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Genetic variability among autotetraploid populations of banana plants derived from wild diploids through chromosome doubling using SSR and molecular markers based on retrotransposons

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Abstract The development of banana cultivars resistant to diseases is the most viable way to minimize crop damages. However, this strategy is limited by the low fertility observed among commercial triploid cultivars, especially those of the Cavendish subgroup. Induced polyploidy has the potential to overcome sterility barriers and allows the development of triploid genotypes with fruit sensory profiles aligned with market demands. This work characterized agronomically five autotetraploid plant populations derived from diploids treated with colchicine. The experiment was conducted using an augmented block design, and 13 agronomic characteristics were evaluated during two production cycles. The sum of ranks proposed by Mulamba and Mock allowed the genotypes Pisang Lilin 8, Malbut 10, Niyarma Yik 2, Pisang Mas 3, and Thong Dok Mak 2 to be selected, which were superior for most of the agronomic characteristics evaluated. SSR and IRAP molecular markers detected variability in the autotetraploid populations, and the analysis of molecular variance supports that approximately 78% of identified variability was contained within the populations studied. Based on the results, it is inferred that the promising autotetraploids can be crossed with diploids to develop secondary triploid hybrids.

Keywords Simple sequence repeats · IRAP · Genetic variability · Colchicine · Diploids

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Introduction

Banana farming is socially and economically important in the tropical and subtropical regions worldwide, especially for small farmers. Bananas are consumed by all

age groups, has high energetic value and various vitamins and minerals, and is the base food of millions of people, in both fresh and processed forms (Castañeda et al. 2017). The banana export industry is a relevant economic factor to many Latin American and Asian countries, such as Costa Rica, Ecuador, and the Philippines. Worldwide banana production in 2017 was approximately 115 million tons cultivated on 5.4 million hectares (FAOSTAT 2018). In 2017, Brazil produced 7.0 million tons of bananas, on approximately 517 thousand hectares, placing it in third place in terms of production worldwide (FAOSTAT 2018).

Among the limiting biological factors in banana production are various phytopathogens (fungi, bacteria, viruses), nematodes, and insects that have negative impacts on production and compromise the final quality of the fruits. The most important fungi are the causative agents of yellow Sigatoka (*Pseudocercospora musicola*, Leach), black Sigatoka (*Pseudocercospora fijiensis*, Morelet), and Fusarium wilt (*Fusarium oxysporum* f. sp. *cubense*). The development of resistant cultivars through crosses between resistant diploids and susceptible commercial cultivars is the most viable way to minimize damages caused by diseases. This strategy is limited by the low fertility observed for commercial triploids, especially those of the Cavendish subgroups and some diploids (Alakonyaa et al. 2018). However, tools such as genetic engineering (cisgenics), induced mutation via gamma ray irradiation, induced in vitro somaclonal variation, somatic hybridization, and induced polyploidy, can be used to improve banana plants (Pestana et al. 2011; Amaral et al. 2015; Borges et al. 2016).

Polyploidy is the presence of more than two complete genomes in the cell nucleus and is an important phenomenon in plant evolution due to an increase or doubling of the basic number of chromosomes (Eng and Ho 2019). Polyploidization affects the genetic and phenotypic constitution of an organism, promoting diversification and environmental adaption of the species, and occurs in at least half of the plants cultivated (Hanzl et al. 2014). This technique has been used to genetically improve various species, including corn, cabbage, and wheat (Aslan et al. 2017; Würschum et al. 2012), as well as bananas (Hamill et al. 1992; Bakry et al. 2007, 2009; Costa et al. 2011; Amaral et al. 2015; Borges et al. 2016).

According to Zhou and Gui (2017), polyploidization increases phenotypic variability, plant vigor, tolerance to biotic and abiotic factors, and adaptability to new environments. Therefore, methods have been developed to double chromosomes, including the use of various antimitotic agents, such as colchicine, trifluralin, oryzalin, and amiprofos-methyl (Van Duren et al. 1996; Ganga et al. 2002; Bakry et al. 2007). Genetic variability induced by polyploidization can be quantified using molecular markers such as simple sequence repeats (SSR) and those associated with families of transposable and retrotransposable elements (IRAP). These markers are widely used in *Musa* spp. diversity studies (Pereira et al. 2012; Pachuau et al. 2014; Rotchanapreeda et al. 2014).

Thus, the objective of this work was to analyze the genetic variability available through doubling chromosomes of autotetraploid banana populations using colchicine, based on agronomic characteristics and SSR and IRAP molecular markers.

Materials and methods

Plant material

Fifty-six autotetraploid plants originated from the wild diploids Malbut, Niyarma Yik, Pisang Mas (subgroup Sucrier), Thong Dok Mak, and Pisang Lilin (subgroup P. Lilin) were evaluated. The plants were from experiments conducted previously to double chromosomes using colchicine (Costa et al. 2011; Pio et al. 2014).

Phenotyping the autotetraploid populations

The experiment was conducted in the Embrapa experimental field, located in Cruz das Almas, BA (12° 40′ 19′ S, 39 °06′ 22′ W′, 220 m above sea level), between July 2015 and September 2017, totaling two production cycles. The climate in the region is humid tropical, with an average temperature of 25 °C, average relative humidity of 72.6%, and average annual rainfall of 1932.3 mm (AGRITEMPO 2018). The soil is a dystrophic yellow latosol that is well drained, deep, and has a medium texture with medium levels of clay. Fertilizer was applied every 6 months based on a soil analysis.

The experimental area was irrigated, using a micro sprinkler system, and the soil was monitored with tensiometers. The experiment was conducted using the augmented block design (Federer 1956), with 56 regular treatments (autotetraploid plants), repeated only once in each of the 12 blocks, and three common treatments, improved diploids 091087-12 and 041054-04 and an autotetraploid genotype derived from Pisang Mas, used

as the control, repeated in each of the blocks. Each parcel (treatments) contained nine plants spaced 2.5 m × 2.0 m. Augmented block consists of a special design where a large number of genotypes can be evaluated at the same time when you are initiating a breeding program and do not have availability of the genotypes being tested. Therefore, you have controls, such as commercial varieties which will become your replicates. This facilitates the installation of the experiment. The controls were exclusively used to estimate the experimental error, which allowed an analysis of variance to be conducted.

The evaluation was carried out for the first and second production cycles and 13 agronomic characteristics were considered: time interval between planting and harvesting (NPH), pseudostem diameter (PSD), plant height (PLH), number of live leaves during flowering and harvesting (LLF and LLH), number of suckers (NSU), bunch and hand weight (BUW and HAW), total number of fruits (NFR), average fruit weight (FRW), fruit length and diameter of the second hand (FLH and FRD), and number of hands (NHA).

