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Molecular Genetics of Sex Determination in Channel Catfish: Studies on *SRY, ZFY,* Bkm, and Human Telomeric Repeats[1]

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ABSTRACT

In amniotes, the banded krait minor (Bkm) minisatellite (GATA), the human telometric sequence $(TTAGGG)_n$, and the Y-specific genes, *ZFY* and *SRY*, are associated with a particular sex. These sequences were studied in the channel catfish, *Ictalurus punctatus*. However, none was sex-specific in catfish; homologs of each were present in males and females. Our data suggest that components of mammalian sex-determining systems may be widespread and shared among the vertebrates in general. Whether those components are involved in sex determination in lower vertebrates or merely represent evolutionary precursors of sex-determining factors in amniotes remains to be determined.

INTRODUCTION

Since 1959, when the Y chromosome was found to determine sex in humans and mice [1, 2], several candidates have been cast in the role of the Y-situated testis-determining gene (*TDF* in humans). The gene for H-Y (male) antigen was a prime candidate [3], but when McLaren et al. [4] reported absence of H-Y in certain male mice, the search for the testis determinant was directed elsewhere. Banded krait minor (Bkm) satellite DNA isolated from the W chromosome of the banded krait, a snake, was found to be widely conserved in evolution and concentrated in the sex-determining region of the mouse Y chromosome [5]. However, Bkm was not male-specific or concentrated on the Y chromosome in other mammals [6].

By deletion mapping, Page et al. [7] identified a region on the human Y chromosome thought to contain all or part of the testis determinant. One of the probes prepared from this region identified a gene that was termed *ZFY* (*zinc-finger Y*) because the nucleotide sequence resembled sequences in the genes of proteins with multiple finger domains [8]. Yet ZFY could not be the primary inducer of the testis because certain XX males with Y-X crossovers lack that portion of the Y that carries *ZFY* [9]; also see [10]. More recently, a gene was identified near the pseudoautosomal pairing region of the human Y chromosome, the area of normal X-Y interchange. This gene, called *SRY,* for *sex-determining region Y,* is conserved and male-specific in mammals and is currently the prime candidate for *TDF* [11, 12].

In contrast to those of mammals, the sex-determining mechanisms of fishes are diverse, labile, and poorly characterized. One approach to the study of sex determination in fish has involved hormonal manipulation of gender, breeding studies, and production of monosex populations and novel combinations of genotype and phenotype, including fertile XY females and YY males. By this approach, heterogamety has been assigned in several groups, including medaka [13], tilapia [14], and certain salmonids [15] and cyprinids [16]. Like most teleost fishes, the channel catfish, *Ictalurus punctatus,* lacks heteromorphic sex chromosomes [17], but the genetics of sex determination in this species has been characterized. These fish have a male-heterogametic-type mechanism (XX/XY) [18], and sex-reversed stocks can be produced readily by administration of hormones [19]. Yet little is known about the molecular genetics of sex determination in catfish or other fishes.

The present study was therefore undertaken to investigate mechanisms of sex determination in fish by evaluation of factors known to be sex-associated in other vertebrates. The specific goals of the study were to determine (1) whether genes corresponding to *ZFY* and *SRY* exist in channel catfish and, if so, whether they are sex-specific; and (2) whether other sex-specific sequences exist in this species.

MATERIALS AND METHODS

Sample Collection and Fish Stocks

Blood samples were drawn into acid-citrate-dextrose (ACD) solution and kept frozen until use. Samples were collected from normal male (XY) and normal female (XX) catfish from a wild population (captured in the Red River, ND) and a domesticated population maintained at Auburn University (Auburn, AL). The latter catfish were of the Kansas strain, under domestication for 70 years [20]. Other populations used in this study were normal males and females maintained at Memphis State University (Memphis, TN); gynogenetically-produced females [21]; and YY males

