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Cytogenetic and Molecular Analysis of the Channel Catfish (Ictalurus Punctatus) Genome.

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CYTOGENETIC AND MOLECULAR ANALYSIS OF THE CHANNEL CATFISH (ICTALURUS PUNCTATUS) GENOME

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ABSTRACT

This research presents studies on cytogenetics and molecular genetics of channel catfish (*Ictalurus punctatus*), the most important fish species cultured in the United States. The goals of this project were to analyze the genome of the channel catfish and to develop a direct method of mapping single-locus genes. A series of techniques were developed to facilitate these studies, including culture of fibroblast cells, preparation of chromosomes from catfish of different ages, staining for nucleolus organizer regions (NOR) and heterochromatin (C-banding), restriction enzyme banding, replication R-banding, simultaneous detection of sister-chromatid exchange (SCE) and C-banding, fluorescent *in-situ* hybridization (FISH) and indirect and direct *in-situ* polymerase chain reaction (ISPCR).

A primary cell line was established from caudal fin tissue. The cell line was fibroblastic, and strongly positive for fibronectin and collagens type I and III in the cytoplasm. These cells maintained a normal karyotype after 42 generations and were positive for the channel catfish gene *Ig H* that codes for immunoglobulin.

Individual chromosomes were identified by location of the NOR and C-banding, and by restriction enzyme and replication R-banding. The 29 chromosome pairs were divided into 8 distinct groups based on morphology and size. Standard C-banding and replication R-banding karyotypes were established, and ideograms were prepared for the first time for this species. A procedure for simultaneous detection of the SCE and C-banding was developed, which may allow measurement of the distance between

exchange sites and centromeres. The baseline occurrence of SCE in the absence of mutagenic materials, were measured to be 3.6 ± 1.6% of chromosomes in each cell.

In addition, FISH and ISPCR procedures were developed for analysis of the single-locus *Ig H* gene on whole-cell, nuclear, and chromosomal preparations. Two copies of the gene were revealed in each positive interphase nucleus. The chromosomal location of the *Ig H* gene was detected. However, the identity of the chromosome remains unknown because the banding pattern was not analyzable after hybridization. Application of the ISPCR in chromosomal mapping is new for fish species and is only in initial stages for higher vertebrates.

INTRODUCTION

Channel catfish is the most important cultured fish species in the United States. Total area in production reached 64,000 ha and 178 million kg of fish were processed in 1991 (Wolters 1993); approximate weight of catfish processed increased to 203 million kg in 1995 (USDA). Despite its significant value in commercial aquaculture, the investment in genetic studies of channel catfish has been relatively low and there is little information on the genomic structure of this species.

Research on channel catfish genetics and breeding began in the late 1960s and early 1970s (Dunham and Smitherman 1987), however, applications of genetic improvement in channel catfish culture have lagged behind genetic improvements made in other farm animal industries. Morphological variations have been found in channel catfish; these include sex, albinism, taillessness, partial tails, triple tails, crooked backs and malformed mouths. None of these are suspected to be due to genetic causes except sex and albinism (Prather 1961; Bondari 1981). Electrophoretic analysis of the protein products of particular gene loci has been used widely to study the genetic structure of fish populations (Issen *et al.* 1988). In channel catfish, protein markers were examined, and found to differentiate among species and stocks of catfish and to measure the changes in gene frequency caused by selection (Dunham and Smitherman 1984; Carmichael *et al.* 1992). Gene-centromere mapping was the first approach used to detect the relative distance between centromere and certain gene loci which encode detectable enzymes (Liu *et al.* 1992). However, the number of suitable loci of

isoenzymes is normally less than 20 for any species, therefore, use of protein markers is not a final solution for developing a high density linkage map.

Genetic mapping using DNA markers has become popular because these markers are generally easier to isolate and characterize. Linkage groups can be postulated by investigating the inheritance patterns of genes or DNA markers (Hallerman and Beckmann 1988). In channel catfish, only a few genes have aroused the interest of research groups; the categories of these genes have been restricted to immunoglobulin genes (Wilson *et al.* 1990; Ghaffari and Lobb 1993; Hayman 1993; Magor 1994), odorant receptor genes (Ngai 1993), growth hormone genes (Tang 1993), and the nucleotide sequence of a precursor to somatostatin (Magzin 1982) and somatostatin (Dixon and Andrews 1985). Alternatively, random markers such as random amplified polymorphic DNA (RAPD), can be used to generate linkage groups of marker DNAs without pre-existing sequence information (Welsh and McClelland 1990).

Another method is to map DNA markers directly to chromosomes by *in-situ* DNA-DNA or RNA-DNA hybridization (Leitch *et al.* 1994). Instead of analyzing the segregation patterns of DNA among offspring, *in-situ* hybridization (ISH) is a method of physical gene mapping by which a gene or gene (linkage) group of DNA markers is assigned directly to a specific chromosome. Location of genes is traced by incubating denatured chromosomal DNA with a labeled nucleic acid probe that contains the DNA sequence corresponding to the gene of interest. Chromosomal sites that have annealed with the probe are identified by autoradiography or fluorescent staining.

The ISH dates back to 1969 when Gall and Pardue demonstrated the detection of amplified ribosomal RNA genes in nuclei of *Xenopus* oocytes. During the past decade, ISH has gone through many refinements to become one of the most important tools in detecting DNA or RNA sequences in tissue samples. The appropriate conditions for various factors of ISH have been established. These factors include tissue fixation, pretreatment, probe size, pre-hybridization, hybridization time and temperature, salt and formamide concentration, and detection systems. One of the major changes was the method for detection of hybridization signals. The original autoradiography has been replaced by fluorescent staining which is more sensitive and convenient, and produces less background signal.

Products and techniques are continually being developed to improve the sensitivity of ISH. The newly emerged technology of *in-situ* polymerase chain reaction (ISPCR) brings new applications of ISH (Gu 1994). The ISPCR is derived from a combination of the traditional *in-situ* hybridization method and the polymerase chain reaction (PCR) used widely by molecular biologists. The latter is capable of amplifying minute quantities of DNA or RNA sequences to billions of identical copies for detection or analysis. Since the first report in 1990 (Hasse *et al.* 1990), ISPCR has undergone rapid development. The input from increasing numbers of investigators has facilitated commercialization of the ISPCR. Thermal cyclers (MJ research Inc. and Perkin Elmer) designed for the ISPCR have become available, and products have been manufactured for use with these research machines (Perkin Elmers). It can be predicted that the ISPCR will soon become a routine molecular tool in research laboratories because of its

automatic, controllable, and convenient features. The application of the ISPCR has mostly been confined to the diagnosis of pathogenic agents such as viruses, with the commonly used materials being tissue sections and whole-cell preparations (Bagasra *et al.* 1992). However, the success of using ISPCR for chromosomal localization of genes has also been reported (Troyer et al. 1994).

Chromosomal *in-situ* PCR detection of genes not only relies on successful primer design, labeling, and control of temperature, but also on good metaphase spreads that have minimal background debris, and recognizable landmarks (or banding patterns) before and after DNA hybridization. Chromosome banding techniques have been developed in humans (Verma and Babu 1989) and other higher vertebrates (Gallagher and Womack 1992), including selective and differential staining techniques. The former reveals specific chromosomal regions such as nucleolus organizer regions (NOR-banding) and heterochromatin (C-banding). Differential staining induces light and dark serial bands along the length of chromosomes subjected to special treatments such as proteolytic enzymes for G-banding, quinacrine staining for Q-banding, and heating for R-banding (the reverse of G- or Q-banding). Differential methods have been indispensable in the unequivocal identification of chromosomes in higher vertebrates.

Fish chromosomes have long been known to be numerous, small and similar in size. In contrast to endothermic vertebrate species it is difficult or even impossible to produce a distinct banding pattern in ectothermic vertebrates including fish (Hellmer *et al.* 1991). Most of the published chromosome banding studies in fishes are restricted to C-banding or NOR characterization (Gold *et al.* 1990), while distinct structural G-, Q-,

and R-banding has been described only for the European eel, *Anguilla anguilla* (Wiberg 1983; Medrano *et al.* 1988). The present data suggest that structural chromosome banding of endothermic vertebrates results from regional differences of base composition (Medrano *et al.* 1988; Schmid and Guttenbach 1988) that can be divided into GC-rich and GC-poor compartments. Ectothermic vertebrates either lack or show only weak compartmentalization of their genomes by base composition (Bernardi 1989).

Many efforts have been made to produce segmented structural differences on fish chromosomes. The most important factors identified are induction of change in constitutional DNA structure by degradation with restriction enzyme (restriction enzyme banding) (Stingo *et al.* 1995) or by chemical labeling of DNA during replication (replication banding) (Delany and Bloom, 1984). Replication banding is the most common method used with ISH, but again only a few successful examples have been reported in fishes (Hellmer *et al* 1991, Pendas *et al.* 1993a).

The diploid chromosome number of channel catfish (2N = 58) has been reported (LeGrande 1981, Wolters *et al* 1981), and confirmed through intergeneric hybridization (LeGrande *et al* 1984, Zhang and Tiersch in review). The nucleolus organizer regions (NOR) has been located on the short arms of a pair of medium-sized submetacentric chromosomes, and this character has been used to examine karyotypes of intergeneric hybrid fishes (Zhang and Tiersch in review). The other chromosomal bands (C-, G-, R-, and Q-) have not been reported in this species, and therefore, identification of individual chromosomes has not been possible to date.

The use of cultured cells is a critical step in replication banding and ISH because it yields less background and better control of timing. In the past, the most commonly used cell types were leukocytes and fibroblasts (Denton 1973). Because fibroblast cells can be isolated from the majority of tissue types, and are accessible from early embryo to adult, they have been widely used in cytogenetics.

The first teleost cell line was established for viral diagnosis (Wolf and Quimby, 1962), and the burgeoning use for cultured fish cells has led to the establishment of cell lines from a wide range of species for the past 30 years (Fryer and Lannan 1994). Although these cell lines were used chiefly for fish virus research (Wolf and Mann 1980), they have also proved useful in studies relating to toxicology (Babich and Borenfreund 1991), carcinogenesis (Hightower and Renfro 1988), gene regulation and expression, and DNA replication and repair (Bols and Lee 1991).

Fish cells are readily propagated *in vitro* using techniques and reagents developed for the culture of mammalian cells (Lannan 1994). Unlike cells of avian or mammalian origin, cultured fish cells require minimal maintenance, and replicate within a broad range of incubation temperatures. The conditions for culture of channel catfish somatic cells including ovary cells (Bowser and Plumb 1980), hepatocytes (Wohlschlag *et al.* 1989), and lymphocytes (Miller *et al.* 1994) have been investigated; this provides an important reference for culturing fibroblast cells of this species.

Briefly, the goal of the current study was to search for a direct method for genomic mapping of channel catfish. *In-situ* PCR would provide a powerful tool for direct localization of single-locus genes or DNA markers on a specific chromosome.

This method is important for channel catfish because location of genes by linkage analysis is difficult at the current stage of technology in this species. Physical mapping of single-locus genes has not been reported from fish species. In a few cases, locations of ribosomal RNA genes in carp (Carman *et al.* 1993) and Atlantic salmon (Pendas *et al.* 1993b; Pendas *et al.* 1994) and other highly repeated fish DNA sequences (Kubota *et al.* 1993) have been investigated by fluorescent *in-situ* hybridization.

The objectives of this study were to: (1) establish a cell line for subsequent cytogenetic studies; (2) develop techniques for analysis and identification of chromosomal structure; (3) establish a standard karyotype for channel catfish, and (4) develop techniques to detect and localize single-locus genes on whole-cells, nuclei, and chromosomal preparations. Results were organized into 5 chapters, and each chapter was prepared as a manuscript for submission to the following journals: The first chapter, 'Development and Characterization of a Primary Fibroblast Cell Line from Channel Catfish,' for *In Vitro* Cellular and Developmental Biology ; the second chapter, 'Genome Characterization of Channel Catfish by location of nucleolus organizer regions, C- and Restriction Enzyme- Banding,' for The Journal of Heredity; the third chapter, 'Replication Banding and Sister-chromatid Exchange of Channel Catfish Chromosomes,' for Cytogenetics and Cell Genetics; the fourth chapter, 'Detection and Localization by *In-situ* Polymerase Chain Reaction of a Channel Catfish Gene Encoding the Immunoglobulin Heavy Chain Constant Region,' for Transactions of the American Fisheries Society, and the fifth chapter, 'Can *In-Situ* Polymerase Chain Reaction and

Replication Banding be Combined for Physical Mapping of the Channel Catfish Genome?,' for Cytobios.

References cited in Introduction

Babich, H., and E. Borenfreund, 1991. Cytotoxicity and genotoxicity assays with cultured fish cells: a review. Toxicology *in Vitro* 5: 91-100.

Bagasra, O., S. P. Hauptman, H. W. Lischner, M. Sachs and R. J. Pomerantz, 1992. Detection of HIV-1 provirus in mononuclear cells by *in-situ* PCR. New England Journal of Medicine 326: 1385-1391.

Bernardi, G., 1989. The isochore organization of the human genome. Annual Review of Genetics 23: 637-661.

Bols, N. C., and L. E. J. Lee, 1991. Technology and uses of cell culture from the tissues and organs of bony fish. Cytotechnology 6: 163-187.

Bondari, K., 1981. A study of abnormal characteristics of channel catfish and blue tilapia. Proceeding of the Annual Southeastern Association of Fisheries and Wildlife Agencies 35: 568-580.

Bowser, P. R., and J. A. Plumb, 1980. Fish cell lines: Establishment of a line from ovaries of channel catfish. *In Vitro* 16: 365-368.

Carman, O., Y. Matsuda, T. Oshiro and F. Takashima, 1993. Mapping of rRNA genes (NOR) in carp chromosomes using the cloned probe of mouse rRNA by FISH analysis. Nippon Suisan Gakkaishi 59: 729.

Carmichael, G. J., M. Schmidt and D. C. Morizot, 1992. Electrophoretic identification of genetic markers in channel catfish and blue catfish by use of low-risk tissues. Transactions of American Fisheries Society 121: 26-35.

Delany, M. E., and S. E. Bloom, 1984. Replication banding patterns in the chromosomes of the rainbow trout. Journal of Heredity 75: 431-434.

Denton, T. E., 1973. *Fish chromosome methodology*. Charles C. Thomas, Springfield, Illinois.

Dixon, J. E., and P. C. Andrews, 1985. Somatostatins of the channel catifsh. Advances in Experimental Medicine and Biology 188: 19-29.

Dunham, R. A., and R. O. Smitherman, 1987. *Genetic breeding of catfish.* Department of Research Information, Alabama Agricultural Experiment Station, Auburn University, Auburn, AL.

Dunham, R. A., and R. O. Smitherman, 1984. *Ancestry and breeding of catfish in the United States.* Alabama Agricultural Experiment Station, Auburn Univ, Auburn, Alabama.

Fryer, J. L., and C. N. Lannan, 1994. Three decades of fish cell culture: a current listing of cell lines derived from fishes. Journal of Tissue Culture Methods 16: 87-94.

Gall, J. G., and M. L. Pardue, 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proceeding of the National Academic Sciences of USA 63: 378-383.

Gallagher, D. S., and J. E. Womack, 1992. Chromosome conservation in the Bovidae. Journal of Heredity 83: 287-298.

Ghaffari, S. H., and C. J. Lobb, 1993. Structure and genomic organization of immunoglobulin light chain in the channel catfish. An unusual genomic organizational pattern of segmental genes. Journal of Immunology 151: 6900-6912.

Gold, J. R., Y. C. Li, N. S. Shipley and P. K. Powers, 1990. Improved methods for working with fish chromosomes with a review of metaphase chromosome banding. Journal of Fish Biology 37: 563-575.

Gu, J., 1994. Principles and applications of *in-situ* PCR. Cell Vision: Journal of Analytical Morphology 1: 8-19.

Hallerman, E. M., and J. S. Beckmann, 1988. DNA-level polymorphism as a tool in fisheries science. Canadian Journal of Fisheries and Aquatic Sciences 45: 1075-1087.

Hasse, A. T., E. F. Retzel and K. A. Staskus, 1990. Amplification and detection of lentiviral DNA inside cells. Proceeding of the National Academic Sciences of USA 87: 4971-4975.

Hayman, J., Russell, S. H. Ghasffari and C. J. Lobb, 1993. Heavy chain joining region segments of the channel catfish: Genomic organization and phylogenetic implications. Journal of Immunology 151: 3587-3596.

Hellmer, A., I. Voiculescu and W. Schempp, 1991. Replication banding studies in 2 cyprinid fishes. Chromosoma 100: 524-531.

Hightower, L. E., and J. L. Renfro, 1988. Recent applications of fish cell culture to biomedical research. The Journal of Experimental Zoology 248: 290-302.

Issen, P. E., J. M. Casselman, G. W. Martin and R. B. Phillips, 1988. Biochemical genetic differentiation of lake trout (*Salvelinus namaycush*) stocks of the great lake region. Canadian Journal of Fisheries Aquatic Sciences 45: 1018-1029.

Kubota, S., M. Kuro-o, S. Mizuno and S. Kohno, 1993. Germ line-restricted, highly repeated DNA sequences and their location in a Japanese hagfish (*Eptatretus okinoseanus*). Chromosoma 102: 163-173.

Lannan, C. N., 1994. Fish cell culture: A protocol for quality control. Journal of Tissue Culture Methods 16: 95-98.

LeGrande, W. H., 1981. Chromosomal evolution in North American catfishes (Siluriformes: Ictaluridae) with particular emphasis on the madtoms, *Noturus*. Copeia 1981: 33-52.

LeGrande, W. H., R. A. Dunham and R. O. Smitherman, 1984. Karyology of three species of catfishes (*Ictaluridae*: *Ictalurus*) and four hybrid combinations. Copeia 1984: 873-878.

Leitch, A. R., T. Schwarzacher, D. Jackson and I. J. Leitch, 1994. *In-situ Hybridization: A practical guide*. Bios Scientific Publishers Limited, Oxford, UK.

Liu, Q., C. A. Goudie, B. A. Simco, K. B. Davis and D. C. Morizot, 1992. Gene-centromere mapping of six enzyme loci in gynogenetic channel catfish. Journal of Heredity 83: 245-248.

Magor, B. G., M. R. Wilson, N. W. Miller, L. W. Clem, D. L. Middleton, and G. W. Warr, 1994. An Ig heavy chain enhancer of the channel catfish *Ictalurus punctatus*: Evolutionary conservation of function but not structure. Journal of Immunology 53: 5556-5563.

Magzin, M., C. D. Minth, C. L. Funckes, R. J. Deschenes, M. A. Tavianini, and J. E. Dixon, 1982. Sequence of a cDNA encoding pancreatic preprosomatostatin-22. Proceeding of the National Academic Sciences of USA 79: 5152-5156.

Medrano, L., G. Bernardi, J. Couturier, B. Dutrillaux and G. Bernardi, 1988. Chromosome banding and genome compartmentalization in fishes. Chromosoma 96: 178-183.

Miller, N. W., V. G. Chinchar and L. W. Clem, 1994. Development of leukocyte cell lines from the channel catfish (*Ictalurus punctatus*). Journal of Tissue Culture Methods 16: 117-123.

Ngai, J., M. M. Dowling, L. Buck, R. Axel and A. Chess, 1993. The family of genes encoding odorant receptors in the channel catfish. Cell 72: 657-666.

Pendas, A. M., P. Moran, J. P. Freije and E. Garcia-Vazquez, 1994. Chromosomal mapping and nucleotide sequence of two tandem repeats of Atlantic salmon 5S rDNA. Cytogenetics and Cell Genetics 67: 31-36.

Pendas, A. M., P. Moran and E. Garciavazquez, 1993a. Replication banding-patterns in Atlantic salmon (*Salmo salar*). Genome 36: 440-444.

Pendas, A. M., P. Moran and E. Garciavazquez, 1993b. Ribosomal-RNA genes are interspersed throughout a heterochromatic chromosome arm in Atlantic salmon. Cytogenetics and Cell Genetics 63: 128-130.

Prather, E. E., 1961. A comparison of production of albino and normal channel catfish. Proceeding of the Annual Conference of Southeast Association of Game and Fisheries Commission 15: 302-303.

Schmid, M., and M. Guttenbach, 1988. Evolutionary diversity of reverse (R) fluorescent chromosome bands in vertebrates. Chromosoma 97: 101-114.

Stingo, V., L. Rocco, G. Odierna and M. Bellitti, 1995. NOR and heterochromatin analysis in two cartilaginous fishes by C-, Ag- and RE (restriction enzyme)-banding. Cytogenetics and Cell Genetics 71: 228-234.

Tang, Y., C. M. Lin, T. T. Chen, H. Kawauchi, R. A. Dunham, and D. A. Powers, 1993. Structure of the channel catfish (*Ictalurus punctatus*) growth hormone gene and its evolutionary implication. Molecular Marine Biology and Biotechnology 2: 198-206.

Troyer, D. L., D. W. Goad, H. Xie, G. A. Rohrer, L. J. Alexander, C. W. Beattie, 1994. Use of direct *in-situ* single copy (DISC) PCR to physically map 5 porcine microsatellites. Cytogenetics and Cell Genetics 67: 199-204.

USDA, 1995. Farm-raised catfish: Quantity processed and prices paid to producers by major processors, and United States imports, 1994-95. The Catfish Journal 10: 22.

Verma, R. S., and A. Babu, 1989. Human Chromosomes: Manual of basic techniques. Pergamon Press, Inc., New York.

Welsh, J., and M. McClelland, 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18: 7213-7218.

Wiberg, U. H., 1983. Sex determination in the European eel (*Auguilla auguilla*, L.). Cytogenetics and Cell Genetics 36: 589-598.

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.

Wohlschlag, N., L. de los Snatos and P. F. Dehn, 1989. Preparation of primary cultures of adult and juvenile catfish (*Ictalurus punctatus*) hepatocytes. Journal of Tissue Culture Methods 12: 53-55.

Wolf, K., and J. A. Mann, 1980. Poikilotherm vertebrate cell lines and viruses: a current listing for fishes. *In Vitro* 16: 168-179.

Wolf, K., and M. C. Quimby, 1962. Established eurythermic line of fish cells *in vitro*. Science 135: 1065-1066.

Wolters, W. R., 1993. Channel catfish breeding and selection programs: constraints and future prospects, pp. 82-95 in *Selective breeding of fishes in Asia and the United States*, edited by K. L. Main, and B. Reynolds. The Oceanic Institute, Honolulu, Hawaii.

Wolters, W. R., C. L. Chrisman and G. S. Libey, 1981. Lymphocyte culture for chromosomal analysis of channel catfish, *Ictalurus puntatus*. Copeia 1981: 503-504.

Zhang, Q., and T. R. Tiersch, in review. Cytogenetic analysis of two intergeneric hybrids of ictalurid catfishes.

CHAPTER 1
DEVELOPMENT AND CHARACTERIZATION OF A PRIMARY FIBROBLAST CELL LINE FROM CHANNEL CATFISH

Introduction

Channel catfish (*Ictalurus punctatus*) is the most important fish species cultured in the United States (Stickney 1993). Research on this species has included the study of disease (Thune *et al.* 1993), physiology (Kang and Caprio 1995), and genetic improvement (Wolters 1993). Techniques for short-term culture of catfish cells support these studies (e.g. Miller and Clem 1988; Wohlschlag and de los Snatos 1989), and several cell lines have been developed for long-term use. The first continuous cell line from channel catfish was an ovary cell line that has been used for about 2 decades in the diagnosis of catfish viruses (Bowser and Plumb 1980). Leukocyte cell lines including monocyte-like cell lines (Vallejo *et al.* 1991), and B cell lines (Miller *et al.* 1994b) were developed more recently and have been used to demonstrate the immune functions of ectothermic animals.

In this study, a primary fibroblast cell line was developed to support cytogenetic and molecular genetic studies. Compared with the ovary cell and leucocyte lines, fibroblast cells can be sampled at embryonic stages and provide material for rapid and early genetic screening. Fibroblast cells yield high-quality chromosomes from mammalian species (Gallagher and Womack 1992) to fishes (Amemiya *et al.* 1984). The objectives of this study were to (1) develop a fibrolast cell line from caudal fin tissue of channel catfish; (2) evaluate effects of basal media and serum supply on growth of the cell line; (3) maintain the cell line by subculture and cryopreservation, and (4)

characterize the cell line by immunocytochemistry, chromosomal analysis, and molecular methods.

Materials and methods

Animals

A total of 10 juvenile channel catfish (body weight: 50 to 200 g) were used in this study. Fish were maintained in an indoor recirculating system at the LSU Aquaculture Research laboratory.

Generation of a primary cell line by explant technique

(1) Solutions and culture media. The washing solution was composed of Ca^{2+}, and Mg^{2+}- free phosphate buffered saline (CMF-PBS), 100 units/ml of penicillin (Gibco BRL, Life Technologies Inc., Gaithersburg, MD), 100 μg/ml of streptomycin (Gibco), and 100 μg/ml of gentamicin (Gibco). Washing medium was composed of Leibovitz L15 medium (Gibco) supplied with 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 100 μg/ml of gentamicin. Three different basal culture media were prepared in this study. The A/L basal medium was composed of a 1:1 mixture of Leibovitz L15 and AIM V (Gibco), 100 units/ml of penicillin, 100 μg/ml of streptomycin, 10 μg/ml of gentamicin, and 1 mg/ml of $NaHCO_3$. The L15 basal medium was composed of Leibovitz L15 medium, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10 μg/ml of gentamicin. The MEM basal medium was composed of Eagle MEM medium (Gibco), 100 units/ml of penicillin, 100 μg/ml of streptomycin, 10 μg/ml of gentamicin, and 25 mM of Hepes buffer (Sigma Chemical Company, St. Louis, MO). All the solutions and media were adjusted to the tonicity of catfish blood plasma by addition

with distilled H_2O at 10% of final volume. Prepartion of catfish serum was based on the previously described procedure of Miller and Clem (1988).

(2) Isolation of fibroblast cells from fin tissue. Fish were anesthetized with MS-222 (Argent Chemical Laboratory, Redmond, WA), and moved in a laminar-flow hood (BBL® Biohazard Cabinet CNSF No. 49, Becton Dickson Company, Cockeysville, MD). The surface of the fish was sterilized with 10% bleach and 70% ethanol. The caudal fin was removed asceptically, placed in a petri dish filled with washing solution, and cut into 1-mm^2 pieces. The tissue fragments were transfered into a 20-ml glass tube and rinsed with washing solution 3 times for 20 min each, on a desktop shaker. At the end of each rinse, the solution was decanted and discarded. The tissue fragments were digested with 0.25% trypsin solution at 3 different temperature and time combinations: 4 °C overnight, 27°C for 2 h, or 37 °C for 1 h.

After digestion, the tissue was centrifuged (Beckman® model TJ-6 centrifuge, Palo Alto, CA) at 350x g for 10 min, and the supernatant removed. A cold A/L complete medium prepared by A/L basal medium and 10% fetal bovine serum (FBS, Gibco) was added to the tube, and sterile forceps were used to seed the digested tissue fragments into each well of a 6-well culture plate (Falcon Plastics, Becton Dickinson Inc., Franklin Lakes, NJ). A coverslip (22 x 22 mm) was placed on the top of tissue to enhance attachment. Prewarmed complete medium (2.5 ml) was added drop by drop to each well. The cultures were incubated (VWR 1820 water jacket incubator, VWR Scientific, Sugarland, TX) at 27 °C in a humidified enviroment supplied with 2% CO_2.

Epithelial and fibroblast-like cells grew out from the tissue fragments 48 h to 72 h after seeding. The monolayer was rinsed with washing solution at Day 5, and fresh medium was added. After cells grew to about 50% confluency, the monolayer was trypsinized and the cells pooled and cultured in 25-cm^2 flasks (Falcon).

The cultures were digested with 0.05%, 0.1%, or 0.25% trypsin when they reached confluency. Cells which detached at different intervals were cultured into different flasks, although only the fibroblast-like cells were retained for subsequent propagation.

(3) Cell line subculture. The cell line developed by the above method was designated as CCf (channel catfish fin tissue) and maintained as follows. The cultures were split 1:4 into subcultures when cells reached confluency. After exmination for sterility, the flasks were placed in a laminar-flow hood and the outer surface of the flasks was sterilized with 70% ethanol. After the spent medium was decanted, the monolayer was rinsed briefly twice with 3 ml CMF-PBS, and 5 ml of 0.25% trypsin solution (pre-warmed to 27 °C) was added to the monolayer. Four ml of tryspsin solution was quickly withdrawn, and 1 ml was left in the flask. Most cells would detach after 10 to 15 min incubation at 27 °C. Three ml (pre-warmed and recovered to 27 °C) of L15 complete medium was added, and the clumps of cells were dessociated by repeated pipetting (5 to 10 times). One ml of the cell suspension was transferred to a fresh flask, and 4 ml of fresh L15 complete medium was added. The flask was sealed and cultured at 27 °C. A sterility test was conducted at passage 20, in which cells were treated same as the above subculture procedure except that antibiotic-free solution or medium was used.

Effects of basal medium, and serum supply

(1) Experimental design. After transfer into 25-cm^2 flasks, cells were passed 2 more times before use in an optimization study. A 3 x 5 factorial arrangement was designed for identifying the optimal growth conditions, with basal media (L15, A/L, or MEM) and serum supplementation (5% catfish serum, 10% catfish serum, 5% catfish serum and 5% FBS, 5% FBS, or 10% FBS) as the two factors. Each treatment included 4 replicates. Cells were seeded into each well of three 24-well plates, each for one medium type, at 2.4 x 10^4 cells/well. The cells were grown at 27 °C in a humidified environment with 2% CO_2 (for A/L medium) or without CO_2 (for L15 or MEM medium). The cultures were harvested 5 days after plating.

(2) Data collection. Viability and cell concentration were calculated by counting live and dead fibroblasts using a dye-exclusion method. Cell samples from each treatment were diluted and stained with 0.1% trypan blue, and within 20 min counted in a hemacytometer at 100x manification using phase-contrast microscopy (Optiphot-2, Nikon Inc., Garden City, NY). Cell concentration was estimated based on the number of cells lying within the eight 1-mm^2 corner squares of a hemacytometer using the following formula:

$$\text{Cell concentration (cells/ml)} = (\text{number of cells}/8) \times \text{dilution factor} \times 10^4$$

(3) Statistical analysis. Cell concentrations were analyzed by two-factor ANOVA with medium (L15, A/L, or MEM) and serum type (5% catfish serum, 10%

catfish serum, 5% catfish serum plus 5% FBS, 5% FBS, or 10% FBS) as the factors. Duncan's multiple means comparison was used to identify differences among treatments, which were considered significantly different when $P \leq 0.05$.

Growth at different plating concentrations

Based on the results of the optimization study, cells were cultured in L15 medium supplied with 5% catfish serum and 5% FBS. Fibroblasts were trypsinized at confluent growth. Cells were diluted to 10^5 cells/ml, 0.7 x 10^4 cell/ml, or 10^3 cell/ml, and were seeded to three 24-well plates (Costar Corp., Cambridge, MA), one at each cell concentration, with 1 ml per well. After 24 h, the plates were removed, and the cells in three wells of each plate were counted. The plates were returned to incubators imediately after removal of the cell samples. Repeated samplings were done at 24-h intervals for the first 5 d, and reduced to once every 2 to 4 d. The sampling stopped after 14 d. Medium was changed when the pH value dropped below 7.

Cell storage and recovery

(1) Cell storage. The monolayers were trypsinized when they reached about 90% confluence. The cells were diluted with 5 ml of cold L15 complete medium, and cell suspensions were transferred to 15-ml tubes. After centrifugation, pellets were resuspended in 2 ml of freshly prepared cryopreservation medium (70% L15 basal medium, 20% FBS, and 10% DMSO), and transferred to sterile cryovials (Corning Inc., Corning, NY) in 1-ml aliquots. The cryovials were wrapped with 5 layers of cotton, covered with foil, and left in a freezer (-80 °C) overnight. The cryovials were stored in liquid nitrogen or left at -80 °C until use.

(2) Cell recovery. The cryovials were removed from the liquid nitrogen, dipped immediately into a beaker filled with warm water (37 °C), and shaken until the last ice crystals diappeared. The vials were opened asceptically and the cell suspensions transferred to 15-ml tubes. The cells were diluted with 10 ml of pre-cooled L15 basal medium (4 °C) with constant stirring to facilitate mixing. After centrifugation, the pellet was resuspended in 1 ml of pre-warmed L15 complete medium, transferred immediately to a 25-cm^2 culture flask, and incubated with another 4 ml of pre-warmed L15 complete medium at 27 °C.

Morphological observation

(1) Light microscopy. Living cells in culture were observed directly with a phase-contrast inverted microscope (Diaphot-TMD, Nikon Inc.). For improved visulization of the cell monolayer, cells were grown on The SuperCell™ Slide (Erie Scientific Company, Portsmouth, NH), fixed in 10% neutral buffered formalin and stained with hematoxylin for 3 to5 min.

(2) Immunocytochemistry. The second passage of cells was grown on coverslips to ~70% confluence, rinsed twice with 0.01 M CMF-PBS (pH 7.4), and fixed with 10% neutral buffered formalin for 10 min. The cells were rinsed twice with PBS and incubated with 0.2% triton X-100 for 5 min. Coverslips were rinsed twice with PBS, immersed in 0.3% H_2O_2 for 5 min, rinsed twice with PBS and incubated in 20% colostrum-free bovine serum (CFBS) (Sigma) for 30 min at room temperature.

The coverslips were incubated with 100 µl of diluted (1:40) rabbit anti-rat monoclonal antibody against the following components: Type I collagen (Chemicon,

Temecula, CA), Type III collagen (Chemicon), and fibronectin (ICN Immuno Biologicals, Costa Mesa, CA). For control, either the primary antibody was omitted or non-immune serum was substituted for the primary antibody. The incubations were performed at room temperature for 1 h. The coverslips were rinsed three times with PBS for 5 min each. The secondary antibody, peroxidase-conjugated goat anti-rabbit antibody (ICN), was added to each coverslip, and incubated at room temperature for 1 h. The coverslips were rinsed three times with PBS for 5 min each. The coverslips were finally stained with diaminobenzidine (DAB), prepared by adding 0.6 mg DAB and 10 µl of 3% H_2O_2 to 1 ml of PBS).

Chromosome analysis

(1) Preparation of metaphase chromosomes. Cultured cells at passages 3, 7, 11, 17, and 21 were used for preparation of chromosomes. Twenty ml of colchicine solution (100 µg/ml in CMF-PBS) were added to each 25-cm^2 flask when cultures reached exponential growth (about 70% confluency), and cells incubated for 1 h. The monolayer was trypsinized and cell masses were broken up by repeated pipetting. The hypotonic treatment, and cold fixation were based on the procedure used for cultured leukocytes (Zhang and Tiersch 1995).

(2) Giemsa and silver staining. For evaluation of general morphology, metaphase spreads were stained with 5% Giemsa solution (freshly made in 0.01 M phosphate buffer, pH 6.8) for 10 to 15 min. The silver staining procedure of Howell and Black (1980) was used to reveal the nucleolus organizer regions (NOR). A mixture of 50% silver nitrate and 2% gelatin solution was prepared immediately before each

experiment and 5 drops of the solution were added to each slide, covered with a coverslip (22 x 60 mm), and incubated at 50 °C until a bright, golden color developed (about 6 to 10 min). Slides were rinsed briefly with deionized water, and dried at room temperature.

(3) Statistical analysis. The chi-square test of homogeneity was used to analyze the percentage of cells with a modal diploid number (2N = 58) at different passages. Treatments were considered significantly different when $P \leq 0.05$.

Polymerase chain reaction

Genomic DNA was extracted using a QIAamp Blood Kit (Qiagen Inc., Clatsworth, CA) from cultured fibroblast cells, and whole blood of channel catfish. Primers of 25 nucleotides in length were synthesized by the LSU Gene Probes and Expression Systems Laboratory at Baton Rouge, and were designed to target the Ch4 exon of the channel catfish *Ig H* gene encoding the immunoglobulin heavy chain constant region gene (Wilson *et al.* 1990). The expected size of the amplified DNA fragment was 303 base pair. The reaction conditions were described previously (Zhang *et al.* 1994).

Results

Generation of a fibroblast cell line by explant technique

Cells grew more aggressively from explanted fin tissue digested at 4 °C than from tissues digested at 27 °C. No attachment was observed in the tissue fragments digested at 37°C. The cultures reached ~40 to 60% confluency between Day 7 and 12. The monolayer did not expand signifcantly after this period. Therefore, it was not advisable to leave cells in culture plates for more than 15 d. Coverslips facilitated the attachment

of tissue fragments, although coverslips interfered with the subsequent rinsing steps and may have affected monolayer expansion. Thus, removal of the coverslips after 7 d is advisable. Differential detachment was able to separate fibroblast-like cells from epithelial-like cells. However, most the epithelial cells disappeared after 3 to 5 passages with no differential digestion with trypsin.

Cells were confluent every 3 to 5 d after being transferred to culture flasks. The cell attachment and propagation in fresh flasks was enhanced when the subcultures were carried out immediately prior to confluency. However, cells could remain viable for more than a month without a change of medium. The cell line was sensitive to trypsinization. Prolonged exposure to trypsin caused the failure of subcultures. The trypsinization of the monolayers should be done at room temperature (~27 °C). The cell line was negative for bacterial and fungal infections at passage 20.

Effects of basal medium and serum supply

No difference was observed among the cultures with three different basal media ($P = 0.95$) (Table 1.1). However, differences were observed among cultures grown in media with different serum types ($P = 0.0001$). There was no interaction between basal medium and serum supply ($P = 0.77$). Cells grown in media with no FBS were not able to attach after culture for 5 d (Table 1.1). The cultures in media supplied with 5% catfish serum and 5% FBS showed the fastest increase in cell number.

Growth at different plating concentrations

Cultures at an initial density of 1.0 x 10^5 cells/ml had a doubling time between 48 and 72 h (Fig. 1.1). Log growth was observed between Day 2 and 5 followed by a brief decline between Day 5 and 6. Growth was restored at Day 7, another period of linear growth was observed between Day 10 and 13. Cultures with an initial density of

Table 1.1. Concentration of viable cells (mean ± SD) of each treatment after culture for 5 d in different basal media and serum types. Values sharing a letter, were not significantly different ($P > 0.05$). Abbreviations: FBS, fetal bovine serum, CCS, channel catfish serum.

