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Bulked segregant analysis of the pirarucu (*Arapaima gigas*) genome for identification of sex-specific molecular markers

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ABSTRACT. *Arapaima gigas* (Osteoglossidae) is one of the largest fish species in the Amazon Basin, attaining lengths of over 2.5 m and weights of over 100 kg. Its flesh is prized, and it has great potential for production in aquaculture systems. However, live pirarucu cannot be reliably sexed visually, even after sexual development, since this species does not have clear external sexual dimorphism. Simple and inexpensive methods for sexing immature pirarucu based on DNA markers would facilitate production of this species in commercial operations. We analyzed *A. gigas* male and female DNA pools with 566 RAPD primers, generating 2609 fragments, with an estimated 1341 segregating polymorphic markers, and an estimated average spacing of 714 kb, which corresponds to less than 0.1% of the species' genome. Two putative sex-specific fragments were initially identified in bulked

samples; but they were not confirmed in a study of individual male and female samples. We suggest that *A. gigas* has developed a non-chromosomal system of sex determination or, alternatively, that the species has undergone a recent loss of the chromosome carrying the sex-determining locus.

Key words: Neotropical fishes; RAPD; Sex determination; Bulked segregant analysis

INTRODUCTION

Pirarucu (*Arapaima gigas*) is found in the Amazon and Araguaia-Tocantins River basins, in a region spanning nine South American countries (Brazil, French Guiana, Suriname, Guyana, Venezuela, Colombia, Ecuador, Peru, and Bolivia). The species is considered to be the largest known fresh water scaled fish, with adults reaching 3 m in length and 200 kg in live weight (Nelson, 1994).

The species has had a major historical role in the diet of Native Americans inhabiting the Amazon region, as evidenced by stratified prehistoric deposits uncovered in archeological sites (Roosevelt et al., 1996). Starting in early 18th century, *A. gigas* has been heavily exploited by commercial fishing, mostly for production of salted and dried meat (Martinelli and Petrere Jr., 1999). Overfishing practices have led to the pirarucu becoming commercially extinct in areas around major Amazonian cities (Bayley and Petrere Jr., 1989). Commercial fishing was banned by the Brazilian government in early 2001, but it is currently permitted under strict guidance of environmental agencies, defining minimum size limits and excluding spawning seasons (Hrbek et al., 2007).

Pirarucu meat is devoid of bones, low in fat and highly valued in local and emerging domestic and international markets. Most of the supply is still derived from wild-caught fish, but the species presents several features that are highly desirable for commercial aquaculture production, such as rapid juvenile growth (yearlings can reach up to 10 kg of live weight) and high carcass yields. However, one of the major hurdles in developing pirarucu culture systems is the production of fry in controlled conditions, since captive breeding is still accomplished by randomly pairing adult males and females in earthen ponds so that they can spontaneously choose a mate, delimit a territory, build a nest, and breed (Chu-Koo et al., 2009).

Neither prepubescent nor adult pirarucu exhibit unambiguous sexual dimorphism. Sexing live adults can only be reliably accomplished around the reproductive phase of development, using ultrasonography, surgical methods (Carreiro et al., 2001), or quantitative metabolite assays (Chu-Koo et al., 2009). Therefore, simple and inexpensive methods for sexing immature pirarucu are needed to help enhance the production of this species in commercial operations.

The identification of sex-specific molecular markers for development of diagnostic assays for sexing prepubescent fish has been accomplished in a number of species: rainbow trout, *Oncorhynchus mykiss* (Iturra et al., 1997); tilapia, *Oreochromis niloticus* (Lee and Donaldson, 2001); African catfish, *Clarias gariepinus* (Kovács et al., 2000); yellow catfish, *Pelteobagrus fulvidraco* (Wang et al., 2009); and turbot, *Psetta maxima* (Casas et al., 2011). A sex-specific molecular marker for *A. gigas* is needed to assist the development and application of innovative

methods for rearing this species on a commercial scale. In this study, we attempted to identify sex-specific molecular markers for pirarucu using bulked segregant analysis (Michelmore et al., 1991), a well-known strategy that has been successfully applied in several instances with similar objectives (Iturra et al., 1997; Kovács et al., 2000; Lee and Donaldson, 2001).