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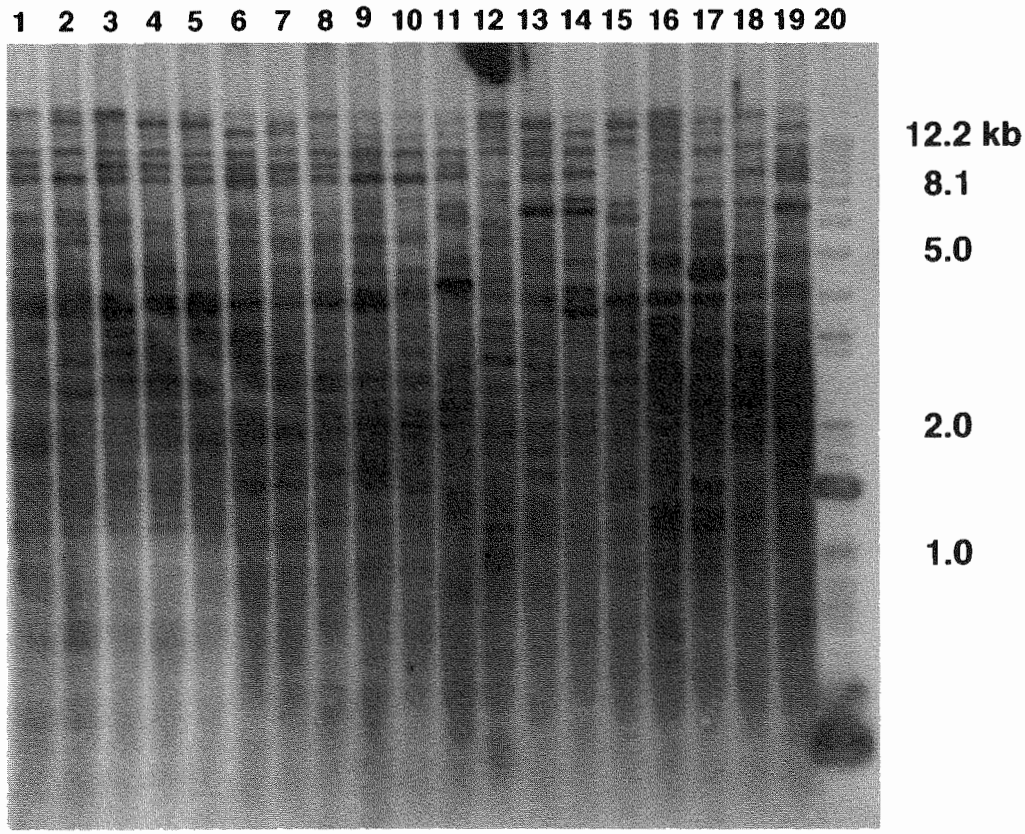

FIG. 1. Southern blot of genomic DNA from 14 male and 6 female channel catfish after hybridization with Bkm. The DNA was digested with the restriction enzyme *Rsa*I. Lanes 1–4, 7, 11, 12, and 17–19 contained DNA from normal males; lanes 5, 6, and 8 contained DNA from YY males. Lanes, 9, 10, and 13–16 contained DNA from females; size markers were placed in lane 20. Fish examined in lanes 1–8 were from Marion, AL, and samples in lanes 9–19 were from a population at Memphis State University.

and sex-reversed XY females [18]. These fish were obtained from the U.S. Fish and Wildlife Service, Southeastern Fish Cultural Laboratory, Marion, AL.

Restriction Analysis and Southern Blot Hybridization

DNA was extracted by use of guanidine-HCl [22] or phenol-chloroform. Seventeen restriction enzymes were used in a preliminary study, and four were chosen for further analysis: *Eco*RI, *Hin*dIII, *Rsa*I, and *Sau*3AI. Restricted DNA was electrophoresed through 0.8% agarose gels at 25 V. The gels were stained with ethidium bromide, illuminated with UV light, and photographed. For Southern blotting, DNA was transferred to nylon filters (Hybond N; Amersham Corp., Arlington Heights, IL) with 10-strength saline sodium citrate (SSC).

Three probes were selected for this study. (1) The Bkm 2(8) probe, consisting mainly of the repeated tetranucleotide, GATA, was obtained from a *Drosophila* genomic library [5]. It was subcloned in plasmid pT7-2 and excised by double digest with *Pst*I and *Bam*HI. (2) The human telomeric repeat sequence $(TTAGGG)_7$ is highly conserved among the vertebrates [23] and displays a male-specific banding pattern in certain strains of mice (C. Bishop, personal communication). (3) A 360-bp fragment of *SRY,* the presumptive testis-determining gene of the human, which includes the 80-amino acid-conserved motif [12, 24], was amplified from DNA of a normal human male by the polymerase chain reaction (PCR; see below). Each of the probes was labeled with ^{32}P, and hybridizations were carried out overnight in Church's buffer at 62°C or higher. Filters were washed three times in single-strength SSC or 0.1-strength SSC for 30 min at 62°C. Autoradiography was carried out at −80°C for 1–5 days.

