博　士（水産科学）　ヤスイ　ジョージ　シゲキ

学 位 論 文 題 名

Studies on restoration of the loach *Misgurnus anguillicaudatus* from cryopreserved sperm and blastomeres by androgenesis and germ-line chimera

（ドジョウにおける精子、割球の凍結保存と
雄性発生、生殖系列キメラを介した個体復活に関する研究）

学位論文内容の要旨

Genebanking is of importance for both farmed and wild species as a means to preserve endangered populations and reconstitute unique genotypes. In fish, a large number of species or strains are now being endangered, and farmed stocks are derived from few genotypes from wild populations. Thus, germplasm preservation may represent a useful tool to increase genetic diversity in broodstock.

Liquid nitrogen cryopreservation is effective for long-term genebanking, but is only applicable for sperm preservation in most species. Cryopreserved sperm is useful for breeding programmes and maintenance of genetic diversity, but the reconstitution of already extinct fish using cryopreserved sperm is very difficult at present because sperm gives rise to inviable haploid progeny after induced androgenesis. Moreover, maternally inherited genotypes and cytoplasmic mitochondrial DNA are not preserved by sperm cryopreservation and thus never restored by androgenesis.

Blastomeres may be also applicable for genebanking because fish genotypes may be reconstituted by a germ-line chimera. Target genotypes may be reconstituted by this method because the host should produce donor gametes in the germ-line chimeras. So, cryopreservation of blastomeres followed by production of germ-line chimeras is considered a useful repository strategy. Cryopreservation of blastomeres was achieved for a number of teleosts, but the successful production of germ-line chimeras from cryopreserved samples has not been achieved yet.

The loach (*Misgurnus anguillicaudatus*) is a fresh-water fish widely distributed in Japan, and the species includes unusual genotypes such as gynogenetically reproducing diploid clones and some polyploids in nature (Arai, 2001, 2003). These fish are interesting for aquaculture as well as academic research, what emphasizes genebanking.

Previously, many technologies including induced androgenesis and production of germ-line chimeras were developed in loach. These procedures may facilitate the restoration of fish genotypes

from cryopreserved sperm and blastomeres, but there is no protocols for cyopreservation in the loach. The objective of the present study was to develop practical protocols for cryopreservation of sperm and blastomeres of the loach, and successful reconstitutution of genotypes through production of androgenenotes and germ-line chimeras from cryopreserved samples.

Cryopreservation of sperm: In this study, a protocol for sperm cryopreservation of loach was optimized by evaluating inseminating dose, thawing temperatures (20, 25 and 30°C for 10s), extenders (loach or cyprinid extenders), internal cryoprotectants (Dimethyl sulfoxide - DMSO, Dimethylacetamide - DMA, glycerol - Gly, ethylene glycol - EG, and methanol – MeOH at 0, 5, 10 and 15%), external cryoprotectants (bovine serum albumin 1 and 2%; sucrose 0.5 and 1%; glucose 0.5 and 1%; glycine 0.5 and 1%). Samples were cryopreserved by slow cooling (-33.3 ± 2.09°C/min) using the cooling tubes method (Yasui *et al.*, 2008). Post-thaw sperm motility was also optimized by activating solutions (distilled water, dechlorinated tap water, 25 mM NaCl and 50 mM NaCl), and finally evaluated by examining the hatchability of the eggs when fertilized with fresh or cryopreserved sperm. After the evaluation of these parameters, sperm cryopreservation was optimized using the following procedure: thawing temperature at 25°C for 10 s; loach or cyprinid extenders; methanol at 10 or 15% as internal cryoprotectants; glycine 0.5% or bovine serum albumin 1% as external cryoprotectants and 50mM NaCl for sperm activation. Using this procedure, the fertilizability of the post-thawed sperm was 47% in comparison to the fresh sperm, at the minimum inseminating dose (687.65 sptz $egg^{-1}mL^{-1}$). Based on this protocol, sperm from other loach species *Lefua nikkonis, M. mizolepis* and *Barbatula toni* were cryopreserved successfully, and the post-thaw motility were 58.33 ± 7.6; 63.3 ± 5.7 and 60.0 ± 5.0, respectively (Yasui *et al.*, 2008, 2009).

