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Genetic variability in banana diploids and nonparametric statistics of fragments associated with natural fruit finger drop

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ABSTRACT. Natural fruit finger drop in bananas is one of the major concerns of producers as well as consumers. The aim of this study was to estimate the genetic diversity among 15 diploid bananas with different levels of resistance to finger drop (RFD) using SSR and ISSR (inter-simple sequence repeat) markers, and assess the possibility of association of bands with RFD variables (v1 and v2) via nonparametric methodologies. Molecular genetic analysis revealed that the dendrogram generated by SSR markers used as co-dominant markers better discriminated the genotypes as to genome constitution, genealogy, and RFD. Although nonparametric tests are limited in terms of inferences, they are very useful when data do not follow normal distribution, such as the case in our study. Bananas are parthenocarpic, which hinders obtainment of representative genetic linkage maps and linkage studies due to small number of segregating populations; a pre-requisite for mapping and QTL studies. Therefore, non-conventional methodologies, such as nonparametric tests, become an attractive

alternative to overcome this barrier. In our study, the nonparametric tests, Kruskal-Wallis and Spearman correlation, revealed negative and positive associations with RFD for 12 bands (7 from ISSR markers and 5 from SSR markers) with high positive and negative correlations. The results show potential for future sequencing of bands, obtaining sequence-characterized amplified region markers, validation, and subsequent possibility for application in marker-assisted selection in banana genetic breeding programs. This is the first report of the use of nonparametric statistics in attempt to evaluate the association between fruit finger drop and ISSR bands in *Musa* spp.

Key words: *Musa* spp; Microsatellites; ISSR; Nonparametric statistics

INTRODUCTION

Bananas are considered one of the most important fruits playing significant social and economic roles worldwide. Brazil is the fifth world producer, with 7.3 million tons produced in 2014, in 500,000 hectares (FAO - Food and Agriculture Organization, 2016). Among the many bottlenecks in banana marketing and causes of substantial post-harvest losses, natural fruit finger drop is one of the major complaints of consumers. This condition is a physiological disorder associated with maturation that, in bananas, is also associated with softening and weakening of the peel in the junction between the fruit and the bunch, called pedicel (Imsabai et al., 2006; Salazar and Serrano, 2013).

Bananas are sold in clusters ranging from 4 to 9 fruits. Therefore, the displacement of individual fruits in the pedicel area is one of the main causes of the decline in the market value and consumer acceptance, generating significant economic impact on producers. Bananas were developed through complex intra- and inter-specific crosses of two diploid species of the genus *Musa*: *Musa acuminata* Colla, with genome A, and *Musa balbisiana* Colla, with genome B. During domestication, crosses produced varying combinations of complete genomes of both parental species as follows: diploids (AA, AB, and BB); triploids (AAA, AAB, and ABB) and tetraploids (AAAA, AAAB, AABB, and ABBB) (Simmonds and Shepherd, 1955), where the A genome is responsible for palatability and taste and the B genome for robustness and disease resistance.

Susceptibility to fruit finger drop varies among cultivars and it was first reported in the triploid Cavendish (AAA) (Hicks, 1934) and banana tetraploids (Marriott, 1980). However, several studies suggest that besides ploidy level, the type of genome also influences susceptibility. Bananas that have the B genome are less prone to fruit dropping when compared to bananas with the A genome (Putra et al., 2010).

This indicates that the alleles of resistance to finger drop (RFD) may be associated with the *Musa balbisiana* species. Studies carried out by Pereira et al. (2004) confirm that the diploid *M. balbisiana* (BB) and triploids with the B genome (ABB and AAB) showed higher resistance to fruit drop when compared to diploids and triploids of *M. acuminata* (AA and AAA) and tetraploids of this same group.

Microsatellite markers have been widely used to assess the genetic diversity in diploid genotypes of *Musa* spp (Creste et al., 2003; Amorim et al., 2009). Microsatellites or simple sequence repeats (SSR) are co-dominant markers with motifs that can vary from 1 to

6 nucleotides repeated in tandem within the genomes of prokaryote and eukaryote organisms. The flanking regions, which are usually highly conserved, are suitable for the development of site-specific primers (Iniguez-Luy et al., 2008).

ISSR markers (inter-simple sequence repeats), which amplify a DNA sequence defined by two inverted microsatellite regions, also have potential for genetic diversity studies in *Musa* (Racharak and Eiadthong, 2007; Lu et al., 2011). Such markers are dominant and highly reproducible with the advantage of generating large quantities of bands, being widely distributed throughout the genome.

One of the strategies to reduce the problem associated with fruit finger drop is the development of resistant cultivars from the selection of progenitors with good combining ability and resistance to this characteristic. In order to improve breeding programs, molecular markers have played a key role in indirect selection of genotypes with resistance to pests and diseases, by associating bands or alleles of interest via nonparametric methods (Mace et al., 2006; Mondal and Badigannavar, 2010; Singh et al., 2013). Although these methods are somewhat limited, with restricted inferences (Whitely and Ball, 2002; Cowan, 1992), they may be used when data do not follow normal distribution and have shown to be useful in association studies, especially for orphan crops that have very limited genomic resources including genetic maps with sufficient marker coverage, which are pre-requisites for a conventional mapping approach. In *Musa* spp this is even more important, since they are parthenocarpic, with very low seeds, and very few individuals can be obtained in segregating populations.

Different banana-breeding programs have produced tri- and tetraploid hybrids obtained from crosses between fertile triploid cultivars or tetraploid hybrids (female parent) and improved diploids (pollen donor) (Crouch et al., 1999; Amorim et al., 2016). Therefore, knowledge of the genetic variability of diploids available for genetic improvement is useful information when selecting parents for crosses between different genotypes (Amorim et al., 2009).

The aim of this study was to estimate the genetic variability among 15 banana diploids with different levels of fruit RFDusing SSR and ISSR markers and assess the possibility of association of fruit finger drop resistance with ISSR and SSR bands via nonparametric statistics.

