Replication Banding and Sister-Chromatid Exchange of Chromosomes of Channel Catfish (*Ictalurus punctatus*)

Q. Zhang, W. R. Wolters, and T. R. Tiersch

A replication banding procedure using fluorouracil (FU) and bromodeoxyuridine (BrdU) was developed for use with cultured leukocytes of channel catfish (*Ictalurus punctatus*). Analyzable banding patterns were produced on chromosomes stained with a fluorochrome plus Giemsa (FPG) method. The consistency of the chromosomal bands was evaluated by computer-assisted image analysis. Banding patterns of representative chromosomes including those bearing nucleolus organizer regions (NOR) were reproducible. A standard RBG-banded karyotype (R bands by BrdU and Giemsa) was established with idiograms of each chromosome. We also developed procedures to study sister-chromatid exchange (SCE) and sister-chromatid differentiation (SCD) in cultured leukocytes, without addition of mutagenic substances. The average occurrence of SCEs in the absence of mutagens was 3.0 ± 1.0 (n = 60) chromosomes per cell and was not significantly different among fish (P = .26).

Studies of fish chromosomes have not been as successful or widespread as those in other vertebrate groups (Gold et al. 1990). A limiting factor has been technical difficulty in obtaining good chromosome spreads, because most species have a large number of small chromosomes. Because of this and minimal compartmentalization of the fish genome by base composition (Medrano et al. 1988), techniques for linear structural banding (e.g., quinacrine, Giemsa, and reverse bands) developed for higher vertebrates (Verma and Babu 1989) have succeeded rarely with fish chromosomes.

Dynamic methods using mitotically active cells, such as replication banding, are an alternative to structural banding. Techniques including RBG (replication bands by bromodeoxyuridine using Giemsa) procedures rely on the incorporation of a base analogue during DNA replication and postfixation modification of chromosome structure in the substituted regions (Ronne 1992). Therefore difficulties that relate to the structure or base composition of fish chromosomes can be bypassed with replication (R) banding. Replication banding of fish chromosomes has been reported in *Carassius auratus* (Zhang and Wu 1985); *Rutilus rutilus* and *Scardinius erythrophthalmus* (Hellmer et al. 1991); *Oncorhynchus mykiss* (Delany and Bloom 1984); *Salmo salar* (Pendas et al. 1993), and *Scorpaena procus* and *Scorpaena notata* (Giles et al. 1988) through intraperitoneal injection of bromodeoxyuridine (BrdU) into living fish. However, banding patterns have been better controlled with the use of cultured cells (Ronne 1992).

Sister-chromatid exchange (SCE) involves the breakage and reunion of chromosomal DNA (presumably between complementary regions) detectable after two or more rounds of replication in the presence of BrdU (Wolff 1982). Analysis of SCE is of interest because there appears to be a correlation between the frequency of SCE and exposure to mutagenic agents such as radiation or chemicals. Therefore SCE analysis can be valuable 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 two consecutive generations yields sister chromatids that can be stained differentially [i.e., sister-chromatid differentiation (SCD)] to identify exchanged segments. Chromatids containing DNA strands with more BrdU incorporation will stain less intensely than chromatids with less incorporation because of the quenching action of BrdU (Verma and Babu 1989).

Computer-assisted chromosome analysis was first reported in fish in the mid-1980s (e.g., Gold et al. 1986). The introduction of newer technology has enabled handling of chromosome images imported directly from the light microscope by video camera (Bauchan and Campbell 1994). Computer-assisted analysis yields objective and quantitative estimates of banded areas of chromosomes (Drets et al. 1992). This technique remains unstudied for application in analysis of the weak bands that have been found in the chromosomes of most fish species.

Channel catfish is the most important food fish cultured in the United States, and genetic study of the species is generating wide interest (Wolters 1993). However, little information is available about chromosome structure, hindering 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 chromosomes, (2) evaluate the consistency of the R-banding technique, (3) establish a standard RBG-banded karyotype and idiogram, and (4) estimate baseline frequency of SCD and SCE in cultured cells.

Material and Methods

Animals

The channel catfish used in this study 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 SCD and SCE.