For the meiotic analysis, immature male flowers, approximately 1.5 cm long, were collected and fixed in Carnoy 3:1 (ethanol: 24 acetic acid – *v*/*v*), for 24 h at room temperature, and then stored in 70% ethanol in a refrigerator in the Plant Tissue Culture Laboratory. To prepare the slides, the anthers were cross-sectioned, lightly crushed to free the microspores into a drop of 2% propionic carmine, covered with a slide, and slightly heated. The number of meiocytes per autotetraploid was then evaluated, considering meiotic abnormalities in the metaphase, anaphase and telophase I and II stages, and in the tetrads. The most representative samples were micro-photographed with an Olympus BX51 microscope. Only the best autotetraploids from each original diploid selected from the Mulamba and Mock sum of ranks index (1978), were analyzed.

Genotyping the autotetraploid populations

Fourteen pairs of SSR primers derived from the wild diploid Calcutta 4 and six combinations of IRAP primers (Creste et al. 2006; Teo et al. 2005) were used to molecularly characterize the autotetraploid populations derived from five wild diploids (Supplementary Table 1). The five original diploids were included in the analysis. The amplification reactions were conducted at a final volume of 25 μL, which included the following: KCl 50 mM, Tris-HCl 10 mM (pH 8.3), MgCl2 2.0 mM, 0.5 μM of each of the dNTPs (dATP, dTTP, dGTP, dCTP) at a concentration of 0.2 mM, 0.5 μM of each primer, 30 ng of genomic DNA, and 1 U of DNA polymerase (Invitrogen, EUA).

Amplifications were carried out with a Perkin Elmer 9700 Thermal Cycler using the following program: one denaturation cycle for 5 min at 94 °C, followed by 35 denaturation cycles of 1 min at 94 °C, 1 min of annealing with a specific temperature for each primer, 1 min of extension at 72 °C, and a final extension of 10 min at 72 °C and ∞ 14 °C. The fragments were separated on 3% ultrapure agarose 1000 gels (Invitrogen) under standard conditions, and the amplified products stained with ethidium bromide, viewed under ultraviolet light, and photographed with UVITEC equipment.

Statistical analysis

The intrablock analysis procedure was adopted (Cochran and Cox 1957; John 1971). This statistical analysis assumed the model was fixed, to consider the effects represented by constants, except for the experimental error. The analyses were conducted using the SAS statistical program (Institute SAS 2010) and the proc glm linear models (procedure for general linear models). The means of the treatments were adjusted by least squares using the "lsmean" model in SAS. Subsequently, the genotypes were classified based on the adjusted means. For each genotype, the sum of the numbers relative to its classification was calculated according the sum of ranks index proposed by Mulamba and Mock (1978):

$$\left(I_{\mathrm{MM}} = \sum_{j=1}^{m} n_{\mathrm{ij}}\right),$$

whereas

I_{MM} is the sum index of the classification,
and
n_{ij} is the classification number of genotypes i in relation to character j.

A principle component analysis (PCA) was conducted to quantify the relevance of the characteristics measured to eliminate redundancies. The selection was based on the mean of each characteristic, from the correlation matrix, and conducted using the princomp procedure in the software SAS v.8.1 (Institute SAS 2010). The PCA analysis

was conducted using the package FactoMineR of the program R (R Core Team 2016).

The agronomic characteristics and molecular data (SSR and IRAP) were analyzed together using the Ward-MLM procedure (Franco et al. 1998). The Ward-MLM clustering method was conducted using the combined matrix obtained with the Gower algorithm (Gower 1971) and the packages StatMatch, cluster, ade4, and NbClust of the program R (R Core Team 2016). The qualitative data (SSR and IRAP) was analyzed using the packages qlcMatrix, ade4, and NbClust of the program R (R Core Team 2016). The molecular variance between and within the autotetraploid populations was explored by AMOVA (analysis of molecular variance) (Excoffier et al. 1992) using the software GENES (Cruz 2006).

Results and discussion

A summary of the analysis of variance for the 13 agronomic characteristics measured for the 59 genotypes (56 autotetraploids and three controls) is presented in Table 1. Based on the *F* test of the analysis of variance there were significant differences for the source of variation in the treatments among the means of the genotypes for all agronomic characteristics evaluated, except for the variable number of live leaves during flowering (LLF).

The eight autotetraploids originating from the wild diploid Pisang Lilin had variation for the number of days between planting and harvesting, with a mean of approximately 500 days; Pisang Lilin 7 was notable for having the lowest values for this parameter. Significant statistical differences were not observed for PSD and PLH among the autotetraploids; however, mean height of the genotypes was similar to the results of Amaral et al. (2015) who measured the original diploid of P. Lilin under the same cultivation conditions. For pseudostem diameter, the mean observed in the present study was greater than that reported by these authors. Plant height is an important characteristic in the improvement of bananas because it is directly related to planting density, management, and productivity. In addition, thicker pseudostems are preferred because plants are less prone to tipping over or breaking due to winds (Amorim et al. 2013). Amaral et al. (2015) evaluated 17 autotetraploids derived from the wild diploid of Pisang Lilin, using colchicine, for different agronomic characteristics. In general, the data corroborate the results of the present study, including the mean height of plants (230.0 cm), number of live leaves during flowering and harvesting (6.1 and 2.8), bunch weight (3.8 kg), and number of hands (4.5). According to the authors, the autotetraploid plants of Pisang Lilin were more fertile compared to the diploid of this genotype, which is considered sterile. This result was confirmed by crossing the autotetraploids of P. Lilin with improved diploids, which resulted in the production of viable seeds. Restoring fertility through induced polyploidy has been reported for other species (Nimura et al. 2006; Rhee et al. 2005; Dunn and Lindstrom 2007).

For the population of autotetraploids obtained through chromosome doubling of the diploid Malbut, statistical differences were observed between the number of live leaves during flowering and harvesting (LLF and LLH) and the number of suckers (NSU). No genotype presented at least eight leaves during flowering, which might be due to possible susceptibility to black and yellow Sigatoka (Table 1). Similar results were observed for the autotetraploids of Niyarma Yik, which is reflected in the low genetic variability for the characters studied.

There was variation for four of the six characteristics evaluated in the autotetraploid population from the Pisang Mas diploid. The mean NPH was approximately 450 days, with an amplitude of variation of approximately 100 days; genotypes 11 and 12 were notable for being the earliest. The autotetraploids 3, 8, 12, 13, and 14 presented more than eight leaves during flowering; however, the same behavior was not observed at harvest (Table 1). There was also variation in the number of suckers, ranging between 1.8 (Pisang Mas 6) and 5.2 (Pisang Mas 11).