Media	Number of replicates	Concentration (x 10^4 cells)
Basal medium		
A/L	46	9.8 ± 13.4 A
L15	45	9.9± 13.3 A
MEM	51	9.2 ± 13.4 A
Serum type		
5% CCS	27	0.4 ± 1.3 D
10% CCS	27	0 D
5% CCS + 5% FBS	27	34.6 ± 8.0 A
5% FBS	30	3.5 ± 0.7 C
10% FBS	31	10.3 ± 4.0 B

0.7 x 10^4 cells/ml did not show an increase in cell numbers until Day 6. Lag growth was observed between Day 6 and 13. Log growth started at Day 14. No growth was observed in cultures with an initial density of 1.0 x 10^3 cells/ml during the examination period (14 d).

Cell storage and recovery

The cryopreserved cells had a recovery as high as 100% after frozen storage for 1 week. The survival was reduced to ~50 to 70% after storing for 3 months at -80 °C. Successful recovery was indicated by the cell attachment in flasks within 2 h.

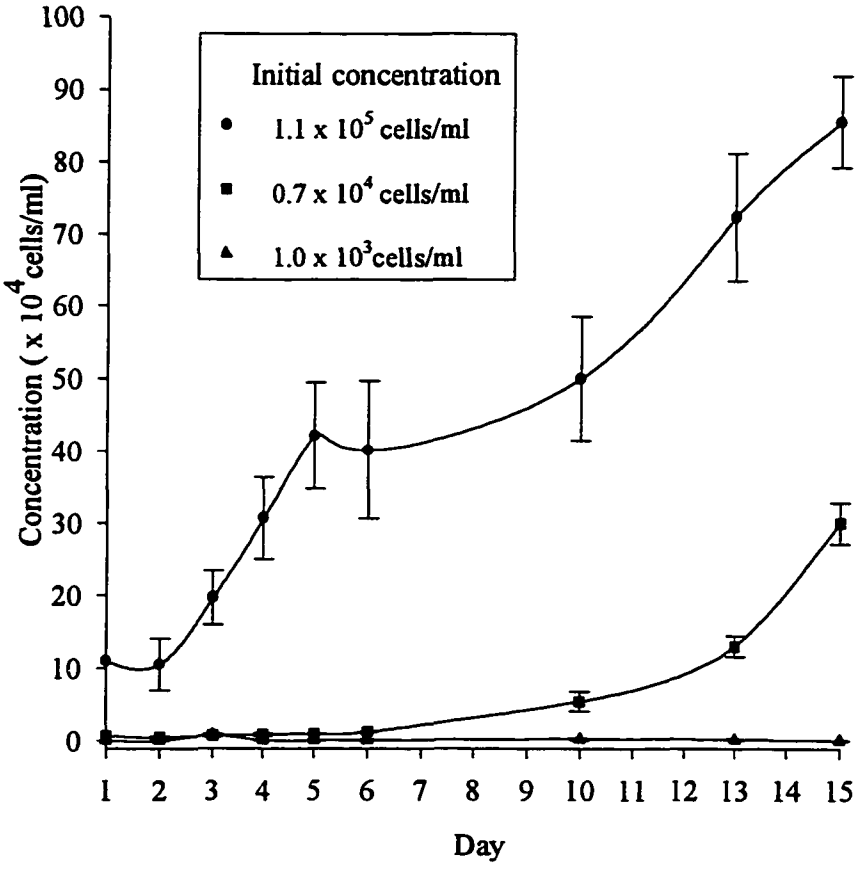


Figure 1.1. Growth of cultured fibroblast cells at three plating densities.

Morphological observation

The established cell line appeared spindle-shaped (Fig. 1.2 a, b, and c) when the cultures were not confluent. The elongation of processes at each pole gradually developed until they contacted with that of adjacent cells. The cells were well spread and randomly oriented. The egg-shaped nuclei were located at the center of the cell bodies (Fig. 1.2 d).

Immunochemical staining for fibronectin demonstrated positive cells (Fig. 1.3 a and b), and that the stain was localized to filamentous structures, but mostly concentrated around the nuclei. The immunochemical staining for Type I collagen (Fig. 1.3 c) and Type III collagen (Fig. 1.3 d) was heavily positive, and in each case the cytoplasmic staining was concentrated on filamentous structures. No staining was observed on slides treated with non-immune primary antibody (Fig. 1.3 e), which demonstrated that the staining was specific to fibronectin, and Type I and Type III collagens. No staining was found on slides prepared without anti-sera (Fig. 1.3 f).

Chromosome analysis

The modal diploid (2N) number of the cultured fibroblast cells was 58 (Fig. 1.4, top, Fig. 1.5a). The percentage of cells with modal chromosome number was between 57 and 64% at diffferent passages, which is usual for chromosomes prepared from fresh cells and primary cultures (LeGrande et al. 1984; Zhang and Tiersch in review). No significant difference ($\chi^2 = 0.5$, df = 4; $P > 0.05$) was found among the passages

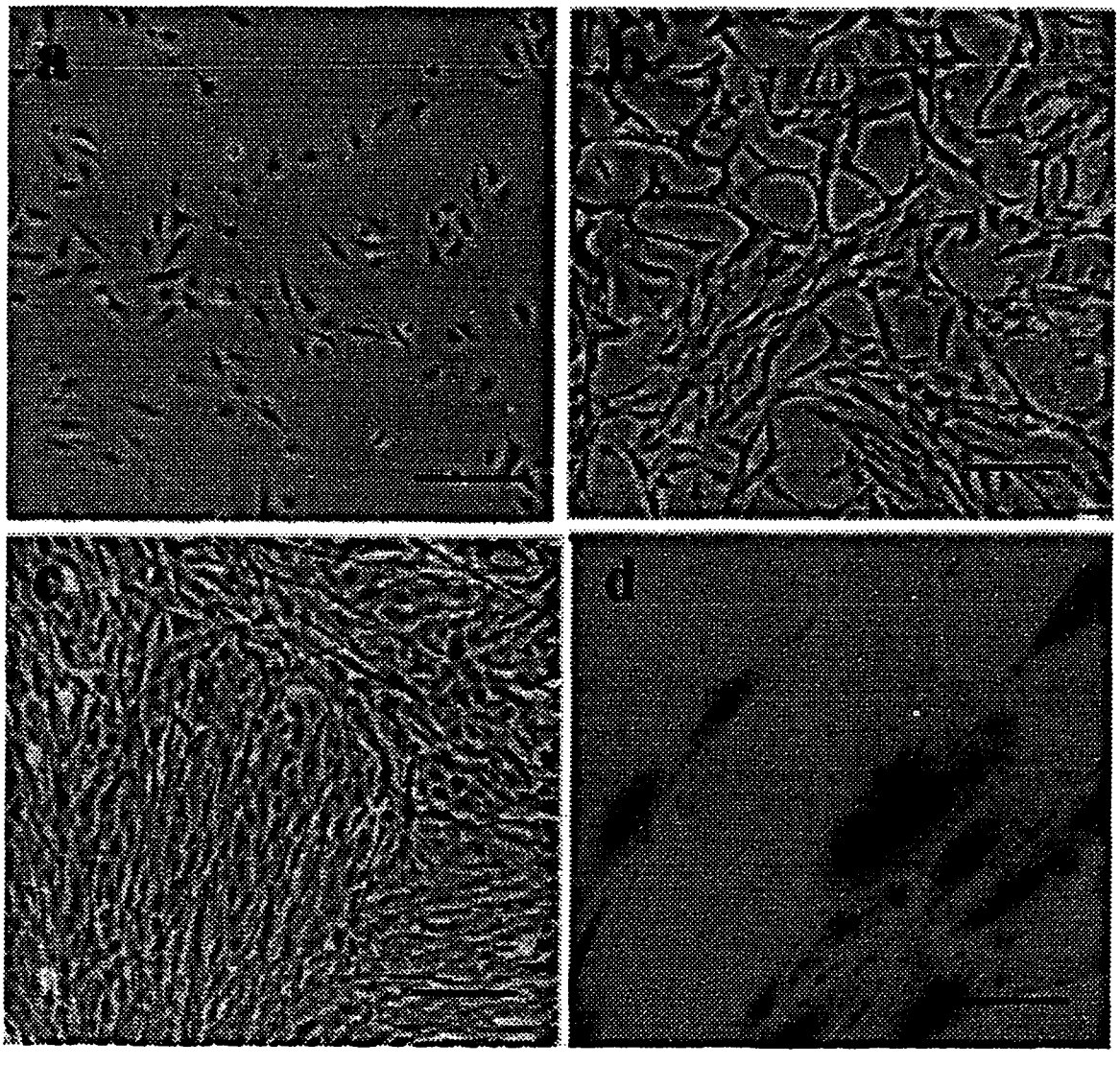


Figure 1.2. Phase-contrast microscopy of cultured CCf cells at 2 h (a), 36 h (b), and 84 h (c) (x 100). Cells were fixed with 10% neutral buffered formalin and stained by hematoxylin, and nuclei of fibroblast cells were located at center of cell bodies (d, x 200).

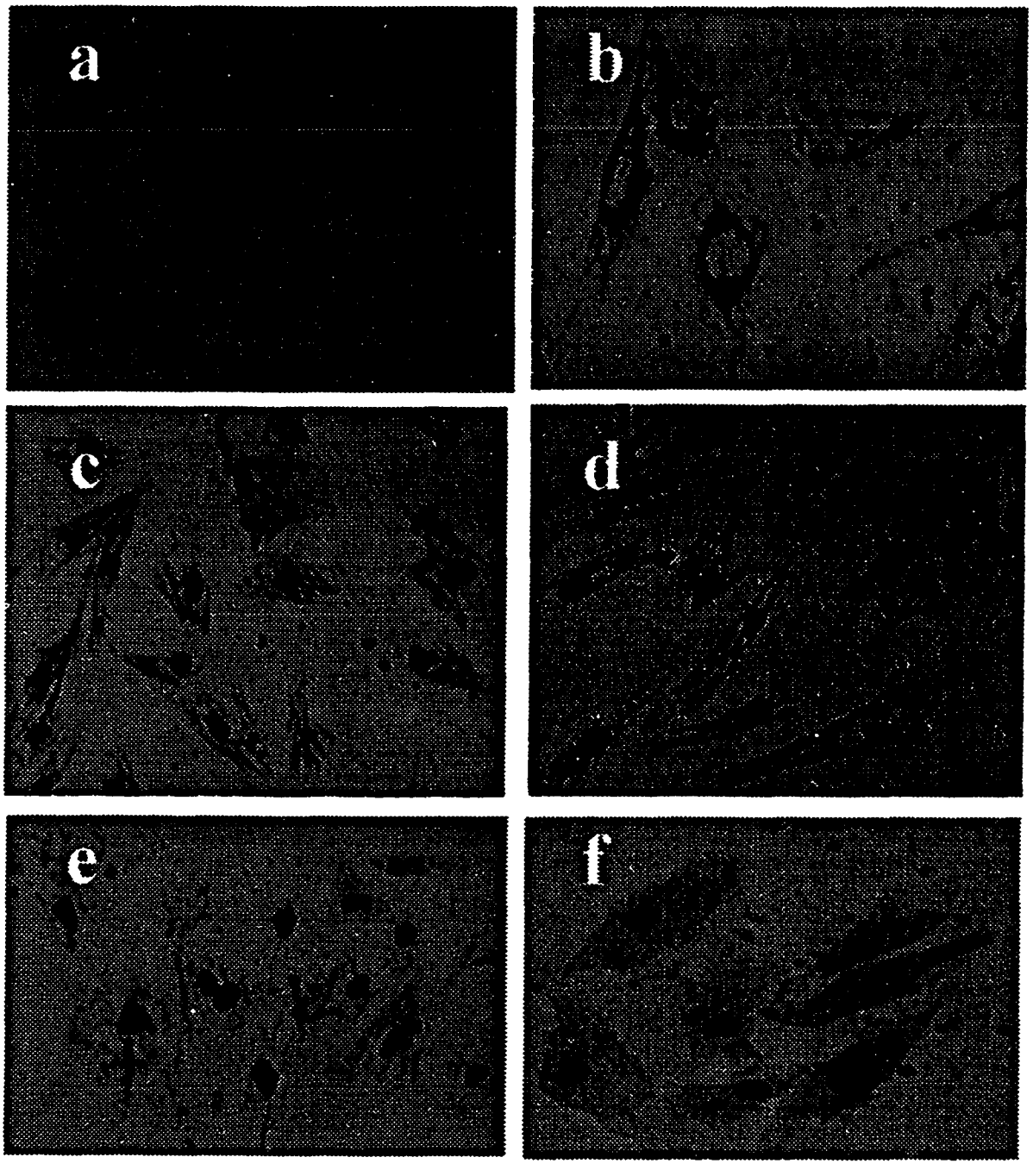


Figure 1.3. Immunochemical staining for fibronectin (a and b), Type I collagen (c) and Type III collagen (d). Negative staining for these cell components was observed when non-immune anti-serum was used to replace the specific antibody in the experiment (e), or when antibodies were omitted (f) (x 100 for a, b, c, d, and e and x 200 for f).

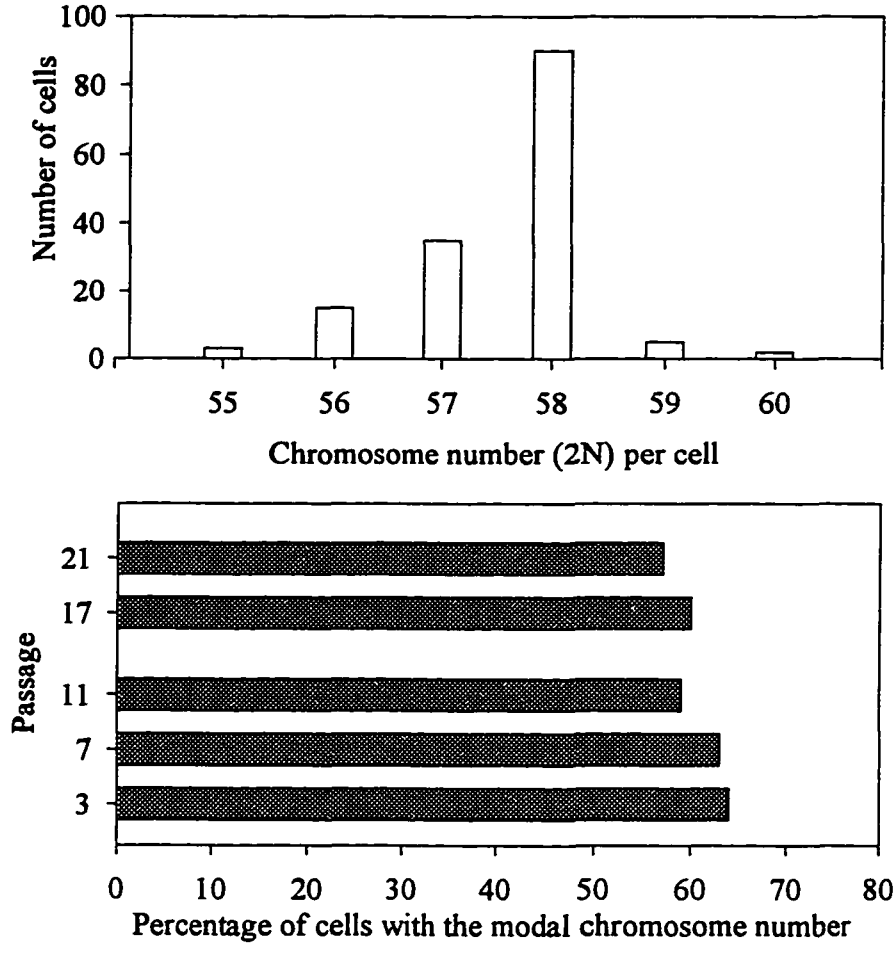


Figure 1.4. Chromosomal analysis of the CCf cell line. Top, frequency distribution of diploid number (2N) chromosomes. Bottom, the percentage of cells with the modal (2N = 58) chromosome number at different passages, which was not significantly different ($\chi^2 = 0.5$, df = 4; $P > 0.05$).

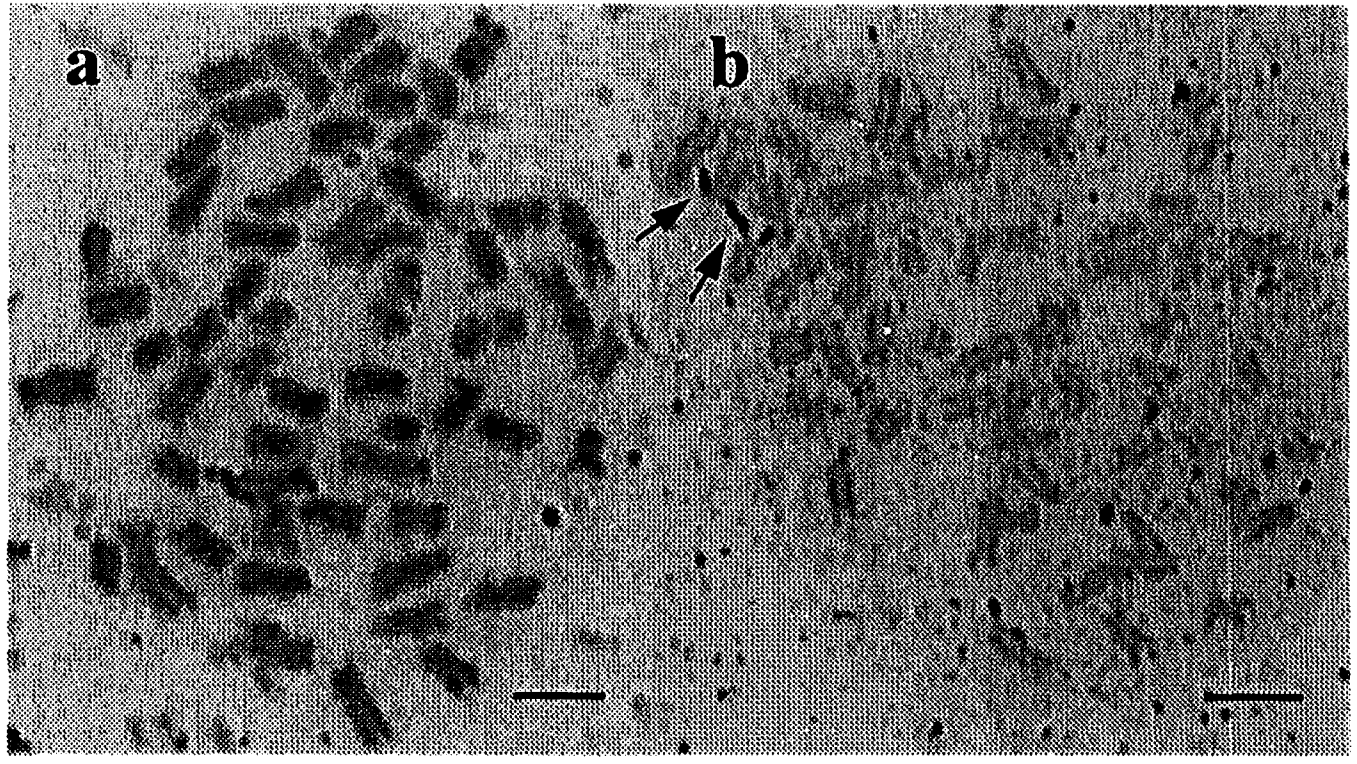


Figure 1.5. Representative metaphase spread prepared from the CCf cell line; Giemsa staining (a) and silver staining (b). The arrowheads point to the location of nucleolus organizer regions (NOR). Bars = 10 μ.

examined (Fig. 1.4, bottom). The nucleolus organizer regions was located on the short arms of a pair of medium-sized submetacentric chromosomes (Fig. 1.5 b).

Polymerase chain reaction

The size of the fragment amplified from DNA isolated from the cultured fibroblast cells (Fig. 1.6, lane c) was the same as the fragment amplified from DNA isoalted from channel catfish blood cells (Fig. 1.6, lane b). No amplification was found in reactions prepared without primers (Fig. 1.6, lane d), or without template DNA (Fig. 1.6, lane e).

Discussion

In this study, I present methods for generation of a primary fibroblast cell line in channel catfish, and conditions for long-term maintanence of this cell line. The cell line was characterized by immunochemical, cytogenetic, and molecular techniques. With minor modifications of this method, I was able to culture fibroblast cells from other ictalurid catfish species such as flathead catfish (*Pylodictis olivaris*), black bullhead catfish (*Ameiurus melas*), and their hybrids with channel catfish (data not shown).

The attachment of explanted tissue to culture vessels is a critical step for primary cultures (Freshney 1994). Common treatments include repeated trypsinization (Noga 1980) for increasing surface adhesion of tissue fragments, or application of physical force such as by a coverglass (Avella *et al.* 1994). Both treatments were helpful in the present study; however, the channel catfish fibroblast cells were sensitive to excessive trypsinization. Tissue fragments digested at 37 °C for 1 h did not yield viable cells for

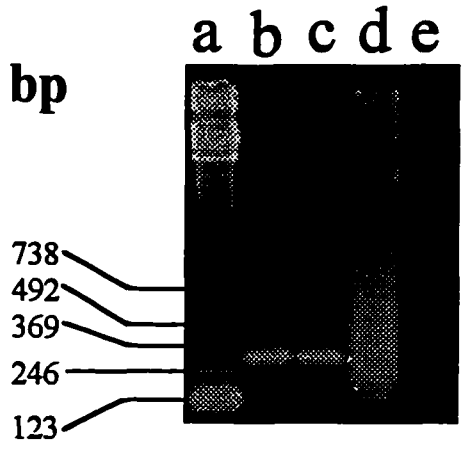


Figure 1.6. Comparison of the PCR products amplified from genomic DNA of CCf cells (lane c) with that isolated from whole blood of channel catfish (lane b), and DNA size marker (lane a). Negative controls were prepared with no primers (lane d) or no template DNA (lane e). The PCR products were separated by electrophesis on a 2% agarose gel in 1x TAE buffer, and the gel was stained with 0.5 μg/ml ethidium bromide for 10 min at room temperature after electrophoresis.

further attachment and growth. The same phenomenon was observed in the subsequent propagation of the cell line, in which monolayer cells treated with warm trypsin (37 °C) solution failed to attach and grow.

All the basal media used are modifications of media designed for use with other cells because no manufacturers have developed media for culture of fish cells. The most widely used media such as RPMI-160 and Dulbecco's modified Eagle medium (DMEM) were effective for culture of catfish after adjusting the osmolarity leukocytes (Faulmann *et al.* 1983; Miller and Clem 1988). More recently, a serum-free medium AIM V, supports a variety of *in vitro* immune functions of catfish leukocytes (Luft *et al.* 1991), and has been used with Leibovitz's L15 medium for culture of different leukocyte cell lines of channel catfish (Miller *et al.* 1994a). For adherent cell types of channel catfish, MEM medium has supported the *in vitro* growth of ovary cells (Bowser and Plumb 1980) and hepatocytes (Wohlschlag *et al.* 1989). In this study, I found that L15 medium supported the growth of fibroblast cells. The cells were cultured with L15 medium in sealed flasks and did not require CO_2 or a humidified environment. Therefore, L15 medium would seem to be a better choice for the CCf cells because of convenience and low cost.

The most critical component of media is usually the serum. Fetal bovine serum is commonly used to establish continuous fish cell lines derived from tissue explants (Fryer and Lannan 1994), including the ovary cell line of channel catfish (Bowser and Plumb 1980). However, FBS did not support the proliferative response of catfish leukocytes (Miller and McKinney 1994), while catfish serum did. In the present study, FBS and

catfish serum were important for culture of CCf cells: FBS was indispensable for the attachment of tissue fragments and subsequent propagation of the CCf cell line, and catfish serum provided enhanced cell growth. Other factors such as culture vessel did not influence the attachment of cells. The CCf cells can grow in Costar, Corning, and Falcon culture flasks with no appreciable difference in growth rate (data not shown). The CCf cells grew most effectively at plating concentrations of 1.0×10^5 cells/ml; cells dispensed at 1.0×10^3 cells/ml failed to form colonies.

It has been known for some time that fibroblasts can synthesize and secrete fibronectin (Hynes and Yamada 1982) and collagen (Porter and Pappas 1959). When CCf cells were permeabilized with Triton X-100, there was an intense staining of fibronectin and collagen in the cytoplasm surrounding the nucleus. The perinuclear staining for fibronectin in CCf cells is similar to what is seen in chick fibroblast cells (Yamada 1978), and the perinuclear staining for collagen parallels seen in permeabilized, cultured rat dental follicle cells (Wise *et al.* 1992) and cultured human fibroblasts (Gay *et al.* 1976).

The diploid number of normal channel catfish is 58 (LeGrande 1981; Wolters *et al.* 1981), and has been verified by intergeneric hybridization (LeGrande *et al.* 1984; Zhang and Tiersch in review). The CCf cells continue to maintain a modal diploid chromosome number of 58. The percentage of cells with this modal number varied from 64% at passage 3 to about 58% at passage 21, the percentage typically observed in karyotyping of fish from natural populations. More significantly, each metaphase spread had one pair of submetacentric NOR-bearing chromosomes, which was found to be a

representative feature of the channel catfish genome (Zhang and Tiersch in review). The ploidy and chromosome morphology of the CCf cell line could change after more passages as was observed in the channel catfish ovary cell line (Bowser and Plumb 1980); however, this remains to be determined.

The CCf cell line has been used in genetic studies, including replication banding, in which cultured cells provide control of timing for blocking and releasing DNA synthesis, and convenience for subsequent removal of chemicals and rinsing. The CCf cell line was used to prepare chromosomes for study of sister-chromatid exchange. The other immediate use of this cell line may include a model for *in vitro* expression of particular transgenes for channel catfish. Fibroblast cells are less differentiated, and therefore can accommodate and express foreign genes easier than specialized cell lines such as a B-cell line (Bouchard *et al.* 1989).

Summary

A primary cell line derived from channel catfish fin tissue (CCf), was developed by explant techniques. The cell line was cultured easily in medium developed for mammalian cells with osmalarity modification and supplementation with catfish serum and FBS. The cell line has been propagated continuously for 25 passages (1:4 dilution per passage), cryopreserved, and recovered successfully at different passages. The cultured cells had fibroblastic morphology, synthesized fibronectin, and Type I and III collagens in the cytoplasm. The cell line possessed a diploid chromosome number and a pair of NOR-bearing medium-sized submetacentric chromosomes, which is typical for

channel catfish. The size of the gene fragment amplified by PCR from DNA of cultured cells was not different from that of DNA from the blood of channel catfish.

References cited in Chapter 1

Amemiya, C. T., J. W. Bickham and J. R. Gold, 1984. A cell culture technique for chromosome preparation in cyprinid fishes. Copeia 1984: 232-235.

Avella, M., J. Berhaut and P. Payan, 1994. Primary cuture of gill epithelial cells from the sea bass *Dicentrarchus labrax*. *In Vitro* Cellular and Developmental Biology 30A: 41-49.

Bouchard, B., B. B. Fuller, S. Vijayasaradhi and A. N. Houghton, 1989. Induction of pigmentation in mouse fibroblasts by expression of human tyrosinase cDNA. Journal of Experimental Medicine 169:2029-2042.

Bowser, P. R., and J. A. Plumb, 1980. Fish cell lines: establishment of a line from ovaries of channel catfish. *In Vitro* 16: 365-368.

Faulmann, E., M. A. Cuchens, C. J. Lobb, N. M. Miller and L. W. Clem, 1983. An effective culture system for studying *in vitro* mitogenic responses of channel catfish lymphocytes. Transactions of the American Fisheries Society 112: 673-679.

Freshney, R. I., 1994. *Culture of animal cells: A manual of basic technique*. Wiley-Liss, New York.

Fryer, J. L., and C. N. Lannan, 1994. Three decades of fish cell culture: a current listing of cell lines derived from fishes. Journal of Tissue Culture Methods 16: 87-94.

Gallagher, D. S., and J. E. Womack, 1992. Chromosome conservation in the Bovidae. Journal of Heredity 83: 287-298.

Gay, S., G. R. Matin, P. K. Muller, R. Timple and K. Kuhn, 1976. Simultaneous ynthesisof Type I and III collagen by fibroblasts in culture. Proceeding of the National Acadeimic Sciences of USA 73: 4037-4040.

Howell, W. M., and D. A. Black, 1980. Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: A one step method. Experientia 36: 1014-1015.

Hynes, R., and K. M. Yamada, 1982. Fibronectins: Multifuctional modular glycoproteins. Journal of Cell Biology 95: 369-377.

Kang, J., and J. Caprio, 1995. *In vivo* responses of single olfactory receptor neurons in the channel catfish, *Ictalurus punctatus*. Journal of Neurophysiology 73: 172-177.

LeGrande, W. H., 1981. Chromosomal evolution in North American catfishes (Siluriformes: Ictaluridae) with particular emphasis on the madtoms, *Noturus*. Copeia 1981: 33-52.

LeGrande, W. H., R. A. Dunham and R. O. Smitherman, 1984. Karyology of three species of catfishes (Ictaluridae: *Ictalurus*) and four hybrid combinations. Copeia 1984: 873-878.

Luft, J. C., L. W. Clem and J. E. Bly, 1991. A serum-free medium for bony fish leukocyte *in vitro* mitogen-induced proliferation. Fish and Shellfish Immunology 1: 233-235.

Miller, N. W., and E. C. McKinney, 1994. *In vitro* culture of fish leukocytes, pp. 339-351 in *Biochemistry and molecular biology of fishes*, edited by Hochachka, and Mommensen. Elsevier Science B. V.

Miller, N. W., V. G. Chinchar and L. W. Clem, 1994a. Development of leukocyte cell lines from the channel catfish (*Ictalurus punctatus*). Journal of Tissue Culture Methods 16: 117-123.

Miller, N. W., M. A. Rycyzyn, M. R. Wilson, G. W. Warr, J. P. Naftel, and L. W. Clem, 1994b. Development and characterization of channel catfish long term B cell lines. Journal of Immunology 152: 2180-2189.

Miller, N. W., and L. W. Clem, 1988. A culture system for mitogen-induced proliferation of channel catfish (*Ictalurus punctatus*) peripheral blood lymphocytes. Journal of Tissue Culture Methods 11: 69-73.

Noga, E. J., 1980. Establishment of primary cell cultures using short-term trypsinization of organ fragments. Journal of Cell Culture Methods 6: 55-56.

Porter, K. R., and G. D. Pappas, 1959. Collagen formation by fibroblasts of the chick embryo dermis. Journal of Biophysics, Biochemistry and Cytology 5: 153-166.

Stickney, R. R., 1993. *Culture of non-salmonid fishes.* CRC Press, Boca Boton, Florida.

Thune, R. L, L. A. Stanley and R. K. Cooper, 1993. Pathogenesis of warm-water bacterial fish pathogens. Annual Review of Fish Diseases, 37-68.

Vallejo, A. N., C. F. Ellsaesser, N. W. Miller and L. W. Clem, 1991. Spontaneous development of functionally active long-term monocyte-like cell lines from channel catfish. *In Vitro* Cellular and Developmental Biology 27A: 279-286.

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.

Wise, G. E., F. Lin and W. Fan, 1992. Culture and characterization of dental follicle cells from rat molars. Cell Tissue Research 267: 483-492.

Wohlschlag, N., L. de los Snatos and P. F. Dehn, 1989. Preparation of primary cultures of adult and juvenile catfish (*Ictalurus punctatus*) hepatocytes. Journal of Tissue Culture Methods 12: 53-55.

Wolters, W. R., 1993. Channel catfish breeding and selection programs: Constraints and future prospects, pp. 82-95 in *Selective breeding of fishes in Asia and the United States*, edited by K. L. Main, and B. Reynolds. The Oceanic Institute, Honolulu, Hawaii.

Wolters, W. R., C. L. Chrisman and G. S. Libey, 1981. Lymphocyte culture for chromosomal analysis of channel catfish, *Ictalurus puntatus*. Copeia 1981: 503-504.

Yamada, K. M., 1978. Immunological characterization of a major transformation-sensitive fibroblast cell surface glycoprotein. Localization, redistribution, and role in cell shape. Journal of Cell Biology 78: 520-541.

Zhang, Q., and T. R. Tiersch, 1995. Cryopreservation of channel catfish (*Ictalurus punctatus*) leukocytes for subsequent cytogenetic analysis. Journal of Fish Biology 47: 1016-1025.

Zhang, Q., and T. R. Tiersch, in review. Cytogenetic analysis of two intergeneric hybrids of ictalurid catfishes.

Zhang, Q., T. R. Tiersch and R. K. Cooper, 1994. Rapid isolation of DNA for genetic screening of catfish by polymerase chain reaction. Transactions of the American Fisheries Society 123: 997-1001.

CHAPTER 2
GENOME CHARACTERIZATION OF CHANNEL CATFISH BY LOCATION OF NUCLEOLUS ORGANIZER REGIONS, C- AND RESTRICTION ENZYME- BANDING

Introduction

The analysis of chromosome morphology is fundamental to genome mapping. In higher vertebrates including humans, a variety of techniques have been developed, which facilitate precise identification of individual chromosomes (Verma and Babu, 1989). Chromatin arrangement along the chromosomes of ectothermic vertebrates such as fishes makes it difficult to obtain the high resolution banding (Bernardi *et al.* 1985; Medrano *et al.* 1988), and the small size and large numbers of fish chromosomes have created additional difficulties in chromosome banding (Thorgaard and Disney 1990).

To date, the techniques which work most successfully with fish chromosomes are staining for nucleolus organizer regions (NOR) (Phillips and Ihssen 1985; Amemiya and Gold 1988) and C-banding (Kiligerman and Bloom 1977a; Gold *et al.* 1986). Detection of the NOR is generally achieved with one of the silver nitrate methods (Howell and Black 1980), although DNA fluorochromes such as chromomycin A3 can differentiate NOR on the chromosomes of many lower vertebrates (Amemiya and Gold 1986; Amemiya and Gold 1987). Silver staining is valuable for identifying the site of active NOR in the chromosome set, while the chromomycin A3 appears to stain active and inactive (non-transcribed) NOR sites in fish chromosomes. Fluorescent *in-situ* hybridization (FISH) using labeled 28S rRNA gene probes is another method to detect

genes associated with the NOR of Atlantic salmon (Pendas *et al.* 1993). Variations in NOR sites were observed both among and within species, enabling phylogenetic studies (Amemiya and Gold, 1988).

The C-banding technique specifically stains constitutive heterochromatin (Arrighi and Hsu 1971), regions of transcriptionally inactive, highly repeated DNA sequences (Peacock *et al.* 1977; John and Miklos 1979). Distribution of C-bands is currently reported for more than 50 species of fishes (Gold *et al.* 1990). The primary emphasis of C-banding has been to document the existence and location of heterochromatin on fish chromosomes. In most cases, C-bands were dispersed throughout the entire chromosome complement (Gold *et al.* 1986; Takai and Ojima 1988; Rab *et al.* 1991) with concentrations on centromeric or telomeric regions. The most extensive C-banding work has been done in salmonid fishes (Phillips and Hartley 1988; Pleyte *et al.* 1989), in which bands were found at interstitial regions and telomeres. However, there are notable exceptions such as *Channa argus*, *C. asiatica*, and *C. maculata* (Li *et al.* 1985), *Symphodus melops* and *S. roissali* (Lopez *et al.* 1989), and many percid species (Mayr *et al.* 1987) in which constitutive heterochromatic regions appeared small, well resolved and primarily centromeric. The C-banding can be useful for identifying homologous chromosomes and, in some cases, for identifying sex chromosomes (Haaf and Schmid 1984). However, C-banding usually does not produce linear patterns on the chromosome arms, which limits usefulness for many other studies such as physical mapping.

The difficulty encountered in serial banding techniques that rely on degradation of structural proteins has forced fish geneticists to employ other techniques such as

restriction enzymes (RE) that recognize and cut specific 4-to-8 base sequences of DNA nucleotides. Digestion of whole chromosomes by REs leads to removal of some DNA fragments, and Giemsa staining can be used to reveal the relative amount and location of the remaining DNA (Miller and Miller 1990). The RE-banding patterns are reproducible (Miller and Miller, 1990), and could produce chromosome banding in species such as fishes that do not band by other methods. Some REs have produced patterns similar to G-banding and modified C-banding in higher vertebrates such as human (Miller *et al.* 1983; Babu and Verma 1986a), mouse (Kaelbling *et al.* 1984), and muntjak (Babu and Verma 1986b; Lima-de-Faria *et al.* 1980). Application of RE-banding techniques in lower vertebrates such as amphibians (Schmid and de Almeida 1988) and fishes (Lloyd and Thorgaard 1988) has yielded some success: reproducible C-band-like patterns have been observed in chromosomes of salmonid fishes (Lloyd and Thorgaard 1988; Hartley 1991a; Hartley 1991b) and cartilaginous fishes (Stingo *et al.* 1995), and with a few cases G-band-like patterns were produced in European eel (Vinas *et al.* 1994).

Although channel catfish (*Ictalurus punctatus*) has become the most important culture species in the United States (Wolters 1993), genetic studies on this species lags behind that of livestock, and even other cultured fishes such as salmonids. The diploid chromosome number of channel catfish is 58 (LeGrande 1981, Wolters *et al.* 1981) and has been confirmed by intergeneric hybridization (LeGrande *et al.* 1984; Zhang and Tiersch in review). This article presents NOR-banding and C-banding of channel catfish chromosomes and the banding patterns of chromosomes treated by ten different restriction enzymes. A computer-based image analysis system was used to automate the chromosome measurements and to assist the process of karyotyping. My objectives

were to: (1) document NOR-phenotype of channel catfish and evaluate its stability among different fishes, (2) document C-banding of channel catfish and use it for homologous pairing, (3) develop a standard karyotype and ideogram of channel catfish, and (4) analyze the banding patterns of chromosomes treated by 10 restriction enzymes.

