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Application of heavy-ion-beam irradiation to breeding large rotifer

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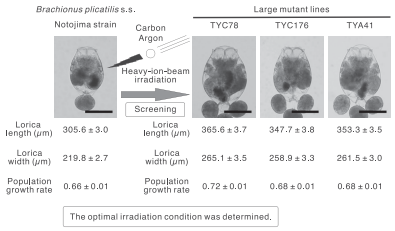
ABSTRACT

In larviculture facilities, rotifers are generally used as an initial food source, while a proper size of live feeds to connect rotifer and *Artemia* associated with fish larval growth is needed. The improper management of feed size and density induces mass mortality and abnormal development of fish larvae. To improve the survival and growth of target larvae, this study applied carbon and argon heavy-ion-beam irradiation in mutation breeding to select rotifer mutants with larger lorica sizes. The optimal irradiation conditions of heavy-ion beam were determined with lethality, reproductivity, mutant frequency, and morphometric characteristics. Among 56 large mutants, TYC78, TYC176, and TYA41 also showed active population growth. In conclusion, (1) heavy-ion-beam irradiation was defined as an efficient tool for mutagenesis of rotifers and (2) the aforementioned 3 lines that have larger lorica length and active population growth may be used as a countermeasure of live feed size gap during fish larviculcure.

Graphical Abstract



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Determination of optimal heavy-ion-beam irradiation conditions to generate mutant rotifers with larger lorica size and population growth.

Keywords: larval feeding, mutagenesis, mutation breeding, optimal condition, rotifera

Abbreviations: *B. plicatilis* sp. complex: *Brachionus plicatilis* species complex; *B. plicatilis* s.s.: *Brachionus plicatilis* sensu stricto; C: carbon; Ar: argon; LET: linear energy transfer; SS-: super small-; S-: small-; L-: large-; RIBF: Radioactive Isotope Beam Factory; psu: practical salinity unit; LD_{50}: 50% lethal dose; *A. vaga: Adineta vaga*; DSB: double-strand breaks

The euryhaline rotifers (*Brachionus plicatilis* species [sp.] complex including 15 cryptic species) are used as an initial feed source for fish larviculture (Fukusho 1983; Lubzens 1987; Lubzens, Tandler and Minkoff 1989; Hagiwara *et al.* 2007) which are divided into 3 morphotypes based on their body sizes: super small, small, and large types (SS-, S-, and L-types, respectively) (Hagiwara *et al.* 1995, 2007; Mills *et al.* 2017). Fish larvae (*Verasper variegatus*, *Seriola quinqueradiata*, *Platycephalus* sp. [Hagiwara *et al.* 2007; Akazawa, Sakakura and Hagiwara 2008], *Takifugu rubripes*, *Plecoglossus altivelis actively*, *Thunnus thynnus orientalis* [Sawada *et al.* 2000], *Sparus aurata* [Fernández-Diaz, Pascual and Yúfera 1994], *Epinephelus bruneus* [Iwasaki *et al.* 2016], *Epinephelus septemfasciatus* [Tanaka *et al.* 2005; Iwasaki *et al.* 2016], and *Hippoglossus hippoglossus* [Olsen *et al.* 2000]) prefer to larger feed items with their growth stages, and thus the feeding scheme of fish larvae is established by their mouth size and rotifer lorica size. For example, live feeds are progressively utilized, SS-, S-, L-type rotifers, and then *Artemia* that is larger than rotifers (Fernández-Diaz, Pascual and Yúfera 1994; Olsen *et al.* 2000; Iwasaki *et al.* 2016). The size distributions of the lorica length of rotifers are 170-190 µm for SS-type, 190-240 µm for S-type, and 240-320 µm for L-type in individuals carrying amictic eggs. The next feed item *Artemia* has a body length of 400-1000 µm (Anderson 1967). Although there is a large size gap between L-type rotifers and *Artemia* nauplii, no feed items for the size gap 320-400 µm has been developed in food scheme of fish larvae. The improper management of live feed size and density induces mass mortality of fish larva and morphological abnormality (Aritaki, Seikai and Kobayasi 1996; Hashimoto *et al.* 2015), and thus a countermeasure of size gap between rotifers and *Artemia* nauplii is required to improve the survival and growth of fish larva. To solve the long-standing problem of size gap, this study employed heavy-ion-beam irradiation generated by RIKEN's Radioactive Isotope Beam Factory (RIBF, Japan) to breed large mutant lines of rotifers. Efficient irradiation conditions of ion beam for rotifer mutagenesis were determined, and subsequently large mutant lines were selected using a large-scale screening developed.

The reproductive mode of rotifers is commonly divided into 2 types: obligate and cyclically parthenogenesis in their life cycle. In aquaculture facilities, intensive mass culture of live feed rotifers is performed via parthenogenetic reproduction to induce their rapid proliferation (Yoshinaga, Hagiwara and Tsukamoto 1999). The euryhaline rotifer *B. plicatilis* sensu stricto (s.s.) is classified as L-type rotifer in which the Notojima strain is the largest among 15 rotifer stocks used in Japan Fisheries Research and Education Agency (Koiso, pers. comm.) and has obligate parthenogenesis. Based on these characteristics, this study employed the Notojima strain to solve the size defects of live feeds with ion-beam irradiation (Hagiwara *et al.* 2001, 2007). The method of mutation breeding with heavy-ion-beam irradiation was conducted, which had been used to establish useful lines from various plants and microorganisms; some of these have been released for commercial use (Nakajo *et al.* 2009; Abe, Ryuto and Fukunishi 2012; Kato *et al.* 2016). The fundamental characteristics of mutagenesis were analyzed in terms of linear energy transfer (LET) values in dry seed of plant irradiated. In addition, the following features were clarified: an appropriate LET for mutagenesis was found to exist (Kazama *et al.* 2008), single-gene disruption is induced by irradiation under these conditions (Hirano *et al.* 2012), and large-scale mutations are induced by large ions in LET (Hirano *et al.* 2012, 2015; Kazama *et al.* 2017); additionally, in microorganisms, deletion mutations are induced by iron-ion irradiation (Ichida *et al.* 2008; Ma *et al.* 2018). These techniques were applied to the present study with carbon- (C) and argon- (Ar) ion beams that induce single-gene disruptions with high frequency and large mutational effects on the genome, respectively. The Notojima strain was irradiated with these heavy-ion beams at different doses. The mutation breeding was performed to determine large mutant rotifer lines with various biological parameters, and the optimal irradiation conditions were estimated. This study created large mutant lines of euryhaline rotifers with morphometric anomaly and active proliferation.

