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May a hybridogenetic complex regenerate the nuclear genome of both sexes of a missing ancestor? First evidence on the occurrence of a nuclear non-hybrid *Squalius alburnoides* (Cyprinidae) female based on DNA sequencing

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Abstract

Based on molecular evidence and on direct observation of gonads and morphology, we describe the occurrence of a female of the hybridogenetic minnow *Squalius alburnoides* bearing the nuclear genome of the paternal ancestor of the complex and the mtDNA of *S. pyrenaicus* (the maternal species). The paternal ancestor is believed to be extinct and the available molecular evidence indicates that it was a species distant from the maternal ancestor and closer to a very different genus (*Anaecypris*). Its nuclear genes were perpetuated through hybrids and through diploid males originated from the hybrids and containing two copies of the paternal genome. The discovery of a diploid female with the pure nuclear genome of the paternal ancestor, even if it represents a very rare occurrence, illustrates a very interesting biological phenomenon: the possibility of re-emergence of an extinct species from its descendent hybrids, although carrying the mtDNA of another species.

Keywords: *Beta-actin, Cyprinidae, extinct ancestor, hybrid, non-sexual,* Squalius alburnoides

Introduction

The Iberian *Squalius alburnoides* (Steindachner, 1866) complex is an exceptionally interesting hybrid system because apart from diploid, triploid, and tetraploid fishes that reproduce mainly by meiotic hybridogenesis, it also includes diploid males whose nuclear genome is non-hybrid and undergoes normal meiosis (reviewed in Alves et al. 2001).

Robalo et al. (2006) showed that the *S. alburnoides* complex resulted from an intergeneric hybridization process, since the nuclear DNA of its ''missing paternal ancestor'' was phylogenetically close to the species *Anaecypris hispanica* Steindachner, 1866—as

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previously suggested in Alves et al. (2001), while the maternally inherited mitochondrial genome is typical of the genus *Squalius* (Alves et al. 2001).

Depending on the species of *Squalius* available in the different drainages where *S. alburnoides* lives, it can form hybrids with *S. pyrenaicus*, *S. carolitertii*, or *S. aradensis* (Alves et al. 1997, 2001; Carmona et al. 1997; Cunha et al. 2004; Pala and Coelho 2005; Sousa-Santos et al. 2006a), a finding confirmed by protein electrophoresis and DNA analysis.

The diploid males carrying two copies of the nuclear genome of the paternal ancestor are morphologically very distinct from the hybrids. They have a narrow body with pointed head, straight dorsal profile and convex ventral profile, terminal mouth with a greater lower jaw so that the large buccal opening is turned slightly upward, deeply forked caudal lobes, (39)40–45 scales in the lateral line, 17–25(26) gillrakers, and a typical non-symmetrical pharyngeal teeth formula of (4)5/4(5) (Collares-Pereira 1984).

All the nuclear non-hybrid specimens from natural populations and experimental crosses so far analysed using genetic and cytogenetic markers were males (see Alves et al. 1998, 2001, 2002; Gromicho and Collares-Pereira 2004). One nuclear non-hybrid female was reported by Carmona (1997), in the river Estena (Guadiana River Basin). In this paper we report the first occurrence of such a non-hybrid female explicitly confirmed with a nuclear marker. This diploid non-hybrid female, also captured in the Guadiana River basin, was morphologically similar to the non-hybrid diploid males. It carried the mtDNA of *S. pyrenaicus* and the nuclear genome of the paternal ancestor.

Methods

The *S. alburnoides* non-hybrid female was electrofished in 2000 in river Caia (Guadiana River basin) and maintained in a tank with other *S. alburnoides* individuals until 2004. The sex of the fish was confirmed by direct observation of the spawning behaviour, extrusion of gametes by applying a mild pressure on the abdomen and *post mortem* examination. To evaluate its readiness to spawn and attractiveness to males, the female was maintained in an outdoor aquarium with four diploid non-hybrid males (confirmed by flow cytometry and *beta-actin* gene sequencing), under natural conditions of light and temperature.

Assignment to a morphological type was made *post mortem* by gillraker and lateral scale counts, inspection of pharyngeal teeth, general body shape, and position of the mouth.