Androgenesis using cryopreserved diploid sperm: The aim of this study was to evaluate the feasibility of cryopreserved diploid sperm as a repository genebank for the loach, along with a restoration strategy utilizing induced-androgenesis. Firstly, we evaluated three types of media for egg inactivation: Hank's saline solution + 0.5% bovine serum albumin (BSA), Ringer's solution + 0.5% BSA, and masu salmon seminal plasma. Haploid and diploid sperm were taken from diploid and tetraploid loaches, respectively, and then cryopreserved using our previous protocol (see above). Fresh and cryopreserved haploid or diploid sperm were then used to fertilize intact or UV-irradiated eggs from wild diploid females. The irradiation media evaluated here successfully maintained the egg quality over 2 hours, but Hank's saline showed improved storage ability and was used for irradiation. Using fresh haploid sperm, the fertilization rate was 63.51 ± 10.68%, decreasing to 28.80 ± 20.06% when irradiated eggs were employed. These progenies were diploid (2n) and haploid (1n), respectively, as confirmed by flow cytometry. For fresh diploid sperm, the fertilization rate was 53.20 ± 2.71% for normal eggs and 34.92 ± 27.12% for inactivated eggs, with respective ploidy status of 3n and 2n. When cryopreserved haploid sperm was used to fertilize normal eggs, a fertilization

rate of 41.83 ± 17.88% was recorded, with diploid progeny. The same sperm source resulted in a fertilization of 28.35 ± 15.01% and haploid progeny when inactivated eggs were utilized. Cryopreserved diploid sperm resulted in triploid progeny and fertilization rate of 21.33 ± 7.14% when normal eggs were used. Using the same sperm source, the fertilization rate of UV-irradiated eggs was only 11.68 ± 6.74% and diploid progeny was obtained. In this putative androgenetic progeny from cryopreserved sperm, all-male inheritance was confirmed by determination of larval morphology and microsatellite genotyping, demonstrating successful reconstitution of the loach from cryopreserved diploid sperm (see Yasui *et al.*, 2010).

Cryopreservation of blastomeres and production of germ-line chimera: An efficient procedure for cryopreservation of loach blastomeres followed by restoration through germ-line chimera formation was established in this study. Blastomeres of the loach were cryopreserved in 250 μL straws in Eagle's minimum essential medium (MEM) with various concentrations of dimethyl-sulfoxide (DMSO; 0, 5, 10, 15 and 20%), and the best concentration was combined with glycerol (1, 2 and 4%) and external cryoprotectants (1 or 2% sucrose; 2, 5 or 10% fetal bovine serum; 1 and 2% bovine serum albumin). Post-thaw viability of the blastomeres was used to optimize cryopreservation conditions. The best post-thaw results indicated that cryopreservation of blastomeres was optimized using 10% DMSO as the cryoprotectant, giving rise to a viability of 65.1 ± 0.6%. This result is lower (P<0.0001) when compared to fresh blastomeres (96.8 ± 0.8%). Later, donor blastomeres were injected with zebrafish GFP-*nos1* 3'UTR mRNA and biotin dextran prior to cryopreservation in the optimal freeze medium. Host embryos were injected with zebrafish DSRed-*nos1* 3'UTR mRNA and reared to the blastula stage. Donor blastomeres were then thawed at 25°C for 10 s and transplanted to the host embryos either immediately or after incubation for 16h at 20°C. Donor and host primordial germ cells (PGCs) migration were visualized with fluorescent imaging during the early stages of embryogenesis, and also by histology in 4-day old embryos. Transplantation of blastomeres immediately after thawing gave lower hatching rates (~3%) and generated a low percentage of germ-line chimeras (~1.1%). In contrast, incubation of cryopreserved sample for 16h followed by transplantation of the GFP-positive blastomeres improved the hatching rate to 90%, and successfully produced presumable germ-line chimeras at a rate of 16.5%. The improved survival rates and germ-line chimerism may be an effective method for genebanking and subsequent reconstitution of endangered fish genotypes (see Yasui *et al.*, accepted).

Conclusion: Protocols for cryopreservation of loach sperm and blastomeres were established in this study. In addition, the loach was reconstituted by induced androgenesis from cyopreserved diploid sperm and by a germ-line chimera. Both reconstitution procedures showed improved survival rates. It was also demonstrated that the new procedure of incubation of post-thaw blastomeres improved survival and production of germ-line chimeras. Sex ratios and gamete maturation of the

reconstituted fish are important characteristics to be studied in later experiments. Despite the potential application for other fish species, several intrinsic characteristics must be examined such as the possibility of induced spawning, genetic inactivation of the egg, the ability to dechorionation, resistance to micromanipulation and other traits.

