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Ploidy Determination in Banana Hybrids through Stomatal Studies and Chloroplast Count

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Stomata are probably a viable taxonomy distinguishing feature. The study of stomata characteristics of synthetic banana hybrids and their parentage has been carried out. Ploidy determination is essential in banana breeding programs to understand the genetic makeup of hybrid plants. The purpose of this study was to determine the ploidy level of hybrids in contrast with the properties of plant stomata in synthetic banana hybrids with those of their parent plants. Therefore, by examining components such as stomata location, number of epidermal cells, number

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of stomata cells, stomata length, width, area, intensity and density, as well as chloroplast count, it is possible to compare and contrast the traits of synthetic banana hybrids and their parentage which have different genomes. The entire mount approach was used to make an incision for the purpose of observing stomata. By characterising the stomata traits of the synthetic hybrids and their parentage, the results were then descriptively and qualitatively assessed. Three different locations on the abaxial of the leaf were taken for the determination. The samples were collected from close to the petiole (R1), middle (R2), and distal end (R3), stomata were observed using the replica method. Results showed that more number of stomata was observed in R2of leaf abaxial. The chloroplast count in pairs of stomatal guard cells from the accessions was also measured. The chloroplast density was determined in pairs of stomatal guard cells from the accessions. Based on morphological descriptions, it was observed that 16.66% of the accessions were diploid, 50% were triploid and 33.33% were tetraploid. When considering the chloroplast count, the accessions were categorized into groups, certain accessions classified as tetraploids, others as triploids, and diploids.

Keywords: Synthetic banana hybrids; taxonomy; ploidy; genome; stomata; chloroplast; abaxial.

ABBREVATIONS

NPH : Non Parthenocarpic Hybrid
YKM : Yangambi
KV : Karpooravalli
% : Percentage
EV : Erachi Vazhai
ZB : Musa accuminata sub sp. zebrina
PL : Pisang Lilin

1. INTRODUCTION

Banana is considered as one of the largest herbaceous plants globally, characterized by their perennial nature, featuring tall aerial shoots that emerge from swollen, fleshy underground corms [1]. The presence of polyploidy in banana species adds significant complexity to the breeding process, primarily due to complications arising from parthenocarpy and sterility issues. Notably, edible banana cultivars exhibit high degree of sterility, making banana breeding a challenging and time-consuming endeavour, as described by Shepherd in 1954 and 1960 [2]. Banana and plantain (*Musa* spp.) are important staple and income-generating fruit crops for millions of people in the tropical and subtropical regions of the world [3]. The size of stomata in bananas was found to be directly proportional to the ploidy level of the plant, as reported by Rowe in 1984, Simmonds in 1948, and Borges in 1971. This means that as the ploidy level increases, the size of stomata also tends to increase. Conversely, stomatal density (number of stomata per unit area) exhibited expected complementary relationship, which means that as ploidy levels increase, stomatal density tends to decrease. This relationship was also observed in the studies mentioned [4]. It's worth noting that there are multiple ploidy levels within the *Musa* spp., as documented by Tenkouano et al. [5]. This diversity in ploidy levels can have significant implications for the stomatal characteristics and overall physiology of different banana varieties.

In banana breeding, a common practice involves transferring valuable genes from diploid banana plants to triploid banana plants through a specific breeding technique known as "3x by 2x crosses" [6]. This type of cross can lead to the generation of a wide variety of offspring with different ploidy levels. These ploidy levels can include diploid (2x), triploid (3x), tetraploid (4x), as well as individuals with aneuploidy (an abnormal number of chromosomes) and hyperploidy (excess of chromosomes), as detailed in the study conducted by Pillay et al. (2002). This approach allows banana breeders to introduce genetic diversity and valuable traits into triploid banana cultivars, which are typically sterile and do not produce seeds. By using these crosses, breeders can work towards developing new banana varieties with improved characteristics and resistance to diseases or environmental stressors[8]. The guard cells are surrounded by, next to, or even directly surround neighbouring cells. The guard cells precisely associate with the neighbouring cells to carry out their functions, which may include two or more [7] (Jezek & Blatt, 2017; Grey et al., 2020). In general, the type, number, density, length, and width of stomata in a leaf are studied in more detail along with the morphology of the surrounding cells [8].