Leukocyte Culture

Leukocytes were isolated by the density centrifugation method (Zhang and Tiersch 1995). Culture media and incubation conditions were described previously (Zhang and Tiersch 1995). 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, Missouri) and 0.5 μg/ml calcium ionophore A23187 (Sigma) (Lin et al. 1992), or with 5 μg/ml concanavalin A (Con A). After 24 h the media with PMA and A23187 were replaced with fresh media containing no mitogens; the cultures with Con A did not require a change of media at this step. Cells were incubated for another 48–72 h until the first round of mitotic activity occurred. Cultures were processed with the following procedures for replication banding and SCE and SCD analysis.

Replication Banding

DNA synthesis of cultured leukocytes was blocked with 5′-fluorouracil (FU; Sigma) at a final concentration 1.0×10^{-7} M. After 17 h 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, Maryland) 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 microliters of colchicine solution (100 μg/ml) was added to each culture at 30 min to 1 h before harvest. The cells were processed by a hypotonic treatment with 0.075 M KCl, followed by cold fixation in a mixture of methanol and acetic acid (3:1).

Chromosome staining was based on the fluorochrome plus Giemsa (FPG) method of Perry and Wolff (1974). After staining with Hoechst 33258 (150 μg/ml) for 25 min at room temperature, slides were placed on a styrofoam board, floated in a 60°C water bath, and irradiated with a long-wave (365 nm) ultraviolet (UV) light (115 V, 60 Hz, 0.3 A; Spectronic Corp., Westbury, New York) for 2 h from a distance of 10 cm. The slides were treated with 2× 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%). The slides were stained finally with 2% Giemsa in 0.01 M phosphate buffer (pH 6.8) for 10 min.

Evaluation of Replication Banding on Marker Chromosomes

Three distinctive chromosomes—1 (the largest chromosome), 6 (the largest metacentric chromosome), and 11 (the chromosome bearing the NOR) (Zhang 1996)—were used as representatives of each spread. The banding patterns of these chromosomes from different cells were compared to examine consistency of the technique. Slides were treated by the FPG procedure, and images of chromosomes were recorded as described below. The slides were destained with the mixture of methanol and acetic acid (3:1), rinsed with distilled water, dehydrated through the series of ethanol solutions, and air dried. The NOR were located using the silver staining procedure of Howell and Black (1980).

Computer-Assisted Chromosome Analysis

The process of karyotyping was assisted by the Optimas® (Bioscan, Inc., Edmonds, Washington) and Kary® (Pro Data, Oslo, Norway) computer software packages. Chromosome images were captured and recorded with an image analysis system (Zhang and Tiersch 1997) directly from a light microscope (Microphot-SA, Nikon Inc., Garden City, New York). For comparison, chromosomes were photographed with Kodak Technical Pan film 2415, the negatives were digitized using a slide scanner (SprintScan 35, Polaroid scanner model CS-2700, Needham Heights, Massachusetts), and the digitized images were stored on a 486 IBM compatible PC with a 1.2 GB hard drive, 8 Mbyte RAM, 2 Mbyte VRAM, and 66 MHz processor. Total lengths and arm lengths of chromosomes were measured by the "line measurement" function of the Optimas® software. The dark and light banding patterns of chromosomes were identified by densitometry and expressed with a luminance plot. The x-axis of the plot represented segments of chromosomes, and the y-axis represented the corresponding grayscale value ranging from 0 (black) to 255 (white). Images of metaphase spreads were imported into the Kary® software, which arranged 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 (%TCL) and cen-

