Cryopreservation of Different Strains of the Euryhaline Rotifer *Brachionus plicatilis* Embryos

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Cryopreservation of different strains of *Brachionus plicatilis* symmetrical stage embryos was conducted. One S-type strain, namely Yashima-S (YS), and three L-type strains, namely Yashima-L (YL), Nagasaki (NG), and Hamana (HA), were used. Symmetrical stage embryos in 10% DMSO-28‰ sea water solution were frozen at -196°C using a two-step freezing procedure; the embryos were first cooled slowly from -5°C to -20°C at a rate of $0.3^{\circ}\text{C}/\text{min}$ and then cooled rapidly by direct transfer to liquid nitrogen. Frozen samples were thawed in a gently stirred water bath at room temperature. A mean survival rate of 36% after 30 days storage in liquid nitrogen of HA strain was significantly lower than YS (55%), YL (58%), and NG (54%), and is due likely to its relatively larger embryo volume. There was no significant difference in the intrinsic rate of increase (r) between strains among clones taken from the cryopreservation or unfrozen control group. The results also indicate that various strains of *B. plicatilis* embryos can be cryopreserved without seriously altering their reproductive capability. The freezing method described could be of great potential in aquaculture and in future studies on rotifer genetics.

Cryopreservation has been widely employed in experimental biology, medicine and animal breeding. Its usefulness in aquaculture, however, has been limited to sperm cryopreservation.^{1,2} Development of cryopreservation techniques for aquaculture could lead to gene banking and effective management of hatchery facilities regardless of the natural spawning season.

Because larval mouth size sets the upper limit of prey size, the size of the rotifer *Brachionus plicatilis* a euryhaline rotifer extensively used as a live food for fish and prawn larvae, is crucial in its utilization. A variety of first-feeding larvae ingests prey sizes between 50 to 100 μm while larvae with large mouths can accommodate a wider size range.³ Body size variation among *B. plicatilis* strains has been reported by Fukusho and Okauchi,⁴ Ito *et al.*,⁵ and Snell and Carillo.⁶ Maintenance of a certain strain with the desired morphological and genetic characteristics is often at risk of contamination, and total collapse of cultures is sometimes unavoidable. In addition, rotifer culture is costly and requires routine work. A reliable cryopreservation

technique for various strains of rotifer could therefore alleviate such problems. In our previous paper,⁷ we demonstrated a successful cryopreservation method for the separated symmetrical stage embryos of *B. plicatilis*. However, the size variation of embryos among strains is a possible important factor which can affect the results of the cryopreservation. This paper describes the cryopreservation of four strains of *B. plicatilis* embryos and evaluates their viability in terms of the intrinsic rate of increase of population after storage in liquid nitrogen.

Materials and Method

One S-type strain, namely Yashima-S (YS), and three L-type strains, namely Yashima-L (YL), Nagasaki (NG), and Hamana (HA), of *B. plicatilis* were used. Each strain was cultured separately in 3 l flasks containing 28‰ and 25°C sea water. Flasks were aerated, and rotifers fed *Nannochloropsis* sp. Density was kept constant at 10 to 50 individuals/ml by renewing the culture every 3 or 4 days. These strains have distinct

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morphological characteristics and have been continuously maintained in the laboratory for three years.

Synchronization of embryo stage and separation of embryos from ovigerous females were done following Toledo and Kurokura.⁷⁾ Briefly, juveniles from the culture stock of each rotifer strain were passed through a 80 μm plankton net and finally collected in 40 μm net. These were separately raised in 2 l flasks following the culture method previously described. Separated juveniles laid their first eggs after 16 to 20 h under such conditions. As soon as a majority of the separated culture laid their first eggs, the rotifers were again collected with a 40 μm net and washed into 20 ml screw cap test tubes. The eggs were separated from ovigerous females by vigorous mixing with a vortex mixer for 10 to 15 min. The test tubes were left to stand for about 10 min to allow the separated embryos to settle. Separated embryos were pipetted out and placed in a watch glass to remove unwanted debris. Collected embryos were raised in 5 ml petri dishes containing filtered sea water through 0.45 μm membrane filter, of which salinity was 28‰, and incubated in a rotary shaker-incubator (Model SCS-R, Sanki Seiki Co.) at 25°C and 50 rpm until used. Separated embryos of all strains reached the symmetrical stage about 7 to 11 h after eggs were laid. Separated embryos of each strain were photographed at 200 $\times$ magnification before freezing for the measurement of embryo volume and the volume ($0.524 \text{ length} \times \text{width}^2$) was calculated following the method of Yufera.⁸⁾ Symmetrical stage embryos were frozen following a two-step freezing procedure described by Toledo and Kurokura.⁷⁾ The embryos were incubated at room temperature in 10% DMSO (v/v) in the filtered sea water for 30 min. Then, the embryos at the density of 100–200 individuals /ml were pipetted into 0.5 ml plastic insemination straws, heat sealed, and immediately frozen in a Program Freezer (FPF, Osaka Sanso Kogyo Ltd.). The straws were cooled from room temperature to -5°C at a rate of $5^\circ\text{C}/\text{min}$, at which point ice seeding was induced by jetting chilled nitrogen gas in the middle of the straws and held for 5 min for temperature equilibration. The straws were then cooled to -20°C at a rate of $0.3^\circ\text{C}/\text{min}$ and held for 5 min before rapid cooling by direct transfer to liquid nitrogen. All samples were stored in liquid nitrogen for at least 30 days

