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M. A. Lysák · M. Doleželová · J. P. Horry
R. Swennen · J. Doležel

Flow cytometric analysis of nuclear DNA content in *Musa*

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Abstract Nuclear genome size variation was studied in *Musa acuminata* (A genome), *Musa balbisiana* (B genome) and a range of triploid clones differing in genomic constitution (i.e. the relative number of A and B genomes). Nuclear DNA content was estimated by flow cytometry of nuclei stained by propidium iodide. The A and B genomes of *Musa* differ in size, the B genome being smaller by 12% on average. No variation in genome size was found among the accessions of *M. balbisiana* (average genome size 537 Mbp). Small, but statistically significant, variation was found among the subspecies and clones of *M. acuminata* (ranging from 591 to 615 Mbp). This difference may relate to the geographical origin of the individual accessions. Larger variation in genome size (8.8%) was found among the triploid *Musa* accessions (ranging from 559 to 613 Mbp). This variation may be due to different genomic constitutions as well as to differences in the size of their A genomes. It is proposed that a comparative analysis of genome size in diploids and triploids may be helpful in identifying putative diploid progenitors of cultivated triploid *Musa* clones. Statistical analysis of data on genome size resulted in a grouping which agreed fairly well with the generally accepted taxonomic classification of *Musa*.

Key words *Musa acuminata* · *Musa balbisiana* · Bananas · Plantains · Flow cytometry · Genome size · Taxonomy

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M. A. Lysák · M. Doleželová · J. Doležel (✉)
De Montfort University Norman Borlaug Centre for Plant Science, Institute of Experimental Botany, Sokolovská 6, CZ 77200 Olomouc, Czech Republic
Fax: +420 68 5228523
E-mail: dolezel@risc.upol.cz; http://www.ueb.cag.cz/olomouc1

J. P. Horry
International Network for the Improvement of Banana and Plantain, Montpellier, France

R. Swennen
Laboratory of Tropical Crop Improvement, Katholieke Universiteit Leuven, Heverlee, Belgium

Introduction

Bananas and plantains (*Musa* spp.) are one of the major export commodities of many developing countries and provide food for millions of people in the tropics and subtropics. Despite its importance, the taxonomy of *Musa* has been studied only to a limited extent. Cheesman (1947) divided the genus into four sections (*Eumusa*, *Rhodochlamys*, *Callimusa* and *Australimusa*) and this classification is still in use. Plantains and most cultivated bananas belong to the section *Eumusa* with a basic chromosome number of $x = 11$. Most cultivars are seed-sterile triploids and their most probable genetic origin at the species level has been discussed by Simmonds (1962). It is believed that they originated as a result of natural inter- and intra-specific hybridization of the diploid *Musa* species, *Musa acuminata* Colla. and *Musa balbisiana* Colla., which possess the genomes A and B, respectively. The taxonomy of triploid cultivars was first studied by Simmonds and Shepherd (1955) using 15 morphological characters that differentiate *M. acuminata* from *M. balbisiana*. These authors classified triploid cultivars into genetic groups based on the relative contribution of their A and B genomes.

The production of bananas is threatened by many diseases caused by pathogenic fungi, bacteria or viruses (Gowen 1995; Robinson 1996). Until now, cross-breeding for improved cultivars has focused mainly on the production of tetraploid hybrids (Ortiz et al. 1995). The re-synthesis of triploids from resistant wild diploid

species and diploid cultivars was first proposed by Stover and Buddenhagen (1986). This is indeed possible and seems to be a promising route for the near future (Bakry and Horry 1994). Breeding would be greatly simplified if the wild progenitors of cultivated bananas and plantains were to be identified. However, until now attempts to do so has provided only limited results. While the genetic variation within *M. balbisiana* seems to be small, *M. acuminata* has diversified into several subspecies and many parthenocarpic landraces. As with triploid cultivars, the origin of the diploid landraces could not be fully elucidated from morphological data alone.