Materials and methods

Animals

Channel catfish used in this experiment were from a population maintained at LSU. They were artificially spawned and reared in indoor recirculating systems (Tiersch *et al.* 1994). The size of fish ranged from 2.5 g (fingerlings) to 1.8 kg (adults). A total of 22 fish were used for preparation of metaphase chromosomes and general karyotyping, of these, 10 were studied for localization of the NOR, and 5 were used for C-banding and RE-banding.

Chromosome preparation

Chromosomes were prepared from primary cultures of leukocytes isolated from peripheral blood of adult fish and from primary cultures of kidney cells or cultured fibroblast cells for fingerlings. Chromosomes also were prepared directly from kidney cells or epithelial cells.

(1) Leukocyte cultures. Leukocytes were isolated from 3 ml of whole blood by density centrifugation (Zhang and Tiersch 1995). Cells were cultured in 25-cm^2 flasks (Falcon Plastics, Becton Dickinson Inc., Franklin Lakes, NJ) with Leibovitz L15 medium (Gibco Laboratory, Grand Island, NY) adjusted to the osmotic pressure of catfish blood plasma (Miller and Clem 1988) and supplied with penicillin (100 μg/ml, Gibco), streptomycin (100 U/ml, Gibco), gentamicin (10 μg/ml, Gibco), Hepes buffer (25 mM,

Sigma Chemical Company, St. Louis, MO), and 10% fetal bovine serum (FBS) (Sigma) and 5% catfish serum. Prepartion of catfish serum was based on the previously described procedure (Miller and Clem 1988). Leukocytes were stimulated for mitosis by addition of 0.05 μg/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) and 0.5 μg/ml calcium ionophore A23187 (Sigma). The flasks were sealed and incubated at 27 °C. After 24 h, the medium was replaced with fresh medium containing no PMA or A23187. The cultures were incubated for 48 to 72 h and mitotic activity was arrested by addition of 0.4 μg/ml colchicine solution and incubation for 30 min to 1 h. The cells were harvested and processed for chromosomes by a standard method: hypotonic treatment with 0.075M KCl and cold fixation with Carnoy's fixative (Zhang and Tiersch 1995).

(2) Culture of kidney cells. Fish were anesthetized with tricaine methanesulfonate (MS-222) (Argent Chemicla Laboratory, Redmond, WA), and placed in a sterile laminar-flow hood (Model 51000-00, LABCONCO, Kansas City, MO). The outer surface of fish was sterilized with 10% bleach followed by 70% ethanol. Kidney tissue (anterior and posterior portions) was removed by sterile dissection and placed in pre-cooled Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (CMF-PBS). The kidney tissues were cut into small fragments, pressed through a 70-μm cell strainer (Falcon) using a sterile syringe plunger, and the cells rinsed with CMF-PBS. The cell suspensions were collected into 15-ml centrifuge tubes and spun at 300x g (Sorvall Centrifuge GLC-2B, DuPont, Wilminton, DE) for 3 to 5 min. The pellets were washed twice with CMF-PBS and resuspended in 1 ml of Leibovitz L15 complete medium (described above). The cells were cultured in the L15 medium under the same conditions used for leukocytes. The mitosis of cultured cells were stimulated by addition of 5 mg/ml

concanavalin A or by addition of PMA and A23187. The remaining steps for preparation of chromosomes were the same as described above.

(3) Culture of fibroblast cells. The methods for culture of channel catfish fibroblast cells have been described in Chapter 1. Chromosomes were prepared from cultures that had been incubated for 3 d and had a 70% to 80% confluence. No mitogens were used. The procedures for preparing chromosomes were the same as described above except that the cell monolayer was trypsinized and broken up through repeated pipetting before hypotonic treatment and fixation.

(4) Chromosome preparation from solid tissues. I used standard methods for preparing chromosomes directly from kidney tissues (LeGrande 1981); however, fish were injected with pokeweed mitogen (PM) to increase the mitotic activity of the kidney cells (Zhang and Tiersch in review). Chromosomes were prepared from epithelial cells of caudal fin based on the method of Kligerman and Bloom (1977b). Larval fish were placed in water containing 0.005% colchicine for 4 h before use.

Chromosome banding

(1) Staining of the Nuclear Organizer Regions. Staining of the NOR was based on the AgNOR procedure of Howell and Black (1980). Slides were covered with a solution of 30% silver nitrate and 1.5% gelatin (Fisher Scientific, Pittsburgh, PA) and incubated at 50 °C for 8 to 10 min. The slides were rinsed briefly with deionized water, and dried at room temperature. The NOR-bearing chromosomes from different spreads were compared by partial karyotyping.

(2) C-banding. The CBG (C-bands by barium hydroxide using Giemsa) banding procedure was based on Sumner's method (1972). Metaphase spreads were

aged on glass slides by incubating at 65 °C for at least 3 d before experiments. The slides were placed in 0.2 N HCl at room temperature for 30 min to 1 h, in 5% $Ba(OH)_2$ at 50 °C for 7 to 10 min, and in 2-x SSC solution at 60 °C for 2 to 4 h. Between steps, the slides were rinsed with deionized water, and air-dried. A complete dehydration series was carried out between the $Ba(OH)_2$ and SSC treatments and before Giemsa staining by dipping slides through 70%, 85%, 95%, and 100% ethanol. Slides were stained with 6% Giemsa (in 0.01 M phosphate buffer, pH 6.8) for 20 min, placed on a slide dryer (Model 77, Fisher Scientific, Pittsburgh, PA) at 40 °C overnight, cleared with xylene (Sigma), and mounted in Permount® solution (Fisher).

(3) Restriction enzyme banding. Ten restriction enzymes were used in this study: *Bam*H I, *Bgl* I, *Eco*R I, *Hin*d III, *Mbo* I, *Msc* I, *Nde* I, *Not* I, *Pvu* II, and *Sau*3A I. The enzymes were diluted to final concentrations of 0.5 to 1.2 U/μl in buffers supplied by the manufacturer (New England Biolabs, Beverly, MA). Each slide was covered with 20 μl of enzyme solution, and incubated in a humid chamber at 37 °C for 3 h. Slides were washed with PBS followed with distilled water, and air-dried. The slides were stained with 5% Giemsa for 20 min. Control slides treated with enzyme buffer but no enzyme were included in each experiment.

(4) Replication banding. Chromosomes were prepared from cultured leukocytes, which was synchronized with fluorouracil and released and labeled with bromodeoxyuridine. The banding pattern was revealed by staining with fluorochrome plus Giemsa. The details for these procedures were described in Chapter 3.

Computer-assisted chromosome analysis

Karyotyping was conducted with the Optimas® and Kary® computer software packages (Bioscan, Inc., Edmonds, WA), as described by Zhang and Tiersch (in review). Total length and arm length of each chromosome was measured. Karyotypes were prepared for unbanded and CBG-banded chromosomes based on the measurements of relative length (or percent of total complement length, %TCL) and centromeric index (CI), which were calculated for each chromosome based on the following formulae:

TCL (%) = (length of the chromosome pair/total comlement length) x 100

CI (%) = (length of short arm/total length of the chromosome) x 100

Chromosome classification was based on Levan's method (1964) in which the CI is 37.5-50% for metacentrics, 25-37.5% for submetacentrics, 12.5-25% for subtelocentrics, and 0-12.5% for telocentrics.

Chromosomes treated with restriction enzymes were sorted by descending order of size and paired based on their banding pattern. The banding pattern of *Hind* III-and *Msc* I-treated chromosomes was analyzed by densitometry; chromosomes treated with *Hind* III were compared with those prepared by replication banding method (Chapter 3). The X- axis of densitomeric plot stands for chromosome segment and Y-axis grayscale value, from 0 (black) to 256 (white).

Ideograms were prepared by the Microsoft PowerPoint® computer software based on the measurements of %TCL and CI, the NOR-, C-, and restriction

enzyme- banding patterns. For high resolution analysis of banding patterns chromosomes were photographed with a microscope-mounted (Microphot-SA, Nikon Inc.) Nikon FX-35DX camera (Nikon Inc., Garden City, NY) and Kodak Technical Pan 2415 film. The negatives were scanned into computer with a slide scanner (SprintScan 35, Poraloid scanner model CS-2700, Needham Heights, MA) for analysis.

Results

The general morphology of metaphase chromosomes was revealed by Giemsa staining (Fig 2.1 a). The NOR were found on a pair of medium-sized of submetacentric chromosomes (Fig 2.1 b) in each metaphase spread, and the %TCL and CI (mean ± SD) of this chromosome were 3.6 ± 0.1 and 34.6 ± 2.0 (Fig. 2.2).

The 29 pairs of homologous chromosomes were divided into 8 groups based on size and centromeric position (Table 2.1, Fig. 2.3 a). For comparison purposes, a

Table 2.1. The grouping system of channel catfish chromosomes.

Group	Size	Morphology	Number of chromosomes
A	Large	Submetacentric	2
B	Large	Subtelocentric	3
C	Medium	Metacentric	3
D	Medium	Submetacentric	5
E	Medium	Subtelocentric	5
F	Medium	Telocentric	2
G	Small	Metacentric	5
H	Small	Submetacentric	4
		Total	29

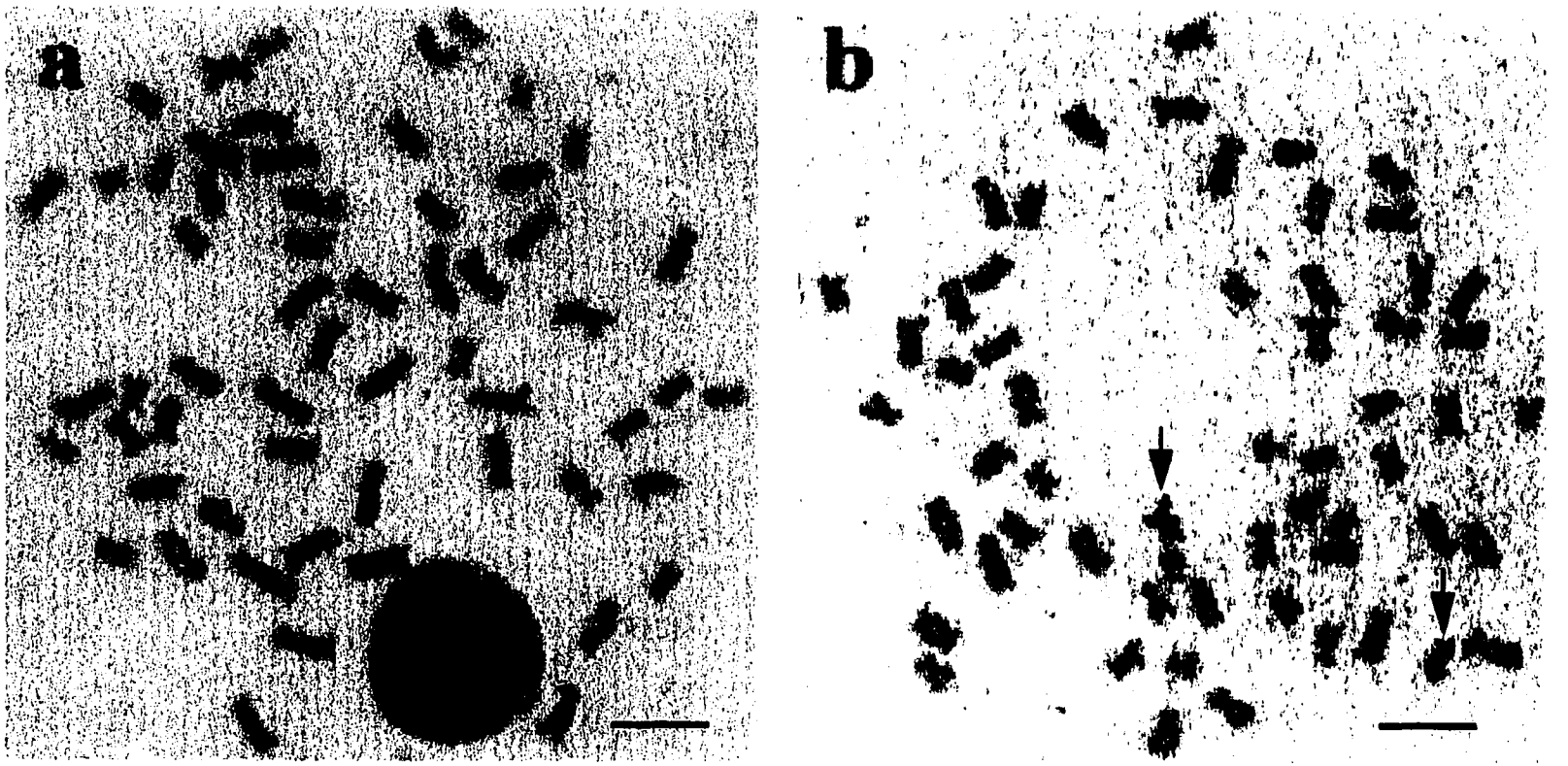
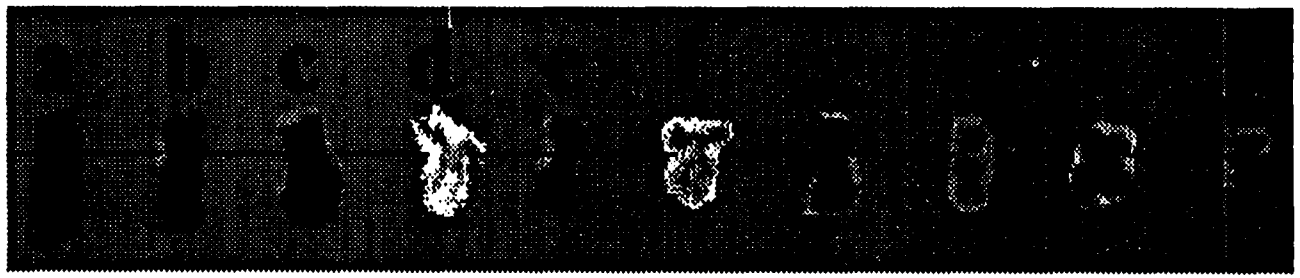


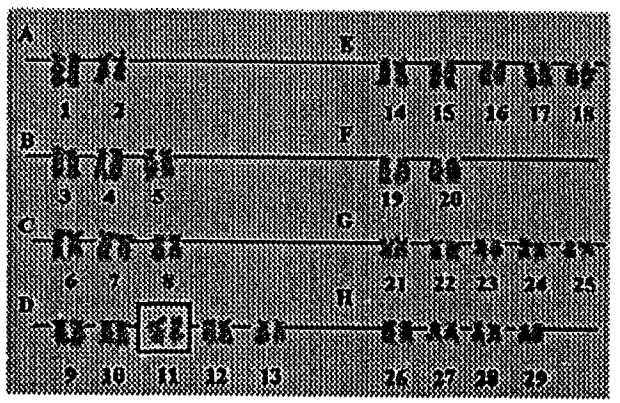
Figure 2.1. Giemsa staining (a) and silver staining (b) of channel catfish metaphase chromosomes. Arrowheads point to location of nucleolus organizer regions. Bars = 10 μ.



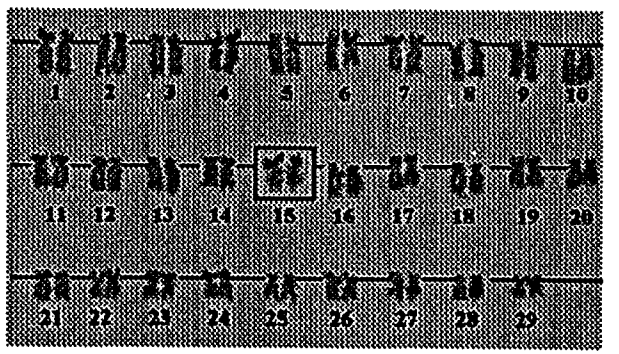
											$\bar{X} \pm SD$
%TCL	3.8	3.7	3.5	3.8	3.5	3.6	3.4	3.4	3.5	3.6	3.6 ± 0.1
CI	35.0	35.2	36.1	35.5	31.9	33.4	35.3	31.0	38.0	34.5	34.6 ± 2.0

Figure 2.2. Partial karyotype and measurements of the NOR-bearing chromosome derived from metaphase spreads of 10 channel catfish. Abbreviations: %TCL = percentage of total complement length; CI = centromeric index (x 1000).

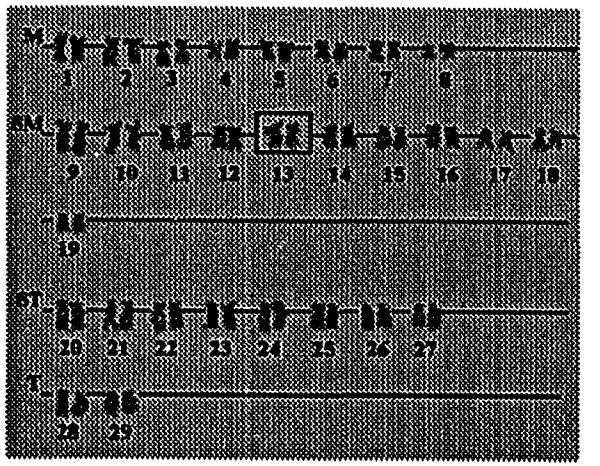
Figure 2.3. A standard karyotype of the channel catfish with indication of the NOR-bearing chromosome. Chromosomes were sorted by centromeric index and size, and divided into 8 groups: A, large submetacentric; B, large subtelocentric; C, medium centromeric; D, medium submetacentric; E, medium subtelocentric; F, telocentric; G, small metacentric; and H, small submetacentric (a). Previous systems used for channel catfish include size-based (b) and morphology-based (c) karyograms prepared from the same spread for comparison. Location of individual chromosomes in the three karyotyping systems are identified (d). The NOR-bearing chromosome (in boxes) is number 11 in (a), 15 in (b), and 13 in (c).



(a)



(b)



(c)

Size and Morphology-based method[1]	Size-based method[2]	Morphology-based method[3]	Size and Morphology-based method[1]	Size-based method[2]	Morphology-based method[3]	Size and Morphology-based method[1]	Size-based method[2]	Morphology - based method[3]
1	1	9	11	15	13	21	22	4
2	4	10	12	19	14	22	26	5
3	3	20	13	20	15	23	27	6
4	2	21	14	8	23	24	23	7
5	5	22	15	9	24	25	29	8
6	6	1	16	12	25	26	21	16
7	7	2	17	13	26	27	25	17
8	17	3	18	18	27	28	24	18
9	11	11	19	10	28	29	28	19
10	14	12	20	16	29			

[1] This study.
[2] Chromosomes were arranged by descending size order.
[3] LeGrande, 1981.

(d)

representative spread was organized by descending size (Fig. 2.3 b) and previously described (LeGrande 1981) morphology and centromere-based (Fig. 2.3 c) systems. The location of each chromosome was different in these three karyotyping systems (Fig. 2.3 d).

The C-banding revealed prominent and highly resolved bands at the centromeres of most chromosomes. It varied little among spreads from different specimens (Fig. 2.4). The smallest metacentric (G25) and submetacentric (H29) chromosomes did not show centromeric C-bands (Fig. 2.5) Chromosome 16 had an identifiable non-centromeric C-band on the short arm (Fig. 2.5). The C-banding patterns agreed between homologous chromosomes (Fig. 2.5). The location of NOR and C-banding was summarized in an ideogram (Fig. 2.6), prepared from 20 metaphase spreads.

Among the 10 restriction enzymes used in this study (Table 2.2), *Bam*H I, *Eco*R I and *Mbo* I produced one or two bands on some of chromosomes, which was similar to what was found in conventional C-banding. However, location of major bands was different and not limited to certain region of the chromosomes. Bands generated by *Bam*H I were more evident in the telomeres whereas bands generated by *Eco*R I were distributed mostly in centromeric regions. Bands created by *Mbo* I were not found on all chromosomes, and were distributed on centromeric and telomeric regions.

The enzymes *Bgl* I and *Pvu* II produced chromosomal bands in interstitial regions. Chromosomes digested by *Bgl* I generally had 2 or more elongated pericentric bands, which sometimes extended to the ends of the chromosomes. The centromeric regions were degraded and faintly stained. In contrast, chromosomes treated by *Pvu* II

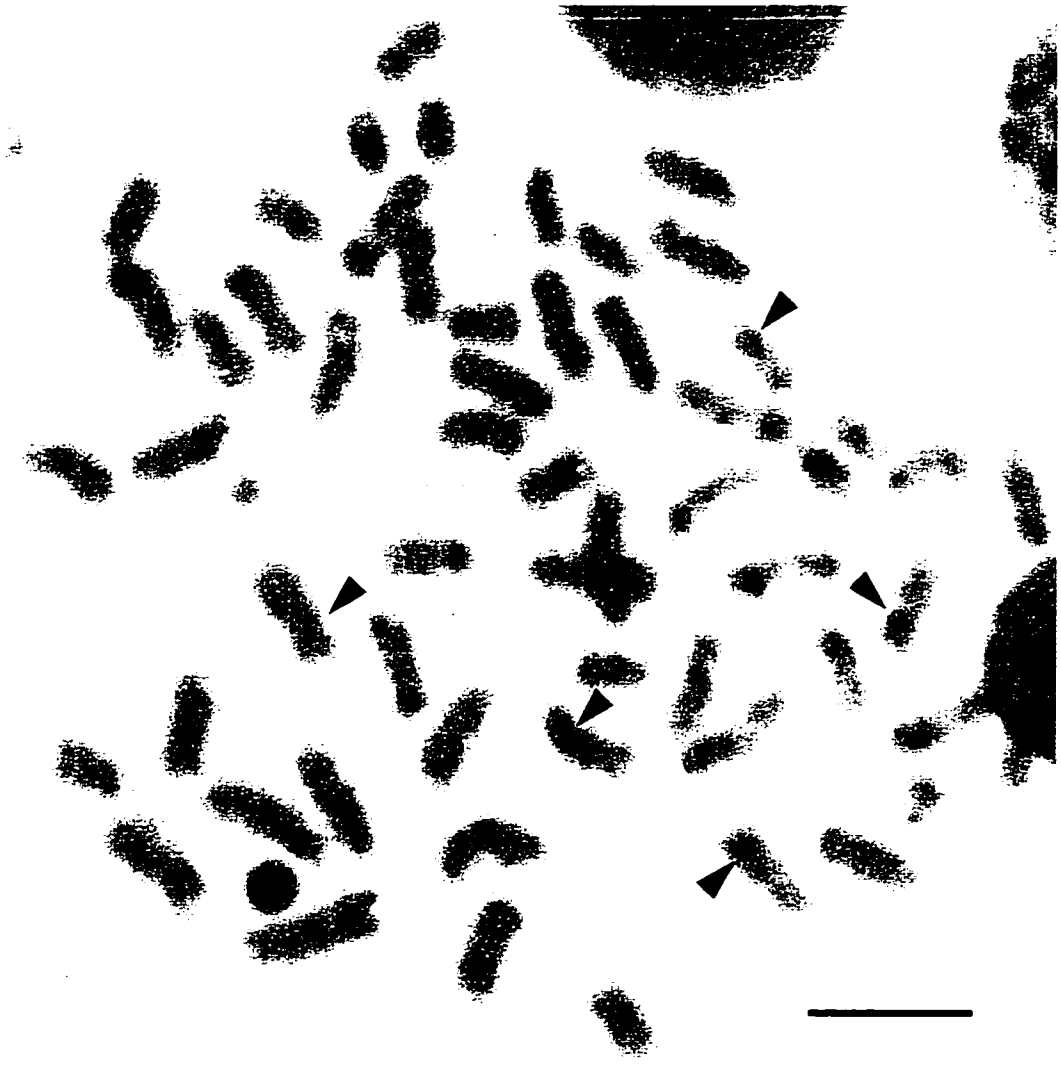


Figure 2.4. Channel catfish chromosomes treated with $Ba(OH)_2$ and stained for heterochromatin. Most chromosomes had a single well-defined C-band (arrowheads) located in the area of the centromere. Bars = 10 μ.

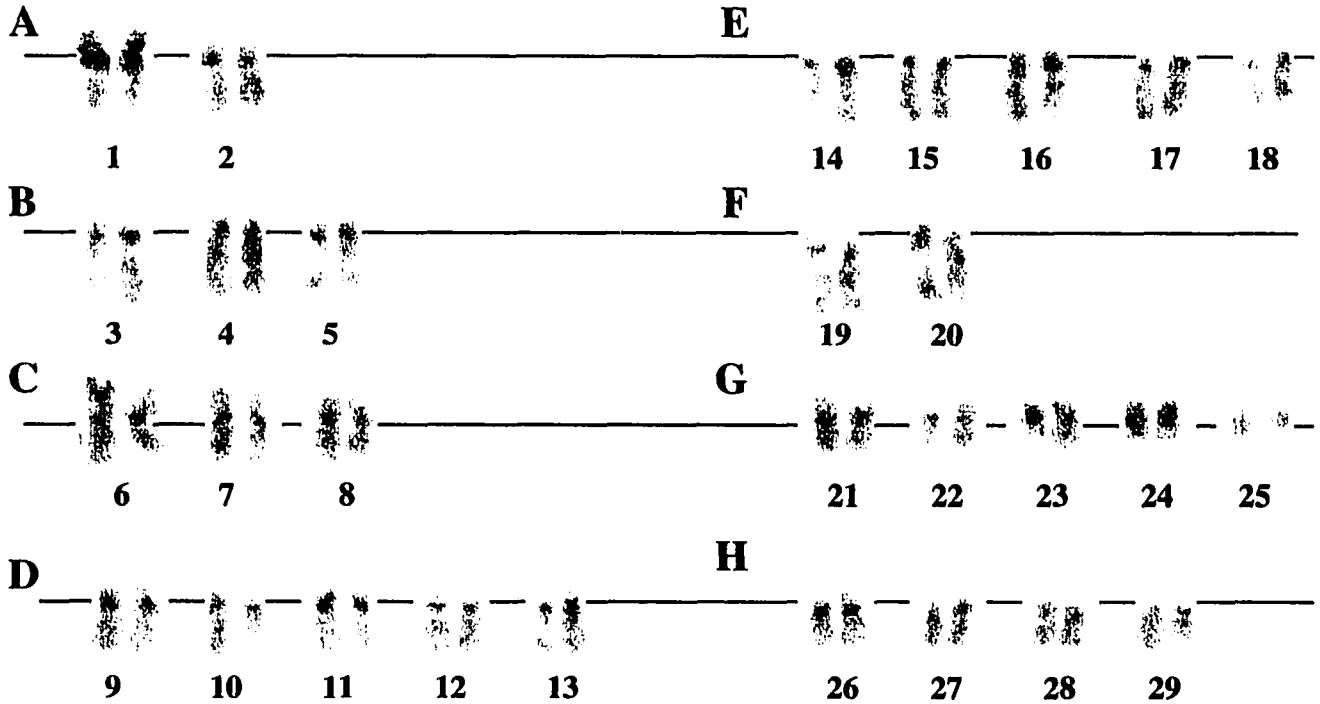


Figure 2.5. A karyotype of CBG-banded (C-bands by barium hydroxide using Giemsa) chromosomes of channel catfish (note: the band on short arm of chromosome 6 is a technical artifact, resulting from overlap with chromosome 20 in the original spread) (x 1000).

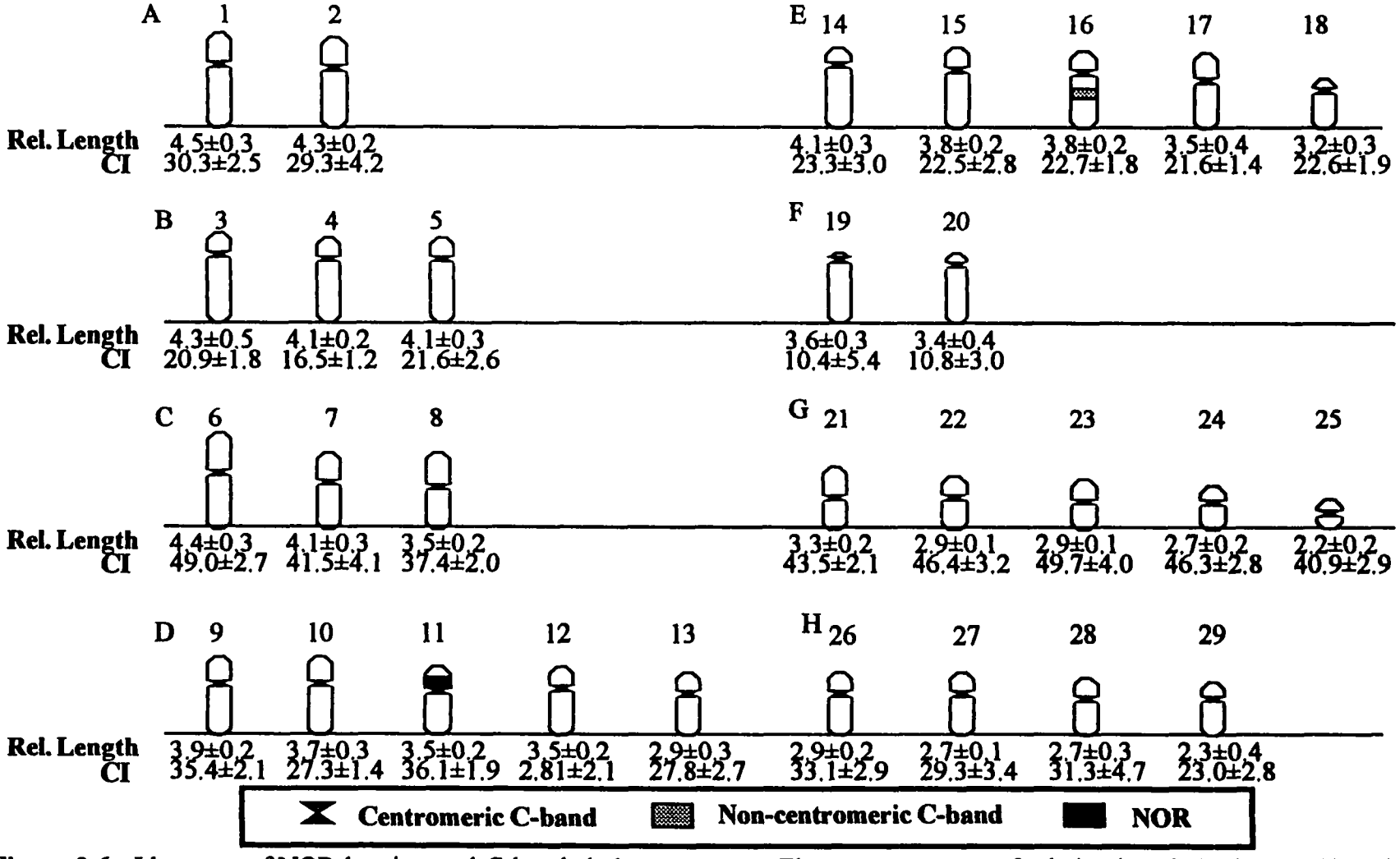


Figure 2.6. Ideogram of NOR-bearing and C-banded chromosomes. The measurements of relative length (Rel. Length) and centromeric index (CI) for each chromosome were derived from data of 20 spreads from 5 channel catfish.

Table 2.2. Banding patterns of channel catfish chromosomes treated with restriction enzymes in comparison with those found in salmonids, amphibians, muntjak, rodents, and humans.

Enzyme	Recognition sequence	Concentration (unit/μl)	Chromosome banding pattern					
			Channel catfish	Salmonids[1]	Amphibians[2]	Muntjac[3]	Rodents[4,5]	Humans[6]
BamH I	GGATCC	1.0	C (telomeric)	-[8]	-	-	-	none
Bgl I	GCCNNNNNGGC	1.2	C (pericentric)	-	-	-	-	-
EcoR I	GAATTC	1.2	C (centromeric)	none	-	none	-	G
Hind III	AAGCTT	1.0	replication band	none	C	-	reverse C, C, G	G
Msc I	TGGCCA	0.3	C (mostly telomeric)	-	-	-	-	-
Mbo I	GATC	0.5	C (centro- or telomeric)	C	-	C	-C	C(G)
Nde I	CATATG	0.5	irregular	-	-	-	-	-
Not I	GCGGCCGC	1.0	none[7]	-	-	-	-	-
Pvu II	CAGCTG	1.0	interstitial	C	-	-	-	C
Sau3A I	GATC	0.4	none	-	-	C	-	-

1. Lloyd and Thorgaard, 1988 ; Hartley, 1991a, Hartley, 1991b.; 2. Schmid and de Almeida, 1988;
3. Lima-de-Faria *et al.*, 1980; Babu and Verma, 1986b; 4. Walker and Providell, 1995;
5. Kaelbling *et al.*, 1984; 6. Miller *et al*, 1983; Miller and Miller, 1990;
7. Uniform staining; 8. Not studied.

resulted in relatively few bands. One other enzyme *Nde* I generated inconsistent banding patterns along chromosomes and resulted in a fuzzy appearance. The enzymes *Not* I and *Sau*3A I did not produce any bands, and the chromosomes exhibited uniform staining with Giemsa.

More information was derived from chromosomes treated with *Hind* III (Figs. 2.7 and 2.8) and *Msc* I (Fig. 2.9). Although centromeric regions of these chromosomes were difficult to identify, there was little difficulty in karyotyping; chromosomes were arranged by descending size and homologous pairs were determined by size and banding patterns. Serial bands were produced on most chromosomes treated by *Hind* III, and the banding pattern was similar to replication R-banding for many chromosomes (Fig. 2.10). The banding patterns were consistent between members of homologous pairs. Chromosomes treated with *Msc* I yielded a clear region on the centromere of many chromosomes. Bands were found mostly on the telomeric regions, and agreed between members of homologous pairs.

Discussion

In this study, a variety of chromosome preparation methods were introduced for cytogenetic study of ictalurid catfishes from embryonic to adult stages. The NOR of channel catfish was localized by comparative study of chromosomes prepared from different specimens. The highly reproducible C-banding and restriction enzyme-banding are reported for first time for this species and for the family Ictaluridae.

Symmetric (or homomorphic) NOR-phenotypes were found in channel catfish and other species such as black bullhead (*Ameiurus melas*) (Zhang and Tiersch in

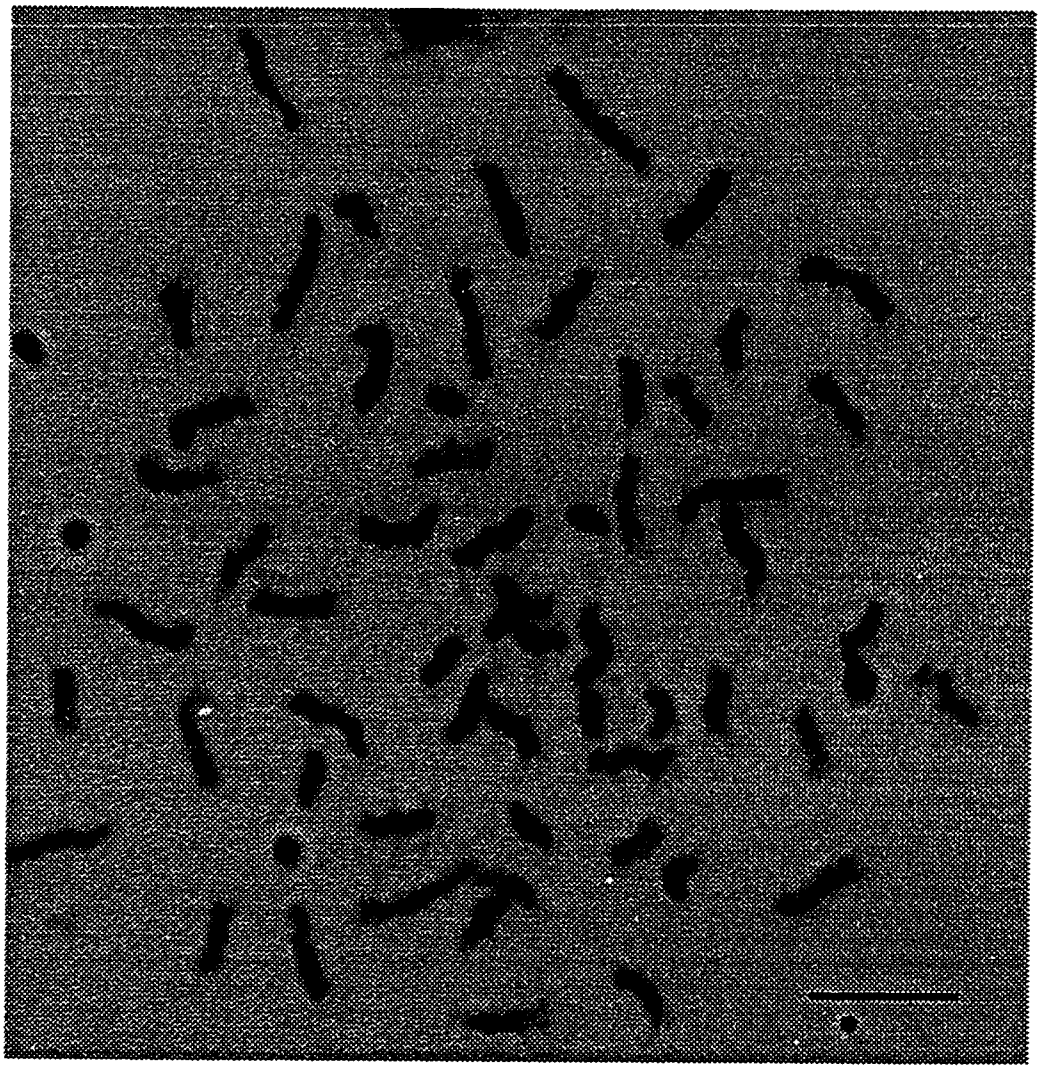


Figure 2.7. Channel catfish chromosomes treated by restriction enzyme *Hind* III. Bar = 10 μ.

