A Microsatellite-Based Genetic Linkage Map for Channel Catfish, *Ictalurus punctatus*

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ABSTRACT

Microsatellite loci were identified in channel catfish gene sequences or random clones from a small insert genomic DNA library. Outbred populations of channel catfish contained an average of eight alleles per locus and an average heterozygosity of 0.70. A genetic linkage map of the channel catfish genome ($N = 29$) was constructed from two reference families. A total of 293 microsatellite loci were polymorphic in one or both families, with an average of 171 informative meioses per locus. Nineteen type I loci, 243 type II loci, and one EST were placed in 32 multipoint linkage groups covering 1958 cM. Nine more type II loci were contained in three two-point linkage groups covering 24.5 cM. Twenty-two type II loci remained unlinked. Multipoint linkage groups ranged in size from 11.9 to 110.5 cM with an average intermarker distance of 8.7 cM. Seven microsatellite loci were closely linked with the sex-determining locus. The microsatellite loci and genetic linkage map will increase the efficiency of selective breeding programs for channel catfish.

THE channel catfish is a freshwater species native to the United States within the Mississippi River valley, states bordering the Gulf of Mexico, and Mexico. Channel catfish stocks have been maintained in state and federal fish hatcheries in the southeastern and midwestern states for several decades to support sport fishing. Research in the 1950s and 1960s led to the development of large-scale channel catfish farming in the southeastern United States (Tucker and Robinson 1990). Production of channel catfish for human consumption is now the largest sector (46%) of commercial finfish production in the United States (FAO 1997), utilizing 78,000 ha of production ponds (USDA 2000). In 2000, 269 million kg of catfish were processed in the United States, a 33% increase from 1995 and 52% increase from 1991 (USDA 2001). Low feed costs, consistent prices paid to producers by processors, successful marketing, and effective industry infrastructure have resulted in steady growth and sustained profitability of the catfish industry.

Research on reproduction, nutrition, and pond management has also supported industry growth, but genetic improvement programs leading to improved catfish lines are only beginning to be applied. Traditional selective breeding programs will serve as the foundation for utilizing catfish quantitative genetic variation for agricultural production (Bondari 1986; Dunham and Smitherman 1987; Wolters and Johnson 1995). There is great potential for the application of genetic improvement techniques such as selective breeding, chromosome manipulation, hybridization, production of monosex groups, and gene transfer to increase catfish production (Gjedrem 1997). Modern molecular techniques will help establish family relationship and pedigrees, and genome maps will help identify genetic markers for traits amenable to selective breeding in aquaculture (Danzmann *et al.* 1999).

Molecular maps of domestic animal genomes provide powerful tools for selective breeding. Initial DNA marker-based genetic linkage maps for aquacultured species, such as tilapia and rainbow trout (Kocher *et al.* 1998; Young *et al.* 1998), relied primarily on random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP) markers. These markers are anonymous DNA sequences that are dominant markers and may be specific to the mapping population. Both RAPD and AFLP markers developed in channel catfish demonstrate low levels of intraspecific polymorphism (Liu *et al.* 1998a,b). An alternative is a catfish linkage map based on microsatellite loci (Waldbieser and Bosworth 1997).

Microsatellite loci are polymorphic DNA sequences containing short tandem repeats. Microsatellite loci appear to be distributed throughout vertebrate genomes and can demonstrate high levels of intraspecific allelic polymorphism. Unique genomic DNA sequence flanking the repeats can be used to identify and further characterize genomic regions surrounding these loci. Genetic linkage maps based on microsatellite markers have been produced for agriculturally important mam-

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malian and avian species such as cattle, swine, sheep, and chickens (Rohrer *et al.* 1996; Kappes *et al.* 1997; de Gortari *et al.* 1998; Groenen *et al.* 2000). Microsatellite-based linkage maps have been produced for rainbow trout (Sakamoto *et al.* 2000) and zebrafish (Gates *et al.* 1999; Shimoda *et al.* 1999), and microsatellite loci were included in the tilapia linkage map (Kocher *et al.* 1998). The current research was designed to produce a microsatellite linkage map of the catfish genome useful for selective breeding in channel catfish populations.

