Transactions of the American Fisheries Society 127:551–559, 1998
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Standardization of the Channel Catfish Karyotype with Localization of Constitutive Heterochromatin and Restriction Enzyme Banding

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Abstract.—Genetic research of fishes is hampered by lack of standardized karyotypes and reliable techniques of chromosome banding. The goal of this study was to develop a standardized karyotype of channel catfish *Ictalurus punctatus* by a variety of banding techniques and computer-assisted analysis. Metaphase chromosomes were prepared from cultured leukocytes and kidney cells of adult and juvenile fish. Silver staining and alkali treatment methods were used to reveal nucleolus organizer regions (NOR) and the location of constitutive heterochromatin (C-bands). Chromosomes were treated with 10 different restriction enzymes, stained with Giemsa, and examined for banding patterns. The chromosomal data were analyzed with two computer software packages. The 29 chromosomes were classified into eight distinct groups based on morphology and size. The NOR were located on a pair of medium-sized submetacentric chromosomes (designated as D-11); this was consistent among cells from different specimens. The C-bands were small and restricted to centromeric regions and were useful for homologous pairing. A standard karyotype of CBG-banded chromosomes (C-bands visualized by treating with barium hydroxide and staining with Giemsa) was developed. The restriction enzyme *Msc* I produced informative banding patterns with dark telomeric bands and clear centromeric regions. The enzyme *Hind* III was most informative, yielding linear banding patterns that were consistent between members of homologous pairs. Our study provides a fundamental step in genome mapping of fishes. Standardization of the channel catfish karyotype and chromosome banding will facilitate physical mapping of genes in this important culture species.

The analysis of chromosome morphology is fundamental to genome mapping. In higher vertebrates including humans, a variety of techniques have been developed to facilitate precise identification of individual chromosomes (Verma and Babu 1989). The diffuse arrangement of chromatin along the chromosomes of fishes makes it difficult to obtain high-resolution banding, and the small size and large numbers of fish chromosomes have created additional difficulties in developing reliable banding techniques (Gold et al. 1990a).

To date, the techniques that have worked most successfully with fish chromosomes are staining for nucleolus organizer regions (NOR) and constitutive heterochromatin (C-banding, Gold et al. 1990a). Distribution of C-bands is currently reported for fewer than 100 species of the estimated 25,000 extant species of fishes. In most cases, C-bands were dispersed throughout the entire chromosome complement (Takai and Ojima 1988; Rab et al. 1991) with concentrations on centromeric or telomeric regions. However, notable exceptions appear in the channid fishes, such as *Channa argus*, *C. asiatica*, and *C. maculata* (Li et al. 1985), and the percid species (Mayr et al. 1987) in which constitutive heterochromatic regions appeared small, well resolved, and primarily centromeric. The C-banding technique can be used to identify homologous chromosomes and, in some cases, sex chromosomes (Haaf and Schmid 1984). However, C-banding usually does not produce linear patterns on the chromosome arms, and this limits its utility for other studies such as physical mapping.

Difficulties encountered in banding techniques that rely on degradation of structural proteins have led fish geneticists to employ other techniques such as digestion with restriction enzymes (REs), which recognize and cleave at specific base sequences of DNA nucleotides. Digestion of whole chromosomes by REs causes removal of some DNA fragments, and Giemsa staining can be used to reveal the relative amount and location of the remaining DNA yielding reproducible patterns in mammalian species (Miller and Miller 1990). Application of RE-banding techniques to fishes has yielded some success. Reproducible C-band-like patterns have been observed in chromosomes of salmonid fishes (Lloyd and Thorgaard 1988; Hart-

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ley 1991b) and cartilaginous fishes (Stingo et al. 1995), and other banding patterns (G-band-like) were produced in European eel *Anguilla anguilla* (Vinas et al. 1994).

