho et al. 2005). Such disparity might be related with the chemical affinity of CMA_3 for GC-rich regions (other than NOR-related) and, on the other hand, with FISH being able to detect smaller clusters of rDNA and also pseudogene copies.

Both rDNA classes can be highly POLYMORPHIC in terms of number and location of sites even among related SPECIES. These patterns are not readily informative regarding PHYLOGENETIC relationships and special attention should be paid to such interpretation of rDNA cytogenetic mapping (Dobigny et al. 2004; Cabral-de-Mello & Martins 2010; but see also Britton-Davidian et al. 2012). Nonetheless, rDNA distribution patterns may provide valuable information on homologies between chromosomal segments, mainly between closely related SPECIES (e.g. Dobigny et al. 2004; Britton-Davidian et al. 2012), and on depicting HYBRID lineages (e.g. Zhu et al. 2006; this dissertation). Despite the great

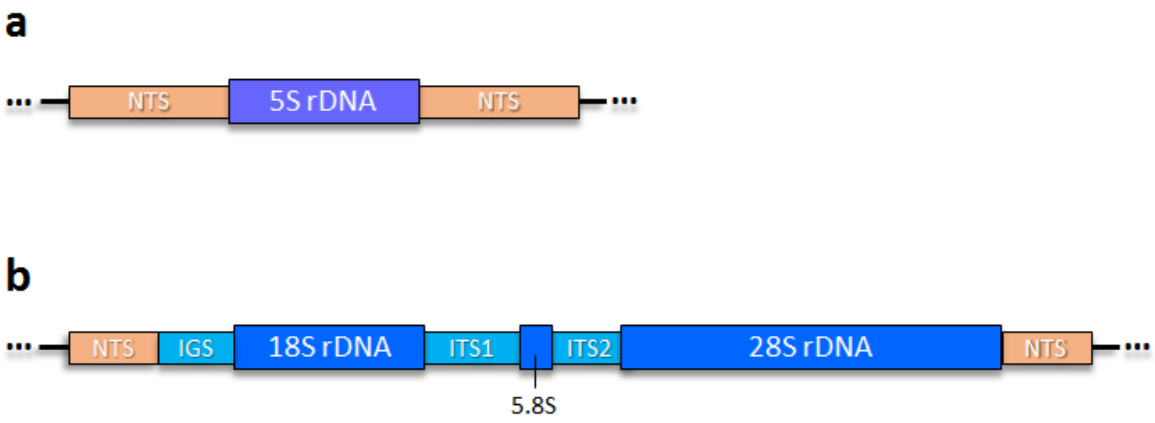

Figure 1.6 Eukaryotic ribosomal genes. Gene segments composing the basic units of the tandem repetitive clusters of (**a**) 5S rDNA and (**b**) 45S rDNA families. NTS = non-transcribed spacer; IGS = intergenic spacer; ITS = internal transcribed spacers.

variation, a single pair of chromosomes bearing each representative of the two rDNA families is considered the PLESIOMORPHIC condition in teleosts (Sola et al. 2003). The most common organization of these gene families indicates a non-associative distribution, with most SPECIES having these sequences clustered on different chromosomes. Recent studies correlate the dynamism of the rDNA clusters with significant intragenomic processes, such as RETROTRANSPOSON activity as demonstrated in the fish species *Erythrinus erythrinus* (Cioffi et al. 2010) or *Astyanax bockmanni* (Silva et al. 2013).

The distribution of these clustered genes were addressed in the characterization of the target SPECIES of the present research and their natural HYBRIDS (**Chapters 2** and **3**) demonstrating greater power of resolution when used in conjunction (double FISH). By these means we were able to: (1) distinguish between the pure parental SPECIES, each with its own pattern of distribution of both rDNAs; (2) portray cases of HYBRIDIZATION even when genetic and/or morphological data failed to detect them; (3) visualize and characterize circumstances of rapid genome instability and reshuffling in these HYBRIDS most likely driven by the HYBRIDIZATION events.

1.3.1.4. Transposable Elements (TEs)

TRANSPOSABLE ELEMENTS (TEs) are repetitive DNA sequences, comprising a group of segments with the capacity to move throughout the chromosomes or transpose between non-homologous sites within the genome (**Figure 1.7**). TEs remained disregarded for a long time since discovered and were viewed as 'junk DNA' or 'selfish DNA' (e.g. Charlesworth et al. 1994, Ferreira et al. 2011b; Hua-Van et al. 2011;

Cioffi & Bertollo 2012), the ultimate parasite: able to propagate itself through VERTICAL TRANSMISSION, intra-genomic TRANSPOSITION and occasionally HORIZONTAL TRANSFER. Since then, TEs have been found abundant in most genomes and, most interestingly, directly implicated in the structure, regulation, diversification and evolution of genomes (e.g. Kidwell 2002; Hua-Van et al. 2011; Cioffi & Bertollo 2012; Arkhipova & Rodriguez 2013; **Figure 1.8**).

Even though TEs multiply independently within the genome, TE insertions are indirectly subject to NATURAL SELECTION. TE distribution in a genome is neither random nor uniform. Besides some rarer site-specific elements, TEs tend to accumulate in neutral or advantageous (positively selected) areas where their potential deleterious impact is reduced: constitutive heterochromatin, telomeric and/or pericentromeric regions, and other low gene density areas. This non-random distribution may reflect true preferential insertion sites or negative SELECTION (against insertions in the euchromatic regions, ectopic TE-mediated RECOMBINATION, or transposition itself; Eickbush & Furano 2002). If an insertion is neutral, its persistence in the population relies on GENETIC DRIFT and demographic parameters (Hua-Van et al. 2011), being actually useful in studies of evolution and HYBRIDIZATION. In fact, HYBRIDIZATION is known to potentially induce TE activation triggering genome-wide reorganization (genetic and epigenetic) or strongly modifying RECOMBINATION patterns (Fontdevilla 2005; Abbott et al. 2013). HYBRIDS were earlier considered as triggers for bursts of transposition (Fontdevilla 2005). TE reactivation usually results from the RECOMBINATION between inactive copies (from each parental

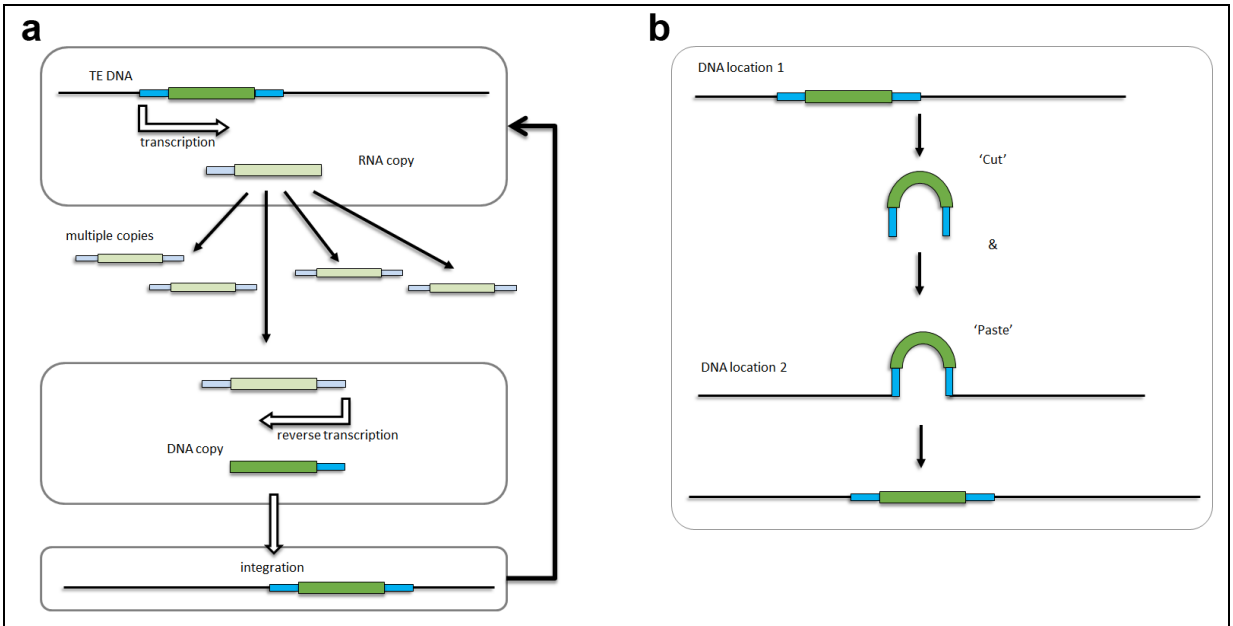

Figure 1.7 Methods of TRANSPOSITION. (**a**) Replicative transposition of RETROELEMENTS and (**b**) 'cut and paste' TRANSPOSITION of class II TRANSPOSABLE ELEMENTS. Adapted from Kidwell & Lisch 1998.

SPECIES) which has been reported and associated with the high RECOMBINATION rates in cases of POLYPLOIDY and HYBRIDIZATION between SYMPATRIC SPECIES (Sharma et al. 2008). In terms of chromosomal rearrangements TE activity may promote more or less dramatic changes (small-scale inversions, deletions, translocations or duplications). On the other hand, DIVERGENCE in TE complements can occur rapidly after HYBRIDIZATION with profound consequences including for GENE FLOW barriers. Extreme cases of massive TE mobilization and MUTATION bursts may result in low HYBRID FITNESS (Abbott et al. 2013). TE activation via HYBRIDIZATION may thus play a critical role during SPECIATION and given the prevalence of HYBRIDIZATION among fishes (Scribner et al. 2001), TE examination may disclose valuable information regarding the processes and the genomes.

TEs abundance in eukaryotic genomes has an apparent correlation with genome size (e.g. from 2.7% in *Takifugu rubripes* to ≥45% in mammalian genomes; Kidwell 2002). RETROELEMENTS are often the main source of TE DNA in eukaryotes (e.g. Arkhipova & Rodriguez 2013) which does not preclude an extraordinary diversity of families (hundreds known to date). Compared to the vast biodiversity found among fish SPECIES (estimated number of 32,500 extant valid SPECIES; Nelson 2006), studies focused on the identification and characterization of TEs in the genomes of fishes are still scarce (Ferreira et al. 2011b). Notwithstanding, all types of TEs can be found in the genomes of fishes (Volff 2005) either clustered or dispersed (e.g. Fischer et al. 2004; Valente et al. 2011). For a long time most of the studies with TEs were restricted to sequence descriptions and presence/absence reports (e.g. Volff et al. 1999, 2000). With the progress in molecular and cytogenetic techniques the interest in PHYSICAL MAPPING these genetic elements has increased significantly.

RETROELEMENTS are also amongst the best studied TEs in fishes, namely the Rex family (Rex1, Rex3 and Rex6). These elements, first described in the fish *Xiphophorus,* have been active during the evolution of several fish lineages being found widespread amongst teleosts (reviewed in Volff 2005). Regarding chromosome mapping, RETROTRANSPOSONS have been investigated in several fish lineages (**Table 1.2**) belonging to the orders Characiformes (Cioffi et al. 2010; Terencio et al. 2012; Silva et al. 2013), Cyprinodontiformes (Nanda et al. 2000), Perciformes (Bryden et al. 1998; Oliveira et al. 1999, 2003; Mandrioli et al. 2001; Harvey et al. 2003; Ozouf-Costaz et al. 2004; Mazzuchelli & Martins 2009; Teixeira et al. 2009; Gross et al. 2010; Fantinatti et al. 2011; Valente et al. 2011), Salmoniformes (Symonová et al. 2013), Siluriformes (Ferreira et al. 2011a; Matoso et al. 2011), and Tetraodontiformes (Mandrioli & Manicardi 2001; Dasilva et al. 2002; Bouneau et al. 2003; Fischer et al. 2004). In cyprinids however, RETROTRANSPOSON sequences have only been described in *Cyprinus carpio*, *Danio rerio* (Volff et al. 1999) and in *Alburnus alburnus* where they were found strongly associated with the giant B

chromosomes (Ziegler et al. 2003); but until this dissertation there has been no study targeting the PHYSICAL MAPPING of these genetic elements to cyprinid genomes (see **Chapter 3.2**).

According to the compilation data (**Table 1.2**), Rex ELEMENTS reveal a clearly differentiated organization from SPECIES to SPECIES suggesting general rearrangements along their dispersion process. TE content and distribution can differ greatly within a same lineage including between closely related SPECIES (e.g. Cioffi et al. 2010; Ferreira et al. 2011b), constituting an important factor in the genomic singularity of SPECIES. TE insertion POLYMORPHISM is common enough to provide an efficient tool for PHYLOGENETIC and population studies, being far more representative of the genetic diversity than phenotypic POLYMORPHISM (reviewed in Hua-Van et al. 2011). Once a TE becomes fixed in a population it is fairly difficult to lose it; hence the importance of following the dynamics of a TE in the genome and/or SPECIES. Despite the countless evidences of the key role of TEs in evolution (e.g. Kidwell 2002; Hua-Van et al. 2011; Cioffi & Bertollo 2012), the impact of TEs at the population level remains poorly explored. Also, data on TE distribution in teleosts may yet be scattered but results already evidenced their participation in the formation/differentiation of sex chromosomes and supernumerary (B) chromosomes, and in the evolution of fish genomes in general.

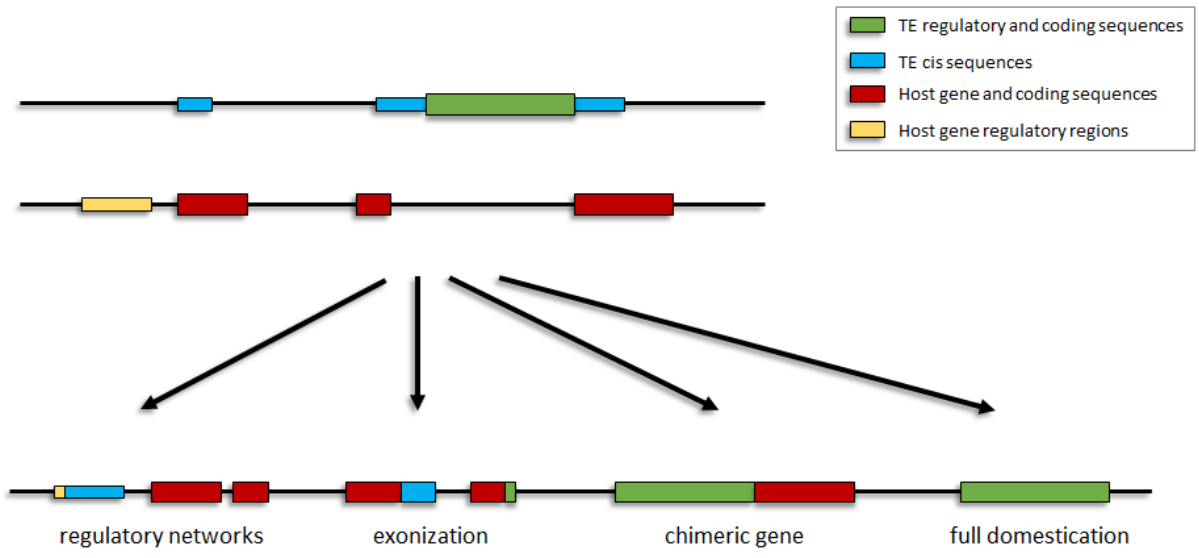

Figure 1.8 Genomic consequences of TEs activity. TE sequences may contribute to gene regulatory regions or coding sequences. Small portions or almost entire elements can be exapted, which can result in new regulations, new genes (domestication), or gene disruption (pseudogenes). Adapted from Hua-van et al. 2011.

Table 1.2 Compilation of studies and main results regarding the distribution of TRANSPOSABLE ELEMENTS (TEs) in the genomes of fish SPECIES.

Order Family	species	TE	distribution	reference
Characiformes				
Characidae	*Astyanax bockmanni*	Rex3	mainly heterochromatin	Silva et al. 2013
Erythrinidae	*Erythrinus erythrinus*	Rex3	dispersed	Cioffi et al. 2010
Prochilodontidae	*Semaprochilodus taeniurus*	Rex1	centromeric, telomeric, chromosome W	Terencio et al. 2012
Cypriniformes				
Cyprinidae	*Alburnus alburnus*	Gypsy, Ty3	B-chromosome	Ziegler et al. 2003
	Anaecypris hispanica *Iberochondrostoma lemmingii* *Iberochondrostoma lusitanicum* *Pseudochondrostoma duriense* *Pseudochondrostoma polylepis*	Rex3	heterochromatin	this dissertation
Cyprinodontiformes				
Poeciliidae	*Xiphophurus maculatus*	XIR LTR-like	Y chromosome	Nanda et al. 2000
Perciformes				
Artedidraconida	*Artedidraco shackletoni*			
Bathydraconida	*Gymnodraco acuticeps* *Gymnodraco victori*			
Bovichtidae	*Bovichtus angustifrons*			
Nototheniidae	*Dissostichus mawsoni* *Notothenia coriiceps* *Patagonotothen tessellata* *Trematomus newnesi* *Trematomus hansoni* *Trematomus bernacchii* *Trematomus pennellii*	Rex1, Rex3, Tc1-like	dispersed, pericentromeric	Capriglioni et al. 2002 Ozouf-Costaz et al. 2004
Channichthyidae	*Chionodraco hamatus* *Neopagetopsis ionah*			
Cichlidae	*Astatotilapia latifasciata*		dispersed, pericentromeric, B-chromosome	Fantinatti et al. 2011
	Astronotus ocellatus		heterochromatin	Mazzuchelli & Martins 2009
	Cichla kelberi	Rex1, Rex3, Rex6	dispersed, heterochromatin	Teixeira et al. 2009
	Chaetobranchus flavescens		heterochromatin	
	Haplochromis obliquidens		dispersed	Valente et al. 2011
	Hemichromis bimaculatus *Heros efasciatus* *Melanochromis auratus*		heterochromatin	
	Oreochromis niloticus	Rex1, Rex3, Rex6, CiLINE2, On2318, ROn-1, Ron-2	dispersed, chromosome 1	Bryden et al. 1998 Oliveira et al. 1999, 2003 Harvey et al. 2003
		On239, Tc1-like	dispersed, centromeric, telomeric	Valente et al. 2011
	Satanoperca jurupari	Rex1, Rex3, Rex6	dispersed, heterochromatin	Valente et al. 2011
	Symphysodon aequifascistus *Symphysodon discus* *Symphysodon haraldi*	Rex3	heterochromatin	Gross et al. 2010
Gobiidae	*Gobius níger*	Mariner-like	NORs	Mandrioli et al. 2001
Salmoniformes				
Salmonidae	*Coregonus albula*	Rex1	dispersed except for one a chromosome	Symonová et al. 2013
	Coregonus fontanae			
Siluriformes				
Loriicaridae	*Hisonotus leucofrenatus* *Paratocinclus maculicauda* *Pseudotocinclus tientensis*	Rex3	dispersed	Ferreira et al. 2011a
Pimelodidae	*Steindachneridion melanodermatum*		telomeric	Matoso et al. 2011
Tetraodontiformes				
Tetraodontidae	*Tetraodon fluviatilis*	Mariner-like	NOR heterochromatin	Mandrioli & Manicardi 2001
	Tetraodon nigroviridis	Rex1, Rex3 Dm-Line Tc1-like Zebulon Tol2 Babar	heterochromatin	Dasilva et al. 2002 Bouneau et al. 2003 Fischer et al. 2004
		Buffy	4–5 chromossomes	

1.3.2. Single Copy Genes

By mapping single copy genes of specific LINKAGE GROUPS, the GENETIC MAP can be linked to the PHYSICAL MAP (i.e. chromosomes). Single copy genes have seldom been mapped in fish genomes (e.g. Nanda et al. 2000; Sola & Gornung 2001). Chromosome mapping of single copy genes usually requires clones from large-insert-libraries (cosmids, PACs, BACs, or YACs) frequently unavailable for non-model SPECIES. One way to solve this limitation is by cross-species comparative analyses – i.e. use clones from a model organism's library in the investigation of a non-model organism – like it has been done for several mammalian orders (reviewed in Ruiz-Herrera et al. 2012); but teleost orders are not as closely related and may therefore disclose additional caveats. Another solution is to prepare a probe cocktail containing two or three genes known to share the same LINKAGE GROUP in model SPECIES (e.g. salmonids, poecilids, zebrafish).

1.3.3. Whole Chromosomes as Probes (WCPs)

Chromosome painting refers to the hybridization of fluorescently labelled chromosome-specific, composite probe pools (WCP) to cytological preparations by which the visualization of individual chromosomes in metaphase or interphase nuclei becomes possible (Ried et al. 1998). Chromosome painting is a common approach in comparative studies allowing for CROSS-SPECIES CHROMOSOME PAINTING on a genome-wide scale. WCPs can be used to trace ancestral chromosome (or chromosomal regions) homeologies (**Figure 1.9**), with the limitation that increasing evolutionary distance between SPECIES reduces resolution power (e.g. Robinson & Yang 2012).

WCPs are usually obtained by microdissection or flow sorting (FACS) followed by DOP-PCR (degenerate oligonucleotide-primed PCR) or whole genome amplification (WGA). Both isolation techniques present additional difficulties regarding their application to fish chromosomes given that fish KARYOTYPES are rather uniform in terms of chromosome size and base content (e.g. Ráb & Collares-Pereira 1995; Sola & Gornung 2001; Arai 2011). For all that, chromosome painting has been applied to a marginal fraction of fish SPECIES. Apart from few exceptions (Henning et al. 2008; Ráb et al. 2008), the vast majority of WCPs from fishes relate to sex chromosomes (e.g. Diniz et al. 2008; Henning et al. 2011; Terencio et al. 2012; Pazian et al. 2013) or B chromosomes (Ziegler et al. 1993). WCPs from a complete set of flow sorted fish chromosomes are available for *Gymnotus carapo* even though some chromosomes could not be resolved (Nagamachi et al. 2010); and more than 500 chromosome-specific BAC clones are available for *Danio rerio* (Freeman et al. 2007). Nonetheless, the demonstration of Kosyaková and

colleagues (2013) by "blindly" microdissecting each chromosome of a single metaphase, may soon allow the generation of chromosome-specific DNA libraries for a variety of other fish SPECIES.

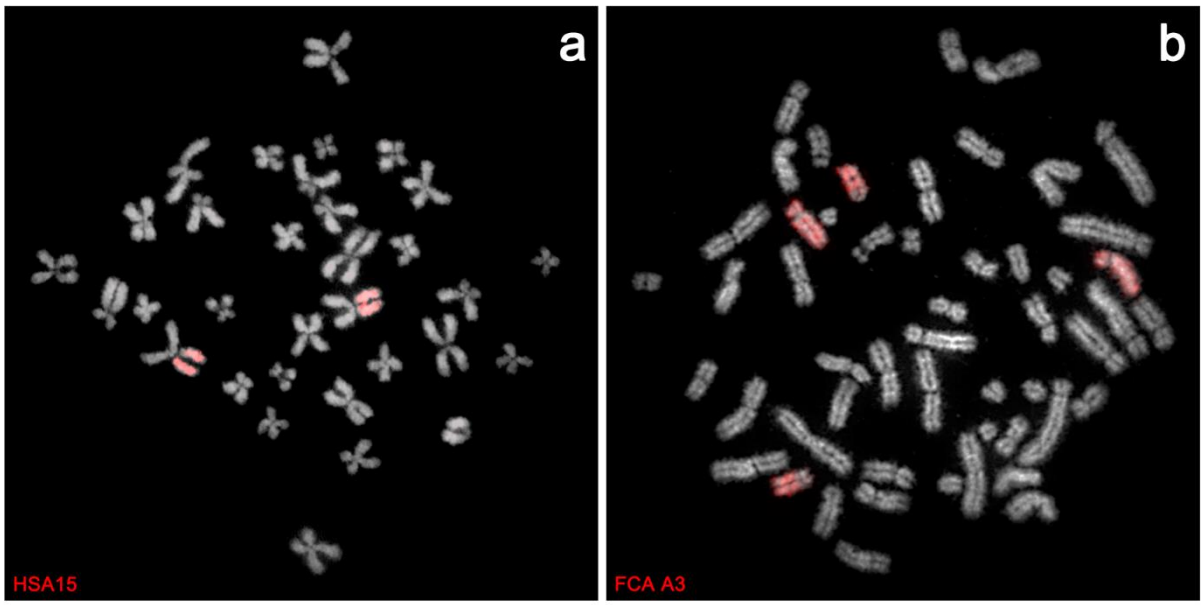

Figure 1.9 ZOO-FISH of (**a**) human chromosome 15 (HSA15) on metaphase chromosomes of pigmy hippopotamus (*Choeropsis liberiensis*; courtesy of Josefina Kjöllerström, CBA/Texas A&M University) and of (**b**) cat chromosome A3 (FCA A3) on metaphase chromosomes of *Genetta genetta granti* (courtesy of Raquel Silva, CBA/Universidade de Trás-os-montes e Alto Douro), evidencing chromosome homeologies and evolutionary rearrangements of a big syntenic block which corresponds to a single whole chromosome in the donor SPECIES.

1.3.4. Whole Genomes as Probes

GENOMIC IN SITU HYBRIDIZATION (GISH) and COMPARATIVE GENOMIC HYBRIDIZATION (CGH) are FISH-derived techniques employing two genomic DNAs as probes (one or both labelled, respectively). These are rather inexpensive means to study genome organisation and sub-chromosomal structure especially among non-model SPECIES for which genome sequence is incomplete or even unavailable. SPECIES-specific probes are useful for quick identification of immature related SPECIES and for the identification of individual parental contribution to interspecific diploid and POLYPLOID HYBRID genomes (reviewed in Marková & Vyskot 2009).

GISH/CGH efficiency is largely based on genome-specific repetitive sequences (Kato et al. 2005). And even though many classes of dispersed repeats are shared among closely related SPECIES, they usually evolve faster than housekeeping genes enabling to differentiate them (Charlesworth et al. 1994). In theory, in GISH/CGH the chromosomal sequences shared between the parental taxa will rapidly block

each other and leave the SPECIES-specific portions (dispersed repetitive sequences such as TRANSPOSABLE ELEMENTS) free to hybridize with one of the two sets of the target (HYBRID) chromosomes (Kato et al. 2005). Therefore, the genetic distance between the parental SPECIES has also an influence in GISH/CGH results.

Notwithstanding, given that genome-specific repeats have frequently a non-random distribution chromosome-specific banding patterns can thus be generated even in fish chromosomes. Although GISH studies have been mostly developed in plants, its application to fish chromosomes already proved useful for basic KARYOTYPE characterization (e.g. Ferreira & Martins 2008), for depicting HYBRID lineages (e.g. Zhu & Gui 2007; Rampin et al. 2012; Knytl et al. 2013) and chromosome elimination in HYBRIDS (e.g. Fujiwara et al. 1997; Sakai et al. 2007), or to provide new perspectives in PHYLOGENETIC and taxonomic studies (e.g. Valente et al. 2009).

In this work, a brief reference to results of CROSS-SPECIES GISH experiments is made in **Chapter 4**, whereas CGH has been employed to investigate HYBRID genomes and unfortunately successful results were only obtained for one specimen (**Chapter 3.1**). Nevertheless results allowed (1) identifying one of the parental SPECIES as the major genomic contributor, (2) proving preferential BACKCROSSING, (3) pinpointing chromosomal regions with both shared and recombined ancestry, (4) recognizing additional translocations besides NOR translocations, and (5) proving that KARYOTYPE similarities typical of subfamily Leuciscinae (Ráb & Collares-Pereira 1995) are restricted to chromosome macrostructure.

1.4. Objectives & Structure of the Dissertation

The main objective of this dissertation was, while using a multi-approach scheme to define and characterize natural AOLxPDU and AOLxPPO HOMOPLOID HYBRIDS, to follow up and understand some processes of genome dynamics in (1) highly conservative Leuciscinae KARYOTYPES (at the macrostructural level) *versus* high SPECIATION rates, and (2) coping mechanisms behind these successful cases of extensive natural HYBRIDIZATION. These HYBRID systems provide an excellent opportunity to shed some light into the processes of (HOMOPLOID) HYBRIDIZATION, ADAPTATION and evolution on a vertebrate context.

Starting from seemingly very similar KARYOTYPES (Pereira et al. 2009, **Appendix II**), the progress on cytogenomics and sequence data allows now for a deeper level of investigation on these genomes and their actual organization. Five specific objectives were then established:

1. The characterization of the pure parental SPECIES in ALLOPATRY, based on molecular cytogenetic tools. The objective was to define SPECIES-specific chromosomal markers in populations with little or no interference from interspecific INTROGRESSION. These markers would then serve as the baseline for (1) a comparative study of Iberian Leuciscinae, and (2) to be used in the survey for HYBRIDS in SYMPATRIC populations;

2. Using such markers, expand the cytogenetic knowledge on Iberian chondrostomine SPECIES occurring in Portugal, for which only conventional cytogenetic data were available or no data at all in some cases;

3. Survey HYBRID ZONES of interest for individuals with admixed ancestry using the same cytogenetic markers and possibly trace genomic rearrangements derived from the HYBRIDIZATION events originating them;

4. PHYSICAL MAPPING of other sets of repetitive DNA to better understand genome organization and restructuring in these SPECIES and their HOMOPLOID HYBRIDS;

5. Correlate results with the evolutionary history of Iberian *Chondrostoma s.l.* and other Leuciscinae, namely the ALLOPOLYPLOID SPECIES COMPLEX of *Squalius alburnoides*, and try to integrate the findings in a more global context of evolutionary potential of HYBRIDS.

To complete our sample on both parental species and putative hybrids, new individuals were captured by electrofishing, fin clipped for genetic purposes, photographed for morphological analysis and immediately released back to the river, unless necessary for new chromosome preparations, and therefore brought to the lab for processing and voucher deposition (MUNHNAC). Chromosome suspensions were either prepared from standard *in vivo* kidney preparations or *in vitro* fibroblast fin cultures (Rodrigues & CollaresPereira 1996). Many individuals and chromosome suspensions were already available at our lab, from previous and continued works on fish cytogenetics, also integrating our sample. Cytogenetic analyses included conventional techniques like Giemsa, Chromomycin A3, Ag-NOR stainings, and C-banding, as well as molecular cytogenetic procedures employing FISH, GISH and CGH techniques.

The present dissertation was structured in six Chapters addressing all the above mentioned specific goals. With the exception of the general Introduction (**Chapter 1**), Discussion (**Chapter 5**) and Final Remarks (**Chapter 6**), the remaining chapters comprise original scientific papers published (**Chapters 2** and **4**), in press (**Chapter 3.1**, uncorrected proofs) or submitted for publication (**Chapter 3.2**) in

indexed, peer-reviewed international journals. Each publication is preceded by a detailed summary of the work in Portuguese. The following section corresponds to a **Glossary** explaining some of the concepts used throughout the text. Finally, in Appendix were gathered all the morphological data resulting from this investigation (**Appendix I**), and three scientific publications also co-authored by the author of this dissertation (**Appendixes II** to **IV**) that, despite not being part of the core of the PhD, were considered of interest as a complement to some of the aspects discussed in **Chapter 5**.

As previously stated, **Chapter 1** concerns key topics to the integration of the following chapters. **Chapter 2** deals with the characterization of the parental SPECIES involved in the two HYBRID systems under analysis, *Achondrostoma oligolepis*, *Pseudochondrostoma duriense* and *P. polylepis* (**objective 1**) while also dealing with the characterization of six other Iberian SPECIES occurring in Portuguese territory (namely, *A. arcasii*, *A. occidentale*, *Iberochondrostoma almacai*, *I. lemmingii*, *I. lusitanicum* and *P. willkommii*) (**objective 2**). In **Chapter 3** HOMOPLOID HYBRIDS are characterized for the first time in a multidisciplinary approach combining morphologic, genetic and cytogenetic markers, using the knowledge available for the parental SPECIES as the comparison point: (1) PHYSICAL MAPPING of both rDNA families, (2) telomeric $(TTAGGG)_n$-repeats, (3) COMPARATIVE GENOMIC HYBRIDIZATION (CGH) (**Chapter 3.1**), and (4), Rex3 RETROELEMENT (**Chapter 3.2**) (**objectives 3** and **4**). **Chapter 4** comprises an overview on fish cytogenetics with emphasis on Iberian HOMOPLOID HYBRIDS but also on other Iberian Leuciscinae, namely the *Squalius alburnoides* polyploid complex). Finally in **Chapter 5**, the various evolutionary implications of the results obtained in this study are debated, making an effort to incorporate theoretical predictions also in comparison to other renowned cases of natural HYBRIDIZATION (HOMOPLOID and POLYPLOID). **Chapter 6** summarizes the main achievements of this dissertation and puts forward some new questions and future prospects on conceivable follow up research.

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Chapter 2

Characterization of the parental species

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Chapter 2.1

Chromosomes of European cyprinid fishes: comparative cytogenetics and chromosomal characteristics of ribosomal DNAs in nine Iberian chondrostomine species (Leuciscinae)

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Resumo

Nove espécies Ibéricas de *Chondrostoma sensu lato* (*s.l.*) foram investigadas com vista à descrição e análise comparativa dos seus cariótipos, com destaque para o mapeamento cromossómico dos genes codificantes para as subunidades de RNA ribossomal (rDNAs). Na literatura encontravam-se já descritos e caracterizados *primers* específicos para os rDNA-5S e 45S, os quais foram utilizados em reacções de PCR para amplificar essas sequências a utilizar como sondas específicas no mapeamento em cromossomas metafásicos, através da técnica de hibridação *in situ* fluorescente (FISH). Foram caracterizadas neste estudo, designadamente, as espécies *Achondrostoma arcasii*, *A. occidentale*, *A. oligolepis*, *Iberochondrostoma almacai*, *I. lemmingii*, *I. lusitanicum*, *Pseudochondrostoma duriense*, *P. polylepis* e *P. willkommii*, todas amostradas em Portugal, quatro das quais correspondentes a endemismos nacionais (i.e. *A. occidentale*, *A. oligolepis*, *I. almacai* e *I. lusitanicum*). Todas as espécies apresentaram um valor diploide de, invariavelmente, 2n = 50 cromossomas e cariótipos característicos de leuciscíneos com seis a sete pares de cromossomas metacêntricos (m), quinze a dezasseis pares de cromossomas submetacênctricos (sm), e três a quatro pares de cromossomas subtelo-acrocêntricos (st-a). Um dos maiores pares do complemento foi diagnosticado como st-a, também típico da subfamília Leuciscinae (ver **Apêndice III**) e, apesar de algumas diferenças de tamanho em determinados pares de cromossomas homólogos, não foram encontrados heteromorfismos que pudessem ser inequivocamente associados à determinação sexual em nenhuma destas espécies. Apesar da maioria destas espécies ter sido anteriormente alvo de uma descrição citogenética convencional (i.e. número diploide, fórmula cromossómica, bandeamento Giemsa), as espécies *A. occidentale* e *P. willkommii* foram caracterizadas pela primeira vez e os dados citogenéticos existentes para as espécies *A. arcasii* e *I. lemmingii* foram revistos e actualizados, com base em metodologias mais modernas e de maior resolução. A despeito das semelhanças descritas e aparentemente conservadas dentro da subfamília Leuciscinae no que respeita à macroestrutura dos cariótipos, foi observada uma considerável variabilidade em termos de número e localização de *clusters* de ambos os marcadores moleculares utilizados neste estudo, especialmente dentro dos géneros *Achondrostoma* e *Iberochondrostoma*. Relativamente ao género *Achondrostoma* apenas foi possível associar tal variabilidade a um politipismo, ou seja, a uma variação entre populações de rios diferentes (em especial da espécie mais comum do género, *A. oligolepis*). No género *Iberochondrostoma* foi possível ir mais além e, paralelamente a uma análise de genética populacional recentemente publicada, traçar a história evolutiva destes *clusters* de genes no seio do género. Em geral, as unidades de rDNA-5S foram localizadas em associação com a região peri-centromérica incluindo parcial ou

totalmente o braço curto de cromossomas st-a. Foram observados quatro a oito sinais positivos independentes destas unidades de genes. Por outro lado, as regiões organizadoras nucleolares (NORs) directamente detectadas pela sonda de rDNA-45S foram observadas em posição terminal no braço curto de cromossomas sm, variando de três a seis unidades independentes, por vezes com grandes polimorfismos de tamanho (i.e. número de cópias) nos cromossomas homólogos. Em nenhuma das espécies se observou a co-localização das duas unidades de genes de rRNA num mesmo cromossoma. Apesar de apenas um par de NORs ser aceite como a condição ancestral para os leuciscíneos, uma variação numérica do género já tinha sido anteriormente confirmada em *I. lusitanicum*, também por FISH. No momento da presente publicação, pensava-se que este polimorfismo pudesse ser característico de Leuciscinae Ibéricos. No entanto, foi reportada uma situação semelhante em Leuciscinae de Itália. Face aos resultados obtidos, foi colocada a hipótese da frequente ocorrência de *bottlenecks* genéticos nas populações que vivem em habitats Mediterrânicos (tipicamente semi-áridos) estarem na origem do extenso polimorfismo observado e da maior probabilidade de fixação de possíveis translocações envolvendo os *clusters* de rDNA.

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Chromosomes of European cyprinid fishes: comparative cytogenetics and chromosomal characteristics of ribosomal DNAs in nine Iberian chondrostomine species (Leuciscinae)

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Abstract Karyotypes and chromosomal features of both minor and major ribosomal RNA genes (rDNA) were investigated in nine Iberian chondrostomine species by fluorescent in situ hybridization (FISH) with 5S and 45S rDNA probes. All species presented invariably diploid values of 2n = 50 and the characteristic leuciscin karyotype pattern with 6–7 metacentric (m), 15–16 submetacentric (sm) and 3–4 subtelo- to acrocentric (st/a) chromosome pairs. The largest chromosome pair of the set was st/a as typical of Leuciscinae and no heteromorphic chromosomes could be unequivocally associated to sex determination. *Achondrostoma occidentale* and *Pseudochondrostoma willkommii* were cytogenetically characterized for the first time while *Achondrostoma arcasii* and *Iberochondrostoma lemmingii* were revisited regarding previous karyotype descriptions. Remarkable variability in number and location was observed for both molecular chromosome markers, especially within *Achondrostoma* and *Iberochondrostoma* genera. Clusters of 5S rDNA were mostly terminally associated to st/a chromosomes varying from four to eight positive signals, whilst NOR sites directly detected by the 45S rDNA probe were identified in sm chromosomes varying from three to six independent clusters. Frequent population bottlenecks in Mediterranean-type semiarid habitats were hypothesized to explain not only such extensive polymorphism which seems unique among leuciscin cyprinids but also the increased probability of fixation of rDNA translocation events.

Keywords Fish cytotaxonomy · Endangered cyprinids · rDNA · Chromosome polymorphisms · FISH · NOR-phenotypes

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Introduction

Historically, a number of species across the western Palearctic Region were assigned to the cyprinid genus *Chondrostoma* Agassiz, 1835 (Elvira 1987, 1997) with highest diversity records in the Balkans and particularly in the Iberian Peninsula. The presence of a well-developed rostral cap above the upper lip and a more or less developed cornified slat covering the lower lip were long considered diagnostic for the genus. However, a recent molecular phylogenetic study on 22 out of 35 described *Chondrostoma* species based on morphological and/or molecular grounds (Robalo et al. 2007) demonstrated that such taxonomy under-represents a deeply branched assemblage which includes five other lineages besides *Chondrostoma*, namely *Protochondrostoma, Pseudochondrostoma, Parachondrostoma, Achondrostoma* and *Iberochondrostoma*.

The chromosomes of chondrostomine species (subfamily Leuciscinae) have been poorly studied (reviewed in Ráb and Collares-Pereira 1995; Monteiro et al. 2009; Pereira et al. 2009; Esmaeili et al. 2010) and available data suggest a very conservative karyotype pattern typically consisting of 6–8 m, 12–16 sm/st and 3–4 st/a pairs of chromosomes (2n = 50), with the largest pair included in the last category and apparently homeologous across genomes of leuciscin cyprinids (Ráb et al. 2008). However, data on Nucleolar

Organizing Regions (NOR)-phenotypes regarding number and location of rDNA sites on chromosomes have been evidencing a higher variability (Rodrigues and Collares-Pereira 1996; Collares-Pereira and Ráb 1999; Kalous et al. 2008; Pereira et al. 2009), a situation not reported among other leuciscins.