Small fin clips were taken from 12 *S. alburnoides* (including the non-hybrid female) and from six *S. pyrenaicus*. Total genomic DNA was extracted by an SDS/proteinase-k based protocol, precipitated with isopropanol and washed with ethanol before re-suspension in water (adapted from Sambrook et al. 1989). DNA samples of the non-hybrid female and of six non-hybrid males from the Guadiana ($N=1$), Tejo ($N=1$), and Quarteira ($N=4$) river basins were genetically analysed for a segment of 927 bp of the nuclear *beta-actin* gene. The amplification process was conducted using the primers BACTFOR and BACTREV (Sousa-Santos et al. 2005). In addition, a total of 1123 bp of the cytochrome *b* (cyt*b*) gene was also amplified using the primers LCB1 (Brito et al. 1997) and HA (Schmidt and Gold 1993) from samples of the non-hybrid female, of six *S. alburnoides* with distinct nuclear genomic constitutions previously assessed by beta-actin sequencing (one non-hybrid AA, two diploid PA, and three triploid PAA hybrids) and of six *S. pyrenaicus* (PP), all from the Guadiana drainage. PCR conditions for both genes followed the procedures described in Sousa-Santos et al. (2005). Each sample was sequenced in both directions with the same primers used for PCR. Sequences were aligned with BioEdit® v.5.0.6 and deposited in

GenBank under the accession numbers: AY943863–65, AY943867, DQ128102, DQ010337, AY943891, DQ335472–481 and DQ350252 (for *beta-actin*); and DQ263227–39 (for cyt*b*). Note that, in the case of the *beta-actin* gene sequences, there are more accession numbers than individuals since some fishes were heterozygous for the analysed fragment and each strand had its own accession number. For a description of the procedure that allows the identification of the two different sequences involved in hybrids, see Sousa-Santos et al. (2005). A non-hybrid nuclear constitution was identified when the chromatograms showed only clear single peaks, a situation that contrasted with the presence of series of double peaks in the chromatograms of *S. alburnoides* that presented a hybrid nuclear genome, as described in Sousa-Santos et al. (2005).

PAUP® 4.0 software (Swofford 1998) was used to calculate the mean percentage of divergence between the cyt*b* gene sequences—defined as the average number of pairwise differences among sequences, expressed as a percentage of the total length of the gene fragment analysed. A parsimony network was constructed with TCS® v1.21 (Clement et al. 2000) using a 95% confidence limit.

Ploidy assessments were made by flow cytometry using fresh fin clips, following an adaptation of the Lamatsch et al. (2000) method developed by M. J. Collares-Pereira et al. (unpublished).

Results

Morphologically, the non-hybrid diploid female presented the same characteristics as all reconstituted non-hybrid males so far described: 45 scales in the lateral line, 18 gillrakers, and a 5/4 pharyngeal teeth formula. Up to now, there are no available estimates of the frequency of these non-hybrid diploid females in natural populations. In our survey, this was the only nuclear non-hybrid female collected, while more than 50 such males were found.

Results of the flow cytometry analysis confirmed the diploidy of this female. The analysis of the fragment of the *beta-actin* gene revealed an unambiguous non-hybrid constitution of the nuclear genome, as shown by the existence of clear single peaks in the chromatogram retrieved from the sequencing process. This female and all the six other nuclear non-hybrid males sequenced shared the same *beta-actin* haplotype (GenBank accession numbers: AY943863–65, AY943867, DQ128102, DQ010337 and DQ350252).

The analysis of the cyt*b* gene showed that the non-hybrid female presented a *S. pyrenaicus*-like mitochondrial genome that was similar to the ones found in the *S. pyrenaicus* and *S. alburnoides* individuals sequenced, differing only by a few mutations (Figure 1). The mean percentage of divergence values between the cyt*b* haplotypes of the non-hybrid female, of the *S. pyrenaicus* and of the remaining *S. alburnoides* individuals is presented in Table I.

This female underwent normal maturation and ovulation, which was demonstrated by the very easy release of mature eggs when a mild pressure was applied to the abdomen of the fish (about 2 weeks before the spawning observations) and by inspection of the gonads and oviducts upon dissection. In addition, it was fully attractive to males. Indeed, the courtship behaviours displayed by the males towards the non-hybrid female were similar to the ones performed towards the much more common hybrid females (Sousa-Santos et al. 2006b) and involved female attraction to the spawning site with quivering, fin shuffling, and manoeuvrings. The female interacted with the courting males and spawned with them several times (the females of *S. alburnoides* do not spawn all the eggs at one time but instead

