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PLOIDY DETERMINATION OF *MUSA* GERMPLASM USING MORPHOLOGICAL DESCRIPTORS AND CHLOROPLAST COUNT IN PAIRS OF STOMATAL GUARD CELLS

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ABSTRACT

Musa species comprise different ploidy natures with most cultivated species being triploid. This results in its sterility and limits improvement. This work was aimed at determining the ploidy nature of *Musa* accessions from identified *Musa* germplasm, using cost-effective procedures. Accessions from *Musa* germplasm of Ebonyi State University, Abakaliki were assessed. To classify their genomes, twenty-six morphological descriptors of both qualitative and quantitative traits were employed. Chloroplast density in pairs of stomatal guard cells of the accessions was also determined. Morphological description showed that germplasm were variants of banana and plantain with 54.55 % of accessions classified as diploid, while 45.45 % were triploid. The chloroplast count showed significant difference at $p \leq 0.05$ with chloroplast number which ranged from 9 to 18. 'Efol red' had mode and mean of 9 and 9.45 ± 0.61, respectively, while Calcutta 4 and PITA 14 had mode of 16, with a mean of 16.70 ± 0.92 for PITA 14. The chloroplast count grouped 18.18 % accessions as tetraploid, 72.73 % as triploid and 9.09 % as diploid. Although morphological characterisation is ideally the first method to adopt while classifying variants, chloroplast characterisation has brought better clarity since its influence by the environment is limited.

Key words: *Musa* species; chloroplast count; accession; germplasm; morphological descriptor

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INTRODUCTION

Musa spp is of great relevance worldwide, due to its commercial and nutritional values. Reports from Hailu *et al.* (2013) showed that the increased awareness of the health benefits in banana consumption has driven its consumption very high in Europe and North America. It is the most consumed tropical fruit in the world. Soleh *et al.* (2022) noted that it is one of the most favoured fruits, and this could be attributed to its sweet flavour and nutritious content. Heslop-Harrison and Schwarzacher (2007) reported that hybridisation and genetic mutation which occur in diverse species and subspecies have enlarged the genetic diversity of banana cultivars and landraces. Conversely, genetic erosion has resulted in plants which are vulnerable to diseases, pests and ecological changes, thereby narrowing the diversity and survival of most species (Perrier *et al*., 2011). There are around 1,940 cultivars of recognised banana in the world (Crichton *et al.*, 2016). In 2012, the global production was estimated at about 140 million metric tonnes (FAOStat, 2014). Bragard *et al.* (2021) estimated the mean annual global production of bananas and plantains from 2014 to 2018 at 115.7 and 38.3 million tonnes, respectively. In 2018 alone, approximately 116 million tonnes of bananas and 40 million tons of plantains were produced (FAOStat, 2020). They reported that *Musa* production does not show corresponding increase with the global human population increase which Bloom (2020) reported to have increased in billions every year for two decades since 1960. With the projected rise in world's population to 9.2 billion by 2050 (Bongaarts, 2009), there is the need to improve on available and highly nutritional crops like *Musa* spp.

To increase the yield of *Musa* spp, ploidy manipulation is advised, as Lopez-Pujol *et al.* (2004) considered it as a valuable tool in genetic improvement of many plants. Different genotypes which were derived from *Musa acuminata* (AA) and *Musa balbisiana* (BB) were classified into different genomic groups including diploids (AA, AB and BB), triploids (AAA, AAB, ABB and BBB) and tetraploids (AAAA, AAAB, AABB and ABBB) (Pollefreys *et al.*, 2004). *Musa* spp have different ploidy levels and genome constitution. Suman *et al.* (2012) noted

that knowledge of ploidy level of *Musa* accessions is vital for breeding, conservation and tissue culture. The fertility of *Musa* accessions is also controlled by its ploidy, because most triploid accessions are sterile while diploids and tetraploids are fertile (Tenkouano *et al.,* 2011). Genomic information aids breeders to decide on the materials to evaluate for varietal development, crop improvement and conservation. It is important that the ploidy and ploidy nature of *Musa* accessions are verified prior to using them for breeding. Feuillet *et al.* (2011) noted that advent of modern DNA sequencing technologies and powerful bioinformatics tools have made the sequencing and assemblage of genomes for economically important crops and their relatives become more common and easy. However, these protocols require high expertise and equipped modern laboratories which are capital-intensive; hence, they are not easily accessible in developing countries. To be able to effectively improve *Musa* spp in developing countries, other protocols which are less capital-demanding and easily understood are recommended for genome determination. This study was aimed at determining the ploidy nature of *Musa* accessions from identified *Musa* germplasm, using morphological descriptors and chloroplast count in pairs of stomatal guard cells.