MATERIALS AND METHODS

Reference families: Two linkage map reference families were crosses between Norris and USDA103 line catfish (origins described in Li *et al.* 1998). Two hundred full-sib offspring from each spawn were maintained in a hatchery until sampling. At 11 months of age, a passive integrated transponder tag was implanted in the dorsal musculature and a blood sample was collected with 1% anticoagulant (0.34 m dipotassium EDTA). Genomic DNA was isolated from 50 μl blood using DNAzol (Molecular Research Center, Cincinnati) and the remaining blood sample was stored at −80°. At 17 months of age, a second blood sample was collected and stored as above, and the sex of the fish was determined by external examination of genitalia.

Microsatellite locus identification: Type II (noncoding) microsatellite loci were isolated from a small insert genomic DNA library. Genomic DNA was digested with *Sau3*AI or *Tsp509*I and 300- to 1500-bp fragments were excised from agarose gels and ligated into pUC19 or pBluescript SK− using standard techniques (Ausubel *et al.* 1995). Competent *Escherichia coli* (DH5α) were transformed with 50 ng of each small insert genomic library and grown on LB agar, and colonies were transferred to nylon membranes (MSI, Westboro, MA). Membranes were screened with alkaline phosphatase-labeled oligonucleotide probes containing repeats of AAT, AAC, AAG, GATA, CAC, AAAT, AAAG, or AAAC (Lifecodes Corporation, Stamford, CT). Filters were hybridized according to the manufacturer's instructions, except the AAT probe was stored in the hybridization solution at 4°, added immediately to the filter, and brought to 37° in a rolling bottle hybridization incubator (P. Cregan, personal communication). Positive hybridization was detected by chemiluminescent exposure of X-ray film.

Plasmid DNA was isolated from positive clones using a Qiaprep spin miniprep kit (QIAGEN, Valencia, CA) and sequenced using a Thermosequenase cycle sequencing kit with Cy5-dye-labeled M13 primers (Amersham Pharmacia Biotech, Arlington Heights, IL). Sequencing products were resolved by denaturing polyacrylamide electrophoresis on an ALFexpress DNA analysis system (Amersham Pharmacia Biotech) and DNA sequence was determined using ALFWin software (Amersham Pharmacia Biotech). The DNA sequences were collected and duplicate clones were identified using FASTA software (Pearson and Lipman 1988). Each sequence was compared with all sequences available in GenBank using the Blast 2.0 program. Matches were considered significant when the smallest sum probability (*P*) was <0.0001 (Altschul *et al.* 1990).

Microsatellite repeats within type I (coding) loci were identified in DNA sequences within GenBank or provided by cooperating scientists. Sequences from type I and type II loci were also compared to a local database containing sequences from channel catfish brain and intestine cDNA libraries using Blast 2.0. Primers flanking microsatellite repeat regions were designed using GeneRunner software (Hastings Software, Hastings-on-Hudson, NY) and synthesized (Research Genetics, Huntsville, AL).

Genotyping: The primer sets were tested on genomic DNA from four unrelated catfish to determine positive PCR amplification using standard conditions. The 15-μl reactions contained 10 mm Tris-HCl (pH 8.3), 50 mm KCl, 1 mm $MgCl_2$, 67 μm dNTPs, 6 pmoles each primer, 0.5 units Taq polymerase (Roche Molecular Biochemicals, Indianapolis), and 40 ng genomic DNA. The reaction profile was 95° for 3 min; 2 cycles of 95° for 1 min and 50° for 30 sec; then 23 cycles of 95° for 30 sec and 50° for 30 sec; and then incubation at 72° for 4 min in a PTC-100 or PTC-200 thermal cycler (MJ Research, Watertown, MA). Reaction products were resolved on a 3% agarose gel stained with ethidium bromide. Reactions that failed to produce an amplification product at 50° annealing temperature were repeated at 45° annealing temperature. One of the primers was resynthesized with a 5′ Cy5 fluorescent label (Research Genetics) for primer sets that provided amplification products.

