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Ploidy level of the banana (*Musa* spp.) accessions at the germplasm collection centre for the East and Central Africa

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Banana Germplasm Collection serves as a source of useful genes for banana breeding. However, insufficient and/or inaccurate information on the ploidy level of the germplasm renders its utilization in breeding difficult. The objective of this study was to determine and validate the ploidy level of 120 banana accessions in the *ex situ* germplasm collection centre for the East and Central Africa, located in Mbarara, Uganda. Flow cytometric analysis of the nuclear DNA content was used to determine the ploidy level of the accessions. Results indicate that accessions: Bura, Diana, Kambani-Rungwe, Paji and Pagatau, and Rungwe that were previously classified as diploids are actually triploids, whereas Selangor previously known to be a diploid is a tetraploid. Accessions such as Galeo, Mwitupemba and Ntindi 1 that were previously classified as triploids were found diploids. GT, FHIA 25 and Muzungu Mwekundu that were considered as tetraploids, were found triploids. The information generated will guide correct placement of these accessions in the regional germplasm collection centre for the East and Central Africa and their utilization in banana breeding.

Key words: Banana germplasm, breeding *ex situ* germplasm collection, flow cytometry, ploidy.

INTRODUCTION

The regional *ex situ* Banana Germplasm Collection (BGC) Centre for the East and Central Africa (ECA), located in Mbarara district, Uganda, was established in 1998 to serve as the banana reference collection for the ECA and to house duplicate banana accessions of the formerly Uganda National Agricultural Research Organization (NARO)-Kawanda banana collection. The collection was re-established in 2008 to serve as a

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back-up and regional repository for genetic improvement of banana and plantain in the region. This was after an agreement reached by the Banana Research Network for East and Southern Africa (BARNESA) steering committee meeting held in Dar-es-Salaam, Tanzania in 2007 (Bioversity International, 2007).

The BGC is managed and maintained by Bioversity International in collaboration with NARO on behalf of BARNESA. Its main goal was to conserve maximum banana diversity from the East African region and to provide genes of desired traits for the banana genetic improvement activities initiated by Bioversity International in early 2009 (Bioversity International, 2010, 2011). Since 2009, the BGC in Mbarara has been enriched through collecting and planting new and unique accessions from both the East African region and International banana research Institutes. In addition, since its re-establishment, the collection has acquired 120 accessions, both local and exotic, with most of the accessions being landraces. Priority for acquisition of new accessions was given to the areas in the region that were not well represented in the BGC in Mbarara (Karamura et al., 2013). After acquisition, the accessions are evaluated for traits of interest such as resistance to pests, diseases, environmental stresses and agronomic attributes. The results of evaluation are thereafter forwarded to breeders for subsequent utilization in the banana breeding programs. Accessions from the collection are also disseminated to different stakeholders, especially students and researchers in the region, particularly to provide a platform for support to people interested in gaining knowledge of identification and description of banana.

A number of ploidy levels exist in *Musa* spp. (Tenkouano et al., 2011). Knowledge of ploidy level in *Musa* accessions is vital for breeding, conservation and tissue culture as they are affected by ploidy (Suman et al., 2012). Accurate determination of ploidy of germplasm has practical implications for breeding a perennial crop like banana that has got a long generation time and extensive land requirements (Pillay et al., 2006). Ploidy level influences fertility of banana. For instance, most triploids are sterile while diploids and tetraploids are fertile (Tenkouano et al., 2011). Depending on ploidy information, breeders are able to decide on the materials to evaluate for banana variety development. Banana breeding usually involves the transfer of useful genes from diploids to triploids by carrying out 3x by 2x crosses. Such a cross can generate a variety of progeny with ploidy levels ranging from diploid, triploid, tetraploid, aneuploidy and hyperploid progeny (Pillay et al., 2002). It is important that the ploidy of banana accessions be verified prior to using them for breeding.

Whereas, the ploidy level of most accessions in the regional *ex situ* BGC in Mbarara was reported (Karamura et al., 2016), the results were not exhaustive. Accessions in this collection are planted in three separate blocks based on three ploidy levels (2x, 3x and 4x). The first block of the collection is planted with diploids, the second; with triploids (excluding the East African Highland bananas and tetraploids, and the third with triploid East African Highland bananas, which are the major local cultivars in the East African region.