MATERIAL AND METHODS

Genetic material

The 15 banana genotypes evaluated included cultivated, improved, and wild diploids and with different levels of RFD, belonging to the banana germplasm collection at Embrapa Mandioca e Fruticultura, Cruz das Almas, BA (Table 1).

Fruit dropping scale and measurements

RFD data were obtained by previous study carried out by Pereira et al. (2004). Values for RFD were the following: 1 for resistant (R) genotypes (>60 N), 2 for moderately resistant (MR; 20-60 N), and 3 for susceptible genotypes (<20 N) (Table 1).

Molecular markers

Thirty SSR markers (Table 2) and 13 ISSR markers (Table 3) were used for the genetic

diversity analysis of 15 banana genotypes with different patterns for RFD. Once the molecular and phenotypic data for RFD were available (Pereira et al., 2004), they were used to verify the presence or not of possible correlation with the variables for RFD, using nonparametric tests.

Table 1. Banana diploid genotypes indicating the genomic group (GG), genealogy, geographical origin, and resistance to finger drop (RFD) and variable 1 (V1) and variable 2 (V2).

Name	GG	Genealogy	Origin	RFD
BB França	BB	Wild diploid[1]	France	Resistant
Butuhan	BB	Wild diploid[1]	Philippines	Resistant
ID028003-01	AA	Improved diploid[2]	Honduras	Resistant
Khai Nai On	AA	Cultivated diploid[3]	Thailand	Resistant
Híbrido AB	AB	Improved diploid	Brazil	M resistant
Ouro	AA	Cultivated diploid	Brazil	M resistant
Tjau Lagada	AA	Cultivated diploid	Costa Rica	M resistant
TH0301	AA	Improved diploid	Brazil	M resistant
Jary Buaya	AA	Cultivated diploid	Honduras	M resistant
Jaran	AA	Improved diploid	Indonesia	M resistant
Lidi	AA	Cultivated diploid	Costa Rica	Susceptible
M53	AA	Improved diploid	Equador	Susceptible
Calcutta 4	AA	Wild diploid	Jamaica	Susceptible
ID013004-04	AA	Improved diploid	Brazil	Susceptible
ID017041-01	AA	Improved diploid	Brazil	Susceptible

[1]Genotype that bears fruit with seeds; [2]genotype obtained by crossing wild diploids; [3]genotypes that bears seedless fruits by parthenocarpy. M resistant = moderately resistant. RFD based on the study of Pereira et al. (2004).

DNA extraction and PCR

Genomic DNA was extracted from young leaves from 15 banana genotypes using the CTAB method (Doyle and Doyle, 1990) (Table 1). Amplification reactions were performed in a final volume of 15 µL containing the following reagents: 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 100 µM of each one f dNTPs (dATP, dTTP, dGTP, and dCTP, dCTP), 0.2 mM of each primer, 1U Taq polymerase (Pharmacia Biotech, USA) and 20 ng genomic DNA.

Thirty microsatellite primers: four from the Ma series (Crouch et al., 1998), five from the AGMI series developed by Lagoda et al. (1998), three from the MaOCEN series (Creste et al., 2006), ten from the CNPMF series (Amorim et al., 2012), and eight from the MASR series (Dr. Ana Ciampi - Embrapa CENARGEN) (Table 2), and thirteen ISSR markers (Table 3), were used for genetic molecular characterization.

Amplifications were performed on the Applied Biosystems thermocycler, using specific annealing temperature (Ta) for each primer. Amplification conditions included one cycle of denaturation for 3 min at 94°C followed by 30 cycles of denaturation for 40 s at 94°C, 40 s Ta specific for each primer, 1 min extension at 72°C, ending with a final extension of 4 min at 72°C and 10°C ∞. Fragments were separated on 3 and 2.5% ultrapure-1000 agarose gel (Invitrogen, Carlsbad, CA, USA) for the SSR and ISSR markers, respectively, under standard conditions, stained with ethidium bromide, visualized under UV light and photo-documented using the UVITEC equipment.

Data analysis

Genetic diversity analysis

For the genetic diversity analysis, three dendrograms were constructed: 1) ISSR

markers, 2) a combined dendrogram using ISSR and SSR markers as dominant markers and 3) using SSR markers as co-dominant markers. Data from ISSR markers were computed as (0) absence and (1) presence of the band, SSR markers as co-dominant markers were computed as base pairs of fragments with the estimated size in base pairs for each allele obtained by the method of reverse mobility based on regression of products of known size of the molecular marker (50 bp) (Fermentas, USA) and for the dendrogram constructed with the combined data, both ISSR and SSR markers were scored as (0) and (1).

The genetic distance matrix was calculated using the Jaccard coefficient for the ISSR and combined ISSR and SSR markers approach and the Nei and Li (1979) coefficient for the SSRs as co-dominant markers. Clusters were generated by the UPGMA (unweighed pair-group method of arithmetic average) method and dendrograms constructed using the Statistica software (Statistica for Windows, 2002). Validation of the clusters was determined by the cophenetic correlation coefficient (CCC) (Sokal and Rohlf, 1962) using the GENES software (Cruz, 2006) and the cut-off established according to criteria suggested by Mingoti (2005).

Nonparametric methodology

Nonparametric tests, although somewhat statistically limited, are used when the data do not follow normal distribution (Cowan, 1992; Whitely and Ball, 2002), such as the case in our study, and therefore were chosen to investigate any possible correlation between bands and phenotypic variation for finger drop in banana fruits since both phenotypic and genotypic data were available.

