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SHORT COMMUNICATION

Construction and Characterization of Two Bacterial Artificial Chromosome Libraries of Grass Carp

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Abstract Bacterial artificial chromosome (BAC) library is an important tool in genomic research. We constructed two libraries from the genomic DNA of grass carp (*Ctenopharyngodon idellus*) as a crucial part of the grass carp genome project. The libraries were constructed in the *Eco*RI and *Hin*dIII sites of the vector CopyControl pCC1BAC. The *Eco*RI library comprised 53,000 positive clones, and approximately 99.94% of the clones contained grass carp nuclear DNA inserts (average size, 139.7 kb) covering 7.4× haploid genome equivalents and 2% empty clones. Similarly, the *Hin*dIII library comprised 52,216 clones with approximately 99.82% probability of finding any genomic fragments containing single-copy genes; the average insert size was 121.5 kb with 2.8% insert-empty clones, thus providing genome coverage of 6.3× haploid genome equivalents of grass carp. We selected gene-specific probes for screening the target gene clones in the *Hin*dIII library. In all, we obtained 31 positive clones, which were identified for every gene, with an average of 6.2 BAC clones per gene probe. Thus, we succeeded in constructing the desired BAC libraries, which should provide an important foundation for future physical mapping and whole-genome sequencing in grass carp.

Keywords Bacterial artificial chromosome · Grass carp · Gene-specific hybridization

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Introduction

Grass carp (*Ctenopharyngodon idellus*) is widely distributed in China, from the plains in Guangdong Province to northeast China except in Xinjiang and Qinghai-Tibet Plateau. Ever since its artificial propagation in 1958, grass carp has gradually become an important fresh water aquaculture species and has been migrated to and cultivated in Asia, Europe, and Africa. In China, grass carp had been a dominant aquaculture species, and its production had reached 3.963 million tons in 2006, accounting for 20% of China's freshwater aquaculture production. However, of late, the development of grass carp aquaculture has been seriously affected due to species degradation and disease. In 2006, due to diseases, the grass carp aquaculture industry's economic losses amounted to USD 0.3 billion. The traditional strategy for genetic selection of grass carp is facing enormous challenges due to the long breeding cycle and the time of 4 to 5 years required for attaining sexual maturity. In fact, thus far, no improved varieties of grass carp have been obtained. Clearly, extensive research on the molecular genetics of grass carp is required for establishing molecular breeding technology systems and accelerating the breeding of improved breeds.

Bacterial artificial chromosome (BAC) libraries provide an invaluable platform for molecular genetics research; these libraries have the advantages of stable maintenance of large DNA fragments and its ease of manipulation (Shizuya

et al. 1992). BAC library is crucial not only for physical mapping, genome analysis, and positional cloning of genes, but also for whole-genome sequencing and comparative genome analysis. Recently, a number of BAC libraries have been constructed and characterized in aquatic animals including flounder (Katagiri et al. 2000), rainbow trout (Katagiri et al. 2001), common carp (Katagiri et al. 2001), tilapia (Katagiri et al. 2001), channel catfish (Quiniou et al. 2003; Wang et al. 2007), salmon (Thorsen et al. 2005), European sea bass (Whitaker et al. 2006), oysters (Cunningham et al. 2006), zhikong scallop (Zhang et al. 2008), and shrimp (Zhang et al. 2010). Based on BAC library, physical maps have been constructed in some important aquaculture species, such as Atlantic salmon (Ng et al. 2005), tilapia (Katagiri et al. 2005), and channel catfish (Xu et al. 2007). BAC-end sequences were used for DNA markers development, comparative genome analysis, and the establishment of conserved syntenis (Xu et al. 2006; Nandi et al. 2007; Wang et al. 2007). Here, the construction of a grass carp BAC library will aid in the physical mapping and assembly of whole-genome sequences and also facilitates DNA-marker exploring and functional gene cloning.

Materials and Methods

Experimental Animal

Blood cells were collected from a male grass carp (Wuhan, Hubei Province, China) using a heparinized syringe.