Azhar et al. (2000) doubled chromosomes of the cultivar Pisang Mas, which is the most appreciated cultivar in Malaysia, using 1% antimitotic colchicine. The study produced 1420 plants, of which 37.5% were confirmed to be autotetraploids using flow cytometry. The autotetraploid seedlings were shorter; the circumference of the pseudostem was higher, and the leaf blades were thinner compared to the original diploid, data that corroborate the results obtained in the present study.

The autotetraploids obtained from the diploid Thong Dok Mak had a mean NPH of 443 days and double the amplitude of variation observed for the population of Pisang Mas. Two autotetraploids had eight or more live leaves during flowering (Thong Dok Mak 7 and 9). As with Pisang Mas, low LLH values were also observed.

Table 1 Average of the thirteen plant characteristics evaluated in 56 banana autotetraploids. Embrapa 2019

	NPH		PSD		PLH		LLF		LLH		NSU		BUW		HAW		NFR		FRW		FLH		FRD		NHA	
P. Lilin 1	540.7	a	11.2	a	199.2	a	3.5	d	1.8	f	1.2	g	1866.1	g	1503.9	f	41.0	f	41.3	a	11.0	a	2.4	e	3.6	g
P. Lilin 2	501.5	a	12.7	a	209.8	a	5.1	d	1.4	g	1.3	g	2055.6	g	1739.7	e	34.7	f	50.3	a	11.5	a	2.4	e	3.7	g
P. Lilin 3	499.2	a	12.7	a	198.8	a	5.7	c	0.2	h	2.0	f	1435.5	g	1118.9	f	25.9	g	44.7	a	10.9	a	2.6	d	3.1	h
P. Lilin 4	521.7	a	12.8	a	210.0	a	6.1	c	1.6	g	2.4	e	2439.0	f	2024.0	e	53.4	e	39.5	a	10.5	a	2.2	f	4.3	e
P. Lilin 5	488.8	a	13.2	a	217.2	a	6.4	c	2.3	e	2.2	e	1941.6	g	1575.7	f	39.0	f	40.0	a	10.7	a	2.5	e	3.8	g
P. Lilin 6	494.3	a	13.0	a	207.8	a	5.5	d	1.4	g	2.1	e	1425.1	g	1195.2	f	32.7	f	34.5	a	9.7	a	2.3	f	4.0	f
P. Lilin 7	443.2	b	13.8	a	222.0	a	5.3	d	1.4	g	2.6	e	3151.0	d	2614.6	d	47.4	e	51.5	a	12.2	a	2.5	e	4.2	f
P. Lilin 8	510.4	a	12.6	a	227.3	a	10.1	a	5.9	a	3.3	d	3574.8	c	3333.4	b	76.8	c	43.7	a	10.2	a	3.1	b	6.1	b
Mean	500.0		12.8		211.5		6.0		2.0		2.1		2236.1		1888.2		43.9		43.2		10.8		2.5		4.1	
Malbut 1	535.0	a	11.1	a	209.6	a	4.2	d	0.7	h	1.0	g	1282.4	g	1071.2	f	30.8	g	36.2	a	10.8	a	2.2	f	3.3	g
Malbut 2	470.1	a	12.5	a	197.7	a	5.6	b	2.4	e	2.7	e	2398.6	e	1949.4	d	40.9	e	44.9	a	11.6	a	2.3	d	3.8	e
Malbut 3	501.3	a	13.0	a	212.0	a	4.6	c	2.0	e	2.2	e	2154.1	f	1782.1	e	35.2	f	44.0	a	10.7	a	2.3	f	3.6	g
Malbut 4	494.3	a	13.0	a	212.3	a	5.0	d	1.6	f	2.4	e	2363.7	f	1891.1	e	35.0	f	47.2	a	11.9	a	2.4	f	3.6	g
Malbut 5	490.5	a	12.8	a	205.6	a	5.5	d	0.8	g	1.8	e	2132.1	f	1689.9	e	26.0	f	48.0	a	11.4	a	2.3	e	2.9	g
Malbut 6	515.9	a	13.7	a	232.5	a	5.4	d	1.9	h	1.9	f	1830.7	f	1557.9	e	48.6	g	36.3	a	10.8	a	2.2	f	4.3	h
Malbut 7	507.4	a	13.2	a	201.6	a	6.1	d	1.9	f	1.5	f	1781.8	g	1479.8	f	46.4	e	36.7	a	10.9	a	2.3	f	4.2	e
Malbut 8	510.3	a	13.7	a	222.8	a	6.5	c	1.7	f	2.4	f	2680.5	g	2309.0	f	62.2	e	37.4	a	12.0	a	2.2	f	4.4	f
Malbut 9	471.4	a	13.8	a	212.3	a	6.6	c	2.5	f	2.1	e	1595.9	e	1305.8	e	42.0	d	33.3	a	10.1	a	2.2	f	4.2	e
Malbut 10	469.8	a	14.0	a	208.8	a	7.0	c	2.3	e	2.6	e	2872.0	g	2518.7	f	54.2	f	50.4	a	11.4	a	2.6	f	4.4	f
Mean	496.6		13.1		211.5		5.7		1.8		2.1		2109.2		1755.5		42.1		41.4		11.2		2.3		3.9	
N. Yik 1	493.5	a	11.5	a	216.1	a	4.0	d	2.1	f	1.1	g	1706.5	g	1351.3	f	46.6	e	31.6	a	10.8	a	2.3	f	3.9	f
N. Yik 2	427.7	b	12.9	a	203.8	a	5.2	d	1.9	f	2.4	e	2223.4	f	1760.9	e	33.4	f	48.4	a	10.7	a	2.5	e	3.5	g
N. Yik 3	520.5	a	12.8	a	214.7	a	5.8	c	1.1	g	1.8	f	2379.4	f	2034.5	e	27.9	g	55.1	a	12.5	a	2.6	e	2.9	h
N. Yik 4	502.8	a	12.9	a	225.4	a	6.0	c	1.9	f	2.2	e	1700.7	g	1438.2	f	48.3	e	31.3	a	10.4	a	2.2	f	3.7	g
Mean	486.1		12.5		215.0		5.3		1.8		1.9		2002.5		1646.2		39.1		41.6		11.1		2.4		3.5	
P. Mas 1	459.0	b	13.2	a	244.2	a	6.5	c	4.0	b	3.8	c	3721.7	c	3038.5	c	79.1	c	39.4	a	9.4	a	2.9	c	5.0	d
P. Mas 2	474.1	b	13.0	a	226.1	a	4.9	b	4.3	f	4.2	c	3014.9	e	2444.1	e	57.6	d	41.4	a	9.3	a	2.8	d	4.0	d
P. Mas 3	414.5	b	13.6	a	242.2	a	8.8	b	4.8	c	4.9	b	4514.6	c	3538.3	b	76.8	c	46.1	a	10.9	a	3.0	b	4.6	d
P. Mas 4	494.0	b	13.0	a	217.0	a	7.6	a	3.0	b	4.0	c	4139.7	c	1837.2	c	65.4	b	28.2	a	11.6	a	3.4	d	4.5	c
P. Mas 5	501.9	b	11.2	a	178.3	a	4.5	a	0.5	b	1.9	c	1403.8	c	1187.8	c	17.7	c	42.0	a	10.6	a	2.4	b	2.7	d
P. Mas 6	512.7	b	12.5	a	203.9	a	6.2	a	2.1	d	1.8	b	1506.5	e	1246.9	e	39.8	d	38.4	a	10.1	a	2.3	e	3.9	e
P. Mas 7	433.7	a	13.5	a	234.4	a	7.9	d	3.1	b	4.4	c	2373.6	d	1999.1	d	56.4	e	36.5	a	8.5	a	2.9	c	3.8	f
P. Mas 8	421.9	b	14.4	a	195.1	a	8.0	a	3.5	b	3.5	b	2785.3	b	2054.1	b	80.9	c	25.3	a	9.3	a	2.9	b	6.4	e
P. Mas 9	495.9	a	11.5	a	216.2	a	7.6	b	2.9	d	3.9	c	2746.5	b	2268.1	e	75.0	d	30.1	a	10.9	a	2.3	a	5.9	e
P. Mas 10	429.7	a	12.5	a	196.2	a	7.6	d	2.0	h	3.9	f	2680.6	g	2137.6	f	66.8	g	32.7	a	9.4	a	2.7	e	5.0	h
P. Mas 11	409.9	a	14.6	a	250.9	a	7.5	c	3.5	f	5.2	f	3846.9	g	3238.1	f	81.7	f	38.4	a	10.1	a	3.0	f	5.6	f

