Localization of BAC clones on mitotic chromosomes of *Musa acuminata* using fluorescence *in situ* hybridization

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Abstract

A bacterial artificial chromosome (BAC) library of banana (*Musa acuminata*) was used to select BAC clones that carry low amounts of repetitive DNA sequences and could be suitable as probes for fluorescence *in situ* hybridization (FISH) on mitotic metaphase chromosomes. Out of eighty randomly selected BAC clones, only one clone gave a single-locus signal on chromosomes of *M. acuminata* cv. Calcutta 4. The clone localized on a chromosome pair that carries a cluster of 5S rRNA genes. The remaining BAC clones gave dispersed FISH signals throughout the genome and/or failed to produce any signal. In order to avoid the excessive hybridization of repetitive DNA sequences, we subcloned nineteen BAC clones and selected their 'low-copy' subclones. Out of them, one subclone gave specific signal in secondary constriction on one chromosome pair; three subclones were localized into centromeric and peri-centromeric regions of all chromosomes. Other subclones were either localized throughout the banana genome or their use did not result in visible FISH signals. The nucleotide sequence analysis revealed that subclones, which localized on different regions of all chromosomes, contained short fragments of various repetitive DNA sequences. The chromosome-specific BAC clone identified in this work increases the number of useful cytogenetic markers for *Musa*.

Additional key words: cytogenetic mapping, chromosome structure, repetitive DNA, ribosomal DNA, subcloning.

Introduction

Bananas and plantains, hereafter collectively called bananas, are perennial giant herbs grown in the tropical and subtropical countries. Bananas belong to genus *Musa*, which has traditionally been divided into four sections based on chromosome number: *Australimusa* (2n = 20), *Callimusa* (2n = 20), which also includes *M. beccarii* (2n = 18), *Eumusa* (2n = 22) and *Rhodochlamys* (2n = 22) (Simmonds and Weatherup 1990). Most of edible cultivars of bananas are parthenocarpic seed sterile vegetatively propagated diploid and polyploid forms of *M. acuminata* (A genome) and hybrids that originated from crosses between *M. acuminata* and *M. balbisiana* (B genome). Both *M. acuminata* and *M. balbisiana* belong to the *Eumusa* section. Other edible cultivars, called Fe´i bananas, belong to the section *Australimusa*. The clonal nature of all cultivars facilities rapid spread of diseases, which are controlled by extensive use of pesticides. Unfortunately, breeding of improved cultivars is hampered by seed sterility, lack of knowledge on the origin of cultivated clones and poor knowledge on genetic diversity within *Musa*, as well as a lack of information on genome structure.

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Abbreviations: BAC - bacterial artificial chromosome; FISH - fluorescence *in situ* hybridization; DAPI - 4',6-diamidino-2-phenylindole; FITC - fluorescein isothiocyanate; PFGE - pulsed field gel electrophoresis.
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Nuclear genome of *Eumusa* species is relatively small (1C ~ 600 Mbp, Doležel *et al*. 1994) and divided to 11 chromosomes, which are small (1 - 2 μm) and similar in morphology. This hampers chromosome identification and evaluation of chromosome structural changes during evolution and breeding. The classical cytogenetic banding techniques for identification of the mitotic banana chromosomes failed (unpublished). One possibility to identify banana chromosomes is the use of chromosome-specific DNA sequences and their cytogenetic mapping by fluorescence *in situ* hybridization (FISH). The most widely used probes for FISH are the ribosomal RNA (rRNA) genes, which are organized in clusters (Long and Dawid 1980, Appels *et al*. 1980, Ellis *et al*. 1988), and other tandem organized repetitive DNA sequences, which result in strong hybridization signals on plant chromosomes (Pedersen *et al*. 1996, Tsujimoto *et al*. 1997, Navrátilová *et al*. 2003).