The identification of interspecific chromosomal homologies is crucial when trying to depict and reconstruct chromosomal evolution involving different taxa. The development of powerful molecular cytogenetic techniques such as FISH and chromosome painting coupled with gene mapping can overcome the limitations of conventional banding analyses in fish chromosomes (e.g. Dobigny et al. 2004). FISH using repetitive DNAs as probes such as ribosomal genes has been widely applied to a variety of organisms, particularly for those with less available banding data including fish species (e.g. Blackman et al. 2000; Sola and Gornung 2001; Vanzela et al. 2002; Cross et al. 2003; Carvalho et al. 2009; Nguyen et al. 2010; Mani et al. 2011; Britton-Davidian et al. 2012). Its usefulness has been proved at diverse levels such as genome organization, phylogenetics and evolution.

In this study, a comparative cytogenetic analysis of nine chondrostomine species occurring in Portuguese freshwaters—*Achondrostoma arcasii* (Steindachner 1866), *Achondrostoma occidentale* (Robalo, Almada, Sousa-Santos, Moreira and Doadrio 2005), *Achondrostoma oligolepis* (Robalo, Doadrio, Almada and Kottelat 2005), *Iberochondrostoma almacai* (Coelho, Mesquita and Collares-Pereira 2005), *Iberochondrostoma lemmingii* (Steindachner 1866), *Iberochondrostoma lusitanicum* (Collares-Pereira 1980), *Pseudochondrostoma duriense* (Coelho 1985), *Pseudochondrostoma polylepis* (Steindachner 1864) and *Pseudochondrostoma willkommii* (Steindachner 1866)—was performed. Two of the species were cytogenetically studied for the first time (*A. occidentale*, *P. willkommii*) and two other (*A. arcasii*, *I. lemmingii*) revisited. DAPI-banding patterns after denaturation required in FISH protocols improved homologues' pairing in karyotype assembly. Combined with 5S rDNA and 45S rDNA markers we also identified the homologies between chromosomes of representatives of the three genera and characterized the actual polymorphic state of NOR-phenotypes on these species' genomes. All data on major and minor ribosomal sites are new for the literature. The cause for the observed extensive variability in ribosomal units' number and location might be related to the occurrence of frequent population bottlenecks that characterize most central and southern Iberian riverine habitats.

Materials and methods

Materials

A total of 29 individuals of nine species belonging to the three new "*Chondrostoma*" genera found in Portuguese inland waters—*Achondrostoma*, *Iberochondrostoma* and *Pseudochondrostoma*—were analyzed. Their numbers, sex and collection sites are listed in Table 1.

Table 1 Specimens analyzed, sex, origin and type of available samples (for preserved material)

Species	No. + sex[a] specimens	Basin–tributary	Date collection	Type of sample	
				Suspension	Slides
A. arcasii	1?	Douro–Maçãs	1994		x
A. occidentale	1?	Sizandro	2011	x	
A. oligolepis	4♂, 4♀	Tejo–Nabão	1994	x	
		Mondego–Mortágua	2007	x	
		Douro–Paiva	2007	x	
		Douro–Sousa	2008	x	
I. almacai	1?	Arade–Monchique	2005		x
I. lemmingii	2♂, 1♀, 2?	Guadiana–Degebe	1994	x	x
		Guadiana–Pias	2008	x	
		Guadiana–Ardila	2011	x	
		Quarteira	2008		
I. lusitanicum	1♂, 2♀, 2?	Samarra	1994, 2005	x	
		Tejo–Raia	2005	x	
		Tejo–Rio Maior	2005	x	
P. duriense	2♂, 1♀, 1?	Douro–Coa	2007	x	
		Douro–Paiva, Tâmega	2008	x	
P. polylepis	1♂, 2♀	Mondego–Mortágua	2007	x	
P. willkommii	1♀	Guadiana–Chança	2011	x	
Total:	29				

[a] ♂ = male, ♀ = female, ? = undifferentiated

Chromosome preparations

Due to the endangered status of most of the species (Rogado et al. 2006) we tried to mainly use chromosome material preserved at −20 °C for several years (some back to 1994) either in the form of mounted slides or cell suspensions in cryopreservation vials filled with methanol:acetic acid (3:1) fixative, with little or no space for air inside (T. Raudsepp, pers. comm.). In any case, chromosome suspensions were either obtained from standard direct kidney preparations and/or mostly from fin fibroblast cultures (Rodrigues and Collares-Pereira 1996) to avoid sacrificing the fishes. Frozen slides were allowed to reach room temperature before opening the slide container in the presence of silica gel (set up before freezing) to avoid moisturizing the chromosomes. Old fixative solutions were freshly replaced after centrifugation every time the suspension was used to prepare slides. Cytoplasm cleaning was improved by acetic acid digestion directly on the chromosome suspensions (50 % v/v) prior to the splash on fully clean and degreased slides pre-washed in sulphochromic mixture (Merck) (Raudsepp and Chowdhary 2008). Chromosome preparations were thermally aged (3 h to overnight incubation at 65 °C), digested with pepsin (0.005 % in HCl 10 mM, 10 min, 37 °C) and RNase (100 μg mL^{-1} in 2× SSC, 1 h, 37 °C), dehydrated in an increasing ethanol series and immediately used for other procedures.

rDNA probes

Two different ribosomal DNA probes were used. The unprocessed 45S rDNA sequence for direct NOR detection was available from the p*Dm*238 clone (Roiha et al. 1981) and the 5S rDNA probe was amplified via PCR. A 50 μL reaction mix was prepared with 1.0 μM of each of the following pair of primers F 5′- TAC GCC CGA TCT CGT CCG ATC -3′ and R 5′- CAG GCT GGT ATG GCC GTA AGC -3′ (Alves-Costa et al. 2008), 1× Taq buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 100 ng of template DNA and 1.25 U Taq polymerase (RBC Bioscience). The expected product with approximately 120 bp was consistently obtained after 32 cycles of amplification and annealing at 58 °C.

PCR and cloning products were ethanol-precipitated before 1 μg of each being labeled by biotin (Invitrogen) (5S rDNA) or digoxigenin (DIG; Roche) (45S rDNA) nick translation kit according to manufacturer's recommendations. Another ethanol precipitation followed, after which they were dissolved in 50 μL hybridization mix [50 % ultrapure deionized Formamide (FA) (Sigma #F9037), 10 % Dextran sulphate, 2× saline sodium citrate (SSC), pH 7.0] to a final working concentration of 20 ng μL^{-1}.

Fluorescence in situ hybridization

Chromosome preparations were denatured for 1 min in 70 % FA/2× SSC (pH 7.0) at 70 °C, immediately passed through a cold ethanol series (70, 70, 90, 100 %) and then air dried. Probes in hybridization mix were denatured at 75 °C for 10 min, chilled on ice for at least 3 min, dropped onto the chromosomes and incubated at 37 °C up to 3 days in a moist chamber with 2× SSC. Post-hybridization washes were performed at 37 °C once in 2× SSC (pH 7.0) and twice in 2× SSC/0.1 % Tween 20 (pH 7.0). Signal detection (1 h, 37 °C) was performed after blocking unspecific hybridization with 3 % bovine serum albumin in phosphate buffered saline (BSA/PBS) for 30 min at 37 °C by incubation with anti-DIG-FITC (fluorescein isothiocyanate) (1:50 in 1 % BSA/PBS) and streptavidin-Cy3 (1:500 in 1 % BSA/PBS), accordingly. Excess of antibodies was washed once in 1× PBS. Slides were mounted with DAPI (4, 6-diamidino-2-phenolindole) (Cambio, UK) and preserved overnight at −20 °C before microscope observation.

Microscopy and image analysis

Preparations were observed under a fluorescence-equipped microscope Olympus BX60 and the gray-scale images were recorded with an ORCA-R^2 (Hammamatsu) cooled digital CCD camera. Digital images were processed and analyzed with Adobe Photoshop® CS5 using tools for contrast manipulation, over-layering and pseudo-coloration equally affecting the whole image. At least fifteen complete metaphases were analyzed per individual. Karyotypes were constructed organizing chromosomes by decreasing sizes into three categories (m, sm and st/a) according to centromere position following the nomenclature of Levan et al. (1964). The fundamental number (NF) was determined considering m and sm chromosomes as bi-armed and st/a as uniarmed elements.

Results

Metaphases from 29 individuals of nine species collected at different tributaries and basins whenever possible within the species' distribution range were comparatively analyzed. The karyotypes (Fig. 1) of the two species here characterized for the first time (*A. occidentale*, *P. willkommii*) and of those two here revisited (*A. arcasii*, *I. lemmingii*) were assembled from FISH results with one or both rDNA probes. Examined specimens from all species had invariably diploid chromosome numbers of 2n = 50 and karyotypes with haploid sets varying between 6 and 7 m + 15 and 16 sm + 3 and 4 st/a (NF = 92–94)

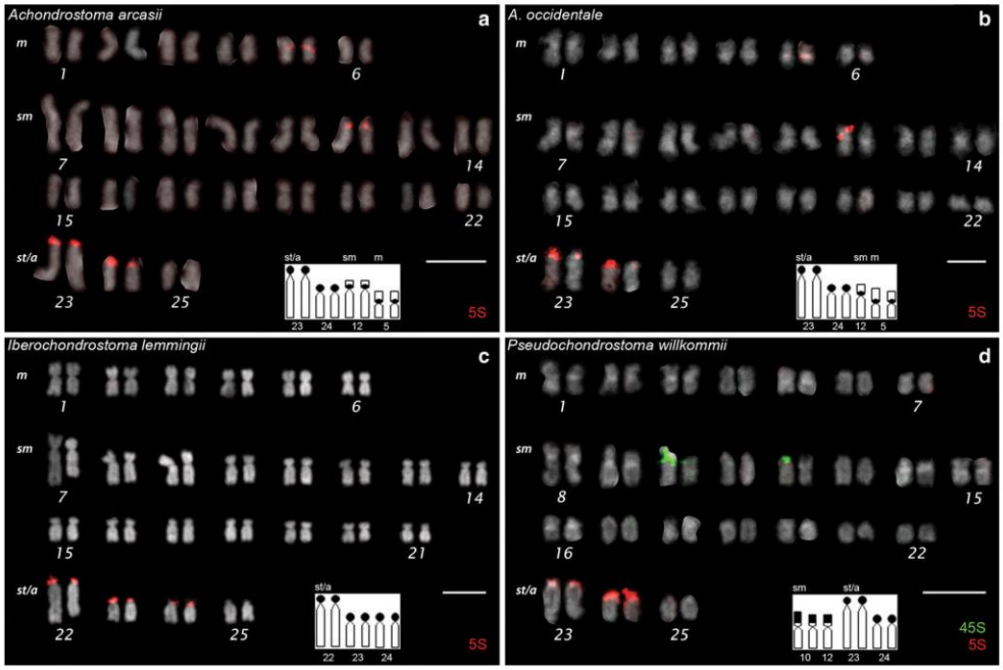

Fig. 1 Karyotypes arranged from mitotic chromosomes after FISH with 45S rDNA (*green*) and 5S rDNA (*red*) on **a** *Achondrostoma arcasii*, **b** *A. occidentale*, **c** *Iberochondrostoma lemmingii* and **d** *Pseudochondrostoma willkommii*. Inset with the respective ideograms of the NOR-bearing chromosomes (black boxes = 45S rDNA, black circles = 5S rDNA), their class (morphology) and pair number. Note the structural differences between some of the most easily identifiable chromosome pairs and the DAPI-banding patterns. Scale bar = 5 μm

as listed in Table 2. No sex-related heteromorphic elements were detected although chromosome size polymorphisms were often observed between homologues throughout the whole sample (Fig. 1).

Positive FISH results were obtained even when using old chromosome suspensions or preparations. However, special handling and pre-treatments were essential to guarantee the quality of the results. Summarized FISH results with both minor and major rDNA probes are given in Table 2.

All 45S rDNA clusters analyzed in seven species were terminally located on the short arms of sm chromosomes (Figs. 1, 2, 3, green probe) between chromosome pairs #9–10 and #11–13 (e.g. Fig. 1d). Polymorphism in size and number was found between and within species being in general population-related. The most common phenotypes were three—in *P. willkommii* (Fig. 1d), *P. duriense* (Fig. 2e), *I. lusitanicum* (Fig. 3e) and *I. lemmingii* (Fig. 3b)—and four—in *P. polylepis* (Fig. 2f), *I. lusitanicum* (Fig. 3f) and *I. almacai* (Fig. 3a) independent clusters. Genome of *A. oligolepis* was characterized by six clusters of these genes (Fig. 2a). *I. lusitanicum* exhibited both most common NOR-phenotypes (Fig. 3e, f) whose distribution seemed to follow a North–South cline variation towards an increase in NOR sites in the southern populations.

Clusters of 5S rDNA analyzed in eight species were in general associated to the short arms of the first two pairs of st/a chromosomes (Figs. 2, 3, red probe), hence considered the basal condition of Iberian chondrostomines. Polymorphism in number was associated with genomes of species within the genus *Achondrostoma* and of *I. lemmingii*. In *A. oligolepis*' genome 5S-phenotypes varied from six (Tejo, Fig. 2b) to seven (Douro, Fig. 2c) and eight (Douro and Mondego, Fig. 2d) FISH-positive signals. The genomes of *A. occidentale* (Fig. 1b) and *A. arcasii* (Fig. 1a) presented seven and eight clusters of 5S rDNA, respectively. The highest number of 5S rDNA clusters in the genome of *I. lemmingii* (six) was found in Northern Guadiana R. populations inhabiting tributaries of its right margin (Fig. 1c). Northern Guadiana R. populations of the left margin together with one individual from Quarteira stream presented the ubiquitous phenotype of only four 5S

Table 2 Comparative analysis of available cytogenetic data on *Chondrostoma s.l.* following the taxonomy proposed by Robalo et al. (2007)

Species	2n	Haploid set	NF	Chromosomal markers (number of signals)				References
				CMA_3	$AgNO_3$	45S rDNA	5S rDNA	
Achondrostoma arcasii	50	8 m + 15sm + 2st/a	96	–	–	–	–	Collares-Pereira (1985)
	50	6 m + 16sm + 3st/a	94	–	–	–	8	Present work
Achondrostoma occidentale	50	6 m + 16sm + 3st/a	94	–	–	–	7	Present work
Achondrostoma oligolepis	50	7 m + 16sm + 2st/a	96	–	–	–	–	Collares-Pereira (1985)
	50	6 m + 16sm + 3st/a	94	4–6	2–4	6	6–8	Pereira et al. (2009), present work
Iberochondrostoma almacai	50	7 m + 15sm + 3st/a	94	3	3	4	–	Monteiro et al. (2009), present work
Iberochondrostoma lemmingii	50	6 m + 16sm + 3st/a	94	–	–	–	–	Collares-Pereira (1985)
	50	6 m + 15sm + 4st/a	92	–	–	3	4–6	Present work
Iberochondrostoma lusitanicum	50	7 m + 15sm + 3st/a	94	2–4	2–3	3–4	4	Monteiro et al. (2009), present work
Chondrostoma nasus	50	19 m/sm + 6a	88	–	–	–	–	Barshiene (1977)
Chondrostoma knerii	50	15 m/sm + 10st/a	80	–	–	–	–	Berberović et al. (1970)
Chondrostoma phoxinus	50	18 m/sm + 7st/a	86	–	–	–	–	Berberović et al. (1970)
Chondrostoma prespense	50	8 m + 13sm + 4st/a	92	2	2	–	–	Bianco et al. (2004)
Chondrostoma regium	52	21sm + 5st/a	94	–	–	–	–	Esmaeili et al. (2010)
Chondrostoma soetta	50	8 m + 7sm + 7st + 3a	94	–	–	–	–	Cataudella et al. (1977)
Parachondrostoma arrigonis	50	8 m + 14sm + 3st/a	94	2	2	–	–	Kalous et al. (2008)
Parachondrostoma toxostoma	50	8 m + 7sm + 7st + 3a	94	–	–	–	–	Cataudella et al. (1977)
Protochondrostoma genei	50	–	–	–	–	–	–	Fontana et al. (1970)
Pseudochondrostoma duriense	50	7 m + 15sm + 3st/a	94	2–4	2	3	4	Pereira et al. (2009), present work
Pseudochondrostoma polylepis	50	7 m + 15sm + 3st/a	94	4	2	4	4	Pereira et al. (2009), present work
Pseudochondrostoma willkommii	50	7 m + 15sm + 3st/a	94	–	–	3	4	Present work

Most of the information concerns to old basic karyotype descriptions based on chromosome formula and/or chromosome and arm numbers, which have weak or no phylogenetic correlation (e.g. Dobigny et al. 2004). Molecular cytogenetic markers have only been recently included substantiating the need for more up-to-date studies in these species

2n diploid number, *m* metacentric, *sm* submetacentric, *st/a* subtelo-acrocentric, *NF* fundamental number

clusters (Fig. 3c); the second examined individual from Quarteira presented an intermediary phenotype with five FISH-positive signals (Fig. 3d). Nevertheless, all *I. lemmingi*'s chromosomes bearing 5S rRNA genes were st/a (pairs #22–24) while polymorphism in chromosome location was also found in *Achondrostoma* species, which integrated pericentromeric signaling at m and sm chromosomes (pairs #5 and #12). Furthermore, this extensive polymorphism in *Achondrostoma* species included also different combinations of 5S rDNA-bearing m and sm chromosomes: 1 m + 1sm (Fig. 2b) and 1 m + 2sm (Fig. 2c) in *A. oligolepis*, 2 m + 1sm (Fig. 1b) in *A. occidentale*, and 2 m + 2sm both in *A. arcasii* (Fig. 1a) and *A. oligolepis* (Fig. 2d), evidencing considerable flexibility of these sequences within these genomes.

Discussion

New karyotype descriptions and comparison with previous cytogenetic studies

In this study *A. occidentale* and *P. willkommii* were cytogenetically characterized for the first time while existing data on chromosome formula and NF of *A. arcasii* and *I. lemmingii* (Collares-Pereira 1985) were updated and corrected. The summary of all data on Iberian endemic chondrostomine species is given in Table 2. Our observations confirm the invariable diploid chromosome number 2n = 50 and the long recognized conservative pattern in karyotype composition of a number of leuciscin cyprinids as well as the presence of relatively small chromosomes

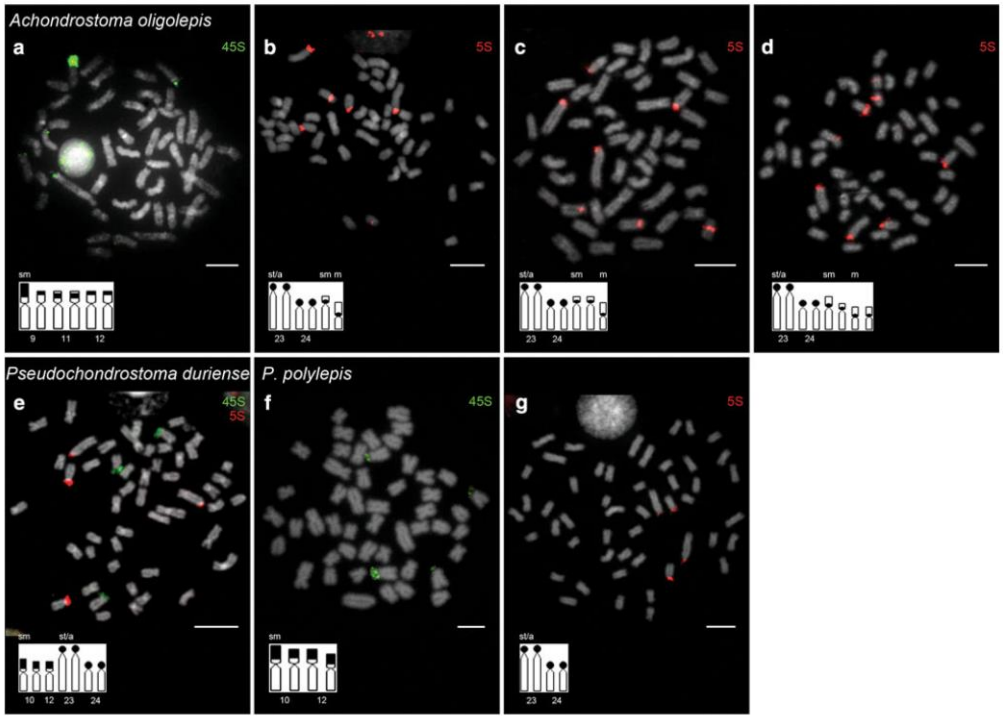

Fig. 2 Phenotypes and polymorphisms for 45S (*green*) and 5S rRNA (*red*) gene clusters mapped onto mitotic chromosomes of **a**–**d** *Achondrostoma oligolepis*, **e** *Pseudochondrostoma duriense* and **f**–**g** *P. polylepis*. Below each metaphase is the respective ideogram representing the chromosomes bearing the ribosomal clusters (*black boxes* = 45S rDNA, *black circles* = 5S rDNA). *Scale bar* = 5 μm

difficult to assign into particular categories (Ráb and Collares-Pereira 1995). The most obvious structural differences in the analyzed karyotypes were restricted to a seventh pair of m chromosomes present in the genomes of straight-mouth nases of the genus *Pseudochondrostoma* and an extra st/a pair in the karyotype of *I. lemmingii*, resulting in the slightly distinct NF value (contrasting with the previously reported value by Collares-Pereira 1985). This seems likely to be caused by peri centromeric inversions (and/or translocations involving centromere repositioning) resulting in the decrease of chromosome arm number.

Karyotyping improved by DAPI-banding together with the combined use of both rDNA markers allowed identifying the homologies between species and performing a comparative analysis with higher level of confidence. Few marker chromosomes have been described for the species under study, one of which—the biggest st/a pair—constitutes the subfamily marker (Ráb et al. 2008). Based on morphology alone, chromosomes such as the remaining st/a, the two biggest sm pairs and, in the karyotypes of some species, the biggest m pair could be easily recognized in metaphase spreads. Additional pairs could be recognized by identifying the NOR-carrying chromosomes.

Characterization and polymorphism of ribosomal DNA in the examined species

The present study applied methods for direct detection of NORs using specific rDNA probes instead of differential indirect staining methods as Ag-impregnation (Howell and Black 1980) and/or GC-specific fluorochromes (Sola et al. 1992). NOR detection by conventional banding protocols has sometimes demonstrated inconsistency to FISH signals in genomes of leuciscin cyprinids (Gromicho et al. 2005). The current FISH results are in agreement with most of the CMA_3 detections available to date (Monteiro et al. 2009; Pereira et al. 2009). Discrepancies were mainly represented by the under-detection of NORs by CMA_3 compared to FISH, which in this case may be due to the contraction of the chromosomes analyzed by both Monteiro et al. (2009)

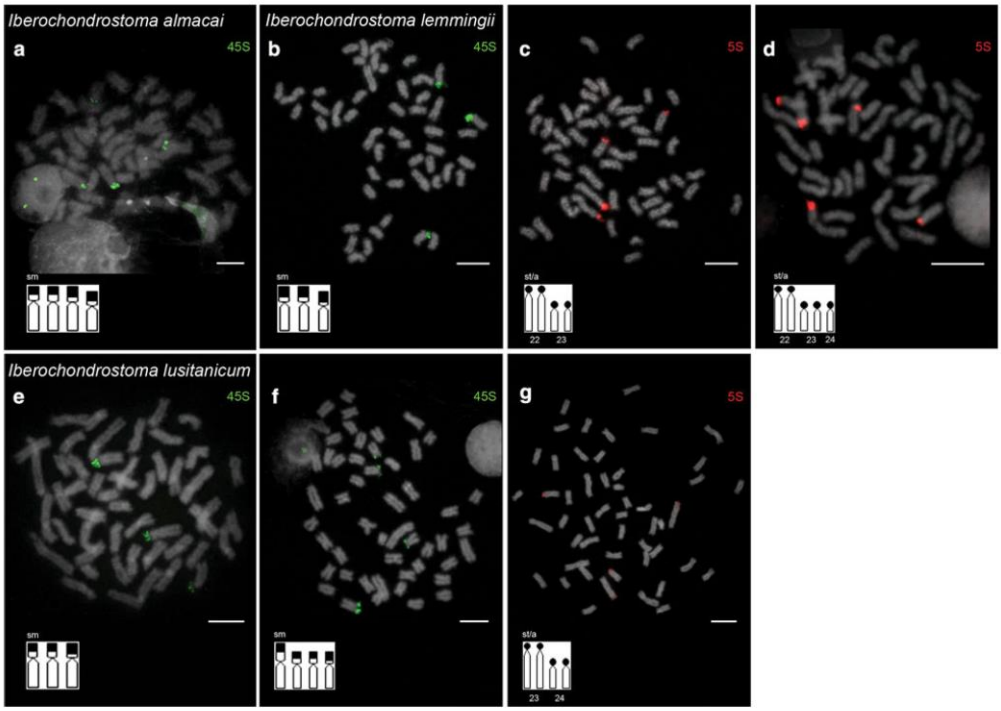

Fig. 3 Phenotypes and polymorphisms for 45S (*green*) and 5S rDNA (*red*) clusters mapped onto mitotic chromosomes of representative species of the genus *Iberochondrostoma*: **a** *I. almacai*, **b–d** *I. lemmingii* and **e–g** *I. lusitanicum*. Inset with ideograms of the NOR-bearing chromosomes (black boxes = 45S rDNA, black circles = 5S rDNA). Scale bar = 5 μm

and Pereira et al. (2009). FISH with specific probes from parts of the rDNA gene cassette can unambiguously detect all NOR clusters, active or non-active, functional or disrupted.

Number and sites of rDNA locations, i.e. NOR-phenotypes, have proven useful in cyprinid cytotaxonomy. Karyotypes of leuciscin cyprinids are usually characterized by a common NOR-phenotype—a single small sm chromosome pair with NORs on the short arms (e.g. Ráb et al. 1996; Collares-Pereira et al. 1998). However, variants have been identified for this cytotaxonomic character within and between genera (e.g. *Aspius aspius*—Ráb et al. 1990; *Phoxinus phoxinus, Eupallasella perenurus* –Boron 2001; *Acanthobrama marmid*—Gaffaroglu et al. 2006; *Telestes ukliva*—Valic et al. 2010), with a higher variability in chondrostomine species than in other leuciscin cyprinids (Rodrigues and Collares-Pereira 1996; Collares-Pereira and Ráb 1999; Kalous et al. 2008; Pereira et al. 2009). Our study confirms this variability and documents a polymorphism involving 2–4 chromosome pairs. Unfortunately, the sample size was quite small to estimate population genetic parameters (such as Hardy–Weinberg balance) regarding the observed polymorphism. Nevertheless, we can hypothesize that frequent population bottlenecks and/or predation in a semiarid environment with fluctuating water levels in the rivers where these fishes occur might significantly influence the distribution patterns of this marker in the populations.

Chromosome mapping of 5S rDNA sites has been recently performed on several fish groups and their tandem repeats were found at one or several chromosomal locations throughout the genome, usually at an interstitial position (Martins and Wasko 2004). The sites and number of 5S genes in genomes of cyprinid fishes are rather poorly known and were analyzed and/or mapped in few species of bitterlings (Inafuku et al. 2000; Fujiwara et al. 2009), polyploid cyprinines (Murakami and Fujitani 1998; Inafuku et al. 2000) and barbines (Singh et al. 2009; Mani et al. 2011). Similarly, only six leuciscin taxa were examined for this character—*Anaecypris hispanica* (Gromicho et al. 2006a), *Leuciscus leuciscus*, *Leuciscus idus*, *Squalius cephalus* (Kirtiklis et al. 2010), and the diploid-polyploid *Squalius alburnoides* complex including its maternal

ancestor *Squaliu pyrenaicus* (Gromicho et al. 2006b)—evidencing 6–8 5S rDNA clusters variable in chromosome type and position. Interestingly, the largest st chromosome pair of the complement bore 5S rDNA sites in all examined species. In an unprecedented NOR survey in Lepidoptera, Nguyen et al. (2010) have described NOR polymorphism in number and chromosomal location even in closely related species, suggesting that rDNA clusters are highly mobile components of the genome. Our study also revealed higher variability for this cytotaxonomic character. All specimens analyzed shared what seems to be the basal 5S rDNA phenotype with four independent clusters terminally located at st/a chromosomes, suggesting this condition as the plesiomorphic state in Iberian chondrostomine species. Derived phenotypes included (1) up to an extra pair of st/a clusters in the karyotype of the most divergent species (*I. lemmingii*), which is somehow congruent with the revised karyotype description considering a fourth pair of st/a chromosomes; and (2) interstitial sites in the karyotypes of the second most diversified genus (*Achondrostoma*), highly variable in number and chromosomal environment, evidencing a noteworthy dynamism and flexibility of these sequences, particularly within *Achondrostoma* genus. The polymorphism observed in *I. lemmingii* may be a straightforward evidence of the population structure across its wide distribution range and diversity of habitats, being congruent with the recent findings on population genetic parameters (Lopes-Cunha et al. 2012). The polymorphism in the genome of *A. oligolepis* seems easier explained by a simultaneous mapping of a class of transposable element highly similar to the 5S rRNA gene as described by Kapitonov and Jurka (2003) or of any other 5S rDNA variant (Martins et al. 2002; Pinhal et al. 2011). Though not yet confirmed by a comparative species sequencing analysis, transposable elements may as well account for the great dynamism of most repetitive sequences, including the rDNAs (e.g. Cioffi et al. 2011). Compared to NOR-phenotypes that are usually very simple or weakly polymorphic in leuciscins, the 5S rDNA phenotypes tend to be much more diverse and their distribution, variability, composition and/or association with transposable elements requires a deeper cytogenetic and/or genomic study in this group, similarly as performed for the neotropical fishes (Martins and Galetti 2001).

Mapping cytogenetic data onto a phylogenetic tree

In the study of Britton-Davidian et al. (2012) considerable NOR variation within and between *Mus musculus* subspecies enabled to precisely track their micro-evolutionary variations corroborating both phylogenetic and biogeographic evidences. The most recent phylogenetic data showed that chondrostomine leuciscins (together with the Balkan genera *Telestes*, *Pseudophoxinus* and *Phoxinellus*) form the monophyletic lineage VI sensu Perea et al. (2010). Their mutual divergence was estimated based on a relaxed molecular clock and in all-genes data set (Fig. 8 in Perea et al. 2010) showing *Iberochondrostoma* as the earliest genus to diverge followed by *Protochondrostoma* + Balkan *Chondrostoma*, then Central-Eastern European and Anatolian *Chondrostoma* and the youngest divergence representing *Achondrostoma* and *Parachondrostoma*. Using this "divergence" concept, we tried to map our cytotaxonomic characters onto the tree (Fig. 4). When analyzed together, the power of the selected markers is highly enhanced, enlightening the relationship between different taxa, primarily among the most closely related. Our results corroborate the phylogenetic data proposed by Perea et al. (2010) for *Chondrostoma s.l.*, resolving some of the past issues mainly at the generic level. Among the least diversified *Pseudochondrostoma* spp., the genome of *P. polylepis* appears as more deviating from the remaining two species evidencing an extra pair of 45S rDNA cluster. Although long apart, the *Achondrostoma* and *Iberochondrostoma* genera come out as the most diversified of the three analyzed. However, the different combinations of cytotypes prevailing in the genomes of their representatives suggest distinct evolutive dynamics. The analysis of *Achondrostoma* spp. is incomplete as the two molecular markers were not successfully mapped in all species and/or the sample size was extremely reduced mainly due to the endangered status of *A. arcasii* and *A. occidentale*. Even so, *A. occidentale* can be pointed out as basal to *A. arcasii* and *A. oligolepis* given that a higher number of independent units of marker sequences were fixed in the latter species. A variety of cytotypes were described for *A. oligolepis* genome, but again sampling was reduced in this species and results cannot be conclusive. Additionally, even though *P. duriense* and *A. oligolepis* from Vouga basin were intentionally left aside given the ongoing extensive hybridization events between them (Aboim et al. 2010), this is not an exclusive scenario of Vouga. Specimens used in this work were assumed as purebred after screening for specific mtDNA and nDNA genes, though one cannot totally exclude the hypothesis of the polymorphism reported here for *A. oligolepis* being affected, to some extent, by past introgression events (Gante et al. 2004). *Iberochondrostoma* appears as the most diversified genus with the highest number of species and present results are consistent with the propositions of *I. lemmingii* as the most divergent species (Perea et al. 2010) and the central unit of evolution within the genus (Robalo et al. 2008; Lopes-Cunha et al. 2012). Both basal and derived cytotypes were described throughout the species distribution range although a sample from Tejo River was not available. Moreover, *I. lusitanicum* appears as the basal

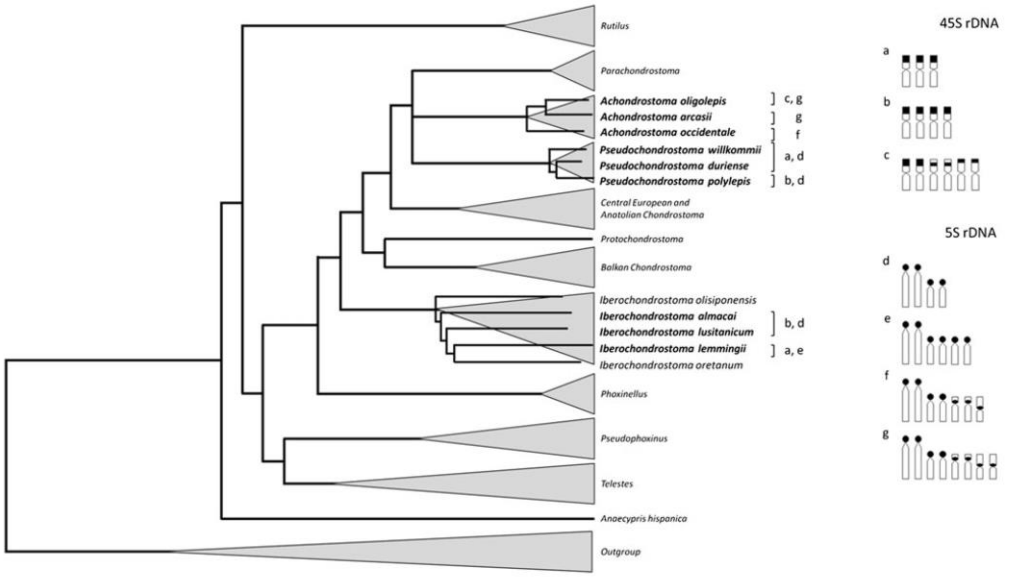

Fig. 4 Schematic representation of rDNA phenotypes (black boxes = 45S rDNA, black circles = 5S rDNA) mapped against the most recent cladogenetic data (Perea et al. 2010) where *grey triangles* account for specific richness and diversity within genera. The represented NOR phenotypes correspond to the highest number of loci and chromosome combination identified for each species included in this work (in bold) (see text for detailed explanation)

taxon within the genus but the insufficient results on its sister-species *I. almacai* prevent a proper comparative analysis to better resolve their relative evolutionary position and relationship.

In conclusion, despite their rare use for phylogenetic purposes, chromosomes may be suitable taxonomic characters despite some inherent limitations. Ribosomal RNA genes have proven their importance in studies of species and population characterization, tracing evolutionary relationships and genome restructuring. Our results were consistent with existing phylogenetic data strengthening the convenience of their usage also for cyprinids. Additionally, every piece of the puzzle we can match will help understanding the big picture of Cyprinidae speciation in Iberian Peninsula.

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Chapter 3

Characterization of natural homoploid hybrids

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Chapter 3.1

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

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Resumo

A hibridação introgressiva sabe-se agora poder agir como um promotor eficaz de diversificação e especiação também nos animais. Compreender os mecanismos por detrás desse processo é actualmente um dos maiores desafios da biologia evolutiva. Foi já proposto que a hibridação possa, por si só, ser responsável por uma rápida reorganização genómica e por rearranjos genómicos mais substanciais do que multiplicações do genoma (poliploidia); por outro lado, os híbridos são tidos como activadores de transposição, um mecanismo que poderá ser responsável pela instabilidade genética e pela reorganização que tipicamente se segue a um processo de hibridação inter-específica. Por sua vez, o estudo de cromossomas mitóticos permite uma boa visualização do genoma podendo oferecer fortes testemunhos de hibridação, recombinação e reorganização genómica, quando se utilizam ferramentas como a hibridação *in situ* fluorescente (FISH) com sondas específicas e a hibridação genómica comparativa (CGH). Os peixes da família Cyprinidae contam com vários exemplos de hibridação natural e são por isso bons candidatos para estudos de hibridação ao nível do genoma. Além disso, não existem muitos estudos de citogenética molecular aplicada a híbridos naturais de peixes. Neste trabalho, o estudo da hibridação entre dois pares de espécies Ibéricas da subfamília Leuciscinae providenciou novos dados relativos a zonas híbridas independentes envolvendo *Achondrostoma oligolepis* (AOL) e *Pseudochondrostoma duriense* (PDU), confirmando ainda a existência de zonas híbridas onde *Achondrostoma oligolepis* é simpátrico com *Pseudochondrostoma polylepis* (PPO). Tendo em consideração o padrão heterogéneo de introgressão encontrado na análise genética comparativa entre duas zonas híbridas envolvendo *A. oligolepis* e *P. duriense*, a adopção de uma abordagem múltipla, combinando marcadores morfológicos, genéticos e citogenómicos para os quais as espécies parentais já haviam sido caracterizadas, aplicada numa ampla selecção de populações, permitiu identificar padrões de mistura genética em todos os peixes amostrados como possíveis híbridos. Os resultados obtidos foram semelhantes em ambos os sistemas AOL×PDU e AOLxPPO. Em geral, tanto os morfotipos híbridos, como os dados de citogenómica e os dados de perfil genético indicaram haver retrocruzamento preferencial, sugerindo ainda AOL como a espécie parental com maior contributo genómico na composição dos genomas híbridos sob análise. Adicionalmente, os resultados implicaram AOL como mais permissivo à introgressão do que qualquer uma das espécies-irmãs PDU ou PPO com quem hibrida. Enquanto híbridos de tipo-PDU ou de tipo-PPO pareceram mais resilientes a modificações do genoma, AOL pareceu estar mais envolvido e ser mais afectado pelos eventos recorrentes de hibridação, uma vez que só foram encontradas translocações cromossómicas em híbridos de tipo-AOL. Todos os híbridos analisados mostraram um extenso polimorfismo de rDNA

aparentemente ausente nas espécies parentais até à data, mas geralmente caindo dentro do limite de possíveis combinações dos genomas parentais. Não obstante, foram ainda detectados fenótipos transgressivos, não explicados por padrões normais de recombinação entre os genomas parentais. Tais padrões incluíram (1) mais *clusters* de rDNA do que o esperado, e (2) a ocorrência de rDNAs 5S e 45S sinténicos. Apesar da associação física entre estas duas famílias de rDNAs ter sido descrita noutras espécies da subfamília, esta sintenia não parece caracterizar nenhuma das espécies de *Chondrostoma s.l.* até agora investigadas para estes marcadores. Os resultados obtidos sugerem uma evolução genómica rápida nos híbridos homoploides, provavelmente responsável pela criação de novas combinações genéticas que permitirão às espécies (antigas ou mais recentes) persistir face a adversidades impostas (ambientais ou genómicas). E ainda que as derradeiras consequências destes acontecimentos aparentemente tão extensivos e recorrentes permaneçam desconhecidas para estes genomas e estas espécies, a aplicação de metodologias mais modernas e de um modo mais amplo (ao nível de todo o genoma) poderá contribuir para esclarecer questões relacionadas com a dinâmica, as causas e o impacto da hibridação inter-específica.

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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 QUOTE PDU and AOL QUOTE PPO complexes. 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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Keywords: Comparative genome hybridization; hybrid zones; introgression; rDNA polymorphism; *Achondrostoma oligolepis*; *Pseudochondrostoma sp.*

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 QUOTE PDU and putative AOL QUOTE 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

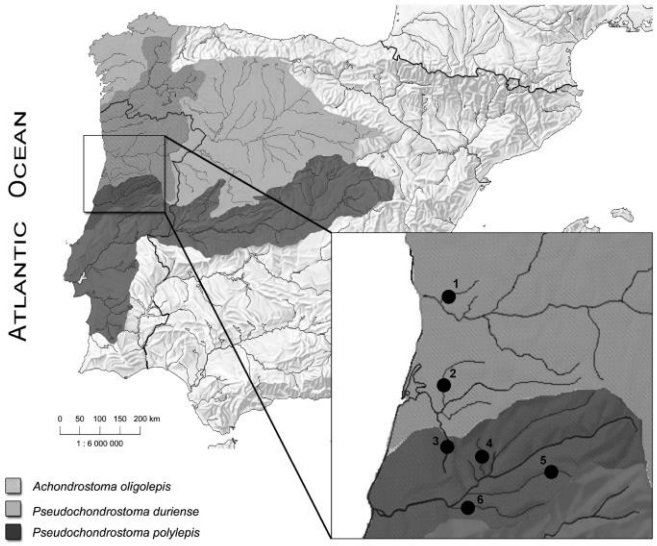

Figure 1 Sampling locations ● across the distribution ranges of ▨ AOL, ▫ PDU and ■ PPO in the Iberian Peninsula. Sampling points: 1—Sousa River (Douro basin), 2—Caima River (Vouga basin) representing AOL QUOTE PDU HZs; 3—Serra River (Vouga basin), 4—Mortágua River, 5—Alva River and 6—Ceira River (Mondego basin) representing AOL QUOTE 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 $_{\text{QUOTE AOL}}$, B $_{\text{QUOTE PDU}}$ or B $_{\text{QUOTE PPO}}$ backcrosses (Supplementary Table S2; see also Supplementary Material).

