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Original article

Construction and characterization of a BAC library from a gynogenetic channel catfish *Ictalurus punctatus*

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Abstract – A bacterial artificial chromosome (BAC) library was constructed by cloning *Hind*III-digested high molecular weight DNA from a gynogenetic channel catfish, *Ictalurus punctatus*, into the vector pBeloBAC11. Approximately 53 500 clones were arrayed in 384-well plates and stored at −80 °C (CCBL1), while clones from a smaller insert size fraction were stored at −80 °C without arraying (CCBL2). Pulsed-field gel electrophoresis of 100 clones after *Not*I digestion revealed an average insert size of 165 kb for CCBL1 and 113 kb for CCBL2. Further characterization of CCBL1 demonstrated that 10% of the clones did not contain an insert. CCBL1 provides a 7.2-fold coverage of the channel catfish haploid genome. PCR-based screening demonstrated that 68 out of 74 unique loci were present in the library. This represents a 92% chance to find a unique sequence. These libraries will be useful for physical mapping of the channel catfish genome, and identification of genes controlling major traits in this economically important species.

bacterial artificial chromosome / catfish / genome / *Ictalurus punctatus*

1. INTRODUCTION

Bacterial artificial chromosome (BAC) libraries have become a widespread tool for maintaining entire genomes as large DNA insert clones due to stability, low levels of chimeric inserts, and ease of manipulation. BAC libraries can be used for gene mapping, cloning, direct DNA sequencing [27], and physical map

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construction [10,16,22]. Since their inception [20], BAC libraries have been developed for many agriculturally important animal species such as cattle [2, 3,6,29,30], goats [19], sheep [26], swine [7,18,21], and chickens [5,31]. Recently, BAC libraries were produced for several commercially important fish species such as the rainbow trout, common carp, tilapia and flounder [12,13].

Channel catfish culture is currently the largest sector (47%) of farmed fish production in North America [8], with 1200 commercial operations and 79 500 ha of production ponds in the United States. Production doubled from 1988 to 1998, and the USA catfish industry now processes 600 million pounds annually [25]. Genetic improvement programs leading to improved catfish lines are beginning to be applied, and linkage and physical maps of the catfish genome can enhance the efficiency of genetic selection programs. The current version of the channel catfish genetic linkage map contains 243 type II (non-coding) and only 20 type I (coding) markers in 32 linkage groups, with an average inter-marker distance of 8.7 cM [28]. However, a physical map of the catfish genome is not yet available. Therefore we constructed and characterized the first channel catfish BAC library. This library will be used for marker identification to improve genome coverage and to increase the number of type I markers on the linkage map, as well as to build a physical map of the channel catfish genome. Ultimately, these resources will aid in the identification of genes controlling economically important traits in catfish.

2. MATERIALS AND METHODS

2.1. Production of BAC clones

The brain of a third-generation gynogenetic catfish [9] was collected after terminal anesthesia in MS-222 (Sigma Chemical Co., St Louis, MO). Brain cells were separated by grinding the tissue gently between the frosted portions of two sterile microscope slides in 6 mL phosphate buffered saline at 4 °C. The cells were passed through a 70 μm cell strainer (Falcon, BD, Franklin Lakes, NJ), collected by centrifugation at 540 g for 15 min, resuspended in 1.65 mL of Buffer L (100 mM EDTA, 20 mM NaCl, 10 mM Tris-HCl pH 8.0) containing 0.6% low melting agarose (InCert agarose, BMA, Rockland, ME), and poured into 75 μL plug molds (1.5 mm × 10 mm × 5 mm, BioRad Laboratories, Hercules, CA). High molecular weight (HMW) DNA was isolated according to previous methods [12,13,20] and stored in 50 mM EDTA at 4 °C. Digestion with *Hind*III and isolation of high molecular weight DNA fractions from pulsed field gels was performed as previously described [12].

The pBeloBac11 vector ([15], ResGen, Huntsville, AL) was prepared using a Qiagen maxiprep kit (Qiagen, Valencia, CA) and purified on a cesium chloride gradient [1]. Restriction digestion, dephosphorylation, and purification was performed as previously described [12]. Linear vector DNA was stored at 4 °C.