Polymorphism and levels of allelic heterozygosity for the cloned loci were determined from fluorescent PCRs performed on random-bred channel catfish and analyzed on the ALFexpress DNA analysis system. One or two microliters of PCR product was mixed with size standards and loading buffer (100 mm bromophenol blue in deionized formamide) so that formamide was 40% of the final volume. The mixture was heated for 3 min at 90° and loaded onto 5% Long Ranger (BioWhittaker Molecular Applications, Rockland, ME) polyacrylamide gels containing 6 m urea and 1× TBE buffer. The products were electrophoresed in 1× TBE running buffer at 1500 V, 60 mA, 25 W, 45° with sampling interval of 1 sec. Gels were reloaded for a maximum of six runs. Allele sizes were determined using AlleleLinks software (Amersham Pharmacia Biotech) with flanking size standards that were 100 bp apart. The genotype data were imported into a relational database (Microsoft Access), and heterozygosity and polymorphism information content values were calculated using Cervus v.1.0B (Marshall *et al.* 1998).

Linkage analysis: Parents and 72 offspring from each reference family were genotyped as above. Allelic data from the two mapping families was converted to CRI-MAP format using macros within Microsoft Access and Microsoft Word. Linkage was determined using CRI-MAP 2.4 (Green *et al.* 1990). Two-point linkage was determined at LOD = 3.0. Multipoint linkage analysis was performed using the ALL and FLIPS options to determine the highest likelihood order of linked markers, which were added in decreasing order of informative meioses. The CHROMPIC option was used to identify unlikely double crossovers, and genotypes producing double crossovers were reamplified and reanalyzed on the ALFexpress to detect potential errors. Spurious linkages were identified and eliminated by comparing the maximum log-likelihood of a marker to its associated linkage group to the log-likelihood at a recombination frequency of 0.5 (Bishop *et al.* 1994; Kappes *et al.* 1997; J. W. Keele, personal communication). Two-point recombination rates were compared between females and males for paired loci in the first 21 linkage groups that were informative in all four parents.

RESULTS

Trinucleotide and tetranucleotide microsatellite loci were cloned from the channel catfish genome and tested for allelic polymorphism in a population of outbred catfish. Oligonucleotide primers were designed for

TABLE 1

Microsatellite loci obtained from genomic DNA library

Polymorphic microsatellite loci in outbred catfish:	313
Average no. alleles per locus:	8
Average heterozygosity for all loci:	0.712
Average heterozygosity for loci with >2 alleles:	0.743
% Loci with HET >0.8:	46.7
% Loci with HET >0.7:	64.5
% Loci with HET >0.6:	75.4

HET, heterozygosity.

445 unique loci, and 394 loci produced a PCR product adequate to warrant synthesis of a fluorescent primer. Automated genotype analysis on polyacrylamide gels revealed that 313 loci (70% of all clones) were polymorphic in random-bred channel catfish (Table 1 and supplemental data at http://www.genetics.org/supplemental/). Polymorphism of microsatellite loci in the channel catfish genome was prevalent, and average heterozygosity over all loci in outbred catfish was 0.70. The loci with AAN and GATA tandem repeats demonstrated the highest levels of allelic polymorphism. Southern blot analysis had revealed abundant CAC repeats in catfish genomic DNA (Bosworth and Wolters 1997); however, most microsatellite clones containing CAC repeats were not polymorphic in the outbred catfish (data not shown).

The map reference families were polymorphic for 11 type I loci containing tandem repeats within existing DNA sequence data (Table 2) and 282 type II loci from the genomic libraries. Six of the type II loci contained DNA sequence similarity to existing genes, and 3 loci contained DNA sequence identical to catfish brain cDNA clones (Table 2). Five type II loci (IpCG0034, IpCG0154, IpCG0188, IpCG0275, and IpCG0296) were associated with short interspersed elements (SINEs) previously identified in the 3′ nontranslated regions of a catfish leukocyte-specific leucine zipper protein and the catfish α-actin gene (Xue *et al.* 1999; Kim *et al.* 2000).