MATERIALS AND METHODS

Study site

The study was carried out at Ebonyi State University, Abakaliki, Ebony, State, Nigeria. Ebonyi State is located in Southeastern Nigeria, bearing coordinate of latitude 6° 19' N and longitude 8° 6' E. The area is characterised by high temperature and rainfall with average monthly temperature of 27˚C (Njoku *et al*., 2015). Rainfall starts appreciably in April and terminates in October, leaving a complete dry period between November and April. The rainfall pattern is bimodal, with its peaks in the months of July and September. The total annual rainfall in the area ranges from 1,500 mm to 2,000 mm, with a mean of 1,800 mm.

Genetic Material

The accessions used were sourced from the germplasm at Ebonyi State University, Abakaliki, Ebonyi State, Nigeria. The germplasm comprised of a total of eleven accessions, which include eight *Musa* landraces collected from states within Southeast Nigeria (‘Agbagba’, ‘Efol red’, ‘Efol’, ‘Owom’, ‘Numbrantor’, ‘Atagafong’, ‘Nblepaul’ and ‘Aging’), two hybrid varieties (PITA 14 and SH3436) and Calcutta 4 which were obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria.

Morphological characterisation of *Musa* germplasm

Characterisation of *Musa* accessions in the *Musa* germplasm of Ebonyi State University, Abakaliki was conducted using morphological descriptors, which were assembled from the modified version of Simmonds and Shepherd (1955). The modification was done by replacing pedicel and bract fading descriptors with thirteen descriptors (pseudostem height, petiole margins, petiole margin clasping, pedicel length, colour fading, male bud length, male bud shape, male rachis position, bunch shape, number of fruits on the mid-hand of the bunch, fruit length at maturity, fruit shape and fruit apex) gotten from descriptors developed by the MusaNet Taxonomy Advisory Group (Taxonomic Advisory Group, 2010). A higher percentage of data was collected between August and September than during the drier condition in October because drought influences the condition of the *Musa* plant.

The accessions were classified by assessing the expression of each of 26 characters shown in Table 1 and scoring 1 for each character that adheres closely with wild *M. acuminata* and 5 for characters with extreme *M. balbisiana* expression. This scoring technique provides for a range of 26 (26 x 1) for wild *M. acuminata* and 130 (26 x 5) for wild *M. balbisiana* species. Intermediate expressions of the characters were assigned scores ranging from 2, 3 to 4 depending on their intensity (Simmonds and Shepherd, 1955). Pure *acuminata* varieties should have scores between 26 and 44 (AA, AAA and AAAA), while pure *M. balbisiana* cultivars should range between 121 and 130. The hybrids were expected to score between 45 and 120 points; while AAB ranges from 45 – 80, AB scores about 85, ABB ranges from 102 – 109 and ABBB scores about 116.

Table 1: Descriptors for *Musa* classification by Simmonds and Shepherd (1955) as modified with descriptors developed by the MusaNet Taxonomy Advisory Group (2010)

	Descriptors	Character	*Musa acuminata* (score: 1)	*Musa acuminata* (score: 2)	Intermediate (score: 3)	*Musa balbisiana* (score: 4)	*Musa balbisiana* (score: 5)
1	Vegetative descriptors	Pseudostem height (m)	≤2	2.1 – 2.3	2.4 – 2.6	2.7 – 2.9	≥3
2		Pseudostem colour	More heavily marked with brown or black blotches	Blotches not too conspicuous	Intermediate	Blotches present but very slight	Blotches slight or absent
3		Petiolar canal	Margin erect or spreading, with scarious wings below, not clasping pseudostem	Wide and erect margin	Straight and erect margin	Margins curved inward but not overlap	Margin enclosed, not winged below, clasping pseudostem
4		Petiole margins	Winged	-	-	-	Not winged
5		Petiole margins clasping	Clasping	-	-	-	Not clasping
6		Peduncle	Usually downy or very hairy (short hairs)	Hair present but not too conspicuous	Intermediate	Hair sparsely present	Glabrous
7		Pedicel length	Short (≤ 10 mm)	11 – 14 mm	15 – 16 mm	17 – 20 mm	Long (≥21 mm)
8	Inflorescence descriptors	Ovules	Two regular rows in each Loculus	-	Three regular rows in each loculus	-	Four irregular rows in each loculus
9		Bract shoulder	Usually high (ratio < 0.28)	Not quite high (ratio = 0.28)	Intermediate (ratio = 0.29)	Not quite low (ratio = 0.30)	Usually low (ratio > 0.30)
10		Bract curling*	Bract reflex and roll back after opening	-	-	-	Bracts lift but do not roll
11		Bract shape	Lanceolate or narrowly ovate, tapering sharply from the shoulder	-	Intermediate	-	Broadly ovate, not tapering sharply
12		Bract apex	Acute	Slightly pointed	Intermediate	Obtuse	Obtuse and split
13		Bract colour	Red, dull purple or yellow outside; pink, dull purple or yellow inside	-	-	-	Distinctive brownish-purple outside; bright crimson inside

