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Genetic Identification of F1 and Post-F1 Serrasalmid Juvenile Hybrids in Brazilian Aquaculture

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Abstract

Juvenile fish trade monitoring is an important task on Brazilian fish farms. However, the identification of juvenile fish through morphological analysis is not feasible, particularly between interspecific hybrids and pure species individuals, making the monitoring of these individuals difficult. Hybrids can be erroneously identified as pure species in breeding facilities, which might reduce production on farms and negatively affect native populations due to escapes or stocking practices. In the present study, we used a multi-approach analysis (molecular and cytogenetic markers) to identify juveniles of three serrasalmid species (*Colossoma macropomum*, *Piaractus mesopotamicus* and *Piaractus brachypomus*) and their hybrids in different stocks purchased from three seed producers in Brazil. The main findings of this study were the detection of intergenus backcrossing between the hybrid ♀ patinga (*P. mesopotamicus*×*P. brachypomus*)×♂ *C. macropomum* and the occurrence of one hybrid triploid individual. This atypical specimen might result from automixis, a mechanism that produces unreduced gametes in some organisms. Moreover, molecular identification indicated that hybrid individuals are traded as pure species or other types of interspecific hybrids, particularly post-F1 individuals. These results show that serrasalmid fish genomes exhibit high genetic heterogeneity, and multi-approach methods and regulators could improve the surveillance of the production and trade of fish species and their hybrids, thereby facilitating the sustainable development of fish farming.

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Introduction

In Brazil, approximately 40 native fish species and 6 interspecific hybrids are cultivated on fish farms [1,2]. The representatives of the Serrasalmidae family, *i.e.*, *Colossoma macropomum* (tambaqui), *Piaractus mesopotamicus* (pacu), *Piaractus brachypomus* (pirapitinga or caranha), and their interspecific hybrids tambacu (female tambaqui×male pacu), patinga (female pacu×male pirapitinga), and tambatinga (female tambaqui×male pirapitinga) correspond to native fish with the largest production in Brazilian aquaculture (56.2 million kg per year) according to IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) [3]. Reciprocal hybrids (*e.g.*, female pacu x male tambaqui) are also viable [4], but these individuals are not typically produced or cultivated on fish farms.

The cultivation of serrasalmids varies among different regions of Brazil [2]. In Southern Brazil, the only species produced is pacu. Tambaqui and pirapitinga, and their hybrids, are not produced in this region because these species cannot tolerate the low temperatures of Southern Brazil. In Northern Brazil, serrasalmid hybrids are produced at a lower rate compared with the pure species tambaqui, the main aquaculture resource in this region. In contrast, hybrids are associated with high production rates in the other regions of Brazil, particularly in the Midwest [3], and the hybrid tambacu has a greater economic importance than other serrasalmid hybrids. This fish group is also widely farmed in other Latin American (Colombia, Venezuela, and Cuba) [5] and Asian (China, Myanmar, Thailand, and Vietnam) countries [6,7].

However, the diversity and zootechnical differences among fish are problematic for the aquaculture industry because pure species or their hybrids can be produced or cultivated as a single species. This inaccuracy primarily reflects the morphological similarity between species, particularly in the case of hybrids and parental species in the juvenile stage. Thus, the use of genetic markers is essential to monitor the production and management of fish hybrid, particularly for the trade between seed suppliers and fish farmers, which is a critical point in the production chain [8].

Currently, PCR-RFLP (polymerase chain reaction - restriction fragment length polymorphism) and multiplex-PCR have been characterized as efficient methods for the rapid and inexpensive identification of hybrids [9–11]. For serrasalmid hybrids, these methodologies facilitate diagnoses based on the combination of single nucleotide variants in the mitochondrial genes, Cytochrome

C Oxidase subunit I (*mt-co1*) and Cytochrome b (*mt-cyb*), with nuclear genes, such as α-Tropomyosin (*tpm1*) and Recombination Activating Gene 2 (*rag2*) [12]. Nuclear diagnostic markers are essential to differentiate hybrids between species, but mitochondrial markers, although haploid, identify the maternal origin of hybrids, and this information is crucial for the assessment of hybridization.

Cytogenetic analysis methods have also been described for the identification of serrasalmid hybrids. Through C-banding and fluorescence *in situ* hybridization (18S ribosomal RNA probe), chromosome markers have facilitated the precise identification of the hybrids tambacu and tambatinga, respectively [13,14]. Although cytogenetic methods have limitations of low throughput because of the effort and time required for data analysis and processing [2], these techniques provide important information to verify ploidy level [13], which cannot be directly assessed through molecular markers.

Despite hybrid vigor in some cases, there are problems associated with the inadequate use of interspecific hybrids for aquaculture production. Occasionally, fish farmers have mistakenly used hybrids as broodstock, as reported for tilapia, catfish, and carp [15–17]. Superior performance or desirable characteristics associated with hybrid vigor might be lost in post-F1 individuals because introgressive hybridization reduces the heterosis obtained in F1 hybrids, and particularly because post-F1 hybrids typically show reduced offspring viability due to high mortality rates [18]. These observations have been previously reported in catfish, where hybrids were used as broodstock [17].

