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Production of gynogenetic diploid *Polyodon spathula* using fertilization with irradiated sperm followed by spontaneous diploidization and distant hybridization caused by heat shock

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Summary

When paddlefish, *Polyodon spathula*, eggs were fertilized with UV irradiated (863 μW cm^{-2}) sperm of Amur sturgeon, *Acipenser schrenckii*, viable gynogenetic diploid progeny with approximately 120 chromosomes occurred due to spontaneous diploidization of the maternal set of chromosomes (SDM). Distant hybridization of *P. spathula* eggs by *A. schrenckii* sperm induced inviable gynogenetic haploid progeny with approximately 60 chromosomes. When eggs gynogenetically activated by heterospecific sperm were heat-shocked with 37°C for 2 min at 18 min post-fertilization, a small proportion (7.8%) of viable gynogenetic diploid progeny appeared. The female inheritance of these gynogenetic diploid progeny was verified by chromosome analyses and microsatellite genotyping.

Introduction

Induced gynogenesis is considered a valuable tool for restoring endangered or extinct species (Saber et al., 2008). The method has been established in sturgeon (Fopp-Bayat et al., 2007) as their threatened status requires urgent conservation efforts (Ludwig, 2008; Zhu et al., 2008). The American paddlefish, *Polyodon spathula*, and the Chinese paddlefish, *Psephurus gladius*, are the only existent species of the family Polyodontidae (Wei et al., 1997; Wu, 2005; Peng et al., 2007). At present, little research on *P. gladius* can be conducted due to its scarcity (Zhang et al., 2009). On the other hand, *P. spathula* is a promising model for studying conservation of the Chinese paddlefish. The technique of gynogenesis induced by heterospecific sperm in *P. spathula* is being used for the conservation and enhancement of them.

Spontaneous diploidization of the maternal chromosome set (SDM) is a well-known natural event in fishes (Cherfas et al., 1991). It can be detected in artificial gynogenesis experiments from the occurrence of normal diploid progeny among haploid controls to which no diploidization treatment was applied (Ezaz et al., 2004). Usually, the environmental shock, thermal, chemical or pressure treatments can easily lead to developmental malformation of the embryo in artificial restoring diploidy of the zygote (Ye and Wu, 2003). Fortunately, SDM induce no actual damage for embryo development (Ezaz et al., 2004). It is therefore of value to use SDM approach to obtain gynogenetic offspring.

Artificially induced SDM has been used successfully to produce gynogens in some teleost species, such as Nile tilapia, *Oreochromis niloticus*, (Ezaz et al., 2004) as well as in white sturgeon, *Acipenser transmontanus*, (Van Eenennaam et al., 1996); sterlet, *Acipenser ruthenus*, and Siberian sturgeon, *Acipenser baerii*, (Fopp-Bayat, 2007).

On the other hand, the use of normal heterospecific sperm rather than UV-irradiated heterospecific sperm to induce egg development is technically easier to handle and greatly increases the efficiency of gynogenesis (Lou, 2001; Chen et al., 2009). Our previous data shows that *P. spathula* eggs initiate gynogenetic haploid development when fertilized with sperm of remotely related heterospecific species, and inviable gynogenetic haploid with approximately 60 chromosomes were produced (Zou et al., 2009). In addition to this preliminary study, the authors want to show more detailed data to conclude gynogenesis by hybridization and SDM in the present manuscript. Therefore, gynogenesis induced in *P. spathula* by fertilization with normal *A. schrenckii* sperm and subsequent heatshock could induce viable gynogenetic diploid progeny.

The aims of the present study were (i) to induce haploidy in *P. spathula* using genetically inactivated *Acipenser schrenckii* sperm and to confirm that normal appearing and 'long-surviving' fish could be due to SDM, (ii) to induce gynogenesis by means of distant hybridization of *P. spathula* eggs, by *A. schrenckii* sperm, then to duplicate chromosomes by heat-shocking the embryos. The successful induction of gynogenetic diploidy would be confirmed by cytogenetic analyses and microsatellite analysis.

Materials and methods

Broodstock and gamete collection

All experiments were conducted at the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Science, in spring 2009 and 2010. Five females of *P. spathula* (10–12 kg), three males of *A. schrenckii* (12–14 kg), and two males of *P. spathula* (9–11 kg) were used for gamete collection. State of maturation was assessed by measuring the oocyte diameter and observing germinal vesicle migration from ovarian biopsies (Mims and Shelton, 2005). Induction of spawning and spermiation was carried out according to the method described by Doroshov et al. (1983). Unfertilized eggs collected by

compressing the female's abdomen were maintained in beakers without water at 15°C. Sperm was drawn into sealed plastic bags with oxygen, and kept at 4°C until use.