学位論文審査の要旨

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Studies on restoration of the loach *Misgurnus anguillicaudatus* from cryopreserved sperm and blastomeres by androgenesis and germ-line chimera

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魚類において、多くの野生種の絶滅が危惧され、人工種苗生産による集団の遺伝的多様性低下も懸念される。遺伝資源保存は多様性を維持するための一つの手段となる。液体窒素を用いた精子の長期凍結保存は多くの魚種で開発され、育種計画と種苗生産に応用されてきた。しかし、人為雄性発生による精子からの個体復活は技術的に染色体倍加が難しく、ミトコンドリアＤＮＡを含む母系遺伝子型は保存・復活できない。胚の割球が保存できれば、生殖系列キメラの作出を介して、遺伝資源を復活しうる可能性があるが、割球凍結保存については研究例が乏しく、凍結した材料から生殖系列キメラを作出した成功例も限られている。本研究では、ドジョウを材料に精子、割球凍結保存の好適条件を明らかにするとともに、凍結保存精子を用いた雄性発生と凍結保存割球を用いた生殖系列キメラ作成による個体復活の諸条件を明らかにし、以下の評価すべき成果を得た。

（１）ドジョウの精子凍結保存の好適な方法としては、ドジョウ用あるいはコイ科魚類用の希釈液、細胞膜透過型(内部)耐凍剤として10あるいは15%メタノール、細胞膜非透過型(外部)耐凍剤として0.5%グリシンあるいは１%牛血清アルブミン(BSA)を用いて凍結保存液を調整すること、解凍条件を 25℃10 秒間とすること、媒精液として 50mMNaCl を用いること、−33.3±2.09℃/分の冷却速度とすることが推奨された。この条件で液体窒素を用いて凍結保存したとこ

ろ、新鮮精子に対して47%の受精率が実現でき(精子量687.65精子/卵/ml)た。また、本条件により他のドジョウ類(カラドジョウ、フクドジョウ等)の精子凍結保存が可能であった。

（2）雄性発生のための卵の保存液として、①Hank's + 0.5%BSA、②リンゲル液+0.5%BSA、③サクラマス精漿を検討したところ、いずれも2時間以上卵質を保持したが、①が最良であった。そこで、①液に卵を入れ、紫外線照射を行い卵核の遺伝的不活性化を試みた。そして、無処理卵、ＵＶ照射卵に、半数体あるいは二倍体の生鮮、凍結保存精子を媒精し、生じる胚の発生成績を調べた。実験に用いた卵と半数体精子は野生型二倍より、二倍体精子はネオ四倍体雄より得た。前者は函館近郊より採集し、後者は野生型二倍体雌の卵に自然四倍体(原産地不明、市場標本)の二倍性精子を受精後、第二極体放出阻止により作成したものである。フローサイトメトリーとマイクロサテライトマーカー型分析により、生鮮・凍結保存半数体精子と無処理卵の受精からは二倍体子孫が、ＵＶ照射卵の受精からは雄性発生半数体が、生鮮・凍結保存二倍体精子と無処理卵の受精からは三倍体子孫が、ＵＶ照射卵の受精からは雄性発生二倍体が生じたことが判明した。すなわち、凍結保存二倍体精子を用いた人為雄性発生により、生存性を有する二倍体ドジョウの個体復活が実現できることが示された。

（3）生殖系列キメラを介した復活に向けて、ドジョウの胚細胞(割球)の凍結保存のための好適条件を検討した。その結果、glycerol、BSA、牛胎児血清等の細胞膜非透過型耐凍剤を一切使わず、10%DMSOを細胞膜透過型の耐凍剤として、割球を250μℓストロー中のMEM培地に入れ液体窒素で凍結する条件が生存率65.1%（生鮮割球生存率96.8%）の良好な成績を与えた。ドナーの胚にゼブラフィッシュのGFP-*nos1* 3'UTRmRNAとbiotin dextranを顕微注射し、その後、凍結保存した。ホストの胚にはDSRed-nos1 3'UTR mRNAを顕微注射し、蛍光の違いによりホスト由来、ドナー由来の始原生殖細胞(PGCs)を区別可能とした。25℃10秒凍結割球を解凍し、その直後あるいは20℃で16時間培養後、ホスト胚に移植した。蛍光実体顕微鏡観察と組織学的観察から、移植したPGCsはホストの生殖隆起領域に移動、到達することが判明し、その観察の結果、解凍直後の移植(孵化率～3%；生殖系列キメラ作成率～1.1%)に比べて、培養後の移植により、より高い90%の孵化率と16.5%の生殖系列キメラ作成率が得られることが判明した。すなわち、凍結割球から得たPGCsの解凍・培養後の移植により、ドジョウ個体の遺伝子型の復活が可能なことが示された。

申請者による以上の研究成果は、魚類をはじめとする水産動物の遺伝資源の保存と復活に寄与する、基礎生物学および水産育種学上重要な成果と評価でき、審査員一同は、申請者が博士（水産科学）の学位を授与される資格のあるものと判定した。