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Induction of meiotic gynogenesis in paddlefish (*Polyodon spathula*) and its confirmation using microsatellite markers

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Summary

A protocol for gynogenesis of *P. spathula* was developed in order to produce inbred lines and to develop a technique to preserve the endangered Chinese paddlefish (*Psephurus gladius*). Diploid gynogenesis was induced in *P. spathula* using ultraviolet (UV)-irradiated Amur sturgeon (*Acipenser schrenckii*) sperm for 4.5-min, 5-min or 5.5-min and subsequent heat shock treatment. UV irradiation of sperm for 5 min at a UV intensity (254 nm) of 863 μw cm^{-2} was the optimum dose to achieve diploid gynogenesis on the basis of observations on hatching rate of eggs. Three microsatellite loci were used to monitor exclusive maternal inheritance of gynogenetic progenies. The results showed all maternal genome among offspring with no paternal genome. The cytogenetic analysis showed that meiotic gynogenetic diploids possessed 120 chromosomes in metaphase plates, while haploid control groups N_{1-3} possessed 60 chromosomes.

Introduction

Gynogenesis is a genome manipulation facilitating the inheritance of maternal genetic material alone, and this technique involves the activation of egg development by genetically inactive spermatozoa and subsequent diploidy restoration (Pandian and Koteeswaran, 1998; Felip et al., 2001). Induced gynogenesis is usually achieved by fertilization of eggs with genetically inactivated sperm using radiation or chemical treatments, or with heterologous sperm that triggers development without any genetic contribution to the egg (Thorgaard, 1983; Ihssen et al., 1990). Controlled gynogenesis is one of the main methods of genome engineering, which can help to solve problems of fish genetics and selection (Cherfas, 1987; Ihssen et al., 1990). Currently, artificial gynogenesis techniques have been developed in some fish species, e.g., common carp (*Cyprinus carpio*) (Komen et al., 1988; Cherfas et al., 1994), silver carp (*Hypophthalmichthys molitrix*) (Xie et al., 2000), turbot (*Scophthalmus maximus*) (Piferrera et al., 2004), and large-scale loach (*Paramisgurnus dabryanus*) (You et al., 2007).

Artificial induction of gynogenesis and polyploidy in Acipenseriformes (sturgeons and paddlefishes) were studied by some authors. The earliest work using artificial techniques for the production of meiotic gynogenetic sturgeon was reported by Romashov et al. (1963). In their study none of the gynogenetic larvae survived beyond 192 days after hatching. A first attempt to induce gynogenesis in *A. transmontanus* was unsuccessfully (Kowtal, 1987). Studies on techniques for production of gynogenetic sturgeons were conducted on various Acipenseriformes species, *A. transmontanus* (Van Eenennaam et al., 1996), *P. spathula* (Mims et al., 1997), *Scaphirhynchus platorynchus* (Mims and Shelton, 1998), *Huso huso and A. persicus* (Pourkazemi et al., 2000). *A. gueldenstaedti* and *A. stellatus* (Recoubratsky et al., 2003), bester (*Huso huso* female × *A. ruthenus* male) (Omoto et al., 2005), *A. ruthenus* and *A. baerii* (Fopp-Bayat et al., 2007), while Saber et al. (2008) successfully induced meiotic gynogenesis in *A. stellatus*.

Gynogenesis has been widely used in the studies of fish genetics and breeding such as establishment of inbred lines, production of all-female fish, and the investigation of sex determination (Van Eenennaam et al., 1999; Felip et al., 2001; Tvedt et al., 2006). However, there are no reports in the literature on applying artificial gynogenesis as a effective tool of conservation and enhancement of endangered species in fish.

The American *P. spathula* and the Chinese *P. gladius* of the Yangtze River are the only surviving species in the family Polyodontidae (Wei et al., 1997; Wu, 2005), the *P. spathula* is considered as a promising model fish for the study of conservation of endangered *P. gladius*. The techniques of gynogenesis induced by heterogenous sperm in American *P. spathula* is conducted as a technic programme for the conservation and enhancement for *P. gladius*. Induced gynogenesis by heterogenous sperm upon of the adult female *P. gladius*, which is a best way to reproduce *P. gladius* and enhance the population.