In the present study, we used PCR-RFLP, multiplex-PCR, and cytogenetic methods to evaluate the juvenile fish trade between seed suppliers and fish farmers in Brazil, focusing on the genetic identification of F1 and post-F1 serrasalmid hybrids. The novelty of this study was the discovery of fertility in the hybrid patinga (female pacu x male pirapitinga), and its use as broodstock in Brazilian aquaculture. Moreover, mistaken trade of hybrid tambacu was detected in fish farms and one post-F1 hybrid was characterized as triploid.

Materials and Methods

We performed the genetic identification of 924 juvenile individuals from eight stocks of live fish purchased from three private Brazilian aquaculture seed producers (herein referred to as SPS, MGS, and SES) (Table 1). All fish farms assessed in this study represent large companies in Brazil. From fish farm SPS, located in São Paulo State (Southeastern Brazil), we analyzed two commercially available stocks, labeled as hybrids tambacu (SPS1) and patinga (SPS2). From fish farm MGS, located in the Minas Gerais State (Southeastern Brazil), we analyzed three commercially produced stocks, labeled as pure tambaqui (MGS1), pacu (MGS2), and hybrid tambacu (MG3). From fish farm SES, located in Sergipe State (Northeastern Brazil), we analyzed three commercially stocks, labeled as pure tambaqui (SES1), hybrids tambatinga (SES2), and tambacu (SES3). The size of the analyzed fish ranged from 5 to 10 cm. We did not notify the producers that the fish would be used for identification purposes.

This study was conducted in strict accordance with the recommendations of the National Council for Control of Animal Experimentation (Brazilian Ministry for Science, Technology and Innovation). The present study was performed under authorization N° 33435-1 issued through ICMBio (Chico Mendes Institute for the Conservation of Biodiversity, Brazilian Ministry for Environment). Fin fragments were collected from each fish under benzocaine anesthesia and all efforts were made to minimize suffering. DNA was extracted from the fin fragments using the Wizard Genomic DNA Purification Kit (Promega) according the manufacturer's protocol. The DNA concentration was assessed against a molecular marker standard (the Low DNA Mass Ladder, Invitrogen) through electrophoresis on a 1% agarose gel.

Table 1. Molecular identification of juvenile serrasalmid fish stocks purchased from different fish farmers.

Fish farm	Stocks purchased	Stock identification	n
SPS (São Paulo State)	tambacu	SPS1	50
	patinga	SPS2	33
MGS (Minas Gerais State)	tambaqui	MGS1	143
	pacu	MGS2	115
	tambacu	MGS3	133
SES (Sergipe State)	tambaqui	SES1	150
	tambatinga	SES2	150
	tambacu	SES3	150

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The samples were genotyped using two methods and different genes, as previously described [12]: 1) multiplex-PCR based on nuclear α-Tropomyosin (*tpm1*) and mitochondrial Cytochrome C Oxidase subunit I (*mt-co1*) genes; and 2) PCR-RFLP using the nuclear Recombination Activating Gene 2 (*rag2*) and mitochondrial Cytochrome b (*mt-cyb*) genes. Both methods provide diagnostic electrophoretic fragments for each parental species and their interspecific hybrids. Diagnostic sizes of the PCR products or restriction fragments are described in the Table 2. The sequences for the primers and restriction enzymes, PCR reagents, reagent concentrations, and reaction conditions were used as previously described [12]. We used multiplex-PCR, followed by PCR-RFLP in subsequent analyses for confirmation. DNA samples from the pure parental species were used as controls for reaction specificity in all experiments. These samples were previously identified through morphological and molecular analyses [12] and obtained from the stock maintained at the Centro Nacional de Pesquisa e Conservação de Peixes Continentais (CEPTA/ICMBio, Pirassununga, São Paulo State, Brazil).

Cytogenetic analysis was also performed to verify the ploidy level in individuals of the SPS2 stock. Chromosomal preparations were obtained according to the methods of Foresti et al. [19]. The chromosome morphology was determined based on the arm ratio consistent with Levan et al. [20], and the chromosomes were subsequently classified as metacentric (m), submetacentric (sm),

Table 2. Sizes of the PCR products or restriction fragments, according to Hashimoto et al. [12].