Clearly, an integrated approach is needed incorporating morphological, cytological and molecular studies to improve the knowledge of *Musa* taxonomy and phylogeny. Recently, molecular markers have been used to analyse the genetic diversity and evolution of the genus (Gawel et al. 1992; Howell et al. 1993; Lanaud et al. 1992; Kaemmer et al. 1997) and with some unexpected results. For instance, Carreel (1994), coupling nuclear, chloroplast and mitochondrial genome analyses, established links between several cultivars and *Musa* species and subspecies, and suggested the involvement of more than two *Musa* species in the origin of some cultivated clones. It is also expected that the development and application of molecular cytogenetics to *Musa* (Doležel 1996; Doleželová et al. 1997) will greatly improve an understanding of the chromosome structure and karyotype variation within this genus. In addition to the karyotype, genome size should also be constant within a population of interbreeding individuals. However, the speciation of *Musa* has been accompanied both by geographical isolation and the frequent occurrence of asexual propagation, which could favour the diversification of genome size.

The nuclear DNA content can be rapidly estimated by flow cytometry (Doležel 1991, 1998). Previously, we have shown that this method is reliable for an estimation of the nuclear DNA content in *Musa* (Doležel et al. 1994). The purpose of the present study was to analyse the extent of nuclear DNA content variation in *Musa* using accessions representing *M. acuminata*, *M. balbisiana* and a range of triploid clones. Furthermore, variation in genome size was assessed and compared with the current system of classification.

Materials and methods

Plant material

Six diploid ($2n = 2x = 22$) and ten triploid ($2n = 3x = 33$) *Musa* species and clones (Table 1) were procured as in vitro rooted plantlets and maintained in the greenhouse.

Chromosome counting

Chromosome numbers were established on slides prepared according to Doležel et al. (1998). Briefly, actively growing roots were treated with 0.05% 8-hydroxyquinoline and fixed in a 3:1 alcohol:acetic mixture. Meristem tips were digested in an enzyme mixture (1% pectinase, 0.5% pectolyase, and 0.5% cellulase) made in 0.1 M citrate buffer (pH 4.7). The suspension of released protoplasts was filtered through a 150-μm nylon mesh and pelleted. The pellet was re-suspended in 75 mM KCl and 7.5 mM EDTA (pH 4) and

Table 1 Taxonomic classification of *Musa* genotypes used in this study

Accession name	ITC Code[a]	Species or group	Subspecies of subgroup	Geographic origin
Diploids				
Niyarma Yik	0269	*acuminata*	*banksii*-derivative	New Guinea
Higa	0428	*acuminata*	*banksii*	New Guinea, Australia
Pisang Mas	0653	AA	Sucrier	Thailand, Malaysia
Pa (Rayong)	0672	*acuminata*	*siamea*	Thailand
(10852)	0094	*balbisiana*		Costa-Rica[b]
Cameroun	0246	*balbisiana*		Cameroon[b]
Triploids				
Gros Michel	1122	AAA	Gros Michel	Thailand, Malaysia, Indonesia[b]
Gran Enano	1256	AAA	Cavendish	Vietnam[b]
Red Dacca	0575	AAA	Red/Green Red	Malaysia
Agbagba	0111	AAB	Plantain (False Horn)	West and Central Africa
Obino l' Ewai	0109	AAB	Plantain (French)	West and Central Africa
Prata	0207	AAB	Pome	Brasil[b]
Silk	0348	AAB	Silk	India
Popoulou	0335	AAB	Popoulou	Polynesia
Maritú	0639	AAB	Iholena	Colombia[c]
Pelipita	0472	ABB		Philippines

[a] ITC Code: code assigned by the INIBAP International Transit Centre (Leuven)
[b] Oldest location known for these accessions. The true geographic origin could not be traced
[c] Possibly introduced from Polynesia in prehistoric times (Langdon 1993)

incubated for 5 min at room temperature. After pelleting, the protoplasts were washed three times with 70% ethanol. Seven microlitres of protoplast suspension was dropped onto a clean ice-cold microscope slide. Shortly before complete drying out, 7 µl of 3 : 1 fixative was added. The slide was then rinsed in 100% ethanol and air-dried at room temperature. Chromosomes were stained in a 3% Giemsa solution in 50 mM of phosphate buffer. Air-dried slides were mounted in Euparal and observed under an Olympus BX60 microscope using a 100×1.35 oil-immersion objective. Images were photographed on Ilford PAN F 50 film using a green optical filter. Chromosome counts were established in four plants of each genotype. In each plant, two slides were observed, each with at least five metaphase plates.

