水 研 機 構 研 報, 第 45 号, 15 − 20, 平 成 29 年
Bull. Jap. Fish. Res. Edu. Agen. No. 45, 15 − 20, 2017

Application of Germplasm Preservation in Breeding Programs for Molluscan Shellfish Aquaculture and Restoration

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Abstract: Germplasm are the genetic materials of germ cells, including gametes, embryos, or larvae. Preservation of germplasm is usually achieved through cryopreservation. The technology of cryopreservation has been applied for human artificial reproduction as a clinical treatment for infertility and for livestock as a tool for breeding programs worldwide. For fish and shellfish, cryopreservation has been studied in more than 200 species for preservation of natural resources and conservation of endangered species. For molluscan shellfish aquaculture, this technology can have the following potential applications: ***1) Preservation of specific lines or strains***. Ongoing breeding programs have yielded specific strains and lines, such as disease resistant oysters. Cryopreservation can be used to preserve these valuable strains and provide gametes for assistance of breeding programs. ***2) Preservation of natural wild populations***. The cryopreserved germplasm of natural populations will act as a repository of genetic diversity and allow for the continued adaptive genetic variation for aquaculture populations through infusion of new material from wild populations. In addition, a germplasm repository of wild populations can provide easy access as study materials for researchers. ***3) Creation of self-fertilization inbred lines***. Inbred lines are one of the most valuable resources for breeding programs but difficult to produce and require years of repeated crossing of brothers and sisters or backcrossing. Most bivalves are protandrous, beginning life as males and changing into females as they age. Therefore, with the techniques of non-lethal sperm collection and cryopreservation, self-fertilized lines can be created by using cryopreserved sperm and oocytes from the same individual after sex reversal; ***4) Preservation of sperm from tetraploid oysters***. Triploid-tetraploid technology is probably the most promising in oyster aquaculture because of the superior traits of triploids. Cryopreservation of sperm from tetraploids can extend the commercialization of triploid-tetraploid technology by the sale of frozen sperm and provide cost savings and security for maintaining tetraploids. In addition, cryopreservation of tetraploids produced each year can offer benefit for maintenance of the tetraploid populations. ***5) Assistance for creation of mutant lines***. Mutant breeding is an effective approach for creation of new strains or lines but seldom used in animal breeding programs because of its low efficiency. Recently, a new approach called TILLING (Targeting Induced Local Lesions in Genomes) was developed in plants for creation of mutant lines. To use this new technique for animal mutant breeding, sperm cryopreservation is an absolutely required technique. So far, this approach has been applied to zebrafish and puffer fish. With the genome sequencing accomplished in oysters, establishment of mutant lines by TILLING and sperm cryopreservation will benefit aquaculture and research on oyster functional genetics, and ***6) Assistance of aquaculture hatchery practice for regular and hybrid seed production***. The cryopreserved germplasm materials can function as a reservoir to meet the need for regular and hybrid seed production. For example, the hard clam hybrid offspring of *Mercenaria mercenaria* with *Mercenaria campechiensis* showed fast growth and higher survival.

Key words: Germplasm, cryopreservation, shellfish aquaculture

2017年２月28日受理（Received on February 28, 2017）
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Introduction

Germplasm is the living genetic resources which are inheritable and can generate new generations, such as seeds for plants, or germ cells, gametes, embryos, or larvae for animals. Preservation of germplasm can serve as repository (seed banks, gene banks or sperm banks) for animal and plant breeding. Usually, germplasm preservation can be achieved through cold storage (for plant seeds) or cryopreservation (for animal germplasm materials). The technology of cryopreservation has been applied for human artificial reproduction as a clinical treatment for infertility (Bernard and Fuller, 1996; Di Santo *et al*., 2012) and for livestock as a tool for breeding programs worldwide (Curry, 2000). For fish and shellfish, cryopreservation has been studied in more than 200 different species with different applications (Tiersch *et al*., 2007), such as preservation of natural resources to maintain biodiversity and conservation of endangered species.