Table 1 (continued)

	NPH		PSD		PLH		LLF		LLH		NSU		BUW		HAW		NFR		FRW		FLH		FRD		NHA	
P. Mas 12	410.3	b	13.2	a	227.4	a	9.9	b	4.1	d	4.3	c	3704.9	f	3096.9	e	93.7	e	33.0	a	8.9	a	2.7	c	5.8	g
P. Mas 13	427.7	b	14.0	a	244.8	a	9.4	b	4.4	c	4.1	c	3802.4	e	3137.0	e	75.8	c	41.2	a	9.9	a	3.1	c	4.9	b
P. Mas 14	414.5	a	13.9	a	199.3	a	9.2	b	3.0	d	4.9	c	2696.0	e	1915.8	e	66.0	c	29.9	a	8.3	a	2.5	f	4.4	c
Mean	450.0		13.1		219.71		7.5		3.2		3.9		3067.0		2367.1		66.6		35.9		9.8		2.8		4.8	
T. D. Mak 1	412.4	b	13.2	a	188.90	a	5.0	d	2.7	g	4.3	c	3047.3	d	2418.30	d	56.7	d	43.0	a	11.4	a	2.62	c	5.7	c
T. D. Mak 2	412.2	b	15.7	a	216.10	a	7.7	d	3.6	c	6.7	b	5729.6	b	4650.30	b	80.8	c	57.7	a	13.0	a	3.12	c	6.3	b
T. D. Mak 3	453.9	a	14.3	a	215.30	a	5.8	d	3.1	f	3.8	b	3106.8	b	2556.30	b	68.0	e	38.9	a	11.7	a	2.46	d	6.0	c
T. D. Mak 4	532.6	b	13.8	a	219.80	a	6.5	d	2.4	e	3.3	c	3652.9	d	3079.10	d	68.9	e	45.3	a	12.3	a	2.68	d	5.7	c
T. D. Mak 5	422.5	b	11.9	a	198.20	a	7.7	c	1.2	g	3.3	a	1528.5	b	1089.20	b	30.8	e	39.1	a	9.4	a	2.42	a	3.6	d
T. D. Mak 6	399.2	b	13.9	a	207.30	a	5.0	c	2.5	e	5.0	b	2123.0	d	1688.80	d	52.7	d	33.2	a	10.7	a	2.32	c	5.2	c
T. D. Mak 7	533.4	b	15.0	a	185.30	a	8.0	b	1.6	d	2.9	b	3315.8	b	2642.60	b	100.5	c	26.4	a	10.0	a	2.04	d	6.5	b
T. D. Mak 8	470.2	b	11.9	a	173.60	a	7.5	c	1.0	c	2.2	c	3425.9	c	3022.30	c	82.0	d	37.1	a	12.5	a	2.18	d	5.7	d
T. D. Mak 9	418.2	b	13.1	a	224.50	a	8.6	c	4.7	g	4.1	c	3594.4	e	2929.60	e	72.8	e	40.7	a	8.5	a	2.97	e	5.1	d
T. D. Mak 10	423.2	a	14.8	a	204.10	a	6.3	c	1.4	d	6.6	e	4570.3	c	3725.50	c	59.1	a	62.0	a	13.9	a	3.29	f	5.1	a
T. D. Mak 11	395.1	b	14.8	a	202.70	a	6.5	c	2.3	e	5.2	b	3024.7	d	2458.70	d	61.3	e	39.4	a	11.4	a	2.88	c	5.8	d
T. D. Mak 12	404.8	b	14.9	a	207.90	a	7.7	c	3.1	f	5.5	c	4106.4	d	3289.10	c	77.3	e	44.3	a	11.8	a	2.70	c	6.2	d
T. D. Mak 13	435.1	b	13.8	a	201.30	a	6.5	c	3.5	e	4.1	c	3837.6	e	3154.60	e	70.0	e	47.6	a	11.7	a	2.74	d	5.6	d
T. D. Mak 14	440.6	b	14.3	a	206.60	a	5.8	d	1.4	f	4.1	c	2893.7	e	2166.20	e	55.0	e	39.1	a	10.8	a	2.51	d	5.4	e
T. D. Mak 15	603.0	b	17.1	a	298.60	a	6.4	b	3.0	c	2.5	a	3856.7	a	3007.60	a	136.1	c	22.1	a	10.2	a	2.03	b	8.6	b
T. D. Mak 16	412.7	b	14.9	a	190.90	a	6.7	c	2.2	f	5.0	c	3316.3	d	2671.20	d	51.6	e	50.5	a	12.0	a	2.81	d	5.2	e
T. D. Mak 17	427.0	b	13.3	a	187.10	a	6.0	c	1.8	d	3.9	c	3376.0	d	2818.80	d	49.0	d	55.7	a	12.1	a	2.90	e	5.1	c
T. D. Mak 18	422.1	a	14.2	a	208.10	a	6.5	c	2.6	e	4.4	d	2662.8	c	1946.90	c	50.0	d	37.9	a	11.5	a	2.69	d	5.3	c
T. D. Mak 19	398.2	b	13.2	a	196.60	a	5.1	b	1.9	g	3.9	c	2761.2	g	2040.50	f	51.7	g	38.5	a	11.3	a	2.60	e	4.8	g
T. D. Mak 20	440.5	b	13.6	a	213.00	a	6.6	d	2.0	e	3.9	b	3246.2	f	2689.2	e	46.9	e	50.5	a	12.9	a	2.7	f	4.5	d
Mean	442.8		14.1		207.30		6.6		2.4		4.2		3358.8		2702.24		66.1		42.5		11.4		2.63		5.6	
Test. 9187*	533.4	a	15.0	a	185.3	a	8.0	b	1.6	g	2.8	d	3316.7	d	2643.6	d	100.5	b	25.8	a	10.5	a	2.0	f	6.5	b
Test. 4154*	470.2	a	11.9	a	173.5	a	7.5	b	1.0	g	2.2	e	3427.5	d	3025.5	c	82.0	c	39.2	a	12.5	a	2.2	f	5.7	c
Test. P. Mas**	418.2	b	13.1	a	224.7	a	8.6	a	4.7	b	4.1	c	3594.2	c	2930.0	c	72.8	c	40.8	a	8.5	a	2.9	b	5.0	d
MS/treat.	3291.0*		2.03*		718.83*		2.81^{ns}		2.62*		2.10*		967.86*		683.68*		740.30*		96.27*		2.81*		0.19*		1.65	
CV (%)	12.70		4.92		5.58		17.90		19.70		15.90		15.40		16.63		12.44		12.52		5.30		5.51		6.74	
Mean/regulars	463.2		13.4		213.8		6.3		2.4		3.4		2837.6		2281.8		55.9		41.51		11.0		2.6		4.7	
Mean/common	473.9		13.3		194.5		8.0		2.3		3.0		3445.4		2864.8		85.1		34.73		10.5		2.4		5.8	