On the other hand, since the sexually maturation of *P. spathula* needs more than 5 years (Mims and Shelton, 2005), to obtain a inbred line of *P. spathula* by sib mating would take several decades and require much of labor and material resources. Modern artificial gynogenetic technology provides a rapid and economic way to establish inbred lines of *P. spathula*. By inducing the gynogenetic development of mature *P. spathula* eggs with UV-irradiated homologous or heterologous sperm and then inhibiting the second polar body release of the gynogenetic eggs, highly pure diploid individual could be obtained (Lou, 2001). After the maturation of the gynogenetic individuals, by reinducing the gynogenesis of mature eggs from a gynogenetic individual, absolute pure line *P. spathula* could be generated. Therefore, artificial gynogenesis and development of a pure line stocks in the *P. spathula* would be of significant benefit for both scientific research and aquaculture.

The major objective of the present study was to induce meiotic gynogenesis in *P. spathula* and apply two methods

(microsatellite DNA and cytogenetic analysis) to confirm the success of this gynogenesis.

Materials and methods

Broodstock and gamete collection

All experiments were carried out at the Experimental Station of Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Science in the spring of 2009. Broodfish were collected from two commercial aquaculture companies (Hubei Hengsheng Industrial Co., Ltd and Hubei Tianxia Xunye Co., Ltd). Female *P. spathula* had an average weight of 10–12 kg, while the average weight of male *P. spathula* and male *A. schrenckii* was 8–10 and 12–14 kg, respectively. Maturation in females was assessed by measuring the diameter of oocytes and observing germinal vesicle migration in ovarian biopsies. Artificial induction of spawning was carried out a hormonal induction (Doroshov et al., 1983; Zou et al., 2009). Unfertilized eggs were collected by pressing the abdomen and maintained in dry beakers. Sperm was drawn from the male into fresh-keeping bags and kept at 4°C in a refrigerator until use. Sperm motility was checked under 400 light microscopy (Leica, Germany) after activation with fresh water.

Experimental design

The experimental treatments we used for the induction of gynogenesis were based on the previous experiment on *A. transmontanus* (Van Eenennaam et al., 1996). A summary of the experimental design is given in Table 1. All experiments included diploid control groups (untreated eggs and sperm): C_1 = female paddlefish (PF) × male PF, to test the viability of eggs. C_2 = female PF × male Amur sturgeon (AS), to test for hybridization potential. Haploid control groups to determine the efficiency of UV inactivation of the sperm (untreated eggs and UV-irradiated sperm): $N_{1–3}$ = PF × irradiated AS (4.5 / 5 / 5.5 min). A triploid control group to assess the efficacy of second polar body retention (temperature-shocked eggs and untreated sperm): N-3 = female PF × male AS (shock), and a treatment designed to induce gynogenesis (UV-irradiated sperm and temperature-shocked eggs): $Gy_{1–3}$ = PF × irradiated AS (4.5 / 5 / 5.5 min) (shock).