Experimental design

Experimentation was conducted on three groups of *P. spathula eggs*: (i) a diploid control group (C) fertilized with *P. spathula* sperm, to test egg viability; (ii) a gynogenetic haploid group (S) fertilized with genetically inactivated *A. schrenckii* sperm, without heat shock treatment, to confirm that normal appearing larvae in haploid group could be due to SDM; and (iii) a gynogenesis by distant hybridization group (Gy) fertilized with normal *A. schrenckii* sperm, with the embryos subjected to heat shock to confirm the feasibility of producing gynogenetic *P. spathula*.

Induction of gynogenesis

The sperm of *A. schrenckii* was divided into two aliquots: irradiated sperm for use with S and untreated sperm for use with Gy. The methods of UV irradiation and parameters of heat shock are described by Zou et al. (2011). In shortly, for the UV irradiation treatment, semen from *A. schrenckii* were diluted 1 : 4 with seminal fluid of the *A. schrenckii* (supernatant from surplus semen by centrifugation (10 ***g*** for 10 min)), The resultant sperm solution (5 ml) was put into Petri dishes (diameter: 2000 mm) to a depth of approximately 200 mm. These dishes were placed on a rotating platform gently 30 cm below the three UV lamps (Philips 30 W), and irradiated for 5 min, which provided an incident light intensity (254 nm) of 863 μW cm^{-2}, as measured by a UV radiometer (UVC-254, Ultra-Violet Products, National). For group S, 1000 ml (approximately 60 000) of eggs were fertilized with 50 ml diluted and irradiated milt (one sperm: four seminal fluid), without heat shock treatment, and the 'fertilized eggs' were activated with 800 ml dechlorinated tapwater and incubated at 18°C. For group Gy, 1000 ml of eggs were fertilized with 50 ml of diluted *A. schrenckii* sperm and, beginning 18 min post-fertilization, subjected to heat shock at 37°C for 2 min, to retain the second polar body. Egg quality was evaluated by insemination with *P. spathula* sperm (C).

Early embryo development was recorded as: fertilization rate (the ratio of live eggs at mid-gastrulation to total eggs 21 h post-fertilization), hatching rate (the ratio of hatched larvae to live eggs at mid-gastrulation 6 days post-fertilization) and survival rate the 30 days (the ratio of viable larvae after 30 days to hatched larvae). Hatched larvae from each treatment were transferred to separate 100 L tanks at 18°C, where they remained until sampling for chromosome characteristics and ploidy determination, and for microsatellite analysis.

Data were analyzed by one-way analysis of variance (ANOVA) with SPSS 13.0 (Chen et al., 2009). All data were expressed as means ± SD, and statistical significance was set at $P < 0.05$.

Identification of gynogenetic fry

Chromosome analysis. Cytogenetic analysis was conducted 60 days post-hatching. Chromosome preparation was derived from fibroblast cells of caudal fin of fry from group C, S, and Gy, cultured according to Freshney (2000). Chromosome preparation was as described by Wang et al. (2004). Ten fry from each group were examined cytogenetically. A mitotic metaphase plate of each specimen was observed and photographed at ×1000 (Nikon, Japan). At least 50 well-spread metaphase plates from each fish were analyzed. In addition, abnormal embryos at the stage of neurulation from group S were used for chromosome preparation, and chromosome preparation was carried out according to the method described by Wu et al.(1981).

Ploidy determination. Ten normal larvae per group were randomly selected for blood sampling 90 days post-hatching. Fifty microlitres of blood was drawn from the caudal vein, mixed immediately with 200 μl phosphate-buffered saline (PBS) (Ph 7.4), and stained with 800 μl 4-6-diamidino-2-phenylindole dihydrochloride (DAPI). After staining for 5 min at 4°C in darkness, nuclear suspensions were subjected to flow cytometry (Beckman Coulter, USA) to measure the relative DNA content. In addition, seven abnormal embryos from group S were also sampled for ploidy assessment according to You et al. (2001). Larvae from group C were used as a diploid standard for the calibration of the cytometer.