Table 1 Specimens analysed in this study

Hybrid complex	*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) 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 genes; 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 QUOTE PDU and AOL QUOTE PPO complexes, 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

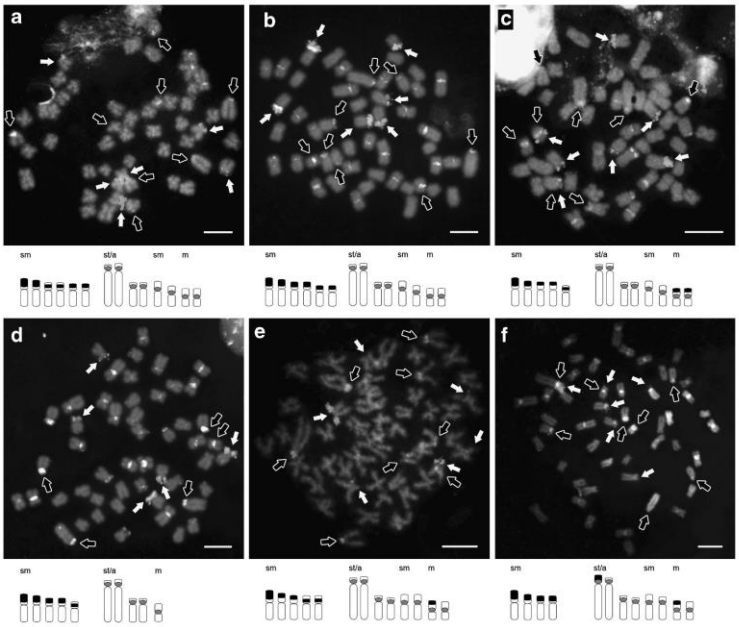

Figure 2 Simultaneous mapping of 5S (red probe, hollow arrows) and 45S rDNA (green probe, white arrows) clusters in specimens with admixed origin from (**a**–**c**) Douro (AOL-like) and (**d**–**f**) Mondego (one PPO- and two AOL-like) basins. Below each metaphase is a partial (unscaled) ideogram of 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.

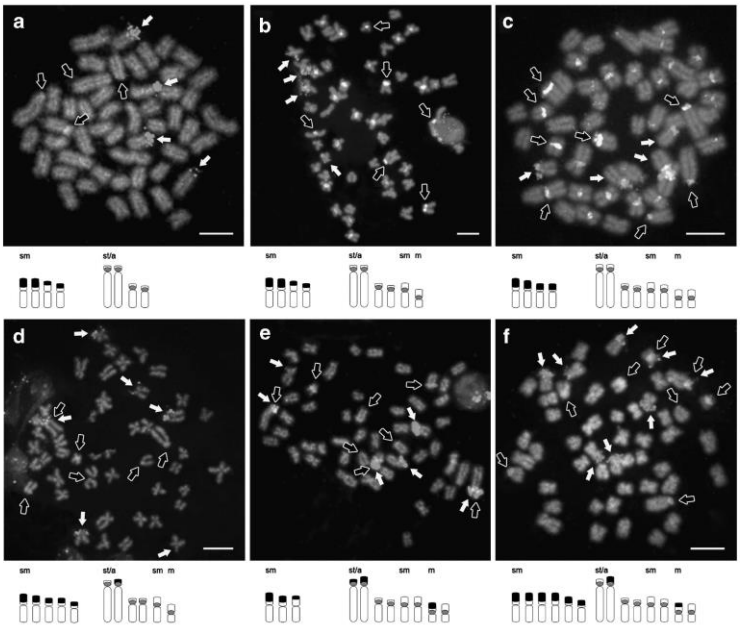

Figure 3 Dual-colour FISH with specific 5S (red probe, hollow arrows) and 45S rDNA probes (green probe, white arrows) in metaphases of (**a–c**) PDU-like and (**d–f**) AOL-like specimens from Vouga basin. Below each metaphase is an (unscaled) ideogram of the rDNA-bearing chromosomes (grey circle ● =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

Only 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 QUOTE PDU were endowed, while confirming the occurrence of AOL QUOTE 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

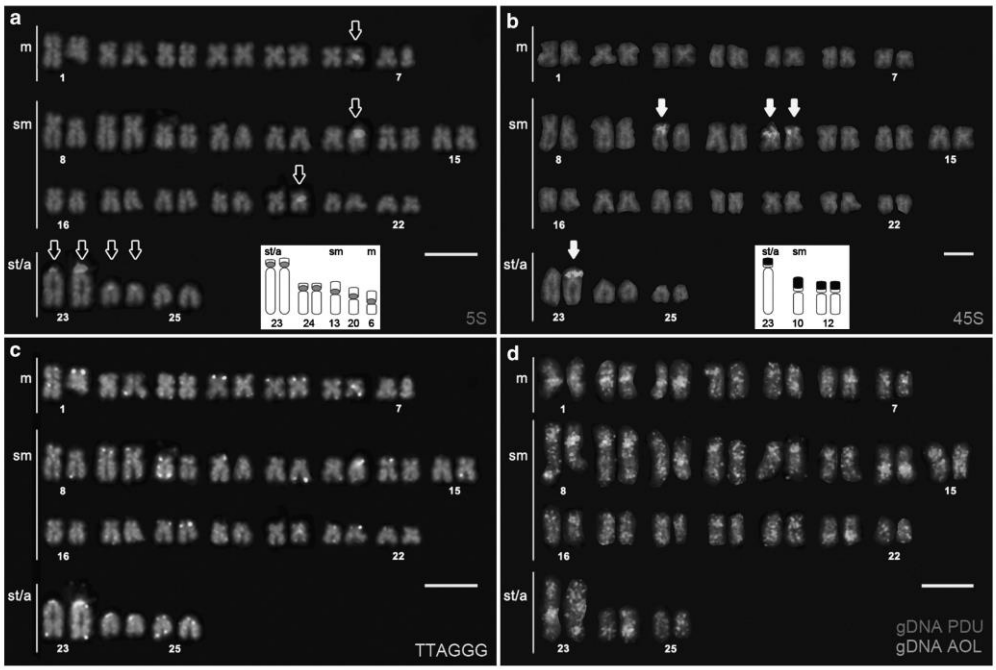

Figure 4 Karyotype of a female AOL-like hybrid from Douro basin after (**a**) FISH with 5S (red probe, hollow arrows) and (**b**) 45S rDNA (green probe, white arrows) sequences, both including inset ideograms of the rDNA-bearing chromosomes (grey circle ● = 5S, black box ■ = 45S); (**c**) FISH with telomeric $(TTAGGG)_n$ 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 chromosome numbers 8, 10 and 23. Bar = 5 μm (online version in colour). 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 QUOTE 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

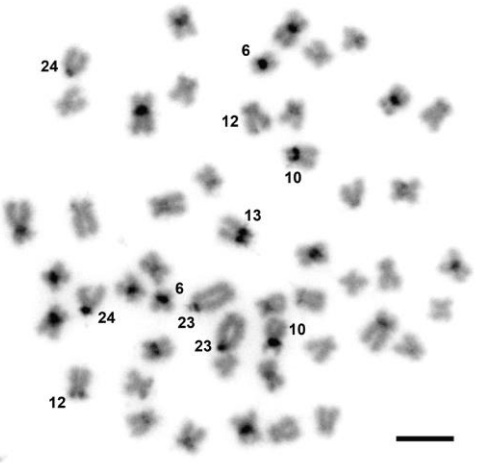

Figure 5 C-banding improved with DAPI counterstaining (CBD)-banded metaphase of a male PDU-like hybrid from Vouga basin evidencing mostly centromeric bands of constitutive heterochromatin. Chromosome pair numbers 6, 10, 12, 13, 23 and 24 are numbered for better paralleling with sequential banding results.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

of conventional cytogenetic markers (that is, 2n, 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 complex 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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Author contributions: CSAP performed all morphological and cytogenetic analyses and drafted the manuscript. MAA performed all genetic analyses and co-drafted the manuscript. PR and MJCP designed the study and co-drafted and revised the text. All authors participated in the discussion of the results and read and agreed to the final version of the manuscript.

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Supplementary Information accompanies this paper on Heredity website (http://www.nature.com/hdy)

Chapter 3.2

Chromosomal mapping of Rex3 evidences ancestral homologies in highly conservative Leuciscinae (Cyprinidae) fish karyotypes and retrotransposon activation in natural hybrids

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submitted

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Resumo

A subfamília de peixes Leuciscinae (família Cyprinidae) é altamente diversa, compreendendo vários taxa de origem híbrida (homoploides e poliploides). Os híbridos homoploides descritos dentro dos *Chondrostoma sensu lato* (*s.l.*), apesar de preservarem as semelhanças cromossómicas ao nível da macroestrutura típicas dos leuciscíneos, foram recentemente caracterizados por uma rápida reestruturação genómica provavelmente mediada por eventos de hibridação iniciais, contando com a transposição de *clusters* de DNA ribossomal (rDNA) e resultando numa grande variedade de combinações presente nos vários híbridos analisados (ver **Capítulo 3.1**). Na tentativa de compreender o papel de algumas sequências repetitivas na diferenciação dos genomas de leuciscíneos Ibéricos, várias espécies foram seleccionadas e examinadas recorrendo essencialmente a técnicas de citogenética molecular (i.e. mapeamento por hibridação *in situ* fluorescente usando como sondas um fragmento do retroelemento Rex3, a fracção do DNA genómico enriquecida em sequências alta- e moderadamente repetitivas – C_0t-1 DNA, e genes ribossomais – 5S e 45S rDNA). Nomeadamente, foram incluídas nesta análise as espécies *Anaecypris hispanica*, *Iberochondrostoma lemmingii*, *I. lusitanicum*, *Pseudochondrostoma duriense*, *P. polylepis,* e híbridos naturais entre *P. polylepis* x *Achondrostoma oligolepis*. O mapeamento cromossómico de Rex3 evidenciou um padrão de acumulação preferencial na região centromérica e em menor extensão na região telomérica de vários cromossomas, correlacionando-se de um modo geral com a distribuição de heterocromatina constitutiva e da fracção de C_0t-1 DNA, mas não tanto com cromossomas portadores dos *clusters* de rDNA. O padrão de Rex3 observado revelou-se bastante pronunciado em pelo menos 10 pares de cromossomas e o facto de ser aparentemente partilhado por todas as espécies analisadas, sugere uma origem anterior ao respectivo processo de diferenciação. Não obstante, foi ainda possível identificar determinados padrões de acumulação espécie-específicos em *Iberochondrostoma lusitanicum*, *Pseudochondrostoma duriense* e *P. polylepis*. Designadamente, no par nº. 12 de *I. lusitanicum*, no par nº. 15 de *P. duriense*, e no par nº. 3 de *P. polylepis*, os quais não co-ocorrem com zonas de heterocromatina constitutiva. É de notar, igualmente, a acumulação de Rex3 na porção distal do maior par de cromossomas st-a, muito provavelmente um padrão também partilhado por todas as espécies da subfamília, como anteriormente proposto num trabalho com sonda específica para este par de cromossomas marcador. Assim como outras classes de sequências repetitivas, também a acumulação de retroelementos foi já demonstrada em cromossomas sexuais. Até à data não foram decisivamente descritos cromossomas sexuais nestes peixes e, se presentes, permanecerão num estado muito precoce de indiferenciação morfológica. Neste estudo foi detectada uma acumulação diferencial de

Rex3 no maior par de cromossomas sm, mas apenas em indivíduos do sexo masculino. No entanto, a insuficiência de fêmeas analisadas com este marcador não permite validar esta ocorrência como estando ligada ao sexo sem um estudo mais alargado. Apesar de uma das espécies parentais dos híbridos homoploides (*A. oligolepis*) não ter sido avaliada para a distribuição de Rex3, o padrão largamente partilhado por todas as restantes espécies permitiu tirar ilacções relativamente aos híbridos. O padrão de acumulação de Rex3 nestes híbridos pareceu seguir basicamente a mesma tendência que nas espécies parentais, ainda que tenham sido detectados mais pares de cromossomas com grandes acumulações deste elemento transponível (pelo menos 15 pares bem demarcados). Este resultado parece indicar haver re-activação de mecanismos de transposição nos híbridos, à semelhança do que foi anteriormente verificado noutros organismos. Alguns destes *clusters* de Rex3 nos híbridos já apareceram correlacionados com *clusters* de rDNA: quer com 45S rDNA que se sabem hoje corresponder a *clusters* translocados, como uma das consequências de hibridação inter-específica (ver **Capítulo 3.1**), quer com alguns dos *clusters* de 5S rDNA, indicando também re-activação do retroelemento Rex3 nestes cromossomas em particular. A filogenia do Rex3 conseguida a partir das sequências anotadas no GenBank (ncbi.nlm.nih.gov/genbank/) com elevada similaridade após análise de BLASTn (ncbi.nlm.nih.gov/blast/) não reflectiu uma filogenia típica / clássica de peixes; mas a distribuição deste elemento já havia demonstrado ser descontínua nos teleósteos e, por outro lado, a sua distribuição parece congruente com uma tendência evolutiva para proteger a sua actividade e para a existência de um sistema regulatório robusto para este tipo de sequências autónomas. Ao se acumularem na heterocromatina, estes elementos reduzem o potencial impacto da sua presença / actividade no genoma do hospedeiro, ao mesmo tempo que evitam forças selectivas negativas, o que permite que se acumulem em grandes *clusters*, como observado. Os elementos transponíveis são considerados uma força dinâmica na regulação e neo-funcionalização genética, na promoção de rearranjos cromossómicos e evolução de genomas, e até especiação, por serem uma fonte de variabilidade genética que aumentam o potencial adaptativo e evolutivo dos genomas que integram. Este estudo permitiu fazer o primeiro mapeamento físico de retroelementos na família Cyprinidae, tendo ajudado a definir possíveis homologias cromossómicas ancestrais, bem como a evidenciar a re-activação de TEs em indivíduos de origem híbrida.

Chromosomal mapping of Rex3 evidences ancestral homologies in highly conservative Leuciscinae (Cyprinidae) fish karyotypes and retrotransposon activation in natural hybrids

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Running title

Rex3 in Iberian Leuciscinae

Abstract

The highly diverse Iberian Leuciscinae comprise many taxa of hybrid origin (homoploid and polyploid). Despite preserving the typical macrostructural chromosome similarities, homoploid hybrids within *Chondrostoma s.l.* were previously characterized by rapid genome restructuring, counting with rDNA transposition resulting in a variety of combinations. To understand the role of repetitive DNAs in the differentiation of Iberian Leuciscinae genomes, a molecular cytogenetic survey was conducted in *Anaecypris hispanica*, *Iberochondrostoma lemmingii*, *I. lusitanicum*, *Pseudochondrostoma duriense*, *P. polylepis* and *P. polylepis*×*Achondrostoma oligolepis* natural hybrids. Rex3 evidenced preferential accumulation to centromeric regions and some telomeres, grossly correlating with constitutive heterochromatin and C_0t-1 DNA, but not particularly with rDNA-bearing chromosomes. The accumulation was obvious in at least 10 chromosome pairs and it seemed to be shared among the different species tested, likely predating their divergence. Nevertheless, species-specific clusters were detected in *I. lusitanicum*, *Pseudochondrostoma duriense*, and *P. polylepis* indicating some differentiation. In the hybrids, at least 15 bi-armed elements had large centromeric accumulation of Rex3 this time also correlating with rDNA clusters. Additionally, strong telomeric signals in the short arms of some chromosomes could be linked to translocated 45S rDNA clusters. Rex3 phylogeny did not render the typical phylogeny of fishes but its distribution pattern is congruent with an evolutionary tendency to protect its activity and a robust regulatory system. This is the first report of retroelement physical mapping in Cyprinidae and it helped defining conceivable ancestral homologies and also recognizing retrotransposon activation in hybrids.

Key words: *Anaecypris hispanica*; *Chondrostoma s.l.* sp., karyotype differentiation, fish hybrids, transposable elements, C_0t-1 DNA.

Introduction

The subfamily Leuciscinae (Cyprinidae) represents a significant part of the South-European ichthyofauna. High biodiversity and an intricate systematics make leuciscines very attractive for the investigation of life history, biogeography and speciation (see e.g. Filipe et al., 2009). Cases of extensive natural hybridization have been reported in Iberia encompassing both homoploid and polyploid systems of hybrid origin (e.g. Aboim et al., 2010; Collares-Pereira and Coelho, 2010).

Leuciscinae karyotypes exhibit quite conservative patterns of diploid values (2n= 48-50), chromosome categories and some chromosome markers (e.g. Ráb and Collares-Pereira, 1995; Ráb et al., 2008; Pereira et al., 2012; references therein). However, recent advent of molecular cytogenetic protocols have demonstrated that such uniformity remains restricted to the macrostructural level (Rossi et al. 2012; Pereira et al., 2013b). Genomes of homoploid hybrids within Iberian *Chondrostoma s.l.* are apparently characterized by rapid genetic restructuring often associated with inter-specific hybrids (Pereira et al., 2013a) where transposable elements may play an important role (e.g. Kidwell, 2002; Fontdevila, 2005; Böhne et al., 2008; Hua-Van et al., 2011; Arkhipova and Rodriguez, 2013). Retrotransposons of the Rex family are widely spread among teleost genomes (Volff et al., 1999, 2000, 2001) and are known to particularly associate with rDNA (e.g. Cioffi et al., 2010; Gross et al., 2010; Symonová et al., 2013).

Although transposable elements are usually silent, bursts of activity and increased copy number can lead to rapid genome diversification between closely related species, as a result of lineage-specific amplification and/or recombination (Hua-Van et al., 2011). Due to their high amplification potential, rapid genome expansions are thought to be mediated by transposon activity, especially under conditions that may disrupt normal operation of transposon control systems, like inter-specific hybridization (Arkhipova and Rodriguez, 2013). In fact, hybridization is known to potentially induce transposon activation triggering genome-wide reorganization (genetic and epigenetic) or strongly modifying recombination patterns (Petrov et al., 1995; O'Neill et al., 1998; Fontdevilla, 2005; Abbott

et al., 2013). As a result, cross incompatibilities between species may arise, potentially constituting a first step towards reproductive isolation (Hua-Van et al., 2011).

To understand and determine the role of repetitive DNAs in the differentiation of Iberian Leuciscinae genomes, a molecular cytogenetic survey was conducted in species *Anaecypris hispanica* (AHI), *Iberochondrostoma lemmingii* (ILE), *I. lusitanicum* (ILU), *Pseudochondrostoma duriense* (PDU), *P. polylepis* (PPO), and PPO × *Achondrostoma oligolepis* (AOL) natural hybrids. The main goals of present study were: (1) to map the chromosomal distribution of Rex3 in these species, (2) to compare it to the distribution of C_0t-1 repetitive DNA fraction of their genomes, (3) to explore the possible transposition (re)activation in the hybrids, and (4) to delineate its association with the translocation of 45S rDNA sites previously identified in these hybrids (see Pereira et al., 2013a). This is the first report of a retroelement physical mapping in Cyprinidae that may contribute to the understanding of whether retrotransposons might be at the basis of genome rearrangements, karyotype differentiation or even speciation.

Materials and Methods

Materials

Data about the individuals used in this study was summarized in Table 1 showing representatives of Iberian Leuciscinae and some of their natural hybrids. All manipulations were performed in accordance with Portuguese guidelines and regulations regarding animal welfare and experimentation (ASAB, 2006).

Genomic DNA was extracted from fin clips or muscle by isopropanol/ethanol precipitation and the set of specific FISH probes used included: (1) the DNA fraction enriched for repetitive sequences – C_0t-1 DNA (Ferreira and Martins, 2008), (2) 5S and 45S ribosomal DNA sequences (Pereira et al., 2012), and (3) a Rex3 fragment PCR-amplified using the pair of primers F3 and R3 originally designed by Volff et al. (1999). All sequences were labelled with DIGoxigenin or BIOtin by nick translation

(Roche) and dissolved in hybmix (50% deionised ultrapure formamide, 10% dextran sulphate, 2x SSC, pH 7.0) to a final concentration of 20 ng.uL^{-1}. Our lab has available a small bank of chromosome preparations obtained and stored throughout the many years of continuous fish cytogenetic studies (from the 80's to present; see also Pereira et al., 2012). C-banding followed Sumner (1972) with DAPI or PI counterstaining. Images were processed as a whole simply using pseudo-colouring, over-layering and brightness/contrast tools. Karyotype assembly followed Levan et al. (1964).

Sequencing and sequence analysis

The PCR-amplified Rex3 fragment was sequenced on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit (Life Technologies). Sequences were subjected to BLASTn analysis (http://blast.ncbi.nlm.nih.gov/blast) in order to determine similarities to sequences deposited in GenBank databases (Benson et al., 2011). The purified fragment was further cloned into pDrive Cloning Vector (Qiagen) and transformed into EZ Competent Cells (Qiagen) for long time storage/access and future deposition in GenBank.

Results

Characterization of the Rex3 fragment

Using the selected pair of Rex3 primers we were able to retrieve a single fragment of approximately 460 base pairs (bp), with no apparent size variation between species (not shown). BLASTn analysis confirmed high homology to: (1) partial sequences of Rex3 retroelement described in *Esox lucius* (91-93%) from Esociformes, *Cyprinus carpio* (87-91%) and *Danio rerio* (83%) from Cypriniformes, *Siniperca chuatsi* (87-88%), *Symphysodon discus* (79%) and *Cichla monoculus* (78%) from Perciformes, *Tetraodon nigroviridis* (83-88%) from Tetraodontiformes, *Fundulus sp.* (85%) from Cyprinodontiformes, and *Polypterus delhezi* (84-86%) and *P. ornatipinnis* (82%) from Polypteriformes (Fig. 1); as well as (2) high similarity to microsatellite sequences of 102-117 bp found in the swamp eel *Monopterus albus* (93%, Synbranchiformes; accession No. EU846210), in the

Cypriniformes common carp *Cyprinus carpio* (92%; accession No. JN750700) and Amur ide *Leuciscus waleckii* (87%; accession No. JN750700), and in the tongue sole *Cynoglossus laevis* (90%, Pleuronectiformes; accession No. EU907166).

Chromosomal distribution of Rex3 retroelement

All genomes examined for Rex3 distribution evidenced a pattern of preferential accumulation to centromeric regions and more moderate on telomeres (Figs. 2a-d, 3a), grossly correlating with blocks of constitutive heterochromatin (Figs. 2f, 3b) and C_0t-1 DNA fraction (Fig. 2e). The clusters were particularly obvious in at least 10 chromosome pairs and the pattern appeared to be shared between the different species tested (Figs. 2a-d); namely, the clusters in metacentric (m) chromosome pairs Nos. 1-2 + 4-6, submetacentric (sm) chromosome pairs 1-2 + 6 + 8 + 10 + 13, and more or less in all subtelo-acrocentric (st-a) chromosome pairs 1-4 (Figs. 2a-d). Few additional distinctive patterns could be recognized in a species-specific mode; particularly, a big interstitial block in the long arm of chromosome pair No. 12 of ILU (Fig. 2b), a big telomeric block in chromosome pair No. 3 of PPO (Fig. 2c), and two clusters in the short arm of chromosome pair No. 15 of PDU (Fig. 2d). Conversely, these bands do not seem to associate with constitutive heterochromatin (not shown) except for PDU (Fig. 2d,f).

In PPOxAOL hybrid genomes, Rex3 distribution appears to agree with the overall centromeric and telomeric patterns of accumulation (Fig. 3a), also correlating with constitutive heterochromatin (Fig. 3b). However, several differences could be found relative to the patterns observed in the parental species: (1) more independent clusters were evident (at least 15), occurring in all m, most of the sm and faintly in the st-a chromosome pairs (Fig. 3a); (2) co-localization with 5S rDNA sites; and (3) conspicuous bands mapped to the short arms of some chromosomes also co-localizing with 45S rDNA clusters (Figs. 3a, c).

Interestingly, the pattern of a double band of Rex3 or other repetitive sequences occasionally present in only one of the homologues in the first sm chromosome pair (Fig.2b,e, 3a) was only observed in male specimens of ILU, PDU and a PPOxAOL hybrid.

Discussion

Rex3 partial sequence

Sequence homology analysis (Fig. 1) demonstrated higher homology to *Esox lucius* (Esociformes, Esocidae; >91%) than to *Cyprinus carpio* or *Danio rerio*, (Cypriniformes, Cyprinidae). Moreover, the levels of sequence homology to the remaining species were very similar (above 78%) despite the phylogenetic interrelationships. Such discrepancy between Rex3 phylogeny and present fish phylogeny was also observed before by Volff et al. (2001) for which several possible explanations were proposed. The most adequate seem to be (1) differences in the evolutionary rates between Rex3 sequence and the host genome, since mobile elements multiply independently within the genome; and/or (2) the operation of multiple mechanisms during Rex3 evolution in fish genomes. Nonetheless, present results suggest little sequence variance since divergence from the *Esox* lineage (at least Late Cretaceous; Nelson, 2006), indirectly pointing to either (1) some sort of positive selection to protect Rex3 activity, as already postulated by Volff et al. (2001); (2) the existence of a robust mechanism of silencing/regulation of Rex3 activity in Leuciscinae (Cyprinidae) preventing its transposition and consequently its differentiation; or possibly (3) a combination of both.

Conserved Rex3 distribution in natural populations

The species analysed in this study had a karyotype composition varying between (6-7) m chromosome pairs, (15-17) sm chromosome pairs and (2-4) st-a chromosome pairs (Fig. 2-3) confirming high level of macrostructural karyotype similarities. Comparative analysis of Rex3 distribution point out possible chromosomal homologies between these long diverged leuciscine species, probably corresponding to

the ancestral condition to all these genera. Assuming the model of vertical transfer, Rex3 genome invasion most certainly preceded their divergence, since it was found quite abundantly even in basal species such as AHI and ILE or ILU (Alburninae is thought to have diverged from Leuciscinae at ca. 12.1 mya, while Iberian *Chondrostoma s.l.* are believed to have originated around 9.4 mya; Perea et al., 2010).

Retrotransposons have been described and mapped in Orders Characiformes (e.g. Cioffi et al., 2010), Cyprinodontiformes (Nanda et al., 2000), Perciformes (e.g. Gross et al., 2010), Salmoniformes (Symonová et al., 2013), Siluriformes (e.g. Ferreira et al., 2011a), and Tetraodontiformes (e.g. Fischer et al. 2004) demonstrating various patterns of genomic distribution from dispersed to clustered. In cyprinids, retrotransposon sequences have only been described in the common carp *Cyprinus carpio*, the zebrafish *Danio rerio* (Volff et al., 1999) and the common bleak *Alburnus alburnus*, with a strong association to the giant B chromosomes found in the latter (Ziegler et al., 2003); but until now there has been no study targeting the physical mapping of these genetic elements to cyprinid genomes. Usually, eukaryotic transposable elements are not randomly distributed along the chromosomes and in agreement Rex3 was generally found concentrated at centromeric and telomeric regions (Figs. 2a-d, 3a). This pattern is especially valid for small genomes like those of evolutionary diploid cyprinid fishes; by accumulating within heterochromatin the impact of its presence or activity on the host genome is reduced, while evading negative selection and allowing for their accumulation in clusters, as observed.

Recent studies have further demonstrated linkage of Rex3 with other classes of repetitive DNA such as 5S rDNA, usually accompanying increased karyotype diversity (e.g. Cioffi et al., 2010; Gross et al., 2010; Symonová et al., 2013). Such association could not be found in the present investigation (not shown; but see Pereira et al., 2013b); Rex3 signals in the 5S rDNA-bearing chromosomes (typically the first 2 pairs of st-a chromosomes; Pereira et al., 2012) were rather faint or small in the (peri)centromeric region (Fig. 2a-d) – the mapping region of 5S rDNA clusters (Pereira et al., 2012).

179 The same was observed for 45S rDNA-bearing chromosomes (e.g. the third sm pair of chromosomes)
180 where Rex3 signals were barely observed (Fig. 2a-d).

181 Conversely, large Rex3 clusters were found at the distal part of the largest st-a chromosome pair, once
182 again co-localizing with heterochromatin (Fig. 2f; but see also Ráb et al., 2008, Monteiro et al., 2009,
183 Pereira et al., 2009) and most likely intercalating with other repetitive sequences (Fig. 2e). In their
184 work with a WCP (whole chromosome paint probe) specific for this st-a chromosome, Ráb et al.
185 (2008) proposed this as the subfamily marker chromosome, likely homologous across this cyprinid
186 lineage and that at least the distal part would be phylogenetically conserved. Accordingly, Rex3
187 accumulation in this particular region likely reflects the same evolutionary history, thus predating the
188 divergence of Leuciscinae subfamily. In fact, Volff et al. (2001) described Rex3 as the most
189 widespread fish retrotransposon with its presence going back as far as 150-200 mya, despite the
190 discontinued distribution.

191 Despite sequence analysis suggested little sequence differentiation (Fig. 1), species-specific patterns of
192 Rex3 accumulation were identified (Fig. 2b-d) that did not associate with heterochromatin. This
193 proves that, even with probable mechanisms of expression regulation, somewhere along the evolution
194 of Iberian species, Rex3 sequences have had the opportunity to transpose and accumulate outside the
195 'comfort areas' of heterochromatin shelter, also indicating independent and rapid divergence of
196 species-specific sequences. However, a comparative sequence analysis between these species is
197 required to corroborate this hypothesis.

198 Among other classes of repetitive sequences, also mobile elements have been demonstrated to
199 accumulate within the sex chromosomes (e.g. Nanda et al., 2000; Cioffi et al., 2010; Ferreira et al.,
200 2011b). Up to date, no sex-related chromosomes have been convincingly identified or characterized in
201 Leuciscinae (e.g. Sola and Gornung, 2001) and, if present, they appear to have remained under a high
202 degree of morphological homology. In the present results differential accumulation of Rex3 between
203 some homologue chromosome pairs was found in male specimens (Figs. 2b,e, 3a). However, mainly
204 due to low sample size of female individuals, these findings must be further validated.

Rex3 expansion in natural homoploid hybrids

Even though one of the parental species involved in the formation of these homoploid hybrids could not be examined (i.e. AOL), Rex3 distribution in the remaining leuciscines seems grossly comparable, thus allowing for inferences to be withdrawn for the hybrids. Immediately, one of the first readings is the increased number of Rex3-bearing chromosomes and the enlarged size of clusters (Fig. 3a). Even without results of expression levels, it seems safe to deduce an apparent proliferation of Rex3 transposition in the hybrids, occurring in most of the bi-armed elements of the chromosomal sets.

Furthermore, to understand if rDNA translocation in the hybrid genomes (Pereira et al., 2013a) could be associated with and possibly be facilitated by the presence of transposable elements, rDNAs were also mapped in the hybrids (Fig. 3c). Previously surveyed hybrids for these markers denote a variety of possible combinations between parental genome contributions, sometimes surpassing it in the form of syntenic associations between both types of rDNA (Pereira et al., 2013a). The particular individual represented in Fig. 3a,c evidenced three translocated clusters of 45S rDNA into chromosomes already bearing 5S rDNA sites (see also Pereira et al., 2012 and Pereira et al., 2013a).

As previously discussed, Rex3 did not particularly correlate with any of the rDNA sites in the species inspected. In the hybrid however, this association seems more conceivable, especially with 5S rDNA clusters (Fig. 3a,c), as described for other fish species (e.g. Cioffi et al., 2010; Gross et al., 2010; Symonová et al., 2013). Even in 45S rDNA-bearing chromosomes thought to be inherited as a whole (e.g. chromosome pair No. 12), telomeric Rex3 co-localizing with the 45S rDNA appears as a possible signature of translocation. The same can be extended to the few homologues' differences (e.g. chromosome pairs Nos. 4 and 7) as a result of rearrangements. Similar to recent demonstrations of stress-activated retrotransposons associated with extensive rDNA multiplication, hybridization-activated transposition and genome rearrangements are likely to occur in other regions of these genomes for which we currently do not possess the means to examine.

Final remarks

Transposable elements are considered a dynamic force in gene regulation and neo-functionalization, chromosome rearrangements, genome evolution, and even speciation (e.g. Kidwell, 2002; Böhne et al., 2008; Hua-Van et al., 2011; Arkhipova and Rodriguez, 2013). By increasing genetic variability, transposable elements also increase the adaptability and evolvability of genomes and species when external conditions change (Hua-Van et al., 2011). Thus, mapping these repetitive sequences on other Leuciscinae representatives – including the Iberian *Achondrostoma* and *Squalius* genera, for example – will allow to support current findings and to better appreciate karyotype differentiation in Leuciscinae. Also, the inclusion of more hybrid forms (both homoploid and polyploid) would greatly benefit the understanding of predicted transposon (re)activation. And unquestionably, to follow up the ongoing work on *Squalius sp.* transcriptomics (Inácio et al., 2012), including these species and homoploid hybrids to understand transposon distribution, regulation and (re)activation in a scenario of genomic, transcriptomic and epigenetic shock subsequent to the hybridization process.

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Ethical statement

All institutional and national guidelines for the care and use of laboratory animals were followed complying with current Portuguese laws.

252 **Conflict of interest**

253 The authors declare no conflict of interest.

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Figures' captions

Figure 1 Rex3 partial sequence alignment with annotated Rex3 partial sequences of high similarity after BLASTn analysis. Sequences are identified by 'species name abbreviation | accession number' as follows: AOL = *Achondrostoma oligolepis*, CCA = *Cyprinus carpio*, CMO = *Cichla monoculus*, DRE = *Danio rerio*, ELU = *Esox lucius*, Fun = *Fundulus sp.*, PDE = *Polypterus delhezi*, POR = *Polypterus ornatipinnis*, SCH = *Siniperca chuatsi*, SDI = *Symphysodon discus*, TNI = *Tetraodon nigroviridis*. Nucleotide similarities are denoted by '.' and gaps introduced for alignment purposes are represented by '–'.

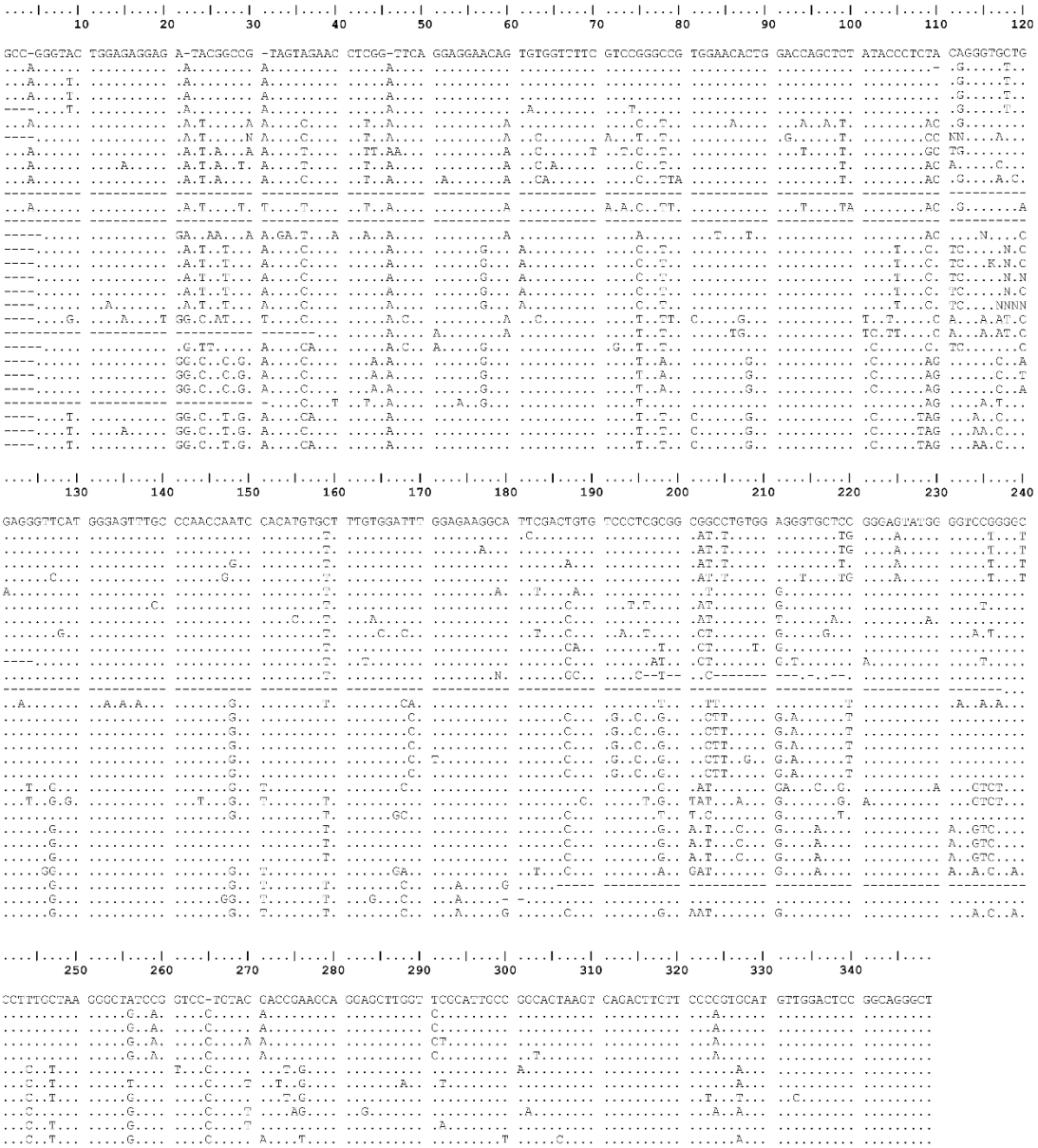
Figure 2 Karyotypes of (**a**) *Anaecypris hispanica*, (**b**) *Iberochondrostoma lusitanicum*, (**c**) *Pseudochondrostoma polylepis*, and (**d-f**) *P. duriense*, arranged after FISH with (**a-d**) Rex3 fragment, (**e**) C_0t-1 DNA fraction and (**f**) C-banding with DAPI counterstaining (negative image). Bar = 5 μm.