Flow cytometric analysis

Approximately 70 mg of *Musa* leaf midrib was harvested and transferred to a glass Petri dish. Approximately 10 mg of soybean leaf tissue (*Glycine max* cv Polanka, 2C = 2.500 pg DNA, Doležel et al. 1994) served as an internal reference standard. This material was chopped up in 1 ml of LB01 buffer (Doležel et al. 1989) which contained 50 µg/ml each of propidium iodide and RNase. The suspension of isolated nuclei was filtered through a 50-µm nylon mesh and the fluorescence of propidium iodide-stained nuclei was analysed with a Partec PAS II flow cytometer (Partec GmbH, Münster, Germany). This instrument was equipped with a high-pressure mercury arc lamp (HBO 100W/2L) and a $40 \times /1.32$ oil-immersion objective. A band-pass filter EM520 was used for excitation, TK560 was employed as a dichroic mirror and a long-pass RG590 served as an emission filter. The gain of the instrument was adjusted so that the G_0/G_1 peak of soybean was approximately on channel 200. The nuclear DNA content (in pg) of *Musa* was estimated as follows:

$$\text{2C nuclear DNA content} = \frac{2.500 \times \text{G}_0/\text{G}_1 \text{ peak mean of } \textit{Musa}}{\text{G}_0/\text{G}_1 \text{ peak mean of } \textit{Glycine}}.$$

Four plants were analysed per genotype and five different leaves were measured in most of the plants. In 20 plants only four healthy leaves were available for analysis. To avoid any bias due to instrument drift, leaves from one plant were measured on different days.

Statistical analysis

Statistical analyses were performed using the NCSS 97 statistical software (Statistical Solutions Ltd, Cork, Ireland). Nested ANOVA and a Bonferroni multi-comparison test were employed to analyse the variation in DNA content. Hierarchical cluster analysis was used to analyse the relationship between *Musa* accessions based on nuclear DNA content. The unweighted pair-group method (UPGM) was chosen for the analysis, using the Manhattan distance as a dissimilarity coefficient. The mean nuclear DNA contents estimated in individual plants were employed as operational units; genotypes were used as clustering groups.

Results

The chromosomes in *Musa* are small (1–2 µm) and are difficult to count on standard squash preparations. To avoid this problem, we have used a protoplast dropping technique which yielded well-spread metaphases suitable for chromosome counting. Chromosome numbers determined in this way confirmed the ploidy levels and chromosome numbers in all accessions analysed.

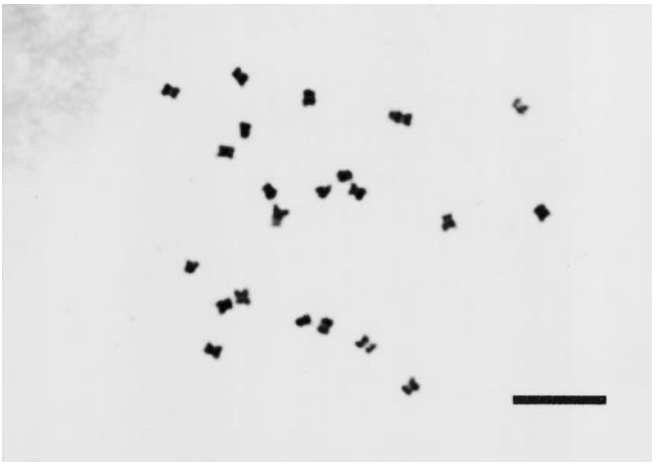

Fig. 1 Mitotic metaphase plate of 'Niyarma Yik' showing $2n = 2x = 22$ chromosomes. Bar = 10 µm

All diploid accessions had 22 chromosomes (Fig. 1) while all triploid genotypes had 33 chromosomes.