PCR

The presence of DNA sequences in catfish corresponding to regions within the *SRY* and *ZFY* genes was tested by use of PCR. Samples of DNA from normal male and female catfish were subjected to 25 cycles of PCR amplification using *Taq* DNA polymerase (Perkin-Elmer Cetus, Irvine, CA). Under conditions recommended by the manufacturer, 500 ng of catfish DNA or 1 μg of human DNA, 100 pmol of each primer, and 2.5 U of *Taq* polymerase were combined in a final volume of 100 μl. The polymerase was added after an

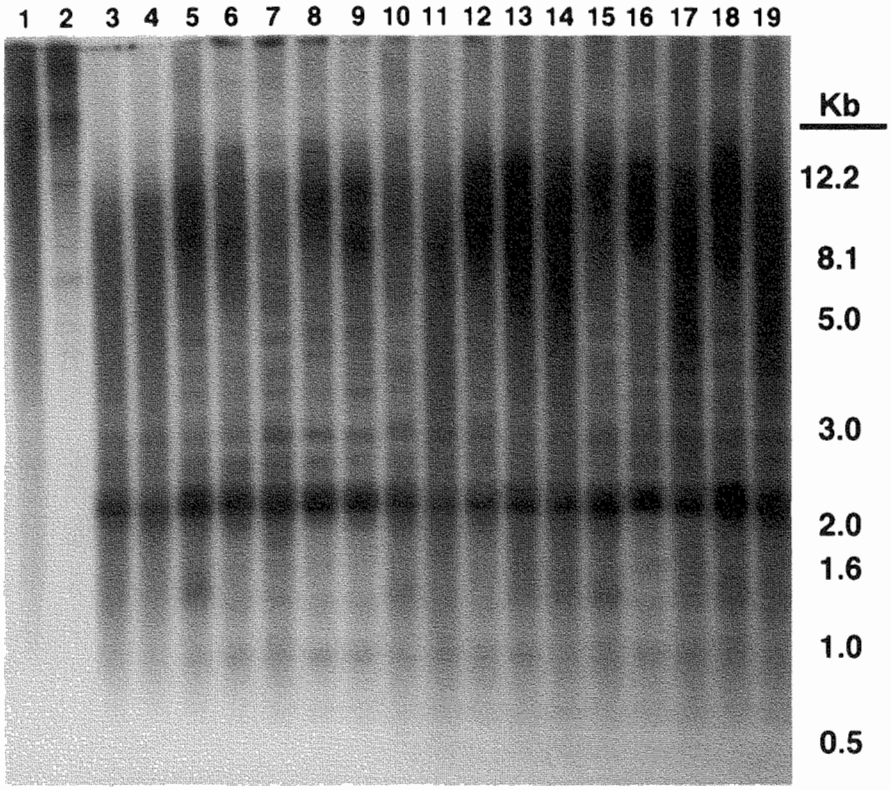

FIG. 2. Southern blot of genomic DNA from male and female mice and channel catfish after hybridization with a human telomeric probe. The DNA was digested with the restriction enzyme *Sau*3AI. DNA from male mouse was loaded into lane 1, and DNA from female mouse was loaded into lane 2. Lanes 3–6 contained DNA from normal (XX) female catfish, lanes 6–10 contained DNA from sex-reversed (XY) females, lanes 12–15 contained DNA from normal males, and lanes 16–19 contained DNA from YY males. All fish were from Marion, AL. Mouse DNA was included as a mammalian control for comparison with catfish DNA; sex-specificity is not seen with this enzyme in this strain of mouse.

initial denaturation step of 95°C for 5 min. The cycle profile included denaturation for 1 min at 94°C; primer annealing for 2 min at 50°C, 51°C, 52°C, or 55°C; and chain extension for 3 min at 72°C. A final cycle included an additional extension for 5 min at 72°C.

For study of *ZFY,* we used two sets of oligonucleotide primers derived from the human zinc-finger exon of the genomic insert of plasmids pDP1065 and pDP1007 [25]. The fragments delineated by the primers were partially overlapping and of similar size (334 bp and 320 bp). One set of the primers was selected from regions where identical sequences were shared between the exons of *ZFY* and a homologous gene, *ZFX*, mapped to the human X chromosome [7]. The primer sequences were (5′ to 3′) TGAATCGCCACCTCTTGGCAGT and TTGTGGTCGCAATGCAAACACT. The other set of primers was selected to enable distinction between *ZFY* and *ZFX.* The primer sequences (and number of base changes) were CCGACACCCGTCGGAACTGAGA (6) and CTCGCACATCTCACACTTATGA (4).

