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Abstract

The hybridogenetic *Squalius alburnoides* fish complex comprises a diversity of forms combining distinct nuclear genomes and ploidies. It resulted from past interspecific hybridizations, incorporating the 'A' parental genome and currently shuffling it with genome copies from sympatric bisexual *Squalius* species. The *S. alburnoides* Quarteira population (Algarve, Portugal), in sympatry with *S. aradensis* ('Q' genotype donor), was addressed in this study. The main goal was to investigate possible intergenomic chromosomal exchanges occurring between the A and the Q parental genomes, in the distinct hybrid forms. Heterospecific exchanges might combine beneficial traits and through introgression, transfer them to the parental species thus improving their evolutionary potential. Two distinct approaches were used:1) Genomic *in situ* hybridization (GISH) applied to mitotic chromosome spreads to identify admixture of fluorescence patterns indicative of such exchanges; and 2) a synaptonemal complexes' analysis to investigative meiotic chromosomes' synapses during gametogenesis. Concerning the first, no clear interchanges were found; since each genome type appeared stained homogeneously according to its own parental origin. Regarding the meiotic approach, striking results were obtained in allotriploid females, which presented the entire 3n=75 chromosome sets in their meiocytes. The pachytene analyses showed no evidences of premeiotic chromosome exclusion or genome endoreduplication, which have been described for most hybrid vertebrates' complexes. A two-phase synaptonemal complex formation appears to take place in those females, however, no clear evidences of heterogenomic synapses were found. While the homospecific chromosome copies appear to engage exclusively in regular synaptonemal complexes, the heterospecific genome seems to synapse itself forming a diversity of irregular associations. Both studies seem to agree in their finding that no obvious intergenomic chromosomal exchanges take place in *S. alburnoides* Quarteira population, thus suggesting the conservation of each genome set integrity upon reproduction.

Keywords: FISH, GISH, Hybridogenetic fish complex, Intergenomic chromosomal exchanges, Synaptonemal complexes

Resumo

Os processos de hibridação interespecífica conduzem geralmente a alterações dos mecanismos reprodutores e, por vezes, à poliploidização. Embora a poliploidização seja um fenómeno bastante frequente no percurso evolutivo das plantas a sua incidência evolutiva nos animais tem sido questionada e considerada rara, particularmente nos vertebrados superiores. No entanto, um aumento considerável de estudos tem levado progressivamente ao reconhecimento da sua importância, questionando a visão clássica de que os híbridos estariam condenados ao insucesso evolutivo, devido à incompatibilidade de distintos genomas de realizarem meiose e produzirem gâmetas balanceados. De salientar que, nos vertebrados inferiores (peixes, anfíbios e répteis), é um fenómeno com considerável incidência provavelmente devido à sua flexibilidade em transitar do processo de reprodução bissexuada para estratégias não-sexuadas. Em peixes, a hibridação é um fenómeno bastante recorrente, em particular nas famílias Cobitidae e Cyprinidae, possivelmente potencializado por factores biológicos (como fertilização externa) e comportamentais (como mecanismos mais incipientes de isolamento pré-zigóticos). O sucesso reprodutor dos híbridos permite a existência de fenómenos de introgressão, viabilizando a transferência de informação genética de uma espécie parental para a outra. Contudo, às estratégias reprodutoras não-sexuadas estão, em geral, associados constrangimentos evolutivos relacionados com a ausência de recombinação genética aquando da produção de gâmetas. Em vertebrados foram descritos diversos mecanismos, alguns dos quais produzem descendência clonal relativamente ao genoma materno, como a partenogénese e a ginogénese e, outros, descendência hemiclonal como a hibridogénese. Um outro mecanismo reprodutor, dependente de esperma, é o caso da hibridogénese meiótica que envolve, à semelhança da hibridogénese clássica, a eliminação de um dos genomas parentais mas, adicionalmente, a segregação e a recombinação entre genomas homoespecíficos. É um processo mais raro e pouco estudado, tendo sido descrito em triplóides do complexo alopoliplóide *Squalius alburnoides*. Com efeito, este complexo hibridogenético é dos que apresenta uma maior variedade de estratégias reprodutoras, contribuindo para a sua elaborada dinâmica e diversidade populacionais. O complexo *S. alburnoides* (família Cyprinidae) compreende um conjunto de pequenos peixes dulciaquícolas com uma vasta distribuição geográfica na Península Ibérica e distintas populações de norte a sul. Resultou da hibridação unidireccional entre fêmeas de *Squalius pyrenaicus* e machos de

uma espécie próxima de *Anaecypris hispanica*. O complexo deve a sua designação ao envolvimento de múltiplas espécies, que conduziram a distintas formas híbridas constituídas por diferentes ploidias e composições genómicas. A sua actual manutenção é conseguida através da incorporação dos genomas das espécies bissexuadas de *Squalius* simpátricas. *S. alburnoides* representa assim um bom sistema modelo para estudar os efeitos da hibridação e da poliploidia nos processos de organização genómica, que poderão criar o panorama adequado para a acção dos mecanismos evolutivos. Na bacia de Quarteira (Algarve, Portugal) o complexo combina o genoma parental 'Q' da espécie *S. aradensis* com o genoma híbrido 'A'. Esta população foi alvo do presente estudo, pretendendo-se averiguar a existência de possíveis trocas entre os genomas 'A' e 'Q' nas distintas formas híbridas. As trocas intergenómicas poderão combinar características benéficas nos genomas híbridos e, através de introgressão, transferi-las às espécies parentais contribuindo para o aumento da sua variabilidade genética e eventual sucesso evolutivo.

Num primeiro trabalho foi caracterizado o cariótipo mitótico de *S. aradensis*, dado que ainda não fora alvo de análises citogenéticas e a sua composição cromossómica era desconhecida. O estudo foi realizado com indivíduos alopátricos ao complexo e foi comprovada a presença do valor diplóide esperado de 50 cromossomas, composto por cinco pares de metacêntricos, 18 pares de submetacêntricos e dois pares de subtelo/acrocêntricos. Três tratamentos distintos vulgarmente utilizados em peixes para identificação das Regiões dos Organizadores Nucleolares revelaram a sua presença no braço curto de um par de cromossomas submetacêntrico. A aplicação sequencial dessas técnicas demonstrou consistência no par marcado e, à semelhança do que está descrito para diversas espécies de Leuciscinae, a existência da condição plesiomórfica do caracter – um par de marcadores nucleolares. A comparação dos cariótipos de machos e fêmeas não evidenciou a existência de cromossomas sexuais diferenciados nesta espécie. Foram ainda estudados cromossomas mitóticos de híbridos através da técnica de hibridação *in situ* genómica (GISH). A utilização da sonda genómica 'A' marcada e de DNA genómico bloqueante 'Q' não marcado revelou que esta técnica é eficaz na identificação da composição genómica de híbridos QA e QAA, tendo sido facilmente reconhecidos o número de cromossomas esperados para cada caso. No entanto, não foram detectadas misturas de padrões de fluorescência, indicativos de potenciais trocas

heteroespecíficas. Os cromossomas apresentaram-se homogeneamente corados, o que permite rejeitar a existência de trocas intergenómicas num nível macroestrutural.

O estudo seguinte foi efectuado em cromossomas meióticos, com o intuito de analisar a formação de sinapses durante a gametogénese dos híbridos. A marcação dos complexos sinaptonémicos permitiu observar ao microscópio electrónico com clareza as fases de zigoteno/paquiteno nos alotriplóides QAA e em indivíduos parentais de *S. aradensis* (QQ). Os complexos sinaptonémicos (CSs) são estruturas proteicas que unem longitudinalmente os cromossomas homólogos durante as primeiras etapas da meiose. O início da formação dos CSs ocorre em zigoteno, após o alinhamento e emparelhamento. O resultado deste processo é, em geral, a formação de bivalentes, que correspondem à sinapse de dois cromossomas homólogos, mediada por um CS maturo, durante a fase de paquiteno. Os resultados obtidos em *S. aradensis* permitiram observar que o processo de formação de sinapse nesta espécie, à semelhança de outros *taxa* com cromossomas pequenos, se inicia numa das regiões cromossómicas sub-distais, estendendo-se depois até à extremidade oposta. Foi também observada a conformação em *bouquet* nesta espécie, a qual corresponde ao agrupamento das regiões teloméricas dos cromossomas numa zona confinada do envelope nuclear. É, regra geral, encontrada na transição entre zigoteno e paquíteno e progressivamente desorganizada à medida que os homólogos emparelham e formam sinapses. Os resultados obtidos nas fêmeas triplóides revelaram que os seus meiócitos apresentam o complemento cromossómico somático, ou seja, 75 cromossomas, em profase I. Isso significa que, ao contrário do que está descrito para a maioria dos complexos híbridos de vertebrados, estas fêmeas de *S. alburnoides* não apresentam exclusão cromossómica nem endoreduplicação genómica premeióticas. Foram, no entanto, encontrados dois panoramas distintos no que diz respeito aos oócitos, sugerindo uma progressão temporal na formação dos complexos sinaptonémicos. Numa primeira etapa parece ocorrer exclusivamente a formação de CSs entre os 50 cromossomas das cópias genómicas homólogas (neste caso entre os homólogos AA, nos híbridos QAA). Nesses núcleos foram encontrados 25 CSs regulares (com perfeito alinhamento dos seus elementos laterais, centrómeros e extremidades teloméricas) dispersos e cerca de 25 univalentes aglomerados num arranjo em *bouquet*. Foram, por outro lado, também observados núcleos em que a percentagem de sinapse é muito elevada, encontrando-se poucos elementos simples. Nestes, a conformação em *bouquet* já não é visível e, aparentemente, o genoma heteroespecífico

forma sinapses não-homólogas entre si mesmo, apresentando uma diversidade de associações irregulares. Este caso parece ocorrer numa fase posterior à primeira mencionada e resultar da necessidade de ultrapassar o *checkpoint* de paquiteno. Sabe-se que, em vários organismos, quando são detectados univalentes até a uma certa fase do ciclo meiótico, este *checkpoint* leva à apoptose das células. Não foram, contudo, detectadas sinapses heteroespecíficas óbvias, as quais poderiam indiciar a ocorrência de recombinação entre os dois genomas distintos.

Os resultados apresentados neste trabalho apontam para uma manutenção da integridade de cada genoma parental na população de *S. alburnoides* da Quarteira, sugerindo que a transmissão dos complementos cromossómicos ocorre em bloco, e que o processo reprodutivo nesta população permite conservar a identidade de cada genoma.

Palavras-chave: FISH, GISH, Complexo hibridogenético de peixes, Trocas cromossómicas intergenómicas, Complexos sinaptonémicos

Contents

General Introduction

One of the most intriguing evolutionary pathways is that resulting from hybridization processes due to its complexity and frequent controversy. Interspecific hybridization often results in changes in modes of reproduction and polyploidization (1). In fact polyploidy, the multiplication of an organism entire chromosome set, constitutes a very significant source of natural genome variation. Two distinct polyploid groups are recognized: *i)* autopolyploids, resulting from chromosome doubling within an individual or from the combination of unreduced gametes from the same genome type, and *ii)* allopolyploids, derived from the hybridization of distinct parental genomes (2). Many hypotheses attempt to explain the prevalence and success of natural allopolyploids, although it is still unclear how organisms cope with the instability resulting from the combination of distinct chromosome sets and which genome reshaping processes account for the evolutionary potential observed in so many groups.

Polyploidy is widely acknowledged as a major process of adaptation and speciation in plants (3). Its incidence amongst animals and its role in the diversification of new evolutionary lineages is more recently being recognized (4-6). Several reasons might explain the low frequency of animal polyploids such as; disruption of gene expression and regulation; interference in sex determination (7) and sterility, particularly severe in the case of allopolyploids due to meiosis' disturbance resulting from the incompatibility of different genomes to perform meiosis and produce viable gametes. Hybrids may overcome common meiotic constrains by switching from a bisexual to a non-sexual reproduction mode, adopting unorthodox reproductive strategies (1). Still considered rare in homeotherms, hybridization and correlated allopolyploidization is known to have occurred extensively in fishes, where 20% of the families have descriptions of natural hybrids (8, 9). The Cyprinidae family presents the highest hybridization incidence currently known (10).

