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Molecular Cytogenetics of *Musa* Species, Cultivars and Hybrids: Location of 18S-5.8S-25S and 5S rDNA and Telomere-like Sequences

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The physical sites of 18S-5.8S-25S and 5S rRNA genes and telomeric sequences in the *Musa* L. genome were localized by fluorescent *in situ* hybridization on mitotic chromosomes of selected lines. A single major intercalary site of the 18S-5.8S-25S rDNA was observed on the short arm of the nucleolar organizing chromosome in each genome. AA and BB genome diploids had a single pair of sites, triploids had three sites while a tetraploid hybrid had four sites. The probe is useful for quick determination of ploidy, even using interphase nuclei from slowly growing tissue culture material. Variation in the intensity of signals was observed among heterogeneous *Musa* lines indicating variation in the number of copies of the 18S-5.8S-25S rRNA genes. Eight subterminal sites of 5S rDNA were observed in Calcutta 4 (AA) while Butohan 2 (BB) had six sites; some were weaker in both genotypes. Triploid lines showed six to nine major sites of 5S rDNA of widely varying intensity and near the limit of detection. The diploid hybrids had five to nine sites of 5S rDNA while the tetraploid hybrid had 11 sites. The telomeric sequence was detected as pairs of dots at the ends of all the chromosomes analysed but no intercalary sequences were seen. The molecular cytogenetic studies of *Musa* using repetitive and single copy DNA probes should yield insight into the genome and its evolution and provide data for *Musa* breeders, as well as generating genetic markers in *Musa*.

Key words: Genome evolution, nucleolar organizing regions, telomeres, *in situ* hybridization, genetic markers, banana, plantain.

INTRODUCTION

Bananas and plantains are important food and cash crops (FAO, 1995) and more molecular and chromosomal information about their genomes is required to complement our knowledge of their breeding and genetics (Rowe, 1984; Bakry *et al*., 1990; Vuylsteke *et al*., 1997). The small size of the genome (Dolezel, Dolezelova and Novak, 1994) and chromosomes (Isobe and Hashimoto, 1994; Osuji, Okoli and Ortiz, 1996*a*) and the difficulty in obtaining dividing cells (Osuji, Okoli and Ortiz, 1996*a*, *b*) have made cytogenetic study difficult. Now methods of molecular cytogenetics are enabling us to characterize the genomes more fully (Osuji *et al*., 1997). The principal edible species are in the section *Eumusa* of the genus *Musa* and comprise *M. acuminata* Colla ($2n = 2x = 22$; A genome; 600 Mbp) and *M. balbisiana* Colla ($2n = 2x = 22$; B genome; 550 Mbp), and their hybrids; the triploid lines ($2n = 3x = 33$) with genome constitutions AAA (dessert or export banana), AAB (plantain) and ABB (cooking banana) are economically important (see Simmonds, 1976; Gowen, 1995).

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Evolution of species, and selection of cultivars, depend on the reorganization and mutation of genes (Stace, 1980) and alteration in the repetitive DNA which makes up a majority of most plant genomes (Heslop-Harrison and Schwarzacher, 1993). The ability to identify individual chromosomes and examine any reorganization, as well as correlate them with marker genes, is valuable for plant breeding. Molecular cytogenetic techniques involving *in situ* hybridization of DNA sequences have proved to be a valuable method to gain insight into the genome.

Ribosomal RNA (rRNA) genes (present as 18S-25S rDNA, comprising the 18S-5.8S-25S rRNA genes and intergenic spacers, and 5S rDNA with the 5S rRNA gene and spacer) are found universally in plants, with multiple copies of the coding sequences and intergenic spacers normally being located at a few discrete chromosomal sites. Over the past decade, examination of the numbers and sizes of the sites of rRNA genes by *in situ* hybridization has contributed insight into the genomes of various plants including members of the Triticeae (de Bustos *et al*., 1996; Vershinin, Alkhimova and Heslop-Harrison, 1996), *Lolium* (Thomas *et al*., 1996), tomato (Kiss, Kis and Solomosy, 1989) and *Vigna* (Galasso *et al*., 1995). Variation of the copy number and distribution of these genes in chromosomal DNA of closely related species reflect their evolutionary