Two sets of primers were used to study *SRY.* One set specified a 360-bp fragment; contained within the sequence of this first fragment was a 258-bp fragment specified by the second set of primers. The primers for the smaller fragment included the ends of the 80-amino acid-conserved motif of *SRY* [12]. The primers for the larger fragment originated outside of the conserved domain, in the flanking region of the open reading frame. Primer sequences for the large fragment were CAGTGTGAAACGGGAGAAAACA and GTACAACCTGTTGTCCAGTTGC; primer sequences for the small fragment were GGCAACGTCCAGGATAGAGTGA and CGGCAGCATCTTCGCCTTCCGA.

The reaction products were analyzed by electrophoresis through 4.0% agarose gels, with ethidium bromide staining, UV illumination, and photography. Samples of DNA from human males and females were included in each test. Reaction mixtures without template DNA were included among the controls.

RESULTS

Restriction Analysis and Southern Blot Hybridization

Restriction fragments were observed in catfish DNA with all enzymes used, but none of the fragments was sex-specific. Considerable hybridization was obtained with the Bkm

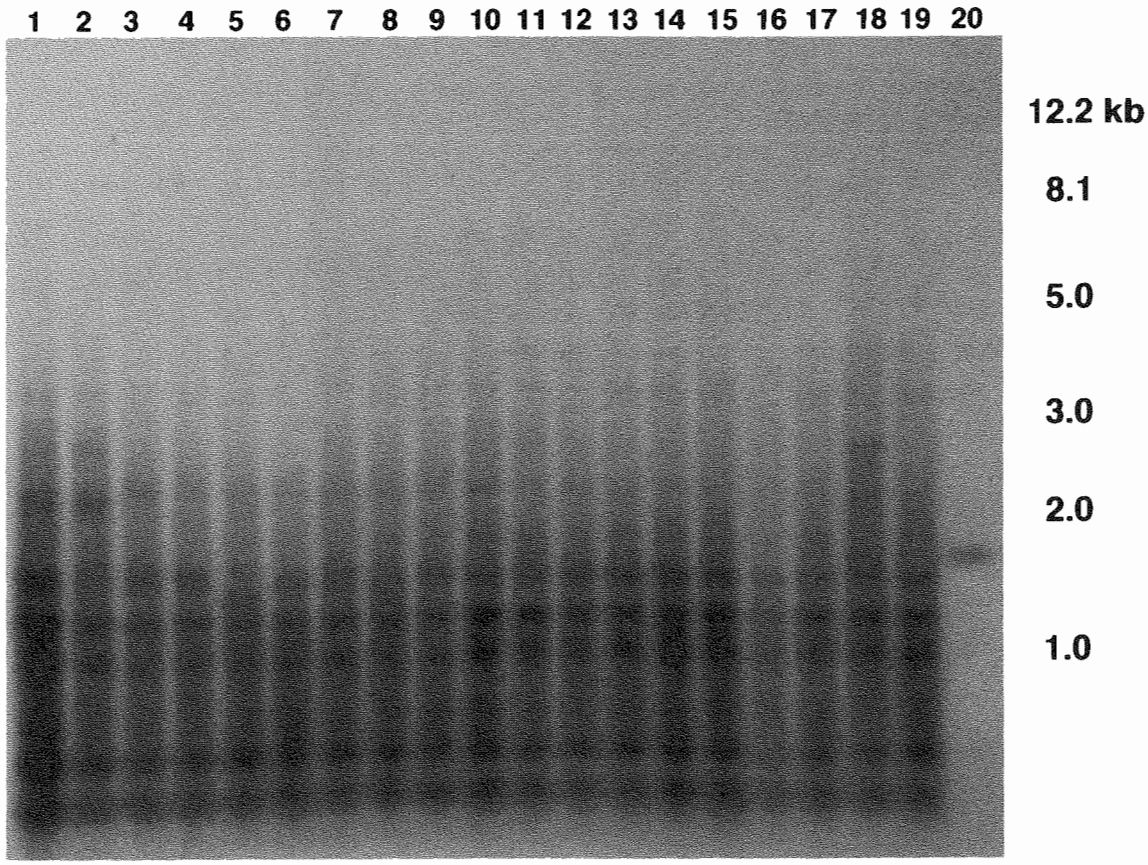

FIG. 3. Southern blot of genomic DNA from 10 male and 9 female channel catfish after hybridization with a probe from the conserved motif of *SRY*. The DNA was digested with the restriction enzyme *Rsa*I. Lanes 1–5 and 11–15 contained DNA from normal males; lanes 6–10 and 16–19 contained DNA from normal females; size markers were placed in lane 20. Fish examined in lanes 1–10 were collected from the Red River, ND; fish in lanes 11–19 were from the Kansas strain kept at Auburn University.

2(8) and human telomeric probes (Figs. 1 and 2), but sex-related differences were not evident. These probes produced distinct fingerprint patterns similar to those described for other oligonucleotide probes [e.g. 26]. The banding pattern produced with Bkm was highly polymorphic and complex. Within-population variation was as great as the variation observed among populations.

