Construction and characterization of BAC libraries for three fish species; rainbow trout, carp and tilapia

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Summary

Bacterial artificial chromosome (BAC) libraries are important tools for genomic research. We have constructed seven genomic BAC libraries from three fish species, rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*) and tilapia (*Oreochromis niloticus*). The two rainbow trout BAC libraries have average insert sizes of 58 and 110 kb. The average size of inserts in the carp BAC library is 160 kb. The average insert sizes of the four tilapia BAC libraries are 65, 105, 145 and 194 kb, respectively. These libraries represent good coverage of each genome (2–64 × coverage). The libraries can be screened by conventional colony hybridization and provide a starting point for the construction of high-density filtres or polymerase chain reaction (PCR) screening approaches. These BAC libraries will facilitate the positional cloning of quantitative trait loci (QTLs) for a variety of economically important traits in these species.

Keywords bacterial artificial chromosome (BAC), carp *Cyprinus carpio*, rainbow trout *Oncorhynchus mykiss*, tilapia *Oreochromis niloticus*.

Introduction

The bacterial artificial chromosome (BAC) was developed as a vector to permit the cloning and stable maintenance of large DNA in *Eschericia coli* (Shizuya *et al.* 1992). As the BAC vector is a single-copy plasmid, the clones appear to be very stable over many generations (Kim *et al.* 1992; Shizuya *et al.* 1992). The BACs can be transformed very efficiently into the *E. coli* host by electroporation. The BAC cloning system has the advantages of high cloning efficiency, smaller size of cloning vector and easy purification by alkaline lysis. BAC libraries have contributed significantly to the genome sequencing projects (Asakawa *et al.* 1997; Dunham *et al.* 1999) and the cloning of disease genes (Kitada *et al.* 1998). The BAC libraries have been constructed for several species of agricultural importance, including cow (Cai *et al.* 1995), chicken (Zimmer & Gibbins 1997) and pig (Suzuki *et al.* 2000). These BAC libraries are being used to positionally clone quantitative trait loci (QTLs) underlying economically important traits in these species.

Following the publication of linkage maps for rainbow trout (Young *et al.* 1998) and tilapia (Kocher *et al.* 1998), it became possible to conduct QTL studies of economic traits in several important aquaculture species. For example, Sakamoto *et al.* (1999) have searched for QTLs associated with spawning time in rainbow trout using 54 microsatellite markers and found eight QTL markers.

Although linkage and QTL-mapping in fish species are now yielding results, the resources for physical mapping and positional cloning are not well developed. Only one BAC library has been reported for an important cultured finfish, the Japanese flounder *Paralichthys olivaceus* (Katagiri *et al.* 2000).

Here we describe the construction of seven new BAC libraries for three fish species: rainbow trout, carp and tilapia. These species are both commercially important as well as popular subjects for basic research. These BAC libraries will facilitate the identification of genes controlling important traits in these species.

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Materials and methods

Sperm were collected from common carp maintained in the Yoshida Research and Training Station, Tokyo University of Fisheries. The rainbow trout sperm were from the RT-101 strain maintained in the Fuji Branch of the Shizuoka Prefectural Fisheries Experimental Station. Tilapia sperm were from the Lake Manzallah strain of *O. niloticus* (individual no. 00-0135-EA1B maintained in the Institute of Aquaculture, Stirling University).

