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Production of inbred larvae through self-fertilization using oocytes and cryopreserved sperm from the same individuals after sex reversal in eastern oyster *Crassostrea virginica*

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Abstract

The eastern oyster *Crassostrea virginica* can change sex which makes self-fertilization possible if sperm can be cryopreserved. In this study, small (~1 year old) and large (~2–3 years old) oysters were biopsied for sperm collection. Survival of the biopsied oysters after 1 year was 50% for small oysters and 17% for large oysters. Oocytes were collected from sex-reversed females, and self-fertilized with cryopreserved sperm. Of the 24 cryopreserved samples, 14 individuals had ≤1% fertility when crossed with oocytes from unrelated females, indicating that the cryopreserved sperm had reduced fertility. The other 10 individuals had a fertility of 39 ± 25% when crossed with oocytes from unrelated females (non-selfing), but showed a significantly lower success of self-fertilization (12 ± 16%) ($P = 0.008$), while aliquots of the same oocytes had a fertilization of 83 ± 11% when crossing with fresh sperm. Larvae were produced at day 3 in the self-fertilized families (12–94% of the fertilized oocytes), and survived to eyed-larvae stage at days 11–14. Genotyping with 9 microsatellite markers confirmed that the larvae resulted from self-fertilization in four families. This study demonstrated the feasibility of creating self-fertilized inbred lines of oysters by use of non-lethal sperm collection and cryopreservation.

Keywords: Eastern oyster, self-fertilization, sperm cryopreservation, inbred line

Introduction

Production and harvest of the eastern oyster *Crassostrea virginica* in the United States have been greatly affected by diseases, coastal degradation and environmental pollution (as exemplified by the Deepwater Horizon oil spill in 2010). Oyster aquaculture calls for genetically improved stocks that possess characteristics suitable for culture such as uniformity, superior growth and disease resistance. While progress has been made in developing oyster breeding programs and biotechnologies (Guo 2004, 2009), more efforts and sophisticated breeding strategies are needed to accelerate breeding to meet the demands of the oyster culture industry.

Inbreeding can be used to create broodstocks with genetic homogeneity. Heterosis can result from crossing of inbred lines, and is a useful approach for genetic improvement (Hedgecock, McGoldrick & Bayne 1995). Besides crossbred vigour, genetic uniformity of inbred lines is also a valuable trait for research and production. In addition, inbred lines would be ideal source materials for constructing reference populations for genetic mapping. Highly inbred individuals are critical for whole-genome sequencing because polymorphism creates problems for genome assembly (Zhang, Fang, Guo, Li, Luo, Xu, Yang, Zhang, Wang, Qi, Xiong, Que, Xie, Holland, Paps, Zhu, Wu, Chen, Wang, Peng, Meng, Yang, Liu, Wen, Zhang, Huang, Zhu, Feng, Mount, Hedgecock, Xu, Liu,

Domazet-Loso, Du, Sun, Zhang, Liu, Cheng, Jiang, Li, Fan, Wang, Fu, Wang, Wang, Zhang, Peng, Li, Li, Wang, Chen, He, Tan, Song, Zheng, Huang, Yang, Du, Chen, Yang, Gaffney, Wang, Luo, She, Ming, Huang, Zhang, Huang, Zhang, Qu, Ni, Miao, Wang, Wang, Steinberg, Wang, Li, Qian, Zhang, Li, Yang, Liu, Wang, Yin & Wang 2012). Thus, the establishment of inbred lines has useful applications in oyster genetics and genomics research as well as in selective breeding. However, when uncontrolled, inbreeding can also impair populations due to inbreeding depression, especially when a new stock is acquired, and the number of animals is small or they are closely related. For example, a disease-resistant oyster line developed in Louisiana (OBOY) was derived from 10 to 20 wild oysters (Leonhardt 2013). With this small population size, founder effects or inbreeding depression could occur. This risk is especially true for oysters because a single breeding pair can produce millions of gametes at each spawning. Nevertheless, rapid and controlled inbreeding can provide a useful tool for oyster breeding.

Inbred lines are easy to produce in plants due to the ease of self-fertilization. However, in animals hermaphrodites are rare, and few of them can be self-fertilized. Inbred animal lines are therefore more difficult to produce and usually require many generations of sib-mating or backcrossing. The eastern oyster can change sex during the spawning season, mostly beginning life as males and changing into females as they age (Galtsoff 1964). This characteristic allows for fertilization by cryopreserved sperm of oocytes from the same oyster after natural sex change, and enables creation of self-fertilized inbred lines. With the usage of non-lethal sperm collection and cryopreservation, it is possible that self-fertilized inbred lines could be created within 2 years and used for breeding programs.