Hybridization with the *SRY*-derived probe produced five or six major bands in a monotonous pattern (Fig. 3). Differences were not detected between sexes or among populations, although polymorphism of certain bands were observed in some cases. Similar patterns of hybridization were observed for gynogenetic, YY, sex-reversed XY, and normal male and female catfish for each of the three probes.

PCR

The primer set selected to distinguish *ZFY* from *ZFX* yielded a single male-specific fragment of about 330 bp at all annealing temperatures in the human. Amplification of the specified sequence was not observed with DNA from human females or from female or male catfish (Fig. 4A). The other primer set—with sequences shared completely by exons of *ZFY* and *ZFX*—yielded fragments of the same size (about 320 bp) in human male and female at all temperatures. A number of other fragments were observed for both sexes of the human at 50°C, but not at higher temperatures. Multiple fragments were amplified from catfish DNA at 50°C and 52°C; two conspicuous fragments of about 510 bp and 600 bp were observed (Fig. 4B), but they were not sex-specific.

Both sets of *SRY* primers yielded a male-specific fragment of expected size (about 260 bp and 360 bp) in human DNA at annealing temperatures that allowed the amplification of fragments in catfish DNA (conspicuous fragments of about 1 kb and 880 bp; Fig. 5, A and B). At these temperatures, fragments were not amplified in human female DNA by use of the primers from outside the conserved motif (Fig. 5B). Sex specificity was not observed in the catfish DNA; identical results were obtained for gynogenetic, XY male, XX female, and YY male catfish (data not shown). It should be noted that the amplified fragments have not been sequenced. Thus amplification of a particular catfish frag-