Although channel catfish *Ictalurus punctatus* has in the last 20 years become the most important food fish cultured in the United States (Wolters 1993), genetic study of this species has lagged well behind that of livestock and other cultured fishes such as salmonids. In early studies the diploid chromosome number of channel catfish was reported to be 56 (Muramoto et al. 1968), or 56 or 58 (Hudson 1976). Modal distribution of diploid number (2N) was later reported as 58 after examination of 62 spreads prepared from kidney cells (LeGrande 1981), and this agreed with the results from a study of cultured leukocytes (Wolters et al. 1981). Research such as genome mapping necessary for genetic improvement of this species is hampered or prevented by the lack of a standardized karyotype and reliable banding techniques. Our objectives were to (1) standardize the unbanded karyotype of channel catfish; (2) localize regions of constitutive heterochromatin by C-banding; (3) develop a standard C-banded karyotype and idiogram, and (4) analyze the banding patterns of chromosomes treated by 10 REs.

Methods

Animals.—Channel catfish used in this experiment were from a research population maintained at Louisiana State University and produced from wild stocks collected in Louisiana during the last 20 years. They were artificially spawned and reared in indoor recirculating systems. Sizes ranged from 2.5 g (juveniles) to 1.8 kg (adults). Twenty-two fish were used for preparation of metaphase chromosomes and general karyotyping. Of these, 10 were studied for localization of the NOR and study of standard karyotype, and 5 were used for C-banding and 5 for RE-banding. Ten metaphase spreads per individual were used for analysis of NOR, C-bands, or RE-bands.

Leukocyte culture and chromosome preparation.—Leukocytes were isolated from adult fish and cultured with procedures reported previously (Zhang and Tiersch 1995). Leukocytes were stimulated for mitosis by the addition of 0.05 μg/mL solution of phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co., St. Louis, Missouri) and 0.5 μg/mL solution of calcium ionophore A23187 (Sigma). The cultures were treated with colchicine (0.4 μg/mL) for 1 h, then harvested and processed for chromosomes by standard methods (Wolters et al. 1981).

Kidney cell culture and chromosome preparation.—Anterior and posterior portions of kidney tissue were removed by sterile dissection and placed in precooled Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (CMF-PBS). Kidney tissues were cut into small fragments and pressed through a 70-μm cell strainer (Falcon Plastics, Becton Dickinson, Inc., Franklin Lakes, New Jersey) with a sterile syringe plunger, and the cells were rinsed with CMF-PBS. The cell suspensions were collected into 15-mL centrifuge tubes and spun at 300 × gravity (Sorvall Centrifuge GLC-2B, DuPont, Wilmington, Delaware) for 3–5 min. The pellets were washed twice with CMF-PBS and resuspended in 1 mL of Leibovitz L15 complete medium composed of L15 basal medium supplemented with fetal bovine serum (5%, volume per volume, v/v), catfish serum (5%, v/v), penicillin (100 units/mL), streptomycin (100 μg/mL), and gentamicin (10 μg/mL). The cells were cultured in the L15 complete medium under the same conditions used for leukocytes. Mitosis was stimulated by the addition of concanavalin A (5 μg/mL) or by addition of PMA and calcium ionophore A23187. The remaining steps for preparation of chromosomes were the same as those used for cultured leukocytes (Wolters et al. 1981).

Staining of the nuclear organizer regions and constitutive heterochromatin.—The nuclear organizer regions (NOR) were revealed by standard silver staining (Gold et al. 1990a). To stain the constitutive heterochromatin (C-bands), metaphase spreads were aged on glass slides by incubating slides at 65°C for at least 3 d before experiments. Slides were placed in 0.2 N HCl at room temperature for 30 min–1 h, in 5% $Ba(OH)_2$ (w/v) at 50°C for 7–10 min, and in 2× SSC (standard sodium citrate) solution at 60°C for 2–4 h. Between steps, the slides were rinsed with deionized water, dehydrated, and air dried. 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, Pennsylvania) at 40°C overnight, cleared with xylene (Sigma), and mounted in Permount solution (Fisher).

Restriction enzyme banding.—Ten restriction enzymes were used in this study: *Bam*H I, *Bgl* I, *Eco*R I, *Hind* III, *Mbo* I, *Msc* I, *Nde* I, *Not* I, *Pvu* II, and *Sau* 3A I. The enzymes were diluted to final concentrations of 0.5–1.2 units/μL in buffers supplied by the manufacturer (New England Biolabs, Beverly, Massachusetts). 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 and then with distilled water and air dried. The slides were stained with 5% Giemsa for 20 min. Control slides treated with buffer without enzyme were included in each experiment.