For molluscan shellfish, the application of germplasm preservation can also serve as germplasm repository for selective breeding (Adams *et al*., 2008), hybrid breeding, commercial seed production, preservation of natural resources, and conservation of endangered species. In addition, due to the characteristic of molluscan shellfish, application of germplasm cryopreservation can be expanded to certain specific areas such as creation of self-fertilization inbred oyster lines (Yang *et al*., 2015), aquaculture of triploid-tetraploids (Dong *et al*., 2005) and possible creation of mutant lines as that for zebrafish (Lawson, 2016).

Materials and Methods

Sample collection

In general, collection of germplasm samples for cryopreservation is based on the reproductive biology of the working species. Currently, most aquaculture molluscan species are bivalves (oysters, clams, scallops, and mussels) and a few gastropods, such as abalones. Those species are primarily free spawning with external fertilization, spiral holoblastic embryo development, and a swimming larval period until metamorphosis. Upon release, gametes are usually activated immediately and ready for fertilization within a few hours.

Germplasm collection methods usually can be: 1) *For gametes*: natural spawning, suspension of dissected gonads, or biopsy of gonads. The last two methods are only useful for certain species, such as some oysters and clams, in which gametes from dissected gonad can remain fertile. 2) *For embryos and swimming larvae*: collection is usually performed by filtering through 25 - μm screen and re-suspending in fresh seawater or buffer.

Viability analysis

For almost all germplasm cryopreservation programs, fertilization and survival have been considered the main criteria for evaluation of cryopreservation success. Additionally, considering the time and resources required for *in vitro* fertilization, other effective and fast viability assays especially for sperm also have been developed and used widely. Viability of fresh or post-thaw sperm can be evaluated by observing motility with a bright-field light microscope and plasm membrane integrity and mitochondria membrane integrity by flow cytometry analysis. For oocytes, viability evaluation can be assisted by observation of the size and morphology besides fertility test. For embryos and larvae, survival and growth can be used for viability evaluation.

Cryopreservation process of germplasm

Cooling rate is one of the most critical factors for success of germplasm cryopreservation, usually varies with germplasm materials and the working species, and is highly related to the volume, materials, and shape of the packaging container. Accurate cooling rate can be achieved by using liquid nitrogen or dry ice with the following methods: 1) Computer-controlled programmable freezer. The cooling process is controlled by spraying of liquid nitrogen which is pre-set by a programmable computer to achieve accurate cooling rates. 2) Home-made cryokits composed of Styrofoam boxes with liquid nitrogen. The cooling rates need to be pre-determined by measuring the temperature with a digital thermocouple, and cooling rates can be adjusted with the depth of liquid nitrogen, size of the

Styrofoam box and floating boat (for holding the samples to be frozen). 3) Others, such as nitrogen vapor in the storage or shipping dewar or a Styrofoam box filled with dry ice. Upon reaching -80 °C, frozen samples can be plunged into liquid nitrogen for long-term storage in storage dewar.

For sample packaging containers for germplasm cryopreservation, many different types have been used for molluscan germplasm cryopreservation in previous publications, such as French straws (0.25 ml, 0.5 ml, and 5 ml), cryovials, and cryo-plastic bags. The choice of packaging containers need to be facilitated with the accuracy of cooling rates, sample biosecurity, high-throughput processing, freezing and thawing process, and efficiency of storage space.

Results and discussion

Since the first report of sperm cryopreservation in the Pacific oyster *Crassostrea gigas* in 1971 (Lannan, 1971), a total of 79 publications have been published on germplasm cryopreservation in molluscan shellfish (Table 1). Those studies were majorly on aquaculture species, including bivalves (oysters, mussels, pearl oysters, scallops, and clams) and gastropods (abalones), and most of those were on oyster species from the Family *Ostreidae* (43 publications). A recent publication reviewed the research on germplasm cryopreservation of oysters with detailed summaries (Hassan *et al*., 2015).