MATERIAL AND METHODS

Sample collection and DNA extraction

Samples were collected from the river system around Santarém (2°26'S; 54°42'W), Pará State, Brazil, from three discrete localities (Santa Maria do Tapará, Ipixuna, and Mucurituba), following the proper governmental authorizations (IBAMA license No. 16623-1, authentication code 37735557). Adult fish were caught, slaughtered, eviscerated, and sexed on the basis of the morphology of the sexual organs. Blood samples from captured animals were collected in EDTA tubes and DNA extractions were performed with a Genomic DNA Isolation Kit (RBC BioAmerica, Miami, FL, USA), according to the manufacturer protocol.

Bulked segregant analysis with random amplified polymorphic DNA (RAPD) primers

DNA samples from 11 females and 12 males were combined in equimolar amounts to form sex-specific bulks, according to procedures previously described (Michelmore et al., 1991). These two bulks were analyzed with a total of 566 RAPD primers (Table 1). PCRs were performed in duplicate with the following reagents: 2 μM primer, 2.5 mM dNTP (each), 25 mM $MgCl_2$, 0.5 U *Taq* polymerase (Promega©), 1X PCR buffer, 3 ng Bulk DNA, in a final volume of 10 μL. The thermal cycling parameters used were: 3 min at 94°C; 40 cycles of 15 s at 95°C, 30 s at 35°C and 1 min at 72°C; with a final step of 7 min at 72°C. PCR products were separated by electrophoresis on 1.5% agarose gels at 140 V for 3 h in 1X TBE buffer. Gels were stained with ethidium bromide, then visualized with a UV transilluminator and photographed with an EagleEye II imaging system (Stratagene). Primers that amplified sex-specific fragments were used to amplify each individual sample included in the initial male and female bulks.

Estimates of the mean number of polymorphic RAPD markers segregating in the samples tested

Twenty-two randomly selected RAPD primers (Operon® OPAB17; OPB08; OPN04, 09, 11-13, 15, 16; OPO07, 08, 10-16, 18-20; OPP08) were used to estimate the mean number of RAPD polymorphic markers segregating in the *A. gigas* samples used in the experiments. DNA samples from each of 12 males and 11 females were amplified separately, and RAPD fragments were separated on agarose gels as previously described. The total number of fragments and the total number of polymorphic fragments generated were determined. In addition, the total number of fragments generated with the 566 primers tested in the sex-specific bulks was also determined.

Table 1. List of the RAPD primers (Operon®) used to analyze *Arapaima gigas* male and female bulked DNA.

Kit	Primer*																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
OPA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPAA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPAB	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPAD	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x			
OPAE	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPAF	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPAG	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x
OPAI		x		x		x			x	x		x	x	x	x	x	x	x	x	x
OPAX	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPB	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPBA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPC	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x		x
OPD	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x
OPE	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPF	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x
OPG	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x
OPH												x				x		x		
OPI	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x
OPJ	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPK	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPL	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPM	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPN	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPO	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPP	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPR	x	x		x		x	x	x		x		x	x	x		x	x	x	x	x
OPS	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPT	x		x	x	x	x	x		x	x		x	x		x	x	x	x	x	x
OPU	x		x			x				x					x	x			x	x
OPV	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x
OPW	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x
OPX	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPY		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
OPZ	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

*Primer sequences available at [http://www.google.com/url?sa=t&rct=j&q=operon%20rapd%20primer%20sequence&source=web&cd=2&ved=0CFwQFjAB&url=http%3A%2F%2Fwww.operon.com%2Fproducts%2Fdownloads%2FOperonsRAPD10merSequences.xls&ei=2Ga0T7aSCoHk9ATWhe36Dw&usg=AFQjCNEmmxkQoQR54d8YbT872IRlsqkpJw].