Sperm and ova treatment

For UV-irradiation, 1 ml of sperm was diluted with 4 ml of seminal fluid of the *A. schrenchill* (supernatant from surplus semen centrifuged at 8000 rpm for 10 min) and put into Petri dishes (diameter: 900 mm) to a depth of approximately 100 mm. These dishes were placed on a rotating platform gently (60 rpm) 30 cm below the three UV lamps (Philips 30 W), 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). Sperm was subjected to three UV-irradiation for three treatments: 4.5 min (Gy_1 for diploid gynogen and N_1 for haploid gynogen), 5 min (Gy_2 for diploid gynogen and N_2 for haploid gynogen) and 5.5 min (Gy_3 for diploid gynogen and N_3 for haploid gynogen). Each treatment had three replicates. The entire UV apparatus was covered in black wooden cabinet to contain the UV light and to prevent photoreactivation of spermatozoan DNA (Flynn et al., 2006). In each treatment, 10 ml of fresh eggs (~600 eggs) were used. Before the insemination time, 100 ml of 18°C water was added to the irradiated sperm suspension and this mixture was immediately added to the eggs. Motility of the irradiated spermatozoa was confirmed microscopically by randomly checking some of the treatment groups after experiments were completed. Three batchs of activated eggs for diploid gynogen ($Gy_{1–3}$) were subjected to heat-shock. These eggs were transferred into boxes with perforated mesh and kept in water at 37°C for 2 min. Heat shocks were applied at 18 min post-fertilization, The temperature in the incubator was constantly monitored. Immediately after treatment, eggs were transferred into 1000 ml Weiss incubators (IRS, Olsztyn, Poland) with their controls in a thermoregulated incubation system at 18°C. Addition of water was considered to be the time of egg activation for all experiments. Survival of developing eggs and viable fry was recorded at different developmental stages: fertilization / gastrulation (21 h after fertilization-a.f.), neurulation (40–55 h a.f.) and hatching (6 days a.f.). Hatched larvae from each individual treatment were transferred to a special system of tanks (100 L each) at a temperature of 18°C, where they remained until sampling for ploidy and microsatellite DNA analysis.

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

Table 1
Experimental design and survival rates at different developmental stage of embryo / larvae for meiotic gynogenesis in *polyodon spathula*. Fertilization rate = (Number of live eggs at mid-gastrulation / Total number of eggs) × 100%, the survival rate at neurulation stage = (Number of live eggs at neurulation stage / Number of live eggs at mid-gastrulation), and hatching rate = (Number of hatched larvae / Number of live eggs at mid-gastrulation)

Group	Species / Sperm irradiation (min)	Shock temperature (°C)	Shock durations (min)	Shock time after activation / fertilization (min)	Fertilization rate (%)	Neurulation (%)	Hatching rate (%)
C_1	PF(-)	–	–	–	85.3 ± 3.7	78.2 ± 2.4	73.4 ± 2.1
C_2	AS(-)	–	–	–	65.4 ± 1.6	0.5 ± 0.03	0.0 ± 0.0
N_1	AS(4.5)	–	–	–	56.3 ± 2.1	28.7 ± 1.7	1.3 ± 0.05
N_2	AS(5)	–	–	–	44.5 ± 1.7	25.3 ± 1.5	1.2 ± 0.03
N_3	AS(5.5)	–	–	–	31.2 ± 1.3	20.5 ± 1.2	1.2 ± 0.01
Gy_1	AS(4.5)	37 ± 0.5	2	18	47.7 ± 1.8	26.9 ± 1.2	18.5 ± 1.0
Gy_2	AS(5)	37 ± 0.5	2	18	56.4 ± 1.2	39.1 ± 1.3	28.7 ± 1.8
Gy_3	AS(5.5)	37 ± 0.5	2	18	43.6 ± 1.4	22.7 ± 1.5	13.2 ± 0.7
3N	AS(-)	37 ± 0.5	2	18	38 ± 0.05	12.5 ± 2.0	7.9 ± 0.6

PF = Paddlefish, AS = Amur sturgeon, C_1 / C_2 = Control (normal PF / AS sperm + normal PF eggs), $N_{1–3}$ = Haploids [(4.5 min / 5 min / 5.5 min irradiated AS sperm + normal PF eggs), $Gy_{1–3}$ = Gynogens [(4.5 min / 5 min / 5.5 min irradiated AS sperm + normal PF eggs) + heat shock], 3N = Triploid [(normal AS sperm + normal PF eggs) + heat shock].

Genetic investigation of gynogenesis effect

Eight pairs of microsatellite primers developed for *P. spathula* (Heist et al., 2002), were used to monitor maternal inheritance. Three loci, namely Psp-28, Psp-29, Psp-32, were finally chosen depend on their high polymorphism (Table 2). Twenty randomly selected larvae from putative gynogenetic groups and controls were sampled at 5 days after hatching and stored in 96% ethanol. Fin-clips from two parental individuals: female *P. spathula*, and male *A. schrenckii* were also stored in 96% ethanol. Offspring DNA (diploid gynogens and diploid control) and their parents' DNA were extracted using the phenol-chloroform technique (Saber et al., 2008).