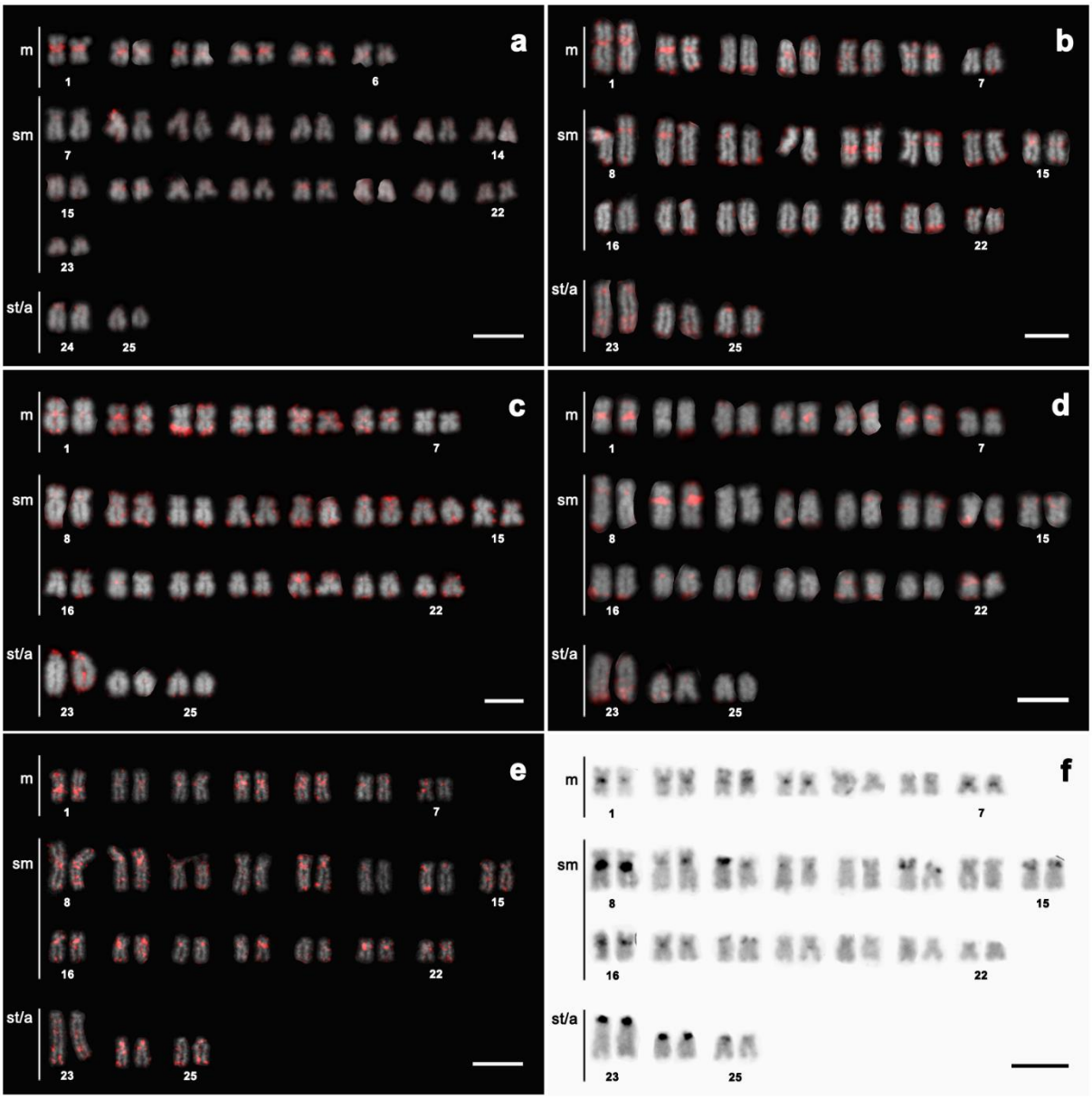
Figure 3 Karyotypes of *P. polylepis* x *A. oligolepis* homoploid hybrids arranged from mitotic chromosomes after (**a**) FISH with Rex3 fragment, (**b**) C-banding with PI counterstaining, and (**c**) dual-colour FISH with 5S and 45S rDNA probes. Bar = 5 μm.

364 **Table 1.** Information regarding the number, sex and location of specimens analysed.

Taxa	Origin	Date of collection	No. and sex of specimens analysed	Fig.
Anaecypris hispanica	Guadiana Basin	1999	1 ♂	2a
Iberochondrostoma lemmingii	Ardila Basin			
	Ardila River	2011	1 ♀	-
Iberochondrostoma lusitanicum	Tejo Basin			
	Raia River	2005	1 ♂	2b
Pseudochondrostoma duriense	Douro Basin			
	Tâmega River	2008	1 ♂, 1 ♀	2d-f
Pseudochondrostoma polylepis	Mondego Basin			
	Ceira River	2007	1 ♂	2c
P. duriense x *A. oligolepis* hybrid	Douro Basin			
	Sousa River	2008	1 ♂	-
P. polylepis x *A. oligolepis* hybrid	Vouga basin			
	Serra River	2008	1 ♂	3a,c
	Mondego Basin			
	Ceira River	2007	1 ♂	3b

365 ♂ = male, ♀ = female

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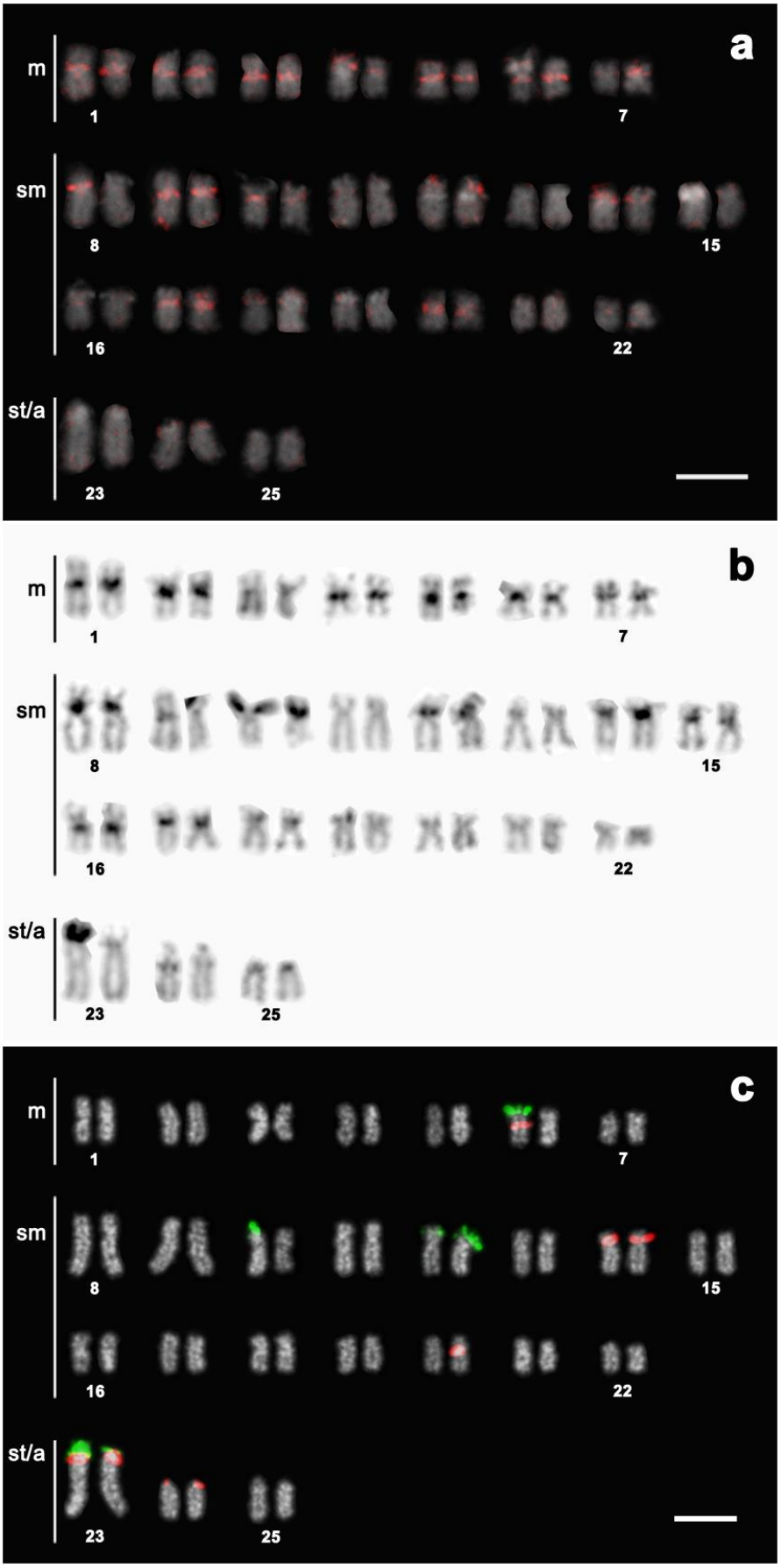
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Chapter 4

Chromosomes of Iberian Leuciscinae (Cyprinidae) revisited: evidence of genome restructuring in homoploid hybrids using dual-color FISH and CGH

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Resumo

Os Leuciscinae Ibéricos têm sido utilizados como modelos para explorar tópicos como a hibridação, a alopoliploidia, as várias formas de reprodução não sexuada e de evolução genómica. Este trabalho centra-se na contribuição da citogenómica como promotora de investigação neste grupo de peixes tão diverso. Foi feita uma revisão de dados de citogenética convencional e de citogenética molecular, facilitando assim a análise comparativa entre as várias espécies de bogas e escalos existentes em cursos de água-doce nacionais. A hibridação natural é um fenómeno bem reconhecido dentro dos géneros do grupo de *Chondrostoma sensu lato* (*s.l.*) e de *Squalius*, apesar de a alopoliploidia só ter sido reportada para estes últimos sem que no entanto sejam conhecidas explicações para uma flexibilidade genómica diferencial. A citogenética de peixes foi muito limitada até há bem puco tempo, por questões técnicas relacionadas essencialmente com a obtenção de um número elevado de preparações cromossómicas de qualidade. Além disso, os peixes caracterizam-se, em geral, pela existência de cromossomas pequenos e em número elevado, muito pouco compartimentalizados em termos estruturais o que, ao contrário do que aconteceu com os homeotérmicos, impediu a aplicação de uma vasta gama de técnicas de bandeamento, dificultando sobremaneira análises comparativas e sistematizadas. A aplicação de ferramentas de hibridação *in situ* fluorescente (FISH) a cromossomas de peixes abriu caminho a novas possibilidades, facilitando por exemplo o reconhecimento de outros pares de cromossomas além dos poucos pares ditos marcadores. A aplicação desta técnica a leuciscíneos Ibéricos permitiu ainda confirmar a condição polimórfica associada aos DNAs ribossomais (rDNAs) e identificar e distinguir entre híbridos e espécies parentais, mesmo em casos de homoploidia. Adicionalmente, foi ainda possível detectar recombinação e instabilidade genómica tanto em híbridos homoploides como em poliploides. Esta reorganização de genoma foi particularmente fácil de despistar nos *clusters* de rDNA testados, corroborando a hipótese de translocação activa de NORs anteriormente proposta para salmonídeos. Todavia foi também possível reconhecer outras regiões potencialmente envolvidas em translocações e/ou duplicações, apesar de menos evidentes utilizando apenas os marcadores actuais. Ainda assim, a multiplicação de *clusters* de rDNA particularmente observada em algumas destas espécies (ver **Capítulo 2**) não parece estar correlacionada com o retroelemento Rex3 (ver **Capítulo 3.2**), que se sabe disperso por várias famílias de teleósteos e que demonstrou já estabelecer uma particular associação com genes ribossomais. Porém, nesta inspecção inicial não foram ainda incluídos híbridos, nos quais se antecipava um aumento nas taxas de transposição, comprovado posteriormente (ver **Capítulo 3.2**). As técnicas de hibridação genómica comparativa (CGH) e de hibridação genómica *in situ* (GISH) são metodologias derivadas da FISH e a sua

aplicação em leuciscíneos Ibéricos permitiu, por um lado, reafirmar a origem híbrida do complexo *Squalius alburnoides* recentemente contestada; e, por outro lado, confirmar que os padrões de cariótipo aparentemente conservados na subfamília Leuciscinae são essencialmente de nível macroestrutural. Experiências de GISH cruzada, i.e., utilizando DNA de uma espécie como sonda e os cromossomas de outra espécie como alvo, revelaram essencialmente homologia ao nível das sequências repetitivas com função estrutural, como centrómeros e telómeros. Como seria de prever, espécies mais aparentadas filogeneticamente apresentaram uma maior fracção de regiões com hibridação positiva do que espécies mais afastadas. Curiosamente, duas das espécies envolvidas em processos de extensa e recorrente hibridação natural (*Achondrostoma oligolepis* e *Pseudochondrostoma duriense*), mostraram mais regiões homólogas do que seria esperado atendendo à sua classificação em géneros diferentes. No entanto, tal semelhança de genomas poderá ser a razão do sucesso da hibridação entre elas, de onde resultam organismos homoploides férteis. Os resultados obtidos neste trabalho permitiram ainda evidenciar a utilidade do mapeamento de DNAs repetitivos sobretudo em espécies não-modelo, de genomas compactos e cariótipos menos variáveis, para os quais não existem dados de sequenciação ou são muito limitados, como é o caso de muitas das linhagens de ciprinídeos.

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Chromosomes of Iberian Leuciscinae (Cyprinidae) Revisited: Evidence of Genome Restructuring in Homoploid Hybrids Using Dual-Color FISH and CGH

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Key Words

CGH/GISH · *Chondrostoma s.l.* · Genome reshuffling Hybridization · Ribosomal DNAs · *Squalius*

Abstract

Iberian Leuciscinae have been used in many studies as models to explore topics such as hybridization, allopolyploidy, modes of reproduction, and evolution. This article focuses on the contribution of cytogenomics to foster research in this group of cyprinid fish. Conventional and molecular banding results were reviewed, facilitating comparative analysis between nase and chub taxa inhabiting Portuguese freshwaters. Hybridization is known to occur within both *Chondrostoma s.l.* and *Squalius* genera although polyploidy has only been reported in the latter; the reasons behind such differential genome flexibility remain unidentified. FISH tools allowed recognizing additional chromosome markers, confirming NOR polymorphism and distinguishing species and their hybrids. Recombination and genome instability were detected in homoploid and polyploid hybrid genomes supporting active NOR transposition. However, the multiplication of rDNAs in these species does not seem to be associated with Rex3 retroelement, though hybrids were not surveyed. CGH and GISH allowed reaffirming the hybrid origin of *S. alburnoides* and confirming that the conservative karyotype patterns within Iberian leuciscines are restricted to the macrostructure. Current data also support the usefulness of mapping repetitive DNAs, especially for nonmodel compact genomes with less variable karyotypes and sequence data resources unavailable, like in many cyprinid lineages.

Challenging systematic and evolutionary issues have been addressed for many vertebrate groups by integrating cytogenetics into genome size and genetic data [e.g. Griffin et al., 2007; Pokorná et al., 2011; Deakin et al., 2012; Svartman and Stanyon, 2012]. Linking cytogenetic to genetic data was greatly enhanced after cytogenetics became a molecular science, allowing looking for the 'signature' of evolutionary processes in the 'chromosomics' era sensu Claussen [2005]. This role was reaffirmed with the culmination of genetic-based approaches and next generation sequencing technologies [Speicher and Carter, 2005; Dobigny and Yang, 2008; Graphodatsky et al., 2012]. Fish biologists, however, have seldom engaged in multidimensional studies including cytogenetic examination to address similar questions [e.g. Jaillon et al., 2004; Freeman et al., 2007; Phillips et al., 2009; Brenna-Hansen et al., 2012; Mazzuchelli et al., 2012].

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The present review on the cytogenetics of Iberian Leuciscinae, a subfamily of Cyprinidae that represents a significant part of the South-European ichthyofauna, focuses on the contribution of cytogenomics to recent advances in fish karyotyping and genome evolution. It is mainly centered in a case-study of homoploid hybridization among 2 pairs of closely related genera within *Chondrostoma s.l.* [Robalo et al., 2007] – hereafter referred to as chondrostomine species or nases as commonly designated, either with straight or arched mouth.

Iberian Leuciscinae

Cyprinidae, likely the most speciose fish (and vertebrate) family, are characterized by a wide biological and ecological plasticity, including at least 8 well-defined lineages: the subfamilies Acheilognathinae, Cyprininae, Cultrinae, Gobioninae, Leuciscinae, Rasborinae, Tincinae, and Xenocyprinae [Mayden et al., 2009]. Despite the broad range of diploid chromosome numbers recorded (from 2n = 42 to 2n = 446), genomes comprise a modal value of 24–25 pairs of small and morphologically similar chromosomes [Ráb and Collares-Pereira, 1995; Mank and Avise, 2006; Arai, 2011]. In general, cyprinids are reasonably well represented in basic karyotype surveys. According to the last database available [Arai, 2011], 26% of the 2,420 described taxa were analyzed, and the range of variation regarding diploid chromosome numbers evidences the occurrence of polyploidy in several lineages [see also Ráb and Collares-Pereira, 1995].

The subfamily Leuciscinae is one of those lineages with some polyploid complexes of hybrid origin (with 3n = 75 and 4n = 100). Even though, leuciscines exhibit quite conservative patterns of diploid values (2n = 48–50), chromosome morphology and few chromosome markers (fig. 1 a–d) [e.g. Ráb and Collares-Pereira, 1995; Ráb et al., 2008; Pereira et al., 2012; references therein]. No sex-related chromosomes have been convincingly characterized, and if present, they appear to have remained under a high degree of morphological homology.

In the Iberian Peninsula, the subfamily contains a total of at least 24 species and 2 diploid-polyploid complexes within the genus *Squalius (S. alburnoides* and *S. palaciosi)* [reviewed in Leunda et al., 2009]. The high levels of species diversity and the intricate taxonomy make leuciscines very attractive to the investigation of life history, biogeography and speciation issues [see e.g. Filipe et al., 2009]. Moreover, many Iberian riverine systems have an intermittent character, and an extensive occurrence of hybridization has been well documented [see e.g. Gante et al., 2004; Gromicho and Collares-Pereira, 2007; Aboim et al., 2010].

Hybridization, a promoter of diversification and speciation in animals [e.g. Seehausen, 2004], results either in organisms with the same number of chromosomes as the parental species (homoploid hybrids) or in organisms with multiple genomes (allopolyploids). The allopolyploid complex of *S. alburnoides* is the best documented [reviewed in Collares-Pereira et al., 2013], but homoploid hybrid leuciscines have long been recognized [e.g. Collares-Pereira and Coelho, 1983; Elvira et al., 1990]. Recently, greater efforts were developed towards understanding the particular cases involving *Achondrostoma oligolepis* and either *Pseudochondrostoma duriense* or its sister-species *P. polylepis* [Gante et al., 2004; Pereira et al., 2009; Aboim et al., 2010].

In general, the occurrence of hybridization is expected to negatively correlate with the levels of genome divergence between parental forms, most likely resulting in unviable or sterile hybrids among more divergent forms [reviewed in Coyne and Orr, 2004]. Actually, homoploid chondrostomine hybrids under study result from intergeneric crosses. However, the phylogenetic revision allocating *Chondrostoma s.l.* into 5 other new genera was recently done [Robalo et al., 2007], and parental species belonged to the same genus until then. The bottom line is that parental species are relatively closely related [Perea et al., 2010], and the resulting hybrids are viable and at least partially fertile, accounting for a high incidence of backcrossing in natural populations [Aboim et al., 2010]. Several independent hybrid zones comprising the same pair of species have been described to date [Aboim et al., 2010], in addition to the recently confirmed hybridization between *A. oligolepis* and *P. polylepis* [Pereira et al., in press]. Based on a combination of mitochondrial and microsatellite markers, each hybrid zone was found to be characterized by distinct bidirectional asymmetric levels of introgression [Aboim et al., 2010].

The Molecular Cytogenetic Approach

Routine cytogenetic procedures easily applicable to higher vertebrates are barely reproducible in lower vertebrates, namely in cyprinid fish, given the lack of genome compartmentalization [e.g. Sola et al., 1981; Medrano et al., 1988; Gold et al., 1990]. Technical improvements to attain high quality preparations [e.g. Henegariu et al., 2001] and higher mitotic indexes [e.g. Rodrigues and Collares-Pereira, 1996; Fujiwara et al., 2001], as well as the optimization of powerful FISH [Phillips, 2001; Sola and Gornung, 2001] and FISH-derived methods [Valente et al., 2009] for fish chromosomes have been decisive to fish cytogenetics.

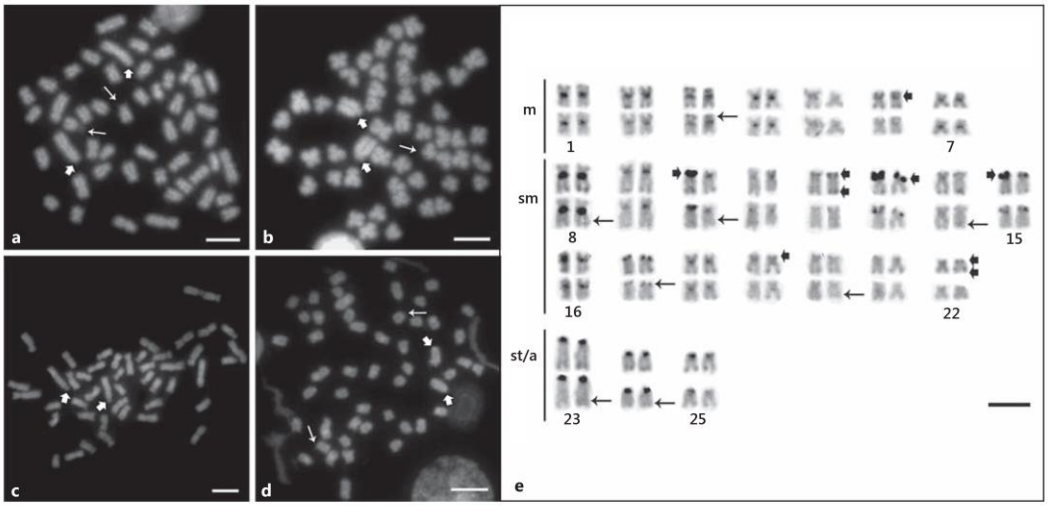

Fig. 1. Metaphase plates of Leuciscinae representatives: **a** *Blicca bjoerkna* female, **b** *Scardinius erythrophtalmus*, **c** *I. lemmingii*, and **d** *P. willkommii* female, showing great chromosome similarity, the DAPI-negative NORs (thin arrows) and the cytotaxonomic subfamily marker (thick arrows). **e** Karyotype of a *P. duriense* female arranged from C-banded chromosomes with distinct fluorochrome counterstaining: propidium iodide (upper row) and AT-specific DAPI (lower row), evidencing mostly centromeric heterochromatin, some noncentromeric heterochromatic bands (thin arrows) and other relevant bands (thick arrows). Bar = 5 μm.

Although far rooted and intensively investigated in Neotropical [e.g. Valente et al., 2009; Cioffi and Bertollo, 2012; and references therein], model [Sola and Gornung, 2001; Freeman et al., 2007] and economically important fish species [e.g. Phillips et al., 2009; Brenna-Hansen et al., 2012], the power of molecular cytogenetics has been extended to a small number of other fish species [e.g. Collares-Pereira and Ráb, 1999; Borón et al., 2009; Pereira et al., 2012; Rampin et al., 2012; Symonová et al., 2013; references therein]. Repetitive DNAs constitute a large fraction of vertebrate genomes and are the most frequently used probes to study fish genomes. They proved to be a prevailing tool in the investigation of chromosomal differentiation, genome structuring and evolution [reviewed in Cioffi and Bertollo, 2012], which is especially relevant for the karyologically conservative leuciscine fishes [Ráb and Collares-Pereira, 1995].

CGH and GISH are FISH-derived techniques suitable for studying hybrid karyotypes using the parental genomic DNAs as probes (either one – GISH – or both labeled – CGH). These techniques of chromosome/genome painting have been applied to several Actinopterygii, namely to species of the families Salmonidae [Fujiwara et al., 1997], Characidae [Zhu and Gui, 2007] and Cichlidae [Valente et al., 2009]. Recently, they were also successfully extended to cyprinids with small genome sizes, like the Iberian Leuciscinae [Rampin et al., 2012]. The individual parental contributions to the genomes of allopolyploids [Bi and Bogart, 2006; Rampin et al., 2012] as well as intergenomic exchanges between chromosomes [e.g. Bi and Bogart, 2006; Rampin et al., 2012] could then be recognized. GISH and CGH are also valuable tools in comparative cytogenetics and in the elucidation of chromosome evolution within and between lineages [e.g. Marková and Vyskot, 2009; Valente et al., 2009]. Genes are no more than discrete units between blocks of repetitive DNA. Therefore, major differences between genomes dwell in the amount and distribution of the repetitive DNA fraction, usually species-specific [Marková and Vyskot, 2009].

The Cytogenetics of Iberian Leuciscinae

Conventional Cytogenetics

Karyologic information and genome size data are only available for half of the Iberian leuciscines recognized so far (http://www.genomesize.com). Evolutionary 'diploid' karyotypes typically consist of 25 pairs of small chromosomes, very similar in size and shape and most of them biarmed with few candidate chromosomes to be the largest of the complement (i.e. 1 subtelocentric to acrocentric, 1–2 submetacentric, 1 metacentric). The karyotype structure in this group varies between 5–7 metacentric, 15–18 submetacentric and 2–4 subtelo- to acrocentric chromosome pairs and fundamental number ranges from 92 to 96 (fig. 1e) [Collares-Pereira, 1985; Ráb and Collares-Pereira, 1995; Collares-Pereira et al., 1998; Gromicho et al., 2006a; Monteiro et al., 2009; Pereira et al., 2009, 2012; Nabais et al., 2013].

Besides basic Giemsa-stained chromosome counts and karyotype morphology [Collares-Pereira, 1985; Ráb and Collares-Pereira, 1995; Collares-Pereira et al., 1998], silver and chromomycin-A_3 bandings added little information to the differentiation of the genomes of these cyprinid fish [Rodrigues and Collares-Pereira, 1996; Gante et al., 2004; Gromicho and Collares-Pereira, 2004; Gromicho et al., 2006a; Monteiro et al., 2009; Pereira et al., 2009]. On one hand, results were consistent with the overall karyotypic pattern of the subfamily, whereas suggesting some degree of divergence by the presence of more than one chromosome pair bearing the NORs. To be precise, up to 3 NOR-bearing chromosome pairs were already described [e.g. Gromicho and Collares-Pereira, 2004], though a single pair has been considered the plesiomorphic character state among leuciscines [see e.g. Ráb and Collares-Pereira, 1995; Collares-Pereira and Ráb, 1999].

Although fishes do not display genome compartmentalization as in higher vertebrates, different fluorochrome counterstainings revealed additional chromosome regions (fig. 1e). These genomes are also usually characterized by a low content of typically centromeric constitutive heterochromatin, occasionally evidencing faint pericentromeric or telomeric bands in some marker chromosomes (fig. 1e) (e.g. large subtelo- to acrocentric and large submetacentric chromosomes) [Monteiro et al., 2009; Pereira et al., 2009].

Molecular Cytogenetics

The major challenge in leuciscines' cytogenetic studies resides in effectively distinguishing species at the karyotype level, fundamental for depicting cases of natural hybridization [for a more detailed discussion, see Pereira et al., in press] and for defining specific management strategies [e.g. Ráb et al., 2007]. As mentioned above, the convenience of FISH procedures offered a great thrust and potential to fish cytogenetics [e.g. Phillips, 2001]. The first case of European leuciscines with multiple NORs was confirmed by FISH with a specific probe for major ribosomal RNA genes (rDNA) [Collares-Pereira and Ráb, 1999], opposing the apparently widely distributed condition of a single pair of NORs on a medium-sized submetacentric chromosome pair. Regarding the minor rDNA clusters, recent studies suggested the large subtelo- to acrocentric chromosome pair as the ancestral 5S-rDNA-bearing chromosome for the entire Leuciscinae, despite revealing greater variation in number and location than 45S rDNA clusters [Pereira et al., 2012; Rossi et al., 2012]. Simultaneous mapping of both minor (5S) and major (45S = 18S + 5.8S + 28S) rDNA families by dual-color FISH provided further advances regarding genome organization in these species.

The combined use of both probes demonstrated increased resolution power allowing to: (1) accurately identify other pairs of chromosomes besides the few marker ones (i.e. the large subtelo- to acrocentric, 1 large metacentric, 1–2 large submetacentric and NOR-bearing chromosome pairs); (2) confirm the polymorphism of NORs in several Iberian [Collares-Pereira and Ráb, 1999; Gromicho et al., 2005, 2006a, b; Pereira et al., 2009, 2012] and non-Iberian leuciscine species [e.g. Bóron et al., 2009; Kirtiklis et al., 2010; Rossi et al., 2012], suggestive of multiple independent origins for the translocation and/or multiplication of rDNA clusters within each lineage; and (3) successfully distinguish species and their hybrids [e.g. Kirtiklis et al., 2010; Pereira et al., in press]. Most vertebrates have minor and major rRNA genes usually located on different chromosomes [e.g. Martins and Wasko, 2004]. Even though colocalization of both gene clusters (syntenies) was reported as common among some evolutionary lineages of Leuciscinae [Gromicho et al., 2006a, b; Schmid et al., 2006; Kirtiklis et al., 2010; Rossi et al., 2012], it seems to be absent in chondrostomines [Pereira et al., 2012].

Interestingly, Iberian *Squalius* species revealed extensive intra- and interindividual variation in number of 28S rDNA clusters compared to a steady number of 5S rDNA sites [e.g. Gromicho et al., 2006b], while Iberian *Chondrostoma s.l.* portrayed species-specific 45S phenotypes and genus-specific 5S phenotypes, both evidencing polytypism with higher incidence in *Achondrostoma* and *Iberochondrostoma* genera [Pereira et al., 2012]. Also in the same work, the use of higher resolution tools allowed

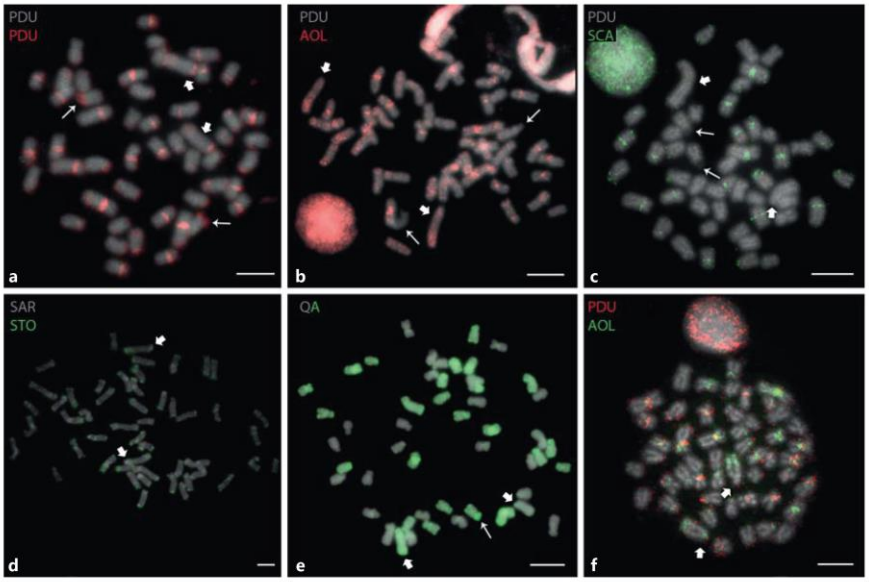

Fig. 2. Metaphase plates of Iberian *Chondrostoma s.l.* (**a–c**, **f**) and *Squalius* representatives (**d**, **e**) analyzed by means of self-GISH (**a**), cross species GISH (**b–e**) and CGH (**f**). DNA (probe) and metaphase (target) donor species are individually indicated as follows: AOL = *A. oligolepis*, PDU = *P. duriense*, SAR = *S. aradensis*, SCA = *S. carolitertii*, STO = *S. torgalensis*, and QA = *S. alburnoides* QA genomotype (= genomic constitution) inspected with genomic DNA from *S. alburnoides* nonhybrid nuclear male (AA genome). The cytotaxonomic subfamily marker (thick arrows) and the DAPI-negative NORs (thin arrows) are also indicated whenever recognizable. Bar = 5 μm.

for the revision and update of cytogenetic data regarding *A. arcasii* and *I. lemmingii* as well as providing the first descriptions for *A. occidentale* and *P. willkommii* [Pereira et al., 2012], increasing the number of Iberian cyprinid fishes cytogenetically characterized.

In an attempt to typify the levels of genome similarity beyond the documented chromosome homogeneity, experiments of cross-species GISH were performed within the 2 most diversified Iberian Leuciscinae lineages *(Squalius* and *Chondrostoma)* [Pereira et al., unpubl. data]. In 2 distinct sets of experiments, genomic DNA from a number of species was individually cross-hybridized against the chromosomes of one of the species *S. aradensis* or *P. duriense* (fig. 2a–d). As it would be expected, closely related species shared a greater fraction of chromosome regions (*P. duriense* vs. *P. duriense* = self-GISH, fig. 2a; *S. torgalensis* vs. *S. aradensis*, fig. 2d) than more divergent species (*S. carolitertii* vs. *P. duriense*, fig. 2c). Remarkably, *P. duriense* and *A. oligolepis* seem to share more chromosome portions than it would be predicted since they likely belong to different genera (fig. 2b). Notwithstanding, such homeologies may be responsible for the success of extensive natural hybridization between them [e.g. Aboim et al., 2010]. It is also worth documenting what seems to be a species-specific pattern of hybridization particularly observed at the DAPI-negative major rDNA clusters of *P. duriense* (fig. 2a–c), even though all species were inspected before for their rDNAs, using the same FISH probe responding positively [Gromicho et al., 2005, 2006b; Pereira et al., 2012; Nabais et al., 2013]. Most of all, differential hybridization patterns prove once more that despite the great overall chromosome and karyotype similarities, chromosome/genome organization is rather different between species. This type of cross-species GISH analyses must be extended and should be reciprocal, in order to withdraw sound information regarding genome structure.

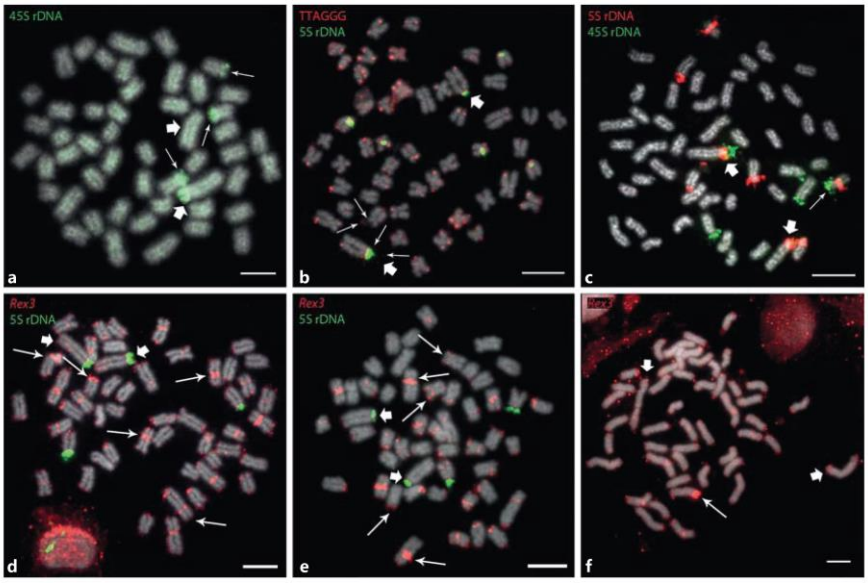

Fig. 3. DAPI-counterstained metaphase chromosomes of Iberian chondrostomine representatives mapped for distinct classes of repetitive DNA. Natural hybrids between *A. oligolepis* and either *P. duriense* (**a**, **b**) or *P. polylepis* (**c**) with translocated 45S rDNA clusters in 1 (**a**, **b**) or 2 (**c**) large subtelo- to acrocentric plus 1 metacentric (thin arrow) chromosome, flanked by telomeric $(TTAGGG)_n$ repeats (**b**, thin arrows; DAPI-negative) and colocalizing with some 5S rDNA clusters (**b**, **c**). Physical mapping of Rex3 retrotransposon in metaphases of *I. lusitanicum* male (**d**), *P. duriense* female (**e**) and *P. polylepis* male (**f**), evidencing particular accumulation in some chromosomes (thin arrows). **d**, **e** Simultaneous detection of Rex3 and 5S rDNA denoting little or no association between both sequences. Thick arrows evidence the cytotaxonomic subfamily marker. Bar = 5 μm.

Natural Hybrids

High levels of natural introgressive hybridizations have been reported within Iberian Leuciscinae, and as previously stated, molecular cytogenetics allowed identifying and distinguishing hybrids from parental species (figs. 2e, f, 3a–c) [e.g. Kirtiklis et al., 2010; Pereira et al., in press]. Even though the 2 families of rDNA have independent chromosome locations in Iberian *Chondrostoma s.l.* [Pereira et al., 2012], some of the chondrostomine homoploid hybrids investigated bear up to 3 units of colocalizing rDNAs (fig. 3c) [Pereira et al., in press]. Besides identifying extensively backcrossed hybrids among independent populations of sympatric species, genetic instability typical for interspecific hybrids was also documented [Fontdevila, 2005; Symonová et al., 2013; Pereira et al., in press]. Results were consistent with the translocation of 45S rDNA clusters, preferentially 'jumping' to 5S-bearing chromosomes (fig. 3b, c).

Telomeric $(TTAGGG)_n$ repeats typically mapped to the terminal regions of each chromosome [Gromicho et al., 2006a, b; Pereira et al., in press], but occasional interstitial telomeric sites commonly associated with recent chromosomal rearrangements [e.g. Phillips and Reed, 1996] were constantly found beneath 45S rDNA units in both parental species and hybrids (fig. 3b) [Pereira et al., in press]. Additionally, 5S rDNA sites were mostly centromeric and intimately associated with heterochromatin-rich (peri)centromeric regions (figs. 1e, 3b–e). The large fraction of flexible and mobile DNAs within heterochromatin [e.g. Cioffi and Bertollo, 2012] may be the reason for a higher plasticity to incorporate foreign jumping sequences like these 45S rDNA clusters. Interstitial telomeric sites have previously been associated with NORs in

salmonids, supporting molecular analyses that suggest ongoing active NOR transposition [Phillips and Reed, 1996], as seems to be the case of the homoploid hybrids of chondrostomines [Pereira et al., in press].

According to Fontdevila [2005], hybrids are triggers of transposition. Transposable elements have been inferred as a dynamic force in gene regulation and neofunctionalization, chromosome rearrangements, genome evolution, and even speciation [Böhne et al., 2008], but the lack of experimental data makes it difficult to prove. The abovementioned newly formed syntenic associations (fig. 3b, c) are distinct evidences of genome instability and reorganization most likely driven by the hybridization events. Retrotransposons of the Rex family are widely spread among teleost genomes and known to particularly associate with rDNA [reviewed in Cioffi and Bertollo, 2012]. Stress-activated retrotransposons have recently been associated with extensive rDNA multiplication and rapid speciation in 2 coregonine species [Symonová et al., 2013]. To determine whether the observed variability of rDNA clusters in these species was due to retrotransposon activity, an initial PCR screening for Rex elements followed by FISH-mapping onto chromosomes of distinct chondrostomine species was performed. Rex3 fragment (~500 bp) showed an essentially centromeric/telomeric distribution, concentrating at certain chromosome pairs (fig. 3d–f) [Pereira et al., unpubl. data]. However, conversely to our expectations, no particular association with rDNA clusters or rDNA-bearing chromosomes was found (e.g. fig. 3d, e). Yet, the distribution of this retroelement remains to be inspected in the hybrids, since some recurrent processes of genome invasion sensu Mallet [2005] may be triggering the (re)activation of transposition [Fontdevila, 2005] that caused the reorganization observed (fig. 3a–c).

Homoploid versus Allopolyploid Hybrids

Even though hybridization is common within Iberian Leuciscinae, polyploidy has only been reported within genus *Squalius* [Collares-Pereira and Coelho, 2010]. Trying to better understand the coping mechanisms behind such differential genome flexibility, GISH or CGH procedures were employed to the allopolyploid *S. alburnoides* complex [Rampin et al., 2012; C Nabais et al., unpubl. data] and the homoploid chondrostomine *A. oligolepis* × *P. duriense* hybrids (fig. 2e, f) [Pereira et al., in press].

The inspection of diploid (QA) and triploid (QAA) hybrids of the confined Quarteira population of *S. alburnoides* complex, using genomic DNA of the nuclear nonhybrid males *S. alburnoides* (AA genome), successfully sorted out respectively one-half (fig. 2e) or two-third (not shown) of the chromosomes as A-inherited, which interestingly appeared of bigger size than Q chromosomes (from the sympatric bisexual species *S. aradensis*, QQ genome). These results corroborated the hybrid origin and generic status of *S. alburnoides*, as reaffirmed by Collares-Pereira and Coelho [2010].

The use of CGH/GISH techniques is not that straightforward in extensively backcrossed homoploid hybrids (fig. 2f). Notwithstanding, results allowed (1) identifying one of the parental species as the major genomic contributor, (2) proving preferential backcrossing, (3) pinpointing shared and recombined chromosomal regions, and (4) recognizing additional translocations [Pereira et al., in press] besides the previously documented (fig. 3a–c). Moreover, results showed once again that the karyotype similarities considered typical for the diverse group of taxa assembled within the subfamily Leuciscinae [Ráb and Collares-Pereira, 1995] are apparent and restricted to chromosome macrostructure.

Final Remarks

Molecular cytogenetics applied to fish allowed overcoming the conventional banding difficulties, with great promise of innovative research as foreseen [Phillips and Reed, 1996; Cioffi and Bertollo, 2012] but most importantly offering the possibility to answer long existing taxonomic and evolutionary questions among others. Studies of interspecific chromosomal, chromatid and/or chromosomal segment homologies and the reconstruction of chromosomal evolution became then possible also for actinopterygians.

The scarcity of sequence data and other resources regarding nonmodel (fish) species make comparative mapping and characterization of repetitive genome fractions even more important than ever thought (i.e. against the odds of the 'selfish' and 'junk' DNA hypotheses) [recently reviewed in Cioffi and Bertollo, 2012]. The pronounced genome plasticity that characterizes most fish genomes in contrast to any other group of vertebrates allows the investigation of several appealing questions on speciation, hybridization, polyploidy, meiosis, epigenetics, sex determination, etc.