The *Squalius alburnoides* fish complex (Cyprinidae) was first described by Collares-Pereira in 1983 (11). Both genetic and cytogenetic studies performed in the last 30 years have demonstrated that it comprises mostly hybrid biotypes with different genomic compositions and ploidies, including diploids (2n=50), triploids (3n=75) and tetraploids (4n=100) and an all-male nuclear non-hybrid lineage (in the southern populations) (reviewed in (12)). Endemic to the Iberian Peninsula, this allopolyploid

complex was originated from unidirectional hybridization events, resulting from crosses between a maternal *S. pyrenaicus* ancestor (P genome) (13) and males from an extinct species closely related to *Anaecypris hispanica* (14, 15). At present, the freshwater *S. alburnoides* complex has a widespread distribution, with distinct populations hybridizing with sympatric bisexual *Squalius* species. Natural populations consist mostly of triploid females (in average 75-85%) however other ploidy forms have been accounting for some variation amongst drainages (reviewed in (16)). The introgression with *Squalius* species introduce genetic variability into the complex thus contributing to the dynamic generational shifting between forms, where parental genomes are lost, gained or replaced (16, 17). The intercrosses replaced the ancestor nuclear P genome in allopatric *Squalius* drainages, leading to the current diversity of genomic compositions, where a latitudinal gradient in species' ranges can be found; in northern populations *S. alburnoides* is sympatric with *S. carolitertii* (C genome), in the central and southern regions with *S. pyrenaicus*, thus maintaining the P genome, and in the southern Quarteira drainage, interspecific crosses occur with *S. aradensis* (Q genome) (13, 18).

S. alburnoides hybrids (both males and females) are fertile and, according to their ploidy level, present several non-sexual reproductive mechanisms. In general, and although gynogenesis has been reported in very rare cases, the syngamy of gametes takes place. In hybrid diploid individuals, clonal transmittance of the parental genome seems to be the most common strategy but hybridogenesis has also been suggested (19). Triploids usually reproduce by meiotic hybridogenesis, but again less commonly, both clonal gamete formation and 'normal' hybridogenesis have been found (16, 20). These reproductive modes presuppose a gametogenetic mechanism where parental chromosomal sets are inherited in block without heterospecific interactions, thus preserving their integral structure. Conversely, studies performed in *Ambystoma* salamander hybrids revealed intergenomic chromosomal exchanges evidenced by non-homologous translocations and homeologous recombination (21, 22). Intergenomic exchanges can be evolutionary beneficial, by combining valuable traits from distinct genomes in hybrids, which through introgression allow their transmission to the parental species, contributing with new variability to their genetic pool.

The case-study of S. alburnoides *population in the Quarteira drainage*

The small independent Quarteira drainage is located in the southwest of Portugal and it is the only known place where *S. alburnoides* complex is sympatric with the bisexual species *Squalius aradensis*. Both have been scarcely studied though it is known that interspecific crosses with *S. aradensis* (Q genome) lead to Q genome introgression at both the nuclear and the mitochondrial levels (18).

The reproductive strategies and overall dynamics of the fish complex in this population just started to be analyzed. Apart from few genetic data, no cytogenetic coverage on *S. alburnoides* complex and *S. aradensis* specimens was performed until now. Nevertheless, studies on other *S. alburnoides* populations have gathered consistent information correlating each forms' genomic composition and the several reproductive strategies adopted (12, 16). In Quarteira, the population is expected to be composed of two distinct diploid forms; nuclear hybrids (QA) and non-hybrid males (AA). Triploids might be either QAA or QQA and eventual tetraploids might combine any proportion of the two distinct parental genomes (18). The hypothetical reproductive strategies are presented in **Figure 1**.

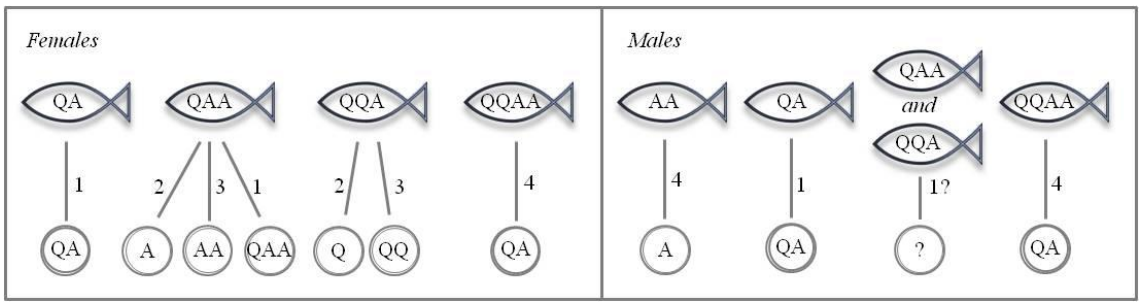

Figure 1. Hypothetical ploidies, genome compositions and gametes' production of *S. alburnoides* in Quarteira population. Not all forms have been found. The reproductive strategies are inferred from studies on distinct *S. alburnoides* populations (bearing P genome type). 1. Clonal gametes' production; 2. meiotic hybridogenesis; 3. hybridogenesis 4. 'normal' meiosis' (based on (12)).

Comparative cytogenetics approach

On the course of evolution a species karyotype can be modified by altering the number, structure and composition of its chromosomes. Cytogenetics allows studying chromosomes' evolution by attempting to detect modifications of the genetic material in

terms of acquisitions, losses and multiple rearrangements, in the form of fusions, fissions, translocations and inversions. An interesting feature of polyploids is their higher tolerance to deletions and their ability to indulge in heterospecific rearrangements involving the transference of a chromosome segment from one parental genome to the other (21-23). In that case, comparative cytogenetics can offer appropriate tools to trace the evolutionary history of extant genomes.

Some of the traditional cytogenetic techniques involve chromosome banding that allows a visualization of genome organization and a disclosure of structural differences in chromosome regions (24). Several chromosome markers have been widely used to obtain a species-specific characterization pattern and on a comparative analytic scale, to identify phylogenetic relationships and genomic organization processes involved in species evolution (e.g. (25)). The very limited success of many common cytogenetic tools in fishes, due to many constrains related to the frequently high number and small size of the chromosomes (reviewed in (26)) and to a weak genome compartmentalization (27), lead to particular focus on very specific markers such as the distribution patterns of constitutive heterochromatin (C-banding) and the number and position of Nucleolus Organizer Regions (NORs). Lately, the development of technical methods with higher resolution, mostly based on fluorescent *in situ* hybridization, such as the comparative chromosome painting, have broaden the study of composition and organization of fish genomes and reinforced the possibility of unravel some important evolutionary mechanisms and taxonomic issues.

Genomic *in situ* hybridization (GISH) allows a comparative genomic study in hybrids. It is particularly useful in allopolyploids by allowing to differentiate chromosomes from distinct parental origins and to check for intergenomic interactions in the form of heterospecific rearrangements. This technique relies on the use of whole genomic DNA (gDNA) labeled as probe, staining chromosomes according to sequence homology (28). Labeled gDNA from one parental species and an excessive amount of unlabelled DNA (usually from the other parental genome) are used to hybridize on chromosomes. The resulting fluorescent pattern can give us information regarding genome evolution, disclosing both recent (intergenomic exchanges) and older events (identifying the most likely parental species).

Meiotic (Synaptonemal complexes) approach

Synaptonemal complexes (SCs) analyses have been widely used in cytogenetic studies to study meiotic chromosome synapses. The SC is an evolutionary well-conserved structure only found during meiotic prophase (29).

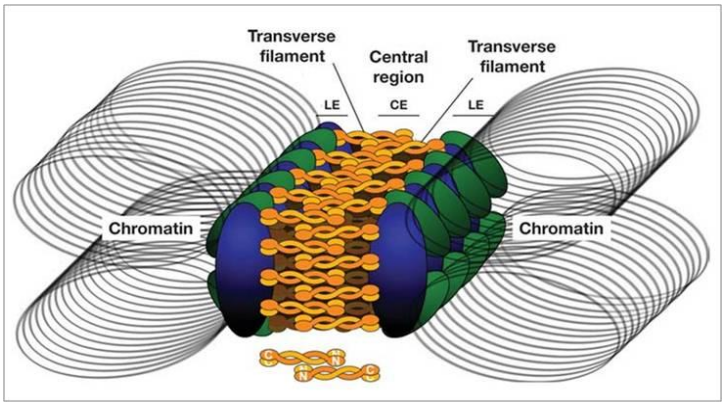

Figure 2. Scheme of a SC structure. The lateral elements (LE), the transverse filaments, the central elements (CE) and the central region mediate the association between chromosomes (adapted from (30)).

It is composed of two outer protein axes (lateral elements) and a third central element, running in parallel (**Figure 2**). This tripartite structure connects paired chromosomes along their longitude (29, 30).

Prophase I is the most relevant meiotic stage, where pairing, recombination and synapsis of homologous chromosomes take place. Synapsis is the outcome of homologues association mediated by a mature SC; the resulting structure constitutes a meiotic bivalent. SC formation is initiated during leptotene, when paired sister chromatids condense along the protein axial element. At zygotene phase, the central element links the axial elements of the two homologues, becoming, in turn, lateral elements. Also in early zygotene, the telomeres of the meiotic chromosomes gather to a confined region of the nuclear envelope, forming a *bouquet* arrangement. This *bouquet* formation is widespread amongst most eukaryotes and was hypothesized to facilitate homologous chromosome pairing and recombination (reviewed in (29, 31)). By the beginning of pachytene, the SC becomes fully matured with the association of perpendicular transverse filaments connecting the lateral and central elements, thus resulting in a continuous SC joining the two homologues. Also during pachytene and in the SC context, crossovers maturate into chiasmata, the physical expression resulting from meiotic recombination. At diplotene, the SC disassembles and homologues separate, except at chiasmata which will hold homologous chromosomes together until metaphase I (31).

In the SC, pachytene chromosomes are less compact than mitotic ones allowing more precise species karyotyping, detection of specific synaptic patterns such as those involved in chromosome rearrangements and abnormalities. Also the heteromorphism of sex chromosomes might be detectable at the level of SCs in the form of atypical synaptic configurations, allowing studying the differentiation and evolution of such chromosomes (29, 32, 33).

SCs have been vastly studied in polyploid genomes, particularly, in triploid vertebrates. A very interesting feature of triploids is their possibility to form trivalents by associating the lateral elements of three distinct chromosomes (34-37). Trivalent formation can involve double synapsis only or, in rare cases, a simultaneously assemblage (triple synapsis) of the three chromosomes partially or fully along their length (36). Trivalent formation usually does not occur by simultaneous pairing of the three chromosomes, but is achieved later at a second less specific phase, not requiring precise chromosome homology (29, 36). Overall, the presence of an extra chromosome set gives rise to non-homologous synapses, derived from a distinct round of SCs formation and sometimes manifested in the form of multivalent synaptic associations. Those usually present irregular configurations, as a consequence of heterosynapses and are often solved later in the meiotic division (29).

Thesis objectives and outline

The main goal of this thesis was addressing the prevalence of genome reshaping in *S. alburnoides,* using distinct but complementary tools, in order to explore the possibility of such intergenomic interactions being held in the complex.

The confined *S. alburnoides* Quarteira population, in sympatry with S. *aradensis*, was used as case-study. As mentioned before, no previous work was ever done on this particular population as regards cytogenetic essays and the same applies to the bisexual species. Therefore, a preliminary characterization of this parental species' chromosomes would be strictly necessary so that its genomic involvement in the complex dynamics could be addressed. The study of potential genomic exchanges was performed, on one hand, on mitotic chromosomes recurring to a whole genomic comparison and, on the other hand, on meiotic chromosomes through synapses' analyses.

The distinct approaches and results are here presented in the form of two separated 'Research Articles'. The first one briefly characterizes *S. aradensis* karyotype and subsequent genomic *in situ* hybridization results against *S. alburnoides* hybrids and both sympatric and allopatric specimens of *S. aradensis*. This work attempts to detect chromosomal exchanges at the level of somatic cells, using the same technique described for *Ambystoma* salamanders and recently developed for *S. alburnoides* populations from Tejo basin (unpublished results). The second article deepens this study by trying to find chromosomal exchanges at the level of prophase I meiotic chromosomes, suggested by non-homologous pairing of interspecific chromosomes in hybrids' gametogenesis.

The last chapter presents some *Concluding Remarks*, aiming to briefly summarize and integrate the main outputs of the present work which gave some interesting insights namely: *i)* on the gametogenetic process in *S. alburnoides* allotriploid females from Quarteira population, and *ii)* on chromosomal data regarding both the local *S. alburnoides* hybrid biotypes and the previously uncharacterized *S. aradensis* species.

Comparative genomic cytogenetics of *Squalius alburnoides* complex in a confined population (Quarteira), with the first description of the karyotype of the parental species *Squalius aradensis*

Abstract: In the confined Quarteira drainage (Algarve, Portugal), the hybridogenetic *Squalius alburnoides* fish complex is sympatric with the bisexual *Squalius aradensis* species, which contributes with Q nuclear genotype to the genomic composition of hybrids. Since there was no previous available data, the mitotic karyotype of *S. aradensis* was ascertained on specimens living in allopatric conditions (from Arade drainage). The species presents a diploid number of 50 chromosomes, composed of five pairs of metacentric, 18 pairs of submetacentric and two pairs of subtelo/accrocentric chromosomes, and a fundamental number of 96. Chromosomes were analyzed by a sequential cytogenetic procedure using fluorescent *in situ* hybridization (FISH) for ribosomal DNA, chromomycin A_3 and silver staining treatments. A consistent positive signal located on the short arms of one submetacentric chromosome pair (*sm*7) was registered. Genomic *in situ* hybridization (GISH) was used on metaphasic chromosomes to identify parental genomes in *S. alburnoides* hybrids and to find evidences of possible intergenomic exchanges. GISH proved to be an effective tool to identify each distinct genome but no visible heterospecific interactions were found, suggesting that the distinct chromosome sets are transmitted in block, thus retaining their integrity.