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relationship as reported for the Triticeae (McIntyre *et al*., 1990; Moore *et al*., 1991; Mukai, Endo and Gill, 1991; Mukai, Nakahara and Yamamoto, 1993; Anamthawat-Jónsson and Heslop-Harrison, 1992) and *Brassica* (Iwabuchi, Itoh and Shimamoto, 1991), and is useful to track linkage groups during evolution (Dubcovsky and Dvorak, 1995). Another highly conserved sequence is found at the physical ends of chromosomes of nearly all plant species. Multiple copies of a motif similar to the seven nucleotide unit TTTAGGG are added by an enzyme, telomerase, incorporating an RNA template, and function in stabilising the chromosome ends and enabling semi-conservative replication of the DNA up to the telomerase-added region. Fuchs, Brandes and Schubert (1995) have reviewed the organization of telomeric sites in various plant species and show a few plant species where intercalary sites are present, or terminal sites are not detected.

In the present work, we aimed to analyse the distribution and organization of the 18S-5.8S-25S and 5S rDNA sequences, as well as the telomeric sequence, in the *Musa* genome, as a contribution to the physical mapping of the genome, and to provide markers for examining chromosomal evolution during plant breeding and speciation.

MATERIALS AND METHODS

The accessions used for this work were obtained from the *Musa* field genebank of the International Institute of Tropical Agriculture (IITA) located at Onne (4° 51′ N, 7° 3′ E and 5 m above sea level), near Port Harcourt in Nigeria (see Swennen, 1990). Sword suckers obtained from these accessions were trimmed and transferred to a glasshouse at the John Innes Centre, UK and maintained at a temperature of 39 ± 6 °C in the day, 11 ± 2 °C at night, and high humidity. The plants include wild diploid *Musa* species (*M. acuminata* 'Calcutta 4'—A genome and *M. balbisiana* 'Butohan 2'—B genome), a diploid dessert banana (Pisang Lilin), two triploid dessert bananas (Valery and Dwarf Cavendish), three plantains (Mbi Egome, Obino l'Ewai and Agbagba), three cooking bananas (Bluggoe, Fougamou and Cardaba) and three plantain hybrids (TMPx 8084-1, AA; TMPx 4698-1, AAAB; and TMPx 2829-62, AA; see Osuji *et al*., 1997). The hybrids are the result of crossing between plantain and the wild diploid banana 'Calcutta 4' (Vuylsteke and Ortiz, 1993).

The method adopted for spreading chromosomes followed Osuji *et al*. (1997). Fresh secondary roots were collected from vigorously growing plants and fixed in 3:1 (v/v) absolute ethanol:acetic acid. No pretreatment was used because additional condensation of the small *Musa* chromosomes was undesirable (Osuji *et al*., 1996*a*). Apices of the fixed secondary roots were excised, washed thrice in 1 × enzyme buffer (0·01 M citric acid-sodium citrate, pH 4·6) for 9 min with shaking to remove fixative, and digested in 1·5 ml enzyme solution containing 2% cellulase (Onuzuka R10, Yakult Honsha Co, Tokyo) and 20% liquid pectinase (from *Aspergillus niger*, Sigma) in enzyme buffer (Schwarzacher *et al*., 1989) for 1 h at 37 °C. Digested root tips were washed thrice with 1 × enzyme buffer and squashed in a drop of 60% glacial acetic acid under a No. 1 (18 × 18 mm) coverslip. Squashing was followed by slight warming over a spirit flame and gentle pressing (Osuji *et al*., 1996*b*). The coverslip was removed after freezing on dry ice and slides were air-dried.

A marked area of each slide containing the chromosome spread was treated with 100 μl of 100 μg ml^{-1} DNase-free RNase in 2 × SSC (20 × SSC: 3 M sodium chloride plus 0·3 M trisodium citrate) solution, covered with a plastic coverslip and incubated at 37 °C for 1 h. Slides were washed three times (for 5 min each) in 2 × SSC (shaking), treated with 10 μg ml^{-1} pepsin, covered with a plastic coverslip, incubated at 37 °C for 30 min and washed for 5 min in 2 × SSC (shaking). Slides were pre-fixed with 4% paraformaldehyde for two × 5 min, washed twice in 2 × SSC (for 5 min), dehydrated in 70% alcohol for two × 5 min and 100% alcohol for 5 min before air drying.

