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Chromosomal location of the 28S ribosomal RNA gene of channel catfish by *in situ* polymerase chain reaction

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This study describes the use of the polymerase chain reaction for physical mapping of fish genes. A 287-base pair (bp) fragment of the 28S ribosomal RNA gene (28S rDNA) of channel catfish *Ictalurus punctatus* was isolated and sequenced with human-derived primers. The nucleotide (nt) sequence of this fragment was 20 bp shorter than that of the corresponding region of the human 28S rDNA. The gene was mapped to chromosomes of channel catfish by fluorescence *in situ* hybridization (FISH) and *in situ* polymerase chain reaction (ISPCR). A major locus and a minor locus of 28S rDNA were found on chromosomes of channel catfish. The major locus was associated with the active nucleolus organizer region (NOR) sites. The minor locus was highly resolved and not detectable by silver staining, suggesting that this locus was not involved in synthesis of ribosomal RNA and possessed fewer copies of 28S rDNA. Both loci contained GC-rich DNA elements that could be components of 28S rDNA repeated units. In this study, a potential method of comparative mapping of the channel catfish genome has been presented by using human-derived oligonucleotide sequences. These data demonstrate that ISPCR is highly specific and will be useful in physical mapping of fish genomes.

Key words: ISPCR; FISH; mapping; chromosome; 28S rDNA; *Ictalurus punctatus*.

INTRODUCTION

The 28S ribosomal RNA gene (28S rDNA) is useful for the initiation of physical genome mapping projects because this gene occurs in multiple copies which improves the ease of detection (Pendas *et al.*, 1993). It has been established that the 28S rDNA is associated with nucleolus organizer regions (NOR) in vertebrates (Long & Dawid, 1980). The NOR can be detected by silver staining for transcriptionally active sites (major loci), or by fluorescent staining with chromomycin A3 to target GC-rich regions of active and inactive 28S rDNA (Amemiya & Gold, 1987). On the other hand, fluorescence *in situ* hybridization (FISH) is a more capable tool for detection of the 28S rDNA within inactive sites (minor loci) of NOR (Pendas *et al.*, 1993).

Detection of multiple-copy DNA sequences by FISH has become popular in the field of molecular genetics, but the sensitivity of the technique has been dependent on probe size, labelling method, and equipment used for detection. *In situ* polymerase chain reaction (ISPCR) is a newly developed technique (Gu, 1994) that can overcome these problems. The use of the ISPCR technique in

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physical mapping has been demonstrated in some mammalian species (Troyer *et al.*, 1994) and described preliminarily in channel catfish *Ictalurus punctatus* (Rafinesque) (Zhang *et al.*, 1997).

However, concerns have been raised about the specificity of this technique, and it has been recommended that an internal positive control be included in each reaction for monitoring the amplification process (Gu, 1994). This is especially important in physical mapping of species without pre-existing genetic information such as channel catfish, the most important food fish cultured in the United States. Therefore it was desirable to test the association between the NOR and the 28S rRNA gene to examine the validity of ISPCR before the technique was applied to detect endogenous DNA sequences or genes foreign to channel catfish (Zhang *et al.*, 1998*a*).

The active NOR of channel catfish has been located on the short arms of a pair of medium-sized submetacentric chromosomes (Zhang & Tiersch, 1998). This phenotype did not vary among preparations from epithelial cells, fibroblast cells, kidney cells or leukocytes (Zhang & Tiersch, 1998) and represents the only gene-level marker known for channel catfish chromosomes. The objectives of the present study were to: (1) amplify and characterize a portion of the channel catfish 28S ribosomal RNA gene; and (2) localize this gene on channel catfish chromosomes by ISPCR and FISH to use the relationship between NOR and 28S rDNA as a test of the specificity of the techniques. The possibility was evaluated further of using this gene as a positive control for ISPCR and the direct use of human-derived primers with channel catfish chromosomes.

MATERIALS AND METHODS

ISOLATION OF GENOMIC DNA

Blood was collected from the caudal vessels of channel catfish and other catfishes (see below) into acid-citrate-dextrose solution (15 : 1, v/v) (Becton-Dickinson vacutainer 4606). Nuclear DNA was isolated from whole blood using a QIAamp blood kit (Qiagen Inc., Chatsworth, CA) and the purity and concentration of DNA was estimated spectrophotometrically using the GeneQuant RNA/DNA calculator (Model 80-2104-98, Pharmacia Biotech, Cambridge, U.K.). Human DNA was extracted from blood by standard phenol-chloroform methods.