To create inbred lines of oysters, two techniques would be required: non-lethal sperm collection and sperm cryopreservation. So far, these techniques have been studied in our laboratory and protocols have been developed and established. Sperm cryopreservation in eastern oysters was first studied in the early 1970s (Hughes 1973), and two more studies followed (Zell, Bamford & Hidu 1979; Paniagua-Chavez & Tiersch 2001). The cryoprotectants used were dimethyl sulphoxide or propylene glycol. The extenders were artificial seawater or calcium-free Hanks' balanced salt solution (Ca-free HBSS) (Hanks 1975). In 2009–2010, we developed a reliable protocol for sperm cryopreservation of eastern oysters with potential for high-throughput application (for large-scale breeding programs) by systematic evaluation of cryoprotectants, cooling rates and thawing temperatures with post-thaw fertilization of 58% for 0.5-mL French straws and 54% for 0.5-mL CBS straws (Yang, Hu, Cuevas-Uribe, Supan, Guo & Tiersch 2012).

For non-lethal sample collection, natural spawning and biopsy were also tested. With 18-ga or 20-ga needles for biopsy after notching, a reasonable amount of sperm ($3.6 \pm 2.1 \times 10^8$ cells) could be obtained from each oyster with a total weight (shell plus meat) of 99.5 ± 23.2 g (mean $\pm$ SD, $N = 20$), shell height of 74.1 ± 6.0 mm and shell length of 60.9 ± 7.2 mm. Fresh sperm motility varied from male to male with an average of $23 \pm 12\%$ ranging from 8% to 44%. After cryopreservation, fertility of post-thaw sperm averaged $20 \pm 22\%$ ranging from 1% to 87% (Yang, Supan, Guo & Tiersch 2013).

In this study, we sought to integrate and extend our previous research for oyster genetic improvement based on creation of self-fertilized inbred lines. The goal of this pilot study was to develop and evaluate methods for creation of inbred lines of eastern oyster by self-fertilization using cryopreserved sperm and oocytes from the same individuals. The objectives were to: (1) collect sperm by non-lethal methods, (2) cryopreserve the sperm samples, (3) culture oysters and collect oocytes after sex change, (4) fertilize the oocytes from sex-reversed females with cryopreserved sperm from the same individual, and (5) confirm self-fertilization by use of genetic markers. This study demonstrated the feasibility of creation of inbred lines through self-fertilization using cryopreserved sperm and oocytes from the same individuals after natural sex change.

Materials and methods

Oysters

The oysters used in this study were wild and collected from an artificial oyster reef in front of the Rutgers Cape Shore Facility, New Jersey in 2010. After transport to the laboratory, oysters were cleaned with fresh water and stored at 4°C for gonad biopsy within 3 days. The control oysters used in fertilization trials were from the NEH strain of Rutgers University. These experiments were performed at the Haskin Shellfish Research Laboratory

(HSRL), Rutgers University (New Jersey, USA), and the motility analysis of thawed sperm was performed at the Aquaculture Research Station of the Louisiana State University Agricultural Center.

Motility analysis

Fresh sperm motility was estimated by viewing movement with a bright-field light microscope (Nikon Eclipse E600; Nikon Instruments Inc., Melville, NY, USA). Post-thaw motility was evaluated using computer-assisted sperm analysis (CASA system, CEROS model; Hamilton Thorne, Inc., Beverly, MA, USA) with 20-μm, 2-cell slides (Hamilton Thorne, Inc.). The parameter settings for CASA were: minimum contrast, 30; minimum cell size, 3 pixels; number of frames for recording, 30; average path (VAP) cut off, 20 μ s^{-1}; straight line (VSL) cut off, 10 μ s^{-1}; static intensity gate, 0.15–2.82; static size gate, 0.72–3.29; elongation gate, 56–99, and slow cell, static. For each sample, at least three CASA measurements of different fields were performed, and the average motility was calculated as the motility for that sample.

Collection of sperm by non-lethal methods

Sperm samples were collected by the methods established in our laboratory after measurement of total weight, shell length and shell height. For each batch of 20–30 oysters, sperm samples were collected by biopsy, processed and cryopreserved. Briefly, oysters were notched on the dorsal side with an 11.4-cm side grinder wheel (0.6-cm width), and a syringe with 20-ga needle was used to biopsy the gonad. Biopsies were limited to three punctures to maximize survival of the oysters, and the gonad samples were released into 300 μL of Ca^{2+}-free HBSS at an osmolality of 650 mOsmol kg^{-1} (Ca-free HBSS650) in 1.5-mL centrifuge tubes. The sex of each oyster was determined by observation of the presence of oocytes or sperm by use of a bright-field microscope (10× magnification). Sperm samples were filtered through a 25-μm screen to remove large pieces of tissue, and were stored on ice for freezing after motility estimation.

Cryopreservation of sperm samples collected by non-lethal method

Sperm cryopreservation was performed based on the protocol developed in 2010 (Yang *et al.* 2012). Briefly, sperm samples were mixed with an equal volume of 20% DMSO in Ca-free HBSS650, and the mixture was packaged into 0.5-mL French straws (IMV Technologies, Paris, France). Because a controlled-rate programmable freezer was not available on-site in NJ, straws were cooled at 20–25°C min^{-1} from 5 to −80°C in nitrogen vapour within a shipping dewar (CX-100; Taylor-Wharton, Theodore, AL, USA). The rate was predetermined by using a data logger (HH147U; Omega Engineering, Inc., Stamford, CT, USA). Briefly, a goblet was held 6–8 cm above the bottom of the shipping dewar with a total of 10 canes with 5 straws loaded in each. The temperature during cooling was monitored with a data logger and thermocouple placed inside a 0.5-mL straw. Frozen samples reaching −80°C were plunged into liquid nitrogen for long-term storage, and shipped overnight in a shipping dewar to the Aquaculture Research Station of the LSU Agricultural Center in Baton Rouge, Louisiana.