Materials and methods

Rotifer culture

The euryhaline rotifer *B. plicatilis* s.s. and the Notojima strains (L-type) provided by the Japan Sea National Fisheries Research Institute Miyazu Lab were cultured with Super fresh Chlorella V12 (Chlorella Industry Co., Ltd.), a highly condensed solution of live *Chlorella* cells. Culture medium (artificial sea water) was prepared by Tetra Marin Salt Pro (Spectrum Brands Japan, Inc.) dissolved in 10 L of Milli-Q water filtered using a 0.22 µm of pore size (Corning 430517). The salinity was adjusted using a salinometer (C-Timvasion) at 18 practical salinity unit (psu). For rotifer feeding, 10 µL of Chlorella V12 (1.4×10^8 cells of *Chlorella*) was diluted with 10 mL of culture medium (1000× dilution). Ten individual rotifers were inoculated into 100 mL bottle with 20 mL culture medium and maintained at 20°C with shaking (70 min^{-1}) under total darkness. The cultures were fed with *Chlorella* ad libitum every 3-4 days.

Heavy-ion-beam irradiation

The rotifers cultured in 600 mL of culture medium were filtered with 40 µm of pore size (Falcon 352340) and then washed with fresh culture medium. After the rotifers acclimated in 10 mL of food suspension, 150 rotifers were inoculated into eighteen 200 µL polymerase chain reaction tubes with 50 µL of culture medium at each irradiation condition. The tubes containing rotifers were placed in the automatic sample irradiation apparatus (Ryuto *et al.* 2006) and irradiated with each heavy-ion beam. Heavy-ion-beam irradiation was performed using C (1.62

Table 1. Frequencies of large mutants established by heavy-ion-beam irradiation

Heavy ion	Dose (Gy)	Total rotifers (No.)	Rotifers with active proliferation (No.)	Reproduction rate (%)	Established mutant lines (No.)	Mutant frequency (%)
C ion	100	240	195	81.3	10	5.1
	150	240	183	76.3	6	3.3
	200	504	391	77.6	13	3.3
	300	504	321	63.7	13	4.0
	400	240	98	40.8	9	9.2
	600	240	60	25.0	1	1.7
	Total	1968	1248	63.4	52	4.2
Ar ion	25	216	197	91.2	1	0.5
	50	216	145	67.1	2	1.4
	75	216	140	64.8	1	0.7
	100	216	84	38.9	0	0.0
	150	216	17	7.9	0	0.0
	Total	1080	583	54.0	4	0.7

GeV, LET = 23 keV/μm) at 6 irradiation doses of 100, 150, 200, 300, 400, and 600 Gy and Ar (3.8 GeV, LET = 312 keV/μm) at 6 irradiation doses of 25, 50, 75, 100, 150, and 200 Gy generated via RIBF (RIKEN, Japan). After irradiation, biological and morphometric characteristics were compared with control groups without irradiation.

Lethality and reproductivity

Four hours after irradiation, each rotifer sample was scaled up to 50 mL with fresh culture medium (18 psu) and then incubated at 15°C. Each irradiated rotifer was individually inoculated into a well of 24-well plate containing 1 mL of fresh culture medium (18 psu) for each irradiation condition. The plates containing rotifers were placed at 20°C with shaking (70 min^{-1}) under total darkness. Lethality (%) of irradiated rotifers was estimated with the number of wells where all individuals died in the 24-well plate. To evaluate the effect of C- or Ar-ion-beam irradiation dose to the rotifers, a correlation chart was plotted between the lapse of time and the average values of lethality rate of 6 plates at each irradiation condition. The active proliferation of irradiated rotifers was judged by over 20 offspring production. The reproduction rate of irradiated rotifers was defined as the ratio of the number of wells with active proliferation to the total number of wells in a similar way. During the large-scale screening for mutant lines, a measurement of lethality and reproduction rate was carried out until steady-state condition of data appeared.

Selection of mutant lines

The total number of inoculated rotifers for each irradiated condition is as follows: 240 rotifers at 100, 150, 400, and 600 Gy; 504 rotifers at 200 and 300 Gy with C-ion beams; and 216 rotifers at 25, 50, 75, 100, and 150 Gy with Ar-ion beams (Table 1). Rotifer selection by measuring the lorica length was too time-consuming to perform in this large-scale screening, and the death of mutant lines due to the deterioration of the culture water became a serious issue. Therefore, visual selection was used for primary, secondary, and tertiary selection in this large-scale screening. Morphometric mutant (large size) was selected with microscopic observation (18.75× of magnification) for the first selection. Wells with more than 100 rotifers propagated were targeted, and 5 individuals with longer lorica length were selected from every well and then individually inoculated into a well containing 1 mL of fresh culture medium (18 psu) for a batch culture. Second selection was performed when each well contained over 100 rotifers. Wells contained the rotifer with longer lorica were selected by visual observation. From a well, 5 individuals with visually longer lorica length were selected under a microscope and each rotifer was individually transferred into a well containing 1 mL of fresh culture medium for a batch culture. When each batch culture showed enough individual numbers, a well with the longest lorica was selected by visual observation from 5 wells as third selection. From a well, 3 individuals with longer lorica length were isolated singly into wells containing 5 mL of fresh culture medium for a batch culture. When each batch culture contained over 5000 rotifers, a well containing the rotifer with the longest lorica and with active proliferation was selected by visual observation from 3 wells as single isolation. Only 1 line was ultimately and independently established from 1 irradiated rotifer. Ten individuals of these samples were transferred into a 100 mL bottle with 30 mL of working volume for the large rotifer candidates. After measuring the lorica length and width of candidates, tested rotifers with a mean lorica length larger than 340 μm were selected as the mutant lines.

Morphometric characteristics of mutant lines

The rotifer size was measured at the exponential growth phase with over 30% of individual proportion with eggs. Lugol's solution (3% iodine, 5% potassium iodide, and 0.7% sodium chloride aqueous solution) was used for the fixation of rotifers carrying amictic eggs to make size observation photographically by Nomarski differential interference microscope with the DP74 digital camera for the BX61 Olympus upright microscope at 100 magnification and measured lorica length and width using the cellSens imaging software (Olympus).

