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Note

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Temperature-dependent Index of Mitotic Interval (τ_0) for Chromosome Manipulation in Korean Rose Bitterling *Rhodeus uyekii*

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Abstract

Eggs of Korean rose bitterling *Rhodeus uyekii* were collected and fertilized to observe temperature-related cleavage rates and mitotic intervals (τ_0). As the water temperature was increased, the slope of first cleavage frequency with elapsed time after fertilization increased, and approximately 30% of fertilized eggs reached first cleavage frequency at every 15 min. At higher temperatures, eggs developed faster and underwent further identical developmental processes. There were strong, negative correlations between τ_0 and water temperatures at all temperatures studied ($Y = -1.225X + 70.05$, $r^2 = 0.988$, where Y is τ_0 and X is temperature).

Key words: Mitotic interval (τ_0), Temperature-dependent, Korean rose bitterling, *Rhodeus uyekii*

Introduction

Korean rose bitterling *Rhodeus uyekii* was determined as an indigenous species of Korea by Mori (1935). Ecological characteristics of Korean rose bitterling were reported by Uchida (1939), and Korean rose bitterling was classified into the genus *Rhodeus* by taxonomic review (Kang et al., 2006). Korean rose bitterling is distributed in rivers that empty into the Korean Western sea and South sea. The spawning season of Korean rose bitterling is from March to May, and the body length of the adult fish is 4-5 cm. Body depth of Korean rose bitterling is not large, and body form has a long egg shape. The value of Korean rose bitterling as an aquarium fish is very high (Kang et al., 2005).

In addition, Korean rose bitterling has a small size, special life habits, and beautiful body color. As such, Korean rose bitterling has generated much interest in researchers. For this, detailed information on their biology, especially early life history and reproductive cycle, effects of environmental factors on reproductive cycle, elongation of the ovipositor, nuptial color component, and effects of diet on nuptial color, have begun to be explored (Ahn, 1995; Kang et al., 2005). Recently, this species has also been considered as a candidate model for Korean research project of National Fisheries Research and Development Institute to address the risks associated with aquatic living modified organisms. Therefore, the producing of sterile triploid Korean rose bitterling stocks is needed for researching DNA transformation operation of Korean rose bitterling.

Triploid Korean rose bitterling have been produced with the purpose of preventing exotic strains from contaminating the local gene pool by unwanted reproduction in natural waters (Kim et al., 1994). More importantly, triploidy will have an important role to play in the regulation of genetically modified strains of Korean rose bitterling, with regard to recent applications of recombinant DNA technology to this species (Nam et al., 1999). Also, tetraploid can providing a convenient way to

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produce large numbers of sterile triploid fish through simple interploidy crosses between tetraploids and diploids (Guo et al., 1996). For these reasons, the development of a practical and convenient method to produce sterile triploid stocks and tetraploid stocks is needed.

The production of polyploid fish (*e.g*., triploids, tetraploid) and fish with uniparental inheritance (gynogenetes) has important applications in both aquaculture and fish research (Mair, 1993). The ability to effectively manipulate ploidy through the application of suitable shocks (temperature, pressure, or chemical) early in egg development requires empirical determination of the shock's magnitude, duration, and time of application (Thorgaard, 1983). In poikilothermic species such as fish, the time of application is dependent on temperature and ploidy manipulation.

Effective, controlled releasing of the second ootid as well as the first cleavage is dependent on the type, intensity, and duration time of treatment (Thorgaard et al., 1981; Onozato and Yamaha, 1983; Thorgaard and Allen, 1987). Thus, control of first cleavage, a means of chromosome engineering, can affect enhancement of aquaculture over the short-term (Thorgaard, 1983). To produce sterile organisms, it can be applied to the induction of triploid. Further, control of first cleavage can be applied to the induction of tetraploidy, mitotic gynogenetic diploidy, and androgenetic diploidy using chromosome engineering. Therefore, to practice effective control of first cleavage, an understanding of the temperature-dependent control of first cleavage is essential (Thorgaard, 1983; Mair, 1993).

In this study, we determined temperature-related cleavage rates or mitotic intervals, measured as the "Dettlaff unit" (τ_0) for winter flounder, in order to establish efficient procedures for chromosome manipulation. The Dettlaff unit is the duration in minutes of one mitotic cycle during early synchronous embryonic cleavage, or the interval between two consecutive cell divisions (Saat and Veersalu, 1996a; Shelton et al., 1997). When measured over a range of temperatures, the relationship of τ_0 to temperature as determined by regression analysis can be used to predict developmental events that are influenced by temperature within a single species and between species with similar spawning biology (Dettlaff, 1986). To date, mitotic intervals (τ_0) have been used to estimate the optimal times for chromosome manipulation in a variety of species such as the paddlefish *Polyodon spathula*, shovelnose sturgeon *Scaphirhynchus platorynchus*, common carp *Cyprinus carpio*, tench *Tinca tinca*, Black plaice *Pleuronectes obscures*, and black crappie *Pomoxis nigromaculatus* (Flajšhans et al., 1993; Shelton and Rothbard, 1993; Mims et al., 1997; Shelton et al., 1997; Gomelsky et al., 2000; Park and Im, 2010). We determined egg development by artificial fertilization as a part of pattern industrialization and also assessed temperature-related τ_0 and cleavage rates to establish the most efficient procedures for chromosome manipulation in Korean rose bitterling.