BAC Vector and Competent Cells

BAC vector CopyControl pCC1BAC (*Hin*dIII/*Eco*RI) kit and TransforMax EPI300 competent cells were bought from Epicentre Biotechnologies (Epicentre, WI, USA).

High-Molecular-Weight DNA Preparation

Blood cells were washed twice by resuspending in phosphate-buffered saline buffer and were then centrifuged at 1,200 rpm for 15 min. The concentration of the blood cells was quantified to 1×10^7–1×10^8 cells/ml in a centrifuge tube; the blood cells were then incubated at 45°C for 15 min and mixed with an equal volume of 1% (*w*/*v*) molten low-melting-point (LMP) agarose. The mixture was then immediately aliquoted into plug molds placed on ice for 30 min. Next, the cells embedded in the LMP agarose plugs were lysed in a lysis buffer containing proteinase K (1 mg/ml) at 50°C for 48 h with a single change of the buffer in between. The DNA plugs were washed in 0.5 M EDTA (pH 9.0–9.3) at 50°C for 1 h and in 0.05 M EDTA (pH 8.0) on ice for 1 h; eight plugs were immersed in 50 ml of 1× Tris-acetate-EDTA (TAE) buffer at 4°C for 3 h and then subjected to pulsed-field gel electrophoresis (PFGE) using the CHEF DRIII system (Bio-Rad, CA, USA) to remove the small DNA fragments and substances in the plugs that might inhibit subsequent restriction enzyme digestion and ligation. The conditions of the PFGE were 1% (*w*/*v*) LMP agarose gel, 6 V/cm, 12.5°C, and a 30-s initial switch time and 30-s final switch time for 16 h in 1× TAE. The plugs were collected from the pulsed-field gel, immersed in 1× Tris-EDTA (TE) buffer (pH 8.0), and stored overnight at 4°C.

BAC Library Construction

In order to construct a BAC library, the optimal partial digestion condition should be determined to obtain a number of high-quality DNA fragments. High-molecular-weight (HMW) DNA was partially digested by varying the amounts of *Eco*RI or *Hin*dIII (0–6U) in 10 min. Three plugs were cut into 24 slices approximately equal in size and twice incubated for 2 h in 8 ml of incubation buffer I (1× *Eco*RI buffer or 1× *Hin*dIII buffer, 4 mM spermidine, 2 mM DTT) on ice. Three slices of plug were transferred into a 1.5-ml tube containing 140 μl of incubation buffer II (1× *Eco*RI buffer or 1× *Hind*III buffer, 2 mM spermidine, 1 mM DTT) plus a varying amount of *Eco*RI or *Hin*dIII. The mixture was then incubated for 2 h on ice and then for 10 min in a water bath maintained at 37°C. The digestions were stopped immediately by transferring the mixture onto ice and adding 20 μl of 0.5 M EDTA (pH 8.0). The digested DNA was analyzed by PFGE on a 1% (*w*/*v*) agarose gel in 1× TAE for 16 h at 6 V/cm and a temperature of 12.5°C (initial switch time, 0.1 s; final switch time, 40 s). The partial digestion condition resulting in a majority of restricted fragments ranging from 100 to 400 kb was selected for large-scale partial digestion.

Large-scale partial digestion of HMW DNA for BAC library construction was performed by using five plugs according to the optimal condition predetermined as above. Size selection was carried out as previously described (Osoegawa et al. 1998). The partially digested DNA was fractionated by PFGE on a 1% (*w*/*v*) agarose gel in 1× TAE for 16 h at 6 V/cm and at 12.5°C (initial switch time, 0.1 s; final switch time, 40 s). The DNA was gently collected with a wide-bore pipette tip and then subjected to second size selection by PFGE in 1× TAE on a 1% (*w*/*v*) agarose gel for 16 h at 6 V/cm and at 12.5 C (initial switch time, 5 s; final switch time, 15 s). The compressed DNA band was excised and recovered by electroelution (Strong et al. 1997). The eluted DNA fragments were dialyzed against 0.5× TE for at least 2 h.