Frequency of large mutant line appearance

The total number of inoculated rotifers for each irradiated condition is described in Table 1. In addition, lines with a mean lorica length >340 μm were selected based on 10% of the elongation rate with the size of control (305.6 ± 3.0 μm). The frequency of this appearance was calculated using the mutant lines showing active proliferation after irradiation: mutant frequency (%) = (number of established mutant lines)/(number of lines with active proliferation) × 100. The frequency was calculated at each irradiation condition.

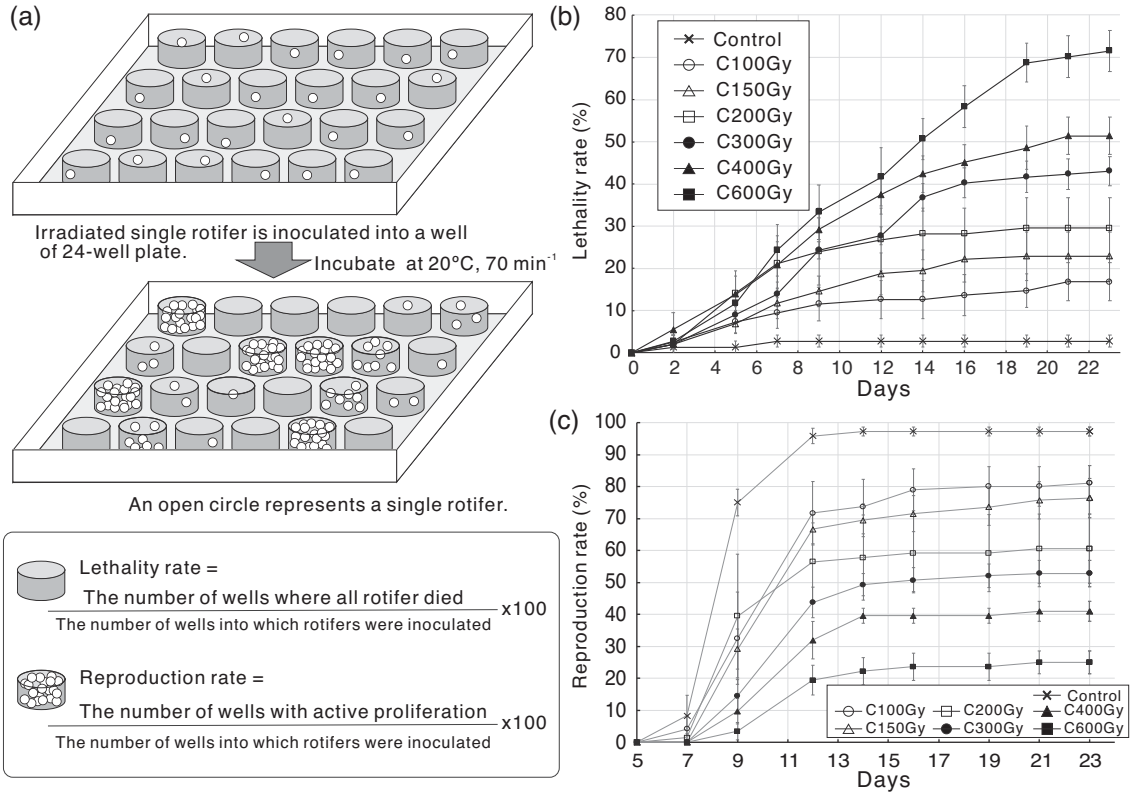


Figure 1. Effect of heavy-ion-beam irradiation to lethality and reproduction rates. (a) The diagram of calculation of lethality and reproduction rate during the culture of irradiated rotifers. (b) The correlation chart between lethality rates and the lapse of time at each irradiation dose using C-ion beam. (c) The correlation chart between reproduction rates and the lapse of time at each irradiation dose using C-ion beam. The average values of lethality or reproduction rates are calculated with results of 6 plates at each irradiation condition. The vertical bars represent standard error.

Statistical analysis of lorica size

The lorica length and width of mutant line were compared with the control group. The sizes of 20 egg-bearing individuals ($n = 20$) (Snell and Carrillo 1984; Fu, Hirayama and Natsukari 1991; Hagiwara *et al.* 1995, 2007) were analyzed using statistical software R version 3.6.1 (R Development Core Team, 2011). Following Bartlett's test (Sendecor and Cochran 1989), the Kruskal–Wallis test (Walpole and Myers 1978; Conover 1999) and Steel's multiple comparison tests (Steel 1960) were performed for significant differences. To evaluate the correlation between lorica length and width, a correlation chart using the average values of lorica length and width was constructed for the control and larger mutant lines (Hada 1938; Roxas 1941; Kazama *et al.* 2012). Outliers were detected by discriminant analysis using Mahalanobis distance via statistical analysis software for Macintosh Ver. 3.0 (Esumi Co., Ltd.) (Mahalanobis 1936). Regression analysis and correlation coefficient were calculated using Excel (Microsoft) spreadsheets after excluding the outliers.

Measurement and statistical analysis of population growth rates

The population growth rate of each large mutant line was observed with 5-mL cultures using 6-well plates. For each large mutant line, every 5 individuals were inoculated into 6 wells of 6-well plate containing 5 mL of fresh culture medium (18 psu). The rotifers were fed with *Chlorella* ad libitum every 2 days, and the total number of rotifers of each well was counted after 5 days. The population growth rate of each well was calculated with the following equation: population growth rate = ln(total population/5 individuals)/5 days) (Yoshinaga, Hagiwara and Tsukamoto 1999; Koiso and Hino 2001). The means and standard errors of the population growth rate of each large mutant line were calculated using data obtained with 6 replications. The population growth rate of each large mutant line was standardized using the control group as the baseline (as shown in Figure 6). To perform comparisons between the control group and each large mutant line, the statistical analysis was performed with R version 3.6.1 for the Bartlett's test (Sendecor and Cochran 1989), followed by Kruskal–Wallis test (Walpole and Myers 1978; Conover 1999) and Steel's multiple comparison tests (Steel 1960).