Increasing the yield of *Musa spp*, commonly known as bananas and plantains can be achieved through ploidy manipulation, which has been recognized as a valuable technique for enhancing the genetics of various plants [9,10]. Different genetic types originating from *Musa*

acuminata (AA) and *Musa balbisiana* (BB) have been categorized into distinct genomic groups. These groups encompass diploids (AA, AB, and BB), triploids (AAA, AAB, ABB, and BBB), and tetraploids (AAAA, AAAB, AABB, and ABBB) [14]. It is worth noting that *Musa* spp exhibit varying levels of ploidy and genome composition [11,12,13,14,15]. (Emshwiller, 2002; Stacy et al., 2002; Beatson et al., 2003; Walker et al., 2005). Flow cytometry has frequently been utilised in ploidy analysis. ^{3} Since it can be used to screen numerous plants quickly and may be applied to any plant tissue, it offers an advantage over the conventional chromosome counting method [16]. Information collected through conventional methods, such as interspecific hybridization, ploidy modifications and seed fertility screening has been supplemented by biotechnological breakthroughs [17] (Wong et al., 2001; Pillay et al., 2002).

Exact knowledge of the ploidy of a variety is important to breeders when attempting to manipulate a multi-ploidy crop such as banana [18,19,20]. 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 [5]. 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 its nature of *Musa* accessions are verified prior to using them for breeding. The advent of modern DNA sequencing technologies and powerful bioinformatics tools has made the sequencing and Assemblage of genomes for economically important crops and their relatives become more common and easy [21]. 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.

Previous research revealed that the genetic group of bananas had different stomata features. Genotype affected the size and density of stomata. In bananas, stomata density had the predicted complementing connection with ploidy level but stomata size had the opposite association. Triploid cultivars were thought to have larger guard cells than diploid cultivars [5]. Additionally, the abaxial and adaxial surfaces of dessert bananas (AA and AAA groups) have different stomata densities. The wax layer was more obvious and organised on the abaxial surface, and the stomata density was higher. Additionally, there is a clear proportional link between banana stomata density and length, with the latter being inversely correlated with density [22]. Stomata profiles may thus be a potential taxonomic distinguishing factor.

2. MATERIALS AND METHODS

2.1 Plant Materials and Leaf Sample Preparation

Plant materials used in this study were 41 banana hybrids representing 3 genomic groups. The leaves sample were obtained from banana field at Tamil Nadu Agricultural University, Coimbatore. Each genomic group consisted of two cultivars or species which were functioning as a replicate. The leave blades were sampled from a mature banana plant, in particular the third leaf from the flag leaf of the inflorescence (at maximum point).

2.2 Stomata Observation

Stomata observation was conducted at ICAR-National Research Centre for Banana, Tiruchirappalli, Tamil Nadu. Stomata profiles were observed using replica method for stomata of opens profile. Stomata with opens profile was collected from the College orchard of Tamil Nadu Agricultural University, Coimbatore, during morning hours (6.00 to 7.00 am). The leaf samples were taken from 3 different locations: lower end near to petiole, middle part and distal end. The lower leaf sample was scraped by using scalpel blade and placed in a slide and the leaf sample was coated with 2-3 drops of acetocarmine solution and coverslip was placed over it. It was made sure that no air bubbles were formed. Then, it was observed under the microscope with 40x magnification. Stomata profile was observed on abaxial/lower surface of leaf sample, and both qualitative and quantitative (morphometric) characteristics were measured.

Table 1. Plant materials used in this study

Female Parent	Male Parent	No. of hybrids
Karpooravalli	NPH-02-01	11
Karpooravalli	Pisang Lillin	11
Karpooravalli	YKM-5	4
Karpooravalli	Erachivazhai	2
Karpooravalli	cv. Rose	12
Karpooravalli	Musa accuminata sub sp. *zebrina*	2
Total seedlings		**42**