Studies of genome organization and evolution will surely benefit from the improvements of cytogenomics applied to fish genomes in general and to cyprinids in particular. Cyprinidae represent both a highly speciose family and the actinopterygians where the incidence of

hybridization and polyploidy seems to be more significant [see e.g. Mable et al., 2011; Collares-Pereira et al., 2013; Ráb and Collares-Pereira, submitted]. The most recent chromosome/genome painting techniques are beginning to empirically demonstrate that genome invasion events are more frequent in cyprinid fish than previously suspected. Additionally, molecular cytogenetics proved capable of further retrieving some cases of genetic admixture over a robust cocktail of genetic markers [e.g. Pereira et al., in press]. Besides significant impact on evolutionary biology and speciation matters, the effects of this new paradigm of the postgenomic era outspreads to the context of biodiversity and conservation [Mallet, 2005; Ráb et al., 2007].

It is worth mentioning that presently, only 34 out of the 204 already cytogenetically analyzed leuciscine species [Esmaeili et al., 2010; Valić et al., 2010; Arai, 2011; Pereira et al., 2012; Rossi et al., 2012; Nabais et al., 2013] were addressed by means of molecular cytogenetics [Rábová et al., 2003; Ocalewicz et al., 2004; Gromicho et al., 2005, 2006a, b; Schmid et al., 2006; Ráb et al., 2008; Kirtiklis et al., 2010; Pereira et al., 2012; Rossi et al., 2012; Nabais et al., 2013]. Therefore, it would be of great value to spread the analysis of the same set of markers over a broad range of phylogenetically related species to, above all, understand evolutionary processes within the distinct cyprinid lineages and sort out the main chromosomal/genome evolution mechanisms. Furthermore, a comparative cytogenomic analysis of both types of hybrid complexes (homoploid and polyploid) might be challenging towards the refinement of how distinctive reproductive strategies (sexual, nonsexual or even both simultaneously, in some cases) and genome reshaping mechanisms may be fuelling evolutionary novelties within leuciscines.

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Chapter 5

Discussion

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5. Discussion

The aim of this chapter is to integrate the results already presented throughout **Chapters 2** to **4** and for which partial discussions were also offered. In this general discussion the various evolutionary implications of these results will be debated in light of theoretical predictions and in comparison to other known cases of natural HYBRIDIZATION (HOMOPLOID and POLYPLOID) in Cyprinidae.

5.1. Chromosome Markers

To study HYBRIDIZATION, diagnostic characters in the taxa of interest must ideally be inferred from localities where taxa do not co-occur and where the influence of HYBRIDIZATION can be ruled out or minimized. In the case-study of HOMOPLOID HYBRIDIZATION between AOLx(PDU/PPO) (2n=50), defining ALLOPATRIC populations is particularly difficult for AOL because (1) co-localization with PDU or PPO occurs across the totality of its distribution range (**Figure 1.2**), and (2) until recently the phylogeography of the genus *Achondrostoma* was not well resolved, with SPECIES undescribed and the range of AOL and its sister-SPECIES AAR (*A. arcasii*) being poorly defined (Doadrio & Carmona 2004; Robalo et al. 2005, 2006, 2007; Doadrio & Elvira 2007). Therefore, in the present work 'ALLOPATRIC' refers to locations where taxa were never sampled together in present or historical collections, whereas 'sympatric' refers to locations where parental taxa and/or putative HYBRIDS have been regularly identified.

One of the methods to appreciate the impact of HYBRIDIZATION on HYBRID genes and genomes is to search for traces of structural rearrangements originated by the merging of two foreign chromosomal sets in a single (HYBRID) nucleus (e.g. Chester et al. 2013). Although Leuciscinae KARYOTYPES are ostensibly very similar (e.g. Ráb & Collares-Pereira 1995), the progress on cytogenomics and sequence data was expected to allow for a deeper investigation on these genomes and their actual organization. Defining parental SPECIES-specific chromosomal markers was necessary to establish a starting point of genomic information and overall, this inquiry was able to fulfil the purposes it was designed for. Namely, it provided chromosomal markers whose distribution and organization can be used to identify and distinguish SPECIES, populations or lineages (**Chapters 2** and **3.2**; **Table 5.1**); it proved valuable for forthcoming surveys on natural HYBRIDIZATION (**Chapter 3.1**); and it delivered better-resolved leuciscine KARYOTYPES, despite the overall macrostructural similarities (**Chapters 2** and **4**).

The largest subtelo-acrocentric (st-a) chromosome pair was one of the first marker chromosomes defined in Leuciscinae, being proposed as the Leuciscinae cytotaxonomic marker (Vasilev 1985; Ráb et al. 2008). Besides being usually the largest on the complement, a clear subterminal band of heterochromatin in the long arms of these chromosomes is frequently found across many Leuciscinae SPECIES (e.g. Bianco et al. 2004; Pereira et al. 2009). Even though this band seemed to be absent in *Alburnus albidus* and *Squalius cephalus* (Bianco et al. 2004), the high homology of this marker across Leuciscinae (including as well *Squalius cephalus*) and probably Phoxininae subfamilies lead us to question the power of CROSS-SPECIES painting on reasoning about KARYOTYPE EVOLUTION in cyprinid fishes (Ráb et al. 2008), appealing for resolution refinements of preserved syntenic blocks in conservative genomes – i.e. sub-chromosomal markers (see e.g. Nie et al. 2012).

Since molecular data in these SPECIES is restricted to mitochondrial and few nuclear genes or microsatellites (e.g. Aboim et al. 2010), the assessment for repetitive sequences allows for a rapid and relatively inexpensive method to define genome organization in fishes (e.g. Cabral-de-Mello & Martins 2010; Cioffi & Bertollo 2012). For this purpose, a set of easily accessible repetitive sequences was selected: ribosomal DNAs (**Chapters 2-4**), $(TTAGGG)_6$-repeats (**Chapter 3.1**), Rex3 RETROTRANSPOSABLE ELEMENT (**Chapter 3.2**), C_0t-1 DNA fraction (**Chapter 4**), and whole genomic DNA in CROSS-SPECIES GISH (**Chapter 4**) or CGH experiments on the HYBRIDS (**Chapters 3.1** and **4**). Some of these sequences or genome fractions were better explored than others mainly due to some technical constraints. Nevertheless, this work represents a broader application of cytogenomic techniques to Iberian cyprinids (but see also Gromicho & Collares-Pereira 2007; Rampin et al. 2012; Collares-Pereira et al. 2013) although the full potential of this approach applied to these taxa is yet to be unveiled. In fact, some of these techniques and principles have also been tentatively applied to other hybrid fishes endemic to the Iberian Peninsula – the *Squalius alburnoides* allopolyploid complex.

5.2. Genome Architecture & Dynamics

5.2.1. Iberian Leuciscinae

Fish genomes usually stand for a great genomic flexibility (Venkatesh 2003) by evidencing a broad range of chromosome numbers (from 2n=12 in the deep-water spark anglemouth *Sigmops bathyphilus*, to 2n=446 in *Ptychobarbus dipogon*; www.fishbase.org) and genome sizes (from 0.35 pg/cell in *Tetraodon nigroviridis* to 132.83 pg/cell in *Protopterus aethiopicus*; www.genomesize.com), a variety of sex determination systems (e.g. Devlin & Nagahama 2002), a higher tolerance to

HYBRIDIZATION and POLYPLOIDIZATION (up to 2n=3-6x in Acipenseridae; Arai 2011; Mable et al. 2011), and accounting for slightly more than one half of the total number of recognized living Vertebrates (Nelson 2006). On the other hand, genome size in teleosts is relatively conserved at the family level (Arai 2011; www.genomesize.com) and Leuciscinae KARYOTYPES seem rather conservative regarding chromosome number (2n=48-50), size, morphology and base composition (e.g. Ráb & Collares-Pereira 1995; Sola & Gornung 2001; Arai 2011), contrasting with the high SPECIATION rates observed not only in Iberian Leuciscinae (e.g. Perea et al. 2010).

At the level of gross chromosomal organization, there is little evidence that chromosomal divergence has proceeded at a rapid rate among leuciscines. SPECIATION events in these fishes typically involve changes in only a small proportion of the genome or changes in genetic regulation that reveal more important than structural changes in determining SPECIATION rates (Avise & Gold 1977). Nevertheless, one cannot exclude the possibility of important genomic changes having occurred beyond current resolution power.

Constitutive heterochromatin among eukaryotes is mainly found on the pericentromeric regions, a pattern usually considered the ancestral character state. Heterochromatin distribution in chondrostomines points to some level of differentiation by evidencing some chromosomes without (or with very few) pericentromeric heterochromatin and some chromosomes with only telomeric accumulation of this element (e.g. Pereira et al. 2009). However, a thorough inspection including more SPECIES within each genus must be performed and chromosome homeologies must be unequivocally recognised before a cross-examination against a PHYLOGENETIC tree is possible in order to better understand the distinct evolutionary trends of differentiation. The same is valid for any available chromosome marker.

The results of this dissertation demonstrated that this uniformity is actually macrostructural, designating lineage- or SPECIES-specific variable features such as (1) number and location of ribosomal DNA units (**Chapter 2**; **Table 5.1**), (2) accumulation of Rex3 RETROTRANSPOSABLE ELEMENT (**Table 5.2**), (3) accumulation of repetitive sequences (**Chapter 3.2**), and (4) to some extent a SPECIES-specific 45S rDNA cluster (**Chapter 4**). Therefore, besides the rearrangements we can suspect but not yet prove to have occurred (i.e. notorious size heteromorphism between some homologue chromosomes), chromosomal modifications underlying SPECIATION in the subfamily Leuciscinae may have involved (1) translocations with multiplication of the rDNA clusters, (2) TRANSPOSITION via mobile elements also with multiplication of the rDNA units, and most likely (3) intrachromosomal rearrangements preserving the overall chromosome structure with minor variations at the total number of meta- (m), submeta- (sm)

or st-a chromosome pairs (see Pereira et al. 2009; Pereira et al. 2012). Other examples of such karyotype macrostructure uniformity but with totally different sub-chromosomal organization have been increasingly documented amongst Characiformes, particularly between fish species of the genus *Symphysodon* (Gross et al. 2010). The authors argued that despite the evolutionary mechanisms preventing major karyotype rearrangements, the genomes of the species examined continued evolving via minor chromosomal variations.

Although a single 45S rDNA cluster terminally located on a pair of small acrocentric chromosomes is believed to represent the PLESIOMORPHIC state in Cyprinidae (Amemiya & Gold 1990), variability in number and/or position of ribosomal genes has been increasingly reported within Leuciscinae, as well as the co-localization of both units (e.g. Gromicho et al. 2006a,b; Kirtiklis et al. 2010; Rossi et al. 2012; present dissertation). Even though, the ANCESTRAL state for Leuciscinae subfamily is believed to comprise a single pair of major rDNA clusters in a medium to small-sized sm chromosome pair (e.g. Ráb & Collares-Pereira 1995) and one pair of minor rDNA in the (peri)centromeric regions or short arms of the largest st-a chromosomes (Rossi et al. 2012), compiled results indicate that rDNA-phenotypes of Eurasian Leuciscinae (**Table 5.1**) may be as variable as in North American leuciscines (e.g. Amemiya & Gold 1990). NORs are usually described as very dynamic regions capable of dissemination within the genome and variants include (1) multiple rDNA clusters, (2) different chromosomal position, (3) different types of rDNA-bearing chromosomes, or (4) loss of rDNA-bearing chromosomes (see e.g. Escobar et al. 2011).

Initially designed to determine whether the POLYMORPHIC state of the rDNAs could be related with TRANSPOSON activity, we assessed the distribution of the Rex3 RETROELEMENT in these genomes, the most abundant family of RETROTRANSPOSONS in fishes (Volff et al. 1999). Rex3 distribution in the examined SPECIES was found compartmentalized with accumulation in pericentromeric regions, suggesting a possible correlation between KARYOTYPE EVOLUTION and RETROTRANSPOSON activity. Correlations between the accumulation of repetitive elements, heterochromatin and chromosome rearrangements have been hypothesized to explain KARYOTYPE differentiation in the fishes *Hoplias malabaricus*, *Erythrinus erythrinus* (Characiformes), *Symphysodon* genus, and *Notothenia coriiceps* (Perciformes) (reviewed in Cioffi & Bertollo 2012). Inversely to recent findings where 18S rDNA (Silva et al. 2013) or 5S rDNA were found intimately associated to Rex3 and an increased KARYOTYPE diversity (e.g. Cioffi et al. 2010; Gross et al. 2010; Symonová et al. 2013), we did not find this correlation with neither of the rDNA families suggesting either (1) another mechanism of TRANSPOSITION or (2) the involvement of another mobile element instead of Rex3.

Interestingly, all of the SPECIES now analysed shared the overall pattern of Rex3 distribution proposing an ancient incorporation of this element in their genomes. This result is not strange considering the broad incidence of Rex3 in teleosts, despite an intermittent distribution (Volff et al. 2001b). Nonetheless, it seems rather interesting to invest in other forms/families of TRANSPOSABLE ELEMENTS for the investigation of genome organization and evolution in fishes in general. To start with, there are many good candidates such as LINEs (e.g. Oliveira et al. 1999), *tc1* and *tc1*-like (e.g. Izsvák et al. 1995), *Ty3-gypsy* (e.g. Volff et al. 2001a), *mariner*-like (e.g. Mandrioli 2000), among others (see e.g. Volff et al. 2003).

As previously mentioned, with the increasing number of SPECIES mapped for these sequences, co-localization of both rDNA gene families has been gradually acknowledged (e.g. Gromicho et al. 2006a,b; Kirtiklis et al. 2010; Rossi et al. 2012) more than it would be expected (e.g. Martins & Wasko 2004). However, such association has not been documented among the chondrostomine SPECIES analysed to date (Pereira et al. 2012) except in cases of HYBRIDIZATION (**Chapter 3.1**; discussed ahead). In one hand, only seven of the 14 evolutionary lineages *sensu* Perea et al. (2010) have so far been surveyed for both these markers (**Table 5.1**). Moreover, data compilation demonstrate a huge gap of results mainly regarding 5S rDNA mapping. On the other hand, one cannot exclude HYBRIDIZATION events as the cause of such interspecific and interpopulation variability. Natural HYBRIDIZATION is a common process among cyprinids (Scribner et al. 2001), and particularly within European Leuciscinae, HYBRIDS (natural or artificial) have been described involving genera *Abramis*, *Alburnoides*, *Alburnus*, *Aspius*, *Blicca*, *Chondrostoma s.l.*, *Leuciscus*, *Pachychilon*, *Phoxinellus*, *Phoxinus*, *Rutilus*, *Scardinius*, *Squalius* and *Vimba* (Treer & Kolak 1994; Scribner et al. 2001). Once again, the importance of including multiple independent datasets must be emphasized, especially if inter-specific HYBRIDIZATION is a possibility among the SPECIES studied. Moreover, single types of data are insufficient for inferring processes, when a number of different mechanisms could have led to the same pattern (e.g. Robinson et al. 2008).

Species	5S rDNA		28S / 45S rDNA		Syntenic associations		Reference
	number of clusters	location	number of clusters	location	number	location	
Lineage II - Alburnine							
Alburnus albidus	-	-	2	2 sm	-		Bianco et al. 2004
Alburnus alburnus	4-6	2 st/a + (1-2) m + (1-2) sm	2	2 sm	0		Schmid et al. 2006
Alburnus filippii	-	-	2	2 st/a	-		Nazari et al. 2009
Alburnus mossulensis	-	-	4	4 sm	-		Yuksel & Gaffaroğlu 2008
Anaecypris hispanica	8	4 st/a + 4 sm	8	4 sm + 4 st/a	8	4 sm + 4 st/a	Gromicho et al. 2006a
Leucaspius delineatus	-	-	2	2 st/a-sm	-		Mayr et al. 1986
Lineage III - Scardinius							
Scardinius acarnanicus	-	-	2	2 sm	-		Bianco et al. 2004
Scardinius erythrophthalmus	6	3 st/a + 3 sm	2	2 sm	2	2 sm	Mayr et al. 1986; Bianco et al. 2004; Pereira et al. unpub.
Scardinus graecus	-	-	2	2 sm	-		Bianco et al. 2004
Scardinius scardafa	-	-	2	2 sm	-		Bianco et al. 2004
Lineage V							
Petroleuciscus borysthenicus	-	-	2	2 sm			Ráb et al. 1996
Squalius alburnoides AA	6	4 st/a + 2 sm	6-8	(2-6) sm + (1-4 st/a)	2-6	4 st/a + 2 sm	Gromicho et al. 2006b
Squalius alburnoides PA	6	4 st/a + 2 sm	8	(3-5) sm + (3-4) st/a	4-6	(2-4) st/a + 2 sm	Gromicho et al. 2006b
Squalius alburnoides PAA	9	6 st/a + 3 sm	12	4 st/a + 8 sm	6	4 st/a + 2 sm	Gromicho et al. 2006b
Squalius alburnoides PPA	9	6 st/a + 3 sm	8	4 sm + 4 st/a	8	4 sm + 4 st/a	Gromicho et al. 2006b
Squalius alburnoides PPAA	12	8 st/a + 4 sm	6-15	9 sm + 6 st/a	10	4 sm + 6 st/a	Gromicho et al. 2006b
Squalius aradensis	-	-	2	2 sm	-		Nabais et al. 2013
Squalius cephalus	6	2 st/a + 2 m + 2 sm	2	2 sm	2	2 sm	Bianco et al. 2004; Kirtiklis et al. 2010
Squalius lucumonis	8	4 st/a + 2 m + 2 sm	2	2 sm	2	2 sm	Rossi et al. 2012
Squalius pyrenaicus	6-7	4 st/a + 2 sm + 1 m	8	4 sm + 4 st/a	6	4 st/a + 2 sm	Gromicho et al. 2006b
	6	4 st/a + 2 m	2	2 sm	2	2 sm	Pazian pers. com.
Squalius squalus	6	4 st/a + 2 m	2	2 sm	0		Rossi et al. 2012
Squalius torgalensis	-	-	2	2 sm	-		Nabais et al. 2013
Lineage VI - Chondrostomine							
Achondrostoma arcasii	8	4 st/a + 2 m + 2 sm	-	-	-		Pereira et al. 2012
Achondrostoma occidentale	7	4 st/a + 2 m + 1 sm	-	-	-		Pereira et al. 2012
Achondrostoma oligolepis	6-8	4 st/a + (1-2) m + (1-2) sm	4-6	4-6 sm	0		Pereira et al. 2012
Iberochondrostoma almacai	-	-	4	4 sm	-		Pereira et al. 2012
Iberochondrostoma lemmingii	4-6	(4-6) st/a	3	3 sm	0		Pereira et al. 2012
Iberochondrostoma lusitanicum	4	4 st/a	3-4	(3-4) sm	0		Pereira et al. 2012
Parachondrostoma arrigonis	-	-	2-8	2-7 sm + 1 st/a	-		Kalous et al. 2008
Pseudochondrostoma duriense	4	4 st/a	3	3 sm	0		Pereira et al. 2012
Pseudochondrostoma polylepis	4	4 st/a	4	4 sm	0		Pereira et al. 2012
Pseudochondrostoma willkommii	4	4 st/a	3	3 sm	0		Pereira et al. 2012
Pseudophoxinus firati	-	-	4	4 sm	-		Karasu et al. 2011
Telestes muticellus	4	2 st/a + 2 m	2	2 sm	0		Rossi et al. 2012
Telestes ukliva	-	-	4	4 sm	-		Valic et al. 2010

Table 5.1 (continued). Summary of the data on rDNA chromosome mapping and syntenic associations between both gene families on Eurasian Leuciscinae. Species where co-localization of both rDNA gene families occur are denoted in *grey*. Denomination of the lineages listed followed Perea et al. 2010.

Species	5S rDNA		28S / 45S rDNA		Syntenic associations		Reference
	number of clusters	location	number of clusters	location	number	location	
Lineage VII							
Rutilus aula	-	-	2	2 sm	-		Bianco et al. 2004
Rutilus prespensis	-	-	2	2 sm	-		Bianco et al. 2004
Rutilus rubilio	6	6 st/a	2	2 sm	0		Rossi et al. 2012
Rutilus rutilus	-	-	2		-		Ráb & Roth 1989
Rutilus virgo	-	-	2		-		Ráb & Roth 1989
Rutilus ylikiensis	-	-	2	2 sm	-		Bianco et al. 2004
Lineage VIII							
Abramis brama	-	-	2	2 st	-		Ocalewicz et al. 2004
Achantobrama marmid	-	-	4	4 sm	-		Gaffaroğlu et al. 2006
Blicca bjoerkna	4	2 st/a + 2 sm	1	1 sm	0		Pereira et al. unpub.
Vimba elongata	-	-	2	2 sm	-		Rabová et al. 2003
Vimba melanops	-	-	2	2 sm	-		Rabová et al. 2003
Vimba vimba	-	-	2	2 sm	-		Rabová et al. 2003
Lineage IX							
Alburnoides bipunctatus	-	-	2	2 sm	-		Nazari et al. 2009
Lineage X							
Leuciscus aspius	-	-	2		-		Ráb et al. 1990
Leuciscus idus	5-6	(4-5) st/a + 1sm	2	2 sm	0		Kirtiklis et al. 2010
Leuciscus leuciscus	8	4 st/a + 4 sm	2	2 sm	1	1 sm	Kirtiklis et al. 2010
Leuciscus waleckii	-	-	2	-	-		Arai 2011
Lineage XII							
Pachychilon macedonicum	-	-	2	2 sm	-		Ráb et al. 2000

m = metacentric, sm = submetacentric, st/a = Subtelo-acrocentric chromosomes, - not determined

5.2.2. Natural Hybrids

Present results discovered the occurrence of syntenic 5S-45S rDNA units as an indicator of inter-specific HYBRIDIZATION between chondrostomine SPECIES in Iberian freshwaters (**Chapters 3.2** and **4**). Such organization in other Leuciscinae was simply regarded as SPECIES-specific evolutionary patterns (Kirtiklis et al. 2010; Rossi et al. 2012), though also put forward as potentially useful to depict cases of natural HYBRIDIZATION (Kirtiklis et al. 2010). Since many of the HYBRIDS among Cyprinidae are fertile, recurrent BACKCROSSING is highly probable resulting in HYBRIDS virtually indistinguishable from their parental taxa (Mallet 2005). Not only do we expect that more SPECIES will soon be evaluated for these markers but we also recommend the re-examination of SPECIES where rDNAs occur in synteny to rule out cases of natural HYBRIDIZATION at the basis of such association.

HYBRIDS inspected during the present work represented a variety of possible combinations between parental genomic contributions resulting in a random assortment of cytonuclear and chromosomal markers distributed among the different HYBRID morphotypes; as well as some TRANSGRESSIVE phenotypes rendered as: (1) more rDNA clusters than possibly inherited from any of the parental forms, and (2) syntenic associations between both gene clusters (**Chapter 3.1**). Cases of TRANSGRESSIVE phenotypes have been reported in other HYBRID cyprinid populations (reviewed in Corse et al. 2012). Similarly to *Gila robusta* complex, present genetic data suggest a decoupling between morphological and cytonuclear markers, possibly pointing to local ADAPTATION and to the role of HYBRIDIZATION in evolution (Gerber et al. 2001).

Obviously, chromosomal repatterning during HYBRIDIZATION and/or evolution may alter the number and position of rDNA sites; but recent studies have shown that the dynamism of the rDNA clusters may be regarded as a strong indicator of significant intragenomic processes (Cioffi & Bertollo 2012) like RETROTRANSPOSON activity, as demonstrated in the fish *Erythrinus erythrinus* (Cioffi et al. 2010). TEs are one of the most fluid components of the genome. Knowing TE's distribution patterns originally present in the parental SPECIES might be highly relevant to understand the nature of changes occurring as a result of HYBRIDIZATION (Arkhipová & Rodriguez 2013). On the other hand, HYBRIDS are long known as activators of TRANSPOSITION in a variety of organisms (reviewed in Fontdevila 2005 and Hua-Van et al. 2011). With that in mind, PHYSICAL MAPPING of the RETROELEMENT Rex3 was applied to mitotic chromosomes of some chondrostomine HYBRIDS revealing gains of hybridization signals at centromeres – other than the original pattern – and short arms of some chromosomes (**Chapter 3.2**). Despite discreet, results seem to corroborate other examples of HYBRID TRANSPOSITION activation. Nevertheless, even without a dramatic copy number increase, a certain level of TE activation may lead to

chromosomal rearrangements which are particularly likely to contribute to rapid genome reshaping in the absence of large-scale TE amplification (reviewed in Arkhipova & Rodriguez 2013). On the other hand, this might characterize the expected and the only viable result in case of TRANSPOSITION activation in HYBRIDS since other possible combinations are most likely deleterious and/or selected against (e.g. non-centromeric accumulation).

Unfortunately, HYBRIDS of first generation (F1) were never found in the studied HYBRID ZONES during present or past sampling campaigns (e.g. from Collares-Pereira & Coelho 1983; Aboim et al. 2010; to present work). Ideally, a F1 HYBRID KARYOTYPE would be composed of 25 chromosomes from one parental SPECIES and 25 chromosomes from the other. By applying GISH or CGH procedures, one would in theory be able to identify the individual contributions by each parent. While the application and the results obtained by these methodologies seem quite straightforward in POLYPLOID individuals (e.g. Rampin et al. 2012), the same is not valid for HOMOPLOID and highly introgressed HYBRIDS (**Chapters 3.1** and **4**). This could either indicate that (1) parental SPECIES are more similar and closer related than expected for an 11-my-divergence time between them (Aboim et al. 2010), so that SPECIES-specific repetitive elements might not have sufficiently differentiated; or that (2) the HYBRIDIZATION process is older than 5 mya, the time-frame considered necessary for the process of genome turnover by means of sequence homogenization; if completed, parental genomes can no longer be resolved by GISH as demonstrated in *Nicotiana* natural ALLOPOLYPLOIDS (Lim et al. 2007).

Indisputably, experimental approaches would significantly contribute to the understanding of HYBRID ZONES and HYBRIDIZATION phenomena, despite the limitation that processes revealed in such controlled and highly simplified systems may not be representative of processes operating in nature (Scribner & Avise 1994). Nevertheless, controlled breeding studies allow (1) to establish important demographic variables difficult to monitor in natural settings (e.g. numbers and genetic composition of founders, levels of exogenous GENE FLOW, and age of the HYBRID population; Scribner & Avise 1994), as well as (2) obtaining precious F1 HYBRIDS to trace genomic restructuring in real time with both progenitor SPECIES available for comparison (see Arkhipova & Rodriguez 2013). Mable (2013) even suggests that greatest insights would come from comparing HOMOPLOID HYBRIDS, ALLOPOLYPLOIDS and AUTOPOLYPLOIDS artificially created from the same parental SPECIES.

5.3. Evolutionary Consequences

The simple fact of two parental genomes co-existing in a single nucleous accounts for both novelty and conflict on an intracellular scale, translating as genomic and transcriptomic shock, i.e. massive genome restructuring and changes in gene expression (e.g. Fontdevila 2005; Hegarty et al. 2006, 2008; Matos et al. 2011; Feldman et al. 2012). Alterations at the chromosome level may explain some of the now observed modifications: intergenomic translocations coupled with possible homeologous RECOMBINATION may yield novel non-additive combinations of parental genes. HYBRIDS may not only provide novelty but accelerate SPECIATION in some cases (reviewed in Abbott et al. 2013 and Soltis 2013).

According to Feldman et al. (2012), the modifications observed in the HYBRIDS/POLYPLOIDS may translate as 'revolutionary' (genetic and epigenetic changes triggered by HYBRIDIZATION and/or POLYPLOIDIZATION) and/or 'evolutionary changes' (post-HYBRIDIZATION/post-POLYPLOIDIZATION genetic changes that contribute to diversity and lead to ADAPTATION). SPECIES-specific demographic factors (e.g. gestation length, offspring birth size, growth rates, size and age at sexual maturity) can dramatically affect the genetic outcomes in HYBRID ZONES (Scribner & Avise 1994), resulting in the current mosaic combination of molecular and morphological characters. Although INTROGRESSION may be highly selective, HYBRIDIZATION has the potential to introduce large sets of new alleles allowing for the acquisition of a genetic architecture otherwise difficult to evolve by sequential accumulation of mutations. A large fraction of this INTROGRESSION is likely to be deleterious, but when large numbers of HYBRIDIZATIONS occur at the same time, the greater the chance that some will pass on to the next generations (Abbott et al. 2013).

The abundant genotypes produced by HYBRID RECOMBINATION should facilitate further exploration of different ecological niches thus contributing to ADAPTATION and SPECIATION, in principle highly dependent on ecological opportunity (Abbott et al. 2013). The expression of extreme phenotypes may lead to (1) population segregation of the HYBRIDS in relation to their parents, by ecological niche separation, for example; and/or (2) to HETEROSIS in which HYBRID phenotypes are more fit than parental ones (Rieseberg et al. 1999; Corse et al. 2012). Onwards, a combination of founder effect, directional INTROGRESSION, and/or SELECTION may result in the total replacement of one genetic marker for another, as observed in other examples of HOMOPLOID HYBRIDIZATION (e.g. Gerber et al. 2001; Costedoat et al. 2005; Broughton et al. 2011). To discriminate among these alternatives, specific investigation to

examine the fitness of each class of HYBRIDS (i.e. morphotype-genotype combination) relative to different environments is then necessary.

Data taken from a single population at one point in time, over multiple populations, or in a time series for single populations can be particularly useful and informative in terms of identifying plausible evolutionary mechanisms driving changes in gene frequency and gene associations over time (mating system, SELECTION, population size, GENETIC DRIFT, and degree of population subdivision). There is a tendency for HYBRID ZONES to move toward regions of low density, low HYBRID unfitness, low dispersal, and in the direction favouring the fitter allele (Scribner et al 2001). For the establishment of a HOMOPLOID HYBRID SPECIES however, genome stabilization must be allowed to proceed for some additional time after REPRODUCTIVE ISOLATION (Buerkle & Rieseberg 2008). HYBRIDS' divergence may be associated to several phenomena like parental genome DNA losses, RETROELEMENT activity and/or intergenomic homogenization (e.g. Lim et al. 2007). In this case-study where HYBRIDIZATION events seem recurrent and extensive (Aboim et al. 2010), ecological barriers between HYBRIDS and parental SPECIES might be altered and/or degraded, so that GENE FLOW back to pure SPECIES never actually ceases (**Chapter 3.1**) and HYBRID genomes will hardly achieve stabilization or SPECIATION.

On the other hand, by preserving the ability to interbreed at least periodically (reproductive plasticity) and sustaining INTROGRESSION, genetic variation is transferred among the different forms generating new genotypes subjected to local selective pressures. By these means, taxa acquire greater flexibility and more rapid ADAPTATION than if variants were derived by mutation alone (Dowling & Secor 1997; Gerber et al. 2001). Under fluctuating environmental conditions like those found in semi-arid Mediterranean habitats (e.g. Magalhães et al. 2002), this process could (1) assure current levels of biodiversity, and/or (2) rapidly enhance biodiversity through the creation of new mosaic taxa. Therefore, and following the examples of *Gila* (Gerber et al. 2001) and *Cyprinella* (Broughton et al. 2011) complexes, the maintenance of reproductive plasticity and INTROGRESSIVE HYBRIDIZATION among chondrostomine taxa may be important for their preservation and/or evolution enabling them to adapt to changing environments.

5.4. To be or not to be polyploid?

As already mentioned, fish genomes seem to undergo and tolerate genetic changes more rapidly and more often than other vertebrate genomes, suggesting that fish genomes are 'plastic' (see Venkatesh 2003). Order Cypriniformes is characterized by the largest diversity of HYBRID and POLYPLOID fishes known to date. Family Cyprinidae contains many HYBRIDIZING and/or POLYPLOID SPECIES and genera (many cyprinid genera are composed of stable POLYPLOID lineages), representing cytogenetic extremes but with relatively conservative modal diploid values (Arai 2011). Within Iberian Leuciscinae, two distinctive types of systems of HYBRIDIZING fish SPECIES can be recognized, resulting in (1) organisms with the same number of chromosomes as the parental SPECIES – exemplified by the HOMOPLOID HYBRIDIZATION between *Achondrostoma oligolepis* and either *Pseudochondrostoma duriense* or *P. polylepis* (e.g. Aboim et al. 2010; present work), or in (2) organisms with ploidy elevation – well represented by the diploid-ALLOPOLYPLOID *Squalius alburnoides* complex (reviewed in Collares-Pereira et al. 2013).

HYBRIDIZATION and POLYPLOIDY are closely related and both may contribute with potential for ADAPTATION to varying environmental conditions (Mable 2013). Both HYBRIDIZATION and POLYPLOIDY can involve dramatic and immediate changes in genome structure which could alter the adaptive responses to environmental change (Mable et al. 2011). Although POLYPLOID genomes increase the cost of replication, they also provide evolutionary advantages, because they present a basis for almost instantaneous SPECIATION (different number of chromosomes) (Abbott et al. 2013; Madlung 2013; Soltis 2013). On the other hand, HYBRIDIZATION alone is thought to induce more substantial genomic rearrangements than genome duplication (e.g. Comai et al. 2003; Hegarty et al. 2006; Buggs et al. 2011; Czypionka et al. 2012). HYBRIDIZATION and POLYPLOIDIZATION are types of 'genomic shock' that could result in dramatic genomic restructuring and set new contexts for gene expression. That in turn, could lead to increased regulatory flexibility or genome destabilization in HOMOPLOID HYBRIDS due to imbalances in gene expression and ultimately cause sterility or mortality (reviewed in Mable 2013).

POLYPLOIDY is most common (but not widespread) among cold-blooded vertebrates therefore directly exposed to changes in their environments; but it does not explain why some SPECIES are POLYPLOID and other not (see Mable et al. 2011). Although knowledge in POLYPLOID plants is much more advanced compared to animals, there is no clear understanding on the factors promoting successful POLYPLOIDIZATION and POLYPLOID SPECIATION in the wild, or on the evolutionary relevance of POLYPLOIDY. Despite significant progress, there still is not enough information to unequivocally answer many unresolved questions on the subject (Madlung 2013; Stöck & Lamatsch 2013).

Although both habitat fragmentation and temperature changes during environmental instabilities are likely accountable for altering SPECIES interactions, which can increase rates of HYBRIDIZATION (Seehausen et al. 2008), the propensity to hybridize seems to be more determined by intrinsic properties (genomic constraints) than by environmental conditions. When genetic divergence between parental SPECIES is low, there may be little chance of major novelties arising in HYBRIDS but, when divergence is high, intrinsic incompatibility may prevent successful HYBRIDIZATION (Abbott et al. 2013). The more closely related are the interacting genomes in the HYBRID, the more likely it is for homeologs to pair, resulting in chromosomal exchanges between the two genomes (Madlung 2013). There is also some evidence that the extent of genomic divergence between hybridizing SPECIES influences the likelihood of diploid or POLYPLOID HYBRID SPECIATION (reviewed in Mable et al. 2011).

Genetic distance and genomic constraints seem to be the key distinguishing factors between both SPECIES complexes considered. The *Squalius alburnoides* complex is composed of HYBRID biotypes with different genomic compositions and ploidies (2n = 50, 3n = 75, and 4n = 100), bisexual and altered sexual modes (reviewed in Collares-Pereira et al. 2013), self-sustained by close interaction with the sympatric bisexual paternal SPECIES, though the paternal ancestor is long extinct. The complex originated from unidirectional HYBRIDIZATION events between *S. pyrenaicus* females and males closely related to the extant species *Anaecypris hispanica*. While the HOMOPLOID chondrostomine HYBRIDS result from crosses between closely related SPECIES – once congeneric (Robalo et al. 2007) – *Squalius alburnoides* originated from the interbreeding of phylogenetically more distant SPECIES – different lineages (*sensu* Perea et al. 2010). Notwithstanding, both complexes are most likely maintained by the genetic compatibility of producing viable HYBRIDS and, in the case of *S. alburnoides*, very successful ones (widespread complex) with great evolutionary potential (reviewed in Collares-Pereira et al. 2013).

While HYBRIDIZATION per se can instantaneously produce distinct taxa through an increase in chromosome number (allopolyploidy) or by altering the modes of reproduction (unisexuality) – e.g. *Squalius alburnoides* complex, INTROGRESSION can only eventually lead to a stable, independent lineage definable by unique combinations of characteristics over time (Dowling & Secor 1997). However, diversification is not instantaneous requiring the recombinant lineages to be isolated from the parental taxa, for a long time enough to evolve and accumulate genetic differences responsible for their independence upon secondary contact (e.g. Dowling & Secor 1997; Buerkle & Rieseberg 2008). The presently investigated HZs of chondrostomine species evidenced continuous bi-directional INTROGRESSION (see Aboim et al. 2010) indicating an earlier or transient stage of the (potential of the) evolutionary process, with the HYBRID SWARM operating as a reservoir of genetic variability and adaptability for times to come.

Table 5.2 Conservation status and strategies of Iberian Leuciscinae (http://www.iucnredlist.org). For species names abbreviations please consult list on page xxi.

species	IUCN conservation status[a]	population trend	threats	conservation measures	obs.	other references
AHI	endangered	decreasing	habitat destruction, river regulation systems, pollution, exotic species	listed in the Annexes II and IV of the European Union Habitats Directive and in Appendix III of the Bern Convention		Collares-Pereira et al. 1999
IAL	critically endangered	decreasing	invasive species, fluctuating habitat conditions	no known conservation projects		Coelho, Mesquita & Collares-Pereira 2005
ILE	vulnerable	decreasing	pollution, habitat destruction, invasive species	listed in the Annex II of the European Union Habitats Directive and in the Appendix III of the Bern Convention		Lopes-Cunha et al. 2012
ILU	critically endangered	not evaluated	habitat destruction	no known conservation projects	pending re-evaluation	Collares-Pereira 1980; Coelho, Mesquita & Collares-Pereira 2005; Gil et al. 2010
IOL	critically endangered	decreasing	habitat destruction, exotic species, pollution	pilot ex-situ breeding program was initiated in July 2010	hybridization with ILU	Gante et al. 2010
AAR	vulnerable	decreasing	habitat destruction, predators, pollution	listed in the Annex II of the European Union Habitats Directive and in the Appendix III of the Bern Convention		Robalo et al. 2006
AOC	endangered	unknown	pollution, habitat destruction	no known conservation projects, pilot captive breeding studies lead to its reintroduction in Alcabrichel River		Gil et al. 2010
AOL	least concern	unknown	pollution, habitat destruction, fluctuating habitat conditions	listed in the Annex II of the European Union Habitats Directive and in the Appendix III of the Bern Convention	hybridization with PDU and PPO	Robalo et al. 2006; this dissertation
PDU	vulnerable	stable	habitat destruction, exotic species	listed in Annex II of the European Union Habitats Directive and Annex III of the Berne Convention as *C. polylepis*	hybridization with AOL	Aboim et al. 2010; this dissertation
PPO	least concern	stable	habitat destruction, exotic species	listed in Appendix II of the European Union Habitats Directive and in Appendix III of the Bern Convention	hybridization with AOL	Aboim et al. 2010 this dissertation
PWI	vulnerable	decreasing	habitat destruction, exotic species	listed in Appendix III of the Bern Convention and Annex II of the European Union Habitats Directive as C. toxostoma		Aboim et al. 2009
SAL	vulnerable	stable	exotic species, river regulation systems	listed in Annex II of the European Union Habitats Directive and in the Appendix III of the Bern Convention	diploid-allopolyploid species complex	Collares-Pereira et al. 2013
SAR	vulnerable	unknown	habitat destruction, drought, exotic species	none	hybridization with SAL	Sousa-Santos et al. 2009
SCA	least concern	unknown	exotic species, river regulation systems	none	hybridization with SAL	
SPY	not listed				hybridization with SAL	
STO	endangered	unknown	exotic species, river regulation systems	none		Magalhães et al. 2002

[a] August 2013

5.5. Management & Conservation Measures

Developmental and genetic explanations for variability in HYBRIDIZATION rates ignore the importance of behavioural decisions to mate, which play the major role in preventing HYBRIDIZATION between sympatric animals. The prevalence of HYBRIDIZATION most likely results from a mixture of behavioural, genetic, and developmental peculiarities of taxa, depending as well on the number of sympatric, closely related SPECIES (Mallet 2005).