Results

Influence of heavy-ion-beam irradiation on lethality and reproduction rate

The employed rotifers survived just after irradiation even with higher doses of C- and Ar-ion beams. During the culture of rotifers irradiated with higher doses, survivors spawned several eggs without hatchability, and their lifespan was shorter than that at lower doses (Figure 1b). As a result, the lethality rate of irradiated rotifers with higher doses increased with the lapse of time and reached to steady state on day 23 for C- (Figure 1b) and on day 22 for Ar-ion beams (data not shown). On the other hand, the reproduction rate of control and irradiated rotifers with lower doses increased with the lapse of time and reached to the steady state for C- (Figure 1c) and for Ar-ion beams (data not shown). The data of day 23 for C and of day 22 for Ar in a steady

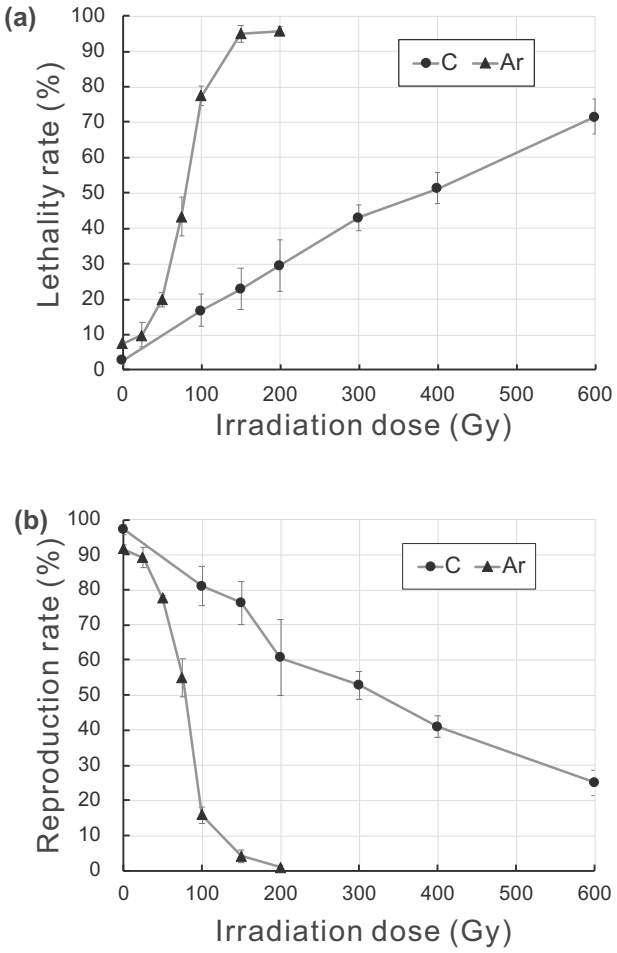


Figure 2. Correlation between heavy-ion-beam irradiation dose and lethality rates, and reproduction rates of irradiated rotifers. (a) Lethality rates with C-ion-beam (●) and Ar-ion-beam irradiation dose (▲). (b) Reproduction rates with C-ion-beam (●) and Ar-ion-beam irradiation dose (▲). Data represent the average values of lethality and reproduction rates with standard error ($n = 6$).

state are replotted on correlation chart between lethality rate and irradiation dose (Figure 2a). The data of reproduction rate are replotted in the same way (Figure 2b). From the correlation chart (Figure 2a and b), the lethality rate increased and reproduction rate decreased in a dose-dependent manner from 100 to 600 Gy C-ion beams. With 600 Gy of irradiation, the lethality rate reached to 71.5% and the reproduction rate was 25.0% as the control showed 2.8% of lethality rate and 97.2% of reproduction rate (Figure 2a and b). The lethality and reproduction rates with 150 Gy Ar-ion beams were 95.0% and 4.2%, respectively (Figure 2a and b). For the irradiation doses to achieve a lethality rate of 43%, the Ar-ion-beam dose was 75 Gy, while the C-ion-beam dose was 300 Gy; for the reproduction rate of 55%, the Ar-ion-beam dose was 75 Gy, while the C-ion-beam dose was 300 Gy. Therefore, the dose of C-ion beam had to be 4 times higher than that of Ar-ion beam to obtain the same influences (Figure 2a and b).

Frequency of appearance of large mutant lines

Using C-ion beams, large mutant lines (Figure 3) were established with appearance frequency from 1.7% to 9.2% at 100-600 Gy (Table 1, the compiled data of all mutant screening). On the other hand, Ar-ion beams induced 0.5%-1.4% of mutant frequency only at lower doses of 25-75 Gy (Table 1). Consequently, 52 large mutant lines were obtained by C- and 4 lines by Ar-ion beams (total 56 lines) (Figure 3 and Table 1).

Morphometric characteristics of large mutant lines

The results of categorizing lorica lengths of the control and the 56 selected larger mutant lines based on irradiation conditions are shown in Figure 4. Mutant lines were classified according to their average lorica length: Class I for 340-350 µm, Class II for 350-360 µm, and Class III for 360-370 µm. Class I corresponds to over 11% elongation, Class II to 15%, and Class III to 18% compared to the lorica length of the controls. The large mutant lines by C-ion beams were categorized as follows: 30 lines of Class I, 19 lines of Class II, and 3 lines of Class III. In the large mutant lines by Ar-ion beams, there were 3 lines in Class I and 1 line in Class II (Figure 4). The lorica lengths of all large mutant lines showed significant differences compared to the control group (305.6 ± 3.0 µm) (Steel's multiple comparison tests, $P < 0.001$, $n = 20$, Figure 4). In addition, the lorica widths of all mutant lines showed significant differences compared to the control group (219.8 ± 2.7 µm) (Steel's multiple comparison tests, $P < 0.001$, $n = 20$, data not shown). The discriminant analyses using Mahalanobis distances were determined outliers as TYC206 and TYC221 lines (Figure 5). The correlation coefficient between 54 mutant lines (except the 2 outliers) and the control group was 0.85 ($P < 0.001$), indicating a strong positive correlation (Figure 5).

Reproductivity of large mutant lines

The population growth rate of the control group was 0.66 ± 0.01, whereas the large mutant lines showed lower or higher rates than the controls; the average values of the population growth rate gradually decreased with increasing irradiation doses (Figure 6, dashed lines). The growth rate was not measured in the 3 mutant lines TYC98, TYC105, and TYC193 since they had exterminated during culture.

The following 3 mutant lines showed a significantly higher population growth rate than the control: TYA41 (Steel's multiple comparison tests, $P < 0.05$), TYC78 ($P < 0.01$), and TYC176 ($P < 0.01$) (Figure 6). On the other hand, 3 lines ($P < 0.05$) and 31 lines ($P < 0.01$) exhibited lower population growth rates than the control (Figure 6).