Culture after sampling and monitoring of oysters for survival and sex changes

After sampling, the oysters were individually placed into a soft nylon net (1.5-cm) with each end sealed and labelled for identification. The packaged oysters were deployed on an intertidal bag-on-rack system at Rutgers Cape Shore experimental site in Delaware Bay, NJ, for grow-out. The oysters were regularly washed and monitored. In June 2, 2011 (after 1 year of culture), the oysters were moved from the grow-out site to the laboratory at HSRL, cleaned with fresh water and individually measured for body weight and shell size. These oysters were notched using an 11.4-cm side grinder wheel on the dorsal side, and gonad samples were biopsied with 20-ga needles for sex identification by microscopic examination of the presence of oocytes or sperm. Females were used for oocyte collection and artificial fertilization, and males were returned to the grow-out site after individual packaging and labelling.

Self-fertilization of oocytes with cryopreserved sperm

Oocyte collection was performed using standard hatchery procedures (Helm & Bourne 2004): mincing of the gonad in 25 ppt filtered seawater, filtering through 100-μm screen to remove large

pieces of tissue, collection on a 20-μm screen, and washing into fresh seawater. Samples of gill and adductor muscle from each dissected female were collected and fixed in 95% ethanol for later genetic analysis. For artificial fertilization, sperm in 0.5-mL French straws were thawed at 40°C for 7–8 s in a water bath, the straws were dried completely with a paper towel, and the sealed end was cut to release the thawed samples into 1.5-mL centrifugation tubes by cutting the other plugged (cotton) end.

For fertilization, sperm (0.3 mL) were mixed with 20 mL of oocyte suspension (concentration of ~20 000 oocytes mL^{-1}) in a 1-L beaker at room temperature (23°C) (the sperm:oocyte ratio was about 500 sperm per oocyte). Additional fresh seawater was added to a volume of 200 mL after 30 min, and to a 1 L final volume after 1 h. Embryos at the 4-cell stage or beyond were counted as fertilized, and the percentage based on total oocytes was used to calculate fertility; two repeated counts were performed for each fertilization group.

In this study, the self-fertilization crosses of each female were performed together with three other single-cross controls. Thus, the four groups were: (1) Oocytes from females crossed with their own cryopreserved sperm (self-fertilization); (2) Oocytes from self-fertilization females crossed with fresh control sperm from non-related males to test the oocyte quality; (3) Oocytes from control females crossed with the cryopreserved sperm to test the quality of cryopreserved sperm; and (4) Oocytes from control females crossed with fresh control sperm to confirm the quality of sperm and oocytes from the control individuals. Because oocyte number in each group was controlled, there were leftover oocytes from each sex-changed female, and all of these were fertilized with the remaining cryopreserved sperm.

Culture of larvae from self-fertilization trials

Culture of fertilized oocytes and larvae were conducted following routine hatchery procedures used in the laboratory. Due to the large number of groups in the fertilization trials, the volume of work needed to be reduced. As such, only self-fertilized groups were cultured beyond hatch. Briefly, oocytes after fertilization were placed in fresh 20 ppt filtered seawater at a density of 100 eggs mL^{-1} for hatching. Swimming larvae (D-stage larvae) were harvested and counted on day 2, and were cultured at a density of 10 larvae mL^{-1} (at most) with single-cell algae (*Isochrysis galbana*) as food. Larval culture was continued with water changes every other day by filtering through proper-sized screens until metamorphosis. At day 2 or 5, about 100 swimming larvae were sampled and fixed in 95% ethanol for genetic confirmation.

Genetic confirmation of self-fertilized families

Pieces of gill tissue (about 1/3 of a single gill lamella) or 1 μL of concentrated larvae (larvae number was estimated as 50) were used for DNA extraction. Genomic DNA was extracted by using the proteinase K digestion method (Wang, Wang, Wang & Guo 2010). Briefly, 50 μL of lysis buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, and 0.5 mg mL^{-1} gelatin) and 1.5 μL of proteinase K (20 mg mL^{-1}) were added to the wells of a 96-well plate, and mixed with samples. The mixtures were incubated at 55°C for 40 min to digest in the lysis buffer, heated at 95°C for 15 min to inactivate proteinase K and cooled to 4°C in a thermocycler (ABI PE GeneAmp 9700; Applied Biosystems, Foster City, CA, USA).