Flow cytometric analysis of isolated nuclei resulted in histograms of the relative nuclear DNA content containing two dominant peaks corresponding to the G_0/G_1 nuclei of *Musa* and *Glycine* (Fig. 2). The 2C nuclear DNA content ranged from 1.108 to 1.274 pg for diploids and from 1.737 to 1.905 pg for triploid accessions. Nested ANOVA indicated significant differences between the nuclear DNA content of individual genotypes ($P < 0.001$). On the other hand, no variation in DNA content between plants within a genotype and between leaves of the same plant was found (Table 2). A Bonferroni multi-comparison test and hierarchical cluster analysis were then used to characterise the variation in nuclear DNA content between the *Musa* species and clones (Table 3, Fig. 3). Table 3 also lists the values of genome size in Mbp, which can then be directly used in molecular studies.

Diploid species and clones

The lowest nuclear DNA content among the *Musa* diploids analysed in this study was found in the two *M. balbisiana* accessions '10852' and 'Cameroun', with a 2C DNA content of 1.108 and 1.121 pg respectively. Both genotypes have a BB genomic constitution and the difference in DNA content between them was not significant (Table 3). Compared to this, the 2C nuclear DNA content estimated for *M. acuminata* and clones with an AA genomic constitution was higher by approximately 12%, the difference between the BB and AA genotypes being statistically significant ($P < 0.01$). However, statistically significant differences in DNA content was also observed between the accessions with an AA genomic constitution (range 1.225–1.274 pg). While three AA genotypes ['Niyarma Yik', 'Pa

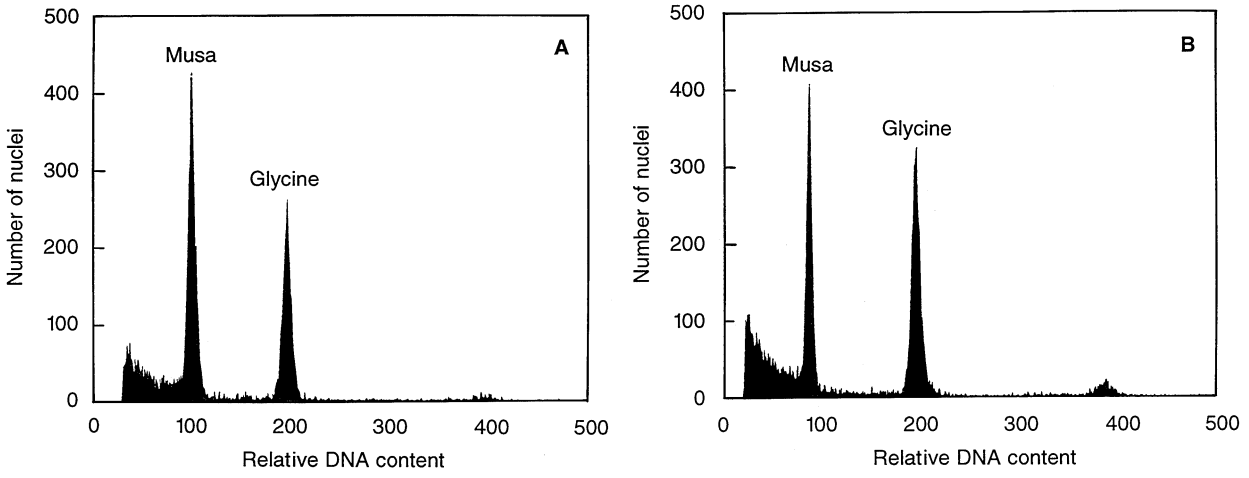

Fig. 2A, B Histograms of relative nuclear DNA content obtained during the analysis of diploid *Musa* genotypes. **A** 'Pisang Mas', 2C = 1.274 pg DNA; **B** *M. balbisiana* (10852), 2C = 1.108 pg DNA. Nuclei isolated from soybean (*G. max*, 2C = 2.500 pg DNA) were used as an internal reference standard. The lower position of the *M. balbisiana* G_0/G_1 peak reflects the lower DNA content of this species

(Rayong)', 'Pisang Mas'] have similar DNA contents, the 'Higa' clone has the lowest DNA content (Table 3).