Microsatellite allele inheritance in two reference families was used to construct a genetic linkage map (Figure 1). A total of 262 loci were placed into 32 multipoint linkage groups at LOD = 3.0 or greater, with an average of 171 informative meioses per locus. The linkage groups ranged in length from 11.9 to 110.5 cM. The 37 markers that mapped to the same location (zero recombinant offspring) were verified to be from different clones, except for IpCG0023 and IpCG0028 (linkage group U3), which were separate tandem repeats derived from the same clone. There were 193 recombinant intervals in 32 linkage groups; 44% were 0.3–4.9 cM, 18% were 5.1–9.9 cM, 20% were 10.2–20.0 cM, and 18% were 20.3–39.8 cM (8.7 cM average). Nine markers were placed into three two-point linkage groups and 22 loci remained unlinked. Seventeen known genes and three expressed sequences were placed on the current map. Seven markers displayed zero or <1% recombination with the putative sex-determining locus (linkage group U6). None of the 5 markers known to be associated with SINEs were on the same linkage group. The average pairwise female:male recombination ratio was 3.18, ranging from 0.07 to 23.5. There was no apparent difference in sex-specific recombination between the two families.

DISCUSSION

The current linkage map contains 32 linkage groups covering 1958 cM (sex averaged) of the channel catfish genome. A further 24.5 cM is contained in three two-point linkage groups. Addition of 252 cM to include telomeric regions (8.7 cM average intermarker distance × 29 linkage groups) increases the estimated total genome size to 2234.5 cM. Catfish erythrocytes contain 2 pg DNA per cell; thus the haploid genome contains $\sim 1 \times 10^9$ bp (Tiersch *et al.* 1990; Tiersch and Goudie 1993), which is equal to 447 kbp per centimorgan. Additional markers are required to condense the existing map into 29 linkage groups corresponding to the number of chromosome pairs in channel catfish (Wolters *et al.* 1981a), and high levels of microsatellite polymorphism in catfish populations support addition of new markers. Genotype analysis of more families will improve map accuracy and increase the probability of detecting marker polymorphism.

Several type I loci were placed on the current genetic map by identification of microsatellite sequences within catfish gene sequences or by sequence similarity of type II loci with orthologous genes. The abundance of microsatellite loci in catfish-expressed sequences (Liu *et al.* 1999; our observations) will aid researchers in the placement of more type I loci on this linkage map. Improvement of the catfish map with type I loci will be necessary for comparative mapping with species for which high density maps exist (Postlethwait *et al.* 1998, 2000; Woods *et al.* 2000).

The sex-specific map distances revealed overall recombination was 3.18-fold more frequent during oogenesis than spermatogenesis in channel catfish, although three linkage groups displayed higher recombination rates in males. Sex-specific differences in recombination have also been observed in other species. The average sex-specific recombination rates in catfish were similar to that of rainbow trout (3.25:1; Sakamoto *et al.* 2000) but higher than the average ratio in humans (1.6:1; Broman *et al.* 1998). Higher female recombination rates near centromeres and higher male recombination rates near telomeres were observed in both humans and rainbow trout, but the current lack of a physical map precluded this analysis in channel catfish.

Seven microsatellite loci were closely linked to the

TABLE 2

Type I and EST loci containing microsatellite polymorphism in channel catfish populations