Previous studies of the *Eumusa* species revealed that 45S rRNA genes were localized in the secondary constriction of one pair of chromosomes, while 5S rRNA genes were localized on 2 - 4 different chromosome pairs (Doleželová *et al*. 1998, Osuji *et al*. 1998, Bartoš *et al*. 2005). Until now, all known banana repetitive DNA sequences were found dispersed on all chromosomes of *M. acuminata* except one part of the retrotransposon monkey and repetitive DNA clone *Radka*14 that localized to secondary constriction (Balint-Kurti *et al*. 2000, Valárik *et al*. 2002).

Other useful cytogenetic markers are clones isolated from large insert DNA libraries, *e.g*. cloned in bacterial artificial chromosomes (BAC) (Hanson *et al*. 1995, Lapitan *et al*. 1997, Kim *et al*. 2002, Mokroš *et al*. 2006). Most of BAC libraries comprise clones carrying inserts 70 - 200 kb long (Vilarinhos *et al*. 2003, Šafář *et al*. 2004, Ortiz-Vazquez *et al*. 2005). If the detection limit of FISH on plant mitotic chromosomes is 10 kb (Lehfer *et al*. 1993, Valárik *et al*. 2004) the inserts of the BAC clones should be easy to detect. Indeed, BAC clones were used successfully as probes for FISH in several plant species (Hanson *et al*. 1995, Jiang *et al*. 1995, Lapitan *et al*. 1997, Zhang *et al*. 2004). At the same time, some studies with BAC-FISH revealed that long inserts contain different types of repetitive DNA sequences that result in dispersed FISH signals (Zhang *et al*. 2004). In order to reduce hybridization of repetitive DNA, various types of blocking DNA and/or 'low-copy' subclones were used (Zhang *et al*. 2004, Janda *et al*. 2006).

Several BAC libraries have been constructed in banana, including a BAC library from *M. acuminata* cv. Calcutta 4 (MA4 BAC library, Vilarinhos *et al*. 2003), BIBAC library of *M. acuminata* cv. Tuu Gia (TGBIBAC library, Ortiz-Vazquez *et al*. 2005) and BAC library of *M. balbisiana* cv. Pisang Klutuk Wulung (MBP BAC library, Šafář *et al*. 2004). Their availability opens a possibility to develop new cytogenetic markers for *Musa*. In this study, we used the MA4 BAC library for identification of the low- and single-copy BAC clones with the aim to develop chromosome-specific cytogenetic markers for *M. acuminata* cv. Calcutta 4.

Materials and methods

Plants: *Musa acuminata* Colla cv. Calcutta 4 ITC 0249 was obtained from the *INIBAP Musa Transit Centre* (ITC, Katholieke Universiteit, Leuven, Belgium) as *in vitro* rooted plants. After transfer to soil, the plants were maintained in a greenhouse.

Identification of 'low-copy' BAC clones: MA4 BAC library was spotted onto nylon membranes *Hybond* N^+ (*AP Biotech*, Piscataway, USA) with the *GeneTAC G3* robot (*Genomic Solutions*, Huntingdon, UK) and the colonies were grown at 37 ºC overnight. The colonies were lysed using 10 % sodiumdodecyl sulphate for 4 min, denatured by 0.5 M NaOH and 1.5 M NaCl for 5 min, neutralized by 0.5 M Tris-HCl and 1.5 M NaCl for 5 min and washed in 2× SSC for additional 5 min at room temperature. Finally the filters were washed with 1 mg cm^{-3} pronase at 40 ºC for 45 min to remove the bacteria residues. DNA was crosslinked using UV cross-linker (*Stratagene*, Cedar Creek, USA). The membranes were screened with a probe for genomic DNA of *M. acuminata*, which was labeled using the *AlkPhos Direct Kit* (*Amersham Biosciences*, Little Chalfont, UK). Hybridization was performed according to manufacturer's instructions. Putative 'low-copy' BAC clones were selected based on weak signals after hybridization with genomic DNA of cv. Calcutta 4. Selected BAC clones were cultured overnight in 100 cm^3 2YT medium (Sambrook *et al*. 1989) supplemented with 15 μg cm^{-3} chloramphenicol. BAC DNA was isolated by alkaline lysis and digested with *Not*I enzyme to estimate insert size of BAC clones. DNA of BAC clones were separated by PFGE on 1 % agarose gel in 0.5× TBE at the following conditions: 6 V switch time 5 - 40 s, angle 120° for 12 h at 14 °C along with the Lambda ladder PFG marker (*New England Biolabs*, Beverly, USA).

