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The interspecific genome structure of cultivated banana, *Musa spp.* revealed by genomic DNA *in situ* hybridization

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Abstract In cultivated banana, *Musa* spp., there are four known genomes, A, B, S, and T. These correspond to the genetic constitutions of wild *Eumusa* species *M. acuminata*, *M. balbisiana*, *M. schizocarpa* and the *Australimusa* species, respectively. Most cultivated clones are triploid or diploid, they have been classified into genomic groups according to chromosome numbers and morphological traits. Genomic *in situ* hybridization (GISH) enabled us to differentiate the chromosomes of these four genomes; however, a distal portion of the chromosomes remained unlabelled. GISH was used to determine the exact genome structure of interspecific cultivated clones. In most cases the results were consistent with the chromosome constitution estimated by means of phenotypic descriptors. The one notable exception, the clone 'Pelipita', has the 8 A and 25 B chromosomes instead of the predicted 11 A and 22 B. GISH also enabled us to determine the chromosome complement of a few clones that could not be classified only on the basis of phenotypic descriptors and chromosome counts. The rDNA sites were located in *Musa* species. They appeared to be often associated with satellites, which can be separated from the chromosomes, representing a potential source of error for chromosome counting using classical techniques.

Key words *Musa* spp · Banana · Genomic *in situ* hybridization · Aneuploidy · Polyploidy · rDNA genes

Introduction

Banana cultivars are derived from natural hybridization between wild diploid *Musa* species. They are diploid, triploid, or tetraploid, the current cultivated clones being mostly triploids. *Musa acuminata* (A genome, 2n=2x=22) is involved in all the cultivars, *M. balbisiana* (B genome, 2n=2x=22) in many of them (Simmonds and Shepherd 1955) and *M. schizocarpa* (S genome, 2n=2x=22) and the *Australimusa* species (T genome, 2n=2x=20) in a few of them (Sharrock1989; Tezenas du Montcel 1989; Carreel 1994). Intercrossing among species and subspecies may have led to the appearance of sterility, a character selected for early on by men in conjunction with parthenocarpy, thereby enabling the production of edible fruits. Classification of cultivated genotypes has been based on their morphological similarities to wild diploid species and their ploidy level (Cheesman 1947, Simmonds and Shepherd 1955), the main groups being AA, AAA, AAB, and ABB. The production of export dessert bananas relies on a very few related genotypes having the AAA genome constitution, while numerous dessert and cooking types with an AAA, AAB (including plantain), or ABB genome constitution are grown for local consumption.

Bananas (and plantains) are susceptible to an ever increasing range of fungal, viral, and insect diseases which, in the absence of locally adapted resistant varieties, can only be controlled by the use of pesticides. Chemical control represents a considerable economic and environmental cost. Thus, there is an urgent need for a wider diversity of genetically improved cultivars with disease resistance, increased productivity, and adaptability to a wider range of growing conditions. It is mainly through genetic improvement that these goals can be achieved. Consequently, breeding programs aimed at broadening the genetic basis and providing new resistance genes have been undertaken using wild and cultivated diploid clones. To this end, a better knowledge of the available genetic diversity and of the origin and genome structure of current cultivars is needed.

Molecular markers that provided useful information and new insight into the classification have been available for several years (Horry and Jay 1988; Lebot et al.1993; Carreel 1994; Carreel et al.1994; Jarret and

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Gawel 1995; Baurens et al.1997; Grapin et al.1998). Genomic *in situ* hybridization (GISH) often enables vizualization of the portion of the genome contributed by each parental species in interspecific hybrids and thus constitutes a powerful complementary tool. It is now the method of choice in plants for testing the origin of natural amphiploids, tracking down the introgression of alien chromosomes, or testing the occurrence of exchange between the genomes involved (Jiang and Gill 1994; Bennett 1995; Gill and Friebe 1998).

In this paper we demonstrate the possibility of differentiating the chromosomes from the four genomes (A, B, S and T) of banana cultivars using GISH. We then report on the use of this technique in investigating the genome structure of interspecific cultivated clones and discuss the potential and limitations of GISH in relation to the small size of the *Musa* genome.