Overall, the germplasm materials cryopreserved include sperm, oocytes, embryos, and larvae, covering almost all of the life forms of germplasm (Table 1). However, no research has been found in molluscan shellfish on cryopreservation of primordial germ cells or gonad tissues, which has been developed in fish to generate gonads through transplantation of germ cells in surrogate or sterile mothers (Okutsu *et al*., 2007; Majhi *et al*., 2009). This is probably because of the advantages of mollusks over finfish for egg, embryo or even larval cryopreservation. For fish, egg or embryo cryopreservation still remains as a huge challenge due to the size, yolk contents, and meroblastic embryo cleavage (Yang and Tiersch,

Table 1. Summary about publications on molluscan shellfish germplasm cryopreservation

Family	Species	Number of publication	Materials for Cryopreservation	Year
Ostreidae	*Crassostrea gigas*	30	Sperm, oocytes, embryos, larvae	1971-2014
	Crassostrea virginica	9	Sperm and larvae	1973-2014
	Saccostrea cucullata			
	Crassostrea tulipa	1	Sperm	1991
	Crassostrea iredalei			
	Crassostrea rhizophorae	1	Sperm and embryos	2005
	Saccostrea glomerata	1	Larvae	2008
	Ostrea edulis	2	Sperm and trochophores	2011, 2012
Mytilidae	*Choromytilus chorus*	1	Embryos	1988
	Mytilus edulis	1	Embryos	1989
	Perna canaliculus	5	Sperm, oocytes, trochophores	2009-2014
	Mytilus galloprovincialis	3	Sperm, trochophores	2009-2013
	Mytilus trossulus	1	Trochophores	2009
Pteriidae	*Pinctada fucata martensii*	6	Sperm and larvae	2003-2012
	Pinctada margaritifera	4	Sperm	2005-2013
Pectinidae	*Argopecten purpuratus*	1	Sperm	2010
	Pecten maximus	1	Sperm	2014
Veneridae	*Tapes philippinarum*	1	Larvae	1992
	Meretrix lusoria	1	Embryos and larvae	1997
Mactridae	*Spisula sachalinensis*	1	Larvae	2008
Haliotidae	*Haliotis gigantean*	1	Sperm	1983
	Haliotis discus	1	Sperm	1983
	Haliotis diversicolor	6	Sperm, oocytes, and embryos	1992-2013
	Haliotis iris	1	Sperm	2000
	Haliotis rufescens	1	Sperm	2005
	Haliotis midae	1	Embryos	2008
	Haliotis laevigata	3	Sperm	2014

2009). For molluscan shellfish, a recent study with the Pacific oyster showed the success of larval cryopreservation, and post-thaw trochophore larvae grew into mature adults and spawned naturally (Suquet *et al*., 2014).

Outlook for future research

Although many publications have reported research on molluscan shellfish germplasm cryopreservation, most research still has limitations that need to be addressed through future research. The limitations could be generalized as: 1) only very small laboratory-scale trials were reported in almost all of the reports; 2) post-thaw fertilization data was not included in most research to test the effectiveness of cryopreservation protocols; 3) no standardized sample packaging containers were used for possible high-throughput application, and 4) most factors in the freezing process were descriptive without quantification for protocol standardization and repetition, such as sperm concentration, sample volume, and cooling rate. These aspects will be the future research directions in this field.

Since the initiation of sperm cryopreservation in the Pacific oyster in 1971, it has been about 40 years. However, cryopreservation of molluscan shellfish species still remains essentially a research activity with little commercial application. For livestock species, sperm cryopreservation has grown into a billion-dollar global industry in about 50 years since its initiation. Compared to livestock and fishes, most aquaculture molluscan shellfish have the potential advantage for oocyte, embryo and larva cryopreservation. Therefore, germplasm cryopreservation will have great application in research projects, breeding programs, commercial industry, and natural resource conservation. Molluscan shellfish have traditionally been a major component of world aquaculture, its production in 2012 (15.2 million tonnes) accounted for about 22.8% of the total (inland and marine) aquaculture production and 60.3% of the world marine aquaculture production (FAO, 2012). It is predictable that germplasm preservation will be a useful technology to enhance and boost the molluscan shellfish industry.