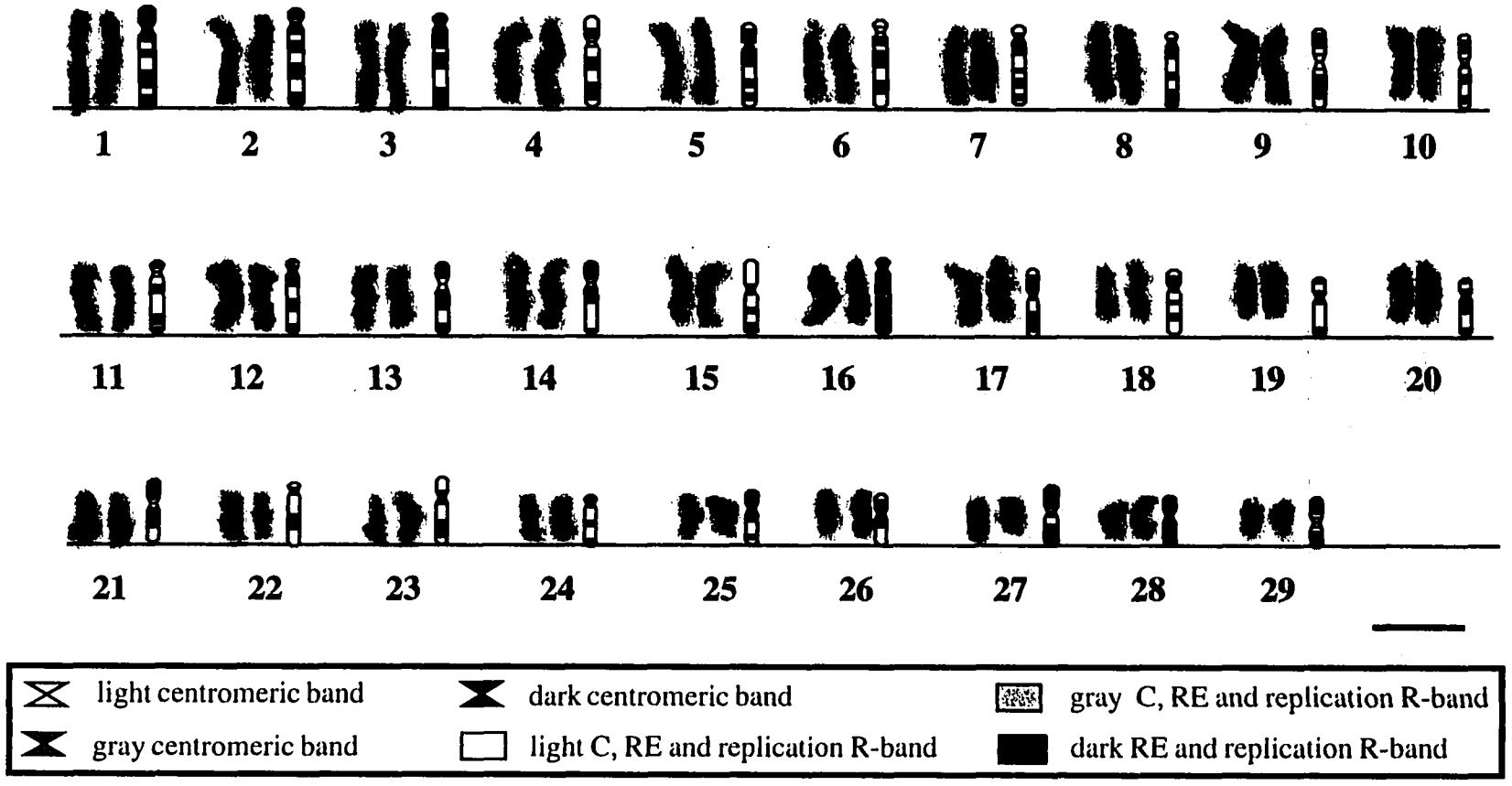


Figure 2.8. A karyotype of channel catfish chromosomes treated with the restriction enzyme *Hind* III. The banded chromosomes were arranged by descending size and ideograms were assembled for each chromosome. Bar = 10 μ.

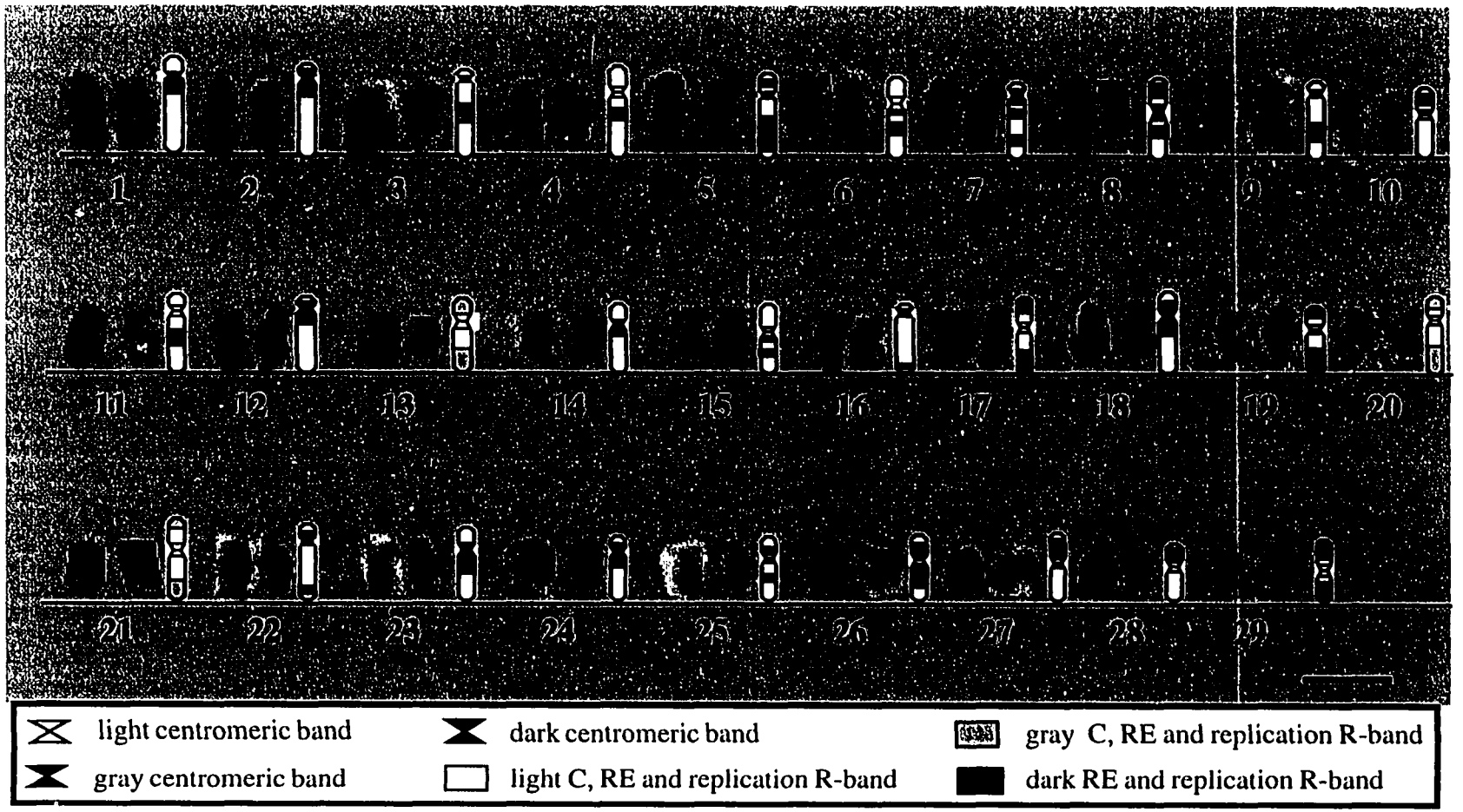
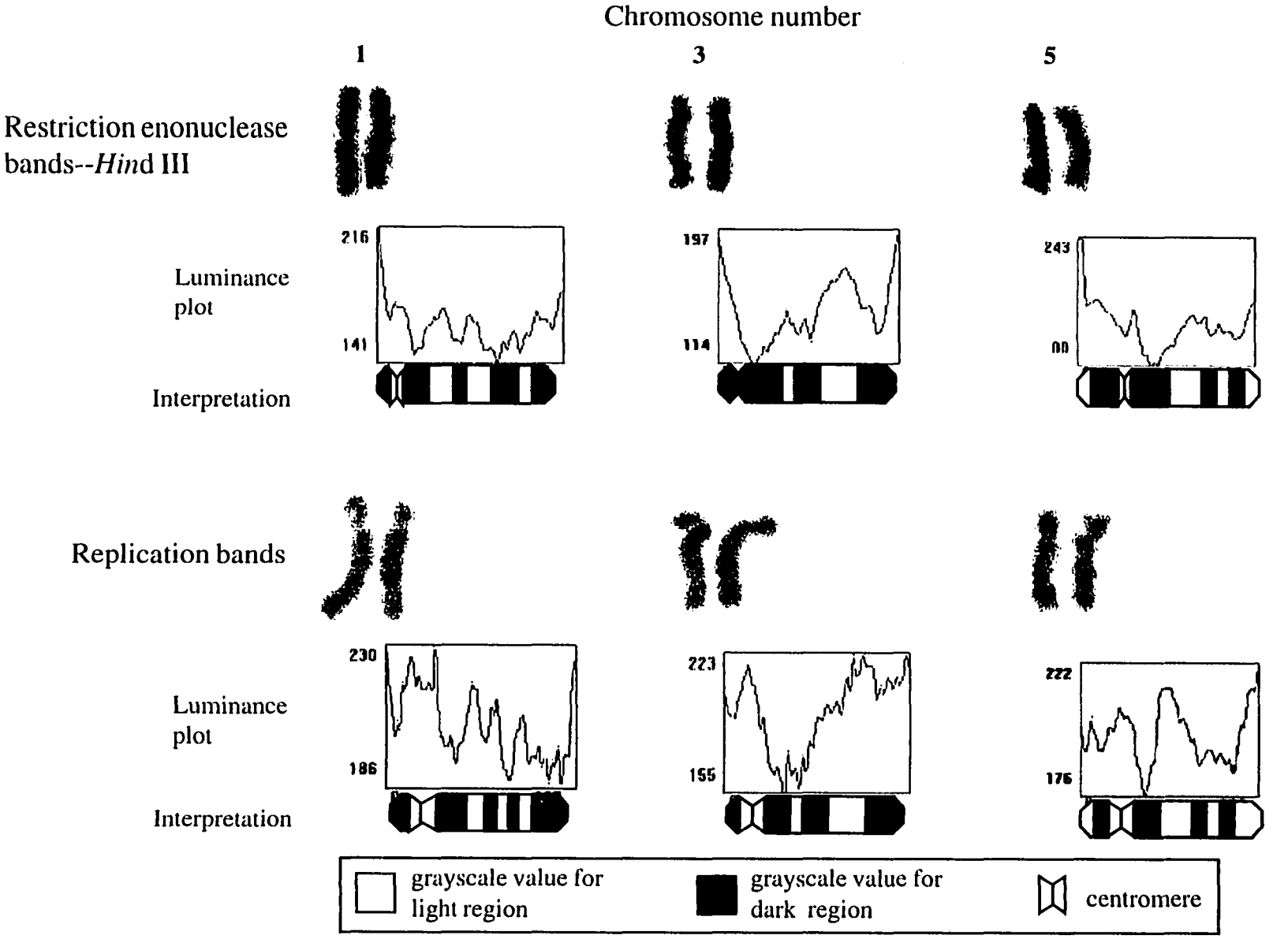


Figure 2.9. A karyotype of *Msc* I-treated channel catfish chromosomes. The banded chromosomes were arranged by descending size and ideograms were assembled for each chromosome. Bar = 10 μ.

Figure 2.10. Comparison of *Hind* III-treated chromosomes with replication R-banded chromosomes. The banding patterns of represented chromosomes (chromosome 1, 3, and 5) were analyzed by densitometry, and the results shown as line charts, in which the X-axis indicates relative grayscale value. Ideograms were created to represent the banding pattern of each chromosome.



review). Because the target molecules of silver staining are proteins associated with transcriptional activity of ribosomal RNA genes, variation in NOR-phenotypes can be caused by functionally inactive NOR sites. In fact, intraspecific variations were detected in ~10-13% of north American cyprinids (Buth *et al.* 1990), presumably from asymmetrical NOR activity. Many other interspecific and intraspecific variations, however, were qualitative and could serve as taxonomic and systematic characters. In the family Ictaluridae, asymmetric NOR activity exists at least in flathead catfish (*Pylodictis olivaris*) (Zhang and Tiersch in review).

The stability of the NOR phenotype in channel catfish was verified by analysis of chromosomes from various cell types (leukocytes, kidney cells, fibroblasts, and epithelial cells) and from specimens of different ages, and substantiated by examination of interspecific hybrids (Zhang and Tiersch in review). Two intergeneric hybrids, between channel catfish (2N = 58) and black bullhead (2N = 60), and between channel catfish and flathead catfish (2N = 56), were produced through artificial fertilization. These hybrids possess odd diploid numbers (2N = 59 or 57) and have unpaired and therefore heteromorphic NOR-bearing chromosomes in each of their metaphase spreads (Zhang and Tiersch in review).

The nomenclature system applied in this study is based on the system used for humans (Passarge 1974), and was useful for identification of non-banded chromosomes of channel catfish. Sorting by descending size regardless of morphology is an old technique for fish chromosomes. It is applicable when centromeres are not identifiable, or when the chromosomes are all of distinct sizes. This method can be done automatically by computer-based image analysis systems. However, the size-based

method did not separate channel catfish chromosomes with distinct morphology because of size overlap between different groups. Sorting by centromeric index reduced standard deviation in averaging data collected from different spreads (data not shown). A centromere-based method has been used on most cytogenetic work of fishes, including channel catfish (LeGrande, 1981). The nomenclature system developed in this study uses a combination of the size and centromere-based methods. Chromosomes with similar centromeric indices were grouped, and then split into subgroups based on size differences.

The C-banding pattern of channel catfish was small and limited to centromeric regions which was similar to results in rainbow trout (*Oncorhynchus mykiss*) (Thorgaard and Disney 1990). There were no distinct secondary bands on any catfish chromosome except E16. The C-bands were prominent and useful for identifying homologous chromosomes. Low abundance of heterochromatin may explain the stable genome size found in this species (Tiersch and Goudie 1993). The genome size of channel catfish was 1.98 pg, and was not different among 14 populations studied (Tiersch *et al.* 1990).

The mechanism of C-banding by alkali treatment has been suggested to result from 2 successive stages: DNA denaturation by HCl and $Ba(OH)_2$ and DNA removal by incubation in 2-x SSC (Verma and Babu 1989). However, it is not known how centromeric DNA was protected from degradation. It was found in this study (data not shown), that packaging of protein in non-centromeric regions could have been eliminated by $Ba(OH)_2$ treatment. This was suggested based on repeated failure in locating the NOR on C-banded chromosomes by silver staining.

The development of restriction enzyme banding was initiated to aid in understanding the mechanisms of traditional chromosome banding at the molecular level. In many cases, the production of enzyme-resistant bands can be interpreted by DNA structure in that region (Miller and Miller 1990). The factors proposed to be responsible for differential staining of metaphase chromosomes following treatment with restriction enzymes are differences in nucleotide sequence along the chromosomes, making some DNA susceptible to digestion (Miller 1983, 1984; Bianchi *et al.* 1985), and/or differences in chromatin structure, making DNA more susceptible to digestion in some regions (Mezzanotte and Ferrucci 1984; Vinas *et al.* 1994). The removal of DNA from chromosomes by REs has been demonstrated by *in-situ* isotope-labeling (Lloyd and Thorgaard 1988). The major limitation of this technique lies in certain structural aspects involving the accessibility of REs to DNA, or in chromatin removal. Therefore, it is sometimes hard to correlate chromosomal DNA composition to RE-banding patterns.

It was found in this study that banding patterns of some restriction enzymes were not correlated with their recognition sequences (Table 2.2); this might be due to different sensitivity of these enzymes to nucleotide modification (methylation) of the recognition sites. For example, the recognition sequences of *Mbo* I, and *Sau*3A I are each GATC, but the resultant banding patterns by these enzymes were distinct. The *Mbo* I produced modified C-bands while the *Sau*3A I did not produce any recognizable bands. Meanwhile, enzymes with different recognition sequences may not be able to produce distinct banding patterns because size difference of DNA fragments removed may not be sufficient to be resolved under light microscope. The enzymes *Bam*H I (6 base-pair

cutter) and *Mbo* I (4 base-pair cutter) share the target sequence element GATC; the banding patterns produced by these two enzymes were not distinguishable.

The most valuable aspect of RE treatment is that it produces reproducible banding patterns. The possibility of generating linear structural banding by restriction enzymes was demonstrated in this study. It has been very difficult to produce such banding on catfish chromosomes. Although numerous G-banding techniques were attempted in this study (e.g., G-bands by trypsin using Giemsa or GTG) (data not shown), inconsistent or no bands were produced. Compared with conventional G-banding, the RE-banding procedure is simpler. From this study it appears that the critical steps are RE concentration, incubation time, temperature, and inclusion of control treatments of restriction buffer without enzymes in each trial. Prolonged incubation with enzyme, or even with buffer only, resulted in a diffuse appearance of chromosomes.

The application of RE-banding on fish chromosomes requires further development. Of the reports addressing this topic, most enzymes produced modified C-bands. Furthermore, there was extensive variation from species to species. For example, *Hin*d III which generated a serial banding pattern for channel catfish in this study, did not produce any bands for rainbow trout (Lloyd and Thorgaard, 1988). The RE-banding could serve as an alternative to replication banding for use in coupling with *in-situ* hybridization (ISH). The target gene-bearing chromosomes could be identified before or after ISH by treatment with approriate restriction enzymes.

References cited in Chapter 2

Amemiya, C. T., and J. R. Gold, 1988. Chromosomal NORs as taxonomic and systematic characters in North American cyprinid fishes. Genetics 76: 81-90.

Amemiya, C. T., and J. R. Gold, 1987. Chromomycin staining of vertebrate chromosomes: enhancement of bandingpatterns by NaOH. Cytobios 49: 147-152.

Amemiya, C. T., and J. R. Gold, 1986. Chromomycin A3 stains nucleolus organizer regions of fish chromosomes. Copeia 1986: 226-231.

Arrighi, S. M., and T. C. Hsu, 1971. Localization of heterochromatin in human chromosomes. Cytogenetics 10: 81-86.

Babu, A., and R. S. Verma, 1986a. Characterization of human chromosomal constitutive heterochromatin. Canadian Journal of Genetics and Cytology 28: 631-645.

Babu, A., and R. S. Verma, 1986b. Expression of heterochromatin by restriction enzyme treatment and distamycin A/DAPI staining of Indian muntjak (*Muntiacus muntjak*) chromosomes. Cytogenetics and Cell Genetics 41: 96-100.

Bernardi, G., B. Olofsson, J. Filispski, M. Zerial, J. Salinas G. Cunt, M. Meunier-Rotival, and F. Rodier, 1985. The mosaic genome of warm blooded vertbrates. Science 228: 953-958.

Bianchi, M. S., N. O. Bianchi, G. E. Pantelias and S. Wolff, 1985. The mechanism and pattern of banding induced by restriction endonucleases in human chromosomes. Chromosoma 91: 131-136.

Buth, D. G., T. E. Dowling and J. R. Gold, 1990. Molecular and cytological investigations, pp. in *The Biology of the Cyprinid Fishes*, edited by I. Winfield, and J. Nelson. Chapman and Hall, London.

Gold, J. R., Y. C. Li, N. S. Shipley and P. K. Powers, 1990. Improved methods for working with fish chromosomes with a review of metaphase chromosome banding. Journal of Fish Biology 37: 563-575.

Gold, J. R., C. T. Amemiya and J. R. Ellison, 1986. Chromosomal heteromatin differentiation in North American cyprinid fishes. Cytologia 51: 557-566.

Haaf, T., and M. Schmid, 1984. An early stage of ZW/ZZ sex chromosome differentiation in *Poecillia sphenops* var. *melanistica* (Poeciliidae, Cyprinodotiformes). Chromosoma 89: 37-41.

Hartley, S. E., 1991a. C, Q, and restriction enzyme banding of the chromosomes in

brook trout (*Salvelinus-fontinalis*) and Arctic charr (*Salvelinus-alpinus*). Hereditas 114: 253-261.

Hartley, S. E., 1991b. Restriction enzyme banding in Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*). Genetical Research 57: 273-278.

Howell, W. M., and D. A. Black, 1980. Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: a one step method. Experientia 36: 1014-1015.

John, B., and G. L. G. Miklos, 1979. Functional aspects of satellite DNA and heterochromatin. International Review of Cytology 58: 1-114.

Kaelbling, M., D. A. Miller and O. J. Miller, 1984. Restriction enzyme banding of mouse metaphase chromosomes. Chromosoma 90: 128-132.

Kiligerman, A. D., and S. E. Bloom, 1977a. Distribution of F-bodies, heterochromatin, and nucleolar organizer in the genome of the central mudminnow, *Umbra limi*. Cytogenetics and Cell Genetics 18: 182-196.

Kligerman, A. D., and S. E. Bloom, 1977b. Rapid chromosome preparations from solid tissues of fishes. Journal of Fisheries Research Board of Canada 34: 266-269.

LeGrande, W. H., 1981. Chromosomal evolution in North American catfishes (Siluriformes: Ictaluridae) with particular emphasis on the madtoms, *Noturus*. Copeia 1981: 33-52.

LeGrande, W. H., R. A. Dunham and R. O. Smitherman, 1984. Karyology of three species of catfishes (Ictaluridae: *Ictalurus*) and four hybrid combinations. Copeia 1984: 873-878.

Levan, A., K. Fredga and A. Sandburg, 1964. Nomenclature for centrameric positions on chromosomes. Hereditas 52: 201-220.

Li, K., Y. C. Li and D. Zhou, 1985. Studies on the karyotypes and C-banding patterns of three species in Channidae (Pisces). Acta Genetica Sinica 12: 470-477.

Lima-de-Faria, A., M. Isaksson and E. Olson, 1980. Action of restriction enzymes on the DNA and chromosomes of *Muntiacus muntjac*. Hereditas 92: 267-273.

Lloyd, M. A., and G. H. Thorgaard, 1988. Restriction enzyme banding of rainbow trout chromosomes. Chromosoma 96: 171-177.

Lopez, J. R., M. C. Alvarez, G. Thode and G. Martinez, 1989. Karyotype divergence in *Symphodus melops* and *S. roissali* (Labridae, Perciformes): C-banded and Ag-NOR karyotypes. Genome 32: 35-39.

Mayr, B., M. Kalat, P. Rab and M. Lambrou, 1987. Band karyotypes and specific types of heterochromatin in several species of European percid fishes (Percedea, Pisces). Genetica 75: 199-205.

Medrano, L., G. Bernardi, J. Couturier, B. Dutrillaux and G. Bernardi, 1988. Chromosome banding and genome compartmentalization in fishes. Chromosoma 96: 178-183.

Mezzanotte, R., and L. Ferrucci, 1984. Alternations induced in mouse chromosomes by restriction endonucleases. Genetica 64: 123-128.

Miller, D. A., and O. J. Miller, 1990. Restriction enzyme banding of metaphase chromosomes, pp. 238-250 in *Trends in chromosome research*, edited by T. Sharma. Narosa Publishing House, Springer-Verlag.

Miller, D. A., Y. C. Choi and O. J. Miller, 1983. Chromosome localization of highly repetitive human DNAs and amplified ribosomal DNA with restriction enzymes. Science 219: 395-397.

Miller, N. W., and L. W. Clem, 1988. A culture system for mitogen-induced proliferation of channel catfish (*Ictalurus punctatus*) peripheral blood lymphocytes. Journal of Tissue Culture Methods 11: 69-73.

Passarge, E., 1974. Analysis of chromosomes in mitosis and evaluation of cytogenetic data, pp. 135-200 in *Methods in human cytogenetics*, edited by H. G. Schwarzacher, and U. Wolf. Springer-Verlag, New York.

Peacock, W., R. Appels, P. Dunsmuir, A. R. Lohe and W. L. Gerlach, 1977. Highly repeated sequences: chromosomal localization and evolutionary conservatism, pp. 494-506 in *International Cell Biology, 1976-1977*, edited by B. R. Brinkley, and K. R. Porter. Rockefeller University Press, New York.

Pendas, A. M., P. Moran and E. Garciavazquez, 1993. Ribosomal-RNA genes are interspersed throughout a heterochromatic chromosome arm in Atlantic salmon. Cytogenetics and Cell Genetics 63: 128-130.

Phillips, R. B., and S. E. Hartley, 1988. Fluorescent banding patterns of the chromosomes of the genus *Salmo*. Genome 30: 193-197.

Phillips, R. B., and P. E. Ihssen, 1985. Chromosome banding in salmonid fish: nucleolar organizer regions in *Salmo* and *Salvelinus*. Canadian Journal of Genetics and Cytology 27: 433-440.

Pleyte, K. A., R. B. Phillips and S. E. Hartley, 1989. Q-band chromosomal polymorphisms in arctic char (*Salvelinus alpinus*). Genome 32: 129-133.

Rab, P., B. Mayr and P. Roth, 1991. Chromosome-banding study of European catfish, *Silurus glanis* (Pisces, Siluridae). Genetica 83: 153-157.

Schmid, M., and C. G. de Almeida, 1988. Chromosome banding in Amphibia. XII. Restriction enzyme banding. Chromosoma 96: 283-290.

Stingo, V., L. Rocco, G. Odierna and M. Bellitti, 1995. NOR and heterochromatin analysis in two cartilaginous fishes by C-, Ag- and RE (restriction enzyme)-banding. Cytogenetics and Cell Genetics 71: 228-234.

Sumner, A. T., 1972. A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75: 304-306.

Takai, A., and Y. Ojima, 1988. Chromosomal distribution of C-banded heterochromatin in cyprinid fishes. Proceedings of the Japan Academy 64B: 49-52.

Thorgaard, G. H., and J. E. Disney, 1990. Chromosome preparation and analysis, pp. 171-187 in *Methods for fish biology*, edited by C. B. Schreck, and P. B. Moyle. American Fisheries Society, Bethesda, Maryland.

Tiersch TR, Goudie CA, and Carmichael GJ, 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.

Tiersch, T. R., and C. A. Goudie, 1993. Inheritance and variation of genome size in half-sib families of hybrid catfishes. The Journal of Heredity 84: 122-125.

Tiersch, T. R., B. A. Simco, K. B. Davis, R. W. Chandler, S. S. Wachtel and G. J. Carmichael, 1990. Stability of genome size among stocks of the channel catfish. Aquaculture 87: 15-22.

Verma, R. S., and A. Babu, 1989. *Human chromosomes: Manual of basic techniques*. Pergamon Press, Inc., New York.

Vinas, A., C. Gomez, P. Martinez and L. Sanchez, 1994. Induction of G-bands on *Anguilla anguilla* chromosomes by the restriction endonuclease *Hae* III, *Hinf* I, and *Mse* I. Cytogenetics and Cell Genetics 65: 79-81.

Walker, L. I., S. G., Providell, 1995. Heterochomatic DNA evolution in Phyllotine rodents: restriction enzyme bands. The Journal of Heredity 86: 240-243.

Wolters, W. R., 1993. Channel catfish breeding and selection programs : constraints and future prospects, pp. 82-95 in *Selective breeding of fishes in Asia and the United States*, edited by K. L. Main, and B. Reynolds. The Oceanic Institute, Honolulu, Hawaii.

Wolters, W. R., C. L. Chrisman and G. S. Libey, 1981. Lymphocyte culture for chromosomal analysis of channel catfish, *Ictalurus puntatus*. Copeia 1981: 503-504.

Zhang, Q., and T. R. Tiersch, 1995. Cryopreservation of leucocytes of channel catfish for subsequent cytogenetic analysis. Journal of Fish Biology 47: 1016-1025.

Zhang, Q., and T. R. Tiersch, in review. Cytogenetic analysis of two intergeneric hybrids of ictalurid catfishes.

CHAPTER 3
REPLICATION BANDING AND SISTER-CHROMATID EXCHANGE OF CHANNEL CATFISH CHROMOSOMES

Introduction

Studies of fish chromosomes have not been as successful or widespread as in other vertebrate groups (Gold *et al.* 1990). Less than 10% of the more than 20,000 extant fish species have been studied cytogenetically (Cau *et al.* 1988). Limiting factors have included technical difficulty in obtaining good chromosome spreads because most species have a large number of small chromosomes (Gold *et al.* 1980). Because of this and low compartmentalization of the fish genome by base composition (Medrano *et al.* 1988), serial (or linear) structural banding (such as Q-, G-, and R-) techniques developed for higher vertebrates that rely on post-fixation modification of chromosome structure (Ronne 1992), have rarely worked with fish chromosomes.

Dynamic chromosome banding such as replication banding is an alternate method to structural banding. It relies on the incorporation of a base analogue during DNA replication, and post-fixation modification of chromosome structure in the substituted regions (Ronne 1992). Therefore, difficulties that relate to the chromosome structure or base composition of fish chromosomes can be bypassed through replication banding. In fact, several attempts at replication banding of fish chromosomes have succeeded, including carp (Zhang and Wu 1985; Hellmer *et al.* 1991), trout (Delany and Bloom 1984; Pendas *et al.* 1993), and scorpion fish (Giles *et al.* 1988).

The base analogue 5'-bromodeoxyuridine (BrdU) used in replication banding can be added to cell cultures simultaneously with inhibitors of DNA synthesis (S-phase) such

as methotrexate, or added after removal of the S-phase inhibitor (Ronne 1992). The incorporation of BrdU at different phases of DNA synthesis phases has resulted in 2 different banding patterns: G-like bands for early phase incorporation, and R-like bands (the reverse of G-bands) for late phase incorporation (Ponce de Leon *et al.* 1992). The replication banding of chromosomes of *Oncorhynchus mykiss* (Delany and Bloom 1984), *Rutilus rutilus* and *Scardinius erythrophthalmus* (Hellmer *et al.* 1991), and *Scorpaena procus* and *Scorpaena notata* (Giles *et al.* 1988) were prepared by intraperitoneal injection of BrdU, which resulted in banding patterns similar to reverse bands (R-bands). However, the banding patterns in all of these species were not confirmed because there was no structural G- or R- banding technique available for comparison. In *Monopterus albus*, chromosomes were prepared from cultured kidney cells treated by standard replication banding methods (Liu 1986, 1988). The resultant patterns were comparable to those received by structural G-banding technique (Liu 1983).

A major limitation of serial banding of fish chromosomes has been poor consistency of banding patterns, especially in structural G-banding (Zhou *et al.* 1989). Consistent replication banding patterns can be prepared from a population of synchronized cells, which results from precise control of timing for the blocking and releasing steps of the procedure (Ronne 1992).

Computer-assisted chromosome analysis was reported in cyprinid fish in the mid-1980's (Gold *et al.* 1986). The introduction of newer technology has enabled handling of chromosome-like images imported directly from the light microscope

(Bauchan and Campbell 1994). Banding patterns identified with the assistance of a computer are much more informative and consistent (Drets *et al.* 1992).

Sister-chromatid exchange (SCE) is the breakage and reunion of DNA presumably between equal positions after 2 or more rounds of replication in the presence of BrdU (Wolff 1982). Analysis of SCEs is of wide interest because there appears to be a good correlation between frequency of SCE and exposure to mutagenic agents such as radiation or chemicals. Therefore, SCE analysis is a valuable method for the study of mutagenesis and environmental toxicology in fish (Kligerman 1979).

Similar to the procedure used in replication banding, the presence of BrdU in cultures of cells for 2 consecutive generations will produce sister chromatids that can be differentially stained (i.e. sister-chromatid differentiation: SCD) to demonstrate the exchanged segments. Chromatids containing DNA strands with more BrdU will stain less intensely than chromatids containing less BrdU incorporation because of the quenching action of BrdU (Verma and Babu, 1989). This method was routinely used to score the occurrence of SCE during 3 rounds of replication and 2 mitotic divisions (or 2 complete cell cycles). However, the SCE occurrence within each cycle was not emphasized. Investigation of SCE occurring during the first cycle (or first-round SCE) could provide information about the timing of SCE, and it could be useful in the study of acute toxicity of environmental mutagens.

The C-banding technique is used to reveal the location of consititutive heterochromatin (Sumner 1972). Channel catfish has a low abundance of heterochomatin, and the small C-bands are restricted to the centromeric regions (Chapter 2). The standard C-banding technique, with little modification, was used to

identify BrdU-incorporated regions of silver carp (*Hypophthalmichthys moritrix*) chromosomes (Zhou *et al.* 1989). Prolonged alkali treatment was found to degrade bands produced by the incorporated BrdU and to maintain intact centromeric bands.

Channel catfish is the most important cultured species in the United States, and genetic study of the species is generating wide interest (Wolters 1993). However, little information is available about chromosome structure, and this hinders basic research, such as gene mapping, in this species. The objectives of this study were to: (1) develop a replication banding procedure for use with channel catfish; (2) evaluate the consistency of the R-banding technique and ideogram; (3) establish a standard RBG-banded karyotype; (4) develop a method for simultaneous identification of SCE, SCD and C-banding, and (5) estimate baseline frequency of SCD and SCE corresponding to the first cell cycle.

Material and methods

Animals

The animals used in this experiment were from a population maintained at LSU and spawned artificially in indoor systems (Tiersch *et al.* 1994). Eight fish (mean ± SD: 210 ± 55 g) were used for isolation of leukocytes and preparation of chromosomes for replication banding. Another five fish (500 ± 45 g) were used for investigation of SCE.

Leukocyte culture

Leukocytes were isolated by the density centrifugation method (Zhang and Tiersch 1995). Culture media and incubation conditions are described in Chapter 2. Mitotic activity of cultured cells was induced by incubating with final concentrations of 0.05 μg/ml phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Company, St.

Louis, MO) and 0.5 μg/ml calcium ionophore A23187 (Sigma), or with 5 μg/ml concanavalin A (Con A). After 24 h, the medium with PE and A23187 was replaced with fresh medium containing no mitogens; the cultures with Con A did not require a change of medium at this step. Cells were incubated for another 48 to 72 h until first mitotic activity occurred. Cultures were processed with the following procedures for replication banding and SCE and SCD analysis.

(1) Synchronization for replication banding. Mitosis of leukocyte cultures was blocked with 5'-fluorouracil (FU; Sigma) at final concentration 1.0×10^{-7} M. After 17 h incubation; the cells were pelleted and rinsed twice with Ca^{2+}- and Mg^{2+}- free phosphate buffered saline (CMF-PBS). The cells were cultured for another 5.5 h in fresh Leibovitz L15 medium (Gibco BRL, Life Technologies Inc., Gaithersburg, MD) with a mixture of 10^{-4} M BrdU, 6×10^{-6} M uridine (Sigma), and 4×10^{-7} M 5'-fluoro-2'-deoxyuridine (FrdU; Sigma). Twenty μl of colchicine solution (100 μl/ml) was added to each culture at 30 min to 1 h before harvest. The cells were processed by a method described previously (Zhang and Tiersch 1995). A simplified procedure (Yan *et al.* 1989) was included for comparison, in which cultures of cells were blocked by an excess of thymidine (0.3 mg/ml final concentration) and released and labeled by BrdU (30 μg/ml final concentration).

(2) Sister chromatid exchange. Bromodeoxyuridine was added to cultures (1.6×10^{-4} M, final concentration) which were incubated for 20 h before harvest. The method for preparation of chromosomes was the same as described above.

Staining procedure

(1) Replication banding. (a) Fluorochrome plus Giemsa (FPG). This follow the method of Perry and Wolff (1974) with a few changes. Slides were placed on a styrofoam board, floated in 60 °C water bath, and irradiated with a long wave (365 nm) UV light (115 volt, 60 Hz, 0.3 Amps, Spectronic Corp., Westbury, NY) for 2 h from a distance of 10 cm. The slides were treated with 2-x SSC for 4 h at 60 °C, rinsed with distilled water (dH_2O) and dehydrated through a series of ethanol solutions (70%, 85%, 95%, and 100%). (b) Hoechst 33258 and actinomycin D double staining. The procedure was derived from those of Jorgenson *et al.* (1978) and Sahar and Latt (1978). The concentration of the Hoechst 33258 (Sigma) working solution was raised to 150 μg/ml in this study. (c) Other fluorescent staining methods. Acridine orange (Ponce de Leon *et al.*, 1992) and propidium iodide were also used in this study (data not shown).

(2) Simultaneous detection of sister-chromatid exchange and C-banding. The procedure was based on the method of C-banding by alkali treatment (Sumner 1972) modified for use with channel catfish (Chapter 2). Slides were treated with 5% $Ba(OH)_2$ and incubated overnight with 2-x SSC and 0.05% Triton X-100 at 60 °C. Prolonged staining in 5% Giemsa was required to reveal C-banding and SCD. Twelve to fifteen spreads from each of five specimens were counted for percentages of SCE and SCD. One-way ANOVA was used to analyze the percentages of SCD or SCE occurrence among individual fish at a significance level 0.05.

(3) Nucleolus Organizer Regions (NOR). The NOR was used as a marker to identify a chromosome (D11) for comparison of replication R-bands among different cells. Slides were treated by the FPG procedure, and images of chromosomes were

recorded for replication R-banding patterns. The slides were destained with Carnoy's fixative, rinsed with dH_2O, dehydrated through the series of ethanol solutions, and air dried. The NOR was located using the silver staining procedure of Howell and Black (1980), with minor modifications described elsewhere (Zhang and Tiersch in review).

Computer-assisted chromosome analysis

The process of karyotyping was assisted by the Optimas® and Kary® computer software packages (Bioscan, Inc., Edmonds, WA). Chromosomal images were captured and recorded by an image analysis system (Zhang and Tiersch in review) directly from a light microscope (Microphot-SA, Nikon Inc., Garden City, NY). For comparison, chromosomes were photographed with Kodak Technical Pan film 2415, and the negatives were digitized using a slide scanner (SprintScan 35, Poraloid scanner model CS-2700, Needham Heights, MA) into the computer for further analysis. Total lengths and arm lengths of chromosomes were measured by the "line measurement" function of Optimas®. The dark-and-light banding patterns of chromosomes were identified by densitometry and expressed with a luminance plot. The X-axis of the plot represents segments of chromosomes, and the Y-axis represents the corresponding grayscale value ranging from 0 (darkest) to 256 (lightest). Images of metaphase spreads were brought into Kary®, which arranges chromosomes in descending order of size by automatic cutting, and pasting to a template.