Discussion

Radiation resistance of rotifers

This study confirmed the strong radiation resistance of adult rotifers of the Notojima strain, which belongs to the *B. plicatilis* s.s. The rotifers showed lethality rate of 77.5% and 71.5% with 100 Gy of Ar-ion- and 600 Gy of C-ion-beam irradiation, respectively (Figure 2a). The radiation resistant of the rotifers was stronger than the following animals. In nematodes, *Caenorhabditis elegans* a well-known model animal, 70% of the eggs died with 50 Gy of C-ion-beam irradiation (Takanami *et al.* 2003). In cultured cells of humans and hamsters, the survival rate was 10^{-2}% or less with 10 Gy of C-ion-beam irradiation (Han *et al.* 1998; Shao, Aoki and Furusawa 2001). Previous studies on the radiation resistance of rotifers have reported in *B. koreanus* (S-type), which belongs to the same *B. plicatilis* sp. complex. The rotifer *B. koreanus* showed a 50% lethal dose (LD_{50}) of 2900 Gy at 24 h and an LD_{50} of 2300 Gy at 96 h after γ-irradiation (Won *et al.* 2016). For the Bdelloid rotifers, the reproduction rate reportedly decreased by about 90% with 1120 Gy of γ-irradiation in *Philodina roseola*

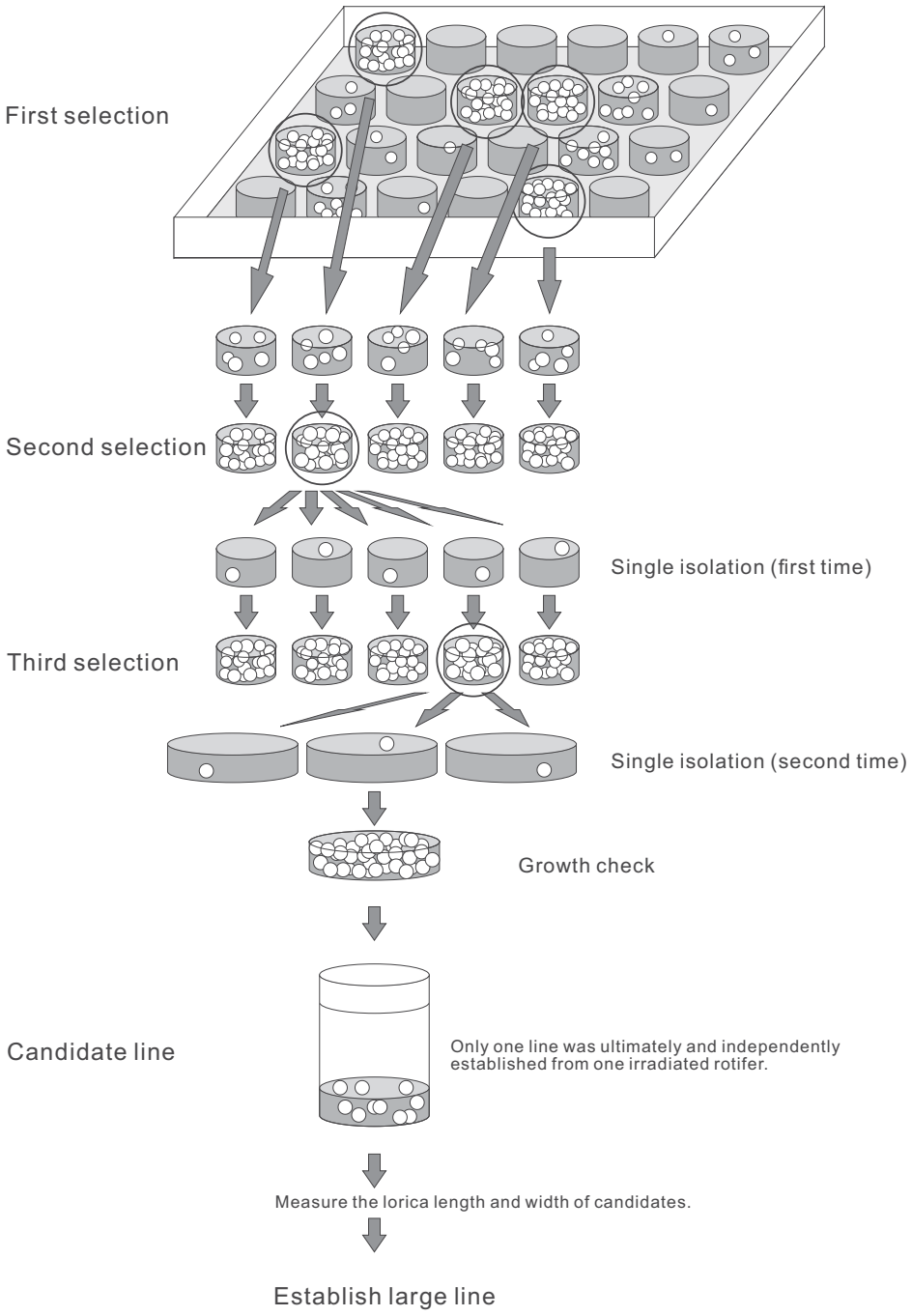


Figure 3. Diagram of selection method of mutant lines. An open circle represents a single rotifer.

(Gladyshev and Meselson 2008) and a decrease of egg production was reported with 5000 Gy of γ-irradiation in *Adineta vaga* (Krisko *et al.* 2012). The problem of radioresistance of bdelloid rotifers have been analyzed and reported that *A. vaga* was far more resistant to ionizing radiation-induced protein carbonylation (Krisko *et al.* 2012; Latta, Tucker and Haney 2019). Even when the genome was damaged into small DNA fragments by proton radiation, *A. vaga* individuals were able to efficiently repair a large amount of DNA double-strand breaks (DSB) (Hespeels *et al.* 2014). After γ-irradiation to *B. koreanus*, Glutathione S-transferase enzyme activity increased dose-dependently with oxidative stress. In addition, the expression of DNA repair-associated genes (such as p53 gene) elevated significantly in response to γ-irradiation (Han *et al.* 2014). In this way, the radiation resistance of rotifers may be caused by an effective system of antioxidant protection, including those required for DSB repair.