		Diagnostic fragment size (bp)		
Method	**Gene**	**pacu**	**tambaqui**	**pirapitinga**
Multiplex-PCR	*mt-co1*	307	435	307 and 610
	tpm1	269	172	131
PCR-RFLP	*mt-cyb*	152 and 513	261 and 405	665
	rag2	750	357 and 393	250 and 500

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subtelocentric (st), and acrocentric (a). Fluorescence *in situ* hybridization (FISH) was performed using the method of Yang et al. [21]. These experiments encompassed all of the genotypes shown below through the molecular identification of the SPS2 stock. The 5S ribosomal RNA (rRNA) gene sequences were PCR amplified from DNA using the primers described by Pendás et al. [22]. To prepare the probe, PCR products of the 5S rRNA gene were labeled with biotin-16-dUTP (Roche) through nick translation (Invitrogen). The chromosomes were counterstained with DAPI (4′,6-diamidino-2-phenylindole, Vector Laboratories). The FISH images were captured and processed using the CytoVision Genus system (Applied Imaging, USA) and a Cohu CCD camera mounted on an Olympus BX-60 microscope.

Results

We obtained the same genotype with all molecular markers in the samples purchased as hybrid tambacu (stocks SPS1, MGS3, and SES3), pure tambaqui (MGS1 and SES1), pacu (MGS2), and hybrid tambatinga (SES2), indicating that these species correspond to hybrid tambacu. The results of the multiplex-PCR analysis of the nuclear marker *tpm1* (Figure 1a) revealed a heterozygous genotype (fragments of 172 and 269 bp), characteristic of hybrid tambacu. Moreover, multiplex-PCR of the mitochondrial marker *mt-co1* (Figure 1b) showed that these hybrids exhibited the genotype of the maternal species tambaqui (fragment of 435 bp), consistent with the identification of these samples as tambacu (♀ tambaqui x ♂ pacu) instead of the reciprocal hybrid paqui (♀ pacu×♂ tambaqui). The results were confirmed through PCR-RFLP using the nuclear *rag2* and mitochondrial *mt-cyb* genes. Thus, the MGS1, MGS2, SES1, and SES2 samples were mislabeled, as these species were actually tambacu.

The results of molecular identification in the SPS2 stock demonstrated that these juveniles likely correspond to post-F1 hybrids, resulting from the backcrossing of the hybrid ♀ patinga (♀ pacu x ♂ pirapitinga) with ♂ tambaqui. Consistent with this hypothesis, all the offspring showed the pacu genotypes for the mitochondrial *mt-co1* (fragment of 307 bp) and *mt-cyb* (fragments of 152 and 513 bp) genes, and segregating genotypes at the nuclear *tpm1* and *rag2* markers, as indicated below (Figures 2 and 3):

- Genotype A (8 individuals): pattern of hybrid tambacu for both nuclear markers *tpm1* (fragments of 172 and 269 bp) and *rag2* (fragments of 357, 393, and 750 bp).
- Genotype B (9 individuals): pattern of hybrid tambatinga for both nuclear markers *tpm1* (131 and 172 bp) and *rag2* (250, 357, 393, and 500 bp).
- Genotype C (6 individuals): nuclear markers of hybrid tambacu for the *tpm1* (172 and 269 bp) gene, and genotype of hybrid tambatinga for the *rag2* (250, 357, 393, and 500 bp) gene.
- Genotype D (9 individuals): opposite nuclear patterns to the genotype C, *i.e.*, genotype of hybrid tambatinga for the *tpm1* (131 and 172 bp) gene, and genotype of hybrid tambacu for the *rag2* (357, 393, and 750 bp) gene.
- Genotype E (1 individual): atypical genotype comprising gene fragments from the three pure species (tambaqui, pacu, and pirapitinga) for the *tpm1* (131, 172, and 269 bp) gene, and genotype of hybrid tambacu for the *rag2* (357, 393, and 750 bp) gene. This unexpected pattern is compatible with a triploid individual, consistent with the cytogenetic results shown below.

According to the Mendelian inheritance, the hypothesized backcross ♀ patinga (♀ pacu x ♂ pirapitinga) x ♂ tambaqui would produce each A–D genotype at 25% among the offspring (Figure 3), and the observed data did not deviate from the null hypothesis, confirmed using the $\chi 2$ test ($p = 0.86$).

The cytogenetic analysis showed a diploid chromosome number of $2n = 54$ for most individuals of the SPS2 stock, with chromosomes presenting morphologies of the types m and sm, excluding one specimen comprising 81 chromosomes (50 metaphases with this chromosome number were counted), suggesting a polyploid individual with three chromosome sets (3n) (Figure 4), and corresponding to genotype E. The occurrence of this individual might reflect a likely meiotic segregation pattern, including a crossing-over between the *tpm1* gene locus and the centromere, but not in the *rag2*-bearing chromosomes, during female oogenesis, with the fusion/retention of the second polar body in the egg, resulting in a triploid individual (Figure 5).