Ploidy level of the accessions that were added to the BGC since 2010 collection missions was determined from accessions' morphological appearance. Studies have however revealed that ploidy level of banana determined primarily by morphological characteristics may not be reliable (Pillay et al., 2003, 2006). Banana ploidy level is determined by other several methods, of which flow cytometry has been found user-friendly, faster and reproducible for screening a large number of accessions (Takayama et al., 2011). In addition to chromosome counting, which is slow and labour intensive, there are other indirect methods such as estimation of stomata size and density, which are not accurate (Vandenhout et al., 1995; Dolezel et al., 1998). The present study assessed the ploidy level of additional 120 accessions in the collection using the flow cytometry method. This was done to verify the ploidy level of these accessions in order to guide their placement in the correct blocks at the collection centre, as well as to provide accurate ploidy information of these 120 banana accessions to breeders.

MATERIALS AND METHODS

Plant material

Fresh midrib tissue samples of approximately 100 mg from cigar leaves of 120 accessions were collected from the *ex situ* BGC in Mbarara. These samples were individually chopped using a sharp razor blade in a disposable Petri dish containing 0.5 ml of cold OTTO I buffer (0.1 M citric acid monohydrate and 0.5% Tween 20) to form a homogenate. An additional 0.5 ml of cold OTTO I was added to the homogenate and mixed thoroughly. The homogenate was filtered through a nylon filter of 50 µm pore size into a polystyrene tube. The samples were incubated for 1-5 min with occasional shaking. Prior to analysis, 2 ml of OTTO II (0.4 M anhydrous disodium phosphate, 4 µg/ml (2 mg/500ml) of 4,6–diamidino-2-phenylindole (DAPI) and 1 µl/ml of β-mercaptoethanol) were added to each sample. This is the staining solution that allows measurement of fluorescence due to the presence of DAPI.

Flow cytometric analysis

Relative fluorescence intensity of stained nuclei was analysed using a Partec Ploidy Analyser (Partec GmbH, MÜnster, Germany) with a mercury arc lamp. The distribution of fluorescence intensities (relative DNA content) obtained after flow cytometric analyses are usually given as channel numbers (arbitrary units). For ploidy screening, the instrument was calibrated using "Calcutta 4" as a reference (standard) diploid (2x) with its peak set at channel 50. TMB4x660K-1 on the other hand was used as the reference tetraploid (4x) with its peak set at channel 100, while Enyeru, an EAHB was used as a reference triploid (3x) with its peak set at channel 75. The peaks of the unknown samples were determined by examining the position of their peaks relative to the reference accessions. All the samples with peaks at channel 50±5 were

Table 1. Expected ploidy levels of the 120 banana accessions before analysis and the observed ploidy levels after analysis using the flow cytometry method.