Locus	Gene	Repeat	Inf.[a] mei.	Primers (5′-3′)	Reference[b]
Acta1	α-Actin	aat	216	ACCATCACCAGAGTCCAGGA GTCCCAGCCATGTATGTG	IpCG0001 (AF228714)
B0102D12	EST from catfish brain cDNA library	gata	144	CCGTTGCCTCAGGATGGAAA ATCAGATGCAGACCAGATCATAG	IpCG0037
B0105H01	EST from catfish brain cDNA library	aaat	211	CACATGGGTGATAAGTGTTGGG GGTGCTATGATAGATGCTCACG	IpCG0013 (Z34530)
B0108B10	EST from catfish brain cDNA library	gaa	288	ACGAATCACAATGCACACCGAA TCTGACCCCAACTTCCTAACA	IpCG0237
B2m	β 2-Microglobulin	at	272	ACTAACGAGGTAATGGATAGAAATG AAAAAGTGGATGACACATAGGG	Criscitiello *et al.* (1998)
CT-53	Leukocyte-specific basic protein	aat	144	GATACGTGTAGGTGCGTGTA CTCCAAGCAGATATTTTTATTA	Chen (1999)
Esr	Estrogen receptor	aat	189	TTGTCATCGTGACCGTGTAC TACGGAGCACAGAACATCAC	Xia *et al.* (1999)
GH	Growth hormone	aat	196	AGATTTGACGTCCTGTGTGTAA TAAATCTCAGCCGTAACCGC	Tang *et al.* (1993)
Hox six6	Homeobox six6	aaat	0	ATTCTAGAGTCGGACCTTACGC AGTCGTTTATGGATTGTGCAGAA	IpCG0098 (AF030281)
IgH	Immunoglobin heavy chain	ca	136	TAGTGATGAGTTCGTATTCTGC AGATTCCAAACCATGTACTGAG	Ghaffari and Lobb (1989)
Icpu-UAA	MHC class 1	ca	215	ATTGCTGTAAAATGGGATTATTAGA TTCAGGAAGTGTGCACGAGG	Antao *et al.* (1999)
Icpu-DAA	MHC class 2α	gt	284	AGACGCTTGACACCAGGACA GACACACACTGGGGAGATCA	Godwin *et al.* (2000)
Icpu-DAB	MHC class 2β	aac/cac	278	CTGTGATTACCTGCTAGATACC TGTGTGAGAGTTCATCTAGGTG	Godwin *et al.* (1997) S. Quiniou (personal comm.)
Isl-2	Insulin gene enhancer protein	aat	0	AGTCTGCTTTGCTCTCGCC GCAGCACAACTACGCAAGATG	IpCG0086 (AK001022)
Nccr-1	Nonspecific cytotoxic cell receptor protein-1	aaac	136	ATAATTTGCACAGTGGACATGG GACCAACACCTTAACCACTG	L. Jaso-Friedmann (personal comm.) (AF159718)
Nramp	Natural resistance associated macrophage protein	ac	142	CCTTCTACAATAAAACCAACATGG CACGTTTAAAAGTTAAAAGGTGCAC	Chen (1999)
Or	Odorant receptor gene cluster	aaat	0	TAATGCCCCAGTATAGTGACAA TCTAGAGTCGGGGGTATACC	IpCG0140 (AF112374)
Otf2	Pou domain, class 2, transcription factor 2	ct	129	AGGTTTAGTGGCACGCGGTC GATCATTAATCAGCTCTTTCCTC	Ross *et al.* (1998)
Pp2a0	Protein phosphatase 2A0 B′ regulatory subunit	aaat	216	AAGTCGAAGAGCACACAGCAC ATCAAAGGCACAGAAGACCATG	IpCG0117 (U38192)
RarB2	Retinoic acid receptor β	cct	94	GAGCGACGGTGCTGTTCAAA AATCACTTACATTACATCTGTTGC	IpCG0206 (X56573)

[a] Informative meioses.
[b] GenBank accession number is in parentheses.