AMPLIFICATION AND SEQUENCE ANALYSIS OF A FRAGMENT OF THE 28S rRNA GENE

A pair of primers were designed (Naito *et al.*, 1992) to target the human 28S rRNA gene (base positions 1542 to 1857) (Gonzalez *et al.*, 1985). The primer sequences were (5′ to 3′) AAACTCTGGTGGAGGTCCGT and CTTACCAAAAGTGGCCCACTA, and were synthesized by the Gene Probes and Expression Systems Laboratory, Louisiana State University, Baton Rouge. Conditions for PCR were: initial denaturation, 94° C for 2 min; 30 cycles of 94° C for 30 s, 55° C for 30 s, and 72° C for 1 min, with a final elongation step of 72° C for 7 min. The PCR products amplified from DNA of channel catfish and human were evaluated by agarose gel electrophoresis, and purified using a QIAquick PCR purification kit (QiaGen Inc.). Nucleotide sequences were determined using the Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA) and the ABI Prism™ 310 Genetic Analyzer (Perkin Elmer). For comparison, we sequenced fragments of 28S rDNA (also produced using the human-derived primers) from flathead catfish *Pylodictis olivaris* (Rafinesque), yellow bullhead *Ameiurus natalis* (Lesueur), and black madtom *Noturus funebris* (Gilbert & Swain) representing the major genera of the

family Ictaluridae, and hardhead catfish *Arius felis* (L.) from the catfish family Ariidae. Probe DNA used in the hybridization step (see below) was prepared by PCR with the same primers with the exception that the primers were labelled with biotin-16-dUTP.

PREPARATIONS OF CHROMOSOMES

Five adult channel catfish were used in this study. Leukocytes were isolated from peripheral blood and cultured in L15 medium (Gibco BRL, Life Technologies Inc., Gaithersburg, MD) supplemented with 5% fetal bovine serum (Gibco) and 5% channel catfish serum (Zhang & Tiersch, 1995). The cultured cells were mitotically stimulated by addition of phorbol ester (Sigma Chemical Company, St Louis, MO) and calcium ionophore A23187 (Sigma). Metaphase chromosomes were prepared by a standard method used for cultured leukocytes of channel catfish (Wolters *et al.*, 1981).

IDENTIFICATION OF NOR-BEARING CHROMOSOMES BY SILVER OR CMA STAINING

Nucleolus organizer regions were revealed by a one-step AgNOR procedure (Howell & Black, 1980). Slides were covered with a solution of 33% silver nitrate and 0·7% gelatin and incubated for 8–10 min at 50° C. For identification of GC-rich regions associated with NOR, slides were stained with chromomycin A3 (0·1 mg ml^{-1}) in a modified McIlvaine's buffer (Amemiya & Gold, 1987).

FLUORESCENCE *IN SITU* HYBRIDIZATION

Chromosomes prepared on two-well, teflon-coated slides (Cel-Line Associates, Inc., Newfield, NJ) were fixed with 2% paraformaldehyde at room temperature for 1 h. The chromosomes were digested with RNase A (100 μg ml^{-1}) in 2 × SSC buffer at 37° C for 1 h. After dehydration with a series of ethanol concentrations (75, 85, 95, and 100%), chromosomal DNA was denatured in 70% formamide at 37° C for 5 min. The slides were chilled immediately in 70% ethanol at − 20° C, and were dehydrated in ethanol and dried in a laminar-flow hood.

The hybridization mixture was composed of biotin-labelled probe (1·2 ng ml^{-1}), 50% deionized formamide, 2 × SSC buffer, 10 × Denhardt's solution, 0·1% sonicated salmon sperm DNA, and 0·1% sodium dodecyl sulphate. The mixture was heated at 95° C for 10 min, and incubated at 37° C for 1 h. Twenty microlitres of the mixture were applied to each well of slides pre-warmed to 37° C, and the coverslips were sealed using clear nail polish. The slides were incubated in a wet box at 37° C for 24–36 h.