Materials and methods

Materials

Plants of ten interspecific cultivars (Table 1) were analyzed, from which the roots were harvested. Total genomic DNA from four wild clones of known AA, BB, SS and TT genomic constitution were extracted to be used as probes. These clones were the cultivar 'Tomolo', PNG 23 related to *M. acuminata banksii* Mueller (AA); *M. balbisiana* Colla (BB) 'Tani' THA021; *M. schizocarpa* Simmonds (SS) PNG 172; and *M. angustigemma* Simmonds (TT) PNG 158. The clones were provided by CIRAD-Guadeloupe and cultivated in a glasshouse in Montpellier.

Chromosome preparations

Roots were harvested from plants cultivated in pots. They were treated in 0.04% hydroxyquinoline for 8 h; 4 h at room temperature and then 4 hours at 4°C. They were fixed for 48 h in ethanol: acetic (3:1) acid and stored in 70% ethanol at 4°C. Chromosome preparations were performed as described in D'Hont et al. (1996).

Fluorescent in situ hybridization (FISH)

In situ hybridization, washes, the detection of biotin with Texas Red and digoxigenin with FITC (fluorescein isothiocyanate), and amplification were performed as described in D'Hont et al. (1996). AA total genomic DNA and rDNA probes (PTA71) were labelled with digoxigenin-11-dUTP and detected with FITC. BB, SS, and TT total genomic DNA were labelled with biotin-14-dUTP and detected with Texas Red. The hybridization mixture (30 µ l per slide) consisted of 50% formamide, 10% dextran sulphate, 2 × SSC, 1% SDS, and 6.5 ng/ml of each parental total genomic DNA probe. In one experiment 5 ng/ml of the rDNA probe was added to the mix. The chromosomes were counterstained with DAPI (4'.6-diamidino-2-phenylindole). The slides were mounted in vectashield antifade solution (Vector Lab) and photographed on Fujicolor 400 print film.

Table 1 Classification of the clones in genomic groups based on ploidy level, morphological and molecular data, and chromosome constitution based on GISH

Clones	Genomic groups	GISH results
Safet Vechi	AB	11A-11B
Wompa	AS	11A-11 S
Karoina	AAT/ATT?	22A-10 T
Laknao	AAB	22A-11B
P. Raja bulu	AAB	22A-11B
Luba	AAB ?	22/23A-10/11B
M. Bouroukou (Plantain)	AAB	21/22A-11/12B
Nyombé (Plantain)	AAB	21/22A-11/12B
Pelipita	ABB	8A-25B
Yawa 2	BATB ?	11A-22B-10 T

Results

Differentiation of chromosomes from the A and B genome

In situ hybridization was carried out on chromosome preparations of 'Safet Vechi', a diploid cultivar which based on morphological characters is expected to be derived from natural hybridization between *M. acuminata* (2n=22) and *M. balbisiana* (2n=22). Digoxigenin-labelled *M. acuminata* genomic DNA was used as one probe, detected by FITC, and biotin-labelled *M. balbisiana* genomic DNA was the second probe, detected by Texas Red. In the hybrid, all the chromosomes were labelled green and red; because of sequence homologies between the genomes of the two species, there was however variation in the intensity of the coloration among the chromosomes. When the two colors were superimposed, sequence differences between the genomes allowed to differentiate two populations of chromosomes. In the preparation, 11 chromosomes fluoresced yellow and thus could be related to *M. acuminata*, whereas 11 fluoresced red-orange and thus could be related to *M. balbisiana* (Fig. 1b and Table 1). One of the A chromosomes (yellow) displayed a red-orange terminal segment associated with a nucleolar constriction, which corresponds to a rDNA site. These highly conserved genes hybridize to DNA of both species. Their detection as red-orange fluorescence, which has been previously described in similar experiments conducted on other plants (D'Hont et al. 1996, Barre et al. 1998), suggests that the red fluorescence is stronger than the green one. In Fig. 1a, the DAPI stain shows the satellite separated from the main part of the chromosome.