PCR reactions mixes were prepared in a total volume of 25 μl with 1 μl (30–50 ng) DNA template, 1 μl (10 μM) each primer, 1 units Taq DNA polymerase, 1 μl (2 mM) each dNTP, Mg^{2+} (25 mM) 1 μl, 10 × Taq buffer 2.5 μl, and re-distilled water was used to bring the reaction mixture to the desired final volume. Amplification consisted of a 5-min denaturation step at 94°C, 30 cycles of 94°C for 30 s, 56–60°C for 30 s, and 72°C for 40 s, followed by a single ten-minute extension step at 72°C. PCR products and reaction buffer were electrophoresed using a 8% polyacrylamide gel, and DNA bands were visualized by the silver staining method (Tegelström, 1986). Every gel analyzing progeny included two lanes containing the appropriate parental microsatellite PCR amplification products. Specific microsatellite profiles for parents were noted and compared to those from experimental groups.

Ten specimens from each gynogenetic group (Gy_1, Gy_2, Gy_3) were analyzed cytogenetically. Ten randomly selected larvae with the different body deformations from each haploid group (N_1, N_2, N_3) were sampled during the yolk sac retention for chromosome preparations. Chromosomes were prepared according to Woznicki et al. (1998) with modifications, at least eight well-spread metaphase plates from each specimen were analyzed.

Results

Optimal conditions for gynogenesis

The survival rate at the gastrulation, neurulation stage and after hatching in studied groups of fish is described in Table 1. Control 1 showed significantly higher fertilization and hatching rate in comparison with other treatment groups. In control 2, *A. schrenckii* spermatozoa appeared to activate the eggs. The embryo could develop to gastrulation, and only few embryos reached to neurulation, but did not produce viable hybrids. In diploid gynogens, Gy_2 showed that the best yields of gynogenotes in comparison with Gy_1 and Gy_3 ($P < 0.05$), Which means *A. schrenckii* sperms irradiated by UV lights at intensity (254 nm) of 863 μw cm^{-2} for 4.5–5.5 min was found to be useful for producing viable putative meiotic diploids, and UV irradiation of sperm for 5 min was the optimum dose to achieve diploid gynogenesis on the basis of observations on hatching rate of eggs. Duration of 2 min for the heat shock at 37°C in the 18 min after fertilization was found to be effective to retain the second polar body and to produce meiotic diploids.

In haploid gynogens, although these groups showed higher fertilization rate, most of embryos died during incubation. The treatments were effective in the production of a 'haploid' offspring group with more than 98% mortality, these larvae displayed features of 'haploid syndrome' (abnormal body shape, open blastopore), it was apparent that 98% the fish had numerous large fluid-filled cysts associated with the internal organs and mesentery and more than 95% died before feeding (Fig. 1).

Table 2
Sequence and specific annealing temperature of microsatellite primers of *Polyodon spathula,* Locus Psp-32 contained an imperfect repeat with numerous short $(GT)_n$ motifs

Locus	Inheritance	Primers(5′-3′)	Temp. (°C)	Longest Repeat	Length of PCR Products (bp)
Psp-28	Disomic	F: CAAAGGCATCCCCTACCAC R:GCTGGACAAAAAGTATGGAGTGC	56	$(GA)_{37}$	224–260
Psp-29	Disomic	F:GGGGTCTAATAAAATCCACCGTTC R: TTGCCTTGTGCTCTGTGTTCC	56	$(GCAC)_6$	195–215
Psp-32	Disomic	F: AATGACTCAGTTGTGTGCTGC R: AAGTGTAGGGGAATCTCACCAG	60	Imp.	173–177