Keywords: Hybrid genomes, FISH, GISH, *Squalius alburnoides* complex, *Squalius aradensis* karyotype

Introduction

The Iberian *Squalius alburnoides* complex is a hybridogenetic cyprinid presenting an incredible population diversity in terms of genomic composition (reviewed in (1)). The complex comprises diploid (2n=50) and polyploid (3n=75 and 4n=100) nuclear hybrids bearing different parental species genomes, as a result of hybridization with distinct bisexual *Squalius* species. Additionally, and only in southern populations, it presents yet an all-male non-hybrid lineage. Despite the clinal variability, *S. alburnoides* triploid females are the most common biotype in nature (2). The distinct forms intercross through sexual and non-sexual reproductive strategies (2, 3). As far as considered, the altered reproductive modes – from clonal transmittance of the parental genome to gynogenesis (in rare cases), hybridogenesis and meiotic hybridogenesis (the most frequent situation) – are constrained by the lack of genetic recombination (2, 3). Only meiotic hybridogenesis has been reported to present recombination and exclusively between the two homospecific genomes (3, 4).

Recent studies on hybrid genomes have been proving them to be more dynamic than expected. Interactions between heterospecific chromosomes in the form of non-homologous exchanges, have been reported in a vertebrates complex (5, 6). Genomic *in situ* hybridization (GISH) allows the comparative genomic analyses of hybrid karyotypes. Labeled genomic DNA (gDNA) from one parental species and an excessive amount of unlabelled DNA (usually from the other parental genome) are used to hybridize on allopolyploids' chromosomes to disclosure their genomic composition and putative rearrangements (7). This molecular approach has been widely applied in plants (8-10) but scarcely used in animals. Successful results were obtained in some fish (11, 12) and amphibian hybrids (5, 6, 13). In the unisexual salamanders of the *Ambystoma* genus GISH revealed intergenomic chromosomal exchanges. These salamanders displayed recombination between non-homologous chromosomes with very well defined genomic regions attributed to one parental species or the other (5). Accordingly, GISH promises to be a useful tool to address genomic evolution in *S. alburnoides*, by revealing the integrity of each parental genome in hybrids.

The 18S-5.8S-28S major ribosomal genes code three of the four RNA molecules constituting ribosomal sub-units. They are found as repetitive copies in tandem at chromosomal regions called Nucleolar Organizing Regions (NORs). During interphase,

ribosomal DNA (rDNA) transcription leads to nucleolus formation, where ribosomal sub-units assemble to form matured ribosomes (14). The chromosomal sites of NORs are very dynamic evolutionarily speaking and have been widely used as cytogenetic markers in fish genomes, evidencing a highly polymorphic phenotype. NORs can be mapped by Fluorescent *in situ* Hybridization (FISH), using a probe for major ribosomal genes, thus localizing every rDNA copy found in the genome, regardless of its activity and functional integrity.

Another common technique long used to identify NORs has been the silver staining (Ag-NOR). Silver nitrate ($AgNO_3$) binds to residual acid proteins allegedly involved in ribosomal genes' transcription in the previous interphasic cycle (15). The lack of correspondence between positive rDNA FISH signals and Ag-NOR has been demonstrate in several species and even in a particular *S. alburnoides* population (16). Several explanations have been suggested for this phenomenon such as the loss of function of rDNA copies due to redundancy, by structural and sequence modifications due to duplications, transpositions and other rearrangements (17). Chromomycin A_3 (CMA_3) is another classical fluorescent marker commonly used to detect GC-rich regions usually associated with NORs (18). It must be taken into consideration however that several studies have reported that multi-CMA_3 positive signals do not necessarily correspond exclusively to NORs (19, 20).

The lack of successful banding techniques usable in fish chromosomes, the easier visualization (physical mapping) of tandem repeated genes and the evolutionary conservation status of ribosomal RNA (rRNA) genes lead to a focus on their study in these species.

Previous studies in *S. alburnoides* were not very conclusive in terms of the dynamics of NORs in a particular population (16, 21). Multichromosomal NOR sites were detected, with stable (always found) and unstable (polymorphic) phenotypes. Most Leuciscinae species only have one NOR-bearing chromosome pair described and this is thus considered the plesiomorphic condition (22). Although somehow expectable for *S. alburnoides* due to its hybrid origin, multiple NOR signals were also detected in some bisexual *Squalius pyrenaicus* specimens using 28S rDNA FISH mapping, accounting for intra-individual variation and failure in Ag-NOR in detecting most rDNA copies as well (16).

This work aimed to study two southern drainages scantily known: Arade and Quarteira, using classical and molecular cytogenetic techniques. In Arade, *S. aradensis* (Q genome) is allopatric to the hybridogenetic complex. Up to now, *S. aradensis* hasn't been the scope of any cytogenetic analysis and has mostly been used in phylogenetic and phylogeographic essays (23-26). Hence, the preliminary characterization of *S. aradensis* karyotype in allopatry was of extreme importance in order to further address its role in the complex dynamics. Routine cytogenetic tools as major rDNA FISH, GC-specific CMA_3 and Ag-NOR staining were sequentially applied to the chromosomes of allopatric *S. aradensis* specimens, in order to study NORs phenotype. In Quarteira population, where *S. aradensis* is sympatric with the *S. alburnoides* complex, this study tested the ability of GISH to distinguish parental genomes in *S. alburnoides* hybrids with known genomic constitution. Ultimately, evidences of intergenomic chromosomal exchanges between Q and A genomes (genome reshaping) would be possibly recovered, manifested by mixed fluorescent staining patterns in hybrid chromosome spreads.

Materials and Methods

Fish sampling and maintenance

Adult specimens were captured by electrofishing in two distinct southern populations; in Arade basin, in September 2010, and in Quarteira drainage, in November 2010. In Ribeira de Odelouca (Arade), allopatric *S. aradensis* were collected whereas in Porto Nobre (Quarteira) both *S. alburnoides* and sympatric *S. aradensis* were sampled. Fish were transported to the laboratory and kept at room temperature in well aerated 25 liters aquaria, with 12 hour light photoperiod.

Ploidy and genome composition screenings'

Individual identification was accomplished through photographic recognition following (27). The genomic composition of each specimen sampled at Quarteira was determined. Combined information from ploidy level, gene sequencing and morphological traits allowed a reliable determination of each individual's genomic constitution. Anesthetized specimens using tricaine mesylate (MS-222, *Sigma*), were used to: *i)* collect blood from the caudal vein and measure erythrocytes' DNA content by flow cytometry, using a Coulter Epics XL cytometer, according to the method

described in (28); *ii)* cut fin clips for cell cultures and for later genomic DNA extraction using a SDS (10% w/v)/proteinase K protocol, followed by isopropanol precipitation and ethanol washes (26). The last step involved the amplification and sequencing of a 935 bp fragment of the beta-actin gene following (29).

Fibroblast cell cultures and chromosomes' preparations

In vitro cellular suspensions were obtained through fibroblast cultures, according to (30), with small modifications. Briefly, fin clips were cut and placed on tissue culture flasks after sterilization in 70% ethanol and 0.1% sodium hypochlorite, and cells were fed with complete culture medium (Leibovitz's L-15 with glutamine (*Gibco*), supplemented with 20% fetal bovine serum (*Gibco*) and 0.5% (v/v) antibiotic/antimycotic (*Sigma*)), and grown at 29ºC for 4-5 weeks. The complete medium was replaced once a week, after a sterile PBS wash. Whenever cell confluence reached 70-80%, cells were treated with trypsin-EDTA (*Gibco*) either for subculture preparation or to harvest cell suspensions. In the later case, cells were incubated with colcemid mitotic inhibitor (0.1% Demecolcine solution, *Sigma*) for 4 hours before trypsinization, settled by centrifugation and submitted to hypotonic treatment with 0.075 M KCl for 14 minutes at 37 ºC. Suspensions were fixed in methanol/acetic acid (3:1 v/v) and stored at -20 ºC. Chromosome spreads were obtained by conventional splashing and selected for cytogenetic procedures.

Classical Cytogenetic Techniques

The generic karyotype characterization of *S. aradensis* was addressed using routine cytogenetic procedures. Fluorescent CMA_3 staining was performed according to (31), with a slide pre-wash in McIlvaine/$MgCl_2$ buffer, one hour incubation with CMA_3 (*Calbiochem*) and Methyl green counterstaining. Ag-NOR detection followed (32) with modifications (33), using Giemsa counterstaining. Some preparations were also treated with buffered 4% Giemsa alone. Whenever possible, slides were destained and used in sequential treatments.

Molecular Cytogenetic Techniques

Two distinct *in situ* hybridization techniques were used: Genomic *in situ* Hybridization (GISH) and major ribosomal DNA Fluorescent *in situ* Hybridization (rDNA FISH). Before conducting the GISH experiments DNA extraction was required.

Total genomic DNA (gDNA) was obtained from muscle as previously described (26). GISH was performed following (5) with few modifications. Allopatric *S. aradensis* 'QQ' gDNA was used as unlabelled blocking DNA by shearing it to fragments of 100-300 bp in size, using the autoclave at 120 ºC for 20 minutes. Whole gDNA was extracted from nuclear non-hybrid *S. alburnoides* fish ('AA' gDNA) and used as probe labeled by nick translation with digoxigenin-11-dUTP, according to the manufacturer's specifications (*Roche).* After product confirmation by agarose gel electrophoresis, both probe and blocking DNA were purified by ethanol precipitation. Blocking DNA was disolved in mQwater, and the probe was resuspended in hybridization mix composed of 50% ultra-pure formamide (*Sigma*), 2x SSC and 10% (w/v) dextran sulfate powder (*Sigma*). For each chromosome preparation, the ratio of 'A' probe to 'Q' blocking DNA used was 1:25 µg DNA. DNA concentrations were determined using Qubit 2.0 Fluorometer (*Invitrogen*). Prior to hybridization the blocking DNA was added to the probe mixture. Slides were denaturated in 70% formamide in 2x SSC at 65ºC for 3 minutes, and immediately dehydrated in an ice-cold ethanol series (70%-90%-100% ethanol) for 7 minutes each and air-dried. The probe mixture was denatured at 75ºC for 10 minutes, immediately placed on ice for another 10 minutes and added to the chromosome preparation. Hybridization was performed in a dark moisture chamber at 37ºC, for 72 hours. Post-hybridization washes were performed at RT, for 7 minutes each: twice in 2x SSC and once in 2x SSC/0.1% $Tween_{20}$ (2XT). As blocking buffer to avoid non-specific binding of the detection antibody, slides were incubated with 3% bovine serum albumin (BSA) for 30 minutes at 37ºC, in a moisture chamber. Anti-digoxigenin antibody conjugated with fluorescein isothicyanate (FITC) (*Roche*) was used to detect the probe signal for an hour and a half incubation at 37ºC in a moisture chamber. The slides were washed twice in 1x PBS at RT for 7 minutes and counterstained with DAPI in antifade solution.

Major rDNA FISH was exclusively used in allopatric *S aradensis*, for karyotype characterization purposes. A rDNA probe containing 18S-5.8S-28S genes plus an intergenic spacer of *D. melanogaster* (pDm 238 clone) was used to localize the repetitive rDNA copies. The probe was labelled by nick translation with digoxigenin-11-dUTP (*Roche*) and disolved in hybridization mix. The pre-hybridization procedures were performed as described above for GISH. Hybridization was allowed to proceed

overnight at 37ºC in a dark moisture chamber. Detection was performed as previously described. Chromosomes were counterstained with DAPI or PI in antifade solution.

Image Processing

Slides were screened in an Olympus BX 60 microscope equipped with a DP50 Olympus CCD camera. All images were processed using *Adobe Photoshop CS4* software.

Chromosomes were arranged in a decreasing size order and classified according to their arm ratios (34) in three morphological groups: metacentric (*m*), submetacentric (*sm*) and sub-telocentric to acrocentric (*st/a*). In order to establish the fundamental number (FN), the chromosomes of the *m* and *sm* groups were considered biarmed and those of group *st/a* were considered uniarmed.

Results

Chromosome spreads were obtained for some of the initially sampled individuals discriminated in **Table 1**.

Table 1. Ploidies, genomic constitutions and sex of the *S. alburnoides* and *S. aradensis* individuals analyzed.

Basin	***Riverside***	***Species***	***Ploidy***	***Genomic Composition***	***Sex***	***No. of specimens***
Arade	Rib.ª Odelouca	*S. aradensis*	2n	QQ	F	1
			2n	QQ	M	5
Quarteira	Porto Nobre	*S. aradensis*	2n	QQ	M	1
		S. alburnoides	3n	QAA	F	2
			2n	QA	M	1

Squalius aradensis (QQ)

Genome characterization of *S. aradensis* was performed on six allopatric specimens. Chromosome counts revealed a diploid number of 50, in 73% of all 757

metaphase spreads analyzed. The remaining results presented hypomodal values, most likely due to technical artifacts.