before thawing.

Frozen samples were thawed by immersing the straws in a gently-stirred water bath at room temperature. Thawed samples were immediately expelled in 5 ml petri dishes and rediluted twice by adding an equal volume of the filtered sea water at 2-min intervals. The embryos were then washed with fresh sea water of the same salinity, transferred to cell well plates (Corning Cell Wells 255820), and placed in the rotary shaker-incubator at 25°C and 50 rpm until hatching. *Nannochloropsis* sp. was provided in each cell well as food for newly-hatched neonates. Survival was expressed as a percentage of the number of hatched embryos relative to the number of embryos recovered.

To evaluate the viability of each strains after cryopreservation, 5 individuals with first laid eggs were pipetted randomly and inoculated in 30 ml Erlenmeyer flasks containing 10 ml of the filtered sea water with 1×10^7 *Nannochloropsis* sp. cells/ml. Subsequently, 5 individuals with first laid eggs separated from the culture stock as described above were treated in the same way and served as the unfrozen control. One ml of *Nannochloropsis* sp. at the density of 1×10^7 cells were added daily to ensure an adequate supply of food. The rotifers were preserved in 1% formalin on the fifth day and the total number of individuals in each flask was counted. Intrinsic rate of increase (r) was calculated according to the formula $dN/dt = rN$, where N and t represent the number of animals and lapsed time in days, respectively.

Statistical analysis was performed using a one-way or two-way ANOVA followed by student's t -test to compare differences between means at $P=0.05$.

Results

Table 1 shows the variation in embryo volume among strains of rotifer. The mean embryo volume of the YS strain ($297 \mu\text{m}^3$) was significantly smaller than YL, NG and HA strains. No significant difference in embryo volume was observed between YL ($367 \mu\text{m}^3$) and NG ($355 \mu\text{m}^3$). HA embryos had the largest volume ($398 \mu\text{m}^3$) among the four strains examined.

The mean survival of symmetrical YS, YL, NG and HA embryos frozen by two-step freezing and stored in liquid nitrogen for 30 days is shown in Table 2. No significant differences in survival

Table 1. Variations in embryo volume among different strains of *B. plicatilis*

Strain	Embryo volume (μm^3)
Yashima-S (YS)	297 ± 6^a
Yashima-L (YL)	367 ± 4^b
Nagasaki (NA)	355 ± 5^b
Hamana (HA)	398 ± 6^c

Values are mean $\pm$ SD of measurements.

^{a,b,c} Means with different superscript are significantly different ($P < 0.05$).

Table 2. Survival of different strains of *B. plicatilis* symmetrical stage embryos 30 days after storage in liquid nitrogen

Strain	Total embryos recovered* ¹	% Survival* ²
Yashima-S (YS)	560 (77–161)	55.0 ± 3.8^a
Yashima-L (YL)	675 (73–183)	58.8 ± 4.6^a
Nagasaki (NG)	578 (88–152)	54.2 ± 2.5^a
Hamana (HA)	519 (71–125)	36.0 ± 2.5^b

*¹ Numbers in parentheses represent the range of embryos recovered per replicate straw.

*² Values are mean $\pm$ SD of five replicate straws.

^{a,b,c} Means with different superscripts are significantly different ($P < 0.05$).

Table 3. Intrinsic rate of increase (r) of different strains of *B. plicatilis* clones from cryopreservation and unfrozen control groups. Values are mean $\pm$ SD of 3 replicates.