These results were used to estimate the average number (n) of polymorphic RAPD markers segregating in the tested samples using the formula:

$$n = x\ (y / z)$$

where *x* is the total number of RAPD fragments amplified in the bulked segregant analysis and *y/z* represents the estimated average proportion of RAPD polymorphic fragments amplified in the samples included in the study, obtained by dividing (y) the number of observed polymorphic fragments by (z) the total number of RAPD fragments obtained.

RESULTS

Bulked segregant analysis

A total of 2609 RAPD fragments were amplified with the 566 primers tested (an aver-

age of 4.6 fragments per primer, Figure 1). Seventy percent of the primers amplified at least one RAPD fragment and 70 primers did not generate any fragments. Two primers amplified putative sex-specific fragments in the bulk evaluation phase. A male-specific fragment of approximately 700 bp was amplified with primer OPAB17 (Figure 2A), and a female-specific fragment of approximately 1500 bp was amplified with primer OPP08 (Figure 2B). The amplification of individual male and female samples with primers OPAB17 (Figure 3) and OPP08 (data not shown) revealed that the previously observed putative sex-specific fragments could actually be amplified from both males and females.

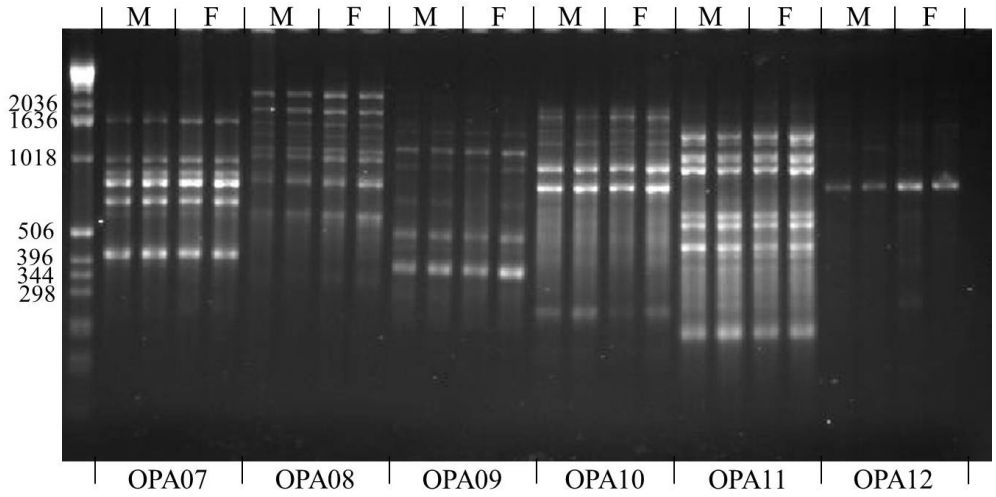

Figure 1. Example of RAPD fragments amplified with different primers from *Arapaima gigas* male (M) and female (F) bulked DNA.