Nine microsatellite markers were selected from a 16-microsatellite multiplex assay developed for parentage assignment in the eastern oyster (Wang *et al.* 2010). The parameters of multiplex PCR were based on the 16-microsatellite multiplex assay and the user manual from the Qiagen Type-it microsatellite PCR kit (Qiagen, Valencia, CA, USA). The polymerase chain reaction (PCR) solution (10 μL) consisted of: 20 ng of template DNA from samples, 1 × Type-it Master Mix, 0.5 pmol of each primer for VIC-RUCV060, FAM-RUCV010, NED-Cvi13, PET-RUCV074, PET-RUCV045, FAM-RUCV114, FAM-RUCV374, and 1.0 pmol of each primer for NED-RUCV131 and VIC-RUCV073. PCR was conducted on a thermocycler (ABI PE GeneAmp 9700), and the cycling protocol consisted of: 95°C for 5 min followed by eight cycles of 95°C for 30 s, 60°C for 90 s, 72°C for 45 s, 14 cycles of 95°C for 30 s, 55°C for 90 s, 72°C for 45 s, eight cycles of 95°C for 30 s, 53°C for 90 s, 72°C for 45 s and a final extension at 60°C for 30 min. PCR products were diluted with 30 μL of deionized water, and 0.5 μL of diluted PCR product was mixed with 10 μL of deionized formamide (Sigma-Aldrich, Co. LLC., St. Louis, MO, USA) and 0.2 μL of size standard (GS-600LIZ, Applied Biosystems). The mixtures were loaded onto a Genetic Analyzer (ABI 3130×l Prism; Applied Biosystems). Allele scoring was performed with GeneMapper v4.0

(Applied Biosystems). The multiplex PCR was repeated once for every sample for confirmation.

Data analysis

Data were analysed with statistical software (SYSTAT 13; Systat Software, Inc., Chicago, IL, USA) and Microsoft Excel (Microsoft 2010) functions. Percentage data were transformed by arcsine square root for normalization. Significance was recognized when the P values were <0.050.

Results

Non-lethal sperm collection from oysters and their size and sex ratio

In total, 139 oysters were processed with notching and gonadal biopsy, and these oysters were grouped into small (estimated as 1-year-old) and large (estimated as 2–3 years old) groups. The small-sized group (N = 116) had an average whole weight of 7.48 ± 7.62 g, shell height of 34.86 ± 7.08 mm, and shell length of 25.09 ± 5.85 mm. The large-sized group (N = 23) had an average whole weight of 57.72 ± 25.59 g, shell height of 86.13 ± 19.63 mm and shell length of 50.13 ± 7.55 mm, which were significantly larger than the small oysters (P = 0.000). The male: female ratio was 87: 29 for the small-sized group, and 13:10 for the large-sized group (Table 1). The analysis for goodness of fit showed that the small-sized group had significantly (P = 0.000) more males (75%) than the large-sized group (50%). The whole body weight and size of males and females were not significantly different in small-sized oysters ($P \geq 0.627$) or in large-sized oysters ($P \geq 0.268$) (Table 1).

Cryopreservation of sperm samples collected by non-lethal methods

For samples collected by gonadal biopsy following notching, sperm production averaged 5.65 ± 4.02 × 10^7 cells per oyster for the small males (N = 87), and 4.22 ± 4.27 × 10^8 cells per oyster for larger males (N = 13). The motility of fresh sperm varied from male to male, from 5% to 70% in small males, and from 20% to 60% for the larger males. After cryopreservation, the post-thaw motility in small males ranged from 0% to 20%, and from 1% to 20% for the larger males.

Table 1 Sex composition (F, female; M, male), body weight (g) and body sizes of small and large eastern oysters *Crassostrea virginica* plus their survival and sex reversal at 1 and 2 years after biopsy for sperm collection and cryopreservation

	Biopsied for sperm collection and cryopreservation, June 15–19, 2010				Survival and sex reversal of biopsied oysters, June 14–18, 2011				Survival and sex reversal of the 23 males that remained as males in 2011, June 14, 2012				
Group	Sex (Number)	Height (mm)	Length (mm)	Weight (g)	Number (%)	Sex (Number)	Height (mm)	Length (mm)	Weight (g)	Number (%)	Sex (Number)	Height (mm)	Length (mm)	Weight (g)
Small (1-year- old)	M (87)	34.81	25.24	7.56	44 (51%)	F (21)	61.25	46.92	33.23					
						M (23)	54.10	41.71	29.11	12 (52%)	F (8)	72.53	52.02	72.53
											M (4)	68.50	51.86	42.25
	F (29)	34.99	24.62	7.46	14 (48%)	F (7)	60.64	47.69	32.13					
						M (7)	61.84	45.84	33.57					
Large (2–3-year- old)	M (13)	84.85	50.00	52.43	2 (15%)	F (2)	94.04	58.85	90.40					
						M (0)	–	–	–					
	F (10)	87.80	50.30	64.59	2 (20%)	F (2)	106.18	60.22	112.80					
						M (0)	–	–	–					

Survival and sex identification of oysters after 1 year of culture

The survival of biopsied oysters after one year of culture (2010–2011) at Cape Shore, New Jersey, was 50% for small oysters (58 of 116), which was higher than that for the large oysters (17%, 4 of 23). The survival of males and females were similar in the small oysters (51% vs. 48%) and large oysters (15% vs. 20%) (Table 1).

