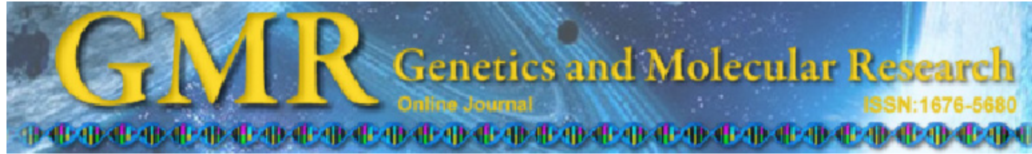

Construction and preliminary characterization of a river buffalo bacterial artificial chromosome library

N.B. Stafuzza[1], C.A. Abbey[2], C.A. Gill[2], J.E. Womack[3] and M.E.J. Amaral[1]

[1]Departamento de Biologia, Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual de São Paulo "Júlio de Mesquita Filho", São José do Rio Preto, SP, Brazil
[2]Department of Animal Science, Texas A&M University, College Station, TX, USA
[3]Department of Veterinary Pathobiology, Texas A&M University, College Station, TX, USA

Corresponding author: M.E.J. Amaral
E-mail: eamaral@ibilce.unesp.br

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ABSTRACT. River buffalo genome analyses have advanced significantly in the last decade, and the genome sequence of *Bubalus bubalis* will be available shortly. Nonetheless, large-insert DNA library resources such as bacterial artificial chromosomes (BAC) are still required for validation and accurate assembly of the genome sequence. We constructed a river buffalo BAC library containing 52,224 clones with an average insert size of 97 kb, representing 1.7 × coverage of the genome. This genomic resource for river buffalo will facilitate further studies in this economically important species allowing for instance, whole genome physical mapping and isolation of genes and gene clusters, contributing to the elucidation of gene organization and identification of regulatory elements.

Key words: BAC library; *Bubalus bubalis*; Cloning; Pulse-field gel electrophoresis

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INTRODUCTION

Among livestock species, the river buffalo (*Bubalus bubalis*) holds great promise and potential for animal agriculture, playing an important role in the worldwide economy through high-quality milk and meat production. Brazil is the largest buffalo-breeding center outside of the Asian continent, holding the largest buffalo herd in the Americas. According to the Food and Agriculture Organization of the United Nations, more than 168 million buffalo live in the world, and approximately one million are located in Brazil (FAO, 2009).

In recent years, the research community has generated buffalo genome resources through cytogenetic studies (Di Meo et al., 2008), whole-genome mapping (Amaral et al., 2008), whole-genome sequencing (Tantia et al., 2011), and studies of the utility of bovine single-nucleotide polymorphism in buffalo (Michelizzi et al., 2011). In Brazil, studies of the river buffalo genome started in 2004 with the construction of a radiation hybrid (RH) panel. This first tool for whole-genome mapping was initially used to generate preliminary maps of individual buffalo chromosomes (Amaral et al., 2007; Goldammer et al., 2007; Miziara et al., 2007; Ianella et al., 2008; Kochan et al., 2008; Stafuzza et al., 2007, 2009) and later to build a buffalo first-generation whole-genome map, assigning the location of 2621 cattle-derived markers (Amaral et al., 2008). Comparisons of these RH maps with the bovine genome sequence revealed target regions containing markers potentially associated with economically important traits.

Currently, several research groups are using next-generation sequencing platforms (Roche 454, Illumina GAIIx) to produce genome sequences for various buffalo breeds (reviewed by Michelizzi et al., 2010). Although these platforms support quick and relatively inexpensive sequencing methods, recent studies of *de novo* assemblies produced by these approaches have indicated a loss of approximately 16% of the genome (Alkan et al., 2011). Large-insert clone libraries, such as those using bacterial artificial chromosomes (BACs), are often considered too costly and laborious to create or maintain for many organisms, but they are still required for accurate genome sequence assembly as well as for experimental validation. The low frequency of chimeric BAC clones in addition to the relatively easy techniques of manipulation and preparation of high molecular weight DNA make these libraries useful tools for exploring eukaryotic genomes.