Computer-assisted chromosome analysis.—Karyotyping was conducted with the Optimas (Bioscan, Inc., Edmonds, Washington) and Kary (Pro Data, Oslo, Norway) computer software packages as described by Zhang (1996). For high-resolution analysis of banding patterns, chromosomes were photographed with a microscope-mounted (Microphot-SA, Nikon, Inc., Garden City, New York) camera (Nikon, Inc., model FX-35DX) and Kodak Technical Pan 2415 film. The negatives were scanned (SprintScan 35, Polaroid scanner model CS-2700, Needham Heights, Massachusetts) for analysis with a resolution of 2,025 pixels per inch. Total length and arm length of each chromosome were measured, and relative length (or percent of total complement length, %TCL) and centromeric index (CI) were calculated for each chromosome based on following formulae: %TCL = (length of the chromosome pair/total complement length) × 100; CI (%) = (length of short arm/total length of the chromosome) × 100. Chromosomes of each type were sorted based on CI and subdivided into groups based on the %TCL values.

Chromosomes treated with enzymes *Hind* III and *Msc* I were sorted by descending order of size and were paired based on banding patterns. Bands generated by these enzymes were further analyzed by microdensitometry (Zhang and Tiersch 1998). The *x*-axis of the microdensitomeric plots identified chromosome segments and the *y*-axis represented relative grayscale values, from 0 (black) to 255 (white). Idiograms were prepared by the Microsoft PowerPoint® computer software, based on the measurements of %TCL and CI, the patterns of NOR-banding, C-banding, and RE-banding.

Results

The 29 pairs of homologous chromosomes were divided into eight groups based on size and centromeric position (Table1; Figure 1a). The NOR were located on a pair of medium-sized submetacentric chromosomes (Figure 1a–1c, inset). For comparison among studies, a representative spread was organized by this nomenclature system (Figure 1a), by the size-based method (Figure 1b) and by morphology-based methods reported previously (Muramoto et al. 1968; LeGrande 1981; Figure 1c). There was considerable variation in the location of chromosomes in these three karyotyping systems (Figure 1d).

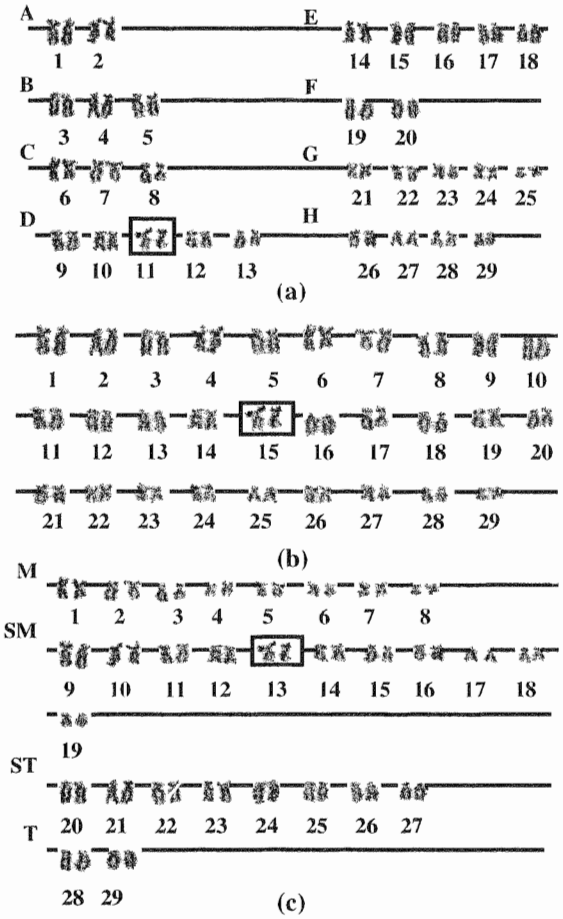
TABLE 1.—The grouping system developed for channel catfish chromosomes in this study.