NPH number of days from planting to harvesting, *PSD* pseudostem diameter (cm), *PLH* plant height (cm), *LLF* number of live leaves during flowering, *LLH* number of live leaves during harvest, *NSU* number of suckers, *BUW* bunch weight (g), *HAW* hand mass (g), *NFR* number of fruits per bunch, *FRW* average fruit mass (g). *FLH* length of fruits in the second hand (cm), *FRD* diameter of fruit in the second hand, *NHA* number of bunches. *Significant at 5% probability; ^{ns} non-significant. Averages followed by the same letter in the columns belong to the same group by the Scott e Knott (1974) test at 5% de probability. *Improved diploid; **Autotetraploid derived from Pisang Mas

The autotetraploid Pisang Lilin 8 was notable for the production parameters BUW, HAW, FRD, and NHA, which significantly differed from the other seven genotypes that originated from the same wild diploid. A similar behavior was observed for Malbut 10 that was superior for the parameters BUW, HAW, and FRW. With the lowest number of autotetraploids, Niyarma Yik exhibited little variation for the parameters measured; however, it is possible to infer the superiority of Niyarma Yik 3 (Table 1).

Pisang Lilin 8 is notable for LLF and LLH, which had ten and six live leaves during flowering and harvesting, respectively. This result is associated with greater resistance to yellow and black Sigatoka, which are diseases present in the experimental area. Black Sigatoka was officially identified in the experimental area in the second half of 2016, making it impossible to analyze the behavior of the autotetraploids against this disease. The complete characterization of the promising autotetraploids for resistance to black sigatoka should be conducted, especially for autotetraploids singled out using the Mulamba and Mock selection index. The autotetraploid Pisang Lilin 8 also had the greatest number of suckers among the genotypes evaluated (Table 1).

The autotetraploid population Pisang Mas, represented by 14 genotypes, exhibited wide variation for the production characters, except for NFR and FLH. For BUW, the mean was 3067 g, varying from 1403 g for Pisang Mas 5 to 4515 g for Pisang Mas 3. Similar behavior was observed for HAW and FRW, of which autotetraploid no. 3 was superior. For the character NHA, Pisang Mas 8 was notable for surpassing the general average by more than 30% (Table 1). Twenty genotypes of Thong Dok Mak, doubled by colchicine, showed wide variation for the production characters. Thong Dok Mak 2 exhibited the highest values for BUW and HAW. Two genotypes had more than 100 fruits per bunch and six or more hands (Thong Dok Mak 7 and 15).

Based on this result, it can be inferred that promising autotetraploids can be selected to use in crosses with the goal of developing secondary triploid hybrids, since there was genetic variation for the characters measured, both among and within populations. According to Amaral et al. (2015), doubling chromosomes changes various characteristics, including leaf architecture, plant height, and size of flowers and fruits.

The choice to double the chromosomes of Pisang Lilin was based on reports in the literature of its resistance of black Sigatoka and Fusarium races 1 and tropical race 4 (TR4), which are relevant diseases to the banana agribusiness (Zuo et al. 2018). The diploid Malbut was selected because it has six times more carotenoids than cultivars of the Cavendish type, 6.87 μg g^{-1} and 1.06 μg g^{-1}, respectively, which could lead to opportunities to develop biofortified triploid cultivars (Amorim et al. 2009). The diploid Niyarma Yik was selected because of reports that it is the probable ancestor of cultivars from the Cavendish subgroup (Perrier et al. 2011; Hippolyte et al. 2012; Christelová et al. 2017). The diploid Pisang Mas was selected for chromosome doubling with the goals of recovering its fertility and using it in crosses, since this cultivar is highly appreciated by both Brazilian and Malaysian consumers (Azhar et al. 2000; Amorim et al. 2013). Thong Dok Mak was selected for polyploidization

Table 2 Mulamba and Mock (1978) I_{MM} index used to select banana autotetraploids