The parental origin of each chromosome in the hybrid could be determined unambiguously. However, the chromosomes were not entirely labelled: a distal portion of variable size, depending on the chromosomes, remained unlabelled, as seen by comparing DAPI staining and GISH results (Fig. 1a, b). In order to verify if this lack of labelling was due to the origin of the genomic DNAs used as probes, we hybridized genomic DNA of 'Safet Vechi' on chromosome preparations of 'Safet Vechi'. The result showed that even under these conditions the

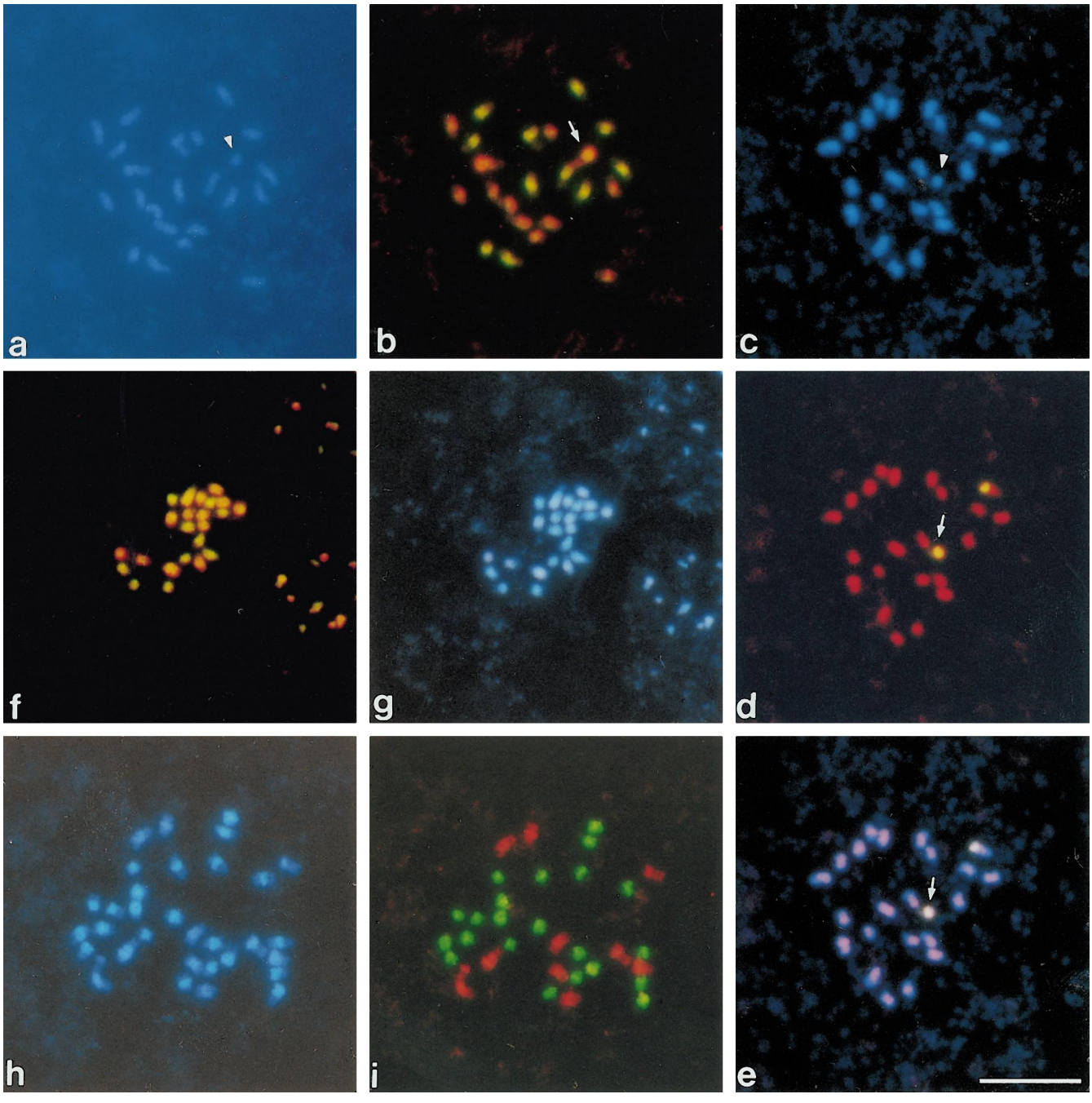

Fig. 1 a–i GISH on somatic metaphase of interspecific *Musa* hybrids. 'Safet vechi' (genome AB) after DAPI (**a**) and after GISH using total DNA from an AA clone revealed with FITC and total DNA from a BB clone revealed with Texas Red (**b**). Safet Vechi after DAPI (**c**), after GISH using 'Safet vechi' total DNA revealed with Texas Red and the rDNA probe PTA71 revealed with FITC (**d**), and after superimposition of DAPI and GISH (**e**). 'Wompa' (genome AS) after DAPI (**g**) and after GISH with total DNA from a AA clone revealed with FITC and total DNA from from a SS clone revealed with Texas Red (**f**). 'Karoina' (genome AAT) after DAPI (**h**) and after GISH using total DNA from a AA clone revealed with FITC and total DNA from a TT clone revealed with Texas Red (**i**). *Arrowheads* indicate satellites separated from the main part of the chromosome, and *arrows* indicate the associated rDNA sites. Bar: 10 µm

distal part of the chromosomes remained unlabelled (Fig. 1c–e). In this experiment two rDNA sites were revealed by the probes PTA71 detected with FITC (yellow), one is associated with a satellite separated from the main part of the chromosome.

Differentiation of chromosomes from the A and S genome

In situ hybridization was carried out on chromosome preparations of 'Wompa', a diploid cultivar shown by molecular markers to be derived from natural hybridization between *M. acuminata* and *M. schizocarpa*. *M.*