In the small-sized group, 44 of the 87 males in 2010 survived after one year of culture, 21 (48%) of them changed into females and 23 of them remained as males (Table 1). No differences were observed in whole body weight and shell size between the sex-changed females and unchanged males ($P \geq 0.184$), nor in the change in total weight and shell size within one year ($P \geq 0.069$). Meanwhile, 14 of the 29 females in 2010 survived to 2011, 7 of them (50%) changed into males and 7 (50%) of them remained females (Table 1). No differences were observed in body weight and shell size between these 'reverted' males and remaining females ($P \geq 0.460$). For the large oysters, 2 of the 13 males in 2010 survived to 2011 and changed into females. Two of the 10 females in 2010 survived and remained as females (Table 1). No comparison was made for the large oysters because of limited sample size (4 surviving individuals).

After another year, 12 of the 23 unchanged males in 2011 from the small oyster group survived to 2012. Among these 8 changed into females, 4 of those remained as males. No differences were observed between the males and females in total weight and shell sizes ($P \geq 0.505$) (Table 1), and no differences were found in the growth of total weight and shell size between the males and females ($P \geq 0.405$).

Production and self-fertilization of oocytes from females with cryopreserved sperm

Oocytes were collected from all sex-changed females in 2011 and 2012 by dissection. In 2011 (one year after biopsy), the 21 females from the small-sized oyster group produced an average of 1 959 507 ± 933 541 oocytes per female ranging from 340 000 to 3 391 500; and the two females from the large-sized oyster group produced 5 088 000 ± 1 927 573 oocytes per female. In 2012 (two years after biopsy), the eight females (all from the small oyster group) produced 2 647 175 ± 2 058 252 oocytes per female ranging from 801 400 to 6 937 800. Correlation analysis showed that oocytes production did not correlate with total body weight or shell size ($P \geq 0.060$).

Self-fertilization with cryopreserved sperm and the controls

In total, there were 31 sex-changed females obtained in two years (23 in 2011, and 8 in 2012). Among these females, oocytes from five of them accidentally did not receive enough hydration time after dissection resulting in poor quality (5–15% fertilization with fresh sperm), and two of them had few cryopreserved samples available. For the other 24 individuals, self-fertilization with cryopreserved sperm varied from 0% to 43% among individuals (Table 2). Cryopreserved sperm from 14 of them showed low fertilization (≤1%, Table 2) when crossed with oocytes from non-related females, indicating that the cryopreserved sperm had reduced fertility. Therefore, the analysis of self-fertilization and comparison with related controls was performed using the 10 individuals whose cryopreserved sperm showed >1% fertility with oocytes from non-self females (Table 2). For these 10 individuals, the post-thaw motility of cryopreserved sperm ranged from 2% to 15% (Table 2). Cryopreserved sperm produced 12 ± 16% self-fertilization (ranging from 1% to 43%), which was significantly lower ($P = 0.008$) than the non-self fertilization they produced (39 ± 25%), non-self fertilization of the same oocytes with fresh control (non-self) sperm (83 ± 11%) ($P = 0.000$) and non-self fertilization of the fresh control oocytes with fresh control sperm (80 ± 19%) ($P = 0.000$).

Fertilized eggs from a total of eight self-fertilized families were cultured for harvest of swimming larvae (Table 2). Survival of fertilized eggs to D-stage larvae harvested at day 2 (about 48 h after fertilization) was 12–94%. Larvae from all families did not survive beyond metamorphosis although eyed-larvae were observed at days 11–14.

Confirmation of paternity and maternity of offspring from self-fertilized families

All nine microsatellites were consistently amplified in all the six parental samples and four of the larval samples, no amplification was observed in the other two larval samples (from oysters 78 and

Table 2 Per cent fertilization with their own cryopreserved sperm (self-fertilization) and larval harvest from female eastern oysters *Crassostrea virginica* after sex reversal and the controls: (1) Fresh oocytes × fresh sperm: oocytes from control females crossed with fresh sperm, this was to confirm the quality of sperm and oocytes from the control individuals; (2) Fresh oocytes × cryopreserved sperm: oocytes from non-self female crossed with cryopreserved sperm, this was to test the quality of cryopreserved sperm; (3) Oocytes from sex-reversed females × fresh sperm: oocytes from sex-reversed females crossed with fresh sperm from non-self males, this was to test the quality of oocytes from sex-reversed females