Digested genomic DNA from two size fractions, 100–150 kb and 150–250 kb, was ligated with the pBeloBAC11 vector, and recombinant molecules were electroporated into competent DH10B cells according to Katagiri *et al.* [12]. The cells were spread onto LB plates containing 12.5 $\mu g \cdot mL^{-1}$ chloramphenicol, 90 $\mu g \cdot mL^{-1}$ isopropylthiogalactoside (Gibco BRL, Gaithersburg, MD) and 90 $\mu g \cdot mL^{-1}$ X-gal (Sigma, St Louis, MO). White colonies from the 150–250 kb insert fraction, CCBL1, were picked using a Flexsys Colony Picker (Genomic Solutions, Ann Arbor, MI) into 80 µL LB/12.5 $\mu g \cdot mL^{-1}$ chloramphenicol/7.5% glycerol in 144 384-well plates. Cultures were grown overnight in a HiGro High Density Shaking incubator (Gene Machines, San Carlos, CA). The arrayed library was replicated twice using the Flexsys Colony Picker, and all replicates were stored at −80 °C. Colonies from the 100–150 kb insert fraction, CCBL2, were harvested by washing the plates with LB/12.5 $\mu g \cdot mL^{-1}$ chloramphenicol/7.5% glycerol. The collected liquid culture was aliquoted and stored at −80 °C for subsequent screening by hybridization.

2.2. Characterization of the BAC library

BAC DNA was isolated from 100 colonies randomly chosen from each fraction of the library. DNA was prepared by standard alkaline lysis with a commercial kit (Qiagen) followed by 0.7% isopropanol precipitation. One microgram of DNA was digested with 5 units of *Not*I (Gibco BRL) at 37 °C for 2 h and separated by pulsed field gel electrophoresis (CHEF-MAPPER, Bio-Rad Laboratories) on 1% Seakem LE agarose gels (BMA) in 0.5X TBE using the following parameters: 6 $V \cdot cm^{-1}$, 120° angle, pulse interval ramping from 5 to 15 s, 15 h at 14 °C. Lambda ladder PFG (New England Biolabs, Beverly, MA) was used as a size marker, and DNA fragment sizes were calculated using GelExpert software (NucleoTech Corp., Hayward, CA).

Fourteen randomly picked BAC clones were grown overnight in 5 mL LB/12.5 $\mu g \cdot mL^{-1}$ chloramphenicol. On the next day (day 1), cultures were diluted 10^{-2} and 1 µL was used to inoculate a new overnight culture. This was repeated on day 2, 3, 4, and 5 until 100 generations had passed. BAC DNA from each clone was prepared as above on day 1 and 6. *Not*I and *Hind*III restriction enzyme digestion patterns of BAC clones were compared at day 1 and day 6.

BAC clones from CCBL1 were pooled 2 ways from each plate for screening by PCR. For the plate pools, each clone (in a 384-well plate) was replicated in 80 µL LB/ 12.5 $\mu g \cdot mL^{-1}$ chloramphenicol media and incubated for 20 h in a HiGro High Density shaking incubator at 400 rpm. The clones from each plate were then pooled, giving a total of 144 plate pools. DNA from each plate pool was extracted by standard alkaline lysis and re-suspended in 100 µL TE buffer. Positive plates were identified by PCR screenings using 0.2 µL of the plate

pools as the template. Primers were designed from microsatellite markers [28] and sequenced channel catfish genes. The 15 μL PCR reaction contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1 mM or 2 mM $MgCl_2$ depending on primer pairs, 400 nM of each primer, 67 μM deoxynucleotides, 0.1% Triton® X-100, and 1 unit Taq polymerase (Promega Corporation, Madison, WI). The PCR cycling protocol was 95 °C, 3 min; 40 cycles of 95 °C, 1 min; 55 °C, 30 s for the type II markers or 40 cycles of 95 °C, 30 s; 55 or 60 °C, 30 s; 72 °C, 1 min for the type I markers; and final extension at 72 °C for 4 min. The products were separated on 2% agarose gels and visualized by ethidium bromide staining.