Group	Size	Morphology	Number of chromosome pairs
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
All			29

The C-banding revealed prominent and highly resolved bands at the centromeres of most chromosomes (Figure 2). Chromosomes were organized into a C-banded karyotype as described above. The smallest metacentric (G-25) and submetacentric (H-29) chromosomes did not show centromeric C-bands. Chromosome E-16 had an identifiable noncentromeric C-band on the short arm. The C-banding patterns agreed between homologous chromosomes. Locations of NOR and C-banding were summarized with an idiogram (Figure 3) prepared from 20 metaphase spreads.

Among the 10 REs used in this study (Table 2), *Bam*H I, *Eco*R I, and *Mbo* I produced only one or two bands on a few chromosomes. Locations of the major bands were not limited to specific regions of the chromosomes. Bands generated by *Bam*H I were most evident at the telomeres, whereas bands generated by *Eco*R I were distributed mostly at centromeric regions. Bands created by *Mbo* I were distributed on centromeric and telomeric regions.

The enzymes *Bgl* I and *Pvu* II produced bands along the length of the chromosomes. Those digested by *Bgl* I generally had two or more elongated bands around the centromeres, and these bands sometimes extended to the ends of the chromosomes. The centromeric regions were degraded and faintly stained. In contrast, chromosomes treated by *Pvu* II yielded relatively few bands. One other enzyme, *Nde* I, generated inconsistent banding patterns and produced a fuzzy appearance. The enzymes *Not* I and *Sau* 3A I did not produce any bands, yielding chromosomes that stained uniformly with Giemsa.



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

1 This study.
2. Chromosomes were arranged with descending order by size.
3. Muramoto et al. 1968; LeGrande, 1981.

(d)

FIGURE 1.—Alternative karyotype systems of the channel catfish with identification of the chromosome bearing the nucleolus organizer region (NOR). **(a)** For this study, chromosomes were sorted by size and centromeric index and divided into eight groups (Table 1). Other karotype systems reported in the literature were prepared from the same spread for direct comparison: **(b)** size-based system and **(c)** morphology-based system. The NOR-bearing chromosomes (in boxes) were (a) D-11, (b) 15, and (c) SM-13. **(d)** The chart shows the location of the individual chromosomes in the three karyotyping systems.

Chromosomes treated with *Hind* III (Figures 4, 5) and *Msc* I (Figure 6) yielded the most information. Banding patterns of these chromosomes were identified by microdensitometry (Figure 4b). Linear bands were produced on most chromosomes treated with *Hind* III. The banding patterns were consistent between members of homologous pairs. Treatment with *Msc* I yielded a clear region on the centromeres of many chromosomes. Bands were found mostly on the telomeric regions and agreed between members of homologous pairs. Because banding techniques often prevented calculation of CIs, chromosomes were arranged by descending size, and homologous pairs were determined by banding patterns.

Discussion

The chromosome nomenclature system applied in this study is based on that used for humans (Verma and Babu 1989) and was useful for identification of nonbanded 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 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 well because of size overlap among different groups. In fact, only 5 of 29 chromosome pairs were consistently identifiable