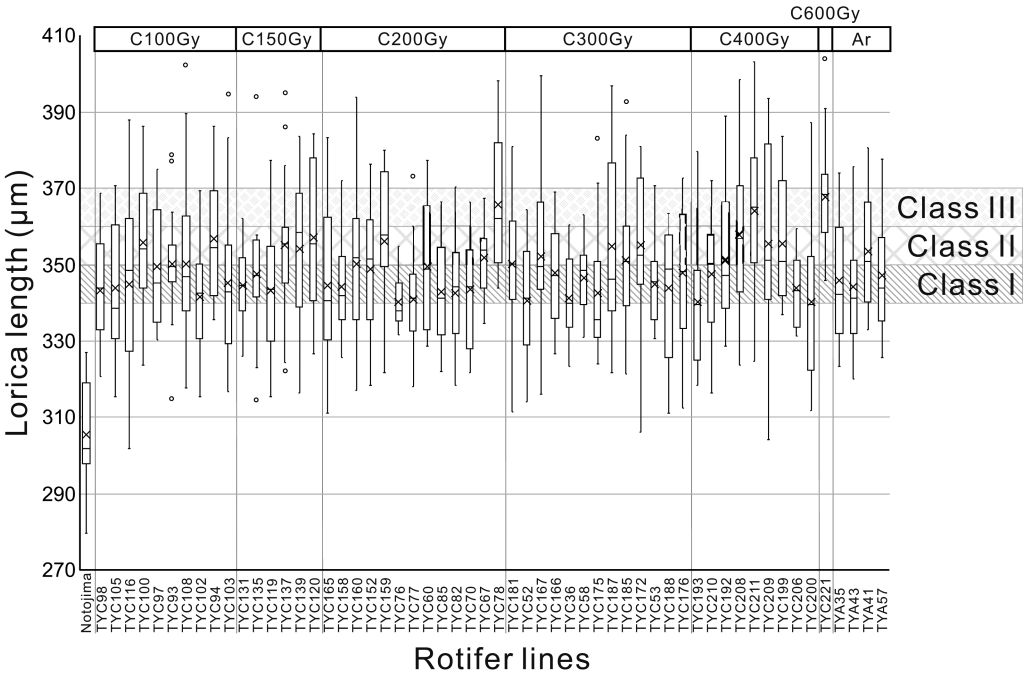


Figure 4. Comparison of lorica lengths between large mutant lines and the control. All mutant lines were significantly different from the control (Steel's multiple comparison tests, $P < 0.001$, $n = 20$). Boxplots indicate the lorica lengths of rotifers ($n = 20$). Mutant lines were separated into classes according to the average lorica length: Class I for 340-350 μm, Class II for 350-360 μm, and Class III for 360-370 μm.

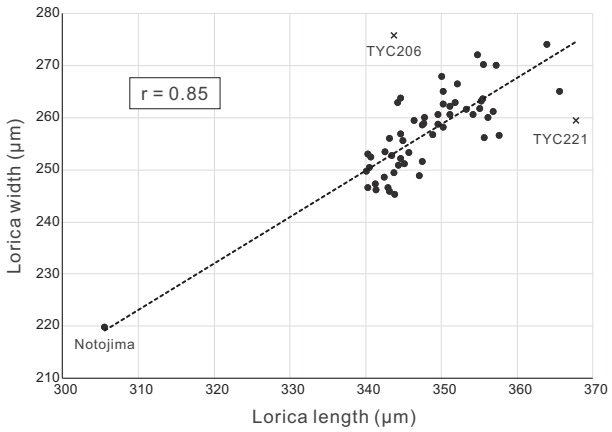


Figure 5. Correlation chart of lorica length and width between large mutant lines and the control. Normal range and outliers (TYC206 and TYC221) are represented • and ×, respectively. Data represent the average values of lorica length and width in each mutant line ($n = 20$). The dashed lines indicate the regression line between 54 mutant lines, excluding the 2 outliers (TYC206 and TYC221 lines), and the control group. r indicates the correlation coefficient.

Appropriate dose of heavy-ion-beam irradiation for rotifers

The reproduction rate should be over 40% to select adequate quantities of independent mutant lines for its application (Shu, Forster and Nakagawa 2012). Irradiations achieved >40% of reproduction rate of the rotifers were at ≤400 Gy of C-ion- and ≤75 Gy of Ar-ion-beam doses (Table 1). The frequency of large mutant lines appearance was higher with C-ion- than Ar-ion-beam irradiation. In addition, there were no significant differences in the frequency of large mutant lines appearance with C-ion-beam irradiation at 100-300 Gy, while the highest frequency was observed at 400 Gy (Table 1). TYC78 line (Class III) irradiated with the C-ion beam at 200 Gy, was selected as the most valuable mutant line based on their higher population growth rate and the largest lorica length compared to those of the control and other mutant lines (Figures 4 and 6). Class II lines, TYC67 line and TYC120 line, were established by 200 and 150 Gy of C-ion beams, respectively. These lines also had a higher population growth rate than the control groups (Figures 4 and 6). From these results, the dose suitable for selecting large mutant lines was determined to be 150-200 Gy of C-ion-beam irradiation. Similarly, TYA41 line was selected from 50 Gy Ar-ion beam irradiated group (Figures 4 and 6) as a Class II lines with a higher population growth rate than that of the control group; this was also determined to be the optimum irradiation dose. In a comparison of the frequency of mutant appearance under the optimum irradiation conditions, the large mutant lines were approximately 2.4 times more efficiently selected with C-ion- than Ar-ion-beam irradiation (Table 1). Collectively, C-ion- is superior to Ar-ion-beam irradiation for the large mutant of rotifers. These results are similar to those found for the irradiation of the model plants, *Arabidopsis* and dry seed of rice (Kazama *et al.* 2008; Hayashi *et al.* 2017; Hayashi *et al.* 2018), which suggest that the greater number of irradiated ion particles was important to achieve a high mutation frequency (Kazama *et al.* 2011).