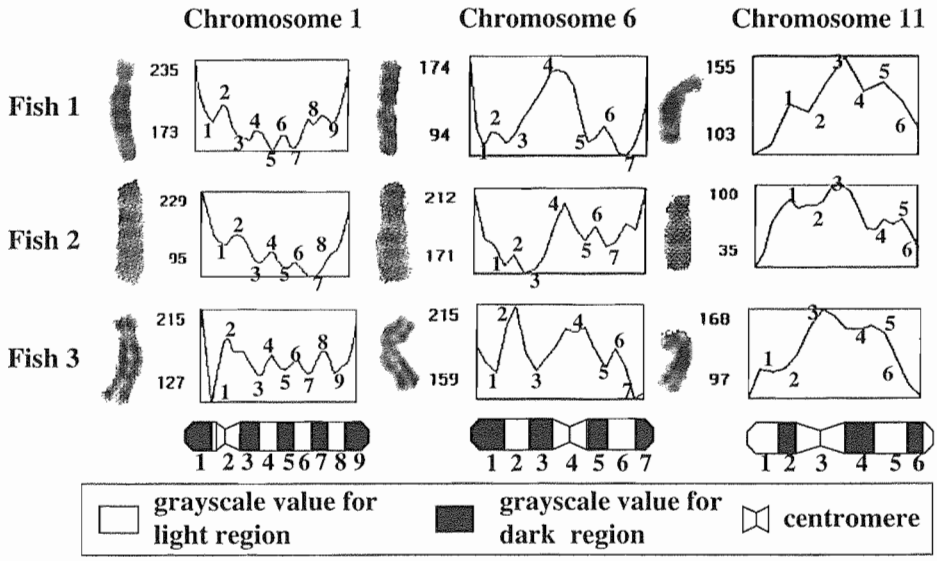

Figure 1. Computer-assisted analysis of replication banding patterns of representative chromosomes. Comparison of the luminance patterns of chromosome 1, 6, and 11 (the NOR-bearing chromosome) from cells of different fish. Arabic numbers indicated on the luminance plots correspond to the specific regions of the chromosome diagrammed within the idiograms.

tromeric index (CI) (Zhang 1996), which were calculated by the following formulas:

$$\%TCL = (\text{total length of each chromosome pair/total length of all chromosomes}) \times 100$$

$$CI\ (\%) = (\text{short-arm length/total length of chromosome}) \times 100.$$

An idiogram was prepared for each chromosome using the Microsoft PowerPoint® software (version 4.0).

Sister Chromatid Differentiation and Exchange

Bromodeoxyuridine (1.6×10^{-4} M final concentration) was added to cultured leukocytes incubated for 20 h before harvest. The method for preparation of chromosomes was the same as described above. Slides were treated with 5% $Ba(OH)_2$ for 7 min at 50°C and incubated overnight with 2× SSC buffer and 0.05% Triton X-100 (Mallinckrodt Specialty Chemicals Co., Paris, Kentucky) at 60°C. Prolonged staining in 5% Giemsa was required to reveal SCD and SCE. Twelve to fifteen spreads from each of five specimens were counted for SCE and SCD. One-way analysis of variance was used to analyze the frequency of occurrence of SCD or SCE among individual fish at a significance level of $P < .05$.

Results

Chromosomes prepared by FU/BrdU replication banding and stained with the FPG method exhibited serial bands with analyzable patterns (Figure 1). Each chromosome pair had a characteristic replication banding pattern and could be identified regardless of the state of contraction (Figure 1). Chromosome 1, the largest in the complement, had one dark band on the short arm and four well-defined dark bands on the long arm. Chromosome 6, the largest metacentric chromosome, had two dark bands on the short arm and two to three on the long arm. Chromosome 11, the NOR-bearing chromosome, possessed one minor dark band on the short arm and one or two dark bands on the long arm, with the major dark band adjacent to the centromere.

Individual chromosomes were identified based on an objective procedure: (1) dividing chromosomes into groups designated as A, B, C, D, E, F, G, or H based on %TCL and CI, and (2) pairing chromosomes within a group according to replication banding patterns (Zhang 1996). The final karyotype (Figure 2) was based on the analysis of 12 spreads and was summarized with an idiogram (Figure 3).

Sister-chromatid differentiation was found on most chromosomes (Figure 4a). However, SCE was observed only on one to five chromosomes per cell (Figure 4a–c). The occurrence of SCD ($P = .07$) was not significantly different among the five fish examined, nor was that of SCE ($P = .26$) (Table 1).