The electrophoretic profile of a total of 208 bands (ISSR - 139 and SSR - 69 bands) computed as (1) and (0) along with the results of analysis for the finger drop variables: v1 = force necessary to release the fruit from the pedicel using a mechanical fruit detacher developed by Cerqueira et al. (2000): resistant genotypes (>60 N), MR genotypes (20-60 N), and resistant genotypes (<20 N); v2: scale proposed by Pereira et al. (2004) where 1 = resistant genotypes, 2 = MR genotypes, and 3 = susceptible genotypes, were submitted to the Spearman correlation and Kruskal- Wallis (Kruskal, 1964)- nonparametric tests. The SAS software (SAS Institute Inc., 2002-2004) was used with the PROC CORR Spearman and PROC NPAR1WAY ANOVA and exact Wilcoxon commands to verify the presence of any correlation between band and variables.

The RFD variable refers to the average of the RFD of the fruit expressed in Newtons (N), meaning the strength applied to the finger drop measurer (equipment) necessary to separate the fruit from the pedicel. Therefore, the higher the number in Newtons, the greater the force necessary to separate the fruit from the pedicel and more resistant is the genotype to finger drop. In contrast, lower numbers are attributed to genotypes more susceptible to finger drop. The scale, which varies from 1 to 3, refers to levels of variable RFD, where more resistant genotypes are given number 1, and more susceptible genotypes, 3 (Pereira et al., 2004).

RESULTS AND DISCUSSION

Genetic diversity analysis using SSR and ISSR markers

Microsatellites and ISSR markers are widely used in *Musa* spp genetic diversity studies (Hippolyte et al., 2012; Lamare and Rao, 2015; Silva et al., 2015). In our study, SSR

and ISSR markers were used to evaluate the genetic diversity of 15 banana diploids with different levels of finger drop resistance.

The electrophoretic profile with microsatellite marker SSR-MASR185 and ISSR marker ISSR-55 is shown in Figure 1A and B.

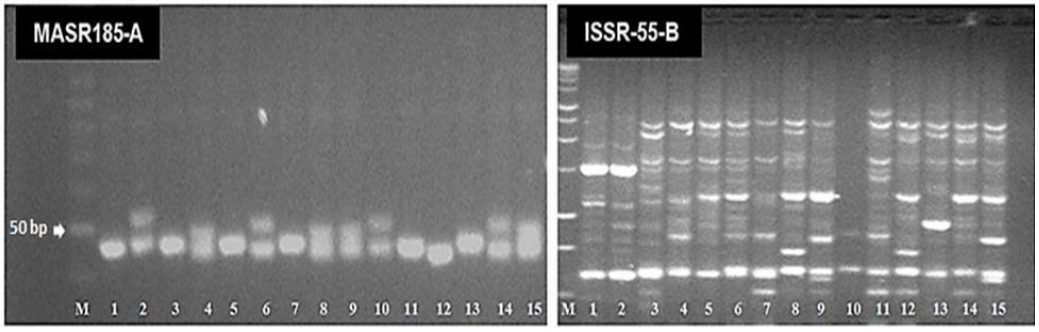

Figure 1. Electrophoretic profile on 3 and 2.5% agarose gels for marker SSR-MASR185 (**A**) and ISSR-55 (**B**), respectively, of 15 banana genotypes used in the study of genetic variability and resistance to finger drop (RFD). *Lane 1* = BB-França; *lanes 2* = Butuhan; *lanes 3* = ID028003-01; *lanes 4* = Khai Nai On; *lanes 5* = Hibrido AB; *lanes 6* = Ouro; *lanes 7* = Tjau Lagada; *lanes 8* = TH0301; *lanes 9* = Jary Buaya; *lanes 10* = Jaran; *lanes 11* = Lidi; *lanes 12* = M53; *lanes 13* = Calcutta-4; *lanes 14* = ID013004-04; *lanes 15* = ID017041-01. *Lane M* = molecular marker - 50 bp (**A**) and 1-kb ladder (Invitrogen) (**B**).

The genetic diversity analysis was evaluated by comparing three dendrograms: i) using 13 ISSR markers, ii) a combined dendrogram with 13 ISSR and 30 SSR markers scored as dominant markers, and iii) 30 SSR markers scored as co-dominant markers (bp of fragments).

From the 30 microsatellite primers assessed, 139 alleles were obtained, with an average of 4.7 alleles per locus. The smallest number of alleles was for primers AGMI 103/104, MASR 148, MASR 154, MASR 153, and CNPMF 3 (2 alleles) and the highest for primer Ma 3/103 (11 alleles) (Table 2). Considering only the results for banana diploids, the average of alleles per locus in our study is in agreement with those found by Amorim et al. (2008) (7.53 alleles) and Amorim et al. (2009) (7.51 alleles).

Ma series (Crouch et al., 1998), AGMI series (Lagoda et al., 1998), MaOCEN (Creste et al., 2006), CNPMF (Amorim et al., 2012), and MASR (Dr. Ana Ciampi - Embrapa CENARGEN).

The polymorphism information content (PIC), which provides an estimate of the discriminatory power of the marker, ranged from 0.16 to 0.86 for primers AGMI 103/104 and Ma 3/103, respectively, averaging 0.52 (Table 3). According to Botstein et al. (1980), markers with PIC values greater than 0.5 are considered highly informative, therefore, primer Ma 3/103 was considered highly polymorphic.

Three dendrograms were constructed: i) using 13 ISSR markers (Figure 2), ii) 30 SSR and 13 ISSR markers scored as (0) and (1) in a combined analysis (Figure 3), and iii) 30 SSR markers scored as co-dominant markers (bp of fragments) (Figure 4). In average, for all three dendrograms, the cophenetic correlation coefficient (CCC), which measures the consistency of the clusters was 0.94, considered adequate for these studies (Vaz Patto et al., 2004).

Table 2. Microsatellites used in genetic diversity and nonparametric test analysis: sequence locus, number of alleles, and polymorphism information content (PIC).