The probes used were pTa71 which has a 9 kb EcoRI fragment containing the 18S-5.8S-25S rDNA genes and intergenic spacer regions isolated from *Triticum aestivum* (Gerlach and Bedbrook, 1979) and pTa794 which has a complete 410 bp 5S gene unit and spacer region isolated from *T. aestivum* (Gerlach and Dyer, 1980). The coding sequences are homologous to all plant species tested so far, but the intergenic spacers are not conserved. The probes pTa71 and pTa794 were labelled with biotin-11-dUTP and digoxigenin-11-dUTP (Boehringer Mannheim), respectively. The telomeric probe, a synthetic oligomer $(CCCTAAA)_6$, was labelled with digoxigenin-11-dUTP by random primer labelling. The hybridization mixture consisted of 20–30 ng of probe DNA (0·5 μl of pTa71 and 1 μl of pTa794, or 1 μl of telomeric clone), 15 μl 100% formamide, 6 μl 50% dextran sulphate, 10% SDS (sodium dodecyl sulphate, in water), 2 μg salmon sperm DNA, 3 μl 20 × SSC in a total volume of 30 μl probe mixture per slide. The mixture was denatured at 70 °C in a water bath for 10 min and cooled in ice for 5 min. Chromosome preparations were treated with hybridization mixture, covered with plastic coverslips and denatured at 80 °C for 5 min in an Omnislide humid chamber and incubated at 37 °C overnight.

After hybridization, preparations were washed for two × 5 min with 2 × SSC, two × 5 min with 20% formamide in 0·1 × SSC (the most stringent wash, allowing sequences with more than 85–90% similarity to remain hybridized), two × 5 min in 2 × SSC, all at 42 °C (shaking), and then two × 5 min in 2 × SSC followed by 4 × SSC/0·02% Tween 20 (detection buffer, shaking) at room temperature. To detect hybridization sites, preparations were first blocked by incubating with 100 μl per slide of 5% w/v bovine serum albumen (BSA) in 4 × SSC/0·02% Tween 20, covered with plastic coverslips at room temperature for 5 min. Hybridization sites were detected by adding 100 μl per slide of detection mixture which contained Cy3-streptavidin and sheep anti-digoxigenin conjugated to fluorescein isothiocyanate (anti-dig-FITC) in BSA and incubating for 1 h at 37 °C. Chromosome preparations were washed twice (5 min each time) in 4 × SSC/Tween 20 and counterstained with 6 μg ml^{-1} DAPI (4′-6-diamidino-2-phenylindole in McIlvaine's citrate buffer, pH 7·0) solution under plastic coverslips for 10 min. Counterstained preparations were

quickly washed in 4 × SSC/Tween 20 and mounted in Antifade solution (Citifluor Glycerol/PBS solution AF1) for observation under Leitz epifluorescence UV microscope with Leitz filter sets (A for DAPI, I2/3 for Cy3, N2 for FITC and Omega Optical Triple bandpass). Photomicrographs were taken on Fujicolor Super HG 400 colour print film, scanned to Kodak PhotoCD, and printed from Adobe Photoshop using only cropping, contrast, colour, overlay and brightness functions which affect the whole image equally. Optimization for publication truncated the range of signal strength in some cases.

RESULTS

18S-25S rDNA

In situ hybridization showed a single 18S-25S major intercalary rDNA site on the short arm of the nucleolar organizing chromosome in each genome; no minor sites were detected consistently and reproducibly (Figs 1–6). The short arms of the chromosomes with the 18S-25S rDNA, seen clearly in prophase nuclei, condensed later than the other chromosome arms, often giving the chromosome an extended appearance. Thus the AA and BB genome diploid species had a single pair of sites (Figs 1 and 2). The triploid hybrids had three sites, and some variation in signal intensity was detected between the three sites indicating different numbers of copies of the 18S-25S rDNA repeat units (Figs 3–5). The tetraploid hybrid TMPx 4698-1 had four 18S-25S rDNA sites (not shown) while the diploid hybrids TMPx 2829-62 (not shown) and TMPx 8084-1 (Fig. 6) had two sites.