The relationship between the diploid accessions, based on nuclear DNA content, is shown in Fig. 3. The BB genotypes are clearly separated from the AA clones. Furthermore, the cluster analysis indicates that 'Niyarma Yik' and 'Pa (Rayong)' are very close, forming a separate cluster together with 'Pisang Mas', whereas the 'Higa' clone is clearly separated from these three AA genotypes.

Triploid cultivars

From a number of existing triploid *Musa* cultivars, three accessions of AAA genomic constitution, six AAB accessions, and one ABB genotype were analysed. As can be seen in Table 3, two of the AAA accessions ('Gran Enano' and 'Gros Michel') had the highest DNA content of all the triploids analysed. Although the difference in DNA content between them was small, it was nevertheless statistically significant ($P < 0.01$). Accessions with a reported AAB genomic constitution had a 2C DNA content ranging from 1.737 to 1.847 pg. One of them ('Obino l'Ewai) was significantly different from the remaining AAB clones. On the other hand, the DNA content estimated for one of the three AAA accessions ('Red Dacca') was not significantly different from that of 'Silk' and 'Popoulou', both with an AAB constitution. The only ABB clone analysed in this study ('Pelipita') had a 2C DNA content of 1.751 pg. However, this value was not statistically different from that of 'Agbagba', with a reported AAB constitution.

The relationship between the triploid accessions based on their nuclear DNA content is shown in Fig. 3. Here, three groups can be clearly distinguished. Starting with the highest DNA content, two AAA accessions ('Gros Michel' and 'Gran Enano') form a separate group. At a lower DNA content level, another group is discriminated consisting of four AAB accessions ('Prata', 'Silk', 'Popoulou', 'Maritú') and one AAA accession ('Red Dacca'). The third group consists of two AAB accessions ('Agbagba', 'Obino l'Ewai') and one ABB accession ('Pelipita').

Table 2 Analysis of data on nuclear DNA content by a nested ANOVA

Source of variation	df	Mean squares	*F*-value
Between genotypes	15	1.85462	8017.54*
Between plants within genotypes	48	0.00023	1.00
Between leaves	236	0.00023	

*$P < 0.01$

Discussion

The results of this study confirmed our previous finding that flow cytometry may be used for the precise and reproducible estimation of nuclear DNA content in *Musa* and that this genus has a small nuclear genome (Doležel et al. 1994, 1997). Our estimation of genome size in *Musa* (range 534–615 Mbp) is significantly lower than the value of 873 Mbp estimated by Arumuganathan and Earle (1991). As the 2C DNA content of 1.81 pg reported by these authors corresponds to the nuclear DNA content of triploids (Table 3) we believe that the discrepancy could be explained by the fact the authors analysed a triploid and not a diploid plant (most of cultivated clones are triploid). On average, the A genome of *Musa* was found

Table 3 Nuclear DNA content, genome size, and Bonferroni's groups of the analysed *Musa* genotypes[a]

Accession name	ITC code[b]	Genome	2C nuclear DNA content [pg]		Mean genome size (Mbp/1C)[c]	Bonferroni's grouping ($P = 0.01$)
			Mean	± SE		
Diploids						
Higa	0428	AA	1.225	0.002	591	A
Niyarma Yik	0269	AA	1.252	0.002	604	B
Pa (Rayong)	0672	AA	1.261	0.004	608	B C
Pisang Mas	0653	AA	1.274	0.002	615	C
M. balbisiana (10852)	0094	BB	1.108	0.005	534	D
Cameroun	0246	BB	1.121	0.003	540	D
Triploids						
Agbagba	0111	AAB	1.737	0.002	559	E
Pelipita	0472	ABB	1.751	0.002	563	E
Obino l'Ewai	0109	AAB	1.777	0.004	572	F
Prata	0207	AAB	1.805	0.002	580	G
Silk	0348	AAB	1.825	0.003	587	G H
Red Dacca	0575	AAA	1.836	0.005	590	H I
Popoulou	0335	AAB	1.842	0.005	592	H I
Maritú	0639	AAB	1.847	0.005	594	I
Gros Michel	1122	AAA	1.881	0.001	605	J
Gran Enano	1256	AAA	1.905	0.005	613	K