Subcloning: Selected BAC clones were either digested with *Pst*I and ligated into pBlueScript SK^+ and transformed to *E. coli* XL1 electro competent cells, or mechanically sheared into fragments 5 - 10 kb long using the *HydroShear* (*GeneMachines*, San Carlos, USA). Sheared DNA fragments were incubated with mung bean nuclease (*New England Biolabs*) for 30 min at 30 ºC, cleaned up by *PCR Rapid Kit* (*Invitek*, Berlin, Germany) and de-phosphorylated with alkaline phosphatase CIP (*New England Biolabs*) at 37 °C for 1 h, and purified. Taq DNA polymerase (2U) and 0.4 M dATP were added to the 0.05 cm^3 of sample and incubated at 72 °C for 30 min to add 3'adenine to the dsDNA (Yuan *et al*. 2003). DNA fragments were then purified, ligated into *pCR-XL-TOPO*

vector and transformed into *One Shot TOP10* electrocompetent *E. coli* cells (*Invitrogen Life Technologies*, Carlsbad, USA). Subclones were plated onto bioassays and recombinant colonies were picked by *GeneTAC G3* robot into 384-well plates. Putative 'low-copy' subclones were selected by hybridization on nylon membranes with a probe for genomic DNA of cv. Calcutta 4.

DNA sequencing and sequence analysis: One BAC subclone that was successfully localized by FISH to a single locus and five additional BAC subclones that gave FISH signals in different regions of all banana chromosomes were sequenced at The University of Arizona (Tucson, USA). The sequenced parts of BAC subclones were assembled by the Staden Sequence Analysis Package (Staden 2000) and edited using the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequence data were compared using the Dotter software (Sonnhammer and Durbin 1995) and searched for homology to sequences in the GeneBank database using *BLAST 2.0.2* (Altschul *et al*. 1997).

Chromosome preparations: Metaphase spreads were prepared as described by Doleželová *et al*. (1998). Actively growing root tips were pre-treated in 0.05 % 8-hydroxyquinoline for 3 h and fixed in 3:1 ethanol : acetic acid. Fixed roots were washed in a solution of 75 mM KCl and 7.5 mM EDTA (pH 4). Meristem tips were digested in a mixture of 2 % pectinase and 2 % cellulase for 90 min at 30 ºC. Protoplast suspension was then filtered through a 150 µm nylon mesh and pelleted. The pellet was resuspended in 75 mM KCl and 7.5 mM EDTA (pH 4) and incubated for 5 min at room temperature. After pelleting, the protoplasts were washed three times with 70 % ethanol, and 0.005 cm^3 of suspension were dropped onto a slide. Shortly before drying out, 0.005 cm^3 of 3:1 fixative were added to the drop to induce protoplast bursting. Finally, the slide was rinsed in 100 % ethanol and air-dried.

Fluorescence *in situ* hybridization (FISH): The hybridization mix consisted of 40 % formamide, 10 % dextran sulfate in 1× SSC, 5 µg cm^{-3} salmon sperm DNA and 1 µg cm^{-3} labeled probe. All probes were labeled by *Dig-11-dUTP* or *Bio14-dATP Nick Translation* (*Amersham Biosciences*) according to manufacturer's instructions. The hybridization mixture was added to slides and denatured at 80 °C for 4 min. The hybridization was carried out at 37 °C overnight. The sites of probe hybridization were detected using anti-digoxigenin-FITC (*Roche Applied Science*, Penzberg, Germany) and *streptavidin-Cy3* (*Vector Laboratories*, Burlingame, USA), and the chromosomes were counterstained with DAPI. The slides were examined with *Olympus AX 70* (Tokyo, Japan) fluorescence microscope and the images of DAPI, FITC and Cy-3 fluorescence were acquired separately with a cooled high-resolution black and white CCD camera. The camera was interfaced to a PC running the *MicroImage* software (*Olympus*).