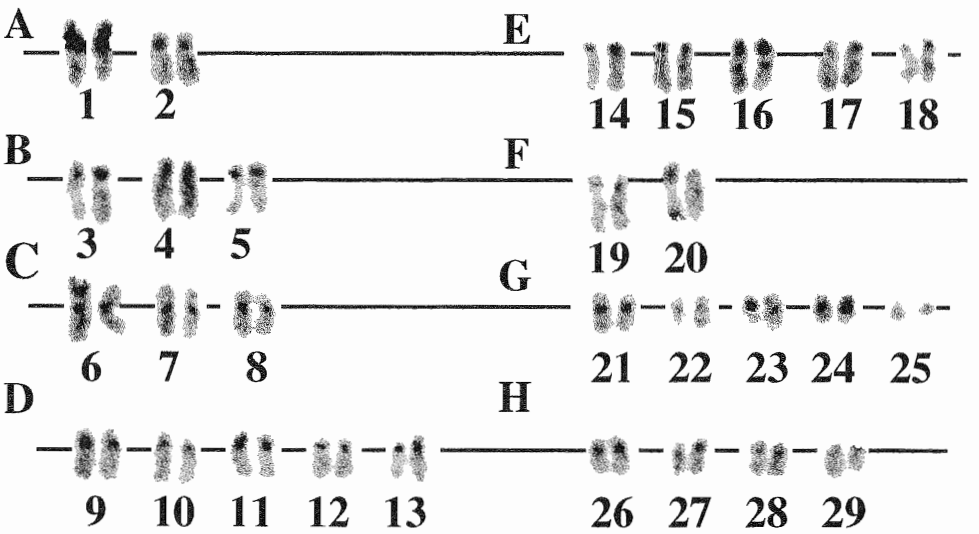


FIGURE 2.—A karyotype of channel catfish chromosomes banded by the CBG method (C-bands visualized by treating with barium hydroxide and staining with Giemsa). The band on the short arm of chromosome C-6 is a technical artifact, resulting from overlap with chromosome F-20 in the original spread.

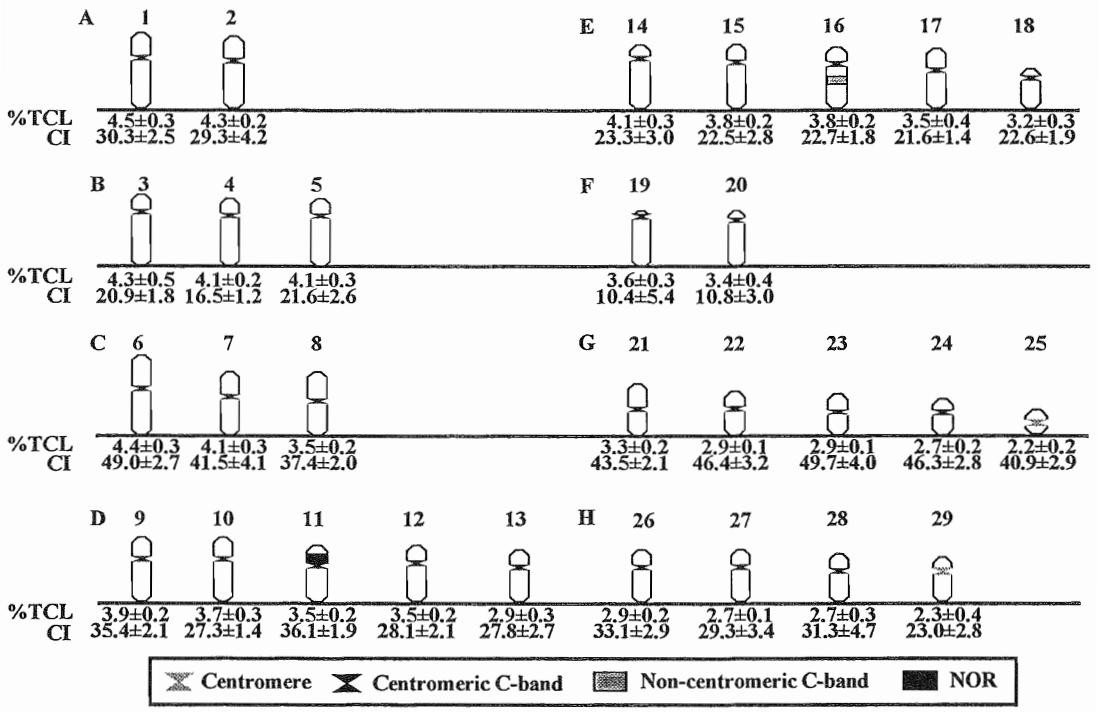


FIGURE 3.—Idiogram of chromosomes bearing the nucleolus organizer region (NOR) and the location of constitutive heterochromatin (C-banding) for channel catfish. The measurements of percent of total chromosome complement (%TCL) and centromeric index (CI) for each chromosome were derived from analysis of 20 spreads obtained from five channel catfish.

TABLE 2.—Banding patterns of channel catfish chromosomes treated with restriction enzymes compared with those found in salmonid fishes.