14		Colour fading	Inside bract colour fades to yellow towards the base	-	-	-	Inside bract colour Continuous to base
15		Bract scars	Prominent	-	-	-	Scarcely prominent
16		Free tepal of male flower	Variably corrugated below tip	-	-	-	Rarely corrugated
17		Male flower Colour	Creamy white	-	-	-	Variably flushed with pink
18		Stigma colour	Orange or rich yellow	-	-	-	Cream, pale yellow or pale pink
19		Male bud length	Short (≤ 20 cm)	20 – 23cm	24 – 26	27 – 30	Long (≥ 30 cm)
20		Male bud shape	Skinny (ratio ≤ 0.45)	0.46 – 0.48	0.49 – 0.51	0.52 – 0.54	Fat (ratio ≥ 0.55)
21		Male rachis position	Falling vertically	At an angle	With a curve	Horizontal or supra-horizontal	Erect
22		Bunch shape	Cylindrical	Truncate (cone shaped)	Asymmetrical	Spiral	Others
23	Fruit descriptors	Number of fruits on the mid-hand of the bunch	≤12	= 13	14 – 15	16	≥17
24		Fruit length (cm) at maturity	≤15 cm	16-20 cm	21-25 cm	26-30 cm	≥31 cm
25		Fruit shape	Straight	Slightly curved	Straight in the distal part	Curved (sharp curve)	Curved in slight 'S' shape (double curvature)
26		Fruit apex	Pointed	Lengthily pointed (like plantain)	Blunt-tipped	Strongly bottle-necked	Rounded

Ploidy determination of field-established accessions based on chloroplast density in stomatal guard cells

The chloroplast density in stomatal guard cells of the accessions was determined using the procedure of Compton *et al.* (1999) with minor modifications. Leaf samples were collected from the second fully expanded leaf of accessions in the field. Three sections evenly distributed along the middle part of the leaves were made. The lower epidermis of the sections was removed with forceps, transferred to a microscope slide and immersed in one drop of 100 % iodine solution. The preparation was covered with a cover glass and allowed to stain for 5 min. All slides were observed under bright field illumination using a LeitzDiaplan binocular microscope at 400x magnification. Ploidy was estimated by counting the number of chloroplasts per guard cell pair of 20 stomata from leaf sections. Accessions were grouped following Krishnaswami and Andal (1977), which grouped genotypes of *Gossypium* into different ploidy levels with an interval of four chloroplasts between ploidy levels. The total number of chloroplasts, mean and mode were recorded and analysed.

RESULTS

Ploidy and genomic distribution of *Musa* accessions using morphological descriptors

The results showed that all the accessions in Ebonyi State University germplasm were variants of banana and plantain with majority of the accessions being classified as diploid ('Efol', 'Owom', 'Numbrantor', 'Atagafong', 'Nblepaul' and 'Aging'), while 'Agbagba', 'Efol red', SH3436, Calcutta 4 and PITA 14 were triploid with two chromosome sets of banana origin. From the keys employed, 'Efol' was scored highest, SH3436 scored least and no accession was purely banana or plantain (Table 2).