			Non-self-fertilization controls			Self-fertilization	Oocyte number and larvae harvested from self-fertilization group						
			(1)	(2)	(3)	Selfing							
Oyster no.	Motility of cryopreserved sperm (%)	Sperm production	Oocytes from non-related females		Oocytes from sex-reversed females		Oocyte number	Day 2 (% of fertilized oocytes)	Day 5	Day 7	Day 9	Day 11	Day 14
27	8	3.06E + 07	–	46	78	1	1 062 000	6000 (56%)	500	–	–	–	–
44	3	3.76E + 07	–	10	65	1	423 000	4000 (94%)	500	–	–	–	–
55	15	4.24E + 07	–	72	82	4	1 656 000	60 000 (90%)	42 000			2 500	800
62	2	5.33E + 07	–	64	85	0	–	–	–	–	–	–	–
63	2	4.61E + 07	–	11	82	0	–	–	–	–	–	–	–
78	15	2.66E + 08	–	64	64	22	690 000	110 000 (72%)	112 500		62 000		1400
75	7	7.50E + 07	52	3	90	1	3 015 000	50 000 (69%)	–	0	–	–	–
17	10	5.00E + 06	85	28	90	16	3 193 000	75 000 (12%)	–	–	4800	50	–
113	12	3.54E + 07	87	56	98	43	2 702 000	520 000 (63%)	218 400	36 000	–	–	2000
103	8	9.87E + 07	96	39	94	35	34 500	9000 (74%)	5600	2000	–	–	–
65	3	8.32E + 07	80	0	79	0	–	–	–	–	–	–	–
66	1	5.53E + 07	88	0	88	0	–	–	–	–	–	–	–
76	9	3.46E + 07	84	1	96	0	–	–	–	–	–	–	–
68	4	1.17E + 08	93	0	27	0	–	–	–	–	–	–	–
87	10	8.25E + 07	91	0	91	0	–	–	–	–	–	–	–
110	6	3.54E + 07	69	1	94	0	–	–	–	–	–	–	–
9	12	1.47E + 08	76	1	97	1	–	–	–	–	–	–	–
3	3	4.64E + 07	87	0	45	0	–	–	–	–	–	–	–
8	6	3.22E + 07	87	1	94	0	–	–	–	–	–	–	–
22	6	5.75E + 07	87	1	93	2	–	–	–	–	–	–	–
37	1	1.24E + 07	92	0	96	0	–	–	–	–	–	–	–
41	2	3.80E + 07	95	0	23	0	–	–	–	–	–	–	–
70	2	4.64E + 07	89	1	98	0	–	–	–	–	–	–	–
90	1	4.64E + 07	97	0	95	0	–	–	–	–	–	–	–

103), due to contaminating material (algal or bacterial aggregates) in the samples. For the amplified samples, all alleles were unambiguously identified and completely consistent with those of the parents (e.g. oyster 75, Fig. 1), indicating that the four larval samples were produced from self-fertilization. As for the two larval samples that failed to amplify, only several of the diagnostic alleles (not all 9 of the markers) were observed.

Discussion

An innovative approach for creation of self-fertilized inbred lines

Inbred lines with high degrees of genetic homogeneity are routinely sought for genetic analyses because they can be used to construct reference families for the mapping of valuable genes or traits. Usually inbred lines of animals, especially vertebrates, are difficult to obtain and require many years of consecutive brother-sister crossings or backcrossing. For example, the International Committee on Standardized Nomenclature for Mice has ruled that a strain of mice can only be considered "inbred" after 20 generations of brother-sister inbreeding (http://www.informatics.jax.org/mgihome) at which point 98.7% of the loci in each animal should be homozygous (Green 1981). With the approaches used in this study, inbred offspring from self-fertilization could reach 98.4% homogeneity within six generations of self-fertilization. This would greatly accelerate oyster breeding programs, and would be an innovative way for establishment of inbred lines.

Some invertebrates are naturally hermaphroditic, such as some snails and scallop species (family Pectinidae). In nature, self-fertilization in hermaphrodites is usually avoided through strategies such as asynchronous maturation or outcrossing (Foighil & Eernisse 1987; Rios, Canales & Pena 1996). Due to the free dispersal of gametes, however, most benthic marine invertebrates such as bivalves usually possess a small chance of self-fertilization

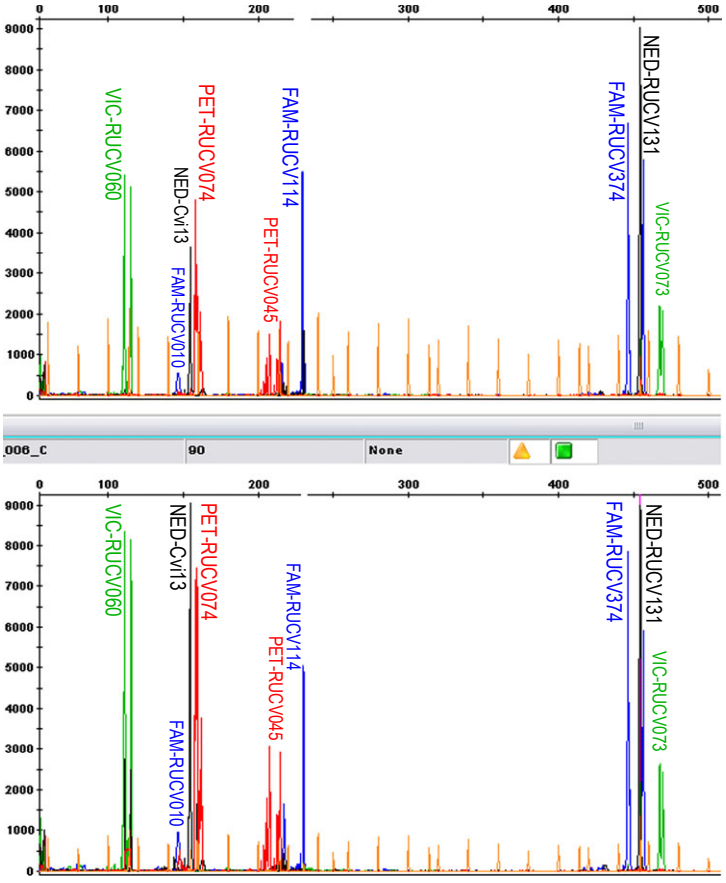

Figure 1 Confirmation of self-fertilization with nine microsatellite markers in larvae from self-fertilized families. The upper panel was the allele profile of oyster 75 and the lower panel was the allele profile of about 50 larvae that resulted from self-fertilization, showing no new alleles in the larvae.