Enzyme	Recognition sequence[a]	Concentration (unit/μL)[b]	Chromosome banding patterns[c]	
			Channel catfish[b]	Salmonids[d]
*Bam*H I	GGATCC	1.0	C (telomeric)	NS
Bgl I	GCCNNNNNGGC	1.2	C (pericentric)	NS
*Eco*R I	GAATTC	1.2	C (centromeric)	None
Hind III	AAGCTT	1.0	Replication band[e]	None
Msc I	TGGCCA	0.3	C (mostly telomeric)	NS
Mbo I	GATC	0.5	C (centromeric or telomeric)	C
Nde I	CATATG	0.5	Irregular	NS
Not I	GCGGCCGC	1.0	None	NS
Pvu II	CAGCTG	1.0	Interstitial	C
Sau 3A I	GATC	0.4	None	NS

[a] G = guanine; A = adenine; T = thymine; C = cytosine; N = any nucleotide.
[b] This study.
[c] C = C-band like; none means uniform staining; NS = not studied.
[d] Lloyd and Thorgaard (1988); Hartley (1991a, 1991b).
[e] Zhang (1996).

by this method. Sorting by centromeric index reduced the standard deviation in averaging data collected from different spreads (data not shown). A centromere-based method has been used in most cytogenetic work on fishes, including channel catfish (Muramoto et al. 1968; LeGrande 1981). This method allowed consistent identification of 9 pairs of chromosomes. The nomenclature system developed in the present study uses a combination of the size and centromere information. Chromosomes with similar centromeric indices were grouped and then split into subgroups based on size differences; this system allowed consistent identification of 15 chromosome pairs.

The constitutive heterochromatin of channel catfish chromosomes was small and limited to cen-

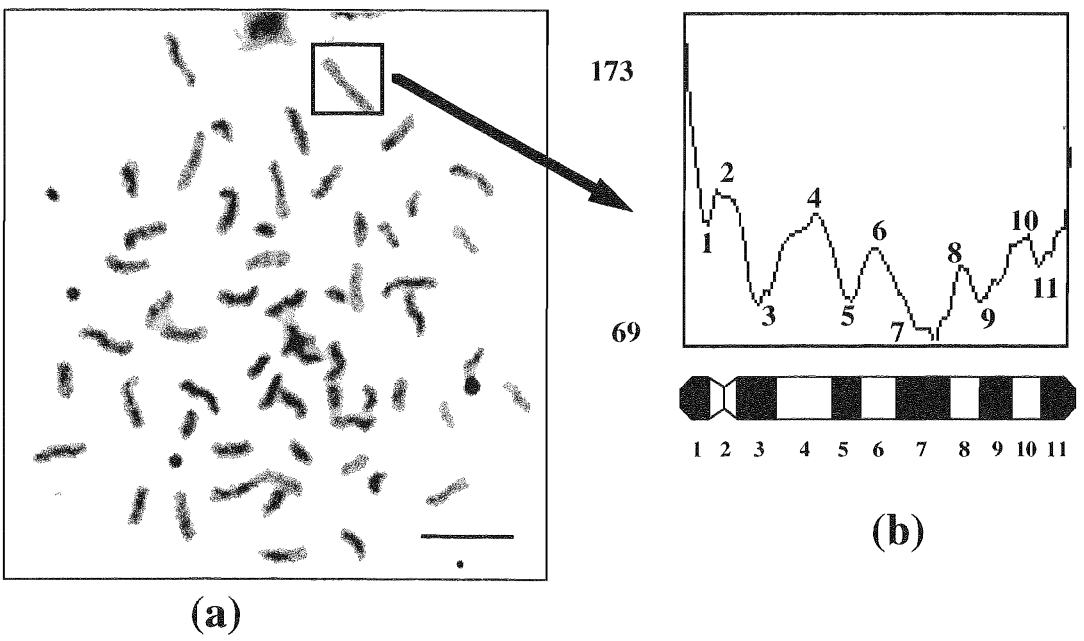


FIGURE 4.—Identification by microdensitometry of banding patterns of restriction-enzyme-treated chromosomes. Chromosomes (A-1 in this example) were **(a)** treated with restriction enzyme *Hind* III and **(b)** depicted with a densitometric plot in which the *x*-axis indicates chromosome segment (numbers 1–11) and the *y*-axis indicates relative grayscale value (from 69 to 173). An idiogram was created to represent the banding pattern (b). In (a), bar = 10 μm.