The most consistent karyotype formula consisted of five pairs of metacentric (*m*), eighteen pairs of submetacentric (*sm*) and two pairs of subtelo/accrocentric (*st/a*) chromosomes (**Figure 1**). This genome composition leads to a high fundamental number (FN=96). No clear distinction between male and female karyotypes was observed, as no evident heteromorphic sex chromosomes were detected.

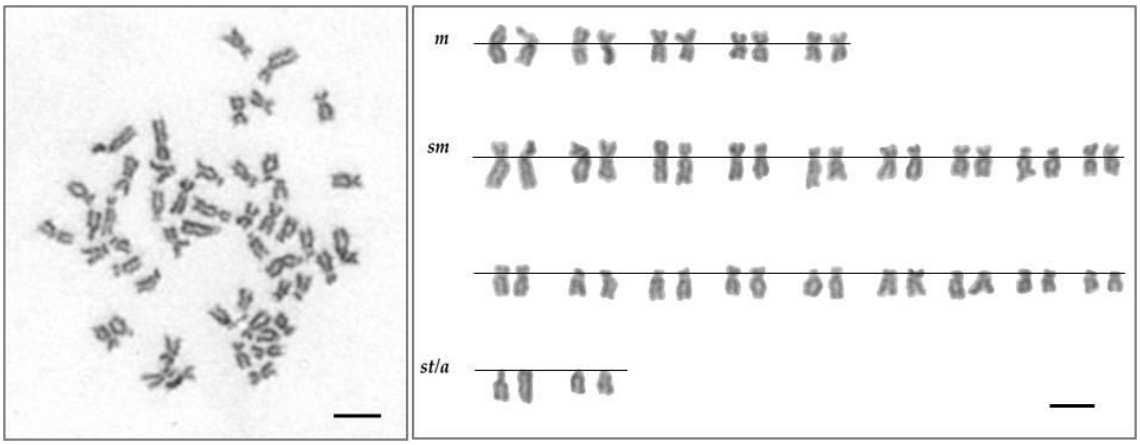

Figure 1. Giemsa stained metaphase and corresponding karyotype of an allopatric *S. aradensis* (QQ) male. Scale bar = 10µm.

The NORs' phenotype was constant throughout all the treatments that consistently stained only one NOR-bearing chromosome pair. The rDNA-positive signal was co-localized to CMA_3- and Ag-positive signals, in the short arm of a submetacentric chromosome pair (*sm*7) as illustrated by sequential staining (**Figure 2**), demonstrating to be GC-rich and transcriptional active. No evidences of multi-chromosomal positive signals were registered.

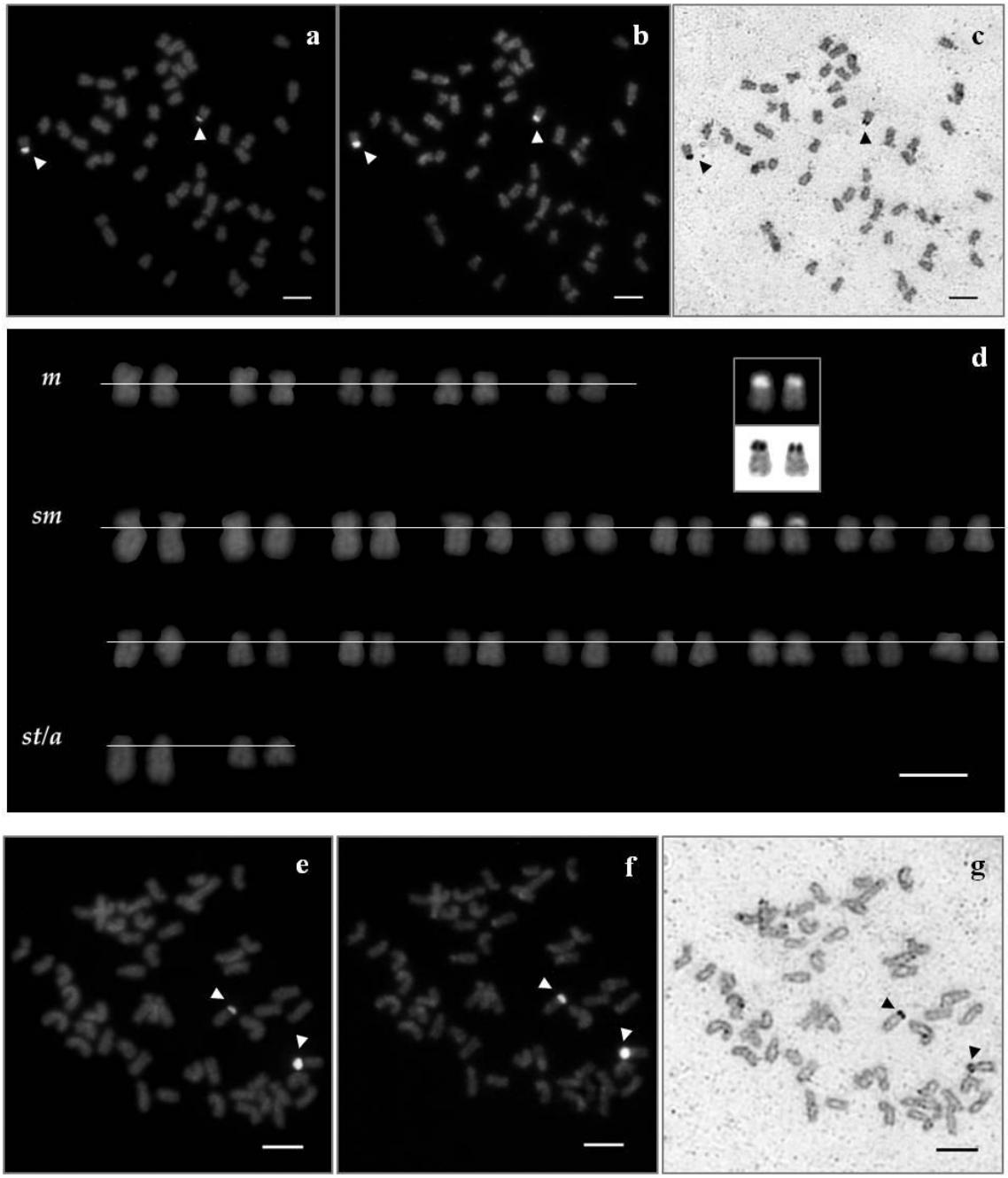

Figure 2. Metaphase spreads of a *S. aradensis* (QQ) (a-c) male and (e-g) female, stained with sequential (a, e) rDNA FISH, (b, f) CMA_3-NOR and (c, g) Ag-NOR treatments. (d) Corresponding karyotype of the same male individual after rDNA FISH, with CMA_3- and Ag-NOR results inset. NORs are indicated by arrowheads. Scale bar = 10µm.

Genomic in situ *Hybridization*

GISH allowed successful discrimination of the chromosome sets of 'A' and 'Q' parental genomes. Both QA (**Figure 3**) and QAA (**Figure 4**) hybrid metaphases presented either chromosomes fully hybridized or lacking hybridization, in agreement with the determined genotype for each individual. In 106 complete chromosome spreads 92% were unequivocally identified.

No evidences of intergenomic exchanges were found in the diploid and triploid hybrid metaphases analyzed. There was no consistent hybridization of the A probe in the heterospecific Q chromosome set, always showing a more or less uniform counterstaining by DAPI. The GISH hybridization pattern was not, however, uniform over the whole chromosome length. The hybridization signals were stronger in the pericentromeric regions, particularly in metacentric chromosomes and in the telomeric regions of many *S. alburnoides* chromosomes, presumably due to a higher amount of constitutive heterochromatin (**Figures 3 and 4**).

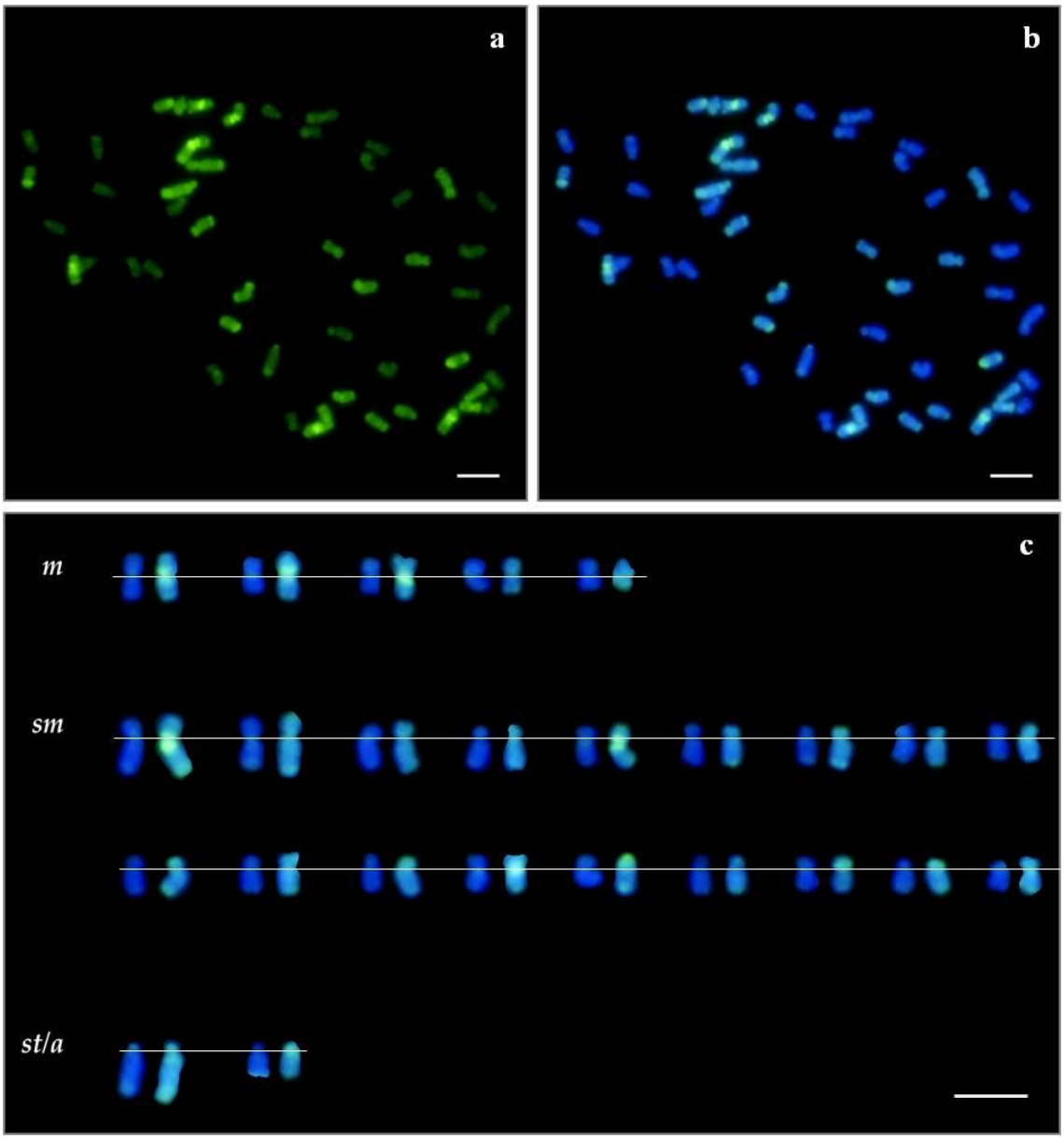

Figure 3. GISH on metaphase chromosomes of *S. alburnoides* QA specimen (**a**) *S. alburnoides* AA genomic probe visualized by yellow-green FITC labeling (**b**) DAPI/FITC merged and corresponding (**c**) karyotype. Scale bar = 10µm.