5S rDNA

Two to four chromosomes per genome carried major 5S rDNA sites, but there was considerable variation in signal strength between sites. The number of sites per genome varied between varieties and because minor sites, seen in many figures, were near the limit of sensitivity of detection or level of background (Figs 1–6). No sites were detected on chromosomes carrying 18S-25S rDNA. One of the strong pairs of 5S loci was a 'double' site, seen to consist of two hybridization sites separated by a short gap along the chromosome (Figs 1–5; most clear in the diploid hybrid TMPx 8084-1, Fig. 6b). In the diploid species, eight subterminal sites of 5S rDNA were observed in Calcutta 4 (AA), while Butohan 2 (BB) showed six 5S rDNA sites (Figs 1 and 2), perhaps with other minor sites near the limit of detection. The diploid cultivar Pisang Lilin (AA, not shown) had five (four major, sub-terminal and one minor, intercalary) 5S rDNA sites.

Triploid lines showed approximately six (Dwarf Cavendish, Fig. 4, and Valery, AAA) to eight (Agbagba, Fig. 3, and Obino l'Ewai, AAB) or nine (Fougamou, ABB, Fig. 5) 5S rDNA sites, but the number of weaker sites varied depending on the variety and hybridization conditions. Among the diploid hybrids, TMPx 8084-1 (Fig. 6) showed five sites of the 5S rDNA and TMPx 2829-62 showed six to eight sites while the highest number of hybridization sites, 11, was seen in the tetraploid hybrid TMPx 4698-1 (not shown).

Telomeric probe

Hybridization of the telomeric sequence was detected as pairs of dots, of varying intensity, at the ends of nearly all the chromosomes analysed (Fig. 7). There was no evidence for intercalary sites of the telomeric sequence.