The concentrations of both the restricted grass carp DNA fragments and pCC1BAC vector were measured by electro-

Table 1 Gene-specific probes used in screening

GenBank accession no.	Gene	Forward primer	Reverse primer
BC163271	TLR1	ATCGTCCACCATTCGTGAGC	CCTCCCTTGATTCTCCGAAT
AY388399	TLR2	ATGGACCTCAGCCAGAATC	GGAAATCTTTGCTTGAACGC
AY616582	TLR3	GGTGTTTCAAGGCTATCGAT	GCCATTTTCCACAATCTAGC
EU551724	TLR4	CTATCTTTCGCTGTATAGTC	GGATAAAAGTGTAGAGCAGC
DQ372601	TLR5	CTTCACCAGTTACAGCAGCT	AGCCATGTTTGGAAATCCTT

phoresis on a 1% (*w*/*v*) agarose gel with standard λDNA of a known concentration. Restricted grass carp DNA fragments were ligated into the cloning-ready vector pCC1BAC at a 4:1 molar ratio of insert to vector molecules.

The ligation mixture (1 μl) was used to transform 20 μl TransforMax EPI300 competent cells by using the BRL Cell-Porator Electroporation System (Gibco, ON, Canada). The transformed cells were immediately resuspended in 1 ml of SOC medium and incubated at 37°C for 1 h with shaking at 225 rpm. Transformants containing the recombinant DNA clones were selected on Lysogeny broth (LB) medium supplemented with 12.5 μg/ml chloramphenicol, 60 μg/ml X-gal, 15 μg/ml IPTG, and grown at 37°C for 16–24 h. The white clones were arrayed into 384-well microtiter plates containing 70 μl LB freezing media and 12.5 μg/ml chloramphenicol, incubated overnight at 37°C, and then stored at −80°C.

BAC Library Characterization

To evaluate the average insert size, we randomly selected 150–250 white BAC clones from each library and isolated the BAC DNA. After *Not*I digestion, the sizes of the inserts were estimated with PFGE.

BAC Library Screening with Gene-Specific Probes

To further validate the genome coverage and test the utility of the library, we screened target genes with gene-specific

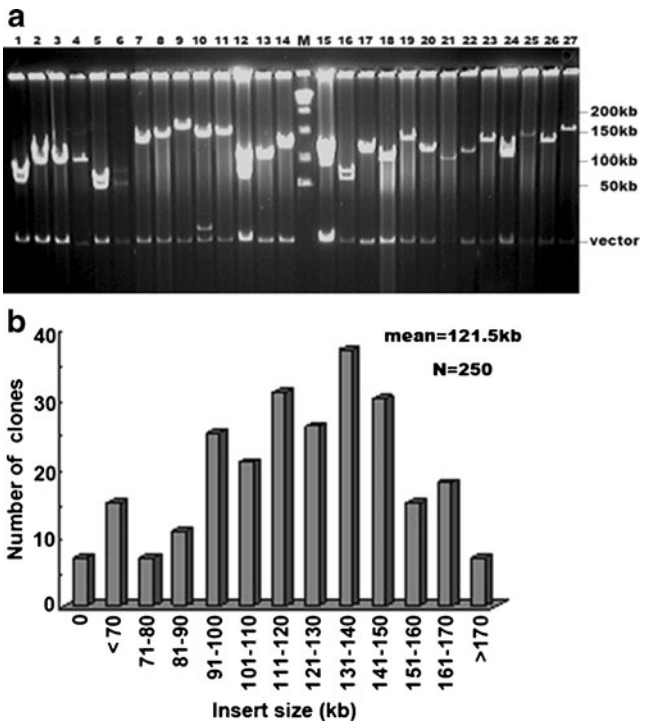

Fig. 1 Analysis of the insert sizes of the *Hin*dIII library. **a** A random sample of white colonies (BACs) selected from the *Hin*dIII library and analyzed on a pulsed-field gel. BAC DNA was digested with *Not*I to release the vector from the insert DNA fragment and then subjected to PFGE. "*M*" represents the lambda Ladder PFG Marker; the *lanes* between the markers are the *Not*I-digested BAC DNAs; and the *band below* is from the pCC1BAC vector. **b** Insert size distribution of the *Hin*dIII BAC library. In all, 250 clones were obtained from the *Hind*III library