Microsatellite analysis. Fin clips from parent specimens (*A. schrenckii* × *P. spathula*) as well as tails of 10 randomly selected larvae per group were sampled 120 days post hatching and stored in 95% ethanol. Total genomic DNA was extracted using the phenol–chloroform method modified after Pourkazemi (1996). Three pairs of microsatellite markers (*Psp-18*, *Psp-29*, *Psp-32*) (Heist et al., 2002) were used. The methods, with modifications, for PCR and amplification were described by Zou et al. (2011).

Results

Fertilization, hatching and survival rates

Fertilization, hatching, and survival rates for each group are shown in Table 1. Treatment group S showed significantly lower fertilization rate in comparison with control group C and Gy ($P < 0.05$). All treatment groups S and Gy showed lower hatching rate in comparison with the control C ($P < 0.05$). Most embryos of the group S displayed features of 'haploid syndrome' (abnormal body shape, open blastopore) and died during hatching. 1.8 ± 0.3% fertilized eggs spontaneously developed into viable diploid larvae with normal appearance. The survival rate of the 30-day-old progeny observed in Gy (75.6 ± 9.2%) was similar to that of C (75.1 ± 18.8%), but the survival rate of S was significantly lower (54.6 ± 7.9%) ($P < 0.05$).

Table 1
Fertilization, hatching, and survival rates of *Polyodon spathula* eggs of experimental group. C, eggs fertilized with *P. spathula* sperm; S, eggs fertilized with genetically inactivated *A. schrenckii* sperm; Gy, eggs fertilized with normal *A. schrenckii* sperm, and embryos then heat shocked

Group	Fertilization rate (%)	Hatching rate (%)	Survival rate (%) at 30 days
C	84.8 ± 10.2	78.2 ± 6.8	75.1 ± 18.8
S	50.3 ± 5.3	1.8 ± 0.3	54.6 ± 7.9
Gy	76.4 ± 9.2	7.8 ± 1.4	75.6 ± 9.2

Note: Fertilization rate: the ratio of live eggs at mid-gastrulation to total eggs 21 h post-fertilization. Hatching rate: the ratio of hatched larvae to live eggs at mid-gastrulation, 6 days post-fertilization. Survival rate: the ratio of viable larvae after 30 days to hatched larvae.

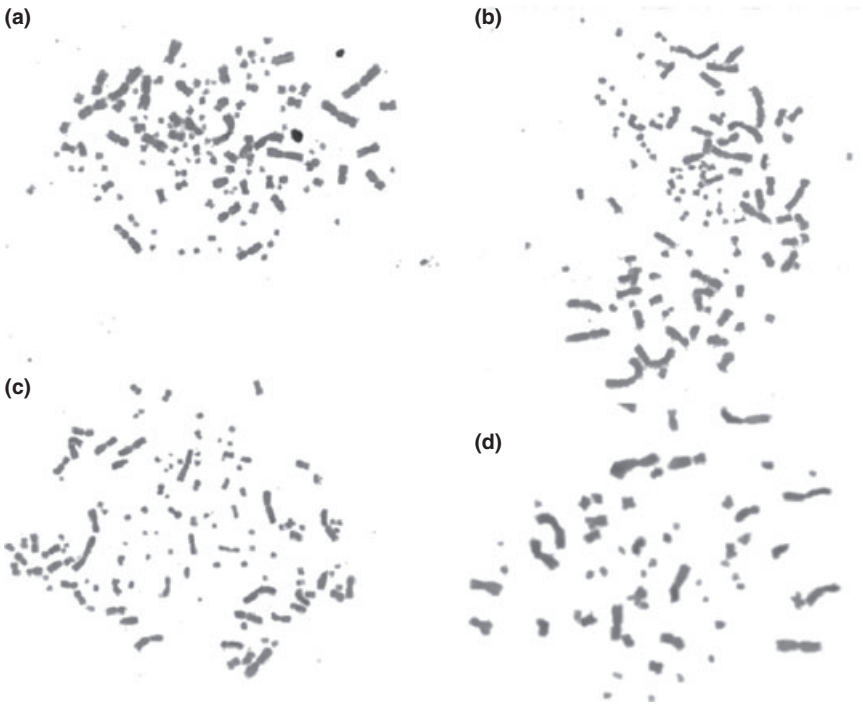

Fig. 1. Metaphase chromosome spreads from (a) normal diploid *Polyodon spathula* with approximately 120 chromosomes in Group C; (b) spontaneous diploid *P. spathula* with approximately 120 chromosomes in Group S; (c) gynogenetic diploid *P. spathula* by hybridization with approximately 120 chromosomes in Group Gy; (d) gynogenetic haploid embryos with approximately 60 chromosomes in Group S. bar = 10 μm

Chromosome and ploidy level analysis

Metaphase plates from C, S, Gy larvae, and the frequency distribution of chromosome numbers are shown in Fig. 1 and Table 2. Groups S and Gy showed diploid chromosome numbers (approximately 120) (Fig. 1b,c), which were same to C (Fig. 1a). However, abnormal embryos in group S showed haploid chromosome numbers (approximately 60) (Fig. 1d).