The protocols for the construction of these BAC libraries were based on our previous work on Japanese flounder (Katagiri *et al.* 2000). The sperm from each fish were embedded in 0.6% low melting agarose plugs ($5 \times 1.5 \times 12$ mm) at a concentration of $1–3 \times 10^8$ cells/ml. The cells inside the agarose plugs were digested in buffer L [100 mM EDTA, 20 mM NaCl, 10 mM Tris–HCl (pH 8.0), 1% N-lauroylsarcosine] containing proteinase K (1 mg/ml) at 50 °C for 2 days with one change of this buffer. Methods for digestion with restriction enzyme *Hin*dIII, pulse field gel electrophoresis (PFGE), isolation and purification of high molecular weight DNA (HMW DNA) are described in Katagiri *et al.* (2000). Fractions corresponding to approximately 50–100 kb and 100–150 kb for rainbow trout DNA, 150–250 kb for carp DNA, and 50–100 kb, 100–150 kb, 150–200 kb and 200–250 kb for tilapia DNA, were isolated for construction of the corresponding BAC library. The purified HMW DNA was ligated to the vector pBAC-*lac* (Asakawa *et al.* 1997) which allows blue/white colony selection. Vector preparation, ligation to insert and electroporation methods are described in Katagiri *et al.* (2000). After electroporation, transformed cells were spread on 500 cm^2 LB agar plates containing chloramphenicol (12.5 μg/ml), X-gal (40 μg/ml) and IPTG (100 μg/ml). The plates were incubated until the colonies had grown to approximately 0.5–1 mm of diameter. The number of colonies and white and blue colony ratios were determined for each plate. Twenty-five millilitres of LB medium containing 7.5% glycerin were then added to each LB agar plate. The LB liquid medium containing the bacterial cells was then collected and mixed with other plates from the same ligation before being aliquoted and stored at −80 °C. The BAC libraries from each DNA size fraction were named as RBL1 (50–100 kb) and RBL2 (100–150 kb) for rainbow trout, CBL for carp (150–250 kb), and TBL1 (50–100 kb), TBL2 (100–150 kb), TBL3 (150–200 kb) and TBL4 (200–250 kb) for tilapia.

To evaluate the average insert size we randomly took 30 white BAC clones from each library and isolated the BAC DNA. After *Not*I digestion, we estimated the size of the inserts by PFGE as described in Katagiri *et al.* (2000).

Screening of the RBL1 with rainbow trout activin βA-1 probe (Ract βA-1) (Tada *et al.* 1998) was carried out to confirm the library quality. Approximately 40 000 white BAC colonies were screened by two rounds of conventional colony hybridization (Glover & Hames 1995). The BAC DNA from positive clones was digested with *Hin*dIII and subjected to Southern blot analysis with Ract βA-1 as described in Katagiri *et al.* (2000). Six BAC libraries, RBL1, CBL, TBL1, TBL2, TBL3, and TBL4, were screened with the ribosomal protein S6 gene (Nam *et al.* 2000) that is thought to be a single copy gene for the determination of the real coverage of these libraries.

Results and discussion

There are several important measures of the quality of a BAC library. First, a very high proportion of clones should have an insert, and the proportion of chimeric clones should be low. Secondly, the insert sizes should be large and homogeneous. Finally, the library should contain a large number of independent clones. We were able to produce high quality BAC libraries which satisfy these criteria by using HMW DNA from sperm. There are several advantages in using the sperm cells. The fish are not injured as a result of dissection. The fresh sperm can be stored in a refrigerator for several days and a cell culture system is not required. Sperm are easily dispersed and embedded in low melting agarose. This allows the proteins of the sperm to be completely digested with proteinase K, improving the separation of the partially digested genomic DNAs by PFGE.

We counted the colony number and determined the white and blue colony ratio of each BAC library (Table 1). The percentages of white colony in 500 colonies of each library were 94 to 99.7%. These high ratios were achieved using ligation conditions in which the molar ratio of insert to vector was 1 : 10 (Katagiri *et al.* 2000). Higher concentrations of insert DNA might have increased the number of colonies recovered, but were not used as this would generate increased numbers of chimeric clones and clones without inserts. The high ratios of white colonies also indicate that the vector was completely digested with *Hin*dIII and thoroughly dephosphorylated.

To estimate insert sizes, we randomly took 30 BACs from each library. After digestion with *Not*I and separation of the fragments by PFGE, we calculated the average insert size of each library (Fig. 1 and Table 1). The insert size distribution of each library reflected the sizes of the fragments used for ligation to the vector (Table 1). There was a good correlation between the size fractions collected from PFGE and the insert sizes in the case of the rainbow trout and tilapia libraries (Fig. 1). The DNA bands of lane 7 of the RBL2 and

Table 1 The characters of seven BAC libraries from three species.