A standard karyotype was prepared by rearranging the chromosomes into groups based on relative length and centromeric index (CI) (Chapter 2) which were calculated by the following formulae:

Relative length (%) = (total length of each chromosome pair/ total length of all chromosomes) x 100.

CI (%) = (short-arm length / total length of chromosome) x 100.

An ideogram was prepared for each chromosome using the Microsoft PowerPoint® computer software. Chromosomes 1, 6, and 11 (NOR-bearing chromosome) were used as representatives of each cell. The banding pattern of these 3 chromosomes from different cells were compared to examine the consistency of this technique.

Results

Chromosomes prepared by the FU/BrdU replication banding procedure and stained with FPG method (Fig. 3.1) exhibited serial bands with good resolution. Chromosomes stained with Hoechst 33258 and actinomycin D showed few bright bands and many faintly-stained bands of low resolution (Fig. 3.2). Chromosomes prepared by the thymidine/BrdU procedure did not show highly resolved bands after staining with FPG (data not shown).

Individual chromosomes were identified based on an objective procedure (Appendix B.4). Each chromosome had a characteristic replication R-banding pattern and could be identified regardless of the state of contraction. This was demonstrated by comparing the banding pattern of representative chromosomes of different cells such as 1, 6, and 11 (NOR-bearing chromosome) (Fig. 3.3) identified by the sequential staining method (Fig. 3.4). The NOR-bearing chromosome possessed 1 major and 1 or 2 minor bands on the long arm, with the major band adjacent to the centromere. Chromosome 1,

Figure 3.1. Channel catfish chromosomes prepared by the fluorouracil-bromodeoxyuridine replication banding procedure, and stained by fluorochrome plus Giemsa to reveal banding patterns. Bar = 10 μ.

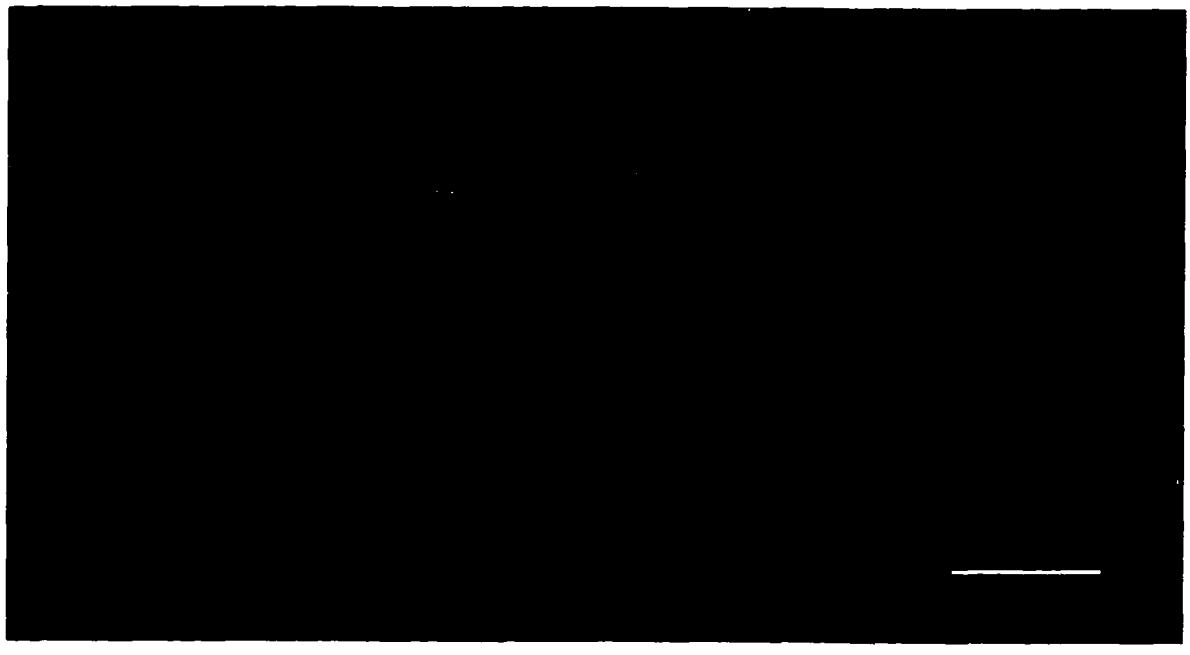
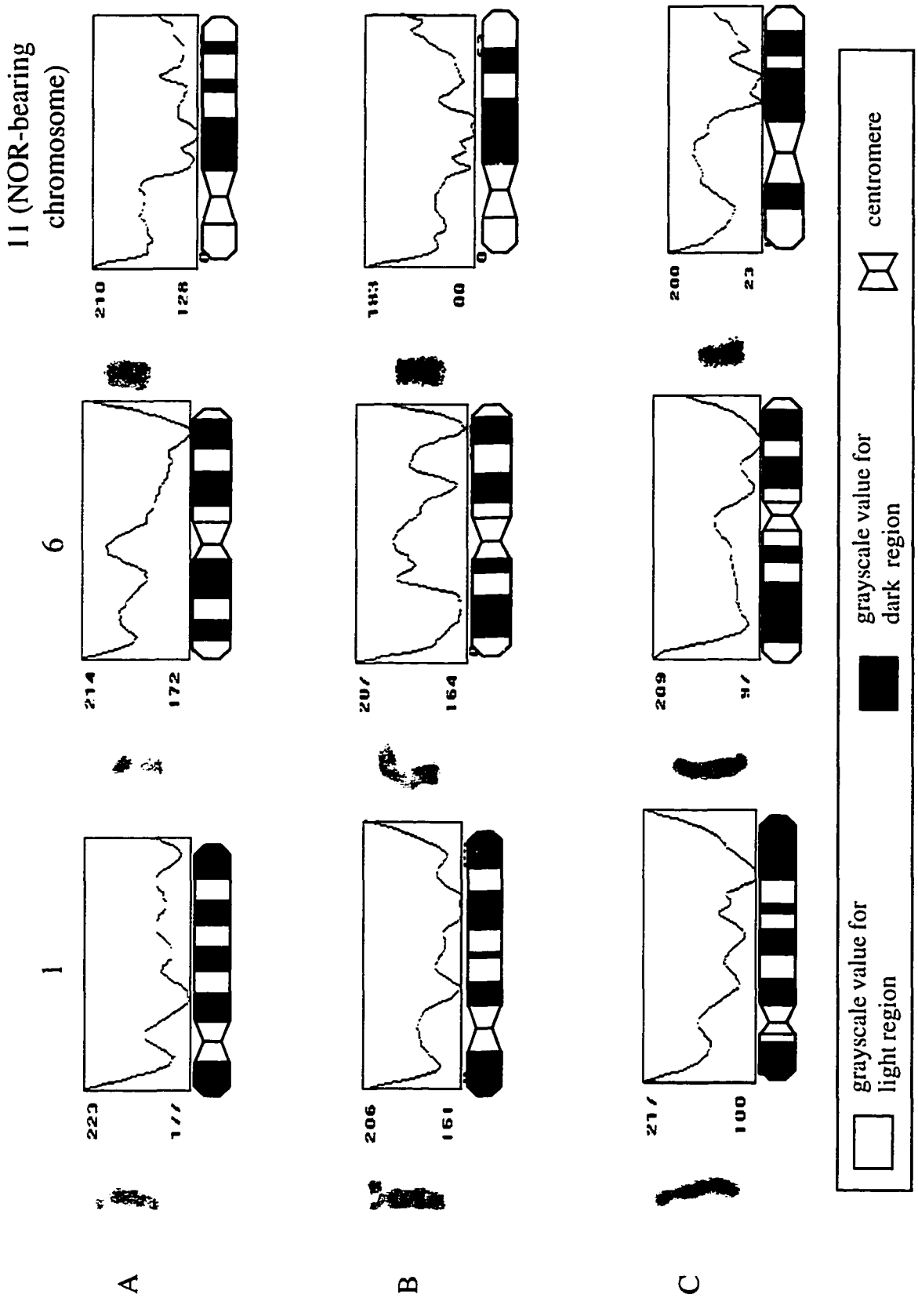


Figure 3.2. Channel catfish chromosomes prepared by the fluorouracil-bromodeoxyuridine replication banding procedure, and stained by Hoechst 33258 and actinomycin D to reveal banding patterns. Bar = 10 μ.

Figure 3.3. Computer-assisted analysis of replication banding patterns of representative chromosomes. Comparison of the luminance patterns of chromosome 1, 6, and NOR-bearing chromosome (11) from cells of different fish individuals (A, B, and C).



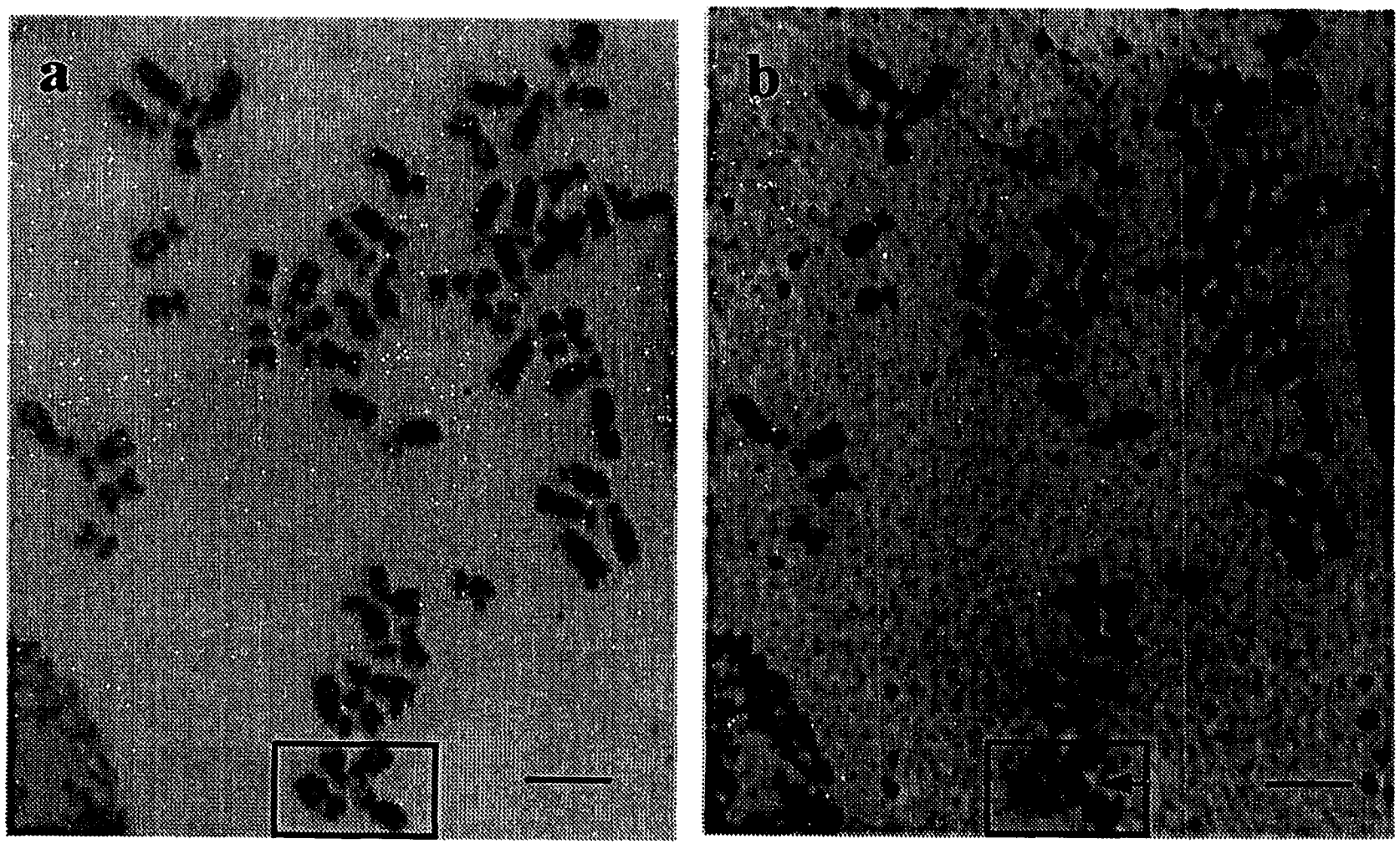


Figure 3.4. Location of NOR-bearing chromosomes (inset) by sequential staining; before (a), and after (b) silver staining. Arrow indicates the NOR-bearing chromosome. Bars = 10 μ.

the largest in the complement, had 1 minor band on the short arm and 4 well-defined bands on the long arm. Another chromosome was readily identifiable: chromosome 6 was the largest metacentric chromosome, and it had 2 bands on the short arm and 2 on the long arm.

The reproducibility of this technique allowed for preparation of a standard karyotype of R-banded chromosomes. The final karyotype (Fig. 3.5) was based on the analysis of 12 spreads and was summarized with an ideogram (Fig. 3.6). The variation between individual karyotypes was not due to banding pattern but mostly to the difficulty in identifying the centromere for some chromosomes.

The SCD was found on most chromosomes (83-100%) in which BrdU was present for 2-round DNA replication or 1 complete cell cycle (Fig 3.7 a, b, and c). However, SCE was observed only on 1 to 4% of the chromosomes. The percentages of SCE ($P = 0.26$) was not significantly different among 5 fish examined (Fig. 3.8), nor was that of SCD ($P = 0.07$). The SCD and C-banding were simultaneously displayed by this method (Fig. 3.9). The C-band was small and resolved, and limited to the centromeric region.

Discussion

In this study, a replication banding technique is presented that yielded reproducible RBG-banding patterns with channel catfish chromosomes; a standard RBG-banded karyotype was established. Also developed was a procedure for simultaneous detection of sister chromatid exchange and C- banding. The breakage sites of SCE chromosomes can potentially be classified by their relative location to the C-banded centromere. These data provide baseline frequencies of SCE and SCD occurring during

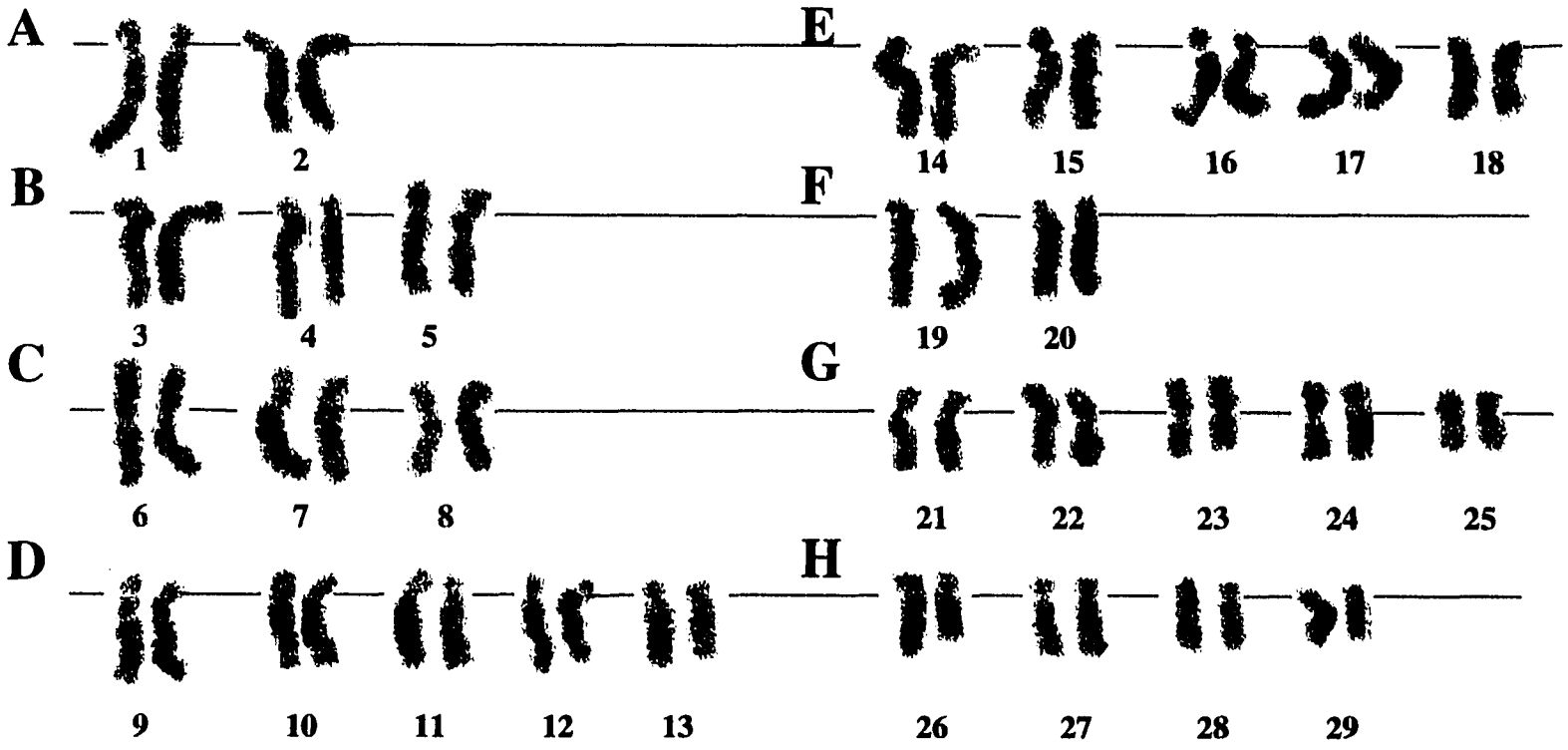


Figure 3.5. A standard RBG-banded karyotype (R-bands by bromodeoxyuridine using Giemsa) of channel catfish. Abbreviations: A, large submetacentric; B, large subtelocentric; C, large and medium metacentric; D, medium submetacentric; E, medium subtelocentric; F, telocentric; G, small metacentric, and H, small submetacentric chromosomes. Bar = 10 μ.

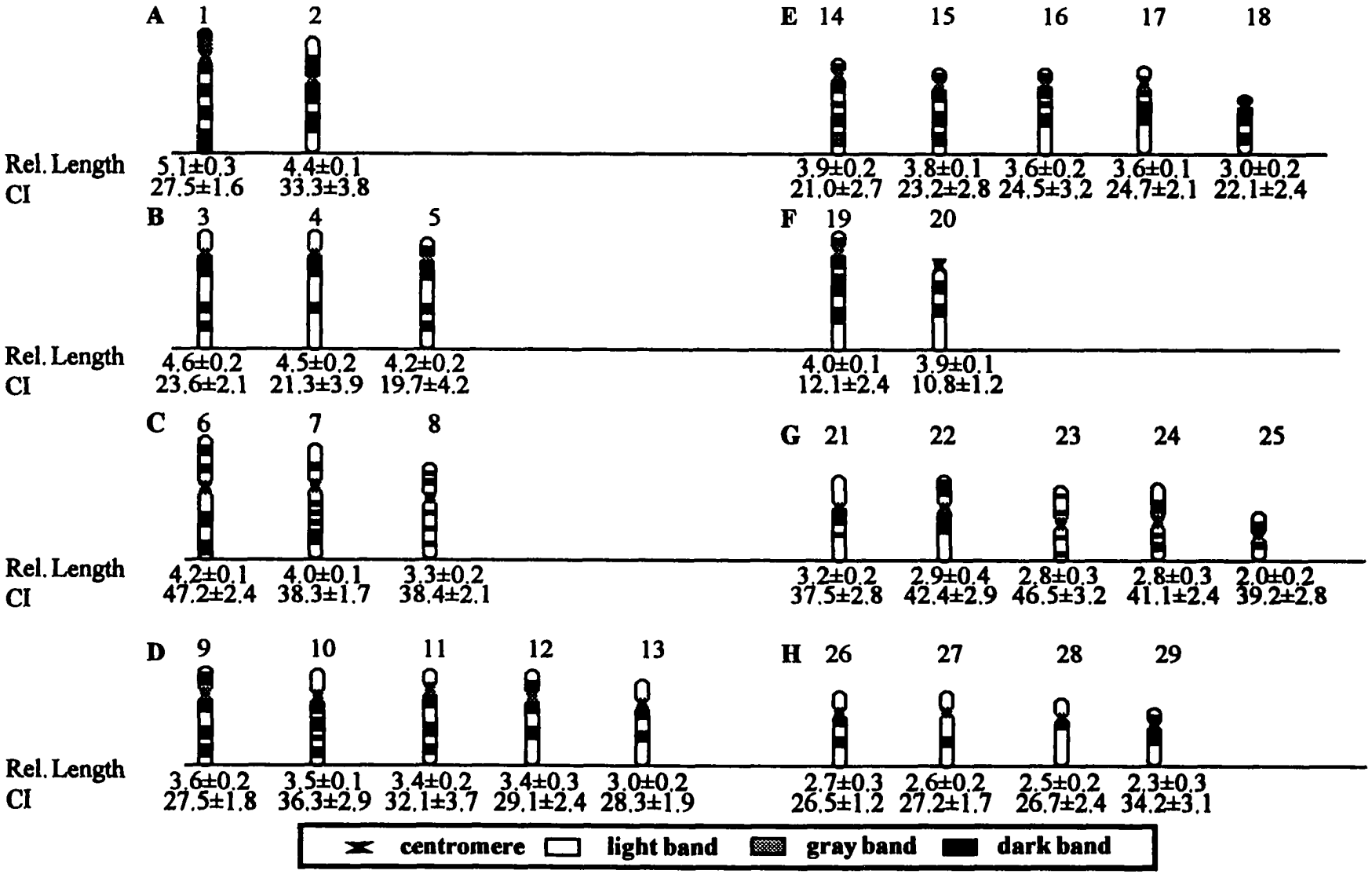


Figure 3.6. An ideogram of RBG-banded karyotype (R-bands by bromodeoxyuridine using Giemsa) of channel catfish. The measurements were taken from chromosomes of 12 different spreads. Abbreviations: Rel. Length = relative length; CI = centromeric index.

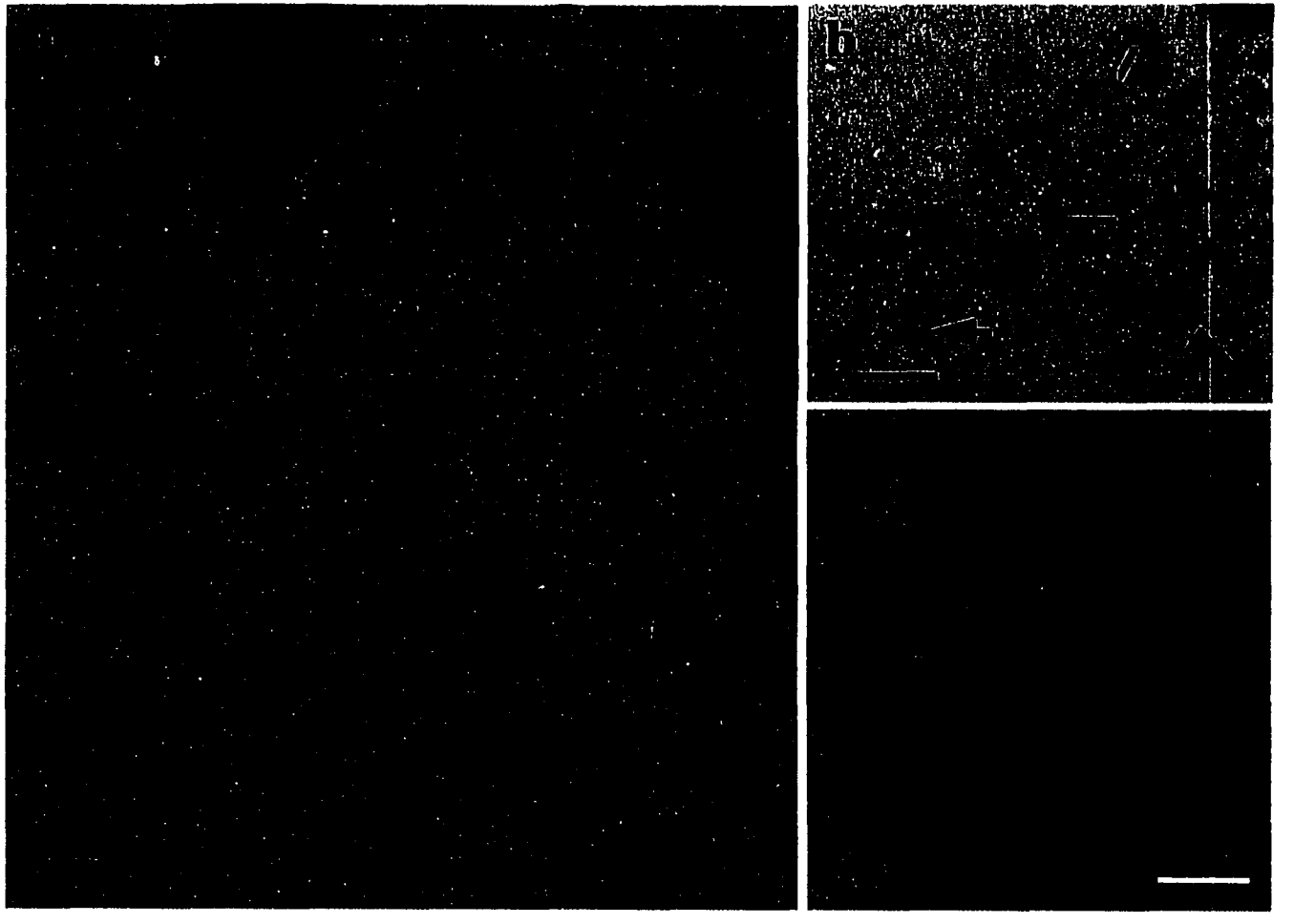


Figure 3.7. Sister-chromatid differentiation (SCD) and exchange (SCE) of channel catfish chromosomes. Arrowheads indicate representative SCE chromosomes; lines indicate position of centromeres. Bars = 10 μ.

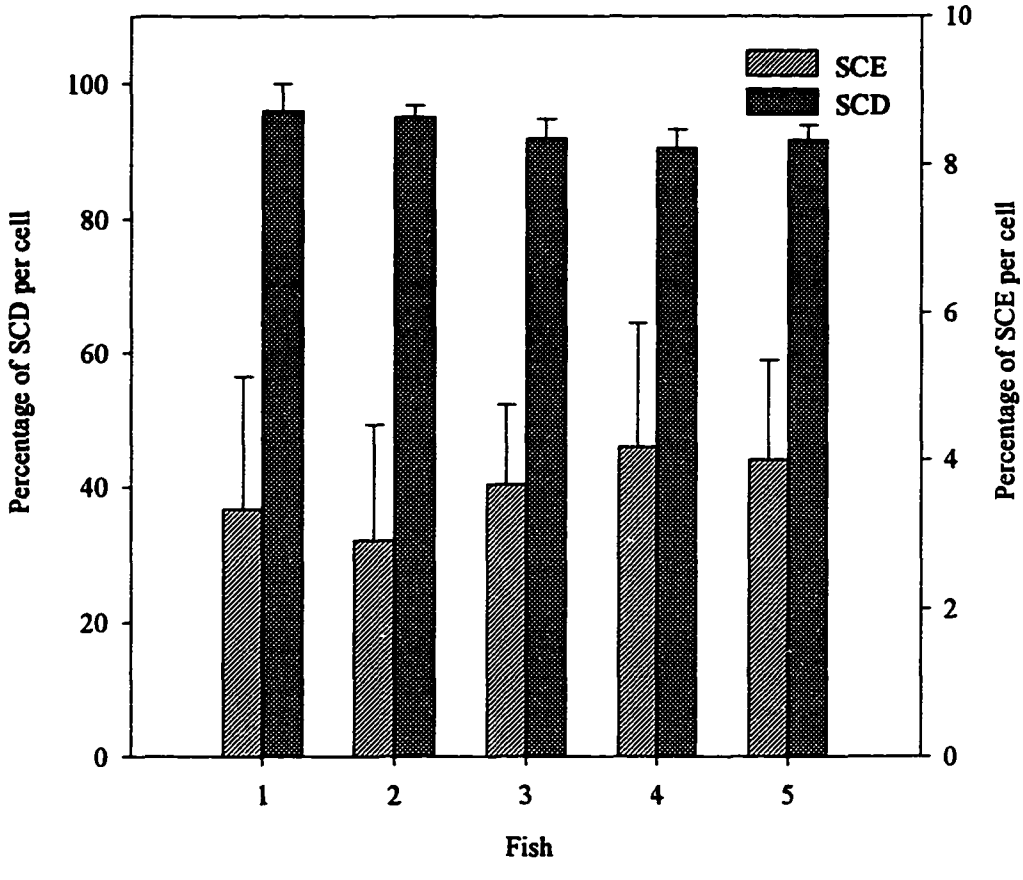


Figure 3.8. The distribution of SCD and SCE occurrence among chromosomes of individual channel catfish. The occurrence of SCE ($P = 0.26$) or SCD ($P = 0.07$) was found to be not significantly different among the 5 individuals examined.

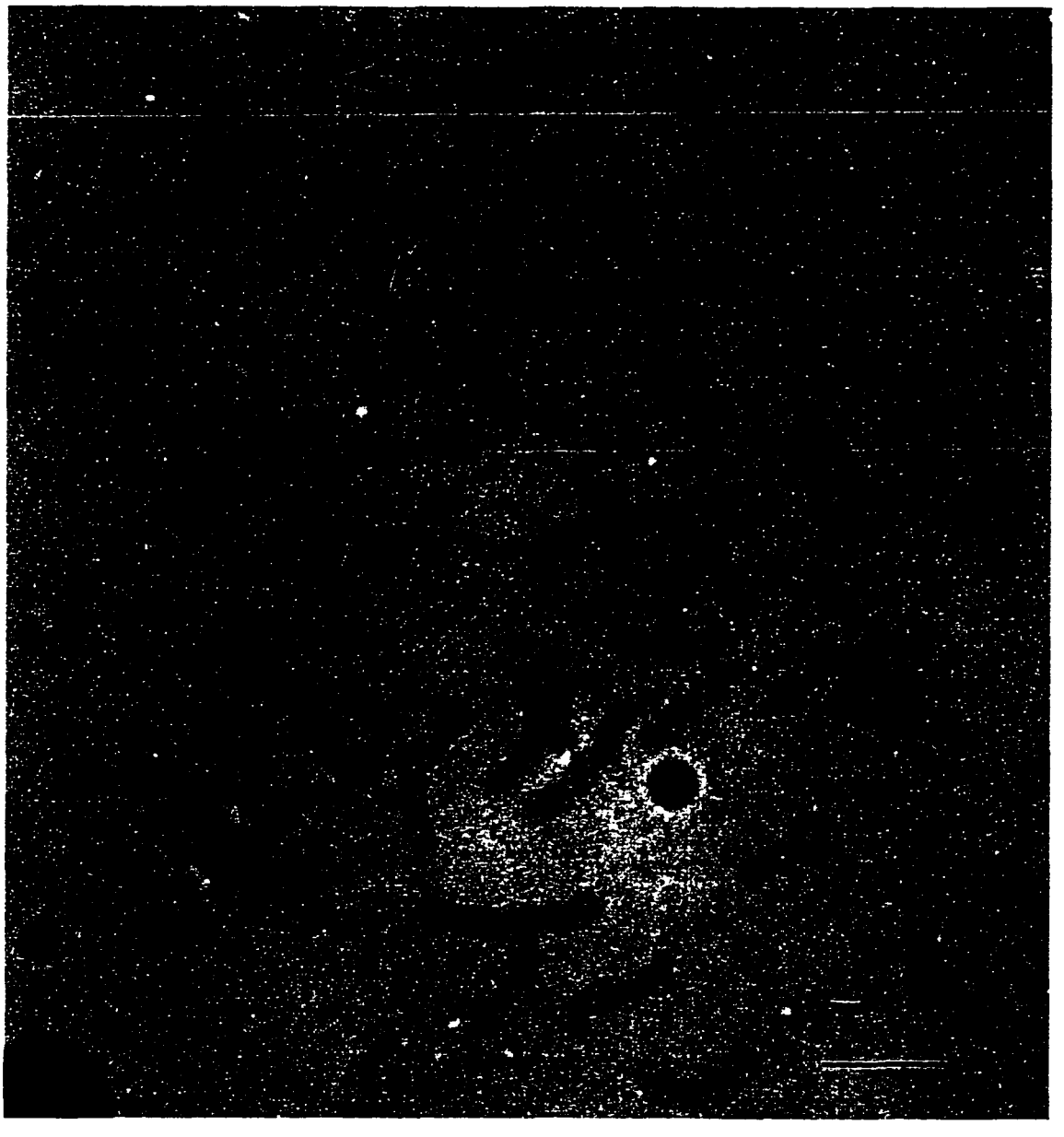


Figure 3.9. Simultaneous detection of sister-chromatid differentiation and C-banding of channel catfish chromosomes. Arrowheads indicate position of representative centromeric C-bands. Bar = 10 μ.

the first 2-rounds of DNA replication or the first cell cycle, which could be used as a reference in toxicological test of the culture environment of this species.

Three factors were critical for the success of the replication R-banding procedure: rapid growth of cultured cells, efficient synchronization of mitosis, and control of intensity of the post-labeling treatment. The dynamic R-bands were generated by differential incorporation of BrdU into replicating DNA segments. The uptake of BrdU is related to the replication status of cultured cells. By addition of mitogens, the number of analyzable spreads can been increased. More importantly, the quality of the replication banding was enhanced by rapid growth of cultured cells. Although Con A, pokeweed mitogen, and phytohemagglutinin M all have proliferative effects on *in vitro* culture of channel catfish leukocytes (Faulmann *et al.* 1983; Miller and Clem, 1988), a mixture of phorbol ester and calcium ionophore was consistent mitogenic for catfish leukocytes (Lin *et al.* 1992). The mode of action of this mixture is different from that of classical mitogens, and it is suspected to mimic intracellular secondary messengers and stimulate the phosphorylation of cellular proteins that in turn regulate cell proliferation.

Consistent replication banding patterns rely on temporal and spatial control of BrdU incorporation, and this can be achieved by cell synchronization. Several treatment schemes were evaluated in this study, including the S-phase inhibitor methotrexate (MTX) and FU, and high doses of thymidine and BrdU (data not shown). Although MTX worked effectively in higher animals (Ronne 1992) and in some fishes such as eel (Liu 1986; Liu 1988), I found that it did not block mitosis in cultured channel catfish leukocytes. I also found that the addition of 0.3 mg/ml of thymidine did not synchronize cells effectively, and resultant spreads were arrested in metaphase, prometaphase, or

even in prophase. A similar result was found in cultures treated with high doses of BrdU (70 μg/ml, data not shown). In contrast, FU was able to block cultures and was easily released by the BrdU-based mixture. Chromosomes were arrested mostly in metaphase and possesed a relative broad shape. The bands were solid, thick, and consistent.

The final consideration of replication banding was the post-labeling treatment. The FPG is a popular staining procedure to reveal BrdU-labeled regions. Various modifications have been developed from the original methods for use in different species. In this study, the intensity of UV irradiation and the duration of 2-x SSC incubation were increased to generate differentiated bands. The double staining method using Hoechst 33258 and actinomycin D was developed originally to stain structural bands on human chromosomes. This direct method was not effective for channel catfish chromosomes, while a similar method using Hoechst staining was able to produce replication bands on salmonid chromosomes (Delany and Bloom 1984). Other direct methods such as staining with acridine orange was able to reveal replication bands in cyprinid fish (Hellmer *et al.* 1991), but they did not work in this study. This probably results from different packaging of DNA and associated protein molecules.

Establishment of an effective evaluation method for replication R-banded chromosomes was another important consideration of this study. Unlike structural bands, most replication bands, especially on fish chromosomes, are continuous and do not have clear borders. Computer-assisted processing of replication R-banded chromosomes was efficient and accurate and results were more informative than subjective methods. Comparisons among different spreads were mediated by analysis of the marker chromosomes, which were identified by morphology and banding patterns, or

by chemical treatment. The NOR-bearing chromosome of channel catfish is inherited stably in the hybrid offspring of channel catfish x flathead catfish (*Pylodictis olivaris*) and channel ctafish x black bullhead (*Ameiurus melas*) (Zhang and Tiersch, in review), and can be used as internal reference to gauge the contraction level of metaphase spreads.

The RBG-banded karyotype was developed for channel catfish based on methods used for human chromosomes (ISCN 1985). Only spreads with broad and solid chromosomes were used for karyotyping, although centromere positions of these chromosomes were difficult to locate without the assistance of image analysis system. On the other hand, elongated chromosomes had well-defined centromeric regions, but the bands on these chromosomes were not analyzable by the naked eye.

In this study, the SCE of channel catfish chromosomes was studied by alkaline treatment followed by prolonged exposure to 2-x SSC buffer. This method allowed the identification of SCEs from the first cell cycle. The occurrence of SCE in the absence of mutagenic treatment is influenced mainly by the level of BrdU substitution in template DNA during the second round of replication (Escalza *et al.* 1985; Cortes *et al.* 1987), and increases with the doses of BrdU and incubation time (Pinero *et al.* 1992). The traditional FPG technique requires about 30% substitution of BrdU in DNA to achieve good chromatid differentiation (Pinero *et al.*, 1992). The current method was designed after failure to reveal SCE or SCD occurred during the first cell cycle using traditional techniques.

A technique for simultaneous detection of SCE or SCD and C-banding was developed in this study. The incorporation of atomic Br derived from BrdU, enhances the binding of DNA with non-histone proteins by formation of additional hydrogen

bonds and makes the BrdU-incorporated chromosomal region more compact and less degradable by alkali treatment (Zhou *et al.* 1989). Based on this principle a modified C-banding method that produces highly resolved centromeric C-bands on channel catfish chromosomes (Chapter 2) was used in this study. However, washing time with 2-x SSC buffer was increased; this was necessary for complete removal of DNA fragments generated by $Ba(OH)_2$.

The development of an *in vitro* BrdU incorporation technique for producing R-banding and SCE could be an important contribution to the genetic study of fishes. The phylogenetic relationships in the family Ictaluridae may be evaluated by studying the replication banding pattern. Homomorphic sex chromosomes, that cannot be identified by morphology in most catfishes, could be distinguishable by examining replication R-bands, as reported in amphibians (Schempp and Schimid 1981).