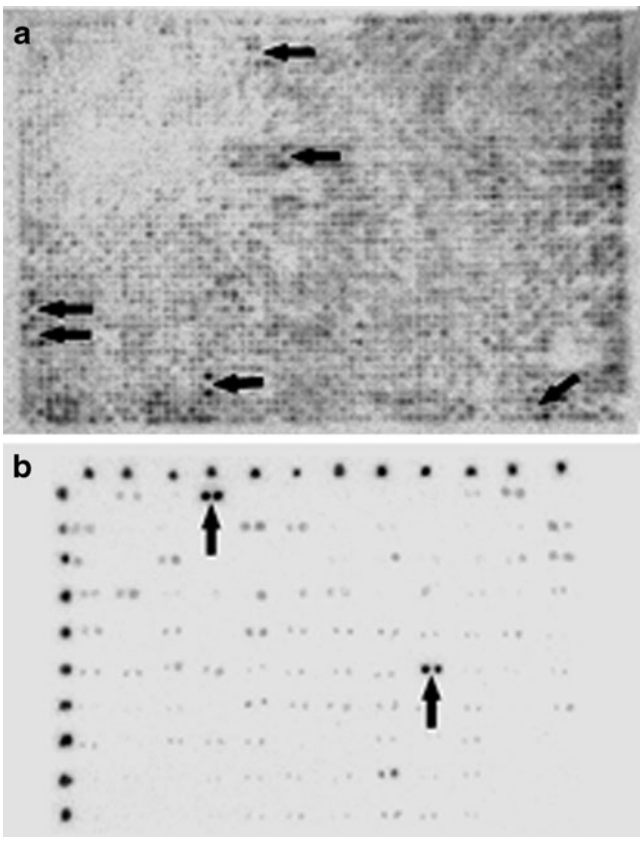

Fig. 2 Library screening with nylon filters. **a** The mixture of probes hybridized with high-density nylon filter. **b** *TLR2* probe hybridized with low-density nylon filter; *arrows* indicate the positive clones

Table 2 Positive clones of gene-specific probes identified from the *Hin*dIII BAC library

Gene	Number of positive clones identified	Positive clones in the library
TLR1	7	011F1, 015L16, 016I13, 040O22, 043H3, 046F9, 048C5
TLR2	2	044J10, 067H21
TLR3	9	028D14, 041G22, 043F2, 046J3, 046H9, 054P11, 056O8, 061O10, 064C6
TLR4	2	033C17, 068M24
TLR5	11	011F10, 15F12, 016F13, 046F9, 046E21, 056P23, 064K11, 064M16, 068D15, 070B3, 091H20

probes. *TLR1~5* gene-specific probes were designed on the basis of the cDNA sequences of zebrafish (Table 1). The mixture of cDNA probes of five genes was hybridized with the entire BAC library embedded on a high-density nylon membrane. Next, we selected the positive clones to construct a new low-density nylon membrane and hybridized it to each gene-specific probe and obtained positive clones for each probe.

Results and Discussion

We constructed two BAC libraries from the genomic DNA of grass carp in the BAC vector pCC1BAC. One library was constructed from the grass carp DNA partially digested with *Eco*RI and the other, from the grass carp DNA partially digested with *Hin*dIII. The two libraries were equivalent to approximately 13.7 haploid genomes of grass carp. The *Eco*RI library comprised 53,000 clones. To estimate the insert size of the *Eco*RI library, we randomly selected 150 clones and analyzed them on pulsed-field gels. The result showed that approximately 99.94% of the clones contained DNA inserts with an average size of 139.7 kb; 84% of the clones had inserts larger than 100 kb (representing 7.4× haploid genome equivalents), and 2% were insert-empty clones. The *Hin*dIII library was assembled from a total of 52,216 clones. We analyzed a random sample of 250 *Hind*III clones on pulsed-field gels to evaluate the inserts of the library. We estimated the clones to have an average insert of 121.5 kb, with 99.82% of them containing inserts greater than 100 kb and providing genome coverage of 6.3×; 2.8% were insert-empty clones (Fig. 1).