Results

Identification of 'low-copy' BAC clones: The availability of three genomic BAC libraries constructed from different banana genomes provides an opportunity to isolate chromosome-specific cytogenetic markers. In this study, we used BAC library from *M. acuminata* cv. Calcutta 4 (MA4 BAC library, Vilarinhos *et al*. 2003) as a source of clones that were tested for FISH on mitotic chromosomes of the same species. With the aim to select out BAC clones containing highly repetitive DNA sequences, sixteen 384-well plates of the MA4 BAC library were spotted onto a nylon membrane and hybridized with a probe for genomic DNA of *M. acuminata*. Based on the intensity of hybridization signals, eighty BAC clones potentially carrying low amounts of repetitive DNA were identified. The analysis of insert size after *Not*I restriction (Fig. 1) showed that all selected BAC clones carried inserts of 70 - 150 kbp. Out of the eighty BAC clones, 12 clones gave no visible signals after FISH on mitotic chromosomes, while 67 BAC clones gave signals distributed on all mitotic chromosomes. Only one BAC clone (2G17) localized to a single locus in subtelomeric region on one pair of mitotic chromosomes of *M. acuminata*.

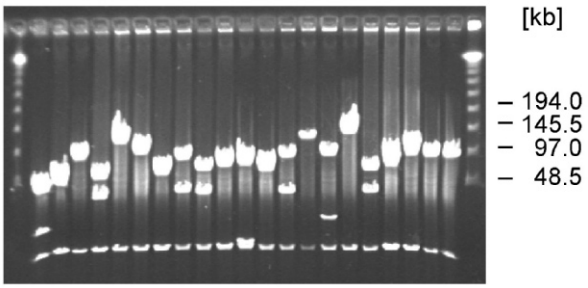

Fig. 1. Determination of BAC insert size. BAC clones from *M. acuminata* cv. Calcutta 4 were isolated using alkaline lysis and digested by *Not*I. Pulse-filed gel electrophoresis with *MidRange PFG Marker I* (*New England Biolabs*) was used to determine insert sizes of individual BAC clones.

Localization of BAC clones on mitotic chromosomes: We then used multicolor FISH to identify the chromosome to which the BAC 2G7 localized. FISH with a probe for 5S rDNA demonstrated that BAC 2G7 localized to one of the 5S rDNA-bearing chromosomes with the 5S rDNA locus in peri-centromeric region (Fig. 2). Most of BAC clones, which gave visible FISH signals, showed dispersed organization in the genome of *M. acuminata*. Due to small

size of banana mitotic chromosomes it was difficult to identify telomeric and sub-telomeric regions as well as centromeric and peri-centromeric regions. This fact hampered the division of BAC clones into specific groups according to the chromosomal distribution of FISH signals. Nevertheless, we have identified BAC clones that hybridized to peri-centromeric regions of all chromosomes (*e.g.* BAC clone 72P16, 70P20 and 6D20) and a BAC clone (3B6) that localized preferentially to telomeric and sub-telomeric regions (Fig. 2).

Organization of BAC subclones on mitotic chromosomes: Six BAC clones (2E2, 2E19, 3B7, 3E7, 2G23 and 2G17) including the chromosome-specific BAC 2G17 were selected for subcloning after digestion with *Pst*I. Other 13 BAC clones (1E20, 1F11, 2H7, 2L19, 3G3, 3H22, 4B4, 4I2, 5K5, 5E12, 6D9, 6D20 and 6E11) were subcloned by mechanical shearing. BAC subclones carrying fragments 5 - 10 kbp long were used as probes for FISH. Subclones of BAC 2G17 (5 and 10 kbp) localized to the same region as the whole BAC clone 2G17 (Fig. 3). 5 kbp-long subclone of BAC 2E2 that gave dispersed signals localized to the secondary constriction only (data not shown). FISH with a 10 kbp-long subclone of BAC 6D9 that gave dispersed signals revealed preferential hybridization in centromeric regions of all chromosomes, with additional weak signals on one arm of several chromosomes (Fig. 3). A 10 kbp subclone of BAC 6D20, which gave strong signals in centromeric regions and weak signals on distal parts of all chromosomes, localized in centromeric regions of all chromosomes (Fig. 3). Subclones of the other 'dispersed' BAC clones gave dispersed signals on all chromosomes similar to those obtained after FISH with whole BAC clones. In some cases FISH with BAC subclones did not result in visible signal on any chromosome.