Genotypes	I_{MM}	Rank	Genotypes	I_{MM}	Rank
Pisang Lilin 1	528	41	Pisang Mas 7	460	33
Pisang Lilin 2	543	45	Pisang Mas 8	338	17
Pisang Lilin 3	563	47	Pisang Mas 9	386	26
Pisang Lilin 4	538	43	Pisang Mas 10	376	22
Pisang Lilin 5	530	42	Pisang Mas 11	320	12
Pisang Lilin 6	579	51	Pisang Mas 12	328	13
Pisang Lilin 7	449	32	Pisang Mas 13	330	15
Pisang Lilin 8	312	9	Pisang Mas 14	383	24
Malbut 1	651	56	Thong Dok Mak 1	306	8
Malbut 2	432	31	*Thong Dok Mak 2*	167	1
Malbut 3	541	44	Thong Dok Mak 3	367	19
Malbut 4	516	40	Thong Dok Mak 4	377	23
Malbut 5	552	46	Thong Dok Mak 5	483	35
Malbut 6	603	54	Thong Dok Mak 6	431	30
Malbut 7	566	48	Thong Dok Mak 7	469	34
Malbut 8	515	39	Thong Dok Mak 8	372	20
Malbut 9	573	50	Thong Dok Mak 9	318	11
Malbut 10	415	28	Thong Dok Mak 10	258	5
Niyarma Yik 1	596	53	Thong Dok Mak 11	317	10
Niyarma Yik 2	496	36	Thong Dok Mak 12	222	2
Niyarma Yik 3	498	37	Thong Dok Mak 13	252	3
Niyarma Yik 4	610	55	Thong Dok Mak 14	426	29
Pisang Mas 1	353	18	Thong Dok Mak 15	505	38
Pisang Mas 2	412	27	Thong Dok Mak 16	258	6
Pisang Mas 3	254	4	Thong Dok Mak 17	284	7
Pisang Mas 4	328	14	Thong Dok Mak 18	383	25
Pisang Mas 5	566	49	Thong Dok Mak 19	373	21
Pisang Mas 6	582	52	Thong Dok Mak 20	331	16

because of its short stature, high number of fruits per bunch and precocity, which are characteristics of interest when considering improving bananas (Brandão et al. 2013).

Due to the large volume of data, selecting the best autotetraploids based on values per se is limited. Thus, the Mulamba and Mock sum of ranks index (1978) was applied, which considered all the characteristics together. The results of this index are presented in Table 2 and reflect the classification by population. One of the advantages of this index is the fact that it does not require the establishment of economic weights or estimates of genotypic and phenotypic variances and covariances. In the classification, the autotetraploids Pisang Lilin 8, Malbut 10, Niyarma Yik 2, Pisang Mas 3, and Thong Dok Mak 2 were selected. These genotypes were superior for most of the agronomic characteristics evaluated in this study and had the lowest I_{MM} values (Table 2). When considering the classification regardless of the population, it was found that of the ten ranked the best, eight were derived from the diploid Thong Dok Mak; the other two were from Pisang Lilin 8 and Pisang Mas 3.

The meiosis analysis of the autotetraploids selected from each population with the Mulamba and Mock (1978), sum of ranks index is presented in Supplementary Table 2. In general, high stability was observed, allowing us to infer that the genotypes have normal meiosis and are fertile, making them ideal for crossing with improved diploids to develop commercial triploid hybrids.

The principal components analysis (PCA) presented in Fig. 1, demonstrates the importance of the agronomic characteristics in the genetic variability observed among the autotetraploids. The total phenotypic variability represented by the components PC1 and PC2 was 62.24%, where PC1 contributed 45.75% and PC2 contributed 19.50%.

According to the analysis, the main variables responsible for the phenotypic variation were the following: BUW with 14.59%, NFR with 13.81%, HAW with 13.81%, and NSU with 11.80%, with NFR and NHA contributing 10.72 and 10.36%, respectively. The variables that contributed the least to the phenotypic variation were FRW (0.33%) and FLH (0.16%) and, therefore, were excluded from the combined Gower analysis. The principal component analysis is presented in Fig. 2. There was a tendency for the autotetraploids derived from the diploid Thong Dok Mak to group, except for Thong Dok Mak 15, located in the quadrant opposite of most of the genotypes. A similar behavior served for the genotypes of Pisang Mas that, except for Pisang Mas 5 and 6, formed a single group. The autotetraploids derived from Niyarma Yik, Malbut, and Pisang Lil were the most dispersed among the quadrants due to their higher genetic variability.

The autotetraploids selected using the Mulamba and Mock (1978) sum of ranks index were dispersed in the PCA, allowing us to infer the possibility of using them

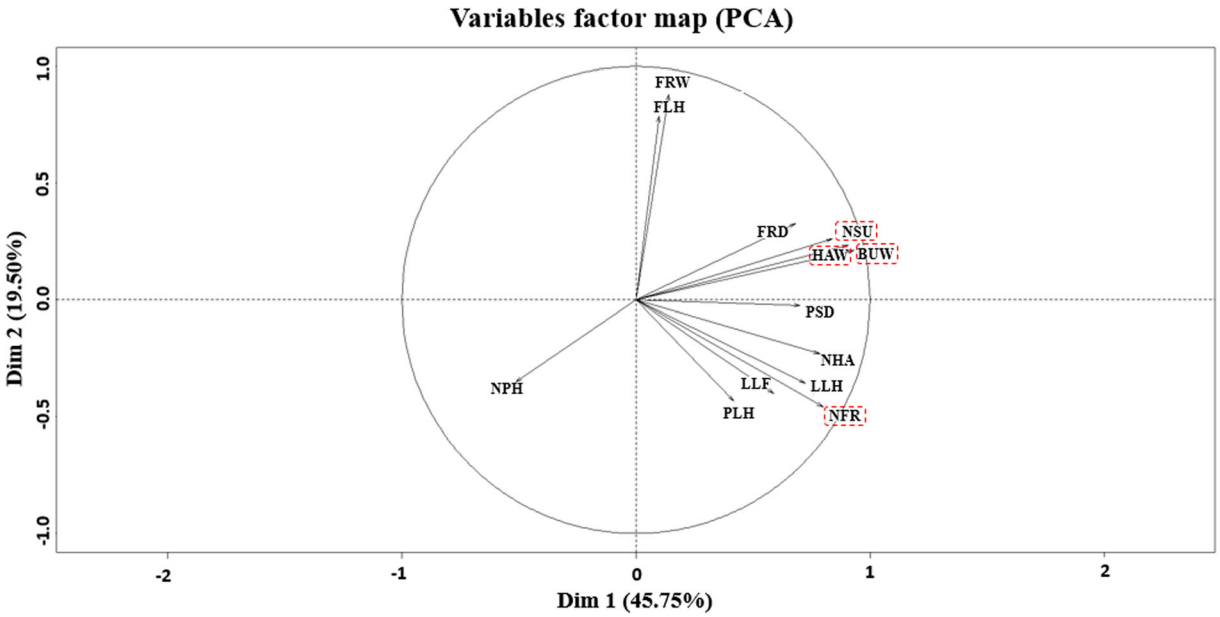

Fig. 1 Principle component analysis (PCA) of the 13 agronomic characteristics measured in autotetraploid banana populations originating from five wild diploids, using the package FactoMineR of the R Program