Discussion

In this study replication banding procedures were developed for channel catfish using an in vitro culture technique. Three factors were critical for the success of the replication banding procedure: rapid growth of cultured cells, efficient arrest and release of the cell cycle, and control of intensity of the postlabeling 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. The number of analyzable spreads was increased by addition of mitogens. Although Con A, pokeweed mitogen, and phytohemagglutinin M all have proliferative effects on in vitro culture of channel catfish leukocytes (Faulmann et al. 1983), a mixture of phorbol ester and calcium ionophore was found to be consistently mitogenic for catfish leukocytes (Lin et al. 1992). Consistent replication banding patterns rely on temporal and spatial control of BrdU incorporation, and

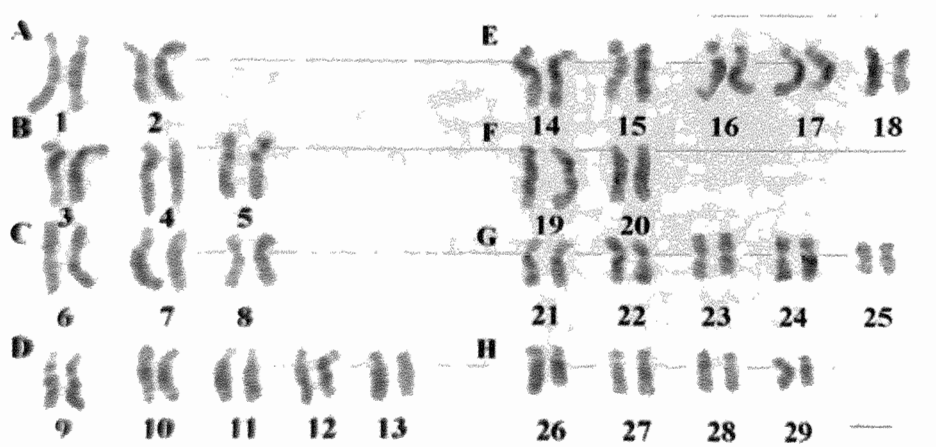

Figure 2. A standard RBG-banded karyotype (R bands by bromodeoxyuridine using Giemsa) of channel catfish. Abbreviations: A, large submetacentric; B, large subtelocentric; C, large and medium metacentric; D, medium submetacentric; E, medium subtelocentric; F, telocentric; G, small metacentric, and H, small submetacentric chromosomes. Bar = 10 μm.