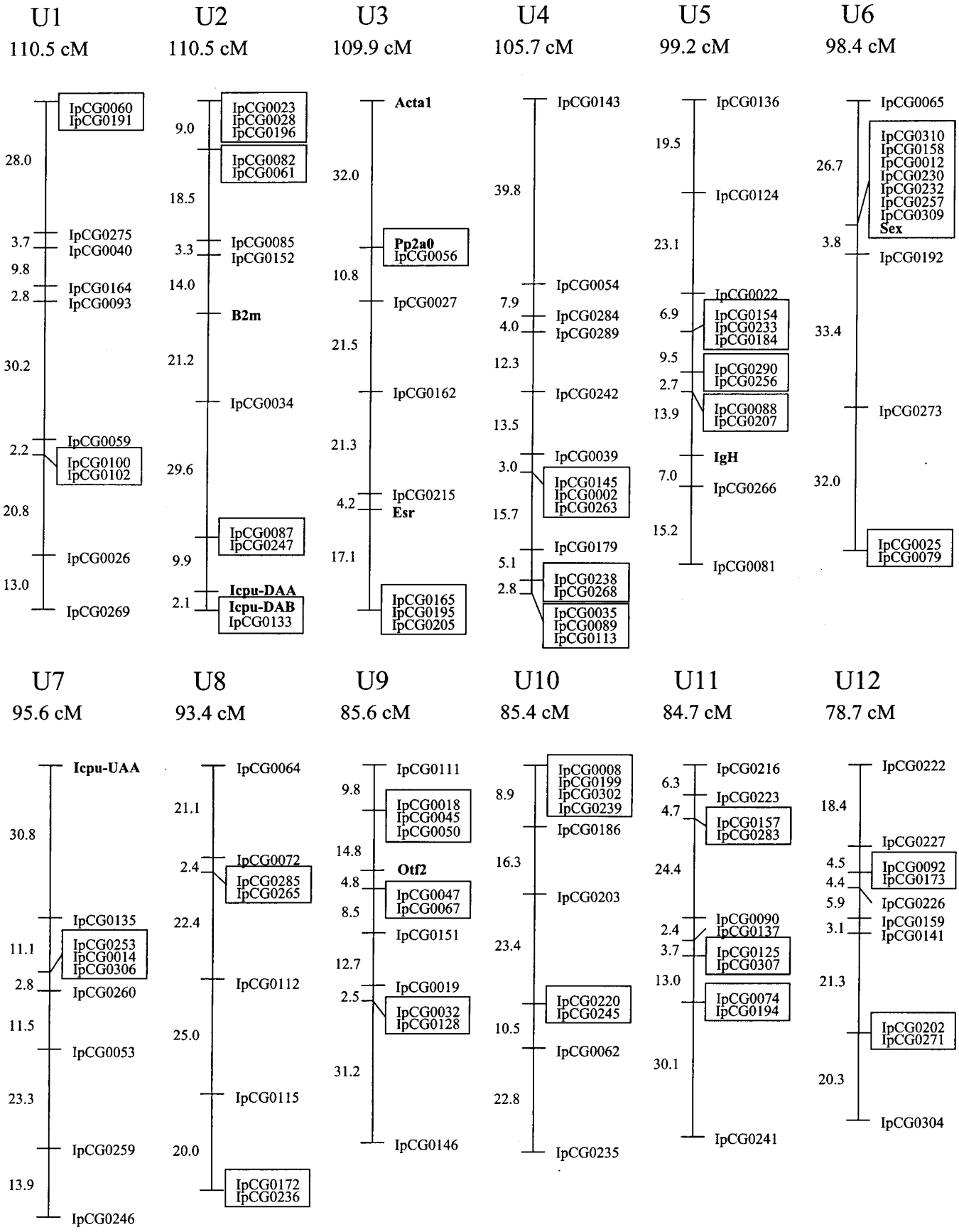

FIGURE 1.—Linkage map of channel catfish genome. Multipoint linkage groups are designated with length of linkage group in centimorgans (cM). Genetic distance (in centimorgans) between markers is shown on left side of linkage group. Markers enclosed by a box are linked within 2 cM. Type I and EST loci are designated in boldface type.