Ploidy characteristics of *Musa* accessions using chloroplast characterisation

The chloroplast count for the eleven accessions showed significance at $p \leq 0.05$ (Table 3). The ploidy distribution determined showed that the chloroplast in guard cell pairs of the accessions ranged from 9 to 18 (Table 4; Plate I). The chloroplast number per guard cell pair in 'Agbagba' ranged from 10 to 14 while the least range of 9-11 chloroplasts was observed in 'Efol red'. 'Efol' and 'Owom' had chloroplast ranges of 12 -14 and 11-14, respectively. SH3436 chloroplast number per guard cell pair ranged from 11-15 whereas an equal range of 11-13 was observed for accessions of 'Numbrantor' and 'Atagafong'. Shortest ranges of 12-13 and 11-12 were observed for 'Nblepaul' and 'Aging', respectively. Calcutta 4 and PITA 14 had chloroplast number per guard cell pair which ranged from 15-17 and 16 to 18, respectively. Guard cell pairs in 'Agbagba' had mode of 10; in 'Efol red' it was 9, while 12 was observed for 'Efol', 'Nblepaul' and 'Aging'. In 'Owom' and SH3436, modes of 14 and 15 were observed, respectively. In 'Numbrantor' and 'Atagafong', their guard cell pair had 11 chloroplasts as their mode whereas 16 was observed for Calcutta 4 and PITA 14. Least mean value of 9.45 was observed for 'Efol red' with very high values of 16.00 and 16.70 observed for Calcutta 4 and PITA 14, respectively. Means of 'Agbagba', 'Numbrantor', 'Atagafong' and 'Aging' were comparatively similar. Also, means of 'Efol', 'Owom' and 'Nblepaul' were similar. SH3436 had a mean value of 13.30. The experiment showed that 'Efol red' may be diploid, 'Agbagba', 'Efol', 'Owom', SH3436, 'Numbrantor', 'Atagafong', 'Nblepaul' and 'Aging' may be triploid while Calcutta 4 and PITA 14 may be tetraploid.

Table 2: Ploidy and genome distribution of *Musa* accessions using morphological descriptors

	26 – 44 (AA, AAA & AAAA)	45 – 80 (AAB)	81 – 100 (AB)	101 – 110 (ABB)	111 – 120 (ABBB)	121 – 130 (BB, BBB & BBBB)
Agbagba	-	62	-	-	-	-
Efol red	-	62	-	-	-	-
Efol	-	-	87	-	-	-
Owom	-	-	85	-	-	-
Sh3436	-	53	-	-	-	-
Numbrantor	-	-	82	-	-	-
Atagafong	-	-	85	-	-	-
Nblepaul	-	-	81	-	-	-
Aging	-	-	81	-	-	-
Calcutta 4	-	60	-	-	-	-
PITA 14	-	71	-	-	-	-

Table 3: ANOVA result for chloroplast count of *Musa* accessions

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	866.309	10	86.631	87.447	0.000
Within Groups	207.050	209	0.991		
Total	1073.359	219			

Significance determined at $p \leq 0.05$

Table 4: Ploidy distribution of accessions through chloroplast count

Serial Number	Accession	Chloroplast number range	Mode	Mean ± Standard deviation	Ploidy level
1	'Agbagba'	10 - 14	10	11.60 ±1.76[a]	3X
2	'Efol red'	9 - 11	9	9.45 ± 0.61[b]	2X
3	'Efol'	12 - 14	12	12.65 ± 0.88[c]	3X
4	'Owom'	11 - 14	14	12.90 ± 1.17[cf]	3X
5	SH3436	11 - 15	15	13.30 ± 1.72[ef]	3X
6	'Numbrantor'	11 - 13	11	11.30 ± 0.57[a]	3X
7	'Atagafong'	11 - 13	11	11.80 ± 0.41[ad]	3X
8	'Nblepaul'	12 - 13	12	12.30 ± 0.47[cd]	3X
9	'Aging'	11 - 12	12	11.55 ± 0.51[a]	3X
10	Calcutta 4	15 - 17	16	16.00 ± 0.73[g]	4X
11	PITA 14	16 - 18	16	16.70 ± 0.92[h]	4X

Mean values were significantly different at $p \leq 0.05$. Least significant difference was determined at 0.05. X represents ploidy. Means with the same superscripts are not significantly different.