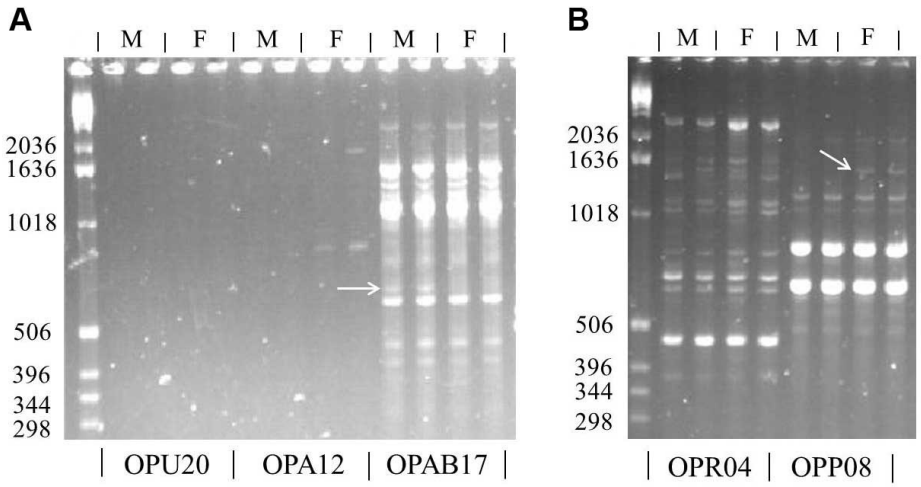

Figure 2. Putative *Arapaima gigas* sex-specific fragments (arrows) amplified from **A.** male (M) and **B.** female (F) bulked DNA with primers OPAB17 and OPP08, respectively.

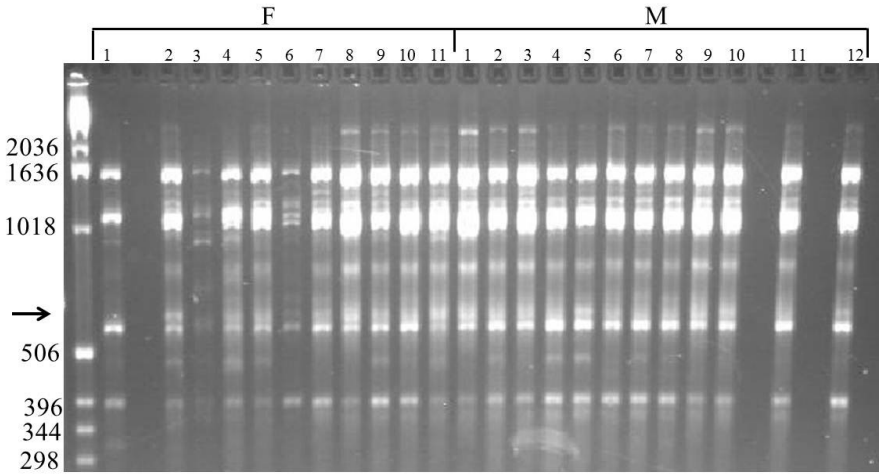

Figure 3. RAPD fragments amplified from *Arapaima gigas* individual male (M) and female (F) samples with primer OPAB17, showing the putative male-specific fragment (arrow) can be observed in samples from both sexes.

Level of RAPD polymorphism in the tested samples and average marker spacing

The 22 primers used for evaluating the level of polymorphism observed in the sampled individuals generated a total of 214 RAPD fragments (z). Of these, 110 fragments (y) were found to be polymorphic in the 23 individuals included in the bulked segregant analysis (Figure 4). The estimated average proportion of polymorphic RAPD fragments amplified in the samples included in the study (y/z) was 51.4%. Considering that a total of 2609 RAPD fragments were amplified in the bulked segregant analysis, we estimate that a total of 1341 polymorphic fragments were segregating in the samples analyzed.