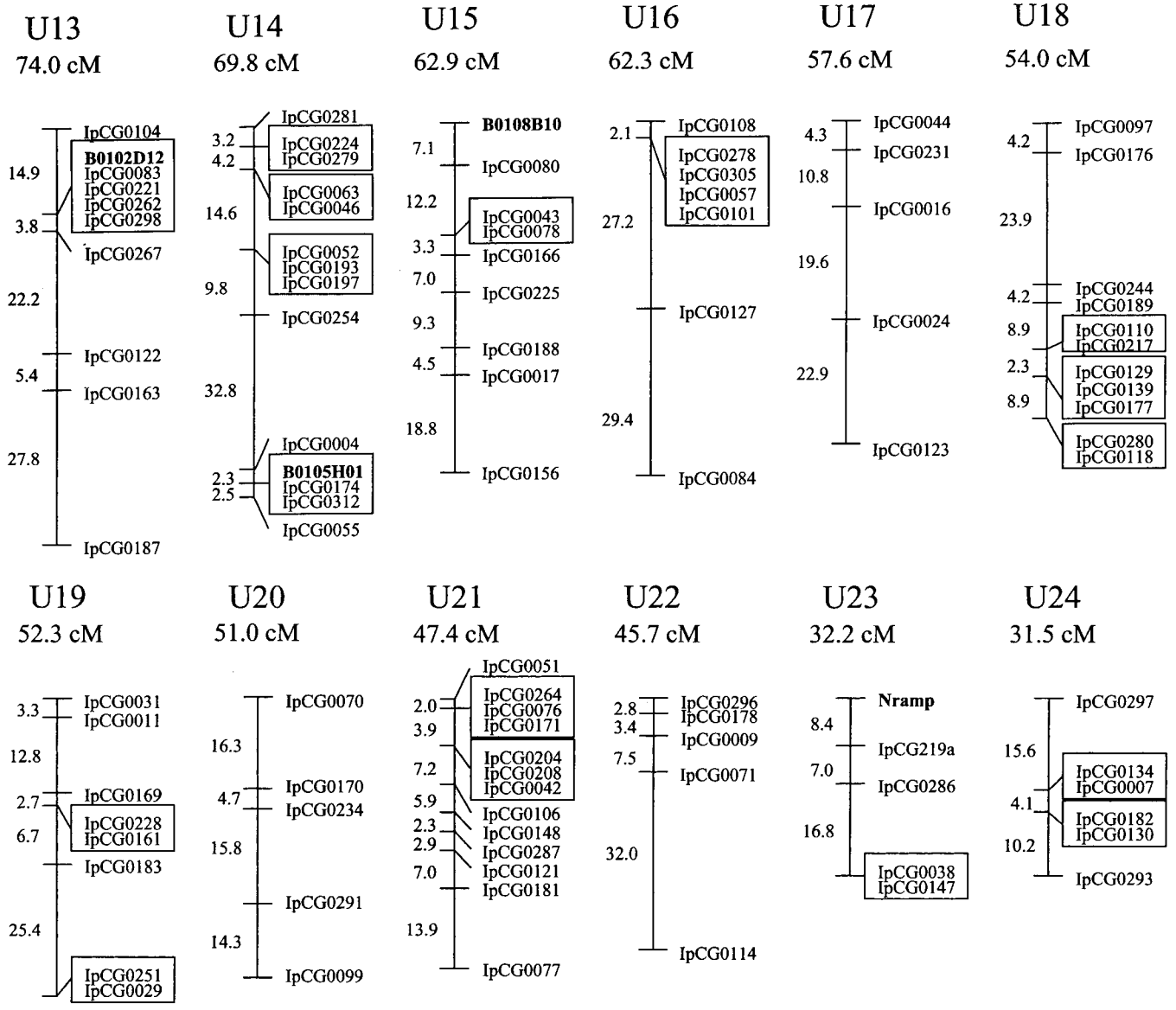

FIGURE 1.—*Continued.*

sex-determining region. Channel catfish exhibit an XY sex-determining system (WOLTERS *et al.* 1981b; DAVIS *et al.* 1990) but do not demonstrate sex chromosome polymorphism. Only glucosephosphate isomerase-B was previously linked to the sex-determining gene via an isozyme polymorphism between channel catfish and blue catfish (LIU *et al.* 1996). Genomic DNA hybridization-based methods did not identify linkage of Zfy, Sry, human telomeric sequence (TTAGGG), or the bkm minisatellite sequences with sex (TIERSCH *et al.* 1992). These seven microsatellite loci can be used as a starting point for fine mapping and identification of the sex-determining locus. To take advantage of improved male growth rates (SIMCO *et al.* 1989), monosex cultures of channel catfish could be more efficiently produced by feminization and rapid identification of XX, XY, and YY fish with a molecular marker (DAVIS *et al.* 1990, 1995).