[a] Statistical analysis was performed using mean values obtained in individual plants ($n = 4$)
[b] ITC code: code assigned by the INIBAP International Transit Centre (Leuven)
[c] One copy of nuclear genome. 1 pg = 965 Mbp (Bennett and Smith 1976)

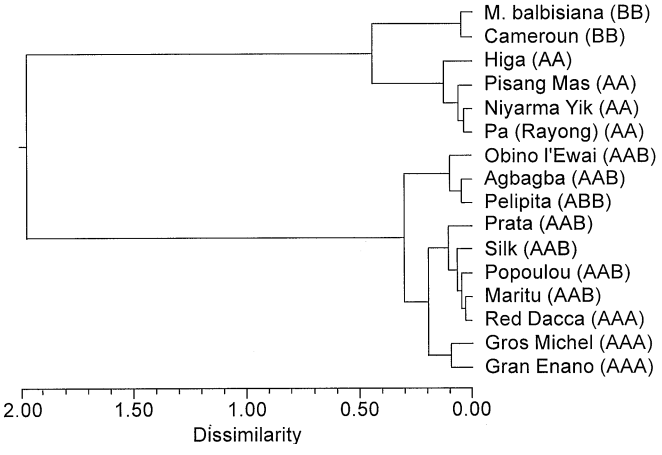

Fig. 3 Cluster analysis of *Musa* species and clones based on nuclear DNA content. The grouping corresponds fairly well with the widely accepted taxonomic classification of the genus *Musa*

to be 12% larger than the B genome. This observation supports earlier results (Doležel et al. 1994) and suggests that genome size can be used to discriminate both genomes. Interestingly, the difference in genome size between *M. acuminata* and *M. balbisiana* agrees with their 10% difference in pollen size (Ortiz 1997).

However, before this parameter can be generally employed, the extent of intraspecific variation in both *Musa* species needs to be analysed. The difference between two randomly chosen accessions with a BB genomic constitution was negligible and statistically non-significant. Although the differences between the AA accessions were small (maximum difference being 3.9%) they were found to be statistically significant. Thus, the analysis of a limited panel of accessions suggests a small intraspecific variation in genome size within *M. acuminata* and related cultivars with an AA genomic constitution. This is surprising when one considers the geographical isolation of some of the *M. acuminata* accessions (e.g. *M. acuminata* subsp. *banksii*) and the vegetative mode of propagation of these genotypes.

The observation of a limited extent of intraspecific variation in genome size in *Musa* contrasts with the large variation that has been observed within some species (Michaelson et al. 1991; Ceccarelli et al. 1992; Graham et al. 1994). Whether the variation reported in these and other studies reflects a large plasticity of the nuclear genome, or rather is due to methodological errors, is still a matter of discussion. For instance, Cavallini and Natali (1990) and Cavallini et al. (1993) observed a large intraspecific genome size variation in *Pisum sativum*. However, this finding was not confirmed by Baranyi and Greilhuber (1996). Similarly, the presence of intraspecific variation in *G. max* reported by Graham et al. (1994) was not confirmed by Greilhuber and Obermayer (1997).

Although the differences in genome size between the AA accessions were small, they were statistically significant. Thus, our analysis clearly discriminated *M. acuminata* ssp. *banksii* ('Higa'), which had the smallest genome, from the rest of the AA accessions. This difference may reflect different areas of origin of individual subspecies and clones. For instance, the original area of

distribution of *M. acuminata* subsp. *banksii* is New Guinea and Australia, whereas subsp. *siamea* originated from South East Asia (Simmonds 1962). However, 'Niyarma Yik' which was collected in New Guinea differs from subsp. *banksii* ('Higa') originating in the same area. It is worth noting that 'Higa' is a wild, non-edible banana whereas 'Niyarma Yik' bears large edible fruits. The latter variety has been subjected to selection and vegetative propagation. The above mentioned results are consistent with the observation made by Carreel (1994) of an identical chloroplast DNA profile in 'Niyarma Yik', 'Pa (Rayong)' and 'Pisang Mas', which differs however from 'Higa'.