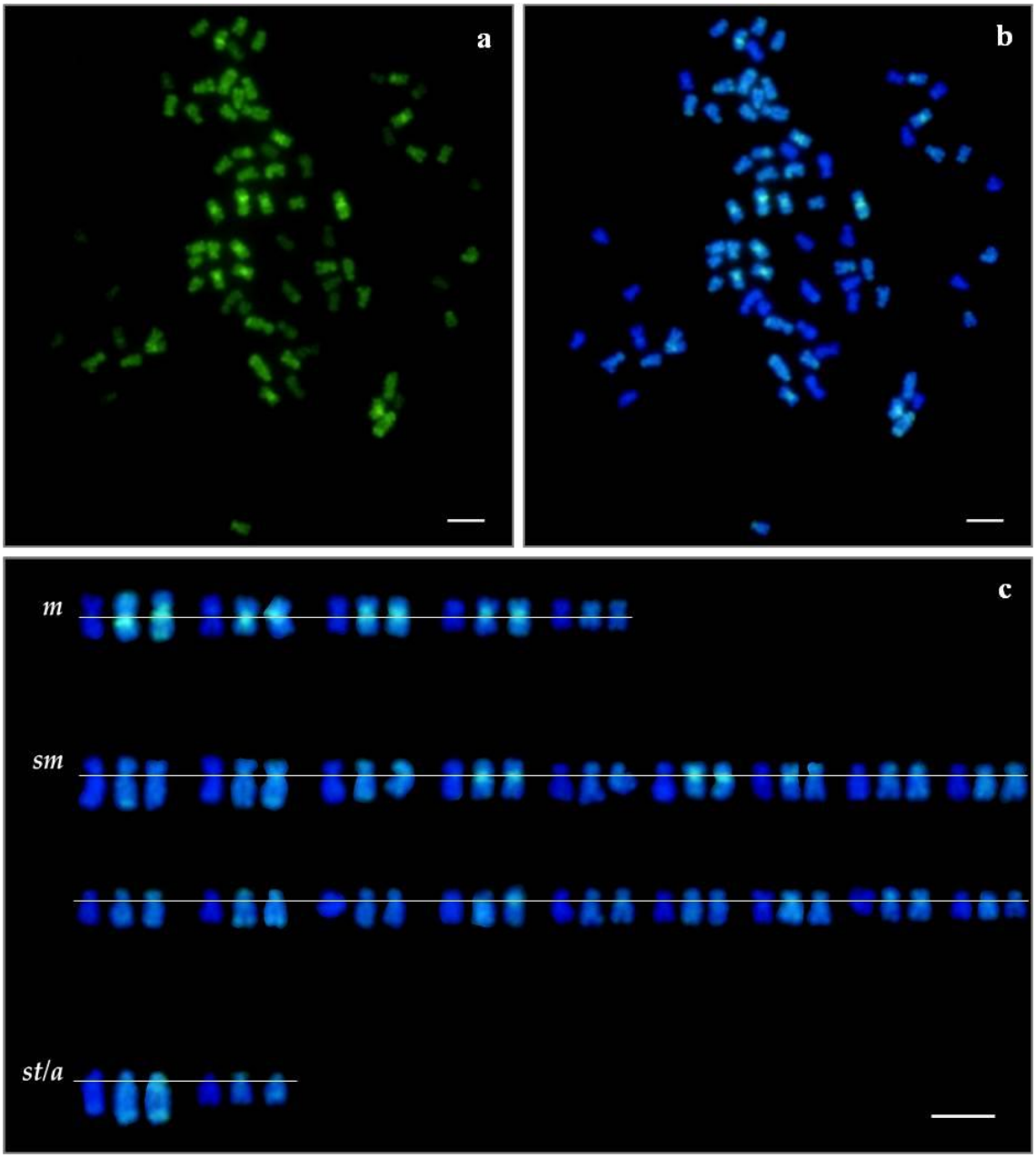

Figure 4. GISH on metaphase chromosomes of *S. alburnoides* QAA specimen (**a**) *S. alburnoides* AA genomic probe visualized by yellow-green FITC labeling hybridization (**b**) DAPI/FITC merged and corresponding (**c**) karyotype. Scale bar = 10µm.

Labelled AA-GISH hybridization onto metaphases of both sympatric and allopatric *S. aradensis* (QQ specimens) did not detect any visible differences. The probe signals were faint and the pattern observed resembled that of the Q chromosomes in *S. alburnoides* hybrids (**Figure 5**).

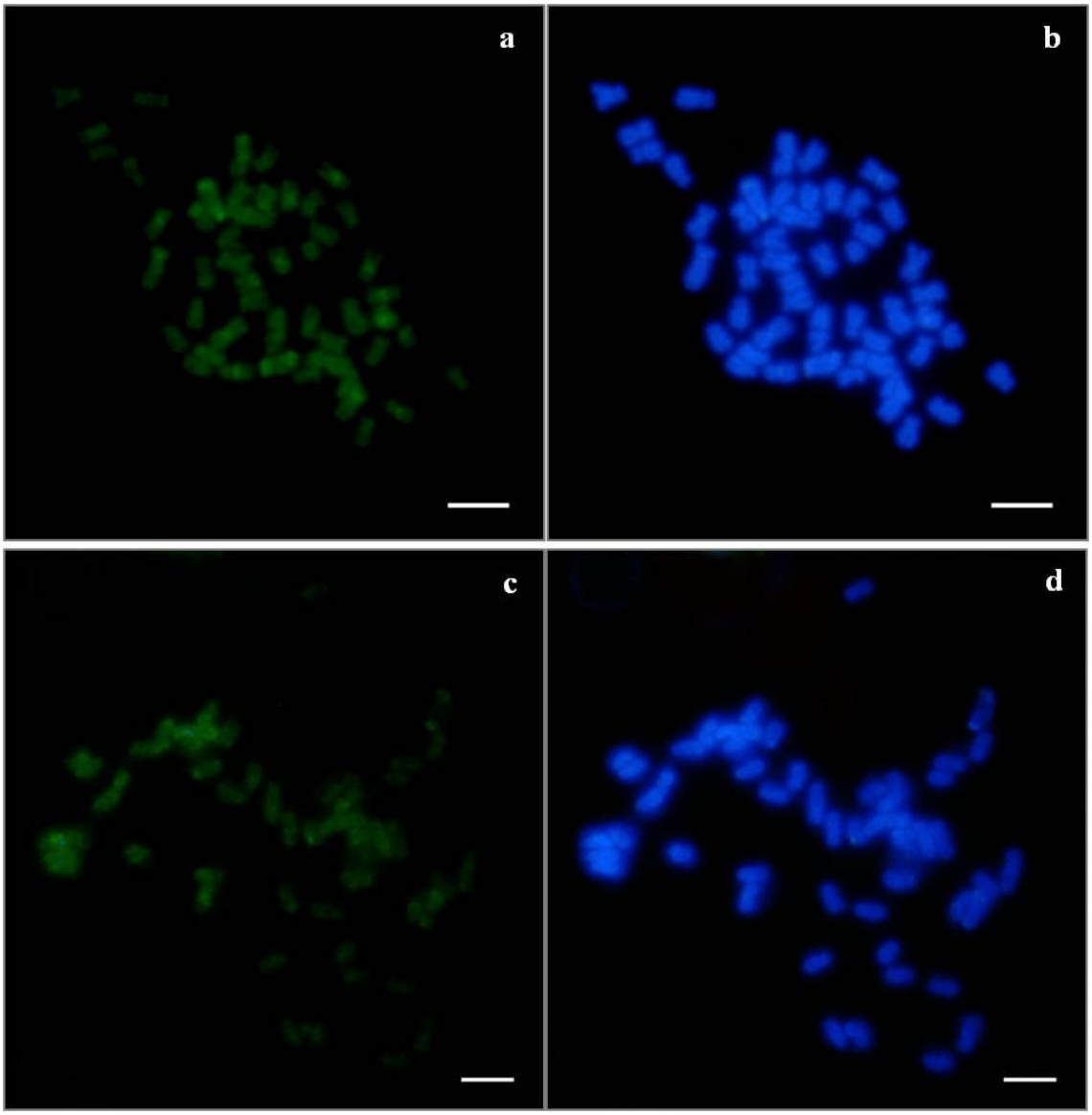

Figure 5. GISH using labeled AA genome probe on chromosomes of (**a**, **b**) allopatric and sympatric **(c**, **d**) *S. aradensis*, (**a, c**) hybridization signal using FITC (**b, d**) DAPI/FITC merged. Scale bar = 10µm.

Discussion

Squalius aradensis karyotype follows what is described for most species of the Leuciscinae subfamily regarding a diploid value of 50 chromosomes and a formula composition of few meta- and subtelo/acrocentric chromosomes, plus many submetacentric ones, with only one NOR-bearing chromosome pair (35).In cyprinids this is assumed to correspond to the primitive character state. Conversely to the closely *related S. pyrenaicus* (16, 21), no NOR polymorphism was found. It is then acceptable to say that this species conserved the plesiomorphic condition (reviewed in (36)).

Similar to most cyprinid species, sex chromosomes if present, apparently remained morphologically undifferentiated, although few studies have proposed the occurrence of heteromorphic sex chromosomes in some other species (36, 37). The results are also in agreement with the synaptonemal complexes analyses, where no clear heteromorphic sex chromosomes were detected (see **Research Article 2)**.

Genomic *in situ* hybridization allowed a successful discrimination of the two distinct parental genomes in both QA and QAA specimens, supporting the use of this technique in the *Squalius alburnoides* complex to unequivocally identify specimens' genomic composition. Notably, this whole-genome approach allowed visualizing the hybrid character of natural *S. alburnoides*, as years of studies have demonstrated. These hybridization results also agree with beta-actin sequencing analyses regarding genomic composition. That is of great interest given the protocol was inferred for *S. alburnoides* P genome-bearing populations (29) and extrapolated for this *S. alburnoides* Q genome-bearing population, proving it to be useful in future studies. The successful application of GISH also suggests that the divergence time between the A and the Q genomes must be over a million years (enough divergence for a differential staining), but should be inferior to 5 million years, otherwise genome turnover would not allow any proper differences to be visible as well (genome homogenization processes, for e.g. by transposable elements activity (38)).

Evidences of interchromosomal exchanges were not found in hybrid genomes, conversely to what has been described in *Ambystoma* salamanders (5) and several polyploid plants (39-41). GISH results in *S. alburnoides* population from Quarteira reported the integrity of the two parental chromosome sets suggesting no interaction between the heterospecific genomes during gametogenesis. QAA hybrids have their GISH results corroborated by synaptonemal complexes analyses, suggesting that during meiotic hybridogenesis synapses are most likely strictly homospecific hence excluding non-homologous recombination (see **Research Article 2**). In such case, genome inheritance is likely to be regular, preserving the integrity of species genome copies and only shuffling the chromosome sets in the distinct *S. alburnoides* forms.

Stronger hybridization signals in pericentromeric chromosome regions just as found in *S. alburnoides* have been described, for example in *Ambystoma* hybrid complex (5), in some Cichlidae (12) and in the hybrid *Pelophylax esculentus* (13), although in the

present case they seem to be more restricted to metacentric chromosomes. Pericentromeric DNA is known to be composed of rapidly evolving repetitive sequences, thus acquiring a species-specific status, as has been pointed in several species (e.g. (12)). Taking that into consideration, GISH can be used as a phylogenetic tool. Though closely related species share common DNA sequences, as evolutionary distance increases the amount of such common sequences decreases. A study by Lim *et al.* (2007) (38) on *Nicotiana* polyploid species was able to disclosure its evolutionary history using both GISH and FISH. Thus by comparing genomic hybridization patterns in distinct species it is possible to associate more closely related specimens where hybridization is more extensive than in species with distant phylogenetic relationships (with lower genome homology).

GISH positive hybridization signals were not noticeably different between sympatric and allopatric *S. aradensis* (QQ). Thus no extended 'A' genomic probe hybridization in both *S. aradensis* populations exists. This accounts for no visible evidences of introgression of 'A' *S. alburnoides* genome into 'Q' genome at the chromosome macrostructural level.

Although the present results did not evidence intergenomic exchanges at the chromosome level, further work should be done, namely by increasing sample size, in order to confirm this supposition. In any case, genomic integrity observed by GISH does not necessarily translate into gene integrity given that recombination might in fact take place but involving such small chromosome regions that these are not visible by means of conventional optical microscopy and/or without recurring to more advanced techniques of molecular cytogenetics (e.g. DNA combing).

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Synaptonemal complexes in the hybridogenetic *Squalius alburnoides* complex: new insights on allopolyploids' gametogenetic processes

Abstract: In the *Squalius alburnoides* fish complex, triploid females reproduce mainly by meiotic hybridogenesis. A synaptonemal complexes (SCs) study was performed in specimens from a confined population (Quarteira) to understand chromosome dynamics during gametogenesis. This approach allowed to examine synapses in hybrids and to compare them to the parental bisexual species *Squalius aradensis*. Results revealed regular synapses in *S. aradensis* and no evidences of sex chromosomes. Synapses' formation pattern was also ascertained. Pachytene analyses in allotriploid females showed that their meiocytes present the complete triploid chromosome set (75 chromosomes) in prophase I. Also, two distinct types of oocytes were observed, suggesting a two-phase synaptic process where, in a first round, exclusively homologous SCs form and univalents of the haploid genome are arranged in a *bouquet* conformation. Later, a second synaptic phase appears to take place and the heterospecific genome forms non-homologous SCs, with multivalent associations. The results disagree with most literature on the meiotic process in non-sexual vertebrates, since both premeiotic chromosome exclusion and whole genome endoduplication have been considered the most likely mechanisms occurring in the gametogenetic processes of allopolyploids. Thus, *S. alburnoides* complex can be an interesting model system to study meiotic regulation whenever hybrid genomes come into contact in the same germinal cell.