The overall evolutionary relevance of natural HYBRIDIZATION is presently unquestionable even though the outcomes may be diverse and not always fully known, for which further investigation work is ever more necessary. It is the case of the HOMOPLOID SPECIES systems included in this work, for which research is still at an embryonic stage. Although HYBRIDIZATION is obviously conditioned by environmental and evolutionary constraints, human activities have the potential to increase its manifestation (see e.g. Scribner et al. 2001) causing formerly REPRODUCTIVELY ISOLATED taxa to come into contact and/or compelling reproductive activities of different SPECIES to smaller areas. HYBRIDIZATION is especially problematic for rare SPECIES; the harmful effects of HYBRIDIZATION, with or without INTROGRESSION, have led to the extinction of many populations and SPECIES (Allendorf et al. 2001). Most of the Iberian Leuciscinae are classified as vulnerable, endangered or critically endangered (**Table 5.2**) and therefore HYBRIDIZATION may have a negative influence on some of these SPECIES. Still, when it comes for the establishment of strategies for biodiversity conservation, HYBRIDIZATION creates additional complications for which there is no consensus plan.

Epifanio & Nielsen (2001) reviewed the significance of HYBRIDIZATION in aquatic systems, raising important questions worth of debate and global awareness regarding HYBRIDS' identification, their impact on natural populations, their ecological and evolutionary role, management and conservation strategies, and legal challenges posed by HYBRIDIZATION. To sum up, Allendorf et al. (2001) concluded that any policy dealing with HYBRIDS must be flexible and recognize that each situation is usually different enough so that general rules are not likely to be effective.

In these particular case-studies, we believe that the present battery of data used for HYBRIDS' identification is powerful enough to detect natural HYBRID SWARMS involving these SPECIES, but their ultimate impact on natural populations remains unknown, their ecological and evolutionary importance remain speculative, and current management/conservation policies only concern the parental SPECIES (also often misdiagnosed) (**Table 5.2**), with complete disregard of natural HYBRIDIZATION or natural HYBRIDS between them. Policy revision is recommended to set appropriate conservation

guidelines dealing with natural HYBRIDIZATION and INTROGRESSION (*sensu* genome invasion), taking into consideration both case scenarios where (1) hybrids may pose as a direct or indirect threat eventually resulting in local species' extinction, or (2) contribute to the generation of evolutionary novelties and ultimately new species.

5.6. References

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Chapter 6

Final Remarks

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Present work focused mainly on the genome organization of two pairs of hybridizing fish species (AOL/PDU and AOL/PPO) previously considered characterized by highly conservative KARYOTYPES, to help understanding KARYOTYPE differentiation at the subfamily level and to characterize genome dynamics and reorganization in their natural hybrids (AOLxPDU and AOLxPPO). This concluding Chapter summarizes the main achievements of this work and puts forward some new questions and future prospects on conceivable follow up investigation.

Overall, present results demonstrated that such KARYOTYPE uniformity within subfamily Leuciscinae remains restricted to the macrostructural level and suggested that the aforementioned homoploid hybrids are characterized by rapid genetic restructuring where TRANSPOSABLE ELEMENTS may play an important role. In particular, concerning the specific aims outlined for this research work:

- Molecular chromosome markers demonstrated that, despite the overall KARYOTYPE similarities in chromosome number, chromosome classes and consequently total number of chromosome arms prevalent across the subfamily Leuciscinae, genome organization is rather different between species as also seen in e.g. *Symphysodon sp.*. Such subliminal differentiation is most likely driven by intra-chromosomal rearrangements and/or translocations, often imperceptible but probably responsible for the small variations in number of chromosome pairs within each category (e.g. a KARYOTYPE formula of 6 m + 16 sm + 3 st/a chromosome pairs in AOL *versus* 7 m + 15 sm + 3 st/a chromosome pairs in PDU and PPO) and for rDNA copy number variation (5S rDNA varying from 4 clusters in PDU and PPO to 6-8 in AOL, and 45S rDNA varying from 3 clusters in PDU, to 3-4 in AOL and 4 in PPO). These results suggest that KARYOTYPE microstructure is expected to be as equally diverse as the variety of species within the family Cyprinidae.
- The set of selected molecular cytogenetic markers revealed adequate enough to distinguish between Iberian genera, between most congeneric species, and retrieving individuals with admixed ancestry from natural HYBRID SWARMS, proving even more powerful when used together with other sets of independent data like morphological and genetic markers. Although fish cytogenetics may be more laborious than genetic or morphological assessments, it demonstrated accurate and reliable in homoploid hybrid identification among highly BACKCROSSED individuals providing extra evidence for RECOMBINATION and INTROGRESSION.
- Chondrostomine homoploid hybrids were apparently characterized by rapid genome restructuring and RETROTRANSPOSON re-activation. Despite highly BACKCROSSED, genome rearrangements were still traceable, mainly by the presence of novel traits like the physical association between 5S and 45S rDNAs, a character state never registered in the parental forms. BACKCROSSING was found preferential with the AOL parent, denoted by all markers (genetic, cytogenetic and morphological) but

only sound supported by new cytogenetic data. Besides, although parental rDNAs did not seem to correlate with Rex3 RETROELEMENT suggesting other means of 5S rDNA propagation within *Achondrostoma* and *Iberochondrostoma* genera, transposed 45S rDNA clusters in the hybrids appeared associated with re-activated copies of Rex3, suggesting its involvement in at least part of the HYBRIDIZATION-mediated genome rearrangements.

- The Iberian Peninsula freshwaters seem a privileged setting for such (genetic, cytogenetic, morphologic, biological) diversity to occur, where the semiarid environment probably contributes for both its incidence and increased probability of fixation in distinct (isolated) populations. In fact, other Leuciscinae species from Mediterranean peninsulas and mainland Europe have also been associated with high levels of diversity, probably due to similar reasons. However, we recommend that natural HYBRIDIZATION involving those fish species should be more prudently considered in the equation of cytogenetic variability that has been documented.

In studies of hybridizing species or merely taxa PHYLOGEOGRAPHY, the integration of independent but complementary methodologies and disciplines like ecology, evolution and genomics is most anticipated. Increasingly integrative approaches should allow us to make more direct associations between POLYTYPISMS, POLYMORPHISMS, and/or the effects of HYBRIDIZATION and POLYPLOIDY on the genome, with the organism responses in its natural environment and hopefully helping to solve many unanswered questions.

In particular for the present case-study:

- It would be of great value to quantify the fertility of the hybrids as well as to examine patterns of chromosome pairing during meiosis (following up the work of Nabais et al. 2012, **Appendix IV**[2]). Adding the possibility of controlled breeding experiments and fry-rearing until cytogenetic analysis could be easily applied, would greatly enhance our knowledge on the rare F1 hybrids regarding interacting parental genomes, modes of inheritance, possible genomic instability and rearrangements;
- Further comparative genomic, cytogenomics, transcriptomic and epigenomic studies of hybrid animals is expected to deliver much progress in the near future. On one hand, understanding TE behaviour in hybrid and polyploid animals seems to be a very promising direction for future investigations. Many reasons have been advanced throughout this dissertation, but also because hybrids with normal KARYOTYPES and anomalous expression patterns have been associated with a

[2] Nabais C, Pereira C, Cuñado N, Collares-Pereira MJ. **2012**. Synaptonemal complexes in the hybridogenetic *Squalius alburnoides* fish complex: new insights on the gametogenesis of allopolyploids. *Cytogenet Genome Res* **138**: 31–35.

weakening in TEs' repression mechanisms. Similarly, small RNA molecules are increasingly emerging as major players in situations when different genomes need to be scanned against each other. Non-coding RNAs provide both fragility (by preserving chromosome breaks) as well as strength and flexibility to chromosomes, and thus may favour new speciation events;

- On grounds of cytogenomics alone, the use of refined probes like (1) BAC genomic or chromosome libraries, or even (2) BAC libraries from other organisms for which sequence data may be more readily available (e.g. the zebrafish *Danio rerio*, the Atlantic salmon *Salmo salar*, or the common carp *Cyprinus carpio*), would allow to quickly develop GENETIC MAPS otherwise difficult to obtain, since standard experimental crosses do not easily apply. (3) Libraries prepared from microdissected chromosomes would be very useful for chromosome mapping since chromosome-specific satellite DNAs, ESTs and eventually QTLs (and HYBRID rearrangements involving all of them) could be traced by FISH.

To sum up, these HYBRID systems have yet a lot to offer in terms of understanding genome plasticity and genome dynamics, as well as evolutionary mechanisms like INTROGRESSION, which are likely to promote ADAPTATION and SPECIATION processes.

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Appendixes

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Appendix I

Morphological characterization data

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The following table gathers morphologic and meristic data concerning each individual analysed throughout the present study, including both museum samples (MUNHAC, Lisbon) and newly sampled individuals, each identified by an individual ID code (lab collection code). Information regarding the origin (Douro, Vouga, Mondego, or Tejo river basins and one lab cross), the overall morphotype and sex is provided; when the morphotype is not typical, a question mark follows the species name. Meristic characters included standard length (SL), number of pored scales in the lateral line (LL), number of scales in the transverse row bellow the lateral line (BLL), number of scales in the transverse row above the lateral line (ALL), number of pharyngeal teeth (PT), number of gill rakers in the first gill arch (GR), number of soft rays in the dorsal fin (DR), and number of soft rays in the anal fin (AR). Morphologic indicators considered mainly mouth-related characters such as shape and position of the mouth and presence/absence of a horny lower lip. Reference values and more detailed information for all of these indicators can be found in **Chapter 1**. You may notice that not all individuals have records for all of the selected characters. This was because (1) many of the newly captured individuals were fin clipped for genetic purposes, delivered back to the river and further analysed via high resolution photograph; (2) others were in very bad state of preservation (lab collection); and (3) others were not fully processed mostly due to time constraints.

basin	ID code	morphotype	sex	meristic characters								mouth		
				SL	LL	BLL	ALL	PT	GR	DR	AR	shape	position	horny lip
Douro	ctm1	P.duriense	♀ ?	9.5	62	5	11		22	8	-	straight	ventral	present
	ctm2	P.duriense	♂ ?	10.4	66	5	11.5		20	8	8	straight	ventral	present
	ctm3	P.duriense	♂ ?	10.0	69	5	11		20	8	8	straight	ventral	present
	ctm4	P.duriense	♀	10.2	71	5	11		21	8	8	straight	ventral	present
	ctm5	P.duriense	♂		70	5	11			8	8	straight	ventral	present
	ctm6	P.duriense	♂ ?		62	5	11			8	8	straight	ventral	present
	ctm7	P.duriense	♂		64	4.5	10.5			8	8	straight	ventral	present
	ctm8	P.duriense	♂		65	5	11			8	8	straight	ventral	present
	ctm9	P.duriense	♀		66	5	11			8	8	straight	ventral	present
	ctm10	P.duriense	♀		68	5	11			8	8	straight	ventral	present
	ctm11	P.duriense	♀		67	5	11			7	7	straight	ventral	present
	cs1	P.duriense	♂?		67	6	11		20	8	-	straight	ventral	present
	cs2	P.duriense	?		65	6	10.5		20	8	8	straight	ventral	present
	cs3	A.oligolepis	♀		44	3.5	8		15?	7	7	elliptic	subterminal	incipient
	cs4	A.oligolepis	♂		42	3	6.5		13	8	8	elliptic	subterminal	incipient
	cs5	A.oligolepis	♂		39	3	6		12	7	6-7	elliptic	subterminal	absent
	cs8	P.duriense ?	?		55	4	9		-	-	-	elliptic	ventral	present
	cs12	A.oligolepis ?	?		57	3.5	10		-	8	-	elliptic	subterminal	present
	cs13	A.oligolepis	♂		39	3.5	6.5		15	7	7	arched	terminal	absent
	cs14	A.oligolepis	♀		39	3	7		13	7	7	arched	terminal	absent
	cs15	P.duriense	♀		66	4.5	11		19	8	7	straight	ventral	present
	cs16	P.duriense	♀		67	5.5	11.5		19	8	8	straight	ventral	present
	cs17	P.duriense	♂?		68	5.5	11			8	8	straight	ventral	present
	cs18	A.oligolepis	♂		38	3	6			7	8	arched	terminal	absent
	cs19	A.oligolepis	♂		38	3	7		12	7	7	elliptic	subterminal	absent
	cs20	A.oligolepis	♂		38	3	6.5		14	8	8	arched	terminal	absent
	cs21	A.oligolepis	♂		36	3	6			7	7	arched	terminal	absent
	cs22	A.oligolepis	♂		38	3	7			7	7	arched	terminal	absent
	cs23	A.oligolepis	♂		37	2	6			7	7	arched	terminal	absent
	cs24	A.oligolepis	♂		38	3	8		11 (inc)	7	7	arched	terminal	absent
	cs25	A.oligolepis	♀		36	3	6			8	8	arched	terminal	absent
	cc1	P.duriense	♀	8.6	70	5	11		21	8	8	straight	ventral	present
	cc2	P.duriense	♂	9.2	71	5	11.5		21	8	8	straight	ventral	present
	cc3	P.duriense	♂	10.5	72	5	11		23	8	8	straight	ventral	present
	cc4	P.duriense	♂	8.7	67	5	11		22	8	8	straight	ventral	present
	Côa 25 out 07	P.duriense	?	11.3	71	5.5	11		19	8	8	straight	ventral	present
	cc5	P.duriense	♀	9.09	67	5	13				8	straight	ventral	present

basin	ID code	morphotype	sex	meristic characters								mouth		
				SL	LL	BLL	ALL	PT	GR	DR	AR	shape	position	horny lip
Douro	cc7	P.duriense	♀	6.69	69	5	11.5				7	straight	ventral	present
	cc8	P.duriense	♂?	6.28		5.5	12			8	8	straight	ventral	present
	cc11	P.duriense	?	6.95	72	4.5	11			8	8	straight	ventral	present
	cc12	P.duriense	♂	5.95	71	5	13			8	8	straight	ventral	present
	cc13	P.duriense	♂?	6.1	67	5.5	12			8	8	straight	ventral	present
	zt1	A.oligolepis ?	?	6.0	38	2	8		13	6	7	arched	terminal	absent
	zt2	A.oligolepis ?	?	5.7	41	3	7		13	7	8	arched	terminal	absent
	zt3	A.oligolepis ?	?	6.1	38	2.5	7.5		20	6	6	elliptic	subterminal	absent
	zt4	A.oligolepis	?	5.5	37	2	7		13	6	6	arched	terminal	absent
	zt5	P.duriense	♂	9.0	74	5	12		22	8	8	straight	ventral	present
	zt6	P.duriense	♂	10.4	66	5.5	12		21	7	7	straight	ventral	present
	zt7	P.duriense	♂	14.0	71	6	12		21	8	7	straight	ventral	present
	zt8	P.duriense ?	♂	10.5	68	6	11		20	7	7	elliptic	ventral	present
	zt9	P.duriense ?	♀	8.2	65	4	11		20	8	7	elliptic	ventral	present
	zt10	P.duriense	♀	8.5	65	5	11		20	7	7	straight	ventral	present
	zt11	P.duriense	?	8.1	66	5	12		21	7	7	straight	ventral	present
	zt12	P.duriense ?	♀	5.3	55	4	11		17	7	7	elliptic	subterminal	present
	zt13	P.duriense ?	♂	8.0	69	5	11		20	7	7	straight	ventral	present
	zt14	P.duriense ?	?	7.3	62	5	10.5		21	7	7	straight	ventral	present
	zt15	A.oligolepis	♀	8.0	40	3	7		13	7	7	arched	terminal	absent
	zt16	P.duriense ?	?	7.8	65	4	10		22	7	7	elliptic	ventral	present
	zt17	A.oligolepis	♀	7.0	38	2.5	7		13	7	7	arched	terminal	absent
	zt18	A.oligolepis	♂	7.2	38	2	7		13	6	6	arched	terminal	absent
	zt19	A.oligolepis ?	♀	5.7	40	2.5	7		14	6	6	arched	terminal	absent
	zt20	A.oligolepis ?	♀	7.6	37	2.5	7		15	6	6	elliptic	subterminal	absent
	zt21	P.duriense ?	♀	10.0	65	5	11		21	7	7	elliptic	ventral	present
	zt22	P.duriense ?	♂	14.6	67	5.5	11		22	7	7	straight	ventral	present
	zt23	A.oligolepis	♂	6.2	36	3	7		14	7	7	arched	terminal	absent
	zt24	A.oligolepis	♂	7.3	40	3	7		12	7	7	elliptic	terminal	absent
	zt25	A.oligolepis	♂	7.1	39	3	7		13	7	7	arched	terminal	absent
	zt26	A.oligolepis	♂	6.6	40	2	6		13	6	6	arched	terminal	absent
	zt27	A.oligolepis	♂	6.4	39	2.5	7.5		12	6	6	arched	terminal	absent
	zt28	P.duriense	♀	13.1	66	5.5	12		22	7	7	elliptic	ventral	present
	zt31	A.oligolepis ?	♀	6.2	40	2	7		16	7	7	arched	terminal	absent
	zt32	P.duriense ?	♂	9.8	71	5	12		20	7	7	straight	ventral	present
	zt33	A.oligolepis	♀	6.4	36	2.5	7		12	7	7	arched	terminal	absent
	zt34	A.oligolepis	♂	5.7	35	3	7		13	7	7	arched	terminal	absent

basin	ID code	morphotype	sex	meristic characters								mouth		
				SL	LL	BLL	ALL	PT	GR	DR	AR	shape	position	horny lip
Douro	zt35	A.oligolepis	?	5.7	36	2.5	6.5		13	6	7	arched	terminal	absent
	zt36	A.oligolepis	♀	6.0	39	3	7		13	7	7	arched	terminal	absent
	zt37	A.oligolepis	?	6.0	36	3	6.5		13	7	7	arched	terminal	absent
	zt39	A.oligolepis	♂	5.7	38		7		12	6	6	arched	terminal	absent
	zt40	A.oligolepis	?	5.0	40	3	6		14	6	7	arched	terminal	absent
	zt41	A.oligolepis ?	?	5.9	51	4	9		17	7	8	elliptic	subterminal	incipient
	zt42	A.oligolepis	?	6.4	39	3	7		12	7	7	arched	terminal	absent
	zt43	A.oligolepis	?	7.0	36	3	7		13	7	8	arched	terminal	absent
	zp1	A.oligolepis	♀?	6.6	39	2	6.5		12	7	7	arched	terminal	absent
	zp2	A.oligolepis	♀?	6.4	35	2	6		12	7	6-7	arched	terminal	absent
	zp3	A.oligolepis	♀?	4.6	35	2	6		13	7	6-7	arched	terminal	absent
	zp4	A.oligolepis	♀?	5.3	36	2	7		13	7	7	arched	terminal	absent
	zp5	A.oligolepis	♀?	7.5	37	2.5	6		12	7	7	elliptic	terminal	absent
	zp6	A.oligolepis	♂?	6.0	36	5.5	7		11	7	6-7	arched	terminal	absent
	zp7	A.oligolepis	♂?	4.9	34	3	6.5		13	7	7	arched	terminal	absent
	zp8	A.oligolepis	♀?	5.0	36	2	6		12	7	7	arched	terminal	absent
	zp9	A.oligolepis	?	4.6	38	2.5	6		12	7		arched	terminal	absent
	zp10	P.duriense	♀?	9.3	71	5	12		21	8	8	straight	ventral	present
	zp11	P.duriense	♀?	9.6	66	5	11		20	7	7	straight	ventral	present
	zp12	P.duriense ?	♀?	9.2	67	5	11		19	8	8	straight	ventral	present
	zp13	A.oligolepis	♀?	8.6	38	3	7		13	7	7	arched	terminal	absent
	zp14	P.duriense	♀?	8.9	69	5	12		20	7	7	straight	ventral	present
	zp15	P.duriense ?	♂?	6.5	73	5	11		19	8	8	straight	ventral	present
	zp16	P.duriense ?	?		66	5-5.5	11			8	8	straight	ventral	present
	zp17	P.duriense	?	5.6	67	4	11			8	-	straight	ventral	present
Vouga	cv1	A.oligolepis	♀?	10.2	39	-	7	5/5	13	7	7	arched	terminal	absent
	cv2	P.duriense	♀?	10.1	64	-	11.5		21	7	7	straight	ventral	present
	cv3	A.oligolepis ?	♀?	6.5	36	-	-	5/5	12	7	7	arched	terminal	absent
	cv4	A.oligolepis ?	?	8.4	36	3	6.5	5/5	12	7	7	elliptic	subterminal	absent
	cv5	P.duriense	♂	10.9	67	-	12	5/5	24	8	-	straight	ventral	present
	cv6	A.oligolepis ?	♂	9.0	38	-	7.5	5/5	13	7	7	arched	terminal	absent
	cv7	A.oligolepis ?	?	6.5	39	2.5	7.5	5/5	15	7	7	elliptic	subterminal	incipient
	cv8	P.duriense	?	9.5	67	6.5	12.5	5/5	20	7	8	straight	ventral	present
	cv9	A.oligolepis ?	♂	10.4	52	-	9.5	5/5	16	8	-	elliptic	subterminal	present
	cv10	P.duriense ?	♀?	8.0	54	-	10.5	5/5	18	8	8	elliptic	subterminal	present
	cv11	A.oligolepis	?	7.0	37	-	7.5		14	7	7	arched	terminal	absent
	cv12	A.oligolepis	♂	6.1	34	-	7		13	7	7	arched	terminal	absent

basin	ID code	morphotype	sex	meristic characters								mouth		
				SL	LL	BLL	ALL	PT	GR	DR	AR	shape	position	horny lip
Vouga	cv13	P.duriense	♂	11.0	67	-	12		21	8	-	straight	ventral	present
	cv14	P.duriense ?	♂	8.7	49	-	9.5	5/5	16	7	8	elliptic	subterminal	present
	cv15	P.duriense ?	♂?	7.0	55	-	10	5/5	17			elliptic	subterminal	present
	cv16	A.oligolepis ?	♂?	6.8	39	-	8.5	5/5	15	7	7	arched	terminal	absent
	cv17	A.oligolepis	♂?	5.8	34	-	7		12	7	7	arched	terminal	absent
	cv18	A.oligolepis	♂	8.5	38	-	7	5/5	12	7	7	arched	terminal	absent
	cv19	P.duriense ?	♂	7.5	52	-	9-10	5/5	18	8	-	elliptic	subterminal	present
	cv20	A.oligolepis	?	6.1	36	-	6.5	5/4	12	7	7	arched	terminal	absent
	cv21	A.oligolepis ?	♂?	7.4	42	3	8	5/5	12	8	8	elliptic	terminal	incipient
	cv22	P.duriense	♂	8.0	60	-	11.5		21	7	7	straight	ventral	present
	cv23	P.duriense ?	?	6.6	57	-	12.5	5/5	19	8	8	straight	ventral	present
	cv24	A.oligolepis	?	6.2	37	2.5	7	5/5	14	7	7	arched	terminal	absent
	cv26	P.duriense ?	?	6.3	47	3.5	8.5	5/5	17	7	7	elliptic	subterminal	incipient
	cv27	A.oligolepis	?	5.9	33	2.5	6.5	5/5	14	7	7	arched	terminal	absent
	cv28	A.oligolepis	?	5.3	36	2.5	6.5	5/5	12	7	7	arched	terminal	absent
	cv29	A.oligolepis	?	4.8	35	-	6.5	5/5	14	7	7	arched	terminal	absent
	cv30	A.oligolepis	?	8.0	37	2.5	6.5	5/5	12	6	7	arched	terminal	absent
	cv31	A.oligolepis	♀	9.0	36	3	6.5	5/5	14	7	7	arched	terminal	absent
	cv32	P.duriense	♂	11.0	66	4.5	11.5	7/5	24	7	9	straight	ventral	present
	cv33	A.oligolepis	♂	8.5	37	3	7.5	5/5	14	7	7	arched	terminal	absent
	cv34	P.polylepis	?	7.6	66	4.5	11	6/5	22	6	6	straight	ventral	present
	cv35	A.oligolepis	♂?	5.1	35	2.5	7.5	5/5	11	8	8	arched	terminal	absent
	cv36	A.oligolepis	♀	7.6	35	2.5	6.5	5/5	12	7	8	arched	terminal	absent
	cv37	A.oligolepis	♀	8.2	37	2.5	6.5	5/5	12	7	6	arched	terminal	absent
	cv38	A.oligolepis	♀	8.7	36	2.5	6.5	5/5	11	7	7	elliptic	subterminal	absent
	cv39	A.oligolepis ?	♂?		39	2.5	7		14	7	7	arched	terminal	absent
	cv40	A.oligolepis	♂		39	2	6			7	7	arched	terminal	absent
	cv41	A.oligolepis	♂		35	2.5	7			8	8	arched	terminal	absent
	cv42	A.oligolepis	♂		38	3	7			7	7	arched	terminal	absent
	cv43	A.oligolepis ?	♂		41	2.5	7			7	7	arched	terminal	absent
	cv44	A.oligolepis	♂		37	3	6.5			7	7	arched	terminal	absent
	cv45	A.oligolepis	♂		35	2.5	7			7	7	arched	terminal	absent
	cv46	A.oligolepis	♂		35	2.5	6			7	8	arched	terminal	absent
	cv47	A.oligolepis	♂		34	2	6.5			7	7	arched	terminal	absent
	Vouga1	A.oligolepis		10.5	36	3	7	5/5	13	7	8	arched	terminal	absent
	Vouga2	A.oligolepis		8.4	34	2	6	5/5	11	7	6	elliptic	subterminal	absent

basin	ID code	morphotype	sex	meristic characters								mouth		
				SL	LL	BLL	ALL	PT	GR	DR	AR	shape	position	horny lip
Volga	cv48	A.oligolepis ?	♀	9.7	50	4	9			7	7			
	cv49	A.oligolepis ?	♂	9.0	54	4	9			8	9			
	cv50	A.oligolepis	♀	8.8	36	2	6.5			7	7			
	cv51	A.oligolepis	♂	7.5	37	2.5	7			8	8			
	cv52	A.oligolepis	♀	7.7	38	2	7			7	7			
	cv53	A.oligolepis	♀	9.3	40	2.5	7			7	7			
	cv54	A.oligolepis	♀	8.5	37	2	7			7	7			
	cv55	A.oligolepis	♀	8.3	37	3	7			7	7			
	cv56	A.oligolepis	-	6.6	36	4	7		11	7	7			
	cv57	A.oligolepis	-	7.1	34	3	6.5		12	7	7			
	cv58	A.oligolepis	-	7.0	35	3	7.5		12	7	7			
	cv59	A.oligolepis	-	6.6	37	3	7.8		-	7	7-8			
	cv60	P.polylepis		9.0	57	5	12		-	8	9	straight	ventral	present
	cv61	A.oligolepis	-	7.2	38	4	6.5			8	6-7			
	cv62	A.oligolepis	-	7.0	35	3	7			7	6			
	cv63	A.oligolepis	-	6.4	39	2.5	6		-	7	7			
	cv64	A.oligolepis	-	7.5	36	4	7			7	7			
	cv65	A.oligolepis	-	9.8	39	3	7		11	7	6-7			
	cv66	P.duriense ?		6.3	58	6	10			8	7-8			
	178	P.duriense ?		8.7	52	5.5	9	5/5	16	8	8	elliptic	ventral	present
	179	A.oligolepis		9.0	41	3.5	8	5/5	13	7	8	elliptic	subterminal	incipient
	180	A.oligolepis ?		7.1	40	3	7.5		16	7	7	arched		incipient
	181	A.oligolepis		7.0	33	2	7	5/4	12	7	8	elliptic	subterminal	incipient
	182	P.duriense ?		5.9	51	5	9	5/5	16	7	6	elliptic	ventral	present
	183	A.oligolepis		6.7	32	2.5	6.5		13	7	7	arched	terminal	absent
	184	A.oligolepis ?		4.6	-	9.5			-	8	7	straight		
	185	A.oligolepis ?		7.4	39	2.5	7.5		14	7	6	elliptic		
	186	A.oligolepis		6.5	40	3.5	7.5		12	7	6	elliptic		
	187	A.oligolepis ?		7.0	40	3	6.5	5/5	16	7	8	elliptic	subterminal	incipient
	188	A.oligolepis		5.0	-	-			12	7	9?	arched	terminal	absent
	189	P.duriense		12.0	71	5	11.5		21	7	7	straight	ventral	present
	190	P.duriense		19.8	75	6	12	-/5	-	8	8	straight	ventral	present
	192	P.duriense		17.5	74	5.5	12.5	5/5	23	-	7	straight	ventral	present
	193	P.duriense		17.0	72	6.5	13	-/5	23	8	8	straight	ventral	present
	194	P.duriense		18.0	70	6	12.5		19	7	7	straight	ventral	present
	195	P.duriense		23.0	72	7	11		22	7	7	straight	ventral	present

basin	ID code	morphotype	sex	meristic characters								mouth		
				SL	LL	BLL	ALL	PT	GR	DR	AR	shape	position	horny lip
Mondego	zd1	A.oligolepis		5.5	35	3	7	5/5	13	7	-	arched	terminal	absent
	zd2	A.oligolepis		7.3	33	2.5	5.5	5/5	13	7	-	arched	terminal	absent
	zd3	A.oligolepis		4.0	38		6.5	5/5	12	7	-	arched	terminal	absent
	zd4	A.oligolepis		6.5	33	2.5	6.5	5/5	10	7	-	arched	terminal	absent
	zd5	A.oligolepis		6.7	34	2.5	6.5	5/5	12	7	-			
	zd6	A.oligolepis		7.7	33	2.5	6.5	5/5	12	7	-	arched	terminal	absent
	zd7	A.oligolepis		5.0	33	2.5	6.5	5/5	12	7	-	arched	terminal	absent
	zd8	A.oligolepis ?		5.0	33		6.5	5/5	11		-	arched	terminal	absent
	zd9	A.oligolepis ?			35	3	6.5	5/5	11		-	arched	terminal	absent
	zd10	A.oligolepis ?		4.3	33		6.5	5/5	13	7	-	arched	terminal	absent
	zd11	A.oligolepis		4.6	33		6.5	5/5	12	7	-	arched	terminal	absent
	zd12	A.oligolepis		4.3	38	3	7	5/5	12		-	arched	terminal	absent
	zd13	A.oligolepis		4.9	37	2.5	6.5	5/5	13		-	arched	terminal	absent
	zd14	A.oligolepis		4.1	37	3	7	5/5	12		-	arched	terminal	absent
	zd15	A.oligolepis		4.1	35	3.0	7.0	5/5	12		-	arched	terminal	absent
	zd16	P.polylepis		11.5	70		11.5	6/5	22	8	-	straight	ventral	present
	zd17	P.polylepis		10.8	70		12.5	6/5	22	8	-	straight	ventral	present
	zd18	P.polylepis		11.0	67		11.5	6/5	23	8	-	straight	ventral	present
	zd19	P.polylepis		10.9	63		12.5	6/5	18	8	-	straight	ventral	present
	zd20	P.polylepis ?		8.8	55		12	5/5	17	7	-	elliptic	ventral	incipient
	zd21	P.polylepis		12.6	69		12.5			8	-	straight	ventral	present
	zd22	A.oligolepis		5.6	35		6.2	5/5	12		-	arched	terminal	absent
	zd23	P.polylepis		10.5	69		11.5	5/5	21	8-9	-	straight	ventral	present
	zd24	P.polylepis		10.9	62	-	11.5	6/5	18	8	-	straight	ventral	present
	zd25	P.polylepis		10.3	65	-	12.5	6/5	23	8	-	straight	ventral	present
	zd26	P.polylepis		9.9	71	5.5	12.5	6/5	23	8	-	straight	ventral	present
	zd27	P.polylepis		10.2	69	5.5	12.5	6/5	24	8	-	straight	ventral	present
	zd28	P.polylepis		9.5	66	5.5	11.5	6/5	-	8	-			
	zd29	P.polylepis		8.0	62		11.0	6/5	20	8	-	straight	ventral	present
	zd30	P.polylepis ?		9.6	62		11.5	6/5	23	8	-	straight	ventral	present
	zd31	P.polylepis		8.8	63	5-6	11.5	6/5	22	8	-	straight	ventral	present
	zd32	P.polylepis		9.0	60	5.5	12.5	6/5	21	8	-	straight	ventral	present
	zd33	P.polylepis		7.8	59	5.0	11.5	6/5	18	8	-	elliptic		present
	zd34	P.polylepis		8.0	60	5.0	11.5	6/5	19	8	-	straight	ventral	present
	zd35	P.polylepis		8.3	61	6.5	12.5	6/5	21	8	-	straight	ventral	present
	zd36	P.polylepis		7.9	59		11.5	6/5	18	8	-	straight	ventral	present
	zd37	A.oligolepis		7.2	38	2.5	6.5	5/5	-	7	-	arched	terminal	absent

basin	ID code	morphotype	sex	meristic characters								mouth		
				SL	LL	BLL	ALL	PT	GR	DR	AR	shape	position	horny lip
Mondego	zd38	A.oligolepis		6.0	35	2	6-7	4/5	12	7	-	arched	terminal	absent
	zd39	A.oligolepis		5.0	34	2.5	6.5	5/5	11	7	-			
	zd40	A.oligolepis		4.5	32	-	6.5	6/5	12	7	-			
	zd42	A.oligolepis		7.5	34	2.5	6.5	5/5	11	7	-	arched	terminal	absent
	zd43	A.oligolepis		7.4	32	2.5	6.5	5/4	12	7	-	arched	terminal	absent
	zd44	A.oligolepis		5.8	35	2.5	6.5	5/5	13	7	-	arched	terminal	absent
	zd45	A.oligolepis ?		7.0	38	2.5	6.5	5/5	13	7	-	arched	terminal	absent
	zd46	A.oligolepis		5.7	33	2	6.5	5/5	12	7	-	arched	terminal	absent
	zd47	A.oligolepis ?		6.3	38	2.5	6.5	5/5	13	7	-	arched	terminal	absent
	zd48	A.oligolepis		6.6	35	2.5	6.5	5/5	11	7	-	arched	terminal	absent
	zd49	A.oligolepis		5.8	32	-	6.5	5/5	-	7	-	elliptic		absent
	zd50	A.oligolepis		7.4	35	-	6.5	5/5	11	7	-	arched	terminal	absent
	zd51	A.oligolepis		6.3	34	-	6.5	5/5	12	7	-	arched	terminal	absent
	zd52	A.oligolepis		6.9	36	2.5	6.5	5/5	12	7	-	arched	terminal	absent
	zd53	A.oligolepis		7.0	34	2.5	6.5	5/5	12	7	-	arched	terminal	absent
	zd54	A.oligolepis		6.6	35	2.5	6.5	5/5	12	7	-	arched	terminal	absent
	zd55	A.oligolepis ?		5.6	34	2.5	6.5	5/5	11	7	-	arched	terminal	absent
	zd56	A.oligolepis ?		6.5	37	3	7	5/5	11	7	-	arched	terminal	absent
	zd57	A.oligolepis ?		5.4	36	-	6.5	5/5	12	7	-	arched	terminal	absent
	zd58	A.oligolepis		4.0	37	2.5	6.5	5/5	12	7	-	arched	terminal	absent
	zd59	A.oligolepis		5.2	35	2.5	6.5	5/5	-	7	-	arched	terminal	absent
	zd61	A.oligolepis ?		5.7	39	3	6			7	6	elliptic	subterminal	absent
	zd62	P.polylepis		13.4	68	5	11	5/5	26	7	8	straight	ventral	present
	zd76	A.oligolepis		7.7	35	2	7	5/5	12	6	6	arched	terminal	absent
	zd77	A.oligolepis		7.5	32	2	7	5/5	11	7	7	elliptic		absent
	Mondego 1	A.oligolepis		7.0	34	3	6	5/5	12			arched	terminal	absent
	Mondego 2	A.oligolepis		7.5	35	3	7	5/5	13	7	6	elliptic	subterminal	absent
	cm1	P. polylepis		9.8	68	5	11			7	9	elliptic		present
	cm2	P. polylepis		10.5	65	5.5	11.5			8	9	straight	ventral	present
	cm3	P. polylepis		11.0	65	5	12			7	9	straight	ventral	present
	cm5	P. polylepis ?		7.5	64	4	11	-	21	7	8	elliptic		present
	cm7	A.oligolepis		4.1	36	3	5?	-	11	6	7	arched	terminal	absent
	cm8	A.oligolepis		4.6	38	-	-	-	11	6	8	arched	terminal	absent
Tejo	ct1	P. polylepis	-	8.3	65	8	12	-	20	8	9	straight	terminal	present
	ct2	P. polylepis	-	17.0	68	6	12	-	23	8	9	straight	terminal	present
	ct3	P. polylepis	-	7.3	65	6	10	-	-	8-9	9	straight	terminal	present

basin	ID code	morphotype	sex	meristic characters								mouth		
				SL	LL	BLL	ALL	PT	GR	DR	AR	shape	position	horny lip
lab cross	cr1.2	F1 hybrid	♀?	4.7	53	4	9		15	7	7	elliptic	subterminal	incipient
	cr1.3	F1 hybrid		5.7	50	4	9		16	7	7	elliptic	subterminal	incipient
	cr1.4	F1 hybrid	♂		49	4.5	9		16	7	7	elliptic	subterminal	incipient
	cr1.10	F1 hybrid		7.56	48	4	8			7	7	elliptic	subterminal	incipient
	cr1.11	F1 hybrid		7.45	51	4	9			7	7	elliptic	subterminal	incipient
	cr1.12	F1 hybrid		9.51	52	4.5	8			7	8	elliptic	subterminal	incipient
	cr1.13	F1 hybrid	♀?	9.23	52	4.5	9		14	8	8	elliptic	subterminal	incipient

Legend

SL	standard size, from the tip of the snout to the caudal peduncle
LL	no. pored scales in the lateral line
BLL	no. scales in the transverse row bellow the lateral line, until the incision of the pelvic fin
ALL	no. scales in the transverse row above the lateral line, until the incision of the dorsal fin
PT	no. pharyngeal teeth, left/right sides
GR	no. gill rakers in the first gill arch
DR	no. soft rays in the dorsal fin
AR	no. soft rays in the anal fin

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Appendix II

Cytogenetic survey of species of two distinct genera of Iberian nases (Cyprinidae, Leuciscinae) that hybridize extensively in nature. I. Evidence of a similar and conserved chromosome pattern with some few species-specific markers at macro-structural level

Carla Sofia A. Pereira • Américo Neto • Maria João Collares-Pereira

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Cytogenetic survey of species of two distinct genera of Iberian nases (Cyprinidae, Leuciscinae) that hybridize extensively in nature. I. Evidence of a similar and conserved chromosome pattern with some few species-specific markers at macro-structural level

Carla Pereira · Américo Neto · Maria João Collares-Pereira

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Abstract *Pseudochondrostoma duriense* and *Pseudochondrostoma polylepis* hybridize extensively with *Achondrostoma oligolepis* in natural populations. In this first survey, karyotypes were comparatively analyzed by C-, AgNOR- and CMA_3-banding procedures in pure (non-introgressed) fish specimens. Leuciscinae pattern was evidenced in the three species: metacentrics and submetacentrics dominance, a big subtelo/acrocentric (marker) chromosome pair and a $2n = 50$; small macro-structural differences were observed. Heterochromatin was centromere-associated. Exceptions were found at *sm*1 and *st*/*a*1 long arms and at *m*1, *sm*3 and *sm*6 short arms. The *st*/*a*1 band was telomeric in the straight-mouth nases and sub-terminal in *A. oligolepis*. Multiple NORs of heterochromatic nature were found in *sm* pairs of the three species. Signals were telomeric except for one pair in *A. oligolepis*. Two to four structural and two functional NORs were found in *P. duriense* and *P. polylepis,* and four to six structural and four functional NORs in *A. oligolepis*. Species-specific markers will prove useful in hybrid zones' cytogenetic characterization and for in-depth studies of genome compatibility-related issues in future studies.

Keywords *Achondrostoma oligolepis* · *Pseudochondrostoma duriense* · *P. polylepis* · C/Ag/CMA_3-bandings · Multiple NORs

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Introduction

Iberian freshwaters are inhabited by several cyprinid species previously assigned to the genus *Chondrostoma* and recently allocated to new and distinct genera (Robalo et al. 2007). Three of them—*Pseudochondrostoma*, *Achondrostoma* and *Iberochondrostoma*—occur in Portugal, and were described to accommodate the straight-mouth nase species (*Pseudochondrostoma duriense*, *Pseudochondrostoma polylepis* and *Pseudochondrostoma willkommii*), and the two remaining genera, several arched-mouth species (see also Coelho et al. 2005).

Both sister-species *P. duriense* and *P. polylepis* extend their distribution ranges to the north-western and central-southern parts of the Peninsula, respectively (Aboim et al. 2009), and occur quite frequently in sympatry with *Achondrostoma oligolepis* (a Portuguese endemic inhabiting the drainages between Lima and Tejo) and *Achondrostoma arcasii* (an Iberian endemic confined to the northeast part of Portugal but extending from Galicia to the Mediterranean slope in Spain—Doadrio 2002).