The source of genome size variation within diploid *M. acuminata* and related diploid clones is not clear. Our data exclude the possibility of chromosome number variation and the occurrence of aneuploidy. While a detailed analysis of chromosome morphology is hindered by the small size of *Musa* chromosomes, recent molecular data provide some clues. For instance, Lanaud et al. (1992) detected variation in rDNA spacer length within the *M. acuminata* complex. In some plant species, variation in the number of telomeric repeats has been described at the intraspecific level (Shippen and McKnight 1998). Furthermore, retrotransposons which may independently replicate offer a potential mechanism for genome size variation (Bennetzen and Kellogg 1997). Clearly, more studies on the *Musa* genome at the molecular level are needed before the mechanism for intraspecific genome size variation may be addressed.

The analysis of nuclear DNA content in triploids indicated a large variation (Table 3). This was expected as triploids may differ by the number of A and B genomes, whose size was shown above to be different. Considering the data on DNA amounts in diploids obtained in the present study, one may estimate the ranges of 2C DNA content for individual triploid groups as follows: BBB (1.662–1.682 pg), ABB (1.721–1.758 pg), AAB (1.779–1.835 pg) and AAA (1.838–1.911 pg). When the data obtained in individual triploids were compared with these intervals, some discrepancies were evident. This was the case for 'Agbagba', whose DNA content suggested an ABB constitution, and 'Red Dacca', whose DNA content was slightly lower than expected for an AAA constitution. Furthermore, the DNA contents of 'Popoulou' and 'Maritú' slightly exceeded the range predicted for an AAB constitution. However, when interpreting these results one should bear in mind that the ranges of DNA content of individual groups were based on the analysis of a relatively small number of diploids.

The cluster analysis based on nuclear DNA content data resulted in a grouping which roughly corresponded with the current taxonomic classification of triploids (Fig. 3). For instance, the cultivated AAA triploids ('Gros Michel' and 'Gran Enano') were clearly separated from the AAB and ABB triploids. However, 'Red Dacca' (subgroup Red/Green Red), with a reported AAA constitution, was found to be closer to the AAB clones. Similarly, 'Obino l'Ewai', and 'Agbagba' (subgroup Plantain), with a reported AAB constitution, were in a cluster well separated from the rest of the AABs (and AAAs). The isolated position of these two plantain cultivars in relation to other AAB and AAA clones corresponds with current morpho-taxonomical classification (Simmonds 1967) and with the results based on an analysis of selected quantitative traits (Osuji et al. 1997). Interestingly, the clone called 'Pelipita', with a reported ABB constitution, was found in the same cluster as 'Obino l'Ewai' and 'Agbagba'.

The differences between the results of our analysis and the currently accepted taxonomic classification of *Musa* may be explained by a presence of A (or B genomes) whose size falls outside of the range estimated in the present study. Furthermore, one cannot exclude the possibility of the amplification of repetitive DNA sequences after the reproductive isolation of vegetatively propagated triploid clones. Taken together, these facts would complicate the use of data on DNA content to estimate the genomic constitution of triploids. Nevertheless, even a limited knowledge of nuclear DNA content may support the results obtained by other methods, and/or stimulate the analysis of accessions with conflicting results, in our quest to identify the origin of cultivated triploids. For instance, considering DNA amount alone, the A genomes of 'Gros Michel' and 'Gran Enano' are more similar in size to those of 'Niyarma Yik', 'Pisang Mas' and 'Pa (Rayong)' than to subsp. *banksii* ('Higa'). On the other hand, the size of the A genome of 'Red Dacca' is similar to that of 'Higa' (Table 3). Further research will be needed to confirm the similarity of genomes in specific diploid and triploid genotypes.

To conclude, we have shown that flow cytometry may be used for the precise estimation of the nuclear-genome size in *Musa*. The results indicated only a small intraspecific variation. Furthermore, the results of a cluster analysis agreed fairly well with the current taxonomic classification. The discrepancies observed may stimulate further research to verify the current classification of some accessions. While the flow cytometric analysis can be used to discriminate between the AA and the BB genotypes of *Musa*, its application to estimate the genomic constitution in polyploids may be complicated by differences between individual genomes.

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