SSR locus name	Sequence (5'-3')	Number of alleles	PIC
AGMI 105/108	F: tcccaacccctgcaaccact R: atgacctgtcgaacatccttt	3	0.46
AGMI 127/128	F: aagttaggrcaagatagtgggatt R: cttttgcaccagttgttagg	4	0.33
AGMI 129/130	F: ggaggcccaacataggaagaggaat R: cataaacgacagtagaaatagcaac	6	0.61
AGMI 103/104	F: acagaatcgctaaccctaatcctca R: ccctttgcgtgcccctaa	2	0.16
AGMI 187/188	F: gcaactttggcagcatttt R: tgatggactcatgtgtacctactat	3	0.39
CNPMF 10	F: cacatcacacgctctgcttc R: tttttcggctgatccaattc	3	0.48
CNPMF 2	F: tgatctcgacgctgcac R: tgacagggcttccacttacag	3	0.23
CNPMF 63	F: ggtgggcaacctgtaatctg R: caccatttgggttttccaac	5	0.73
CNPMF 14	F: catcgaggatgcacatcaag R: ccaaaagagccacgattcag	7	0.59
CNPMF 37	F: gagccgtggctgtcactaag R: tatactctcgatcaccgggc	8	0.77
CNPMF 60	F: tgaaatctgaaccctggtgg R: acgcacacacacacacaatg	7	0.75
CNPMF 20	F: cctcgcacatcaacccttac R: catgatcaccatttcctccc	6	0.51
CNPMF 19	F: gtgttcgagagctttcagcc R: agaacaatcaagccagcagc	4	0.63
CNPMF 32	F: aggcttcgaccacaaactcc R: agcgttctcgttccaatcac	4	0.53
CNPMF 3	F: gggccaaccacatgatctac R: actcgagcacaaatggaacc	2	0.24
Ma 1/17	F: aggcggggaatcggtaga R: ggcgggagacagatggagt	7	0.73
Ma 3/103	F: tcgcctctctttagctctg R: tgttggaggatctgagattg	11	0.86
Ma 1/24	F: gagcccattaagctgaaca R: ccgacagtcaacatacaataca	6	0.65
Ma 1/27	F: tgaatcccaagtttggtcaag R: caaaacactgtccccatctc	6	0.75
MaOCEN 3	F: ggaggaaatggaggtcaaca R: ttcgggataggaggaggag	3	0.35
MaOCEN 1	F: tctcaggaagggcaacaatc R: ggaccaaagggaaagaaacc	6	0.74
MaOCEN 10	F: ggaagaaagaagtggagaatgaa R: tgaaatggataaggcagaagaa	3	0.29
MASR 189	F: gatggttcgtccgtcagatt R: cacagtcaccaaatccatcg	7	0.78
MASR 166	F: cgagtccgaagtcgcttcta R: ttgagcttgtgcctcctttt	4	0.67
MASR185	F: gacactgctccacaaaccct R: gcttcttcgggtgtctgttc	5	0.69
MASR 148	F: gcaagtgtggcaactgagaa R: cagcctgcccgaattatta	2	0.37
MASR 165	F: ggttggcgtacgtgaagagt R: cgctgttgccaacgtagata	4	0.49
MASR 154	F: gagaggatcgaggaaaaggg R: acggtgctgaaatatccagg	2	0.20
MASR 153	F: ccgcccatcctcgattacat R: gaataaaccatacaccgaggtaaa	2	0.36
MASR 149	F: tcgtcaggtctgtatgcgag R: ctgcaagaggacatcaaacaag	4	0.39
Total		139	-
Average		4.7	0.52

Table 3. Thirteen ISSR primers used in genetic diversity and nonparametric test analysis: primer name, sequence, and total number of bands.

Primer name	Sequence (5'-3')
ISSR2-DiCA3'G	CACACACACACACACAG
ISSR11-DiGA3'C	GAGAGAGAGAGAGAGAC
ISSR12 -DiGA3'RC	GAGAGAGAGAGAGAGARC
ISSR34-TriCAG3'RC	CAGCAGCAGCAGCAGRC
ISSR45-TriTGT3'YC	TGTTGTTGTTGTTGTYC
ISSR46-TriTGT5'CR	CRTGTTGTTGTTGTTGT
ISSR 50-TriAAG 3'RC	AAGAAGAAGAAGAAGRC
ISSR-55-TriACA 3'RC	ACAACAACAACAACARC
ISSR56-TriACT 3'RC	ACTACTACTACTACTRC
ISSR71-TriTCT 3'RC	TCTTCTTCTTCTTCTRC
ISSR72-TriTCC 3'RC	TCCTCCTCCTCCTCCRC
ISSR85-TriCCG 3'RC	CCGCCGCCGCCGCCGRC
ISSR88-TriCGC 3'RC	CGCCGCCGCCGCCGCRC

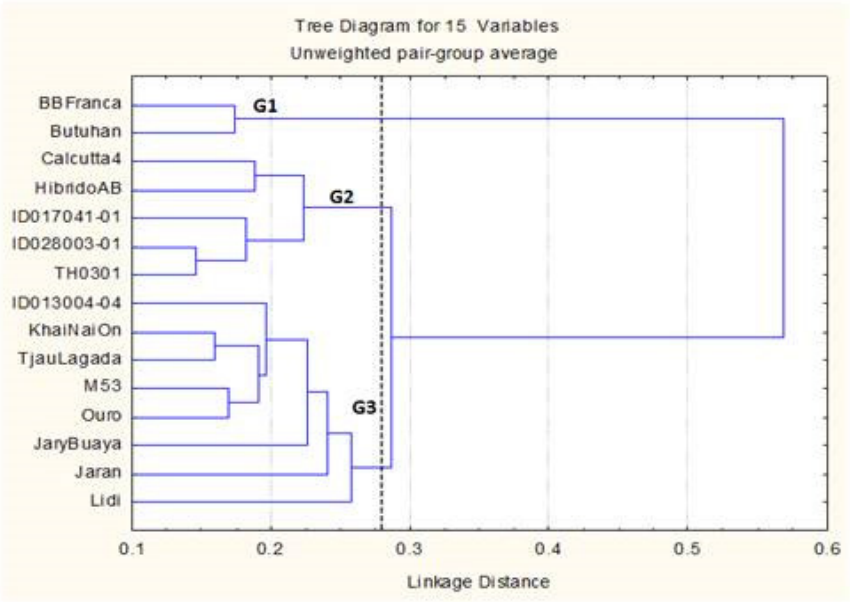

Figure 2. Dendrogram generated with 15 banana genotypes with different patterns of fruit drop resistance using 139 bands from 13 ISSR markers: (0) absence and (1) as presence of band. The distance matrix was calculated using the Jaccard coefficient index and clusters formed by the UPGMA method using the Statisitica software (Statistica for Windows, 2002). The cut-off was based on the criteria proposed by Mingoti (2005).