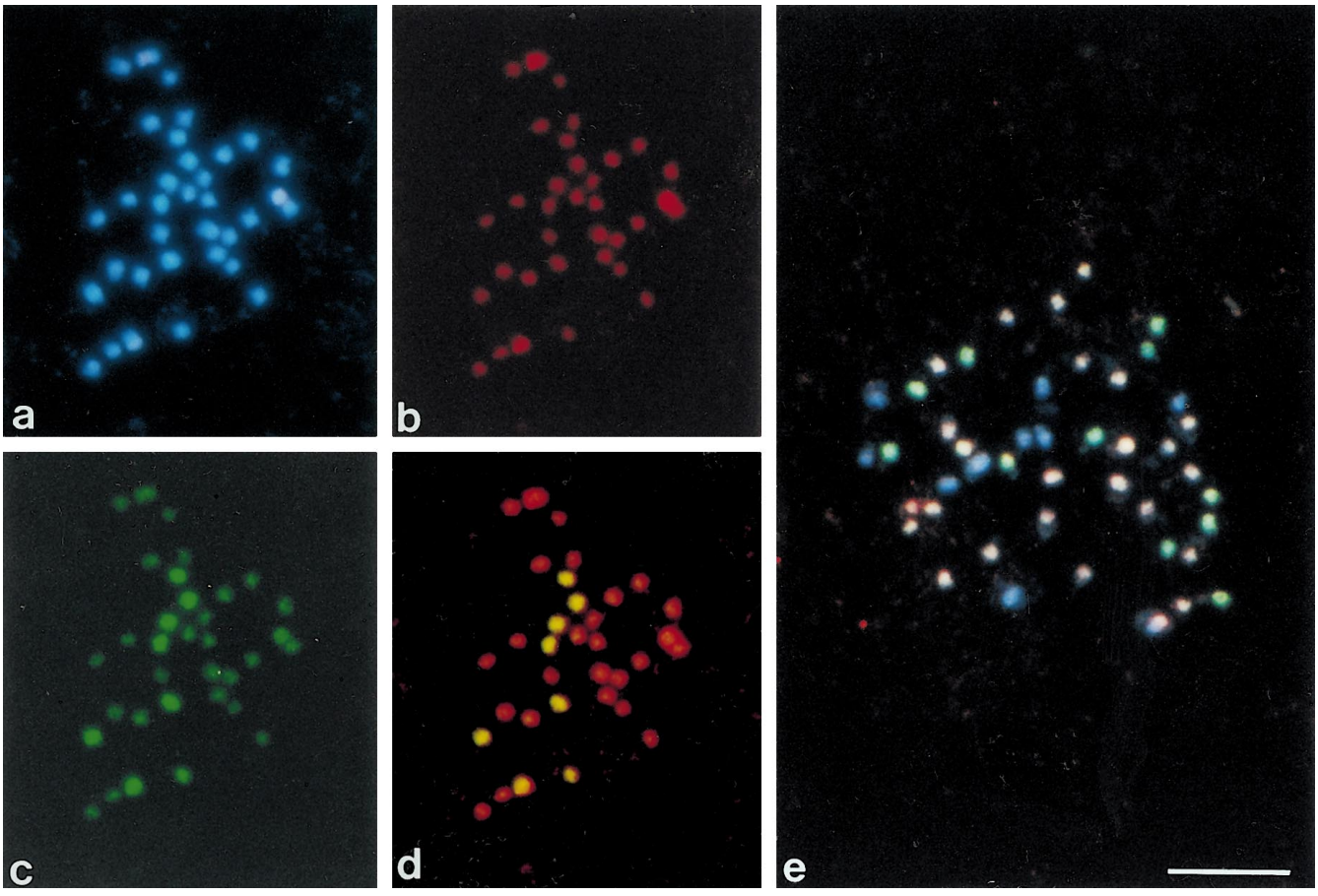

Fig. 2 GISH on somatic metaphase of cultivated interspecific *Musa* hybrids. 'Pelipita' (genomic group AAB) after DAPI (**a**), after GISH using total DNA from a BB clone revealed with Texas Red (**b**), after GISH using total DNA from a AA clone revealed with FITC (**c**), and after superimposition of **b** and **c** (**d**). 'Yawa 2' (genome ABBT) after superimposition of DAPI and GISH using total DNA from a AA clone revealed with FITC and total DNA from a BB clone revealed with Texas Red (**e**). Bar: 10 µm

acuminata genomic DNA was used as one probe, revealed with FITC, and *M. schizocarpa* genomic DNA was the second probe, revealed with Texas Red. In the hybrid all the chromosomes were labelled by the two parental probes (in green and red) with only a weak variation in intensity between chromosomes. However, when the two colors were superimposed, two populations of chromosomes were differentiated. Eleven chromosomes appeared yellow-orange and can be related to *M. acuminata* and 11 appeared red-orange and can be related to *M. schizocarpa* (Fig. 1f and Table 1). One satellite appeared strongly labelled in red and separated from the main part of a S chromosome.

Differentiation of chromosomes from the A and T genome

In situ hybridization was carried out on chromosome preparations of 'Karoina', a triploid cultivar classified ATT or AAT on the basis of morphological characters and molecular markers and thus probably derived from natural hybridization between *M. acuminata* and an *Australimusa* species. *M. acuminata* genomic DNA was used as one probe, revealed with FITC, and *M. augustigemma* genomic DNA was the second probe, revealed with Texas Red (Fig. 1h, i and Table 1). Very weak cross-hybridization was observed, and two very different populations of chromosomes were observed on the superimposed picture. Twenty-two chromosomes appeared green and can be related to *M. acuminata*, and 10 appeared red and can be related to *Australimusa* species. This cultivar can thus be classified AAT. The chromosomes from the T genome appeared larger than the ones from the A genome, and a comparison of GISH and DAPI staining (Fig 1h ,i) showed that the former were more extensively labelled.