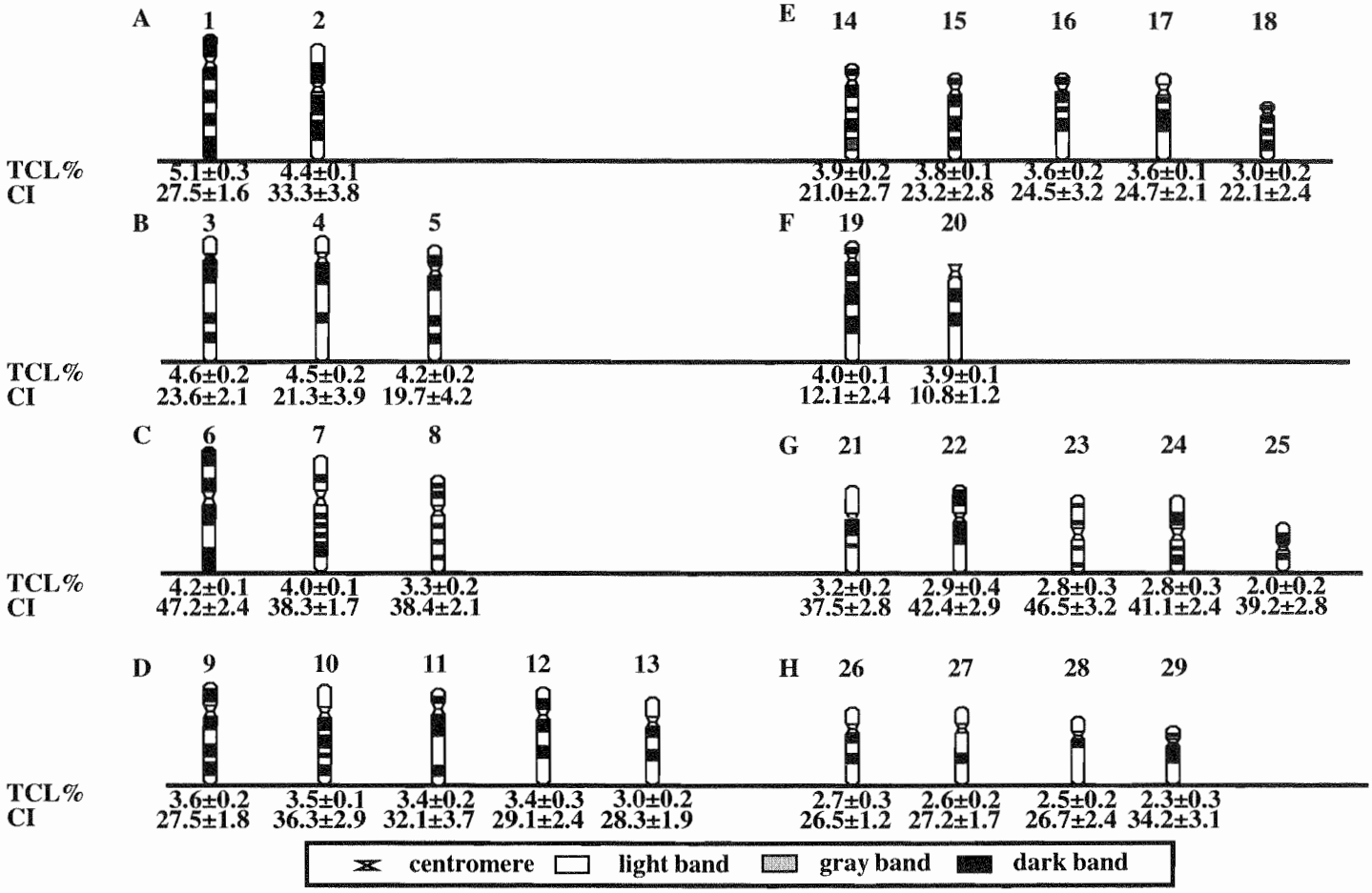

Figure 3. An ideogram of RBG-banded karyotype (R bands by bromodeoxyuridine using Giemsa) of channel catfish. The measurements were taken from chromosomes of 12 different spreads. Abbreviations: %TCL = percentage of total complement length, or relative length; CI = centromeric index.

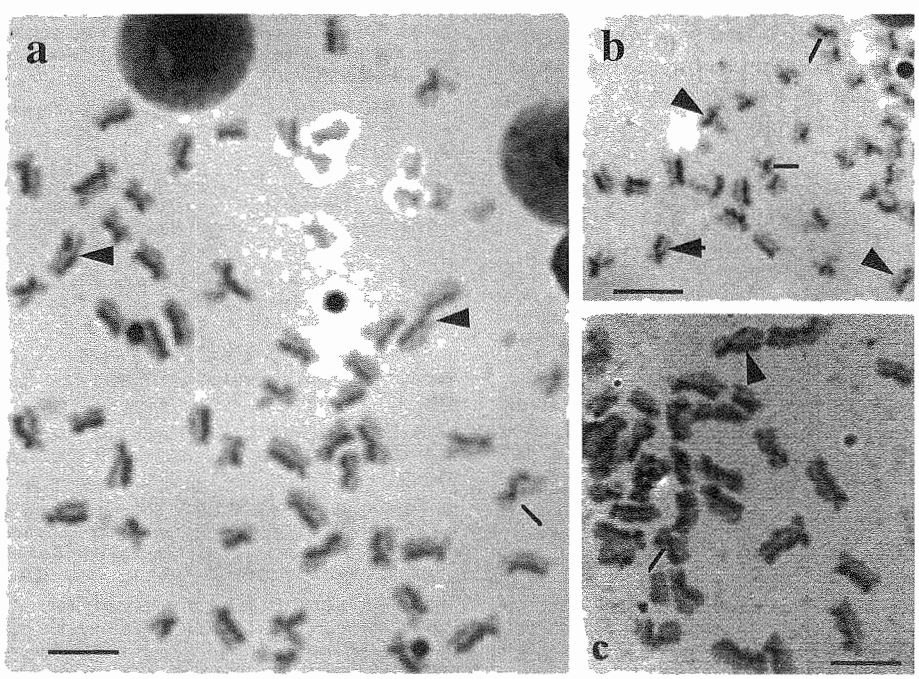

Figure 4. Sister-chromatid differentiation (SCD) and exchange (SCE) of channel catfish chromosomes. Arrows indicate representative SCE chromosomes; lines indicate position of centromeres. Bars = 10 μm.