The FISH analysis of the specimens in the SPS2 stock revealed 5S rRNA clusters in the subcentromeric region of four chromosomes, some of which were non-homologous, as revealed through differences in morphology/size and the positions of the hybridization signals (Figures 4a and 4b). In two chromosomes, the genes were located on the long arms (major clusters), and in the other two chromosomes, the genes were located on the short arms (minor clusters). We observed different combinations of these chromosomes in the analyzed individuals, further suggesting the occurrence of post-F1 individuals. Moreover, the individual previously identified as triploid was characterized with six 5S rRNA clusters: three chromosomes with FISH signals in the subcentromeric region of the long arms (major clusters), and the other three chromosomes with signals in the short arms (minor clusters) (Figure 4c). No correlation was observed between the different chromosomes carrying 5S rRNA clusters with the

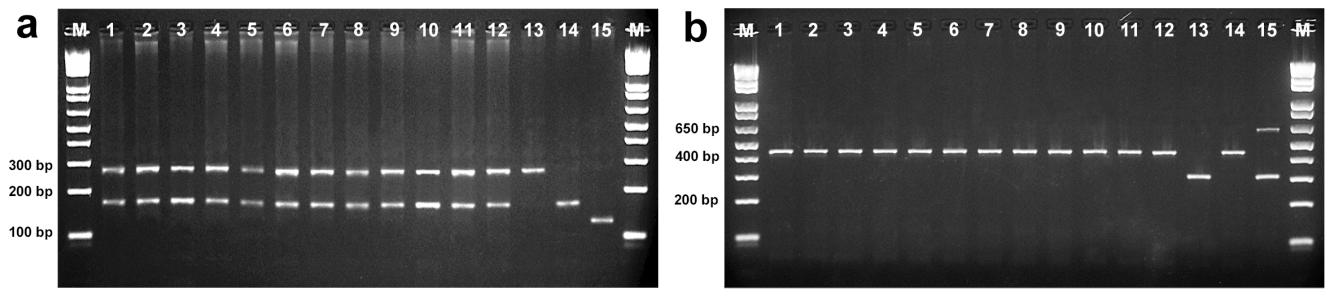

Figure 1. Molecular identification of the samples SPS1, MGS1, MGS2, MGS3, SES1, SES2, and SES3 using the nuclear *tpm1* (a) and mitochondrial *mt-co1* (b) genes in multiplex-PCR. Lanes 1–12, genotypes of hybrid tambacu (♀ tambaqui x ♂ pacu); and lanes 13, 14, and 15, control DNA samples from the pure pacu, tambaqui, and pirapitinga species, respectively; M, 1 Kb Plus DNA Ladder.
doi:10.1371/journal.pone.0089902.g001

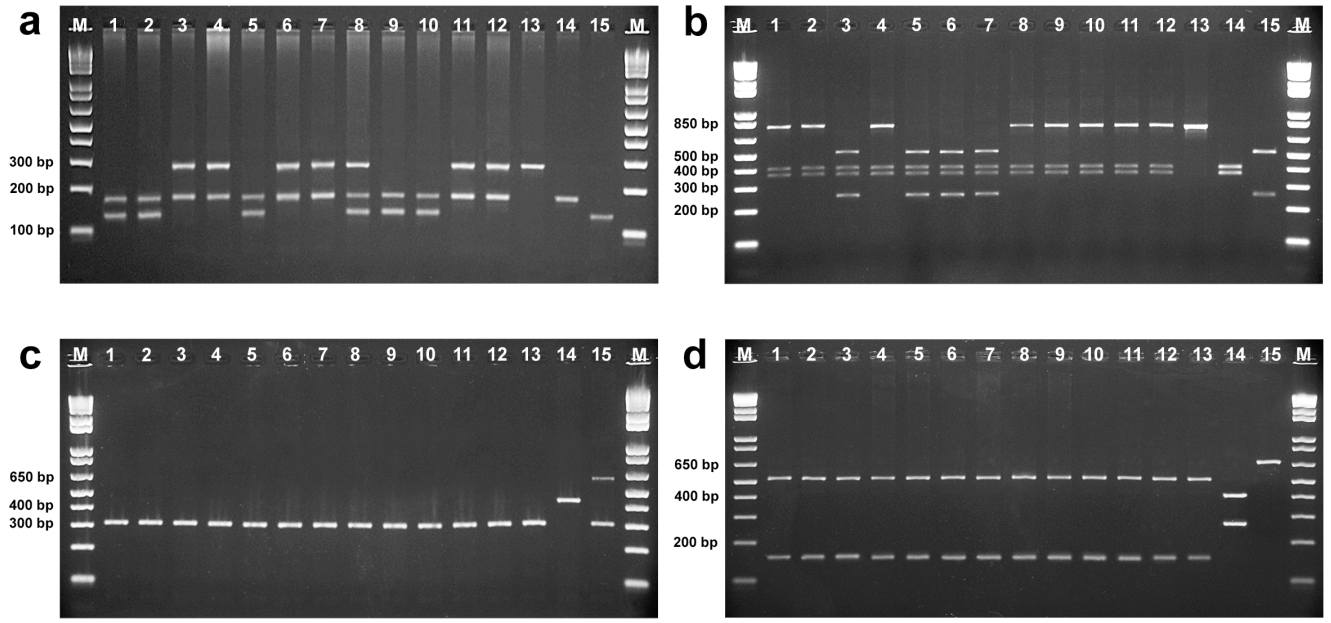

Figure 2. Molecular identification of the SPS2 stock using the nuclear *tpm1* (a) and *rag2* (b) genes, and mitochondrial *mt-co1* (c) and *mt-cyb* (d) genes. Lanes 4, 11, and 12, genotype A; lane 5, genotype B; lanes 3, 6, and 7, genotype C; lanes 1, 2, 9, and 10, genotype D; lane 8, genotype E; and lanes 13, 14, and 15, control DNA samples from the pure pacu, tambaqui, and pirapitinga species, respectively; M, 1 Kb Plus DNA Ladder.
doi:10.1371/journal.pone.0089902.g002