Identification of the genome constitution of banana and plantain cultivars

In situ hybridization using *M. acuminata* genomic DNA as one probe, revealed with FITC, and *M. balbisiana* genomic DNA as a second probe revealed with Texas red, was carried out on a few banana and plantain cultivars in order to investigate their genomic constitution. The results are summarized in Table 1. For 'Pelipita', a cultivar

classified ABB, we identified 8 A and 25 B chromosomes instead of the 11 A and 22 B expected (Fig. 2a–d). For the three AAB banana cultivars, 'Laknao', 'P. raja bulu', and 'Luba' we identified, as expected, 22 A and 11 B chromosomes. For the two AAB plantain cultivars, 'M. bouroukou' and 'Nyombé', we identified around 21 A and 12 B chromosomes. For the last three clones, the quality of the preparation did not allow us to determine the exact number of A and B chromosomes (+/- 1 chromosome). Indeed, when the shape of the chromosomes is not well-defined, rDNA sites are difficult to indentify and thus satellites can be confused with whole chromosomes.

Identification of the trispecific genome structure of cultivar 'Yawa 2'

The A, B, and T genomes have been shown to be involved in cv 'Yawa 2' based on molecular markers. Hybridization was thus carried out on 'Yawa 2' chromosome preparations using *M. acuminata* total DNA as one probe, revealed with FITC, and *M. balbisiana* total DNA as a second probe, revealed with Texas red. Since the T genome showed very weak cross hybridization with genome A and, thus, by extension supposedly with genome B, no probe was used for genome T. Superimposition of FITC, Texas Red, and DAPI revealed the presence of three chromosome populations (Fig. 2e). Eleven chromosomes appeared green and can be related to *M. acuminata*, 22 chromosomes appeared red and can be related *M. balbisiana* and 10 unlabelled chromosomes appeared blue and can be related to *Australimusa* species.

Discussion

Using genomic *in situ* hybridization in interspecific hybrids, we demonstrated the possibility to differentiate the chromosomes of the four genomes (A, B, S and T) included in cultivated bananas. This follows a first report on the resolution of the A and B genomes by GISH (Osuji et al. 1997).

Classifications based on morphological descriptions (Simmonds and Weatherup 1990) and molecular markers (Carreel 1994) place *M. schizocarpa* very close to *M. acuminata*, with *M. balbisiana* being more distant. On the same basis *Australimusa* species (genome T) appear far apart from these three *Eumusa* species and have a different basic chromosome number of n=10 instead of the n=11 of the *Eumusa* species (Cheesman 1947). The distance between the species is in agreement with the color contrast observed between the chromosomes in hybrid preparations after GISH. Very little cross-hybridization was observed between the A and T genome; this results in a highly contrasted differentiation of the chromosomes. In contrast, important cross-hybridization between the two genomes was observed in the AB and AS hybrids, and the chromosomes were detected by both parental probes. However, variation in the respective intensity of labelling with each probe was observed among A and B chromosomes and to a lesser extent among A and S chromosomes. Superimposition of the two probes enabled two categories of chromosomes to be distinguished, with less contrast between the A and S chromosomes than between the A and B chromosomes.

The sizes of the A, B, S, and T genomes have been determined by flow cytometry (genomes A and B, Dolezel et al. 1994; genomes A, B, S and T, C. Jenny unpublished data). The T genome appears to be the largest followed by the S genome, the A genome and the B genome. According to Jenny (unpublished data) their sizes are 1.27 pg/2 C for the T genome (2n=20), 1.18 pg/2 C for the genome S (2n=22), 1.11 pg/2 C for 2n=22 for the A genome (2n=22), and 1.03 pg/2 C for the B genome (2n=22). These differences in DNA amount appear to be related to the size of the chromosomes observed at the metaphase stage. The A, S; and B banana chromosomes are not entirely labelled following GISH; a distal part of variable size, depending on the chromosome, remained unlabelled. The presence of these unlabelled chromosome ends appears to be related to genome sizes. We have already observed this phenomena in *Coffea* (Barre et al. 1998), and it has also been reported in other species with small genomes, *Brassica* (Fahleson et al. 1997) and *Rubus* (Lim et al. 1998). In our study, only the chromosomes of the largest genome, namely the genome T, were entirely labelled. The critical genome size below which difficulties may be encountered for obtaining an even GISH labelling seems to be around 0.6 pg/basic genome. This fits well with the idea that GISH is essentially based on the detection of repeated sequences whose amount constitutes the major source of variation in basic genome size in plants.

