DNA from Cryopreserved Fish Sperm

Lynn Pittman-Cooley and Terrence R. Tiersch

Introduction

As cryopreservation of fish sperm becomes increasingly applied, it creates new opportunities for genetic study of fishes. However, methods for the isolation of DNA from cryopreserved fish sperm have received only limited study (e.g. Cummings and Thorgaard 1994). Procedures for cryopreservation of fish sperm involve the use of cryoprotectant molecules and specific cooling rates to control the formation of ice crystals and the level of dehydration in cells during freezing. It is important to evaluate the effect of cryopreservation procedures on the purity and yield of DNA from fish sperm with respect to the long-term value of samples stored in germplasm repositories.

Cryopreserved sperm can be used for artificial spawning, and genetic analysis of frozen samples would be useful in marker-assisted broodstock selection for genetic improvement of aquaculture species. Cryopreserved sperm allows conservation of genetic resources in endangered species and control of genetic diversity in artificial propagation programs. Identification of rare alleles in frozen sperm could provide genetic markers for monitoring of stock enhancement programs in wild fishes. Isolation of DNA from cryopreserved sperm offers verification by genetic analysis of the source of sperm from high-value fish such as koi carp *Cyprinus carpio* that can be worth thousands of dollars apiece based on coloration and markings.

Therefore, this study was designed to develop procedures for DNA isolation from fish sperm cryopreserved for use in fertilization (Pittman-Cooley and Tiersch, 1999). Our objectives were to: 1) evaluate the effect of cryoprotectants, freezing rate, and storage temperature on the purity and yield of DNA isolated from cryopreserved sperm of channel catfish *Ictalurus punctatus*; 2) evaluate utility of the isolated DNA for use with the polymerase chain reaction (PCR), and 3) evaluate DNA isolation procedures for use with cryopreserved sperm of other fishes.

Sperm Collection and Refrigerated Storage

Sperm were manually stripped from four species of fish: spotted seatrout *Cynoscion nebulosus*, black drum *Pogonias cromis*, koi carp, and a federally listed endangered species, the bonytail chub *Gila elegans*. Because sperm cannot be stripped from channel catfish, testes were surgically removed and crushed. Sperm were suspended in Hanks' balanced salt solution (HBSS) prepared with reagent-grade chemicals (Sigma Chemical Company, St. Louis, Missouri) (Tiersch et al. 1994). Because fish sperm become motile when diluted in water, the osmotic pressure of solutions was measured by vapor pressure osmometer (model 5500, Wescor Corp., Logan, Utah) and the osmolality was adjusted to levels (Bates et al. 1996) that prevented activation of sperm in the freshwater species (300 mOsmol/Kg) and marine species (200 mOsmol/Kg) studied. For estimation of sperm motility, 2 µL of sperm were activated

Pittman-Cooley, L. and T. R. Tiersch. Isolation of DNA from Cryopreserved Fish Sperm. In: *Cryopreservation in Aquatic Species*. Tiersch, T. R. and P. M. Mazik, Editors. World Aquaculture Society, Baton Rogue, Louisiana. Pp. 310-313.

with 20 µL of distilled or salt water, and percent motility was estimated using dark-field microscopy (100-X). Only sperm samples with >50% motility were used for study.

Cryopreservation

Sperm samples from channel catfish (0.5 mL) were stored fresh at 4 °C (without cryoprotectant), or frozen at -20 °C (without cryoprotectant) or at -196 °C (with or without cryoprotectant). Samples receiving cryoprotectant were suspended in 10% solutions (final concentration) of DMSO (Sigma), glycerol (Sigma), or methanol (Fisher Scientific, Pittsburgh, Pennsylvania) and frozen at in 0.5-mL straws (IMV International Corp., Minneapolis, Minnesota) sealed with polyvinylchloride powder (IMV). An equivalent amount of sperm (1.6×10^7 cells) contained in testicular tissue (calculated as 8 mg by weight) was placed in screw-capped cryogenic tubes (2.0 mL) and plunged directly into LN_2 as a control treatment for storage without HBSS. Sperm were stripped from the four other species and frozen in 0.5-mL straws using procedures established previously for each species in our laboratory (e.g. Wayman et al. 1996, 1997) and were stored in LN_2. Sperm samples were thawed by placing straws directly into a water bath (40 °C) for 7 sec (Christensen and Tiersch 1996). Motility was evaluated within 5 min after thawing.