In general, the three dendrograms were not able to separate the genotypes according to their finger drop resistance patterns, except for groups G1 (Figures 2 and 3), where both genotypes are resistant to finger drop, and G1 (Figure 4), with both genotypes susceptible to finger drop, improved diploids, ID017041-01, and ID013004-04, and G3 comprised of R and MR genotypes (Figure 4).

The dissimilarity distances for the dendrogram generated by 139 ISSR markers (Figure 2), varied from 0.15 to 0.61%, with the most similar genotypes being TH0301 and ID0280031 and the most dissimilar, BB França and Jaran, and Jary Buaya and Butuhan. This dendrogram

separated the gentoypes into three major groups: G1: BB França and Butuhan (BB); G2: ID017041-01(AA), ID028003-01 (AA), TH0301 (AA), Calcutta 4 (AA) and Híbrido AB, and G3: ID013004-04 (AA), Khai Nai On (AA), Tjau Lagada (AA), M53 (AA), Ouro (AA), Jary Buaya (AA), Jaran (AA), and Lidi (AA). Although groups G1 and G3 separated the genotypes according to their genomes, BB and AA, respectively, only G1 separated them according to genomic constitution and RFD: BB França and Butuhan.

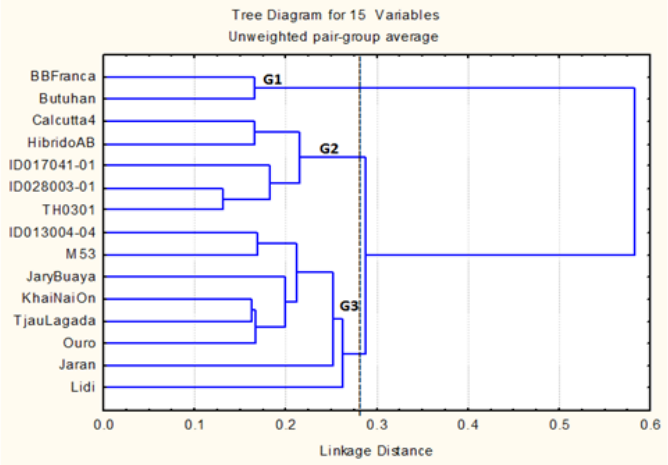

Figure 3. Dendrogram generated with 15 banana genotypes with different patterns of fruit drop resistance using 208 bands from 13 ISSR markers (139) and 30 SSR markers (69) scored as dominant markers: (0) absence and (1) as presence of band. The distance matrix was calculated using the Jaccard coefficient index and clusters formed by the UPGMA method using the Statistica software (Statistica for Windows, 2002). The cut-off was based on the criteria proposed by Mingoti (2005).

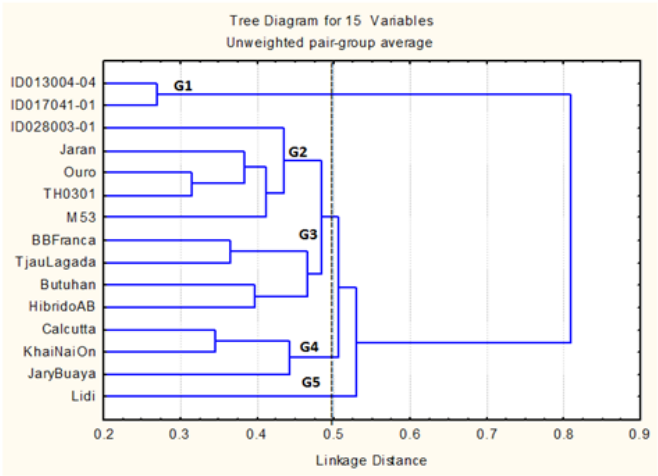

Figure 4. Dendrogram generated with 15 banana genotypes with different levels of resistance to finger drop (RFD) using 139 SSR polymorphic bands with scores based on bp of fragments. The distance matrix was calculated using the Nei and Li (1979) coefficient and clusters formed by the UPGMA method using the Statistica software (Statistica for Windows, 2002). The cut-off was based on the criteria proposed by Mingoti (2005).

Group G3 presented the largest number of moderately resistant genotypes: Jaran (AA), Jary Buaya (AA), Ouro (AA), Tjau Lagada (AA), - and susceptible: Lidi (AA), ID013004-04 (AA), M53 (AA), also grouping a genotype considered resistant, Khai Nai On (AA). ID013004-04 and Calcutta-4 in this same group are interesting, because Calcutta-4 was used as grandmother in the cross with ID013004-04. ID028003-01 (AA) and Calcutta-4 also in the same group can be explained because Calcutta-4 is one of the parents of ID280031.

The Lidi (AA), susceptible genotype, although clustered in group 3, remained separated from the other genotypes. This is justified once this genotype is known to present translocation and inversion events, which makes it very distinct among *Musa* genotypes (Shepherd, 1999).