(Wanguemert, Pezzuto & Borzone 2000; Martinez, Mettifogo, Perez & Callejas 2007; Liu, Alabi & Pearce 2008; Petersen, Ibarra, Ramirez & May 2008).

In oysters of the genus of *Crassostrea*, hermaphrodites are rare, and occurrence varies with age and environment (Galtsoff 1964; Kennedy, Newell & Eble 1996; Guo, Hedgecock, Hershberger, Cooper & Allen 1998). Therefore, it is not practical to create selfing inbred lines by using natural hermaphrodites. The use of non-lethal sperm collection and cryopreservation represents an innovative method for the creation self-fertilized inbred lines. This approach has never been reported for eastern oysters. Self-fertilization with cryopreserved sperm has been attempted with the Pacific oyster *Crassostrea gigas* (Lannan 1971), although there was no genetic confirmation or efforts to create inbred lines. In the present study, larvae were cultured to eyed-stage and genetically confirmed as being the offspring from self-fertilization. The failure to culture the larvae beyond metamorphosis was likely due to problems with use of 20-l buckets. Typical hatchery batches of larvae often fail in this limited culture system. Even so, our results demonstrate this approach was feasible for establishment of inbred lines in oysters. Based on the current preliminary results, the calculation of oyster numbers necessary at each step of the approach based on cumulative mortality can be estimated from this study (Fig. 2). For example, to produce five self-fertilized inbred lines, it would be necessary to consider starting with about 500 oysters as an initial population for sperm collection.

Sex reversal of oysters was bidirectional and was not related to body size

In many bivalve species, alternate or simultaneous hermaphroditism is common. The oviparous oyster species of the genus *Crassostrea* are usually not hermaphroditic, whereas the larviparous oysters of the genus *Ostrea* are bisexual, in which gonads can contain functional ova and spermatozoa simultaneously, and can undergo rhythmical changes in sex maturity (Galtsoff 1964). For *Crassostrea* oysters, the primary gonad is bisexual

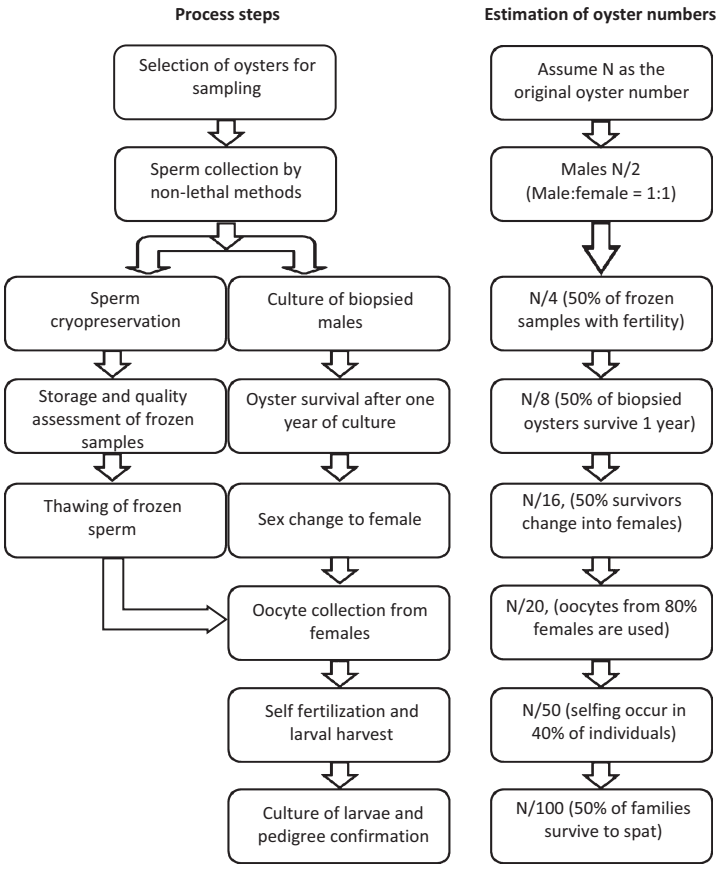

Figure 2 The schematic flow chart for creation of self-fertilized lines by using non-lethal sperm collection and cryopreservation in eastern oysters *Crassostrea virginica* and estimation of the relative numbers of oysters required at each step.

as spermatogonia and ovogonia can each be found in the same follicles. These oysters usually began as males and change into females with ageing (Galtsoff 1964). In the present study, the group of small oysters sampled in 2010 had 75% males and 25% females, and these oysters were most likely in their first spawning season based on their size (Galtsoff 1964; Kennedy *et al.* 1996). Therefore, a question can be asked: Do all eastern oysters begin as males? This question requires more investigation by tracking gametogenesis of individual oysters during their initial sex differentiation (Choi, Powell, Lewis & Ray 1994).