Fish	Library name	White colony (%)	Average insert size (kb)	Insert size distribution (kb)	Clone number	Haploid genome coverage[1]	Colony hybridization No. of positive clones/ no. of used clones
Rainbow trout	RBL1	99.0	58	35–80	380 000	6.7	4/40 000[2], 2/125 000[3]
	RBL2	98.3	110	90–140	160 000	5.3	ND
Carp	CBL	98.7	160	120–230	23 000	2	11/100 000
Tilapia	TBL1	99.7	65	30–115	120 000	6	2/55 000
	TBL2	99.7	105	75–135	800 000	64.6	1/20 000
	TBL3	98.3	145	75–170	100 000	11.2	4/60 000
	TBL4	94.0	194	170–220	40 000	6	7/55 000

[1]The haploid genome coverages of three fish BAC libraries were calculated from Hinegardner & Rosen (1972).
[2]DNA probe was Ract-β1.
[3]DNA probe was ribosomal protein S6.

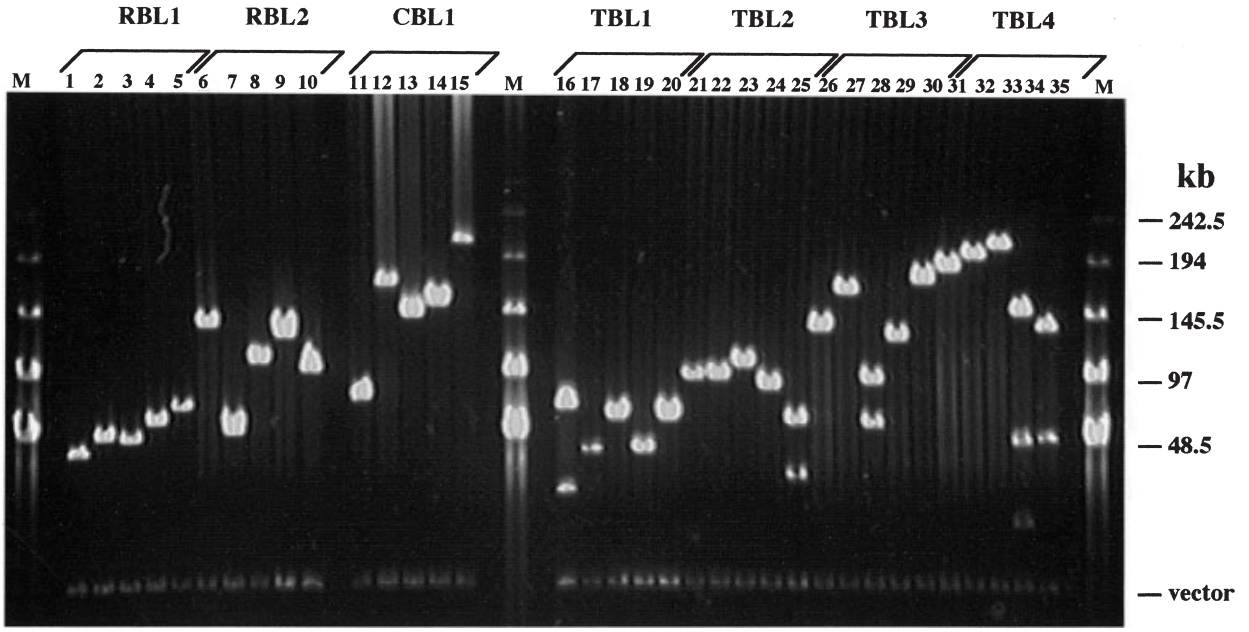

Figure 1 PFGE analysis of insert sizes of representative clones from seven fish BAC library (representing three species) after digestion with *Not*I. Five randomly selected clones are shown for each libraries. Lanes 1–5, RBL1; lanes 6–10, RBL2; lanes 11–15, CBL; lanes 16–20, TBL1; lanes 21–25, TBL2; lanes 26–30, TBL3; lanes 31–35, TBL4; lane M, λladder.

lane 11 of the CBL are seen near 50 and 70 kb, respectively. However, each of these two BAC has two overlapping bands of the same size. We confirmed this by electrophoresing undigested DNA from lanes 7 and 11 and comparing with lanes 6, 8–10 and 12–15. The BACs of lanes 7 and 11 were similar sizes with other lanes of each library (data not shown). From these results, the sizes of the BACs of lanes 7 and 11 were calculated to be approximately 100 and 140 kb.