The dendrogram generated by ISSR markers only presented similar results obtained for the combined analysis for the total of 208 ISSR and SSR scored as dominant markers (Figure 3) for all three groups, in which the genetic distances varied from 0.13 to 0.61, but were able to better separate the more distant genotypes. The most similar genotypes were TH030-1 and ID028003-01, and the most dissimilar: Jaran and BB França, Jary Buaya and Butuhan, BB França and ID013004-04, BB França and Jary Buaya, ID017041-01 and BB França, M53 and BB França, and Butuhan and M53. For the G2 cluster in these two dendrograms (Figures 2 and 3), Cacutta 4 is one of the progenitors of the improved diploids, ID028003-01 and TH030-1, which is in agreement as to their genealogy.

In the dendrogram generated by 30 SSR markers, scored as co-dominant markers (Figure 4), the genotypes were separated into 5 groups with distances varying from 0.27 to 0.89. This demonstrates that the SSR markers used as co-dominant markers have a much greater discrimination power (Creste et al., 2003). This dendrogram (Figure 4) separated the genotypes into G1: ID013004-04 and ID017041-01, G2: ID280031, Jaran, Ouro, TH0301 and M53, G3: BB França, Tjau Lagada, Butuhan and Hibrido AB, G4: Calcutta4, Khai Nan On and Jary Buaya, and G5: Lidi.

The most similar genotypes were the improved diploids in G1, ID013004-04 (AA) and ID017041-01 (AA), both susceptible to finger drop. This is in agreement with the fact that they share the same mother and grandmother, Madang, respectively, in their genealogy. The most dissimilar genotypes were Tjau Lagada (BB) and ID013004-04 (AA), 0.89. G2 only separated the genotypes according to their genomic constitution. G3 is represented by both MR and R genotypes to finger drop. G4 only separated the genotypes according to their genomic constitution and Lidi was separated in G5. This separate group for Lidi was expected as also mentioned earlier for the other two dendrograms, due to its unique translocations and inversions being considered a unique genotype (Shepherd, 1999). Thus, the cluster analysis did not show a perfect separation of the genotypes according to finger drop resistance, or genealogy. Similar results were obtained by Creste et al. (2003), whose study based on microsatellite primers did not show a perfect separation between cultivated diploids, wild, and improved hybrids. The authors noted that some genotypes were grouped according to geographical origins, while others had no other relationship.

Amorim et al. (2008) using SSR markers and diploid genotypes, also found no complete separation among improved, cultivated, and wild hybrids, and some diploids were grouped based only in their geographical origin. In this study, there was no separation of genotypes based solely on geographic origin, but genotypes were grouped based on their genomic constitution.

Even though none of the three dendrograms showed perfect separation regarding finger drop resistance/susceptibility, the one generated by SSR markers as co-dominant markers

better discriminated the genotypes regarding genomic constitution, genealogy, and RFD. This information is important, since these diploids are used in the banana genetic breeding program aiming to develop fruits with RFD.

Nonparametric tests analysis

Although the *Musa* spp genome has been recently sequenced (D'Hont et al., 2012), bananas are still considered an orphan crop. Bananas are made up by complex genomic combinations (genomes A and B), are parthenocarpic, and this characteristic alone hinders obtainig representative genetic linkage maps (Shepherd, 1999). Therefore, non-conventional methodologies, such as nonparametric tests, become attractive to overcome the lack of pre-requisites for conventional studies, such as obtainment of segregating populations for QTL association studies. Nonparametric tests using the Spearman correlation procedure and Kruskal-Wallis are widely found in the literature (Mace et al., 2006; Mondal and Badigannavar, 2010; Singh et al., 2013).

Mace et al. (2006) used nonparametric tests in 22 groundnut genotypes with differing levels of resistance to rust using 23 SSR markers. The results showed that a total of eight SSR loci were associated with rust resistance through Kruskal-Wallis and AMOVA tests. Their results also demonstrated that the SSR loci identified on the diverse set of 22 genotypes indicate that non-traditional methodologies, such as nonparametric tests, in comparison to traditional ones, such as QTL analysis undertaken on a segregating F2 and RIL populations of 4 ICRIST mapping populations for rust resistance, can be employed to associate genomic regions with traits of importance.

Mondal and Badigannavar (2010) also used nonparametric tests to study association of SSR markers linked to rust and late leaf spot resistance in a set of 20 cultivated groundnut genotypes. Three and four SSR alleles were found associated with rust and late leaf spot resistance, respectively.

Nonparametric tests were also used by Singh et al. (2013) with screening of 24 polymorphic SSRs in 36 cultivated pigeon pea with different levels of resistance to Fusarium wilt. Kruskal-Wallis and simple regression detected significant association of six SSR markers with Fusarium wilt resistance.

Although finger drop may be considered a quantitative characteristic, in our study, nonparametric tests were carried out in order to investigate possible association among 208 ISSR (139) and SSR (69) bands and two variables of resistance to fruit finger drop: v1: force I Newtons (N) necessary to detach the fruit from the pedicel using a mechanical detacher and v2: a scale varying from 1-3, where 1 = resistant to finger drop, 2 = moderately resistant to finger drop, and 3 = susceptible to finger drop, developed by Pereira et al. (2004). The v1 variable varied between 101.2 N for the most resistant genotype (BB França) to 5.7 for the most susceptible (Lidi).

For the entire analysis, correlation values varied from -0.05803 to 0.79772, but only higher correlations (above 70%) were taken into consideration (Table 4). Correlations were only found for the v1 variable, and the highest 12 correlations are presented in Table 4, which varied from -0.71005 to +0.79772. The highest correlation with the v1 variable was for marker/bands ISSR2B6 and MASR189B3, +0.79772. A positive correlation means that the presence of the band (1) is possibly linked to the v1 variable (Newton force to detach the fruit from the pedicel using a mechanical fruit detacher; Pereira et al., 2004). Seven bands

presented negative correlation, which means that the absence of the band would identify possible resistant genotypes to finger drop.