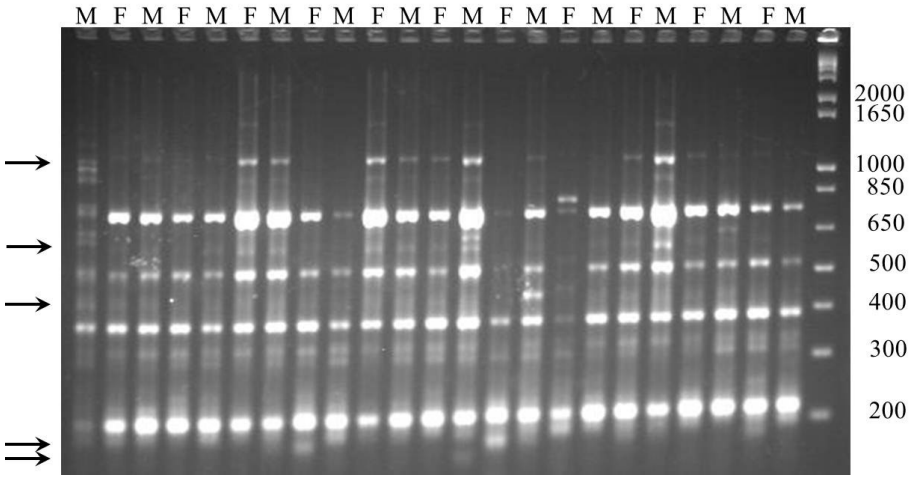

Figure 4. RAPD fragments amplified from *Arapaima gigas* individual male (M) and female (F) samples with primer OPN16. Arrows indicate polymorphic fragments.

Considering that the haploid nuclear content of *A. gigas* has been estimated as 0.98 pg (Hinegardner and Rosen, 1972), we estimate that the pirarucu genome has a total length of 958.44 Mbp [(0.978 x 10^3 Mbp/pg) x 0.98 pg], using the formula proposed by Dolezel et al. (2003). Thus, we can infer that the bulked segregant analysis resulted in the mapping of polymorphic RAPD fragments with an average spacing of 714 kbp, assuming that the fragments were randomly distributed throughout the species' genome.

DISCUSSION

The development of simple and efficient methods for sexing prepubescent fish is an essential step for appropriate broodstock management of species that do not exhibit well-defined sexual dimorphism. The inability to identify the sex of juveniles prevents early assortment of appropriate proportions of males and females for establishing optimal selection and matting strategies. This issue is particularly critical for larger species, because fewer individuals tend to be kept as broodstock for reasons of production efficiency. High levels of behavior-induced stress caused by inadequate management of breeding animals, due to the allocation of disproportionate numbers of females to males in breeding tanks, can jeopardize the production of fry (Carreiro et al., 2011). In species such as sturgeon (*Huso huso*), where sex-specific molecular markers have not been identified (Keyvanshokooh et al., 2007), surgical (Falahatkar et al., 2011) and non-invasive (Masoudifard et al., 2011) methods have been developed for sexing fish. This has enabled discrete management practices for males and females, which are raised for the production of meat and eggs (caviar), respectively (Falahatkar et al., 2011).

Different methods have been developed to generate monosex populations of several fish species, such as induced sex reversal of alevins by hormonal treatments, and induced gynogenesis through thermal or hyperbaric shocking of fertilized eggs (Pandian and Koteeswaran, 1998; Lee and Donaldson, 2001). In some cases, these methods have been widely adopted by producers as a consequence of the obtained gains in productivity and profitability (Beardmore et al., 2001). However, the effective use of these technologies is also highly dependent on diagnostic methods for the identification of "supermales" (YY) or "superfemales" (WW) for use as broodstock.

A number of different sex determination systems, ranging from genetic to environmental sex determination, have been reported for different species of fish. Genetic sex determination systems vary from exclusively chromosomal with heterogametic males (XY) or females (ZW), to systems influenced by autosomal genes, or of polygenic nature (Devlin and Nagahama, 2002). In addition, different sex determination systems have been found in species from the same genus, such as the tilapia *Oreochromis* sp, where classic male heterogametic (*O. niloticus)* and female heterogametic (*O. aureus*) species have been reported (Campos-Ramos et al., 2001). Even though genetic factors probably regulate sex determination in most fish species, only a small proportion of teleosts studied actually show distinct sex chromosomes that can be morphologically identified by standard cytogenetic analysis (Arkhipchuk, 1995). Nevertheless, heteromorphic sex chromosomes have been observed in more than 176 species of fish from different groups (Devlin and Nagahama, 2002); this includes species belonging to the family comprising *A. gigas*, such as *Osteoglossum bicirrhosum* (Uyeno and Miller, 1971). However, *A. gigas* has been shown to lack differentiated sex chromosomes (Marques et al., 2006) indicating that, if the species presents a chromosomal sex determination system, the

differentiated genomic region associated with the sex determining locus (SDL) is not large enough to be morphologically identified through standard karyotype analysis.