this can be achieved by synchronization of cell populations. Several treatments were evaluated in this study, including use of the reversible S-phase inhibitors methotrexate (MTX) and FU, and high doses of thymidine and BrdU (data not shown). Although MTX has worked effectively in higher animals (Ronne 1992) and in some fishes such as eel (Liu 1988), it did not block mitosis in cultured channel catfish leukocytes. We also found that the addition of 0.3 mg/ml of thymidine did not synchronize cells effectively, and a similar result was found in cultures treated with high doses (70 μg/ml) of BrdU. In contrast, FU was able to block cultures and was easily released by the BrdU-based mixture. The resultant spreads were arrested mostly in prometaphase.

The final consideration of replication banding was the postlabeling 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 a prolonged incuba-

Table 1. The occurrence of sister-chromatid differentiation (SCD) and exchange (SCE) in chromosomes of channel catfish ($2N = 58$)

Fish	Number of chromosomes per spread	
	SCD	SCE
1	56 ± 1	3 ± 1
2	55 ± 1	2 ± 1
3	56 ± 2	2 ± 1
4	58 ± 1	4 ± 1
5	57 ± 1	2 ± 1
Mean ± SD	57 ± 2	3 ± 1

The number of chromosomes per cell (mean ± SD) bearing SCD ($P = .07$) or SCE ($P = .26$) were not significantly different among the five individuals examined.

tion with 2× SSC was utilized to generate differentiated bands. The double-staining method using Hoechst 33258 and actinomycin D was developed originally to identify 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 rainbow trout chromosomes (Delany and Bloom 1984). Other direct methods such as staining with acridine orange revealed replication bands in cyprinid fishes (Hellmer et al. 1991), but did not produce reproducible bands in this study, presumably resulting from a 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-banded chromosomes was efficient and reproducible, and the results were more informative than those of subjective methods. Comparisons among different spreads were enabled by use of marker chromosomes, which were identified by distinctive morphology and banding patterns. The chromosomes bearing the NOR of channel catfish were consistently located on short arms of a pair of medium-sized submetacentrics (Zhang 1996) and could be used as an internal reference to gauge the contraction level of metaphase spreads. The variation found among individual karyotypes was not due to differences in banding pattern but was mostly due to the difficulty in identifying centromeric regions of chromosomes.

In this study the SCE of channel catfish chromosomes were produced by alkaline treatment followed by prolonged exposure to 2× SSC buffer. The incorporation of atomic Br derived from BrdU enhances the binding of DNA with nonhistone 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 (Zhang 1996) was used in this study. Our method (designed after failure to reveal SCE or SCD with routine FPG staining techniques) was found to be effective for use in analyzing chromosomes incorporated with low levels of BrdU.

To our knowledge, this is the first report of a cytogenetic map of channel catfish providing a foundation for physical mapping in this species. Fluorescence in situ hybridization (FISH) is a common technique for identification of chromosomal location of DNA sequences. Simultaneous recognition of hybridization signal and identification of individual chromosomes has been reported in mammalian species with techniques combining replication banding and FISH (Larramendy et al. 1993). Techniques allowing use of in situ polymerase chain reaction for detection of a single-locus gene have been developed for channel catfish (Zhang et al. 1997), although individual identification of chromosomes remains problematic. Further studies are required to standardize these techniques for physical mapping in this species. The development of an in vitro BrdU incorporation technique for producing replication banding and SCE could allow analysis of phylogenetic relationships or identification of catfish sex chromosomes as reported in amphibians (Schempp and Schmid 1981).

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