Keywords: Allopolyploid, Meiotic hybridogenesis, Synaptonemal complexes, Gametogenesis, *Squalius alburnoides, Squalius aradensis*

Introduction

In the last 20 years an increasing number of studies have been demonstrating that unorthodox modes of reproduction in vertebrates are not just an odd curiosity apart from the evolutionary mainstream (1). In fact, among vertebrates, non-sexual reproduction has been fairly described in fishes, amphibians and reptiles (1, 2). These deviations to sexual reproduction seem to be, in general, linked with hybridization events: the combination of distinct genomes from two genetically distinct parental species (3). Interspecific hybridization typically skews the sex-ratio in the hybrids toward females and alters gametogenesis in a way that the reproductive strategies adopted usually involve little or no genetic recombination (4).

Squalius alburnoides is an allopolyploid fish complex, endemic to the Iberian Peninsula. It consists of diploid (2n=50) and polyploid (3n=75 and 4n=100) forms with sexual and mainly non-sexual modes of reproduction (reviewed in (5)). Natural populations differ considerably in terms of biotypes and ploidies found, depending upon ongoing hybridization with sympatric bisexual *Squalius* species, namely with *S. aradensis* in the small southern population of Quarteira (herein addressed). In most populations there is a triploid female dominance, sustaining the most representative altered mode of reproduction within the complex; meiotic hybridogenesis (2, 6, 7). In hybridogenesis only part of the hybrid genome, derived from one of the parental species, is transmitted to the gamete, while the other parental genome is discarded and restored in each generation through fertilization (2). In *S. alburnoides* triploids however, the meiotic hybridogenesis process involves not only the exclusion of the heterospecific genome, but additionally, random segregation and recombination between the two homospecific genomes takes place, leading to the production of haploid gametes (2, 4).

Genetic studies performed upon experimental crosses (4, 8) allowed a clearer understanding of *S. alburnoides* reproductive pathways, explaining its continuous generational shifting between distinct genomic forms. Breeding experiments, with DNA fingerprinting of both parents and their progeny, demonstrated that triploid mothers produce haploid (and rarely diploid or even clonal gametes (9)) gametes, which are then fertilized by a male sperm donor (10). Offspring resulting from those crosses evidenced recombination of the non excluded maternal genomes (whereas the heterospecific genotype was never inherited) suggesting that along the maturation process of the

oocytes, at least part of meiosis was retained (4, 8). Alves *et al.* (1998) (4) suggested that results agreed with the patterns observed in allotriploid *Pelophylax esculentus* (11) where the two homospecific genomes should form regular synapsis and undergo meiosis, after the elimination of the unmatched genome. However, the actual meiotic process has never been studied in *S. alburnoides* complex. Meiotic studies in other vertebrates' complexes were mostly performed in meiotic figures and not in synaptonemal complexes (e.g. (12-14).

Synaptonemal complexes (SCs) analyses have been widely used in cytogenetic studies to address the process of meiotic chromosome synapses, because of the high-resolution allowed by electron microscopy (15). The SC is an evolutionary well-conserved structure only found on meiotic prophase chromosomes (16). It presents a tripartite proteinaceous organization, around 100nm width, connecting paired chromosomes along their longitude (17). It is composed of two outer protein axes (lateral elements) and a third central element, running in parallel (16, 17).

In general, SC formation begins at the sub-distal regions of the chromosomes, but additional interstitial synaptic initiation sites may also form, particularly in genomes with large chromosomes. Nevertheless, there is a considerable flexibility regarding this process and for instance, asynchronous of bivalent formation within a nucleus is common. Studies have revealed that synaptic process is largely variable from one organism to another (16).

Overall, synapsis occurs preferentially between homologous chromosomes. In theory, the number of synaptonemal complexes (SCs) at a meiotic prophase nucleus corresponds to the species haploid chromosome number. However SCs can also form between common regions of non-homologous chromosomes, but in that case synapsis tends to be irregular and often, instead of bivalents, multivalent associations form involving more than two chromosomes. Several studies have seen that only chiasmate bivalents are present at metaphase I, suggesting that, in general, crossing over is restricted to homologous synapses and doesn't occur between non-homologous chromosomes (16).

SCs have vastly been used to study non-diploid vertebrates, in particular triploid genomes. A very interesting feature of triploids is their possibility to form trivalents by associating the lateral elements of three distinct chromosomes (18-21). The presence of

an extra chromosome set, usually results in non-homologous synapsis, sometimes in the form of multivalent associations. In general, multivalent associations do not present full synapses, covering only a small fraction of the entire chromosome length instead (20), probably due to partial homology and to the synaptic adjustment mechanism. This process takes place by late pachytene, regardless of homology, and involves changes in the synaptic configuration, allowing both heterosynapsis and pairing of homologous regions that escaped synapsis at zygotene, thus resulting in SC configurations with irregular synaptic shapes (20). With respect to fishes, trivalent association is more frequently found amongst autotriploids for e.g. in *Trichomycterus davisi* (21) and in *Scophthalmus maximus* (20).

The study of SCs can be accomplished either by 3D reconstruction of meiocytes or applying 2D surface techniques, easily visible by protein marking methods such as silver staining (15, 16). In the present work a surface spreading technique was used for visualization by electron microscopy. This technique is advantageous in allowing analysis of a large number of nuclei and it is particularly useful to study meiotic pairing behavior and the structural organization of synapsis in polyploids (16). The goal is to understand how organisms cope with extra chromosome sets to produce viable gametes.

This work presents a preliminary meiotic study in *S. alburnoides* triploid females and in male and female *S. aradensis*. The aim was to address chromosome synaptic behavior by observation of SCs during gametogenesis. The goal was accomplished for allotriploid females with a QAA genomic composition, belonging to a confined *S. alburnoides* population located in Quarteira (Algarve, Portugal) in sympatry with *S. aradensis* (presenting the 'Q' genome type).

Materials and Methods

Fish sampling and maintenance

Adult fish were captured by electrofishing in November 2010 in the southern Quarteira population. All specimens of *S. alburnoides* and sympatric *S. aradensis* were collected at Porto Nobre (Quarteira river basin). Fish were transported to the laboratory and kept at room temperature in well aerated 25 liters aquaria, with 12 hour light photoperiod. In February 2011, according to data on these species reproductive season and plausible period for favorable meiocyte maturation (22), the water temperature was

artificially raised to 22ºC and the daily feeding intake supplemented with frozen *Artemia salina*.

Ploidy and genomic composition screening

Individual identification was accomplished through photographic recognition according to the method described in (23). Specimens' ploidy was assessed measuring the DNA content of erythrocytes (RBC) by flow cytometry, using a Coulter Epics XL cytometer and following the technique used in (24). Fin clips were also collected and used for DNA extraction (25), to further determine specimens' genomic profile, by sequencing and analyzing a 935 bp fragment of the beta-actin gene, as described in (26). Together with morphological traits, the previous characterization methods offered a reliable genomic composition inference for each individual (see **Research Article 1**).

Synaptonemal Complex Preparations

Several experiments were performed throughout the month of March, due to the uncertainty of the accurate meiocyte maturation period prone to SCs observation. Adult fish were sacrificed with an overdose of tricaine mesylate anesthetic solution (MS-222, *Sigma*). Gonads were then dissected and macroscopically classified as male or female. SCs spreading preparation was conducted according to the protocol described by (27).

Gonads were washed in sterile PBS and then transferred to Dulbecco's medium (*Gibco*: with 4.5g/L D-glucose, with L-glutamine and without sodium pyruvate) containing 2 mmol/L EDTA and 0.1% bovine serum albumin, and cleaned from fat and adjacent tissues. Small portions of the gonads were homogenized to obtain a cloudy cell suspension, which was then centrifuged at 1500 rpm for 10 minutes to settle and discard the majority of the supernatant containing tissue blocks. Fresh Dulbecco's medium was added to accomplish a proper cell dilution and was kept on ice until performing the surface-spreading technique. For later electron microscopy analysis, the slides were coated with a plastic film, produced by dipping them in a 1% (w/v) solution of plastic solved in chloroform. One drop of the cell suspension was applied to the coated microscope slide. Two drops of spreading medium (60 mmol/L phosphate buffer, 1 mmol/L EDTA, 0.03% Triton X-100, pH 7.5) were added on top, mixed with the suspension and allowed to settle for 6-8 minutes. Several drops of paraformaldehyde fixative (4% (w/v), pH 8.9) were added and mixed by tilting the slide in different

directions. Slides were dried overnight on a warm plate heated around 37ºC, then washed in water for 30 seconds, and air dried in a vertical position. Preparations were stained using a nylon mesh impregnated with 40% silver nitrate solution and incubated in a moisture chamber at 45ºC for 30 minutes, until turning to a caramel coloration. Slides were washed in distilled water and air dried in a vertical position. The preparations were scanned under a bright field optical microscope to locate areas with well-spread SCs structures, which were then cut, floated off of the slide in distilled water and picked up with 50-mesh copper electron microscope grids.

Image processing

Photographs were captured using a Jeol 1200 electron microscope. Images were analyzed with *Adobe Photoshop* CS4 software.

Results

Successful pachytene SCs results were obtained for two QAA triploid *S. alburnoides* females and three *S. aradensis* specimens; two males and one female (**Table 1**). Most slides presented few or no SCs spreads per specimen.

Table 1. Ploidies, genomic constitutions and sex of the *S. alburnoides* and *S. aradensis* specimens analyzed.

Fish Identification	***Ploidy***	***Genotype***	***Sex***
*S. alburnoides*1	3n	QAA	Female
*S. alburnoides*2	3n	QAA	Female
*S. aradensis*1	2n	QQ	Female
*S. aradensis*2	2n	QQ	Male
*S. aradensis*3	2n	QQ	Male

Squalius aradensis

Results were very scarce for *S. aradensis*. Only one pachytene nuclei for each male and female specimen were successfully analyzed, yet they granted a general idea of the

synaptic process in this QQ genome species. Twenty five perfectly synapsed bivalents were found, confirming the diploid condition of 2n=50 chromosomes described from somatic karyotype (See **Research Article 1**) and no evidence of heteromorphic sex chromosomes (**Figure 1**).

The *bouquet* formation was found in this species, as illustrated in a male's SC spread (incomplete, with twenty three bivalents) (**Figure 1b**). This chromosome arrangement should be retained throughout pachytene, suggesting its stability. Bivalents revealed a clear centromere demarcation, as well as thickened telomeric ends, both characteristic of a typical SC formation. These aspects were identified by the stronger staining resulting from attached proteins. Synapsis was regular; SCs were formed along the entire homologous chromosomes' lengths, presenting constant width and the centromeres perfectly aligned (**Figure 1c**).

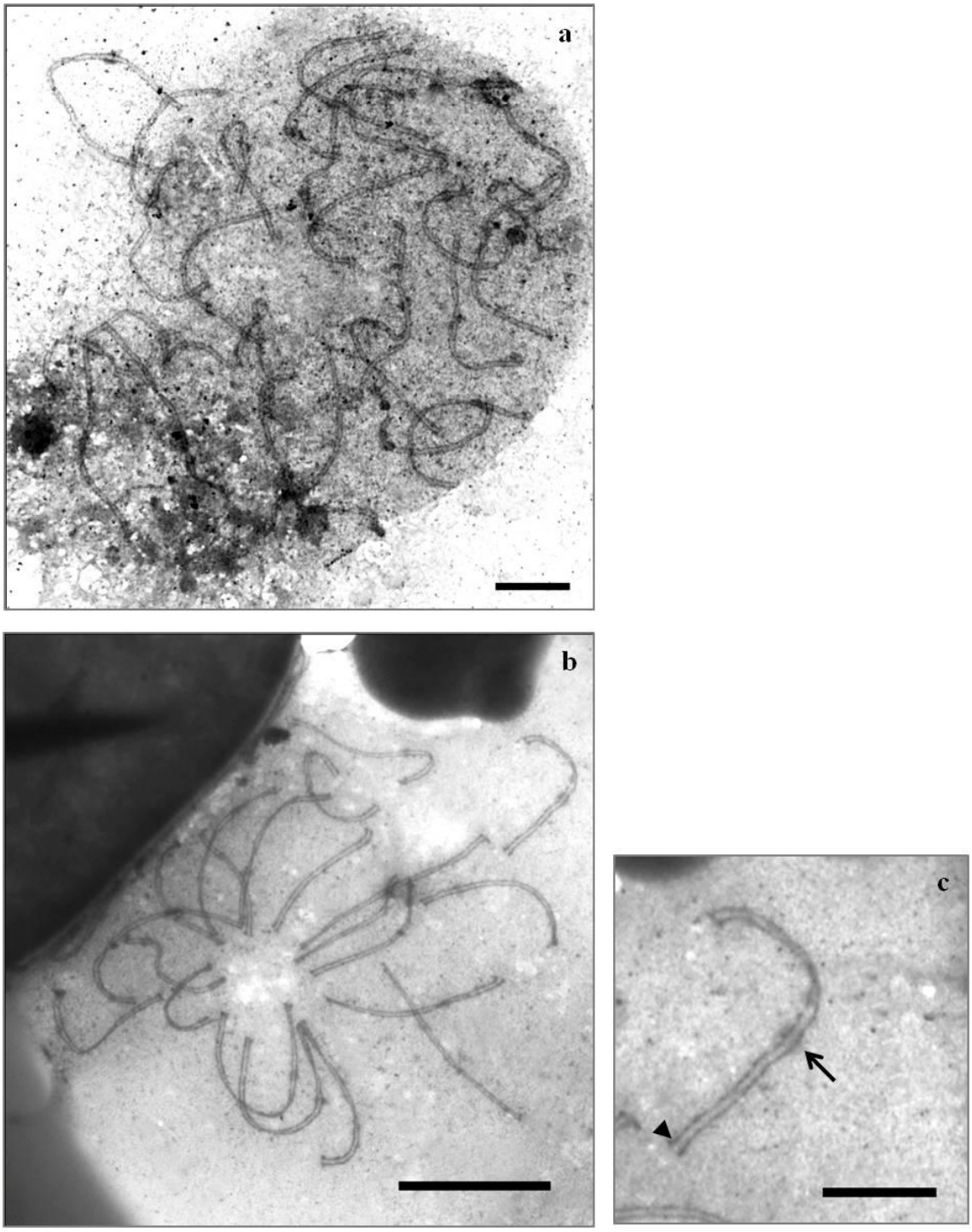

Figure 1. Electron micrographs of two pachytene nuclei from *S. aradensis* (**a**) female presenting 25 and **(b**) male presenting 23 homologous bivalents (incomplete). Scale bars = 5µm. In detail, (**c**) a regular bivalent formation revealing characteristics of a typical SC: a clear centromere (arrow) and the thickening at telomeric ends (arrow head). Scale bar = 2µm.