IN SITU POLYMERASE CHAIN REACTION

A detailed procedure for detection of genes of channel catfish by ISPCR was reported previously (Zhang *et al.*, 1997). Reaction mixtures were prepared in the same manner as for liquid-phase PCR including use of biotin-labelled primers. Twenty microlitres of the mixture were applied to each well containing nuclei and chromosomes. The slides were subjected to 15 cycles on a thermal cycler (MJ Research, Inc., Watertown, MA) with the same parameter settings as for liquid-phase PCR (above).

DETECTION OF HYBRIDIZATION SIGNAL

Slides were rinsed sequentially with 50% formamide in 2 × SSC (v/v) for 2 min, 2 × SSC twice for 5 min at 37° C, and 0.2% Tween 20 in 4 × SSC for 5 min at room temperature. The slides were blocked with solution PNM (0·1 M phosphate buffer, 0·1% Nonidet P-40, 5% nonfat dry milk, and 0·02% sodium azide) for 10 min. Slides were stained with avidin-fluorescein isothiocyanate (FITC) (Boehringer Mannheim Co.; 1 : 200 in solution PNM) for 1 h at 37° C. Slides were rinsed three times with solution PN (0·1 M phosphate buffer and 0·1% nonidet P-40) for 5 min each. Slides were stained with biotinylated anti-avidin (Vector Laboratories, Burlingame, CA, 1 : 100 in solution PNM) at 37° C for 1 h. Slides were rinsed and blocked using the procedure described above, stained again with avidin-FITC, and rinsed with solution PN. Slides were counterstained with propidium iodide (PI) (0·5 μg ml^{-1}) prepared in an antifading medium (100 mg

p-phenylenediamine in 100 ml glycerol, pH 11). Fluorescent images of chromosomes were recorded with light microscopy procedures described below.

To examine the relationship between the hybridization signals and NOR, slides were rinsed gently with 2 × SSC, and left in the buffer for 20 min. The slides were dehydrated through a concentration series (70, 80, 95 and 100%) of ethanol, and stained with silver nitrate as described above.

LIGHT MICROSCOPY

For silver staining, chromosomal images were analysed by a computer-based image analysis technique (Zhang & Tiersch, 1998). Fluorescent images of chromosomes stained by CMA, or FITC and PI, were examined under a fluorescence microscope (Microphot-SA, Nikon Inc.) equipped with filters for FITC and chromomycin A3 (excitation wavelengths of 480 nm), and propidium iodide (excitation wavelength of 535 nm). Fluorescent images of nuclei and chromosomes were photographed using Kodak Ektachrome (400 ASA) colour slide film.

RESULTS AND DISCUSSION

AMPLIFICATION OF 28S rDNA SEQUENCE OF CATFISHES WITH HUMAN-DERIVED PRIMERS

The primers were designed to anneal at conserved regions of the gene and to amplify a variable region. The target fragment of the 28S rRNA gene comprised ~288 base pairs (bp) in four species of catfishes of the family Ictaluridae (which contains the channel catfish), and was about 20 bp shorter than the corresponding region of the human 28S rDNA (Gonzalez *et al.*, 1985) (Fig. 1). The fragment comprised 296 bp in a single catfish species of the family Ariidae, and was 11 bp shorter than that of human (Fig. 1). The variations occurred mostly in the central region of the catfish fragments, corresponding to the region between bases 1674 and 1700 of the human 28S rDNA (Fig. 1).

Large variation in length and nucleotide (nt) sequence are characteristic of 28S rRNAs among or within various species (Gonzalez *et al.*, 1985; Naito *et al.*, 1992). Nucleotide dissimilarity in the variable region averaged 8·6% among different species of *Xenopus* (Ajuh & Maden, 1990) and 3·7% among individual humans (Gonzalez *et al.*, 1985). In this study, we found dissimilarity in the variable region was ~14% among catfishes and human, while among species of the Ictaluridae the dissimilarity was 0·8–2·4%. These observations support our contention that primers designed from the nt sequence of human 28S rDNA were able to recognize and allow amplification of a corresponding gene in five species representing two families of catfishes.