BAC libraries have been constructed for several species of agricultural importance, including cattle (Cai et al., 1995; Zhu et al., 1999; Buitkamp et al., 2000; Warren et al., 2000; Eggen et al., 2001; Fujisaki et al., 2002), goat (Schibler et al., 1998), horse (Godard et al., 1998), pig (Rogel-Gaillard et al., 1999; Anderson et al., 2000; Suzuki et al., 2000; Fahrenkrug et al., 2001; Jeon et al., 2003; Liu et al., 2010), and sheep (Gill et al., 1999; Vaiman et al., 1999). Although large-insert DNA libraries from closely related ruminant species such as cattle, goat, and sheep have been used for *in situ* hybridization experiments in buffalo (Di Meo et al., 2005, 2008; Perucatti et al., 2009), a buffalo library is necessary for whole-genome physical mapping and isolation of genes and gene clusters as well as for the identification of regulatory elements. Herein we describe the construction of a river buffalo BAC library, a key resource for comparative gene-mapping studies, sequence assembly, and characterization of quantitative trait loci through positional cloning.

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MATERIAL AND METHODS

Preparation of high molecular weight DNA

A male fibroblast culture was established from a skin biopsy to construct the RH panel described elsewhere (Amaral et al., 2007). Fibroblasts from this cell line were the source of DNA for BAC library construction. Fibroblast cultures were prepared using standard cytogenetic techniques with culture flasks. After incubation with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) for 5 min at 37°C, the concentration of cells in suspension was determined using a hemocytometer chamber under an optical microscope. The cell concentration was adjusted to 3 x 10^7 cells/mL using phosphate-buffered saline. The cell suspension was mixed with an equal volume of molten 1% low-melting-temperature agarose and poured into plastic plug molds (Bio-Rad, Hercules, CA, USA). Embedded cells were lysed in 3 changes of ESP buffer (25 mM EDTA, 1% sodium-lauryl-sarcosine, 0.5 mg/mL proteinase K, pH 9.0) at 50°C with shaking at 25 rpm for 24 h/wash. The plugs were washed 3 times (1 h/wash) with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) plus 1 mM phenylmethylsulfonyl fluoride and then 3 times (1 h/wash) with TE at room temperature.

Partial digestion of genomic DNA and size selection

Buffalo genomic high molecular weight DNA partial digestions were optimized on one-fourth of a plug by varying the concentration (0.25-4.0 U) of *Hin*dIII (New England Biolabs, Ipswich, MA, USA) and time of digestion (2-15 min). Optimal sizes were generated with 0.25 U of *Hin*dIII when plugs were incubated at 37°C for 5 min. Before digestion, the plugs were equilibrated in 2 changes of 45 µL *Hin*dIII 1X digestion buffer for 1 h each. The digestions were stopped with the addition of 50 mM EDTA. The partially digested DNA was subjected to pulsed-field gel electrophoresis (PFGE) on a 1% low-melting agarose gel using a CHEF Mapper®XA System (Bio-Rad) in 1X TAE buffer (40 mM Tris, 30 mM acetate, 1 mM EDTA, pH 8.0), 6 V/cm, with a 90-s pulse for 20 h at 14°C.

Agarose slices corresponding to sizes ranging from 70 to 300 kb were excised from the gel and subjected to PFGE on a 1% low-melting agarose gel under the parameters described above for a second size selection to eliminate trapped small fragments comigrating with the selected fraction. The agarose slices with the targeted DNA sizes (70 to 300 kb) were melted at 68°C for 8 min, incubated at 45°C for 5 min, and digested with 1 U GELase (Epicentre Technologies, Madison, WI, USA) per 300 mg gel slice at 45°C for 30 min.