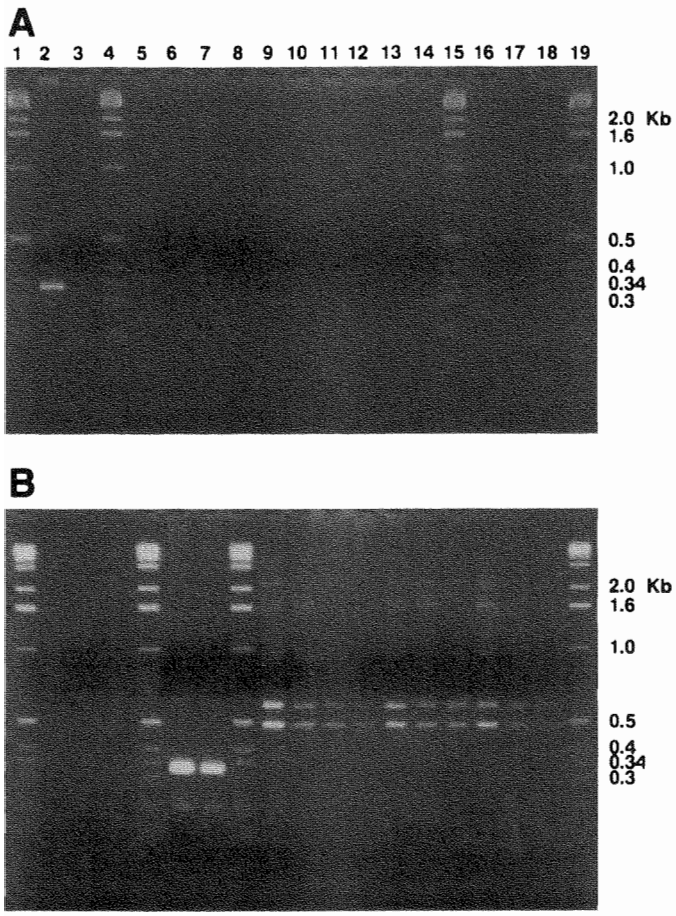

FIG. 4. Analysis by PCR of *ZFY* and *ZFX* and of homologous sequences in DNA of normal male and female of humans and catfish. A) Oligonucleotide primers were specific for *ZFY*. Lanes 1, 4, 15, and 19 contained size markers; lane 2, DNA from human male; lane 3, DNA from human female; lanes 5–9, DNA from catfish females; lanes 10–14, DNA from catfish males. Lane 17 was a negative control (no template DNA in reaction), and lanes 16 and 18 were not loaded. The Y-specific fragment in human male DNA was 334 bp. The reaction was performed at 50°C annealing temperature. B) Oligonucleotide primers specified a region shared completely by *ZFY* and *ZFX*. Lanes 1, 5, 8, and 19 contained size markers; lane 6, DNA from human male; lane 7, DNA from human female; lanes 9–13, DNA from catfish females; lanes 14–18, DNA from catfish males. Lane 3 was a negative control (no template DNA in reaction), and lanes 2 and 4 were not loaded. The fragment seen in male and female human was 320 bp. Fragments from catfish DNA appeared at 510 bp and 600 bp. The reaction was performed at 50°C annealing temperature. The fish examined were from Marion, AL.

ment with mammalian *SRY* primers indicates strong homology with primer DNA, and not necessarily with sequences between the primers.

DISCUSSION

Bkm Minisatellite DNA

The Bkm minisatellite hybridizes to DNA from a wide variety of eukaryotic organisms including invertebrates and vertebrates. A cluster of Bkm sequences was localized in the sex-determining region of the mouse Y chromosome and in the region of an aberrant Y responsible for sex-reversed XX*Sxr* mice, which develop as phenotypic, though sterile, males [27]. Moreover, Bkm DNA is associated with the W chromosome in certain birds and in snakes, including those without heteromorphic sex chromosomes [28].

We identified multiple polymorphic Bkm fragments in the catfish, none of which was related to sex. Similar results have been reported for the hybridization of Bkm with DNA from rainbow trout [29]. The role of Bkm in sex determination thus remains uncertain. Given the high level of Bkm polymorphism in catfish, trout, and other vertebrates [e.g. 30], this minisatellite nevertheless may provide markers for DNA fingerprinting of fish.

Human Telomere Sequence

The tandemly repeated hexanucleotide $(TTAGGG)_n$ was detected by in situ hybridization at the telomeres of all chromosomes in 91 vertebrate species representing bony fish, amphibians, reptiles, birds, and mammals [23]. Although hybridization of $(TTAGGG)_7$ to DNA from some strains of mice identifies a male-specific fragment, the $(TTAGGG)_7$ probe revealed a pattern of hybridization in catfish DNA similar to that seen in other vertebrates; sex-specific hybridization was not observed. The patterns observed were polymorphic, and thus $(TTAGGG)_n$, like Bkm, could prove useful for fingerprint analysis.

ZFY Gene

A sequence from *ZFY* has been found to hybridize to sequences on the Y chromosome of every eutherian mammal tested. Inasmuch as the DNA sequence of this gene coded for a zinc-finger protein, it could function as a DNA regulatory element. A homologous sequence, *ZFX,* was found on the human X chromosome, and a mechanism of dosage compensation similar to that of *Drosophila* and certain nematodes was favored among four hypotheses to explain the possible action of this gene [7].

Sequences homologous to *ZFY* were found in both sexes in reptiles regardless of the sex-determining mechanism by Bull et al. [31] and in both sexes in sturgeon and trout by Ferreiro et al. [32]. In our PCR, *ZFY*-specific primers did not generate amplified fragments from catfish DNA, whereas primers specifying a region shared completely by *ZFY* and *ZFX* did. Sequences corresponding to *ZFX* were more strictly conserved among mammals than were sequences corresponding to *ZFY* [7], and greater conservation of sequences corresponding to *ZFX* could also be the case in catfish. This would seem to agree with "Ohno's Law," according to which the X and Y chromosomes diverged from an ancestral autosomal pair; the Y chromosome lost most of its genetic material to become a specialized sex-determinant, whereas genes on the X chromosome were more or less conserved throughout mammalian evolution [33].