A high coverage of the library is critical for screening of any particular genes. Hinegardner & Rosen (1972) reported the total cellular DNA content of 275 different fish species, estimating the haploid genome size of carp as 1870 Mb and that of tilapia as 1320 Mb. Although the DNA content of rainbow trout has not been reported, the genome size of two other salmonids, *Oncorhynchus kisutch* and *Oncorhynchus tshawytscha*, were calculated to be 3300 and 3630 Mb, respectively. We therefore assume that the haploid genome size of rainbow trout is approximately 3500 Mb. Based on the average insert size, the total number of clones of each library, and the haploid genome size of each fish, we estimated the coverage of the seven BAC libraries (Table 1). The RBL1 and RBL2 provide approximately 6.3- and 5.0-fold coverage of the

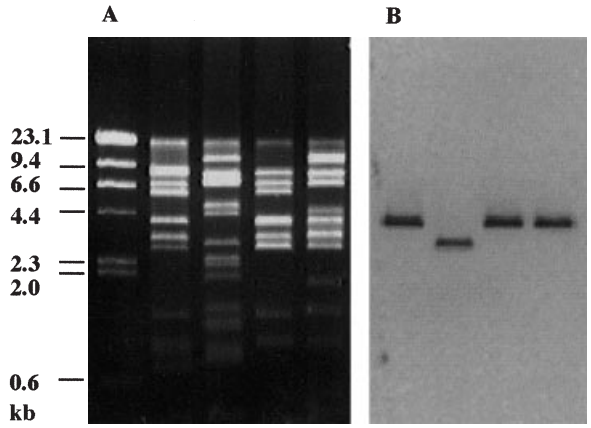

Figure 2 Agarose gel electrophoresis (a) and Southern blot hybridization (b) of four BAC clones (RBL1) screened by activin βA after digestion with *Hin*dIII. Left lane of panel A shows the λ*Hin*dIII digestion.

rainbow trout genome. The CBL provides 2.0-fold coverage of the carp genome. The four BAC libraries of tilapia cover the haploid genome 5.7 and 63.6 times. We picked 36 860 clones (an approximately 3-fold coverage of the haploid genome) from TBL3 and 33 400 clones (an approximately 5-fold coverage of the haploid genome) from TBL4 and stored them in 384-well microtiter plates in order to construct rapid screening systems (Asakawa *et al.* 1997).

To confirm the utility of these libraries, we carried out screening of the RBL1 with a probe for the activin βA gene (Tada *et al.* 1998). We identified four BAC clones hybridizing to the probe as expected from the library coverage. These BACs were purified and digested with *Hin*dIII. The agarose gel (Fig. 2a) clearly demonstrates that each clone has a unique restriction pattern, and the Southern blot (Fig. 2b) confirms that each contains the activin βA gene. We also conducted colony hybridization using six BAC libraries, RBL1, CBL, TBL1, TBL2, TBL3, and TBL4, with a probe for ribosomal protein S6, for estimating the real coverage of these libraries. The results are shown in Table 1. The ratios of positive clones of colony hybridization of each of the BAC libraries were approximately those expected from the calculated haploid genome coverage from Hinegardner & Rosen (1972).

The construction of BAC libraries requires specialized equipment and skills, and investment of 2–3 months in the laboratory work. Our BAC libraries will be made available to interested researchers upon request. We expect these libraries will facilitate comparative gene mapping and positional cloning of QTLs in these important aquaculture species.

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