Materials and Methods

On June 2 2011, Korean rose bitterling *Rhodeus uyekii*, 15 females and 15 males, were collected from the Inland Aquaculture Research Center of National Fisheries Research and Development Institute in Jinhae, Korea, and then transported to the Fishery Genetics and Breeding Science Laboratory, Korea Maritime University, Korea. One day after transport, we started feeding newly hatched brine shrimp, *Artemia salina* nauplii (Inve premium; Inve, Salt Lake City UT, USA) collected from aquaria to the fish every afternoon. The fish were fed a commercial artificial diet (NRD 3/5; Inve, Salt Lake City, UT, USA) as a nutritional supplement every evening. Further, 30% of the culture water in the aquarium was exchanged everyday, and water temperatures were maintained at 20 ± 0.5°C.

On June 8, 2011, eggs and sperms of Korean rose bitterling were collected from 30 females and 30 males; 40 eggs collected from 2 females and sperms collected from 2 males were artificially fertilized by a wet process. In a triplicate experiment, 200 fertilized eggs were collected from 10 females and 10 males.

To assess the temperature-dependency of the first cleavage and mitotic interval (τ_0), the water temperatures were maintained using temperature-controlled water baths set at 12, 16, 20, and 24°C. Samples were generally taken at 15 min intervals and fixed with 5% neutral formalin solution (50 mL of formalin, 3.25 g $Na_2HPO_4 \cdot 12H_2O$, 2.25 g KH_2PO_4, 950 mL DW) at 4°C before observation. We measured the diameter of 10 fertilized eggs at 50× magnification under an optical microscope (Axiostar plus; Zeiss, Germany) and microscope camera (Axiocam MR, Zeiss, Germany). This experiment was performed in triplicate.

The time of appearance of the first cleavage furrow was recorded and used as the starting point for the timing of subsequent cell divisions. The times at which approximately 10% of the developing embryos reached the two- (τ_I) (Fig. 1A) and eight-cell (τ_{III}) (Fig. 1B) stages were recorded. A value of 10% was selected according to the recommendation of Ignat'eva (1975). Mean mitotic cycle intervals (τ_0) were calculated as $\tau_0 = (\tau_{III} - \tau_I)/2$. The relationship between the mean mitotic interval and water temperature was examined by simple linear regression.

Results and Discussion

The mean diameter of the Korean rose bitterling *Rhodeus uyekii* eggs was 3.8 ± 0.04 mm, and this value was similar to that of rose bitterling and oily bitterling (Kim and Park, 1985; Suzuki and Jeon, 1988). Eggs of *Rhodeus* are ovoid, inadhesive, and demersal, and the eggs of several other species are ovoid, inadhesive, and demersal, including those of rosy bitterling *R. ocellatus* and oily bitterling *Acheilognathus Koreensis* (Kim and Park, 1985; Suzuki and Jeon, 1988). Ko-

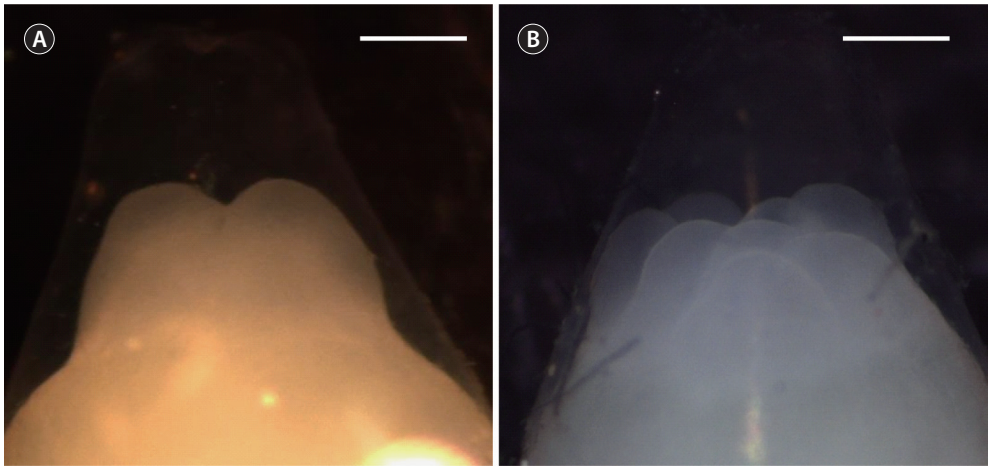

Fig. 1. External morphology of egg development in Korean rose bitterling, *Rhodeus uyekii*. (A) 2-cell stage. (B) 8-cell stage. Scale bars = 200 μm.

rean rose bitterling eggs do not contain oil globules (Park and Park, 1986).

