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Blocking the 1st Cleavage in Mud Loach, *Misgurnus mizolepis*

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Blocking the 1st mitotic cleavage was performed in mud loach (*Misgurnus mizolepis*) using UV-irradiated cyprinid loach (*M. anguillicaudatus*) sperm and thermal shocks. Optimum UV range for inactivation of cyprinid loach sperm was between 3,150 to 4,050 ergs/mm^2. Heat shock treatment (41℃ for 3 mins) with various treatment initiation times ranged from 22 to 50 min post insemination resulted wide range of success for induced gynogenesis. Best result was obtained when haploid eggs were shocked at 28 min after insemination (corresponding to metaphase division of the 1st cleavage); 26% of total eggs inseminated were viable diploid gynogens. The hatching success and early survival of the both meiotic and mitotic gynogenetic groups were significantly lower than those of control crosses ($P < 0.05$). Maternal origin of induced gynogenetic mud loach was verified by multi-locus DNA fingerprinting.

Key words : Blocking the 1st mitosis, Induced gynogenesis, Mud loach

Introduction

Induced chromosome doubling of haploid genome by blocking the 1st cleavage allows induced parthenogenesis without any contribution of genetic materials from the other sex. This technique has been given much attention as a potential way for (1) genetic improvement of aquatic species enabling rapid establishment of highly inbred broodstock (Komen et al., 1991; Palti et al., 1997), (2) investigation of genetic sex determination mechanism of fish and production of mono-sex population (Galbreath et al., 1994; Pongthana et al., 1995), and (3) mapping genes relative to its centromeres (Thorgaard et al., 1983; Thompson and Scott, 1984; Mair, 1993).

Mud loach (*Misgurnus mizolepis*), an important species both for food and ceremonial works by Buddhists in Korea. About 4,000 MT of this species are produced in Korea every year both by culture and capture (Kim et al., 1994), and the domestic market for mud loach rapidly expands in recent years. Although this species has been a subject for many genetic manipulations including triploidy (Kim et al., 1994), interspecific hybridization (Kim et al., 1995), sex control (Kim et al., 1997; Nam et al., 1998), and recently transgenesis (Nam et al., 1999), no study for development of techniques of blocking the 1st cleavage for induced gynogenesis has been reported yet. The combining of this technique and such genetic manipulations would have important implications, with respect to the establishment of valuable isogenic lines with desired characteristics.

The objective of this study is to develope an

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optimized protocol for blocking the 1st mitotic division in mud loach in order to evaluate the potential usefulness of inbred mud loach lines.

Materials and Methods

Fish and gamete collection

The gametes were obtained from the brood-stock maintained in Fish Genetic Engineering laboratory, Pukyong National University. Induced spawning were performed using IP injections of HCG (6-8 IU/g body weight for mud loach females and 2 IU/g body weight cyprinid loach males) as described previously (Kim et al., 1995).

Inactivation of the sperm and induction of gynogenetic haploid

The sperm from cyprinid loach (*M. anguillicaudatus*) was diluted 1:10 with ice-cold 0.85% NaCl solution and irradiated with various dosages of UV irradiation (0 to 9,000 ergs/mm^2). An aliquot of 330 eggs was mixed with each lot of sperm (200 $\mu\ell$) and activated by adding water. The moment of water addition was taken as the insemination (fertilization) time. The optimal amount of UV was determined by examining both the incidence of haploidy and survival of embryo at 24 hours post insemination (just before hatching). Each treatment had four replicate groups.

Mitotic diploid gynogenesis

The effect of heat shock applied at different phases of the first cleavage was also monitored. The eggs inseminated with UV-irradiated sperm (3,870 ergs/mm^2) were incubated at 25℃ and heat-shocked (41℃ for 3 min) at 2-min intervals between 22 and 50 min after fertilization. Hatching success and early survival up to the 1st feeding were evaluated as described above with 5 replicated groups for each treatment. The phase of the first cleavage showing the best yield was located by histological examination of one-celled embryos. The developing embryos were fixed in Bouins fluid. Groups of 50 eggs from each sample were analyzed using conventional histological techniques.