The rDNA sites in the different clones studied often appeared to be associated with the secondary nucleolar constrictions that delimited satellites. Quite often these satellites break away from the main part of the chromosomes. They may then be confused with whole, small chromosomes. This may explains at least partially, why aneuploidy is frequently reported in bananas (Cheesman and Larter 1935; Sandoval et al. 1996; Shepherd and Da Silva 1996). Clones are often reported to have a few additional chromosomes, usually between 1 and 2 for diploid clones and between 1 and 3 for triploid clones (Jenny et al. 1997). These apparent supernumerary chromosomes could correspond to a separated satellite, whose number can be at the most one per basic genome. After GISH, satelites appeared strongly labelled red-orange due to the presence of rDNA sites; thus, they can be distinguished from whole chromosomes.

GISH enables to differentiate chromosomes of the A, B, S, and T genomes. However, the fact that the chromosomes are not entirely labelled following GISH is an important limitation to the technique since it probably prevents, at least in most cases, the detection of potential interspecific intra-chromosomal exchanges. This could perhaps be solved by the development of repeated spe-

cies-specific telomeric or subtelomeric probes, as reported in other species such as sugarcane (Alix et al. 1998). However, these sequences must be infrequent since the lack of GISH labelling most probably reflects a lack of repeated sequences in the distal part of the chromosome.

Despite this limitation, GISH has a great potential to specify the chromosome constitution of current interspecific banana cultivated clones and new breeding materials. In the cultivated clones studied, GISH results were in most of the cases consistent with the chromosome constitution estimated through phenotypic descriptors. However, in one case, that of clone 'Pelipita', we identified 8 A and 25 B chromosomes instead of the expected 11 A and 22 B chromosomes. This demonstrates that important meiosis irregularities can happen. In other cases, where phenotypic descriptors are inefficient in classifying cultivars, GISH efficiently complements molecular markers to determine their genome constitutions. It is only recently that it has been confirmed using molecular markers that the species *M. schizocarpa* and species of the *Australimusa* section have contributed to the origin of some cultivars (Carreel 1994). However, it was not possible to determine in which proportion due to the difficulties in revealing alleles present on the differents hom(oe)ologous chromosomes using molecular markers. For instance Carreel (1994) showed that the S genome was involved to some extent in the cultivar 'Wompa'. GISH confirmed this result and demonstrated that the S genome contributed a full set of S chromosomes to this cultivar. In the same way, Carreel (1994) demonstrated, using molecular markers, that the T genome was involved in 'Karoina' and 'Yawa 2', but he could not determine to what extent. GISH showed that one basic set of T chromosomes is present in 'Karoina' and 'Yawa 2' and established their genome constitution as AAT and ABBT, respectively. Regarding Luba, a clone first classified as AB, GISH results confirmed the molecular markers data which suggested that it is a triploid of AAB constitution.

Further refinement in molecular cytogenetics will be useful in banana by providing physical markers. Our study pointed out one rDNA locus per basic genome in the *Eumusa* species studied. The rDNA locus could be mapped on the genetic map available (Fauré et al. 1993). This would allow the assignment of the chromosomes carrying an rDNA site to one particular linkage group and provide a first bridge between physical and genetic maps. The development of similar bridges (repeated sequences, BAC,...) for the various linkage groups will enable different chromosomes to be assigned to the respective linkage groups and will efficiently complement genetic mapping efforts. This would open the way for the investigation of structural rearrangements which are reported to be frequent in banana (K. Shepherd, unpublished data).These rearrangements lead to important meiosis irregularities and irregular chromosome transmission and may be involved in the development of sterility, a prerequisite for fruit production. Having access to structural polymorphism will thus provide additional criteria for selecting wild diploids among disease resistance donors for the creation of new triploid banana cultivars.

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