For the Row-Column pools, plates 1–40 were replicated into four 96-deep well plates each containing 600 μL LB/chloramphenicol, grown 16 h, and each pooled into one 96-deep well box. Row pools were prepared from 200 μL/well and column pools from 300 μL/well for each box and DNA were extracted as above. The pools were screened by PCR as above except 35 cycles were used. Clones from positive row/column addresses were grown individually from the original 384-well plate and screened by PCR as above using 30 cycles.

Ten CCBL1 clones that did not contain an insert after restriction digestion analysis were grown overnight in 5 mL LB/chloramphenicol. The BAC DNA was prepared as above with the addition of 100 μL procipitate (LigoChem Inc., Fairfield, NJ) to the neutralization buffer during the alkaline lysis protocol and all steps were performed at RT. Precipitated DNA was air-dried and resuspended in 30 μL of water. The clones were sequenced with T7 and M13 reverse primers using ABI PRISM® BigDye™ Terminator chemistry on an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) in the USDA, ARS, Mid-South Area Genomics Laboratory. Two hundred nanograms of DNA was used and the cycling conditions were as follow: 95 °C, 4 min and 99 cycles of 95 °C, 30 s; 50 °C, 20 s; 60 °C for 4 min.

3. RESULTS AND DISCUSSION

A channel catfish BAC library was produced from gynogenetic fish brain tissue. Potentially reduced DNA sequence variation in gynogenetic catfish will assist the identification of multiple copy genes due to a reduced number of alleles. Also, reduced allelic variation at restriction enzyme recognition sites will assist BAC contig assembly by restriction fingerprinting. The CCBL1 clones were arrayed into 144 384-well plates representing 55 296 BAC clones. On average, 12 wells per plate did not grow (3.1%) leaving approximately 53 500 BAC clones. The average insert size for CCBL1 clones was 165 kb with 98% of the inserts ranging between 140 and 205 kb (Figs. 1 and 2). Approximately 10% of CCBL1 clones were empty. The average insert size for CCBL2 clones was 113 kb with 98% of the inserts ranging between 90

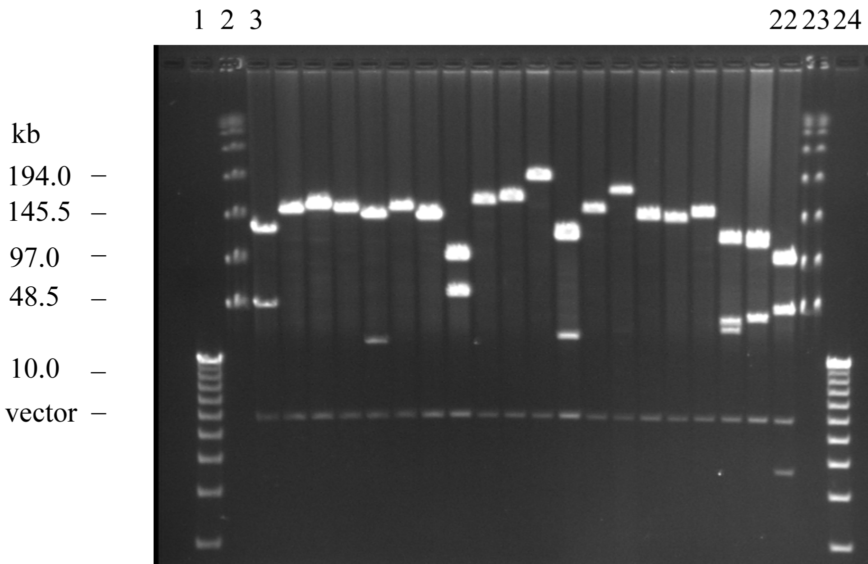

Figure 1. Representative analysis of channel catfish CCBL1 clones by pulsed field gel electrophoresis. Lanes 1 and 24: 1 kb plus markers. Lanes 2 and 23: Bacteriophage lambda concatamers. Lanes 3–22: randomly selected BAC clones digested with *Not*I.