CMA STAINING REVEALED GC-RICH REGIONS OF NOR

Silver staining, which detects proteins associated with transcriptional activity, revealed NORs on telomeric regions of the short arms of one pair of medium-sized submetacentric chromosomes [Fig. 2(a)]. Chromomycin A3 (CMA), which stains GC-rich regions of the NOR, revealed a minor NOR site located between the centromere and the end of the long arm of a subtelocentric chromosome [Fig. 2(b)]. Major NOR sites detectable by silver staining possessed a higher proportion of GC-rich DNA elements, and staining of these loci were ~10 times more intense than that of the minor loci.

Human	agcggtcctg	acgtgcaaat	cggtcgtccg	acctgggtat	aggggcgaaa	ga-ctaatcg	aaccatctag	70
Channel catfish	agcggtcctg	acgtgcaaat	cggtcgtccg	acctgggtat	aggggcgaaa	gagctaatcg	aaccatctag	
Flathead catfish	agcggtcctg	acgtgcaaat	cggtcgtccg	acctgggtat	aggggcgaaa	gagctaatcg	aaccatctag	
Yellow bullhead	agcggtcctg	acgtgcaaat	cggtcgtccg	acctgggtat	aggggcgaaa	gagctaatcg	aaccatctag	
Black madtom	agcggtcctg	acgtgcaaat	cggtcgtccg	acctgggtat	aggggcgaaa	gagctaatcg	aaccatctag	
Hardhead catfish	agcggtcctg	acgtgcaaat	cggtcgtccg	acctgggtat	aggggcgaaa	gagctaatcg	aaccatctag	
Ambiguity	....	....	....	....	....	..*.......	....	
Human	tagctggttc	cctccgaagt	ttccctcagg	a-tagctggc	gctctcgcag	acccgacgca	ccccgccac	140
Channel catfish	tagctggttc	cctccgaagt	ttccctcagg	antagctggc	actc----gt	acc-------	---------	
Flathead catfish	tagctggttc	cctccgaagt	ttccctcagg	antagctggc	actc----ag	acc-------	---------	
Yellow bullhead	tagctggttc	cctccgaagt	ttccctcagg	antagctggc	actc------	-------gga	c--------c	
Black madtom	tagctggttc	cctccgaagt	ttccctcagg	antagctggc	actc------	-------gga	c--------c	
Hardhead catfish	tagctggttc	cctccgaagt	ttccctcagg	antagctggc	gctc--g-ag	a----a---a	-----gcttt	
Ambiguity	....	....	....	.*........	*...******	....	....	
Human	gcagttttat	ccggtaaagc	gaatgattag	aggtcttggg	gccgaaacga	-tctcaacct	attctcaaac	210
Channel catfish	tcagttttat	ccggtaaagc	taatgactag	aggcattggg	gccgaaacga	ntctcaacct	attctcaaac	
Flathead catfish	tcagttttat	ccggtaaagc	taatgactag	aggcattggg	gccgaaacga	-tctcaacct	attctcaaac	
Yellow bullhead	tcagttttat	ccggtaaagc	taatgactag	aggcattggg	gccgaaacga	gtctcaacct	attctcaaac	
Black madtom	tcagttttat	ccggtaaagc	taatgactag	aggcattggg	gccgaaacga	gtctcaacct	attctcaaac	
Hardhead catfish	gcagttttat	ccggtaaagc	taatgactag	aggccttggg	gccgaaacga	gtctcaacct	attctcaaac	
Ambiguity	*.........	....	*.....*...	...**.....	....	*..........	....	
Human	tttaaatggg	taagaagccc	ggctcgctgg	cgtggagccg	ggcgtggaat	gc-gagtgcc		270
Channel catfish	tttaaatggg	taagaagccc	ggctcgctgg	cttggagccg	ggcatggaat	ga-gagtgcc		
Flathead catfish	tttaaatggg	taagaagccc	ggctcgctgg	cttggagccg	ggcatggaat	ga-gagtgcc		
Yellow bullhead	tttaaatggg	atagaagccc	ggctcgctgg	cttggagccg	ggcatggaat	gaagattgcc		
Black madtom	tttaaatggg	aaagaagccc	ggctcgctgg	cttggagccg	ggcatggaat	ga-gattgcc		
Hardhead catfish	tttaaatggg	aaagaagccc	ggctcgctgg	cttggagccg	ggcatggaat	gcgagccgcc		
Ambiguity	....	**........	....	.*........	...*......	.*****....		