References cited in Chapter 3

Bauchan, G. R., and T. A. Campbell, 1994. Use of an image-analysis system to karyotype diploid Alfalfa (*Medicago sativa L*). Journal of Heredity 85: 18-22.

Cau, A., S. Salvadori, A. M. Deiana, J. L. Bella and R. Mezzanotte, 1988. The characterization of *Muraena helena L.* mitotic chromosomes: karyotype C-banding, NOR and *in-situ* digestion with endonucleases. Cytogenetics and Cell Genetics 47: 223-226.

Cortes, F., W. F. Morgan and S. Wolff, 1987. Effects of exogenous thymidine on sister-chromatid exchange frequency in chinese hamster ovary cells with bromodeoxyuridine and chlorodeoxyuridine-substituted chromosomes. Mutation Research 192: 277-282.

Delany, M. E., and S. E. Bloom, 1984. Replication banding patterns in the chromosomes of the rainbow trout. Journal of Heredity 75: 431-434.

Drets, M. E., G. Obe, F. J. Monteverde, G. A. Folle, I. I. Medina, M. G. De Galvez, J. E. Duarte, and B. H. Mechoso, 1992. Computerized graphic and light microscopic analyses of T-banded chromosome segments of chinese-hamster ovary cells and human-lymphocytes. Biologisches Zentralblatt 111: 204-214.

Escalza, P., F. Cortes and B. Schvartzman, 1985. Induction of sister-chromatid exchnage (SCEs) by 5-fluorodeoxyuridine. The role of 5-bromodeoxyuridine incorporated into parental DNA. Mutation Research 151: 77-82.

Faulmann, E., M. A. Cuchens, C. J. Lobb, N. M. Miller and L. W. Clem, 1983. An effective culture system for studying *in vitro* mitogenic responses of channel catfish lymphocytes. Transactions of the American Fisheries Society 112: 673-679.

Giles, V., G. Thode and M. C. Alvvarez, 1988. Early replication bands in two scorpion fishes, *Scorpaena porcus* and *S. notata* (Order Scorpanneiformes). Cytogenetics and Cell Genetics 47: 80-83.

Gold, J. R., Y. C. Li, N. S. Shipley and P. K. Powers, 1990. Improved methods for working with fish chromosomes with a review of metaphase chromosome banding. Journal of Fish Biology 37: 563-575.

Gold, J. R., C. T. Amemiya and J. R. Ellison, 1986. Chromosomal heteromatin differentiation in North American cyprinid fishes. Cytologia 51: 557-566.

Gold, J. R., W. J. Karel and M. R. Strand, 1980. Chromosome formulae of north American fishes. The Progressive Fish-Culturist, 42: 10-23.

Hellmer, A., I. Voiculescu and W. Schempp, 1991. Replication banding studies in 2 cyprinid fishes. Chromosoma 100: 524-531.

Howell, W. M., and D. A. Black, 1980. Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: a one step method. Experientia 36: 1014-1015.

ISCN, 1985. *An international system for human cytogenetic nomenclature*. Karger, Basel, New York.

Jorgenson, K. F., J. H. van de Sande and C. C. Lin, 1978. The use of base pair specific DNA binding agents as affinity labels for the study of mammalian chromosomes. Chromosoma 68: 287-302.

Kligerman, A. D., 1979. Introduction of sister chromatid exchanges in the central mudminnow following *in vivo* exposure to mutagenic agents. Mutation Research 64: 205-217.

Lin, G. L., C. F. Ellsaesser, L. W. Clem and N. W. Miller, 1992. Phobol ester/calcium ionophore activate fish leukocytes and induce long-term cultures. Development and Comparative Immunology 16: 153-163.

Liu, L., 1988. Analysis of high-resolution G-banding pattern in fish chromosomes treated by BrdU. Acata Genetica Sinica 15: 117-121.

Liu, L., 1986. Studies on making fish chromosomes elongated with high resolution G-banding, pp. 910-917 in *Indo-Pacific Fish Biology: Proceedings of the Second International Conference on Indo-Pacific Fishes*, edited by T. Uyeno, R. Arai, T. Tanchi and K. Matsuura. Ichthyological Society of Japan, Tokyo, Japan.

Liu, L., 1983. Studies on the chromosomes G-banding patterns in rice-field eels (*Monopterus albus* Zuiew). Acta Genetica Sinica 10: 230-234.

Medrano, L., G. Bernardi, J. Couturier, B. Dutrillaux and G. Bernardi, 1988. Chromosome banding and genome compartmentalization in fishes. Chromosoma 96: 178-183.

Miller, N. W., and L. W. Clem, 1988. A culture system for mitogen-induced proliferation of channel catfish (*Ictalurus punctatus*) peripheral blood lymphocytes. Journal of Tissue Culture Methods 11: 69-73.

Pendas, A. M., P. Moran and E. Garciavazquez, 1993. Replication banding-patterns in Atlantic salmon (*Salmo salar*). Genome 36: 440-444.

Perry, P., and S. Wolff, 1974. New Giemsa method for differential staining of sister chromatids. Nature 251: 156-158.

Pinero, J., P. Daza, P. Escalza and F. Cortes, 1992. Influence of low doses of BrdU and estimation of spontaneous SCE in CHO chromosomes: three-way differential staining and an immunoperoxidase method. Chromosoma 102: 66-70.

Ponce de Leon, F. A., Y. Li and Z. Weng, 1992. Early and late replicative chromosomal banding patterns of *Gallus domesticus*. Journal of Heredity 83: 36-42.

Ronne, M., 1992. Synchronization, banding and *in-situ* hybridization: A short laboratory manual. *In Vivo* 6: 49-58.

Sahar, E., and S. A. Latt, 1978. Enhancement binding patterns in human metaphase chromosomes by energy transfer. Proceeding of the National Academic Sciences of USA 75: 5650-5654.

Schempp, W., and M. Schmid, 1981. Chromosome banding in Amphibia. VI. BrdU-replication patterns in Anura and demonstration of XX/XY sex chromosomes in *Rana esculenta*. Chromosoma 83: 697-710.

Sumner, A. T., 1972. A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75: 304-306.

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. Transaction of American Fisheries Society 123: 580-586.

Verma, R. S., and A. Babu, 1989. *Human chromosomes: Manual of basic techniques.* Pergamon Press, Inc., New York.

Wolff, S., 1982. *Sister chromatid exchange*. John Wiley and Sons, New York.

Wolters, W. R., 1993. Channel catfish breeding and selection programs: constraints and future prospects, pp. 82-95 in *Selective breeding of fishes in Asia and the United States*, edited by K. L. Main, and B. Reynolds. The Oceanic Institute, Honolulu, Hawaii.

Yan, J., S. Liu, X. Zhang and M. Zhang, 1989. The application of cell synchronization to chromosome banding of fish and the tentative karyotype analysis of *Ctenopharyngodon idellus*. Zoological Research 10: 123-128.

Zhang, Q., and T. R. Tiersch, 1995. Cryopreservation of leucocytes of channel catfish for subsequent cytogenetic analysis. Journal of Fish Biology 47: 1016-1025.

Zhang, Q., and T. R. Tiersch, in review. Cytogenetic analysis of two intergeneric hybrids of ictalurid catfishes.

Zhang, R., and H. Wu, 1985. A study of sex chromosome in *Carassius auratus* by BrdU-Hoechst 33258-Giemsa techniques. Acta Genetica Sinica 12: 373-378.

Zhou, M., Y. Li, T. Zhou and X. Yu, 1989. A BrdU-BSG method for G-banding in fish chromosomes and an ideogram of G-banded karyotype of silver carp. Acata Genetic Sinica 16: 184-187.

CHAPTER 4
DETECTION AND LOCALIZATION BY *IN-SITU* POLYMERASE CHAIN REACTION OF A CHANNEL CATFISH GENE ENCODING THE IMMUNOGLOBULIN HEAVY CHAIN CONSTANT REGION

Introduction

In-situ hybridization (ISH) involves a hybridization reaction between a labeled nucleotide probe and a complementary strand of target DNA or RNA in tissue sections or in intact cells (Leitch *et al.* 1994). Many refinements were made in the past decade, that have changed the original ISH (Gall and Pardue 1969) into a technique that provides fast, precise and sensitive localization of one copy per cell (Lawrence 1990). The major technical variations of ISH include probe length, and the types of labeling and detection methods. The size of probes can range from 25 to 500 nucleotides for DNA, and from hundreds to thousands of nucleotides for RNA (Leitch *et al.* 1994). Probes can be labeled with radioisotopes (Chang *et al.* 1988), biotin, digoxigenin (Chen 1994), chemiluminescent (Xie and Troyer 1996), or fluorescent markers.

Fluorescent *in-situ* hybridization (FISH) is a consistent technique for visualizing DNA within cells, interphase nuclei, and extended chromatin fibers (Lawrence 1990). Although the innovation of FISH has enabled mapping of single-locus genes (Lawrence *et al.* 1990), an inherent limitation to this technique is that it requires multiple copies of identical DNA or RNA sequences for detection (Nouvo 1992). Fewer copies require a secondary signal amplification for detection. For this reason, FISH has been employed to study mRNA present in high copy numbers (de Bault and Wang 1995), or highly

repeated DNA sequences (Kubota *et al.* 1993; Pendas *et al.* 1993). This difficulty has been gradually overcome with introduction of the polymerase chain reaction (PCR) into morphological and diagnostic fields (Gu 1994). The PCR technique is capable of producing billions of copies of target DNA, but these products do not stay associated with the target tissue or chromosome structure. A combination of ISH with PCR has enabled detection of single-copies of DNA or RNA (Anderson 1995). Currently, applications of *in-situ* PCR (ISPCR) are confined mainly to the detection of foreign or mutated genes (Bagasra and Pomerantz 1993, Yin *et al.* 1994).

Direct and indirect methods are two important variations developed for the ISPCR (Gu 1994). The direct ISPCR uses primers or free nucleotides labeled with biotin, digoxigenin, or protein molecules that allow direct visualization of targets of interest after amplification. This procedure is more straightforward and easier to perform. The indirect method starts with the PCR amplification followed by hybridization with labeled probes. This procedure is more complicated but the hybridization step provides additional check on specificity of the PCR amplification.

In this study, I chose the *Ig H* gene (encoding the immunoglobulin heavy chain constant region) of channel catfish as a target. The nucleotide sequence of this gene and its mRNA splicing pattern have been reported (Wilson *et al.* 1990). The objectives of this study were to develop an ISPCR procedure for detecting genetic material using intact cells of channel catfish, to expand the use of ISPCR for gene localization in interphase nuclei, and to evaluate the validity and specificity of the ISPCR procedure.

Materials and methods

Animals

Channel catfish were obtained from a stock at LSU, and spawned in an indoor recirculating system. Five healthy fish (mean ± SD, 550 ± 58 g) were used for periodic blood sampling in this study.

Microscope slides

Two-well microscope slides with a teflon-coated border (Cel-line, Newfield, NJ) were used. Slides were autoclaved and coated with a 2% 3'-aminopropyltriethoxysilane (AES) (Sigma Chemical Company, St. Louis, MO) solution made in acetone.

Isolation of leukocytes

Leukocytes were isolated from peripheral blood of channel catfish using the density centrifugation method (Zhang and Tiersch 1995). The isolated cells were resuspended in Ca^{2+}- and Mg^{2+}- free phosphate buffered saline (CMF-PBS) at 1.0×10^5, 1.0×10^6, and 1.0×10^7 cells/ml. Ten ml of the final cell concentrations were placed in wells of the coated slides, and the slides were dried overnight in a laminar hood.

Preparation of interphase nuclei

The isolated leukocytes were cultured in L15 medium (Gibco BRL., Life Technologies Inc., Gaithersburg, MD) supplied with 10% fetal bovine serum (Gibco) and 5% catfish serum. Concanavalin A was added to culture medium for stimulation of mitosis. Cells were harvested after 3 d of culture, and interphase nuclei were prepared with the same procedure used for preparation of catfish chromosomes (Zhang and Tiersch 1995).

Extraction of genomic DNA

Blood was collected from the caudal vessels of catfish into acid-citrate-dextrose solution (Becton-Dickinson vacutainer 4606). Genomic DNA was extracted from whole blood using the QIAamp blood kit (Qiagen Inc., Chatsworth, CA). The purity and concentration of DNA was estimated using the GeneQuant RNA/DNA calculator (model 80-2104-98, Pharmacia Biotech, Cambridge, England).

Preparation of biotin-labeled probe DNA

Probe DNA was synthesized by PCR using primers labeled with biotin-16-dUTP. Primers were synthesized by the LSU Gene Probes and Expression Systems Laboratory, and were designed to target the Ch4 exon of the *Ig H* gene. The sequences were: TCCCCAAGGTTTACTTGCTCGCTCC and CGATGGATCTGGATATTGGCGCAC (5' to 3'), which yields a DNA fragment of 303 base pairs (bp) from genomic DNA of channel catfish. The PCR reaction conditions were described previously (Zhang *et al.* 1994), and the PCR product was purified with QIAquick spin PCR purification kit (Qiagen Inc., Chatsworth, CA). Purity and yield were estimated by the method described above.

In-situ amplification and hybridization

The ISPCR procedure for whole-cell preparations was based on methods used for diagnosis of viral genes in human cells (Bagasra *et al.* 1994). The ISPCR procedure for preparations of interphase nuclei was based on standard FISH techniques (Ward *et al.* 1994).

(1) Slide pretreatment. (a) Cells. Slides were placed on a heat block (Thermolyne Dri-bath, Model 17615, Dubuque, Iowa) for 90 s at 105 °C and fixed in 2%

paraformaldehyde (PFA, Sigma) solution for 8 h. The slides were rinsed once with 3-x PBS, and twice with 1-x PBS for 10 min each with constant stirring. After treating with 0.3% H_2O_2 (Sigma) overnight, the slides were rinsed twice with 1-x PBS for 10 min. The cells were digested with proteinase K (6 μg/ml) (Amresco, Solon, OH) at room temperature for 1 h and heated at 95 °C for 1 min to denature the proteinase. The slides were rinsed with 1-x PBS and distilled water (dH_2O), dehydrated through a series of 70% to 100% ethanol (EtOH), and dried in a laminar hood. (b) Interphase nuclei. The steps remained the same as above with the following changes; the slides were not fixed with PFA and were treated with 0.3% H_2O_2 solution for 8 h at room temperature. Instead of proteinase treatment, slides were incubated with DNase-free RNase A (200 μg/ml) (Calbiochem® Corp., La Jolla, CA) for 1 h at 37 °C.

(2) Pre-denaturation (for interphase nuclei only). Slides were treated with 70% deionized formamide (Sigma) and 2-x SSC for 2 min at 70 °C and placed immediately into 70% EtOH for 5 min at -20 °C. Slides were dehydrated through a series of 80% to 100% EtOH and dried in a laminar-flow hood.

(3) *In-situ* PCR. Reaction mixtures were prepared in the same way as for liquid-phase PCR. Biotin-labeled primers were used for direct ISPCR, and unlabeled primers were used for indirect ISPCR. Twenty μl of reaction mixture was applied to each well containing whole cells or nuclei. To prevent evaporation, each well was sealed with coverglass (22 x 22 mm) attached with vacuum grease along its inner edge. The edge of the coverglass was sealed with clear nail polish. The cell preparations were subjected to 20 cycles on a thermal cycler (MJ Research, Inc., Watertown, MA), and the

chromosome preparations were subjected to 10 to 15 cycles of the same parameters used for liquid-phase PCR.

(4) Post-hybridization (for indirect PCR only). After *in-situ* PCR, slides were dipped in 100% EtOH for 2 min and the coverglass was removed. Slides were rinsed with 2-x SSC and dH_2O, dehydrated with a series of 70% to 100% ethanol solutions, and dried in a laminar hood. The hybridization mixture included the following components: biotin-labeled probe (1.2 ng/ml), 50% deionized formamide, 2-x SSC buffer, 10-x Denhardt's solution, 0.1% sonicated salmon sperm DNA, and 0.1% sodium sodecyl sulfate (SDS). Twenty ml of hybridization mixture was applied to each well, and sealed using the method described above. Hybridization was performed on the thermal cycler: slides were heated for 5 min at 95 °C and cooled gradually to 37 °C. Slides were incubated at 37 °C overnight.

(5) Detection of hybridization signal. (a) Enzyme-mediated method (for whole cells only). After hybridization, slides were rinsed twice with 1-x PBS for 10 min. The slides were blocked by 1% bovine serum albumin (BSA) (Sigma) for 30 min with constant agitation at room temperature. Fifteen ml of streptavidin-conjugated peroxidase (10 mg/ml, Sigma) were added to each well, and were sealed with a coverglass and incubated for 1 h at 37 °C. The slides were rinsed 3 times with 1-x PBS for 10 min, and stained with 5% 3-amino-9-ethyl-carbazole (AEC, Sigma) for 10 to 30 min at 37 °C. The slides were rinsed immediately with tap water, and air dried. (b) Fluorochrome-based method. After hybridization, slides were rinsed sequentially with 50% formamide in 2-x SSC (v/v) for 2 min at room temperature, 2-x SSC twice for 5 min at 37 °C, and 0.2% Tween 20 in 4-x SSC for 5 min at room temperature. Slides

were blocked with 1% BSA, 4-x SSC, and 0.05% Triton X-100 (v/v) for 15 to 30 min at room temperature. Slides were stained with avidin-fluorescein isothiocyanate (FITC) (Boehringer Mannheim Co., 1:200 in 4-x SSC and 1% BSA) for 1 h at 37 °C. Slides were rinsed twice with 2-x SSC for 10 min at 37 °C, and 0.2 % Tween 20 in 4-x SSC for 5 min at 37 °C, and 0.2% Tween 20 in 4-x SSC for 5 min at room temperature. Slides were counterstained with propidium iodide (5 μg/ml) prepared in antifade medium (100 mg *p*-phenylenediamine in 100 ml glycerol, pH 11).

(6) Image analysis. Cell and chromosome preparations were examined under a fluorescence microscope (Microphot-SA, Nikon Inc.) equipped with fluorescence filters for FITC (excitation wavelengths 420-490 nm) and propidium iodide (excitation wavelengths 330-380 nm). The images of AEC-stained cells were recorded and analyzed directly by image analysis. Fluorescent images of cells and chromosomes were photographed using Kodak Ektachrome (400 ASA) color slide film. The slides were scanned into the computer with a slide scanner (SprintScan 35, Polaroid scanner model CS-2700, Needham Heights, MA) for further analysis.

Validation and control

(1) Internal control. Fetal donkey dermal (FDD) cells were used as an internal negative control in this study. The FDD cells were mixed with catfish leukocytes at ratios of 1:3, 2:2, and 3:1, and the concentration was adjusted to 1.0 x 10^6 cell/ml. The sizes of FDD and catfish leukocytes were measured by the "area measurement" function of the Optimas® software, and a student's t-test was used to evaluate size difference of these cell types at significance level 0.05. The PCR was performed as above except that the annealing temperature was raised to 65 °C to maximize specificity for catfish cells.

(2) Liquid-phase PCR. Liquid-phase PCR was included in each experiment as a control. The other PCR-related controls were reactions prepared without template DNA, primers, or polymerase.

(3) Examination of biotin-labeled probe DNA by dot blot assay. The incorporation of biotin into probe DNA is the prerequisite for hybridization. A routine procedure for detecting biotin incorporation was followed (Leitch *et al.* 1994) with modification for use with peroxidase-based detection systems. Nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) were soaked in 1-x PBS for 5 min, and blotted dry between filter paper sheets. Five ml each of the following DNA solutions were loaded on the membrane: unlabeled probe, labeled but not purified, and labeled and purified. The membranes were left in a laminar hood until completely dried and transferred into 1-x PBS for 1 min. The membrane was blocked with 1% BSA and stained by the streptavidin-peroxidase-AEC (color-based) development method as described above.

(4) DNA sequencing. Sequences of probe DNA, and PCR products amplified during liquid-phase PCR were analyzed by using the cycle sequencing ready reaction kit (Perkin Elmer, Foster City, CA) and the ABI Prism™ 310 Genetic Analyzer (Perkin Elmer). Sequences were imported into PC/Gene computer software package (IntellGenetics, Inc., Mountainview, CA); final sequence was determined with complementary information from positive and negative strands and compared with published sequences.

Results

Overall procedure considerations

In general, the goal of the ISPCR procedure on whole-cell preparations (Fig. 4.1) was to render cell membranes permeable to the reaction components, yet impermeable to the PCR products. The goal of the ISPCR procedure on nuclear preparations was to increase the sensitivity of target amplification and retention of PCR products. The proteinase digestion, which was considered to be the most critical step for cell preparations, could be affected by cell concentration. Cell suspensions dispensed at 1.0×10^5 to 10^6 cells/ml had better digestion efficiency than suspensions distributed at 1.0×10^7 cells/ml (Fig. 4.2). The target gene in positive cells was amplified and the products were retained within cells stained with yellow-green (FITC) fluorescence. The negative cells were stained orange red with propidium iodide (a counterstaining) because of no amplification or no PCR products retaining within cells. The ISPCR performed on interphase nuclei included similar procedures but with several different steps (Fig. 4.1). Instead of proteinase digestion, slides were treated with RNase A to increase the sensitivity of target binding. Predenaturation was another critical step in the protocol of interphase nuclei, but it was not necessary for cell preparation. The washing after hybridization for cell preparations could be performed more times or at higher temperature than for nuclear preparations.

In-situ amplification on cell preparations

Based on the above results, cell suspensions were adjusted to 1.0×10^6 cells/ml. The intracellular PCR products were detected by a streptavidin-peroxidase-AEC color development method (Fig. 4.3), or by avidin-FITC based fluorescent staining (Fig. 4.4).

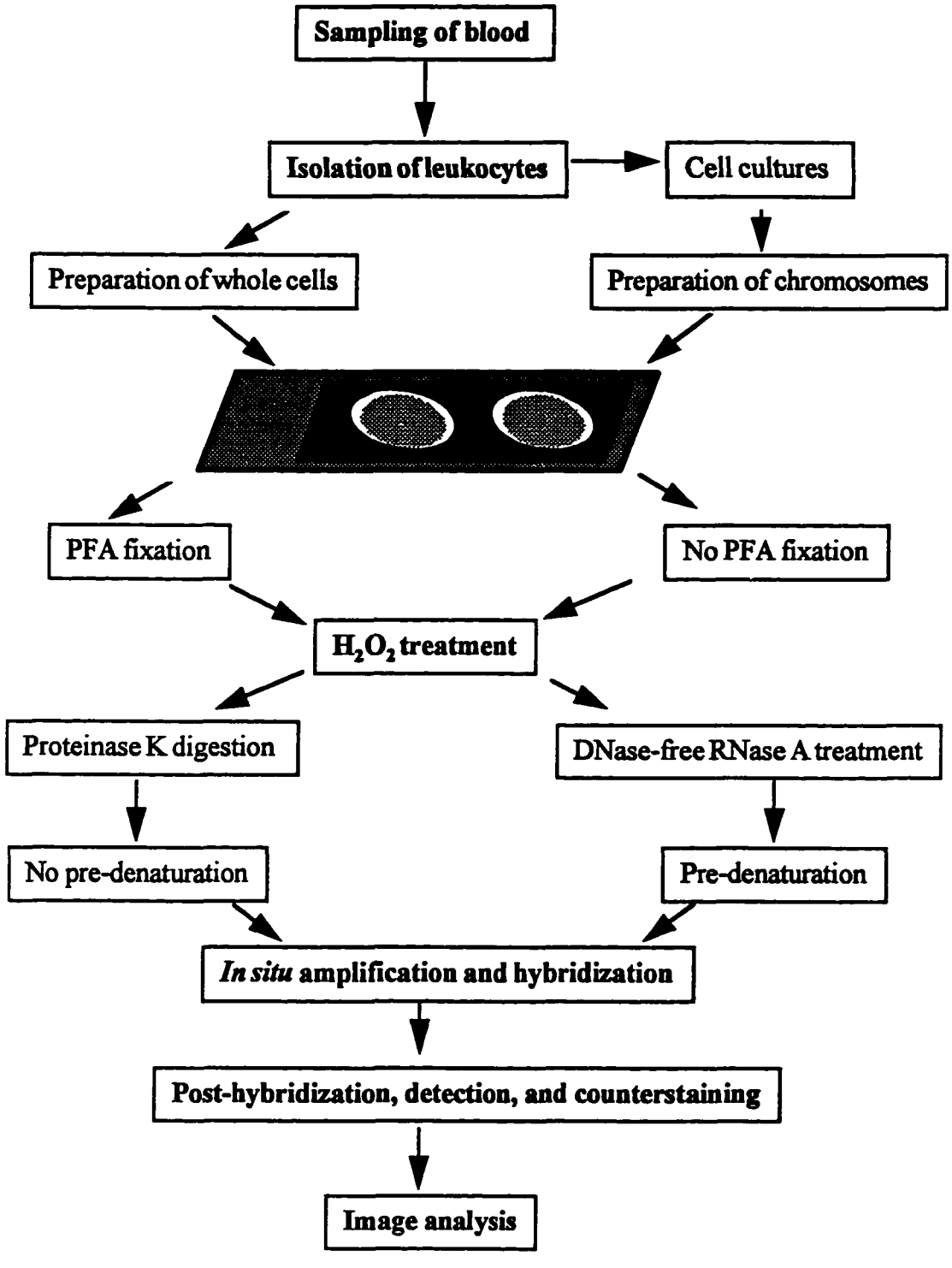


Figure 4.1. A diagram *in-situ* polymerase chain reaction (ISPCR) procedure. Left, preparation of whole cells; center, common steps; right, preparation of interphase nuclei.

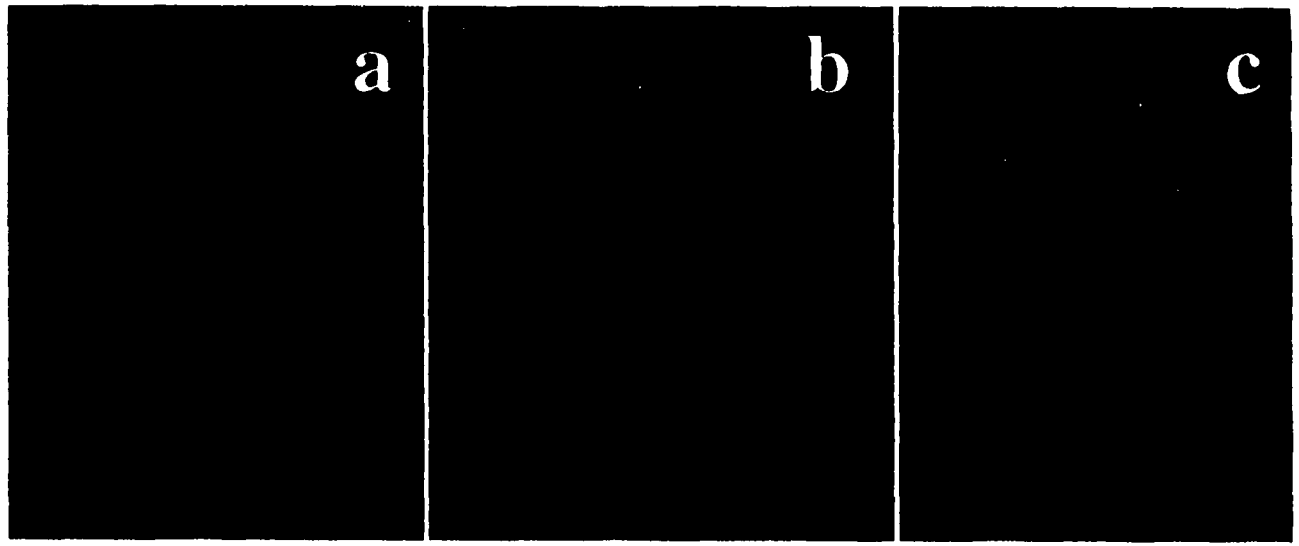


Figure 4.2. *In-situ* PCR amplification of DNA from intact catfish leukocytes prepared at different concentrations: 1.0 x 10^5 cells/ml (a); 1.0 x 10^6 cells/ml (b), and 1.0 x10^7 cells/ml (c). Intracellular PCR products were detected by fluorescent staining with avidin-FITC (yellow fluorescence), and cells were counterstained with propidium iodide (red fluorescence). Proteinase digestion of the cells of highest density was not sufficient, and consequently no amplification signal (yellow fluorescence) was detected; only the counterstaining color (orange red) was observed (c) (x 1000). Note: the original color was yellow in (a) and (b) and orange red in (c).

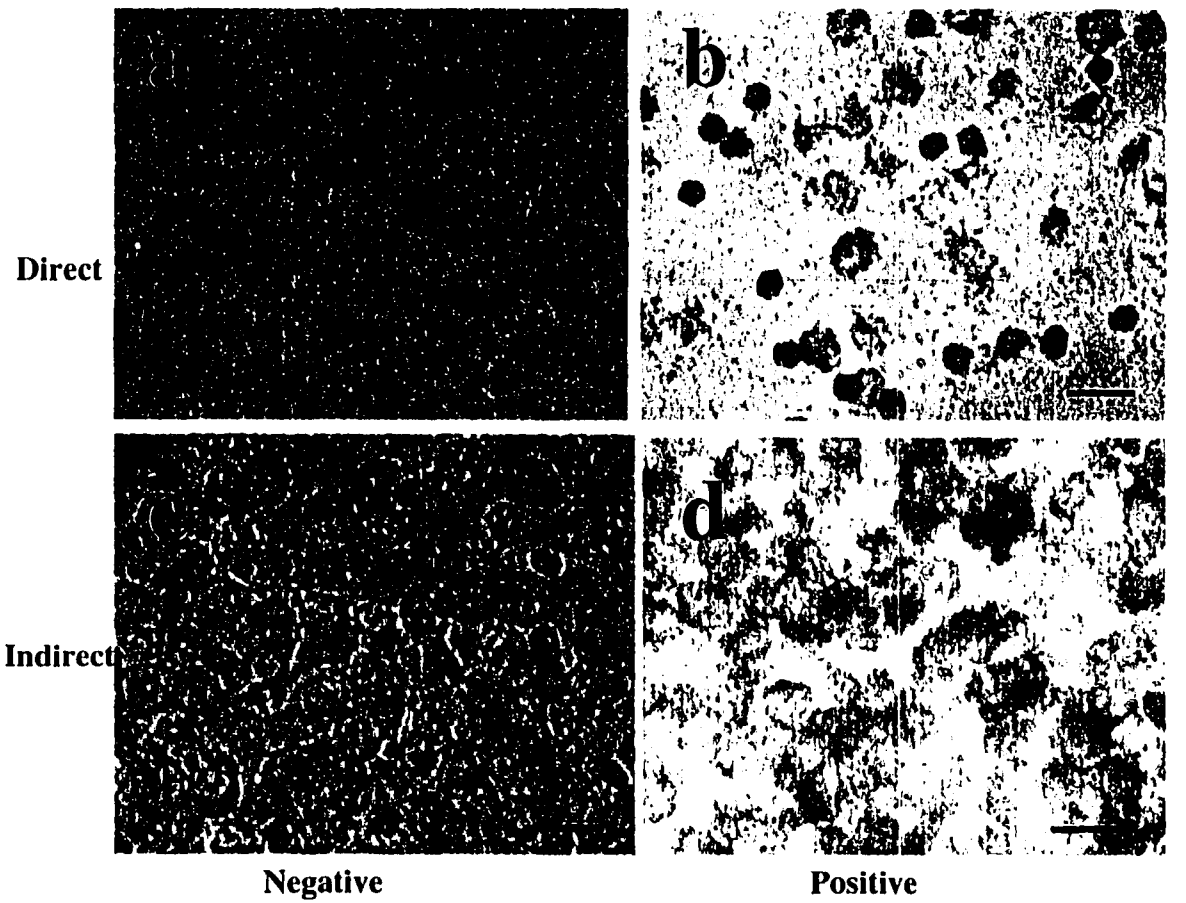


Figure 4.3. *In-situ* PCR amplification of DNA from catfish leukocytes with unlabeled primers as negative control (a), or with biotin-labeled primers (b). Intracellular PCR products were detected directly by the streptavidin-peroxidase color development method (x 1000). An indirect ISPCR procedure was included for comparison, in which cells were amplified using unlabeled primers, and intracellular PCR products were hybridized with biotin-labeled (d), or with unlabeled probe DNA as control (c) (x 1000). No intracellular PCR products were detected within cells from the controls (a and c); only outlines of these cells were seen.

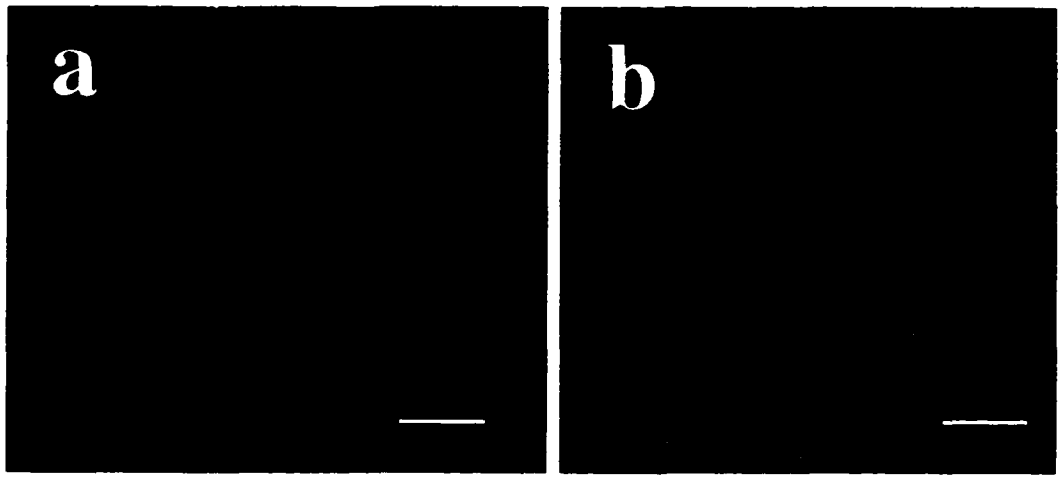


Figure 4.4 *In-situ* PCR amplification of DNA from catfish leukocytes with unlabeled primers as negative control (a), or with biotin-labeled primers (b) (x 1000). Intracellular PCR products were detected by the avidin-FITC-based fluorescence method (yellow fluorescence), and cells were counterstained with propidium iodide (red). The original color was orange-red in (a) and yellow in (b).

For the color development method, the direct ISPCR procedure was first applied. The controls, amplified by unlabeled primers, were negatively stained with AEC (Fig. 4.3 a). Therefore, only the outlines of cells were visible. Cells retaining the PCR products amplified with biotin-labeled primers were stained intensely in the nucleus and cytoplasm (Fig. 4.3 b). These results were verified by an indirect ISPCR procedure. The control cells, in which the *Ig H* gene was amplified with unlabeled primers and hybridized with unlabeled probe, were not stained (Fig. 4.3 c). Cells on the treatment slides were amplified for the *Ig H* gene with unlabeled primers but hybridized with labeled probe, which provided heavy staining with AEC (Fig. 4.3 d).

For the fluorescent method, the control cells maintaining the PCR products amplified by unlabeled primers, were not stained by FITC and displayed only propidium iodide counterstaining (Fig. 4.4 a). The cells with the products amplified by biotin-labeled primers were stained brightly by avidin-FITC, and very little counterstaining was observed (Fig. 4.4 b).

In-situ amplification on interphase nuclei

Direct (Fig 4.5 a) and indirect procedures (Fig. 4.5 b and c) were used to detect the intranuclear location of the *Ig H* gene. Two copies of the target gene were found in each of the positive interphase nuclei for both methods.

Validation and Control

The *in-situ* amplification for the channel catfish *IgH* gene was conducted on channel catfish leukocytes (Fig. 4.6 a), on FDD cells (Fig. 4.6 d), and on a mixture of channel catfish leukocytes and FDD cells (Fig. 4.6 b and c). The yellow-green

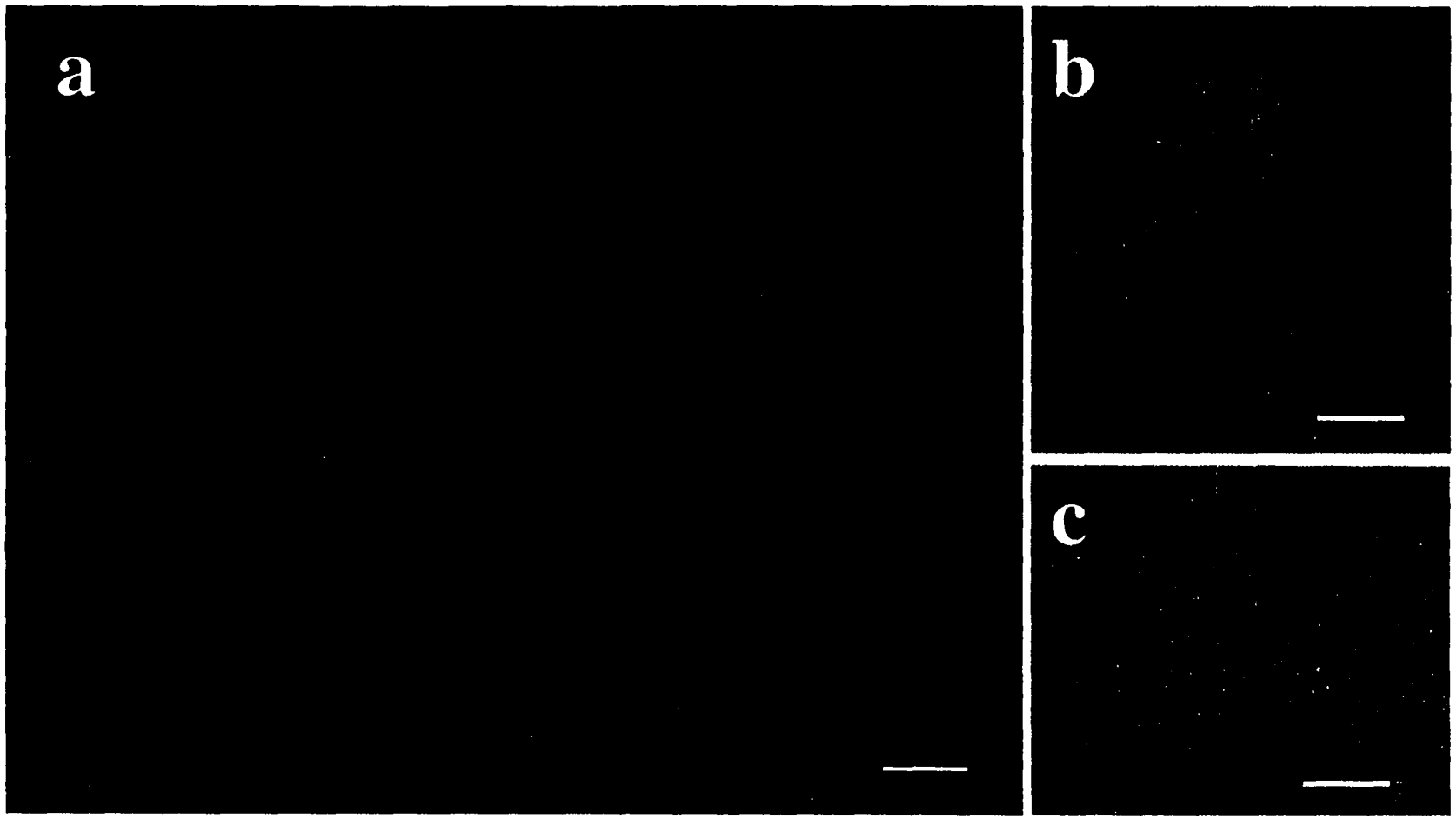


Figure 4.5. Localization on interphase nuclei of the channel catfish gene *Ig H* encoding the immunoglobulin heavy chain constant region by direct (a) and indirect (b and c) *in-situ* PCR methods (x 1000). For comparison, images of interphase nuclei were viewed with fluorescent filters for propidium iodide (a) and FITC (b). Each positive nucleus was found to have 2 copies of the target gene (clearly seen in b and c).