The genetic linkage map will be useful for marker-assisted selection in channel catfish. High levels of allelic polymorphism and phenotypic variation in research and commercial catfish populations (WALDBIESER and BOSWORTH 1997) will benefit marker-assisted selective breeding programs. Seventy percent of the polymorphic microsatellite markers contained a polymorphic information content of 0.6 or greater, so it is likely a high percentage of new markers placed on the map will be useful for selection programs. Microsatellite polymorphism has already proven useful for fish identification and determination of spawn parentage in channel catfish (WALDBIESER and WOLTERS 1999). High levels of fecundity (tens of thousands of offspring per full-sib family) and multiple sire parentage will assist researchers to estimate genetic components of variance and to identify quantitative trait loci. Marker-assisted introgres-

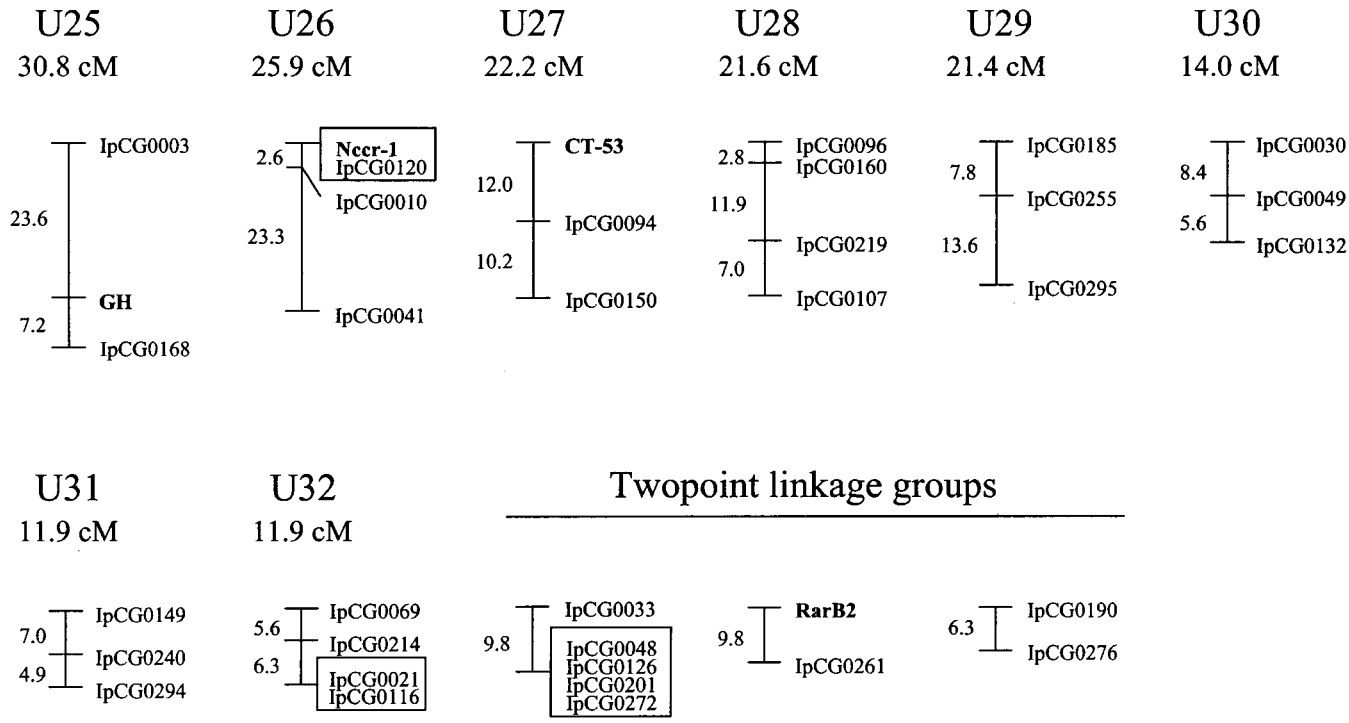

FIGURE 1.—*Continued.*

sion of beneficial alleles from blue catfish (WOLTERS *et al.* 1996; DUNHAM and ARGUE 2000) can also enhance important production traits such as filet yield and innate disease resistance in select strains of channel catfish. These refined breeding technologies can be applied much earlier in the development of genetically improved catfish lines than for select lines of traditional agricultural species.

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