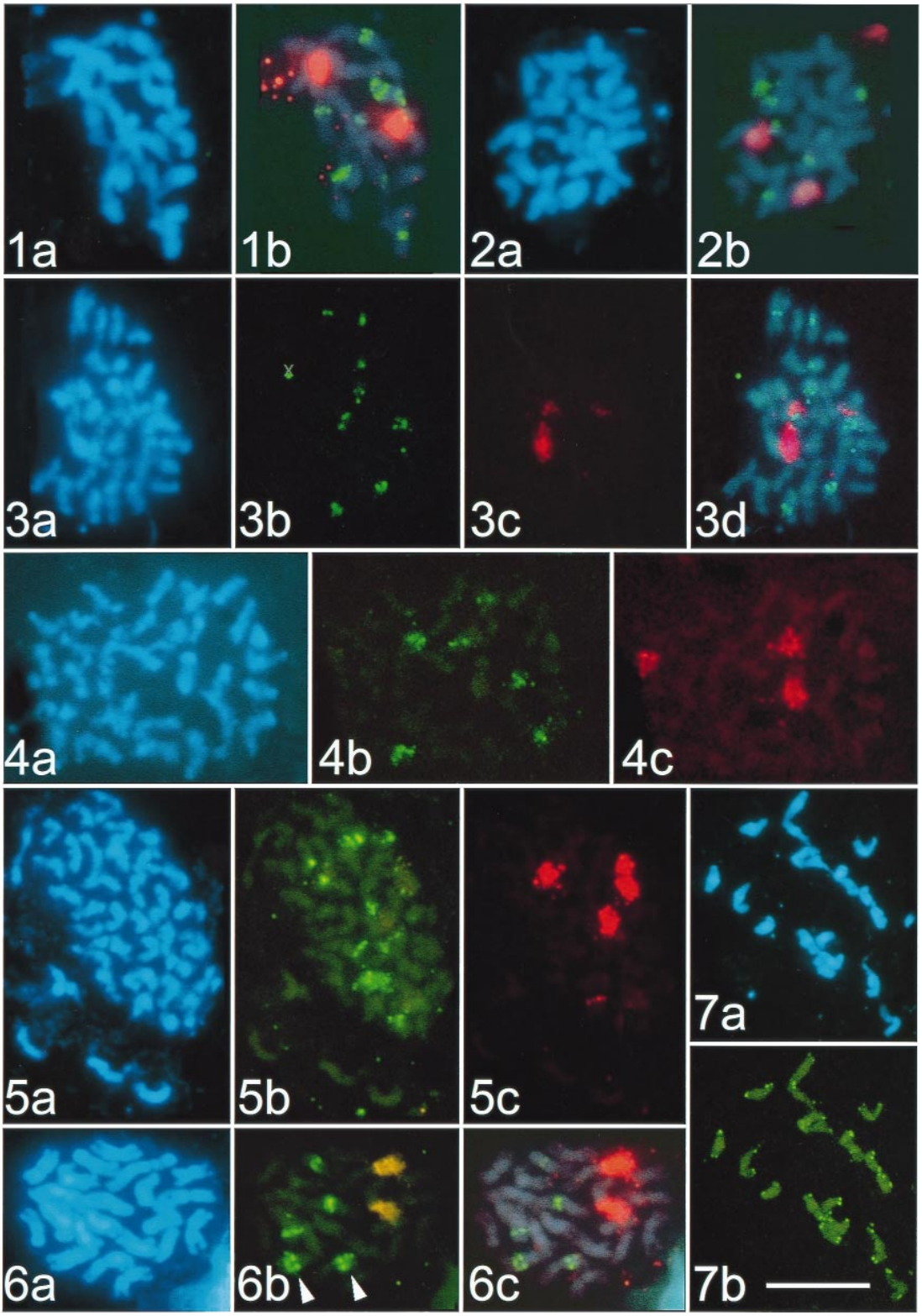
DISCUSSION

The results presented extend our knowledge of the organization of conserved repetitive DNA sequences into the large neo-topical taxonomic order Zingiberales. 18S-25S and 5S rDNA (Figs 1–6) from bread wheat and telomeric sequences (Fig. 7) characterized in many other species, show strong hybridization to *Musa* chromosomes. The sequences provide useful chromosome markers and information about the evolution and variability of the chromosomes.

Only one of the 11 chromosomes per genome of the two *Musa* species analysed carried an 18S-25S rDNA site reproducibly detected by *in situ* hybridization (Figs 1–6). Among the sample of plant species examined so far, no correlation of chromosome number, chromosome size, or taxonomic position, with number of 18S-25S rDNA sites, is evident. For example, rye ($2n = 14$) has only a single pair of sites, while the related barley (also $2n = 14$) has six pairs of sites, two major and four minor (Leitch and Heslop-Harrison, 1992). Among the legumes, *Vigna unguiculata* ($2n = 2x = 22$) had four major and one minor pair of 18S-25S rDNA sites (Galasso *et al*., 1995), while *Vicia faba* ($2n = 2x = 12$ large chromosomes) has only a single major pair (Pearce *et al*., 1996).

Diploid (as normally considered) *Brassica* species also have variable numbers of 18S-25S rDNA sites: two in *B. nigra* ($2n = 16$) and five in *B. campestris* ($2n = 20$) per haploid genome (Maluszynska and Heslop-Harrison, 1993). Thus the observation of a single pair of sites on the 11 chromosomes of each genome of *Musa* is low but probably not exceptional. If molecular polymorphisms can be found, the sequence will provide a useful anchor point to correlate one pair of *Musa* chromosomes with the corresponding linkage group in the genetic map (Faure *et al*., 1993) of the bananas. The 18S-25S rDNA probe may also be used to assess ploidy quickly by hybridization to interphase nuclei —including in tissue culture—without the need for chromosome counting or DNA measurement.