Flow cytometry

To confirm the successful restoration of diploidy, ploidy of presumptive gynogenetic individuals was checked using WinBryte HS flow cytometer (BioRad, USA). The cell suspensions (from blood cells or larval cells) were prepared according to protocol provided by manufacturer. For blood samples, 3-5 $\mu\ell$ of heparinized whole blood (about 1.5 $\times$ 10^6 cells) were directly added to reconstituted Kinesis-50 kit (BioRad) containing 50 μg/mℓ propidium iodide (PI), and incubated for 1 hour at 4℃ in the dark. For larval cells and for small fry, the samples was homogenized in PBS (pH 7.8), followed by passages through G26 gauge needle and 15 μm mesh, and then stained with PI as described above. Relative DNA amount was estimated using the human white blood cell as an internal control.

Multi-locus DNA fingerprinting

Quality control and verification of induced gynogenesis were performed based on comparing multi-locus DNA fingerprint profiles of parents and randomly taken gynogenetic progenies. Genomic DNA was isolated from whole blood using conventional SDS/proteinase K method as described by Nam et al. (1999). Samples were digested in a solution of 50 mM

Tris, 5 mM EDTA, pH 8.0, 150 mM NaCl, 0.5% SDS, 200 μg/mℓ proteinase K at 50℃ for several hours to overnight, and DNA was purified with TE (10 mM Tris, 1 mM EDTA pH 8.0)-saturated phenol (pH 8.0) and phenol/chloroform (1:1) extractions. Five μg of purified DNA was digested with Hinf I (10 unit) for 12 hours and separated on 0.9% agarose gel. The gel was de-purinated by 0.2 N HCl for 10 min, denaturated by 1.5 M NaCl and 1 N NaOH for 45 min and then neutralized by 1 M Tris pH 8.0 and 1.5 M NaCl for 45 min. The DNA in gel was transferred to a positively charged nylon membrane (Boehringer Mannheim, Germany) using a capillary method under 20X SSC, and fixed with UV cross linker. The membrane was probed with $(GACA)_4$ oligonucleotide that had been labeled with digoxygenine-11-dUTP (Synthetic Genetics Inc., USA). Prehybridization, hybridization and stringent washes were also carried out according to the procedures as described by Gross et al. (1994). Detection of hybridized signals was performed with a non-isotopic labeling and detection kit according to the manufacturers recommendation (BM, Germany).

Statistics

The differences in fertilization, survival of embryo, hatching success, early survival of hatched larvae and yields of gynogens were assessed by ANOVA test. The difference was considered to be significant at $P < 0.05$.

Results

Hertwig effect and induction of gynogenetic haploidy

Survival of embryo at 24 hours post insemination (just before hatching) and incidence of haploidy were quite different depending on the amount of UV irradiated with typical Hertwig effect. The optimum UV range was between 3,150 to 4,050 ergs/mm^2 (Fig. 1) in which more than 60% haploid embryos could be obtained from the total eggs inseminated. Most, but not all, haploid fish could also hatch but died before the 1st feeding. Exceptionally a few fish from haploid groups survived after 3 days post hatching, but all of them didn't take the feed supplied and died within 5 days after hatching. Haploid fish showed typical haploid syndrome such as distorted body and short underdeveloped tails (photographs not shown).

Inhibition of the 1st mitotic division by heat shock

Hatching success and yield of viable gynogen (estimated as percentage of survived fish up to the 1st feeding out of total eggs inseminated) were strongly affected by different initial treatment timings of heat shock treatment for blocking the 1st mitotic division. Two peaks in both percent hatching and yield of gynogen

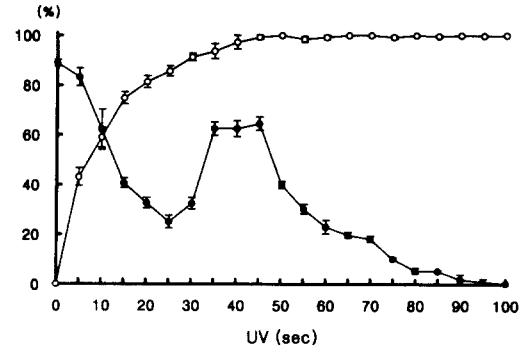

Fig. 1. Mean survival at 24 hours post insemination (closed circles) and haploid incidence (open circles) of mud loach eggs inseminated with UV-irradiated cyprinid loach sperm. The amount of UV per second is 90 ergs/mm^2.