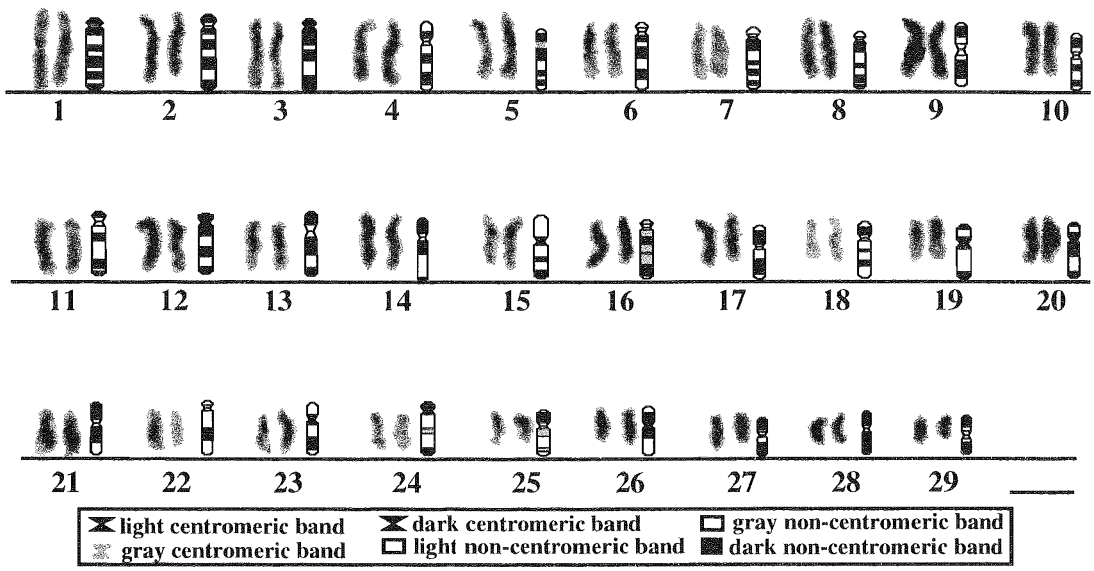


FIGURE 5.—A karyotype of channel catfish chromosomes treated with the restriction enzyme *Hind* III. The chromosomes were arranged by descending size and paired by banding patterns, and idiograms were developed for each chromosome pair. Bar = 10 μm.

tromeric regions, unlike results observed in cyprinid fishes (Gold et al. 1986). Distinct secondary bands were absent on all catfish chromosomes except E-16. The C-bands were prominent and useful for identifying homologous chromosomes. The low abundance of heterochromatin may explain the stable genome size found in the ictalurid catfishes (Tiersch and Goudie 1993). The genome size of channel catfish was 1.98 ± 0.01 pg and was not different among 14 populations studied (Tiersch et al. 1990), representing the smallest level of genome size variation (~2.3%) described in a vertebrate species. On the other hand, heterochromatin can account for 35–39% of the total genome