Chart 1. Classification of stomata index

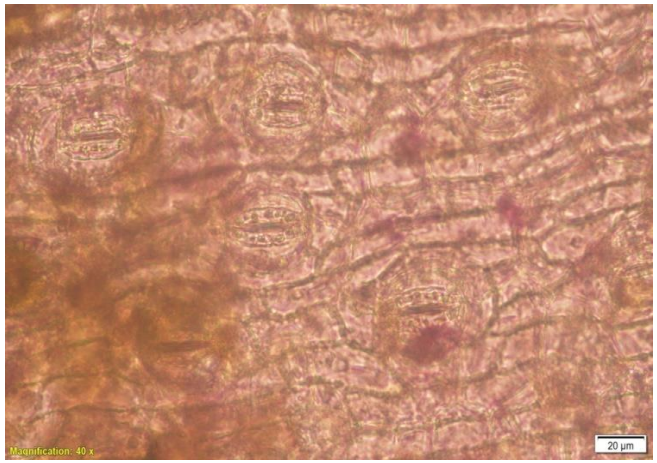
Ploidy	Stomatal density (No. of stomata/mm²)	Stomatal size (µm²)
	Saithiamoorthy (1973)	Vandenhout et al. [23]
Diploids	40.00-50.00	1250.00
Triploids	30.00-40.00	1250-1840
Tetraploids	9.00-15.20	1840.00

Table 2. Assessment of ploidy level in phase I hybrids by stomatal and chloroplast studies

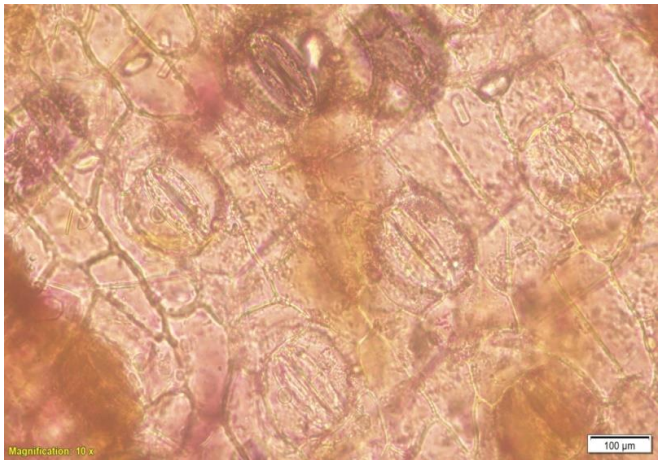
S. No	Hybrids	Stomatal Size (µm²)	Stomatal Density (no./mm²)	Stomatal Intensity	Chloroplast	Ploidy*
1	KV X NPH-02-01	948.88	272.11	0.09	8.13	2
2	KV X NPH-02-01	950.76	293.88	0.10	8.67	2
3	KV X NPH-02-01	956.52	302.04	0.11	8.73	2
4	KV X NPH-02-01	935.32	280.27	0.10	8.87	2
5	KV X NPH-02-01	1129.66	185.03	0.07	8.67	2
6	KV X NPH-02-01	1019.48	210.88	0.08	8.60	2
7	KV X NPH-02-01	890.27	231.29	0.08	8.60	2
8	KV X NPH-02-01	895.78	254.42	0.09	9.13	2
9	KV X NPH-02-01	1037.95	243.54	0.08	8.67	2
10	KV X NPH-02-01	872.29	236.73	0.08	9.13	2
11	KV X NPH-02-01	875.53	257.14	0.09	9.00	2

S. No	Hybrids	Stomatal Size (µm²)	Stomatal Density (no./mm²)	Stomatal Intensity	Chloroplast	Ploidy*
12	KVX EV-1	1589.25	152.38	0.06	10.40	3
13	KVX EV-2	1641.24	164.63	0.06	8.27	3
14	KV X ZB-1	1776.28	180.96	0.08	8.93	4
15	KV X ZB-2	1679.54	170.07	0.07	8.33	4
16	KV X YKM-5	1538.69	125.17	0.05	7.93	4
17	KV X YKM-5	1645.02	148.30	0.06	9.13	4
18	KV X YKM-5	1367.86	174.15	0.07	9.40	4
19	KV X YKM-5	1810.95	140.14	0.06	8.73	4
20	KV X PL	1476.13	145.58	0.06	6.93	3
21	KV X PL	1436.81	165.99	0.06	9.87	3
22	KV X PL	1366.84	179.59	0.07	10.07	3
23	KV X PL	1398.31	178.23	0.07	8.73	3
24	KV X PL	1571.23	145.58	0.06	7.60	3
25	KV X PL	1715.51	138.78	0.05	8.80	3
26	KV X PL	1625.67	172.79	0.06	8.80	3
27	KV X PL	1713.87	163.27	0.06	8.87	3
28	KV X PL	1483.44	163.27	0.06	8.53	3
29	KV X PL	1538.71	160.54	0.07	8.80	3
30	KV X PL	1551.72	152.38	0.06	8.53	3
31	KV X ROSE	1112.59	174.15	0.08	9.33	3
32	KV X ROSE	1697.64	178.23	0.08	8.40	3
33	KV X ROSE	1682.72	178.23	0.07	8.87	3
34	KV X ROSE	1552.83	180.95	0.08	7.73	3
35	KV X ROSE	1330.83	170.07	0.08	7.87	3
36	KV X ROSE	1447.85	178.23	0.07	8.13	3
37	KV X ROSE	1552.26	161.90	0.07	8.33	3
38	KV X ROSE	1274.57	153.74	0.06	7.60	3
39	KV X ROSE	1942.88	115.65	0.05	8.60	3
40	KV X ROSE	1283.29	126.53	0.05	8.73	3
41	KV X ROSE	1643.65	123.81	0.05	9.07	3
42	KV X ROSE	1547.15	187.29	0.06	9.13	3
	Mean	1393.04	183.76	0.07	8.68	