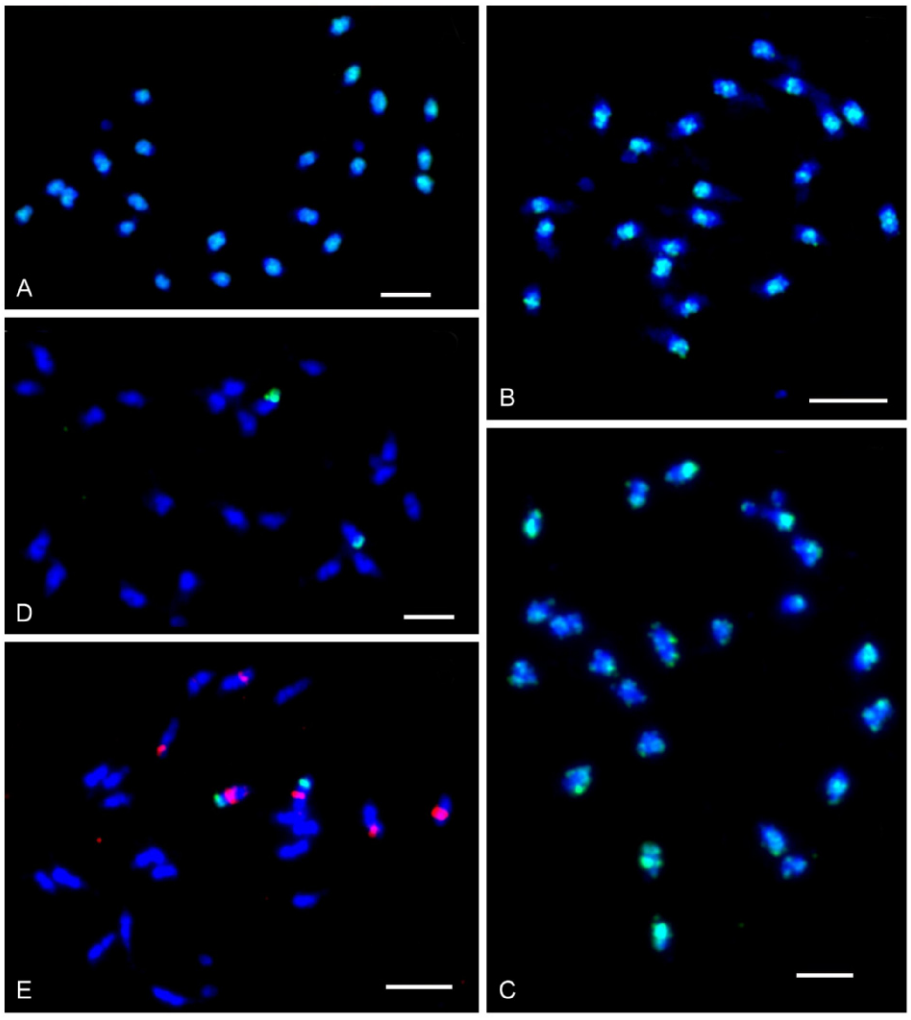

Fig. 2. Localization of putative 'low-copy' BAC clones on mitotic chromosomes of *M. acuminata* cv. Calcutta 4 (2n = 22). FISH with BAC MA4_9D8 resulted in dispersed signals on all chromosomes (*A*), BAC clone MA4_72P16 localized to pericentromeric regions of all chromosomes (*B*) and BAC clone MA4_3B6 preferentially localized to subtelomeric regions (*C*). BAC clone MA4_2G17 gave a single locus-specific signal on one pair chromosomes (*D*), which carried one cluster of 5S rRNA genes (E). BAC clones were labeled by digoxigenin (green signals) and 5S rDNA was labeled by biotin (red signals). *Bar* = 5 μm.