As shown in Fig. 2, the eggs of Korean rose bitterling showed faster development at higher temperature. The times at which eggs reached the one-cell stage at 12°C, 16°C, 20°C, and 24°C were 150 min, 135 min, 120 min, and 100 min, respectively. As the water temperature was increased, the slope of the first cleavage frequency with elapsed time after fertilization increased, and approximately 30% of fertilized eggs reached first cleavage frequency every 15 min. As shown in Fig. 3, the mitotic intervals at 12°C, 16°C, 20°C, and 24°C were 52 min, 48 min, 44.5 min, and 40 min, respectively, and there were strong negative correlations between mitotic intervals (τ_0) and water temperature at all temperatures ($Y = -1.225X + 70.05$, $r^2 = 0.988$ where Y is τ_0 and X is temperature).

In an attempt to determine temperature-related cleavage rates and τ_0, we found that Korean rose bitterling eggs underwent cleavage within the temperature range of 12 to 24°C. Based on our results, we determined that Korean rose bitterling eggs showed faster development and decreased mitotic intervals with increasing water temperature, which indicates strong negative correlations between τ_0 and water temperature. Although mitotic intervals and hatching time after fertilization were different, the trend of temperature-dependent τ_0 in Korean rose bitterling is similar to those in black plaice *Pleuronectes obscures*, winter flounder *Pseudopleuronectes americanus*, far eastern catfish *Silurus asotus*, greenling *Hexagrammos otakii*, baltic herring *Clupea harengus membras*, perch *Perca fluviatilis*, and ruffe *Gymnocephalus cernuus* (Saat and Veersalu, 1996a, 1996b; Park and Im, 2001; Park and Johnson, 2002; Park et al., 2006; Park and Im, 2010).

In addition, the relationships between mitotic interval and water temperature in fish were typically curvilinear, indicating that temperatures were within the range in which the species

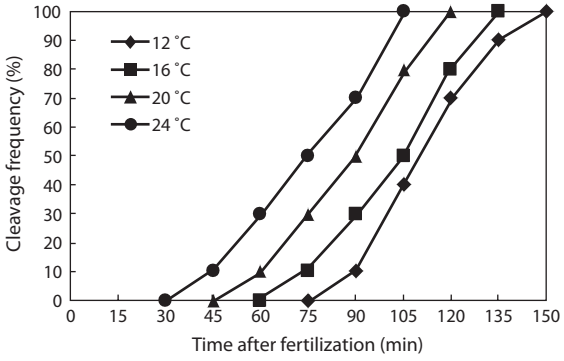

Fig. 2. The percentages of Korean rose bitterling *Rhodeus uyekii* eggs developed to anaphase of the first cleavage at four different temperatures with time.

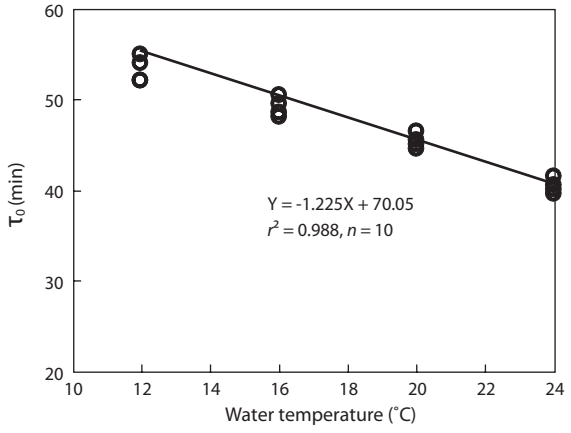

Fig. 3. Mitotic intervals (τ_0, Y) for Korean rose bitterling *Rhodeus uyekii* as functions of temperature (X). Temperatures used are within the normal range for spawning and early development for this species. Eggs from three females were fertilized with pooled sperm from one male and distributed among the temperature treatments. The experiments were performed three times.

of fish naturally spawn and develop (Saat and Veersalu, 1996a, 1996b; Park and Im, 2001; Park and Johnson, 2002; Park et al., 2006; Park and Im, 2010). The linear response of the τ_0 against temperature attained in this study is in accordance with a study on the developmental rate for black carp, winter flounder, and black plaice (Shelton and Rothbard, 1993; Park and Johnson, 2002; Park and Im, 2010). However, additional observations are needed. The available data suggest that the curves of the dependency of τ_0 on temperature are highly species-specific. The species-specificity of the development rate can be used to identify the taxonomic ranges of different fish species. Considering the identity of the mitotic events and the short time intervals (τ_0), the chromosome manipulations in Korean rose bitterling would be most efficient at temperatures between 16 and 20°C.

Therefore, this study demonstrated obvious specific and clear differences in the time of the first cleavage and mitotic intervals at different temperatures in Korean rose bitterling. Data obtained will be useful for the development of an optimal treatment protocol for chromosome manipulation. Further, data on egg development will be valuable in the form of a biological aquaculture database. The results of this study and the investigations into seeding production by artificial fertilization, induction of triploid for producing sterile organisms, and induction of tetraploid, mitotic gynogenetic diploid, and androgenetic diploid in Korean rose bitterling will aid future research.

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