were found in treated groups; 28 min and 42 min after insemination. The group treated with 28 min of initial treatment time showed the best result: the overall yield was 26.2% of viable diploid gynogen out of total eggs inseminated (Fig. 2). The histological location of one-celled embryos revealed that the phase showing the highest yield (28 min post insemination) was corresponding to metaphase stage of the 1st cleavage (Fig. 3).

Flow cytometry

The flow cytometry successfully revealed the ploidy levels of presumptive gynogenetic haploid and diploid individuals. The mean channel numbers of gynogenetic haploid and diploid group were 21.1±1.0 and 42.3±1.7, respectively. It was clearly matched to expected genome size of haploid (1.4 pg/cell) and diploid mud loach (2.8 pg/cell), respectively (Blacklidge, 1997) (Fig. 4).

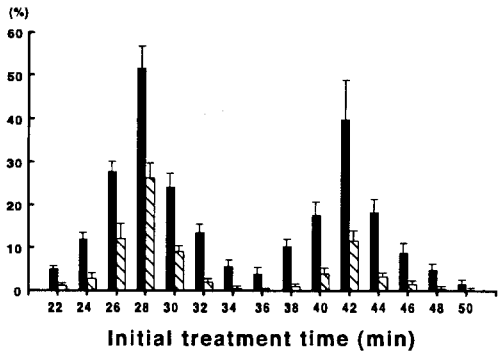

Fig. 2. Effects of various initial treatment times for blocking the first cleavage division by heat shock (41℃ for 3 min) on hatching success (black bars) and yield of gynogenetic diploids (gray bars). The yield of gynogenetic diploids was estimated as percentage of surviving fish up to the 1st feeding out of total eggs inseminated. Each bar represent the mean of 5 replicates and the standard deviations are indicated by T bars.

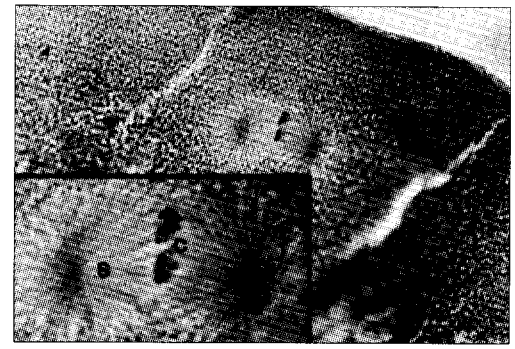

Fig. 3. Histological location of one-celled embryo at 28 mins post insemination at 25℃. C, chromosomes; S, spindle fiber; SP, spindle pore.

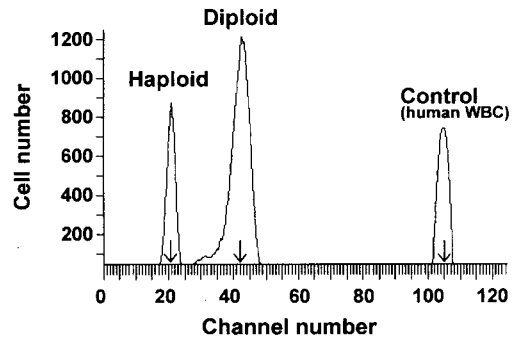

Fig. 4. Histograms showing the DNA content profiles of gynogenetic haploid, diploid and human white blood cell control, as revealed by flow cytometric analysis.

Multi-locus DNA fingerprint profiles of gynogens

A number of loci were detected with the multi-locus DNA fingerprinting $(GACA)_4$ probe in combination with the restriction enzyme Hinf I. In general, there was concordance in DNA fingerprint profiles between the mother and her gynogenetic progenies; most clearly identifiable bands in gynogenetically derived progenies were observed in the mother. No notable indication of paternal transmission was found in any meiogynes and mitogynes. Several putative diagnostic bands were also detected (Fig. 5).