Table 3 shows the DNA contents and ploidy levels of the progeny in Group C, S, and Gy. In control diploid group C, gynogenetic diploid Groups S and Gy, all progeny were diploids. However, abnormal embryos in Group S showed haploid. DNA content of diploid in group C larvae to haploid was approximately two times.

Microsatellite DNA analysis

The results of microsatellite analysis obtained no genetic contribution from the paternal genome (*A. schrenckii*) in groups S and Gy. The locus *Psp-29* was the most reliable for genetic verification of fish from the present experiment, because at this locus the mare of the gynogenetic diploids was characterized by all alleles being different from those of

Table 2
Frequency distribution of chromosome numbers from Group C, S, Gy larvae and S abnormal embryos. Group C: normal diploid *Polyodon spathula*; Group S (S_1): spontaneous diploid *Polyodon spathula*; Group S (S_2): haploid embryo of *Polyodon spathula*; and Group Gy: gynogenetic diploid *Polyodon spathula* by hybridization

Group	Sample number	Frequency distribution of chromosome (numbers)				Metaphase counts	Modal (%)
C	10	>115 (42)	115–119 (58)	120 (430)	121–124 (28)	558	77
S(S_1)	10	>115 (46)	115–119 (63)	120 (375)	121–124 (39)	523	71
S(S_2)	8	>55 (31)	56–59 (54)	60 (335)	61–65 (28)	448	74
Gy	10	>115 (42)	115–119 (65)	120 (486)	121–124 (30)	623	78

Note: S_1, spontaneous diploids in Group S; S_2, haploid embryos in Group S.

Table 3
DNA contents and ploidy levels of larvae in Group C, S (S_1, S_2), and Gy. Group C: normal diploid *Polyodon spathula*; Group S (S_1): spontaneous diploid *Polyodon spathula*; Group S (S_2): haploid embryo of *Polyodon spathula*; and Group Gy: gynogenetic diploid *Polyodon spathula* by hybridization. DNA values from normal diploid *P. spathula* in Group C was set as '200'and used as a standard

Group	Sample number	Average channel number ± SD	CV (%)	DNA contents ±SD (pg per N)	Ploidy type
C	10	200.0 ± 0.86	1.63 ± 0.04	3.50 ± 0.32	Diploid
S(S_1)	10	204.2 ± 0.91	2.12 ± 0.07	3.57 ± 0.43	Diploid
S(S_2)	7	101.8 ± 0.54	0.98 ± 0.02	1.78 ± 0.19	Haploid
Gy	10	205.7 ± 0.99	2.48 ± 0.08	3.59 ± 0.47	Diploid

Note: S_1, spontaneous diploids in Group S; S_2, haploid embryos in Group S.

Table 4
Microsatellite genotypes at three loci in progeny of Group C, S and Gy. Group C: normal diploid *Polyodon spathula*; Group S: spontaneous diploid *Polyodon spathula*; and Group Gy: gynogenetic diploid *Polyodon spathula* by hybridization

Locus	Group	Male *P. spathula* genotype	Male *A. schrenckii* genotype	Female genotype	Microsatellite genotypes in progeny (numbers)		
Psp-18	C	164 / 180	–	164 / 185	164 / 164 (3)	164 / 185 (8)	185 / 180 (9)
	S	–	150 / 158	164 / 185	164 / 164 (3)	185 / 185 (4)	165 / 185 (13)
	Gy	–	150 / 158	164 / 185	164 / 164 (2)	185 / 185 (4)	165 / 185 (14)
Psp-29	C	198 / 210	–	195 / 215	195 / 198 (5)	195 / 210 (7)	215 / 210 (8)
	S	–	190 / 190	195 / 215	195 / 195 (5)	195 / 215 (11)	215 / 215 (4)
	Gy	–	190 / 190	195 / 215	195 / 195 (7)	195 / 215 (10)	215 / 215 (3)
Psp-32	C	165 / 200	–	170 / 200	170 / 165 (9)	170 / 200 (8)	200 / 200 (3)
	S	–	120 / 140	170 / 200	170 / 170 (2)	170 / 200 (10)	200 / 200 (8)
	Gy	–	120 / 140	170 / 200	170 / 170 (5)	170 / 200 (9)	200 / 200 (6)