Regular synapses were also found in the female's pachytene nucleus (**Figure 1a**). Interestingly, at first sight, female SCs appeared longer than male ones (**Figure 1a** and

Figure 1b, respectively). However, a larger sample and accurate measurements are required to support this speculation.

Records of a mid-late zygotene stage were obtained in a male spread, allowing observing some of the initial steps of the synaptic process. This was particularly useful to understand the formation of SCs amongst these fishes. Asynchrony of the SCs formation was evident, because differences in the progression of synapsis were observed within a same nucleus (**Figure 2**). Chromosomes appeared to start their alignment over a significant length, as seen by the proximity of the two axial elements along an extensive longitude (nearly becoming parallel) (**Figure 2a**). Synapses seemed to form near chromosome ends, after homologues' alignment and pairing (arrow head in **Figure 2a**). It most likely begins on one of the two sub-distal regions of the paired homologues (**Figure 2b**) and then extend to the opposite end (**Figure 2c)** until it is completed **(Figure 2d**).

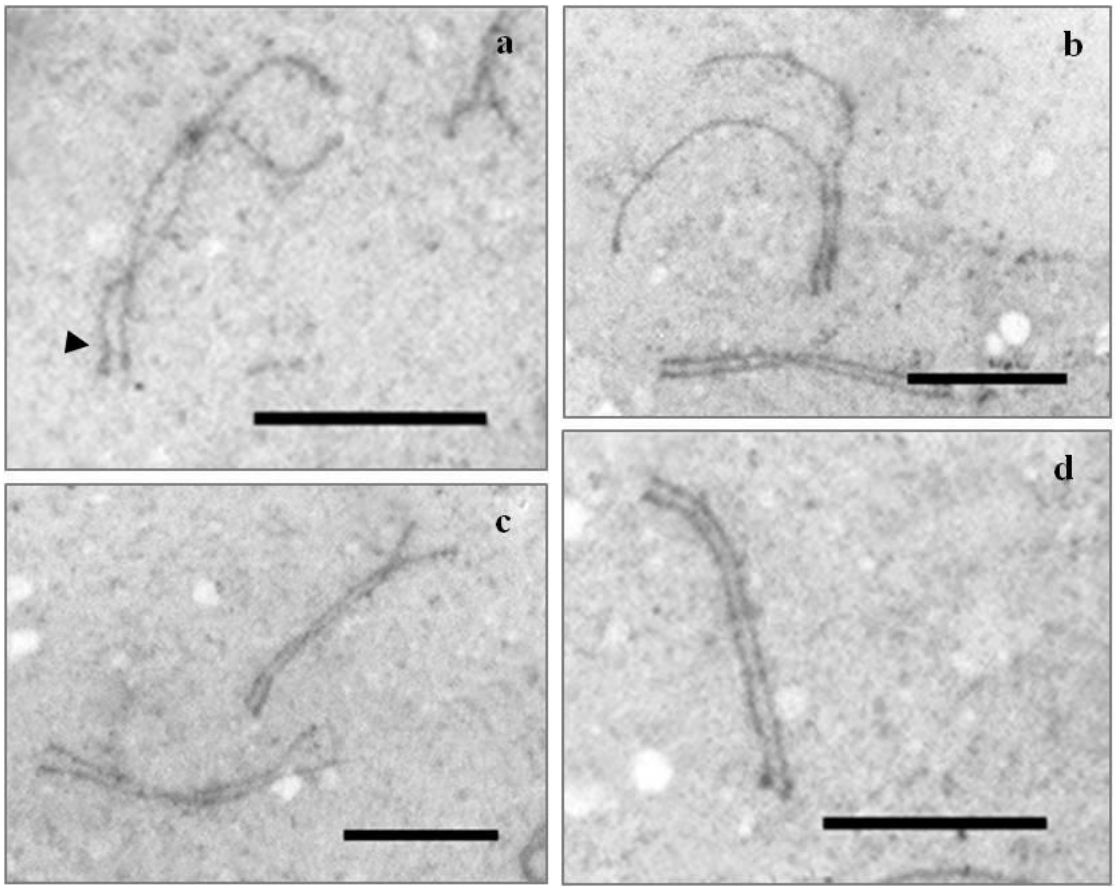

Figure 2. Electron micrographs details of synapse formation in *S. aradensis* step-by-step: (**a**) chromosomes' alignment and the presumptive synapsis initiation site near a distal region (arrow head); (**b**) SC progressively being established between two homologues; (**c**) synapsis nearly complete, except at one or both chromosome ends; (**d**) complete synapsis. Scale bar = 2µm.

Squalius alburnoides complex

Twenty three pachytene nuclei were fully analyzed amongst a total of 36 examined by electron microscopy for triploid *S. alburnoides* females, seven nuclei in one female (*S. alburnoides*1) and 16 in the other one (*S. alburnoides*2).

Allotriploid QAA oocytes presented 75 pachytene chromosomes (the triploid value). However, two distinct case scenarios were found: *i)* either 25 regular homologous bivalents, plus 25 single elements in the form of univalents (78% of the cases) (**Figure 3a-c**) or *ii)* much more than 25 bivalents with almost all chromosomes (at least >75%) forming synapses (22% of the cells) (**Figure 4**). The most frequent case where 50 chromosomes engaged in regular SCs associations, supports the parsimonious explanation that in allotriploid females with a QAA genomic composition, the two A genomes form perfect homologous synapses (**Figure 3d**), while the heterospecific Q genome, from a distinct parental genome, remains as single elements. It is relevant to notice that while the homospecific SCs are found scattered around the nuclei, the heterospecific univalents remain closely together, sometimes even in a *bouquet* arrangement (**Figure 3b**). The second case demonstrates the occurrence of irregular non-homologous synapsis supposedly among the heterospecific chromosomal set, where multivalent associations between the 25 Q chromosomes were also found, presenting uneven SCs' width as a result of unsynaptic regions (**Figure 5**). The transition from the *bouquet* organization to a phase where synapses were mixed in a disordered manner suggests a shift to a more advanced pachytene time frame (**Figure 4**).

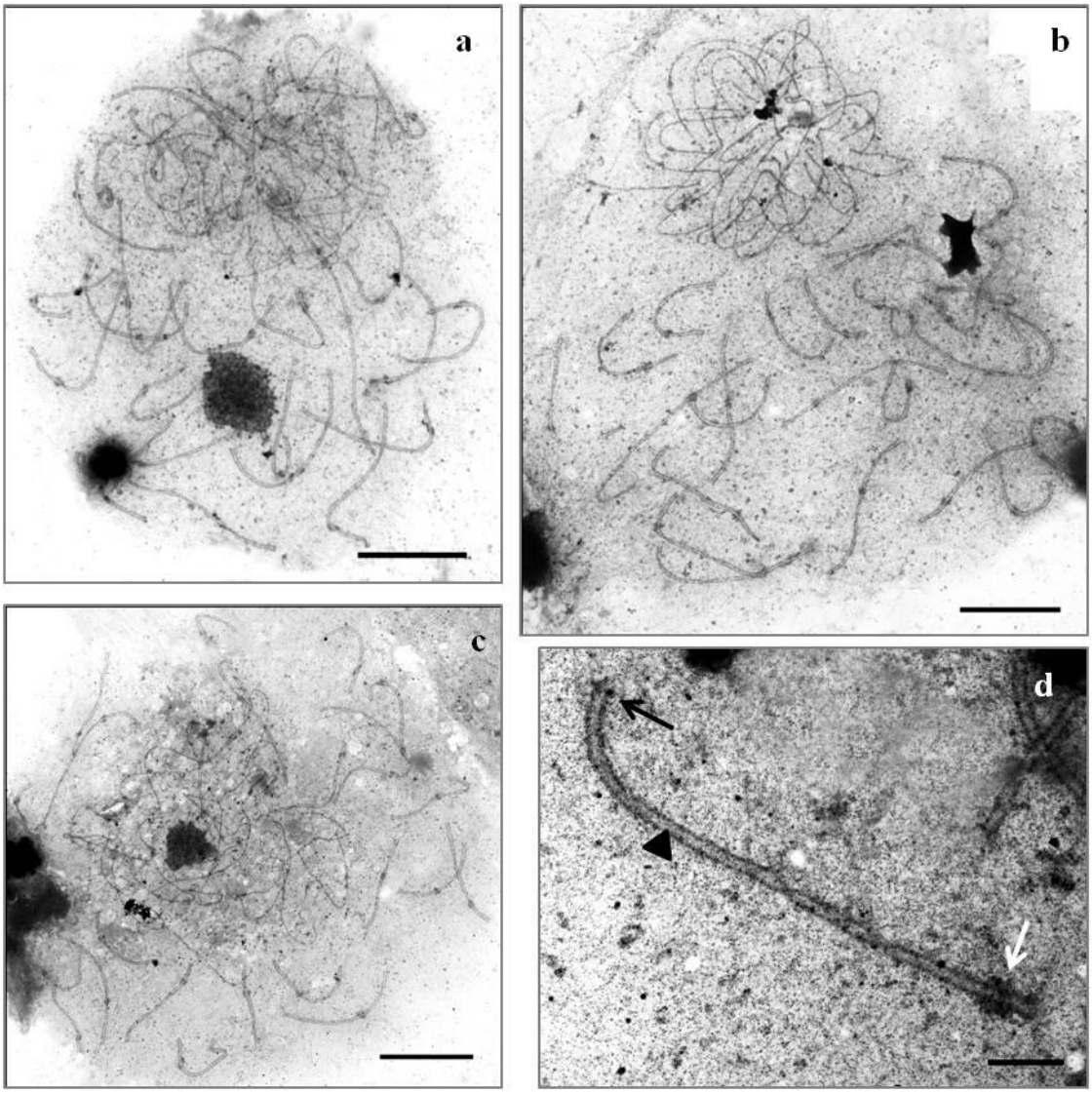

Figure 3. Electron micrographs of complete pachytene nuclei from distinct triploid QAA *S. alburrnoides* females: **(a-c)** 25 bivalents resulting from homologous synapsis between AA genome and 25 univalents corresponding to Q genome. Scale bar = 5µm. **(d)** *S. alburnoides* triploid females' detail of a presumably '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 (arrow head). Scale bar = 1µm.