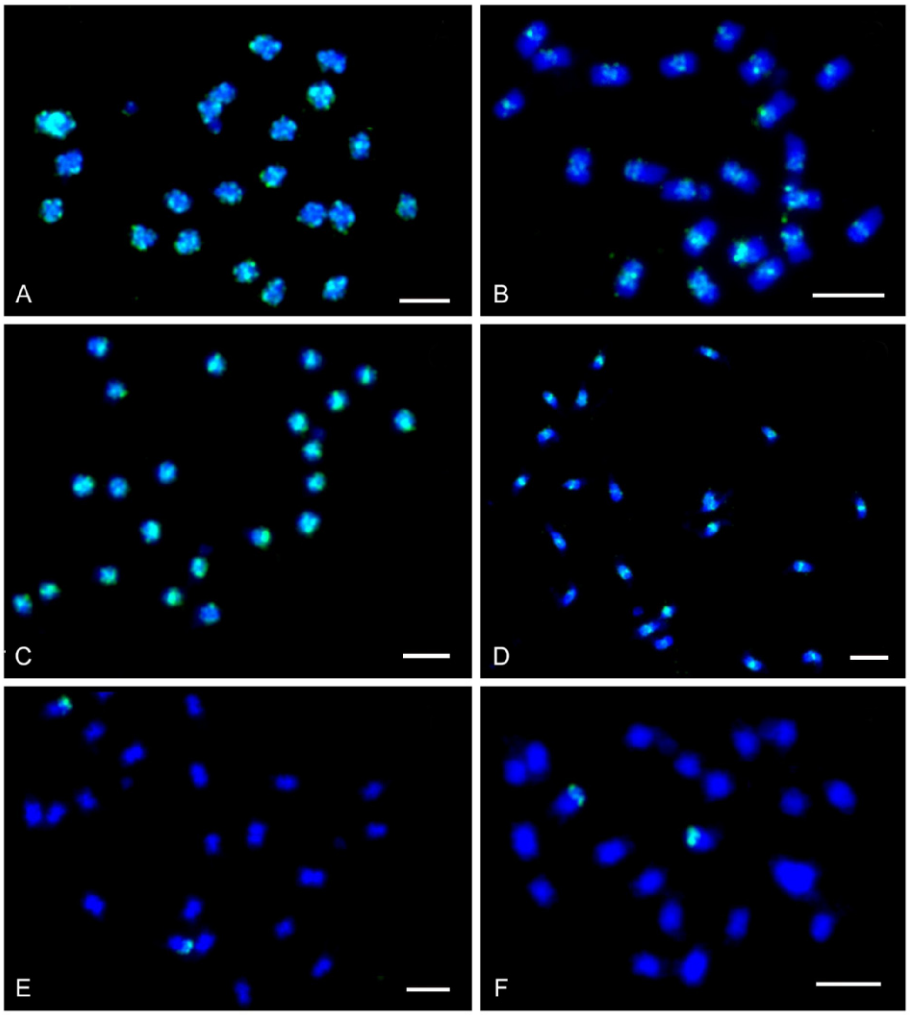

Fig. 3. Localization of BAC clones on mitotic chromosomes of *M. acuminata* cv. Calcutta 4. FISH with BAC clone MA4_9D8 resulted in dispersed signals along all chromosomes (*A*). A subclone of MA4_6D9 (6D9/J2) localized to centromeric regions of all chromosomes with additional weak hybridization signals on one arm of several chromosomes (*B*). BAC clone MA4_6D20 gave visible FISH signals in centromeric and peri-centromeric regions (*C*). Subclone of BAC MA4_6D20 (6D20/3) localized to centromeric regions of all chromosomes (*D*). *E* - a subclone of BAC MA4_2G17 (2G17/14) mapped to the same region as the whole BAC clone (*F*). *Bar* = 5 μm.

Table 1. Genomic distribution and sequence homology of selected BAC subclones (SSP - smallest sum probability).