the sire. Alleles at 195 bp and 215 bp were observed at locus *Psp-29* in the female *P. spathula* (the parent of gynogenetic offspring) while allele 190 bp was characteristic for the male sperm donor at the same locus (Table 4). Similarly, microsatellite locus *Psp-32* in groups S, Gy, and C showed the maternal genome in all samples. At locus *Psp-18*, two alleles of 164 and 185 bp were observed in S and Gy, similar to the maternal fish, while in the paternal *A. schrenckii*, two alleles (150 and 158 bp) were found.

Discussion

Techniques for chromosome manipulation have been successfully applied to induce diploid gynogenesis in *P. spathula* using UV-irradiated heterospecific sperm to activate eggs, followed by second polar body restoration treatment as described by Mims et al. (1997) and Zou et al. (2011). In the present study, gynogenetic *P. spathula* larvae were also produced by (i) spontaneous diploidization of the maternal chromosome set after initiation of gynogenesis by fertilization with genetically inactived *A. schrenckii* sperm; and (ii) distant hybridization in *P. spathula*, by fertilizing their eggs with normal *A. schrenckii* sperm, then heat-shocking the embryos to duplicate chromosomes, which are reported for the first time in *P. spathula*.

At present, the low incidence of SDM has been observed during induction of gynogenetic haploid white sturgeon (Van Eenennaam et al., 1996), sterlet (Fopp-Bayat and Woznicki, 2007; Fopp-Bayat et al., 2007), and Siberian sturgeon (Fopp-Bayat, 2007). The mechanism of SDM induction has been studied (Thompson et al., 1981; Flajshans et al., 1993; Cherfas et al., 1995), and is most commonly associated with suppression of the second meiotic division (Ezaz et al., 2004). However, cytogenetic confirmation of diploid status had not been provided in fish. The present paper is the first well-documented case of spontaneous gynogenesis following egg fertilization with genetically inactivated heterospecific sperm.

Without irradiation, *A. schrenckii* spermatozoa appeared to activate the *P. spathula* eggs (up to gastrulation), but did not result in viable hybrids, chromosome analysis showed that the embryos were haploids with approximately 60 chromosomes (Zou et al., 2011). Additionally, it has been observed from cytology of *P. spathula* (♀) × *A. schrenckii* (♂), that, at insemination, an *A. schrenckii* sperm entered a *P. spathula* egg, and its nucleus was condensed near the female pronucleus but did not fuse with it (data not shown). The sperm merely activated egg development and the embryos developed into haploids, which eliminated the possibility that the genome of heterospecific sperm affected gynogenetic progeny. Therefore, gynogenetic diploid *P. spathula* induced by fertilization with normal *A. schrenckii* sperm and subsequent heatshock can be successfully.

Since *A. schrenckii* has 238 ± 8 chromosomes and is considered as a functional tetraploid species (Ludwig et al., 2001), while *P. spathula* has approximately 120 chromosomes and is considered diploid (Ludwig et al., 2001; Mims and Shelton, 2005), it is straightforward to identify gynogenetic fry through chromosome analysis or counting alleles. All gynogens tested in our study possessed maternal characteristics with a diploid chromosome complement of approximately 120 chromosomes. Therefore, gynogenetic fry were precisely identified by chromosome and ploidy level analysis. Although gynogenesis can be proven indirectly by restoring diploidy, exclusive maternal inheritance should be confirmed with molecular markers (Morishima et al., 2001; Flynn et al., 2006; Tvedt et al., 2006). In the present study, microsatellite DNA analysis was applied for verification of unipaternal inheritance in the gynogenetic diploid group of fish. All the analyzed gynogenetic diploids in the experimental fish possessed only maternal genotypes.

In summary, both spontaneous gynogenesis and distant hybridization followed by heatshock induced gynogenesis in *P. spathula* were effective methods. The results of the present study demonstrated that gynogenesis of *P. spathula* holds great potential for population enhancement, and will encourage future research.

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