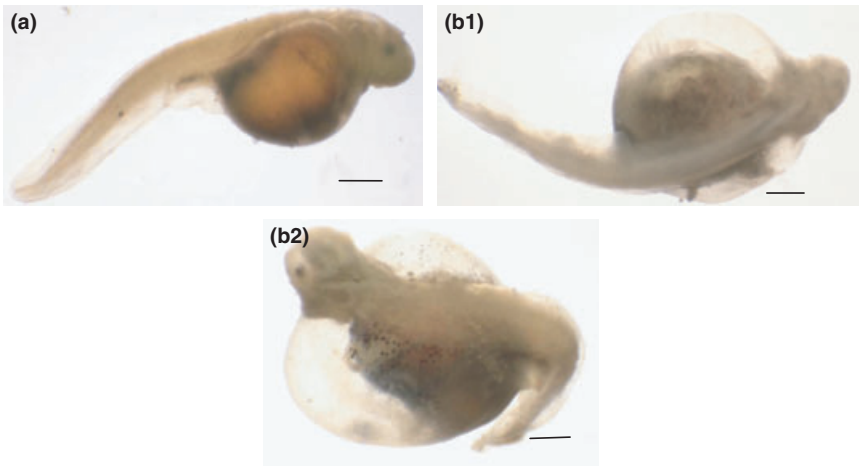

Fig. 1. Hatching *Polyodon spathula* larvae of diploid gynogens group (a: normal) and haploid group (b1, b2: abnormal). Scale section = 8 mm × 8 mm.

Microsatellite and cytogenetic analysis

Maternal inheritance in gynogenesis was confirmed using three microsatellite loci: Psp-28, Psp-29 and Psp-32 (Table 3). Comparison of microsatellite Psp-28 in one locus in all diploid gynogens with the maternal genotype revealed the maternal genome in all samples (two alleles, 240, 250) and no paternal genome. The same results, obtained from comparison of diploid gynogens with their parents using Psp-32 and Psp-29 microsatellite markers showed only the maternal genome among diploid genome offspring.

The cytogenetic analysis confirmed that the chromosome number in all studied gynogenetic individuals was diploid (~120 chromosomes in metaphase plates) (Fig. 2a), 60 chromosomes (Fig. 2b) from haploid group N_{1-3} were observed, The chromosome complements of *P. spathula* haploids have never been described before.

Discussion

In this study, a protocol to produce gynogenetic *P. spathula* was developed, involving a combination of UV irradiation of the *A. schrenckii* sperm and the subsequent application of a heat shock to retain the second polar body of 'fertilized' eggs, and this approach was effective in producing viable, diploid gynogenotes. In most gynogenesis studies, fresh homogeneous sperm was used to activate eggs to develop (Lou, 2001; Flynn et al., 2006). Gynogenetic diploids are difficult to distinguish from normal diploids when uses homogeneous sperm in case when the sperm was not completely inactivated by UV light. Gynogenetic diploid fry were successfully obtained by inducing *P. spathula* eggs with *A. schrenckii* sperm. Since the *A. schrenckii* has 238 ± 8 chromosomes (Song et al., 1997; Chen, 2007) and the *P. spathula* has 120 (Mims and Shelton, 2005), it is straight-forward to identify gynogenetic fry through chromosome analysis (Fopp-Bayat et al., 2007; Xu et al., 2007). In addition, without irradiation treatment, *A. schrenckii* spermatozoa appeared to activate the *P. spathula* eggs develop up to gastrulation, however this group (C_2) did not result in any viable hybrids, which eliminated possibility that genome of heterologous sperm disturbed gynogenetic progenies. Therefore, gynogenetic fry induced by heterogeneous sperm are readily identified with high precision, so using heterogeneous sperm with a different chromosome number for gynogenetic induction has become a new method to study gynogenesis in fish (Wu and Lou, 1999; Lou, 2001).

In the present study, The high mortality and occurrence of deformities in the gynogenetic haploid group were comfirmed, and the haploid embryos obtained displayed the same 'haploid syndrome' as was described previously (Van Eenennaam et al., 1996) in *A. transmontanus*, *A. ruthenus* and *A. baerii* (Fopp-Bayat et al., 2007). Meanwhile, a few normal hatching larvae were also occasionally observed in the haploid treatment group, It seems likely that a few normal hatching larvae found in haploid treatment groups were, in fact, diploids due not to insufficient irradiation, but rather, spontaneous diploidization, which was also detected in haploid control group of induction gynogenesis in *A. baerii* (Fopp-Bayat, 2007) and Bester (*H. huso* female × *A. ruthenus* male) (Omoto et al., 2005).