FIG. 1. Comparison of partial nucleotide sequences of 28S ribosomal RNA gene (28S rDNA) from human and five catfish species. Nucleotide sequences of a fragment of the 28S rDNA isolated from human, channel catfish (Genebank accession no. AF056008) flathead catfish (accession no. AF056009), yellow bullhead (accession no. AF056010), black madtom (accession no. AF056011), and hardhead catfish (accession no. AF056012). These sequences did not include the regions of the upstream and downstream primers. Dots represent matched nucleotide positions, stars represent unmatched nucleotide positions, and dashed lines represent gaps among positions.

The NOR were usually found to be associated with heterochromatin in chromosomes of cyprinid (Amemiya & Gold, 1988) and salmonid fishes (Pendas *et al.*, 1993). In previous studies (Zhang & Tiersch, 1998) the channel catfish had a low abundance of heterochromatin, restricted to the centromeric regions of chromosomes. In the present study, neither of the NOR loci were associated with regions of centromeric heterochromatin. The results from these chromosome banding techniques indicate that there are two types of NOR in channel catfish, major and minor loci, and that the major loci are transcriptionally active and the minor loci are not.

LOCALIZATION OF THE 28S rRNA GENE IN THE CHANNEL CATFISH GENOME

Two types of loci of 28S rDNA were detected by FISH [Fig. 3(a)] and *in situ* PCR [Fig. 3(b)]. The major loci were located on the short arm of a submetacentric chromosome in all spreads examined, and had a staining area 10–20 times larger than that of the minor loci. The minor loci were resolved

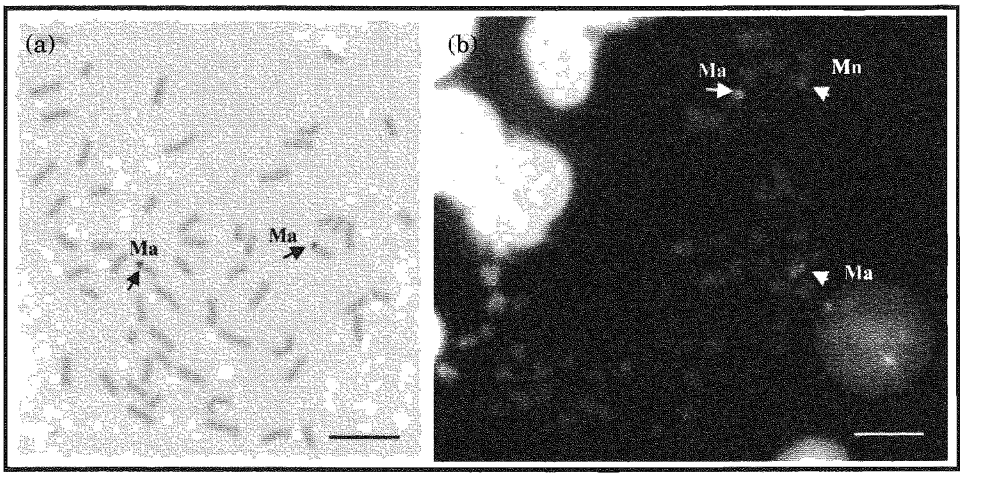

FIG. 2. Analysis of the sites of nucleolus organizer region in channel catfish by (a) silver staining, and (b) chromomycin A3 staining. Arrowheads indicate location of NOR. Ma, Major locus; Mn, minor locus. Scale bars = 10 μm.