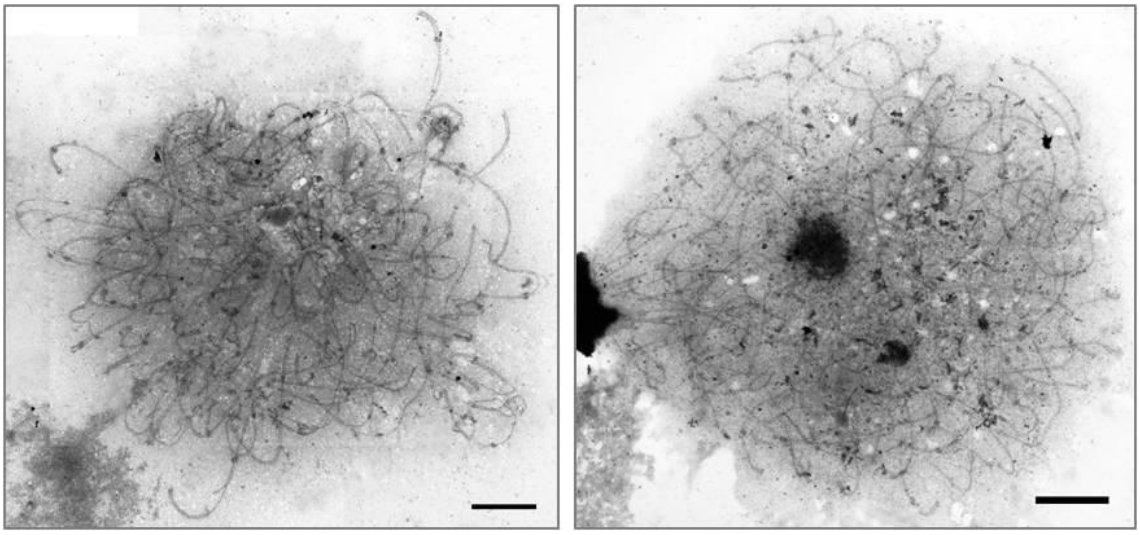

Figure 4. Electron micrographs of complete pachytene nuclei from distinct triploid QAA females presenting both homologous (between AA genomes) and non-homologous (Q genome) synapsis. Scale bar = 5µm.

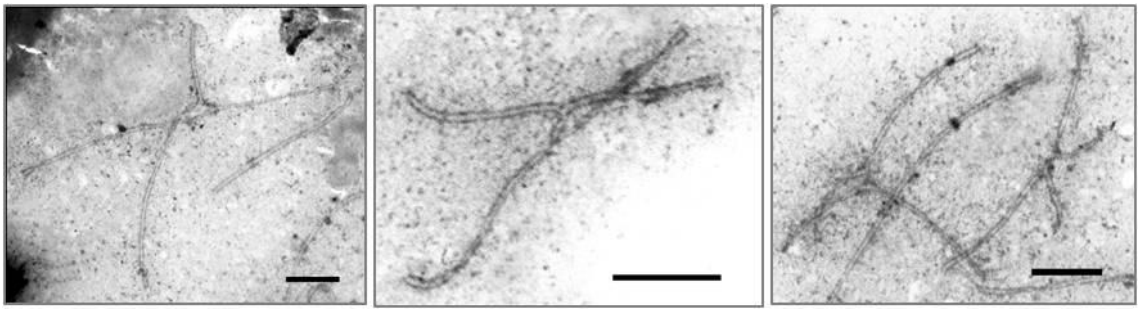

Figure 5. Electron micrograph details of a QAA *S. alburnoides* pachytene nucleus evidencing non-homologous synapsis involving the Q genome in the form of multivalent associations. Scale bar = 2µm.

Differences in the overall individual results obtained for the two triploid females might be correlated to their sexual maturation stage. Considering the percentage of cells with high synaptic profile, *S. alburnoides*1 was in a more advanced stage of maturation than *S. alburnoides*2, whose cells were largely in the stage where Q genome remains unsynaptic. These results are congruent with the macroscopic appearance of their gonads, with the more mature female presenting more yellowish and bigger oocytes (data not shown).

Discussion

The absence of SCs results for the majority of specimens processed suggests that most likely March was not the best period for the desired gonads' maturation stage and thus few cells in an early meiotic stage were present. For further meiotic studies in these species, January or February may be the recommended period of time.

Both male and female *S. aradensis* synapses proved to be regular, with centromeres and telomeric ends perfectly aligned. . Sex differences in SC length, though interesting, might be due to distinct pachytene stages and only precise measurements of fully formed pachytene SC in a large number of male and female meiocytes could confirm it. This phenomenon has been described in zebrafish (28) and more recently in *Tilapia* (29) but it is particularly known in mammals, namely in humans (30). Differences in SC length have been attributed to distinct pachytene chromatin compaction and has been also suggested that inter-sex variation may depend upon the number of chromatin loops that associate with the meiotic chromosome core (30).

Pairing and synapsis in *S. aradensis* appear to begin exclusively in sub-distal chromosome regions and extend towards the middle. This situation has been described in several vertebrates and is common in animals with small chromosomes like fish species (15, 20, 27).

The presence of a *bouquet* stage allowed supporting a time-line in triploid meiocytes. Knowing this arrangement can be maintained throughout pachytene, but is still corresponding to an early stage (**Figure 3**), suggests that a subsequent meiotic phase, where that configuration is lost, follows (**Figure 4**). According to this, synapsis in allotriploid females seems to present a two-phase formation, where 25 homologous bivalents first form and as they become fully assembled and mature, disperse in the nucleus. These SCs are regular with constant width between lateral elements and centromeres well aligned. The 25 heterospecific univalents linger together in a somewhat *bouquet* arrangement. As meiosis proceeds, the single elements seem to pair among themselves (second synaptic phase), forming not only non-homologous bivalents, but also multivalent associations. It is relevant to mention that no triple synapses were observed.

These suppositions are based upon pachytene nuclei organization and on the distinct results of the two triploid females. Macroscopic analysis of the gonads suggests that one female was more sexually matured, with darker (yellowish) and larger oocytes than the other, with whitish smaller eggs (data not shown). While the first one had a higher percentage of synapsis formation (with multivalent association), the second revealed a larger proportion of cells with 25 bivalents plus 25 univalents. This agrees with the proposed time progression, with the first synaptic round more frequently found in the less mature female, and the second round most common in the more sexually mature one.

A two-phase synapsis formation has been widely described both in polyploid plants and animals, as well as in several species with different chromosome abnormalities (reviewed in (16, 31)). The second phase seems to require the formation of the largest percentage of synapses possible, in order to overcome the pachytene checkpoint (32). In mammals, this checkpoint is very strict, and leads to apoptosis when asynaptic chromosomes are present (33). Non-homologous associations could result from SC formation between chromosomal regions with common DNA sequences (such as short homologous repetitive sequences) found on chromosomes of the single copy genome.

Considering that triploid females produce haploid gametes by means of meiotic hybridogenesis, their heterospecific genome must be excluded. The results obtained prove that this elimination process occurs after pachytene. Meiosis, with recombination, should proceed between the AA homologous genomes.

Previous experimental crosses performed in *S. alburnoides* carrying 'P genome', found no evidences of recombination between the heterospecific genomes (in that case, between P and A on PAA females), but recombination between the AA copies was reported (4, 8). This supports the formation of AA synapses in the QAA females herein analyzed and evidences a distinct meiotic dynamics for the Q genome, which seems never to engage in SCs formation with A genome.

The heterospecific exclusion might happen during anaphase I; for instance, chromosomes might be lost in the first polar body or be retained in the equatorial plate (due to misassembling to the meiotic spindle), while the homologous genome is segregated and proceeds to the second meiotic division. It is likely that recombination only occurs between A genome copies during pachytene. During diplotene, the SC

dissembles and homologous A chromosomes remain united by their chiasmata (which resulted from previous recombination events), whereas Q chromosomes might disperse and be lost since they theoretically do not form chiasmata to hold them together.

Several distinct mechanisms are proposed for a wide range of hybrid organisms to overcome the presence of a foreigner genome copy and be able to produce viable gametes. The present results, however, counteract other vertebrates' meiotic dynamics because *S. alburnoides* meiotic hybridogenesis lacks a premeiotic chromosome exclusion or genome endoreduplication in their gametogenetic process.

In the allotriploid fish *Poeciliopsis 2monacha-lucida* (and *monacha-2lucida*) the oogonium chromosome number is endomitotically raised to hexaploidy after which meiotic reduction occurs (34). The same is found in the *Ambystoma* salamander complex, where allotriploid females present chromosome doubling (without cytokinesis) prior to meiosis and neither trivalents nor univalents have been found. Besides, instead of forming homologous synapses, chromosomes presumably pair with their copy forming a triploid number of pseudobivalents and, as a result, produce gametes with somatic ploidy levels (35, 36). But conversely to triploid *S. alburnoides*, their oocytes do not suffer karyogamy and the triploid level is, this way, retained throughout the life cycle.

Another scenario occurs in male triploid Batura toads (*Bufo pseudoraddei baturae*), where a premeiotic elimination of one of the three chromosome sets, followed by meiosis to produce haploid gametes has been proposed. Females, however, produce diploid gametes either by chromosome exclusion followed by endomitosis and reduction or by initial duplication of only half the genome followed by reduction, being the last mechanism the most likely one (12). A parental chromosome exclusion (in general, the set from a paternal origin) is fairly well described in diploid hybrid vertebrates undergoing hybridogenesis [*e.g.* diploid *Pelophylax esculentus* (36-38) and diploid *Poeciliopsis monacha-lucida* (39)].

Allotriploid loach *Misgurnus anguillicaudatus* females, similarly to *S. alburnoides* triploid females (9), are able to produce triploid and haploid eggs simultaneously. The underlying mechanism for triploid eggs production in loaches was suggested to be premeiotic endomitosis, while the haploid eggs were produced by chromosome elimination during meiosis. In this case the authors proposed that some chromosomes

were cytologically eliminated, and the remaining ones underwent the two meiotic divisions (14). This could also be the case of *S. alburnoides*. Upon SC dissemblance in diplotene, the haploid genome should return to the univalent form and without proper kinetochores to attach to, the spindle does not assemble with Q chromosomes which are therefore lost. However, recent studies on triploid *M. anguillicaudatus* revealed only a diploid number of bivalents and no evidences of univalents in the germinal vesicles. This lead to the proposal that, like in Batura toads, one genome is presumably lost before reaching pachytene stage (13).

Even though consisting of an unorthodox reproductive strategy, meiotic hybridogenesis can be evolutionary advantageous because it retains the most important meiotic benefit: recombination. Considering that most likely the two homospecific copies come from different progenitors, the advantages are assured. It prevents the loss of heterozygosity commonly associated to non-sexual species. Therefore, the *S. alburnoides* complex is likely to have found a mechanism to avoid meiotic constrains resulting from interspecific hybridization, suggesting the flexibility of this system in changing gametogenesis regulation.

This is the first study on *S. alburnoides'* meiotic processes. Although preliminary, it allowed some disclosure of a specific and distinct gametogenetic mode in this allopolyploid complex of cyprinid fishes. Nevertheless more studies are necessary to deepen the understanding of gametogenesis in *S. alburnoides*, covering a broader sample and investigating the mechanism in other hybrid forms. For instance, in the case of diploid hybrids (e.g. QA), where clonal gametes are produced, a premeiotic endoreduplication is expected to be found.

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Concluding Remarks

The main goals proposed for this thesis were mostly accomplished. Knowledge of *S. aradensis* karyotype might be useful for future cytotaxonomic studies involving this and other closely related bisexual species. Genomic *in situ* hybridization (GISH) proved to be a reliable technique to identify genomic composition in *S. alburnoides* hybrids. It could also be used as a taxonomic tool to enlighten genomic relationships between species. The first synaptonemal complexes (SCs) results were striking by showing the use of this technique in *S. alburnoides* complex, with such diversity in reproductive modes and also, by illustrating that, conversely to what happens in most described hybrid gametogenesis, there is neither a genome endoreduplication nor genome exclusion prior to meiosis in allotriploid females. However, the process leading to the heterospecific genome elimination (for oocyte haploidization) remains to be clarified. For this, the subsequent meiotic stages need to be specifically analyzed, following the complete process step by step.

The results presented in both research articles do not support the occurrence of intergenomic chromosomal exchanges between A and Q genomes. However, the small sample size analyzed must be taken into consideration. These particular studies are generally confined to a small sample due to the need to sacrifice specimens (particularly, in the synaptonemal complexes study). When performed in species presenting an alarming conservation status like the case (38), those constrains are aggravated. The sampling must be reasonably handled and whenever possible, the specimens must be returned to the field. In this case, the sample size was also affected by fibroblast cultures usual constrains; mainly contaminations and/or low cell growth (in the first work); and by imprecise germinal cells' maturation (in the second study). All these situations accounted for the small replicates' number, thus results were obtained for only part of the sampled individuals.

New questions have arisen with the SCs work not only concerning the process of meiotic hybridogenesis (as previously mentioned) but also the interest in studying the meiotic process in other *S. alburnoides* hybrids (namely in QQA females and in QA specimens from both genders), which could provide new insights on the dynamics and possible genomic interactions within the hybridogenetic complex. It is thus relevant to

follow meiosis in a larger sample and in the distinct populations' biotypes, not only in pachytene but also in other stages.

The need to examine more specimens is also valid for the GISH study. There is a possibility that other specimens and distinct *S. alburnoides* populations behave differently, thus retrieving new and relevant information. The fact that no exchanges were observed in the sample analyzed does not necessarily mean that genome reshaping is not taking place in *S. alburnoides* complex. Further exploring this topic is yet very important to keep on trying to understand genomic evolution of vertebrate allopolyploid complexes and, on that perspective, *S. alburnoides* constitutes a particularly interesting model system.

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