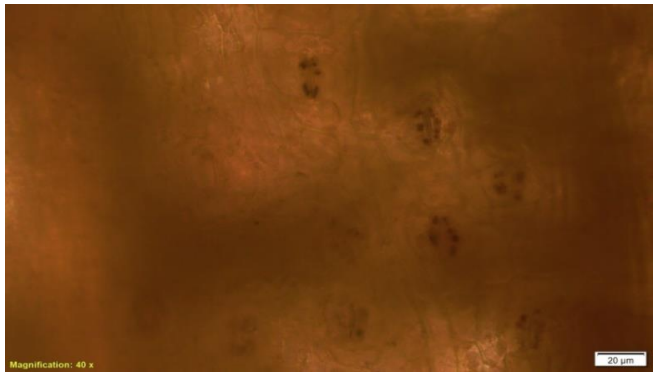
*Note: * 2 : Diploid; 3: Triploid; 4: Tetraploid*



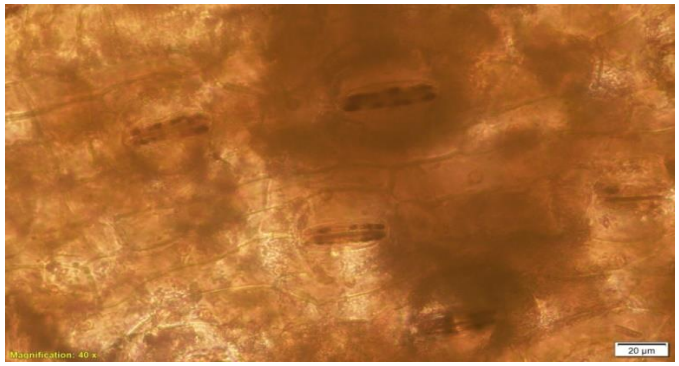
1.a KV X NPH-02-01



1.b KV X YKM - 5



1.c KV X PL



1.d KV X YKM-5

Fig. 1. Microscopic view of stomata of banana hybrids at 40x magnification

Stomata density is calculated by the formula (Lestari, 2006):

- Stomata density = $\frac{Number\ of\ Stomata}{"Stomata's\ Wide\ Field\ of\ View"}$
- Wide field of view = photo length x photo width

The observation of the stomata index on leaves is carried out with the following formula:

- Stomata index $= \frac{Number\ of\ stomata}{Number\ of\ epidermal\ cells + Number\ of\ stomata}$
- Stomatal intensity= no of stomata /(no of epidermal cells + no of stomata)

2.3 Chloroplast Studies

The leaf sample was taken from the middle part of the leaf blade near to petiole. The leaf surface was coated with 2-3 drops of Iodine solution after placing it in a slide and the coverslip was placed above the sample. It was made sure that no bubbles were formed. Then the coated sample was left for 2-3 minutes. Later, it was observed under the microscope with 40x magnification. Chloroplast profile was observed on abaxial/ lower surface of leaf sample. Chloroplast count was taken. Iodine solution is used as a staining solution, so that the chloroplast can be easily count from stomata.