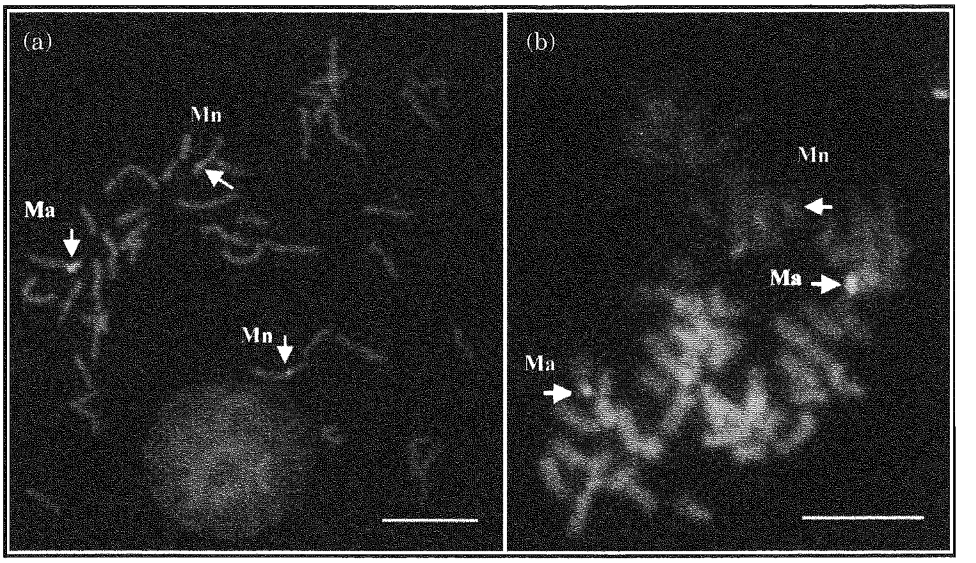

FIG. 3. Localization of the 28S ribosomal RNA genes by fluorescence *in situ* hybridization (a) and *in situ* polymerase chain reaction (b). Arrowheads indicate location of 28S rDNA. Ma, Major locus; Mn, minor locus. Scale bars = 10 μm.

clearly as a spot between the centromere and the end of the long arm of a subtelocentric chromosome [Fig. 3(a)]. This chromosome had a relative length of 4·4% and centromeric index of 23%, which identified it as a chromosome in group B based on a previously established karyogram (Zhang *et al.*, 1998*b*).

Neither type of loci was associated with centromeric regions, although the results of CMA staining indicated that these loci contained GC-rich DNA elements. Highly repetitive DNA elements are distributed within intergenic

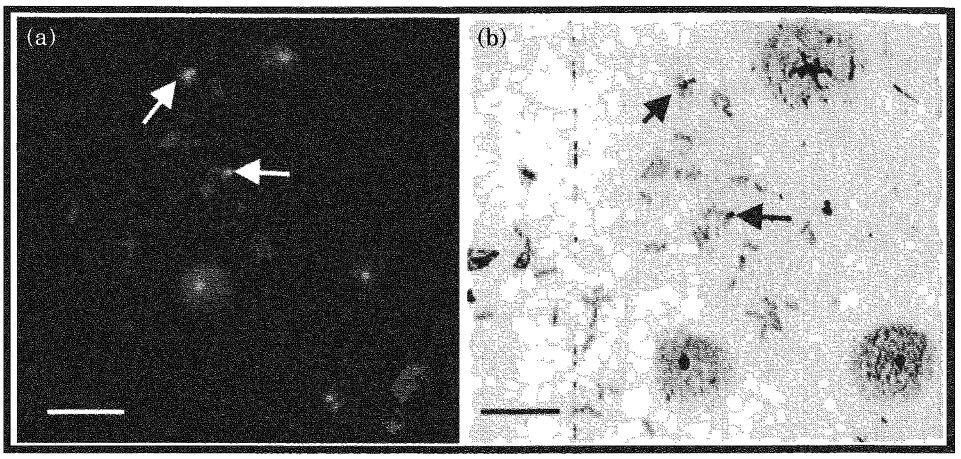

FIG. 4. Relationship between the 28S rRNA gene loci and the nucleolus organizer region (NOR) of channel catfish. The same chromosome spread was subjected to *in situ* polymerase chain reaction (a), followed by staining with silver nitrate (b). Arrowheads indicate location of 28S rDNA and NOR. Scale bars = 10 μm.

spacers of repeated units in salmonid fishes such as lake trout *Salvelinus namaycush* (Walbaum) resulting in a multiplicity of ribosomal RNA genes and polymorphism of NOR phenotypes (Zhuo *et al.*, 1995). This relationship could result from unequal chromosomal rearrangement occurring on heterochromatic arms (Amemiya & Gold, 1988; Pendas *et al.*, 1993), a condition likely for autotetraploid species such as salmonids. On the other hand, channel catfish have a low abundance of heterochromatin (Zhang & Tiersch, 1998) and a small, stable genome size (Tiersch *et al.*, 1990; Tiersch & Goudie, 1993). These results demonstrate that the major and minor NORs identified by the chromosome staining techniques, were also identified by the ISPCR and FISH techniques. This indicates that the 28S rDNA was being mapped to the specific chromosomal location of the NOR.