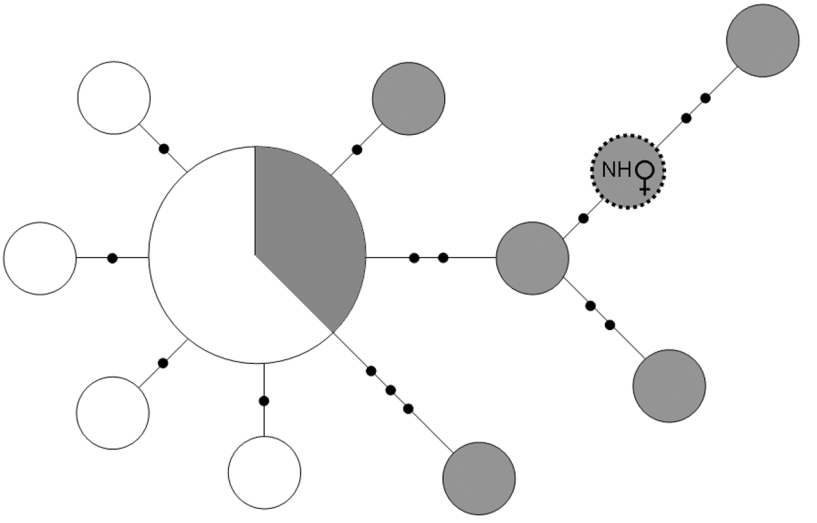

Figure 1. Minimum spanning network among cyt*b* haplotypes. The majority of the haplotypes (represented by circles) are exclusive of *Squalius alburnoides* (in grey) and of *S. pyrenaicus* (in white) individuals, except for the central one which is a haplotype shared between one *S. alburnoides* and two *S. pyrenaicus* individuals. NH indicates the haplotype of the non-hybrid female. The number of mutations between haplotypes is represented by small black dots.

Table I. Mean percentage of divergence between cyt*b* gene haplotypes from the non-hybrid female, *Squalius alburnoides*, and *S. pyrenaicus* from Guadiana (the intraspecific mean percentage of divergence values are presented in the diagonal).

		Non-hybrid female	*S. pyrenaicus*	*S. alburnoides*
Non-hybrid female	$N=1$	–		
S. pyrenaicus	$N=6$	0.33 ± 0.05	0.13 ± 0.05	
S. alburnoides	$N=6$	0.25 ± 0.10	0.28 ± 0.16	0.34 ± 0.13

make repeated spawnings that may extend for an entire day; Sousa-Santos et al. 2006b). Eggs had a normal appearance and the development reached the stage where embryos with already fully pigmented eyes were visible moving actively in the egg capsule. Unfortunately, all the progeny was lost due to a fungal infection.

Discussion

Although the possibility of occurrence of females with a non-hybrid nuclear genome within the *S. alburnoides* complex has been confirmed, the mechanism responsible for the production of these apparently rare reconstituted non-hybrid females is as yet unclear. The fertilization of haploid A oocytes (produced by PAA hybridogenetic females) by A sperm of nuclear non-hybrid AA males has always generated male offspring in all experimental crosses analysed to date (Alves et al. 1998; Gromicho and Collares-Pereira 2004). Studies based on such crosses have shown that around 3% of hybrid females may reproduce by gynogenesis (Alves et al. 2001), and that the most common female's biotype in southern drainages—the triploids PAA—generally produces haploid gametes (A) by a meiotic hybridogenetic process. However, there is also evidence that PAA females may, in addition

to these reduced eggs, produce gametes with partially (AA) or totally (PAA) unreduced genomes (Alves et al. 2004).

In addition to the fertilization of A eggs by A sperm, two other possibilities ending up in the production of these rare nuclear non-hybrid females may be hypothetically considered: a gynogenetic development process of diploid AA oocytes produced by PAA females and eventually, the fertilization by A sperm of reduced A oocytes after P-genome extrusion in PA females, although they generally transmit their genome clonally to offspring (see Alves et al. 2001; Gromicho and Collares-Pereira 2004).

From a general evolutionary perspective, and regardless of the mechanism involved, the most relevant aspect of the finding here reported is that it highlights the possibility of the reconstitution of the nuclear genome of an extinct species, whose genes had been perpetuated in hybrids for many generations. The only mark of the past hybridization history that remains is apparently the mtDNA inherited from the maternal species from which the hybrids originated. However, before we can achieve a full assessment of the significance of this finding, it will be necessary to investigate whether these nuclear non-hybrid diploid females are genetically distinct from the corresponding males or are mere developmental deviations of what are in fact genetically males. In addition, it will be of paramount importance for the study of this fish complex to investigate what kind of gametes these reconstituted females originate. If they prove to display normal meiosis, if their gametes combined with the corresponding males produce fish of both sexes, and if they reach sufficient frequency in nature to have a reasonable chance of mating with reconstituted males of the same genetic makeup, they could originate a viable lineage fully independent from the hybrids. Indeed, the observation that the AA progeny of nuclear non-hybrid diploid males is composed only by males, as mentioned above, may be affected by the fact that they mate with fish of hybrid origin. It may well be that if they mate with AA females they may produce progeny of both sexes, as their ancestors had to do. The rarity of these females may simply be due to the fact that the hybrids are so more abundant that the AA males will mate with hybrids on most occasions.

There is still another feature that makes *S. alburnoides* a very remarkable case among the hybridogenetic vertebrates so far investigated: in several of its forms there are males and/or females which include meiosis and recombination in their gametogenetic processes (Alves et al. 2001), giving the complex the capability of maintaining a high level of genetic variability which is unavailable to most other vertebrate hybrids so far described.

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