Multiple 5S rDNA sites, of varying strength, were detected in the *Musa* species and hybrids; such multiple sites have been detected in all other species analysed using *in situ* hybridization. The variation in number of sites observed in *Musa* probably reflects both loss or gain of sites, and amplification or reduction of number of gene copies at individual loci (around the threshold of detection). The process is presumably continuing within both diploid species



FIGS 1–7. *In situ* hybridization to chromosomes of *Musa* (banana and plantain) species, cultivars and hybrids. Blue DAPI staining shows chromosomes while in Figs 1–6, red and green signals show sites of hybridization of 18S-25S rDNA and 5S rDNA, respectively. In some micrographs, possible minor sites of 5S rDNA are visible. Scale bar = 10 μm. Fig. 1. *Musa acuminata* 'Calcutta 4' ($2n = 2x = 22$, AA genomes) with two sites of 18S-25S rDNA and eight major sites of 5S rDNA including a double site on one pair of chromosome arms. Fig. 2. *Musa balbisiana* 'Butohan 2' ($2n = 2x = 22$, BB genomes) with two sites of 18S-25S rDNA and six major sites of 5S rDNA. Fig. 3. Plantain cultivar 'Agbagba' ($2n = 3x = 33$, AAB genomes) with three sites of 18S-25S rDNA and eight sites of 5S rDNA, including the pair of double sites.

and hybrids. The significance of 'double' sites of 5S rDNA loci in the *Musa* lines is unknown; similar sites have been found in *Hordeum* species analysed with a similar resolution of *in situ* hybridization (shown but not noted by de Bustos *et al*., 1996; Taketa, Harrison and Heslop-Harrison, pers. comm.). It will be interesting to discover more about the genetic linkage of the sites and whether both can be active.

As with the 18S-25S rDNA sites, hybridization of the 5S rDNA probes has considerable potential for chromosome identification as in barley (Leitch and Heslop-Harrison, 1993), and hence for following individual chromosomes through breeding programmes, particularly in combination with total genomic DNA probes (Osuji *et al*., 1997) and linking the genetic and physical chromosome maps.

The ends of *Musa* chromosomes showed strong homology to the synthetic telomeric oligonucleotide sequence (CCCTAAA), and no intercalary concentrations of this sequence. The terminal sites were small, and copies of the sequence are likely to be present on all chromosome arms. Changes in the behaviour of the telomeric sequence are important during hybridization, cell culture and evolution. In barley, chromosomes may break but are then restabilized (healed) by addition of the telomere sequence (Wang, Lapitan and Tsuchiya, 1992). Both in mammals (Harley, Futcher and Greider, 1990) and barley (Kilian, Stiff and Kleinhofs, 1995), there is evidence that the number of copies of the telomere motif changes during tissue culture, and this may relate to the loss of chromosomes or deletion of genes near chromosome ends during culture. In plant lines derived from hybrids, with additional chromosome fragments, the fragments may be unstable and perhaps lack telomere sequences (Schmidt *et al*., 1997).

Molecular cytogenetic investigations of plant genomes using repetitive and single-copy DNA probes is proving valuable for both fundamental and applied aspects of studies of genome evolution and plant breeding. Their application to *Musa* species, cultivars and artificial hybrids adds to our knowledge of the genome and its continuing evolution, particularly under the stresses of intensive breeding, tissue culture and disease in commercial plantations. With the many challenges in breeding of banana and plantains, including the use of hybrids, resistance to disease including banana streak virus (BSV) and tissue culture leading to somaclonal variation (Vuylsteke, Swennen and De Langhe, 1991), knowledge about repetitive sequences including rDNA, the telomere and their variability and applicability as chromosome and genetic markers is valuable. In particular, the use of the 18S-25S rDNA probe to determine ploidy in slowly dividing tissue culture material may be valuable.

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x, Stain precipitate. Fig. 4. Dessert banana cultivar 'Dwarf Cavendish' ($2n = 3x = 33$, AAA genomes) with three sites of 18S-25S rDNA and six sites of 5S rDNA. Fig. 5. Cooking banana cultivar 'Fougamou' ($2n = 3x = 33$, ABB genomes) with three sites of 18S-25S rDNA and about nine sites of 5S rDNA. The small red 18S-25S site (lower centre) was not seen in other metaphases, and probably represents displaced signal. Fig. 6. Plantain hybrid TMPx 8084-1 ($2n = 2x = 22$, AA genomes) with two sites of 18S-25S rDNA and five sites of 5S rDNA. Arrowheads show double sites of 5S hybridization on single chromosome arms. Fig. 7. *In situ* hybridization of the telomere sequence (CCCTAAA) to chromosomes of the wild diploid banana 'Calcutta 4', showing a pair of sites at the ends of most arms of the 22 chromosomes (green signal).

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