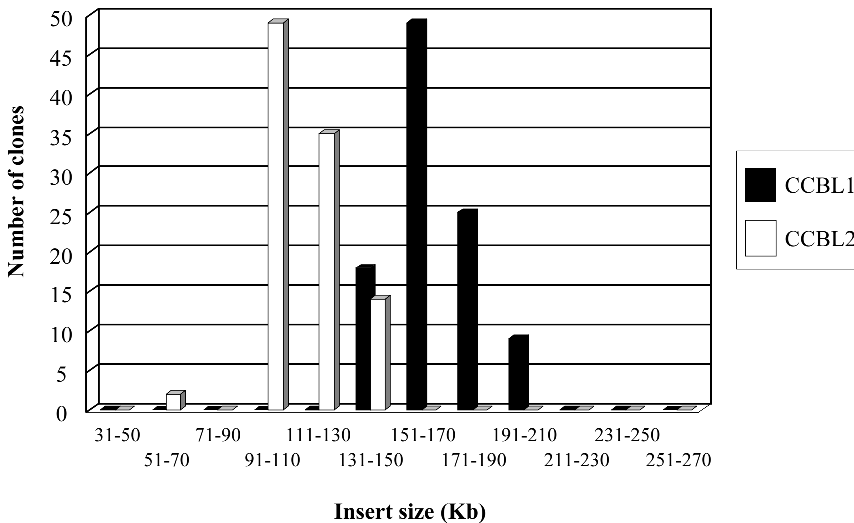

Figure 2. Size distribution of 100 randomly selected clones from the CCBL1 and CCBL2 size fractions.

and 150 kb (Fig. 2). Seven percent of CCBL2 clones contained no inserts. The removal of DNA less than 50 kb in size after the first electrophoresis of HMW DNA and size selection after the second electrophoresis permitted efficient production of these large insert libraries with a narrow size distribution, *i.e.* the elimination of potential small insert clones allowed for an increased transformation efficiency of larger insert BAC clones (Fig. 2). This was similar to the use of a reverse electrophoresis step to remove smaller DNA fragments described by Osoegawa *et al.* [17].

Clone stability was assayed by serial culture and restriction enzyme digestion. *Not*I and *Hind*III restriction digest patterns of 14 randomly chosen BAC clones were compared before and after 100 generations in serial growth. No apparent rearrangements were observed between day 1 and day 6 (data not shown) which confirmed that catfish BAC clones, like BAC clones of other species, are stable in culture [3, 14, 20, 30].

Non-specific digestion of the vector, resulting in a non-functional lacZ subunit upon vector re-ligation, could inhibit blue/white selection [7, 17]. Sequence analysis of 10 empty CCBL1 clones demonstrated no alteration of the lacZ gene region (data not shown), so the presence of empty clones in the library was probably due to a low level of precipitated X-gal in the colony.

The haploid genome of catfish is estimated to be 1.1×10^9 bp [11, 24, 23] thus, based on the number of clones and average insert size, CCBL1 contained a 7.2-fold catfish haploid genome equivalent. Genome coverage of CCBL1 was also estimated by screening all 144 plate pools for 22 type II microsatellite markers (Tab. I). All type II markers used to screen the BAC library demonstrated single-locus Mendelian inheritance [28]. The CCBL1 fraction contained, on average, 7.8 ± 5.1 BAC clones per marker. This is a conservative estimate since there could be more than one positive BAC clone per plate. While these results were consistent with the calculated genome coverage, some areas of the genome may be over- or under-represented due to variation in *Hind*III sites in the selected range of insert sizes, or the inability to clone/maintain certain catfish genomic regions in *E. coli*.