Table 4. Nonparametric data, Spearman correlation, Kruskal Wallis test and 2 values for 10 ISSR bands associated with the resistance to finger drop (RFD) variable.

Marker/band	Variable (v1)	Spearman (rs)	χ^2	Pr > χ^2
ISSR50B13	v1	-0.71005**	5.0417*	0.0247
Ma1/27B1	v1	-0.71005**	5.0417*	0.0247
MASR148B1	v1	-0.71714**	5.1429*	0.0223
ISSR2B7	v1	-0.71714**	5.1429*	0.0233
ISSR50B7	v1	-0.75056**	5.6333**	0.0176
MASR185B1	v1	-0.75056**	5.6333**	0.0176
MASR185B2	v1	-0.77460**	6.0000**	0.0143
ISSR2B8	v1	+0.67082*	4.5000*	0.0339
ISSR71B3	v1	+0.69631*	4.3636*	0.0367
ISSR45B2	v1	+0.75593*	4.0000*	0.0400
ISSR2B6	v1	+0.79772**	5.7273**	0.0167
MASR189B3	v1	+0.79772**	5.7273**	0.0167

**Significant at 1% probability. *Significant at 5% probability. (χ^2, chi square test). v1 = force in Newtons to detach fruit from pedicel using a mechanical fruit detacher (Pereira et al., 2004).

Our study shows that markers ISSR50, ISSR2, and SSR: MASR185 are interesting because one single marker yielded 2, 3, and 2 bands possibly linked to finger drop resistance, respectively.

Although nonparametric tests are limited, enabling restricted inferences, these tests were most appropriate to use in our data, since it did not follow normal distribution. In our study, the results were surprisingly positive, since no correlation was expected due to the possible quantitative nature of the finger drop variables. However, our study shows that high (negative and positive) significant correlations were found and may be explored in banana-breeding programs aiming the development of bananas more resistant to finger drop, a major concern among consumers.

Given the presence or absence of the bands and negative and positive correlations, it is possible to select in favor or against the presence of bands and make up a combination of these bands to be used in marker-assisted selection. The ideal is to use a combination of all bands in order to increase the chances of success for use in marker-assisted selection.

Sequencing of these bands will allow the design of sequence characterized amplified region markers. This will be the next step towards the validation of data and possible use in marker-assisted selection for this characteristic. These markers may then be used in combinations in order to best convey results during the validation process. It is worth mentioning that this is the first report of the use of nonparametric tests to study association of markers with finger drop resistance in bananas, and validation of these markers will enable banana genetic breeding programs worldwide to overcome one of the major bottlenecks in the banana-breeding program, which concerns finger drop of the fruit - reducing market value.

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REFERENCES

Amorim EP, Reis RV, Santos-Serejo JA, Amorim VBO, et al. (2008). Variabilidade genética estimada entre diplóides de banana por meio de marcadores microssatélites. *Pesqui. Agropecu. Bras.* 43: 1045-1052. https://doi.org/10.1590/S0100-204X2008000800014

Amorim EP, Lessa LS, Ledo CAS, Amorim VBO, et al. (2009). Caracterização agronômica e molecular de genótipos diploides melhorados de bananeira. *Rev. Bras. Frutic.* 31: 154-161. https://doi.org/10.1590/S0100-29452009000100022

Amorim EP, Silva PH, Ferreira CF, Amorim VBO, et al. (2012). New microsatellite markers for bananas (*Musa* spp). *Genet. Mol. Res.* 11: 1093-1098. https://doi.org/10.4238/2012.April.27.8

Amorim EP, Amorim VBO, Santos-Serejo JA and Silva SO (2016). O Agronegócio da Banana. 1ed.Brasília. *Embrapa* 1: 171-200.

Botstein D, White RL, Skolnick M and Davis RW (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* 32: 314-331.

Cerqueira ARC, Silva O and Medina VM (2000). Avaliação da resistência à queda de frutos de bananeira (*Musa* spp.) por diferentes métodos. In: Simpósio Brasileiro de Melhoramento de Fruteiras 2, Viçosa. Resumos nos anais do Simposio Brasileiro de Melhoramento, 175.

Cowan AR (1992). Nonparametric Event Study Tests. *Rev. Quant. Finance Account.* 2: 343-358. https://doi.org/10.1007/BF00939016

Creste S, Tulman Neto A, Silva SO and Figueira A (2003). Genetic characterization of banana cultivars (*Musa* spp.) from Brazil using microsatellite markers. *Euphytica* 132: 259-268. https://doi.org/10.1023/A:1025047421843

Creste S, Benatti T, Orsi MR, Risterucci A-M, et al. (2006). Isolation and characterization of microsatellite loci from a commercial cultivar of *Musa acuminata. Mol. Ecol. Notes* 6: 303-306. https://doi.org/10.1111/j.1471-8286.2005.01209.x

Crouch HK, Crouch JH, Jarret RL, Cregan PB, et al. (1998). Segregation at microsatellite loci in haploid and diploid gametes of *Musa. Crop Sci.* 38: 211-217. https://doi.org/10.2135/cropsci1998.0011183X003800010035x

Crouch JH, Crough HK, Tenkouano A and Ortiz R (1999). VNTR- based diversity analysis of 2x and 4x full-sib *Musa* hybrids. *Electron. J. Biotechnol.* 2: 99-108. https://doi.org/10.2225/vol2-issue3-fulltext-1

Cruz CD (2006). Programa GENES: aplicativo computacional em genética e estatística. UFV, Viçosa, 442.

D'Hont A, Denoeud F, Aury JM, Baurens FC, et al. (2012). The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. *Nature* 488: 213-217 https://doi.org/10.1038/nature11241.

Doyle JJ and Doyle JL (1990). Isolation of plant DNA from fresh tissue. *Focus* 12: 13-15.

FAO - Food and Agriculture Organization (2016). FAO statistical databases. Available at [http://www.faostat.fao.org]. Accessed October 10, 2016.