DNA Isolation

For isolation of DNA, sperm were lysed with 3% b-mercaptoethanol (Amresco, Solon, Ohio) and 2% SDS (Sigma) at 37 °C for 30 min. Samples of DNA were extracted using a mixture of phenol, chloroform and isoamyl alcohol (25:24:1) (Amresco), followed by another chloroform extraction. Proteinase K (Amresco) was added to a final concentration of 100 µg/mL, and samples were incubated at 50 °C for 1 to 3 hr. The DNA samples were extracted with phenol and chloroform, extracted again with chloroform, precipitated by adding 0.1-X volume of 3M sodium acetate (EM Science, Gibbstown, New Jersey) and 0.7-X volume of isopropanol (Mallinckrodt, Chesterfield, Missouri). Samples were centrifuged at 10,000 x g for 10 min, and pellets were resuspended in 100 µL of TE buffer composed of 10 mM Tris (Sigma) and 0.1 mM EDTA (Amresco). All samples were suspended at the same final volume (100 µL), and therefore yields were compared using concentration values. Absorbance was measured for DNA samples at 260 nm and 280 nm to determine yield (concentration) and purity.

Polymerase Chain Reaction

Analyses were performed with primers developed from channel catfish DNA, targeting the CH4 exon of the channel catfish gene encoding the constant region of the immunoglobulin M heavy chain (Thongpan et al. 1997). Primers were designed from the published DNA sequence (Wilson et al. 1990), and the expected size of the band amplified by PCR was 303 base pairs (bp). DNA samples were amplified using a Model PTC-100 thermal cycler (MJ Research, Watertown, Massachusetts). Reaction mixtures (100 µL) consisted of 0.5 mg template DNA, 200 mM of each dNTP, 0.3 mM of the CH4 primers, 1.2-X reaction buffer, and 4 units (1 µL) of Vent® polymerase (New England

Biolabs, Beverly, Massachusetts). Samples were initially denatured at 95 °C for 5 min, followed by 30 cycles of: denaturing at 95 °C for 30 sec, annealing at 61 °C for 30 sec, and extending at 72 °C for 1 min. A final extension step of 5 min at 72 °C was performed. Products of PCR were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide (0.5 mg/mL), visualized on an UV transilluminator (Model 3-3500, Fotodyne, New Berlin, Wisconsin) and photograghed. Polymerase chain reaction was not conducted on species other than channel catfish.

Statistical Analysis

Two separate analyses were performed. The first compared samples stored at three temperatures: refrigerated at 4 °C, or frozen at -20 °C or at -196 °C. The second analysis addressed comparisons among the cryopreserved samples only (-196 °C storage). These treatments included samples stored in 10% DMSO, 10% methanol, 10% glycerol, and a control group to which no cryoprotectant was added. One-way analysis of variance (Excel 5.0, Microsoft Corporation, Redmond, Washington) was used to test for differences in the amount or purity of DNA isolated. Duncan's multiple range test was used for pair-wise comparisons. The level for statistical significance was set at $P < 0.05$.

Results and Discussion

The concentration of DNA obtained from samples of fresh sperm was significantly higher than that obtained from the different freezing treatments. There was large variation overall in DNA concentration (range: 16 to 811 mg/mL), but even the samples with the lowest DNA concentrations (e.g. <50 mg/mL) yielded successful PCR amplification. Six of the lowest nine concentrations were from the groups cryopreserved in DMSO or glycerol. It remains to be determined if this is related to the observation that methanol is superior to DMSO and glycerol as a cryoprotectant for channel catfish sperm (Tiersch et al. 1994). The reason for the decreased yield from cryopreserved sperm in relation to fresh sperm is unclear, but sperm samples cryopreserved in 0.5-mL straws for use in artificial spawning yielded sufficient DNA for genetic analysis. This indicates that sperm frozen for breeding purposes can be used for genetic study without special preparation. There was no significant difference in purity among fresh and frozen samples regardless of freezing rate or storage temperature.