Morphological features of large mutant lines

The lorica length of the large mutant lines was ranged 320-400 μm (Figure 4). Although the selection criterion in the screening used enlargement of lorica length, it was possible to select mutants based on lorica widths which were also significantly wider than those of the controls (Figure 5). In the correlation chart of lorica length and width of 54 large mutant lines and the control group, the correlation coefficient was distributed in the vicinity of the regression line, and the coefficient showed

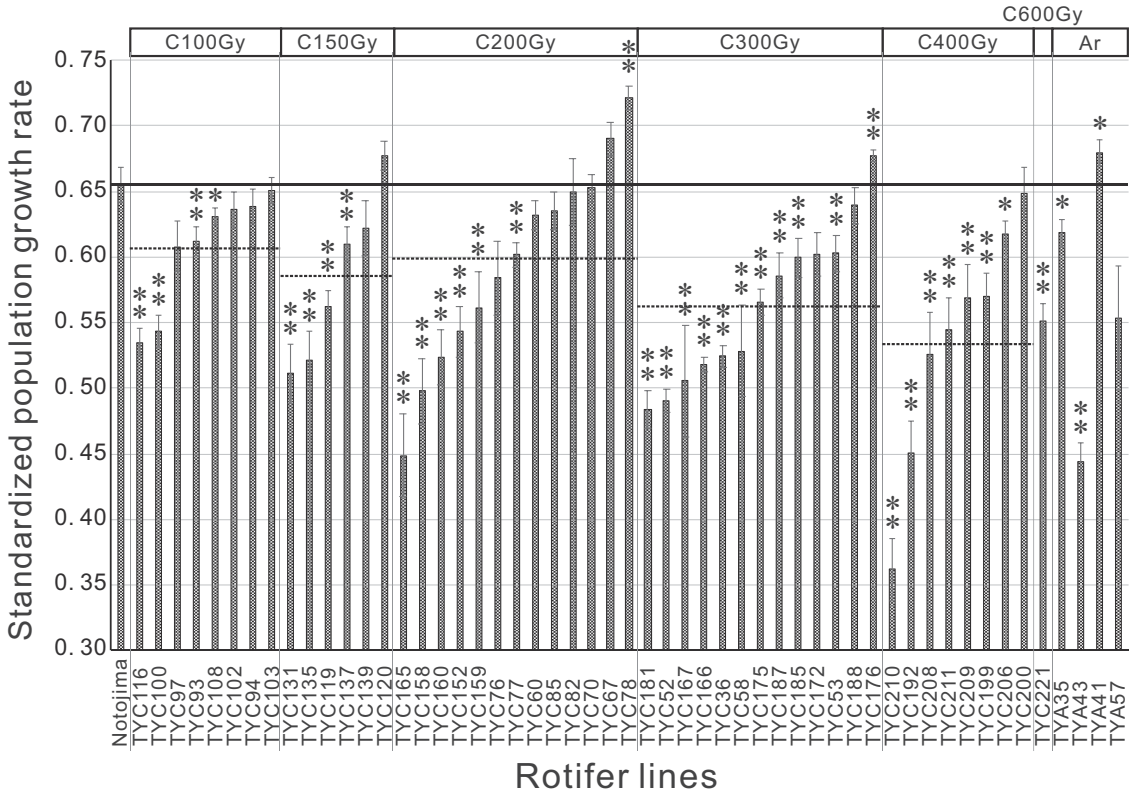


Figure 6. Comparison of population growth rates between large mutant lines and the control. Population growth rates were measured for each line, rates were standardized using the control group as baseline, with the average values and standard errors. The straight line shows the population growth rate of the control as an index. The dashed line shows the average population growth rate for each irradiation dose. Asterisks indicate significant differences of mutant lines compared to the control (Steel's multiple comparison tests, $P < 0.05$, $n = 6$).

that there was a strong positive correlation at 0.85 ($P < 0.001$), demonstrating that enlargement occurred while a normal shape was maintained (Figure 5). This suggests the existence of a scaling mechanism in the Notojima strain (Ben-Zvi, Shilo and Barkai 2011; Inomata *et al.* 2013). Because the ratio of lorica width to length (aspect ratio) is constant in rotifer strains of the *B. plicatilis* sp. complex (Hagiwara *et al.* 2007), it is likely that the scaling mechanism was conserved during the evolutionary process and therefore, may function even when the body is enlarged owing to mutations. However, 2 large mutant lines, judged as outliers in Figure 5, exhibited different morphological modification. The aspect ratio in the control group was 1.35 ± 0.01 (Figure 7a) whereas 1.25 ± 0.01 (Figure 7b1) in the TYC206 line which means morphological anomalies of relatively wider lorica widths than the analogous form. The aspect ratio was 1.42 ± 0.01 in TYC221 line (Figure 7b2) which means morphological differences of relatively longer lorica lengths.

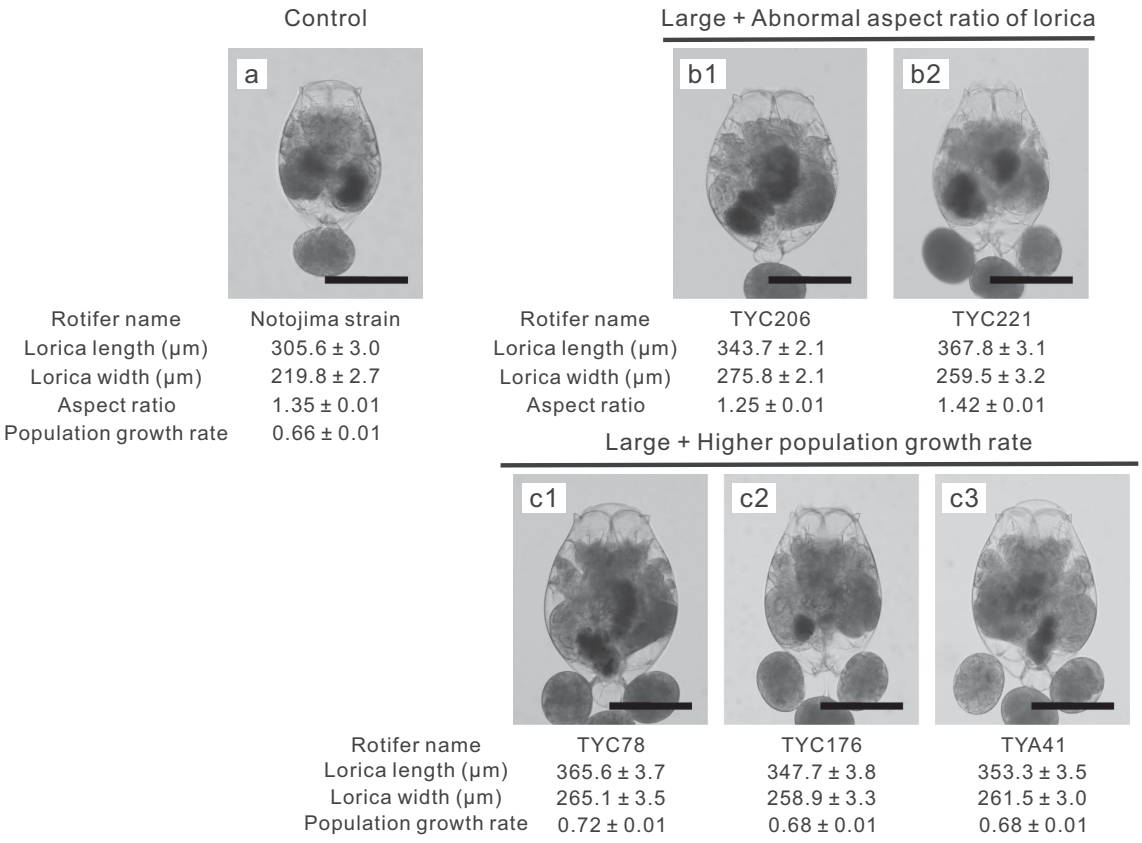
Population growth of larger mutant lines