BAC subclone	Genomic distribution (FISH)	DNA sequence	GenBank accession No.	SSP
2G17/14	Chromosome-specific	*Musa balbisiana* clone MBP_81C12	AC186754	$1e^{-49}$
6D20/3	Centromeric regions of all chromosomes	*Musa balbisiana* clone MBP_71C19	AP009325	$1e^{-168}$
6D9/J2	Centromeric regions of all chromosomes and weak signals on one arm of several chromosomes	transposon related region - copia like	AC186756	$1e^{-138}$
5K5/C14	Centromeric and pericentromeric signals on all chromosomes	copia like DNA sequence	AC186752	$1e^{-95}$
2E19/10	Dispersed signals on all chromosomes	Putative cyclase family protein of rice	NM001068058	$2e^{-24}$
5E12/A16	Dispersed signals on all chromosomes	*Musa acuminata* clone *Radka*8 and clone *Radka*9	AF399948 AF399938	$2e^{-135}$ $1e^{-127}$

Sequence analysis of selected subclones: BAC subclone 2G17/14 that localized to single locus was selected for sequencing and more than 13 kbp of nucleotide sequence was obtained. The sequences were assembled using contig assembly program (*CAP*, *BioEdit*) into five different contigs with lengths ranging from 1000 to 1526 bp. Out of

them, only 504 bp long part of one contig showed homology with *M. balbisiana* clone MBP_81C12 deposited in GenBank (Table 1). The remaining sequences did not show homology to any known DNA sequence.

Three subclones 6D20/3, 6D9/J2 and 5K5/C14 which gave centromeric and/or peri-centromeric signals on all banana chromosomes were sequenced. More than 7 kbp of nucleotide sequences of BAC subclone 6D20/3 were assembled into two contigs, 800 and 861 bp long. One contig was fully homologous to a non-annotated sequence of *M. balbisiana* clones MBP 94I16 and MBP 71C19, and *M. acuminata* sequence MuG9 (Aert *et al*. 2004). A 408 bp fragment of the other contig of 6D20/3 was homologous to other parts of BAC clones MBP 71C19 and MBP 94I16 and also showed homology to non-annotated sequence of MUSA8 clone that carries repeat family of cacao swollen shoot badnavirus (Ndowora *et al*. 1999). 2.6 kbp of nucleotide sequences of subclone 6D9/J2 were assembled into one 1242 bp long contig. Its 600 bp region showed homology to retrotransposon-related region of copia retrotransposon. Similarly, a 600 bp part of the sub-BAC 5K5/C14 was homologous to copia-like sequence (Table 1).

More than 6.5 and 9.8 kbp of nucleotide sequences were obtained from subclones 2E19/10 and 5E12/A16, respectively. FISH with both subclones resulted in dispersed signals on all chromosomes. Nucleotide sequences of sub-BAC 2E19/10 were assembled into 3 contigs. One contig showed weak homology ($2e^{-24}$) to putative cyclase family protein of rice. Nucleotide sequences of BAC subclone 5E12/A16 were assembled into 2 contigs, which showed homology to different parts of already sequenced *Musa* BAC clones (http://www.tigr.org/tdb/e2k1/maa1/) and also to *Radka*8 and *Radka*9 repetitive DNA sequences (Table 1).

Discussion

Previous studies showed that BAC libraries were powerful tools for isolation of locus-specific markers, and the so called BAC-FISH technique was used successfully to localize chromosome-specific BAC clones (Hanson *et al*. 1995, Lapitan *et al*. 1997, Kim *et al*. 2002). BAC clones anchored to genetic maps were used as cytogenetic markers to compare genetic and cytogenetic maps and to study chromosome translocations in different plant species (Song *et al.* 2000, Kim *et al.* 2002, Lengerová *et al.* 2004). Hasterok *et al.* (2006) showed a possibility to localize heterologous BAC clones for physical mapping in *Brachypodium distachyon*. An attractive possibility is the localization of pooled repeat-free PCR products from BAC clones (Lamb *et al.* 2007).