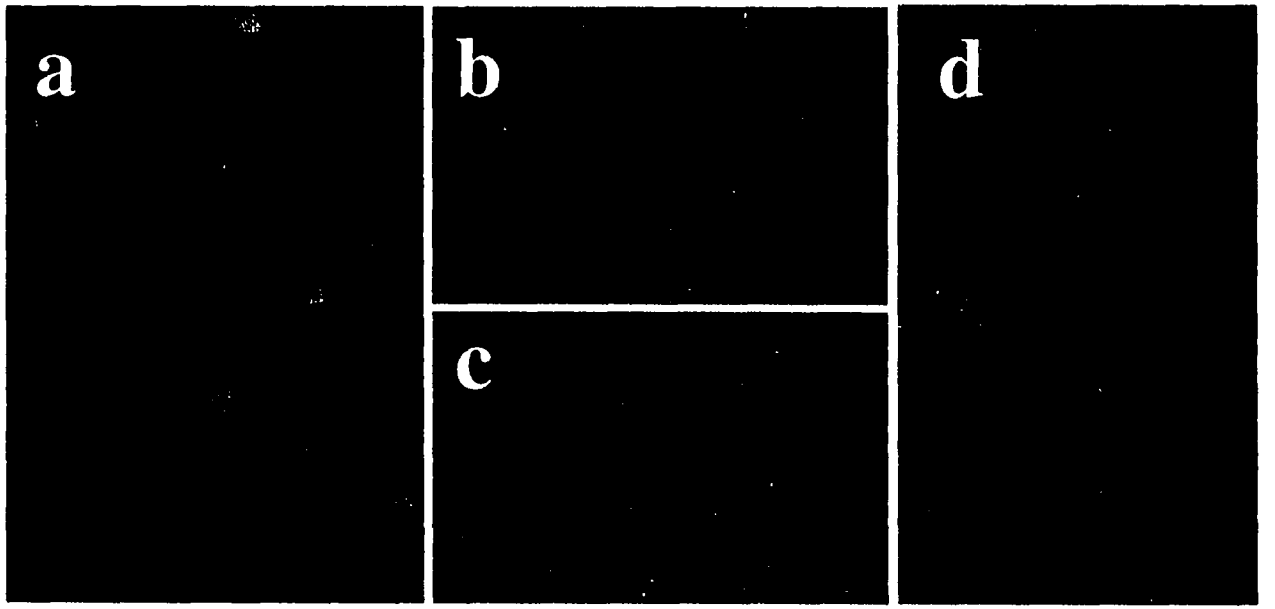


Figure 4.6. *In-situ* PCR was performed on channel catfish leukocytes (a), fetal donkey dermal (FDD) cells (d), and mixtures of channel catfish leukocytes and FDD cells (b and c). Intracellular PCR products were detected by the avidin-FITC-based fluorescence method (yellow fluorescence), and cells were counterstained by propidium iodide (red fluorescence). Yellow fluorescence appeared only within the catfish leukocytes, which were significantly smaller than the FDD cells (note: the original color was yellow on channel catfish leukocytes and red on FDD cells) (x 1000).

fluorescence (FITC) appeared only on channel catfish leukocytes which were significantly smaller ($P = 0.001$) than FDD cells.

The sizes of the probe and the liquid-phase PCR product were about the 303 bp fragment as expected (Fig. 4.7.a). No product was detected from DNA extracted from FDD cells, or from the reactions in which no template DNA, polymerase, or primers were added (Fig 4.7 a).

Biotin incorporation was detected in the probe DNA synthesized by PCR (Fig. 4.7 b). The AEC-staining for purified probe was less intense than that for unpurified probe. Negative staining was found on PCR product amplified by unlabeled primers.

The nulceotide sequence of 307 bp of the PCR-amplified *Ig H* gene fragment was compared with the published sequence (Fig. 4.7 c). A 100% agreement was found in alignment between the primer sequences.

Discussion

In this study, an *in-situ* PCR method was developed for detecting a single-locus gene within intact channel catfish leukocytes and nuclei. The specificity of the ISPCR procedure was verified by use of internal negative controls for the cell preparation. The identical nature of the synthesized probe DNA, and PCR product acquired during ISPCR, was verified by gel electrophoresis, dot-blot assay and DNA sequencing.

The ISPCR procedure was easy for use with whole cell preparations. The most important aspects of this technique were microscope slide preparation, control of proteinase digestion, *in-situ* amplification, and post-hybridization treatment. The use of

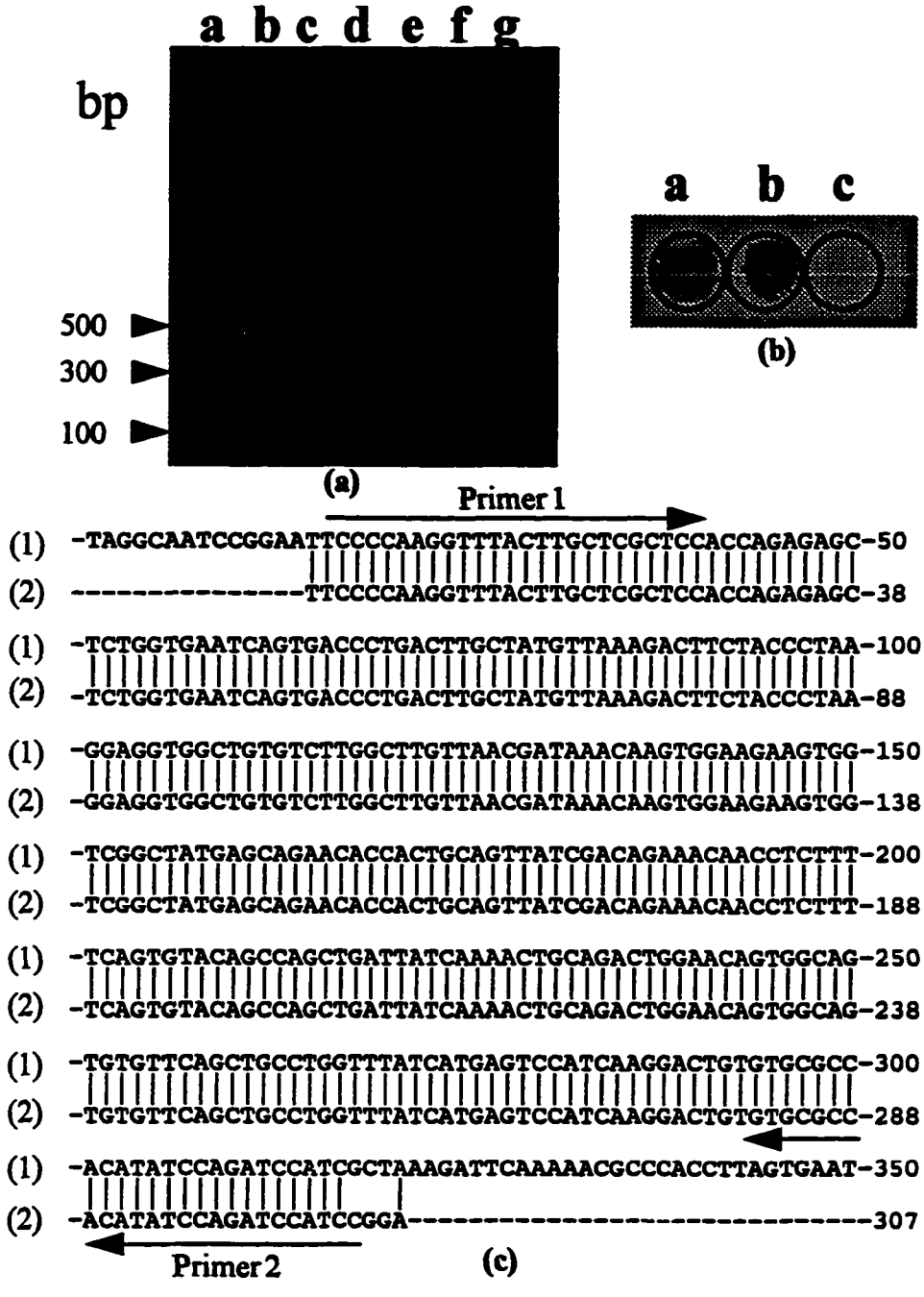


Figure 4.7. Analysis of PCR-amplified DNA fragments by agarose gel (2%) electrophoresis; biotin incorporation was examined by dot-blot assay, and nucleotide sequences of the PCR products were determined by DNA sequencing. The size of PCR products and probe DNA were about 303 bp as expected (A: DNA marker, a; channel catfish DNA, b; probe DNA, c; DNA from fetal donkey dermal cells, d; no template DNA, e; no polymerase, f; and no primers, g). Biotin incorporation was detected on probe DNA by the dot-blot assay (B: biotin-labeled and purified probe, a; biotin-labeled and unpurified probe, b; and unlabeled probe, c). The nucleotide sequence of the PCR-amplified fragment of the *Ig H* gene was determined; the nucleotide sequence between two primers completely agreed with the published sequence (C: Wilson *et al.*, 1990, 1; this study, 2).

coated slides was important to keep cells from detaching during treatments. However, uneven coating or remnant coating materials on slides will interfere with the visualization of target images in subsequent steps. In a previously described method (Bagasra *et al.* 1994), slides were rinsed with water immediately after coating with AES. In this study, better results were obtained when 100% acetone was used to clean the freshly coated slides.

The digestion of cell preparations with proteinase was perhaps the most important step of the entire procedure. Excessive digestion resulted in leakage of intracellular PCR products to the background and adjacent cells and increased non-specific staining. Insufficient digestion reduced penetration of primers and reactants and resulted in false negative results. A criteria for timing of digestion the appearance of "peppery dots" on cell membranes, established in previous methods (Bagasra *et al.* 1994), was only visible under 1000x magnification for catfish leukocytes. Therefore, an improved approach needs to be developed for monitoring the digestion process in these cells.

In-situ amplification was the most delicate step of the procedure. A variety of methods have been investigated for prevention of evaporation during thermocycling (Stapleton *et al.* 1994; Vogel and Kell 1994), and commercial products such as Probe-Clip® (Grace Bio-Labs, Pontiac, MI) are manufactured for this use. However, the sealing method used in this study was more secure, and the results more reproducible.

The continuous process of heating and cooling reduced the visibility of cells and caused morphological change, and thermocycling for 30 or more cycles resulted in the breakage of some cell membranes. The ISPCR amplification for 15 to 20 cycles

generated sufficient copies of target DNA for detection and maintained morphological integrity of the leukocytes.

Amplification in the indirect ISPCR was followed by hybridization with biotin-labeled probe. The indirect ISPCR is a widely accepted procedure because the hybridization step is a confirmation of the specificity of *in-situ* amplification. However, excessive hybridization resulting in heavy staining by AEC has been found to affects the visualization of target cells. In this study, 2 different signal detection methods were compared: color development and fluorescent staining. The buffering system affected the efficiency of detection. The 1-x PBS enhanced streptavidin-AEC staining and reduced background noise, while 2-x SSC increased the efficiency of fluorescent staining.

Interpretations of interphase and metaphase mapping was established for human (Lawrence *et al*, 1988; Lawrence *et al.* 1990), and can be used to explain what I found in this study. Prior to DNA synthesis, interphase nuclei possess G_1 (2C) DNA content, so the 2 copies of *Ig H* gene found in each interphase nucleus should correspond to the copy carried on each of 2 homologous chromosomes. Correspondingly, the metaphase nucleus has G_2 + M (4C) DNA content and each homologous chromosome should bear 2 copies of the *Ig H* gene aligned side-by-side on sister chromatids. In many cases (e.g. Lawrence 1990) including the current study, however, these 2 adjacent copies were too close to be resolved separately.

The ISPCR techniques developed in this study can be applied for the diagnosis of genetic materials foreign to the genome of normal channel catfish, which is especially useful in transgenic studies. The integration of transferred genes in the genome of host

fish can be investigated from embryonic to adult stages. Besides the physical locations, the activity of these genes can be detected by reverse transcription (RT)-ISPCR which has been an important tool for studying the expression of viral genes in AIDS patients. Other studies such as direct linkage of mutated genes to the resultant phenotypes, can also be facilitated by the ISPCR.

References cited in Chapter 4

Anderson, V. M., 1995. *In-situ* PCR: New frontier for histopathologists, pp. 131-144 in *In-situ PCR and related technology*, edited by J. Gu. Eaton Publishing Co., Natic, MA.

Bagasra, O., T. Seshamma, J. Hansen, L. Bobroski, P. Saikumari, J. P. Pestaner, R. J. Pomerantz, 1994. Application of *in-situ* PCR methods in molecular biology: I. Details of methodology for general use. Cell Vision: Journal of Analytical Morphology 1: 324-335.

Bagasra, O., and R. J. Pomerantz, 1993. Human immunodeficiency virus type I provirus is demonstrated in peripheral blood monocytes *in vivo*: A study utilizing an *in-situ* polymerase chain reaction. AIDS Research and Human Retroviruses 9: 69-76.

Chang, H. C., D. J. Jolly, E. Biro, O. W. Jones and T. Friedmann, 1988. Chromosomal sublocalization of human gene sequences by *in-situ* hybridization. Journal of Tissue Culture methods 11: 1-7.

Chen, T. R., 1994. Fluorescence *in-situ* hybridization (FISH): detection of biotin- and digoxigenin-labeled signals on chromosomes. Journal of Tissue Culture methods 16: 39-47.

de Bault, L. E., and B. L. Wang, 1995. Oligonucleotide-primed *in-situ* transcription and immunogold-silver staining systems: Localization of mRNAs in tissues and cells, pp. 99-112 in *In-situ PCR and related technology*, edited by J. Gu. Eaton Publishing Co., Natick, MA.

Gall, J. G., and M. L. Pardue, 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proceeding of the National Academic Sciences of USA 63: 378-383.

Gu, J., 1994. Principles and applications of *in-situ* PCR. Cell Vision: Journal of Analytical Morphology 1: 8-19.

Kubota, S., M. Kuro-o, S. Mizuno and S. Kohno, 1993. Germ line-restricted, highly repeated DNA sequences and their location in a Japanese hagfish (*Eptatretus okinoseanus*). Chromosoma 102: 163-173.

Lawrence, J. B., 1990. A fluorescence *in-situ* hybridization approach for gene mapping and the study of nuclear organization, pp. 1-38 in *Genetic and physical mapping*, edited by K. E. Davies, and S. M. Tilghman. Cold Spring Harbor Laboratory Press.

Lawrence, J. B., R. H. Singer and J. A. McNeil, 1990. Interphase and metaphase resolution of different distances within the human dystrophin gene. Science 249: 928-932.

Lawrence , J. B., C. A. Villnave and R. H. Singer, 1988. Sensitive, high-resolution chromatin and chromosome mapping *in-situ*: Presence and orietation of two closely integrated copies of EBV in a lymphoma cell line. Cell 52: 51.

Leitch, A. R., T. Schwarzacher, D. Jackson and I. J. Leitch, 1994. *In-situ Hybridization: a practical guide*. Bios Scientific Publishers Limited, Oxford, UK.

Nouvo, G. J., 1992. *PCR in-situ hybridization: Protocols and applications*. Raven Press, New York.

Pendas, A. M., P. Moran and E. Garciavazquez, 1993. Ribosomal-RNA genes are interspersed throughout a heterochromatic chromosome arm in Atlantic salmon. Cytogenetics and Cell Genetics 63: 128-130.

Stapleton, M. J., M. C. Levin and S. Jacobson, 1994. DNA amplification within cells on slides: Advances in evaporation and temperature control. Cell Vision: Journal of Analytical Morphology 1: 177-181.

Vogel, P., and W. M. Kell, 1994. A method for controlling evaporation losses from specimens on glass slides during *in-situ* PCR. Cell Vision: Journal of Analytical Morphology 1: 336-338.

Ward, D. C., J. Menninger, J. Lieman, T. Desai, A. Banks, A. Boyle, P. Bray-Ward and T. Haaf, 1994. Integration of the physical, genetic and cytogenetic maps of human chromosomes: Implications for the development od diagnostic DNA probes. Cell Vision: Journal of Analytical Morphology 1: 61-66.

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.

Xie, H., and D. L. Troyer, 1996. Chemiluminenscent detection of unique sequences on chromosomes afetr on-slide PCR. BioTechniques 20: 54-56.

Yin, J., M. G. Kaplitt and D. W. Pfaff, 1994. *In-situ* PCR and *in vivo* detection of foreign gene expression in rat brain. Cell Vision: Journal of Analytical Morphology 1: 58-59.

Zhang, Q., and T. R. Tiersch, 1995. Cryopreservation of channel catfish (*Ictalurus punctatus*) leukocytes for subsequent cytogenetic analysis. Journal of Fish Biology 47: 1016-1025.

Zhang, Q., T. R. Tiersch and R. K. Cooper, 1994. Rapid isolation of DNA for genetic screening of catfish by polymerase chain reaction. Transactions of the American Fisheries Society 123: 997-1001.

CHAPTER 5
CAN *IN-SITU* POLYMERASE CHAIN REACTION AND REPLICATION BANDING BE COMBINED FOR PHYSICAL MAPPING OF THE CHANNEL CATFISH GENOME?

Introduction

Application of *in-situ* hybridization (ISH) to the direct localization of single-locus sequences on metaphase chromosomes began (Gerhard *et al.* 1981; Harper *et al.* 1981) with autoradiography. General acceptance of ISH in physical mapping occurred during the past decade, led by the development of non-isotopic labeling techniques and by use of fluorescent or enzymatic reporters (Bauman *et al.* 1980). Fluorescent *in-situ* hybridization (FISH) has now become a standard method for chromosomal localization of genes (Trask *et al.* 1991; Ronne 1992) in higher vertebrates including humans (Chen 1994), mice (Matsuda *et al.* 1992), cattle (Iannuzzi *et al.* 1993), and birds such as chickens (Rauen *et al.* 1994). Except for use in cytogenetics and gene mapping, FISH is most commonly used for diagnosis of chromosomal abnormalities in prenatal examination (Claussen *et al.* 1993) and for the study of tumor biology (Criel *et al.* 1994).

However, an inherent limitation to the FISH technique is that multiple copies of a target sequence are required for detection (Nouvo 1992). *In-situ* polymerase chain reaction (ISPCR) is a newly developed technology that allows multiplication of target DNA sequences to billions of copies. Although ISPCR is theoretically usable in physical mapping, information on this topic is limited to technical notes (Gosden and Hanratty 1993; Xie and Troyer 1996) and only a few have addressed chromosomal mapping (Troyer *et al.* 1994a; Troyer *et al.* 1994b). This is because it is hard to preserve

chromosome morphology in thermal cycling and difficult to retain PCR products *in-situ*, preventing excessive non-specific hybridization signals.

The identification of chromosome banding patterns is an additional and required step of physical mapping. Several procedures have been proposed to allow the simultaneous observation of chromosome bands and hybridization signals, such as the use of alkaline antifading, and counterstaining solution (Lemieux *et al.* 1992) and 4', 6-diamidino-2-phenylindole (DAPI) banding (Heng and Tsui 1993). Alternatively, hybridization signals and banding pattern can be displayed in 2 successive steps. (Viegas-Pequignot 1992; Larramendy *et al.* 1993).

Compared with higher vertebrates, little information is available about application of ISH for gene mapping and cytogenetic studies of fish species. Ribosomal RNA genes (Carman *et al.* 1993; Pendas *et al.* 1993b; Pendas *et al.* 1994) and other highly repeated DNA sequences (Kubota *et al.* 1993) have been investigated in a few cases. Greater difficulty can be expected in applying ISPCR to fish species because fish chromosomes are small and numerous. Techniques for identifying individual chromosomes are available only in a few species (Hellmer *et al.* 1991; Chapter 3). Most fish species do not have well differentiated chromosome bands, and therefore, simultaneous display of hybridization signals and banding patterns can be difficult to achieve.

In this study, an ISPCR method was tested for chromosomal localization of the channel catfish *Ig H* gene encoding the immunoglobulin heavy chain constant region (Wilson *et al.* 1990). The objectives of this study were to: (1) identify the location of the *Ig H* gene on channel catfish chromosomes, and (2) to analyze the identity *Ig H*-bearing chromosome by simultaneous and sequential staining methods.

Materials and methods

Preparation of metaphase chromosomes

Leukocytes were isolated from peripheral blood of channel catfish and cultured in L15 medium (Gibco BRL, Life Technologies Inc., Gaithersburg, MD) supplied with 10% fetal bovine serum (Gibco) and 5% catfish serum. Phorbol ester (Sigma Chemical Company, St. Louis, MO) and calcium ionophore A23287 (Sigma) were added for stimulation of mitotic activity (Lin *et al.* 1992). Metaphase chromosomes were prepared based on a replication banding procedure and stained by the fluorochrome plus Giemsa (FPG) method to reveal banding patterns (Chapter 3).

Chromosome length was measured by the "line measurement" function of the Optimas® computer software package (BioScan Inc., Edmonds, WA), and the relative length (percent of total complement length: %TCL) was calculated by the method described elsewhere (Zhang and Tiersch in review).

In-situ PCR amplification and hybridization

Chromosomes were prepared on slides with a teflon-coated border (Cel-line, Newfield, NJ). Pretreatments including RNase A digestion and denaturation of chromosomal DNA, were based on a procedure used for interphase nuclei (Chapter 4). The primers were synthesized with biotin labeling at the 3'-ends. The primer sequences and the conditions for the ISPCR amplification have been described in Chapter 4.

Detection of hybridization signals and chromosome bands

The hybridization signals were detetected by staining with avidin-fluorescein isothiocyanate (FITC) (Chapter 4). Slides were counterstained with propidium iodide (5 mg/ml) prepared in an alkaline antifade medium (100 mg *p*-phenylenediamine in

100 ml glycerol, pH 11) for simultaneous production of chromosome bands. For the sequential detection method, after photographing the hybridization signals, slides were rinsed in 2-x SSC buffer to remove the antifading medium. Slides were baked at 65 °C overnight and processed by the fluorochrome plus Giemsa (FPG) method for displaying chromosome bands (Chapter 3).

Fluorescent *in-situ* hybridization (FISH)

A standard FISH procedure (Ward *et al.* 1994) was performed on chromosome preparations for comparison with the ISPCR technique. The hybridization was performed at 37 °C in a humid box for 36 to 48 h. The subsequent treatments were the same as used in the ISPCR.

Results

The chromosomal location of the *IgH* gene was revealed by the ISPCR procedure (Fig. 5.1 a and b), and was consistently found on the telomeric position of a particular chromosome. The fluorescent counterstaining (propidium iodide in alkaline medium) did not reveal the chromosome banding pattern, however, neither the sequential treatment by FPG method which was performed after the ISPCR. The replication R-bands visible before ISPCR (Fig. 5.2 a) became weak and were not analyzable after thermal cycling (Fig. 5.2 b).

The chromosomal location of the *Ig H* gene displayed by standard FISH method was shown in Figure 5.1 c. The morphology of the *Ig H*-bearing chromosome was

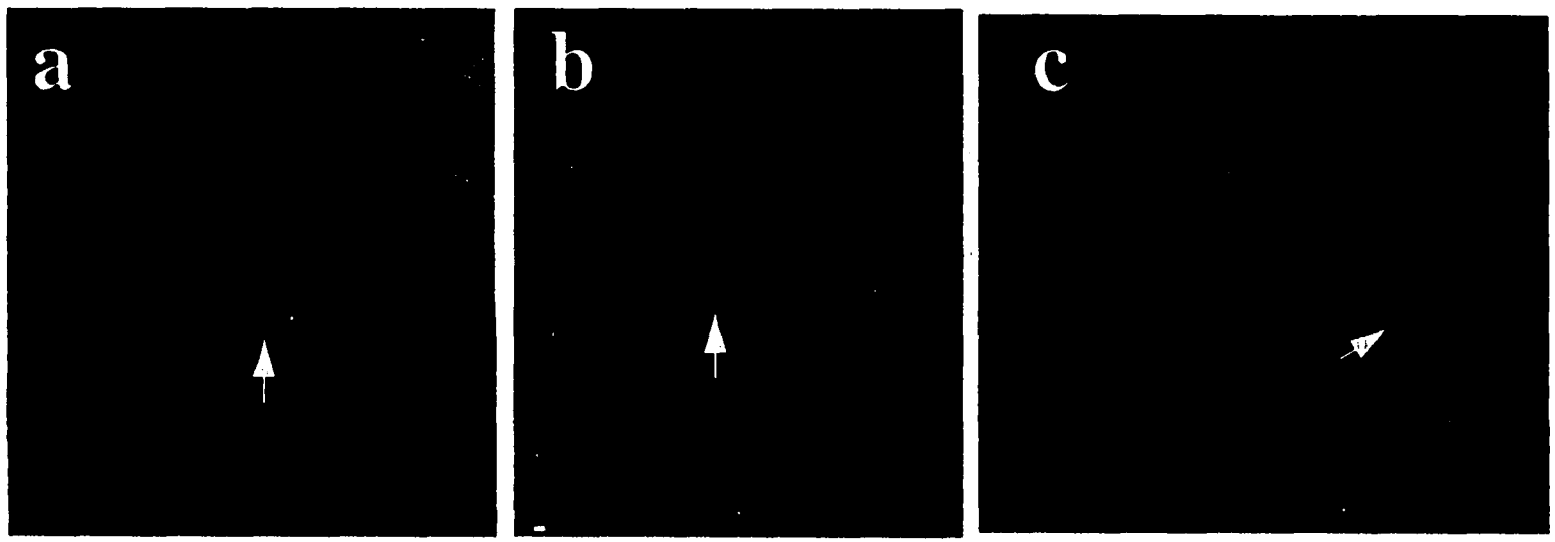


Figure 5.1. Chromosomal location of the channel catfish *Ig H* gene by direct *in-situ* PCR (a and b, x 1000). Standard fluorescence *in-situ* hybridization procedure was included for verification (c, x 1000). Arrowheads indicate location of the target gene.

Figure 5.2. Replication banding of channel catfish chromosomes prepared by the fluorochrome plus Giemsa staining method: before (a), or after *in-situ* PCR amplification (b) (x 1000). The R-bands were not recognizable after *in-situ* PCR amplification. Bars = 10 μ.

similar to that identified by the ISPCR procedure, but the chromosome remained anonymous.

The relative length of the anonymous *Ig H*-bearing chromosome was 3.2 ± 0.2% (n = 10), which was identified to be in the groups D, E, G, or H based on my proposed chromosome classification system of channel catfish (Chapter 2). Further identification requires knowledge of centromere position.

Discussion

In this study, the channel catfish *Ig H* gene was mapped to a preliminary chromosome location. However, the identity of this chromosome remains to be established. Chromosome banding patterns could not be revealed by counterstaining with alkaline propidium iodide, or by subsequent staining with the fluorochrome plus Giemsa (FPG) method because bands become too weak to be analyzed after the ISPCR or FISH.

Replication banding is an important chromosome banding technique for coupling with ISH (Viegas-Pequignot, 1992), and this technique is well developed for mammalian species (Ronne 1992). However, only a limited number of examples have been reported for fish species including cyprinid fish (Zhang and Wu 1985; Hellmer *et al.* 1991), salmonid fish (Delany and Bloom 1984; Pendas *et al.* 1993a), and scorpaenid fish (Giles *et al.* 1988). In another study (Chapter 3) it was found that replication banding of channel catfish chromosomes can only be revealed by harsh treatment such as the FPG technique. Fluorescent stainings including acridine orange, propidium iodide, Hoechst 33258 and actinomycin D, were not able to produce analyzable R-bands. Overall, the

replication banding pattern of fish species is not as highly resolvable as seen in higher vertebrates such as human (Verma and Babu 1989).

Although many procedures have been developed for simultaneous detection of replication R-bands and hybridization signals in mammalian species, most are not applicable for channel catfish chromosomes. These fluorescent procedures do not yield banding patterns on chromosomes without ISPCR or FISH treatments. Therefore, I used a sequential detection method. Unfortunately, the banding pattern was extremely weak and not analyzable after performing hybridization procedures.

This problem could be a cumulative effect resulting from repeated treatments by heat and salt (buffer) in the ISPCR or FISH. The contrast of chromosomes was poor after 20 or more cycles of thermocycling, and the images were difficult to separate from background. Therefore, the number of cycles was reduced to between 10 and 15, and the counterstaining concentration of propidium iodide was increased. Although the contrast of chromosomal images was increased, the intensity of the hybridization signal (FITC yellow-green fluorescence) was weakened. High concentration of salt solution was good for removal of non-specific binding, but it reduced the visibility of chromosome objects.

Chromosomal DNA treated for replication procedures can present another problem for subsequent PCR amplification although this problem was not encountered in the present study. The UV irradiation may induce fragmentation or dimerization of target template DNA and result in the negative amplification. The replacement of BrdU in the position of thymidine of template DNA may cause non-specific amplifiaction and

mismatches in the PCR products. This problem could become serious for target DNA of large size or with AT-rich regions.

Potential solutions for low visibility of replication banding patterns may include densitometric analysis of chromosomes or immunochemical methods. The densitometric method has been applied for analysis of chromosome banding patterns (Chapters 2 and 3), and was highly sensitive for location of weak and continuous chromosome bands (Fig. 5.3). The BrdU-incorporated regions of chromosomes were revealed by immunochemical methods based on the use of anti-BrdU antibody and immunoperoxidase (Pinero *et al.* 1993). The antibody can detect the incorporation of BrdU in chromosomes prepared by BrdU-based replication banding procedures. Therefore, this method could be used to display R-bands of chromosomes after thermocycling treatment.

Restriction enzyme banding or chromosome painting by repetitive DNA probes could be used as alternatives to replication banding for use with the ISPCR. Restriction enzymes (RE) are a group of enzymes that recognize and cut specific sequences of DNA nucleotides. Digestion of whole chromosomes by RE leads to removal of some DNA fragments, and Giemsa staining can be used to reveal the relative amount and location of the remaining DNA (Miller and Miller 1990). This technique was able to produce a serial banding pattern for channel catfish chromosomes (Chapter 2). Repetitive DNA probes such as probes specific for telemeres or centromeres have been used to generate chromosome-specific markers by FISH (Cox *et al.* 1993, Hagemann *et al.* 1993). By

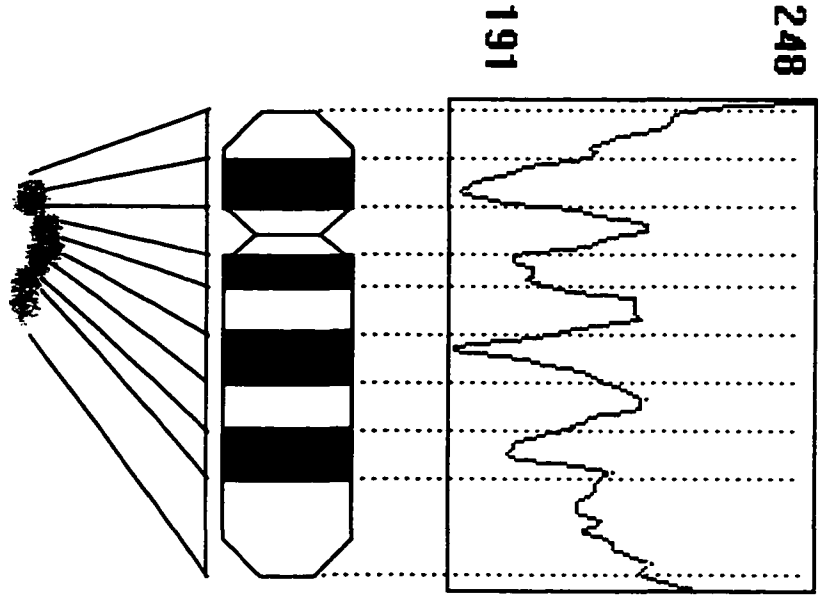


Figure 5.3. Analysis of chromosome banding patterns by densitometry. The banding pattern (left) was expressed as a luminance plot (right), in which the X axis indicates chromosome segments and the Y axis indicates relative grayscale value. The ideogram (middle) was created for the chromosome based on the luminance plot.

dual-color probing it may be possible to visualize target genes and chromosome markers simultaneously.

Application of the ISPCR technique in chromosomal mapping is still in its infancy for fish species and higher vertebrates including humans. To my knowledge, this is first report on physical mapping of a single-locus gene in a fish species. The routine use of the ISPCR technique awaits improvement of the procedure and supportive studies on the methodology of chromosome identification.

References cited in Chapter 5

Bauman, J. G. J., J. Wiegant, P. Borst and P. van Duijn, 1980. A new method for fluorescnce microscopical localization of specific DNA sequences by *in-situ* hybridization of fluorochrome-labeled RNA. Experimental Cell Research 128: 485-490.

Carman, O., Y. Matsuda, T. Oshiro and F. Takashima, 1993. Mapping of rRNA genes (NOR) in carp chromosomes using the cloned probe of mouse rRNA by FISH analysis. Nippon Suisan Gakkaishi 59: 729.

Chen, T. R., 1994. Fluorescence *in-situ* hybridization (FISH): detection of biotin- and digoxigenin-labeled signals on chromosomes. Journal of Tissue Culture methods 16: 39-47.

Claussen, U., R. Ulmer, E. Beinder and H. J. Voigt, 1993. Rapid karyotyping in prenatal-diagnosis - a comparative-study of the pipette method and the *in-situ* technique for chromosome harvesting. Prenatal Diagnosis 13: 1085-1093.

Cox, A. V., S. T. Bennett, A. S. Parokonny, A. Kenton, M. A. Callimassia, M. D. Bennett, 1993. Comparison of plant telomere locations using a PCR-generated synthetic probe. Annals of Botany 72: 239-247.

Criel, A., I. Wlodarska, P. Meeus, M. Stul, A. Louwagie, A. Van Hoof, M. Hidajat, C. Mecucci, and H. Van Den Berghe, 1994. Trisomy-12 is uncommon in typical chronic lymphocytic leukemias. British Journal of Haematology 87: 523-528.

Delany, M. E., and S. E. Bloom, 1984. Replication banding patterns in the chromosomes of the rainbow trout. Journal of Heredity 75: 431-434.

Gerhard, D. S., E. S. Kawasaki, F. C. Bancroft and P. Szabo, 1981. Localization of a unique gene by direct hybridization *in-situ*. Proceeding of the National Academic Sciences of USA 78: 3755-3759.

Giles, V., G. Thode and M. C. Alvvarez, 1988. Early replication bands in two scorpion fishes, *Scorpaena porcus* and *S. notata* (Order Scorpanneiformes). Cytogenetics and Cell Genetics 47: 80-83.

Gosden, J., and D. Hanratty, 1993. PCR *in-situ*: A rapid alternative to the *in-situ* hybridization for mapping short, low copy number sequences without isotopes. BioTechniques 15: 78-80.

Hagemann, S., B. Scheer and D. Schweizer, 1993. Repetitive sequences in the genome of Anemone blanda: Identification of tandem arrays and of dispersed repeats. Chromosoma 102: 312-324.

Harper, M. E., A. Ullrich and G. R. Saunders, 1981. Localization of the human insulin gene to the distal end of the short arm of chromosome 11. Proceeding of the National Academic Sciences of USA 78: 4458-4460.

Hellmer, A., I. Voiculescu and W. Schempp, 1991. Replication banding studies in 2 cyprinid fishes. Chromosoma 100: 524-531.

Heng, H. H. Q., and L. C. Tsui, 1993. Modes of DAPI banding and simultaneous *in-situ* hybridization. Chromosoma 102: 325-332.

Iannuzzi, L., D. S. Gallagher, A. M. Ryan, G. P. Di Meo and J. E. Womack, 1993. Chromosomal localization of omega and trophoblast interferon genes in cattle and river buffalo by sequential R-banding and fluorescent *in-situ* hybridization. Cytogenetics and Cell Genetics 62: 224-227.

Kubota, S., M. Kuro-o, S. Mizuno and S. Kohno, 1993. Germ line-restricted, highly repeated DNA sequences and their location in a Japanese hagfish (*Eptatretus okinoseanus*). Chromosoma 102: 163-173.

Larramendy, M. L., S. J. Nylund, E. Armstrong and S. Knuutila, 1993. Simultaneous detection of high-resolution R-banding and fluorescence *in-situ* hybridization signals after fluorouracil-induced cellular synchronization. Hereditas 119: 89-94.