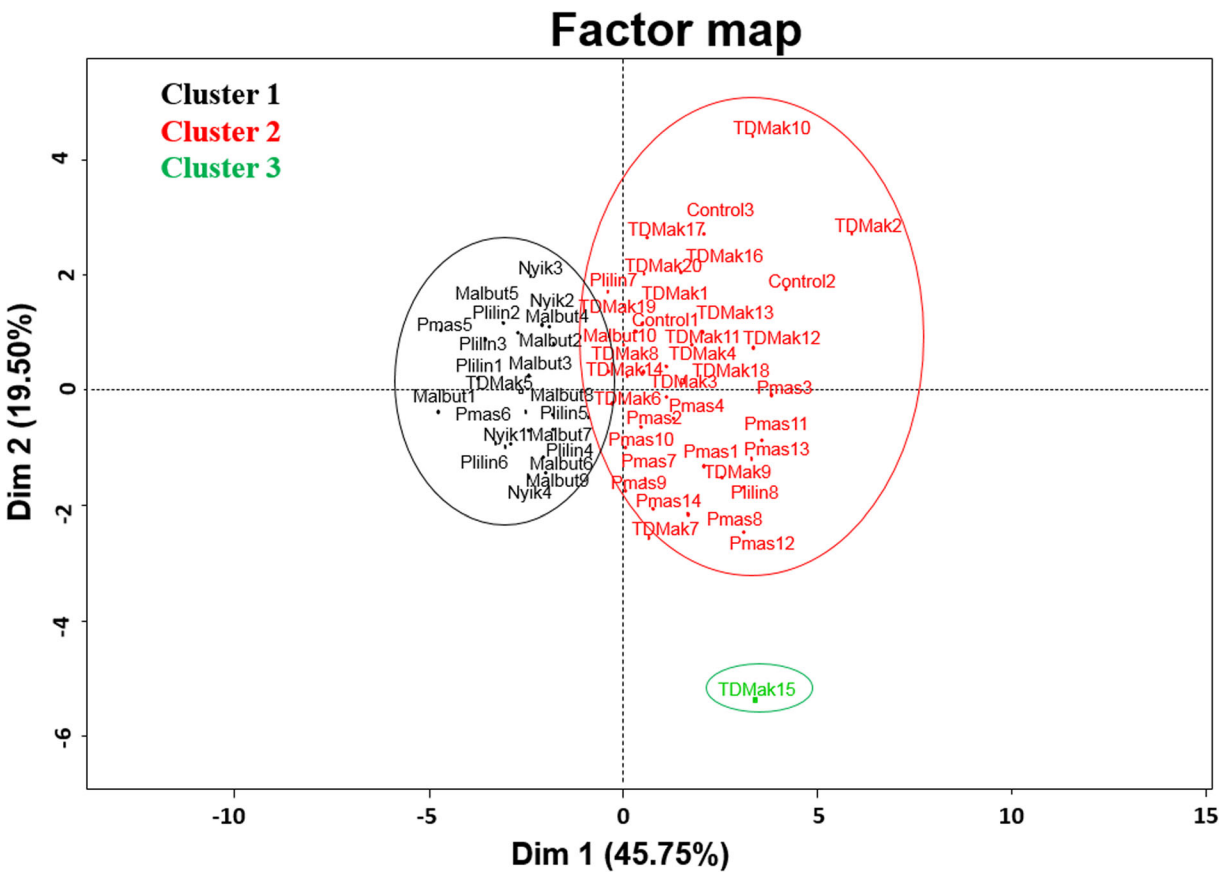

Fig. 2 Dispersion graph of autotetraploid banana genotypes, originating from five wild diploids, based on the principal component analysis (PCA) using the package FactoMineR of the program R (R Core Team 2016)

in crosses to develop commercial triploid cultivars. This result supports the criteria adopted for selecting wild diploids for chromosome doubling: genetic similarity with commercial cultivars, potential for biofortification, and resistance to diseases. Figure 3 a shows the dendrogram of the genetic similarity based on the combined analysis (13 agronomic characteristics and 25 SSR and 42 IRAP molecular markers) obtained using the Ward-MLM method. This analysis did not consider the FRW and FLH variables due to their low contribution to the phenotypic variation. The correlation coefficient values (ccc) was high ($r = 0.85$, $p < 0.0001$, 10.000 permutations) and adequate, since values of $r \geq 0.56$ are considered ideal and reflect good fitness with the genetic similarity values (Vaz Patto et al. 2004).

Except for genotypes Thong Dok Mak 4 (G1) and Thong Dok Mak 10 (G3), the autotetraploids of this population were mostly grouped in G2. The same behavior was observed in the population derived from the wild diploid Niyarma Yik, which was in G3. The autotetraploids of Pisang Lilin, Malbut, and Pisang Mas grouped hierarchically in G4, each with populations derived from close original diploids, except for Pisang Mas 5 and 6, Malbut 3, and Pisang Lilin 8 (Fig. 3a).

The wild diploids (marked in the dendrogram with a zero after their name (e.g., PLilin0), were included in the diversity analysis that only used the molecular markers (SSR and IRAP), so they could be genetically compared with the derived autotetraploid populations (Fig. 3b). Four groups were formed: G1 with only Thong Dok Mak 4; G2 with all of the autotetraploids of the populations derived from Pisang Mas, Pisang Lilin, and Malbut (except Malbut 2); G3 with the diploid Malbut (not doubled); and G4 with the autotetraploids derived from Thong Dok Mak and Niyarma Yik, as well as their original diploids. The Malbut diploid was notable for not grouping with its derived autotetraploids. These results reinforce the importance of inducing polyploidy