Microsatellite DNA marker technology is a powerful approach for confirmation of the exclusively maternal transmission and in the estimation of recombination rates in gynogens (Flynn et al., 2006; Tvedt et al., 2006), In this study, microsatellite DNA analysis used can be applied for genetic identification of individual *P. spathula* after genome manipulation, the results of microsatellite DNA analysis showed that there was no genetic contribution from the paternal genome in the gynogenetic group and a complete maternal genome among diploid gynogens, and thus it is unambiguously demonstrated that gynogenesis in the *P. spathula* was induced successfully. In addition, the ploidy level of gynogenetic *P. spathula* (2n) was confirmed base on study of the chromosome number.

Table 3
Alleles segregation (in base pairs) and alleles frequence at three microsatellite DNA loci in gynogenetic progeny of *Polyodon spathula*

Locus	Male genotype	Female genotype	Phenotypes distribution in meiotic diploids (n = 20)		Inheritance	Alleles frequency in gynogenetic progeny
Psp-28	266, 274	240, 250	240, 250	20 / 20	Disomic	f(240) = 0.5; f(250) = 0.5
Psp-29	190, 190 or 190, null	195, 215	195, 215	20 / 20	Disomic	f(195) = 0.5; f(215) = 0.5
Psp-32	120, 140	170, 200	170, 200	20 / 20	Disomic	f(170) = 0.5; f(200) = 0.5

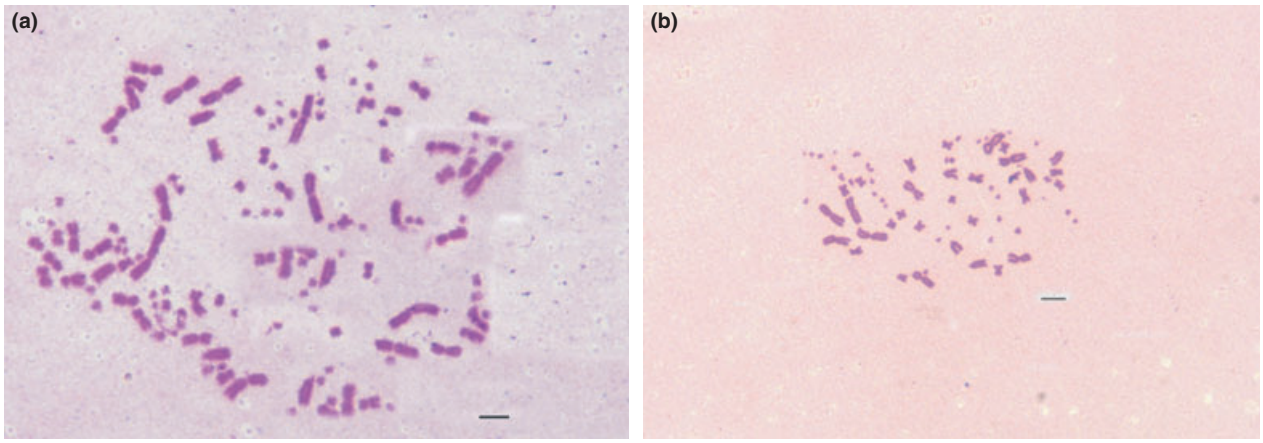

Fig. 2. Metaphase chromosome set from genogenetic diploid (a) haploid (b) embryos in *Polyodon spathula*. (a) diploid gynogenetic specimen with ~120 chromosomes. (b) Haploid specimen with ~60 chromosomes. Scale bar equals10 μm.

The results of the present study, have practical importance and may be useful for the future research focused on inducing gynogenesis in *P. gladius* to preserve genotypes or owns GeneBank. Furthermore, growth performance and gonadal development of gynogenotes will be tested in later studies.

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