The present study took into account all of the aforementioned issues and was designed to identify sex-specific RAPD fragments, if *A. gigas* has a classic male or female heterogametic sex determination system. Compared to other methods that have been used previously for identifying sequences present and absent from a genome, bulked segregant analysis with RAPD markers is a simple, low-cost approach, which has been used successfully in several recent studies aimed at identifying sex-specific markers in a number of species (Iturra et al., 1997; Lee and Donaldson, 2001; Gebler et al., 2007; Mariotti et al., 2009; Casas et al., 2011). Even in the current context where sequencing and resequencing of complete genomes are becoming widely accessible due to the greater capacity and reduced cost conferred by recent technological advances (Metzker, 2010), this methodology is still a proven approach with great potential to provide positive results in similar studies. Despite not identifying sex-specific markers for *A. gigas*, our estimates of genome coverage with segregating polymorphic markers revealed that, if this species actually has a chromosomal sex determination system, the SDL must be contained within a genomic region of minimal proportions, containing less than 714 kb; this would be equivalent to less than 0.1% of the species' genome.

The degenerative processes of content reduction and loss of gene function in the heterogametic chromosome have been widely described and are consensually accepted as the result of the recombination suppression caused by the acquisition of the sex-determining gene by an autosome, followed by the accumulation of mutations (Rice, 1987; Charlesworth and Charlesworth, 2000; Steinemann and Steinemann, 2000; Lahn et al, 2001; Graves, 1995, 2006; Graves et al., 2006). *A. gigas* belongs to one of the oldest groups of teleosts, which originated in the Jurassic period (Lavoué and Sullivan, 2004). Therefore, if this species has a classical chromosomal male or female heterogametic sex determination system (XY or ZW), the sex chromosomes should have accumulated significant changes over the last 150 million years, as observed in other species (Marshall Graves and Shetty, 2001; Marshall Graves, 2008). Conversely, the duplication that led to the formation of the SDL in the medaka (*Oryzias latipes*) was calculated to have occurred ~10 million years ago (Kondo et al., 2004), and the region was shown to have accumulated only 258 kbp over this period. This is equivalent to ~0.03% of the species' genome, in what is considered to be the youngest vertebrate Y chromosome known with an identified sex-determining gene (Kondo et al., 2006).

The lack of large genomic differences associated with the *A. gigas* SDL could be a consequence of the species having developed a non-chromosomal system of sex determination, which could be influenced by polygenic and/or environmental factors. Alternatively, it is also possible that *A. gigas* has undergone a recent loss of the chromosome carrying the SDL, as reported by Marshall Graves and Shetty (2001) in two mammals of the genus *Elliobus*. These two species of rodent have completely lost the Y chromosome and have no *SRY* gene, the consensual mammalian SDL (Just et al., 1995). Marshall Graves and Shetty (2001) suggested that perhaps a new sex-determining gene has arisen in these species, leading to the redundancy of *SRY* and the Y chromosome itself, which completely degraded over time.

Analyses of the structure of synaptonemal complexes have been used to identify the sex-specific regions of fish meiotic chromosome pairings that could not be identified by traditional karyotype analysis (Carrasco et al., 1999; Campos-Ramos et al., 2001). This approach could be used to evaluate the *A. gigas* genome for the presence of heterogametic

regions. Furthermore, experiments involving crosses between sex-reversed fish, generated by hormonal treatment or produced by artificial induction of gynogenesis, have been used extensively to elucidate the mechanism of sex determination of a large number of fish species (Lee and Donaldson, 2001; Xiang-Shan et al., 2010). Such experiments are also likely to help elucidate the mechanism of sex determination of *A. gigas*, and could lead to the development of a diagnostic method for sexing prepubescent fish, which would have a significant impact on the development of the commercial aquaculture of this species.

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