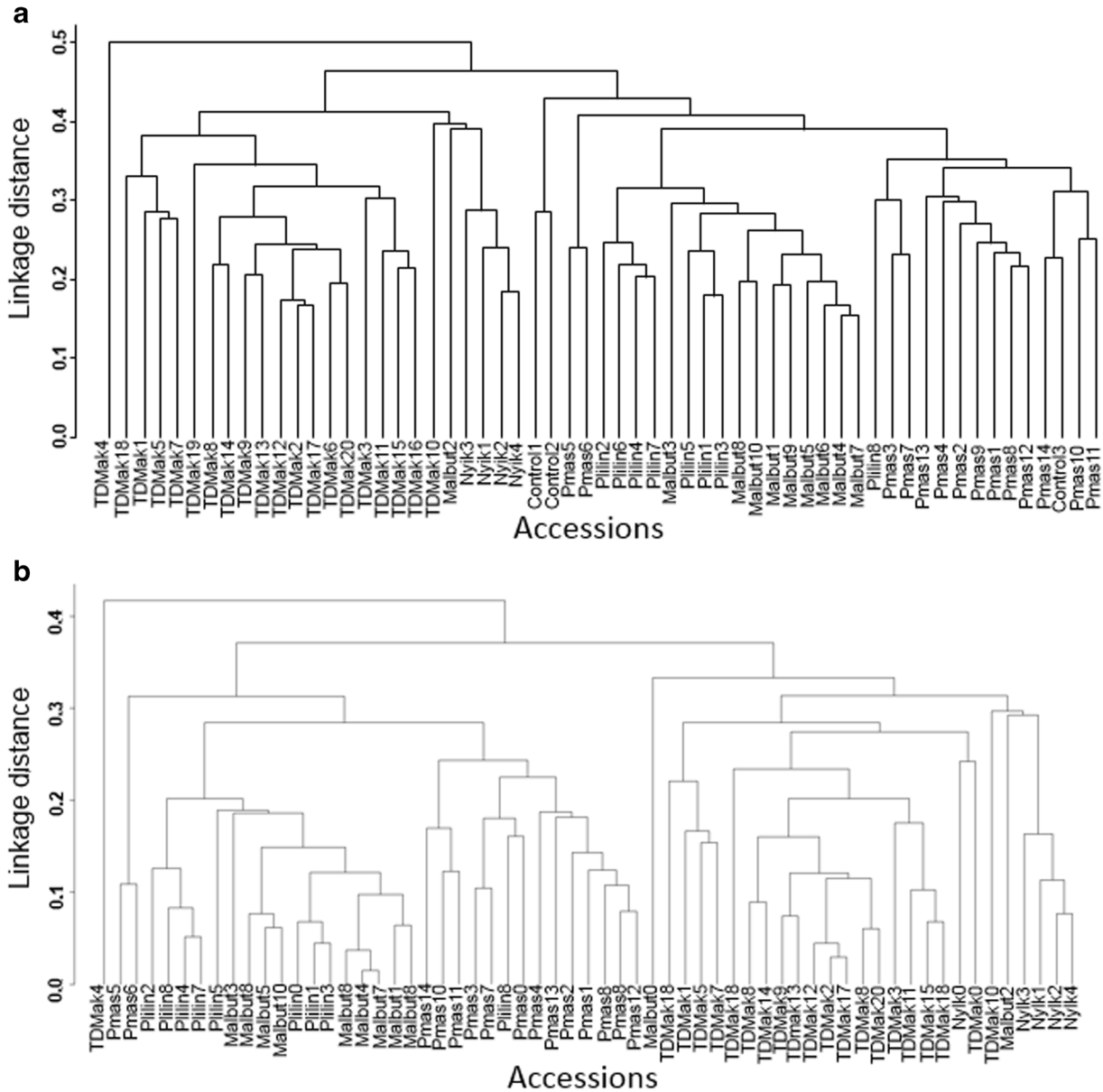

Fig. 3 Genetic variability among autotetraploid banana populations originating from five wild diploids. **a** The Ward-MLM clustering method was used with a combined matrix by the Gower algorithm using agronomic characteristics and molecular markers. **b** Genetic variability estimated using SSR and IRAP molecular markers

level as a tool to increase the genetic base of *Musa* spp., considering the genetic variability detected in the autotetraploid populations compared to the original diploids.

The analysis of molecular variance showed that approximately 78% of the variability identified by the SSR and IRAP markers was within the autotetraploid populations derived from the wild diploids. The Φ_{ST} statistic, which is equivalent to the proportion of genetic diversity attributed to the differences among populations, corroborates the AMOVA results (Supplementary Table 3).

AMOVA analysis has been used to calculate diversity in autopolyploid populations (Mermans and Liu 2018) and tetraploids from cotton treated with colchicine (Rauf et al. 2006). In our work, AMOVA showed that the difference was more within than between populations, with 62.67 and

37.32, respectively. The $\phi ST = 0.37$ demonstrates moderate differentiation among the populations studied. This result is optimistic as an exploratory analysis since it shows that the colchicine treatment was able to also create variability that can be explored by breeders, given the straight genetic base in *Musa* spp.

Hamill et al. (1992) produced autotetraploids using the diploid SH3362 (resistant to Foc RT4) treated with colchicine and reported that they were more susceptible to cold compared to the original diploid and also maintained their resistance to *Fusarium oxysporum* s. sp. *cubense* tropical race 4. Van Duren et al. (1996) doubled the chromosomes of the diploid SH3362 using the same antimitotic agent. According to these authors, the autotetraploids generated had great improvement potential based on the low heterozygosity observed for the original diploid and the resistance to *Fusarium* tropical race 4. Rodrigues et al. (2011) doubled the chromosomes of the improved diploids 013018-01 and 086094-04 that are resistant to *Fusarium oxysporum* f. sp. *cubense* race 1. According to these authors, the autotetraploids produced have potential for use in crosses to develop commercial triploids hybrids. The results also showed that the genotypes had regular meiosis, which suggests female fertility.

Bakry et al. (2007) doubled the chromosomes of 25 wild diploids with colchicine and produced autotetraploids that were agronomically evaluated for various production cycles under field conditions and observed reduced vigor and production of suckers, especially for the diploids for cooking. All of the plants flowered under tropical conditions and were used in crosses to develop triploids. Jenny et al. (2013) crossed an autotetraploid, obtained through doubling the chromosomes of the diploid Kunnan, with wild diploids of *Malaccensis*, IDN110 and *Musa balbisiana* (CMR and P. Klutuk) and obtained the triploid progenies AAB (38–Kunnan × Malaccensis; 9–Kunnan × IDN 110) and ABB (15–Kunnan × CMR; 8–Kunnan × P. Klutuk). Most of the triploid hybrids exhibited parthenocarpy, increased bunch weight, and resistance to black sigatoka.

Therefore, the autotetraploids selected will be used in crosses with elite diploids developed by Embrapa, with the goal of creating triploid progenies to potentially select genotypes with characteristics aligned with the demands of producers and consumers.

Conclusions

Five autotetraploids selected using the Mulamba and Mock index have potential for use in crosses to develop triploid cultivars.

The SSR and IRAP molecular markers are efficient to quantify the genetic variability resulting from polyploidization via colchicine of wild banana diploids.

The analysis of molecular variance of the autotetraploid populations derived from five wild banana diploids allowed most of the genetic variability to be inferred within the populations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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