In PCR analysis, a DNA fragment of 300 bp in length was amplified from channel catfish DNA by use of the CH4 exon primers at a success rate of 90%. This success rate is based on the total number of DNA isolations examined in the study. It should be noted, however, that DNA from every fish was successfully amplified. Nucleic acid was isolated from cryopreserved sperm of the four other fish species with comparable yield and purity as that obtained from sperm of channel catfish.

Fish sperm is a readily available source of DNA and provides a number of benefits when cryopreserved. Unlike collection of blood or other tissues, collection of sperm is non-invasive in most cases. Sperm collection can often be incorporated into existing study programs, thus minimizing the cost of data collection. We have demonstrated in this study that use of various cryoprotectants and freezing temperatures allowed sufficient quantities of DNA to be isolated from cryopreserved fish sperm for

genetic analysis. Successful isolation of DNA from cryopreserved sperm of rainbow trout *Oncorhynchus mykiss* has been reported (Cummings and Thorgaard 1994). Thus, isolation of DNA from cryopreserved sperm would seem broadly applicable among fish species, and offers the potential for analysis years after sample collection.

Acknowledgements

This project was supported in part by grants from the U.S. Department of Agriculture, The U.S. Bureau of Reclamation, and the Louisiana Sea Grant College Program. This manuscript was approved for publication by the Director of the Louisiana Agricultural Experiment Station as number 99-66-0350.

References

Bates, M. C., W. R. Wayman and T. R. Tiersch. 1996. Effect of osmotic pressure on the activation and storage of channel catfish sperm. Transactions of the American Fisheries Society 125:798-802.

Christensen, J. M. and T. R. Tiersch. 1996. Cryopreservation of channel catfish spermatozoa: Effect of cryoprotectant, straw size, and formulation of extender. Theriogenology 47:639-645.

Cummings, S. A. and G. H. Thorgaard. 1994. Extraction of DNA from fish blood and sperm. BioTechniques 17:427-429.

Pittman-Cooley, L. and T. R. Tiersch. (1999). Cryopreserved fish sperm as a source of DNA for genetic analysis. The Progressive Fish-Culturist. In press.

Thongpan, A., M. Mingmuang, S. Thinchant, R. Cooper, T. Tiersch and K. Mongkonpunya. 1997. Genomic identification of catfish species by polymerase chain reaction and restriction enzyme analysis of the gene encoding the immunoglobulin M heavy chain constant region. Aquaculture 156:129-137.

Tiersch, T. R., C. A. Goudie and G. J. Carmichael. 1994. Cryopreservation of channel catfish sperm: Storage in cryoprotectants, fertilization trials, and growth of channel catfish produced with cryopreserved sperm. Transactions of the American Fisheries Society 123:580-586.

Wayman, W. R., R. G. Thomas and T. R. Tiersch. 1996. Cryopreservation of sperm of the spotted seatrout (*Cynoscion nebulosis*). Gulf Research Reports 9:183-188.

Wayman, W. R., R. G. Thomas and T. R. Tiersch. 1997. Refrigerated storage and cryopreservation of black drum *(Pogonias cromis)* spermatozoa. Theriogenology 47:1519-1529.

Wilson, M. R., A. Marcuz, F. Ginkel, N. W. Miller, L. W. Clem, D. Middleton and G. W. Warr. 1990. The immunoglobulin M heavy chain constant region gene of the channel catfish, *Ictalurus punctatus*: An unusual mRNA splice pattern produces the membrane form of the molecule. Nucleic Acid Research 18:5227-5233.