Lemieux, N., B. Dutrillaux and E. Viegaspequignot, 1992. A simple method for simultaneous R-banding or G-banding and fluorescence *in-situ* hybridization of small single-copy genes. Cytogenetics and Cell Genetics 59: 311-312.

Lin, G. L., C. F. Ellsaesser, L. W. Clem and N. W. Miller, 1992. Phobol ester/calcium ionophore activate fish leukocytes and induce long-term cultures. Development and Comparative Immunology 16: 153-163.

Matsuda, Y., Y. N. Harada, S. Natsuume-Sakai, K. Lee, T. Shiomi, V. M. Chapman, 1992. Location of the mouse complement factor H gene (*cfh*) by FISH analysis and replication R-banding. Cytogenetics and Cell Genetics 61: 282-285.

Miller, D. A., and O. J. Miller, 1990. Restriction enzyme banding of metaphase chromosomes, pp. 238-250 in *Trends in chromosome research*, edited by T. Sharma. Narosa Publishing House, Springer-Verlag.

Nouvo, G. J., 1992. *PCR in-situ hybridization: Protocols and applications*. Raven Press, New York.

Pendas, A. M., P. Moran, J. P. Freije and E. Garcia-Vazquez, 1994. Chromosomal mapping and nucleotide sequence of two tandem repeats of Atlantic salmon 5S rDNA. Cytogenetics and Cell Genetics 67: 31-36.

Pendas, A. M., P. Moran and E. Garciavazquez, 1993a. Replication banding-patterns in Atlantic salmon (*Salmo salar*). Genome 36: 440-444.

Pendas, A. M., P. Moran and E. Garciavazquez, 1993b. Ribosomal-RNA genes are interspersed throughout a heterochromatic chromosome arm in Atlantic salmon. Cytogenetics and Cell Genetics 63: 128-130.

Pinero, J., T. Ortiz and F. Cortes, 1993. 3-way differentiation of chinese-hamster ovary chromosomes by immunoperoxidase technique using a monoclonal antibromodeoxyuridine antibody. Biotechnic and Histochemistry 68: 215-221.

Rauen, K. A., C. D. S. Le Ciel, U. K. Abbottt and N. J. Hutchison, 1994. Localization of the chicken PgK gene to chromosome 4p by fluorescent *in-situ* hybridization. The Journal of Heredity 85: 147-150.

Ronne, M., 1992. Synchronization, banding and *in-situ* hybridization: A short laboratory manual. *In Vivo* 6: 49-58.

Trask, B. J., H. Massa, S. Kenwrick and J. Gitschier, 1991. Mapping of human-chromosome Xq28 by 2-color fluorescence *in-situ* hybridization of DNA-sequences to interphase cell-nuclei. American Journal of Human Genetics 48: 1-15.

Troyer, D. L., D. W. Goad, H. Xie, G. A. Rohrer, L. J. Alexander, C. W. Beattie, 1994a. Use of direct *in-situ* single copy (DISC) PCR to physically map 5 porcine microsatellites. Cytogenetics and Cell Genetics 67: 199-204.

Troyer, D. L., H. Xie, D. W. Goad and D. Z. Skinner, 1994b. Use of a new technique to map the porcine alpha interferon gene to chromosome 1. Mammalian Genome 5: 112-114.

Verma, R. S., and A. Babu, 1989. *Human Chromosomes: Manual of basic techniques.* Pergamon Press, Inc., New York.

Viegas-Pequignot, E., 1992. *In-situ* hybridization to chromosomes with biotinated probes, pp. 135-153 in *In-situ hybridization: A practical approach*, edited by D. G. Wilkinson. IRL Press at Oxford University Press, New York.

Ward, D. C., J. Menninger, J. Lieman, T. Desai, A. Banks, A. Boyle, P. Bray-Ward and T. Haaf, 1994. Integration of the physical, genetic and cytogenetic maps of human chromosomes: Implications for the development of diagnostic DNA probes. Cell Vision: Journal of Analytical Morphology 1: 61-66.

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.

Xie, H., and D. L. Troyer, 1996. Chemiluminenscent detection of unique sequences on chromosomes afetr on-slide PCR. BioTechniques 20: 54-56.

Zhang, Q., and T. R. Tiersch, in review. Cytogenetic analysis of two intergeneric hybrids of ictalurid catfishes.

Zhang, R., and H. Wu, 1985. A study of sex chromosome in *Carassius auratus* by BrdU-Hoechst 33258-Giemsa techniques. Acta Genetica Sinica 12: 373-378.

SUMMARY

The goal of this project was to begin analyzing the genome of the channel catfish and to develop a method for direct gene mapping in this species. A series of techniques were developed to facilitate these studies, including culture of fibroblast cells, preparation of chromosomes from channel catfish of different ages, staining for nucleolus organizer regions (NOR) and heterochromatin (C-banding), restriction enzyme banding, replication banding, simultaneous detection of sister-chromatid exchange and C-banding, FISH, and indirect and direct *in-situ* polymerase chain reaction. This resulted in development and characterization of a primary fibroblast cell line, establishment of standard karyotypes of unbanded, CBG-banded (C-banding by barium hydroxide using Giemsa), and RBG-banded (replication R-banding by bromodeoxyuridine using Giemsa) chromosomes, and detection and localization of a single-locus gene in whole cells, interphase nuclei and metaphase chromosomes.

A primary fibroblast cell line, designated as CCf, was established, which provides a model for *in vitro* study in this species. The CCf cell line was generated from caudal fin tissue by the explant technique. The attachment of explanted tissue fragments inside of culture vessels was enhanced by cold digestion with trypsin and application of external force with a coverglass. The isolated fibroblast cells were easily cultured in medium developed for mammalian cells (with reduced osmolarity and addition of catfish serum). Leibovitz L15 medium supplemented with 5% fetal bovine serum and 5% catfish serum provided the growth requirement of the cells; this culture technique was convenient

and inexpensive. The cell line was propagated continuously, and cryopreserved and recovered successfully at different passages.

The doubling time of the cell line at a plating concentration of 1.0×10^5 cell/ml was around 56 h. The cultured cells were spindle-shaped with 2 elongate poles, and each cell had an egg-shaped nucleus located at the center of the cell body. Cells synthesized fibronectin, and Type I and III collagens in their cytoplasm. The cell line possessed a diploid chromosome number of 58 and a pair of NOR-bearing medium-sized submetacentric chromosomes, which are typical to metaphase spreads of channel catfish. A fragment of the immunoglobulin constant region *Ig H* gene was amplified by polymerase chain reaction (PCR) from DNA of cultured cells, the size of which (303 bp) did not differ from that amplified from DNA of blood.

Structure of individual chromosomes was analyzed by Giemsa staining, location of the NOR, C-banding, restriction enzyme and replication banding. A standard procedure was established to construct karyotypes resulting from different banding techniques (Appendix B.4). Metaphase chromosomes were prepared by a variety of techniques: temporary culture of leukocytes and kidney cells, long-term culture of fibroblast cell, and direct preparation from solid tissues. A centromeric index and morphology-based karyotype, consisting of 8 identifiable groups, was developed for channel catfish. The NOR of channel catfish was located on the short arm of chromosome D11. Channel catfish had a low abundance of heterochromatin, limited to centromeric regions. Members of each homologous pair agreed in their C-banding patterns. A standard karyotype of CBG banding (C-bands by barium hydroxide using Giemsa) was developed for this species.

Digestion of chromosomal DNA by restriction enzymes produced patterns most similar to C-banding but with different locations. Of these enzymes the *Msc* I produced the most informative banding pattern. The enzymes *Hind* III yielded a reproducible serial banding pattern which was comparable to that produced by replication banding. However, there was no correlation between the banding patterns and the recognition sequences of the enzymes used in this sudy.

Replication banding was prepared from cultured leukocytes which were synchronized with fluorouracil and released and labeled with bromodeoxyuridine (BrdU). The banding pattern was revealed by staining with fluorochrome plus Giemsa. Individual chromosomes were identifiable by their unique banding patterns. The patterns were evaluated by a densitometric method with the assistance of a computer-based image analysis system. The replication banding of representative chromosomes was consistent among different cells. A standard RBG karyotype (replication R-banding by bromodeoxyuridine using Giemsa) of channel catfish was developed, and ideograms were prepared for each chromosome. Overall, chromosomal markers created by the NOR, C, RE, and replication bandingtechniques have allowed to idendify individual chromosomes of channel catfish (Fig. S.1).

Chromosomes displaying sister-chromatid exchanges (SCE) were prepared from cultured leukocytes labeled with BrdU for 20 h. The baseline occurrence of sister-chromatid exchange (SCE) for the first cell cycle was about 3.6% of the entire complement. Prolonged treatment of chromosomes with 2-x SSC enabled simultaneous

Figure S.1. A comprehensive diagram of channel catfish chromosomes treated by silver staining for the nucleolus organizer regions (NOR), C-banding (C), restriction enzymes *Msc* I (M) and *Hin*d III (H), and replication R-banding (R).

detection of SCE and SCD (sister-chromatid differentiation). This technique may allow measurements of the distance between exchange sites and centromeres.

Direct mapping of single-locus genes has been difficult by standard *in-situ* hybridization techniques because of the low abundance of target copies. The on-slide amplification by PCR can incease the copy numbers of target genes into the billions, which enhances the signal strength of probe molecules. By standard FISH and direct and indirect *in-situ* PCR (ISPCR) methods, the *Ig H* gene was detected within intact channel catfish leukocytes and interphase nuclei. The ISPCR technique allowed us to locate the *Ig H* gene on a chromosome. The specificity of the ISPCR procedure was verified by inclusion of an internal negative control for the cell preparations. The synthesized probe and PCR product acquired during ISPCR were identical, about 303 bp as expected. The incorporation of biotin into the probe was verified by dot-blot assay. The nucleotide sequence of these fragments agreed with the published sequence.

Application of the ISPCR in chromosomal mapping is still in the initial stages for fish species and higher vertebrates including humans. To my knowledge, this is first report on physical mapping of single-locus gene from fishes. Routine use of the ISPCR technique awaits improvement of the procedure and development of supportive studies such as fluorescent banding techniques for fish chromosomes.

APPENDIX A
DATA COLLECTION AND ANALYSIS

A.1. Growth (mean ± SD) of the cultured fibroblast cells at three plating densities (h = hour; d = day).

Time	Total cell number (x 10^4 cells/ml)		
	Treatment 1	Treatment 2	Treatment 3
0 h	0.1± 0.0	0.7 ± 0.0	11.0 ± 0.0
24 h	< 0.1	0.5 ± 0.1	10.5 ± 3.6
48 h	0.8 ± 0.2	0.8 ± 0.2	19.7 ± 3.7
76 h	0.1 ± 0.1	0.9 ± 0.2	30.7 ± 5.7
4 d	0.1 ± 0	0.9 ± 0.1	42.1 ± 7.3
5 d	0.1 ± 0.1	1.2 ± 0	40.1 ± 9.5
9 d	0.3 ± 0.1	5.4 ± 1.4	50.0 ± 8.5
12 d	0.2 ± 0.1	13.0 ± 1.4	72.3 ± 8.9
14 d	0.1 ± 0.1	30.0 ± 2.8	85.5 ± 6.3

A.2. Sampling of fibroblast cells cultured in different basal media and serum supplements. Abbreviations: CCS, channel catfish serum; FBS, fetal bovine serum.

Basal medium	Serum type	Cell number (x 10^4) well 1	well 2	well 3
L15	5% CCS	0	0	4.5
L15	5% CCS	0	0	3.5
L15	5% CCS	0	0	4
L15	10% CCS	0	0	0
L15	10% CCS	0	0	0
L15	10% CCS	0	0	0
L15	5% CCfs and 5% FBS	37.0	30.5	54.5
L15	5% CCS and 5% FBS	31.0	24.0	32.5
L15	5% CCS and 5% FBS	28.0	21.0	42.0
L15	5% FBS	2.4	3.3	4.5
L15	5% FBS	3.2	3.1	3.5
L15	5% FBS	2.7	2.5	4
L 15	10% FBS	14.5	13.0	8.5
L 15	10% FBS	13.5	13.5	7.5
L 15	10% FBS	10.5	12	13
A/L	5% CCS	0	0	0
A/L	5% CCS	0	0	0
A/L	5% CCS	0	0	0
A/L	10% CCS	0	0	0
A/L	10% CCS	0	0	0
A/L	10% CCS	0	0	0
A/L	5% CCS and 5% FBS	35.4	31.8	44.4
A/L	5% CCS and 5% FBS	26.4	24.0	41.4
A/L	5% CCS and 5% FBS	34.2	37.8	36.6
A/L	5% FBS	3.3	3.9	3.4
A/L	5% FBS	3.2	4.7	2.3
A/L	10% FBS	12.0	13.8	7.2
A/L	10% FBS	16.2	12.0	6.0
A/L	10% FBS	4.8	14.4	10.2
A/L	10% FBS	-	-	10.8
MEM	5% CCS	0	0	0
MEM	5% CCS	0	0	0
MEM	10% CCS	0	0	0
MEM	10% CCS	0	0	0
MEM	5% CCS and 5% FBS	36.4	44	31.4
MEM	5% CCS and 5% FBS	27.2	48.8	26.6
MEM	5% FBS	3.3	3.9	3.4
MEM	5% FBS	3.8	4.5	3.7

(Table A.2 Con'd).

Basal medium	Serum type	Cell number (x 10^4) well 1	well 2	well 3
MEM	5% FBS	3.2	4.7	2.3
MEM	5% FBS	3.8	3.7	3.6
MEM	10% FBS	2.7	6.1	1.6
MEM	10% FBS	13.6	5.5	12.0
MEM	10% FBS	14.5	5.4	13.1
MEM	10% FBS	13.9	5.8	12.6

A.3. Analysis of variance among fibroblast cells cultured with different basal media and serum supplements.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Serum	4	22787.4	5696.9	340.3	0.0001
Medium	2	1.7	0.9	0.05	0.95
Serum*Medium	8	81.3	10.1	0.61	0.77
Error	127	2125.7	16.7		

A.4. Diploid chromosome numbers of cultured fibroblast cells at different passages.

Passage	Diploid chromosome number 55	56	57	58 (mode)	59	60	% mode
3	1	2	7	19	1	0	64
7	0	4	6	19	1	0	63
11	0	3	8	17	1	1	59
17	1	5	5	18	1	0	60
21	1	1	9	17	1	1	57
Pooled	3	15	35	90	5	2	

A.5. Homogenesity analysis by chi-square of the numbers of cultured fibroblast cells with modal chromosomal number of different passages*.

Passage	Number of modal cells (A)	Number of non-modal cells (a)	Total (n)	p (= A/n)	d (= p*A)
3	19	11	30	0.63	11.97
7	19	11	30	0.63	11.97
11	17	13	30	0.57	9.69
17	18	12	30	0.60	10.80
21	17	13	30	0.57	9.69
Total	90	60	150 (N)		54.12
				P = 90/150 = 0.6	D = P*90 = 54

*$\chi^2 = (\Sigma d - D)/[P*(1-P)] = (54.12-54)/[0.6*(1-0.6)] = 0.12/0.24 = 0.5$ with df = 4
(note: $\chi^2_{4,\,0.05} = 9.49$).

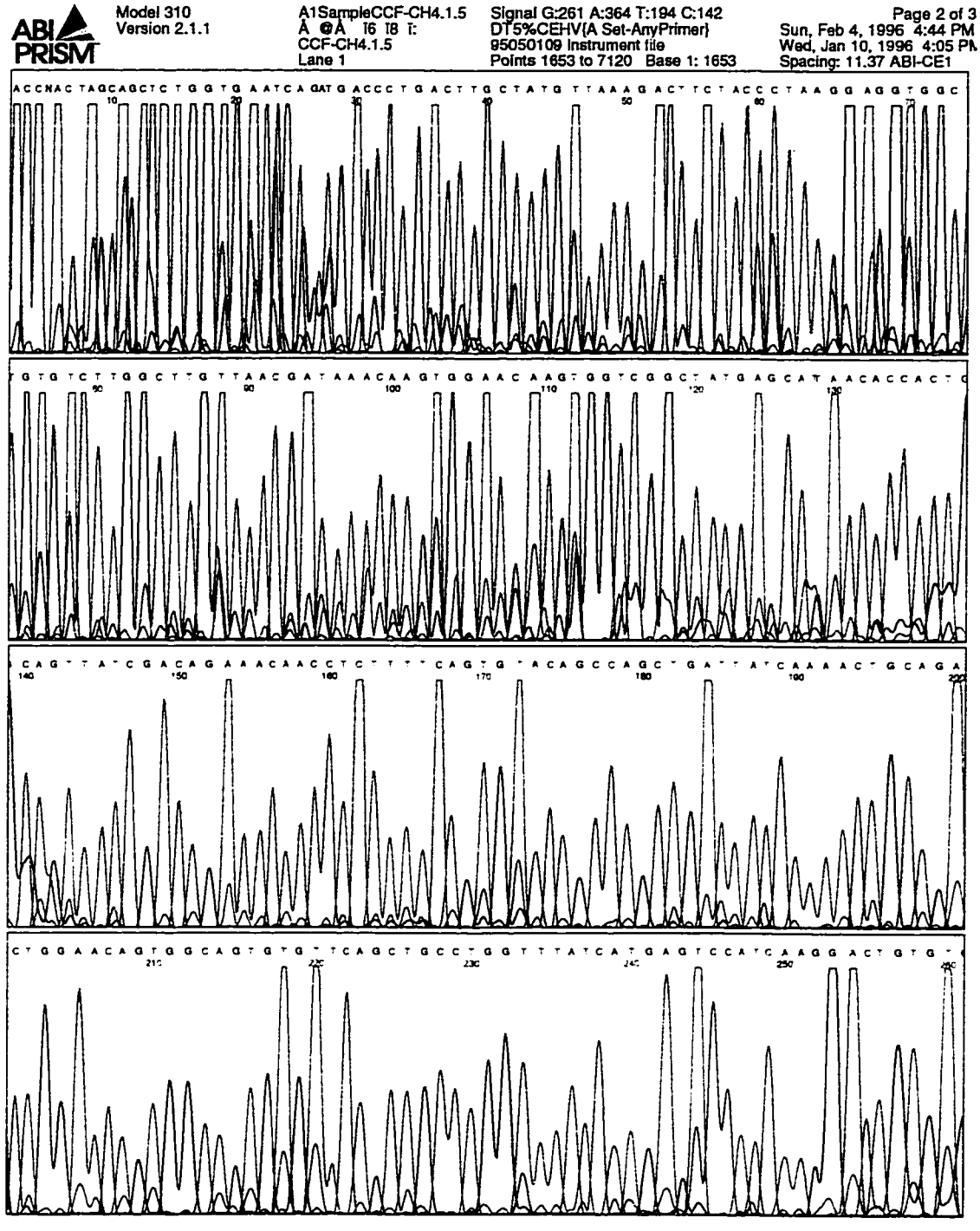
A.6. The occurrence (percent of total complement) of sister-chromatid exchange (SCE) and differentiation (SCD) within cells from five different fish.

Fish 1		Fish 2		Fish 3		Fish 4		Fish 5	
SCE	SCD	SCE	SCD	SCE	SCD	SCE	SCD	SCE	SCD
4	92	3	93	5	96	2	93	3	86
3	93	4	94	3	97	4	94	4	94
5	94	4	92	2	95	5	93	3	92
4	96	3	91	1	96	4	92	6	89
7	98	5	95	0	83	3	91	5	87
1.7	96	7	92	4	98	4	93	4	89
1.7	93	3	86	5	98	4	86	3	93
5	96	2	87	3	95	3	89	2	91
2	96	5	95	4	98	5	92	3	92
3	95	4	94	3	97	6	91	4	91
3	97	3	93	3	97	8	93	3	87
0	94	5	90	4	100	2	92	4	94
4	95	-	-	1	96	-	-	-	-

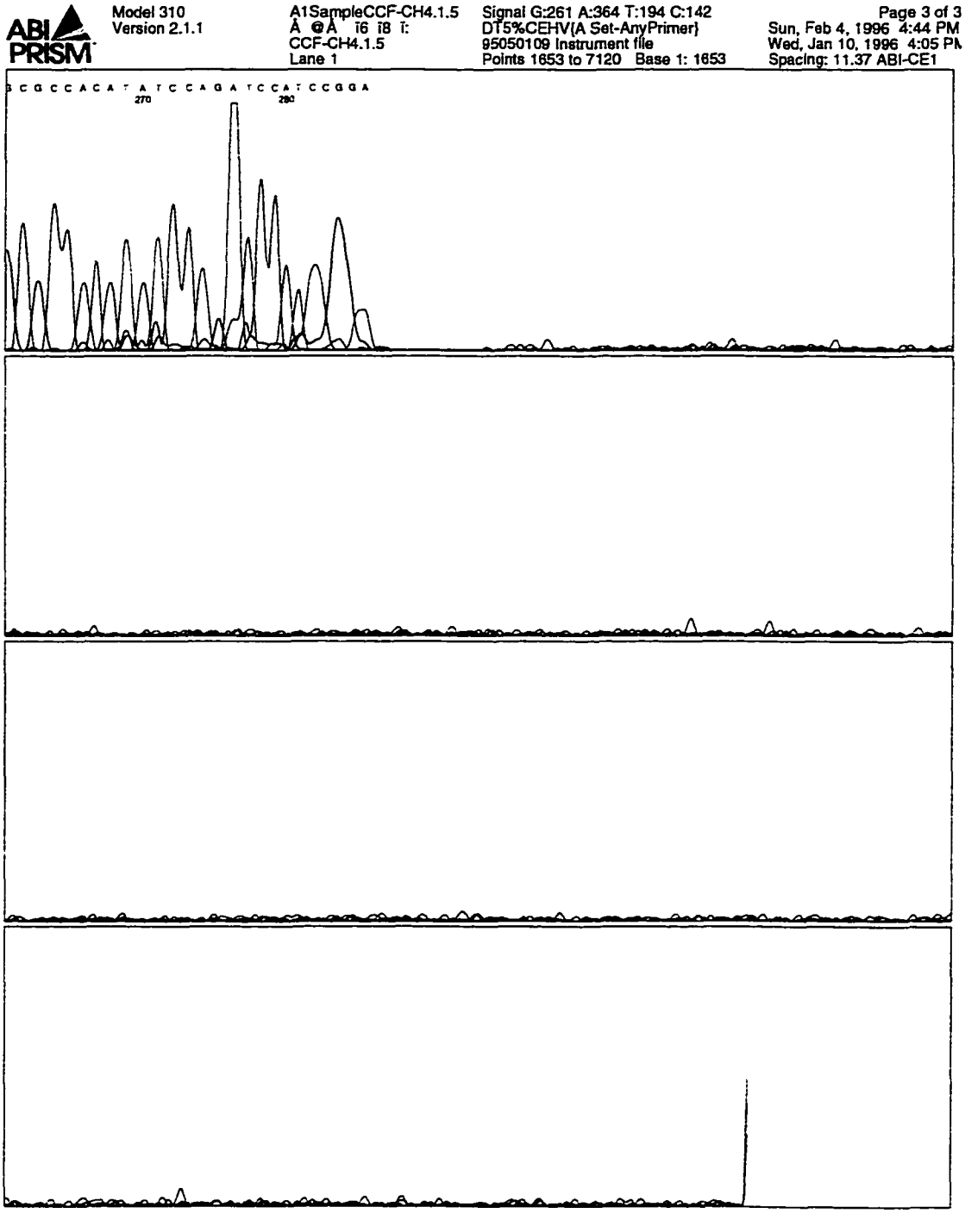
A.7. Computer-assisted measurement of the areas (in computer unit or CU) of catfish leukocytes and fetal donkey dermal cells.

Catfish leukocytes	Fetal donkey dermal cells
0.24	0.65
0.25	0.68
0.2	0.63
0.26	0.56
0.15	0.58
0.21	0.65
0.24	0.47
0.23	0.56
0.25	0.65
0.24	0.64
0.16	0.63
0.23	0.62
0.25	0.65
0.24	0.64
0.28	0.67
0.26	0.59
0.26	0.58
0.24	0.62
0.24	0.64
0.26	0.65
Mean = 0.23 ± 0	Mean = 0.62 ± 0

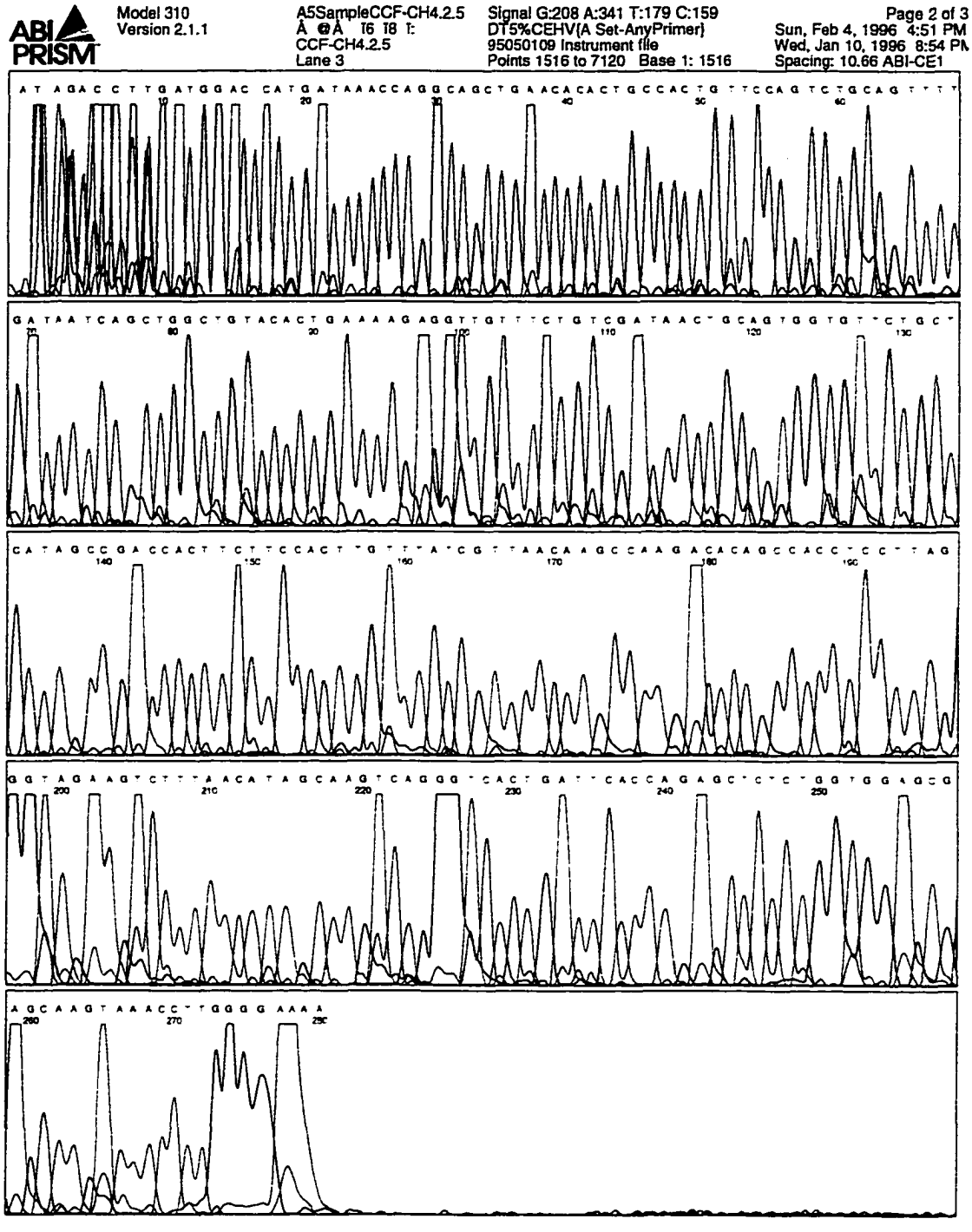
A.8. Nucleotide sequence analysis of the PCR products amplified from the channel catfish *Ig H* gene.



(Figure A.8 Con'd).



(Figure A.8 Con'd).



APPENDIX B
STANDARD OPERATING PROCEDURES

B.1. Buffers and solutions

A. 10-x PBS stock solution (pH 7.2-7.4)

1. Dissolve 20.5 g NaH_2PO_4 H_2O and 179.9 g Na_2HPO_4 $7H_2O$ (or 95.5 g Na_2HPO_4) in about 4 liters of double distilled water;
2. Adjust to the required pH (7.2-7.4). Add 701.3 g NaCl and make up to a total volume of 8 liters;
   (Note: The stock solution is diluted at 1:10 ratio for preparation of 1-x PBS,. Final concentration of buffer should be 0.01 M phosphate and 0.15 M NaCl).

B. 20-x SSC

1. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 80 mL of water;
2. Adjust the pH to 7.0 with a few drops of 10 N solution of NaOH;
3. Bring the volume to 1 liter with water;
4. Sterilize by autoclaving.
   (Note: the same method is used for preparation of 2-x SSC).

C. 5% $Ba(OH)_2$

1. Dissolve 5 g of $Ba(OH)_2.8H_2O$ in 100 ml of distilled water with stirring for 20 min;
2. Filter before use.

D. Mixture of bromodeoxyuridine (BrdU), fluorodeoxyuridine (FrdU), and uridine solution

1. Prepare the FrdU stock solution as the following and store frozen in dark vials:

FrdU	1 mg
distilled water	10 ml

2. Prepare the uridine stock solution as the following and store frozen:

Uridine	1 mg
Distilled water	1 ml

3. Mix the BrdU with the FrdU and uridine stock solutions as follows:

BrdU	3 mg
FrdU stock solution	0.1 ml
Uridine stock solution	0.2 ml
Distilled water	0.7 ml

4. Store the solution in dark vials at -20 °C.

E. 50-x Denhardt's solution (stored at -20 °C)

1. Prepare the following chemicals;

Ficoll (Type 400, Pharmacia)	5 g
Polyvinylpyrrolidone	5 g
BSA (Fraction V, Sigma)	5 g

2. Add water to 500 ml, and store at -20 °C.

F. 6. 2% Paraformaldehyde

1. Take 12 g paraformaldehyde and add to 600 ml 1 x PBS;
2. Heat at 65 °C for 10 min; when the solution starts to clear, add 4 drops of 10 N NaOH and stir;
3. Adjust to neutral pH and cool to room temperature;
4. Filter on Whatman's No. 1 paper.

G. AEC (3-amino-9ethyl-carbazole) staining solution

1. AEC stock solution

(1). Dissolve powder from Sigma in PBS to make a solution of 1 mg/ml;
(2). Dilute this solution in sterile PBS at a 1:30 ratio immediately before use.

2. 50 mM acetate buffer (pH 5.0)

(1). Add 74 ml of 0.2 N acetic acid and 176 ml of 0.2 M sodium acetate to 1 liter of deionized water and mix.

3. AEC working solution

(1). Mix the following solutions;

50 mM acetate buffer	5 ml
AEC stock solution	250 µl
30% H_2O_2	25 µl

(2). Make fresh before each use, and keep the solution in the dark.

B.2. Isolation of catfish leukocytes

1. Three ml of whole blood aree drawn aseptically from the caudal vessels into heparinized syringes and transferred to 10-ml vaccutainers;
2. Whole blood is diluted 1:1 with Ca^{2+}- and Mg^{2+}- free phosphate buffered saline (CMF-PBS);
3. Three ml of each of two different densities of ficoll-hypaque solution are layered in a 15-ml centrifuge tube: HISTOPAQUE-1.119 (Sigma Chemical Co., St. Louis, MO) is placed below a layer of HISTOPAQUE-1.083 (Sigma);
4. Six ml of diluted whole blood are placed on top of the HISTOPAQUE layers;
5. The tubes are centrifuged at 450 x g for 30 minutes at room temperature;
6. After centrifugation, the leucocyte-rich layer (second from the top) is removed and washed three times with CMF-PBS by centrifuging at 80 x g for 5-7 min.
7. After the final wash, the cell pellet was resuspended in 1 ml of Leibivotz L15 complete medium.

B.3. Preparation of metaphase chromosomes from cultured cells

1. Twenty ml of colchicine solution (100 μg/ml in CMF-PBS) is added to each culture;
2. After 30 min to 1 h, cells are transferred with culture medium into a 15-ml centrifuge tube, and centrifuged at 80 x g for 5 min;
3. Cell pellets are resuspended in 1 ml of 1-x PBS;
4. Five to ten ml of 0.075 M (or 0.56%) KCl are added slowly, and the tube is left for 25 min at room temperature;
5. At the end of hypotonic treatment, 1 ml of cold Carnoy's fixative (3:1 methanol-acetic acid) is added;
6. The tube is centrifuged at 200x g for 5 min, and the supernatant is removed;
7. The cells are fixed three times with Carnoy's fixatives for 30 min each at -20 °C;.
8. After the last fixation, cells are resuspended in 3 to 5 ml of cold fiative;
9. Cells are dropped onto cold, wet microscope slides, dried at 40 °C overnight.

B.4. The procedure for identification of channel catfish chromosomes and development of a standard karyotype (Chapter 3 and Summary).

Steps of karyotyping		
1. Scan chromosomes;	**Identification methods**	**Final groups**
1.1. Sort by size (%TCL);	2.3 - 5.0%	A = large metacentrics; 1-2. B = large subtelocentrics; 3-5.
1.2. Divide into preliminary groups based on size (%TCL);	Large = 4.0-5.0% Medium = 3.0-4.0% Small = 2.0-3.0%	C = large and medium metacentrics; 6-8. D = medium submetacentrics; 9-13.
1.3. Rearrange by centromeric index (% CI);	Metacentri = 37.5-50% Submetacentric = 25-37.5% Subtelocentric = 12.5-25% Telocentric = 0-12.5%	E = medium subtelocentrics; 14-18. F = telocentrics; 19-20. G = small metacentrics; 21-25.
1.4. Assign to final groups:		H = small submetacentrics; 26-29.
2. Chromosome banding	**Chromosome**	**Banding pattern**
2.1. the NOR-band	D11	p, 1 band;
2.2. C-banding	All chromosomes	Centromeric band
	E16	q, 1 non-centromeric band
2.3. Replication R-bands	A1	p, 1 band; q, 4 bands;
	A2	p, 1 band; q, 2 bands;
	B3	p, no band; q, 3 bands;
	B4	p, no band; q, 2 bands;
	B.5	p, 1 band; q, 3 bands;
	C6	p, 2 bands; q, 2 bands;
	C7	p, 1 band; q, 4 bands;
	C8	p, 2 bands; q, 3 bands;
	D9	p, 1 band; q, 3 bands;
	D10	p, no band; q, 4 bands;
	D11	p, no band; q, 2-3 bands;
	D12	p, 1 band; q, 2 bands;
	D13	p, no band; q, 2 bands;
	E14	p, 1 band; q, 3-4 bands;
	E15	p, 1 band; q, 3 bands;
	E16	p, 1 band; q, 3 bands;
	E17	p, no band; q, 2 bands;
	E18	p, 1 band; q, 3 bands;
	F19	p, 1 band; q, 3bands;
	F20	p, no band; q, 2 bands;
	G21	p, no band; q, 2 bands;
	G22	p, 1 band; q, 1 band;
	G23	p, 2 bands; q, 2 bands;
	G24	p, 1 band; q, 2 bands;
	G25	p, 1 band; q, 1 band;
	H26	p, no band; q, 2 bands;
	H27	p, no band; q, 1 band;
	H28	p, no band; q, 1 band;
	H29	p, 1 band; q, 1 band

B.5. Coating of microscope slides with 3'-aminopropyltriethoxysilane (AES).

1. Place slides in 0.2 N HCl for 3 to 4 d;
2. Rinse slides in deinoized water for up to 2 h and then dry with a kimwipe;
3. Soak slides in acetone for 10 min;
4. Dip slides in 2% (v/v) AES in acetone for 1 min;
5. Rinse thoroughly in acetone;
6. Dry in laminar hood overnight.

B.6. Reaction mixture for the polymerase chain reaction

Template DNA (~15 ng/μl)	3 μl
10x PCR buffer	10 μl
$MgCl_2$ solution (25 mM)	8 μl
dNTP mixture (1.0 mM each of dATP, dTTP, dGTP, and dCTP)	8 μl
DMSO	1 μl
Primer-1 (50 μM)	1 μl
Primer-2 (50μM)	1 μl
Taq DNA polymerase (5 Units/μl)	0.5 μl
ddH_2O	67.5 μl
Total	**100 ml**

The thermal cycler is programed as follows: 95°C denaturation, 59°C annealing, and 72°C DNA elongation, for 30 s each with an initial denaturation step of 2 min at 95°C. The reactants for the *in-situ* PCR (or solid-phase PCR) is prepared the same as described above but without addition of template DNA.

VITA

Quiyang Zhang was born in Huian, Fujain, China, on April 12, 1963. He had three sisters and three brothers. During the years of his childhood, China was suffering from the Cultural Revolution, and it was difficult for any parent to support such a large family. Quiyang learned from his father how to struggle through difficulties while he was still a child. He attended XiHu Elementary School at the age of six, and moved to HeShan Middle School at the age of eleven. His excellent performance at school persuaded his father to allow him to continue his studies until graduation. In 1979, he passed the National Standard Test and entered the Department of Biology at Xiamen (Amoy) University. He graduated with a Bachelor of Science in Zoology in 1983, and moved to Xiamen Fisheries College (XFC) where he met Ling Zhang, and they married in 1987. He started at XFC as a teaching assistant, and was promoted to a lecturer in 1989. His research activities during that time included diseases of aquatic animals and artificial reproduction of penaeid shrimps. In August, 1990, he was admitted to the Graduate School at Louisiana State University. He studied fish nutrition in the Aquaculture Program of the School of Forestry, Wildlife, and Fisheries with an award of a full-time graduate assistantship, and received his Master of Science in 1993. He studied aquaculture genetics at LSU while working toward the Doctor of Philosophy degree in Wildlife and Fisheries Science supported in part by a Cooperative Agreement with USDA-ARS.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Quiyang Zhang

Major Field: Wildlife and Fisheries Science

Title of Dissertation: Cytogenetic and Molecular Analysis of the Channel Catfish (Ictalurus punctatus) Genome

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

April 19, 1996