3. RESULTS AND DISCUSSION

3.1 Assessment of Ploidy Status

The results revealed that, a different banana crosses (hybrids) includes KV X NPH-02-01, KV X EV, KV X ZB, KV X YKM-5, KV X PL and KV X ROSE were investigated, each revealing distinct ploidy chromosome sets. These findings shed light on the genetic diversity and ploidy variations within the banana breeding program. The cross between KV X NPH-02-01 displayed a Diploid ploidy level, indicative of its genetic makeup. In contrast, the cross KVX EV exhibited a Triploid ploidy, reflecting the chromosome composition of this banana hybrid. A different result emerged for the cross KV X ZB, which demonstrated a tetraploid ploidy level, suggesting a unique genetic profile within this breeding combination. Similarly, the cross KV X YKM-5 also displayed a tetraploid ploidy. Intriguingly, two other crosses, KV X PL and KV X ROSE, both exhibited a triploid ploidy level. These findings highlight the potential diversity in ploidy levels among banana crosses and offer valuable insights into the genetic complexities within the banana breeding program. The variation in ploidy levels among these banana crosses underscores the importance of understanding the genetic diversity within banana species for effective breeding and crop improvement strategies. These results contribute to the ongoing efforts to develop new banana cultivars with desirable traits and enhanced genetic resilience.

3.2 Assessment of Stomatal Studies

3.2.1 Stomatal size(µm²)

In the present investigation of various banana hybrids for stomatal sizes, the results showed significant variations in stomatal sizes, offering valuable insights into the anatomical characteristics of these hybrids. For the hybrid KV X NPH-02-01, the largest stomatal size was recorded to be 1431.93 µm² in R1, while the smallest size was measured at 586.13 µm² in R3. This discrepancy in stomatal size underscores the diversity within this hybrid's stomatal morphology. In the case of the KVX EV hybrid, the largest stomatal size was recorded to be 1835.29 µm² in R1, with the smallest size at 1453.85 µm² in R3. These variations highlight the dynamic nature of stomatal characteristics within this hybrid. Similarly, the hybrid KV X ZB displayed a range in stomatal sizes, with the largest at 1857.34 in R1 and the smallest at 1563.12 µm² in R3, revealing the plasticity of stomatal features. The hybrid KV X YKM-5 exhibited the largest stomatal size at 1876.29 in R1, while the smallest size was found to be 1207.30 µm² in R1, indicating differences in stomatal dimensions within the same replicate. In the case of the KV X PL hybrid, the largest recorded stomatal size was recorded to be 1862.70966 µm² in R1, while the smallest was 1261.51 µm² in R3, further emphasizing the variability in stomatal traits. Finally, the hybrid KV X ROSE demonstrated a notably wide range in stomatal sizes, with the largest at 2322.29 µm² in R2 and the smallest at 1052.73 µm² in R1. These findings underscore the complex nature of stomatal morphology within this particular hybrid. Overall, the diverse stomatal sizes observed among these banana hybrids illuminate the genetic and anatomical diversity present within banana varieties. The highest range of stomatal size was found in R2 (1427.46 µm²) and lowest is found in R3 (1338.36 µm²). This understanding

is critical for breeding programs aiming to develop banana cultivars with desired physiological traits and improved environmental resilience.

3.2.2 Stomatal density (No. of stomata/mm²)

In the case of the hybrid KV X NPH-02-01, the highest stomatal density was recorded in R1 (330.61), while the lowest density was observed R1 (146.93), highlighting the diversity within this hybrid's stomatal distribution. For the hybrid KVX EV, the highest stomatal density was noted at 183.67 in R3, with the lowest density at 122.44 in R1. These variations underscore the dynamic nature of stomatal characteristics within this hybrid. Similarly, the hybrid KV X ZB displayed a range in stomatal densities, with the highest at 220.40 in R3 and the lowest at 122.45 in R1, revealing the plasticity of stomatal features. The hybrid KV X YKM-5 exhibited the highest stomatal density at 191.83 in R3, while the lowest density was found to be 114.28 in R1, indicating differences in stomatal distribution within the same replicate. In the case of the KV X PL hybrid, the highest recorded stomatal density was 208.16 in R3, while the lowest was 126.53 in R2, further emphasizing the variability in stomatal traits. Finally, the hybrid KV X ROSE demonstrated a notably wide range in stomatal densities, with the highest at 263.91 in R3 and the lowest at 89.79 in R3. These findings underscore the complex nature of stomatal morphology within this particular hybrid. In summary, the varying stomatal densities observed in these banana hybrids shed light on the genetic and anatomical diversity inherent in banana cultivars. This knowledge holds great importance for breeding initiatives focused on creating banana varieties with specific physiological traits and enhanced adaptability to environmental conditions.