S/N	Accession name	Expected ploidy	Observed Ploidy	CV (%)	S/N	Accession name	Expected ploidy	Observed ploidy	CV (%)
1	Rwoyalwansega	3x	3x	5.7	61	Butuhan	2x	2x	5.6
2	Ilalyi	3x	3x	4.5	62	Mjenga Michael	2x	2x	5.4
3	Cula	3x	3x	4.6	63	Gashulie	3x	3x	5.0
4	Namutobisho	3x	3x	3.9	64	Nalwezinga	3x	3x	2.5
5	Luholele	3x	3x	4.3	65	Kasenene	3x	3x	5.4
6	Haahaa	3x	3x	3.5	66	Kambani-Rungwe	2x	3x	6.7
7	Kalasa	3x	3x	5.3	67	Bitambi	3x	3x	7.7
8	Mlema	3x	3x	4.5	68	Kirun	2x	2x	6.3
9	GT	4x	3x	4.7	69	Cultivar Foce	2x	3x	6.1
10	Ntindi 1	3x	2x	6.7	70	Mlambichi	2x	2x	6.5
11	Logiri 1	3x	3x	6.7	71	Pisang Mas	2x	2x	5.6
12	Paji	2x	3x	4.6	72	Oruhuna	3x	3x	6.4
13	Ntebwe	3x	3x	6.5	73	Obutsipa	3x	3x	6.6
14	Kabila	3x	3x	6.1	74	Katejurantamere	3x	3x	6.0
15	Kikonjekonje	3x	3x	5.3	75	Inyumbu	3x	3x	7.5
16	Ekitabwila	3x	3x	4.9	76	Mlelembo	2x	2x	6.5
17	Kanjabu	3x	3x	5.2	77	SH-3362	2x	2x	8.2
18	Engotte	3x	3x	5.2	78	Ingoromora	3x	3x	5.1
19	PV 0344	4x	4x	5.5	79	Paka	2x	2x	6.9
20	Babyesala	3x	3x	6.6	80	9722-1	2x	2x	8.8
21	Opu (Nyakisangani)	3x	3x	5.2	81	Kabana 6H	3x	3x	4.1
22	Nyamabere	3x	3x	5.3	82	Kahuma	3x	3x	5.9
23	Short Gros Michel	3x	3x	6.9	83	Ndiibwabalangira	3x	3x	5.1
24	Ibwi	3x	3x	7.3	84	Nyalambya	3x	3x	4.5
25	FHIA 25	4x	3x	6.7	85	Galeo	3x	2x	9.3
26	Kitarasa	3x	3x	6.4	86	Green Red	3x	3x	6.5
27	TMB x 25511/2	3x	4x	4.8	87	Logiri2	3x	3x	7.1
28	Menvu	3x	3x	6.6	88	Enkongo	3x	3x	5.7
29	Suu	3x	3x	9.2	89	Intariho	3x	3x	6.6
30	Marimbi	3x	3x	4.8	90	548/4 PITA 1	3x	3x	6.4
31	Ensika	3x	3x	6.0	91	Nyerere	3x	3x	5.3
32	Selangor	2x	4x	5.5	92	Nyamahwa	3x	3x	6.2
33	Mbiya	3x	3x	4.5	93	Maganya	3x	3x	5.7
34	Kikundi	3x	3x	6.8	94	Eti Kehel	2x	2x	6.9
35	Enyanshenyi	3x	3x	6.3	95	Halahala	2x	2x	7.3

Table 1. Contd.

36	Nansaba	3x	3x	6.3	96	Pitu	2x	2x	8.1
37	548/9/Pita 2	3x	4x	3.9	97	Ndyali	2x	2x	7.7
38	Saba	3x	3x	4.3	98	Red bogoya	3x	3x	6.2
39	Galeo	3x	2x	8.9	99	Namaliga	3x	3x	7.2
40	Itoke	3x	3x	3.9	100	Bura	2x	3x	6.9
41	Nyarwewunzika	3x	3x	9.0	101	Enyamanshari	3x	3x	5.7
42	M17	3x	3x	5.9	102	Enyamara	3x	3x	6.4
43	Majabaga	3x	3x	3.7	103	Entudde	3x	3x	4.3
44	Kijakazi	3x	3x	6.0	104	Inyarwanda	3x	3x	4.9
45	Enyabongere	3x	3x	3.6	105	Pagatau	2x	3x	5.2
46	KM5	3x	3x	5.3	106	Siira (-EA)	3x	3x	8.1
47	Morong Princesa	2x	2x	8.9	107	Namwezi	3x	3x	5.8
48	Poyo	3x	3x	7.0	108	TMB x 1378/Bita 2	4x	4x	4.5
49	Mwitupemba	3x	2x	6.3	109	Kitombo	3x	3x	4.5
50	Muvubo	3x	3x	6.5	110	Nakinyika	3x	3x	5.4
51	1652-4	3x	3x	4.7	111	Kattabunyonyi	3x	3x	6.4
52	8386S-19	3x	3x	5.8	112	Umburasika	3x	3x	6.3
53	Mtahato	3x	3x	4.9	113	Mukazi-alanda	3x	3x	6.8
54	Rutare	3x	3x	5.4	114	Empuramura	3x	3x	5.4
55	Ntindi 2	3x	3x	7.4	115	Nakhaki	3x	3x	5.4
56	TMB x 5295-1/Bita 3	4x	4x	4.3	116	Nante	3x	3x	4.1
57	Mzungu Mwekundu	4x	3x	6.0	117	Inkurura	3x	3x	4.4
58	Isha	3x	3x	5.6	118	Namunwe	3x	3x	5.6
59	Diana	2x	3x	5.0	119	Enyanja	3x	3x	5.7
60	TT2	2x	2x	6.9	120	Nabuyobyo	3x	3x	5.6

considered diploids, while those at channel 75±5 were triploids and those at channel 100±5 were tetraploids.