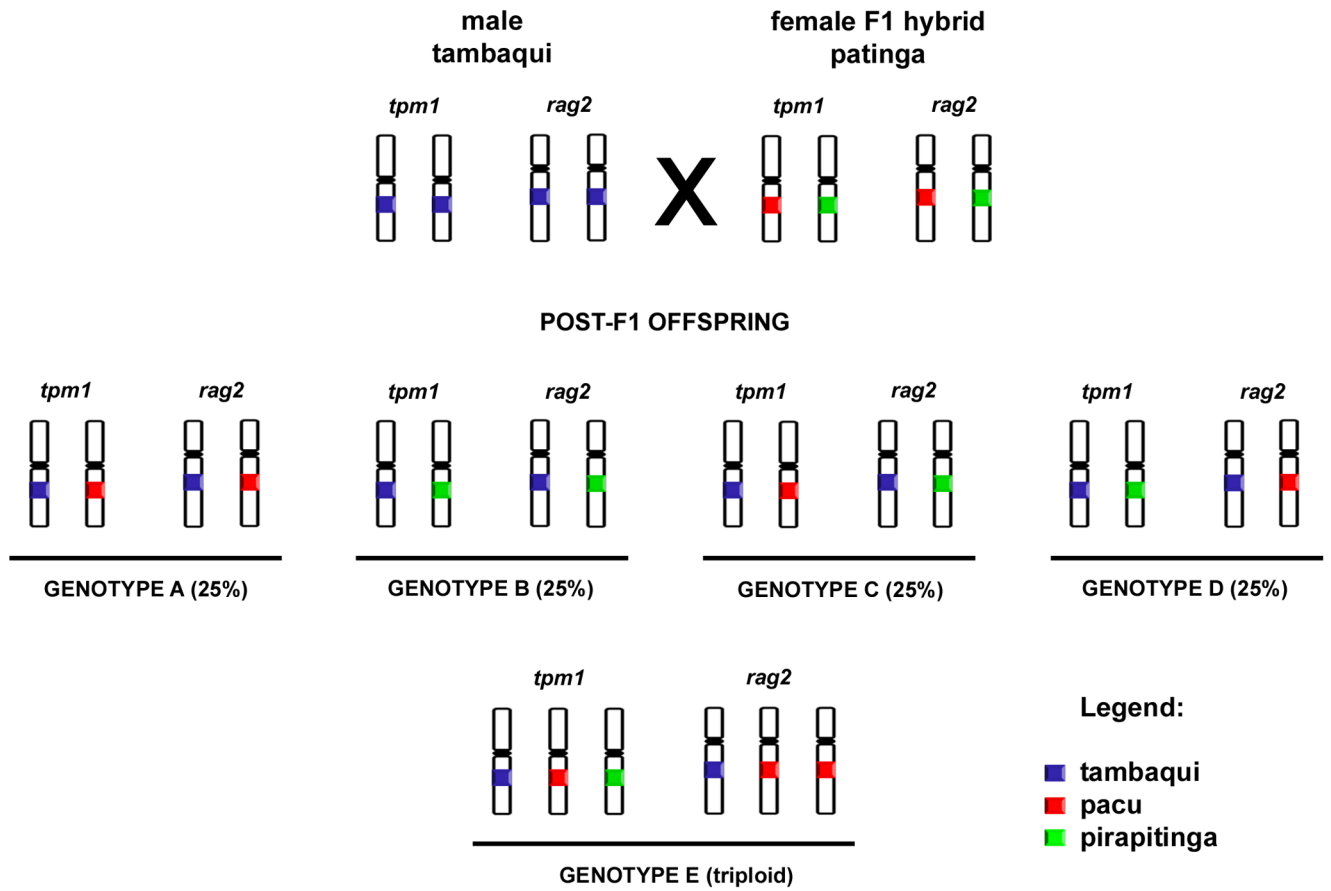

Figure 3. Schematic representation of the backcrossing between ♂ tambaqui x ♀ patinga (♀ pacu x ♂ pirapitinga), demonstrating the *tpm1* and *rag2* gene loci and the expected probability of each genotype.
doi:10.1371/journal.pone.0089902.g003