Ligation and transformation conditions

Approximately 100 ng size-selected DNA was ligated with 10 ng *Hin*dIII-digested pBeloBAC11 (Kim et al. 1996) at a 1:8 molar ratio, using 3 U T4 DNA ligase (New England Biolabs) at room temperature for 16 h. One and a half microliters of the ligation were used to transform 20 µL ElectroMAX™ DH10B competent cells (Invitrogen, Rockville, MD, USA) using an electroporator (Eppendorf Multiporator®; 2000 V) in 1-mm-wide electroporation cuvettes. The cells were grown in 1 mL super optimal broth with catabolic repressor (SOC) medium (Invitrogen) at 37°C with shaking at 200 rpm for 50 min and then plated onto

Luria-Bertani (LB) agar plates containing 12.5 µg/mL chloramphenicol, 100 µg/mL isopropyl-beta-D-thiogalactopyranoside (IPTG), and 50 µg/mL 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal).

The plates were incubated at 37°C for 20 h, and white recombinant BAC clones were picked with sterile toothpicks and transferred to individual wells of a 384-well microtiter plate containing 80 µL 1X LB freezing media [36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM sodium citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, 4.4% v/v glycerol, and 12.5 µg/mL chloramphenicol] per well. The microtiter plate was incubated for 16 h at 37°C and then stored at -80°C.

Evaluation of the average insert size and BAC library pooling

The average insert size of the BAC library was determined using PFGE after digestion with the restriction enzyme *Not*I (New England Biolabs). Two hundred and forty randomly selected BAC clones were grown in 5 mL of LB media containing 12.5 µg/mL chloramphenicol. The BAC DNA was isolated using standard alkaline lysis methods (Sambrook et al., 1989). The purified BAC DNA was digested with 5 U *Not*I at 37°C for 2 h.

PFGE followed, using a 1% low-melting agarose gel in 1X Tris-borate-EDTA, with a switch time ramping from 1.4 to 13.5 s/pulse and 6 V/cm at 14°C for 16 h. The gel was stained with ethidium bromide and photographed. The insert sizes were estimated through comparison with the MidRangeII marker (New England Biolabs). To minimize the number of PCRs needed to isolate an individual clone containing a sequence of interest, the buffalo BAC library was pooled three-dimensionally.

Single-plate DNA pools were prepared by combining 50 µL an overnight culture from all 384 BAC clones composing one plate. Using a 96-well grid format, clones from a single 384-well plate were grown as a set of four 96-well plates in 1 mL LB media containing 12.5 µg/mL chloramphenicol and incubated at 37°C for 18 h with shaking at 200 rpm. Superpools of DNA that contained DNA from 3072 BAC clones were made by pooling 8 single-plate pools.

PCR-based screening of the buffalo BAC library was carried out to isolate selected markers, and to date, a total of 4 markers have been screened. The denatured crude lysate from each pool set was used as a template for PCR. Once a superpool was identified, the 8 single pools corresponding to each superpool were screened to identify the positive single plate, and then the 40 pools making up one plate of row and columns were screened to identify a single BAC containing the marker of interest.

RESULTS

Seventeen superpools corresponding to 52,224 clones were prepared from the buffalo BAC library. Insert sizes ranged from 25 to 250 kb (Figure 1) with an average of 97 kb, which corresponds to approximately 1.7 genome equivalents assuming a buffalo genome size of 3000 megabases. Therefore, an 82% chance exists of finding any given sequence in the library. BAC DNA was extracted from 240 randomly selected clones, digested with *Not*I, and submitted to PFGE with the MidRangeII marker (New England Biolabs) to determine the insert sizes. The clones were scored for insert size in 10-kb intervals, and only 3 clones selected for sizing were without an insert, suggesting that 1.25% of the clones in the library are empty.