A high coverage of the library is critical for the screening of any particular gene. To validate the genome coverage of the library and test their utility for gene characterization, positioning cloning, and genome physical mapping, we screened the clones of the *Hin*dIII BAC library gridded onto high-density nylon membrane using the mixture probe designed from a part of the *TLR1-5* gene sequences. One hundred and eighteen positive clones were obtained. By further hybridization with the gene-specific probes, respectively, we identified 2–11 positive clones for each gene probe and a total of 31 BAC clones for the five gene probes with an average of 6.2 positive clones per gene probe (Fig. 2 and Table 2). The grass carp *TLR1–5* gene sequences were obtained by directly sequencing the positive BAC clones. The GenBank accession numbers of grass carp *TLR1–5* gene are FJ542041, FJ542042, DQ864497, FJ542043, and GQ844688, respectively.

Grass carp is an important representative species of cyprinid fish and an ideal model species for genome research. This species has a chromosome number of 48 ($2n$=48, XY type; Li et al. 1985); its genome size is approximately 1,000 Mb, which is between the genome size of the zebrafish (1,700 Mb) (Postlethwait et al. 1994) and that of the medaka (800 Mb) (Ishikawa 2000). In this study, we used the male grass carp genomic DNA to construct two BAC libraries that covered all the genomic information, including the X and Y sex chromosomes. The construction of a grass carp genomic BAC library will provide an important platform for the implementation of the grass carp genomic project (Table 3).

A BAC library is the basis for constructing a physical map, and genome coverage is the key factor in determining the physical map quality. Previous studies have shown that a clonal coverage of 6.0–8.0 genome equivalents was sufficient for the development of a genome-wide physical map of approximately 95% genome coverage. The main reason for the lack of some genomic information is the heterogeneity distribution of the restriction endonuclease sites in the genome (Tao et al. 2001). In the genome, DNA fragments are too small (<40 kb) at the dense restriction sites regions and too large (>400 kb) at the sparse restriction sites regions. In the above-mentioned studies,

Table 3 Summary of the two grass carp BAC libraries

Library name	Cloning vector	Restriction enzyme used	Total clones	Insert-empty clones (%)	Average insert size (kb)	Genomic coverage
*Eco*R I library	pCC1BAC	*Eco*RI	53,000	2	139.7	7.4×
Hind III library	pCC1BAC	*Hind* III	52,216	2.8	121.5	6.3×

these two DNA fragments were not recovered after gel electrophoresis; this is an inherent defect that is identified while building a physical mapping using a single BAC library. Hence, it has been suggested that complementary BAC libraries be constructed and that physical genome map coverage be enhanced by using different DNA restriction fragments (Tao et al. 2001). In this study, we established *Eco*RI and *Hin*dIII BAC libraries of the grass carp with a total genomic coverage of 13.7× (Table 3). These two BAC libraries are expected to aid in building a high-genome coverage physical map.

The toll-like receptor (TLR) family plays an important role in the innate immune system in mammals (O'Neill 2008). Recently, a number of specific *TLRs* were found in teleosts and had some unique functions (Matsuo et al. 2008; Sepulcre et al. 2009). These specific *TLRs* might have a close relationship with the unique living environment for fish and the evolutionary process of vertebrates (Oshiumi et al. 2008). Studies on TLR in teleost, especially in the commercial fish species, are increasingly being conducted (Hirono et al. 2004; Tsujita et al. 2004; Baoprasertkul et al. 2007). In this study, we obtained 31 *TLR1-5* positive clones from the *Hin*dIII BAC library by using gene-specific probes. The mean number of positive clones per gene probe (6.2) is consistent with the value of 6.3 positive clones per gene probe that was predicted using calculations based on the clone insert size, number of clones, and genome size. Our results showed that the BAC library we constructed was expected to match the quality requirements. Moreover, the clone of the grass carp *TLR1-5* and its sequence data lay the foundation for further studies on gene function and its application in molecular breeding of the grass carp.

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