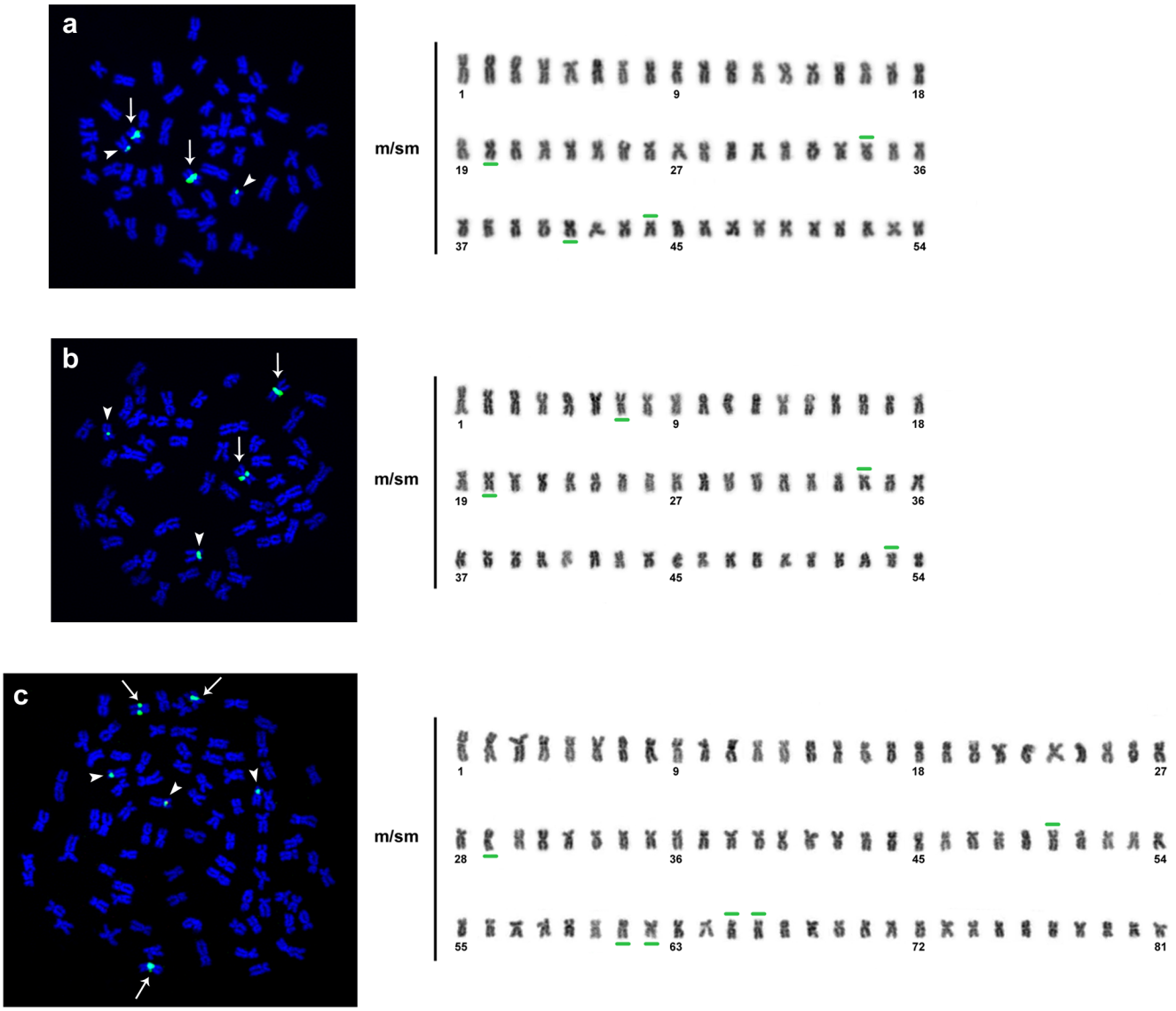

Figure 4. Metaphases and the respective karyotypes of individuals of the SPS2 stock, showing the chromosome location of the 5S rRNA clusters. The arrows and arrowheads indicate the 5S rRNA major and minor clusters, respectively, in metaphase. In the karyotypes, the green bars indicate the chromosomes bearing 5S rRNA genes and their respective locations (long or short arms). (a) an individual of genotype A, (b) an individual of genotype B, and (c) an individual of genotype E.
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genotypes of the nuclear markers *tpm1* and *rag2*, thus suggesting independent segregation between these markers.

Discussion

Hybrids between tambaqui and pacu are popular in Brazil, as these hybrids combine the robustness and faster growth rate of tambaqui with the low temperature resistance of pacu [23,24]. The technology required for the reproduction of hybrid tambacu through hormonal induction has been well established and widely used in farming systems, making cultivation easier than with other hybrids and even pure species. This effect might explain the results obtained in the present study, in which the hybrid tambacu was traded as other hybrids and pure species. The admixture of different types of fish is another problem observed on fish farms [12], where stocks of serrasalmid juvenile fish comprised up to five types of fish, including pure species and hybrid individuals. However, these mislabeling activities represent a fraud to the market and are not productive for cultivation and aquaculture because different hybrids and pure species have specific zootechnical characteristics and economic values [2].