3.2.3 Stomatal intensity

For the hybrid KV X NPH-02-01, the highest stomatal intensity was recorded at 0.13 in R3, while the lowest intensity was observed at 0.04 in R1. These findings highlight the considerable range in stomatal intensity within this particular hybrid. In the case of the KVX EV hybrid, the highest stomatal intensity was noted at 0.07 in R3, with the lowest intensity at 0.04 in R2. These variations underscore the dynamic nature of stomatal intensity within this hybrid. Similarly, the hybrid KV X ZB displayed variations in stomatal intensity, with the highest at 0.09 in R3 and the lowest at 0.05 in R2, reflecting the plasticity of stomatal features. The hybrid KV X YKM-5 exhibited the highest stomatal intensity at 0.07 in R3, while the lowest intensity was found to be 0.051 in R2, indicating differences in stomatal intensity within the same replicate. In the case of the KV X PL hybrid, the highest recorded stomatal intensity was 0.09 in R3, while the lowest was 0.04 in R2, further emphasizing the variability in stomatal traits. Finally, the hybrid KV X ROSE demonstrated variations in stomatal intensity, with the highest at 0.10 in R3 and the lowest at 0.03 in R3. These findings underscore the complex nature of stomatal intensity within this particular hybrid. Overall, the diverse stomatal intensities observed among these banana hybrids highlight the genetic and anatomical complexity present within banana varieties. Understanding these variations is crucial for breeding programs aiming to develop banana cultivars with specific physiological traits and improved environmental adaptability.

3.3 Chloroplast Studies

For the hybrid KV X NPH-02-01, the highest chloroplast count was recorded at 11.4 in R2, while the lowest count was observed at 6.4 in R3. These findings indicate a range in chloroplast numbers within this hybrid, with some replicates having a higher abundance of chloroplasts than others. Similarly, the hybrid KVX EV displayed variations in chloroplast count, with the highest count at 10.6 in R1 and the lowest at 6.8 in R1. These variations suggest differences in chloroplast content between replicates. The hybrid KV X ZB exhibited variations in chloroplast count as well, with the highest count at 10.6 in R2 and the lowest at 6.8 in R1. These results highlight the variability in chloroplast numbers among different replicates. In the case of the KV X YKM-5 hybrid, the highest chloroplast count was 10.8 in R2, while the lowest count was 7.0 in R1. These differences in chloroplast numbers indicate variations in chloroplast content within this hybrid. The hybrid KV X PL showed significant variations in chloroplast count, with the highest recorded count at 13.2 in R2 and the lowest at 6.2 in R3. These findings underscore the substantial range in chloroplast abundance within this particular hybrid. Finally, the hybrid KV X ROSE demonstrated variations in chloroplast count, with the highest at 13.0 in R2 and the lowest at 5.8 in R1. These results highlight the diverse chloroplast numbers observed in different replicates of this hybrid.

4. CONCLUSION

From the result of investigation, it was concluded that the initial step in investigating the genetic diversity of a population, as proposed by Buitrago-Bitar et al*.* [24], involves morphological characterization, which entails the examination of fundamental attributes like color, shape, odor, and texture [25]. Despite the existence of morphological, biochemical, and molecular descriptors for bananas [23], legal varietal identification has predominantly relied upon morphological characteristics, as indicated by Lombard et al*.,* (1999), Priolli et al*.,* (2002), and Rocha et al*.,* (2002). The challenge lies in the differentiation of closely related accessions using exclusively morphological descriptors due to the high susceptibility of these traits to environmental influences. Furthermore, the need for varying durations of assessment, with some evaluations occurring during the later stages of development, contributes to the complexity of this differentiation process. The ploidy levels of both phase I hybrids were determined using an assessment of stomatal density. In the case of phase I hybrids, a total of 41 hybrids were examined for their genomic status. The results showed that KV X NPH-02-01of 11 samples were diploid (with genomes denoted as AA and AB), KV X EV (2), KV X PL (11), KV X ROSE (12) were triploid (with genomes AAA and AAB), KV X ZB (2) and KV X YKM-5 (4) were tetraploid (with genome AABB). This information reveals the ploidy levels of these hybrids, which is essential for understanding their genetic characteristics and potential applications in breeding programs or research [26,27].

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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