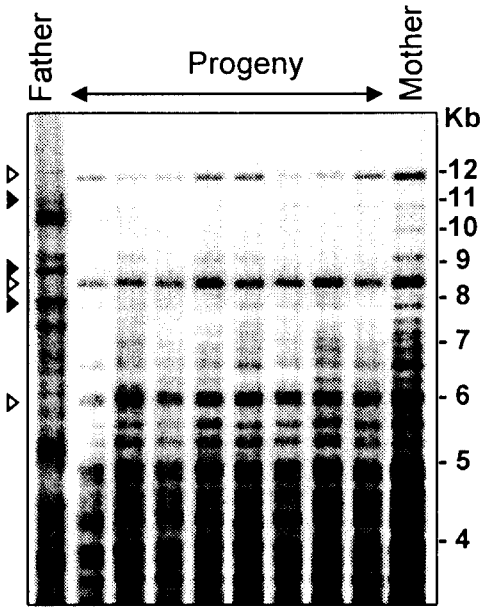

Fig. 5. Multi-locus DNA fingerprint profiles of gynogenetic progeny and their parents. Closed and open arrow heads indicate the putative diagnostic bands from father and mother parents, respectively.

Discussion

The optimum amount of UV for inactivation of cyprinid loach sperm in present study was from 3,150 to 4,050 ergs/mm^2, which is different with the result of previous report on this species (Suzuki et al., 1985; Arai et al., 1993). It might be probably due to the preparation and dilution of sperm, or to the different treatment conditions including UV irradiating equipment. The morphology of haploid mud loach is similar to that of haploid cyprinid loach previously described (Suzuki et al., 1985).

The present treatment of heat shock (41℃ for 3 min) was reliable for producing viable diploid mitotic gynogens of mud loach, although fine-tuning of heat treatment such as duration and temperature is remained to be studied. The initial treatment timing was critical for success of gynogen production via heat shock, as in the cases of many previous reports (Komen et al., 1991; Cherfas et al., 1993 & 1994; Gomelsky et al., 1998). Histological location of the most effective phase during the 1st mitosis for heat shock indicated that the period was metaphase of the 1st cleavage. Our observation coincides with the earlier ones of common carp (prometaphase to metaphase, Komen et al., 1991) and ornamental koi carp (metaphase, Cherfas et al., 1994). The bimodal patterns (28 and 42 min after fertilization) observed in our heat shock treatment might reflect the impact on the karyokinesis and cytokinesis during the 1st cleavage. In the present study, blocking the karyokinesis was more effective than that of cytokinesis, which is similar to previous observations made in the carps (Komen et al., 1991; Cherfas et al., 1993).

The status of the gynogenetic progeny was successfully verified with multiple-locus DNA fingerprinting and several potential diagnostic bands effectively proved the gynogenetic status of both meiogynes and mitogynes. It indicated no observable contribution from male parent. The evaluation of the success of gynogenesis using DNA fingerprinting has been reported in tilapia (Carter et al., 1991) and also in African catfish (Volckaert et al., 1994). Further research will be made to evaluate the performances and reproductive physiology of gynogens produced by blocking the 1st cleavage in order to examine their potential usefulness for establishment of clonal lines.

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References

Arai, K., K. Matsubara and R. Suzuki, 1993. Production of polyploids and viable gynogens using spontaneously occurring tetraploid loach, *Misgurnus anguillicaudatus*. Aquaculture, 117 : 227-235.

Blacklidge, K. H., 1997. Genome quantitation and analysis in fishes. Ph. D. thesis, Purdue University, Indiana. pp.74.

Carter, R. E., G. C. Mair, D. O. F. Skibinski, D. T. Parkin and J. A. Beardmore, 1991. The application of DNA fingerprinting in the analysis of gynogenesis in tilapia. Aquaculture, 95 : 41-52.

Cherfas, N. B., G. Hulata and O. Kozinsky, 1993. Induced diploid gynogenesis and polyploidy in ornamental (koi) carp, *Cyprinus carpio* L. 2. Timing of heat shock during the first cleavage. Aquaculture, 111 : 281-290.