Some natural intergeneric hybrids involving distinct species of these genera were firstly described based on morphological grounds (e.g., Collares-Pereira and Coelho 1983; Elvira et al. 1990). The former study was at Távora River (Douro drainage) and suggested the existence of recurrent backcrossing between *P. duriense* and *A. arcasii*, since some traits evidenced character displacement towards *A. arcasii*. This putative introgression was later revisited (Gante et al. 2004) using morphology, *mt*DNA (cytochrome *b*), allozymes and Nucleolus Organizer Region (NOR) phenotypes and allowed to evidence: (1) that *A. oligolepis* (=*C. macrolepidotum*) was the species involved in the interspecific crosses with *P. duriense*, instead of *A. arcasii*; and (2) that there were different levels of trait

introgression for nuclear traits but not mtDNA introgression, questioning either an equal fitness for males and females, or eventually non-random backcross mating.

However, because of the reduced sample size and the marginal levels of significance for some tests, Gante et al. (2004) results were considered only indicative of such preliminary observations. Thus, a more complete survey started on this (Douro) and other known hybrid zones (in Vouga and Mondego drainages) involving the same complex of species (*P. duriense* and *A. oligolepis* in Vouga, and *P. polylepis* and *A. oligolepis* in Mondego), using specimens with known genetic profile (mitochondrial and nuclear DNA analyses) to verify the putative existence of chromosome markers that would allow distinguishing non-introgressed (pure parentals) from introgressed specimens in such hybrid zones.

Indeed, fish cytogenetics is becoming very valuable in complementing distinct areas of research such as genomics, evolution and conservation (Pisano et al. 2007). Conversely to the several banding techniques used in higher vertebrates, longitudinal banding procedures have been rarely successful in fish chromosome studies mainly due to the lack of genome compartmentalization (Sumner 1990), and were replaced by banding techniques that detect local segments/markers, either the nucleolar organizing regions (NOR-banding) or the constitutive heterochromatin regions (C-banding), which are species-specific (e.g., Rábová et al. 2003; Sola et al. 2003; Bianco et al. 2004; Gromicho et al. 2006; Ueda 2007; Boron et al. 2009).

Therefore, the present work aimed to comparatively describe the karyotypes of *P. duriense* and *P. polylepis*, and to revisit the previous description of *A. oligolepis* based on standard Giemsa coloration (Collares-Pereira 1985), using now C-, $AgNO_3$- and CMA_3-banding procedures. Besides, willing to find potential species-specific markers, this study also intended to establish a baseline for the cytogenetic characterization of the existing and previously mentioned hybrid zones.

Materials and methods

Specimens were caught by electrofishing in Douro, Vouga (*P. duriense* and *A. oligolepis*) and Mondego (*P. polylepis* and *A. oligolepis*) drainages (Table 1), brought alive to the lab and kept under well aerated conditions till further analysis. Fish were sacrificed with an overdose of the anaesthetic MS222 (Sandoz) in accordance to the recommended ethic guidelines (ASAB 1998). Specimens' identity was confirmed by genetic profile assessment (*cyt* b and 10 microsatellites sequencing) in order to exclude any real or potential introgressed specimens (either at mitochondrial or nuclear level).

A total of 41 fish (21 *A. oligolepis*, 11 *P. duriense* and 10 *P. polylepis*) were used. Chromosomes were prepared from cephalic kidney and also occasionally from fibroblast fin culture (Rodrigues and Collares-Pereira 1996). To address the presence and localization of constitutive heterochromatin, C-banding followed the procedure of Sumner (1972) using DAPI staining. NORs' detection included the classical fluorescent staining with the GC-specific chromomycin A_3 (CMA_3) performed as described by Sola et al. (1992). Some of the metaphases were destained and sequentially examined in buffered Giemsa (4%, 7 min) and then Ag-stained using the method of Howell and Black (1980) with Gold and Ellison (1982) modifications. About 10–15 metaphases per specimen were screened in an Olympus BX60 equipped with a DP50 Olympus digital camera. NOR-phenotypes were established according to the number and location of CMA_3- and Ag-signals. Karyotypes were prepared using the software UTHSCSA Image Tool version 3.0 and CorelDraw Suite 12, by considering three morphological groups: metacentric (*m*), submetacentric (*sm*) and subtelocentric to acrocentric (*st/a*) chromosomes in a decreasing order of size according to arm ratio (Levan et al. 1964) after measuring in MicroMeasure version 3.3. To estimate the fundamental number (FN) value, *m* and *sm* chromosomes were scored as bi-armed and *st/a* as uni-armed.

Results

Chromosome counts for every specimen was invariably $2n = 50$ with a FN = 94. Karyotype similarities were remarkable and differences were mainly restricted to the number of metacentric (*m*) and submetacentric (*sm*) chromosome pairs. Three subtelocentric/acrocentric (*st/a*) chromosome pairs, including the largest chromosome of the set—the first *st/a* pair—were always present. The observed haploid formulas were 6*m*:16*sm*:3*st/a* for *A. oligolepis* and 7*m*:15*sm*:3*st/a* for both *P. duriense* and *P. polylepis* (Figs. 1, 2). A slight heteromorphism not linked to sex differentiation was found in some of the bigger chromosome pairs in the three species.

In general, constitutive heterochromatin was associated with centromeric and pericentromeric regions (Fig. 1a–c). Exceptions to this pattern were particularly found for the three species in *st/a*1 and *sm*1 chromosomes' long arms and in some *m* and *sm* chromosomes' short arms, namely *m*1, *sm*3 and *sm*6 pairs. The extent and/or the exact location of the *st/a*1 band differed between *A. oligolepis* and the straight-mouth nases being, respectively, sub-terminal and terminal. The *m*1 band appeared in *A. oligolepis*' individuals only.

Table 1 Origin and number of individuals used for each species' cytogenetic analysis, with the variation of NOR-phenotypes according with species and cytogenetic technique (CMA_3+ or Ag-staining)

Species	Drainage	No. fish	CMA_3 positive signals (%)										
			No. plates	1	2	3	4	5	6	7	8	9	10	11
A. oligolepis	Douro	7	44	2.27	6.82	22.73	36.36	29.55			2.27			
	Vouga	6	56				8.93	8.93	44.64	14.29	23.21			
	Mondego	8	17				23.53		41.18		17.65	11.76		5.88
P. duriense	Douro	8	169	5.33	36.09	26.63	19.53	2.96	9.47					
	Vouga	3	90			10.00	75.56	10.00	4.44					
P. polylepis	Mondego	10	26		3.85	15.38	76.92		3.85					
	Total	42	402											

Species	Drainage	No. fish	Ag positive signals (%)						
			No. plates	1	2	3	4	5	6
A. oligolepis	Douro	7	36	11.11	38.89	36.11	11.11	2.78	
	Vouga	6	12			16.67	83.33		
	Mondego	8	8	37.50	25.00		12.50		25.00
P. duriense	Douro	8	154	24.03	44.16	20.13	9.09	2.60	
	Vouga	3	10	10.00	90.00				
P. polylepis	Mondego	10	23	8.70	86.96	4.35			
Total	42	243							

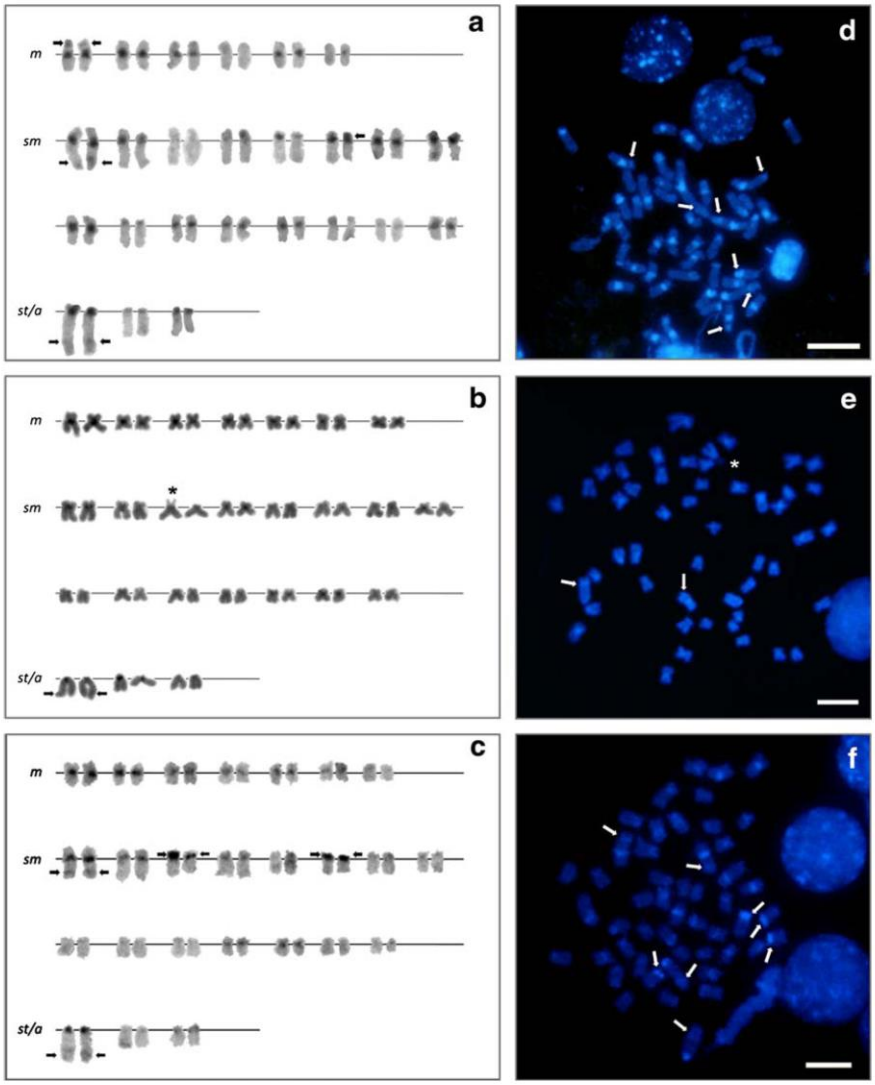

Fig. 1 C-banded karyotypes of *A. oligolepis* (**a**), *P. duriense* (**b**) and *P. polylepis* (**c**) specimens and corresponding DAPI-stained metaphases (**d–f**) evidencing distinct heterochromatic blocks (*arrows* ⇨). The *asterisk* (*) indicates a special feature found only in *P. duriense* individuals so far. *Scale bar* corresponds to 5 μm

NOR detection by CMA_3-staining evidenced more often four (Douro) to six (Vouga and Mondego) positive signals in *A. oligolepis* mostly corresponding to *sm*3, *sm*5 and *sm*6 chromosome pairs. All of the signals were distally located in the short arms of the chromosomes except for that in the 5th *sm* pair which was interstitial for all the studied populations (Fig. 2a). *P. duriense* presented two (Douro) to four (Vouga) CMA_3-positive signals located at the

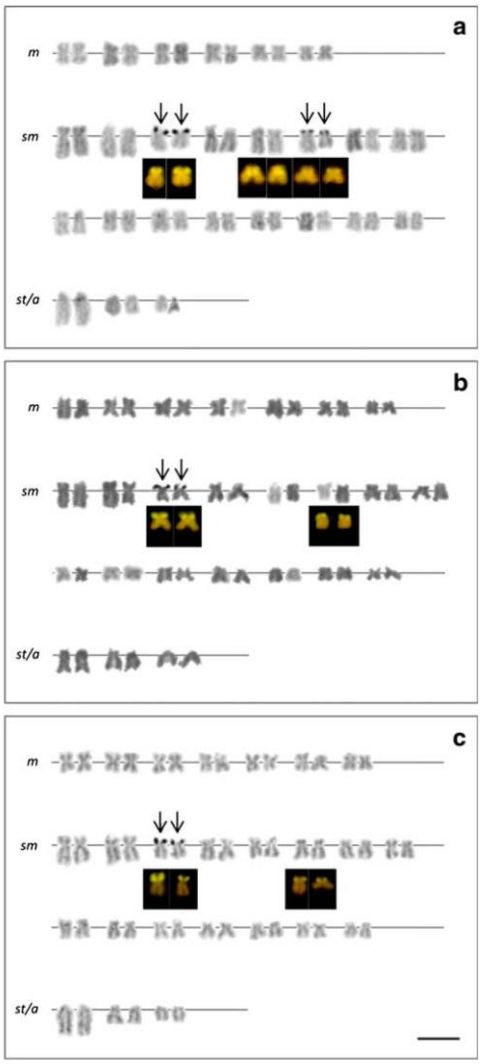

Fig. 2 Ag-banded karyotypes of *A. oligolepis* (**a**), *P. duriense* (**b**) and *P. polylepis* (**c**) specimens evidencing functional (Ag-stained; *arrows*) and structural (CMA_3-stained; *second row*) NORs. *Scale bar* corresponds to 5 μm

telomeres of the short arms of the 3rd and 6th *sm* chromosome pairs (Fig. 2b) and *P. polylepis* evidenced in general four signals with this same location (Fig. 2c).

Ag-staining revealed more frequently two pairs of functional terminal NORs for *A. oligolepis* (*sm*3 and *sm*6; Fig. 2a) whilst for *P. duriense* and *P. polylepis* there was generally just one pair (*sm*3; Fig. 2b, c, respectively).

Relevant inter-population differences could be observed for *A. oligolepis* and *P. duriense* evidencing distinct polymorphic NOR-phenotypes. In general, Douro's populations showed less CMA_3-positive signals than Vouga's populations (Table 1).

Inter-species variations were also evident especially between the arched- and the straight-mouth species. *A. oligolepis* individuals were in general more variable presenting from one to eleven CMA_3-marked chromosomes per metaphasic plate, while *P. duriense* and *P. polylepis* specimens ranged from one to six marked chromosomes. Regarding Ag-staining results, the variation range was identical between species ranging from one to six marked chromosomes (Table 1).

Discussion

The results obtained for the three species confirmed the nearly invariable leuciscine condition of $2n = 50$ chromosomes and characteristic karyotypes composed of approximately six to eight pairs of *m* and three to four pairs of *st/a* chromosomes (Klinkhardt et al. 1995; Ráb and Collares-Pereira 1995). The biggest pair of the complement was allocated to the *st/a* set of chromosomes, also typical of Eurasian Leuciscinae as firstly documented by Vasil'ev (1985) and repeatedly evidenced in other cyprinid studies (Ráb and Collares-Pereira 1995 and references therein; e.g., Bianco et al. 2004; Boron et al. 2009).

Revisiting the karyotype description of *A. oligolepis* by Collares-Pereira (1985) a distinct haploid formula was found, due to the relocation of one *m* and one *sm* chromosome pairs into the *sm* and *st/a* sets, respectively. The analysis of such small-size chromosomes is quite difficult (see review in Ráb and Collares-Pereira 1995) and the C-banding procedure allowed a more precise assignment of the centromeric regions. The three species share the characteristic of four medium-sized and two small-sized *m* chromosomes with the exception of an extra pair in the straight-mouth nases which seemed bigger than the rest of them in some cases (dependent of the chromosomes' image quality).

Chromosome (small) size and level of contraction made it difficult to address interspecies variability regarding the distribution patterns of constitutive heterochromatin. The quantitative and positional changes in constitutive heterochromatin observed by C-banding have been suggested to be amongst the most important speciation factors (e.g., Ueda 2007) and useful in cytotaxonomy of cyprinids (Ren et al. 1992). As expected, heterochromatin was found associated to centromeric and pericentromeric regions. The

observed exceptions associated to *sm* chromosomes seemed related to NOR genes' location revealing a heterochromatic nature of these regions in the analysed species. The putative marker block in the big *st/a* pair was shorter and sub-terminal in *A. oligolepis* whereas terminal in *P. duriense* and *P. polylepis*. This chromosome pair was recently proven to have a highly homologous and phylogenetically conserved distal region in a variety of Leuciscinae species (Ráb et al. 2008) and the present C-banding results suggest its AT-rich repetitive DNA character. The lighter short arm of *sm*3 chromosome pair in *P. duriense* (Fig. 1b, e) might be due to late-replicating DNA instead of euchromatin itself. Additionally, the *m*1 heterochromatic block may be also pointed out as a putative *A. oligolepis*-specific marker (Fig. 1a).

Even insufficiently analyzed in Eurasian taxa, one NOR-bearing chromosome pair (single pattern) is present in most studied leuciscins and has been considered a plesiomorphic character (revised by Ráb and Collares-Pereira 1995; Ráb et al. 2007). Despite the apparent constancy observed to date in the sub-family, multichromosomal locations (multiple NORs) have been also described in Eurasian and North-American species and considered a derived character. In European species they were only reported for *Iberochondrostoma lusitanicum* (Rodrigues and Collares-Pereira 1996; Collares-Pereira and Ráb 1999), *Iberochondrostoma almacai* (Monteiro et al. 2009), *Eupallasella perenurus* (Boron et al. 1997; Boron 2001), *Phoxinus phoxinus* (Boron 2001) and more recently for the female specimen of *Parachondrostoma arrigonis* (Kalous et al. 2008) and for *Leuciscus leuciscus* (Boron et al. 2009), as well as for the three now studied species *A. oligolepis*, *P. duriense* (see also Gante et al. 2004) and *P. polylepis*. Thus, the species of the new Iberian *Chondrostoma* genera defined by Robalo et al. (2007) although apparently retaining the common leuciscin karyotype pattern including an ubiquitous middle-size *sm* NOR-bearing pair of chromosomes, exhibit more differentiated NOR-phenotypes demanding an in-depth investigation with molecular tools (Ráb et al. 2007; Boron et al. 2009) in a wider range of congeneric taxa.

CMA_3- and Ag-signals were positively correlated and as expected, more structural (CMA_3-) than functional (Ag-positive) NORs were observed. Whether this is a consequence of GC-rich regions and/or inactive (silenced) NORs still need to be addressed by fluorescence in situ hybridization (FISH) with specific probes.

The performed analyses evidenced an identical co-localization of two pairs of structural NORs (*sm*3 and *sm*6) with one of them functionally active in the three species (*sm*3) and the other only active in *A. oligolepis* (*sm*6). Additionally, *A. oligolepis* specimens presented a pair of interstitial structural NOR (*sm*5). Gante et al. (2004) reported a double-NOR-phenotype in a single chromosome pair of *A. oligolepis* individuals suggesting the occurrence of inversion events. The present situation may represent a relic of that double-NOR condition reported by Gante et al. (2004) split into two pairs of chromosomes (*sm*5 and *sm*6) suggesting rearrangements by translocation. Such double-NORs were found in homozygosity in pure *A. oligolepis* specimens from a more southern drainage (Alcoa River, Gante and Collares-Pereira, unpublished data) and were described as a fixed population trait, similarly to what was observed in *I. lusitanicum* and interpreted as the result of a putative negative selection for the heterozygotic condition (Rodrigues and Collares-Pereira 1996; Collares-Pereira and Ráb 1999). Heterozygosity for the double-NOR condition also found by Gante et al. (2004) in *A. oligolepis*, *P. duriense* and hybrid specimens were tentatively explained as a consequence of past bidirectional introgression processes. Notwithstanding, Ueda (2007) considered that karyotypes may express chromosomal aberrations promoted during growth and cellular differentiation at early development stages that consequently may result in considerable and rapid chromosomal changes.

Despite the high inter-population polymorphism observed in the three studied species, in general, Douro individuals presented less structural NORs than Vouga's specimens which may suggest the existence of different selective forces acting in each basin. Even though, *A. oligolepis* presented the highest rate of variability (Table 1) which may result from ancient admixture events between populations as suggested for the double-NOR condition in Alcoa River specimens (Gante et al. 2004).

Together with inter- and intra-population polymorphisms in number and size of NORs, some intra-individual variations were observed. This would not be expected unless independent structural changes occurred from cell to cell (see Galetti et al. 1995).

To conclude, knowing in advance the genetic profiles of all specimens through the analysis of several nuclear and *mt*DNA markers was highly important for this baseline cytogenetic study. It allowed finding that, although both straight-mouth nases have a quite similar karyotype at a macro-structural level, it is slightly distinct from the other parental species of hybrid zones (*A. oligolepis*). Thus, the species-specific chromosome markers now obtained may be used for depicting non-pure (introgressed) specimens in population surveys. Looking for further insights into such hybrid karyotypes by the application of molecular techniques with a much higher resolution power (e.g., GISH) might allow defining the ongoing inter-genome reshaping mechanisms inside hybrid zones. Moreover, understanding such genome dynamics in introgressed versus non-introgressed fish by moving towards the joint utilization of phylogenetic, genetic, cytogenetic and spatial data is expected to contribute to identify evolutionary diversification mechanisms that

favor the broadening of intraspecific polymorphisms and the evolution of phenotypic plasticity in ecological traits.

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Appendix III

Chromosome studies of European cyprinid fishes: interspecific homology of leuciscine cytotaxonomic marker – the largest subtelocentric chromosome pair as revealed by cross-species painting

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Chromosome studies of European cyprinid fishes: interspecific homology of leuciscine cytotaxonomic marker—the largest subtelocentric chromosome pair as revealed by cross-species painting

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Key words: chromosome painting, fish cytogenetics, karyotype uniformity, leuciscine cyprinids

Abstract

Leuciscine cyprinids possess a nearly invariant diploid number (2n=50) with an extremely uniform karyotype comprising of 8 pairs of metacentric, 13–15 pairs of submetacentric and 2–4 pairs of subtelocentric (st) to acrocentric (a) chromosomes. The largest pair is characteristically an st/a element—the 'leuciscine' cytotaxonomic marker. Previously, the interspecific homology of this chromosome pair could not be assessed owing to the inability to produce euchromatic or serial banding patterns. In the present study, we used laser-microdissection (15–20 copies of the marker chromosome) to construct a whole chromosome probe (WCP) from the marker chromosome of the roach *Rutilus rutilus* to ascertain the interspecific homology of marker chromosomes by cross-species in-situ hybridization. WCP was hybridized to chromosomes of widely distributed (*Abramis brama*, *Alburnoides bipunctatus*, *Alburnus alburnus*, *Aspius aspius*, *Ballerus ballerus*, *B. sapa*, *Blicca bjoerkna*, *Chondrostoma nasus*, *Leucaspius delineatus*, *Leuciscus leuciscus*, *L. idus*, *R. rutilus*, *Scardinius erythrophthalmus*, *Squalius cephalus*, *and Vimba vimba*) and Iberian endemic species (*Achondrostoma oligolepis*, *Iberochondrostoma almacai*, *I. lusitanicum*, *Pseudochondrostoma duriense*, *S. alburnoides and S. pyrenaicus*). Cross-species in-situ hybridization to chromosomes of *Phoxinus phoxinus*, a representative of leuciscine sister lineage, showed the same pattern as in all of the leuciscins. The probe consistently hybridized to the distal part of the short arm of the marker chromosome, indicating sequence homology.

Abbreviations

DAPI	4′, 6-diamidino-2-phenolindole	G-, R-, Q- bands	Giemsa-stained euchromatic bands, reverse-Giemsa bands, fluorescent bands with quinacrine
dNTP	deoxyribonucleotide triphosphate	IAPG	Institute of Animal Physiology and Genetics
dUTP	deoxyuridine triphosphate		
DOP-PCR	degenerate oligoprimers polymerase chain reaction	m, sm, st, a chromosomes	metacentric, submetacentric, subtelocentric, acrocentric chromosomes
FISH	fluorescence in-situ hybridization		

NOR	nucleolar organizer region
PALM	photoactivated localization microscopy
SSC	standard sodium citrate
Tris	2-amino-2-(hydroxymethyl)-1, 3-propanediol
UL-FC	University of Lisboa – Faculty of Sciences
WCP	whole-chromosome probe
Zoo-FISH	cross-species chromosome fluorescent in-situ hybridization

Introduction

Unlike the chromosomes of homoiothermic vertebrates, the chromosomes of ectothermic vertebrates generally do not routinely show euchromatic, serial banding patterns (G-, R-, Q- bands) after treatment with G-banding methods (using trypsin digestion as described by Seabright 1971; incubation in hot 2× SSC according to Sumner *et al.* 1971) or staining with base-specific fluorochromes (Medrano *et al.* 1988, Sumner 1990). Thus, banding procedures developed to investigate chromosomes of higher vertebrates (especially birds and mammals) are not or are only rarely reproducible with fish chromosomes (e.g. Blaxhall 1983, Gold *et al.* 1990, Yu *et al.* 1994). The structural basis of euchromatic or serial banding patterns is associated with the compartmentalization of the genome of higher vertebrates into AT-rich and GC-rich isochores, which is generally not present in the chromosomes of fishes, most amphibians and reptiles (Medrano *et al.* 1988, Schmid & Guttenbach 1988, Schmid *et al.* 1990, Sumner 1990). As a result, the ability to identify chromosomal homologies (e.g. Dobigny *et al.* 2004) and reconstruct the chromosomal evolution in fishes using interspecific homology of chromosome, chromatid and/or chromosomal segments is hampered. However, the ability to microdissect indvidual, correctly identified chromosomes and to prepare whole chromosome probes (WCP) (e.g. Kubíčková *et al.* 2002) combined with in-situ hybridization (chromosome painting, Zoo-FISH) (Rens *et al.* 2006) can overcome the banding difficulties, allowing for studies of interspecific chromosomal homologies in fishes.

Chromosome painting of fish chromosomes has been used only rarely. Fujiwara *et al.* (1997) studied chromosome elimination in embryogenesis of salmonid hybrids. Zhu *et al.* (2006) used FISH to confirm ploidy levels in diploid and triploid crucian carp. FISH identifed interspecific varaibility of NOR-bearing chromosomes in gymnotiform electric fishes (Nagamachi *et al.* 2007) and sex chromosomes and their possible (non)homologies in salmonids (Reed *et al.* 1995, Phillips *et al.* 2001, 2007). To the best of our knowledge, however, no other study has used WCP for cross-species in-situ hybridization to examine inter-specific homologies in fish chromosomes.

Representatives of nearly all leuciscine genera examined to date (e.g. *Abramis*, *Achondrostoma*, *Alburnoides*, *Alburnus*, *Anaecypris*, *Aspius*, *Ballerus*, *Blicca*, *Chondrostoma*, *Delminichthys*, *Iberochondrostoma*, *Leucaspius*, *Leuciscus*, *Pachychilon*, *Parachondrostoma*, *Petroleuciscus*, *Phoxinellus*, *Pseudaspius*, *Pseudochondrostoma*, *Rutilus*, *Scardinius*, *Squalius*, *Telestes*, *Tribolodon*, *Vimba*; Ráb & Collares-Pereira 1995, Bianco *et al.* 2004 and references therein) possess a nearly invariable diploid chromosome number (2n=50) with extremely uniform karyotypes comprised of 8 pairs of metacentric (m), 13–15 pairs of submetacentric (sm) and 2–4 pairs of subtelocentric (st) to acrocentric (a) chromosomes. The largest pair of their complement is characteristically st/a element–the 'leuciscine' cytotaxonomic marker. Presence of this marker was noted by previous researchers who pioneered the descriptive cytogenetics of cyprinids (Vasil'ev 1985).

As a continuation of our cytogenetic studies of leuciscine cyprinid fishes (e.g. Rábová *et al.* 2003, Gromicho *et al.* 2006 and references therein), we used laser-microdissection to produce a WCP for the 'leuciscine' marker chromosome of the roach, *Rutilus rutilus*. Cross-species in-situ hybridization was used to examine chromosomes of (1) 14 widely distributed European species, (2) 6 Iberian endemic species and (3) brook minnow, *Phoxinus phoxinus*, a representative of sister lineage of Leuciscinae (Cunha *et al.* 2002), to identify interspecific homologies.

Material and methods

Chromosome preparations

The number, sex and location of specimens analysed are shown in Table 1. The specimens examined were not deposited as vouchers. Standard direct procedures for chromosome preparation from cephalic kidney or fin fibroblast culture (Gromicho *et al.*

Painting with leuciscine cytotaxonomic chromosome marker

Table 1. The number, sex and location of specimens analyzed

Species	Location	Number and sex of analysed specimens	Figure
Abramis brama	Dyje R., Danube basin	1 ♀	2a
Blicca bjoerkna	Dyje R., Dabube basin	3 ♂	1, 2b
Ballerus ballerus	Dyje R., Danube basin	3 ♂	2c
B. sapa	Dyje R., Danube basin	5 ♂	2d
Vimba vimba	Vltava R., Elbe basin	3 ♀	2e
Alburnus alburnus	Elbe R.	1 ♂	2f
Alburnoides bipunctatus	Laborec R., Tisza basin	1 ♀	2g
Rutilus rutilus	Sázava R., Elbe basin	5 ♂	2h
Scardinius erythrophthalmus	Dyje R. Danube basin	10 juveniles	2i
Aspius aspius	Elbe R.	1 juvenile	2j
Leucaspius delineatus	Sázava R., Elbe basin	3 ♂ , 4 ♀	2k
Chondrostoma nasus	Laborec R., Tisza basin	2 juveniles	2l
Achondrostoma oligolepis	Ceira R., Mondego basin	1 ♀	2m
Iberochondrostoma almacai	Monchique R., Arade basin	1 juvenile	2n
Iberochondrostoma lusitanicum	Maior R., Tejo basin	1 juvenile	2o
Pseudochondrostoma duriense	Caima R., Vouga basin	1 ♂	2p
Leuciscus idus	Elbe R.	2 ♂	2q
L. leuciscus	Sázava R., Elbe basin	1 ♀	2r
Squalius cephalus	Sázava R., Elbe basin	3 ♂	2s
S. pyrenaicus	Ardila R., Guadiana basin	1 juvenile	2t
S. alburnoides	Ardila R., Guadiana basin	1 ♂	2u
Phoxinus phoxinus	Vltava R., Elbe basin	1 ♂	2v

2006) were used. 'Valid Animal Use Protocols' were in force at IAPG and UL-FC during this study.

The isolation of metaphase marker chromosome

The protocol for laser microdissection using the PALM MicroLaser system followed exactly that described in detail by Kubíčková *et al.* (2002). Briefly, the membrane was cut around the easily identifiable marker chromosome (Figure 1) from metaphase plates of common roach, *R. rutilus* using a laser microbeam with the minimum energy possible. The microdissected chromosome was catapulted directly into the cap of a PCR tube containing 2 µl PCR oil. Between 15 and 20 copies of the marker chromosome were dissolved in 20 µl 10 mmol/L Tris-HCl, pH 8.8. After closing the tube with a cap containing copies of the marker chromosome, the sample was spun down by centrifugation.

Preparation of probe

Initial DOP-PCR of microdissected chromosomes was performed in a thermal cycler in a reaction mixture composed of 60 mmol/L Tris-HCl, pH 8.8, 15 mmol/L $(NH_4)_2SO_4$, 3.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 1.6 µmol/L of primer 5′-CCG ACT CGA GNN NNN NAT GTG G- 3′ (Telenius *et al.* 1992), 0.05% W-1, and 2 U Taq polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) in a final volume of 40 µl.

The probe was labelled in a second DOP-PCR reaction using SpectrumOrange-dUTP (Vysis, Richmond, UK) in a 20 µl (final volume) mixture containing 2 µl of primary PCR product as template, 50 mmol/L Tris-HCl, pH 9.3, 15 mmol/L $(NH_4)_2SO_4$, 2.25 mmol/L $MgCl_2$, 0.1% Tween, 1 U LA polymerases Mix (Top-Bio, Czech Republic), 1.6 µmol/L of primer 5′-CCG ACT CGA GNN NNN NAT GTG G- 3′, 0.2 mmol/L of each dNTP and 0.02 mml/L SpectrumOrange-dUTP. The PCR conditions for both primary DOP-PCR and DOP-labelling-PCR were as described by Kubíčková *et al.* (2002).

Fluorescence in-situ hybridization

Hybridization mixture (10 µl final volume) was composed of 0.8 µl labelled probe, 50% formamide in 2× SSC, 10% dextran sulfate, 7 µg salmon sperm DNA and 1.5 µg competitor DNA. The hybridization mixture was denatured at 72°C for 10 min and then reannealed at 37°C for 80 min. Slides were denatured

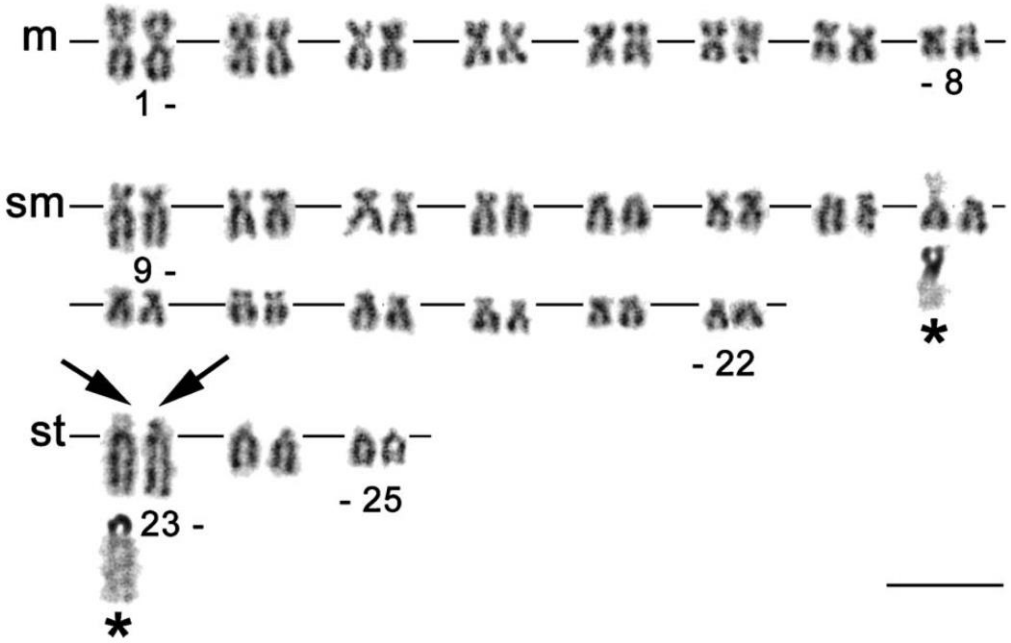

Figure 1. Karyotype of male silver bream, *Blicca bjoerkna*, arranged from Giemsa-stained chromosomes showing the general pattern of leuciscine karyotypes including the marker st chromosome (no. 23, arrows). Included are sequentially Ag-stained chromosomes (asterisks) documenting rare translocation of NOR sites from the standard NOR-bearing, medium-sized sm chromosome to the terminal position in the short arms of the marker chromosome: Bar represents 5 μm.

in 70% formamide in 2× SSC, pH 7.0, for 2 min and then dehydrated in an EtOH series. The hybridization reaction was performed overnight in a moist chamber at 37°C. Posthybridization washes were done twice in 50% formamide in 2× SSC, pH 7.0 at 42°C for 5 min, then in 0.1× SSC, pH 7.0 at 42°C for 5 min, and finally in Tris–NaCl–Tween 20 buffer at 42°C for 5 min. Slides were counterstained with DAPI (4′, 6-diamidino-2-phenolindole) in mounting medium (Cambio, UK) and then covered with a cover slide and sealed using nail polish.

Microscopy and image processing

Preparations were observed with an AX70 (Olympus) microscope equipped with standard fluorescence filter sets. Gray-scale hybridization signals on chromosomes and/or DAPI counterstained chromosomes were recorded by a cooled digital DP30VW Olympus camera separately using Ikaros software (Metasystems, Germany). Digital images were then pseudo-coloured (blue for DAPI, red for SpectrumOrange), superimposed, and processed with Adobe Photoshop, version 10. The chromosomes were classified using the nomenclature proposed by Levan *et al.* (1964).

Results

The karyotype uniformity of leuciscine cyprinid fishes is demonstrated in Figure 1, where the karyotype of silver bream (*Blicca bjoerkna*, the same individual as used for FISH with WCP, Figure 2b) is arranged from Giemsa-stained chromosomes. This karytope indisputably shows the leuciscine marker chromosome—the large pair of st chromosomes (no. 23). Moreover, this specimen is heterozygous for a unique translocation of the nucleolar organizer regions (NORs), from one NOR-bearing, medium-sized sm homologue to a terminal position in the

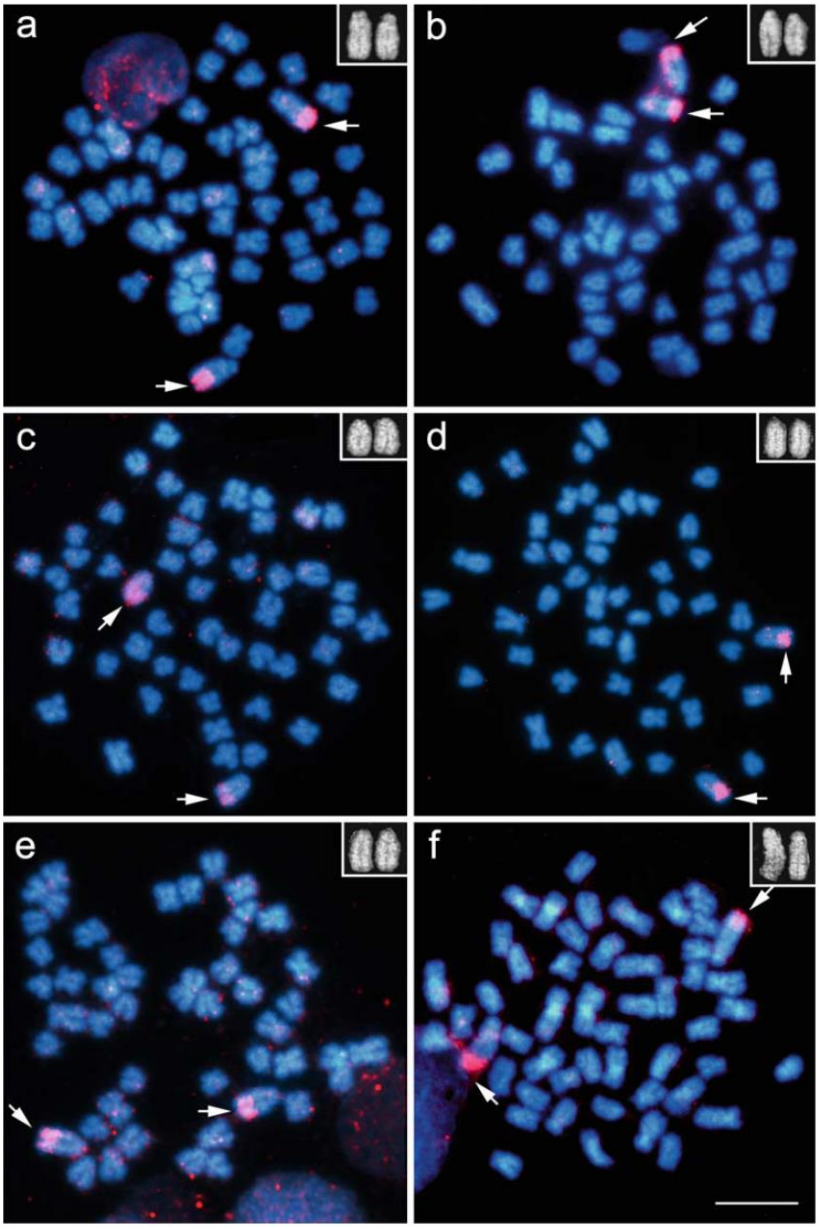

Figure 2. Cross-species chromosome painting with roach WCP of the chromosomes of *Abramis brama* **(a)**, *Blicca bjoerkna* **(b)**, *Ballerus ballerus* **(c)**, *B. sapa* **(d)**, *Vimba vimba* **(e)**, *Alburnus alburnus* **(f)**, *Alburnoides bipuctatus* **(g)**, *Rutilus rutilus* **(h)**, *Scardinius erythrophthalmus* **(i)**, *Aspius aspius* **(j)**, *Leucaspius delineatus* **(k)**, *Chondrostoma nasus* **(l)**, *Achondrostoma oligolepis* **(m)**, *Iberochondrostoma almacai* **(n)**, *I. lusitanicum* **(o)**, *Pseudochondrostoma duriense* **(p)**, *Leuciscus idus* **(q)**, *L. leuciscus* **(r)**, *Squalius cephalus* **(s)**, *S. pyrenaicus* **(t)**, *S. alburnoides* **(u)** and *Phoxinus phoxinus* **(v)**. Positive hybridization signals are shown by arrows; DAPI-counterstained marker chromosome from each metaphase is shown in inset (upper right) in each species. Bar represents 5 μm.