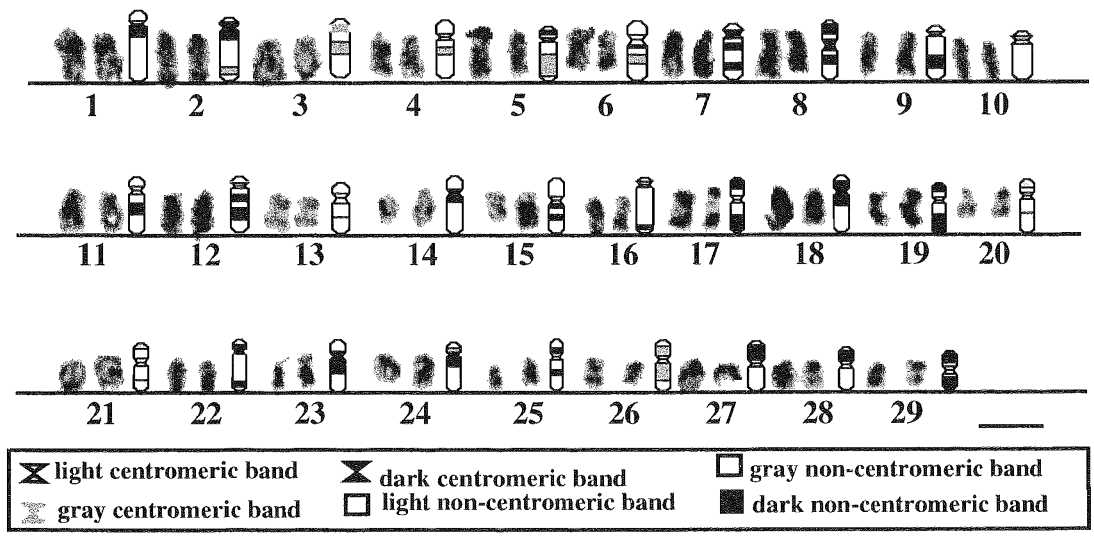


FIGURE 6.—A karyotype of channel catfish chromosomes treated with restriction enzyme *Msc* I. The chromosomes were arranged by descending size and paired by banding patterns, and idiograms were developed for each chromosome pair. Bar = 10 μm.

of some cyprinid fishes (Gold et al. 1986), and variation of genome sizes within populations of cyprinid fishes can be as high as 13.5% (Gold et al. 1990b).

The development of RE-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 REs are differences in nucleotide sequence along the chromosomes (Bianchi et al. 1985) or differences in chromatin structure, making DNA more susceptible to digestion in some regions (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 DNA to REs or in chromatin removal. Therefore, RE-banding patterns are not always correlated with chromosomal DNA composition.

We found that banding patterns of some REs were not correlated with their recognition sequence (see Table 2), which might be due to differential sensitivity of these enzymes to necleotide 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 of these enzymes were different. The enzyme *Mbo* I produced modified C-bands, whereas *Sau* 3A I did not produce any recognizable bands. The site sensitive to methylation is A (adenine) for *Mbo* I and C (cytosine) for *Sau* 3A I. Furthermore, enzymes with different recognition sequences may not produce distinct banding patterns because size differences of the DNA fragments removed may not be resolvable under the light microscope. The enzymes *Bam*H I (a 6-base-pair recognition sequence) and *Mbo* I (a 4-base-pair recognition sequence) 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 was demonstrated in this study with the enzyme *Hind* III. Although numerous G-banding techniques were attempted in this study (e.g., G-bands by trypsin with Giemsa staining; data not shown), banding was inconsistent or absent. The RE-banding procedure was simpler than conventional G-banding methods. However, difficulties were encountered in identifying centromeres of RE-treated chromosomes because uniform banding patterns were not present on these regions. This ambiguity prevented karyotyping of these chromosomes with the size and morphology-based method. The application of RE-banding to fish karyotyping requires further development. Of the reports addressing this topic, most enzymes have produced modified C-bands, although results varied greatly from species to species. For example, *Hind* III, which generated a linear banding pattern for channel catfish in this study, did not produce any bands for rainbow trout *Oncorhynchus mykiss* (Lloyd and Thorgaard 1988).

Computer-assisted karyotyping, as shown in this study, provides an important approach to identifying the weak banding patterns generated by many restriction enzymes. This technique will be helpful to fish cytogeneticists because there are no banding techniques capable of producing a highly resolved linear pattern for teleost species. Furthermore, an automated karyotyping system can shorten the time required for traditional methods and minimize measurement errors.

Our study provides a fundamental but important step in genome mapping of a commercially valuable fish species. Standardization of the channel catfish karyotype will provide basic information for cytogenetic studies and facilitate the physical mapping of genes. The unique C-banding pattern on each chromosomes helped in identifying homologous pairs. Regardless of its weakness in resolving centromeres, the RE-banding can be used to generate markers for each chromosome to produce a highly resolved serial pattern.

Acknowledgments

Technical assistance was provided by J. Ratcliff. We thank J. Avault, R. Chabreck, R. Cooper, D. Shih, M. Stine, and W. Wolters for critical review. This study was funded in part by U.S. Department of Agriculture (USDA), Agricultural Research Service, cooperative agreements 6402-31000-002-02S, USDA special grant 93-34310-9057, and the Louisiana Catfish Promotion and Research Board. This manuscript was approved by the Director of the Louisiana Agricultural Experiment Station as manuscript 96-22-0159.

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Received May 20, 1997
Accepted December 4, 1997