THE MAJOR 28S rDNA LOCUS WAS DIRECTLY ASSOCIATED WITH ACTIVE NOR

The major 28S rDNA loci [Fig. 4(a)] revealed by ISPCR were associated with the active NOR loci detected by the AgNOR techniques on the same chromosomes after ISPCR [Fig. 4(b)]. The size of the hybridization signal was equivalent to that of the silver staining, indicating that this locus of rDNA was actively involved in rRNA synthesis. It should be noted that the particular chromosome (identified as D-11) and intensity of silver staining observed after ISPCR were not different from silver staining of spreads not used for ISPCR (this was not a false positive caused by the PCR reaction). This experiment was performed to verify directly that the same chromosome was identified by separate techniques as a major locus of the 28S rDNA.

COMPARISON OF RESULTS DERIVED FROM DIFFERENT TECHNIQUES

Although the major 28S rDNA loci were detectable by the AgNOR, CMA, FISH and ISPCR techniques, the minor loci were not detectable by AgNOR,

TABLE I. Localization of nucleolus organizer region (NOR) and associated 28S ribosomal RNA gene by different techniques*

Locus	Features	AgNOR	CMA	FISH	ISPCR
Major†	Occurrence	20 of 20 spreads	20 of 20 spreads	16 of 16 spreads	18 of 18 spreads
	Signal intensity§	+++	+++	+++	+++
Minor‡	Occurrence	0 of 20 spreads	6 of 20 spreads	12 of 16 spreads	16 of 18 spreads
	Signal intensity	None	+	+	++

*AgNOR, Staining by silver nitrate; CMA, staining by chromomycin A3 (GC-rich region); FISH, fluorescence *in situ* hybridization, and ISPCR, *in situ* polymerase chain reaction.

†Transcriptionally active.

‡Transcriptionally inactive, or sometimes active.

§+++, Very strong; ++, strong, and +, weak.

and were difficult to visualize with CMA (Table I). However, the copy number of 28S rDNA in the minor locus was sufficient for detection by FISH and the signal was highly resolved. *In situ* PCR increased the signal for 28S rDNA in the minor loci. However, contrast of the chromosomes and hybridization signals was reduced due to degradation during thermal cycling, which was reported by Zhang *et al.* (1997). Therefore, ISPCR techniques did not show an advantage over FISH for detecting multiple-copy genes such as 28S rDNA.

IMPLICATIONS AND POTENTIAL APPLICATIONS

Primers derived from a human gene were capable of amplifying a corresponding gene from five species of catfishes. These primers were successful as well for *in situ* amplification on catfish chromosomes. Although the 28S rDNA is highly conservative among animal species, conserved regions of other genes exist and the sequence information in these regions is sufficient for design of primers. Thus, physical mapping of channel catfish genes could be performed with sequence information obtained from mammalian species. This offers significant benefits to be derived from comparative studies, and demonstrates a direct application of human research to the study of aquaculture species.

The 28S rDNA, because of its association with the NOR, was informative for verifying specific amplification by ISPCR and provides a useful internal positive control for mapping studies. This is especially useful for economically important species such as channel catfish that are poorly characterized genetically. Given the intrinsic difficulties of cytogenetic analysis in fishes, the association between NOR and 28S rDNA may be the only gene-level marker available to test physical mapping techniques.

It was demonstrated that *in situ* PCR was highly specific for detection of DNA sequences on catfish chromosomes, indicating that ISPCR is a powerful tool for physical mapping in difficult-to-study taxa such as fishes. Traditional breeding studies and linkage mapping are hampered by the 3–4-year generation interval in channel catfish. Physical mapping of genes by ISPCR offers rapid analysis of linkage groups and quantitative trait loci in a single generation.

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