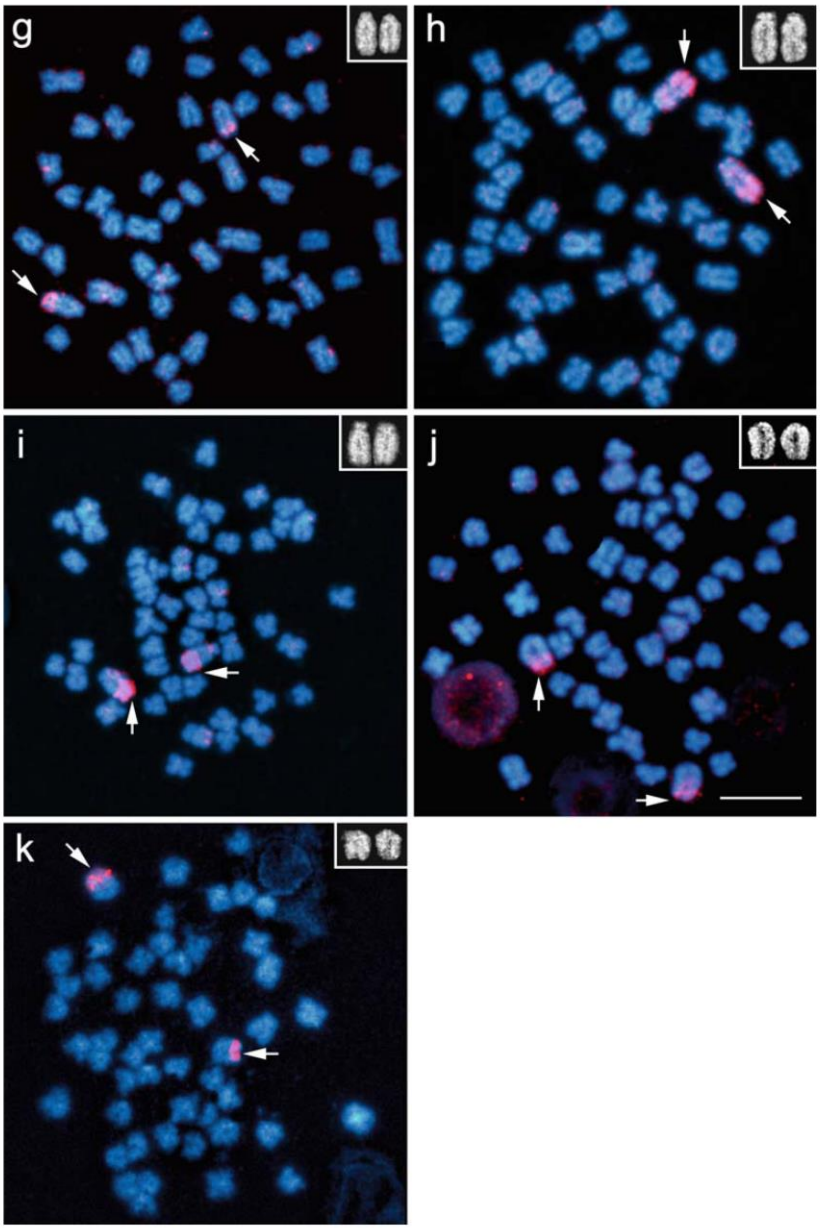

Figure 2. (Continued).

Painting with leuciscine cytotaxonomic chromosome marker

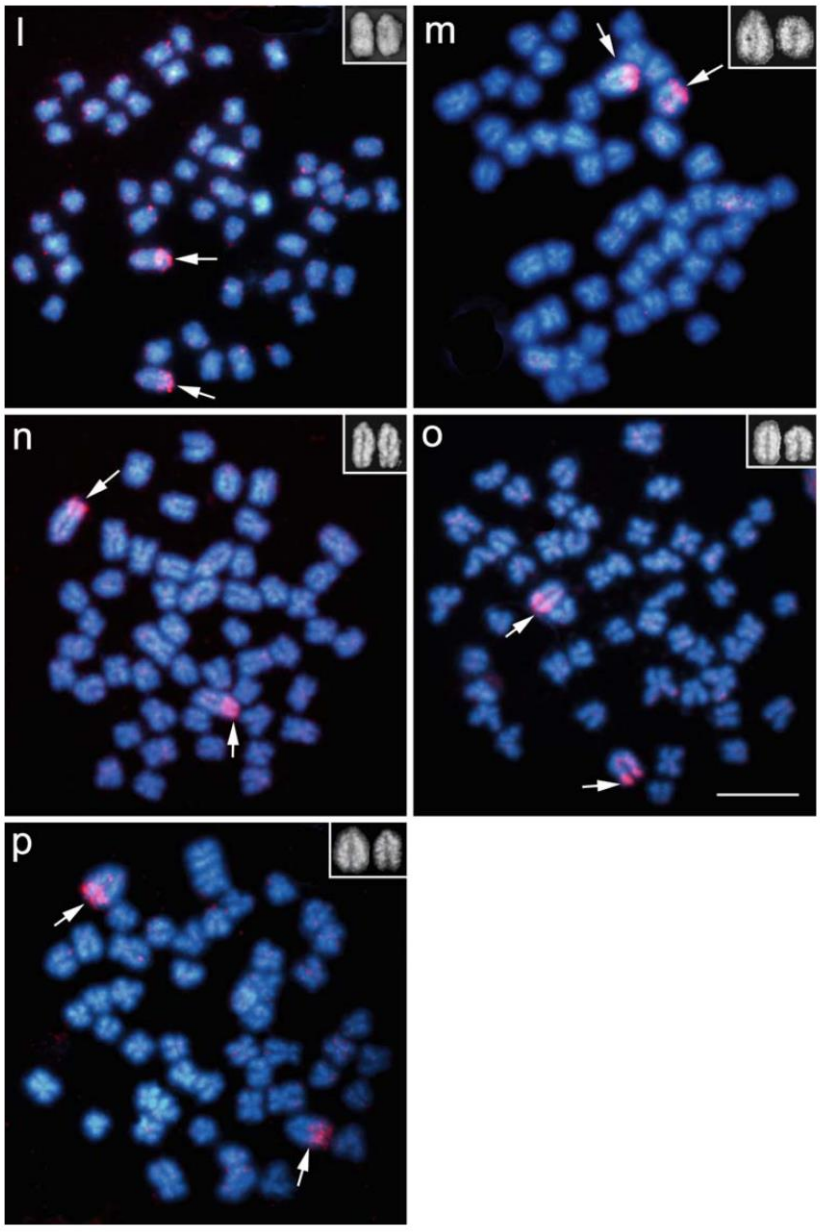

Figure 2. (Continued).

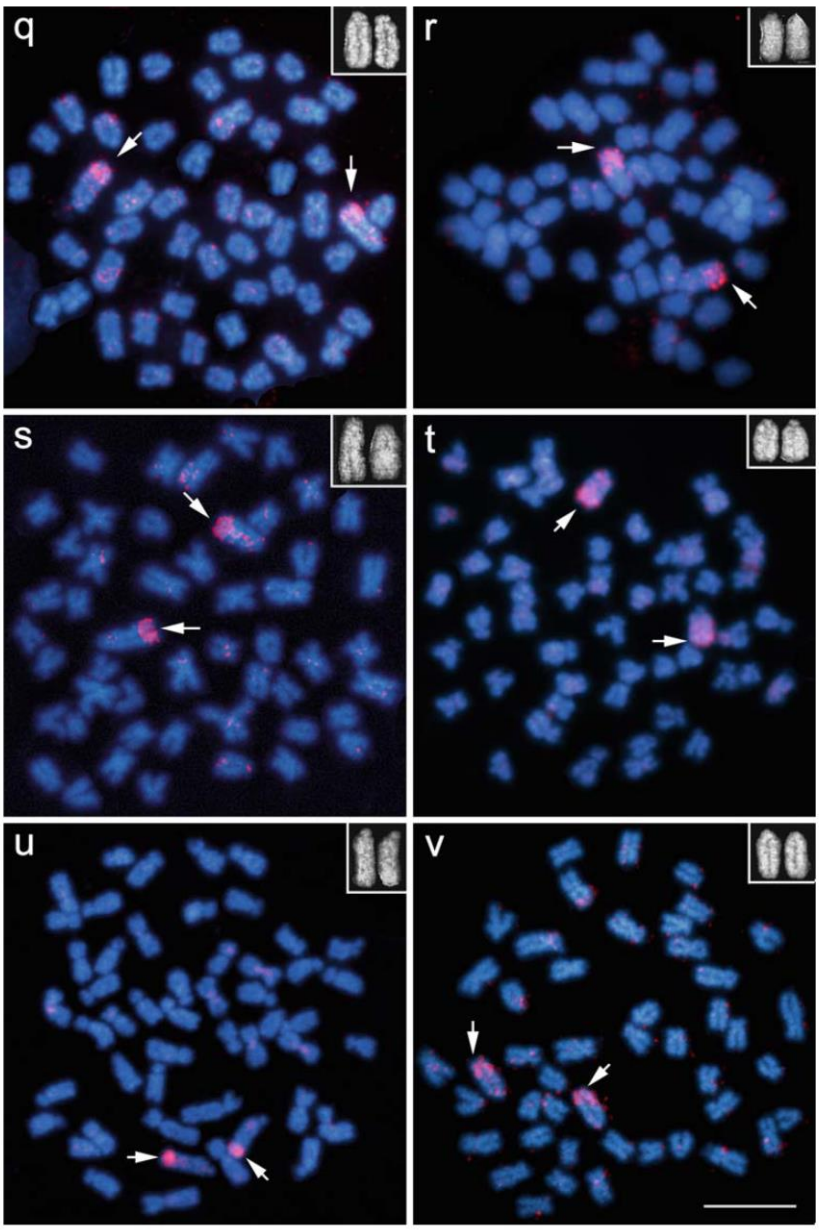

Figure 2. (Continued).

short arms of the st marker chromosome. The presence of a medium-sized sm NOR-bearing chromosomal pair is ubiquitous among cytogenetically analysed leuciscine species.

The DAPI-stained marker st chromosomal pair from each analysed species is shown in insets in Figures 2a–v. Hybridization of WCP to the chromosomes of 20 leuciscine species demonstrated nearly the same pattern in each case (Figures 2a–t). The WCP hybridized to the distal third of the long arm of the marker chromosome pair. Hybridization signals were not detected in any other chromosomes.

Owing to the small size of the marker chromosome, it was nearly impossible to quantify interspecific differences. However, closer inspection revealed some qualitative differences. In several species (*A. brama* (Figure 2a), *B. bjoerkna* (2b), *R. rutilus* (2h), *S. erythrophthalmus* (2i), *I. lusitanicum* (2o), *L. idus* (2q), *S. pyrenaicus* (2t)), very weak hybridization signal was present close to the pericentromeric region. In all species, the size of main hybridization signal was approximately equal in size except in the spirlin, *A. alburnoides* (Figure 2g) where one homologue displayed a distinctly smaller hybridized region. Interestingly, the same hybridization pattern (distal third of longer arm of the largest st chromosome pair) was observed in the chromosomes of brook minnow *P. phoxinus*, a representative of sister lineage of Leuciscinae.

Discussion

In the present study we tested the applicability of laser microdissection and cross-species hybridization for analysis of small fish chromosomes. Cyprinid fishes are characterized by the presence of relatively small chromosomes with their centromere positions ranging gradually from median to nearly terminal, making it difficult to assign some chromosomes to particular chromosomal categories and thus making correct identification of individual chromosomes nearly impossible (Ráb & Collares-Pereira 1995). The exceptions are the NOR-bearing elements and distinct marker chromosomes. Such a marker chromosome pair is present in the karyotypes of all leuciscine species. We chose this chromosome for laser microdissection because it can easily be identified in the metaphases, it provides a sufficient amount of DNA, and positive hybridization of the target chromosome with the WCP is very easy to detect. Chromosome preparations from the roach *R. rutilus* were used for production of the probe because of the ease of manipulating these fishes to stimulate higher mitotic activity, resulting in the preparation of high-quality chromosomes for laser microdissection. Zoo FISH experiments attempted to sample the existing leuciscine diversity across phylogenetic lineages (Zardoya & Doadrio 1999) and specifically to include both widely distributed and endemic species. Positive hybridization signal was detected in the distal third of the marker chromosome in all representatives of leuciscins and one representative of phoxinins, with practically no detectable interspecific differences. The fact that the WCP derived from *R. rutilus* hybridized only to the distal third of the marker chromosome in this and the related species may indicate that not all of the dissected chromosomal DNA was amplified during the first round of DOP-PCR. Modification of the DOP-PCR conditions (e.g. use of alternative degenerate primers) and/or production of two separate probes from distal and proximal parts of the marker chromosome would likely resolve this problem.

Hybridization results clearly demonstrate that at least the distal third of the marker chromosome is homologous across the genomes of a variety of species. Given the external karyotypic and chromosomal uniformity within this group of fishes, the portion of the marker chromosome that did not hybridize with the WCP clearly has a different sequence composition. Hellmer *et al.* (1991) studied the organization of chromosomal replication in two phylogenetically closely related leuciscine species (roach *R. rutilus*, and rudd *S. erythrophthalmus)*. This study found that at least 12 pairs of chromosomes, including the marker chromosome, significantly differed in replication banding patterns. Although no other study of replication patterns in leuciscins (and/or phoxinins) is available, the results of Hellmer *et al.* (1991) suggest that there are internal chromosomal rearrangments between the species.

Our results with cross-species painting show that the 'leuciscine' marker chromosome is likely homologous across this cyprinid lineage and at least the distal part is phylogenetically conserved. Such a conclusion may also apply to chromosomes of the phoxinin lineage of cyprinids (a group less cytogenetically explored than leuciscins), in which at least the European representatives possess karyotypes

similar to leuciscins, with one remarkably large pair of st chromosomes (Boroń 2001).

Positive hybridization signal in the karyotype of brook minnow *P. phoxinus* is indicative of ancient chromosomal homologies. This finding is consistent with the recently raised hypothesis that the present-day karyotype structures of evolutionarily diploid cyprinids (and cobitoids) are of ancient origin (Bohlen *et al.* 2008, personal communication). This hypothesis can be further addressed by more extensive cross-species chromosome painting studies. The apparent high conservation of the distal part of the marker chromosome, not only across examined leuciscin taxa but probably also in phoxinins might raise the more general question whether the cross-species painting/Zoo-FISH approach can contribute further to uncovering various aspects of chromosome/karyotype evolution in cyprinid fishes. If karyotypes and/or their parts are indeed conserved, any interchromosomal rearrangements between species using WCPs would not be observed because there are in fact none. However, WCPs can directly demonstrate conservation of karyotypes and/or their segments, whereas other markers used in descriptive fish cytogenetics (diploid chromosome number, number of chromosome arms, chromosomal morphology and applicable banding patterns) provide only indirect evidence. Therefore, Zoo-FISH with WCPs is a substantial methodological improvement over standard staining and/or banding methods, especially when WCPs are used to chromosomes of distantly related taxa or groups (e.g. Graphodatsky 2007 and references therein) where, however, the phylogenetic relationships of examined taxa have to be known for correction interpretation of the results correctly.

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Appendix IV

Synaptonemal complexes in the hybridogenetic *Squalius alburnoides* fish complex: new insights on the gametogenesis of allopolyploids

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Synaptonemal Complexes in the Hybridogenetic *Squalius alburnoides* Fish Complex: New Insights on the Gametogenesis of Allopolyploids

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Key Words
Chromosome elimination · Cyprinidae · Meiotic hybridogenesis · Natural hybrids · Synapsis · Vertebrates

Abstract
In the *Squalius alburnoides* fish complex, allotriploid females (3n = 75) reproduce mostly by meiotic hybridogenesis, producing haploid gametes by means of the elimination of the heterospecific chromosome set and recombination between the 2 homospecific genomes. A synaptonemal complexes (SCs) analysis was performed in specimens from a confined southern population (Quarteira, Portugal) to understand chromosome dynamics during gametogenesis. The comparative study between hybrid females with QAA genome composition and the parental bisexual species *Squalius aradensis* (2n = 50, QQ genome) evidenced: (i) that allotriploid meiocytes comprise the complete chromosome set (75 chromosomes) in prophase I, proving the heterospecific genome (Q) is only excluded after pachytene stage, and (ii) a 2-phase synaptic process where initially, exclusively homologous SCs form and the unmatched univalents remain in a bouquet conformation, followed by the establishment of extensive non-homologous SCs with multivalent associations among the later. These findings disagree with most literature concerning the meiotic process in allotriploid vertebrates, since the most accountable mechanisms (premeiotic exclusion of the unmatched chromosome set and whole genome endoduplication) were not observed.

An increasing number of studies over the last 20 years have been demonstrating that interspecific hybridization and polyploidization in animal species can be relevant mechanisms for diversification and origin of new evolutionary lineages [Dowling and Secor, 1997; Otto and Whitton, 2000; Barton, 2001; Comai, 2005; Mallet, 2007]. Unorthodox modes of reproduction in vertebrates are not just an odd curiosity apart from evolutionary mainstream and are usually linked to hybridization events [Schultz, 1969; Dawley, 1989; and for recent review Neaves and Baumann, 2011]. Such events usually challenge bisexual organisms with the need to alter their gametogenetic process to cope with the presence of distinct genome sets and successfully produce fertile gametes. To overcome the constraints imposed by meiotic recombination and chromosome segregation, hybrid individuals,

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particularly lower vertebrates, have found distinct solutions like adopting non-recombinant reproductive modes (parthenogenesis, gynogenesis or hybridogenesis) frequently associated with ploidy elevation [Schultz, 1969; Dawley, 1989; Choleva et al., 2012].

One of the most intricate allopolyploid systems known in vertebrates is the Iberian minnow *Squalius alburnoides* [Gregory and Mable, 2005], comprising hybrid biotypes with different genomic compositions and ploidies (diploids: 2n = 50, triploids: 3n = 75, and tetraploids: 4n = 100) [reviewed in Gromicho and Collares-Pereira, 2007; Collares-Pereira and Coelho, 2010]. The complex was originated by unidirectional hybridization events, resulting from crosses between *S. pyrenaicus* females (PP genome) and males closely related to *Anaecypris hispanica* (AA genome). It is widely distributed with natural populations dominated by triploid females (in average 75–85% of sampled fish), despite some drainage-specific variations in population structure. *S. alburnoides* is sympatric with the congeneric species *S. carolitertii* (CC genome) in northern populations, *S. pyrenaicus* (maintaining the ancestral P genome) in central and southern regions and with *S. aradensis* (QQ genome) in the southern Quarteira drainage. These bisexual species bring genetic variability into the complex through introgression with distinct biotypes contributing to a generational shifting where parental (nuclear) genomes are dynamically lost, gained or replaced [Alves et al., 2001; reviewed in Collares-Pereira and Coelho, 2010].

The most common mode of reproduction within this complex is meiotic hybridogenesis, characteristic of allotriploid females of all studied *S. alburnoides* populations [Collares-Pereira and Coelho, 2010]. This gametogenetic process was depicted using distinct molecular markers to compare inheritance profiles of both parents and progenies in numerous experimental crosses [Alves et al., 1998, 2001; Crespo-López et al., 2006]. Because these females typically produce haploid gametes – diploid or clonal unreduced eggs were very rarely observed – see Alves et al. [2004], meiotic hybridogenesis was considered to also involve 2 distinct steps, as proposed for *Pelophylax esculentus* allotriploids [Christiansen and Reyer, 2009]: (1) the pre-meiotic extrusion of the heterospecific chromosome set and (2) the recombination and random segregation of the 2 homospecific genomes.

Meiotic studies in allopolyploid vertebrate complexes are scarce and were essentially based on lampbrush chromosomes or on metaphase I chromosome configurations [Macgregor and Uzzell, 1964; Cimino, 1972a, b; Günther et al., 1979; Guerrini et al., 1997; Stöck et al., 2002, 2012; Morishima et al., 2008], but interestingly not on synaptonemal complexes (SCs). These are highly regulated and tripartite protein structures strictly meiotic and involved in synapsis, recombination and segregation of homologous chromosomes. The formation of synapses between common regions of non-homologous chromosomes was also found, mainly in plants, though arrangements tend to be irregular and multivalent associations might be observed [Zickler and Kleckner, 1999; Page and Hawley, 2004].

The meiotic process was never examined in *S. alburnoides* complex, and the first analysis on SCs of a naturally occurring allopolyploid vertebrate is herein presented. The main goal was to address the dynamics of the distinct genomes in the germ line of allotriploid females, namely, understanding how and when the exclusion of the heterospecific chromosome set takes place, using a cytogenetic approach allowing the detailed visualization of the first meiotic stages. The study was undertaken in *S. alburnoides* triploid females with a QAA genomic composition (very likely resulting from the fertilization of QA eggs by haploid A sperm) from the confined population of Quarteira (south Portugal), where they coexist with *S. aradensis* specimens (2n = 50, QQ genomic composition) [Sousa-Santos et al., 2006], here used as controls.

Materials and Methods

In the beginning of November 2010, adult fish were captured by electrofishing in the Quarteira river basin (south Portugal). Specimens of *S. alburnoides* complex and of the sympatric *S. aradensis* were transported to the laboratory and kept at room temperature in well-aerated 25-liter aquaria, under a 12-hour light photoperiod. In February 2011, according to data on their annual reproductive season and plausible period for favorable meiocytes' maturation [Ribeiro et al., 2003], the water temperature was artificially raised to 22°C and the daily feeding intake was supplemented with frozen *Artemia salina*. All procedures were developed in accordance to the recommended ethic guidelines [ASAB, 2006].

Individual identification was accomplished following Morgado-Santos et al. [2010] and the ploidy of each specimen was assessed by erythrocytes (RBC) flow cytometry, according to Próspero and Collares-Pereira [2000]. Individual genomic profile was determined as described in Sousa-Santos et al. [2005]. Gonads were dissected and macroscopically classified as male or female. SCs preparations were conducted following the method of surface spreading developed by Cuñado et al. [2000] on both male and female gonads. Photographs were captured using a Jeol 1200 electron microscope and processed with Adobe Photoshop CS4 software.

Results

SCs spreads were obtained for 2 allotriploid *S. alburnoides* females (3n = 75, QAA) and 3 *S. aradensis* specimens (2 males, 1 female; 2n = 50; QQ). The synapses in *S. aradensis* were regularly composed of 25 completely synapsed bivalents showing SCs with constant width and centromeres perfectly aligned (data not shown). Twenty-three (out of 36) pachytene nuclei were fully analyzed for the allotriploid *S. alburnoides* females. Their oocytes exhibited 75 pachytene chromosomes (= somatic triploid value) represented by the axial/lateral elements. However, 2 different scenarios were found: (a) either 25 regular bivalents plus 25 single axial elements corresponding to univalents (78% of the cells) (fig. 1a, c), or (b) more than 25 bivalents, with the majority of chromosomes (at least >75%) forming SCs (22% of the results) (fig. 1e).

The most parsimonious explanation places these scenarios as 2 distinct pachytene stages that oocytes of allotriploid females (QAA) go through. In zygotene-early pachytene, the 2 homospecific copies form perfect homologous synapses, while the heterospecific Q genome remains as single elements (fig. 1a, c). The homologous SCs are regular, with a constant width between lateral elements and the centromeres well aligned (fig. 1b). It is relevant to notice that while the homospecific SCs were found scattered around the nuclei, the heterospecific univalents remained closely assembled, sometimes even in a bouquet configuration (fig. 1a, c, d). Later, in a second phase, irregular non-homologous synapses seem to take place among the heterospecific chromosome set (fig. 1e). Multivalent associations were also found at this step, with SCs presenting uneven width as a result of unsynaptic regions (fig. 1f, g). The transition from the bouquet organization to a phase where synapses were mixed in a disordered manner suggests a shift towards a more advanced pachytene timeframe, a scenario also put forward by the macroscopic observation of the gonads' maturation stages (data not shown).

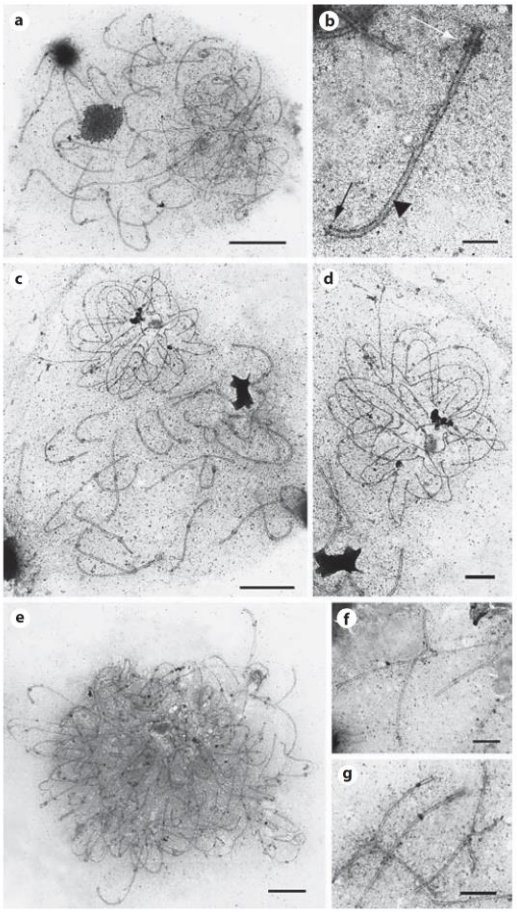

Fig. 1. Electron micrographs of pachytene nuclei from distinct triploid (QAA) *S. alburnoides* females: **a**, **c** 25 homologous bivalents presumably between AA genome copies and 25 univalents corresponding to Q genome. Scale bar = 5 μm. **b** Detail of a seemingly AA regular bivalent, representing the characteristic SC: a clear centromere (white arrow), the thickening at telomeric ends (black arrow) and the central element running in the middle (arrowhead). Scale bar = 1 μm. **d** Detail of the bouquet arrangement of the Q univalents in **c**. Scale bar = 2 μm. **e** Nucleus displaying both homologous (presumably between AA genomes) and non-homologous (most likely Q genome) synapses. Scale bar = 5 μm. **f**, **g** Details evidencing non-homologous synapses probably involving the Q genome in the form of multivalent associations. Scale bar = 2 μm.

Discussion

Despite many studies on the reproduction of allopolyploid vertebrates, little is known about their meiotic mechanisms. Knowing the dynamics of the distinct chromosome sets during gametogenesis is crucial to understand how these organisms cope with unmatched genome copies and retain their evolutionary potential. In this study, the bouquet conformation found in distinct

stages of a number of meiocytes allowed supporting a timeline in allotriploid meiosis. Knowing that the bouquet arrangement can be maintained until pachytene, though still representing an earlier stage, implies a subsequent meiotic phase where such configuration is lost [Scherthan et al., 1996; Zickler and Kleckner, 1998; Scherthan, 2007]. Our results suggest the formation of synapsis in 2 distinct phases in *S. alburnoides* allotriploid females. It starts with the regular pairing of the 50 homologous AA chromosomes (fig. 1a–c), dispersing in the nucleus as they become fully assembled and mature. At this stage, the remaining 25 heterospecific univalents (Q genome) linger in a bouquet-like arrangement (fig. 1d). As meiosis proceeds, univalents seem to synapse with themselves forming not only non-homologous bivalents, but also multivalent associations (fig. 1f, g) most likely related to the coupling of regions with common repetitive DNA sequences, as seen in plants [Zickler and Kleckner, 1999; Page and Hawley, 2004].

A 2-phase-synapsis formation has been widely described both in allopolyploid plants and animals, including several species with different chromosome abnormalities [McClintock, 1933; Rasmussen and Holm, 1980; Gillies, 1989; Cuñado et al., 2002]. The second phase seems to require the highest formation of synapses possible; in order to overcome the pachytene checkpoint [Roeder and Bailis, 2000], a surveillance control ensuring the correct events take place in the meiotic cell, arresting or delaying the cycle in response to defects in cellular processes [Hartwell and Weinert, 1989]. In mammals, the presence of asynapsed chromosomes at pachytene triggers cell removal via a p53-independent apoptotic pathway [Burgoyne et al., 2009].

Evidences of allelic recombination were found between the AA copies of allotriploid PAA females of *S. alburnoides* (carrying 'P genome' of the parental species *S. pyrenaicus*), but never between the heterospecific genomes [Alves et al., 1998; Crespo-López et al., 2006]. The same is expected in QAA females, supporting the idea that initial synapses are restricted to A genome copies and confirming a distinct meiotic dynamics for the heterospecific Q chromosomes.

Considering that *S. alburnoides* allotriploid females typically produce haploid gametes by means of meiotic hybridogenesis [Alves et al., 2004], the heterospecific fraction of their genomes must be excluded at some point of gametogenesis. Mechanisms relying on a pre-meiotic chromosome exclusion and/or genome endoduplication have been proposed for other allotriploids to overcome the presence of a foreign genome copy and be able to produce fertile gametes [Macgregor and Uzzell, 1964; Cimino, 1972a; Graf and Müller, 1979; Günther et al., 1979; Ogielska, 1994; Guerrini et al., 1997; Stöck et al., 2002, 2012; Morishima et al. 2008; Christiansen and Reyer, 2009], but the present results evidenced that the chromosome elimination process occurs only after pachytene in this Iberian fish complex.

By late diplotene/early diakinesis, the SCs are fully degraded and the homologues (in this case, A chromosomes) held together by chiasmata alone [Rasmussen and Holm, 1980] are correctly oriented and subsequently segregated. Theoretically, Q chromosomes which did not form chiasmata to hold them together might, therefore, disperse and be lost. We suggest the heterospecific elimination to occur during anaphase I, eventually in the first polar body.

This first study on *S. alburnoides* meiosis allowed some disclosure on a specific and distinct gametogenetic process. Albeit an unorthodox reproductive strategy, meiotic hybridogenesis can be evolutionary advantageous in retaining the most important meiotic benefit: recombination. Considering the most likely zygote formation process in such QAA individuals with the 2 homospecific genome copies coming from both progenitors, the advantages are in preventing the loss of heterozygosity commonly associated with non-recombinant modes of reproduction. However, the full process leading to the heterospecific genome elimination (for oocyte haploidization) in the complex remains to be investigated. The subsequent gametogenetic stages need to be specifically analyzed, if possible using a broader sample and triploids from other populations, since they might provide important clues regarding the flexibility of hybrid systems in changing gametogenetic processes to regulate sexual compatibility.

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Glossary

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A

ADAPTATION – or ADAPTIVE TRAIT, is a trait with a current functional role in the life history of an organism that is maintained and evolved by means of NATURAL SELECTION.

ADAPTIVE DIVERGENCE – populations exposed to different ecological environments diverge for traits influencing survival and reproduction; by diverging, gene flow between populations is reduced since immigrants become less fit than residents and because hybrids perform poorly in either environment.

ADAPTIVE RADIATION – evolution of ecological and phenotypic diversity within a rapidly multiplying lineage.

ALLOPATRIC SPECIATION – see SPECIATION.

ALLOPOLYPLOIDY – having more than two chromosome sets derived from different species as a result of the multiplication of the number of chromosomes in a HYBRID lineage following interspecific HYBRIDIZATION.

ANCESTRAL POLYMORPHISM – a situation in which incomplete LINEAGE SORTING of gene copies over generations might lead to fixation of alleles in descendant species whose genealogical structure does not reflect true species PHYLOGENY.

APOMORPHY – the innovative state of character; also known as derived state.

AUTOPOLYPLOIDY – having more than two chromosome sets derived from a single species; can arise spontaneously or from the fusion of unreduced gametes.

B

BACKCROSSING – when hybrids mate with parental forms.

BIOGEOGRAPHY – the study of the distribution of species and ecosystems in a geographic space, through a geological time.

C

CHROMOSOMAL or PHYSICAL MAPS – method of assigning DNA fragments to chromosomes by means of FISH procedures without the need for complete sequencing data.

COALESCENCE THEORY – statistical model applied to studies of POPULATION GENETICS attempting to trace all alleles of a particular gene shared by all members of a population to the most recent common ancestor.

COMPARATIVE GENOMIC HYBRIDIZATION (CGH) – a FISH derived technique based on the simultaneous application of two differentially-labelled genomic DNAs as probes.

CONTACT ZONES – see HYBRID ZONES.

CROSS SPECIES GISH – see ZOO-FISH.

D

DIVERGENCE – see ADAPTIVE DIVERGENCE.

DRIFT – see GENETIC DRIFT.

E

EVOLUTIONARY ECOLOGICAL GENOMICS – The recognition that evolution can happen on an ecological timescale has prompted the integration of ecology and evolution, while easier access to high-throughput sequencing technologies has increased the number of genetic non-model species entering the 'omics' era.

F

FITNESS – a central concept in NATURAL SELECTION, it can be defined by the reproductive success of an organism or species.

G

GENE FLOW – the transfer of alleles or genes from one population to another; also known as gene migration.

GENETIC DRIFT – a change in allele frequencies caused by random sampling.

GENETIC MAP – see LINKAGE MAP.

GENOMIC IN SITU HYBRIDIZATION (GISH) – a FISH-derived technique based in the application of a labelled genomic DNA as probe, in the presence of an excess of unlabelled competitor DNA to suppress common repetitive sequences.

H

HAPLOTYPE – a combination of alleles at adjacent loci on a chromosome that are inherited together.

HEMIPLASY – HOMOPLASY-like outcomes introduced by LINEAGE SORTING.

HETEROLOGOUS FISH – when probe DNA and target chromatin DNA are from different species.

HETEROKARYOTYPE – in this context, referring to a HYBRID KARYOTYPE composed of at least half a set of each parental species' chromosomes.

HETEROSIS – HYBRIDS' condition or fitness raised beyond the state observed in the parental forms

HOMOLOGOUS FISH – when probe and target DNAs are from the same species.

HOMOPLASY – structural similarity among characters thought to have originated independently as a result of convergent or parallel evolution; also considered as PHYLOGENETIC noise.

HOMOPLOID HYBRID SPECIATION – the process by which an independent lineage arises through HYBRIDIZATION and the combination of parental genomes, without an increase in chromosome number (ploidy).

HORIZONTAL TRANSFER – the transfer of genetic material from one organism to another organism that is not its offspring, usually by means of host-pathogen interactions.

HYBRID – in the strict sense, the first offspring resulting from interbreeding between differentiated taxa.

HYBRIDIZATION – reproduction between members of genetically distinct populations yielding exclusively unviable or infertile offspring.

HYBRID SPECIATION – the origin of a new evolutionary lineage from the HYBRIDIZATION between existing lineages, that is at least partially reproductively isolated from both parental lineages and demonstrates a distinct evolutionary and ecological trajectory.

HYBRID SWARM – population of HYBRIDS that has survived beyond the initial hybrid generations, with interbreeding between HYBRID individuals and BACKCROSSING with its parental types.

HYBRID ZONES – geographic regions where genetically distinct populations or species come into contact, mate, and produce HYBRIDS.

I

INTROGRESSIVE HYBRIDIZATION – reproduction between members of genetically distinct populations, producing offspring with mixed ancestry as a result of directional (reciprocal or not) genetic exchanges between the hybridizing taxa.

K

KARYOEVOLUTION – evolution that results in changes to the KARYOTYPE of organisms caused by changes in chromosome characteristics (number, structure, etc.).

KARYOTYPE – the number and type of chromosomes constituting the complete set of chromosomes characteristic of a cell line, an individual or a species, based on chromosome length, centromere position, banding patterns, sex determining chromosomes, and other physical characteristics.

L

LINEAGE SORTING – the evolutionary process whereby multiple gene lineages in an ancestral species are eventually replaced by lineages unique to each descendant species.

LINKAGE GROUP – all of the genes on a single chromosome, inherited as a group.

LINKAGE MAP – a GENETIC MAP revealing the position of its genes relative to each other based on recombination frequencies, rather than a specific physical distance along each chromosome. Genetically linked loci are physically less susceptible to recombination and thus improbable to be separated onto different chromatids being inherited together during meiosis.

M

METAPOPULATION – a group of spatially separated populations of the same species interacting at some level.

MORPHOTYPE – a group of different morphological types of individuals of the same species in a population.

MUTATION – source of genetic variation in the form of new alleles.

N

NATURAL SELECTION – a key mechanism of evolution concerning the gradual natural process by which biological traits become more or less common in a population as a function of the effect of inherited traits on the differential reproductive success of organisms interacting with their environment.

P

PARAPATRIC SPECIATION – see SPECIATION.

PHYLOGENETICS – the study of evolutionary relationships among groups of organisms by means of comparative analyses of molecular sequencing and/or morphological data, resulting in hypothetical evolutionary history between taxa and/or taxonomic groups.

PHYLOGEOGRAPHY – the study of the historical processes that may be responsible for the contemporary geographic distributions of individuals in light of the patterns associated with a gene genealogy.

PHYSICAL MAP – see CHROMOSOMAL MAP.

PLESIOMORPHY – a character state inferred to have been retained from its ancestors; also known as the ancestral state.

POLYMORPHISM – when two or more clearly different phenotypes exist in the same population of a species.

POLYPLOIDY – having more than two paired ancestral chromosome sets.

POLYTYPISM – a special type of POLYMORPHISM concerning one species with genetically distinct populations (overall or average gene frequencies and not necessarily the presence of any gene or allele in one population absent in the other) in different geographic areas.

POPULATION GENETICS – the study of allele frequency distribution and alteration under the influence of the four main evolutionary processes: NATURAL SELECTION, GENETIC DRIFT, MUTATION and GENE FLOW but also taking in consideration factors as RECOMBINATION, population subdivision and POPULATION STRUCTURE, in attempt to explain and predict phenomena as ADAPTATION and SPECIATION.

POPULATION STRUCTURE – population-specific patterns in the genetic constitution of the individuals within that population, characterised by their genotypes and/or allele frequencies.

Q

QUANTITATIVE TRAIT LOCI (QTL) – stretches of DNA containing or linked to the genes that underlie a quantitative trait (usually phenotype-related).

R

RECOMBINATION – genetic exchange between homologous chromosomes during meiosis. The further a loci is from the centromere the greater is its susceptibility or the probability to undergo recombination.

REINFORCEMENT – usually referring to reinforcement of REPRODUCTIVE ISOLATION.

REPRODUCTIVE ISOLATION – mechanisms, behaviours and physiological processes that prevent the members of two different species that cross or mate from producing offspring, or which ensure that any offspring that may be produced is not fertile.

RETROELEMENTS, RETROTRANSPOSONS or RETROPOSONS – class I transposable elements that transpose via an RNA intermediate subsequently copied into cDNA by a REVERSE TRANSCRIPTASE and integrated into a new genomic site (retrotransposition).

REVERSE TRANSCRIPTASE – an enzyme used to generate complementary DNA (cDNA) from an RNA template, by a process termed reverse transcription.

S

SELF-GISH – synonym of HOMOLOGOUS GISH, is based in the use of a labelled genomic DNA to hybridize on the chromosomes of the same donor species; usually used as a positive control.

SELECTION – see NATURAL SELECTION.

SPECIATION – evolutionary divergence of subsets of one ancestral species into two different species. It can be SYMPATRIC – in the absence of geographic isolation; PARAPATRIC – in the absence of geographical barriers to gene flow, resulting in incipient species that occupy adjacent areas; or ALLOPATRIC – under strict conditions of geographic isolation.

SPECIES – separately evolving gene pools, unevenly distributed but interconnected in a reproductive community allowing for gene recombination.

SPECIES COMPLEX – a group of closely related species, interdependent on each other to reproduce and subsist, owing to their usually incomplete REPRODUCTIVE ISOLATION and/or altered modes of reproduction.

STASIPATRIC SPECIATION – chromosomal SPECIATION model, by which chromosomal changes promote HYBRID dysfunction or UNDERDOMINANCE of HETEROKARYOTYPIC individuals.

SYMPATRIC SPECIATION – see SPECIATION.

SYNAPOMORPHY – a trait shared by two or more taxa and inferred to have been present in their most recent common ancestor but absent from its own ancestor.

T

TRANSGRESSIVE SEGREGATION – generation of phenotypes in segregating hybrid populations that are extreme relative to either parental taxa phenotypes.

TRANSPOSABLE ELEMENTS or TRANSPOSON – genetic entities with the ability to change their chromosomal location. See RETROELEMENTS.

TRANSPOSITION – the transfer of genetic material between chromosomes or organisms in a manner other than recombination or traditional reproduction, usually the method by which mobile elements change their location in a genome. See also HORIZONTAL GENE TRANSFER.

U

UNDERDOMINANCE – SELECTION against the mean of a population distribution, causing disruptive SELECTION and DIVERGENT genotypes.

Z

ZOO-FISH – synonym of CROSS SPECIES GISH or HETEROLOGOUS GISH, is based in the use of a labelled genomic DNA or WCP of one species to hybridize on the chromosomes of another species.