Cherfas, N. B., Y. Peretz, N. Ben-Dom, B. Gomelsky and G. Hulata, 1994. Induced diploid gynogenesis and polyploidy in the ornamental (koi) carp *Cyprinus carpio* L. 3. Optimization of heat-shock timing during the 2nd meiotic division and the 1st cleavage. Theor. Appl. Genet., 89 : 281-286.

Galbreath, P. F., W. St. Jean, V. Anderson and G. H. Thorgaard, 1994. Freshwater performance of all-female diploid and triploid Atlantic salmon. Aquaculture, 128 : 41-49.

Gomelsky, B., N. B. Cherfas, A. Gissis and G. Hulata, 1998. Induced diploid gynogenesis in white bass. Prog. Fish Cult., 60 : 288-292.

Gross, M. L., A. R. Kapuscinski and A. J. Faras, 1994. Nest-specific DNA fingerprints of smallmouth bass in lake Opeongo, Ontario. Trans. Am. Fish. Soc., 123 : 449-459.

Kim, D. S., J. -Y. Jo and T. -Y. Lee, 1994. Induction of triploidy in mud loach (*Misgurnus mizolepis*) and its effect on gonad development and growth. Aquaculture, 120 : 263-270.

Kim, D. S., Y. K. Nam and I. -S. Park, 1995. Survival and karyological analysis of reciprocal diploid and triploid hybrids between mud loach (*Misgurnus mizolepis*) and cyprinid loach (*Misgurnus anguillicaudatus*). Aquaculture, 135 : 257-265.

Kim, D. S., Y. K. Nam and J. -Y. Jo, 1997. Effect of oestradiol-17β immersion treatments on sex reversal of mud loach, *Misgurnus mizolepis* (Gunther). Aquaculture Res., 28 : 941-946.

Komen, J., A. B. J. Bongers, C. J. J. Richter, W. B. van Muiswinkel and E. A. Huisman, 1991. Gynogenesis in common carp (*Cyprinus carpio* L.). II. The production of homozygous gynogenetic clones and F1 hybrids. Aquaculture, 92 : 127-142.

Mair, G. C., 1993. Chromosome-set manipulation in tilapia techniques, problems and prospects. Aquaculture, 111 : 227-244.

Nam, Y. K., C. H. Noh and D. S. Kim, 1998. Effect of 17α-methyltestosterone immersion treatments on sex reversal of mud loach, *Misgurnus mizolepis*. Fisheries Sci., 64 : 914-917.

Nam, Y. K., C. H. Noh and D. S. Kim, 1999. Transmission and expression of an integrated reporter construct in three generations of transgenic mud loach (*Misgurnus mizolepis*). Aquaculture, 172 : 229-245.

Palti, Y., J. J. Li and G. H. Thorgaard, 1997. Improved efficiency of heat and pressure shocks for producing gynogenetic rainbow trout. Prog. Fish Cult., 59 : 1-13.

Pongthana, N., D. J. Penman, J. Karnasuta and B. J. McAndrew, 1995. Induced gynogenesis in the silver barb (*Puntius gonionotus* Bleeker) and evidence for female homogamety. Aquaculture, 135 : 267-276.

Suzuki, R., T. Oshiro and T. Nakanishi, 1985. Survival, growth and fertility of gynogenetic diploids induced in cyprinid loach, *Misgurnus anguillicaudatus*. Aquaculture, 48 : 45-55.

Thompson, D. and A. P. Scott, 1984. An analysis

of recombination data in gynogenetic diploid rainbow trout. Heredity, 53 : 441-452.

Thorgaard, G. H., F. W. Allendorf and K. L. Knudsen, 1983. Gene-centromere mapping in the rainbow trout: high interference over long map distances. Genetics, 103 : 771-783.

Volckaert, F. A. M., P. H. A. Galbusera, B. A. S. Hellemans, C. Van den Haute, D. Vanstaen and F. Ollevier, 1994. Gynogenesis in the African catfish (*Clarias gariepinus*). I. Induction of meiogynogenesis with thermal and pressure shocks. Aquaculture, 128 : 221-233.