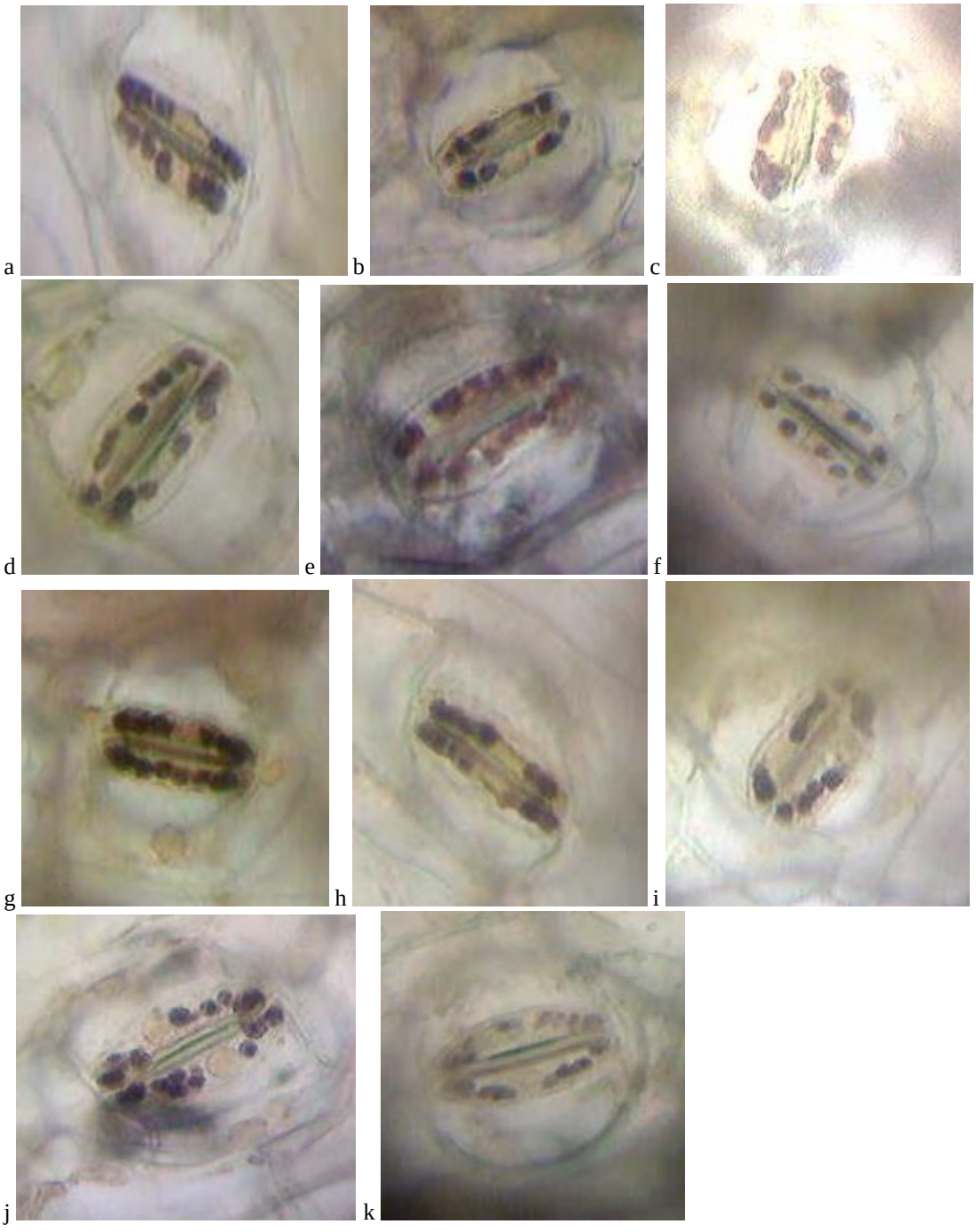

Plate I: Pictorial view of chloroplast distribution in guard cells of *Musa* accessions

Legend: a = 'Agbagba'; b = 'Efol red'; c = 'Efol'; d = 'Owom'; e = SH3436; f = 'Numbrantor'; g = 'Atagafong'; h = 'Nblepaul'; i = 'Aging'; j = Calcutta 4; k = PITA 14.
(Source: © Ukwueze, C. K. 2022)

DISCUSSION

Morphological characterisation is the first step, according to Buitrago-Bitar *et al.* (2020), to study the genetic variability of a population that possesses key features like colour, shape, smell and texture. Although there are morphological, biochemical and molecular descriptors for bananas, but whenever varietal identification demanded by law is considered, only morphological descriptors have been employed to characterise cultivars (Lombard *et al.,* 1999; Priolli *et al.,* 2002; Rocha *et al.,* 2002). Difficulty in character differentiation of much related accessions using only morphological descriptors is high. This is because most of the traits are influenced by the environment and also require varied time for usage since some evaluations are done at late stage of development.