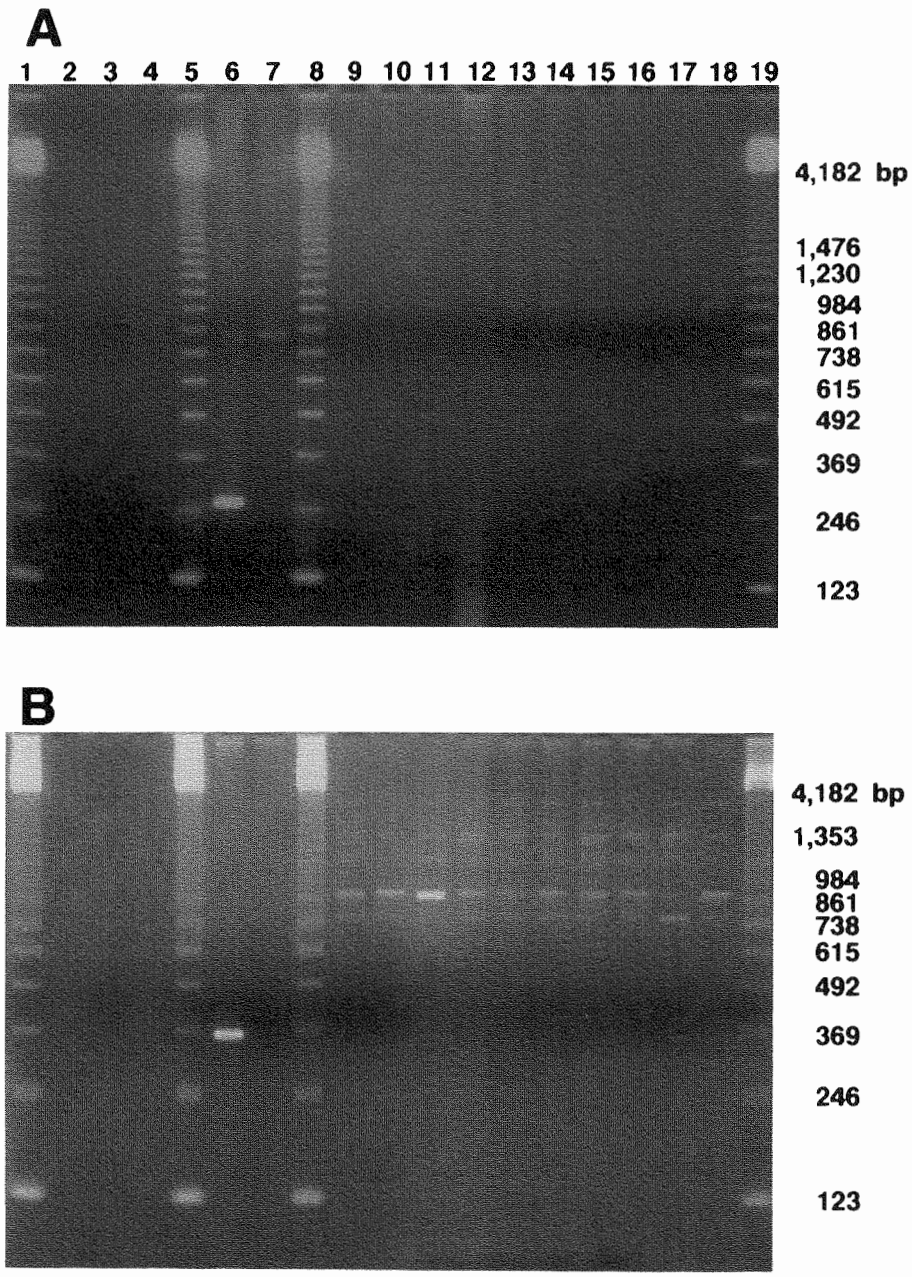

FIG. 5. Analysis by PCR of *SRY* and of homologous sequences in DNA from normal male and female humans and catfish. A) Oligonucleotide primers were specific for the conserved motif of *SRY*. Lanes 1, 5, 8, and 19 contained size markers; lane 6, DNA from human male; lane 7, DNA from human female; lanes 9–13, DNA from catfish females; lanes 14–18, DNA from catfish males. Lane 3 was a negative control (no template DNA in reaction), and lanes 2 and 4 were not loaded. The Y-specific fragment in human male DNA is 258 bp. Fragments from catfish DNA appeared at about 1 kb. Possible technical failure occurred in lane 12 (catfish female). The reaction was performed at 51°C annealing temperature. B) Oligonucleotide primers spanned flanking regions and the conserved motif of *SRY*. Lane designations are the same as noted above. The fragment seen in male human DNA is 360 bp. A conspicuous fragment amplified from the catfish DNA appeared at about 880 bp. The reaction was performed at 50°C annealing temperature. The fish examined were from Marion, AL.