Strain	Unfrozen control	Cryopreserved
Yashima-S (YS)	0.55 ± 0.03	0.51 ± 0.03
Yashima-L (YL)	0.52 ± 0.02	0.49 ± 0.02
Nagasaki (NG)	0.50 ± 0.03	0.48 ± 0.02
Hamana (HA)	0.52 ± 0.01	0.47 ± 0.02

rates (54–59%) were observed among embryos of YS, YL and NG strains. However, survival of HA embryos (36%) after freezing was significantly lower than the other three strains.

Post-thaw survivors were observed to swim and feed actively on *Nannochloropsis* sp. The mean intrinsic rate (Table 3) of population increase (r) of clones from the cryopreserved strains ranged from 0.50 (NG) to 0.55 (YS). Two-way ANOVA revealed no significant differences in r among strains within the cryopreserved and unfrozen control groups. However, taken as groups, r decreased significantly between the two groups.

Discussion

The present results confirm our previous

report⁷⁾ and indicate that symmetrical stage embryos of different strains of *B. plicatilis* embryos can be cryopreserved. However, survival of HA strain symmetrical embryos was significantly lower than YS, YL and NG strains (Table 2). A possible reason for the lower survival of HA strain symmetrical stage embryos may be related to its relatively bigger embryo size (Table 1). An increase in embryo size proportionally reduces the cooling rate required to avoid intracellular freezing which is known to reduce survival in a variety of cells cooled at supra-optimal rate.⁹⁾ In the present study, symmetrical stage embryos of all strains examined were cooled at $0.3^\circ\text{C}/\text{min}$ from -5°C to -20°C before being plunged into liquid nitrogen. It is most likely that HA strain embryos require a cooling rate lower than $0.3^\circ\text{C}/\text{min}$ before being immersed in liquid nitrogen to allow sufficient time for dehydration and to reduce intracellular freezing. Most of the HA strain embryos were observed to be morphologically damaged after thawing as a consequence of intracellular freezing as shown in mouse embryos.¹⁰⁾ Further experiments using rotifer embryos with extreme volume sizes are needed to confirm this point.

The behavior of neonates from cryopreserved embryos was comparable to that of the unfrozen control group. Neonates appeared to swim and feed actively on *Nannochloropsis* sp. and reproduced normally by parthenogenesis. There was no significant difference in the intrinsic rate of increase (r) among strains within the cryopreserved and unfrozen controls (Table 3). This result agrees well with the data of Rumengan and Hirayama¹¹⁾ who observed similar population growth patterns among 13 strains of *B. plicatilis* collected from different geographical locations, and raised in controlled conditions between 20°C and 24°C . In contrast, Snell and Carillott reported variations of r among *B. plicatilis* strains with a range of 0.55 (Austria strain) to 1.06 (Spain strain) collected from various sites. It is likely that differences obtained by various investigators may be due to different rotifer strains and methodologies used.

Cryopreservation of *B. plicatilis* by other workers^{12,13,14)} had limited success. In most cases, rotifer individuals were taken from the culture stock for freezing, whereas, only separated symmetrical stage embryos were employed in this study. Symmetrical stage *B. plicatilis* embryos incubated in 10% DMSO-sea water solution

have been reported to have a higher freezing tolerance when compared with other embryonic stages.¹⁴⁾ The present results also concur with Euteneuer *et al.*¹³⁾ who demonstrated that frozen amictic eggs survive better than adults or neonates.

While mammalian embryo preservation is rapidly developing,¹⁵⁾ advance in aquatic embryo preservation is relatively slow.^{2,16,17)} In the preservation techniques of *B. plicatilis* strains, Lubenz *et al.*¹⁸⁾ proposed a technique in which amictic females were preserved at 4°C in refrigerator. Their technique needs intricate handling during the preservation period at present and cost problems were anticipated for commercial use on a large scale. On the contrary, cryopreservation technique needs no handling during the preservation period except the supply of liquid nitrogen and greater merits can be expected. We have shown in this paper that separated symmetrical stage embryos of YS, YL, NG and HA strain *B. plicatilis* can survive storage in liquid nitrogen for at least 30 days without serious changes in their reproductive capability. Although longer storage in liquid nitrogen should be tested, we do not foresee any change in viability since biological activity of cells which can survive freezing below -130°C ceases⁹⁾. No further loss of viability was observed in mouse embryos stored at -196°C for 27 to 29 months.¹⁰⁾ By following or modifying the cryopreservation method described in this study, clones of a specific strain of *B. plicatilis* having the desired genetic and morphological characteristics can be conveniently stored in liquid nitrogen without altering their viability. Therefore, the present results have great potential in fish and prawn hatchery management.

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