RESULTS AND DISCUSSION

The coefficients of variation for the samples analysed were less than 10% (Table 1), indicating the quality of preparation of samples and reliability of the results. Ploidy levels of the 120 accessions analyzed are shown in Table 1. Most of the accessions were triploids (83%), followed by diploids (16%) and tetraploids (1%). Following these results, accessions were reallocated to their respective correct blocks at the regional germplasm collection centre as shown in Table 2.

It is interesting to note that Selangor (*Musa acuminata*) previously reported as a diploid (Pillay et al., 2006; de Jesus et al., 2013), displayed tetraploid nuclei in the present study (Figure 1). The inter-study result differences could be ascribed to the presence of different cytotypes in banana. Possibly chromosome counting from a large number of accessions from a wide geographical range is necessary to determine the existence of cytotypes in Selangor. Changes in ploidy levels in bananas may occur when plants are maintained under *in vitro* conditions (Dolezelova et al., 2005).

Banana taxonomists have always assigned

Table 2. Properly rearranged *Musa* accessions based on the confirmed ploidy levels after ploidy analysis.

S/N	Accession	Ploidy level	Blocks where accessions were before determination of ploidy level	Blocks where accessions are after determination of ploidy level
1	Diana	3x	Block 1	Block 2
2	Bura	3x	Block 1	Block 2
3	Kambani Rungwe	3x	Block 1	Block 2
4	Pagatau	3x	Block 1	Block 2
5	Galeo	2x	Block 2	Block 1
6	Ntindi 1	2x	Block 2	Block 1
7	Mwitu Pemba	2x	Block 2	Block 1
8	Mzungu Mwekundu	3x	Block 2	Block 2
9	GT (tetraploid)	3x	Block 2	Block 2
10	Cultivar Foce	3x	Block 2	Block 2
11	FHIA 25	3x	Block 2	Block 2
12	Selangor	4x	Block 2	Block 2