Special attention should be given when hybrids are sold as pure species, as demonstrated in the present study for the stocks of tambaqui (MGS1 and SES1) and pacu (MGS2). The results showed that in addition to fraud, fish farmers are not aware of the potential biological and environmental risks represented by hybrids, whose impact could affect the aquaculture industry and threaten native species, as previously described in other species, such as tilapia, catfish, and trout [25–28]. Moreover, the same problems have been observed for other Brazilian fish farms in several States (São Paulo, Minas Gerais, Piauí, Sergipe, and Pará) [12,29], indicating that could be a common practice in the aquaculture industry.

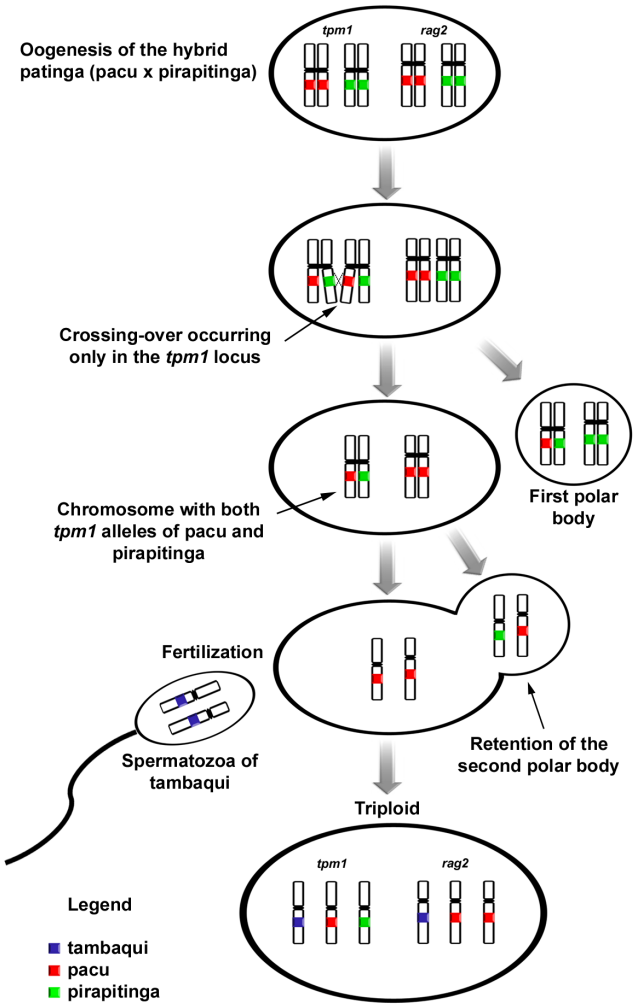

Figure 5. Schematic representation of the events of crossing-over and the fusion/retention of the second polar body, which likely generated the triploid individual of genotype E.
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In addition, the results of the molecular characterization in the present study provided clear evidence that individuals of female patinga are fertile, similar to the hybrids tambacu and tambatinga [18,30]. Hence, due to the difficulty of morphological identification, these hybrids can be erroneously used as broodstock on Brazilian fish farms, which is not productive for aquaculture and represents even higher risks [15,31]. The negative effects of post-F1 individuals include the dilution or loss of the desirable characteristics, resulting from hybrid vigor [32], low hatching rate and high mortality level [17,18]. Therefore, the data obtained in the present study show that molecular tools should be applied in breeding facilities to ensure the integrity of pure stocks used on the Brazilian fish farms, as previously demonstrated [18], where even post-F1 hybrids have been observed in the farmed broodstock of catfish.

Serrasalmid hybrids have also been detected in the natural environment and some authors have suggested that these individuals are the consequence of aquaculture activities [12,33,34]. The presence of fertile hybrids on the farms observed in the present study reveals the potential risks of these farming practices to wild populations. Introgressive hybridization poses a threat to the genetic integrity of pure species, which might result in a single hybrid population, as reported in trout, catfish, and tilapia [27,35–37]. In Brazil, this situation has also been described for hybrids between the Neotropical catfish species *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum* [38]. Genetic analysis of these species have revealed a high frequency of hybrids, including post-F1 generation individuals in the Mogi Guaçu (50%) and Aquidauana (30%) rivers, where the majority of the Brazilian fish farms are located, suggesting that hybrids might be introduced from farmed stocks [38]. The results obtained from the present study should be complemented with the analysis of wild populations to confirm whether hybrids in natural environments result from farm escapees or natural hybridization. This point is critical for the development of government regulations to achieve sustainable and environmentally respected aquaculture.