In addition, it has been reported that females tend to be larger than males at their first spawning season (Needler 1941), and a correlation exists between the development of oocytes in the primary bisexual gonad and the rate of body growth (Coe 1934). In this study, no differences were found in total weight and shell sizes between males and females in any of the 3 years studied, and none were found in the gain of total weight and shell sizes within a single year. Our sampling was limited and we recognize that a large body of data exist in support of larger body sizes in females (Kim & Powell 1998). More investigation is needed to clarify this by measuring and tracking same-age oysters that originate from the same parents.

In this study, sex changes were observed to be bidirectional from male to female and from female to male with similar ratios. This observation agrees with a previous study of eastern oysters (Needler 1941), and another for Pacific oysters (Lango-Reynoso, Chavez-Villaba & Le Pennec 2006). It has been suggested that sex change may be under some genetic control in the eastern oyster (Haley 1977) and the Pacific oyster (Guo *et al.* 1998). However, no detailed evidence has been reported to show that sex change is controlled by specific factors (e.g. genetic or environmental factors), and such control was not evident in our data and those of Galtsoff (1964).

Potential for inbreeding depression in self-fertilized families

Inbreeding depression can result in reduced fitness, and commonly occurs in inbred populations and families, but does not inevitably occur in all organisms. Hermaphroditic species often exhibit lower degrees of inbreeding depression than do outcrossing species because repeated generations of possible self-fertilization are thought to reduce deleterious homozygous alleles from populations (Charlesworth & Charlesworth 1987; Crnokrak & Roff 1999). In hermaphroditic scallops, self-fertilized inbred lines have been produced in species such as *Pecten maximus* (Beaumont & Budd 1983), *Argopecten circularis* (Ibarra, Cruz & Romero 1995), *Argopecten purpuratus* (Winkler & Estevez 2003; Toro, Montoya & Martinez 2009; Toro, Montoya, Martinez, Gutierrez & Vergara 2010; Concha, Figueroa & Winkler 2011), *Argopecten irradians* (Zheng, Zhang, Guo & Liu 2008) and *Argopecten irradians concentricus* (Liu, Liu & Sun 2011). Genetic analysis in all these studies indicated that some degree of inbreeding depression was present in most of self-fertilized families, but not all of them. In the present study, the level of fertilization between gametes from the same individual was significantly lower than the control fertilizations in which oocytes from different individuals were crossed with cryopreserved sperm, suggesting the presence of certain mechanisms to limit self-fertilization. These mechanisms may involve sperm-oocyte recognition or interaction, rather than inbreeding depression. Further studies are needed to follow larval growth and metamorphosis, spat development and adult performance to evaluate the effects of self-fertilization in *Crassostrea*.

Improvement of this approach is necessary for creation of inbred lines

In this study, the larvae from self-fertilized families did not survive beyond metamorphosis. Larval culture could be a major cause, and the small number of larvae available from the start also contributed to this problem. Practical solutions could come from increasing the amount of sperm sampled by non-lethal collection. This can be achieved by using larger oysters or improving their gamete production by conditioning. In this study, the oysters used for sperm collection in 2010 averaged 7.48 g, and were taken directly from the wild site at the peak of spawning season. Increasing the post-thaw fertility of cryopreserved sperm could also help. Assurance of sperm quality and the use of proper protocols for sperm cryopreservation are two aspects to increase post-thaw fertility. The use of oysters at their peak maturity by indoor conditioning may be employed for this purpose. Optimization of cryopreservation protocols can assure the output of post-thaw sperm fertility

(Tiersch 2011). In this study, the cooling of sperm samples was performed by placing the sample canister in nitrogen vapour at a specific position in a shipping dewar that was pre-set by measuring the cooling rate at that point. However, the actual cooling rates could vary depending on factors such as how many times the shipping dewar was opened. A programmable freezer could be used to assure the cooling rates to overcome this shortcoming. Finally, the use of a large number of oysters for non-lethal sperm sampling at the beginning can assure that sufficient sex-changed females would be available for self-fertilization. This approach involved a series of steps, and at each step the number of available oysters decreased (Fig. 2), therefore the oyster population sampled at the beginning should be of sufficient size to assure the future availability of sex-changed individuals for oocyte collection and self-fertilization.

Thus, the establishment of inbred lines by self-fertilization is possible with the following protocol: collection of sperm samples of good quality and sufficient quantity, survival of male oysters after sampling, culture for 1–2 years to allow sex reversal into females, collection of oocytes of sufficient quality and quantity, self-fertilization and care of larvae in sufficient numbers to ensure survival beyond metamorphosis. At each step, losses accumulate, with each generation leaving a certain percentage of the original starting number. Therefore, consideration must be given to ensure that sufficient numbers of oysters are employed at the beginning. In addition, during inbreeding some proportion of the lines could die out from homogeneity of recessive lethal genes before a reasonably high degree of inbreeding has been attained. Consequently, the overall program should start with a sufficient number of different lines, and other strategies such as sister-brother crossing may combined to maintain inbred lines in later generations. Also, the genetic background of the starting populations or lines should be considered.

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