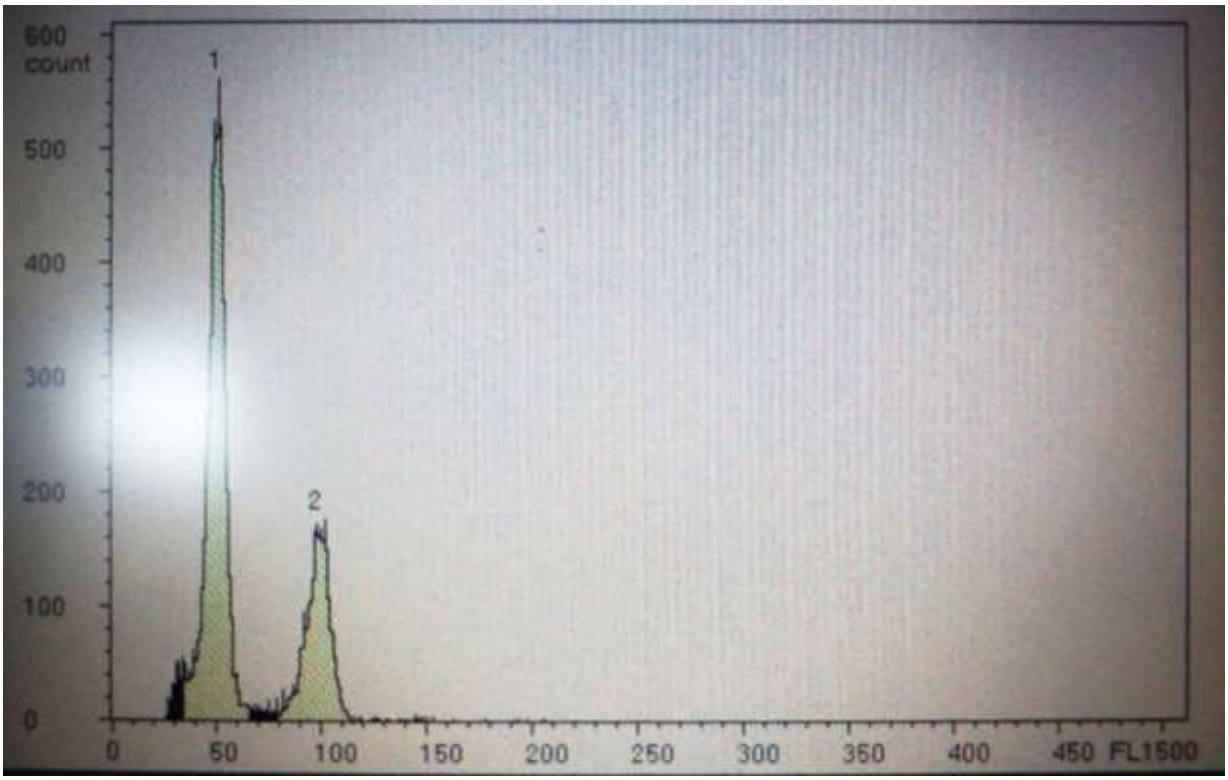

Figure 1. Histogram showing Selangor as a tetraploid with its peak at channel 100 and Calcutta 4, a diploid (control genotype) with its peak set channel at 50.

ploidy levels to different accessions on the basis of morphological traits such as leaf orientation, and on the basis of physiological, cellular and biochemical aspects (Mustafa, 2013). Polyploids such as bananas are often apparent by their distinct and robust morphology (Briggs and Walters, 1984). However, diploids are delicate in nature, lean in size and even perish in harsh weather conditions. Following this system, plants with erect leaves are considered diploids while those with drooping leaves as tetraploids, and those with an intermediate leaf orientation as triploids. This method of ploidy determination is subjective and not always reliable. It

becomes even more unreliable when evaluating hybrids from a breeding program that consist of a mixture of ploidy levels including aneuploids.

The various indirect methods of determining banana ploidy level, for example by estimating stomata size and density (Vandenhout et al., 1995) or measurement of pollen grain sizes were reported (Tenkouano et al., 1998). While these methods depend on statistical analysis to determine ploidy, they are not accurate because the measured parameters are greatly influenced by changes in the growth environment (Xu and Zhuo, 2008). Therefore, chromosome counting remains the only accurate method of ploidy level determination in banana. However, the method requires cell synchronization to metaphase stage for easy visualization of chromosomes. The technique is not routinely used because it is labour intensive and obscure by the low quality of squash slide preparations (Dolezel et al., 1998). The ploidy of plants with large chromosomes can easily be determined by chromosome counting but bananas present a challenge due to its small chromosomes which are always hard to spread out during squash preparations (Dolezel et al., 1998; Pillay and Tenkouano, 2011). Flow cytometry is a user-friendly technique, considering the fact that it is faster and reproducible for screening large number of accessions.

With the flow cytometry ploidy analysis methods, the banana ploidy level is determined by measuring the cell nuclear DNA content and subsequently comparing the relative position of the sample peak to that of the reference accession. With this approach, synchronization of cells is not required. Cells in G_1 and G_2 phases can be differentiated easily including aneuploids. The amount of fluorescence given off by the cell nucleus is directly proportional to the DNA content, which in turn positively correlates with the number of chromosomes. Therefore, increase in ploidy level is perceived as a shift in peak position to the right. This method together with chromosome counting was used to confirm chromosome number of Sukali ndizi, which for a long time was reported as diploid AB instead of triploid AAB (Pillay et al., 2003). Unfortunately, plant cytogenetic does not appear to be the *forte* of many researchers since the advent of molecular biology, yet it is key in answering basic questions for breeders. Misallocation of ploidy levels to different accessions remains a challenge in banana germplasm collections, which calls for deliberate efforts to embrace cytogenetic tools.

Using flow cytometry, previous studies have shown inconsistencies in ploidy levels of banana accessions whose ploidy was determined based entirely on morphological traits (de Jesus et al., 2013; Dolezel et al., 1994; Irish et al., 2009; Pillay et al., 2006; Nsabimana and van Staden, 2006). Knowledge of the ploidy of bananas is valuable for banana breeding schemes as it involves interploidy crosses leading to several possible ploidy levels in the progeny. Flow cytometry provides a rapid way of determining ploidy levels in this crop.

Conflict of Interests

The authors have not declared any conflict of interests.

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