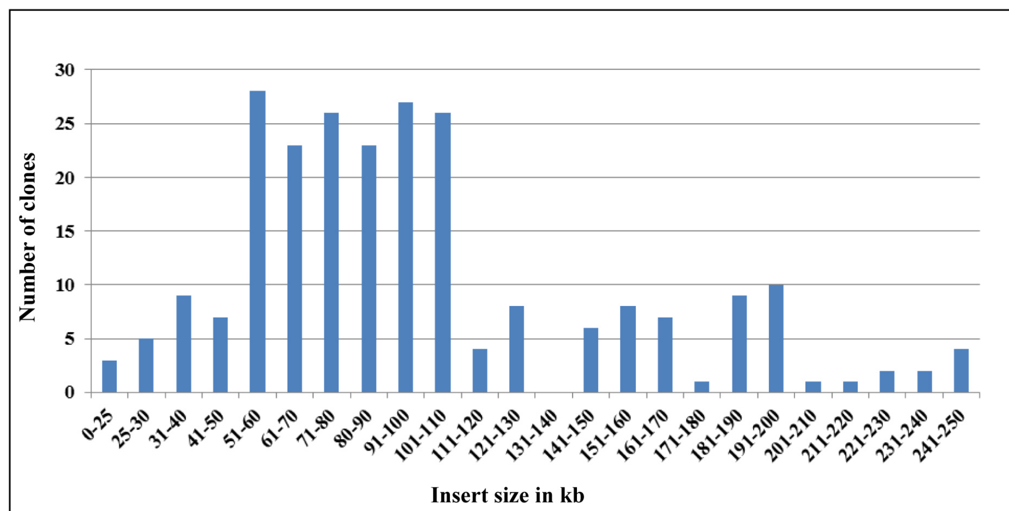

Figure 1. Insert size distribution of buffalo bacterial artificial clones chromosomes.

A preliminary screen of the buffalo BAC library was performed with 3 superpools (9216 clones) using PCR primers from markers previously mapped on the buffalo major histocompatibility complex RH map (DRB2, DQA2, DYB, and DMA) (Rodrigues Filho et al., 2008). One positive superpool was found for the DMA loci. In the next step, eight single pools-representing DNA from each of the 8 plates in the positive superpool-were screened. The intersection of rows and columns identified the location of the positive BAC clone for the DMA loci. This clone could then be used for the physical mapping and characterization of the buffalo major histocompatibility complex region.

DISCUSSION

Brazil was the first country to construct a genomic tool, a RH panel, for whole-genome mapping of river buffalo (Amaral et al., 2007). To facilitate genomic studies in this species, we constructed a new tool: a BAC library. The combination of the existing river buffalo RH maps and the results that can be generated with the BAC library will allow for high-resolution characterization of target genomic regions that can be applied, for instance, to understand better the genetic variation of the river buffalo breeds in Brazil and contribute to the development of new strategies for buffalo genetic improvement programs.

The BAC library described herein contributes to the increase in coverage of the Bovidae family genome. Added to the existing bovine (Cai et al., 1995; Zhu et al., 1999; Buitkamp et al., 2000; Warren et al., 2000; Eggen et al., 2001; Fujisaki et al., 2002), ovine (Gill et al., 1999; Vaiman et al., 1999), and caprine (Schibler et al., 1998) BAC libraries, this library provides improved genome-equivalent coverage of the bovid genomes available.

The insert sizes of the buffalo BAC library, averaging 97 kb, were smaller than expected, with 30% of the verified clones sized smaller than the minimal size of the DNA that was selected to make the library (70 kb). The smaller than expected insert sizes resulted in an

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81% chance of identifying a buffalo BAC clone containing the sequence of interest. Expanding the number of clones in the buffalo BAC library is necessary to ensure a 99% chance of finding any target sequence, which is required mainly for functional studies in which an exact buffalo sequence is required.

The buffalo BAC library was constructed from male DNA; thus, both X and Y chromosomes are represented, but each sex chromosome is underrepresented because only one copy of each is present in the genome with 2 copies of every autosome. This characteristic must be considered when using the library to screen genes on the X and the Y chromosomes that are not part of the pseudo-autosomal region.

The buffalo BAC library described herein will be valuable for the validation and accurate assembly of the buffalo genome sequences, the physical mapping of the buffalo genome, and the construction of minimum tiling paths for region-specific resequencing. The buffalo BAC library will also allow for the investigation of genome organization, gene regulation, and chromosome evolution in closely related species, such as cattle, sheep, and goat, and is available for collaborative research by contacting the authors.

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