Based on morphological descriptors used, the eleven accessions studied were classified into two different ploidy groups, diploid (AB) and triploid (AAB), both being hybrids of plantain and banana with a similar range of scores. This is not in line with the findings of Buitrago-Bitar *et al.* (2020) who reported that the 57 morphological descriptors proposed by IPGRI (1996) characterised 12 *Musa* cultivars into five cultivars with banana genome (A) and seven cultivars with plantain genome (B). In a comparitive study which involved morphological and molecular characterisation by Cruz-Cárdenas *et al.* (2017), incomplete separation of A and B genomes by the markers was reported, though they concluded that both morphological and molecular markers are needed to complement each other for clarity. In this work, six score ranges were set for the accessions to be classed, but the accessions were distributed into only two score ranges. This was because the accessions had similar scores and majority of the descriptors were influenced by environment, hence, highly unstable. Similar observation was made by Batte *et al.* (2018), who characterised 11 cultivars using 31 descriptors. The result showed that cultivars had similar scores and the descriptors used were not suitable to distinguish between the cultivars studied. They attributed the result to high instability shown by the descriptors during scoring. However, Javed *et al.* (2002) also characterised 16 populations of Malaysian wild *M. acuminata* with the help of 46 morphological characters and discovered that the quantitative characters were not stable. Due to instability in quantitative morphological descriptors, Batte *et al.* (2018) proposed and demonstrated that stable characters should be considered a *priori* for any cultivar classification. Therefore, a good morphological descriptor should be stable, distinctly identifiable and heritable across generations. Present result indicated that there is no pure banana or plantain, which could be due to mutations over a long period of time, or according to Simmonds (1962), it could result from inter- and intra-specific hybridisation, hence, producing hybrids. According to Karamura *et al.* (1998), diploids AA yielded AAA triploids by meiotic chromosome restitution while interspecific hybridisation between AA types (and perhaps AAA) and *M. balbisiana* (BB) produced various AAB and ABB types of today. The morphological ploidy grouping of some accessions is in accordance with other works, as seen for 'Agbagba' accession (Pillay *et al.,* 2006). In other accessions, their morphological ploidy grouping is not in support of previous researches, as seen in PITA 14 (Bakry *et al.,* 2009) and Calcutta 4 (Crouch *et al.,* 1999).

Simmonds (1973) reported that *Musa* spp have three ploidy levels in various combinations of the A (*M. acuminata*) and B (*B. balbisiana*) genomes, being diploids with 22 chromosomes (2x), triploids with 33 (3x) and tetraploids (4x). The challenge is in recognising the ploidy status of *Musa* accessions, although Tang *et al.* (2010) reported chloroplast count as an effective method for identifying ploidy. Savitsky (1964) reported that the limits of the number of chloroplasts between diploids and triploids, and between triploids and tetraploids is overlapping. The corresponding increment of ploidy level in response to chloroplast increase is in agreement with Talebi *et al.* (2017), whose work on *Agastache foeniculum* (anise hyssop) showed a correlated increase of ploidy number with chloroplast number. Grouh *et al.* (2011) showed that this increase is related to plant species and tissues that were employed while determining the chloroplast number. Kerdsuwan and Te-chato (2012) reported that the chloroplast numbers of tetraploid plants of *Rhynchostylis gigantean* were lower than that of diploid plants, showing a negative correlation between chloroplast number and ploidy level. The ranges of chloroplast seen in stomatal guard cell pair in this study is different from the range reported by Oselebe *et al.* (2006), in which their diploid and triploid had mean chloroplast number of 16.9 and 33.5, respectively. They reported a corresponding chloroplast decrease

as ploidy decreased. The ploidy grouping of Calcutta 4 as triploid is contrary to earlier report by Crouch *et al.* (1999) that it is diploid. These variations, based on reports by Oselebe *et al.* (2006) and Crouch *et al.* (1999), could be attributed to mutations and cross-fertilisation that might have taken place across time on the *Musa* accessions.

CONCLUSION

Genomic classification of *Musa* germplasm of the University has given insight into which breeding programme to adopt while improving on the accessions. The morphological descriptors employed were helpful, but its influence by the environment produced narrowed variation among accessions. Hence, the need for chloroplast descriptions which has limited impact from environment and is capable of producing a replicable result.

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