The combination of molecular markers and cytogenetic techniques was essential for the identification of a triploid individual in the present study and to confirm the hybridization processes. Cytogenetic information is considered a suitable tool to verify ploidy level and analyze parental chromosome sets in hybrids [4,39,40]. In previous studies, pure species of tambaqui, pacu, and pirapitinga were characterized by a diploid chromosome number of 54 m/sm chromosomes [13,14]. The individuals cytogenetically analyzed in this study also showed $2n = 54$ chromosomes, with the exception of the sample corresponding to genotype E, which was characterized by triploidy (3n). This type of event was also described for an individual of the hybrid tambacu [13] and for a post-F1 specimen resulting from backcrossing between ♀ pacu x ♂ tambacu (♀ tambaqui x ♂ pacu), which also generated gynogenetic individuals [18].

Spontaneous triploid fish can be explained by several cytological mechanisms that produce unreduced diploid gametes [41]. Premeiotic endomitosis is characterized by genome doubling without cytokinesis before meiotic division, followed by two quasinormal divisions. In apomixis, meiosis is repressed in the first division, and the oocyte is produced through mitosis, without the recombination and segregation of the homologous chromosomes. However, in both mechanisms, the unreduced gametes are isogenic, *i.e.*, genetically identical to the parent without genetic variation of the resulting eggs [41,42]. Premeiotic endomitosis and apomixis occurs in several fish species [43,44], including interspecific hybrids [45], but cannot explain the triploid event observed in the present study, as genetic variations were detected in the molecular analyses.

Alternatively, these results are consistent with a cytological mechanism similar to automixis, in which meiosis is maintained and diploid gametes are generated after meiosis through the fusion/retention of the polar body. This phenomenon has been well documented in the triploid offspring of poeciliid interspecific hybrids [42]. The resulting triploid products are not genetically identical to the parental genome, as segregation and recombination result in nonidentical homologous chromosomes [42], explaining the atypical genotype E observed herein. However, further studies are needed to evaluate whether this triploid event is due to automixis or whether hybridization facilitates polyploidization, as shown in other species [46].

Consistent with the data obtained in this study, the presence of 5S rRNA clusters in two pairs of chromosomes in diploid individuals is a common characteristic in fish genomes [47,48]. However, the results of the present study demonstrated the occurrence of different combinations of chromosomes bearing 5S rRNA sites, suggesting these combinations were inherited from chromosomes of distinct species.

The molecular and cytogenetic data obtained herein are consistent with the hypothesis of the fertility of hybrid patinga.

However, the presence of additional post-F1 hybrids (F2 or advanced backcrosses) cannot be ruled out at least for some specimens because of the small number of nuclear markers used in this study. According to Boecklen and Horward [49], more than 70 nuclear markers are needed for the reliable differentiation between pure species and advanced hybrid crosses or backcrosses. Thus, the acquisition of additional markers based on nuclear genes is necessary for the identification of serrasalmid hybrids. The combination of next-generation sequencing technologies with restriction enzyme analyses simplifies genome sequencing to obtain deeper coverage at particular sites, thus facilitating the identification of thousands of SNPs (single nucleotide polymorphisms) at low cost [50–52], as Hohenlohe et al. [53] and Amish et al. [54] demonstrated through the identification of thousands of SNPs for the accurate detection of hybrids between *Oncorhynchus mykiss* and *Oncorhynchus clarkii lewisi*.

In Brazil, most aquaculture establishments are not licensed and there is little proposed legislation regulating fish breeding [8]. In contrast, some countries have legislation that specifically addresses issues concerning hybridization: California (USA) has laws prohibiting the hybridization of fish without a proper license [2,55]. Thus, specific laws should be implemented in Brazil to address the problems of the uncontrolled trade and management of the fish hybrids detected in several studies [12,17,29,38]. Moreover, confinement measures are indispensable to avoid the widespread dissemination of fish hybrids [2], particularly physical and reproductive measures required for transgenic fish [56,57].

Consistent with Hashimoto et al. [12], the results obtained in the present study show that genetic tools should be applied to monitor the trade of juvenile fish hybrids, representing a preventive measure for the sustainable development of the aquaculture industry, particularly because serrasalmid hybrids are fertile (*e.g.*, hybrid patinga) and hybrid tambacu can be erroneously traded as pure species. In conclusion, this multi-approach analysis (molecular, cytogenetic, and FISH methods) was useful for the detection of hybridization and the results provided new insights concerning the genome plasticity of serrasalmid species, including the occurrence of intergenus backcrossing between ♀ patinga (♀ pacu x ♂ pirapitinga) x ♂ tambaqui and the presence of an post-F1 hybrid triploid, likely derived from similar mechanisms of unreduced gametes in automixis described for other fish hybrids [42].

Author Contributions

Conceived and designed the experiments: DTH JAS FPF. Performed the experiments: DTH FPF. Analyzed the data: DTH FF PM FPF. Contributed reagents/materials/analysis tools: DTH JAS FF FPF. Wrote the paper: DTH JAS FF PM FPF.

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