The average values for each irradiation dose (Figure 6, dashed line) gradually decreased over 200 Gy of C-ion beams. The mutant 34 lines (61% of 56 mutant lines) showed significantly lower population growth rates compared with the control group at 0.66 ± 0.01; mutants generally had low proliferation rates (Koike *et al.* 2002; Rakwal *et al.* 2008; Arase *et al.* 2011; Isono *et al.* 2015). Nevertheless, several mutant lines established in this study had high proliferation abilities, and 3 lines (5%) exhibited significantly higher population growth rates than the control. These phenomena indicated that the diversity and effectiveness of mutagenesis by heavy-ion-beam irradiation.

Feeding management is important for the survival and growth of larvae during rearing fish larva. Active proliferation and a larger size are important factors for excellent live feeds and may contribute to the improvement of rearing fish larva performance. TYC78, TYC176, and TYA41 were not only larger lorica length (Figures 4 and 7c1-c3) but also higher proliferative activities (Figures 6 and 7c1-c3) than the control. Therefore, they are potential candidates for resolving the large size gap between rotifers and *Artemia* nauplii in fish larviculture.

"Forward genetics" applications

In this study, we evaluated efficiency of larger mutant lines with morphological and reproductive parameters. The established lines stably maintained their phenotype for at least 3 years from the irradiation. This suggests that genetic variation in the established mutant lines is stably passed onto the next generation. This means that "forward genetics" can be performed by analyzing the specific genes associated with mutant lines, a technique which may be applicable to forward genetics in *A. vaga* (Flot *et al.* 2013), *B. plicatilis* (Han *et al.* 2019) and *B. calyciflorus* (Kim *et al.* 2018) of the Monogononta and Bdelloidea lineages, which have previously been subjected to whole-genome sequencing. In particular, a genome-wide sequence analysis of NH1L strains belonging to the same *B. plicatilis* s.s. as the Notojima strain was reported by Han *et al.* in 2019 (Han *et al.* 2019), and a genome-wide sequence analysis of *B. calyciflorus* of the genus *Brachionus*



Rotifer name	Notojima strain
Lorica length (μm)	305.6 ± 3.0
Lorica width (μm)	219.8 ± 2.7
Aspect ratio	1.35 ± 0.01
Population growth rate	0.66 ± 0.01

Rotifer name	TYC206	TYC221
Lorica length (μm)	343.7 ± 2.1	367.8 ± 3.1
Lorica width (μm)	275.8 ± 2.1	259.5 ± 3.2
Aspect ratio	1.25 ± 0.01	1.42 ± 0.01

Rotifer name	TYC78	TYC176	TYA41
Lorica length (μm)	365.6 ± 3.7	347.7 ± 3.8	353.3 ± 3.5
Lorica width (μm)	265.1 ± 3.5	258.9 ± 3.3	261.5 ± 3.0
Population growth rate	0.72 ± 0.01	0.68 ± 0.01	0.68 ± 0.01

Figure 7. Photographs of rotifer lines with morphological or proliferative difference and the control. (a) control rotifer. (b1) and (b2) Morphological mutants with abnormal aspect ratio (TYC206, TYC221). (c1)-(c3) Proliferative mutants (TYC78, TYC176 and TYA41) with higher population growth rates than the control. Values are mean ± standard errors, and images of rotifer lines are of individuals with the average lorica lengths. Scale bars represent 200 μm.

was reported by Kim *et al.* (2018), indicating that application of the selection techniques used in this study can introduce forward genetics into the genus *Brachionus*.

Conclusion

In this study, C-ion-beam irradiation was efficiently mutagenic compared to Ar-ion-beam irradiation for the Notojima strains of *B. plicatilis* s.s. Based on our method for selecting larger mutant lines via a large-scale screening, 52 large mutant lines (mutation frequency of 4.2%) were established from 1968 irradiated individual rotifers using C-ion beams (Table 1), and 4 lines were established from 1080 irradiated individual rotifers with Ar-ion beams (mutation frequency of 0.7%), leading to a total of 56 large mutant lines (Table 1). Among them, 3 (6%) mutant lines from C-ion-beam irradiation group had ≥18% of the average lorica lengths compared to those of the controls. Moreover, C-ion beam of 200 Gy, which produced TYC78, was the optimal irradiation condition for the rotifer *B. plicatilis* s.s. (Figures 4 and 6). Three lines (TYC78, TYC176 and TYA41) were 1.1-1.2 times larger in size and had significantly higher population growth rate than the controls. Consequently, the irradiation conditions that preserved the reproductive activity and high frequency of mutant appearance were estimated at 200 Gy of C-ion- and at 50 Gy of Ar-ion-beam irradiation. Thus, these mutant lines are suitable to solve the feed size gap between rotifers and *Artemia* nauplii and should improve the marine fish larviculture system.

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Author contribution

T.K. conceived this study. K.T. and T.A. designed this study. M.K., T.K., and N.T. cultured rotifer and prepared samples for irradiation experiments. To obtain mutants strains, the heavy-ion irradiation to a wild strain was performed by K.T. and T.A., K.T., M.Y., and K.I. performed the experiments including lethality rate, population growth rate, and mutant screening. K.T., H.I., and T.A. analyzed the results and contributed to the discussions. A.H. and M.K. supervised this study. K.T. and H.J.K. wrote the manuscript with the assistance from all authors.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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