SRY Gene

The *SRY* gene encodes a testis-specific transcript; it shares homology with a mating-type protein from yeast, and it has a conserved DNA-binding motif present in the nuclear high-mobility-group proteins, HMG1 and HMG2 [12]. Mutations, including single-base substitutions and a four-nucleotide deletion, were found within the conserved DNA-binding motif of *SRY* in three human XY females [34, 35]. The cor-

responding gene in the mouse, *Sry,* was deleted from the Y chromosome in certain XY female mice [11], and expression of *Sry* was observed in the embryonic gonad during the stage in which the testis begins to form [36]. These observations, among others, strongly support a role in primary sex determination for mammalian genes corresponding to *SRY,* and in fact embryonic injection of a 14-kb sequence including the murine *Sry* is sufficient to induce differentiation of testes in some transgenic XX males of the mouse [37].

In the catfish, multiple cross-hybridizing fragments were detected by Southern blotting with a probe including the conserved DNA-binding motif of *SRY,* and in PCR, multiple fragments were amplified by use of primers specifying and flanking the conserved motif. A similar situation has been described in the mouse; in addition to the Y sequence, at least four autosomal loci were reported to carry the conserved *SRY* motif [11]. Moreover, sequences corresponding to the conserved motif of *SRY* were observed in 23 other nonmammalian species representing four vertebrate classes [24]. Sex-specificity was not associated with the fragments identified in the catfish, and it remains to be determined whether sequences cross-hybridizing to *SRY* are involved in sex determination in fish or other nonmammals.

Sex Determination in Channel Catfish

Hormonal feminization is complete in male-to-female sex-reversed channel catfish, and the feminized adults are fertile, although virilization with hormones has not yet been achieved in channel catfish. All-female populations were produced by feeding with androgen-treated pellets during the first 21 days after yolk-sac absorption [19]. Evidently half the offspring were sex-reversed genotypic males. Mating of females from the all-female populations with normal males generated progeny sex ratios significantly different from 1:1 in nine of fifteen test crosses [18]. These data were consistent with the numbers expected for the mating of XY females and XY males within a male-heterogametic-type system, and suggested the occurrence of anonymous YY males in the progeny.

The production of all-female offspring by induction of gynogenesis [21] provides additional evidence for male heterogamety in this species. The XX/XY system should be considered as a model, however, because heteromorphic sex chromosomes have not been found in channel catfish, nor have male-female differences in genome size [38], although XX/XY sex chromosomes are known to exist in another catfish, *Noturus taylori* [17]. Male-female differences in expression of H-Y antigen also could not be detected. Serological H-Y—found in every vertebrate tested, usually in the heterogametic sex—was present in the catfish. However, despite the fact that H-Y is determined by a Y-situated gene in mammals, H-Y was not sex-specific in catfish: mammalian H-Y antibody reacted with cells from males and females [39]. A similar situation was described in other fish of the superorder Ostariophysii by Müller and Wolf [40], suggesting occurrence in both sexes of a precursor gene for H-Y in those species.

If heteromorphic sex chromosomes are evolving in this species, differences were not detectable with Bkm or the telomeric probes. The lack of sex-specificity of H-Y, *ZFY,* and *SRY* homologs suggests that the sex-determining mechanism of catfish is less specialized than that of mammals and that both sexes of the fish retain the information required for development as male or female (this certainly is the case for XY and YY males; XX males have not been produced). Alternatively, none of these sequences is involved in sex determination in catfish, or there is a testis determinant on the catfish Y chromosome that remains undiscovered.

At present, no procedure is available for identification of the sex genotype in channel catfish, apart from gross phenotype, except for breeding and analysis of progeny sex ratios. Further studies might include the localization of sequences homologous to *ZFY* and *SRY* on specific chromosomes by in situ hybridization, and the application of DNA probes derived from the catfish genome.

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