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(1) Fuller B., Lane N., and Benson E., 2004: Life in the Frozen State, CRC Press, New York, 672pp.

Cryobiology is a research field encompassing disciplines including mathematics, biophysics, cell biology, molecular biology, and metabolism physiology. This book brings together the knowledge about cryobiology from these aspects into one platform for readers to understand this research, and is probably the first major textbook on cryobiology. It includes four themes with a total of 23 chapters, and the four themes are Fundamental Aspects, Life and Death at Low Temperatures, Freezing and Banking of Living Resources, and Medical Applications.

(2) Polge C., Smith A. U., and Parkes A. S., 1949: Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* **164**, 666.

This is the first paper to report the successful cryopreservation and cold-drying of sperm from fowl by using glycerol as cryoprotectant. The finding in this one-page short report opened the door for human sperm cryopreservation which is now a major clinical treatment for infertility, and bull sperm cryopreservation which is now a huge industry for its breeding programs worldwide. For fish, the first report was published after this report in 1953 in herring (Blaxter J. H. S., 1953: Sperm storage and cross-fertilization of spring and autumn spawning herring. *Nature*, **172**, 1189 – 1190), and this preliminary communication showed that it was possible to cross-fertilize the two spawning 'types' of herring *Clupea harengus* found in the north-east Atlantic by using the cryopreserved sperm.

(3) Tiersch T. R. and Green C. C., 2011: Cryopreservation in Aquatic Species, World Aquaculture Society, Baton Rouge, LA, pp.1034.

This is the 2nd version of the book published electronically by the World Aquaculture Society (www.was.org). The first version is the volume 7 in the series of publication "Advances in World Aquaculture" published in 2000. The revised version is designed as a comprehensive single compendium of information on cryopreservation in aquatic species, and provides a broad overview of the principles, procedure and perspectives which are necessary for development and application of cryopreservation technology. It includes 101 chapters (compared to the 55 in the first edition) organized into 11 sections. This book can assist with teaching, research and program development, and is available for readers to purchase through the World Aquaculture Society with a very affordable price for students and researchers.

(4) Yang H., Hu E., Cuevas-Uribe R., Supan J., Guo X., and Tiersch T. R., 2012: High-throughput sperm cryopreservation of eastern oyster *Crassostrea virginica*. *Aquaculture* **344-349**, 223–230.

This study provided a reliable protocol for sperm cryopreservation in the eastern oyster *Crassostrea virginica* with potential for high-throughput processing. In this study, DMSO yielded the highest post-thaw motility at a cooling rate of 20 °C/min when thawed at 30 or 40 °C among the three tested cryoprotectants. Further evaluation of cooling rates of 10, 15, 20, 25 and 30 °C /min showed that 20 or 25 °C /min yielded the highest post-thaw motility (34 ± 5%) and fertility (77 ± 12%) for French straws and

CBS straws (28 ± 3% and 69 ± 14%). Equilibration times of 10 to 60 min did not cause significant differences in post-thaw motility when freezing with 10% DMSO at a cooling rate of 25 degrees C/min. Also, sperm concentrations ranging from 1×10^8 to 1×10^9 cells/ml at freezing did not cause significant differences in post-thaw motility. Finally, after thawing, sperm cryopreserved from 16 males with this protocol showed 58 ± 24% fertility (from 18 to 86%) for French straws, and 54 ± 21% fertility for CBS straws (from 18 to 95%). Overall, this research provided an outline template for developing a basic protocol for sperm cryopreservation for any other molluscan shellfish species.

(5) Yang H., Wang Y., Guo X., and Tiersch T. R., 2015: Production of inbred larvae through self-fertilization using oocytes and cryopreserved sperm from the same individuals after sex reversal in eastern oyster *Crassostrea virginica*. *Aquac. Res.* **46**, 2153-2165

The authors in this study reported the production of self-fertilized larvae in eastern oyster *Crassostrea virginica* for the first time, and demonstrated the feasibility of creating self-fertilized inbred lines by use of non-lethal sperm collection and cryopreservation. 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, and sex reversal was observed bidirectional (from female to male and also from male to female). 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 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.