Grapin A, Noyer JL, Carreel F, Dambier D, et al. (1998). Diploid *Musa acuminata* genetic diversity assayed with sequence-tagged microsatellite sites. *Electrophoresis* 19: 1374-1380. https://doi.org/10.1002/elps.1150190829

Hicks EW (1934). Finger dropping from bunches of Australian Cavendish Bananas. *J. Counc. Sci. Ind. Res.* 7: 165-168.

Hippolyte I, Jenny C, Gardes L, Bakry F, et al. (2012). Foundation characteristics of edible Musa triploids revealed from allelic distribution of SSR markers. *Ann. Bot.* 109: 937-951. https://doi.org/10.1093/aob/mcs010

Imsabai W, Saichol K and Doorn W (2006). Physiological and biochemical changes during banana ripening and finger drop. *Postharvest Biol. Technol.* 39: 211-216. https://doi.org/10.1016/j.postharvbio.2005.10.001

Iniguez-Luy FL, Voort AV and Osborn TC (2008). Development of a set of public SSR markers derived from genomic sequence of a rapid cycling *Brassica oleracea* L. genotype. *Theor. Appl. Genet.* 117: 977-985. https://doi.org/10.1007/s00122-008-0837-9

Kruskal JB (1964). Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. *Psychometrika* 29: 1-27. https://doi.org/10.1007/BF02289565

Lagoda PJL (1998) Diploid *Musa acuminata* genetic diversity assayed with sequence-tagged microsatellite sites. *Electrophoresis* 19: 1374-1380.

Lamare A and Rao SR (2015). Efficacy of RAPD, ISSR and DAMD markers in assessment of genetic variability and population structure of wild Musa acuminata colla. *Physiol. Mol. Biol. Plants* 21: 349-358. https://doi.org/10.1007/s12298-015-0295-1

Lu Y, Zhang X, Pu J, Qi Y, et al. (2011). Molecular assessment of genetic identity and genetic stability in banana cultivars (*Musa* spp.) from China using ISSR markers. *Aust. J. Crop Sci.* 5: 25-31.

Mace ES, Phong EDT, Upadhyaya ES, Chandra S, et al. (2006). SSR analysis of cultivated groundnut (*Arachis hypogaea* L.) germplasm resistant to rust and late leaf spot diseases. *Euphytica* 152: 317-330. https://doi.org/10.1007/s10681-006-9218-0

Marriott J (1980). Bananas - physiology and biochemistry of storage and ripening for optimum quality. *Crit. Rev. Food Sci. Nutr.* 13: 41-88. https://doi.org/10.1080/10408398009527284

Mingoti AS (2005). Análise de dados através de métodos de estatística multivariada: uma abordagem aplicada. UFMG, Belo Horizonte, 295.

Mondal S and Badigannavar AM (2010). Molecular diversity and association of SSR markers to rust and late leaf spot resistance in cultivated groundnut (*Arachis hypogaea* L.). *Plant Breed.* 129: 68-71. https://doi.org/10.1111/j.1439-0523.2009.01635.x

Nei M and Li W-H (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* 76: 5269-5273. https://doi.org/10.1073/pnas.76.10.5269

Pereira MCT, Salomão LCC, Silva SO, Cecon PR, et al. (2004). Suscetibilidade à queda natural e caracterização dos frutos de diversos genótipos de bananeiras. *Rev. Bras. Frutic.* 26: 499-502. https://doi.org/10.1590/S0100-29452004000300030

Putra ETS, Zakaria W, Abdullah NAP and Saleh G (2010). Weak neck of Musa sp. cv. 'Rastali': a review on its genetics, crop nutrition, and postharvest. *J. Agron.* 2: 45-51.

Racharak P and Eiadthong W (2007). Genetic relationship among subspecies of *Musa acuminate* Colla and A-genome consisting edible cultivated bananas assayed with ISSR markers. *Songklanakarin J. Sci. Technol.* 29: 1479-1489.

Salazar BM and Serrano EP (2013). Etiology and Postharvest Control of Finger Drop Disorder in 'Cuarenta Dias' Banana (*Musa acuminata* AA Group). *Philipp. Agric. Sci.* 96: 163-171.

SAS Institute Inc. (2002-2004). Cary, NC: SAS Institute Inc.

Shepherd K (1999). Cytogenetics of the genus *Musa*. International Network for the Improvement of Banana and Plantain, Montpellier, France.

Silva PRO, Jesus ON, Creste S, Figueira A, et al. (2015). Evaluation of microsatellite loci from libraries derived from the wild diploid 'Calcutta 4' and 'Ouro' banana cultivars. *Genet. Mol. Res.* 14: 11410-11428. https://doi.org/10.4238/2015.September.25.9

Simmonds NW and Shepherd K (1955). The taxonomy and origins of the cultivated bananas. The journal of the Linean Society of London, London, 55: 302-312.

Singh AK, Rai VP, Chand R, Singh RP, et al. (2013). Genetic diversity studies and identification of SSR markers associated with *Fusarium* wilt (*Fusarium udum*) resistance in cultivated pigeonpea (*Cajanus cajan*). *J. Genet.* 92: 273-280. https://doi.org/10.1007/s12041-013-0266-7

Sokal RR and Rohlf FJ (1962). The comparison of dendrograms by objective methods. *Taxon* 11: 33-40. https://doi.org/10.2307/1217208

Statistica for Windows (2002). Computer program manual. Version 6. Tulsa: Statsoft, 1CD-ROM.

Vaz Patto MC, Satovic Z, Pêgo S and Fevereiro P (2004). Assessing the genetic diversity of Portuguese maize germplasm using microsatellite markers. *Euphytica* 137: 63-72. https://doi.org/10.1023/B:EUPH.0000040503.48448.97

Whitley E and Ball J (2002). Statistics review 6: Nonparametric methods. *Crit. Care* 6: 509-513. https://doi.org/10.1186/cc1820