Despite the relatively small size of banana nuclear genome, and hence a lower proportion of DNA repeats, isolation of chromosome-specific clones from a genomic BAC library was not efficient. To the best of our knowledge, until now, only four BAC clones have been mapped to banana mitotic chromosomes by FISH (Vilarinhos *et al.* 2006). The reason for the low efficacy of BAC-FISH on mitotic metaphase chromosomes of *Musa* is not clear and contrasts with the relative success in other species (Lapitan *et al*. 1997, Kim *et al*. 2002, Mokroš *et al*. 2006). One explanation could be a particular molecular organization of *Musa* chromosomes with many dispersed DNA repeats. While many sequenced BAC clones were shown to contain various classes of dispersed repeats, such has as the *Radka* sequences (Valárik *et al.* 2002, Aert *et al.* 2004), this hypothesis needs verification.

In the present work, we examined eighty putative low-copy BAC clones and only one of them (BAC 2G17) localized to a single locus. Strong FISH signals are typical for tandem organized repetitive sequences or tandem organized genes. We suspect that BAC 2G17 carries a specific tandem repeat or tandem organized genes, *e.g*., a gene family. Other banana BAC clones localized to all mitotic chromosomes. Dispersed signals after FISH with most of the BAC clones were clearly due to the presence of repetitive DNA sequences. Although we attempted to select out clones carrying repetitive DNA, the detection limit of Southern hybridization presumably did not permit elimination of BAC clones with one or a few copies of different types of repeats. Sequence data of randomly selected BAC clones of *M. acuminata* (MuG9 and MuH9) showed that gene-rich BAC clones contain parts of repetitive DNA sequences, mainly the *Radka* sequences (Aert *et al.* 2004). The *Radka* sequences are known to be dispersed throughout the banana genome (Valárik *et al.* 2002).

Banana BAC clones that gave no visible signals after FISH on mitotic chromosomes could carry chloroplast and/or mitochondrial DNA. Contamination of organelle DNA in the MA4 BAC library was estimated to be 1.5 % (Vilarinhos *et al.* 2003). However, in our work the percentage of BAC clones with no visible signals on banana mitotic chromosomes after FISH was ten times higher. This increase could be due to the removal of highly repetitive BAC clones after hybridization with genomic DNA. Other reason for the failure of FISH with some BAC clones could be the presence of DNA sequences coming from chromosomes regions not accessible to hybridization. Low accessibility could be due to high compactness of chromatin of mitotic metaphase chromosomes. If this is the case, a solution could be to perform FISH on less condensed meiotic (pachytene) chromosomes as has been done in other species (Lysák *et al.* 2001). However, until now a reliable method for pachytene FISH in *Musa* has not been reported.

With the aim to obtain DNA fragments free of repetitive DNA, we subcloned selected BAC clones. Despite relatively short inserts of subclones (5 - 10 kbp), only one subclone derived from a ‘dispersed’ BAC localized to a single locus. Other subclones localized to all

banana chromosomes and/or they did not give visible signals after FISH. These observations could be explained in a similar way as the results obtained with the whole BAC clones.

Selected BAC subclones were sequenced with the aim to understand the nature of their FISH patterns. Subclones 6D9/J2 and 5K5/C14 showed homology to different parts of copia-like repetitive element. The presence of different parts of the same type of repetitive DNA sequence correlated with the similar FISH pattern. Subclone 5E12/10 that gave strong dispersed FISH signals carries DNA sequence homologous with *Radka*8 and *Radka*9 repeats, which gave strong dispersed hybridization FISH signals on mitotic chromosomes (Valárik *et al.* 2002). Interestingly, homology was found also for the short part of subclone 2G17. The subclone carries a 504 bp part with homology to unknown DNA sequence of *M. balbisiana* BAC clone MBP_81C12. Better characterization of nucleotide sequence of the single-locus BAC clone would require sequencing the entire clone.

To conclude, we have identified one chromosome-specific BAC clone. This increases the number of useful cytogenetic markers for *Musa* that can be used to study genome organization and its changes during evolution and breeding. At the same time our results point to the low efficacy of developing cytogenetic markers from genomic BAC libraries. Considering the urgent need for more cytogenetic markers, other strategies will have to be pursued to reach this goal.

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