The CCBL1 fraction was screened for 27 catfish genes and individual BAC clones were identified for all genes (Tab. II). It was also screened for type II markers representing all channel catfish linkage groups [28] and positive plate pools were identified for 45 out of the 51 type II markers (Tab. I). Overall, these results predicted a 92% chance of finding a single copy sequence in CCBL1. The smaller insert CCBL2 fraction and a recent BAC library based on *Eco*RI digestion from a diploid catfish [4] will be useful to complement the genomic coverage of CCBL1. All 32 linkage groups [28] were represented in CCBL1, and it will be a useful resource for the integration of linkage and physical maps for channel catfish.

Table I. Detection of loci in CCBL1 by PCR screening.

LG[a]	Locus	Present	No. plates[b]	LG[a]	Locus	Present	No. plates[b]
U1	IpCG0164	+	7	U14	IpCG0281	+	
U1	IpCG0191	−		U15	IpCG0166	+	
U2	IpCG0196	+		U15	IpCG0237	+	9
U2	*B2M*	+		U16	IpCG0108	+	4
U3	IpCG0001	+		U17	IpCG0044	+	17
U3	*Acta*	+		U18	IpCG0176	+	2
U4	IpCG0035	+	8	U19	IpCG0169	+	5
U4	IpCG0054	−		U19	*IGF-1*	+	
U4	IpCG0143	+		U20	IpCG0070	+	
U4	IpCG0284	+		U21	IpCG0051	+	14
U5	IpCG0124	+		U22	IpCG0296	+	
U5	IpCG0136	+		U23	IpCG0038	+	3
U5	*IgH*	+		U24	IpCG0297	+	5
U6	IpCG0065	+		U25	IpCG0003	+	
U6	IpCG0310	+	8	U26	IpCG0010	−	
U7	IpCG0135	+	4	U26	IpCG0041	+	
U7	*MHC Iα*	+		U26	IpCG0120	+	5
U8	IpCG0064	+		U27	IpCG0094	+	2
U8	IpCG0285	+	13	U27	IpCG0150	−	
U9	IpCG0032	+		U28	IpCG0096	+	10
U9	IpCG0111	−		U29	IpCG0185	+	
U10	IpCG0199	+	7	U29	IpCG0255	+	22
U11	IpCG0157	+	2	U30	IpCG0049	+	8
U11	IpCG0216	+		U31	IpCG0149	+	
U12	IpCG0173	+		U31	IpCG0240	+	6
U12	IpCG0222	+		U32	IpCG0069	+	
U13	IpCG0104	+	10	U32	IpCG0214	−	
U14	IpCG0193	+		U32	IpCG0021	+	

[a] Linkage group; [b] selected markers were used to screen all 144 plate pools.
Avg. number of plates per marker = 7.8 (SD = 5.1).

4. CONCLUSION

The CCBL1 fraction of the BAC library provided 7.2-fold coverage of the channel catfish genome. Screening with type I and II markers indicated a good coverage of the catfish genome with this library. Direct sequencing of BAC clones has revealed microsatellite repeats in several genes, which should prove useful for placing type I markers on the channel catfish linkage map.

Table II. Genes identified in CCBL1 by PCR screening of pooled clones.

Gene	Accession number
α-actin (*Acta*)	AF228714
β-actin	D. Nonneman, Pers. Comm.
GAPDH	D. Nonneman, Pers. Comm.
CYP 11	AF063836
CYP 17	AF063837
Estrogen receptor alpha	AF061275
FSH receptor	AF285182
LH receptor	AF285181
IGF-1	L. Clay, Pers. Comm.
Somatotropin	AF267989
Proinsulin	T. Mommsen, Pers. Comm.
Somatostatin 14	V00607
Somatostatin 22	J00945
Follistatin	B. Bosworth, Pers. Comm.
MyoD	B. Bosworth, Pers. Comm.
Myogenin	B. Bosworth, Pers. Comm.
Myostatin	AF396747
PACAP	AF321243
Nramp	AF400108
NPY	AF267164
MHC I α	AF053549
β2-Microglobulin (*B2M*)	AF016042
CD 45	E. Kountikov, Pers. Comm.
Ig H	AF068137
TCR β	U39193
TCR γ	E Bengten, Pers. Comm.
Novel immune type receptor	AF397467

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